DOCTOR OF PHILOSOPHY

Aspects of bacterial disease prevention and control in penaeid prawns

Alabi, Abayomi Olusegun Olanrewaju

Award date:
1997

Awarding institution:
Bangor University

Aspects of bacterial disease prevention and control in penaeid prawns

A thesis submitted to the University of Wales

By

Abayomi Olusegun Olanrewaju Alabi

B.Sc. (University of Lagos, Nigeria),

M.Sc. (University of Lagos, Nigeria)

in candidature for the degree of Philosophiae Doctor

School of Ocean Sciences,

University College of North Wales

University of Wales,

Bangor, Menai Bridge.

August, 1997.

SOME PARTS
EXCLUDED
UNDER
INSTRUCTION
FROM THE
UNIVERSITY

Acknowledgments

I would like to express my gratitude to all the people who helped make this study possible. My thanks go to my two supervisors: Drs. D. A. Jones and J. W. Latchford. They corrected many copies of this thesis, and exhibited a lot of help, patience and guidance over the past three years. My gratitude also goes to Dr. Simon Webster of the School of Biological Sciences for his help in making use of the micro-titre plate reader and Dr. Norma Blackstock for help with the historesin embedding and sectioning.

I am grateful to all the people at the School of Ocean Sciences, especially John and Susanna Rowlands, Fernando and Maria Ribiero, and Karen Abey for both their friendship and their help. Thanks are also due to all the academic and technical staff in the SOS for always being prepared to help.

I am indebted to the Nigerian Federal Scholarship Board for allowing me to get a higher degree

I would like to thank my siblings and their partners for all their support and encouragement, My special friends, SRN Funlola Adeyi, Mr. Mimiko and Mr. Ajayi. Most of all, I want to thank my heavenly father..Jesus.

This work was funded by the British Commonwealth Scholarship Commission of the Association of Commonwealth Universities.

Dedicated to my parents

and to 'Bisola

Abstract

Bacterial concentrations in natural sea water were significantly reduced by 5μm filtration and subsequent treatment with ozone and UV light led to further reductions in total viable counts of bacteria and autoclaving water gave complete sterility. However, with the addition of artificial diets, such treated water was recolonised very rapidly and within 24h, these water samples had significantly higher bacteria counts than the 5μm filtered sea water treatment ($f = 21.30$; $p < 0.001$). Further treatment of 5μm filtered sea water also led to changes in the bacterial composition of the water. In ozonated and UV light irradiated water samples, the proportions of presumptive *Vibrio sp.* increased in the 24h following treatment while in 5μm filtered and natural sea water samples, the proportions of presumptive *Vibrio sp.* fell over the same period. When *Penaeus indicus* PZ1 stage larvae were reared to PL1 stage in 5μm filtered sea water, good larval survival was obtained irrespective of whether live or microencapsulated artificial diets were fed. In contrast, larvae raised in autoclaved, ozonated and UV light irradiated water exhibited significantly lower survival when raised on live diets and often showed complete mortalities when fed on microencapsulated diets.

In periods of poor sea water quality, additions of bacteria from algal cultures to PZ1 stage larvae fed algae had no effect on larval survival to M1 stage for larvae reared in 5μm filtered sea water or autoclaved water. In contrast, additions of these bacteria to larvae reared in 5μm filtered sea water and fed on MED, led to a significant increase in larval survival. This effect was absent when larvae were reared in autoclaved water. Addition of bacteria from algal cultures were found to inhibit counts of presumed viable *Vibrio sp.* while having no effects on total viable counts.

No significant differences were observed in the percentage of soluble protein leached from microencapsulated diets and microparticulate diets when incubated up to 48h in bacterial laden and sterile water indicating that leaching is independent of microbial activity up to 48h. However broken microcapsules gave higher total viable bacterial counts over 48h in 5μm filtered sea water when compared with intact microcapsules.

Significant levels of protection were conferred on larvae when either fresh or freeze-dried vaccines were administered by immersion, but not when such vaccines were administered orally. The degree of protection offered was correlated with the virulence of the pathogen from which the vaccine was made.

Enhanced protection given by vaccines produced from the more virulent strains was not wholly due to activation of the prophenoloxidase system since such vaccines induced less stimulation of the prophenoloxidase system than less virulent strains.

Untreated plasma of *Penaeus vannamei* significantly enhanced *Escherichia coli* growth compared with sea water nutrient medium. In contrast, plasma from vaccinated prawns exhibited antibacterial activity detectable up to 7d after vaccination.

Exposure to a mixture of fungicides implicated in the initiation of the taura syndrome disease (TS), had no effects on the immune competence of *P. indicus* juveniles following live, *in vivo* pathogenic challenge. In addition, no effects on growth, growth rates, moulting rates or survival of postlarval and early juvenile prawns were observed following exposure to the fungicides. Prawns exhibited no gross or histopathological symptoms characteristic of TS.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

CHAPTER 5: EFFECTS OF VACCINES ON ASPECTS OF THE IMMUNE SYSTEM OF *PENAEUS VANNAMEI*

CONTRIBUTING MATERIAL

Parts of the work carried out in this study have contributed to the following:

Alabi, A. O., E. Yudiati, D. A. Jones and J. W. Latchford. 1996: Bacterial levels in penaeid larval cultures. In: Book of Abstracts, World Aquaculture '96, January 29-February 2, Queen Sirikit National Convention Centre, Bangkok, Thailand. pp. 4. World Aquaculture Society, Baton Rouge.

Alabi, A. O., E. Yudiati, D. A. Jones and J. W. Latchford. 1997: Bacterial levels in penaeid larval cultures. In: Diseases in Asian Aquaculture. (Eds: Flegel, T. W. and MacRae, I. H.). in press. Fish Health Section, Asian Fisheries Society, Manila.

Alabi, A. O., Z. Che Cob, D. A. Jones and J. W. Latchford. (Accepted): Microbial effects on larval growth and survival of microencapsulated diets as a total replacement for algae in the culture of *P. indicus* larvae. Aquaculture International.

Latchford, J. W., S. B. Prayitno and A. O. Alabi. 1995: The use of vaccines and immunostimulants in the culture of penaeid shrimp. In: Larvi '95 - Fish and Shellfish larviculture symposium. (Eds: Lavens, P., Jaspers, E. and Roelants, I.). pp. 506. European Aquaculture Society, Gent, Belgium.

Latchford, J. W., S. B. Prayitno and A. O. Alabi. 1996: The use of vaccines in the culture of penaeid prawns. Journal of Shellfish Research. 15(2): 456.

Parts of the penaeid larval rearing trials performed in water with different methods of pre treatment (Chapter 3) were performed with the assistance of Ms. E. Yudiati and such have been included in her M.Sc. thesis.

CHAPTER 1

INTRODUCTION

The aim of this general introduction is to review the incidence of bacterial diseases with special emphasis on those caused by members of the genus *Vibrio* on penaeid prawn culture and its impact on production world wide. Current methods of control are also summarised including the use of antibiotics and chemotherapeutants, environmental methods, use of probiotics and vaccines and immunostimulants. This is to place in context, the research aims of the early part of the thesis which were to investigate methods of water pre-treatment on bacterial populations and growth in penaeid larval cultures and to measure the effectiveness of various vaccines and delivery methods on combating bacterial diseases in prawns. The latter part of the review centres on the immune system in penaeid prawns as a precursor to studies on various aspects of the immune systems in *Penaeus vannamei* juveniles with emphasis on the induction of cell free antibacterial activity in *P. vannamei.*

In a separate study, the effect of a fungicide cocktail implicated in the Taura syndrome disease of penaeid prawns (a disease syndrome of uncertain aetiology), is tested in *Penaeus indicus* to investigate the effects of the fungicides on various aspects of the biology of the prawns.

The demand for fish and shell fish continues to grow rapidly. In order to keep up with predicted increases in the world population, the maximum sustainable yields from established capture fisheries of 100 million tonnes (Chamberlain, 1993) which have been predicted by the end of the decade, still leaves a large shortfall which can only be bridged by aquaculture. Indeed, in 1990 came the first decline in fisheries since 1976, a drop of about 3%, suggesting the approaching limits of production from "wild" marine resources. In the case of marine prawn supply this limit may already have been reached as Csavas (1994), reported a plateau in 1985 of around 1.9 million tonnes although a massive increase in cultured prawns kept world supply expanding (Csavas, 1994; New, 1997). Figure 1.1

Prawns have attracted a considerable amount of interest world-wide. They are a gourmet food in the form of table prawns and smaller species can be dried and used as condiments in the flavouring of food (Sagua, 1980). They have also been used as laboratory animals for the study of morphology and taxonomy of crustaceans. Prawn industries provide one of the major sources of income in developing countries such as Ecuador, Bangladesh and the Philippines (Landesman, 1994; New, 1997). Rosenberry (1988) reported over forty countries with 31,000 prawn farms cultivating 765,000 hectares of ponds. Such farms require a dependable supply of post-larval prawns but availability from the wild is very unreliable and at best,

seasonal, usually depending on environmental factors and the levels of over fishing which reduce stocks of gravid females (Jones, 1988). Such wild post larvae may be carriers of bacterial and viral diseases which become expressed in the intensive conditions of prawn farming as well as being often mixed with other unwanted species and pests and separation is a difficult and laborious process. Hatchery reared seed are on the other hand, available all year round and of known quality.

FIGURE 1.1: GLOBAL PRODUCTION OF CAPTURED AND CULTURED PRAWNS

Data from FAO 1975, 1980, 1982, 1992.

Source : Csavas (1994)

Bacterial disease

Larval disease is one of the most important factors affecting such hatchery production and bacterial disease problems have contributed significantly to larval production losses (Shewbart *et al.*, 1972; Lightner, 1983; 1988; Takahashi *et al.*, 1984; 1985a; b; Sunaryanto and Mariam, 1986; Egusa *et al.*, 1988; Baticados *et al.*, 1990; Karunasagar *et al.*, 1994; Mohney *et al.*, 1994).

Generally, animals kept in artificial culture conditions experience stress at one time or another and become more susceptible to disease (Lightner, 1988). In most of the major prawn farming regions of the world, prawn hatchery and farm production losses due to disease have risen and bacterial disease problems have contributed to the closure of many farms and hatcheries (Shewbart *et al.*, 1972; Lightner, 1988; Lavilla-Pitogo *et al.*, 1990; Brock and Leamaster, 1992; Karunasagar *et al.*, 1994). The determinants of disease have often been represented as a triad of: the animal, the pathogen and the environment. The animal and pathogen co-exist in an environment which may favour either (Thrusfield, 1986). The infectivity and virulence of the pathogen is opposed by the susceptibility or resistance of the host and the incidence of health or disease depends on the balance of all three factors.

In many disease outbreaks there is an interplay of several causal factors making timely identification of the controlling factors problematical (Sunaryanto and Mariam, 1986; Brock and Lightner, 1990; Mohney *et al.*, 1994; Prayitno, 1994; Prayitno and Latchford, 1995). The very nature of some farms (high prawn density per unit volume of water) encourages the development and transmission of many diseases (Lightner, 1992). Lin (1989) and Chen (1990) cited by Lee and Wickins (1992) stated that in Taiwan, it was the prawn farmers themselves who suffered the greatest setbacks when in 1988, environmental degradation brought about by their own activities resulted in severe disease outbreaks, the near collapse of the industry, and lowered production from 80,000 tonnes to 24,000 tonnes.

Bacteria are common inhabitants of the marine environment and form a micro-flora in guts of healthy prawns (Ruangpan, 1982; Lightner, 1988; 1992; Lavilla-Pitogo *et al.*, 1990; Lightner *et al.*, 1992; Owens *et al.*, 1992; Mohney *et al.*, 1994; Prayitno, 1994). Bacterial diseases often occur in association with other diseases caused by factors such as water quality, fungi, protozoans and viral infections.

Bacterial disease can cause up to 100% mortality in penaeid larval cultures with members of the genus *Vibrio* recognised as causing the most serious diseases (Shewbart *et al.*, 1972; Takahashi *et al.*, 1984; 1985a; b; Sunaryanto and Mariam, 1986; Egusa *et al.*, 1988; Lightner, 1988; Baticados *et al.*, 1990; Brock and Lightner, 1990; Lavilla-Pitogo *et al.*, 1990; Mohney *et al.*, 1994). Sano and Fukada (1987) cited by Brock and Leamaster (1992), reported that in 1984, vibriosis caused an annual production loss of 3.4% (70.2 tonnes) of total production of kuruma prawns in Japan.

Gross clinical signs exhibited by prawns affected in epizootics of vibriosis in adults, included lethargic swimming abilities and lying motionless at the pond edges. Such severely affected prawns lost the escape reflex, had heavily melanized cuticles and may or may not have been heavily fouled by cuticular epibionts. Less severely affected prawns were pale and opaque (Takahashi *et al.*, 1984; 1985b; Anderson *et al.*, 1988; de la Peña *et al.*, 1993). Gills of affected prawns were often brown in colour and the prawns showed red colouration. In addition, there were slight, melanised erosions of the uropods, pleopods and pereiopods and affected prawns showed empty stomachs and midguts (Anderson *et al.*, 1988). Affected juveniles displayed cloudiness of the muscle in the 6^{th} abdominal segment (Takahashi *et al.*, 1985b) and black or brown spots in the gills and lymphoid organs (Takahashi *et al.*, 1985b; Anderson *et al.*, 1988; de la Peña *et al.*, 1993).

Histological examination of affected prawns revealed extensive necrosis and bacterial invasion of the lymphoid organ with multiple melanized haemocytic nodules. Such nodules were most commonly composed of a bacterial colony in the centre surrounded by a melanized zone and multiple layers of haemocytes attempting to encapsulate the bacterial colony. Nodules were also observed albeit to a lesser extent, in other tissues such as heart, gills, hepatopancreas, gonads and muscle (Takahashi *et al.*, 1985b; Anderson *et al.*, 1988; Egusa *et al.*, 1988; de la Peña *et al.*, 1993).

Affected post larvae displayed cloudiness of the hepatopancreas (Takahashi *et al.*, 1984) while affected larvae were lethargic (Lavilla-Pitogo *et al.*, 1990). They became opaque especially in the thoracic region. These larvae settled to the bottom and made intermittent, very weak swimming movements.

Light microscopic observations revealed densely packed motile bacteria in the gut and haemocoel. (Lavilla-Pitogo *et al.*, 1990).

In fish, many *Vibrio sp.* have been reported as primary pathogens capable of causing disease at low concentrations (Egidius, 1987). However, attempts to reproduce bacterial diseases in prawns using *Vibrio sp* isolated from such diseased prawns do not always produce experimental infection except when massive doses are injected (Sae-oui *et. al.*, 1987; Lightner, 1988; 1992; Sahul Hameed *et al.*, 1996) supporting suggestions that most *Vibrio sp.* are opportunistic pathogens of prawns. Many opportunistic species of *Vibrio* have been isolated from diseased prawns (Anderson *et al.*, 1987; Nash *et al.*, 1992). These bacteria apparently establish lethal infections as a result of other primary conditions such as other infectious diseases, nutritional diseases, extreme environmental stress and wounds (Lightner, 1983; 1988; Lee and Wickins, 1992; Nash *et al.*, 1992). Despite the apparent opportunistic nature of most *Vibrio sp.* pathogens of penaeid prawns, some more recently occurring disease syndromes have been caused by *Vibrio sp.* which behave more like primary pathogens than opportunistic invaders and cause disease at very low concentrations (Cipriani *et al.*, 1980; Lavilla-Pitogo *et al.*, 1990; Lightner, 1992; de la Peña *et al.*, 1993). Cipriani *et. al.*, (1980) suggested that bacteria with lipolytic activity can initiate lesions in the non-chitinous epicuticle of shrimps allowing the entrance of chitinoclastic forms, and Lavilla-Pitogo *et al.*, (1990) isolated a chitinase and lipase producing strain of *V. harveyi* implicated in a severe vibriosis epizootic which occurred in the Philippines in 1987.

Since the recent and rapid intensification of prawn farming, luminescent bacteria been recognised as causing significant disease problems in prawn hatcheries. Such diseases have caused heavy mortalities, low post-larval production and the temporary shutdown of some prawn hatcheries in many prawn producing countries (Shewbart *et al.*, 1972; Takahashi *et al.*, 1984; 1985b; Sunaryanto and Mariam, 1986; Baticados, 1988; Lavilla-Pitogo *et al.*, 1990). In 1991, massive luminous bacterial disease outbreaks occurred in Indonesia, causing a reduction of almost 70% in prawn post-larvae production resulting in losses of US$ 8.1 billion (Prayitno, 1994). As a result, the price of postlarvae ready to be reared in on-growing ponds doubled from US$ 0.50 per thousand to US $1.00 per thousand postlarvae (PL) -15 stage prawns. Luminous vibriosis outbreaks in Indonesia and the Philippines appear to be linked to changes in salinities and pH of the water as they have been reported to peak during the rainy season (Sunaryanto and Mariam, 1986; Prayitno, 1994; Prayitno and Latchford, 1995).

A rare acid-fast bacterial infection in prawns has been reported in *P. vannamei* by some authors (Lightner, 1985; Krol *et al.*, 1989), caused by a mycobacterial, gram positive, acid fast bacterium. Affected tissues contain melanized multifocal nodular lesions with acid fast debris. *Mycobacterium* infection has also been reported in *M. rosenbergii.* (Brock *et al.*, 1986). and in the crayfish *(Austropotambius pallipes)* together with norcadia infection.

Disease control

Rapid identification of bacterial pathogens in an intensive culture system is essential for appropriate disease control strategies. In fish, enzyme-linked imunosorbent assays (ELISA) have been used to diagnose and confirm furunculosis in moribund fish (Adams and Thompson, 1990; Oku *et. al.*, 1987), and prawns (Adams, 1991a; b). However, while such methods are useful in fish disease control, the opportunistic nature of most *Vibrio sp.* pathogens of prawns makes them less so in prawn culture situations.

Control methods against bacterial disease may be directed against any one or a combination of the components of the disease triad discussed above. Methods currently used which are directed against the pathogens include:

Antibiotics / Chemotherapeutants

Antibiotics are widely used in aquaculture (Camper and McFeters, 1979; Castille and Lawrence, 1986; Brown, 1989; Baticados *et al.*, 1990; Baticados and Paclibare, 1992; Williams *et al.*, 1992; Limsuwan, 1993). In penaeid prawn culture, antimicrobials are the most commonly used class of therapeutant and chloramphenicol, furacin, furazolidone and oxytetracycline have been reported to be effective in combating bacterial disease in prawns in laboratory trials (Takahashi *et al.*, 1985a; Sunaryanto and Mariam, 1986; de la Peña *et al.*, 1993).

Brown (1989) reported the prophylactic use of low levels of antibiotics in hatcheries in Ecuador rather than occasional application of higher levels of antibiotics to cure problems which have already occurred. However, (Williams *et al.*, 1992) reported on the declining usefulness of oxytetracycline due to increasing resistance by many target strains of bacteria. Other authors (Baticados *et al.*, 1990; de la Peña *et al.*, 1993), have also reported the low sensitivity of bacteria to oxytetracycline. The more powerful antibiotic, chloramphenicol has been used widely in larval culture as a result of its broad spectrum action (Brown, 1989; Williams *et al.*, 1992; Primavera *et al.*, 1993). Empirical doses for administration have even been established (Baticados *et al.*, 1990). Despite this, Baticados *et al.* (1990), reported varied responses of bacteria to chloramphenicol. In addition, its potential toxicity to humans (it has been reported to cause irreversible aplastic anaemia in humans (Brown, 1989), as well as the potential for development of chloramphenicol resistant human pathogens should limit its use., The genus *Vibrio*, one of the bacterial genera most targeted by the addition of antibiotics to culture water, includes some potent bacteria which associate with humans and shellfish such as *Vibrio cholerae* which causes cholera in humans and is commonly associated with planktonic crustacean copepods on which crustacean shellfish feed. Of greater importance is the realisation that a resistant species of bacteria can transmit its resistance to an antibiotic to a different bacterial species by genes contained on plasmids. There is a danger that, while antibiotic resistance builds up within human pathogens in the sea, such antibiotic resistance may be transferred to normal human gut bacteria if antibiotic resistant bacteria are ingested (Brown, 1989)., Research on the effectiveness of applying antibiotics to reduce mortalities in hatcheries is limited. Brown (1989) reported a *Pecten maximus* (bivalve) hatchery in which, a significant drop in numbers of *Vibrio*-like bacteria in the culture water occurred following treatment with chloramphenicol but, one of the twenty-one strains present at the end of the trial was resistant to chloramphenicol.

Apart from the immediate dangers of encouraging antibiotic resistance and possible toxicity toward the larvae, the regular use of therapeutic agents encourages the propagation of weak and substandard larvae. In addition an initial dependence on drug use usually leads to the need for continuous application on an increasing basis in order to maintain the required effect thus raising costs. Samuelsen *et. al.* (1994) have reported reduced antibiotic efficacies after as little as one month's use.

Finally, antibiotic persistence in animal tissue and sediments has been reported (Jacobsen and Berglind, 1988; Jacobsen, 1989; Samuelsen, 1989; Coyne *et al.*, 1994; Kerry *et al.*, 1995; Capone *et al.*, 1996). Indiscriminate use would therefore, increase the risks of encouraging antibiotic resistance in a wide variety of bacterial strains.

Other chemotherapeutants such as sodium hypochlorite, malachite green, formalin, hydrogen peroxide, iodine and chlorine have also been used to control bacteria (Castille and Lawrence, 1986; Lee and Wickins, 1992; Primavera *et al.*, 1993; Boyd, 1996). In using chemotherapeutants, the mere knowledge of a particular compound's toxic level to penaeid prawns is insufficient to establish its potential for use

(Castille and Lawrence, 1986; Baticados *et al.*, 1990; Williams *et al.*, 1992). Ideally, this information (toxic level) must be viewed along with the compound's relative efficiency against target disease organisms. The toxicity and efficacy vary depending on the drug used, larval stages, species and the target organism (Castille and Lawrence, 1986) and only use of data on all factors will allow estimation of a drug's safety margin (Mohney *et al.*, 1994). To do this in a disease situation is costly and time consuming. Baticados *et al.*, (1990) concluded that chemical control of luminous vibriosis in prawn hatcheries was of limited use based on the efficacy of the readily available drugs, the development of antibiotic resistance by the bacteria, and the limited tolerance of the prawn larvae to the drugs.

Environmental control methods

Several researchers (Sunaryanto and Mariam, 1986; Baticados, 1988; Prayitno and Latchford, 1995) reported increased incidents of luminous bacterial disease epizootics observed in Indonesia during the rainy season and some authors (Baticados, 1988; Prayitno, 1994; Prayitno and Latchford, 1995), observed that low salinity and neutral to acidic pH medium significantly increased the virulence of one of the causative *V. harveyi* strains. These authors suggested that control methods such as increasing salinities and pH values during rainy seasons could prevent the occurrence of such diseases. Kaspar and Tamplin (1993) demonstrated an influence of temperature on the tolerance to unfavourable salinities of several strains of *V. vulnificus* usually associated with oysters. Correlations occurred between decreasing temperatures and increasing salinities (above optimal ranges) respectively, with increasing mortalities. However, reckless use of environmental methods of pathogen control may simply select for potential pathogens which may be more virulent under the new environmental conditions and use must be based on a sound knowledge of possible effects on both the target pathogen as well as other possible pathogens which might be affected. Mohney *et al.* (1994) reported that a massive epizootic of vibriosis in Ecuador in 1989 - 1990 was caused by *Vibrio sp.* isolates with higher salinity optima and postulated that these bacteria became more prominent at the higher than usual salinity and nutrient levels obtained after a prolonged period of drought.

Several authors (Anderson *et al.*, 1988; Brock and Leamaster, 1992) reported that of all the methods of disease management tried, the most successful method found for preventing mortalities in subsequent production cycles at affected farms was a reduction in pond biomass by partial harvesting and increased water exchanges once mortalities began or in the last 4 weeks of the grow-out cycle. Also important were the changes in the pond preparation between production cycles. In addition to the usual draining and drying of ponds until the bottom cracked, excessive detritus was physically removed and quicklime (CaO) was applied at the rate of 0.5kg.m^{-2} of pond bottom. In addition, some researchers (Baticados *et al.*, 1990) have suggested that prevention with particular attention on rigorous water management and sanitation to prevent entry of potentially pathogenic bacteria, is the best method of control.

Some of the more widespread methods currently used to disinfect or partially sterilise rearing water before entry into the facilities include use of ozone, chlorination and ultraviolet light irradiation (Murchelano *et al.*, 1975; Colberg and Lingg, 1978; Wedemeyer *et. al.*, 1982; Harris *et al.*, 1987; Paller and Lewis, 1988; Reid and Arnold, 1992; 1994; Liltved and Landfald, 1993; Liltved *et al.*, 1995; Boyd, 1996).

Use of probiotics

An alternative method of controlling pathogenic bacterial populations through modification of the environmental component of the disease triad, may be the use of probiotic bacterial strains which control the concentrations of potential pathogens by competitive exclusion. Addition of a non-pathogenic strain of *V. alginolyticus* to *P. vannamei* post-larval cultures (Garriques and Arevalo, 1995), resulted in increased survival rates and growth over controls and tanks with antibiotic prophylaxis. In addition, Douillet and Langdon (1994) have reported enhancement of *Crassostrea gigas* larval survival by addition of a probiotic strain of bacteria. Other researchers (Gomes, 1992), have reported that the maintenance of a balanced bacterial community enhances penaeid larval cultures possibly by preventing development of potential pathogens. Brown (1989), reports an unquantified hatchery practice in Taiwan in which, growth of harmless bacteria were encouraged by the addition of algae to prawn cultures grown in the dark and enrichment of the water with higher unsaturated fatty acids (HUFA) compounds.

The probiotics appear to exert their effects in a variety of ways: Intriago and Jones, (1993) have reported that *Flexibacter* strain Inp3 provided a source of food for *Artemia* and assisted in the digestion of algae. Similarly, Garriques and Arevalo (1995) have reported increased growth and survival in *P. vannamei* post larvae following additions of a probiotic strain of bacteria. In addition Kaspar and Tamplin (1993) reported that while addition of a rifampin resistant strain of *V. vulnificus* to sterile water resulted in a 2 log increase in bacterial counts over 14d, addition of the same strain to natural sea water caused a decline in bacterial numbers to undetectable levels suggesting competition by biotic factors present in the water. This concept of naturally balanced bacterial populations as being beneficial is enhanced by the results obtained by Douillet and Langdon (1994) who showed that additions of a probiotic strain of bacteria enhanced growth in axenic cultures of larval *C. gigas* but not in xenic cultures.

Probiotic effects observed may yet be due to a combination of factors: early penaeid larval stages establish their bacterial flora in a non-selective way and the gut microflora of *P. japonicus* larvae have been shown to generically mirror the environmental microflora (Yasuda and Kitao, 1980). As a result, in addition to competitive control of potential pathogens, the properties of the bacterial flora are important. Establishment of a large amount of bacteria producing extracellular digestive enzymes may therefore facilitate nutrition. McDonald *et al* (1986) demonstrated the contribution of bacteria in the nutrition of the host *Solea solea* by producing vitamins and enzymes. Yazawa *et al.*, (1988) reported the presence of eicosapentanoic acid producing bacteria in the intestines of certain oily fish, a potential source of essential fatty acids for the fish's dietary requirements. Jones *et. al.*, (1997) have reported that incorporation of an *E.*

coli strain genetically engineered for enhanced protease production into *P. indicus* post larval diets significantly enhanced growth rates.

The maintenance of a stable gut microflora in penaeid prawns is difficult as the fore and hind guts are lined with cuticle which is shed during moulting. Established microflora associated with the fore and hind guts would therefore, be lost during each moult cycle. The non-selective mode of re-establishing the new gut microflora, emphasises the requirement for a constant balance in the environmental microflora.

As explained previously, knowledge of the biochemical characteristics of involved bacteria is essential in attempts to use probiotics. Lightner (1992), reported that the addition of 20kg.ha^{-1} of sucrose to prawn ponds with developing sea-gull syndrome (SGS)-vibriosis provided an effective reduction in the luminous bacteria and prawn losses. The suggested reason was that *V. parahaemolyticus*, the most common isolate from SGS-vibriosis infected prawns, cannot utilise sucrose whereas, other apparently less pathogenic *Vibrio* species, can utilise the sugar as well as the high levels of nitrogen (urea and/or nitrate) for their growth, thus "blooming" and removing available nutrients needed by *V. parahaemolyticus* for growth.

Despite the wide diversity of bacterial species and strains present in natural bacterial communities, species or strains antagonistic towards potential pathogens may be few. Gatesoupe (1990) has reported that in a bacterial community obtained in a turbot larval hatchery, competitive control of a pathogenic strain of *Aeromonas sp* was due to a single non-pathogenic *V. alginolyticus* strain.

Vaccines/Immunostimulants

Vaccination is the process of inducing protective immunological memory in an organism. Hence, if the correct antigen is presented to the correct branch of the immune system, an immune response will be elicited which will display immunological memory of the antigen. Vaccines already play an important role in the farming of fish (Holm and Jorgensen, 1987; Hjeltnes *et. al.*, 1989; Norqvist *et al.* 1989; Lillehaug, 1991; Iida and Wakabayashi, 1992; Nikl *et. al.*, 1993).

Despite the realisation that unlike fish, decapods do not possess the adaptive immune system which responds to vaccination (Lackie, 1980; Klein, 1984; Ratcliff *et al.*, 1985; Smith and Chisholm, 1992), significant protection against pathogens has been reported in prawns following prior exposure to killed or live, non-pathogenic bacterial cells (Itami *et al.*, 1989; 1992; Adams, 1991b; Prayitno, 1994). Classically, vaccines are prepared from specific pathogens to give protection against specific diseases (Amos, 1981). Although some partial specificity has been reported in the activity of prawn vaccines, the protection afforded has been demonstrated to be due to the overall enhancement of the immune system (immunostimulation) (Itami *et al.*, 1989; Adams, 1991b; Song and Hsieh, 1994; Sung *et al.*, 1996).

Microbial cell wall components (lipopolysaccharides (LPS) and beta glucans (β-glucans) of gram negative bacteria and fungi respectively), and crude bacterial preparations have been shown to enhance disease resistance in prawns (Adams, 1991b; Vargas-Albores *et al.*, 1993a; b; Song and Hsieh, 1994; Vargas-Albores, 1995; Sung *et al.*, 1996).

Johansson and Soderhall (1989) suggested that beta-glucan protection is probably due in part to its activation of the prophenoloxidase (proPO) system which represents the terminal component of a complex cascade of enzymes which function in non-self recognition and host defence (Söderhäll, 1982; Ashida and Söderhäll, 1984; Söderhäll and Cerenius, 1992). These mechanisms include bactericidal activity and phagocytosis.

The Immune System:

Increased disease susceptibility is usually a consequence of impairment of the immune system resulting from environmental factors and may be very important at the population level. This makes a detailed understanding of the immune system imperative. Despite the abundance of literature available on the diseases of prawns, the immune system in penaeid prawns has not received much attention, and there are few reports in the literature on the action of the immune system and how its manipulation may help in the defence against infection.

The possible prophylactic use of immunostimulants in situations known to result in larval stress and hence, increased susceptibilities to disease (such as during periods of increased pathogen abundance like spring blooms or developmental phases of particular vulnerability to disease), offers potential manipulative advantages in prawn hatcheries. Even though most disease causing bacteria are opportunistic pathogens, their presence may still create conditions of reduced performance and sub epizootic mortalities. Latchford *et al.*, (1995) reported that immersion vaccination of *P. monodon* postlarvae with killed *V. harveyi* sufficiently increased the resistance of such postlarvae to counteract such conditions.

Disease Resistance:

Research into prawn immune systems have been focused mainly on processes mediated by haemocytes: Phagocytosis, encapsulation, melanisation and coagulation (Itami *et al.*, 1989; Omori *et al.*, 1989; Hose *et al.*, 1990; Martin *et al.*, 1993; 1996; Vargas-Albores *et al.*, 1993a; b; Song and Hsieh, 1994).

Haemocytosis, an increase in the number of haemocytes, is considered to be a prerequisite of phagocytosis and encapsulation since an increase in the number of blood cells contributes towards these processes (Hose *et al.*, 1992). In fact, some studies have already documented enhanced haematopoiesis in decapods infected with a virus (Lightner, 1983) and a fungus (Hose *et al.*, 1984) although no categorisation of the types of haemocytes produced were performed. However, other workers (Martin *et al.*, 1993; 1996; Hauton *et al.*, 1997), have reported a marked haemocytopaenia following bacterial injection and increased haematopoiesis only after 72-96h following injection. Despite the depleted number of circulating haemocytes, fresh challenges by injected bacteria gave the same initial effective clearance of bacteria (Martin *et al.*, 1993). These authors suggested that the steps involved in initial depletion of injected bacteria were independent of circulating haemocyte counts and depended instead, on physiochemical adherence of bacteria to the haemocoel linings where circulating granulocytes would then

attach and phagocytose the bacteria. These authors described histological sections showing bacteria to be attached to the haemocoel where they were subsequently surrounded by small granular haemocytes.

Although the clearance of foreign particles and the associated haemocytopaenia and enhanced haemopoiesis has been described, less attention has been paid to bacterial dynamics *in vivo* following injection in both "immune" and naïve prawns.

Knowledge of the bacterial dynamics *in vitro* and *in vivo* involved in the resistance of naive and "immunized" penaeid prawns to infection by a normally pathogenic bacterium following vaccination may provide a model for studies of prawn antibacterial immunity.

Recognition of foreign bodies is a prerequisite for the initiation of immune processes. In crustaceans, the actual processes of foreign recognition are still unclear. Some researchers (Smith and Söderhäll, 1983; Ashida and Söderhäll, 1984, Söderhäll *et. al.*, 1986; Adams, 1991b) have suggested this to be due to cell bound opsonic factors associated with the proPO system. This system has been shown to be specifically activated by β-glucans and LPS (Hose and Martin, 1989; Vargas-Albores *et al.*, 1993a; b)., Other researchers, (Vargas-Albores *et al.*, 1993a; b), have attributed this to lectins present in prawn plasma. Lectins exist not only as opsonizing molecules (Vasta, 1987; Vargas-Albores *et. al.*, 1993a; b; Vargas-Albores 1995) but also, as carbohydrate receptors on the membrane of the haemocytes (Renwrantz and Stahmer, 1983; Vasta, 1987; Renwrantz, 1990). Considerable variation in PO activity has been reported (Sung *et al.*, 1996) following proPO activation of *P. monodon* haemocyte lysates by different immunostimulants (β-glucans, LPS and *Vibrio* antigen). Similarly, Song and Hsieh (1994) have reported differences in stimulation of *P. monodon* haemocytes using different immunostimulants. The antigenic capacities of immunostimulant preparations are determined by their abilities to stimulate the immune system. These are related to bacterial surface characteristics which are sometimes related to virulence determination. However, while in fish, the superiority of vaccines from more virulent bacteria over less virulent strains of the same species have been recognised (Trust *et al.*, 1982; McCarthy *et al.*, 1983; Olivier *et al.*, 1985; Loghothetis and Austin, 1996), no attempts have been made to determine the vaccine potentials of various bacterial species in prawns.

Apart from these immediate cellular responses to microbial infection, some studies have reported the acquisition of a longer lasting immune state in penaeid prawns following prior exposure to killed bacterial cells or immunostimulants (Itami *et al.*, 1989; Song and Sung, 1990; Adams, 1991b; Sung *et. al.*, 1991; 1996; Prayitno, 1994).

In insects, the development of the acquired immune state corresponds temporally with the synthesis of several proteins that appear in the haemolymph (Faye and Wyatt, 1980; Hoffman *et al.*, 1981; Engström *et al.*, 1984; Dunn *et al.*, 1985; Flyg *et al.*, 1987; Kaaya *et al.*, 1987; Natori, 1987). Such proteins include lysozyme which has been demonstrated in the haemolymph of several insects (Hultmark *et al.*, 1980; Dunn *et al.*, 1985; Flyg *et al.*, 1987; Kaaya *et al.*, 1987) and bivalves, (Cheng and Rodrick, 1974; Foley and Cheng, 1977; Cheng *et al.*, 1980) as well as other antibacterial proteins. Attempts to demonstrate lysozyme

activity in prawns have so far, been equivocal. Some authors (Guzmán *et al.*, 1993) have reported haemolytic activity in cell free haemolymph of *Penaeus vannamei* while, other researchers (Noga *et al.*, (1996) have been unsuccessful. Some reports have been made of antibacterial activity in plasma of both vaccinated (Adams, 1991b; Sung *et al.*, 1996) and naïve (Noga *et al.*, 1996) prawns. However, such reports must be interpreted with caution as a result of the different methods used in obtaining the plasma used. Some authors obtained plasma after allowing withdrawn haemolymph to clot and homogenised the clot before centrifuging and testing for antibacterial activity. Such methods would obviously lead to the release of cell bound antibacterial components into the haemolymph and such contamination may account for some of the results obtained. In addition, few attempts have been made towards either quantitative or qualitative analyses of these observed antibacterial factors and their significance to overall long term disease control strategies has been largely overlooked.

Significant protection has been reported following pathogenic challenge when prawns have been vaccinated by immersion (Itami *et al.*, 1989; Adams, 1991b; Prayitno, 1994; Sung *et al.*, 1994; 1996), but not orally. Oral delivery of vaccines is the more desirable method for vaccination and immune stimulation of larval prawns due to its ease of administration, their fragile nature and the large quantities of animals to be treated. However, levels of protection obtained after oral vaccination in fish is usually lower when compared to injection or immersion modes of vaccination (Lillehaug, 1989a; b; Dunn *et al.*, 1990). Oral administration of immunostimulants in diets has been reported to enhance growth (Song and Sung, 1990) and survival (Itami and Takahashi, 1991) in *P. monodon* larvae and post larvae. However, such studies were either not followed by pathogenic challenge (Itami and Takahashi, 1991), or such challenges failed (Song and Sung, 1990). Some researchers (Sung *et al.*, 1994; Sung and Song, 1996) studied the uptake of *Vibrio* components in *P. monodon* after immersion and found that particulate matter can enter via the gills and digestive system and quickly (within 5min) be distributed to the hepatopancreas and haemopoieteic tissue. Sung and Sung, (1996) suggested that absorption of *Vibrio sp.* antigen delivered was similar whether delivered orally or by immersion. However, tissue location of *Vibrio* antigen in the digestive system may not imply final functionality of the antigenic properties of the vaccine. In model studies on antigen uptake from the gut of fish, Jenkins (1994) reported that much of the proteins was degraded and the antigenic properties lost. This finding shows that development of a good oral vaccine is dependent on proper protection from gastric destruction.

A knowledge of the duration of immunostimulant efficacy is essential in practical prawn farming. However, much work is still needed to produce enough documentation of practical formulas and application procedures. Sung *et al.*, (1994) reported that *in vivo* protection against *V. vulnificus* pathogens lasted up till 10d after immersion vaccination of *P. monodon* postlarvae in a β-glucan suspension. In *in vitro* studies, Sung *et. al.*, (1996) reported that antibacterial activity obtained in *P. monodon* plasma after immunostimulation with *Vibrio* antigen was only apparent between 6 and 12h following vaccination, while when animals were vaccinated with zymosan and β- glucan, the antibacterial activity obtained extended

from 3h to 24h. These differences are probably due to the different efficacies of the stimulatory substances in enhancing disease resistance. In contrast, Adams (1991b) reported the persistence of induced bactericidins in *P. monodon* plasma for up to 5d after vaccination.

Pesticides are common pollutants of aquaculture environments and one of the most serious diseases recently affecting prawn culture is the Taura syndrome (TS) disease (Jimenez, 1992; Lightner, 1996), which has been variously suggested to be caused by either a virus or a mixture of fungicides running off from land based cultivation sites. Prawn cellular defence mechanisms have been suggested as indicators of pesticide pollution (Weeks-Perkins *et al.*, 1995), and methyl parathion based insecticides have been reported to significantly reduce haemocyte chemotaxis and phagocytic activities (Tangtrongpiros *et al.*, 1997) and haemocyte respiratory bursts (Bodhiaksha and Weeks-Perkins, 1994) in *P. monodon*. However, the effect of these reported decreases in circulating haemocyte counts on the overall immune state of the prawns is unclear. As reported above, Martin *et al.*, (1993) have shown that initial reduction of injected bacteria in *Sicyonia ingentis* were independent of the counts of circulating haemocytes and depended instead, on physiochemical adherence of the invading micro-organisms to the haemocoel linings. However, *in vivo* pathogenic challenges were not performed on these prawns. In an attempt to address the question of TS, the effects of a mixture of TS implicated fungicides on various biological functions of a different species of prawn from that currently affected by TS were investigated. The effects of live *in vivo* pathogenic challenges on survival of prawns exposed to TS implicated fungicides were also. assessed

The aims of this study

The aims of this study were:

1 to obtain a method for environmental control of bacteria potentially pathogenic to *P. indicus* larvae.

2 to obtain a method for vaccine preservation and oral delivery and to assess the efficacy of cross-protection induced upon vaccination.

3 to study the duration and persistence of cell-free antibacterial activity in plasma of vaccinated prawns and to isolate and identify new proteins synthesized upon vaccination.

4 to establish the aetiology of Taura syndrome and the effects of fungicides on reduction of immunocompetence and various aspects of the biology of *Penaeus indicus*.

CHAPTER 2

MATERIALS AND METHODS

Bacterial culture media

Several culture media were used for the culture and identification of bacteria used in this study. All media were autoclaved at 121°C and 1.055g.mm^{-2} for 15min before use unless otherwise stated.

Modified sea water complex (SWC) medium (Reichelt and Baumann, 1973)

(Medium for general bacterial culture)

Yeast extract	3.0g
Bacto-peptone	5.0g
Glycerol	3.0ml
Filtered sea water	750.0ml
Distilled water	250.0ml
pH	7.2 - 7.3

TCBS (Thiosulphate citrate bile salt sucrose) agar

(selective medium for *Vibrio sp.*)

TCBS powder	88.0g
25‰ filtered, sterile sea water	1L

Prepared by heating gently over a low flame until boiled prior to use.

Pseudomonas base medium (lab M)- selective medium for Pseudomonas sp.

Pseudomonas agar base	48.4g
glycerol	10.0ml
deionised water	1L
pH	7.2 - 7.3

A lyophilised antibiotic supplement (x108 C.F.C) was added after the autoclaved base medium had been cooled to around 47°C.

Unless otherwise stated, total viable counts (TVC) of bacteria were estimated by making ten-fold serial dilutions of an aliquot (usually 1ml) of the required sample in sterile saline and spreading 50 or 100µl

aliquots of each dilution on SWC agar plates. The plates were incubated at 28°C for 24 or 48h and the TVC of bacteria per ml of the original sample was estimated from the number of bacterial colonies grown.

Diets

Protozoea (PZ) stage artificial diets used were the microencapsulated diet (MED), Frippak crustacean algal replacement (CAR) or a commercial microparticulate diet (MPD). The mysis (M1 - M3) stage and M3 - post-larval (PL-1) stage diets used were the MED crustacean diet numbers 2 and 3 (CD2 and CD3) respectively, (INVE Aquaculture, Belgium). The live diets used were a mixture of the algae *Skeletonema costatum* and *Tetraselmis chuii* fed at concentrations of 45 and 25 cells.μl^{-1} respectively to the PZ1 - PZ3 stages, reduced to 15 and 10 cells.μl^{-1} respectively at the M1 to PL1 stages when supplementation with *Artemia* at 2 nauplii.ml^{-1} commenced.

Prawn Rearing Methods

Unless otherwise stated, PZ1 stage larvae of *Penaeus indicus* spawned in the School of Ocean Sciences, were reared in 2L round-bottomed flasks (RBFs) at a temperature of 28°C ± 2°C and a stocking density of 100 larvae.L^{-1}. Air was supplied through pipes attached to glass rods and they were disinfected prior to use by a 30min immersion in 5 - 10% sodium hypochlorite and then thoroughly rinsed with sterile water. The flask necks were plugged with sterile wads of cotton wool to prevent airborne contamination and sterile air was supplied through 0.2μm filters (GELMAN). Rearing water was changed (100%) daily unless otherwise stated. Larvae were fed daily with either live diets as described above, or MED at the following rates: PZ1 - PZ3, M1 - M3 and M3 - PL1 stages were fed concentrations of 4, 6 and 8 mg.L^{-1}.day^{-1} respectively of CAR, CD2 and CD3. Daily *Artemia* supplementation at 2 nauplii.ml^{-1} was added from M1 - PL1 stages.

Bacterial strains

Pathogenic *Vibrio harveyi* strains BP03, BP04, BP05 and a less pathogenic strain: IN7, isolated from healthy *Penaeus monodon* in Indonesia (Prayitno, 1994) were obtained from the School of Ocean Sciences. *Vibrio harveyi* strain COL2 isolated from diseased *P. monodon* was a kind gift from INVE Aquaculture. The strains were maintained on TCBS agar plates at 4°C and cultured in SWC at 28°C for 24h before use.

Vaccine production

Log phase cultures of all the gram negative bacteria listed above were killed by addition to 0.5% of 100% formalin. The cultures were incubated with shaking, at 25°C for 12h. The formalin killed bacteria were centrifuged at 11000 rpm for 2min. The resulting pellet was then rinsed and re-suspended in sterile saline. This cycle of centrifugation and resuspension was repeated three times and the final pellet was suspended

in sterile saline to the required concentrations before use. Samples to be freeze-dried were aseptically poured into sterile tubes, and flash-frozen in liquid nitrogen before being freeze-dried. Before use, the freeze dried pellets were redissolved in sterile saline and sonicated gently in a bath sonicator to completely re-suspend the killed cells without disruption of cell integrity.

A sterility check was performed by streaking 1ml of the rinsed samples on SWC agar plates and incubating at 28°C for 24h to check for bacterial growth. Samples showing bacterial growth were discarded.

Humoral And Cellular Immunity Assays

Bacterial species used and their preparation:-

In both *in vitro* and *in vivo* bacterial killing assays, and for stimulation of the proPO system of naïve *P. vannamei* juveniles, non-pathogenic *Vibrio harveyi* strain DPEX, and pathogenic *Vibrio harveyi* strains BP03, BP04, BP05 and IN7 were obtained, prepared and stored as described above. In addition *Escherichia coli* strain XL1-Blue MRF′ (Stratagene), resistant to 15μg.ml^{-1} tetracycline and with a recombinant plasmid providing resistance to 100μg.ml^{-1} ampicillin, was maintained on LB slants and was grown in SWC at 37°C for 24h before use. *Aeromonas hydrophila* strain PM from diseased *Penaeus monodon* (Prayitno, 1994), were stored on SWC agar plates at 4°C and cultured in SWC medium. Lyophilised *Micrococcus lysodeikticus* (*luteus*) (Sigma), was similarly cultured in SWC medium at 28°C for 24h before use.

Vaccines were made by exposing log-phase cultures of the relevant bacterium to 0.5% formalin and incubating at 20 - 25°C with gentle shaking for 12h as described above.

Measurement of soluble protein

Unless otherwise stated, measurement of soluble protein content was by the bicinchoninic acid reaction method (Smith *et al.*, 1985; Shihabi and Dyer, 1988), and used bovine serum albumin (BSA) as standard.

Statistical Analysis Of Data

All data obtained were tested for normality using the Anderson-Darling test for normality and homogeneity of variance using Bartlett's test for homogeneity of variance in normal distributions, and Levene's test for homogeneity of variance in continuous distributions. Where these conditions were satisfied, parametric tests such as T-tests, analyses of variance (ANOVA) and the general linear model (GLM) were used in data analysis. Where ANOVA suggested the occurrence of significant differences, second order comparisons such as Tukey's and Scheffe's pairwise comparisons were included to identify the souce of the differences. In situations where the conditions of normality or homogeneity were violated, the non-parametric alternatives such as the Kruskal-Wallis tests and Mann-Whitney Rank sum tests were used (Sokal and Rohlf, 1995). Except where otherwise stated, significant levels of difference was set at $P<0.05$.

CHAPTER 3

EFFECTS OF WATER PRE TREATMENT ON SURVIVAL OF PENAEID LARVAE

Introduction

Prawn pathogens have been reported as part of the normal microflora in natural aquatic systems and bacterial disease in prawn hatcheries has mainly been attributed to excessive levels of a particular pathogen in the environment. This usually results from environmental changes which favour the development of the pathogen (Baticados, 1988; Gatesoupe, 1990; de la Peña *et al.*, 1993; Mohney *et al.*, 1994; Griffith, 1995; Prayitno and Latchford, 1995). As hatchery water supplies are a major route for the introduction of potentially pathogenic bacteria into prawn culture facilities (Baticados *et al.*, 1990; Lavilla-Pitogo *et al.*, 1990; Primavera *et al.*, 1993), a wide range of methods are employed to prevent, or at least limit the number of potential pathogens in water supplies. However, absolute sterility of rearing water is very difficult to achieve in laboratory situations and impossible in commercial prawn hatcheries.

Antibiotics are the most common class of therapeutants used to control pathogen concentrations (Camper and McFeters, 1979; Takahashi *et al.*, 1985a; Castille and Lawrence, 1986; Sunaryanto and Mariam, 1986; Baticados *et al.*, 1990; Baticados and Paclibare, 1992; Bell, 1992; Williams *et al.*, 1992; de la Peña *et al.*, 1993; Limsuwan, 1993; Primavera *et al.*, 1993). However, reduced efficacies have been reported for some antibiotics after as little as one month's use (Samuelsen *et al.*, 1994). In addition, Baticados *et al.*, (1990) has reported varied antibacterial responses to various antibiotics. Antibiotics often persist for long periods in both animal tissues (Jacobsen, 1989) and sediments (Jacobsen and Berglind, 1988; Samuelsen, 1989; Coyne *et al.*, 1994; Kerry *et al.*, 1995; Capone *et al.*, 1996). Antibiotic treatment may also result in prawns that are unfit for human consumption and more seriously, in the development of antibiotic resistance (Brown, 1989; Baticados *et al.*, 1990; Williams *et al.*, 1992), a problem exacerbated by the possibility of plasmid mediated transfer of antibiotic resistance between these bacteria and potential human pathogens.

Other chemotherapeutants such as sodium hypochlorite, malachite green, formaldehyde, hydrogen peroxide and iodine have been used (Castille and Lawrence, 1986; Lee and Wickins, 1992; Primavera *et al.*, 1993; Boyd, 1996), but often depend on the host having a higher tolerance threshold level to the substance than the target bacteria. These differences are often marginal and in addition, depend on various physiological and environmental factors involved. Furthermore, after disinfection of the water, these compounds must be volatilised to prevent toxicity to the larvae thus leaving the culture water exposed to recolonisation by bacteria. Dupree (1981) concluded that some other widely used pre-treatment

methods such as ultraviolet light irradiation (UVT), ozonation (O_3) and in certain instances, chlorine were more suitable pre-treatment methods.

Chlorination has been widely used in disinfecting rearing water (Boyd, 1996). However, chlorine residues are toxic to animals and some evidence has also suggested that chlorination may cause the formation of carcinogenic compounds (Murphy *et al.*, 1975; Ward and DeGraeve, 1978; Jolley *et al.*, 1982). As a result, increasing interest has been concentrated on alternative methods of disinfection such as UVT and O_3. However, UVT efficiencies are determined by the size, species and growth stage of bacteria, as well as water clarity (Murchelano *et al.*, 1975; Chang *et al.*, 1985; Harris *et al.*, 1987). Bacteria are inactivated by absorption of UVT by the DNA which forms links between successive pyrimidine bases to form dimers in the DNA. These thymine dimers distort the formation of the double helix and inhibit normal DNA replication (Harris *et al.*, 1987). However, the reversal (photoreactivation) of such UVT-induced mutation by visible light or high wavelength light dependent enzymes (which can split the thymine dimers) has been reported (Jagger, 1967; Levine and Thiel, 1987; Liltved and Landfald, 1993). Dark repair of UVT induced damage using two enzymes which excise the dimers and replace the excised regions by copying the undamaged strand have also been reported (Jagger, 1967; Harm, 1968; Masek and Sedliakova, 1977).

In addition to being toxic to bacteria (Colberg and Lingg, 1978; Wedemeyer *et al.*, 1982; Liltved *et al.*, 1995), ozonation improves water quality through the oxidation of ammonia and nitrite (Colberg and Lingg, 1978; Paller and Lewis, 1988) and the oxidation of dissolved organics (Paller and Lewis, 1988; Reid and Arnold, 1994). However, ozone and its intermediates are highly reactive and even small amounts of organic matter in the water causes a rapid dissipation (Broadwater *et al.*, 1973; Liltved *et al.*, 1995) . In addition residual ozone is highly toxic and has been reported to cause gill epithelial damage and death of *Onchorhynchus mykiss* exposed to as little as 0.0093 mg $O_3.L^{-1}$ (Wedemeyer *et al.*, 1982). Despite this, Reid and Arnold (1994) reported that *P. vannamei* could tolerate 24h exposure to residual ozone concentrations of 1.0mg.L^{-1} compared to fish (red drum, *Sciaenops ocellatus*) which could only tolerate 0.1mg.L^{-1}. The authors attributed this to the chitinous outer layer on prawn gills which protected the prawns from the oxidative properties of ozone.

As bacteria are re-introduced into culture water from a wide variety of sources including the diet and the larvae themselves (Nicholas *et al.*, 1989; Gatesoupe, 1990; Dehasque *et al.*, 1991; Verdonck *et al.*, 1994), other promising methods seek to control the levels of potential pathogens by the use of other bacteria as probionts (microbial culture suspensions or crude microbial extracts which contribute to microbial balance (Parker, 1974)), (Douillet and Langdon, 1994; Bergh, 1995; Garriques and Arevalo, 1995). Some researchers (Gomes, 1992; Alabi *et al.*, 1997), have also suggested that the maintenance of the natural balance of bacterial populations may benefit penaeid larval cultures. Indeed, Ottogalli (1991; 1992) successfully reared penaeid larvae without algae and exclusively on MED by deliberately including bacteria. In bivalves, Douillet and Langdon (1994) have reported enhancement of *Crassostrea gigas* larval survival due to the presence of naturally occurring microflora present in sea water and concluded that this

explained why addition of a probiotic strain of bacteria to rearing water enhanced survival of axenic algal-fed larvae but not the survival of xenic algal fed larvae.

The method by which such probiotics facilitate bacterial balance is unclear. While bacteriocin-like substances have been purified from *Vibrio sp.* (Schwinghamer and Rockwell, 1978; Hoyt and Sizemore, 1982), which inhibited growth of other bacterial strains in a simulated enteric environment, it conferred no competitive advantages in free-living situations. Competitive exclusion of pathogenic bacteria has been obtained by some researchers (Austin *et al.*, 1995; Garriques and Arevalo, 1995). Other researchers (Salvesen *et al.*, 1995; Skjermo *et al.*, 1997), using the principle that aged water will contain a higher proportion of non-opportunistic bacteria as opposed to the faster growing opportunistic bacteria, obtained enhanced growth and survival of turbot larvae grown in aged "matured" water along with a depression in TVCs. Bergh (1995) has suggested that *Vibrio sp* inhibition exhibited by bacteria associated with larval *Hippoglossus hippoglossus* was via inhibition of colonisation and proliferation of pathogenic intestinal bacteria. The enhanced growth reported by most researchers (Salvesen *et al.*, 1995; Skjermo *et al.*, 1997) upon addition of probiotics also suggests a nutritional contribution from the bacteria which provide essential nutrients. In addition, Skjermo *et al.* (1997) suggested that turbot larvae in microbially matured water benefit from a commensal gut microflora which may also improve digestion and nutrition in the early first phase of feeding. Furthermore, Intriago and Jones (1993) have demonstrated the nutritional contribution of *Flexibacter* strain Inp3 to *Artemia* diets.

Control of bacterial populations by the use of algae (Bell *et al.*, 1974; Kogure *et al.*, 1979; Kellam and Walker, 1989; Austin and Day, 1990; Austin *et al.*, 1992) as well as macroalgal extracts (Reichelt and Borowitzka, 1984; Kellam and Walker, 1989; de Nys *et al.*, 1995; Givskov *et al.*, 1996; Gram *et al.*, 1996) and bacterial swarming co-ordination (Ziebell *et al.*, 1977) have been reported. This emphasises their potential uses in maintaining the balance of bacterial populations. However, some researchers have also reported enhancement as well as suppression of bacterial populations by algae (Bell *et al.*, 1974; Kogure *et al.*, 1979) indicating that algae: bacteria interactions are species specific.

This chapter examines the effect of complete and partial pre-sterilisation of culture water on the composition and bacterial counts and relates results to survival of *P. indicus* larvae fed with live and artificial diets. The possible use of algal and bacterial exudates as antibacterial substances for bacterial control in hatcheries as well as the effects of using microbially matured water on the survival of *P. indicus* larvae is also investigated.

Materials And Methods

Diets used:

PZ1 to PZ3 and M stage larvae were fed MED diets as described in chapter 2. Live diets used were mixed algae (*Skeletonema costatum* and *Tetraselmis chuii*) *Artemia* nauplii were supplemented as described in chapter 2.

Tests on pre-treatment of water and bacterial growth

Tests were carried out using natural raw sea water (RSW) from the Menai Strait which was filtered to 5μm (5μmSW) and further treated in one of the following ways: either ozonated (O_3) (Sander ozonizer, model 25) using fine pore air stones for 15min at an ozone concentration of $10mg.O_3.h^{-1}$, passed through an ultraviolet lamp (UVT) of intensity $18\mu W.cm^{-2}$ at a rate of $1L.min^{-1}$ or autoclaved (AuW) as described in chapter 2. Controls received no further treatment (5μmSW). To investigate bacterial dynamics with live diets, an additional control treatment consisting of AuW water + mixed algae was included. *Artemia*, rinsed three times in sterile saline, were added at the concentration of 5 nauplii.ml^{-1} to this treatment at 24h. The TVC of bacteria in the *Artemia* suspension after the third, final rinse was determined by homogenising a small volume usually 2ml, of the *Artemia* suspension with a sterile glass piston homogeniser followed by serial dilution in sterile saline and plating on SWC agar, before addition to the culture water. Residual ozone in the O_3 samples was air-stripped by bubbling 0.2μm filtered air through the samples for 24h prior to addition of the diets. The different water samples were put into sterile, acid washed 2L RBFs held at 28° C ± 2°C with moderate aeration to completely mix the added diets. Air pipes and stones used were disinfected prior to use, with sodium hypochlorite as described in chapter 2. CAR feed was added at $4mg.L^{-1}.day^{-1}$ to the 5μmSW and UVT water treatments immediately after treatment while diet addition to the O_3 and AuW treatments started 24h after treatment to allow for air stripping of residual ozone and cooling of the autoclaved water respectively.

Bacterial counts were taken at 0, 24, 48 and 72h by removing 1ml samples from each of the treatments, making ten-fold serial dilutions with sterile sea water, and spreading 50μl aliquots of each dilution on SWC agar plates. These plates were incubated at 28°C for 48h and the TVC of bacteria per ml were determined from the colony counts.

Changes in the bacterial composition of treated water

200ml of sea water treated as above (except for the AuW treatment) were incubated with shaking, at 28°C and samples were withdrawn at 0 and 24h for plating on SWC and TCBS agar + 25‰ sterile sea water Preliminary identification of different bacterial colonies growing on both SWC and TCBS agar from all treatment groups was carried out using morphological criteria (see Appendix 3.2). Representative colonies

of presumed *Vibrio sp.* were re-streaked onto fresh SWC agar plates and incubated for 24h at 28°C. Single colonies from these plates were transferred to API 20 NE (BioMérieux) test strips using the manufacturer's protocol and incubated at 30°C for a 24h reading. Inconclusive readings were re-incubated for an additional 24h for a second, definitive reading.

Larval rearing methods:

PZ1 stage larvae of *P. indicus* were stocked as described in chapter 2, in 2L RBFs in either UVT, O_3, AuW or 5μmSW. Larvae were fed either MED or mixed algae as described in chapter 2. Rearing water was not changed for the duration of the experiments. Surviving larvae were counted daily and TVC was determined at every larval stage. All treatments were triplicated.

Use of bacteria from algal cultures as probiotics

These experiments and subsequent probiotic investigations involving larval survival were carried out in periods of bad water quality (May, 1996), following spring diatom and *Phaeocystis pouchetti* blooms in the Menai Strait when the water contained high concentrations of organic nutrients and had bacterial numbers above 3.0×10^6 cells.cm^{-3} due to algal decay (Blight *et al.*, 1995)

Abbreviation codes used in these tests were:

1. Water treatments were either 5μmSW (SW) or autoclaved water (AuW).
2. Diet treatments were either mixed algae (A) or MED
3. The addition or not of bacteria from algal culturesl populations was indicated either by +B (added bacteria) or -B (no added bacteria).

PZ1 stage larvae of *P. indicus* were reared in 5μm filtered sea water and fed as follows:

a. Mixed algae (*Skeletonema costatum* and *Tetraselmis chuii*) from xenic algal cultures at the rate of 45 and 25 cells.μl^{-1} respectively (SWA+B).
b. Mixed algae as in treatment group (a), from which the culture water and most of the associated bacteria had been removed by filtering out the algae on 3μm membrane filters and vortexing the membranes in sterile saline for 2 - 3min. to re-suspend the algae which were then fed to the prawns (SWA-B).
c MED (CAR) at the rate of 4mg.L^{-1}.day^{-1} with the addition of algal culture medium containing bacteria which had been removed from treatment group (b) above. To avoid overfilling the larval rearing tanks, The volume of algal culture medium was reduced when necessary, by concentrating the associated bacteria on 0.22μm membrane filters (Nuclepore) before being fed to the larvae (SWMED+B).
d. MED (CAR) at the rate of 4mg.L^{-1}.day^{-1} (SWMED-B).

Control prawns were raised in autoclaved, 5μmSW and fed using the same treatments as above. Abbreviation codes for the different treatments are similar to those above with autoclaved (AuW) replacing the 5μmSW codes :

a (AuWA+B).

b (AuWA-B).

c (AuWMED+B).

d (AuWMED-B).

Surviving larvae were counted and growth was measured at the M1 stage, TVCs and PVVC were taken at 0h (when the larvae were stocked) and the end of the experiments.

To determine the effects of algal exudates on larvae growth and survival, supplementary larval rearing trials were performed in AuW fed CAR with the addition of algal culture water which had been 3μm filtered to remove the algal cells (AuWMED+A+B). While in the second treatment, the bacteria in the algae culture water were retained on 0.22μm membrane filters, washed twice and resuspended in sterile saline before being added to the CAR fed larval cultures (AuWMED-A+B). Controls had 0.22μm filtered and autoclaved algal culture water added to 5μm filtered water and negative controls were with autoclaved sea water alone.

Larval survival in matured water

Water maturation tests were carried out to determine the effects of microbial maturation of sea water on penaeid larval growth and survival: 5μmSW was stored with aeration for three weeks in 20L carboys at an ambient temperature of 28 - 31°C. The temperature of the water was allowed to gradually rise to ambient levels (OW). Control treatments consisted of similar volumes of water with the temperature rapidly increased over 24h from 10°C to 28°C using a 50W "visitherm" VTH50 immersion heater (Aquarium systems) set to 28°C (NW). Positive controls were with OW to which was added bacteria from mixed algal cultures which had been 3μm filtered to remove algal cells (OW + B) The TVC of bacteria added with each of the algal species was determined on SWC. PZ1 stage larvae of *P. indicus* were reared to M1 stage in the above water treatments and fed solely on MED. Growth was measured as increase in total lengths using an eyepiece graticle. and the number of surviving larvae were counted at the end of the experiment. Bacterial numbers were estimated at the beginning and the end of the experiments.

Larval survival in cultures inoculated with preserved bacteria.

In additional tests, water samples from a culture of *P. indicus* PZ2 stage larvae reared in 5μmSW and fed on CAR were stored at -20°C in sterile (40% (v/v)) glycerol. Two litres of AuW were inoculated with 50μl of this mixture and aerated for 24h prior to addition of *P. indicus* PZ1 stage larvae at the rate of 100 larvae.L^{-1}. Controls used 5μmSW without added bacteria. A negative control group consisted of AuW with no added bacteria. Rearing water was not changed during the tests and larvae were fed CAR at 4mg.L^{-1}.day^{-1}. Surviving larvae were counted at the M1 stage. TVCs and PVVC counts were taken at the beginning and the end of the trials.

Bacterial Antagonism tests

Various *Vibrio sp.* isolates from healthy *P. vannamei* larval cultures were obtained from Centro de Investigacion in Alimentacion y Desarrollo (CIAD) Mexico, and screened for their potential uses as probiotics:

In preliminary tests, the isolates were spotted onto SWC agar plates and grown for 24h. Sloppy SWC agar containing 0.5% agar was then prepared and seeded with a 10 fold dilution of each one of *V. harveyi* strains BP03, BP04 and BP05, well mixed and poured onto the spotted plates. The plates were incubated for a further 24h and then examined for zones of clearing in the sloppy agar around the spotted isolates. Isolates which appeared to display zones of clearing were then further tested for bacterial antagonism using both well and disk diffusion methods. In the well diffusion methods, log-phase cultures of the probiotics in SWC were harvested by centrifugation and the supernatants were filter sterilised (0.2μm), into sterile tubes on ice. 0.2mm wells were made in SWC agar plates seeded with each one of the above named pathogens using hollow glass rods sterilised with 70% alcohol and subsequent flaming. 20μl aliquots of filtered bacterial supernatants were then added into each well. The plates were incubated for 48h and examined for zones of clearing. In confirmatory tests, sterile 2mm diameter filter paper disks were placed on agar plates spread with log phase cultures of the pathogens used. 10μl of bacterial exudates were then added onto each disk and the plates incubated for 48h and later examined for zones of clearance around the disks. Each individual test was triplicated.

The isolates were further tested for antibacterial activity using the method described by Austin *et al.*, (1995). Thus, SWC plates were inoculated with parallel streaks of *V. harveyi* strains BP03, BP04 and BP05, and the probionts were streaked at right angles across the lines of the inoculum. The plates were then incubated at 28°C for 48 and examined for zones of growth interruption or overgrowth of the pathogen which would indicate antagonism.

Results

Water pre-treatment and bacterial growth

Five micron filtration reduced TVC in RSW by about 82.20% to a mean of $1.46 \times 10^4 \pm 1.56 \times 10^3$ cfu.ml^{-1}. An upper tailed T-test on the logarithmic transformation of TVC revealed this reduction to be significant ($p = 0.0004$; $t = -13.87$; 95% CI = 3.76 to 8.28 cfu.ml^{-1}) (Appendices 3.1 and 3.1a).

Immediately after further pre-treatment, TVC in the O_3 and UVT treatments were significantly lower than TVC in the 5μmSW treatment (ANOVA: $f = 75.83$, $p < 0.001$) (Appendices 3.1, 3.1b and Figure 3.1). Further pre-treatment with O_3 was more effective than the UVT treatment with reductions of about 98.79% (mean = $9.90 \times 10^2 \pm 1.35 \times 10^3$ cfu.ml^{-1}) and about 96.70% (mean = $2.71 \times 10^3 \pm 1.51 \times 10^3$ cfu.ml^{-1}) respectively compared to RSW. However, following air-stripping of the residual ozone for 24h, TVC increased from a mean of $9.90 \times 10^2 \pm 1.35 \times 10^3$ to $1.21 \times 10^5 \pm 1.58 \times 10^4$ cfu.ml^{-1} before the addition of diets (Figure 3.1). At this time, ANOVA, followed by Tukey's pairwise comparisons revealed significant differences in log TVC values obtained in all the treatments with bacterial concentrations in the UVT treatment being significantly lower (mean log TVC = 3.39 ± 0.234 cfu.ml^{-1}) than obtained in the 5μmSW treatment (mean log TVC = 4.16 ± 0.048 cfu.ml^{-1}) which in turn, was significantly lower than log TVC obtained in the O_3 treatment (mean log TVC = 5.08 ± 0.058 cfu.ml^{-1}) ($f = 106.55$; $p < 0.001$) (Appendices 3.1 and 3.1c). Autoclave treatment led to total elimination of bacteria.

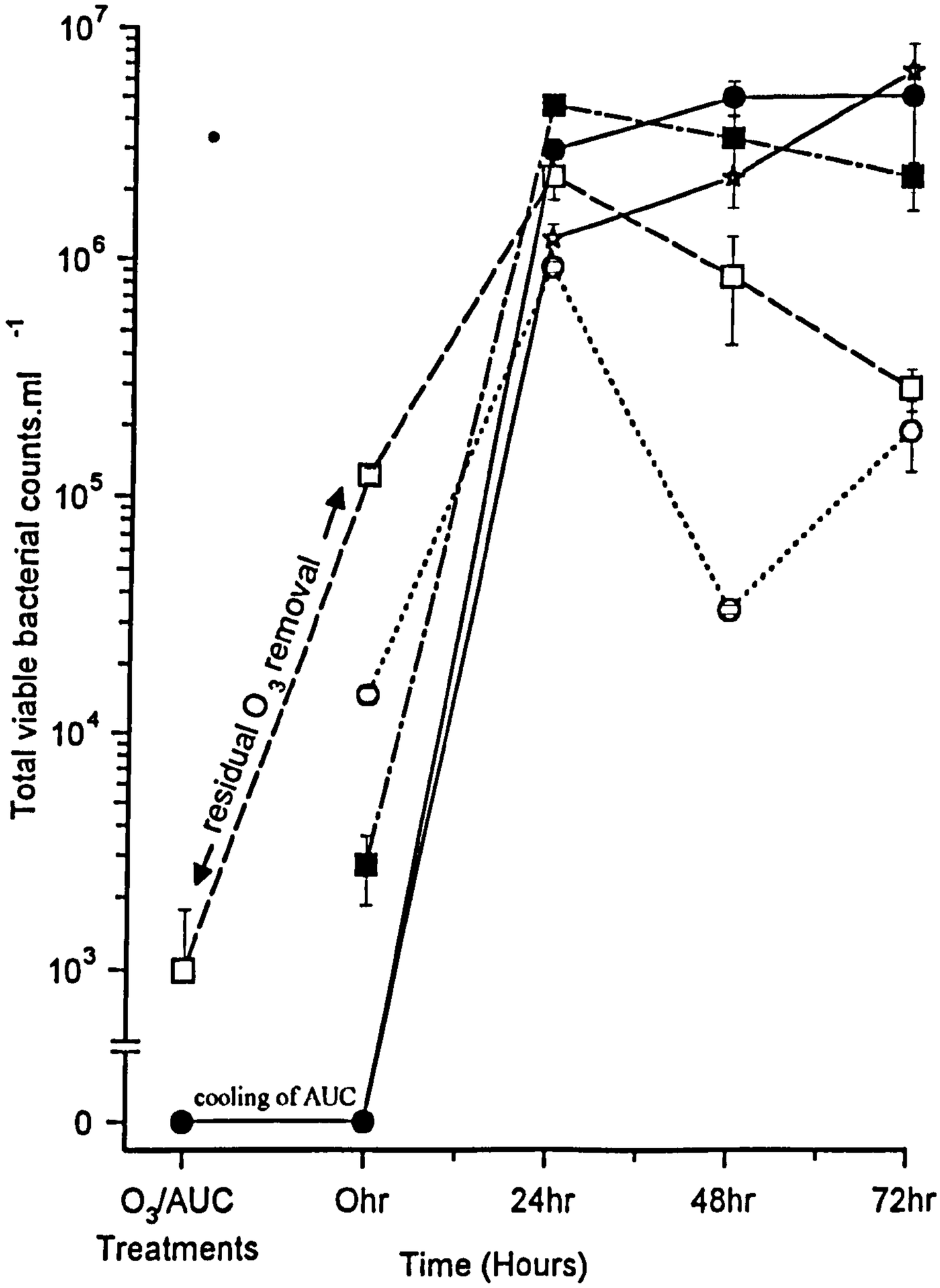
The TVC of bacteria added to the AuW+live feed treatment with the algae was estimated to give a final mean of $1.15 \times 10^4 \pm 5.25 \times 10^2$ cfu.ml^{-1} at 0h (Appendix 3.1). At 24h after diet addition, there were no significant differences in TVC between the 5μmSW (mean log TVC = 5.96 ± 0.0422 cfu.ml^{-1}) and the AuW+live feed treatments (mean log TVC = 6.07 ± 0.13 cfu.ml^{-1}). ANOVA showed that both treatments had significantly lower log TVC compared to the O_3, UVT and AuW treatments which had mean log TVC values of 6.33 ± 0.18; 6.65 ± 0.07 and 6.47 ± 0.03cfu.ml^{-1} respectively (ANOVA: $f = 21.30$, $p < 0.001$) (Appendices 3.1 and 3.1d).

However, an increase in TVC was observed in the AuW+live feed treatment following the addition of *Artemia* nauplii into this treatment at 24h. Although very few bacteria were added with the *Artemia* suspensions (final TVC in 2L cultures = $7.85 \times 10^1 \pm 2.09 \times 10^1$ cfu.ml^{-1} (Appendix 3.1), at 48h, mean log TVC obtained in the AuW+live feed treatment (mean = 6.35 ± 0.04 cfu.ml^{-1}) was significantly higher than that obtained in the 5μmSW treatment (mean = 4.52 ± 0.03 cfu.ml^{-1}) in addition, it was not significantly different from log TVCs obtained in the O_3, UVT and AuW treatments with mean log TVCs (± SD) of 5.69 ± 0.67, 6.68 ± 0.02, and 6.47 ± 0.45 cfu.ml^{-1} respectively (ANOVA: $f = 17.54$; $p < 0.001$) (Appendices 3.1, 3.1e and Figure 3.1).

