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# Effects of *Pseudomonas* sp. on the Water Quality and Bacterial Flora of a Giant Tiger Prawn (*Penaeus monodon*) Hatchery

Wen-Yu Liu[1*], Guang-Hsiung Kou[2] and Shiu-Nan Chen[2]

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## ABSTRACT

The present experiment investigated the effects of aquatic bacteria, *Pseudomonas* sp., on the water quality and bacterial flora in hatching ponds of giant tiger prawn (*Penaeus monodon*). In giant tiger prawn larval rearing waters where bactericides have not been applied, the count of colony forming units in 1 ml of rearing water (CFU/ml) may reach a range of $4.6 \times 10^4 \sim 2.56 \times 10^{12}$. Addition of a suspension of viable *Pseudomonas* sp. to this rearing water did not affect the total viable bacterial count ($P>0.05$). However, *Pseudomonas* sp. was able to inhibit the growth of *Vibrio* in the rearing waters since its quantity in the two experimental ponds was lower than that in the control.

Prior to hatchery operation, the bacterial genera detected in the experimental pond water were metabolically inactive species (e.g. *Acinetobacter, Moraxella* and *Kingella*). Active strains (e.g. *Aeromonas, Haemophilus, Salmonella*, and *Vibrio*) dominated the bacterial flora when artificial feed was introduced. In the case of the occurrence of mass mortality, the majority of metabolically active species that existed in the rearing water were the pathogenic bacteria *V. parahaemolyticus* and *V. alginolyticus*.

From $PL_4$ to $PL_9$ stages, when *Pseudomonas* sp. suspension of 300 ml/day ($1.0 \times 10^9$ CFU/ml was added into the rearing water, the unionic ammonia-N ($NH_3$-N) concentration was lower in the experimental ponds than in the control. Furthermore, the concentration of $NH_3$-N in the experimental ponds did not affect the survival rate (r=1:-0.533 & 1:-0.5742), but the situation appeared to be exactly opposite in case of the control (r=1:-0.926). A significant difference was hence observed from the $M_3$ to $PL_9$ between the survival rates of prawn larvae in treated and control ponds ($P<0.05$).

***Key words:*** *Pseudomonas sp., bioaugmentation, bacterial flora, giant tiger prawn (Penaeus monodon) hatchery.*

## INTRODUCTION

Some commercial profitable bacteria have been used to reduce the concentration of total ammonia nitrogen (TAN) in channel catfish ponds (Boyd *et al.*, 1984; Chiayvareesajja and Boyd, 1993). *Pseudomonas* sp. has been reported to inhibit *Enterobacter* sp. in the aquarium (Bianchi *et al.*, 1992). Moreover, PM-4 strain (Maeda and Nogami, 1992) and *Tetraselmis suecica* have also been observed to inhibit the growth of pathogenic strains of *Vibrio* spp (Austin and Day, 1990).

Heterotrophic bacterial flora, especially pathogenic *Vibrio*, had been correlated to the mass mortality of giant tiger shrimp larvae (Liu *et al.*, 1994; Lavilla-Pitogo *et al.*, 1990). In order to resolve this problem, bactericides were commonly used in shrimp hatcheries by most of farmers in Taiwan. However, long-term treatment of antibiotics or drugs may result in the development of drug-resistant bacterial strains

[1] Department of Aquaculture, Taiwan Fisheries Research Institute, Keelung, Taiwan, 202
[2] Department of Zoology, National Taiwan University, Taipei, Taiwan 106
* Corresponding author.

(Nygaard *et al.*, 1992; Kerry *et al.*, 1994). On the other hand, the deterioration of water quality is a serious problem in giant tiger prawn hatcheries (Chen *et al.*, 1986). Hence, improving the quality of rearing water is also a very important task for giant prawn larvae producers.

In this paper, *Pseudomonas* sp. has been selected as a profitable bacteria and its ability to reduce nitrogen waste and inhibit the growth of pathogenic bacteria in the larval rearing water of giant tiger prawn were investigated.

