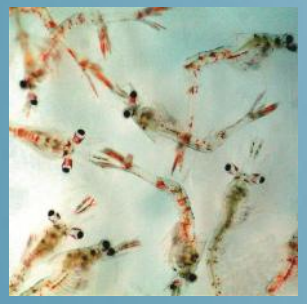
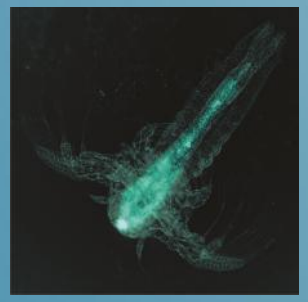
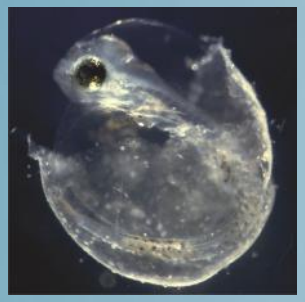
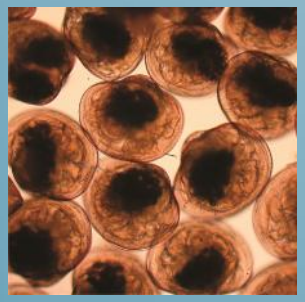




larvi 2013

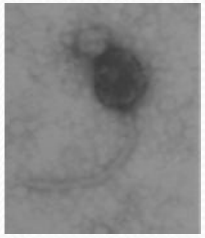
6th fish & shellfish larviculture symposium

Bacteriophage application as a management strategy in hatcheries - a review

Indrani Karunasagar

ghent university, belgium, 2-5 september 2013

Bacteriophage application as a management strategy in shrimp hatcheries



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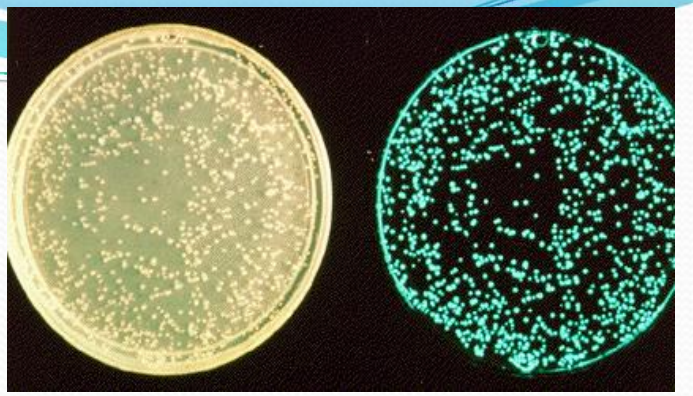
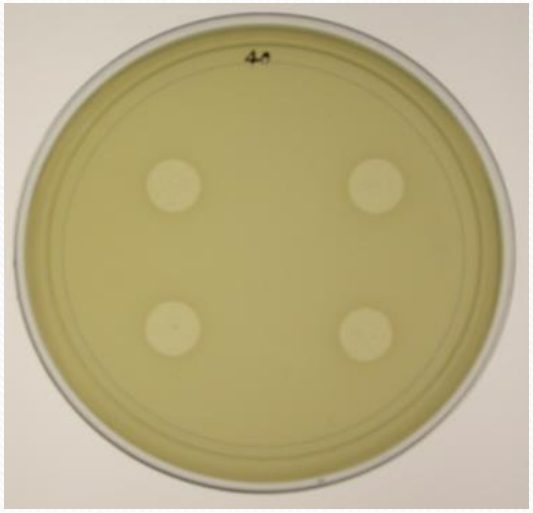
Grateful thanks to Larvanet and the organizers for the funding support to participate in LARVI 13


31 7 2004





ELSEVIER

Aquaculture 128 (1994) 203–209

Aquaculture

Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection

I. Karunasagar*, R. Pai, G.R. Malathi, Indrani Karunasagar

Department of Fishery Microbiology, University of Agricultural Sciences, College of Fisheries, Mangalore 575002, India

Accepted 21 August 1994

Abstract

The cause of mass mortality in *Penaeus monodon* larvae in a hatchery was investigated. Antibiotic-resistant *Vibrio harveyi* could be isolated from all the infected larvae. These bacteria were absent in healthy eggs and nauplii. Although the intake seawater had *V. harveyi*, these strains were sensitive to antibiotics. The results suggest that antibiotic-resistant *V. harveyi* had been colonising larval tanks. The isolates from moribund larvae showed much lower LD_{50} values than isolates from natural seawater, thus indicating their higher virulence.

Keywords: Antibiotic-resistant bacteria; *Vibrio harveyi*; *Penaeus monodon*; Diseases and their control — crustaceans

ELSEVIER

Aquaculture 140 (1996) 241–245

Aquaculture

Biofilm formation by *Vibrio harveyi* on surfaces

I. Karunasagar *, S.K. Otta, Indrani Karunasagar

Department of Fishery Microbiology, University of Agricultural Sciences, College of Fisheries, Mangalore-575 002, India

Accepted 26 September 1995

Abstract

The role of biofilm in the survival and persistence of the bacterial shrimp pathogen *Vibrio harveyi* and its possible role in perpetuating infection in shrimp hatcheries was studied. *Vibrio harveyi* formed biofilms on all three substrates tested: cement slab, high density polyethylene (HDPE) plastic and steel coupons. Cell density was highest on the plastic surface followed by the cement slab and the steel surface. Biofilm on the three surfaces also exhibited differential sensitivity to the sanitiser chlorine, maximum resistance being found on the concrete slab followed by plastic and steel coupons. Planktonic cells were sensitive to short exposure to low levels of chlorine. Biofilm formation occurred even in the presence of the antibiotics chloramphenicol and tetracycline, both added to the medium at 50 ppm.

Keywords: Biofilm; *Vibrio harveyi*; Substrate; Sanitiser; Chlorine; Antibiotic

Luminous Bacterial Disease- Harveyi clade

- **Problem in shrimp hatcheries & farms**
- **Causative agent : *Vibrios***
- **Autochthonous flora of coastal waters**
- **Association with crustaceans**
- **Animals show luminescence**
- **Bacteria also show luminescence**

Other diseases caused by bacteria of *Harveyi* clade - EMS

New shrimp disease – a global threat

Early Mortality Syndrome (EMS) /

Acute Hepatopancreatic Necrosis Syndrome

Identified as a member of *Harveyi* clade related to *V. parahaemolyticus*

CONTROL MEASURES

A major challenge

- **Antibiotics & chemicals - ineffective**
  - resistance to many agents.
  - residues in products
  - environmental concerns on spread of resistance

- **Bacteria persist in hatchery environment as biofilms - surfaces like tanks, pipes etc.**

- **Biofilm bacteria several times more resistant to sanitisers and antibiotics than normal planktonic bacteria.**

Need of the hour- to look at alternative solutions

Phage Therapy – A Novel Approach

INTRODUCTION

What are phages ?

- viruses that infect bacteria

- have lytic and lysogenic life cycle

- lytic phages are good candidates for antibacterial therapy

- highly specific to one (rarely another) bacterial species

- nontoxic to animals and plants

EMERGENCE OF PATHOGENIC BACTERIA RESISTANT TO MOST OF THE ANTIMICROBIAL AGENTS HAS BECOME A CRITICAL PROBLEM

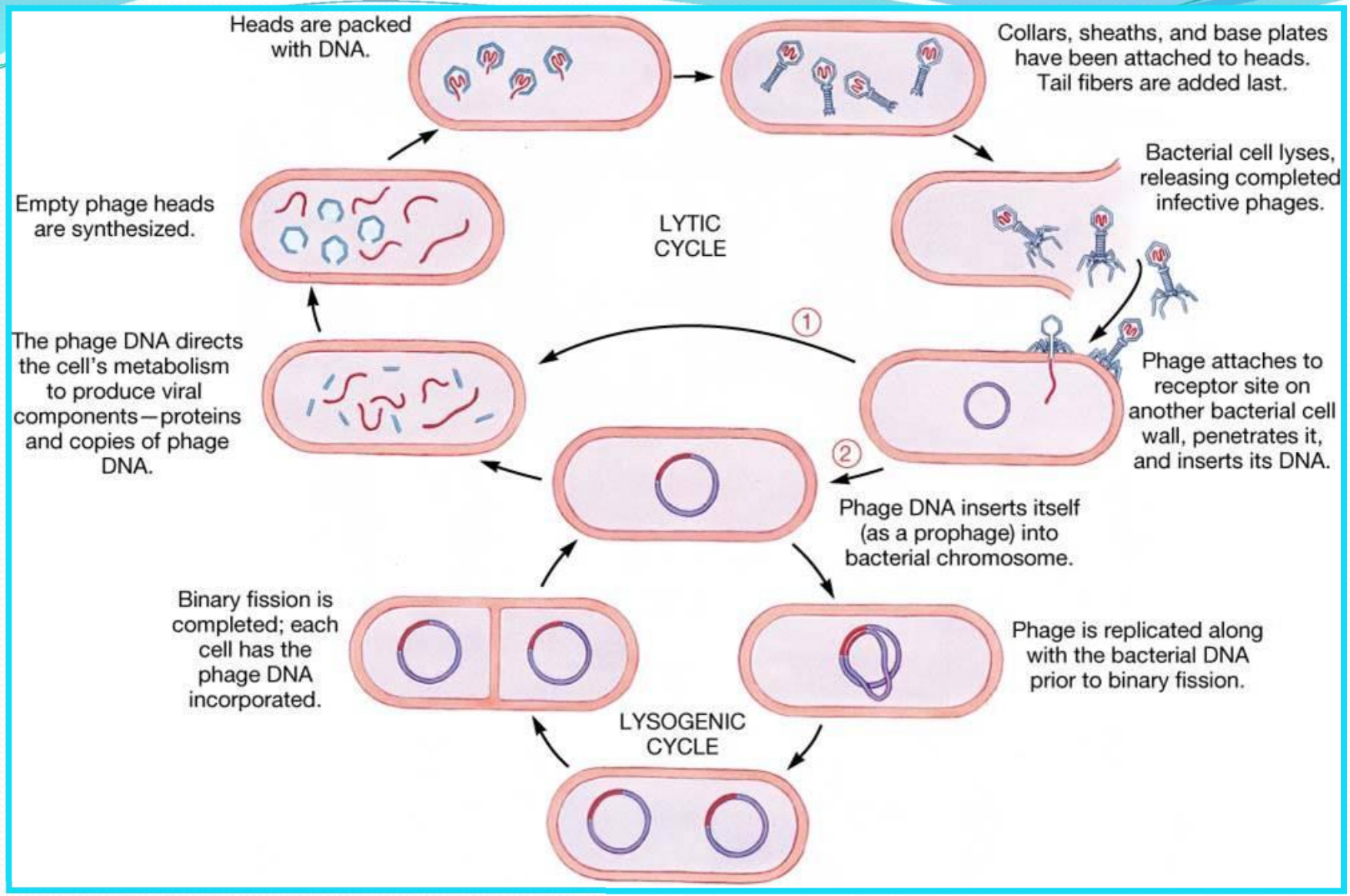


Fig.- Bacteriophage life cycle

Source:http://faculty.irsc.edu/FACULTY/TFischer/images/bacteriophage life cycle.jpg

Attributes of phages that supports its therapeutic response

The issue	Limitations of antibiotics	Advantages of phages
Fate of drug molecule	Metabolic destruction of molecule as it works	Exponential growth
Concentration of the drug	High conc is required	All or none effect
Resistance by bacteria	Antibiotics become obsolete over time	Co-evolve to overcome bacterial mutation
Spread of bacterial resistance	Broad spectrum	Host specific, do not cross species boundaries

Phage Therapy Program

Sample Collection

Phage Isolation

E. coli

Swine
Cattle

Salmonella

Swine
Poultry
Cattle

PHAGE BANK

Coli-Pro and Salmo-Pro

Efficacy

Characterization

Safety

Manufacture

Market

Use of phages to control aquatic diseases is promising

Why ?

