



Screening and Developing of Probiotic Bacteria from the Environment of Culture Shrimps

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Outline of my talk

- **Bacteria and shrimp culture**
- Isolation and identification of bacteria stains
- Screening of Probiotic Bacteria for penaeid shrimp
- Developing of Probiotic agent for shrimp culture



bacteria and shrimp culture

- Symbiotic bacteria of shrimp
(intestinal tract, gills, or skin)
- Ambient bacteria in water
- Soil bacteria in sediment



Symbiotic bacteria of shrimp

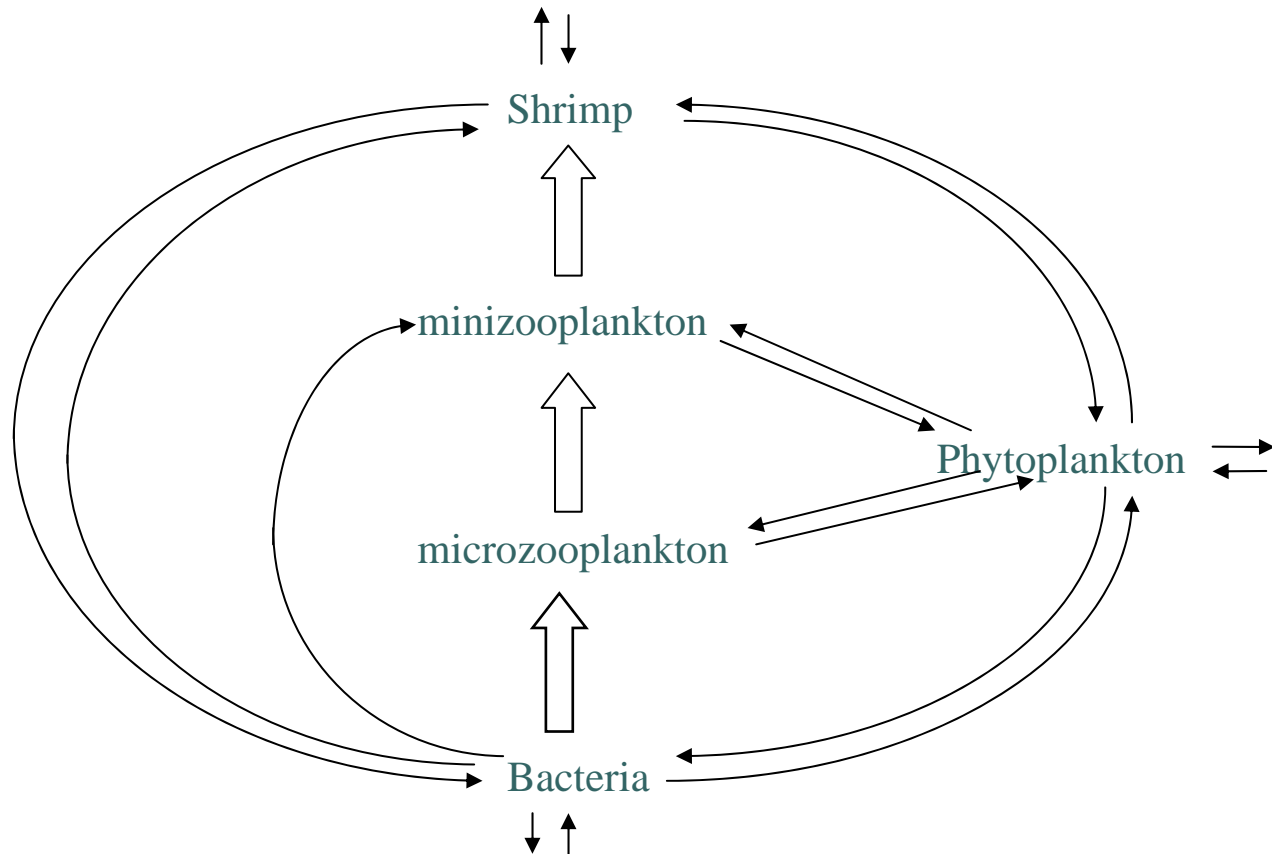
- The total number of culturable bacteria is about 10^5 - 10^{12} cfu/g
- It can benefit the shrimp's healthy by production Inhibitory compounds, competition for chemicals or available energy, by competition for adhesion sites, and by enhancement of the immune response.
- Therefore it not only can aid in feed's digestion, but can prevent pathogens from proliferating in the intestinal tract.

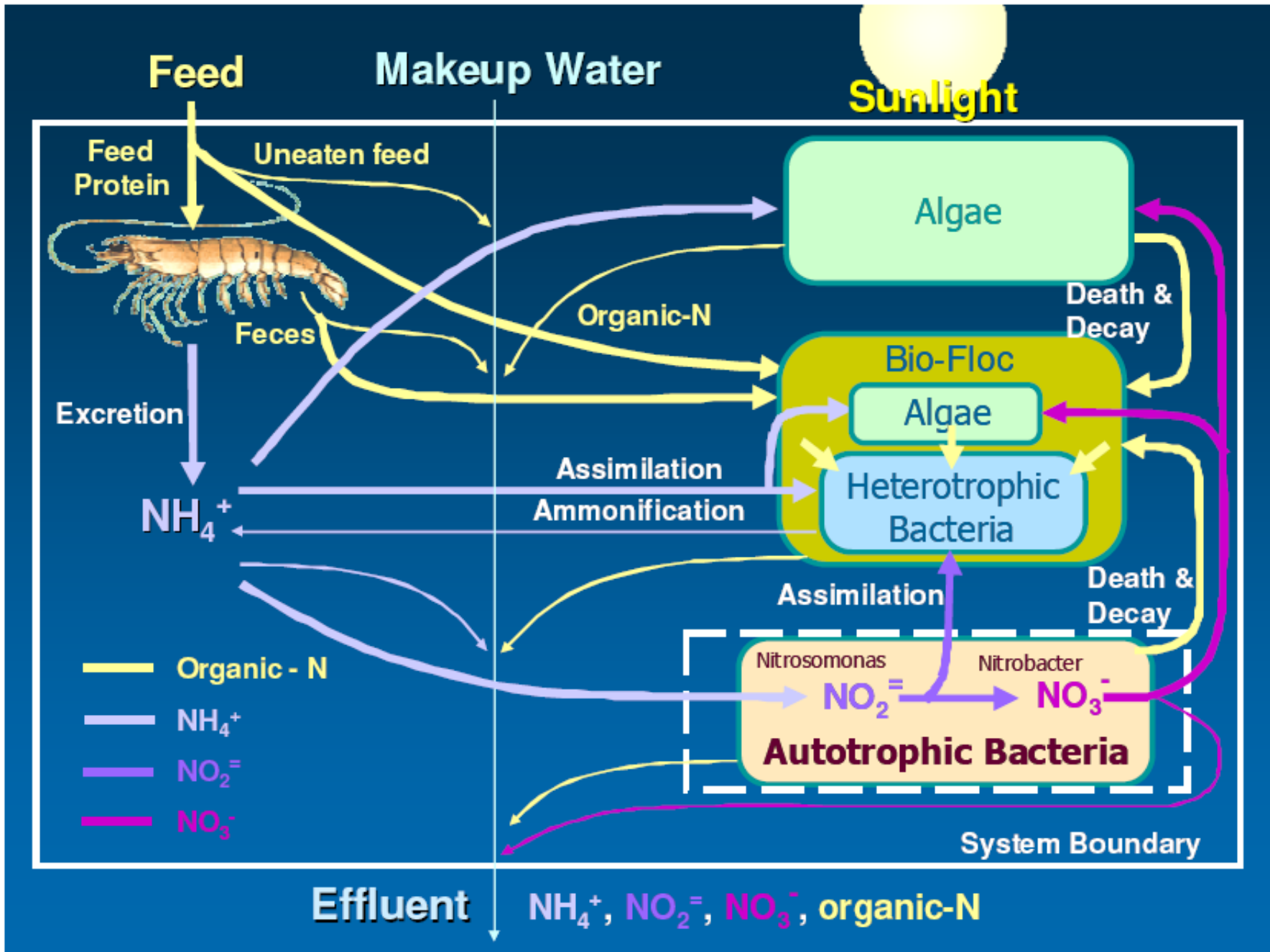


Ambient bacteria in water

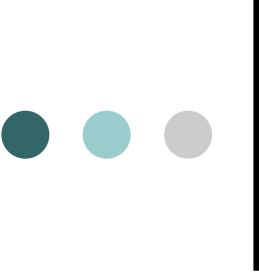
- The total number of culturable bacteria is about 10^6 - 10^8 cfu/mL
- the bacterial production fluctuated from 0.5-50 gC/L.d
- carbon/nitrogen balance and nitrogen removal processes

main food chain & micro food cycle in shrimp pond





By Yoram Avnimelech, 1999

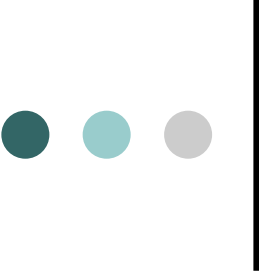


Bio-folc technology is a viable option for intensive or even extensive aquaculture production . It helps to maintain good water quality without the need for special water quality control compartment. In addition, it saves on feed inputs and expenditure and reduces environmental pollution.



Soil bacteria in sediment

- The total number of **culturable bacteria is about 10^5 - 10^7 cfu/g**
- Among of them, autotrophic bacteria is more than 99%, and heterotrophic bacteria is less than 1%

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- bacteria would be found to be useful both as food and as biological control agents of shrimp disease and activators of the rate of nutrient regeneration in aquaculture.
 - *Vibrio alginolyticus* has been employed as a probiotic in many Ecuadoran shrimp hatcheries since late 1992. As a result, hatchery down time was reduced from approximately 7 days per month to less than 21 days annually, while production volumes increased by 35%.



