

**Department for International Development**  
**Strategy for Research on Renewable Natural Resources**  
**RD1 (12/96)**

**Control of Bacterial Disease in Small Scale Fresh-Water Aquaculture**  
**Project R7054**

**Project Completion Report**  
**December 1998 - May 1999**

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## Scotland

## 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

	<u>Planned</u>	<u>Actual</u>
<b>START DATE:</b>	1/12/97	1/12/97
<b>FINISH DATE:</b>	31/5/99	31/5/99
<b>TOTAL COST:</b>	£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<sup>1, 2</sup>. 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<sup>3, 4</sup>. The innate immune defences of animals kept in good conditions can withstand a degree of bacterial challenge, but readily succumb to virulent pathogens<sup>5, 6, 7</sup>. Continued exposure to less virulent pathogens is also detrimental, and fish under-perform if held in poor quality water<sup>7</sup>. 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<sup>8, 9,10</sup>. A small study on tropical farmed freshwater fish showed similar findings<sup>11</sup>. 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<sup>1</sup>

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

**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<sup>2</sup>, which on average is equivalent to 9 adult fish per m<sup>2</sup>. 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<sup>2</sup>. 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<sup>3</sup>. 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<sup>2</sup>. 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 Thanh 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 Thanh Village, in Can Tho 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<sup>2</sup> in area, and was stocked at around 10 fish per m<sup>2</sup>. 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<sup>2</sup> in size with a stocking density estimated at 40 fish per m<sup>2</sup>. 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<sup>3</sup>, and were stocked at a density of 50 fish per m<sup>3</sup>. 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<sup>3</sup>. 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 used 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 led 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<sup>2</sup>, but this varies depending on the system, with thresholds of 20 or 30 fish m<sup>2</sup>. 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 liaise 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<sup>0</sup>C compared with a usual temperature of 22 to 25<sup>0</sup>C. 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<sup>14</sup>, 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°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°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<sup>-1</sup> on *As* and between  $10^3$  and  $10^7$  cfu ml<sup>-1</sup> on TSA in water sampled from the four sites. A range of bacterial species was recovered, but most remained unspiciated.

In most freshwater systems the ideal alkalinity levels are between 20 to 300 mg L<sup>-1</sup> 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  $< 0.1 \text{ mg L}^{-1}$ , 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 is not known 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  $10,000 \text{ mg L}^{-1}$ . 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 effect, since cycle 2 was carried out in the dry season, while cycle 1 analyses were 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<sup>-1</sup>.

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<sub>3</sub> 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)<sup>15</sup> 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 µm nylon mesh into a universal containing 2 ml of Leibovitz-15 medium (L-15) (Sigma U.K), 10 Units ml<sup>-1</sup> 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 ( $1 \text{ mgml}^{-1}$ ) 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  $\mu\text{l}$  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.<sup>16, 17</sup> 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 or not. It was therefore discontinued 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 its 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 cycle 1. 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 losses due to disease. In Vietnam, sandgoby 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 exacerbated 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 so, 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°C (air temperature in the wet lab). The reduced temperature experiment was carried out in an air-conditioned aquarium.

The challenge organism, *Aeromonas hydrophila* (T<sub>4</sub>) 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 BioMerieux, France). A bacterial suspension ( $1 \times 10^8$  cfu ml<sup>-1</sup>) was passaged twice intraperitoneally through adult fish, and recovered from the liver of dead animals. Bacteria were prepared at  $1 \times 10^5$  cfu ml<sup>-1</sup> 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 inflicted 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 stressors 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*, mortalities 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 known 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 placed 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 in replicate tanks (60 x 90 x 60cm<sup>3</sup>) (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<sub>1</sub>/GFGT<sub>2</sub>) and fish from the poor farm, maintained in good water quality (PFGT<sub>1</sub>, PFGT<sub>2</sub>)]. 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<sub>1</sub>/GFPT<sub>2</sub>) and fish from the poor farm, maintained in poor water quality (PFPT<sub>1</sub>, PFPT<sub>2</sub>)].

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<sup>0</sup> 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 mortality 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, ( $\chi^2_1 = 0.802$ ,  $P = 0.370$ ). 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  
January 1999 - March 1999

DFID Funded Project R7504- Bacterial Disease in Fresh Water Fish  
Dr. Kim Thompson and Ms. Margaret Crumlish  
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## 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.



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## **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 single water parameter could be identified which continuously influenced 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

<i>As:</i>	<i>Aeromonas</i> selective medium
AAHRI:	Aquatic Animal Health Research Institute
CFU:	colony-forming units
CO <sub>2</sub> :	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 <sub>2</sub> :	Nitrite
NO <sub>3</sub> :	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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