At 72h, there were no significant differences in TVC obtained between the 5μmSW and O_3 water treatments with mean log TVCs of 5.22 ± 0.26 cfu.ml^{-1} and 5.43 ± 0.14 cfu.ml^{-1} respectively. In addition,

ANOVA revealed that both treatments had significantly lower log TVCs compared to the other treatments with mean log TVCs of 6.34 ± 0.12, 6.44 ± 0.61 and 6.81 ± 0.06cfu.ml^{-1} for the UVT, AuW and AuW+live feed treatments respectively ($f = 14.61$, $p < 0.001$) (Appendices 3.1, 3.1f and Figure 3.1).

FIGURE 3.1: TOTAL VIABLE BACTERIAL LEVELS IN WATER WITH DIFFERENT PRE-TREATMENTS WHEN FED LIVE AND ARTIFICIAL PENAEID LARVAL DIETS



O = 5μm filtered sea water

☆ = Autoclaved water (AUC) + live feed

● = Autoclaved water (AUC) + microcapsules

□ = Ozonated water (O_3)

■ = U/V treated water

Raw sea water was 5μm filtered, further treated as shown above and fed with microencapsulated diets and live diets. Live diet addition was of *S. costatum* and *T. chuii* (at 45 and 25 cells.μl^{-1} respectively) at the 0h and *Artemia* nauplii (5 nauplii.ml^{-1}) at 24h Diet addition started at 0h. Residual O_3 was air-stripped and AuW treatments cooling was carried out for 24h prior to diet addition. Each data point represents the mean value of three replicate readings. Error bars indicate the standard errors of the mean.

Data calculated from Appendices 3.1 and 3.1a - f

Bacterial composition of treated water

Appendix 3.2 shows the results of the morphological identification of the bacteria obtained from the water treated as described in the materials and methods section of this chapter and Table 3.1 shows the results of the bacterial profiling tests for presumptive *Vibrio sp.* The only presumptive species of *Vibrio* that could be identified with confidence (confidence levels ranged from 23.60% to 99.20%) by the API 20 NE test was *V. alginolyticus*. In all the treatments, there was variation in the TVC of the replicates used, but generally the PVVC (expressed as a percentage of the TVC) was higher in the O_3 and UVT treated waters than in the 5µmSW and RSW at 24h. These increased from 0.08 ± 0.11% and 0% in O_3 and UVT treated water respectively at 0h, to 0.36 ± 0.19% and 1.58 ± 1.56% respectively at 24h (Figure 3.2). In contrast, in RSW and 5µmSW treatments the proportion of PVVC decreased from 4.68 ± 1.58% and 3.07 ± 2.30% respectively to 0.07 ± 0.02% and 0.16 ± 0.13% at 24h (Figure 3.2).

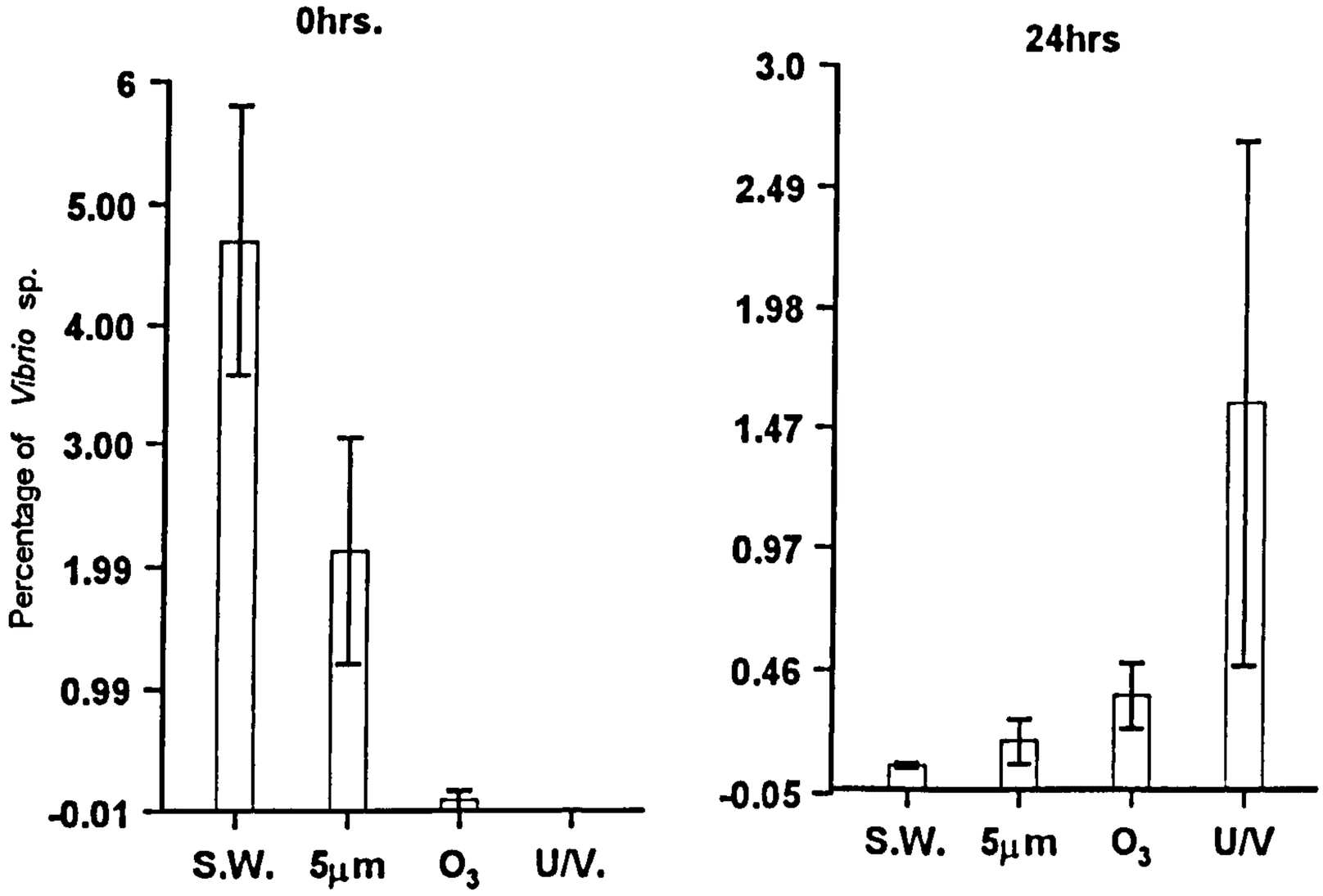
Table 3.1: API-20 NE results for 24h growth of presumptive *Vibrio sp.* bacteria isolated from water and grown on *Vibrio sp.* specific plates 24h following different methods of sea water pre-treatment.

Bacterial characteristics	Bacterial isolates								
	reactions/enzymes	1	2	3	4	5	6	7	8
Colony Shapes		Green, circular, entire, with thin, transparent edge	Blue-green dots around 1mm diameter	Green with wide, creamy umbra, circular, entire around 9 5mm diameter	Yellow, circular, curled around 8 2mm	Yellow, circular, entire around 6 65mm	Yellow, circular, lobate edges around 2.2mm diameter	Green, irregular	Greenish cream, circular, entire
Gram stain	retention of safranin	-	-	-	-	-	-	-	-
Growth on TCBS		+	+	+	+	+	+	+	+
NO3	reduction of nitrates to nitrites/ nitrogen	+	+	+	+	+	+	+	+
TRP	Indole production	+	+	+	+	+	+	+	+
GLU	acidification	+	+	+	+	+	+	+	+

Bacterial characteristics	Bacterial isolates								
	reactions/enzymes	1	2	3	4	5	6	7	8
ADH	arginine dihydrolase	-	-	-	-	-	-	-	-
URE	urease	-	-	-	-	-	-	-	-
EC	hydrolysis (β-glucosidase)	+	+	+	+	+	+	+	+
GEL	hydrolysis (protease)	-	+	+	-	+	+	+	+
PNG	β-galactosidase	+	-	-	-	-	-	-	-
GLU	assimilation	+	+	+	-	+	+	+	+
ARA	assimilation	-	+	-	-	-	+	-	-
MNE	assimilation	-	+	-	-	-	-	-	-
MAN	assimilation	-	+	+	-	+	+	+	+
NAG	assimilation	-	+	+	-	+	+	+	+
MAL	assimilation	-	+	+	-	+	+	+	+
GNT	assimilation	-	+	+	-	+	+	+	+
CAP	assimilation	-	-	-	-	-	-	-	-
ADI	assimilation	-	+	-	-	-	-	+	-
MLT	assimilation	+	+	+	+	+	+	+	+
CIT	assimilation	-	-	+	-	-	-	-	-
PAC	assimilation	-	-	-	-	-	-	-	-
OX	cytochrome oxidase	-	-	+	+	-	-	-	+
24h reading		-	-	+	+	+	+	-	+
48h reading		+	+	-	-	-	-	+	-
Closest species		V. alg	V. alg	V. alg	*	V. alg	V. alg	V. alg	V. alg
Confidence				91 3%		99 2%	91 7%		97 3%

+ = positive reaction
-- = negative reaction
v. alg. = *Vibrio alginolyticus*
* = *Inconclusive*

FIGURE 3.2: PROPORTIONS OF PRESUMPTIVE *VIBRIO SP.* (ON TCBS AGAR) IDENTIFIED IN TREATED WATER SAMPLES AT 0H AND 24H AFTER TREATMENT.

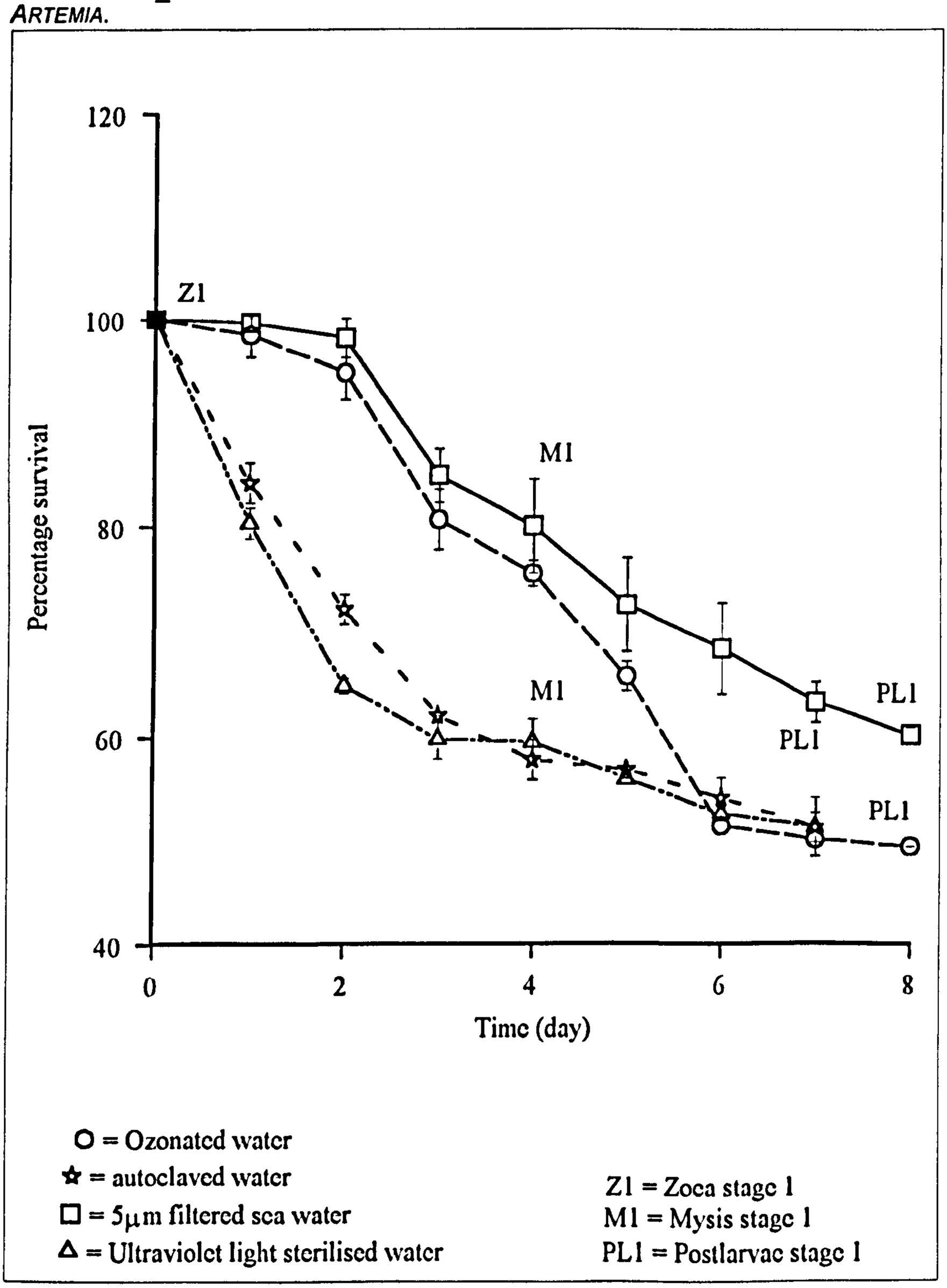


Error bars denote the standard errors of the means. The x-axis include negative values to permit visualisation of zero values. Data calculated from Appendix 3.2.

Larval survival

The survival of *P. indicus* larvae fed on live diets in pre-sterilised and partially sterilised water is shown in Figure 3.3. Final survivals to PL1 stage were 49.67 ± 0.29%, 60.17 ± 0.29%, 51.33 ± 0.58% and 51.33 ± 1.16% for the O_3, 5µmSW, UVT and AuW treatments respectively (Appendix 3.3 and Figure 3.3). ANOVA on the arcsine transformations of the percentage survival data revealed that larval survival obtained to PL1 stage in the 5µmSW treatment was significantly higher than larval survival obtained in all the other treatments ($f = 157.23$; $p < 0.001$) (Appendices 3.3 and 3.3a). Survival in the O_3 treatment was also significantly higher than in the UVT and AuW treatments until day 5 ($f = 508.49$; $p < 0.001$) (Appendices 3.3 and 3.3b and Figure 3.3) after which, there were no significant differences in larval survival between these three treatments.

FIGURE 3.3: THE EFFECT OF SEA WATER TREATMENT ON SURVIVAL OF *P. INDICUS* LARVAE FED ON MIXED ALGAE AND *ARTEMIA*.

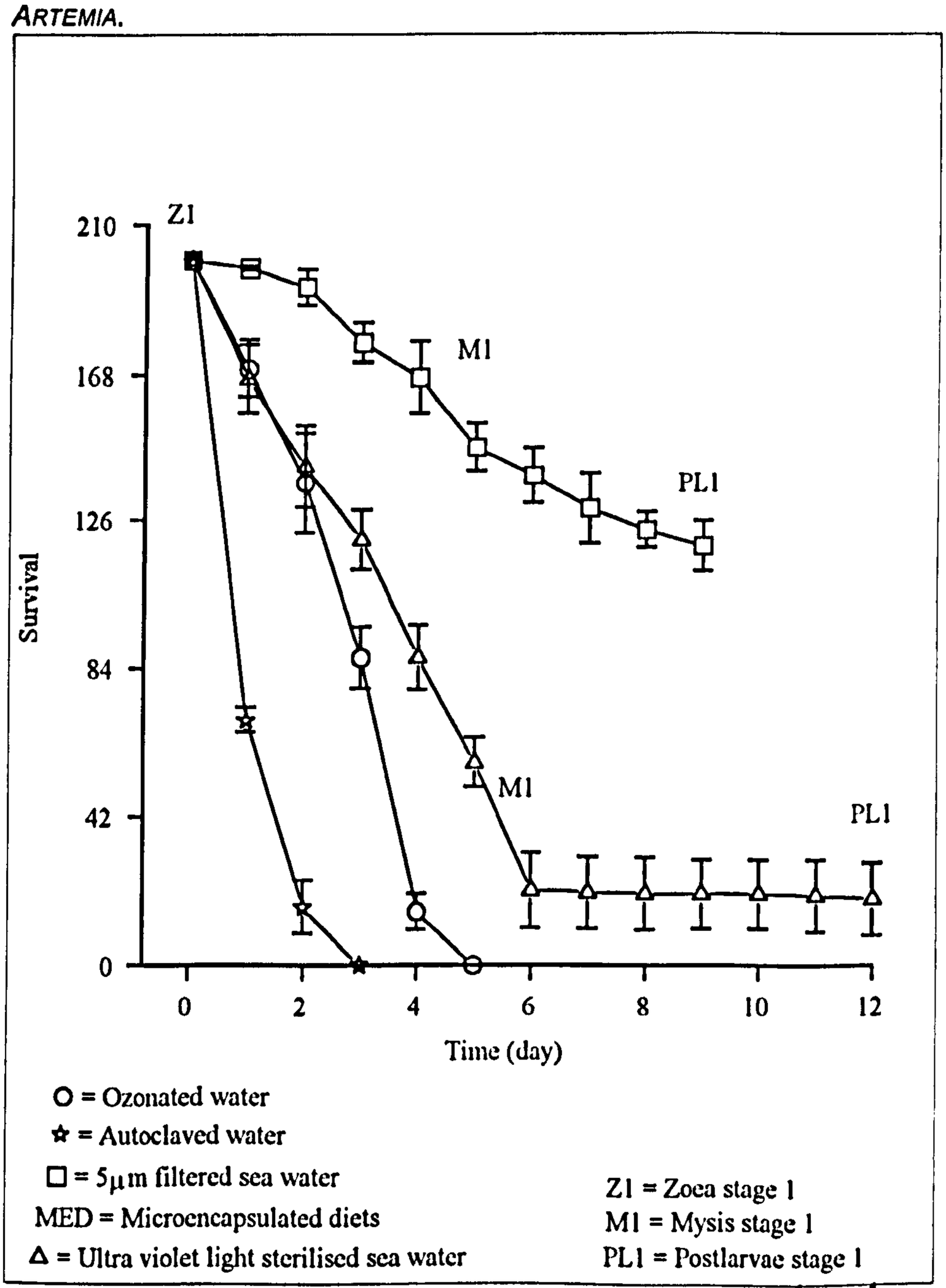


Larvae were fed mixed algae, *Skeletonema costatum* and *Tetraselmis chuii* at 45 and 25 cells.μl^{-1} respectively daily at the Z1-Z3 stage and this was reduced to 15 and 10 cells.μl^{-1} respectively from the M1 - PL1 stages when *Artemia* supplementation at 2 nauplii.ml^{-1} began. Each data point represents the mean value of three replicates. Error bars denote the standard errors of the means.
Data were calculated from Appendices 3.3, 3.3a and 3.3b.

Larval survival in the water treatments which were fed on MED showed a greater variation between treatments. Mean percentage larval survivals to PL1 stage obtained were 0% (O_3 and AuW treatments); 9.17 ± 5.01% and 59.00 ± 3.61% for the UVT and 5μmSW water treatments respectively (Appendix 3.3 and Figure 3.4). After only 2d when all treatments still had surviving larvae, ANOVA indicated that survival

of larvae reared in 5μmSW (mean = 96.17 ± 2.52%), was significantly higher than larval survival obtained in all the other treatments (means = 68.33 ± 7.32%; 70.67 ± 6.03% and 8.17 ± 3.75% for the O_3, UVT and AuW treatments respectively.) (ANOVA: f 108.54; $p < 0.001$) (Appendices 3.3 and 3.3c). Mass mortalities occurred in the AuW and O_3 water treatments with all the larvae dying in 3 and 5d respectively. Apart from the 5μmSW treatment, only the UVT treatment had larvae surviving to PL1 stage. These larvae had retarded development when compared to those in the 5μmSW treatment which reached PL1 stage 3 days earlier and in addition, had significantly higher survival to PL1 (59.00 ± 3.61%) compared to the UVT treatment (9.17 ± 5.01%) ($p = 0.0001$; $t = 13.93$; 95% CI = 41.87 to 60.28%) (Appendices 3.3 and 3.3d).

FIGURE 3.4. THE EFFECT OF SEA WATER TREATMENT ON SURVIVAL OF *P. INDICUS* LARVAE FED ON MED AND *ARTEMIA*.

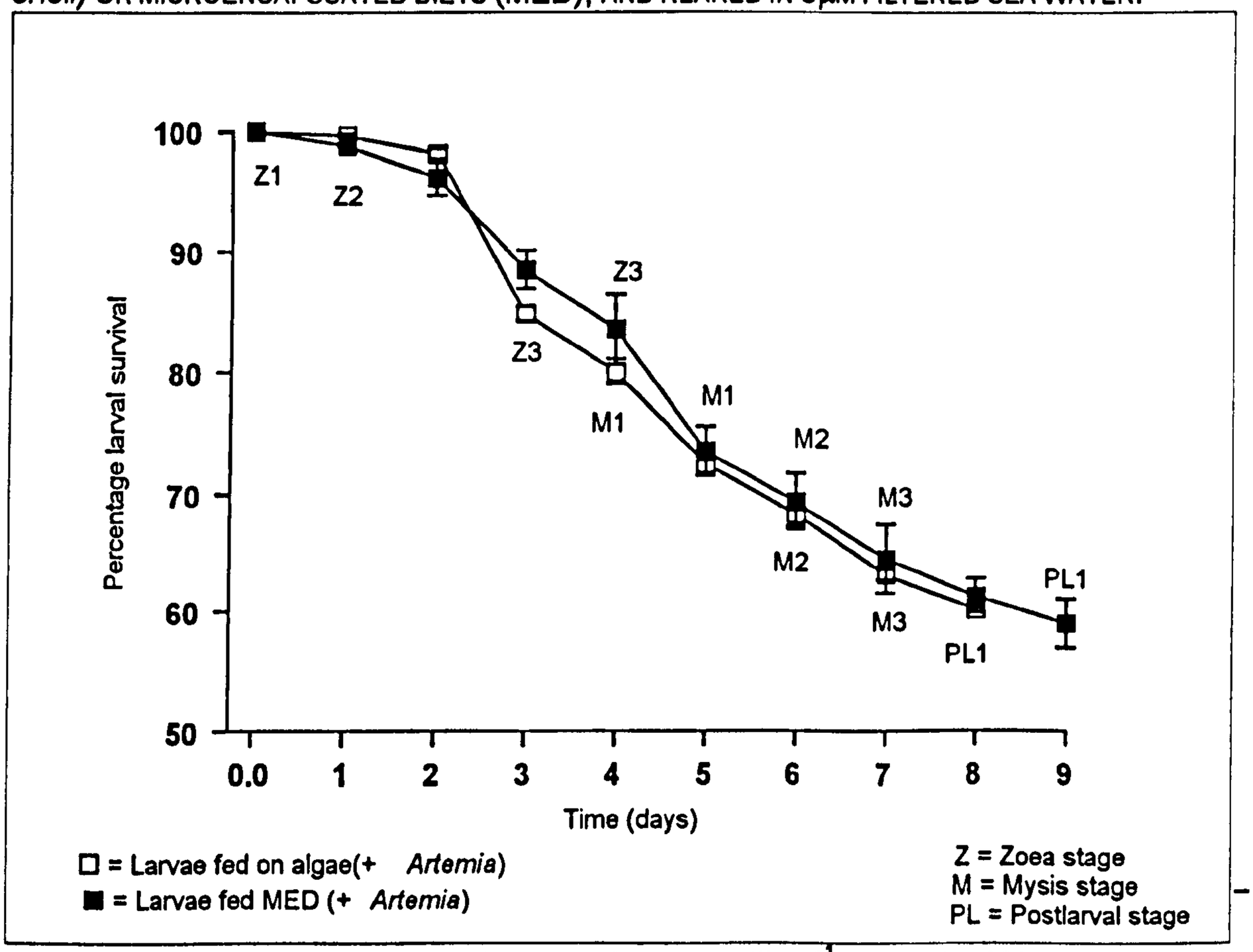


Larvae were fed Frippak MED at the rates of 4, 6 and 8 $mg.L^{-1}.day^{-1}$ at the Z1 - Z3, M1 - M3 and M3 - PL1 stages respectively. *Artemia* supplementation at 2 $nauplii.ml^{-1}$, began at the M1 stage. Each data point is

the mean of three replicate values. Error bars show the standard error of the mean. Data was calculated from Appendices 3.3 and 3.3c.

There was no significant difference in percentage larval survival between larvae fed MED and live diets controls when both were reared in 5μmSW (t = 0.55; p = 0.32, 95% CI = -9.65 to 12.47%) (Appendices 3.3, 3.3e and Figure 3.5). However, a difference occurs in their development with the algal fed larvae developing a day faster than the MED fed larvae from Z-3 to PL-1 stages (Figure 3.5).

FIGURE 3.5: SURVIVAL OF *P. INDICUS* LARVAE FED ON MIXED ALGAE (*SKELETONEMA COSTATUM* AND *TETRASELMIS CHUII*) OR MICROENCAPSUATED DIETS (MED), AND REARED IN 5μM FILTERED SEA WATER.



Both groups were supplemented with *Artemia* (2 nauplii.ml^{-1}) from the M1 stage onwards and reared in 5μm filtered natural sea water . Each data point represents the mean value of three replicates. Error bars denote the standard errors of the means. Data was calculated from Appendices 3.3 and 3.3e.

Larval survival using bacteria from algal cultures as probiotics

Mean percentage larval survival (± standard deviation) of *P. indicus* larvae in the various diet treatments is shown in Table 3.2. Final survivals ranged from 0% (AuWMED-B; AuWMED+B) to 94.17 ± 4.75% (AuWA-B). In the treatment groups fed on algae, variation within replicates in the 5μmSW treatments was less (95% CI = -9.59 to -2.50%) (Appendix 3.4a) compared to the AuW treatments (95% CI = -43.50 to 37.46%) (Appendix 3.4b). Despite this, lower tailed 2-sample T-tests showed that survival to M1 stage was similar

within water treatment groups fed on algae and the inclusion or not of bacteria with the algae seems to have no effect on larval survival (AuW treatment groups: p = 0.58; t = -0.22; 95% CI = -43.50 to 37.46%) (Appendices 3.4 and 3.4b). and 5μmSW treatment groups: (p = 1.0; t = - 4.76; 95% CI = -9.56 to -2.50%) (Appendices 3.4 and 3.4b).

Larval survivals in all treatments fed algae were also higher than larval survivals in the treatments fed MED where survivals showed greater variation with mean survivals ranging from 0% (AuWMED-B; AuWMED+B) to 47.70 ± 37.10% (SWMED+B) (Table 3.2). In addition, unlike in the algal fed groups, the addition of bacteria from algal cultures to 5μmSW enhanced larval survival in MED fed treatments.

Though percentage larval survivals obtained in the SWMED+B were higher (mean = 47.70 ± 37.1%), compared with percentage survivals in the SWMED-B treatment (mean = 3.00 ± 2.29%), The large variability within replicates (SD = ± 37.10%), led to a lower tailed t-test not giving a significant difference (p = 0.088, t = - 2.05, 95% CI = -99.95 to 52.27%) (Appendices 3.4 and 3.4c).

Raising larvae on artificial feeds in autoclaved water with (AuWMED+B) or without (AuWMED-B) added bacteria from algal cultures resulted in complete culture collapse (Appendix 3.4 and Table 3.2).

Table 3.2. Mean percentage survival rates to mysis 1 stage of *P. indicus* larvae grown in 5μm filtered sea water (5μmSW) and autoclaved seawater (AuW fed with different live and artificial diet treatments.

	WATER TREATMENT	
FEED	*AUTOCLAVED SEA WATER(AuW)*	*5μM FILTERED SEA WATER(5μmSW)*
AI +B	92.67 ± 6.79 Aa	88.33 ± 0.29 Aa
AI -B	94.17 ± 4.75 Aa	91.00 ± 0.87 Aa
MED ***-B***	0 Ba	3.00 ± 2.29 Bb
MED ***+B***	0 Ba	47.70 ± 37.1 Cb

Live diets were mixed algae, *Skeletonema costatum* and *Tetraselmis chuii* (***AI***) fed at concentrations of 45 and 25 cells.μl^{-1}.day^{-1} respectively and artificial diets (MED) were CAR fed at 4mg.L^{-1}.day^{-1}. Each diet treatment either had added bacteria (***+B***) from xenic algal cultures or not (***-B***). Controls used autoclaved sea water (AuW) with the same diet treatments. 100% = 200 larvae Any common letter indicates no significant difference (α = 0.05), upper case for vertical comparisons, lower case for horizontal comparisons. Each value is the mean of three replicate values.
Data calculated from Appendices 3.4 and 3.4a -c

Only the water treatments fed on algae had larvae moulting to the M1 stage at the termination of the trial.

Furthermore, the addition of bacteria from algal cultures to MED fed cultures enhanced larval development with the SWMED+B treatment at the PZ3 and the SWMED-B treatment at the PZ2 larval stages respectively at the cessation of the trials.

Table 3.3 shows final lengths (in mm) obtained in the different water and diet treatment groups from an initial mean length of 0.87 ± 0.04 mm at the PZ1 stage. Lengths obtained (Appendix 3.4) did not exhibit a normal distribution and so, a non-parametric alternative to T- tests (the Mann-Whitney U tests) were performed The SWA+B treatment had significantly higher final lengths (mean = 3.38 ± 0.31mm) compared

to the AuWA+B treatment group (mean = 2.87 ± 0.39mm) (Upper tailed U test: point estimate = 0.640mm, w = 1158.5, 95% CI = 0.2800 to 0.7999mm) (Appendix 3.5a). In the treatments fed on algae without added bacteria, Final lengths obtained in the SWA-B (mean = 3.27 ± 0.23mm), was not significantly higher than final lengths obtained in the AuWA-B treatment (mean = 3.26 ± 0.30mm) (Upper tailed U test: point estimate = -0.0400mm, w = 874.5, 95% CI = -1600 to 0.0801mm) (Appendix 3.5b).

In the surviving treatments fed on MED, the addition of bacteria from algal cultures significantly enhanced growth. From the same initial mean length of 0.88 ± 0.04mm, the SWMED+B treatment group had significantly higher final total lengths (mean = 2.10 ± 0.43mm) compared to the SWMED-B treatment group (mean = 1.70 ± 0.10mm) (Upper tailed U test: point estimate = 0.5400mm, w = 511.5, 95% CI = 0.0999 to 0.7601mm) (Appendix 3.5c).

TABLE 3.3. Mean growth (in mm) to mysis 1 stage of *P. indicus* larvae grown in 5µm filtered sea water (5µmSW) fed with different live and artificial diet treatments.

	TREATMENT	
FEED	AUTOCLAVED SEA WATER(A*u*W)	5µM FILTERED SEA WATER(5µmSW)
Al +B	2.82 ± 0.58 Aa	3.38 ± 0.06 Ba
Al -B	3.26 ± 0.30 Ab	3.26 ± 0.24 Ab
MED ***+B***	0	2.10 ± 0.43 c
MED ***-B***	0	1.7 ± 0.10 d

Live diets were mixed algae, *Skeletonema costatum* and *Tetraselmis chuii* (***Al***) fed at concentrations of 45 and 25 cells.μl^{-1}.day^{-1}, and artificial diets (MED) were CAR fed at 4mg.L^{-1}.day^{-1}. Each diet treatment either had added bacteria (***+B***) from xenic algal cultures or not (***-B***). Controls are with autoclaved sea water (AuW) with the same diet treatments. Any common letter indicates no significant difference (α = 0.05), upper case for vertical comparisons, lower case for horizontal comparisons. Each value is the mean of three replicate values.
Data calculated from Appendices 3.5 and 3.5 a - c.

ANOVA on the log transformations of final TVC obtained in all the different water and diet treatments used also reveals significant differences at the end of the experiments (Table 3.4). Within both the 5µmSW and AuW treatment groups, the addition of algae with or without the associated bacteria, consistently gave lower log TVCs compared to MED fed treatments although these differences were not always significant (ANOVA: f = 5.43; p = 0.002) (Appendices 3.6, 3.6a and Table 3.4). Generally, log TVCs were higher in the treatment groups with autoclaved water in contrast to corresponding 5µmSW treatment groups (Table 3.4). The addition of bacteria from algal cultures sometimes led to a significant lowering of log TVC within each water treatment group.

Analysis of variance carried out on the PVVC concentrations obtained revealed significant differences occurring between some of the treatments (ANOVA: f = 5.91; p = 0.002) (Appendices 3.6, 3.6b and Table 3.5). Once more, PVVC within water treatment groups was lower in groups with added algae compared to

MED fed treatments. In addition, apart from the SWA+B which had higher PVVC.ml^{-1} compared to the SWA-B treatment, the addition of bacteria from algal cultures to diet treatments gave lower PVVC.ml^{-1} when compared with equivalent treatments without added bacteria (Table 3.5).

Table 3.4. Mean log TVC (± SD) of bacteria.ml^{-1} in cultures at mysis 1 stage of *P. indicus* larvae grown in both autoclaved and 5μm filtered sea water and fed with different live and artificial diet treatments.

DIET TREATMENT	*COMPARISON CODE LETTERS*	*Number of Replicates*	*MEAN LOG TVC PER ML*	*COMPARISONS*
AuWAI +B	*A*	3	5.36 ± 0.17	ABcdEFGH
AuWAI -B	*B*	3	5.45 ± 0.10	ABcDEFGH
AuWMED -B	*C*	3	6.22 ± 0.11	abCDefgH
AuWMED +B	*D*	3	6.01 ± 0.47	aBCDefGH
SWAI +B	*E*	3	5.12 ± 0.23	ABcdEFGH
SWAI -B	*F*	3	4.86 ± 0.23	ABcdEFgh
SWMED -B	*G*	3	5.58 ± 0.28	ABCDEfGH
SWMED +B	*H*	3	5.65 ± 0.64	ABCDEfGH

AuWAI = Autoclaved water (AuW) + Algae (AI)
AuWMED = Autoclaved water .(AuW) + MED (CAR).
SWAI = 5μm filtered sea water (SW)+ Algae (AI)
SWMED = 5μm filtered sea water (SW) + MED (CAR)
Each diet treatment either had added bacteria (+B) from xenic algal cultures or not (-B)
A, B, C, D, E, F, G and *H* = comparison code letters for the different diet treatments respectively
Readings with the same code letter both in upper case characters denotes no significant difference (α = 0.05). Two readings with the same code letters in both upper and lower case characters indicates a significant difference. Each data point is the mean value of three replicates
Data calculated from Appendices 3.6 and 3.6a

Table 3.5. Mean total viable presumptive *Vibrio sp.* (PVVC) counts of bacteria.ml^{-1} in cultures at mysis 1 stage of *P. indicus* larvae grown in both autoclaved and 5μm filtered sea water and fed with different live and artificial diet treatments.

DIET TREATMENT	*COMPARISON CODE LETTERS*	*Number of Replicates*	*MEAN PVVC PER ML*	*COMPARISONS*
AuWAI+B	*A*	3	$2.4 \times 10^3 \pm 8.94 \times 10^2$	ABcDEFGH
AuWAI-B	*B*	3	$2.54 \times 10^3 \pm 5.68 \times 10^2$	ABcDEFgH
AuWMED-B	*C*	3	$6.10 \times 10^3 \pm 4.0 \times 10^2$	abCdEfgh
AuWMED+B	*D*	3	$2.23 \times 10^3 \pm 8.54 \times 10^2$	ABcDEFGH
SWAI+B	*E*	3	$4.07 \times 10^3 \pm 1.75 \times 10^3$	ABCDEfgh
SWAI-B	*F*	3	$1.69 \times 10^3 \pm 6.03 \times 10^2$	ABcDeFgH
SWMED-B	*G*	3	$3.63 \times 10^2 \pm 1.08 \times 10^2$	AbcDefGH
SWMED+B	*H*	3	$1.88 \times 10^3 \pm 2.59 \times 10^3$	ABcDeFGH

AuWAI = Autoclaved water (AuW) + Algae (AI)
AuWMED = Autoclaved water (AuW)+ MED (CAR).
SWAI = 5μm filtered sea water (SW) + Algae (AI)
SWMED = 5μm filtered sea water (SW) + MED (CAR)
Each diet treatment either had added bacteria (+B) from xenic algal cultures or not (-B)
A, B, C, D, E, F, G and *H* = comparison code letters for the different diet treatments respectively

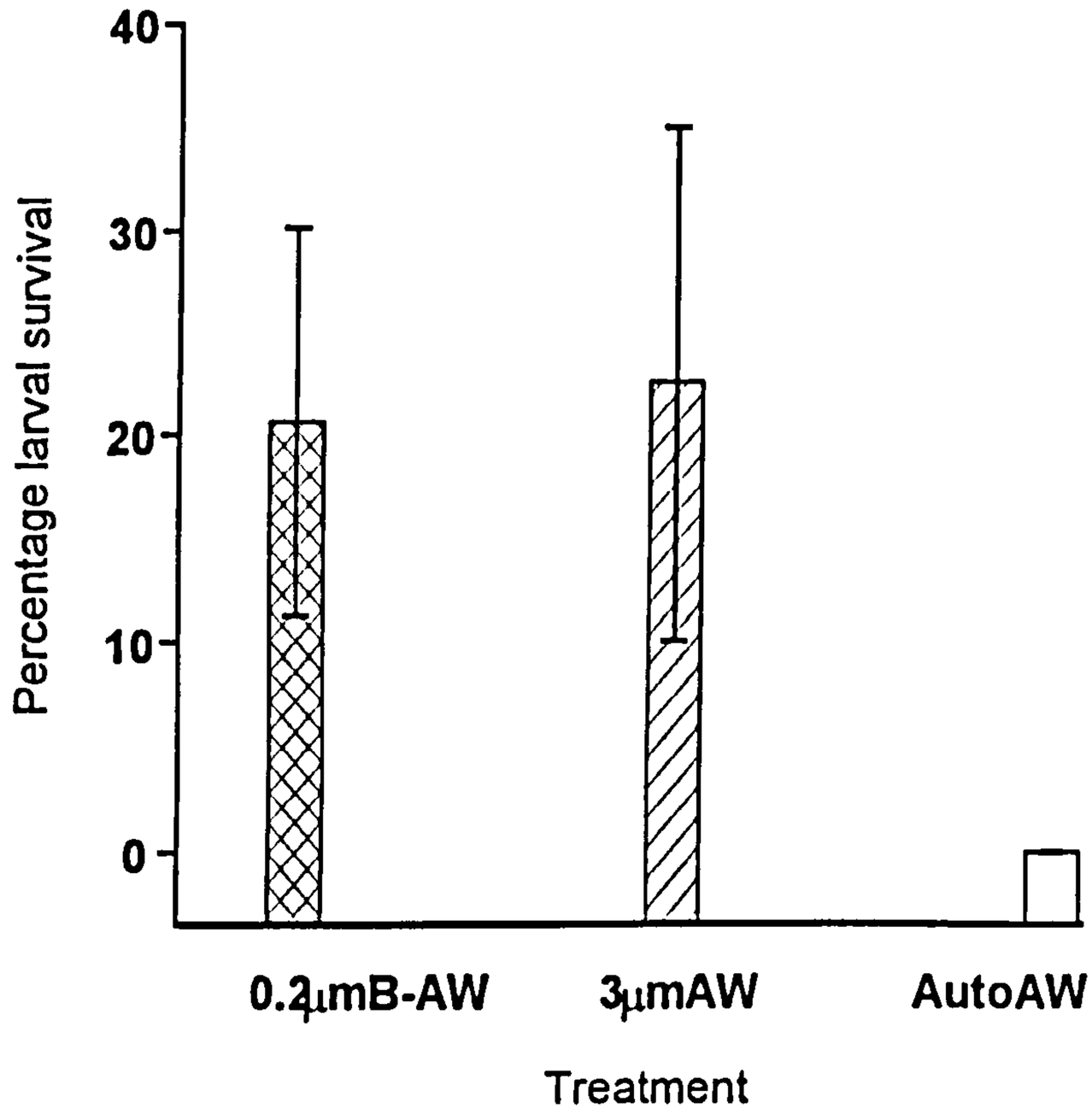
Readings with the same code letter both in upper case characters denotes no significant difference (α = 0.05). Two readings with the same code letters in both upper and lower case characters indicates a

significant difference. Each data point is the mean value of three replicates. Data calculated from Appendices 3.6 and 3.6b

The effects of the presence or absence of algal exudates on larval survival in periods of poor water quality is further illustrated in Figure 3.6. When 3μm filtered algae culture water was added to sterile water, larval survival to M1 ranged from 6.00 to 47.00% (mean = 22.50 ± 21.60%). When the algal exudates were passed through 0.22μm filters and the retained bacteria added to sterile water, percentage survivals ranged from 5.5 to 38.0% (mean = 20.67 ± 16.82%). A two tailed T-test revealed that these differences were not significant (p = 0.90, t = -0.13; 95% CI = -45.73 to 41.69%) (Appendix 3.6c). In contrast, complete mortalities were obtained in all replicates of the controls when the algal culture water added was autoclaved.

In the treatments with surviving larvae, final lengths were similar and from an initial mean length of 2.69 ± 0.08mm showed an increase in length of 169.01% (final mean length = 4.55 ± 0.19mm) in the treatment with added bacteria from algal cultures from which the exudates had previously been removed. In the 3μ mSW treatment, this increase was 159.51 % (mean final length = 4.30 ± 0.27mm). Nevertheless, a two-sample Mann-Whitney U test revealed these differences to be significant (point estimate = 0.2667mm; w = 812.5, 95% CI = 0.1333 to 0.4000mm) (Appendix 3.6d and Table 3.6).

FIGURE 3.6. PERCENTAGE SURVIVAL TO M1 STAGE OF *P. INDICUS* LARVAE FED WHOLLY ON MICROENCAPSULATED DIETS (CAR).



Larvae were fed MED at 4mg.L^{-1}.day^{-1} in autoclaved sea water to which had been added 3µm filtered mixed algal (*Skeletonema costatum* and *Tetraselmis chuii*) culture water (3µmAW), bacteria which had been 0.22µm filtered from algal culture water (0.22µmB-AW) and autoclaved 3µm filtered algal water (AutoAW). 100% = 200 larvae. Each data point is the mean of three replicate values. Error bars denote the standard errors of the mean.

Table 3.6: Initial, final lengths and percentage increases in total lengths of PZ2 stage *Penaeus indicus* larvae fed wholly on microencapsulated diets (CAR)

Treatment	*N*	*Length (± SD)*	*Percentage increase*
Start	15	2.69 ± 0.08^{a}	
3µm AW	25	4.30 ± 0.27^{b}	159.51
0.22µmB-AW	25	4.55 ± 0.19^{c}	169.01

Larvae were fed MED at 4mg.L^{-1}.day^{-1} in autoclaved sea water to which had been added 3µm filtered mixed algal (*Skeletonema costatum* and *Tetraselmis chuii*) culture water (3µmAW) and bacteria which had been 0.22µm filtered from algal culture water (0.22µmB-AW). Each value is the mean of three replicate values (± standard deviation). Comparison is vertical. Values with different superscripts are significantly different. Data calculated from Appendices 3.6d and f using the Kruskal Wallis test followed by Dunn's pairwise comparisons.

Larval survival in bacterially matured water

Lower tailed 2-sample T-tests performed on the arcsine transformations of percentage larval survival obtained in tests on the bacterial maturation of culture water revealed significant differences ($p = 0.035$; $t = -2.45$; 95% CI = -37.70 to 2.40%) (Appendix 3.7) in number of surviving larvae when raised in OW and OW+B. Mean survivals obtained were 3.83 ± 1.53% and 21.67 ± 12.29% for the OW and OW+B treatments respectively. Use of NW led to complete culture collapse (Table 3.7).

Larvae in the OW+B treatment also developed faster compared to the OW treatment which was two larval developmental stages behind (PZ2) at the completion of the test when the OW+B treatment was at the M1 stage.

Mean log TVC and PVVC obtained per ml are also shown in Table 3.7. The TVC of bacterial cells added to the OW+B treatment with the algae was $2.64 \times 10^4 \pm 1.71 \times 10^3$ cfu.ml^{-1} and this gave an initial total mean TVC of $6.74 \times 10^4 \pm 1.98 \times 10^3$ cfu.ml^{-1} in contrast to OW with an initial TVC of $4.10 \times 10^4 \pm 1.98 \times 10^3$ cfu.ml^{-1}. The NW treatment had the lowest initial TVC with $6.70 \times 10^3 \pm 1.56 \times 10^3$ cfu.ml^{-1}.

The NW treatment also exhibited the greatest increase in TVC (856.72%) to $6.41 \times 10^4 \pm 6.30 \times 10^4$ cfu.ml^{-1} and a PVVC increase of 103.47% to $4.11 \times 10^3 \pm 2.84 \times 10^3$ cfu.ml^{-1}. This is in contrast to the OW with TVC and PVVC increases of 42.11% to $5.83 \times 10^4 \pm 7.83 \times 10^4$ cfu.ml^{-1} (for TVC) and 43.27% to $8.30 \times 10^3 \pm 6.20 \times 10^3$cfu.ml^{-1} (for PVVC). The OW+B treatment also showed an increase in TVC of 3.73% to $9.00 \times 10^4 \pm 1.23 \times 10^5$cfu.ml^{-1} but showed a reduction in PVVC of 50.98% to $2.24 \times 10^3 \pm 1.40 \times 10^3$ cfu.ml^{-1}. The final concentrations of PVVCs obtained (expressed as percentages of TVC) were thus 2.49%, 14.3% and 6.42% for the OW+B, OW and NW treatments respectively (Appendix 3.7).

The OW+B treatment displayed a lower mean log PVVC value (3.29 ± 0.29 cfu.ml^{-1}) compared to the OW and NW treatments with mean log PVVC values of 3.55 ± 0.29 cfu.ml^{-1} and 3.47 ± 0.40 cfu.ml^{-1} respectively at the termination of the trials (Table 3.7). Despite this, ANOVA showed no significant differences in both final log TVC ($f = 0.06$; $p = 0.944$) and log PVVC ($f = 0.48$; $p = 0.639$) (Appendices 3.7, 3.7b and 3.7c respectively) between all treatments.

Table 3.7:Mean percentage survival and mean log TVC and PVVC (± SD) obtained after rearing *Penaeus indicus* PZ1 stage larvae in 5µm filtered sea water which was either heated up within 24h (NW) or allowed to age and heat up gradually for 3 weeks (OW) or OW with added bacteria filtered from axenic mixed algal cultures (OW+B).

Treatment	*N*	*Old water*	*Old water+ added bacteria*	*New water*
Survival (%)	3	3.83 ± 1.53	21.67 ± 12.29	0
Mean growth (mm)	3	1.70 ± 0.01	2.20 ± 0.27	0
Log TVC (cfu.ml^{-1})	3	4.36 ± 0.81	4.57 ± 0.76	4.35 ± 1.05
PVVC (cfu.ml^{-1})	3	3.47 ± 0.40	3.29 ± 0.29	3.55 ± 0.29

Data calculated from Appendices, 3.7 and 3.7a - c.

Larval survival in cultures with preserved bacteria

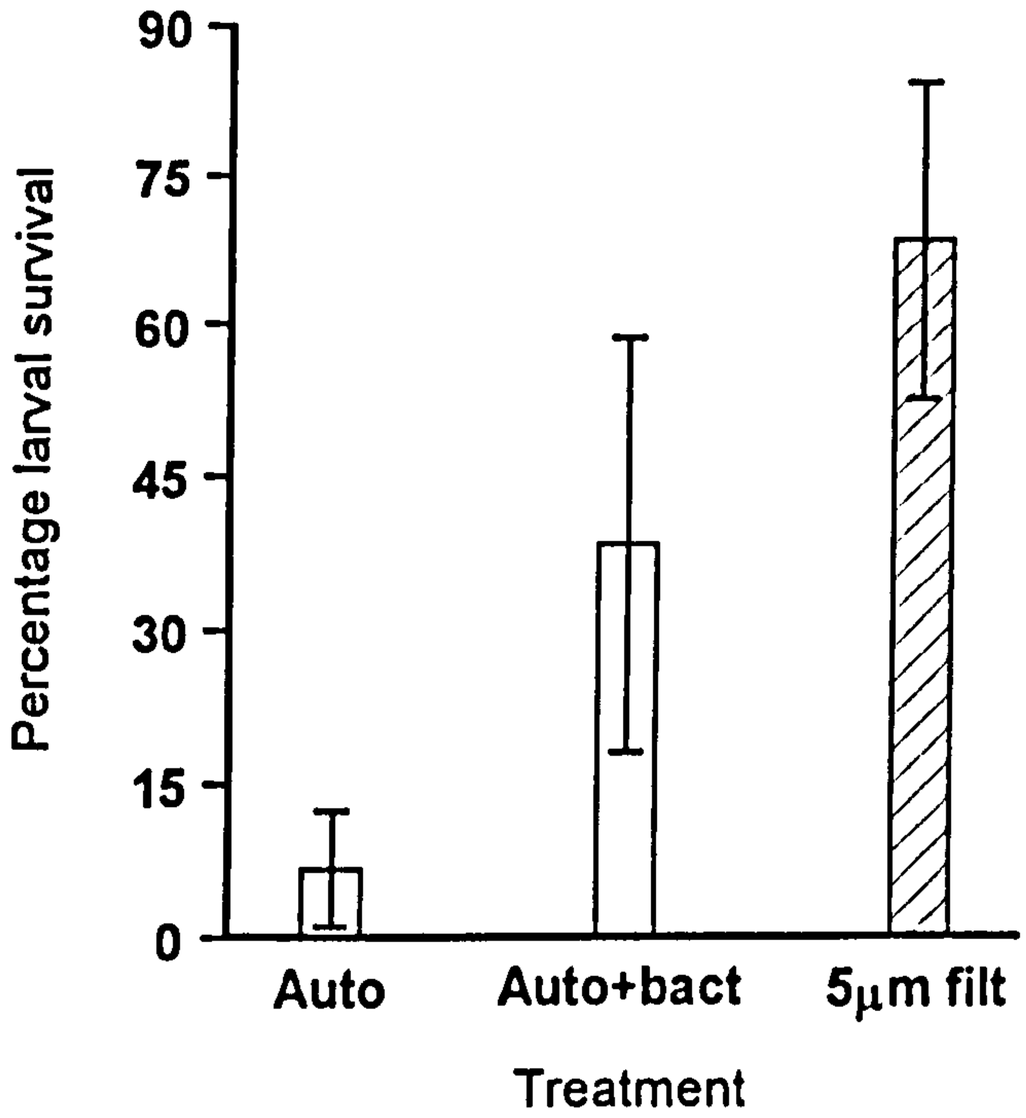
Figure 3.7 shows percentage survival of *P. indicus* larvae fed on MED and raised in AuW, 5µmSW and AuW to which preserved bacteria had been added. Mean TVCs in the treatment with added bacteria and the 5µmSW were 2.67 x 10^4 ± 2.89 x 10^3 cfu.ml^{-1} and 6.50 x 10^4 ± 1.80 x 10^4 cfu.ml^{-1} respectively and there were no detected PVVCs in all the treatments before larvae were stocked (Appendix 3.8).

Final TVCs in the 5µmSW treatment was significantly lower (mean = 6.60 x 10^4 ± 5.30 x 10^3cfu.ml^{-1}) compared to the AuW+B treatment (mean = 1.77 x 10^5 ± 3.06 x10^4 cfu.ml^{-1}) which in turn, was significantly lower than TVC obtained in the AuW treatment (mean = 4.90 x 10^5 ± 2.00 x 10^4 cfu.ml^{-1}). Analysis of variance on the log transformations of the TVC gave values of: f = 108.37; p < 0.001 (Appendix 3.8b).

Survival to M1 stage was more variable in the AuW and AuW+B treatments ranging from 0 - 9% and 0 to 34.5% respectively. This was in contrast to larval survivals obtained in the 5µmSW treatment which ranged from 22 - 49%. However, as a result of the large variability in the larval survival obtained in the AuW+B treatment, ANOVA revealed no significant differences in larval survival between this treatment and 5µmSW treatment (f = 4.04; p = 0.077) (Appendix 3.8a) These indicated that while 5µm filtration gave lower TVC and higher larval survivals, addition of preserved bacteria to AuW also significantly lowered TVC and encouraged larval survival.

Although the AuW treatment had higher PVVC (mean = 4.90 x 10^3 ± 4.40 x 10^3cfu.ml^{-1}) (Appendix 3.8c), there was PVVC growth on only one of three replicate TCBS plates in the AuW+preserved bacteria treatment and this "within treatments" variability did not permit statistical comparisons.
There was no PVVC growth observed on plates from the 5µmSW treatments at the termination of the trials.

FIGURE 3.7. PERCENTAGE SURVIVAL TO MYSIS 1 STAGE OF *P. INDICUS* LARVAE FED SOLELY ON MICROENCAPSULATED DIETS (CAR) IN AUTOCLAVED SEA WATER, AUTOCLAVED SEA WATER TO WHICH PRESERVED BACTERIA FROM A SUCCESSFUL LARVAL CULTURE FED EXCLUSIVELY ON MED WERE ADDED AND 5µM FILTERED NATURAL SEA WATER.



Auto = autoclaved water
5µm filt = 5µm filtered sea water
Auto+bact = autoclaved water + added bacteria from a culture with an established bacterial population

Larvae were fed at 4mg.L^{-1}.day^{-1}. 100% = 200 larvae. Each data point is the mean of three replicate values. Error bars denote the standard errors of the mean.
Data calculated from Appendices 3.8 and 3.8a - b.

Bacterial antagonism tests

Several strains of *Vibrio sp.* (HL32; HL40; HL51; HL56; HL57; HL58; HL59; HL61 and HL62) were tested for their ability to inhibit the growth of *V. harveyi* strains BP03, BP04, and BP05. From Table 3.8 it can be seen that the well and disk diffusion methods gave different results. While no zones of growth inhibition were observed in the disk diffusion methods, three isolates (HL57, HL58 and HL40) in the well diffusion methods gave zones of inhibition against BP05. Similarly, results obtained in the cross-streaking tests were equivocal with all the probiotics tested overgrowing the pathogens in some lanes, and being overgrown in others.

Table 3.8: Inhibition of *Vibrio harveyi* strain BP05 growth around both disks and wells with bacterial supernatants after 24h incubation.

Potential probiont	*Replicate*	*Well diffusion method*	*disk diffusion method*
HL32	A	0	0
	B	0	0
	C	0	0
HL40	A	10.85mm	0
	B	0	0
	C	0	0
HL51	A	0	0
	B	0	0
	C	0	0
HL56	A	0	0
	B	0	0
	C	0	0
HL57	A	11.45	0
	B	7.76	0
	C	0	0
HL58	A	7.25	0
	B	7.0	0
	C	0	0
HL59	A	0	0
	B	0	0
	C	0	0
HL61	A	0	0
	B	0	0
	C	0	0
HL62	A	0	0
	B	0	0
	C	0	0

Inhibition was measured as the diameters of the clear zones with no bacterial growth around the disks

Discussion

A significant reduction in TVC was observed when raw sea water was 5μm filtered (Figure 3.1). This was to be expected as bacteria in the sea have been found to be predominantly attached to both microscopic and macroscopic particle surfaces (Hermansson *et al.*, 1987). Indeed, high bacterial counts of over 10^8 bacteria.g^{-1} have been recorded for marine sediments (Kaneko *et al.*, 1978) and some authors have reported detrital aggregates to harbour bacterial communities up to five orders of magnitude higher than those found in the surrounding sea water (Alldredge *et al.*, 1986; Alldredge and Cohen, 1987). Retention of these particles on the 5μm filters would consequently reduce the concentration of bacteria getting through. Transferring this to a commercial scale may however not give similar results as Prieur and Caval (1979) reported obtaining frequently higher bacterial concentrations after 5μm filtration and attributed this to build up of bacterial populations in the filtration system which escaped periodically into the culture system.

Apart from the AuW treatment, other methods of pre-treatment attempted did not lead to complete elimination of bacteria. This persistence of low levels of bacteria in treated water is very likely due to the ability of bacteria to form clumps and/or adhere to particles and thereby avoid lethal doses of ozone as suggested by Katzenelson *et al.*, (1974). *Bacillus sp.* spores have also been reported to remain viable after ozonation (Colberg and Lingg, 1978) and Ishizaki *et al.*, (1986) suggested that this resistance was realised by retarding ozone penetration to the active sites.

In addition to oxidation of degradable organic matter, ozonation also oxidises recalcitrant organic compounds to degradable organic matter (Otte and Rosenthal, 1979; Liltved *et al.*, 1995). The production and presence of such biodegradable organic material in the residual broth also exerts an ozone demand (Broadwater *et al.*, 1973) resulting in less efficient disinfection of water. This reduction in efficiency as well as the presence of more soluble organic substrates for bacterial growth in the residual broth would explain the exponential increase in TVC in ozonated water during the first 24h after O_3 treatment and residual O_3 removal.

Bacterial repair of UV light induced damage has been reported (Jagger, 1967; Masek and Sedliakova, 1977; Barker, 1986; Harris *et al.*, 1987; Levine and Thiel, 1987; Liltved and Landfald, 1993), and while UVT treatment initially significantly reduced the TVC, the cultures grew again very rapidly. Mevel (1979) reported that in such treated water bacterial concentrations were sometimes greater than those observed before UV treatment. The persistence of low levels of bacteria observed after UVT treatment may be explained by the differential sensitivities of different bacterial species to UVT (Harris *et al.*, 1987; Liltved *et al.*, 1995). In addition, Wardell *et al.*, (1986) found that while UV irradiation gave 100% kill against single species cultures of *Desulfovibrio desulphuricans*, it was less efficient against indigenous heterotrophic bacteria.

Though autoclaving water led to complete sterility, bacteria were re-introduced with both the algae and the MED added.

The lower TVCs obtained in the AuW+live diets as opposed to the MED fed cultures at 24h appears to either reflect the probiotic potential of the balanced bacterial community included with the algae as suggested by Alabi *et. al.*, (1997) or it may be due to the inhibitory effect of algae or algal extracts on bacterial growth as has been suggested by several authors (Bell *et al.*, 1974; Kogure *et al.*, 1979; Austin and Day, 1990; Austin *et al.*, 1992) or both. The more rapid increases in TVC observed in the AuW+live diet treatment following *Artemia* addition was also, probably due to the metabolic activities of the *Artemia* nauplii which would disturb the microbial environment. Partial sterilisation may thus modify bacterial communities, upset dynamic population balances and select for certain bacterial species which may lead to the collapse of larval cultures. Some authors (Doubleday *et al.*, 1977; Lehrbach *et al.*, 1977) have reported enhanced mutabilities in various bacterial strains following UV treatment. Other authors (Murchelano *et al.*, 1975; Prieur and Caval, 1979) have also found that while water pre-treatment modified the generic composition of the microflora, it caused no permanent changes in bacterial concentration (Mevel, 1979; Prieur and Caval, 1979). In contrast, 5μmSW retains the balanced bacterial population originating from natural sea water and though exhibiting the highest TVC immediately following treatment, this balance enabled 5μmSW to subsequently exhibit lower TVCs as opposed to the treated water samples.

The low presumptive *Vibrio sp.* counts obtained immediately after water treatment at 0h, can be attributed to limitation by the selective nutrients present in the media as well as slow recovery of the bacterial cells from damage caused by the pre-treatment processes. Damaged cells have exacting nutrient requirements for growth and have been reported to be sensitive to inhibitory effects in selective media which detects only those organisms strong enough to grow in the medium provided (Bissonnette *et al.*, 1975; Camper and McFeters, 1979). The ability of bacteria to repair UVT induced (Jagger, 1967; Masek and Sedliakova, 1977; Ziebell *et al.*, 1977; Barker, 1986; Harris *et al.*, 1987; Levine and Thiel, 1987; Liltved and Landfald, 1993), chlorine induced (Camper and McFeters, 1979), and environmental stress induced (Bissonnette *et al.*, 1975; Ebo *et al.*, 1992) damage has been reported.

By 24h however, either these bacteria are sufficiently recovered to utilise the nutrients provided, or there is more rapid replication of the surviving culturable cells (Weichart *et al.*, 1992) and these potential pathogens occur at much higher proportions of the TVCs in the treated water samples compared to the 5μ mSW samples where their proportions actually declined. This again emphasises the importance of the balance of the bacterial population.

Obviously, most disinfection methods lower total bacterial loads only temporarily in open culture systems (Murchelano *et al.*, 1975; Prieur and Caval, 1979). Apart from altering the generic composition of the bacterial community, this process may also modify surviving bacteria so that they not only dominate, but may also become more virulent. While this hypothesis requires further study, biochemical modification of bacterial pigmentation has been reported (Murchelano *et al.*, 1975; Bullock and Stuckey, 1977) and

marked changes in virulence have been reported in environmentally stressed bacterial pathogens (Baticados, 1988; Mohney *et al.*, 1994; Prayitno, 1994; Prayitno and Latchford, 1995) which may support the hypothesis.

The good survival rates obtained with larvae fed on algae in all water treatments (Figure 3.3) suggests that although the larvae can adapt to all pre-treatment methods if fed with live feed, rearing in 5μmSW gives significantly better larval survival than rearing in O_3, UVT and AuW treated water. It is suggested that this is due to the retention of a well balanced bacterial population from natural sea water in contrast to other treatments where the bacterial populations may have been modified by the treatment processes and remain unbalanced. This agrees with the results of Douillet and Langdon (1994) who also reported enhancement of *Crassostrea gigas* larval survival by naturally occurring microflora present in sea water.
When live algal feeds were replaced by MED, the effects of pre-treatment of culture water were more apparent (Figure 3.4). Cultures in which pre-treatment of water was attempted to reduce bacterial levels in culture water gave poor survival when the larvae were fed on artificial diets. The only exception was when 5μmSW was used with these diets. This also emphases the importance of maintaining the natural bacterial community balance. The more obvious effects of water pre-treatment on larval survival in the MED fed treatments seems to result from the absence of balance in the bacterial communities present. This is in contrast to the less apparent effects observed with live feeds which can be attributed at least to an extent, to the introduction of more or less balanced bacterial populations with the algae which may compensate for the modification caused by the pre-treatment processes. This also corresponds well with results obtained by Ottogalli, (1991; 1992) using a similar sea water filtration method.

Bacterial enhancement of penaeid larval nutrition has been suggested for *P. monodon* (Amjad and Jones, 1989), and this has also been demonstrated for *C. gigas* (Douillet and Langdon, 1994). However, Baker and Bradnam (1976) report that while detritivores digest over half of the bacteria ingested, these bacteria are not as quantitatively important as other components of the detrital food material. In present work, the higher concentrations of bacteria reached in all pre-treated culture water samples as opposed to the lower counts in 5μmSW (Figure 3.1), suggest that nutrition supplied by bacteria are unlikely to be the cause of the better survival achieved in 5μmSW.

As observed from the probiotic assays, additions of bacterial populations from established algal cultures to *P. indicus* larvae fed on MED and grown in 5μmSW (SWMED+B) during periods of poor water quality, resulted in enhanced larval growth and survival in contrast to the poor survival obtained with SWMED-B and in the AuW cultures fed MED (AuWMED+B and AuWMED-B). In agreement with Ottogalli (1991; 1992), this also demonstrates that the presence of a balanced bacterial population is necessary to achieve growth and survival of penaeid larvae when fed MED in the absence of microalgae. The absence of natural populations of bacteria from sea water in AuW cultures fed MED resulted in total mortalities irrespective of the addition or not of bacteria from algal cultures. However, addition of similar quantities of bacteria from

algal cultures to larvae fed MED in 5μmSW (containing natural populations of bacteria) produced a significant improvement in survival (Table 3.2) when compared to the AuW treatments fed MED. In addition, despite having a high variability, addition of bacteria from algal cultures to SWMED treatments led to an improvement in mean larval survival with values of 3.00% and 47.70% obtained for survival to M1 stage in the SWMED-B and SWMED+B treatments respectively. This suggests that the addition of bacteria from algal cultures may enhance or correct for the seasonal variations in natural sea water quality which has been described by some authors (Helm, 1971; Blight *et al.*, 1995) and agrees with Douillet and Langdon, (1994) who also reported that seasonal variations in bacterial species composition did not affect enhancement of larval *Crassostrea gigas* growth following additions of a probiotic strain (CA2) of bacteria. As a result of the dynamic nature of the phenomenon involved (competitive exclusion), the beneficial effects of probiotics would naturally be expected to be variable.

When penaeid larvae were fed on live algae, there were no differences in the high survival levels obtained when reared in AuW and 5μmSW treatments nor when bacteria were added or removed (Table 3.2). While this improved growth and survival of larvae on live algae might be attributed to nutrition, Kumlu and Jones (1995) have demonstrated that live algal supplements of as few as 15 cells.μl^{-1} added to MED can give growth and survival equal to that achieved on full algal rations. They demonstrated that the contribution made by live algae is in stimulating larval enzyme secretion and hence facilitating digestion of MED. These authors also calculated that the energetic contribution made by 15 cells.μl^{-1} could not account for the increase in larval production achieved. Larval survival levels on MED in the absence of algae (50.75%) (Kumlu and Jones, 1995) were very similar to those obtained in present work. In contrast to MED only fed larvae, the addition of bacteria from algal cultures had no effect on survival of larvae fed on live algae (Table 3.2) which suggests that apart from stimulating enzyme secretion, the presence of live algae may also control the bacterial balance in penaeid cultures. Addition of bacteria from algal cultures has little effect on TVC, but appears to depress PVVC in cultures fed MED (Table 3.5).

Although the control of bacterial populations by microalgae (Bell *et al.*, 1974; Kogure *et al.*, 1979; Kellam and Walker, 1989; Austin and Day, 1990; Austin *et al.*, 1992) and macroalgal extracts (Reichelt and Borowitzka, 1984; Kellam and Walker, 1989; de Nys *et al.*, 1995; Givskov *et al.*, 1996; Gram *et al.*, 1996) have been reported, the efficacies obtained vary between different bacterial species.

The overall suppression of bacterial levels in the presence of live algae may depend on the continuous production of algal metabolites inhibiting bacterial growth as Austin and Day (1990) found that the inhibitory action of spray dried *Tetraselmis suecica* rapidly declined after 5h. de Nys *et al.*, (1995) have also reported an increase in the minimum inhibitory concentrations of *Delisea pulchira* extracts effective against the marine bacterium SW8 to increase from 10ng.ml^{-1} to 10μg.ml^{-1} over 22.5h. This reduction in activity would also account for the lower effectiveness of bacteria filtered from algal cultures in promoting survival of larvae fed MED in the absence of live algae. The concept of algal exudates regulating bacterial concentrations is supported by the enhanced larval survival observed when algal water containing algal

exudates was added to 5μmSW compared to controls in which the algal exudates added had been destroyed by autoclaving (Figure 3.6). Austin and Day (1990) similarly observed that antibacterial activity in spray dried *Tetraselmis suecica* was lost after exposure to temperatures over 60°C.

The method by which algae or algal extracts may exert this bacteriostatic effect is unknown. Apart from inhibiting bacterial growth, (Austin and Day, 1990; Austin *et al.*, 1992; de Nys *et al.*, 1995) algal extracts have also been reported to have inhibitory effects on bacterial swarming co-ordination thereby reducing the abilities of potential pathogens to colonize surfaces and establish disease (Givskov *et al.*, 1996; Gram *et al.*, 1996). However, algae-bacteria interactions are species specific and stimulation as well as inhibition of various bacterial species by algae and algal extracts have been reported (*Bell et al.*, 1974; Kogure *et al.*, 1979). Although a degree of antibiosis may be exhibited, large scale screening programs of algal extracts showing *in vitro* antibacterial activity subsequently revealed most of the extracts to be inactive *in vivo* (Reichelt and Borowitzka, 1984). Despite this, Austin *et al.*, (1992) obtained significantly increased survivals of *Salmo salar* L. following prophylactic treatment with 1% spray dried *T. suecica* incorporated into the diet and subsequent challenge by pathogens and they suggested a possible relationship of the algal metabolites of *T. suecica* to glucans which have been reported to enhance the non-specific immune systems of both prawns (Vargas-Albores *et al.*, 1993a; Song and Hsieh, 1994; Vargas-Albores, 1995; Sung *et al.*, 1996) and salmon (Robertsen *et al.*, 1990; Raa *et al.*, 1992). However if this were the case in present work, the mortalities observed in the (AuWMED+B) treatment (Table 3.2) should have been avoided. This suggests an environmental rather than an immunological role for the algal exudates in maintaining the balance of bacterial communities as the enhanced survival in the (SWMED+B) shows. This is further confirmed by the increased survival observed when balanced bacterial population samples from previous successful larval cultures are inoculated in sterile sea water (Figure 3.7). The increased larval survival obtained confirms that when a balanced natural bacterial community exists in natural sea water, live algae may be totally replaced by MED (Ottogalli, 1991; 1992). If this balance is disturbed as in the aftermath of algal blooms (Helm, 1971; Utting and Helm, 1985; Blight *et al.*, 1995), supplementation of MED with live algae or algal exudates is necessary.

Bacterially matured culture water (Table 3.2) also promoted better larval survival as opposed to new water which was rapidly heated up to the required temperature. Though many heterotrophic bacteria have been reported to synthesise several heat shock proteins upon temperature abuse (Allan *et al.*, 1988; Cloutier *et al.*, 1992), the protection afforded is not total (Cloutier *et al.*, 1992) and also varies between species and strains.

The rapid temperature change from 10 to 28°C might have imposed severe heat shock on the bacterial populations leading to an imbalance in the bacterial community. The more gradual increase in temperature of the OW treatment on the other hand, might have led to a more balanced community. Murchelano *et al.* (1975) reported no change in generic diversity of bacteria in stored sea water in an oyster hatchery over 15d despite an increase of 3 orders of magnitude from 10^4 to 10^7 cfu.ml^{-1}. Certain authors, (Salvesen *et*

al., 1995; Skjermo *et al.*, 1997) have suggested that microbially maturing water encourages the growth of slower-growing non-opportunistic bacterial species at the expense of the faster growing and presumably pathogenic opportunistic species. In this study however, survival obtained in the OW though higher compared to the total mortality obtained in the NW, does not agree with their findings. As this assay was carried out during the period of poor water quality in the Menai strait, it is possible that the bacterial maturation of the water was insufficient to overcome the bad water quality. Additions of bacteria from algal cultures to OW (OW+B) significantly improved larval survival (Table 3.2) and growth. This enhanced larval development was also observed in probiotic tests (Table 3.3). Though the reasons for the observed growth enhancement are unclear, They may reflect the addition of a more balanced bacterial community together with some algal exudates which control the potential pathogens.

Bacterial isolates tested for antagonism against known pathogenic isolates as possible probionts revealed varying results (Table 3.6). The well diffusion methods gave varied results among replicates with the isolates HL40, HL57 and HL58 against the pathogen, *V. harveyi* strain BP05. Despite this, confirmatory tests using disk diffusion methods revealed no such zones of antibacterial activity. Taken together with the huge variations observed within replicates, these results suggest that zones of clearance observed in the well diffusion methods were probably caused by technical errors in the experimental set up possibly resulting from inadequate cooling of the sterilised tubes used in making the wells which would have killed the bacteria mixed with the agar. We may have failed to obtain a potential probiotic strain as only seven strains were tested in this study. In addition, this study was restricted to bacteria isolated on only one medium. The stability and activity of inhibitory metabolites produced by bacteria is highly dependent on incubation conditions, making reproduction difficult (Olsson *et al.*, 1992). Lemos *et al.*, (1985) found only 38 out of 200 epiphytic isolates had antagonistic activity towards other bacteria. However, the potential ability for selective pre-emptive inhibition of potential pathogens in larval prawns offered by these bacterial probiotics emphasise the need for further studies on the mode and types of competitive advantage offered.

CHAPTER 4

STABILITY OF MICROENCAPSULATED DIETS, IMMERSION AND ORAL VACCINATION AND CROSS VACCINATION TESTS

Introduction

Bacterial disease problems are commonly encountered in prawn hatcheries and rearing facilities (Shewbart *et al.*, 1972; Lightner, 1983; 1988; Takahashi *et al.*, 1984; 1985a; b; Sunaryanto and Mariam, 1986; Egusa *et al.*, 1988; Baticados *et al.*, 1990; Lavilla-Pitogo *et al.*, 1990; Brock and Leamaster, 1992; Lee and Wickins, 1992; Mohney *et al.*, 1994). Total rearing water sterility due to pre-treatment is both impossible (Mevel, 1979; Wardell *et al.*, 1986; Boyd, 1996; Alabi *et al.*, 1997) and even undesirable (Brown, 1989; Gomes, 1992; Alabi *et al.*, 1997) such that potentially pathogenic bacteria are introduced into the rearing water and may occasionally cause problems under the conditions of intensive aquaculture. Initial treatment of these diseases is often uneconomical involving increased production costs such as reduced stocking densities and decreasing water carrying capacities (Andersen *et al.*, 1988; Brock and Leamaster, 1992). Should such methods fail however, a direct method of post-infection therapy is the use of antibiotics (Takahashi *et al.*, 1985a; Castille and Lawrence, 1986; Sunaryanto and Mariam, 1986; Baticados *et al.*, 1990; Baticados and Paclibare, 1992; Bell, 1992; Williams *et al.*, 1992; de la Peña *et al.*, 1993; Limsuwan, 1993; Primavera *et al.*, 1993). There are several problems associated with antibiotic use including potential environmental hazards (Jacobsen. and Berglind, 1988; Coyne *et al.*, 1994; Kerry *et al.*, 1995; Capone *et al.*, 1996) and the possible spread of antibiotic resistance (Brown, 1989; Samuelsen, 1989; Baticados *et al.*, 1990; Baticados and Paclibare, 1992; Williams *et al.*, 1992). Use of some antibiotics in association with artificial diets has been reported to cause faeces aggregation on penaeid larvae leading to heavy mortalities (Moullac *et al.*, 1992). This suggests a possible role of antibiotics in digestive mechanisms especially in the secretion of the peritrophic membrane as well as on digestive enzymes. Additionally, Rikjers *et al.*, (1981) have reported that some of the more commonly used antibiotics suppress the immune responses in carp.