- **Both bacteria and phages are in suspension similar to the lab conditions.**

- **Natural phages are evolved to be successful in liquid medium**

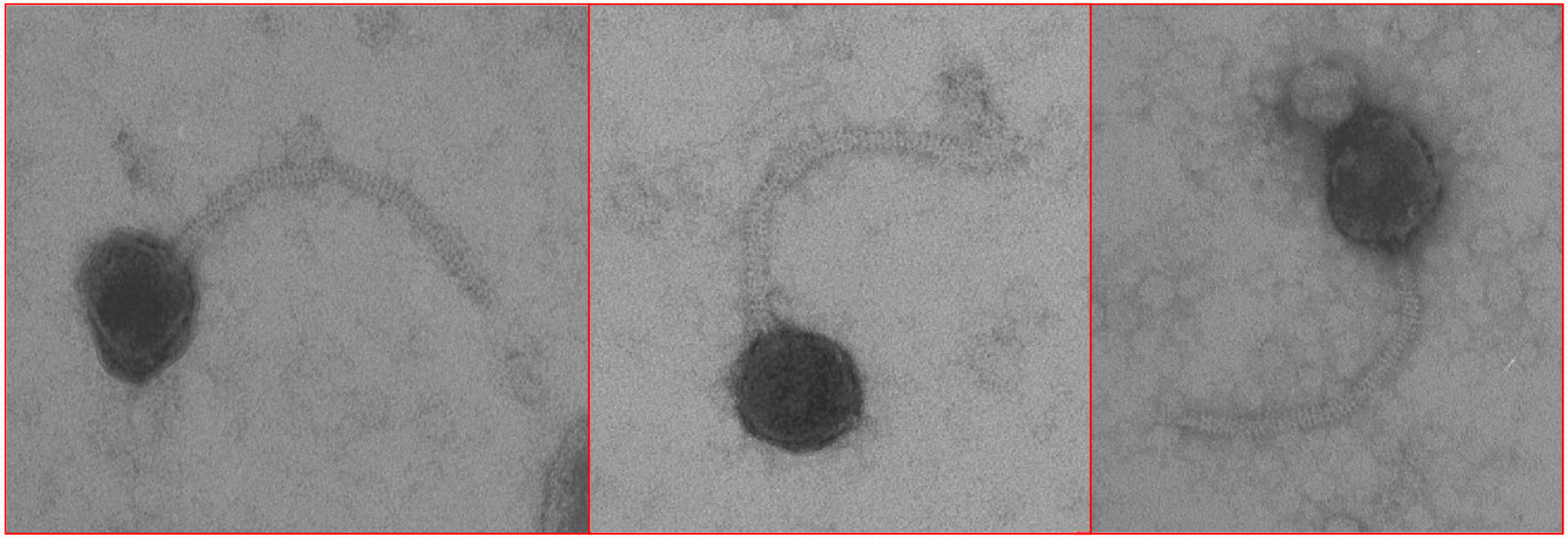
- **Therapeutic phage can have intimate contact with the pathogens of fish, crustacea and molluscs**

Advantages of Phage as a Biocontrol Agent

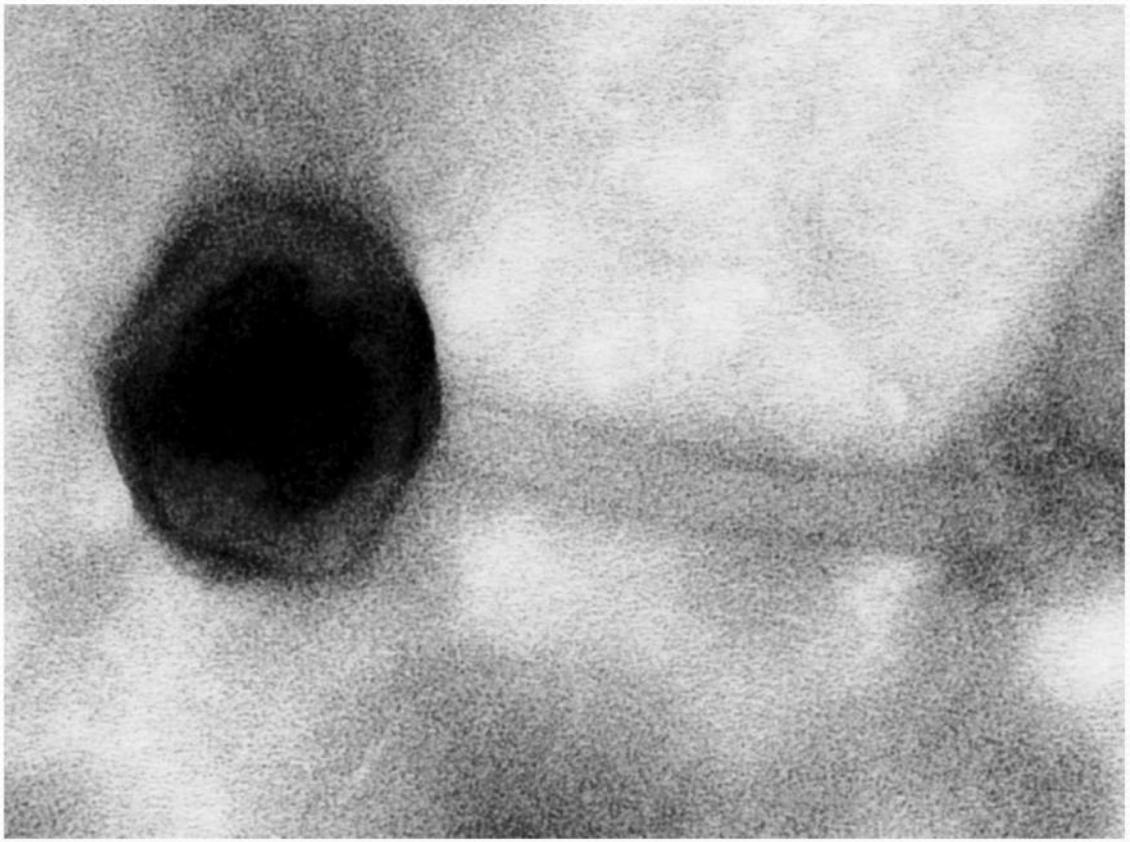
- **Normal inhabitant of marine environment**
- **Specific**
- **Once host population disappears, bacteriophages also disappear**
- **Harmless to other normal flora, do not affect useful bacteria associated with larvae, animals or pond**

Therefore, an ecofriendly management measure

TEM of V.harveyi phage



Electron micrograph of negatively stained *V.harveyi* Phage



V. harveyi phage- Myovirus

Phage titre values obtained at different hours after infection by semi-solid agar overlay technique

Hours	Titre value (pfu/ml)
1	10^3
2	10^5
3	10^{10}
4	10^{11} - optimum
5	10^{11} - optimum
6	10^{10}
7	10^8
8	10^5

Phage isolates with respective host bacteria, source, plaque size and genome size

Phages isolates	Host bacteria[a]	Source	Plaque size (diameter, mm)	Genome size[b] (Kb)
Viha1	VH 017	hatchery water	3-5	94
Viha2	VH 020	hatchery water	3-5	94
Viha3	VH 025	hatchery water	4-6	70
Viha4	VH 042	creek water	1-3	85
Viha5	VH 102	hatchery water	0.5-1	83
Viha6	VH 036	hatchery water	1-2	60
Viha7	VH 039	hatchery water	5-6	44

[a]Bacterial isolates from our own culture collection
[b]Approximate size of genome estimated by RFLP pattern using Kodak 1D software

Shivu et al., 2007; Environmental Microbiology

Morphological features of *V. harveyi* phages

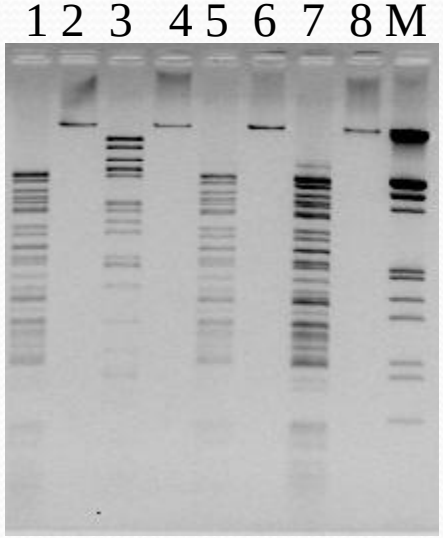
Phage	Family	Head diam (nm)	Tail length (nm)	Tail diam (nm)	Additional features			
					Collar	Base plate	Tail pins	Terminal bulb
Viha1	*Siphoviridae*	56±5	176±9	9±1	-	-	-	+
Viha2	*Siphoviridae*	53±3	200±18	8±1	-	-	-	+
Viha3	*Siphoviridae*	56±5	211±22	9±1	-	-	-	+
Viha4	*Myoviridae*	114±9	192±22	24±3	+	+	+	-
Viha5	*Siphoviridae*	92±6	175±19	19±2	-	-	-	+
Viha6	*Siphoviridae*	48±5	126±12	11±1	-	-	-	+
Viha7	*Siphoviridae*	58±3	194±16	9.5±1	-	-	-	+

[A] The values are the means of nine independent measurements for different phage particles.

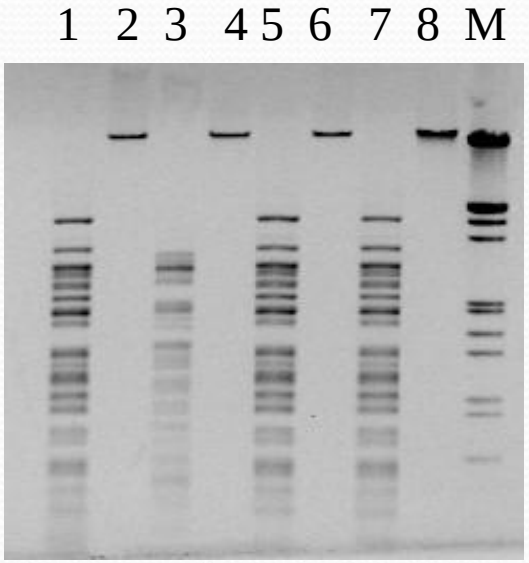
***Shivu et al.*, 2007; Environmental Microbiology**

