Department for International Development

Strategy for Research on Renewable Natural Resources

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Control of Bacterial Disease in Small Scale Fresh-Water Aquaculture

Project R7054

Project Completion Report

December 1998 - May 1999

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RNRRS PROJECT COMPLETION SUMMARY SHEET

DATE REPORT COMPLETED: 15/7/99

PROJECT NUMBER: R7054

RNRRS PROGRAMME: Aquaculture

PROGRAMME MANAGER: Professor James Muir

RNRRS PROGRAMME PURPOSE: Reduce the impact of fish disease

RNRRS PRODUCTION SYSTEM: Aquaculture

COMMODITY BASE: Freshwater fish

BENEFICIARIES: 'The rural poor'

TARGET INSTITUTIONS: Aquatic Animal Health Research Institute (AAHRI), Bangkok,Thailand; College of Agriculture, CanTho University, Vietnam

GEOGRAPHIC FOCUS: Southeast Asia

	Planned	Actual
START DATE:	1/12/97	1/12/97
FINISH DATE:	31/5/99	31/5/99
TOTAL COST:	£99 223	

Fresh-water Aquaculture is a valuable contributor to nutritional and income demands of rural and peri-urban populations within developing countries. A range of aquatic disease conditions, in which bacterial agents are a major element, can seriously affect its productivity and development potential. Continuous low level losses from either opportunistic or facultative bacterial disease outbreaks not only affect production but are economically devastating for rural farmers. Sustainable aquaculture requires effective management of the local ecosystem to achieve optimal productivity[1, 2.] However, in many inland systems, increasing organic enrichment in turn, influences bacterial loads and enhances potential disease risks. A wide spectrum of bacterial species can be detected, predominantly opportunistic pathogens[3, 4.] The innate immune defences of animals kept in good conditions can withstand a degree of bacterial challenge, but readily succumb to virulent pathogens[5, 6,7.] Continued exposure to less virulent pathogens is also detrimental, and fish under-perform if held in poor quality water[7]. To offset losses, farmers often increase stocking densities thus promoting stress-associated diseases and exacerbating the situation. Conventional assessment techniques for bacterial loads and stock conditions are complex and expensive, and a means of understanding the

environment and bacterial load/disease risk relationship is required. A simple method to detect and warn of dangerous conditions, and the consequent development of simple and reliable management measures could have a fundamental impact on reducing bacterial diseases and improving productivity.

This proposal is based on a hypothesis developed from an unexpected finding resulting from research carried out under (ODA) R5998 on farmed tropical frogs (*Rana rugulosa*) in Thailand, in which a high level of bacteria was frequently observed in macrophages isolated from clinically healthy animals[8, 9,10.] A small study on tropical farmed freshwater fish showed similar findings[11]. No overt signs of disease were present and recovery of bacteria from the animals was difficult, but could be achieved after a 24-hour broth enrichment step. Potential opportunistic pathogens found ubiquitously in the aquatic rearing conditions of both fish and frogs were recovered and also identified within macrophages. Thus, if macrophage bacterial isolation could be an accurate determinant of both rearing environment and potential disease risk, it could be possible to develop better understanding of management options and their impacts. Further, the presence of bacteria in fish tissue macrophages may lead to fundamental reappraisals of the process of disease initiation and the host defence mechanisms under such culture conditions.

The work presented here represents an 18-month study to examine the relationship between environmental conditions, bacterial load in the water and bacteria levels in tissue macrophages of a range of clinically healthy freshwater fish species, farmed in a range of culture systems in Thailand and Vietnam. Preliminary assessment was made of the clinical significance of the macrophage bacterial load. The aim of this work was to improve production in fresh-water aquaculture through the control of clinical bacterial disease and subclinical infection, and to identify management practices most effective in promoting fish health.

1 Project purpose:

1.1.1 Co-ordinate findings from small scale fresh water sites in Vietnam and Thailand defining relationship between pond/water management, environmental bacterial load, disease frequency, treatment and outcomes, and macrophage-bacteria interaction in fish.

1.1.2 Linked laboratory studies on the bacterial load in fish phagocytic cells and in the water, the effect of different environmental stressors on the outgrowth of "carried" bacteria; and the effect of the presence of bacteria of moderate to severe virulence.

1.2.1 Develop dissemination material and conduct national level workshops for farmers, extension officers and health managers and a Regional workshop for Aquatic resource managers/regional aquatic health specialists/ planning and policy staff.

1.2.2 Prepare follow-up study plan to elucidate the clinical significance of the findings in Phase 1.

2 Outputs:

2.1.1 QUESTIONNAIRE AND SURVEY

(Experimental data are presented in Appendix 2)

2.1.1.1 Questionnaire

Institute of Aquaculture (IOA), Stirling

Staff Dr K. Thompson- project manager[1]

Ms M. Crumlish- research fellow

A questionnaire was designed at the IOA in consultation with a statistician, to assess annual production, management strategies and disease status of a variety of small-scale fresh water farms in Vietnam and Thailand (Appendix I). Four farm sites were chosen in both of these countries, based on the information obtained from the questionnaire. The farms were monitored in a field-based survey, the aim of which was to examine the relationship between farm management, the bacterial load in the environment, disease outbreaks, bacteria and macrophage interactions and treatments used to control disease. Farm sites were visited once a month for four months throughout each sampling cycle and two sampling cycles were conducted at different times of the year to examine seasonal influences on the water system.

Researchers from IOA visited partners in Thailand and Vietnam at the start of the project to help initiate the survey and demonstrate standard operating procedures (SOP) developed at IOA for sampling in the field.

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[1] In June of 1998 Dr. V. Inglis (original project manager) retired from the project and its management was subsequently transferred to Dr. K. Thompson.

Aquatic Animal Health Research Institute (AAHRI), Thailand

Staff Dr. Supranee Chinabut- project supervisor

Dr T. Somsiri-project supervisor

Ms Yonprapa -research assistant

Ms Anintita- research assistant

Although, many farmers were initially approached, relatively few wanted to be involved in the survey conducted by AARHI. Some farmers feared that repeated sampling would result in stress-related problems within their stock, with fish not feeding for up to a week after being disturbed. The farmers looked this as an unnecessary economic loss of productivity. The research team from AARHI was able to select four farmers from the results of the questionnaire, who were willing to participate in the survey. The farms chosen were all hybrid catfish (*Clarias gariepinus* x C. *batrachus*) producers, located in different locations around Bangkok (Samutprakarn, Suanprikthai, Supanburi and Patumthani) (Table 2.1). These sites were also selected since they were within 2 to 3 h from AAHRI. The distance of the farm from the laboratory was believed to be a major constraint for analysing sampled material when the farms were originally selected.

The farms chosen differed both in terms of size and production (Table 2.2), and in their management practise (Table 2.3 and Table 2.4). The questionnaire provided information relating to the farm's stocking density and production rate (Table 2.2), the feeding regime practised on the farms (Table 2.3), the frequency and impact of disease outbreaks (Table 2.4a), and treatments applied by the farmer to control or prevent disease problems (Table 2.4b). The farms were classified as good, intermediate or poor, based on both the questionnaire and on the Thai research teams' impression of the farmers husbandry skills, the frequency of disease outbreaks and the smell and colour of the water at the site. The farm in Patumthani was selected to represent a good farm, while the site at Suanprikthai was classified as poor. The other two sites were regarded as intermediate farms (Table 2.1).

Farm 1:

The farm in Lumsai District, Patumthani Province, owned by Mr Supote, was considered a good site and consisted of a large single-family unit, with 14 ponds. The estimated annual production of catfish at this site was around 20 tons. The stocking density was approximately thirty-six fingerlings per m^2, which on average is equivalent to 9 adult fish per m^2. The fish produced for market, were

predominantly fed chicken waste and never given trash fish or feed supplements after they were 20 days old. The level of disease outbreaks was considered low at this site, and no chemical or antibiotic treatment was required. No disease outbreaks reported on this farm during the course of sampling, with the exception of jaundice disease when the fish were 4 months old. The farmer controlled this with reduced feeding and increased water changes.

Farm 2:

The farm selected in Suanprikthai District, Patumthani Province, was classified as a poor farm. It was a single-family unit comprising of 2 ponds owned by Mr Phone and had an estimated annual production of 4 tons. The stocking density of the ponds was believed to be around 22 fingerlings per m^2. Fish were produced mainly for family consumption, but some were sold locally. Juvenile fish were sometime fed commercial pellets, but generally, trash fish and animal waste were used to feed young and adult catfish alike. Only one disease outbreak occurred at the site in the 12-month period prior to commencing the survey, which appeared to affect the digestive system of the animal. No etiological agent was, however, isolated. Traditional remedies were sometimes used, but never antibiotics. The farmer harvested his stock 2 months into the second sampling cycle and no further sampling was performed at the site after this time.

Farm 3:

The site in Kokcotoa District, Supanburi Province was a single-family unit, owned by Mr Prapas, and consisted of 6 ponds. It was a polyculture farm, culturing Nile tilapia, catfish and chicken, and was considered as one of the intermediate sites. It had a stocking density of 20-23 fingerlings per m^3. The estimated annual catfish production from this farm of 4 to 5 tons was marketed locally. Juvenile fish were initially fed commercial pellets and later maintained on trash chicken waste. Only one major disease outbreak was recorded in the 12-month period prior to commencing the survey result in a 10 % loss of stock. Clinical signs of the disease included swollen abdomen, back ulcers and dead necrotic tissue around the mouth. However, no etiological agent was confirmed. Chemicals and antibiotics (administered in the feed for 3 to 5 days) have both been applied to treat such disease problems.

Farm 4:

The farm chosen in SumutPrakarn Province, as the other intermediate site, was a single-family unit owned by Mr Phaitoon and consisted of 2 ponds. The estimated annual production of the farm was 4.5 tons of which was marketed locally. The farmer's stocking density was estimated to be 20 to 23 fingerlings per m^2. Young fish were initially fed commercial pellets, but after one month post-hatch, they were maintained on trash animal waste, to which was sometimes added a feed-supplement. Two disease outbreaks occurred at the site in the 12-month period prior to commencing the survey in March 1998. Disease outbreaks were recorded in January (1998) with clinical signs of ascites and ulcers. Signs of fin rot and gill swelling were evident in the second outbreak. Both juvenile and adult fish were affected and an estimated 10% of stock was lost due to these episodes. Chemicals and antibiotics were both applied at the site during disease outbreaks.

College of Agriculture, CanTho University, Vietnam

Staff Mrs Dung- project supervisor

Ms Hoa- research assistant

Ms Nok- research assistant

The Vietnamese team also selected four farms based on the questionnaire, to be included in their monitoring programme. The sites chosen were representative of the range of freshwater aquaculture systems currently practised in the south of Vietnam. At the start of the project, the research team from CanTho University and IOA visited a number of farms to establish the type of culture systems available, and which would be appropriate for the survey. The range of farming systems within sampling distance from the laboratory was more extensive than seen around AARHI in Thailand. Farmers at some of the sites visited, answered the questionnaire, the information of which is provided in Tables 2.5a and 2.5b. The Vietnamese group however, later selected four completely different farms for subsequent sampling visits (Table 2.6).

The farms selected by the CanTho research team were chosen firstly because the farmer was willing to participate in the study and allow staff to sample their stock, and secondly the farmers were generally keen to obtain information and advice on disease outbreaks at their site. When fish were healthy however, they often did not want the fish to be disturbed. The collaboration and relationship with participating fish farmers in Vietnam was much stronger than experienced with the catfish farmers in Thailand. The location of the farm was also considered, as they needed to be situated within working distance from the laboratory.

The farms selected for monitoring were situated in different provinces and the type of farm system examined here varied considerably. They included a large cage culture site producing catfish (*Pangasius bocourti*) at Chau Doc, a small cage culture facility at Dong Thap farming sandgoby, a monoculture pond at Thanh Quoi Village producing snakeheads and a polyculture site at Chau Thanh Village consisting of fish, pigs and rice (Table 2.6). The polyculture site was stocked with a variety of fish species including Indian carp, common carp, silver carp, tilapia, gourami, silver barb and catfish. The farms were graded good, intermediate or poor, again based on answers obtained from the questionnaire and the teams' opinion of the water quality (smell and water colour), the farmer's husbandry skills and production at each farm. The polyculture facility at Chau Thanh was considered as a intermediate to good farm, while the farms at Dong Thap and Thanh Quoi were considered as intermediate to poor. The large cage site at Chau Doc was classified as a good farm. Initially, a snakehead farm at Tranh Quoi was chosen to represent a poor farm, but after experiencing large-scale losses at this site, the farmer harvested his fish and sampling had to be discontinued. The etiological agent of the disease outbreak remains unidentified. A similar farm at the same location (Farm 2 as indicated in Table 2.6), also producing snakehead fish, was chosen as a replacement and was sampled throughout the second sampling cycle. It was classified as an intermediate to poor farm

The disease history of these sites is shown in Table 2.8

Farm 1

The polyculture pond site located at Chau Tranh Village, in CanTho Province, was classified as a good to intermediate farm. The farm was a large family complex, the produce of which was destined for local market. There was one pond at this site, 500 m^2 in area, and was stocked at around 10 fish per m^2. The farmer mixed fish of different size and age together within the pond. During the first sampling cycle, Indian carp, Tilapia spp. and kissing gourami were cultured. However, in January 1999, wild snakeskin and additional kissing gourami were caught and added to the species cultured within the pond. The fish were maintained on vegetables, and pig and human waste. All species of fish were apparently healthy when examined by the research team during their sampling visits. No disease outbreaks were recorded at this farm.

Farm 2

A monoculture pond site was chosen at Thanh Quoi Village, CanTho Province and represented an intermediate to poor quality farm. It was a large family complex with 2 ponds, 500 m^2 in size with a stocking density estimated at 40 fish per m^2. Snakehead fish cultured here, were for family consumption and local market, and were harvested once they reached 400 to 600 g. Since the size of the fish in the pond was irregular, the farmer was able to select fish throughout the year for market. The fish were maintained on trash fish and appeared healthy. Outbreaks of epizootic ulcerative syndrome (EUS) have been recorded at this site.

Farm 3

The small cage culture site was a large family complex, located in Dong Thap Province. The cages were 3 x 5 x 1 m^3, and were stocked at a density of 50 fish per m^3. This site was also regarded as an intermediate to poor quality farm. The farmer harvested his stock (sandgoby) for local market once they reached 200 g or more. The smaller fish were left to grow and were collected with the next harvest. No disease outbreaks were reported during the first or second sampling cycles at this site, although disease outbreaks occurred on neighbouring sandgoby farms during September to December, and March to April. No further information regarding the clinical signs or the etiological agent of the disease outbreaks was available.

Farm 4

The large cage culture at Chau Doc Province was reported to be company owned on the questionnaire. This may have been misinterpreted since these cages are usually Government owned; or rather farmers receive from the Government to buy their farms. The family lived on top of the cages which, generally measure 15 x 6 x 4.5 m and were stocked at a density of 120 fish per m^3. The *P. bocourti,* cultured here, were harvested for market between 800 to 1200 g in weight. The farmer harvested the cages in January and April 1999. Usually only one cage is harvested at a time, since the farmer staggered the size of his stock. Although the farmer did not experience any disease outbreaks, he did report that the fish developed red-coloured fins, possibly haemorrhages, during harvesting and was most likely caused by handling. The process of sampling disturbed the fish, and they would not eat afterward. Therefore, different cages were sampled at each visit visits, but still on the same farm.

Establishing the exact stocking density at some sites was difficult since the farmers were often unaware of their stocking density, or even the amount of fish use to stock the pond initially. The ponds were stocked with seed produced either on site or by neighbours, or were bought locally at the market. The farmer often used wild fish to supplement his pond, with the result that wild fish numbers are now in decline and this is a major concern in Vietnam. Attention is currently focusing on artificial breeding programmes

VAC polyculture farming systems were encouraged 5 years ago and their numbers are still increasing throughout the Mekong Delta. Over 50 % of the freshwater farms within the Mekong Delta region are now polyculture farms sites. A variety of animals are farmed in these systems, including pigs, chickens and buffalo. Disease problems are generally less than other farming systems, but the fact that human waste is put into the ponds may be of concern for human health. The main disease problems associated with these systems tend to be due to water quality or EUS, which can occur early in the wet season during April and May, or at the end of the wet season in November and December. It is common for farmers to culture a wide range of fish species within the pond, such as silver barb, carp, common carp and tilapia. Farmers often supplement their ponds with wild caught fish, and this may be carried out on a daily basis. Wild fish are thought to taste better and are more resilient than cultured fish.

Disease outbreaks are less frequent in polyculture farming systems compared with monoculture farms, simply because the stocking densities maintained in these sites are much lower. However, it would appear that some polyculture farmers are now progressing to semi-intensive or intensive monoculture practices, and this has lead to concerns about water resources both for the farms and the local community. The Department of Fisheries has recommended that optimal stocking densities should balance the input and output of the water resource. Recommended stocking densities are 10 fish m^2, but this varies depending on the system, with thresholds of 20 or 30 fish m^2. Local scientific research staff rather than the extension officers, usually gives advice on disease to farmers in the Mekong Delta region. This most frequently relates to the stocking densities of their ponds, but also covers other issues such as husbandry practices and disease prevention. In general, larvae tend to be more susceptible to obligate pathogens compared with juvenile or adult fish, which appear more vulnerable to opportunistic infections.

All fish sampled appeared healthy, with no obvious signs of disease, but it was difficult to ascertain whether sampled fish were infected with parasites, a big problem in freshwater aquaculture systems in Vietnam. The large cage culture site and the polyculture farm were generally disease-free

during the sampling cycles discussed below, but the sandgoby and the snakehead farms did experience some disease outbreaks. Before sampling, research staff would liase with the farmer, and if disease problems were evident, the fish were treated, and sampling would then take place once the fish appeared healthy. Guava leaves are often used to treat bacterial and fungal diseases at these sites. Disease outbreaks frequently occur on the farms in the Mekong Delta at the end of the wet season. This starts around April/ May and continues until September/ October. Disease outbreaks are also associated with the dry season, which begins around December/January, and may result for the decrease in water temperatures, possibly because fish become immunocompromised at lower temperatures, this being a well-established phenomenon. In 1998, the average temperature was 17^0C compared with a usual temperature of 22 to 25^0C. General disease outbreaks identified in the region included 'red-spot', which could be treated with antibiotics, and white patch disease, for which there is no effective treatment. Farmers normal buy antibiotics used to treat human aliments, from the local pharmacist.

2.1.1.2 Survey

Initial work at IOA involved the development of sampling methods, which could be easily performed in the field. A method for the isolation of head kidney macrophages from fish was developed under laboratory conditions and from which a farmed-based sampling technique was devised. SOP for sampling in the field (see Appendix 3), were set up for use by collaborators in Thailand and Vietnam, which describe how to: (1) isolate head kidney macrophages from the fish; (2) determine the number of macrophages which contained bacteria in their cytoplasm; (3) measure water quality parameters and (4) estimate the level of viable bacterial recovered from water samples. Extensive training was giving in these procedures to the research staff at both AARHI and CanTho University before sampling commenced. The four farms in both Vietnam and Thailand were sampled four times over two seasons. Head kidney macrophages were isolated from fish at the pond-side using the sampling technique developed at IOA. Water, sampled at each farm to assess its quality, were taken back to the laboratory for processing. Water quality analysis was contracted out to other laboratories within the collaborating institutes, involved in routinely analysis. This was an expensive venture however. Bacterial levels in the water were determined from colony forming units (CFU)

prepared at the pond side on tryptone soya agar (TSA) and *Aeromonas* selective (As) media. Plates were cultured for 24 to 48 h at 28°C, back at the laboratory.

Water quality analyses

Water quality is an important consideration here[14], not only with respect to fish health, but also for establishing conditions, which promote high levels of bacterial growth within the pond. The parameters, which influence water quality interact directly with each other, and can affect the environment of the cultured fish. Physical, chemical and biological parameters are important when considering the dynamics of the fishponds. These are discussed in depth in Appendix 6.

The temperature and pH of farm water can vary over the course of the day. It was only possible to measure these parameters once a day at the time of sampling the fish. This restricts interpretation of the water quality data, but was a foreseen limitation at the time of planning the study. Samples were taken and analysed so as to establish trends between the different farming systems over the two sampling cycles and water temperature appeared to be constant throughout each sampling cycle (29 to 30^{0}C).

AAHRI

Water quality parameters measured at each of the four sites included temperature, pH, alkalinity, ammonia, nitrites, TSS and turbidity. Values for these parameters varied considerably between the four farming systems and between the individual farms on different sampling days. Water temperature and pH remained relatively constant between the water systems of the different farms throughout sampling (29 to 30^{0}C and pH 6.9 to 7.2, respectively).

Only slight variations were observed over the first sampling cycle in the levels of viable bacteria recovered from water of the individual farms. The level of bacteria recovered ranged from between 10^3 and 10^5 cfu ml^{-1} on *As* and between 10^3 and 10^7 cfu ml^{-1} on TSA in water sampled from the four sites. A range of bacterial species was recovered, but most remained unspeciated.

In most freshwater systems the ideal alkalinity levels are between 20 to 300 mg L^{-1} and the water alkalinity levels of farms cycle 1, were found to be within this range, except Farm 3 (intermediate grade). In cycle 2, the alkalinity levels were higher than ideal for all ponds sampled.

The amount of ammonia was low in all of the ponds initially, but started to increase by the third sampling point of cycle 1. A similar trend was noted in cycle two at Farm 4. There are many reasons for this, but it is likely due to over-feeding. If farmers over-fed their fish, the decomposition

of waste feed can increase the amount of ammonia. In cycle 2, the ammonia values in the four ponds differed dramatically, not only from each other, but also between sampling points within a cycle 1. Water temperature and pH did not differ between the two cycles.

The nitrite levels were low for all farms in cycle 1 and were within the recommended range of ? 0.1mg L^{-1}, while levels seen during cycle 2 were greater than seen during cycle 1. These levels were high at all four farms, but particularly at Farm 4, during the second sampling. Again this may have been due to increased feeding and low oxygen levels in the pond, but this in not know with certainty, since DO was not measured. *Clarias* spp., are very tolerant to higher levels of nitrites compared with other sensitive species such as salmonids.

It is not unusual for catfish species to live in waters with very high levels of suspended solids of up to 10,000 ml L^{-1}. However, the level of TSS in all ponds was low both, in cycle 1 except Farm 1, and in cycle 2 for all of the four farms.

The viable bacterial counts in cycle 2 were slightly higher than seen in cycle 1, but in general the total viable bacterial counts were low for all farms, except in cycle 2 during the first sampling at Farm 2. Levels had decreased by the second sampling, however. The reason for this was not clear, but may have been related to a reduced feeding rate or increasing water changes, which in turn reduced the amount of viable bacteria in the water. The water quality parameters followed similar trends between all the farms throughout cycle 1, but differed to each other throughout cycle 2. This may have been a seasonal affect, since cycle 2 was carried out in the dry season, while cycle 1 analyses was performed during the wet season.

CanTho University

The first cycle of sampling for the survey was completed between March and September 1998. It was not possible to collect samples between May and July 1998, as the Vietnamese group had a problem obtaining chemical reagents. Sampling was, therefore, delayed until August and September 1998. The second cycle of the farming systems started in December 1998 and was completed in April 1999. The sampling regime was performed as described for the Thai sites.

Water quality parameters measured at each site, included temperature, pH, transparency, dissolved oxygen, total nitrates, total phosphates, TSS and organic and inorganic suspended solids (OSS and IOSS, respectively). A wide range of results was obtained for the different farms, but only slight variations occurred within sites between the different sampling days. Temperature and pH, however, appeared similar between the farming systems throughout the sampling period.

The amount of viable bacteria in sampled water was determined and these appeared similar between the farming systems, with bacterial counts on TSA and As agar ranged from between 10^2 and 10^4 cfu ml^{-1}.

The water quality parameters measured at CanTho University differed compared with those analysed at AARHI. This was limited by the facilities at the two research institutes, but as many parameters as measured as possible. However, no water quality data is available for cycle 1 monitoring of the farms in Vietnam. Since the farming systems examined in Vietnam are different to each other, their water quality could not be compared directly. Water quality data obtained at each sampling point was pooled for each farm and their water quality statistically assessed on this basis.

