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SID 5 Research Project Final Report

- **Note**

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  A SID 5A form must be completed where a project is paid on a monthly basis or against quarterly invoices. No SID 5A is required where payments are made at milestone points. When a SID 5A is required, no SID 5 form will be accepted without the accompanying SID 5A.

- This form is in Word format and the boxes may be expanded or reduced, as appropriate.

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Project identification

1. Defra Project code: FC1164-SARF001

2. Project title:

   Preventation and management of Ichthyophthirius multifiliis

3. Contractor organisation(s):

   Institute of Aquaculture
   University of Stirling
   Stirling
   FK9 4LA

4. Total Defra project costs: £ 30,049

5. Project: start date ..... 01 January 2005

   end date ..... 31 July 2005

6. It is Defra's intention to publish this form.
Please confirm your agreement to do so.....**YES** ☒ **NO** ☐

(a) When preparing SID 5s contractors should bear in mind that Defra intends that they be made public. They should be written in a clear and concise manner and represent a full account of the research project which someone not closely associated with the project can follow.

Defra recognises that in a small minority of cases there may be information, such as intellectual property or commercially confidential data, used in or generated by the research project, which should not be disclosed. In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain. Where it is impossible to complete the Final Report without including references to any sensitive or confidential data, the information should be included and section (b) completed. NB: only in exceptional circumstances will Defra expect contractors to give a "No" answer.

In all cases, reasons for withholding information must be fully in line with exemptions under the Environmental Information Regulations or the Freedom of Information Act 2000.

(b) If you have answered NO, please explain why the Final report should not be released into public domain

n / a

Executive Summary

7. The executive summary must not exceed 2 sides in total of A4 and should be understandable to the intelligent non-scientist. It should cover the main objectives, methods and findings of the research, together with any other significant events and options for new work.

The ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876, "Ich" or whitespot disease is recognised to be one of the most pathogenic diseases of wild and cultured freshwater fish. Mortalities result when large numbers of the mature parasitic trophont stage exit the host causing disruption to the epidermis and interfere with the osmotic balance of the host. Heavy *I. multifiliis* infections are strongly correlated with high water temperatures and low rainfalls which facilitate the development and multiplication rates of the parasite within a farm system. Infections persist at low levels over the winter months and increase in severity with rising spring temperatures. Outbreaks result from a massive multiplication within farm sites as opposed to a large influx of parasites from connecting water supplies. *Ichthyophthirius multifiliis* has now overtaken proliferative kidney disease as the number one parasite problem within the UK trout industry and accounts for an estimated 2-5% loss in annual production (~360-900 tons) amounting to £2 million in lost revenue. The EU ban on the use of malachite green and dimetradizole as efficacious treatments in food fish, has left the industry without suitable replacements for the treatment of *I. multifiliis.* At present, only formalin, chloramine-T and a number of disinfectants that include the active agents hydrogen peroxide and sodium percarbonate are permitted for use against *I. multifiliis* in the EU. The activity of these chemicals is limited and they are effective only in the killing of the external stages of the parasite present in the water column during the period of the chemical bath necessitating multiple baths to control outbreaks.

The objectives of the current 7-month project were to test the efficacy of three novel approaches to control and treat outbreaks of *I. multifiliis* in British rainbow trout, *Oncorhynchus mykiss,* farms. Specifically, these approaches were a) to conduct field and laboratory trials with a novel in-feed nutriceutical; b) optimise the chemical constituents of a "raceway treatment" and conduct laboratory and field trials to assess its action on different stages of the parasite's life-cycle; and c) build and test a mechanical modular system, install it within a working hatchery system and assess its efficacy over a period when the likelihood of an *I. multifiliis* outbreak was high.

Trials by other authors demonstrated the *in vitro* efficacy of the nutriceutical caprylic acid against the marine ciliate protozoan *Cryptocaryon irritans* and against coccidia in piglets in *in vivo* trials. A 10-day regime of a 2.5ppm caprylic acid medicated diet fed to *O. mykiss* experimentally infected with *I. multifiliis* effected a 67.65% reduction in the number of trophonts

surviving on fish when compared to a set of triplicate controls (p = 0.001). The trials were repeated using diets containing nominal 10ppm and 25ppm caprylic acid and a third diet containing 2.5ppm caprylic acid mixed with 1.25ppm Citrox BC. A 12-day regime of medicated feed prior to infection continued by a further 6 days after infection failed to elicit a reduction in establishing trophont numbers in any of the test groups. Additional trials with oregano and salt did, however, produce better results. A 12-day regime containing 1.62ppt oregano oil reduced trophont numbers by ~30% (43.5 ± 12.6 on the oregano group *cf* 62.0 ± 21.4 trophonts per fish on the control group). This reduction was statistically significant (p = 0.0015). *In vitro* trials with salt were also performed. The use of salt is favoured over formalin as a treatment when water temperatures exceed 20^{o}C, however, salt baths for longer than ~8hrs on a farm are difficult to implement. A series of 4-hour trials using salt at concentrations ranging from 0-35ppt found that concentrations of 15ppt or above were effective in killing all the tomonts in the *in vitro* trials. Following this work, this regime has now been adopted for use on farms for controlling *I. multifiliis* in the water column in large culture systems (large raceways, ponds etc).

The second objective of the project was to investigate the efficacy of a paint, Sealant-X, in reducing the number of tomonts able to encyst upon it thereby reducing the number of developing cysts and the number of theronts surviving to reinfect fish. Earlier trials had produced mixed results so trials with a new formulation of Sealant-X were tested and found to reduce establishing infections by 17.94%, however, no statistically significant differences between the groups was observed (p = 0.2707).

In the production of the mechanical device, a series of supporting experiments were necessary to support its development. These included experiments on tomont settlement behaviour, the speed of parasite development and the minimum forces needed to dislodge cysts. To investigate tomont settlement, tomonts were dropped into an aquarium from which it was determined that 72.6% (n = 98) of tomonts settled on the bottom of the aquarium while 27.4% settled within 3.2cm of the bottom of the tank. The minimum suction pressure required to remove encysted cysts was determined by calculating the volume of water expelled from a syringe and the time taken to dislodge the cyst. Repeat trials (n = 6) determined that a minimum pressure of 0.001675 Pa was required to dislodge cysts. Further work on cyst development at a range of temperatures and pHs was conducted to determine the shortest development time and, therefore, the frequency with which the device should be used in farm raceways. Trials found that cyst development was fastest at pH7 (24.3 hrs from tomont to theront at 22^{o}C) which slowed as the pH moved away, in either direction. Trials, however, showed that as the water temperature rises above 22^{o}C, the device should then be used twice a day, at least seven hours apart to remove the majority of cysts as the time taken from tomont to theront takes ~18.5hrs at 24^{o}C and pH7.

The primary mechanical device or "System*Ich*" consists of a special suction head connected to a pump that is used to vacuum the bottom of raceways to remove parasite cysts. A secondary and equally necessary device was the use of a low adhesion polymer sheeting (Pisces Wsp20-05) that was used to line concrete raceways. A series of polymers / coatings (n = 6) were tested to elucidate the most efficacious in preventing cyst attachment. Two were found to kill 100% of tomonts in *in vitro* trials, however, the high cost of one, Ecosea Cuproguard^{TM}, would be prohibitive for use in fish farms, and the second, Sealant-X (already tested under Objective 2 of this project) was found to produce less than satisfactory results. The third best performer of those tested, Pisces Wsp20-05, killed 90.21% of the tomonts tested in the *in vitro* trials and was chosen for use in the field trials.

Field trials were conducted on a commercial trout farm in the south of Scotland over a period of three months (mid-May until mid-August 2005). Six raceways (6m long × 1m wide × 1m deep) were used, three of which were lined with the 6mm thick polymer sheeting (Pisces Wsp20-05). Each lined raceway was vacuumed once per day (one pass up the raceway and one pass back); the control raceways were brushed as normal. Every two weeks, twenty fish from each raceway were sampled and the total number of trophonts on each fish were determined. Statistical analysis confirmed the efficacy of the System*Ich* device in reducing establishing trophonts numbers by 99.4+% (p<0.0001).

Following the fifth field sample (8th July), trophont numbers on the control fish were dangerously high and were treated with a series of formalin treatments to control numbers. Four formalin treatments were administered over the next 10 days in both the test and the control raceways. It was questioned what were the benefits of the polymer sheeting lining raceways and what additional reductions were afforded through the use of the device. At this point the use of

the suction head was also withheld. After the use of the suction head was withheld, trophont numbers on the fish in the lined raceways slowly increased but remained lower than levels on the control fish suggesting that the lining is also able to reduce the number of cysts settling and surviving in raceways by about 39%. Survival analysis applied to the mortality data over this latter phase (25 days) of the field trial showed that the lined raceways also had lower total mortalities with only 3% of the total stock dying *c.f.* 6% in the control raceways. Over the course of the trial mortalities were 2× higher in the control raceways than in the treated (1662 morts in the control raceways *c.f.* 858 morts in the test raceways).

The benefits of System*Ich* device is that it is a non-chemical approach that uses a combination of a suction head and a special polymer lining (Pisces Wsp20-05) to remove cysts, uneaten food pellets and faeces. The time taken to use this device is no longer than that currently used to brush raceways but is more efficient in reducing the number of trophonts per fish (99.4+%) and we believe is less stressful. It is also believed that additional benefits result from the use of lined raceways include: a cleaner environment for the stock, more efficient removal of faeces, improved water quality, lower bacterial loads and reduced fin abrasion. It is believed that as the concrete is sealed, the levels of other fish pathogens in the system will be lower and trials to test this are underway.

Project Report to Defra

8. As a guide this report should be no longer than 20 sides of A4. This report is to provide Defra with details of the outputs of the research project for internal purposes; to meet the terms of the contract; and to allow Defra to publish details of the outputs to meet Environmental Information Regulation or Freedom of Information obligations. This short report to Defra does not preclude contractors from also seeking to publish a full, formal scientific report/paper in an appropriate scientific or other journal/publication. Indeed, Defra actively encourages such publications as part of the contract terms. The report to Defra should include:

- the scientific objectives as set out in the contract;
- the extent to which the objectives set out in the contract have been met;
- details of methods used and the results obtained, including statistical analysis (if appropriate);
- a discussion of the results and their reliability;
- the main implications of the findings;
- possible future work; and
- any action resulting from the research (e.g. IP, Knowledge Transfer).