## MATERIALS AND METHODS

### Analytical methods

### Bacterial quantity and genus identification

Water sample for bacterial analysis was collected every day at 8 a.m., when the larvae have metamorphosed into another stage of development. In this study, larval metamorphosis occurred daily from hatching until the post larval stage ($PL_9$).

The total count of viable bacteria was indicated in terms of colony forming units per milliliter (CFU/ml) of rearing water. Bacteria were enumerated from the surface of cultured marine agar 2216 (Difco) after spread plating for 7 days at 23 ℃ . Four colonies were randomly selected from a plate with colony counts ranging from 30-300 for isolation and identification (Greenberg *et al.*, 1992). The 192 strains were identified to the genus level based on the 52 morphological and biochemical characteristics (Liu *et al.*, 1995) from Bergey's Manual of Systematic Bacteriology (Krieg, 1984) and Textbook of Diagnostic Microbiology (Koneman, 1988). The commercial kit, Biolog Non-clinical and the API system, were used to identify the pathogenic species. The definition of inac tive and active metabolism of isolate is quoted from Chang *et al.* (1996).

### Water quality

Water temperature, dissolved oxygen (DO), and pH were determined with a portable and changeable glass-electrode meter (Ciba-Coring). Salinities were detected with a salinometer (Osawa). TAN was measured by the phenolhypochlorite method (Grasshoff *et al.*, 1983) and the fraction of unionized ammonia-N ($NH_3$-N) calculated from the Bower-Bidwell's equation based on Whitefield's theory (Whitfield, 1974). The measuring methods of Nitrite-N ($NO_2$-N), hydrogen sulfide ($H_2S$), and total phosphorus ($PO_3$-P) were in accordance with those described by Greenberg *et al.* (1992). Redox potential (R/O) was determined using a glass electrode connected to a pH meter (Coring). Total organic carbon (TOC) and the demand for biochemical oxygen ($BOD_5$) were determined every five days using the TOC (O.I.C.) and $BOD_5$ meter (WTW 1020), respectively. At the fully aerated condition, two-liter water sample was collected thrice every day. A two meter plastic pipe sampler was used and samples were collected from a distance of fifteen centimeters above the pond bottom.

### Pond system and food regime

Three cement ponds, belonging to a private hatchery in Southern Taiwan and having a capacity of 60-tons each, were used in this experiment. The hatching system was similar to the one described previously by Liao (1984).

Underground seawater was pumped from the sandy layers of the coastal area. Pumped water was then filtered through a 200-mesh plankton net prior to being stocked into the storage ponds, where it was fully aerated with a blower. Spawners imported from Southeast Asia were used for the production of fertilized eggs. Following morphogenesis, the nauplii were transferred to the hatching ponds at a stocking density of 4,000,000/pond. The hatching ponds were constantly aerated and water temperature of 31.9-35 ℃ and salinity of 31-35 ‰ were maintained.

The average survival rate (%) of tiger prawn larvae in sixteen stages was calculated from a one liter sample of pond water collected three times per day during the course of experiment. When the prawn

larvae metamorphosed from zoea I ($Z_1$) to zoea III ($Z_3$), the diatom *Skeletolema* was added to the ponds. In $Z_3$ stage, several types of artificial feeds were also provided until the larvae metamorphosed to $PL_9$. Newly hatched Artemia nauplii were fed from Mysis III ($M_3$) to $PL_9$. Water exchange was not done until the post larval stage.

### Bacterial augmentation

A bacterial strain of *Pseudomonas* sp. was isolated from the pond water of a kuruma shrimp (*Penaeus japonicus*) farm located in north of Taiwan. The experimental ponds were treated with 300 ml/day ($1.0 \times 10^9$ CFU/ml) of this bacterial suspension, which was cultured in tryptic soy agar (TSA, Difco) for 24 hours. Scraping out the thick bacterial pad from the surface of agar plate, then mixed and homogenized the bacterial suspension with properly volume of saline. The suspension was added daily to the experimental ponds starting the $Z_3$ stage until $PL_9$. A volume of the suspension was added into the rearing water to obtain a final concentration of $5.0 \times 10^3$ CFU/ml.

