# DEVELOPING STRATEGIES FOR THE CONTROL OF *ICHTHYOPHTHIRIUS MULTIFILIIS* FOUQUET, 1876 (CILIOPHORA)

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SARA M. PICÓN CAMACHO

(BSC, MSC)

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INSTITUTE OF AQUACULTURE

UNIVERSITY OF STIRLING

*To Eric Leclercq, for your love and support.*

*Thank you for believing in me*

# DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

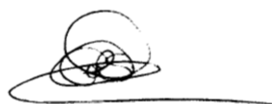
CANDIDATE NAME: SARA PICON CAMACHO

SIGNATURE:

DATE: 20/ 05/2011

SUPERVISOR NAME: ANDREW P. SHINN

SIGNATURE:



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# ABSTRACT

The intensification of freshwater aquaculture worldwide has facilitated the propagation of the parasitic ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876 commonly known as "fish white spot" or "Ich". *Ichthyophthirius multifiliis* infections lead to high mortalities, generating significant economic losses in most cultured freshwater fish species worldwide. Until recently, malachite green was the chemical treatment traditionally used to control *I. multifiliis* infections. Its reclassification as carcinogenic to humans and its subsequent ban for use in food fish has left the industry without any suitable treatments. Currently, in-bath formaldehyde and sodium chloride treatments are the most common option used in farm systems to control *I. multifliis* infections. Given their low efficacy, however, they are not considered as sustainable long–term options. There is, therefore, an urgent necessity to find efficacious alternatives for controlling *I. multifliis* infections. The general aim of this research project was to improve the management of *I. multifiliis* infections in order to develop more comprehensive, environmentally friendly and sustainable therapeutic strategies for use in freshwater food fish aquaculture.

The present PhD-thesis present first a literature review chapter providing an overview and critical assessment of chemotherapeutants and physical interventions tested within the last 30 years against *I. multifiliis* infections. The experimental worked consisted of a number of *in vitro* and *in vivo* trials were conducted using experimental scale flow-through, static tank systems and commercial scale raceways within a rainbow trout hatchery, in addition to molecular work on different isolates of the parasite. The results of this research are organised into three experimental chapters which describe the testing of

chemical and non-chemical treatments against *I. multifiliis* infections and work undertaken to determine the most suitable molecular markers to identify *I. multifiliis* isolates.

In the first experimental chapters, the possibility of efficiently controlling *I. multifliis* infections through the administration of novel environmentally-friendly chemical treatments (*e.g.* bronopol and peracetic acid-based products) was investigated. The results clearly showed that bronopol and peractic acid-based products have a strong biocidal/cytotoxic effect against all free-living stages of *I. multifiliis* (*e.g*. tomonts, cysts and theronts). The administration of high concentrations of bronopol (*e.g.* 20, 50 and 100 mg $L^{-1}$) over short periods of exposure (*e.g.* 30 min) significantly reduced the survival of tomonts, cysts and theronts and delayed the development of *I. multifiliis* tomonts and cysts. Prolonged low concentrations of bronopol (*e.g.* 1 mg $L^{-1}$) greatly reduced the survival of infective theronts, although such treatment did not affect the ability of surviving theronts to subsequently infect a host. When tested *in vivo*, the continuous prolonged exposure (*e.g.* 27 days) of low concentrations of bronopol (*e.g.* 2 and 5 mg $L^{-1}$) had an impact on the population dynamics of *I. multifiliis,* this being demonstrated by a significant reduction in the number of trophonts developing within the fish. Low concentrations of bronopol (*e.g.* 2 mg $L^{-1}$) administrated as a preventive treatment prior to infection also proved to be very successful at reducing the colonisation success of *I. multifiliis*. Peracetic acid administrated at low concentrations (*e.g.* 8, 12 and 15 mg $L^{-1}$) over a short window of exposure (*e.g.* 1 h) displayed a strong biocidal effect against all the free-living stages of *I. multifiliis* (*e.g.* tomonts, cysts and theronts). The bronopol and peracetic acid-based products tested here both appear to be capable of disrupting the development of

the cyst stage of *I. multifiliis* which is seldom reported for chemotherapeutants currently used against this parasite. These results suggest that bronopol and peracetic acid-based products have a place in the arsenal of treatment options for controlling *I. multifiliis* infections in commercial aquaculture systems.

The use of a mechanical device or a biological control agent to remove the cyst stage of *I. multifiliis* and the impact of such control on the population dynamics and the levels of infection of fish were also investigated. The results revealed that tomonts preferentially settle and encyst on the base of culture systems and on biofilm–covered substrates. The survival of the tomont stage is greatly affected by the composition of the substrate upon which it settles and is significantly lower on polypropylene-based plastic. The lining of raceways in a commercial rainbow trout hatchery with a low-adhesion polymer created a smooth surface facilitating the dislodgement and elimination of the cyst stage of *I. multifilis* by natural flushing or brushing. The physical removal of the cyst stage alone, through the use of a mechanical device or substrate detrivorous/algae feeder as a biological control agent, significantly reduced the propagation of *I. multifiliis* to a low level of infection without the need to deploy an additional chemical treatment. These studies demonstrate that the cyst is a key stage in the dynamics of *I. multifiliis* infection and its removal from the fish culture systems could constitute an effective and simple mean of managing *I. multifiliis* infections.

The third experimental chapter explores the utilisation of molecular marker to characterise different isolates of *I. multifiliis*. The results highlight the unsuitability of the rDNA region (ITS-1 and ITS-2) and the strong potential of the mtDNA (COI) as molecular markers to discriminate isolates of *I. multifiliis* from distant geographical locations. It is suggested that genetic “barcoding” using

mtDNA is the most effective method to identify *I. multifiliis* isolates. Importantly, genetic “barcoding” could allow associating *I. multifiliis* strains or geographical isolates with particular properties as regards their ecophysiology, pathogenicity and sensitivity to treatment, in order to improve the management of *I. multifiliis* infections according to the specific genetic isolate encountered.

This research project demonstrates the efficacy of a range of new approaches against the propagation of *I. multifiliis*. Together, our findings contribute towards the development of a more effective and integrated system for managing *I. multifliis* infections in farm systems. The utilisation of physical methods and of environmentally friendly chemotherapeutants holds great potential for the control of *I. multifiliis* infections in organic fish production and in a broader context to any freshwater food fish farms affected by *I. multifiliis*.

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# CHAPTER 1

# GENERAL INTRODUCTION

The following introduction starts with an overview of the state of world aquaculture highlighting the importance of freshwater fish farming. It continues by focusing on the situation in the United Kingdom (U.K.) where the aquaculture production is clearly dominated by two single species: Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* Walbaum. Finally, this introduction provides an overview of the current knowledge on the ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876, commonly known as the freshwater "white spot" or "Ich". The worldwide distribution of this parasitic disease is described and its significance within the U.K. rainbow trout industry is underlined before addressing the taxonomy, life-cycle and control methods for *I. multifiliis* infections within the food-fish aquaculture industry.

The expansion of freshwater aquaculture worldwide and the intensification of the production systems have facilitated the propagation of *I. multifiliis* infections which generate significant economic losses every year in most cultured fish species worldwide. Traditionally, malachite green was the universal chemical treatment used to control *I. multifiliis* infections in both ornamental and fish-food aquaculture. However, the reclassification of malachite green as carcinogenic to humans and its subsequent ban throughout the European Union and USA for use in fish produced for human consumption has left the fish-food industry without an effective treatment (Matthews, 2005; Dickerson, 2006; Noga, 2010). There is therefore an urgent necessity to find efficacious alternatives to malachite green for controlling *I. multifliis* infections in farmed fish.

The present PhD thesis explores a range of new treatments and practical management strategies to assist freshwater farm managers in the control of *I. multifiliis* infections. It is anticipated that the present results will find application in

commercial freshwater rainbow trout and salmon facilities in the U.K. but also in the broader sense, to any freshwater farmed food-fish species susceptible to *I. multifiliis*. In addition, part of this thesis investigates the identification of new molecular markers to identify possible intra-specific variation in *I. multifiliis* populations and the possible implications of intra-specific variation in the management of *I. multifiliis* infections.

## 1. World finfish aquaculture production

The aquaculture sector has shown a strong and steady annual growth rate of 6.5% between 2002 and 2007 which contrasts with the volume of captured fish production that remained stable over this period (FAO, 2009a). The global aquaculture production of fish, crustaceans and molluscs reached 50.3 million tonnes in 2007 for a value of USD 87 million and contributed by 36% in volume and 49% in value to the worldwide fishery production (FAO, 2009a). The aquaculture production was largely dominated by freshwater fish (26.8 million tonnes) followed by diadromous fish (3.3 million tonnes) accounting for 83.8% and 10.4% respectively of the global aquaculture fish production. Amongst the first 30 aquatic species (fish, crustaceans and molluscs) produced, a total of 7 cyprinids were found and the group of species accounted overall for 38% in volume of the global aquaculture production. Besides cyprinids, the main single fish species produced were Nile tilapia, *Oreochromis niloticus* (2.1 million tonnes, rank 9) and Atlantic salmon, *Salmo salar* (1.4 million tonnes; rank 11)(Figure1).

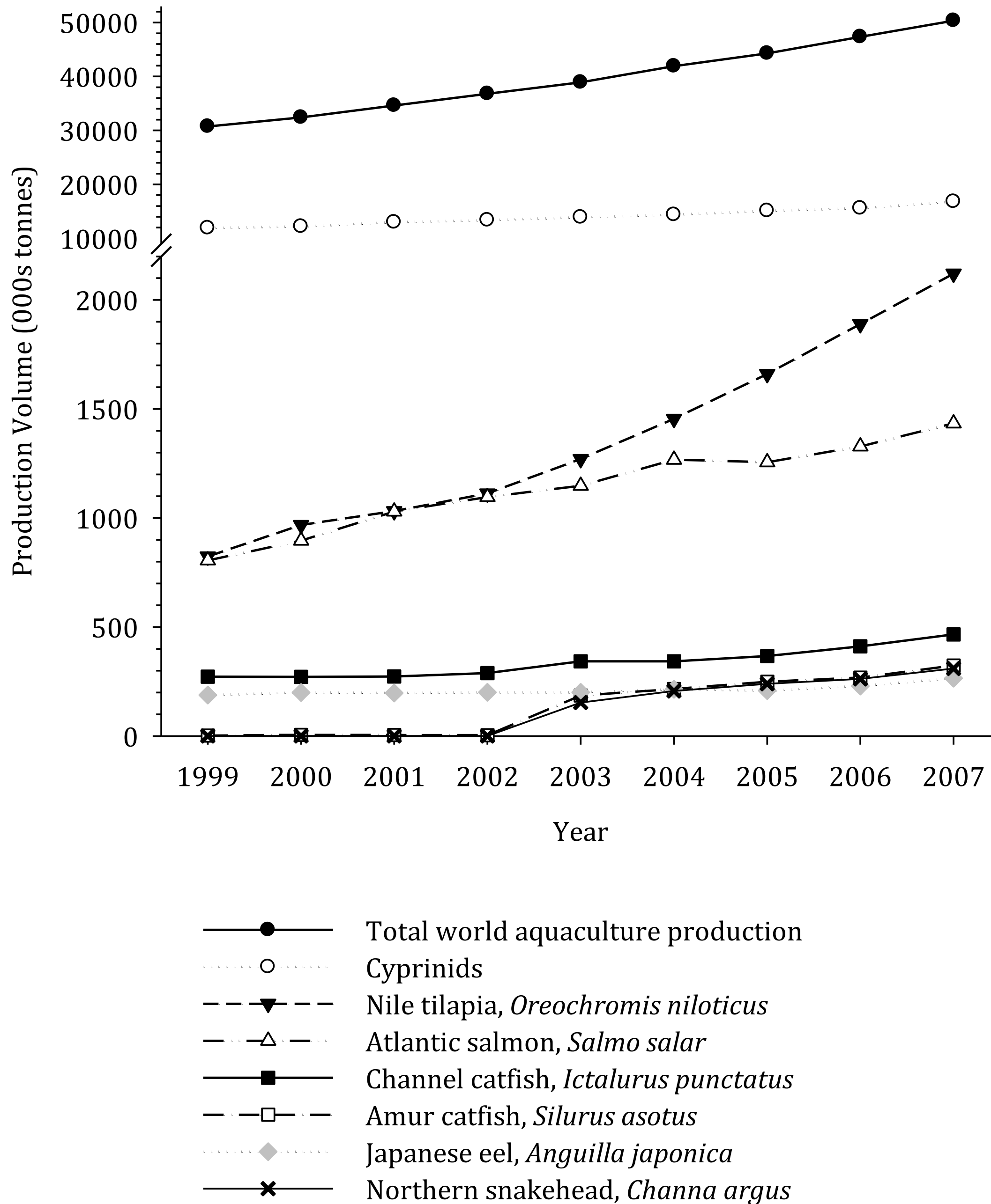


**Figure 1.** Total world aquaculture production and major freshwater finfish species production from 1998-2006. Production is expressed as thousands of tonnes. Data obtained from FAO (2009a).

Despite the strong growth of the global aquaculture sector within the last few decades, it is estimated that the global aquaculture volume must increase by 40 million tonnes by 2030 in order to meet the projected increase in demand for fish protein, as estimated from the current trend in the world human population

(FAO, 2009b). As highlighted in the FAO report (2009b), the global increase in the demand for fish protein is likely to be further accentuated by a number of factors. First, there is a current trend towards a reduction in the price of the major aquaculture species (cyprinids, tilapia, salmon and shrimp) due to more efficient production systems and the higher volume produced. In addition, there is an increasing awareness of the health benefits of eating fish containing high levels of unsaturated fatty-acid (omega-3 and omega-6) and essential fatty acids (EFA). Accordingly in some inland areas such as Eastern Europe where poultry, pig and beef are still the major sources of protein, it is forecasted that the proportion of fish in the diets will increase. Finally, in developing countries where the production of terrestrial farmed meat can be naturally restricted or comparatively expensive such as in West African coastal countries, fish can provide up to 50% of the protein intake. However, the limited availability of infrastructures compromises the inland transportation of freshly captured fish. Freshwater aquaculture is therefore increasingly regarded and often promoted as a highly beneficial activity in developing countries worldwide, providing sustainable development and local wealth, protein sources and health promoting nutrients (FAO, 2009b).

**2. Aquaculture in the United Kingdom**

In 2008, the British aquaculture sector generated approximately £600 million pounds and was largely dominated by finfish, valued at £500 million for a total volume of 144.000 tonnes. Over 98% of the national finfish aquacultural production was derived from two species: Atlantic salmon (129,000 tonnes; 89.7%) and rainbow trout (12,000 tonnes; 8.5%) (Reese, 2010) (Figure 2). Other

finfish species produced from aquaculture in the United Kingdom in 2008 were cod (1,822 tonnes), brown trout (70.8 tonnes), sea bass (20 tonnes), turbot (19.5 tonnes) and Nile tilapia (15.5 tonnes) (Reese, 2010) (Figure 3).

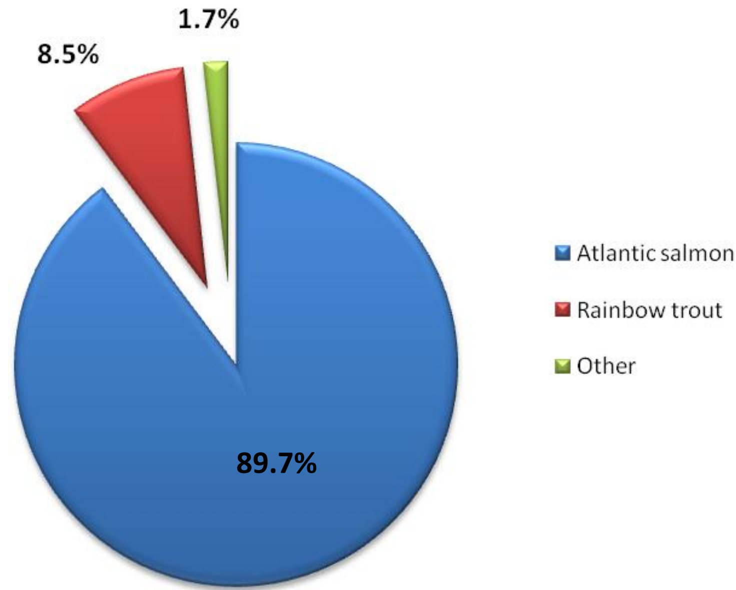


**Figure 2.** Relative contribution of Atlantic salmon, rainbow trout and other finfish species to the British finfish aquacultural sector in 2008. Production is expressed as percentage by weight of the total production. Data obtained from Reese (2010).

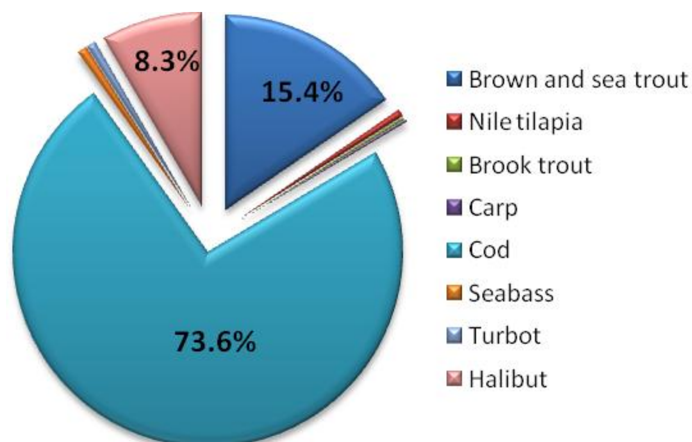


**Figure 3.** Relative contribution of finfish species other than Atlantic salmon and rainbow trout to the British finfish aquaculture sector in 2008. Production is expressed as percentage by weight of the total production. Data obtained from Reese (2010).

Within the United Kingdom, Scotland is the largest aquacultural producer accounting for, in 2008, 80% of the volume (144,079 tonnes) of the national output, followed by England and Wales (14%, 24,891 tonnes) and Northern Ireland (6%, 10,872 tonnes) (Reese, 2010). In Scotland, the freshwater production of Atlantic salmon smolts has been fluctuating over the last ten years (1998-2008) and showed an historical peak of 47.5 million smolts produced in 2006. In comparison, the volume of Scottish rainbow trout has been increasing from 4,913 tonnes in 1998 to 7,670 tonnes in 2008 (+ 56.1%) (Marine Scotland, 2009) (Figure 4). In 2008 in Scotland, there were a total of 31 companies and 66 sites registered for the production of rainbow trout and of 38 companies and 130 sites involved in the freshwater production of Atlantic salmon (Marine Scotland, 2009).

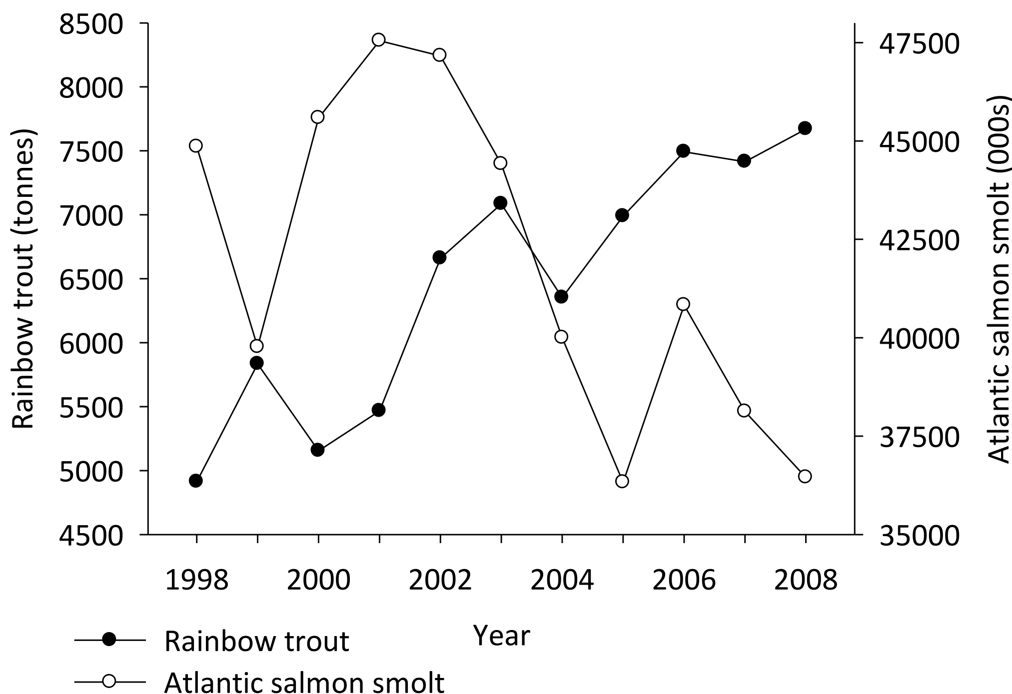


**Figure 4.** Production of Atlantic salmon smolt and rainbow trout in Scotland from 1998 to 2008. Data are expressed in thousands of tonnes in the production of Atlantic salmon smolts and in tonnes in rainbow trout production (Marine Scotland, 2009).

## 3. *Ichthyophthirius multifiliis*

The expansion and often intensification of aquaculture worldwide has lead to the rapid spread of certain diseases by the introduction of new hosts and through the transportation of live infected fish (Guo & Woo, 2009). Protozoan parasites are some of the most harmful parasitic diseases infecting freshwater teleosts (Scholz, 1999). Among these, ciliated protozoans cause significant losses in freshwater aquaculture, the most important species being *Chilodonella* sp., *Trichodina* sp. and *I. multifiliis* (Lom & Dyková, 1992).

### 3.1. Host and distribution of *Ichthyophthirius multifiliis* disease

#### 3.1.1. Worldwide distribution

*Ichthyophthirius multifiliis*, the causative organism of so-called "fish whitespot disease" has been hypothesised to have originated from carp culture in the Far East (Dashu & Lieng-Siang, 1960; Hines & Spira, 1974a) and is currently among the most widespread parasitic diseases in freshwater (Matthews, 2005). This parasite is associated with high mortalities and important economical losses in a wide range of cultured species in both the ornamental (Ling *et al.*, 1991) and fish-food industries (Valtonen & Koskivaara, 1994; Rintamäki-Kinnunen & Valtonen, 1997). In addition, it has been recorded in the wild causing heavy mortalities in lake and river fish populations (Wurtsbaugh & Tapia, 1988; Valtonen & Koskivaara, 1994; Aguilar *et al*., 2005; Maceda-Veiga *et al*., 2009).

It is the ability of this ciliate to adapt to different host species and environments that has facilitated its worldwide spread. *Ichthyophthirius multifiliis* is found from sub-Artic to tropical freshwater environments (Dickerson, 1994; Matthews, 2005; Dickerson, 2006). As reflected by its worldwide distribution, *I.*

*multifiliis* is characterised by very low host specificity and seems able to infect all freshwater teleost species. Although there can be different levels of resistance between different host species, no freshwater teleost has, to date, been shown to have complete resistance to this parasite (Dickerson, 1994; Matthews, 2005; Dickerson, 2006). In particular, *I. multifiliis* has been recorded to infect all farmed freshwater fish species such as carp species (*Cyprinus* spp.), channel catfish (*Ictalurus punctatus* Rafinesque), tilapia species (*Oreochromis* spp.), European eel (*Anguilla anguilla* L.) and various salmonid species (*e.g.* Atlantic salmon, rainbow trout and brown trout, *Salmo trutta* L.) (Paperna, 1991; Valtonen & Koskivaara, 1994; Noble & Summerfelt, 1996; Buchmann & Bresciani, 1997; Rintamäki-Kinnunen & Valtonen, 1997; Münderle *et al*., 2004; Matthews, 2005; Dickerson, 2006; El-Sayed, 2006; Jørgensen *et al*., 2009).

#### 3.1.2. Importance of *I. multifiliis* disease in the United Kingdom

Within the United Kingdom, white spot disease has been recorded to infect all freshwater salmonid species and it has been estimated by the British Trout Association (BTA) that approximately 60% of rainbow trout farms in the U.K. suffer *I. multifiliis* infections every year (BTA, personal communication). Accordingly, the Department of Environment, Food and Rural Affairs (Defra) recognized the ciliate protozoan *I. multifiliis* as the most common parasitic disease in rainbow trout farms (Defra; http://defra.gov.uk; website visited 7th October 2010). Similarly, a survey of forty-four rainbow trout farms across the British Isles found *I. multifiliis* as the most prevalent parasite with 32% of sites having an infection followed by *Ichthyobodo* sp. (11.6%), *Gyrodactylus* sp. (2.2%), *Diplostomum spathacaeum* L. (1.7%) and *Chilodonella* sp. (1.1%) (MacIntyre,

2008).

**3.2. Taxonomy**

*Ichthyophthirius multifiliis* is a protozoan belonging to the Phylum Ciliophora Doflein, 1901 and to the Class Oligohymenophorea. Members of the Phylum Ciliophora are considered as highly organised protists that are characterised, amongst other features, by their ciliary organelles, and the possession of one to several sets of nuclei (dualism: micronucleus and macronucleus) used for reproduction. Ciliates are capable of asexual (binary division) and/or sexual reproduction (autogamy or conjugation) (Lom & Dykova, 1992; Dickerson, 2006). The micronucleus contains a germ line used for sexual exchange of DNA whereas the macronucleus is used for the production of RNA to support vegetative cell growth and cell proliferation. For example in *Tetrahymena* sp., when two mating cells undergo sexual reproduction, the exchange of haploid micronuclei results in the development of a new macronucleus for later cell proliferation (Prescott, 1994). *Ichthyophthirius multifiliis* is characterized by having only two nuclei: the vegetative macronucleus and the reproductive micronucleus. However in this species, only asexual reproduction has been documented and the possibility for sexual reproduction remains unclear (Ewing *et al.*, 1988; Lom & Dyková, 1992; Noe & Dickerson, 1995; O'Donoghue, 2005).

*Ichthyophthirius multifiliis* is considered to be a single species but several studies have suggested the occurrence of different strains. Two main physiological strains of *I. multifiliis* were proposed and discriminated based on their water temperature range: a coldwater (7-11°C) and warm water (13-16°C) strain (Nigrelli *et al.*, 1976). A broader range of abiotic features *e.g.* including salinity tolerance, have also been used to discriminate different strains of *I. multifiliis*

(Aihua & Buchmann, 2001). More recently using molecular techniques, the existence of intra-specific variation using surface antigens has been demonstrated (Simon & Schmidt, 2007). To date, eleven different isolates or strains of *I. multifiliis* belonging to five different types of antigens have been described (Dickerson *et al*., 1993; Dickerson & Clark, 1998; Dickerson, 2006). Interestingly, the presence of genetic variation between strains has been hypothesised to be related to geographical regions adapted to specific fish hosts and water temperature ranges (Lom & Dyková, 1992). The importance of these strains is that they exhibit very diverse levels of infectivity or virulence (Elsayed *et al*., 2006; Swennes *et al*., 2007; Ling *et al.*, 2009) and susceptibility when exposed to chemical treatments (Straus & Meinelt, 2009; Straus *et al*., 2009). Straus & Meinelt (2009) demonstrated differences in toxicity between two isolates of *I. multifiliis* obtained from two different fish species to peracetic acid treatments, whereas Straus *et al.* (2009) observed different responses to copper sulphate treatments by isolates from distant localities. However, research to identify and characterise strains of *I. multifiliis* has been scarcely begun.

### 3.3. Biology and life-cycle of *I. multifiliis*

*Ichthyophthirius multifiliis* has a direct life-cycle that comprises four different life stages (Matthews, 2005) (Figure 5). (1) The parasitic stage or trophont develops within the epithelium of the fish gills and skin (including fins) on which it feeds. (2) Each trophont ultimately develops into a single tomont that exits from the host as a free-swimming organism which then settles on a substrate and encysts. (3) The cyst produced corresponds to the reproductive stage of the parasite, with each cyst undergoing binary division (asexual reproduction) and producing up to 3,000

daughter cells called tomites that quickly differentiate into (4) the free-swimming infective stage or theronts (Wagner, 1960; Lom & Dyková, 1992; Matthews, 2005).

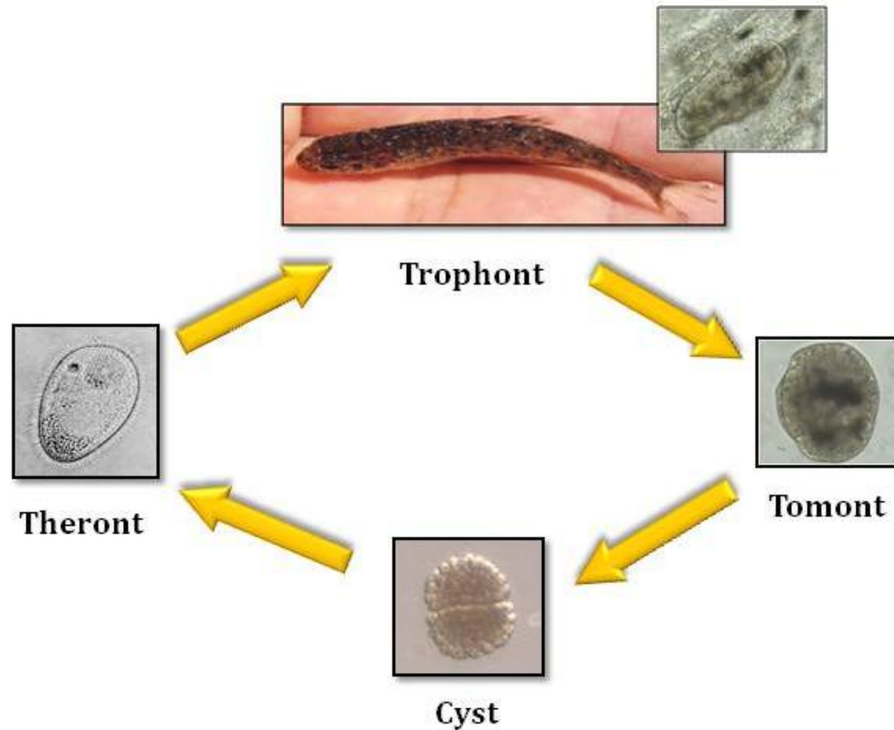


**Figure 5.** Schematic representation of the life-cycle of *I. multifiliis* Fouquet, 1876.

### 3.3.1. Trophonts

The prime morphological feature of the parasitic stage is a structure called the "apical perforatorium" that allows the rapid (less than 5 min) penetration of the parasite into the epithelium of the host fish (Canella & Rocchi-Canella, 1976; Ewing *et al*., 1985; Ewing & Kocan, 1992; Lom & Dyková, 1992). Upon penetration, the parasite rotates and physically forces its way into the fish thereby creating lesions and cell debris that are ingested and used as a source of energy (Canella & Rocchi-Canella, 1976). The duration required for the trophont to conclude feeding and exit the fish host as a free-swimming tomont has been shown to be affected by a number of factors including primarily water temperature but also host species and

host body-region (Dickerson, 2006). In particular, the trophont stage within the fish epidermis was shown to be inversely correlated to the water temperature, ranging from twenty days at 7°C to seven days at 20°C (Aihua & Buchmann, 2001). The importance of this life-stage as a growth or feeding stage is demonstrated by the fact that the trophont naturally exits its host at a minimum size of 85µm (Ewing *et al*., 1986). These large white spots in the epithelium are characteristic, allowing easy identification for diagnostic purposes (Figure 6). Ultimately, it is the development and departure of trophonts from the fish host which generate significant mortalities, creating lesions (Ewing *et al*., 1985; 1986) leading to osmoregulatory imbalance and respiratory dysfunctions (Hines & Spira, 1973, 1974 a, b; Tumbol *et al*., 2001) and to secondary infections by bacteria and fungi (Antychowicz *et al*., 1992; Matthews, 2005) .

**3.3.2. Tomonts**

The free-swimming tomont stage that exits the fish is sourrounded by cilia, providing its motile capacity, and presents a large, horse-shoe shaped macronucleus (Ewing & Kocan, 1992). The tomont actively swims (swimming speed average = 2.212 ± 0.342 mm $s^{-1}$; Araño Puig, 2004) in the water column for a brief period of time (3 h at 15°C; 1 h at 21-23°C) then settles on the substrate to encyst (Wagner, 1960; Shinn *et al*., unpublished). The tomont was suggested to be capable of developing into the cyst stage on any kind of substrate (Dickerson, 2006). To date, little is known about the tomont settlement behaviour only that they preferentially encyst on light- couloured substrates (Nickell & Ewing, 1989).

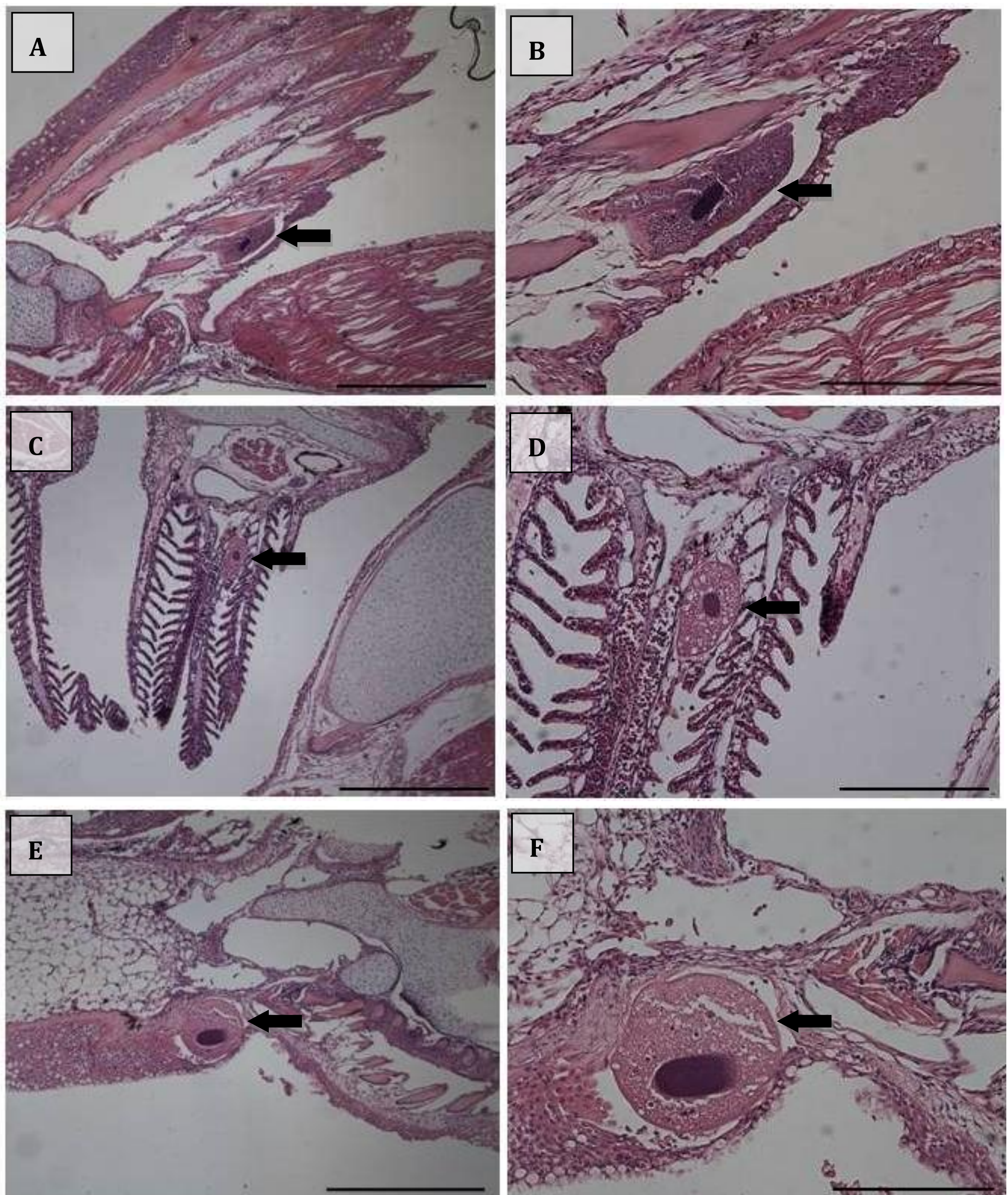


**Figure 6.** Hematoxylin and eosin stained histological tissue sections through the fins, gills and skin of rainbow trout fingerlings infected with *Ichthyophthirius multifiliis* Fouquet, 1876. A & B. Fin sections. C & D. Gill sections. E & F. Skin sections. Scale bar = 500 µm. Black arrows show the location of the trophont stage.

### 3.3.3. Cysts

As the tomont encysts, it produces a sticky mucus coating, apparently released from the secretory mucocysts present within its cytoplasm (Ewing & Kocan, 1992). The cyst's coat consists of two distinct gelatinous layers protecting this life-stage against the entry of fungi and /or bacteria (Ewing *et al*., 1983). The cyst divides by binary palintomic division and produces up to 1,000-3,000 daughter cells called tomites (Wagner, 1960; Lom & Dyková, 1992). Both the number and size of tomites produced by a cyst was shown to be proportional to the size of the initial tomont but also to the ambient water temperature (Canella & Rochhi-Canella, 1976).

### 3.3.4. Theronts

Tomites that are liberated into the water column quickly differentiate into free-swimming, infective theronts. The process of differentiation from a tomite to a theront remains unclear but is characterized by the acquisition of a pyriform to fusiform shape and the development of buccal apparatus and an "apical perforatorium" (Dickerson, 2006). The theront must find a host during its relatively short life-span to survive and complete its life-cycle. The life-span of this infective stage was shown to last a maximum of 92 h under low water temperature and to be inversely proportional to the ambient water temperature (Wagner, 1960; Aihua & Buchmann, 2001). In addition, the infectivity of the theront was shown to vary over its life-span (Lom & Dyková, 1992). Using a population of theronts maintained at 23-24°C, these authors calculated that the theront infectivity was around 34% for the first 12 h following its release from the cyst which then decreased to 1% after 20 h while the maximum theront life-span recorded in this

warm water experiment was 30 h. In order to allow the theront to actively find a fish host, it is surrounded by short length cilia and a single larger caudal cilium that was suggested to assist in maintaining its direction (McCartney *et al*., 1985; Kozel, 1986; Matthews, 1996). The theront direction appears to be dictated by a positive phototaxis, which arguably facilitates the encounter and infection of a fish host (Whali *et al*., 1991). Theronts response to a light stimulus might be facilitated by the "Organelle of Lieberkühn", a photoreceptor found in some ciliates and present only in the theront stage of *I. multifiliis* (Ewing & Kocan, 1992; Dickerson, 2006; Sun *et al*., 2009). In addition to its positive phototaxis, the theront exhibits a range of host-finding swimming behaviours which in some cases, is stimulated by specific chemical host cues (Haas *et al*., 1999). Theronts have also been shown to be chemoattracted by sera from a wide range of both freshwater and marine teleosts species which confirms the low-host specificity of *I. multifiliis* (Buchmann & Nielsen, 1999).

The importance of water temperature on the developmental rate of *I. multifiliis* has been previously documented within all individual life-stages of the parasite as previously discussed. Accordingly, the time for the parasite to complete its life-cycle (corresponding to one infection wave) is much faster under high water temperatures than under lower temperatures (Wagner, 1960; Nigrelli *et al*., 1976; Noe & Dickerson, 1995; Dickerson, 2006). This was confirmed in a preliminary trial as part of the research for this thesis. In the trial, *I. multifilis* from a single isolate from Scotland survived, multiplied and developed from the tomont to the theront stage at 4°C in 124 hours (~5 days) and at 24°C in 18 hours (Figure 7).

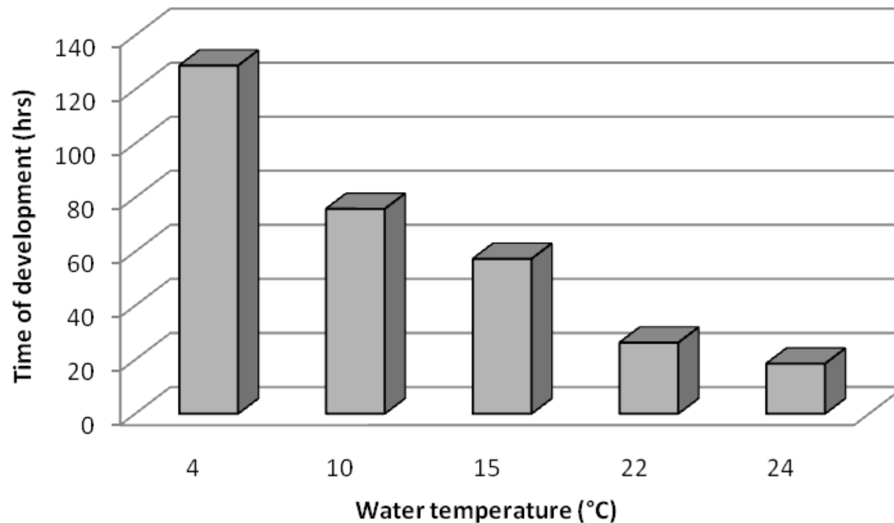


**Figure 7.** Time of development (hrs) from the tomont to the theront stage in relationship to the water temperature (°C). Data collected in the current study.