References to published material

9. This section should be used to record links (hypertext links where possible) or references to other published material generated by, or relating to this project.

References

Hirazawa, N., Oshima, S. & Hata, K. (2001) *In vitro* assessment of the antiparasitic effect of caprylic acid against several fish parasites. *Aquaculture*, **200** (3-4), 251-258.

Hirazawa, N., Oshima, S., Hara, T., Mitsuboshi, T. & Hata, K. (2001) Antiparasitic effect of medium-chain fatty acids against the ciliate *Cryptocaryon irritans* infestation in the red sea bream *Pagrus major*. *Aquaculture*, **198** (3-4), 219-228.

Juglal, S., Govinden, R. & Odhav, B. (2002) Spice oils for the control of co-occurring mycotoxin-producing fungi. *Journal of Food Protection*, **65** (4), 683-687.

Marounek, M., Skrivanova, E. & Skrivanova, V. (2004) A note on the effect of caprylic acid and triacylglycerols of caprylic and capric acid on growth rate and shedding of coccidia oocysts in weaned piglets. *Journal of Animal and Feed Sciences*, **13** (2), 269-274.

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SID 5A Supplementary Information to Final Project Report

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Project identification

1. **Is the completed SID 5 attached.....YES** ☒ **NO** ☐
   This form is to be used in conjunction with the SID 5 form for those projects NOT paid at milestone points.
   Important: unless both parts of the SID 5 and 5A are completed and submitted together, the Final Report (SID 5) will NOT be accepted.

2. Project title
   Prevention and management of Ichthyophthirius multifiliis

3. Defra Project code: FC1164/SARF1

4. Defra Project Manager: Dr Mark James

5. Name and address of contractor:
   Institute of Aquaculture
   University of Stirling
   Stirling
   Postcode FK9 4LA

6. Contractor's Project Manager: Andrew Shinn

7. Project: start date 01/01/2005
   end date 31/07/2005

8. Final year costs: **approved** expenditure ..... £ 30049
   actual expenditure ..... £ 30049

9. Total project costs/total staff input:
   total **approved** expenditure ..... £ 30049
   total **actual** expenditure ..... £ 30049
   ***approved** staff input ..... £ 1.0
   *__actual__ staff input ..... £ 1.0
   Staff years of direct science effort

10. Is there any Intellectual Property arising from this project which is suitable for commercial exploitation .....YES ☒ NO ☐
    This requires a YES/NO answer only. All other details of any Intellectual Property must be included under the Scientific Report in the SID 5 or in an accompanying Annex.

Scientific objectives

11. List the scientific objectives as set out in the contract. If necessary these can be expressed in abbreviated form. Indicate where amendments have been agreed with the Defra Project Manager, giving the date of amendment.

The main objective of the project was to test the efficacy of three novel mechanisms for the control of *I. multifiliis* in trout farms by first of all conducting laboratory trials to optimise their use before assessing their performance under field test conditions.

Specifically, the project objectives were as follows:

1) Build and test a mechanical modular system at the Institute of Aquaculture and then test its efficacy in killing the different stages of laboratory reared populations of *I. multifiliis*.

2) Install the mechanical system within the hatchery unit of a working farm site with a history of *I. multifiliis* and assess its action over the summer period when the likelihood of an outbreak is high.

3) Optimise the chemical constituents of the "raceway treatment" and conduct laboratory and field trials to assess its action on different stages of the parasite's life-cycle. Trials will define the activity of the treatment (duration and efficacy of the anti-protozoal action) under differing environmental conditions.

4) Conduct field and laboratory trials with the novel in-feed nutriceutical to establish an effective dose and regime for its use.

Milestones

12. List the milestones for the final year.

It is the **responsibility of the contractor** to check fully that **all** milestones have been met and to provide a detailed explanation if this has not proved possible.

Milestone		Target date	Milestones met	
Number	Title		In full	On time
01/01	Construction and testing of the mechanical modular system	01/06/05	YES	YES
01/02	Installation and field testing of the mechanical system	01/09/05	YES	YES
01/03	Assessment of a novel raceway treatment	01/09/05	YES	YES
01/04	Chemotherapy trials using a novel in-feed nutriceutical	01/09/05	YES	YES

13. If any milestones have not been met in the final year or were late, please give an explanation below.

All the milestones of the project have been met.

Declaration

14. I declare that the information I have given in forms SID 5 and 5A is correct to the best of my knowledge and belief.

Name	Andrew Shinn	Date	20/09/05
Position held	Lecturer		

Objective 1: *In vivo* studies on the efficacy of selected nutriceutical compounds against stages of *I. multifiliis*

Nutriceutical compounds used for testing

Two nutriceuticals, a herbal premix containing oregano oil (Uncle Ted's Organics Ltd.) and caprylic acid (Mackie Pharmaceuticals), were selected for the *in vivo* trials (only the use of caprylic acid, however, was outlined in the initial proposal). The inclusion of the oregano trial represents an additional trial that was conducted at no extra cost to the funding body. The herbal premix containing oregano oil, also contained variable amounts of the two phenols, carvacol and thymol, both of which have been shown to be effective against a range of pathogenic and non-pathogenic micro-organisms (Juglal, Govinden & Odhav, 2002). Caprylic acid is a medium chain fatty acid found naturally in coconut oil and butter. *In vitro* and *in vivo* tests using caprylic acid against the theronts of the marine ciliate protozoan *Cryptocaryon irritans* Brown, 1951 were effective in lowering the number of trophonts infecting fish (Hirazawa *et al.*, 2001a,b). Similarly, work conducted by Marounek *et al.* (2004) were able to reduce the number of coccidia shed by piglets that had received a diet enriched with 5-10g caprylic acid / kg commercial feed. In addition to the preparation of diets containing different concentrations of caprylic acid, a further formulation was made incorporating the immunostimulant Citrox BC (Mackie Pharmaceuticals) which is made from a mix of bioflavonoids, ascorbic, malic and citric acids. Although this product is reported to have a wide range of uses, it is primarily used in the aquaculture industry as a treatment against *Saprolegnia* and furunculosis.

***In vivo* trial using a herbal premix containing oregano oil**

Feed preparation

A diet containing the herbal premix was prepared by grinding a commercial trout feed (Trouw) using a blender and adding the herbal premix at a rate of 2g/kg (as per the manufacturer's instructions for inclusion into poultry feed). Once mixed, the diet was re-pelleted using a California Pellet Mill CL2 to obtain pellets measuring 1.2 mm in size. Excess dust was removed from the diet by using a 500 micron mesh filter before use.

Source of I. multifiliis to infect the experimental rainbow trout

Tomonts were harvested using a plastic pipette as they naturally exited heavily infected rainbow trout (*O. mykiss*) collected from a commercial farm site. Tomonts were collected in batches (~30+) and maintained in washed 10ml Petri dishes containing dechlorinated, tank-conditioned water. Cysts were incubated in a Binder environmental chamber at 14°C until theronts were released.

Experimental design

A total of 180 rainbow trout averaging 5g in weight were held together in a 100 litre aerated tank within a constant temperature room at the Institute of Aquaculture. Fish were acclimated for a period of three days during which they were fed an unmedicated 2% body weight day per day ration. On day 4, the fish were exposed to ~100 theronts of *I. multifiliis* per fish overnight. Approximately 12 hours later, the fish were randomly allocated to one of six 30 litre, aerated tanks. Feeding was withheld on day 1 post-infection, but was then given in two rations, at 9am and 5pm, for the next twelve days. Fifty percent of the water in each tank was changed daily and were maintained at 12:12 L:D and 14°C over the entire experimental period (see Figure 1.1). The

experimental fish were carefully observed during feeding and any uneaten pellets and faeces were carefully siphoned from the tanks after an hour. The uneaten pellets from each tank were carefully collected onto pre-weighed filter papers and then dried in a 50°C oven overnight, then reweighed to calculate the proportion of uneaten food.

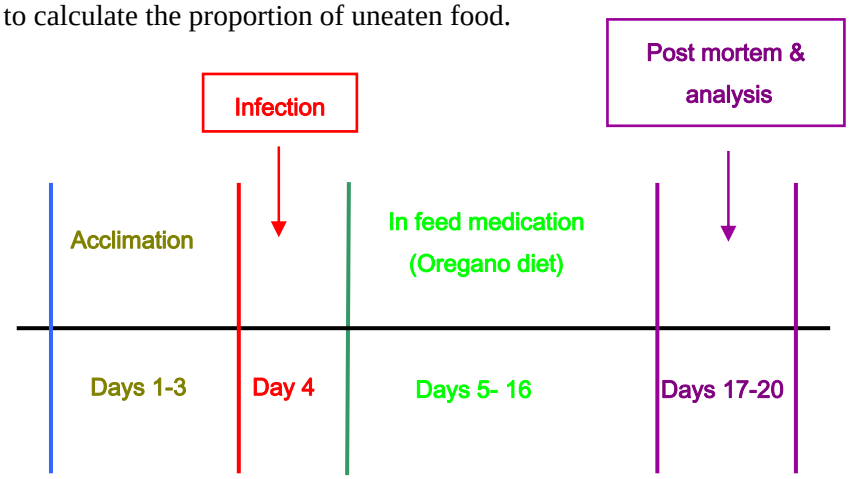
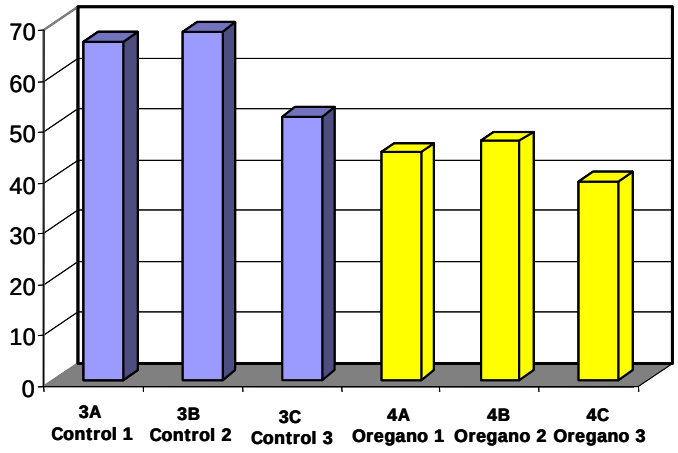


Figure 1.1: Design of the experiment to assess the efficacy of the oregano oil enriched diet against infections of *I. multifiliis.*

On day 17 of the trial a sub-sample of 10 fish were removed from each tank, killed by an overdose of anaesthetic and then the total number of trophonts on the gills, fins and body surface were counted with the aid of an Olympus SZ30 dissecting microscope. The results were then subjected to statistical analysis.

