8. Project Report to Defra

There are three main objectives of the programme of work as described in contract FC1183.

Objective 1. Assess the susceptibility of English and Welsh salmonids to *G. salaris*.

Objective 2. Investigate the population growth of *G. salaris* under field and laboratory conditions.

Objective 3. Conduct further *G. salaris*-based experimentation in support of UK contingency planning.

There are over 430 species of *Gyrodactylus*, small ectoparasitic monogenean worms principally infecting fish, some species of which are highly pathogenic. While most species of *Gyrodactylus* are non-pathogenic, causing little harm to their hosts, other species like *Gyrodactylus salaris* Malmberg, 1957, which is an OIE (Office International des Epizooties) – listed pathogen of Atlantic salmon, *Salmo salar* L., has led to a catastrophic decimation in the size of the juvenile salmon population in over 40 Norwegian rivers (Bakke, Cable & Harris, 2007). Uncontrolled increases in the size of the parasite population on resident salmon populations have necessitated extreme measures such as the use of the biocide rotenone to kill-out entire river systems, to remove the entire fish population within a river and the parasite (Bakke *et al*., 2007). Given the impact that *G. salaris* has had in Norway and elsewhere in Scandinavia and Russia (Rintamäki, 1989; Ieshko *et al*., 1995; Alenäs, 1998; Alenäs, Malmberg & Carlstrand, 1998) many European states including the UK now have mandatory surveillance programmes screening wild salmonid populations (*i.e.* brown trout *Salmo trutta* L., Arctic charr *Salvelinus alpinus* (L.), grayling *Thymallus thymallus* (L.), Atlantic salmon, *etc*) for the presence of notifiable pathogens including *G. salaris*. The current project set out to make a significant contribution to national *G. salaris* contingency planning by determining the responses of different strains of *G. salaris* on different English and Welsh salmonids to pathogenic strains of *G. salaris*. The study also aims to assess the extent to which laboratory conditions might affect the results of infection experiments, and gauge whether extrapolation from existing results is appropriate for UK contingency planning.

All the three objectives listed above were investigated although trials with *G. salaris* under field conditions were not possible and this will be discussed under Objective 2.

Objective 1: To assess the susceptibility of English and Welsh salmonids to *G. salaris*

The recent reports of *G. salaris* in Italy (Paladini *et al*., 2009) and Poland (Rokicka *et al*., 2009) purportedly linked to the movement of salmonid stocks across borders emphasises the biosecurity risk this pathogen poses. The UK is, currently, a recognised *G. salaris*-free state (Platten *et al*., 1994; Shinn *et al*., 1995), and given the value of its respective salmonid industries, it is important the UK's *G. salaris*-free status is upheld.

Existing UK dispersion models and contingency plans for its containment are based on the assumption that British stocks of Atlantic salmon, *S. salar*, would be vulnerable to *G. salaris* and therefore at risk; that brown trout would be entirely resistant to infection and unaffected; and that grayling would be relatively resistant. Brown trout and grayling, following models determined for Scandinavian populations of these hosts (see review in Bakke *et al.*, 2007), are thought to harbour low level infections for few weeks, not displaying the exponential increase in numbers seen on Atlantic salmon. Native UK stocks of brown trout and grayling, however, have been separated from their Scandinavian counterparts since the last period of glaciation, and their relative patterns of susceptibility and / or resistance may therefore differ to those expected. Should differences be demonstrated, then this would require a substantial redrafting of current contingency plans and a redesign and analysis of dispersion models.

English and Welsh grayling are commonly infected with *Gyrodactylus thymalli* Žitňan, 1960, a congener morphologically similar to and commonly confused with *G. salaris*. It has been suggested that *G. thymalli* may be conspecific with *G. salaris* (see Hansen *et al*., 2006; OIE, 2009), however, Scandinavian grayling are unable to support experimental infections of *G. salaris* suggesting that the *G. thymalli* is not conspecific with *G. salaris* (see Soleng & Bakke, 2001). Given the debate regarding their conspecificity, that *G. thymalli* exists within the UK and that the UK has been separated from mainland Europe for ~200,000 years (Gupta *et al*., 2007), the inclusion and experimental exposure of British grayling to *G. salaris* was important.

Assumptions that UK salmon are susceptible to *G. salaris* are derived from an earlier study by Bakke & MacKenzie (1993), which tested the susceptibility of several Scottish populations of Atlantic salmon (*i.e.* from the Rivers Shin and Conon) to Norwegian *G. salaris* strains. The experimental exposure of other British salmonids (*i.e.* brown trout, grayling *etc*) to *G. salaris* had not been conducted prior to the current study. To ensure that *G. salaris* infections on English and Welsh salmonids follow the same infection dynamics as their Scandinavian counterparts and that current national *G. salaris* contingency plans within England and Wales are appropriate, it was

imperative that these trials were conducted to ensure complete confidence in the existing contingency policy. Objective 1 of Defra project FC1183, therefore, set out to verify whether these assumptions were correct. To determine this, Atlantic salmon, brown trout and grayling eggs, stripped from wild fish, were reared in UK Government Environment Agency run hatcheries and then transported to a secure research facility of the Norwegian Veterinary Institute (NVI) in Oslo, Norway for experimental challenge with a strain of *G. salaris* known to be pathogenic to Norwegian salmon.

This objective represented the biggest challenge in terms of sorting authorisations out from the Norwegian and British governments to transport fish from one country to another. Given the UK's bacterial kidney disease (BKD) status, assessments and clearance from the Chief Veterinary Officer in both the UK and Norway, were sought before the fish were transported. The entire process was conducted in collaboration with colleagues throughout Defra, the Environment Agency and Cefas (Weymouth).

Experimental methodology

In January 2012, approximately 70 Atlantic salmon from the Welsh River Dee, 70 brown trout from the English River Tyne, and 70 grayling from the English River Nidd were shipped to the Norwegian Veterinary Institute in Oslo. Fish were subsequently acclimated for one week in flow through (12 L h^{-1}) fibreglass tanks (1 m × 1 m × 1 m) using Oslo tap water delivered at a constant 11±1°C with additional aeration. Thirty fish from each population were randomly sampled and allocated to a tank for experimental exposure to the Norwegian *G. salaris* haplotype A, a strain known to be pathogenic to *S. salar* (see Hansen *et al*., 2003). Ten individuals of Atlantic salmon from the River Lærdalselva, a population known to be susceptible to *G. salaris*, were used as a control. Each batch of fish were infected by exposing them to *G. salaris*-infected fins excised from heavily infected, laboratory held, Norwegian salmon for a period of 24 hours. The population of *G. salaris* that was used originated from the River Fusta and correspond to haplotype A following Hansen *et al*. (2003). Following the exposure period, each fish was lightly anaesthetised in Finquel® Vet. 100% (50 mg Finquel L^{-1}), tattooed with a unique mark using alcian blue (40 mg/ml^{-1}), and the total number of *G. salaris* on each fin and body zone was counted. Each fish was then randomly assigned to one of three recovery tanks (5 L circular, flow-through 200 ml min^{-1} tanks). Each population (n = 10, juvenile fish) was tested in triplicate, with the exception of the control, which is used as a standard control model within the *G. salaris* aquarium at NVI. Seven days later, each tank of fish were anaesthetised and the number of *G. salaris* on each individually marked fish determined. The fish were sampled after one week and then every 14 days thereafter.

Results of the susceptibility challenge

The dynamics of *G. salaris* infection on each population of fish was followed over a total of 110 days and the results are presented in Figures 1-4. The results show that the population of Welsh salmon from the River Dee are very susceptible to *G. salaris* infection (average intensity ~4000 parasites/fish in 40 d; see Figure 1) when compared against the Norwegian control tank of fish which had a mean intensity of ~2000 parasites/fish over the same time period (Figure 2).