Use of low levels of other chemotherapeutic agents such as chlorine and formalin are also unlikely to have much impact on disease causing organisms (Primavera *et al.*, 1993; Boyd, 1996) since the concentrations required to kill the pathogens are detrimental to larval survival (Boyd, 1996).

With these problems, research has focused on alternative methods of disease control such as the use of vaccines and immunostimulants of microbial origin (Itami *et al.*, 1989; Song and Sung, 1990; Adams,

1991b; Sung *et al.*, 1991; 1996; Prayitno, 1994). These methods attempt to stimulate the immune system and are therefore more suited for immunoprophylaxis rather than post-infection therapy. Such medication has been given to prawn larvae either orally by mixing with the food (Song and Sung, 1990; Itami and Takahashi, 1991) or by immersion (Prayitno, 1994). While both immersion and oral modes of vaccination are suitable for prawn larvae, oral vaccination is the more practical, removing the need to manipulate animals and is less time consuming. A potential drawback in the use of oral vaccination however, is the reduced efficiency due to the apparent destruction of the vaccines in the digestive system reported in fish (Jenkins *et al.*, 1994). Despite this, successful use of oral vaccines and immunostimulants has been described in fish (Raa *et al.*, 1992; Nikl *et al.*, 1993) but not prawns.

Larval feed design and stability is of vital importance in the oral administration of medicines. Most artificial diets available include processed natural products such as freeze-dried algae and yeasts or microbound particles using a wide range of gels such as agar, gelatin, zein, carrageenan or curing processes such as pH or formalin (Jones, 1988). However, the resultant particles exhibit poor particle stability and leach rapidly (Jones *et al.*, 1987; Jones, 1988). Microencapsulated diets (MED) (Jones *et al.*, 1984; 1987) were designed to overcome such problems of disintegration of larval prawn diets in water and have been widely used in penaeid larval culture (Ottogalli, 1991; 1992; Jones *et al.*, 1993). Recent reports (Muir and Sutton, 1994) have however, questioned the stability of MED suggesting that they are rapidly degraded by bacteria and their use in larval cultures may provide an oral route of entry for potentially pathogenic bacteria into the larvae.

As the use of such diets if this were true, would be self defeating in attempts to control potential pathogens, the initial aim of these experiments was to investigate the stability of MED, to test the efficacy of microencapsulation in reducing bacterial concentrations in culture waters and to establish the extent of microbial participation in the degradation of MED. In addition, the efficacies of oral and immersion modes of vaccination in conferring protection against diseases are compared.

In many disease outbreaks, there is an interplay of several causal factors which makes identification of the controlling factors difficult (Sunaryanto and Mariam, 1986; Lightner, 1988; Brock and Lightner, 1990; Mohney *et al.*, 1994; Prayitno, 1994; Prayitno and Latchford, 1995). It would be impractical to produce vaccines for every possible pathogen which might cause disease and hence, it would be desirable to produce a multivalent vaccine capable of offering resistance against a wide range of pathogens. The final part of this chapter investigates the possibilities of vaccine preservation by lyophilisation as well as the prospects of multiple cross-protection being conferred on larvae by such vaccines.

Materials And Methods.

Diets:

Protozoea (PZ) stage diets used were the micro-encapsulated diet (MED), Frippak Crustacean Algal Replacement (CAR) and a commercial micro-particulate diet (MPD). The mysis (M) stage diet used was the MED Frippak Crustacean diet number 2 (CD2), (INVE Aquaculture, Belgium). The live diets used were a mixture of the algae fed as described in chapter 2, until the larvae reached the M1 stage where the experiments were stopped.

Effect Of Diet Stability On Leaching Rates And Bacterial Loading Of Culture Water

Determination of total protein content, and protein leaching rates of larval diets

Samples of each of CAR, CD2 and MPD diets were dried to constant weight at 60°C over 24h and small (about 1mg) samples of each were weighed onto previously precombusted strips of aluminium foil. The total nitrogen content of the diets were determined in an elemental analyser (Roboprep-CN Biological Sample Converter; Europa Scientific) using acetinilide as a standard. Each sample was replicated five times. Protein content was estimated by multiplying nitrogen content by a conversion factor of 6.25 (Osborne and Voogt, 1978; Jobling, 1983).

To assess the effect of bacteria on protein leaching rates, 2L RBFs containing 2L of 5μm filtered water from *Penaeus indicus* post-larval recirculatory system cultures were inoculated with $1g.L^{-1}$ of either CAR, CD2 or MPD diets. These inclusion rates were much higher than the $4mg.L^{-1}$ (increase = x 250) and $7mg.L^{-1}$ (increase = x 143) recommended by the MED manufacturers for the PZ and M stage diets respectively to facilitate detection of small amounts of leached proteins. Controls consisted of autoclaved water with identical diet concentrations. Negative controls contained both 5μmSW and AuW with no added feeds. Flasks were incubated at 28°C ± 1°C with moderate aeration to allow full mixing of the diets. After incubation for 0, 1, 2, 4, 6, 9, 12, 24 and 48h intervals, 2ml samples were removed from each treatment for determination of dissolved protein as described in chapter 2. The amount of protein leached from each diet treatment was expressed as a percentage of the total protein content of the diet added. The protein concentration in the unfed controls was expressed as mg of soluble protein present per ml of solution. Each treatment was triplicated.

Percentage of total organic material in diets

In initial tests to determine total organic content of the diets, CAR, CD2 and MPD diets, were dried to constant weight at 60°C and 1g of each diet was measured into pre-weighed crucibles which were then combusted in a muffle furnace at 400 - 500°C for 7h. The final weights of the crucibles and remaining ash

were then measured and the initial weights of the crucibles were subtracted. The weight of the organic content of the feeds was then expressed as a percentage of the total amount of feed initially added.

Diet stability

In tests to assess the stability of the diets in water, 1g each of the feeds was weighed into sterile, 250ml conical flasks and 200ml of distilled water was added to each flask. The flask necks were plugged with cotton wool and incubated in a shaking incubator at 28°C ± 1°C and 150rpm. The flasks were removed after 6h and the water drained onto pre-weighed filter papers and the diets dried to constant weight at 60° C. The differences between the initial and final weights was taken as the amount that had been lost to the water and was expressed as percentage loss of the dietary solubles from the organic portion of the diets per hour.

Bacterial growth

To determine the increase in bacterial loading of culture waters due to added diets, RBFs, each with 2L of 5μmSW were inoculated with the manufacturer's maximum recommended concentrations of the PZ and M stage MED, and the PZ stage MPD diets. These corresponded to 4mg.L^{-1} and 7mg.L^{-1} for the PZ and M stage diets respectively. Controls consisted of 5μmSW without added diets. The flasks were incubated at 28°C ± 1°C with moderate aeration. To obtain TVC, the flasks were well shaken and 1ml samples were removed at 0, 6, 12, 24 and 48h, serially diluted, and 50μl aliquots of each dilution were spread onto SWC agar plates. The agar plates were incubated at 28°C for 48h and the TVC per ml were calculated from the resulting bacterial growth.

Bacterial attachment to diets

The TVC of bacteria associated with the diet particles were determined by removing 50ml aliquots from each treatment at 6, 12, 24 and 48h which was then filtered through a 3μm membrane filter (Nuclepore) to retain only the diet particles and associated bacteria. The bacterial counts in the filtrates were determined by taking 1ml aliquots from each filtrate, serially diluting in sterile saline, and spreading onto SWC agar plates. The filter membranes were rinsed three times with 200ml of sterile sea water each time and re-suspended in 10ml of sterile saline, vigorously vortex shaken for 3min to dislodge the diet particles and serially diluted in sterile saline. Fifty microliter aliquots of each dilution were then spread on SWC agar plates and the TVC of bacteria associated with the diets in the original sample volume was estimated using the formula

$$x = \frac{a}{b}$$

where x = TVC associated with diet particles.ml^{-1} in original sample

a = initial volume of water removed from samples (50ml)

b = final volume of solution (10ml)

The precision of this method was verified by matching the sum of the viable bacteria associated with the food particles and the viable bacteria in the filtrates to the TVC ± 2 x 10^3cfu.ml^{-1}.

The efficacy of microencapsulation in reduction of bacterial concentrations

In additional tests to assess the efficacy of microencapsulation in reducing bacterial loading, 3g of CAR was suspended in 150ml of distilled water and the capsules broken by sonication for 45min. The mixture was then divided into three 250ml conical flasks to give a final concentration of 0.5g.L^{-1} in 5μmSW from a *P. indicus* post-larval rearing tank. Controls used whole capsules (0.5g. L^{-1}) in similarly treated water. The flasks were incubated with shaking, at 28°C ± 1°C and TVC were taken at 0, 3, 6, 12, 24 and 48h.

Immersion Vaccination

Larval vaccination was carried out using a modification of the immersion method of Prayitno (1994). Both fresh and freeze-dried vaccines were used and were added to the cultures of PZ1 stage larvae of *P. indicus* in circular tanks with dimensions (25cm x 40cm) (radius x height) and 40L of water to give about 2.07 x 10^7 cells.ml^{-1}. The larvae are left immersed in vaccines for 6 - 8h after which they were transferred into autoclaved water and stocked at 100 larvae.L^{-1} into rectangular stacking boxes (38.00cm x 28.50cm x 21.50cm) (length x breadth x height).

Larvae were fed mixed algae and 100% water change was carried out daily. Controls consisted of unvaccinated larvae which had otherwise been subjected to the same treatments.

Pathogenic challenge was carried out 48h after vaccination and for these, previously vaccinated or control group (unvaccinated) larvae are stocked into 2L RBFs at 100 larvae.L^{-1} at 25‰ and 28°C ± 2°C. Log growth phase cultures of pathogenic bacteria cultured in SWC medium were harvested by centrifugation, rinsed twice and resuspended in sterile saline before being added to larval cultures to give a final concentration of around 1.24 x 10^6 cells.ml^{-1}.

Culture waters were not changed for the duration of the immersion challenge and surviving larvae were counted after 48h. Controls consisted of unvaccinated and challenged larvae while positive (general) controls were with unvaccinated and unchallenged larvae.

Oral vaccination:

Lyophilised *Vibrio harveyi* strain BP04 used in this assay was kindly incorporated into CAR diets by INVE Aquaculture at the rate of 0.05%, a concentration reported by Itami and Takahashi (1991) to enhance growth and survival of *Penaeus monodon* larvae. Controls consisted of CAR with no added vaccines.

Penaeus indicus PZ-1 stage larvae were reared in groups in rectangular stacking boxes (38cm x 28.50cm x 21.50cm) (length x breadth x height) in autoclave sterilised water with a temperature of 28°C, a salinity of 25‰ and a stocking density of 100 larvae.L^{-1}. Larvae were fed either CAR alone or CAR + vaccine at

4mg.L^{-1}.day^{-1}. The food was fed 4 times a day, at 6h intervals. Rearing water was changed daily to maintain good water quality. After two days, the larvae from each treatment were divided into 2L RBFs at a density of 100.L^{-1}.

Challenge was carried out using two different strains of *V. harveyi*: a highly virulent strain (BP04) and a less virulent strain (IN7) at a concentration of 1.54 x 10^6cells.ml^{-1}. Controls were with the larvae fed CAR alone and positive (general) controls were fed CAR and were unchallenged.

TVC were taken daily for the duration of the challenge tests and additional bacterial controls involving only the CAR+vaccine and pathogenic bacteria were included to assess any direct effect between the bacteria and any active ingredient in the feeds.

Results

Total protein content, diet stability and leaching rates of protein from diets

Total protein content of the diets determined from nitrogen content analyses gave averages of 525.90 ± 19.39μg, 553.95 ± 21.72μg and 455.51 ± 7.72μg per mg of diet for the CAR, CD2 and MPD diets respectively (Appendix 4.1). ANOVA, followed by Tukeys' pair wise comparisons revealed that the MPD diet had a significantly lower protein content compared to the two MED diets ($f = 42.51$; $p < 0.001$) (Appendix 4.1a).

Investigation of diet stability by percentage loss of dietary solubles in sterile water indicates that all the diets lose over 37.00% of their dietary soluble contents into the culture water within 6h (Table 4.1a) with the MED PZ stage diet leaching the least (mean = 6.24 ± 0.21%.h^{-1}) followed by the M stage MED diet (mean = 6.43 ± 0.12%.h^{-1}). The highest leaching rates occurred in the MPD diet (mean = 9.60 ± 0.61%.h^{-1}) (Table 4.1a). ANOVA on the arcsine transformations of the percentage nutrient loss per hour revealed that the two MED diets lost significantly less dietary solubles compared to the MPD diet ($f = 82.19$; $p < 0.001$) (Table 4.1b),

Protein leach rates showed high variability within treatments, but were on average, generally lower in the 5 μmSW samples compared to the AuW samples within treatment groups up to 9h (CAR and CD2) (Appendices, 4.2 and 4.2a to x and Figure 4.1). For the MPD diet treatment, these differences lasted up till 4h when leach rates were significantly lower in the 5μmSW filtered treatment (mean percentage soluble protein leached = 52.98 ± 2.78μg.mg^{-1} protein) than in the AuW treatment (mean percentage soluble protein leached = 63.88 ± 5.70μg.mg^{-1} protein). ($p = 0.045$; $t = 2.87$; 95% CI = 0.40 to 26.48%) (Appendix 4.2l). Apart from this, T-tests on the arc sine transformations of percentage protein leached from both the AuW and 5μmSW treatments within each diet group showed no significant differences up to 48h (Appendices 4.2, 4.2a-x).

Table 4.1a:Mean percentage nutrient leach (± standard deviations) of different microencapsulated (CAR and CD2) and microparticulate (MPD) larval diets in autoclaved water.
A

Diet	*Weight of feed left after 6 hours (g)*	*Amount of nutrients leached (g)*	*% of nutrients leached expressed as a percentage of average amount of organic matter in 1 g.*	*% of nutrients leached out per hour*
CAR A	0.66 ± 0.012	0.34 ± 0.012	37.40 ± 1.277	6.24 ± 0.214
CD2 A	0.65 ± 0.006	0.35 ± 0.007	38.55 ± 0.724	6.43 ± 0.123
MPD A	0.45 ± 0.03	0.55 ± 0.035	57.55 ± 3.65	9.6 ± 0.608

Table 4.1b shows the results of the Scheffe's pairwise comparisons on arcsine transformations of percentage nutrient leach per hour from each of the diets in sterile distilled water.

B Analysis of variance and Scheffe's pair wise comparisons: Units = Asin (%)

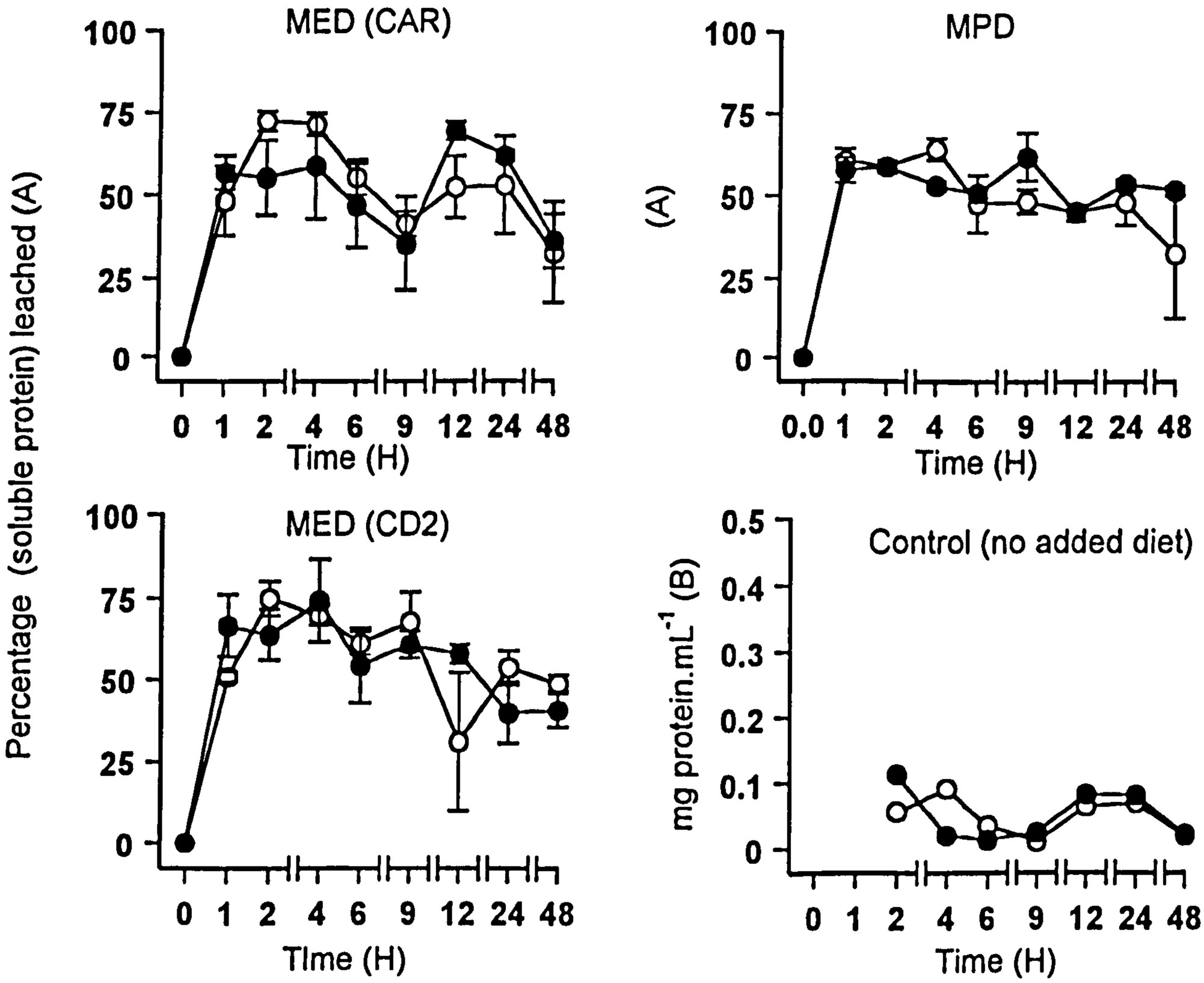
Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*N*	*Mean*	*StDev*
Treatments	2	0.114634	0.057317	94.31	<0.001	CAR	3	0.38338	0.01375
Error	6	0.003647	0.000608			CD2	3	0.39579	0.00784
Total	8	0.118281				MPD	3	0.62876	0.03966

Scheffe's pair wise comparisons

Diet	*Diet*	*diffmean*	*SE*	*low_ci*	*up_ci*
CAR	CD2	-0.0019300	0.0025690	-0.0106694	0.0068094
CAR	MPD	-0.0338400	0.0028723	-0.0436109*	-0.0240691*
CD2	MPD	-0.0319100	0.0028723	-0.0416809*	-0.0221391*

Comparison is horizontal. * denotes a significant difference at the 95% confidence interval

FIGURE 4.1: SOLUBLE PROTEIN LEACHED IN 5µM FILTERED SEA WATER (●)AND AUTOCLAVED SEA WATER SAMPLES(O) FROM 1G.L^{-1} OF THE MICRO-ENCAPSULATED DIETS, (CAR AND CD2) AND THE MICRO-PARTICULATE DIET (MPD)



Protein concentrations were expressed as percentage of total protein content of diets (A). Controls without added diets and soluble protein concentration expressed as total soluble protein (mg) content per ml of culture water (B). 100% = 525.90µg, 553.95µg and 455.51µg of protein for CAR, CD2 and MPD diets respectively. Each data point represents the mean value of three replicates. Error bars denote the standard errors of the mean. Data calculated from Appendix 4.2.

Bacterial growth in 5µm filtered sea water fed with microencapsulated and microparticulate diets

Mean log TVCs obtained in 5µmSW were lowest in the unfed control treatments for the duration of the trial (Table 4.2). Despite this, ANOVA followed by Tukey's pairwise comparisons revealed these differences to be significant only up to 6h for the PZ stage diets and 12h for the M stage diet. At 6h, there were no significant differences in log TVC obtained between the MED treatments. In addition, both had significantly higher log TVCs compared to the MPD treatment which in turn had significantly higher log TVC than in the unfed controls ($f = 31.85$; $p < 0.001$) (Appendices 4.3, 4.3a and Table 4.2).

At 12h, apart from the CD2 treatment which still had significantly higher log TVC than the controls, there were no significant differences in log TVC between the other diet treatments and the control (ANOVA: $f = 4.43$; $p = 0.04$) (Appendix 4.3b). After 12h, there were no significant differences in TVCs between all the treatments and the controls without added diets up to 48h ($f = 0.82$; $p = 0.519$) (Appendices 4.3c and d), suggesting that TVC obtained was dependent on both protein content and leach rates from the diets.

Bacterial attachment to diets

In all the treatments, mean log TVC attached to added diets showed an increase up to 24h, followed by a slight decrease of 0.24 log units, 0.53 log units, 0.46 log units and 0.66 log units for the CAR, CD2, MPD and control diet treatments respectively between 24 and 48h (Appendix 4.4 and Table 4.3). At 6h and 12h, the lowest log TVCs obtained were in the unfed controls (means = 3.54 ± 0.16 and 4.28 ± 0.18 cfu.ml^{-1} respectively). Mean log TVCs obtained in the other diet treatments at these times were (CAR = 4.46 ± 0.45 and 4.98 ± 0.09 cfu.ml^{-1}), (CD2 = 4.27 ± 0.14 and 5.07 ± 0.17 cfu.ml^{-1}) and (MPD = 4.07 ± 0.26 and 4.98 ± 0.14 cfu.ml^{-1}) at 6 and 12h respectively (Appendix 4.4 and Table 4.3).

ANOVA followed by Tukey's pairwise comparisons showed no significant differences in the log TVC per ml of bacteria attached to the diet particles at 6h. In addition, (except for the MPD treatment), log TVC of bacteria associated with the diet treatments were significantly higher than log TVC associated with other particles obtained in the controls at this time ($f = 6.07$; $p = 0.019$) (Appendix 4.4a). However by 12h, there were no significant differences in log TVC obtained between all the diet treatments though they all had significantly higher log TVC when compared to the controls ($f = 18.61$; $p = 0.001$) (Appendix 4.4b).

At 24 and 48h, the CD2 diet treatment exhibited the lowest log TVCs (means = 5.15 ± 0.10 and 4.63 ± 0.46 cfu.ml^{-1} respectively) compared to the CAR, MPD and control treatments which had mean log TVCs of (5.32 ± 0.05 and 5.09 ± 0.09 cfu.ml^{-1}), (5.18 ± 0.11 and 4.72 ± 0.17 cfu.ml^{-1}) and (5.38 ± 0.04 and 4.72 ± 0.34 cfu.ml^{-1}) at 24 and 48h respectively. At 24h, the log TVC in the CD2 diet treatment was significantly lower compared to the controls (ANOVA: $f = 5.07$; $p = 0.030$) (Appendix 4.4c) after which time, there were no significant differences in TVC between all groups up to 48h (ANOVA: $f = 1.40$; $p = 0.318$). (Appendix 4.4d and Table 4.3).

Table 4.2: Mean log total viable counts (TVC) (± SD) of bacteria.ml^{-1} in 5µm filtered sea water to which, different microencapsulated (CAR and CD2) diets and a microparticulate (MPD) diet, were added.

	FEED TREATMENT			
TIME (HOURS)	CAR A	CD2 B	MPD C	CONTROL D
0	4.6160 ± 0.295	4.6160 ± 0.295	4.6160 ± 0.295	4.6160 ± 0.295
6	5.8564 ± 0.0769 ABcd	5.9542 ± 0.1407 ABcd	5.5164 ± 0.1115 abCD	5.1476 ± 0.1108 abcD
12	6.3686 ± 0.0930 ABCD	6.5560 ± 0.0854 ABCd	6.4801 ± 0.1513 ABCD	6.2320 ± 0.1225 AbCD
24	6.4722 ± 0.0274 ABCD	6.5007 ± 0.0475 ABCD	6.5170 ± 0.0290 ABCD	6.4193 ± 0.0477 ABCD
48	5.4981 ± 0.0953 ABCD	5.4691 ± 0.1016 ABCD	5.4850 ± 0.215 ABCD	5.2913 ± 0.269 ABCD

A, ***B***, and ***C*** = comparison code letters for CAR, CD2, and MPD diet treatments respectively. ***D*** = Control treatment with 5µm filtered sea water but no added diet. Readings with the same code letter both in upper case characters denotes no significant difference ($\alpha = 0.05$). Readings with the same code letters in both upper and lower case characters indicates a significant difference. Each data point is the mean value of three replicates
Data calculated from Appendices 4.3 and 4.3a-d.

Table 4.3: Mean log TVC (± SD) of bacteria ml^{-1} attached to different microencapsulated (CAR and CD2) diets and a microparticulate (MPD) diet in 5µm filtered sea water.

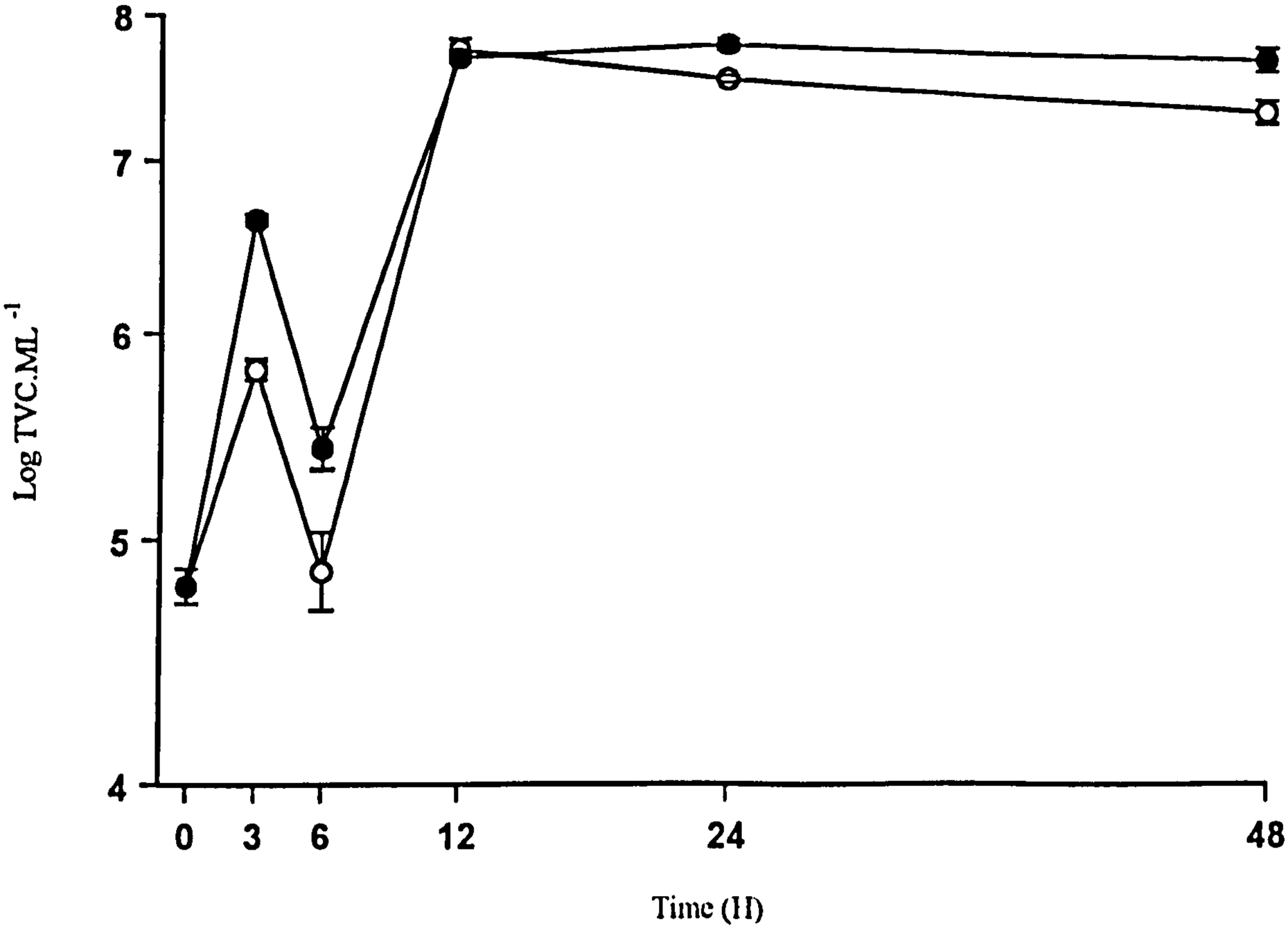
	DIET TREATMENT			
TIME (HOURS)	CAR A	CD2 B	MPD C	CONTROL D
6H	4.462 ± 0.453 ABCd	4.2734 ± 0.1366 ABCd	4.067 ± 0.257 ABCD	3.5355 ± 0.1608 abCD
12H	4.9834 ± 0.0926 ABCd	5.0672 ± 0.1676 ABCd	4.9817 ± 0.1428 ABCd	4.275 ± 0.177 abcD
24H	5.3244 ± 0.0549 ABCD	5.1544 ± 0.1047 ABCd	5.1818 ± 0.1071 ABCD	5.3756 ± 0.0448 AbCD
48H	5.0893 ± 0.0901 ABCD	4.629 ± 0.458 ABCD	4.7180 ± 0.1675 ABCD	4.717 ± 0.336 ABCD

A, ***B***, and ***C*** = comparison code letters for CAR, CD2, and MPD diet treatments respectively. ***D*** = Control treatment with 5µm filtered sea water but no added diet. Readings with the same code letter both in upper case characters denotes no significant difference ($\alpha = 0.05$). Readings with the same code letters in both upper and lower case characters indicates a significant difference. Each data point is the mean value of three replicates
Data calculated from Appendices 4.4 and 4.4a-d.

There was an increase in log TVCs obtained in 5µmSW inoculated with both sonicated and intact microcapsules (Appendix 4.5 and Figure 4.2). However, the treatment group with sonicated MED showed the greater increase (about 6755.21%) from an initial concentration of $6.04 \times 10^4 \pm 1.91 \times 10^4$ cfu.ml^{-1} to $4.32 \times 10^6 \pm 5.95 \times 10^5$ cfu.ml^{-1} cells.ml^{-1} within 3h. This contrasted with the intact microcapsule group which increased by about 1017.71% to $6.51 \times 10^5 \pm 1.41 \times 10^5$cfu.ml^{-1} during the same period.
After the initial exponential growth phase, both treatment groups showed a slight (about 1 log) reduction in bacterial numbers at 6h followed by a greater (about 2.5 log) increase in both treatments which was maintained until 48h (Figure 4.2). Lower tailed T-tests on the differences in the log TVCs per ml obtained in both groups over 48h, revealed that log TVCs were significantly lower in the water sample containing intact

MED compared to the treatment containing broken MED throughout the experimental period apart from the 12h interval where there were no significant differences in log TVC ($p = 0.75$, $t = 0.75$; 95% CI = 0.69 to 1.90cfu.ml^{-1}) (Appendices 4.5, 4.5a - e).

FIGURE 4.2: LOG TOTAL VIABLE COUNTS (TVC) OF BACTERIA IN 5µM FILTERED SEA WATER FROM POST-LARVAL *P. INDICUS* CULTURES TO WHICH INTACT (O)AND BROKEN (●)MICROENCAPSULATED DIETS (CAR) WERE ADDED AT 0.5G.L^{-1}.



CAR was broken by sonication for 45min. Controls were with intact (unbroken) microcapsules. Each data point represents the mean value of three replicates. Error bars denote the standard errors of the mean. Data calculated from Appendices 4.5 and 4.5 a-e

Vaccination and pathogenic challenge

Table 4.4 shows the effects of immersion vaccination of PZ-1 stage larvae of *P. indicus* with formalin-killed *V. harveyi* strains COL2, BP05 and IN7 followed by pathogenic challenge with live bacteria of the same strains. In all the cases, the lowest percentage survivals were obtained with the unvaccinated and challenged controls (Table 4.4). ANOVA on the arcsine transformations of the percentage survival values, revealed no significant differences in survivals obtained with the unvaccinated and unchallenged (general) controls, and the vaccinated treatments (Table 4.4) In addition, the general controls exhibited significantly

higher percentage survival values compared to the challenged controls (COL2: f = 14.11; p = 0.005), (IN7: f = 7.06; p = 0.027) (BP05: f = 36.91; p < 0.001) (Appendices 4.6a - c and Table 4.4).

In contrast to results obtained with the *V. harveyi* strain BP05 vaccine, despite having higher mean percentage larval survival values when compared to the challenged controls, treatments vaccinated with *V. harveyi* strains COL2 and IN7 also exhibited large standard deviations from the means (Table 4.4). This variability resulted in no statistically significant differences being observed between the vaccinated and control treatments in these groups by the ANOVA, and reveals differences in vaccine efficacies.

Vaccine preservation

In experiments performed using fresh and lyophilised *Vibrio harveyi* strain BP05 vaccines, percentage larval survivals obtained were 44.17% and 41.00% for larvae vaccinated with fresh and lyophilised vaccines respectively. Larval survival with lyophilised vaccines showed greater variability (SD = ± 7.00%) compared to the survival obtained with fresh vaccines (SD = ± 3.75%) (Table 4.5). Percentage larval survival was lowest with unvaccinated but challenged larvae (mean survival = 6.50 ± 4.82%).

ANOVA on the arc sine transformations of the percentage survival values obtained revealed no differences in the protection offered to larvae whether vaccinated with fresh or freeze-dried vaccines and larval survivals in both vaccinated treatments were significantly higher than larval survival obtained in the unvaccinated but challenged controls (f = 42.12, p < 0.001) (Table 4.5), indicating that lyophilisation of the vaccines did not affect its efficacy.

Table 4.4: Effect of formalin killed vaccines administered by immersion on the survival (%) of *Penaeus indicus* PZ3 stage larvae 48h after challenge with different strains of *Vibrio harveyi*. Controls were unvaccinated but challenged and general controls were unvaccinated and unchallenged.

Strain	*Vaccinated survival (%)*	*Unvaccinated survival (%)*	*General controls (%) unvaccinated and unchallenged*
COL2	47.30 ± 36.0^{a}	6.33 ± 7.77^{a}	94.67 ± 4.73^{b}
IN7	78.80 ± 11.30^{ab}	63.80 ± 6.83^{a}	89.00 ± 4.58^{b}
BP05	44.17 ± 3.75^{a}	6.50 ± 4.82^{b}	40.83 ± 4.07^{a}

Comparison is horizontal. Values with the same superscripts are not significantly different (α = 0.05).
Data calculated from Appendices4.6 and 4.6 a - c.

Table 4.5: The survival (%) of prawn larvae and one-way analysis of variance on arcsine percentage survival conferred on *P. indicus* PZ3 stage larvae after vaccination with fresh and lyophilized *V. harveyi* BP05 vaccines and subsequent pathogenic challenge by *V. harveyi* BP05

N	*Fresh vaccines*	*Freeze-dried*	*controls (unvaccinated)*
3	44.17 ± 3.75	41.00 ± 7.00	6.50 ± 4.82

Analysis of variance

Source	*DF*	*SS*	*MS*	*F*	*p*	*Vaccine*	*N*	*Mean*	*StDev*
Treatment	2	0.28373	0.14187	42.12	<0.001	Fresh	3	0.46	0.04
Error	6	0.02021	0.00337			Freeze dried	3	0.42	0.08
Total	8	0.30394				controls	3	0.07	0.05

Tukey's pair wise comparisons

Row		*Code.*	*Code*	*Diffmean*	*Low_ci*	*Up_ci*
Fresh vaccines	1	1	2	0.03436	-0.111100	0.17982
Freeze-dried vaccines	2	1	3	0.39265	0.247190*	0.53811*
Unvaccinated controls	3	2	3	0.35829	0.212830*	0.50375*

Comparison is horizontal. * indicates a significant difference ($\alpha = 0.05$).

Cross-protection tests

Significant cross-protection was offered by *V. harveyi* strain BP05 against subsequent challenge by other strains of *V. harveyi* and the percentage survivals obtained (means = 46.17%, 42.50% and 41.00%) after challenge by *V. harveyi* strains IN7, BP04 and BP05 respectively, were significantly higher than those obtained for the unvaccinated controls (means = 23.17%, 23.67% and 6.50% respectively). Variance ratios (f) and p values obtained were : f = 10.60, p = 0.011; f = 13.16, p = 0.006 and f = 4.51, p = 0.064 for *V. harveyi* strains IN7, BP04 and BP05 respectively (Appendices 4.7a - c and Table 4.6).

Results of further comparison of multivalent protection offered using a more virulent strain (BP04) and a less virulent strain (IN7) (Prayitno, 1994) of *V. harveyi* are shown in Table 4.7. While cross-protection was obtained, the more virulent strain (BP04), conferred significantly more protection onto vaccinated larvae. Mean percentage survival values of 47.50%, 68.50%, 52.17% and 60.17% were obtained for larvae challenged by strains BP04, IN7, BP03 and BP05 respectively after prior vaccination with strain BP04. This contrasted well with the mean percentage larval survivals of 37.33%, 50.50%, 43.70% and 38.17% respectively, obtained following challenge with the above bacteria after prior vaccination with strain IN7. However, these differences were not always significant (Appendices 4.8, 4.8a - d and Table 4.7).

Table 4.6: Effect of freeze-dried formalin killed *V. harveyi* BP05 vaccine on the survival (%) of *P. indicus* PZ3 larvae 48h after challenge with *V. harveyi* strains IN7, BP04 and BP05

Strain	*N*	*Vaccinated*	*unvaccinated*	*controls (unchallenged)*
IN7	3	46.17 ± 2.75^a	23.17 ± 10.20^b	40.83 ± 4.07^a
BP04	3	42.50 ± 6.06^a	23.67 ± 4.25^b	40.83 ± 4.07^a
BP05	3	41.00 ± 7.00^a	6.50 ± 4.82^b	40.83 ± 4.07^a

Comparison is horizontal. Values with the same superscripts are not significantly different ($\alpha = 0.05$). Data calculated from Appendices 4.7 and 4.7a - c.

Table 4.7: Survival (%) of *P. indicus* PZ3 stage larvae which had been vaccinated either by *V. harveyi* strain BP04 or strain IN7 and then challenged by *V. harveyi* strain IN7, BP03, BP04 or BP05.

Challenge bacterial strain	*N*	*BP04*	*IN7*	*Control (Challenged)*	*control (unchallenged)*
BP04	3	47.50 ± 10.30^{a}	37.33 ± 1.26^{a}	23.00 ± 3.61^{b}	76.7 ± 4.86^{c}
IN7	3	68.50 ± 3.50^{a}	50.50 ± 1.50^{b}	35.50 ± 3.50^{c}	76.7 ± 4.86^{a}
BP03	3	52.17 ± 14.29^{a}	43.7 ± 3.62^{ad}	21.50 ± 5.00^{bd}	76.7 ± 4.86^{c}
BP05	3	60.17 ± 13.60^{a}	38.17 ± 2.08^{bd}	31.33 ± 2.08^{cd}	76.7 ± 4.86^{a}

Comparison is horizontal. Values with the same superscripts are not significantly different ($\alpha = 0.05$). Data calculated from Appendices 4.8 and 4.8a - d.

Oral vaccination

The percentage survival values obtained when larvae were orally vaccinated with *Vibrio harveyi* strain IN7, were similar in both the vaccinated (mean = 39.82 ± 15.85%), test (challenged) control (mean = 51.59 ± 6.96%) and general (unchallenged) control (mean = 42.88 ± 19.5%) groups, and ANOVA on the arc sine transformations of the percentage survival data, revealed no significant differences between all treatment groups. ($f = 0.48$, $p = 0.64$) (Appendices 4.9, 4.9b-c and Table 4.8), reflecting the relative avirulence of this strain.

On the other hand, subsequent challenge with the more virulent strain (BP04) gave lower percentage survivals of 65.50 ± 9.67%, 0.83 ± 0.76% and 0.33 ± 0.58% following oral vaccination for the general controls, vaccinated and test control groups respectively. In this case, while ANOVA showed that there was no significant difference in survival between the vaccinated and the test controls (Table 4.9), survival obtained in both of these treatments was significantly lower ($f = 88.47$, $p < 0.001$) (Appendices 4.10 and 4.10b) compared to the unchallenged (general) controls. These indicated that in contrast to immersion methods of vaccination, no protection was conferred upon larvae by oral vaccination.

Log TVCs obtained in both treatments showed no significant differences in concentration for the duration of the experiments. However, log TVC in the bacterial controls (diet + vaccine + pathogen) which were added to check whether there was any direct effect between the diet and the pathogens was significantly higher than in the other treatments probably due to the nutrient leach from the uneaten diets added.

ANOVA on log transfomations of TVC values gave variance ratios (f) and p values of: $f = 27.26$; $p < 0.001$ and $f = 12.66$; $p = 0.005$ for larval cultures vaccinated with strains IN7 and BP04 vaccines respectively at 48h. (Appendices 4.9b and 4.10a).

Table 4.8: Larval survival (mean % ± SD) and log TVCs obtained in *P. indicus* larval cultures at various times following oral vaccination by *V. harveyi* strain BP04 vaccine and subsequent challenge by *V. harveyi* strain IN7

Day	*car+vac*	*cont*	*Gen. cont*	*Bact+Vac*
1	6.19	6.19	*	6.19
2	6.22 ± 0.10^{a}	6.30 ± 0.07^{a}	6.07 ± 0.15^{a}	7.24 ± 0.31^{b}
3	5.42 ± 0.15^{a}	5.64 ± 0.17^{a}	5.58 ± 0.27^{a}	6.87 ± 0.26^{b}
% survival (Day3)	39.82 ± 15.85^{a}	51.59 ± 6.96^{a}	42.88 ± 19.5^{a}	

Comparison is horizontal. Values with the same superscripts are not significantly different ($\alpha = 0.05$). Data calculated from Appendices 4.9 and 4.9a - c.

Table 4.9: Larval survival (mean % ± SD) and log TVCs obtained in *P. indicus* larval cultures 48h after challenge by *V. harveyi* strain BP04 which followed 48h of oral vaccination by *V. harveyi* strain BP04 vaccine.

Treatment	*Gen. Cont.*	*Car+VAC*	*Cont*	*Bact.+Vac*
Mean log TVC	5.21 ± 0.06^{a}	6.48 ± 0.25^{b}	6.50 ± 0.18^{b}	7.40 ± 0.41^{c}
% survival	65.50 ± 9.76^{a}	0.83 ± 0.76^{b}	0.33 ± 0.58^{b}	

Comparison is horizontal. Values with the same superscripts are not significantly different ($\alpha = 0.05$). Data calculated from Appendices 4.10 and 4.10a and b.

Discussion

In the analysis of total diet proteins, some error may have been introduced by the use of a nitrogen to protein conversion factor of 6.25 which is derived from fish proteins (Osborne and Voogt, 1978; Jobling, 1983). However, the calculated values (means = 52.5% and 55.4% of dry weight) for the CAR and CD2 diets respectively, are not greatly different from the total protein contents claimed by the manufacturer (55.0%).

Although the initial percentage soluble protein leaching rates were higher over the first hour in the 5μmSW (bacteria containing) treatments fed MED diets compared to the AuW treatments, after this time, leach levels were generally lower in the 5μmSW samples compared to AuW samples. These lower readings are very likely due to the presence of bacteria in these 5μmSW treatments which utilise the soluble protein as soon as it becomes available leading to lower soluble protein measurements. The initial increase in percentage protein leach rates within 1h observed in the 5μmSW treatment fed MED may also have been protein leached from the diet before utilization by the available bacteria.

The smaller differences in percentage soluble protein leached between the 5μmSW and AuW groups obtained in the MPD diet treatment from 0 to 2h, may be explained by the significantly lower total protein content of this diet (455.51$\mu g.mg^{-1}$) compared to MED diets with 525.90$\mu g.mg^{-1}$ and 553.95$\mu g.mg^{-1}$ for the PZ and M stage diets respectively. Due to the high protein leach rate of the MPD (60.41% in 1h) as well as the leach rates of total dietary solubles, most of the available protein content of this diet would have already been leached into the water, thus leading to the smaller differences observed in the percentages of soluble protein content leached.

No significant differences were observed in leaching rates when all diets were incubated in sterile or bacterial contaminated water samples over 48h. Despite sterility checks on the AuW samples to ensure initial sterility, introduction of bacteria into the cultures with the feed may have occurred. Even if this had occurred however, they appeared not to have contributed much to the protein consumption as up to 6h, the AuW displayed slightly higher soluble protein values compared to the 5μmSW treatments. Thus, there does not appear to be any evidence that bacteria are responsible for increasing leach rates from MED or MPD over 48h.

The higher total protein content of the MED diets compared to the MPD diet is reflected in the significantly higher TVCs obtained when compared to the MPD treatment (Table 4.2). Though the MPD treatment had significantly lower TVCs compared to the MED diets at this time, this was not due to better stability and/or reduced leach rates as the diet stability tests showed above, but may be due to the lower content of soluble protein. The occurrence of significantly higher TVCs in the CD2 diet compared to the other treatments at 12h can also be attributed to the higher concentration of diet added (7$mg.L^{-1}$) compared to the other two (PZ stage) diets at 4$mg.L^{-1}$ each. As after 6h and 12h respectively, there were no significant differences in

TVCs between all the diet treatments and the controls, the rate of bacterial growth in a medium is likely to depend on the rate at which soluble nutrients become available.

TVC attached to diet particles also shows a similar trend in that there were no significant differences between the MPD and control treatments at 6h again, suggesting that most of the nutrients in the MPD treatment were probably already in solution supplying enough nutrients for the free-living bacteria. Increased adhesion has been reported to be a consequence of nutrient depletion observed in several marine bacteria (Dawson *et al.*, 1981; Kjelleberg and Hermansson, 1984; Albright *et al.*, 1986; Hermansson *et al.*, 1987). The higher TVCs attached to the MED diets at 6h would therefore confirm better the stability of and less leaching by these diets (MED) compared to the MPD. This also agrees with Hermansson *et al.*, (1987) who hypothesise that, in a growth deficient medium, non-growing small cells may be transferred by active or passive processes to a particle in the vicinity where adsorbed dissolved nutrients (ZoBell, 1943; Neihof and Loeb, 1972) are found to allow growth. By 12h, there were no significant differences in TVC of bacteria attached to all diets. Again, the higher TVCs observed attached to diet particles in the MED CD2 treatment reflects the comparatively higher amounts of CD2 diet (7mg.L^{-1}) added initially. There were no significant differences between all the treatments and the control at 24 and 48h. Although no diets were added to the controls, this may be explained by increased bacterial adhesion to other bacterial cells as well as to inorganic microparticles which passed through the 5μm filter.

Bacterial adhesion to non-metabolites and inanimate surfaces has been reported (ZoBell, 1943; Pedersen, 1982; Kjelleberg and Hermansson, 1984; Hermansson *et al.*, 1987; Karunasagar *et al.*, 1995) and through interaction with or, adhesion to non-metabolites, increased bacterial activity has been stimulated (Conn and Conn, 1940; Bigger and Nelson, 1941). Furthermore, Fletcher and Floodgate (1973) demonstrated production of a secondary adhesive, fibrous reticular polysaccharide by bacteria attached to solid surfaces. These processes would lead to increases in both the general TVCs as well as the TVC of bacteria attached to the inanimate surfaces being retained on the 3μm filters in the control groups with no added diets.

The more rapid increase in TVCs in treatments with broken MED compared to the intact MED groups reflects the higher amounts of nutrients which were immediately available to the bacteria and confirm that microencapsulation initially reduces the rate of loss of solubles and assists in the reduction of TVC.

Significant protection was conferred on the prawn larvae by some of the formalin-killed vaccines tested. This confirms previous work by Prayitno (1994) who tested the efficacy of vaccination of larval *P. indicus* against luminous bacterial pathogens. With one exception (Itami and Takahashi, 1991), contemporary work on penaeid vaccination has focused mainly on post larval to adult prawns (Itami *et al.*, 1989; 1992; Song and Sung, 1990; Sung *et al.*, 1991) and in challenge tests, vaccinated animals were challenged by exposure to the virulent pathogens from which the vaccines have been prepared. Results have however been less than conclusive with some authors (Itami *et al.*, 1989) reporting reduced mortalities on challenge

while for others (Sung *et al.*, 1991) there was no protection offered. Similar results were obtained in this study with significant protection being offered by some strains of *V. harveyi* but not by other strains.
In any case, field confirmation of such laboratory tests is considerably more difficult as prawn larvae are affected by a myriad of disease pathogens and environmental variables which make it difficult to ascribe mortality to a single pathogen (Lightner, 1988; Brock and Lightner, 1990; Brock and Leamaster, 1992). As a result, the cross-protection conferred on larvae demonstrated in present work due to vaccination by other strains of *V. harveyi* is particularly relevant as there is no immediate prospect of a vaccine against some of the ubiquitous opportunistic pathogens to which farmed larvae are vulnerable. This incidence of cross-protection also confirms the wide spread agreement about the lack of an adaptive immune response which responds to vaccination in crustaceans (Lackie, 1980; Klein, 1984; Ratcliff *et al.*, 1985; Smith and Chisholm, 1992).

Vaccines are classically directed against the acquired immune system of organisms which is characterised by the ability to produce antibodies against specific epitopes of solubilised molecules (antigens) or other microbial components. Such systems also display immunological memories against previously encountered invaders (Amos, 1981). This makes the use of the term "vaccination" in connection with prawns a misnomer. The mechanism by which such "vaccination" confers protection on prawns is still poorly understood but is known to be due to the augmentation of the non-specific immune system in which case, "immunostimulation" would be a more descriptive term (as described in chapter 5).

This view is supported by the observation that in cross-protection tests, survival of larvae vaccinated with the more pathogenic *Vibrio harveyi* BP04, gave better survival against a range of other pathogenic *Vibrio harveyi* strains when compared to a less pathogenic strain, IN7. It even gave larvae better protection against challenge by the IN7 strain than larvae vaccinated with a vaccine made from the IN7 strain.
The reasons for the apparent differences in vaccine efficacies obtained here are unknown, but the consistency of increased protection achieved against all tested strains makes it unlikely to be due to random variation. While further study is required to fully elucidate the reasons for these differences, it may be related to the amount and/or types of recognised cross-reacting antigens on the cell walls. Indeed in fish, differences in surface characteristics of different *Aeromonas salmonicida* (Trust *et al.*, 1982; Kay *et al.*, 1988; Chu *et al.*, 1991) and *A. hydrophila* (Loghothetis and Austin, 1996) strains has been shown to be associated with virulence determination. Such surface characteristics are thought to be involved in foreign recognition. Comparisons of the vaccine potentials of such bacterial strains has revealed their superiority as fish immunogens over non-virulent strains (Trust *et al.*, 1982; McCarthy *et al.*, 1983; Olivier *et al.*, 1985; Loghothetis and Austin, 1996). Such may be the case in prawns where humoral lectins (Adams, 1991b; Vargas-Albores *et al.*, 1993b; Vargas-Albores, 1995) may act both as recognition proteins and opsonins.

Itami *et al.*, (1989) found no significant differences in the increased protection conferred on *P. monodon* adults whether vaccinated by injection, immersion or spray methods. However, vaccination by injection

and spray methods are not appropriate for prawn larvae due to their small sizes, delicate nature and the large amounts of larvae usually involved. Immersion vaccination has been shown to work in larvae (Prayitno, 1994) and this has been confirmed in present work. Unlike immersion vaccination however, oral vaccination of *P. indicus* larvae in this study failed to offer protection to the larvae against subsequent pathogenic challenge. While similar oral administration of *Vibrio sp.* bacterin to larval (Itami and Takahashi, 1991) and post larval (Song and Sung, 1990) *P. monodon* have been reported to enhance growth, they were either not followed by pathogenic challenge (Itami and Takahashi, 1991) or no protection was observed following such challenge. This is in good agreement with the results obtained in this study following oral vaccination.

Apart from the lack of a protective effect, pathogenic challenges can fail for many reasons. These include the route of pathogenic challenge, virulence of the pathogen, environmental and physiological factors affecting both pathogen and hosts and vaccine preparation and delivery. Taking into consideration, that no successful report has yet been made of orally obtained larval protection from vaccines, it seems possible that the vaccines are not absorbed in a functional state from the digestive system for although Sung and Song (1996) suggested that that absorption and digestion of *Vibrio* bacterin are similar whether delivered orally or by immersion, this observation was made with adult prawns.

No growth measurements were taken with the oral vaccination trials in this study, however the improved growth reported by other researchers following post larval vaccination (Song and Sung, 1990) is enigmatic and seems to suggest an effect on the digestive rather than immune systems. Detritivores have been reported to digest over half of the bacteria ingested (Baker and Bradnam, 1976). Similarly, Intriago and Jones (1993) have reported the growth of *Artemia* to sub adult stage when fed *Flexibacter* strain Inp3. In addition, Jones *et al.*, (1997) have reported improvements in growth of *P. indicus* post larvae when a recombinant, protease producing *E. coli* strain was incorporated into their diets.

In this study, the significantly higher survivals observed in the unchallenged (general control) group as opposed to the almost complete mortalities obtained in the CAR+VAC (test) and CAR (control) groups when orally vaccinated and challenged with pathogenic *V. harveyi* strain BP04, demonstrates the failure of oral vaccination in conferring enhanced protection on larvae. In addition when challenged with a less virulent pathogen (IN7), the CAR+VAC (test treatment) had the lowest survival (mean = 39.82%) as opposed to the CAR (challenged control) (mean = 51.59%) and the unchallenged (general control) (42.88%) groups although these differences were not statistically significant. However, it is worth noting that larvae in this study were at the PZ3 larval stage as opposed to the older (PL30) stage (Song and Sung, 1990) prawns used by other researchers who have reported a stimulation of growth. At such an early developmental stage, a deficiency in secretion of digestive enzymes occurs (Jones *et al.*, 1992; Kumlu and Jones, 1995) related to the undeveloped hepatopancreas which progresses in development after the PZ3/M1 stages (Lovett and Felder, 1990; Abubakr, 1991; Abubakr and Jones, 1992). Despite this, Itami and Takahashi (1991) obtained significantly enhanced larval survival of PZ3 - M1 stage *P. monodon* fed

MED with incorporated killed *Vibrio sp.* cells. The reported differences obtained when compared to this study, can however be explained by these researchers supplementing their MED diets with mixed algae *Skeletonema sp.* and *Merosilla sp.* at 1,250 cells.ml^{-1}. Algal supplementation of artificial diets has been reported to enhance enzyme secretion, digestibility and growth of penaeid larvae (Kanazawa *et al.*, 1977; Amjad and Jones, 1992; Amjad *et al.*, 1992; Kamarudin, 1992; LeVay *et al.*, 1993; Kumlu and Jones, 1995). This enhanced digestibility as a result of the algal supplementation would facilitate better nutritional utilisation of the MED and the bacterial cells by the early larvae.

Analysis of the log TVCs obtained in the oral vaccination tests did not reveal any direct effect of the diets on the pathogens as the log TVCs in the bacterial control groups were significantly higher compared to log TVCs in all the other groups through out the experiments, ruling out a direct inhibition of bacterial growth by vaccines incorporated into the diets.

In view of failure of the oral methods of vaccination it is gratifying that vaccines preserved by lyophilization, proved effective in immersion vaccination and presents a method of obtaining a consistent supply of defined immunostimulants for the vaccination of larval prawns.

CHAPTER 5

EFFECTS OF VACCINES ON ASPECTS OF THE IMMUNE SYSTEM OF *PENAEUS VANNAMEI*

Introduction

Incidents of bacterial disease epizootics on prawn farms and hatcheries have been well documented (Takahashi *et al.*, 1985a; b; Sunaryanto and Mariam, 1986; Sae-oui *et al.*, 1987; Baticados, 1988; Egusa *et al.*, 1988; Lightner, 1988; Baticados *et al.*, 1990; Brock and Lightner, 1990; Lavilla-Pitogo *et al.*, 1990; Ottogalli, 1991; Brock and Leamaster, 1992; de la Peña *et al.*, 1993; Mohney *et al.*, 1994), with bacteria of the genus *Vibrio* being implicated in the majority of cases. Most of the bacteria causing diseases have been reported as part of the normal prawn microflora (Vanderzant *et al.*, 1971; Yasuda and Kitao, 1980; Ruangpan, 1982; Lightner, 1985; 1988; 1992; Lavilla-Pitogo *et al.*, 1990; Owens *et al.*, 1992; Mohney *et al.*, 1994; Prayitno, 1994) and in nature, penaeid prawns, with their open circulatory systems must have the ability to rapidly kill and clear invasive or opportunistic microbes which manage to penetrate the structural and chemical defences of the cuticle and get into the haemolymph. Increased susceptibility to disease found in farmed prawns, occurs as a consequence of impairment of the immune defence mechanisms resulting from the physical and environmental abuse characteristic of most intensive aquaculture systems. With the growing importance of disease in prawn culture systems, a detailed knowledge of the functioning of its immune system is essential in disease control and treatment.

Vertebrate-like antibody precursors have not yet been demonstrated in invertebrates, and it is widely accepted that invertebrates are incapable of an adaptive immune response (Lackie, 1980; Klein, 1984; Ratcliff *et al.*, 1985; Smith and Chisholm, 1992). Despite this, immunostimulation of prawns using products of microbial origin, has been reported to enhance resistance against disease causing pathogens (Adams, 1991b; Prayitno, 1994; Latchford *et al.*, 1995; Sung *et al.*, 1996). Both humoral and cellular factors have been implicated in these resistance procedures (Itami *et al.*, 1989; Adams, 1991b; Vargas-Albores *et al.*, 1993b; Vargas-Albores, 1995; Sung *et al.*, 1996) and research has been primarily focused on the cellular processes of immunity especially with regards to generation of reactive oxygen species (Song and Hsieh, 1994; Sung *et al.*, 1996), phagocytosis (Hose and Martin, 1989; Vargas-Albores, 1995), encapsulation (Hose and Martin, 1989), coagulation (Omori *et al.*, 1989) and multimeric systems such as the coagulation system and the pro-phenoloxidase (proPO) enzyme activation system which has been identified as being at least, partly responsible for non-self recognition, opsonisation and phagocytosis of foreign bodies in crustaceans (Söderhäll and Unestam, 1979; Söderhäll, 1982; Smith and Söderhäll, 1983; Ashida and

Söderhäll, 1984; Hose *et al.*, 1987; Chisholm and Smith, 1992; Vargas-Albores *et al.*, 1993a; Vargas-Albores, 1995; Sung *et al.*, 1996).

Phenoloxidase (PO, O-diophenol-oxygen oxidoreductase; E.C. 1.10.3.1), is the major enzyme involved in this process and is essential in the production of melanin during infection and wound healing. Occurring in crustaceans as its inactive pro-enzyme (Söderhäll *et al.*, 1986; Hose *et al.*, 1987; Vargas-Albores *et al.*, 1993a), this system has been shown to be specifically activated by microbial cells (Smith and Söderhäll, 1983; Ashida and Söderhäll, 1984; Vargas-Albores *et al.*, 1993a; Vargas-Albores, 1995; Sung *et al.*, 1996).

Recognition of "foreigness" is a first step in defence processes but in crustaceans, the actual processes of foreign recognition are still unclear. It has been variously suggested to be due to products of proPO activation (Smith and Söderhäll, 1983; Ashida and Söderhäll, 1984; Söderhäll *et. al.*, 1986; Adams, 1991b). or agglutinins ("Lectins": sugar-binding proteins or glyco-proteins of non-immune origin which precipitate glycoconjugates or agglutinates cells excluding carbohydrate binding enzymes and toxins (Goldstein *et al.*, 1980)). Lectins have been found on invertebrate haemocyte surfaces (Renwrantz and Stahmer, 1983; Vasta, 1987; Renwrantz, 1990) and plasma (Vasta, 1987; Vargas-Albores *et al.*, 1993b; Vargas-Albores, 1995) which can bind to potentially dangerous bacteria and fungi or their cell wall components. In addition, Hose and Martin (1989) have reported enhanced phagocytosis following prior opsonisation of bacteria with cell free plasma. Similarly, Vargas-Albores, (1995) reported increased phagocytic capacities by *Penaeus californiensis* haemocytes following incubation of erythrocytes with LPS-binding agglutinin isolated from brown prawn haemolymph, suggesting that the most important role of invertebrate agglutinins in immune defence is in opsonically enhancing phagocytosis rather than agglutination or precipitation. However, Goldenberg and Greenbury (1983), have found the opsonising and agglutinating activities in *Homarus americanus* to be separate.

In fresh water crayfish, some researchers (Smith and Söderhäll, 1983; Söderhäll *et al.*, 1986) have suggested that bacterial endotoxins or cell wall products stimulate the labile deposit cells (granulocytes in this case), to release proPO granules into the plasma where they become activated and coat the microbes thus enhancing phagocytosis. This suggestion however, may not apply to penaeid prawns as it contradicts the findings by Hose and Martin (1989) of attachment of proPO granules to bacterial clumps only after 3h incubation when many of the phagocytes had lysed after phagocytosis. In addition, (Vargas-Albores, 1995) has found LPS and β-1,3-glucan recognition factors in *Penaeus californiensis* plasma and reported that recognition proteins (agglutinins) activate cellular activities after prior reaction with microbial substances, (LPS, or β-1,3-glucans). While further study on the molecular basis of opsonisation and antimicrobial defences is required, it seems likely, as suggested by Smith and Chisholm (1992), that antimicrobial defence lies with a range of factors rather than a single broad spectrum factor.

In decapods, bacterial clearance accompanied by a marked haemocytopaenia, has been reported following injection of live or dead bacteria (Martin *et al.*, 1993; 1996; Hauton *et al.*, 1997). A series of interesting

experiments have been performed using radio labelled bacteria (Martin *et al.*, 1993) and indirect fluorescent antibody techniques (Sung and Song, 1996) to follow bacterial clearance from the haemolymph. Bacteria are removed to some tissues (principally the gills and haematopoietic tissue) where they are eliminated by fixed phagocytes. The exact method of disposal here is unclear but Martin *et al.*, (1996) and Hauton *et al.*, (1997) have reported an increase in phagocytic activity after sequestration of bacteria. Particle engulfment is an energy requiring process and this "respiratory burst" generates a series of microbicidal substances that either inhibit microbial activities, or completely digests the micro-organisms (Song and Hsieh, 1994). It has also been suggested that encapsulated organisms may be killed by the toxic effects of melanin and the other intermediate by-products of proPO system activation, or lysosomal enzymes contained in some haemocytes (Hose *et al.*, 1987).

Antimicrobial factors represent another component of the invertebrate immune response (Ratcliff *et al.*, 1985). Lysozyme-like activity has already been demonstrated in the haemolymph of several insects (Hultmark *et al.*, 1980; Engström *et al.*, 1984; Kaaya *et al.*, 1987) and bivalves (Cheng and Rodrick, 1974; Foley and Cheng, 1977; Cheng *et al.*, 1980). Results of attempts to demonstrate lysozyme activity in penaeid prawns have so far, been equivocal. Some authors (Guzmán *et al.,* 1993) have reported haemolytic activity in cell free haemolymph of *Penaeus vannamei* while, other researchers (Noga *et al.*, (1996) have been unsuccessful. Antibacterial factors with properties clearly different from those of lysozyme have also, been reported in insects (Faye and Wyatt, 1980; DeVerno *et al.*, 1984; Engström *et al.*, 1984; Yoshida and Ashida, 1986; Boman and Hultmark, 1987).

Apart from these immediate responses to microbial infection, a few studies have described the acquisition of a longer lasting "immune" state following exposure of prawns to killed *Vibrio sp.* (Itami *et al.*, 1989; Adams, 1991b; Prayitno, 1994) cells and immunostimulants (Sung *et al.*, 1996). However, no analyses of protein composition of prawn plasma after injection has yet been performed and the significance of these long term resistance factors to overall disease resistance has largely been overlooked.

The resistance of naive and "immunized" penaeid prawns to infection by a normally pathogenic bacterium following prophylaxis may provide a model for studies of prawn antibacterial immunity.

Knowledge of bacterial dynamics *in vitro* and *in vivo* in such prawns, coupled with studies of humoral and cellular defence mechanisms and bacterial virulence factors may offer increased manipulative advantages in prawn growing regions.

In this chapter, the elicitation and duration of induced antibacterial activity is investigated and related to the *in vitro* and *in vivo* destruction of several bacterial species by immunized and naïve plasma of *P. vannamei.* The capacity of different bacterial species to activate the proPO system in both *P. indicus* and *P. vannamei* is also assessed. In addition, to observe possible new proteins produced, analytical electrophoresis of both immunized and naive plasma was conducted in poly-acrylamide gels using the anionic detergent, sodium dodecyl sulphate (SDS) with Bicin-Tris together with heat to denature the proteins before running these on discontinuous gels.

Materials And Methods

Prawns used

Penaeus indicus juveniles used were 2nd generation captive offspring previously spawned in the School of Ocean Sciences and grown in recirculatory raceways to juvenile stage. *Penaeus vannamei* post-larvae were obtained from Centro de Investigacion in Alimentacion y desarrollo (CIAD) Mexico, and grown to a mean weight of 9.41g in a recirculatory raceway at 28°C ± 2°C with 31 - 33‰ salinity. To avoid inadvertent stimulation of prawn immune systems by additives in formulated diets, they were fed only with cut squid and lugworms. Only apparently healthy, intermoult animals were used.

Anticoagulant solution for haemolymph collection - Shrimp salt solution (SSS) (Vargas-Albores *et al.*, 1993a)

NaCl	450mM
KCl	10mM
EDTA.Na_2	10mM
HEPES	10mM
pH	7.3
Ratio of haemolymph to anticoagulant	1:2

Cacodylate buffer used in phenoloxidase determination

Na.cacodylate	10mM
$CaCL_2$	10mM
pH	7.0

Bacterial species used and vaccine preparation

Non-virulent *Escherichia coli* strain XL1-Blue MRF', *Vibrio harveyi* strain DPEX, and pathogenic *Vibrio harveyi* strains BP03, BP04, BP05 and IN7 as well as *Aeromonas hydrophila* strain PM were obtained and prepared as described in Chapter 2. Lyophilised gram positive *Micrococcus lysodeikticus* (*luteus*) (Sigma) was similarly prepared.

Formalin-killed vaccines were made from the relevant bacteria.

Haemolymph Extraction and Separation:

Haemolymph was removed from the heart of juvenile *P. vannamei* using a 27 gauge needle into a 1ml syringe containing 2 volumes of ice cold shrimp salt solution (SSS) (Vargas-Albores *et al.*, 1993a). The procedures were carried out on ice and subsequent manipulations were carried out in a cold room at 4°C. The withdrawn blood was centrifuged in an Eppendorf microfuge at 13000g for 2min. The supernatant was

passed through a 0.45μm PVDF Acrodisc LC13 filter (Gelman) and stored at -177°C for no more than 4 weeks before use.

Assays of prophenoloxidase activity

To obtain the inactive prophenoloxidase (proPO) enzyme containing granules from the haemocytes, the haemocyte pellets obtained after decanting the plasma supernatants were resuspended in SSS and washed again. They were finally resuspended in 3ml of sodium cacodylate, the haemocyte containing tubes were fixed inside icetrays and the mixture homogenised using a glass piston homogeniser (Pierce). The resulting suspension was centrifuged for 20min at 43000g and 4°C to pellet the cell debris. The supernatant, called haemocyte lysate (HLS), was the enzyme source and was stored at -177°C for up to 4 weeks before use.

Incubation procedures were performed in 96 well microtitre plates with 50μl of HLS mixed with 50μl of each of all the gram negative bacteria listed above which had been formalin killed, lyophilised and resuspended ($2mg.ml^{-1}$) in sodium cacodylate (CAC) buffer. Blanks were with CAC buffer alone and controls consisted of zymosan (Sigma) resuspended ($2mg.ml^{-1}$) in CAC buffer + 50μl HLS. Additional controls against spontaneous oxidation of the substrate alone consisted of CAC buffer, L-Dopa and zymosan. Plasma controls were also included to ensure that no accidental activation of the HLS occurred during the procedure. Samples with plasma showing proPO activity were discarded. The samples were incubated at room temperature (20°C) for 65min after which 100μl of L-3,4-Dihydroxyphenyl-alanine (L-Dopa) dissolved ($3mg.ml^{-1}$) in CAC buffer, was added to each well and absorbance values at 490nm were taken at 5min intervals over 20min in a microplate reader (Biokinetics Reader EL-340, BIO-TEK Instruments). One unit of proPO activity was expressed as an increase in absorbance of 0.001mg $protein^{-1}.min^{-1}$.

Bacterial growth in plasma from naïve prawns

To assess the bacterial growth in plasma from untreated (naïve) juveniles, 25μl of *Escherichia coli* suspension (mean total TVC = 6.91×10^5 cfu) were mixed with 150μl of plasma and 25μl of SSS. In control samples, 150μl of plasma was replaced by 150μl of SWC medium. Each treatment was triplicated. Samples were incubated in sterile 1.5ml eppendorf tubes. Fifty microliter aliquots from each sample were spread on SWC agar plates at 0h to determine initial bacterial concentration. All the samples were incubated with gentle shaking, at 31°C for 3h after which the tubes were rapidly chilled in an ice-tray, suitably diluted in sterile saline and 50μl of each sample was again spread on agar plates. Plates were incubated at 37°C for 24h and the TVC were determined.

Assays of antibacterial and lysozyme activity in cell free haemolymph of vaccinated *Penaeus vannamei*

Preliminary tests were performed to check the effects of the addition of 1mg.ml^{-1} of hen egg white lysozyme (Sigma) to *Escherichia coli* suspensions. Twenty five microlitres of *Escherichia coli* suspension (mean total TVC = 6.91 X 10^5 cfu) was incubated with 25µl of lysozyme (11.2mg.ml^{-1} in sterile SSS), 25µl of SWC and 125µl of SSS. The controls consisted of 25µl of *Escherichia coli* suspension, 25 µl of SWC and 150µl of SSS.

Forty eight hour cultures of *Vibrio harveyi* strain DPEX were centrifuged at 11000g (5min, 25°C) to pellet the cells. The pellet was re-suspended in sterile SSS (pH = 7.3), rinsed thrice and resuspended in SSS. One hundred microliters of the final suspension containing 2.215 x 10^5 to 2.998 x 10^5 cells at room temperature, were injected into the ventral sinus of the first abdominal segment of each replicate prawn. Controls were placebo injected with 100µl of sterile SSS and negative controls were uninjected. Test prawns were kept three each, in net covered baskets with dimensions of 31.5cm x 21.6cm x 20.1cm (length x breadth x height) suspended in a *Penaeus vannamei* rearing raceway as above. Haemolymph was removed at 0, 6, 12, 24 and 48h, 4d 7d, 14d and 21d and cell-free plasma was obtained as described above.

Log-phase *Escherichia coli* and lyophilised *Micrococcus luteus* grown in SWC (pH = 6.4) were harvested by centrifugation (400g; 25°C; 2min) and washed once in sterile SSS + 0.1M phosphate buffer (pH = 6.4). The bacterial pellets were resuspended in sterile SSS, well shaken and kept on ice. A stock solution of 11.2mg.ml^{-1} lysozyme was prepared in 0.1M phosphate buffered SSS. Incubation mixtures were prepared in sterile 1.5ml eppendorf tubes on ice. For the anti *E. coli* assays, 25µl of *E. coli* suspension was mixed with 150µl of plasma from either bacteria injected or placebo injected prawns with 25µl of SWC and 25µl of lysozyme. Controls consisted of plasma from uninjected prawns. Contamination controls were included in which the bacterial suspension was replaced with SSS and the 150µl of SSS was replaced with 150µl of naïve plasma. The addition of 25µl lysozyme brought the final lysozyme concentration in the mixtures to 1mg.ml^{-1}. Antibacterial activity was regarded as a decrease in the TVC compared to the initial TVC and ratio of change in TVC (R) was calculated as

$$R = \frac{A_t - A_o}{A_o}$$

with A_o and A_t being the TVC at the beginning and the end of the reactions respectively. One unit of antimicrobial activity was computed as a difference of 0.01 between R(control) and R(treatment).

Lysozyme assays were similarly carried out with *Micrococcus luteus* replacing the *E. coli* used in the antibacterial assays and the hen egg white lysozyme was excluded. Lysozyme activity was similarly regarded as a decrease in TVC compared to the initial TVC and where this occurred, ratio of change in TVC (R) was calculated as

$$R = \frac{A_t - A_0}{A_0}$$

and lysozyme activity (units) was computed as R(control) - R(treatment).