Results

The counts, expressed as the average number of trophonts per fish for each tank, are presented in Figure 1.2. Following normality and homogeneity of variances tests, the data were subjected to an ANOVA and Tukey-Kramer Multiple Comparisons Test which gave a significant result (P=0.014). An unpaired t-test applied to pooled data also gave a significant result (P=0.0015)

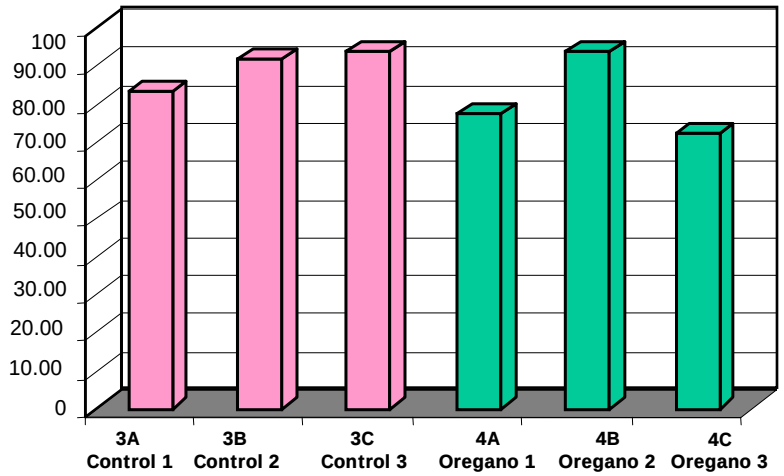


Tank name	Number of trophonts per fish
3A	66.43 ± 20.06
3B	68.29 ± 23.31
3C	51.29 ± 19.48
4A	44.71 ± 13.56
4B	47.0 ± 9.75
4C	38.86 ± 14.48

Figure 1.2: Graph of the average number of trophonts surviving on fish receiving a 12-day course of 2ppt oregano oil premix against a control set of tanks which received a normal pelleted feed.

with the average number of trophonts surviving on the fish receiving the oregano medicated feed being 30% lower than the control group (43.52 ± 12.61 trophonts per fish (oregano group) cf. 62.0 ± 21.41 trophonts per fish (control group)).

The percentage of the diet ingested was calculated from the number of uneaten pellets that were collected one hour after each ration (Figure 1.3). The results show that across the 12 day medication period 70+% of the ration was ingested in each tank (av. 89.76 ± 5.68% in the control group and an av. 81.23 ± 11.40% in the oregano group). This suggests that an ingested dose of 1.62ppt oregano enriched diet over 12 days was sufficient to give significant results and a 29.81% reduction in the number of trophonts surviving on them.



Tank name	Percentage of feed assimilated (%)
3A	83.30 ± 20.04
3B	92.01 ± 7.85
3C	93.97 ± 4.01
4A	77.38 ± 13.03
4B	94.06 ± 7.09
4C	72.25 ± 20.50

Figure 1.3: Graph of the average proportion of a control and oregano enriched diet, expressed as a percentage that was ingested over a 12-day period.

In vivo trial using the nutriceutical caprylic acid

Diet preparation

Three different caprylic acid enriched diets were prepared for *in vivo* assessment using a 99% pure formulation: 10ppm caprylic acid, 25ppm caprylic acid and a diet containing 1.25ppm Citrox BC plus 2.5ppm caprylic acid. All three diets were prepared by mixing the calculated amount of caprylic acid with cod liver oil in a ratio of 1:30 and then spraying it onto a commercial trout pellet feed (Trouw). To ensure that the bioactive compound was not lost on addition into water, 5g samples of the 10ppm and 25ppm caprylic acid enriched diets were added to 5 litres of water and left for 5 minutes. The pellets were then collected by siphoning from the tank and the amount of caprylic acid remaining within them was determined following lipid extraction using a Tecator Soxtec System. Lipid analysis revealed that there was little loss of caprylic acid from the pellets with values of 9.75ppm and 24.23ppm being recovered for the 10ppm and the 25ppm caprylic acid diets respectively.

Experimental design

Twelve 30 litre static tanks were set-up within a constant temperature room maintained at 14^{o}C and 12:12 L:D at the Institute of Aquaculture. The fish were acclimated for 4 days on a standard

commercial trout pellet at 2% body weight per day prior to the start of the trial. The fish were then fed the appropriate diet for 11 days following the procedure outlined for the oregano trial. Feeding was withheld on day 16 when the fish were infected by the addition of theronts of *I. multifiliis* to each tank. Feeding with the medicated diet continued on day 17 until day 22 after which the 10 fish from each tank were killed by an overdose of anaesthetic and the total number of trophonts on each fish were counted and subjected to statistical analysis. The key events in the experimental procedure are summarised in Figure 1.4.

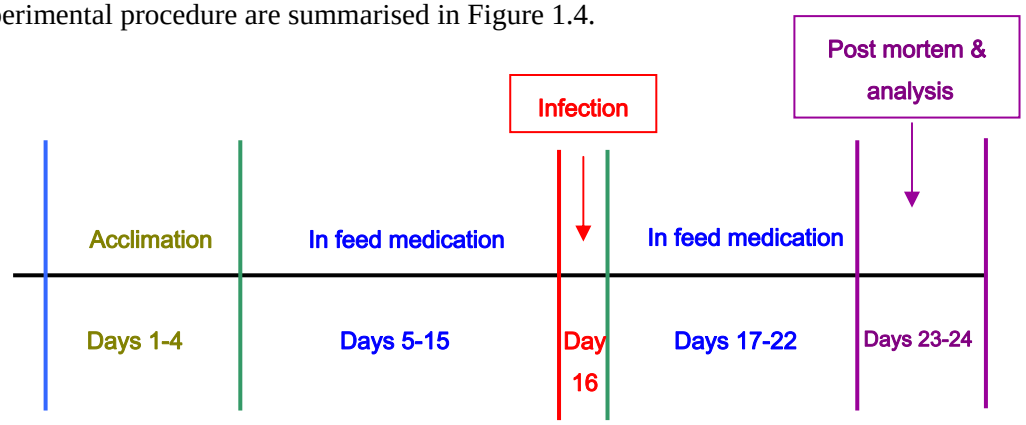
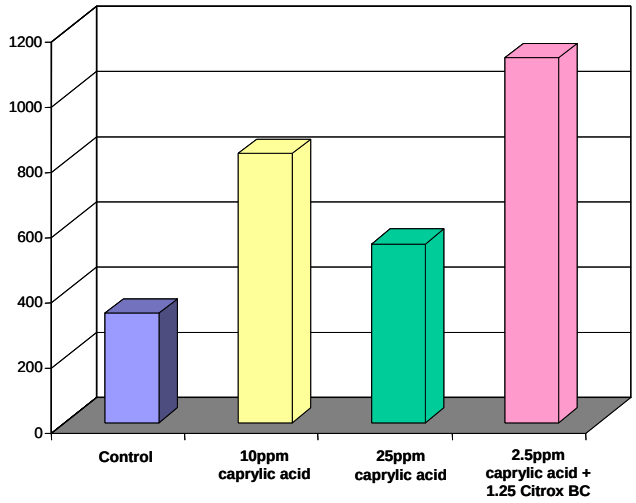


Figure 1.4: Design of the experiment to assess the efficacy of the three caprylic acid enriched diets (10ppm, 25ppm and 2.5ppm + 1.25ppm Citrox BC) against infections of *I. multifiliis.*

Results

Post-mortem examination and the counting of the trophonts began on day 7 post-infection (day 23 post-start). The results of the average number of trophonts surviving on each fish are shown in Figure 1.5. The results were surprising in that the number of trophonts surviving in groups having received a caprylic acid diet were higher than the control set, thus suggesting that the caprylic acid offered no protection against the infection of *I. multifiliis* theronts. The graphic presented in Figure 1.6 shows that the actual doses consumed over the experimental period were 8.27ppm (10ppm group), 17.94ppm (25ppm group) and 2.15ppm (2.5ppm + 1.25ppm Citrox BC group).

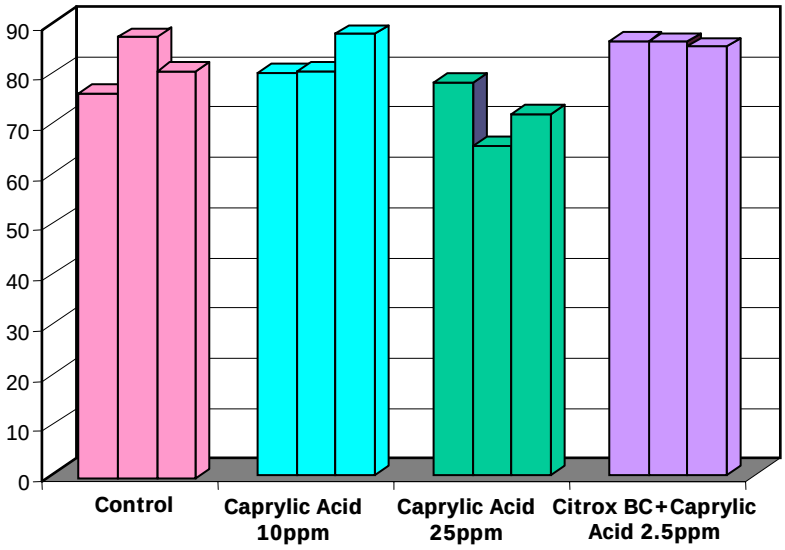


Tank Name	Av nos. of trophonts per fish
Control	336
10ppm CA	825
25ppm CA	549
2.5ppm CA + 1.25ppm Citrox BC	1119

Figure 1.5: Graph illustrating the average number of trophonts of *I. multifiliis* per fish (pooled data) fed a medicated diet of either 0ppm, 10ppm caprylic acid, 25ppm caprylic acid or 2.5ppm caprylic acid + 1.25ppm Citrox BC.