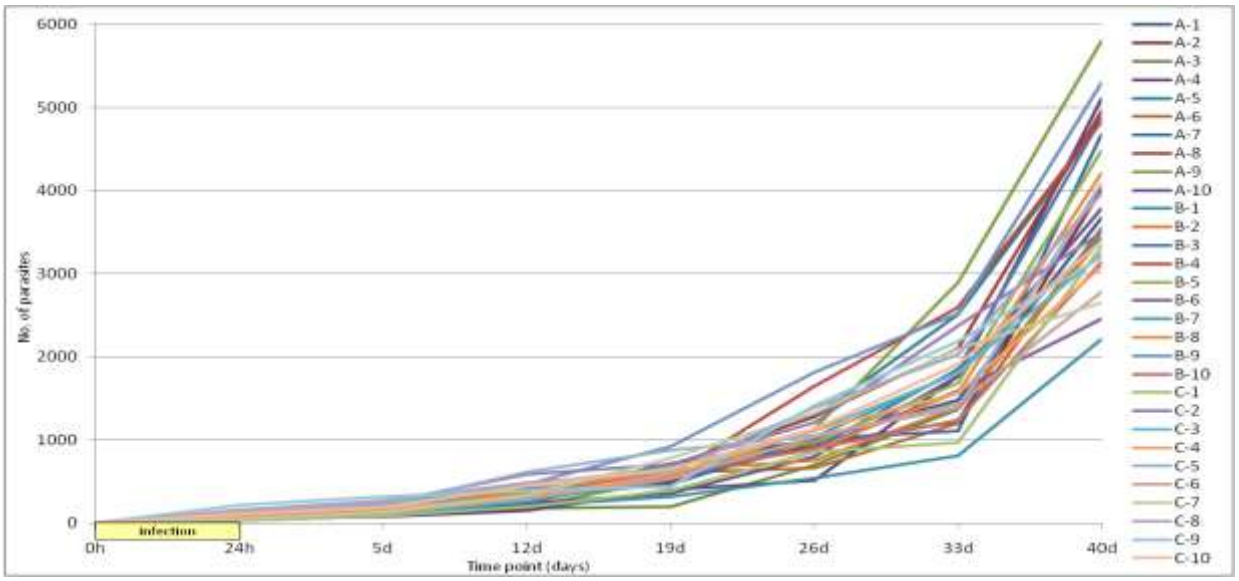


Figure 1. An experimental infection of *Gyrodactylus salaris* Malmberg, 1957 on individual Atlantic salmon (n = 30) originating from the River Dee in Wales. Parasite numbers rapidly increased to ~4000 *G. salaris* per fish by day 40 post-infection at which point the experiment had to be terminated on welfare concerns for the fish.

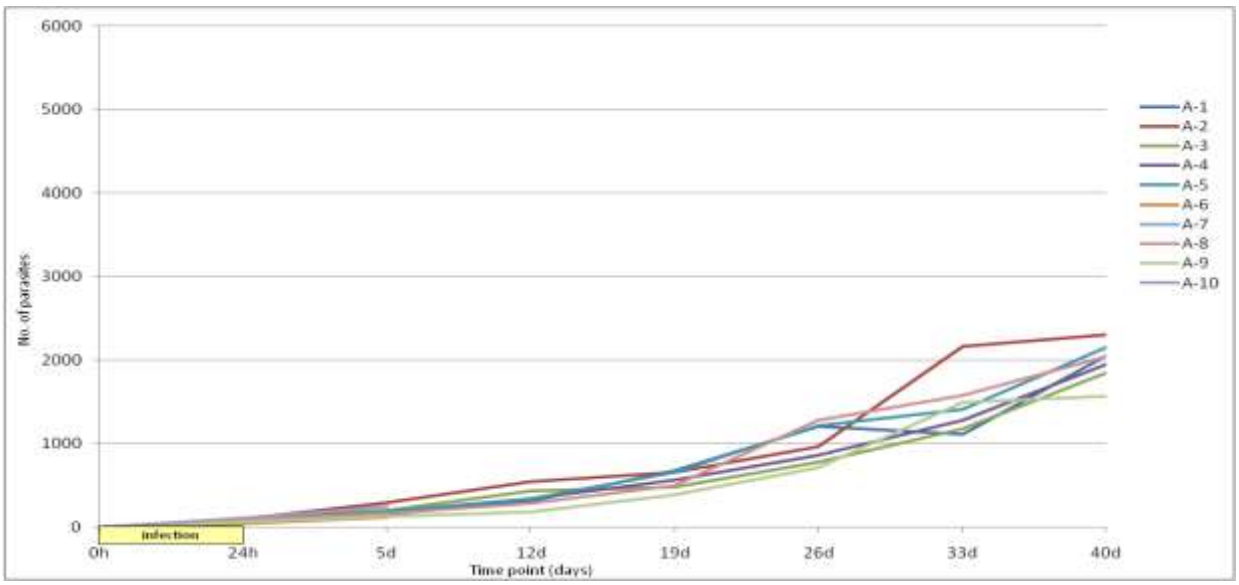


Figure 2. An infection of *Gyrodactylus salaris* on individual Atlantic salmon from the River Lærdalselva, Norway (n = 10). The graph is shown on the same scale as that presented for Figure 1. The number of *G. salaris* increased to ~2000 parasites per fish within the same 40 day period.

The infection *G. salaris* on the brown trout from the River Tyne peaked after ~12 days (mean intensity 145.9 parasites/fish; Figure 3), whilst that on the River Nidd grayling peaked after ~19 days (mean intensity 252.6 parasites/fish; Figure 4). Thereafter, both hosts were able to respond and reduce the size of parasitic infection. The *G. salaris* infection had almost disappeared on both sets of fish by the time the experiment was terminated on day 110 post-infection.

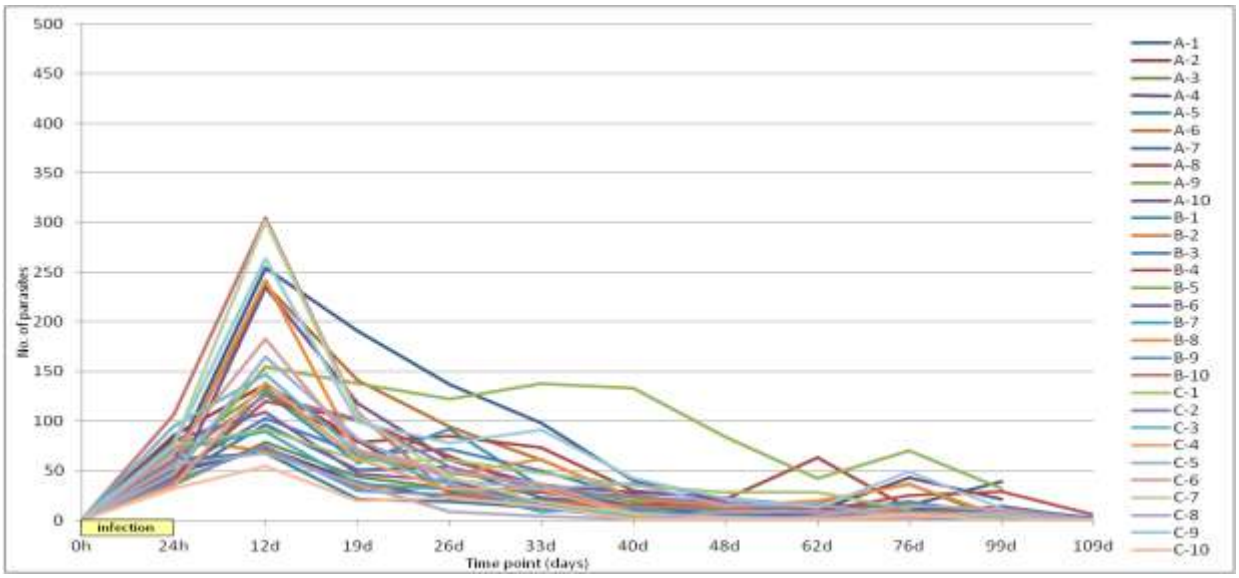


Figure 3. The population dynamics of *Gyrodactylus salaris* (Fusta strain haplotype A) on individual brown trout (n = 30) from the English River Tyne . Infections peaked at day 12 post-infection (p.i.) with a smaller subsequent peak around day 26 p.i. This population of trout, however, were able to carry a *G. salaris* infection for at least 110 days (*i.e.* 7 of the 30 fish were still infected with 1-6 *G. salaris* each) when the experiment was terminated.

