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SID 5 Research Project Final Report

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Project identification

1. Defra Project code: FC1173

2. Project title:
   A study of the aetiology and control of rainbow trout gastroenteritis (RTGE).

3. Contractor organisation(s):
   Prof. James F. Turnbull
   University of Stirling
   Stirling
   UK
   FK9 4LA

4. Total Defra project costs (agreed fixed price): £ 30000

5. Project: start date ..... 01 October 2005
   end date ..... 01 March 2009

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 In these cases, such information should be detailed in a separate annex (not to be published) so that the SID 5 can be placed in the public domain.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

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.

Trout farming in the UK is an important source of rural employment, high quality healthy food and supports angling one of the UKs most popular participation sport. Rainbow Trout GastroEnteritis (RTGE) has caused the death of large number of farmed trout in the UK and other European trout farming industries. The disease was associated with "*Candidatus* arthromitus" a segmented filamentous bacterium (SFB) but the role of SFB in the development of the disease was not known. This project aimed to support the long term sustainability of the trout farming industry through studying the cause and effects of RTGE and developing control strategies in consultation with the industry.

Throughout the project we received a great deal of support and co-operation from individual farmers and industry representatives; this included financial aid, their time, effort and advice. The project was conducted in two phases: the first was a preliminary investigation to determine if further work and investment were justified. In the first phase it was necessary to conduct a review of the literature to utilize all existing information. Although there had previously been very few scientific papers published on RTGE, there were similar or related conditions in other animals. Unfortunately this was not very productive since relevant information was limited but this demonstrated the necessity for further research. Samples were taken from affected and unaffected fish in the same farms to describe the nature of the pathology associate with RTGE and examine for any obvious infectious agents such as bacteria or viruses. Other initial preparatory work was completed and at the end of the first phase it was agreed that continued funding was justified.

The second phase successfully combined those disciplines, techniques or methods that were most appropriate to understanding RTGE. These included epidemiology (the collection and statistical analysis of information from real outbreaks of the disease); microbiology (the study of microorganisms associated with the disease including bacteria and viruses and their genetic material); histopathology (the interpretation of thin stained sections of tissue for evidence of disease processes); electron microscopy (the application of extremely high magnifications to examine surface or cross sections of minute pieces of tissue); tissue culture (using cells grown in artificial media to detect the presence of viruses or toxins) and finally blood biochemistry (the study of blood constituents to examine the effects of the disease on the vital functions of the fish).

Initially, before the time of the year when the RTGE occurs, data were collected from previous outbreaks by interviewing farmers and examining production records. This information demonstrated that the condition was a problem of the large intensive farms producing fish for human consumption and that the number of affected sites was increasing i.e. the disease was spreading. There was no obvious association with source of feed, age of fish or location. This part of the study also allowed us to identify those sites most likely to suffer from RTGE, thereby helping the next part of the study.

Several farms (12) were studied throughout the period of greatest risk (over the summer). Farmers were trained to improve detection of the problem and collection of data, water and biological samples from affected and unaffected fish. With the data collected we were able to describe the impact, appearance, existing control strategies and pattern of spread RTGE within affected farms. We were also able to refine the definition of RTGE to make it easier to identify it conclusively either on farms or through laboratory samples. The main finding was that RTGE behaved like an infectious disease obviously spreading both within affected ponds and throughout affected farms and there was a

period of roughly 20 to 25 days between introduction of the suspected infectious agent and the outbreak of clinical disease. It also seemed that high levels of feeding and stressful events, such as handling, increased the risk of an outbreak and that existing treatments were either ineffective or only reduced the severity of an outbreak. These treatments did not stop or prevent further outbreaks of the disease.

SFB associated with RTGE were found in the intestine of affected as well as apparently normal fish, although numbers of SFB were much higher in most affected fish. Nevertheless it would appear that the mere presence of the bacteria is not enough on its own to cause the disease. Observations on the intestine and blood biochemistry demonstrated that the fish died due to loss of protein from the intestine. No evidence was found of viral infection or toxins in the gut of affected fish. It was planned but not possible to conduct experiments to artificially reproduce the disease and this should be a priority for future studies.

After analysis of all other parts of the study was completed, the information obtained was discussed with farmers and industry representatives to develop best practice guidelines for prevention of reduction of impact of RTGE. These guidelines were circulated to all the participating farmers as well as to all members of the British Trout Association and presented at several conferences and meetings of farmers, fish vets and academics. No data have yet been collected on the application of these guidelines and this should also be a priority for future studies.

This was a successful project using a multidisciplinary approach and working with stakeholders to understand a complex disease and propose strategies to reduce its impact.

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).

Context of project

Rainbow Trout Gastro Enteritis (RTGE) or Summer Enteritis is an economically damaging, emerging disease of the European trout farming industry and has been recorded in the UK, France, Spain and Italy. Segmented Filamentous Bacteria (SFB) are the suspected cause of this condition although only three papers had been published on the disease at the start of the project.

One carried out in France by Michel et al. (2002) recorded daily mortalities of 0.5 to 1% over a 15 to 30 day period. Mortalities occurred once water temperatures reached 15ºC and appeared to be triggered by stress factors such as climatic changes, handling or movement operations. Fish generally exhibited lethargy and reduced appetite, accumulations of mucoid faeces within the ponds were also noted. Some fish developed dark striping of the flanks with or without nervous signs. Macroscopic necropsy findings were of haemorrhage and oedema of the intestinal wall whilst histologically the lumen contained mucoid material and large numbers of filamentous organisms. Unsuccessful attempts were made to culture the organisms.

Another study, also from France (Urdaci et al. 2001), used genetic probes specific for RNA sequences of SFB present in other animal species. These probes bound to the filamentous organisms found in the intestines of affected trout and it was concluded that these organisms were of the same group as rat, mouse and chicken SFBs, provisionally named the '*Candidatus* arthromitus' group and related to Clostridium phylogenic group I.

The third paper is a description of the first RTGE diagnosis in the UK (Branson, 2003).

SFBs are a group of autochthonous, strictly anaerobic, spore-forming, Gram-positive bacteria that have been recorded in many unrelated species including termites, cockroaches, frogs, ducks, cats, sheep and humans but have never been cultured in vitro (cited in Klaasen et al. 1992, Talham et al. 1999). In general these bacteria are considered beneficial commensals and work in mice has implicated them in the development and stimulation of the intestinal immune system (Umesaki et al. 1999, Talham et al. 1999). In mice stressors such as overcrowding, high temperatures and continuous lighting result in decreased numbers of intestinal SFBs (Klaasen et al. 1992).

Conversely some reports link the presence of SFBs in birds with diarrhoea, malabsorption and gas/fluid filled intestines (cited in Urdaci et al. 2001). However in one of these cases (stunting syndrome in turkey poults) an underlying virus was subsequently demonstrated as being responsible (Ali et al. 1997) and in others it has been suggested that the colonization of SFBs is related to diet, environmental stress and antimicrobial drugs rather than true pathogenesis (Goodwin et al. 1991).

The situation with regards to RTGE and the presence of SFBs remains similarly confused. A direct causal relationship between presence of these organisms and the clinical signs has not been demonstrated. Thus it is entirely plausible that the action of a primary agent, be it viral, toxicological, bacterial etc, results in an alteration of the local intestinal environment that is not only deleterious with regard to fish health but also favours massive overgrowth of non pathogenic filamentous commensals.

As far as we are aware the only current research projects into RTGE are under the emerging diseases project at CEFAS Weymouth and this project. It would appear that research initiatives in mainland Europe have not been continued.

Scientific objectives and extent to which these have been met

The aim of the project was to identify potential control strategies for RTGE through the investigation of the epidemiological, pathogenic and aetiological aspects of this syndrome to support the sustainability of UK trout farming by controlling RTGE. The project was divided in two phases, with the following objectives:

FIRST PHASE (1 year)

Phase 1-01 Recruitment of post-graduate researcher
The candidate will be initially enrolled as an MPhil student (one year research degree) and if continued funding is available the student will transfer to PhD registration at the end of the first year.
Completed in full (01/09/2005). Delays in countersigning resulted in the researcher being recruited two months later than the milestone set initially.

Phase 1-02. A literature review of information related to RTGE, both directly and indirectly
A detailed review of the literature available on RTGE will be conducted, as well as any literature that could have an special relevance, for example information about the normal physiology of fish gut, SFB in other species, similar conditions found in fish or other species, potentially useful methods for this study and others.
Completed in full and on time (01/04/2006).

Phase 1-03 Clinical samples/Preliminary analysis completed
Histopathology, haematology and serum biochemistry will be undertaken to provide information on the sequential pathology and cause of death. These techniques will also allow detailed screening of tissue for evidence of previously overlooked pathogens that may be responsible for or contribute to the onset of RTGE. During the trial, samples will also be taken for bacteriology and previously published PCR primers will be tested against samples from UK fish.
Completed in full and on time (01/03/2006).

Phase 1-04 Epidemiology
Case control data will be collected from affected and matched unaffected farms, through structured and semi-structured questionnaires. The unit of interest will be the rearing unit (cage, tank or pond). Data will initially be screened visually and through univariable analysis then multivariate logistic regression with clinical RTGE, as the outcome variable. Survival analysis will be used to model time dependent covariates.
Completed in full, but not on time. This was due to lack of time and availability of specific mortality data from the affected pond sites. The time allocated to this task was used in the development of a protocol for the collection of production data, and the design of retrospective and prospective epidemiological studies. Also, the links with the UK rainbow trout farming industry which were essential for the second phase of the project.

Phase 1-05 Field case definition
This objective will determine the validity of diagnosis made by farmers based on clinical signs and to develop a robust field case definition.
Completed in full and on time (01/03/2006)

Phase 1-06 First review prior to approval of SARF funding
A meeting will be held with Dr M James to present the progress during the data and sample collection phase of the project and discuss the need for SARF funding. This meeting would be attended by representatives of the BTA.
(This review meeting was held at the IoA on September 2005 with 1-07)

Phase 1-07 Second review prior to approval of continued Defra funding
A meeting will be held with both BTA and DEFRA representatives to review the project and discuss the need and the potential for continued funding.
(This review meeting was held at the IoA on September 2005 with 1-06)

SECOND PHASE (2.5 years)

Initially it was inappropriate to define specific activities or milestones for the second phase since these were dependent on the findings from the first phase. These objectives with their milestones were agreed during the review meeting where further funding was discussed and approved (Phase 1-06; Phase 1-07). The objectives set for this phase were the following:

Phase 2-01. Retrospective study
This will be a survey of a wide selection of the industry including both BTA and non-BTA members from existing contact lists (Defra AW1204). The retrospective study will serve a variety of functions. It will validate the current working case definition and identify affected farms. At a national level it will produce definitive evidence for the distribution and severity of RTGE in the UK trout industry. It will also identify seasonal patterns and examine a number of major farm level risk factors. Findings will be triangulated through meetings with, FRS Aberdeen, CEFAS Weymouth; the major trout feed company (Skretting UK) and members of the Fish Veterinary Society.
Completed in full and on time (01/07/2006)

Phase 2-02. Prospective study
This will involve a smaller number of farms including affected and unaffected sites. It will use a longitudinal design collecting data on a weekly basis, with farm visits at the start and during the study to enhance compliance. A wide range of background information will be collected at the start of the study and then weekly management and mortality data will be collected either by email or phone. This will require that participating farmers examine a proportion of their mortalities to determine the prevalence of RTGE. Analysis of the data may include a variety of multivariable techniques including survival analysis. It may be necessary to use a case control analysis if RTGE only affects a small number of sites in 2006 (less than 10).

