IMPROVING PRAWN HATCHERY PRODUCTION BY REDUCING LOSSES DUE TO BACTERIAL DISEASES

Fishing Industry Research and Development Council
Project number 89/78

FINAL REPORT

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EXECUTIVE SUMMARY

Study objectives

(i) Investigate relationships between bacterial diseases of larval prawns, bacterial population densities in water, and other water quality parameters in commercial hatchery cultures.

(ii) Assess the usefulness of simple methods for estimating population densities of total bacteria and common bacterial pathogens in water in larval culture tanks. These methods could be used routinely to improve hygiene standards in prawn hatcheries as well as in hatcheries associated with other forms of aquaculture.

(iii) Identify bacteria associated with important bacterial diseases in larval prawns.

(iv) Establish a bioassay system to allow maintenance of larvae for experimental procedures.

(v) Using this bioassay system and results obtained under Objectives (i) and (iii), experimentally reproduce important bacterial diseases of larval prawns, thereby confirming the validity of the results.

Collaborating Institutions

The collaborative project involved NSW Agriculture &Fisheries and Queensland Department of Primary Industries.

Results

(i) Bacterial necrosis was the only bacterial disease syndrome seen during monitoring of 8 larval production runs at 2 commercial hatcheries during the study. Results indicated a possible causal relationship between concentrations of one or more components of the "presumptive Vibrio" populations in rearer tank water, as detected by dip slide TCBS agar, and outbreaks of bacterial necrosis. The source of these putative pathogens and the means by which they induce lesions was not determined.

There was no evidence that outbreaks of bacterial necrosis were causally associated with changes in other variables measured, including concentrations in rearer water of total bacteria, total heterotrophic bacteria, unionised ammonia, nitrite or nitrate.

There was no convincing evidence that antibiotics, at concentrations used at commercial hatcheries during the study, were effective in reducing concentrations of the putatively pathogenic presumptive Vibrios.

(ii) Dip slides proved to be a convenient method of indicating bacterial concentrations in larval rearing tank water, *Artemia* cultures and algal cultures. The technique is simple and all necessary facilities are available in most hatcheries. As most of the recognised pathogens of larval penaeids grow on TCBS medium, the technique could be refined by using dip slides with this medium on both sides. This would reduce costs while providing the security of a replicate sample.

While providing useful information on hatchery hygiene and serving as an indicator for

possible outbreaks of bacterial necrosis, such monitoring does not appear to have predictive value. In our study, counts of more than 100 colonies on TCBS were associated with bacterial necrosis prevalence >10% on the sampling day and/or the following day in 25 (48%) of 52 instances. By contrast, counts of less than 100 colonies on TCBS agar were recorded the day before and/or on the same day as a bacterial necrosis prevalence of >10% in only 5 (5%) of 96 instances. Given the requirement for 24 hour incubation before colony numbers can be counted, any predictive value is lost; within the same period, hatchery operators can more accurately determine the disease status of the larvae by direct examination.
The epifluorescence count did not appear to be a useful predictor of changes in disease status. It was not possible to differentiate different types of bacteria except on morphological grounds and we found the technique time-consuming and difficult to control. The equipment required is not normally available in hatcheries.

(iii) Thirteen different species of bacteria were identified from larval rearing water containing diseased larvae, and from algae and brine shrimp cultures. The most common bacteria was *Vibrio alginolyticus*. This bacteria was subsequently found not to cause disease when larvae in the bioassay system were challenged. Nine different bacteria were cultured and identified from bacterial necrosis lesions on diseased larvae. No single species was common, but included *Vibrio tubiashi* and *Vibrio alginolyticus*.

(iv) It was found that there was a wide variation in survival between batches of larvae used in the bioassay system, the mean survival at postlarvae ranged from 1.3 to 34.7%. There was also a wide range of survivals between individual one litre larval cultures making it hard to determine statistically significant differences in survival and disease prevalence between treatment groups. The bioassay system studies did show that low nutrition, trauma and high stocking densities increased the level of larval disease and probably reduced larval survival.

Directions for Future Research Activities

The study findings indicate the need for further research into possible causal relationships betweeen presumptive Vibrio concentrations in rearer water and outbreaks of bacterial necrosis in penaeid prawn larvae. Findings also demonstrate the continuing need for researchers and diagnosticians to provide a rational basis for antibiotic usage in prawn hatcheries and the requirement to investigate more effective control methods. Possible bacterial pathogens need to be isolated from a wider range of geographical locations, identified and their pathogenicity conclusively demonstrated. The role of bacterial biofilms on pipe and tank surfaces need to be investigated. Using information from hatchery monitoring in combination with pathogenic isolates, the pathogenesis of bacterial diseases in larvae needs to demonstrated in the bioassay system so that important factors involved in the initiation of disease can be conclusively identified. There is a clear need for identification of probiotic bacteria and other biological control agents, with the aim of introducing new methods and technologies to commercial prawn hatcheries so that healthy prawn larvae can be produced more sustainably.

ABSTRACT

Bacterial necrosis was the only bacterial disease syndrome seen during monitoring of 8 larval production runs at 2 commercial hatcheries during the study. Results indicated a possible causal relationship between concentrations of one or more components of the "presumptive Vibrio" populations in rearer tank water, as detected by dip slide TCBS agar, and outbreaks of bacterial necrosis. The source of these putative pathogens and the means by which they induce lesions was not determined.
There was no evidence that outbreaks of bacterial necrosis were causally associated with changes in other variables measured, including concentrations in rearer water of total bacteria, total heterotrophic bacteria, unionised ammonia, nitrite or nitrate.
There was no convincing evidence that antibiotics, in the concentrations used at he commercial hatcheries during the study, were effective in reducing concentrations of the putatively pathogenic presumptive Vibrios.

Dip slides proved to be a convenient method of monitoring bacterial concentrations in larval rearing tank water, *Artemia* cultures and algal cultures. While providing useful information on hatchery hygiene and serving as an indicator for possible outbreaks of bacterial necrosis, the 1-day incubation requirement means such monitoring has no predictive value; during the 2-day risk period relevant to the dip slide result, hatchery operators can more accurately determine the disease status of the larvae by direct examination.
The epifluorescence technique did not appear to be a useful predictor of changes in larval disease status.
Thirteen species of bacteria were identified from larval rearing water containing diseased larvae, and from algae and brine shrimp culture samples. The most common isolate was *Vibrio alginolyticus*. This organism was subsequently found not to cause disease when larvae in the bioassay system were challenged. Nine bacterial species were isolated and identified from bacterial necrosis lesions on diseased larvae. No single species was common, but included *Vibrio tubiashi* and *Vibrio alginolyticus*.

In order to study the relationship between rearing environment and bacterial pathogens, a small-scale larval rearing system was developed. It was based on one litre Imhoff cones in a temperature-controlled water bath. *Penaeus monodon* larvae were used for all trials. Ultrafiltered, u/v sterilised seawater was exchanged daily in the larval cultures. *Chaetoceros gracilus* and Frippak microencapsulated diets were the source of food for the larvae. It was found that between batch and between replicate survival was highly variable. This reduced the sensitivity with which differences between treatments could be detected on analysis. The bioassay system studies showed that low nutrition, trauma (heavy aeration) and high stocking densities increased the prevalence of bacterial necrosis lesions and tended to reduce larval survival. In the one trial where treatment larval cultures were exposed to a daily cold-water shock, survival appeared to increase.

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1. INTRODUCTION

1.1 Background

Marine prawn farming is a rapidly growing aquaculture industry. From 1987, two years prior to this project beginning, production of marine prawns was 71 tonnes (Curtis 1988; Maguire *et al* 1988) and this rose to 594 tonnes in 1989/90 (O'Sullivan 1991). Smaller increases in production have continued since then. In Queensland alone, 715 tonnes were produced in 1991/92 (Lobegeiger and Barlow 1992). This farm production depends entirely on the supply of post-larvae from hatcheries to stock the earthen grow-out ponds. Hatchery production in Australia has at times failed to fulfill the quantities, quality and time of supply expectations of the farms. Before 1989/90, ponds often remained empty because postlarvae were not available (Kenway 1991). Inconsistent hatchery production and mass larval mortalities can be attributed to bacteria and bacterial diseases, as well as to spawner quality, difficulties in supplying live food organisms to the larvae and poor water quality. Although the importance of bacterial diseases is recognised, the mechanisms by which bacteria cause such problems is poorly understood. Consequently, many of the larval rearing systems developed for penaeid prawns depend on the routine addition of antibiotics to the culture water to ensure larval survival (AQUACOP 1983; Liao 1985). The continued use of antibiotics in larval rearing is not sustainable. Antibiotic-resistant strains of bacteria develop, forcing the closure or extended `dry-out' of hatcheries. The inhalation of aerosols containing antibiotic-resistant bacteria or antibiotics by hatchery workers is a public health risk. Disposal of medicated water may pose environmental risks. Essentially, the dependence on antibiotics implies the larval rearing system, or our management of it, must be improved.

The aim of a hatchery is to produce and supply good quality postlarvae to farms on time and in the numbers required. Without this consistency of supply, prawn ponds will not be fully utilised and, as a consequence, farmers will not optimise their return on capital invested. There was, and still is, considerable interest by Australian prawn hatchery operators in strategies to avoid acute mass mortalities of larval cultures. Operators would like to be able to monitor and/or manipulate bacteria with a view to minimising losses due to bacterial diseases.

Except for the recent work of Lavilla-Pitogo and colleagues (1990;1992) in the Philippines, Muir (1992) in Australia and Flegel (1993) in Thailand, there has been very little research published on bacterial diseases of penaeid prawn larvae. When this project was proposed, little information was available on monitoring bacterial populations in prawn hatcheries or on predicting disease outbreaks. The specific bacteria associated with larval mortalities or the lesions on the larvae had not ever been identified. There was a real need to investigate the ecology of bacteria in prawn hatcheries, to determine how bacteria can be monitored easily, to identify primary infectious agents, and to identify other factors associated with the major bacterial diseases of prawn larvae.

This project arose from separate proposals submitted to the Fishing Industry Research and Development Council by staff at Regional Veterinary Laboratory (RVL), Wollongbar, NSW Agricultural & Fisheries and Animal Research Institute, Queensland Department of Primary Industries (QDPI). Both proposals addressed the problem of larval survival in

prawn hatcheries. On the advise of FIRDC, the two groups then developed a collaborative proposal which included aspects of the two original proposals. R. Callinan was then NSW Agriculture & Fisheries' specialist fish pathologist. He, together with G. Fraser, a veterinary pathologist, were situated in an ideal position to service the prawn hatcheries and farms in northern NSW. Both scientists had previously investigated disease events in prawn hatcheries. RVL Wollongbar is fully equipped to undertake detailed investigations and research in animal diseases. I. Anderson is the QDPI specialist fish pathologist. He has a background of working in tropical aquaculture and research in prawn diseases. His co-researcher, L. Eaves has extensive experience as a diagnostic and research bacteriologist in animal disease. Her involvement in this project began a dedicated role as the fish bacteriologist within QDPI. ARI is the main central animal health research facility in Queensland. It has laboratories for each of the major disciplines in animal health research and facilities to hold and maintain aquatic animals. QDPI staff and facilities at the Bribie Island Aquaculture Research Centre and the Southern Fisheries Centre were available for advice and support on larval rearing methods, larval nutrition and system designs. In 1990, I. Anderson was transferred to the Oonoonba Veterinary Laboratory (OVL) in Townsville. Similar to RVL, Wollongbar, OVL is well equipped for research on the diseases of aquatic animals and FIRDC had no objections to some aspects of the project continuing at the new location. There was the possibility of working with two prawn hatcheries located close to Townsville. R. Callinan committed 40% of his time to the project, I. Anderson and L. Eaves committed 33% and G. Fraser 10%. Thus 1.16 full-time-equivalent (FTE) graduate scientists were used throughout the project. Half of the time of a QDPI technician assisted, and project funds provided additional technical support to RVL (half a laboratory assistant) and ARI (half a scientific assistant).

At least two commercial hatcheries were proposed for monitoring during the project. Initially the Ballina Prawn Farm hatchery at Evans Head participated. When it closed after 2 larval production runs had been monitored, the Sea Ag hatchery at Ballina participated, collaborating in monitoring of a further 6 runs; this hatchery then also closed. The subsequent decision by the Gold Coast Marine Prawn Hatchery not to participate in the project, and the closure of two hatcheries close to Townsville, limited the number of hatchery sites that could be monitored during the project. Of the 18 hatcheries in operation in Australia at the start of the project, only six remained at the end (Kenway 1991).

The proposed plan of operation of the project was summarised as:

Year 1:RVL: Monitoring bacterial population densities.
ARI: Preliminary bacterial taxonomy.
Development of experimental bioassay system.

Year 2:RVL: Monitoring bacterial population densities.
Study of bacterial disease outbreaks.
ARI: Bacterial taxonomy.
OVL: Experimental use of bioassay system.
Study of bacterial disease outbreaks.

Year 3:RVL: Study of bacterial disease outbreaks.
Analysis of monitoring results.

ARI: As in Year 2.
OVL: As in Year 2.

The project began on 24 July 1989 with the appointment of temporary project staff. It officially ended in June 1992, but in fact work continued till the end of 1992.

1.2 Objectives

i) Investigate relationships between bacterial diseases of larval prawns, bacterial population densities in water, and other water quality parameters in commercial hatchery cultures.

ii) Assess the usefulness of simple methods for estimating population densities of total bacteria and common bacterial pathogens in water in larval culture tanks. These methods could be used routinely to improve hygiene standards in prawn hatcheries as well as in hatcheries associated with other forms of aquaculture.

iii) Identify bacteria associated with important bacterial diseases in larval prawns.

iv) Establish a bioassay system to allow maintenance of larvae for experimental procedures.

v) Using this bioassay system and results obtained under Objectives i) and iii), experimentally reproduce important bacterial diseases of larval prawns, thereby confirming the validity of the results.

1.3 Literature Review

Bacteria have been implicated as causes of massive mortalities in cultures of larvae and postlarvae in marine prawn hatcheries (AQUACOP 1977; Lavilla-Pitogo *et al* 1990; Natividad and Lightner 1992; Chen 1992; Lightner 1993). Most evidence implicating bacteria, although indirect, is convincing, being extrapolated from findings in older prawns. Evidence that bacteria cause disease in prawn larvae includes the detection of large numbers of bacteria on the lesions of affected larvae (AQUACOP 1977: Anderson *et al* 1990), histological observation of bacteria within moribund larvae (AQUACOP 1977; Brock 1983; Lightner 1985), and successful treatment or prevention of diseases by antibacterial chemicals (eg. Delves-Broughton and Poupard 1976; AQUACOP 1977; Liao 1985; Lightner 1985). Filipino workers have recently proven (by fulfilling Kochs postulates) a bacterial cause for serious mortalities associated with luminescent larvae (Lavilla-Pitogo et al, 1990).
Bacterial diseases of prawn larvae may be differentiated into several distinct syndromes or conditions. Unfortunately, the causes of very few of these syndromes are fully understood and most appear to have a complex aetiology (Bell and Lightner, 1992).

Luminous vibriosis
Luminous vibriosis is a disease characterised by affected larvae and early postlarvae exhibiting a greenish luminescence at night (Sunaryanto and Mariam, 1986; Larvilla-Pitogo *et al* 1990). The disease is widespread and has been reported from Thailand (Sae-oui *et al* 1987), Indonesia (Sunaryanto and Mariam 1986), Philippines (Lavilla-Pitogo *et al* 1990), Australia (Owens and Hall-Mendelin 1990), China and Ecuador (Bell and Lightner 1992). Total mortalities of affected populations are seen in hatcheries. Clinically the luminescent larvae or postlarvae swim weakly, sink to the bottom, they have an opaque, whitish body, stop feeding and may swim abnormally (Sunaryanto and Mariam 1986; Lavilla-Pitogo *et al*

1990; Bell and Lightner 1992). When diseased larvae are examined microscopically, a dense mat of bacteria is seen to cover the mouth and the lining of the stomach. Bacteria appear to eventually spread to the haemolymph and then throughout the body. Melanised mouth appendages, oesophagus and stomach cuticle have also been reported to be associated with luminous bacteria. The motile gram-negative bacteria *Vibrio harveyi*, *V. splendidus* and *V. albensis* have been isolated from diseased larvae, but *V. harveyi* is now considered the primary cause of luminous vibriosis. Lavilla-Pitogo and colleagues (1990) reproduced the disease by exposing healthy larvae and postlarvae to 10^2 *V. harveyi* cells per ml and then reisolated the bacteria in pure culture. This indicates quite clearly *V. harveyi* is a primary pathogen. The most effective treatment of an outbreak is reported to be an increased water exchange to 80 to 90% daily (Baticados *et al* 1990). The most likely source of *V. harveyi* is the seawater pumped into the hatchery (Larvilla-Pitogo *et al* 1990). More recently Lavillo-Pitogo *et al* (1992) found *V. harveyi* in the midgut of female spawners. They suggested the spawners and their faeces should be separated from the eggs as soon as possible after spawning to prevent transmission of infection to the larval rearing water.

Filamentous Bacterial Disease

Fouling of the surface of larvae and post-larvae by long filamentous bacteria can cause mortalities. Low levels of surface infestations are harmless, but if heavy, mortalities of 30-100% can be seen (Barkate *et al* 1974: Lightner 1988). A number of different ages and species of marine crustaceans can be affected including penaeid prawns (Fisher 1977). *Leucothrix mucor* is usually responsible for the fouling, although other genera of filamentous and chain-forming bacteria can be involved (Lightner 1985, 1988). Their role in disease is indirect and involves mechanical entanglement of the larvae to impair feeding and swimming, or obstruction of the respiratory surface (Fisher 1977). Acute mortalities of larvae and post-larvae have been recorded when periods of low dissolved oxygen concentrations in rearing water, or following molting, handling etc coincide with heavy infestations (Lightner 1988). Clinically affected larvae grow slowly, feed poorly and have impaired swimming and molting when fouling becomes serious; numerous filamentous bacteria attached to the gills and cuticle are easily seen on microscopic examination of affected larvae. The bacteria are ubiquitous and enter the hatchery with pumped sea-water or broodstock (Fisher 1977; Lightner 1983). There is a clear association between filamentous bacterial disease and high levels of dissolved nutrients in the larval culture water, since these constitute the agents' nutrient source. As orgainic loads, phosphorus and/or nitrate levels increase in water, so will the numbers of *L. mucor* on the larvae. Treatment of the disease is possible (Lightner and Supplee 1976), but good water exchange, tank cleaning and water quality maintenance will prevent outbreaks (Baticados *et al* 1990; Lightner, 1988).

Bacterial Necrosis

From our experience, the most common bacterial syndrome in prawn larvae from hatchery rearing tanks is bacterial necrosis (BN), which is characterised by multifocal, melanised lesions on the cuticle of setae and appendages. Affected larvae become weak, cannot moult completely and die (AQUACOP 1977; Lightner 1985). In young larvae (zoeal stages) mortalities develop rapidly and all larvae in a rearing tank may die within 24 hours (AQUACOP 1977, Tareen 1982). Bell and Lightner (1992) do not separate the syndrome BN from a non-specific bacterial syndrome, larval vibriosis; it appears BN may progress to

systemic infection, or toxins absorbed from the bacteria on the surface may have a systemic effect. There have been no reports in the literature of isolations of bacteria from bacterial necrosis lesions. On histology, numerous Gram-negative rod-shaped bacteria can be seen at the site of damage. This evidence, together with the success of antibiotics (AQUACOP 1977; Tareen 1982, Lightner 1988) in preventing and controlling mortalities, implies an important role for bacteria in BN. There could well be other factors that predispose larvae to BN, although AQUACOP (1977) found chlorination or UV treatment of the water were not enough to prevent the disease.

Larval Bacterial Septicaemia (Vibriosis)

Bell and Lightner (1992) use the name non-specific bacterial syndrome to encompass this group of bacterial infections. It is probable that a number of different causes ultimately lead to a fatal infection characterised by the presence of numerous bacteria throughout the haemolymph and organs. Affected larvae typically stop eating, become lethargic and die. Microscopically, numerous Gram-negative, rod-shaped bacteria are found throughout the body. Signs of other diseases may also be present. Surprisingly little research has been done on the aetiology of vibriosis in penaeid prawn larvae. With the exception of Muir (1990), reports of the bacteria which cause the disease use information extrapolated from older, juvenile prawns (Lightner 1988). *Vibrio alginolyticus, V. anguillarum, V. parahaemolyticus, Vibrio* spp., *Pseudomonas* spp. and others have been isolated from the haemolymph and organs of juvenile and adult prawns with systemic infections and septicaemia (Barkate 1973; Vanderzant *et al* 1970; Delves-Broughton and Poupard 1976; Lightner and Lewis 1975; Lightner *et al* 1984; Anderson *et al* 1988).

In his recent study Muir (1990) isolated a range of bacteria from moribund *P. monodon* larvae. These were most commonly *Vibrio* sp., *V. damsela, V. tubiashii, V. harveyi* and *Pseudomonas* sp. Subsequent reinfection studies found *V. damsela* and *Vibrio* sp. could invade the larvae and their tissues. Toxins from *V. harveyi, V. damsela* and *Vibrio* sp were lethal. As *V. damsela* caused mortalities when larvae were exposed to doses of 10^3 cells per ml, Muir (1990) considered it a primary pathogen. Unfortunately, the bacteria were not reisolated from moribund larvae to confirm Kochs' Postulates. Lightner (1983,1988) argues, because the bacterial species implicated (but not proven) as causes of vibriosis in larvae are considered part of the natural flora, the infections are secondary, occurring after other infectious diseases, nutritional disease, extreme stress, wounding etc. Muir (1990) found that low and variable rearing water temperatures and salinity, decreased pH, and high ammonia and nitrate concentrations were correlated with larval mortalities. In turn, the high bacterial counts in rearing tank water (10^6 - 10^7 cells/ml) correlated with the high ammonia and nitrate levels.

While the pathogenesis of these infections in penaeid prawn larvae is not known, environmental factors probably play a role, and stressed larvae may be more susceptible to systemic infections (Flegel *et al* 1993). In addition, environmental changes may themselves be lethal. For example, ammonia and other nitrogenous compounds which accumulate in closed culture systems may reach toxic concentrations. Acute toxicity of ammonia and nitrite ions for *P. monodon* has been demonstrated experimentally (Chin and Chen 1987; Chen and Chin, 1988). Nitrate is also suspected of being toxic to *P. monodon* larvae (Muir *et al* 1991).

Recommended disease prevention methods involve lowering nutrient levels and total bacterial counts in the rearing tank water, good hatchery hygiene and avoidance of trauma to larvae (Lightner, 1988; Muir 1990). Despite this, many hatcheries still rely on the

routine addition of antibiotics to rearing water. However, antibiotics are of limited use in controlling bacterial diseases in prawn hatcheries because of the restricted efficacy of readily available compounds, the limited tolerance of prawn larvae to therapeutic concentrations and the development of resistant bacterial strains (Baticados *et al* 1990). Antibiotic use and losses due to bacterial disease may be reduced if pathogenic bacterial concentrations in larval cultures could be monitored using simple methods. A modification of the Dip-Slide (Media Makers) used by medical technologists to enumerate bacteria in fluids (Claesson and Holmlund 1986), may allow hatchery technologists to monitor concentrations of potentially pathogenic bacteria, detect sources of bacterial contamination and to administer antibiotics more judiciously.

Research on the normal bacterial flora of healthy penaeid prawn larvae is very limited, as are studies on the bacterial ecology of prawn hatcheries. Bacterial levels in *P. monodon* larval cultures have been reported in the range of $1.0x10^2$ to $9.0x10^5$ (Llobrera and Gacutan, 1977) and $9.5x10^2$ to $1.9x10^7$ cells per ml (Muir 1990); Yasuda and Kitao (1980) found bacteria in *P. japonicus* larval rearing water reached levels between 10^3 and 10^4 cells/ml except during the protozoeal stage when counts increase to $1.8x10^5$. Bacterial counts of 10^3 to 10^4 have been reported for *P. stylirostris* larval rearing water (Lewis *et al* 1982).

In the only report of bacterial levels associated directly with penaeid prawn larvae, Muir (1990) found total bacterial numbers ranging from 2.4×10^4 to 7.0×10^9 per gram of larval tissue. Muir (1990) also reported on the number of bacteria found in intake seawater, spawning tank water, algal cultures, *Artemia* cultures and in hatchery aerosols.

The majority of bacteria isolated from larval rearing tank water and the digestive tract or tissues of healthy penaeid larvae belong to the genera *Vibrio, Listonella, Pseudomonas, Aeromonas, Photobacterium* and *Flavobacterium* (Yasuda and Kitao 1980; Lewis *et al* 1982; Muir 1990). In contrast, Llobrera and Gacutan (1977) report 79% of isolates from the larval rearing water were Gram-positive, mostly *Micrococcus* and *Staphylococcus*.

2. MATERIALS AND METHODS

Factors suspected of contributing to outbreaks of bacterial diseases were monitored daily through larval and postlarval stages during production of 8 *P. monodon* larval batches at 2 commercial hatcheries.

2.1 Monitoring hatchery management

Records of management procedures, including feeding, water exchange, health examination and antibiotic administration, were maintained by hatchery technologists on a supplied, standard questionnaire (Appendix I).

2.2 Water quality analysis

One litre samples of larval culture water were collected each morning prior to feeding and water exchange and filtered under vacuum through 0.45µm cellulose acetate filters. The filtered water was frozen in 30ml aliquots and subsequently analysed for ammonia, nitrite and nitrate concentrations according to the methods of Ryle, Mueller and Gentien (1981). All glassware used in the hatchery and laboratory was acid washed.

2.3 Monitoring bacterial population densities in hatcheries

Dip-Slides

Dip-Slides (Media Makers), coated on one side with marine agar (Zobell, 1941), which supports growth of marine heterotrophic bacteria, and on the reverse side with Thiosulphate Citrate Bile salts Sucrose (TCBS) agar, which supports growth of "presumptive" *Vibrio* spp., were used to assess bacterial concentrations. Bacterial concentrations in larval rearing tank water were measured each morning prior to feeding and water exchange. Bacterial concentrations in algal cultures and *Artemia* cultures were measured immediately before feeding. At each sampling, two (replicate) dip slides were briefly dipped into the vessel, drained and returned to the sterile case. The procedure was repeated using a 1/100 dilution in sterile sea water of rearing tank water, algal culture or *Artemia* culture. After 24 hours incubation at approximately 22^{0}C, hatchery technologists graded colony numbers on each medium into 5 classes, 0, 10, 100, 1000 and >1000, representing 0, 1-10, 11-100, 101-1000, and greater than 1000 colonies respectively. Slides were then stored at 5^{0}C for 3-4 days before dispatch to the laboratory where the counts were checked and dominant organisms selected for preliminary identification using standard bacteriological methods. Selected isolates were subsequently submitted to ARI for definitive identification (see 2.4 below).