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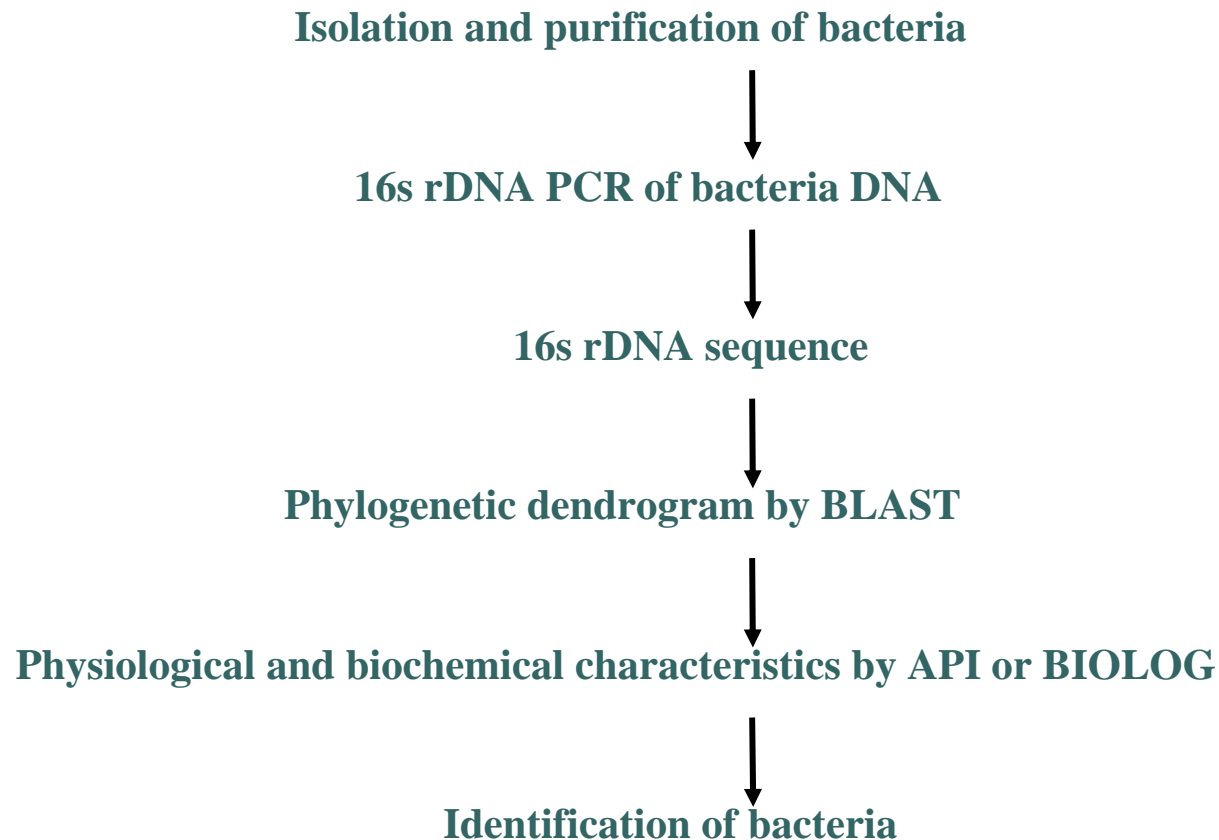


Putative probiotics isolated from shrimp or from its ambient environment

<i>Strain number</i>	<i>amount</i>	<i>time</i>	<i>source</i>
PC060701-50	50	July, 2006	<i>Penaeus chinensis</i> , intestine; Qingdao
LV060801-15	15	Aug., 2006	<i>Litopenaeus vannamei</i> , intestine; Qingdao
PC070451-67	17	Apr., 2007	<i>Penaeus chinensis</i> , intestine; Weifang
PC070668-76	9	June, 2007	<i>Penaeus chinensis</i> , intestine; Qingdao
PC080601-07	7	June, 2008	<i>Penaeus chinensis</i> , hatching water; Bingzhou
PC080708-21	14	July, 2008	<i>Penaeus chinensis</i> , culture water; Dongyin,

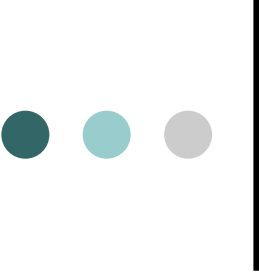


The basic prototype of Identification





PC070461的API 20E反应结果

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- There are 28 stains had been classified and identified by 16S rDNA sequence, API 20E and BIOLOG.
 - Among of them, 24 stains were identified to species but 4 were identified to genus only.
 - And 25 stains are gram positive stains, 3 stains are gram negative stains.

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- **This 28 stains are belong to 10 genus as below:**

Vibrio sp., V. crassostreae, V. harveyi, V. alginolyticus, V. campbellii

Bacillus firmus, B. thuringiensis

Ochrobactrum sp., O. anthropi,

Exiguobacterium aestuarii

Sphingomonas sp.

Pseudoalteromonas elyakovii

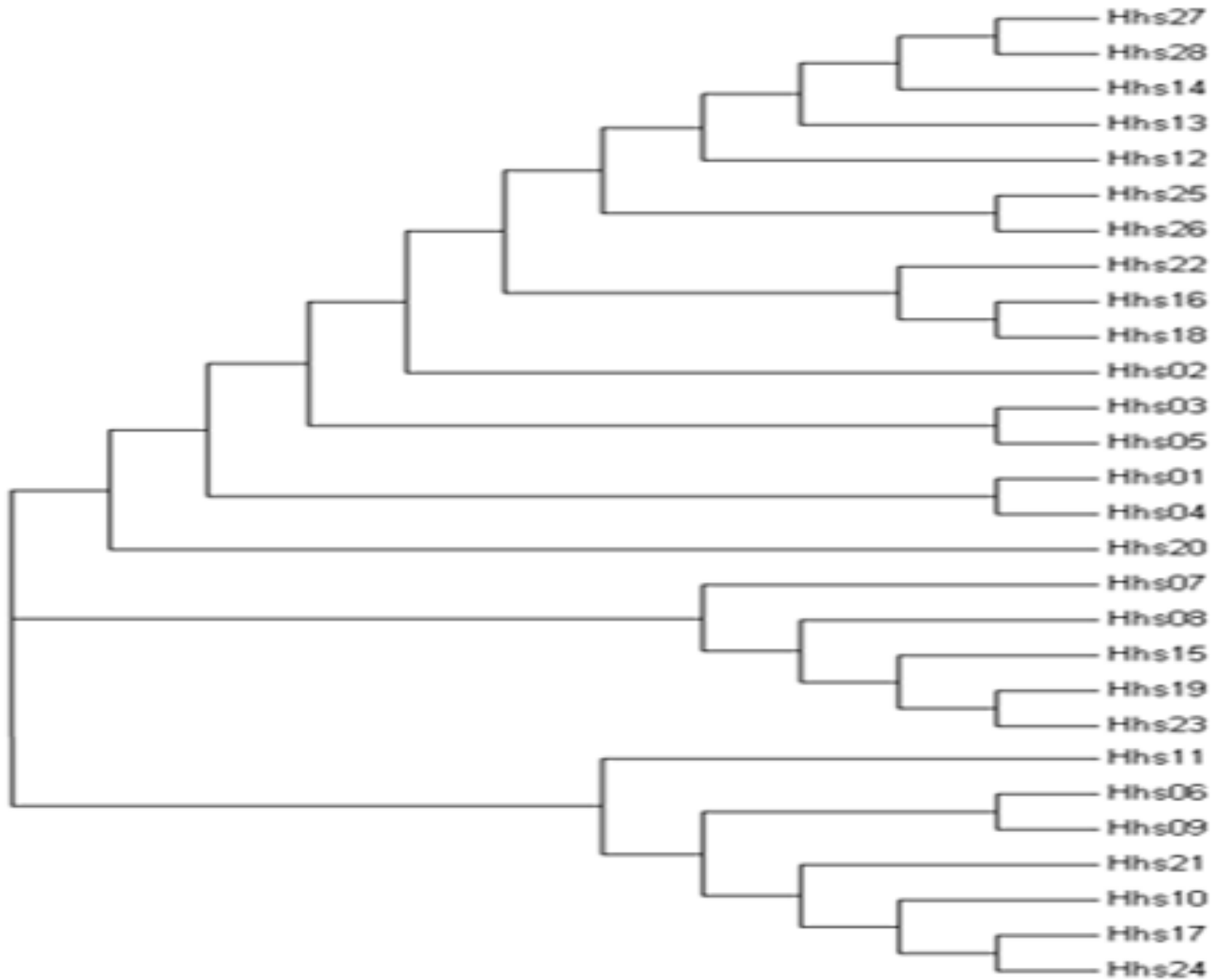
Tenacibaculum discolor

Photobacterium damsela

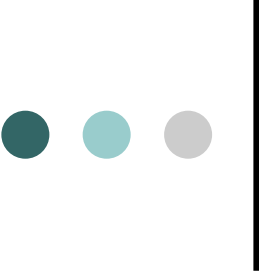
Ensifer adhaerens

Stenotrophomonas maltophilia

I



Phylogenetic tree based on 16S rDNA sequences of 28 stains

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- The optimum culture medium of 6 stains (LV060810, LV060806, PC070477, PC070568, PC070573, PC070460) were detected at last, by Single-factor test and uniform design.



Optimum medium composition of the intestinal symbiotic bacteria

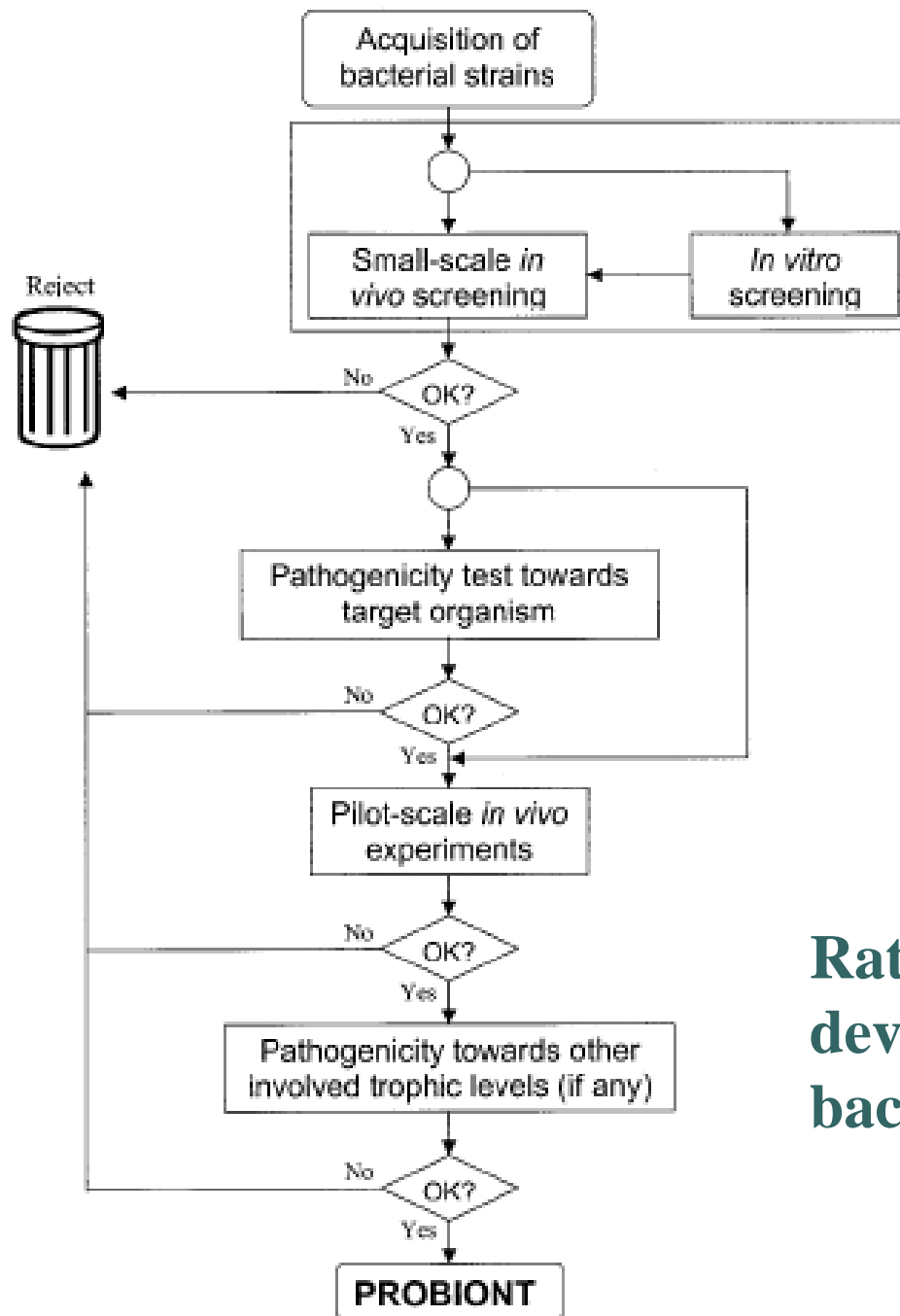
(%, except pH)