The amount of DO in the water was lowest in the polyculture farm and highest in the large cages. This was not surprising, as large cage culture facility was the only farm, which had aerators, and these were used once a day. It was interesting to note that the amount of DO was low in the monoculture farm at sample point 4, which corresponds to increased organic suspended solids at this time. The COD for all of the farms was similar and low except in the small cages at sample point 3, where increased levels of COD were observed. All farms had low amount of nutrients present in their water. Although this would strict primary production, the possibility of algal blooms developing and phytoplankton levels increasing were reduced. The TSS of the farms was high, particularly at the polyculture site. This was expected, as the TSS appeared to be primarily organic material, probably resulting from the fish waste, and pig and human faeces, used to feed the fish. The lowest level of TSS was found in the large cage culture facilities. Again, this was expected, since although it had high stocking densities, water exchange due to river flowing, was greater compared to that of the small cages and pond sites. Hence, most waste material was removed form the cage by the flowing water. The amount of TSS was similar between in the monoculture pond and the small cages, and the level of suspended solids increased at all farms as the transparency of the water decreased.

The water quality data alone was not sufficient to grade the farms. The data obtained in cycle 2 was as expected for each farming system. It would have been useful to have had water quality data for cycle 1, as this would have provided a comparison between the two seasons. Water exchange is important because it removes nutrients and phytoplankton from the system, removes toxic waste (NH_3 and nitrites), maintains salinity levels and may be used to substitute aeration. Water exchange in the large cages cannot be estimated, but the flow of the river in which they are situated was running reasonable quickly, while the small cages, located in canals had a much slower

flow through them. The farmer at the snakehead farm changed the water of his pond by 30% in the dry season, over 2 to 3 days on the 15th and 30th of each month. This corresponded with lunar cycle and high tide. In the dry season, it is often difficult to carry out water changes of the pond, but in the wet season no water changes were needed, because of the excess rainfall. In the polyculture system water was changed once a week by up to 20-30% of its volume.

The large cage culture system appeared to have the best water quality, with high oxygen content (5 to 8 ppm) and produce 100 to 200 tonnes of fish per cage. The cages were about 4 to 5 m deep.

Relationship between water quality and percentage of macrophages containing bacteria

Initially, macrophage cell isolation had been performed using a conventional laboratory-based method as described by Secombes (1990)[15] in which a discontinuous Percoll gradient was used to isolate the cells. Difficulties were encountered with this method for sampling the fish held at farm sites. The animals either had to be transported to the laboratory for analysis, or their kidney tissue had to be removed at the farm and this taken to the laboratory for macrophage isolation. Fish are exposed to a variety of environmental stressors under transportation, which in turn can influence the disease status of the animal and thus the number of bacteria that may be found within their macrophages. Alternatively, bacterial contamination can occur during tissue isolation at the farm site, and by the time the head kidney has arrived at the laboratory for analysis, contaminating bacteria have multiplied. This, consequently, can affect the levels of bacteria found within the macrophages isolated in the laboratory. Keeping kidney samples cool during transportation is also a problem, especially during the hot season when temperatures may reach as high as 40°C. Hence, a simple isolation procedure was developed to overcome such problems, whereby macrophages could be collected from the head kidney of the fish at the pond-side. No centrifugation is required during the method, so it can be easily performed at the farm. The technique is suitable for use in tropical climates and provides sufficient yields of macrophages, free from bacterial contamination.

The isolation technique described here is a modification to that used by Secombes (1990). All materials and reagents were aseptically prepared prior to sampling and the sterility of the reagents was tested by streaking them onto TSA and *As* medium. Macrophage suspensions were prepared by teasing head kidney tissue through a 100 ?m nylon mesh into a universal containing 2 ml of Leibovitz-15 medium (L-15) (Sigma U.K), 10 Units ml^{-1} of heparin and 0.1 % (v/v) of either

foetal calf serum or naive catfish serum. Two circles were drawn with a PAP pen (Sigma U.K) onto glass microscope slides. The slides were placed in a glass Petri dish and a bacteriology loop of a fibronectin (Sigma U.K) solution (1 $mgml^{-1}$) was added to one of the circles. Fibronectin was applied to promote the attachment of the macrophages to the microscope slide. After incubating the slides for 15 min with the fibronectin solution, they were washed with sterile saline (0.85 % w/v) and 200 ?1 of the cell suspension added to each circle. Two slides were prepared for each fish. The slides were incubated for 30 min in the Petri dish, after which unattached cells (e.g. erythrocytes and lymphocytes) were removed by washing the slides 3 times with sterile saline. Excess moisture was removed from the slides by tapping them onto a tissue. Adhering macrophages were fixed by incubating the slides for 30 s in the alcohol fixative from a Rapi-Diff II staining kit (Lamb Pharmaceuticals, UK) or with laboratory grade ethanol (80% v/v). Both fixatives proved suitable for this purpose. The slides were immediately stained for 1 min in both the eosin-based and then the Giemsa-based dyes of the Rapi-Diff II kit. The slides were washed twice with normal tap water, tapped dry and taken back to the laboratory for examination by light microscopy under oil-immersion.

High levels of macrophages attached to the microscope slides after 30-min incubation. Cells were clearly visible after staining and little cell lysis occurred upon fixation. It was important to use fresh fixative on each sampling day and stain the microscope preparations immediately as substantial cell lysis otherwise occurred. Although the initial cell suspension was of a mixed leukocyte population, the majority of cells that attached to the slides were macrophages. The number of macrophages, which attached was determined by counting the cells observed in four random fields of view, on each of the two circles per slide. The average number of adhering macrophages was then calculated per fish, where as many as 10 macrophages or more could be seen in any single field of view.

There are many advantages with this method compared to the conventional technique. Firstly, it is possible to process several samples at the same time and secondly, bacterial contamination is greatly reduced as a result of the short sampling time.

Effect of Fibronectin on Macrophages Attachment

A variety of substances have been used to enhance the attachment of fish phagocytes to substrate surfaces.[16, 17] Fibronectin was used to facilitate the attachment of macrophages to the glass slides. Firstly, macrophages from a population of laboratory catfish were tested and it was found that the

mean number of macrophages (? SD) which attached to the slides in the presence of fibronectin was 42 ? 5 (n = 12), while significantly fewer macrophages attached when fibronectin was not used, 37 ? 7 (n = 12). When the effectiveness of fibronectin was compared in the field sampling of the survey, data was analysed using a paired T-Test, where P ? 0.05 was considered significant.

AARHI

Macrophages attachment to the microscope slides was determined for every fish sampled from each of the four farms in Thailand during the first cycle of pond visits. The numbers of macrophages, which attached when fibronectin was used, are presented in Table 2.9. It can be seen that it had no effect on the number macrophages which, attached with any of the fish examined. Levels of attached macrophages were similar between catfish sampled from the different farms, regardless of whether fibronectin was used of not. It was therefore discontinue after the first cycle, especially since it is a relatively expensive chemical and added a further 15 mins to the incubation procedure.

CanTho University

The Vietnamese did not use Fibronectin during the first cycle of samplings due to technical difficulties, but it effectiveness at increasing macrophage attachment was examined during the second cycle of farm visits. Again no significant difference was found in the number of cells attached, when fibronectin was used compared to when it was not used (Table 2.10).

Relationship between viable bacterial water counts, water quality and percentage macrophages containing bacteria

The relationship between the water quality parameters and the percentage of macrophages with bacteria were examined using Pearson Product Moment Correlation Test, p? 0.05.

AARHI

Head kidney macrophages were sampled from six catfish at each site on each sampling day and the level of resident bacteria within the cells determined. The number of fish containing bacteria within their macrophages generally ranged from between 2 and 6 animals, although the animals appeared clinically healthy. The percentage of macrophages containing bacteria was generally low, with levels typically between 0 and 4%.

Macrophage attached to the glass slides and which contained bacteria in their cytoplasm were expressed as a percentage of total cells counted. In Thailand, 200 cells were counted in each circle of the microscope slide for each fish sampled. This was carried out in cycle 1 and 2, however, during the first cycle, problems were encountered with the cell fixation with many cells appearing lysed. This was dramatically reduced by changing the fixative of the Rapi-Diff II kit to 80% (v/v) ethanol. Cell lysis was further reduced by fixing and staining the macrophage preparations at the pond-site. This was only carried out for the second cycle. Data presented here was analysed using a Pearson Product Moment Correlation, where P ? 0.05 was considered significant.

At Farm 1, the good farm, no significant relationships were found between the bacterial counts in the water, the percentage macrophages with bacterial or the water quality parameters during cycle 1. However, by cycle 2, there was an increase in the number of macrophages with bacteria, but a decrease in the number of viable bacterial in the water compared with cycle 1, although, a significantly positive relationship was found between the bacterial water counts and the percentage of macrophages with bacteria. However, since bacterial levels in the water were lower in cycle 2 compared with cycle 1 where no significant relationship seen, this would suggest that other factors may be influencing the increased number of macrophages which contain bacteria in cycle 2. A significantly positive relationship was found in the second cycle between water temperature, TSS and turbidity levels in the water and the percentage of macrophages, which contained bacteria. Therefore, it may be that the increase in these water quality conditions has positively influenced the number of macrophages with bacteria.

At Farm 2, classified as the poorest farm by AARHI research staff, a significantly positive correlation was found in both cycles between the bacterial water counts and the percentage macrophages with bacteria. In cycle 1, however, there was no significant effect of the water quality, whereas in cycle 2 significantly positive and negative relationships were found between some of the water parameters and the levels of macrophages with bacteria.

At Farm 3, termed as an intermediate farm, a statistically significant relationship was found between the percentage of macrophages with bacteria and the bacterial counts found in the water during in cycle 1. The water quality parameters did not appear to affect the percentage bacteria in the water as no significant relationships were found. However, in cycle 2, although there was no significant relationship between the macrophages with bacteria and the bacterial counts in the water,

the water quality parameters did have highly both significantly positive and negative relationships between macrophages with bacteria. It was expected that there would be no relationship between macrophages with bacteria and bacterial counts in the water, as the level of bacterial in the water was lower in cycle 2, compared with cycle 1. The level of macrophages with bacteria was similar between the two cycles, however.

Farm 4 was an intermediate farm and had no significant relationships between the bacterial counts in the water, the percentage macrophages with bacteria or the water quality parameters. This farming environment appeared to be relatively stable over the two cycles.

CanTho University

Bacteria were observed in the macrophages isolated from the head kidney of the various fish species cultured at the four sites. Initially, the level of bacteria and cell counts obtained by the Vietnamese appeared high, but this was due to a misinterpretation of cell types within the macrophage sample. Re-examination of the samples yielded lower numbers of macrophages containing bacteria and ranged from between 0 and 20 % of the cells examined. This was representative of the general situation found at all farm sites, except for the catfish cultured in the large cage facilities at Chau Doc, which had low levels of bacteria in their cells. The percentage of macrophages with internalised bacteria was greater in the different fish species examined in Vietnam compared with those sampled in Thailand. This may reflect the differences between the farming systems employed in the two countries or may indicate species, which are more susceptible to potential disease problems. The number of macrophages with resident bacteria was always much lower in the catfish (*Clarias* spp.) than the other species examined.

The percentage of macrophages with bacteria was determined for fish sampled both in cycle 1 and cycle 2, and were analysed using a Pearson Product Moment Correlation.

In farm 1, the polyculture farm, no significant relationships were found between the percentage macrophages with bacteria and the bacterial level in the water for both cycles 1 or 2. Similarly, water quality parameters did not appear to influence the level of macrophages with bacteria.

In farm 2, the monoculture farm, no significant relationships were found between the macrophages and the bacterial level in the water in cycle1. Also, there was no correlation between any of the water quality data and the percentage of macrophages with bacteria in cycle 1. However,

in cycle 2 a negative correlation was found which indicated that as the percentage of macrophages with bacteria increased, the level of bacteria in the water decreased. There was no influence by any of the water quality parameters except for the chemical oxygen demand in cycle 2, where a positive relationship was detected between this and the percentage macrophages with bacteria.

In the small cage farm, (farm 3) a significantly positive relationship was found between the percentage macrophages with bacteria, the bacterial load in the water and the level of dissolved oxygen, in cycle 1. However, by cycle 2 a negative relationship was found whereby as the percentage of macrophages with bacteria increased the total number of viable bacteria in the water decreased. In cycle 2 the bacterial water counts were lower than in cycle 1, but the percentage of macrophages with bacteria were similar between the 2 cycles. No significant relationships were found between the macrophages with bacteria and the water quality parameters measured.

In the large cage farm (Farm 4), a positive correlation was found in cycle 1 for the percentage macrophages with bacteria and the viable bacterial counts in the water. Only water pH and water temperature appeared to have any significant relationships between the percentage macrophages with bacteria in cycle 1. However, by cycle 2 no significant influence could be found on the percentage macrophages by the water quality data and a significant negative relationship was found between the percentage macrophages and the bacterial counts in the water. This was not surprising; since the bacterial counts in the water were much lower in cycle 2 samples compared with cycle 1 samples.

The fish species sampled in Thailand were restricted to catfish farms, the choice of which was based on convenience and ease of sampling, since catfish farms were abundant around the surrounding Bangkok area. This species is regarded as being more resilient to disease than other cultured species and normally live in ponds of poor water quality. They can frequently be found buried in the mud at the bottom of the pond, where they undoubtedly encounter high levels of bacteria. Initially, the site had to be located near to the laboratory so sampled material could be taken back to the laboratory for analysis. With the development of the macrophage field sampling technique, sampling can now be performed in the field and the time restriction for analysis has removed. Most of the fish farms within easy location of Bangkok are intensive rather than extensive farming systems, and are large commercial enterprises compared with the extensive systems seen in rural aquaculture in South East Asia. This explains why snakehead farmers were less than willing to participate in the survey, so as not to jeopardise their profits. There appeared to be very little difference between water

quality and productivity of the farms chosen, with similar environmental conditions seen at all four sites. Interestingly, water quality followed very similar trends between farms throughout the wet season, and much more variation in measured parameters was seen between farms during the dry season. Trends in water quality did not appear to be related to the grade of the farm. It should be stressed that the degree of difference seen between the four farms, based on the classification "good and bad", did not appear to be that different.

Positive significant relationships were found between bacterial counts in the water, water quality and the percentage of macrophages with bacteria, implying that as bacterial levels in the water, or the water quality parameters increased, so the level of bacteria in the macrophages increased. However, negative relationships were also noted between the quality of water and levels of bacteria within the macrophages, inferring that as water parameters increased, so the level of bacteria in the cells decreased. There did not appear to be any particular trend between this relationship, and no single water parameter could be identified which continuously influencing the level of bacteria within the cells. This was mainly because an insufficient number of farm sites were sampled to allow any firm conclusion to be established. The reason for the positive/ negative relationships seen here must be a results of stimulation or immuno-suppression of the macrophages by their environment, so as to enhance or hinder their control of invading bacteria. Extending sampling to further sites would highlight the parameters, which influence macrophage levels with bacteria. The species of bacteria present in the water column and how it compares with those seen within the macrophage of the fish is also important. The assault of bacteria on the fish will depend on species present in the fishes' environment. Levels of viable bacteria in the water were determined using TSA and *As* medium. The later selectively identifies *Aeromonads*, which were low and similar between farms.

Fish husbandry practised at the four sites was generally good, with few recommendations required to improve them, but one would be for the farmer at the poor-site to improve the diet of his stock by feeding commercial pellets rather than trash animal waste.

A greater choice of farming systems and species cultured was available around CanTho compared with Bangkok. It was therefore, decided to examine a range of freshwater fish species, farmed in a variety of culture systems in Vietnam. Farmers were generally willing to participate in the survey in Vietnam compared with Thailand. Water parameters measured were similar between the farms and did not represent the extremes between the environmental conditions that were anticipated. As water quality analysis was only performed during the dry season, it was not possible

to examine seasonal influences on environmental conditions in Vietnam, or establish if similar trends in water quality were obtained to those seen at the farms in Thailand during the wet season. Water quality analysis at CanTho was restricted, and no alkalinity, ammonia, and nitrite levels are available, only total nitrogen levels. Differences were seen in the DO and COD, and TSS levels between sites. Both highly significant positive and negative correlations were found between bacterial counts in the water, water quality and the level of macrophages with bacteria. Again there did not appear to be a trend to this pattern between the four sites sampled.

Farmers frequently change production to match market requirements, so as to obtain optimal prices for their harvest, but in doing so often experience loses due to disease. In Vietnam, sandgody currently has a high market price, but this species is particularly susceptible to "red spot disease" and "white patch disease". Little is known about the severity of bacterial disease in either Thailand or Vietnam and of its impact on rural aquaculture in South East Asia. The frequency and extent of disease outbreaks and etiological agents involved are often unknown. Such information is vital if bacterial diseases are to be controlled effectively. In Vietnam, the diagnostic laboratory performed no bacterial identification. Diagnosis was made on clinical signs, and appearance of bacterial colonies on agar and whether the recovered bacterium was Gram positive or negative.

Some suggestions of how productivity might be improved at the Vietnamese sites are included. The small culture cage, farming sandgoby, was an intermediate/bad farm. The diet and water quality conditions seen at this site were the poorest recorded between the four farms, possible because the site is situated in a small canal. Therefore during the dry season, when the survey was carried out, the water in the canal was very low making water exchange difficult. Intensive culture, as seen here, is generally more susceptible to disease problems through higher stocking density. The fact that sandgoby are more susceptible to disease than other cultured species exasperated the situation. Stocking density on the site was very high and should be reduced, and the cage should be at a depth of 1 m to improve water exchange.

The polyculture farm was classified as a good to intermediate site: The farmer at this site should focus on the water quality of his farm, by ensuring that the organic load from the pigs, (which goes directly into the pond) balances with the requirement of his pond system. The bacterial counts in the water were also higher at this site. The type of fish species included in polyculture systems is also important, as they may not be as able to utilise the waste material efficiently. Advice at this farm has previously been given as to which species of fish to stock, reduce stocking densities and how to remove water hyacinth from the pond.

The farmer at the monoculture site, culturing snakeheads (an intermediate/bad farm) used predominately poor-quality trash fish to feed his stock, which was an unsuitable diet for this species. As the number of snakehead farms has increased, the price of the trash fish has also increased, but its availability has decreased. Therefore, the farmers have started using marine trash fish, which is often inappropriately stored resulting in high bacterial loads, toxins and added preservatives in the feed. Environmental conditions in this farm need to be improved, as the water quality at this site was regarded as poor.

The large cage site, shown to be a good farm with high productivity had low incidences of disease outbreaks. There was good aeration and water exchange at this site and needs little improvement. Large cage culture is concentrated in the Chau Doc area and makes up less than 10% of aquaculture in Vietnam.

No direct comparison could be made between the farming systems in Vietnam and Thailand. The species of catfish farmed in Vietnam *(P. bocourti*) was of a different genus to those farmed in Thailand (*Clarias gariepinus* x C. *batrachus*). While bacteria were observed in head kidney macrophages from all different species of apparently healthy farmed fish, examined by the Vietnamese team, the percentage of macrophages with resident bacteria was much lower in catfish compared with the other species examined. It remains to be determined if the presence of the bacteria is simply due to natural bacterial clearance by the macrophages. However, the presence of bacteria within the cells, caused by to the presence of high bacterial loads within the animals environment, may in fact, immuno-compromised the animals to the point where they then easily succumb to opportunistic infections. Many of the bacteria isolated from the macrophages have been identified as *Aeromonad* and *Psuedomonad* Spp. The clinical significance of these bacteria still remains to be determined. The macrophage cell isolation technique provides a 'snap shot' of macrophage population sampled from fish at the pond side. It is therefore anticipated that may be useful for monitoring and evaluating the health status of farmed fish at the farm sites.

2.1.2 Laboratory-based studies

IOA

The possibility of differentiating between live and dead bacteria within the macrophages was examined at IOA using a commercially available kit (Baclight LIVE/DEAD kit: Molecular Probes Ltd, Netherlands). It was possible to distinguish between live and dead bacteria with the kit, as

dead bacteria appeared red and live bacteria green under UV light. The difference between the bacteria was clearly seen in water samples experimentally "spiked" with a mixture of bacterial species. The technique has not yet been adapted for the field since water samples need to be transported back to the laboratory for examination under a fluorescent microscope and in doing do, the bacterial population would undoubtedly change during transportation. So far, viable bacterial counts made at the pond-side produce more reliable results. The LIVE/DEAD kit was used in preliminary studies at IOA to distinguish between viable and dead bacteria within the macrophages. Further optimisation of the technique is required, however, since presently fluorescence emitted by the stained bacteria is too strong to allow differentiation of live and dead bacteria within the cells. Potentially, this technique is very useful for establishing if the bacteria are resident within the macrophages or are simply present due to natural clearing of bacteria from the fishes system by the macrophages.

AARHI

Laboratory experiments were conducted in the wet laboratory at AAHRI using catfish (*Clarias gariepinus*) to investigate firstly the effect of stress on disease susceptibility and secondly the effect of environmental conditions on the bacteria load within the macrophages. These studies were used as a preliminary examination of the clinical significance of the macrophage bacterial load.

Effect of stress on disease susceptibility

The aims of this experiment were firstly to examine the effects of a variety of stressors on the disease susceptibility of hybrid catfish, and secondly to establish if the resident bacteria within their macrophages, often opportunistic in nature, predisposed the animal to disease when subjected to stressful events.

Stressors chosen are ones commonly encountered by fish during their culture and include: poor water quality (no water exchange); reduced water temperature (20°C); increased water temperature (35°C); transportation stress for 1 h; removing fish from water for 2 h and high stocking density (10 fish per tank). These were applied before artificially challenging the animals with a bacterial pathogen.

The fish, approximately 13-cm in length, were fed daily on a commercial pellet and given 70% water changes every 2 days. Before stressing them, the surface of the fish was scored (0.5 cm in length) with a sterile needle, sufficient to remove skin, but not to draw blood. A control group of

animals was also scored. Each stress experimental consisted of four groups: (1) a control group (which received no bacterial challenge or stress); (2) bacterial challenge only; (3) stress only and (4) a combination of bacterial challenge and stress. Fish (five fish per tank and five replicate tanks per group) were placed into glass tanks containing 20 L of freshwater at 28^0C (air temperature in the wet lab). The reduced temperature experiment was carried out in an air-conditioned aquarium.

The challenge organism, *Aeromonas hydrophila* (T_4) was obtained for a bacterial collection housed at IOA. Bacteria were cultured on TSA and identified by primary (Gram stain, shape, motility, oxidase, oxidation/fermentation) and secondary biochemical tests (API 20E BioMeriuex, France). A bacterial suspension (1×10^8 cfu ml^{-1}) was passaged twice intraperitoneally through adult fish, and recovered from the liver of dead animals. Bacteria were prepared at 1×10^5 cfu ml^{-1} for the challenge, the concentration of which was verified by CFU. After stress (group 3 and 4 fish), bacterial challenge was administered by bath to groups 2 and 4 for 1 h, after which time the water was changed. All animals were fed as before and water changed every two days, except for poor water quality trial. Fish were monitored twice daily for seven days, mortalities recorded and bacterial swabs prepared from the liver of dead fish. Specific mortalities were confirmed as described above.

Mortality levels between the various experimental groups were found to differ (Table 2.27a) and were still occurring in some of the tanks at the end of the trial on day 7, while in other tanks no further mortalities occurred after day 2 (Table 2.28). The bacterial colonies recovered from the liver of dead fish were all confirmed as *A. hydrophila* and the only bacterial species recovered. Some control animals also died, due to wounds inflected as a result of fighting. Catfish have sharp barbs at the end of their pelvic fins, and these often inflict damage on other animals in the same tank.

The data was analysed using a Chi-Square Test (where P? 0.05 was considered statistically significant) and levels of significance are shown in Table 2.27b. When fish were subjected to stress and bacterial challenge, differences were seen in the effect that the various stressors had on disease susceptibility, and mortalities were higher than those seen when unstressed fish were challenged. Fish subjected to low water temperature had the highest level of mortalities when exposed to *A. hydrophila* (84%), followed by poor water quality (80%), and high stocking density (76%), (this value may also include stress due to fighting), and high water temperature (64%). The lowest level of mortalities was recorded in fish subjected to transport stress (24%). These animals were transported in an air-conditioned van, and this may have reduced the impact of the transportation stress.

The effect of the different stessors on the mortality of unchallenged fish varied considerably (Table 2.27a). Low water temperatures with no bacterial challenge produced the highest levels of mortalities amongst group 3 fish. Since the fish were not challenged with *A. hydrophila,* mortalites that resulted, may have been a consequence of bacteria present within their macrophages. Macrophages were unfortunately not examined for the presence of bacteria, so no correlation could be made between mortalities and bacteria resident with the macrophages of the animal. However, as the next experiment will show, many of the bacteria isolated from the macrophages were opportunistic bacteria. The effect of the stressors applied here, especially reduced temperature, may have increased the animals' susceptibility to these opportunistic pathogens, especially since reduced temperature is know to immunocompromise the animal.