Restriction digestion using different enzymes

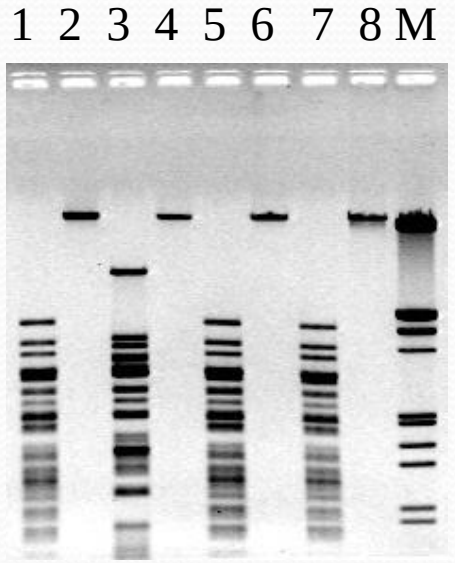
***Hind* III**



***Hinc* II**



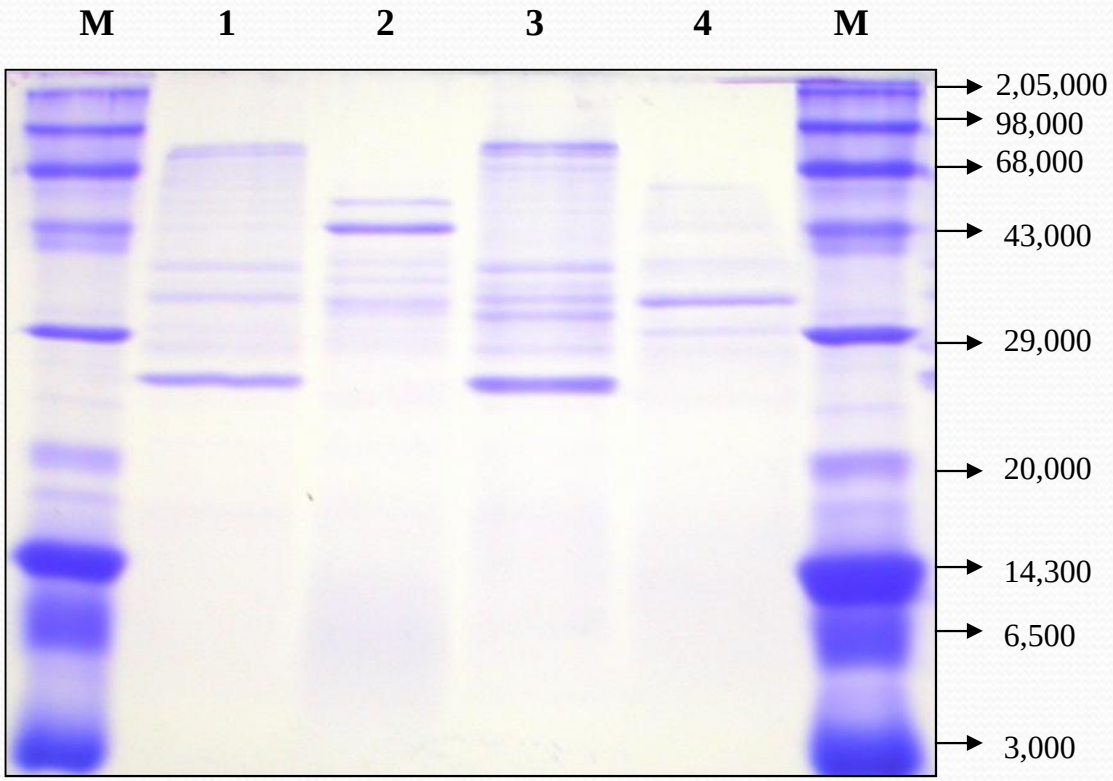
***HPA* 1**



Lane 1-KP; Lane 3-AP; Lane 5-VP; Lane 7-SP; Lane 2,4,6,8-negative controls; Lane M-Lambda DNA *Eco* R1 *Hind* III double digest

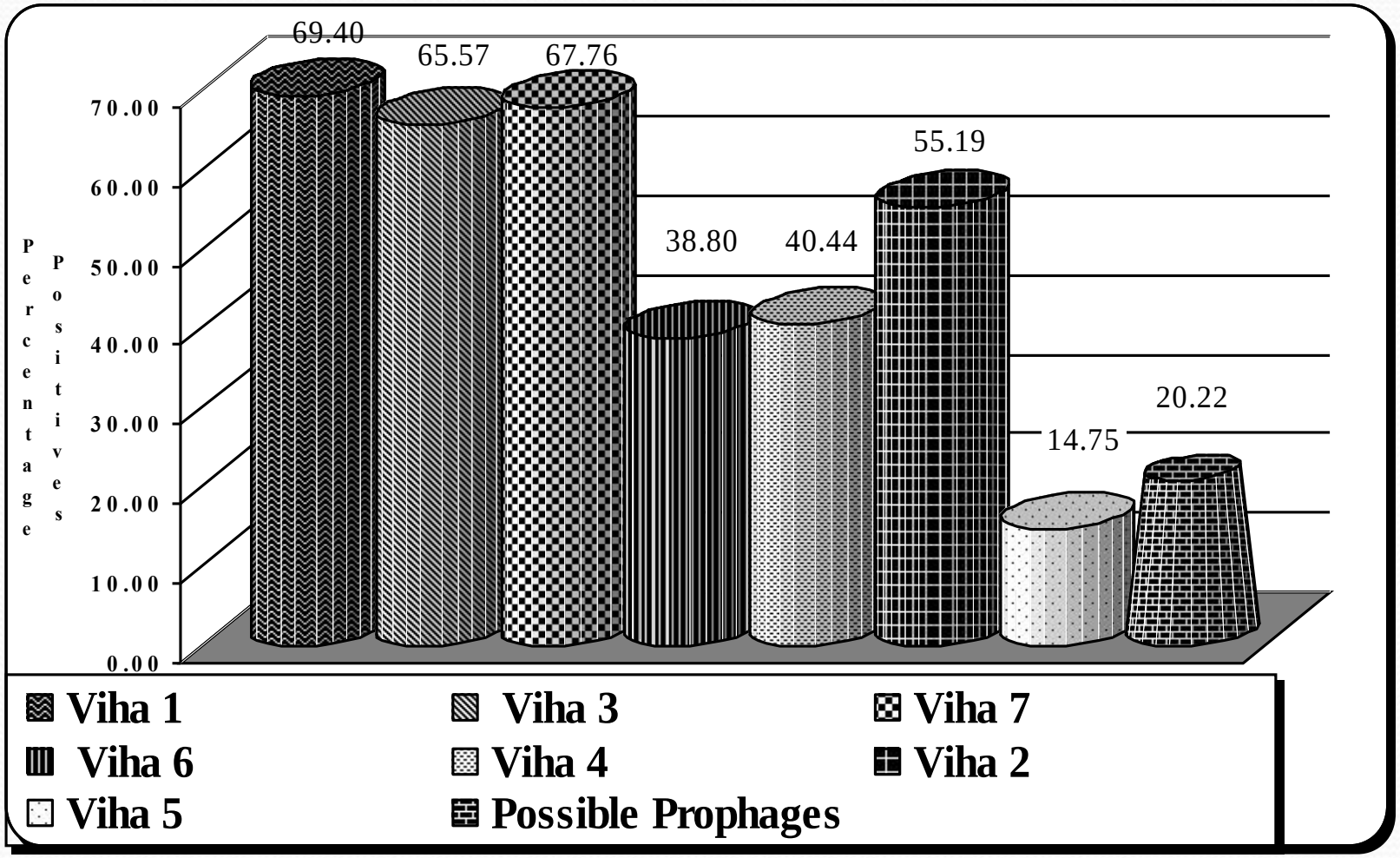
***Shivu et al*., 2007; Environmental Microbiology**

SDS-PAGE of bacteriophage structural protein



Lane 1 to 4: Different phage proteins

Lytic spectrum of *V. harveyi* phages



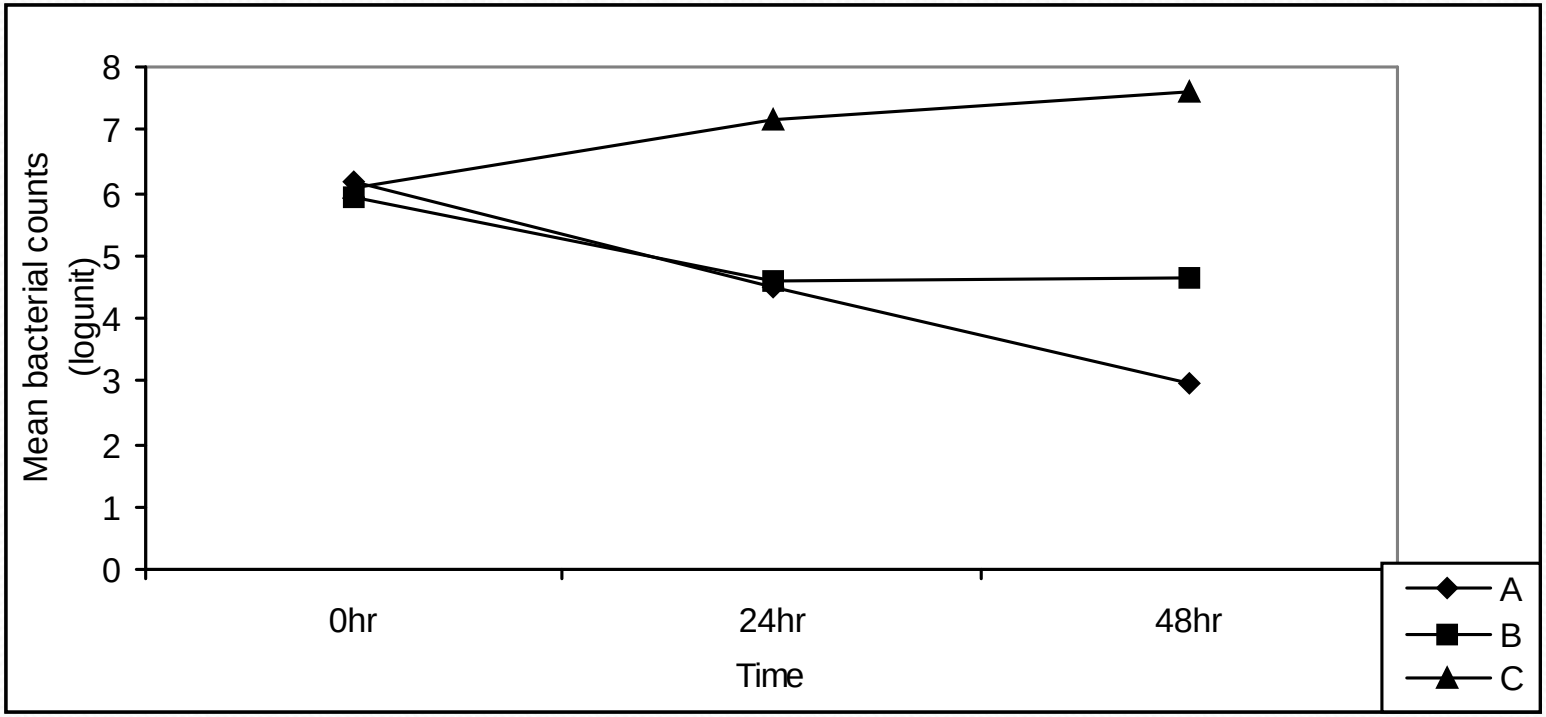
Shivu et al., 2007; Environmental Microbiology

Bacteriophage therapy in laboratory microcosm

Beakers	Time interval	Dose of phage	TPC (cfu/ml)	LBC (cfu/ml)	Larval Survival (%)
A Test	Initial	100 «l	$1.37x10^6$	$7.80x10^5$	100
	After 24 h	100 «l	$1.21x10^6$	$4.29x10^4$	100
	After 48 h	Nil	$9.80x10^5$	$1.20x10^2$	80
B Test	Initial	100 «l	$1.02x10^6$	$1.36x10^6$	100
	After 24 h	Nil	$1.29x10^6$	$9.30x10^5$	80
	After 48 h	Nil	$7.30x10^6$	$8.90x10^5$	40
C Control	Initial	Nil	$4.29x10^6$	$1.78x10^6$	100
	After 24 h	Nil	$9.20x10^6$	$4.68x10^5$	75
	After 48 h	Nil	$3.90x10^6$	$1.03x10^5$	10