	LV060810	LV060806	PC070477	PC070568	PC070573	PC070460
Sucrose	1.0	1.2	0.8	1.3		
Glucose					1.2	0.8
Tryptone	0.8	0.6	0.4	1.0	1.0	1.0
Beef Extract	0.8	0.8	0.4	0.5	0.3	0.2
Yeast Extract	0.2	0.2	0.6	0.2	0.5	0.5
$C_6H_5Na_3O_7 \cdot 2H_2O$	0.08	0.08	0.10	0.10	0.08	0.09
K_2HPO_4	0.06	0.02	0.06	0.10	0.02	0.06
KH_2PO_4		0.04		0.02	0.01	
$MgSO_4$	0.1	0.06	0.04	0.06	0.04	0.10
pH	7.2	7.8	7.2	6.0	7.2	7.2
inoculum size	7	5	3	5	9	7



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Rationale for screening and developing of probiotic bacteria

From Laurent Erschuere, 2000



Screening and Preselection of Putative Probiotics

- In vitro antagonism tests
- Evaluation of pathogenicity of selected Strains
- Small-scale tests in order to evaluate the effect of potential probiotic on shrimp



In vitro antagonism tests

- There are four stains which were isolated from the intestine of *Litopenaeus vannamei* showing antagonism against three pathogenic indicator vibrios, *Vibrio anguillarum*, *V. harveyi*, and *V. parahaemolyticus*.
- Their inhibitory activities were tested by solid diffusion assay and liquid blend assay.



The inhibitory of strains to indicating bacteria

Indicator strains	Inhibitory/Diameter of inhibitory zone (mm)			
	LV060806	LV060808	LV060810	LV060815
<i>Vibrio anguillarum</i>	mantle/9	inhibit/6	mantle/8	-
<i>Vibrio harveyi</i>	inhibit/2	-	inhibit/3	inhibit/3
<i>Vibrio parahaemolyticus</i>	-	-	inhibit/3	inhibit/5
<i>Staphylococcus aureus</i>	inhibit/5	inhibit/6	inhibit/2	-
<i>Micrococcus cohn</i>	mantle/7	mantle/5	-	-



Evaluation of Pathogenicity of Selected Strains (I)

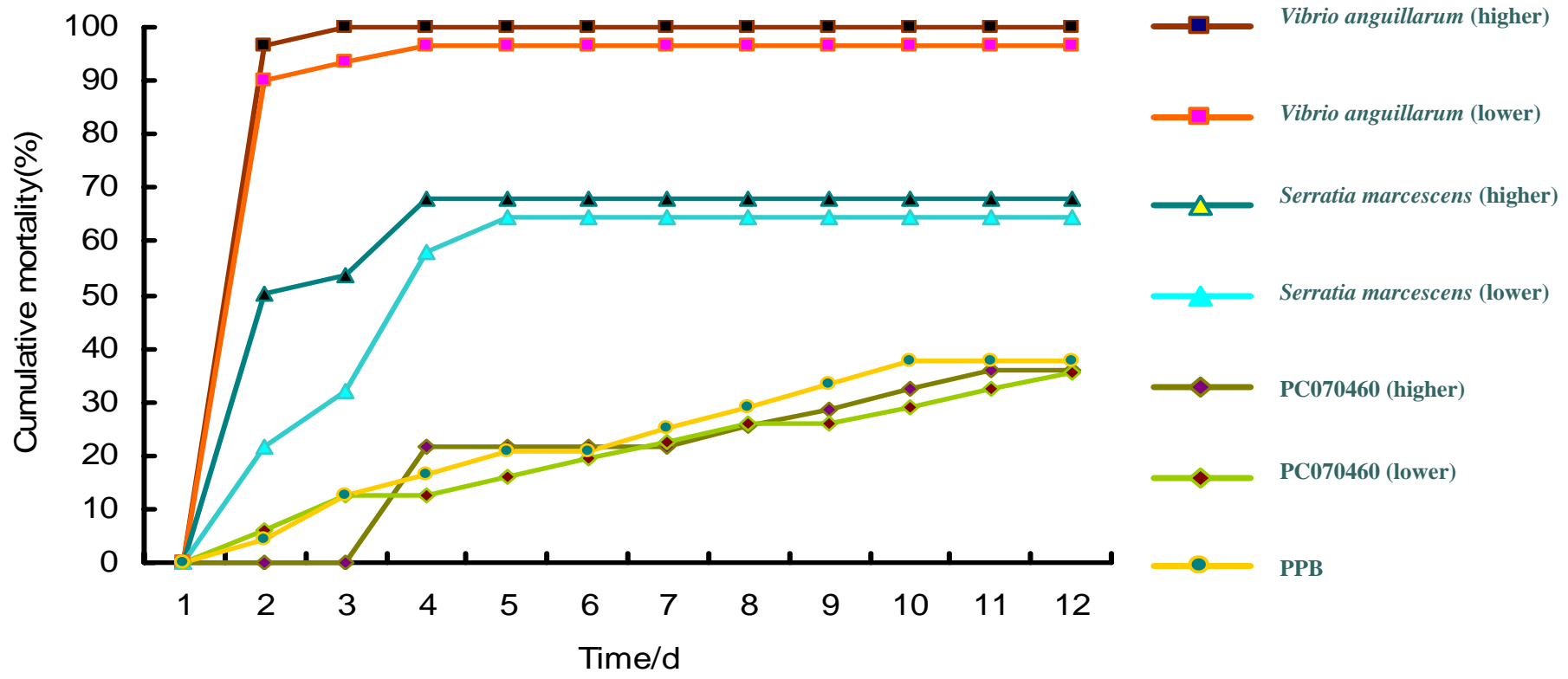
○ Shrimp: *Penaeus chinensis*

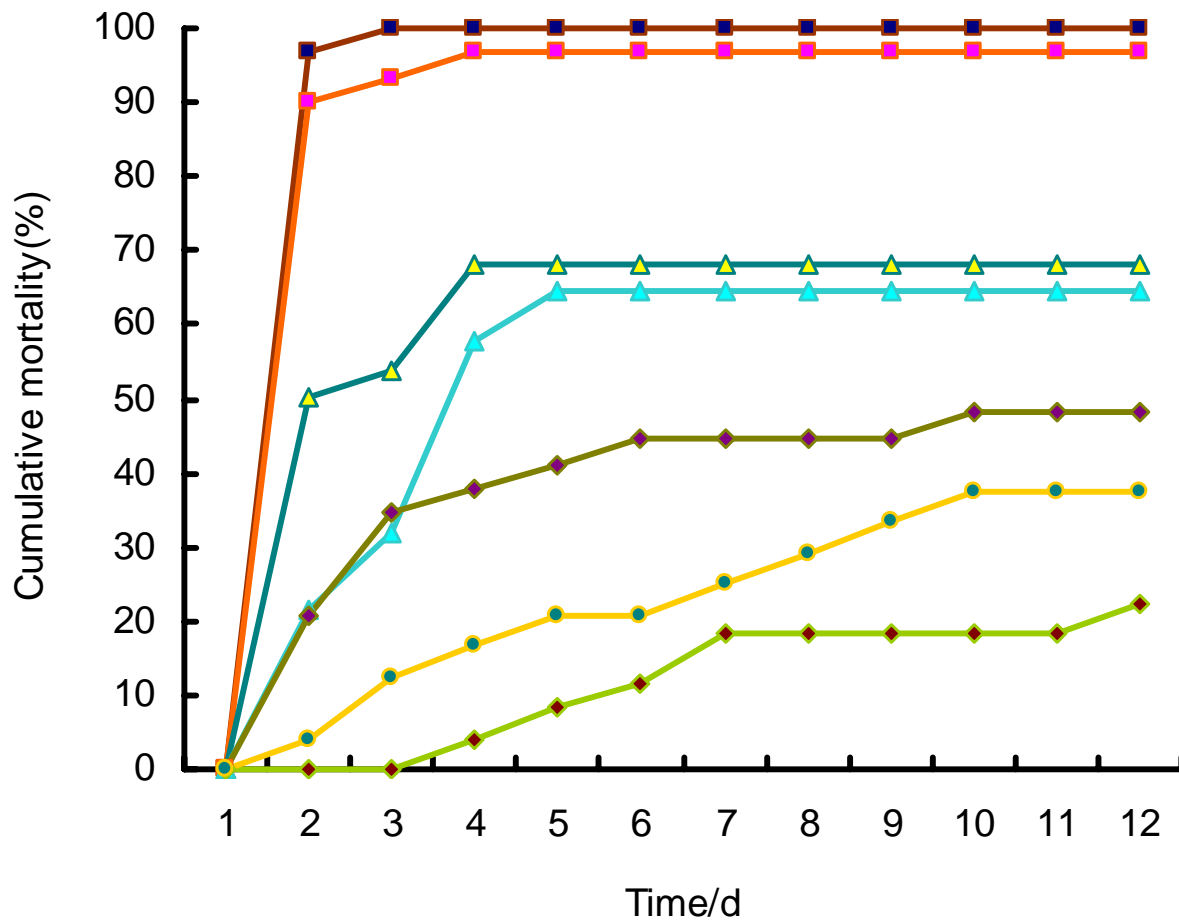
Selected strains: PC070460、PC070461、
PC070462、PC070463、PC070368、
PC070370、PC070373、PC070374、
PC070375、PC070376

Pathogenic bacteria: *Vibrio anguillarum*
(virulent stains)