The study will identify pond or cage level risk factors such as proximity to another affected cage/unit, source of fish, recent grading, stocking density, growth rate and efficacy of control strategies with more power than the retrospective study. It will also provide an opportunity to collect biological samples.
Completed in full and on time (01/05/2007)

Phase 2-03 Aetiology
This objective is subdivided in four sub-objectives:

2-3.1. SFB
The presence of these bacteria in affected and unaffected animals will be examined using a molecular test for the specific detection of SFB DNA. Once tested for specificity and sensitivity, this technique will be used to explore the relationship between and potential reservoirs of infection, the clinical disease and the SFB. Electron microscopy will also be employed to further investigate the interaction between the SFB and the intestinal mucosa.
Completed in full and on time (01/06/2007)

2-3.2. Anaerobic bacterial culture – SFB
Through the link with Prof. Taylor at the University of Glasgow veterinary college attempts will be made to isolate the spores and culture the SFB. This will include examination of the micro-environment within the fish gut. While it may be possible to conduct some preliminary studies during 2006 time has been specifically allocated to this task in 2007.
(Not completed. This task was not undertaken, since other aspects were thought to take priority. Also some of the molecular work reduced the necessity for anaerobic culture of the bacteria (Phase 2-03.1). This was discussed during the review meeting at the IoA May 2008 and was a agreed by the project monitoring officer.

2-3.3. Other agents
Standard histopathological samples will be analysed and routine bacteriology employed to further investigate any differences between affected and unaffected fish. Initially, toxic causes were to be examined through gavage of filtered gut material and the use of monoclonal antibodies, but the use of cytotoxic assays was proposed as a preliminary test of the toxicity of the intestinal contents of RTGE-affected fish during the project review meeting held on May 2008 at the IoA.
Completed in full and on time (01/02/2008)

2-3.4. Viral agents
The PhD student will visit CEFAS Weymouth for a period of up to 6 weeks during 2007 to explore possible viral aetiology. This placement has been agreed with Dr Irvine and Dr Verner-Jeffries but the specific timing has still to be finalised. To ensure the usefulness of the visit, a set of samples will be sent to CEFAS for preliminary virological analysis at CEFAS Weymouth.
Completed in full and on time (01/10/2007)

Phase 2-04. Experimental reproduction of RTGE
This objective will involve an attempt at the experimental transmission of RTGE through gavage of intestinal contents from RTGE-affected fish. Protocols from stunting syndrome in turkeys may be adapted for this purpose.
Not completed. This work was not undertaken, due to a review of the home office licence that took place during the whole of the period when fresh samples were available. This was discussed at the final review meeting IoA Feb 2009.

Phase 2-05. Pathogenesis
The exact cause of death in RTGE has not yet been defined but osmotic imbalance and protein loss into the intestine are potential candidates. The blood osmolarity of affected and unaffected fish will be studied as will the protein content of the blood and gut contents.
Completed in full and on time (01/10/2008)

Phase 2-06. Farm based control strategies
As relevant information becomes available best practice guidelines will be developed during the study and discussed with farmers and fish veterinarians with a view to setting up on-farm trials, to test the strategies.
Completed in full and on time (01/04/2009)

Phase 2-07. Reporting
Normal reporting procedures will be followed with annual reports on the first of April and a final report at the end of the project. Findings will be reported to DEFRA, SARF and to participating farms through reports protecting the identity of participating farms. The results will also be made available to the BTA and prepared for peer review publication.
(Yearly review meetings took place at the IoA on Nov 2006, May 2007, May 2008 and Feb 2009. All the scientific information generated by this study has been compiled in five draft manuscripts, of which one has already been accepted for publication).

Details of the methods used and the results obtained

FIRST PHASE (year 1)

Phase 1-03 Clinical samples/Preliminary analysis completed

Five sampling trips were made from the 6th of September 2005 to the 7th of October 2005. During these trips three rainbow trout farms were visited, and it was found that the cage site pre-selected for the trial did not suffer RTGE at significant levels during the summer of 2005. Another cage site with a previous history of RTGE problems was visited with the same results. Finally, an earth pond site affected with RTGE was located in Central Scotland, and samples were taken. Interestingly, the salt-feed treatment was being implemented, but did not seem to have any significant effect on the presence of RTGE. The outbreak was starting to decline at the time the farm was identified.

Several rainbow trout sampled from the affected site showed gross and histopatological signs typical of RTGE as well as microscopic presence of SFB. These samples were analyzed using histology, scanning electron microscopy, transmission electron microscopy, bacteriology and a molecular technique.

Several observations and conclusions were made from the study of these samples:

Histology: Haematoxylin and eosin staining revealed the presence of SFB in both pyloric caeca and lower intestine of RTGE affected trout. These SFB were intimately associated with intestinal mucosa, although no apparent host reaction was observed. The intestinal contents were composed mainly of sloughed off epithelial cells within a proteinaceous matrix. Tissue Gram stains demonstrated that the SFB were Gram variable. This has been reported in SFB in other species and has been related to the developmental stage of the bacteria (Michel et al., 2002).

Scanning electron microscopy: SEM photographs taken during the study show a close interaction with the rainbow trout intestinal epithelium. This type of interaction has not been previously reported, although it was suspected from parallels with other species. Interestingly, severe generalized mucosal blebbing, an indication of mucosal response to the bacteria was observed.
Transmission electron microscopy: This technique is being used to try and clarify the nature of this interaction between the SFB and the mucosa. The attempts to visualize the interaction of SFB with the mucosa have been unsuccessful, although SFB have been pictured and their ultra-structure described.

Bacteriology: Comparison between the bacterial flora of RTGE affected and apparently healthy fish were carried out, using standard microbiological techniques of culture and identification. Gram -, Gram + and Gram variable organisms were isolated from fish intestines using these techniques. They were identified to genus level and the information collected was analyzed, and the preliminary observation was made that the intestinal bacterial diversity of fish in the RTGE affected sites where samples were taken was lower than that one found in the fish from the site with RTGE, and within this site, the intestinal bacterial species diversity was lower in fish presenting signs of RTGE than in apparently healthy fish. These results include only bacteria easily cultured in standard media.
No SFB were isolated with conventional techniques and anaerobic techniques were tested unsuccessfully for the isolation of SFB.

PCR: A molecular technique for the identification of "Candidatus arthromitus" 16S rDNA from paraffin embedded intestinal tissue using published SFB specific primers (Urdaci et al., 2001), was tested on samples that contain SFB, as well as samples from apparently healthy fish with no evidence of SFB. One of the four published primer combinations amplified a DNA fragment in the SFB positive samples (SFB779F-SFB1380R) and not in tissue from apparently healthy fish. This fragment presented the expected molecular weight for that primer combination.
The PCR reaction was then optimized for different parameters, including annealing temperature and the use of a nested or consecutive PCR technique.

Phase 1-05 Field case definition

A working field case definition was developed that would appear to differentiate RTGE from other potential differential diagnoses. This will be further tested during the epidemiological studies. The case definition was:

"RTGE is a condition of rainbow trout, during periods of warmer water temperatures (exact range has not yet been defined), associated with intermittent mortalities up to or even exceeding 0.5% per day. The lower intestine is swollen, oedematous and reddened and the contents of the gut are yellow and gelatinous."

SECOND PHASE (year 2-3.5)

Phase 2-01. Retrospective study

A retrospective epidemiological cross-sectional study of RTGE on UK rainbow trout farms was conducted from April to June 2006. The data collected related to the years 2000 (first report of RTGE in the UK Branson, 2003) to 2005. All data were collected by questionnaire completed through a phone, interview, e-mail or post. Farms were only considered positive for RTGE when diagnosed by at least one independent fish health expert. Independent experts were Skretting or Institute of Aquaculture Fish Veterinarians.

In this study the unit of interest was the whole site, and the survey aimed to identify:

- RTGE affected farms.
- Extent and severity of RTGE, i.e. distribution and prevalence.
- Risk factors associated with RTGE around which hypotheses could be generated and further research targeted.
- UK rainbow trout industry's perception of the working case definition of RTGE at the fish level.

A farm was only considered to be RTGE positive if RTGE had been confirmed by an independent health expert at any time between 2000 and 2005.

The closed non-scientific questions included in the survey were derived from literature on RTGE and Candidatus Arthromitus as well as an understanding of the routine management procedures of rainbow trout farms. Information was also collected both on the views of the farmers regarding the case definitions for RTGE and their confidence in their own ability to identify the condition. After the preliminary design the questionnaire was piloted by fish farm managers and scientists. After further amendment the questionnaire was piloted in two rainbow trout sites (not included in the study) and assessed for how long it took, nature of data collected and absence of ambiguity. The process allowed the identification of problems with the design, which were then eliminated in the definitive version of the questionnaire. A copy of the final questionnaire can be found in Appendix I.

Of 126 sites initially contacted, 84 sites agreed to take part in the study. These sites produced 13,539t of the total 15,917t production (85%) of the UK rainbow trout production in 2004 (Defra report). Figure 1 is a map of the participating sites. With the exception of the approximate location of participating sites all the data collected will remain confidential and no specific site details will be released without prior permission of the manager.

Figure 1 Map of the rainbow trout farms (n=84) participating in the Rainbow Trout GastroEnteritis (RTGE) retrospective epidemiological cross-sectional study.

A database was created in Epi Info software (CDC) and used to collate all survey data. Each variable was tested against the outcome (i.e. RTGE presence on the site) in univariable and stratified analysis using the same software and subsequently in multivariable analysis with Statistica (StatSoft corp).

Results of the survey

The survey found a total of 11 RTGE confirmed cases (13.1% of 84 sites) from 2000 to 2005. The number of sites with RTGE during each one of the years is displayed in Figure 2. The prevalence of RTGE positive sites in the respondents to the survey ranged from 2.4% during 2000 to 8.3% during the years 2003 and 2005. At the start of the project a description of the disease was circulated through the BTA, however, of the 84 respondents to the survey only 15 (17.9%) were confident in the recognition of RTGE. Therefore while we can be confident that the positive cases were real cases of RTGE we can have less confidence for the unaffected sites. In the prospective study fish were examined form both affected and unaffected sites to confirm their status.

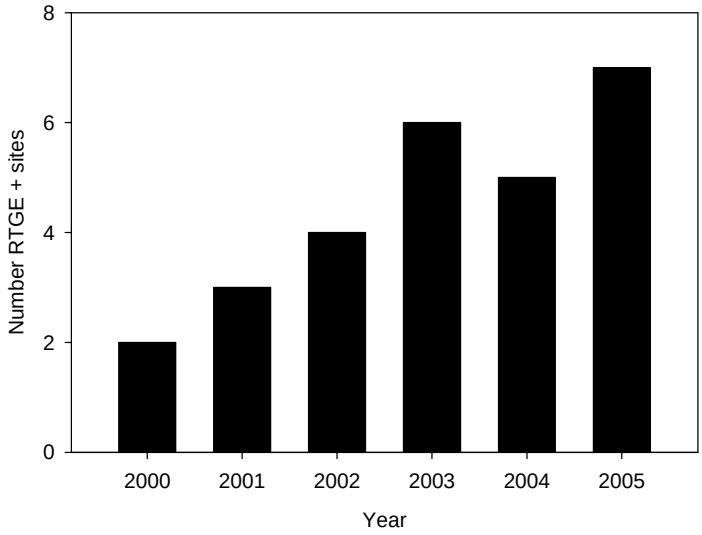

Figure 2 The total number of RTGE positive cases per year for all the participant sites of the RTGE retrospective study (84 sites in total).