Epifluorescence

The method of Moriarty and Chandrika (1986) was used to monitor total bacterial concentrations in larval rearing tank water. Samples were collected each morning prior to feeding and water exchange. The aliquot size tested was dependent on the number of bacteria present and was adjusted by trial and error until approximately 20 to 40 bacteria were counted in each grid field.

2.4 Laboratory examination of larvae

Each morning prior to feeding and water exchange, aliquots of rearer culture water were passed through a 200µm screen until approximately 150 larvae had been collected. Larvae were washed from the screen into Davidson's fixative and held for later examination under a dissecting microscope, where prevalence of abnormalities, including bacterial necrosis,

was determined. Samples were subsequently processed for histopathology using standard procedures.

2.5 Analysis of results of hatchery project component

Data for each larval batch were transferred from hatchery data sheets to computer spreadsheets. From these, graphs representing all relevant interactions were generated and assessed visually for possible causal associations.

2.6 Bacterial Identification Techniques

2.6.1 *Introduction*

Genera within the family *Vibrionaceae* comprise the predominant bacterial flora found in the marine environment. There have been several taxonomic studies of the Vibrionaceae and related species, and the number of recognized species in the genera *Vibrio*, *Photobacterium*, *Aeromonas* and *Pleisomonas* has risen from five in 1974 to 41 at this time. This number will increase as specific names are assigned to more phenotypic groups. The identification of bacteria isolated from the hatcheries and larvae was based on a range of tests used by Bryant *et al* (1986a) for the numerical classification of Vibrionaceae. Bryant *et al* (1986b) constructed a computer matrix for the probabilistic identification of species in the Vibrionaceae based on this data. Of the 111 tests used in the previous study, Bryant *et al* (1986b) selected 30 tests to form a basis for routine identification, and a further 51 tests for the separation of 38 phenons of Vibrionaceae at a level of 0.9999 or greater. This programme, the probabilistic identification of bacteria (PIB), was purchased and installed for the identification of prawn hatchery isolates of Vibrionaceae. Seventy-three of the 81 tests in the identification matrix were set up, together with a further 16 auxiliary tests.

2.6.2 *Bacterial identification tests*

Isolates were cultivated on Trypticase Soy Agar to which was added Electrolyte Supplement Solution (ESS) to satisfy the ionic growth requirements for marine bacteria (Furniss *et al* (1978). They were grown initially at 25°C for 18 - 24 hours, then tested as below to confirm they were Vibrionaceae:

Gram stain - For determination of cellular morphology and confirmation that the cultures were gram negative

Oxidase test - Oxidase Reagent Droppers (Becton Dickinson), containing 0.5ml tetramethyl-p-phenylene diamine dihydrochloride, to ensure they were oxidase positive

Motility - A 1% tryptone water broth (with NaCl final concentration = 3%) was inoculated and examined for motility by phase contrast microscopy following overnight incubation

O/F reaction - Two steamed and cooled marine oxidation/fermentation tubes (casein hydrolysate 0.1%, yeast extract 0.01%, $MgSO_4.7H_2O$ 0.3%, $CaCl_2.2H_2O$ 0.15%, NaCl 0.8%, KCl 1.0%, Phenol Red 0.001%, agar 0.3% and glucose 1.0%) were stab inoculated with the isolate and one was subsequently covered with sterile molten vaseline.
The tubes were examined for evidence of fermentative growth with or without the presence of gas following overnight incubation.

The following tests were carried out on the confirmed Vibrionaceae. All incubations were

carried out at 25°C unless otherwise stated.

Salt tolerance - Isolates were tested for their ability to grow in a 1% tryptone water containing 0, 3, 6, 8, 10 and 12% (w/v) final concentration of NaCl as described by Lee *et al* (1979).

Indole production - Isolates were tested as described by Furniss *et al* (1978) using Kovacs reagent (Cowan 1974).

Swarming - Marine Agar (Furniss *et al* (1978) was prepared and spot inoculated with the isolates. Plates were examined for swarming after overnight incubation.

Antibiotic sensitivity - Resistance to O/129 (10 and 150 g), Ampicillin 10 g and Polymyxin 50 iu was determined plates of blood agar base with added ESS. Discs for O/129 and Ampicillin testing were purchased (Oxoid) and Polymyxin discs were prepared, dried and stored at -5°C. Sensitivity test plates were dried at 37°C for 30 minutes just prior to use. A saline suspension of an overnight culture was prepared and adjusted to a turbidity equal to Mc Farlane tube 0.5. This was spread over the surface of the dried plate. When the plates were dry, the test discs were pressed onto the surface of the agar. The plates were examined for resistance to the antibiotic after overnight incubation.

Decarboxylation - Decarboxylation of arginine, lysine and ornithine were determined by modifying the method of Møller (1955) with the addition of 0.02% KH_2PO_4 to the medium as a buffer. Tests were read after 1 - 2 days incubation.

Nitrate reduction - ESS and 0.1% agar was added to the medium of Lee *et al* (1979) providing a semi-solid agar deep. After 3 - 5 days incubation, the cultures were tested as described by Lee *et al* (1979).

Gluconate - The method of Cowan (1974) was used with the addition of ESS to the medium. Cultures were tested with Clinitest tablets (Ames & Co.).

ONPG test - The method of Lee and Donovan (1985) was used.

Urea hydrolysis - The method of Cowan (1974) was used with the addition of ESS to the medium.

Voges Proskauer test - The method of Lee *et al* (1979) was used with the addition of ESS to the medium.

Sugar fermentation - Fermentation of L-arabinose, arbutin, inositol, D-mannitol, mannose, trehalose, salicin, sorbitol, sucrose and glucose was tested in peptone water sugars with ESS using bromcresol purple as an indicator as described by Lee and Donovan (1985). A Durham tube was added to the glucose medium to detect gas production.

Luminescence -The medium of Baumann (1984) was used and plates were read as described by Lee *et al* (1979).

Tellurite inhibition - Cultures were spotted onto TSA plus ESS plates containing 0.0005% potassium tellurite and examined for growth after 1 - 2 days incubation.

Haemolysis - Plates containing 5% sheep blood in a blood agar base were spot inoculated with isolates and haemolysis read as described by Furniss *et al* (1978).

Enzymes - Alginase, chitinase, DNAse, lecithinase, amylase and lipase (Tween 20 and Tween 80)activity was tested as described by Furniss *et al* (1978) using blood agar base supplemented with ESS in place of marine agar.

Aesculin hydrolysis -The method of Lee and Donovan (1985) was used with the addition of ESS to the medium.

Casein hydrolysis - The method of Cowan (1974) was used with the addition of ESS to the medium.

Xanthine degradation - The method of Cowan (1974) was used with the addition of ESS to the medium.

Gelatin hydrolysis - The method of West and Colwell (1981) was used with the addition of ESS to the medium. Plates were spot inoculated and gelatinase activity read as a zone of opacity around the inoculum after 1 - 2 days incubation. This was confirmed by flooding the plate with 30% Trichloracetic acid (Cowan 1974).

Carbon utilisation - A range of carbon substrates were added to the basal medium agar of Baumann (1984) at a final concentration of 0.2% (sugars) and 0.1% (other substrates). Substrates tested were L-arabinose, cellobiose, fructose, galactose, glucose, mannose, maltose, melibiose, lactose, melizitose, sucrose, trehalose, D-xylose, ethanol, glycerol, inositol, 1-propanol, D-sorbitol, D-galacturonate, gluconate, glucuronate, amygdalin, arbutin, L-citrulline, L-hydroxyproline, L-leucine, D-glucosamine, N-acetylglucosamine, glutarate, DL-3-hydroxybutyrate, α-ketoglutarate, malonate, succinate, L-rhamnose, D-mannitol, γ-aminobutyrate, pyruvate and putrescein. Tests were interpreted as described by Lee *et al* (1979).

Temperature tolerance - Isolates were tested for growth at 4°C, 25°C, 30°C, 37°C and 42°C.

The API 20E and the API 20NE were modified by the addition of 2.0% NaCl for identification of non-fermentative isolates.

2.7 Experimental Bioassay System

2.7.1 *Introduction*

The aim of the bioassay system was to have a small scale larval rearing system in which a number of replicated larval cultures could be reared to post-larvae 1 or 2 with a good survival and in which many experimental variables were controlled. The basis from which the bioassay system was developed was the small scale system described by Wilkenfeld,

Lawrence and Kuban (1986). Major adaptions were need to get the system to suit our purposes.

2.7.2 *Supply of larvae (for experimentation)*

Consistent supply of newly hatched prawn nauplii remained problematic throughout the project. A single species, *Penaeus monodon* was always used, but from a variety of sources. Nauplii III/IV were collected from spawnings in commercial hatcheries in Innisfail and Mackay, packed in oxygenated plastic bags and transported in buses to Townsville. Nauplii III to VI were collected from the Mariculture Unit, Australian Institute of Marine Science (AIMS) controlled breeding program. The final method of obtaining nauplii was by purchase of broodstock female prawns from a commercial broodstock supplier in Cairns. The broodstock were flown from Cairns to Townsville, then transported and held in the AIMS broodstock tanks. These broodstock were induced to spawn by eyestalk ablation. Gonad involution occurred in some of the broodstock from Cairns. Nauplii III to VI were collected and transported to the laboratory.
All nauplii were held at the laboratory for acclimatisation. Salinity was be adjusted to that used in the bioassay system. Counting and transfer to the bioassay system was done when the larvae were nauplii VI or protozoea I, some 55 to 60 hours after hatching.
Consequently, the larvae used in each trial in the bioassay system were from a single wild-caught female, but were genetically different between each trial. Marked variations in batches of nauplii were apparent in their vigour to first feeding as protozoea and survival to postlarvae.

2.7.3 *Structure*

The entire bioassay system was housed in a constant temperature room run at 24-26°C. One litre, graduated Imhoff cones (height 45.5cm, diameter at top 12.5cm) were used as the larval culture container. These were supported by a perspex sheet with 8cm diameter holes cut in it and mounted on a PVC pipe stand. The perspex sheet and the cones were placed in a one tonne Reln tank (2.31 x 1.17 x 0.5m). The tank was raised to a height where it was convenient to work on the cones. Water was added to the tank to the level of the raised perspex sheet and where 28cm of the cone was submerged. Two 300W aquarium heaters were used to heat the water bath to 29°C. A submersible fountain pump (Platypus P450) was used to ensure complete circulation of water and an even temperature throughout the water bath. An Externa IV air pump (14W) was used to provide air to each of the cones. The air was filtered through an autoclavable 0.3mm filter (Bacterial air vent, Gelman Sciences) before passing through gang valves which adjusted the air flow for each cone. Sterile plastic tubing and 1ml disposable pipettes delivered air to the bottom point of the cone. To prevent aerosol droplet transfer between cones, each was covered by a piece of perspex. There was a small notch to allow entry of the airline.

2.7.4 *Seawater (for experimentation)*

All seawater originated from the seawater system of the Mariculture Unit, AIMS and was transported to the laboratory in tanks. It was held in a 5 tonne storage tank under aeration until needed. Salinity varied from time to time, falling in the range of 28 to 33ppt. Seawater required each day for the bioassay system was transferred to a 50 litre plastic barrel and heated overnight to 32°C. Prior to use in the larval cultures, the seawater was pumped through a series of cartridge filters (10, 5, 1, 0.6 and 0.2μm) and then through a 25W Acry-tec ultra-violet steriliser. This seawater was used immediately for that day's

requirements.

Initially bacteria-free seawater could not be produced by this filter system. Trials eventually led to a schedule where freshwater was used to flush out seawater each day after use and where the filters and housings were disinfected every three days with sodium metabisulphite and citric acid. When not in use the system was dried out and the 0.6 and 0.2mm filter cartridges had to be held in a -20°C freezer to maintain their integrity.

2.7.5 *Feed supply*

The larvae in the bioassay system were fed with the marine diatom *Chaetoceros gracilus* and commercial microencapsualted diets; Crustacean Algal Replacement (CAR) and Crustacean Diet number 2 (CD2), Frippak.

2.7.5.1 *Algal production*

An initial stock culture of *Chaetoceros gracilus* was purchased from CSIRO, Hobart but failed to grow. The stock *Ch. gracilus* used for all the trials originated from AIMS. The medium FE_2 was used for all algal cultures. Several 250ml stock cultures of *Ch. gracilus* were maintained in the laboratory using ¼ FE_2. Every 7 days 50ml of the stock was transferred to a fresh 200ml of medium.

Algal culture shelves, similar to that described by Fox (1983), were constructed to grow the *Ch. gracilus*. Illumination on a 12 hour light:12 dark cycle was provided by paired 36W cool white fluorescent tubes mounted at the back of each shelf. 50ml of stock culture was transferred to 200ml FE_2 in a screw top flat bottom flask on day one. On day 4 this culture was transferred to 5 litres of FE_2 in a 5L Pyrex culture bottle. These 5L cultures were aerated via a modified stopper (Fox 1983). The aeration was supplied by aquarium air pumps via a 0.8mm disposable syringe filter (Minisart NML, Sartorius) and an autoclavable 0.3mm filter (Bacterial air vent, Gelmen Sciences) in series. After 3 days of growth, when the cell density reached 10^5-10^6 per ml, the culture was ready to be harvested, concentrated and fed to the larvae. Axenic, aseptic algal culture techniques were used at all times. The media would be dispensed into the culture flasks and bottles, then autoclaved. The modified stoppers for aeration of the 5L bottles were autoclaved prior to use. All transfers were done in laminar flow cabinets.

Each day of a trial, 1- 1.5 litres of *Ch. gracilus* culture was concentrated by filtering through a 10mm mesh Nylex filter cloth which had been glued over the end of a 1 litre plastic beaker with the bottom cut out. The culture in the 5L bottle was replenished with 1L of sterile fresh FE_2. Each 5L culture was harvested for 3 consecutive days before it was discarded. These procedures were also done in a laminar flow cabinet.

The number of algal cells in the algal concentrate would be determined in an improved Neubauer counting chamber. The volume of algal concentrate to be added to each larval culture was then calculated.

2.7.5.2 *Microencapsulated diet*

The two microencapsulated diets (MED) were held in darkened containers in a -20°C freezer. Pre-weighted amounts were prepared and sealed in individual aluminium foil packets. The pre-weighted amounts would be added to sterile seawater immediately prior to feeding. Once a standard rearing protocol was established a 12mg per 10ml concentration of CAR and a 20mg per 10ml concentration of CD2 was used to feed the larval cultures.

2.7.6 *Standard rearing protocol*

Six larval rearing trials were required before suitable techniques and procedures were established.

Preparation of the system consisted of disinfection of cones, airlines etc in 60mg chlorine/L for at least 24 hours. The equipment was then rinsed and air dried for 24 hours.
Each cone was filled with 1 L of filtered seawater. 100 (or 200) nauplii VI or protozoea I larvae were counted and added to the cone. Counting was done by transferring individual larvae by fine pipettes from a petri dish containing larvae to a beaker of filtered seawater. The larvae in the beaker were then added to the cone. Airlines and 1ml pipettes were then set in each cone and the airflow adjusted to a rate just sufficient to mix the water. Algae was added to the required number. The cone was then covered.
Each day the seawater in each cone was changed once, usually in the morning around 9.00am. The larvae were filtered from the old seawater into a 1L plastic beaker with 100mm mesh Nylex filter cloth glued over the cut out bottom. The cone was rinsed with filtered seawater, but checked for any retained larvae, then filled with 1L of fresh, filtered seawater. The larvae were then gently washed off the filter cloth and returned to the cone. At no time were the larvae allowed to dry out, most of the time they remained immersed in water. After the water was exchanged in all of the cones the required amount of algal concentrate would be added. Feeding of the MED was done twice daily at 8.00am (before the water exchange) and 6.00pm, or four times a day at 8.00am, 10.00am, 2.00pm and 6.00pm depending on the trial. The CAR diet would be fed from day 1 or 2 or when the larvae were protozoea I. CAR would be stopped on day 6 or when the larvae molted to mysis I and be replaced with CD2. The volume of MED in sterile seawater fed at each larval stage is given in Table 2.
Treatment and control groups comprised four, six or, more usually seven replicated cones. Each cone was assigned randomly to a treatment or control group for each trial.

Table 2: The feeding schedule for micro-encapsulated diets CAR and CD2 used in the standard larval rearing protocol in the bioassay system for each cone.

DAY	STAGE*1	MED*2	
		CAR(ml)	CD2(ml)
0	NI-NIII	-	-
1	NIII-PI	(0.5)?	-
2	PI	0.14	-
3	PI-PII	0.15	-
4	PII	0.2	-
5	PIII	0.3	-
6	PIII-MI	0.3	-
7	MI	-	0.35
8	MII	-	0.35
9	MIII	-	0.4
10	MIII-PL1	-	0.45
11	PL1	-	0.45

*1 N = nauplii, P = protozoea, M = mysis, PL = postlarvae
*2 Suspension in sterile seawater with 12mg CAR/10ml and 20mg CD2/10ml concentrations.

2.7.7 *Counting larvae and lesions*

Counting larvae was done every two or three days. Larvae filtered onto the Nylex filter cloth were washed into a petri dish. The larvae were counted as they were individually transferred to a beaker of filtered seawater, then placed back in the cone.
The percentage prevalence of bacterial necrosis or other lesions were calculated by examination of preserved larvae under a dissecting microscope. Typically, three randomly selected cones would be removed from each treatment or control group once the larvae were protozoea III or mysis I (around day 5). The larvae would be concentrated and preserved in 10% buffered neutral formalin. Larvae from the remaining cones would be preserved once the trial ended.

2.7.8 *Viable bacterial counts*

The numbers of viable aerobic heterotrophic bacteria in the larval culture water of the bioassay were examined on two occasions to get information on bacterial dynamics in the cones. A spread plate counting method with duplicates was used. Plates were made of 4% marine agar (Difco) and dilution blanks were sterile saline (1.5%) solutions. 0.1ml was added to each agar plate and spread. Plates were incubated overnight at 25°C. Those plates with <300 or >30 colonies were counted. Samples from the cones being monitored

were taken hourly after water exchange with fresh filtered seawater. Two larval cultures in Trial B (see below) were monitored initially. A control cone and one each from the two treatment groups in Trial L (see below, Bacterial Challenge) were then examined.

2.7.9 *Larval rearing trials*

2.7.9.1 *Level of nutrition*

Using a standard stocking density of 100 nauplii per 1L cone at first stocking, the effect of various levels of nutrition were compared. Over five rearing trials the following feeding schedules were used:

Trial		*Ch. gracilus* cells x 1,000 per ml	Number of MED feeds per day	Plane of Nutrition
A.	Control	100	4	High
	Treatment One	50	4	Low
	Treatment Two	20	1	Very Low
B.	Control	100	2	High
	Treatment	50	4	Low
C.	Control	80	4	High
	Treatment	50	2	Low
D.	Control	80	4	High
	Treatment	50	2	Low
E.	Control	80	4	High
	Treatment	50	2	Low

2.7.9.2 *Level of aeration*

There have been suggestions that aeration of larval cultures that is too strong will damage the cuticle of the larvae increasing their susceptibility to bacterial infections. Normal aeration in the bioassay system was very gentle, just sufficient to mix water, food particles and larvae while ensuring no 'dead spots' occurred in the cones. Three trials examined the effect of heavy aeration on larval survival and lesion prevalence (F, G and H). Aeration levels were increased in the treatment group to were the surface of the water in the cone 'boiled' violently. Feeding levels were 80,000 *Ch. gracilus* cells/ml/day and four MED feeds per day. Initial stocking densities were 100 nauplii per litre.

2.7.9.3 *Stocking densities*

Two levels of stocking density were compared in two trials (I and J). The control level was 100 nauplii per litre. Treatment cones had 200 nauplii per litre stocked on day one of the rearing trial. Normal aeration was used. Feeding levels were 80,000 *Ch. gracilus* per ml per day and four MED feeds per day.

2.7.9.4 *Cold water shock*

One trial was done to examine the effect of a stress in increasing the susceptibility of larvae to bacterial diseases (Trial K). A daily cold shock was used to stress larvae. After filtration of the heated seawater a disinfected plastic bucket with 15 litres of seawater would be placed in a 2°C chiller room to cool. This seawater at temperatures of 20-24°C was used as the fresh seawater at water exchange for the treatment cones.

2.7.9.5 *Bacterial challenge (Trial L)*

A bacterial isolate from a diseased larval culture, *Vibrio alginolyticus*, was used to challenge larvae in an attempt to cause disease. The bacteria was stored in aliquots of broth culture at -20°C. This aliquot was inoculated into Vibrio starter culture media (L.E. Eaves, pers. comm.), incubated at 25°C for 24 hours and then washed twice in 1.5% saline solution. The bacterial pellet was finally resuspended in 1.5% saline solution to a known density for addition to larval cultures. On day four of the larval rearing period when the larvae were protozoea II, 0.1 mls of the *V. alginolyticus* suspension was added to one group of cones and 1.2 mls to another. This gave *V. alginolyticus* concentrations of 10^2 cells/ml and 10^6 cells/ml respectively. A control group had no bacteria added. Initial stocking densities were 100 nauplii per litre. Feeding levels were 80,000 *Ch. gracilus* cells per ml per day and four MED feeds per day. Standard rearing procedures were followed prior and subsequent to the addition of *V. alginolyticus*.

2.7.9.6 *Statistical analysis of larval rearing trial results.*

Mean survival of control and treatment cones on the day of sampling were compared by a standard analysis of variance.

3. RESULTS

3.1 Hatchery monitoring program

3.1.1 *Disease prevalence*

Bacterial necrosis was the only bacterial syndrome affecting larvae or postlarvae seen during the study. Bacterial necrosis with melanization of the cuticle and bacterial colonization occurred in all 8 monitored larval batches. Lesions were most common on the appendages and sometimes resulted in partial amputation or stubbing. Maximum prevalence for each batch ranged from 5% to 49%. The prevalence of necrosis fluctuated rapidly from day to day, indicating either death or recovery of affected prawns (Figs1-8). The course of these infections remains uncertain because concentrations, and hence mortality rates, of prawns could not be reliably determined, particularly in the postlarval stages. While the prevalence of bacterial necrosis in all larval cultures was usually highest during the postlarval phase, affected larvae were seen in most runs as early as mysis stage.

3.1.2 *Bacteriology*

Larval rearing tank water

With some experience, the dip slides were easy to read and there was good agreement between duplicate slides. Initially some hatchery technicians had difficulty distinguishing bacterial colonies from blemishes on the surface of the dip slides. Care also had to be taken to avoid overlooking confluent growth. Rarely, one of the two slides failed to support growth while profuse growth occurred on its replicate.

In undiluted larval rearing tank water, bacterial colonies on marine agar were often confluent and were recorded as >1000. On TCBS, counts greater than 1000 were less common. Such counts were seen only after prawns had entered the postlarval stage. On 25 of the 30 occasions when 10% or more of larvae had bacterial necrosis, the TCBS count on that day and/or the preceding day exceeded 100 colonies (Table 1).

The response of TCBS counts to antibiotic additions to larval rearing water was erratic, with no clear pattern emerging.

Relationships between TCBS dip slide counts, prevalence of bacterial necrosis and antibiotic usage are illustrated in Figs.1-8.

Table 1.

Association between TCBS Dip-Slide Counts and prevalence of bacterial necrosis

Larval Batch	Necrosis > 10%		Necrosis < 10%	
	TCBS+*	TCBS-**	TCBS+*	TCBS-**
1	2	0	1	11
2	1	2	6	6
3	3	0	12	12
4	13	2	2	12
5	0	0	1	15
6	3	0	1	14
7	0	1	2	10
8	3	0	2	11
Total:	**25**	**5**	**27**	**91**

*TCBS counts > 100 on day before and/or on same day as above prevalence occurred.
**TCBS counts <100 on day before and/or on same day as above prevalence occurred.

We were frequently unable to recover organisms from TCBS medium after incubated dip slides had been held at 5°C for 3 or more days.
Twenty two selected dip slide isolates from rearer tanks were submitted to ARI for identification.

Total bacterial concentrations obtained by the epifluorescence technique ranged from 2.0 x 10^6 to 4.5 x 10^8 organisms/ml; most concentrations were within the range 1-9 x 10^7/ml (Figs 9-16). Concentrations in all larval batches were usually highest during the postlarval period. Antibiotic administration did not always reduce bacterial concentrations.

Algal and Artemia cultures
Bacterial concentrations in algal cultures were remarkably variable, and most counts of cultures however fell within the range 10-1000 on marine agar medium. Few of these organisms grew on TCBS and on only two occasions were 1000 or more colonies counted on that medium. Two days after feeding one of these algal cultures there was an increased TCBS dip slide count on water from the larval culture (Fig 24) and an increase in the prevalence of bacterial necrosis followed (Fig 8).
Bacterial counts from undiluted *Artemia* cultures were usually confluent on both media. When one hundred-fold dilutions were cultured, counts were usually in the 10 to >1000 range on marine agar and 0 to 1000 on TCBS.
Relationships between TCBS dip slide counts for rearer water, algal cultures and *Artemia* cultures are shown in figures 17-24.
Sixteen selected dip slide isolates from algal or *Artemia* cultures were submitted to ARI for identification.

3.1.3 Water chemistry
Relationships betweeen bacterial necrosis and concentrations of un-ionised ammonia nitrogen, nitrite and nitrate in rearer water are shown in figures 25-32, 33-40 and 41-48 respectively; 96 hour LC_{50} concentrations for ammonia nitrogen and for nitrite at each larval and postlarval stage (Chin and Chen 1987; Chen and Chin, 1988) are included.
The maximum values obtained for un-ionised ammonia nitrogen (NH_3-N), nitrite nitrogen and nitrate nitrogen were respectively 0.04, 0.02 and 1.3 mg/l.