In vitro bacterial killing assays with plasma from vaccinated and naïve prawns

Penaeus vannamei juveniles (mean wt. = 9.41g) were injected with 100µl of thrice washed vaccines of *Vibrio harveyi* strains BPO4, DPEX and *Escherichia coli* strain XL1-Blue MRF`. All vaccines were suspended in sterile SSS to give a wet weight concentration of about 10.875mg.ml^{-1} which corresponded to total CFUs of 7.46 x 10^6, 7.38 x 10^6 and 9.68 x 10^6 per 100µl respectively before injection. Placebo controls were injected with sterile SSS and negative controls were not injected at all. Haemolymph was removed from the prawns' hearts after 48h and pooled. The haemocytes were spun down in an eppendorf microfuge, and the supernatants passed through a 0.45µm (Gelman) filter and stored at -177°C for no more than 4 weeks until used.

Challenge tests were carried out in sterile 1.5ml eppendorf tubes and the test mixtures were: 200µl of plasma obtained from prawns from each vaccine treatment and the controls, were reacted with 100µl of live *Escherichia coli* suspension (mean total cfu = 5.35 x 10^5). A contamination control consisting of plasma from naïve prawns + SSS was included. The bacterial cells in each group were sensitised by the addition of 25µl of lysozyme which gave a final concentration of 1mg.ml^{-1}.

Fifty microlitre samples were removed at 0, 3, 6, 12 and 24h and plated after suitable dilution. Antibacterial activity was recorded as survival index (SI) values (Wardlaw and Unkles, 1978) calculated as

$$SI = \frac{\text{CFU at end}}{\text{CFU at start}} \times 100$$

with a SI value greater than 100 indicating growth and one lower than 100 indicating antibacterial activity.

Measurement of haemolymph volume

The haemolymph volume of *Penaeus vannamei* juveniles was estimated using the inulin dilution method of Levenbook (1958). ^{14}C Inulin carboxyl -^{14}C, 1.4 mCi.g^{-1} (Sigma) was diluted with sterile SSS to give a solution containing 6.0 x 10^4 disintegrations per minute (dpm).µl^{-1}. *Penaeus vannamei* juveniles (n = 12; weight range = 2.10 to 13.90g), were injected with 50µl of this inulin solution and held at 28°C. At 1, 2 and 3h, 40µl of haemolymph were removed from the pleopod base of the first abdominal segment of each prawn using a 20µl auto-pipette equipped with an ultra thin microtip. One hundred microlitres of a tissue solubilizer (Soluene 350, Packard Canberra) were added to the removed haemolymph and the mixture was made up to 4015.00µl in a high ionic strength liquid scintillation cocktail (Hionic Flour, Packard Canberra). A set of standards were made up in SSS to reflect final dilutions in volumes of 1950 to 4950µl. Blanks consisted of haemolymph from uninjected prawns and SSS for the samples and the standards respectively. Radioactivity was measured using a Beckman LS7500 liquid scintillation counter.

In vivo bacterial killing assays in vaccinated prawns

Lyophilised formalin - killed vaccines of *Vibrio harveyi* strains BP04 (virulent) and DPEX (avirulent), and *Escherichia coli* Strain XL1-Blue MRF' were resuspended in SSS and the concentrations of each was adjusted to 7.26μg.ml^{-1}. One hundred microliters of the final vaccine suspension corresponding to total cfus of ≈ 7.38 x 10^6, 7.46 x 10^6 and 9.68 x 10^6 for *Vibrio harveyi* strains DPEX and BP04 and *Escherichia coli* strain XL 1-Blue MRF' respectively, was injected into the ventral sinus of the first abdominal segment of *Penaeus vannamei* juveniles (mean weight = 9.41 ± 2.23g). Vaccinated prawns were stocked according to vaccines administered, at 0.15 prawns.L^{-1}, in 20L of water in rectangular stacking boxes with dimensions of 33.50 x 24.00 x 14.00cm (length x breadth x height).

Each vaccine treatment group was triplicated. Bacterial challenge tests were carried out 48h after vaccinations by injecting 100μl of each of the live bacteria from which the vaccines used were made by injections into the ventral abdominal sinus of the target prawn. Concentrations of injected bacteria were ≈ 7.38 x 10^6, 7.46 x 10^6 and 9.68 x 10^6 total cfus for *V. harveyi* strains DPEX and BP04 and *E. coli* strain XL 1-Blue MRF` respectively.

At 3h, 8h, 12h, 24h and 48h, 10μl of haemolymph was removed from the pleopod base of the first abdominal segment of each prawn using a 10μl auto-pipette (Eppendorf Reference) equipped with an ultra thin microtip. The withdrawn haemolymph was immediately serially diluted in sterile SSS, and spread on SWC agar plates.

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) electrophoresis of proteins

Protein electrophoresis was carried out according to the method of Laemmli (1970)

Chemicals and Reagents

Laemmli buffer (Laemmli, 1970)

0.0625M Tris-HCL (pH 6.8)

2% SDS

10%Glycerol

5% mercaptoethanol

0.001%bromophenol blue

Resolving gels

Table 5.0: Showing the quantities of reagents used to make different concentrations of SDS-Page resolving gels.

Reagents	*15% Gel*	*10% Gel*	*8.5% Gel*
Distilled water	2.69ml	4.35ml	4.85ml
29% Acrylamide, 1% Bisacrylamide	5.00ml	3.33ml	2.83ml
500mM Bicin-Tris pH 8.3	2ml	2ml	2ml
10% Sodium dodecyl Sulphate	100µl	100µl	100µl
2.5% Ammonium persulphate	200µl	200µl	200µl
TEMED	16µl	16µl	16µl

Stacking gel

29% Acrylamide, 1%Bis-acrylamide	500ml
10%SDS	30µl
0.2M Tris-HCl	1.5ml
distilled water	1.0ml
Temed	3.0µl
2.5% APS	15µl
pH	6.8

Running buffer

Tris base	0.025M
glycine	0.192M
SDS	0.1%
pH	8.3

Staining solution (Coomasie blue)

Methanol	20ml
Glacial acetic acid	5ml
Distilled water	25ml
Coomasie brilliant blue	0.1%

Silver staining reagents (Sigma)

Fixing solution

Glacial acetic acid	90ml

Ethanol	270ml
Distilled water	540ml

Stop solution

Glacial acetic acid	3.0ml
Deionised water	297ml

Development solution

Formaldehyde	0.02% in deionised water
Sodium carbonate	2.50% in deionised water

Photographic reducing solution. (Switzer *et al.*, 1979).

Solution A:

NaCl	37g
CuSo4	37g
deionised water	850ml

Concentrated ammonium hydroxide was added until a deep blue precipitate was formed and then dissolved. The solution was then made up to 1L with deionised water.

Solution B:

Sodium thiosulphate	436g
Deionised water	1L

Equal volumes of solutions A and B were mixed together, and the final solution was diluted 1:3 (v/v) with deionised water before use.

Gel preparation

A mini PAGE gel apparatus was used for SDS-PAGE. The gel plates chamber and spacers were cleaned with ethanol prior to use. The apparatus, plates and spacers were assembled according to the manufacturer's instructions. The resolving gel was prepared by gently mixing together the ingredients taking care to avoid the formation of air bubbles. The resolving gel was then poured into the chamber until it reached a level of 2cm below the top of the rabbit eared plate. An overlay of propan-2-ol was immediately poured onto the top of the resolving gel to ensure a perfectly level surface and to prevent diffusion of oxygen (which retards polymerisation) into the gel.

After polymerisation, the propan-2-ol was poured off, the chamber rinsed with distilled water and excess distilled water was blotted with a paper towel. The stacking gel mix was then poured on top of the resolving

gel and the stacking gel combs were immediately inserted avoiding the formation of air bubbles, and the gel left to polymerise.

Sample preparation

Cell free haemolymph was obtained from vaccinated, placebo injected, wounded and naïve *Penaeus vannamei* juveniles. Protein concentrations were determined as described in Chapter 2 and all samples were adjusted to similar values (about 3$\mu g.\mu l^{-1}$), using human serum albumin (HSA) as standard. The samples were mixed 1:1 with laemmli buffer heated at 100°C for 5min to denature the proteins after which, they were stored at -20 C until used.

Electrophoresis of protein samples

The gel system was assembled according to the manufacturer's instructions. Running buffer was then added to the top and bottom buffer chambers and samples carefully injected into the wells. Unused wells received an equal amount of gel-loading buffer. The gels were run through the stacking gel at 15 volts, increased to 25-30 volts when the bromophenol blue marker entered the resolving gel. The run was terminated when the marker reached the bottom of the gel. The gel was removed, stained for at least 4h in Coomasie brilliant blue stain and then destained with frequent changes, in a mixture of glacial acetic acid, methanol and distilled water (ratio 1 : 4 : 5 (v/v/v) respectively).

Coomassie Blue stained gels were subsequently overstained with silver using the AG-5 silver stain kit (Sigma) following the manufacturer's protocol:

Pre-stained gels were rinsed with deionised water for 10min three times, followed by subsequent equilibration in 0.1% silver nitrate solution for 30min. The gels were rapidly rinsed in deionised water, 300ml of development solution were poured over the gel, and gently agigated while watching for the development of the protein bands.

After the development of the bands, the development solution was poured off and the reaction stopped with 1% glacial acetic acid solution for 5min followed by three 10min rinses in distilled water. Gels were placed in photographic reducer solution for 5 - 10sec., followed by a rapid 1min tap water rinse. Gels were subsequently rinsed three times in deionised water (10min each) and stored in deionised water.

Results

Stimulation of bacterial growth in naïve plasma

Upper tailed 2-sample T-tests on the results of the preliminary assays of the logarithmic conversion of increase in TVC of *E. coli* when incubated in plasma from naïve juveniles of *P. vannamei* showed a significant increase ($p = 0.0002$; $t = 11.37$; 95% CI = 1.333 to 1.56 cfu) (Appendix 5.1) in TVC when incubated for 3h in plasma (mean log TVC = 6.37 ± 0.02) compared to controls incubated in SWC (mean log TVC = 6.21 ± 0.01) indicating that *P. vannamei* plasma constitutes a richer nutritive medium for bacterial growth than SWC.

Effects of addition of lysozyme to *Escherichia coli* suspensions

Lower tailed T-tests on the effects of pre-sensitisation of *E. coli* suspensions by addition of 1mg.ml^{-1} of lysozyme revealed no significant differences in TVCs obtained after 3h incubation ($p = 0.13$; $t = -1.32$; 95% CI = 0.59 to 1.21 cfu) (Appendix 5.2), with mean log total TVC values of 6.10 ± 0.08 and 6.18 ± 0.05 for the treatments with and without added lysozyme respectively indicating that addition of 1mg.ml^{-1} of hen egg white lysozyme has no significant effect on bacterial growth.

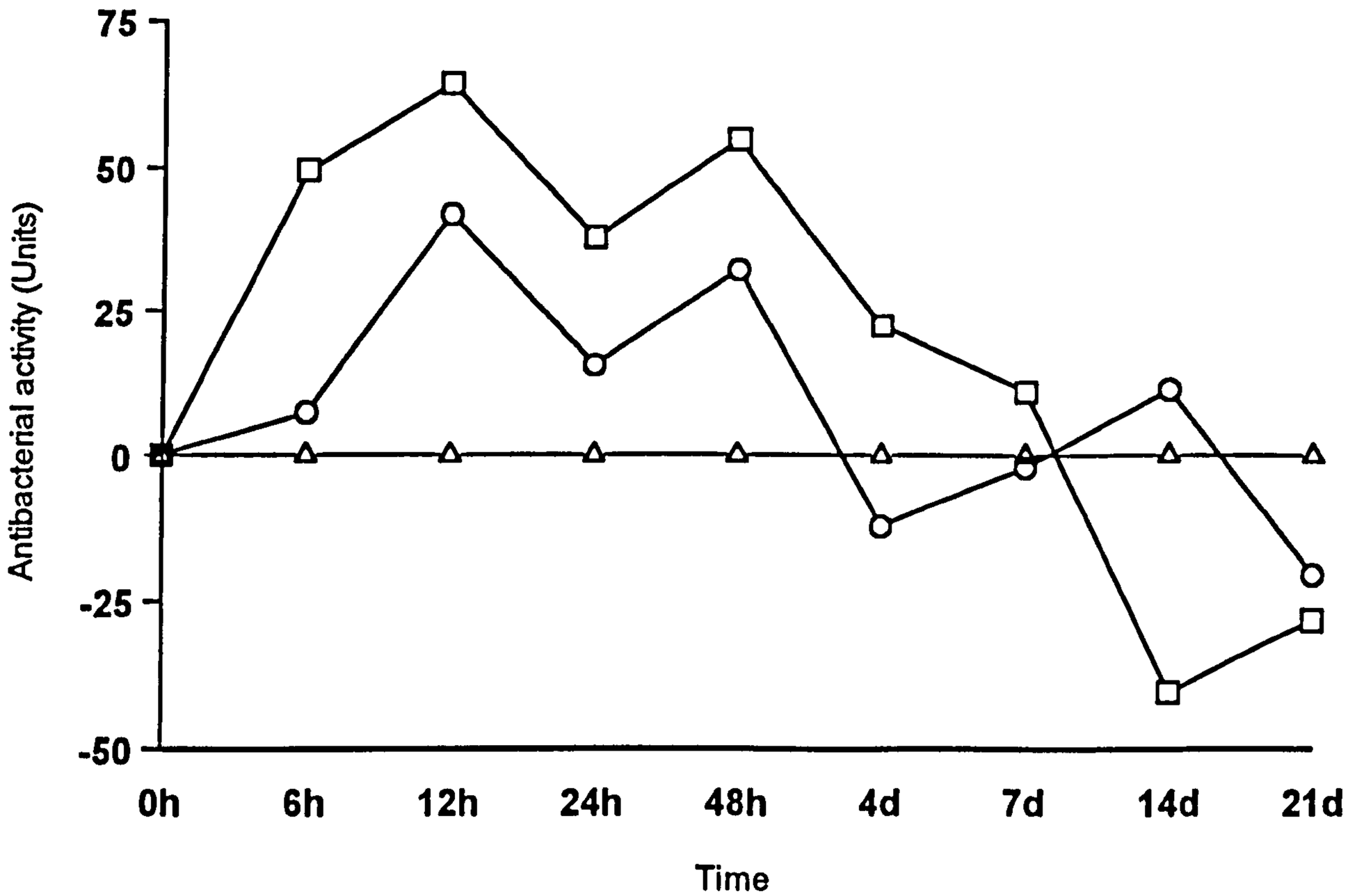
Measurement of antibacterial activity in plasma of vaccinated *P. vannamei*

Anti *E. coli* activity is shown in Figure 5.1. Upper tailed 2-sample T-tests revealed that the log TVCs of *E. coli* obtained after 3h incubation in naive plasma samples were significantly higher (mean log TVC = 5.96 ± 0.06) compared to the log TVCs at 0h (mean log TVC = 5.84 ± 0.01) ($p = 0.0071$; $t = 3.69$; 95% CI = 1.09 to 1.63 cfu) (Appendix 5.3), indicating that the plasma of naïve *P. vannamei* contains no background antibacterial activity when analysed under present conditions.

In contrast to this, a marked antibacterial activity appears within 6h in both vaccinated and placebo injected juveniles and rises to maximum values at 12h (Figure 5.1). Antibacterial activity was detectable until 7d after vaccination when ANOVA revealed no significant differences in antibacterial activities obtained in the vaccinated treatment and those of the placebo injected and control groups ($f = 0.47$; $p = 0.644$) (Appendices 5.4, 5.4a - h). At all times when antibacterial activity was detected, the levels were higher in the vaccinated treatments compared to the placebo injected treatments and the untreated controls although these differences were not always significant (Appendices 5.4, 5.4a - h and Figure 5.1), suggesting that antibacterial activity observed in prawn plasma was stimulated in the prawns not only by vaccination, but also by wounding.

FIGURE 5.1. EFFECT OF VACCINATION ON ANTIBACTERIAL ACTIVITY IN PLASMA OF *P. VANNAMEI.*

Anti *E. coli* activity in sera of vaccinated (□), placebo injected (○) and uninjected (△) juveniles of *Penaeus vannamei* over time



Data calculated from appendices 5.4 and 5.4a-h

Measurement of lysozyme activity in plasma of vaccinated *P. vannamei*

In lysozyme assays, log TVCs of *Micrococcus luteus* obtained in all the treatments (vaccinated, placebo injected and controls) after 3h incubation, were consistently significantly higher than the initial log TVCs at 0h (Appendices 5.5, 5.5a - h and Table 5.1), indicating a lack of lysozyme activity in all the treatments. Despite this, at 6h, plasma from vaccinated prawns exhibited a definite suppression of *Micrococcus luteus* growth with log TVCs obtained in the vaccinated treatment (mean log TVC = 4.36 ± 0.25) not being significantly higher than initial log TVCs (mean log TVC = 4.03 ± 019). In addition, 6h after vaccination, the concentration of *M. luteus* in vaccinated plasma after 3h incubation was significantly lower when compared to both the placebo (mean TVC = 4.66 ± 0.05), and the control (mean TVC = 4.88 ± 0.18) plasma treatments (ANOVA: f = 13.80; p = 0.001) (Appendix 5.5a). After 6h, all treatment log TVCs were significantly higher than initial log TVCs up to 48h (Appendices 5.5 and 5.5d and Table 5.1), where only the placebo injected treatment was not significantly higher than the initial log TVCs (f = 11.08; p = 0.002).

Table 5.1: Log TVC of *Micrococcus luteus* cells after 3h incubation in plasma withdrawn at different times from vaccinated (Vac), placebo injected (Plac) and naive (Control) prawns for 3h. "Start" is the initial log concentration of *M. luteus* cells added.

Time	*Vac*	*Plac*	*Control*	*Start*
0h				
6h	4.3634 ± 0.2492 ABcD	4.6558 ± 0.0455 ABCd	4.8790 ± 0.1822 aBCd	4.0157 ± 0.2080 AbcD
12h	4.7473 ± 0.0626 ABCd	4.5702 ± 0.2321 ABCd	4.8790 ± 0.1822 ABCd	4.0157 ± 0.2080 abcD
24h	4.7571 ± 0.0669 ABCd	4.7048 ± 0.0539 ABCd	4.8790 ± 0.1822 ABCd	4.0157 ± 0.2080 abcD
48h	4.6858 ± 0.1144 ABCd	4.5205 ± 0.2813 ABCD	4.8790 ± 0.1822 ABCd	4.0157 ± 0.2080 aBcD
4d	4.3464 ± 0.2302 ABcD	4.6180 ± 0.0238 ABCd	4.8790 ± 0.1822 aBCd	4.0157 ± 0.2080 AbcD
7d	4.3045 ± 0.1949 ABcD	4.3295 ± 0.0455 ABcD	4.8790 ± 0.1822 abCd	4.0157 ± 0.2080 ABcD
14d	4.6061 ± 0.1277 ABCd	4.6209 ± 0.1222 ABCd	4.8790 ± 0.1822 ABCd	4.0157 ± 0.2080 abcD
21d	4.7891 ± 0.6935 ABCD	4.1103 ± 0.0861 ABCD	4.8790 ± 0.1822 ABCD	4.0157 ± 0.2080 ABCD

ABCD = comparison code letters for vaccinated, placebo injected, naive plasma and start values respectively. Comparison is horizontal. Any two treatments with the same letter in both upper and lower case characters are significantly different. (α = 0.05). Statistical analysis was by analysis of variance followed by Tukey's pairwise comparisons. Data analysed from Appendix 5.5.

Measurement of *in vitro* killing activity in plasma of vaccinated *P. vannamei*

Results of the *in-vitro E. coli* killing assays in plasma from prawns injected with vaccines made from various bacterial strains (Figure 5.2), revealed decreases in survival index (SI) values at 3h in all the vaccinated treatments apart from the *V. harveyi* strain BP04 treatment which exhibited a slight increase (increase = 6.46%) (SI value = 106.46 ± 10.98%). The unvaccinated (control) treatment exhibited a larger increase (increase = 27.36%) (SI value = 127.36 ± 9.30%) over the same time interval. SI values obtained for the other treatments were 93.11 ± 8.24%, 98.5 ± 17.7%, and 68.8 ± 26.4 % for the DPEX, SSS and *E. coli* vaccinated plasma respectively. As the values contained percentage values higher than 100%, arcsine transformations were not possible and hence, ANOVA, followed by Tukey's pairwise comparisons, were performed on log transformations of surviving TVC values (Appendix 5.6). This revealed no significant differences in *in vitro* killing of the bacteria in all the treatments at 3h, apart from the *E. coli* treatment which was significantly reduced in concentration compared to the control ($f = 5.20$; $p = 0.016$) (Appendices 5.6 and 5.6b).

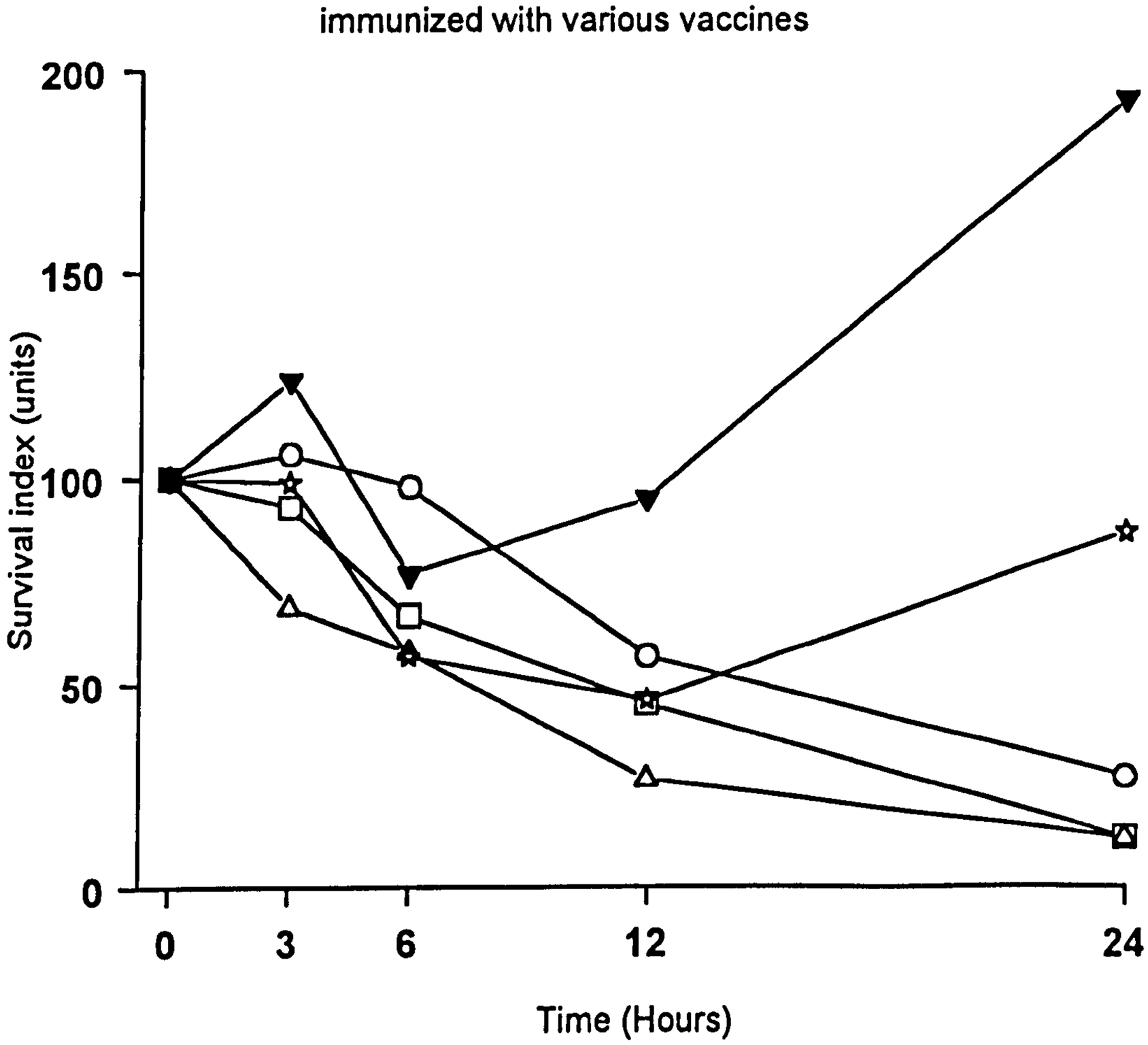
Following this, the SI values fell consistently in all vaccinated treatments up to 24h where SI values of 11.92 ± 5.23%, 11.94 ± 3.64% and 27.25 ± 7.78% were obtained for the *E. coli* and *V. harveyi* strains DPEX and BP04 treatment respectively. SI values obtained in the placebo injected (SSS) and control treatments decreased only up to 6h (control: SI = 76.51 ± 6.73%) and 12h (SSS: SI = 45.98 ± 3.23%) after which they both exhibited rises in SI values terminating at 24h with SI values of 86.7 ± 34.3% and 192.5 ±

51.2% for the SSS and control treatments respectively (Figure 5.2 and Appendices 5.6 and 5.6a). ANOVA at this time, revealed no significant differences in survival of *E. coli* incubated in plasma from prawns vaccinated with the *Vibrio harveyi* strains BP04 and DPEX as well as *E. coli* strain XL1-Blue MRF`. In addition, bacteria incubated in all three vaccinated plasma displayed significantly lower concentrations when compared to bacteria incubated in SSS and naïve plasma controls respectively. The concentrations of surviving bacteria in these two treatments were not significantly different from each other (ANOVA: f = 35.80; p< 0.001) (Appendix 5.6and 5.6e).

In vitro activation of the prophenoloxidase activating systems of naive *Penaeus vannamei* juveniles by formalin-killed vaccines from different bacterial species

Penaeus indicus cell free plasma were consistently contaminated by activated PO as evidenced by the higher PO activation values obtained for the plasma (Appendix 5.7a). Haemocyte lysates (HLS) from *P. indicus* were therefore discarded. Scatterplot analyses of results of the kinetic assays of phenoloxidase activation in haemocytes of naïve *P. vannamei* juveniles over 20min by the different bacterial strains used consistently displayed low R^2 (goodness of fit values) (Appendices 5.7b, and 5.7l - t), indicating that the increases in absorbencies observed over time are not linear. PO activation values were therefore, calculated per mg of protein, per min for each time interval (Table 5.2). Results revealed that for all the bacterial species tested, PO activation detected was higher in the initial 5min (after the addition of L-Dopa) compared to subsequent times after which, the activities generally levelled out or declined (Table 5.2). The highest PO activation was recorded upon stimulation by *Vibrio harveyi* strain IN7, followed by *Aeromonas hydrophila*, *Vibrio harveyi* strain BP04, zymosan and *V. harveyi* strains DPEX, BP03 and BP05 with mean values of 26.57 ± 3.20, 22.75 ± 5.30, 20.76 ± 3.32, 15.28 ± 9.84, 12.63 ± 2.01, 11.79 ± 0.29 and 11.29 ± 4.20 units respectively. *Escherichia coli* gave the lowest mean PO activity value of 5.65 ± 1.60 units over 5min (Table 5.2). This revealed that activation of the proPO system was independent of bacterial virulence.

FIGURE 5.2: SURVIVAL INDEX VALUES OF *ESCHERICHIA COLI* STRAIN XL1-BLUE MRF′ SUSPENSIONS VARIOUS TIMES AFTER INCUBATION IN PLASMA FROM *PENAEUS VANNAMEI* JUVENILES WHICH HAD BEEN PREVIOUSLY VACCINATED BY INJECTION WITH 100µL OF A 10.88MG.ML^{-1} (WET WEIGHT) SUSPENSION OF *VIBRIO HARVEYI* STRAINS BPO4 (VIRULENT) AND DPEX (AVIRULENT) AND *ESCHERICHIA COLI* STRAIN XL1-BLUE MRF′ IN SHRIMP SALT SOLUTION (SSS). INJURY CONTROLS WERE INJECTED WITH 100µL OF SSS AND PLASMA CONTROLS WERE UNINJECTED.



Haemolymph was removed from prawns and plasma separated from the haemocytes after 48h. Data calculated from Appendices 5.6 and 5.6a.

Table 5.2 : Phenoloxidase activity (mean ± SD) exhibited by haemocyte lysates of *Penaeus vannamei* elicited on exposure to *Aeromonas hydrophila*, *Escherichia coli*, various strains of *Vibrio harveyi* and zymosan.

Elicitor	*N*	*5 min*	*10 min*	*15 min*	*20 min*
zymosan	3	15.280 ± 9.840	7.720 ± 5.460	5.650 ± 3.870	4.530 ± 3.060
Aeromonas hydrophila	3	22.750 ±5.280	13.120 ± 4.060	9.470 ± 2.900	7.060 ± 2.460
Vibrio harveyi strain BP03	3	11.791 ± 0.288	6.311 ± 0.801	4.318 ± 1.089	3.155 ± 0.829
Vibrio harveyi strain BP04	3	20.760 ± 3.320	12.622 ± 0.761	8.410 ± 1.840	6.103 ± 1.079
Vibrio harveyi strain BP05	3	11.290 ± 4.240	5.980 ± 2.640	4.152 ± 1.448	3.529 ± 1.229
Vibrio harveyi strain DPEX	3	12.620 ± 2.010	6.145 ± 1.007	4.650 ± 0.724	3.405 ± 0.437
Vibrio harveyi strain IN7	3	26.570 ± 3.200	15.030 ± 1.770	10.795 ± 1.685	8.262 ± 1.645
Escherichia coli strain XL 1 Blue MRF`	3	5.647 ± 1.602	2.330 ± 2.260	1.270 ± 2.160	-0.080 ± 1.820
Cell free plasma	3	3.820 ± 12.820	-2.820 ± 6.430	-4.150 ± 4.510	-4.940 ± 3.580
Control	3	-16.109 ±1.037	-15.279 ±1.254	-13.729 ±0.852	-13.120 ± 0.708

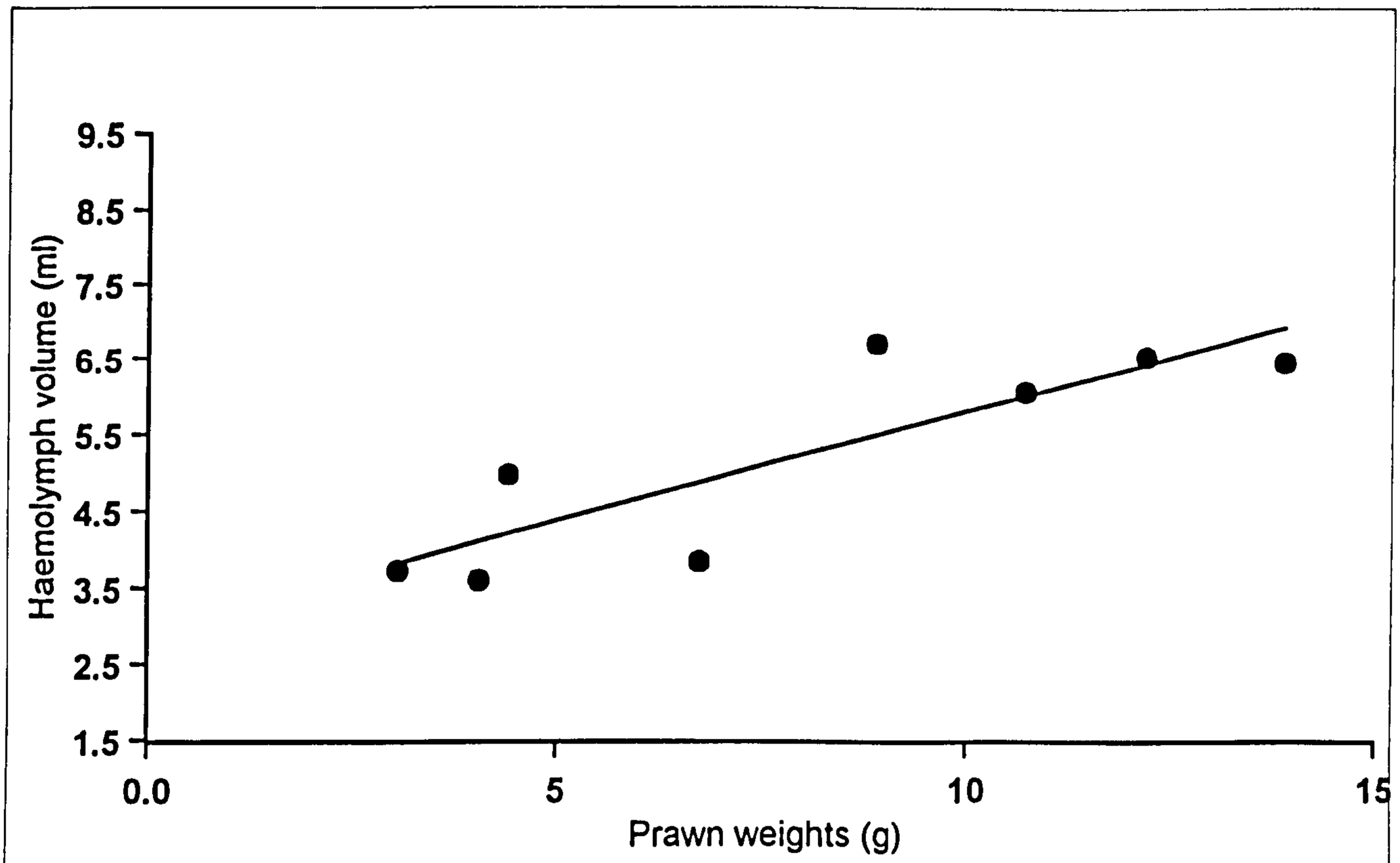
50µl of haemocyte lysate from naïve *Penaeus vannamei* was reacted for 1h with 50µl of 2mg.ml^{-1} of each of the listed bacteria suspended in sodium cacodylate buffer and 100µl of L-Dopa was added. proPO activation was measured as dopa chrome formation by absorbance at 490nm at 5min intervals over 20min. One unit of proPO activity is expressed as an increase in absorbance of 0.001.mg protein^{-1}.min^{-1} Data calculated from Appendix 5.7.

Estimation of the initial concentration of injected bacteria:

The dilution of injected ^{14}C (in dpm), is shown in Appendix 5.8. Total dpm obtained in the standards gave a linear correlation with a prepared set of standard volumes (r^2 = 92.6) Variance ratios (f) and p values obtained were: (f = 25.05, p = 0.038) (Appendix 5.8a), and normal plots of the residuals and the fits gave a straight line. Haemolymph volumes in the sample prawns were estimated from the total dpm (Appendix 5.8).

Regression of estimated haemolymph volume against body weight of sample prawns indicated a linear equation with r^2 = 71.9, and had f statistic and p values being: f = 15.33 p = 0.008 (Appendix 5.8b and Figure 5.3). In addition, normal plots of the residuals and the fits gave a straight line. The volume (± SD) of haemolymph corresponding to the mean weight of the prawns used in *in vivo* killing assays was estimated from this regression line (Appendix 5.9) and this was used to estimate the initial mean concentrations of bacteria following injection.

FIGURE 5.3: REGRESSION OF ESTIMATED HAEMOLYMPH VOLUME (ML) AGAINST BODY WEIGHT (G) OF *PENAEUS VANNAMEI* JUVENILES



Haemolymph volume = 2.95 + 0.28(Prawn weights)

F = 15.33, p = 0.008; R-squared = 0.719

Prawns were injected with 50µl of a solution of ^{14}C inulin (6.0 x 10^4 dpm) and 40µl samples were removed after 3h and the dilution of injected ^{14}C measured in a liquid scintillation counter. Data calculated from Appendices 5.8 and 5.8a to c.

In-vivo killing assays:

The mean weights of prawns used (9.41 ± 2.23g), corresponded to an estimated volume of 5.63 ± 0.63ml (Appendices 5.8, 5.8a and b, and 5.9). Initial concentration of injected bacteria (see materials and methods section of this chapter) after dilution in haemolymph was therefore 1.31 x 10^3, 1.32 x 10^3 and 1.72 x 10^3 cfu.µl^{-1} which corresponded to initial log values of 3.117, 3.121 and 3.2355 cells.µl^{-1} for prawns injected with *Vibrio harveyi* strains DPEX and BP04 and *Escherichia coli* strain XL1-Blue MRF` respectively.

In all the treatments, injection of bacteria into the haemolymph was followed by a generally significant reduction in the mean TVC of bacteria within 3h (Table 5.3). Prior immunostimulation with vaccines was not implicated in these initial decreases as ANOVA revealed no differences in the log concentrations of bacteria obtained between vaccinated and placebo injected prawns per µl in all the treatments after 3h. F-statistic and probability values obtained were: f = 1.01, p = 0.437; f = 2.54, p = 0.130; and f = 1.28, p = 0.345 for the prawns in treatments challenged with *Vibrio harveyi* strains DPEX and BP04 and *Escherichia*

coli strain XL1-Blue MRF` respectively (Appendix 5.9b). Table 5.4 shows the log TVC of bacteria surviving in *P. vannamei* haemolymph at various times after injection.

Table 5.3: Lower tailed one sample T-Tests on the mean survival in (log) bacterial numbers of cells of *Vibrio harveyi* strains BP04 and DPEX and *Escherichia coli* strain XL1-BLUE MRF` per microlitre 3h after injection into *P. vannamei* juveniles which had been previously vaccinated with formalin killed cells of *V. harveyi* strains BP04 or DPEX or *E. coli* strain XL1-Blue MRF. Controls were vaccinated with a placebo: shrimp salt solution (SSS).

Challenged by	*Vaccinated with*	*N*	*Mean*	*StDev*	*SE Mean*	*T*	*P value*	*Significant reduction ?*
DPEX	SSS	3	2.163	0.355	0.205	-4.66	0.022	yes
DPEX	DPEX	3	2.834	0.237	0.137	-2.07	0.087	no
DPEX	BP04	3	2.567	0.260	0.150	-3.66	0.034	yes
DPEX	*E. coli*	3	2.440	0.343	0.198	-3.42	0.038	yes
E. coli	SSS	3	2.8612	0.1697	0.0980	-3.82	0.031	yes
E. coli	DPEX	3	2.6196	0.3334	0.1925	-3.20	0.043	yes
E. coli	BP04	3	2.9052	0.1015	0.0586	-5.64	0.015	yes
E. coli	*E. coli*	3	2.4280	0.5604	0.3235	-2.50	0.065	no
BP04	SSS	3	2.487	0.199	0.115	-5.53	0.016	yes
BP04	DPEX	3	2.702	0.064	0.037	-11.33	0.0038	yes
BP04	BP04	3	2.632	0.170	0.098	-4.97	0.019	yes
BP04	*E. coli*	3	2.640	0.162	0.093	-5.14	0.018	yes

Logarithmic transformations (log) of bacterial counts.μl^{-1}, were compared to initial mean log bacterial counts of 3.117, 3.121 and 3.2355 cells.μl^{-1} for prawns injected with *Vibrio harveyi* strains DPEX and BP04 and *Escherichia coli* strain XL-1 blue MRF` respectively. Data calculated from Appendices 5.8, 5.8a and b, and 5.9.

The initial decline phase lasted from 3 to 8h (Table 5.4). Following this, a second phase of bacterial multiplication occurs the duration (8h to 12h) of which, is both strain and vaccine dependent. These multiplication phases generally lasted only up to 8h in prawns vaccinated with bacterin from *V. harveyi* strains BP04 and DPEX in contrast to prawns vaccinated with the placebo and *E. coli* bacterin where these multiplication phase generally lasted up to 12h. Bacterial concentrations then declined in all vaccinated treatments up to 24h after which, this decline either continued or bacterial multiplication occurred presumably leading to eventual death.

When prawns were vaccinated with *V. harveyi* strain BP04 bacterin, and challenged with non virulent *V. harveyi* strain DPEX or *E. coli*, the second phase of bacterial multiplication was absent as the initial decline continued up to 24h after which, it either continued (DPEX bacterin) or bacterial multiplication occurred (E. *coli* bacterin) (Table 5.4). The absence of a second phase of bacterial multiplication observed here, apparently confirms its superiority as a vaccine over *E. coli* and *V. harveyi* strain DPEX.

SDS-PAGE Protein electrophoresis.

The protein profiles of vaccinated, placebo injected, wounded and naïve *P. vannamei* plasma are shown in Figures 5.3 and 5.4. These did not reveal any new proteins in any of the tested plasma.

Table 5.4: Log survival (mean ± SD) of cells of *Vibrio harveyi* strains BP04 and DPEX and *Escherichia coli* strain XL1-BLUE MRF`` at various times after injection into *P. vannamei* juveniles which had been previously vaccinated with formalin killed cells of *V. harveyi* strains BP04 or DPEX or *E. coli* strain XL-1 blue MRF. Controls were vaccinated with a placebo: shrimp salt solution (SSS).

Time	*Vaccinated with*	*N*	*Challenged by BP04*	*Challenged by DPEX*	*Challenged by E. coli*
3hr	SSS	3	2.487 ± 0.199	2.163 ± 0.355	2.8612 ± 0.1697
	DPEX	3	2.7018 ± 0.0640	2.834 ± 0.237	2.620 ± 0.333
	BP04	3	2.6322 ± 0.1701	2.567 ± 0.260	2.9052 ± 0.1015
	E. coli	3	2.6401 ± 0.1618	2.440 ± 0.343	2.428 ± 0.560
8hr	SSS	3	3.0400 ± 0.2700	2.1592 ± 0.1175	3.2920 ± 0.5510
	DPEX	3	3.0820 ± 0.3140	2.787 ± 0.288	2.7280 ± 0.1970
	BP04	3	3.2040 ± 0.4040	2.536 ± 0.878	2.6690 ± 0.4860
	E. coli	3	2.5340 ± 0.2110	2.845 ± 0.577	3.0260 ± 0.3520
12hr	SSS	3	3.2430 ± 0.5210	3.005 ± 0.207	3.8460 ± 0.7140
	DPEX	3	2.8310 ± 0.2230	2.675 ± 0.443	3.1597 ± 0.0776
	BP04	3	2.4390 ± 0.3310	2.415 ± 0.680	2.5210 ± 0.2680
	E. coli	3	2.9350 ± 0.5440	2.958 ± 0.197	3.1635 ± 0.0602
24hr	SSS	3	2.2850 ± 0.4050	2.097 ± 0.306	2.3040 ± 0.2980
	DPEX	3	2.5490 ± 0.4420	2.703 ± 0.832	2.6881 ± 0.1279
	BP04	3	2.5300 ± 0.6180	2.244 ± 0.787	2.1490 ± 0.0154
	E. coli	3	2.3620 ± 0.3120	2.304 ± 0.298	All dead
48hr	SSS	3	2.5420 ± 0.9800	2.111 ± 0.467	2.4290 ± 0.9610
	DPEX	3	1.6559 ± 0.0485	2.9144 ± 0.1177	2.2914 ± 0.0931
	BP04	3	2.4860 ± 0.5820	1.811 ± 0.378	3.1842 ± 0.1671
	E. coli	3	3.3085 ± 0.1179	3.394 ± 0.184	All dead

Data calculated from Appendix 5.9 and 5.9a to c.

FIGURE 5.3: TEN PERCENT SDS-PAGE GEL SHOWING THE PROTEIN PROFILES OF PLASMA FROM *P. VANNAMEI* JUVENILES WHICH HAD BEEN EITHER VACCINATED (LANE A), PLACEBO INJECTED (LANE B) OR WOUNDED (LANE C). LANE D CONTAINS THE NAIVE CONTROLS. STANDARDS (S) USED WERE THE DALTON MARK VII-L (SIGMA).

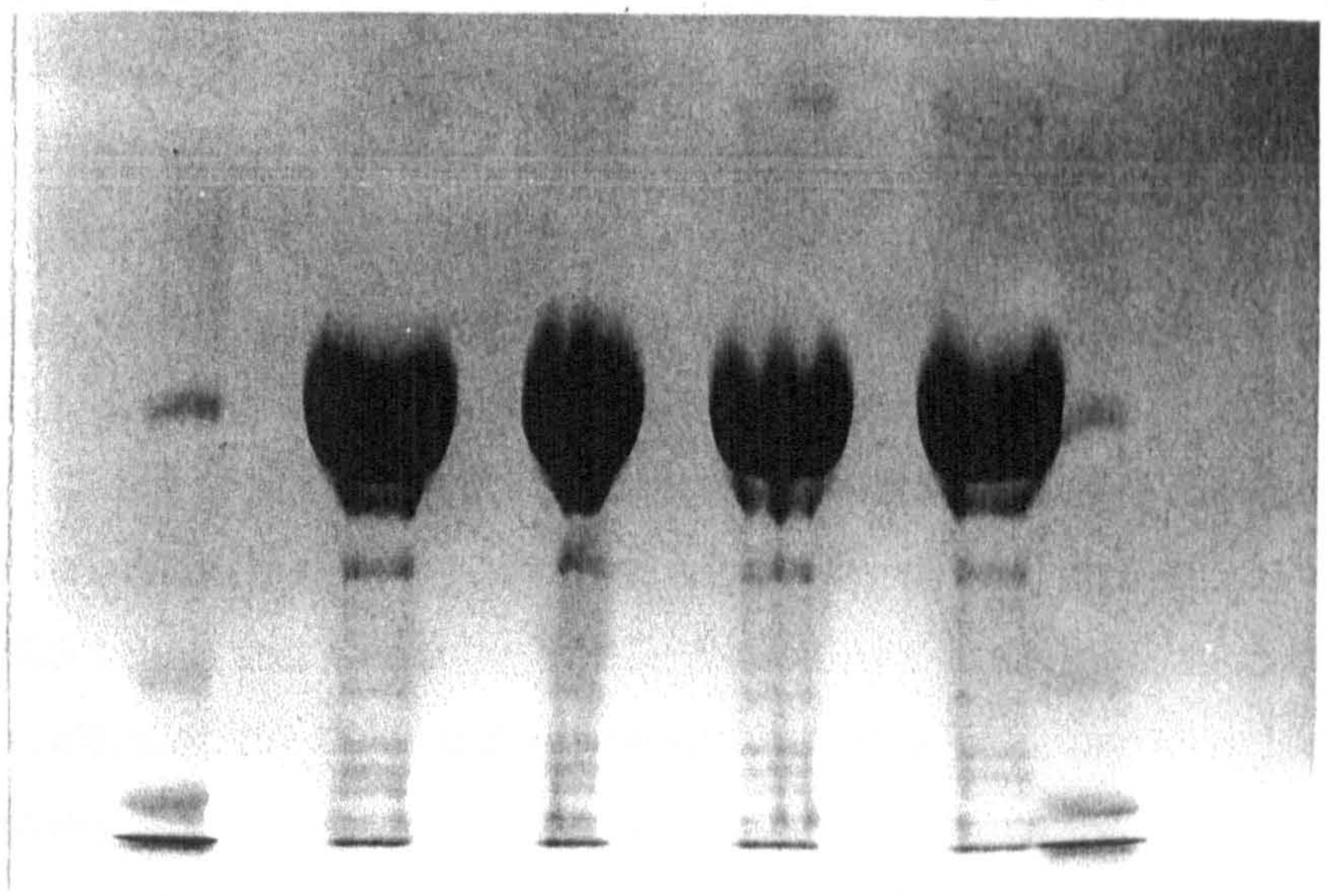
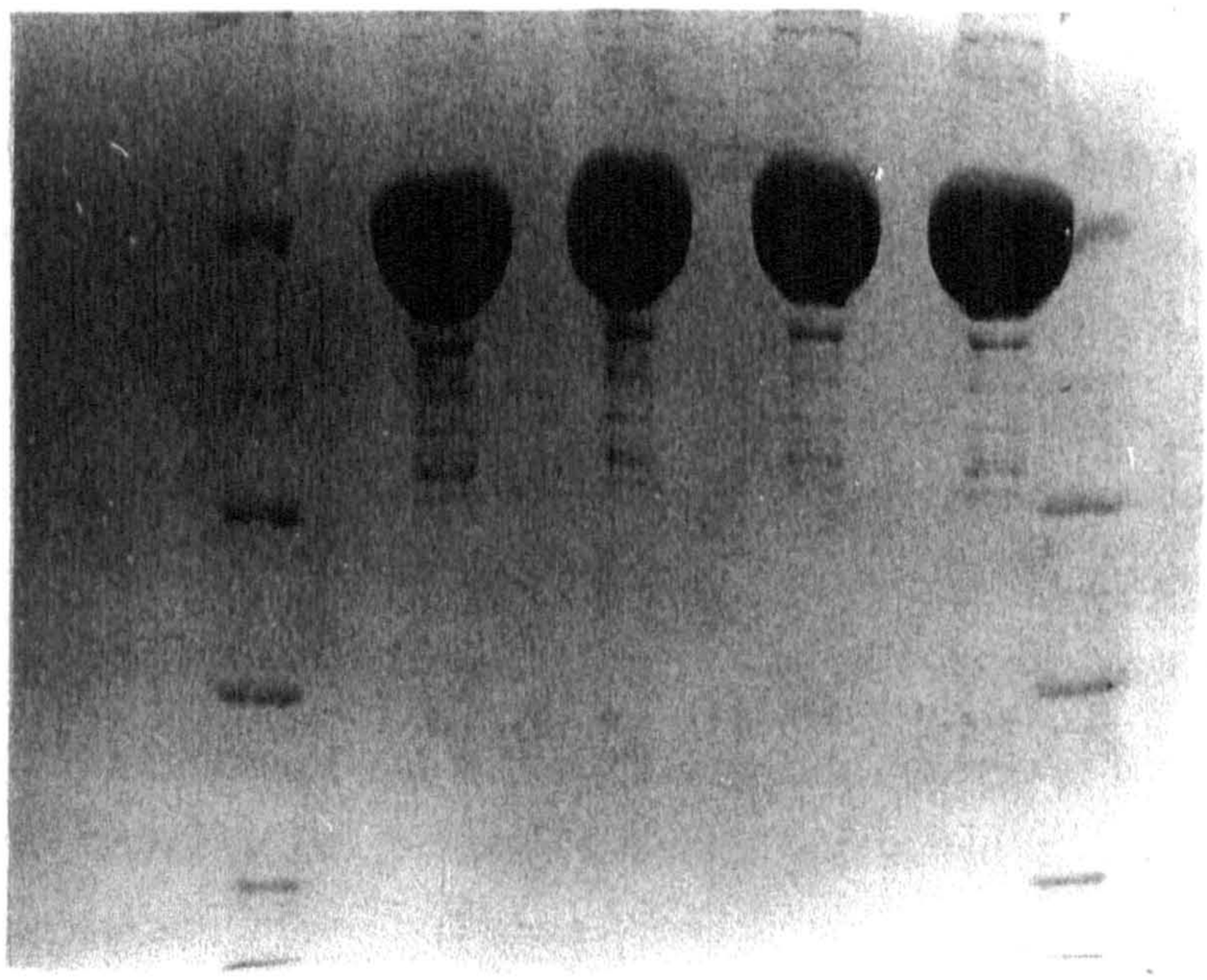


FIGURE 5.4: FIFTEEN PERCENT SDS-PAGE GEL SHOWING THE PROTEIN PROFILES OF PLASMA FROM *P. VANNAMEI* JUVENILES WHICH HAD BEEN EITHER VACCINATED (LANE A), PLACEBO INJECTED (LANE B) OR WOUNDED (LANE C). LANE D CONTAINS THE NAIVE CONTROLS. STANDARDS (S) USED WERE THE DALTON MARK VII-L (SIGMA)..



Discussion

Results obtained in the assays of growth of *E. coli* incubated in naïve plasma reveal that *P. vannamei* plasma constitutes a rich nutritive medium for bacterial growth *in vitro*. This is in contrast to the results obtained by Noga *et al.* (1996) who obtained antibacterial activity in haemolymph of unstimulated *P. setiferus*. This discrepancy may indicate a difference in the inherent toxicities of naive *P. vannamei* and *P. setiferus* plasma to bacteria. It could also be due to procedural differences. The previous researchers used whole haemolymph which was allowed to clot before crushing, centrifugation and subsequent use in antibacterial assays. Clotting in crustaceans involves plasma gelation by cell bound coagulogen (Durliat and Vranck, 1981; Omori *et al.*, 1989) the exocytosis of which, provides mechanisms for the simultaneous release of other bioactive molecules and the triggering of the proPO activating system (Smith and Chisholm, 1992; Söderhäll and Cerenius, 1992). In addition, homogenisation of the clot would break the haemocytes, also releasing the cellular antibacterial factors which may have led to the observed increase in antibacterial activity.

No apparent effects of incubating *E. coli* suspensions with 1mg.ml^{-1} of lysozyme on growth were observed after incubation for 3h. Lysozyme cleaves the β1,4 glycosidic bonds in the cell walls of gram-positive bacteria and with gram negative bacteria it weakens, but does not rupture the cell wall thereby making the bacteria more sensitive to antimicrobial action (Vaara, 1992). The difficulty of detecting antibacterial activity in prawn haemolymph is usually because the concentration of the active agent is too low to detect with the method used. Sung *et al.* (1996) detected antibacterial activity in stimulated *P. monodon* only after concentrating the plasma by a factor of three. However, the present study demonstrates that by pre-conditioning the target bacteria with lysozyme treatment, its sensitivity to the antimicrobial substances present is enhanced and direct evidence of antimicrobial activity can be obtained.

The antibacterial activity observed in placebo treated animals indicates that not only vaccination but also, wounding has the ability to induce antibacterial activity in prawns. Similar observations have been made by Adams (1991b) who detected bactericidins in *P. monodon* controls injected with saline and suggested that it may have been a non-specific response to injection stress. However, in contrast to results obtained by the previous author, the amount of antibacterial activity detected in present work was lower in all placebo treated animals compared to vaccinated animals. The duration of antibacterial activity suggests that the immune response in *P. vannamei* juveniles is of limited term, being evident only for 7d.

Though lysozyme activity was not detected in all the treatments tested, there was a brief period of significant growth stasis for *Micrococcus luteus* in vaccinated plasma 6h after administration. Having confirmed the requirement of hen egg white lysozyme in sensitisation of gram negative bacteria to activity by antibacterial factors, it is possible that the responsible factor (lysozyme-like factor), works in synergy with other antibacterial factors making a brief appearance only to condition the bacteria for subsequent activity by antibacterial factors.

The anti *E. coli* activity observed in *P. vannamei* plasma was not due to inherent toxicity (or other factors) in the plasma since it has been demonstrated that this bacterium grows well in cell-free haemolymph of naïve juveniles (this study). The bacterial killing obtained, is thus attributed to antibacterial factors produced following vaccination. *In vitro* killing assays reveal partial specificity by these antibacterial factors with the lowest initial SI. values obtained with the treatment vaccinated with *E. coli*, closely followed by the DPEX treatment. Although there were no differences in SI values obtained after 24h between these two, the lowest initial (3h) SI value was obtained in the plasma vaccinated with *E. coli* compared to the *V. harveyi* strains DPEX and BPO4 as well as the placebo (SSS) vaccinated plasma and this difference persisted up to 12h. This type of partial specificity has been reported for *P. monodon* bacterins (Adams, 1991b). The initial decrease in SI values followed by an increase up to 24h observed in the SSS treatment may be explained by the fact that the antibacterial factors produced on wounding, are much lower than those produced by the vaccinated juveniles and hence are more readily adsorbed by some of the added bacteria leaving others to grow. Similar results were obtained by Mori and Stewart (1978) who reported loss of bactericidal activity in plasma of vaccinated *Homarus americanus* following adsorption with *Pseudomonas perolens* and *Aerococcus viridans* (var) *homari.*

In the present study proPO activation was measured *in vitro* in unstimulated prawns and the activation values given by zymosan (15.28 units) are not greatly different from the lowest values obtained with the pathogenic *Vibrio harveyi* strain BP05 (11.29 units). Highest stimulation was observed for the relatively non-pathogenic *Vibrio harveyi* strain IN7 (26.57 units). *Escherichia coli* vaccines gave the lowest activation of proPO in *P. vannamei* HLS (5.65 units). Other prawn researchers measured PO activity after immunostimulation (Sung *et al.*, 1996), and found that *Vibrio* antigen produced the lowest stimulation of the proPO system in comparison to zymosan and β-glucans. These authors also had naïve control HLS giving proPO activity values of 5.04, 2.30 and 1.65 units following activation by *Vibrio* antigen, zymosan and β-glucans respectively. The reasons why the proportionate stimulatory effects of the *Vibrio* antigen should reduce after immunostimulation compared to those for zymosan and β-glucans which increase are unclear and were not explained by these authors. In addition, which of the immunostimulants was used as an elicitor was not stated. The lower proPO activity values obtained in naïve HLS by these researchers as compared to this study may be attributed to a number of factors: The lower concentration of L-Dopa substrate used (1.60 $mg.ml^{-1}$) as opposed to 3.00 $mg.ml^{-1}$ used in present work as well as the different reaction times used. In the present study, elicitors were incubated with the HLS at 20°C for 65min and then reacted with L-dopa at 5min intervals for 20min, while Sung *et. al.*, (1996) incubated the elicitors with the HLS for 15 min at 37°C and then reacted with HLS for 1min.

The observed higher stimulatory effect on the proPO system by the relatively non-pathogenic *Vibrio harveyi* strain IN7 as opposed to the more pathogenic strains BP03, BP04 and BP05, suggests that the higher protection conferred on *P. indicus* larvae due to vaccination (see chapter 4) by vaccines from a more virulent *Vibrio sp.* strain as opposed to a less virulent one, is not entirely due to the stimulation of the proPO system. Since the more pathogenic strains did not give proPO activation values as high as those

given by the less pathogenic strain IN7. It suggests the presence of some other recognition and antimicrobial factors which must be present in the plasma. This view is further supported by the observation that despite the very low stimulation of the PO system given by *Escherichia coli* (PO activation units = 5.65), in *in-vitro* killing assays, immune plasma from prawns vaccinated with this *E. coli* strain exhibited SI values initially lower than and finally, not being significantly different from those exhibited by *V. harveyi* strain DPEX (PO activation units = 12.62) and *V. harveyi* strain BP04 (PO activation units = 20.76).

Curiously, injection of *P. vannamei* juveniles with formalin killed *E. coli* cells and subsequent challenge by non-pathogenic *E. coli* produced total mortalities in *in vivo* tests after 24h. Toxic shock or bacterial septicaemia are unlikely to be the reasons for this as the *E. coli* strain had been specifically engineered to be non-pathogenic to humans (Bullock *et al.*, 1987), and in preliminary tests of virulence, had proven to be non-pathogenic to prawn larvae as well. In addition, the other prawns in the same treatment challenged with live *E. coli* exhibiting similar levels of mean TVC (mean log TVC = 3.16 and 3.85 cfu in the *Vibrio harveyi* strain DPEX and placebo injected samples respectively) did not succumb to bacterial septicaemia. It may be that the animals were overwhelmed by injection stress or physical damage during the injection process.

In vivo killing assays reveal an initial significant reduction in the mean TVC of all injected strains of bacteria over 3h, which sometimes lasted up to 8h. Since naïve haemolymph has been demonstrated to be non-bactericidal and the magnitude of the observed initial (3h) decline in the TVC was at least similar for both placebo injected and vaccinated prawns in all the treatments, the initial rapid reduction in the mean TVC of injected bacteria may be due to the primary cellular mechanisms of immune defence: phagocytosis, nodule formation and agglutination. This also implies an active role for haemocytes in the induction of the longer lasting humoral immune response.

This initial reduction was followed by a second phase of bacterial multiplication, the length of which was strain and vaccine dependent, generally lasting up to 12h when vaccination was carried out using SSS and *Escherichia coli* bacterin, and up to 8h when vaccination was with bacterin from *V. harveyi* strain DPEX and also when prawns vaccinated with bacterin from the more virulent *V. harveyi* strain BP04 were challenged by live *Vibrio harveyi* strain BPO4. This decline phase was then followed by either a further reduction or a multiplication in the TVC which will presumably lead to eventual prawn death.

Antibacterial activity appears at 8h (usually in prawns vaccinated with bacterin from *V. harveyi* strains BP04 and DPEX), leading to a slight reduction in bacterial numbers. This antibacterial activity is more evident in all treatments at 12h when a more discernible decline is evident, reflecting differences in the efficacies of the various vaccines. This period is in agreement with the period taken to attain maximal antibacterial activity as time course experiments in this study have revealed.

After the initial decline in bacterial numbers, subsequent more effective bacterial killing exhibited by the treatments vaccinated with *V. harveyi* strains BPO4, DPEX and *E. coli* in that order, as compared to the

placebo injected prawns, results from pre-existing antibacterial factors present in immune haemolymph. In addition, the absence of the secondary phase of bacterial multiplication observed when prawns are vaccinated with *V. harveyi* strain BP04 and challenged by non-pathogenic *V. harveyi* strain DPEX or *E. coli*, reflects the superior efficacy of vaccines made from this strain in conferring protection. This agrees with the observations in chapter 4 where, following larval vaccination, vaccines from a more virulent strain of pathogenic *Vibrio sp.* conferred better upon larvae against a wider range of *Vibrio sp.* strains compared to vaccines made from non or less virulent *Vibrio sp.* strains.

In contrast to reports by some other researchers (Sung and Song, 1996; Sung *et al.*, 1996), total clearance of bacteria was not observed from the haemolymph of *P. vannamei*. The reasons for this may be that the clearance of bacteria from haemolymph in our experiments was not as efficient as reported by these authors probably reflecting the differences in either species or methods of challenge or both. However Adams (1991b), working with the same species as the previous authors, has reported low levels of injected and immersed *Vibrio alginolyticus* to persist in *P. monodon* haemolymph up to 5d after application.

It seems likely that the initial cellular responses maintain the bacterial concentrations at low levels while *de novo* synthesis of antibacterial factors (possibly mediated by a factor or factors released from the haemocytes) is proceeding. A intriguing avenue for further study involving injection of a protein synthesis inhibitor along with some test bacteria should give a conclusive indication on *de novo* synthesis of immune proteins.

The absence of new polypeptide bands on the SDS-PAGE gels may not conclusively discount *in-vivo* antibacterial protein production. Faye and Wyatt (1980) reported that in *Cecropia* silkmoth pupae, immune proteins constitute too little a part of the total normal haemolymph proteins to be detectable. In studies where antibacterial activity has been detected, plasma was either concentrated by a factor of 3 (Sung *et al.*, 1996) or the test bacteria were pre-sensitised with lysozyme (this study). To avoid overloading gels, only 10µl of plasma samples containing 30µg of total plasma protein was loaded into each well. This may not have been enough to be detected by present methods. Both Coomassie Brilliant Blue which has been reported to be capable of detecting at least 0.1µg of protein (Sambrook *et al.*, 1989) and the more sensitive silver staining method employed (minimum concentration = 10-50ng), did not elicit a response. Two dimensional denaturing gels as well as non-denaturing separation of both naïve and immune proteins on acid PAGE gels followed by antibacterial assays on the separated proteins may prove conclusive.

CHAPTER 6

EFFECT OF FUNGICIDES IMPLICATED IN TAURA SYNDROME ON GROWTH, MOULTING RATES AND DISEASE RESISTANCE OF *PENAEUS INDICUS*.

Introduction

Taura syndrome (TS) disease of cultured prawns was first reported in Ecuador around the southern banks of the river Taura (Jimenez, 1992), typically affecting early juvenile *P. vannamei* between 0.05 to 2g (Jimenez, 1992; Lightner, 1996). However, since then reports have been made of TS affected prawns in Hawaii, Honduras, Mexico, Texas, Peru, Columbia, Guatemala, Brazil and Nicaragua (Lightner, 1996).

The gross clinical signs of TS include lack of appetite, lethargy and opaque musculature after moulting associated with a reduction in shell hardening. Taura syndrome affected prawns also display a pale pink to reddish colouration due to expansion of red chromatophores mainly in the uropods, telson and pleopods (Jimenez, 1992; Lightner, 1996).

Taura syndrome consists of two histopathological phases, the acute (peracute) and the chronic phases. In haematoxylin and eosin (H & E) preparations, the acute stage of TS is characterised by multifocal necrosis of the cuticular epithelium and subcutis of the general body cuticle, gills and appendages and in the cuticular linings of the fore and hindguts (Jimenez, 1992; Jimenez *et al.*, 1995; Lightner *et al.*, 1995; Lightner, 1996). The cytoplasm of affected cells display an increased eosinophilia, and nuclear pyknosis and karyorrhexis. Animals which survive the acute phase display a chronic (or recovery) phase of TS which is characterised by lesions under the cuticle, which may. or may not be infected by opportunistic pathogenic bacteria, large haemocytic infiltration and melanization.

The aetiology of TS remains a contentious issue. It has been variously suggested to either be caused by a virus (Hasson *et al.*, 1995; Lightner *et al.*, 1995; Bonami *et al.*, 1997) or due to a toxic aetiology caused by run off of fungicides used to combat the fungal disease *Sigatoka negra* in banana plantations (Jimenez, 1992; Lightner and Redman, 1994; Lightner *et al.*, 1994; Intriago *et al.*, 1995) into prawn ponds. The main fungicides implicated in this are the ergosterol synthesis inhibitors "Tilt 250 EC" (trademark of Ciba Geigy for propiconazole: (1-[[2-(2,4-dichlorophenyl)-4-propyl-1, 3-dioxolan-2-yl] methyl]-1H-1, 2,4-triazole)) and "Calixin" (trade mark of BASF for tridemorph: (C_{11}-C_{14} 4-alkyl-2,6-dimethylmorpholine)).

Some researchers (Hasson *et al.*, 1995; Overstreet *et al.*, 1997) have reported successful completion of Rivers' (1937) postulate which states that the etiological agent must be isolated from a diseased host and passed via cell-free extracts to a susceptible host in which the disease is later manifested successfully before the agent is considered to be a virus or virus-like organism. In addition several researchers (Brock *et al.*, 1995; Hasson *et al.*, 1995; Lightner *et al.*, 1995; Bonami *et al.*, 1997) have identified a virus belonging to the family Picornaviridae as being responsible for TS. However, some differences occurred in the gross symptoms observed (Hasson *et al.*, 1995), and those reported previously for TS such as a lack of red colouration of the abdomen and tail fans due to expansion of red chromatophores and lethargic swimming to the surface followed by a dorsal to ventral rotating movement and then sinking back down to the bottom. In addition, TS-like lesions were observed in the haematopoietic nodules unlike previously reported for TS where the haematopoietic nodules were unaffected.

Following this, proponents of a toxic aetiology have also suggested histopathological differences between TS affected prawns in Ecuador and subsequent episodes identified as TS. Jimenez *et. al.*, (1995) presented both light and electron microscopic evidence showing the reported viral diseases to be associated with intracellular rickettsia-like bacteria similar to the causal agent of infectious hypodermal and haematopoietic necrosis virus (IHHNV) (Frelier *et al.*, 1992). This was in contrast to the histopathological symptoms observed in the original TS affected prawns in which, lesions were not associated with bacteria.

Multifocal necrosis of the cuticle is no longer a unique characteristic of TS and has been reported in *P. japonicus* to be caused by a virus tentatively identified as belonging to the family Polydnaviridae (Inouye *et al.*, 1994; Momoyama *et al.*, 1994; Nakano *et al.*, 1994). In addition, bacterial shell disease lesions (Cipriani *et al.*, 1980) have been reported to be indistinguishable from lesions obtained in the chronic phase of TS (Lightner *et al.*, 1995).

Alvarez and Friedl (1992) have reported that the fungicide Triforine, was lethal to about half the haemocyte population of *Crassostrea virginica*, and also caused a slight inhibition of phagocytosis in the remaining haemocytes. In prawns, methyl parathion insecticides have been reported to significantly reduce chemotaxis and phagocytic activities in juvenile *P. monodon* (Tangtrongpiros *et al.*, 1997). Obviously, the goal of maximising yield by minimising mortality rates through stimulation of the prawn immune system as discussed in chapter 5, would not be achieved if the immune function is compromised.

The aim of the present study was therefore to investigate the effects of the two major fungicides on various aspects of the biological functions of *P. indicus*, a different species of penaeid prawn from those which have been widely affected by TS. In addition, the effect of fungicide incorporation into the diets on reduction of immunocompetence is assayed using a direct injection of pathogenic bacteria into the prawns.

Materials And Methods

Juvenile *Penaeus indicus* used in the taura syndrome assays were 2nd generation captive offspring raised in the School of Ocean Sciences.

Fungicide and diet preparation:

The fungicides "Tilt 250 EC" (trademark of Ciba Geigy for propiconazole: (1-[[2-(2,4-dichlorophenyl)-4-propyl-1, 3-dioxolan-2-yl] methyl]-1H-1, 2,4-triazole)) and "Calixin" (trade mark of BASF for tridemorph: (C_{11}-C_{14} 4-alkyl-2,6-dimethylmorpholine)) were dissolved separately in water and mixed with the fish oil fraction of diet A (Table 6.0) to give fungicide concentrations of 1000, 2500 and 5000μg active fungicide ingredient per kg each (Appendix 6.1) before mixing with the rest of the diets. Final concentrations of combined fungicide cocktail with active ingredient ratios of 1 : 1, in diets produced were 0μg.kg^{-1}(Diet A), 2000μg.kg^{-1} (Diet B), 5000μg.kg^{-1} (Diet C) and 10000μg.kg^{-1} (Diet D). These diets were chosen deliberately to be much higher than levels reported by Intriago *et. al.*, (1995) who used levels of 0, 5, 50 and 100μg of fungicide cocktail per kg diet to elicit the symptoms of the TS in *P. vannamei*.

Effects of fungicides on prawn survival

Prawns were stocked at 0.3 prawn.L^{-1} in 100L of water in circular tanks of dimensions 55.00 x 33.00cm (radius x height). Temperature ranged from 23.5 - 27°C and salinity from 17 - 22‰. Prawns were divided into 4 groups and fed either Diet A, Diet B, Diet C or Diet D.

Diet addition was at 10% of the prawns' body weight per day and 100% water change was performed daily. Taura syndrome is observed within 14 - 40d of stocking juvenile prawns in ponds (Jimenez, 1992; Lightner *et al.*, 1995) , and in experimental infections, Intriago *et. al.*, (1995) reported expression of TS in *P. vannamei* fed with 5 to 100μg of active ingredient of a 1/1 fungicide cocktail mix of tilt and calixin within 15d. In addition, TS expression in *P. vannamei* has been reported within 5 - 7d after commencement of feeding with TS infected tissue (Brock *et. al.*, 1995; Lotz 1997) as well as injection of a cell-free suspension of infected tissue (Lotz, 1997). As a result, in this study, survival was recorded daily for 28d and prawn weights were measured at the end of the trials.

At least 10% of the prawns from each treatment were subsequently fixed and preserved for histological sectioning.

Effects of fungicides on growth and moulting rate:

To facilitate better growth discrimination, early *Penaeus indicus* juveniles (mean weight = 0.13g) were stocked at 3 prawns.L^{-1} in 1L conical flasks at 27°C ± 2°C and fed with diet D. Controls were fed on diet A. Diets were given at 10% body weight three times daily and 100% water change was carried out daily. Moulting rates were monitored by marking the cephalothorax of each prawn using non-toxic permanent

markers (Pentel pen, N60) and these were used to observe moulting rates. The cephalothorax of freshly moulted prawns were re-marked immediately after each moult. Prawn weights were recorded daily for 14d and cumulative moults were taken over 14d.

Effects of fungicides on prawn resistance to bacterial challenge

In subsequent assays to test the susceptibility of prawns fed on the different diets to bacterial infection, randomly selected individuals from the above taura syndrome tests were stocked at 0.5 prawn.L^{-1} in 20L of sea water in plastic rectangular stacking boxes with dimensions of 38cm x 30cm x 21cm (length x breadth x height) and a temperature of 25°C ± 2°C and a salinity of 22 - 25‰. Pathogenic challenge was carried out by injection of 100µl of *V. harveyi* BP03 containing 7.0 x 10^3 cells into the lateral muscles of the 6th abdominal segments. This concentration of injected bacteria was higher than the 1.9 x 10^3 to 2.6 x 10^3 reported by Itami *et al.* (1989) to cause mortalities in experimentally infected *P. japonicus*. Rearing water was changed (100%) daily and surviving prawns were counted daily for 7d.

Table 6.0: 44.60 % protein content diet used to feed *Penaeus indicus* juveniles in Taura syndrome tests

Feed Ingredient	*Percentage (%)*
Fish meal	55
Wheat meal	23
Vitamin mix	2
Fish oil	0.75
Rice/Starch	13.05
Kaolin	2
Mineral mix	2
Carboxymethylcellulose	2
water	0.2
total	100

Histological examination of prawns

Fixation:

Prawns were fixed in Davidson's fixative

Davidson's fixative

95% Ethyl alcohol	330ml
100% Formalin	220ml
Glacial acetic acid	115ml
distilled water	335ml

0.1ml fixative is injected into each of the following regions:

1. Laterally in the hepatopancreas proper,
2. In the region anterior to the hepatopancreas
3. Laterally, in the anterior abdominal region and
4. Laterally in the posterior abdominal region

An equivalent of 5 - 10% of the prawn's body weight was injected until all signs of life ceased. The cuticle was immediately slit mid-laterally from the sixth abdominal segment to the base of the rostrum and taking care not to cut too deeply into the underlying tissue. Prawns were then re-immersed into the remaining fixative for at least 48h at room temperature. Fixed prawns were then transferred into 50% ethyl alcohol for storage until embedding.

Embedding

Two methods of embedding were performed on preserved prawns :

Method 1: Epoxy resin embedding

Wax :Resin

Dodecyl succinic anhydride (DDSA)	9.5g
N-Methyl dimethyl	0.5g
Dibutyl phthalate	1ml

After mixing, the resin was prepared by heating for 5min in a 60°C oven after which, it was removed, stirred, and re-heated at 60°C for 60min before use.