RTGE positive cases from 2000 to 2005 were located in two distinct areas, namely the south of England and Scotland. The 4 RTGE cases in the south of England were clustered in a small area whereas the 7 cases in Scotland were more widely distributed. All the RTGE cases in England were river based farming systems, whereas in Scotland both river-based and loch-based systems were found. Table I shows all the participant sites and RTGE cases by region from 2000 to 2005. No RTGE cases were found in Wales. All the RTGE cases were table producing sites, and only one of them was also producing for restocking. All cases were producing more than the median production of 70 tonnes/year and used externally supplied fry.

Table I Number of participant sites in the RTGE retrospective epidemiological study divided by region and presence or absence of RTGE from 2000 to 2005

Region	RTGE absent (%)	RTGE present (%)	Total
England	55 (93%)	4 (7%)	59
Scotland	12 (63%)	7 (37%)	19
Wales	6 (100%)	0 (0%)	6
Total	73	11	84

Univariable analysis identified significant risk factors for RTGE. However, all RTGE positive sites had above average (median) production of 70t/year and several of the risk factors were associated with level of production as shown by correlation analysis. This led to conduct stratified analysis, whereby the dataset was divided through the median (70 Tonnes/year) and the analyses were only conducted in the higher stratum. Following this analysis seventeen variables were identified as possible risk factors, as listed in Table II.

Table II Potential risk factors for the presence of RTGE from 2000 to 2005 identified during stratified univariable analysis of data from RTGE retrospective epidemiological survey in the high productivity stratum (>70 Tonnes/year), with a cut-off point of $p<0.05$.

Dichotomous variable (variable type)	Crude odds ratio	95% confidence interval (T)	P-value (FE)
Fry source 3 (yes/no)	18.0	3.3–97.8	<0.001
Use of processing plant 1 or 2 (yes/no)	18.8	3.2–110.3	<0.001
Mean production (high/low)*	18.2	2.0–161.4	0.002
Fry source 12 (yes/no)	17.1	1.6–178.1	0.01
Fry source 16 (yes/no)	17.1	1.6–178.1	0.01
Automated and/or demand feeders (yes/no)	11.3	1.0–123.2	0.05
Diploid vs. triploid	8.2	1.0–72.4	0.03
ERM I.P. vaccination†	6	1.4–26.6	0.02
Fry source 8 (yes/no)	3.1	1.7–39.5	0.01
Aeration vs. oxygenation	0.2	0.05–0.9	0.04
Earth substrate‡	0.2	0.1–1.0	0.05
Production for restocking only	0.1	0.01–0.8	0.01

(T) Taylor series, (FE) Fisher exact. *High> 217 tonnes/year. †: ERM = Enteric red mouth disease; I.P. = Intraperitoneal. ‡: Only applies to river-based sites

Continuous variable (measure unit)	Median (mean) RTGE cases	Median (mean) non-RTGE cases	P-value (KW)
Water use (million gallons/day)*	20 (21.5)	5 (9.5)	0.002
Mean production (tonnes/year)	300 (452.7)	155 (244.1)	0.003
Trout residence time (months)	8 (9.2)	12 (14.3)	0.005
Trout weight at harvest (g)	400 (390)	600 (823.4)	0.02
Maximum water temperature (°C)	20 (21.4)	18.5 (18.3)	0.05

(KW) = Kruskal–Wallis analysis; * Only applies to river based sites

Variables not significantly associated with RTGE were not included in this table.

Further analysis showed that none of the fry sources were used by all the RTGE positive farms. More information is necessary to investigate fry source as a potential risk factor in the prospective study and the fry sources were not considered for

Unconditional Logistic regression was used for multivariable analysis (outcome or dependent variable RTGE cases). The best fit model identified two variables strongly associated with RTGE cases, as shown in Table III.

Table III Logistic regression model of variables associated with RTGE cases.

Variables	Odds ratio (95% C.I.)	Z-statistic	P
Use of processing plant 1 or 2 (yes/no)	24.93(2.33–266.87)	2.66	0.008
Trout residence time (months)	0.59(0.59–0.97)	−2.08	0.04
Constant		1.25	0.21

−2*Log Likelihood=25.6, Degrees of Freedom=2, $P<0.001$.

These two variables generated the best model, but a significant correlation with other variables associated with RTGE and the presence of other acceptable models suggested that these variables may also be interpreted as indicators of a specific type of site. Both these factors are related with high intensity production, normally for the table market with lower turnover times and strictly controlled feeding regimes.

An analysis was conducted to examine proximity to an affected farm as a potential risk factor for RTGE. The occurrence of RTGE for sites less than 5 miles distant and in the same watercourse was examined. All the sites fulfilling those conditions were also RTGE cases. This suggested that the spread between farms via water was a possibility.

This retrospective study suffered from lack of detail regarding specific batches and some concerns regarding the true nature of unaffected farms. This study was necessary to look for large obvious risk factors and potential control strategies for RTGE and while it did not identify major risk factors or suggested control strategies it increased our understanding and informed our approach to the prospective study and other aspects of the project. Of the original hypotheses it is possible to say: RTGE is not confined to a restricted area, water type or farm system. There is clustering of cases but there is insufficient data to determine if there is spread between sites. No single source of fry could account for all the outbreaks of RTGE but this requires further investigation in the prospective study. Egg source would not appear to be associated with RTGE. All the affected farms use a palliative feeding strategy and while it is not yet possible to say if this reduces the severity of an outbreak it does not prevent outbreaks. No other control strategy has eliminated the condition from an affected site.

Especially relevant was the fact that RTGE was present in the UK in at least 11 sites from 2000 to 2005 and that it is likely that its prevalence in the UK did increase during this period. In the UK, RTGE was associated with high productivity and production of fish for the portion size market.

Phase 2-02. Prospective study

A prospective epidemiological study on RTGE in the UK was set up during the summer of 2006. 17 farms were initially included in the study, including all the sites that had a previous history of RTGE from 2000 to 2005 as possible cases (a total number of 11 farms, identified through the retrospective epidemiological study) and also including several negative control farms that were not affected by RTGE in the past. In the pre-sampling phase, controls were matched with possible cases in yearly production in Tonnes as well as system type. The study was designed to include as many positive sites as practicably possible and data collection took place from June to November 2006 aiming to include complete RTGE outbreaks in the sites selected as suggested by previous knowledge of the syndrome.

The case definition used for the study was: *"RTGE is a condition of rainbow trout, associated with daily mortalities of 0.5% or more and present during the summer. Affected moribund fish usually present a lighter colouration as well as a generally swollen appearance externally, while internally, their lower intestine is distended, congested, oedematous and has a yellow viscous content. Other organs appear apparently unchanged"*. This case definition was created from literature searches (Branson 2003; Michel *et al.* 2002; Urdaci *et al.* 2001) and the knowledge acquired during the preliminary phase of the project and facilitated the selection of RTGE cases based on gross lesions.

All the participating sites were visited before the start of the study and trained in the case definition (Appendix II) to ensure consistency of RTGE mortality recording. Afterwards, visits were made to each site a minimum of two times during the period of the study. The purpose of the visits, as well as to collect data, was to confirm farmers' reports of RTGE and collect negative and positive biological samples to be used in the aetiological studies of RTGE. It is noteworthy that RTGE was observed in 13 sites during the summer of 2006, six cases more than the seven detected in 2005 by the retrospective epidemiological study. This occurrence could be explained either by a previously undetected presence of RTGE in UK farms (perhaps it had been present with low impact to the production, not warranting the involvement of an independent fish health expert) or, on the other hand, a real increase in the number of RTGE cases per year in the UK. It is noteworthy that bacteriological analysis of the samples taken by the researcher did not confirm the diagnosis of RTGE on one of the 13 sites were RTGE was recorded by site staff and therefore, this site was not included in the rest of the analysis.

Production data was extracted from farming management software as well as from on-farm paper records and collated in a single database for convenience of analysis. Several variables were created to facilitate the analysis of the data (Table IV).

The analysis aimed to describe and examine epidemiological patterns found within RTGE positive sites and therefore, only the data from 12 positive sites was included in this study, which was conducted at the unit level for reference to intra-site epidemiology of RTGE. A productive unit was defined as a population of rainbow trout which were stocked in the same cage/tank/pond/raceway on one site at the same time and units were considered cases when RTGE presence had been recorded in them at any time during the period of the study. Table V displays a summary of the characteristics of all the units stocked in these sites during the period of the study, irrespective of their RTGE status.

General descriptive analyses were performed in the totality of the sample for reference to the whole sites studied, although for further statistical analysis, a selection process was applied in order to minimize possible confounding. All apparently healthy units were selected to ensure that only units belonging to the population at risk were included as reference (i.e. only units at risk of becoming RTGE positive). In order to achieve this, only units that had been stocked continuously for at least a month, had been fed at any point during the study and produced for the table market were included in the analysis. These criteria also eliminated units that were used for fish transfer management (i.e. grading, harvest, etc.). With regards to the selection of RTGE cases, only positive units that suffered significant mortalities and where RTGE was not mixed simultaneously with another disease conditions were considered for the analysis.

Table IV. All variables recorded during a RTGE prospective longitudinal study conducted between June and October 2006 in 12 RTGE positive sites in the UK. Each variable was recorded daily for each one of the stocked units in all sites. Variables are presented by category and possible values for each one are displayed.

Category	*Variable*	*Possible Values*
Outcome Variables	RTGE presence	Yes/No
	RTGE cumulative mortality (%)	Continuous, Min, Max, Mean
Site Management	Number of days stocked	Continuous, Min, Max, Mean
	Aeration System	Yes/No
	Oxygenation System	Yes/No
	System Type	Cages Ponds Raceways Tanks
	Feeding system	Automated Demand Hand
	Feed input*	Continuous, Min, Max, Mean
	Feed type	1 to 14
	Pellet size (mm)	Continuous, Min, Max, Mean
	Treatments	Starvation In-feed NaCl mixed on site Commercial in-feed NaCl In-feed Liquid Paraffin In-feed Oxytetracycline Other in-feed Antibiotic Chloramin T in water + In-feed Liquid paraffin Chloramin T in water Formalin in water
	Number of Movements	Continuous, Min, Max, Mean
	Fish transfer	RTGE to RTGE RTGE to Healthy Healthy to RTGE Healthy to Healthy Stocking Harvest
	Contiguity	Contiguity to RTGE cases Downstream to RTGE cases**
	Water Usage (number of times)	Continuous**
Environment	Water temperature (°C)	Continuous, Min, Max, Mean
	Bottom material	Concrete Earth Fibreglass Metal Net
Fish Variables	Total biomass (kg)	Continuous, Min, Max, Mean
	Mean Weight (g)	Continuous, Min, Max, Mean
	Stocking Density (kg/m^3)	Continuous, Min, Max, Mean
	Source of fry	1 to 34
Other Mortality Causes	Bacterial Kidney Disease (BKD)	Yes/No
	Costiasis	Yes/No
	Enteric Red Mouth (ERM)	Yes/No
	Furunculosis	Yes/No
	Handling mortalities	Yes/No
	Predation	Yes/No
	Proliferative Kidney Disease	Yes/No
	Rainbow Trout Fry	Yes/No
	Sleeping Disease (SD)	Yes/No
	Unspecified mortalities	Yes/No
	White Spot ("Ich")	Yes/No

* Feed input was expressed as a percentage of the individual fish weight; **: Only applies to land-based sites

Table V. Summary statistics of all stocked productive units (cage/tank/pond/raceway) from 12 RTGE+ sites included in a longitudinal epidemiological study on the presence and impact of RTGE at the unit level.