Vinod *et al*., 2006; Aquaculture

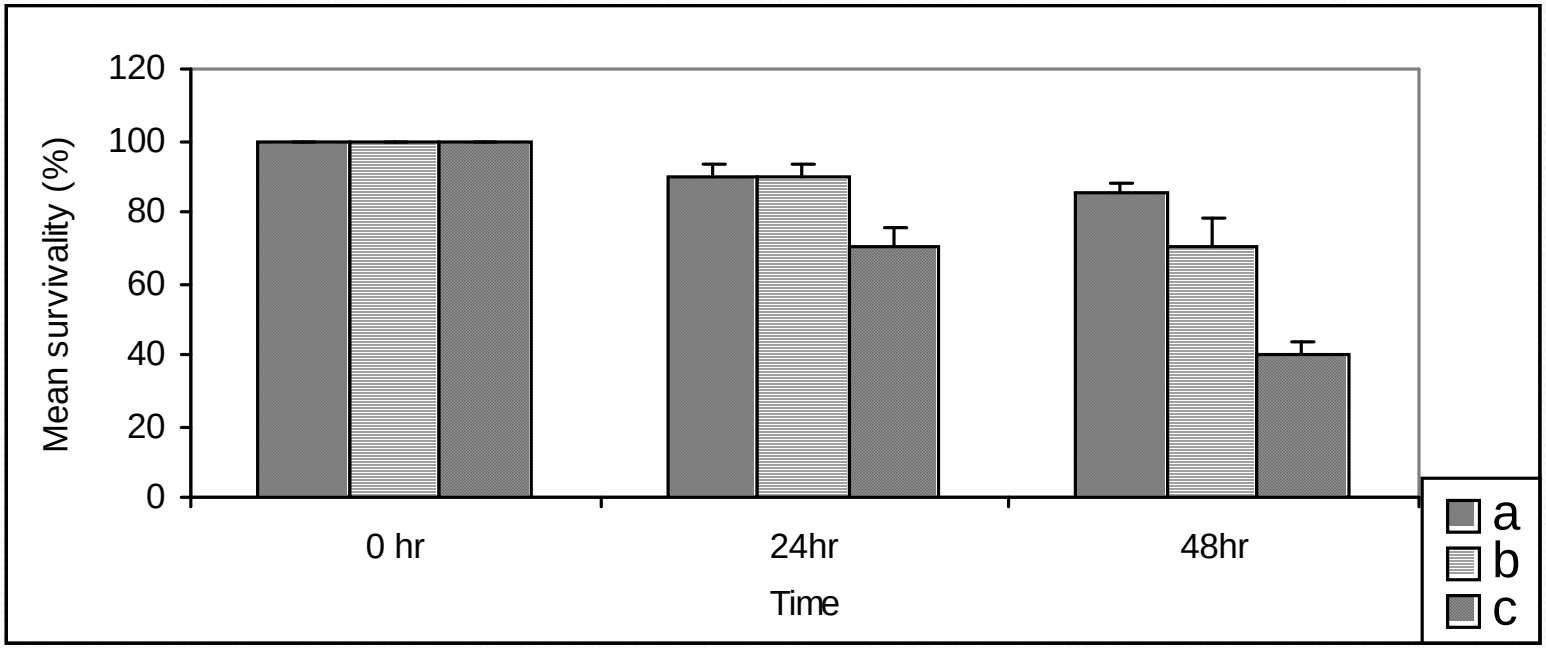
Mean luminous bacterial counts for 3 replicate tanks for 48 hr after being challenged with strains of pathogenic *Vibrio harveyi* and treated with bacteriophage



a- treated with two dosage of 100 µl phage for every 24 hr
b- treated with one dosage of 100 µl phage
c- control

Vinod *et al*., 2006; Aquaculture

Mean survival of *Penaeus monodon* larvae and standard error for 3 replicate tanks for 48 hr after being challenged with strains of pathogenic *Vibrio harveyi* and treated with bacteriophage

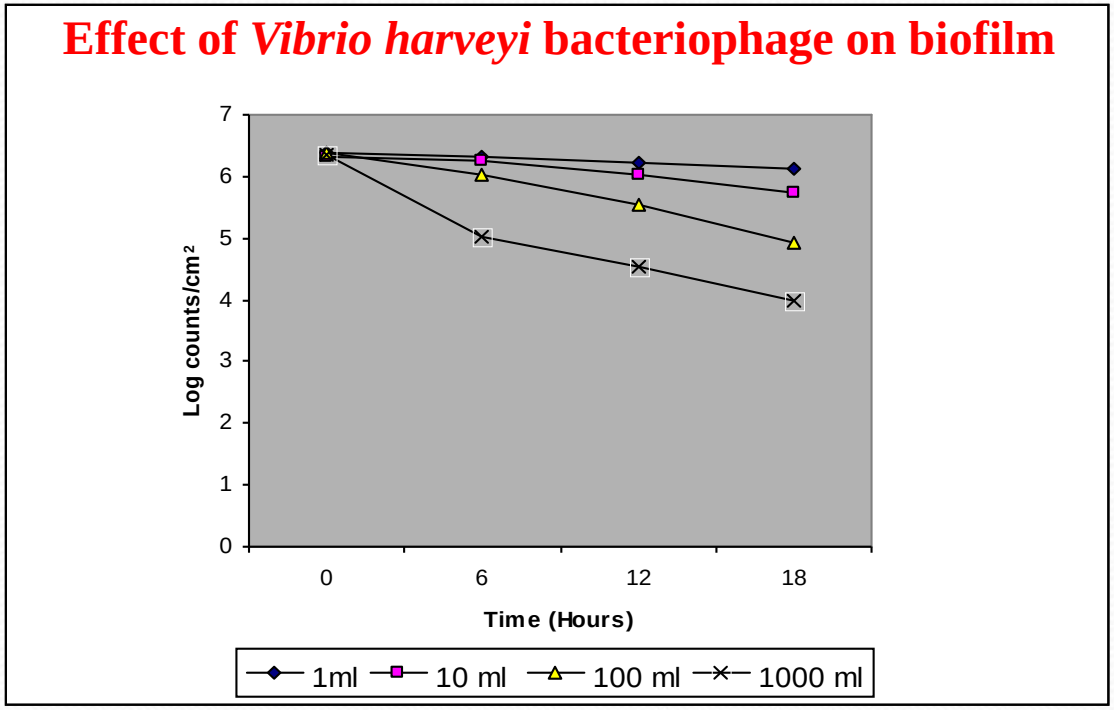


a- treated with two dosage of 100 µl phage for every 24 hr
b- treated with one dosage of 100 µl phage
c- control

Vinod *et al*., 2006; Aquaculture

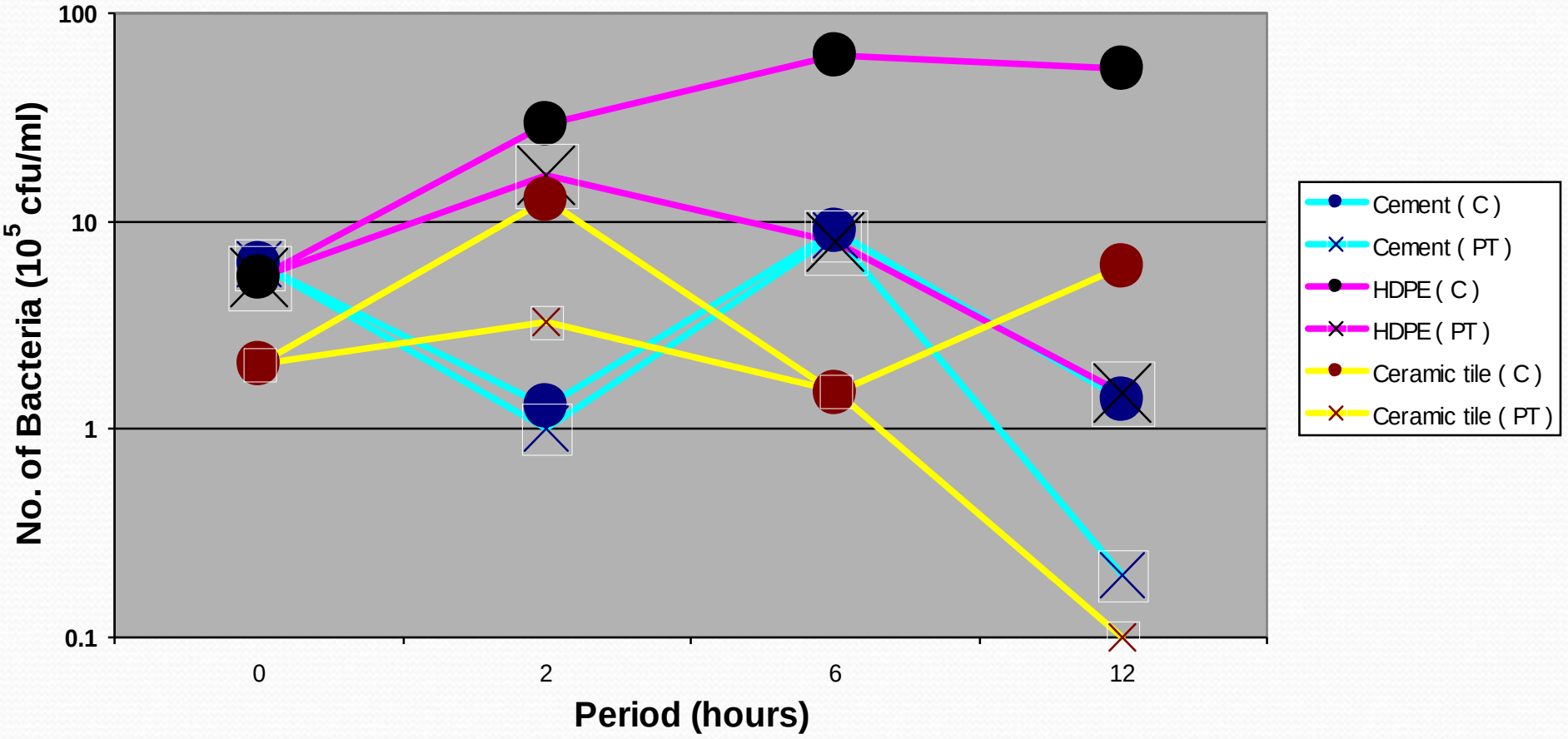
Effect of *Vibrio harveyi* bacteriophage on biofilm

Vol of phage Time (hr)	1«l	10«l	100«l	1000«l	Control
0	2.36 ò 10^6	2.16 ò 10^6	2.43 ò 10^6	2.19 ò 10^6	2.81ò10^6
6	2.06ò 10^6	1.75 ò 10^6	1.06 ò 10^6	1.06 ò 10^5	2.87ò10^6
12	1.76 ò 10^6	1.03 ò 10^6	3.9 ò 10^5	3.5 ò 10^4	2.78ò10^6
18	1.38ò 10^6	5.5 ò 10^5	8.3 ò 10^4	9.4 ò 10^3	2.74ò10^6