Discussion

The nutriceutical trials conducted within this part of the project suggests that when fish infected with *I. multifiliis* are given a 12-day course of an oregano oil enriched diet (~1.6ppt), there is a statistically significant reduction of 29.81% in infection level. The *in vivo* trials with caprylic acid were, however, disappointing and does not demonstrate the same effect on *I. multifiliis* as found by Hirazawa *et al*. (2001b) on *Cryptocaryon*. Despite all their experimental fish dying, they concluded that the fish groups fed a 75ppm caprylic acid diet harboured lower infection levels of *Cryptocaryon irritans*.



Tank Name	Percentage of feed assimilated (%)
Control 1	76.42 ± 14.96
Control 2	87.75 ± 20.51
Control 3	80.86 ± 20.77
10ppm CA	80.05 ± 25.25
10ppm CA	80.29 ± 27.31
10ppm CA	87.72 ± 11.86
25ppm CA	78.06 ± 29.84
25ppm CA	65.50 ± 26.82
25ppm CA	71.75 ± 27.81
CBC + CA	86.31 ± 8.98
CBC + CA	86.17 ± 19.39
CBC + CA	85.21 ± 17.29

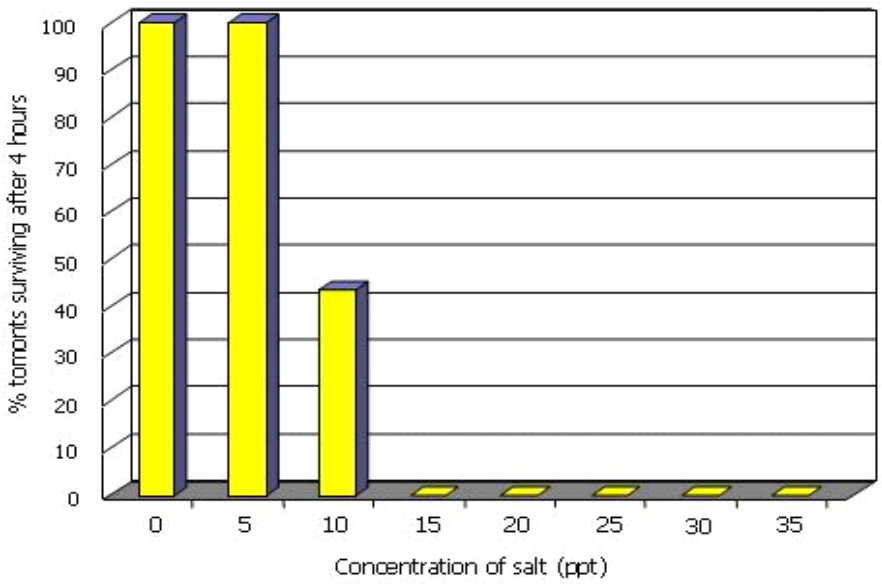
Figure 1.6: Graph of the average proportion of the appropriate caprylic acid enriched diet, expressed as a percentage, ingested over the experimental period. Abbreviations: CA = caprylic acid; CBC = Citrox BC.

Chemotherapy trials using salt

A series of chemotherapy trials using salt were also conducted to supplement the nutriceutical trials. These trials were not outlined in the original project proposal but were conducted alongside the main project objectives to develop farm husbandry practices for the control of *I. multifiliis* outbreaks on commercial trout farms. Current farm practices use multiple 1-hour, 200ppm formalin treatments to target the free-living stages of the parasite in the water column and reduce the number surviving for re-infection. However, formalin becomes more problematic at water temperatures above 20^{o}C as it removes oxygen from the water, therefore, the use of other compounds is preferrable. Salt is routinely used for a variety of fish health conditions including the water treatment of *I. multifiliis*. Given that there are practical problems in administering chemical treatments for periods of greater than ~8 hours, our farm partner asked if we could determine a concentration of salt (NaCl) that would give efficacious results within a 4-hour administration period.

Source of Ichthyophthirius multifiliis and challenge design

Tomonts naturally exiting from heavily infected 5g *O. mykiss* were collected by pipette and placed in batches of 5+ tomonts/dish into washed 20ml plastic Petri dishes in as little water as possible. Concentrations of 0, 5, 10, 15, 20, 25, 30 and 35 ppt salt were made up in volumetric flasks using 15°C carbon-filtered, dechlorinated tank conditioned water and 15ml of the appropriate salt concentration was added to a dish with tomonts. All trials were made up in triplicate and maintained in an incubator at 15°C and observed at 2 and 4 hours post-start.



Salt Concentration	Percentage of trophonts surviving after 4 h
0	100
5	100
10	43.5
15	0
20	0
25	0
30	0
35	0

Figure 1.7: The percentage of tomonts surviving after a four hour exposure to a range of salt (NaCl) concentrations ranging from 0-35ppt.

Results

The results presented in Figure 1.7 show that the tomonts were tolerant of low concentrations of salt (0 and 5ppt) and could encyst and start to divide after four hours. After 2-hours in 10ppt salt, tomonts were still alive but had not attached to the bottom of the dish and displayed little ciliary movement. After 4 hours, ~66% of the tomonts had died, while those that remained alive were in a shriveled, unattached state. At higher salt concentrations (15-35ppt), all the tomonts were shriveled after 2 hours and were dead within 4 hours. The farm now routinely uses 15ppt for 4 hours for the treatment of *I. multifiliis* when conditions for the use of formalin are unfavourable (i.e. 20+°C) and perceive this to be effective.

Objective 2. To optimize the chemical constituents of a "raceway treatment" and conduct laboratory and field trials to assess its action on different stages of the parasite's life-cycle.

The findings of an earlier DEFRA-SARF-BTA funded project (CARD FC1158) identified a paint (Sealant-X) that when used in *in vitro* laboratory trials killed 100% of the tomonts attempting to settle on it. This compound was tested under field conditions, but the results obtained were inconclusive. Although the average burden of trophonts on the fish in the painted raceways were ~40% lower than the fish in the control (unpainted) raceways, the results were not consistent

between all the raceways that were tested. Following discussions with the manufacturer, a second formulation was prepared and tested under laboratory conditions in this project.

Experimental design

Two large tanks measuring 75cm (wide) × 80cm (long) × 35cm (deep) were sub-divided using foam PVC to give three raceways measuring 25cm (wide) × 66cm (long) × 21cm (deep) in each tank (Figure 2.1). Three of these raceways were then painted with the new formulation of Sealant-X following the manufacturer's guidelines. The tanks were then allowed to dry for 2 days and then ran for three days to season them before adding fish to the system. Each raceway was covered with netting under a 12:12 L:D light regime in a constant temperature room (15^{o}C) at the Institute of Aquaculture. The water flow rate in each tank was balanced and maintained at 15^{o}C and 0.5 litres / min, a level previously determined as allowing trophonts to successfully settle within the system. A total of 240 *O. mykiss* (av. 8g) were randomly distributed across the 6 raceways (40 per raceway). The fish were fed once a day (1% body weight / fish /day) and any uneaten pellets and faeces siphoned out 1-hour after feeding. The fish were acclimated in the tanks for 7 days prior to parasite exposure.

To infect fish, tomonts were harvested as they naturally exited heavily infected *O. mykiss*. Tomonts were maintained in dechlorinated, tank-conditioned water in a 15^{o}C Binder environmental chamber until they released their theronts. Fish were infected via the water supply by adding theronts to the header tank (ca. 500,000). Fish were post-mortemed on days 10 and 24 post-infection to: 1) assess the level of infection establishing when infective stages of the parasite enter the system; and 2) to assess the efficacy of the paint in disrupting settlement and the level of re-infection.

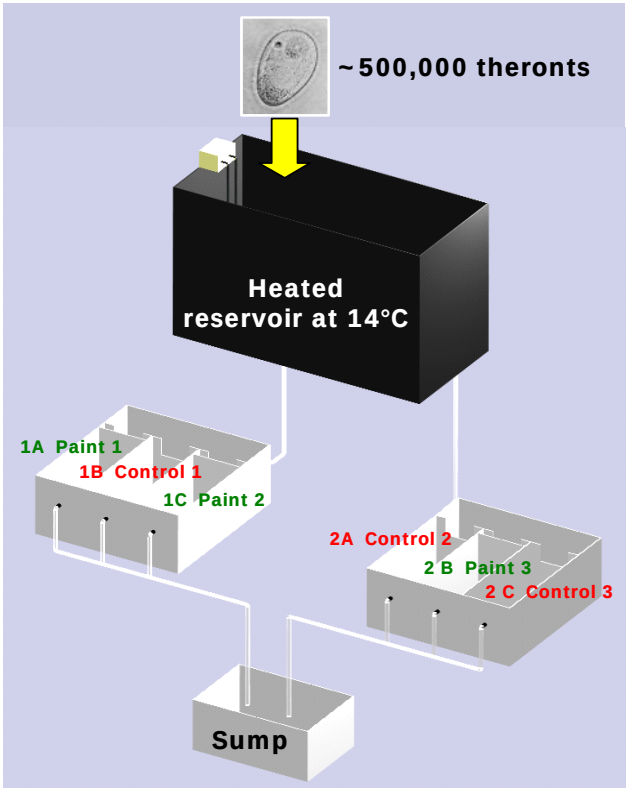
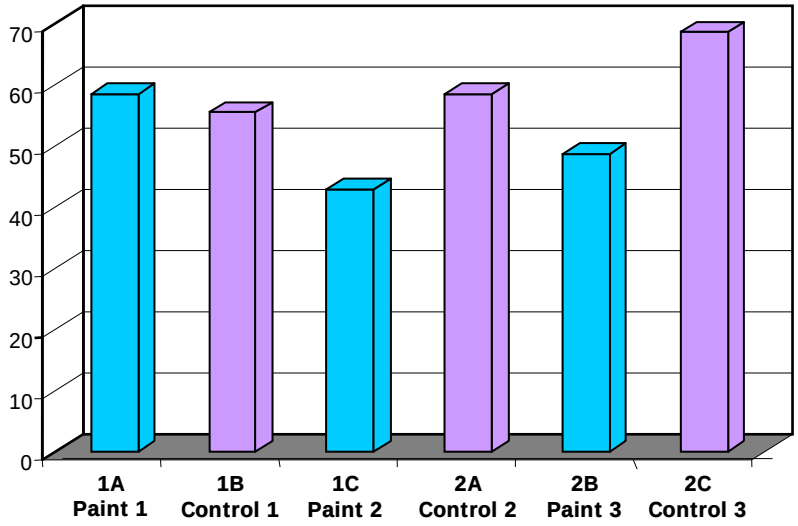


Figure 2.1: The experimental set-up used to assess the efficacy of the paint Sealant-X on disrupting *I. multifiliis* tomont settlement behaviour and their subsequent infection success.