	Min	Mean	Max	SD
Stocking days (d)	4	113.9	153	38.3
N° Fish	97	30430.4	148474	26758.3
Mean Fish Weight (g)	4	144.8	2859	225.0
Stocking Density (kg/m^3)	0.3	17.6	63	13.0
Water Temperature (°C)	7	15.2	24	0.9
N° Times water is used	1	1.3	8	0.9
N° fish movements*	0	8.8	63	10.1

*Includes all movements in or out of the units. SD: Standard deviation

RESULTS OF THE LONGITUDINAL STUDY

RTGE impact at the site level and site characteristics: The 12 sites included in the study were distributed across the UK and RTGE was observed and recorded in 164 productive units across all of these sites, representing 39% of all stocked units. The number of units affected by RTGE in each site varied from 2(5.7%) to 44(95.7%). Fish losses due to RTGE totalled 61.4 Tonnes across all the sites and represented 27% of the weight of all the mortalities during the period of the study. Total cumulative mortalities due to RTGE averaged 4.7%(0.02-77.9%) in affected units and daily RTGE mortalities averaged 0.3%(0.002-21.9%). Significant differences were found in cumulative mortalities due to RTGE between river based (21%) and lake based sites (17%).

RTGE presentation at the unit level: First RTGE recordings were made in June in English sites and in Mid-July in Scottish sites. RTGE was present in both Scottish and English sites until the end of October.

Of all the units where RTGE was recorded during the period of the study, 73 units were selected as cases for further analysis as explained above. RTGE represented 53.8% of the total number of mortalities recorded in these units from June to October 2006, although mortalities due to other disease conditions were also observed, including parasitic, bacterial and viral diseases of common appearance during the summer period. A summary of the characteristics of selected cases is shown in Table VI.

Table VI. Summary statistics of 73 RTGE affected units from 12 RTGE+ sites included in the prospective epidemiological study. These units did present a significant number of mortalities by RTGE and RTGE outbreaks were never mixed with other conditions simultaneously.

Variable	Min	Mean	Max	SD
Number of RTGE Outbreaks	1	1.2	2	0.4
Total Number of Days Stocked (d)	42	125.5	153	30.0
Total Number of Fish Movements*	0	10.4	63	10.5
Stocking Density (kg/m^3)†	0.8	25.9	58.9	12.7
Fish Mean Weight (g)†	16	216.5	564	103.9
Water Temperature (°C)†	9.8	15.9	22.0	2.0
Water Temperature (°C)‡	12.0	16.1	20.7	1.7

SD: Standard deviation. * All movements in or out of the units. † During RTGE outbreaks. ‡ During first day RTGE outbreaks.

RTGE was observed in 32 ponds and 7 raceways in earth based sites and in 34 cages in water based sites. It is noteworthy that RTGE was observed twice in tanks as a cause of background mortalities mixed with an outbreak of Proliferative Kidney Disease (PKD), but it was never observed as an isolated condition in this system type and therefore not selected for further analysis.

The average length of recorded RTGE outbreaks was 25.5 days, although the total duration of the outbreaks could be from 3 to 63 days. The analysis of the relative % of RTGE mortalities along time in the selected units revealed a temporal epidemic pattern involving a lower primary peak of mortality numbers followed by a higher secondary peak or else a single mortality peak only as can be observed in Figure 1. These two presentations were not clearly delimited in several of the longer outbreaks, but this occurrence was exceptional.

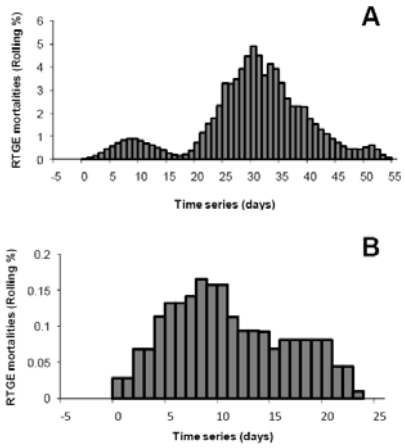

Figure 1. RTGE mortality curves of two selected units from the prospective epidemiological study. Mortalities are expressed as the percentage of the rolling average of mortalities to clearly illustrate mortality patterns. The time variable is expressed in days and day 0 is the first day of RTGE outbreaks. A: Primary peak in mortalities followed by a higher secondary peak (arrows); B: Single mortality peak (arrow).

RTGE incubation period: The average time elapsed between stocking of previously empty units to RTGE onset was 23.7 days before the outbreaks (4 to 48 days; SD=11.7).This analysis did not consider intra-site transfers and a histogram of the frequencies within this time series is displayed in Figure 4. The average time between repeated outbreaks was 22.6 days (19 to 26 days; SD=2.2). This information allowed suggesting that the maximum time of incubation and disease course for RTGE could be expected to normally last from 20 to 25 days. Experimental transmission is necessary to confirm this observation.

Pattern of Spread: Both fish transfer data and contiguity to RTGE cases were analysed in order to describe the spread pattern of RTGE within the sites studied.

The results of the initial analysis of the fish transfer data in respect to the RTGE presence in the outgoing and receiving units are shown in Table VII. For this analysis, all fish transfers were categorized according to the RTGE status of the outgoing and receiving unit and then the odd ratios of the receiving unit depending on the RTGE status of the outgoing unit were analysed. Only fish transfers involving selected units were used in the analysis and only transfers previous to RTGE onset were considered for RTGE positive receiving units (i.e. only potentially infectious transfers).

A total number of 935 intra-site fish transfers were examined during this analysis and a significant increase in the odds of becoming RTGE+ after receiving fish from a case were observed in river based sites, whereas the differences observed were not significant in lake based sites. Interestingly, a significant difference was observed when both site types were analyzed together though stratified analysis.

TableVII. Results of the initial analysis of intra-site fish transfers. The analysis was applied separately to river based sites and lake based sites. The frequencies of each movement type are displayed before the results of the analysis for reference.

	INTRA-SITE FISH MOVEMENT FREQUENCIES						
	Healthy to Healthy	***RTGE to Healthy***	***Healthy to RTGE***	***RTGE to RTGE***	***Odds ratio****	***95% CI***	***P (FE)***
Land Based	375	140	26	34	3.5	1.96-6.27	<0.001
Water Based	224	45	79	12	0.76	0.36-1.57	0.5

*Odds of being RTGE positive after incoming fish transfer from a RTGE positive unit. FE: Fisher Exact (threshold P<0.05)

Following this analysis, time series analysis of fish transfer data from RTGE cases was conducted in order to assess the relative risk of fish transfers from RTGE cases depending on the stage of the outbreak. To achieve this, movements were divided along the time axis in periods of 15 days. All the fish transfers 7 weeks before and after the outbreak were used as a reference control group and the fish transfers of all the other time periods where compared individually against this reference. There were no fish movements out of RTGE affected units during the outbreak in any lake based site, and this made the time series analysis impossible for this site type. On the other hand, movements were made during RTGE outbreak in earth based sites and it was found that the relative risk of the fish transfer was only higher if fish were transferred during the outbreak in the outgoing unit, as shown in Figure 4. This observation suggests fish transfer from affected units undergoing RTGE outbreaks are an important source of horizontal spread of RTGE in a site and should be avoided.

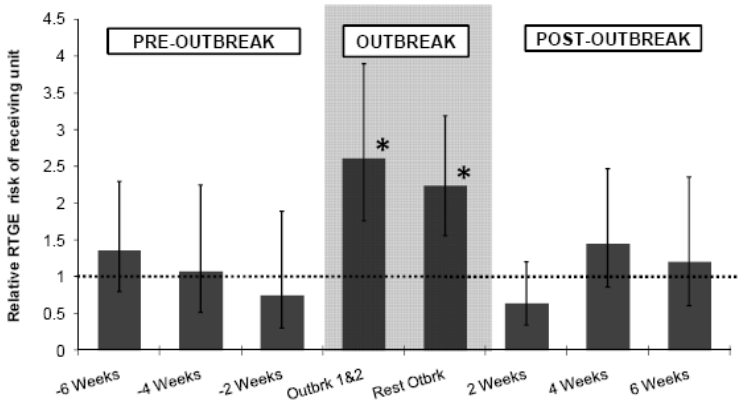

Figure 4. Graph displaying the variations in the relative risk of receiving units after intake of a fish transfer from an RTGE case, depending on the timing of the transfer relative to the stage of the RTGE outbreak in the outgoing unit. Time-series points which showed significant difference in Yates corrected test are highlighted with a star (P<0.001 in both cases). Upper and lower Cornfield confidence intervals are displayed for each time-series point and a dotted line is plotted at relative risk=1 for visual reference.

Also part of the study on the pattern of spread of RTGE was the analysis of the sites layout in respect to the spatial spread of RTGE along time. For this analysis, all the units within a site were classified as contiguous to an RTGE case or non contiguous, as well as downstream to a case or not (for river based sites). Only positive units that were contiguous or downstream to cases before their own outbreak were considered and index cases for each site were not included. The results of this analysis can be observed in Table VIII.

Significantly increased odds of a unit becoming RTGE positive were observed for both earth and water based sites when they were contiguous to previous positive cases. Also, analysis of earth based sites did show increased odds if negative units were located downstream to RTGE cases (OR=5.1(1.1-27.2) P=0.03) but not if they were located upstream (OR=2.2 (0.7-7.3) P=0.2).

TableVIII. Results of univariable analysis of the odds of an apparently healthy unit becoming RTGE positive if it was contiguous to a RTGE case. The analysis was applied separately to river based sites and lake based sites and then stratified analysis was applied to all site types together.

	CONTIGUITY TO RTGE CASES						
	Contiguous and RTGE+	*Contiguous and healthy*	*Non contiguous and RTGE+*	*Non contiguous and healthy*	*Odds ratio**	*95% CI*	*P(FE)*
Land based	28	15	1	13	24.8	2.8-546.1	<0.001
Water	8	6	6	29	6.4	1.4-32.9	0.01
All Sites**	36	21	7	42	11.1	3.2-36.0	<0.001

*Odds of becoming RTGE positive after contiguity to a RTGE positive unit. **Stratified by site type using Mantel-Haenzel adjusted odds ratio. FE: Fisher exact test (Confidence level p<0.05).