Appendages were dehydrated for at least 30min through each of a steadily increasing alcohol series i.e.: 50, 70%, 90% and 100% and at least 90min in absolute alcohol. Samples were then transferred to a 1:1 (v/v) of propanol and resin for at least 7d. The samples were then transferred into absolute resin for at least 48h before embedding.

The samples were removed and put into small plastic tubes containers to provide support while the tissue was properly aligned. More resin was then added until the tissue is totally covered and the tubes kept at 60°C until the resin sets. The part of the resin containing the tissue is then cut with a hack saw and trimmed with a hacksaw before sections are cut.

Method 2: Historesin embedding:

Infiltration procedures were carried out at 4°C: Tissues dehydrated in 100% alcohol were placed in a 1:1 (v/v) mixture of alcohol and historesin infiltration solution (HIS) for at least 3h after which, the mixture was discarded and replaced with HIS for up to 48h. Following this, the HLS was refreshed and the tissues left to infiltrate for at least 7d.

Embedding was carried out in polythene moulds: The infiltrated tissues were placed on small agar blocks within the polythene moulds (for support), covered with a embedding mixture consisting of a 15:1 (v/v) mix of HLS and hardener and left to polymerise overnight. When the blocks are hardened, they are trimmed to shape and stuck onto a perspex chuck with cyanoacrylate adhesive.

Attempts to trim blocks made from epoxy resin wax revealed that the resin had failed to penetrate the cuticle. As a result, microtomy and subsequent slide preparation were carried out on tissues infiltrated with HIS.

Three and a half microlitre thick sections of the blocks were cut using a glass knife in a LKG Bromma microtome. Cut sections were immediately placed in a floatation water bath (40°C), from where, they were picked up on labelled microscope slides pre-coated with a thin layer of albumen. The slides were dried for several days on a slide warming table before being stained.

Staining

Two method of staining were performed:

Method 1:

Haematoxylin and Eosin (H and E) stain

Haematoxylin:	
Haematoxylin	6g
Sodium iodate	0.6g
Aluminium sulphate	52.8g
Distilled water	690ml
Ethylene glycol	250ml
Glacial acetic acid	60ml

Eosin	
Eosin Y (Alcoholic: (CI 45389)	0.5g
95% Ethanol	100ml
Glacial acetic acid	2drops

Cut and dried sections on slides, were stained in Ehrlich's haematoxylin for 30min after which they were washed in running tap water until the sections turned blue. This was verified under the microscope to see that the nuclei had been stained blue. If necessary, differentiation was performed by a quick dip in 0.5% HCl followed by further washing in running tap water until the sections turned blue again.

The sections are then stained in 0.5% Eosin for 10min and washed in running water to remove the excess stain. The slides were then dried on a hot plate and mounted in DePeX mountant (Gurr) (DPX).

Method 2:

Feulgen stain for DNA

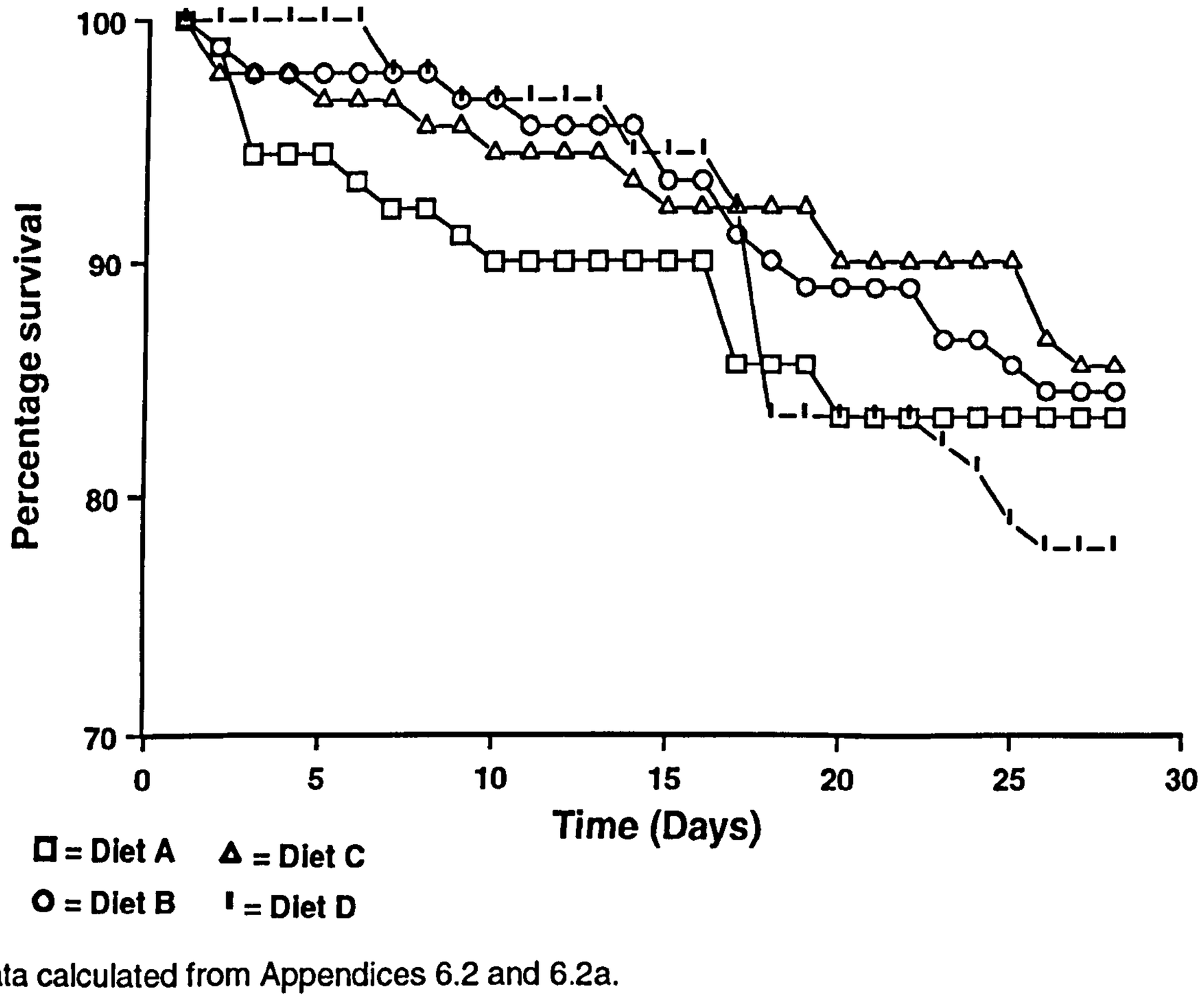
Sections dried on slides, were hydrolysed in 1N HCl at 60°C for 20min, rinsed in distilled water and then stained in periodic acid Schiff's reagent in the dark at room temperature for 60min. The sections were washed in running water for 5min, dried on a hot plate, and then mounted in DPX.

Results

Growth and survival experiments

Figure 6.1 shows the survival of *Penaeus indicus* juveniles after rearing for 28d with the various diet treatments. Mean percentage survivals obtained were 83.33 ± 12.02%, 84.44 ± 1.92%, 85.56 ± 11.71% and 77.78 ± 15.03% for the diets A, B, C and D respectively. Analysis of variance on the arc sine conversions of percentage survival values showed no significant differences either at the 95 or 99% confidence intervals (f = 0.23, p = 0.874) (Appendices 6.2 and 6.2a, and Figure 6.1).

FIGURE 6.1: MEAN PERCENTAGE SURVIVALS (± SD) OBTAINED WHEN *PENAEUS INDICUS* JUVENILES WERE REARED ON DIET A (TABLE 6.0), SUPPLEMENTED WITH 0µG (DIET A), 2000µG (DIET B), 5000µG (DIET C) AND 10000µG (DIET D) OF MIXED FUNGICIDE COCKTAIL (TRIDEMORPH AND PROPICONAZOLE) PER KG OF DIET.



Data calculated from Appendices 6.2 and 6.2a.

Similarly, although diet A (0µg of fungicides) displayed the highest final mean weight of 1.10 ± 0.33g when compared to mean weights of 1.06 ± 0.28g, 1.07 ± 0.38g and 1.00 ± 0.32g obtained for the diets B, C and D respectively, (Table 6.1), Analysis of variance did not reveal these differences to be significant (f = 0.78; p = 0.509) (Appendices 6.3 and 6.3a and Table 6.1). Both results suggested that addition of a fungicide

cocktail containing up to 10000µg of active ingredients per kg of diet did not affect growth and survival of *Penaeus indicus* juveniles

Table 6.1: Mean final weight (± SD) obtained after 28 days of rearing *Penaeus indicus* juveniles (initial mean weight = 0.36g). Juveniles were fed on an artificial diet, supplemented with 0µg, 2000µg, 5000µg and 10000µg of mixed fungicide cocktail (tridemorph and propiconazole) per kg of diet (called Diets A, B, C and D respectively).

Variable	*N*	*Mean*	*StDev*	*Percentage increase*
Diet A	47	1.10[a]	0.3284	206.70
Diet B	58	1.06 [a]	0.2803	195.81
Diet C	61	1.07 [a]	0.3835	196.67
Diet D	41	1.00 [a]	0.3246	177.03

Comparison is vertical, Values with the same superscripts are not significantly different (α = 0.05). Data calculated from Appendices 6.3 and 6.3a

Pathogenic challenge

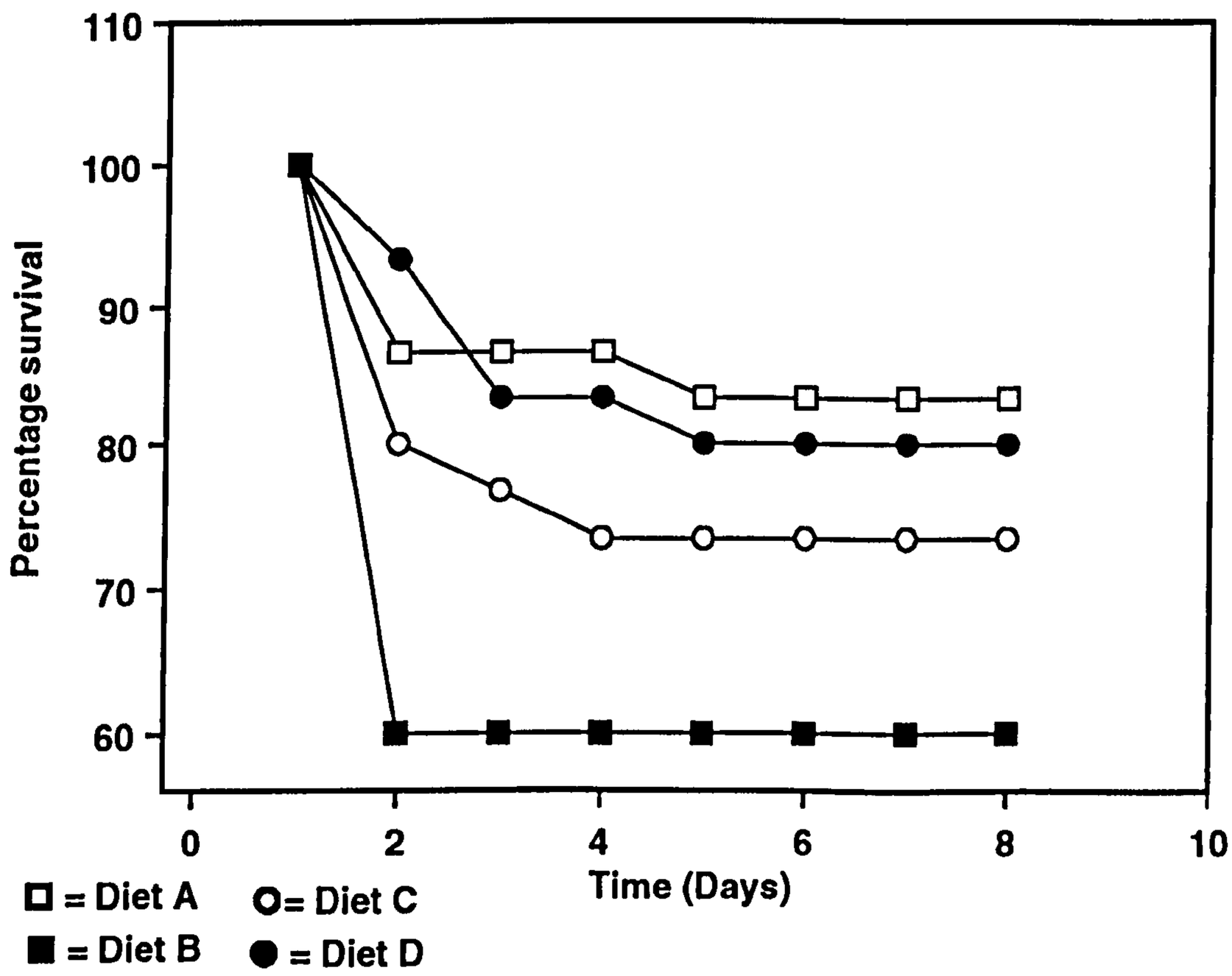
Pathogenic bacterial challenge following 28 days of feeding *Penaeus indicus* juveniles with diets containing varying concentrations of fungicides, gave no significant decreases in survival among all the diet treatments. The highest survival obtained 8d after bacterial injection was in diet A fed juveniles (Mean = 83.33 ± 15.28%) compared with mean survival values of 60.00 ± 0.00%, 73.33 ± 15.28% and 80.00 ± 20.0% obtained with treatments fed with diets B, C and D respectively. (Figure 6.2). Despite this, analysis of variance performed on the arc sine transformations of the percentage survival data obtained, gave no significant differences among all the diet treatments (f = 1.09; p = 0.407) (Appendices 6.4 and 6.4a - g and Figure 6.2). This also indicates that there is no apparent reduction in immune competence of *Penaeus indicus* juveniles after being fed for 28d with diets containing up to 10000µg of active ingredient of mixed fungicides (tilt and calixin) per kg of diet.

Growth and moulting rates

Figure 6.3 shows the growth of early *Penaeus indicus* juveniles fed on diets A and D over 14d. Mean final weights obtained were 0.1827 ± 0.04g and 0.1883 ± 0.04g for the treatments fed diets A and D respectively. Upper tailed T-tests revealed no significant differences in the final weights obtained (t = -0.30; p = 0.61; 95% CI = -0.046 to 0.035g) (Appendices 6.5 and 6.5a). Growth rates (Figure 6.3), were calculated using the square root transformation of increases in weight over time. The transformed growth rates, estimated from the slopes of linear regression lines, were slightly higher in treatments fed on diet D (0.016 ± 0.01g.d^{-1}) compared to the diet A treatment (0.014 ± 0.01g.d^{-1}) (Appendices 6.5 and 6.5b, and Figure 6.3). Comparing these growth rates using the general linear model of ANOVA (GLM), did not reveal these differences to be significant (f = 0.45; p = 0.502) (Appendix 6.5d).

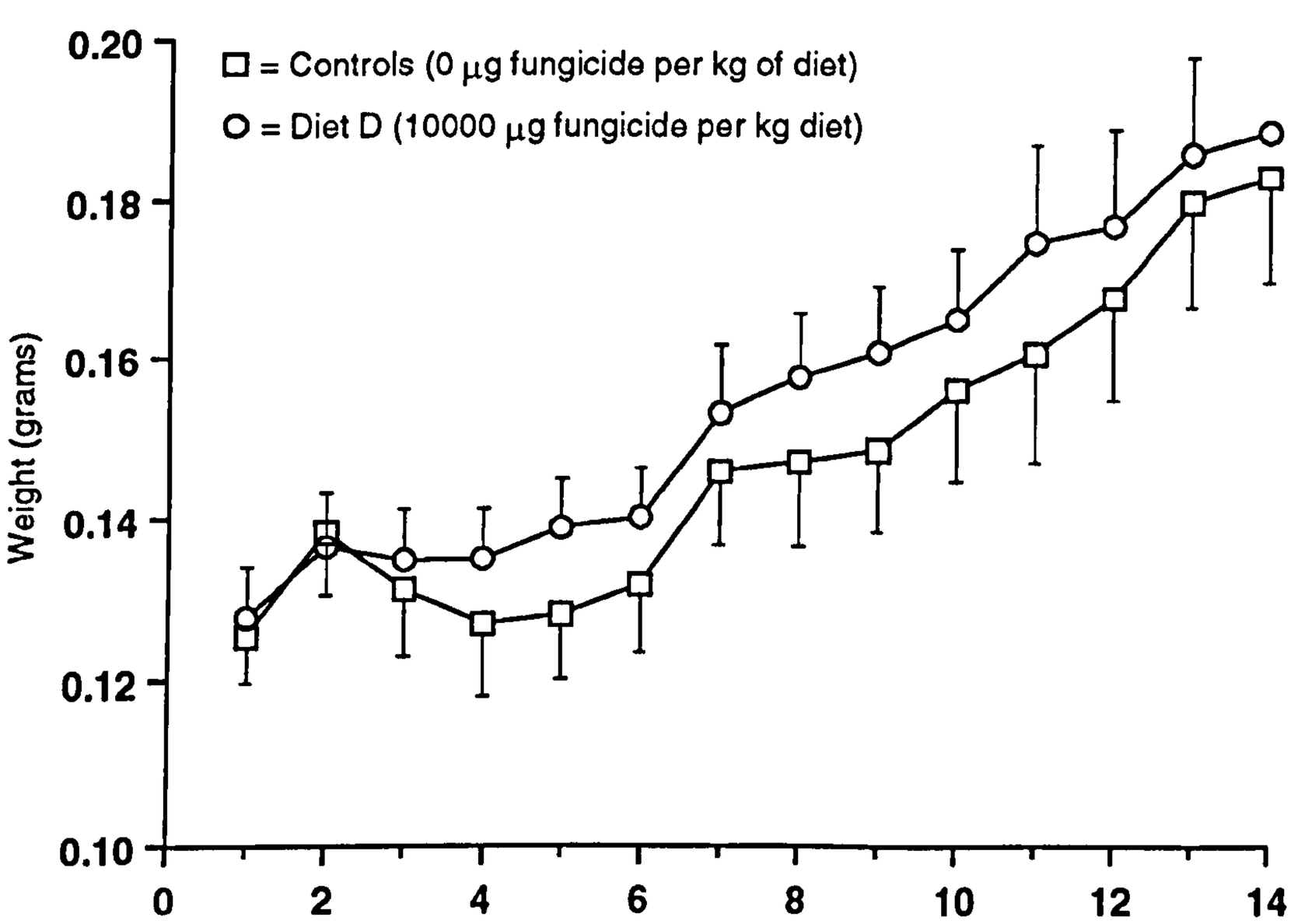
Total number of moults recorded in both treatments over 14d were 25 and 27 for diets A and D respectively. Figure 6.4 shows the number of moults recorded for the two treatments presented as cumulative moult counts obtained over 14d. The moulting rates were estimated as the slopes (b). Comparisons of the slopes obtained using GLM revealed no significant differences in moulting rates obtained in both treatments (f = 0.66; p = 0.426) (Appendices 6.5 and 6.6a, and Figure 6.4).

FIGURE 6.2: SURVIVAL OF *PENAEUS INDICUS* JUVENILES INJECTED WITH PATHOGENIC *VIBRIO HARVEYI* STRAIN BP03



Juveniles had previously been raised for 28 days on diet a (Table 6.0) supplemented with 0µg (diet A), 2000µg (diet B), 5000µg (diet C) and 10000µg(diet D) of mixed fungicide (tridemorph and propiconazole) cocktail per kg of diet. Data calculated from Appendices 6.4 and 6.4a - g

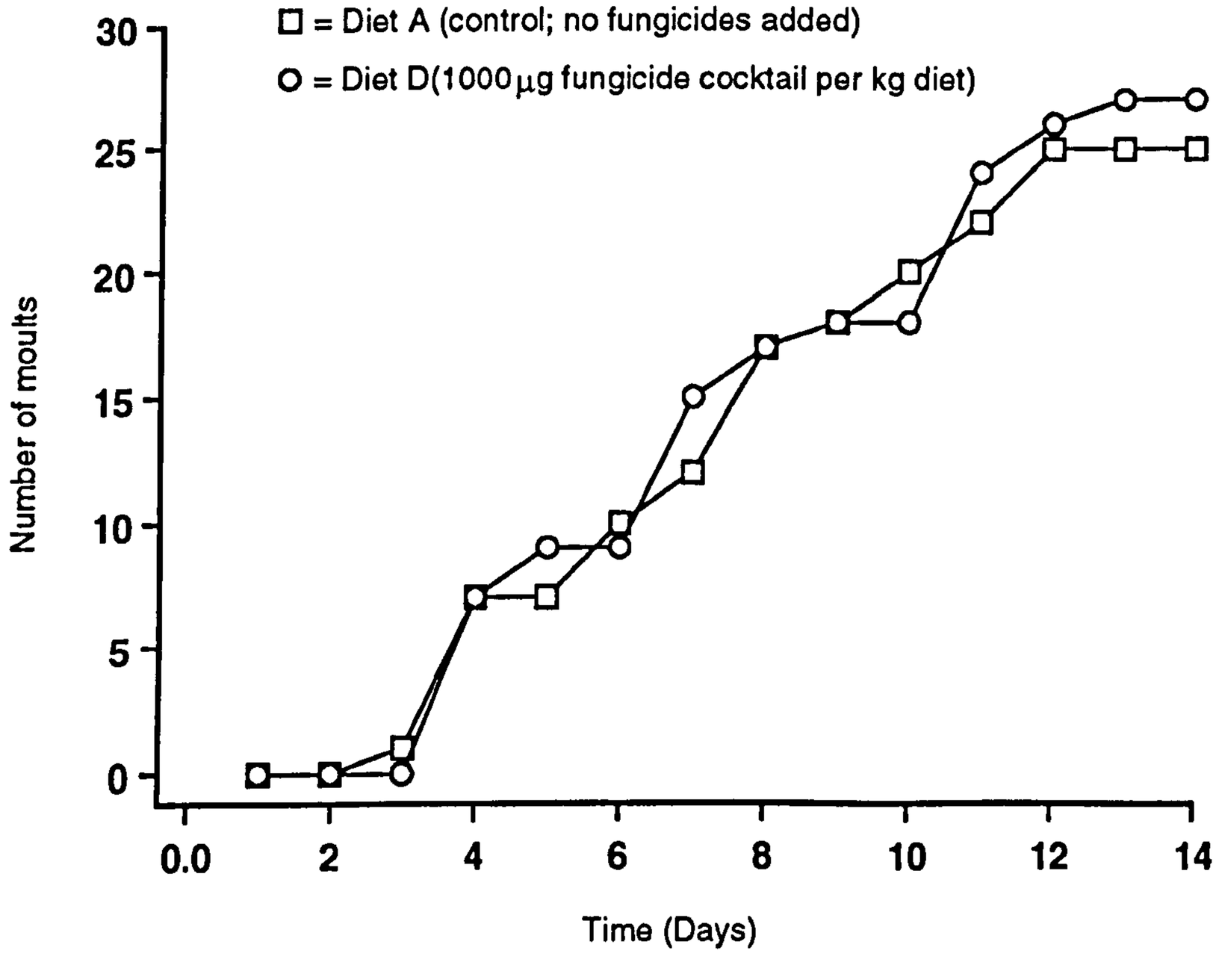
FIGURE 6.3: GROWTH (G) OF *PENAEUS INDICUS* EARLY JUVENILES FED ON AN ARTIFICIAL DIET, SUPPLEMENTED WITH 10000µG OF MIXED FUNGICIDE (TRIDEMORPH AND PROPICONAZOLE) COCKTAIL PER KG OF DIET (DIET D). CONTROLS (DIET A) HAD NO ADDED FUNGICIDES.



Data calculated from Appendices 6.5 and 6.5b.

FIGURE 6.4: CUMULATIVE MOULTS OF *PENAEUS INDICUS* EARLY JUVENILES FED ON AN ARTIFICIAL DIET SUPPLEMENTED WITH 10000µG OF MIXED FUNGICIDE (TRIDEMORPH AND PROPICONAZOLE) COCKTAIL PER KG OF DIET (DIET D). CONTROLS (DIET A) HAD NO ADDED FUNGICIDES.

Cumulative moults in early juveniles of *P. indicus* fed different concentrations of fungicides

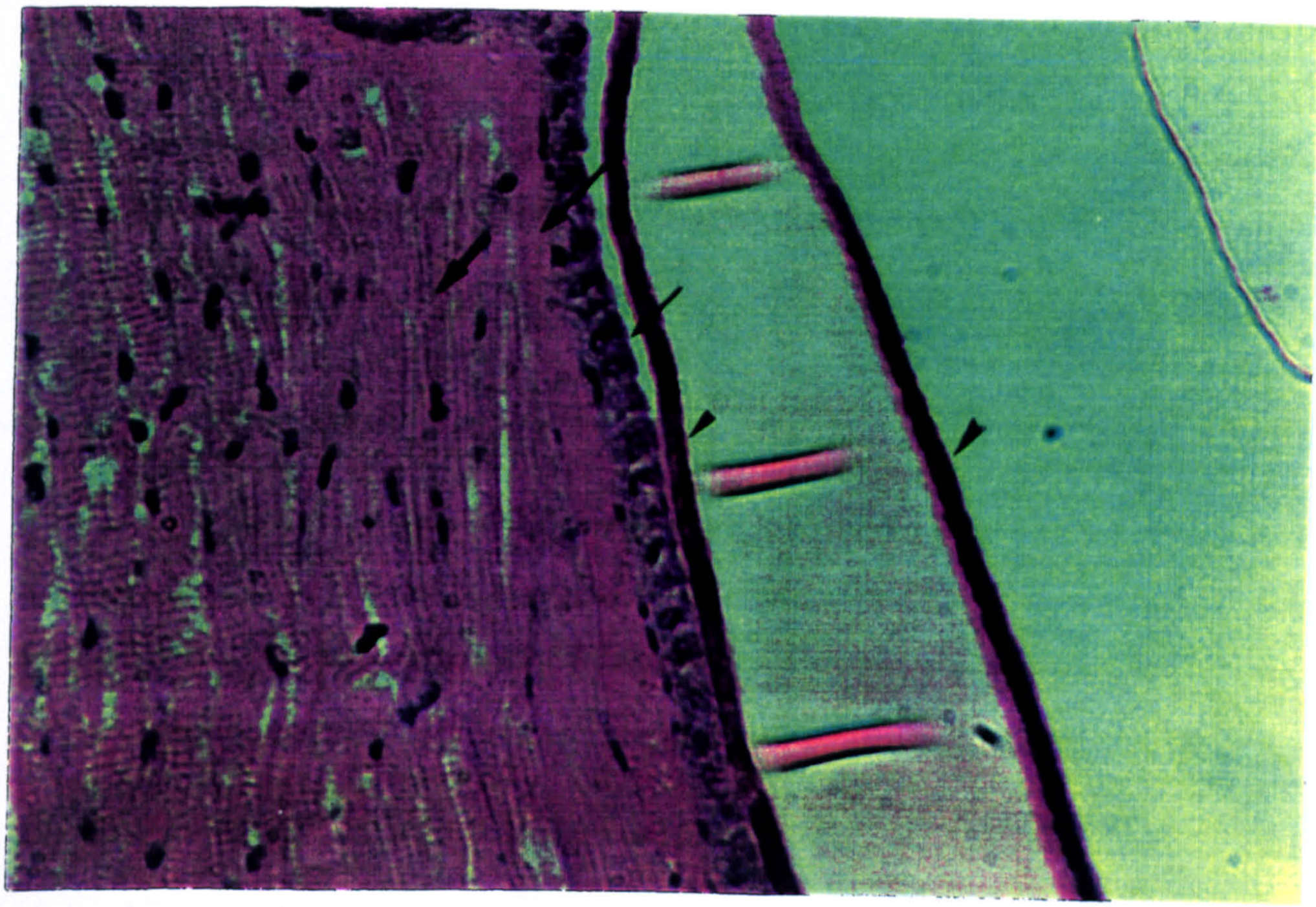


Data calculated from Appendices 6.5 and 6.6a.

Histology

Figure 6.3 shows a longitudinal histological section through an appendage of a prawn fixed from diet treatment D. At least 10 sections were examined from each block sectioned and at least 3 prawns from each diet treatment were sectioned after fixing. No Taura syndrome like lesions were observed in any of the treatments examined. This contrasts with results by Intriago *et al.*, (1995) (Figure 6.4) which shows prominent TS-like lesions reportedly induced in *P. vannamei* juveniles after 15d feeding with much lower fungicide concentration (5 - 100µg of active ingredient.kg^{-1} diet).

FIGURE 6.3: LONGITUDINAL SECTION THROUGH A PLEOPOD OF *P. INDICUS* JUVENILE REARED ON AN ARTIFICIAL DIET 10000µG OF MIXED FUNGICIDE COCKTAIL (TRIDEMORPH AND PROPICONAZOLE) PER KG OF DIET. FOR 28D.



The old cuticle (bold arrow head) is being replaced by a newer cuticle (thin arrowhead) the epithelial cells (thin arrow) lie under the new cuticle. Longitudinal 3.5µm section through the pleopod of a juvenile P. indicus after 28d feeding on an artificial diet containing 10000µg (active ingredient) of a mixture of fungicides per kg of diet. The basement membrane is not apparent and the circular (thin arrow) and longitudinal (bold arrow) muscle layers lie under the epithelial cells. Sections were fixed in Davidson's fixative and preserved in 50% alcohol. Sections were stained with Haematoxylin and Oesin. Magnification 400X

The cuticle has been artifactually separated from the epithelial cells by the fixation process and is not in range. Small arrow heads show prominent areas of lesions in the columnar epithelial cells. The basement membrane (thick arrow head) is separated from the underlying circular (thin arrow) and longitudinal (bold arrow) muscle layers. Longitudinal 3.5µm section through the dorsal abdominal cuticle of a juvenile *P. vannamei* after 14d feeding on an artificial diet containing 5 - 100µg (active ingredient) of a mixture of fungicides per kg of diet. Sections were fixed in Davidson's fixative and preserved in 50% alcohol. Sections were stained with Haematoxylin and Oesin. Magnification 400X

Slide was supplied courtesy of Intraigo *et al.*, (1995)

Discussion

Pesticides have been reported to affect penaeid prawn enzyme systems (Srinivasulu Reddy and Ramana Rao, 1988; 1991; Srinivasulu Reddy *et al.*, 1991) and an inhibition of prawn digestibility by fungicides has been suggested to be a possible mode of action of fungicides on juvenile prawns (Intriago *et al.*, 1995). These authors argue that this, correlated with the higher protein requirements of smaller *Penaeus vannamei* (Smith *et al.*, 1985), should lead to the more apparent effects evident in young juveniles and they report inhibition of *P. vannamei* digestibility by concentrations as low as 300ng of active ingredient of a 1:1 cocktail of tilt and calixin per kg. To facilitate replication of TS symptoms, unusually high concentrations of fungicides (2000 to 10000µg of active ingredient per kg) were used in the present study, and very early *P. indicus* juveniles (mean weight = 0.13 ± 0.02g) were used in the growth rate assays. Despite this, no effects were observed on growth rates in early juveniles when fed 0 and 10000µg fungicides.kg^{-1} diet thus discounting any apparent effects of the fungicide cocktail on *P. indicus* digestibility.

Moulting of crustaceans is affected by extrinsic factors like temperature, salinity, light intensity and pollutants as well as intrinsic factors such as nutrition (Kleinholz, 1985). Ranga Rao and Doughtie (1984) reported formation of lesions about the articulations of appendages in the grass prawn associated with severe degenerative changes in the subcuticular epithelium which appeared to interfere with the normal premoult cuticle formation when exposed to several pesticides. Similarly, Lightner and Redman (1994) proposed that TS is caused by a substance which stops moulting by inhibition of calcium and/or phosphate metabolism in the prawn cuticular epithelial cells. Since tridemorph and propiconazole inhibit ergosterol synthesis in target fungi and as the moulting hormone in prawns, ecdysone, is also a sterol structurally similar to ergosterol (Greineisen, 1994), Lightner *et. al.*, (1994) suggested that they may interfere with the moulting process. However, this seems unlikely as penaeid prawns cannot synthesise the sterol ring and have a dietary requirement for this (Teshima *et al.*, 1975; 1989; Kanazawa *et al.*, 1988). This is supported by results obtained in this study which revealed no differences in growth and moulting rates of juvenile prawns and discounts theories of effects of fungicides on *P. indicus* cuticle synthesis.

Though TS is reportedly expressed in field conditions within 14 - 40d of stocking (Brock *et al.*, 1995; Lightner *et al.*, 1995). Intriago *et. al.*, (1995) has reported experimental expression of TS lesions within 15d following oral administration of a fungicide cocktail (5 to 100µg.kg^{-1}). In addition, Brock *et. al.*, (1995) has also reported experimental expression of TS lesions within 5 to 6d in prawns fed with chopped TS positive tissues. However, despite allowing sufficient time for experimental TS expression (28d) in this study, none of the gross signs symptomatic of the peracute or chronic phases of TS were observed in any of the diet treatments. In addition, light microscopic examination of histological samples from all treatments did not

reveal any histopathological lesions or necrosis of the cuticular epithelium and subcutis symptomatic of TS in any of the diet treatments.

Pesticides have been reported to reduce the immunocompetence of oysters (Alvarez and Friedl, 1992) and prawns (Tangtrongpiros *et al.*, 1997) *in vitro*. Though these authors did not perform subsequent live bacterial challenge, the effects of such observed reductions on the immune defence functions would be expected to be an overall decrease in prawn resistance to opportunistic pathogens. No phagocytic measurements were taken in this study and direct challenge of *P. indicus* by pathogenic *V. harveyi* strain BPO3, gave no significant differences in mortalities. This probably reflects the different modes of action of the different fungicides. Different modes of activity of various pesticides on prawns have been reported by (Ranga Rao and Doughtie, 1984)

In the present study, addition of very high concentrations (2000 to 10000µg of a (1:1) cocktail of tridemorph and propiconazole active ingredients per kg of diet) did not lead to any apparent changes in growth, moulting rates, survivals and susceptibilities to pathogenic bacterial challenge of early *P. indicus* juveniles. While this seems to suggest that these fungicides have no effect on the prawns, several important factors prevent conclusive discounting of the toxic aetiology theory:

1. Though TS has been reported in a wide range of salinity conditions, it has been reported to be more prevalent during the rainy seasons when the salinities of the affected regions drop to near 0‰ (Intriago *et al.*, 1995; 1996; Lightner *et al.*, 1995). Increasing lethality of the pesticide (Aroclor^{R}) has been reported with decreasing salinities in *P. aztecus* (Nimmo and Bahner, 1974). Intriago *et. al.* (1996) have suggested that osmoregulation of juvenile prawns was compromised at low salinities and (Intriago *et al.*, 1995) have suggested an accumulative effect of low salinities, osmoregulation and fungicide pollution to be the cause of TS. *Penaeus vannamei*, the affected species in most cases is very tolerant of low salinities and grows faster at salinities of 5 to 15% (Bray *et al.*, 1994). Intriago *et. al.* (1996) reported 50% mortality in *P. vannamei* fed on chopped TS positive tissues when reared in water of 2‰ salinity in contrast to prawns reared in water with salinities of 35‰ were there were no recorded mortalities. The *P. indicus* juveniles used in this study grow best at 20 - 30‰ and find salinities lower than 10‰ lethal (Kumlu and Jones, 1994). As a result, the salinities used in this study ranged from 17 to 22‰ (Appendix 6.2).

2. The effects of the interactions between latent viral infections and fungicides needs to be properly investigated. Couch (1974), has suggested that the exposure of *P. duorarum* to several pesticides may facilitate or enhance the expression of latent viral infections. The main victim of TS (*P. vannamei*), co-exists with IHHNV with no apparent symptoms (Bell and Lightner, 1984) and some studies have used IHHNV expression as an indication of stress in *P. vannamei* (Lightner *et al.*, 1995). In addition, some researchers (Kalagayan *et al.*, 1991; Browdy *et al.*, 1993) have related runt deformity syndrome (RDS)

to increasing incidence of IHHNV. It may be argued also that as our *P. indicus* populations are IHHNV free, they would therefore, avoid the effects of any possible interaction between fungicides and the virus.

3. It may be also argued that *P. indicus* may simply be resistant to the effects of the fungicides. In the case of infectious disease aetiologies, Bell and Lightner (1984) have reported the resistance of *P. vannamei* to IHHNV which caused massive mortalities to *P. stylirostris*. While this remains conceivable in the case of infectious diseases, A toxic aetiology should exclude this possibility.

In conclusion, an effect of the tested fungicides was expected on various biological functions of *P. vannamei*, but none was observed. While the aetiological agent still remains doubtful, further studies with a more low salinity tolerant species in conditions more closely resembling those which occur in areas where TS has been reported may help clarify the aetiology of TS.

More importantly, from the point of view of this study, *in vivo* studies on the effects of other fungicides with different modes of activity from the ergosterol inhibitors on the impairment of the immune system needs to be investigated.

CHAPTER 7

CONCLUSIONS

Loss of cultured prawns due to disease caused by pathogenic agents has had and continues to have a very significant effect on prawn production world wide and the present study contributes to the understanding of the disease and immunological processes necessary for formulation of disease control strategies for penaeid prawn larvae.

Treatment of 5µm filtered water with O_3, UVT or AuW significantly reduced TVC counts immediately following pre-treatment. However, these reductions were only temporary and final concentrations of bacteria were higher than those obtained in 5µmSW. In addition, the proportion of presumed *Vibrio sp.* counts increased in the treated water groups over 24h in contrast to PVVC in raw sea water and 5µmSW where the PVVC proportions declined. These results illustrate the importance of maintaining the balance of microbial populations in culture systems.

Various methods are used to pre-treat rearing water in commercial hatchery operations in attempts to prevent or limit the entry of potential bacterial pathogens. However, absolute sterility is impossible to obtain in such systems and generic, specific and strain differences among the target heterotrophic bacteria ensure that different methods of water pre treatment attempted to lower TVC, will also lead to generic changes in the microbial composition of the water. Such changes have been reported by several authors (Murchelano *et al.*, 1975; Prieur and Caval, 1979) following similar water treatment regimes. Thus, the pre-treatment processes affect the microbial balance in a way that may favour the development of a potential pathogen previously kept in control by the balance of the bacterial community. Gatesoupe (1990) reported that when a shift occurred in the competitive microbial balance obtained between a pathogenic strain of *Aeromonas sp.* and non-pathogenic *Vibrio alginolyticus* in larval turbot cultures which favoured the pathogen, this usually led to increased concentrations of pathogens compared to the nonpathogen and preceded mass larval mortalities.

In addition, the rationale behind attempts to raise prawn larvae in environments devoid of pathogens is questionable as eventually, all animals will need to develop resistance to pathogens in their environment or lose the selective advantages of natural selection.

Although maintenance of the natural microbial community is generally beneficial for prawn survival, the natural microbial community itself is sometimes disturbed due to seasonal changes in water quality as in the aftermath of diatom and *Phaeocytis pouchetti* blooms (Blight *et al.*, 1995). In such periods of poor

water quality, addition of bacteria or algal extracts from algal cultures to larval cultures fed MED and reared in 5µmSW corrected for deteriorations in sea water quality. It is important to note that feeding larvae on live algae with or without added bacteria from algal cultures, gave a more sustained enhancement of larval survival. In addition, bacteria from algal cultures added to larval cultures appears to depress PVVC levels in MED fed cultures. This suggests a role for bacteria from algal cultures and algal extracts in maintaining the bacterial balance in the culture water. The more sustained enhancement of larval survival obtained when fed algae is not thought to be due to nutritional contribution by the algae as Kumlu and Jones (1995) have demonstrated that addition of a low concentration of algal cells to MED fed larval cultures stimulates enzyme production and facilitates digestion of MED. They also calculated that the energetic contribution of algal cells is insufficient to account for the increase in survival obtained. Rather, it is suggested that the enhanced larval survival obtained is due to the continuous production of algal metabolites (or extracts) which contribute to maintenance of the bacterial balance of the water.

The exact methods by which algae or algal extracts exert this bacteriostatic effect are largely unknown although it would appear that they probably act as probionts enhancing the growth of non pathogenic competitors of *Vibrio sp.* Regardless of the mode of operation, these findings contribute to the advantages of using such algal species as larval diets.

Further study is needed to identify such potentially beneficial algal species and the exact mode of operation.

The higher concentrations of bacteria obtained in all pre-treated culture water samples when compared to 5µm filtered water, also indicates that enhanced nutrition due to bacteria is not likely to be the cause of the better larval survival observed when larvae were reared in 5µmSW. It is suggested that the enhanced survival obtained in 5µmSW is due to natural bacterial balance which encourages higher survival as a result of competitive exclusion or control of potential pathogens. Similar strategies have been used in poultry where oral application of a beneficial bacterial broth obtained from the intestines of adult hens to young chickens leads to the more benign bacterial species taking up all the available attachment sites in the young and thus, excluding the more virulent *Salmonella sp.* (Schleifer, 1985; Mead and Impey, 1986). In prawns, Yasuda and Kitao (1980) have reported similarities between intestinal bacteria in *P. japonicus* and the species of bacteria in the surrounding environment.

Regardless of the method of water pre-treatment attempted, bacterial recolonisation occurred rapidly and the growing concern about improper and prolonged use of antibiotics coupled with the development of drug resistance among bacterial pathogens increases the need for urgent research into possible use of vaccines to stimulate the prawn immune system for defence against disease. In the present work, the efficacy of immersion methods of vaccination used by others (Itami *et al.*, 1989; Song and Sung, 1990; Sung *et al.*, 1991; Prayitno, 1994) was confirmed. In contrast, oral vaccination of larvae attempted using both virulent (BP04) and avirulent (IN7) strains of *Vibrio harveyi* as subsequent challenge pathogens, failed. Reasons for these failures are unclear but it is possible that:

1. Destruction of vaccine components occurred during the diet production.
2. Prawn larvae are unable to absorb the vaccine in a functional form due to digestive breakdown.
3. Prawns are unable to sufficiently digest the MED as a result of digestive enzyme deficiency in early larval stages.

While further work needs to be done to identify and overcome the problems associated with oral delivery of vaccines, the success of immersion methods of vaccinating penaeid larvae (Prayitno, 1994; This study) may offer an alternative to the use of antibiotics. However, for vaccines to be widely accepted as an alternative to antibiotics, they must be at least as efficient as antibiotics in addition to being competitively priced or the use of the latter will continue. The successful protection of penaeid larvae against subsequent infection obtained in laboratory trials still has to be repeated on a commercial scale. A commercial prawn vaccine (PMB: Penaeid Multivalent Bacterin, Argent Chemicals Ltd.) has recently become available. However, in addition to the high cost (1mg = $170.00), there is no scientific evidence as to its efficacy.

The general consensus concerning the lack of an adaptive immune response in crustaceans is confirmed by the high incidence of cross-protection observed following immersion vaccination and subsequent challenge with different strains of *Vibrio harveyi*. When vaccine efficacies were compared, vaccines made from the more virulent strain of *Vibrio harveyi* (BP04), consistently conferred higher levels of protection upon larvae compared to the less pathogenic (IN7) strain. This may be due at least in part, to differences in cell surface characteristics responsible for recognition of "foreigness" by the prawn immune system. Several researchers (Trust *et al.*, 1982; Kay *et al.*, 1988; Chu *et al.*, 1991; Loghothetis and Austin, 1996) have suggested an association between bacterial virulence and differences in cell surface characteristics. The superiority in vaccine potentials of more virulent bacterial strains over non virulent strains has also been recognised (Trust *et al.*, 1982; McCarthy *et al.*, 1983; Olivier *et al.*, 1985; Loghothetis and Austin, 1996)

The cell free plasma of *Penaeus vannamei* is not inherently toxic to bacteria and provides a rich nutrient medium for *E. coli* growth.

This study shows that protection and cross-protection was conferred to prawn larvae following prior exposure to killed pathogenic or live non-pathogenic bacteria. Detection and quantification of this antibacterial activity has been problematic, usually due to the low concentrations of the active ingredients present. However, this study shows that preconditioning target bacteria with lysozyme increases sensitivity to the antimicrobial substances present, enabling direct evidence and quantification of antimicrobial activity.

Antibacterial activity was observed in both vaccinated and placebo injected prawns, indicating that wounding also has the ability to induce antibacterial activity in prawns, although this antibacterial activity was lower than obtained in vaccinated prawns. Antibacterial activity was only present for 7d, reflecting its limited duration. This finding has profound implications for disease control in penaeid culture systems.

Thus vaccines may for instance, be applied prophylactically to coincide with an anticipated period of increased stress (and therefore, increased disease susceptibility) in prawns.

Several authors have reported improved growth and survival in vaccinated penaeid prawns even though such prawns were not challenged by pathogens (Song and Sung, 1990; Latchford *et al.*, 1995). Although disease causing bacteria in prawns are generally opportunistic, they may still cause conditions of reduced performance and some mortalities even when environmental conditions are relatively good. In such cases, the knowledge of the duration of the immune response may also be important in determining the timing of "booster" vaccinations, which would enhance the resistance levels sufficiently to abolish such infections. However, the biological costs of continual activation of the prawn immune system by such booster vaccinations needs to be properly considered. For instance, it is known that a marked haemocytopaenia accompanies injection of bacteria in penaeid species (Martin *et al.*, 1993; 1996) with haemocyte levels returning to preinjection levels only after 72 - 96h. Though this depletion was demonstrated to have no deleterious effect on subsequent bacterial clearance or defence functions, crustacean haemocytes have been demonstrated to perform a variety of physiological functions including carbohydrate metabolism, lipoprotein or amino-acid transport and storage, and hardening of the exoskeleton (Bachau, 1981; Hose *et. al.*, 1992), and the effects of having such decreased levels of haemocytes on other body functions is unknown. In such situations, the use of probiotics or microbially balanced water may help keep pathogens in check until there is a requirement for the administration of vaccines.

Although lysozyme was not detected in all the tested treatments, there was a brief period of growth stasis for *Micrococcus luteus* in vaccinated plasma 6h after administration. Having demonstrated the requirement of lysozyme for quantification of antibacterial activity *in vitro*, it is suggested that the responsible factor (lysozyme-like factor) works in synergy with other antimicrobial actors and only needs to make a brief appearance to sensitise the bacteria for subsequent killing by other antibacterial compounds.

The antibacterial activity observed appears to be a multicomponent system of many pathways as discussed in Chapter 5. The more pathogenic strains of *V. harveyi* did not give PO activation values as high as those given by the less pathogenic strain IN7. As higher protection was conferred to larvae by vaccines made from more virulent bacterial strains as opposed to the less virulent strains, the presence of some other recognition and antimicrobial factors located in the plasma is suggested. This view is further supported by the observation that despite the very low stimulation of the proPO system given by *Escherichia coli* (PO activation units = 5.65), in *in-vitro* killing assays with cell-free plasma, It exhibited SI values initially lower than and finally not being significantly different from those exhibited by *V. harveyi* strains DPEX (PO units = 12.62), and BP04 (PO units = 20.76).

The relationship of haemolymph volumes of *P. vannamei* juveniles between 3.05 and 13.90g to weights was determined by using the dilution of radioactive ^{14}C, and this revealed a positive linear correlation of

haemolymph volume with prawn weights. This was used to determine the initial dilution of bacteria injected into *P. vannamei* juveniles for *in vivo* killing assays.

When the survival of different species of bacteria injected into prawns previously exposed to various vaccines were observed *in vivo*, there was an initial significant reduction in the mean TVC of bacteria over the first 3 to 8h. This indicated the functionality of the immediate cellular disease responses: phagocytosis, nodule formation and agglutination, and occurred in all treatments. This decline was followed by a period of bacterial multiplication the duration of which is suggested to be both strain and vaccine dependent. When prawns were vaccinated with the non-pathogenic *E. coli* and the placebo, this multiplication phase lasted up to 12h. Vaccinating with both pathogenic and non-pathogenic strains of *V. harveyi* (BP04, and DPEX respectively), gave multiplication phases lasting only up to 8h, reflecting the differences in vaccine efficacies.

It is suggested that the initial cellular responses serve to keep bacterial concentrations at a low level until the appearance of antibacterial activity. Time of appearance and efficacy of synthesised antibacterial compounds appears to depend on vaccine efficiencies, appearing at 8h in BP04 and DPEX vaccinated and 12h in SSS and *E. coli* vaccinated prawns.

Increased disease susceptibility may well be a consequence of impairment of the immune system resulting from environmental factors. Several researchers have reported effects of pesticides on different aspects of prawn enzyme systems (Srinivasulu Reddy and Ramana Rao, 1988; 1991; Srinivasulu Reddy *et al.*, 1991; Tangtrongpiros *et al.*, 1997). However, although an effect of the tested fungicides (tridemorph and propiconazole) was expected on various biological functions of *P. indicus*, none was observed. Further studies on the effects of other fungicides with different modes of activity from the ergosterol inhibitors tested on impairment of various biological functions of prawns with specific attention to the immune system needs to be investigated.

The determinants of disease may be represented as a triad consisting of animal, pathogen and environment. The animal and the pathogen co-exist in an environment which may favour either. The infectivity and virulence of the pathogen is opposed by the susceptibility and infectiousness of the animal. The incidence of disease then depends on the balance of these factors. For some diseases, such as those involving obligate pathogens, the environmental component of the triad is of little consequence, while for others, the environment has a profound effect on disease development.

Disease control methods may therefore, be directed against any one component or a combination of components. This study has revealed some possible approaches to control bacterial diseases in prawn hatcheries using a combination of factors affecting all three components determining disease.

1. Maintenance of the natural balance in bacterial communities present by 5µm filtration of the natural sea water and reducing or totally stopping the use of pre-treatment techniques which may modify the generic composition of the water as well as enabling increased virulence in the pathogens.

2. Identification and use of algal species which control bacterial levels as well as acting as larval diets.
3. Identification of more virulent bacterial species as potential vaccines. In addition the time of vaccination and possible booster vaccinations is important. This study has revealed that initial vaccination persists only for 7d and further work is required to determine the efficacy of booster vaccinations.

REFERENCES

Abubakr, M. A. 1991: Studies on the decapod larval gut in relation to diet. Ph.D. thesis. University of Wales, North Wales, Bangor, Menai Bridge, Gwynedd, UK. 230p.

Abubakr, M. A. and D. A. Jones. 1992: Functional morphology and ultrastructure of the anterior mid-gut diverticulae of larvae of *Penaeus monodon* Fabricus, 1798 (Decapoda, Nanantia). Crustaceana. 62:142-158.

Adams, A. and K. Thompson. 1990: Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of *Aeromonas salmonicida* in fish tissue. Journal of aquatic animal health. 2:281-288.

Adams, A. 1991a: Detection of *Vibrio parahaemolyticus* biotype *alginolyticus* in penaeid shrimps using an amplified enzyme-linked immunosorbent assay. Aquaculture. 93:101-108.

---. 1991b: Response of penaeid shrimp to exposure to *Vibrio* species. Fish and Shellfish Immunology. 1:59-70.

Alabi, A. O., E. Yudiati, D. A. Jones and J. W. Latchford. 1997: Bacterial levels in penaeid larval cultures. In: Diseases in Asian Aquaculture. (Eds: Flegel, T. W. and MacRae, I. H.). in press. Fish Health Section, Asian Fisheries Society, Manila.

Albright, L. J., S. K. McCrae and B. E. May. 1986: Attached and free-floating bacterioplankton in Howe Sound, British Columbia, a coastal marine fjord-embayment. Applied and Environmental Microbiology. 51:614-621.

Allan, B., M. Linseman, L. MacDonald, J. S. Lam and A. M. Kropinski. 1988: Heat shock response of *Pseudomonas aeruginosa*. Journal of Bacteriology. 170(8):3668-3674.

Alldredge, A. L. and Y. Cohen. 1987: Can microscale chemical patches persist in the sea? Microelectrode study of marine snow, fecal pellets. Science. 235:689-691.

Alldredge, A. L.., J. J. Cole and D. A. Caron. 1986: Production of heterotrophic bacteria inhabiting macroscopic organic aggregates (marine snow) from surface waters. Limnology and Oceanography. 31:68-78.

Alvarez, M. R. and F. E. Friedl. 1992: Effects of a fungicide on *in vitro* hemocyte viability, phagocytosis and attachment in the American oyster, *Crassostrea virginica*. Aquaculture. 107:135-140.

Amjad, S. and D. A. Jones. 1989: A comparison of the stability of some feeds used in penaeid larval culture. Journal of the World Aquaculture Society. 20(1):Aquaculture communiques. 12A.

---. 1992: An evaluation of artificial diets used in the culture of penaeid shrimp larvae *Penaeus monodon* (Fabricus). Pakistan Journal of Zoology. 24(2):135-142.

Amjad, S., D. A. Jones and K. Chitravedivelu. 1992: Advances in penaeid larval feed technology. In: Proceedings of seminar on new technologies in aquaculture. (Eds: Cheah, S. H. and Saidin, H. J.). pp. 29-45. Malaysian Fisheries Society, Serdang, Selagor, Malaysia.

Amos, W. M. G. 1981: Basic immunology. Butterworths, London, Boston. 188pp.

Anderson, I. G., M. Sharriff, G. Nash and M. Ash. 1987: Mortalities of juvenile shrimp, *Penaeus monodon*, associated with *Penaeus monodon*, Cytoplasmic reo-like virus and rickettsial and bacterial infections from Malaysian brackish water ponds. Asian Fisheries Science. 1:47.

Anderson, I. G., M. N. Shamsudin, M. Shariff and G. Nash. 1988: Bacterial septicaemia in juvenile tiger shrimp, *Penaeus monodon*, cultured in Malaysian brackish water ponds. Asian Fisheries Science. 2:93-108.

Ashida, M. and K. Söderhäll. 1984: The prophenoloxidase activating system in crayfish. Comparative Biochemistry and Physiology. 77B(1):21-26.

Austin, B. and J. G. Day. 1990: Inhibition of prawn pathogenic *Vibrio sp.* by a commercial spray-dried preparation of *Tetraselmis suecica*. Aquaculture. 90:389-392.

Austin, B., E. Baudet and M. Stobie. 1992: Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. Journal of Fish Diseases. 15:55-61.

Austin, B., F. Stuckey, P. A. W. Robertson, I. Effendi and D. R. W. Griffiths. 1995: A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalli*. Journal of Fish Diseases. 18:93-96.

Bachau, A. G. 1981: Crustaceans. In: Invertebrate blood cells, Vol. 2. pp. 386-420. Academic press, New York.

Baker, J. H. and L. A. Bradnam. 1976: The role of bacteria in the nutrition of aquatic detritivores. Oecologia. 24:95-104.

Barker, R. M. 1986: Epidemic strains of *Salmonella agona* differentiated by phage restriction and u.v-protection markers on Col1b plasmids. Letters in Applied Microbiology. 3:101-104.

Baticados, M. C. L. 1988: Control of luminous bacterial infection in prawn hatcheries. SEAFDEC Asian Aquaculture. X(1):10-11.

Baticados, M. C. L. and J. O. Paclibare. 1992: The use of chemotherapeutic agents in aquaculture in the Philippines. In: Diseases in Asian aquaculture 1. Proceedings of the first symposium on disease in Asian aquaculture. (Eds: Shariff, M., Subasinghe, R. P. and Arthur, J. R.). pp. 531-546. Fish Health Section, Asian Fisheries Society, The Philippines.

Baticados, M. C. L.., C. R. Lavilla-Pitogo, E. R. Cruz-Lacierda, L. D. de la Pena and N. A. Sunaz. 1990: Studies on the chemical control of luminous bacteria *Vibrio harveyi* and *Vibrio splendidus* isolated from diseased *Penaeus monodon* larvae and rearing water. Diseases of Aquatic Organisms. 9:133-139.

Bell, T. A. 1992: Principals of shrimp culture chemotherapy. In: Proceedings of the special session on shrimp farming. (Ed: Wyban, J.). pp. 227-237. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Bell, T. A. and D. V. Lightner. 1984: IHHNV virus: Infectivity and pathogenicity studies in *Penaeus stylirostris* and *Penaeus vannamei*. Aquaculture. 38:185-194.

Bell, W. H., J. M. Lang and R. Mitchell. 1974: Selective stimulation of marine bacteria by algal extracellular products. Limnology and Oceanography. 19:833-839.

Bergh, Ý. 1995: Bacteria associated with early life stages of halibut, *Hippoglossus hippoglossus* L., inhibit growth of a pathogenic *Vibrio sp.* Journal of Fish Diseases. 18:31-40.

Bigger, J. W. and J. H. Nelson. 1941: The growth of coliform bacilli in distilled water. Journal of Pathology and Bacteriology. 53:189-206.

Bissonnette, G. K., J. J. Jezeski, G. A. McFeters and D. G. Stuart. 1975: Influence of environmental stress on enumeration of indicator bacteria from natural waters. Applied Microbiology. 29(2):186-193.

Blight, S. P., T. L. Bentley, D. Lefevre, C. Robinson, R. Rodrigues, J. Rowlands and P. J. leB. Williams. 1995: Phasing of autotrophic and heterotrophic plankton metabolism in a temperate coastal ecosystem. Marine Ecology Progress Series. 128:61-75.

Bodhiaksha, N. and B. A. Weeks-Perkins. 1994: The effects of methyl parathion on phagocytosis and

respiratory burst activity of tiger shrimp (*Penaeus monodon*) phagocytes. In: Modulators of fish immune responses. (Eds: Stolen, J. S. and Fletcher, T. C.). pp. 11-22. SOS Publications, Fair Haven, NJ, USA.

Boman, H. G. and D. Hultmark. 1987: Cell-free immunity in insects. Annual Review of Microbiology. 41:103-126.

Bonami, J. R., K. W. Hasson, J. Mari, B. T. Poulos and D. V. Lightner. 1997: Taura syndrome of marine penaeid shrimp: Characterization of the viral agent. Journal of General Virology. 78(2):313-319.

Boyd, C. E. 1996: Chlorination and water quality in aquaculture ponds. World Aquaculture. 27(3):41-45.

Bray, W. A., A. L. Lawrence and J. R. Leung-Trujillo. 1994: The effect of salinity on growth and survival of *Penaeus vannamei*, with observations on the interaction of IHHN virus and salinity. Aquaculture. 122(2-3):133-146.

Broadwater, W. T., R. C. Hoehn and P. H. King. 1973: Sensitivity of 3 selected species to ozone. Applied Microbiology. 26:391-393.

Brock, J. A. and B. Leamaster. 1992: A look at the principal bacterial, fungal and parasitic diseases of farmed shrimp. In: Proceedings of the special session on shrimp farming. World Aquaculture Society, Baton Rouge, LA. USA.

Brock, J. A. and D. V. Lightner. 1990: Microbial pathogens and diseases of marine crustaceans. In: Diseases of marine animals. (Ed: Kinne, O.). pp. 245-349. John Wiley and sons, New York.

Brock, J. A., L. K. Nakagawa and R. J. Shimojo. 1986: Infection of a cultured freshwater prawn, *Macrobrachium rosenbergii* de Man (Crustacea: Decapoda) by *Mycobacterium spp.*, Runyon group 11. Journal of Fish Diseases. 9:319-324.

Brock, J. A., R. Gose, D. V. Lightner and K. Hasson. 1995: An overview on Taura Syndrome, an important disease of farmed *Penaeus vannamei.* In: Swimming through troubled water: Proceedings of the special session on shrimp farming Aquaculture '95. (Eds: Browdy, C. L. and Hopkins, J. S.). pp. 84-94. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Browdy, C. L., J. D. Holloway, C. O. King, A. D. Strokesa, J. S. Hopkins and P. A. Sandifer. 1993: IHHN virus and intensive culture of *Penaeus vannamei*: Effects of stocking density and water exchange rates. Journal of Crustacean Biology. 13:87-94.

Brown, J. H. 1989: Antibiotics: Their use and abuse in aquaculture. World Aquaculture. 20(2):34-43.

Bullock, J. M. and H. M. Stuckey. 1977: Ultraviolet treatment of water for destruction of five gram-negative bacteria pathogenic to fishes. Journal of the Fisheries Research Board of Canada. 34:1244-1249.

Bullock, W. O., J. M. Fernandez and J. M. Short. 1987: XL1-blue: A high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. Biotechniques. 5:376-379.

Camper, A. K. and G. A. McFeters. 1979: Chlorine injury and the enumeration of waterborne coliform bacteria. Applied and Environmental Microbiology. 37(3):633-641.

Capone, D. G., D. P. Weston, V. Miller and C. Shoemaker. 1996: Antibacterial residues in marine sediments and invertebrates following chemotherapy in aquaculture. Aquaculture. 145:55-75.

Castille, F. L. and A. L. Lawrence. 1986: The toxicity of erythromycin, minocycline, malachite green and formalin to nauplii of the shrimp *Penaeus stylirostris*. Journal of the World Aquaculture Society. 17(1-4):13-18.

Chamberlain, G. W. 1993: Aquaculture trends and feed projections. World Aquaculture. 24(1):19-30.

Chang, J. C. H., S. F. Ossoff, D. C. Lobe, M. H. Dorfman, C. M. Dumais, R. G. Qualls and J. D. Johnson. 1985: UV inactivation of pathogenic and indicator micro-organisms. Applied and Environmental Microbiology. 49:1361-1365.

Cheng, T. C. and G. E. Rodrick. 1974: Identification and characterization of lysozyme from the hemolymph of the soft-shelled clam *Mya arenaria*. Biological Bulletin. 147:311-320.

Cheng, T. C., J. W. Huang, H. Karadogan, L. R. Renwrantz and T. P. Yoshino. 1980: Separation of oyster hemocytes by density gradient centrifugation and identification of their surface receptors. Journal of Invertebrate Pathology. 36:35-40.

Chisholm, J. R. S. and V. J. Smith. 1992: Antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas*. Journal of the Marine Biological Association of the United Kingdom. 72:529-542.

Chu, S., S. Cavaignac, J. Feutrier, B. M. Phipps, M. Kostrzynska, W. W. Kay and T. J. Trust. 1991: Structure of the tetragonal surface virulence array protein and gene of *Aeromonas salmonicida*. J. Biol. Chem. 266:15258-15265.

Cipriani, G. R., R. S. Wheeler and R. K. Sizemore. 1980: Characterization of brown spot disease of Gulf coast shrimp. Journal of Invertebrate Pathology. 36:255-263.

Cloutier, J., D. Prévost, P. Nadeau and H. Antoun. 1992: Heat and cold shock protein synthesis in arctic and temperate strains of rhizobia. Applied and Environmental Microbiology. 58(9):2846-2853.

Colberg, P. J. and A. J. Lingg. 1978: Effect of ozonation on microbial fish pathogens, ammonia, nitrate, nitrite and BOD in simulated reuse hatchery water. Journal of the Fisheries Research Board of Canada. 35:1290-1296.

Conn, H. J. and J. E. Conn. 1940: The stimulating effect of colloids upon the growth of certain bacteria. Journal of Bacteriology. 39:99-100.

Couch, J. A. 1974: An enzootic polyhedrosis virus of pink shrimp: ultrastructure, prevalence and enhancement. Journal of Invertebrate Pathology. 24:311-331.

Coyne, R. M., M. Hiney, B. O'Connor, J. Kerry, D. Cazabon and P. Smith. 1994: Concentration and persistence of oxytetracycline in sediments under a marine salmon farm. Aquaculture. 119:25-40.

Csavas, I. 1994: Important factors in the success of shrimp farming. World Aquaculture. 25(1):34-56.

Dawson, M. P., B. A. Humphrey and K. C. Marshall. 1981: Adhesion: a tactic in the survival strategy of a marine vibrio during starvation. Current Microbiology. 6:195-199.

Dehasque, M., L. Verdonck, P. Sorgeloos, J. Swings, P. Légér and K. Kersters. 1991: Determination of the bacterial contamination in live food production systems in marine fish hatcheries in southern Europe. In: Larvi '91-Fish and crustacean larviculture symposium. (Eds: Lavens, P., Sorgeloos, P., Jaspers, E. and Ollevier, F.). pp 399-402. European Aquaculture Society Special Publication No. 15, Gent, Belgium.

de la Peña, L. D., T. Tamaki, K. Momoyama, T. Nakai and K. Muroga. 1993: Characteristics of the causative bacterium of vibriosis in the kuruma prawn, *Penaeus japonicus*. Aquaculture. 115:1-12.

de Nys, R., P. D. Steinberg, P. Willemsen, S. A. Dworjanyn, C. L. Gabelish and R. J. King. 1995: Broad spectrum effects of secondary metabolites from the red alga *Delisea pulchra*in antifouling assays. Biofouling. 8:259-271.

DeVerno, P. J., J. S. Chadwick, W. P. Aston and G. B. Dunphy. 1984: The *in vitro* generation of an antimicrobial activity from the fat body and haemolymph of non-immunized larvae of *Galleria mellonella*. Developmental and Comparative Immunology. 8:537-546.

Doubleday, O. P., M. H. L. Green and B. A. Bridges. 1977: Spontaneous and ultraviolet-induced mutation

In *Escherichia coli*: Interaction between plasmid and tif-1 mutator effects. Journal of General Microbiology. 101:163-166.

Douillet, P. A. and C. J. Langdon. 1994: Use of a probiotic for the culture of larvae of the pacific oyster (*Crassostrea gigas* Thunberg). Aquaculture. 119:25-40.

Dunn, A. Polk, D. J. Scarrett, G. Olivier, S. Lall and M. F. A. Gossen. 1990: Vaccines in aquaculture: the search for an efficient delivery system. Aquacultural Engineering. 9:23-32.

Dunn, W. Dai, M. R. Kanost and C. Geng. 1985: Soluble peptidoglycan fragments stimulate antibacterial protein synthesis by fat body from *Manduca sexta*. Developmental and Comparative Immunology. 9:559-568.

Dupree, H. K. 1981: An overview of the various techniques to control infectious diseases in water supplies and in water reuse aquacultural systems. In: Proceedings of the bio-engineering symposium for fish culture. (Eds: Allen, L. J. and Kinney, E. C.). pp. 83-89. Fish Culture Section, American Fisheries Society, Bethesda, Maryland.

Durliat, M. and R. Vranck. 1981: Action of various anticoagulants on hemolymphs of lobsters and spiny lobsters. Biological Bulletin. 160:55-68.

Ebo, Budu-A., S. Toora, R. F. Ablett and J. Smith. 1992: Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured *Listeria monocytogenes* cells. Applied and Environmental Microbiology. 58(9):3177-3179.

Egidius, E. 1987: Vibriosis: pathogenicity and pathology. A review. Aquaculture. 67:15-28.

Egusa, S., Y. Takahashi, T. Itami and K. Momoyama. 1988: Histopathology of vibriosis in the kuruma shrimp, *Penaeus japonicus* Bate. Fish Pathology. 23:59-65.

Engström, P., A. Carlsson, Å. Engström, Z. Tao and H. Bennich. 1984: The antibacterial effect of attacins from the silk moth *Hyalophora cecropia* is directed against the outer membrane of *Escherichia coli*. The EMBO Journal. 3(13):3347-3351.

Faye, I. and G. R. Wyatt. 1980: The synthesis of antibacterial proteins in isolated fat body from *Cecropia* silkmoth pupae. Experientia. 36:1325-1326.

Fletcher, M. and G. D. Floodgate. 1973: An electron-microscope demonstration of an acidic polysaccharide involved in the adhesion of a marine bacterium to solid surfaces. Journal of General Microbiology. 74:325-

334.

Flyg, C., G. Dalhammer, B. Rasmusson and H. G. Boman. 1987: Insect immunity: inducible antibacterial activity in *Drosophila*. Insect Biochemistry. 17(1):153-160.

Foley, D. A. and T. C. Cheng. 1977: Degranulation and other changes of molluscan granulocytes associated phagocytosis. Journal of Invertebrate Pathology. 29:321-325.

Frelier, P. F., R. F. Sis, T. A. Bell and D. H. Lewis. 1992: Microscopic and ultrastuctural studies of necrotising hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) cultured in Texas. Veterinary Pathology. 29:269-277.

Garriques, D. and G. Arevalo. 1995: An evaluation of the production and use of a live bacterial isolate to manipulate the microbial flora in the commercial production of *Penaeus vannamei* post larvae in Ecuador. In: Swimming through troubled water, Proceedings of the special session on shrimp farming, Aquaculture '95. (Eds: Browdy, C. L. and Hopkins, J. S.). pp. 53-59. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Gatesoupe, François-J. 1990: The continuous feeding of turbot larvae *Scophtalmus maximus* and the control of bacterial environment of rotifers. Aquaculture. 89:139-148.

Givskov, M., R. de Nys, M. Manefield, L. Gram, R. I. A. Maximilien, L. E. O. Eberl, S. Molin, P. D. Steinberg and S. Kjelleberg. 1996: Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. Journal of Bacteriology. 178(22):6618-6622.

Goldenberg, P. Z. and A. H. Greenberg. 1983: Functional heterogeneity of carbohydrate-binding hemolymph proteins: evidence of a non-agglutinating opsonin in *Homarus americanus*. Journal of Invertebrate Pathology. 42:33-41.

Goldstein, I. J., R. C. Hughes, M. Monsigny, T. Osawa and N. Sharon. 1980: What should be called a lectin? Nature. 285:66.

Gomes, L. A. 1992: Zen and the art of aquafarming, a close look at Taiwanese aquaculture practices. World Aquaculture. 23(3):18-26.

Gram, L., R. deNys, R. I. A. Maximilien, M. Givskov, P. D. Steinberg and S. Kjelleberg. 1996: Inhibitory effects of secondary metabolites from the red alga *Delisea pulchra* on swarming motility of *proteus mirabilis*. Applied and Environmental Microbiology. 62(11):4284-4287.

Greineisen, M. L. 1994: Recent advances in our knowledge of ecdysteroid biosynthesis in insects and crustaceans. Insect Biochemistry and Molecular Biology. 24:115-132.

Griffith, D. R. W. 1995: Microbiology and the role of probiotics in Ecuadorian shrimp hatcheries. In: Larvi '95 - Fish and Shellfish larviculture symposium. (Eds: Lavens, P., Jasers, E. and Roelants, I.). pp. 478. European Aquaculture Society, Gent, Belgium.

Guzmán, María-A., J. L. Ochoa and F. Vargas-Albores. 1993: Haemolytic activity in the brownshrimp (*Penaeus californiensis* Holmes) haemolymph. Comparative Biochemistry and Physiology. 106A(2):271-275.

Harm, W. 1968: Dark repair of photorepairable UV lesions in *Escherichia coli*. Mutation Research. 6:25-35.

Harris, G. D., D. Adams, D. L. Sorensen and M. S. Curtis. 1987: Ultraviolet inactivation of selected bacteria and viruses with photoreactivation of the bacteria. Water Research. 21(6):687-692.

Hasson, K. W., D. V. Lightner, B. T. Poulos, R. M. Redman, B. L. White, J. A. Brock and J. R. Bonami. 1995: Taura Syndrome in *Penaeus vannamei*. Demonstration of a viral aetiology. Diseases of Aquatic Organisms. 23(2):115-126.

Hauton, C., J. A. Williams and L. E. Hawkins. 1997: The effects of a live *in vivo* pathogenic infection on aspects of the immunocompetence of the common shore crab, *Carcinus maenas* (L.). Journal of Experimental Marine Biology and Ecology. 211:115-128.

Helm, M. M. 1971: The effect of sea water quality on the laboratory culture of *Ostrea edulis* L. larvae. ICES C.M. 1971/K:28, 8pp. (mimeo). .

Hermansson, M., G. W. Joes and S. Kjelleberg. 1987: Frequency of antibiotic and heavy metal resistance and plasmids in bacteria of the marine air-water interphase. Applied and Environmental Microbiology. 53:2338-2342.

Hjeltnes, B., K. Andersen, Hans-M. Ellingsen. 1989: Vaccination against *Vibrio salmonicida*. The effect of different routes of administration and of revaccination. Aquaculture. 83:1-6.

Hoffman, J., D. Hultmark and H. G. Boman. 1981: Insect immunity: *Galleria mellonella* and other Lepidoptera have cecropia-P-9-like factors active against Gram negative bacteria. Insect Biochemistry. 11:537-548.

Holm, K. O. and T. Jorgensen. 1987: A successful vaccination of Atlantic salmon, *Salmo salar* L. against

"Hitra" disease or cold water vibriosis. Journal of Fish Diseases. 10:85-90.

Hose, J. E. and G. G. Martin. 1989: Defence functions of granulocytes in the ridgeback prawn *Sicyonia ingentis*. Journal of Invertebrate Pathology. 53:335-346.

Hose, J. E., D. V. Lightner, R. M. Redman and D. A. Danald. 1984: Observations on the pathogenesis of the imperfect fungus, *Fusarium solani*, in the California brown shrimp, *Penaeus californiensis*. Journal of Invertebrate Pathology. 44:292-303.

Hose, J. E., G. G. Martin, V. A. Nguyen, J. Lucas and T. Rosenstein. 1987: Cytochemical features of shrimp haemocytes. Biological Bulletin. 173:178-187.

Hose, J. E., G. G. Martin and A. S. Gerard. 1990: A decapod hemocyte classification scheme integrating morphology, cytochemistry and function. Biological Bulletin. 178:33-45.

Hose, J. E., G. G. Martin, S. A. M. Tiu and N. McKrell. 1992: Patterns of hemocyte production and release throughout the molt cycle in the penaeid shrimp *Sicyonia ingentis*. Biological Bulletin. 183:185-199.

Hoyt, P. R. and R. K. Sizemore. 1982: Competitive dominance by a bacteriocin-producing *Vibrio harveyi* strain. Applied and Environmental Microbiology. 44(3):653-658.