The statistical methods applied herein were selected from SAS/STATS Guide for Personal Computers, Version 6.03, SAS Institute Cary, NC.

## RESULTS AND DISCUSSION

### Bacterial count from rearing water

Bacterial count revealed no significant difference between the treated and control ponds ($P>0.05$). The counts were found at a range of $4.6 \times 10^4$ to $2.56 \times 10^{12}$ (CFU/ml) in the present study, which may be due to the large amount of artificial feeds supplied from $Z_3$ until the last stage of $PL_9$.

The viable bacteria count (CFU/ml) was, however, never observed to exceed $10^5$ in the nauplius stage (Llobrera and Gacutan, 1977). Lin *et al.* (1988) reported counts of $10^3$~$10^6$ from $N_6$ to $PL_4$ stages in rearing waters of giant tiger shrimp hatching ponds. Liu *et al.* have also reported that the bacterial number in a sample of rearing water obtained from the larval pond of a giant tiger prawn ranged from $10^3$ to $10^6$ CFU/ml (Liu *et al.*, 1994).

### Comparison of bacterial dynamics, Vibrio species, and survival rates at different metamorphic stages

#### $N_6$ to $Z_3$

Pathogenic *Vibrio* spp was not observed in the hatching ponds when the larvae were at the $N_6$ to $Z_3$ stages (Fig. 1A). The results also showed that during this period, both the bacterial flora of the experimental and control ponds were dominated by inactive bacterial strains. No significant difference in the survival rates was observed in this period ($P>0.05$).

#### $M_1$ to $PL_1$

During this period, bacterial flora changed gradually from inactive to active genera (Fig. 1B). The dominant bacterial genus obtained in the pond at $PL_1$ stage was observed to be active strains. In addition, pathogenic *Vibrio* also simultaneously started to appear in both experimental and control ponds. Starting at the $M_3$ stage, the survival rate of larval shrimp in the experimental ponds was found to be higher than that in the control while the amount of *Vibrio* in the experimental ponds was lower as compared to the control (Table 2). *Vibrio* species in the pond water included *V. parahaemolyticus* and *V. alginolyticus*.

#### $PL_2$ to $PL_4$

Although the two inactive and active types of bacterial genera developed simultaneously in this period, active strains were dominant in the control pond (Fig 1C). *Moraxella* occurred at the $PL_4$ stage. It is important to note that the *Vibrio* strains appeared simultaneously in the $PL_3$ stage; however, its amount was less in the experimental ponds. The dominant *Vibrio* species present in the control pond was *V. alginolyticus*. The survival rates of prawn larvae were found to be higher in the experimental ponds as compared to the control.

#### $PL_5$ to $PL_9$

The highest amount of Vibrio was

Table 1. Survival rate (%) from the $M_3$ to $PL_9$ stages of giant tiger prawn (*Penaeus monodon*) larvae from three hatching ponds

| Hatching pond | Metamorphic stages and survival rate (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $M_3$ | $P_1$ | $P_2$ | $P_3$ | $P_4$ | $P_5$ | $P_6$ | $P_7$ | $P_8$ | $P_9$ | Mean ± sd |
| Exp. 1 | 78 | 70 | 66 | 62 | 59 | 52 | 44 | 37 | 25 | 20 | 51.3 ± 19.38 |
| Exp. 2 | 65 | 50 | 47 | 45 | 42 | 38 | 29 | 20 | 14 | 7 | 35.7 ± 17.97 |
| Control | 61 | 35 | 33 | 29 | 26 | 22 | 19 | 15 | 9 | 0 | 24.9 ± 16.67 |

One-way ANOVA: F=5.4091; P<0.05.