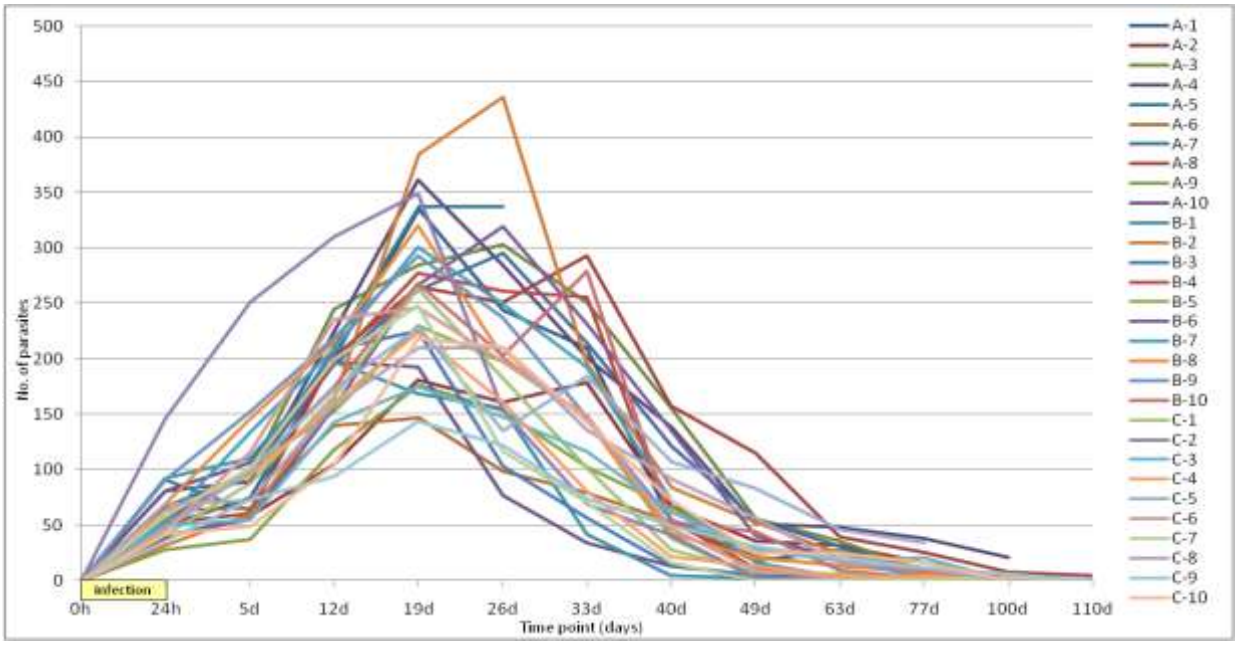


Figure 4. The number of *Gyrodactylus salaris* (Fusta strain haplotype A) on individual grayling (n = 30) from the English River Nidd infected under laboratory conditions. Infections peak on day 19

post-infection (p.i.) with a subsequent smaller peak around day 33. By the time the experiment was terminated on day 110 p.i., only two fish were still infected, one with five *G. salaris*, the other fish with a single parasite.

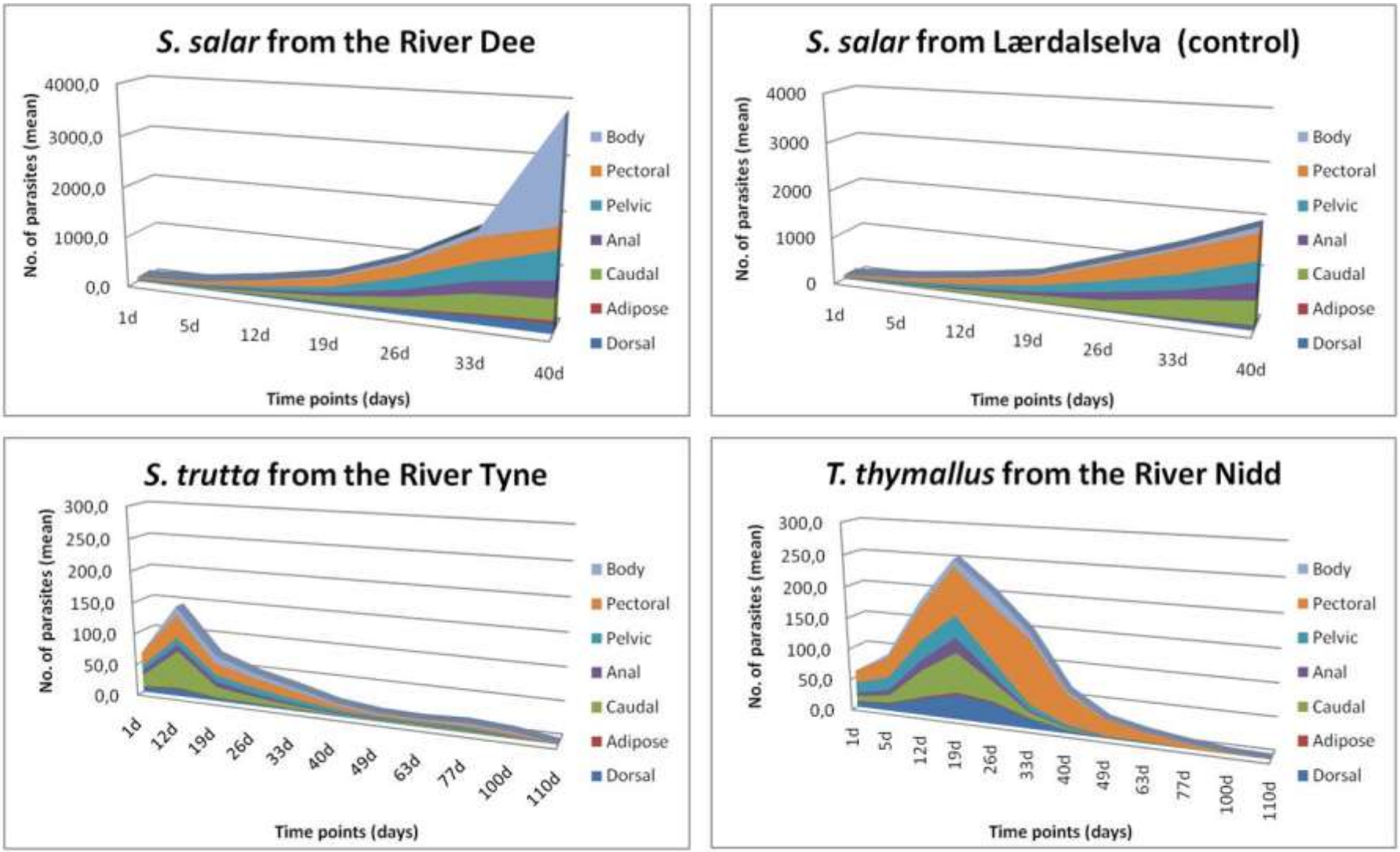


Figure 5. The distribution of *Gyrodactylus salaris* Malmberg, 1957 on the fins and body of Atlantic salmon from the Welsh River Dee (n =30) and the River Lærdalselva, Norway (n =10), brown trout from the English River Tyne (n = 30) and on a population of English grayling from the River Nidd (n =30) throughout the duration of the experiment.

The mean parasite burdens and the range of parasites at each sampling time point on each of the four populations of fish are shown in Table 1. Figure 5 shows the average distribution of the *G. salaris* across the body and fins of each fish species throughout the experimental infection. The graphs show the importance of the fins, as the preferred site of infection. Numbers on the Welsh salmon from the River Dee suggest when the infection reaches ~2000 *G. salaris* per fish, there are no further increases on the fins, however, numbers on the body subsequently rapidly increase. Buchmann & Bresciani (1998), suggested that this distribution on the host may be a consequence of differing mucous cell densities in different parts of the fish (Pickering, 1974) and that the parasites avoid the mucous cell rich areas during the response phase and escape localised immune reactions (Richards & Chubb, 1996; Buchmann & Bresciani, 1997; Buchmann & Uldal, 1997; Buchmann, 1998a,b).

Table 1. Intensity of *Gyrodactylus salaris* (River Fusta haplotype A strain) infection on Welsh salmon and English grayling and brown trout and compared to a Norwegian salmon control. The mean intensity ±1 standard deviation and the range in parentheses is presented for each host and for each sampling date post-infection (p.i.). Given the high number of *G. salaris* seen on both the Welsh and Norwegian salmon stocks on day 40 p.i., there were concerns for the welfare of the fish and the experiment was terminated. Although some grayling and brown trout were still infected on day 110 p.i., the experiment was terminated.