To complement these findings, survival analysis was used to analyse the differences in the time of onset of RTGE between units that were contiguous (or downstream) to cases compared to units that were not. This analysis confirmed a significantly faster onset of RTGE in units contiguous to RTGE cases from the first day of the study, irrespectively of the site types. This was also the case for units located downstream to RTGE cases in river based sites. The results of the survival analysis are displayed in Figure 5.

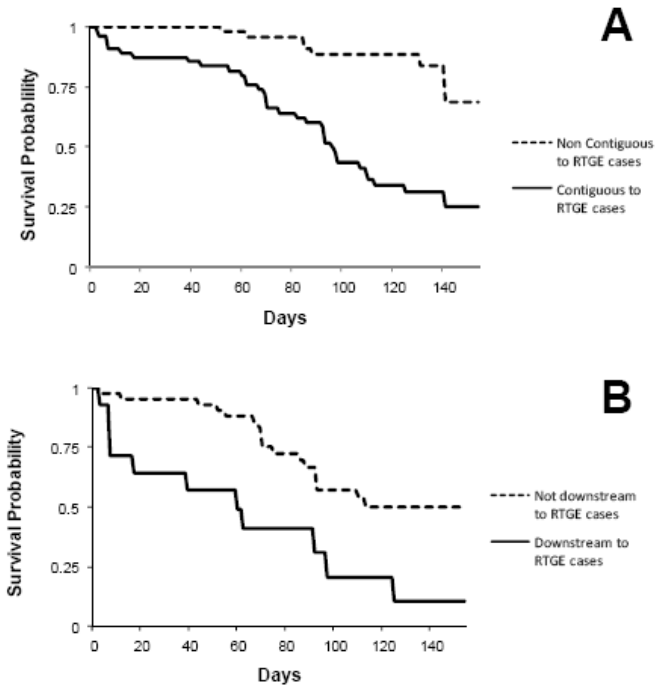

Figure 5. Survival probability plots obtained by Kaplan Meier analysis of the timing of RTGE onset with respect to the first day of a longitudinal epidemiology study on the presence and prevalence of RTGE in 12 rainbow trout producing sites. A: Applies to both water and earth based sites and illustrates a significantly higher probability of becoming RTGE positive earlier for units that were contiguous to RTGE cases (Wilcoxon=24.16; P<0.001). B: Applies to earth based sites only and illustrates a significantly higher probability of units located downstream to cases to become RTGE cases earlier than units located elsewhere (Wilcoxon=10.65; P<0.001).

To conclude the analysis on the pattern of spread, multivariable models were built in order to assess the effect of these sources of transmission together. This analysis used selected productive units where both contiguity and fish transfer data were available (cases: 15; controls: 32). Both contiguity to cases and fish transfers from RTGE positive units were identified as risk factors for RTGE by multivariable logistic regression, as shown in Table VIX.

TableVIX. Multivariable logistic regression model of the two sources of transmission of RTGE studied (Contiguity to cases and intra-site fish transfers from RTGE affected units). The outcome considered was RTGE unit=Yes/No.

Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Contiguous to RTGE case (Yes/No)	8.3(1.8-38.6)	2.7	0.007
Received fish from RTGE (Yes/No)	8.9(1.5-54.2)	2.4	0.017
CONSTANT	*	-3.4	<0.001

-2*Log Likelihood=42.9, Degrees of Freedom=2, p<0.001

Concluding, the analysis of the spread pattern of RTGE within a site has revealed an important role of fish transfers and contiguity to cases in the horizontal spread of RTGE within affected sites. These two findings together confirm an infectious nature of RTGE and strongly suggest transmission via water as a possibility.

Identification of risk factors linked to RTGE at the unit level: To achieve this purpose, a case control study was designed using the data available from selected RTGE affected and apparently healthy units.

Cases were matched to controls in system type (cage/pond/raceway/tank) and in geographical location (England/Scotland). System type was chosen due to its confounding association with several management variables and to account for the differences found between earth and water based sites, whereas geographical location (England/Scotland) was chosen for its association with all the environmental variables, as shown by preliminary analyses in both cases. A ratio of two controls per case was established for all matches by random selection of cases and controls within each match, leading to a final number of 43 cases and 86 controls. All variables were then summarized for the whole period of the study: For continuous variables, minimum, maximum and average values were calculated and included in the analysis both as continuous and categorized through the median value.

Analyses were performed using and conditional multivariable logistic regression, a technique that takes into account the case-control matches when interpreting the data. This technique was chosen to try and minimize the confounding effect of the match variables as much as possible.

Finally, two multivariable models were built to explain both environmental and management variables associated with RTGE. These models are shown in tables X and XI below.

Table X. Conditional multivariable logistic regression model including environmental variables significantly associated with the presence of RTGE.

Environmental Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Mean stocking density (kg/m^3)	23.9 (1.3-444.7)	2.1	0.03
Mortalities due to predation (Yes/No)	19.3(1.2-300.3)	2.1	0.03
Mean temperature of water (°C)	15.8 (1.1-218.0)	2.1	0.04
Contiguous to RTGE case (Yes/No)	11.1 (1.2-104.1)	2.1	0.03

-2*Log Likelihood=26.3, Degrees of Freedom=4, P<0.001

Table XI. Conditional multivariable logistic regression model including management variables significantly associated with the presence of RTGE.

Management Variables	*Odds Ratio (95% C.I.)*	*Z*	*P*
Received fish from RTGE cases (Yes/No)	5.5 (1.7-18.2)	2.8	0.005
Mean daily Feed Input per Fish (High/Low)*	5.4 (1.7-17.2)	0.6	0.005
Mortalities due to handling (Yes/No)	3.0 (1.1-8.7)	0.5	0.04
No use of aeration	0.2 (0.04-0.7)	-2.4	0.02

*Categorised through median (=0.9% of individual fish weight). -2*Log Likelihood=67.6, Degrees of Freedom=4, p<0.001

These models apply to both river and lake-based sites and confirm the findings from previous analysis taking into account the interactions between the selected variables, as well as matching the cases to the controls. Two different models have helped to explain the data from two different perspectives. The model containing environmental variables indicates that situations where higher stress levels can be expected are significantly linked with the presence of RTGE in affected units, as well as confirming previous observations on the effect of higher water temperature and the possibility of transfer via water. The second model includes variables related to the site management and represents practices significantly associated with RTGE presence in affected units and suggests that generally higher feed input, fish handling and intra-site transfer of RTGE affected fish are all linked with the presence and spread of RTGE. The protective effect of the lack of aeration systems in the units may be related with both the spread of the condition or else be an indirect effect of other characteristics of the units where aeration was used in the chosen sites.

Finally, we fitted general linear models to data from the period when the outbreaks where active to identify factors associated with a higher impact of RTGE (expressed as the natural logarithm of the daily cumulative mortalities). For these models, site was included as a random factor to account for variability due to this variable. The final model is shown in Table XI.

Table XI. General linear model of the variables associated with the natural logarithm of the RTGE cumulative mortalities (n=69).

Model term	*Factor Type*	*Coefficient*	*SE*	*P*
Site	Random			<0.001
Ln Outbreak Length (Days)	Covariate	1.21	0.12	<0.001
Ln Mean (Daily Feed Input (kg)/Fish Number)	Covariate	0.54	0.13	<0.001
Constant		1.18	0.93	0.21

Adjusted R^2=85.5%. SE: Standard error

The results of the GLM identified a significant influence of higher daily feed input/fish during outbreaks. This was observed after adjustment for confounding by outbreak length, and suggested that reducing the feeding input during RTGE outbreaks could help to reduce the impact that this condition has on affected units.

Phase 2-3. Aetiology

A total of 874 fish were sampled between June and September 2006, of which 220 were consistent with the RTGE case definition at the fish level. Sampling was designed from a descriptive epidemiological viewpoint, where a minimum sample size of 30 fish samples were taken from each sampled production unit, enabling the detection of 10% prevalence with 95% confidence level. Fish samples were taken from each participating farm depending on its RTGE status grouped as follows:

RTGE positive farms (Three groups of samples):

- RTGE moribund fish (as many as practicable).
- Thirty apparently healthy fish cohabiting with RTGE affected, randomly selected.
- Thirty fish randomly selected fish from an apparently healthy unit (Negative controls)

RTGE negative farms:

Thirty randomly selected fish from a randomly selected unit (Negative controls).

The aim of the sampling was to create a sample library that would enable an epidemiological approach to the analysis of the results as well as to provide enough flexibility to research different aspects of the syndrome in an effective way. Additional samples were taken for transmission and scanning electron microscopy analysis (including distal intestine as well as pyloric caeca) and viral screening.

Phase 2-3.1. SFB aetiology

A preliminary experiment was conducted before a larger scale molecular epidemiology experiment would be started, in collaboration with CEFAS. The purpose of the experiment was to use DNA extracted from paraffin embedded tissue samples collected from rainbow trout sites during the summer of 2006 for the specific detection of Candidatus *arthromitus* with a nested PCR technique. The ultimate objective of the experiment was to describe the distribution of SFB in the pyloric caeca and distal intestine of fish affected and non-affected by RTGE in a preliminary manner using 16S genetic fingerprinting techniques in conjunction with histological techniques.

Firstly, DNA was extracted from 1µm thick sections of the tissue following a protocol developed at the IoA (Crumlish *et al.* 2007) and from fresh intestinal contents using standard protocols. A nested PCR was then performed on the extracted DNA consisting of two steps. The first step utilized eubacterial primers 20F and 1500R (universal for all eubacteria), whereas for the second step SFB specific primers were used (SFB779F and SFB1500R). Both these primers target the *rrs* gene of the 16S rDNA of eubacteria and SFB respectively and have been designed during previous work with eubacteria (Amann *et al.* 1990) and SFB (Urdaci *et al.* 2001). To confirm these results, one positive PCR sample was randomly chosen from each fish group, the DNA was purified using a Qiaquick DNA purification kit (Qiagen, UK) and then sequenced using a Beckman Coulter CEQ 8800 sequencer (UK).

The results of the analysis demonstrated the presence of SFB DNA in fresh and paraffin embedded samples of the following experimental groups: (a) Moribund fish showing RTGE; (b) apparently healthy fish cohabiting with fish with RTGE; (c): fish from an unaffected unit from an affected farm and (d) fish sampled from a random unit in an apparently unaffected farm. Six distal intestine samples and six pyloric caeca samples were selected from each group of fish and the sequencing of the PCR products was consistent with that one of trout "*C.* arthromitus" (Figures 6 and 7).

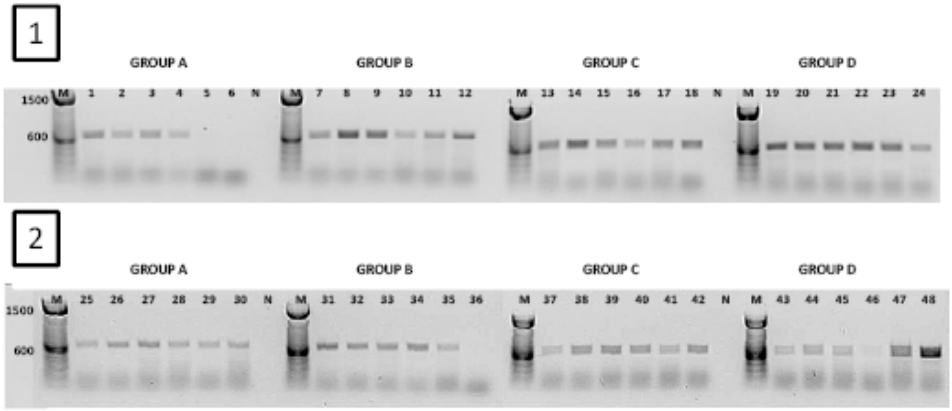

Figure 6. PCR products from paraffin wax-embedded tissues. Group A: RTGE-affected fish; Group B: Apparently healthy fish cohabiting with affected; Group C: Fish from an apparently healthy unit in an affected site; Group D: healthy fish from a random unit in an unaffected site. (n=6 samples per group; gel 1: distal intestine samples; gel 2: pyloric caeca samples; M: marker; N: negative control).