Hultmark, D., H. Steiner, T. Rasmuson and H. G. Boman. 1980: Purification and properties of three inducible bactericidal proteins from haemolymph of immunized pupae of *Hyalophora cecropia*. European Journal of Biochemistry. 106:7-16.

Iida, T. and H. Wakabayashi. 1992: Preliminarytrial of vaccination against edwarsiellosis of hirame (Japanese flounder), Paralichthys olivaceus. In: Diseases in Asian Aquaculture 1. (Eds: Shariff, M., Subasinghe, R. P. and Arthur, J. R.). pp. 407-412. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Inouye, K. S., N. Miwa, H. Oseko, H. Nakano, T. Kimura, K. Momoyama and M. Hiraoka. 1994: Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus* in Japan in 1993: electron microscopic evidence of the causative virus. Fish Pathology. 29:149-158.

Intriago, P. and D. A. Jones. 1993: Bacteria as food for *Artemia*. Aquaculture. 113:115-127.

Intriago, P., R. Jimenez, M. Machuca, R. Barniol, E. Krauss and X. Salvador. 1995: Taura Syndrome, a toxicity syndrome of *Penaeus vannamei* in Ecuador. In: Book of Abstracts, World Aquaculture '95,

February 1-4, San Diego, USA. pp. 143. World Aquaculture Society, Baton Rouge.

Intriago, P., R. Jimenez, M. Machuca, R. Barniol, E. Krauss and X. Salvador. 1996: Survival and concentration of biogenic amines in *Penaeus vannamei* juveniles fed on different concentrations of Taura syndrome shrimp added to the diet. In: Book of abstracts, World Aquaculture '96, January 29-February 2, Queen Sirikit national convention centre, Bangkok, Thailand. pp. 175. World Aquaculture Society, Baton Rouge.

Ishizaki, K., N. Shinriki and H. Matsuyama. 1986: Inactivation of *Bacillus* spores by gaseous ozone. Journal of Applied Bacteriology. 60:67-72.

Itami, T. and Y. Takahashi. 1991: Survival of larval giant tiger prawns *Penaeus monodon* after addition of killed *Vibrio* cells to a microencapsulated diet. Journal of Aquatic Animal Health. 3:151-152.

Itami, T., Y. Takahashi and Y. Nakamura. 1989: Efficacy of vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus*. Journal of Aquatic Animal Health. 1:238-242.

Itami, T., Y. Yan and Y. Takahashi. 1992: Studies on vaccination against vibriosis in cultured kuruma prawns *Penaeus japonicus* 2. Effects of different vaccine preparations and oral vaccination efficiency. Journal of the Shimonoseki University of Fisheries. 40(3):139-144.

Jacobsen, M. D. 1989: Withdrawal times of freshwater rainbow trout *Salmo gairdneri* Richardson, after treatment with oxalinic acid, oxytetracycline and trimetoprim. Journal of Fish Diseases. 12:29-36.

Jacobsen, P. and L. Berglind. 1988: Persistence of oxytetracycline in sediments from fish farms. Aquaculture. 70:365-370.

Jagger, J. 1967: Introduction to research in ultraviolet photobiology. Prentice-Hall Inc., N,J., USA. 164.

Jenkins, P. G., A. B. Wrathmell, J. E. Harris and A. L. Pulsford. 1994: Systemic and mucosal immune responses to enterically delivered antigen in *Oreochromis mossambicus*. Fish and Shellfish Immunology. 4:255-271.

Jimenez, R. 1992: Sindrome de Taura (resumen). Aquacultura del Ecuador. 1:1-16.

Jimenez, R., M. Machuca and L. de Barniol. 1995: Taura syndrome: epizootiology and histopathology of a mortality toxicity syndrome of penaeid shrimp in Ecuador. In: Book of Abstracts, World Aquaculture '95, February 1-4, San Diego, USA. pp. 147. World Aquaculture Society, Baton Rouge,.

Jobling, M. 1983: A short review and critique of the methodology used in fish growth and nutrition. Journal of Fish Biology. 23:685-703.

Johansson, M. W. and K. Söderhäll. 1989: Cellular immunity in crustaceans and the proPO system. Parasitology Today. 5(6):171-176.

Jolley, R. L., R. B. Cumming, N. E. Lee, J. E. Thompson and L. R. Lewis. 1982: Micropollutants produced by disinfection of wastewater effluents. Water Science Technology. 14:45-59.

Jones, D. A. 1988: Tropical prawn culture. University of Wales Review of Science and Technology. 4:51-58.

Jones, D. A., D. L. Holland and S. Jabborie. 1984: Current status of microencapsulated diets for aquaculture. Applied Biochemistry and Biotechnology. 10:275-288.

Jones, D. A., K. Kurmaly and A. Arshad. 1987: Penaeid shrimp hatchery trials using microencapsulated diets. Aquaculture. 64:133-146.

Jones, D. A., L. LeVay and M. S. Kamarudin. 1992: Feeding and nutritional requirements of penaeid shrimp larvae. Memorias I Congreso Ecuatoriano de Acuicultura. :45-52.

Jones, D. A., M. S. Kamarudin and L. LeVay. 1993: The potential for replacement of live feeds in larval culture. Journal of the World Aquaculture Society. 24(2):199-201.

Jones, D. A., M. Freeman, J. Latchford, N. Misciatelli, F. A. L. T. Ribeiro and S. C. Rowlands. 1997: Progress in the use of artificial feeds in penaeid larval culture. In: Abstracts of the 2nd Asia-Pacific marine biotechnology Conference and 3d Asia- Pacific conference on algal biotechnology, May 7-10, 1997, Phuket. (Eds: National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Aquatic Resources Research Institute, Chulalongkorn University, King Mongkut's Institute of Technology Thonburi). pp 107. Phuket, Thailand.

Kaaya, G. P., C. Flyg and H. G. Boman. 1987: Insect immunity: induction of cecropin and attacin-like antibacterial factors in the haemolymph of *Glossina morsitans morsitans*. Insect Biochemistry. 17(2):309-315.

Kalagayan, H., D. Godin, R. Kanna, G. Hagino, J. Sweeny, J. Wyban and J. Brock. 1991: IHHN virus as an etiological factor in runt-deformity syndome (RDS) of juvenile *Penaeus vannamei* cultured in Hawaii. Journal of the World Aquaculture Society. 22(4):235-243.

Kamarudin, M. S. 1992. Studies on the digestive physiology of crustacean larvae. Ph.D. thesis. University of Wales. 190 pp.

Kanazawa, A., S. Teshima and S. Tokiwa. 1977: Nutritional requirements of prawn - VII. Effect of dietary lipids on growth. Bulletin of the Japanese Society of Scientific Fisheries. 43:849-856.

Kanazawa, A., L. Chim and A. Laubier. 1988: Tissue uptake of radioactive cholesterol in the prawn *Penaeus japonicus* Bate during induced ovarian maturation. Aquatic Living Resouces. 1:85-91.

Kaneko, T., G. Roubal and R. Atlas. 1978: Bacterial populations in the Beaufort Sea. Arctic. 31:91-107.

Karunasagar, I., R. Pai, G. R. Malathi and I. Karunasagar. 1994: Mass mortality of *Penaeus monodon* larvae due to antibiotic resistant *Vibrio harveyi* infection. Aquaculture. 128:203-209.

Karunasagar, I., S. K. Otta and I. Karunasagar. 1995: Biofilm formation by *Vibrio harveyi* on surfaces. Aquaculture. 140:241-245.

Kaspar, C. W. and M. L. Tamplin. 1993: Effects of temperature and salinity on the survival of *Vibrio vulnificus* in sea water and shellfish. Applied and Environmental Microbiology. 59(8):2425-2429.

Katzenelson, E., B. Kletter and I. Shuval. 1974: Inactivation kinetics of viruses and bacteria in water by use of ozone. Journal of American Water Works Association. 66:725-729.

Kay, W. W., B. M. Phipps, R. A. Garduño and T. J. Trust. 1988: The A-layer of *Aeromonas salmonicida*: Organisation and functions. In: Crystalline bacterial cell surface layers. (Eds: Sleytr, U. B., Messner, P., Pum, D. and Sra, M.). pp. 44-50. Springer-Verlag, Berlin.

Kellam, S. J. and J. M. Walker. 1989: Antibacterial activity from marine microalgae in laboratory culture. British Phycological Journal. 24:191-194.

Kerry, J., M. Hiney, R. Coyne, S. NicGabhaim, D. Gilroy, D. Cazabon and P. Smith. 1995: Fish feed as a source of oxytetracycline resistant bacteria in the sediments under fish farms. Aquaculture. 131:101-113.

Kjelleberg, S. and M. Hermansson. 1984: Starvation-induced effects on bacterial surface characteristics. Applied and Environmental Microbiology. 48:487-503.

Klein, J. 1984: Are invertebrates capable of anticipatory immune responses? Scandinavian Journal of Immunology. 29:499-505.

Kleinholz, L. H. 1985: Biochemistry of crustacean hormones. In: The biology of crustacea: Integument pigments and hormonal process. (Eds: Bliss, D. S. and Mantel, L. H.). pp. 464-522. Academic Press, New York.

Kogure, K., U. Simidu and N. Taga. 1979: The effect of *Skeletonema costatum* (Grev.) Cleve on the growth of marine bacteria. Journal of Experimental Marine Biology and Ecology. 36:201-215.

Krol, R., W. E. Hawkins, . Vogelbein and R. M. Overstreet. 1989: Histopathology and ultrastructure of the hemocytic response to an acid fast bacterial infection in cultured *Penaeus vannamei*. Journal of Aquatic Animal Health. 1:37-42.

Kumlu, M. and D. A. Jones. 1994: Salinity tolerance of hatchery-reared postlarvae of *Penaeus indicus* H. Milne Edwards originating from India. Aquaculture. 130:287-296.

---. 1995: The effect of live and artificial diets on growth, survival and trypsin activity in larvae of *Penaeus indicus*. Journal of the World Aquaculture Society. 26(4):406-415.

Lackie, A. M. 1980: Invertebrate immunity. Parasitology. 80:393-412.

Laemmli, U. K. 1970: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature. 227:680-685.

Landesman, L. 1994. Negative impacts of coastal aquaculture development. World Aquaculture. 25(2):12-17.

Latchford, J. W., S. B. Prayitno and A. O. Alabi. 1995: The use of vaccines and immunostimulants in the culture of penaeid shrimp. In: Larvi'95 - Fish and Shellfish larviculture symposium. In: Larvi '95 - Fish and Shellfish larviculture symposium. (Eds: Lavens, P., Jaspers, E. and Roelants, I.). pp. 506. European Aquaculture Society, Gent, Belgium.

Lavilla-Pitogo, C. R., M. C. L. Baticados, E. R. Cruz-Lacierda and L. D. de la Pena. 1990: Occurrence of luminous bacterial disease of *Penaeus monodon* larvae in the Philippines. Aquaculture. 91:1-13.

Lee, D. O. C. and J. F. Wickins. 1992: Crustacean farming. Blackwell Scientific Publications, Melbourne Paris, Berlin, Vienna. 329pp.

Lehrbach, P., A. H. C. Kung and B. T. O. Lee. 1977: Loss of ultraviolet light protection and enhanced ultraviolet light-induced mutability in *Pseudomonas aeruginosa* carrying mutant R plasmid. Journal of General Microbiology. 101:135-141.

Lemos, M. L., A. E. Toranzo and J. L. Barja. 1985: Antibiotic activity of epiphytic bacteria isolated from intertidal seaweeds. Microbial Ecology. 11:149-163.

LeVay, L., A. Rodriguez, M. S. Kamarudin and D. A. Jones. 1993: Influence of live and artificial diets on tissue composition and trypsin activity in *Penaeus japonicus* larvae. Aquaculture. 118:287-297.

Levenbook, L. 1958: Intracellular water of larval tissues of the southern armyworm as determined by the use of C14-carboxyl-inulin. Journal of Cellular and Comparative Physiology. 52:329-339.

Levine, E. and T. Thiel. 1987: UV-Inducible DNA repair in the cyanobacteria *Anabaena sp.* Journal of Bacteriology. 169(9):3988-3993.

Lightner, D. V. 1983: Diseases of cultured penaeid shrimp. In: CRC handbook of mariculture. (Ed: McVey, J. P.). pp. 289-320. CRC Press Inc., Boca Raton, Fl.

---. 1985: A review of the diseases of cultured penaeid shrimps and prawns with emphasis on the recent discoveries and developments. In: Proceedings of the first international conference on the culture of penaeid prawns/shrimps. (Eds: Taki, Y., Primavera, J. H. and Llobrera, J. A.). pp. 79-103. SEAFDEC Aquaculture department, Illoilo city, The Philippines.

---. 1988: Vibrio disease. In: Disease diagnosis and control in North American marine aquaculture. (Eds: Sindermann, C. J. and Lightner, D. V.). pp. 42-47. Elsevier publications, Amsterdam, Oxford, New York, Tokyo.

---. 1992: Shrimp pathology: Major diseases of concern to the farming industry in the Americas. Memorias I Congreso Ecuatoriano de Acuicultura. 1992:177-195.

---. 1996: Taura syndrome virus (TSV). In: A handbook of pathology and diagnostic procedures for diseases of penaeid shrimp. (Ed: Lightner, D.V.). pp. 3/1-3/12. World Aquaculture Society, Baton Rouge.

Lightner, D. V. and R. M. Redman. 1994: Histopathology and ultrastructural studies of Taura syndrome, a putative toxicity syndrome of penaeid shrimp. In: Book of Abstracts, World Aquaculture '94, January 14-18, New Orleans. (Ed: World Aquaculture Society). pp. 227. Louisiana State University, Baton Rouge.

Lightner, D. V., R. M. Redman and J. R. Bonami. 1992: Morphological evidence for a single bacterial aetiology in Texas necrotizing hepatopancreatitis in *Penaeus vannamei* Crustacea: Decapoda). Diseases of Aquatic Organisms. 13:235-239.

Lightner, D. V., L. S. Jones and G. W. Ware 1994: Proceedings of the Taura Syndrome workshop:. executive summary, submitted reports and transcribed notes. January 21-22, 1994. University of Arizona, Tucson. .

Lightner, D. V., R. M. Redman, K. W. Hasson and C. R. Pantoja. 1995: Taura Syndrome in *Penaeus vannamei* (Crustacea, Decapoda)-gross signs, histopathology and ultrastructure. Diseases of Aquatic Organisms. 21(1):53-59.

Lillehaug, A. 1989a: Oral immunization of rainbow trout, *Salmo gairdneri* Richardson, against vibriosis with vaccines protected against digestive degradation. Journal of Fish Diseases. 12:579-584.

---. 1989b: A survey on different procedures used for vaccinating salmonids against vibriosis in Norwegian fish-farming. Aquaculture. 83:217-226.

---. 1991: Vaccinating salmonids against furunculosis. Canadian Translation of Fisheries and Aquatic Sciences. 102(10):673-674.

Liltved, H. and B. Landfald. 1993: UV inactivation and photoreactivation of bacterial fish pathogens. In: Fish Farming Technology. (Eds: Reinetsen, Dahle, Jýrgensen and Tvinnereim). pp. 77-83. Balkema, Rotterdam.

Liltved, H., H. Hektoen and H. Efraimsen. 1995: Inactivation of bacterial and viral fish pathogens by ozonation or UV irradiation in water of different salinity. Aquacultural Engineering. 14:107-122.

Limsuwan, C. 1993: Diseases of black tiger shrimp, *Penaeus monodon* Fabricus in Thailand. In: Technical bulletin. (Ed: Akiyama, D. M.). pp. 1-15. American Soy bean Association, Singapore.

Loghothetis, P. N. and B. Austin. 1996: Variations in antigenicity of *Aeromonas hydrophila* strains in rainbow trout (*Oncorhychus mykiss*, Walbaum): an association with surface characteristics. Fish and Shellfish Immunology. 6:47-55.

Lotz, J.M. 1997: Effect of host size on virulence of taura virus to the marine shrimp *Penaeus vannamei* (Crustacea: Penaeidae)..Diseases of Aquatic Organisms. 30:45-51.

Lovett, D. L. and D. L. Felder. 1990: Ontogenic change in digestive enzyme activity of larval and postlarval white shrimp *Penaeus setiferus* (Crustacea, Decapoda, Penaeidae). Biological Bulletin. 178:144-159.

Martin, G. G., D. Poole, C. Poole, J. E. Hose, M. Arias, L. Reynolds, N. McKrell and A. Whang. 1993: Clearance of bacteria injected into the haemolymph of the penaeid shrimp, *Sicyonia ingentis*. Journal of

Invertebrate Pathology. 62:308-312.

Martin, G. G., J. E. Hose, G. Minka and S. Rosenberg. 1996: Clearance of bacteria injected into the hemolymph of the ridgeback prawn *Sicyonia ingentis* (Crustacea, Decapoda) - role of hematopoietic tissue. Journal of Morphology. 227(2):227-233.

Masek, F. and M. Sedliakova. 1977: Pyrimidine dimer excision and DNA degradation during liquid holding recovery in ultraviolet irradiated *Escherichia coli* k12 uvr^+ rec. Journal of General Microbiology. 101:131-133.

McCarthy, D. H., D. F. Amend, K. A. Johnson and J. A. Bloom. 1983: *Aeromonas salmonicida*: determination of an antigen associated with protective immunity and evaluation of an experimental bacterin. Journal of Fish Diseases. 6:155-174.

McDonald, N.L., J. R. Stark and B. Austin. 1986: Bacterial microflora in the gastro-intestinal tract of Dover sole, *Solea solea* with emphasis on the possible role of bacteria in the nutrition of the host. FEMS Microbiology Letters. 35:37-38.

Mead, G. C. and Impey, C. S. 1986: Current progress in reducing salmonella colonization of poultry by "competitive exclusion". Journal of Applied Bacteriology, Symposium Supplement 1986, 67S-75S.

Mevel, G. 1979: Contrôle bactériologique quantitatif de l'eau de mer utilisée pour les élevages de larves de bivalves. Rapp. D.E.A. Océanographie biologique, Brest, 71pp. (miméo).

Mohney, L. L., D. V. Lightner and T. A. Bell. 1994: An epizootic of vibriosis in Ecuadorian pond-reared *Penaeus vannamei* Boone (Crustacea: Decapoda). Journal of the World Aquaculture Society. 25(1):116-125.

Momoyama, K., M. Hiraoka, H. Nakano, H. Koube, K. Inouye and N. Oseko. 1994: Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus* in Japan in 1993: Histopathological study. Fish Pathology. 29:141-148.

Mori, K. and J. E. Stewart. 1978: The hemolymph bactercidin of American lobster (*Homarus americanus*): adsorption and activation. Journal of the Fisheries Research Board of Canada. 35:1504-1507.

Moullac, G. L., .Aquacop, P. Roy and A. V. Wormhoudt. 1992: Effects of trophic and prophylactic factors on some digestive enzymatic activities of *Penaeus vannamei* larvae. Memorias I Congreso Ecuatoriano de Acuicultura. 1992:81-85.

Muir, P. R. and D. C. Sutton. 1994: Bacterial degradation of microencapsulated foods used in larval culture. Journal of the World Aquaculture Society. 25:371-378.

Murchelano, R. A., C. Brown and J. Bishop. 1975: Quantitative and qualitative studies of bacteria isolated from sea water used in the laboratory culture of the American oyster, *Crassostrea virginica*. Journal of the Fisheries Research Board of Canada. 32:738-745.

Murphy, K., C. Zaloum and D. Fuford. 1975: Effect of chlorination practice on soluble organics. Water Research. 9:389-396.

Nakano, H., H. Koube, S. Umezawa, K. Momoyama, M. Hiraoka, K. Inouye and N. Oseko. 1994: Mass mortalities of cultured kuruma shrimp, *Penaeus japonicus* in Japan in 1993: Epizootiological survey and infection trials. Fish Pathology. 29:135-139.

Nash, G., C. Nithimathachoke, C. Tungmandi, A. Arkarjamorn, P. Prathanpipat and P. Ruamthareesub. 1992: Vibriosis and its control in pond-reared *Penaeus monodon* in Thailand. In: Diseases in Asian Aquaculture 1. (Eds: Shariff, M., Subasinghe, R. P. and Arthur, J. R.). pp. 143-156. Fish Health Section, Asian Fisheries Society, Manila, Philippines.

Natori, S. 1987: Hemolymph proteins participating in the defence system of *Sarcophaga peregrina*. Molecular Entomology. :369-378.

Neihof, R. and G. Loeb. 1972: Molecular fouling of surfaces in seawater. In: Proceedings of the 3rd International congress on marine corrosion and fouling. (Ed: Gaithersburg, M. D.). pp. 710-718. 2 - 6 Oct. 1972.

New, M. B. 1997: The global emergence of tropical aquaculture. Aquaculture Europe 21(4): 41-49.

Nicholas, J. L., E. Robic and D. Ansquer. 1989a: Bacterial flora associated with a trophic chain consisting of microalgae, rotifers and turbot larvae: influence of bacteria on larval survival. Aquaculture. 83:237-248.

Nikl, L., T. P. T. Evelyn and L. J. Albright. 1993: Trials with an orally and immersion-administrated ß-1,3 glucan as an immunoprophylactic against *Aeromonas salmonicida* in juvenile chinook salmon *Oncorhynchus tshamytscha*. Diseases of Aquatic Organisms. 17:191-196.

Nimmo, D. R. and L. H. Bahner. 1974: Some physiological consequences of polychlorinated biphenyl and salinity stress in penaeid shrimp. In: Pollution and physiology of marine organisms. (Eds: Vernberg, F. J. and Vernberg, W. B.). pp. 427-443. Academic Press, New York.

Noga, E. J., T. A. Arroll, R. A. Bullis and L. Khoo. 1996: Antibacterial activity in hemolymph of white shrimp, *Penaeus setiferus*. Journal of Marine Biotechnology. 4:181-184.

Norqvist, A., A. Hagstom and H. Wolf-Watz. 1989: Protection of rainbow trout against vibriosis and furunculosis by the use of attenuated strains of *Vibrio anguillarum*. Applied and Environmental Microbiology. 55(6):1400-1405.

Oku, Y., Y. Uesaka, T. Hirayama and S. Hazanow. 1987. Development of highly sensitive bead -Elisa to detect bacterial toxins. Microbial Immunology. 32(8):807-816.

Olivier, G., T. P. T. Evelyn and R. Lallier. 1985: Immunogenicity of vaccines from a virulent and an avirulent strain of *Aeromonas salmonicida*. Journal of Fish Diseases. 8:43-55.

Olsson, J. C., A. Westerdahl, P. Conway and S. Kjelleberg. 1992: Intestinal colonisation potential of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*) associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology. 58:551-556.

Omori, S. A., G. G. Martin and E. J. Hose. 1989: Morphology of hemocyte lysis and clotting in the ridgeback prawn, *Sicyonia ingentis*. Cell and Tissue Research. 255:117-123.

Osborne, D. R. and P. Voogt. 1978: The analysis of nutrients in foods. Academic press, London. 251p.

Otte, G. and H. Rosenthal. 1979: Management of closed brackish-water system for high density fish culture by biological and chemical water treatment. Aquaculture. 18:169-181.

Ottogalli, L. 1991: Total substitution of microparticles for algae for *Penaeus stylirostris* larval rearing in New Caledonia. Journal of the World Aquaculture Society. 22:46A.

---. 1992: Nueva gestion del agua en las crias de Peneidos en Nueva Caledonia. Memorias I Congreso Ecuatoriano de Acuicultura. 1992:87-91.

Overstreet, R. M., D. V. Lightner, K. W. Hasson, S. McIlwain and J. M. Lotz. 1997: Susceptibility to Taura syndrome of some penaeid shrimp species native to the Gulf of Mexico and the south-eastern United States. Journal of Invertebrate Pathology. 69(2):165-176.

Owens, L., P. Muir, D. Sutton and M. Wingfield. 1992: The pathology of microbial diseases in tropical crustacea. In: Disease in Asian Aquaculture. (Eds: Shariff, M., Subasinghe, R. P. and Arthur, J. R.). pp. 165-172. Fish Health Section, Asian Fisheries Society, The Philippines.

Paller, M. H. and W. Lewis. M. 1988: Use of ozone and fluidized-bed biofilters for increased ammonia removal and fish loading rates. The Progressive Fish Culturist. 50:141-147.

Parker, R. B. 1974: Probiotics, the other half of the antibiotics story. Animal Nutrition and Health. 29:4-8.

Pedersen, K. 1982: Factors regulating microbial biofilm development in a system with slowly flowing seawater. Applied and Environmental Microbiology. 44(5):1196-1204.

Prayitno, S. B. 1994. Studies of bacteria causing prawn disease in Indonesia with special emphasis on luminous bacterial disease. Ph.D. thesis. University of Wales, Bangor. 250 pp.

Prayitno, S. B. and J. W. Latchford. 1995: Experimental infections of crustaceans with luminous bacteria related to Photobacterium and Vibrio: Effect of salinity and pH on infectiosity. Aquaculture. 132:105-112.

Prieur, D. and J. P. Caval. 1979: Bacteriological and physico-chemical analysis in a bivalve hatchery: techniques and preliminary results. Aquaculture. 17:359-374.

Primavera, J. H., C. R. Lavilla-Pitogo, J. M. Ladja and M. R. Dela-Pena. 1993: A survey of chemical and biological products used in intensive prawn farms in the Philippines. Marine Pollution Bulletin. 26(1):35-40.

Raa, J., G. Roerstad, R. Engstad and B. Robertsen. 1992: The use of immunostimulants to increase resistance of aquatic organisms to microbial infections. In: Diseases in Asian 1 Aquaculture. (Eds: Shariff, M., Subasinghe, R. P. and Arthur, J. R.). pp. 39-50. Fish Health Section, Asian Fisheries Society, The Philippines.

Ranga Rao, K. and D. G. Doughtie. 1984: Histopathological changes in grass shrimp exposed to chromium, pentachlorophenol and dithiocarbamates. Marine Environmental Research. 14:371-395.

Ratcliff, N. A., A. F. Rowley, S. W. Fitzgerald and C. P. Rhodes. 1985: Invertebrate immunity: basic concepts and recent advances. International Review of Cytology. 97:183-350.

Reichelt, J. L. and L. Baumann. 1973: Taxonomy of the marine luminous bacteria. Archives Microbiology. 94:283-330.

Reichelt, J. L. and M. A. Borowitzka. 1984: Antimicrobial activity from marine algae: Results of a large-scale screening programme. Hydrobiologia. 116/117:158-168.

Reid, B. and C. R. Arnold. 1992: The intensive culture of the penaeid shrimp *Penaeus vannamei* Boone in a recirculatory raceway system. Journal of the World Aquaculture Society. 23(2):146-153.

---. 1994: Use of ozone for water treatment in recirculating-water raceway systems. The Progressive Fish Culturist. 56:47-50.

Renwrantz, L. 1990: Internal defence system of *Mytilus edulis*. In: Studies in neuroscience, neurobiology of *Mytilus edulis*. (Ed: Stephano, G. B.). pp. 256-275. Manchester university press, Manchester.

Renwrantz, L. and A. Stahmer. 1983: Opsonizing properties of an isolated hemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of hemocytes from *Mytilus edulis*. Journal of Comparative Physiology. 149:535-546.

Rikjers, G. T., R. van Oosterom and W. B. van Muiswinkel. 1981: The immune system of cyprinid fish. Oxytetracycline and the regulation of humoral immunity in carp (*Cyprinus carpio*). Veterinary Immunology and Immunopathology. 2:281-290.

Rivers, T. M. 1937: Viruses and Koch's postulate. Journal of Bacteriology. 33:1-12.

Robertsen, B., G. RÝrstad, R. Engstad and J. Raa. 1990: Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. Journal of Fish Diseases. 13:391-400.

Rosenberry, B. 1988: World shrimp farming. Aquaculture Digest. (Ed: Rosenberry, B). pp. 1-24. San Diego, U.S.A.

Ruangpan, L. 1982: Diseases and parasites of *P. monodon* Fabricus. In: Asian fish health bibliography and abstracts 1: South east Asia. (Ed: Arthur, J. R.). pp. 188. Fish Health Section, Asian Fisheries Society, The Philippines.

Sae-oui, D., A. Tansutapanit and L. Ruangpan. 1987: *Vibrio harveyi* a causative agent from white shrimp nauplii, *Penaeus merguiensis*. In: Asian fish health bibliography and abstracts. (Ed: Arthur, J. R.). pp. 190. . Fish Health Section special publications, Asian Fisheries Society, The Philippines.

Sagua, V. O. 1980: Observation on the ecology and some aspects of the reproductive biology of the small white shrimp *Palaemon hastatus* Aurivillus (Crustacea: Palaemonidae) in the Lagos area, Nigeria. Bulletin de II.F.A.N. 42(2):280-295.

Sahul Hameed, A. S., P. V. Rao, J. J. Farmer, F. W. Hickman-Brenner and G. R. Fanning. 1996: Characteristics and pathogenicity of a *Vibrio campbellii*-like bacterium affecting hatchery-reared *Penaeus indicus* (Milne Edwards, 1937) larvae. Aquaculture Research. 27:853-863.

Salvesen, I., J. Skjermo and O. Vadstein. 1995: Application of microbially matured water during first feeding of turbot (*Scophthalmus maximus* L.). In: Larvi '95-Fish and shellfish larviculture symposium. (Eds: Lavens, P., Jaspers, E. and Roelants, I.). pp. 484-487. European Aquaculture Society, special publication No.24, Gent, Belgium.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989: Molecular cloning, A laboratory manual. Cold Spring Harbour Laboratory Press, New York. F9.

Samuelsen, O. B. 1989: Degradation of oxytetracycline in seawater at two different temperatures and light intensities, and the persistence of oxytetracycline in the sediment from a fish farm. Aquaculture. 83:7-16.

Samuelsen, O. B., B. T. Lunestad, A. Ervik and S. Fjelde. 1994: Stability of antibacterial agents in an artificial marine aquaculture sediment studied under laboratory conditions. Aquaculture. 126:283-290.

Schleifer, J. H. 1985: A review of the efficiency and mechanism of competitive exclusion for the control of salmonella in poultry. World's Poultry Science Journal. 41:72-83.

Schwinghamer, E. A. and J. Rockwell. 1978: Competitive advantage of bacteriocin and phage-producing strains of *Rhizobium trifolli* in mixed culture. Soil Biology and Biochemistry. 10:303-371.

Shewbart, K. L., W. L. Mies and P. D. Ludwig. 1972: Identification and quantitative analysis of the amino acids present in protein of the brown shrimp, *Penaeus aztecus*. Marine Biology. 16:64-67.

Shihabi, Z. and D. Dyer. 1988: Protein analysis with bicinchoninic acid. Annals of Clinical and Laboratory Science. 18(3):235-239.

Skjermo, J., I. Salvesen, G. Øie, Y. Olsen and O. Vadstein. 1997: Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. Aquaculture International. 5:13-28.

Smith, L. L., P. G. Lee, A. L. Lawrence and K. Strawn. 1985: Growth and digestibility by the three sizes of *Penaeus vannamei* Boone: effects of dietary protein level and protein source. Aquaculture. 46:85-96.

Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartener, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk. 1985: Measurement of protein using bicinchoninic acid. Analytical Biochemistry. 150:76-85.

Smith, V. J. and J. R. S. Chisholm. 1992: Non-cellular immunity in crustaceans. Fish and Shellfish

Immunology. 2:1-31.

Smith, V. J. and K. Söderhäll. 1983: ß-1,3 glucan activation of crustacean hemocytes *in vitro* and *in vivo*. Biological Bulletin. 164:299-314.

Söderhäll, K. 1982: Prophenoloxidase activating system and melanization - A recognition mechanism of arthropods? A review. Developmental and Comparative Immunology. 6:601-611.

Söderhäll, K. and L. Cerenius. 1992: Crustacean Immunity. Annual Review of Fish Diseases. :3-23.

Söderhäll, K. and T. Unestam. 1979: Activation of serum prophenoloxidase in arthropod immunity. The specificity of cell wall glucan activation and activation by purified fungal glycoproteins of crayfish phenoloxidase. Canadian Journal of Microbiology. 25:406-414.

Söderhäll, K., V. J. Smith and M. W. Johansson. 1986: Exocytosis and uptake of bacteria by isolated haemocyte populations of two crustaceans: evidence for cellular co-operation in the defence reactions of arthropods. Cell and Tissue Research. 245:43-49.

Sokal, R. R. and Rohlf, F. J. 1995. Biometry. The principles and practice of statistics in biological research. Third edition. W. H. Freeman and Company. New York. 887pp.

Song, Y. and Y. Hsieh. 1994: Immunostimulation of tiger shrimp (*Penaeus monodon*) hemocytes for generation of microbicidal substances. Analysis of reactive oxygen species. Developmental and Comparative Immunology. 18(3):201-209.

Song, Y. L. and H. H. Sung. 1990: Enhancement of growth in tiger shrimp (*Penaeus monodon*) by bacterin prepared from *Vibrio vulnificus*. Bulletin of the European Association of Fish Pathology. 10:98-99.

Srinivasulu Reddy, M. and K. V. Ramana Rao. 1988: Effects of technical and commercial grade phophamidon on the carbohydrate metabolism in selected tissues of penaeid prawn, *Metapenaeus monoceros* (Fabricus). Bulletin of Environmental Contamination and Toxicology. 40:389-395.

---. 1991: Methylparathion, carbaryl and aldrin impact on nitrogen metabolism of prawn, *Penaeus indicus*. Biochemistry International. 23:389-396.

Srinivasulu Reddy, M., P. Jayaprada and K. V. Ramana Rao. 1991: Toxic impact of Aldrin on acid and alkaline phosphatase activity of penaeid prawn, *Metapenaeus monoceros: In vitro* study. Bulletin of Environmental Contamination and Toxicology. 46:479-484.

Sunaryanto, A. and A. Mariam. 1986: Occurrence of a pathogenic bacteria causing luminescence in penaeid larvae in Indonesian hatcheries. Bulletin of the Brackishwater Aquaculture Development Centre. 8(2):64-70.

Sung, Hung-H. and Yen-L. Song. 1996: Tissue location of *Vibrio* antigen delivered by immersion to tiger shrimp (*Penaeus monodon*). Aquaculture. 145:41-54.

Sung, H. H., Y. L. Song and G. H. Kou. 1991: Potential uses of bacterin to prevent shrimp vibriosis. Fish and Shellfish Immunology. 1:311-312.

Sung, H. H., Y. L. Yang and Y. L. Song. 1996: Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulation. Journal of Crustacean Biology. 16(2):278-284.

Switzer, R. C. 111,, C. R. Merril, and S. Shifrin. 1979: A highly sensitive silver stain for detecting proteins and peptides in polyacrylamide gels. Analytical Biochemistry. 98:231.

Takahashi, Y., H. Nagoya and K. Momoyama. 1984: Pathogenicity and characteristics of *Vibrio sp.* isolated from diseased postlarvae of kuruma prawn, *Penaeus japonicus* Bate. Journal of the Shimonoseki University of Fisheries. 32:23-31.

Takahashi, Y., T. Itami, A. Nakagawa, H. Nishimura and T. Abe. 1985a: Therapeutic effects of oxytetracycline trial tablets against vibriosis in cultured kuruma prawns *Penaeus japonicus* Bate. Bulletin of the Japanese Society of Scientific Fisheries. 51:1639-1643.

Takahashi, Y., Y. Shimoyama and K. Momoyama. 1985b: Pathogenicity and characteristics of *Vibrio sp* isolated from diseased postlarvae of Kuruma prawn, *Penaeus japonicus* Bate. Bulletin of the Japanese Society of Scientific Fisheries. 51:721-730.

Tangtrongpiros, J., S. Piyatratitivorakul, R. Rattanaphanee, N. Chaisiri, V. Panichkriengkrai, S. Kanchanapangka, S. Srichairat, N. Chansue, A. Tawatsin, W. Koeypudsa, P. Ruantongee and S. Rungsupa. 1997: Effects of methyl parathion on shellfish culture important to the economy of Thailand. In: Shrimp biotechnology in Thailand. BIOTEC publication 2/2540 (Eds: Flegel, T. W., Menasveta, P. and Paisrnrat, S.). pp. 229-244. National Centre for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand.

Teshima, S. I., H. J. Ceccaldi, J. Patrois and A. Kanazawa. 1975: Bioconversion of demosterol to cholesterol at various stages of the molting cycle in *Palaemon serratus* Pennant, Crustacea Decapoda. Comparative Biochemistry and Physiology. 50(B):485-489.

Teshima, S. I., A. Kanazawa and S. Koshio. 1989: Nutritive value of sitosterol for the prawn *Penaeus japonicus*. Nippon Suisan Gakkaishi. 55:153-157.

Thrusfield, M. 1986: Veterinary epidemiology. Butterworths, London. 260pp.

Trust, T. J., W. W. Kay, E. E. Ishiguro, J. T. Buckley and T. W. Pearson. 1982: Properties of A-protein, a virulence factor on the surface of *Aeromonas salmonicida*. In: Immunology and immunization of fish. (Eds: van Muiswinkel, W. B. and Cooper, E. L.). pp. 178-180. Developmental and Comparative Immunology, Supplement 2.

Utting, S. D. and M. M. Helm. 1985: Improvement of sea water quality by physical and chemical pre-treatment in a bivalve hatchery. Aquaculture. 44:133-144.

Vaara, M. 1992: Agents that increase the permeability of the outer membrane. Microbiological Review. 56:395-411.

Vanderzant, C., R. Nickelson and P. W. Judkins. 1971: Microbial flora of pond-reared shrimp *Penaeus aztecus*. Applied Microbiology. 21:916-921.

Vargas-Albores, F. 1995: The defence system of brown shrimp (*Penaeus californiensis*): Humoral recognition and cellular responses. Journal of Marine Biotechnology. 1995:153-156.

Vargas-Albores, F., María-A. Guzmán and José-L. Ochoa. 1993a: An anticoagulant solution for haemolymph collection and prophenoloxidase studies of penaeid shrimp (*Penaeus californiensis*). Comparative Biochemistry and Physiology. 16A:299-303.

---. 1993b: A lippolysaccharide-binding agglutinin isolated from brown shrimp (*Penaeus californiensis* Holmes) haemolymph. Comparative Biochemistry and Physiology. 104B(2):407-413.

Vasta, G. R. 1987: Serum and hemocyte-associated lectins of the oyster *Crassostrea virginica*. In: Lectins. (Eds: Býg-hansen, T. C. and van Driessche, E.). pp. 677. Walter de Gruyter, New York.

Verdonck, L., J. Swings and K. Kersters. 1994: Variability of the microbial environment of rotifer *Brachionis plicatilis* and *Artemia* production systems. Journal of the World Aquaculture Society. 25(1):55-59.

Ward, R. W. and G. M. DeGraeve. 1978: Residual toxicity of several disinfectants in domestic wastewater. Journal. Water Pollution Control Federation. 50:46-60.

Wardell, J. N., N. S. Battersby and D. J. Stewart. 1986: A note on the control of sulphate-reducing bacteria

in seawater by u.v. irradiation. Journal of Applied Bacteriology. 60:73-76.

Wardlaw, A. C. and S. E. Unkles. 1978: Bactericidal activity of coelomic fluid from the sea urchin *Echinus esculentus*. Journal of Invertebrate Pathology. 32:25-34.

Wedemeyer, R. C., N. C. Nelson and W. T. Yasutake. 1982: Physiological and biochemical aspects of ozone toxicity to rainbow trout (*Salmo gairdneri*). Journal of the Fisheries Research Board of Canada. 36:605-614.

Weeks-Perkins, B. A., N. Chansue and D. Wong-Verelle. 1995: Assays of immune function in shrimp phagocytes: Techniques used as indicators of pesticide exposure. In: Techniques in fish immunology-4, Immunology and pathology of aquatic invertebrates. (Eds: Stolen, J. S., Fletcher, T. C., Smith, S. A., Zelikoff, S., Kaattari, S. L., Anderson, R. S., Söderhäll, K. and Weeks-Perkins, B. A.). pp. 223-231. SOS Publications, Fair Haven, NJ, USA.

Weichart, D., J. D. Oliver and S. Kjellebeg. 1992: Low temperature induced nonculturability and killing of *Vibrio vulnificus*. FEMS Microbiology Letters. 100:205-210.

Williams, R. R., T. A. Bell and D. V. Lightner. 1992: Shrimp antimicrobial testing 11. Toxicity testing and safety determination for twelve antimicrobials with penaeid shrimp larvae. Journal of Aquatic Animal Health. 4:262-270.

Yasuda, K. and T. Kitao. 1980: Bacterial flora in the digestive tract of prawns, *Penaeus japonicus* Bate. Aquaculture. 19:229-234.

Yazawa, K., K. Araki, K. Watanabe, C. Ishikawa, A. Inove, K. Kundo, S. Watabe, K. Hashimoto. 1988: Eicosapentanoic acid productivity of bacteria isolated from fish intestines. Nippon Suisan Gakkaishi. 54(10):1835-1838.

Yoshida, H. and M. Ashida. 1986: Microbial activation of two serine enzymes and propenoloxidase in the plasma fraction of hemolymph of the silkworm, *Bombyx mori*. Insect Biochemistry. 16(3):539-545.

Ziebell, R., F. P. Imray and D. G. Macphee. 1977: DNA Degradation in wild-type or repair-deficient strains of *Salmonella typhimurium* exposed to ultraviolet light or photodynamic treatment. Journal of General Microbiology. 101:143-149.

ZoBell, C. E. 1943: The effect of solid surfaces upon bacterial activity. Journal of Bacteriology. 46:39-56.

APPENDICES

Appendix 3.1:Log TVC.ml^{-1} of bacteria found in raw sea water (RSW) after various pre-treatments

Treatment	*Log TVC O_3 (0h)**	*oh*	*24h*	*48h*	*72h*
RSW*		4.81954			
		4.93450			
		4.97313			
0_3	3.40312	5.01703	6.42488	5.99388	5.35793
0_3	2.61278	5.09691	6.12385	4.92428	5.59550
0_3	1.47712	5.13033	6.44404	6.15836	5.34242
UVT		3.64345	6.71265	6.69020	6.37658
UVT		3.18184	6.67852	6.65706	6.20952
UVT		3.34242	6.57054	6.69723	6.43775
5µm		4.19312	5.91593	4.50515	4.95424
5µm		4.10721	6.00000	4.50515	5.22531
5µm		4.18752	5.96473	4.55630	5.47422
Auto			6.43136	6.81823	7.07188
Auto			6.50106	6.63649	6.40824
Auto			6.46835	5.95904	5.84386
Auto+Live	1.88081[a*]	4.03941**	6.19033	6.39093	6.84261
Auto+Live	2.00217[a*]	4.07918**	5.93852	6.32015	6.73719
Auto+Live	1.77085[a*]	4.06070**	6.08279	6.33646	6.83506

- 0_3 = ozonated water fed MED
- UVT = Ultraviolet light irradiated water fed MED
- 5µm = 5µm filtered seawater fed MED
- Auto = Autoclaved sea water fed MED
- Auto + Live feed = autoclaved sea water fed mixed algae and *Artemia*.
- The 0_3 treatment was air-stripped for 24h to remove residual 0_3 and TVC immediately after treatment was identified by *. Concentration of bacteria in mixed algal cultures added to the auto+live feed treatment at 0h are given by **. Concentration of bacteria added with the rinsed *Artemia* nauplii to the Auto+live feed treatment at 24h is given by[a*].

Appendix 3.1a: Upper tailed two sample T-Test and confidence intervals (C.I.) on log TVC obtained in raw sea water (RSW) after 5µm filtration (5µmSW)

95% C.I. for mean 1 - mean 2: (0.575, 0.918 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1. RSW	3	4.9091	0.0799	0.046
2. 5µmSW	3	4.1626	0.0481	0.028

T-Test mean 1= mean 2 (vs >): T= 13.87 P=0.0004 DF= 3

Appendix 3.1b: Analysis of variance on TVC of bacteria present per ml in 5µm filtered sea water (5µmSW) samples which had been ozonated (O_3) or UV light treated (UVT). Controls were with 5µmSW. TVC was taken immediately after the different pre-treatment methods were performed.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	329627712	16481386	75.83	<0.001
Error	6	13041425	2173571		
Total	8	342669152			

Treatment	*Code*	*N*	*Mean*	*StDev*
0_3	1	3	990	1347
UVT	2	3	2707	1505
5µmSW	3	3	14600	1562

Tukey's pairwise comparisons

Row		Code.	Code,	Diffmean	Low_ci	Up_ci
O_3	1	1	2	-1717	-5411.2	1977.16
UVT	2	1	3	-13610	-17304.2*	-9915.84*
5µm	3	2	3	-11893	-15587.2*	-8198.84*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.1c: Analysis of variance on TVC of bacteria present per ml in 5µm filtered sea water (5µmSW) samples which had been ozonated (O_3) or UV light induced (UVT). Controls were with 5µmSW. TVC was taken after residual O_3 had been air stripped for 24h and immediately after the different pre-treatment methods were performed for the other treatments.

Source	DF	SS	MS	F	P
Treatment	2	4.3058	2.1529	106.55	<0.001
Error	6	0.1212	0.0202		
Total	8	4.4270			

Treatment	Code	N	Mean	StDev
O_3	1	3	5.0814	0.0582
UVT	2	3	3.3892	0.2343
5µmSW	3	3	4.1626	0.0481

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1. O_3	1	2	1.6922	1.33607*	2.04833*
2.UVT	1	3	0.9188	0.56267*	1.27493*
3. 5µmSW	2	3	-0.7734	-1.12953*	-0.41727*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.1d: Analysis of variance on TVC of bacteria present per ml in AuW, UVT, O_3 or 5µmSW, 24h after addition of 4mg.ml^{-1} of MED. AuW +live feed treatments were fed mixed algae with the concentrations of added bacteria as shown in Appendix 3.1.

Source	DF	SS	MS	F	p
Treatment	4	0.9663	0.2416	21.30	<0.001
Error	10	0.1134	0.0113		
Total	14	1.0798			

Treatment	code	N	Mean	StDev
O_3	1	3	6.3309	0.1796
UVT	2	3	6.6539	0.0742
5µmSW	3	3	5.9602	0.0422
Auto	4	3	6.4669	0.0349
Auto+live	5	3	6.0705	0.1264

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1.	1	2	-0.323000	-0.608385*	-0.037615*
2	1	3	0.370700	0.085315*	0.656086*
3	1	4	-0.136000	-0.421385	0.149386
4	1	5	0.260400	-0.024985	0.545786
5	2	3	0.693700	0.408315*	0.979086*
6	2	4	0.187000	-0.098385	0.472386
7	2	5	0.583400	0.298015*	0.868786*
8	3	4	-0.506700	-0.792085*	-0.221315*
9	3	5	-0.110300	-0.395685	0.175085
10	4	5	0.396400	0.111015*	0.681785*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.1e: Analysis of variance on TVC of bacteria present per ml in AuW, UVT, O_3 or 5µmSW, 48h after addition of 4mg.ml^{-1} of MED. AuW+live feed treatments were fed mixed algae at 0h and at 24h, 5nauplii.ml^{-1} of Artemia were added. The concentrations of bacteria added with the live diets are shown in Appendix 3.1.

Source	DF	SS	MS	F	p	Level	code	N	Mean	StDev

Treatment	4	9.213	2.303	17.54	<0.001	0_3	1	3	5.6922	0.6701
Error	10	1.313	0.131			UVT	2	3	6.6815	0.0215
Total	14	10.526				5µmSW	3	3	4.5222	0.0295
						Auto	4	3	6.4713	0.4528
						Auto+live	5	3	6.3492	0.0371

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.98930	-1.96099*	-0.01761*
2	1	3	1.17000	0.19831*	2.14169*
3	1	4	-0.77910	-1.75079	0.19259
4	1	5	-0.65700	-1.62869	0.31469
5	2	3	2.15930	1.18761*	3.13099*
6	2	4	0.21020	-0.76149	1.18189
7	2	5	0.33230	-0.63939	1.30399
8	3	4	-1.94910	-2.92079*	-0.97741*
9	3	5	-1.82700	-2.79869*	-0.85531*
10	4	5	0.12210	-0.84959*	1.09379*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.1f: Analysis of variance on TVC of bacteria present per ml in AuW, UVT, O_3 or 5µ mSW, 72h after addition of 4mg.ml^{-1} of MED. AuW+live feed treatments were fed mixed algae at oh and at 24h, 5nauplii.ml^{-1} of Artemia were added. The concentrations of bacteria added with the live diets are shown in Appendix 3.1.

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*code*	*N*	*Mean*	*StDev*
Treatment	4	5.6467	1.4117	14.61	<0.001	0_3	1	3	5.4320	0.1418
Error	10	0.9660	0.0966			UVT	2	3	6.3413	0.1181
Total	14	6.6127				5µmSW	3	3	5.2179	0.2601
						Auto	4	3	6.4413	0.6147
						Auto+live	5	3	6.8050	0.0588

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.90930	-1.74371*	-0.07489*
2	1	3	0.21410	-0.62031	1.04851
3	1	4	-1.00930	-1.84371*	-0.17489*
4	1	5	-1.37300	-2.20741*	-0.53859*
5	2	3	1.12340	0.28899*	1.95781*
6	2	4	-0.10000	-0.93441	0.73441
7	2	5	-0.46370	-1.29811	0.37071
8	3	4	-1.22340	-2.05781*	-0.38899*
9	3	5	-1.58710	-2.42151*	-0.75269*
10	4	5	-0.36370	-1.19811	0.47071

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.2: Morphological separation of bacteria from different sea water treatments at 0h and 24h after treatment.

Time	TVC.ml^{-1}	Replicate A	Replicate B	Replicate C	PVVC.ml^{-1}	Replicate A	Replicate B	Replicate C
RSW (0h)	Oblong, small cream dots	3.7 x 10^{6}	3.4 x 10^{6}	5.0 x 10^{6}	Bluish green, 1 - 1.55mm diameter circular dots	1 x 10^{1}	1 x 10^{1}	1 x 10^{1}
	circular, entire, medium sized translucent, smooth	4.0 x 10^{1}		2 x 10^{1}	lighter bluish green dot 0.85mm	1 x 10^{1}		

	Round, deep creamy dots, entire	5.4×10^{2}			Yellowish, circular, lobed, 2.90mm diameter		1×10^{1}	
	bright yellow, entire, small round dots	2×10^{1}	2.2×10^{2}	1.2×10^{2}	lighter bluish green, circular dot 1.0mm diameter		1×10^{1}	
	Cream, with oblong, deeper core	1×10^{1}		8×10^{1}	Dark green nucleus with wide, yellow halo, circular, curled, 8.80mm diameter			1×10^{1}
	irregular, creamy, smooth edges	1×10^{1}			Creamy, green, circular dot, 0.60mm diameter			3×10^{1}
	Bright yellow, round, medium sized, entire		1×10^{1}					
	translucent, creamy, circular, entire, medium size		8×10^{1}					
	deep creamy., circular, entire		9×10^{1}	1.2×10^{2}				
	circular, light creamy edges, wide brown core, smooth, entire		1×10^{1}	2×10^{1}				
	very light cream, round, entire		3.2×10^{2}					
	yellowish oblong dots			6×10^{1}				
	transparent, small, yellow dots			8×10^{1}				
	other small, creamy dots	4.61×10^{3}	6.39×10^{3}	1.2×10^{2}				
	Total	6.3×10^{3}	7.46×10^{3}	7.29×10^{3}		2×10^{2}	3×10^{2}	5×10^{2}
5µmSW (0h)	Big, round, Smooth, creamy	1×10^{1}		1.1×10^{2}	Yellow, circular, curled, 1.1mm diameter	1×10^{1}		
	circular, entire, medium sized transluscent, smooth	4×10^{1}	6×10^{1}	1.4×10^{2}	Bluish green circular, entire, dot, 0.8mm diameter	1×10^{1}		
	Irregular, light, creamy, filamentous edges	1×10^{1}	4×10^{1}		Large, round colony in centre of plate 34.35mm diameter, lobed		1×10^{1}	1×10^{1}
	Oblong, small cream dots	6×10^{2}	2.2×10^{2}	5.4×10^{2}	blue-green colony, irregular		1×10^{1}	
	translucently creamy, bean shaped with oblong, deeper core	4×10^{1}		1×10^{2}				
	Yellow, irregular, small	1×10^{1}	1×10^{1}					
	circular, light creamy edges, wide brown core, smooth, entire	1×10^{1}						
	other small, creamy dots	5.3×10^{3}	2.51×10^{3}	3.1×10^{2}				
	Yellowish creamy round dots		3×10^{1}					
	other dots		6×10^{2}	6.04×10^{3}				
	yellowish oblong dots			5×10^{1}				
	Total	6.02×10^{3}	3.48×10^{3}	7.33×10^{3}		2×10^{2}	2×10^{2}	1×10^{1}
O_3 (0h)	Light creamy dots, circular	4.10×10^{2}		2.6×10^{2}	Large, round colony in centre of plate, 22.7mm diameter, lobed		1×10^{1}	
	creamy, circular dots	2.8×10^{2}						

	Creamy, circular, entire, with three concentric rings	3.0×10^{1}						
	Yellow dots	6×10^{1}	3.4×10^{6}	1.7×10^{6}				
	Curled, curled, creamy	2×10^{1}	2×10^{1}					
	translucently creamy, bean shaped with oblong, deeper core	1×101	7×10^{1}	3×10^{1}				
	other small, creamy dots	1.77×10^{5}	3.6×10^{5}	3.5×10^{5}				
	Bright yellow, round, circular, medium sized, entire		1×10^{1}					
	circular, entire, medium sized translucently creamy smooth		7×10^{1}	7×10^{1}				
	Medium circular, entire, creamy		5×10^{1}	3×10^{1}				
	Small creamy, with one side curled and the other straight		1×10^{1}	3×10^{1}				
	Yellow, circular, eroded edges			1×10^{1}				
	Total	2.58×10^{5}	4.17×10^{5}	4.10×10^{5}			1×10^{1}	
UVT (0h)	Small, yellow, curled, curled	3×10^{1}		3×10^{1}				
	Bright yellow, round, circular, medium sized, entire	3×10^{1}						
	Light creamy, circular, entire	3×10^{1}	2×10^{1}	3×10^{1}				
	creamy circular dots	2.8×10^{6}	2.5×10^{6}	2.4×10^{6}				
	Light cream dots, circular	1.9×10^{6}		1×10^{6}				
	Transparent, small, yellow dots	1×10^{6}	2×10^{1}					
	creamy, curled, curled, small	1×10^{1}	1×10^{1}	1×10^{1}				
	Myceloid		2×10^{1}					
	Circular, entire, deep creamy, medium		2×101					
	translucently creamy, bean shaped with oblong, deeper core		1×101					
	Yellow, ellipse, small			1×10^{1}				
	Total	6.7×10^{6}	5.6×10^{6}	4.2×10^{6}				
RSW (24h)	Yellow, circular, entire	1×10^{5}			Yellow, small dots, colonies = 0.8- 2.7mm	3.2×10^{6}		4.7×10^{6}
	Cream, with slight yellowish tint, circular, entire, medium	1×10^{7}	4×10^{5}		Creamy, green colonies, irregular, large	3×10^{1}	2×10^{6}	
	translucent, light cream, circular, entire	1.4×10^{7}	6×10^{5}		Green, irregular, entire, 0.65 - 1.9mm diameter	2.1×10^{6}		
	Oblong, slightly yellowish cream dots	7.5×10^{7}			large, oblong, lobed, 41.1mm diameter	1×10^{1}		

	Glassy yellow dots	5×10^{5}	3.2×10^{4}	8×10^{5}	Yellow, irregular, smooth, 7.65mm		1×10^{2}	
	Yellow core with light cream umbra	1×10^{5}			yellow, circular, lobed, 1.6 - 2.2mm		3×10^{2}	
	other cream dots	1.15×10^{5}	1.16×10^{5}	7.76×10^{5}	Green, circular, entire. 4.6 - 6.2mm		1×10^{2}	1×10^{1}
	translucent, slightly creamy, circular	2.4×10^{4}		3×10^{5}	Yellow, kidney shaped, lobed, 2.15mm		1×10^{2}	
	Kidney shaped, creamy, with outer edge lighter coloured and inner darker	6×10^{5}			Yellow, irregular, lobed		1×10^{2}	
	slightly creamy, almost circular, entire		5×10^{5}	1×10^{5}	Yellow, large central colony, lobed		1×10^{2}	2×10^{1}
	Yellow oblong dots		1.54×10^{4}	4.2×10^{4}	Yellow, circular entire, 4.6 - 6.15mm			3×10^{1}
	Brownish, circular, entire		1×10^{5}		Green, circular entire, with transparent edges, 3.3 – 4.65mm			2×10^{1}
	Cream, circular, entire			7×10^{5}	Creamy green colony, circular, entire, 8.25mm			1×10^{1}
	Very light cream, (almost transparent)			5.10×10^{4}	green dots			1.1×10^{2}
	circular, entire, faint, small							
	Cream, small, irregular, wavy			1×10^{5}				
	Total	1.29×10^{5}	1.22×10^{5}	8.89×10^{5}		5.70×10^{2}	1.0×10^{5}	6.7×10^{2}
5µmSW (24h)	Orange, circular, entire, medium	2×10^{5}			Wide green cores with creamy edges, circular, entire, 5.7 - 10.45mm diameter	8×10^{1}	2×10^{2}	
	creamy, circular, entire	1.7×10^{4}	1.1×10^{4}	1.9×10^{4}	greenish cream colonies, more cream than green, circular, entire, 4.5- 6.5mm diameter	3×10^{1}		
	Faintly yellowish wide core with translucent umbra, circular, entire	1×10^{5}		3×10^{5}	Greenish cream, irregular, smooth, 9.45 - 11.67mm diameter	3×10^{1}	2×10^{2}	
	Creamy core with translucent umbra, circular, eroded	1×10^{5}			light greenish, circular, entire, 1.45 - 3.85mm diameter	1×10^{2}		1.5×10^{2}
	Transparent, creamy, circular, entire	2.2×10^{4}	1.2×10^{4}	2.2×10^{4}	Yellow colonies1.0 - 1.25mm diameter	5×10^{1}		
	three concentric circles of translucent creamy, translucent circular, entire	1×10^{5}			yellow dots smaller than 0.85mm	3×10^{1}		2.4×10^{2}
	brownish, circular, entire	3×10^{5}		2×10^{5}	Fainter, yellow colonies, circular, entire around 2.15 - 2.85mm	5×10^{1}		2×10^{1}
	transparent, irregular, inner core totally transparent, irregular, entire edges with some eroded areas	1×10^{5}			yellow, irregular, undulate around 3.75 - 4.85mm diameter	9×10^{1}	1×10^{2}	1×10^{1}
	creamy, similar to previous one	1×10^{5}			green, circular, entire, 0.9 - 17mm diameter	8×10^{1}	8×10^{2}	

	Yellowish, oblong dots	2.6 x 10^{7}	2 x 10^{5}/3.2 x 10^{4}	3.3 x 10^{7}	blue-green colonies around 0.85mm diameter	6 x 10^{1}		
	other cream dots	1.1 x 10^{6}	1.12 x 10^{6}	9.6 x 10^{5}	Light green colonies with transparent thin umbras, irregular, undulate	4 x 10^{1}	1 x 10^{4}	
	Light creamy, mid sized, circular, entire		1.1 x 10^{7}		Yellow colonies, circular, lobed, 2.25 - 4.3mm		1.1 x 10^{6}	2 x 10^{1}
	Light creamy dot		8.2 x 10^{7}	1.28 x 10^{6}	Yellow, oblong, from 0.85 - 1.7mm		4 x 10^{4}	
	slightly creamy, almost circular, entire		1.3 x 10^{7}		green dots		1.5 x 10^{6}	2.8 x 10^{4}
	cream, curled, undulate			2 x 10^{6}	Creamy green, curled, curled, 5.95 - 9.15mm			5 x 10^{1}
					Green, circular, curled			1 x 10^{1}
					Creamy green, small dots 0.65 - 1.35mm			2.3 x 10^{4}
					Yellow, circular, curled, with thin, creamy edge , Around 3.55mm			2 x 10^{1}
					Creamy yellow, almost circular, entire, (i.e, bean shaped) 3.25 - 4.4mm			3 x 10^{1}
	Total	1.19 x 10^{6}	1.27 x 10^{6}	1.17 x 10	Total	6.4 x 102	4.4 x 103	1.06 x 103
O_3 (24h)	yellowish, circular, undulate	1 x 10^{6}			Scattered around the edges, green and yellow	5 x 10^{4}	1.6 x 10^{6}	6.0 x 10^{4}
	cream, circular, entire	6 x 10^{6}	7 x 10^{6}					
	glassy cream, circular, lobate	1 x 10^{6}						
	transparent ream, circular, entire	3 x 10^{6}						
	creamy, kidney shaped. Oblong deeper core	4 x 10^{6}						
	oblong cream dots	3.8 x 10^{7}						
	translucent, very light creamy dots	3.2 x 10^{7}						
	Glassy, yellow dots	3.2 x 10^{7}	7 x 10^{6}					
	other creamy dot	4.04 x 10^{6}	2.7 x 10^{6}					
	Yellowish creamy, circular, curled		1 x 10^{6}					
	cream, curled, curled		1 x 10^{6}					
	Total	5.21 x 10^{6}	2.87 x 10^{6}	1.48 x 10^{6}		5 x 10^{4}	1.6 x 10^{6}	6.0 x 10^{4}
UVT (24h)	Creamy, circular, entire	1 x 10^{7}		8 x 10^{6}	Blue-green dots, 0.5 - 1.25mm	3.46 x 10^{6}	4.7 x 10^{6}	9.1 x 10^{6}
	Whitish, milky cream, circular, entire	1 x 10^{6}	1.7 x 104	5 x 103	Green, circular, entire, 2.7 - 6.0mm	2.6 x 10^{4}	1.9 x 10^{4}	
	Transparent, circular, entire	9 x 10^{6}	2.7 x 10^{7}	1.3 x 10^{7}	green, irregular	1.3 x 10^{4}	3 x 10^{1}	
	yellow dots	2 x 10^{6}	8 x 10^{6}	2 x 10^{6}	green, circular, with transparent Umbra, around 4.75 - 5.65mm	1 x 10^{1}	1 x 10^{1}	1 x 10^{4}
	other cream dots	7.88 x 10^{6}	9.57 x 10^{6}	2.64 x 10^{6}	yellow dots			7 x 10^{4}
	Yellow, circular, entire		3 x 10^{6}		Green, circular, entire 4.4 - 9.35mm			1.5 x 10^{6}
	creamy, concentrically ringed surface, circular, entire		3 x 10^{6}		Creamy, circular, curled, 5.8 - 9.00mm			4 x 10^{4}

	Transparent creamy dots		1.4 x 10^{4}	1.5 x 10^{4}				
	Creamy, circular, eroded		7 x 10^{3}	1.0 x 10^{3}				
	creamy, kidney shaped. Oblong deeper core			4 x 10^{3}				
	Total	8.10 x 10^{3}	1.03 x 10^{3}	3.12 x 10^{3}		3.86 x 10^{3}	4.93 x 10^{3}	1.18 x 10^{4}

Appendix 3.3: Survival of Penaeus indicus larvae fed on live or artificial diets and reared in 5μmSW, O_3 UVT or AuW pre-treated water samples to PL-1 stage

		Live diets				*Artificial diets*			
day	replicate	5μmSW	O_3	UVT	AuW	5μmSW	O_3	UVT	AuW
0	1	200	200	200	200	200	200	200	200
0	2	200	200	200	200	200	200	200	200
0	3	200	200	200	200	200	200	200	200
1	1	200	198	162	170	198	178	175	73
1	2	199	198	160	168	198	169	169	69
1	3	199	195	160	167	197	162	156	66
2	1	198	192	130	145	197	150	154	24
2	2	196	189	130	145	193	139	140	16
2	3	195	188	129	143	187	121	130	9
3	1	172	164	121	124	182	96	128	0
3	2	169	160	120	124	178	85	121	0
3	3	168	160	118	124	171	79	111	0
4	1	163	152	120	117	176	20	96	
4	2	161	150	120	116	169	15	86	
4	3	156	151	117	114	156	10	78	
5	1	148	132	112	114	152	0	64	
5	2	146	132	112	114	150	0	58	
5	3	141	130	111	113	139	0	50	
6	1	140	103	106	110	146		31	
6	2	137	103	105	108	140		22	
6	3	133	103	105	107	130		10	
7	1	128	101	104	104	138		30	
7	2	126	100	102	104	131		21	
7	3	125	100	102	100	118		10	
8	1	120	99			127		30	
8	2	120	99			124		20	
8	3	121	100			117		10	
9	1					124		29	
9	2					120		20	
9	3					110		10	
10	1							29	
10	2							20	
10	3							10	
11	1							29	
11	2							20	
11	3							9	
12	1							28	
12	2							19	
12	3							8	

***Appendix 3.3a: One-Way Analysis of Variance on the arc sine transformation of survival to PL-1 stage of* Penaeus indicus *larvae reared in water with different pre-treatments and fed on algae and* Artemia**

Source	*DF*	*SS*	*MS*	*F*	*P*
Treatment	3	0.0294502	0.0098167	157.23	<0.001
Error	8	0.0004995	0.0000624		
Total	11	0.0299497			

Treatment	*Code*	*N*	*Mean*	*StDev*
5µmSW	1	3	0.64559	0.00362
O_3	2	3	0.51976	0.00333
UVT	3	3	0.53907	0.00674
AuW	4	3	0.53910	0.01342

Tukey's pairwise comparisons (units = arc sine %)

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.125830	0.105170*	0.146490*
2	1	3	0.106520	0.085860*	0.127180*
3	1	4	0.106490	0.085830*	0.127150*
4	2	3	-0.019310	-0.039970	0.001350
5	2	4	-0.019340	-0.040000	0.001320
6	3	4	-0.000030	-0.020690	0.020630

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

***Appendix 3.3b: One-Way Analysis of Variance on the arc sine transformation of percentage survival of Penaeus indicus larvae after 5days rearing in water with different pretreatments. Larvae were fed live diets (mixed algae and* Artemia)**

Source	*DF*	*SS*	*MS*	*F*	*P*
Treatments	2	0.0280570	0.0140285	508.49	<0.001
Error	6	0.0001655	0.0000276		
Total	8	0.0282226			

Level	*Code*	*N*	*Mean*	*StDev*
0_3	1	3	0.71641	0.00764
UV	2	3	0.59238	0.00348
AuW	3	3	0.60448	0.00351

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.124030	0.110866*	0.137194*
2	1	3	0.111930	0.098766*	0.125094*
3	2	3	-0.012100	-0.025264	0.001064

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.3c: One-way analysis of variance on the arc sine transformation of survival to PL-1 stage of Penaeus indicus larvae fed on MED and reared in water with different pre-treatments

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	2.25960	0.75320	108.54	<0.001
Error	8	0.05551	0.00694		
Total	11	2.31511			

Level	*Code*	*N*	*Mean*	*StDev*
5µmSW	1	3	1.3037	0.0946
O_3	2	3	0.7554	0.0998
UVT	3	3	0.7873	0.0862
AuW	4	3	0.0818	0.0377

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.5483	0.33042*	0.76618*
2	1	3	0.5164	0.29852*	0.73428*
3	1	4	1.2219	1.00402*	1.43978*
4	2	3	-0.0319	-0.24978	0.18598
5	2	4	0.6736	0.45572*	0.89148*
6	3	4	0.7055	0.48762*	0.92338*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.3d: Upper tailed two sample T-Test and confidence intervals on survival of Penaeus indicus larvae reared in 5µmSW and UVT treated water to PL-1 stage

95% C.I. for mu 1 - mu 2: (0.432, 0.647 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1. 5umSW	3	0.6315	0.0444	0.026
2. UVT	3	0.0919	0.0503	0.029

T-Test mu 1 = mu 2 (vs >): T= 13.93 P=0.0001 DF= 4

Appendix 3.3e: Upper tailed two sample T-Test and confidence intervals for survival of Penaeus indicus larvae reared in 5µm filtered water and fed either live or artificial diets

95% C.I. for mu 1 - mu 2: (-0.0966, 0.125 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1. Live diets	3	0.64559	0.00362	0.0021
2. Artificial diets	3	0.6315	0.0444	0.026

T-Test mu 1 = mu 2 (vs >): T= 0.55 P=0.32 DF= 2

Appendix 3.4: Survival to M1 stage and increase in lengths (mm) of Penaeus indicus larvae reared in 5µm filtered sea water (SW) and autoclaved sea water (AuW). Larvae were fed either mixed algae (A) or MED (CAR) with (+B) or without (-B) added bacteria which had been filtered from axenic algal cultures

		LENGTHS (mm)						
Treatment	*survival*	*AuWA+B*	*SWA+B*	*AuWA-B*	*SWA-B*	*SWCAR*	*SWCAR+B*	*Start*
AuA+B	198	2.56	3.64	3.64	2.65	1.52	2.28	0.92
AuA+B	171	2.56	3.56	3.68	3.00	1.54	1.80	0.92
AuA+B	187	2.84	3.24	3.44	3.16	1.60	2.20	0.90
SWA+B	176	2.76	3.32	3.44	3.36	1.70	1.64	0.86
SWA+B	177	3.32	3.60	3.40	3.16	1.80	1.68	0.90
SWA+B	177	3.68	3.64	3.48	3.20	1.64	2.24	0.82
AuA-B	198	2.72	2.68	3.52	3.20	1.74	1.56	0.88
AuA-B	179	2.68	3.72	3.48	3.08	1.74	2.16	0.82
AuA-B	188	2.84	3.56	3.32	2.84	1.78	1.68	0.84
SWA-B	181	2.64	3.36	3.20	3.52		1.60	
SWA-B	181	2.72	3.60	2.60	3.00		2.48	
SWA-B	184	2.52	3.56	2.88	3.44		2.56	
SWCAR-B	7	3.20	3.44	2.76	3.24		2.60	
SWCAR-B	1	2.56	2.88	3.24	3.16		2.32	
SWCAR-B	10	2.68	3.60	3.24	3.08		2.56	
SWCAR+B	151	2.68	3.56	3.48	3.28		2.48	
SWCAR+B	124	2.60	3.40	3.28	3.20		1.72	
SWCAR+B	11	2.68	3.52	3.36	3.44		2.52	
AuWAR-B	0	2.32	3.60	3.44	3.40		2.64	
AuCAR-B	0	3.28	2.52	3.24	3.32		1.68	
AuCAR-B	0	3.32	3.40	3.24	3.48		2.28	
AuCAR+B	0	3.36	3.32	3.48	3.32		2.36	
AuCAR+B	0	2.32	3.56	3.08	3.60		2.36	
AuCAR+B	0	3.56	3.48	3.04	3.64		1.88	
		3.60	3.44	3.44	3.52		1.84	
		2.64	3.36	2.52	3.12			
		2.60	2.68	2.76	3.32			
		3.04	3.28	3.36	3.40			

		3.32	3.40	3.40	3.60
		2.64		3.44	3.28

Appendix 3.4a: Upper tailed two sample T-Test and confidence intervals for survival of Penaeus indicus larvae to M1 stage when reared in 5µmSW and fed on mixed algae with (SWA+B) or without (SWA-B) added bacteria filtered from the algae.

95% C.I. for mu 1 - mu 2: (-0.0960, -0.025 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1. SWA+B	3	1.08295	0.00614	0.0035
2. SWA-B	3	1.1436	0.0212	0.012

T-Test mu 1 = mu 2 (vs >): T= -4.76 P=1.0 DF= 4

Appendix 3.4b: Upper tailed two sample T-Test and confidence intervals for survival of Penaeus indicus larvae to M1 stage when reared in autoclaved water and fed on mixed algae with (AA+B) or without (AA-B) added bacteria filtered from the algae.

95% C.I. for mu 1 - mu 2: (-0.45, 0.384 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1. AA+B	3	1.221	0.202	0.12
2. AA-B	3	1.253	0.163	0.094

T-Test mu 1 = mu 2 (vs >): T= -0.22 P=0.58 DF= 4

Appendix 3.4c: Two sample T - tests and confidence intervals on the Arcsine transformations of percentage larval survival of M1 stage Penaeus indicus reared in 5µm filtered sea water and fed on CAR with (+B) or without (-B) added bacteria filtered from axenic agal cultures.