Results

After 10 days, 10 fish from each raceway were post-mortemed and the total number of trophonts on their gills, fins and body surfaces were counted and subjected to statistical analysis. The first analysis confirmed that the infection level in each raceway was equal and that there was no difference in the number of trophonts on each group of fish. A second sample of 10 fish was taken from each raceway on day 24. The results presented in Figure 2.2 show that the number of trophonts on the fish in the control raceways were slightly higher (av. 61.33 trophonts per fish) than those on the fish in the painted raceways (av. 50.33 trophonts per fish) at the second sample point. The data was normally distributed and a Tukey parametric test (SPSS v.11) confirmed that there was no statistical significant difference between the two groups of fish ($p = 0.2707$). It was concluded that this formulation of the paint was not effective in reducing subsequent *I. multifiliis* infections in raceways.



Tank Name	Number of trophonts per fish
Paint 1A	59 ± 16.17
Control 1B	56 ± 14.54
Paint 1C	43 ± 15.29
Control 2A	59 ± 28.45
Paint 2B	49 ± 16.22
Control 2C	69 ± 19.82

Figure 2.2: The graph shows the average number of trophonts found on the second sample of experimental fish in painted and in control raceways.

Objective 3: Construction of a mechanical modular system and efficiacy under field conditions.

The objective of this part of the project was to construct and test a mechanical modular control system, install it within a working hatchery system and assess its efficacy over a time period when the likelihood of an *I. multifiliis* outbreak was high. Given the short time of funding available (7 months) and to ensure that a mechanical device was constructed in time to test on a commercial farm site from the start of May onwards, we sought the engineering expertise of Pisces Engineering Ltd. Through a series of consultations, the designs of the device were discussed and adapted. However, before the prototype system could be built, it was necessary to obtain key information on the behaviour and biology of the parasite. This information would be used in the design features of the beta product used for field testing. The additional information that was required included:

i) the survival of tomonts on a range of different polymer linings
ii) tomont settlement behaviour
iii) the determination of the minimum pressures required to dislodge cysts
iv) development rates of cysts at a range of different temperatures and pHs

The testing of a range of polymer lining experiments

These trials were conducted in addition to the paint experiments detailed under Objective 2. The objective of this part of the work was to identify a polymer lining that would either kill tomonts on contact or represent an unfavourable surface such that tomonts do not settle, thus causing them to use up vital energy reserves in searching for a suitable substrate on which to settle. Tomonts of *I. multifiliis* that do not settle are unlikely to encyst and undergo binary fission. It was hypothesised that if the settlement process could be interferred with, then this could form part of a strategy for the control of *I. multifiliis* within farm environments. To investigate this, tomonts exiting infected laboratory held *O. mykiss* were harvested and then dropped in batches into Petri dishes lined with one of the polymers listed in the table below. All the polymers were soaked in dechlorinated tank-conditioned water for a minimum of 24 hours before testing to ensure that there was no chemical finish on the products that may affect the results. The Petri dishes were then maintained in a 15^{o}C Binder environmental chamber and examined periodically to determine the number of alive and dead tomonts. The results are detailed below:

Table 3.1. The efficacy of various polymer linings in their ability to prevent tomonts of *I. multifiliis* settling on them and encysting. The percentage of tomonts dying when tested with each polymer lining are presented.

Product	Nos of trophonts tested	% kill
Crystal polystyrene (Petri)	102	9.78
Pisces Wsp20-05	102	90.21
Polyethylene-based polymer	102	76.49
Chlorvar-chlorinated rubber	15	46.60
Sealant-X	30	100.00
Ecosea Cuproguard^{TM}	30	100.00

The results show that two polymers, Sealant-X and Ecosea Cuproguard™, killed all the tomonts that they were exposed to. Of these, the results of Sealant-X are discussed in detail under Objective 2 ("*the testing of a raceway treatment*"). Ecosea Cuproguard is used as a marine lining on yachts to prevent barnacle growth and was tested here to determine its action on tomont settlement. Although effective in *in vitro* trials, the high costs of this product are prohibitive and realistically prevent its use in large farm sites. This latter product was not tested further, but did serve as a positive control against which to measure the activity of the other lining materials. The next most efficacious product tested was the polymer Pisces Wsp20-05 produced by Pisces Engineering, which killed 90.21% of the tomonts exposed to it in the *in vitro* trials. As the Sealant-X paint results proved inconclusive, it was decided that the field trials would use the Wsp20-05 lining, a rigid polymer that comes in sheets that can be welded together and bolted to the raceway walls. This product creates a smooth, flat, hardwearing, uniform surface over which the mechanical device can operate. As the entire raceway is lined, there are additional benefits in that there are no cavities in the concrete for the cysts to settle in and the smooth walls, it is believed, may reduce fin erosion of the stock. The field results of using Pisces Wsp20-05 are discussed in further detail under the sub-section "*Mechanical device*" (see also Figure 3.2).

Tomont settlement behaviour

This experiment was designed to determine the behaviour of tomonts in a small-scale aquaria to ascertain settlement locations. Experimental glass aquaria measuring 14cm (length) × 14cm (width) × 14cm (depth) were constructed and glued together using a silicon-based product. The aquaria were then washed and soaked for 24 hours with 15^{o}C dechlorinated, tank-conditioned water before filling them with a fresh aliquot of tank water. Tomonts of *I. multifiliis* were then harvested as they naturally exited infected *O. mykiss* and dropped in to the glass aquaria and allowed to settle. After five hours at 15^{o}C, the tomonts had settled, encysted and begun to divide. The aquaria were then carefully screened and the position of each cyst was recorded. The experiment found that 72.6% of tomonts (n = 98) settled on the bottom of the aquaria while 27.4% (n = 37) settled within 3.2cm of the bottom of the aquaria. Given that the majority of tomonts encyst on the bottom of the aquaria, it could be expected that a similar or higher percentage of tomonts (i.e. 72.6+%) would also encyst on the bottom of a commercial farm raceway. This finding was essential in the design of the mechanical device in determining which surfaces would need to be cleaned by the device.

The conclusion of this experiment is that a device cleaning the bottom of the raceway and / or the walls 3-5cm above the bottom and removing cysts should have significant impacts on the recruitment and subsequent infection dynamics of *I. multifiliis* within farm raceways.

The minimum pressure required to dislodge cysts

To calculate the minimum suction pressures required by the device to remove cysts of *I. multifiliis* from the bottom of the raceway the following experiment was set-up. Tomonts were collected as they naturally exited laboratory infected *O. mykiss* and were allowed to encyst in a Petri dish containing dechlorinated, tank-conditioned water. Once the tomonts had encysted and reached the two cell stage, water was expelled from a 5ml syringe to dislodge the parasites. By determining the volume expelled and the time taken to dislodge the cyst, the values could be used in the formulae given below to determine the minimum pressures needed to dislodge cysts from plastic Petri dishes.

$$\text{Pressure} = \frac{\text{Mass (kg)}}{\text{Length (m)*Time (sec)}^2} = \text{Pa (SI)}$$

$$d_{H20} = \frac{\text{Mass}}{\text{Volume}} = 1 \text{ g/ml}$$

1ml in the syringe = 0.5cm
1 Pa = 0.00075 mm Hg

From repeat experiments (n = 6), a mean pressure value of 0.001675 Pa was determined as the minimum pressure required to dislodge cysts. This value was used in the design of the mechanical device to ensure that the suction pressure over the entire vacuum area was exceeded.

Development rates of cysts at a range of different temperatures and pHs

To work out the frequency with which the device should be used during the field trials on a farm site, it was necessary to determine the development rate of all the external stages of the parasite (from tomont through cyst to theront) at a range of temperatures and pHs. Part of the work was determined under a previous DEFRA-SARF-BTA grant (CARD FC1158) but the work was extended here to provide a complete data set. The graphs below (Figure 3.1) show the development of cysts at pH7 at a range of different temperatures, and at 22°C under a range of different pHs.