Time points (days)	***Salmo salar* (R. Dee, Wales)**	***Salmo salar* (control) Laerdalselva, Norway**	***Salmo trutta fario* (R. Tyne, England)**	***Thymallus thymallus* (R. Nidd, England)**
1	88.0 ± 44.9 (28–215)	80.9 ± 20.9 (47–110)	65.3 ± 35.5 (32–221)	60.7 ± 24.0 (28–149)
5	157.4 ± 61.4 (76–314)	183.4 ± 53.6 (114–291)	-	93.4 ± 40.9 (37–251)
12	343.6 ± 116.5 (151–615)	349.1 ± 113.1 (184–544)	145.9 ± 73.6 (55–305)	182.5 ± 46.2 (94–310)
19	581.6 ± 156.9 (200–923)	560.4 ± 111.6 (385–679)	74.0 ± 39.4 (20–191)	252.6 ± 64.3 (144–385)
26	1043.5 ± 296.2 (511–1812)	1003.1 ± 231.3 (714–1284)	52.0 ± 30.5 (9–137)	206.7 ± 81.1 (77–436)
33	1741.5 ± 510.2 (810–2890)	1459.7 ± 352.6 (1114–2165)	37.3 ± 30.0 (4–138)	151.1 ± 73.0 (34–293)
40	3850.7 ± 898.2 (2210–5805)	1988.9 ± 233.5 (1570–2300)	20.7 ± 24.3 (1–133)	66.1 ± 42.6 (5–158)
49	-	-	13.1 ± 15.6 (0–84)	29.7 ± 26.7 (0–115)
63	-	-	11.8 ± 13.2 (0–63)	16.9 ± 14.7 (0–48)
77	-	-	14.5 ± 16.7 (0–70)	8.5 ± 10.2 (0–38)
100	-	-	10.6 ± 9.9 (0–39)	2.1 ± 4.5 (0–21)
110	-	-	0.9 ± 1.6 (0–6)	0.3 ± 1.2 (0–5)

Discussion

Current national contingency plans in the UK assume that the dynamics of *G. salaris* infection on native English and Welsh salmonids will follow those already modelled in Scandinavia. These Scandinavian studies suggest that Atlantic strains of salmon are susceptible to infection, whilst grayling are innately resistant but *G. salaris* can survive and reproduce on Scandinavian grayling for 143 days and that brown trout are entirely resistant to infection.

The infection of *G. salaris* on the Welsh population of salmon followed the expected infection trajectory with average infections rising to 4000 parasites per fish in just 40 days. The rate of parasite increase (~137% d^{-1}), however, was markedly faster that of the infection on the Norwegian control group of salmon (*i.e.* 66% d^{-1}). The study of Bakke & MacKenzie (1993) subjected two populations of Scottish Atlantic salmon (*i.e.* Shin and Conon) to *G. salaris* and found these populations to be highly susceptible to infection. The strain of *G. salaris* used for this trial originated from the River Figga most likely corresponding to haplotype A (though not stated, this is interpreted from the map of haplotype distribution presented in Hansen *et al.*, 2003). The current trial also used a strain of *G. salaris* from the River Fusta corresponding to haplotype A. The current experiment confirms that Welsh salmon are also susceptible to *G. salaris* haplotype A. Likewise the infections of *G. salaris* on grayling were not completely outside the expected response with a low level of parasites remaining on fish for the duration of the 110 day experiment. Only two out of the 30 grayling, however, were still infected at the end of the trial. Earlier trials with Scandinavian populations of grayling using the Lierelva strain of *G. salaris* suggested that infections could persist for anything between 35 (Soleng & Bakke, 2001) and 143 days (Sterud *et al.*, 2002). There was value, therefore, in determining the response of English grayling to infection. The finding that English grayling can carry infections for long periods of time gives cause for concern in that they may play a role in extending the infection window to susceptible hosts.

Perhaps the most interesting finding from the current trial is the infection of *G. salaris* on the population of brown trout from the River Tyne. Prior to this study, brown trout had been considered resistant to *G. salaris* infection. Jansen & Bakke (1995), for example, infecting both single and pooled samples of brown trout with the strain of *G. salaris* from the River Lierelva (haplotype F) found that fish could carry an infection for up to 50 days. The current study found that when a pool of brown trout were each given an initial infection of ~70 *G. salaris* per fish, then the *G. salaris* infections on these fish persisted for at least 110 days, when the experiment was terminated. Of these, 7 of the 30 fish were still infected with between 1 and 6 parasites each. Brown trout parr naturally infected with a low intensity of *G. salaris*, however, have been reported by a number of authors (Tanum, 1983; Mo, 1988; Malmberg & Malmberg, 1991; Johnsen & Jensen,

1992). The studies by Tanum (1983) and Mo (1988) also demonstrated that brown trout were able to maintain their *G. salaris* infections when cohabited with infected salmon. A study by Bakke *et al*. (1999), found that brown trout held in isolation eliminated their *G. salaris* infections in less than two weeks suggesting that they could be innately resistant. The current study, however, found that English brown trout can carry an infection for 110 days, and this finding appears to contradict those of Bakke *et al*. (1999). Most of Bakke and co-workers experimental studies result from using *G. salaris* "originating" from the River Lierelva, *i.e.* haplotype F according to the study of Hansen *et al.* (2003). The strain of *G. salaris* used in the current study, however, was derived from the River Fusta (Vefsna region) corresponding to haplotype A. It is not possible, therefore, to ascertain whether the observed brown responses between the two trials result from genetic differences in trout or in the strain of *G. salaris* used. The results from Tanum (1983) and Mo's (1988) studies are interesting, when the work of Harris *et al.* (2000) is also considered who found that immunosuppression of brown trout made them susceptible to *G. salaris* infection. Although every precaution was taken to ensure fish welfare was upheld throughout the duration of the current susceptibility trial, the level of stress placed upon the brown trout during their transportation from the UK to Norway and in their new holding tanks is not known. Whilst the 110 day period of infection may not accurately reflect how British populations of brown trout in the wild would respond to *G. salaris* if introduced into the UK, the trial has shown that the River Tyne population of trout are able to manage infections and keep numbers to a low level. Although there were no *G. salaris*-related brown trout mortalities, the concern is that populations of brown trout under stress may extend the period over which individuals carry an infection, therefore, increasing the possible risk of parasite transfer to other fish species. Whilst brown trout are able to resist infections and keep *G. salaris* numbers to a "low" level (<50 parasites fish^{-1}), the term "resistance" needs to be considered carefully in the role that brown trout could play in the event of a *G. salaris* introduction.

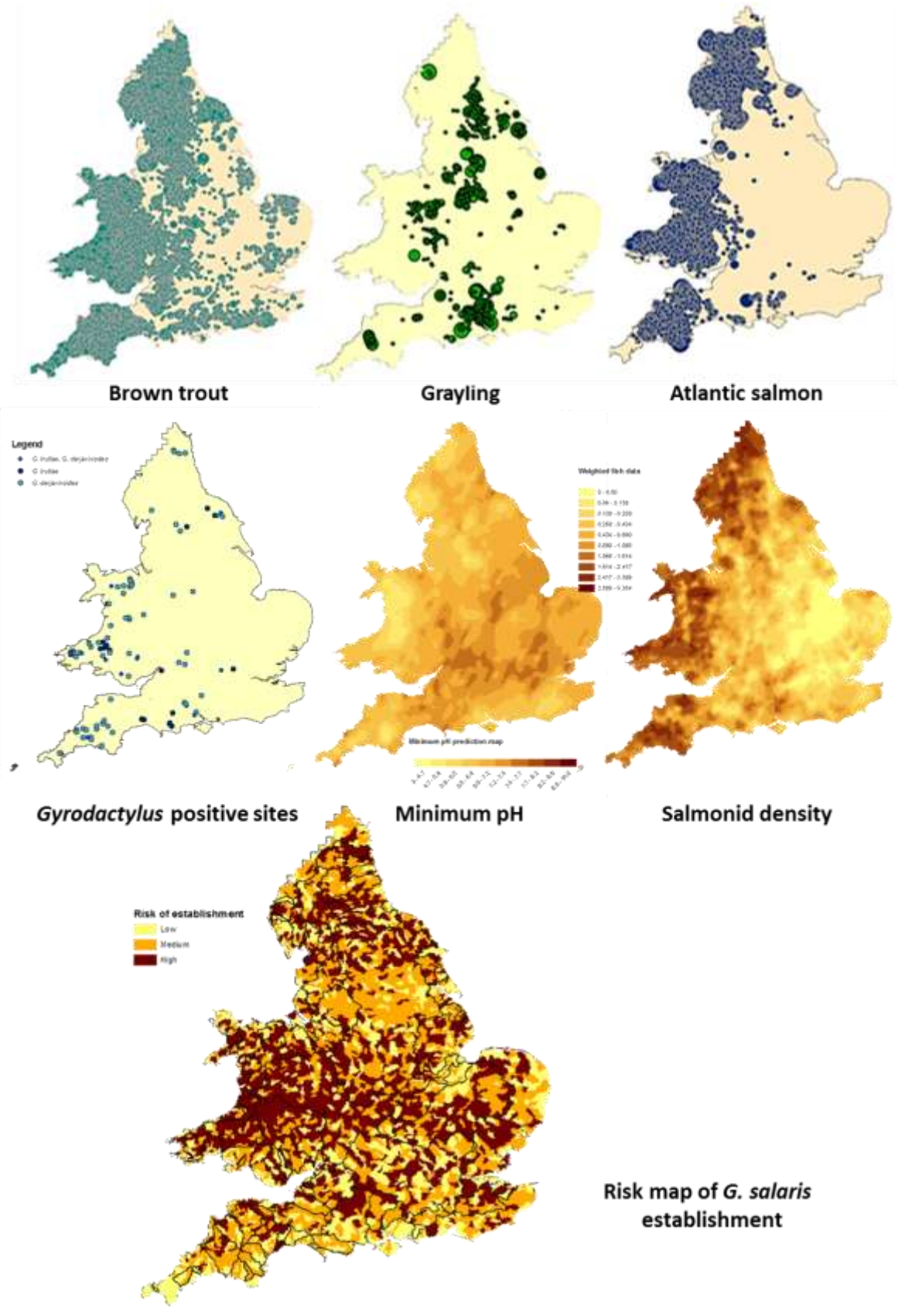
The findings from this trial are significant in that they demonstrate that Welsh salmon are also susceptible to *G. salaris*, that grayling respond in a similar manner to their Scandinavian counterparts and carry infections for up 110 days, and that English brown trout are also resistant but can harbour infections for longer than those reported for Norwegian populations *i.e.* 110+ days as opposed to 50 days. These extended windows of infection and the interpretation of "resistance" within the context of national contingency planning and subsequent management in the event of a *G. salaris* outbreak, however, requires careful scrutiny.