Table 2. Comparison of the amount of *Vibrio* from 192 bacterial isolates occurred in different metamorphic stages

| Hatching pond | metamorphic stages | | | | | | | | sum |
|---|---|---|---|---|---|---|---|---|---|
| | $M_3$ | $PL_1$ | $PL_2$ | $PL_3$ | $PL_4$ | $PL_5$ | $PL_6$ | $PL_8$ | |
| Exp. 1 | 2 | 2 | 1 | 3 | - | 4 | 4 | 1 | 17 |
| Exp. 2 | - | 2 | - | 3 | - | 4 | 4 | 2 | 15 |
| Control | - | 4 | 2 | 3 | 2 | 4 | 4 | 3 | 22 |

Remark: the vibrio's quantities obtained from that the last *vibrio* number (CFU/ml) counted after 10 fold series dilution and no significant difference of bacterial count between the experimental and control ponds (P>0.05).

observed in this period (Fig. 1D; Table 2). The survival rate of prawn larvae in the control pond was comparatively lower than that in the experimental ponds (Table 1). The predominant species of *Vibrio* was *V. alginolyticus* and the abundance of inactive and active strains were similar from $PL_5$ to $PL_9$ stages (Fig. 1D).

### Interrelationship between *Vibrio* count and survival rate

No pathogenic species of *Vibrio* were found in the ponds at $M_3$ stage and the survival rates of prawn larvae were higher than 61% in this period (Table 1). The rapid increase of *Vibrio* count significantly affected the survival rates of prawn larvae $PL_5$ to $PL_6$ stages. It is, therefore, concluded that a near zero correlation exists between the survival rates observed from the experimental ponds and *Vibrio* counts (r=1:-0.25&-0.16). The control pond showed a statistically incomplete negative correlation (r=1:-0.48). Significant differences were observed in the survival rate of shrimp larvae stages (P<0.05) from $PL_1$ to $PL_9$ and the quantity of *Vibrio* between the experimental and control ponds.

A spray-dried green microalgae (*Tetraselmis suecica*) preparation had been observed to rapidly inhibit activities of the pathogenic strains of *Vibrio* from cultured prawns (Austin and Day, 1990). Bianchi *et al.* (1992) suggested that *Pseudomonas* sp. may positively inhibit the growth of *Enterobacter*. Meada and Nogami (1989) have also reported that the concentration of bacterial suspension up to $10^6$ per milliliter rearing water may be beneficial for inhibiting the growth of pathogenic strains. The present study revealed that an average concentration of $10^3$ of *Pseudomonas* sp. per milliliter rearing water contributed significantly to the inhibition of *Vibrio* growth in the prawn hatching ponds (Table 2).

### Water quality

During the course of the present experiment, the average pH value was between 8.0-8.4 for both the experimental and control ponds (Fig. 2A). Concentrations of DO in both treated and control ponds were