Effect of good and poor water conditions on the percentage of macrophages which contain bacteria

A second experiment was set up to examine the relationship between water quality and the bacterial load within the macrophages of the fish. Fish originating from farms of either good or poor water quality was maintained under laboratory conditions, some of which were placed in good quality water and some in poor water quality. Macrophages of the animals were examined for over a month to see if levels of resident bacteria increased or decreased when fish were place in water of differing quality.

One population of catfish was purchased from the 'poor' farm included in the Thai sampling survey above (Farm 2). Fish representing the 'good' farm were from stocks held at AAHRI. Fish from each group were placed for replicate tanks (60 x 90 x 60cm^3) (25 fish per tank), and were acclimated for 3 days before the start of the experiment. Water changes (50%) and excess food was removed every 2 days from two tanks of each group [fish from the good farm, maintained in good water quality ($GFGT_1$/$GFGT_2$) and fish from the poor farm, maintained in good water quality ($PFGT_1$, $PFGT_2$)]. The other set of fish was given 50% water changes every 7 days and excess food left in tank [fish from the good farm, maintained in poor water quality ($GFPT_1$/$GFPT_2$) and fish from the poor farm, maintained in poor water quality ($PFPT_1$, $PFPT_2$)].

Three fish were sampled from each tank on day 1, 2, 3, 5, 8, 12, 20, 28, macrophages from one of the head kidneys were isolated, as indicated in Appendix 3 and inspected for the presence of bacteria. The other anterior kidney was sampled onto TSA and AS plates. These were incubated

at 28^0 C, examined 24 to 48 h later and any bacterial growth recorded. A loop of macerated kidney suspension was also streaked directly onto TSA and AS plates.

The percentage of macrophages with bacteria was relatively constant in the good farm fish, when they were maintained in good water quality (Figure 2.16a). However, fish from the good farm, kept in water of poor quality had only slightly higher percentages of macrophages with bacteria by comparison (Figure 2.16a). In the latter, the percentage of macrophages with bacteria fluctuated between sample days.

A similar percentage of macrophages with bacteria were found in all fish sampled from the poor farm on day 1 to 5, but after this the percentage of cells with bacteria started to increase (Figure 2.16b). The fish kept in the poor water conditions generally had a higher percentage of macrophages, which contained bacteria, compared with the fish kept in the good water conditions. These levels were similar over the first 4 days of the trial; however, levels started to increase after this time in fish originating from the poor farm. It was anticipated that fish from the 'poor' farm would have higher levels of macrophages containing bacteria compared with fish from the 'good' site, due to higher speculated level of bacteria in the water of the 'poor' farm. Unfortunately, bacterial counts were not determined at the start of the experiment for the site where the fish originated.

The percentage of macrophages with bacteria fluctuated in fish maintained in both the good and the poor tanks over the course of the trial, and these levels did not appear to be influenced by the water conditions the fish were maintained in during the experiment. What is important is the fish from the 'good' farm generally had lower levels of macrophages with bacteria lower compared with fish from the poor site, throughout the course of the trial. Possibly the macrophages of these fish were more readily stimulated on exposure to higher levels of bacteria, and were thus able to ingest and kill bacteria present in the experimental tank. Macrophages from fish of the poor site did not appear as able to cope with the higher level of bacteria present in the water, possible because their killing ability was reduced due to their constant exposure to high bacteria levels on the farms at which they were previously held.

Viable bacterial counts were only determined for the tank water on days 12 and 20 (Table 2.29), and higher levels were found on day 12 compared with day 20 (Table 2.29). Unfortunately the lack of water quality data meant that statistical analysis of the relationship between water quality and bacterial level within the macrophages was limited. It was too expensive for the Thai's to analyse the large amount of water quality analysis generated in this experiment. However, when the

percentage of macrophages with bacteria and the viable CFU of the water were analysed using a Mann-Whitney Sum Rank Test, there was a non-significant trend for the PFGT1 and PFGT2 to have more bacteria in the water and fewer macrophages contained visible bacteria ($P = 0.343$). No relationship was established between the bacterial counts in the water and the percentage macrophages with bacteria, but increased levels of bacteria were observed in the macrophages. It may be that the homeostasis of the fish was disturbed, due to changes in the water quality experience here compared with the farm site.

Bacteria recovered from tank water, head kidney and macrophage suspensions were speciated using primary and secondary biochemical bacteriology tests. A range of bacterial species was identified from the recovered bacteria (Table 2.30). Not all fish sampled gave bacterial growth from their head kidney or their macrophage suspension. However, where there was bacterial growth, a variety of different bacterial species were identified, although only to genus level. It was interesting to note that no bacterial growth was obtained from fish sampled from tanks GFGT2 of PFGT2 and fish sampled from these tanks had very low percentages of macrophages with visible bacteria throughout the experimental period.

Some fish died over the course of the experiment, and bacteria, which grew from liver swabs of the dead animals, were identified as *Aeromonas* spp. Dead fish frequently had extensive lesions to their body surface, possibly due to fighting and this may have increased their susceptibility to infection. More dead fish were recovered from animals obtained from the 'poor' farm and kept in poor water conditions, compared with fish of other tanks. Bacteria were recovered from head kidney of many fish, especially fish from tanks GFPT2, PFPT1 and PFPT2. Bacterial growth was obtained from macrophage suspensions plated directly on to TSA from fish of tanks GFGT1 and PFGT1. The percentage of macrophages with bacteria, in fish from both of these tanks was higher than compared with the fish from GFGT2 and PFGT2. This may have reflected the number of live bacteria present in the macerated cell suspension not yet killed by the macrophages, and hence were able to grow on the TSA plates.

Future work should focus on the viability of the internalised bacteria visible within the macrophages. This would provide insight into the basis of the bacteria within the macrophages (where they are simply being cleared from the fish, or whether they are dormant within the cells). It would also help to establish if the macrophages are able to cope with high bacterial loads they may encounter within their aquatic environment and whether the activity of macrophages is stimulated but the bacteria, or in some way comprised.

CanTho University

The Vietnamese research team also completed two laboratory experiments, similar to the studies undertaken in Thailand, Ms. Crumlish, on one of her visits to CanTho. The first experiment investigated the effect of stress on disease susceptibility in farmed catfish under laboratory conditions. However, the only stressor applied was that of removing fish from water for 2 h then challenge them with *A. hydrophila*. The highest level of mortalities occurred in fish subjected to stress and bacteria (40%); with fish administered only bacterial or stress having morality rate of 32% and 28% respectively.

The percentage of cumulative mortalities and the day at which mortalities ceased are presented in Table 2.31. No significant difference was found in the percentage mortalities between any of the treated groups, ($?^2_1$ = 0.802, P = 0.370). Unfortunately, the bacteriology laboratory at CanTho did not have the expertise to identify the bacterial species recovered from the liver of dead fish. However, samples grew on TSA and colonies were small and cream coloured, similar to the appearance T_4 colonies. No significant difference was found in the percentage mortalities between experimental groups. No untreated control group was included in the experiment and fish were given insufficient acclimation time to their new environment.

A second experiment, in which the influence of water quality on the bacterial load of fish macrophages was examined. The results of this study were not available in time to be included in this report, but will be analysed at a later date. The research team has collected all the necessary water analysis for this experiment.

2.2.1 Dissemination of Results

IOA

In the first quarter of the project Dr. Inglis and Ms. Crumlish visited Vietnam and Thailand to meet the collaborating research teams and to discuss the goals of the project. Ms. Crumlish provided training in field sampling techniques during this time for the relevant research staff at AARHI and CanTho University. The techniques included basic bacteriology and immunology, which were incorporated into the sampling regimes of the survey. These techniques are now routinely practised within the collaborating research institutes. SOPs for the sampling regime, set up by Ms. Crumlish at the IOA, were introduced during the training sections.

AARHI and CanTho University

The regional workshops intended for Phase I of DFID project R7054 have been rescheduled for Phase 2 of the project. The workshops will provide training in field sampling techniques and in the assessment of the health status of farmed fish.

3. Contribution of outputs to project goal:

The project purpose was to improve production in fresh-water aquaculture through the control of clinical bacterial disease and subclinical infection. The incidence of bacteria within macrophages was firmly established, and although levels were generally low, most macrophage preparations examined contained bacteria. Both highly significant positive and negative correlations were found between bacterial counts in the water, water quality and the level of macrophages with bacteria, but no signal water parameter could be identified which continuously influencing the level of bacteria within the cells. Preliminary assessment using laboratory-based studies suggested that fish did succumb to opportunistic infections when subjected to stressful events. What was clearly highlighted from the study was the lack of baseline information relating to the occurrence and severity of bacterial disease outbreaks in either Thailand or Vietnam, and their impact on rural Aquaculture in South East Asia, and without which effective control strategies are hampered.

4. Publications:

A number of papers have been prepared relating to the work conducted in DFID project R7054.

(1) Sampling for Macrophage Cells at the Pond Side by Ms. Crumlish was published in the AAHRI newsletter (July 1998).

(2) Development of a sampling method for isolation of head kidney macrophages at the pond-side has been submitted to Journal of Fish Diseases.

(3) A literature review entitled *Intracellular Fish Pathogens* is in preparation for submission to Journal of Fish Diseases, a preliminary draft of which is included here (Appendix 4).

(4) "Intracellular pathogens in fish disease" is in preparation for submission to the Indian Association of Microbiologists.

Further publication will be prepared from the field data and the experimental studies

5. Internal reports:

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Valerie Inglis and Ms. Margaret Crumlish
First Quarterly Report
January 1998 - March 1998

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Kim Thompson and Ms. Margaret Crumlish
Second Quarterly Report
April 1998 - June 1998

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Kim Thompson and Ms. Margaret Crumlish
Third Quarterly Report
July 1998 - September 1998

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Kim Thompson and Ms. Margaret Crumlish
Fourth Quarterly Report
October 1998 - December 1998

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Kim Thompson and Ms. Margaret Crumlish
First Quarterly Report
January1999 - March 1999

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish
Dr. Kim Thompson and Ms. Margaret Crumlish
Annual Report
December 1998 - March 1999

6. Other dissemination of results:

Ms. Crumlish presented an oral paper at the Fifth Asian Fisheries Forum, Chaing Mai, Thailand, in November 1998, entitled *Observations on the Macrophage Cell Characteristics from Fish in Various Farming Systems*

7. Follow-up indicated/planned:

2.2.2 Phase 2

IOA

A concept note entitled *Control of Bacterial Disease in Small-Scale Fresh Water Aquaculture* was submitted and approved by DFID and is a continuation of DFID project (R7054). The project memorandum is recently been submitted.

8. Authors of this report:

DR K.D. THOMPSON

MS. MARGARET CRUMLISH

EXECUTIVE SUMMARY

Fresh-water aquaculture is a valuable contributor to nutritional and income demands of rural and peri-urban populations within developing countries. Continuous low level losses from either opportunistic or facultative bacterial disease outbreaks not only affect production but are economically devastating for rural farmers. Sustainable Aquaculture requires effective management of the local ecosystem to achieve optimal productivity.

The purpose of this project was to improve production in fresh-water Aquaculture through the control of clinical bacterial disease and subclinical infection. The work represents an 18-month study to examine the relationship between environmental conditions, bacterial load in the water and levels of tissue macrophages with bacteria isolated from a range of clinically healthy freshwater fish species, farmed in a range of culture systems in Thailand and Vietnam. A field-based sampling technique was developed to provide a quick and simple method for isolating macrophages at the pond-side.

The sites chosen in Thailand were all catfish farms to allow comparison between the same fish species cultured in different farming systems. Most fish farms located around Bangkok are intensive rather than extensive farming systems, and are large commercial enterprises compared with the extensive systems seen in rural Aquaculture in South East Asia. There appeared to be little difference between water quality and productivity in the farms chosen, with similar environmental conditions seen at all four sites. The water quality followed very similar trends between farms throughout the wet season, but larger variations in measured parameters during the dry season. Both highly significant positive and negative correlations were found between bacterial counts in the water, water quality and the level of macrophages with bacteria, but no signal water parameter could be identified which continuously influencing the level of bacteria within the cells.

A greater choice of farming systems and cultured species were available around CanTho, Vietnam, compared with Bangkok and a range of freshwater fish species, farmed in a variety of culture systems was, therefore examined in Vietnam. Both positive and negative significant correlations were found between bacterial counts in the water, water quality and the percentage of macrophages with bacteria. Again there did not appear to be a trend to this pattern between the four sites sampled.

The incidence of bacteria within macrophages was firmly established, and although levels were generally low, most macrophage preparations examined contained bacteria. Fish cultured in these systems co-exist with the systemic bacteria, appearing to be free from disease, though

implications for incipient disease are not clear. It remains to be established if bacteria present in the macrophages are simply due to natural bacterial clearance, or whether in fact the presence of the bacteria immuno-compromised the animals to the point where they easily succumb to opportunistic infections. Many of the bacteria were opportunistic pathogens, and as such may pre-dispose the animal to disease. The clinical significance of the macrophage bacterial load still remains to be determined, although preliminary assessment using laboratory based studies suggested that fish did succumb to opportunistic infections under stress. If this link is established, their presence may be used as an indicator to predict potential disease outbreaks.

What was clearly highlighted in the study the lack of baseline information relating to the occurrence and severity of bacterial disease outbreaks in either Thailand or Vietnam, and the impact they have on rural Aquaculture in South East Asia. The frequency and extent of disease outbreaks and etiological agents involved are generally unknown. Such information is vital if bacterial diseases are to be controlled effectively in the region.

ABBREVIATIONS

As:	*Aeromonas* selective medium
AAHRI:	Aquatic Animal Health Research Institute
CFU:	colony-forming units
CO_2:	carbon dioxide
COD:	chemical oxygen demand
DFID:	Department for International Development
DO:	dissolved oxygen
EUS:	Epizootic ulcerative syndrome
IOA:	Institute of Aquaculture
IOSS:	organic suspended solids
n/a	no answer
NO_2:	Nitrite
NO_3:	Nitrate
ODA:	Overseas Development Agency
OSS:	organic suspended solids
SOP:	standard operating procedures
TN:	total nitrogen
TP:	total phosphorous
TSA:	tryptone soya agar
TSS:	total suspended solids

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Appendix 1: Questionnaire

Bacterial Disease in Fresh-water Aquaculture: Stirling - CanTho Link.

1. Date: Month .... Day .... Year ....

SITE

Farmer's name: ....

2. Province: .... District: .... Village: ....

3. Single Family Unit? ☐

Large Family Complex? ☐

Company Owned? ☐

4. Monoculture: ☐

Polyculture:
- ?? With animals. ☐
- ?? With animals and non-rice crops. ☐
- ?? With animals and rice. ☐
- ?? Informal e.g. garden pond. ☐

PRODUCTION

5. Number of ponds: ☐

Total number of cages on farm (if appropriate): ☐

Are fish produced for:
- ?? Own needs? ☐
- ?? Market? ☐

6. Estimate yearly production in Kilos (or Dong):

FISH

7. Buy seed and grow-on? ☐

Produce own? ☐

Species of fish cultured?...

8. Feeding:

	Always	Usually	Sometimes	Never
?? Commercial feed pellets				
?? Trash fish				
?? Trash animal waste				
?? Supplements used				

Others Please Specify ....

DISEASE

9 Number of disease outbreaks in the last 12 months. [ ]

Losses – estimate percentage e.g. 10-30%. [ ]

Cause(s) of death:...

10. Confirmed by a laboratory?

Yes	No

11. Months when disease(s) are most likely to occur? ....

11. Treatments used:

	Always	Usually	Sometimes	Never
?? Traditional.				
?? Chemical.				
?? Antibiotics				

Give brief details of each, if possible:...

11. Preventative routine:

	Always	Usually	Sometimes	Never
?? Traditional.				
?? Chemicals.				
?? Drugs.				
?? Vaccines.				

Give brief details of each, if possible:...

SUBJECTIVE, IMPRESSION OF DATA COLLECTOR.

14. Environment of farm. Good 1 2 3 4 5 Bad

15. Water quality. Good 1 2 3 4 5 Bad

Distance from laboratory (in hours) [ ]

Code number to match results for bacterial water count and fish studies:..............................

Data Collector: ....

Appendix 2: Experimental data collected during DFID project R7054

Table 2.1: Hybrid catfish (*Clarias gariepinus* x C. *batrachus*) farms selected by AARHI

Farm N^{o}	Farmer	Place	Grade
1	Mr Supote	Lumsai District, Patumthani Province	Good
2	Mr Pone	Suanprikthai District, Patumthani Province	Poor
3	Mr Prapas	Kokcotoa District, Supanburi Province	Intermediate
4	Mr Phaitoon	SumutPrakarn Province	Intermediate

Aquatic Animal Health Research Institute (AAHRI), Bangkok, Thailand

Table 2.2: Production data for selected Thai farms

Farm	Unit	Culture	N^{0} Ponds	Production Outlet	Annual Production	N^{0} Fingerlings (m^{2})
1	Single Family	Mono	14	Market	20 tons	36
2	Single Family	Mono	2	Market/Family	4 tons	22
3	Single Family	Poly*	6	Market	4 tons/crop	20-23
4	Single Family	Mono	2	Market	4.5 tons	33-48

* Farm also produces chickens and Nile tilapia

Table 2.3: Feeding Regimes

Farm	Seed	Diet				
		Commercial Pellets	Trash Fish	Trash Animal Waste	Supplement	Comments
1	Own	Sometimes	Never	Always	Never	Pellets until fish were 20 days old, then switched to chicken waste
2	Bought	Sometimes	Never	Never	Never	Pellets first month only
3	Bought	Sometimes	Never	Always	Never	Pellets first month only
4	Bought	Usually	n/a	Usually	Sometimes	Occasionally fed bread

n/a = no answer given

Table 2.4a: Disease outbreaks on Thai farms

Farm	N° of disease outbreaks	Losses (%)	Cause of Death	Diagnosis	Time
1	n/a	n/a	jaundice disease[1]	farmer	fish 4 months old
2	1	n/a	digestive problems [2]	farmer	January
3	1	50%	swollen abdomen, back ulcers, necrotic tissue at the mouth	farmer	January
4	2	10	fin rot, gill swelling	farmer/ laboratory	wet season

[1]This was actually described by the farmer as a yellow body colour

[2] Farmer described clinical signs associated with digestive problems

n/a: no answer

Table 2.4b: Treatments by Thai farmers

Farm	Treatment		
	Traditional	Chemical	Antibiotic
1	Always	Never	Never
2	Sometimes	Never	Never
3[1]	Always	Usually	Sometimes
4[2]	n/a	Sometimes	Sometimes

Farm	Preventative Treatment			
	Traditional	Chemical	Drugs	Vaccine
1	Usually	Never	Never	Never
2	Never	Never	Never	Never
3	Always	Sometimes	Never	Never
4	Never	Never	Never	Never

[1] Farmer occasionally applied formalin when disease problems occur

[2] Farmer applied chemical, mostly seolite (lime), and gave antibiotics during wet season

n/a: no answer

Table 2.5a Background information from initial farm visits in Vietnam

Farm	Area	Unit/Culture	Pond/Cage N^{o}	Production outlet	Annual production	Species farmed	Seed
1	Omon district, CanTho Province	Large Family/Mono	3	Market	20 million VND	Sandgoby	Bought
2	Omon district, CanTho Province	Large Family/Mono	2	Market	20kg	Sandgoby	Bought
3	Chau Thang district, CanTho Province	Large Family/Mono	n/a	Market	5-6 million VND	n/a[1]	Bought
4	Cahu Thanh district, CanTho Province	Large Family /Mono	5	Market	n/a	Giant gourmai	Own
5	CanTho Province	Single family /Mono	1	Market	n/a	n/a[1]	Bought

[1] The farmer was either uncertain of the fish species as there was a mixture of fish included in the pond, or no information was given because the farmer frequently changed species.

n/a: no answer; VND: Vietnamese Dung

Table 2.5b: Disease outbreaks and treatments from farms initially visited Vietnam

Farm	Disease outbreaks/ Losses (%)	Cause/ Diagnosis*	Treatments	Prevention	Comment
1	3 in last 12 months, lost 70-80% stock	poor water quality/ farmer	antibiotics always, traditional or chemical sometimes	Always drugs, traditional or chemicals sometimes	Farm judged as poor, with bad water quality
2	1 or 2 in Jan-Feb, lost 5% stock	Red Spot Disease/ farmer	never antibiotics, traditional or chemical sometimes	salt sometimes	intermediate
3	n/a lost 7% stock	broken head, swollen kidney/ farmer	antibiotics sometimes	sometimes drugs	intermediate to poor
4	n/a lost 20-30% stock	respiratory, gill problems/ laboratory, Farmer	antibiotics sometimes	sometimes drugs	intermediate to good
5	none, 3 years before lost 5% stock	EUS during Nov-Dec/ farmer	always antibiotics	usually drugs	intermediate to poor

*Disease diagnosed from clinical signs by farmer, or by laboratory. n/a: no answer

Table 2.6: Farm selected by CanTho University for repeated sampling visits

Farm N^{o}	Farm Type	Location	Grade
1	polyculture pond	Chau Thanh village, CanTho Province	Good/intermediate
2	monoculture pond	Thanh Quoi village, CanTho Province	Intermediate/poor
3	small cage	Dong Thap Province	Intermediate/poor
4	large cage	Chau Doc Province	Good

Table 2.7 Information on the four farm sites selected by the Vietnamese for monitoring

Farm	Facility	Culture	Pond/cage N^{o}	Annual production	Seed	Species cultured	Market
1	Large Family Complex	Poly	1	n/a	Bought	Indian carp, tilapia spp. kissing gourmai	local
2	Large Family Complex	Mono	2	n/a	Bought	Snakehead	self/local
3	Large Family Complex	Mono	2	n/a	Own	Sandgoby	local
4	[1]Company Owned	Mono	2	n/a	Own	*Pangasius bocourti*	loacl

[1] may be an interpretation error, since these cages are normally owned by Government or farmers receive a Government loan

n/a: not answered

Table 2.8 Feeding regimes, disease outbreaks, treatments and preventative measures used at Vietnamese sampling sites

Farm	Feeding	Diseases /Losses	Outbreaks	Diagnosis	Treatment	Prevention
1	usually vegetables	none	none	none	never	never
2	usually trash fish	EUS/ 5%	September	farmer/ laboratory	sometimes chemicals and antibiotics	[1]sometimes vaccines
3	usually trash fish	none	none	none	sometimes antibiotics	[1]sometimes chemicals/ vaccines
4[2]	usually trash fish	none	none	none	sometimes antibiotics	[1]sometimes drugs/ vaccines

[1] No additional information available on vaccines administered

[2] Antibiotics (flumequine) administered prophylactically

Water quality results from Thailand

Farm 1 (Good farm)

This site was sampling on 2.12.98; 6.1.99; 4.2.99 and 23.2.99 during the second cycle of the survey[2]. The temperature was relatively stable throughout this time, with a slight decrease in January and early February. The pH was also fairly stable throughout this period. Alkalinity was high in December and January and fluctuated from low to high in early February and late February, respectively (Figure 2.1b). The nitrite levels were all low and of a similar level, but the ammonia levels fluctuated dramatically between the different sampling times, with the highest level recorded in January and the lowest in February (Figure 2.2b and 2.3b). The turbidity of the water was similar throughout the cycle (Figure 2.5b) and TSS were also stable throughout, with the lowest value recorded in late February (Figure 2.6b). The bacterial counts of the water increased from January to late February (Figure 2.6b).

Farm 2 (Poor farm)

This site was sampling on 29.12.98 and 13.1.99. There were only two sampling, however, as the farmer harvested his fish after the second visit. The temperature decreased by 3^0C from December to January, the alkalinity of the water was higher in January (Figure 2.1b), but the pH and TSS (Figure 2.5b) of the water were similar between sampling points. Nitrite levels were low and stable between the samplings (Figure 2.3b), but the ammonia levels fluctuated dramatically, with high levels recorded in December and very low levels in January (Figure 2.2b). Water turbidity was also low in January (Figure 2.4b). There was no real variation between the TSA or As counts between the two sampling times (Figure 2.6b).