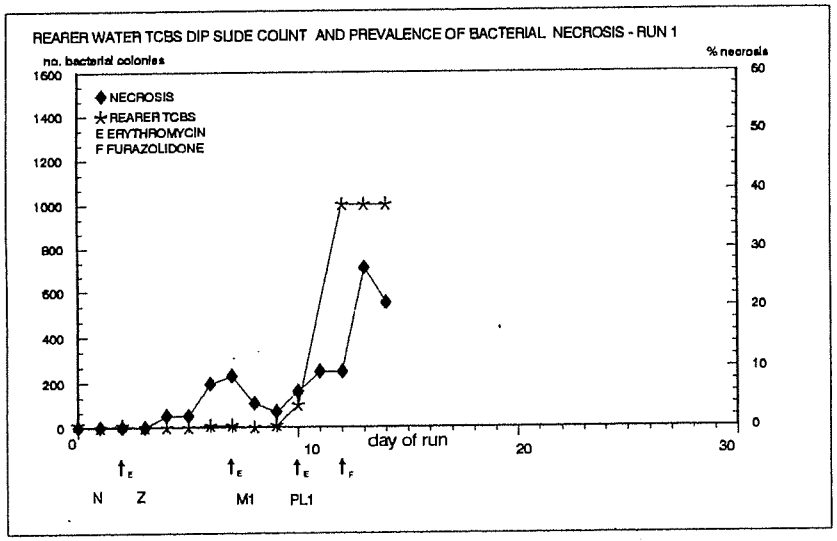

Fig.1

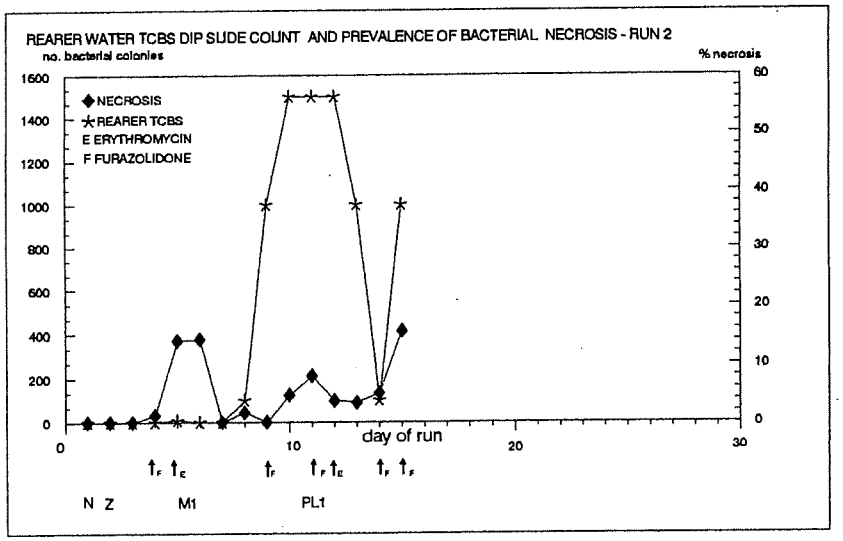

Fig. 2

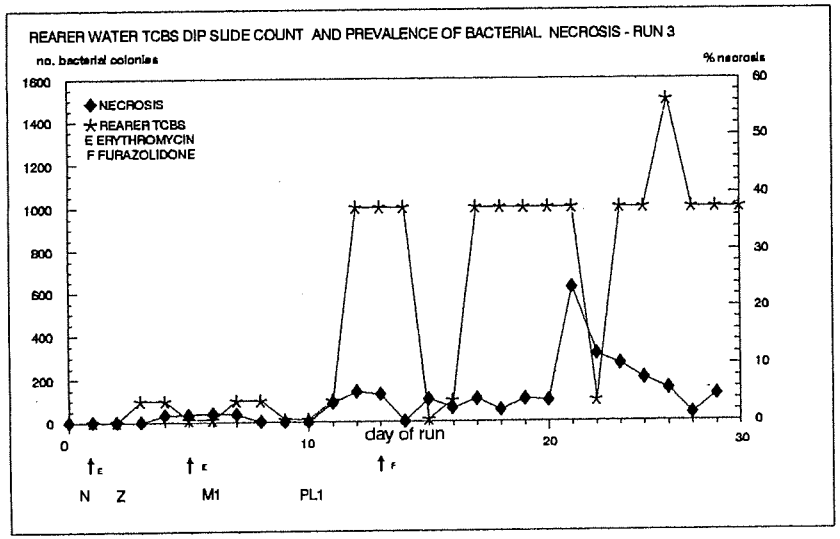

Fig. 3

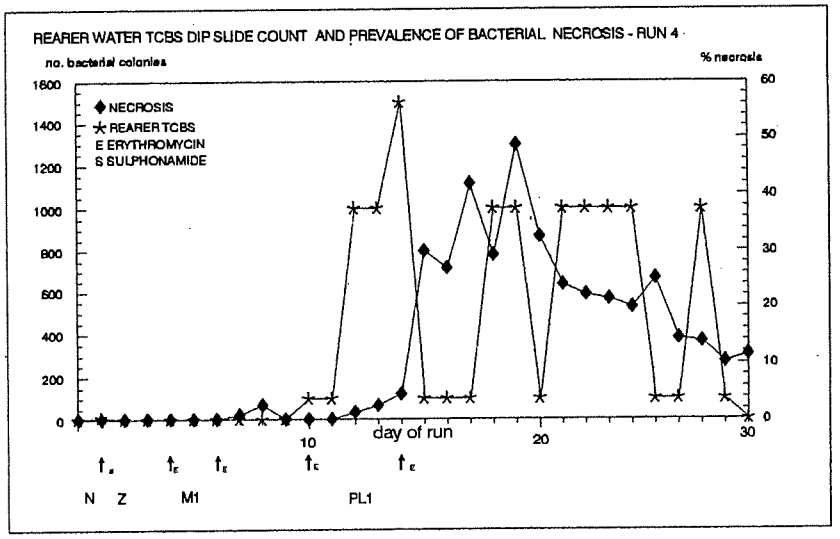

Fig. 4

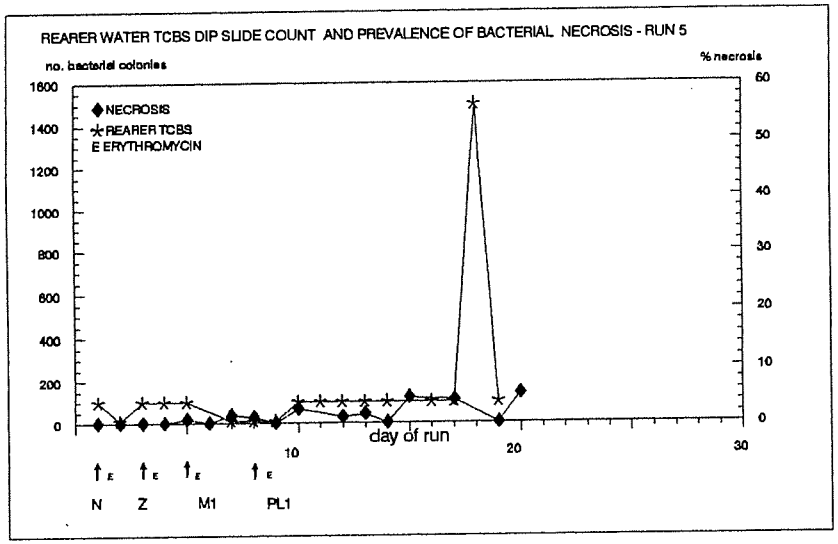

Fig.5

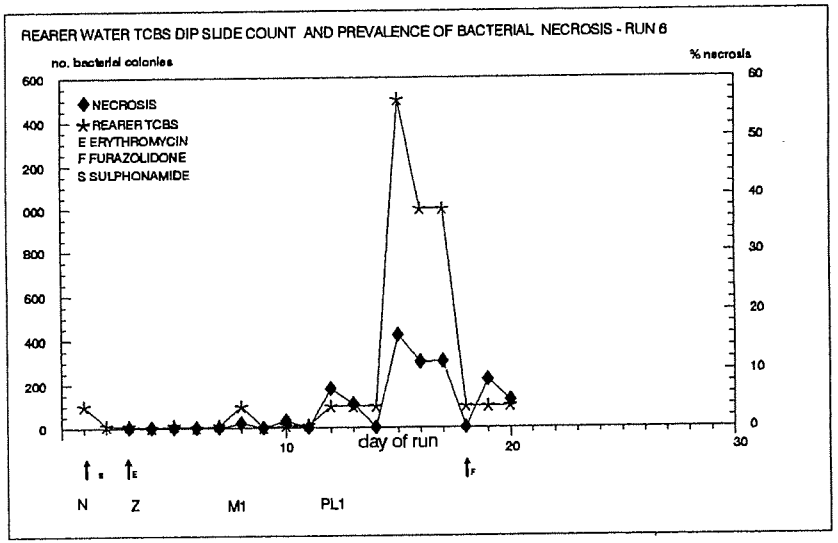

Fig. 6

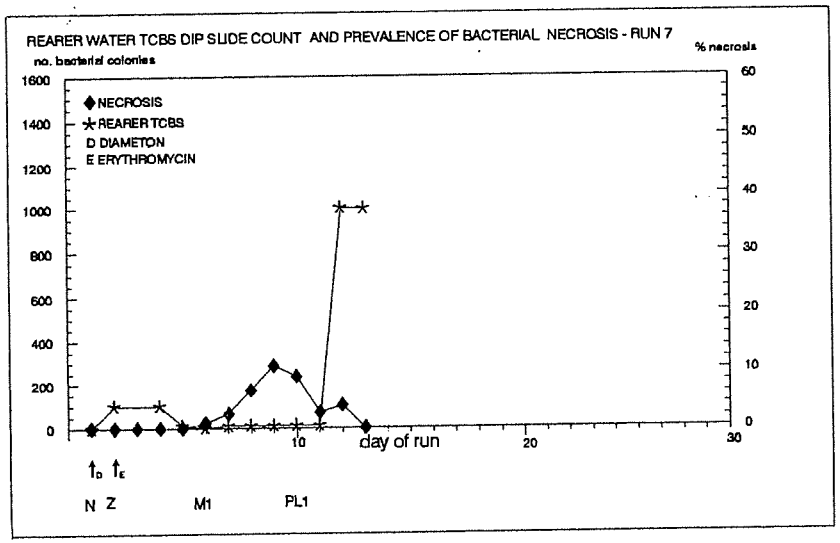

Fig. 7

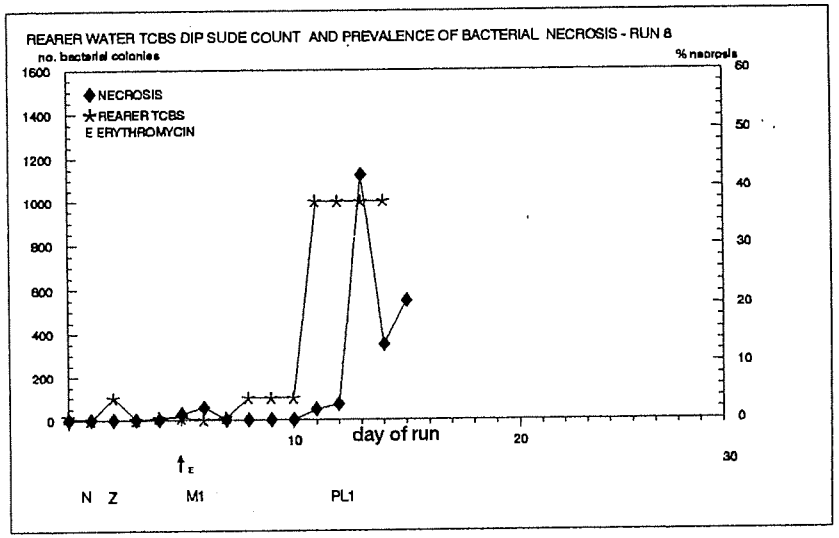

Fig. 8

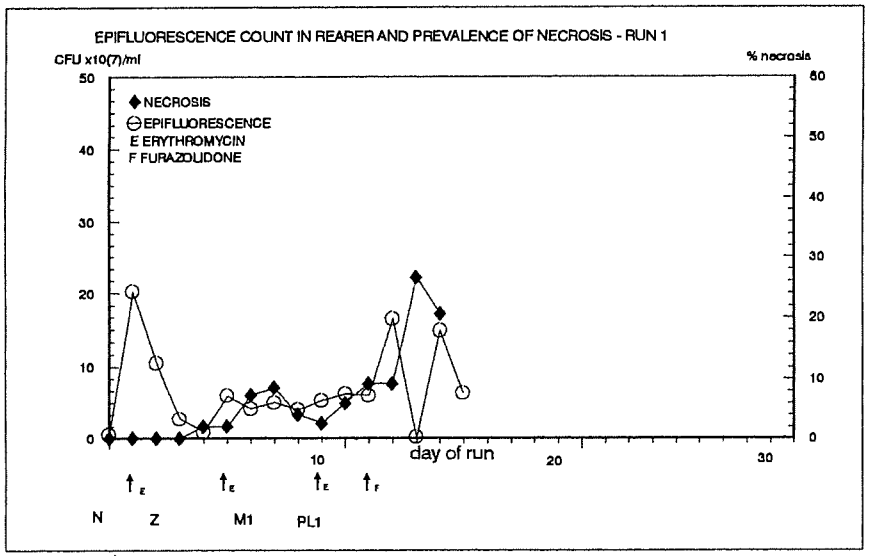

Fig. 9

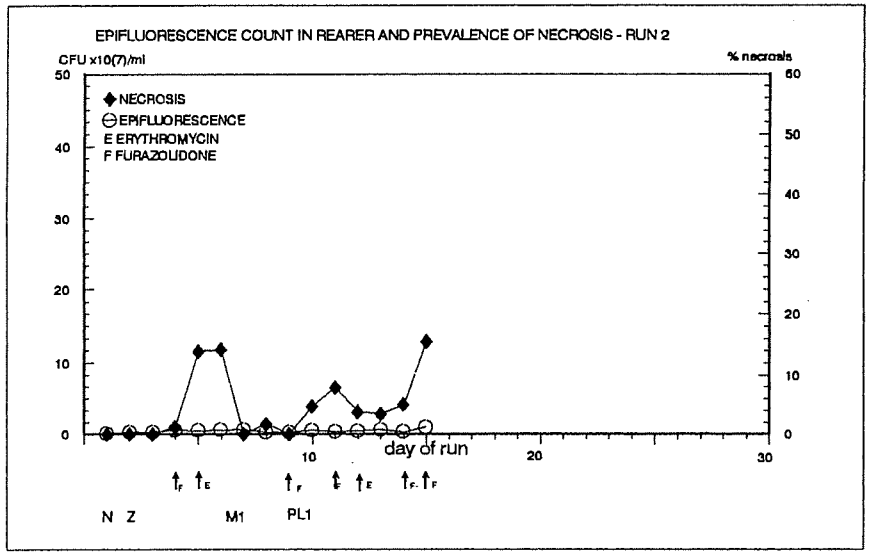

Fig. 10

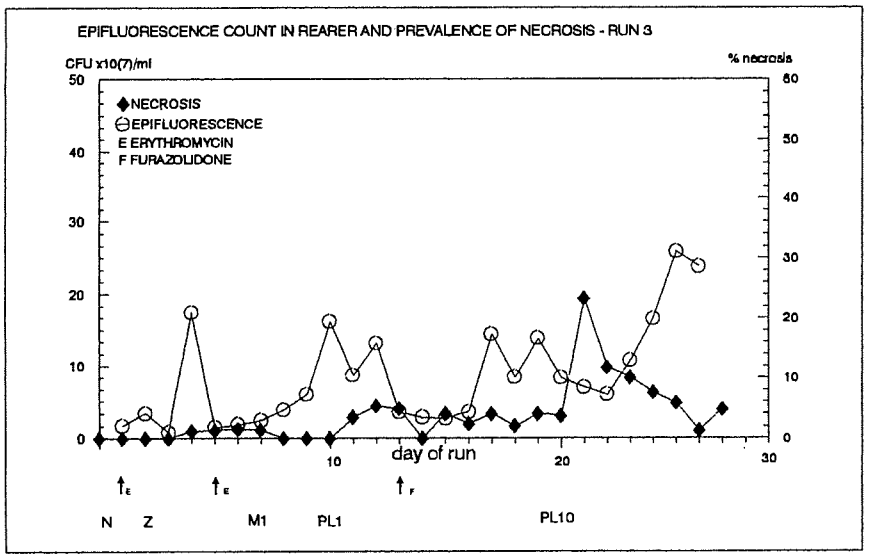

Fig. 11

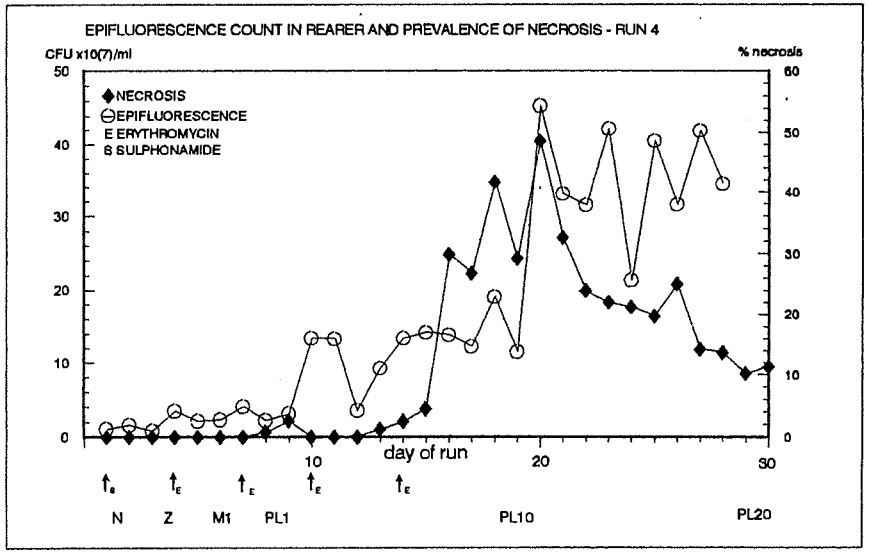

Fig. 12

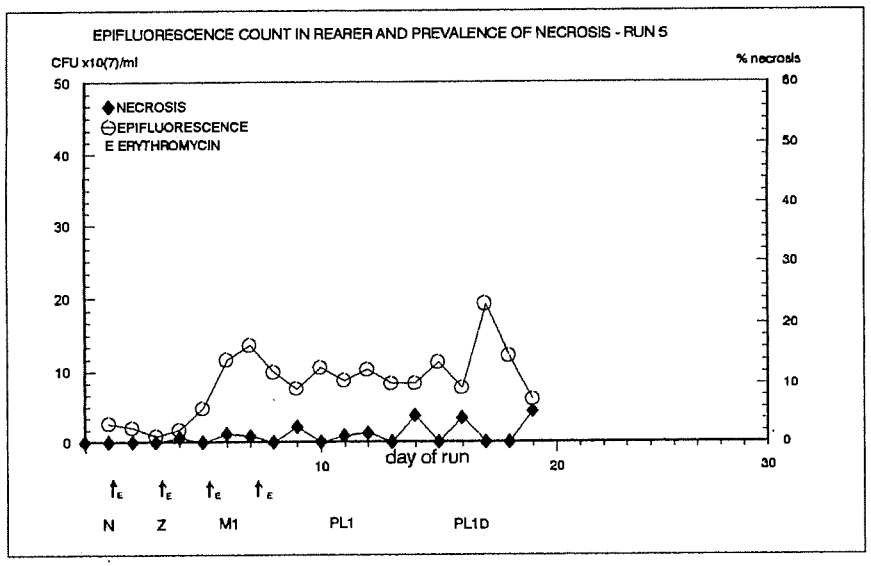

Fig. 13

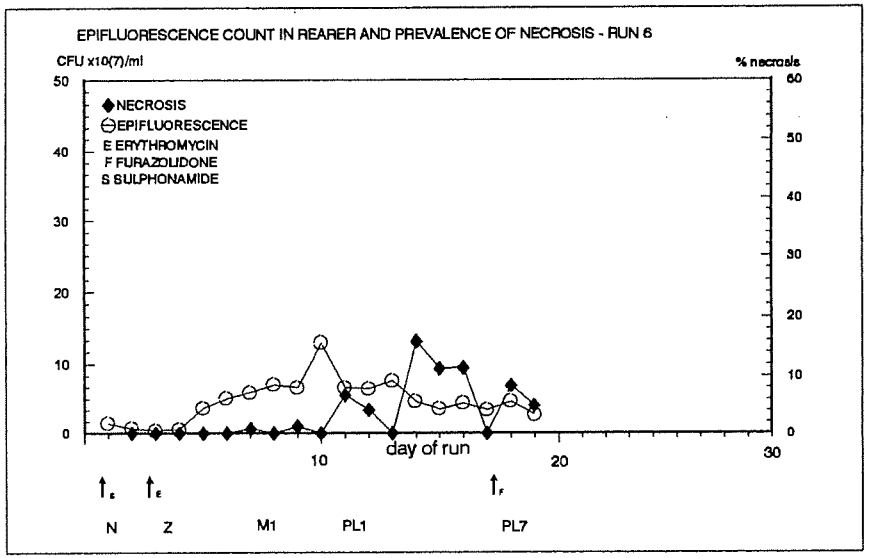

Fig. 14

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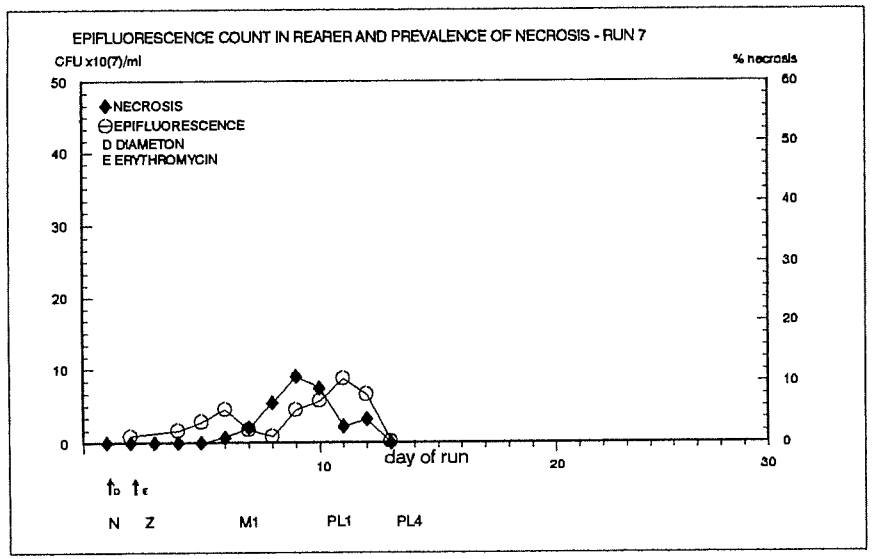

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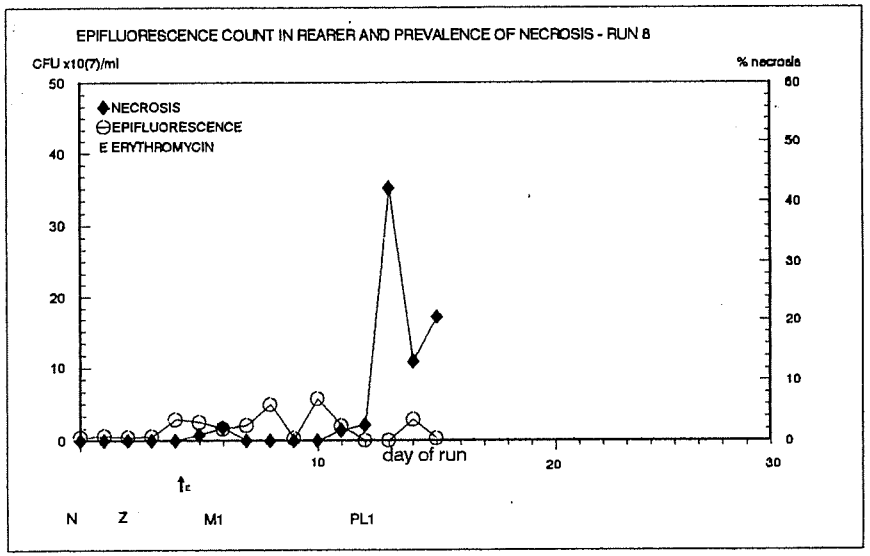

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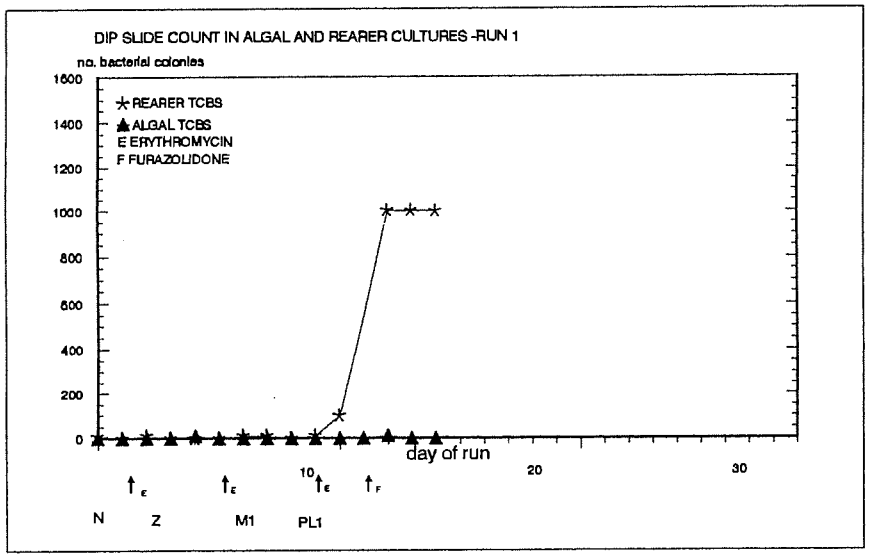

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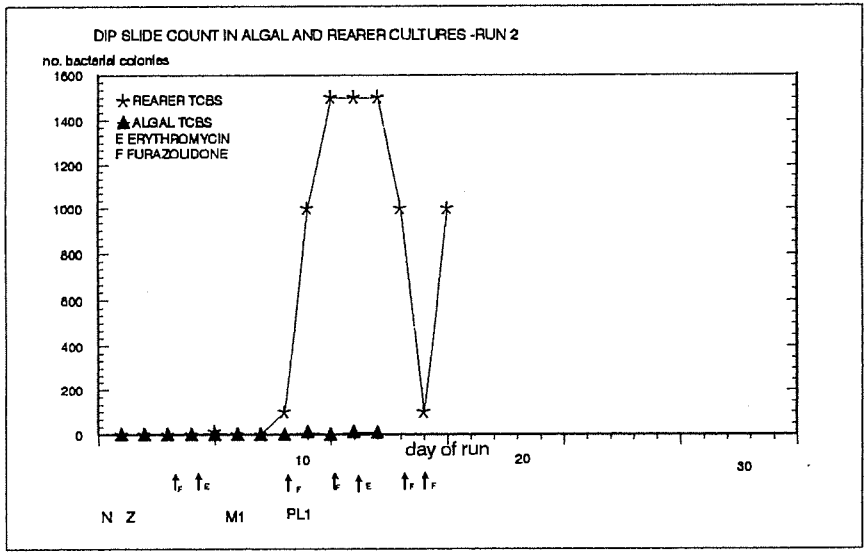