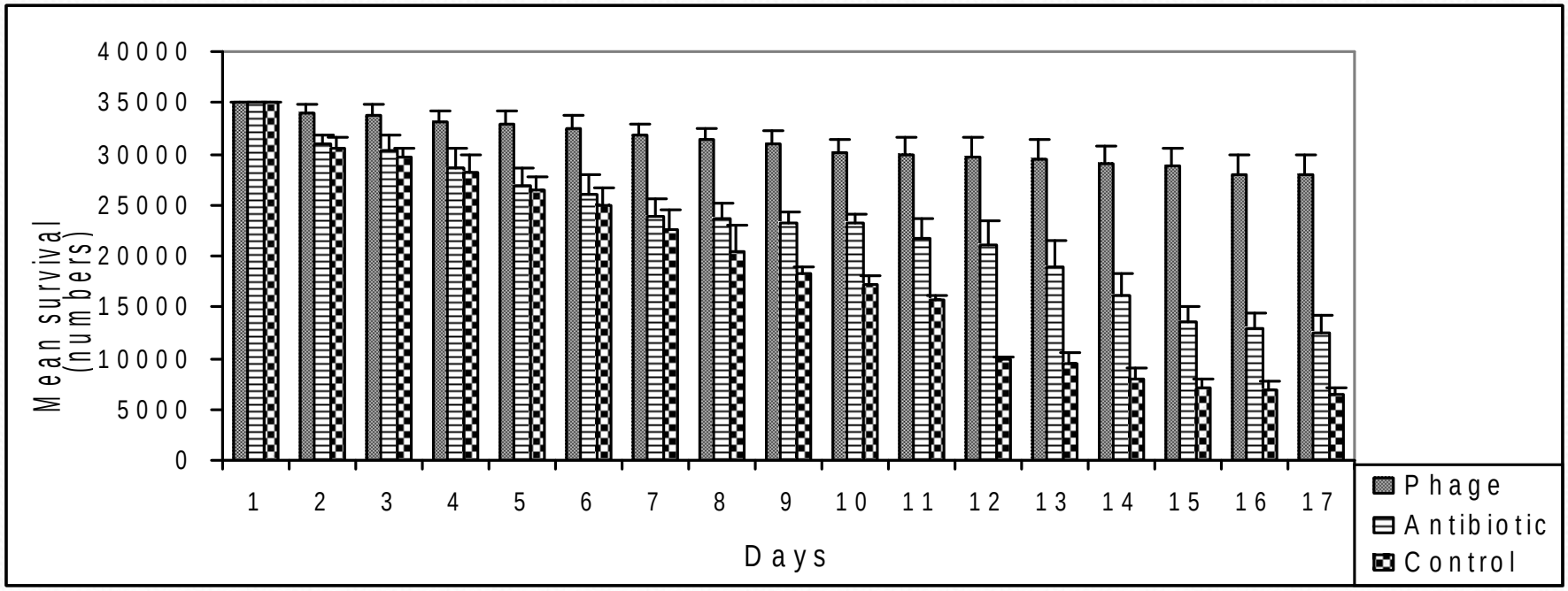


Karunasagar *et al*., 2007; Aquaculture

Effect of Phage Treatment on *V. harveyi* Biofilm Cells on Various Surfaces

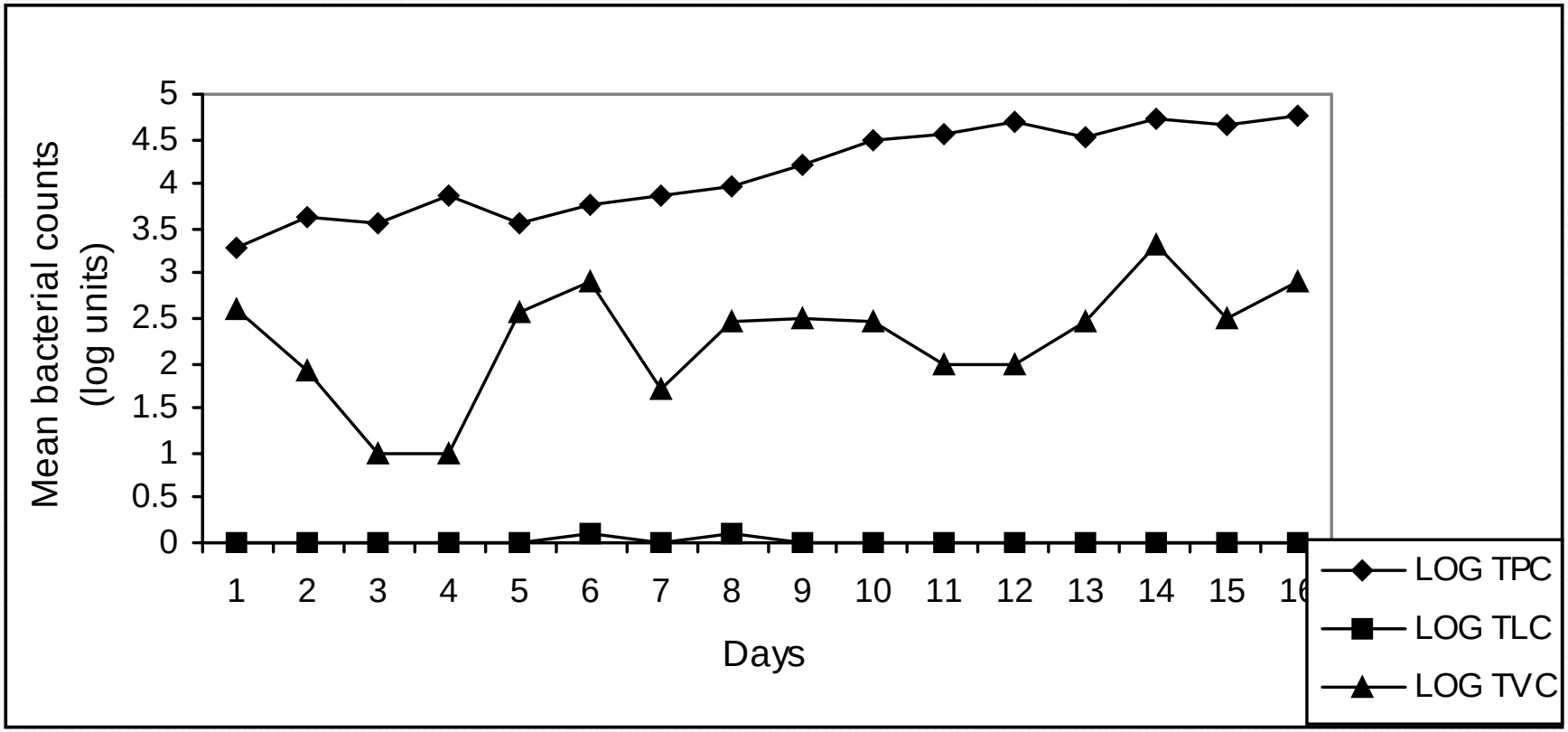


Mean survival of *Penaeus monodon* larvae and standard error for 3 replicate tanks of 35000 nauplii larvae 17 reared for days (from zoea to post larvae) with 2 different treatments (Bacteriophage and antibiotic) and a control.



Vinod *et al*., 2006; Aquaculture

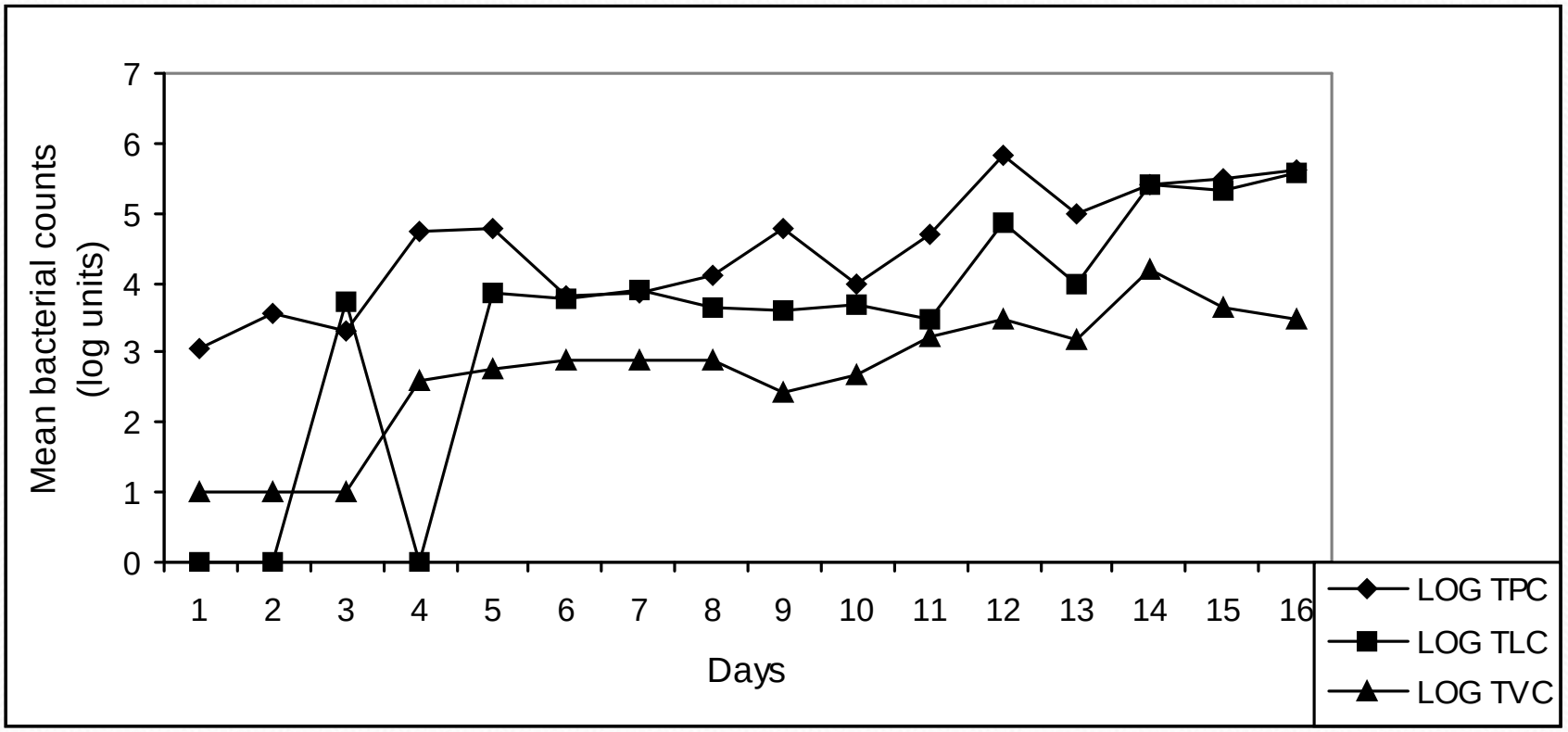
Mean bacterial counts of 3 replicate tanks treated with Bacteriophage



TPC- total plate count, TLC- Total luminous bacterial count, TVC- total Vibrio count

Vinod *et al*., 2006; Aquaculture

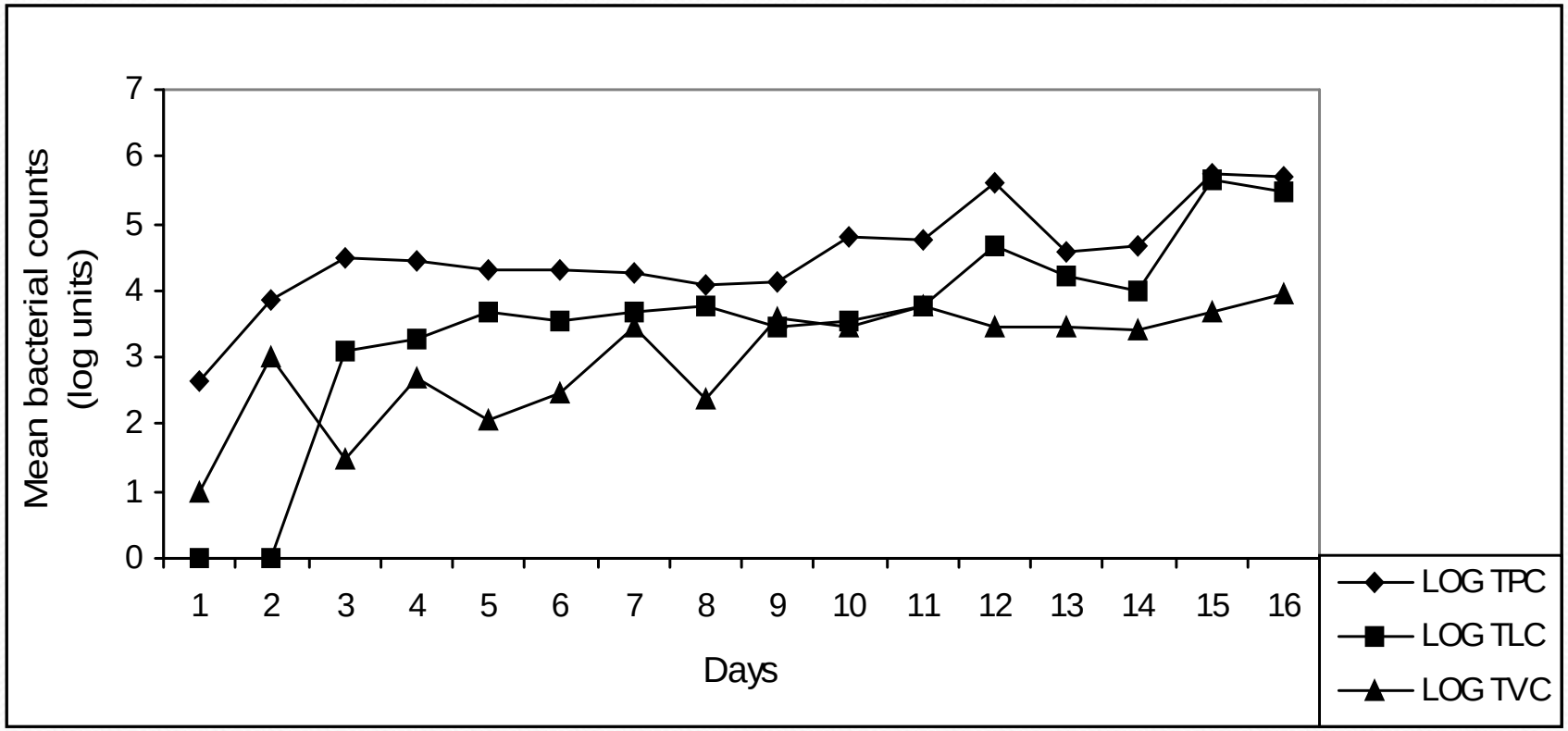
Mean bacterial counts of 3 replicate tanks treated with antibiotic



TPC- total plate count, TLC- Total luminous bacterial count, TVC- total Vibrio count

Vinod *et al*., 2006; Aquaculture

Mean bacterial counts of 3 replicate untreated tanks (control)



TPC- total plate count, TLC- Total luminous bacterial count, TVC- total Vibrio count.