Farm 3 (Intermediate farm)

This farm was visited on 16.2.98, 20.1.99, 17.2.99 and 3.3.99 for second cycle sampling. The temperature increased slightly from December to March (0.5 - 1^0C). Water alkalinity was high in all samples taken (Figure 2.1b), but the pH was around 7 throughout the cycle. The TSS was also high, but similar between the four sample times (Figure 2.5b). The nitrite (Figure 2.3b) and ammonia levels (Figure 2.2b) increased in February and March, while and the water turbidity was highest in February and lower

[2] Dates of first cycle sampling are not available for this report.

again by March. The bacterial counts in the water samples increased from December to march with the highest levels recorded on As media in March. (Figure 2.6b)

Farm 4

(Intermediate farm)

Samples were taken on 23.12.98; 27.1.99; 10.2.99 and 25.2.99 t this location for the second cycle. The water temperature was similar throughout these times, and increased by only 1^0C from December to late February. Water alkalinity was high, but stable between the sampling period (Figure 2.1b), and the TSS of the water was high in both January and February (Figure 2.5b). Nitrite levels in the water were fairly stable, but lower in January compared with December and February (Figure 2.3b), while ammonia levels were low in January and early February, but higher in December and late February (Figure 2.2b). The turbidity measurement was similar over the four samplings, except in late February where it was very low (Figure 2.4b). The level of CFU obtained on TSA increased in January, but were reduced again by February (Figure 2.6b). However, the As counts increased in January and remained high into February.

Alkalinity of the water was determined for all four farms at each sampling point for both sampling cycles. In cycle 1, the alkalinity of the water was found to be similar between the four farms, except for Mr. Prapas' farm (farm 3). By the four sampling of the first cycle, the alkalinity of the water at this site was almost double that of the other three farms (Figure 2.1a). These levels were still high in the second cycle, and the levels measured at the other three farms were generally higher than found during the first cycle. This was particularly evident at the fourth sampling point of the second cycle (Figure 2.1b).

The level of ammonia in the water was similar at the four sites over the course of the first sampling cycle. The values obtained at the first and second sampling point were low for all four farms (Figure 2.2a). By the third sampling point, however, the level of ammonium in the water had started to increase, and by fourth sampling, the ammonia content of the water at Mr. Prapas' farm (Farm 3) was more than twice that measured at the other three sites (Figure 2.2a). Farm 3 had the lowest level of ammonia throughout the four sampling periods of the second cycle, when compared with the other three farms (Figure 2.2b). The ammonia levels of the water at the four farms appeared to fluctuate

dramatically over the course of the second sampling cycle (Figure 2.2b). The water on Mr Phaitoons' farm (Farm 4) had the highest ammonia level at the end of this cycle.

Nitrite levels were found to be low in the water of all four farms during the first sampling cycle (Figure 2.3a). The nitrite levels in the water were also low at the farms at the start of the second sampling cycle, but did start to rise by the second sampling point, with the highest levels of nitrite found at Mr. Phaitoons' farm (Farm 4)(Figure 2.3b).

The TSS in pond water, measured at each site, was found to be generally very low throughout cycle 1, with the exception of Mr. Supotes' farm (Farm 1) where very high TSS were recorded at the second sampling point of this cycle (Figure 2.4a). By the second cycle of sampling, the TSS values for all ponds were very low and remained low for the duration of cycle 2 (Figure 2.4b).

Low levels of water turbidity were found on the first 2 samplings of cycle 1 at all farm sites, but by the third sampling, the turbidity of water measured at Mr. Phone (Farm 2) and Mr. Phaitoons (Farm 4) farms' were relatively high compared with the other two sites (Figure 2.5a). However, in cycle 2 all of the farms had low turbidity levels, which remained similar throughout the sample points in cycle 2 (Figure 2.5b).

The viable bacterial counts of water from each site were determined as colony forming units (CFU) made on tryptone soya agar (TSA), and were prepared at the same time as the fish were sampled. Bacterial counts were low at all farm sites, throughout cycle 1 (Figure 2.6a). The viable bacterial counts of the water sampled during cycle 2 were low and did not differ between the four farms, except for Farm 2 in the first sampling of the cycle (Figure 2.6b). By the second sampling point, bacterial levels on this farm were reduced to levels similar to the other 3 farms (Figure 2.6b).

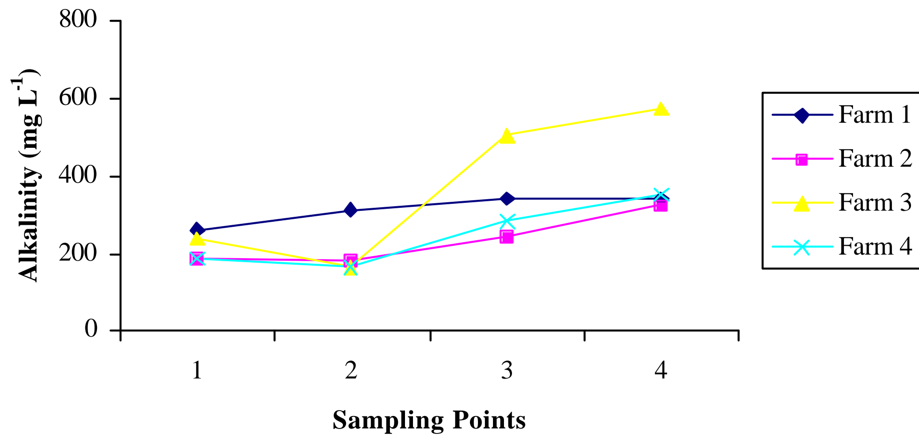

Figure 2.1a: Alkalinity of water on the four Thai farms during the first sampling cycle

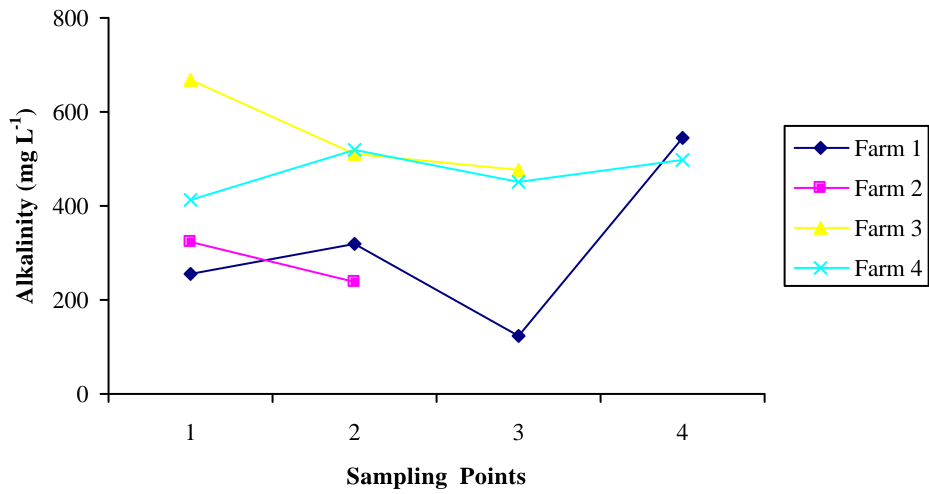

Figure2.1b: Alkalinity of water on the four Thai farms during the second sampling cycle

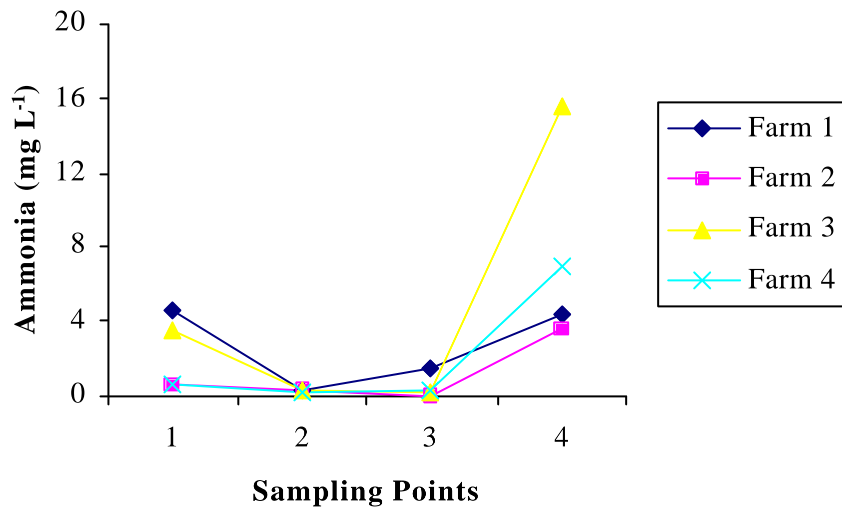

Figure 2.2a Ammonia levels in water on the four Thai farms during the first sampling cycle

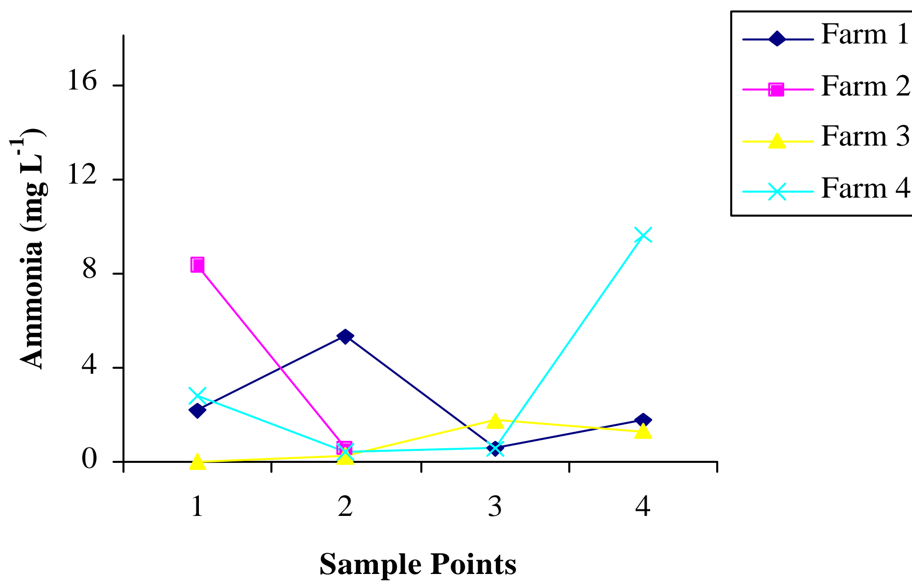

Figure 2.2b Ammonia levels in water on the four Thai farms during the second sampling cycle

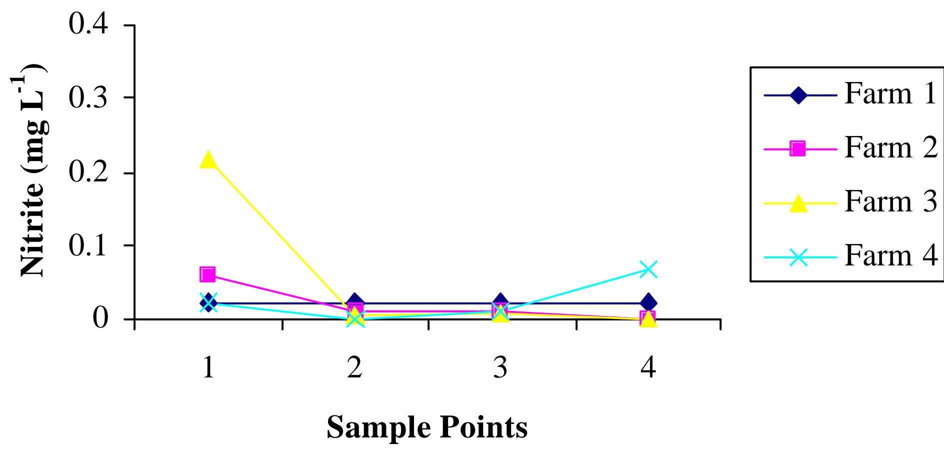

Figure 2.3a Nitrite levels in water on the four Thai farms during the first sampling cycle

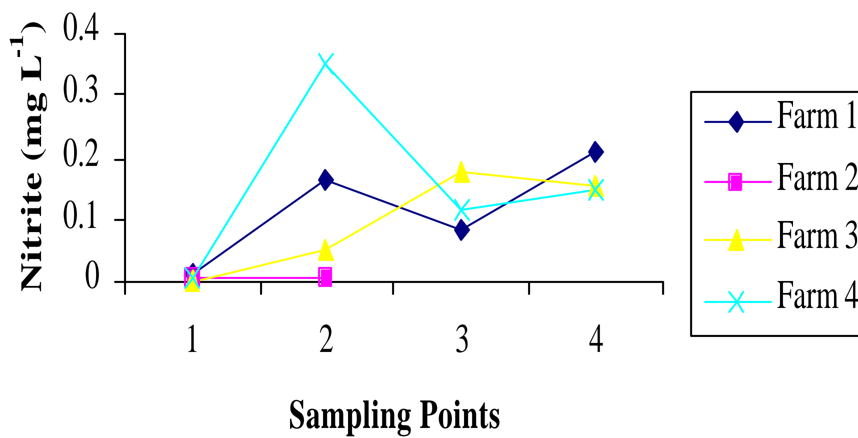

Figure2.3 b Nitrite levels in water on the four Thai farms during the second sampling cycle

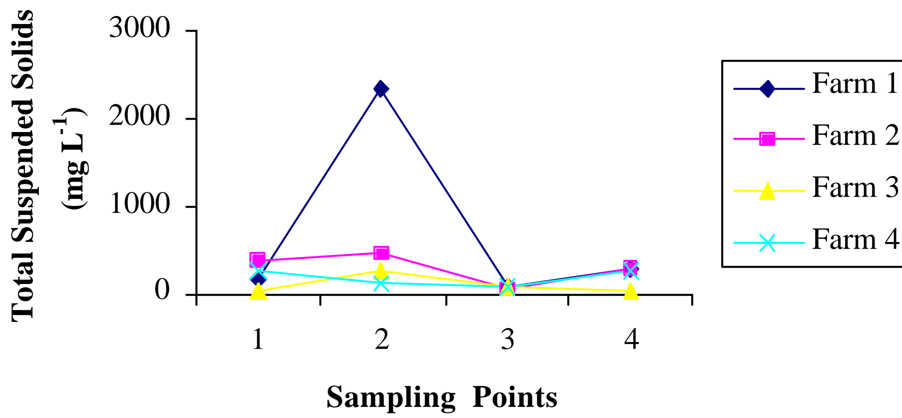

Figure 2.4a Total suspended solids in water on the four Thai farms during the first sampling cycle

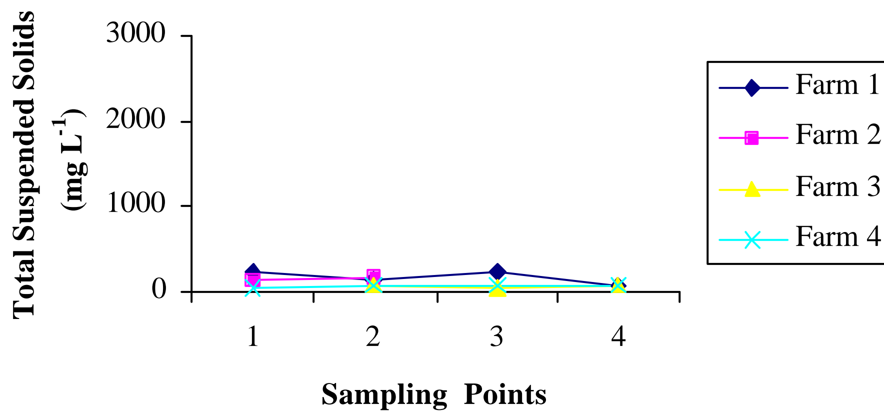

Figure 2.4b Total suspended solids in water on the four Thai farms during the second sampling cycle

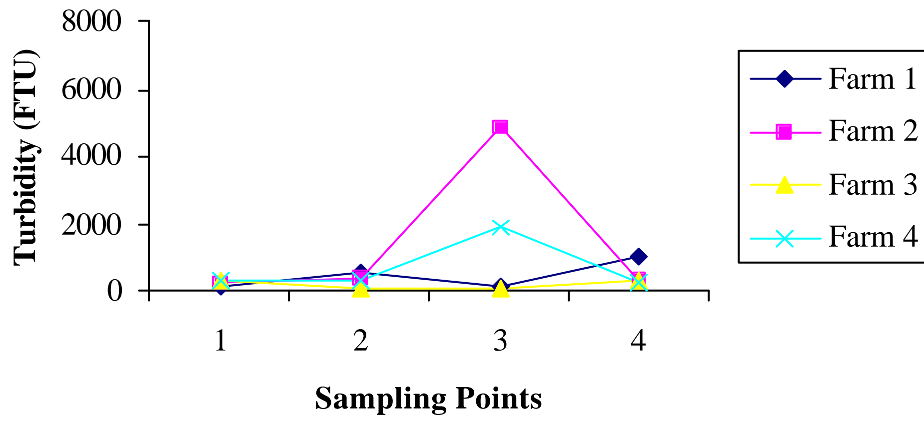

Figure 2.5a Water turbidity on the four Thai farms during the first sampling cycle

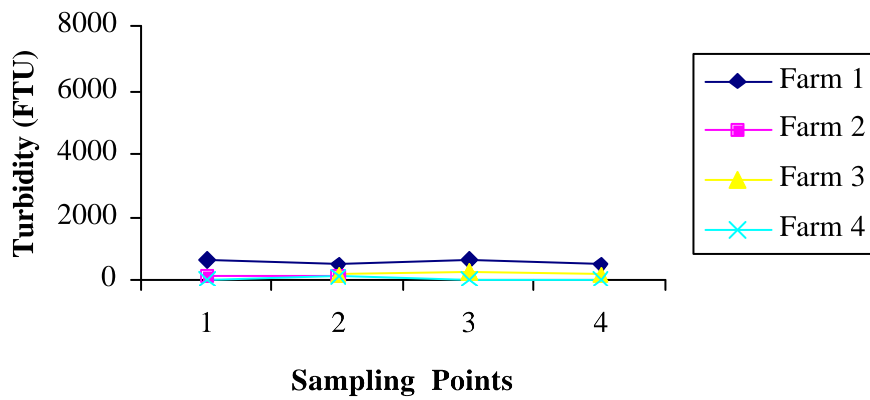

Figure2.5b Water turbidity on the four Thai farms during the second sampling cycle

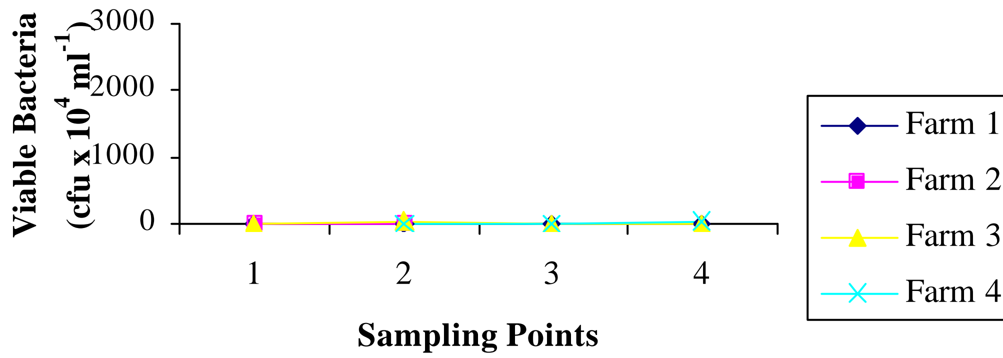

Figure2.6a Viable bacterial counts in the water of the four Thai farms during the first sampling cycle

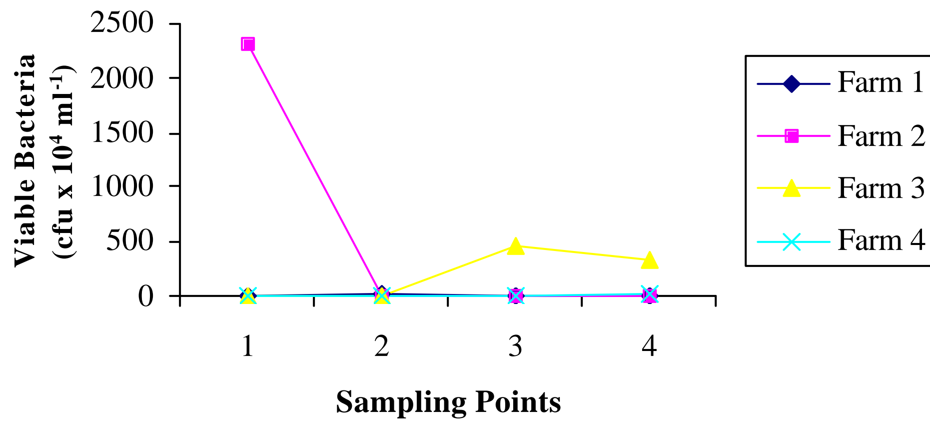

Figure 2.6b Viable bacterial counts in the water of the four Thai farms during the second sampling cycle

Water quality results from Vietnam

Farm 1 (Polyculture Farm)

The amount of available nutrients and primary production rate in this polyculture pond was low in all of the samples taken during cycle 2 (Figure 2.7). The chemical oxygen demand (COD) fluctuated throughout the sampling times whereas the DO or amount of available oxygen was relatively constant during cycle 2 (Figure 2.7).

The level of IOSS in the water samples taken from the polyculture farm were low (Figure 2.8), whereas the TSS and the OSS levels were similar and showed a similar trend (Figure 2.8a). There did not appear to be an inverse trend in the TSS and transparency levels (Figure 2.8b), and it may have been that other particles were obscuring the visibility in this pond rather than solely organic matter.

Farm 2 (Monoculture Farm)

In Figure 2.9, there was a low level of total nitrogen (TN) and total phosphorous (TP) found in the monoculture pond system. This indicated that there was a low level of available nutrients and a low rate of primary production potential (Figure 2.9). The amount of COD decreased slightly at sample point 2, which corresponded with a slight increase in TN and TP levels. The amount of DO decreased over time (Figure 2.9).

There was an obvious inverse trend in the amount of TSS (Figure 2.10b) and the level of transparency (Figure 2.10a). The amount of TSS appeared to most consist of OSS material rather than IOSS material as shown by the data presented in Figure 2.10b.

Farm 3 (Small Cages)

The DO and the COD had similar trends in the samples taken in cycle 2 for the small cage farm. The amount of DO was relatively stable throughout the sampling time, while TN and TP levels were low (Figure 2.11).

A similar trend was seen in TSS levels and the amount of OSS found in the water. The IOSS levels was very low throughout sampling compared with the amount of OSS found, suggesting that the TSS were predominantly OSS (Figure 2.12a). The amount of TSS found in the water was inversely related to the transparency levels as shown in Figure 2.12b.

Farm 4 (Large Cages)

The amount of primary production potential and available nutrients in the large cages was low, as indicated by the TP and TN levels (Figure 2.13). Although the COD was relatively stable throughout the sampling points in the large cages, there was an increase in the amount of DO at sample point 3, indicating that the cages were supersaturated with oxygen (Figure 2.13).

The amount of IOSS was very low compared with the OSS of the large cages (Figure 2.14a). There was a similar trend between the TSS and the OSS as shown in Figure 2.14a, which suggested that most of the TSS found in the large cages consisted of OSS material. There was a clear inverse relationship between the level of transparency (Figure 2.14b) and the amount of TSS in the large cages (Figure 2.14a).

Viable Bacterial Count in the Water

The amount of total bacterial colonies that were found in the water column in each of the four ponds was measured for cycle 1 (Figure 2.15a) and cycle 2 (Figure 2.15b). The bacterial counts were higher in all four farms in cycle 1 (Figure 2.15a) compared with cycle 2 (Figure 2.15b). The large cages had the lowest levels for both cycle 1 and 2, and while the polyculture farm had the highest level in cycle 1, the levels in the monoculture farm were highest in cycle two (Figure 2.15b). The level of bacteria in the water decreased over cycle 1 and by sampling point 4, all farms had low levels of bacteria in their ponds. Whereas, in cycle 2 there was an increase in bacterial colonies in the water, peaked in the monoculture and polyculture farms at sample point 2, but decreased after this time (Figure 2.15b).