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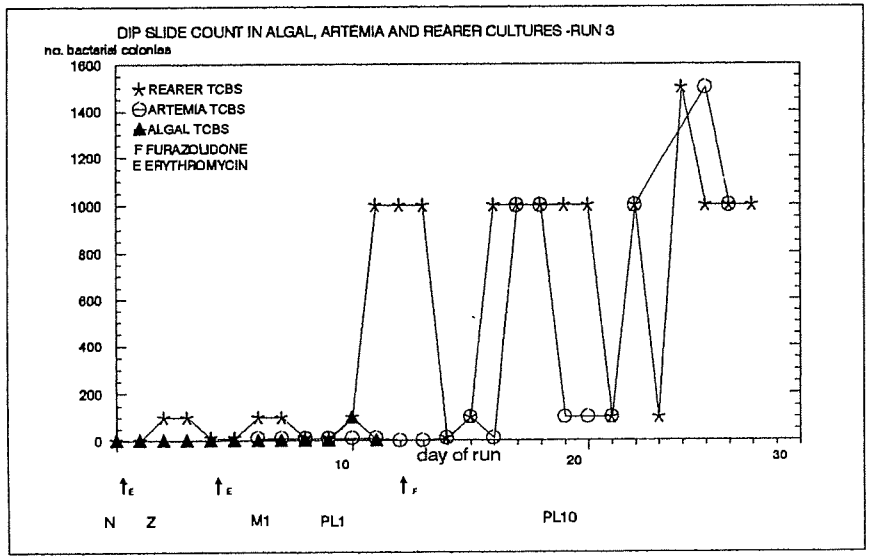

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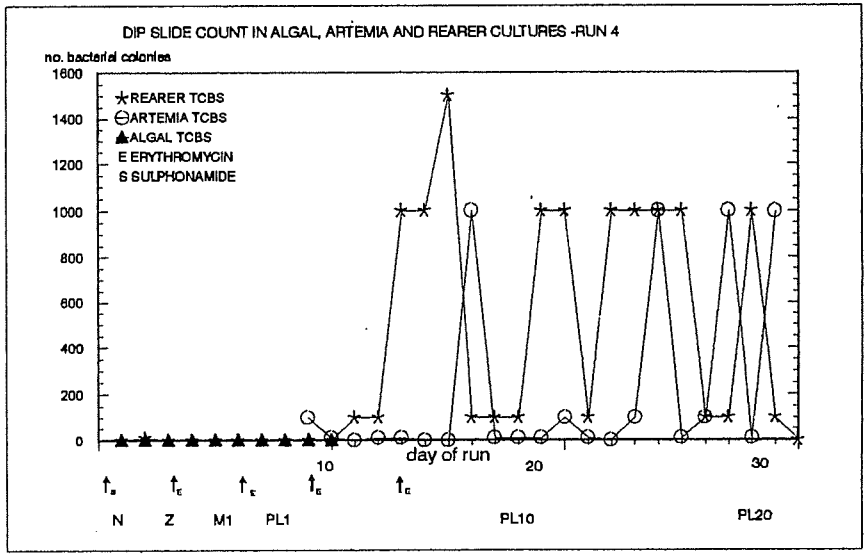

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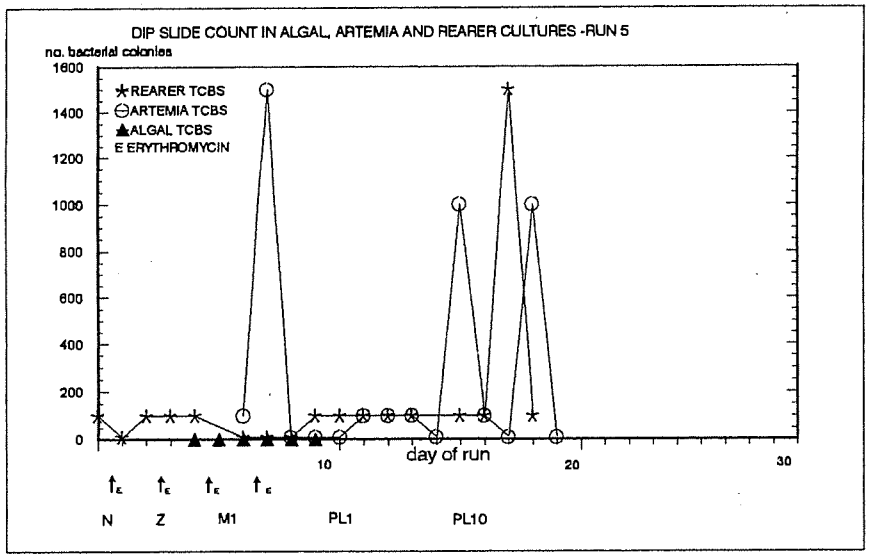

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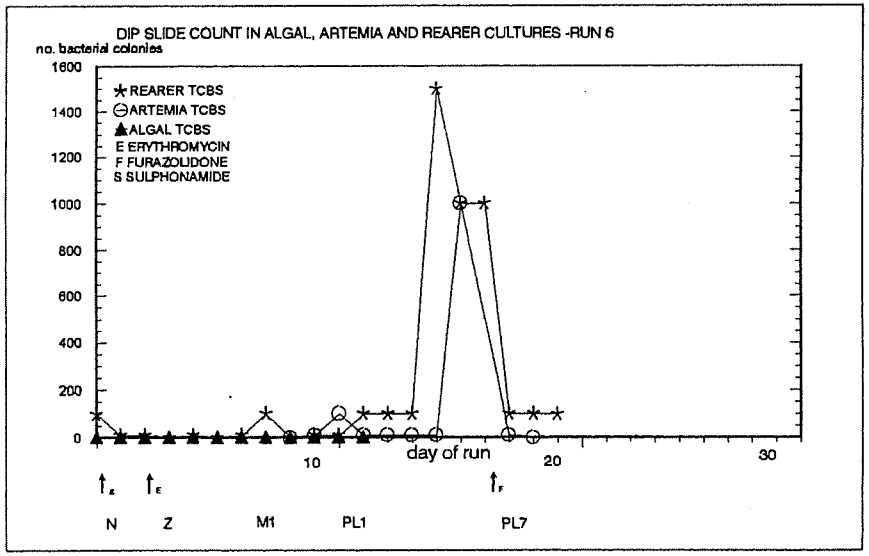

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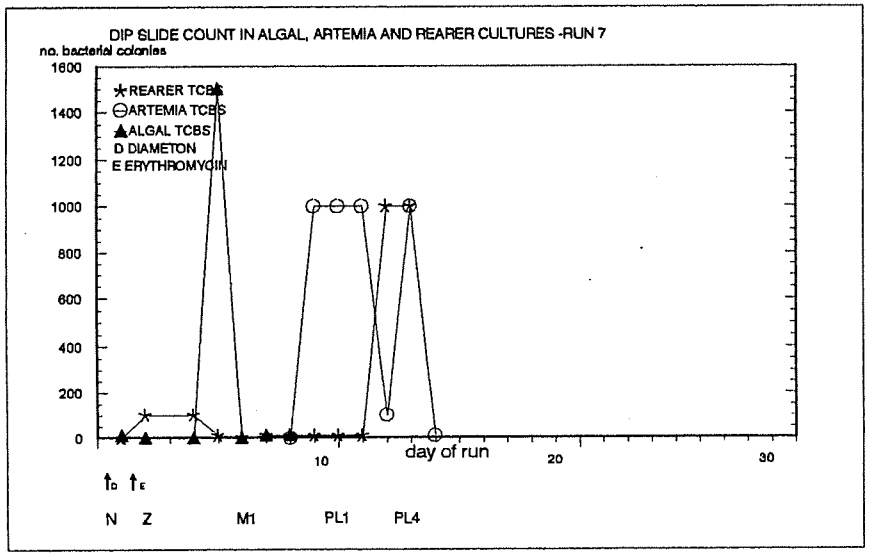

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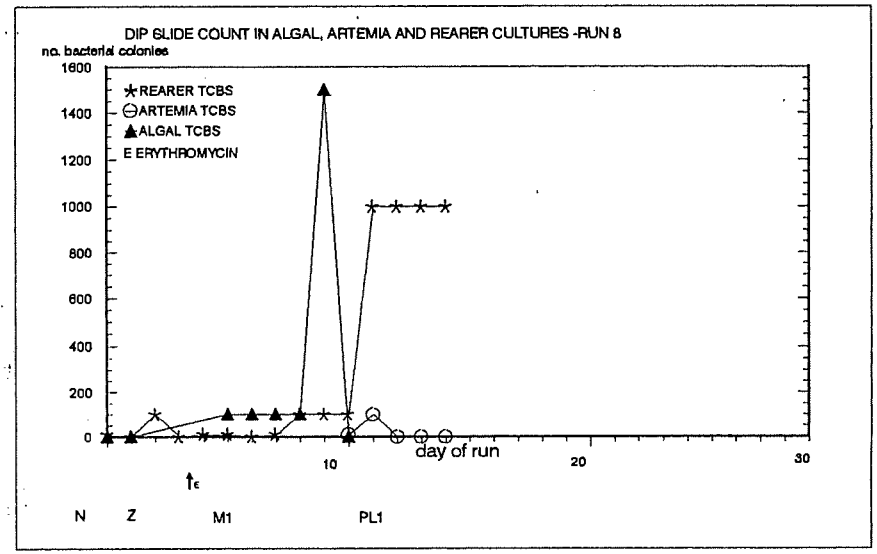

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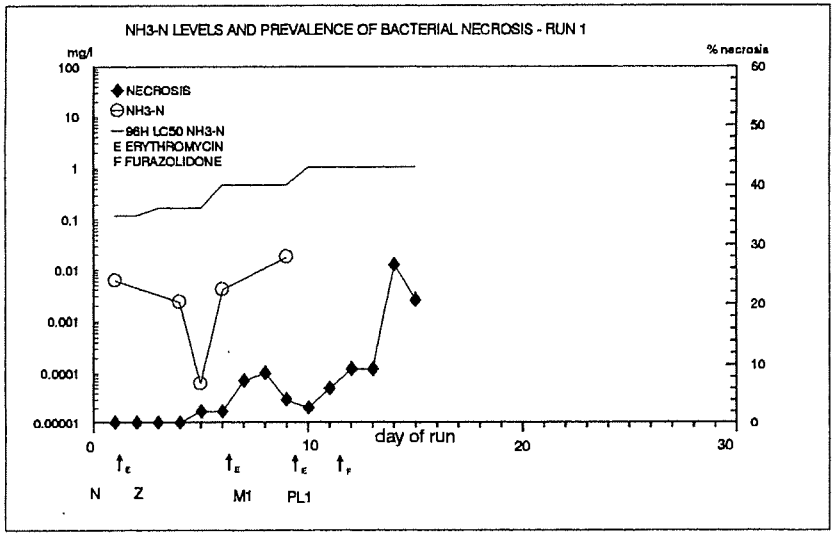

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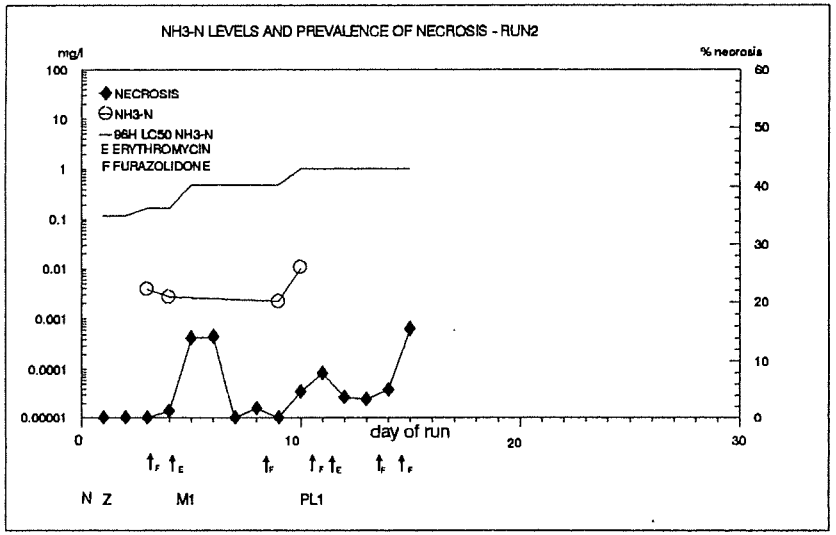

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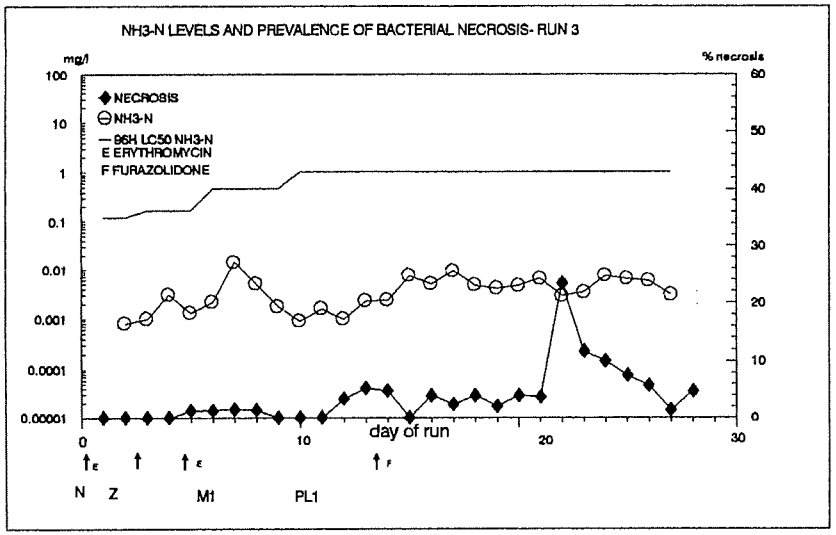

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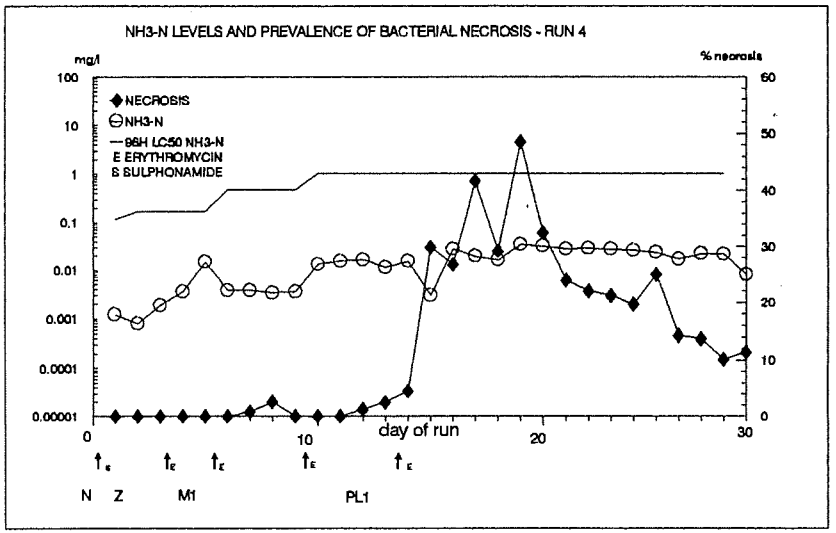

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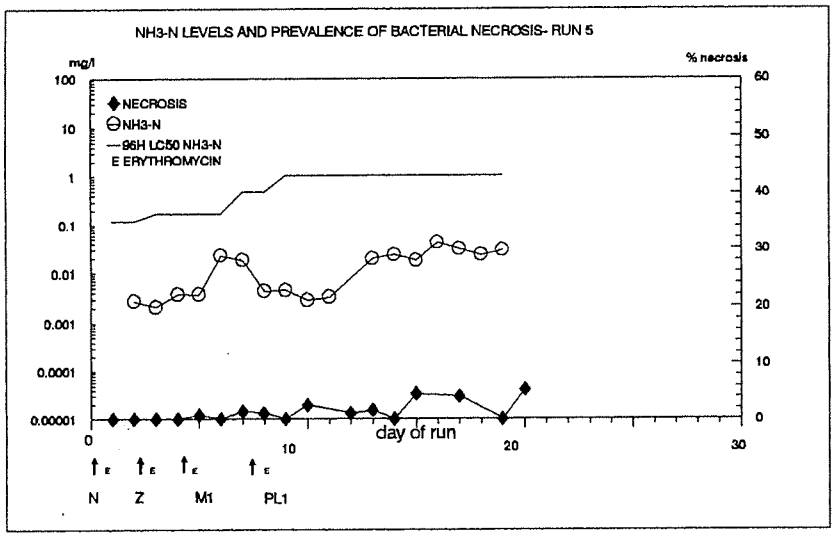

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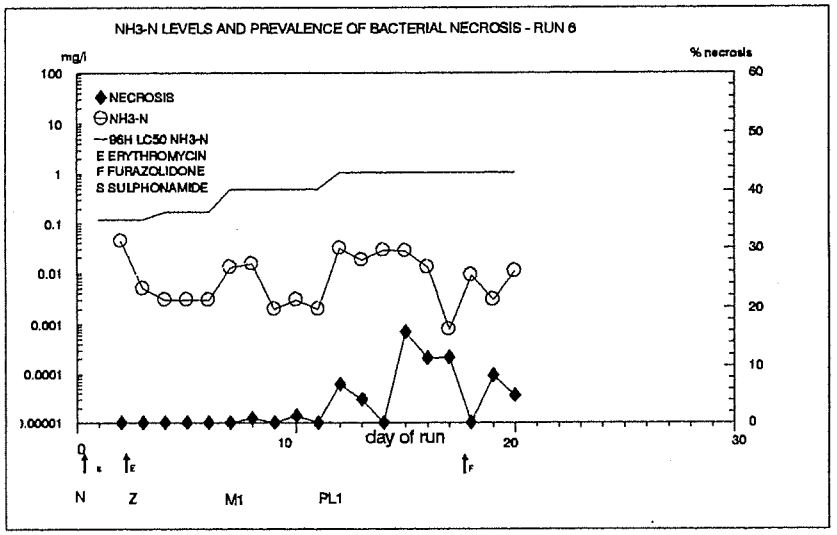

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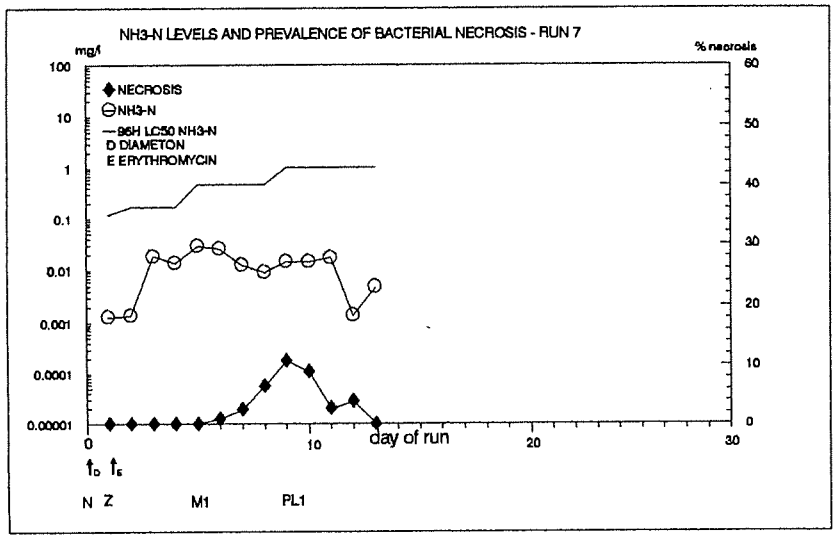

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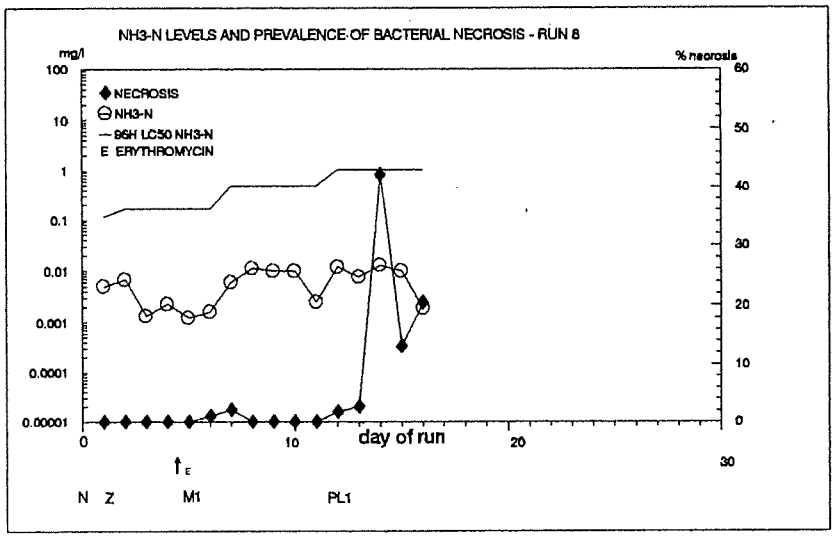

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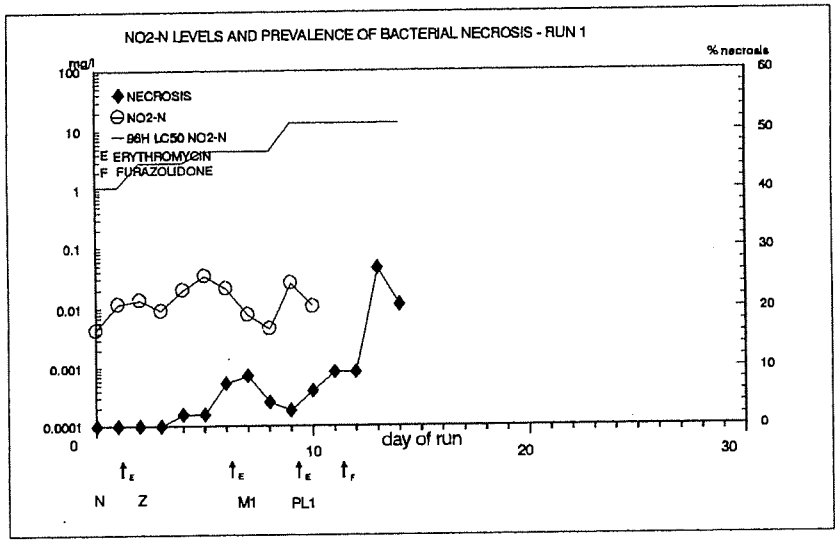

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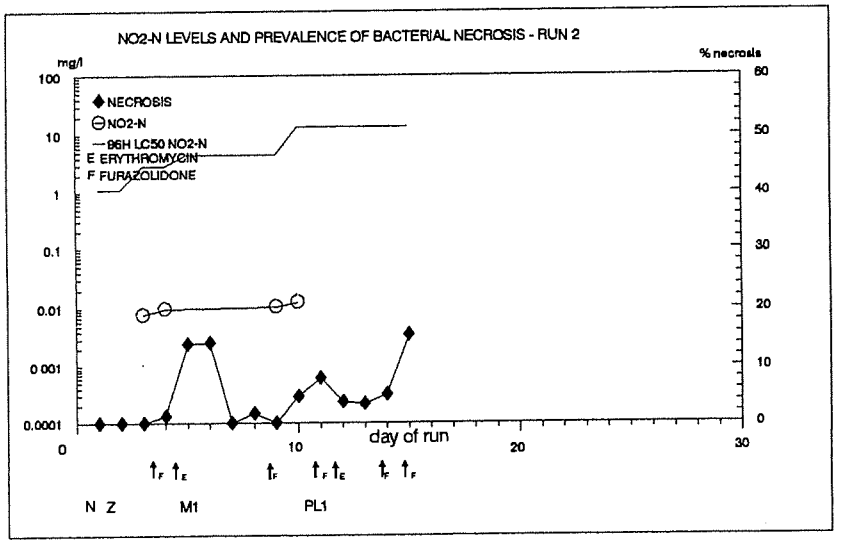

Fig. 34

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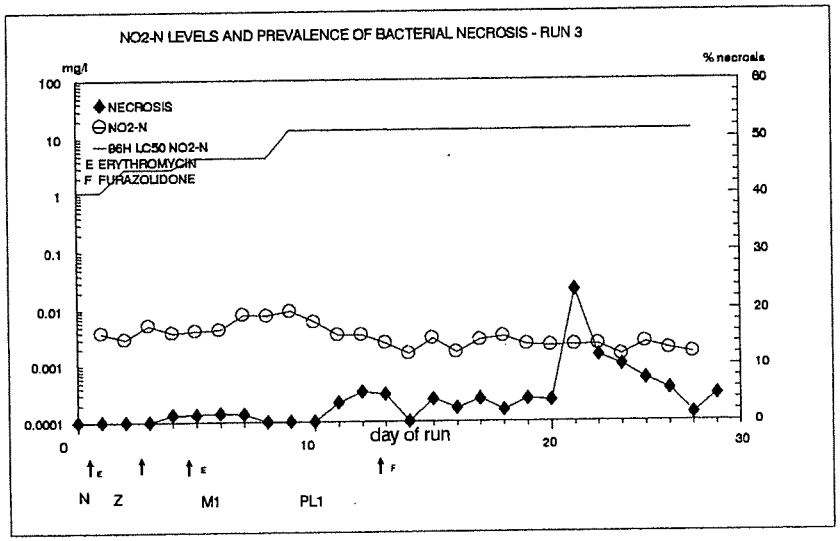

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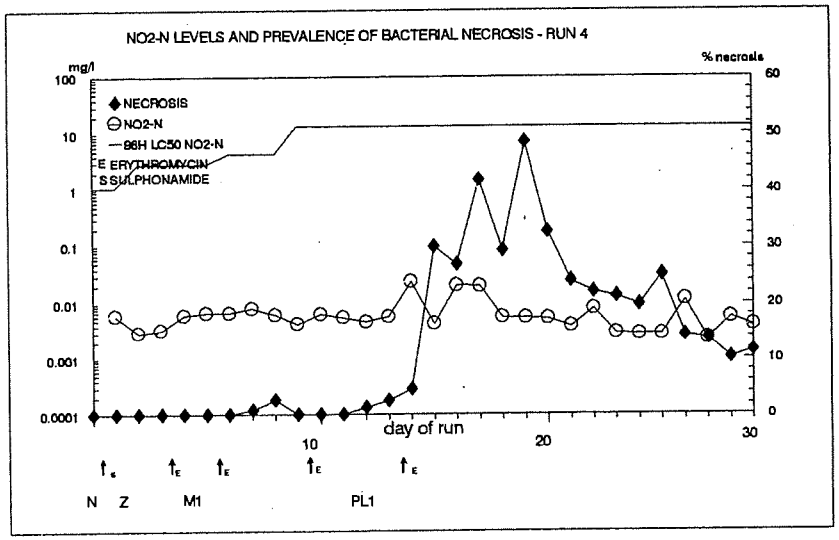