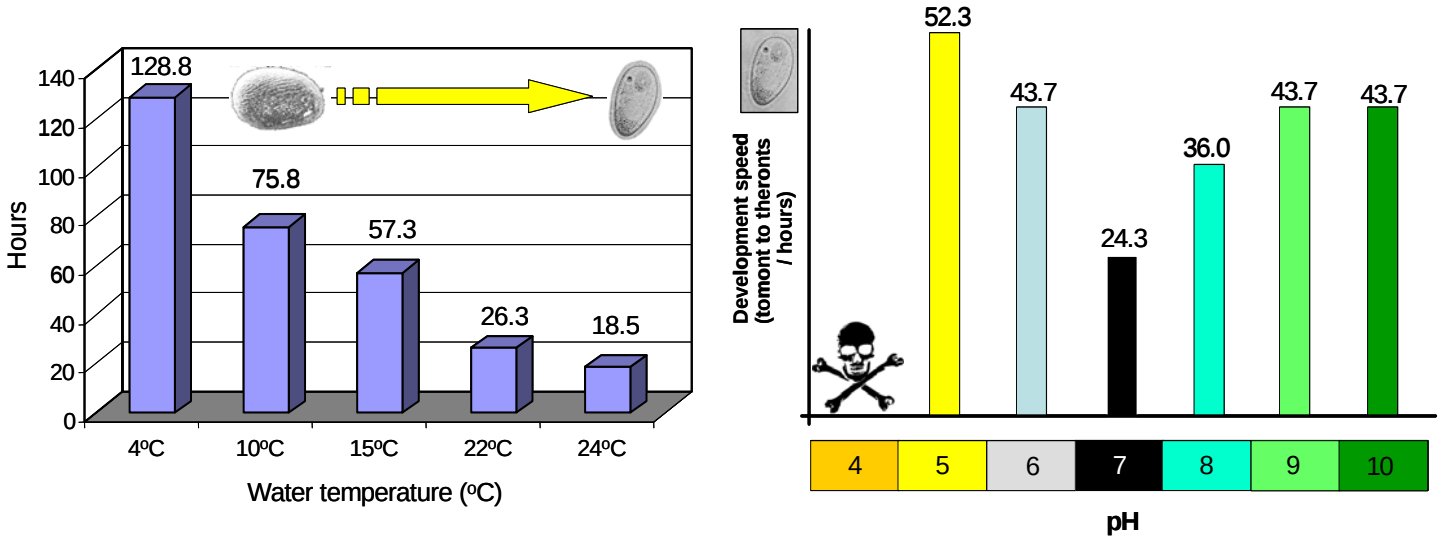


Figure 3.1. The development of cysts at pH7 at a range of different water temperatures and the development of cysts at 22°C at a range of different pHs. The time at which the theronts are released from the cysts are given.

The results show that if the mechanical device were used once a day at temperatures below 22°C it should remove cysts before they have released their theronts. However, once the temperature exceeds 22°C it is advisable to use the mechanical device twice a day (at least 7 hours apart) to remove cysts from raceways before they can release their theronts. From the pH experiment it can be seen that the development of the cysts is fastest at pH7 and slows as the pH moves away, in either direction.

Mechanical device

The information from the experiments detailed above was used in the construction of the beta product for field-testing. The primary mechanical device consists of a special suction head connected to a pump that is used to vacuum the bottom of raceways to remove parasite cysts (Figure 3.3). A secondary and equally necessary device was the use of the low adhesion polymer sheeting (Pisces Wsp20-05) that was used to line concrete raceways (Figure 3.2). This polymer reduced the problems of cyst adhesion and the potential for cysts to settle and develop in the cracks and pores of the rough concrete surface. Latex impressions of the concrete raceway showed that were numerous refugia for tomonts to settle in and encyst. It was thought that daily brushing of the raceways was unlikely to remove the majority of the cysts with the result that infection levels would quickly rise to dangerous levels. By lining raceways to give a smooth surface, the mechanical device with its wide suction head could clean the entire bottom surface efficiently (Figure 3.4).

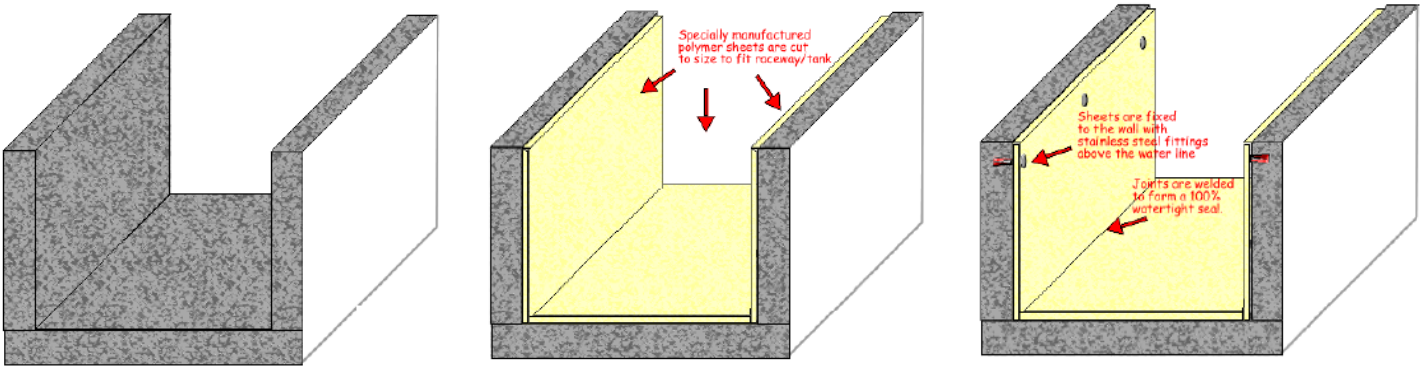


Figure 3.2. The lining of concrete raceways with polymer Pisces Wsp20-05. The sheets are cut to size, the joints welded together to give a watertight seal and then bolted to the concrete above the water line.

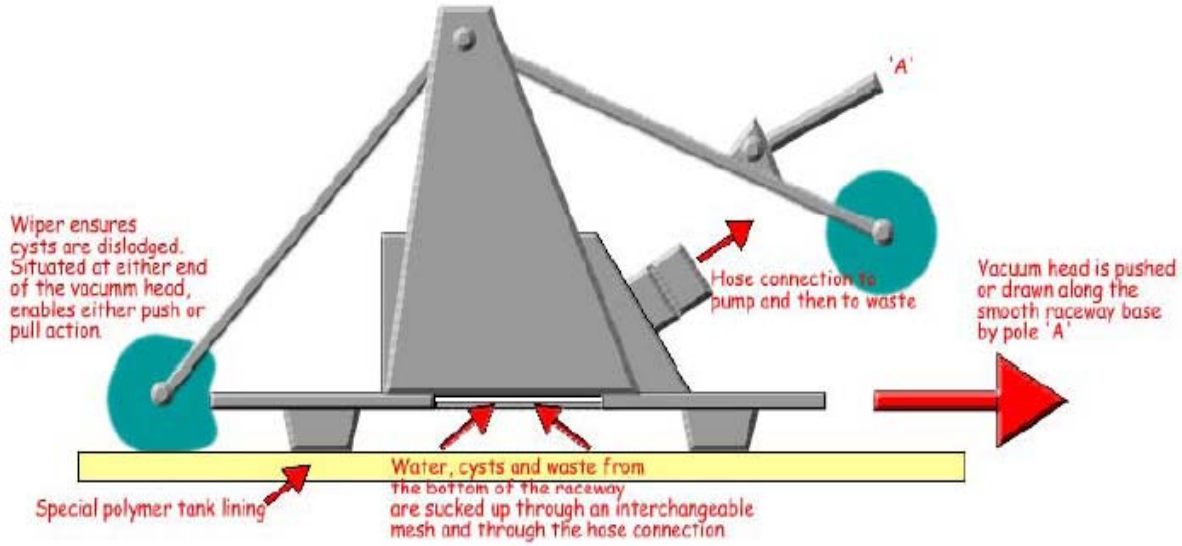


Figure 3.3. The mechanical device or "System*Ich*" uses a special suction head connected to a pump that is used to vacuum the bottom of raceways removing the cysts of *I. multifiliis*, faeces and any uneaten food pellets. Trailing wipers ensure that the surface is thoroughly cleaned.

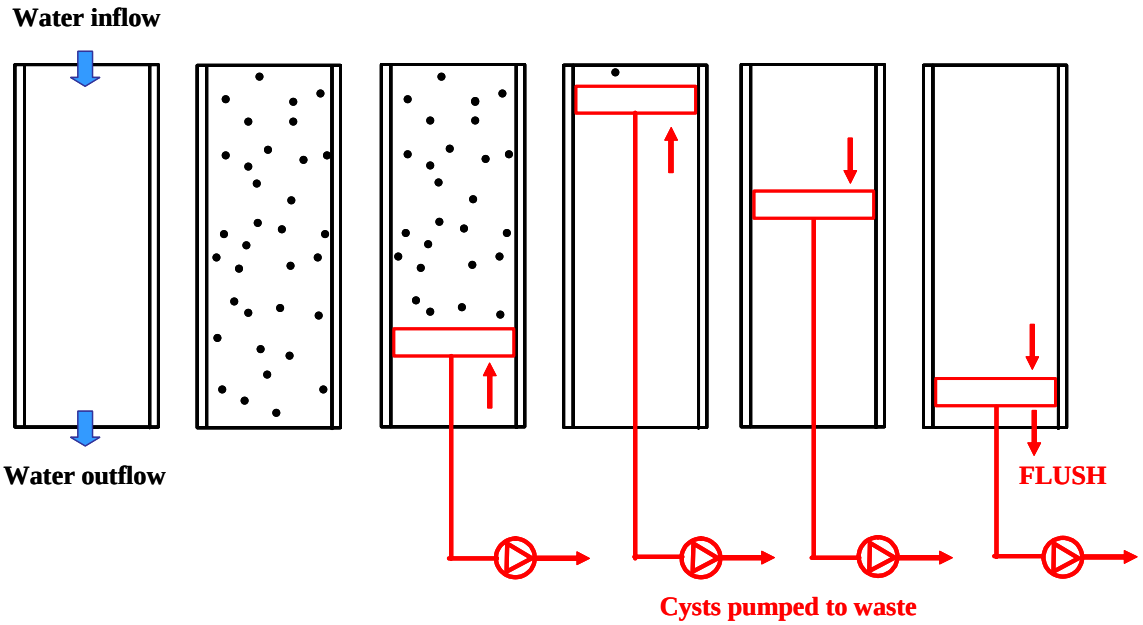


Figure 3.4. The use of the mechanical device in farm raceways. The device is pushed towards the water inlet and then dragged back to the outlet screen. The wipers tilt accordingly so that they always trail the suction head. Any material that is not drawn up the suction head is drawn to the outlet screen where it will either by drawn away by the water current or removed as part of normal husbandry.