95% C.I. for mu 1 - mu 2: (-1.538, 0.55 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE*
1 (-B)	3	0.0300	0.0229	0.013
2 (+B)	3	0.526	0.419	0.24

T-Test mu 1 = mu 2 (vs <): T= -2.05 P=0.088 DF= 2

Appendix 3.5a: Two sample Mann-Whitney Test and confidence intervals on final lengths of Penaeus indicus larvae fed on mixed, axenic algae and reared in 5µm filtered (SWA+B) or autoclaved (AuWA+B) sea water

95.0 Percent C.I. for ETA1-ETA2 is (0.2800 to 0.7999mm)

S.W.A+B	*N* = *29*	*Median* = *3.44*
AuWA+B	N = 30	Median = 2.70

Point estimate for ETA1-ETA2 is 0.6400
W = 1158.5
Test of ETA1 = ETA2 vs. ETA1 > ETA2 is significant at 0.0000
The test is significant at 0.0000 (adjusted for ties)

Appendix 3.5b: Two sample Mann-Whitney Test and confidence intervals for final lengths of Penaeus indicus larvae fed on algae which had been 3µm filtered and rinsed with sterile saline to remove the associated bacteria and reared in 5µm filtered (SWA-B) and autoclaved (AA-B) sea water

95.2 Percent C.I. for ETA1-ETA2 is (-0.1600 to 0.0801mm)

S.W.A	*B*	*N*	=	*30*	*Median*	=	*3.28*
AutoA	B	N	=	30	Median	=	3.36

Point estimate for ETA1-ETA2 is -0.0400
W = 874.5
Test of ETA1 = ETA2 vs. ETA1 > ETA2
Cannot reject since W is < 915.0

Appendix 3.5c: Two sample Mann-Whitney Test and Confidence Interval on final lengths of Penaeus indicus larvae fed on MED and raised in 5µm filtered sea water with (SWMED+B) or without (SWMED-B) added bacteria from mixed algal cultures

95.4 Percent C.I. for ETA1-ETA2 is (0.0999 to 0.7601mm)

SWCAR+B	*N*	=	*25*	*Median*	=	*2.24*
S.W.CAR	N	=	9	Median	=	1.70

Point estimate for ETA1-ETA2 is 0.5400
W = 511.5
Test of ETA1 = ETA2 vs. ETA1 > ETA2 is significant at 0.0021
The test is significant at 0.0020 (adjusted for ties)

Appendix 3.6: Final TVC and PVVC concentrations (cfu.ml^{-1}) obtained when P. indicus PZ1 stage larvae were reared to M1 stage in either 5µm filtered (5µmSW) or autoclaved (AuW) sea water and fed on mixed algae or a microencapsulated diet (MED, with (+Bact) or without (-Bact) the addition of bacteria filtered from algal cultures..

Water treatment	*Diet*	*TVC (cfu.ml^{-1})*	*PVVC (cfu.ml^{-1})*
AuW	Algae + Bact	2.34×10^5	2.95×10^3
		1.56×10^5	1.37×10^3
		3.35×10^5	2.89×10^3
AuW	Algae - Bact.	2.47×10^5	3.09×10^3
		3.62×10^5	2.58×10^3
		2.42×10^5	1.96×10^3
AuW	MED-Bact	2.21×10^6	*
		1.35×10^6	6.50×10^3
		1.52×10^6	5.70×10^3
AuW	MED + Bact	1.35×10^6	1.28×10^3
		3.06×10^5	2.49×10^3
		2.55×10^6	2.93×10^3
5µmSW	Algae + Bact	8.18×10^4	4.23×10^3
		1.17×10^5	5.74×10^3
		2.37×10^5	2.26×10^3
5µmSW	Algae - Bact	7.66×10^4	2.34×10^3
		1.19×10^5	1.58×10^3
		4.14×10^4	1.15×10^3
5µmSW	MED - Bact	1.89×10^5	4.7×10^2
		*	*
		6.78×10^5	2.55×10^2
5µmSW	MED + Bact	5.75×10^5	8.5×10^1
		9.16×10^4	7.1×10^2
		1.67×10^6	4.85×10^3

Appendix 3.6a: One-way analysis of variance on log TVC .ml^{-1} obtained in larval cultures raised in autoclaved (AuW) or 5µm filtered (SW) sea water fed with algae (A) or artificial (MED) diets and with (+B) or without (-B) added bacteria obtained from mixed algal cultures.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	7	4.118	0.588	5.43	0.002
Error	16	1.733	0.108		
Total	23	5.851			

Treatment	*code*	*N*	*Mean*	*StDev*
AuA+B	1	3	5.3623	0.1662
AuA-B	2	3	5.4454	0.0985
AuMED+B	3	3	6.0071	0.4718
AuMED-B	4	3	6.2186	0.1120
SWA+B	5	3	5.1192	0.2346
SWA-B	6	3	4.8589	0.2303
SWMED-B	7	3	5.5801	0.2813
SWMED+B	8	3	5.6481	0.6378

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.08310	-1.01281	0.84661
2	1	3	-0.64480	-1.57451	0.28491
3	1	4	-0.85630	-1.78601	0.07341
4	1	5	0.24310	-0.68661	1.17281
5	1	6	0.50340	-0.42631	1.43311
6	1	7	-0.21780	-1.14751	0.71191
7	1	8	-0.28580	-1.21551	0.64391
8	2	3	-0.56170	-1.49141	0.36801
9	2	4	-0.77320	-1.70291	0.15651
10	2	5	0.32620	-0.60351	1.25591
11	2	6	0.58650	-0.34321	1.51621
12	2	7	-0.13470	-1.06441	0.79501
13	2	8	-0.20270	-1.13241	0.72701
14	3	4	-0.21150	-1.14121	0.71821
15	3	5	0.88790	-0.04181	1.81761
16	3	6	1.14820	0.21849*	2.07791*
17	3	7	0.42700	-0.50271	1.35671
18	3	8	0.35900	-0.57071	1.28871
19	4	5	1.09940	0.16969*	2.02911*
20	4	6	1.35970	0.42999*	2.28941*
21	4	7	0.63850	-0.29121	1.56821
22	4	8	0.57050	-0.35921	1.50021
23	5	6	0.26030	-0.66941	1.19001
24	5	7	-0.46090	-1.39061	0.46881
25	5	8	-0.52890	-1.45861	0.40081
26	6	7	-0.72120	-1.65091	0.20851
27	6	8	-0.78920	-1.71891	0.14051
28	7	8	-0.06800	-0.99771	0.86171

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.6b: One-way analysis of variance on log PVVC.ml^{-1} obtained in larval cultures raised in autoclaved (AuW) or 5µ m filtered (SW) seawater , fed with algae (A) or artificial (MED) diets and with (+B) or without (-B) added bacteria obtained from mixed algal cultures.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	7	62778860	8968409	5.91	0.002
Error	16	24299592	1518724		

Treatment	*Code*	*N*	*Mean*	*StDev*
AuA+B	1	3	2402	894
AuA-B	2	3	2542	568

Total	23	87078456	AuMED+B	3	3	2233	854
			AuMED-B	4	3	6100	400
			SWA+B	5	3	4075	1748
			SWA-B	6	3	1688	605
			SWMED-B	7	3	363	108
			SWMED+B	8	3	1882	2590

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-140	-3626.38	3346.38
2	1	3	169	-3317.38	3655.38
3	1	4	-3698	-7184.38*	-211.62*
4	1	5	-1673	-5159.38	1813.38
5	1	6	714	-2772.38	4200.38
6	1	7	2039	-1447.38	5525.38
7	1	8	520	-2966.38	4006.38
8	2	3	309	-3177.38	3795.38
9	2	4	-3558	-7044.38*	-71.62*
10	2	5	-1533	-5019.38	1953.38
11	2	6	854	-2632.38	4340.38
12	2	7	2179	-1307.38	5665.38
13	2	8	660	-2826.38	4146.38
14	3	4	-3867	-7353.38*	-380.62*
15	3	5	-1842	-5328.38	1644.38
16	3	6	545	-2941.38	4031.38
17	3	7	1870	-1616.38	5356.38
18	3	8	351	-3135.38	3837.38
19	4	5	2025	-1461.38	5511.38
20	4	6	4412	925.62*	7898.38*
21	4	7	5737	2250.62*	9223.38*
22	4	8	4218	731.62*	7704.38*
23	5	6	2387	-1099.38	5873.38
24	5	7	3712	225.62*	7198.38*
25	5	8	2193	-1293.38	5679.38
26	6	7	1325	-2161.38	4811.38
27	6	8	-194	-3680.38	3292.38
28	7	8	-1519	-5005.38	1967.38

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.6c: Two sample T-test and confidence intervals on arc sine percentage survival of P. indicus mysis stage larvae reared in sterile water with added 3µm filtered algae culture water (3.0µmAW) algae culture water which was 0.2µm filtered to retain the associated bacteria which were then added to autoclaved water (0.2µmAW)

95% C.I. for mu 0.2µm - mu 3.0µm: (-0.475, 0.43 Asin %)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
0.2µmAw	3	0.210	0.169	0.097
3.0µmAW	3	0.232	0.227	0.13

T-Test mu 0.2µm = mu 3.0µm (vs not =): T= -0.13 P=0.90 DF= 4

Appendix 3.6d: Initial and final lengths of M1 stage Penaeus indicus larvae fed wholly on microencapsulated diets (CAR) Larvae were fed at 4mg.l-1.day-1 in autoclaved sea water to which had been added 3µm filtered mixed algal (Skeletonema costatum and Tetraselmis chuii) culture water (3µmAW) and bacteria which had been 0.22µm filtered from algal culture water (0.22µmB-AW)

0.22µmB-A	*3µmAW*	*Start*
4.7	4.1	2.7
4.3	4.4	2.7
4.7	4.1	2.7
4.5	4.5	2.6
4.9	4.7	2.7
4.3	4.8	2.7
4.5	4.5	2.8
4.5	4.1	2.8
4.5	4.7	2.9
4.5	4.7	2.7
4.7	4.1	2.6
4.6	4.1	2.6
4.5	4.1	2.7
4.5	4.1	2.6
4.3	4.1	2.7
4.3	4.2	
4.5	4.3	
4.3	4.1	
4.5	4.1	
4.5	3.7	
4.7	4.4	
4.7	4.4	
4.6	4.2	
4.9	4.7	
4.8	4.3	

Appendix 3.6e: Two sample Mann-Whitney test and confidence intervals on final lengths of M1 stage Penaeus indicus larvae fed wholly on microencapsulated diets (CAR) Larvae were fed at 4mg.l^{-1}.day^{-1} in autoclaved sea water to which had been added 3µm filtered mixed algal (Skeletonema costatum and Tetraselmis chuii) culture water (3µmAW) and bacteria which had been 0.22µm filtered from algal culture water (0.22µmB-AW)

95.2 Percent C.I. for ETA1-ETA2 is (0.1333 to 0.4000mm)

0.22mmB-	*N*	=	*25*	*Median*	=	*4.5333*
3mmAW	N	=	25	Median	=	4.2000

Point estimate for ETA1-ETA2 is 0.2667
W = 812.5
Test of ETA1 = ETA2 vs. ETA1 ~= ETA2 is significant at 0.0007
The test is significant at 0.0007 (adjusted for ties)

Appendix 3.6f: Kruskall-Wallis tests and Dunn's pairwise comparisons on the initial and final lengths of M1 stage Penaeus indicus larvae fed wholly on microencapsulated diets (CAR) Larvae were fed at 4mg.L^{-1}.day^{-1} in autoclaved sea water to which had been added 3µm filtered mixed algal (Skeletonema costatum and Tetraselmis chuii) culture water (3µmAW) and bacteria which had been 0.22µm filtered from algal culture water (0.22µmB-AW)

Kruskal-Wallis Test

LEVEL	*NOBS*	*MEDIAN (mm)*	*AVE.RANK*	*Z-VALUE*
1	25	4.533	47.5	4.89
2	25	4.200	33.5	0.17
3	15	2.667	8.0	-5.84
OVERALL	65		33.0	

H = 40.94 d.f. = 2 p = 0.000
H = 41.14 d.f. = 2 p = 0.000 (adjusted for ties)

Dunn's pairwise comparisons

Row	COMP	ARING	ABSDIFF	ST. DEV	DIFF/SD
1	1	2	14.0	5.34790	2.61785
2	1	3	39.5	6.17522	6.39653
3	2	3	25.5	6.17522	4.12941

K66 2.39398

If the above statistic (K66) is less than any of the values in the DIFF/SD column then that pair is significantly different .

Appendix 3.7: Percentage survivals of P. indicus larvae reared in 5µm filtered sea water which was either rapidly heated up within 24h (NW), allowed to age and heat up to ambient temperatures gradually (OW) or OW with added bacteria from mixed algal cultures, associated total viable counts (TVC) and presumed* Vibrio sp. *(PVVC) bacteria.

Treatment	Replicate	percentage survival
old water	A	2.5
	B	3.5
	C	5.5
Old water + algal bacteria	A	35.5
	B	17.5
	C	12
New Water	A	0
	B	0
	C	0

PVVC (log cfu.ml^{-1})	treatment		TVC (log cfu.ml^{-1})	at start	pvvc(start)	
3.28892	1	nw	3.14613	3.74819	3.30535	nw
3.86451	1	nw	4.80209	3.89209	*	nw
3.48714	1	nw	5.10517	4.62737	*	ow
4.16304	2	ow	3.55630	4.59770	*	ow
3.32015	2	ow	5.17026	5.46538	3.26007	sk
3.91698	2	ow	4.36549	5.50515		tet
3.57229	3	ow+b	4.50515			
2.99564	3	ow+b	3.84510			
3.29667	3	ow+b	5.36361			

Appendix 3.7a: Two Sample T-Test and confidence intervals for survival to M1 stage of Penaeus indicus larvae reared in aged 5µm filtered sea water without (OW) or with (OW+B) added bacteria from algal cultures

95% C.I. for mu 1 - mu 2: (-0.3866, 0.024 Asin %)

Treatment	N	Mean	StDev	SE Mean
1. OW	3	0.0383	0.0153	0.0088
2. OW+B	3	0.220	0.127	0.073

T-Test mu 1 = mu 2 (vs <): T= -2.45 P=0.035 DF= 4

Appendix 3.7b: Analysis of Variance on log TVC obtained per ml after rearing Penaeus indicus larvae in 5µm filtered sea water which was either heated up within 24h (NW) or allowed to age and heat up gradually for 3weeks (OW) or OW with added bacteria from mixed algal cultures

Source	DF	SS	MS	F	p	Level	Code	N	Mean	StDev
Treatment	2	0.092	0.046	0.06	0.944	1	1	3	4.3511	1.0545
Error	6	4.686	0.781			2	2	3	4.3640	0.8070
Total	8	4.778				3	3	3	4.5713	0.7614

Appendix 3.7c: Analysis of variance on log PVVC per ml obtained after rearing Penaeus indicus larvae in 5µm filtered sea water which was either heated up within 24h (NW) or allowed to age and heat up gradually for 3weeks (OW) or OW with added bacteria from mixed algal cultures

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	2	0.105	0.053	0.48	0.639	OW	1	3	3.4668	0.3977
Error	6	0.654	0.109			OW+B	32	3	3.2882	0.2884
Total	8	0.759				NW	3	3	3.5469	0.2924

Appendix 3.8: Survival and bacterial counts in cultures of PZ-1 stage Penaeus indicus larvae raised to M1 stage in autoclaved water (AuW) with (+B) or without (-B) added preserved bacteria which had been taken from a previously successful PZ-2 stage culture of P. indicus larvae. Controls were reared in 5µm filtered sea water (5µmSW)

Treatment	N	Initial number of larvae	Final survival	Bacterial counts	Initial bacterial count in AuW	initial bacterial count in AuW +B	Inital bacterial count in 5umSW	Final bacterial count in AuW	Final bacterial count in AuW+B	Final bacterial count in 5 µmSW
AuW	A	200	2	TVC.ML^{-1}	0	2.5×10^{7}	5.0×10^{7}	4.9×10^{5}	2.1×10^{5}	6.4×10^{7}
AuW	B	200	18	TVC.ML^{-1}	0	2.5×10^{7}	6.0×10^{7}	4.7×10^{5}	1.7×10^{5}	6.2×10^{7}
AuW	C	200	0	TVC.ML^{-1}	0	3.0×10^{7}	8.5×10^{7}	5.1×10^{5}	1.5×10^{5}	7.2×10^{7}
AuW+PB	A	200	0					*	*	*
AuW+PB	B	200	69	PVVC.ML^{-1}	0	0	0	8.5×10^{5}	$<1.2 \times 10^{5}$	$<1.2 \times 10^{5}$
AuW+PB	C	200	46	PVVC.ML^{-1}	0	0	0	6.2×10^{5}	$<1.2 \times 10^{5}$	$<1.2 \times 10^{5}$
5µmSW	A	200	63	PVVC.ML^{-1}	0	0	0	$<1.2 \times 10^{5}$	1.2×10^{5}	$<1.2 \times 10^{5}$
5µmSW	B	200	44							
5µmSW	C	200	98							

Appendix 3.8a: One-way analysis of variance on survival of Penaeus indicus larvae raised in autoclaved water (AuW) with (+B) or without added preserved bacteria which had been taken from a previously successful PZ-2 stage culture of P. indicus larvae. Controls were reared in 5µm filtered sea water (5µmSW)

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
C3	2	0.1518	0.0759	4.04	0.077	AUtoW	1	3	0.0334	0.0494
Error	6	0.1126	0.0188			Auto+B	2	3	0.1948	0.1791
Total	8	0.2643				5µm SW	3	3	0.3515	0.1476

Appendix 3.8b: - One-way analysis of variance on log TVC of bacteria obtained per ml when Penaeus indicus larvae were raised in autoclaved water (AuW) with (+B) or without added

preserved bacteria which had been taken from a previously successful PZ-2 stage culture of P. indicus larvae. Controls were reared in 5µm filtered sea water (5µmSW)

Source	*DF*	*SS*	*MS*	*F*	*p*	*Level*	*Code*	*N*	*Mean*	*StDev*
C3	2	1.28549	0.64275	108.37	<0.001	AW	1	3	5.6900	0.0177
Error	6	0.03559	0.00593			AW+B	2	3	5.2429	0.0739
Total	8	1.32108				5µmSW	3	3	4.7644	0.1097

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.4471	0.254145*	0.64006*
2	1	3	0.9256	0.732645*	1.11856*
3	2	3	0.4785	0.285545*	0.67146*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 3.8b:Descriptive Statistics on log PVVC obtained in waters to which preserved bacteria had been added.

Variable	Treatment	N	Mean	std. dev
PVVC	AUto	3	2.57394	2.23015
	Auto+B	2	2.0000	2.0000

Appendix 4.1:Results of Carbon: Nitrogen analysis of artificial diets used in diet stability and nutrient leaching rate tests

Sample	*sample weight (mg)*	*µg of carbon*	*µg of Nitrogen*	*Weight (%) carbon*	*Weight (%) Nitrogen*	*µg protein /mg sample*
CD2a	1.12	532.7	95.55	47.56	8.5	533.20
CD2b	1.07	524.8	99.58	49.04	9.3	581.66
CD2c	1.14	564.4	104.32	49.51	9.1	571.93
CD2d	0.92	433.9	78.94	47.16	8.6	536.28
CD2e	1.4	664.6	122.44	47.47	8.7	546.61
MPDa	1.43	800.6	105.53	55.95	7.4	461.23
MPDb	0.91	496.5	64.54	54.56	7.1	443.27
MPDc	1.20	665.1	86.91	55.42	7.2	452.66
MPDd	1.00	564.4	73.84	56.44	7.4	461.50
MPDe	1.62	892.7	118.95	55.10	7.3	458.91
CARa	1.28	600.0	110.71	46.87	8.6	540.58
CARb	1.00	480.9	88.31	48.09	8.8	551.94
CARc	1.38	603.9	112.16	43.76	8.1	507.97
CARd	1.42	635.5	117.76	44.69	8.3	518.31
CARe	1.12	501.2	91.52	44.75	8.2	510.71

Appendix 4.1a: One-Way Analysis of Variance on total protein content of CAR, CD2 and MPD diets

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	2	25712	12856	42.51	<0.001	CD2	1	5	553.93	21.72
Error	12	3629	302			MPD	2	5	455.51	7.72
Total	14	29341				CAR	3	5	525.90	19.38

Tukey's pairwise comparisons

Code.	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	2	98.4200	69.1205*	127.719*

1	3	28.0300	-1.2695	57.329
2	3	-70.3900	-99.6895*	-41.091*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.2: Percentage soluble protein leached when 1g.L^{-1} of each of the MED, CAR and CD2 and the MPD were incubated in 5µm filtered sea water. Controls consisted of the same concentrations of diets in autoclaved sea water. Additional controls were with AuW and 5µmSW without added diets.

Time(H)	carAuW	car5µmSW	cd2AuW	cd25µmSW	mpdAuW	mpd5µmSW	No dieta(AuW)	No diets (5µmSW)	Time(H) (For no diets)
0	0.0000	0.0000	0.0000	0.000	0.0000	0.0000	*	*	*
0	0.0000	0.0000	0.0000	0.000	0.0000	0.0000	*	*	*
0	0.0000	0.0000	0.0000	0.000	0.0000	0.0000	*	*	*
1	45.0465	46.5867	52.5152	80.876	67.4842	49.8338	*	*	*
1	31.9642	61.7986	47.7854	48.382	58.1321	61.7105	*	*	*
1	67.4651	61.2472	52.2263	69.972	55.6075	61.0080	0.000	0.000	0
2	66.5524	51.2834	69.0875	75.605	61.0080	55.9807	0.000	0.000	0
2	73.0935	37.8778	69.9721	66.254	55.6075	57.4076	0.000	0.000	0
2	77.1437	76.2120	84.7753	48.977	58.8346	62.0837	0.000	0.000	1
4	73.7781	74.9190	70.0443	61.921	59.2737	52.4682	0.000	0.000	1
4	74.9190	26.6210	74.3769	98.929	70.2503	50.4924	0.000	0.000	1
4	64.6509	74.3485	64.0869	61.018	62.1276	55.9807	0.088	0.121	2
6	55.9040	54.0025	53.7969	69.142	62.1276	50.9314	0.023	0.104	2
6	45.6359	22.0574	64.9896	61.921	47.4189	53.1268	0.121	0.021	4
6	64.0804	64.0804	64.4480	31.773	32.9298	48.0775	0.060	0.018	4
9	49.0586	63.1297	53.9774	60.477	44.5650	49.1752	0.008	0.014	6
9	36.6989	23.9589	84.3059	67.878	44.7845	74.2019	0.060	0.011	6
9	38.2201	18.4445	64.4480	53.617	55.3221	60.8104	0.009	0.023	9
12	69.0243	68.4539	-5.9574	55.242	48.7361	44.7845	0.011	0.024	9
12	49.6291	73.9682	67.6974	54.519	45.2236	46.5408	0.121	0.023	12
12	37.8398	64.6509	30.8700	63.546	40.3939	43.6869	0.006	0.140	12
24	64.0804	58.3760	45.4927	47.118	35.1251	52.4682	0.121	0.140	24
24	23.5786	62.1789	51.6306	51.270	50.2728	50.9314	0.016	0.021	24
24	70.5455	64.0804	63.5453	21.122	57.7354	55.9807	0.019	0.016	48
48	62.7494	49.4389	45.1316	50.728	49.3947	53.7854	0.019	0.026	48
48	14.6416	21.1066	53.4358	33.578	-7.2446	48.7361			
48	19.5854	37.2694	47.4785	37.369	54.8830	51.3705			

Appendix 4.2a: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 1h.

95% C.I. for mu AuW - mu 5umSW: (-0.47, 0.284 Asin %)

Treatment	N	Means	StDev	SE Mean
AuW	3	0.511	0.211	0.12
5umSW	3	0.603	0.103	0.059

T-Test mu AuW = mu 5umSW (vs not =): T= -0.68 P=0.53 DF= 4

Appendix 4.2b: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 1h.

95% C.I. for mu AuW - mu 5umSW: (-0.564, 0.15 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.5335	0.0306	0.018
5umSW	3	0.741	0.221	0.13

T-Test mu AuW = mu 5umSW (vs not =): T= -1.61 P=0.18 DF= 4

Appendix 4.2c: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 1h.

95% C.I. for mu AuW - mu 5umSW: (-0.146, 0.218 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.6503	0.0799	0.046
5umSW	3	0.6143	0.0803	0.046

T-Test mu AuW = mu 5umSW (vs not =): T= 0.55 P=0.61 DF= 4

Appendix 4.2d: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 2h.

95% C.I. for mu AuW - mu 5umSW: (-0.199, 0.62 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.8097	0.0769	0.044
5umSW	3	0.598	0.245	0.14

T-Test mu AuW = mu 5umSW (vs not =): T= 1.43 P=0.23 DF= 4

Appendix 4.2e: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 2h.

95% C.I. for mu AuW - mu 5umSW: (-0.207, 0.51 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.850	0.140	0.081
5umSW	3	0.698	0.174	0.10

T-Test mu AuW = mu 5umSW (vs not =): T= 1.18 P=0.30 DF= 4

Appendix 4.2f: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 2h.

95% C.I. for mu AuW - mu 5umSW: (-0.083, 0.083 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.6249	0.0334	0.019
5umSW	3	0.6251	0.0396	0.023

T-Test mu AuW = mu 5umSW (vs not =): T= -0.01 P=0.99 DF= 4

Appendix 4.2g: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 4h.

95% C.I. for mu AuW - mu 5umSW: (-0.404, 0.69 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.7932	0.0786	0.045
5um	3	0.652	0.331	0.19

SW

T-Test mu AuW = mu 5umSW (vs not =): T= 0.72 P=0.51 DF= 4

Appendix 4.2h: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 4h.

95% C.I. for mu AuW - mu 5umSW: (-0.861, 0.57 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.7701	0.0717	0.041
5umSW	3	0.916	0.440	0.25

T-Test mu AuW = mu 5umSW (vs not =): T= -0.57 P=0.60 DF= 4

Appendix 4.2i: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 4h.

95% C.I. for mu AuW - mu 5umSW: (0.004, 0.268 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.6946	0.0752	0.043
5umS W	3	0.5586	0.0329	0.019

T-Test mu AuW = mu 5umSW (vs not =): T= 2.87 P=0.045 DF= 4

Appendix 4.2j: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 6h.

95% C.I. for mu AuW - mu 5umSW: (-0.340, 0.52 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.588	0.111	0.064
5umSW	3	0.496	0.245	0.14

T-Test mu AuW = mu 5umSW (vs not =): T= 0.59 P=0.59 DF= 4

Appendix 4.2k: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 6h.

95% C.I. for mu AuW - mu 5umSW: (-0.318, 0.47 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.6586	0.0785	0.045
5umSW	3	0.585	0.231	0.13

T-Test mu AuW = mu 5umSW (vs not =): T= 0.52 P=0.63 DF= 4

Appendix 4.2l: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 6h.

95% C.I. for mu AuW - mu 5umSW: (-0.305, 0.241 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.500	0.167	0.097
5umSW	3	0.5320	0.0294	0.017

T-Test mu AuW = mu 5umSW (vs not =): T= -0.33 P=0.76 DF= 4

Appendix 4.2m: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 9h.

95% C.I. for mu AuW - mu 5umSW: (-0.396, 0.51 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.4269	0.0748	0.043
5umSW	3	0.370	0.273	0.16

T-Test mu AuW = mu 5umSW (vs not =): T= 0.35 P=0.75 DF= 4

Appendix 4.2n: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 9h.

95% C.I. for mu AuW - mu 5umSW: (-0.28, 0.488 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.758	0.222	0.13
5umSW	3	0.6538	0.0902	0.052

T-Test mu AuW = mu 5umSW (vs not =): T= 0.75 P=0.49 DF= 4

Appendix 4.2o: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 9h.

95% C.I. for mu AuW - mu 5umSW: (-0.447, 0.119 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.5042	0.0711	0.041
5umSW	3	0.668	0.161	0.093

T-Test mu AuW = mu 5umSW (vs not =): T= -1.61 P=0.18 DF= 4

Appendix 4.2p: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 12h

95% C.I. for mu AuW - mu 5umSW: (-0.70, 0.291 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.556	0.190	0.11
5umSW	3	0.7632	0.0653	0.038

T-Test mu AuW = mu 5umSW (vs not =): T= -1.79 P=0.22 DF= 2

Appendix 4.2q: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 12h

95% C.I. for mu AuW - mu 5umSW: (-1.29, 0.726 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.333	0.402	0.23
5umSW	3	0.6168	0.0623	0.036

T-Test mu AuW = mu 5umSW (vs not =): T= -1.21 P=0.35 DF= 2

Appendix 4.2r: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 12h

95% C.I. for mu AuW - mu 5umSW: (-0.125, 0.1208 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.4647	0.0468	0.027
5umSW	3	0.4669	0.0161	0.0093

T-Test mu AuW = mu 5umSW (vs not =): T= -0.07 P=0.95 DF= 2

Appendix 4.2s: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 24h

95% C.I. for mu AuW - mu 5umSW: (-0.82, 0.642 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.572	0.293	0.17
5umSW	3	0.6633	0.0367	0.021

T-Test mu AuW = mu 5umSW (vs not =): T= -0.53 P=0.65 DF= 2

Appendix 4.2t: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 24h

95% C.I. for mu AuW - mu 5umSW: (-0.179, 0.49 Asin %)

N	*Mean*	*StDev*	*SE*	*Mean*
AuW	3	0.568	0.110	0.064
5umSW	3	0.414	0.176	0.10

T-Test mu AuW = mu 5umSW (vs not =): T= 1.28 P=0.27 DF= 4

Appendix 4.2u: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 24h

95% C.I. for mu AuW - mu 5umSW: (-0.275, 0.155 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.500	0.130	0.075
5umSW	3	0.5603	0.0307	0.018

T-Test mu AuW = mu 5umSW (vs not =): T= -0.78 P=0.48 DF= 4

Appendix 4.2v: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CAR was incubated in 5µmSW and AuW for 48h

95% C.I. for mu AuW - mu 5umSW: (-0.56, 0.500 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.341	0.293	0.17
5umSW	3	0.371	0.153	0.088

T-Test mu AuW = mu 5umSW (vs not =): T= -0.16 P=0.88 DF= 4

Appendix 4.2w: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of CD2 was incubated in 5µmSW and AuW for 48h

95% C.I. for mu AuW - mu 5umSW: (-0.089, 0.268 Asin %)

N	Mean	StDev	SE	Mean
AuW	3	0.5089	0.0493	0.028
5umSW	3	0.4191	0.0998	0.058

T-Test mu AuW = mu 5umSW (vs not =): T= 1.40 P=0.24 DF= 4

Appendix 4.2x: Two sample T-test and confidence intervals obtained on the arc sine transformations of percentage soluble protein leached per mg of total protein content when 1g.L^{-1} of MPD was incubated in 5µmSW and AuW for 48h

95% C.I. for mu AuW - mu 5umSW: (-1.09, 0.701 Asin %)

Treatment	N	Means	StDev	SE Mean
AuW	3	0.342	0.360	0.21
5umSW	3	0.5388	0.0294	0.017

T-Test mu AuW = mu 5umSW (vs not =): T= -0.94 P=0.44 DF= 2

Appendix 4.3:Log TVC of bacteria obtained at in 5µm filtered sea water to which were added the microencapsulated diets, CAR and CD2 and the microparticulate diet, MPD. Controls were with 5µm filtered sea water with no added diets.

Time (H)	CAR	CD2	MPD	Control
0	4.61700	4.61700	4.61700	4.61700
0	4.3200	4.3200	4.3200	4.3200
0	4.9100	4.9100	4.9100	4.9100
6	5.84361	6.02890	5.40654	5.06819
6	5.93892	6.04171	5.51322	5.27416
6	5.78675	5.79183	5.62941	5.10037
12	6.47129	6.63246	6.64933	6.19312
12	6.29003	6.57171	6.35793	6.13354
12	6.34439	6.46389	6.43297	6.36922
24	6.45484	6.55388	6.55023	6.43616
24	6.50379	6.46240	6.50379	6.36549
24	6.45788	6.48572	6.49693	6.45637
48	5.47712	5.44716	5.38021	5.34242
48	5.60206	5.38021	5.73239	5.00000

48	5.41497	5.57978	5.34242	5.53148

Appendix 4.3a: One-Way analysis of variance on log TVC of bacteria in water with different artificial diets 6h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	1.2044	0.4015	31.85	< 0.001
Error	8	0.1008	0.0126		
Total	11	1.3052			

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	5.8564	0.0769
CD2	2	3	5.9541	0.1407
MPD	3	3	5.5164	0.1115
Control	4	3	5.1476	0.1108

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.097700	-0.391278	0.19588
2	1	3	0.340000	0.046423*	0.63358*
3	1	4	0.708800	0.415222*	1.00238*
4	2	3	0.437700	0.144123*	0.73128*
5	2	4	0.806500	0.512922*	1.10008*
6	3	4	0.368800	0.075222*	0.66238*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.3b: One-Way Analysis of Variance on log TVC of bacteria obtained in cultures with different artificial diets 12h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.1789	0.0596	4.43	0.041
Error	8	0.1077	0.0135		
Total	11	0.2866			

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	6.3686	0.0930
CD2	2	3	6.5560	0.0854
MPD	3	3	6.4801	0.1513
Cont.	4	4	6.2320	0.1225

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.187400	-0.491282	0.116481
2	1	3	-0.111500	-0.415382	0.192381
3	1	4	0.136600	-0.167282	0.440482
4	2	3	0.075900	-0.227982	0.379782
5	2	4	0.324000	0.020119*	0.627882*
6	3	4	0.248100	-0.055781	0.551982

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.3c: One-Way Analysis of Variance on log TVC of bacteria obtained in water treatments with different artificial diets 24h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.01652	0.00551	3.59	0.066
Error	8	0.01226	0.00153		
Total	11	0.02878			

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	6.4722	0.0274
CD2	2	3	6.5007	0.0475
MPD	3	3	6.5170	0.0290
Cont.	4	3	6.4193	0.0477

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.0285001	-0.091147	0.034147
2	1	3	-0.0448003	-0.107447	0.017847
3	1	4	0.0528998	-0.009747	0.115547
4	2	3	-0.0163002	-0.078947	0.046347

5	2	4	0.0813999	0.018753*	0.144047*
6	3	4	0.0977001	0.035053*	0.160347*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.3d: One-Way Analysis of Variance on log TVC of bacteria in water treatments with different artificial diets 48h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.0848	0.0283	0.82	0.519
Error	8	0.2765	0.0346		
Total	11	0.3613			

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	5.4981	0.0953
CD2	2	3	5.4691	0.1016
MPD	3	3	5.4850	0.2151
Cont.	4	3	5.2913	0.2694

Appendix 4.4:Log TVC of bacteria attached to the microencapsulated diets, CAR and CD2 and the microparticulate diet, MPD in 5µm filtered sea water. Controls were with 5µm filtered sea water with no added diets.

Time (H)	*CAR*	*CD2*	*MPD*	*Control*
6	4.22608	4.29226	4.28780	3.70415
6	4.98444	4.39967	3.78462	3.51851
6	4.17447	4.12840	4.12710	3.38382
12	4.93802	5.01284	5.14301	4.39794
12	4.92221	5.25527	4.87157	4.35411
12	5.08991	4.93349	4.93044	4.07188
24	5.27554	5.21484	5.27416	5.33041
24	5.38382	5.03342	5.20683	5.37658
24	5.31387	5.21484	5.06446	5.41996
48	5.19590	4.94939	4.58092	4.33244
48	5.07188	4.83378	4.66839	4.86094
48	5.00000	4.10380	4.90472	4.95713

Appendix 4.4a: One-Way analysis of variance on log TVC of bacteria attached to different artificial diet particles 6h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	1.4390	0.4797	6.07	0.019
Error	8	0.6324	0.0791		
Total	11	2.0715			

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	4.4617	0.4535
CD2	2	3	4.2734	0.1366
MPD	3	3	4.0665	0.2570
Cont.	4	3	3.5355	0.1608

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.188300	-0.547273	0.92387
2	1	3	0.395200	-0.340373	1.13077
3	1	4	0.926200	0.190627*	1.66177*
4	2	3	0.206900	-0.528673	0.94247
5	2	4	0.737900	0.002327*	1.47347*
6	3	4	0.531000	-0.204573	1.26657

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.4b: One-Way Analysis of Variance on log TVC of bacteria attached to different artificial diet particles 12h after diet addition.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	1.2335	0.4112	18.61	0.001

Treatment	*Code*	*N*	*Mean*	*StDev*
CAR	1	3	4.9834	0.0926

Error	8	0.1768	0.0221		CD2	2	3	5.0672	0.1676
Total	11	1.4103			MPD	3	3	4.9817	0.1428
					Cont.	4	3	4.2746	0.1770

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.083800	-0.472607	0.30501
2	1	3	0.001700	-0.387107	0.39051
3	1	4	0.708800	0.319993*	1.09761*
4	2	3	0.085500	-0.303306	0.47431
5	2	4	0.792600	0.403793*	1.18141*
6	3	4	0.707100	0.318293*	1.09591*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.4c: One-way analysis of variance on log TVC of bacteria attached to different artificial diet particles 24h after diet addition

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	0.10437	0.03479	5.07	0.030	CAR	1	3	5.3244	0.0549
Error	8	0.05491	0.00686			CD2	2	3	5.1544	0.1047
Total	11	0.15928				MPD	3	3	5.1818	0.1071
						Cont.	4	3	5.3756	0.0448

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.170000	-0.046620	0.386621
2	1	3	0.142600	-0.074020	0.359221
3	1	4	-0.051200	-0.267820	0.165421
4	2	3	-0.027400	-0.244021	0.189221
5	2	4	-0.221200	-0.437821*	-0.004579*
6	3	4	-0.193800	-0.410421	0.022821

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.4d: One-way analysis of variance on log TVC of bacteria attached to different artificial diet particles 48h after diet addition.

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	0.3780	0.1260	1.40	0.313	CAR	1	3	5.0893	0.0991
Error	8	0.7224	0.0903			CD2	2	3	4.6290	0.4585
Total	11	1.1005				MPD	3	3	4.7180	0.1675
						Cont.	4	3	4.7168	0.3364

Appendix 4.5: Log TVCs of bacteria obtained after addition of microcapsules broken by sonication to 5μm filtered sea water. Controls were with intact microcapsules.

Time (H)	*Control*	*Sonicated*
0	4.79239	4.79239
0	4.92428	4.92428
0	4.66276	4.66276
3	5.76492	6.67578
3	5.74663	6.56110
3	5.91062	6.66181
6	5.16435	5.48001
6	4.57978	5.55145

6	4.83251	5.22011
12	7.60531	7.72997
12	7.81157	7.71517
12	7.82607	7.62014
24	7.57749	7.74115
24	7.50651	7.85733
24	7.53403	7.74429
48	7.40483	7.49415
48	7.15229	7.75205
48	7.35603	7.73239

Appendix 4.5a: Lower tailed two sample T test and confidence intervals on log TVC obtained 3h after microcapsules broken by sonication were added to 5µm filtered sea water. Controls were with intact microcapsules.

95% C.I. for mu 1 - mu 2: (-1.001, -0.650 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1 Control	3	5.8074	0.0899	0.052
2 Sonicated	3	6.6329	0.0626	0.036

T-Test mu 1 = mu 2 (vs <): T= -13.06 P=0.0001 DF= 4

Appendix 4.5b: Lower tailed two sample T test and confidence intervals on log TVC obtained 6h after microcapsules broken by sonication were added to 5µm filtered sea water. Controls were with intact microcapsules.

95% C.I. for mu 1 - mu 2: (-1.11, -0.01 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1 Control	3	4.859	0.293	0.17
2 Sonicated	3	5.417	0.174	0.10

T-Test mu 1 = mu 2 (vs <): T= -2.83 P=0.024 DF= 4

Appendix 4.5c: Lower tailed two sample T test and confidence intervals on log TVC obtained 12h after microcapsules broken by sonication were added to 5µm filtered sea water. Controls were with intact microcapsules.

95% C.I. for mu 1 - mu 2: (-0.161, 0.279 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1 Control	3	7.748	0.123	0.071
2 Sonicated	3	7.6884	0.0596	0.034

T-Test mu 1 = mu 2 (vs <): T= 0.75 P=0.75 DF= 4

Appendix 4.5d: Lower tailed two sample T test and confidence intervals on log TVC obtained 24h after microcapsules broken by sonication were added to 5µm filtered sea water. Controls were with intact microcapsules.

95% C.I. for mu 1 - mu 2: (-0.362, -0.121 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1 Control	3	7.5393	0.0358	0.021
2 Sonicated	3	7.7809	0.0662	0.038

T-Test mu 1 = mu 2 (vs <): T= -5.56 P=0.0026 DF= 4

Appendix 4.5e: Lower tailed two sample T test and confidence intervals on log TVC obtained 48h after microcapsules broken by sonication were added to 5µm filtered sea water. Controls were with intact microcapsules.

95% C.I. for mu 1 - mu 2: (-0.670, -0.040 log cfu.ml^{-1})

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
1 Control	3	7.304	0.134	0.077
2 Sonicated	3	7.660	0.144	0.083

T-Test mu 1 = mu 2 (vs <): T= -3.13 P=0.018 DF= 4

Appendix 4.6: Percentage survival of Penaeus indicus PZ3 stage larvae following immersion vaccination with formalin-killed cells of* Vibrio harveyi *strains IN7, BP05 and COL2. Larvae were subsequently challenged 48h after vaccination by immersion with the corresponding live* Vibrio harveyi *strain. Controls were unvaccinated but challenges. General controls were unvaccinated and unchallenged.

Treatment	*Replicate*	*IN7*	*BP05*	*COL2*
Vaccinated	A	67.0	49.0	11.0
	B	80.0	36.0	48.0
	C	89.5	38.0	83.0
Control	A	67.0	10	0.0
	B	68.5	8.5	15.0
	C	56.0	1.0	4.0
Gen. control	A	85.0	38.0	93.0
	B	88.0	39.0	91.0
	C	94.0	45.5	100.0

***Appendix 4.7 Percentage survival of P. indicus PZ3 stage larvae vaccinated with lyophilised* V. harveyi *strain BP05 and subsequently challenged with various strains of* V. harveyi**

Challenge bacteria	*Replicate*	*survival (%) of vaccinated larvae*	*survival (%) of unvaccinated larvae*
Vibrio harveyi strain IN7	A	46.0	32.0
Vibrio harveyi strain IN7	B	49.0	25.5
Vibrio harveyi strain IN7	C	43.5	12.0
Vibrio harveyi strain BP04	A	43.5	28.0
Vibrio harveyi strain BP04	B	48.0	19.5
Vibrio harveyi strain BP04	C	36.0	23.5
Vibrio harveyi strain BP05	A	49.0	10.0
Vibrio harveyi strain *Vibrio harveyi* strain BP05	B	36.0	8.5
Vibrio harveyi strain BP05	C	38.0	1.0
Control	A	38.0	35.0
control	B	39.0	33.0
control	C	45.5	37.5

Appendix 4.8: Percentage survival of Penaeus indicus PZ3 stage larvae following immersion vaccination with formalin-killed cells of either* Vibrio harveyi *strain IN7 or BP04. Larvae were subsequently challenged 48h after vaccination by immersion with the live* Vibrio harveyi *strains BP03, BP04, BP05 and IN7. General controls were unvaccinated and unchallenged.

Challenge bacterium	*Replicate*	*vaccinated with BP04*	*Vaccinated with IN7*	*General control*
Vibrio harveyi strain BP04	A	36.5	37.5	75.5
Vibrio harveyi strain BP04	B	57.0	38.5	72.5
Vibrio harveyi strain BP04	C	48.5	36.0	82.0
Vibrio harveyi strain IN7	A	65.0	49.0	
Vibrio harveyi strain IN7	B	68.5	50.5	
Vibrio harveyi strain IN7	C	72.0	52.0	

Vibrio harveyi strain BP03	A	46.0	39.5
Vibrio harveyi strain BP03	B	42.0	45.5
Vibrio harveyi strain BP03	C	68.5	46.0
Vibrio harveyi strain BP05	A	67.0	36.5
Vibrio harveyi strain BP05	B	44.5	37.5
Vibrio harveyi strain BP05	C	69.0	40.5

Appendix 4.6a: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae vaccinated and subsequently challenged with* V. harveyi *strain COL2

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	2.3507	1.1754	14.11	0.005
Error	6	0.5000	0.0833		
Total	8	2.8507			

Treatments	*Code*	*N*	*Mean*	*StDev*
Vaccinated	1	3	0.5300	0.4352
Controls	2	3	0.0635	0.0780
Gen. cont	3	3	1.3028	0.2335

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.4665	-0.25669	1.18969
2	1	3	-0.7728	-1.49599*	-0.04961*
3	2	3	-1.2393	-1.96249*	-0.51611*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.6b: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae vaccinated and subsequently challenged with* V. harveyi *strain IN7

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	0.2538	0.1269	7.06	0.027
Error	6	0.1079	0.0180		
Total	8	0.3617			

Treatment	*Code*	*N*	*Mean*	*StDev*
Vaccinated	1	3	0.9233	0.1871
Controls	2	3	0.6944	0.0872
Gen. cont	3	3	1.1048	0.1063

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.2289	-0.107275	0.565075
2	1	3	-0.1815	-0.517675	0.154675
3	2	3	-0.4104	-0.746575*	-0.074225*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.6c: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae vaccinated and subsequently challenged with* V. harveyi *strain BP05

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	0.25501	0.12750	36.91	<0.001
Error	6	0.02073	0.00345		
Total	8	0.27573			

Treatment	*Code*	*N*	*Mean*	*StDev*
Vacc	1	3	0.42338	0.07757
Control	2	3	0.06509	0.04830
Gen. cont	3	3	0.42093	0.04488

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.35829	0.211113*	0.505467*
2	1	3	0.00245	-0.144727	0.149627
3	2	3	-0.35584	-0.503017*	-0.208663*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.7a: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae following vaccination with lyophilised* V. harveyi *strain BP05 and subsequent challenge by* V. harveyi *strain IN7

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	0.09844	0.04922	10.60	0.011
Error	6	0.02787	0.00465		
Total	8	0.12631			

Level	*Code*	*N*	*Mean*	*StDev*
Vacc	1	3	0.48004	0.03108
Unvacc	2	3	0.23462	0.10467
Cont	3	3	0.42093	0.04488

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.24542	0.074554*	0.416286*
2	1	3	0.05911	-0.111756	0.229976
3	2	3	-0.18631	-0.357176*	-0.015444*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.7b: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae following vaccination with lyophilised* V. harveyi *strain BP05 and subsequent challenge by* V. harveyi *strain BP04

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	0.07364	0.03682	13.16	0.006
Error	6	0.01679	0.00280		
Total	8	0.09043			

Treatment	*Code*	*N*	*Mean*	*StDev*
Vacc	1	3	0.43965	0.06680
Unvacc	2	3	0.23909	0.04380
Cont	3	3	0.42093	0.04488

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.20056	0.067971*	0.333149*
2	1	3	0.01872	-0.113869	0.151309
3	2	3	-0.18184	-0.314429*	-0.049251*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.7c: One-way analysis of variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae following vaccination with lyophilised* V. harveyi *strain BP05 and subsequent challenge by* V. harveyi *strain BP05

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	2	0.1972	0.0986	4.51	0.064
Error	6	0.1312	0.0219		
Total	8	0.3284			

Level	*Code*	*N*	*Mean*	*StDev*
Vacc	1	3	0.3035	0.2475
Unvacc	2	3	0.0651	0.0483
Cont	3	3	0.4209	0.0449

Appendix 4.8a: One-way analysis of variance on arcsine percentage survival of* Penaeus indicus *PZ3 stage larvae vaccinated by* V. harveyi *strains BP04 and IN7 and challenged by* V. harveyi *strain BP04

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.68063	0.22688	42.88	<0.001
Error	8	0.04233	0.00529		
Total	11	0.72296			

Vaccine	*Code*	*N*	*Mean*	*StDev*
BP04	1	3	0.49550	0.11682
IN7	2	3	0.38262	0.01356
Cont	3	3	0.23218	0.03700
Gen. Cont.	4	3	0.87603	0.07723

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.11288	-0.013122	0.238882
2	1	3	0.26332	0.1373188*	0.389322*

3	1	4	-0.38053	-0.506532*	-0.254528*
4	2	3	0.15044	0.024438*	0.276442*
5	2	4	-0.49341	-0.619412*	-0.367408*
6	3	4	-0.64385	-0.769852*	-0.517848*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.8b: One-way analysis of variance on arcsine percentage survival of Penaeus indicus PZ3 stage larvae vaccinated by* V. harveyi *strains BP04 and IN7 and challenged by* V. harveyi *strain IN7

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.47276	0.15759	63.13	<0.001
Error	8	0.01997	0.00250		
Total	11	0.49273			

Treatment	*Code*	*N*	*Mean*	*StDev*
BP04	1	3	0.75533	0.04811
IN7	2	3	0.52944	0.01738
Cont	3	3	0.36309	0.03745
Gen. Cont.	4	3	0.87603	0.07723

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.22589	0.095120*	0.356660*
2	1	3	0.39224	0.261470*	0.523010*
3	1	4	-0.12070	-0.251470	0.010070
4	2	3	0.16635	0.035580*	0.297120*
5	2	4	-0.34659	-0.477360*	-0.215820*
6	3	4	-0.51294	-0.643710*	-0.382170*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.8c One-way analysis of variance on arcsine percentage survival of Penaeus indicus PZ3 stage larvae vaccinated by* V. harveyi *strains BP04 and IN7 and challenged by* V. harveyi *strain BP03

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.6740	0.2247	22.28	<0.001
Error	8	0.0807	0.0101		
Total	11	0.7546			

Treatment	*Code*	*N*	*Mean*	*StDev*
BP04	1	3	0.5553	0.1740
IN7	2	3	0.4503	0.0383
Cont.	3	3	0.2169	0.0512
Gen. Cont.	4	3	0.8760	0.0772

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.1050	-0.157844	0.367844
2	1	3	0.3384	0.075556*	0.601244*
3	1	4	-0.3207	-0.583544*	-0.057856*
4	2	3	0.2334	-0.029444	0.496244
5	2	4	-0.4257	-0.688544*	-0.162856*
6	3	4	-0.6591	-0.921944*	-0.396256*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.8d: One-way analysis of variance on arcsine percentage survival of Penaeus indicus PZ3 stage larvae vaccinated by* V. harveyi *strains BP04 and IN7 and challenged by* V. harveyi *strain BP05

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.58479	0.19493	22.58	<0.001
Error	8	0.06907	0.00863		
Total	11	0.65385			

Treatment	*Code*	*N*	*Mean*	*StDev*
BP04	1	3	0.65229	0.16607
IN7	2	3	0.39167	0.02257
Cont.	3	3	0.31875	0.02189
Gen. Cont.	4	3	0.87603	0.07723

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	0.26062	0.017655*	0.503585*
2	1	3	0.33354	0.090575*	0.576505*
3	1	4	-0.22374	-0.466705	0.019225
4	2	3	0.07292	-0.170045	0.315885
5	2	4	-0.48436	-0.727325*	-0.241395*
6	3	4	-0.55728	-0.800245*	-0.314315*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.9: Bacterial counts (cfu.ml^{-1}) and larval survivals (%) in cultures of P. indicus larvae orally vaccinated by* V. harveyi *IN7 and challenged 48h after vaccination.

Treatment	*Replicate*	*Day 1*	*Day 2*	*Day 3*	*Survivals (Day3)*
CAR + vaccine	A	1.54×10^6	1.26×10^6	2.94×10^5	44.67
	B	1.54×10^6	1.78×10^6	3.40×10^5	52.67
	C	1.54×10^6	2.0×10^6	1.76×10^5	22.11
Control (Challenged)	A	1.54×10^6	1.66×10^6	2.76×10^5	55.61
	B	1.54×10^6	2.32×10^6	5.20×10^5	55.61
	C	1.54×10^6	2.08×10^6	5.80×10^5	43.55
Control (Unchallenged)	A	0	1.66×10^6	6.70×10^5	59.63
	B	0	8.16×10^5	4.22×10^5	47.57
	C	0	1.21×10^6	1.96×10^5	21.44
Bacteria + immunostimulant (without larvae)	A	1.54×10^6	$3.0 \times 10'$	3.26×10^6	
	B	1.54×10^6	$1.19 \times 10'$	4.10×10^6	
	C	1.54×10^6	7.50×10^6	3.92×10^6	
Bacteria + vaccine (without larvae)	A	1.54×10^6	$1.31 \times 10'$	$1.43 \times 10'$	
	B	1.54×10^6	$2.95 \times 10'$	6.12×10^6	
	C	1.54×10^6	$1.37 \times 10'$	4.42×10^6	

Appendix 4.9a: One-way analysis of variance on Arc sine transformations of percentage survivals obtained in 24h cultures of P. indicus PZ3 stage larvae orally vaccinated (Vac) by* V. harveyi *IN7, with challenged controls (cont) and unchallenged controls (Gen. cont)

Source	*DF*	*SS*	*MS*	*F*	*p*	*Level*	*N*	*Mean*	*StDev*
Factor	2	0.0268	0.0134	0.49	0.635	CAR+VAC	3	0.4136	0.1713
Error	6	0.1641	0.0273			CONT	3	0.5433	0.0803
Total	8	0.1909				GENCONT	3	0.4502	0.2150

Appendix 4.9b: One-way analysis of variance on log TVCs obtained in 24h cultures of* P. indicus *PZ3 stage larvae orally vaccinated (Vac) by* V. harveyi *IN7, with challenged controls (cont), unchallenged controls (Gen. cont) and a separate bacteria control group which consisted of vac + added pathogen without any larvae (Bact. cont)

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	2.5362	0.8454	42.52	<0.001	Vac	1	3	6.2173	0.1044
Error	8	0.1591	0.0199			Cont	2	3	6.3012	0.0741
Total	11	2.6953				Gen. cont	3	3	6.0715	0.1545
						Bact. cont	4	3	7.2413	0.1982

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	-0.0839	-0.45285	0.285047
2	1	3	0.1458	-0.22315	0.514747
3	1	4	-1.0240	-1.39295*	-0.655053*
4	2	3	0.2297	-0.13925	0.598647
5	2	4	-0.9401	-1.30905*	-0.571153*
6	3	4	-1.1698	-1.53875*	-0.800853*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval..

Appendix 4.9c: One-way analysis of variance on log TVCs obtained in 48h cultures of* P. indicus *PZ3 stage larvae orally vaccinated (Vac) by* V. harveyi *IN7, with challenged controls (cont), unchallenged controls (Gen. cont) and a separate bacteria control group which consisted of vac + added pathogen without any larvae (Bact. cont)

Source	DF	SS	MS	F	p
Treatment	3	3.9844	1.3281	27.26	<0.001
Error	8	0.3898	0.0487		
Total	11	4.3742			

Treatment	Code	N	Mean	StDev
Vac	1	3	5.4151	0.1502
Cont	2	3	5.6401	0.1741
Gen. cont	3	3	5.5812	0.2696
Bact. cont	4	3	6.8625	0.2633

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	-0.22500	-0.80217	0.352168
2	1	3	-0.16610	-0.74327	0.411068
3	1	4	-1.44740	-2.02457*	-0.870232*
4	2	3	0.05890	-0.51827	0.636068
5	2	4	-1.22240	-1.79957*	-0.645232*
6	3	4	-1.28130	-1.85847*	-0.704132*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.10a: One-way analysis of variance on log TVCs obtained in 48h cultures of P. indicus PZ3 stage larvae orally vaccinated (Vac) by* V. harveyi *BP04, with challenged controls (cont), unchallenged controls (Gen. cont) and a separate bacteria control group which consisted of vac + added pathogen without any larvae (Bact. cont)

Source	DF	SS	MS	F	p
Treatment	3	4.886	1.629	12.66	0.005
Error	6	0.772	0.129		
Total	9	5.658			

Treatment	Code	N	Mean	StDev
Gen cont	1	2	5.2055	0.0839
cont	2	3	6.4834	0.2503
Vac	3	3	6.4980	0.1821
Bact. cont	4	2	7.3962	0.7570

Tukey's pairwise comparisons

Row	code.	code,	diffmean	SE	low_ci	up_ci
1	1	2	-1.27790	0.327872	-2.51651*	-0.03929*
2	1	3	-1.29250	0.327872	-2.53111*	-0.05389*
3	1	4	-2.19070	0.359166	-3.54753*	-0.83387*
4	2	3	-0.01460	0.293258	-1.12245	1.09325
5	2	4	-0.91280	0.327872	-2.15141	0.32581
6	3	4	-0.89820	0.327872	-2.13681	0.34041

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.10b: One-way analysis of Variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae orally vaccinated and subsequently challenged by* Vibrio harveyi *BP04

Source	DF	SS	MS	F	p
Treatment	2	1.01784	0.50892	88.47	<0.001
Error	6	0.03452	0.00575		
Total	8	1.05236			

Treatments	Code	N	Mean	StDev
old car (cont)	1	3	0.71921	0.13102
old car(infected)	2	3	0.00833	0.00764
car+vaccine(infected)	3	3	0.00333	0.00577

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	0.71088	0.520876*	0.900884*
2	1	3	0.71588	0.525876*	0.905884*
3	2	3	0.00500	-0.185004	0.195004

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 4.9c: One-way analysis of Variance on arcsine percentage survival of* P. indicus *PZ3 stage larvae orally vaccinated and subsequently challenged by* Vibrio harveyi *IN7

Source	DF	SS	MS	F	p
Treatments	2	0.0259	0.0130	0.48	0.643
Error	6	0.1632	0.0272		
Total	8	0.1891			

Treatment	Code	N	Mean	StDev
Car+vaccine	1	3	0.4132	0.1719
Control	2	3	0.5403	0.0798
Gen.control	3	3	0.4478	0.2137

Appendix 4.10: Bacterial counts and larval survival (%) in cultures of* P. indicus *larvae orally vaccinated by* V. harveyi *BP04 and challenged 48h after vaccination.

Treatment	Replicate	DAY 1	DAY 3	survival (day3)
Unchallenged control	A	0	1.84×10^5	56.00
	B	0	1.40×10^5	75.50
	C	0	1.54×10^5	65.00
Challenged control	A	1.24×10^6	2.32×10^6	1
	B	1.24×10^6	5.90×10^6	1.5
	C	1.24×10^6	2.06×10^6	0
CAR+vaccine (challenged)	A	1.24×10^6	3.98×10^6	1
	B	1.24×10^6	1.94×10^6	0
	C	1.24×10^6	4.04×10^6	0
Feed (CAR + vaccine) (without larvae)	A	1.24×10^6	8.54×10^6	
	B	1.24×10^6	7.26×10^6	
	C	1.24×10^6	1.54×10^6	

Appendix 5.1: Upper tailed two sample T-test and confidence interval on the log TVCs of Escherichia coli when incubated in P. vannamei plasma and in SWC

95% C.I. for mu 1 - mu 2: (0.123, 0.2017 log cfu)

Treatment	N	Mean	StDev	SE Mean
1. Plasma	3	6.3707	0.0231	0.013
2. SWC	3	6.20856	0.00868	0.0050

T-Test mu 1 = mu 2 (vs >): T= 11.37 P=0.0002 DF= 4

Appendix 5.2: lower tailed two sample T test on log TVC of* Escherichia coli *grown in SWC medium for 3h with (+L) and without (-L) the addition of 1mg.ml^{-1} of hen egg white lysozyme.

95% C.I. for mu 1 - mu 2: (-0.232, 0.082 log cfu)

Treatment	N	Mean	StDev	SE Mean
1. +L	3	6.1009	0.0818	0.047
2. -L	3	6.1758	0.0542	0.031

T-Test mu 1 = mu 2 (vs <): T= -1.32 P=0.13 DF= 4

Appendix 5.3: Upper tailed two-sample t-test and confidence interval on differences obtained between initial log TVC and log TVC after 3h when incubated in naive plasma from P. vannamei

95% C.I. for mu 1 - mu 2: (0.038, 0.2114 log cfu)

Treatment	N	Mean	StDev	SE Mean
1. 3h	4	5.9640	0.0562	0.028
2. 0h	3	5.8394	0.0119	0.0069

T-Test mu 1 = mu 2 (vs >): T= 3.69 P=0.0071 DF= 5

Appendix 5.4: Survival ratios of* Escherichia coli *and antibacterial activities of* P. vannamei *plasma which had been vaccinated, placebo injected and naïve (uninjected)

Time	Survival ratio vaccinated	Survival ratio placebo	Survival ratio Control	Antimicrobial activity (vaccinated)	Antimicrobial activity (Placebo)	control
0h	0.0173366	0.0173366	0.0173366	0.00000	0.00000	0
0h	0.0311327	0.0311327	0.0311327	0.00000	0.00000	0
0h	0.0248394	0.0248394	0.0248394	0.00000	0.00000	0
6h	-0.0075815	0.0111071	0.0173366	2.49181	0.62294	0
6h	-0.0078201	0.0155942	0.0311327	3.89528	1.55385	0
6h	-0.0286636	0.0223314	0.0248394	5.35030	0.25080	0
12h	-0.0298860	-0.0229335	0.0141036	4.39896	3.70371	0
12h	-0.0467876	0.0106910	0.0311327	7.79204	2.04417	0
12h	-0.0286636	-0.0305871	0.0248394	5.35030	5.54265	0
24h	-0.0023661	0.0071838	0.0353598	3.77259	2.81760	0
24h	-0.0020133	0.0092284	0.0227761	2.47894	1.35477	0
24h	0.0046922	0.0272657	0.0152270	1.05348	-1.20387	0
48h	-0.0014559	0.0228407	0.0353598	3.68157	1.25191	0
48h	-0.0232584	-0.0221230	0.0099633	3.32216	3.20863	0
48h	-0.0141865	0.0078574	0.0152270	2.94135	0.73696	0
4d	0.0034670	0.0166301	0.0173366	1.38695	0.07064	0
4d	0.0110522	0.0304422	0.0227761	1.17239	-0.76661	0
4d	0.0058949	0.0338983	0.0332129	2.73179	-0.06854	0
7d	0.0275843	0.0169842	0.0269689	-0.06153	0.99847	0
7d	-0.0097022	0.0383317	0.0311327	4.08350	-0.71989	0
7d	0.0105207	-0.0048932	0.0120007	0.14800	1.68939	0
14d	0.0562804	0.0254082	0.0173366	-3.89438	-0.80716	0
14d	0.0324946	0.0218474	0.0311327	-0.13619	0.92853	0
14d	0.0385274	0.0038794	0.0248394	-1.36880	2.09600	0
21d	0.0486017	0.0444439	0.0141036	-3.44982	-3.03403	0
21d	0.0487865	0.0221582	0.0131831	-3.56034	-0.89752	0
21d	0.0122750	0.0365787	0.0248394	1.25644	-1.17393	0

Appendix 5.4a: One way analysis of variance on log survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 6h after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	DF	SS	MS	F	p
Treatment	2	0.0025592	0.0012796	16.98	0.003
Error	6	0.0004522	0.0000754		

Treatment	Code	N	Mean	StDev
VAC	1	3	-1.5E-02	0.012103
PLAC	2	3	0.016344	0.005650

Total	8	0.0030115				Cont	3	3	0.024436	0.006907

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.031344	-0.0531018*	-0.0095862*
2	1	3	-0.039436	-0.0611938*	-0.0176782*
3	2	3	-0.008092	-0.0298498	0.0136658

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.4b: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 12h after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont)

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	2	0.005269	0.002635	12.00	0.008	Vac	1	3	-0.03511	0.01013
Error	6	0.001318	0.000220			Plac	2	3	-0.01428	0.02196
Total	8	0.006587				Control	3	3	0.02336	0.00861

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.02083	-0.0579955	0.0163355
2	1	3	-0.05847	-0.0956355*	-0.0213045*
3	2	3	-0.03764	-0.0748055*	-0.0004745*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.4c: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 24h after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	2	0.0009753	0.0004877	7.11	0.026	Vac	1	3	0.000408	0.003721
Error	6	0.0004113	0.0000686			Plac	2	3	0.019778	0.009401
Total	8	0.0013866				Cont	3	3	0.024454	0.010171

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.019370	-0.0401235	0.0013835
2	1	3	-0.024046	-0.0447995*	-0.0032925*
3	2	3	-0.004676	-0.0254295	0.0160775

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.4d: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 48h after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	2	0.001650	0.000825	3.00	0.125	Vac	1	3	-0.01297	0.01095
Error	6	0.001648	0.000275			Plac	2	3	0.00286	0.02289
Total	8	0.003297				Cont	3	3	0.02018	0.01340

Appendix 5.4e: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in P. vannamei plasma 4d after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	2	0.0007250	0.0003625	6.65	0.030	Vac	1	3	0.006805	0.003874
Error	6	0.0003272	0.0000545			Plac	2	3	0.026990	0.009137
Total	8	0.0010522				Cont	3	3	0.024442	0.008068

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	-0.020185	-0.03868318	-0.0016869*
2	1	3	-0.017637	-0.0361351	0.0008611
3	2	3	0.002548	-0.0159501	0.0210461

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.4f: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in P. vannamei plasma 7d after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	2	0.000290	0.000145	0.47	0.644	Vac	1	3	0.00947	0.01867
Error	6	0.001834	0.000306			Plac	2	3	0.01681	0.02161
Total	8	0.002124				Cont	3	3	0.02337	0.01006

Appendix 5.4g: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 14d after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	2	0.001023	0.000512	4.60	0.062	vac	1	3	0.04243	0.01236
Error	6	0.000668	0.000111			Plac	2	3	0.01704	0.01154
Total	8	0.001691				Cont	3	3	0.02444	0.00691

Appendix 5.4h: One way analysis of variance on survival ratio (SR) index of* Escherichia coli *log tvc after 3h incubation in* P. vannamei *plasma 21d after it had either been vaccinated (VAC), placebo injected (P) or naïve (Cont).

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	2	0.000662	0.000331	1.62	0.273	VAC	1	3	0.03655	0.02103
Error	6	0.001224	0.000204			PLAC	2	3	0.03439	0.01130
Total	8	0.001886				Cont	3	3	0.01738	0.00648

Appendix 5.5: Log survival of* Micrococcus luteus *cell incubated in* Penaeus vannamei *plasma which had been vaccinated, placebo injected and naïve (uninjected).

Time	vaccinated	placebo	start	control
0h	4.18184	4.18184	4.18184	4.18184
0h	3.81954	3.81954	3.81954	3.81954
0h	4.08279	4.08279	4.08279	4.08279
0h				4.80821
6h	4.44871	4.68305	4.18184	4.75051
6h	4.08279	4.60423	3.81954	5.14922
6h	4.55871	4.68305	4.08279	4.80821
6h				4.80821
12h	4.68305	4.30320	4.18184	4.75051
12h	4.75051	4.68305	3.81954	5.14922
12h	4.80821	4.72428	4.08279	4.80821

12h				4.80821
24h	4.71850	4.64542	4.18184	4.75051
24h	4.83442	4.75051	3.81954	5.14922
24h	4.71850	4.71850	4.08279	4.80821
24h				4.80821
48h	4.55871	4.60423	4.18184	4.75051
48h	4.78032	4.20683	3.81954	5.14922
48h	4.71850	4.75051	4.08279	4.80821
48h				4.80821
4d	4.44871	4.64542	4.18184	4.75051
4d	4.50786	4.60423	3.81954	5.14922
4d	4.08279	4.60423	4.08279	4.80821
4d				4.80821
7d	4.38202	4.30320	4.18184	4.75051
7d	4.44871	4.38202	3.81954	5.14922
7d	4.08279	4.30320	4.08279	4.80821
7d				4.80821
14d	4.75051	4.75051	4.18184	4.75051
14d	4.50786	4.50786	3.81954	5.14922
14d	4.55991	4.60423	4.08279	4.80821
14d				4.80821
21d	5.55630	4.04139	4.18184	4.75051
21d	4.60423	4.08279	3.81954	5.14922
21d	4.20683	4.20683	4.08279	4.80821
21d				4.80821

Appendix 5.5a: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 6h after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatments	3	1.3706	0.4569	13.80	0.001
Error	9	0.2980	0.0331		
Total	12	1.6686			

Treatment	*Code*	*N*	*Mean*	*StDev*
Vaccine	1	3	4.3634	0.2492
Placebo	2	3	4.6568	0.0455
Start	3	3	4.0281	0.1872
Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	*code.*	*code,*	*diffmean*	*SE*	*low_ci*	*up_ci*
1	1	2	-0.293400	0.148549	-0.79907	0.21227
2	1	3	0.335300	0.148549	-0.17037	0.84097
3	1	4	-0.515600	0.138954	-0.98861*	-0.04259*
4	2	3	0.628700	0.148549	0.12303*	1.13437*
5	2	4	-0.222200	0.138954	-0.69521	0.25081
6	3	4	-0.850900	0.138954	-1.32391*	-0.37789*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5b: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn12h after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatments	3	1.3558	0.4519	14.26	0.001
Error	9	0.2853	0.0317		
Total	12	1.6411			

Treatment	*Code*	*N*	*Mean*	*StDev*
Vaccine	1	3	4.7473	0.0626
Placebo	2	3	4.5702	0.2321
Start	3	3	4.0281	0.1872
Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	code.	code,	diffmean	SE	low_ci	up_ci
1	1	2	0.177100	0.145373	-0.31776	0.67196
2	1	3	0.719200	0.145373	0.22434*	1.21406*
3	1	4	-0.131700	0.135984	-0.59460	0.33120
4	2	3	0.542100	0.145373	0.04724*	1.03696*
5	2	4	-0.308800	0.135984	-0.77170	0.15410
6	3	4	-0.850900	0.135984	-1.31380*	-0.38800*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5c: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 24h after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	DF	SS	MS	F	p
Treatments	3	1.3972	0.4657	22.73	<0.001
Error	9	0.1844	0.0205		
Total	12	1.5816			

Treatment	Code	N	Mean	StDev
Vaccine	1	3	4.7571	0.0669
Placebo	2	3	4.7048	0.0539
Start	3	3	4.0281	0.1872
Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	code.	code,	diffmean	SE	low_ci	up_ci
1	1	2	0.0523	0.116905	-0.34565	0.45025
2	1	3	0.7290	0.116905	0.33105*	1.12695*
3	1	4	-0.1219	0.109354	-0.49415	0.25035
4	2	3	0.6767	0.116905	0.27875*	1.07465*
5	2	4	-0.1742	0.109354	-0.54645	0.19805
6	3	4	-0.8509	0.109354	-1.22315*	-0.47865*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5d: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 48h after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	DF	SS	MS	F	p
Treatments	3	1.3079	0.4360	11.08	0.002
Error	9	0.3541	0.0393		
Total	12	1.6621			

Treatment	Code	N	Mean	StDev
Vaccine	1	3	4.6858	0.1144
Placebo	2	3	4.5205	0.2813
Start	3	3	4.0281	0.1872
Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	code.	code,	diffmean	SE	low_ci	up_ci
1	1	2	0.1653	0.161864	-0.38570	0.71630
2	1	3	0.6577	0.161864	0.10670*	1.20870*
3	1	4	-0.1932	0.151410	-0.70861	0.32221
4	2	3	0.4924	0.161864	-0.05860	1.04340
5	2	4	-0.3585	0.151410	-0.87391	0.15691
6	3	4	-0.8509	0.151410	-1.36631*	-0.33549*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5e: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 4d after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev

Treatments	3	1.3553	0.4518	14.69	0.001	Vaccine	1	3	4.3465	0.2302
Error	9	0.2768	0.0308			Placebo	2	3	4.6180	0.0238
Total	12	1.6322				Start	3	3	4.0281	0.1872
						Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	*code.*	*code,*	*diffmean*	*SE*	*low_ci*	*up_ci*
1	1	2	-0.2715	0.143295	-0.75928	0.21628
2	1	3	0.3184	0.143295	-0.16938	0.80618
3	1	4	-0.5325	0.134040	-0.98878*	-0.07622*
4	2	3	0.5899	0.143295	0.10212*	1.07768*
5	2	4	-0.2610	0.134040	-0.71728	0.19528
6	3	4	-0.8509	0.134040	-1.30718*	-0.39462*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5f: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 7d after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatments	3	1.3682	0.4561	16.43	0.001	Vaccine	1	3	4.3045	0.1949
Error	9	0.2498	0.0278			Placebo	2	3	4.3295	0.0455
Total	12	1.6180				Start	3	3	4.0281	0.1872
						Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	*code.*	*code,*	*diffmean*	*SE*	*low_ci*	*up_ci*
1	1	2	-0.025000	0.136137	-0.48842	0.438420
2	1	3	0.276400	0.136137	-0.18702	0.739820
3	1	4	-0.574500	0.127345	-1.00799*	-0.141011*
4	2	3	0.301400	0.136137	-0.16202	0.764820
5	2	4	-0.549500	0.127345	-0.98299*	-0.116011*
6	3	4	-0.850900	0.127345	-1.28439*	-0.417411*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5g: One-way analysis of variance on log TVC of* Micrococcus luteus *cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 14d after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatments	3	1.2735	0.4245	16.46	0.001	Vaccine	1	3	4.6061	0.1277
Error	9	0.2322	0.0258			Placebo	2	3	4.6209	0.1222
Total	12	1.5057				Start	3	3	4.0281	0.1872
						Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	*code.*	*code,*	*diffmean*	*SE*	*low_ci*	*up_ci*
1	1	2	-0.014800	0.131149	-0.46124	0.43164
2	1	3	0.578000	0.131149	0.13156*	1.02444*
3	1	4	-0.272900	0.122678	-0.69051	0.14471
4	2	3	0.592800	0.131149	0.14636*	1.03924*
5	2	4	-0.258100	0.122678	-0.67571	0.15951
6	3	4	-0.850900	0.122678	-1.26851*	-0.43329*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.5h: One-way analysis of variance on log TVC of Micrococcus luteus cells surviving after 3h incubation in* Penaeus vannamei *plasma withdrawn 21d after the prawns had been vaccinated (VAC), placebo injected (PLAC) or naïve (Cont).