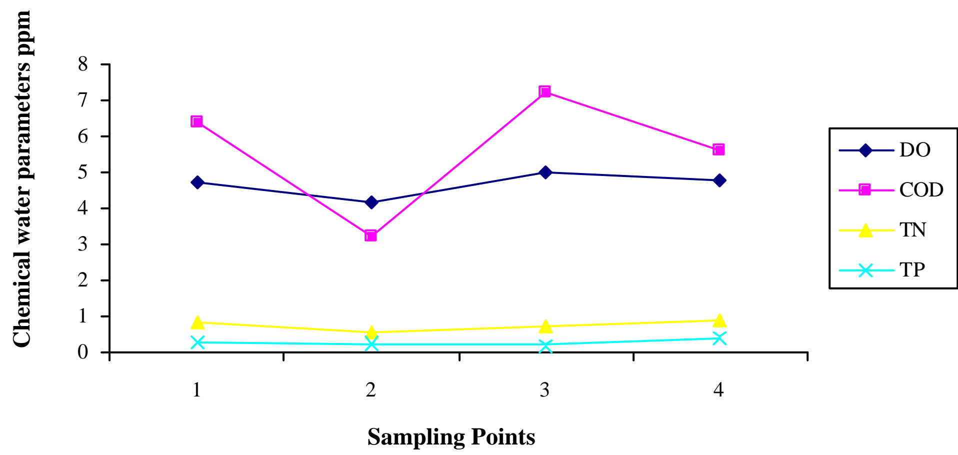

Figure 2.7 Chemical Water Parameters for Polyculture Farm, Cycle2, Vietnam

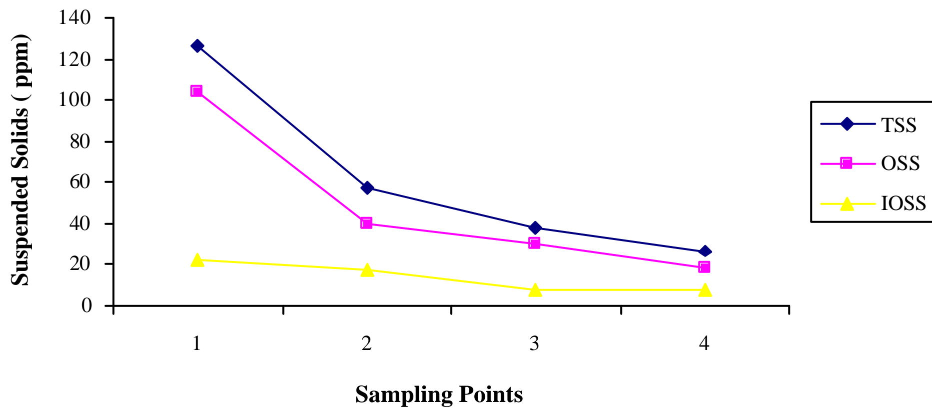

Figure 2. 8a Suspended Solids in water of Polyculture Farm, Cycle 2, Vietnam

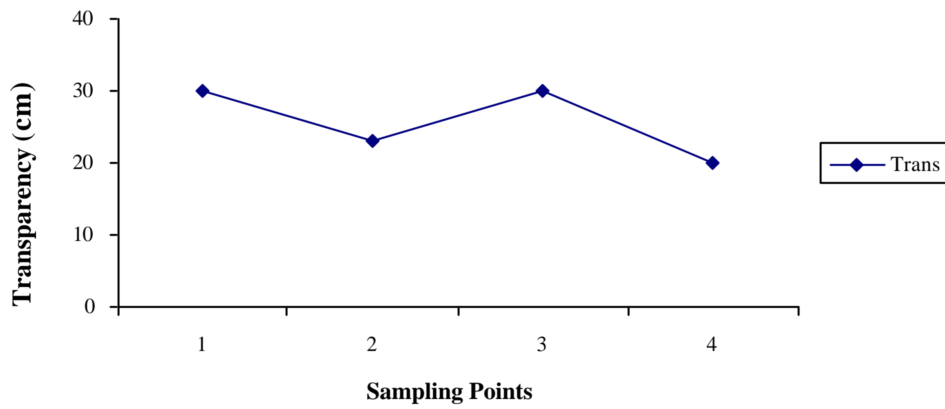

Figure 2.8b Transparency Level in water of Polyculture Farm, Cycle 2, Vietnam

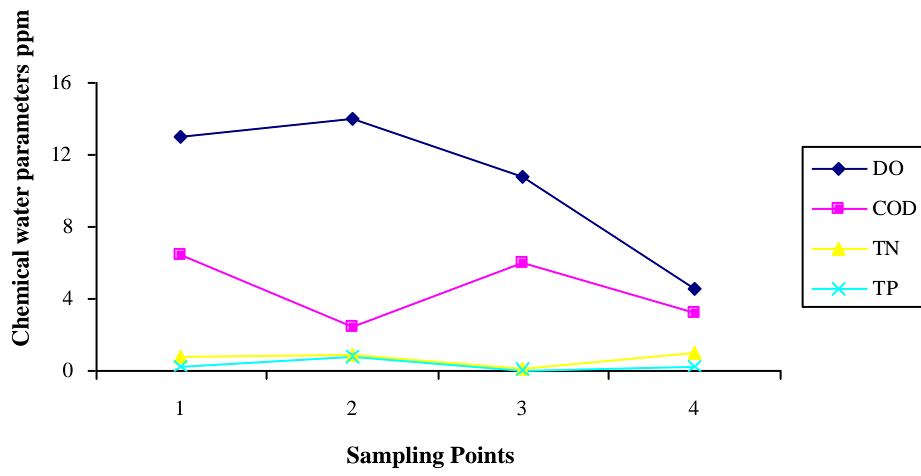

Figure 2.9 Chemical Water Parameters for the Monoculture Pond, Cycle 2, Vietnam

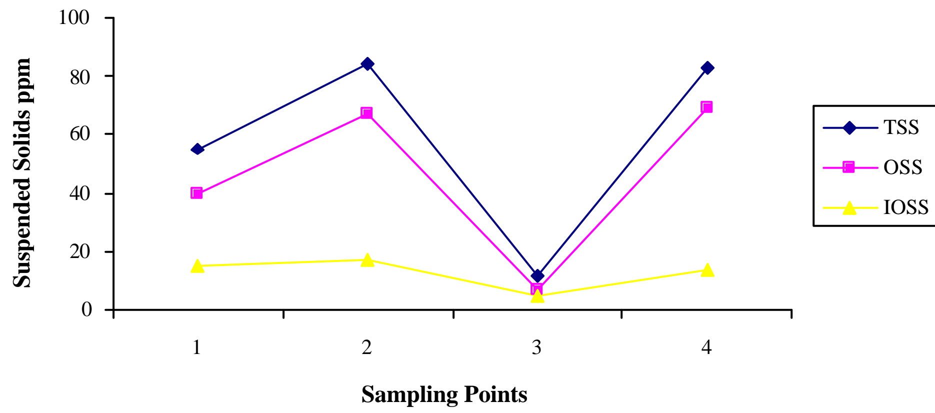

Figure 2.10a Total Suspended Solids in water of Monoculture Farm, Cycle 2, Vietnam

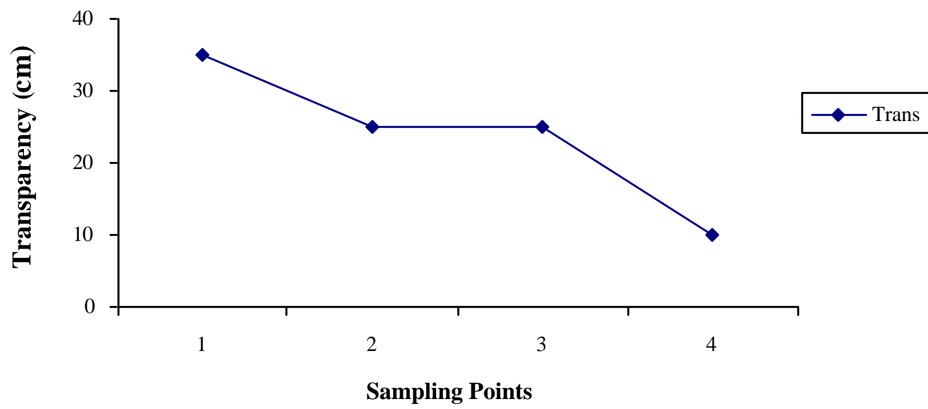

Figure 2.10b Transparency Level in water of Monoculture Pond, Cycle 2, Vietnam

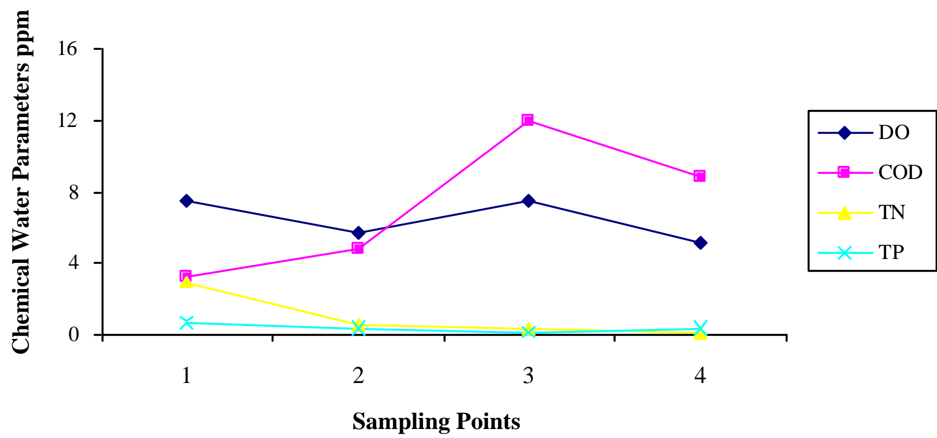

Figure 2.11 Chemical Water Quality in Small Cages, Cycle 2, Vietnam

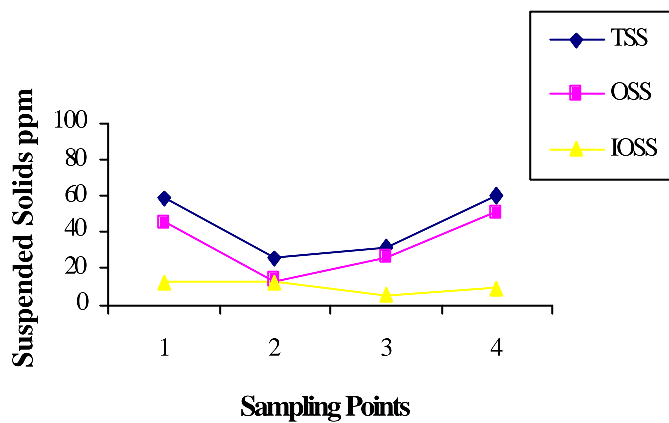

Figure 12.2a Amount of Suspended Solids ***in water of Small Cages,*** *Cycle 2, Vietnam*

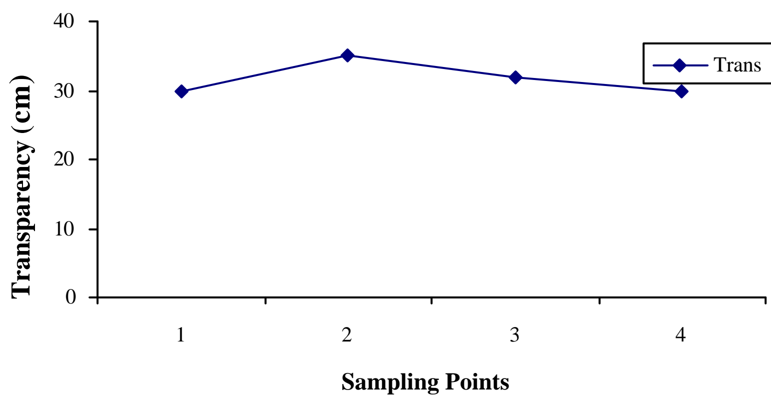

Figure 2.12b Transparency in water of Small Cages, Cycle 2, Vietnam

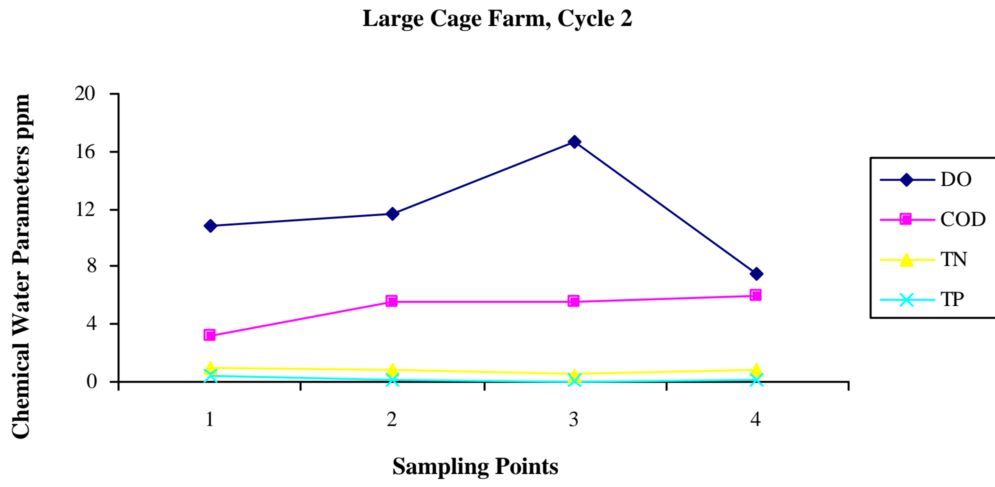

Figure 13 Chemical Water Parameters for Large Cages, Cycle 2, Vietnam

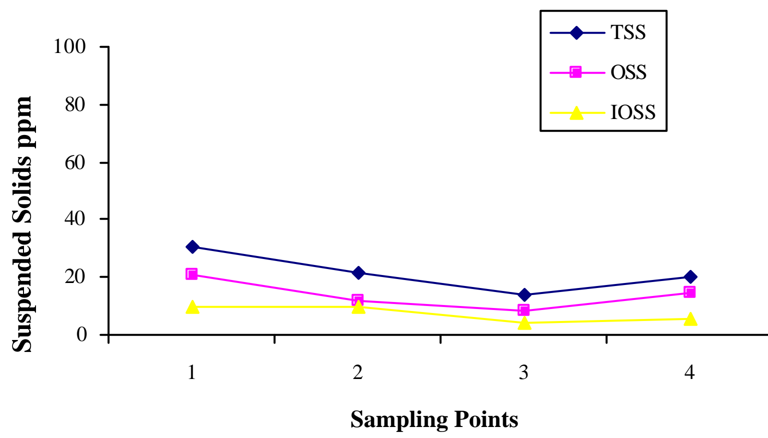

Figure 2.14a Amount of Suspended Solids ***in water of*** *Large cages, Cycle 2, Vietnam*

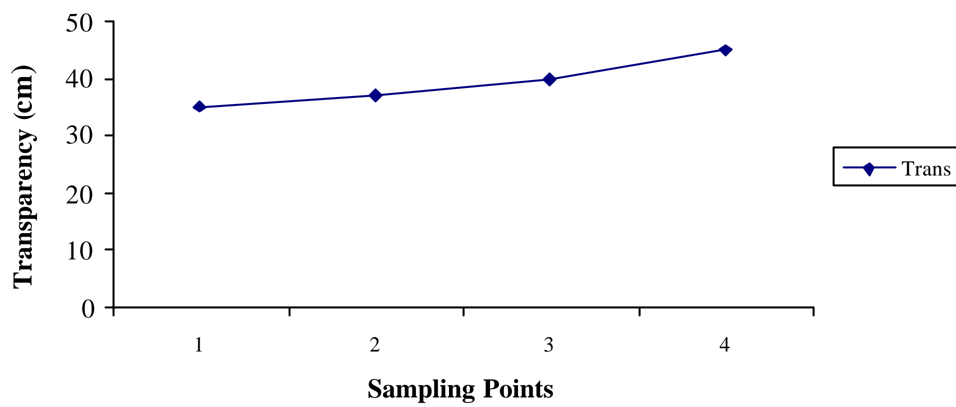

Figure 2.14b Transparency in water of Large Cages, Cycle 2, Vietnam

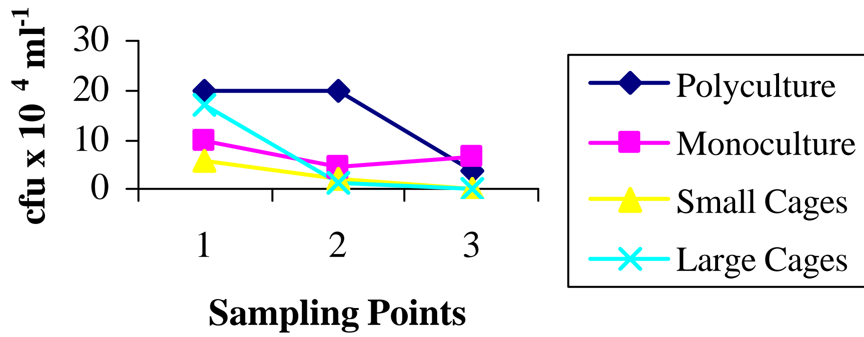

Figure 15a Total Viable Bacterial Counts in Water for Four Farms, Cycle 1 Vietnam

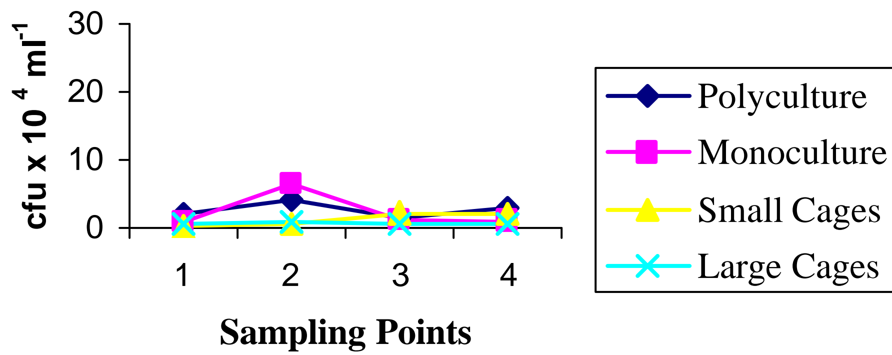

Figure 15b Total Viable Bacterial Counts in Water for Four Farms, Cycle 2, Vietnam

Table 2.9. Attached of catfish macrophages, sampled in Thailand using fibronectin

Sampling date	Sample Size (n)	[1]Mean N^0 ? SD		*P
		(+F)	(-F)	
Farm 1				0.187
May 98	6	25 ?7	25 ?7	
June 98	6	27 ?5	23 ?10	
July 98	6	33 ?15	24 ?16	
Sept. 98	6	26 ?12	24 ?11	
Farm 2				0.413
June 98	6	19 ?8	17 ?8	
Early August 98	6	14 ?3	13 ?3	
Late August 98	6	4 ?4	4 ?4	
Farm 3				0.348
May 98	6	29 ?4	28 ?4	
June 98	6	16 ?3	17 ?4	
July 98	6	14 ?10	15 ?14	
August 98	6	10 ?10	11 ?8	
Farm 4				0.452
June 98	6	22 ?6	22 ?3	
July 98	6	23 ?2	22 ?2	
August 98	6	17 ?6	16 ?4	
Sept. 98	6	13 ?5	15 ?5	

[1] Number of attached macrophages in 10 fields of view, average of two cycles per fish for six fish ? SD: standard deviation, +F: with fibronectin, -F: without fibronectin, *Significant at P? 0.05 using Student t test

Table 2.10 Attached of fish macrophages, sampled from Vietnamese farms using fibronectin

Sample date	Sample Size (n)	[1]Mean N^0 ? SD		*P
		(+F)	(-F)	
Farm 1 (Polyculture)				0.648
Early January 99	6	No data	4 ? 1	
Late January. 99	6	1 ? 0	2 ? 1	
March 99	6	6 ? 3	6 ? 2	
April 99	6	8 ? 11	8 ? 12	
Farm 2 (Monoculture)				0.218
December. 98	6	No data	3 ? 1	
January. 99	6	2 ? 2	3 ? 3	
March 99	6	4 ? 3	4 ? 2	
April 99	6	4 ? 3	2 ? 1	
Farm 3 (Small Cage)				0.442
December. 98	6	7 ? 14	8 ? 12	
January. 99	6	25 ? 13	5 ? 5	
March 99	6	16 ? 14	16 ? 15	
April 99	6	10 ? 4	10 ? 3	
Farm 4 (Large Cage)				0.306
December. 98	6	No data	1 ? 0	
January. 99	6	No data	8 ? 3	
Early March 99	6	7 ? 3	6 ? 3	
Late March 99	6	4 ? 2	2 ? 1	

[1] Number of attached macrophages in 10 fields of view, average of two cycles per fish for six fish ? SD: standard deviation, +F: with fibronectin, -F: without fibronectin, *Significant at P? 0.05 using Student t test

Relationship between bacterial level in macrophages, water quality and bacterial counts in the Thai ponds

Farm 1 (Mr Supote)

The percentage of macrophages, which had bacteria visible in their cytoplasm, is indicated in Table 2.11(cycle 1 and 2). No relationship was found between the number of viable bacteria in the water and the percentage of macrophages containing bacteria in cycle 1 (P = 0.866). However, a positive correlation was obtained between these variables in cycle 2, (P = 0.012).

No significant relationships were found between any of the water quality parameters measured and the percentage of macrophages containing bacteria in cycle 1 (Table 2.12a). In cycle 2, however, a statistically significant positive relationship was found between water temperature, TSS or water turbidity and the percentage of macrophages containing bacteria (Table 2.12b). A significant negative correlation was found between the percentage of macrophages with bacteria and the level of nitrites in the water (Table 2.12b).

Farm 2 (Mr Phone)

The percentage of macrophages, which contained bacteria, are shown in Table 2.13 (cycle 1 and 2). A positive correlation was found between the number of viable bacteria in the water and the percentage of macrophages with bacteria in their cytoplasm for both cycle 1 (P = 0.024) and cycle 2 (P = 0.003).

A significant relationship was found in cycle 1 between some of the water quality parameters measured at this site and the percentage of macrophages containing bacteria (Table 2.14). A significantly negative correlation was found between the percentage of macrophages with bacteria and the amount of alkalinity and ammonia in the water, while a significantly positive correlation was detected in the level of nitrates and the percentage of macrophages with bacteria (Table 2.14). In cycle 2, water temperature, pH, alkalinity, ammonia, nitrate and turbidity all had a significantly positive correlation with the percentage of macrophages with bacteria (P = 0.003), whereas a significantly negative correlation was found between the TSS (P = 0.003).

Farm 3 (Mr Phaitoon)

The viable bacterial counts from the water samples and the percentage of macrophages with bacteria visible when sampled from the head kidney of the fish were calculated for cycle 1 and 2 (Table 2.15). A negative correlation was found between the bacterial counts in the water and the percentage of macrophages with bacteria for cycle 1 samples taken at farm 3, ($P= 0.021$). However, no significant relationship was detected in the cycle 2 samples ($P = 0.058$).

No significant relationships were found between the percentage of macrophages with bacteria and the water quality samples taken during cycle 1 (Table 2.16a). In cycle 2, however, a significantly positive relationship was found between the percentage of macrophages with bacteria and water temperature, and ammonia and nitrates levels (Table 2.16b). A highly significant positive correlation was also found between TSS and the percentage of macrophages with bacteria (Table 2.16b), but a highly significant negative correlation with turbidity (Table 2.16b).

Farm 4 (Mr Prapas)

The percentage of macrophages with bacteria in their cytoplasm and the total viable bacterial counts from the water are shown in Table 2.17 (cycle 1 and 2) during visits to Farm 4. No significant relationships were found in cycle 1 ($P = 0.141$) or cycle 2 ($P = 0.928$) between these parameters when analysed

At Farm 4, no significant relationships were found between the percentage of macrophages with bacteria and the water quality parameters measured in cycle 1 or 2 (Table 2.18).