Objective 2. To investigate the population growth of *G. salaris* under field and laboratory conditions – investigating the effect of water chemistry on the dynamics of *G. salaris* infection.

UK contingency plans are currently based on a large number of experimental studies conducted within Scandinavia. These include the implicit assumption that the responses, in terms of the pattern of *G. salaris* infection and population growth, observed in the laboratory environment accurately reflect the dynamics of infection in the wild. If the experimental data is to be used to inform national contingency planning, then it is imperative that the influence of water chemistry on *G. salaris* infections is investigated. Current contingency plans, however, are based on an understanding of *G. salaris* population dynamics largely derived from a single experimental protocol carried out using a common domestic water supply in Oslo. It is known, however, that subtle deviations in water composition can affect the course of gyrodactylid infection, notably the addition of aluminium sulphate which is being trialled as a remedial measure for the control of *G. salaris* within infected watercourses (Soleng *et al*., 1999; Poléo *et al.*, 2004). Sub-lethal levels of cadmium and zinc typically found in UK domestic tap water (*i.e.* <5µg L^{-1}) were also found to impact significantly on the pattern of population growth of *Gyrodactylus turnbulli* Harris, 1986 (see Carter, 2003; Gheorghiu *et al*., 2007).

This part of the study had been designed to investigate the population dynamics of one strain of *G. salaris* on one population of Atlantic salmon held in three different water compositions. There were, however, unforeseen practical reasons why this aspect of the study was not conducted as originally planned. Access to aquarium space, facilities and infected animals to conduct this experiment in Norway within the allocated budget was not possible. To try and fulfil the research objectives of this part of the project, the work focused on the current distribution of *Gyrodactylus* species infecting salmonids in the UK and the water chemistry of *Gyrodactylus* positive sites. To achieve this, there was a collaboration with researchers working in the same research organisation on FC1175 "Development of a risk evaluation system for the establishment of *Gyrodactylus salaris* in English and Welsh river systems". A Geographic Information System (GIS) based risk map was produced which related to the results of gyrodactylid surveys to environmental datasets. From these, salmonid density, pH and distance to mouth of the river emerged as key factors in the establishment of *Gyrodactylus*. These were used in the construction of a risk map for *G. salaris* establishment across England and Wales (see Figure 6).

Figure 6. A risk map for the establishment of *Gyrodactylus salaris* in England and Wales. The distribution of brown trout, grayling and Atlantic salmon across England and Wales is shown in relation to the distribution of *Gyrodactylus* positive sites, average salmonid density and the pH of aquatic sites.



Use of tannic acid in preventing the establishment / survival of *Gyrodactylus*

Given the apparent importance of water chemistry in the establishment of *Gyrodactylus*, this objective set out to explore the influence of different tannins and humic substances on the survival of gyrodactylids. This natural organic material is produced from decaying vegetation and their concentration as dissolved organic carbon (DOC) in water works can, at least, range from 4.3 to 14.5 mg L^{-1} (Sharp, Parsons & Jefferson, 2006). Here we present our investigations using tannic acid both as a substance preventing the establishment of *Gyrodactylus* and as a control agent.

Tannic acid ($C_{76}H_{52}O_{46}$) is a polyphenol that is ubiquitous in plants, including tea. Its astringent properties are used in the formulation of several pharmaceutical anti-diarrhoeal, haemostatic and anti-hemorrhoidal products (Ashok & Upadhyaya, 2012). Tannins, by way of generalisation, have the ability to inhibit enzymes, precipitate proteins and to scavenge free-radicals; given these properties their use as anti-viral (*e.g.* HIV, see Lin *et al*., 2004), anti-bacterial (*e.g. Staphylococcus aureus* and *Helicobacter pylori*, see Akiyama *et al*., 2001; Funatogawa *et al*., 2004) and anti-parasitic (*e.g. Leishmania*, see Kolodziej & Kiderlen, 2005) agents have been explored.

Methodology

A 1mM solution of tannic acid (Sigma-Aldrich, Steinheim, Germany) was prepared using 0.2 µm filtered, dechlorinated Oslo tap water at 6±1ºC and then serially diluted to give concentrations of 0.5; 0.25; 0.1 mM and a control (0 mM). Fifty *G. salaris* haplotype F (Lierelva strain) were placed in each concentration, run in triplicate, including a control set.

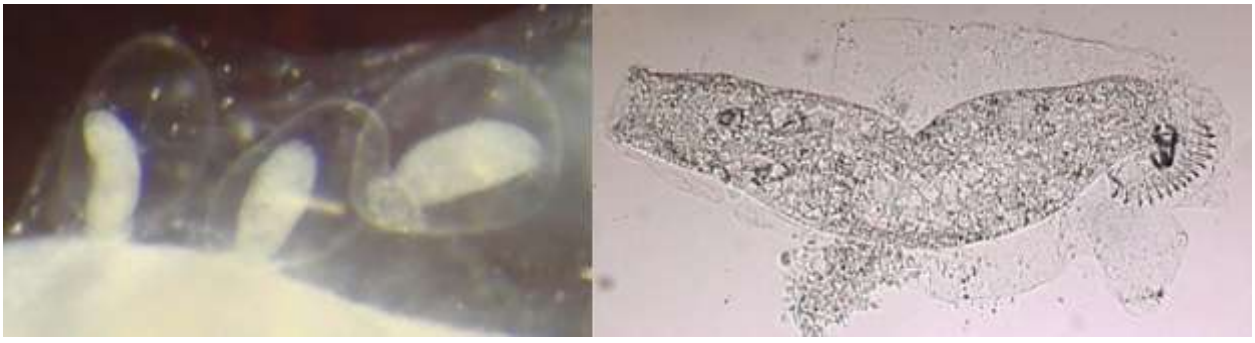


Figure 7. The effect of 0.5mM tannic acid on *Gyrodactylus salaris* which causes swelling and lifting off of the tegument.

The table 2 shows the efficacy of tannic acid in killing *G. salaris* haplotype F (Lierelva strain) when continuously exposed to tannic acid demonstrating that low doses of tannic acid result in high percentage kills. A repeat trial using 0.5mM tannic acid for only 10 mins resulted in a 90% parasite mortality within the first hour post-exposure (results not shown). The results suggest that tannic

acid may prove effective in killing *Gyrodactylus*, however, further *in vitro* and *in vivo* trials are now required to explore the full potential of this compound as a treatment agent.