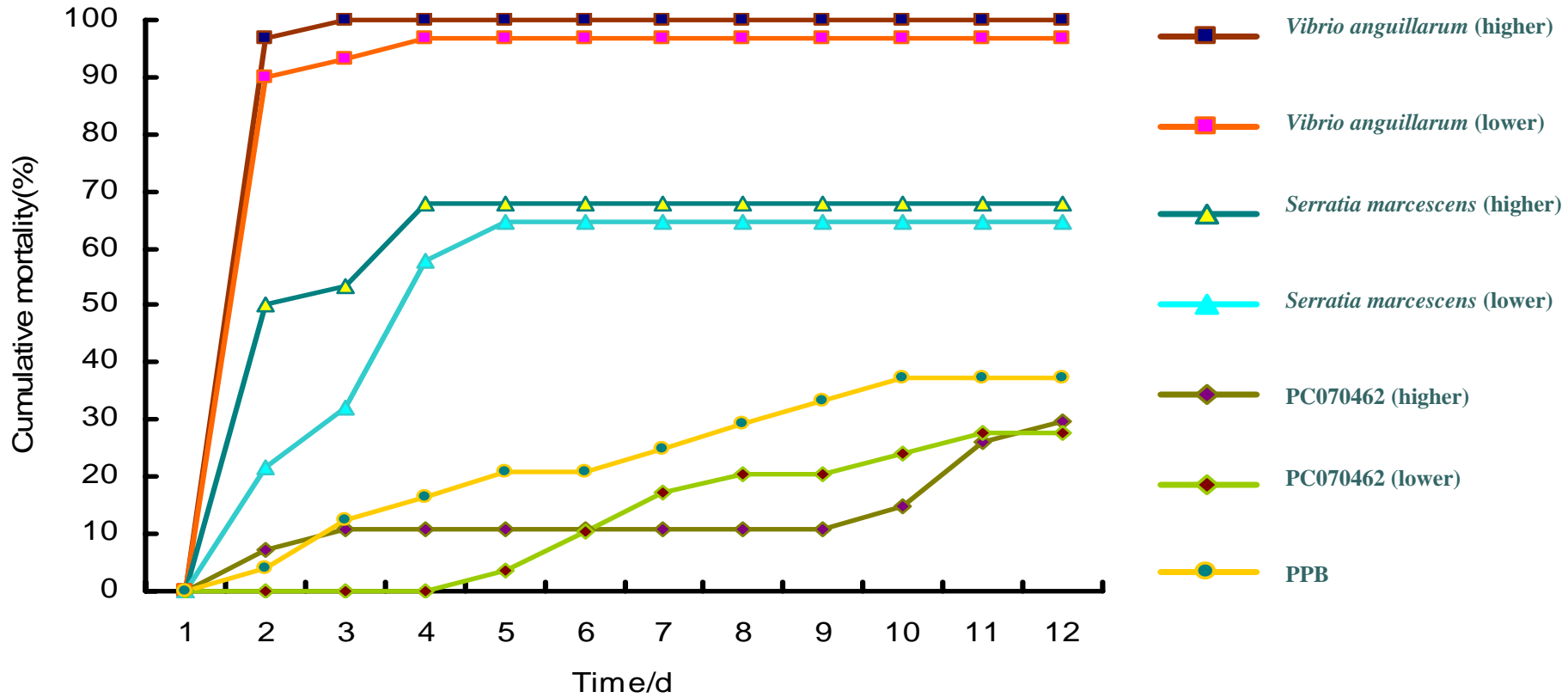
Serratia marcescens
(attenuate stains)

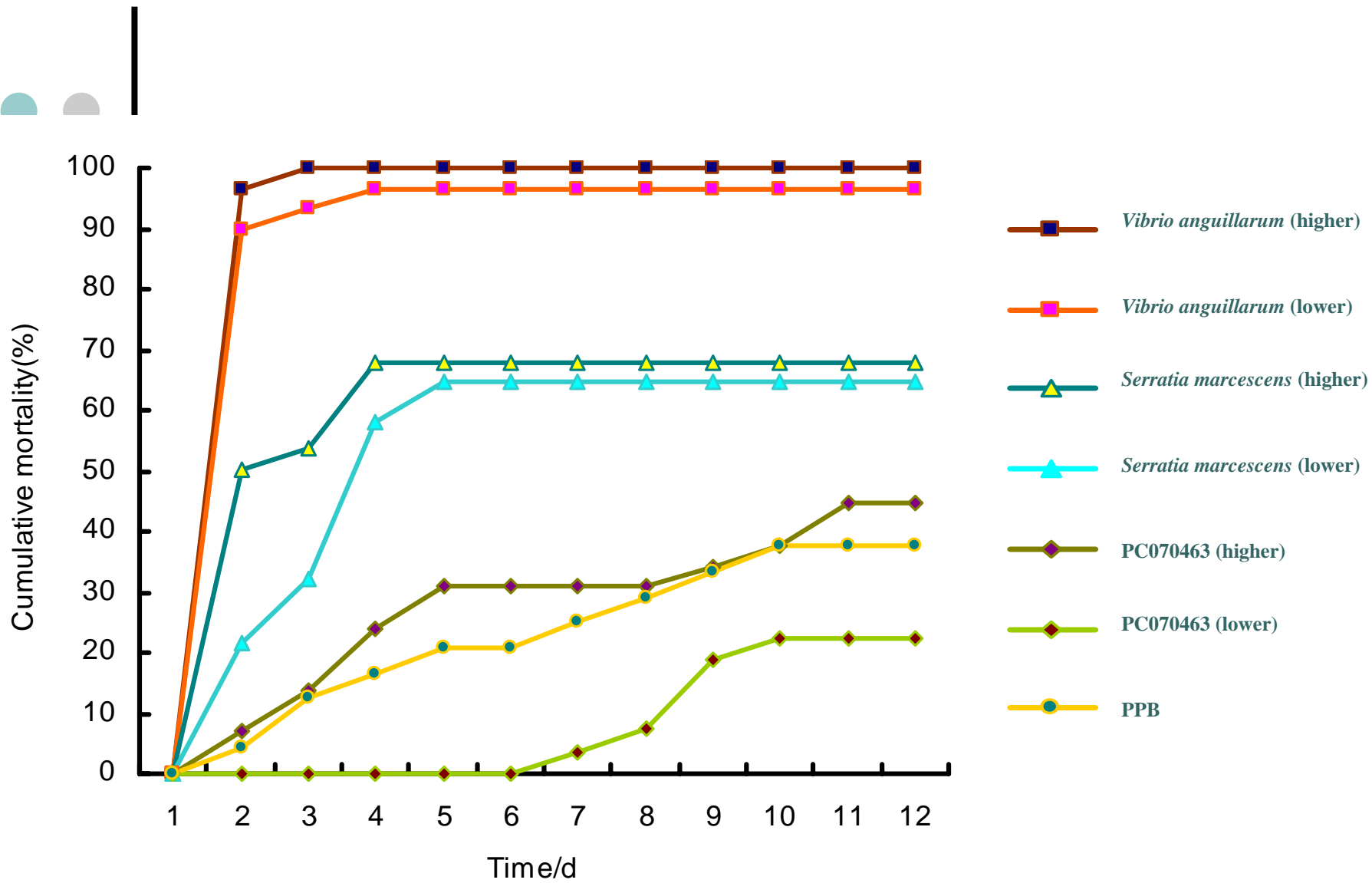
Challenge dose: 10^6 cells/ml (low dosage)
 10^7 cells/ml (high dosage)
inject, 50 μ l/shrimp