Table 2.11a: Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 1 during first sampling cycle in Thailand

Date	[1](+F)		[1](-F)		CFU (x10^4 ml^{-1})	
	% Cells	Range	% Cells	Range	TSA	AS
May 98	0.5 ?0.5	0 – 1.25	0.2 ?0.4	0 - 1	1.7	0.5
June 98	0.3 ?0.3	0 – 0.75	0.1 ?0.2	0 - 0.5	230	no data
July 98	0.4 ?0.3	0 – 1	0.2 ?0.1	0 - 0.25	6.5	4
Sept. 98	0.2 ?0.2	0 – 0.5	0.2 ?0.2	0 - 0.5	4	0.8

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.11b Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 1 during second sampling cycle in Thailand

Date	% cells with bacteria[1]	Range	CFU (x10^4 ml^{-1})	
			TSA	AS
December 98	2.9 ?0.5	2.3 - 3.3	11	0.2
January 99	1.4 ?1	1 - 2.6	0.9	0.001
Early February 99	1.6 ?0.6	0.75 - 2.35	5.9	0.08
Late February 99	1.3 ?0.8	0.8 - 2.4	4	1

CFU = colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.12a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 1 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter Measured	P Value
pH	0.603
Alkalinity	0.7625
Ammonia	0.8813
Nitrate	0.6929
Total Suspended Solids	0.349
Turbidity	0.496

Table 2.12b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 1 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter Measured	P Value
Temperature	0.0391
Alkalinity	0.308
Ammonia	0.431
Nitrite	0.00296
Total Suspended Solids	0.0170
Turbidity	0.0200

Table 2.13a Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 2 during first sampling cycle in Thailand

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
May 98	no data	no data	no data	no data	no data	no data
June 98	0.7 ?0.9	0 - 2.75	1.4 ?1.3	0 - 3.8	4	0.3
Early August 98	0.6 ?0.4	0 - 1.75	0.6 ?0.3	0.25 - 1	5.4	0.5
Late August 98	0.04 ?0.1	0 - 0.25	0.04 ?0.1	0 - 0.25	0.8	0.05

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

Table 2.13b Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 2 during second sampling cycle in Thailand

Date	% cells with bacteria[1]	Range	CFU ($x10^4$ ml^{-1})	
			TSA	AS
December 98	2.7 ?0.7	2.2 - 3.9	5.1	0.1
January 99	1.3 ?0.5	0.75 - 1.9	2	0.3

CFU = colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

Table 2.14: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 2 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
pH	0.857
Alkalinity	0.00807
Ammonia	0.0452
Nitrate	0.0255
Total Suspended Solids	0.182
Turbidity	0.884

Table 2.15a Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 3 during first sampling cycle in Thailand

Date	(+F)		(-F)		CFU (x10^4 ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
May 98	0.4 ?0.3	0 - 0.75	0.1 ?0.3	0 - 0.75	7	0.2
June 98	0.7 ?0.5	0 - 1.25	0.5 ?0.4	0 - 1	6	0.4
July 98	0.5 ? 0.4	0 - 0.75	0.3 ?0.6	0 - 1.5	1.4	0.08
August 98	0.07 ?0.11	0 - 0.25	0.08 ?0.2	0 - 0.5	454	81

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.15b Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 3 during second sampling cycle in Thailand

Date	% cells with bacteria[1]	Range	CFU (x10^4 ml^{-1})	
			TSA	AS
December 98	0.4 ?0.2	0.1 - 0.6	2.3	0.09
January 99	0.3 ?0.2	0.1 - 0.5	21	0.3
Early February 99	0.4 ?0.3	0.1 - 0.4	4.1	0.3
Late February 99	2.6 ?1	1.8 - 3.8	1.2	0.8

CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.16a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 3 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.156
pH	0.251
Alkalinity	0.366
Ammonia	0.157
Nitrate	0.399
Total Suspended Solids	0.0762
Turbidity	0.0626

Table 2.16b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 3 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.0111
pH	0.0364
Alkalinity	0.0128
Ammonia	0.475
Nitrate	0.227
Total Suspended Solids	0.0000000346
Turbidity	0.000469

Table 2.17a Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 4 during first sampling cycle in Thailand

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
June 98	0.4 ?0.4	0.2 - 1.25	0.2 ?0.2	0 - 0.5	0.5	0.2
July 98	0.9 ?0.7	0 - 1.75	0.7 ?0.7	0 - 1.75	1.8	0.2
August 98	0.3 ?0.4	0 - 1	0.7 ?0.6	0 - 1.5	18	0.03
Sept. 98	0.2 ?0.3	0 - 0.75	0.4 ?0.7	0 - 1.75	330	300

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.17b Percentage of catfish head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 4 during second sampling cycle in Thailand

Date	% cells with bacteria[1]	Range	CFU ($x10^4$ ml^{-1})	
			TSA	AS
December 98	0.3 ?0.2	0 - 0.6	no data	no data
January 99	0.2 ?0.2	0 - 0.7	1.1	0.3
February 99	2 ?3	0.8 - 2	13.2	3.5
March 99	0.9 ?1	0 - 0.7	24.8	22.2

CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.18a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 4 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.144
pH	0.9456
Alkalinity	0.7845
Ammonia	0.607
Nitrate	0.400
Total Suspended Solids	0.149
Turbidity	0.578

Table 2.18b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 4 in Thailand, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.6970
pH	0.209
Alkalinity	0.259
Ammonia	0.102
Nitrate	0.433
Total Suspended Solids	0.325
Turbidity	0.307

Relationship between bacterial level in macrophages, water quality and bacterial counts in the Vietnamese ponds

Farm 1 (Polyculture)

No relationship was found between the number of viable bacteria in the water and the percentage of macrophages which contained bacteria in cycles 1 (P = 0.438) or cycle 2 (P = 0.052). No significant relationships were found between the percentage macrophages with bacteria and the water quality parameters (Table 2.20) over the two cycles when statistically analysed

Farm 2 (Monoculture)

The percentage of macrophages with internalised bacteria are presented together with the viable bacterial counts in the water determined during cycles 1and 2, in Table 2.21. No significant relationships were found in cycle 1 (P = 0.395), but in cycle 2, a significant negative correlation was found between the number of bacterial in the water and the percentage of macrophages with bacteria (P= 0.004).

No significant relationships were found between the water quality data and the percentage of macrophages with bacteria in both cycles (Table 2.22), except for COD in cycle 2, where a significantly positive correlation was found.

Farm 3 (Small Cage)

The percentage of macrophages with bacteria and the total viable bacterial counts in the water samples were calculated for cycles 1and 2 (Table 2.23). In cycle 1 a significant positive correlation between the percentage of macrophages containing bacteria and level of bacteria in the water (P = 0.043) and in cycle 2 a significant negative correlation was found (P = 0.005).

In the small cage farm sampled during cycle 1, only dissolved oxygen had a significantly positive correlation between the percentage of macrophages with bacteria. The remaining water quality tests had no significant relationship with bacterial levels (Table 2.44a), while in cycle 2, no statistically significant relationships were found (Table 2.24b).

Farm 4 (Large Cage)

The percentage of macrophages with bacteria and the viable bacterial colony counts from the water samples were determined in cycles 1 and 2 (Table 2.25). A highly significant positive correlation was found between the mean macrophages with bacteria and the water quality parameters tested (P = 0.000007) in cycle 1, while in cycle 2, a statistically significant negative correlation was found (P = 0.028).

A significantly negative correlation was found between the temperature and the percentage of macrophages with bacteria in the first cycle (Table2.26a). A significantly positive correlation was found between all other parameters and the percentage of macrophages with bacteria, except for the pH of the water where no significant relationship was found. No significant relationships were found between the percentage of macrophages with bacteria and the water quality parameters measured during cycle 2 (Table 2.26b).

Table 2.19a Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 1 during first sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
February 98*	no data	no data	28 ? 10	16 - 40	20	14
Sept. 98	11 ? 12	0 - 33	11 ? 13	0 - 32	20	no data
Oct. 98	12 ? 11	0 - 27	13 ? 9	0 - 23	4	1.7

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for four fish ? standard deviation (SD)

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

Table 2.19b Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 1 during second sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
December 98	13 ? 8	0 - 20	12 ? 9	0 - 25	1.7	0.3
January 99	6 ? 4	1 - 12	4 ? 3	2 - 9	4	0.6
March 99	6 ? 4	0 - 11	5 ? 3	0 - 9	1.3	0.1
April 99	9 ? 2	6 - 12	9 ? 3	7 - 15	2.8	0.28

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.20a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 1 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.7992
pH	0.7992
Dissolved Oxygen	0.7992
Transparency	0.7992

Only two samples were available from the data collected in cycle 1 (August and October 1998) and one of the variables did not differ, therefore the P values were the same for all parameters.

Table 2.20b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 1 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	**P Value**
Temperature	0.120
pH	0.420
Dissolved Oxygen	0.317
Chemical Oxygen Demand	0.583
Transparency	0.831
Total Nitrogen	0.846
Total Phosphates	0.414
Total Suspended Solids	0.367
Organic Suspended Solids	0.337
Inorganic Suspended Solids	0.623

Table 2.21a Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 2 during first sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
March 98	no data	no data	13 ?13	5 - 36	10	3
Sept. 98	10 ?7	0 - 18	8 ?6	3 - 20	4.5	no data
Oct. 98	10 ?7	0 - 19	10 ?7	3 - 20	6.6	3.7

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

Table 2.21b Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 2 during second sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
December 98	no data	no data	5 ?5	0 - 14	0.8	0.1
January 99	no data	no data	1 ?1	4.5 - 13.5	6.4	450
Early April 99	5 ?3	3 - 10	5 ?2	3 - 9	1	0.43
Late April 99	4 ?2	0 - 6	3 ?3	0 - 6	0.9	0.3

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

Table 2.22a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 2 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
pH	0.8299
Dissolved Oxygen	0.8299
Transparency	0.8299

Only two samples were available for statistical analyses from cycle 1 (September and October 1998) and one of the variable did not change between the sampling points, therefore the P values was similar.

Table 2.22b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 2 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.364
pH	0.0695
Dissolved Oxygen	0.9351
Chemical Oxygen Demand	0.0410
Transparency	0.620
Total Nitrogen	0.355
Total Phosphorous	0.103
Total Suspended Solids	0.219
Organic Suspended Solids	0.212
Inorganic Suspended Solids	0.342

Table 2.23a Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 3 during first sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
March 98	no data	no data	14 ?7	7 - 26	17	0.06
August.98	12 ?6	1 - 18	8 ?5	2 - 16	2.1	no data
Oct. 98	7 ?2	4 - 10	6 ?4	3 - 14	3	0.1

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

Table 2.23b Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 3 during second sampling cycle in Vietnam

Date	(+F)		(-F)		CFU ($x10^4$ ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
December 98	no data	no data	10 ?7	10.7 - 18	0.1	0.014
January 99	7 ?4	2 - 13	7 ?6	0 - 18	0.4	0.02
March 99	8 ?4	5 - 14	6 ?2	3 - 9	2.1	0.46
April 99	5 ?4	0 - 6	4 ?4	0 - 12	2	0.4

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ? standard deviation (SD)

Table 2.24a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 3 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature[2]	0.8059
pH[2]	0.170
Dissolved Oxygen[2]	0.0391
Chemical Oxygen Demand[1]	0.118
Total Nitrogen[1]	0.118
Total Phosporous[1]	0.118
Total Suspended Solids[1]	0.118
Organic Suspended Solids[1]	0.118
Inorganic Suspended Solids[1]	0.118
Transparency[2]	0.148

[1]Data was available for two samples only (August and October 1998) in cycle 1.

[2]Data was only available for the months March, August and October 1998 in cycle 1

Table 2.24b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 3 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.684
Dissolved Oxygen	0.3431
Chemical Oxygen Demand	0.275
Transparency	0.9859
Total Nitrogen	0.0710
Total Phosphorous	0.243
Total Suspended Solids	0.9492
Organic Suspended Solids	0.9481
Inorganic Suspended Solids	0.444

Table 2.25a Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 4 during first sampling cycle in Vietnam

Date	(+F)		(-F)		CFU (x10^4 ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
March98	no data	no data	19 ?4	14 - 22	6	0.084
Sept. 98	0 ?0	0	1.3 ?0.8	0 - 3	1.4	no data
Oct. 98	2.5 ?1.5	0 - 4	6 ?3	3 - 12	0.05	0.04

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

Table 2.25b Percentage of head kidney macrophages, which contained bacteria upon sampling and the total viable bacterial counts of the water of Farm 4 during second sampling cycle in Vietnam

Date	(+F)		(-F)		CFU (x10^4 ml^{-1})	
	% Cells[1]	Range	% Cells[1]	Range	TSA	AS
January 99	no data	no data	7 ?3	5 - 12	0.42	0.12
January 99	1 ?2	0 - 4	3 ?3	0 - 8	0.8	0.06
March 99	7 ?6	1 - 15	7 ?3	3 - 11	0.5	0.13
April 99	6 ?2	3 - 14	7 ?10	1 - 6	0.5	0.03

+F: with fibronectin; -F: without fibronectin; CFU: colony forming units; TSA: tryptic soya agar: AS: *Aeromonas* select agar

[1]percentage of macrophages containing bacteria, mean of 200 macrophages counted per fish for six fish ?standard deviation (SD)

Table 2.26a: Statistical Analysis of relationship between water quality obtained during the first sampling cycle of Farm 4 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.0172
pH	0.0172
Dissolved Oxygen	0.395

No more data were available for analyses in cycle 1

Table 2.26b: Statistical Analysis of relationship between water quality obtained during the second sampling cycle of Farm 4 in Vietnam, and percentage of fish head kidney macrophages, which contain bacteria

Parameter	P Value
Temperature	0.700
pH	0.899
Dissolved Oxygen	0.202
Chemical Oxygen Demand	0.740
Transparency	0.655
Total Nitrogen	0.819
Total Phosphorous	0.821
Total Suspended Solids	0.999268
Organic Suspended Solids	0.760
Inorganic Suspended Solids	0.483

Laboratory experiments- AARHI (Experiment 1)

Table 2.27a: Cumulative Mortalities (%) of fish subjected to various stressors prior to an artificial bacterial challenge

Stressor	Cumulative Mortalities (%)			
	Control	Bacteria Only	Stress Only	Combined*
Temp (35^0C)	24	52	64	64
(20^0C)	16	68	20	84
HSD	n/a	52	42	76
No water change	32	76	32	80
Transportation	4	28	4	24
Removed from water	n/a	4	20	36

*= stress and bacterial challenge, N/A = data not available, HSD = high stocking density

Table 2.27b Statistical Analyses of Cumulative Mortalities in stress experiment performed at AARHI

Stressor	P Value	?^{2} Value	Comment
Temperature (35^{0}C)	0.014	?$^2_3$ = 10.684	Highly significant increase in mortality between the control and experimental groups
	0.607	?$^2_2$ = 1	No significant difference between bacteria, stress and combined group
Temp. (20^{0}C)	< 0.000	?$^2_3$ = 35.126	Highly significant increase in mortality between all experimental and control groups
	0.185	?$^2_1$ = 1.754	No significant difference in the percentage mortality between the bacteria and combined group
High stocking density	0.059	?$^2_2$ = 5.669	No significant difference between treated groups
No water change	< 0.000	?$^2_3$ = 21.455	Highly significant difference between the controls and treated groups
Transportation	0.022	?$^2_3$ = 9647	Significant increase in mortality between control and treated groups
Removed from Water	0.018	?$^2_2$= 8	Significant increase in mortality found between the bacteria and combined groups

?^{2} : Chi squared value, P: probability

Table 2.28 Number of day after which mortalities ceased after fish were subected to different stressors

Experiment & Tank	End of Mortalities (Days)
High Temperature (35°C)	
[1]Control	2
[2]Bacteria Only	3
[3]Stress Only	2
[4]Combined	2
Low Temperature (20°C)	
Control	2
Bacteria Only	3
Stress Only	3
Combined	2
High stocking density	
Control	N/A
Bacteria Only	2
Stress Only	4
Combined	3
No Water Change	
Control	6
Bacteria Only	4
Stress Only	6
Combined	6
Transportation	
Control	4
Bacteria Only	2
Stress Only	7
Combined	3
Removed from Water	
Control	N/A
Bacteria Only	6
Stress Only	7
Combined	3

N/A = data not available

[1] control group (no bacterial challenge or stress); [2] bacterial challenge only; [3] stress only; [4] a combination of bacterial challenge and stress.

Laboratory experiments- AARHI (Experiment 2)

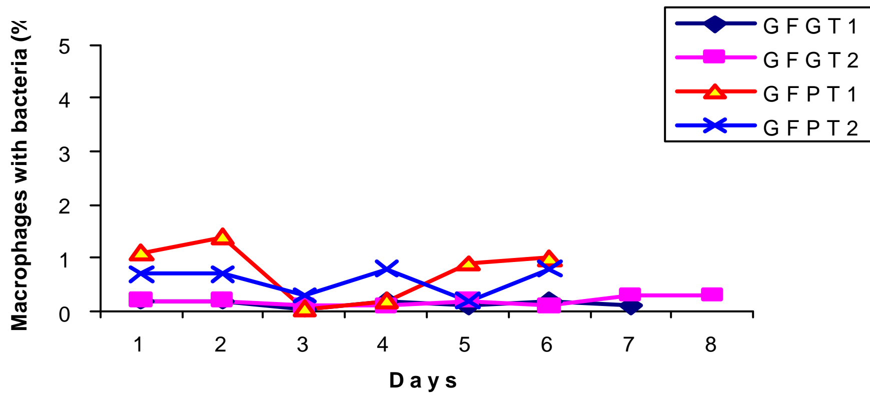

Figure 2.16a Percentage of macrophages isolated from fish obtained from the good farm, which contained with bacteria

GFGT1 = good farm good tank1, GFGT2 = good farm good tank 2, GFPT1 = good farm poor tank 1, GFPT2 = good farm poor tank 2.

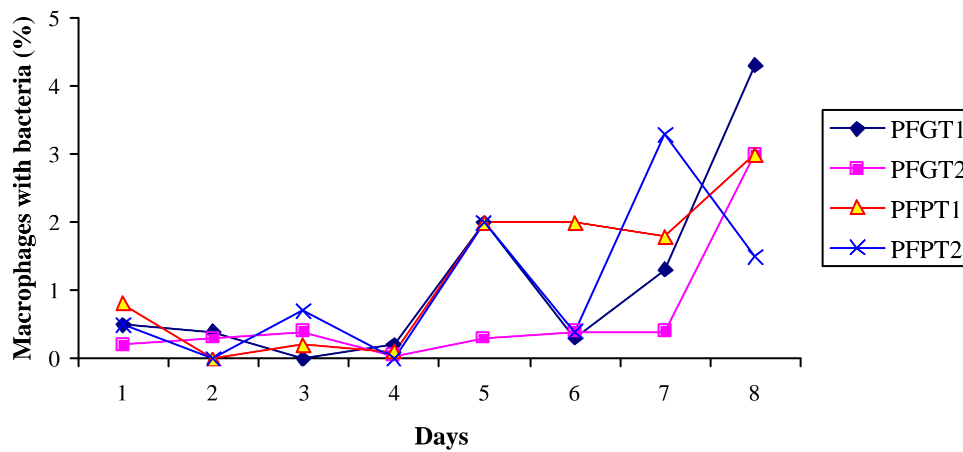

Figure 2.16b Percentage of macrophages isolated from fish obtained from the good farm, which contained with Bacteria

PFGT1 = poor farm good tank 1, PFGT2 = poor farm good tank 2, PFPT1 = poor farm poor tank 1, PFPT2 = poor farm poor tank 2.

Table 2.29. Bacterial levels in tank water of experiment 2 (AARHI)

Tank	Sample Day	*N^0 Colonies x 10^4 ml^{-1}
PFGT1	12	13.2
PFGT1	20	0.048
PFGT2	12	3.25
PFGT2	20	0.053
PFPT1	12	0.48
PFPT1	20	0.037
PFPT2	12	4.41
PFPT2	20	0.019

*On TSA

Table 2.30 Identification of bacterial species recovered from bacterial swabs

GFGT1	GFGT2	GFPT1	GFPT2	PFGT1	PFGT2	PFPT1	PFPT2
3 (m)		42 (m)	11 (M)	2 (m)		% (M)	18 (T)
15 (M)			27 (T)	4 (M)		45 (m)	32 (T)
56 (M)			31 (T)	25 (M)		58 (T)	68 (m)
59 (T)			60 (m)			63 (T)	76 (T)
			73 (T)			66 (m)	

m: swab prepared from mort,

M: swab prepared from macrophage suspension,

T: swab prepared from head kidney direct

Bacterial colonies shown numerically were identified to genus level:

motile *Aeromonas* spp- 2, 3, 15, 42, 45, 56, 66, 68, 73

Bacteroides sp.- 4

Acinebacter spp.- 5, 18, 32, 76

Enterobacteriaceae sp.- 11

Micrococcus sp.- 25, 27

Yersinia sp.- 31

alkali *Psuedomonas* sp.- 58

Eikenella sp. (often confused with *Bacteroides* sp.)- 60

Achromobacter sp.- 63

Laboratory experiments- CanTho (Experiment 1)

Table 2.31: Cumulative Mortalities (%) of fish subjected to various stressor prior to an artificial bacterial challenge

Experimental Group	Percentage Mortality (%)	Day
Combined*	40	3
Bacterial Alone	32	4
Stress Alone	28	6

*= stress and bacterial challenge

Appendix 3- Standard operating procedures

Protocols

Field Sampling

Outline of Field Sampling in Vietnam & Thailand.

1. Collect water samples to measure the bacterial counts in the water. Use the spread plates method. Take the water sample and serially dilute the original in sterile saline (1:10 dilution) and then take 0.1 mls of the bacterial suspension from the water at the appropriate dilution and drop on TSA and Aeromonas selective media plates. Flame a bacteriology glass spreader, allow it to cool and then spread the bacterial solution evenly around the plates. Seal the lid and then take back to the laboratory and incubate at 28^0 C and check growth after 24 and 48 hours. Count the colonies grown and adjust to colony forming units (cfu) ml^{-1}.

2. Remove the fish from the pond/cage and once the fish is anaesthetised, complete a health assessment of every individual animal, external and internally. Take note or photographs of anything unusual.

3. Remove the head kidney aseptically and place a small section onto TSA media and streak using a flame sterile, but cooled bacteriology loop. Take the plates back to the laboratory and incubate at 28^0 C and look for any colony growth after 24 and 48 hours. Take note of any colonies recovered and the type of growth (pure, mixed). If the same type of colony is recovered repeatedly, try to identify using primary and if possible, secondary bacteriology identification tests.

4. Take the remaining section of the head kidney and place in 2 mls of L-15 supplemented media (with Foetal Calf Serum and heparin) and macerate this through a sterile 100 ? m mesh using a 1-ml syringe. This gives the mixed cell suspension.

5. On each slide there should be two marked circles, where one has been coated in fibronectin solution and the other is without fibronectin solution. Add 200 ?l of the mixed cell suspension to each circle on the microscope slide and incubate in a humid chamber for 30 minutes. After this time remove the unattached cells (lymphocytes, erythrocytes) by washing the slide three times with sterile saline. Fix the slide in alcohol (80% ethanol) for 30 seconds and then immediately stain the slides using the Rapi-Diff II stain for 1 minute per stain. Produce two microscope slides per fish.

6. With the remaining cell suspension streak a sterile, cooled bacteriology loopful onto a TSA plate and take back to the laboratory and incubate at 28^0 C and check colony growth after 24-48 hours.

7. Take water samples to measure chemical water parameters at each sample time. The water quality laboratory can do this.

8. At the time of sampling measure the pH and temperature of the water in the farm.

Additional Notes

1. Wear gloves at all times when sampling the fish.
2. Heat sterilise the instruments and keep them in 70% alcohol when not in use in the field.
3. Check all reagents for possible bacterial contamination after being in the field and at the laboratory on a weekly basis.
4. Ask the farmer if anything unusual has happened since the last sample time and take note on the questionnaire, such as harvesting, applying chemical or antibiotics, and changing husbandry techniques or disease outbreaks.

Standard Operating Procedures (SOP)

These were produced and left in place at the laboratory in Vietnam. The Thai research group had more research experience in this area and required reminders and only additional training in unfamiliar topics.

Isolation of Serum and Plasma from Tropical Cultured Fish

1. If serum is required DO NOT USE an anticoagulant.
2. If an anticoagulant is used then take a small volume of heparin into the needle BEFORE BLEEDING the fish.
3. Bleed the fish by caudal vessel using sterile needle and syringe and transfer blood immediately to sterile Eppendorff.
4. If serum is required then leave the blood in the Eppendorff for 2 hours (maximum) at room temperature (20 ? 2^0C). To remove the clot, centrifuge the serum at 6500 rpm for 5 minutes in a microcentrifuge and then remove the serum and place in a sterile Eppendorff. This can be used immediately or frozen at -20^0 C until required.
5. If plasma is wanted then once the blood is in the Eppendorff centrifuge at 6500 rpm for 5 minutes in a microcentrifuge after collecting the blood. This can be used immediately or stored frozen at -20^0 C until required.