Table 2. The treatment efficacy of 0-1mM tannic acid against *Gyrodactylus salaris* (Lierelva strain haplotype F) against time (h) when continuously exposed.

***G. salaris* Lierelva strain haplotype F continuous exposure (% dead)**				
DOSE (mM)	**1h**	**2h**	**3h**	**4h**
0 (control)	0	0	0	0
0.075	0	70	83.3	100
0.1	50	96.7	100	100
0.25	73.3	100	100	100
0.5	100	100	100	100
1	100	100	100	100

Objective 3. Further *G. salaris*-based experimentation in support of UK contingency planning

Under this objective two principle lines of investigation were conducted. The first approach was to explore alternative means of controlling *G. salaris* using chemicals and the second approach was to explore the infection process using microarray.

Alternative means of controlling* G. salaris *using chemical agents

Gyrodactylus salaris infections in Norway are currently managed through the use of the biocide rotenone and / or aluminium sulphate. With the constant threat of *G. salaris* entering UK waterways and the lack of any effective treatment, other than the total eradication of all river fauna using rotenone, it is important that investment is made now to explore new management strategies and if necessary new chemical treatments that will specifically target *Gyrodactylus* infections. At the same time, much debate within the UK surrounds the use of both rotenone and aluminium sulphate for the treatment of *G. salaris*. The use of both chemicals are linked to human health concerns. Rotenone has been suggested to be responsible for behavioural and pathological symptoms of Parkinson's disease (Giasson & Lee, 2000; Newhouse *et al*., 2004; Cannon *et al*., 2009), whilst the use of aluminium sulphate has been identified as a risk factor in the development of Alzheimer's Disease (WHO, 1998). This objective, therefore, set to explore the utility of other chemical agents in controlling infections of *G. salaris*. There was some overlap in this objective with that of Defra project FC1175 which explored the use of platyhelminth specific compounds (*e.g.* octopamines) to interfere with the transmission process and infection of new hosts (see Defra final report FC1175; Brooker *et al*., 2011). A series of chemical trials were conducted but only the results of one compound, bronopol, marketed under the trade name of Pyceze^{TM} (Novartis Animal Vaccines Ltd.), will be reported on in detail here.

Bronopol (2-bromo-2-nitropropane-1,3-diol) is a broad-spectrum disinfectant, has been demonstrated to cause membrane damage in microbial organisms through the inhibition of membrane bound enzymes (Stretton & Manson, 1973; Shepherd *et al*., 1988) and has been shown to be effective in the control of other ectoparasitic species like the ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876 (Picon-Camacho *et al*., 2012; Shinn *et al*., 2012).

Experimental animals (parasites and hosts)

The efficacy of all chemicals were initially tested in the UK, *in vitro*, against *Gyrodactylus arcuatus* Bychowsky, 1933, a species parasitising 3-spine sticklebacks (*Gasterosteus aculeatus* L.), and then subsequently against *G. salaris* in Norway. *Gyrodactylus arcuatus* was selected as the species for chemical assessment in the UK because this species is a common ectoparasite of 3-spine sticklebacks and easily acquired, but also because this species is found in a wide range of aquatic habitats from freshwater to marine and therefore represents a good model for testing.

Forty specimens of 3-spined sticklebacks (total length 3-6 cm) naturally infected with *G. arcuatus* were collected from a tributary of the River Allan in Stirlingshire and transported to the Institute of Aquaculture, University of Stirling, UK. The fish were held in small tank (60 × 30 × 40 cm) with oxygen under 12 h light : 12 h dark photoperiod regime at 6±1°C and fed on bloodworms for one month to allow parasite numbers on the captive held fish to increase.

For the trials using *G. salaris* conducted in Natural History Museum, Department of Zoology, University of Oslo (Norway), two populations of Atlantic salmon (total length 10-15 cm; weight 5-20 g) from the rivers Glitra and Lierelva were sampled and subsequently maintained in 200 L aquaria supplied with 6±1ºC dechlorinated Oslo tap water. The fish were fed *ad libitum* with a commercial pellet food, and kept under a photoperiod regime of 12 h light : 12 h dark. The fish from the River Glitra were a stock of fish that had been maintained in the aquarium for a period of 5 months, whilst the population of salmon collected from the Lierelva had been kept in the University of Oslo research aquarium for a period of 2 years.

Chemical exposure procedure

Two sets of trials were conducted: a continuous exposure trial and 1 hour exposure to bronopol trial. For the continuous chemical exposure trial, a fresh batch of bronopol was prepared in the following concentrations 0, 25, 50, 100, 150, 250, 375, 500, 625 and 750 ppm bronopol using water feeding the experimental tanks filtered through a 0.2µm filter. For the trials, a heavily *Gyrodactylus*-infected fish was killed using a UK Home Office Schedule 1 method and then small pieces of fin, each with 10 *Gyrodactylus* specimens attached were removed and placed into a 5 cm Petri dish containing the relevant concentration of bronopol maintained at 6±1°C. Each

concentration was tested in triplicate and the parasites in each dish were assessed each hour and the number of dead and alive parasites determined. Three populations of *Gyrodactylus* were tested, *G. arcuatus* from *G. aculeatus* from the River Allan, *G. salaris* Lierelva strain haplotype F reared on Lierelva stock and the same *G. salaris* strain but reared on Glitra stock. For the 1 hour exposure trial, only three doses of bronopol were tested *i.e.* 25, 50, 100 ppm on the two stocks of *G. salaris* (n = 50 parasites per replicate).

Results

The results of the bronopol trials are shown in Tables 3 and 4 where it can be seen that the action of bronopol on *G. salaris* (only the results for the *G. salaris* maintained on Glitra salmon are shown but both sets of results were similar) was markedly better than on *G. arcuatus*.

Table 3. The treatment efficacy of 0-750 ppm (mg L^{-1}) bronopol (Pyceze) against *Gyrodactylus arcuatus* Bychowsky, 1933 against time (h) when continuously exposed.

***G. arcuatus* from *G. aculeatus* from the River Allan, UK - continuous exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	5	10	15	15	15	15	15	100
50	0	0	0	0	5	5	5	100
100	0	0	0	5	10	10	15	100
150	0	0	0	0	0	0	20	100
250	0	0	0	10	10	30	70	100
375	5	5	5	15	15	70	95	100
500	5	10	80	90	100	100	100	100
625	5	5	90	90	100	100	100	100
750	5	75	95	100	100	100	100	100

Table 4. The treatment efficacy of 0-750 ppm (mg L^{-1}) bronopol (Pyceze) against *Gyrodactylus salaris* Malmberg, 1957 (Lierelva strain haplotype F) against time (h) when continuously exposed.

***G. salaris* from *S. salar* from the River Glitra, Norway - continuous exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	0	0	5	35	40	55	70	100
50	0	25	40	50	65	80	95	100
100	20	25	85	100	100	100	100	100
150	30	40	85	100	100	100	100	100
250	45	70	100	100	100	100	100	100
375	20	70	100	100	100	100	100	100
500	50	100	100	100	100	100	100	100
625	100	100	100	100	100	100	100	100
750	100	100	100	100	100	100	100	100

The trial results suggest that the LC50 for *G. salaris* was ~50 ppm bronopol for 4 h whilst the LC50 for *G. arcuatus* was considerably higher at ~375 ppm for 5 h. The results for the one hour bronopol exposure trial on the survival of *G. salaris*, after which the bronopol was replaced with fresh, dechlorinated, filtered Oslo tap water are shown in Table 5.

Table 5. The treatment efficacy of 0-100 ppm (mg L^{-1}) bronopol (Pyceze) against *Gyrodactylus salaris* Malmberg, 1957 (Lierelva strain haplotype F) against time (h) when parasites are exposed for just one hour after which the bronopol was replaced with fresh, dechlorinated, filtered Oslo tap water.

***G. salaris* from *S. salar* from the River Glitra, Norway - 1h exposure (% dead)**								
DOSE (ppm)	**1h**	**2h**	**3h**	**4h**	**5h**	**6h**	**7h**	**24h**
0 (control)	0	0	0	0	0	0	0	0
25	0	0	6	17	40	48	51	78
50	7	15	21	39	63	70	83	100
100	9	19	27	47	67	100	100	100

On-going trials, are repeating this work, looking at a larger number of replicates to confirm the concentrations and time windows of exposures.