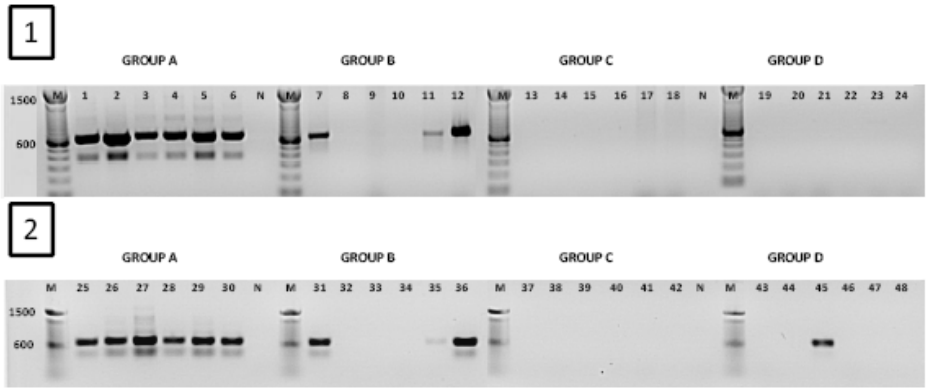

Figure 7. PCR products from fresh digestive contents. Group A: RTGE-affected fish; Group B: Apparently healthy fish cohabiting with affected; Group C: Fish from an apparently healthy unit in an affected site; Group D: healthy fish from a random unit in an unaffected site. (n=6 samples per group; gel 1: distal intestine samples; gel 2: pyloric caeca samples; M: marker; N: negative control).

In order to complement these primary findings of the main experimental design, four more experimental groups were included in the study: (1) fry from a UK hatchery (Hatchery A) that supplied fish to RTGE-positive sites, (2) fry from a UK hatchery (Hatchery B) that supplied fish to sites in which RTGE had never been reported, (3) fry from Northern Ireland (Hatchery C), and (4) ongrowing rainbow trout from Northern Ireland (Site C). None of these sites had had RTGE reported in the past. For these additional groups, analyses were performed in paraffin embedded distal intestine samples only and these samples were processed using the same protocol.

After the analysis, none of the Northern Ireland trout samples were positive for SFB DNA (data not shown), but positive samples were observed in the fish from both Hatchery A and Hatchery B, as shown in Figure 8 below.

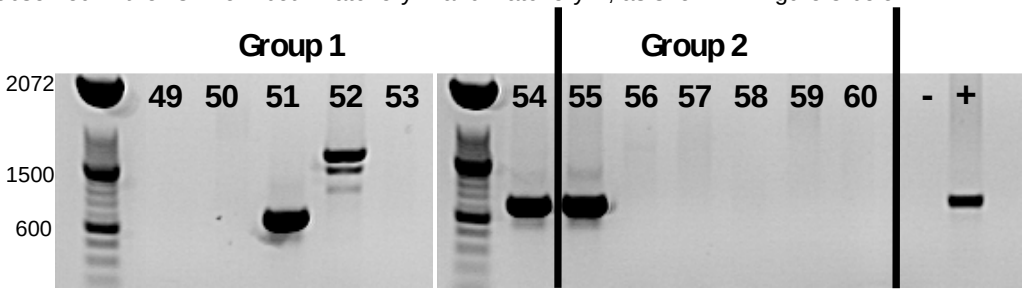

Figure 8. PCR amplified products from a "*Candidatus* arthromitus" specific nested PCR. These samples originated from Hatchery A and Hatchery B, both RTGE negative at the time of sampling. Hatchery A (group 1) supplied fish to RTGE + and RTGE - sites, whereas Hatchery B (group 2) only supplied fish to RTGE – sites. -: Negative control (No template DNA); +: Positive control (Section known to contain C. arthromitus DNA).

All the PCR products sequenced for both parts of the study presented a consistency of 98-100% to the published sequence of trout Candidatus arthromitus sequence (Urdaci et al. 2001).

In order to complement the results of the molecular study, all the paraffin embedded tissues from all experimental groups used for DNA extraction were sectioned at 5µm and stained with both haematoxylin/eosin and gram stains. Each section was then examined using light microscopy for the presence or absence of the following: Autolytic changes in the tissue, consistency of any pathological changes observed in the section with the current histopathological description of RTGE (*i.e.* severe congestion of the intestinal wall accompanied by epithelial detachment and enterocyte necrosis (Michel *et al.* 2002)), presence of Gram-positive filamentous bacteria and, if present, a description of these bacteria (segmented appearance, Gram variability and interaction/association with enterocytes).

Gram variable segmented filamentous bacteria were observed in several tissue sections, including RTGE fish and apparently healthy trout. These bacteria were not observed in any of the distal intestine samples of any fish with RTGE and evidence of severe tissue lysis was observed in these sections. This was not the case with pyloric caeca from the same fish, where autolytic changes were less pronounced and Gram variable segmented filamentous bacteria were always present in large numbers both attached to the mucosal layer of the intestine as well as apparently free within the lumen (Figure 9).

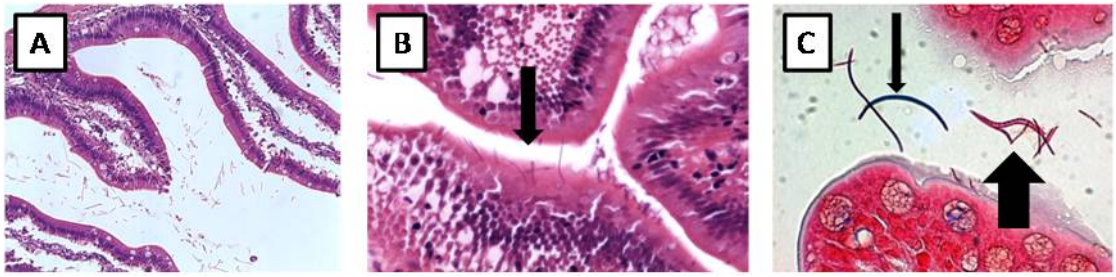

Figure 9. Histopathological images of the digestive system of rainbow trout (*Oncorhynchus mykiss*) affected with rainbow trout gastroenteritis (RTGE). A: Large numbers of filamentous bacteria occupy the lumen of pyloric caeca and epithelial detachment is observed (H&E; x100). B: Filamentous bacteria (arrow) are closely associated with the epithelium of pyloric caeca (H&E; x200). C: The filamentous bacteria are clearly segmented (wider arrow) and display Gram variability: Gram positive (thinner arrow, blue) and Gram negative (wider arrow, red) depending on the individual, in several occasions both staining patterns were observed in a single bacterium (Gram counterstained with Neutral Red; x1000). All these sections were positive in "*Candidatus* arthromitus" specific PCR.

Regarding the presence of SFB within the digestive system of apparently healthy fish, Gram variable filamentous bacteria were observed in the distal intestine of two fish cohabiting with RTGE affected individuals and one fry from hatchery A (Figure 4). In this case, these bacteria were never observed interacting closely with the enterocytes, although close interaction with feed particles was observed in several occasions. SFB were not present in large numbers in any of these samples (Figure 10).

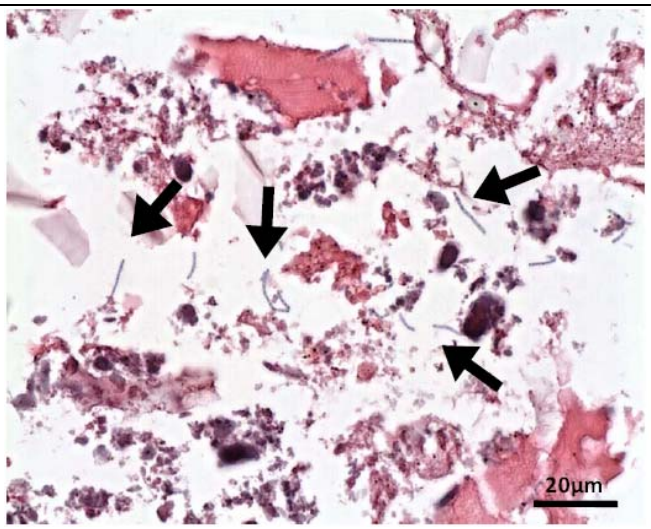

Figure 10. Histopathological images of distal intestine of apparently healthy rainbow trout (*Oncorhynchus mykiss*) displaying Segmented Filamentous Bacteria (SFB) presence. Several SFB (arrows) can be observed amongst feed particles in the intestinal lumen of rainbow trout fry from hatchery A (H&E;x200). All these sections were positive for "*Candidatus* arthromitus" DNA presence in PCR.

In conclusion, this study reported the presence of SFB DNA in apparently healthy and RTGE affected rainbow trout. In addition, parallel histopathological observations confirmed the presence of gram variable segmented filamentous organisms in RTGE affected and clinically healthy rainbow trout and suggested that pyloric caeca are the preferred site for visualizing SFB in trout with RTGE. The results of this study suggest that the presence of SFB is not invariably associated with clinical disease and that more information is required to understand the role of these organisms.

Phase 2-3.3. Other agents

The hypothetical presence of a toxin was studied by a cytotoxicity assay, where cell lines were exposed directly to different dilutions of filtered intestinal contents from RTGE affected and apparently healthy rainbow trout. The cell lines used were from rainbow trout gonad (RTG-2) and were chosen from a range of fish cell lines available at the IoA after a preliminary study using two samples, one from a RTGE affected and one from an apparently healthy fish. During this preliminary study the appropriate titration range and inoculation method were also established. Titration values ranged from 1:125 to 1:16000 for the former and preformed inoculation was the selected method in the later.

For the final experiment, thawed intestinal content samples from three RTGE+ fish and three apparently healthy fish from the same location were inoculated in a 96 well plate with preformed RTG-2 cells in 150µl L15 media supplemented with 2% Foetal Bovine Serum (the layout and titrations can be observed in Figure 11). Intestinal contents were kept on ice throughout the process and diluted at 1:50 before they were filtered through a 45µm pore size filter. Preliminary titrations were made in a secondary plate in a manner that ensured the appropriate final titration when 100µl were added to the 150µl of media in the wells. After inoculation, the plate was sealed and incubated at 20°C with no additional CO_2 added and the cells in each well were observed daily for a period of 21 days for the presence of toxicity. A blind freeze-thaw passage was performed for all RTGE + samples, as a secondary screening for viral presence.