Passaging Bacteria to Increase Virluence Properties

When a bacterial culture has been kept for any length of time on an agar based medium, it can loos important characteristics such as virulence. Therefore, before using a stored bacterial culture it is important to enhance these characteristics that may have been lost. This is usually done by introducing a high concentration of the bacterial suspension into control healthy fish and recovering a pure bacterial culture from the internal organs. The concentration of the bacteria should be so that the fish will die or at least be moribund, twenty-four hours post bacterial administration. A pure culture of the bacteria that has been introduced to the fish should be recovered from the internal organs (liver, kidney and spleen) of the infected fish and the whole technique repeated. This is called **passasge** and it helps to enhance the virulence properties of the bacteria.

Material and Methods

1. The route of bacterial administration is more rapid by intraperitoneal (i.p) injection using a sterile needle and syringe.
2. Prepare the bacterial suspension at 1 x 10^8 cfu ml^{-1} (see **Bacterial Challenge**) and inject by i.p into 2 clinically healthy fish. The volume of the bacteria added will differ depending on the size of the fish and the bacterial species used.
3. Leave the fish in the tank as per normal with no food for 24 hours. The next day, sample the internal organs of the dead or moribund fish to isolate a pure culture of the bacterial species given.
4. To do this, aseptically remove a small section of the liver, spleen and kidney and streak this directly onto a tryptone soya agar plate (TSA) using a flamed and cooled bacteriology loop.

5. Incubate the TSA plate for 24 hours at optimal temperature for the bacterium (usually 28^0C).
6. Check the purity of the growth the next day and aseptically remove some colonies using a sterile bacteriology loop and place the colonies into (approximately 10 mls) tryptone soya broth (TSB) to enhance growth of the bacteria. Incubate for 24 hours at optimal temperature.
7. The following day the bacterial suspension in TSB should be centrifuged and the supernatant removed and replaced with same volume of sterile saline. This should be repeated twice and the bacterial suspension in sterile saline measured spectrophotomically to obtain a culture concentration of 1×10^8 cfu ml^{-1}. This should then be injected by i.p into another two clinically healthy fish. The fish should be placed into a tank and left for 24 hours. After this time the fish should be dead or moribund and again sample these fish to recover a pure culture of the bacteria that was introduced.
8. Repeat steps 4 and 5.
9. Finally, identify the pure bacterial culture that has been passaged using primary and if possible secondary bacteriology identification tests and then when satisfied that the results indicate your bacterial species, use this bacterial strain as a virulent bacterial strain for the appropriate experimental purposes (usually bacterial challenge studies).

Standard Curve for Measuring Bacterial Concentration in Suspension

Preparation of a standard curve using a spectrophotometer can produce an accurate and reliable method to determine the exact concentration of bacteria in suspension. This is particularly useful method when doing bacterial challenge studies.

Equipment

Spectrophotometer (wavelength 540 or 610nm)
Clean cuvettes

Sterile Pasteur pipettes
Latex gloves
Sterile test tubes (with lids) or Universals
Sterile saline
TSB
TSA plates
Microliter (?l) Gilson pipettes and sterile tips
Millilitre (ml) Gilson pipette and sterile tips

Method

1. Grow bacteria in a volume of TSB for 24 hours at optimal temperature (this is to log-phase growth).

1. Switch on the spectrophotometer and allow to stabilise at room temperature with the required settings in place.
2. Use sterile saline as Blank.
3. In a separate cuvette put 1.5 to 2 mls (this will depend on the cuvette volume) of the original bacterial suspension at log-phase growth and measure the optical density. This tells the original optical density.
4. Dilute this original suspension with sterile saline until the optical density of the suspension reads 0.9. It is very important to do this SLOWLY and carefully, as it is very easy to dilute the suspension too much. Measure the optical density and record the result.

5. In seven test-tubes of universals have 4.5 mls of sterile saline already prepared and add 0.5 mls of the bacterial suspension measured at 0.9 optical density into the first test-tube (this is 10^{-1} dilution). Invert and the take 0.5 mls of the 10^{-1} dilution and add to the next test-tube (10^{-2}). Complete the serial dilution's in the way until you have reached 10^{-7} and remember and change the pipette tip between dilutions.
6. Once the serial dilutions are made, take the dilution's 10^{-4} to 10^{-7} and complete drop counts onto TSA plates for each dilution.
7. Drop counts include separating the TSA plate into six equal sections (like a pie) and putting a 20?1 drop into each section on the TSA plate. The plates are then incubated for 24 hours at 28^{0}C and the number of colonies counted.
8. Steps 5 to 8 are repeated for all of the optical densities listed in Table 1.

Table 1 Results Table for Standard Curve Data

OD Wanted	OD Actual	Dilution Factor Counted	N^{0} Colonies ml^{-1}
0.9			
0.8			
0.7			
0.6			
0.5			
0.4			
0.3			
0.2			
0.1			

OD = optical density

Once the number of colonies have been calculated (Figure 1), these values can be plotted on a line graph and then this is a template to be used when a bacterial suspension at a critical concentration is required.

Figure 1 Calculation

Average number of colonies X 50 X dilution factor = number of colonies ml^{-1}

Bacterial Challenge by Bath Administration

Bath administration is the more natural and easier route to challenge a large number of fish. The fish should be checked for health and acclimated to the laboratory before given the challenge. It is recommended that the bacterial strain be passaged before being used in any bacterial challenge work.

1. Grow a pure culture of the bacterial suspension on TSA plate at 28^0C for 24 hours. If the culture has been kept in storage then it should be identified before being used to confirm the bacterial species.
2. Take some colonies from the TSA plate and place directly into a volume TSB and incubate for 24 hours at 28^0C. This is log-phase growth.
3. Once the bacterial suspension is at log-phase growth then centrifuge the suspension at 3000 to 3500 rpm for 15 mins and resuspend the pellet in sterile saline. Repeat this again. The bacterial suspension has now been washed twice and is ready for use.
4. Measure the optical density of the suspension to give 1×10^8 cfu ml^{-1}as detailed in the **Standard Curve** protocol and verify the concentration by completing drop counts. This also verifies that the bacteria are viable.
5. There is now a working bacterial suspension kept in sterile saline, which can be added to the bath challenge tanks.
6. The amount of bacterial suspension required would depend on the amount of water in the tanks and the concentration required.

An example of Bacterial Challenge Calculation

Bacterial concentration required = 1×10^5 cfu ml^{-1}

Volume of water in tanks = 10 L (this is equal to 10,000 ml)

Number of bacterial to be added =

Volume of water x concentration required

= 10,000 mls x 10^5 = 10^4 x 10^5 = 10^9

Therefore, add 1 ml of 10^9cfu ml^{-1} to 10 L of water per tank to give 1 x 10^5 cfu ml^{-1}

HOWEVER

If there is only 1 x 10^8 cfu ml^{-1} then add **10 mls of 1 x 10^8 cfu ml^{-1} , which is equal to 1 ml of 1 x 10^9 cfu ml^{-1}**

Appendix 4

A literature review entitled *Intracellular Fish Pathogens* is in preparation for submission to Journal of Fish Diseases, a **preliminary** draft of which is included here

Intracellular Bacterial Pathogens of Fish

Abstract

In cultured teleost species, large-scale losses from bacterial disease continue to occur. Studies have identified the virulence factors associated with highly pathogenic strains of bacteria and investigated the interaction between pathogen and the immune response of fish species. Particular interest has focused on identifying the mechanisms by which, intracellular bacterial pathogens of fish are able to evade the immune response and cause disease. There are six known intracellular pathogens of both wild and cultured fish species, which are able not only to survive, but also replicate within the hostile environment of the immune functioning cells of the host. Thriving within these cells is a very specialised evasion strategy adopted by the bacteria that, results in slow detection of the pathogen and extreme difficulty in applying treatments, due to the intracellular nature of the bacteria. The six bacterial species and the evasion mechanisms employed by these intracellular fish pathogens are discussed within this review.

Immune response to intracellular and extracellular pathogens

The immune system of vertebrate species has evolved into both innate and adaptive defence mechanisms, that are able to cope with a wide array of infectious agents. There are numerous effector systems, which the immune system employs to destroy the different pathogens. These include neutralisation, where antibodies bind to pathogens and prevent the pathogens attaching to the host cells. Phagocytosis, ultimately results in the destruction of the pathogen ingested by the phagocytes. The immune system of the host also utilises cytotoxic reactions by lymphocytes and natural killer cells and apoptosis, which are directed against whole cells that are too large for phagocytosis to be effective (Roitt, Brostoff and Male 1998).

There are fundamental differences in the type of immune response activated and the appropriate reaction will depend on whether the encounter is with an extracellular or intracellular pathogen. In mammals, interaction with an intracellular pathogen can trigger two types of response from the immune system of the host: T lymphocytes of the adaptive immune response may destroy the infected cell directly by cytotoxicity, or they may activate the infected cell which will then destroy the pathogen (Roitt *et al.* 1998). Organisms ingested by phagocytic cells can be stimulated by chemical messengers released from the T lymphocytes to enhance production of microbicidal substances. The phagocytic cells are of two basic cell types, generally referred to as the mononuclear phagocytes and the polymorphonucleated granulocytes. The later have a lobed, irregular shaped nucleus and are divided into neutrophils, eosinophils and basophils, depending on the staining properties of their cytoplasmic granules (Roitt *et al.* 1998). The mononuclear phagocytes are sometimes referred to as 'professional' phagocytes and this reflects their main function. They also serve as antigen presenting cells by processing and presenting antigens to T lymphocytes and can be found in the blood stream (monocytes) or in the tissues (macrophages). Macrophage activation is a complex process, but these cells produce a first line means of defence against pathogenic invasion in vertebrate species. Macrophages posses many microbicidal mechanisms such as production of reactive oxygen intermediates, generation of an array of toxic substances all lethal for microbes.

Evasion strategies of pathogens and how they escape from an immune response

Due to the efficient immune response of the vertebrate host, some microbial pathogens have evolved evasion strategies to avoid destruction and prolong survival within the host. Virulent pathogens have associated factors, which help facilitate their entry and spread within a host. These virulence factors enable the microbes to override a defence reaction and initiate disease. Various properties have been associated with virulence, and these will differ depending on the microbe involved. A capsule is associated with high virulence and can prolong the survival of the organism within the host by inhibiting phagocytosis and avoiding deposition on the surface of the microbe of opsonic factors produced by the host. Many virulent bacteria can produce toxins, which are generally

species-specific to the microbe, and these are released by the bacteria post-entry into the host and facilitate the spread of infection by causing tissue lysis and cell death.

Therefore, bacterial virulence factors can enhance survival of the microbe within the host, but some micro-organisms have evolved more specialised survival mechanisms and these are able to live and replicate within the cells of the host. Some intracellular pathogens exist within the phagocytes but this is such a hostile environment for the microbes that it is difficult to understand fully, the range of mechanisms that must be employed to enable survival of the microbes. Some intracellular bacteria avoid the bactericidal environment of the phagocytes completely by inducing uptake by normally non-phagocytic cells. Other intracellular species are able to 'switch off' production of interleukins and other chemical messengers produced by the phagocytic cells, hence they avoid destruction as the phagocytic cells do not receive the appropriate chemical signal to become activated and produce bactericidal enzymes.

Intracellular pathogens of fish

The work presented in this review will only focus on the recognised intracellular bacterial pathogens that have caused disease in wild and cultured fish species. There are six bacterial species that have been identified as intracellular pathogens of various fish species. These include *Mycobacteria spp., Nocardia spp., Pasteurella spp., Renibacterium salmoninarum, Streptococci spp. and Rickettsia spp.*

Mycobacteriosis

Mycobacterial infections in fish are commonly referred to as fish tuberculosis or mycobacteriosis. It is an important bacterial infection in commercial fish species and disease outbreaks have been reported world-wide in over 150 different species of marine, brackish and freshwater fish. Prevalence of infection has reached 15% in monitored wild fish populations and 100% in fish cultured in intensive systems (Smith 1996). Mycobacteriosis is a systemic and chronic disease, producing various clinical signs. These have included listlessness, emaciation, skin discolouration, exophthalmia, scale loss, presence of nodules as well as fin necrosis (Frerichs 1993). This bacterial disease affects both temperate and tropical freshwater and marine animals. Some animals such as salmonids do not always readily display external clinical signs but

internally microscopic grey-white granulomas have been found in parenchymatous tissue such as liver, kidney and spleen (Frerichs 1993). The microbes that cause mycobacteriosis are described as Gram positive, acid-fast, non-motile, non-sporing, pleomorphic rods. They range from 1-4 x 0.2-0.6 ?m in size and are slow growing microbes, usually taking 2 to 3 weeks to culture in the laboratory. There are several strains causing fish tuberculosis including *Mycobacterium marinum*, *M. fortuitum* and *M.chelonae*. Transmission of mycobacterial infections is thought to occur by ingestion of contaminated food or aquatic detritus, however, invasion by the microbes may also occur if the host skin or gills are damaged.

Nocardiosis

Nocardiosis is a long established intracellular bacterial pathogen of both freshwater and marine cold and tropical fish species (Valdez and Conroy 1963). It has been described as an important pathogen of Japanese cultured yellowtail (*Seriola quinqueradiata*), (Kawatsu, Homma, Kawaguchi 1976). The clinical signs of a chronic infection with *Nocardia* spp. can include inactivity, skin discolouration, emaciation and latterly the appearance of nodules externally and internally (Frerichs 1993). The bacterium *Nocardia kamachi* can cause high numbers of mortalities in young cultured yellowtails, which are particularly susceptible to infection. Two species of Nocardia have been studied in fish: *N. kampachi* and *N. asteroides*. They are biochemically very similar but differences occur between sugar fermentation in *N. kapachi* (Kusuda and Taki 1973). They are Gram positive, branching, filamentous bacilli and can be isolated and grown on standard bacteriology agar media in the laboratory at 20^0 to 30^0C.

Pasteurellosis

Pasteurellaceae is a family consisting of three genera: *Pasteurella, Haemophilus* and *Actinobacillus* spp. The causative agent of pasteurellosis is the bacterium *Pasteurella piscicida*, which is a particularly important pathogen of Japanese cultured marine fish (Kitao 1993a). The clinical signs of pasteurellosis vary, as in acute cases the external body colour only may darken (Tung, Tsai, Ho, Huang and Chen 1985) and internally, small white granuolmas may occur in the kidney and spleen. *Pasteurella piscicida* is a Gram negative short rod which exhibits bipolar staining and is non-motile, non-spore forming and without a capsule. Outbreaks of disease have been associated with high

water temperatures and low salinity after heavy rains, and have resulted in wide-scale losses of stock (Kitao 1993a). Geographically, it is a particular problem in Japan and in some areas of America and Taiwan, where transmission of the microbe is thought to occur horizontally (Kitao 1993a).

Bacterial Kidney Disease (BKD)

The aetiological agent of bacterial kidney disease is the Gram positive, non-motile, bacillus *Renibacterium salmoninarum*. This is a serious infection of cultured (Earp, Ellis and Ordal 1953) and wild salmonids (Mitchum, Sherman and Baxter 1979). It was first recognised in wild Atlantic salmon (*Salmo salar*) in Scotland (Smith 1964) but has now spread to many parts of the United States and Canada causing infection in trout as well as salmon. The disease itself is very slow growing and more often than not, fatal. Reported clinical signs have included anaemia, exophthalmia, abdominal distention and shallow ulcers on the skin, but these are not always readily apparent in all infected animals. Internally there may be cavitation of the muscle, internal haemorrhage as well as the appearance of creamy-white granulomatous lesions in the kidney (Evelyn 1993). The aetiological agent of BKD is the small gram positive, non-acid fast, non-motile, rod *R. salmoninarum*. This pathogen is able to enter and survive in fish macrophages and have been found in the eggs of an infected female host (Evelyn 1993). It is described as an obligate fish pathogen as the bacterium does not appear to be able to survive outside the host.

Streptococcal infections

Streptococcal septicaemia was first reported in 1957 occurring in cultured rainbow trout (*Oncorhynkiss. mykiss*) in Japan (Hoshina, Sano and Morimoto 1958). Since then it has caused disease problems in various freshwater and marine spp. (Kitao 1993b). Fish have been both naturally and experimentally infected, where a specific host susceptibility to streptococcal infection was identified as, trout suffered heavy mortalities compared with tilapia (*Tilapia sparrmanii*) and carp (*Cyprinus carpio*) which were rarely affected (Kitao 1993b). The pathogen belongs to a large and complex genus of Gram positive cocci. They are non-motile and non-spore forming but some do posses capsules. They affect a range of fish species resulting in a variety of clinical signs including, erratic swimming, change of body colour, exophthalmia, haemorrhages and ulceration of the external body surface. Internally, they affect the liver, spleen and

sometimes the kidney and heart, causing discoloration, spleenomegaly and necrotic lesions. The mechanisms of pathogenicity of Streptococci have not yet been fully evaluated but high virulence has been associated with an exotoxin produced by the bacterium (Watson 1960).

Rickettsia

Rickettsial-like organisms have been shown to cause serious disease problems in cultured salmonids and is particularly prevalent in Chile (Bravo and Campos 1989). These organisms have only been tentatively classified as belonging to the Order Rickettsiales, but becaue the taxonomy has not yet been clearly established they are mostly described as rickettsial-like organisms (RLO) (Fryer and Lannan 1994). This small obligate pathogen produced a septicaemic condition recognised as 'coho salmon syndrome' or 'huito' disease. Diagnosis of infected individuals is not simple as similar to BKD infected animals, fish suffering from rickettsial infection do not always display overt clinical signs. Nevertheless, some animals do display gross signs such as pale gills and skin lesions. The spleen, liver and kidney in infected fish may also become swollen and discoloured (Turnbull 1993). The rickettsia-like organisms are often found in the cytoplasm of the phagocytic cells but have also been observed histologically as free in the tissues. This obligate intracellular pathogen is described as a small, basophilic coccus and may occur singularly or in diffuse groups. Rickettsial-like organisms have been grown in fish cells lines *in vitro* but attempts to grow the pathogen on artificial bacteriology media has proved futile (Turnbull 1993).

Immune response to intracellular pathogens

Mycobacteria spp.

Experimental infection of rainbow trout with *M. tuberculosis* produced a delayed-type hypersensitivity reaction. This is a T-cell response and as suggested by the nomenclature it is a slow response to the microbial antigens (Bartos and Sommer 1981).

Nocardia spp.

There are few published studies on the immune response of fish to *nocardia*. However, Kusuda and Nakagawa (1978) did find high serum agglutinating antibody levels in

experimentally infected yellowtails. The antibody response was detected 6 weeks post-treatment with heat or formalin-killed bacteria (Frerichs 1993).

***Pasteurella* spp.**

A study by Arijo, Borrego, Zorilla, Balebona and Morinigo (1998) investigated the role of the capsule in the bacterial species *Photobacterium damsela* subspecies *piscicida* (formerly known as *P. piscicida*). The capsulated bacterial strains were more readily ingested than the non-capsulated bacterial strains and when the non-capsulated bacteria were induced to synthesis a capsule there was a significant reduction in the percentage phagocytosis by gilthead (*Sparus aurata*, L) macrophages. Therefore it appeared that the presence of a capsule severely inhibited macrophage ingestion and killing when measured *in vitro*. Injection of formalin inactivated culture of *P. piscicida* to sea bass produced agglutinating antibodies for 2 to 4 weeks, post-injection. The presence of the antibodies was persisted in the sera of injected fish for 3 to 3.5 months as detected with ELISA test (Mazzolini, Ceschia, Giorgetti, Magni, Passera, Danielis and Marotta 1996).

BKD

Experimental infection of rainbow trout leukocyte cultures *in vitro* with *R. salmoninarum* demonstrated a prolonged survival of the bacterium within the mononuclear phagocytes (Guttenberger, Duimstra, Rohovec and Fryer 1997). Durability of the microbial cell wall enhanced survival within the cells as significant bacterial losses only started to occur 96 hours after experimental infection. A study by Baudin, Santos, Rivas, Skarmeta and Barja (1993) reported increased resistance to killing when the bacterium was incubated with fish macrophages *in vitro*. Untreated bacteria were able to survive for 3 to 4 days compared with serum treated microbes, which remained intact for 7 days.

***Streptococci* spp.**

No detailed studies on the immune response of fish infected with streptococci could be found in the present day literature.

***Rickettsia* spp.**

Few studies have managed to investigate fully the immune response of the host when infected with RLO. These are difficult bacterial species to work with as they are not easily cultured or maintained in the laboratory *in vitro* (Fryer and Lannan 1994).

Interaction of intracellular bacterial pathogens and macrophages

There are two types of phagocytic cells identified in all evolutionary advanced vertebrates: the circulatory granulocytes, predominantly neutrophils, and the tissue-dwelling macrophage cells (Roitt *et al.* 1998). Although both cell types are able to ingest bacteria in teleosts, macrophage cells are more prominent in the uptake and destruction of bacterial species compared with the neutrophils (Ellis 1982, Ainsworth 1992). Fish macrophage cells are able to kill ingested microbes through production of reactive oxygen intermediates (Chung and Secombes 1988) in a similar manner as described for mammalian macrophages (Roitt *et al.* 1998). The macrophage cells in fish are found more intermingled throughout the tissues than in mammals. They are particularly high in concentration within the underlying tissues of the intestine, gills and in the anterior kidney, spleen and heart.

***Mycobacteria* spp.**

Many mechanisms mediate the attachment and ingestion of *Mycobacteria* spp. by the professional phagocytes. In man, studies have shown that complement receptors particularly C1 and C3 have an important function in regulating the attachment and uptake of bacteria. Mycobacterial attachment and internalisation is also mediated by a mannose receptor and binding to the mannose receptor is associated with highly virulent strains of bacteria (Sinai and Joiner 1997). It is general accepted that the mycobacterial phagosome does not fuse with lysosomes within the phagocyte (Sinai and Joiner 1997). An electron microscopy study by Armstrong and Hart (1971) demonstrated that phagosomes containing live bacteria did not fuse with lysosomes, and nearly all of the damaged bacteria were contained within cells where phagolysosomal fusion had occurred.

Goren, Hart, Young and Armstrong (1976) reported that addition of sulfatides (sulpholipid components of the cell wall) of *M. tuberculosis*, when added to

macrophages *in vitro*, inhibited fusion of yeast phagosomes with lysosomes. Follow-up studies indicated that addition of sulfatides and other colloids to macrophages *in vitro* could generally render lysosomes incapable of fusing but the extent of inhibition depended on the assay used. Only live bacteria were able to inhibit fusion as dead bacteria, even in neighbouring phagosomes of the same macrophage did not have this effect (Goren *et al.* 1976). Ammonia secretions produced by the bacterium *M. tuberculosis*, were also shown to inhibit fusion (Gordon, Hart and Young, 1980).

Nocardia *spp.*

As *nocardia* spp. are very difficult to grow *in vitro*, there have been few laboratory based studies investigating the immune response of the host or the interaction of the host phagocytes with the bacterium. However, a Japanese study did report that infected yellowtail fish had higher numbers of lymphocytes and granulocytes accumulated at the site of infection and many of the bacteria were phagocytosed with 72 hours post-infection (Kusuda, Kimura and Hamaguchi 1989).

Pasteurella *spp.*

The intracellular bacterium *P. piscicida* is able to enter and replicate within the macrophage cells of infected fish species. The phagocytes readily ingest it, where it can grow in large numbers to eventually destroy the cell and further invade the host (Evelyn 1996). Experimental infection of Japanese yellow tail with *P. piscicida* resulted in accumulation of the macrophages in the spleen, gills, kidney and liver. The macrophage cells were observed to be nectrotic and enlarged with numerous intact bacteria. The numbers of bacteria contained within the enlarged macrophages of the gills were enough to impair respiration (Nelson, Kawahara, Kawai, Kusuda 1989).

A trend was found between high production of superoxide anion and high levels of bactericidal activity by fish macrophages infected with *P. piscicida*, when measured *in vitro* (Skarmeta, Bandin, Santos and Toranzo 1995). However, additional bactericidal factors may also be involved either directly or indirectly to enhance the uptake and destruction of bacteria in macrophages.