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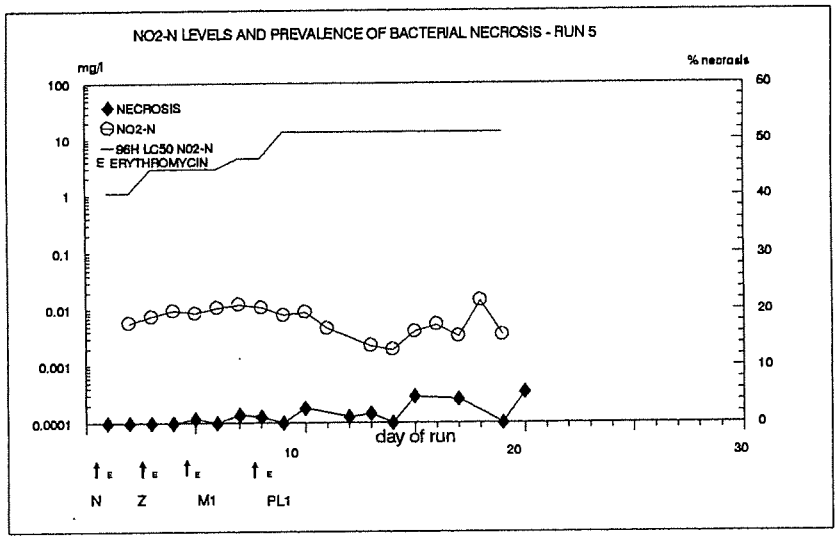

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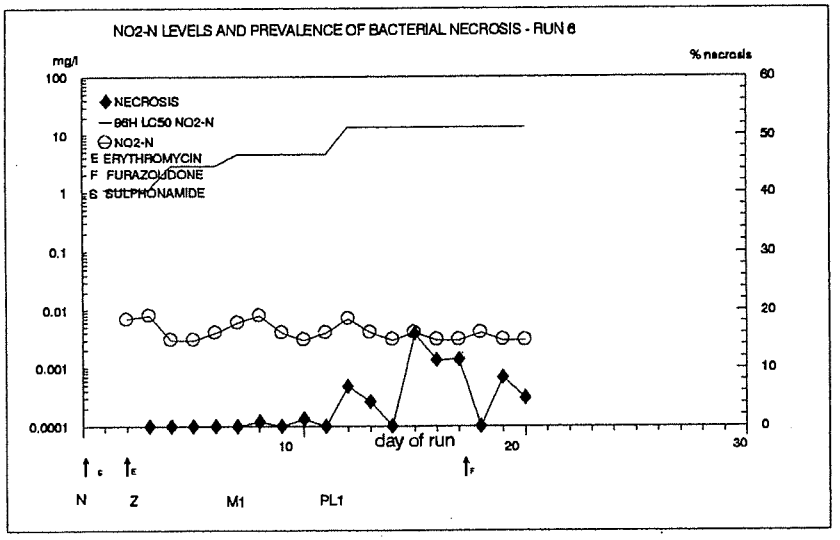

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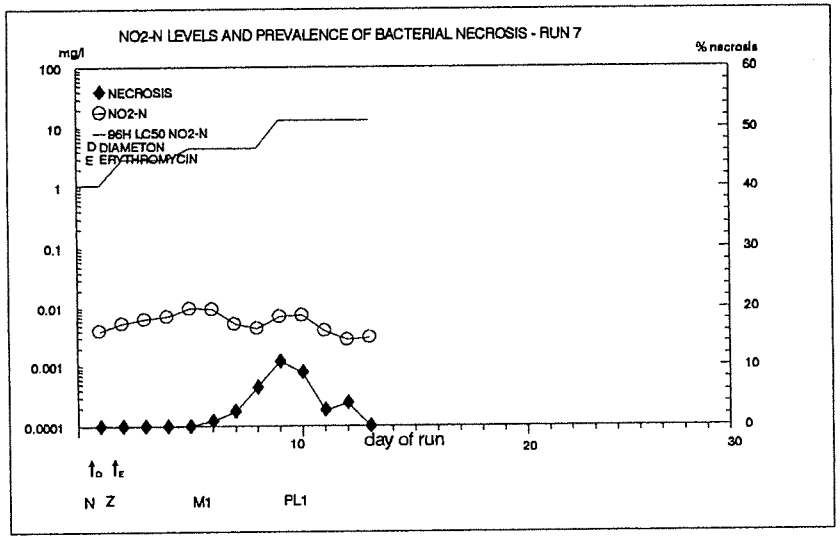

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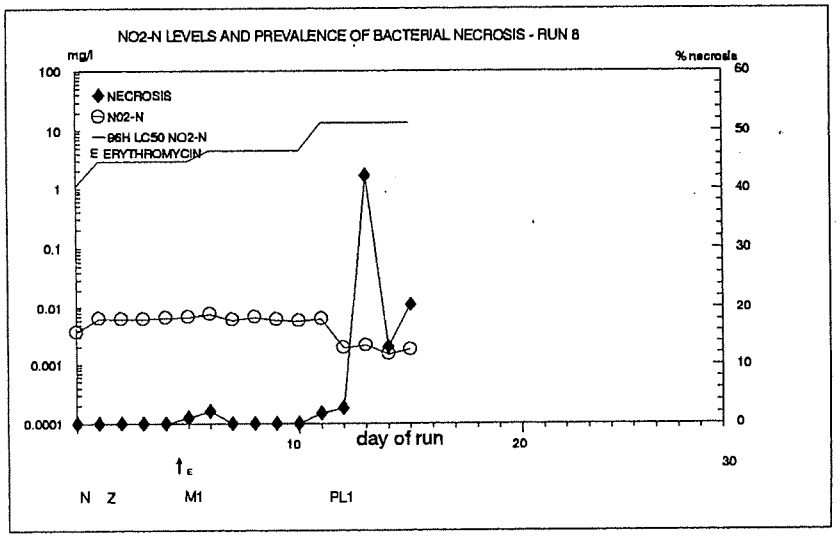

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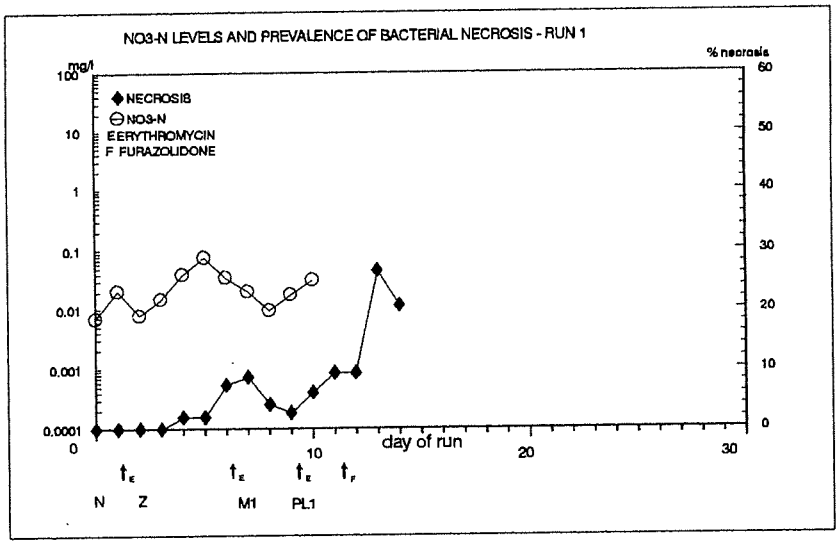

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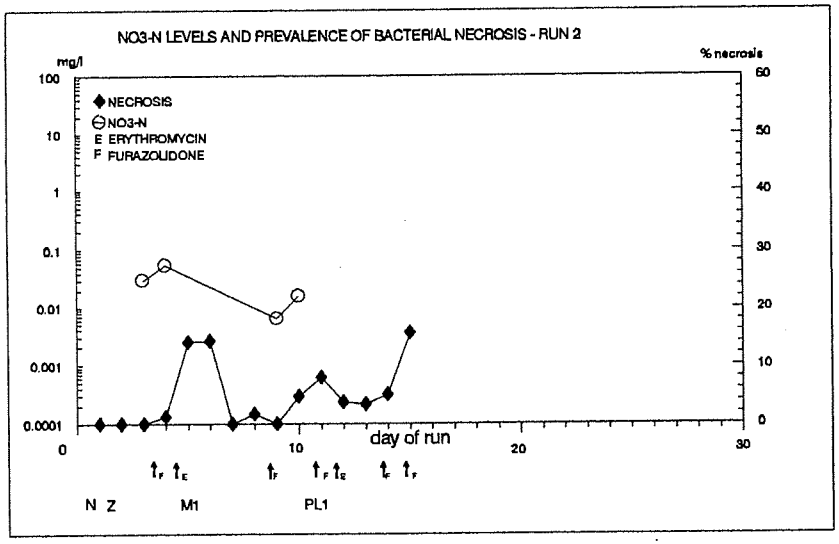

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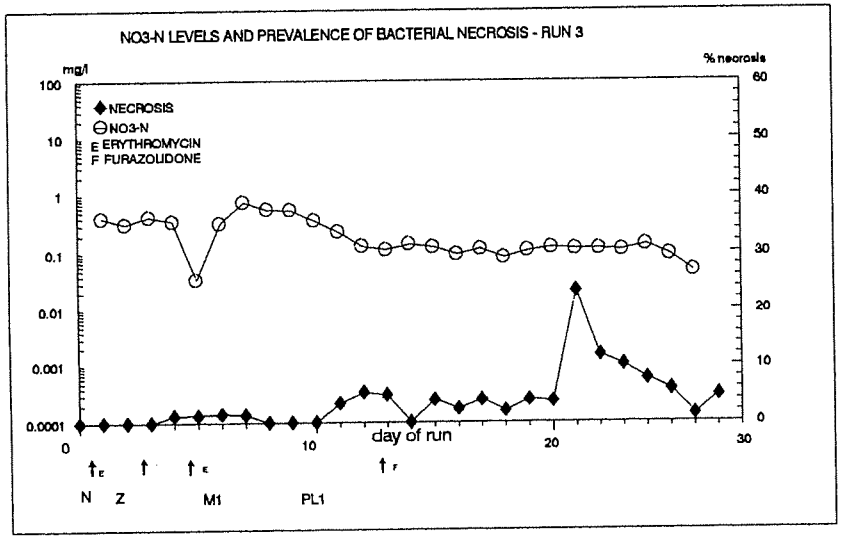

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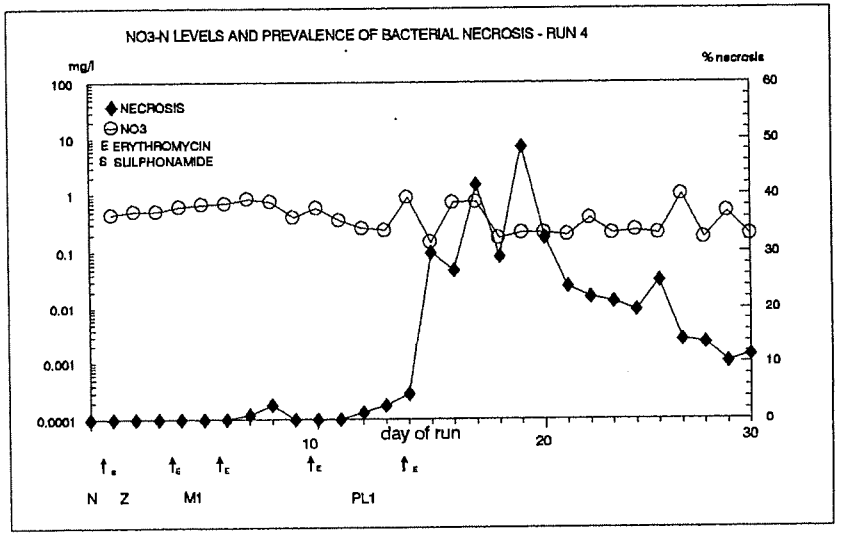

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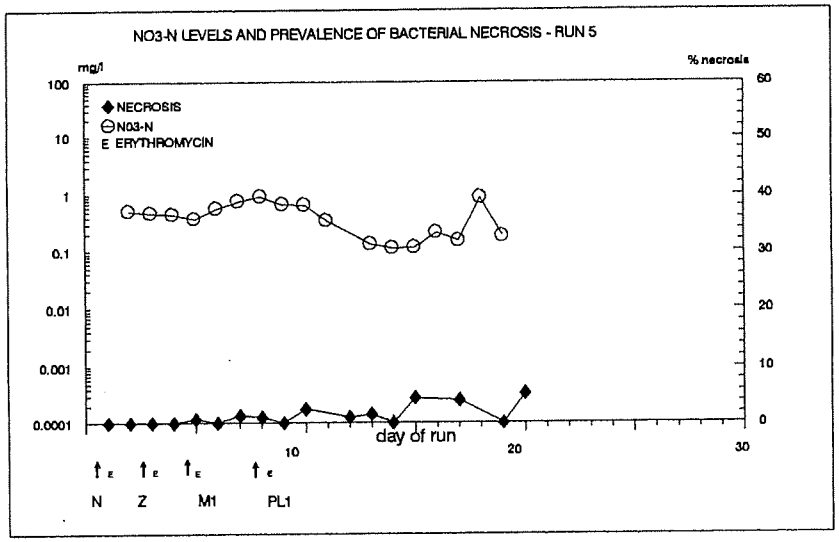

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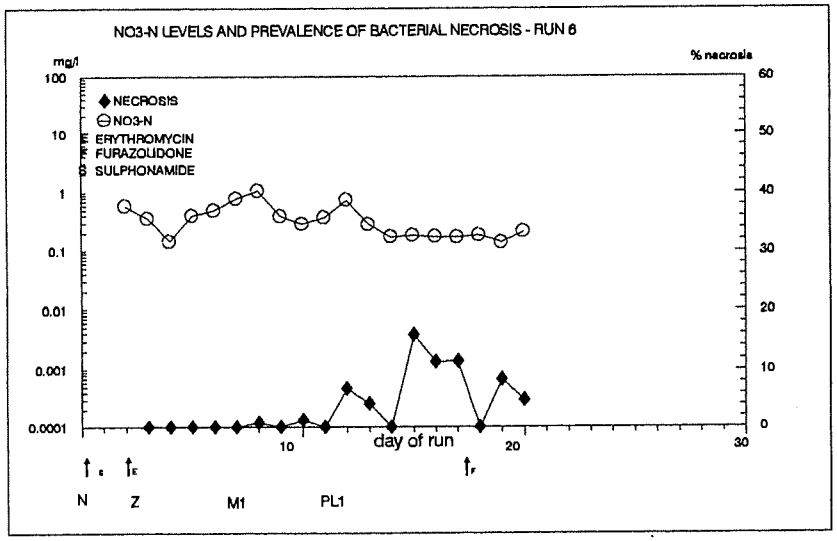

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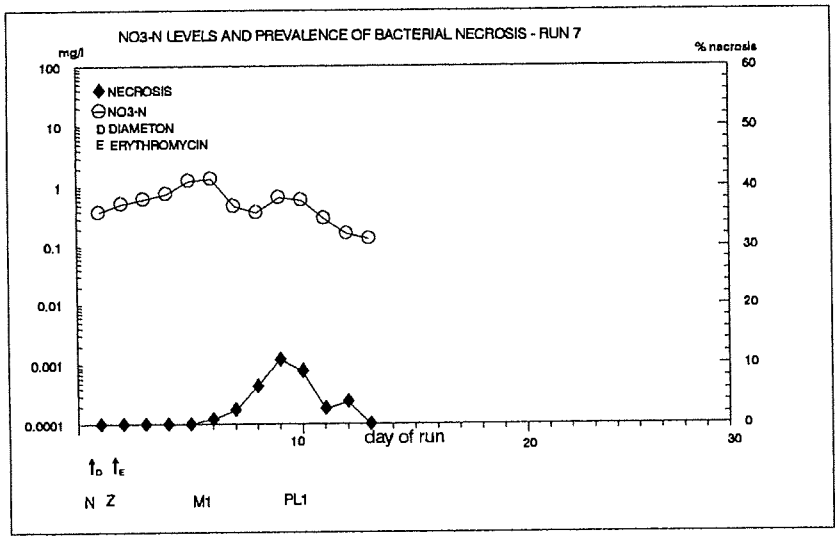

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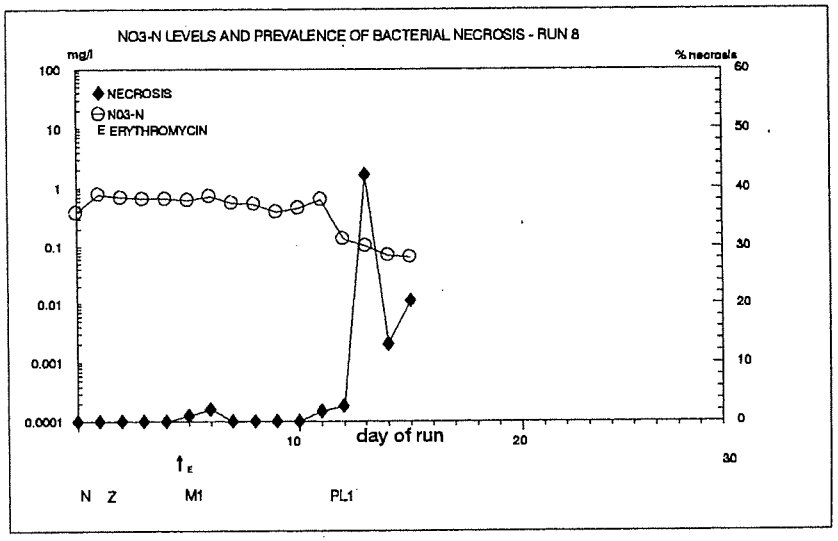

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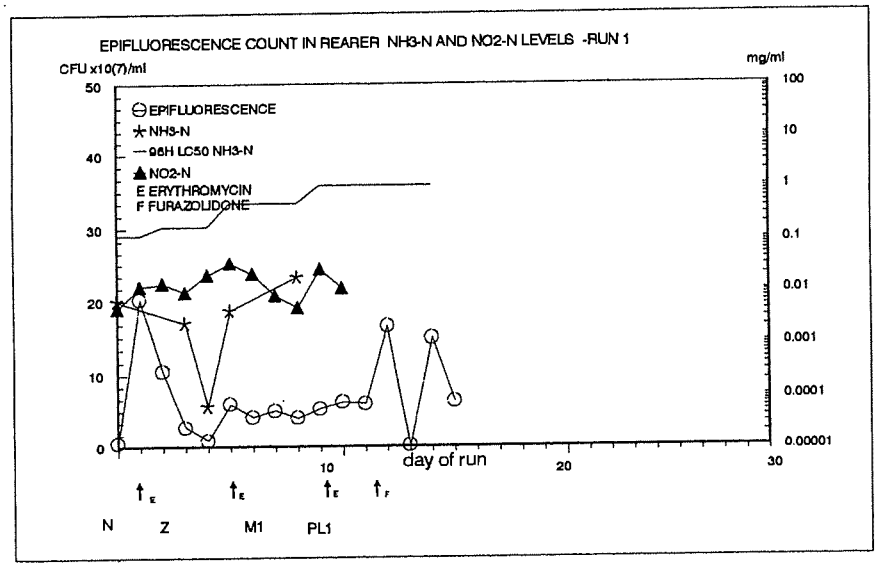

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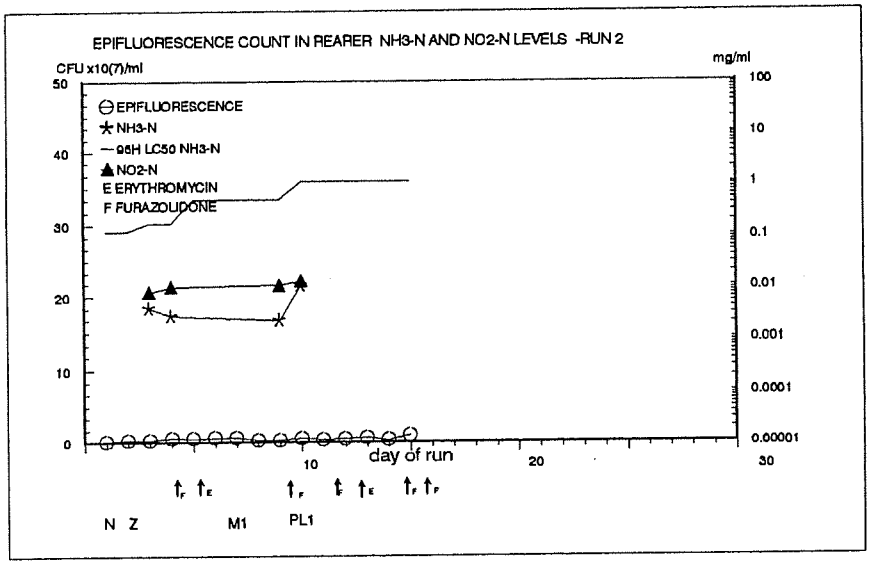

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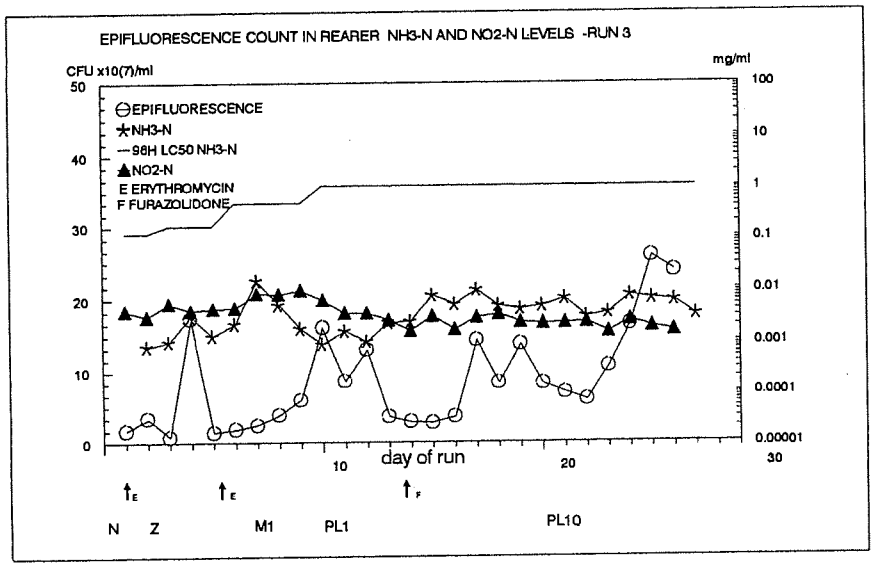

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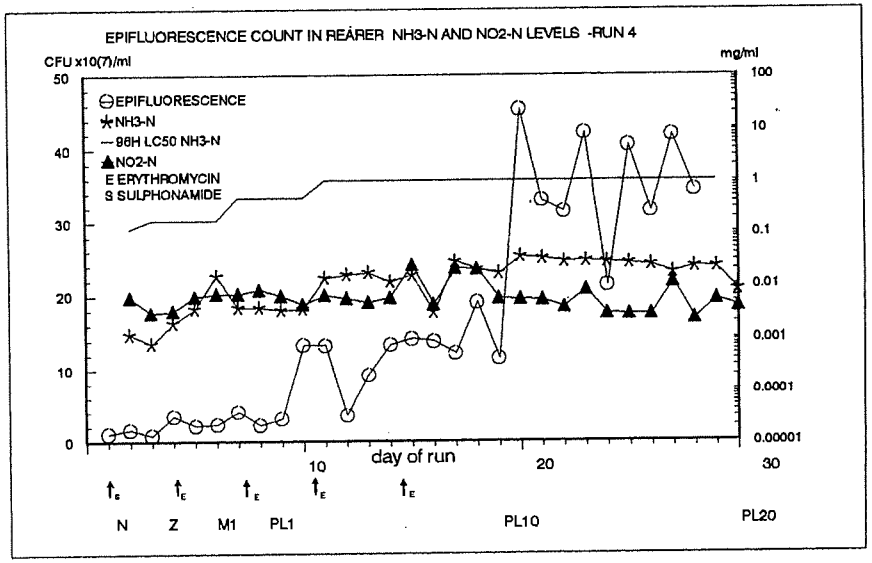

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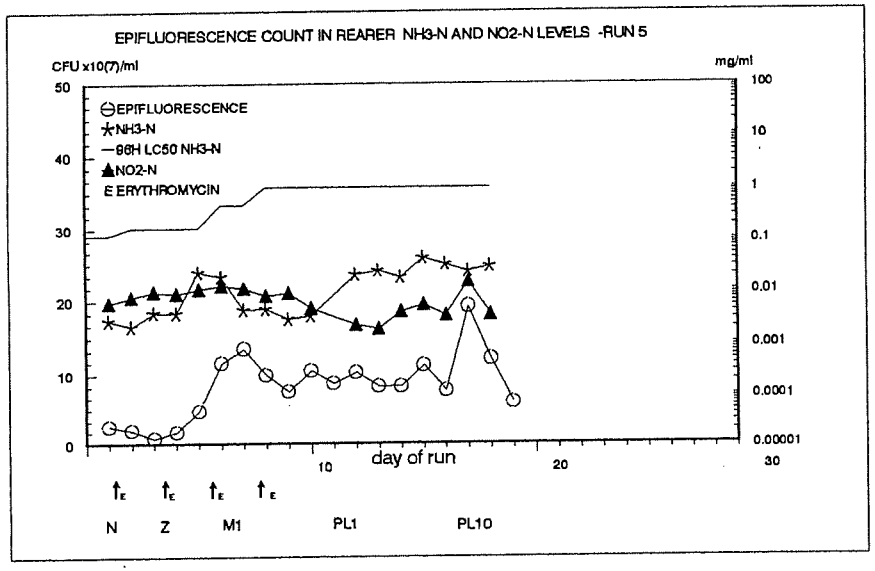