This strong effect of temperature on the population dynamics of *I. multifiliis* is responsible for a important seasonal component to this year-round infection (Marcos-López *et al.*, 2010). The seasonality of infections, related to the increase of water temperature at the beginning of the spring or early summer on temperate latitudes, but also the period required for the parasite to complete a full life-cycle under its local conditions, are therefore key factors on establishing effective control programs against *I. multifiliis* (Valtonen & Koskivaara, 1994).

### 3.4 Current research on key aspects of the biology and life-cycle of *I. multifiliis*

There are still many aspects of the biology of *I. multifiliis* that are not fully understood; amongst these are the possibility of sexual reproduction and the role of recently discovered endosymbiotic bacteria in the cytoplasm.

*Ichthyophthirius multifiliis* presents the nuclear dualism characteristic of

other ciliates capable of alternating between sexual and asexual reproduction such as *Tetrahymena* sp. (Orias, 1998). This nuclear organization is composed of two functionally distinct nuclei: a polyploid macronucleous (MAC) containing the "somatic" genome and the diploid micronucleus (MIC) containing the "germline" genome that is transcriptionally silent and instrumental for sexual reproduction (Riley & Katz, 2001; Motl & Chalker, 2009). However, only asexual reproduction by binary division at the cyst stage has been documented in *I. multifiliis* (Lom & Dyková, 1992). The senescence of an *I. multifiliis* infection after a maximum of 50 cycles (Houghton & Matthews, 1990) or 2-3 years (Xu & Klesius; 2004) of laboratory-maintained cultures and the presence of aggregated trophonts within the fish skin (Matthews, 1996) indicates the possibility of sexual reproduction (Ewing *et al.*, 1988; Noe & Dickerson, 1995). It has therefore been hypothesised that theronts could act as mating types and that sexual reproduction could take place within the fish host, allowing cell rejuvenation.

The presence of Gram-negative endosymbiotic bacteria in the cytoplasm of all the stages of *I. multifiliis* belonging to the Classes Rickettsiales and Sphingobacteriales (Sun *et al*., 2009) and their association with glycogen granules (Lobo-da-Cunha & Azevedo, 1988) suggest that these bacteria may play an important role in the life-cycle of *I. multifiliis*.

### 3.5. Control methods

The most efficient treatment against *I. multifiliis* to date was the chemical compound malachite green that effectively treated both the free-swimming stages (tomonts and theronts) and the feeding/parasitic stage (trophonts) within the fish epithelia (epidermis and gills) (Leteux & Meyer, 1972; Wahli *et al*., 1993; Tieman &

Goodwin, 2001; Buchman *et al*., 2003). Malachite green was shown to be very successful as a bath treatment when administrated on its own or in combination with formaldehyde (Guest, 1983; Wahli *et al*., 1993). The coloured salt and carbinolbase form of malachite green incorporated into the feed at 1.2 g $kg^{-1}$ feed for 10 days also provided in effective in-feed treatment by significantly reducing the number of trophonts establishing on the fish (Ruider *et al.*, 1997).

The application of malachite green remains a common chemotherapy in the ornamental fish industry however, in fish for human consumption it has been banned since 1991 by the Food and Drug Administration (FDA) in the US (Chang *et al*., 2001) and since 2000 by the European Union (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC). Since the early 80's it has been well established that malachite green could have carcinogenic and mutagenic effect in humans (Meyer & Jorgenson, 1983; Srivastava *et al*., 2004). In fish, malachite green accumulates in a number of tissues such as the liver, kidney, muscle and skin (Poe & Wilson, 1983) where it persists for long periods of time (minimum withdrawal period: 600 degree days) (Alderman & Clifton-Hadley, 1993). In addition, repeated treatments with malachite green increase its accumulation in tissues (Alderman, 1985).

In the last ten years, there has been extensive research to find efficacious replacement therapies to control *I. multifiliis* infections in farmed fish destined for the food industry. The different treatment approaches that have been investigated can be categorized as physical methods, chemical methods and vaccine development.

**3.5.1. Physical methods**

High water temperatures above 30°C are lethal to *I. multifiliis* (Lom & Dyková,

1992; Duarte *et al.*, 1993; Noga, 2010). Increasing the water temperature to 32°C for a period of 5 days has proven to be effective in eliminating *I. multifiliis* infections in tropical fish species (Matthews, 2005). In flow through systems, a flow rate increase (>85 cm $min^{-1}$ with a water turnover rate of >2.1 $h^{-1}$) was shown to greatly reduce the severity of *I. multifiliis* infections, arguably by flushing the parasite away from the rearing system (Bodensteiner *et al.*, 2000). In the ornamental fish industry, the transfer of infected fish (every 5-7 days) to infection-free aquaria was similarly effective as it would break the parasite life-cycle, by reducing the number of theronts able to infect the fish (Brown & Gratzek, 1980; Dickerson, 2006; Noga, 2010). This approach combined with a reduction of the stocking densities of infected fish was applied with a degree of success in an intensive rainbow trout hatchery (BTA hatchery manager, personal communication). Finally, brushing the bottom of culture tanks / systems has been demonstrated to be an efficient method to remove *I. multifiliis* stages (Noga, 2010) as this disturbs the cyst stage freeing it from the substrate, compromising the probability of its survival and its effective multiplication into numerous infective theronts. Biological controls have been demonstrated to successfully reduce the number of external parasites in aquaculture. For instance the utilisation of *Labroides* sp. in seawater salmon farms to control sea lice (*Lepeophtheirus salmonis* Krøyer, 1837 and *Caligus elongatus* von Nordmann, 1832) infections (Deady *et al*., 1995). However to date no biological control agent has been tested to manage *I. multifiliis* infections. Finally, the utilisation of UV light (91900 μW s $cm^{-2}$) in recirculating systems is able to successfully remove the theront stage from the water column (Gratzek *et al.*, 1983).

The aforementioned methods have limited practical application on large-

scale farm systems where the number of infected fish can reach thousands, particularly over the spring-summer periods when infection levels increase. The intensive production of farmed fish is often concomitant with a limited availability of water and tank space and reduction of labour intensive management practices.

**3.5.2. Chemical treatments**

According to Noga (2010), the application of formaldehyde treatments is one of the most widespread methods to control *I. multifiliis* infections in farm systems. Formaldehyde treatments are typically applied as short-duration or prolonged bath treatment at, *e.g.* 160-250 ppm for 1 hour in flow-through systems and at 15-25 ppm for a longer period of time in ponds with lower water exchange rates (Brown & Gratzek, 1980). However, formaldehyde treatments over the summer period have many side effects such as reducing the oxygen available in the water (Cross, 1972) as well as compromising fish mucus production (Buchmann *et al.*, 2004). These latter authors demonstrated that rainbow trout exposed to formaldehyde concentrations of 200-300 ppm for 1 hour, showed a significant reduction in mucus production, making the fish more susceptible to secondary infections by fungi and bacteria. Importantly, there are raising concerns regarding the environmental impacts of discharging the biocide formaldehyde into the environment, in addition to general safety of the workers handling large volumes of formaldehyde (Wooster *et al.*, 2005; Pedersen *et al.*, 2009). Formaldehyde was re-classified by the WHO International Agency for Research on Cancer as "carcinogenic to humans" in 2004 (WHO IARC, 2006) and it is expected that its application will not be permitted to treat fish-food in the near future.

Sodium chloride as common salt, is the second most frequently used

treatment against *I. multifiliis* infections. Some strains of *I. multifiliis* tolerate concentrations of salt up to 1 g $l^{-1}$ (Noga, 2010) such that the application of a minimum of 5 g $l^{-1}$ has been proven to reduce theront survival (Aihua & Buchmann, 2001; Shinn, unpublished data). Salt has been demonstrated to be weakly efficacious as an overall treatment given that multiple or long-term bath applications are often required (Selosse & Rowland, 1990; Miron *et al.*, 2003; Lahnsteiner & Weismann, 2007; Balta *et al.*, 2008; Mifsud & Rowland, 2008). In euryhaline fish species such as eels, channel catfish, tilapia and salmonids, the application of salt at concentrations of between 7-20 g $l^{-1}$ has been suggested to be the most effective (Matthews, 2005). Of note, salt has also been tested as an in-feed treatment but had no effect on the parasitic load (trophonts counts) establishing in fish when used at 1.2-6% by weight of the diet for 30 day period (Garcia *et al*., 2007).

Even if the in-bath application of salt could help to recover the osmotic imbalance created by the trophonts exiting the fish, its use is restricted by the salinity tolerance of the host species. Salt treatments are, however, poorly effective at the maximum concentrations tolerated by stenohaline freshwater teleost species (Selosse & Rowland, 1990; Miron *et al.*, 2003; Lahnsteiner & Weismann, 2007; Balta *et al.*, 2008; Mifsud & Rowland, 2008).

Other treatments used to treat the free-swimming stages of *I. multifiliis* are, amongst others: copper sulphate, especially in channel catfish cultured in ponds in the U.S.A. (Schlenk *et al*., 1998; Tieman & Goodwin, 2001; Straus, 2008), potassium permanganate (Straus & Griffin, 2001; Straus & Griffin, 2002; Balta *et al*., 2008), chloramine-T (Shinn *et al*., 2001, 2003a; Rahkonen & Koski, 2002) and sodium percarbonate (Buchmann *et al*., 2003; Heinecke & Buchmann, 2009; Bruzio &

Buchmann, 2010; see Chapter 2).

Only few chemicals are available to disrupt the trophont development, the main one being toltrazuril. Toltrazuril administered in bath treatments at 10μg $ml^{-1}$ for a period of 4 h per day and for 4 consecutive days, significantly reduced the number of trophonts on infected fish but was not able to kill the theront stage (Melhorn *et al.*, 1988). Quinine administrated as in-feed at 5 g $kg^{-1}$ feed for 7 to 10 days was also able to reduce the number of trophonts but was unpalatable to the fish (Schmahl *et al.*, 1996). No other chemical treatments have been identified as effective against the trophont stage of *I. multifiliis.* This is most likely due to the fact that the trophont is physically protected by the overlying layer of epithelium and fish mucus (Post & Vesley, 1983). Similarly, there is no chemical treatment described as being able to disrupt the development of the cyst which is protected from external aggression by an external coating layer (Ewing *et al.*, 1983).

Following the ban of malachite green, no chemical treatments effective against all stages of *I. multifiliis* (on and off-the fish) have been identified or licenced. The limited number of alternative chemotherapeutants appears to remove exclusively and only partially the free-swimming stages (tomonts and theronts) from the water column with no reported effects on the other life-stages. Repeated treatments are therefore required, which have an inherent labour cost and compromise fish homeostasis and growth performance. The chemotherapeutic strategies presently available are not satisfactory and, in the case of formaldehyde, not sustainable in the longer-term.

### 3.5.3. Vaccine development

The main objective of using vaccination as a control method is to induce a specific

long-term protection against a target disease. A number of vaccines are currently commercialised against some of the most important bacterial and viral diseases in farmed fish such as, *e.g.* vibriosis and furunculosis (Press & Lillehaug, 1995; Hastein *et al*., 2005; Sommerset *et al*., 2005; Bravo & Midtlyng, 2007). However, to date there is no effective vaccine against any of the most important parasitic diseases of fish, including *I. multifiliis* (Sommerset *et al.*, 2005).

It is well known that fish naturally exposed to a certain level of *I. multifiliis* infection are able to acquire protective immunity which can last from several months to a year (Hines & Spira, 1974c; Burkart *et al*., 1990; Matthews, 1994). This knowledge has stimulated efforts towards the development of vaccine against *I. multifiliis*. Preliminary trials have shown that the duration and the level of protection of the fish is closely related to the intensity of exposure and to the type of material used to enhance immunoprotection: *i.e.* the use of live or dead parasites (Dickerson, 2006). Although formalin-fixed theronts, cilia of theronts and sonicated trophonts provided a certain degree of protection, the level of protection remained low and was significantly inferior than when using live parasites to immunise the fish (Alishahi & Buchmann, 2006; Xu *et al.*, 2008a; Zhang *et al*., 2009). The utilisation of live parasites for vaccination is not economically viable on a commercial scale due to the impossibility of raising large amounts of parasite *in vitro* (fish host are required) and, in fact, is not an option due to the absence of non-virulent *I. multifiliis* strains for challenging the fish (Sigh, 2003). In order to uncover these bottlenecks, the use of the free-living ciliate *Tetrahymena* sp., which is typically easily cultured, in its ability to induce immunity against *I. multifiliis,* has been explored. Some authors were successful at inducing immunity against *I. multifiliis* by intraperitoneal injection or by bath vaccination using live cells and

/or by using cilia harvested from *Tetrahymena* sp. (Goven *et al*., 1981; Ling *et al*., 1993a), others, however, reported low levels of protection using these approaches (Sigh & Buchmann, 2002). These discrepancies suggest that there might be specific antibodies or innate factors exclusive to *I. multifiliis*. The identification and production of protective antigens currently appears as the most feasible and realistic strategy towards the production of commercial vaccines against *I. multifiliis*. In that aim, the named i-antigens (immobilisation antigens) of *I. multifiliis* that are defined as "antigens which are recognised by immobilisation and agglutination antibodies *in vitro* assays" (Sigh, 2003) are currently under investigation. The i-antigens are proteins associated with the cilia membrane with molecular masses ranging from 40 to 60 KDa in size (Dickerson, 1993). Antigenic variation occurs in most ciliates, including parasitic species like *I. multifiliis* and *Plasmodium* sp. and also in free-living species such as *Tetrahymena* sp. and *Paramecium* sp. In parasitic ciliates like *I. multifiliis* i-antigens have been demonstrated to induce the protective immunity in fish (Wang & Dickerson, 2002). It is also suggested that i-antigens could be involved in signal transduction (Simon & Schmidt, 2007) allowing, *e.g.* an invading theront to recognize and prematurely exit a previously immunised host (Cross & Matthews, 1992). Therefore, cross-linking i-antigens might trigger a signal on the parasite to exit the host before it is immobilised by fish antibodies. In this case, i-antigens would act as chemo- or mechanosensory receptors to detect and escape the immune system of fish (Clark & Dickerson, 1997). This represents a novel mechanism of immunity where the parasite is not killed by the antibodies but alternatively is forced to exit the fish host (Clark & Dickerson, 1997).

Different isolates *of I. multifiliis* contain i-antigens of different sizes and, to

date, ten isolates of *I. multifiliis* have been separated into five serotypes according to their i-antigen profile (Dickerson & Clark, 1998). Although it is a promising avenue for the future vaccination against *I. multifiliis,* the role of i-antigens is still not fully understood and it seems to be other factors involved in raising protective immunity against this parasite. This is highlighted by the fact that fish naturally immunised following a challenge with a live isolate of *I. multifiliis* appeared protected against all the serotypes while fish vaccinated using a specific purified i-antigen were immunised only against that particular strain (Dickerson *et al*., 1993; Leff *et al*., 1994; Clark & Dickerson, 1995; Wang *et al*., 2002).

The development of vaccines against *I. multifiliis* is presently going towards the utilisation of DNA vaccines encoding i-antigens (Sigh, 2004; Lorenzen & La Patra, 2005). However, this management strategy is still at an early experimental stage and much research remains to be done in order to elucidate the role of i-antigens and investigate other *I. multifiliis* factors involved in the host fish immune response (Matthews, 2005; Dickerson, 2006).

## 4. Justification and aim of the thesis

Hulme *et al*. (2002) hypothesized that the annual average air temperature in the U.K. could increase by up to 0.5-1.5°C by 2020 and by 2-3.5°C by 2080 while changes in the rainfall profile is likely to lead to wetter winters and drier summers. Should this occur, this alteration in air temperature will have not only a direct effect on the physiology and immune system of fish (Bowden *et al.*, 2007) but also on the propagation of diseases that have a temperature-dependant development rate such as in *I. multifiliis.*

*Ichthyophthirius multifiliis* is able to develop at low temperatures (4°C) but

rapid outbreaks and high mortalities mainly occur in salmonid farms subjected to water temperatures over 15°C. Infections of *I. multifiliis* can occur all-year round but they have a strong seasonal component (spring-summer period) (Marcos-López *et al.*, 2010) that could be significantly extended and intensified by an increase in run-off water temperature.

Following the ban preventing the use of malachite green, no satisfactory protocols are presently in place for the prevention or effective treatment of *I. multifiliis* infections in food-fish aquacultural systems. In addition, the most widely used alternative treatments are formaldehyde-based chemotherapeutants which are increasingly recognized as potential health and safety hazards for those handling them, with additional negative impacts on the fish's homeostatic state and the environment into which they are discharged. The administration of formaldehyde does not therefore appear to be a long-term sustainable possibility for the control *I. multifiliis* infections. To date, no candidate chemotherapeutants have been identified as being effective against all the different life-stages of *I. multifiliis*.

Ideally, effective candidate compounds should easily degrade in the water and should be applied following well-defined protocols according to their toxicity on both the host and parasite and to the dynamic of the parasite population. It can also be hypothesized that treatments maintaining a low level of infection would permit the acquisition of a degree of immunity against *I. multifiliis*, as opposed to situations where a rapid outbreak of the disease is allowed following infections. A range of physical treatments have been identified but no strategy that is applicable for use in commercial-scale, intensive production systems are currently available. In addition to these, no study that we are aware of, has addressed the potential of

using biological controls to manage *I. multifiliis* infections in fish production systems. Such disease management strategies have shown promising results not only in terrestrial farming systems but also in aquacultural systems such as reducing salmon lice infections in Atlantic salmon sea cages.

Finally, the presence of different strains of *I. multifiliis* exhibiting diverse levels of pathogenecity and susceptibility to treatments highlight the need to identify suitable molecular markers, which might contribute towards the development of a strain reference bank for *I. multifiliis* allowing the discrimination between strains of *I. multifiliis* and providing a strong analytical tool to support epidemiological studies.

The specific objectives of the thesis were:

1. To provide a general overview and critical assessment of chemotherapeutants and physical interventions tested within the last 30 years to highlight the most effective and promising options against *I. multifiliis* infections (**Chapter 2, Paper I**).
2. To determine the anti-protozoan efficacy of a bronopol-based compound, *in vitro,* on the free-living stages of *I. multifiliis* (tomonts, cysts and theronts) (**Chapter 3, Paper II**).
3. To evaluate the low-dose, continuous application of a bronopol-based compound in reducing the colonisation success of *I. multifiliis* on rainbow trout fingerlings (**Chapter 3, Paper III**).
4. To assess the *in vitro* efficacy of a peracetic acid-based compound on the viability of the free-living stages of *I. multifiliis* (tomonts, cysts and theronts) (**Chapter 3, Paper IV**).

5. To assess the efficacy of controlling *I. multifiliis* infections by the removal of the cyst stage using a novel mechanical device in combination with a low adhesion polymer tested in a commercial rainbow trout hatchery (**Chapter 4, Paper V**).
6. To examine the utility of a bottom-algae feeder *Glyptoperichthys gibbiceps* Kner, 1854 as a biological control at reducing *I. multifiliis* infections through the removal of the cyst stage (**Chapter 4, Paper VI**).
7. To investigate the use of a ribosomal and a mitochondrial molecular marker to discriminate different strains of *I. multifiliis* (**Chapter 4, Paper VII**).

# CHAPTER 2

# LITERATURE REVIEW

# CHAPTER 2

## PAPER I

*REVIEW ARTICLE*

**An assessment of the use of chemotherapeutants and physical interventions in the treatment of *Ichthyophthirius multifiliis* Fouquet, 1876, a protozoan parasite of freshwater fish**

**Picón-Camacho, S. M.[a]**, Marcos-Lopez, M.[a], Bron, J.E.[a] & Shinn, A.P.[a]

[a]*Institute of Aquaculture, University of Stirling, FK9 4LA Stirling, United Kingdom*

**Ready to be submitted to *Diseases of Aquatic Organisms***

**Contributions:** This manuscript was compiled and written in full by the author of this thesis with advice and thorough proofreading by the co-authors.

**Keywords**: *Ichthyophthirius multifiliis*, white spot, chemotherapeutant, treatment, ciliate, parasite.

**Abstract**

Infection by the ciliate protozoan *Ichthyophthirius multifiliis* Fouquet, 1876 causes significant economic losses in freshwater aquaculture worldwide. Following the introduction of a ban on the use of malachite green for treating fish to be used for human consumption, imposed by the European Union and United States, there has been extensive new research aimed at identifying suitable replacements. Here we make a critical assessment of the chemotherapeutants and management strategies, which have been tested or are currently employed and evaluate their possible application in farm systems. Of the current, most commonly used treatments, formaldehyde, copper sulphate and potassium permanganate are not sustainable options. The use of the purportedly, more environmentally friendly compounds such as humunic acid (10%), potassium ferrate (VI), peracetic acid and bronopol-based compounds have been recently tested and represent promising alternatives because of their demonstrable efficacy *in vivo* and their low toxicity to fish and humans. Further investigation, however, is required to optimise the most efficacious treatments to establish precise protocols of administration to minimize the volume of chemotherapeutant added whilst ensuring their maximum performance. At the same time, there needs to be a greater emphasis placed on the utilisation of management strategies and non-chemical interventions focusing on the removal of free-swimming stages and cysts of *I. multifiliis* from farm culture systems as potential promising environmentally-friendly alternatives in the control of *I. multifiliis* infections.

## 1. Introduction

The freshwater protozoan parasite of fish, *Ichthyophthirius multifiliis* Fouquet,

1876, also known as fish whitespot, continues to impact wild and cultured fish populations worldwide and places an additional economic burden on global freshwater finfish aquaculture. It has been nearly 30 years since major reviews were published examining the use of chemotherapeutants for the control of *I. multifiliis* (Cross, 1972; Hoffman & Meyer, 1974; Herwig, 1979) and since that time, particularly in view of the EU and United States ban imposed in 2000 and 1991 on the use of malachite green to treat fish for food production, there has been extensive research worldwide focusing on the provision of more efficient and environmentally friendly products and management techniques for controlling *I. multifiliis* infections. This paper seeks to provide an overview and assessment of those chemotherapeutic and physical interventions, which are currently employed or have been tested for efficacy since the three earlier reviews were published.

The ciliate protozoan *I. multifiliis* is one of the most important freshwater diseases affecting the aquaculture and ornamental fish industries. It has low host specificity, infecting a wide range of fish species, including commercially important species such as channel catfish (*Ictalurus punctatus* Rafinesque, 1818) and rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) (Valtonen & Koskivaara, 1994; Noble & Summerfelt, 1996; Buchmann & Bresciani, 1997; Rintamäki-Kinnunen & Valtonen, 1997; Matthews, 2005; Jørgensen *et al*., 2009). It has a direct life-cycle, which is temperature dependant such that the warmer the water temperature the faster the life-cycle completes. The life-cycle involves four different stages: (1) the trophont, which resides within the surface epithelium of gills, fins and other body surfaces; (2) the tomont, a free-swimming stage that exits the fish and settles on the substrate to become the encysted stage (3), which in turn repeatedly divides by binary fission to produce tomites which are released to the water column. Tomites

differentiate into the infective stage (4) the theront, which needs to find a host within a 92 h window to successfully complete the life-cycle by penetrating the epidermis and developing into the trophont stage before it dies (Lom & Dyková, 1992; Matthews, 2005).

Currently, the most common form of treatment to control this ciliate in farm systems is the use of trough-based bath treatments over short exposure times (*e.g.* 30 min - 4 h) which target the free-swimming stages of the parasite (*i.e.* tomonts and theronts). The other two stages, the trophont and the cyst are protected underneath the host surface epithelium (Post & Vesley, 1983) and the cyst coat (Ewing *et al*., 1983) respectively; therefore they are rarely susceptible to treatments.

Historically, malachite green (MG) was commonly used for the control of *I. multifiliis* and a range of other fish diseases (Srivastava *et al*., 2004) due to its demonstrable efficacy, low cost, ready availability, high stability during storage and high solubility in water (Schnick, 1988; Henderson *et al.,* 1997). This organic (triphenylmethane) dye was favoured for the control of *I. multifiliis* infections because of its high efficacy against both the free-swimming stages (tomonts and theronts) of the parasite and the feeding parasite stage (trophont) within the fish's epithelium (Wahli *et al*., 1993; Tieman & Goodwin, 2001; Buchman *et al*., 2003). However, the ecotoxicological effects of MG and its derivatives (mainly leucomalachite) are well documented at including details relating to its cytotoxicity, carcinogenecity, mutagenecity, induction of chromosomal fractures, teratogenecity and respiratory toxicity (Culp & Beland, 1996; Srivastava *et al.,* 2004). Malachite green and its derivatives, however, are highly persistent in the environment, bioaccumulating in the ecosystem and fish tissues (Henderson *et al.,*

1997). Although the use of MG has never been licensed by the US Food and Drug Administration (FDA), its use in food product was initially permitted under an 'investigational new animal drug' status (Alderman, 1985). This status was revoked in 1991 and MG was listed as a priority chemical for toxicity and carcinogenicity testing (Culp & Beland, 1996; Culp, 2004). Similarly in Canada, the use of MG and the presence of its derivatives in food animals are not permitted and its continued use was advised against in 1992 when MG was classified as a class II health hazard (Canadian Food Inspection Agency 2010). Its use within the European Union was subsequently banned in 2000 under EC directive 90/676/EEC; article 14, regulation 2377/90/EEC.

As a consequence of the widespread ban and enforced restrictions imposed on the use of MG and the concern regarding the presence of its derivatives in food-products (Herberer, 2009), there has been extensive research in the last few decades focusing on the provision of alternative, effective and environmentally friendly products and management techniques for controlling *I. multifiliis* infections. Despite the global effort, no such management strategies have yet emerged. There is a strong commercial and scientific need for providing a critical summary of candidates and currently applied chemotherapy but also the potential of other management strategies against *I. multifiliis* infections. As such, it has been nearly 30 years since the use of chemotherapeutants for the control of *I. multifiliis* was reviewed in three independent works (Cross, 1972; Hoffman & Meyer, 1974; Herwig, 1979).

This paper reviews current knowledge on the chemotherapies (compound, dose, duration and efficacy) and physical interventions employed or tested against *I. multifiliis* since the three earlier reviews. The aim of this work is to facilitate

information recovery from the original research and to assist identifying the most suitable therapy against *I. multifiliis* while highlighting the most promising treatments for further research and application in farm systems.

**2. Assessment of currently applied chemotherapies**

A large number of compounds have been tested for efficacy against *I. multifiliis* although relatively few of them have been widely deployed to provide effective control under field conditions. Appendix 1 provides a detailed list of 109 compounds used to control *I. multifiliis* under laboratory or field conditions from 1980 onwards. Of those used under field conditions, the most commonly used treatments are: chloramine-T, copper sulphate, formaldehyde, metronidazole, potassium permanganate and toltrazuril (Dickerson, 2006; Noga, 2010). Whilst malachite green was the most extensively employed treatment prior to 2000 eliminating the tomont, theront and trophont stages, its use has been largely discontinued for food fish, particularly in the EU and the United States.

Formaldehyde has been proven to be very effective at eliminating the free-living stages of the parasite (*i.e.* tomonts, cysts and theronts) (Wahli *et al*., 1993; Shinn *et al.,* 2005; Lahnsteiner & Weismann, 2007; Heinecke & Buchmann, 2009), however, when used for *in vivo* baths, fish survival is compromised (Wahli *et al.,* 1993; Tieman Goodwin, 2001). Currently, formaldehyde is one of the most commonly used treatments to control *I. multifiliis* infections in aquaculture systems (Noga, 2010). However, efficiency is achieved only at high concentrations which are repeatedly applied (*i.e.* 100 mg $L^{-1}$ for 30 min to 1 h over 10 consecutive days in salmonid farms) such that, in flow-through systems with rapid water turn-over as used for *e.g.* in the intensive production of salmonid, high volumes are

required. In addition, the use of formaldehyde has many reported side effects such as reducing the oxygen available in the water by 1 ppm for each 5 ppm of formaldehyde that is used (Cross, 1972; Pillay & Kutty, 2005). This can be particularly problematic in summer when increasing water temperatures accelerates the completion of the life-cycle of *I. multifiliis* which is also concomitant with a reduction in the oxygen holding capacity of the water. Importantly, formaldehyde has been shown to affect the production of mucus in fish (Buchmann *et al.,* 2004). These latter authors demonstrated that *O. mykiss* exposed to formaldehyde at concentrations of 200-300 ppm for 1 h had a reduced mucus production and were more susceptible to secondary infections by fungi and bacteria. Accordingly when applied *in vivo* in the form of baths, fish survival can be compromised (*e.g.* toxicity observed in *O. mykiss* exposed to two treatments of 25 and 100 mg $L^{-1}$ of formaldehyde for 1 h on days 9 and 12 post-infection) (Wahli *et al.,* 1993). Importantly, the effect of water quality parameters on the toxicity of formaldehyde to fish and to *I. multifiliis* remains poorly characterized (Meinelt *et al.,* 2005). Although formaldehyde is an approved aquacultural therapeutic within the EU (Schlotfeld, 1993; 1998), in 2004 it was reclassified by the WHO International Agency for Research on Cancer as “carcinogenic to humans” (WHO IARC, 2006). Even though it is quickly metabolised by aquatic organisms and holds a low potential for bioaccumulation (Hohreiter & Rigg, 2001**;** Duffort *et al.,* 2005), it can be envisaged that formaldehyde might be banned due to the hazard it poses to workers handling large volumes of the chemical (Wooster *et al.*, 2005). Given the high volumes of formaldehyde required in a typical farm treatment and the potential toxic risks to both fish stock and the farm workers handling it, the future of formaldehyde as a long-term acceptable and sustainable chemotherapy regime

looks unlikely.

Sodium chloride (salt) is the second most commonly product used for the treatment of *I. multifiliis* infections. The application of a minimum of 2.5 g $L^{-1}$ has been proven to reduce the theront and tomont survival (Aihua & Buchmann, 2001; Shinn *et al.,* 2005; Lahnsteiner & Weismann, 2007). The bath application of salt could help to recover the osmotic imbalance and loss of salts created by mature trophonts exiting the fish, however, multiple, daily treatments are required for the control of *I. multifiliis*. A treatment regime of 1-5 g $L^{-1}$ salt applied continuously for a minimum period of 7 to 32 days was able to reduce the number of trophonts establishing on fish (Selosse & Rowland, 1990; Miron *et al.,* 2003; Lahnsteiner & Weismann, 2007; Balta *et al.,* 2008; Mifsud & Rowland, 2008). Higher concentrations of salt (*e.g.* 15-20 g $L^{-1}$) over short periods of exposure (*e.g.* 20 - 60 min) were not able to reduce the level of infection (Lahnsteiner & Weismann, 2007; Balta *et al.,* 2008). The incorporation of salt in to fish feed has also been explored with contradictory results. Rahkonen & Koski (2002) reported a reduction in infection levels when salt was incorporated at a level of 0.3-1.0% and fed for 3 to 11 days. Garcia *et al*. (2007), however, did not observe any significant reduction in parasite burdens when fish were fed a diet containing 1.2-6.0% salt for a period of 30 days. The use of salt does not appear to represent an economically viable and safe treatment option for infected stenohaline freshwater fish species such as common carp (Noga, 2010). The use of this compound has been suggested to have greater potential in euryhaline fish species such as eels and tilapia raised in open, artificial systems with the application of salt at concentrations of 7-20 g $L^{-1}$ (Matthews, 2005).

Copper sulphate has been shown to be effective at eliminating *I. multifiliis* in

a range of fish species when used at low concentrations (Ling *et al.*, 1993b; Schlenk *et al.,* 1998; Goodwin & Straus, 2006; Straus, 2008; Rowland *et al.,* 2009). However, long periods of exposure can lead to toxicity, gill damage and growth suppression (Cardeilhac & Whitaker, 1988; Moore, 2005; Rábago-Castro *et al.,* 2006). Copper has a very low therapeutic index (Boyd, 2005) and its toxicity to both fish host and *I. multifiliis* is known to vary widely with water chemistry parameters, particularly water alkalinity and hardness (Deilhac & Whitaker, 1988; Strauss, 2008; Strauss *et al.,* 2009). Copper sulphate is a recognized algaecide and is known to be toxic to a wide range of invertebrate organisms including rotifers, cladocerans and copepods (Boyd, 1990). When added to pond systems, there is a risk of phytoplankton mortality which consequentially might result in lower oxygen levels at night which in turn compromises the trophic chain on which the fish stock might rely (Noga, 2010). It is vital therefore that its use on small sub-sample of the fish stock in the local water is determined before it is applied on a large scale basis. Particular care should be taken when using this compound in green water pond systems. Future research should be aimed at identifying the range of water quality parameters and concentrations within which this compound is effective against *I. multifiliis* infections and can be safely administered without a risk to fish.

Potassium permanganate ($KMnO_4$) is also commonly used against *I. multifiliis*, mainly in farm pond systems (Brown & Gratzek, 1980; Noga, 2010). Low concentrations (*e.g.* 0.8-1.0 mg $L^{-1}$) over short periods of exposure (30 min to 4 h) were able to eliminate the theront stage in the water column (Straus & Griffin, 2001). When tested *in vivo*, low concentrations (*e.g.* 0.25- 2 mg $L^{-1}$) require longer periods of exposure (continuously from 6 to 20 days) to significantly decrease the number of trophonts per fish (Straus & Griffin, 2001; Tieman & Goodwin, 2001;

Straus & Griffin, 2002). The application of higher concentrations (*e.g.* 10-20 mg $L^{-1}$) for 30 min was found to be toxic to treated fish (Balta *et al.,* 2008). Potassium permanganate oxidizes organic matter, reducing dissolved oxygen levels, its effects are notable when used particulary in ponds. This compound has a low therapeutic index and can be very toxic when used in waters of a high pH when it can precipitate on gills leading to high mortalities (Tucker, 1987; Dolezelova *et al.,* 2009; Noga, 2010). Potassium permanganate treatments against *I. multifiliis* show very low efficacy at concentrations that are not toxic to fish and therefore large quantities of compound and continuous application are often required to manage infections.

Chloramine-T is an organic chlorine compound, specifically a sodium salt that when mixed with water is a very strong disinfectant (Treves-Brown, 2000; Noga, 2010). When used to treat *I. multifiliis* stages, chloramine-T has been found to be very effective *in vitro* for the treatment of both the tomont and theront stages (Shinn *et al.,* 2001). *In vivo*, however, chloramine-T was effective only when administered at high concentrations (*e.g.* 100 mg $L^{-1}$ for 30 min daily for 10 days) (Shinn *et al.,* 2001; Tieman & Goodwin, 2001; Rahkonen & Koski, 2002; Shinn *et al.,* 2003a; Rintamäki-Kinnunen *et al.,* 2005a; Balta *et al.,* 2008). The administration of high doses of chloramine-T can inflict damage to the gill epithelia and has been reported to affect the development of the swim bladder in young fry (Sanabria *et al.,* 2009). The average lethal time (LT50) for a dose of 50 mg $L^{-1}$ chloramine-T was determined to be 166.8 min (Powell & Harris, 2004). Although these latter authors suggested that the toxicity of chloramine-T to freshwater Atlantic salmon, *Salmo salar* L., was as sensitive as *O. mykiss,* and more sensitive than *I. punctatus,* the latter showed histopathological changes when exposed to a daily 3 h 80 mg $L^{-1}$

static immersion bath (Gaikowski *et al.,* 2009). Future work, therefore, should explore the efficacy of using daily 30 min baths of chloramine-T ranging between 30 to 80 mg $L^{-1}$ over a period of 10 consecutive days (or the full duration of the parasite life-cycle as dictated by the local water temperature).

Hydrogen peroxide is a powerful oxidiser which has been used under field conditions to control *I. multifiliis*. High doses, however, can cause gill damage leading to fish mortality (especially at high temperatures) (Schmidt *et al.*, 2006; Noga, 2010). Its use *in vitro* tests against free-living stages of *I. multifiliis*, however, were disappointing (Lahnsteiner & Weismann, 2007; Shinn *et al.,* 2005), with a 100 mg $L^{-1}$ treatment for 1 h effecting only a 15% mortality of theronts (Shinn *et al.,* unpublished). It is not surprising, therefore, that a 20 day regime of 25 mg $L^{-1}$ hydrogen peroxide failed to bring about a reduction in the number of trophonts on stock which consequentially resulted in high mortalities (Tieman & Goodwin, 2001).

Metronidazole has been shown to be very successful at reducing the number of trophonts on infected fish when incorporated into the fish feed diet (Tojo-Rodriguez & Santamarina-Fernandez, 2001; Tokşen & Nemli, 2010). This compound, however, is listed as "possibly carcinogenic to humans" by the WHO and is currently banned by the EU and USA for use in animal feed and in USA specifically for animals destined for human consumption. In ornamental fish however, its incorporation in feed has been shown to be effective. Its future use as a potential treatment is no longer considered.

The triazinetrione derivative coccidiostat Toltrazuril has been shown to be effective against the tomont stage in *in vitro* trials (Schmahl *et al.,* 1989; Tojo-Rodriguez *et al.,* 1994). However, when administrated *in vivo* it is either ineffective

(Schmahl *et al.,* 1989; Tojo-Rodriguez *et al.,* 1994) or toxic to the fish (From *et al.,* 1992).

**3. The potential of alternative chemical compounds**

Despite recent extensive research to explore the utility of alternative environmentally friendly chemical compounds, only few new compounds have been shown to display some efficacy at reducing *I. multifiliis* infections *in vivo* (see Appendix 1). Of the compounds administrated as in-bath treatments that have been identified, acetic acid (4%), bronopol, peracetic acid-based products, combinations of peracetic acid and formaldehyde, huminic acid (10%) and potassium ferrate (IV) displayed a good level of efficacy when tested *in vivo*. Of these, acetic acid (4%) is widely used in Turkey to control protozoan infections (Kayis *et al*., 2009). When tested *in vivo* against *I. multifiliis*, a single short dip bath of 10 ml $L^{-1}$ for 3 min was able to reduce the trophont burden on treated fish (Balta *et al.,* 2008).

Bronopol, a chemical compound already licensed for use as an aquacultural chemotherapeutant, applied at low concentrations (*e.g.* 2 and 5 mg $L^{-1}$) over a long period of exposure (*e.g.* 27 days) has been demonstrated to be highly effective against the free-swimming stages of *I. multifiliis*, as well as reducing the number of trophonts subsequently establishing in successive waves of infection (Shinn *et al.,* 2010; Picón-Camacho *et al.,* 2010a). Bronopol does not accumulate in fish tissues or in the environment; a withdrawal period after its administration, therefore, is not required (Novartis, 2002). It presents no serious toxicological hazard to humans (Bryce *et al.,* 1978) or to fish (Pottinger & Day, 1999), and, it degrades very quickly, especially when exposed to high intensity UV light (Noga, 2010).

Bronopol-based product shows strong potential to manage *I. multifiliis* infections on farm systems. However timing of deployment according the parasite population dynamic and optimal treatment concentration remain to be optimised for this product to constitute an economically viable solution in commercial scale aquaculture.

Formulations of peracetic acid (PAA), hydrogen peroxide and acetic acid have proven able to kill the tomont stage after within 48 h of exposure at concentration of 0.8 - 0.9 mg $L^{-1}$. Importantly, cysts recently attached to the substrate were also killed following a 12 h exposure to 1 - 3 mg $L^{-1}$ to PAA solutions (Meinelt *et al.,* 2009). When used *in vivo*, formulations containing a high proportion of PAA were also able to reduce the number of trophonts on infected fish (Rintamäki-Kinnunen *et al.,* 2005a; Sudová *et al*., 2010). Adding peroctanoic acid to a PAA formulation, further improved the anti-protozoal activity of the solution (Bruzio & Buchmann, 2010), such that cyst stages were killed after 60 min exposure (Picón-Camacho *et al.,* 2010b). PAA's stability, however, has been shown to be closely linked to a range of water quality parameters (*i.e.* temperature, organic matter content and pH) (Pedersen *et al.,* 2009). In addition, the degradation of PAA over time must be assessed and taken into account to establish the most effective treatment regime to use on site. The efficacy of PAA, notably against the cyst and trophont stages, however, highlights the potential of this compound as a treatment against *I. multifiliis*.

Low concentrations of huminic acid (10%) (100-150 µl $L^{-1}$) were found to disrupt the development of tomonts, however, when the same concentrations were used *in vivo*, the results were inconsistent and appeared to be dependent on water temperature and the treatment regime used (Lahnsteiner & Weismann, 2007).