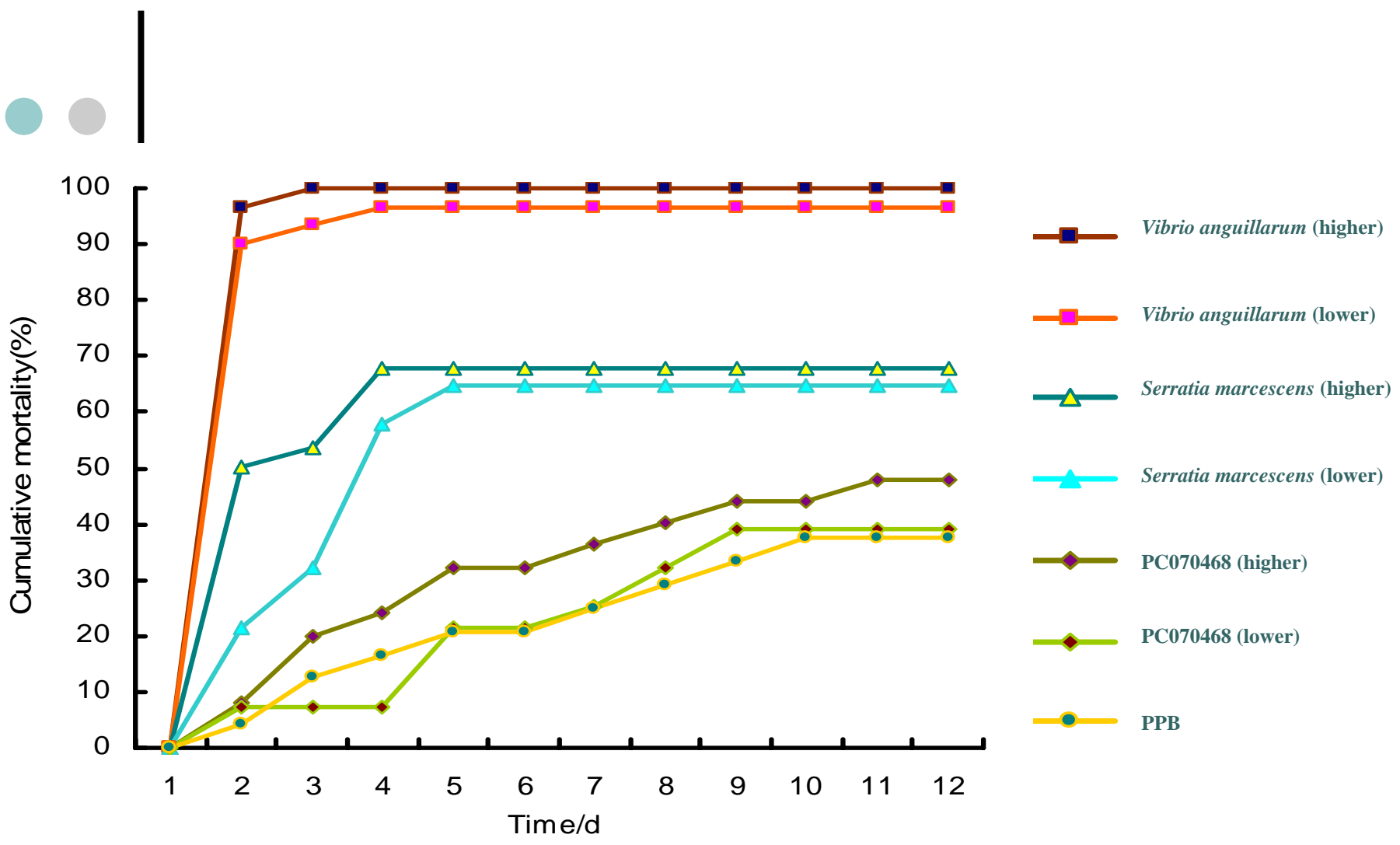


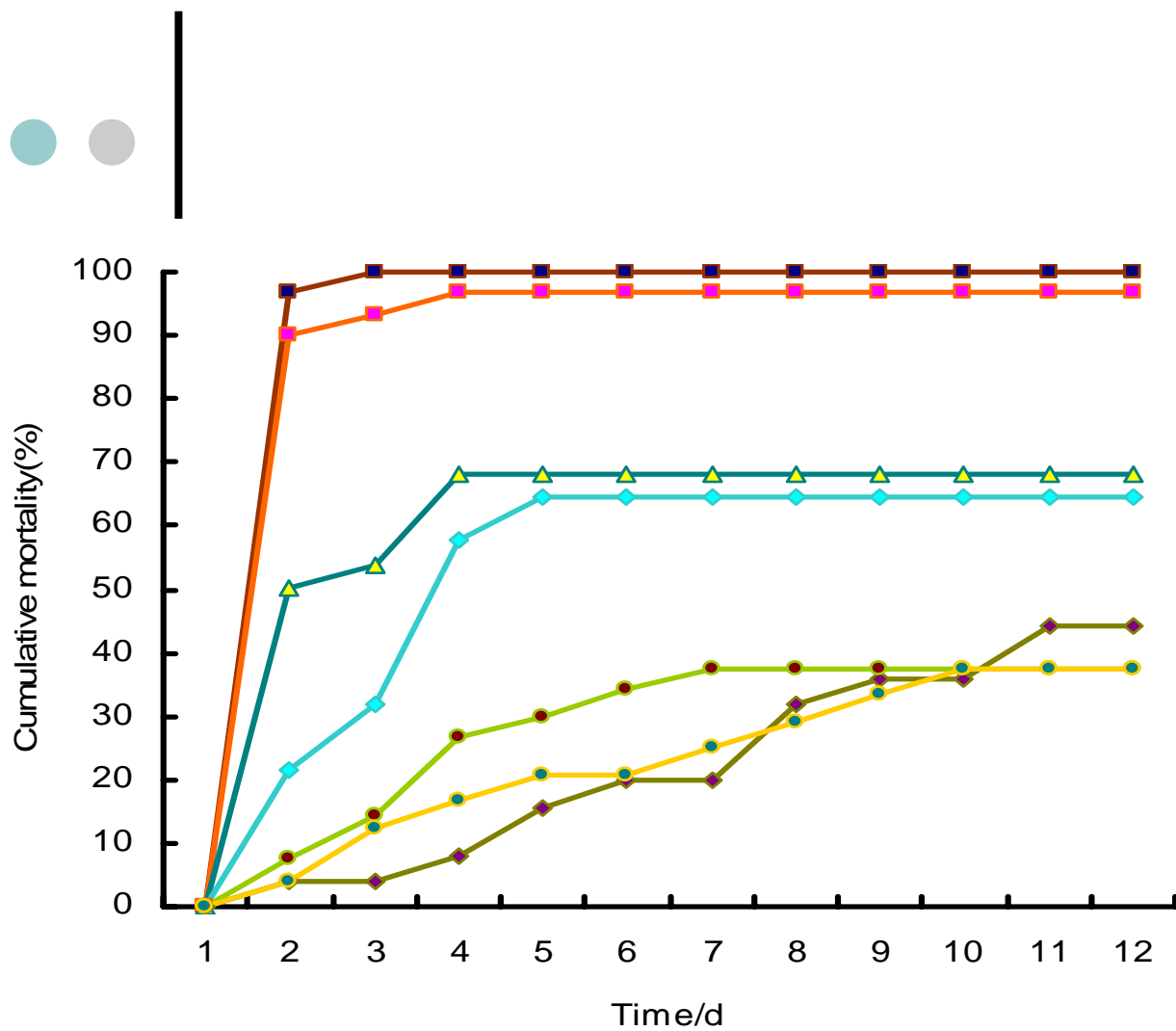


- Vibrio anguillarum* (higher)
- Vibrio anguillarum* (lower)
- Serratia marcescens* (higher)
- Serratia marcescens* (lower)
- PC070461 (higher)
- PC070461 (lower)
- PPB