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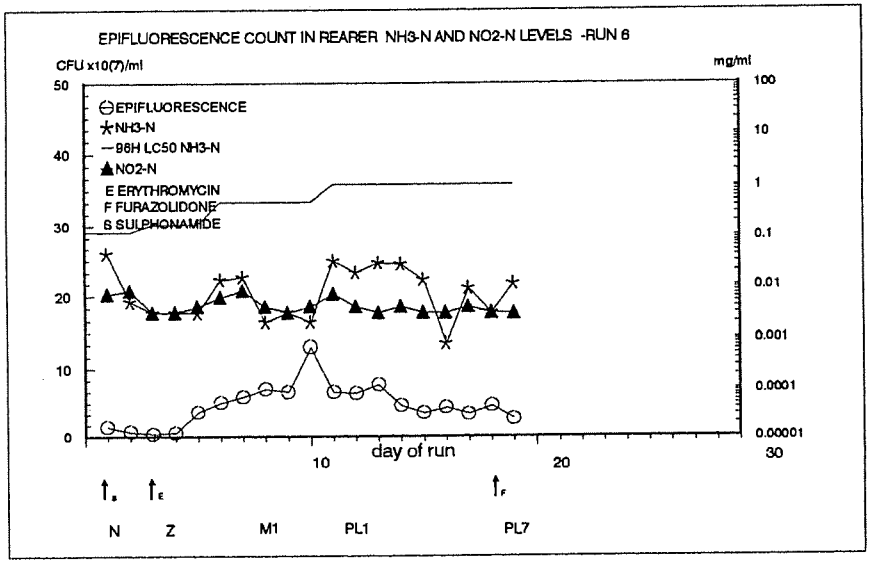

Fig. 54

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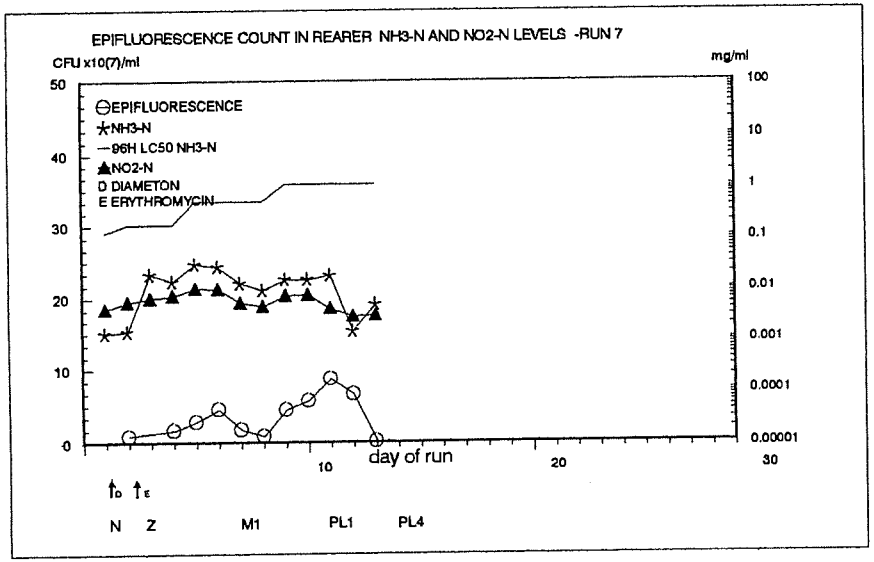

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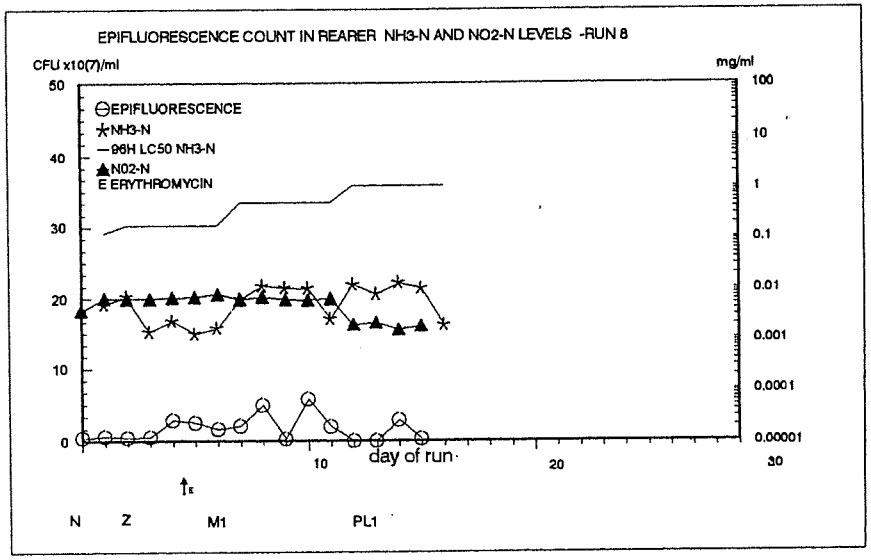


Fig. 56

3.2 Bacterial Identification

3.2.1 Hatchery monitoring program

The bacterial population of the water was monitored daily throughout the developmental stages of the prawn larvae. Samples were taken from the prawn rearer tanks as well as the algal and brine shrimp (*Artemia* sp.) cultures used to feed the larvae. Dip slides of TCBS medium and marine agar were used for bacterial counts. When disease was suspected in a larval culture, the dominant bacterial type on the dip slides from the days just prior to the problem was removed and sent to ARI for identification. The identification and source of the isolates together with the developmental stage of the larvae are listed below.

Hatchery A

Source	Larval Stage	Bacterial Identity
Batch 1:		
Rearer tank	PL 5,6	*Pseudomonas paucimobilis*
Rearer tank	PL 5,6	*Vibrio* species - (nearest to *V. tubiashi*)
Rearer tank	PL 5,6	*Vibrio* species - (nearest to *V. anguillarum*)
Rearer tank	MI	*V. harveyi*
Batch 2:		
Rearer tank	ZIII/MI	*V. vulnificus*
Algal culture	ZIII/MI	*Pseudomonas species*
Rearer tank	MIII/PL1	*V. vulnificus*

Hatchery B

Source	Larval Stage	Bacterial Identity
Batch 3:		
Rearer tank	MIII	*V. tubiashi*
Rearer tank	MIII	*V. pelegius I*
Rearer tank	MIII	*V. alginolyticus*
Rearer tank	MIII	*V. harveyi*
Rearer tank	MIII	*V. harveyi*
Rearer tank	MIII	*Vibrio* species - (nearest to *V. harveyi*)

Batch 4:

Rearer tank	PL5	*Aeromonas sobria*
Rearer tank	PL5	*Aer. sobria*
Rearer tank	PL7	*V. alginolyticus*
Rearer tank	PL16	*V. alginolyticus*
Rearer tank	PL16	*V. alginolyticus*
Artemia culture	PL6	*V. alginolyticus*
Artemia culture	PL7	*V. alginolyticus*
Artemia culture	PL17	*V. alginolyticus*
Artemia culture	PL17	*V. alginolyticus*
Artemia culture	PL19	*V. alginolyticus*

Batch 5:

Artemia culture	MIII/PLI	*V. alginolyticus*
Artemia culture	MIII/PL1	*V. splendidus* II
Artemia culture	PL10	*Vibrio* species - (nearest to *V. anguillarum*)
Artemia culture	PL10	*V. alginolyticus*

Batch 6:

Artemia culture	PL 6	*V. alginolyticus*
Artemia culture	PL 6	*V. alginolyticus*
Rearer tank	PL15	*Vibrio* species - (nearest to *V. anguillarum*)
		V. alginolytics

Batch 7:

Algal culture	MI	*V. alginolyticus*
Algal culture	PL 2	*V. alginolyticus*

Batch 8:

Algal culture	MIII	*V. proteolyticus*
Artemia culture	PL 2	*Vibrio* species - (nearest to *V. anguillarum*)
Rearer tank	PL 3	*V. alginolyticus*
Rearer tank	PL 3	*V. alginolyticus*
Rearer tank	PL 4	*v. alginolyticus*

3.2.2. Bioassay system

Where diseased larvae were observed in the larval cultures in the bioassay system, they were removed and appendages with necrotic lesions were removed and lesions cultured bacteriologically.

Bacteria identified from the melanised lesions and the site of the lesion are listed below.

Site	Identification
Trial B-PL2	
Rostrum tip	*V. tubiashi*
Rostrum tip	*V. tubiashi*
Melanized antenna	*Vibrio* species - (nearest to *V. tubiashi*)
Melanized antenna	*Vibrio* species - (nearest to *V. tubiashi*)
Trial D-MIII larvae	
Melanized pereiopod	*Deleya* species
Melanized rostrum tip	*Deleya* species
Trial D-PIII larvae	*Aeromonas hydrophila, Pseudomonas* sp.
Pereiopod	*Pseud. paucimobilis*
Antenna tip	*Pseud. paucimobilis*
Rostrum tip	*Pseudomonas* sp.
Rostrum tip	*Aeromonas hydrophila, Pseudomonas* sp.
Uropod tip	
Trial E-MIII larvae	
Uropod tip	*V. tubiashi*-like
Peripod tip	*V. tubiashi*-like
Antenna tip	*V. tubiashi*-like
Trial J-MI larvae	
Antenna tip	*V. alginolyticus*
Antenna tip	*V. alginolyticus*
Antenna tip	*Ps. paucimobilis*
Antenna tip	*Alcaligenes* species

3.3 Bacterial growth in the larval culture water of the bioassay system.

The actual viable bacterial counts (VBC) measured in the larval culture water of cones in trial B and L, over time, are given in Table 3.3.1, while Graphs 3.3.1 and 3.3.2 show these results graphically.

It is probable the filtration system was not supplying bacteria-free water in either trial or that bacterial (VBC) counts increased rapidly immediately on transfer of water into the cones.

After VBC increased rapidly to around 10^6/ml by 4-5 hours, they appeared to stabilise at that level or drop to around 2-5 x 10^5/ml. Generally the effect of additional bacteria did not cause a clear change in VBC counts in Trial L culture water. The high fluctuating background counts could well have masked the additional bacteria.

3.4 Larval rearing trials in the bioassay system

Throughout the project we found the quality (strength, vitality and viability) of each batch of *P. monodon* nauplii very variable. In some trials survival was around 30-40% after ten days, while other batches were at very low numbers (1-2%) by this time. Graph 3.4.1 gives the mean survival of control cultures throughout the trials. The age at which we found each larval stage is also indicated on this graph.
Another problem of the bioassay system trials was the great variation in survival in the replicated cultures within a treatment. This made it difficult to reveal significant (statistically) differences in all of the trials. In spite of this some inferences can be made by observing the trends in survival and prevalence of bacterial necrosis lesions recorded.

3.4.1 *Level of nutrition*

The mean survivals of larvae on each sampling day for Trials A, B, C, D and E are listed in Table 3.4.1. The very low plane of nutrition in Trial A did significantly lower survival as compared to the controls (Graph 3.4.2). Significant low survivals of larvae on a low plane of nutrition were also seen in Trial C (Graph 3.4.3). There were no significant differences observed in Trials B (Graph 3.4.2), D (Graph 3.4.3) and E (Graph 3.4.4). Though a trend for the larval cultures on a low plane of nutrition to have lower mean survivals was apparent in Trial D and E.

3.4.2 *Level of aeration*

The mean survival of larvae on each sampling day for Trials F, G and H are listed in Table 3.4.2. In Trial F heavily aerated larval cultures had significantly better survival than the controls (Graphs 3.4.5) while the reverse occurred in Trial G (Graph 3.4.5). There was no significant difference in survival of larvae in Trial H (Graph 3.4.6), but a trend for lower survival in heavily aerated cultures was apparent.

3.4.3 *Stocking densities*

The mean survivals of larvae on each sampling day for Trials I and J are listed in Table 3.4.3. A significant lower mean survival for high density larval cultures was recorded on day in Trial J (Graph 3.4.7). The trend in both trials was for a lower mean survival in those larval cultures initially stocked at 200 nauplii per litre.

3.4.4 *Cold water shock*

The mean survivals of larvae on each sampling day for Trial K are listed in Table 3.4.4. Significantly better survival were recorded in larval cultures exposed to the cold shock (Graph 3.4.8).

3.4.5 *Bacterial challenge*

The mean survivals of larvae on each sampling day for Trial L are listed in Table 3.4.5. Over all survival of larval cultures was very high in this trial and no significant differences between the control and treatment cultures was recorded (Graph 3.4.9).

3.4.6 *Prevalence of bacterial necrosis lesions*

As all control/treatment groups and trials were not sampled for examination to determine

lesion prevalence, only general observations could be made. Results are shown in Graph 3.4.10. With the exception of the sample on Day 6 of Trial D, all examinations of larvae in Trials E, F, H, I, J and K revealed that the prevalence of bacterial necrosis lesions was highest in treatment cultures. This was apparent whether they were exposed to a low plane of nutrition, heavy aeration, high stocking density or cold water shock. The results from Trial L were not as clear cut. Certainly there were more larvae with lesions in the two treatment groups by Day 8.

Table 3.3.1: The viable aerobic, heterotrophic bacterial numbers found in the larval culture water of cones in the bioassay system. The counts are an average of duplicate plates.

I. From Trial B (Day 2, Protozoea I)

Fresh filtered seawater had a bacterial count >5.0 x 10^3 /ml

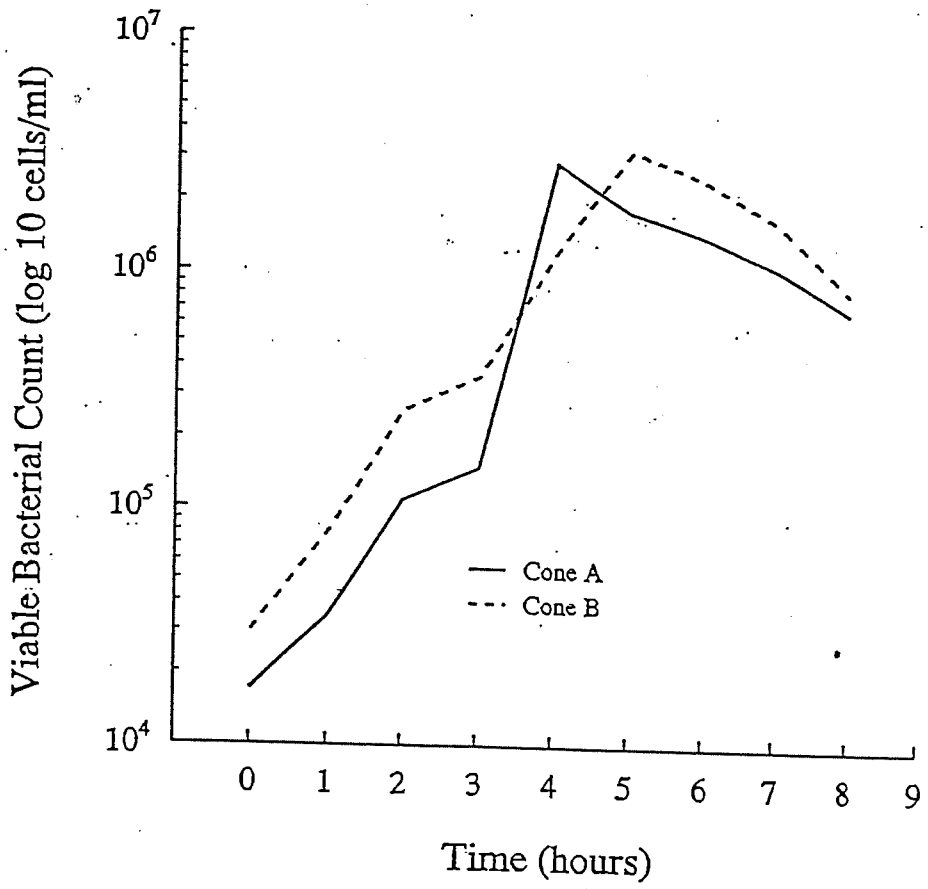
TIME (Hours)	**CONE A** cells/ml	**CONE B** cells/ml
0	1.7 x 10^4	3.0 x 10^4
1	3.5 x 10^4	8.0 x 10^4
2	1.1 x 10^5	2.6 x 10^5
3	1.5 x 10^5	3.6 x 10^5
4	2.9 x 10^6	1.2 x 10^6
5	1.8 x 10^6	3.3 x 10^6
6	NT*	NT
7	NT*	NT
8	7.0 x 10^5	8.5 x 10^5

II. From Trial L (Day 4, Protozoea II)

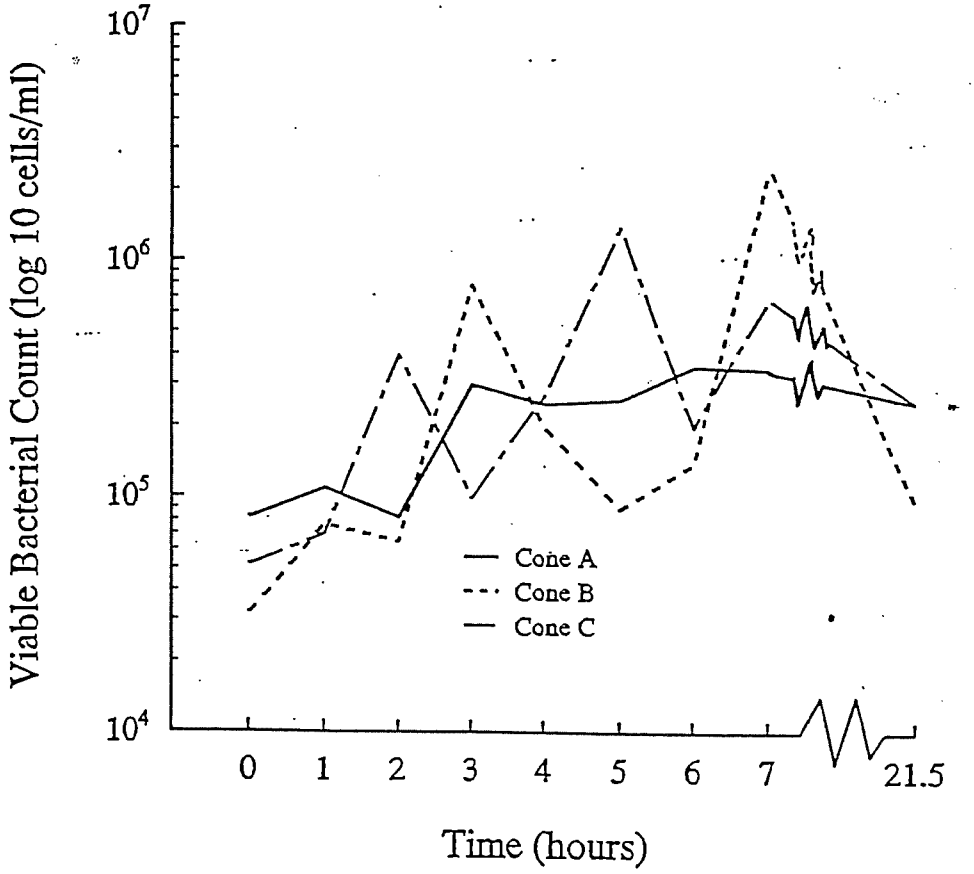
TIME (Hours)	**CONE A** control cells/ml	**CONE B** 10^2 cells/ml added*2	**CONE C** 10^6 cells/ml added
0	8.2 x 10^4	3.2 x 10^4	5.2 x 10^4
1	1.1 x 10^5	7.7 x 10^4	7.0 x 10^4
2	8.2 x 10^4	6.5 x 10^4	4.0 x 10^5
3	3.0 x 10^5	7.9 x 10^5	1.0 x 10^5
4	2.5 x 10^5	2.0 x 10^5	2.7 x 10^5
5	2.6 x 10^5	9.0 x 10^4	1.4 x 10^6
6	3.6 x 10^5	1.4 x 10^5	2.0 x 10^5
7	3.5 x 10^5	2.5 x 10^6	7.0 x 10^5
21.5	2.6 x 10^5	1.0 x 10^5	2.6 x 10^5

*NT = not tested

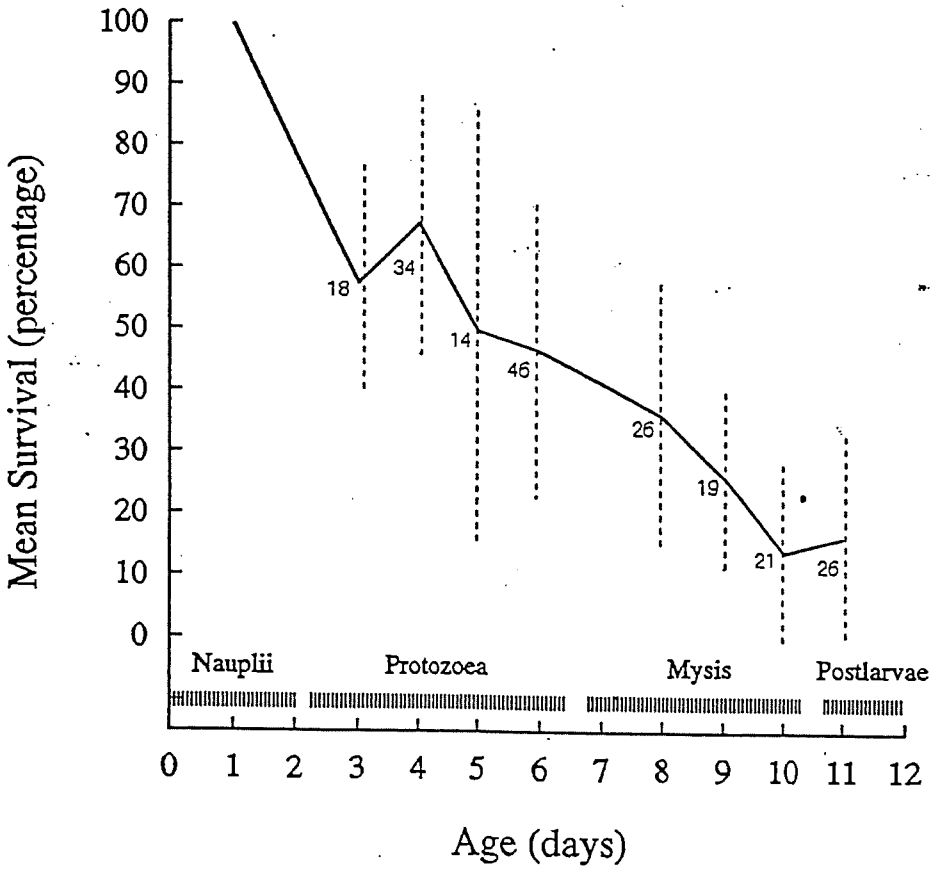
*2 The suspension of *Vibrio alginolyticus* was added 100 minutes after Time 0.



Graph 3.3.1: The viable aerobic, heterotrophic bacterial numbers found in the larval culture water of two cones (A and B) in the bioassay system on Day 2 of Trial B. The fresh filtered seawater supply had a bacterial count of >5.0 x 10^3 per ml.



Graph 3.3.2: The viable aerobic, heterotrophic bacterial numbers found in the larval culture water on Day 4 of Trial L. Cone A is a control larval culture, Cone B had 10^2 *Vibrio alginolyticus* per ml added and Cone C had 10^6 *V. alginolyticus* per ml added. The bacteria were added at 1.5 hours.



Graph 3.4.1: The average survival of larvae in one litre Imhoff cones used as control cultures in Trials A through L. One ± SD given for each data point. The number at the data point is *n*. The usual stage of the larvae in the trials is also indicated along the *x*-axis.

TABLE 3.4.1: The survival of larvae in Trials A to E where the level of nutrition was varied.