Ling *et al.* (2010) demonstrated that 4.8 mg $L^{-1}$ potassium ferrate (VI) administrated for 2 h was very effective *in vitro*, in killing theronts. When the same dose was used as an *in vivo* continuous bath treatment for 3 days, it resulted in an 80% redution in the number of trophonts on the treated fish. An increase in concentration to 19.2 mg $L^{-1}$ applied for 3 days managed to completely eradicate the infection from the fish stock suggesting that potassium ferrate (VI) is very successful at disrupting trophont development. Potassium ferrate (VI) is an environmentally friendly, strong oxidising agent (Ma & Liu, 2002), that is less toxic to fish and humans than closely related potassium salts such as potassium permanganate (Ling *et al.,* 2010). The effectiveness and degradation rate of potassium ferrate (VI) in the aquatic environment, however, is strongly linked to pH and water temperature (Johnson & Sharma, 1999) and these must be considered when establishing a treatment regime based on its use.

Of the bath chemicals that have investigated in recent years, potassium ferrate (VI) and the peracetic acid and bronopol-based compounds all possess potential as promising alternatives to current chemotherapies for the control of *I. multifiliis* infections.

Of the in-feed treatments described in Appendix 1, the compounds with the highest apparent efficacy *in vivo* in controlling *I. multifiliis* infections are amprolium hydrochloride, vitamin C, quinine, SalarBec, salinomycim sodium and secnidazole.

Shinn *et al.* (2003b) found that two anti-coccidiostats, amprolium hydrochloride and salinomycim sodium, when incorporated into a commercial feed were able to significantly reduce the number of trophonts establishing on fish. *In vitro* trials with amprolium hydrochloride were disappointing (Farley & Heckmann,

1980; Tojo-Rodriguez *et al.,* 1994; Shinn *et al,.* 2001) but treatment with 100 mg $L^{-1}$ for an hour compromised the survival of the cyst stage ultimately killing 85-90% of the cysts (Shinn *et al.,* 2001). Incorporation of 1g per kilo of feed given over 8 days post-infection did not manage to reduce the trophont burden on fish (Tojo-Rodriguez *et al.,* 1994). However, a dose 63 ppm amprolium hydrochloride given 10 days prior the infection reduced the number of trophonts subsequently establishing on fish by up to 78% when compared to the control group (Shinn *et al.*, 2003b). Salinomycin sodium has only been tested *in vivo* with promising results (Shinn *et al.*, 2003b). Infected fish fed a diet containing 47-63 ppm salinomycim sodium for a period of 10 days were found to have a significant reduction (80-93%) in the number of trophonts on them at the end of the trial when compared to the control groups (Shinn *et al.,* 2003b). The same authors also tested SalarBec, a blend of Vitamin C, E and B (Miles *et al.,* 2001). When medicated feed containing 0.32% SalarBec was fed to fish for a period of 10 days prior to infection with *I. multifiliis,* a 65% reduction in the number of trophonts surviving on challenged fish was found (Shinn *et al.,* 2005).

Vitamin C on its own or in combination with Vitamin E has also been tested with success *in vivo* (Wahli *et al.*, 1985; Wahli *et al.,* 1995, Walhi *et al.,* 1998). Quinine when incorporated into feed at a rate of 5 g per kg feed and given over a period of 7 to 10 days effected the complete elimination of *I. multifiliis* on medicated fish (Schmahl *et al.,* 1996). Medicated fish using vitamin C and quinine, however, showed some growth suppression as a result of decreased food intake.

Finally, secnidazole is a an antibiotic which has been shown to reduce *I. multifilis* infections when incorporated into feed and presented at 24-36 mg $kg^{-1}$ body $weight^{-1}$ (Tokşen & Nemli, 2010) or 40 g per kg of feed for ten days (Tojo-

Rodriguez & Santamarina-Fernandez, 2001). While secnidazole appeared to be effective, the cost of using it on a large commercial scale would be prohibitive (Noga, 2010).

Although the utilisation of medicated feed appears to be an efficient, targeted strategy for reducing trophont burdens, there are a number of disadvantages which centre on the unavailability of commercially prepared medicated feed, unpalatability issues and the general inappetance displayed by heavily infected fish. Further research, therefore, is required to optimise the presentation and appetance of each medicated feed, to establish how each is metabolised and the clearance rate of each anti-*I. multifiliis* compound.

**4. The emergence of natural extracts**

Some new and alternative treatments include the utilisation of plant extracts such as those from garlic, *Allium sativum* L., which showed promising results when tested *in vitro* (Buchmann *et al*., 2003). However, when incorporated in-feed and tested *in vivo* this extract did not manage to significantly reduce infection levels when compared to the control groups (Shinn *et al.,* unpublished). Other natural products such as those from papaya *Carica papaya* L. and the velvet bean *Mucuna pruriens* L. were successful when tested *in vitro* and *in vivo* against tomonts and trophonts (Ekamen *et al.,* 2004). The use of probiotics (*e.g. Aeromonas sobria*) has proven to be very effective at reducing infections in medicated fish (Pieters *et al.*, 2008). Recent research by Yao *et al.* (2010) using the extract from *Macleaya cordata* Willd has shown high efficacy in *in vitro* trials against tomonts and an important trophont reduction (*e.g.* 75-97%) when administrated *in vivo* at low concentrations (*e.g.* 0.6 - 0.9 mg $L^{-1}$) for 48 h.

There is therefore considerable potential for the use of such natural products to control *I. multifiliis* infections; however *in vivo* trials carried out under field trial conditions are a critical requirement prior wider deployment of such treatments.

**5. Non-chemical based management strategies**

In the last few years, a wide range of management strategies (see Appendix 2) have been tested to control *I. multifiliis*. Some of these non-chemical therapies have the potential to successfully reduce *I. multifiliis* infections in addition to chemotherapy or in specific aquacultural systems such as recirculation aquaculture systems.

Farley & Heckmann (1980) used “electrotherapy” as a possible treatment to control whitespot infections. Whilst there was some tomont mortality following exposure to short pulses of electricity (5 sec), it seems that this was probably due to water hydrolysis rather than lysis of the parasite. It was concluded that the amperage necessary to disrupt trophonts within the fish epidermis would be too high and lethal to the fish.

The utilisation of a single UV lamp (91900 μW s $cm^{-2}$) has, in contrast, successfully managed to reduce the mortality of fish infected with *I. multifiliis* in a close recirculation system by controlling the spread of *I. multifiliis* stages between tanks (Gratzek *et al.,* 1983).

The mechanical filtration of inlet water, considering that the size of theronts ranges from 57.4 × 28.6 μm (at 5°C) and 28.6 × 20.0 μm (at 30°C), is not a suitable method to prevent the entry of the parasite to farm systems (Aihua & Buchmann, 2001). Nonetheless, a combination of an 80 μm mesh followed by a treatment of sodium percarbonate prevented tomonts from entering the system and killed

theronts (Heinecke & Buchmann, 2009).

Bodensteiner *et al*. (2000) demonstrated that increasing the flow rate and water turnover in fish farms above 85 cm $min^{-1}$ and 2.1 L $h^{-1}$ managed to reduce infection levels by flushing the free-swimming stages of the parasite out of the system. However, since water availability in farms can fluctuate greatly over the year, often reducing significantly over the summer months when water temperature increases with concomitant increases in *I. multifiliis* infections, this cannot always provide a viable control solution.

Finally, Shinn *et al.* (2009) have recently demonstrated that the combination of regular cleaning with a vacuum cleaning head and the use of a low adhesion polymer to line rainbow trout raceways is able to remove cysts and reduce infection levels by up to 99.55% when compared to control groups. Notwithstanding, none of the management strategies described above have been adopted so far in a commercial fish farm context.

**6. Conclusion**

Currently, the most frequent method employed to control *I. multifiliis* infections in farm systems is the use of in-bath chemical treatments. Because of its asynchronous life-cycle and the continuous release into the water column of different stages (Lom & Dyková, 1992; Matthews, 2005), multiple applications are usually required over long periods of time, especially during the summer when water temperatures can rise considerably. Large quantities of chemicals are frequently used (*i.e.* formaldehyde and sodium chloride) when the infections levels are high. Such treatments are therefore expensive and not environmentally friendly due to the toxicity of large doses of chemical recognised as being effective.

In the present overview we have assessed the efficacy and practicality of a wide range of chemical products and management strategies that are potentially available to be used in farm systems. However, there remain considerable difficulties in comparing efficacies between products, since no standardised methods are employed across the stakeholder community for culturing the parasite, assessing viability of the theront stage and infecting fish. The greatest current discrepancy in determining the efficacy of a treatment follows from the counting method employed for enumerating the trophont stage in *in vivo* studies. Some researchers only consider the trophonts present on skin scrapes or gills while others take into the account direct observations of the number of visible trophonts present in skin, fins and gills. In addition to these methodological variations, there is the fact that different strains / genotypes of *I. multifiliis* can behave very differently in terms of infectivity (Elsayed *et al.*, 2006; Swennes *et al*., 2007; Ling *et al.*, 2009), host specificity and susceptibility to treatments (Straus & Meinelt, 2009; Straus *et al*., 2009). Hence, a chemical treatment demonstrated to successfully eliminate one strain might not exhibit the same efficacy when applied to treat a different one.

From this study, chemical treatments remain the principal method for controlling *I. multifiliis* infections in aquaculture, despite numerous attempts to develop and implement physical and farm management-based alternatives. With the introduction of a ban on the use of malachite green in food-fish and a likely future ban on the use of formaldehyde, options for effective chemotherapeutant treatment remain severely depleted. For these reasons, considerable research has been conducted to develop new chemotherapeutants or screen existing compounds, both natural and synthesised, for efficaciousness against one or more

stages of this parasite. New products, where deployed, will need to be derived from sustainable sources and of themselves be more environmentally friendly and more suitable for use in food-fish than previous compounds. As part of the attempt to reduce the use of chemotherapeutants, both new deployment strategies *e.g.* extended low dose treatments and strategies helping to reduce initial infection levels *e.g.* flow control or breeding fish for resistance, need to be considered.

**Appendix 1.** Chemotherapeutic treatments tested against infections of *Ichthyophthirius multifiliis* Fouquet, 1876. A compound is regarded as being effective if it kills ≥50% of the stages under test. Mortality refers to the parasite stages unless otherwise stated.

| Compound | Dose | Host / parasite stage | Efficacy | Reference |
|---|---|---|---|---|
| **Acaprin (1, 3-di-6-quinolylurea)** | | | | |
| *In vitro* | 200 mg $l^{-1}$ for 2 h | Tomonts | Effective - 62.5% mortality after 1-2 h<br>60% of surviving tomonts develop normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 200 mg $l^{-1}$ for 3 h | *Oncorhynchus mykiss* | Not effective - all trophonts developed normally † | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 500 mg $kg^{-1}$ for 8 d | | Not effective - all trophonts developed normally † | |
| | 40g $kg^{-1}$ for 10 d | | Not effective - no details | Tojo-Rodriguez & Santamarina -Fernandez (2001) |
| **Acetic acid (4%)** | | | | |
| *In vivo* - bath | 10 ml $l^{-1}$ for 3 min | *O. mykiss*<br>*Salvelinus fontinalis*<br>*Salmo trutta* | Effective - reduction on the number of trophonts on treated fish but no details given | Balta *et al.* (2008) |
| ***Allium sativum* (garlic extract)** | | | | |
| *In vitro* | 0.5 mg $l^{-1}$ for 1.5, 3 & 15 h | Theronts | Not effective - <50% mortality | Buchmann *et al*. (2003) |
| | 2.5 mg $l^{-1}$ for 1.5, 3 & 15 h | | Not effective - <50% mortality | |
| | 12.5 mg $l^{-1}$ for 1.5, 3 & 15 h | | Not effective - <50% mortality | |
| | 62.5 mg $l^{-1}$ for 1.5 & 3 h | | Not effective - <50% mortality | |
| | 62.5 mg $l^{-1}$ for 15 h | | Effective - >50% mortality | |
| | 312.5 mg $l^{-1}$ for 1.5 h | | Not effective - <50% mortality | |
| | 312.5 mg $l^{-1}$ for 3 & 15 h | | Effective - >50% mortality | |
| | 1562.5 mg $l^{-1}$ for 3 & 15 h | | Effective - >50% mortality | |
| | 30 mg $l^{-1}$ for 24 h | Tomocysts | Not effective - 13% mortality | Buchmann *et al*. (2003) |
| | 117 mg $l^{-1}$ for 24 h | | Effective - 53% mortality | |
| | 570 mg $l^{-1}$ for 24 h | | Effective - 100% mortality | |
| **Amphotericin B (dissolved in Na-desoxycholate)** | | | | |
| *In vitro* | 0.25 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 24 h | Wahli *et al.* (1993) |
| | 2.5 mg $l^{-1}$ for 24 h | | Effective - 100% mortality after 1 h | |
| | 0.25 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | |

| | | | | |
|---|---|---|---|---|
| | 2.5 mg l$^{-1}$ for 24 h | | Effective - 100% mortality after 24 h | |
| | 0.25 mg l$^{-1}$ for 3 h | Theronts | Effective - 100% mortality after1 h | |
| | 2.5 mg l$^{-1}$ for 3 h | | Effective - 100% mortality after 5min | |
| *In vivo* - bath | 0.25 mg l$^{-1}$ - 2× for 1 h<br>1$^{st}$: day 9 p.i, 2$^{nd}$: day 12 p.i | *O. mykiss* | Not effective - no details | Wahli *et al.* (1993) |
| **Amprolium hydrochloride (1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2-picolinium chloride hydrochloride) commercialised as Amprolmix** | | | | |
| *In vitro* | 20 mg l$^{-1}$ for 1 h | Tomonts | Not effective - 10% mortality | Shinn *et al.* (unpublished) |
| | 50 mg l$^{-1}$ for 1 h | | Not effective - 10% mortality | |
| | 100 mg l$^{-1}$ for 1 h | | Not effective - 90% mortality | |
| | 200 mg l$^{-1}$ for 2 h | Tomont | Not effective - 0% mortality after 2 h; tomonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| | 1000 mg l$^{-1}$ for 48 h | | Not effective - survival not affected | Farley & Heckmann (1980) |
| | 100 mg l$^{-1}$ for 15 h | Cysts | Effective - 85% mortality | Shinn *et al.* (2001) |
| | 100 mg l$^{-1}$ for 41 h | | Effective - 90% mortality | |
| | 20 mg l$^{-1}$ for 1 h | Theronts | Not effective - 22.4% mortality | Shinn *et al.* (unpublished) |
| | 50 mg l$^{-1}$ for 1 h | | Not effective - 20.4% mortality | |
| | 100 mg l$^{-1}$ for 1 h | | Not effective - 22.3% mortality | Shinn *et al.* (2001) |
| *In vivo* - bath | 200 mg l$^{-1}$ for 3h day 6 p.i. | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg kg$^{-1}$ for 8 d p.i. | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| | 75 ppm for 10 d prior inf. | *O. mykiss* | Effective - 63% reduction in trophont numbers | Shinn *et al.* (2001) |
| | 63 ppm for 10 d prior inf. | *O. mykiss* | Effective - 77.6% reduction in trophont numbers | Shinn *et al.* (2003b) |
| | 75 ppm for 10 d p.i. | | Not effective - 32.2% reduction in trophont numbers | |
| | 104 ppm for 10 d p.i. | | Effective - 62% reduction in trophont numbers | |
| **Amprolmix** | | | | |
| See entry for amprolium hydrochloride | | | | |
| ***Aeromonas sobria*** | | | | |
| *In vivo* - In-feed | 10$^{8}$ cells g$^{-1}$ feed for 14 d | *O. mykiss* | Effective in not that there was no mortality of treated fish | Pieters *et al.* (2008) |
| **Aquahumin** | | | | |
| See entry for huminic acid (10% solution) | | | | |
| **Ascorbate-2-phosphate (vitamin C)** | | | | |

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|---|---|---|---|---|
| *In vivo* - In-feed | 5000 mg $kg^{-1}$ feed for 9 d | *O. mykiss* | Effective but 2-16% of medicated fish died | Wahli *et al.* (1985) |
| | 50 mg 200 $kg^{-1}$ feed | *O. mykiss* | Effective - reduction in trophont numbers but no details | Wahli *et al.* (1995) |
| | 50 mg 2000 $kg^{-1}$ feed | | Effective - reduction in trophont numbers but no details | |
| | 1-3 g $kg^{-1}$ feed for 1 week-1 month | Not specified | Effective - no details | Rahkonen & Koski (2002) |
| **Ascorbate-2-phosphate (Vitamin C) + d-l-alpha-tocopheryl acetate (Vitamin E) complex diet** | | | | |
| *In vivo* - In- feed for 7 weeks | | | | |
| | 0 + 1.8 mg $kg^{-1}$ feed | *O. mykiss* | Not effective - 44% of medicated fish died | Walhi *et al.* (1998) |
| | 4.3 + 771.0 mg $kg^{-1}$ feed | | Effective but ~20% of medicated fish died | |
| | 24.8 + 34.0 mg $kg^{-1}$ feed | | Not effective - 62% of medicated fish died | |
| | 27.5 + 776.0 mg $kg^{-1}$ feed | | Effective but ~20% of medicated fish died | |
| | 2065.0 + 2.5 mg $kg^{-1}$ feed | | Effective but < 20% of medicated fish died | |
| | 2093.3 + 30.8 mg $kg^{-1}$ feed | | Effective but ~20% of medicated fish died | |
| | 2025.0 + 754.3 mg $kg^{-1}$ feed | | Effective but < 20% of medicated fish died | |
| **Ascorbyl phosphate** | | | | |
| *In vivo* - In-feed | 50 mg 2000 $kg^{-1}$ feed | *O. mykiss* | Effective - reduction in trophont numbers but no details | Wahli *et al.* (1995) |
| **Baycox** | | | | |
| See entry for toltrazuril | | | | |
| **Bithionol** | | | | |
| *In vivo* - In feed | 40 g $kg^{-1}$ of feed for 10 d | *O. mykiss* | Not effective - 68% fish with high number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Bronopol (2-bromo-2-nitropropane-1, 3-diol)** | | | | |
| *In vitro* | 20 mg $l^{-1}$ for 30 min | Tomonts | Effective - 76.2% mortality | Shinn *et al.* (2010) |
| | 50 mg $l^{-1}$ for 30 min | | Effective - 97.2% mortality | |
| | 100 mg $l^{-1}$ for 30 min | | Effective - 100% mortality | |
| | 20 mg $l^{-1}$ for 30 min | Cysts | Not effective - 3.3% mortality; cyst development delayed | Shinn *et al.* (2010) |
| | 50 mg $l^{-1}$ for 30 min | | Not effective - 10% mortality; cyst development delayed | |
| | 0.1 mg $l^{-1}$ for 12h | Theronts | Not effective - 7.15% mortality | Shinn *et al.* (2010) |
| | 0.1 mg $l^{-1}$ for 24 h | | Not effective - 31.55% mortality | |
| | 0.1 mg $l^{-1}$ for 36 h | | Not effective - 31.66 % mortality | |
| | 0.1 mg $l^{-1}$ for 48 h | | Not effective - 18% mortality | |
| | 0.25 mg $l^{-1}$ for 12h | | Not effective - 14.03% mortality | |
| | 0.25 mg $l^{-1}$ for 24 h | | Not effective - 30.95% mortality | |

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|---|---|---|---|---|---|
| | | 0.25 mg l$^{-1}$ for 36 h | | Not effective - 40.0% mortality | |
| | | 0.25 mg l$^{-1}$ for 48 h | | Not effective - 34.84% mortality | |
| | | 0.5 mg l$^{-1}$ for 12h | | Not effective - 22.38% mortality | |
| | | 0.5 mg l$^{-1}$ for 24 h | | Not effective - 38.77% mortality | |
| | | 0.5 mg l$^{-1}$ for 36 h | | Not effective - 26.51% mortality | |
| | | 0.5 mg l$^{-1}$ for 48 h | | Effective - 59.21% mortality | |
| | | 0.75 mg l$^{-1}$ for 12h | | Not effective - 8.88% mortality | |
| | | 0.75 mg l$^{-1}$ for 24 h | | Not effective - 40.00% mortality | |
| | | 0.75 mg l$^{-1}$ for 36 h | | Not effective - 26.51% mortality | |
| | | 0.75 mg l$^{-1}$ for 48 h | | Effective - 68.57% mortality | |
| | | 1 mg l$^{-1}$ for 12 h | | Not effective - 13.88% mortality | |
| | | 1 mg l$^{-1}$ for 24 h | | Not effective - 37.93% mortality | |
| | | 1 mg l$^{-1}$ for 36 h | | Not effective - 44.44 % mortality | |
| | | 1 mg l$^{-1}$ for 48 h | | Effective – 75.00% mortality | |
| | | 1 mg l$^{-1}$ for 12 h | | Effective - 70.84% mortality | |
| | | 1 mg l$^{-1}$ for 24 h | | Effective - 100% mortality | |
| | | 20 mg l$^{-1}$ for 30 min | Theronts | Not effective - 18.5% mortality | Shinn *et al.* (2010) |
| | | 50 mg l$^{-1}$ for 30 min | | Not effective - 31.3% mortality | |
| | | 100 mg l$^{-1}$ for 30 min | | Effective - 51.7% mortality | |
| | | 100 mg l$^{-1}$ for 30 min | | Effective - 50% mortality; all dead after 43 h | Shinn *et al.* (2010) |
| | *In vivo* - bath | 1 mg l$^{-1}$ for 36 d p.i | *O. mykiss* | Not effective - number of trophonts increased on treated groups | Picón-Camacho *et al.* (2010) |
| | | 2 mg l$^{-1}$ for 36 d p.i | | Effective - 46% reduction in trophont numbers on the 2$^{nd}$ wave of infection; 83% reduction in trophonts numbers on 3$^{rd}$ wave | |
| | | 2 mg l$^{-1}$ 24 h prior inf. & 72 h p.i. | | Effective - 35-48% trophont reduction on treated groups | |
| | | 5mg l$^{-1}$ for 36 d p.i | | Effective - 83% reduction in trophont numbers on the 2$^{nd}$ wave of infection; 97% reduction in trophont numbers on 3$^{rd}$ wave | |
| | | 50 mg l$^{-1}$ daily for 1h for 10 d | *O. mykiss* | Not effective - no reduction in trophont numbers on treated fish | Shinn *et al.* (2003a) |
| | | 50 mg l$^{-1}$ for 30 min for 10 d (alternate days) | *O. mykiss* | Not effective - no significant reduction in trophont numbers | Shinn *et al.* (unpublished) |
| | | 100 mg l$^{-1}$ daily for 30 min for 10 d | | Not effective - 33.3% reduction in trophont numbers | Shinn *et al.* (2003a) |
| | | 100 mg l$^{-1}$ daily for 30 min for 10 d | | Effective - 81.1% reduction in trophont numbers | |
| | | 100 mg l$^{-1}$ for 1 h on day 7 | | Not effective - no reduction in trophont numbers on treated fish | |
| ***Brochothrix thermosphacta*** | | | | | |

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|---|---|---|---|---|
| *In vivo* - In-feed | $10^{10}$ cells $g^{-1}$ feed for 14 d | *O. mykiss* | Not effective - 98% mortality on treated fish | Pieters *et al.* (2008) |
| **Cadmium chloride** | | | | |
| *In vitro* | 0.005 ppm for 18-22 h | Theronts | Not effective - 0% mortality | Bisharyan *et al.* (2003) |
| | 0.05 ppm for 10 min, 1 & 5 h | | Not effective - 0% mortality | |
| | 0.05 ppm for 18-22 h | | Effective - 50-90% mortality | |
| | 0.5 ppm for 10 min &1 h | | Not effective - 0% mortality | |
| | 0.5 ppm for 5 h | | Effective - 50-90% mortality | |
| | 0.5 ppm for 18-22 h | | Effective - 100% mortality | |
| | 5 ppm for 10 min | | Not effective - reduction in swimming velocity | |
| | 5 ppm for 1 h | | Effective - 50-90% mortality | |
| | 5 ppm for 5 h | | Effective - 100% mortality | |
| | 50 ppm for 10 min | | Not effective - reduction in swimming velocity | |
| | 50 ppm for 1 h | | Effective - 100% mortality | |
| | 500 ppm for 10 min | | Effective - 100% mortality | |
| ***Carica papaya* (papaya)** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 3 h | Trophonts* | Not effective - 0% mortality | Ekamen *et al.* (2004) |
| | 100 mg $l^{-1}$ for 6 h | | Not effective - 10% mortality | |
| | 150 mg $l^{-1}$ for 3 h | | Not effective - 5% mortality | |
| | 150 mg $l^{-1}$ for 6 h | | Effective - 55% mortality | |
| | 200 mg $l^{-1}$ for 3 h | | Not effective - 25% mortality | |
| | 200 mg $l^{-1}$ for 6 h | | Effective - 100% mortality | |
| | 250 mg $l^{-1}$ for 3 h | | Effective - 90% mortality | |
| | 250 mg $l^{-1}$ for 6 h | | Effective - 100% mortality | |
| *In vivo* - bath | 200 mg $l^{-1}$ for 92 h | *Carassius a. auratus* | Effective - 89% reduction in trophont number on the skin and fins | Ekamen *et al.* (2004) |
| | 250 mg $l^{-1}$ for 92 h | | Effective - 92% reduction in trophont number on the skin and fins | |
| **Chloramine-T (sodium p-toluenesulfonchloramide)** | | | | |
| *In vitro* | 5 mg $l^{-1}$ for 1 h | Tomonts | Effective - 100% mortality | Shinn *et al.* (2001) |
| | 50 mg $l^{-1}$ for 15 min | Theronts | Effective - 100% mortality | |
| *In vivo* - bath | 1 mg $l^{-1}$ daily for 11 d | *I. punctatus* | Not effective - 100% mortality on treated fish | Tieman & Goodwin (2001) |
| | 2 mg $l^{-1}$ daily for 11 d | | Not effective - 100% mortality on treated fish | |
| | 5 mg $l^{-1}$ daily for 11 d | | Not effective - 100% mortality on treated fish | |
| | 5, 10 & 15 mg $l^{-1}$for 1 h | *O. mykiss*<br>*S. fontinalis*<br>*S. trutta* | Not effective - no details | Balta *et al.* (2008) |
| | 10 mg $l^{-1}$ for 6 h | *O. mykiss* | Not effective - no reduction in trophont numbers | Shinn *et al.* (unpublished) |
| | 14 mg $l^{-1}$ 3 times a week | *Salmo salar* | Not effective - parasite numbers increased over infection period | Rintamäki-Kinnunen |

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| | for 3 weeks | | | *et al.*(2005a) |
| | 16 mg $l^{-1}$ 3 times a week for 2 weeks | | Trial inconclusive - low parasite numbers across all groups | Rintamäki-Kinnunen *et al.*(2005a) |
| | 100 mg $l^{-1}$ for 30 min 4 times a day for 10 d | *O. mykiss* | Effective - 93% reduction in trophont numbers | Shinn *et al.* (2001) |
| | 100 mg $l^{-1}$ for 1 h on day 7 p.i. | *O. mykiss* | Not effective - 14% reduction in trophont numbers | Shinn *et al.* (2003a) |
| | 100 mg $l^{-1}$ for 6 h on day 7 p.i. | | Not effective - no reduction in trophont numbers | |
| | 100 mg $l^{-1}$ daily for 30 min for 10 d | | Effective - 90.5% reduction in trophont numbers | |
| | 100 mg $l^{-1}$ daily for 30 min for 10 d | | Effective - 97.3% reduction in trophont numbers | |
| | 100 mg $l^{-1}$ for 30 min 4 times over 10 d | | Effective - significant reduction in 50% of the tanks | Rahkonen & Koski (2002) |
| **Chloramine-T (sodium p-toluenesulfonchloramide) + formaldehyde** | | | | |
| *In vivo* - bath | 10 + 100 mg $l^{-1}$ 3 times a week for 4 weeks | *S. salar* | Not effective - no details | Rintamäki-Kinnunen *et al.*(2005a) |
| | 8 + 125 mg $l^{-1}$ 3 times a week for 5 weeks | | Effective - no details | Rintamäki-Kinnunen *et al.* (2005b) |
| **Chloramphenicol (D (-) threo-2,2-dichloro-N-[hydroxy-α(hydroxymethyl)-p-nitrophenethyl] acetamide)** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 3 h | Theronts | Not effective - no details | Wahli *et al.* (1993) |
| | 100 mg $l^{-1}$ for 24 h | Adults** | Not effective - no details | |
| | 100 mg $l^{-1}$ for 24 h | Cysts | Not effective - no details | |
| **Chloroquine** | | | | |
| *In vitro* | 200 mg $l^{-1}$ for 2 h | Tomonts | Effective - 50% mortality after 2 h. Tomonts surviving develop normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 200 mg $l^{-1}$ for 3 h | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg $kg^{-1}$ for 8 d | | Not effective - all trophonts developed normally | |
| | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Not effective - high numbers of trophonts on all treated fish | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Chlortetracycline** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 1 h | Wahli *et al.* (1993) |
| | 100 mg $l^{-1}$ for 3 h | Theronts | Effective - 100% mortality after 5 min | |
| | 100 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | |
| *In vivo* - bath | 100 mg $l^{-1}$- 2× for 1h 1st: day 9 p.i, 2nd: day 12 p.i | *O. mykiss* | Not effective - no details | Wahli *et al.* (1993) |
| In-feed | 75 mg $kg^{-1}$ fish for 10 d | | Not effective - no details | |

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| **Citrocide** | | | | |
| *In vivo* - In-feed | 10 ppm for 7 d p.i. | *O. mykiss* | Partially effective - ~40% reduction in trophont numbers | Shinn *et al.* (2005) |
| **Citrox BC** | | | | |
| *In vivo* - In-feed | 10 ppm for 7 d p.i. | *O. mykiss* | Not effective - 25% reduction in trophont numbers | Shinn *et al.* (2005) |
| **Clopidol (3,5-dichloro-2,6-dimethyl-4-pyridinol) commercialised as Coyden** | | | | |
| *In vivo* - In-feed | 65 ppm for 10 d prior inf. | *O. mykiss* | Not effective - 35.2% reduction in trophont numbers | Shinn *et al.* (2003a) |
| | 92 ppm for 10 d prior inf. | | Not effective - 20.1% reduction in trophont numbers | |
| | 72 ppm for 10 d prior inf. | | Not effective - 35.6% reduction in trophont numbers | Shinn *et al.* (unpublished) |
| **Copper sulphate ($CuSO_4$)** | | | | |
| *In vitro* | 55 µg $l^{-1}$ for 24 h | Tomites | Not effective - 100% manage to infect *C. auratus* | Ling *et al*. (1993b) |
| | 110 µg $l^{-1}$ for 24 h | | Not effective - 90% manage to infect *C. auratus* | |
| | 160 µg $l^{-1}$ for 24 h | | Effective - tomites inactive but 20% manage to infect *C. auratus* | |
| | 255 µg $l^{-1}$ for 24 h | | Effective - 100% mortality | |
| | 220 µg $l^{-1}$ for 24 h | | Effective - 100% mortality | |
| | 0.027 mg Cu $l^{-1}$ as $CuSO_4$ for 3h (alkalinity 48 mg $l^{-1}$) | Theronts | Effective - 50% mortality | Goodwin & Straus (2006) |
| | 0.028 mg nonchelated liquid $CuSO_4$ $l^{-1}$ for 3 h (alkalinity 48 mg $l^{-1}$) | | Effective - 50% mortality | |
| | 0.027 mg Cu $l^{-1}$ as $CuSO_4$for 3h (alkalinity 48 mg $l^{-1}$) | | Effective - 50% mortality | |
| | 0.05 mg Cu $l^{-1}$ as $CuSO_4$ for 3h (alkalinity 48 mg $l^{-1}$) | | Effective - 95% mortality | |
| | 0.05 mg nonchelated liquid $CuSO_4$ $l^{-1}$ for 2h (alkalinity 48 mg $l^{-1}$) | | Effective - 95% mortality | |
| | 0.056 Cu $l^{-1}$ as $CuSO_4$ for 3h (alkalinity 243 mg $l^{-1}$) | | Effective - 50% mortality | |
| | 0.053 mg nonchelated liquid $CuSO_4$ $l^{-1}$ for 3 h (alkalinity 243 mg $l^{-1}$) | | Effective - 50% mortality | |
| | 0.075 mg Cu $l^{-1}$ as $CuSO_4$ for 3h (alkalinity 243 mg $l^{-1}$) | | Effective - ~95% mortality | |
| | 0.075 mg nonchelated liquid $CuSO_4$ $l^{-1}$ for 3 h (alkalinity 243 mg $l^{-1}$) | | Effective - ~ 95% mortality | |
| | <0.25 mg $l^{-1}$ Cu $l^{-1}$ as $CuSO_4$ or nonchelated liquid $CuSO_4$ $l^{-1}$ up to 1h (alkalinity 48 and 243 mg $l^{-1}$) | | Not effective - no reduction in theront survival | |
| *In vivo* - bath | 0.05 mg $l^{-1}$ for 10 d | *I. punctatus* | Not effective - 100% of infected fish died on day 10 | Schlenk *et al.* (1998) |

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|---|---|---|---|---|
| | 0.05 mg $l^{-1}$ daily for 17 d | *B. bidyanus* | Not effective - treated fish remained infected | Rowland *et al.* (2009) |
| | 0.1 mg $l^{-1}$ for 10 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 0.1 mg $l^{-1}$ for 8 d | | Not effective - all treated fish died on day 13 | |
| | 0.1 mg $l^{-1}$ daily for 17 d | *B. bidyanus* | Effective - treated fish free of trophonts | Rowland *et al.* (2009) |
| | 0.20 mg $l^{-1}$ daily for 14 d | | Effective - treated fish free of trophonts | |
| | 0.25 mg $l^{-1}$ daily for 14 d | | Effective - treated fish free of trophonts | |
| | 255 µg $Cu^{+2}$ $l^{-1}$ for 1 week | *C. auratus* | Effective but 11.1% of infected fish died | Ling *et al*. (1993b) |
| | 255 µg $Cu^{+2}$ $l^{-1}$ for 2 weeks | | Effective but 33.3% of infected fish died fish | |
| | 255 µg $Cu^{+2}$ $l^{-1}$ for 3 weeks | | Not effective - 44.4% of infected fish died | |
| | 288 µg $Cu^{+2}$ $l^{-1}$ for 15 min | | Not effective - 100% of infected fish died | |
| | 288 µg $Cu^{+2}$ $l^{-1}$ for 30 min | | Not effective - 66.70% of infected fish died | |
| | 288 µg $Cu^{+2}$ $l^{-1}$ for 60 min | | Not effective - 44.4% of infected fish died | |
| | 288 µg $Cu^{+2}$ $l^{-1}$ for 2 h | | Effective but 11.1% of infected fish died | |
| | 0.4 mg $l^{-1}$ for 8 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 0.5 mg $l^{-1}$ for 10 d | | Effective - no trophonts found on treated fish | |
| | 0.5 mg $l^{-1}$ daily for 17 d | *B. bidyanus* | Not effective - 100% of infected fish died† | Rowland *et al.* (2009) |
| | 0.8 mg $l^{-1}$ for 8 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 1 mg $l^{-1}$ for 10 d | | Effective - no trophonts found on treated fish | |
| | 1 mg $l^{-1}$ daily for 17 d | *B. bidyanus* | Not effective - 100% of infected fish died† | Rowland *et al.* (2009) |
| (static tanks) | 1 mg $l^{-1}$ daily for 11 d (alkalinity 68 mg $l^{-1}$) | *I. punctatus* | Not effective - 80 - 100% of the treated infected fish died | Tieman & Goodwin (2001) |
| | 1 mg $l^{-1}$ daily for 11 d (alkalinity 180 & 250 mg $l^{-1}$) | | Not effective - 80% of the treated infected fish died | |
| | 1 mg $l^{-1}$ alternate day for 11 d (alkalinity 150 mg $l^{-1}$) | | Not effective - all the treated infected fish died | |
| | 1.1 mg $l^{-1}$ (8 d trial) Treatment: d1, 3, 5 & 7 | *I. punctatus* | Effective - 15% of the treated infected fish died | Straus (2008) |
| | 1.2 mg $l^{-1}$ for 8 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 1.5 mg $l^{-1}$ for 10 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| (static tanks) | 1.5 mg $l^{-1}$ daily for 11 d | *I. punctatus* | Not effective - 40% of the treated infected fish died | Tieman & Goodwin (2001) |

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|---|---|---|---|---|
| | (alkalinity 68 mg $l^{-1}$) | | (treated fish remain infected) | |
| | 1.5 mg $l^{-1}$ daily for 11 d | | Not effective - all the treated infected fish died | |
| | (alkalinity 180 & 250 mg $l^{-1}$) | | | |
| | 1.5 mg $l^{-1}$ alternate day for 11 d | | Not effective - all the treated infected fish died | |
| | (alkalinity 50 mg $l^{-1}$) | | | |
| | 1.6 mg $l^{-1}$ for 8 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 2.0 mg $l^{-1}$ for 10 d | *I. punctatus* | Effective - no trophonts found on treated fish | Schlenk *et al.* (1998) |
| | 2.2 mg $l^{-1}$(8 d trial) | *I. punctatus* | Not effective - 81.7% of the treated infected fish died | Straus (2008) |
| | Treatment: d1, 3, 5 & 7 | | | |
| | 3.3 mg $l^{-1}$(8 d trial) | | Not effective - 98.3% of the treated infected fish died | |
| | Treatment: d1, 3, 5 & 7 | | | |
| | 4.4 mg $l^{-1}$(8 d trial) | | Not effective - 96.7% of the treated infected fish died | |
| | Treatment: d1, 3, 5 & 7 | | | |
| **Coyden** | | | | |
| See entry clopidol (3, 5-dichloro-2, 6-dimethyl-4-pyridinol) | | | | |
| **Decoquinate** | | | | |
| *In vivo* - In-feed | 100 ppm 10d prior to infection | *O. mykiss* | Not effective - no reduction in trophont numbers on treated fish | Shinn *et al.* (2003a) |
| **Desirox** | | | | |
| See entry for peracetic acid + acetic acid + hydrogen peroxide based formulations (13% PAA + 20% AA+ 20% $H_2O_2$) | | | | |
| **Detarox** | | | | |
| See entry for peracetic acid + acetic acid + hydrogen peroxide based formulations | | | | |
| **Diethylcarbamazine** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Partially effective - lower trophont counts observed in skin scrapes (60% of treated fish free of infection) | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Dimetridazole (DMZ)** | | | | |
| *In vitro* | 5 mg $l^{-1}$ for 3 h | Theronts | Not effective - no details | Wahli *et al.* (1993) |
| | 5 mg $l^{-1}$ for 24 h | Adults** | Not effective - no details | |
| | 5 mg $l^{-1}$ for 24 h | Cysts | Not effective - no details | |
| *In vivo* - bath | 28 mg / feed mixed with lactate (Emetryl ®) for 10 d | *O. mykiss* | Effective - no visible signs of infection by day 7 p.i. | Rapp (1995) |
| In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Not effective - 85% of treated fish have high numbers of trophonts | Tojo-Rodriguez & |