Vinod *et al.*, 2006; Aquaculture

Issues in phage therapy

- **Standardization of the dose of phage to be applied under various environmental variables**

- **Salinity (ppt)** **20, 25 and 30**

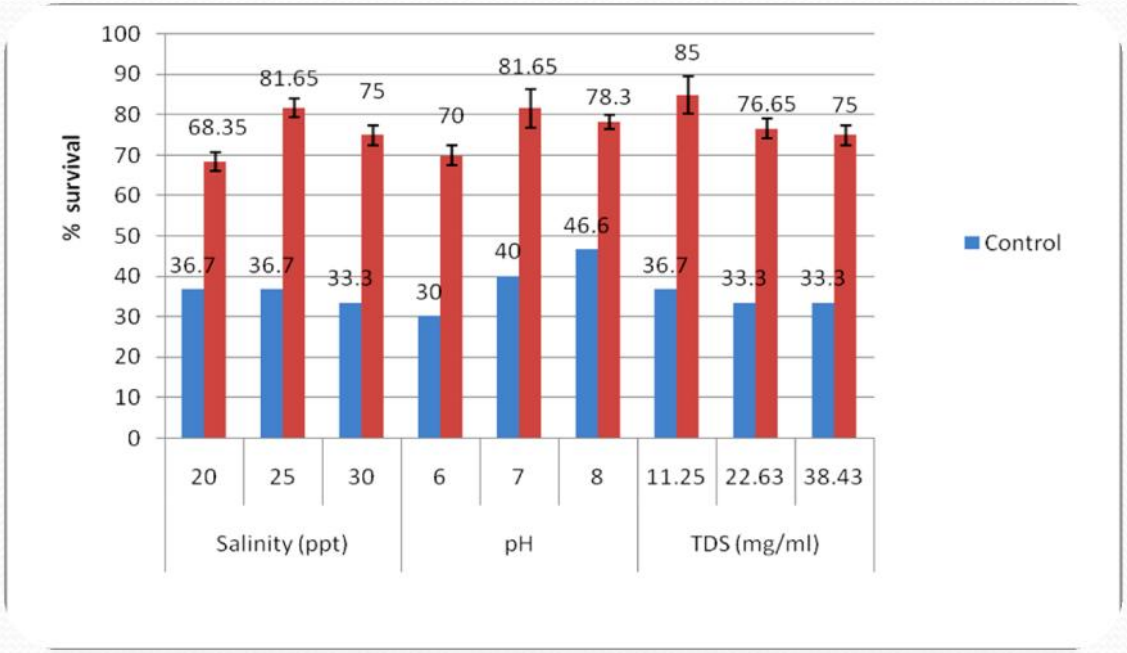
- **Temperature** **20°C, 30°C and 37°C**

- **pH** **6, 7 and 8**

- **Total dissolved solids**

Survival of *P. monodon* larvae both in control and phage treated troughs under different water quality parameters

	Salinity			pH			TDS		
	20	25	30	6	7	8	11.25	2.63	38.43
Control 1	100	100	100	100	100	100	100	100	100
Control 2	36.7	36.7	33.3	30.0	40.0	46.6	36.7	33.3	33.32
Phage treated (Mean±SD)	68.35± 2.333	81.65± 2.333	75± 2.404	70± 4.667	81.65± 2.333	78.3± 2.404	85± 2.404	76.65± 4.738	75± 2.404

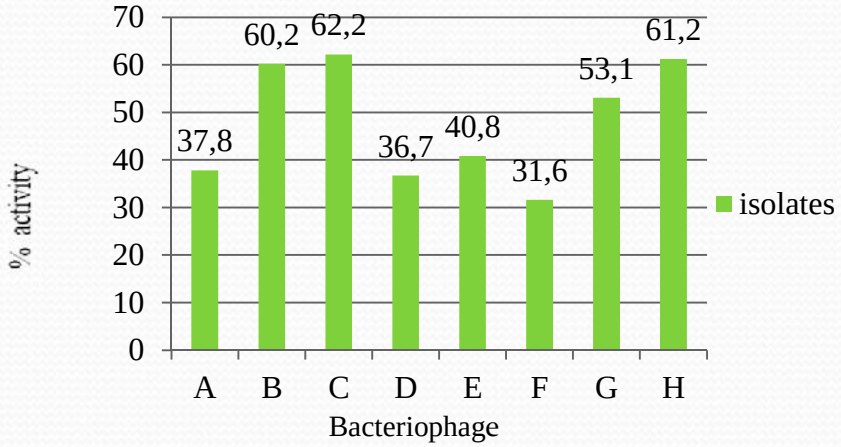


Application of lytic phages for the bio-control of *Vibrio parahaemolyticus*

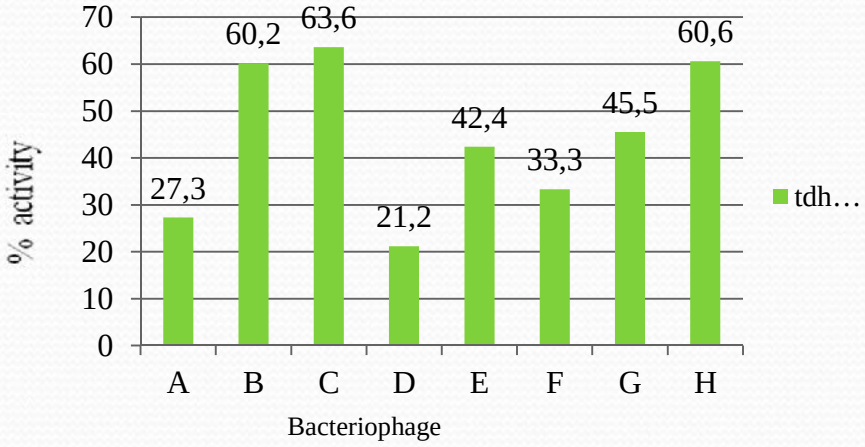
Sampling sites	Number of samples analyzed	Phages isolated
Seafood from harbor	6	2
Seafood from market	10	4
Estuarine water	8	0
Seawater	13	2
Total	37	8

Phage Name	Host range (in percentage)			
	Total Vp (n=98)	*tdh*⁺ Vp (n=33)	*trh*⁺ Vp (n=61)	*tdh* ⁺ & *trh* ⁺ Vp (n=15)
VpPA	37.8	27.3	34.4	33.3
VpPB	60.2	60.2	67.2	86.7
VpPC	62.2	63.6	65.6	73.3
VpPD	36.7	21.2	39.3	26.7
VpPE	40.8	42.4	45.9	53.3
VpPF	31.6	33.3	31.2	46.7
VpPG	53.1	45.5	55.7	60
VpPH	61.2	60.6	68.9	86.7

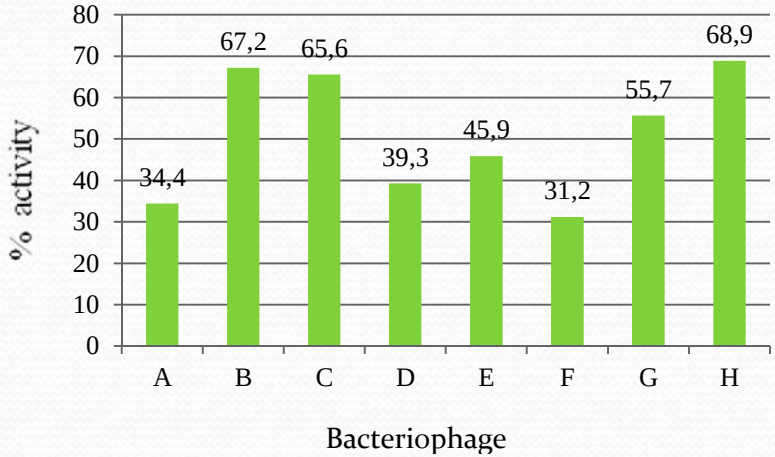
Lytic activity of various phages against *V. parahaemolyticus (tlh for total Vp n=98)*



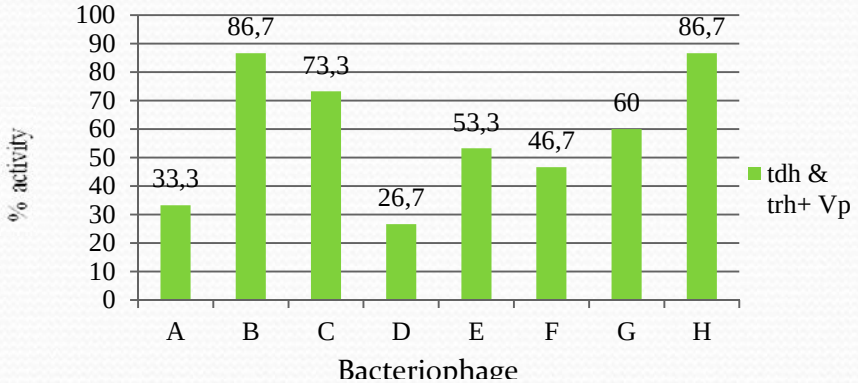
Lytic activity of various phages against *tdh* positive *V. parahaemolyticus (n=33)*



Lytic activity of various *V. parahaemolyticus* phages against *trh* positive *V. parahaemolyticus (n =61)*



Lytic activity of various *V. parahaemolyticus* phages against *tdh, trh* positive *V. parahaemolyticus (n=15)*



Phage therapy in Aquaculture – Lysozyme helps overcome phage resistance

- **Role of lysozyme on phage activity :**
  - **Lysozyme alone**
  - **Phage alone**
  - **Lysozyme and phage together**