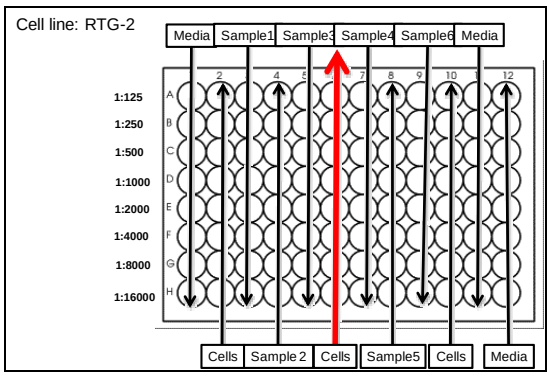

Figure11. Layout of the 96 micro-well plate used in the cytotoxicity assay for assessment of comparative toxicity between the intestinal contents of RTGE affected and apparently healthy rainbow trout. The titrations used can be observed on the left of the plate. An empty column was left between healthy and affected fish to minimize cross contamination. Rows with cells only were used as control references to assess changes in test wells.

The results obtained from the assay demonstrated higher toxicity of the intestinal contents of apparently healthy fish to the RTG-2 cell lines, and cellular death was observed in all the wells at 1/100 for apparently healthy fish, whereas RTGE+ samples did not cause total cellular death in any case. All surviving cells started to degrade at day 20 of observation, likely due to exhaustion of the nutrients within the culture media. Figure 12 displays the number of days to death of each one of the experimental groups.

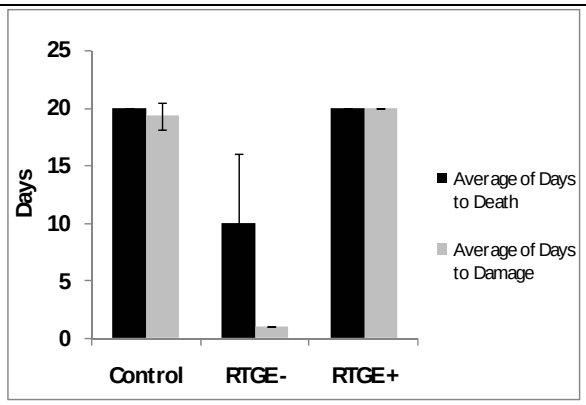

Figure12. Number of days to total cellular death and observed cellular damage for all experimental groups included in the cytotoxicity assay. Error bars display the standard deviation for each experimental group (n=3). These values correspond to the lowest titration used in the experiment only (1:125).

Other changes observed in the cells included syncitial formation and intracellular vacuolation, which could be observed to differing degrees for both experimental groups and controls. Freeze-thaw passages did not show viral presence.

Concluding, the results of the toxin studies have not elucidated the clear presence of a toxin, although the higher toxicity to the cell lines shown by intestinal contents from apparently healthy fish could be interpreted as an indication of active digestion, which would be absent in RTGE affected fish. The results of this study do not warrant the use of monoclonal antibodies against a range of toxins.

Phase 2-3.3. Viral agents
Intestinal samples from RTGE affected and unaffected fish were placed in transport media and sent to CEFAS Weymouth screening of viral presence by exposing different cell lines to a homogenate of intestinal tissue and its contents. The experiment identified no viral infection in any of the cell lines tested; these results will be reported by CEFAS.

Phase 2-05. Pathogenesis
All gross signs from rainbow trout sampled during the summer of 2006 were collated in a database and subjected to descriptive analysis, during which gross signs, presence of other conditions, digestive pH and haematocrit were examined for refinement of the case definition as well as to base hypothesis on the pathogenesis of RTGE.

Case definition assessment: The database included 152 RTGE + fish and 152 matched apparently healthy controls. The analysis of the gross presentation and bacteriological examination of rainbow trout with signs consistent with the previous case definition revealed issues related to mixed conditions and assessed less frequent gross signs included in the presentation of RTGE. This information was used to identify RTGE affected fish without concurrent disease for pathogenesis studies.

Mixed conditions: Secondary bacterial infections were found in several fish consistent with the case definition of RTGE and a total number of 23(15%) fish were positive for kidney bacteriology. After basic bacteriology identification it was established that most of these fish were likely to have been infected with *Aeromonas salmonicida* (18(78%)) and *Yersinia ruckeri* (3(13%)). These fish were excluded from the case definition by only including fish that were negative for kidney bacteriology using conventional bacteriology techniques (Tryptone Soya Agar incubated at 22°C).
Another disease condition frequently found in conjunction with RTGE during the summer of 2006 was Proliferative Kidney Disease (PKD) and 16 fish (11%) consistent with RTGE case definition also presented enlarged kidneys consistent with PKD. This presentation was usually accompanied by the presence of pale gills, splenomegaly, hepatomegaly and a significantly reduced Packed Cell Volume (PCV) when compared to apparently healthy fish ($P<0.001$ KW). These fish were also excluded from the case definition by only including fish where non digestive organs looked apparently normal.

Gross presentation: The frequency of the presentation of pathological changes in fish consistent with the case definition is summarized in Table XII.

Table XII. **Summary of pathological changes observed in rainbow trout consistent with the original case definition of RTGE. The relative percentage and association with moribund fish is also included.**

	Description	Frequency (%)	Relative % Moribund*	OR (95% CI)	P (FE)
External Gross Signs	Swollen Appearance	107 (70%)	96%	18.8 (5.9-60.1)	<0.001
	Lighter Colouration	81 (53%)	100%	N/A	<0.001
	Striping of Flanks	47 (31%)	98%	12.2 (1.6-93.4)	0.001
	Gill Pallor	9 (6%)	89%	1.5 (0.2-12.2)	0.6
	Darker Colouration	7 (5%)	100%	N/A	<0.001
	Haemorrhagic Gills	5 (3%)	100%	N/A	<0.001
	Skin Lesions	1 (1%)	100%	N/A	<0.001
Internal Gross Signs	Pyl. Caeca Congestion	97 (64%)	90%	2.7 (1.1-6.6)	0.03
	Gastric Dilation	61 (40%)	98%	19.1 (2.5-146.2)	<0.001
	Enlarged Kidney	16 (11%)	81%	0.8 (0.2-2.9)	0.4
	Splenomegaly	16 (11%)	88%	1.3 (0.3-6.0)	0.5
	Hepatic Pallor	8 (5%)	88%	1.3 (0.2-10.9)	0.6
	Hepatomegaly	5 (3%)	80%	0.7 (0.1-6.6)	0.6
	Hepatic Haemorrhage	5 (3%)	80%	0.7 (0.1-6.6)	0.6

* Relative to the total number of fish where each pathological change was observed. OR: Odds ratio of the association with moribund RTGE + fish; 95% CI: 95% Confidence Intervals; FE: Fisher Exact.

Further cluster analysis (Figure 13) revealed three presentations associated with RTGE+ fish identified with the case definition.

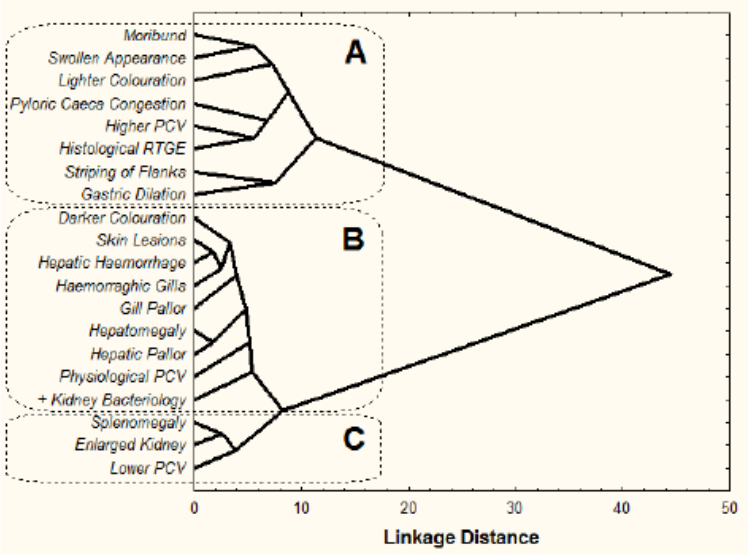

Figure 13. Cluster analysis of laboratory tests and gross signs presented by rainbow trout with RTGE. Gross signs part of the case definition used are not included. Gross signs observed in cluster A were present in 31-75% of the fish, those of cluster B in 11-16% and those of cluster C in 1-6% (n=152).

Cluster A was comprised of pathological changes associated with moribund RTGE+ fish (Table XII), and cluster B and C were associated with concurrent conditions. This additional knowledge was used to sample RTGE+ fish without concurrent conditions for the analysis of the pathogenic mechanisms of RTGE, using apparently healthy cohabiting fish as reference.

Preliminary analysis of digestive pH: Intestinal and gastric pH measurements were made for a group of RTGE affected fish (n=6) and apparently healthy (n=29) using a portable flat pH meter (Horiba Twin™, HORIBA, Japan). The result of these preliminary analyses suggested that lower gastric pH is associated with the presence or not of feed contents (Kruskal Wallis P<0.001), rather than presence or absence of RTGE (Kruskal Wallis P=0.13 KW). Intestinal pH doesn't appear to be affected by the presence or absence of either feed nor RTGE (Kruskal Wallis P=0.71 KW). The results obtained from this analysis did not warrant further specific search for differences on digestive pH variations in RTGE affected and unaffected fish.

Biochemistry analysis: Several observations made during the collection of samples, including gastric dilation with fluid contents and generally swollen appearance of affected fish led to consider the hypothesis of osmotic imbalance as the possible cause of death of RTGE fish. In order to test this hypothesis, biochemistry and PCV values were analysed.

Blood samples were taken from the caudal vein using a heparinised syringe and centrifuged at 3000g for 10 m to extract plasma, which was then transferred to a cryovial and snap frozen in liquid nitrogen for preservation. Previously, a small part of the blood sample was used to fill in a heparinised haematocrit tube, which was centrifuged at 20000g for 5 m. PCV was measured and recorded for reference.

Samples were taken from fish belonging to the following experimental groups: (a) 10 RTGE affected fish; (b) 10 clinically healthy cohabiting fish from the same location and sampled simultaneously; (c) 10 clinically healthy fish from a different site. The inclusion of negative controls as a reference was necessary, due to a lack of haematological and biochemical reference values in the literature. These samples were sent to the Scottish Agriculture Research centre for routine biochemistry analysis on total protein and albumin.

Once the results from these tests were available, globulin concentrations were calculated by subtracting the albumin value from the total protein figure for each fish. Finally, albumin/globulin ratios were calculated. Albumin values include low molecular weight proteins and Globulins include high molecular weight proteins. The average values for each group of each one of these parameters are shown in Table XIII. The differences found between these groups were assessed using Kruskal-Wallis.

Table XIII. Comparative values of biochemical parameters of blood plasma from apparently healthy fish RTGE-affected fish.