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|---|---|---|---|---|---|
| | | | | | Santamarina-Fernandez (2001) |
| **Diminazine aceturate** | | | | | |
| | *In vitro* | 100 mg $l^{-1}$ for 2 h | Tomonts | Effective - 75% mortality after 2 h | Tojo-Rodriguez *et al.* (1994) |
| | | 200 mg $l^{-1}$ for 2 h | | Effective - 100% mortality after 45 min | |
| | *In vivo* - bath | 100 mg $l^{-1}$ for 3 h | *O. mykiss* | Not effective - all tomonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| | In-feed | 1000 mg $kg^{-1}$ for 8 d | | Not effective - all tomonts developed normally | |
| **Elancoban** | | | | | |
| | See entry for monensin sodium | | | | |
| **Emetryl ®** | | | | | |
| | See entry for dimetrazole | | | | |
| **Enheptin (2-amino-5-nitrothiazole) diluted in ethyl alcohol and acetone** | | | | | |
| | *In vitro* | 100 mg $l^{-1}$ for 2 min | Trophozoites* | Effective - 100% mortality after 2 h post exposure | Post & Vesley (1983) |
| **Formaldehyde** | | | | | |
| | *In vitro* | 10 µl $l^{-1}$ for 10 h | Trophonts* | Not effective - 7% mortality | Lahnsteiner & Weismann (2007) |
| | | 45 µl $l^{-1}$ for 10 h | | Effective - 7% mortality, no viable theronts produced | |
| | | 55 µl $l^{-1}$ for 10 h | | Effective - 100% mortality | |
| | | 25 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 24 h | Wahli *et al.* (1993) |
| | | 32 mg $l^{-1}$ for < 2 h 30 min | Tomonts | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | | 64 mg $l^{-1}$ for < 1 h | | Effective - 100% mortality | |
| | | 100 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 1 h | Wahli *et al.* (1993) |
| | | 128 mg $l^{-1}$ for < 15 min | Tomonts | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | | 25 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | Wahli *et al.* (1993) |
| | | 100 mg $l^{-1}$ for 24 h | | Effective - 100% mortality after 24 h | |
| | | 8 mg $l^{-1}$ for 2 h 30 min (21-22 ºC) | Theronts | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | | 8 mg $l^{-1}$ for 5 h (11-12ºC) | | Effective - 100% mortality | |
| | | 10 mg $l^{-1}$ for 1h | | Not effective - ~5% mortality | Shinn *et al.* (2005) |
| | | 16 mg $l^{-1}$ for ~2 h (11-12ºC) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | | 16 mg $l^{-1}$ for ~1 h 5 min (21-22ºC) | | Effective - 100% mortality | |
| | | 25 mg $l^{-1}$ for 3 h | | Effective -100% mortality after 30 min | Wahli *et al.* (1993) |

| | | | | |
|---|---|---|---|---|
| | 32 mg $l^{-1}$ for ~1 h 5 min (11-12ºC) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 32 mg $l^{-1}$ for ~<50 min (21-22ºC) | | Effective - 100% mortality | |
| | 50 mg $l^{-1}$ for 1h | | Not effective - ~10% mortality | Shinn *et al.* (2005) |
| | 64 mg $l^{-1}$ for <50 min (11-12ºC) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 64 mg $l^{-1}$ for 15 min (21-22 ºC) | | Effective - 100% mortality | |
| | 100 mg $l^{-1}$ for 1h | | Not effective - ~3% mortality | Shinn *et al.* (2005) |
| | 100 mg $l^{-1}$ for 3 h | | Effective - 100% mortality after 30 min | Wahli *et al.* (1993) |
| | 200 mg $l^{-1}$ for 1h | | Not effective - ~40% mortality | Shinn *et al.* (2005) |
| *In vivo* - bath | 10 µl $l^{-1}$ for 6 h for 5 d<br>(18 °C, 24 h intervals) | *O. mykiss* | Not effective - 0% survival of treated fish on d1 & 3 | Lahnsteiner & Weismann (2007) |
| | 40 µl $l^{-1}$ for 4 h for 5 d<br>(18 °C, 24 h intervals) | | Not effective - 30% survival of treated fish on d 1;<br>0% survival on d 3 | |
| | 80 µl $l^{-1}$ for 1 h for 5 d<br>(18 °C, 24 h intervals) | | Effective - 50% survival on treated fish on d1 & 3<br>No trophonts seen on fish on d 1 & 3 | |
| | 80 µl $l^{-1}$ for 2 h for 5 d | *C. carpio* | Effective - all treated fish survived; low number of trophonts on d1 & 3 | Lahnsteiner & Weismann (2007) |
| | 110 µl $l^{-1}$ for 2 h for 5 d | | Effective - all treated fish survived; no trophonts on d1 & 3 | |
| | 110 µl $l^{-1}$ for 1 h for 5 d<br>(18 °C, 24 h intervals) | *O. mykiss* | Effective - 90% survival of treated fish on d1 & 3<br>No trophonts on d1 & 3 | |
| | 110 µl $l^{-1}$ for 1 h for 5 d<br>(10 °C, 48 h intervals) | | Effective - 100% survival of treated fish on d1& 3;<br>no trophonts on d 1 & 3 | Lahnsteiner & Weismann (2007) |
| | 110 µl $l^{-1}$ for 1 h for 5 d<br>(18 °C, 24 h intervals) | | Effective - 100% survival of treated fish on d1& 3;<br>no trophonts on d 1 & 3 | |
| | 110 µl $l^{-1}$ for 1 h for 5 d<br>(18 °C, 48 h intervals) | | Not effective - 50% and 10% survival of treated fish on d1 & 3;<br>heavy infections on d 1 & 3 | |
| | 110 µl $l^{-1}$ for 1 h for 5 d<br>(25 °C, 24 h intervals) | | Not effective - 0% survival of treated fish on d1 & 3;<br>heavy infections on d 1 & 3 | |
| | 110 µl $l^{-1}$ for 12 h (25 °C) | *O. mykiss* | Not effective - 100% and 10% survival of treated fish on d1 & 3;<br>Moderate and heavy infections on d1 & 3 | |
| | 110 µl $l^{-1}$ for 3× 1 h for 5 d<br>(25°C, 24 h intervals) | | Not effective - 70% and 0% survival of treated fish on d1 & 3;<br>Moderate infection on d1 | |
| | 110 µl $l^{-1}$ for 5× 1 h for 5 d<br>(25°C, 24 h intervals) | | Not effective - 30% and 0% survival of treated fish on d1& 3;<br>Moderate infection on d1 | |
| | 110 µl $l^{-1}$ for 7× 1 h for 5 d | | Not effective - 0% and 0% survival of treated fish on d1 & 3; | |

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| | (25°C, 24 h intervals) | | heavy infection on d1 | |
| | 110 µl l$^{-1}$ for 12 h (18 °C) | | Not effective - 100% and 10% survival of treated fish on d1 & 3 medium and very heavy infections on d1 & 3 | |
| | 110 µl l$^{-1}$ for 3× 1 h for 5 d (18°C, 24 h intervals) | | Not effective - 100% and 40% survival of treated fish on d1 & 3; low and heavy infections on d1 & 3 | |
| | 110 µl l$^{-1}$ for 5× 1 h for 5 d (18°C, 24 h intervals) | | Effective - 100% survival of treated fish on d1 & 3; no infection on d1 & 3 | |
| | 110 µl l$^{-1}$ for 7× 1 h for 5 d (18°C, 24 h intervals) | | Effective - 100% survival of treated fish on d1 & 3; no infection on d1 & 3 | |
| | 0.1, 0.15 & 0.2 ml l$^{-1}$ for 1 h | *O. mykiss* *S. fontinalis* *S. trutta* | Effective - reduction in the number of trophonts on treated fish | Balta *et al.* (2008) |
| | 10 mg l$^{-1}$for 17 d | *B. bidyanus* | Not effective - all treated infected fish died | Rowland *et al.* (2009) |
| | 20 mg l$^{-1}$for 17 d | | Not effective - all treated fish still infected | |
| | 25 mg l$^{-1}$- 2× for 1 h 1$^{st}$: day 9 p.i, 2$^{nd}$: day 12 p.i | *O. mykiss* | Not effective - not specified but fish survival compromised | Wahli *et al.* (1993) |
| | 25 mg l$^{-1}$for 4 h for 4 d week $^{-1}$ | *I. punctatus* | Not effective - 40-70% of treated infected fish died | Bodensteiner *et al.* (2000) |
| (static tanks) | 25 mgl$^{-1}$ alternate days for 20 d | *I. punctatus* | Not effective - all treated infected fish died | Tieman & Goodwin (2001) |
| | 25 mgl$^{-1}$ daily for 20 d | | Partially effective - 20-60% of treated infected fish died | |
| | 25 mg l$^{-1}$ 3-4 times a week for 6 weeks | *S. salar* | Not effective - parasite load increased on treated fish | Rintamäki- Kinnunen *et al.* (2005b) |
| | 25 mg l$^{-1}$ 3-4 times a week for 6 weeks | *S.trutta* | Not effective - parasite load increased on treated fish | Rintamäki- Kinnunen *et al.* (2005b) |
| | 30 mg l$^{-1}$for 17 d | *B. bidyanus* | Effective - no trophonts found on treated fish | Rowland *et al.* (2009) |
| | 50 mgl$^{-1}$ alternate day for 20 d | *I. punctatus* | Not effective - all treated infected fish died | Tieman & Goodwin (2001) |
| | 50 mgl$^{-1}$ daily for 20 d | | Partially effective - 20-60% of treated infected fish died | |
| | 50 mg l$^{-1}$ 3-4 times a week for 6 weeks | *S. salar* | Not effective - parasite load increased on treated fish | Rintamäki- Kinnunen *et al.* (2005b) |
| | 50 mg l$^{-1}$ 3-4 times a week | *S.trutta* | Not effective - parasite load increased on treated fish | |
| | 100 mg l$^{-1}$- 2× for 1 h 1$^{st}$: day 9 p.i, 2$^{nd}$: day 12 p.i | *O. mykiss* | Not effective - not specified but fish survival compromised | Wahli *et al.* (1993) |
| (static tanks) | 100 mgl$^{-1}$ daily for 20 d | *I. punctatus* | Not effective - all treated infected fish died | Tieman & Goodwin (2001) |

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| (flow through) | 100 mg$l^{-1}$ alternate day for 20 d | | Not effective - all treated infected fish died † | |
| | 60- 250 mg $l^{-1}$ for 20 min- 1h | Not specified | Effective - efficacy though not specified | Rahkonen & Koski (2002) |
| **Formaldehyde + Desirox (13% peracetic acid, 20% acetic acid and 20% hydrogen peroxide)** | | | | |
| *In vivo* - bath | 25 + 10 mg $l^{-1}$ 3-4 times a week for 4 weeks | *S. salar* | Effective - parasite load reduced on treated fish | Rintamäki- Kinnunen *et al.* (2005b) |
| | 50 + 10 mg $l^{-1}$ 3-4 times a week for 4 weeks | | Effective - parasite load reduced on treated fish | |
| | 100 + 10 mg $l^{-1}$ 3 times a week for 4 weeks | *S. salar* | Trial inconclusive - details missing | Rintamäki- Kinnunen *et al.* (2005a) |
| | 123 + 8 mg $l^{-1}$ 4 times a week for 5 weeks | | Not effective - parasite load increased on treated fish | Rintamäki- Kinnunen *et al.* (2005a) |
| **Formaldehyde + hydrogen peroxide** | | | | |
| *In vivo* - bath | 100 + 100 mg $l^{-1}$ 3 times a week for 3 weeks | | Trial inconclusive - details missing | Rintamäki- Kinnunen *et al.* (2005a) |
| **Formaldehyde + malachite green** | | | | |
| *In vitro* | 25 + 0.1 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 24 h | Wahli *et al.* (1993) |
| | 100 + 0.4 mg $l^{-1}$ for 24 h | | Effective - 100% mortality after 24 h | |
| | 25 + 0.1 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | |
| | 100 + 0.4 mg $l^{-1}$ for 24 h | | Effective - 100% mortality after 24 h | |
| | 25 + 0.1 mg $l^{-1}$ for 3 h | Theronts | Effective - 100% mortality after 30 min | |
| | 100 + 0.4 mg $l^{-1}$ for 3 h | | Effective - 100% mortality after 5 min | |
| *In vivo* - bath | 25 + 0.05 mg $l^{-1}$ | *Cichla ocellaris* | Effective - efficacy not specified | Guest (1983) |
| | 25 + 0.1 mg $l^{-1}$– 2× for 1 h $1^{st}$: day 9 p.i, $2^{nd}$: day 12 p.i | *O. mykiss* | Partially effective against the parasites - efficacy not specified | Wahli *et al.* (1993) |
| | 100 + 0.4 mg $l^{-1}$– 2× for 1 h $1^{st}$: day 9 p.i, $2^{nd}$: day 12 p.i | | Partially effective against the parasites - efficacy not specified | |
| | 225 + 0.83 mg $l^{-1}$ for 3 times a week for 3 weeks | *S. salar* | Partially effective - small increases in parasite burdens observed over infection period | Rintamäki- Kinnunen *et al.* (2005a) |
| | 225 + 0.83 mg $l^{-1}$ for 3 times a week for 2 weeks | | Trial inconclusive - low parasite numbers across all groups | |
| **Furacin ([[(5-nitrofuran-2-yl) methylideneamino] urea)** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 2 min | Trophozoites* | Effective - 30% mortality after 12 h, 80% after 24 h post exposure | Post & Vesley (1983) |
| **Furazolidone (mixed with ethanol)** | | | | |

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|---|---|---|---|---|
| *In vitro* | 100 mg $l^{-1}$ for 24 h | Adults** | Not effective - no details | Wahli *et al.* (1993) |
| | 100 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | |
| | 100 mg $l^{-1}$ for 3 h | Theronts | Not effective - no details | |
| *In vivo* - bath | 25 mg $l^{-1}$– 2× for 1 h<br>$1^{st}$: day 9 p.i, $2^{nd}$: day 12 p.i | *O. mykiss* | Not effective - no details | Wahli *et al.* (1993) |
| In-feed | 50 mg $kg^{-1}$ fish for 10 d | | Not effective - no details | |
| **Furoxone (3-[(5-nitrofuran-2-yl) methylideneamino]-1,3-oxazolidin-2-one) diluted in ethyl alcohol and acetone** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 2 min | Trophozoites* | Not effective - 0% mortality after 24 h post exposure | Post & Vesley (1983) |
| **β- Glucan (from *Saccharomyces cerevisiae*)** | | | | |
| *In vivo* | 0.2% for 14 d prior inf. | *O. mykiss* | Not effective - 14% trophont reduction | Lauridsen & Buchmann (2010) |
| | 0.2% for 35 d prior inf. | | Not effective - 18% trophont reduction | |
| **HbβP-1 (peptide from the β-haemoglobin peptide family)** | | | | |
| *In vitro* | 12.5 μg $ml^{-1}$ for 5 min 21s | Trophonts*(323 μm) | Effective -100% mortality | Ullal *et al.* (2008) |
| | 12.5 μg $ml^{-1}$ for 1 min 35 s | (222 μm) | Effective - 100% mortality | |
| | 12.5 μg $ml^{-1}$ for 2 min 50 s | (500 μm) | Effective - 100% mortality | |
| | 25 μg $ml^{-1}$ for 6 min 36 s | (323 μm) | Effective - 100% mortality | |
| | 25 μg $ml^{-1}$ for 1 min 54 s | (231 μm) | Effective - 100% mortality | |
| | 25 μg $ml^{-1}$ for 4 min 14 s | (519 μm) | Effective - 100% mortality | |
| | 50 μg $ml^{-1}$ for 4 min 3 s | (323 μm) | Effective - 100% mortality | |
| | 100 μg $ml^{-1}$ for 3 min 18 s | (323 μm) | Effective - 100% mortality | |
| | 200 μg $ml^{-1}$ for 3 min 15 s | (323 μm) | Effective - 100% mortality | |
| **Huminic acid (10% solution) commercially sold as Aquahumin** | | | | |
| *In vitro* | 50 μl $l^{-1}$ for 10 h | Trophonts* | Not effective - 10% mortality | Lahnsteiner & Weismann (2007) |
| | 100 μl $l^{-1}$ for 10 h | | Effective - 77% mortality | |
| | 150 μl $l^{-1}$ for 10 h | | Effective - 90% mortality, no theronts produced by surviving trophonts | |
| | 200 μl $l^{-1}$ for 10 h | | Effective - 100% mortality | |
| *In vivo* - bath<br>(daily for 5 d) | 100 μl $l^{-1}$ for 2 h for 5 d | *O. mykiss* | Effective - 90% survival of treated fish; no infections d1 & 3 | Lahnsteiner & Weismann (2007) |
| | 100 μl $l^{-1}$ for 4 h for 5 d | *C. carpio* | Not effective - 100% and 60% survival of treated fish on d1& 3; medium and heavy infections on d1 & 3 | |
| | 150 μl $l^{-1}$ for 2 h for 5 d | *O. mykiss* | Effective - 100% survival of treated fish; no infections d1 & 3 | |
| | 150 μl $l^{-1}$ for 4 h for 5 d | *C. carpio* | Not effective - 100% and 70% survival of treated fish on d1& 3; medium and heavy infections on d 1 & 3 on treated fish | |
| | 150 μl $l^{-1}$ for 2 h for 5 d<br>(10°C, 48 h interval) | *O. mykiss* | Effective - 100% survival of treated fish; no infections d1 & 3 | |

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| | 150 µl $l^{-1}$ for 2 h for 5 d (18°C, 24 h interval) | | Effective - 70% survival of treated fish; no infections d1 & 3 | |
| | 150 µl $l^{-1}$ for 2 h for 5 d (18°C, 48 h interval) | | Not effective - 0% survival on treated fish | |
| | 150 µl $l^{-1}$ for 2 h for 5 d (25°C, 24 h interval) | | Not effective - 0% survival of treated fish; heavy infections on d1 &3 on treated fish | |
| | 200 µl $l^{-1}$ for 4 h for 5 d | *C. carpio* | Not effective - 100% and 60% survival of treated fish on d1 & 3; medium and heavy infections on d 1 & 3 on treated fish | |
| **Hydrogen peroxide ($H_2O_2$)** | | | | |
| *In vitro* | < 50 mg $l^{-1}$ for 10 h | Trophonts* | Not effective - trophonts developed normally | Lahnsteiner & Weismann (2007) |
| | 10 mg $l^{-1}$ for 1 h | Theronts | Not effective - ~10% mortality | Shinn *et al.* (2005) |
| | 50 mg $l^{-1}$ for 1 h | | Not effective - ~5% mortality | |
| | 100 mg $l^{-1}$ for 1 h | | Not effective - ~5% mortality | |
| | 200 mg $l^{-1}$ for 1 h | | Not effective - ~15% mortality; 20% mortality | Shinn *et al.* (unpublished) |
| *In vivo* - bath | 25 mg $l^{-1}$daily for 20 d (flow through) | *I. punctatus* | Not effective - 80% of treated infected fish died | Tieman & Goodwin (2001) |
| | 25 mg $l^{-1}$daily for 20 d (static tanks) | | Not effective - all treated infected fish died | |
| **Hydrogen peroxide + acetic acid based formulation commercialized as Perotan** | | | | |
| *In vitro* | 50 µl $l^{-1}$ for 10 h | Trophonts* | Not effective - 7% mortality | Lahnsteiner & Weismann (2007) |
| | 100 µl $l^{-1}$ for 10 h | | Effective - 100% mortality | |
| **Incimaxx Aquatic** | | | | |
| See entry for peracetic acid + acetic acid + hydrogen peroxide + peroctanoic acid based formulation | | | | |
| **Iodine** | | | | |
| *In vivo* - bath (static tanks) | 0.25 mg $l^{-1}$ daily for 11 d | *I. punctatus* | Not effective - all treated infected fish died | Tieman & Goodwin (2001) |
| | 0.50 mg $l^{-1}$ daily for 11 d | | Not effective - all treated infected fish died | |
| | 1.00 mg $l^{-1}$ daily for 11 d | | Not effective - all treated infected fish died | |
| | 1.00 mg $l^{-1}$ for 11 d (alternate days) | | Not effective - all treated infected fish died | |
| **Ivermectin commercialised as Ivomec** | | | | |
| *In vitro* | <50 mg $l^{-1}$ for 10 h | Trophonts* | Not effective - trophonts developed normally | Lahnsteiner & Weismann (2007) |
| **Ivomec** | | | | |
| See entry for Ivermectin | | | | |
| **Ketoconazole** | | | | |
| *In vitro* | 200 mg $l^{-1}$ for 2 h | | Effective - 0% mortality after 2 h but tomonts do not develop | Tojo-Rodriguez *et al.* (1994) |

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|---|---|---|---|---|
| *In vivo* - bath | 200 mg $l^{-1}$ for 3 h | *O. mykiss* | Not effective - all trophonts develop normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg $kg^{-1}$ for 8 d | | Not effective - toxic to the fish; all trophonts develop normally | |
| | 40 g $kg^{-1}$ for 10 d | *O. mykiss* | Effective - 50% of medicated fish with low numbers of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Levamisole** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ for 10 d | *O. mykiss* | Effective - 40% of medicated fish with low numbers of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| ***Macleaya cordata* (active compound sanguinarine)** | | | | |
| *In vitro* | 60 mg $l^{-1}$ for 4 h | Trophonts* | Not effective - 32.5% mortality | Yao *et al.* (2010) |
| (Butanol extract) | 80 mg $l^{-1}$ for 4 h | | Not effective - 47.5% mortality | |
| | 100 mg $l^{-1}$ for 4 h | | Effective - 65% mortality | |
| | 120 mg $l^{-1}$ for 4 h | | Effective - 70% mortality | |
| (Chloroform extract) | | | | |
| | 30 mg $l^{-1}$ for 4 h | | Effective - 67.5% mortality | |
| | 50 mg $l^{-1}$ for 4 h | | Effective - 82.5% mortality | |
| | 70 mg $l^{-1}$ for 4 h | | Effective - 100% mortality | |
| | 90 mg $l^{-1}$ for 4 h | | Effective - 100% mortality | |
| (Ethyl acetate extract) | | | | |
| | 60 mg $l^{-1}$ for 4 h | | Not effective - 37.5% mortality | |
| | 80 mg $l^{-1}$ for 4 h | | Effective - 52.5% mortality | |
| | 100 mg $l^{-1}$ for 4 h | | Effective - 80% mortality | |
| | 120 mg $l^{-1}$ for 4 h | | Effective - 80% mortality | |
| (Petroleum ether extract) | | | | |
| | 60 mg $l^{-1}$ for 4 h | | Not effective - 27.5% mortality | |
| | 80 mg $l^{-1}$ for 4 h | | Not effective - 30% mortality | |
| | 100 mg $l^{-1}$ for 4 h | | Not effective - 30% mortality | |
| | 120 mg $l^{-1}$ for 4 h | | Not effective - 40% mortality | |
| (Water extract) | | | | |
| | 60 mg $l^{-1}$ for 4 h | | Not effective - 7.5% mortality | |
| | 80 mg $l^{-1}$ for 4 h | | Not effective - 17.5% mortality | |
| | 100 mg $l^{-1}$ for 4 h | | Not effective - 25% mortality | |
| | 120 mg $l^{-1}$ for 4 h | | Not effective - 27.5% mortality | |
| (Fractions from the chloroform extract) | | | | |
| (Fraction A) | 10 mg $l^{-1}$ for 4 h | Trophonts* | Not effective - 12.5% mortality | Yao *et al.* (2010) |
| | 20 mg $l^{-1}$ for 4 h | | Not effective - 15% mortality | |
| | 30 mg $l^{-1}$ for 4 h | | Not effective - 22.5% mortality | |
| (Fraction B) | 10 mg $l^{-1}$ for 4 h | | Not effective - 12.5% mortality | |
| | 20 mg $l^{-1}$ for 4 h | | Not effective - 20% mortality | |
| | 30 mg $l^{-1}$ for 4 h | | Not effective - 25% mortality | |
| (Fraction C) | 10 mg $l^{-1}$ for 4 h | | Not effective - 42.5% mortality | |

| Treatment | Method | Dose | Stage / host | Outcome | Reference |
|---|---|---|---|---|---|
| | | 20 mg $l^{-1}$ for 4 h | | Not effective - 67.5% mortality | |
| | | 30 mg $l^{-1}$ for 4 h | | Not effective - 87.5% mortality | |
| | (Fraction D) | 5 mg $l^{-1}$ for 4 h | | Effective - 90% mortality | |
| | | 9 mg $l^{-1}$ for 4 h | | Effective -100% mortality | |
| | | 11 mg $l^{-1}$ for 4 h | | Effective -100% mortality | |
| | (Fraction E) | 10 mg $l^{-1}$ for 4 h | | Effective - 55% mortality | |
| | | 20 mg $l^{-1}$ for 4 h | | Effective -77.5% mortality | |
| | | 30 mg $l^{-1}$ for 4 h | | Effective - 92.5% mortality | |
| | (Fraction F) | 10 mg $l^{-1}$ for 4 h | | Not effective - 7.5% mortality | |
| | | 20 mg $l^{-1}$ for 4 h | | Not effective - 20% mortality | |
| | | 30 mg $l^{-1}$ for 4 h | | Not effective - 25% mortality | |
| | (Fraction G) | 10 mg $l^{-1}$ for 4 h | | Not effective - 5% mortality | |
| | | 20 mg $l^{-1}$ for 4 h | | Not effective - 7.5% mortality | |
| | | 30 mg $l^{-1}$ for 4 h | | Not effective - 15% mortality | |
| | (Compounds from Fraction D) | | | | |
| | (Compound I) | 5 mg $l^{-1}$ for 4 h | Trophonts* | Not effective - 17.5% mortality | |
| | | 7 mg $l^{-1}$ for 4 h | | Not effective -22.5% mortality | |
| | | 9 mg $l^{-1}$ for 4 h | | Not effective - 22.5% mortality | |
| | (Compound II) | 5 mg $l^{-1}$ for 4 h | | Not effective - 22.5% mortality | |
| | | 7 mg $l^{-1}$ for 4 h | | Not effective - 30% mortality | |
| | | 9 mg $l^{-1}$ for 4 h | | Not effective - 37.5% mortality | |
| | (Compound III) | 0.5 mg $l^{-1}$ for 4 h | | Effective - 87.5% mortality | |
| | | 0.9 mg $l^{-1}$ for 4 h | | Effective - 100% mortality | |
| | | 1.3 mg $l^{-1}$ for 4 h | | Effective - 100% mortality | |
| | (Compound IV) | 5 mg $l^{-1}$ for 4 h | | Not effective - 5% mortality | |
| | | 7 mg $l^{-1}$ for 4 h | | Not effective -7.5% mortality | |
| | | 9 mg $l^{-1}$ for 4 h | | Not effective - 22.5% mortality | |
| | *In vivo* - bath | | | | |
| | (Compound I) | 0.2 mg $l^{-1}$ for 48 h | *Ctenopharyngodon idella* | Not effective - 16.1% trophont reduction on treated fish | |
| | | 0.3 mg $l^{-1}$ for 48 h | | Not effective - 17.3% trophont reduction on treated fish | |
| | | 0.4 mg $l^{-1}$ for 48 h | | Not effective - 32.9% trophont reduction on treated fish | |
| | | 0.5 mg $l^{-1}$ for 48 h | | Not effective - 53.9% trophont reduction on treated fish | |
| | | 0.6 mg $l^{-1}$ for 48 h | | Effective - 75.3% trophont reduction on treated fish | |
| | | 0.7 mg $l^{-1}$ for 48 h | | Effective - 82.3% trophont reduction on treated fish | |
| | | 0.8 mg $l^{-1}$ for 48 h | | Effective - 89.4% trophont reduction on treated fish | |
| | | 0.9 mg $l^{-1}$ for 48 h | | Effective - 96.8% trophont reduction on treated fish | |
| **Malachite green** | | | | | |
| | *In vitro* | 1 mg $l^{-1}$ for 24 h | Adults** | Effective - 100% mortality after 1 h | Wahli *et al.* (1993) |
| | | 0.15 mg $l^{-1}$ for 24 h | Tomocysts | Effective - 100% mortality | Buchman *et al.*(2003) |

| | | | | | |
|---|---|---|---|---|---|
| | | 1 mg $l^{-1}$ for 24 h | Cysts | Effective - 100% mortality after 24 h | Wahli *et al.* (1993) |
| | | 0.004 mg $l^{-1}$ for 1.5, 3 & 15 h | Theronts | Not effective - <50% mortality | Buchman *et al*. (2003) |
| | | 0.02 mg $l^{-1}$ for 1.5, 3 & 15 h | | Not effective - <50% mortality | |
| | | 0.10 mg $l^{-1}$ for 1.5 & 3 h | | Not effective - <50% mortality | |
| | | 0.10 mg $l^{-1}$ for 15 h | | Effective - >50% mortality | |
| | | 1 mg $l^{-1}$ for 3 h | | Effective -100% mortality after 1 h | Wahli *et al.* (1993) |
| | | 50 mg $l^{-1}$ for 15 h | | Effective - >50% mortality | Buchman *et al*. (2003) |
| | | 50 mg $l^{-1}$ for 1.5, 3 & 15 h | | Effective - >50% mortality | |
| | *In vivo* - bath | 25 mg $l^{-1}$- 2× for 1h<br>1st: day 9 p.i, 2nd: day 12 p.i | *O. mykiss* | Effective - no details | Wahli *et al.* (1993) |
| | | 0.1 mg $l^{-1}$ daily for 20 d (static tanks) | *I. punctatus* | Effective - no trophonts on treated fish but toxic to fish<br>Experiment stopped on d 9 | Tieman & Goodwin (2001) |
| | | 0.1 mg $l^{-1}$ daily for 20 d (flow through) | | Effective - no trophonts on treated fish; 0% fish mortality | Tieman & Goodwin (2001) |
| **Malachite green (coloured salt)** | | | | | |
| | *In vivo* - In-feed | 1.2 g $kg^{-1}$ feed for 10 d (fish species with stomach) | *Paracheirodon axelrodi*<br>*Hyphessobrycon herbertaxelrodi*<br>*Hyphessobrycon flammeus*<br>*Hasemania nana*<br>*Aequidens pulcher* | Effective - medicated fish free of trophonts by d 5-8 | Ruider *et al.* (1997) |
| | | 1.2 g $kg^{-1}$ feed for 10 d (fish species without stomach) | *Xiphophorous helleri*<br>*Barbus tetrazona tetrazona*<br>*Xiphophorus maculatus* | Effective - medicated fish free of trophonts by d 7-8 | Ruider *et al.* (1997) |
| **Malachite green (carbinolbase)** | | | | | |
| | *In vivo* - In-feed | 1.2 g $kg^{-1}$ feed for 10 d (fish species with stomach) | *P. axelrodi*<br>*H. herbertaxelrodi*<br>*H. flammeus*<br>*H. nana*<br>*A. pulcher* | Effective - medicated fish free of trophonts by d 6-8 | Ruider *et al.* (1997) |
| | | 1.2 g $kg^{-1}$ feed for 10 d (fish species without stomach) | *X. helleri*<br>*B. tetrazona tetrazona*<br>*X. maculatus* | Effective - medicated fish free of trophonts by d 6-7 | Ruider *et al.* (1997) |
| **Malachite green (leucoform)** | | | | | |
| | *In vivo* - In-feed | 1.2 g $kg^{-1}$ feed for 10 d (fish species with stomach) | *P. axelrodi*<br>*H. herbertaxelrodi* | Not effective - all medicated fish died across d 4-10 | Ruider *et al.* (1997) |

| | | | | |
|---|---|---|---|---|
| | 1.2 g kg$^{-1}$ feed for 10 d (fish species without stomach) | *H. flammeus* *H. nana* *A. pulcher* *X. helleri* *B. tetrazona tetrazona* *X. maculatus* | Not effective - all medicated fish died across d 4-10 | Ruider *et al.* (1997) |
| **Malachite green (p-dimethylaminobenzophenone)** | | | | |
| *In vivo* - In-feed | 1177 mg kg$^{-1}$ feed for 6 d | *H. flammeus* *B. tetrazona tetrazona* *X. helleri* | Not effective - no reduction in trophont numbers on treated fish | Ruider *et al.* (1997) |
| **Malachite green (N,N-dimethylaniline)** | | | | |
| *In vivo* - In-feed | 316 mg kg$^{-1}$ feed for 4 d | *H. nana* *X. helleri* *X. maculatus* | Not effective - no reduction in trophont numbers on treated fish | Ruider *et al.* (1997) |
| **Methylene blue** | | | | |
| *In vitro* | 100 mgl$^{-1}$ for 2 min | | Effective - 100% mortality after 12 h | Post & Vesely (1983) |
| *In vivo* - bath | 2 mg l$^{-1}$daily for 20 d (static tanks) | *I. punctatus* | Effective - no trophonts on treated fish but 20% fish mortality† | Tieman & Goodwin (2001) |
| | Alternate days for 20 d: 2 mg l$^{-1}$& 100 mg l$^{-1}$ formaldehyde (static tanks) | | Not effective - no trophonts on treated fish but 40-70% fish mortality† | Tieman & Goodwin (2001) |
| **Metronidazole** | | | | |
| *In vitro* | 25 mg l$^{-1}$ for 3 h | Theronts | Not effective - no details | Wahli *et al.* (1993) |
| | 25 mg l$^{-1}$ for 24 h | Adults** | Not effective - no details | |
| | 25 mg l$^{-1}$ for 24 h | Cysts | Not effective - no details | |
| *In vivo* - In-feed | 7.5 mg kg$^{-1}$ b.w for 7 d | Salmonids not specified | Effective - no details | Rahkonen & Koski (2002) |
| | 24 mg kg$^{-1}$ b.w for 10 d | *C. auratus* | Effective - no trophonts on treated fish | Tokşen & Nemli (2010) |
| | 36 mg kg$^{-1}$ b.w for 10 d | | Effective - no trophonts on treated fish | |
| | 40 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Effective - 35% of medicated fish were free of infection | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **N-methylglucamine** | | | | |
| *In vitro* | 200 mg l$^{-1}$ for 2 h | Tomonts | Not effective - 12.5% mortality after 2 h; surviving tomonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 200 mg l$^{-1}$ for 6 d | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg kg$^{-1}$ for 8 d | | Not effective - all trophonts developed normally | |
| | 40 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Not effective – all medicated fish had high numbers of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **MinnFinn™** | | | | |

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| | See entry for peracetic Acid (PAA) + acetic acid + hydrogen peroxide based formulation (4.5% PAA + 9% AA + 22% $H_2O_2$) | | | |
| **Monensin sodium based formulation sold as commercial product as Elancoban** | | | | |
| *In vitro* | 20 mg $l^{-1}$ for 1 h | Trophonts* | Effective - 100% mortality after 14.5 h of exposure | Shinn *et al.* (unpublished) |
| | 50 mg $l^{-1}$ for 1 h | | Effective - 100% mortality after 14.5 h of exposure | |
| | 100 mg $l^{-1}$ for 1 h | Trophonts* | Effective - 100% mortality | Shinn *et al.* (2001) |
| | 20 mg $l^{-1}$ for 1 h | Theronts | Not effective - 27.4% mortality | Shinn *et al.* (unpublished) |
| | 50 mg $l^{-1}$ for 1 h | | Not effective - 36.8% mortality | |
| | 100 mg $l^{-1}$ for 1 h | | Partially effective - 48.2% mortality | Shinn *et al.* (2001) |
| *In vivo* - In-feed | 2 ppm for 10 d | | Not effective - no significant reduction in trophont numbers | Shinn *et al.* (unpublished) |
| | 5 ppm for 10 d | | Not effective - no significant reduction in trophont numbers | |
| | 10 ppm for 10 d | | Not effective - no significant reduction in trophont numbers | |
| | 100 ppm for 10 d prior to inf. | *O. mykiss* | Not effective - no reduction in trophont numbers | Shinn *et al.* (2003a) |
| ***Mucuna pruriens* (velvet bean)** | | | | |
| *In vitro* | 100 mg $l^{-1}$ for 3 h | Trophonts* | Not effective - 0% mortality | Ekamen *et al.* (2004) |
| | 100mg $l^{-1}$ for 6 h | | Effective - 65% mortality | |
| | 150 mg $l^{-1}$ for 3 h | | Not effective - 25% mortality | |
| | 150 mg $l^{-1}$ for 6 h | | Effective - 100% mortality | |
| | 200 mg $l^{-1}$ for 3 h | | Not effective - 35% mortality | |
| | 200 mg $l^{-1}$ for 6 h | | Effective - 100% mortality | |
| *In vivo* - bath | 100 mg $l^{-1}$ for 72 h | *C. a. auratus* | Effective - 59% reduction in trophonts on the skin/ 60% on the gills | Ekamen *et al.* (2004) |
| | 150 mg $l^{-1}$ for 72 h | | Effective - 79% reduction in trophonts on the skin/ 83% on the gills | |
| | 200 mg $l^{-1}$ for 72 h | | Effective - 92% reduction in trophonts on the skin/ 91% on the gills | |
| **Na-desoxycholate** | | | | |
| *In vitro* | 25 mg $l^{-1}$ for 3 h | Theronts | Not effective - no details | Wahli *et al.* (1993) |
| | 25 mg $l^{-1}$ for 24 h | Adults** | Not effective - no details | |
| | 25 mg $l^{-1}$ for 24 h | Cysts | Not effective - no details | |
| **Netobimin** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Effective - 75% of medicated fish with moderate number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Nicarbazin** | | | | |
| *In vivo* - In-feed | 100 ppm for 10 d prior inf. | *O. mykiss* | Not effective - no reduction in trophont numbers on treated fish | Shinn *et al.* (2003a) |
| **Niridazole** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Effective - 90% of medicated fish with low numbers of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Nitroscanate** | | | | |

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|---|---|---|---|---|
| *In vivo* - In-feed | 40 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Effective - 50% of medicated fish had no trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **N-methylglucamine** | | | | |
| *In vivo* - In-feed | 40 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Not effective - all medicated fish had high numbers of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Ornidazole** | | | | |
| *In vivo* - In-feed | 24 mg kg$^{-1}$ b.w for 10 d | *C. auratus* | Effective - no trophonts on the treated groups | Tokşen & Nemli (2010) |
| | 36 mg kg$^{-1}$ b.w for 10 d | | Effective - no trophonts on the treated groups | |
| **Oxytetracycline** | | | | |
| *In vitro* | 100 mg l$^{-1}$ for 3 h | Theronts | Not effective - medium survival rate after 3 h | Wahli *et al.* (1993) |
| | 100 mg l$^{-1}$ for 24 h | Adults** | Not effective - high survival rate after 24 h | |
| | 100 mg l$^{-1}$ for 24 h | cysts | Not effective - high survival rate after 24 h | |
| **Paromomycin** | | | | |
| *In vitro* | 200 mg l$^{-1}$ for 2 h | | Effective - 75% mortality after 2 h, surviving tomonts reproduce normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 200 mg l$^{-1}$ for 3 h | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg kg$^{-1}$ for 8 d | | Not effective - all trophonts developed normally | |
| **Peracetic acid (PAA) + acetic acid + hydrogen peroxide based formulation (40%PAA + 25% AA+ 15% $H_2O_2$) sold as commercial product as Wofasteril®** | | | | |
| *In vitro* | 0.4 mg l$^{-1}$ for 48 h | Tomonts | Not effective - 21% mortality | Meinelt *et al.* (2009) |
| | 0.5 mg l$^{-1}$ for 48 h | | Not effective - 20% mortality | |
| | 0.6 mg l$^{-1}$ for 48 h | | Not effective - 39% mortality | |
| | 0.7 mg l$^{-1}$ for 48 h | | Not effective - ~40% mortality | |
| | 0.8 mg l$^{-1}$ for 48 h | | Effective - ~75% mortality | |
| | 0.9 mg l$^{-1}$ for 48 h | | Effective - 82% mortality | |
| | 0.5 mg l$^{-1}$ for 12 h | Cysts (<2.5 h) | Not effective - 42% mortality | Meinelt *et al.* (2009) |
| | 1 mg l$^{-1}$ for 12 h | | Effective - 75% mortality | |
| | 2 mg l$^{-1}$ for 12 h | | Effective - 98% mortality | |
| | 3 mg l$^{-1}$ for 12 h | | Effective - >99% mortality | |
| | 0.5 mg l$^{-1}$ for 2 h | Cysts (24 h+) | Not effective - all trophonts developed normally | Meinelt *et al.* (2009) |
| | 0.5 mg l$^{-1}$ for 4 h | | Not effective - all trophonts developed normally | |
| | 1 mg l$^{-1}$ for 2 h | | Not effective - all trophonts developed normally | |
| | 1 mg l$^{-1}$ for 4 h | | Not effective - all trophonts developed normally | |
| | 2 mg l$^{-1}$ for 2 h | | Not effective - all trophonts developed normally | |
| | 2 mg l-1 for 4 h | | Not effective - all trophonts developed normally | |
| | 2.5 mg l$^{-1}$ for 2 h | | Not effective - all trophonts developed normally | |
| | 3 mg l$^{-1}$ for 2 h | | Not effective - all trophonts developed normally | |
| | 2.5 mg l$^{-1}$ for 4 h | | Not effective - all trophonts developed normally | |