Discussion

Bronopol is used extensively throughout the UK for the control of the oomycete *Saprolegnia parasitica* (see Pottinger & Day, 1999); for the ciliated protozoan *I. multifiliis* (see Picon-Camacho *et al*., 2012; Shinn *et al*., 2012), and elsewhere for the dinoflagellate *Amyloodinium ocellatum*, the causative agent of "velvet disease" (Roberts-Thomson, 2007). Although while these results demonstrate that bronopol could be used to control infections of *G. salaris* in confined aquaria, this does not mean that this advocates its use in river systems as there are a plethora of logistic, economic and environmental considerations to take in to account. This does, however, take important steps towards investigating alternative control agents for use in the event of an outbreak.

i) Microarray of the* Gyrodactylus *infection process

A study by Kuusela *et al.* (2007) suggests that *G. salaris* originally arose from hybridisation between two Baltic *G. thymalli* strains, which raises the interesting possibility that further hybridisation events between UK gyrodactylids could lead to the emergence of new pathogenic forms. In the event of an introduction of *G. salaris* into the UK, there is the possibility of further hybridisation events. The likelihood of this happening and the potential pathogenicity of any new strains arising is an area of research which remains largely unexplored but has important implications for national contingency planning. Very little, however, is known during *G. salaris*

infections on susceptible populations of Atlantic salmon. The current work, therefore, set out to conduct an infection, to take salmon samples at key time points and then using a microarray-based approach attempt to determine which genes and processes are being up or down regulated at each time point. Identifying these processes may provide critical insights into the host-parasite interaction, how the host responds throughout the infection and how *G. salaris* possibly circumvents its host's responses.

Methodological approach

A total of 240 specimens of Atlantic salmon (total length 10-15 cm; weight 5-20 g), originally from the River Alta, were transferred to and reared in the Natural History Museum, Department of Zoology, University of Oslo, Norway. The fish were maintained in 200 L aquaria containing dechlorinated Oslo tap water, fed *ad libitum* with a commercial pellet food, and kept under a 12 h light : 12 h dark photoperiod regime. After a week of acclimation, the fish were treated on three consecutive days with 0.2% NaCl solution for four hours to remove any ectoparasitic infections.

The fish were subsequently randomly assigned to one of four 50 L tanks (60 fish each) – 2 test tanks and 2 controls. The fish in the two test tanks were infected with *G. salaris* Lierelva strain haplotype F following a standard protocol for infection, such that each fish acquired ~50 *G. salaris* within the 24 hour infection period. Twelve fish from each of the 4 tanks were then sampled on days 0, 7, 14 and 28 post-infection. Each fish was processed individually; killed by an overdose of anaesthetic, followed by severing of the spinal cord. At post-mortem, the pectoral and pelvic fins, spleen and head kidney, were removed and immediately fixed in RNA*later* for RNA analysis. As this project forms part of a PhD programme of research, at the time that this report was submitted, the RNA from all 192 fish (12 fish per tank per time point) had been extracted and purified following standard laboratory protocols. The RNA from the 12 fish from each tank collected at each time point were then randomly divided into three RNA pools (4 fish each), to minimise individual variation in RNA pools, and then subjected to microarray analysis to determine which processes were being up and down regulated in response to the parasite at each of the time points in the infection. The microarray data is complex and is currently being processed. While it is evident that a number of systems are being up and down regulated, these require some interpretation and these will be reported on once the full analysis is complete. By examining these processes and obtaining a clearer understanding of what makes *G. salaris* pathogenic, it is hoped that we will then be able to implement control measures to either protect the host or reduce the impact that this pathogen has, thereby contributing to national contingency plans and management strategies in the event of its introduction into the country.

References

Akiyama, H., Kazuyasu, F., Yamasaki, O., Oono, T., Iwatsuki, K. (2001). Antibacterial action of several tannins against *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*, 48: 487–491.

Alenäs, I. (1998). *Gyrodactylus salaris* på lax i svenska vattendrag och laxproblematiken på svenska västkysten. *Vann*, 1: 135–142. [In Swedish].

Alenäs, I., Malmberg, G., Carlstrand, H. (1998). Undersökningar av *Gyrodactylus salaris* på lax i Ätrans vattensystem, Falkenbergs kommun under fem år 1991–1995. *Miljö-og hälsoskyddskontoret, Falkenbergs kommun, Rapport 1998*, 1: 1–12 [In Swedish].

Ashok, P.K., Upadhyaya, K. (2012). Tannins are astringent. *Journal of Pharmacognosy and Phytochemistry*, 1 (3): 51–56.

Bakke, T.A., MacKenzie, K. (1993). Comparative susceptibility of native Scottish and Norwegian stocks of Atlantic salmon, *Salmo salar* L., to *Gyrodactylus salaris* Malmberg: laboratory experiments. *Fisheries Research*, 17: 69–85.

Bakke, T.A., Soleng, A., Harris, P.D. (1999). The susceptibility of Atlantic salmon (*Salmo salar* L.) × brown trout (*Salmo trutta* L.) hybrids to *Gyrodactylus salaris* Malmberg and G*yrodactylus derjavini* Mikailov. *Parasitology*, 119: 467–481.

Brooker, A.J., Grano Maldonado, M.I., Irving, S., Bron, J.E., Longshaw, M., Shinn, A.P. (2011). The effect of muscle receptor agonists on the behaviour and transmission of *Gyrodactylus* spp. *Parasites & Vectors*, 4: 207.

Buchmann, K. (1998a). Binding and lethal effect of complement from *Oncorhynchus mykiss* on *Gyrodactylus derjavini* (Platyhelminthes: Monogenea). *Diseases of Aquatic Organisms*, 32: 195–200.

Buchmann, K. (1998b). Some histochemical characteristics of the mucus microenvironment in four salmonids with different susceptibilities to gyrodactylid infections. *Journal of Helminthology*, 72: 101–107.

Buchmann, K., Bresciani, J. (1997). Parasitic infections in pond-reared rainbow trout *Oncorhynchus mykiss* in Denmark. *Diseases of Aquatic Organisms*, 28: 125–138.

Buchmann, K., Bresciani, J. (1998). Microenvironment of *Gyrodactylus derjavini*: association between mucous cell density and microhabitat selection. *Parasitology Research*, 84: 17–24.

Buchmann, K. Uldal, A. (1997). *Gyrodactylus derjavini* infections in four salmonids: comparative host susceptibility and site selection of parasites. *Diseases of Aquatic Organisms*, 28: 201–209.

Cannon, J.R., Tapias, V.M., Na, H.M., Honick, A.S., Drolet, R.E., Greenamyre, J.T. (2009). A highly reproducible rotenone model of Parkinson's disease. *Neurobiology of Disease*, 34 (2): 279–290.

Carter, P.K.L. (2003) *The impact of low concentration of cadmium on host-monogenean interactions.* PhD Thesis University of Stirling 408pp.

Funatogawa, K., Hayashi, S., Shimomura, H., Yoshida, T., Hatano, T., Ito, H., Iría, Y. (2004). Antibacterial activity of hydrolysable tannins derived from medicinal plants against *Helicobacter pylori*. *Microbiology and Immunology*, 48 (4): 251–261.

Gheorgiu, C., Cable, J., Marcogliese, D.J., Scott, M. (2007). Effects of waterborne zinc on reproduction, survival and morpholometrics of *Gyrodactylus turnbulli* (Monogenea) on guppies (*Poecilia reticulata*). *International Journal for Parasitology*, 37: 375–381.

Giasson, B.I., Lee, V.M.-Y. (2000). A new link between pesticides and Parkinson's disease. *Nature Neuroscience*, 3 (12):1227–1228.

Gupta, S., Collier, J.S., Palmer-Felgate, A., Potter, G. (2007). Catastrophic flooding origin of shelf valley systems in the English Channel. *Nature*, 448: 342–345.

Hansen, H., Bachmann, L., Bakke, T.A. (2003). Mitochondrial DNA variation of *Gyrodactylus* spp. (Monogenea, Gyrodactylidae) populations infecting Atlantic salmon, grayling, and rainbow trout in Norway and Sweden. *International Journal for Parasitology*, 33: 1471–1478.

Hansen, H., Martinsen, L., Bakke, T.A., Bachmann, L. (2006) The incongruence of nuclear and mitochondrial DNA variation supports conspecificity of the monogenean parasites *Gyrodactylus salaris* and *G. thymalli*. *Parasitology*, 133: 639-650.

Harris, P.D., Soleng, A., Bakke, T.A. (2000). Increased susceptibility of salmonids to the monogenean Gyrodactylus salaris following administration of hydrocortisone acetate. *Parasitology*, 120: 57–64.