Source	DF	SS	MS	F	p
Treatments	3	1.946	0.649	5.09	0.025
Error	9	1.146	0.127		
Total	12	3.092			

Treatment	Code	N	Mean	StDev
Vaccine	1	3	4.7891	0.6935
Placebo	2	3	4.1103	0.0861
Start	3	3	4.0281	0.1872
Control	4	4	4.8790	0.1822

Scheffe's pairwise comparisons

Row	code.	code,	diffmean	SE	low_ci	up_ci
1	1	2	0.6788	0.290975	-0.31170	1.66930
2	1	3	0.7610	0.290975	-0.22950	1.75150
3	1	4	-0.0899	0.272183	-1.01643	0.83663
4	2	3	0.0822	0.290975	-0.90830	1.07270
5	2	4	-0.7687	0.272183	-1.69523	0.15783
6	3	4	-0.8509	0.272183	-1.77743	0.07563

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.6: Log TVCs of surviving* Escherichia coli *cells incubated in vitro in* P. vannamei *plasma which had been vaccinated with formalin killed cells of* Vibrio harveyi *strains BP04 and DPEX and* E. coli. *Placebo controls were injected with sterile shrimp salt solution. Controls consisted of uninjected plasma and general controls were incubated in SWC)

Time (H)	DPEX	BP04	SSS	E. coli	control	Gen. control
0	5.39070	5.39070	5.39070	5.39070	5.39070	5.39070
0	5.41337	5.41337	5.41337	5.41337	5.41337	5.41337
0	5.39620	5.39620	5.39620	5.39620	5.39620	5.39620
3	5.35079	5.46153	5.45800	5.37788	5.53015	5.51695
3	5.34776	5.39458	5.32432	5.06388	5.50705	5.47080
3	5.40537	5.42107	5.38422	5.20708	5.47592	5.50547
6	5.14288	5.46138	5.17715	5.10368	5.24794	5.45026
6	5.28177	5.34928	5.19128	5.12705	5.27843	5.46401
6	5.24143	5.34528	5.08214	5.24794	5.32171	5.41850
12	5.14160	5.21747	5.01959	4.80703	5.37317	5.62887
12	5.06388	5.08277	5.10328	4.86759	5.46130	5.69888
12	4.93394	5.16377	5.06300	4.76895	5.27615	5.90886
24	4.44846	4.70423	5.46619	4.38527	5.75039	5.99227
24	4.61232	4.96725	5.37036	4.31745	5.74494	5.81097
24	4.32951	4.80064	5.10067	4.64803	5.52309	5.66402

Appendix 5.6a: Survival index values of* Escherichia coli *cells incubated in vitro in* P. vannamei *plasma which had been vaccinated with formalin killed cells of BP04, DPEX and* E. coli. *Placebo controls were injected with sterile shrimp salt solution. Controls consisted of uninjected plasma and general controls were incubated in SWC.

DPEX	BP04	SSS	E. coli	Controls	Gen controls
91.222	117.715	116.764	97.0915	137.864	133.739
85.980	95.767	81.462	44.7215	124.076	114.138
102.135	105.895	97.279	64.6960	120.150	128.609
56.518	117.676	61.158	51.6393	71.986	114.701
73.858	86.281	59.967	51.7233	73.294	112.368
70.022	88.937	48.522	71.0794	84.238	105.268
56.351	67.107	42.549	26.0814	96.044	173.051
44.721	46.709	48.968	28.4591	111.668	192.980

34.494	58.555	46.431	23.5913	75.849	325.579
11.423	20.584	118.985	9.8757	228.926	399.551
15.811	35.800	90.572	8.0182	214.573	249.808
8.577	25.377	50.637	17.8580	133.935	185.276

Appendix 5.6b: One-Way Analysis of Variance on log TVC of* E. coli *strain XL1-Blue MRF` surviving after 3h incubation in plasma from* P. vannamei *which had been variously vaccinated with vaccines made from* Vibrio harveyi *strains BP04 and DPEX,* E. coli *strain XL1_Blue MRF`, and a placebo treatment of SSS. Controls were plasma from unvaccinated prawns.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	4	0.13375	0.03344	5.20	0.016
Error	10	0.06425	0.00642		
Total	14	0.19800			

Level	*Code*	*N*	*Mean*	*StDev*
DPEX	1	3	5.3680	0.0324
BPO4	2	3	5.4257	0.0337
SSS	3	3	5.3888	0.0670
E. coli	4	3	5.2163	0.1572
Cont	5	3	5.5044	0.0272

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.057700	-0.272810	0.157409
2	1	3	-0.020800	-0.235910	0.194310
3	1	4	0.151700	-0.063410	0.366810
4	1	5	-0.136400	-0.351509	0.078710
5	2	3	0.036900	-0.178210	0.252010
6	2	4	0.209400	-0.005709	0.424510
7	2	5	-0.078700	-0.293809	0.136410
8	3	4	0.172500	-0.042609	0.387610
9	3	5	-0.115600	-0.330709	0.099510
10	4	5	-0.288100	-0.503209*	-0.072990*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.6c: One-Way Analysis of Variance on log TVC of* E. coli *strain XL1-Blue MRF` surviving after 6h incubation in plasma from* P. vannamei *which had been variously vaccinated with vaccines made from* Vibrio harveyi *strains BP04 and DPEX,* E. coli *strain XL1_Blue MRF`, and a placebo treatment of SSS. Controls were plasma from unvaccinated prawns.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	4	0.11340	0.02835	6.97	0.006
Error	10	0.04068	0.00407		
Total	14	0.15408			

Level	*Code*	*N*	*Mean*	*StDev*
DPEX	1	3	5.2220	0.0714
BPO4	2	3	5.3853	0.0659
SSS	3	3	5.1502	0.0594
E. coli	4	3	5.1596	0.0774
Cont	5	3	5.2827	0.0371

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.163300	-0.334573	0.007973
2	1	3	0.071800	-0.099473	0.243073
3	1	4	0.062400	-0.108873	0.233674
4	1	5	-0.060700	-0.231973	0.110573
5	2	3	0.235100	0.063827*	0.406374*
6	2	4	0.225700	0.054427*	0.396974*
7	2	5	0.102600	-0.068673	0.273873
8	3	4	-0.009400	-0.180673	0.161873
9	3	5	-0.132500	-0.303773	0.038773

10	4	5	-0.123100	-0.294374	0.048173

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.6d: One-Way Analysis of Variance on log TVC of* E. coli *strain XL1-Blue MRF` surviving after 12h incubation in plasma from* P. vannamei *which had been variously vaccinated with vaccines made from* Vibrio harveyi *strains BP04 and DPEX,* E. coli *strain XL1_Blue MRF`, and a placebo treatment of SSS. Controls were plasma from unvaccinated prawns.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	4	0.48380	0.12095	21.29	<0.001
Error	10	0.05682	0.00568		
Total	14	0.54061			

Level	*Code*	*N*	*Mean*	*StDev*
DPEX	1	3	5.0465	0.1049
BPO4	2	3	5.1547	0.0678
SSS	3	3	5.0620	0.0419
E. coli	4	3	4.8145	0.0497
Cont	5	3	5.3702	0.0926

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.108200	-0.310532	0.094133
2	1	3	-0.015500	-0.217832	0.186833
3	1	4	0.232000	0.029667*	0.434333*
4	1	5	-0.323700	-0.526033*	-0.121367*
5	2	3	0.092700	-0.109633	0.295033
6	2	4	0.340200	0.137867*	0.542533*
7	2	5	-0.215500	-0.417833*	-0.013167*
8	3	4	0.247500	0.045167*	0.449833*
9	3	5	-0.308200	-0.510533*	-0.105867*
10	4	5	-0.555700	-0.758033*	-0.353367*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.6e: One-Way Analysis of Variance on log TVC of* E. coli *strain XL1-Blue MRF` surviving after 24h incubation in plasma from* P. vannamei *which had been variously vaccinated with vaccines made from* Vibrio harveyi *strains BP04 and DPEX,* E. coli *strain XL1_Blue MRF`, and a placebo treatment of SSS. Controls were plasma from unvaccinated prawns.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	4	3.4680	0.8670	35.80	<0.001
Error	10	0.2422	0.0242		
Total	14	3.7102			

Level	*code*	*N*	*Mean*	*StDev*
DPEX	1	3	4.4634	0.1420
BPO4	2	3	4.8240	0.1331
SSS	3	3	5.3124	0.1895
E. coli	4	3	4.4503	0.1746
Cont	5	3	5.6728	0.1297

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.36060	-0.77824	0.05704
2	1	3	-0.84900	-1.26664*	-0.43136*
3	1	4	0.01310	-0.40454	0.43074
4	1	5	-1.20940	-1.62704*	-0.79176*
5	2	3	-0.48840	-0.90604*	-0.07076*
6	2	4	0.37370	-0.04394	0.79134
7	2	5	-0.84880	-1.26644*	-0.43116*
8	3	4	0.86210	0.44446*	1.27974*
9	3	5	-0.36040	-0.77804	0.05724
10	4	5	-1.22250	-1.64014*	-0.80486*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.7a: Absorbance values (490nm) obtained in prophenoloxidase activation tests on activation of* Penaeus indicus *haemocyte lysate at different times after activation by* Aeromonas hydrophila *(AH),* Escherichia coli *(EC), zymosan (ZY) and* Vibrio harveyi *strains BP03, BP04, BP05, IN7 and DPEX. Controls consisted of HLS + sodium cacodylate buffer.

Time (min)	AH	ZY	IN7	EC	BP03	BP04	BP05	DPEX	Plasma	Control
0	0.238	0.181	0.463	0.305	0.257	0.227	0.359	0.214	0.226	0.003
0	0.242	0.192	0.463	0.312	0.259	0.226	0.370	0.211	0.235	0.003
0	0.236	0.215	0.460	0.310	0.275	0.225	0.379	0.212	0.217	0.002
0	0.235	0.176	0.458	0.313	0.264	0.215	0.360	0.207	*	*
5	0.253	0.182	0.474	0.310	0.274	0.224	0.368	0.228	0.257	0.004
5	0.253	0.195	0.476	0.316	0.269	0.234	0.378	0.220	0.268	0.007
5	0.249	0.208	0.466	0.312	0.276	0.234	0.381	0.224	0.237	0.009
5	0.243	0.185	0.447	0.311	0.266	0.211	0.371	0.196	*	*
10	0.253	0.183	0.480	0.312	0.283	0.229	0.373	0.233	0.271	0.006
10	0.257	0.200	0.478	0.319	0.272	0.233	0.384	0.225	0.277	0.008
10	0.254	0.210	0.472	0.312	0.276	0.233	0.384	0.225	0.247	0.012
10	0.245	0.181	0.450	0.317	0.272	0.226	0.378	0.207	*	*
15	0.252	0.183	0.484	0.314	0.285	0.231	0.376	0.235	0.281	0.006
15	0.257	0.201	0.480	0.320	0.274	0.235	0.386	0.227	0.285	0.010
15	0.253	0.213	0.474	0.314	0.277	0.234	0.388	0.228	0.257	0.012
15	0.244	0.181	0.454	0.314	0.273	0.226	0.377	0.212	*	*
20	0.251	0.183	0.486	0.314	0.285	0.231	0.378	0.233	0.290	0.007
20	0.261	0.203	0.483	0.322	0.275	0.239	0.387	0.224	0.288	0.010
20	0.254	0.217	0.477	0.316	0.236	0.236	0.389	0.228	0.263	0.013
20	0.242	0.182	0.457	0.314	0.230	0.230	0.378	0.211	*	*

Protein content of the plasma was adjusted to about 70.83µg.ml^{-1} before commencement of the assays. In order to obtain the proPO activation values per mg of protein, the increases in absorbance values were multiplied by 14.12 (to get 1mg of protein) and as 1 unit of proPO activation was defined as the increase in absorbance of 0.001, the product was multiplied by 1000 (to obtain proPO units).

Appendix 5.7b: Absorbance values (490nm) obtained in prophenoloxidase activation tests on activation of* Penaeus vannamei *haemocyte lysate at different times after activation by* Aeromonas hydrophila *(AH),* Escherichia coli *(EC), zymosan (ZY) and* Vibrio harveyi *strains BP03, BP04, BP05, IN7 and DPEX. Controls consisted of HLS + sodium cacodylate buffer.

Time (min)	ZY	AH	BP03	BP04	BP05	DPEX	IN7	EC	Plasma	Control
0	0.204	0.299	0.315	0.322	0.393	0.240	0.268	0.243	0.307	-0.017
0	0.207	0.317	0.303	0.334	0.404	0.244	0.277	0.244	0.197	-0.020
0	0.208	0.292	0.311	0.335	0.406	0.242	0.300	0.278	0.231	-0.023
5	0.256	0.356	0.339	0.358	0.406	0.261	0.326	0.258	0.328	-0.047
5	0.234	0.361	0.326	0.374	0.430	0.270	0.323	0.253	0.221	-0.054
5	0.221	0.328	0.335	0.384	0.435	0.271	0.356	0.288	0.209	-0.056
10	0.260	0.359	0.344	0.376	0.405	0.261	0.336	0.262	0.317	-0.073
10	0.229	0.381	0.327	0.384	0.436	0.273	0.336	0.252	0.193	-0.086
10	0.223	0.326	0.334	0.383	0.434	0.266	0.354	0.279	0.191	-0.085
15	0.264	0.364	0.348	0.383	0.408	0.263	0.342	0.264	0.305	-0.094
15	0.234	0.386	0.328	0.386	0.435	0.275	0.344	0.251	0.179	-0.107
15	0.223	0.329	0.331	0.374	0.435	0.272	0.354	0.273	0.176	-0.107
20	0.268	0.369	0.348	0.376	0.410	0.264	0.346	0.258	0.290	-0.116
20	0.235	0.383	0.325	0.388	0.439	0.275	0.346	0.241	0.167	-0.130
20	0.225	0.326	0.332	0.374	0.439	0.269	0.352	0.264	0.159	-0.130

Protein content of the plasma was adjusted to about 0.40mg.ml^{-1} before commencement of the assays. In order to obtain the proPO activation values per mg of protein, the increases in absorbance values were multiplied by 2.5 (to get 1mg of protein) and as 1 unit of proPO activation was defined as the increase in absorbance of 0.001the product was multiplied by 1000 (to obtain proPO units).

Appendix 5.7d: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by* Aeromonas hydrophila *over 20min

The regression equation is: AH = 0.242 + 0.000610 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.24250	0.002389	101.5	<0.001	Regression	1	0.000372	0.000372	9.78	0.006
time	0.00061	0.000195	3.13	0.006	Error	18	0.000685	0.000038		
					Total	19	0.001057			

s = 0.006168 R-sq = 35.2% R-sq(adj) = 31.6%

Appendix 5.7e: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by zymosan over 20min

The regression equation is: ZY = 0.191 + 0.000250 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.19105	0.005362	35.63	<0.001	Regression	1	0.0000625	0.0000625	0.33	0.575
time	0.00025	0.000438	0.57	0.575	Error	18	0.0034505	0.0001917		
					Total	19	0.0035130			

s = 0.01385 R-sq = 1.8% R-sq(adj) = 0.0%

Appendix 5.7f: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by* Vibrio harveyi *strain IN7 over 20min

The regression equation is: IN7 = 0.462 + 0.000735 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.461750	0.004244	108.80	<0.001	Regression	1	0.0005402	0.0005402	4.50	0.048
time	0.0007350	0.0003465	2.12	0.048	Error	18	0.0021616	0.0001201		
					Total	19	0.0027018			

s = 0.01096 R-sq = 20.0% R-sq(adj) = 15.6%

Appendix 5.7g: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by* Escherichia coli *strain XL-1 Blue MRF over 20min

The regression equation is: *E. coli* = 0.311 + 0.000325 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.310600	0.001207	257.26	<0.001	Regression	1	0.00010563	0.00010563	10.87	0.004
time	0.000325	0.00009858	3.30	0.004	Error	18	0.00017492	0.00000972		
					Total	19	0.00028055			

s = 0.003117 R-sq = 37.6% R-sq(adj) = 34.2%

Appendix 5.7h: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by* Vibrio harveyi *strain BP03 over 20min

The regression equation is: BP03 = 0.271 - 0.000170 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*

Constant	0.270600	0.005690	47.56	<0.001	Regression	1	0.0000289	0.0000289	0.13	0.719
time	-0.0001700	0.0004646	-0.37	0.719	Error	18	0.0038849	0.0002158		
					Total	19	0.0039138			

s = 0.01469 R-sq = 0.7% R-sq(adj) = 0.0%

Appendix 5.7i: Regression Analysis on increase of absorbance value due to activation of Penaeus indicus haemocyte lysate by* Vibrio harveyi *strain BP04 over 20min

The regression equation is: BP04 = 0.223 + 0.000545 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.223500	0.002228	100.29	<0.001	Regression	1	0.00029703	0.00029703	8.97	0.008
time	0.0005450	0.0001820	3.00	0.008	Error	18	0.00059592	0.00003311		
					Total	19	0.00089295			

s = 0.005754 R-sq = 33.3% R-sq(adj) = 29.6%

Appendix 5.7j: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by Vibrio harveyi strain BP05 over 20min

The regression equation is: BP05 = 0.369 + 0.000785 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.369350	0.002495	148.04	<0.001	Regression	1	0.00061622	0.00061622	14.85	0.001
time	0.0007850	0.0002037	3.85	0.001	Error	18	0.00074698	0.00004150		
					Total	19	0.00136320			

s = 0.006442 R-sq = 45.2% R-sq(adj) = 42.2%

Appendix 5.7k: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by* Vibrio harveyi *strain DPEX over 20min

The regression equation is: DPEX = 0.213 + 0.000690 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*	*SOURCE*	*DF*	*SS*	*MS*	*F*	*p*
Constant	0.213100	0.003708	57.47	<0.001	Regression	1	0.00047610	0.00047610	5.19	0.035
time	0.0006900	0.0003028	2.28	0.035	Error	18	0.00164990	0.00009166		
					Total	19	0.00212600			

s = 0.009574 R-sq = 22.4% R-sq(adj) = 18.1%

Appendix 5.7l: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *plasma by zymosan over 20min

The regression equation is: plasma = 0.234 + 0.00258 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.234133	0.006308	37.12	<0.001
time	0.0025800	0.0005151	5.01	<0.001

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0049923	0.0049923	25.09	<0.001
Error	13	0.0025866	0.0001990		
Total	14	0.0075789			

s = 0.01411 R-sq = 65.9% R-sq(adj) = 63.2%

Appendix 5.7k: Regression Analysis on increase of absorbance value due to activation of* Penaeus indicus *haemocyte lysate by control over 20min

The regression equation is: cont-in = 0.00400 + 0.000347 time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.004000	0.001134	3.53	0.004
time	0.00034667	0.00009260	3.74	0.002

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.000090133	0.000090133	14.02	0.002
Error	13	0.00008360	0.000006431		
Total	14	0.000173733			

s = 0.002536 R-sq = 51.9% R-sq(adj) = 48.2%

Appendix 5.7l: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by zymosan over 20min

The regression equation is: ZY = 0.218 + 0.00152 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.217533	0.008117	26.80	<0.001
Time	0.0015200	0.0006627	2.29	0.039

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0017328	0.0017328	5.26	0.039
Error	13	0.0042821	0.0003294		
Total	14	0.0060149			

s = 0.01815 R-sq = 28.8% R-sq(adj) = 23.3%

Appendix 5.7m: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Aeromonas hydrophila *over 20min

The regression equation is: AH = 0.320 + 0.00249 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.32013	0.01126	28.43	<0.001
Time	0.0024933	0.0009196	2.71	0.018

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0046625	0.0046625	7.35	0.018
Error	13	0.0082444	0.0006342		
Total	14	0.0129069			

s = 0.02518 R-sq = 36.1% R-sq(adj) = 31.2%

Appendix 5.7n: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Vibrio harveyi strain *BP03 over 20min

The regression equation is: BP03 = 0.319 + 0.00106 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.319133	0.004798	66.52	<0.001
Time	0.0010600	0.0003917	2.71	0.018

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0008427	0.0008427	7.32	0.018
Error	13	0.0014962	0.0001151		
Total	14	0.0023389			

s = 0.01073 R-sq = 36.0% R-sq(adj) = 31.1%

Appendix 5.7o: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Vibrio harveyi strain *BP04 over 20min

The regression equation is: BP04 = 0.347 + 0.00214 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.347333	0.006726	51.64	<0.001
Time	0.002140	0.0005491	3.90	0.002

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0034347	0.0034347	15.19	0.002
Error	13	0.0029402	0.0002262		
Total	14	0.0063749			

s = 0.01504 R-sq = 53.9% R-sq(adj) = 50.3%

Appendix 5.7p: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Vibrio harveyi strain *BP05 over 20min

The regression equation is: BP05 = 0.409 + 0.00118 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.409200	0.006488	63.08	<0.001
Time	0.0011800	0.0005297	2.23	0.044

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0010443	0.0010443	4.96	0.044
Error	13	0.0027357	0.0002104		
Total	14	0.0037800			

s = 0.01451 R-sq = 27.6% R-sq(adj) = 22.1%

Appendix 5.7q: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Vibrio harveyi strain *DPEX over 20min

The regression equation is: DPEX = 0.252 + 0.00115 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.251600	0.003884	64.77	<0.001
Time	0.0011467	0.0003172	3.62	0.003

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.00098613	0.00098613	13.07	0.003
Error	13	0.00098080	0.00007545		
Total	14	0.00196693			

s = 0.008686 R-sq = 50.1% R-sq(adj) = 46.3%

Appendix 5.7r: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Vibrio harveyi strain *IN7 over 20min

The regression equation is: IN7 = 0.302 + 0.00289 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.301800	0.008410	35.88	<0.001
Time	0.0028867	0.0006867	4.20	0.001

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0062496	0.0062496	17.67	0.001
Error	13	0.0045977	0.0003537		
Total	14	0.0108473			

s = 0.01881 R-sq = 57.6% R-sq(adj) = 54.4%

Appendix 5.7s: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *haemocyte lysate by* Escherichia coli *strain XL-1 Blue MRF over 20min

The regression equation is: *E. coli* = 0.262 -0.000100 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.261533	0.006528	40.06	<0.001
Time	-0.0001000	0.0005330	-0.19	0.854

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.0000075	0.0000075	0.04	0.854
Error	13	0.0027702	0.0002131		
Total	14	0.0027777			

s = 0.01460 R-sq = 0.3% R-sq(adj) = 0.0%

Appendix 5.7t: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *plasma by zymosan over 20min

The regression equation is: plasma = 0.254 - 0.00224 Time

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	0.25373	0.02705	9.38	<0.001
Time	-0.002240	0.002209	-1.01	0.329

SOURCE	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	0.003763	0.003763	1.03	0.329
Error	13	0.047566	0.003659		
Total	14	0.051329			

s = 0.06049 R-sq = 7.3% R-sq(adj) = 0.2%

Appendix 5.7t: Regression Analysis on increase of absorbance value due to activation of* Penaeus vannamei *plasma by Sodium cacodylate (controls) over 20min

The regression equation is: control = - 0.0241 - 0.00522 Time

Predictor	Coef	Stdev	t ratio	p
Constant	0.024133	0.002990	8.07	<0.001
Time	0.0052200	0.0002441	21.38	<0.001

SOURCE	DF	SS	MS	F	p
Regression	1	0.020436	0.020436	457.24	<0.001
Error	13	0.000581	0.000045		
Total	14	0.021017			

s = 0.006685 R-sq = 97.2% R-sq(adj) = 97.0%

Appendix 5.8: Haemolymph volumes of* Penaeus vannamei *of different weights using radioactive carbon[14] inulin dilutions obtained at I, 2 and 3h following injection into the ventral body sinuses.

	Radioactivity levels (disintegrations per minute) (dpm					*estimated haemolymph volume (µl)*		
Weights (g)	*1hr*	*2hr*	*3hr*	*std vol (µl)*	*standard sd*	*1hr*	*2hr*	*3hr*
2.10	8123.8	17326.8	*	1950	51252.7	6044.06	5097.19	*
12.20	5011.8	4435.0	3489.9	2950	34058.0	6364.24	6423.59	6.52083
3.80	35329.4	37270.5	*	3950	26741.3	3244.95	3045.23	*
13.90	5611.5	5317.3	4278.6	4950	21293.7	6302.54	6332.81	6.43968
4.05	12877.0	48468.4	31975.9			5555.02	1893.11	3.58998
1.80	53389.9	*	*			1386.75	*	*
4.40	33645.8	15136.5	18561.6			3418.17	5322.54	4.97014
10.73	9937.7	11337.8	8066.8			5857.43	5713.38	6.04992
3.05	49107.4	37171.2	30760.1			1827.37	3055.45	3.71507
8.90	3093.0	2119.7	1806.0			6561.67	6661.81	6.69408
6.75	36544.0	34003.1	29651.1			3119.98	3381.41	3.82917
8.60	18222.6	17071.4	13814.1			5005.02	5123.46	*

* = dead prawn.

***Appendix 5.8a: Regression analyses of standard dilutions of ^{14}C inulin against standard volumes. haemolymph volumes against prawn weights of* Penaeus vannamei**

The regression equation is
dpm = 66868 - 9.72 std vol

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	66868	7042	9.50	0.011
stdvol	-9.719	1.942	-5.01	0.038

Analysis of Variance

Source	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	472330752	472330752	25.05	0.038
Error	2	37705740	18852870		
Total	3	510036480			

s = 4342 R-sq = 92.6% R-sq(adj) = 88.9%

Appendix 5.8b: Regression analyses of estimated* Penaeus vannamei *haemolymph volumes against prawn weights

The regression equation is
Volume = 2.95 + 0.284 weight

Predictor	*Coef*	*Stdev*	*t-ratio*	*p*
Constant	2.9533	0.6425	4.60	0.004
weight	0.28419	0.07257	3.92	0.008

Analysis of Variance

Source	*DF*	*SS*	*MS*	*F*	*p*
Regression	1	9.3151	9.3151	15.33	0.008
Error	6	3.6447	0.6075		
Total	7	12.9598			

s = 0.7794 R-sq = 71.9% R-sq(adj) = 67.2%

Appendix 5.9: Estimated haemolymph volumes of* Penaeus vannamei *juveniles used in* in vivo *bacterial killing assays. Volumes of haemolymph were estimated from the regression analysis in Appendix 5.8b.

weights (g)	*volumes (ml)*	*weights (g)*	*volumes (ml)*
5.4	4.49399	7.8	5.17671
8.1	5.26206	11.8	6.31459
11.4	6.20080	8.25	5.30473
13.4	6.76974	9.30	5.60342
15.4	7.33868	9.60	5.68876
10.1	5.83099	11.80	6.31460
9.2	5.57497	10.70	6.00168
10.9	6.05857	7.60	5.11982
8.2	5.29050	7.90	5.20516
5.2	4.43709	10.10	5.83099
6.9	4.92069	8.00	5.23361
10.5	5.94478	8.50	5.37584
9.2	5.57497	9.00	5.51808
9.8	5.74565		

Appendix 5.9a: Log TVC of* Vibrio harveyi *strain BP04 present in-vivo in haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Vaccine	*3h*	*8h*	*12h*	*24h*	*48h*
SSS	2.71181	2.74036	3.51587	2.52892	1.48144
SSS	2.41162	3.11394	3.57054	1.81757	2.73239
SSS	2.33646	3.26482	2.64246	2.50920	3.41330
DPEX	2.67210	3.02119	2.78958	2.11727	1.60638
DPEX	2.77525	2.80346	3.07188	3.00000	1.65801
DPEX	2.65801	3.42160	2.63246	2.52892	1.70329
BP04	2.74036	3.65992	2.26007	3.23045	2.87622
BP04	2.72016	2.89376	2.23553	2.29447	2.76418
BP04	2.43616	3.05690	2.82086	2.06446	1.81757
E. coli	2.77159	2.29447	3.55509	2.57864	3.20683
E. coli	2.68931	2.69461	2.53529	2.50243	3.28103
E. coli	2.45939	2.61172	2.71600	2.00432	3.43775

Appendix 5.9b: Log TVC of* Vibrio harveyi *strain DPEX present in-vivo in haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Vaccine	*3h*	*8hr*	*12h*	*24h*	*48h*
SSS	2.57287	2.29447	3.24304	2.39270	2.52892
SSS	1.95856	2.08279	2.89098	2.11727	2.19866
SSS	1.95856	2.10037	2.87967	1.78247	1.60638
DPEX	2.93651	3.02531	2.16435	3.12057	2.77887
DPEX	3.00260	2.86747	2.94890	1.74507	2.99123
DPEX	2.56229	2.46687	2.91275	3.24304	2.97313
BP04	2.65801	1.54900	1.90741	2.36549	2.14922
BP04	2.77012	2.82737	3.18752	1.40312	1.87967
BP04	2.27416	3.23045	2.14922	2.96332	1.40312
E. coli	2.04532	3.49554	3.13988	2.30535	3.26482
E. coli	2.61700	2.39270	2.74896	2.60097	3.60423
E. coli	2.65801	2.64738	2.98453	2.00432	3.31175

Appendix 5.9c: Log TVC of* E. coli *present in-vivo in haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Vaccine	*3h*	*8h*	*12h*	*24h*	*48h*
SSS	*2.66745*	3.20412	4.11394	2.30535	2.06446
SSS	2.93247	3.88138	3.03743	2.60097	1.70329
SSS	2.98363	2.78958	4.38739	2.00432	3.51851
DPEX	*2.23553*	2.91540	3.23805	2.55509	2.20952
DPEX	2.78958	2.52244	3.15836	2.81023	2.39270
DPEX	2.83378	2.74507	3.08279	2.69897	2.27184
BP04	*2.97955*	2.38382	2.81757	2.16435	3.10037
BP04	2.94645	2.39270	2.45179	2.14922	3.07555
BP04	2.78958	3.23045	2.29447	2.13354	3.37658
E. coli	*1.78247*	3.41664	3.14613		
E. coli	2.71181	2.92840	3.23045		
E. coli	2.78958	2.73239	3.11394		

Appendix 5.9d: One-way analysis of variance on log TVC of* Vibrio harveyi *strain BP04 present 3h after in-vivo injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.0748	0.0249	1.01	0.437
Error	8	0.1973	0.0247		
Total	11	0.2721			

Treatment	*Code*	*N*	*Mean*	*StDev*
SSS	1	3	2.4866	0.1986
DPEX	2	3	2.7018	0.0640
BP04	3	3	2.6322	0.1701
E. coli	4	3	2.6401	0.1618

Appendix 5.9e: One-way analysis of variance on log TVC of* Vibrio harveyi *strain BP04 present 8h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	0.7869	0.2623	2.77	0.111	SSS	1	3	3.0397	0.2700
Error	8	0.7574	0.0947			DPEX	2	3	3.0821	0.3135
Total	11	1.5442				BP04	3	3	3.2035	0.4036
						E. coli	4	3	2.5336	0.2112

Appendix 5.9f: One-way analysis of variance on log TVC of* Vibrio harveyi *strain BP04 present 12h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	0.992	0.331	1.82	0.222	SSS	1	3	3.2430	0.5208
Error	8	1.453	0.182			DPEX	2	3	2.8313	0.2227
Total	11	2.445				BP04	3	3	2.4388	0.3311
						E. coli	4	3	2.9355	0.5442

Appendix 5.9g: One-way analysis of variance on log TVC of* Vibrio harveyi *strain BP04 present 24h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF`` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	0.149	0.050	0.24	0.868	SSS	1	3	2.2852	0.4051
Error	8	1.676	0.209			DPEX	2	3	2.5487	0.4417
Total	11	1.825				BP04	3	3	2.5298	0.6176
						E. coli	4	3	2.3618	0.3119

Appendix 5.9h: One-way analysis of variance on log TVC of* Vibrio harveyi *strain BP04 present 48h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF`` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	4.105	1.368	4.16	0.047	SSS	1	3	2.5424	0.9798
Error	8	2.629	0.329			DPEX	2	3	1.6559	0.0485
Total	11	6.734				BP04	3	3	2.4860	0.5816
						E. coli	4	3	3.3085	0.1179

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	0.88650	-0.61365	2.38665

2	1	3	0.05640	-1.44375	1.55655
3	1	4	-0.76610	-2.26625	0.73405
4	2	3	-0.83010	-2.33025	0.67005
5	2	4	-1.65260	-3.15275*	-0.15245*
6	3	4	-0.82250	-2.32265	0.67765

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.9i: One-way analysis of variance on log TVC of* Vibrio harveyi *strain DPEX present 3h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.6987	0.2329	2.54	0.130
Error	8	0.7343	0.0918		
Total	11	1.4330			

Treatment	*Code*	*N*	*Mean*	*StDev*
SSS	1	3	2.1633	0.3547
DPEX	2	3	2.8338	0.2374
BP04	3	3	2.5674	0.2601
E. coli	4	3	2.4401	0.3425

Appendix 5.9j: One-way analysis of variance on log TVC of* Vibrio harveyi *strain DPEX present 8h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.876	0.292	0.97	0.452
Error	8	2.402	0.300		
Total	11	3.278			

Treatment	*Code*	*N*	*Mean*	*StDev*
SSS	1	3	2.1592	0.1175
DPEX	2	3	2.7865	0.2879
BP04	3	3	2.5356	0.8779
E. coli	4	3	2.8452	0.5774

Appendix 5.9k: One-way analysis of variance on log TVC of* Vibrio harveyi *strain DPEX present 12h after in-vivo injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.676	0.225	1.22	0.364
Error	8	1.480	0.185		
Total	11	2.156			

Treatment	*Code*	*N*	*Mean*	*StDev*
SSS	1	3	3.0046	0.2066
DPEX	2	3	2.6753	0.4429
BP04	3	3	2.4147	0.6801
E. coli	4	3	2.9578	0.1968

Appendix 5.9l: One-way analysis of variance on log TVC of* Vibrio harveyi *strain DPEX present 24h after in-vivo injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF' *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*

Treatment	3	0.603	0.201	0.54	0.669	SSS	1	3	2.0975	0.3056
Error	8	2.988	0.373			DPEX	2	3	2.7029	0.8318
Total	11	3.591				BP04	3	3	2.2440	0.7872
						E. coli	4	3	2.3035	0.2983

Appendix 5.9m: One-way analysis of variance on log TVC of* Vibrio harveyi *strain DPEX present 48h after in-vivo injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	4.750	1.583	15.49	0.001	SSS	1	3	2.1113	0.4674
Error	8	0.818	0.102			DPEX	2	3	2.9144	0.1177
Total	11	5.568				BP04	3	3	1.8107	0.3778
						E. coli	4	3	3.3936	0.1839

Tukey's pairwise comparisons

Row	*Code.*	*Code,*	*Diffmean*	*Low_ci*	*Up_ci*
1	1	2	-0.8031	-1.63839	0.03219
2	1	3	0.3006	-0.53469	1.13589
3	1	4	-1.2823	-2.11759*	-0.44701*
4	2	3	1.1037	0.26841*	1.93899*
5	2	4	-0.4792	-1.31449	0.35609
6	3	4	-1.5829	-2.41819*	-0.74761*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.9n: One-way analysis of variance on log TVC of* Escherichia coli *strain XL-1 Blue MRF` present 3h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatmen t*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	0.446	0.149	1.28	0.345	SSS	1	3	2.8612	0.1697
Error	8	0.928	0.116			DPEX	2	3	2.6196	0.3334
Total	11	1.374				BP04	3	3	2.9052	0.1015
						E. coli	4	3	2.4280	0.5604

Appendix 5.9o: One-way analysis of variance on log TVC of* Escherichia coli *strain XL-1 Blue MRF` present 8h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF`` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	0.747	0.249	1.42	0.308	SSS	1	3	3.2917	0.5511
Error	8	1.406	0.176			DPEX	2	3	2.7276	0.1971
Total	11	2.154				BP04	3	3	2.6690	0.4863
						E. coli	4	3	3.0258	0.3524

Appendix 5.9p: One-way analysis of variance on log TVC of* Escherichia coli *strain XL-1 Blue MRF` present 12h after* in-vivo *injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	2.635	0.878	5.94	0.020	SSS	1	3	3.8463	0.7137
Error	8	1.182	0.148			DPEX	2	3	3.1597	0.0776
Total	11	3.817				BP04	3	3	2.5213	0.2684
						E. coli	4	3	3.1635	0.0602

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	0.68660	-0.31956	1.69276
2	1	3	1.32500	0.31884*	2.33116*
3	1	4	0.68280	-0.32336	1.68896
4	2	3	0.63840	-0.36776	1.64456
5	2	4	-0.00380	-1.00996	1.00236
6	3	4	-0.64220	-1.64836	0.36396

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.9q: One-way analysis of variance on log TVC of* Escherichia coli *strain XL-1 Blue MFR` present 24h after in-vivo injection into haemolymph of Penaeus vannamei juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	N	Mean	StDev
Treatment	2	0.4623	0.2312	6.57	0.031	SSS	3	2.3035	0.2983
Error	6	0.2112	0.0352			DPEX	3	2.6881	0.1279
Total	8	0.6735				BP04	3	2.1490	0.0154
						E. coli			

Tukey's pairwise comparisons

Row	Code.	Code,	Diffmean	Low_ci	Up_ci
1	1	2	-0.3846	-0.854711	0.08551
2	1	3	0.1545	-0.315611	0.62461
3	2	3	0.5391	0.068989*	1.00921*

Comparison is horizontal, * indicates a significant difference at the 95% confidence interval.

Appendix 5.9r: One-way analysis of variance on log TVC of* Escherichia coli *strain XL-1 Blue MRF` present 48h after in-vivo injection into haemolymph of* Penaeus vannamei *juveniles which had been previously vaccinated with either* Vibrio harveyi *strains BP04 (virulent) or DPEX (avirulent), or* Escherichia coli *strain* XL1-Blue MRF` *(avirulent). Controls were injected with a placebo treatment consisting of equal volumes of shrimp salt solution (SSS) at various times after injection

Source	DF	SS	MS	F	p	Treatment	N	Mean	StDev
Treatment	2	1.387	0.693	2.17	0.196	SSS	3	2.4288	0.9609
Error	6	1.920	0.320			DPEX	3	2.2914	0.0931
Total	8	3.306				BP04	3	3.1842	0.1671

Appendix 6.1: Calculations of Active ingredient concentrations in the fungicides.

Tilt (250EC (25% active ingredient)

250EC = (1000µl of tilt contains 250µl of active ingredient (25% of 1000)).
Specific gravity = 1.27. i.e: 1ml of Tilt weighs 1mg
Now, since specific gravity of distilled water = 1.00,
Tilt is 27% heavier than distilled water. So, we must subtract the 27% to get the actual weight of the active ingredient in 1ml.

Since 1ml of tilt weighs 250mg, removing 27% from it leaves us with an active ingredient of 182.5mg.ml^{-1}.

Calixin (78% active ingredient (tridemorph) and specific gravity of 0.86)

Calixin is 0.14% lighter than distilled water. So we must add the 14% to get the actual weight of the active ingredient in 1ml.

Since 1ml of tridemorph weighs 780mg, then we would have to add 14% (109.2) giving us an active ingredient concentration of 889.20mg.ml^{-1} of calixin.

Calculation of concentrations of fungicides added per kg of diet.

Each fungicide was added at 1000, 2500 and 5000µg.kg^{-1}. To give a cocktail of 2000, 5000 and 10000µg.kg^{-1} respectively.

Tilt
1ml (Tilt) 182.5mg active ingredient and
4ml (Tilt) 730.0mg active ingredient

adding 96ml distilled water,
100ml (mixture) = 730mg
1ml = 7300µg
since,
7300µg = 1000µl (mixture)
1000µg = 136.99µl (mixture),
2500µg = 342.47µl (mixture) and
5000µg = 684.93µl(mixture)

Calixin
1ml (calixin) = 889.20mg active ingredient
adding 249ml distilled water,

250ml = 889.20mg and
1ml = 3.56mg
since,
3556.80µg = 1000µl (mixture)
1000µg = 281.15µl (mixture)
2500µg = 702.88µl (mixture)
5000µg = 1405.75µl (mixture)

Appendix 6.2: Percentage survivals of* Penaeus indicus *juveniles after 28 days feeding on diets containing a 1:1(vol:vol) cocktail of the fungicides, tridemorph and propiconazole added at 0µg, 2000µg, 5000µg and 10000µg.kg-1 of diet, called Diets A, B, C and D respectively.

Day	*Diet A*	*Diet B*	*Diet C*	*Diet D*
1	100.000	100.000	100.000	100.000
1	100.000	100.000	100.000	100.000

1	100.000	100.000	100.000	100.000
2	96.667	100.000	100.000	100.000
2	100.000	96.667	93.333	100.000
2	100.000	100.000	100.000	100.000
3	86.667	96.667	100.000	100.000
3	96.667	96.667	93.333	100.000
3	100.000	100.000	100.000	100.000
4	86.667	96.667	100.000	100.000
4	96.667	96.667	93.333	100.000
4	100.000	100.000	100.000	100.000
5	86.667	96.667	100.000	100.000
5	96.667	96.667	93.333	100.000
5	100.000	100.000	96.667	100.000
6	83.333	96.667	100.000	100.000
6	96.667	96.667	93.333	100.000
6	100.000	100.000	96.667	100.000
7	80.000	96.667	100.000	96.667
7	96.667	96.667	93.333	100.000
7	100.000	100.000	96.667	96.667
8	80.000	96.667	100.000	96.667
8	96.667	96.667	93.333	100.000
8	100.000	100.000	93.333	96.667
9	80.000	93.333	100.000	96.667
9	93.333	96.667	93.333	96.667
9	100.000	100.000	93.333	96.667
10	80.000	93.333	100.000	96.667
10	93.333	96.667	90.000	96.667
10	96.667	100.000	93.333	96.667
11	80.000	90.000	100.000	96.667
11	93.333	96.667	90.000	96.667
11	96.667	100.000	93.333	96.667
12	80.000	90.000	100.000	96.667
12	93.333	96.667	90.000	96.667
12	96.667	100.000	93.333	96.667
13	80.000	90.000	100.000	96.667
13	93.333	96.667	90.000	96.667
13	96.667	100.000	93.333	96.667
14	80.000	90.000	100.000	96.667
14	93.333	96.667	90.000	90.000
14	96.667	100.000	90.000	96.667
15	80.000	90.000	100.000	96.667
15	93.333	93.333	86.667	90.000
15	96.667	96.667	90.000	96.667
16	80.000	90.000	100.000	96.667
16	93.333	93.333	86.667	90.000
16	96.667	96.667	90.000	96.667
17	73.333	90.000	100.000	96.667
17	90.000	86.667	86.667	86.667
17	93.333	96.667	90.000	93.333
18	73.333	86.667	100.000	66.667
18	90.000	86.667	86.667	86.667
18	93.333	96.667	90.000	96.667
19	73.333	86.667	100.000	66.667
19	90.000	86.667	86.667	86.667
19	93.333	93.333	90.000	96.667
20	70.000	86.667	100.000	66.667
20	86.667	86.667	80.000	86.667
20	93.333	93.333	90.000	96.667
21	70.000	86.667	100.000	66.667

21	86.667	86.667	80.000	86.667
21	93.333	93.333	90.000	96.667
22	70.000	86.667	100.000	66.667
22	86.667	86.667	80.000	86.667
22	93.333	93.333	90.000	96.667
23	70.000	86.667	100.000	66.667
23	86.667	86.667	80.000	86.667
23	93.333	86.667	90.000	93.333
24	70.000	86.667	100.000	66.667
24	86.667	86.667	80.000	86.667
24	93.333	86.667	90.000	90.000
25	70.000	86.667	100.000	66.667
25	86.667	86.667	80.000	76.667
25	93.333	83.333	90.000	93.333
26	70.000	83.333	96.667	66.667
26	86.667	86.667	73.333	73.333
26	93.333	83.333	90.000	93.333
27	70.000	83.333	96.667	63.333
27	86.667	86.667	73.333	76.667
27	93.333	83.333	86.667	93.333
28	70.000	83.333	96.667	63.333
28	86.667	86.667	73.333	76.667
28	93.333	83.333	86.667	93.333

Appendix 6.2a: One-way analysis of variance on the arc sine transformations of percentage survival obtained with* Penaeus indicus *juveniles after 28 days of feeding on diets containing a 1:1(vol:vol) cocktail of the fungicides, tridemorph and propiconazole added at 0μg, 2000μg, 5000μg and 10000μg.kg^{-1} of diet, called Diets A, B, C and D respectively.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.0303	0.0101	0.23	0.874
Error	8	0.3537	0.0442		
Total	11	0.3840			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	3	3	1.0092	0.2168
Diet B	3	3	1.0062	0.0366
Diet C	3	3	1.0612	0.2446
Diet D	3	3	0.9210	0.2621

Appendix 6.3: Final weights obtained after feeding* Penaeus indicus *juveniles on diet A (Table 6.0) supplemented with 0μg (Diet A), 2000μg (Diet B), 5000μg (Diet C) and 10000μg (Diet D) per kg of a 1:1(vol:vol) cocktail of the fungicides, tridemorph and propiconazole.

Diet A	*Diet B*	*Diet C*	*Diet D*
1.300	1.033	1.500	1.231
0.934	1.605	1.576	0.546
1.355	1.108	1.264	1.779
1.028	1.245	1.159	0.951
0.943	0.965	1.417	1.131
1.400	1.199	1.360	1.127
0.836	1.386	0.951	1.106
1.273	1.084	0.832	1.400
0.936	0.925	0.997	1.115
1.232	0.871	1.046	1.113
1.087	1.000	0.874	0.345
1.312	1.078	0.826	0.946
0.583	1.539	0.500	1.523
1.085	0.951	1.677	1.665
1.106	1.314	1.268	0.442
1.060	0.957	0.758	0.347
1.100	1.325	1.254	1.606

1.020	1.040	1.300	0.782
1.095	0.425	1.292	0.781
1.039	1.295	0.591	0.922
0.732	1.441	0.571	0.773
1.466	1.052	0.336	0.893
0.708	1.118	1.390	1.162
1.322	1.206	1.136	1.064
1.083	1.034	1.700	1.096
0.988	1.042	1.162	0.500
0.315	1.129	1.506	1.168
0.600	1.466	1.091	0.686
0.738	0.780	2.048	0.956
0.827	0.778	2.077	1.287
1.404	1.052	0.677	0.930
1.293	1.647	1.215	0.777
0.985	1.116	1.016	1.010
1.200	0.895	0.418	0.839
1.821	0.978	0.413	1.025
1.916	0.987	1.526	1.070
1.274	1.325	1.100	1.121
1.462	1.040	0.880	0.979
1.430	0.425	1.206	0.967
0.682	1.213	0.988	0.692
0.758	0.582	0.810	1.035
0.700	1.602	0.421	
0.925	0.845	1.165	
1.239	0.906	1.030	
1.256	1.149	1.107	
1.833	0.867	0.871	
1.213	1.673	0.400	
	1.222	1.177	
	1.066	0.998	
	0.785	1.231	
	0.552	1.136	
	0.803	1.172	
	1.300	1.071	
	0.817	1.614	
	0.770	1.283	
	0.828	0.974	
	1.042	0.770	
	0.886	0.557	
		0.664	
		1.007	
		0.791	

Appendix 6.3a: One-way analysis of variance of final weights obtained after feeding* Penaeus indicus *juveniles on diet A (Table 6.0) supplemented with 0μg (Diet A), 2000μg (Diet B), 5000μg (Diet C) and 10000μg (Diet D) per kg of a 1:1(vol:vol) cocktail of the fungicides, tridemorph and propiconazole.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.258	0.086	0.78	0.509
Error	203	22.480	0.111		
Total	206	22.737			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	1	47	1.1041	0.3284
Diet B	2	58	1.0649	0.2803
Diet C	3	61	1.0680	0.3835
Diet D	4	41	0.9973	0.3246

Appendix 6.4: Percentage survival values obtained when Penaeus indicus juveniles previously fed on a diet containing 0ug, 2000µg, 5000µg and 10000µg of mixed fungicide cocktail (tridemorph and propiconazole) per kg of diet for 28 days, were subsequently challenged by injection of 100µl of pathogenic* Vibrio harveyi *strain BP03 containing 10^3 cells.

Day	Diet A	Diet B	Diet C	Diet D
1	100	100	100	100
1	100	100	100	100
1	100	100	100	100
2	100	60	70	80
2	90	60	90	100
2	70	60	80	100
3	100	60	70	70
3	90	60	90	80
3	70	60	70	100
4	100	60	70	70
4	90	60	90	80
4	70	60	60	100
5	100	60	70	60
5	80	60	90	80
5	70	60	60	100
6	100	60	70	60
6	80	60	90	80
6	70	60	60	100
7	100	60	70	60
7	80	60	90	80
7	70	60	60	100
8	100	60	70	60
8	80	60	90	80
8	70	60	60	100

Appendix 6.4a: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 1 after challenge.

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	0.8381	0.2794	3.42	0.073	Diet A	1	3	1.1553	0.3989
Error	8	0.6539	0.0817			Diet B	2	3	0.6435	0.0000
Total	11	1.4920				Diet C	3	3	0.9408	0.1726
						Diet D	4	3	1.3563	0.3715

Appendix 6.4b: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 3 after challenge.

Source	DF	SS	MS	F	p	Treatment	Code	N	Mean	StDev
Treatment	3	0.4785	0.1595	1.69	0.245	Diet A	1	3	1.1553	0.3989
Error	8	0.7539	0.0942			Diet B	2	3	0.6435	0.0000
Total	11	1.2324				Diet C	3	3	0.8902	0.1988
						Diet D	4	3	1.0912	0.4223

Appendix 6.4c: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 4 after challenge.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.4973	0.1658	1.67	0.250
Error	8	0.7958	0.0995		
Total	11	1.2931			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	1	3	1.1553	0.3989
Diet B	2	3	0.6435	0.0000
Diet C	3	3	0.8462	0.2459
Diet D	4	3	1.0912	0.4223

Appendix 6.4d: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 5 after challenge.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.380	0.127	1.09	0.407
Error	8	0.929	0.116		
Total	11	1.309			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	1	3	1.0912	0.4223
Diet B	2	3	0.6435	0.0000
Diet C	3	3	0.8462	0.2459
Diet D	4	3	1.0472	0.4751

Appendix 6.4e: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 6 after challenge.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.380	0.127	1.09	0.407
Error	8	0.929	0.116		
Total	11	1.309			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	1	3	1.0912	0.4223
Diet B	2	3	0.6435	0.0000
Diet C	3	3	0.8462	0.2459
Diet D	4	3	1.0472	0.4751

Appendix 6.4f: One-way analysis of variance on arc sine transformation of survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 7 after challenge.

Source	*DF*	*SS*	*MS*	*F*	*p*
Treatment	3	0.380	0.127	1.09	0.407
Error	8	0.929	0.116		
Total	11	1.309			

Treatment	*Code*	*N*	*Mean*	*StDev*
Diet A	1	3	1.0912	0.4223
Diet B	2	3	0.6435	0.0000
Diet C	3	3	0.8462	0.2459
Diet D	4	3	1.0472	0.4751

Appendix 6.4g: One-way analysis of variance on the arc sine transformations of percentage survival of* Penaeus indicus *juveniles fed on diet a (Table 6.0) supplemented with a cocktail of the fungicides, tridemorph and propiconazole at 0µg, 2000µg, 5000µg and 10000µg per kg of diets (called Diets A, B, C and D respectively) for 28 days and subsequently challenged by a challenge of 10^3 cfu of pathogenic* Vibrio harveyi *strain BP03. Day 8 after challenge.

Source	*DF*	*SS*	*MS*	*F*	*p*	*Treatment*	*Code*	*N*	*Mean*	*StDev*
Treatment	3	0.380	0.127	1.09	0.407	Diet A	1	3	1.0912	0.4223
Error	8	0.929	0.116			Diet B	2	3	0.6435	0.0000
Total	11	1.309				Diet C	3	3	0.8462	0.2459
						Diet D	4	3	1.0472	0.4751

Appendix 6.5: Weights (g), increases in weight and moulting rates obtained when early juveniles of* Penaeus indicus *are fed with diet a (table 6.0) supplemented with 10000µg of mixed fungicides (tridemorph and propiconazole) cocktail (Diet D). Controls (Diet A) had no added fungicides.

Days	*Diet A*	*Diet D*	*Increase in weight (Diet A)*	*Increase in weight (Diet D)*
1	0.1366	0.1148	0.0000	0.000000
1	0.1017	0.1440	0.0000	0.000000
1	0.1048	0.1189	0.0000	0.000000
1	0.1480	0.1154	0.0000	0.000000
1	0.1319	0.1032	0.0000	0.000000
1	0.1417	0.1390	0.0000	0.000000
1	0.1077	0.1552	0.0000	0.000000
1	0.1317	0.1474	0.0000	0.000000
1	0.1240	0.1107	0.0000	0.000000
2	0.1013	0.1279	0.0049	-0.006200
2	0.1421	0.1595	0.0065	0.006500
2	0.1338	0.1296	0.0064	0.016800
2	0.1415	0.1530	0.0106	0.012500
2	0.1820	0.1505	0.0102	0.002900
2	0.1112	0.1582	-0.0015	0.019200
2	0.1586	0.1086	-0.0064	-0.002200
2	0.1338	0.1061	0.0021	0.012100
2	0.1402	0.1357	0.0098	0.018900
3	0.1500	0.1456	0.0134	-0.006200
3	0.1481	0.1624	0.0054	0.012300
3	0.1518	0.1518	0.0146	0.008600
3	0.1640	0.1213	0.0160	0.005900
3	0.1157	0.1563	0.0162	0.013200
3	0.1194	0.1275	0.0101	0.012800
3	0.0903*	0.1086	-0.0174	-0.009600
3	0.1071	0.1164	-0.0160	0.015000
3	0.1339	0.1234	0.0099	0.012700
4	0.1720*	0.1458	0.0107	0.003800
4	0.1393	0.1442*	0.0049	0.000200
4	0.1294*	0.1492*	-0.0009	0.020400
4	0.0904	0.1200	0.0240	0.004600
4	0.1096*	0.1035*	0.0074	0.000300
4	0.1437*	0.1393*	0.0020	0.010200
4	0.1473	0.1186*	-0.0173	-0.009400
4	0.1066*	0.1664*	-0.0221	0.019000
4	0.1039*	0.1279*	0.0054	0.017200
5	0.1561	0.1407*	0.0195	0.008400
5	0.1094	0.1490	0.0077	0.005000
5	0.1071	0.1533	0.0023	0.029100
5	0.0938	0.1278*	0.0168	0.012400

5	0.1137	0.1695	0.0054	0.005300
5	0.1314	0.1480	-0.0034	0.014300
5	0.1648	0.1232	-0.0139	-0.014500
5	0.1373	0.1085	-0.0180	0.022100
5	0.1383	0.1300	0.0074	0.019300
6	0.1538	0.1283	0.0172	0.011400
6	0.1526*	0.1744	0.0124	0.007000
6	0.1391*	0.1471	0.0015	0.028200
6	0.0971*	0.1262	0.0223	0.012900
6	0.1145	0.1101	0.0207	0.006900
6	0.1375	0.1489	-0.0026	0.009900
6	0.1703	0.1431	-0.0106	-0.012100
6	0.1141	0.1510	-0.0172	0.000300
6	0.1063	0.1325	0.0135	0.021800
7	0.1903*	0.1490	0.0329	0.020000
7	0.1604	0.2002*	0.0159	0.022400
7	0.1409	0.1615*	0.0024	0.042600
7	0.1038*	0.1385*	0.0423	0.023100
7	0.1180	0.1664*	0.0285	0.010100
7	0.1072	0.1413*	0.0041	0.034300
7	0.1695	0.1348	-0.0039	-0.006200
7	0.1176	0.1133	-0.0137	0.052800
7	0.1458	0.1733*	0.0169	0.030600
8	0.1793*	0.1421	0.0427	0.027100
8	0.1281*	0.1216*	0.0264	0.025400
8	0.1533*	0.1799	0.0029	0.027500
8	0.2007	0.1419*	0.0527	0.026700
8	0.1334*	0.2024	0.0343	0.018400
8	0.1077*	0.1464	0.0116	0.040900
8	0.1062	0.1476	-0.0015	-0.007600
8	0.1662	0.1694	0.0017	0.055000
8	0.1493	0.1663	0.0253	0.055600
9	0.1125*	0.1565*	0.0414	0.026900
9	0.1319	0.2080	0.0300	0.030400
9	0.1513	0.1675	0.0041	0.027500
9	0.2016	0.1417	0.0536	0.028300
9	0.1317	0.1251	0.0373	0.021900
9	0.1089	0.1464	0.0105	0.043600
9	0.1780	0.1437	0.0048	0.001300
9	0.1692	0.1744	0.0002	0.060600
9	0.1522	0.1826	0.0273	0.056800
10	0.2120	0.1486	0.0485	0.031600
10	0.1843*	0.2142	0.0345	0.034200
10	0.1640*	0.1727	0.0037	0.032300
10	0.1167	0.1589	0.0640	0.033200
10	0.1362	0.1782	0.0524	0.021200
10	0.1085	0.1893	0.0223	0.050300
10	0.1851	0.1464	0.0090	0.003700
10	0.1418	0.1244	0.0101	0.066800
10	0.1549	0.1512	0.0309	0.062000
11	0.2326*	0.1534*	0.0637	0.026000
11	0.1430	0.1262	0.0335	0.054400
11	0.1648	0.1572*	0.0033	0.038300
11	0.2003*	0.1661	0.0846	0.038000
11	0.1352	0.2420*	0.0560	0.023000
11	0.1543	0.2071*	0.0231	0.068100
11	0.1180	0.1408	0.0103	0.010900
11	0.1879	0.1984*	0.0113	0.094600
11	0.1081	0.1802*	0.0303	0.069500

12	0.2395	0.1452*	0.0696	0.030400
12	0.1532*	0.1299*	0.0352	0.063300
12	0.1628*	0.1564	1.0000	0.037500
12	0.2062	0.1563	0.0915	0.040900
12	0.1369*	0.2073	0.0564	0.026700
12	0.1732	0.1793	0.0315	0.065000
12	0.1188	0.1667	0.0111	0.011500
12	0.1883	0.2449	0.0215	0.097500
12	*	0.2040	0.0388	0.068600
13	0.2100	0.1658	0.0734	0.047100
13	0.1449	0.2516	0.0432	0.062500
13	0.1978	0.2215	1.0000	0.046100
13	0.2514	0.1619	0.1034	0.050400
13	0.1641	0.2065	0.0627	0.028800
13	0.1808	0.1988	0.0561	0.082500
13	0.1198	0.1669*	0.0121	0.011700
13	0.1964	0.1320	0.0324	0.104200
13	dead	0.1650	0.0568	0.088100
14	0.2186	0.1594	0.0820	0.044600
14	0.2018	0.1420	0.0421	0.067900
14	0.1872	0.1725	1.0000	0.053600
14	0.2543	0.1631	0.1063	0.047700
14	0.1438	0.2573	0.0699	0.038800
14	0.1766	0.2185	0.0455	0.079500
14	0.1233	0.1806	0.0156	0.025400
14	0.1561	0.2119	0.0244	0.109900
14	dead	0.1895	0.0526	0.078800

* identifies a newly moulted prawn.

Appendix 6.5a: Upper tailed two-sample T-test and confidence intervals obtained on final weights of* Penaeus indicus *juveniles fed for 14 days on diet a (Table 6.0) with 10000ug of fungicide cocktail (tridemorph and propiconazole added per kg of diet (Diet D). controls had no added diet (Diet A).

95% C.I. for mu dieta - mu dietd: (-0.046, 0.035g)

Treatment	*N*	*Mean*	*StDev*	*SE Mean*
Diet A	8	0.1827	0.0424	0.015
Diet D	9	0.1883	0.0356	0.012

T-Test mu dieta = mu dietd (vs >): T= -0.30 P=0.61 DF= 15

Appendix 6.5b: General linear model regression analysis and T-tests on differences in growth rates of* Penaeus indicus *juveniles fed for 14 days on diet a (Table 6.0) with 10000ug of fungicide cocktail (tridemorph and propiconazole added per kg of diet (Diet D). Controls (Diet A), had no added fungicides.

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
Day	1	0.82200	0.81887	0.81887	280.09	<0.001
Treatment	1	0.01575	0.00068	0.00068	0.23	0.629
Treatment*day	1	0.00133	0.00133	0.00133	0.45	0.502
Error	220	0.64319	0.64319	0.00292		
Total	223	1.48227				

T-test

Term	*Coeff*	*Stdev*	*t-value*	*P*
Constant	0.030313	0.007776	3.90	<0.001
Day	0.014881	0.000889	16.74	<0.001
Day*diet A	-0.000599	0.000889	-0.67	0.502
Day*diet D	0.000599	0.000889	0.67	0.502

Appendix 6.6a: General linear model regression analysis and T-tests on differences in moulting rates of* Penaeus indicus *juveniles fed for 14 days on diet a (Table 6.0) with 10000μ g of fungicide cocktail (tridemorph and propiconazole) added per kg of diet (Diet D). Controls (Diet A), had no added fungicides.

Source	*DF*	*Seq SS*	*Adj SS*	*Adj MS*	*F*	*P*
time	1	2400.05	2400.05	2400.05	795.65	<0.001
treatmn	1	2.29	0.27	0.27	0.09	0.766
treatmn*time	1	1.98	1.98	1.98	0.66	0.426
Error	24	72.40	72.40	3.02		
Total	27	2476.71				

T-test

Term	*Coeff*	*Stdev*	*t-value*	*P*
Const	-3.4396	0.6933	-4.96	<0.001
time	2.29670	0.08142	28.21	<0.001
time*Diet A	-0.06593	0.08142	-0.81	0.426
time*Diet D	0.06593	0.08142	0.81	0.426

CONTRIBUTING MATERIAL

larvi '95

fish & shellfish larviculture symposium

gent, belgium, september 3-7, 1995

editors

patrick lavens
edmonde jaspers
ivo roelants

european aquaculture society
special publication no. 24
gent, belgium
august, 1995

LARVI '95 - FISH & SHELLFISH LARVICULTURE SYMPOSIUM
P. Lavens, E. Jaspers, and I. Roelants (Eds)
European Aquaculture Society, Special Publication No. 24, Gent, Belgium. 1995.

THE USE OF VACCINES AND IMMUNOSTIMULANTS IN THE CULTURE OF PENAEID SHRIMPS

J.W. Latchford[1], S.B. Prayitno[2], and A. Alabi[1]

[1] School of Ocean Sciences, University of Wales Bangor, Menai Bridge, Gwynedd, UK
[2] Dept. of Fisheries, Diponegoro University, Semarang 50242, Indonesia

Abstract

The increased incidence of diseases in cultured shrimps coupled with a growing awareness of the problems of the use of antibiotics in controlling such diseases has led to the development of alternative methods of disease control. Vaccines against several strains of luminous and non-luminous bacterial pathogens were tested for their efficacy in both small scale and commercial scale culture systems of *P. indicus* and *P. monodon* larvae and postlarvae. Formalin-killed bacterins and vaccines consisting of live attenuated strains of pathogenic bacteria produced by UV light mutagenesis gave a significant ($P<0.5$) degree of protection against subsequent infection by virulent pathogens when compared to nonvaccinated controls in small scale culture systems. Field trials of vaccines on a commercial scale using larval and early postlarval *P. monodon* were carried out in Indonesia. Following vaccination, vaccinated animals and non-vaccinated controls were grown on for a period of 17 weeks. The vaccinated animals showed a significantly higher survival rate (69.25%) than the non-vaccinated controls (27.5%) and a significantly enhanced growth rate. The incorporation of immunostimulants in artificial diets also increased the survival rate of larvae and postlarvae subsequently exposed to virulent bacterial pathogens indicating that both vaccines and immunostimulants offer a degree of protection against disease. To determine the mechanism by which vaccines confer protection against bacterial diseases juvenile *P. monodon* and *P. indicus* were vaccinated. The numbers and activity of haemocytes in the haemolymph of vaccinated animals was analysed and compared to that of non-vaccinated animals. Early findings indicate that the vaccines used in this study may confer a degree of protection upon inoculated animals due to their effect on the haemocytes in a similar fashion as has been suggested for immunostimulants such as beta linked 1-3 glucans.

WORLD AQUACULTURE '96

Book of Abstracts

Hosted by Thailand Department of Fisheries and the Chulabhorn Research Institute

THE 1996 ANNUAL MEETING OF THE WORLD AQUACULTURE SOCIETY

JANUARY 29 - FEBRUARY 2, 1996
QUEEN SIRIKIT NATIONAL CONVENTION CENTER
BANGKOK, THAILAND

Sponsors List:

• ASEAN • ASEAN Fisheries Foundation • Asian Development Bank • SEAFDEC • INFOFISH
• Network of Aquaculture Centres in Asia-Pacific • United Nations (ESCAP, FAO, Mekong Secretariat)
• U.S. Department of Agriculture • *Seafood Leader* magazine • Asian Institute of Technology
• IAAEM International Association of Aquaculture Economics and Management
• Thai Food Processors' Association • Thai Frozen Foods Association
• Fish Health Section of Asian Fisheries Society

BACTERIAL LEVELS IN PENAEID LARVAL CULTURE

A.O. Alabi, E. Yudiati, D.A. Jones* and J. Latchford

School of Ocean Sciences
University of Wales
Bangor, Menai Bridge
Gwynedd LL59, 5EY
UNITED KINGDOM

Rearing water is a major source for the introduction and distribution of disease in shrimp hatcheries, but

absolute sterility is very difficult and often impossible to achieve in commercial shrimp hatcheries despite the wide range of methods employed to limit and reduce the number of bacteria occurring in hatchery water supplies. Chemotherapeutants depend on a host having a higher tolerance threshold level to the substance than the target organism. However, these differences are often marginal and depend also on the physiological state of the larvae and as a result, their use is not encouraged. Bacterial resistance has also been reported due to the widespread and indiscriminate use of antibiotics. Other pre-treatment methods widely used are filtration, ultra-violet (U/V) light irradiation and ozonation. All these methods aim to reduce or eliminate bacteria in the hatchery however, there are conflicting reports on the beneficial (Ottogalli, 1991; 1992) or harmful (Muir and Sutton, 1994) effects of bacteria in penaeid larval culture.

The aims of this study are to determine the effectiveness of different methods of pre-treatment of sea water for penaeid larval culture, and to evaluate their impact upon penaeid larvae fed artificial and live feeds.

Initial experiments measured the total viable bacterial concentration (TVC) for live feeds and microencapsulated feeds incubated in 3µm filtered natural and hatchery seawater. Results at 24 hours and 28°C, show TVC in U/V sterilised water with artificial feed to be significantly higher than all other treatments apart from the treatment with autoclaved water. TVC in 3µm filtered seawater with artificial feed was also found to be significantly lower than all other treatments except the treaments with live feed in water of 35‰ salinity. TVC in all treatments increased by about two orders of magnitude over 48 hours resulting in bacterial levels of 10^5 to 10^6 colony forming units (cfu)/ml. In contrast, in 3µm filtered seawater with no added feed, bacterial numbers decreased to 10^4 cfu/ml by 48 hours.

Larval culture experiments were then conducted with *P. indicus* in autoclaved, U/V and ozone treated water and 3µm filtered natural seawater using the same live and microencapsulated feeds. Survival to post-larvae (PL)1 (60%) was not significantly different between larvae fed on live algae and microencapsulated feed as a total substitute for algae in 3µm filtered seawater. In addition, TVC remained at 10^4 to 10^5 cfu/ml.

Other water treatments gave more variable results. Despite water sterilisation using the above methods, all larval cultures showed a TVC of 10^6 - 10^7 cfu/ml within 48 hours. Larval survival to Mysis 1 with microencapsulated diets and live algae ranged from 20 - 59% and 0 - 89%, respectively. Sterilisation or partial sterilisation of seawater appears to encourage the selective development of bacterial communities which differ from those found in natural seawater. These may cause the collapse of larval cultures, even when those are fed on live algae. However, the use of natural seawater filtered to 3µm allows the total replacement of algae with microencapsulated feeds.

KEYWORDS: Penaeid shrimp culture; Hatchery production; Microencapsulation

ournal of Shellfish Research, Vol. 15, No. 2, 447–464, 1996.

ABSTRACTS OF TECHNICAL PAPERS

Presented at the 16th Annual Meeting

MILFORD AQUACULTURE SEMINAR

Milford, Connecticut

February 26–28, 1996

MS 60

Bacterial Levels in Penaeid Larval Cultures

ALABI, A.O.[1]. E. YUDIATI[2], D.A. JONES[1] AND J.W. LATCHFORD[1]

[1]*School of Ocean Sciences, University of Wales, Bangor, Menai-Bridge Gwynedd, LL59 5EY, UK.*

[2]*Diponegoro University, Semarang 50242, Indonesia.*

ABSTRACT

Total Viable Counts (TVC) of bacteria in 3 - 5 mm filtered natural seawater were significantly lower than in ozonated (O_3), ultraviolet light-treated (UVT) and autoclaved (AUC) water up to 48 h after the addition of microencapsulated prawn feeds (MED). TVC in all treatments, except the 3 - 5mm filtered sea water, increased by about 2 orders of magnitude over 48 h, resulting in bacterial levels of 10^5 to 10^6 Colony Forming Units (cfu).ml^{-1}. When Protozoea-1 (PZ1) stage *Penaeus indicus* larvae were cultured in 5 µm filtered water, survival to post-larvae (PL)1 (60%), was not significantly different ($\mu = 0.05$) between larvae fed on live diets and on MED as a total substitute. TVC remained low at 10^4 to 10^5 cfu.ml^{-1}. PZ1 larvae raised in O_3, UVS and AUC water showed significantly lower survival of 50 - 51% at PL1 when fed on live diets. When MED was used to replace live diets using these three water pre-treatments, survival of the larvae to PL1 fell to 0 - 10%. In addition, TVC increased to 10^6 to 10^7 cfu.ml^{-1} after 48 h. The percentage of presumptive *Vibrio* sp. increased over 24 h in seawater treated with UVS and O_3, but decreased in 3 - 5mm filtered and unfiltered natural sea water (SW). Pre-treatment or partial sterilisation of culture water appears to adversely affect the survival of *P. indicus* larvae fed artificial feeds, possibly by disturbing the natural sea water bacterial population balance. The use of 5mm filtered sea water allowed the total replacement of algae with MED without any significant affect on penaeid larval survival.

A.O. Alabi, E. Yudiati, D.A. Jones and J.W. Latchford (1997).Bacterial Levels in Penaeid Larval Cultures. *In* Diseases in Asian Aquaculture III. T. Flegel, I. H. MacRae (eds.), Fish Health Section, Asian Fisheries Society, Manila.

Microbial effects on larval growth and survival of microencapsulated diets as a total replacement for algae in the culture of *P. indicus* larvae

Alabi, A.O[a]., Che Cob, Z[b]. Jones D.A[a]. and Latchford J.W.

School of Ocean Sciences,

University of Wales, Bangor, Menai-Bridge

Anglesey, LL59 5EY

Accepted
Submitted for publication to Aquaculture International

Microbial effects on larval growth and survival of microencapsulated diets as a total replacement for algae in the culture of *P. indicus* larvae

Alabi, A.O[a]., Che Cob, Z[b]. Jones D.A[a]. and Latchford J.W.

School of Ocean Sciences,

University of Wales, Bangor, Menai-Bridge

Anglesey, LL59 5EY

[a] Corresponding authors

[b] Current address: Matriculation Centre

National University of Malaysia

43600 Bangi,

Selangor, Malaysia

Abstract

Intact microencapsulated protozoeal diets (MED), added to 5μm filtered hatchery water at the recommended concentration of 4mg.l^{-1} gave no significant difference in bacterial numbers after 6h, over a 48h period compared to control groups of 5μm filtered and autoclaved hatchery water. Disruption of the microcapsules resulted in a significant increase in bacterial numbers up to 12h compared to controls with intact microcapsules. Protein leach rates were similar in sterile water and 5μm filtered water indicating that the leaching was independent of microbial activity. MED leached significantly less dietary solubles compared to a microparticulate diet (MPD). There was no evidence to suggest significant attachment of bacteria to diet particles for at least 48h.

In low quality water after algae blooms, survival of *Penaeus indicus* larvae was poor when reared in both sterile and 5μm filtered water. Addition of bacteria filtered from xenic algal cultures together with the algal exudates to such water gave significant improvements in survival to larvae fed MED in 5μm filtered water but not in autoclaved culture water.

Live algal diets promote high larval survival and growth irrespective of the inclusion bacteria.

It is suggested that algal exudates act as selective bacteriocides and total replacement of algae with MED requires a balanced bacterial community which can be attained at times, from natural sea water or when the natural bacterial community balance is disturbed, corrected by the use of algal exudates.

GLOSSARY OF TERMS.

Acid fast	Bacteria which upon being stained with a red dye (usually carbol fuschin) still retain the red colouration after decolourization in acid-alcohol.
Aetiology (etiology)	Cause of disease.
Eosinophilia	The process of staining (usually protoplasmic granules) red with the dye eosin.
Furunculosis	Bacterial disease (usually of salmonid fishes), marked by ulcers resembling boils.
Haemocytosis	An increase in the number of haemocytes in the haemolymph.
Haematopoeisis	The process of forming new haemocytes.
Haemocoel	The primary body cavity of metazoan invertebrates containing circulatory fluid (haemolymph).
Haemocytopaenia	An abnormal dimunition in the number of haemocytes in the haemolymph.
Karyorrhexis	The breakup of the chromatin of the nucleus into darkly staining granules.
Lectins	Proteins or glycoproteins of non-immune origin which exact carbohydrate binding specificity.
Lesion	Wound or morbid change anywhere in the body.
Lyophilisation	Freeze-drying.
Naive	Not having taken or recieved a particular drug or treatment.
Necrosis	Death of a cell (or group of cells) while still part of a living body.
Opsonin	A substance (usually in blood plasma) which renders invading micro-organisms more susceptible to phagocytosis.
Phagocytosis	Destruction of tissue cells or microbes by the action of phagocytes.
Pyknosis	Shrinkage of stainable material of a nucleus into a deeply staining knot (usually a feature of cell degeneration).
Septicaemia	The invasion of the body tissue by pathogenic bacteria and their multiplication therein.