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|---|---|---|---|
| 0.04 mg $l^{-1}$ for 1-4 h | Theronts | Not effective - 0-5% mortality | Straus & Meinelt (2009) |
| 0.08 mg $l^{-1}$ for 1-4 h | | Not effective – 0-5% (trial 1); 10-20% mortality (trial 2) | |
| 0.12 mg $l^{-1}$ for 1-4 h | | Not effective – 5-10% (trial 1); 20-30% mortality (trial 2) | |
| 0.16 mg $l^{-1}$ for 1-3 h | | Not effective - 20-40% mortality | |
| 0.16 mg $l^{-1}$ for 4 h | | Effective - 50% mortality | |
| 0.20 mg $l^{-1}$ for 1 h | | Not effective - 40% mortality | |
| 0.20 mg $l^{-1}$ for 1-4 h | | Not effective - 30% mortality | |
| 0.20 mg $l^{-1}$ for 2-4 h | | Effective - 50% mortality | |
| 0.24 mg $l^{-1}$ for 1-4 h | | Not effective - 30% mortality (trial 1); 60% mortality (trial 2) | |
| 0.28 mg $l^{-1}$ for 1-4 h | | Effective - 50 % mortality (trial 1); 70-80 % mortality (trial 2) | |
| 0.32 mg $l^{-1}$ for 1-4 h | | Effective - 60% mortality (trial 1); 80-90% mortality (trial 2) | |
| 0.36 mg $l^{-1}$ for 1-4 h | | Effective - 70% mortality (trial 1); 80-95% mortality (trial 2) | |
| 0.40 mg $l^{-1}$ for 1-4 h | | Effective - 80% mortality (trial 1); 90-95% mortality (trial 2) | |
| *In vivo* - bath (40% PAA solution) | | | |
| 1 mg $l^{-1}$ for 4 d | *C. carpio* | Effective - significant reduction on the number of trophonts on treated fish | Sudová *et al.* (2010) |
| **Peracetic Acid (PAA) + hydrogen peroxide + based formulation (4.5% PAA + 22% $H_2O_2$ + 9% AA) sold as the commercial product as MinnFinn™** | | | |
| *In vitro* | | | |
| (theronts from *Notemigonus crysoleucas*) | | | |
| 0.0225 mg $l^{-1}$ for1-4 h | | Not effective - 0% mortality | Straus & Meinelt (2009) |
| 0.0450 mg $l^{-1}$ for 1-4 h | | Not effective - 0% mortality | |
| 0.0675 mg $l^{-1}$ for 1-4 h | | Not effective - 5% mortality | |
| 0.0900 mg $l^{-1}$ for 1-4 h | | Not effective - 5-20% mortality | |
| 0.1125 mg $l^{-1}$ for1-4 h | | Not effective - 20-40% mortality | |
| 0.1350 mg $l^{-1}$ for 1 h | | Not effective - 30% mortality | |
| 0.1350 mg $l^{-1}$ for 2-4 h | | Effective - 50% mortality | |
| 0.1575 mg $l^{-1}$ for 1 h | | Not effective - 40% mortality | |
| 0.1575 mg $l^{-1}$ for 2-4 h | | Effective - 60% mortality | |
| 0.1800 mg $l^{-1}$ for 1 h | | Not effective - 40% mortality | |
| 0.1800 mg $l^{-1}$ for 2-4 h | | Effective - 60% mortality | |
| 0.2025 mg $l^{-1}$ for 1 h | | Not effective - 40 % mortality | |
| 0.2025 mg $l^{-1}$ for 2-4 h | | Effective - 70% mortality | |
| 0.2250 mg $l^{-1}$ for 1-4 h | | Effective - 50-70% mortality | |
| (theronts from *Xiphophorus hellerii*) | | | |
| 0.0225 mg $l^{-1}$ for1-4 h | | Not effective - 0-10% mortality | |
| 0.0450 mg $l^{-1}$ for 1-4 h | | Not effective - 10% mortality | |
| 0.0675 mg $l^{-1}$ for 1-4 h | | Not effective - 10-20% mortality | |
| 0.0900 mg $l^{-1}$ for 1-4 h | | Not effective - 10-30% mortality | |
| 0.1125 mg $l^{-1}$ for1-3 h | | Not effective - 20-40% mortality | |
| 0.1125 mg $l^{-1}$ for4 h | | Effective - 50% mortality | |
| 0.1350 mg $l^{-1}$ for 1 h | | Not effective - 40% mortality | |

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|---|---|---|---|---|
| | 0.1350 mg $l^{-1}$ for 2-4 h | | Effective - 50% mortality | |
| | 0.1575 mg $l^{-1}$ for 1-4 h | | Effective - 50-60% mortality | |
| | 0.1800 mg $l^{-1}$ for 1-4 h | | Effective - 60-80% mortality | |
| | 0.2025 mg $l^{-1}$ for 1-4 h | | Effective - 60-90 % mortality | |
| | 0.2250 mg $l^{-1}$ for 1-4 h | | Effective - 70-95% mortality | |
| **Peracetic acid + acetic acid + hydrogen peroxide based formulation sold as the commercial product Detarox** | | | | |
| *In vivo* - bath | 10 mg $l^{-1}$ for 25-45min<br>$2^{nd}$ treatment after 4-7 d | Salmonids - not specified | Effective - no details | Rhakonen & Koski (2002) |
| **Peracetic acid + acetic acid + hydrogen peroxide based formulation (13% PAA + 20% AA+ 20% $H_2O_2$) sold as commercial product Per Aqua** | | | | |
| *In vitro* | 0.08 mg $l^{-1}$ for 30 min up to 60 min | Tomonts | Not effective - 0-20% mortality | Bruzio & Buchmann (2010) |
| | 0.08 mg $l^{-1}$ for 1.5 h up to 4.5 h | | Not effective - 30-40% mortality | |
| | 0.8 mg $l^{-1}$ for 15 min | | Not effective - 20% mortality | |
| | 0.8 mg $l^{-1}$ for 30 min | | Effective - ~90% mortality | |
| | 0.8 mg $l^{-1}$ for 45 min | | Effective - ~100% mortality | |
| | 0.8 mg $l^{-1}$ for 15 min | Theronts | Effective - 100% mortality | |
| | 8 mg $l^{-1}$ for 15 min | | Effective – 100% mortality | |
| *In vivo* - bath | 40 mg $l^{-1}$ 3 times a week for 3 weeks | *S. salar* | Not effective - infections rose throughout the infection period | Rintamäki-Kinnunen *et al.* (2005a) |
| **Peracetic acid + acetic acid + hydrogen peroxide based formulation (13% PAA + 20% AA+ 20% $H_2O_2$) sold as commercial product Desirox + formaldehyde** | | | | |
| *In vivo* - bath | 10 + 100 mg $l^{-1}$ 3 times a week for 4 weeks | *S. salar* | Trial inconclusive - details missing | Rintamäki-Kinnunen *et al.* (2005a) |
| **Peracetic acid + acetic acid+ hydrogen peroxide + peroctanoic acid based formulation sold as commercial product Incimaxx Aquatic** | | | | |
| *In vitro* | 0.08 mg $l^{-1}$ for 30 min up to 4.5 h | Tomonts | Not effective - ~15-20% mortality | |
| | 0.8 mg $l^{-1}$ for 15 min | | Not effective - 40% mortality | Bruzio & Buchmann (2010) |
| | 0.8 mg $l^{-1}$ for 30 min | | Effective - 80% mortality | |
| | 0.8 mg $l^{-1}$ for 45 min | | Effective - 100% mortality | |
| | 8 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | Picón-Camacho *et al.* (2010) |
| | 12 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| | 15 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| | 8 mg $l^{-1}$ for 1 h | Cysts | Effective - 100% mortality | Picón-Camacho *et al.* (2010) |
| | 12 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| | 15 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| | 0.8 mg $l^{-1}$ for 15 min | Theronts | Effective - 100% mortality | Bruzio & Buchmann (2010) |
| | 8 mg $l^{-1}$ for 15 min | | Effective – 100% mortality | |

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|---|---|---|---|---|
| | 8 mg $l^{-1}$ for 1 h | | Effective - 98.3% mortality | Picón-Camacho *et al.* (2010) |
| | 12 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| | 15 mg $l^{-1}$ for 1 h | | Effective - 100% mortality | |
| **Per Aqua** | | | | |
| See entry for peracetic acid + acetic acid + hydrogen peroxide based formulations (13% PAA + 20% AA+ 20% $H_2O_2$) | | | | |
| **Perotan** | | | | |
| See entry for hydrogen peroxide + acetic acid based formulations | | | | |
| **Piscidin 2 (antimicrobial polypeptide)** | | | | |
| *In vitro* | 12.5 µg $ml^{-1}$ for 9 min 46s | Trophonts* | Effective – 100% mortality | Ullal *et al.* (2008) |
| | 25 µg $ml^{-1}$ for 7 min 33s | | Effective – 100% mortality | |
| | 50 µg $ml^{-1}$ for 6 min | | Effective – 100% mortality | |
| | 100 µg $ml^{-1}$ for 6 min 15s | | Effective – 100% mortality | |
| | 200 µg $ml^{-1}$ for 6 min 25s | | Effective – 100% mortality | |
| | 3.1 µg $ml^{-1}$ for 4 h | Theronts | Not effective - 0% killed, but less active than those in the controls | Colorni *et al.* (2008) |
| | 6.3 µg $ml^{-1}$ for 10 min | | Effective - 100% mortality | |
| | 12.5 µg $ml^{-1}$ for 10 min | | Effective - 100% mortality | |
| | 100 µg $ml^{-1}$ for 5 min | | Effective - 100% mortality | |
| **Piperazine** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Effective - 55% of the medicated fish had a low number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Potassium ferrate (VI)** | | | | |
| *In vitro* | 0.096 for 30 min up to 4 h | Theronts | Not effective - 0% mortality | Ling *et al.* (2010) |
| | 0.96 mg $l^{-1}$ for 30 min and 1h | | Not effective - 0% mortality | |
| | 0.96 mg $l^{-1}$ for 2 h | | Not effective - 14% mortality | |
| | 0.96 mg $l^{-1}$ for 4 h | | Not effective - 25% mortality | |
| | 1.92 mg $l^{-1}$ for 30 min | | Not effective - 0% mortality | |
| | 1.92 mg $l^{-1}$ for 1 h | | Not effective - 20% mortality | |
| | 1.92 mg $l^{-1}$ for 2 h | | Not effective - 24% mortality | |
| | 1.92 mg $l^{-1}$ for 4 h | | Effective - 56% mortality | |
| | 4.80 mg $l^{-1}$ for 30 min | | Not effective - 38% mortality | |
| | 4.80 mg $l^{-1}$for 1 h | | Effective - 58% mortality | |
| | 4.80 mg $l^{-1}$for 2 h | | Effective - 100% mortality | |
| | 9.60 mg $l^{-1}$for 30 min | | Effective - 50% mortality | |
| | 9.60 mg $l^{-1}$for 1 h | | Effective - 64% mortality | |
| | 9.60 mg $l^{-1}$for 2 h | | Effective - 100% mortality | |
| | 14.40 mg $l^{-1}$for 30 min | | Effective - 59% mortality | |
| | 14.40 mg $l^{-1}$for 1 h | | Effective - 100% mortality | |

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|---|---|---|---|---|
| | 19.20 mg $l^{-1}$for 30 min | | Effective - 69% mortality | |
| | 19.20 mg $l^{-1}$for 1 h | | Effective - 100% mortality | |
| | 24.00 mg $l^{-1}$for 30 min | | Effective - 100% mortality | |
| | 48.00 mg $l^{-1}$for 30 min | | Effective - 100% mortality | |
| *In vivo* | 1.92 mg $l^{-1}$ for 3 d | *C. auratus* | Effective - 71.94% trophont reduction | Ling *et al.* (2010) |
| | 4.80 mg $l^{-1}$ for 3 d | | Effective - 80.30% trophont reduction | |
| | 9.60 mg $l^{-1}$ for 3 d | | Effective - 83.39% trophont reduction | |
| | 19.20 mg $l^{-1}$ for 3 d | | Effective - 100% trophont reduction | |
| **Potassium permanganate ($KMnO_4$)** | | | | |
| *In vitro* | 0.1- 0.5 mg $l^{-1}$ for 15 up to 45 min | Theronts | Not effective - no details | Straus & Griffin (2001) |
| | 0.6 mg $l^{-1}$ for 15 min up to 4 h | | Not effective - 0-1% mortality | |
| | 0.7 mg $l^{-1}$ for 15 min up to 4 h | | Not effective - 5-10% mortality | |
| | 0.8 mg $l^{-1}$ for 15 min | | Not effective - 0% mortality | |
| | 0.8 mg $l^{-1}$ for 30 min up to 4 h | | Effective - 70% mortality | |
| | 0.9 mg $l^{-1}$ for 15 min | | Not effective - 0% mortality | |
| | 0.9 mg $l^{-1}$ for 30min up to 4 h | | Effective - 90-95% mortality | |
| | 1.0 mg $l^{-1}$ for 15 min | | Not effective - 0% mortality | |
| | 1.0 mg $l^{-1}$ for 30 min up to 4 h | | Effective - 95 - >99% mortality | |
| *In vivo* - bath | 0.25 mg $l^{-1}$ for 6 d | *I. punctatus* | Effective - low number of trophonts on treated fish | Straus & Griffin (2001) |
| | 0.50 mg $l^{-1}$ for 6 d | | Effective - low number of trophonts on treated fish | |
| | 0.75 mg $l^{-1}$ for 6 d | | Effective - low number of trophonts on treated fish | |
| | 1.0 mg $l^{-1}$ for 6 d | | Effective - No trophonts on treated fish | |
| | 2 mg $l^{-1}$daily for 20 d (static tanks and flow through) | *I. punctatus* | Not effective - all treated infected fish died; trophonts present | Tieman & Goodwin (2001) |
| | 0.25 mg $l^{-1}$ for 8 d | *Tilapia aurea* | Effective - No trophonts on treated fish | Straus & Griffin (2001) |
| | 0.50 mg $l^{-1}$ for 8 d | | Effective - No trophonts on treated fish | |
| | 0.5 mg $l^{-1}$ daily for 10 d | *I. punctatus* | Not effective - high number of trophonts on treated fish | Straus & Griffin (2002) |
| | 0.75 mg $l^{-1}$ daily for 10 d | | Not effective - high number of trophonts on treated fish | |
| | 1.0 mg $l^{-1}$ daily for 10 d | | Effective - no trophonts on treated fish | |
| | 1.25 mg $l^{-1}$ daily for 10 d | | Effective - no trophonts on treated fish | |
| | 1.25 mg $l^{-1}$ daily for 10 d | | Not effective - toxic to treated fish | |
| | 4 mg $l^{-1}$ 3 times a week for 2 weeks | *S. salar* | Trial inconclusive - low parasite numbers across all groups | Rintamäki-Kinnunen *et al.* (2005a) |
| | 10 - 20 mg $l^{-1}$ for 30 min | *O. mykiss* *S. fontinalis* | Effective - there was a reduction in trophont number on treated fish but was toxic to all three fish species | Balta *et al.* (2008) |

| | | | | |
|---|---|---|---|---|
| | | *S. trutta* | | |
| **Potassium permanganate ($KMnO_4$) + dimetrazole** | | | | |
| *In vivo* - bath + in-feed | 3 mg l$^{-1}$ every 2nd d, 5 times + dimetrazole in feed 28 mg/fish for 10 d | *O. mykiss* | Effective - no signs of infection by d 7 | Rapp (1995) |
| **Potassium persulfate + sodium dodecylbenzosulfonate + malic acid + sulfamic acid based formulation commercialised as Virkon S** | | | | |
| *In vitro* | 1 mg l$^{-1}$ for 1 h | Theronts | Not effective - ~8% mortality | Shinn *et al.* (2005) |
| | 10 mg l$^{-1}$ for 1 h | | Effective - ~55% mortality | |
| | 100 mg l$^{-1}$ for 1 h | | Not effective - 60% mortality† | |
| | < 50 µl l$^{-1}$ for 10 h | Trophonts* | Not effective - trophonts develop normally | Lahnsteiner & Weismann (2007) |
| *In vivo* - bath | 10 µl l$^{-1}$ for 2 h for 5 d | *O. mykiss* | Not effective - none of the treated fish survived | Lahnsteiner & Weismann (2007) |
| | 10 µl l$^{-1}$ for 4 h for 5 d | *C. carpio* | Not effective - 90% of the treated fish had survived on d 1; 50% were still alive by d 3 but had a high number of trophonts | |
| **Pyceze™** | | | | |
| See entry for bronopol | | | | |
| **Quinacrine** | | | | |
| *In vitro* | 200 mg l$^{-1}$ for 2 h | Tomonts | Partially effective - 50% mortality after 2 h; 50% of tomonts develop normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 100 mg l$^{-1}$ for 3 h, d 6 p.i. | *O. mykiss* | Not effective - 50% fish mortality; all trophonts developed normally | |
| In-feed | 500 mg kg$^{-1}$ for 8 d | | Not effective - all trophonts developed normally; signs of feed rejection from d 4 | |
| **Quinine** | | | | |
| *In vivo* - In-feed | 5 g kg$^{-1}$ feed for 7 d | *Poecilia sphenops* | Effective - 100% elimination of trophonts | Schmahl *et al.* (1996) |
| | 5 g kg$^{-1}$ feed for 8 d | *X. helleri* | Effective - 100% elimination of trophonts | |
| | 5 g kg$^{-1}$ feed for 10 d | *Hyphessobrycon herbertaxelrodi* | Effective - 100% elimination of trophonts | |
| **1,3-di-6-quinolylurea** | | | | |
| *In vivo* - In-feed | 40 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Not effective – all treated fish had a high number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Ronidizole (1-methyl-2-carboxymethyl-5-nitroimidazole)** | | | | |
| *In vitro* | 250 mg l$^{-1}$ for 1h - 48 h | Trophozites* | Not effective - 0% mortality | Farley & Heckmann (1980) |
| | 500 mg l$^{-1}$ for 1h - 4 h | | Not effective - 0% mortality | |
| | 500 mg l$^{-1}$ for 8 h | | Effective - 25% mortality | |
| | 500 mg l$^{-1}$ for 24 h | | Effective - 65.5% mortality (3 repeat trials) | |
| | 500 mg l$^{-1}$ for 48 h | | Effective - 65.5% mortality | |

| | | | | |
|---|---|---|---|---|
| | 750 mg $l^{-1}$ for 1 h | | Not effective - 0% mortality | |
| | 750 mg $l^{-1}$ for 2 h | | Not effective - 2.5% mortality | |
| | 750 mg $l^{-1}$ for 4 h | | Not effective - 47.5% mortality | |
| | 750 mg $l^{-1}$ for 8 h | | Effective - 82.5% mortality | |
| | 750 mg $l^{-1}$ for 24 h | | Effective - 87.5% mortality (trial 1); 100% mortality (trial 2) | |
| | 500 mg $l^{-1}$ for 1 h | Tomites | Not effective - 10% mortality | Farley & Heckmann (1980) |
| | 500 mg $l^{-1}$ for 2 h | | Not effective - 23% mortality | |
| | 500 mg $l^{-1}$ for 4, 8 & 24 h | | Effective - 55%, 90% and 96% mortality respectively | |
| | 750 mg $l^{-1}$ for 1 h | | Not effective - 19% mortality | |
| | 750 mg $l^{-1}$ for 2, 4, 8 & 24 h | | Effective - 51.5%, 77.5%, 79% and 96.5% mortality respectively | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Not effective - 100% medicated fish high number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **SalarBec** | | | | |
| *In vivo* - In-feed | 0.32% for 10 d prior infection | *O. mykiss* | Effective - 65% trophont reduction | Shinn *et al.* (2005) |
| **Salinomycin sodium** | | | | |
| *In vivo* - In-feed | 38 ppm for 10 d p.i. | *O. mykiss* | Effective - 80.2% trophonts reduction | Shinn *et al.* (2003b) |
| | 43 ppm for 10 d p.i. | | Effective - 71.9% trophonts reduction | |
| | 47 ppm for 10 d p.i. | | Effective - 93.3% trophonts reduction | |
| **Secnidazole** | | | | |
| *In vivo* - In-feed | 24 mg $kg^{-1}$ b.w for 10 d | *C. auratus* | Effective - no trophonts on treated fish | Tokşen & Nemli (2010) |
| | 36 mg $kg^{-1}$ b.w for 10 d | | Effective - no trophonts on treated fish | |
| | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Effective - 75% medicated fish free of infection | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Silver nitrate** | | | | |
| *In vitro* | 0.67 mg $l^{-1}$ | Tomites | Effective - 100% mortality in less than 15 sec | Farley & Heckmann (1980) |
| **Sodium carbonate peroxyhydrate** | | | | |
| *In vivo* - bath | 60-90 mg $l^{-1}$ daily for 30-1 h for 4-6 d | Not specified | Not effective – no details | Rahkonen & Koski (2002) |
| **Sodium chloride (NaCl)** | | | | |
| *In vitro* | 2.5 g $l^{-1}$ for 24 h | Theronts | Effective - ~50% mortality | Shinn *et al.* (2005) |
| | 5 g $l^{-1}$ for 24 h | | Effective - ~95% mortality | |
| | 10 g $l^{-1}$ for 24 h | | Effective - ~98% mortality | |
| | 15 g $l^{-1}$ for 10 h | Trophonts* | Not effective – 0% mortality | Lahnsteiner & Weismann (2007) |
| | 20 g $l^{-1}$ for 10 h | | Effective – 100% mortality | |

| | | | | |
|---|---|---|---|---|
| *In vivo* - bath | 1 g l$^{-1}$ for 12 d | *B. bidyanus* | 100% theronts killed; trophonts 57% lower than controls*<br>(8.7 ± 15.1% fish survive *vs* 0% of control fish) | Mifsud & Rowland (2008) |
| | 1 g l$^{-1}$ for 16 d | | Theronts 74.9% and trophonts 89.8% lower than controls*<br>(96.7 ± 4.7% fish survive *vs* 66.7 ± 47.1% of control fish) | |
| | 2 g l$^{-1}$ for 12 d | | 100% theronts and tomonts mortality*<br>(96.7 ± 4.7% fish survive *vs* 0 % of control fish) | |
| | 2 g l$^{-1}$ for 16 d | | 100% theronts and tomonts mortality*<br>(96.7 ± 4.7% fish survive *vs* 66.7 ± 47.1% of control fish) | |
| | 3 g l$^{-1}$ for 16 d | | 100% theronts and tomonts mortality*<br>(100% fish survive *vs* 66.7 ± 47.1% of control fish) | |
| | 3 g l$^{-1}$ daily for 20 d (static tanks) | *I. punctatus* | Not effective – all treated fish died | Tieman & Goodwin (2001) |
| | 4 g l$^{-1}$ for 45 d | *Rhamdia quelen* | 24.7% of the treated fish survived *versus* 8.1% of the control fish | Miron *et al.* (2003) |
| | 4 g l$^{-1}$ daily for 30 d | *R. quelen* | Effective - reduction in the number of trophonts on treated fish | Garcia *et al.* (2007) |
| | 5 g l$^{-1}$ for 14 d at 11-18°C | *B. bidyanus* | Effective - no trophonts visible on treated fish | Selosse & Rowland (1990) |
| | 5 g l$^{-1}$ for 8 d at 24°C | *B. bidyanus* | Effective - no trophonts visible on treated fish | |
| | 5 g l$^{-1}$ for 7 d at 19-22°C | *Maccullochella peeli* | Effective - no trophonts visible on treated fish | |
| | 5 g l$^{-1}$ for 7 d at 23-26°C | *Tandanus tandanus* | Effective - no trophonts visible on treated fish | |
| | 10 and 15 g l$^{-1}$ for 20 min | *O. mykiss*<br>*S. fontinalis*<br>*S. trutta* | Not effective - no details | Balta *et al.* (2008) |
| | 20 g l$^{-1}$ for 1 h for 5 d | *O. mykiss* | Partially effective - 60% of treated fish survived, no infections on d 1 but a high infection on d 3 | Lahnsteiner & Weismann (2007) |
| | 20 g l$^{-1}$ for 1 h for 5 d | *C. carpio* | Not effective - 0% survival on treated fish†; no infections d 1 but high infection on d 3 | |
| | 20 g l$^{-1}$ for 20 min | *O. mykiss*<br>*S. fontinalis*<br>*S. trutta* | Effective - reduction in the number of trophotns on treated fish | Balta *et al.* (2008) |
| In-feed | 0.3-1.0% feed for 3-11 d | *C. carpio* | Effective - no details | Rahkonen & Koski (2002) |
| | 1.2 % feed for 30 d | *R. quelen* | Not effective - high mortality within the treated groups | Garcia *et al.* (2007) |
| | 2.5 % feed for 30 d | | Not effective - high mortality within the treated groups | |
| | 5.0 % feed for 30 d | | Not effective - high mortality on treated groups | |
| | 6.0 % feed for 30 d | | Not effective - high mortality on treated groups | |

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|---|---|---|---|---|
| **Sodium percarbonate** | | | | |
| *In vitro* | 12.5 mg l$^{-1}$ for 3 h | Theronts / cysts | Effective on theronts but not effective on cysts | Jensen *et al.* (2001) |
| | 12.5 mg l$^{-1}$ for 24 h | Tomocysts | Not effective - 11% mortality | |
| | 13 mg l$^{-1}$ for few hours (no details) | | Effective on theronts but not effective on cysts | |
| | 63 mg l$^{-1}$ for 1 h | | Effective on theronts but not effective on cysts | Buchmann *et al*. (2003) |
| | 512 mg l$^{-1}$ for <1 h | Tomonts | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 256 mg l$^{-1}$ for <1 h 30 min | | Effective - 100% mortality | |
| | 128 mg l$^{-1}$ for <4 h 15 min | | Effective - 100% mortality | |
| | 0.5 mg l$^{-1}$ for 1.5, 3 & 15 h | Theronts | Not effective - <50% mortality | Buchman *et al.* (2003) |
| | 2.5 mg l$^{-1}$ for 1.5, 3 & 15 h | | Not effective - <50% mortality | |
| | 8 mg l$^{-1}$ for 30-60 min | | Not effective - ~0% mortality | Bruzio & Buchmann (2010) |
| | 8 mg l$^{-1}$ for 1.5 h | | Not effective - ~40% mortality | |
| | 8 mg l$^{-1}$ for 2 h | | Effective - ~70% mortality | |
| | 8 mg l$^{-1}$ for 2.5 h | | Effective - ~70% mortality | |
| | 8 mg l$^{-1}$ for 3 h | | Effective - ~100% mortality | |
| | 8 mg l$^{-1}$ for ~5 h (11-12°C) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 8 mg l$^{-1}$ for ~2 h 20 min (21-22°C) | | Effective - 100% mortality | |
| | 12.5 mg l$^{-1}$ for 1.5 h | | Not effective - <50% mortality | Buchman *et al.* (2003) |
| | 12.5 mg l$^{-1}$ for 3 & 15 h | | Effective - >50% mortality | |
| | 16 mg l$^{-1}$ for ~1 h 20 min (11-12°C) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 16 mg l$^{-1}$ for ~1 h 40 min (21-22°C) | | Effective - 100% mortality | |
| | 32 mg l$^{-1}$ for ~1 h 10 min (11-12°C) | | Effective - 100% mortality | |
| | 32 mg l$^{-1}$ for ~30 min (21-22°C) | | Effective - 100% mortality | |
| | 62.5 mg l$^{-1}$ for 1.5, 3 & 15 h | | Effective - >50% mortality | Buchman *et al.* (2003) |
| | 64 mg l$^{-1}$ for ~30 min (11-12°C) | | Effective - 100% mortality | Heinecke & Buchmann (2009) |
| | 64 mg l$^{-1}$ for ~10 min (21-22°C) | | Effective - 100% mortality | |
| | 312.5 mg l$^{-1}$ for 1.5, 3 & 15 h | | Effective - >50% mortality | Buchman *et al.* (2003) |
| | 1562.5 mg l$^{-1}$ for 1.5, 3 & 15 h | | Effective - >50% mortality | |
| *In vivo* - bath | Concentration not specified for 20 min every 2$^{nd}$ and 3$^{rd}$ day | *O. mykiss* | Not effective - no details | Rahkonen & Koski (2002) |
| **Sulfachlorpyrazine** | | | | |

| | | | | |
|---|---|---|---|---|
| *In vitro* | 100 mg $l^{-1}$ for 3 h | Theronts | Not effective - high survival rate after 3 h | Wahli *et al.* (1993) |
| | 100 mg $l^{-1}$ for 24 h | Adults** | Not effective - high survival rate after 24 h | |
| | 100 mg $l^{-1}$ for 24 h | Cysts | Not effective - high survival rate after 24 h | |
| **Sulfaquinoxaline** | | | | |
| *In vitro* | 200 mg $l^{-1}$ for 2 h | | Not effective - 12.5% mortality after 2 h; tomonts surviving treatment developed normally | Tojo-Rodriguez *et al.* (1994) |
| *In vivo* - bath | 200 mg $l^{-1}$for 3 h, day 6 p.i. | *O. mykiss* | Not effective - all tomonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| In-feed | 1000 mg $kg^{-1}$ for 8 d | | Not effective - all tomonts developed normally; 4% fish mortality on day 6 p.i.; no feed unpalatability | |
| **Thiophanate** | | | | |
| *In vivo* - In-feed | 40 g $kg^{-1}$ feed for 10 d | *O. mykiss* | Not effective - 100% medicated fish high number of trophonts | Tojo-Rodriguez & Santamarina-Fernandez (2001) |
| **Toltrazuril** | | | | |
| *In vitro* | 10 µg $ml^{-1}$ for 2 h | Tomonts | Effective - 100% mortality | Schmahl *et al.* (1989) |
| | 200 mg $l^{-1}$ for 2 h | | Effective - 0% mortality but tomonts did not develop | Tojo-Rodriguez *et al.* (1994) |
| | <50 mg $l^{-1}$ for 10 h | Trophonts* | Not effective - trophonts developed normally | Lahnsteiner & Weismann (2007) |
| | 10 µg $ml^{-1}$ for 2 h | Theronts | Not effective - 0% mortality | Schmahl *et al.* (1989) |
| *In vivo* - bath | d 1 10 mg $l^{-1}$ (2 h)<br>d 2 & d 3 20 mg $l^{-1}$ (1 h) | Various spp. | Trophonts affected but not theronts | Mehlhorn *et al.* (1988) |
| | d 1 10 mg / l (4 h)<br>d 3 & d 5 10 mg / l (4 h) | Various spp. | 100% trophonts killed but theronts not affected | Mehlhorn *et al.* (1988) |
| | 1 µg $ml^{-1}$ for 4.5 h | *A. rostrata* | Not effective - no details | Schmahl *et al.* (1989) |
| | 5 µg $ml^{-1}$ for 4.5 h | *A. rostrata* | Not effective - one third of the parasites dropped off the fish within 24 h. New infections established within 2 d | |
| | 10 µg $ml^{-1}$ for 2 h | | Effective - two thirds of the parasites dropped off the fish within 24 h, fish were free from new infections over the following 14 days | |
| | 10 µg $ml^{-1}$, 2 h ($1^{st}$ d)<br>20 µg $ml^{-1}$, 1 h ($2^{nd}$ d)<br>20 µg $ml^{-1}$, 2 h ($3^{rd}$ d) | *A. rostrata* | Effective - no details | Schmahl *et al.* (1989) |
| | 5 mg $l^{-1}$ | *O. mykiss* | Not effective - toxic, after 5 h 100% fish mortality | From *et al.* (1992) |
| | 10 mg $l^{-1}$ | | Not effective - toxic, after 3.5 h 100% fish mortality | |
| | 20 mg $l^{-1}$ | | Not effective - toxic, after 2 h 100% fish mortality | |
| | 50 mg $l^{-1}$ | | Not effective - toxic, after 2 h 100% fish mortality | |
| | 200 mg $l^{-1}$for 3 h, day 6 p.i. | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |

| | | | | |
|---|---|---|---|---|
| In-feed | 1000 mg kg$^{-1}$ for 8 d | *O. mykiss* | Not effective - all trophonts developed normally | Tojo-Rodriguez *et al.* (1994) |
| **Tramisol (6S)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3]thiazole)** | | | | |
| *In vitro* | 100 mg l$^{-1}$ for 2 min | Trophozoites* | Effective - 50% mortality after 2 h; 100% mortality post exposure | Post & Vesley (1983) |
| **Tricaine methanesulfonate (TM)** | | | | |
| *In vitro* | 50 mg l$^{-1}$ buffered with Na $CaCO_3$ (time of exposure not specified) | Tomonts | Not effective - 4.9% mortality | Xu *et al.* (2008b) |
| | 50 mg l$^{-1}$ not buffered (time of exposure not specified) | | Not effective - 1.1% mortality | |
| | 150 mg l$^{-1}$ buffered with Na $CaCO_3$ for 2-3 min | | Not effective - 1.8% mortality | |
| | 150 mg l$^{-1}$ buffered with Na $CaCO_3$ (time of exposure not specified) | | Not effective - 9.2% mortality | |
| | 150 mg l$^{-1}$ not buffered for 2-3 min | | Not effective - 6.1% mortality | |
| | 150 mg l$^{-1}$ not buffered (time of exposure not specified) | | Not effective - 9.9% mortality | |
| | 300 mg l$^{-1}$ buffered with Na $CaCO_3$ (time of exposure not specified) | | Not effective - 7.3% mortality | |
| | 300 mg l$^{-1}$ not buffered (time of exposure not specified) | | Effective - 100% mortality | |
| **Triclabendazole (5- choloro-6-(2, 3- dichlorophenoxy) -2- methylthio-1H- benzimidazole)** | | | | |
| *In vivo* - In-feed | 20g kg$^{-1}$ feed for 10 d | *O. mykiss* | Not effective - 100% medicated fish with >50 trophonts | Luzardo-Álavarez *et al.* (2003) |
| **Triclabendazole + β- cyclodextrin (ratio 1:2)** | | | | |
| *In vivo* - In-feed | 10 g kg$^{-1}$ feed for 10 d | *O. mykiss* | Effective - 58% reduction in trophont number compared to control | Luzardo-Álavarez *et al.* (2003) |
| | 20g kg$^{-1}$ feed for 10 d | | Effective - 42% reduction in trophont number compared to control | |
| **Vitamin C** | | | | |
| See entry for ascorbate-2-phosphate | | | | |
| **Vitamin E** | | | | |
| See entry for d-1-alpha-tocopheryl acetate | | | | |
| **Violet C** | | | | |
| *In vivo* - bath | 0.01 mg l$^{-1}$ for 2 d | *C. carpio* | Not effective - not specified | Kurovskaya (2005) |
| | 0.02 mg l$^{-1}$ for 6 d | | Effective - no trophonts on treated fish | |
| | 50 mg l$^{-1}$for 30 min | | Effective - no trophonts on treated fish | |
| **Virkon S** | | | | |
| See the entry for potassium persulfate + sodium dodecylbenzosulfonate + malic acid + sulfamic acid based formulation | | | | |

**Wofasteril®**

See entry peracetic Acid (PAA) + hydrogen peroxide + acetic acid (40%PAA + 15% $H_2O_2$ + 25% AA)

**Abbreviations:** d: days; h: hours; inf.: infection; p.i.: post-infection; *authors use the term "throphont / trophozites" for the free-swimming stage which exited the fish host; ** authors used the term "adults" for the free-swimming stage which exited the fish host; †: toxic to fish; [a] carp / trout / eels/ ornamental fish.

**Appendix 2**. Management strategies tested against infections of *Ichthyophthirius multifiliis* Fouquet, 1876. A strategy is regarded as being effective if it kills ≥50% of the stages under test. Mortality refers to the parasite stages unless otherwise stated.

**Electrotherapy**

*In vitro* - Trophozoides*

| Electrode type | Volts per 2.5 cm separation | Current | Duration (s) | Efficacy | Reference |
|---|---|---|---|---|---|
| Carbon | 55-150 | 150-350 | 5 | Not efficient –14.35% mortality after 24 h | Farley & Heckmann (1980) |
| Carbon | 104-150 | 200-350 | 5 | Not efficient –7.09% mortality after 24 h | |
| Carbon | 150 | | 3 | Efficient – 100%mortality | |
| Carbon | 150 | | 3 | Efficient – 100% mortality | |
| Carbon | 250 | | 3 | Efficient – 100% mortality | |
| Carbon | 350 | | 3 | Efficient – 100% mortality | |
| Carbon | 350 | | 3 | Efficient – 100% mortality | |
| Copper | 88-115 | 135-200 | 5 | Efficient – 100% mortality after 24 h | |
| Copper | 115 | 135 | 5 | Efficient – 100% mortality after 24 h | |
| Steel hardware cloth | 150-240 | 160-400 | 5 | Not efficient –2.99% mortality after 24 h | |
| Steel hardware cloth | 150 | 340 | 5 | Not efficient –0.87% mortality after 24 h | |

**Mechanical filtration**

*In vitro* - tomonts

| Mesh size (µm) | Efficacy | Reference |
|---|---|---|
| 500 | Not efficient - 0% tomonts filtered out | Heinecke & Buchmann (2009) |
| 300 | Not efficient - 6% tomonts filtered out | |
| 160 | Not efficient - 22% tomonts filtered out | |
| 80 | Efficient – 100% tomonts filtered out | |

**Mechanical removal of the cysts**

*In vitro* – tomonts

| Lining surface | Efficacy | Reference |
|---|---|---|
| Crystal polysterin | Not efficient – 9.8% mortality | Shinn *et al.* (2009) |
| Polypropylene – based plastic | Efficient – 90.2% mortality | |
| Polyethylene – based plastic | Efficient – 76.5% mortality | |
| Chlovar chlorinated rubber | Not efficient – 46.6% mortality | |

*In vivo* – commercial raceways in *O. mykiss* hatchery
(Suction head + lining of the bottom of the raceways)

| Visit number | Efficacy | Reference |
|---|---|---|
| 1 (after 2 weeks) | No infection in control and experimental raceways | Shinn *et al.* (2009) |
| 2 (after 4 weeks) | No infection in control and experimental raceways | |

| | | |
|---|---|---|
| 3 (after 6 weeks) | Low infection levels in both control and experimental raceways | |
| 4 (after 8 weeks) | Effective – 92% reduction in trophont numbers compared to the control | |
| 5 (after 10 weeks) | Effective – 99% reduction in trophont numbers compared to the control | |
| (Suction head stopped, only lining of the bottom of the raceways) | | |
| 6 (after 12 weeks) | Effective – 54% reduction in trophont numbers compared to the control | |

**UV light**

*In vivo*- fish species not specified

| **Number of ultraviolet light bulbs used** | **Efficacy** | |
|---|---|---|
| 0 | Not efficient – 82.81% fish mortality | Gratzek *et al.* (1983) |
| 1 | Efficient – 1.33% fish mortality | |
| 2 | Efficient – 0.7% fish mortality | |

**Water flow**

*In vivo* - experimental raceways of *I. punctatus* fingerlings

| **Fish density (no. L$^{-1}$)** | **Flow rate (L min$^{-1}$)** | **Velocity (cm min$^{-1}$)** | **Turn-over (no. h$^{-1}$)** | **Efficacy** | |
|---|---|---|---|---|---|
| 0.33 | 5 | 4.1 | 0.5 | Not effective – 100% mortality of infected fish | Bodensteiner *et al.* (2000) |
| 0.25 | 15 | 12.2 | 1.5 | Not effective – 52% mortality of infected fish | |
| 0.25 | 25 | 20.3 | 2.5 | Effective – 14% mortality of infected fish | |
| 0.33-0.66 | 5 | 4.1 | 0.5 | Not effective - 100% mortality of infected fish | |
| 0.33-0.66 | 25 | 20.3 | 2.5 | Effective – 9% mortality of treated fish | |
| 0.33-0.66 | 45 | 36.5 | 4.5 | Effective – 7% mortality of treated fish | |

*In vivo* - production raceways of *I. punctatus* fingerlings

| | | | | | |
|---|---|---|---|---|---|
| 0.89-1.29 | >2800 | >85 | >2.1 | Effective – no trophonts observed | |
| 0.71-1.40 | >2800 | >85 | >2.1 | Effective – no trophonts observed | |

**Abbreviations:** s: seconds, *****authors use the term "trophozites" for the free-swimming stage which exited the fish host.

# CHAPTER 3

# TESTING OF NOVEL CHEMICAL TREATMENTS

# CHAPTER 3

## PAPER II

*RESEARCH ARTICLE*

**The anti-protozoal activity of bronopol on the key life-stages of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora)**

Shinn, A.P.[a], **Picón-Camacho, S.M.[a]**, Bron, J.E.[a], Conway, D.[a], Yoon, G.H.[a,b], Guo, F.C.[c,d] & Taylor, N.G.H.[a,e]

[a]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, United Kingdom*
[b]*Ministry of Fisheries Wealth, Muscat, Sultanate of Oman*
[c]*Novartis Animal Vaccines Ltd., 4 Warner Drive, Springwood Industrial Estate, Braintree, Essex CM7 2YW, United Kingdom*
[d]*Novartis Animal Vaccines Animal Health Canada Inc., Aqua Health Business, 797 Victoria Road, Route 116, Victoria, PE COA 2GO, Canada*
[e]*Cefas Weymouth Lab., Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, United Kingdom*

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**Abstract**

The utility of bronopol (2-bromo-2-nitropropane-1, 3-diol), licensed under the trade name of Pyceze™ (Novartis Animal Vaccines Ltd.), as a biocide for the treatment of *Ichthyophthirius multifiliis* Fouquet, 1876 was explored through a series of *in vitro* and *in vivo* trials. *In vitro* exposure to bronopol (0, 20, 50 and 100 mg $L^{-1}$) for 30 min was observed to kill 0%, 76.2%, 97.2% and 100% of tomonts within the window of exposure. For the tomonts surviving the bronopol treatment, subsequent development was delayed. The time from free-swimming tomont until the release of theronts ranged from 28.3 h for 0 mg $L^{-1}$ to 70 h for parasites in 20 and 50 mg $L^{-1}$ bronopol, these concentrations also caused asymmetric cyst cell division. Exposure of *I. multifiliis* cysts (min. 8 cell stage) to 100 mg $L^{-1}$ bronopol for 30 min killed 50% of the cysts by the end of chemical treatment and 100% of cysts within the following 42.5 h. Lower doses of bronopol were less effective in killing cysts (3.3% of cysts were killed in 20 mg $L^{-1}$; 10% in 50 mg $L^{-1}$), but delayed the release of theronts (25.7 h for 0 mg $L^{-1}$ to 46.2 h for parasites exposed to 20-50 mg $L^{-1}$). A 30 min exposure to 100 mg $L^{-1}$ bronopol was effective at killing 51.7% of the theronts within the window of exposure.