Trial A:

DAY	STAGE*	NUMBER OF REPLICATES PER TREATMENT	NUMBER OF LARVAE		
			CONTROL	LOW FEED	VERY LOW FEED
			mean ±1SE*2	mean ±1SE	mean ±1SE
3	PI	4	60.5 ± 17.3	49.3 ± 13.9	48.6 ± 9.9
6	PIII/MI	4	48.8 ± 10.8	37.0 ± 15.3	24.4 ± 9.8
9	MIII	4	37.8 ± 12.2	30.8 ± 14.7	13.8 ± 7.4
11	PL1	4	32.5 ± 16.6	27.0 ± 16.7	9.8 ± 6.0

Trial B:

			CONTROL mean ±1SE	LOW FEED mean ±1SE
4	PII	6	33.3 ± 7.4	32.7 ± 3.4
6	PIII	6	28.8 ± 6.9	29.8 ± 3.8
8	MII	6	26.3 ± 6.7	27.5 ± 4.2
9	MII/III	6	25.7 ± 6.8	26.2 ± 3.3
10	MIII	6	24.5 ± 7.6	24.5 ± 3.9
11	MIII/PL	6	23.2 ± 7.9	23.0 ± 5.2
12	PL1	6	20.5 ± 6.8	21.5 ± 8.1
13	PL2	6	20.2 ± 7.2	15.5 ± 8.4

Trial C:

3	PI/II	7	39.9 ± 6.2	39.1 ± 6.9
5	PIII	7	16.0 ± 5.4	18.3 ± 4.4
6	MI	3	9.0 ± 4.4	7.0 ± 1.7
9	MII/III	4	4.5 ± 1.3	0.3 ± 0.5
10	MIII	4	1.8 ± 1.5	0
11	PL1	4	1.8 ± 1.5	0

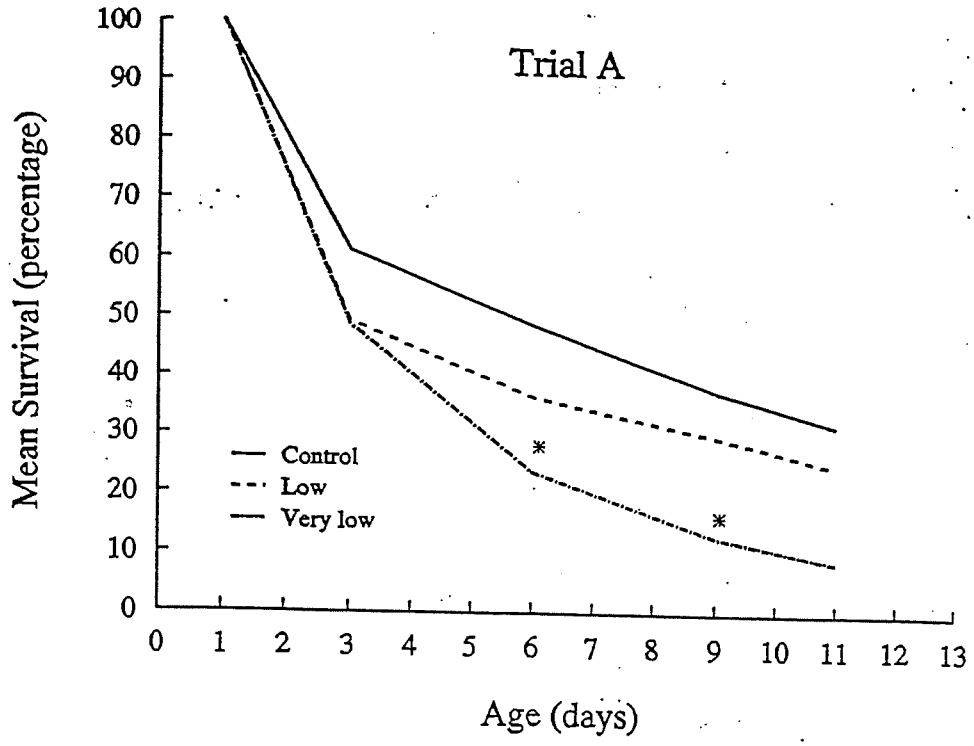
Trial D:

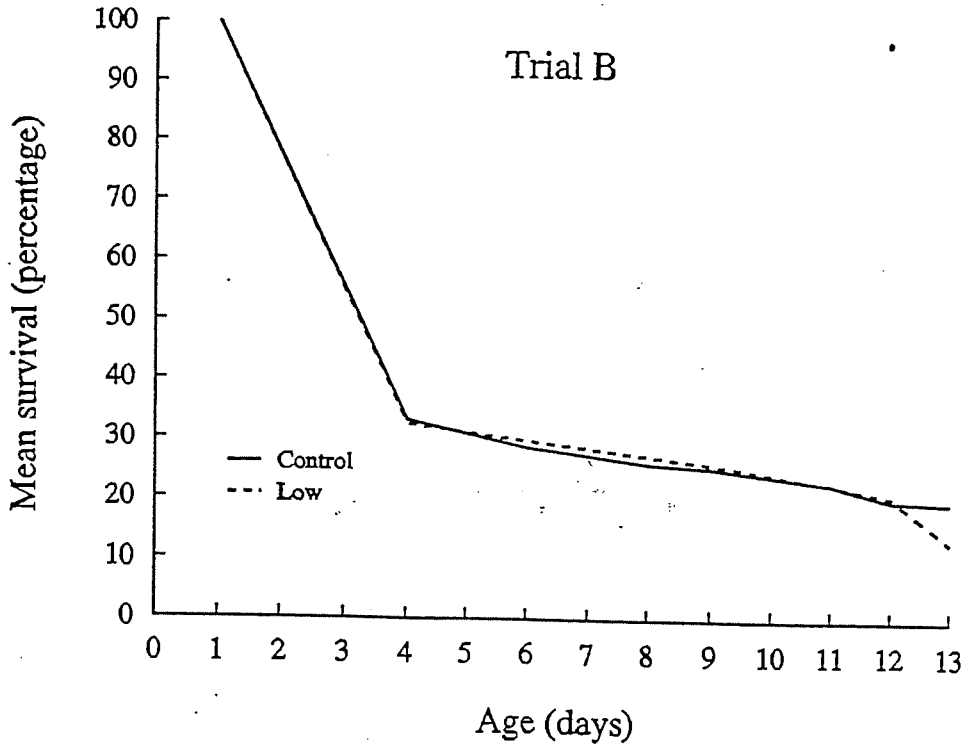
4	PII	7	57.1 ± 8.2	50.7 ± 17.3
6	PIII	7	34.4 ± 18.8	28.9 ± 8.7
8	MI/III	4	29.0 ± 17.5	13.5 ± 16.3
10	MIII	4	7.3 ± 7.6	3.3 ± 5.8
11	MIII/PL	4	3.5 ± 3.4	1.8 ± 3.5

Trial E:

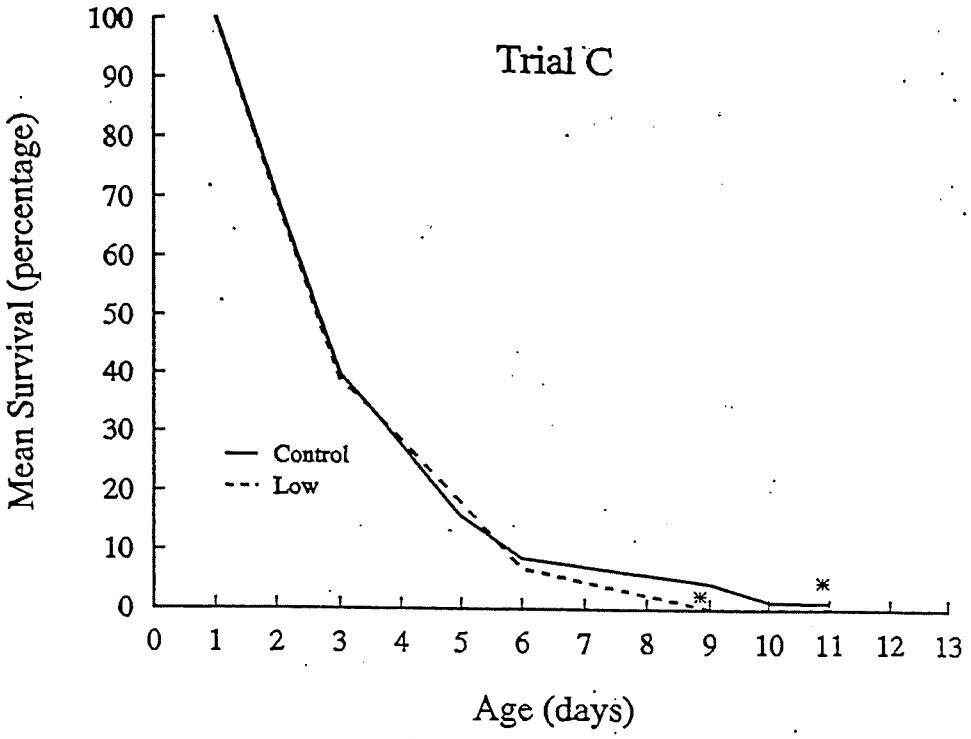
4	PI/II	7	72.9 ± 12.6	73.1 ± 10.0
6	PII/III	7	61.0 ± 13.5	46.4 ± 15.0
8	MI	7	54.5 ± 15.2	30.5 ± 22.8
11	MIII/PL	7	33.8 ± 17.3	17.5 ± 12.8
12	PL2	7	25.5 ± 7.9	11.8 ± 7.5

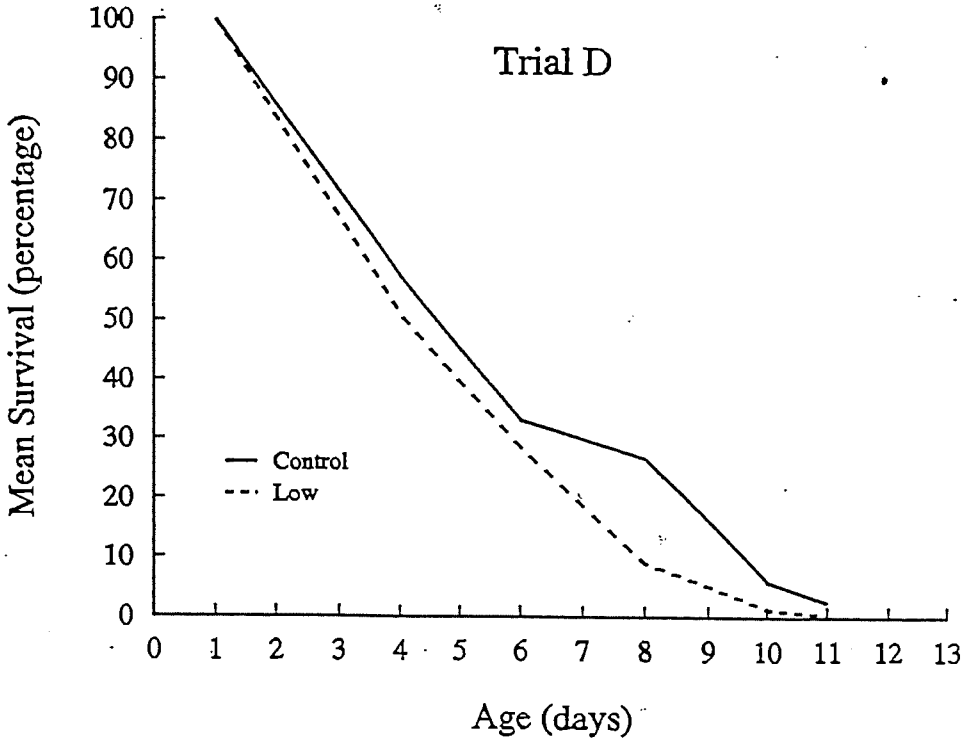
* P = protozoea, M = mysis, PL = postlarvae
*2 SE = standard error



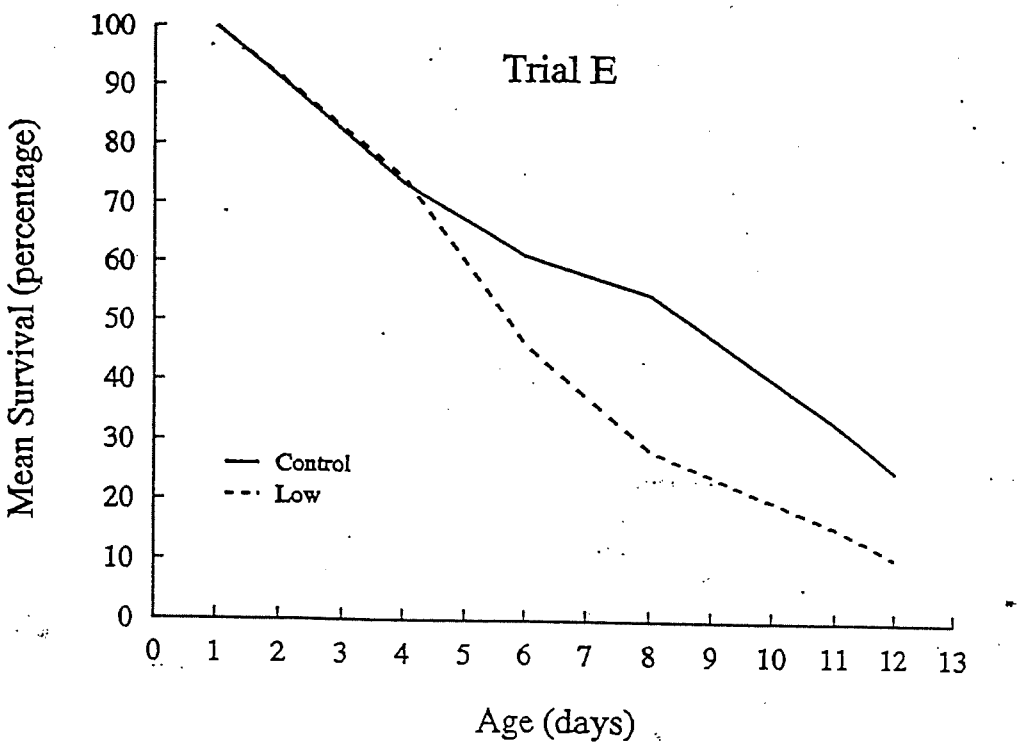


Graph 3.4.2: The survival (equivalent means) of larvae in experimental culture trials A and B where the level of nutrition was low. An asterisk marks a mean survival in treatment cultures significantly different (at 95% confidence) to that of control cultures on that day.





Graph 3.4.3: The survival (equivalent means) of larvae in experimental culture trials C and D where the level of nutrition was low. An asterisk marks a mean survival in treatment cultures significantly different (at 95% confidence) to that of control cultures on that day.

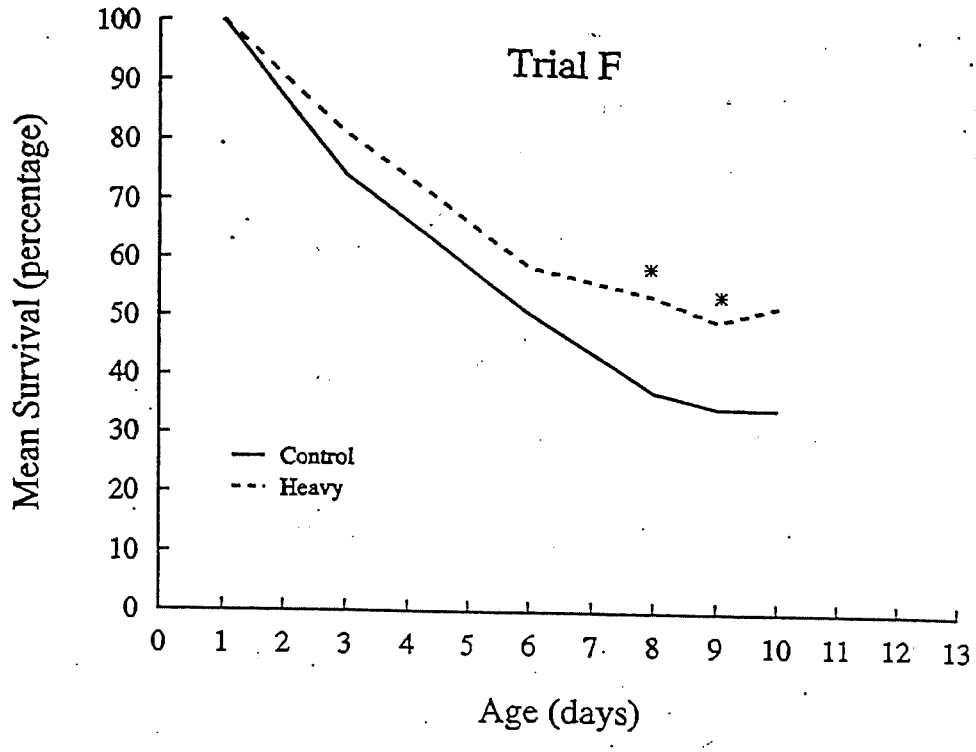


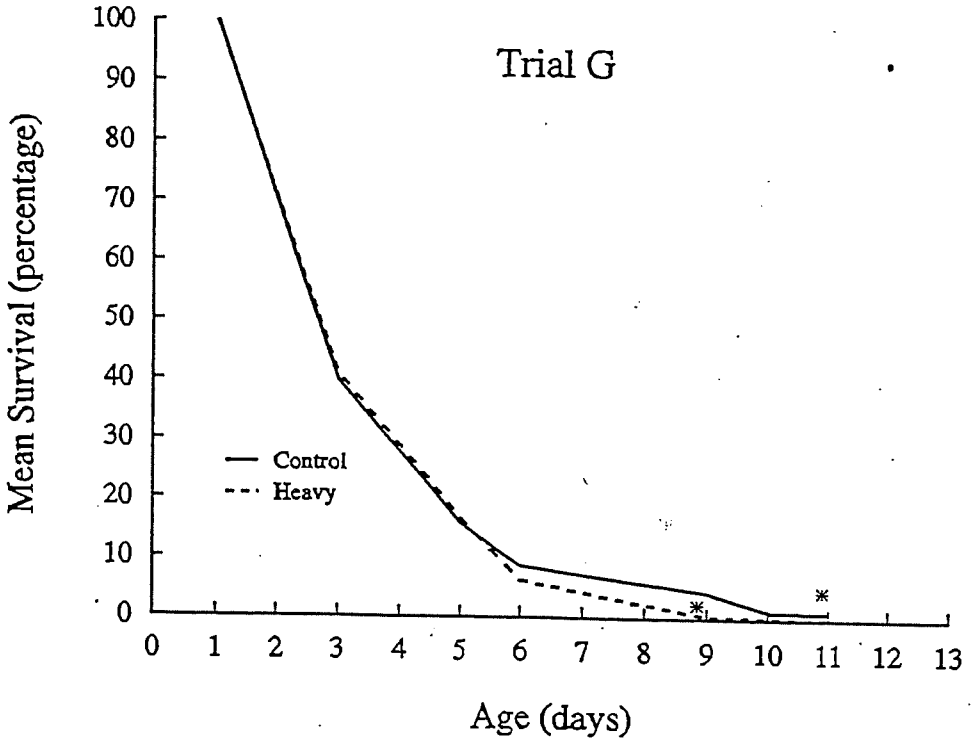
Graph 3.4.4: The survival (equivalent means) of larvae in experimental culture trial E where the level of nutrition was low.

TABLE 3.4.2: The survival of larvae in Trials F to H where the level of aeration was varied.

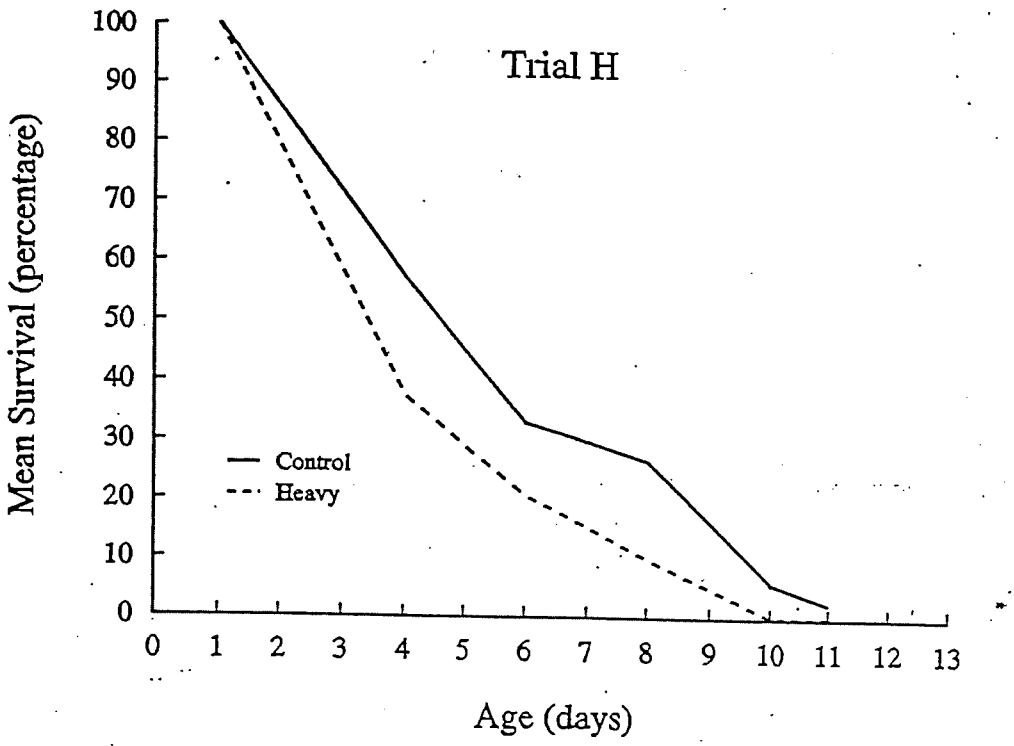
DAY	STAGE*	NUMBER OF REPLICATES PER TREATMENT	NUMBER OF LARVAE	
Trial F:			CONTROL mean ±1SE*2	HEAVY AERATION mean ±1SE
3	PII	7	73.6 ± 9.9	80.4 ± 9.0
6	PIII/MI	7	50.4 ± 11.0	58.3 ± 10.6
8	MII	5	37.4 ± 8.9	53.6 ± 9.1
9	MII/III	5	35.0 ± 9.7	49.5 ± 8.9
10	MIII/PL	3	34.7 ± 8.5	52.0 ± 7.0
Trial G:				
3	PI/II	7	39.9 ± 6.2	41.1 ± 9.8
5	PIII	7	16.0 ± 5.4	17.1 ± 6.3
6	MI	3	9.0 ± 4.4	6.3 ± 2.5
9	MII	4	45. ± 1.3	0.8 ± 1.0
10	MIII	4	1.8 ± 1.5	0.5 ± 0.8
11	PL	4	1.8 ± 1.5	0
Trial H:				
4	PII	7	57.1 ± 8.2	39.0 ± 18.3
6	PIII	7	34.4 ± 18.8	22.9 ± 22.3
8	MI/II	4	29.0 ± 17.5	15.3 ± 21.4
10	MIII/PL	4	7.3 ± 7.6	0.5 ± 1.0
11	PL	4	3.5 ± 3.4	0.5 ± 1.0

* P = protozea, M = mysis, PL = postlarvae
*2 SE = standard error





Graph 3.4.5: The survival (equivalent means) of larvae in experimental culture trials F and G where the level of aeration was heavy. An asterisk marks a mean survival in treatment cultures significantly different (at 95% confidence) to that of control cultures on that day.

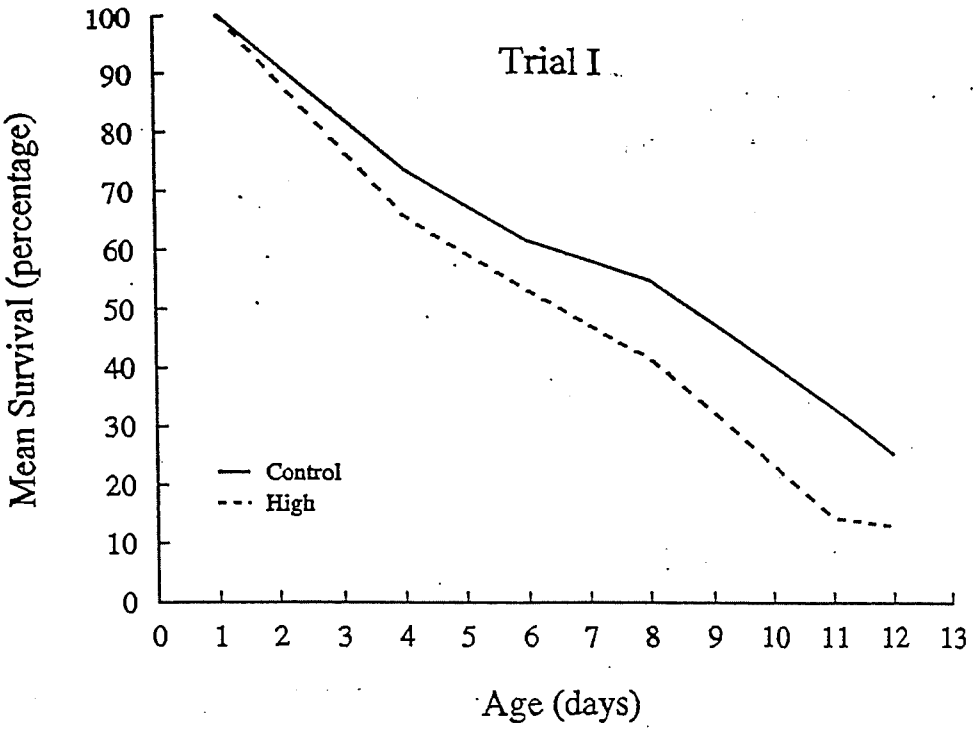


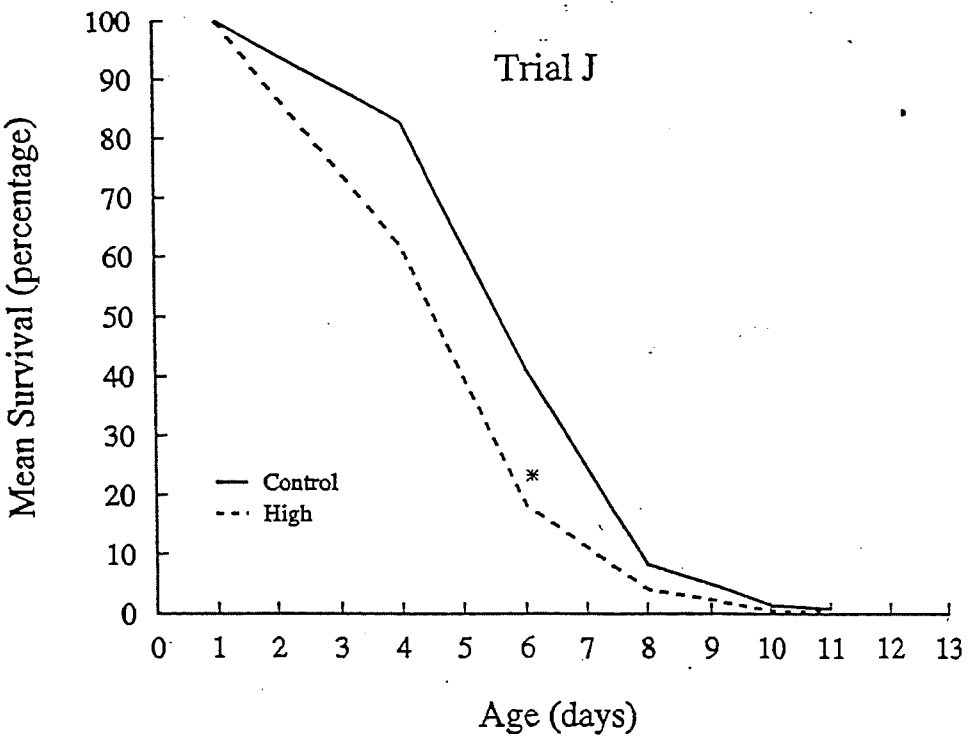
Graph 3.4.6: The survival (equivalent means) of larvae in experimental culture trial H where aeration was heavy.

TABLE 3.4.3: The survival of larvae in Trials I and J where the stocking density was varied.

DAY	STAGE*	NUMBER OF REPLICATES PER TREATMENT	NUMBER OF LARVAE	
			CONTROL mean ±1SE*2	HIGH DENSITY mean ±1SE
Trial I:				
4	PI/II	7	72.9 ± 12.6	65.5 ± 6.3
6	PII/III	7	61.0 ± 13.5	52.9 ± 8.3
8	PIII/MI	4	54.5 ± 15.2	41.8 ± 13.0
11	MIII/PL	4	33.8 ± 17.3	31.3 ± 20.7
12	PL1	4	25.5 ± 7.9	14.4 ± 10.7
Trial J:				
4	PII	7	81.4 ± 11.6	61.4 ± 21.0
6	MI	7	41.6 ± 22.9	18.7 ± 6.0
8	MII	4	8.8 ± 4.9	5.1 ± 5.2
10	MIII/PL	4	2.0 ± 1.8	0.5 ± 0.1
11	PL1	4	1.3 ± 1.0	0.5 ± 0.1

* P = protozoea, M = mysis, PL = postlarvae
*2 SE = standard error





Graph 3.4.7: The survival (equivalent means) of larvae in experimental culture trials I and J where the stocking density was double that of control cultures (200 per litre on Day One against 100 per litre). An asterisk marks a mean survival in treatment cultures significantly different (at 95% confidence) to that of control cultures on that day.