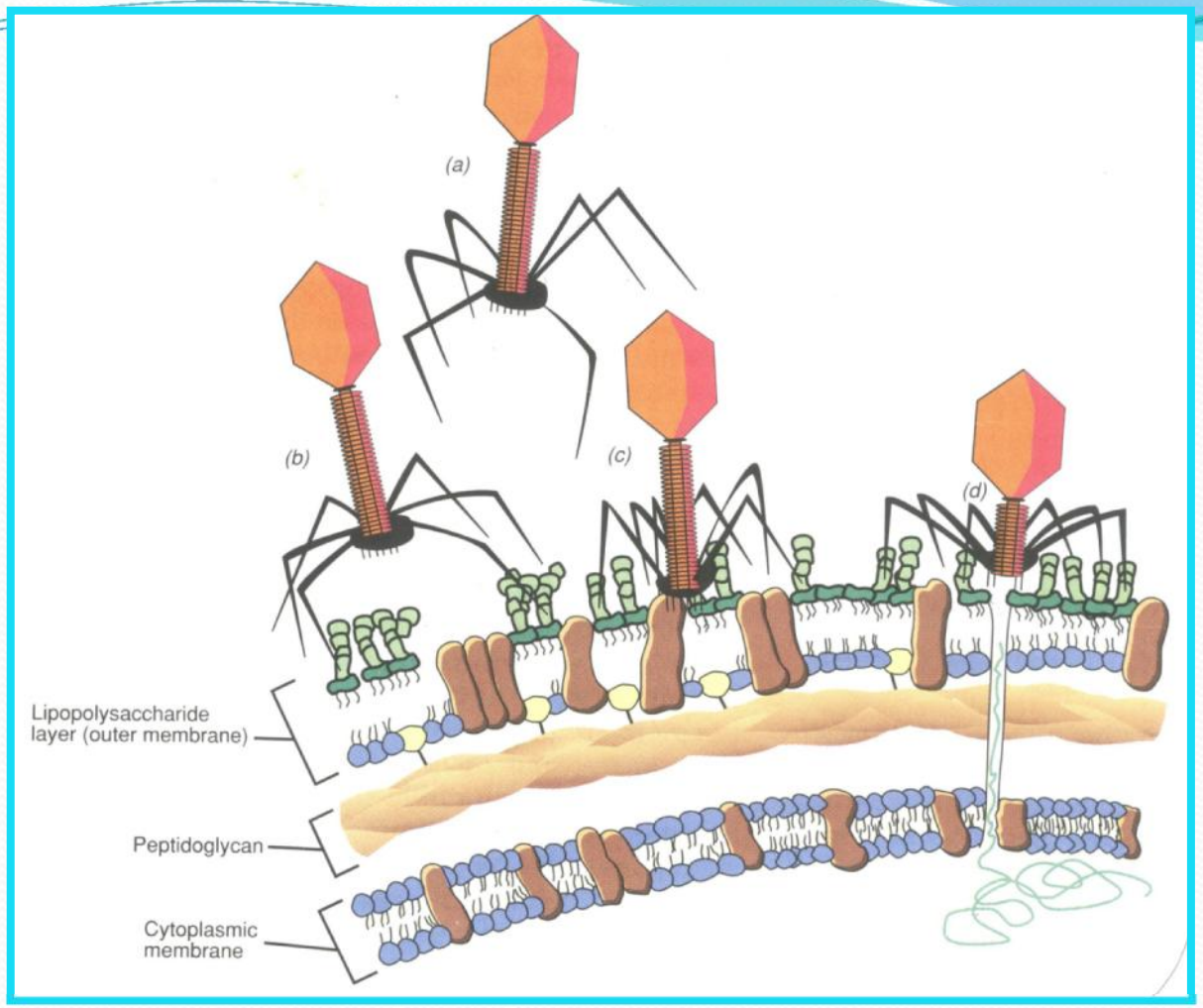


Fig. - Attachment of bacteriophage particle to cell wall of bacteria

Source: Madigan *et al.*, 1997

Madigan *et al.*, 1997

The penetration of phage DNA inside the bacteria is promoted by lysozyme produced by the phage

Tyagi *et al.*, 2007

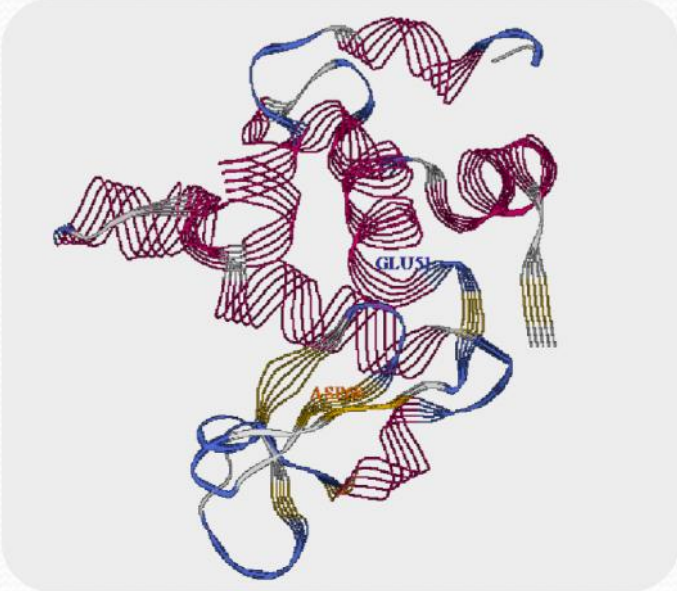
Recombinant lysozyme expressed from black tiger shrimp
Reduced *V. harveyi* in sea water by 3 log units
in 1 hour

We surmised that phage penetration might increase in the presence of our recombinant shrimp lysozyme.

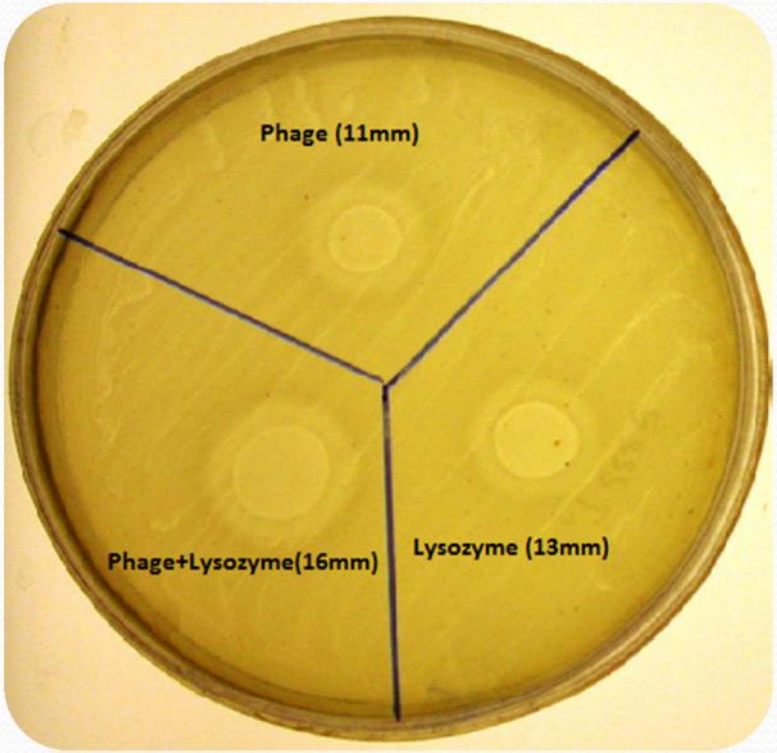
Expression of the recombinant shrimp lysozyme

Tyagi *et al.*, 2007

- **Recombinant *E. coli* grown in 200 ml of LB broth until the OD_{600} was 0.5-0.7**
- **1mM concentrations of IPTG added and incubated for 4 hr at 37˚C with constant agitation at 150 rpm**
- **Cells harvested by centrifugation at 11,000 × g for 5 min**
- **Polyacrylamide gel for electrophoresis performed**



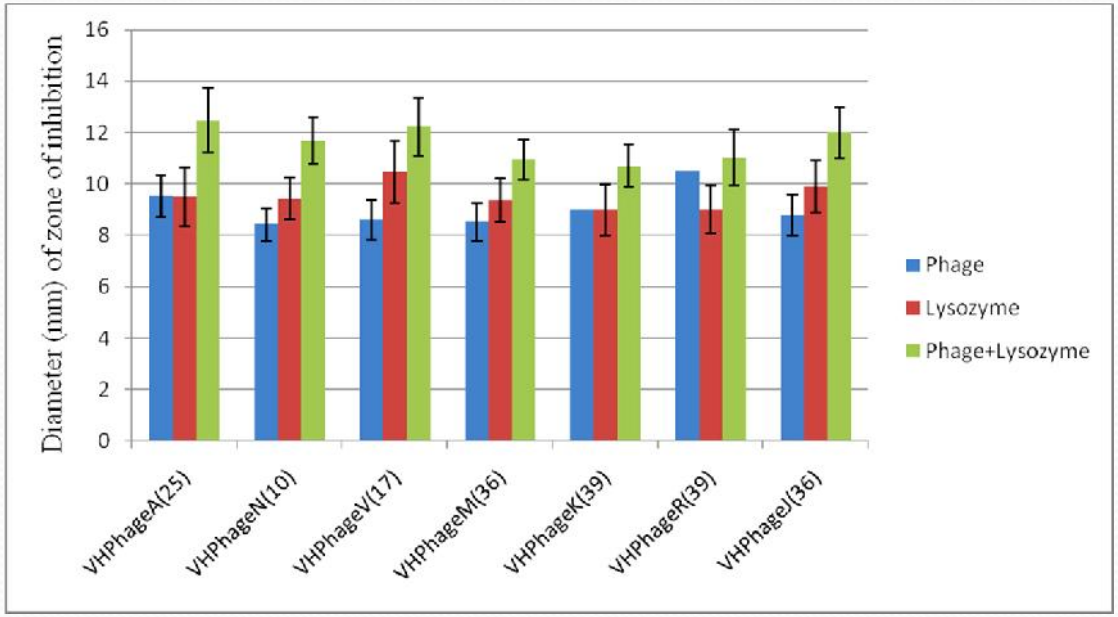
3 D structure of shrimp lysozyme



Zone of inhibition on Solid phase assay by phage alone, lysozyme alone and phage + lysozyme together.

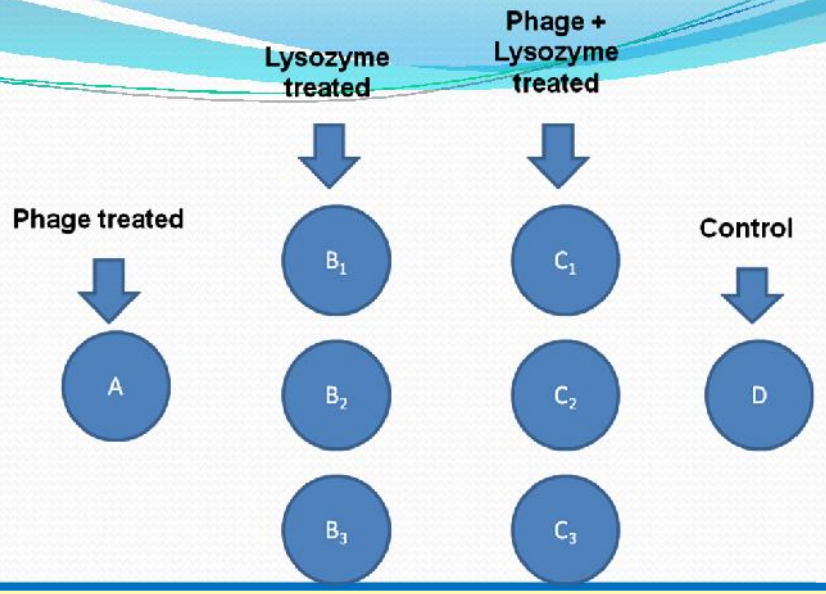
Zone of inhibition (Mean ±SD of diameter) produced on *V. harveyi* lawn of various isolates (n=87) by phage alone , lysozyme alone and phage+lysozyme together

	Phage (Mean±SD)	Lysozyme (Mean±SD)	Phage+Lysozyme (Mean ±SD)
VHPhageA(25)	9.53±0.81	9.5±1.14	12.48±1.25
VHPhageN(10)	8.42±0.65	9.43±0.83	11.68±0.9
VHPhageV(17)	8.61±0.77	10.46±1.23	12.23±1.12
VHPhageM(36)	8.52±0.73	9.37±0.84	10.96±0.77
VHPhageK(39)	9±0	9±0.99	10.71±0.81
VHPhageR(39)	10.5±0	9.01±0.93	11.02±1.09
VHPhageJ(36)	8.81±0.8	9.91±1.02	12±1.01