Field trials using the mechanical device

Field trials were conducted on a commercial trout farm in the south of Scotland (name withheld) over a period of three months (mid-May until mid-August 2005). Six raceways (6m long × 1m wide × 1m deep) were used, three of which were lined with the 6mm thick polymer sheeting (Pisces Wsp20-05). The linings were installed by Pisces Engineering Ltd. The other three raceways were used as the controls. Each raceway was stocked with ~3000 five gram *O. mykiss* fry and maintained on a standard pellet feed. Each lined raceway was vacuumed once per day (one pass up the raceway and one pass back); the control raceways were brushed as normal. Every two weeks, twenty fish from each raceway were sampled and the total number of trophonts on the fins, gills and body surface were determined within the Parasitology Laboratory at the Institute of Aquaculture. The results were subjected to statistical analysis.

In addition to the samples taken from the hatchery raceways, a sample of 20 fish from one of the outside raceways (OR) were also taken every two weeks to monitor the infection dynamics in other parts of the farm.

Results of the field trial using the mechanical device

The fortnightly sampling of stock from one raceway (OR) outside the hatchery unit showed that *I. multifiliis* infections appeared in April with 12% of stock infected with 0.1 trophonts per fish (Figure 3.5). As water temperatures rose, infection levels increased exponentially with the number of trophonts on each fish increasing 50-fold throughout the month of May. At this point it was necessary to implement chemical treatments to control numbers.

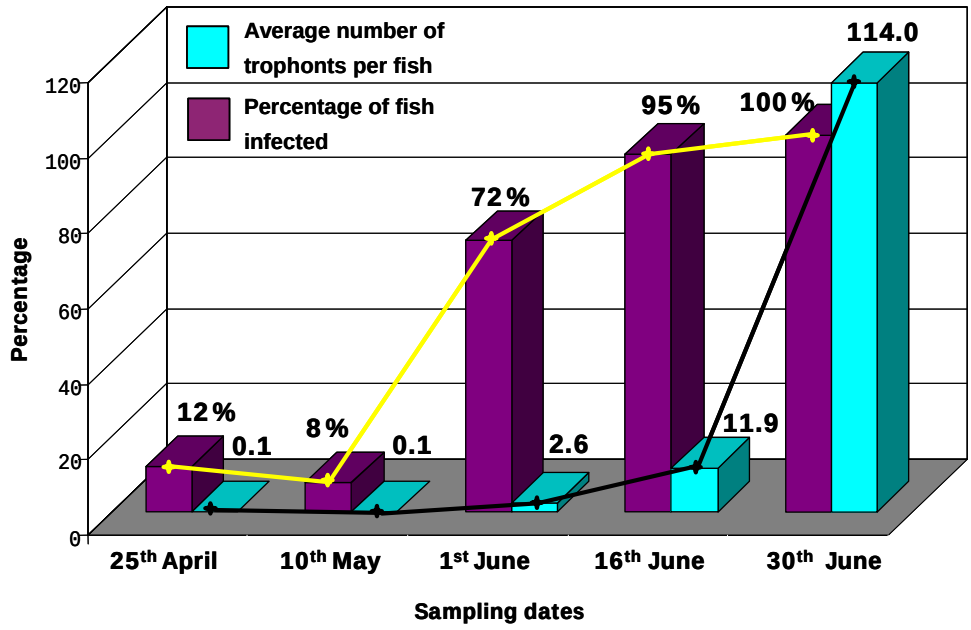


Figure 3.5. The infection dynamics of *I. multifiliis* on juvenile *O. mykiss* in a commercial farm raceway. At each time point the percentage of fish infected and the average number of trophonts per fish is shown.

The fish within the hatchery raceways were stocked on the 10th May; at this time point all the fish were uninfected (Figure 3.6). A sample of 20 fish per raceway at each time point was taken.

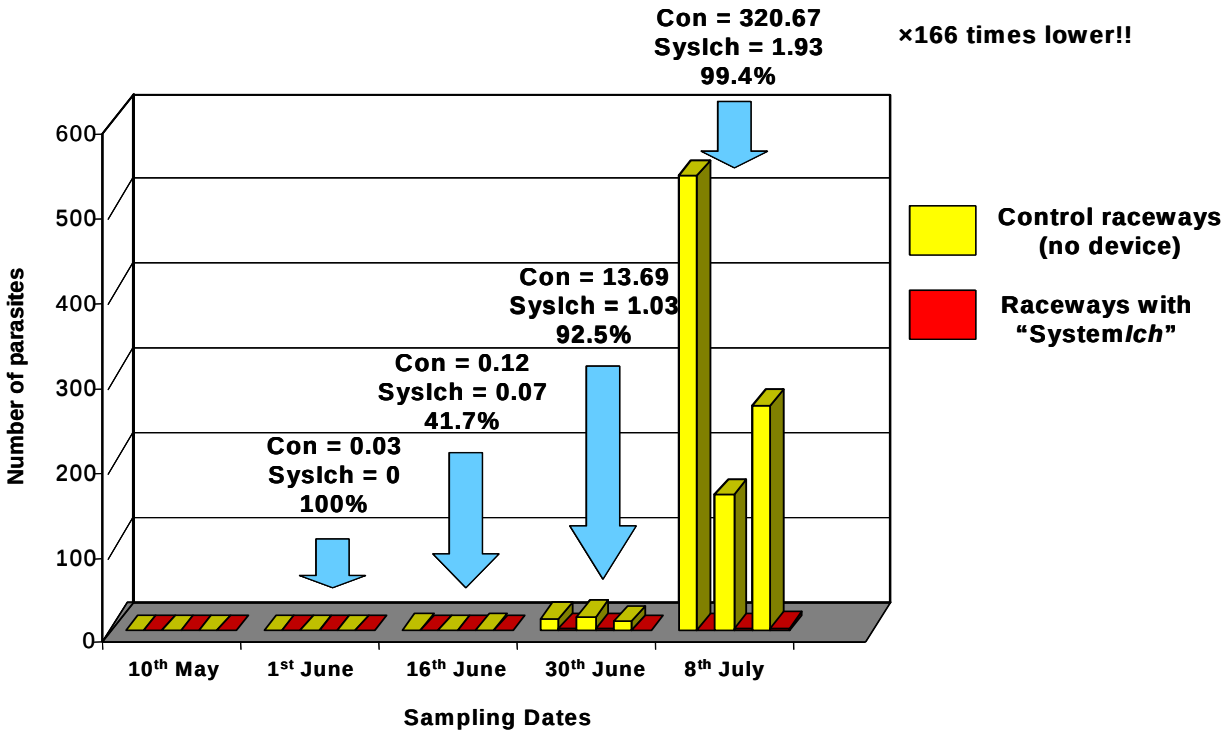


Figure 3.6. The infection dynamics of *I. multifiliis* within the experimental raceways within a commercial hatchery. The average number of trophonts per fish is shown for the control raceways and those using the mechanical device (System*Ich* or "SysIch"). The percentage reduction in trophont numbers through the use of the System*Ich* device are also presented.

The data presented in Figure 3.6 shows that while fish in the control raceways showed the same exponential increase in numbers seen for the outside raceways displayed in Figure 3.5, the fish in the raceways using the System*Ich* device are held at under 2 trophonts per fish. The trophonts on the System*Ich* fish may represent infections arising from theronts entering the raceway via the incoming water current or, are the result of the few trophonts settling and encysting above the clearance zone of the device. The field trial was able to demonstrate the efficacy of the System*Ich* device in removing cysts and reducing establishing trophonts numbers by 99.4+%. Significant differences between the test and control groups were found in the 4th (30th June; $p<0.0001$) and 5th samples (8th July; $p<0.0001$).

Following the combined trials of using both the device and the lining, it was questioned what were the benefits of the polymer sheeting lining raceways and what additional reductions were afforded through the use of the device. Following the 8th July sample, trophont numbers on the control fish were dangerously high and in the interests of health and welfare, it was necessary to implement a series of formalin treatments to control numbers. Four formalin treatments were given over the next 10 days in both the test and the control raceways. At this point the use of the suction head was also withheld (Figure 3.7).