BKD

It is the unique chemical structure of the bacterial cell wall that has been attributed towards the intracellular survival of BKD and progression of disease (Gutenberger 1993). A study by Guttenberger (1993), observed that non-opsonised *R. salmoninarum* was readily ingested by rainbow trout macrophages and the ingested bacteria survived for several days and eventually replicated within the infected cells. The macrophages of infected fish did produce a respiratory burst activity but this did not kill the ingested bacteria (Bandin, Ellis, Barja and Secombes 1993). A protein identified as p57 on the surface of *R. salmoninarum* may aid survival of the bacteria within macrophages, as this has been reported as an inhibitor of respiratory burst activity (Kaattari, Chen, Turaga and Wiens 1988). In the study by Guttenberger (1993) the bacteria were observed to escape from the phagosomes into the surrounding cell cytoplasm. Therefore, escape into the cell cytoplasm to avoid phagolysosomal fusion would be another very efficient evasion strategy.

No detailed information was found about the interaction of fish macrophages and streptococci or rickettsial-like organisms in the present day literature.

Although protection of the bacteria from the destructive immune response is afforded by dwelling within the macrophages, the vacuolar membrane of non-fusogenic vacuoles restricts the access of the bacteria to the nutrient-rich cytoplasm (Sinai and Joiner 1997). Nevertheless, intracellular pathogens are able to survive and replicate within the phagocytes and therefore must be able to obtain nutrients for growth. It may be that these intracellular microbes have modified the vacuole membrane in some manner to allow access to the nutrients and metabolites, which are necessary for microbial growth. This has not yet been elucidated at the molecular level (Sinai and Joiner 1997).

Available treatments

In aquatic systems, over-application and combined chemotherapy has resulted in antibiotic resistant bacterial strains and hindered future treatments. Although other preventative measures (such as traditional treatments and physical barriers to the culture system) are currently in operation, antibiotic treatments are still applied and in certain circumstances, they are the only option available, but their use against intracellular

pathogens has been limited. To be effective, antibiotics must penetrate the cell and be retained in the infected subcellular compartments. Here, they need to be able to express their activity against the infectious agent. The ?-lactams are ineffective against intracellular pathogens because they do not concentrate within the cell. Aminoglycosides can penetrate the cell but at a very slow rate and they may accumulate in the lysosomes, where the acidic pH value destroys their activity. Lincosamides, macroglides and fluorquinolones all accumulate within the cell, but display various activity due to their deposition within organelles (Tulkens 1991).

***Mycobacteria* spp.**

Treatment of mycobacterial infections is difficult due to the pathogen being intracellular and a slow growing organism when cultured in the laboratory. However, addition of chloramine B or T at 10 mgL^{-1} for twenty-four hours to a tank of naturally infected animals did control the spread of the infection (van Duijin 1981). The effects of two treatments applied in combination and singularly were investigated as possible therapeutants against mycobacteriosis in sea bass (*Dicentrarchus labrax*). The two agents were streptomycin and allicin (as a garlic extract) and were given to the infected fish by injection. The results indicated that injection of streptomycin produced a regression of splenic lesions, where the granulomas appeared reduced in size and number (Colorni, Avtalion, Knibb, Berger, Colorni and Timan 1998). However, application of streptomycin appeared to immunosuppress the fish as was shown by a sudden increase in antibody titres when the treatment was stopped. Also the bacterium could be recovered from surviving fish at the end of the experiment, therefore, further work is still required to elucidate the actual applied use of streptomycin and allicin as potential treatments against mycobacteriosis (Colorni *et al.* 1998).

Nocardia *spp.*

Van Duijn (1981) reported a successful treatment of infected fish after administering a combination of sulphisoxazole and deoxycycline or monocycline by oral or parenteral routes. Kusuda and Nakagawa (1978) experimentally infected Japanese yellowtail with heat-killed nocardiae and these fish developed high serum agglutinating antibody levels within six weeks post-bacterial challenge. The authors suggested that vaccination may be a possible means of treatment but a reliable treatment of fish infected with nocardia is

not yet available because in most circumstances, experimental infection has proved difficult (Snieszko, Bullock, Dunbar and Pettijohn 1964). This may suggest that nocardiae may only be pathogenic under certain strict environmental conditions and monitoring the environmental conditions of the cultured stock may prove to be more beneficial compared with extensive laboratory studies involving treatments of experimentally infected fish. A study by Chen and Wang (1993) investigated the potential use of several anti-microbial agents against 16 isolates of *N. asteroides in vitro*.

Pastuerella *spp.*

Ampicillin has been investigated as a potential treatment against pastuerellosis, as this antibiotic can enter the phagocyte. A range of antibiotics have been applied in the past to treat outbreaks of pasteurellosis but multiple drug-resistant bacterial strains carrying transferable plasmids have been isolated from Japanese yellowtail farms, one year after treatment (Aoki and Kitao 1985). A florfenicol resistant gene (pp-flo) was found to be derived from a transferable R-plasmid of P. piscicida (Kim and Aoki 1996). Although a commercially recognised vaccine is not yet available, several studies in Japan have shown the use of laboratory produced vaccines effective against pasteurellosis (Wakabayashi, Toyoda and Egusa 1977, Kusuda, Ninomiya, Hamaguchi and Muraoka 1988). Following good husbandry regimes and reducing overcrowding has been shown to control the onset and establishment of pseudotuberculosis by *P. piscicida*.

BKD

An extensive search by Austin (1985) demonstrated the potential use of a range of macroglide antibiotics such as erthyromycin phosphate against BKD infections. Treatment with antibiotics is difficult even with substances that are able to penetrate and enter the macrophage cells, as the concentration of erythomycin administered orally to treat infected fish was found to be much higher than the recommended *in vitro* minimum lethal concentration (Evelyn, Ketcheson and Prosperi-Porta 1986). The effect of erthyromycin thiocyanate on the survival of BKD infected fish was investigated. The antibiotic was administered as a feed additive and a standard therapeutic dose of 100 mg kg^{-1} bodyweight for 28 days significantly enhanced the survival of experimentally infected yearling chinook salmon (*Oncorhynchus tshawytscha*), (Peters and Moffitt 1996).

Vaccination against BKD has been investigated but the progress is gradual due to the slow response of vaccinated animals to produce agglutins against the bacterium (Evelyn 1971). A more positive response to vaccination has been demonstrated after a single intraperitoneal injection, but this is not only time consuming but also costly. Therefore, at present the most effect treatment method is avoidance of the microbe within the culture facilities, which is not simple because the pathogen can be transmitted horizontally as well as vertically.

Streptococci *spp.*

Antibiotic treatment with erthromycin to cultured yellowtails and rainbow trout was effective against streptococcal infection (Katae, Kouno, Shimizu, Kusuda, Taniguchi, Shiomitsu and Hasegawa 1980 and Kitao, Iwata and Ohta 1987). Other types of antibiotics have been reported as potential treatments but antibiotic resistance quickly followed due to over-application of combined antibiotic treatments. Streptococci have been found in mud and the surrounding water thus making preventative measures very difficult. However, by reducing stressful situations such as overcrowding and overfeeding (as infected feed is a suspected route of infection) may help to reduce the severity of outbreaks (Kitao 1993b).

Rickettsia *spp.*

Treatment of rickettsial infection has proved perplexing because the transmission pathway in fish is still undetermined. Animals may be infected artificially by injection and in such cases the spread of infection appears to be horizontal, however, vertical transmission may also occur as rickettsial-like organisms have been detected in the ovaries and testes of infected fish. This has yet to be shown to occur in every diseased animal (Turnbull 1993). Prevention of spread is complex as infected fish do not always display clinical signs of disease, but animals in culture facilities are routinely screened for the presence of these small obligate pathogens.

Dormant bacteria and carrier status

If the growth conditions for the bacteria decrease, some species are able to lie dormant until the conditions improve. These bacteria are usually spore-forming where the endospore contains a dehydrated form of the microbe, which is still viable but difficult

to culture under standard bacteriology conditions. The aquatic bacterium *Legionella pneumophila* is the causative agent of Leigionnaires' disease in man. When nutrients are low in the aquatic environment, *L. pneumophila* can enter a viable but non-culturalble state (Steinert, Emoedy, Amann and Hacker 1997). This is intracellular pathogen enters a non-replicating but viable state where the bacteria are still able to infect human monocytes (Steinert *et al.* 1997). Providing a dormant stage when the environmental conditions are hostile, or nutrients are low will enhance survival of the bacterium and should not affect the virulence characteristics associated.

The intracellular pathogen *P. piscicida* was found to enter a dormant but infective stage in both sea water and sediment (Magarinos, Romalde, Barja and Toranzo 1994). Margarinos *et al.* (1994), found that *P. piscicida* could survive in the sediment or water for 6 to 12 days depending on the strain and type of microcosm and during the starvation period the metabolic activity of the microbes was reduced by more than 80%. No difference was found in the biochemical, physiological and serological characteristics of the bacteria when in the dormant stage compared with viable bacteria but the dormant organisms were much smaller in size, but were still infective and pathogenic (Magarinos *et al.* 1994).

Viable bacteria recovered from clinically healthy animals

Tropical farmed frogs

During an ODA-funded study investigating the innate defence mechanisms of tropical farmed frogs, *Rana rugulosa*, bacteria were observed in the macrophage cells isolated from apparently healthy animals. Therefore, a ten-week study was conducted at the Aquatic Animal Health Research Institute, (AAHRI) Thailand to investigate this finding further. The animals were bred and reared within the wet laboratory at AAHRI but were maintained within similar environmental conditions as to those found on the frog farms in Thailand. None of the frogs included in this work or previously in the study investigating the innate defence mechanisms did any of the frogs have gross signs of pathology. All frogs had an appetite and increased in weight and size, the skin was intact and moist, the eyes were clear and bright and they responded positively to external stimuli. No bacteria were recovered or grown on tryptone soya agar (TSA) plates when

the internal organs were sampled, using routine bacteriology methods (Frerichs and Millar 1993).

During viable macrophage cell counts, motile bacteria were observed by light microscopy but they were difficult to recover using standard microbiology methods, however, colonies were grown on TSA plates after a 24-hour broth enrichment step. This produced in a wide range of bacterial colonies where the dominant colony type was selected and a pure culture identified by primary (Gram, shape, oxidase, motility and oxidation/fermentation) and biochemical (API 20E BioMeriuex UK) identification tests, following the methods in Frerichs and Millar (1993). The results from the 10-week study found that all of the frogs sampled had bacteria visible in their macrophage preparations and identification of the bacterial colonies recovered produced mixed species, commonly grouped as, Gram negative, motile rods. Further biochemical analysis of the dominant bacterial growth yielded, produced a range of opportunistic pathogens, ubiquitously found within the surrounding aqueous environment of the cultured tropical frogs. Observation of the alcohol fixed and stained macrophage cells attached to glass slides showed that a large number of macrophages had bacteria inside the cytoplasm. Bacteria were also observed extracellularly, and these were thought to have originated from lysed macrophage cells that had ruptured during the initial isolation process.

This was an unusual finding as it is general accepted that clinically healthy animals do not harbour high bacterial loads within their cells or tissues, without displaying signs of disease. However, a study by Haynes, Harding, Keniski and Cohen (1992) found that a laboratory population of clinically healthy frogs, *Xenopus laevis*, had a small cocco-baccillus bacterium associated not with the macrophage cells but with the splenic lymphocytes. Haynes *et al.* (1992) also reported difficulty in recovering the bacterium but it was evident and spread naturally throughout the laboratory populations. No associated gross pathology was observed in the *X. laevis* infected with the bacteria and the authors did not identify the bacterial species to genus or species level.

The clinical significance of finding bacteria associated with the splenic macrophages from the farmed *R. rugulosa* was unknown, but worthy of further investigation. It was also thought to be profitable to find out whether fish species, cultured in Thailand and

kept in similar conditions to the farmed frogs also had bacteria visible in their macrophages.

Tropical farmed fish

Small-scale freshwater fish farmers in Thailand often culture fish and frog species interchangeably. This is because the market price for aquatic species fluctuates widely and so the most economically viable species will be cultured at that time. Although small changes are made, in general, the culture conditions on the farms do not vary to accommodate the different species farmed. A study was conducted to investigate the presence of bacteria in macrophages isolated from the head kidney of cultured freshwater hybrid catfish (*Clarias gariepinus* x *C. batrachus*).

Five farms in different provinces of Thailand including Bangkok, Kalasin, Supanburi, Patumthani and Lopburi were selected and the fish sampled. This involved a brief examination of the external and internal organs of the animals before a small section of the head kidney from the catfish was aseptically taken and streaked directly onto TSA plates. This was completed at the pond-side to investigate the ease of bacterial recovery from the internal organs of clinically healthy farmed fish. Bacterial growth was checked up to 48 hours later. Fish (four to six animals per farm) were then taken back to the wet laboratory at AAHRI and sampled for bacterial recovery as detailed for the pond-side. The macrophage cells were then isolated from the head kidney using the method of Secombes (1990) on different density gradients and a purified macrophage suspension was placed on a cleaned microscope slide and the cells left to attach for 2 hours at 22^0C.

The unattached cells such as erthyrocytes and lymphocytes were removed by washing and the cells fixed and stained. Observation of these slides by light microscopy under oil immersion showed many macrophages with activated morphology (large, spread, some vacuoles present) and bacteria internalised within the cytoplasm. Most cells had bacteria present, and often there were more that one bacterium inside the cytoplasm. A lot of bacteria were observed extracellularly, as shown previously with the frog work.

When the internal organs of the fish were sampled at the pond side and in the laboratory after transportation only non-specific growth was recorded. Pure bacterial growth was recovered from the purified macrophage suspensions sampled in the laboratory at AAHRI after a 24-hour broth resuscitation step. The same species of bacteria was not

recovered from the fish sampled but a range of Gram negative, motile, bacilli were recovered and identified from the macrophage suspensions (Frerichs and Millar 1993). It was very interesting to note that a single specific pathogen was not recovered but instead a range of potential opportunistic microbes, commonly found in the aqueous environment of the fish.

The farmed fresh-water frog and fish studies produced similar results. A high number of macrophage cells isolated from the spleen and head kidney respectively, had bacteria present in the cytoplasm of the cells. Although the bacteria were clearly visible under light microscopy they were difficult to recover without an enrichment step. Nevertheless, preliminary identification of the bacterial species recovered produced a wide range of potential opportunistic bacteria, commonly found in the aqueous environment. Although the clinical significance was unknown, a possible relationship was suggested between the bacterial load in the water an the presence of bacteria in the macrophages. A brief study was conducted to investigate the presence of bacteria in laboratory bred and reared fish species.

Laboratory fish populations

Work was carried out at Institute of Aquaculture, Stirling to investigate the presence of bacteria isolated from the head kidney macrophages of clinically healthy laboratory fish populations. These animals had been bred and maintained under laboratory conditions. The aquatic species included tropical and cold water animals, where macrophage cells were isolated from head kidney of various fish species (salmon, trout, catfish, nile tilapia, redbelly tilapia) using the method of Secombes (1990).

Prior to macrophage cell isolation, the animals were observed for any external or internal signs of disease and then a small section of the head kidney was aseptically taken and streaked directly onto a TSA plate and incubated for 24 hours at 22^0C. Any bacterial growth recovered was recorded and this was done to investigate the ease of bacterial recovery from the internal organs of these laboratory fish. After macrophage isolation, alcohol-fixed and stained macrophage suspensions were observed under oil immersion by light microscopy to look at the macrophage cells morphology as well as observe the presence of bacteria in the macrophages. A small volume of the macrophage suspension received a 24-hour broth resuscitation step before being

streaked onto an TSA plate and incubated at 22^0C and examined after 24 hours and any bacterial growth was recorded and pure colonies identified using the method of Frerichs and Millar (1993).

This produced very interesting results as bacteria were observed in the macrophages of only a few of the tialpia and none were seen in the macrophage cells isolated, fixed and stained from the other tropical and temperate fish species examined. The number of macrophages with bacteria was very low and again there was difficulty in recovering and growing the bacteria on agar plates without a broth resuscitation step, however, bacteria visible in the macrophage suspensions.

Conclusion and Summary

?? Intracellular bacteria have evolved similar evasion strategies in fish species as detailed for mammals.

?? Survival mechanisms of all of the intracellular bacteria have not yet been elucidated and further work is required. However, from the current day knowledge a range of evasion strategies are employed by the intracellular pathogens of fish and similarities have been observed between the bacterial evasion methods adopted in mammals and fish.

?? Due to the intracellular nature of these pathogens, treatment is difficult therefore, other management strategies on fish farms have been applied.

?? Cultured tropical fish and frogs in Thailand appear to have some bacteria in their macrophages. The number of animals with bacteria and the bacterial load within the cells isolated was higher in tropical farmed frogs and fish, compared with the laboratory fish. No bacteria were recovered or observed in the macrophages isolated from the cold water fish species.

?? The bacteria observed in the macrophages isolated from the tropical farmed frogs and fish were not determined alive or dead from the work conducted and further studies are required to investigate this. It may be that the bacterial presence results in an immunocompromised status or the bacteria may indeed be priming the macrophages to help clear the opportunistic pathogens frequently found in the aquatic environment.

?? Due to the range of bacterial species recovered and the fact that a single faculative pathogen has not been found associated with the macrophages may suggest that the bacterial species isolated and identified have originated from the environment, as potential opportunistic pathogens only were found.

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Appendix 6

Background information relating to water quality [1]

Pond water originates as precipitation, and contains small amounts of inorganic and organic materials. It is also saturated with carbon dioxide (CO_2), making it slightly acidic. Run-off, rainwater that has passed through soil and rocks, becomes enriched with different minerals. This enters the water systems feeding the ponds.

The parameters, which influence water quality interact directly with each other, and can affect the environment of the cultured fish. Physical, chemical and biological parameters are important when considering the dynamics of the fish ponds. The amount of nutrients available, the alkalinity, and hardness of the water influence plant productivity, which, in turn, has direct impact on the food chain of aquatic animals. Although, the diet of most cultured species is supplemented, there is a highly positive correlation between the level of plant growth and the productivity of aquatic system.

Temperature is very important, firstly because many biochemical processes within the pond are temperature-dependant, and secondly fish are poikilothermic, with their internal body temperatures reflecting that of their environment. This has important implications for the immune response of the fish and hence their ability to fight infection. The pond temperature closely follows that of the air, and so is influenced by both season and location. The temperature and pH of farm water can vary over the course of the day. It was only possible to measure these parameters once a day at the time of sampling the fish. This restricts interpretation of the water quality data, but was a foreseen limitation at the time of planning the study. Samples were taken and analysed so as to establish trends between the different farming systems over the two sampling cycles and water temperature appeared to be constant throughout each sampling cycle (29 TO 30^0C).

The alkalinity of the water refers to the amount of tritratable bases (bicarbonate, carbonate, ammonia, hydroxide, phosphate silicate and some organic salts) present. In fish culture systems, it is usually bicarbonate, carbonate or both which dictate the level of alkalinity of the water. High alkalinity can reflect excessive evaporation of the water, whereby ions, present in the water, are concentrated. Water of low alkalinity can be vulnerable to pH fluctuations because of the low levels of buffering ions present. Low

[1] 15. Boyd, C.E (1996) Water Quality in Ponds for Aquaculture pp 477, Shrimp Mart (Thai) Co. Ltd., Thailand.

pH, in turn, can be harmful to the fish, although both high and low pH can cause gill damage, and thus impairing respiration. Water of low alkalinity is usually associated with sandy or organic areas. At the start of the wet season, water pH can fall dramatically as a result of acid rain, and run-off from the land enriched with pesticides.

CO_2 is necessary for photosynthesis by aquatic plants, and levels within the water are related to pH, temperature and alkalinity. Dissolved oxygen (DO), the amount of soluble oxygen present in the water, is related to temperature; DO decrease as water temperature increase. In natural waters, DO concentrations are constantly changing and biological parameters have a bigger impact on the DO than physical ones. The chemical oxygen demand (COD) is a measure of how much oxidisable organic material is present. Plants produce oxygen from photosynthesis, which in turn is controlled by temperature, light, available nutrients, plants species and density, and water turbulence. Plants and animals both require oxygen. In ponds where there is a high rate of photosynthesis and low levels of respiration, CO_2 levels will be low and DO concentrations will be high. If the rate of respiration is greater (i.e. the oxygen demand increases) than that of photosynthesis, the amount of DO will decrease and CO_2 levels will increase. The amount of oxygen required by the culture system depends on the species cultured, size of the fish, food intake, activity, water temperature and the available DO and levels of 5 mg L^{-1}or more are required by most fish, however, this value depends on the species cultured. Some species of fish can survive at 3 mg L^{-1} DO, while others need 7 mg L^{-1} or more.

The amount of light penetration into the pond is regulated by the turbidity of the water, the presence of suspended or colloidal particles. High water turbidity due to phytoplankton is useful as this reduces underwater weeds, and increases fish production by acting as an additional food source. High turbidity due to suspended particles such as clay is bad for both fish and the water system. However, turbidity levels in ponds are generally low. In culture ponds, the amount of available nutrients and phytoplankton increase during the warmer months. High levels of suspended soil can impair the respiration of the fish and limit the amount of available light, thus reducing photosynthesis, which may in turn, reduces the DO levels and increase CO_2 levels. The colour of the water is a subjective indictor, commonly used by farmers to assess the water quality of their system. This is really a measure of the turbidity, and if water is clear then there is little primary production present (vegetation and phytoplankton) and if it is too high, DO levels may be low. The amount of total suspended solids (TSS)

includes the level of organic (OSS) and inorganic suspended solids (IOSS). Suspended solids can include feed waste, phytoplankton, faecal material and the IOSS may arise from clay or other such particles. The amount of suspended solids in the water is of concern as these particles can cause gill damage to the fish and induce stress as by impairing respiration.

Nitrogen and phosphorous levels indicate the amount of available nutrients and primary production potential present within the pond. Phosphorous is an essential metabolic nutrient and all waters contains organic phosphorous. Inorganic forms, however, are present as polyphosphates from fertiliser. Concentrations of phosphorous within water systems are usually low (5 to 20 ?g L^{-1}). Water contains both inorganic nitrogen, as a result of nitrogen fixation, and organic nitrogen from the decay of organic matter. Organic nitrogen exists as amino groups in protein, which are deaminated by microbes to release ammonia (NH_3) into the environment. Ammonia dissociates to form ammonium ions ($NH_3 + H^+ = NH_4^+ = OH^-$). The ratio of NH_4^+ to NH_3 increases with pH and water temperature. Analytical procedures measure total NH_3, which contains a mixture of both unionised (NH_3) and ionised (NH_4^+) ions, both of which can be used by aquatic plants or nitrified to nitrate. The oxidation of NH_4^+ is a potential source of acidity in the aquatic environment. In the presence of low oxygen levels, microbes use nitrate or other oxidised forms of nitrogen for respiration. The rate of nitrogen fixation in ponds is unknown, but is considered to be substantial. The major source of NH_3 in fish ponds is from excretion. Although both NH_3 and NH_4^+ are toxic to fish, of the two NH_3 is more so. If concentrations of NH_3 increase within the water, NH_3 excretion by the animal decreases, and NH_3 levels in the blood and tissues of the animal increases. This results in increased blood pH, which in turn affects enzyme-catalyzed reactions and membrane stability. Increased levels of NH_3 result in higher oxygen consumption by tissues, gill damage and reduced oxygen transport by the circulatory system. Chronic NH_3 exposure can lead to increased disease susceptibility and poor growth rates. Daily fluctuations of pH within the culture systems can affect the level of NH_3.

Nitrite (NO_2) levels in the water of fish ponds is also important. NO_2 is an intermediate product formed during the nitrification of NH_3 to nitrate (NO_3) and is toxic to fish and other aquatic organisms. Concentrations within freshwater systems are usually low, unless there are high levels of ammonia present. When absorbed by the fish, NO_2 reacts with heamaglobin to form methhaemaglobin, and this is unable to

transported oxygen. Thus, NO_2 toxicity causes anaemia, and is sometimes referred to 'brown blood disease' because of the brown colour of the blood resulting from the methaemaglobin. Sub-lethal concentrations of NO_2 increase disease susceptibility of fish. The levels of NO_2 in the fish are influenced by water pH, animal size, previous exposure, nutritional status, any current infections and DO levels of the water.

In culture systems additional feed is supplied to the fish to enhance growth and this generally increases the concentration of nitrogen, phosphorous and organic matter in the system. This may then result in an increase in oxygen demand, which will increase concentration of NH_3, NO_2 and CO_2 in the system. An efficient farming system should consider such factors and how they interact and influence one another and how to maintain manageable levels which will not adversely affect the fish stocked.