Long exposures to 1 mg $L^{-1}$ bronopol (up to 48 h) were successful in significantly reducing the number of theronts of *I. multifiliis* surviving after 12 h (controls = 100% survival; 1 mg $L^{-1}$ exposed = 29.16% survival) and 24 h (controls = 73.99% survival; 1 mg $L^{-1}$ = 0% survival). The infection potential of theronts surviving a 12 and 24 h treatment with 1 mg $L^{-1}$ bronopol was demonstrated by their continued ability to infect naïve hosts. These theronts were able to find hosts, penetrate and develop into trophonts. There was, however, a significant reduction in the colonisation success of the treated theronts when compared to the control

groups. The findings of this study demonstrate that bronopol (Pyceze™) is able to impact on the survival of the external stages of *I. multifiliis* (*i.e*. tomonts, cysts and theronts) in the water column suggesting that bronopol may serve a role in the efficacious chemotherapeutic control of *I. multifiliis* infections.

## 1. Introduction

The ciliate protozoan parasite *Ichthyophthirius multifiliis*, commonly known as "whitespot" or "Ich", demonstrates low host specificity, and can cause substantial mortality in freshwater fish populations (McCallum, 1982; Wahli *et al.*, 1991; Matthews, 1994; Buchmann *et al.*, 2001). The parasite has a direct life-cycle that comprises four stages: a parasitic trophont (1) that sits within the host's epidermis, an exiting free-swimming tomont (2), a cyst (3) within which parasite division occurs, tomites which are released from the cyst and subsequently differentiate into infective free-swimming theronts (4) (Matthews, 2005). Host pathology occurs principally when the trophont exits the fish. This causes respiratory stress and osmoregulatory dysfunction (Hines & Spira, 1973, 1974 a, b; Tumbol *et al.*, 2001). Secondary bacterial or fungal infections of the exit wounds may also occur, increasing the likelihood of mortality (Antychowicz *et al*., 1992; Matthews, 2005).

There are relatively few effective control strategies for the management of *I. multifiliis* infections in farm, pond or open systems. Such infections, if left untreated, can result in significant losses from wild and commercially reared fish populations (Shinn *et al.*, 2009). In the UK, this parasite is a major problem for the trout farming industry. Over 60% of British trout farms report infections of *I. multifiliis* each year, resulting in an overall loss of fish amounting to an estimated

£2 million (British Trout Association *pers. comm.*). Whilst malachite green was traditionally used to treat *I. multifiliis* infections in the farm environment, its potential harmful impacts upon human health led to its use in food fish being banned by the European Union (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC). Although formaldehyde is commonly used as an alternative treatment to malachite green, this has not been licensed for this purpose and in 2004 was reclassified by the WHO International Agency for Research on Cancer (IARC) as "carcinogenic to humans" (WHO IARC, 2006). Although chloramine-T is registered under the biocides directive (98/8/EC) and is used to treat the free-living stages of the parasite by disinfecting the water in which the fish live (Cross & Hursey, 1973; Shinn *et al.*, 2001) and is permitted for use in aquaculture in the UK, it is not currently licensed for the direct treatment of fish in the US (Haneke & Masten, 2002). Sodium chloride (NaCl) baths have also been shown to be efficacious against the parasite, though large quantities are required to treat commercial-scale production facilities (Miron *et al.*, 2003; Mifsud & Rowland, 2008). None of these potential alternatives demonstrate the same efficacy as malachite green (Rintamäki-Kinnunen & Valtonen, 1997). Although other chemicals have demonstrable effects against the parasite, either as an in-feed or bath treatments (Mehlhorn *et al.*, 1988; Schmahl *et al.*, 1989, 1996; Schlenk *et al.*, 1998; Tojo-Rodriguez & Santamarina-Fernandez, 2001; Straus & Griffin, 2002; Buchmann *et al.*, 2003; Shinn *et al.*, 2003 a,b; Ekamen *et al.*, 2004; Straus, 2008; Sudová *et al.*, 2010), they are not currently licensed either as a medicine or biocide for this purpose. Consequently, until effective licensed chemical alternatives are available, the control of *I. multifiliis* on fish farms must centre on the use of husbandry practices such as the use of fast water flow rates, filtration and other

mechanical methods to remove the free-living stages of the parasite (Bodensteiner *et al.*, 2000; Picón- Camacho *et al.*, 2007; Heinecke & Buchmann, 2009; Shinn *et al.*, 2009).

Although no licensed products currently exist to treat trout infected with *I. multifiliis*, products authorised for the treatment of other pathogens in trout could be prescribed under the veterinary cascade, if it was thought they could be efficacious. Bronopol (2-bromo-2-nitropropane-1, 3-diol), a broad spectrum disinfectant, has been demonstrated to cause membrane damage in microbial organisms through the inhibition of membrane bound enzymes (Stretton & Mason, 1973; Shepherd *et al.*, 1988). Under the trade name of Pyceze™ (Novartis Animal Vaccines Ltd.), bronopol is currently licensed for use against *Saprolegnia parasitica* (Coker) infections in rainbow trout, *Oncorhynchus mykiss* Walbaum and Atlantic salmon, *Salmo salar* L. Given this background, this study therefore aimed to test the efficacy of bronopol as a possible alternative treatment for use against both the free-living and parasitic stages of *I. multifiliis* infecting *O. mykiss*.

The current study was set out to assess: 1) the *in vitro* cytotoxic activity of 0-100 mg $L^{-1}$ doses of bronopol (30 min) against the tomonts, cysts and theronts of *I. multifiliis*; 2) the potential of long duration (up to 48 h), low dose (0.1-1.0 mg $L^{-1}$), treatment exposure on the survival of theronts; 3) the treatment efficacy of a continuous dose of 1 mg $L^{-1}$ bronopol applied across the external development of *I. multifiliis* (*i.e.* from tomonts to theronts); and, 4) the infection potential of theronts pre-treated with 1 mg $L^{-1}$ bronopol.

## 2. Materials and methods

### 2.1. Source of *I. multifiliis*

Batches of *I. multifiliis* tomonts were collected as they naturally exited a laboratory maintained culture of infected juvenile, *O. mykiss.* The culture of *I. multifiliis* originated from a naturally infected *O. mykiss* stock held on a commercial facility fed by the River Bladnoch, Cree Estuary, Scotland.

### 2.2. Collection of the *I. multifiliis* stages

Tomonts were collected and placed, in batches of 30, into washed Petri dishes containing carbon-filtered tank water and were either used immediately or maintained in a WTB Binder Labortechnik GmbH (Germany) precision environmental chamber maintained at 15°C ± 1°C and 12:12 (L:D) until they reached either the cyst (*i.e.* minimum 8 cell stage) or theront stages (*i.e.* after ~23-30 h).

### 2.3. Chemotherapy trials

Each concentration of bronopol was made up fresh using Pyceze™ (bronopol BP 50% *w/v*; batch no. P698/011, Novartis Animal Vaccines Ltd.) and dechlorinated tank water at 15°C and used within 60 min of the start of each trial. Each test solution was tested in triplicate against a minimum set of 3 control dishes.

Tomonts were tested by placing them in batches of 10 tomonts per 10 ml Petri dish, carefully removing most of the water, rinsing them in the appropriate dose of bronopol to be used, and then adding 5 ml of the appropriate bronopol test solution. Following chemical addition, the Petri dishes were immediately transferred to a 15°C incubator. After the exposure period, the bronopol was

replaced with two changes of tank water. The first change was used as a rinse to remove any remaining chemical and the second change was used for subsequent parasite maintenance. The efficacy of each dose of bronopol was assessed by monitoring the Petri dishes until all the tomonts had either died or continued their development until they had released theronts.

For the cyst trials, tomonts were collected as above but were maintained in the 15°C incubator for a minimum of 3 h. Tomonts were classified as cysts when all parasites had settled on the bottom of the test vessel, produced a cyst coat and were at the minimum 8-cell stage of division. The parasites were then exposed to the appropriate dose of bronopol and processed as described for the tomonts.

For the theront trials, tomonts were collected as above and maintained in the 15°C incubator until the parasites had developed through the cyst stage and had released their theronts. The theronts were maintained for at least 12 h prior to the start of the experiment to ensure they were "infective" or "mature". Theronts were treated by placing 2.5 ml of the theront culture into a Petri dish and then adding the nominal dose of bronopol.

After the exposure period, the chemical was replaced by two changes of 15ºC tank water by inserting a fine 20 μm mesh filter and carefully siphoning out the old medium using a 1000 μl micropipette fitted with wide bore tips. The number of live *versus* dead theronts was determined in 1 ml samples taken from the bronopol treated theront culture. The theronts in each 1 ml aliquot were stained by adding 100 μl of 0.01% neutral red stain and then fixing the sample 5 min later with 200 μl 10% neutral buffered formalin. The efficacy of the chemical was assessed by comparing the number of viable (red) and dead (colourless) theronts as determined using a Sedgwick-Rafter chamber on an Olympus BX51

compound microscope at ×10 magnification.

**Experiment 1: *In vitro* exposure of *I. multifiliis* tomonts, cysts and theronts to 0-100 mg $L^{-1}$ bronopol for 30 min**

Tomonts, cysts and theronts were treated with 0, 20, 50 and 100 mg $L^{-1}$ bronopol at 15ºC for 30 min after which the chemical in the tomont and cyst dishes was replaced with two changes of tank water. The efficacy of the different doses of bronopol in killing theronts was determined at the end of the treatment window *i.e.* after 30 min exposure.

**Experiment 2: *In vitro* exposure of *I. multifiliis* theronts to 0.1-1 mg $L^{-1}$ bronopol for up to 48 h**

A total of 125 10 ml Petri dishes were set-up each containing 2.5 ml theront culture and the appropriate concentration of bronopol, which on addition to the theront culture gave a working concentration of 0, 0.1, 0.25, 0.5, 0.75 and 1 mg $L^{-1}$ bronopol. The bronopol solution in each dish was replaced every 12 h with a fresh batch of bronopol, following removal of the old solution as described above. The number of live theronts in each dish was determined at 12, 24, 36 and 48 h. Five replicate dishes were set-up for each concentration and each time point. The counts from each dish were subjected to statistical analysis using an analysis of covariance through a general linear model (SPSS versio 18.0; IBM, USA) and 2 × 2 contingency tables (StatCalc Epi Info ™ 6, CDC, 1993).

**Experiment 3: Efficacy of a continuous dose of 1 mg $L^{-1}$ bronopol applied across the external development of *I. multifiliis***

To assess the cumulative efficacy of bronopol across all the external stages of *I. multifiliis* (*i.e.* tomonts to theronts), batches of 30 tomonts per Petri dish were treated with 1 mg $L^{-1}$ bronopol which was replaced every 12 h. Each dish was assessed every 12 h and the data from the theront counts were then subjected to statistical analysis using 2 × 2 contingency tables (StatCalc Epi Info ™ 6, CDC, 1993).

**Experiment 4: To determine whether theronts surviving a bronopol treatment are able to subsequently infect fish**

Twenty one 120 ml Petri dishes, each containing 75 ml of theront culture, were treated with either 0 or 1 mg $L^{-1}$ bronopol for 0 h (3 × 0 mg $L^{-1}$ bronopol), 12 h (3 × 1 mg $L^{-1}$ bronopol; 3 × 0 mg $L^{-1}$ bronopol) and 24 h (6 × 1 mg $L^{-1}$ bronopol; 6 × 0 mg $L^{-1}$ bronopol). Of the theront culture dishes maintained for 24h, the chemical in three of the 1 mg $L^{-1}$ bronopol treated dishes was refreshed at 12 h, as was the water in three of the 0 mg $L^{-1}$ bronopol dishes. The medium in the other three 0 and 1 mg $L^{-1}$ bronopol dishes were not refreshed at 12 h. At the end of each time period, the number of live *versus* dead theronts was determined in two 1 ml aliquots sampled from each Petri dish. Fifty ml of the remaining theront culture was then used to challenge naïve *O. mykiss*. Those theront cultures where the medium was not replaced after 24 h were not used for the subsequent infection challenge. This trial was set out to establish whether the duration of treatment and / or refreshing the medium in the dishes at 12 h reduced the survival of theronts and their ability to infect fish.

For the infection trial, fifteen replicate 10 litre tanks were set up in a 15ºC constant temperature room, each tank containing ten ~5g *O. mykiss* fingerlings. Nine tanks (3 tanks per group) were challenged with untreated theronts collected at the start of the experiment (0 mg $L^{-1}$ bronopol-$T_0$), 12 h (0 mg $L^{-1}$ bronopol-$T_{12}$) and 24 h (0 mg $L^{-1}$ bronopol-$T_{24}$) post-start of the experiment. Fish in a further six tanks (3 tanks per group) were challenged with theronts pre-exposed to either 1mg $L^{-1}$ bronopol for 12 h (1 mg $L^{-1}$ bronopol-$T_{12}$) or for 24 h (1 mg $L^{-1}$ bronopol-$T_{24\ \text{refreshed at 12 h}}$). The fish in each tank were exposed to the relevant batches of theronts for 3 h under static conditions, in the dark with aeration, before the water supply (0.5 l / min) to each tank was resumed. Fish were then maintained for 10 d at 15°C on a 2% body weight $d^{-1}$ ration of commercial diet (Skretting, UK). At the end of the 10 day period, the fish were euthanised by an overdose of 1 ml $L^{-1}$ 2-phenoxyethanol (MERCK, Germany) and the total number of trophonts on the fins, gills and entire body surface was determined. Data for theronts surviving chemical treatment were subjected to statistical analysis using 2 × 2 contingency tables (Fishers Exact test) (StatCalc Epi Info ™ 6, CDC, 1993).

## 3. Results

### Experiment 1: *In vitro* exposure of *I. multifiliis* tomonts, cysts and theronts to 0 - 100 mg $L^{-1}$ bronopol for 30 min

*a) Tomonts*

For the tomonts (n = 42) exposed to 20 mg $L^{-1}$ bronopol for 30 min, 76.2% of the tomonts burst whilst the remaining 23.8% underwent asymmetric division to release their theronts after 70 h. For the tomonts (n = 44) exposed to 50 mg $L^{-1}$ (30 min), all the tomonts became stationary within 15 min following the addition

of the chemical. After 60 min (chemical replaced with tank water after 30 min), 61.4% of the tomonts had burst. Those surviving continued to develop and release their theronts after ~47 h post-start. Tomonts (n = 45) exposed to 100 mg $L^{-1}$ bronopol, ceased movement 5 min after the addition of the chemical and displayed little ciliary movement with 30-40% of the tomonts developing small projections over their surface. All tomonts had burst by the end of the 30 min exposure. The tomonts (n = 40) in the control group by comparison (0 mg $L^{-1}$), developed normally and released their theronts after ~28 h.

This trial found that doses of 50 mg $L^{-1}$ bronopol or lower for 30 min are partially successful in killing *I. multifiliis* tomonts (*i.e.* 61.4-76.2%) while a dose of 100 mg $L^{-1}$ bronopol for 30 min was successful in killing all tomonts.

*b) Cysts*

Cysts (n = 30) exposed to 20 mg $L^{-1}$ bronopol appeared to be unaffected by the treatment and began to release their theronts after ~23 h. Similarly, those exposed to 50 mg $L^{-1}$ bronopol (n = 30) also appeared to be largely unaffected by the treatment although there was an apparent delay in their development and subsequent release of theronts which were noted ~43 h post-start. Fifty percent of the cysts exposed to 100 mg $L^{-1}$ bronopol were killed 60 min post-start. The remaining cysts were dead ~43 h post-start. The control cysts (0 mg $L^{-1}$; n = 30) released their theronts ~23 h post-start.

Doses of 20-50 mg $L^{-1}$ bronopol for 30 min did not impact on the survival of cysts although the latter dose did affect the speed of development and the subsequent release of theronts. Treating cysts with a dose of 100 mg $L^{-1}$ bronopol was effective in killing all cysts although the effect was not immediate.

*c) Theronts*

The number of theronts surviving each dose of bronopol displayed a clear dose response, survival decreasing with the increasing concentration of bronopol used. The number of theronts surviving each dose, given as the mean percentage followed by the standard deviation and range in parentheses were 96.4 ± 3.38% (93.3-100%) for 0 mg $L^{-1}$; 81.7 ± 3.75% (77.9-85.4%) for 20 mg $L^{-1}$; 68.7 ± 2.63% (65.7-70.3%) for 50 mg $L^{-1}$ and 48.3 ± 21.65% (24.6-67.0%) for those theronts exposed to 100 mg $L^{-1}$ bronopol for 30 min.

**Experiment 2: *In vitro* exposure of *I. multifiliis* theronts to 0.1-1 mg $L^{-1}$ bronopol for up to 48 h**

Table 1 shows a graded response in theront mortality with both exposure time and the concentration of bronopol used. An analysis of covariance through a general linear model confirmed that there were significant differences with both time ($p \leq 0.0001$) and concentration ($p \leq 0.001$). Similarly, 2 × 2 contingency tables confirmed that there were significant differences ($p \leq 0.001$) between the treated and control groups both before (crude relative risk = 3.3) and after adjusting for the effect of time (Mantel-Haenszel adjusted relative risk = 3.94). The calculation of crude relative risk ignores the effects of time by pooling all data (control *vs* 1 mg $L^{-1}$ bronopol) and calculates that there is a 3.3 fold increase in the likelihood of a theront dying in the treatment group. The adjusted relative risk adjusts for any confounding effect that time may have on the outcome. The adjusted value of 3.94 (or a 3.94 fold increase in the likelihood of theronts dying over those in the control group) suggests that there is only a small but significant contribution of exposure time.

**Table 1:** The mean (± standard deviation) percentage of live theronts in replicate 1 ml aliquots taken from culture dishes containing bronopol (Pyceze™) doses ranging from 0.1 mg $L^{-1}$ to 1 mg $L^{-1}$ against a control group (0 mg $L^{-1}$ bronopol) of dishes containing conditioned tank water.

| **Bronopol** | **Time (h)** | | | | |
|---|---|---|---|---|---|
| **(mg $L^{-1}$)** | **0** | **12** | **24** | **36** | **48** |
| **0.00** | 91.50±3.46 | 96.65±1.83 | 89.54±2.17 | 87.14±2.02 | 95.32±6.63 |
| **0.10** | | 92.85±4.72 | 68.45±3.68 | 68.34±2.35 | 82.00±2.83 |
| **0.25** | | 85.97±6.99 | 69.05±3.37 | 60.00±14.14 | 65.16±2.14 |
| **0.50** | | 77.62±7.88 | 61.23±3.41 | 73.49±13.92 | 40.79±13.02 |
| **0.75** | | 91.12±1.19 | 60.00±0.00 | 73.49±13.92 | 31.43±16.16 |
| **1.00** | | 86.12±3.73 | 62.07±4.88 | 55.56±7.86 | 25.00±35.36 |

**Experiment 3: Efficacy of a continuous dose of 1 mg $L^{-1}$ bronopol applied across the external development of *I. multifiliis***

The effects of a continuous dose of 1 mg $L^{-1}$ bronopol on the external stages of *I. multifiliis* from tomont up to and including theront release are shown in Table 2. The results show that there were was a total of 37 dead tomonts removed from the bronopol test vessels as opposed to 10 tomonts removed from the control vessels over the 48 h period when tomonts were present. Although 50 mature tomonts were added to each vessel at the start of the experiment, further sub-divisions, notably within the bronopol vessels, provided more than 50 stages (tomonts and cysts) in the latter counts (36 h onwards). No statistical analysis was applied to the dead tomont count data.

Table 2 gives the accumulated effect of 1 mg $L^{-1}$ bronopol *versus* a triplicate set of controls presented as the percentage of theronts live at time points 36-60 h post-start. Using 2 × 2 contingency tables (Fishers Exact test) confirmed that there were significant differences between the 0 mg $L^{-1}$ (control) and the 1 mg $L^{-1}$

bronopol dose at 36 h (p = 0.000000001; odds ratio = 11.94; range 9.97-14.30), 48 h (p = 0.000000001; odds ratio = 5.90; range 5.00-6.95) and 60 h post-start (p = 0.00000001; odds ratio = 7.96; range = 6.24-10.17). The crude odds ratio for the pooled data set (36 h + 48 h + 60 h data) is 7.53 which reveals that the odds of the parasite dying are 7.53 times greater in the 1 mg $L^{-1}$ bronopol treatment group than in the control group. After adjusting for the effect of time, the Mantel-Haenszel adjusted odds ratio was determined to be 8.3. This shows that the effect of time confounded with the treatment and after adjustment, the impact of theronts dying in 1 mg $L^{-1}$ bronopol were 8.3 times greater than in the control group.

**Experiment 4: To determine whether theronts surviving a bronopol treatment are able to subsequently infect fish**

The percentage counts of live and dead theronts from the 1 ml aliquots in each of the test Petri dishes are shown in Table 3. Using 2 × 2 contingency tables (Fishers Exact test) confirmed that there were significant differences between the 0 mg $L^{-1}$ and the 1 mg $L^{-1}$ dose at both 12 h (p = 0.0001; odds ratio = 197.5; range 26.09-4109.28) and 24 hours (p = 0.0001; odds ratio = 67.76; range 9.23-1388.22). The odds ratio for the pooled data set (12 h + 24 h data) is 19.53 indicating that the odds of the parasite dying are 19.53 times greater in the treatment group than in the control group. After adjusting for the effect of time the Mantel-Haenszel adjusted odds ratio was determined to be 111.84. This shows that the effect of time confounded with the treatment and after adjustment, the impact of the treatment was greater leading to the odds of the parasite dying being 111.84 times greater in the treatment group than in the control group.

**Table 2:** The cytotoxic activity of 1 mg $L^{-1}$ bronopol (Pyceze™) against the external stages of *Ichthyophthirus multifiliis* against time (h) when presented as a continuous exposure with the chemical replaced every 12 h.

| Time(h) | 0 mg $L^{-1}$ bronopol | 1 mg $L^{-1}$ bronopol |
|---|---|---|
| **0** | Total of 150 tomonts added | Total of 150 tomonts added |
| **12** | 148 cysts (98.7%) at 8-32+ cell stage; 2 free-swimming (1.3%) | 112 cysts (74.7%) at 8-32+ cell stage; 5 abnormally dividing cysts (3.3%); 32 free-swimming tomonts (21.3%); 1 dead tomont (0.7%)* |
| **24** | 148 cysts (98.7%) at 128+ cell stage; 21 free-swimming tomonts (0.65%); 21 dead tomonts (0.65%)* | 131 cysts (87.3%) at 128+ cell stage; 9 free-swimming tomonts (5.68%)†; 10 dead tomonts (6.32%)*† |
| **36** | Theronts (88.50 ± 3.56% alive)2; 41 free-swimming tomonts (0.44%)†; 21 dead tomonts (0.22%)*† | Theronts (38.83 ± 9.75% alive)3; 6 free-swimming tomonts (1.89%)† - 3 show asymmetric division (0.95%)†; 9 dead tomonts (2.84%)*† |
| **48** | Theronts (76.33 ± 10.29% alive)4; 61 free-swimming tomonts (0.22%)†; 61 dead tomonts (0.22%)*† | Theronts (35.67 ± 6.15% alive)5; 41 free-swimming tomonts (0.54%)†; 171 dead tomonts (2.3%)*† |
| **60** | Theronts (65.67 ± 16.08% alive) | Theronts (20.67 ± 5.13% alive)7; 41 dead tomonts (0.54%)† |
| **72** | Theronts (0% alive) | Theronts (0% alive) |

Footnotes: [1]The appearance of additional free-swimming tomonts suggests further division events prior to encystment; [2]Total count = 1588 theronts; [3]Total count = 2364 theronts; [4]Total count = 1506 theronts; [5]Total count = 1397 theronts; [6]Total count = 616 theronts; [7]Total count = 845 theronts; *removed from the dishes; †percentages adjusted for the removal of dead stages.

**Table 3:** The percentage of live theronts (mean ± standard deviation) following exposure to either 1 mg $L^{-1}$ bronopol (Pyceze™) or to tank water (0 mg $L^{-1}$) for either 12 or 24 h and whether the chemical was replaced (R) or not (NR). Counts were derived from two 1 ml aliquots from each triplicate set of dishes.

| Time (h) | Bronopol (mg $L^{-1}$) | Live theronts (%) |
|---|---|---|
| **0** | 0 | 96.32 ± 3.89 |
| **12** | 0 | 100.00 ± 0.00 |
| **24** | 0 (R) | 73.99 ± 28.59 |
| **24** | 0 (NR) | 94.86 ± 1.15 |
| | | |
| **12** | 1 | 29.16 ± 5.96 |
| **24** | 1 (R) | 0.00 ± 0.00 |
| **24** | 1 (NR) | 0.00 ± 0.00 |

Following respective treatments, each batch of theronts was added to its respective replicate tanks containing naïve fish. All the fish were euthanised 10 days after infection and the total number of trophonts on the gills and all body surfaces was determined *post-mortem* (Table 4). The trophont data between replicates were not subjected to statistical analysis as the initial starting doses of theronts were not equal and therefore cannot be compared. This trial was used only to assess the ability of bronopol treated theronts to infect fish. The trial did demonstrate, however, that theronts surviving a 1 mg $L^{-1}$ pre-treatment with bronopol for either 12 or 24 h were still able to infect naïve fish.

**Table 4.** The subsequent colonisation success of theronts pre-exposed to either 0 mg $L^{-1}$ or 1 mg $L^{-1}$ bronopol (Pyceze™) for either 0, 12 or 24 h as determined by the number of trophonts (mean number ± standard deviation) present on *Oncorhynchus mykiss* (7 fish per replicate) 10 days post-challenge.

| Time (h) | Bronopol (mg $L^{-1}$) | No. of trophonts/ fish | Fish no. |
|---|---|---|---|
| **0** | 0 | 123.23 ± 58.05 | 20 |
| **12** | 0 | 44.48 ± 15.12 | 21 |
| **24 (R)** | 0 | 113.97 ± 164.64 | 21 |
| | | | |
| **12** | 1 | 7.00 ± 8.58 | 21 |
| **24 (R)** | 1 | 37.60 ± 43.25 | 20 |

## 4. Discussion

Short exposures (*i.e.* 30 min) to high doses of bronopol (*i.e.* 20, 50 and 100 mg $L^{-1}$) appear to have a marked effect on the survival of the tomont, cyst and theront stages of *I. multifiliis*. The cytotoxic activity of low concentrations of bronopol (*i.e.* 0.1-1 mg $L^{-1}$) over longer periods of exposure (*i.e.* up 48 h) are also able to reduce the survival of theronts (*e.g.* 0.1 mg $L^{-1}$ bronopol for 48 h reduced the number of theronts surviving by ~13% when compared to the control group). The low proportion of theronts surviving a 1 mg $L^{-1}$ bronopol treatment, however, was still able to infect fish.

Prolonged, low doses of bronopol (*i.e.* 1 mg $L^{-1}$) had notable effects on the survival and subsequent development of tomonts and cysts and upon the release of theronts. A low proportion of tomonts, for example, failed to settle immediately but continued swimming and then later showed abnormal division prior to settlement. There was a notable difference in the number of dead tomonts removed from the two groups (1.09% from the control group and 12.7% from the 1 mg $L^{-1}$ bronopol-exposed group). Similarly, there was a marked difference in the

percentage of theronts surviving chemical exposure (*i.e*. 88.5% of the control theronts *versus* 38.83% of the 1 mg $L^{-1}$ treated theronts). Although a large number of tomonts were collected for this trial (*i.e.* 150 per group) and randomly assigned to one of the six experimental dishes, it is not possible to say whether the bronopol treatment caused an increase in division and hence in the number of theronts released from each cyst (*i.e.* ~79,400 theronts in the control population *versus* 118,200 theronts in the 1 mg $L^{-1}$ bronopol-treated population) or whether there was a skewed distribution in the size of tomonts subsequently releasing theronts (a single tomont can release between 50-3000 theronts) in each population. Caution should be therefore used when comparing the number of theronts in different treatment groups as the number of parasites released from each cyst can vary widely from 250 to 3,000 tomites (Wagner, 1960; Lom & Dyková, 1992; Mathews, 2005).

If a 12 h exposure to 1 mg $L^{-1}$ bronopol causes a 60+% reduction in theront numbers (not including the impacts on tomonts), then this represents a significant improvement over many other currently used water treatment products. For example, 200 mg $L^{-1}$ formaldehyde for 1 h kills 40% of theronts; 200 mg $L^{-1}$ hydrogen peroxide for 1 h - 15%; 10 mg $L^{-1}$ Virkon S for 1h - 50%; 5 ppt salt for 24 h - 90% and 20 mg $L^{-1}$ chloramine T for 15 min kills 20% of theronts (Shinn *et al*., unpublished results). As tomonts are continuously released from the fish and theronts from cysts (Lom & Dyková, 1992; Matthews, 2005), short term bath treatment (~ 1 h) and / or flush treatments are relatively ineffective as they only remove the stages present in the water column at the time of treatment. One appropriate water treatment strategy is therefore to employ a biocide that is effective at low doses, can be administered over long periods to give protection, is

economically competitive and has a low environmental impact. From the present study, continuous exposure to bronopol would target exiting trophonts, cysts and theronts, could interfere with parasite settlement and the production of the cyst coat and might prolong cell division and delay the infection process, in addition to causing direct mortality.

The capability of theronts to survive a bronopol exposure and be able to infect a fish host was assessed by exposing the theronts to a continuous, low dose (*i.e.* 1 mg $L^{-1}$) of bronopol over a 48 h period and then infecting naïve fish hosts. This trial demonstrated that the exposure of theronts to low levels of bronopol (1 mg $L^{-1}$) has a significant effect in reducing the number of theronts surviving after both 12 h (100% control cf. 29.16% 1 mg $L^{-1}$) and 24 h (73.99% control cf. 0% 1 mg $L^{-1}$). Statistical analysis confirms that theront exposure to 1 mg $L^{-1}$ bronopol increases their likelihood of dying approximately 20-fold and after adjusting for the effects age-related mortality (time) this then increases the odds likelihood of death in the treatment group by over 100-fold. As theront penetration is very rapid, the likelihood of a theront penetrating a host within a farm raceway is very high. Therefore for a chemical to be effective it would have to demonstrate a quick action that either kills the parasite or renders it unable to infect a host. A concentration of 1 mg $L^{-1}$ of bronopol kills ~ 29% of theronts within 12 h and 100% within 24 h. Those theronts that do survive bronopol exposure are able to go on to infect fish.

The current study has shown that low doses of bronopol (*i.e.* 1 mg $L^{-1}$) over long periods of exposure (*i.e.* 48 h) have subtle effects on the development and survival of the tomonts and theronts of *I. multifiliis*. However, to ensure a 100% kill, one experimental approach might be to increase the dose used in a continuous

bath regime (*i.e*. a minimum bronopol concentration of 2 mg $L^{-1}$), exploring the action of bronopol on the entire external phase of the parasite life-cycle (exiting trophont to penetrating theront). Data from such study could then be used to design trials where low doses of chemical are deployed for longer periods than are currently used. While continuous deployment of chemotherapeutant in commercial-scale systems would be prohibitive (*i.e.* 24 h per day), it is not unreasonable to expect that similar volumes to those currently used in a 30-60 min treatment if administered at a low dose over longer periods (*i.e.* 4 h+) could cause greater reductions in parasite numbers than those achieved by the high dose, short duration treatments currently employed.

In conclusion, the data from this study demonstrates the efficacy of bronopol (Pyceze™) in killing all three external stages of *I. multifiliis* (*i.e.* tomonts, cysts and theronts). The success of this compound in reducing established infections in rainbow trout requires testing under field or near-field conditions to confirm the results of the current *in vitro* study.

**Acknowledgments**

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# CHAPTER 3

## PAPER III

*RESEARCH ARTICLE*

**Effects of continuous exposure to low doses of bronopol on the infection dynamics of *Ichthyophthirius multifiliis* (Ciliophora), parasitising rainbow trout (*Oncorhynchus mykiss* Walbaum)**

**Picón-Camacho, S.M.[a*],** Taylor, N.G.H.[a,b], Bron, J.E.[a], Guo, F.C.[c,d] & Shinn, A.P.[a]

[a]*Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, United Kingdom*
[b]*Cefas Weymouth Lab., Barrack Road, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom*
[c]*Novartis Animal Vaccines Ltd., 4 Warner Drive, Springwood Ind. Est., Braintree, Essex CM7 2YW, United Kingdom*
[d]*Novartis Animal Vaccines Animal Health Canada Inc., Aqua Health Business, 797 Victoria Road, Route 116, Victoria, PE COA 2GO, Canada*

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**Keywords**: *Ichthyophthirius multifiliis*, bronopol, Pyceze™, chemotherapy, management, aquaculture.

**Abstract**

*Ichthyophthirius multifiliis* Fouquet, 1876 infections on intensively reared stocks of fish can increase rapidly, which if left unmanaged, can result in the heavy loss of stock. The present study explores the efficacy of continuous low doses (1, 2 and 5 mg $L^{-1}$) of bronopol (marketed as Pyceze™, Novartis Animal Vaccines Ltd.) in reducing the number of trophonts establishing on juvenile *O. mykiss* held under small scale culture conditions. The effect of bronopol on the colonisation success of theronts added to the water inflow feeding a fish system was investigated by starting the administration of 2 mg $L^{-1}$ bronopol 24 h prior to the start of infection and continuing the deployment of this concentration through the experimental challenge and for 72 h following the introduction of theronts. The effect of bronopol on exiting trophonts through their external development up to and including their infection as theronts was also assessed through the delivery of 1 mg $L^{-1}$, 2 mg $L^{-1}$ and 5 mg $L^{-1}$ bronopol at days 10-36 post-infection. The trial showed that a nominal dose of 2 mg $L^{-1}$ bronopol administered prior to infection significantly reduced the number of theronts surviving in the water column at the time of the initial challenge by 35-48% ($p < 0.05$). Similarly, nominal doses of 2 and 5 mg $L^{-1}$ bronopol administered as the first wave of mature *I. multifiliis* trophonts exited fish developed externally, reduced the number of trophonts establishing on fish as the second cycle of infection by between 46-83% ($p<0.05$; $p<0.001$). Continuous application of 2 and 5 mg $L^{-1}$ bronopol throughout the second and third cycles of *I. multifiliis* infection gave further reductions of between 83-97% ($p<0.05$; $p<0.001$). The number of trophonts on the fish in the control tanks and those treated with 1 mg $L^{-1}$ and 2 mg $L^{-1}$ (pre-infection), by comparison, were observed to increase with successive cycles of infection. From tank trials, this

study therefore, demonstrates that the strategic, long duration, deployment of low doses of bronopol can significantly reduce the number of trophonts establishing on fish and may have a role in farm strategies to manage *I. multifiliis* infections.

## 1. Introduction

Chemotherapeutant treatments that have been employed for the reduction and management of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora) can be divided principally into two groups: those that target and have an effect on the parasitic trophont stage within the host's epithelium, and those targeting the free-living external stages of the parasite *i.e.* the tomonts, cysts and theronts (Matthews, 2005). Historically, the use of malachite green alone and in combination with formaldehyde, was considered the most successful approach in the control of *I. multifiliis* infections in farm systems (Guest, 1983; Whali *et al*., 1993). The ban on the use of malachite green to treat fish for human consumption in Europe (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC) and the reclassification of formaldehyde by the WHO International Agency for Research on Cancer as "carcinogenic to humans" (WHO IARC, 2006) makes necessary the identifycation and development of efficacious replacements. A wide range of chemotherapeutants administered as bath treatments are commonly used to control *I. multifiliis*, infections, including chloramine-T (Shinn *et al*., 2001; Tieman & Goodwin, 2001; Rahkonen & Koski, 2002; Shinn *et al*., 2003a), copper sulphate (Schlenk *et al.*, 1998; Tieman & Goodwin, 2001; Straus, 2008), formaldehyde (Wahli *et al.*, 1993; Heinecke & Buchmann, 2009) hydrogen peroxide, $KMnO_4$ (Straus & Griffin, 2001; Straus & Griffin, 2002, Balta *et al*., 2008), sodium chloride (Selosse & Rowland, 1990; Miron *et al.*, 2003; Mifsud & Rowland, 2008) and

toltrazuril (Mehlhorn *et al.*, 1988; Schmahl *et al.*, 1989; Tojo-Rodriguez *et al.*, 1994). All of these treatments have risks associated with their use (Altinok, 2004; Lauren & MacDonald, 1986; Straus & Tucker, 1993; Noga, 2010). A number of issues are also associated with the discharge of these chemotherapeutants (*e.g.* formaldehyde and sodium chloride) especially during periods of high temperature, when large quantities may be required to reduce infections levels of *I. multfiliis* in farm systems and water supplies may be restricted.

In addition to these chemical approaches, strategies for the physical removal of external stages of the parasite have also been suggested to be successful in reducing infections. Water filtration (80 μm) combined with the use of sodium percarbonate (SPC) was found to be effective at filtering out all tomonts from the water column while 8 mg $L^{-1}$ SPC at 11-12ºC or 32 mg $L^{-1}$ SPC at 21-22ºC for 30-35 min was effective at killing all theronts (Heinecke & Buchmann, 2009). Similarly, Shinn *et al.* (2009) were able to reduce the number of trophonts establishing on rainbow trout, *Oncorhynchus mykiss* (Walbaum) in a commercial-scale trial by 99.55%, using a combination of a vacuum device and a low-adhesion polymer tank lining for the daily removal of cysts from the bottom of the culture system.

From the literature, the continuous deployment of chemicals appears to be an effective alternative strategy for the removal of the external stages of *I. multifiliis* (see Chapter II, Paper I). Bronopol (2-bromo-2-nitropropane-1,3-diol) licenced as Pyceze™ is primarily used in the aquaculture industry for the control of *Saprolegnia parasitica* (Coker) infections of salmonid eggs (Pottinger & Day, 1999; Aller-Gancedo & Fregeneda-Grandes, 2007). The current study set out to establish the efficacy of continuous low doses of bronopol (1, 2 and 5 mg $L^{-1}$) in reducing the number of external stages of *I. multifiliis* and therefore the number of trophonts

subsequently establishing on juvenile *O. mykiss* held under small scale culture conditions during successive waves of infection. The study also set out to determine the effect of bronopol on the colonisation success of theronts entering the system by administering doses of bronopol (2 mg $L^{-1}$) at the time of initial infection.

## 2. Materials and methods

### 2.1. Experimental tank design

A fifteen tank experimental system, each tank measuring 66 cm (l) × 24.5 cm (w) × 20 cm (d) (32.24 litre volume), was constructed within a temperature-controlled aquarium facility set at 14°C ± 1°C in the Institute of Aquaculture, University of Stirling (U.K.). Incoming mains water was dechlorinated by passing it through an ELGA C960 carbon filter. Dechlorinated water passed into two 200 litre header tanks, linked in series by a pair of 54 mm pipes, within which the water was aerated and heated to 15°C ± 1°C using a 5 kilowatt heater (Howden, Scotland). Heated water then passed into the trial tanks via PVC piping. The flow to tanks was regulated using taps fitted to the inflow of each tank and monitored by measuring the outflow rate (litres $min^{-1}$) for each tank. Water flow was measured twice per day (9:00 and 18:00) and adjusted, when necessary, to 0.5 litre $min^{-1}$. A constant head of water, and therefore pressure and flow rate to each tank, was maintained by using an excess flow of incoming water into the first header tank and fitting two overflow pipes to carry the excess water away. Each tank was randomly allocated fifty ~5 g rainbow trout (mean stocking density 7.93 kg $m^{-3}$) taken from a single stock of fish collected from a commercial trout hatchery with no history of *I. multifiliis*, within the Central Scotland area. The fish were acclimated for a period of

fourteen days prior to the start of the experiment.