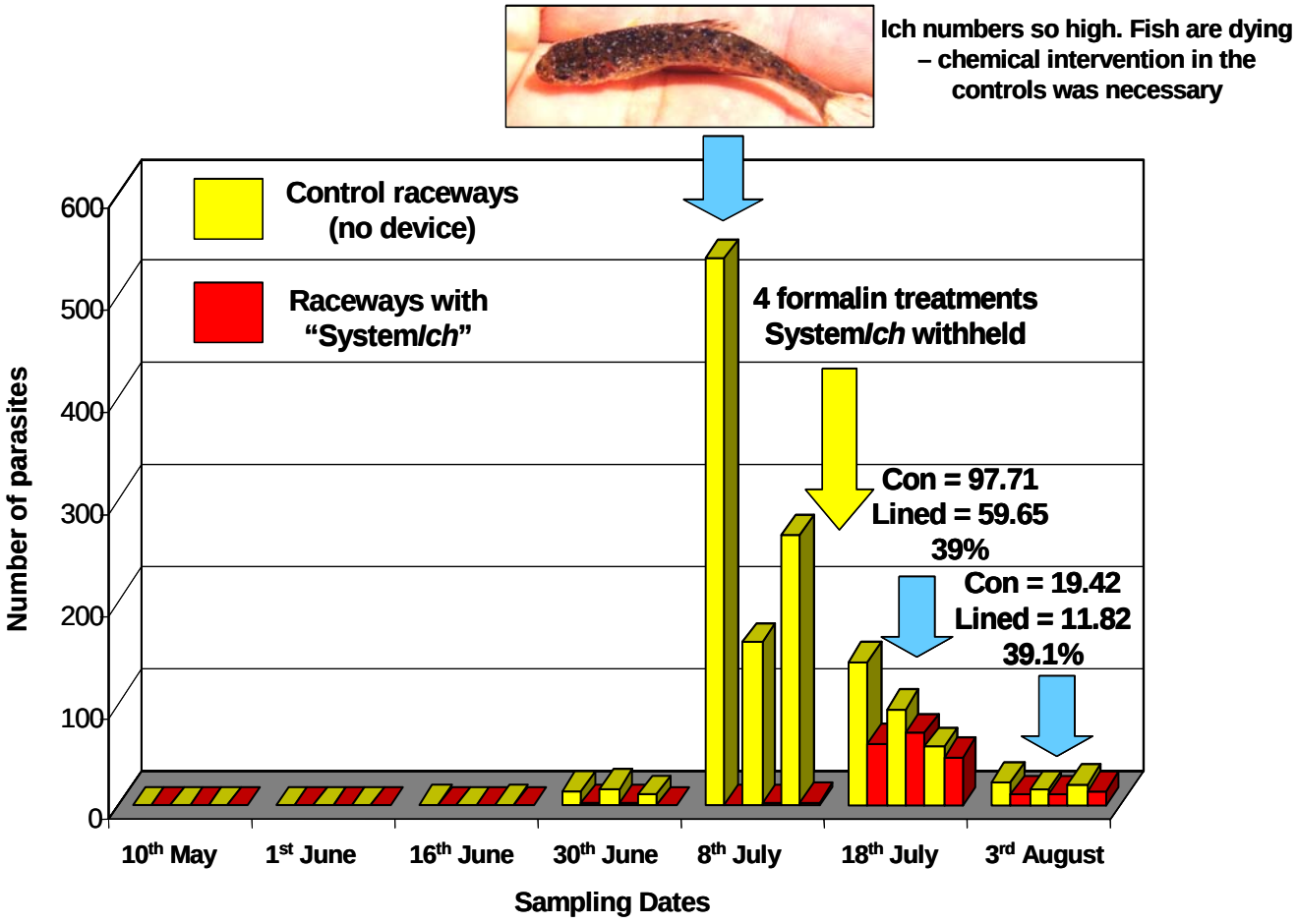


Figure 3.7. The dynamics of *I. multifiliis* in the hatchery raceways after the use of the System*Ich* device was withheld. The average number of trophonts per fish is shown for the control raceways and those lined with the polymer sheeting Pisces Wsp20-05 "Lined"). The percentage reduction in trophont numbers through the use of the polymer lining are also presented.

From Figure 3.7, it can be seen that the formalin treatments were successful in reducing trophont numbers of the fish held in the unlined (control) raceways. It can also be seen that trophont numbers on the fish in the lined raceways slowly increase after the use of the suction head was withheld (18th July; p=0.0988). As the trial continued, heavier rainfall in August and lower temperatures impacted upon the *I. multifiliis* dynamics, which began to decrease. The trial also suggests that the lining was able to significantly reduce the number of cysts settling and surviving by about 39% (3rd August; p=0.0091).

Given the short time available for this study (7 months), both components of the mechanical device, the suction head and the lining, had to be explored over the course of one farm infection. The full benefits of using the device in decreasing the number of fish morts, however, have not been fully explored. Figure 3.8 shows the number of morts recorded on the two groups of fish (control and test) against the average number of trophonts determined on each stock of fish. The graph suggests that there was no real difference in the number of morts during the period when the device was used. The device, however, was stopped at a point when trophont numbers on the control fish were near their peak (300+) and at a point when they kill fish. By following the curves it can be seen that the control fish continue to die despite the formalin treatments and the altered environmental conditions over the course of the trial twice as many fish died in the control raceways as did in the treated raceways. The reason for the high number of morts in the test raceways on the 21st July is possibly linked to a quick rise in water temperature and the exit of a large number of trophonts. It is important to note that although the test raceways did not need treating, they were treated with formalin alongside the control raceways to standardise experimental conditions.

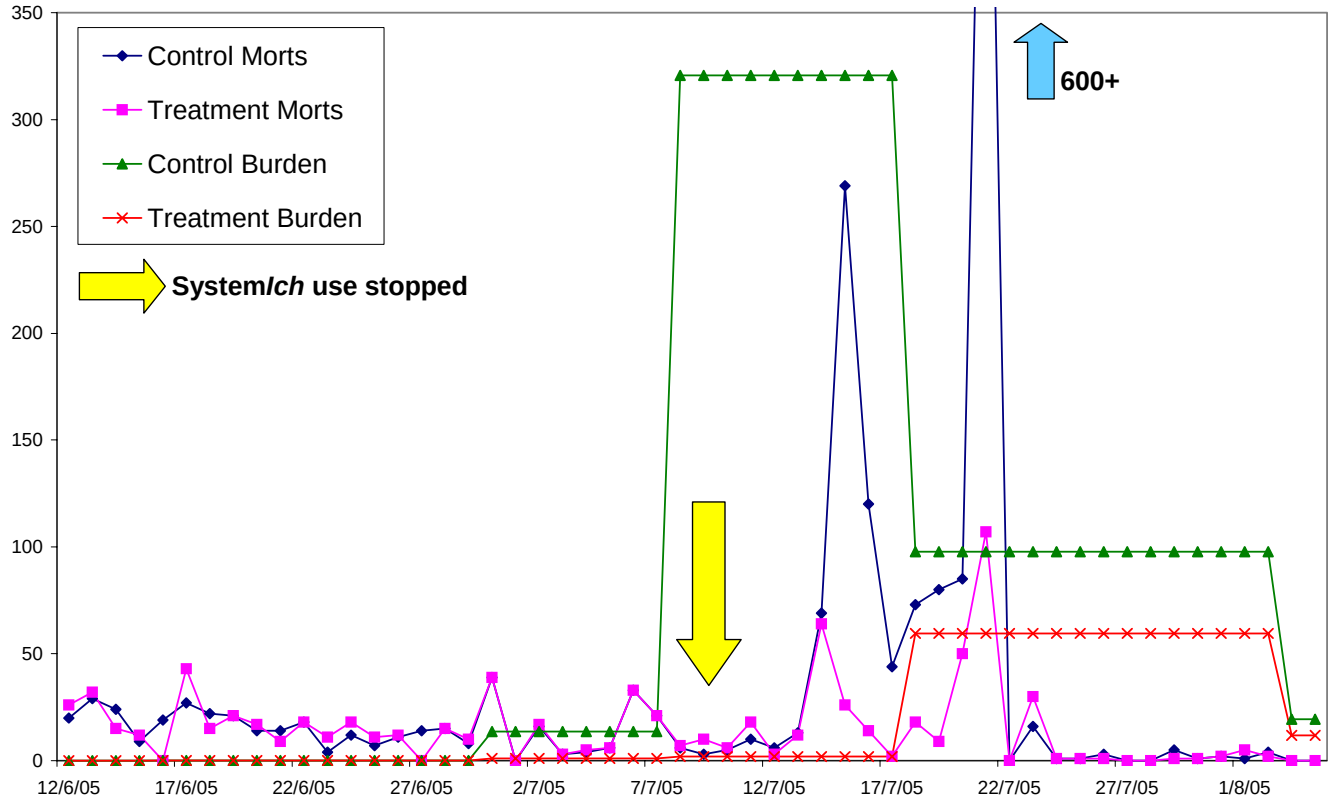


Figure 3.8. The daily number of morts removed from the test (Lining + device) and control raceways over the experimental period (pooled data). The average number of *I. multifiliis* trophonts per fish is also shown.

The benefits of using the System*Ich* suction head and lining Pisces Wsp20-05, however, can be visualised by calculating a stock survival curve over the post-device experimental period (Figure 3.9). The results show that 6% of the entire stock was lost in the control raceways over the period 10th July to the 3rd August (after the use of the suction head was stopped) while only 3% of stock was lost from the treated raceways. If the device and the lining had been used continuously over the entire *I. multifiliis* infection period, it is hypothesized that further stock losses would have been prevented.

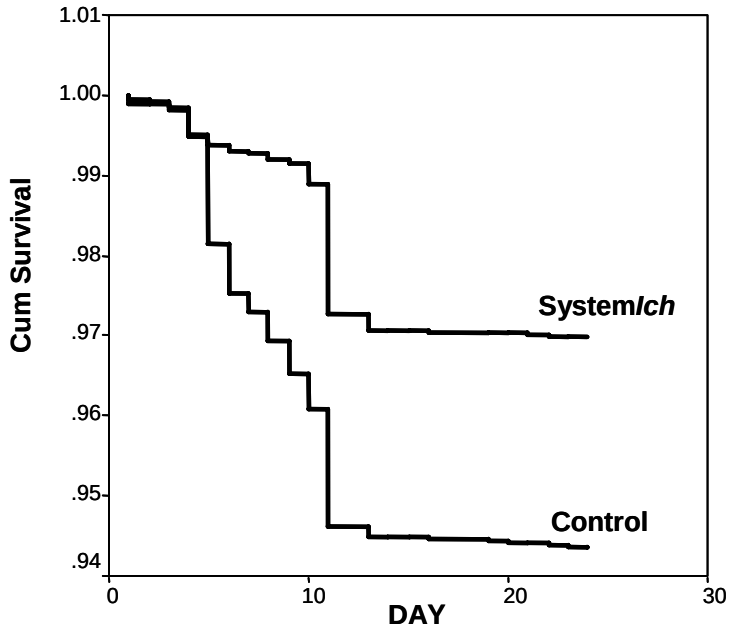


Figure 3.9. Survival curve of fish stock within unlined control raceways and raceways lined with the System*Ich* polymer lining Pisces Wsp20-05 over the 25 day post-device use period. Figures are based on entire stock surviving in the raceway at the end of the trial and the differences are a result of using the lining only.

Conclusion

Ichthyophthirius multifiliis is global disease that has been estimated to kill 5-10% of all freshwater fish annually. To control this parasite, multiple chemical treatments are often used to reduce the number of infective theronts in the water column. There is, however, serious concern regarding chemical use and the impact on the environment and there is an urgent need to find a non-chemical, environmentally sound alternative to controlling this fish pathogen.

The System*Ich* device is a non-chemical approach that uses a combination of a suction head and a special polymer lining (Pisces Wsp20-05) to remove cysts, uneaten food pellets and faeces. The two components when used in combination could reduce current trophont loads on infected fish by 99.4+% and lead to a 2-fold reduction in total mortalities. It is also believed that additional benefits result from the use of lined raceways, including: a cleaner environment for the stock, better removal of faeces, improved water quality, lower bacterial loads and reduced fin abrasion. It is believed that as the concrete is sealed, the levels of other fish pathogens in the system will be lower and trials to test this are planned.

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