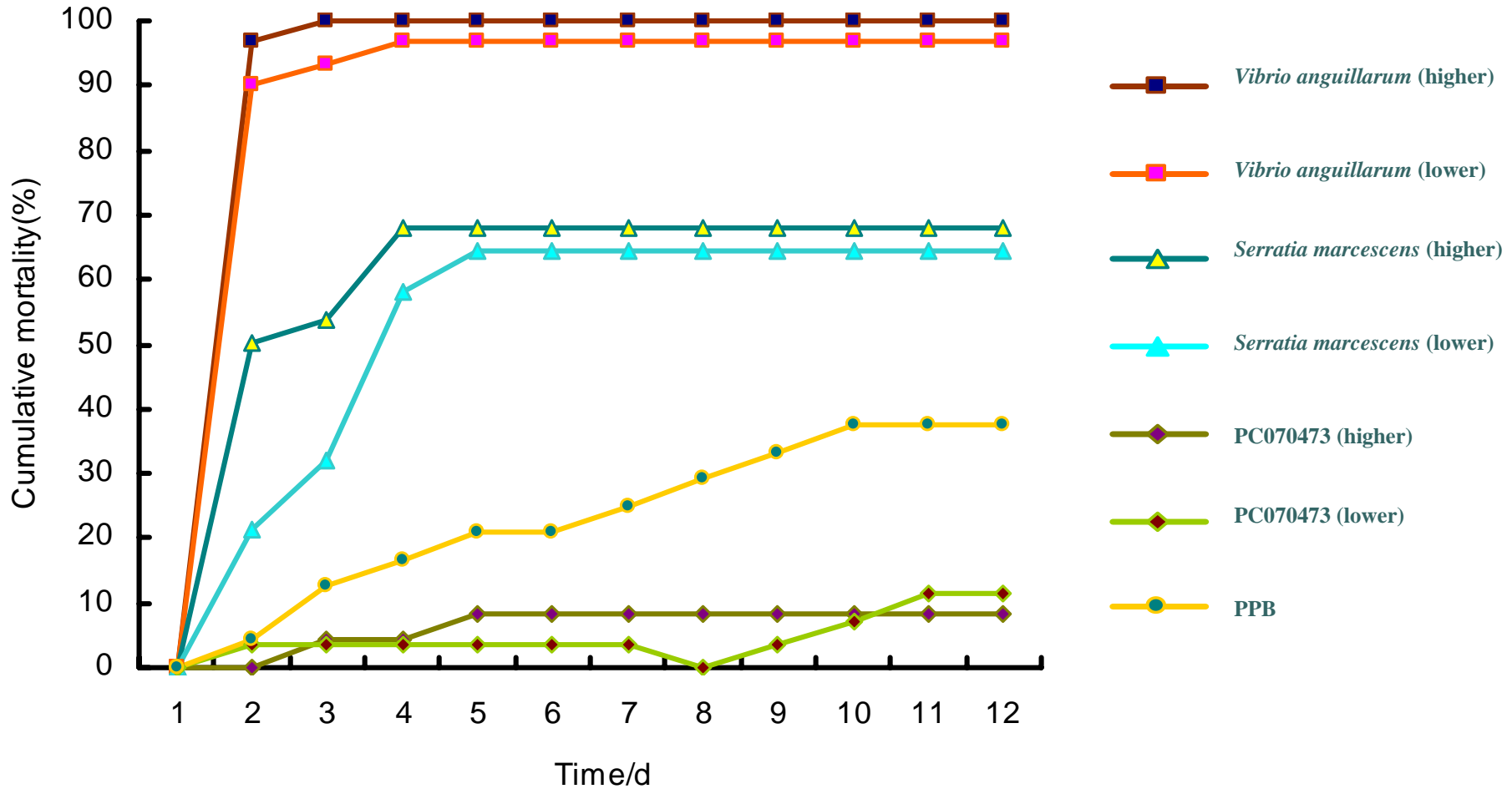


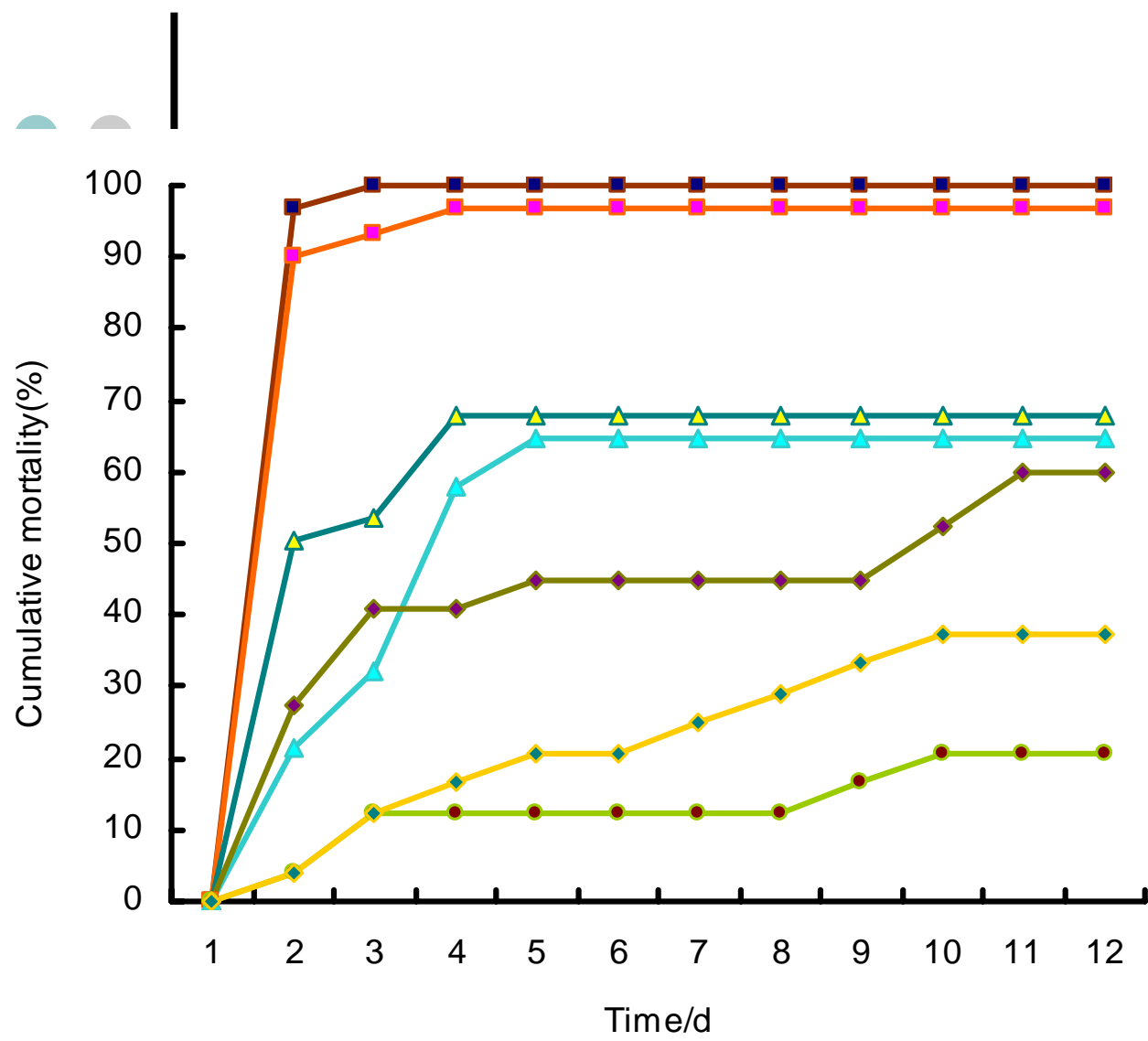




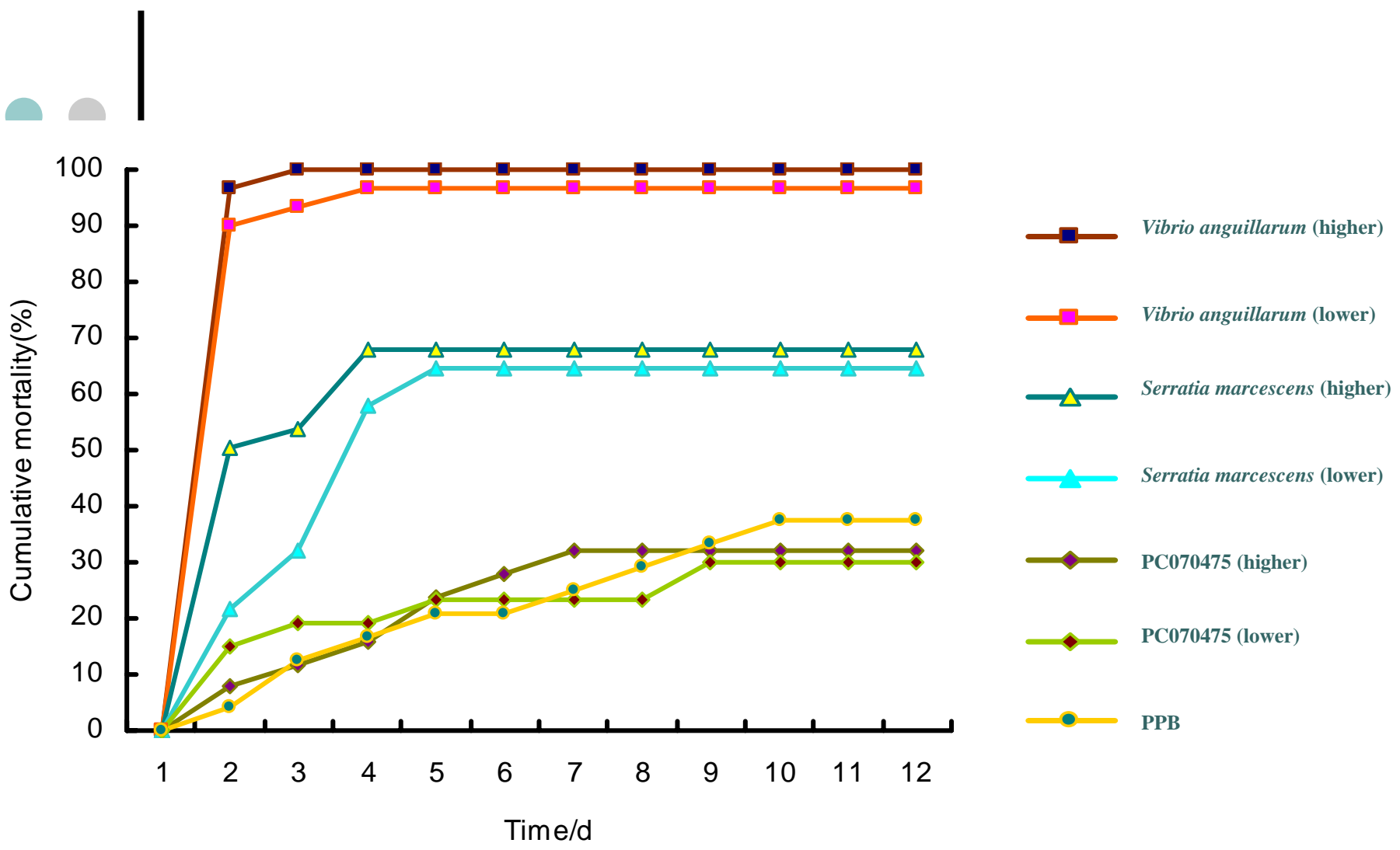


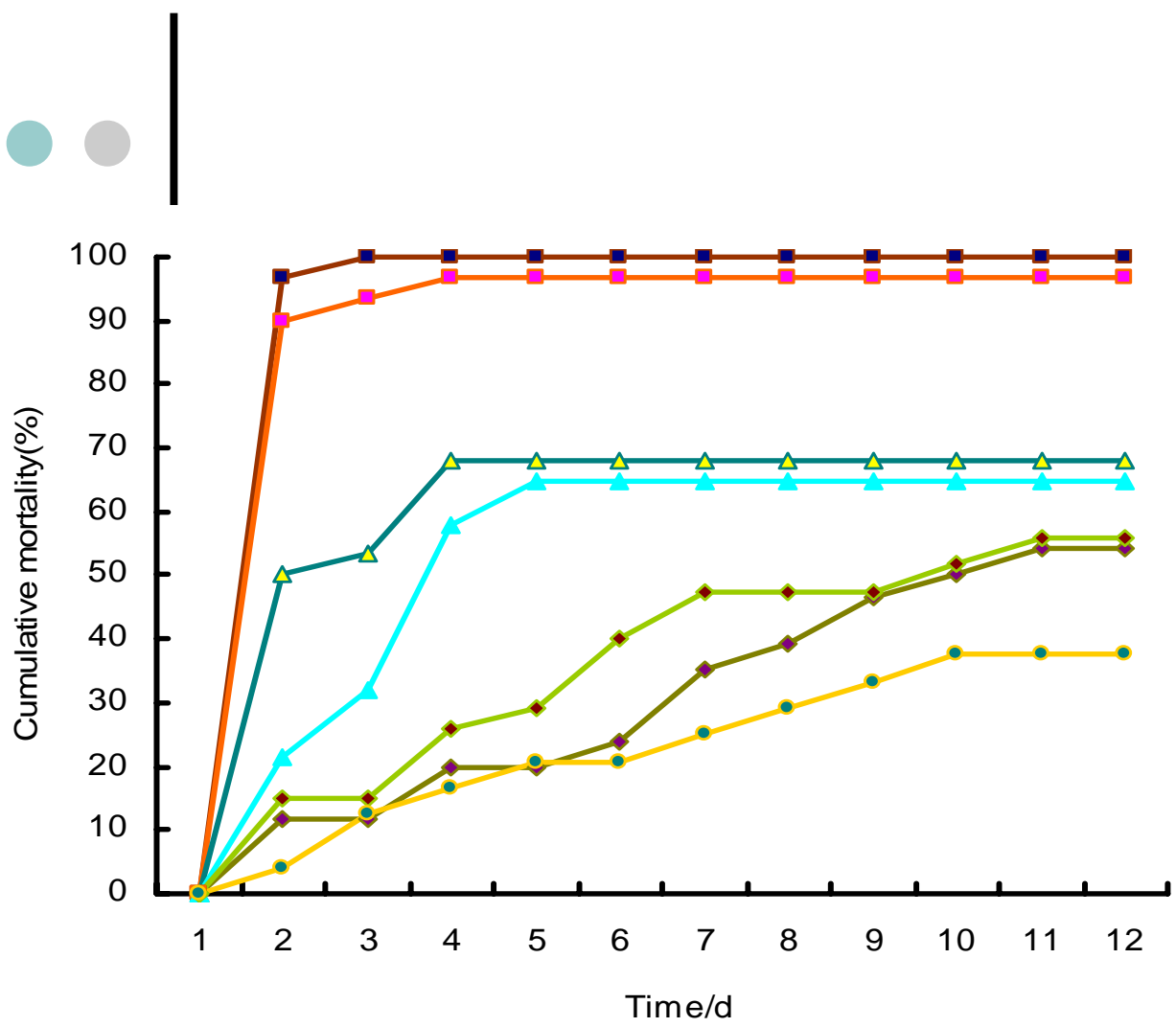
- Vibrio anguillarum* (higher)
- Vibrio anguillarum* (lower)
- Serratia marcescens* (higher)
- Serratia marcescens* (lower)
- PC070470 (higher)
- PC070470 (lower)
- PPB





- Vibrio anguillarum* (higher)
- Vibrio anguillarum* (lower)
- Serratia marcescens* (higher)
- Serratia marcescens* (lower)
- PC070474 (higher)
- PC070474 (lower)
- PPB





- Vibrio anguillarum* (higher)
- Vibrio anguillarum* (lower)
- Serratia marcescens* (higher)
- Serratia marcescens* (lower)
- PC070476 (higher)
- PC070476 (lower)
- PPB



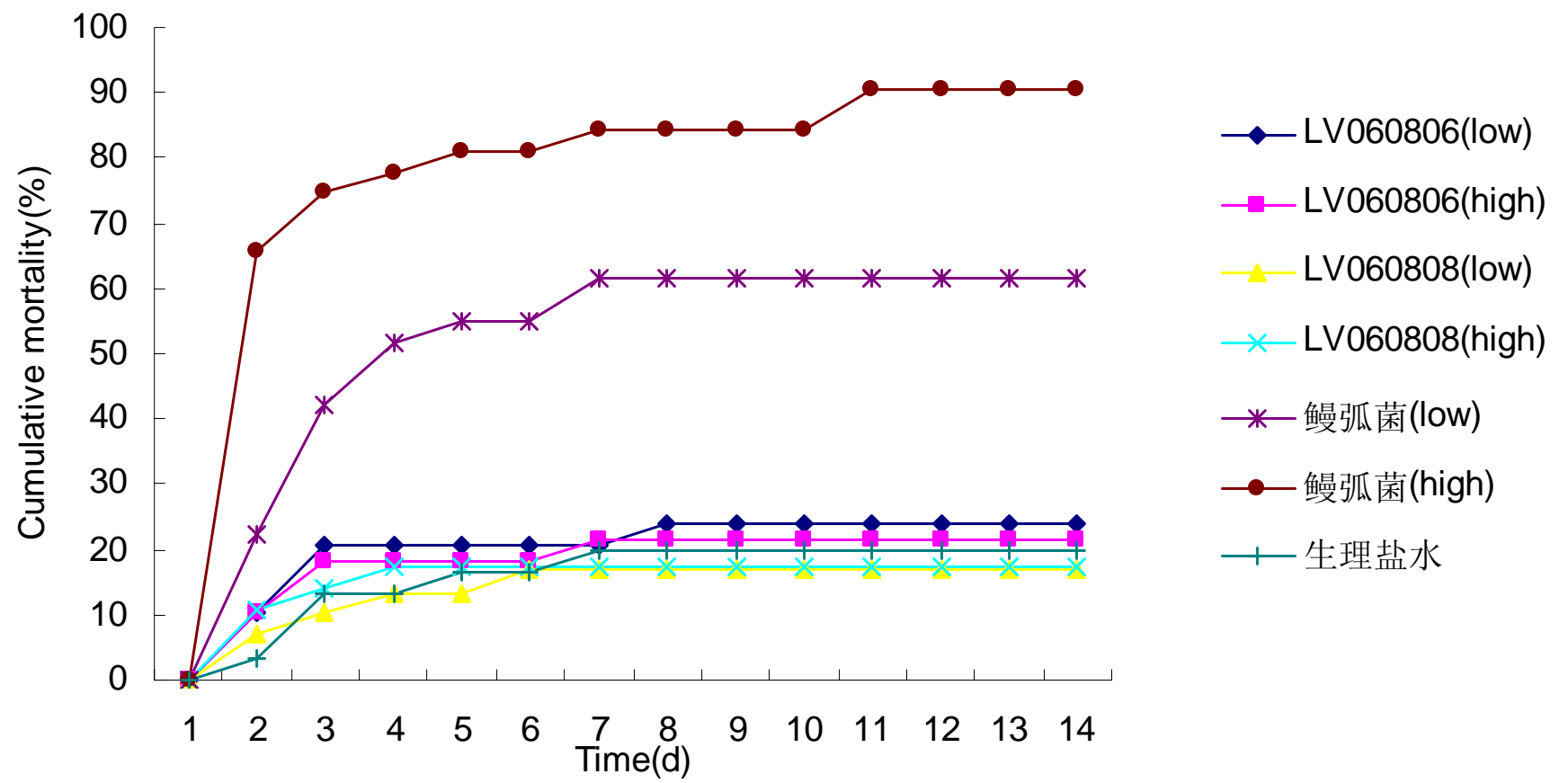
Evaluation of Pathogenicity of Selected Strains (II)