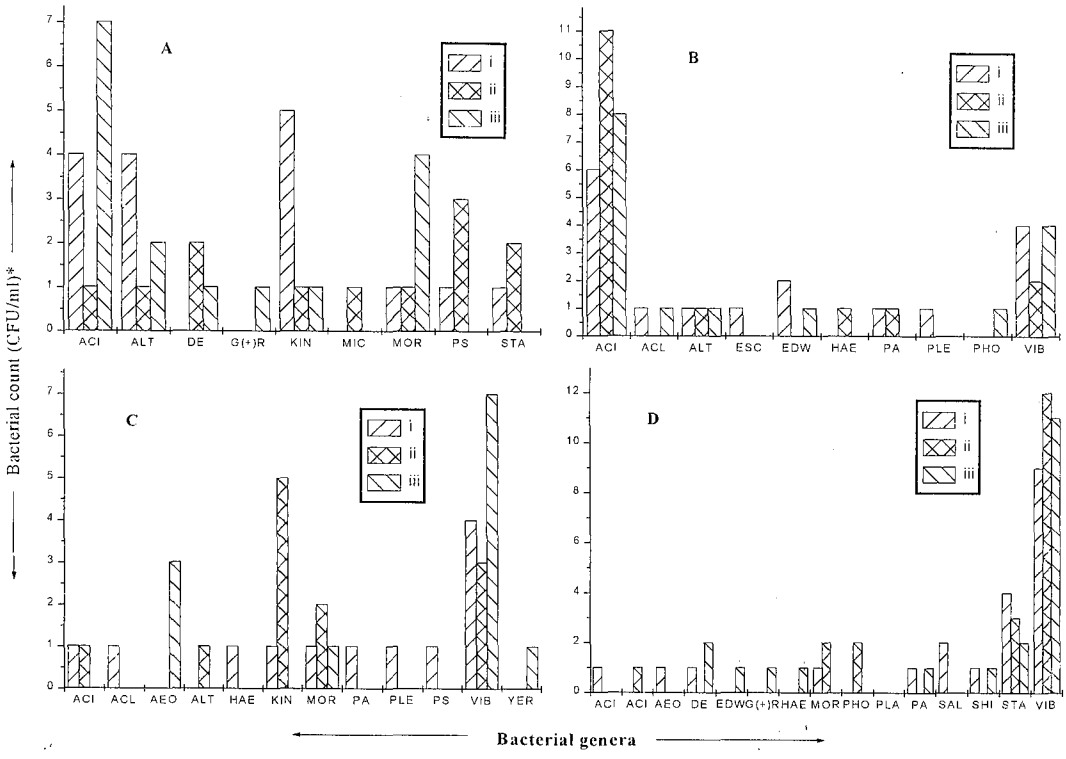


Fig. 1. Bacterial distribution from 192 isolates (CFU/ml) observed in the hatching ponds during larval stages of giant tiger prawn (*Penaeus monodon*). A: from 6th nauplius to 3rd zoea; B: from 1st mysis to 1st post larvae; C: from 2nd to 4th post larvae; D: from 5th to 9th post larvae. ACI: *Acinetobacter*; ACL: *Alcaligenes*; AEO: *Aeromonas*; ALT: *Alteromonas*; DE: *Deinococcus*; EDW: *Edwardsella*; ESC: *Escherichia*; G(+)R: Gram stain(+) rod; HAE: *Haemophilus*; KIN: *Kingella*; MOR: *Moraxella*; MIC: *Micrococcus*; PA: *Pasteurella*; PHO: *Photobacterium*; PLA: *Planococcus*; PLE: *Plesiomonas*; PS: *Pseudomonas*; SAL: *Salmonella*; SHI: *Shigella*; STA: *Staphylococcus*; VIB: *Vibrio*; YER: *Yersinia*. i and ii: experimental ponds; iii: control pond; *: with 10 fold series dilution to $10^{10}$ per milliliter of pond water and no significant difference of bacterial count between the exeperimental and control ponds (P>0.05).

not lower than 5 mg/l. Salinity varied in the range of 31-35 ‰. R/O (*Eh*) did not significantly differ between the treated (54-113 mV) and the control ponds (43-121 mV).

The concentration of $NH_3$-N in both the treated and control ponds continuously increased with the culture period (Fig. 2E). A significant difference in $NH_3$-N was observed between the experimental and control ponds from $PL_4$ to $PL_9$ stages ($P < 0.05$). Statistically, a negative correlation (r=1:-0.926) was found between the concentration of $NH_3$-N and the survival rate. The $NO_2$-N was not maintained at a low level prior to $PL_7$ and its highest concentration was found to be 0.17 mg/l.

The statistical results on concentrations of $H_2S$ are 0.01-0.25 mg/l in the experimental and 0.01-0.12 mg/l in the control ponds, respectively. The fraction of $PO_3$-P was enhanced but its concentration fluctuated depending on the larval stage. Its highest concentration was at 0.82 and 0.89 mg/l in the treated and control ponds, respectively (Fig. 2B).

There was no significant variation in the $BOD_5$ between the treated (3->40 mg/l) and control (6.1-31 mg/l; Fig. 2D) ponds. Furthermore, there was no correlation between $BOD_5$ and bacterial count expressed in CFU/ml.