Experimental setup

Activity in seawater



Eight flasks –Each with 100ml sterilized sea water taken containing

***V. harveyi* (final count of 1.19 × 10^6 /ml)**

+

- **A: Phage - 15µl of bacteriophage (1.41×10^9 pfu/ml)**
- **B: Lysozyme treated - 25µl(B_1), 50µl (B_2)and 100µl (B_3) of TSL (1.6mg/ml conc.)**
- **C: Phage + Lysozyme treated with 25µl(C_1), 50µl (C_2) and 100µl (C_3) of TSL (1.6mg/ml conc.)**
- **D: *V. harveyi* control**

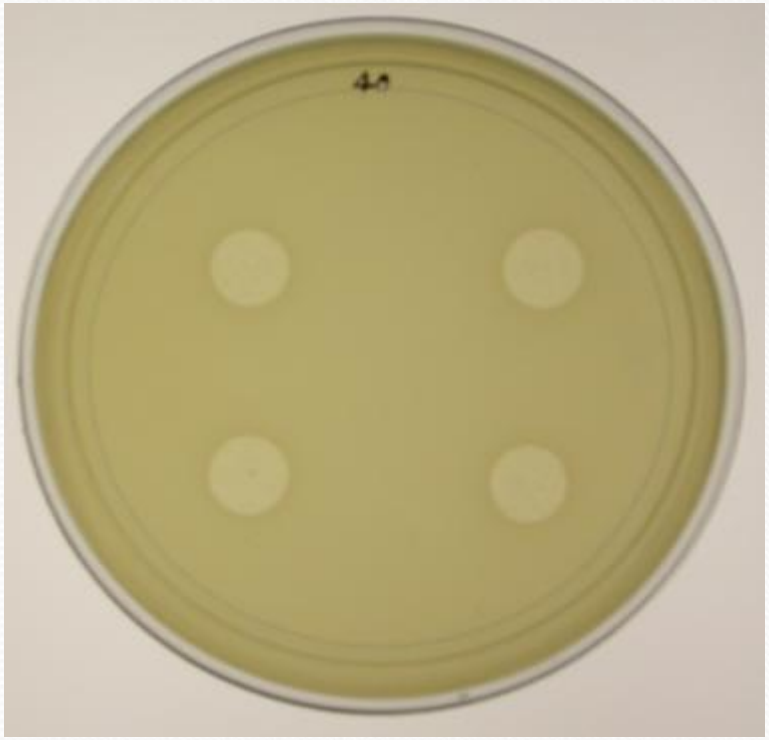
0 h, 1 h, 2 h, 4 h and 24 h intervals : total plate count (TPC) determined

Phage isolates with respective host bacteria and source

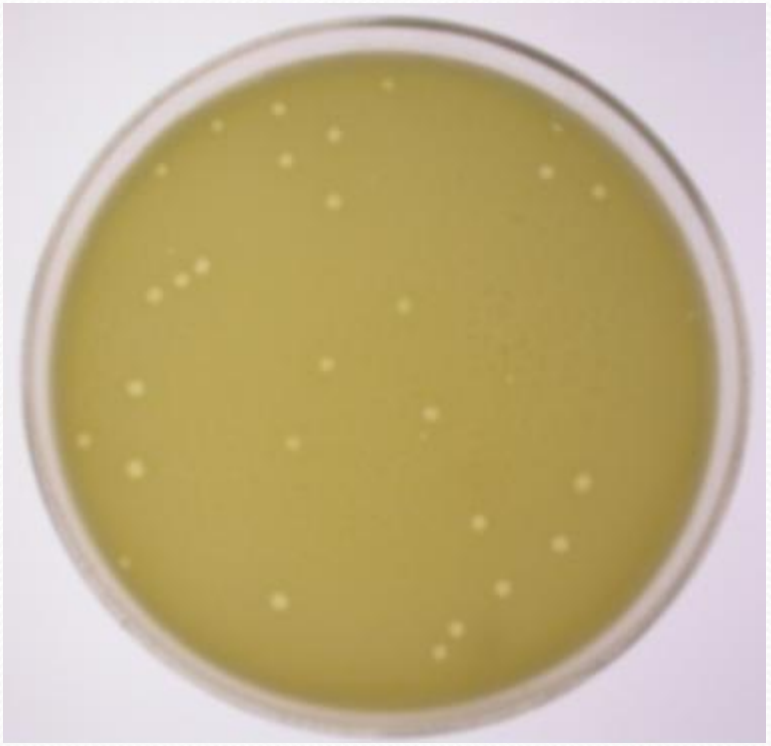
Phages	Host bacteria[a]	Source
Vf	*V. fischeri*	Shrimp farm water
Va	*V. alginolyticus*	Shrimp hatchery water
Vh	*V. harveyi*	Shrimp hatchery water
Vp	*V. parahaemolyticus*	Oysters
Vv	*V. vulnificus*	Oysters

[a]Bacterial isolates from our own culture collection

Zones of clearing due to phage isolate from *V. parahaemolyticus*



Plaques formed by *V. parahaemolyticus* phage on soft agar



Does chitin have some influence on phage adsorption and in turn reducing the bacterial load? Experimental study

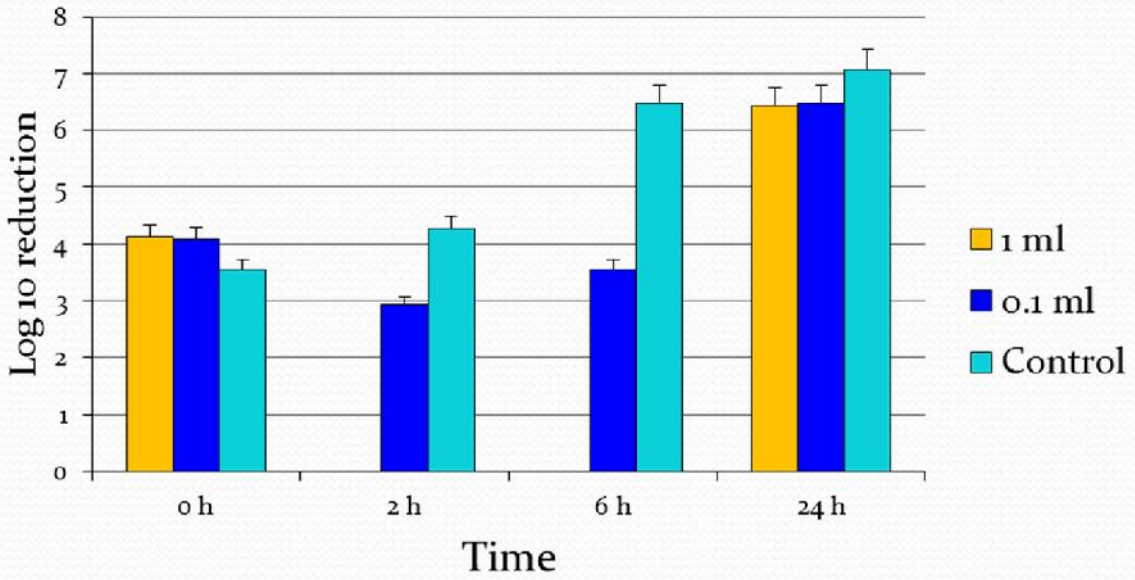
Vf phage- chitin had no influence

Vh phage - 2 log reduction

Va phage – Count nil at 2h and 6h after phage treatment indicating that chitin has some influence on phage activity by providing actively growing bacteria.

At 24 h *V. alginolyticus* count increased : perhaps due to the phages being adsorbed on the surface of lysed bacterial cells

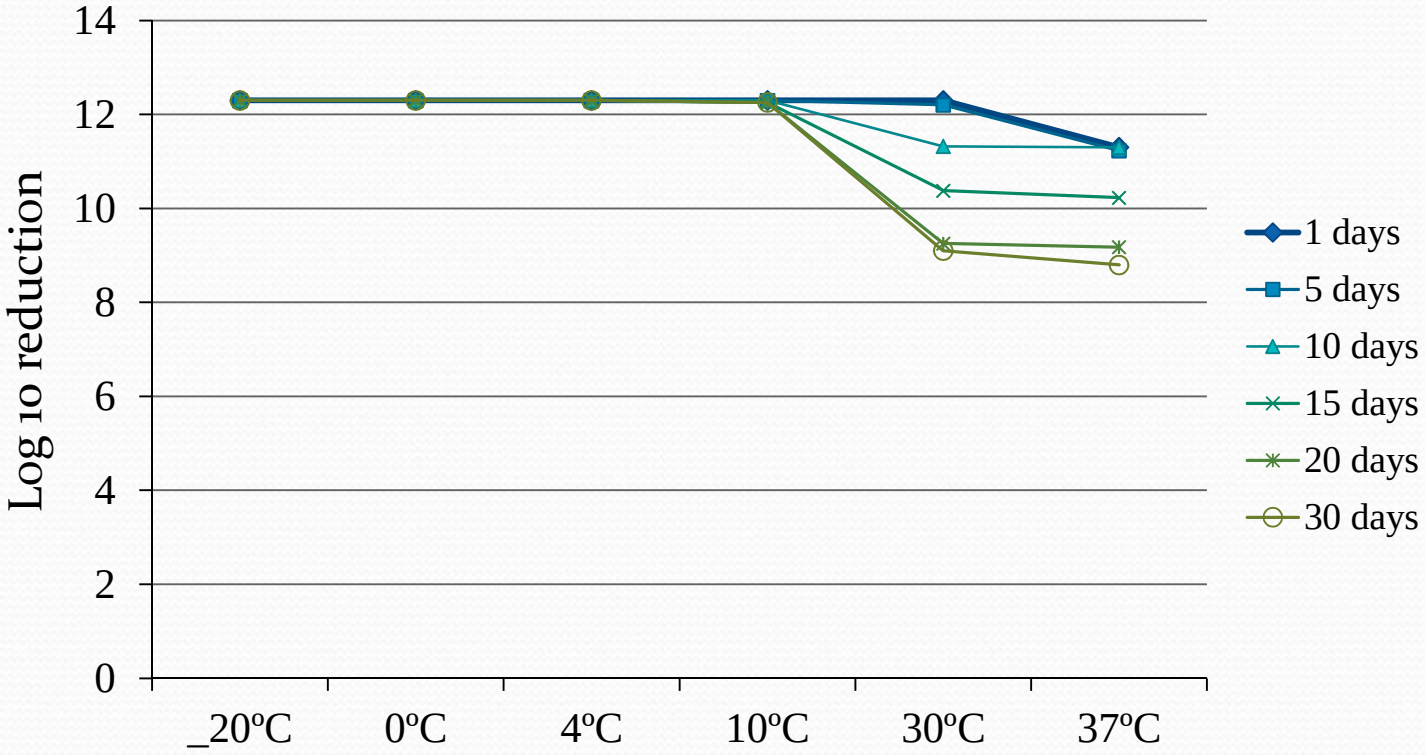
V. alginolyticus phage with addition of 0.1g chitin



Tub	Phage dose	Hours after phage treatment cfu/ml on TCBS			
		0h	2h	6h	24h
A	1 ml	1.36×10^4	-	-	2.7×10^6
B	0.1 ml	1.21×10^4	8.5×10^2	3.5×10^3	3.0×10^6
C	Control	3.45×10^3	1.87×10^4	3.0×10^6	1.17×10^7

- Addition of chitin brought about higher reduction in vibrio counts by phage
- Activity was dose dependent

Titre values of phage at different storage temperatures



No change in titer of phage at low storage temperatures
Only at 30 and 37^{0}C , reduction in titer observed.

Results demonstrate promise for transport and field application

APPLICATION

- **As prophylactic to prevent build up of vibrio pathogens in hatcheries.**
- **To treat luminous bacterial disease in hatcheries and ponds.**
- **To treat broodstock, eggs, nauplii by dipping in phage**
- **To tackle biofilm formation by vibrios**

DO AQUACULTURE ENVIRONMENTS FAVOUR LYSOGENY?

Generally higher percentage of lysogens are found in isolates from oligotrophic environments (Jiang and Paul, 1998)

Lysogeny may not be favoured in environments of aquaculture systems

Phage therapy with bacteriophages lacking putative virulence genes would be safe

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The Microbes of Mangalore

Thank you