○ Shrimp: *Litopenaeus vannamei*

Selected strains: LV060806, LV060808
LV060810, LV060815

Pathogenic bacteria: *Vibrio anguillarum*

Challenge dose: 10^4 cells/ml (lower dosage)
 10^6 cells/ml (higher dosage)
inject, 50 μ l/shrimp





Evaluation of Potential Probiotic Effects on shrimp (I)

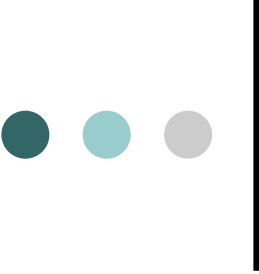
- shrimp: *Litopenaeus vannamei*

group A: fed the control diet

group B: fed the diet with 0.1% (w/w) live bacteria

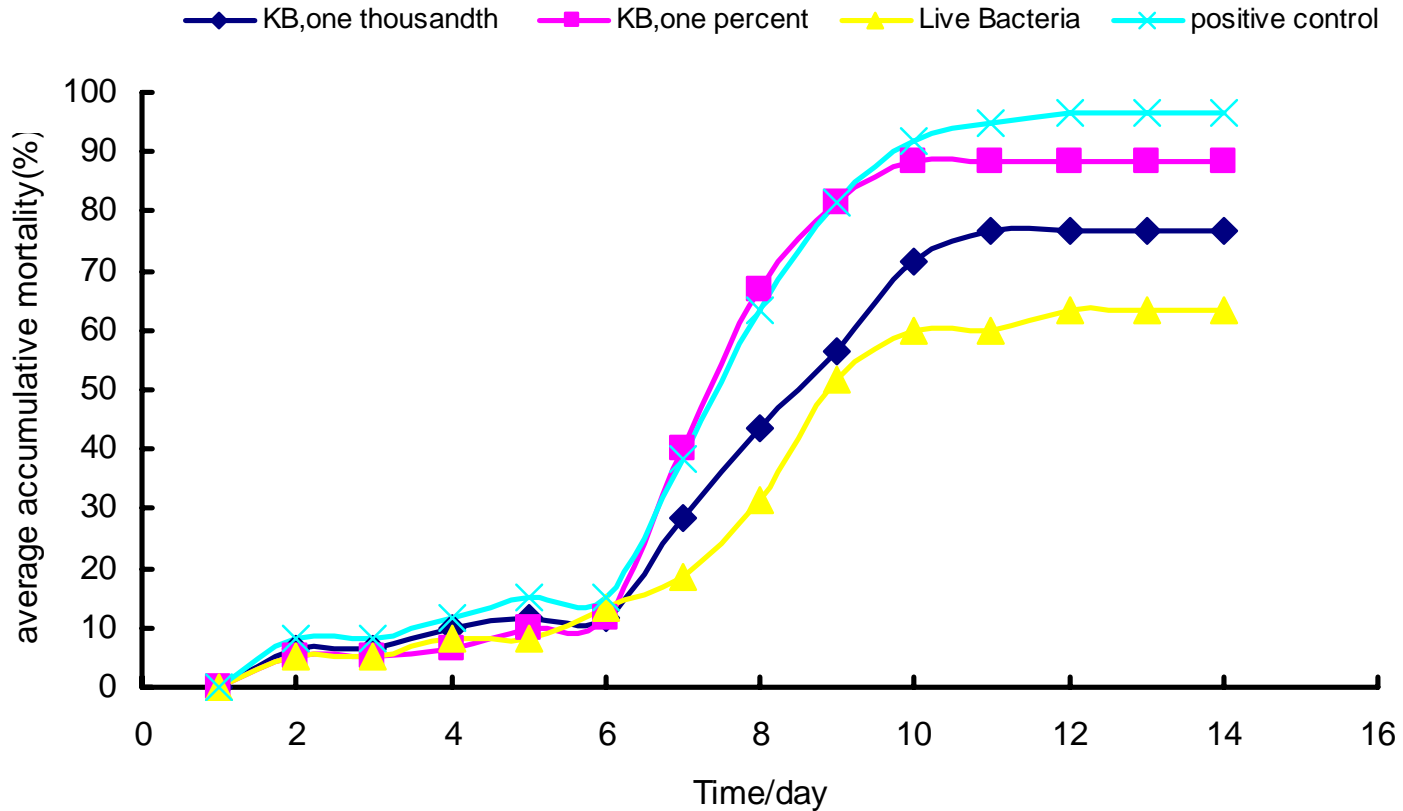
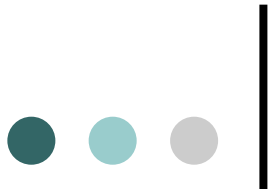
group C: fed the diet with 0.1% (w/w) killed bacteria

group D: fed the diet with 1% (w/w) killed bacteria

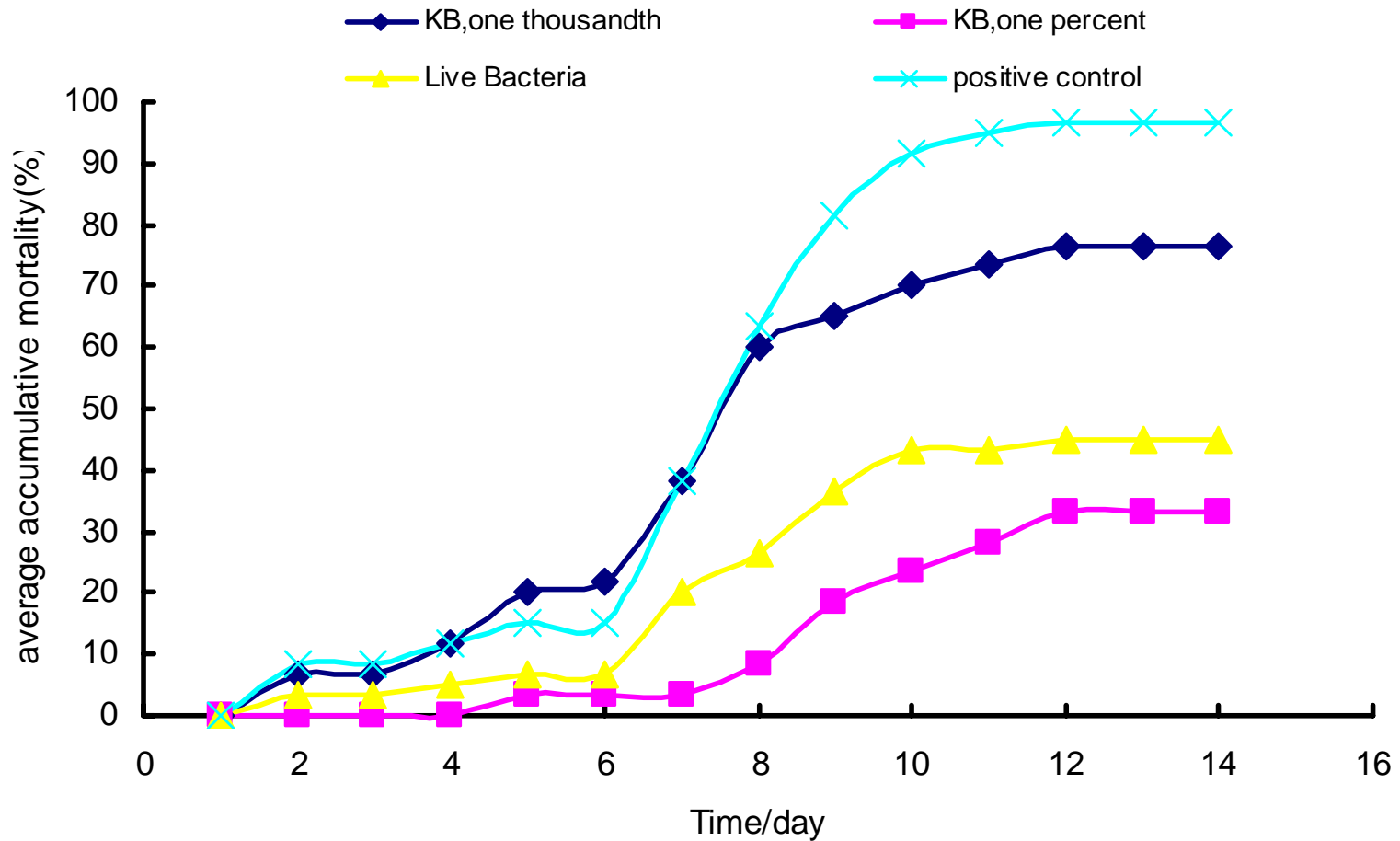


Sample's strategy

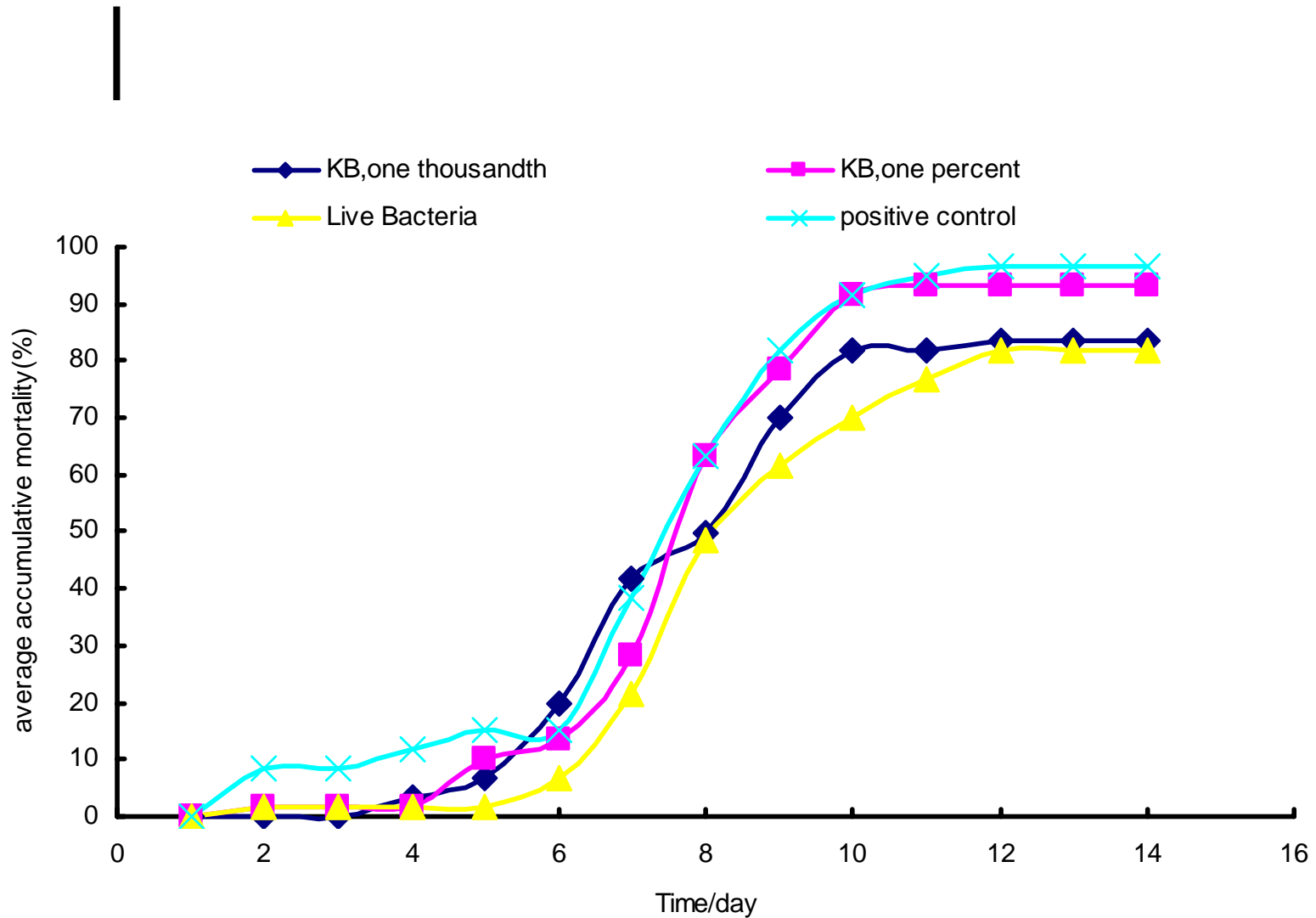
- The shrimps of every group are sampled every five days, and hemolymph are sampled for gene expression, such as Toll receptor, lipopolysaccharide and beta-1,3-glucan binding protein, lysozyme and antimicrobial et al, serum are sampled for detecting enzymatic activity, such as PO, AKP, ACP et al, and gills are sampled for virus detection;
- The shrimps will be challenged by White spot syndrome virus, after 3 weeks past, through oral route.