The concentration of TOC measured in

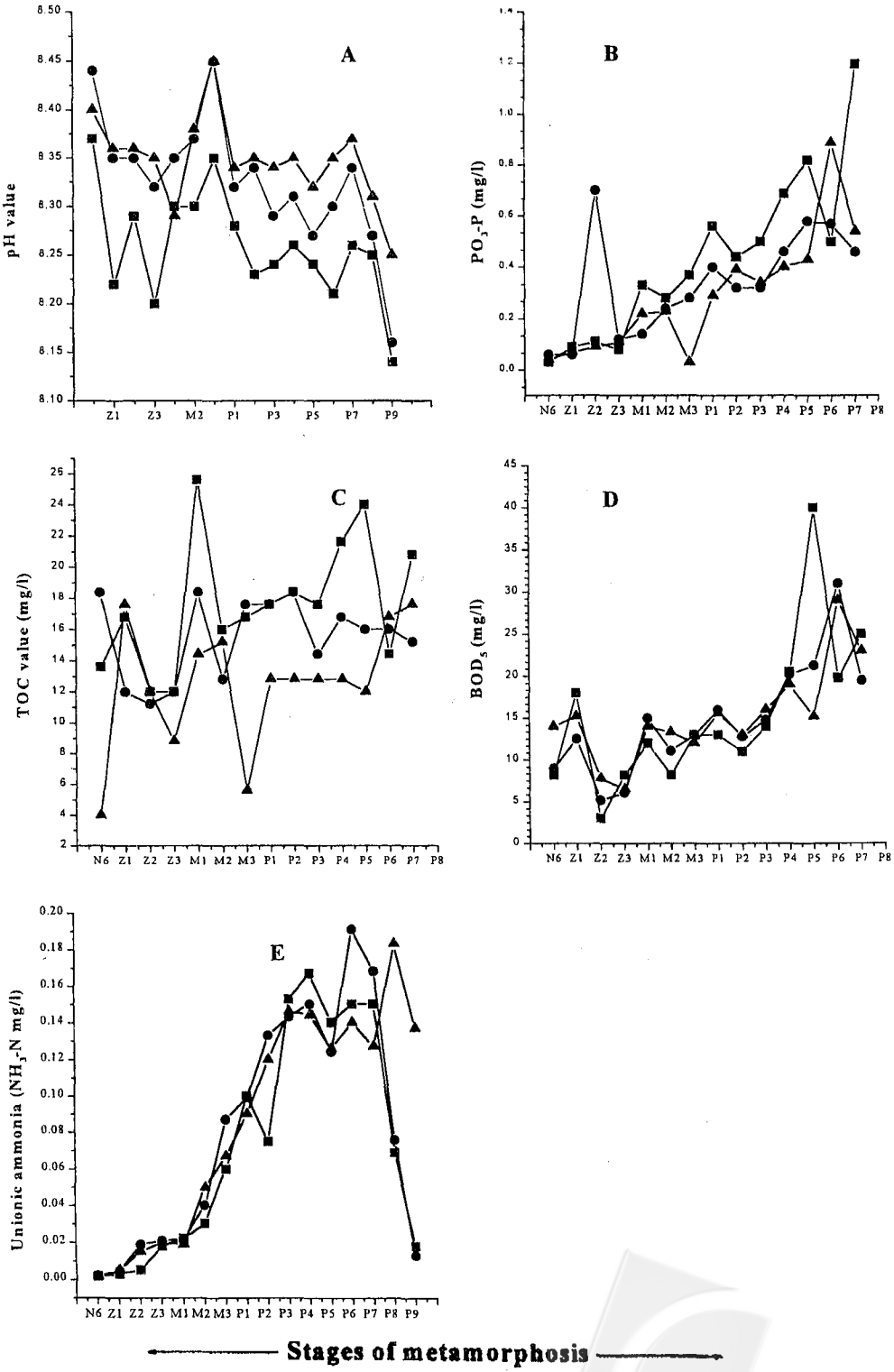


Fig. 2. Variations of pH and concentrations of $BOD_5$, $PO\text{-}P_3$, TOC, and $NH_3$-N during the experimental period. — ■ — & — ● — : as experimental ponds; — ▲ — : control pond.

the experimental and control ponds appeared to fluctuate (Fig. 2C), but statistically, no significant difference was observed among these ponds ($P>0.05$). It may be concluded from these results that no correlation existed between TOC and bacterial count.