**2.2. Delivery of bronopol to experimental tanks**

A 12-channel peristaltic pump (202F Watson-Marlow Inc., USA) and 2 mm Teflon tubing was used to deliver appropriate doses into triplicate tanks per dose. Doses tested comprised 1 mg $L^{-1}$, 2 mg $L^{-1}$ (defined as 2 mg $L^{-1}$ pre-infection or 2 mg $L^{-1}$ post-infection), 5 mg $L^{-1}$ and 0 mg $L^{-1}$ (no treatment, control) from a series of stock solutions containing the appropriate concentration of bronopol. At the start of the experiment, each tank received an initial dose of bronopol necessary to bring it to the required concentration before the pump was switched on. Ten litres of each concentration of bronopol (Pyceze™ BP 50% *w/v*) was made up fresh twice per day (10:00 and 22:00) to ensure that there was no deterioration in the activity of the bronopol added to the system.

The development of the *I. multifiliis* cysts and theronts maintained in culture (see section below) were carefully monitored. On the day before the theront challenge, the peristaltic pump delivering 2 mg $L^{-1}$ bronopol to the three 2 mg $L^{-1}$ pre-infection tanks was started. The addition of bronopol to these tanks continued for a further 3 days post-infection to cover the time period free-living theronts are able to survive. The aim of pre-treating tanks was to investigate the impact of bronopol in reducing initial or colonising infections of *I. multifiliis* theronts entering culture systems. The remaining triplicate sets of tanks, (0 mg $L^{-1}$ controls; 1 mg $L^{-1}$; 2 mg $L^{-1}$ post-infection; 5 mg $L^{-1}$) were exposed to theronts of *I. multifiliis* in the absence of bronopol. On day 9 post-infection, the peristaltic pump was switched on and the appropriate dose of bronopol was delivered continuously to all the test groups. Fresh batches of bronopol stock solutions were made up and replaced

every 12 hours and the pump was run continuously for the remainder of the experiment (~27 days).

### 2.3. Bronopol dose confirmation studies

The dose of bronopol delivered to each tank was assessed at the three sampling points during the experimental period: 10-11 days post infection, 23-24 days post infection and 35-36 days post infection. On each sampling occasion, a 100 ml water sample was taken from each tank and a second sample taken one hour later. Each sample was taken using new 125 ml amber glass Winchester bottles, which were rinsed once in the relevant tank water before a sample was collected from the mid-point (depth and position) in each tank. Immediately after sampling, the water samples were stabilised by the addition of 50 µl of 85% orthophosphoric acid solution. Each bottle was stored at 4°C until they were shipped in a chilled container by courier to Novartis Tiergesundheit AG, Switzerland for analysis.

### 2.4. Experimental infection with theronts of *I. multifiliis*

To establish a culture of *I. multifiliis*, a sample of naturally infected *O. mykiss*, held on a commercial trout facility on the River Bladnoch was used. Tomonts were harvested as they naturally exited stock fish and the resulting theronts were used to infect a second, naïve, batch of ~ 5 g *O. mykiss* to amplify the number of parasites required for the initial theront challenge. Harvested tomonts were collected in batches of 30 and placed in pre-washed 10 ml plastic Petri dishes containing 7 ml 15°C dechlorinated tank water. Tomonts were maintained at 15°C ± 1°C in an environmental cabinet (WTB Binder Labortechnik GmbH (Germany); 12 L: 12D) until the tomonts had encysted and released their theronts (usually within 23-30

h). The number of theronts generated by each amplification cycle was determined and confirmed by the use of the vital stain, 0.5% neutral red (5 µl stain per 1 ml of theront culture) placed in a Sedgewick Rafter counting chamber.

To infect the fish, the water flow leaving the main header tank was stopped and the peristaltic pump feeding the three 2 mg $L^{-1}$ pre-infection tanks was switched off. A total of 70 ml of theront culture (154 theronts $ml^{-1}$; ~200 theronts $fish^{-1}$) was carefully added to each tank at the inflow pipe. The tanks were then maintained under static conditions for 2 h in the dark before the water flow was switched back on and the peristaltic pump restarted. Aeration was maintained via an airstone, which was positioned close to the outflow pipe during the experimental exposure, and the fish were monitored every 30 min throughout the infection period and for 5 h post-infection. Following infection, the system was maintained and adjusted daily to ensure that a water temperature of 15°C ± 1 °C and an outlet flow rate of 0.5 L $min^{-1}$ was maintained.

**2.5. Post-mortem examination**

Following the experimental challenge, the first sub-sample of fish was taken on days 9-10 post-infection with 9 fish being removed from each tank and killed by an overdose of 10 ml 10 $L^{-1}$ 2-phenoxyethanol (MERCK, Germany). The gills were then removed and the number of *I. multifiliis* trophonts on all the body surfaces and the gills were assessed using an Olympus SZ30 dissecting microscope at ×4 magnification. Further sub-samples of 9 fish were taken on day 23-24 and on day 35-36 post-infection, these timings coinciding with the second and third waves of infection using the in-stage timings reported by Matthews (2005) for the life-cycle of *I. multifiliis* at 15°C.

### 2.6. Statistical analysis

The number of trophonts on each fish at each sampling occasion was recorded and then analysed using Minitab version 15 (Minitab Inc., USA) and SPSS version 18.0 (IBM, USA) to assess the effects of bronopol treatment. The trophonts counts were subjected to an Anderson-Darling normality test and Levene's test for homogeneity of variance. To adjust the data to a normal distribution the data were subjected to a log 10 transformation.

Differences between treatments and between replicates (tanks) from each treatment were assessed using a one-way factorial nested general linear model (GLM). In case of significant differences between the different bronopol concentrations multiple comparisons were made using a *post-hoc* Tukey's test.

## 3. Results

### 3.1. Infection level

The trophont burdens on the experimental fish were determined at 3 time points, 10 days after initial infection, when only one triplicate set of tanks received bronopol treatment, and then on days 23 and 35 post-infection to determine the relative increase or increase in numbers during the second and third waves of *I. multifiliis* infection in the presence of varying concentrations of bronopol continuously delivered to each tank (Table 1).

*First sub-sample (10 days post-infection)*

On day 10 post-infection, 9 fish from each tank were sampled. The raw counts and the mean number of trophonts per fish and treatment group are presented in Table 1. A

one-way nested GLM showed that there were significant differences in the number of trophonts between tank replicates (p = 0.006) but not between treatments (p = 0.154). The data from each treatment were pooled and a Tukey-Kramer *post-hoc* multiple comparisons test revealed significant differences for the 2 mg $L^{-1}$ pre-infection group of fish with respect to the levels of three of the other groups (2 mg $L^{-1}$ post-infection, 5 mg $L^{-1}$ and the 0 mg $L^{-1}$ control groups) that had not received a dose of bronopol at this stage (Figure 1).

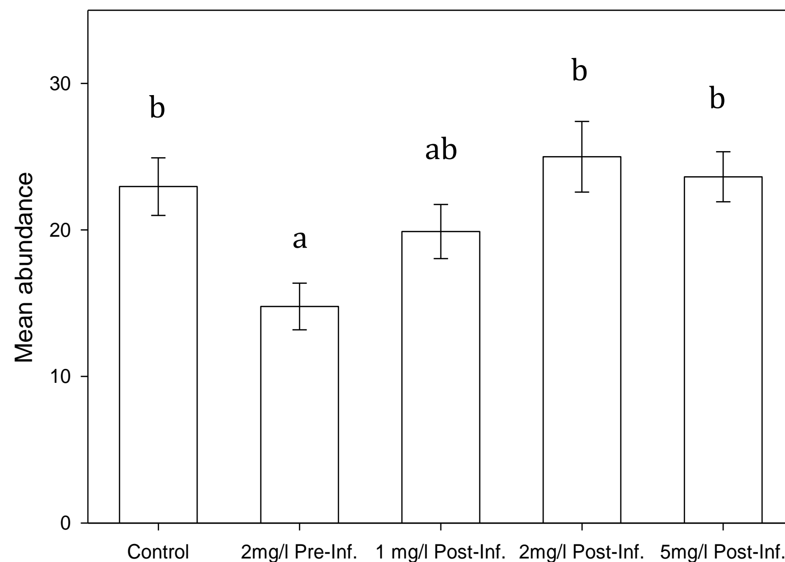


**Figure 1**. Mean abundance ± S.E. of trophonts of *I. multifiliis* on each fish in each group of fish (pooled data) sampled on the first sampling point (10-11 days post-infection). Different letters over each bar show significant differences ($p<0.05$) among the different concentrations of bronopol.

**Table 1.** Mean number of trophonts±1 S.D. per fish in each test group determined at the first (10-11 days post infection), second (23-24 days post-infection.) and third (35-36 days post infection) sampling points. *No fish survived the trophonts exiting the second wave of infection in this replicate due to the high levels of parasite burden. Treat.: treatment; Rep.: replicate.

| Treat. | 0 mg $L^{-1}$ bronopol post- infection | | | 2mg $L^{-1}$ bronopol pre- infection | | | 1mg $L^{-1}$ bronopol post- infection | | | 2mg $L^{-1}$ bronopol post-infection | | | 5mg $L^{-1}$ bronopol post-infection | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tank | **Rep. 1** | **Rep. 2** | **Rep. 3** | **Rep. 1** | **Rep. 2** | **Rep. 3** | **Rep. 1** | **Rep. 2** | **Rep. 3** | **Rep. 1** | **Rep. 2** | **Rep. 3** | **Rep. 1** | **Rep. 2** | **Rep. 3** |
| • **First sampling point (10-11 days post-infection)** | | | | | | | | | | | | | | | |
| Mean ±SD | 21.3 ±8.4 | 21.2 ±10.2 | 26.3 ±11.7 | 17.6 ±8.7 | 8.8 ±6.3 | 16.1 ±5.2 | 14.0 ±5.6 | 18.6 ±9.9 | 27.1 ±8.3 | 22.3 ±11.1 | 31.8 ±13.4 | 20.9 ±11.3 | 27.9 ±8.7 | 20.9 ±7.9 | 22.1 ±9.4 |
| Average | **23.0±10.1** | | | **14.8±7.7** | | | **19.9±9.6** | | | **25.0±12.5** | | | **23.6±8.9** | | |
| • **Second sampling point (23-24 days post-infection)** | | | | | | | | | | | | | | | |
| Mean ±SD | 204.6 ±102.8 | 262.2 ±178.2 | 427.4 ±223.3 | 393.1 ±274.5 | 58.6 ±35.7 | 239.6 ±148.6 | 254.0 ±166.2 | 552.9 ±286.2 | 377.9 ±218.1 | 258.6 ±96.0 | 108.0 ±47.8 | 184.3 ±75.8 | 10.9 ±4.1 | 84.8 ±37.4 | 93.0 ±57.2 |
| Average | **342.0±298.78** | | | **230.4±223.1** | | | **396.3±255.6** | | | **183.6±96.0** | | | **67.0±53.4** | | |
| • **Third sampling point (35-36 days post- infection)** | | | | | | | | | | | | | | | |
| Mean ±SD | 1328.7 ±1458.5 | 482.22 ±743.26 | * | 2508.9 ±1918.5 | 111.2 ±59.7 | 262.0 ±499.3 | 376.6 ±380.6 | 1947.5 ±1949.4 | 1357.1 ±1491.4 | 262.0 ±499.3 | 145.3 ±174.3 | 184.4 ±149.6 | 13.3 ±3.1 | 31.6 ±23.8 | 23.8 ±31.3 |
| Average | **947.8±1240.5** | | | **1016.0±1609.2** | | | **1177.8±1523.6** | | | **151.9±173.1** | | | **22.8±26.0** | | |

*Second sub-sample (23 days post-infection)*

The raw data and the mean number of trophonts per fish and per treatment group determined from the second sub-sample of fish (23 days post-infection) is shown in Table 1. The one-way nested GLM showed significant differences within the replicates ($p<0.001$) but not between treatments ($p = 0.061$). A Tukey-Kramer *post-hoc* test for multiple comparisons revealed significant differences between the 5 mg $L^{-1}$ and the remaining groups (0 mg $L^{-1}$, 2 mg $L^{-1}$ pre-infection and 2 mg $L^{-1}$ post-infection) whereas no significant differences were found between the 2 mg $L^{-1}$ pre-infection and the 2 mg $L^{-1}$ post-infection groups (Figure 2).

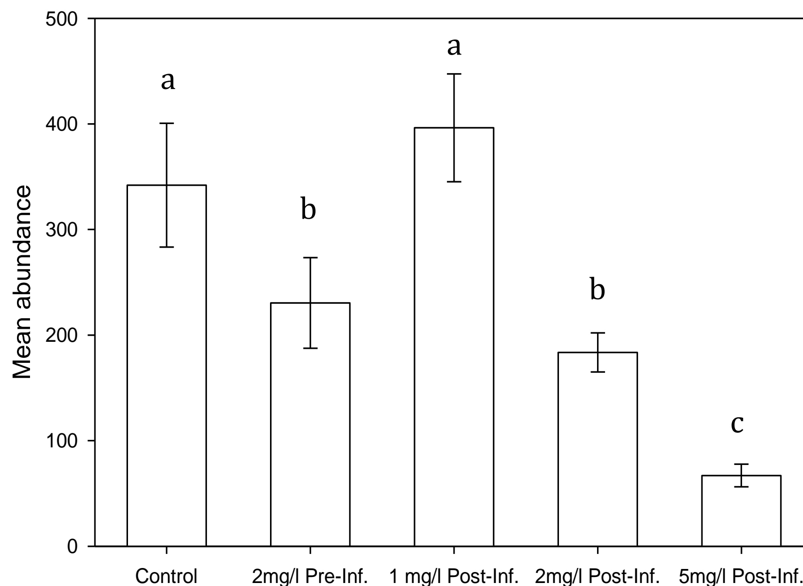


**Figure 2.** Mean abundance ± S.E. of trophonts of *I. multifiliis* on each fish in each group of fish (pooled data) sampled on the second sampling point (23-24 post infection). Different letters over each bar show significant differences ($p<0.05$) among the different concentrations of bronopol.

*Third sub-sample (35 days post-infection)*

The raw data and the mean number of trophonts per fish per treatment group on

the third sub-sample (35 days post-infection) of fish are shown in Table 1. A one-way nested GLM indicated that there were significant differences in the number of trophonts between the treatments ($p<0.05$) as well as between the replicates ($p<0.001$). A Tukey-Kramer *post-hoc* multiple comparisons test revealed significant differences between the 2 mg $L^{-1}$ post-infection (84% reduction when compared to the control) and 5 mg $L^{-1}$ (98% reduction when compared to the control) when compared to the rest of the treatment groups (0 mg $L^{-1}$ and 1 mg $L^{-1}$ post-infection; 2 mg $L^{-1}$ pre-infection) (Figure 3).

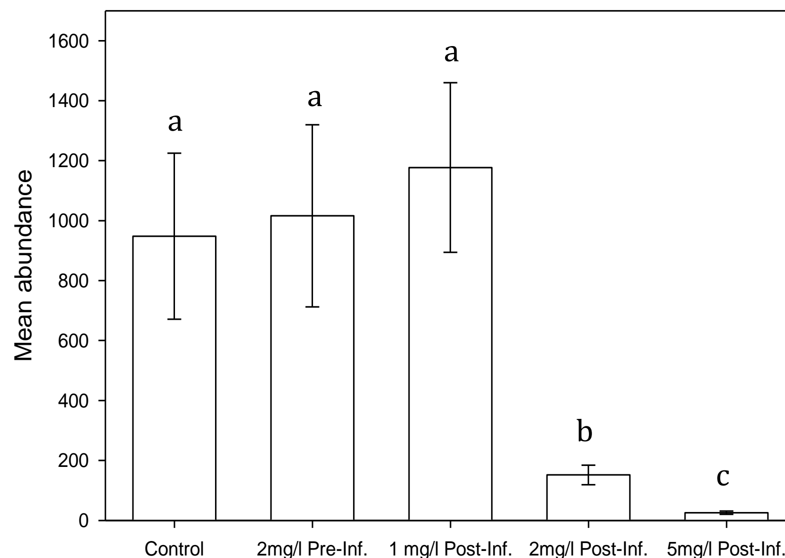


**Figure 3**. Mean abundance ± S.E. of trophonts of *I. multifiliis* on each group of fish (pooled data) sampled on the third sampling occasion (35-36 days post-infection). Different letters over each bar show significant differences ($p<0.05$) among the different concentrations of bronopol.

### 3.2. Bronopol dose confirmation study

The concentration of bronopol (Pyceze™) in each tank was determined from replicate samples taken on three occasions in the experiment, on days 10, 24 and 35 post-infection. Analysed concentrations were close to the targeted nominal doses (data not

shown), except for slight deviations most likely result of localised variations in the water flow rate delivered to each tank. The flow rates to each tank were adjusted twice a day to 0.5 L min$^{-1}$ to ensure that fluctuations were minimised and the target doses met.

**3.3. Fish mortalities**

Across the 35 day trial, there were low numbers of mortalities within the treatment groups (*i.e.* average number of fish lost across the 12 treatment tanks was 5.63 ± 3.07), with the heaviest mortalities occurring within the triplicate set of control tanks (average number of fish lost was 17.66 ± 11.93). These losses were most likely the result of the large number of trophonts exiting fish from the second wave of infection (see Table 1).

**4. Discussion**

Since the U.K. ban on the use of malachite green in 2000, there has been a concerted effort to identify suitable replacement agents or strategies for the effective control and management of *I. multifiliis* infections in commercial farm systems (Shinn *et al.*, 2009). Currently formaldehyde is the most common treatment deployed to treat *I. multifiliis* infections in trout farms. In 2004, formaldehyde was reclassified by the WHO International Agency for Cancer Research as "carcinogenic to humans" (WHO IARC, 2006), thus it is possible that its use elsewhere, including the aquaculture industry, will be reviewed in the near future. The continuous application of low doses of bronopol (Pyceze™) in the current trial (*i.e.* 2-5 mg L$^{-1}$) resulted in significant reductions in the number of *I. multifiliis* stages surviving and able to re-infect. Bronopol is a broad spectrum

disinfectant that has been demonstrated to cause membrane damage to microbial organisms *e.g. Escherichia coli* Migula, 1895 and *Pseudomonas aeruginosa* Migula, 1900, through inhibition of the thiol enzymes in cell membranes (Croshaw *et al.*, 1964; Stretton & Mason, 1973; Saito & Onoda, 1974; Shepherd *et al.*, 1988) and has been widely used in food production, water disinfection and incorporated into healthcare products (Kumanova *et al.*, 1989). Within the aquaculture industry, bronopol, under the product name Pyceze™ (LC50 96 h for *O. mykiss* is 41.2 mg $L^{-1}$; Novartis Report, 2006) is licensed for the disinfection of salmonid eggs and for the treatment against the oomycete pathogen *Saprolegnia parasitica* (Pottinger & Day, 1999; Aller-Gancedo & Fregeneda-Grandes, 2007). In addition to its anti-mould properties, bronopol has been successfully used in the broad spectrum control of bacterial infections on the eggs of several commercially important marine fish species including *Gadus morhua* L. and *Melanogrammus aeglefinus* L. (Treasurer *et al.*, 2005; Birkbeck *et al.*, 2006). In line with the current results are those reported from the dinoflagellate *Amyloodinium ocellatum* Brown, 1931. The prolonged exposure to bronopol was shown to significally impact the cell division of the tomont stage of *A. ocellatum* and the subsequent release of dinospores, but not the number of trophonts surviving and developing within the fish host (Roberts-Thomson, 2007).

*In vitro* work by Shinn *et al.* (2010) using a 1 mg $L^{-1}$ continuous dose of bronopol reduced the number of theronts surviving after 24 h by 30.68% and after 48 h by 73.77% when compared to the control groups. The continuous exposure of tomonts to 1 mg $L^{-1}$ bronopol during their external development phase reduced the number of tomonts surviving by 19.28%, causing asymmetric division in those encysting, and reducing the number of theronts that were alive at 36 h by 56.12%.

Based on those findings, it was deemed appropriate to employ a minimal exposure dose of 2 mg $L^{-1}$ in the current trial.

The current trial demonstrated that the use of a nominal dose of 2 mg $L^{-1}$ bronopol (actual ~2.45 mg $L^{-1}$) prior to and throughout the infection of the experimental fish with theronts of *I. multifiliis* significantly reduced the number of trophonts establishing on treated fish by 35-48% (14.78 ± 7.65 on the 2 mg $L^{-1}$ exposed fish; 22.96 ± 10.07 on the 0 mg $L^{-1}$ control fish; Table 1, Figure 1). Similarly, nominal doses of 2 mg $L^{-1}$ (actual ~2.07 mg $L^{-1}$) and 5 mg $L^{-1}$ (actual ~7.95 mg $L^{-1}$) in the second wave of infection reduced the number of trophonts establishing on fish by between 46 and 83%, while doses during the third wave of infection gave further reductions of between 83 and 97% (Table 1, Figures 2-3). The latter result shows that the trophont burden on the fish increases with successive waves of infection 0 mg $L^{-1}$, 1 mg $L^{-1}$ and 2 mg $L^{-1}$ pre-infection groups (Table 1). Levels of infection in the 2 mg $L^{-1}$ and 5mg $L^{-1}$ post-infection groups, however, after rising briefly during the second wave of infection, were significantly lower during the third wave of infection and were below those observed during the second wave of infection (Table 1, Figures 2-3). The experimental design had hoped to follow a fourth wave of infection, providing the health and welfare of the experimental fish were not compromised, however, the high numbers of trophonts observed on fish in the 0 mg $L^{-1}$, 1 mg $L^{-1}$ and the 2 mg $L^{-1}$ pre-infection tanks were in excess of 947 trophonts per fish (Table 1) and the trial was therefore terminated at this point.

Current farm practices involve the use of daily, short duration (30-60 min) high dose bath treatments which, in the case of formaldehyde, are administered over a 10 day period to manage infections. These treatments, however, are only

effective in removing the free-living stages of *I. multifiliis* (*i.e.* tomonts, tomites and theronts) during the window of chemical exposure. The cysts and trophonts of *I. multifiliis* appear to be largely unaffected by formaldehyde hence such treatments appear to have little impact on the dynamics of *I. multifiliis* infection on farms (unpublished data). The work of Shinn *et al.* (2009) demonstrates in this respect that during short periods of warm weather, the number of *I. multifiliis* trophonts establishing on cultured fish can increase exponentially, with burdens on fish rising sharply from 13.69 ± 6.57 trophonts fish$^{-1}$ to 361.88 ± 260.75 trophonts fish$^{-1}$ in just 2 weeks.

Bodensteiner *et al.* (2000) demonstrated that infections could be managed (*i.e.* mortalities <10%) by increasing the rate of water turnover in infected systems to 4.5 / h (min water velocity 20.3 cm min$^{-1}$). This, however, is based on the assumption that water is not a constrained resource. For farm sites with limited resources, however, dropping the water level to achieve the required water velocity is not advised as this only serves to concentrate both the fish and parasite together.

Increasing the window of chemical exposure targets a greater proportion of the parasite stages released from the fish and subsequently the number surviving and able to re-infect the fish. A farm management strategy which focuses on the development of methods for the deployment of low dose chemotherapeutants at strategic time points in the infection cycle and over greater time periods (*i.e.* >60 min d$^{-1}$) could provide a key contribution to white spot control. If managed carefully, the total chemical used need not exceed quantities currently employed; therefore satisfying regulatory bodies, but could potentially prove cost effective by reducing the number of fish lost and the number of treatment events required

during periods of warm weather. From tank trials, the current study has demonstrated that continuous low dose exposure to bronopol (Pyceze™) is capable of reducing numbers of *I. multifiliis* both on and off fish. It is therefore concluded that low doses of bronopol used for periods of greater than 60 min $day^{-1}$ may have the potential to fulfil a role in farm strategies to manage *I. multifiliis* infections.

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# CHAPTER 3

## PAPER IV

*SHORT-COMMUNICATION*

# ***In vitro*** **assessment of the chemotherapeutic action of a specific hydrogen peroxide, peracetic, acetic and peroctanoic acid based formulation against the free-living stages of *Ichthyophthirius multifiliis* (Ciliophora)**

**Picón-Camacho, S.M.**[a], Marcos-Lopez, M.[a], Beljean, A.[b], Debeaume, S.[c] & Shinn, A.P.[a]

[a]*Institute of Aquaculture, University of Stirling, FK9 4LA, Stirling, United Kingdom*
[b]*Schering-Plough Intervet, 92 Rue Baudin, 92307 Levallois Perret Cedex, France*
[c]*ECOLAB S.N.C., 8, Rue Rouget-de-Lisle, 92442 Issy-les-Moulineaux, France.*

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**Keywords**: *Ichthyophthirius multifiliis*, chemotherapy, hydrogen peroxide, peracetic acid, acetic acid, Incimaxx Aquatic.

## 1. Introduction

*Ichthyophthirius multifiliis* Fouquet, 1876, commonly known as "white spot", is a ciliate protozoan that reputedly infects all freshwater fish species and result in significant economic losses to the food and ornamental fish industry (Ling *et al*., 1991; Valtonen & Koskivaara, 1994; Rintamäki-Kinnunen & Valtonen, 1997).

There are four key developmental stages within the life-cycle: the sub-epithelial, parasitic trophont (1) which, when mature, exits the host as a free-swimming tomont (2). Tomonts quickly settle on an appropriate substrate, encyst (3) and then undergo binary fission to produce tomites. Released tomites rapidly differentiate into theronts (4) which become infective (Lom & Dyková, 1992; Matthews, 2005).

Growing concerns regarding environmental pollution and human health impacts have lead to a tightening of regulations concerning chemotherapy applications in aquaculture. Classically, the most effective compound for use against an infection of *I. multifiliis*, which includes cytotoxic action against the trophonts, was malachite green (Leteux & Meyer, 1972; Whali *et al*., 1993; Tieman & Goodwin, 2001; Buchmann *et al*., 2003). Although still widely used in the ornamental industry, the use of malachite green in food fish production has been banned in Europe (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC), the United States and elsewhere given its teratogenic potential (Meinelt *et al*., 2009). In its place, other broad spectrum chemotherapeutants including formaldehyde, hydrogen peroxide, potassium permanganate, sodium chloride, chloramine T, copper sulphate and peracetic acid based formulations, have been commonly used with mixed success (Meinelt *et al*., 2007; Meinelt *et al*., 2009; Straus & Meinelt, 2009; Sudová *et al*., 2010). While these compounds are able to

remove the free-swimming stages in the water column during the window of chemical exposure, they have limited success, if any, against the cysts and the trophonts situated beneath the host's epithelium. Consequentially, multiple bath treatments are required to manage the dynamics of infection. This lack of efficacy for use in the food fish industry has lead to the search for and the development of new, replacement chemotherapeutic agents.

The current study set out to determine the efficacy of Incimaxx Aquatic (ECOLAB, France) a commercially available hydrogen peroxide, peracetic, acetic and peroctanoic acid based formulation (HPPAPA). This product is currently licensed for use in freshwater and marine aquaculture for the broad spectrum control of bacterial, fungal and parasitic infections (ECOLAB, 2005). The objective of the present study was to determine the *in vitro* toxicity of this specific HPPAPA product over a range of doses (8-15 mg $L^{-1}$ for 60 min) against the three principal free-living stages of *I. multifiliis i.e.* tomonts, cysts and theronts.

## 2. Materials and methods

### 2.1. Source of *I. multifiliis*

Tomonts of *I. multifiliis* were harvested as they naturally exited from a laboratory maintained culture of infected rainbow trout, *Oncorhynchus mykiss* (Walbaum), and then placed in batches of 20-30 tomonts in 10 ml diameter Petri dishes (Sterilin, U.K.) containing dechlorinated water at 15$^{o}$C. The source of *I. multifiliis* used for the trial originated from naturally infected *O. mykiss* stock held on a commercial trout facility in Scotland (name withheld).

### 2.2. Chemotherapy trials

The tomonts were either used directly, being randomly allocated to one of the test vessels for immediate chemotherapeutant exposure (*i.e.* treatment of tomonts), or were placed in 10 ml Petri dishes containing dechlorinated water and placed in a WTB Binder Labortechnik GmbH (Germany) precision environmental chamber and then maintained at 15ºC and 12:12 (L: D) until the parasites had encysted and reached, at least, the 8 cell stage or had differentiated into theronts (after approx. 36-48 h). Each concentration of the HPPAPA based product (8, 12 and 15 mg $L^{-1}$) was made up fresh using dechlorinated water at 15ºC and used within 60 min of preparation. Each test solution was tested in triplicate against a set of 4 control dishes.

For the tomont trials, parasites were collected and placed in batches of 10 per Petri dish in 5 ml dechlorinated water at 15ºC. Then, 5 ml of a 2× dose of the relative concentration of HPPAPA (*i.e.* 16, 24 and 30 mg $L^{-1}$) was added so that the nominal dose was obtained (*i.e.* 8, 12 and 15 mg $L^{-1}$). Following chemical addition, the Petri dishes were immediately transferred to a WTB Binder Labortechnik GmbH (Germany) precision environmental chamber maintained at 15ºC and 12:12 (L: D). After 60 min, the condition of the parasites was evaluated before the concentration of HPPAA in each dish was replaced by two changes of 15ºC dechlorinated water by inserting a fine 20 μm mesh filter and carefully siphoning out the old medium using a 1000 μl micropipette fitted with wide bore tips. The efficacy of each dose of HPPAPA was assessed by comparing the number of live stages against dead ones in each Petri dish against a control set of dishes after its replacement with dechlorinated water.

For the cyst trials, tomonts were collected and maintained as above until

the parasites had encysted and had reached, at least, the 8 cell stage. The cysts were then exposed to the appropriate chemical dose of HPPAPA for 60 min before the chemical was replaced and monitored as described above. The efficacy of each dose of HPPAPA was assessed by monitoring the Petri dishes until all the cysts had either died or continued their development until they had released their theronts.

For the theront trials, tomonts were collected and maintained as above for approximately 2 days until the cysts had released their stages. The dishes were maintained for a further 12 h prior to the start of the experiment to ensure that all the tomites had differentiated into theronts and were infective. Aliquots of 2 ml containing mature theronts were placed into new 10 ml Petri dishes using wide bore micropipette tips and exposed to 2 ml of 2× the nominal concentration of HPPAPA. After 60 min, the viability of the theronts was determined by enumerating the number of dead and live theronts in two 1 ml aliquots of theront culture taken from each dish and dispensed as twenty 50 µl droplets using a wide bore micropipette tip onto a glass slide and examining them at ×4 magnification on an Olympus SZ40 dissecting microscope.

**2.3. Statistical analysis**

For each parasite stage, the mortality data is given as a mean percentage ± standard error. The percentage data were transformed using an arcsin function and then analysed using a one-way ANOVA. Significant differences ($p \leq 0.05$) between treatments were identified using a Tukey-Kramer multiple comparisons test. All the analyses were carried out using the software SPSS version 18.0 (IBM, USA).

## 3. Results

The current study demonstrates that the administration of an 8, 12 or 15 mg $L^{-1}$ dose of a specific HPPAPA based product for 60 min kills near all free-living stages of *I. multifiliis* (*i.e*. theronts, tomonts and cysts) within the window of treatment (see Table 1).

**Table 1.** Percentage of the theront mortality ± 1 S. E. of the free-living stages of *I. multifiliis* (*i.e.* tomonts, cysts and theronts) exposed for 60 min to 8, 12 or 15 mg $L^{-1}$ of a specific formulation of hydrogen peroxide, peracetic, acetic and peroctanoic acid (Incimaxx Aquatic). The numbers given in brackets represent the number of dead individuals recorded out of the total number exposed to the relative concentration of chemical product. *shows significant differences (p≤0.001) compared against the control group; n= number of counts per replicate. †The mortalities observed in the tomont and theront control groups fall within acceptable limits given the longevity of each parasite stage (*i.e.* ≤ 20%).

| Percentage mortality at different concentrations of HPPAA | | | | |
|---|---|---|---|---|
| **Treatment / *I. multifliis* stage** | **0 mg $L^{-1}$ (n=8)** | **8 mg $L^{-1}$ (n=6)** | **12 mg $L^{-1}$ (n=6)** | **15 mg $L^{-1}$ (n=6)** |
| **Tomont** | 7.40 ± 6.27 (4 / 54)† | 100 ± 0.0 (26 / 26)* | 100 ± 0.0 (29 / 29)* | 100 ± 0.0 (26 / 26)* |
| **Cyst** | 0 ± 0.0 (0 / 59) | 100 ± 0.0 (27 / 27)* | 100 ± 0.0 (29 / 29)* | 100 ± 0.0 (29 / 29)* |
| **Theront** | 15.13 ± 6.89 (84 / 652)† | 98.30 ± 2.31 (320 / 326)* | 100 ± 0.0 (264 / 264)* | 100 ± 0.0 (197 / 197)* |

## 4. Discussion

HPPAPA as the commercial product Incimaxx Aquatic is currently licensed for use in France and is under consideration in Greece, Norway and the United Kingdom. Using Behrens & Karber's (1935) method, the HPPAPA $LC_{50}$ (24 h) for *O. mykiss* was determined to be 8.163 ppm (ECOLAB, 2005). The hydrogen peroxide and

carboxylic acid-based components of the formulation make it a powerful oxidizing agent that has had demonstrable effects against a broad spectrum of bacterial and fungal pathogens of freshwater fish. Of particular note, is its reported efficacy against *Aeromonas salmonicida* Lehmann et Neumann, 1896, *Flavobacterium* spp., *Flexibacteria* sp*., Lactococcus* sp., *Yersinia* spp., *Vibrio anguillarum* Bergeman, 1909, the monogenean *Gyrodactylus* spp. and the oomycete *Saprolegnia parasitica* (ECOLAB, 2005).

The constituent components of Incimaxx Aquatic have been used separately with mixed success against the free-living stages of *I. multifiliis*. A dose of 200 mg $L^{-1}$ hydrogen peroxide was found to kill ~15% of theronts within the 1 h window of treatment (Shinn, unpublished) while daily treatments of 25 mg $L^{-1}$ for 20 days was unsuccessful in preventing the total loss of infected fish (Tieman & Goodwin, 2001). The use of peracetic (PAA) and acetic acid (AA) based products against certain free-living stages, however, appears to be more effective. Straus & Meinelt (2009) used a 40% formulation of PAA and 25% of AA (Wofasteril® E400, KESLA PHARMAWOLFEN GmbH, Greppin, Germany) to kill 95% of theronts when applied as a 0.40 mg $L^{-1}$ dose for 3 h. Using the same formulation, Meinelt *et al.* (2009) were able to kill 100% of tomonts using a dose of 2 mg $L^{-1}$ PAA within 48 h. The exposure of cysts to 2.5 mg $L^{-1}$ for 4 h, however, did not kill the cysts or appear to significantly reduce the number of theronts subsequently emerging from cysts. Subsequently, the study of Sudová *et al.* (2010) experimentally challenged *Cyprinus carpio* L. with theronts and then continuously administered 1 mg $L^{-1}$ PAA (40%) for 4 days and found a significant reduction in the number of trophonts pre- and post-PAA treatment. Peroctanoic acid as treatment of *I. multifiliis* has not been tested on its own.

The use of the HPPAPA formulation in the current study, demonstrates that a dose of 8 mg $L^{-1}$ for 60 min significantly reduced ($P<0.001$) the number of free-living stages of *I. multifiliis* within the window of chemical application. Importantly, the 8 mg $L^{-1}$ dose was able to kill all cysts and prevent the release of theronts. Given that the development of trophonts ranges from 14 days at 15°C (Shinn *et al*., unpublished data) to 5 days at 21°C (Ewing & Kocan, 1986) and that the external development of tomonts to the release of tomites from the cyst takes 57.3 h at 15°C to 18.5 h at 24°C (Shinn *et al.*, unpublished data). The strategic deployment of daily 60 min treatments with the HPPAPA product Incimaxx Aquatic at temperatures below ~22.5°C may produce significant reductions in the number of external *I. multifiliis* stages that survive and are able to infect fish. Ongoing studies are currently investigating regimes using low doses of this product in reducing parasite burdens on fish already infected with *I. multifiliis*.

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# CHAPTER 4

# TESTING OF NON-CHEMICAL TREATMENTS

# CHAPTER 4

## PAPER V

*RESEARCH ARTICLE*

**Mechanical control of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora) in a rainbow trout hatchery**

Shinn, A.P.[a], **Picon-Camacho, S.M.[a]**, Bawden, R.[b] & Taylor, N.G.H.[a,c]

[a]*Institute of Aquaculture, University of Stirling, Stirling, FK9 4LA, United Kingdom*

[b]*Pisces Engineering Ltd., Ballochallan, Cambusmore, Callander, FK17 8LJ,United Kingdom*

[c]*Cefas, Weymouth Lab;, Barrack Road, The Nothe, Weymouth, Dorset DT4 8UB, UK*

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**Contributions:** The present manuscript was compiled and written in full by Dr. Andrew Shinn with the help from Dr. Nicholas Taylor and the candidate. The candidate has been actively involved in the *in vitro* trials, design of the mechanical device, sampling, parasite counting and data analysis with the assistance of Drs. Andrew Shinn and Nicholas Taylor. Support on the mechanical device design was provided by Robert Bawden. Support on site (commercial rainbow trout hatchery) was provided by Mark Davies.

**Keywords**: *Ichthyophthirius multifiliis*, white spot, mechanical control, protozoan, parasite control, *Oncorhynchus mykiss*.

**Abstract**

*Ichthyophthirius multifiliis*, more commonly known as 'white spot' is recognised as one of the most pathogenic diseases of wild and cultured freshwater fish. In trout hatchery systems infections can quickly establish and result in high mortality if left unregulated. Current control centres around the use of regular bath treatments with formaldehyde, however, treatments are not always effective and are labour intensive. Environmentally safe, non-chemical alternatives are required. This paper describes the performance of a mechanical system developed to remove cysts from commercial trout raceways. The system consists of two parts: a specially designed suction head connected to a pump that is used to vacuum the bottom of hatchery raceways, and a low-adhesion polymer raceway lining. Following tests on a series of potential linings, the most efficacious product in preventing settlement of cysts was used to line three raceways in a commercial rainbow trout (*Oncorhynchus mykiss*) hatchery. Over a period of three months, lined raceways were vacuumed on a daily basis. The three control raceways were maintained according to the farms normal husbandry procedures. Approximately thirty fish were sampled from each raceway at fortnightly intervals, and the abundance of the parasite in each raceway determined. The mechanical system led to a significant reduction in the abundance of the parasite in the test raceways, with a mean abundance of 361.88 ± 260.75 parasites per fish in control raceways compared with 1.64 ± 1.59 parasites per fish in the test raceways at the peak of the infection. Additionally fish survival was significantly higher in test raceways over control, with a mean of 84.5% of the stock surviving in the test raceways compared with only 70.6% in the controls by trial end.

## 1. Introduction

The mechanical prevention and control of pathogens is often overlooked, but is long established and has proven to be effective under many different circumstances. Obvious examples include the use of flea/knit combs to reduce the burden of fleas and head lice, mosquito nets to prevent malaria and barrier contraceptives such as condoms to avoid the transmission of many sexually transmitted diseases. Less well known examples also exist to control parasitic infections found in aquaculture. Control of infections by species of *Argulus* Müller, 1785 in carp culture has been achieved through the use of removable substrates upon which the parasite lays its eggs. These substrates are removed and cleaned before the parasite can hatch, thus reducing recruitment to the system (Bauer, 1959; Hoffman, 1977). Control of species of eye fluke, *Diplostomum* von Nordmann, 1832 and *Tylodelphys* Diesing, 1850 by mechanical filtration and electrical grids have also been described by Larsen *et al.* (2005) and Schäperclaus (1991) respectively. Another example is through the dislodgement and removal of mobile stages of sea lice, *Lepeophtheirus salmonis* Krøyer, 1837 and *Caligus elongatus* von Nordmann, 1832, from salmon cages through the use of pump systems (Anonymous, 1996).

*Ichthyophthirius multifiliis* Fouquet, 1876 is the greatest cause of disease attributed mortality in the UK trout industry, accounting for an estimated 2-5% loss in annual production (c.360-900 tons), amounting to ~£2 million in lost revenue (*pers. comm.* British Trout Association). This ciliate protozoan is highly pathogenic and can cause the disease “white spot” in both wild and cultured freshwater fish (Matthews, 2005). The parasite has a temperature dependant direct life-cycle. An infective theront stage penetrates the epidermis of the fish

before turning into the feeding trophont stage. The parasite exits the host as a tomont which encysts on a suitable substrate within the environment prior to undergoing binary fission and the release of the next generation of theronts.

Control of this prolific and highly pathogenic parasite is difficult and few effective chemical control measures are available for the trout industry to use, partly due to the potential consequences to human health posed through the use of chemotherapy on animals intended to enter the food chain. Safe, cost effective and environmentally friendly alternatives to control are required and mechanical methods may provide such an option.