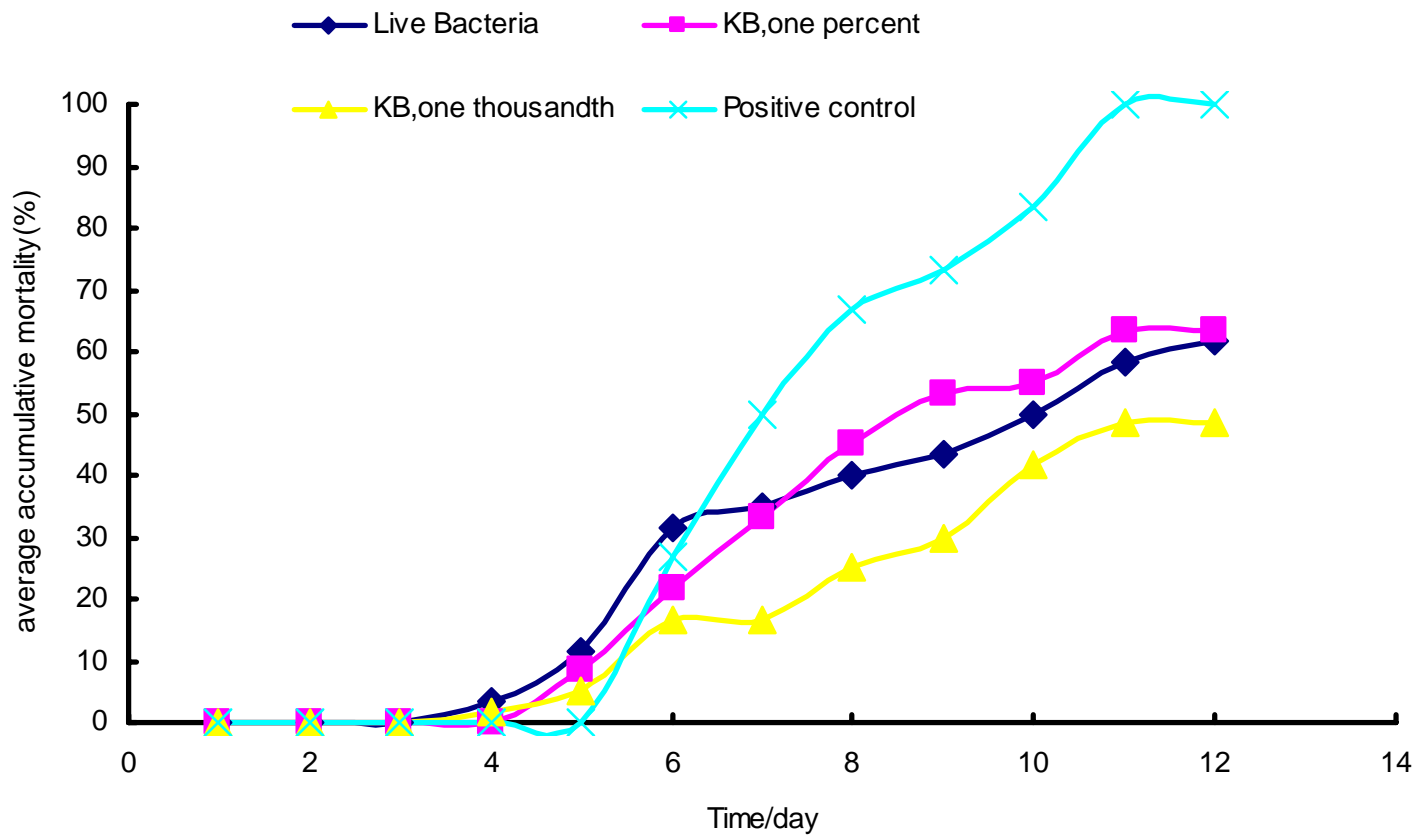
The accumulative mortality of *Litopenaeus vannamei* which were infected by WSSV, after feeding selected bacteria PC070460



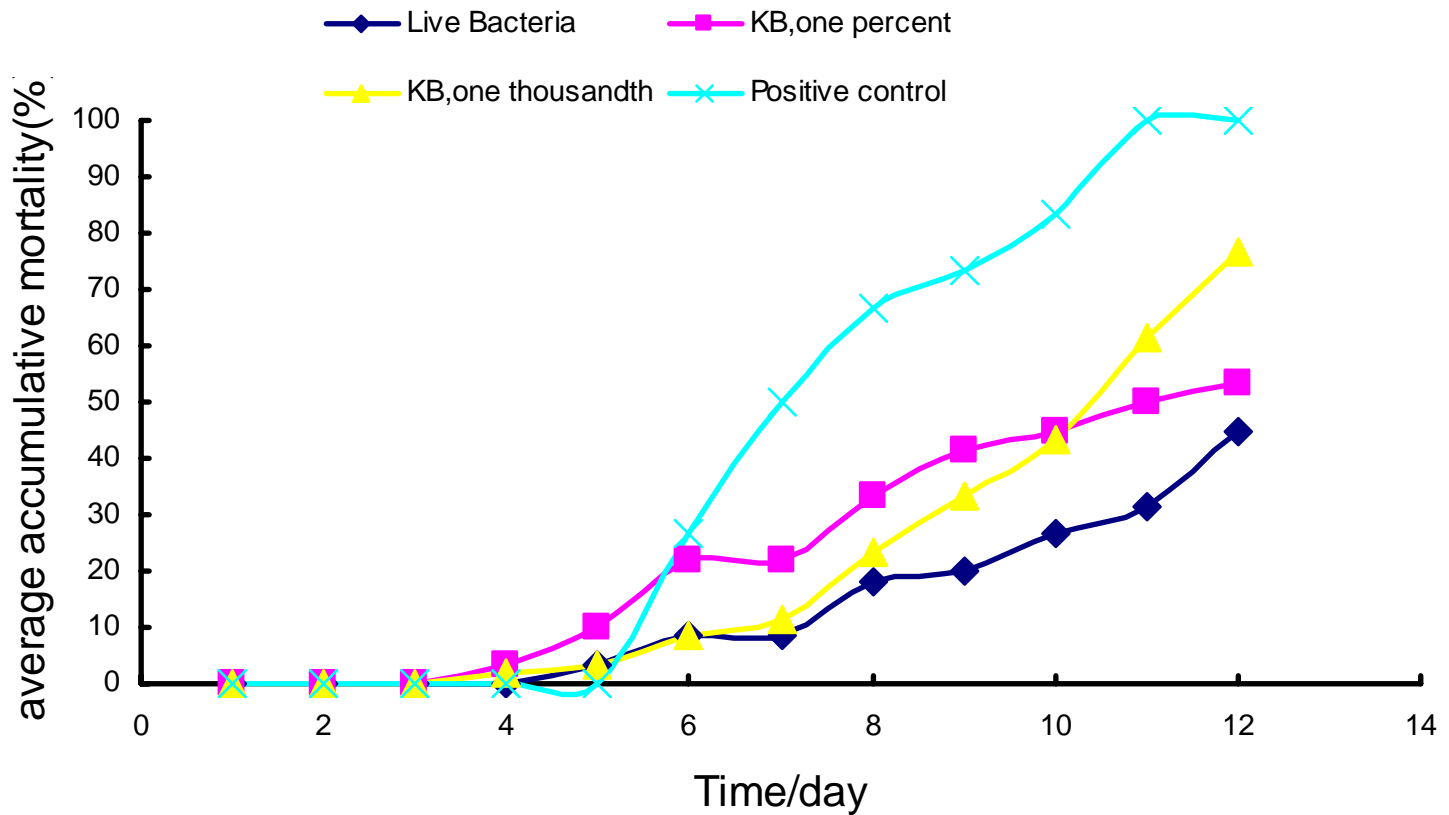
The accumulative mortality of *Litopenaeus vannamei* which were infected by WSSV, after feeding selected bacteria PC070573



The accumulative mortality of *Litopenaeus vannamei* which were infected by WSSV, after feeding selected bacteria LV060810



The accumulative mortality of *Litopenaeus vannamei* which were infected by WSSV, after feeding selected bacteria PC070463



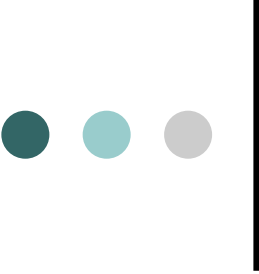
The accumulative mortality of *Litopenaeus vannamei* which were infected by WSSV, after feeding selected bacteria PC070465



Evaluation of Potential Probiotic Effects on shrimp (II)

- shrimp: *Penaeus chinensis*

Every bacteria group was fed the diet with 3×10^8 cells/g, and the negative control group was fed with the diet without bacteria



Sample's strategy

- The shrimps of every group are sampled every ten days, to weight body's weight and detecting the total number of cultured bacteria of intestine trace and the total number and strains of dominant bacteria;
- The shrimps will be challenged by White spot syndrome virus, after 4 weeks past, through oral route



Next work

- **Screening live bacteria, killed bacteria or component of bacteria in order to develop micro-ecological agent or immunostimulants for shrimp culture**
- **Mass production, economic evaluation, and evaluation of compliance with legislation**
- **Development of monitoring tools**

THANK YOU



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