VARIABLE	*RTGE– Mean (SE)*	*RTGE+ (a) Mean (SE)*	*RTGE+ (b) Mean (SE)*	*P (KW)*	*Reference values**
Sodium (mmol/l)	146.5 (2.4)	-	89.4 (1.5)	<0.001*	123-164†
Chloride (mmol/l)	123.1 (2.2)	-	45.5 (2.0)	<0.001*	120-147†
Potassium (mmol/l)	3.3 (0.2)	-	3.3 (0.2)	0.9095	3.3-3.5†
Total Protein (g/l)	43 (1.5)	83.1 (3.5)	-	<0.001*	28-60§
Albumin (g/l)	18.6 (0.7)	32.2 (1.9)	-	<0.001*	17-19§
Globulin (g/l)	24.4 (1.1)	50.9 (2.7)	-	<0.001*	5-41§
Albumin/Globulin (g/g)	0.8 (0.03)	0.6 (0.04)	-	0.02*	N/A

SE: Standard Error. (a) & (b): RTGE+ experimental groups. KW: Kruskal-Wallis. All groups n=10 fish. †: Powell (2006). §: Bowser (1993)

The results of the analysis also revealed significantly increased CPV values in RTGE affected fish (P<0.001, not shown in the table).

Overall, the results of these tests did suggest haemoconcentration due to reduced plasma volume, as shown by significant higher PCV and an increase in total protein concentration. The albumin/globulin ratio was significantly lower in RTGE affected fish, and this reflects a relative reduction of plasmatic proteins of small molecular weight, whereas bigger molecular weight proteins may have remained constant, been lost at a slower pace or indeed increased. The latter possibility is unlikely, and this presentation is consistent with a protein losing enteropathy where small molecular weight proteins are lost to the intestinal lumen after severe disruption of the osmotic barrier in the digestive mucosal surface. This disruption leads to fluid entering the fish vascular compartment from the environment as freshwater fish are hyperosmotic. This fluid then moves from the vascular compartment to the interstitial space (*i.e.* rest of the tissues) due to a decrease in blood osmolarity following the loss of plasma albumin. The fluid transfer results in the generalized swollen appearance observed in RTGE affected fish and it is very likely the cause of death. This information also suggests that the reason behind gastric dilation may be an increased water oral intake, which otherwise does never happen in freshwater fish, resulting from thirst receptors being triggered by a decreased circulating volume.

This pathogenic picture is consistent with the effectiveness of in-feed salt treatment, a control strategy that has been targeted to RTGE. In-feed salt would effectively support the fish by equalising the osmotic pressures between the intestinal and vascular compartment, whereas in-feed liquid paraffin would reduce fluid loss to the intestinal compartment by creating of a temporary waterproof layer over the damaged intestinal mucosa. Other possible mechanisms of action of these two treatments cannot be excluded with the knowledge available on the aetiology and pathogenesis of RTGE.

Histological and electron microscopic observations have confirmed both damage and osmotic imbalance at the intestinal mucosa level, as shown in Figures 13 and 14 below.

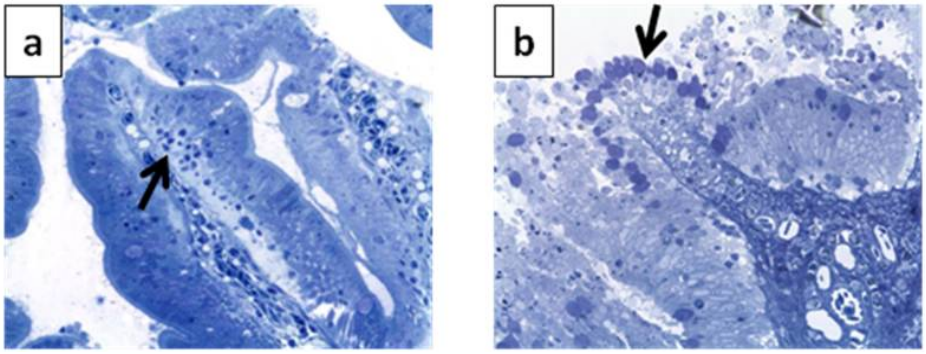

Figure 13. Toloudine blue stained histological pictures displaying damage to the intestinal epithelium in RTGE affected fish. (a): Basal hydropic degeneration (arrow) followed by (b): generalized epithelial detachment (arrow).

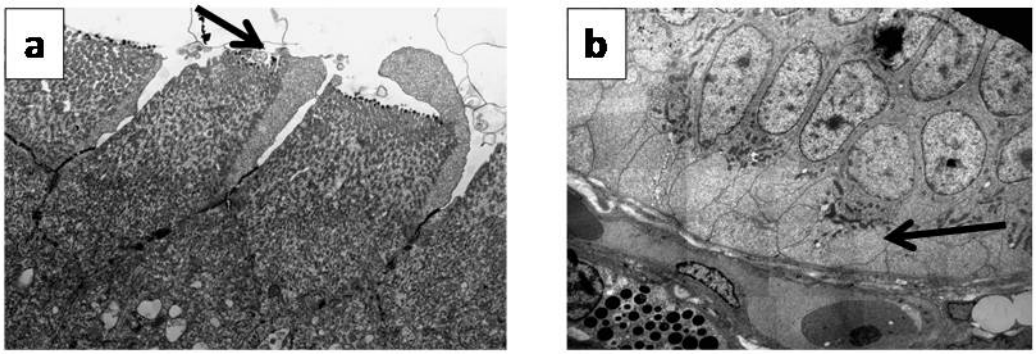

Figure 14. Transmission electron microscope pictures displaying ultrastructural osmotic damage in the intestinal epithelium. (a): apical blebbing, i.e. cellular oedema of enterocytes (arrow), with compromise of the integrity of the microvilli (b): basal hydropic degeneration of enterocytes (arrow), with cytoplasmic dilution and loss of structure.

Phase 2-06. Farm based control strategies

After the analysis of all the other parts of the study was complete, the information gained was used to develop best practice guidelines (Appendix III), which were circulated to all the participating farmers as well as all the members of the BTA.

Discussion of the reliability of the results and the main implications of these findings

This study has used a multidisciplinary approach to the study of RTGE which has generated scientific information related to different aspects of this syndrome, including its epidemiology, pathogenesis and aetiology. Both the experimental design and techniques used in all parts of the study had been previously used successfully for the research of animal disease, both in fish and other species. Additionally, all aspects of data collection, fish sampling and processing of the samples were exhaustively tested before each experiment to ensure consistency and practicability of the approach used. Whenever a technical issue was identified, it was resolved before starting the experiment. For these reasons, we are confident in these results as presented.

This work has revealed RTGE is a significant problem for the UK rainbow trout industry, especially to large producers for the table market. At the unit level, RTGE behaves as an infectious disease and movement of fish within the sites played an important role in its spread within affected sites. Several management and environmental variables associated with its presence were identified, as well as its main pathogenic mechanism. All of this information is directly relevant to the control of this disease, and has been compiled in a list of practical control strategies that can be applied at the farms affected (Appendix III), including:

- Isolation of units affected with RTGE. Do not move the fish unless absolutely necessary.
- If possible avoid reuse of water from units suffering from RTGE.
- In-feed salt is most effective when it is commenced prior to an outbreak and maintained throughout its duration.
- Avoid very high levels of feeding if possible and reduce feeding at the first sign of an outbreak.

This study has also revealed that the presence of SFB DNA does not always result in clinical RTGE, suggesting that its involvement in the aetiology may be multifactorial or it may not be the aetiological agent at all. More research will be required to clarify this.

Possible future work

Although this study has contributed with valuable scientific information on RTGE, this condition still warrants further investigation. Of special interest would be to test the possibility of transmission of RTGE by gavage of filtered and unfiltered intestinal contents from RTGE-affected fish. These experiments would allow testing the hypothesis of a bacterial versus viral putative infectious agent as well as the investigation of the sequential pathology of RTGE. Further efforts should also be made to promote the uptake of the control strategies proposed as a result of this study and to test them on affected sites through field intervention studies. The identification of the aetiology of RTGE is directly relevant to its control and the use of gavage, gradient centrifugation or culture independent techniques may help to identify the causal agent of this syndrome.

Actions resulting from the research (e.g. IP, Knowledge Transfer)

The findings of this project have been widely disseminated and discussed with a variety of stakeholders. A list of control strategies was developed in collaboration with farmers and the BTA (Appendix III). Our intentions were to test the effectiveness of these strategies in field intervention trials but the strategy was not adopted by any farms last year, largely due to changes in the business structure of the industry. However, the BTA will circulate again the advice before the high risk period this year and further discussion with farmers is ongoing to examine the practicality of the control strategies and the reasons for lack of uptake last year.

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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.

Publications

-Del-Pozo, J., 2009, "A study of the Aetiology and Control of Rainbow Trout Gastro Enteritis" PhD thesis. University of Stirling (UK). The viva examination will take place on the 15th March 2009.

-del Pozo, J., Crumlish, M., Ferguson, H. W., Turnbull, J.F.T., "A Retrospective Cross-Sectional Study on Rainbow Trout Gastroenteritis (RTGE) in the UK.", In-press, Aquaculture 2009

Manuscripts ready for submision

-del Pozo, J., Crumlish, M., Ferguson, H. W., Turnbull, J.F.T., "Prospective Longitudinal Study of "*Candidatus* arthromitus" Associated Rainbow Trout Gastroenteritis in the UK." Ready for submission, 2009

-del Pozo, J., Crumlish, M., Ferguson, H. W., Turnbull, J.F.T., "Histopathology & Ultrastructure of "*Candidatus* arthromitus"-Associated Rainbow Trout Gastroenteritis." Ready for submission, 2009

-del Pozo, J., Crumlish, M., Ferguson, H. W., Turnbull, J.F.T., "A Study of Gross, Histological and Blood Biochemical Changes in Rainbow Trout (*Oncorhynchus mykiss* W.) with Rainbow Trout GastroEnteritis (RTGE)." Ready for submission, 2009

-del Pozo, J., Crumlish, M., Ferguson, H. W., Turnbull, J.F.T., "A Comparative Molecular Study of the Presence of "*Candidatus* arthromitus" in the Digestive System of Healthy Rainbow Trout *Oncorhynchus mykiss* (Walbaum) Affected with Rainbow Trout Gastroenteritis (RTGE)." Ready for submission, 2009

Posters and Presentations

-del Pozo, J., Crumlish, M., Ferguson, H.W., Turnbull, J.F., "Update on RTGE". Fish Veterinary Society scientific meeting, Southampton, UK, November 2008 (Oral presentation)

-del Pozo, J., Crumlish, M., Ferguson, H.W., Turnbull, J.F., "Use of multidisciplinary approaches to the research of an emerging disease: A review of recent advances in the knowledge on rainbow trout gastroenteritis". Institute of Aquaculture PhD Research Conference, Stirling, UK, October 2008 (Oral presentation)

-del Pozo, J., Crumlish, M., Ferguson, H.W., Turnbull, J.F., "Transmission and scanning electron microscopic observations on the aetiology and pathogenesis of Rainbow Trout GastroEnteritis (RTGE)". American Fisheries Society (Fish Health Section) Annual Meeting, Charlottetown, Canada, July 2008 (Poster presentation)

-del Pozo, J., Crumlish, M., Ferguson, H.W., Turnbull, J.F., "A longitudinal prospective study of risk factors associated with the presence and severity of Rainbow Trout GastroEnteritis (RTGE)". American Fisheries Society (Fish Health Section) Annual Meeting, Charlottetown, Canada, July 2008 (Oral presentation)

-del Pozo, J., Verner Jeffreys, D., "Current knowledge on Rainbow Trout GastroEnteritis (RTGE)." Fish Veterinary Society Annual Meeting, Southampton, UK, November 2006 (Oral Presentation)