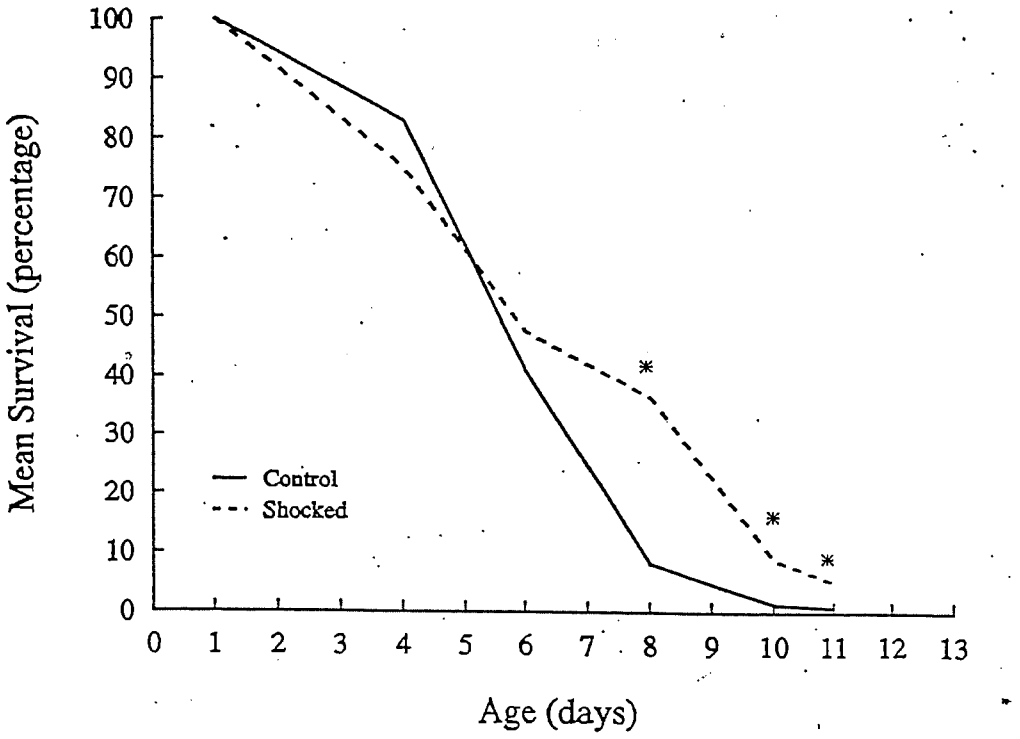
TABLE 3.4.4: The survival of larvae in Trial K where the larvae were exposed to a daily cold shock.

DAY	STAGE*	NUMBER OF REPLICATES PER TREATMENT	NUMBER OF LARVAE	
			CONTROL mean ±1SE*2	COLD SHOCKED mean ±1SE
Trial K:				
4	PII	7	81.4 ± 11.6	74.1 ± 11.7
6	MI	7	41.6 ± 22.9	47.9 ± 17.4
8	MII	4	8.8 ± 4.9	37.0 ± 14.6
10	MIII/PL	4	2.0 ± 1.8	10.8 ± 9.7
11	PL1	4	1.3 ± 1.0	5.8 ± 3.7

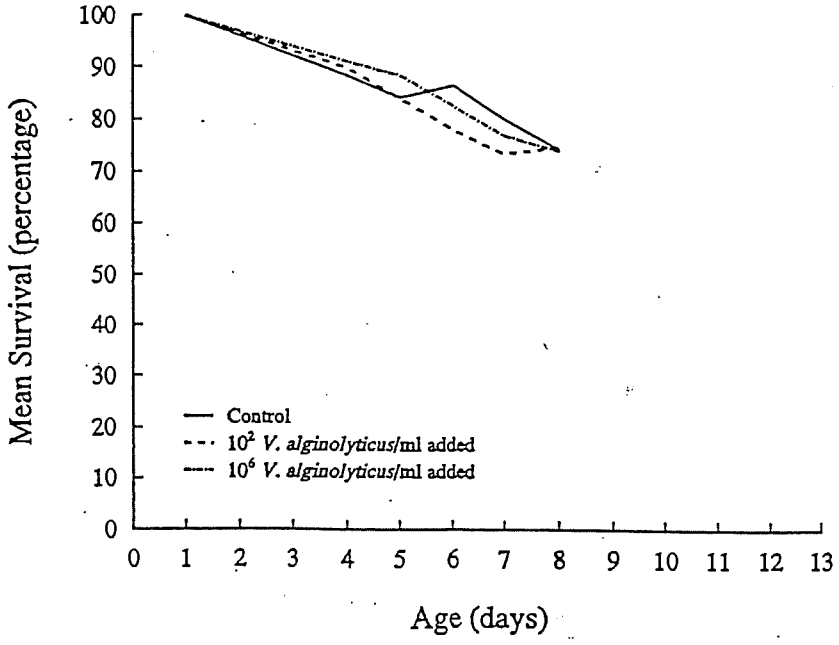
TABLE 3.4.5: The survival of larvae in Trial L where the larvae where exposed to added *Vibrio alginolyticus* on Day 4.

DAY	STAGE*	NUMBER OF REPLICATES PER TREATMENT	NUMBER OF LARVAE		
			CONTROL mean ±1SE*2	10^2 cells/ml mean ±1SE	10^6 cells/ml mean ±1SE
Trial L:					
4	PII	7	87.1 ± 9.3	89.0 ± 8.0	90.0 ± 7.5
5	PIII	7	83.6 ± 8.7	83.0 ± 8.1	87.6 ± 8.9
6	PIII	5	86.4 ± 6.0	77.8 ± 8.2	82.0 ± 10.3
7	MI	3	80.0 ± 7.2	73.3 ± 9.0	76.7 ± 10.4
8	MII	3	74.3 ± 8.1	74.7 ± 10.1	74.0 ± 8.5

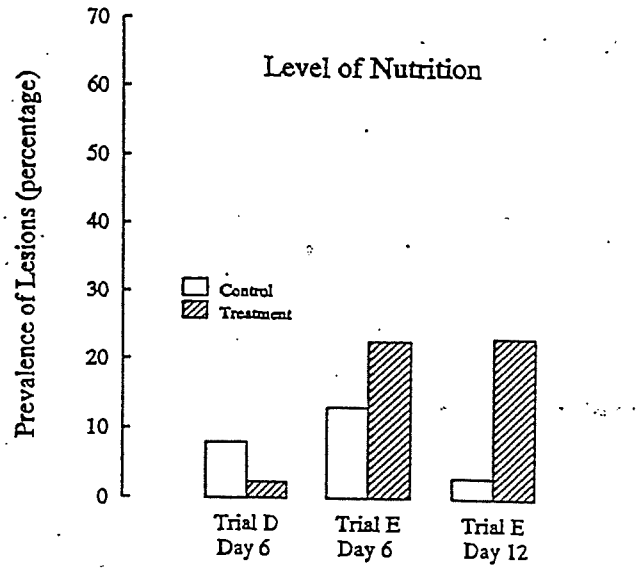
* P = protozoea, M = mysis, PL = postlarvae
*2 SE = standard error

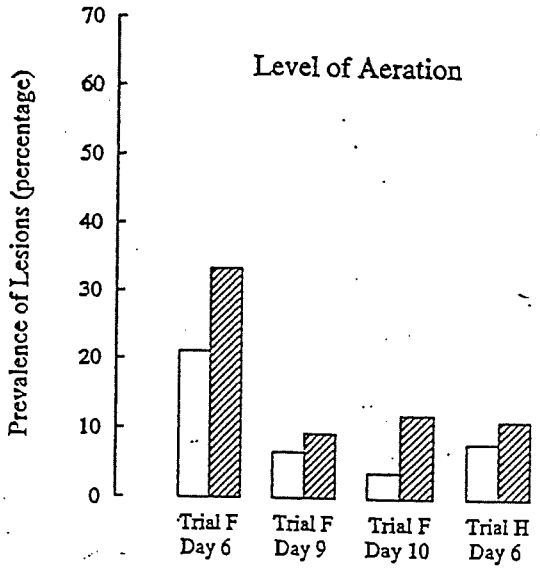


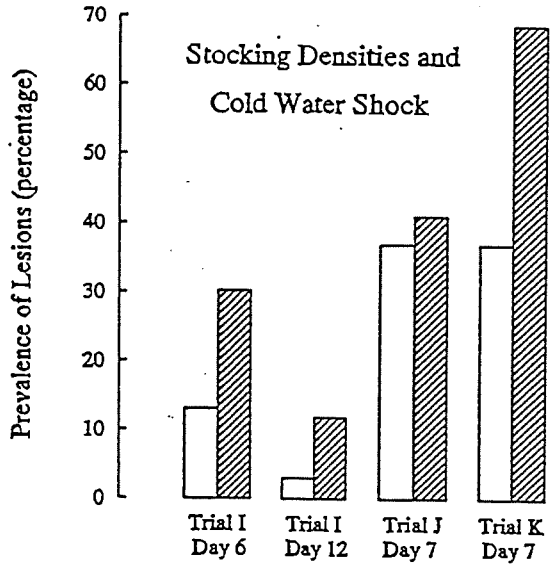
Graph 3.4.8: The survival (equivalent means) of larvae in experimental culture trial K where the larvae were exposed to a cold shock each day. An asterisk marks a mean survival in treatment cultures significantly different (at 95% confidence) to that of control cultures that day.

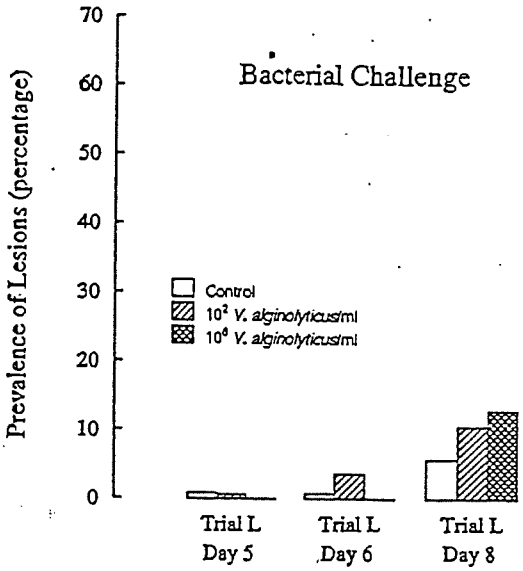


Graph 3.4.9: The survival (equivalent means) of larvae in experimental culture trial L where 10^2 *Vibrio alginolyticus* cells per ml or 10^6 *V. alginolyticus* cells per ml were added on Day 4.









Graph 3.4.10: The prevalence of bacterial necrosis lesions on larvae in various trials and at various ages. The prevalence in control larval cultures is always compared to those larval cultures exposed to the treatment.

4. DISCUSSION

Pathogenesis of bacterial necrosis

Of the 4 currently recognised bacterial syndromes affecting penaeid prawn larvae, only bacterial necrosis was detected in the 8 larval production batches studied. Results clearly indicated a possible causal relationship between concentrations of one or more components of the "presumptive Vibrio" populations in rearer tank water and outbreaks of bacterial necrosis. Counts in rearer tank water of 100 or more colonies on dip slide TCBS agar were recorded the day before and/or on the same day as a bacterial necrosis outbreak (prevalence >10%) in 25 (48%) of 52 instances. By contrast, counts of less than 100 colonies on TCBS agar were recorded the day before and/or on the same day as a bacterial necrosis outbreak in only 5 (5%) of 96 instances.

The source of these putative pathogens and the means by which they induce lesions remains undetermined. In many instances, TCBS counts in rearer water remained >100 for several consecutive days, despite daily water exchanges and only occasional evidence that either algal cultures or *Artemia* cultures were, at any time during the study, a significant source of these bacteria. It is possible that the presumptive Vibrios multiplied in situ in the tanks in response to changing nutrient levels, which were not measured in the study. These bacteria may have been derived from the intestinal tracts of the larvae and postlarvae themselves (Yasuda and Kitao 1980). Alternatively, these bacteria may have colonised, and then been released from, surfaces in the tank or water supply system, which were not sampled during the study. It is unlikely they were present in the concentrations detected in the filtered (usually to a nominal 1μm) seawater supplied to the rearer tanks.

Various antibiotics were used, apparently arbitrarily, on a routine, prophylactic basis during production of all batches monitored during the study. There was no convincing evidence they were effective in reducing concentrations of the putatively pathogenic presumptive Vibrios.

No other causal factors for bacterial necrosis were identified from the data. Epifluorescence estimates of total bacterial concentrations, and dip slide marine agar counts of heterotrophic bacteria, in rearer tank water failed to show evidence of causal relationships with bacterial necrosis. Un-ionized ammonia and nitrite concentrations in rearer tank water were consistently below those reported as toxic for *P. monodon* (Chen and Chin, 1988; Chin and Chen, 1987). For both chemicals, these authors suggested "safe levels" which are 10% of the experimentally determined or extrapolated 96-h LC_{50} for each larval or postlarval stage. In our study, these "safe levels" for nitrite were never exceeded and only rarely did the un-ionized ammonia level approach the "safe level". Contrary to the findings of Muir *et al* (1990), which indicated that NO_3-N concentrations as low as 1 mg/l are toxic to *P. monodon* larvae, in our study hatchery operators reported no significant mortalities in larval cultures with NO_3-N concentrations up to 1.1mg/l. In one larval culture, NO_3-N concentration reached 1.3 mg/l and this culture subsequently suffered a heavy reduction in larval concentrations. However, hatchery technicians considered this was due to a management-related accident, rather than disease. There was no evidence that increased NO_3-N concentrations led to increased prevalence of bacterial necrosis.

To our knowledge, there are no published accounts of comparable studies of the

pathogenesis of bacterial necrosis in penaeid prawn hatcheries. In studies of luminescent vibriosis, a bacterial septicaemia, Lavilla-Pitogo *et al* (1990) showed that concentrations of *V. harveyi* cells $>10^2$/ml in rearer water were associated with outbreaks. This increase in bacterial concentration was attributed to increased concentrations of nutrient in rearer water. There have been two studies examining bacterial associations with mortalities in larval Penaeus monodon. Such mortality rates may often be composite and relatively crude variables, representing mortalities due to a number of separate causes, bacterial and other, within and between larval production runs. Muir (1990) correlated larval mortalities with low and variable rearer water temperatures and salinity and decreased pH. We found no evidence of causal relationships between changes in rearer water temperature or salinity and bacterial necrosis. Water temperatures and salinities during our study remained within acceptable limits (ref) Flegel *et al* (1993) failed to show a relationship between larval mortalities and presumptive Vibrio concentrations in rearer water.

Usefulness of dip slides in predicting bacterial disease outbreaks

Dip slides proved to be a convenient method of indicating bacterial concentrations in larval rearing tank water, *Artemia* cultures and algal cultures. The technique is simple and all necessary facilities are available in most hatcheries. As most of the recognised pathogens of larval penaeids grow on TCBS medium, the technique could be refined by using dip slides with this medium on both sides. This would reduce costs while providing the security of a replicate sample.

While providing useful information on hatchery hygiene and serving as an indicator for possible outbreaks of bacterial necrosis, such monitoring does not appear to have predictive value. For example, in our study, counts of more than 100 colonies on TCBS were associated with bacterial necrosis prevalence >10% on the sampling day and/or the following day in 25 (48%) of 52 instances. By contrast, counts of less than 100 colonies on TCBS agar were recorded the day before and/or on the same day as a bacterial necrosis prevalence of >10% in only 5 (5%) of 96 instances. Given the requirement for 24 hour incubation before colony numbers can be counted, any predictive value is lost; within the same period, hatchery operators can more accurately determine the disease status of the larvae by direct examination.

The epifluorescence technique did not appear to be a useful predictor of changes in disease status. It was not possible to differentiate different types of bacteria except on morphological grounds and we found the technique time-consuming and difficult to control. The equipment required is not normally available in hatcheries.

Consistent with the suggestion of Chen (1993), the results also indicated that *Artemia* cultures are often heavily contaminated with *Vibrionaceae* and may be a source of potentially pathogenic bacteria in larval culture water. However, feeding of contaminated *Artemia* cultures during the present study did not usually result in a measurable increase, either immediate or following a lag period, in presumptive Vibrios in rearer water. In order to minimise the risk of introducing *Artemia*-associated pathogens, prawn hatchery technicians have developed washing methods which may reduce surface bacterial contamination of *Artemia* cultures; however, antibiotic treatment may be necessary to reduce gut bacterial concentrations.

Lewis *et al* (1988) have shown survival of larval pacific oysters *Crassostrea gigas* is

improved when bacterial concentrations in algal cultures are reduced by water filtration. In our study, algal cultures were usually less frequent sources of contamination than the *Artemia* cultures.
Further study of relationships between bacterial pathogens and outbreaks of bacterial disease in prawn hatcheries is required. In particular, the sources of infection for each of the major syndromes must be determined. Bacteria from early lesions on diseased prawns must be recovered and identified. Possible relationships between concentrations of these bacteria in rearer culture water, surfaces in the rearer tank and water supply system, *Artemia* cultures, algal cultures and, in particular, larval and postlarval prawn intestinal tracts, must be elucidated so that rational control and prevention measures can be put in place.

Bacteria associated with bacterial disease.

Little previous information is available on the specific bacteria associated with bacterial diseases of prawn larvae. Lavilla-Pitogo *et al* (1990) found *V. harveyi* in rearing tank water of larval cultures exhibiting luminous vibriosis. Earlier work from the Philippines (Llobrera and Gacuton, 1977) did not identify specific bacteria in the rearing water of larval cultures, but found bacteria in normal cultures included Gram-positive bacteria (mostly *Micrococcus* and *Staphylococcus*) and *Acinetobacter, Flavobacterium* and *Moraxella.* This is quite different from the flora identified in this study. *Vibrio* species dominated, mostly *V. alginolyticus.* Muir (1990) found that isolates from larval culture water included a range of *Vibrio* bacteria but no single type was common. In his study many of the *Vibrios* did not match any previously described species. The same occurred in this study. The taxonomy of tropical marine *Vibrios* is a complex and difficult area. *V. alginolyticus* was chosen as the challenge organism for use in the bioassay system as it was the most frequently encountered organism in the hatchery monitoring. Its failure to produce any significant larval mortality could indicate it is not a pathogen of prawn larvae, although a common bacteria in the hatchery environment.

Bacteria identified from external bacterial necrosis lesions on larvae were typically *Vibrios* with *V. tubiashi* and *V. alginolyticus* common. This is similar to Muir's findings of *V. harveyi, V. tubiashi* and *V. alginolyticus* being the most commonly isolated bacteria from diseased larvae (whole larvae preparations). However a relatively wide range of other organisms was identified and there needs to be further work to define the role of the whole range of bacterial isolates.

While it is clear the causative infectious agent of luminous vibriosis is *V. harveyi* (Lavilla-Pitogo *et al*, 1990), the bacterial aetiology of bacterial necrosis and larval bacterial septicaemia is still not clearly defined. Muir (1990) showed that *V. damsela* and a *Vibrio* sp. could invade larval tissues, also that toxins from *V. harveyi, V. damsela* and *Vibrio* sp. were lethal to larvae in acute pathogenicity tests. However none of these bacteria were shown to reproduce the diseases as seen in hatcheries. One can speculate that some bacterial pathogens do not directly affect larvae and are not normally found on/in larvae. This group of organisms may release toxins into the culture water which exert a toxic action on the larvae. Further work to describe the specific bacteria which are pathogens is required.

Without the clear identification of pathogens (and the pathogenesis of the diseases) the development of specific control measures may be difficult.

Larval rearing trials in the bioassay system.

There are many general recommendations on the conditions which should be maintained in prawn larval cultures if high survival to postlarvae is to be achieved. Most emphasis is on nutrition and water quality, but recommendations for hygiene have also been provided. With the exception of luminous vibriosis, there has been no research directly linking specific environmental factors to increased susceptibility to bacterial diseases in prawn larvae. This understanding is critical, together with monitoring hatchery cultures for causal relationships and the identification of the specific pathogenic agent(s), if appropriate control methods are to be developed. To do research on the relationship between the rearing environment and possible bacterial pathogens it appeared necessary to develop an experimental scale larval rearing system. Fundamental to any study of bacterial pathogens is the requirement to fulfil Kock's Postulates. That is, the organism isolated from a diseased animal must be reintroduced to a healthy animal to reproduce the same disease. Obviously this would be an undesirable experiment in a commercial hatchery operation. The use of the bioassay system was problematic. It took time to define an appropriate rearing schedule and there were some problems which would need to be resolved in future studies using the bioassay system. One problem was the supply of high quality nauplii. While the experimental design depended on comparison of control and treatment cultures for each batch of larvae, the large between batch survival rates was undesirable. Secondly there was a high between replicate culture variation. This considerably reduced the sensitivity with which differences between treatments could be detected on analysis. Finally, background heterotrophic bacterial counts were high (10^5 VBC/ml), even when bacteria-free culture water and disinfected cones were supplied daily. In future use of the bioassay system an on-site spawning unit would be essential, as will further refinement of the bioassay system to reduce between replicate variations in survival. As prawn larvae cannot be reared abiotically (in the absence of all bacteria) there will always be bacteria in the gut available to re-establish a bacterial flora, even in sterile rearing water, the problem of background bacteria will remain. Ideally these bacteria will be managed or monitored continuously.

In the context of these constraints, the trials in the bioassay system did indicate some useful findings. It seems obvious that low nutrition levels would reduce survivals, although the trend for an increased prevalence of bacterial necrosis lesions indicates nutrition could affect larval resistance to infections. Trauma to larvae has been considered a predisposing factor in bacterial diseases in hatcheries. High aeration was used as a trauma in three trials. In one trial survival was higher with heavy aeration, while it tended to be lower in the other two trials. An equivocal result but the role of trauma was supported by the trend for higher lesion prevalence in heavily aerated cultures. There was a trend for lower survival at high stocking densities. This could result from the lower water quality with the more rapid build up of metabolites in the highly stocked cultures or result from an increased incidence of disease (highly stocked cultures did tend to have a higher

prevalence of lesions) with the greater ease of transmission of bacteria from larvae to larvae. The significantly better survival of larvae that were cold-shocked daily is contrary to expectations. Muir (1990) observed that low temperatures were a probable cause of larval mortality. Further trials would need to be done to determine if this result is repeated.

In the one trial where larvae were challenged with *V. alginolyticus*, no increased mortality of larvae was observed. The implication is that this isolate was not pathogenic. It could also be due to inhibition of its growth by other heterotrophic bacteria in the culture water. As the bacterial suspension added was washed, there would have been no pre-existing toxins added at the time of inoculation. With the growth inhibition no more toxin would have been produced. Future research will need to screen potential pathogens for acute pathogenic effects as well as comparing the effects of different numbers of inoculated bacteria, washed cells and cell-free extracts before an organism is excluded from consideration as a pathogen.

5. CONCLUSIONS

Hatchery monitoring results indicated a possible causal relationship between concentrations of one or more components of the "presumptive Vibrio" populations in rearer tank water, as detected by dip slide TCBS agar, and outbreaks of bacterial necrosis. The source of these putative pathogens and the means by which they induce lesions was not determined.

There was no evidence that outbreaks of bacterial necrosis were causally associated with changes in other variables measured, including concentrations in rearer water of total bacteria, total heterotrophic bacteria, unionised ammonia, nitrite or nitrate.

There was no convincing evidence that antibiotics, at concentrations used at commercial hatcheries during the study, were effective in reducing concentrations of the putatively pathogenic presumptive Vibrios.

Dip slides proved to be a convenient method of indicating bacterial concentrations in larval rearing tank water, *Artemia* cultures and algal cultures. The technique is simple and all necessary facilities are available in most hatcheries. While providing useful information on hatchery hygiene and serving as an indicator for possible outbreaks of bacterial necrosis, such monitoring does not appear to have predictive value.

The epifluorescence technique did not appear to be a useful predictor of changes in disease status.We found the technique time-consuming and difficult to control; the equipment required is not normally available in hatcheries.

The bacteria identified from larval rearing water, algae and brine shrimp cultures, and from bacterial necrosis lesions added to the small base of knowledge which defines the potential bacterial pathogens of prawn larvae. The findings support the view that *Vibrio* bacteria constitute the main group of disease-causing organisms. There still remains the possibility that bacteria in the genera *Pseudomonas* and *Aeromonas* have a role in causing disease.

The bioassay system is not yet an ideal experimental system to study relationships between rearing environment, bacterial pathogens and larval disease. Its use did indicate poor larval nutrition, trauma and high stocking densities increase the prevalence of bacterial necrosis and probably reduce larval survival. With further refinement the bioassay system will continue to be a useful tool to determine the pathogenesis of bacterial diseases of prawn larvae and to evaluate control methods.

The study findings indicate the need for further research into possible causal relationships between presumptive *Vibrio* concentrations in rearer water and outbreaks of bacterial necrosis in penaeid prawn larvae. Findings also demonstrate the continuing need for researchers and diagnosticians to provide a rational basis for antibiotic usage in prawn hatcheries and the requirement to investigate more effective control methods. Possible bacterial pathogens need to be isolated from a wider range of geographical locations, identified and their pathogenicity conclusively demonstrated. The role of bacterial biofilms on pipe and tank surfaces needs to be investigated. Using information from hatchery monitoring and the isolated pathogenic bacteria, the pathogenesis of bacterial diseases in larvae needs to be demonstrated in a bioassay system. In this way important factors involved in the initiation of disease can be identified. There is a clear need for identification of probiotic bacteria and other biological control agents, in order to introduce new methods and technologies to commercial prawn hatcheries so that healthy prawn larvae can be produced more sustainably.

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8. APPENDIX

8. APPENDIX

Time am/pm

Larval batch:	Larval stages
Date:	Larval density (no/l)
	No. aliquots for > 150 larvae

Time am/pm

Direct examination (500ml sample)

Estimated: No. examined
: % dead
: % deformed
: % normal motility
: % reduced motility

Organic matter (high/medium/low)

Time am/pm

Microscopic examination

Total examined

Number with : cuticle damage (incl. appendages)
: abnormal conformation
- describe abnormality

: peritrichs
: fungi
: other (describe)

Gut filling (average % in sample- 0/25/50/75/100)

Time am/pm

Rearer water temperature

Rearer water volume (tonnes)

Rearer water exchange (tonnes)

Time am/pm

Water treatment

Antibiotic : name
: amount (g)

EDTA : amount (g)

Algal cultures

Species: _____

	Batch:		Age:		Batch:		Age:		Batch:		Age:	
Time fed am/pm												
Cells/ml												
Condition												

Species: _____

	Batch:		Age:		Batch:		Age:		Batch:		Age:	
Time fed am/pm												
Cells/ml												
Condition												

Rearer

Algal species: _____ Cells/ml

Time am/pm				

Algal species: _____ Cells/ml

Time am/pm				

Artemia culture

Strain (fresh): _____

Strain (frozen): _____

	Time am/pm					
Fresh (no. fed)						
Frozen (no. fed)						

Rearer

Artemia no./ml: before addition

: after addition

Time am/pm					

Artificial food

	Time am/pm					
B.P. (g added)						
A.S. (g added)						
Other (g added)						