Ieshko, E.P., Berland, B., Shulman, B., Bristow, G., Shurov, I. (1995). Parasites of Atlantic salmon parr in some Karelian rivers flowing into the White Sea. *Abstract Fourth International Symposium of Ichthyoparasitology, Munich, Germany, October 3rd–7th*, p. 100.

Jansen, P.A., Bakke, T.A. (1995). Susceptibility of brown trout to *Gyrodactylus salaris* (Monogenea) under experimental conditions. *Journal of Fish Biology*, 46: 415–422.

Johnsen, B.O., Jensen, A.J. (1991). The *Gyrodactylus* story in Norway. *Aquaculture*, 98: 289–302.

Kolodziej, H., Kiderlen, A.F. (2005). Antileishmanial activity and immune modulatory effects of tannins and related compounds on *Leishmania* parasitised RAW 264.7 cells. *Phytochemistry*, 66: 2056–2071.

Kuusela, J., Zietara, M.S., Lumme, J. (2007) Hybrid origin of Baltic salmon-specific parasite *Gyrodactylus salaris*: a model for speciation by host switch for hemiclonal organisms. *Molecular Ecology*, 16: 5234–5245.

Lin L.U., Shu-wen L., Shi-bo J., Shu-guang W. (2004). Tannin inhibits HIV-1 entry by targeting gp41. *Acta Pharmacologica Sinica*, 25 (2): 213–218.

Malmberg, G., Malmberg, M. (1991). Investigations for *Gyrodactylus* on salmonids in natural waters and fish farms during the periods 1951–72 and 1986–May 1991. *Information från Söttvattenslaboratoriet-Drottningholm: Fiskeristyrelsens sötvattenslaboratorium*, 2: 1–30. [In Swedish, English summary].

McHugh, W.S., Shinn, A., Kay, J.W. (2000). Discrimination of the notifiable pathogen *Gyrodactylus salaris* from *G. thymalli* (Monogenea) using statistical classifiers applied to morphometric data. *Parasitology*, 121: 315–323.

Mo, T.A. (1988). *Gyrodactylus*undersøkelsene av fisk i forbindelse med rotenon-behandlingen av Skibotnelva i august 1988. *Gyrodactylus*undersøkelsene ved Zoologisk museum, *Universitetet i Oslo Rapport*, 5: 1–14 [in Norwegian].

Newhouse, K., Hsuan, S.-L., Chang, S.H., Cai, B., Wang, Y., Xia, Z. (2004) Rotenone-induced apoptosis is mediated by p38 and JNK MAP kinases in human dopaminergic Sh-SY5Y cells. *Toxicological Sciences*, 79: 137–146.

OIE (2009). Manual of diagnostic tests for aquatic animals 2009 (Chapter 2.3.3). Gyrodactylosis (*Gyrodactylus salaris*). Available from: http://www.oie.int/eng/normes/fmanual/2.3.03_Gyrodactylosis.pdf>.

Paladini, G., Gustinelli, A., Fioravanti, M.L., Hansen, H., Shinn, A.P. (2009). The first report of *Gyrodactylus salaris* Malmberg, 1957 (Platyhelminthes, Monogenea) on Italian cultured stocks of rainbow trout (*Oncorhynchus mykiss* Walbaum). *Veterinary Parasitology*, 165: 290–297.

Pickering, A.D. (1974). The distribution of mucuos cells in the epidermis of the brown trout *Salmo trutta* (L.) and the char *Salvelinus alpinus* (L.). *Journal of Fish Biology*, 6: 111–118.

Picón-Camacho, S.M., Taylor, N.G.H., Bron, J.E., Hunter, R., Guo, F.C., Shinn, A.P. (2012). Effects of long duration, low dose bronopol exposure on the control of *Ichthyophthirius multifiliis* (Ciliophora), parasitising rainbow trout (*Oncorhynchus mykiss* Walbaum). *Veterinary Parasitology*, 186: 237–244.

Platten, M., McLoughlin, M., Shinn, A.P. (1994). Distribution and identification of gyrodactylid species in fish farms and rivers in Northern Ireland. *Veterinary Record*, 135: 411–412.

Poléo, A.B.S., Schjolden, J., Hansen, H., Bakke, T.A., Mo, T.A., Rosseland, B.O., Lydersen, E. (2004). The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology*, 128: 169–177.

Pottinger, T.G., Day, J. (1999). A *Saprolegnia parasitica* challenge system for rainbow trout: assessment of Pyceze as an anti-fungal control agent for both fish and ova. *Diseases of Aquatic Organisms*, 36: 129–141.

Richards, G.R., Chubb, J.C. (1996). Host response to initial and challenge infections, following treatment, of *Gyrodactylus bullatarudis* and *G. turnbulli* (Monogenea) on the guppy (*Poecilia reticulata*). *Parasitology Research*, 82: 242–247.

Rintamäki, P. (1989). *Gyrodactylus salaris* at a fish farm in northern Finland. In: *Parasites of Freshwater Fishes in North-west Europe* (O.N. Bauer, ed.), pp. 123–130. Petrozavodsk: Institute of Biology, USSR Academy of Sciences, Karelian Branch.

Roberts-Thomson, A. (2007). *Managing velvet disease in marine fish hatcheries*. PhD Thesis. School of Molecular and Microbial Sciences, University of Queensland, Australia.

Rokicka, M., Lumme, J., Ziętara, M.S. (2007). Identification of *Gyrodactylus* ectoparasites in Polish salmonid farms by PCR-RFLP of the nuclear ITS segment of ribosomal DNA (Monogenea, Gyrodactylidae). *Acta Parasitologica*, 52: 185–195.

Sharp, E., Parsons, S.A., Jefferson, B. (2006). Seasonal variations in natural organic matter and its impact on coagulation in water treatment. *Science of the Total Environment*, 363: 183–194.

Shepherd, J.A., Waigh, R.D., Gilbert, P. (1988). Antibacterial action of 2-bromo-2-nitropropane-1,3-diol (bronopol). *Antimicrobial Agents and Chemotherapy*, 32: 1693–1698.

Shinn, A.P., Sommerville, C., Gibson, D.I. (1995). Distribution and characterization of species of *Gyrodactylus* Nordmann, 1832 (Monogenea) parasitizing salmonids in UK, and their discrimination from *G. salaris* Malmberg, 1957. *Journal of Natural History*, 29: 1383–1402.

Shinn, A.P., Hansen, H., Olstad, K., Bachmann, L., Bakke, T.A. (2004). The use of morphometric characters to discriminate specimens of laboratory-reared and wild populations of *Gyrodactylus salaris* and *G. thymalli* (Monogenea). *Folia Parasitologica*, 51: 239–252.

Shinn, A.P., Picón-Camacho, S., Bron, J.E., Conway, D., Yoon, G.H., Hunter, R., Guo, F.C., Taylor, N.G.H. (2012). The anti-protozoal activity of bronopol on the key life-stages of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora). *Veterinary Parasitology*, 186: 229–236.

Soleng, A., Poléo, A.B.S., Alstad, N.E.W., Bakke, T.A. (1999). Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology*, 119: 19–25.

Soleng, A., Bakke, T.A. (2001). The susceptibility of grayling (*Thymallus thymallus*) to experimental infections with the monogenean *Gyrodactylus salaris*. *International Journal for Parasitology* 31: 793–797.

Sterud, E., Mo, T.A., Collins, C.M., Cunningham, C.O. (2002). The use of host specificity, pathogenicity and molecular markers to differentiate between *Gyrodactylus salaris* Malmberg, 1957 and *G. thymalli* Žitňan, 1960 (Monogenea, Gyrodactylidae). *Parasitology*, 124: 203–213.

Stretton, R.J., Manson, T.W. (1973). Some aspects of the mode of action of the antibacterial compound bronopol (2-bromo-2-nitropropane-1,3-diol). *Journal of Applied Bacteriology*, 36: 61–76.

Tanum, K. (1983). Studier av taksonomi og vertsforhold hos *Gyrodactylus*-arter på laksefisk av slektene *Salmo* og *Salvelinus* i Norge. Hovedfagsoppgave i spesiell zoologi, parasittologi. University of Oslo, Norway, p. 99.

World Health Organization (1998) Guidelines for drinking-water quality. 2nd ed. Addendum to Vol. 2. *Health criteria and other supporting information.* WHO, Geneva.