Due to large variations in pH, DO, $H_2S$, and salinity, no apparent differences could be noted between the treated and control ponds ($P>0.05$). Fry (1987) has reported that the concentration of $BOD_5$ and TOC are related to the microbial activities and are generally increased by numerous bacteria. In contrast, no correlation between the bacterial count (CFU/ml) and the concentrations of $BOD_5$ and TOC was found in the present study in both treated as well as control ponds. Commercially available bioagents failed to show beneficial effects in terms of reduction of TAN in channel catfish ponds (Boyd *et al.*, 1984; Chiayvareesajja and Boyd, 1993). The water quality could not also be improved by bacterial suspension if the concentration of the bacterial agent was lower by 10-100 folds than the bacterial flora in the pond (Boyd *et al.*, 1984). The present study, however, revealed that at a concentration of $5.0 \times 10^3$ (CFU/ml), *Pseudomonas* sp. could improve the water quality and inhibit *Vibrio* abundance in the prawn hatching ponds. *Vibrio* did not become dominant until the last day of the experiment.

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# 添加假單胞菌(*Pseudomonas* sp.)菌液對草蝦(*Penaeus monodon*)苗繁殖場水質及細菌相之影響

劉文御[1*]・郭光雄[2]・陳秀男[2]

(2000年6月16日收件；2000年8月4日接受)

本研究嘗試利用養蝦池中分離出來的一株假單胞菌控制草蝦苗繁殖場池水中的氨氮濃度及抑制病原性弧菌的數量，藉以增加蝦苗的活存率。

在未使用藥物的情形下，飼水中的總生菌數爲$4.6 \times 10^4$-$2.56 \times 10^{12}$ CFU/ml以上，將假單胞菌投入試驗組的蝦苗池後與控制組比較，兩者飼育水中的總生菌數並無差異($P>0.05$)，因此投入活菌後對水中的總生菌數並無影響。在未使用人工餌料之前，試驗組飼育水中的菌相大多以代謝不活潑菌爲優勢種，其中最主要的菌屬包括：*Acinetobacter*，*Moraxella*及*Kingella*等，但在使用人工餌料之後，水中的菌相開始轉變成代謝活潑型的菌屬，其中代表性的菌屬有：*Aeromonas*，*Vibrio*，*Alteromonas*，*Haemophilus*及*Salmonella*等。

當蝦幼苗發生大量死亡之時，水中代謝活潑菌株中以病原性弧菌爲優勢種，其中又以*Vibrio parahaemolyticus*，*V. alginolyticus*出現最多。在試驗組中的弧菌數量明顯的低於控制組，因此，試驗菌株對於抑制弧菌的數量有明顯的功效。自眼幼蟲第三期開始至後期幼苗第九期止，以*Pseudomonas* sp.菌液每日投入池水中300毫升($1.0 \times 10^9$)，可以降低試驗組後期幼苗第四期至第九期($PL_4$-$PL_9$)時飼育水中非離子化氨($NH_3$-N)的濃度，其與控制組比較有明顯的差異($P<0.05$)。此外，試驗組池水中非離子化氨的濃度與活存率間呈不完全負相關($r=1$:-0.533 & 1:-0.5742)；相反的，在控制組則呈完全負相關($r=1$:-0.926)。而且蝦苗的活存率在試驗組與控制組間雖無明顯的差異($P>0.05$)，然而自後期幼苗第一期開始至第九期的活存率則有明顯差異($P<0.05$)，試驗組的活存率明顯的高於控制組。

[1] 台灣省水產試驗所水產養殖系
[2] 國立台灣大學動物學系
* 通訊作者