In order to prevent stress and damage to the host fish, the most obvious points in the life-cycle of *I. multifiliis* to target by mechanical means are the free living stages. Heinecke & Buchmann (2009), in laboratory trials, used a combination of water filtration and chemotherapy to control the free-living stages of *I. multifiliis*. Tomonts were removed from the water column using 80 μm mesh and then a 60 minute treatment of 16-32 mg $L^{-1}$ sodium percarbonate (11-22$^{o}$C) was used to kill all the theronts. The mesh would not, however, prevent theronts which measure 28.6-57.4 μm (length) × 20.0-28.6 μm (wide) (Aihua & Buchmann, 2001) from entering systems. As most current, licensed chemotherapeutants only kill the tomont and theront stages free in the water column at the time of the treatment, their impact in preventing infection is questioned. As *I. multifiliis* infections within farm sites appear to result from an on-site multiplication and not from a massive invasion of theronts entering the culture system via the water supply (current authors, unpublished data), the preferred control strategy must be deployed within the culture system. However, due to the small size, the large number and the short period infective theronts spend in the water column, the

relatively large, sedentary reproductive cysts provide a more obvious target for control by a mechanical method.

Concrete raceways are a commonly used system of rearing trout in the UK. Fish farmers often brush the bottom of raceways on a regular basis in an attempt to remove *I. multifiliis* cysts and settled waste from the system. However, due to the porous nature and rugose finish of concrete, it is likely that numerous surface pockets exist in which the tomonts can encyst and develop, thus casting doubt on the effectiveness of brushing raceways to reduce the burden of the parasite.

This paper aims to assess the potential of controlling *I. multifiliis* infections using engineering solutions. Specifically this study evaluates whether a mechanical control system could significantly reduce the abundance of the parasite and mortality attributed to it in raceway systems, by reducing parasite encystment, and using a suction device to remove the parasite from the system.

## 2. Materials and methods

### 2.1 Tomont settlement behaviour

To gain an indication of the proportion of tomonts likely to encyst on the sides or bottom of raceway systems, tomonts of *I. multifiliis* were collected using a glass pipette as they exited 5 g rainbow trout, *Oncorhynchus mykiss* (Walbaum), held in a tank maintained 15$^{o}$C. The tomonts were transferred to, and released at the surface of a glass aquarium (dimensions: 14cm (l) × 14cm (w) × 14cm (h)) containing 2.7 litres of dechlorinated, tank conditioned water held at 15$^{o}$C and containing no fish. A total of 135 tomonts were transferred to the tank. Five hours after transfer, the tank was drained and dismantled so as the glass panels could be easily screened to determine the number of tomonts successfully encysting on the

bottom and side panels. Panels were examined at ×4 magnification using an Olympus SZ30 dissecting microscope and the position of cysts recorded.

**2.2 Tomont survival on a range of raceway linings**

In order to reduce parasite settlement on concrete raceways and to produce a smooth surface to reduce wear and increase the ease of use of the mechanical device, it was necessary to apply a hardwearing polymer lining to the inside of treated raceways. Three potential linings were tested to determine the ability of the parasite to successfully encyst upon them: a polypropylene based plastic, a polyethylene-based plastic and Chlorvar chlorinated rubber (Teamac).

Crystal polystyrene Petri dishes were used as a negative control, as previous studies by the authors had established a high encystment success by the parasite on this plastic. Treatment Petri dishes were lined with each of the test polymers and left to soak in dechlorinated tank-conditioned water for a minimum of 24 hours before testing to ensure that there was no chemical finish on the products that may affect tomont survival. Petri dishes were filled with dechlorinated, tank conditioned water held at 15°C and 30 tomonts harvested using the method described above were added to each dish. The Petri dishes were then maintained at 15°C in a Binder environmental chamber and examined periodically to determine the number of alive and dead tomonts. The experiment was conducted in triplicate and terminated when either all the tomonts had either encysted or died.

**2.3 The mechanical device: design and construction**

The primary mechanical device consisted of a special suction head connected to a

pump that was used to vacuum the bottom of raceways to remove parasite stages (Figure 1). The device was submerged and then pushed, via a rod, towards the water inlet and then dragged back to the outlet screen. A wiper the width of the raceway trailed the raceway bottom behind the suction head. Any material that was not drawn up the suction head was drawn to the outlet screen where it was either drawn away by the water current or removed as part of normal husbandry. In addition to the suction head, low adhesion polymer sheeting was used to line three test concrete raceways (6m (l) × 1m (w) × 1m (d)) in a commercial hatchery. The sheets of polymer were cut to size, the joints welded together to give a watertight seal and then bolted to the concrete above the water line using stainless steel fittings.

**2.4 Field trials using the mechanical device**

The field trial was conducted at a commercial *O. mykiss* farm in the south of Scotland over a three month period (10$^{th}$ May to 10$^{th}$ August 2005). Six consecutive raceways (dimensions: 6m (l) × 1m (w) × 1m (d)) were used. Three were lined with the 6mm thick polymer sheeting described above and the remaining three were left unlined as controls. The raceways were divided into three blocks of two and the treated and control raceway randomly selected within each block. Water levels and flow rates in the raceways were equalised.

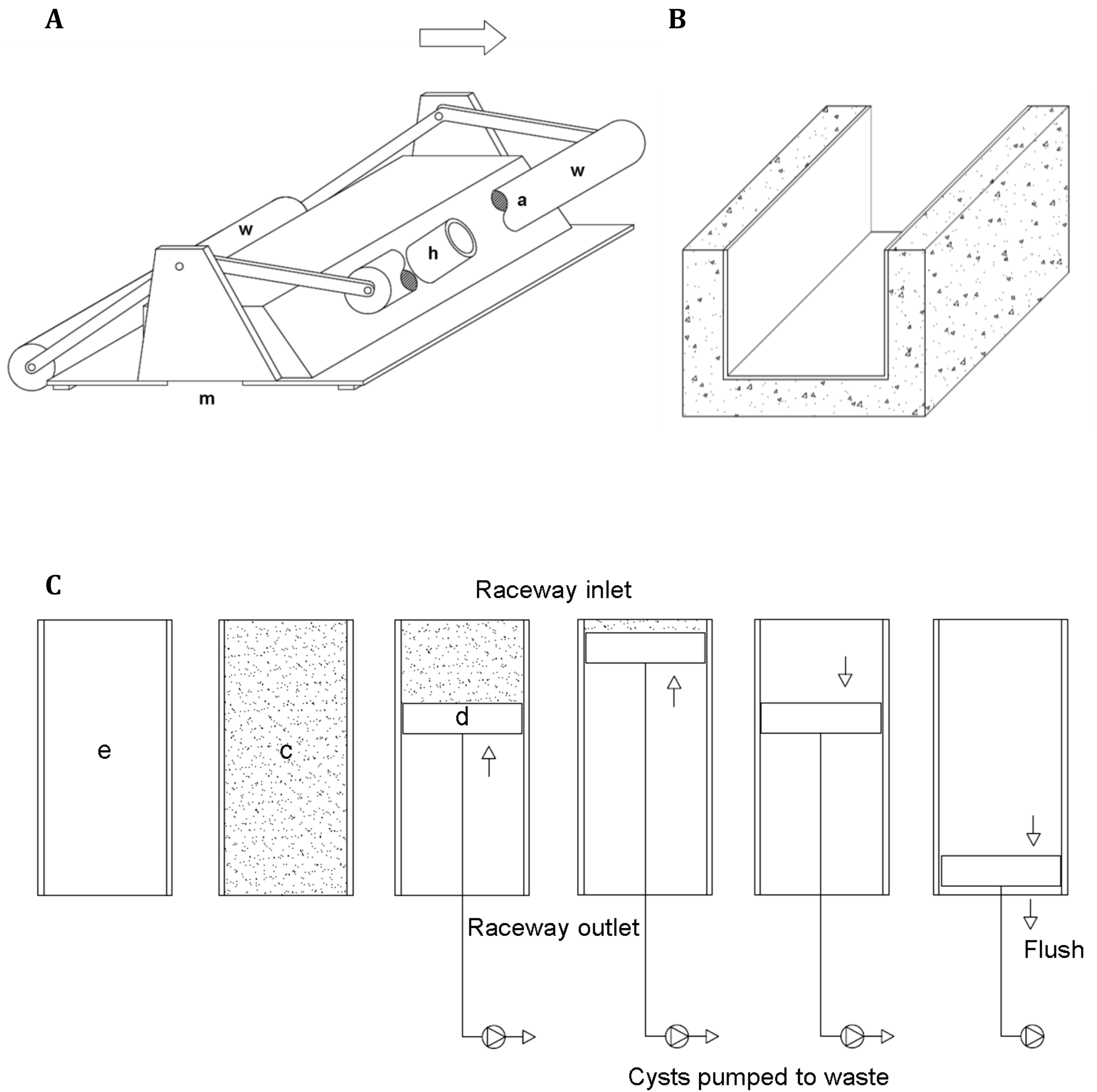


**Figure 1**: (A) The mechanical device used for the removal of *I. multifiliis* cysts from commercial-scale rainbow trout raceways. The device operates over a smooth coating applied to the internal face of the raceways. The arrow indicates the direction of the device being pulled towards the operator. a = vacuum head is pushed or drawn along the smooth raceway base by a pole attached to the top of one of the wiper blades (attachment point not shown); h = hose connection to the pump and then to waste; m = water, cysts and waste from the bottom of the raceway are sucked up through an interchangeable mesh and through the hose connection; w = wipers positioned at either end ensure that cysts are dislodged; situated at either end of the vacuum head enables them to be engaged in either a pulling or pushing action. (B) Polymer sheets are cut to size to fit the raceway. The sheets are fixed to the walls of the raceway with stainless steel fittings above the water line. The joints of the polymer sheets are welded to form a 100% watertight seal. (C) Use of the mechanical device in the raceways. The device is pushed towards the water inlet and then drawn back towards the outlet. *I. multifiliis* cysts are sucked up and pumped away to waste. c = raceway with *I. multifiliis* cysts on the base; d = mechanical device; e = empty raceway.

Each of the six raceways were stocked with circa 5000, 5g *O. mykiss* on the 10th May 2005. This gave a starting stock density of 16.7kg $m^{-3}$ in each raceway. Fish in each raceway were fed and maintained according to standard husbandry practices for the farm. Lined raceways were vacuumed once per day using one upstream pass followed by one downstream pass (water was discharged to land in order to prevent unnecessary levels of parasite or detritus entering the river system). Control raceways were brushed on a daily basis as part of the farm's normal husbandry practices.

At fortnightly intervals fish were sampled from each raceway. Where time permitted a sample size of approximately 30 fish per raceway were taken. During periods of heavy infection, a sample of 30 fish was not always possible due to limited resources and time constraints. Sampling was conducted by making one sweep with a 30cm × 30 cm hand net. The first 30 fish to be counted out of the net were placed in a bucket of water containing a sufficient dose of 200 ppm 2-phenoxyethanol to cause death by over anaesthesia. These fish were then screened individually at ×4 magnification using an Olympus SZ30 dissecting microscope and the total number of trophonts on the fins, gills and the entire body surface were counted.

**2.5 Statistical analysis**

The number of trophonts per fish were plotted using Excel 2000 (Microsoft, USA) for each raceway and sample point to establish trends and assess the distribution of the data. Based on this assessment, the data were analysed to identify differences between raceways at each sample point using a Generalised Linear Model (GLM) that assumed a Poisson error distribution in R version 2.7.0. (R

Development Core Team, 2005). A nested model, with sample visits nested within raceways was used. Mortality data recorded daily by the fish farmer for each raceway were analysed using Cox regression and Kaplan-Meier survival curves in Stata version 9.2 (StataCorp, USA) to compare mortality rates between treated and control raceways. An event was defined as mortality attributed by the fish farmer to *I. multifiliis*. Any fish assessed to have died through other causes during the study were censored from the population.

## 3. Results

### 3.1 Tomont settlement behaviour

The experiment found that 72.6% of tomonts (n = 98) settled on the bottom of the aquaria while the remaining 27.4% (n = 37) settled within 3.2 cm of the bottom. All tomonts successfully encysted within five hours.

### 3.2 Tomont survival on a range of polymer linings

Results of the tomont settlement experiment are presented in Table 1. All three polymers tested reduced the amount of settlement observed when compared to the control polymer (Crystal polystyrene). The Pisces Polymer Wsp 20-25 reduced settlement by the greatest amount (*i.e.* 9.8 %; Table 1), and was therefore used to line the test raceways in the subsequent field trial of the mechanical device.

**Table 1.** Settlement success (%) of *I. multifiliis* tomonts on different polymersubstrates expressed as the mean ± standard deviation.

| Lining | Settlement success (%) |
|---|---|
| Control - Crystal polystyrene | 90.2 ± 4.1 |
| Pisces polymer Wsp 20-05 | 9.8 ± 4.6 |
| Polyethylene-based plastic | 23.5 ± 7.9 |
| Chlorvar chlorinated rubber | 53.4 ± 5.1 |

### 3.3 Field trials using the mechanical device

The dynamics of the parasite in each of the study raceways is summarised in Figure 2 and Table 2. Statistically significant differences between raceways at each visit are detailed in Table 3.

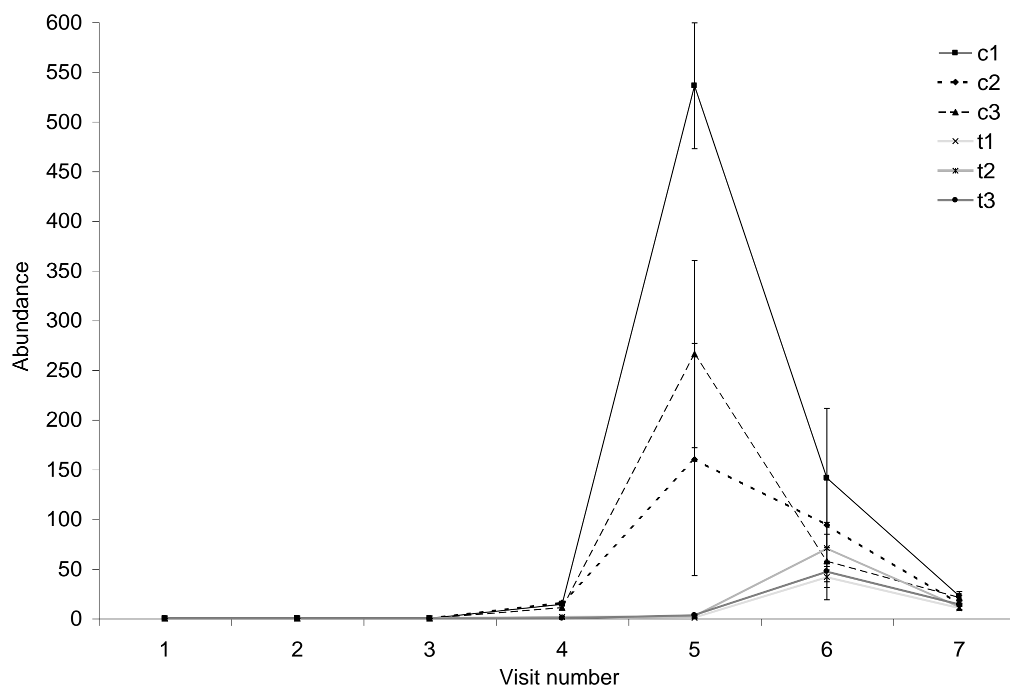


**Figure 2.** The abundance of *I. multifiliis* on rainbow trout in control raceways (c) and raceways treated (t) with the mechanical device. Visits were separated by two weeks. Vertical bars show standard errors

**Table 2.** The prevalence and abundance of *Ichthyophthirius multifiliis* trophonts in control and treated raceways at each sample visit. Figures represent the mean ± standard deviation. The number of fish infected and the range of trophonts per fish are shown in parentheses. Use of the suction head was withheld in the three test raceways after the fifth visit to the farm site (*i.e.* after 10 weeks post-start).

| Visit | Treated 1 | Treated 2 | Treated 3 | Average | Control 1 | Control 2 | Control 3 | Average |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 | 0.00 | 0.00 | 0.00 | 0.08 ± 0.28 (8%; 0-1) | 0.00 | 0.00 | 0.03 ± 0.16 (2.6%; 0-1) |
| 2 | 0.00 | 0.00 | 0.00 | 0.00 | 0.10 ± 0.31 (10%; 0-1) | 0.00 | 0.00 | 0.03 ± 0.18 (3.3%; 0-1) |
| 3 | 0.06 ± 0.40 (5%; 0-1) | 0.11 ± 0.31 (10%; 0-1) | 0.06 ± 0.22 (5%; 0-1) | 0.07 ± 0.25 (7%; 0-1) | 0.22 ± 0.40 (15%; 0-1) | 0.06 ± 0.22 (5%; 0-1) | 0.11 ± 0.30 (15%; 0-1) | 0.12 ± 0.32 (11.67%; 0-1) |
| 4 | 1.53 ± 2.80 (47%; 0-11) | 1.44 ± 2.06 (50%; 0-7) | 0.13 ± 0.35 (13%; 0-1) | 1.04 ± 2.08 (37%; 0-11) | 14.33 ± 5.79 (100%; 5-26) | 15.80 ± 6.71 (100%; 5-23) | 10.93 ± 6.61 (100%; 3-24) | 13.69 ± 6.57 (100%; 3-26) |
| 5 | 0.60 ± 0.84 (30%; 0-2) | 2.00 ± 1.83 (100%; 1-6) | 3.20 ± 0.84 (100%: 2-4) | 1.64 ± 1.59 (68%; 0-6) | 536.00 ± 179.14 (100%; 287-833) | 160.00 ± 261.5 (100%: 19-626) | 266.00 ± 188.51 (100%: 87-405) | 361.88 ± 260.75 (100%; 19-833) |
| | | **Use of suction head stopped** | | | | | | |
| 6 | 60.83 ± 29.57 (100%; 5-97) | 70.77 ± 41.87 (100%; 38-171) | 47.33 ± 25.55 (100%; 12-78) | 53.52 ± 36.34 (100%; 5-171) | 141.38 ± 280.53 (100%; 2-1149) | 94.06 ± 186.56 (94.2%; 0-698) | 57.70 ± 123.06 (100%; 2-404) | 103.21 ± 213.76 (98%; 0-1149) |
| 7 | 10.33 ± 9.29 (80%; 0-25) | 11.20 ± 8.72 (87%; 0-27) | 13.93 ± 6.08 (100%; 5-28) | 11.82 ± 8.11 (89%; 0-28) | 22.60 ± 17.13 (93%; 0-55) | 14.73 ± 9.85 (93%; 0-35) | 20.93 ± 10.46 (100%; 1-37) | 19.42 ± 13.07 (96%; 0-55) |

**Table 3.** Results of a GLM comparing the abundance of *I. multifiliis* on rainbow trout in control raceways (c) and raceways treated (t) with the mechanical device at each sample visit. Visits show raceways that had significantly ($p<0.05$) higher (>) or lower (<) abundance than the reference raceway in the left column. ns = no significant differences observed.

| | Visit number | | | | | | |
|---|---|---|---|---|---|---|---|
| **Raceway** | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **Control 1** | ns | ns | ns | <t1,t2,t3 | <c2,c3,t1,t2,t3 | <c2,c3,t1,t2,t3 | <c2,t1,t2,t3 |
| **Control 2** | ns | ns | ns | <t1,t2,t3 | <t1,t2,t3, >c1,c3 | <c3,t1,t3, >c1 | >c1 |
| **Control 3** | ns | ns | ns | <t1,t2,t3 | <c2,t1,t2,t3 | <t1, >c1,c2 | <t1,t2,t3 |
| **Treated 1** | ns | ns | ns | >c1,c2,c3, <t3 | >c1,c2,c3,t3 | >c1,c2,c3,t2 | >c1,c3,t3 |
| **Treated 2** | ns | ns | ns | >c1,c2,c3, <t3 | >c1,c2,c3 | >c1, <t1,t3 | >c1,c3,t3 |
| **Treated 3** | ns | ns | ns | >c1,c2,c3 <t1,t2 | >c1,c2,c3 <t1 | >c1,c2 | >c1,c3, <t1,t2 |

The parasite was observed in low abundance in all raceways at visit 3 and 4 (Figure 2), however the abundance of trophonts on the fish sampled during visit 4 were significantly higher in all three control raceways than in the treated raceways (Table 3). By visit 5 (10 weeks post-start), there had been a large increase in the abundance of the parasite in all three control raceways but not in the treated raceways (Figure 2). The abundance in the control raceways at visit 5 was significantly higher than in the treated (Table 2). Significant differences in abundance were also observed between some of the treated and control raceways (Table 2). Between visits 5 and 6 the fish farmer conducted formaldehyde treatments on all six of the raceways due to the high parasite burden observed in the controls. Due to time and resource constraints, the farmer was unable to use the mechanical device for the remainder of the study period.

By visit 6 the parasites abundance had reduced in the control raceways and

increased in the raceways previously exposed to mechanical. At this visit, control 1 had a significantly higher abundance of the parasite than all three treated raceways (Tables 2 and 3). Control 2 had a significantly higher abundance of the parasite than treated raceways 1 and 3, and control 3 had a significantly higher abundance of the parasite than treated raceway 1 (Tables 2 and 3). The abundance of the parasite was lower in all 6 raceways by visit 7 than visit 6. Controls 1 and 3 had significantly higher numbers than all three treated raceways, however no significant differences were observed between control 2 and any of the treated raceways (Tables 2 and 3).

Kaplan-Meier curves to compare mortality attributed to *I. multifiliis* infections in each of the test raceways are displayed in Figure 3. All three treated raceways had lower levels of mortality than the control raceways. Total mortality by the end of the trial ranged between 11 and 22% (mean = 15.5%) in the treated raceways, and 24 to 33% (mean = 29.4%) in the controls. Analysis by Cox regression showed the likelihood of fish dying because of *I. multifiliis* infections over the course of the study was significantly higher in all three control raceways than in treated raceways 1 and 2. No significant difference was observed between control 1 and treated raceway 1, however controls 2 and 3 showed a significantly higher likelihood of mortality than treated raceway 1.

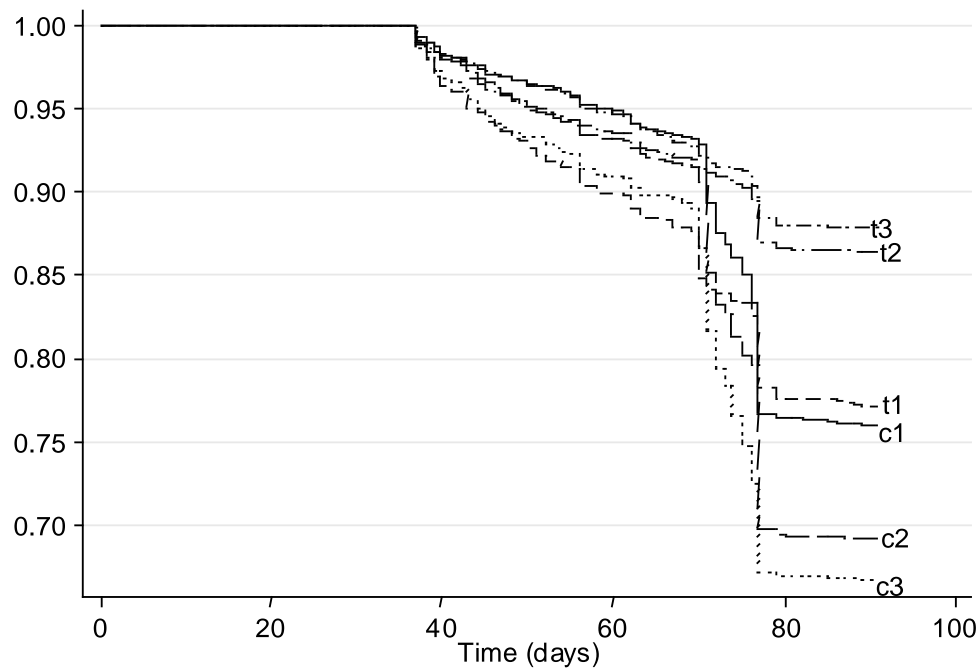


**Figure 3.** Mortality curves for rainbow trout dying of *I. multifiliis* infection in control raceways (c1-3) and raceways treated (t1-3) with the mechanical device. The study was terminated at day 91.

## 4. Discussion

Infections by *I. multifiliis* are a serious problem for many sectors within the global freshwater aquaculture industry (Matthews, 2005). In the UK, this protozoan is a major problem within the trout farming sector (*pers. comm.* British Trout Association) as controlling infections is difficult due to the continuous release of infectious stages into the water column and the protection the parasitic stage receives from the hosts' epithelium against most water treatments (Post & Vesley, 1983; Shinn *et al.*, 2003 a, b). For many years the control of *I. multifiliis* in these systems has centred on the use of bath treatments containing a combination of formaldehyde and malachite green, which was perceived to be one of the few treatments that could affect the parasitic trophont stage as well as exiting tomonts and free-swimming infective theronts (Walhi *et al.*, 1993). To date, however, no peer-review research appears to have been published that investigates mechanical

control of the parasite. This study aimed to develop and evaluate a mechanical system to control *I. multifiliis* infections in raceway systems during periods of reduced water availability.

Investigations into different polymer linings demonstrated that not all polymers are conducive to successful encystment, and the polypropylene-based plastic was shown to be especially effective at reducing settlement. Although this is unlikely to be feasible in pond culture, the lining of tank and raceway systems with low adhesion polymers is likely to be practical and investigations into the effect of lining alone without mechanical removal is warranted.

Use of the combination of the polypropylene low adhesion based plastic and the suction device produced a clear change in both the parasite dynamics and host survival between treated and control raceways. In control raceways, parasite numbers were observed to increase at circa eight weeks (4 visits) post stocking, with the height of the infection occurring at circa ten weeks (visit 5). Shortly after this time, repeated formaldehyde treatments were applied to all raceways in an attempt to reduce the parasite burden. Although at 12 weeks post-stocking (visit 6), it appeared that the treatments had been effective in reducing the parasite burden in the control raceways, this period coincided with high fish mortality rates in the control raceways. Fish farmer records detailing examination of these mortalities suggest that the mortality occurred in the most heavily infected fish, thus causing the abundance to appear reduced by week 14 (visit 7). This may also suggest that the use of formaldehyde in the absence of malachite green (or other compounds) has little effect on burdens of *I. multifiliis*, which is in accordance with the findings of Walhi *et al.* (1993).

In treated raceways, parasite abundance remained below ten parasites per

fish until the use of the suction head ceased after week 10 (visit 5). After this, parasite abundance increased and peaked at circa week 12 (visit 6), which suggests that use of the low adhesion polymer in the absence of the use of the suction head is insufficient to prevent the establishment and multiplication of *I. multifiliis* infections. Parasite abundance in these treated raceways did, however, remain lower than observed in the control raceways.

Use of the system was not just effective at reducing the parasite burden, but also significantly improved survival. It is believed that mortality attributed to *I. multifiliis* infection occurs due to osmoregulatory and respiratory dysfunction that occurs when large numbers of the parasite exit the host skin or gill epithelium (Hines & Spira, 1973, 1974 a, b). Although low rates of mortality attributed to *I. multifiliis* were observed in all raceways from around day 38 post-stocking, this rate did not increase until day 69 post-stocking, which was around the time infection was observed to peak in the control raceways. From this point on the mortality rates were greater in the control raceways than in the treated. On average, mortality was 13.9% greater in the control raceways than in the treated raceways, which equates to around 695 more fish surviving in each of the treated raceways by the end of the trial based on the stock densities that were used.

After week 10 (visit 5) use of the suction head was stopped providing an insight as to how important the mechanical removal of the cysts was, and highlights the importance of continuous use in order to manage the parasite population. Obviously it could be argued that the increased abundance of *I. multifiliis* observed after use of the suction head was stopped could have occurred had its use continued, but based on the results up to this time the authors feel confident this was not the case.

This trial was used as a proof of concept to test the potential of mechanical control measures in the control of *I. multifiliis* infections. Only one opportunity was available to test the concept in a commercial setting and as a consequence the system was over engineered so as to increase the likelihood of the system being effective. Settlement trials demonstrated that in a static water system the majority of trophonts encyst on the bottom of a system. This would suggest that it is not necessary to line the sides of the raceways with low adhesion polymers and that just coating the bottom may be sufficient. Fitting the polymer itself required specialist knowledge and tools in order to obtain a secure hardwearing fit. Following the completion of the trial, the commercial facility replaced the polymer and sealed each raceway with a non-toxic, self-levelling paint. The farm reported that the benefits were consistent with the use of the polymer (M. Davies, personal communication).

The frequency at which the device is used should also be optimised for the temperature conditions. In this trial, the suction head was used in each raceway once a day. At water temperatures of less than 22°C this should be sufficient for effective control, as it takes over 24 hours for the parasite to be released from a cyst (Matthews, 1994). The use of the suction head should be at around the same time each day to ensure the 24 hour period does not elapse, and should the temperature rise above 22°C the suction head should be used twice a day with at least 6 hours between treatments. For the system to be effective, its use must be incorporated into a farm's standard husbandry regime. Although its use may seem labour intensive there are likely to be other savings in terms of staff time such as no requirement for brushing raceways (often done on a daily basis) and reduced application of treatments such as formaldehyde. Additionally, the system appeared

to be very effective at removing fish mortalities from the raceways, thus further reducing staff time.

In addition to reduced impact of infections by *I. multifiliis*, there are likely to be several other potential benefits associated with the use of this system. Reduced chemical use has obvious environmental benefits, but also reduces the likelihood of resistant strains of pathogen emerging and reduces the exposure of the users to potentially hazardous products. By discharging the water pumped through the suction head to land, not only is the amount of parasite introduced to the river system receiving farm effluent reduced, but also the amount of solid waste entering the river. Discharge of suspended solids from fish farms is an area of high environmental concern, and in England and Wales, the Environment Agency monitor fish farm effluent to ensure the levels discharged are not too high. The effective removal of detritus from raceways is likely to have benefits to water quality, and the reduction of surface area gained through providing a smooth surface instead of rough concrete may reduce the habitat for opportunistic microorganisms to colonise.

## 5. Conclusions

This study provides proof of concept that mechanical methods can be effective at controlling pathogens within aquaculture systems. It is hoped that the concepts put forward in this study can be developed to provide an effective alternative to the control of *I. multifiliis* by chemical treatment, and that with further development it may be incorporated into standard farm husbandry in raceway systems. Although installation of such a system on a fish farm will pose an initial economic outlay, it is hoped that the benefits and savings gained through improved

fish health and general farm husbandry, and reduced requirement for chemical treatments will mean such costs are rapidly recouped. Studies are currently underway to establish whether this form of control can be developed and applied to other aquatic systems and pathogens.

**Acknowledgements**

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# CHAPTER 4

## PAPER VI

*RESEARCH ARTICLE*

**An examination of the utility of the leopard pleco (*Glyptoperichthys gibbiceps*, Kner, 1854) in the control of *Ichthyophthirius multifiliis* Fouquet, 1876**

**Picón-Camacho, S.M.[a],** Bron, J.E.[a] and Shinn, A.P.[a]

[a]*Institute of Aquaculture, University of Stirling, FK9 4LA, Stirling (United Kingdom)*

**In Preparation**

**Contributions:** This manuscript was compiled and written in full by the author of this thesis with advice and thorough proofreading by the co-authors.

**Keywords:** *Ichthyophthirius multifiliis*, pest management, biological control, leopard pleco.

**Abstract**

The ciliate freshwater parasite *Icthyophthirius multifiliis* Fouquet, 1876 is considered to be one of the most important parasitic diseases in freshwater aquaculture worldwide. After the ban of malachite green to treat fish for human consumption, there are only few alternatives to control *I. multifiliis* infections in the fish industry. Currently, in-bath chemical treatments are commonly used target exlusively the free-swimming stages of the parasite. These chemical protocols are partially efficient and repeated applications are often required. In the present study we demonstrate the selective settlement behaviour of the tomont stage towards biofilm-covered substrates and for the first time the potential of using a substrate algae feeder (leopard pleco, *Glyptoperichthys gibbiceps*) to control *I. multifiliis* infections. The utilisation of a biological achived a reduction of 63% on the number of trophonts on the stock fish compared to the control groups. These biological strategies could ultimately control *I. multifliis* infections in farm systems and potentially reduce the application of chemical interventions.

## 1. Introduction

Following the ban upon the use of malachite green in the European Union, Canada and the United States for treating fish destined for human consumption (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC; Srivastava *et al.,* 2004; Martínez-Bueno *et al.,* 2010), there has been concerted effort to identify appropriate alternatives for the management of external parasites in commercial scale aquaculture systems.

The most common therapeutic strategy is the repeated application of chemical-bath treatments in an attempt to cure an infection deemed as excessively

high, during a parasite outbreak (Noga, 2010). The repeated application of a specific chemical treatment has economic ramifications but also potential impacts on the fish homeostasis and marketability while often constituting a risk of hazard to the local environment and workers alike. Many chemotherapeutants currently in use are still under scrutiny with regard to their human and environmental toxicology (WHO IARC 2006; Noga, 2010) while, in practice, toxicity to the fish stock is a significant flaw often restricting chemical application and efficacy (Cardeilhac & Whitaker, 1988; Wahli *et al*., 1993; Moore, 2005; Balta *et al*., 2008). In addition, repeated chemotherapy may lead to the development of resistance within the parasite population (Jones *et al*., 1992; Fallang *et al*., 2004; Lees *et al*., 2008). Accordingly, there is a strong interest in identifying efficacious non-chemical therapies to control parasitic infections in commercial aquaculture (Noga, 2010).

In recent years, there have been a number of attempts to include biological controls as part of a wider management strategy against aquatic parasites. A biological control has been defined as “the use of living organisms to suppress the population density or impact of a specific pest organism, making it less abundant or less damaging than it would otherwise be” (Eilenberg *et al*., 2001). Biological controls have been widely used for pest management in terrestrial agriculture (Gerson *et al*., 2003; Shah & Pell, 2003) and the removal of aquatic ectoparasites by associate species, *e.g.* cleaner wrasse (*Labroides* spp.) is well characterised in wild fish populations (Côte, 2000). However, this biological approach to pest management remains to date poorly exploited in commercial aquaculture (Barber & Poulin, 2002). One of the most well-known examples is the utilisation of wrasse (corkwing wrasse, *Symphodus melops* L. and goldsinny wrasse, *Ctenolabrus*

*rupestris* L.) to control infections of parasitic caligid copepods (*Lepeophtherius salmonis* Krøyer, 1837 and *Caligus elongatus* Nordmann, 1832) in the marine phase of Atlantic salmon, *Salmo salar*, farming (Deady *et al*., 1995; Rae, 2002; Kent & Poppe, 2002). Other examples include the use of bluestreak cleaner wrasse (*Labroides dimidiatus* Valenciennes) against infections of the monogenean *Benedenia lolo* Yamaguti, 1968 on blackeye thicklip (*Hemigymnus melapterus* Bloch) (Grutter *et al.,* 2002) and tropical cleaner fish (wrasse and gobies) to control *Neobenedenia melleni* MacCallum, 1927 infecting marine-cultured Florida hybrid red tilapia (*Oreochromis mossambicus* x *Oreochromis niloticus*) (Cowell *et al*., 1993).

*Ichthyophthirius multifiliis* Fouquet, 1876 is considered to be one of the most significant protozoan parasites of freshwater fish, infecting ornamental and food fish alike worldwide including commercially important aquaculture species such as tilapia (*Oreochromis* spp.), common carp (*Cyprinus carpio* L.), channel catfish (*Ictalurus punctatus* Rafinesque) and various salmonid species (*e.g. Oncorhynchus mykiss* Walbaum and *Salmo salar* L.) (Paperna, 1991; Valtonen & Koskivaara, 1994; Noble & Summerfelt, 1996; Buchmann & Bresciani, 1997; Rintamäki-Kinnunen & Valtonen, 1997; Münderle *et al*., 2004; Matthews, 2005; Dickerson, 2006; El-Sayed, 2006; Jørgensen *et al*., 2009). This ciliate protozoan parasite has a direct and temperature-dependant life-cycle characterized by a parasitic stage (trophont) that feeds within the epithelial tissues (skin, fins and gills) and can reach up to 1 mm in diameter (Lom & Dyková, 1992). *Ichthyophthirius multifiliis* has two free-swimming stages (tomonts and theronts) and a reproductive stage (cyst). The tomont is covered in cilia and, upon release from the fish host, swims briefly (~1 h at 21-23 °C) and then settles on an

appropriate substrate. The settled tomont, then encysts and undergoes binary division to produce 250 to 3,000 daughter cells (tomites) (Lom & Dyková, 1992; Wagner, 1960) that are released into the water column and differentiate into the theront or infective stage and must rapidly find a host or die (Matthews, 2005; Dickerson, 2006). The most common therapies against this parasite are repeated bath treatments of either formaldehyde or sodium chloride for infections in salmonid species in Europe (Rintamäki- Kinnunen *et al*., 2005b; Lahnsteiner & Weismann, 2007) and of either copper sulphate or potassium permanganate to treat channel catfish (*Ictalurus punctatus* Rafinesque, 1818) and silver perch (*Bidyanus bidyanus* Mitchell, 1838) in the United States and Australia (Tieman & Goodwin, 2001; Straus & Griffin, 2002; Rowland *et al*., 2009). These treatments are effective against the free-swimming stages of *I. multifiliis* (tomonts and theronts) but are largely ineffective against the reproductive cyst stage which is protected by a wall coat (Ewing *et al*., 1983).

No studies that we are aware of have explored the potential of using a biological control against *I. multifiliis*. The use of a cleaner wrasse, *L. dimidiatus*, has, however, been shown to have limited efficacy in controlling infections of the marine white spot species *Cryptocaryon irritans* Brown, 1951 (Grutter, 2002). It has been suggested that this difference was due to the cleaner fish removing only a small proportion of the *C. irritans* population on the host, and specifically, theronts in the process of penetrating or having just penetrated the host or mature tomonts exiting the host epithelium (Grutter, 2002). This could also be expected for *I. multifiliis* as the parasitic trophont stage of both species are well protected within the host's epidermis (Post & Vesley, 1983). However, it was recently demonstrated that the systematic removal or disruption of the cyst stage proved to be a highly

effective strategy in reducing the magnitude of *I. multifiliis* infections in a commercial scale fish rearing system (Shinn *et al*., 2009). In this study, which did not use chemotherapeutants, the use of a mechanical device to vacuum the bottom of commercial raceways that had been sealed and lined with a polypropylene-based plastic within a rainbow trout hatchery were observed to reduce the level of *I. multifiliis* infection on the fish by approximately 99% when compared to untreated control group after 10 weeks (Shinn *et al*., 2009). These dramatic results were supported by the fact that, within a static aquarium, approximately 75% of tomonts settle and encyst on the bottom whereas 25% are found attached to the sides of the vessel and within the first 3.2 cm of the base (Shinn *et al*., 2009). The positive impact of removing or disrupting the cyst stage of *I. multifiliis* is not surprising as each cyst can produce up to 3,000 infective theronts (Wagner, 1960). Although little is known about the tomont's settlement behaviour, the attachment of *I. multifiliis* cyst to the culture enclosure makes it a likely collateral target to substrate feeders, detrivorous species and algae grazers alike. It can be hypothesized therefore that *I. multifiliis* cysts are susceptible to ingestion by certain bottom feeding or substrate grazing fish species or alternatively that parasite development might be disrupted if it is dislodged and / or resuspended in to the water column by the fish's feeding activities thereby facilitating its evacuation from the rearing environment in flow-through systems.

The tropical freshwater species *Glyptoperichthys gibbiceps* Kner, 1854 commonly known as the "leopard pleco", originates from the middle and upper Amazon and Orinoco basins (Ferraris, 2007), has a temperature optima ranging from 23 °C to 27 °C and can reach a maximum size of 60 cm and have a life-span of 10 to 15 years (Froese &. Pauly, 2010). It is a nocturnal algae / detrivorous

substrate feeder that is frequently selected by aquarists for its capacity in helping to maintain a clean tank environment. In addition, *G. gibbiceps* is, as other members of the armoured catfish Loricariidae family, characterized by bony plates covering its body surface (Montoya-Burgos *et al*., 1998). This suggests the possibility of a physical defense against the penetration of the infective *I. multifiliis* theront, hence a low susceptibility and / or sensitivity to the disease. The algal grazing behaviour and the skin structure of this species make it a strong candidate in tropical and sub-tropical environments for the biological control of *I. multifiliis* by removal of the cyst stage. However, no previous studies have assessed the susceptibility of *G. gibbiceps* to *I. multifiliis* and the possibility of using this species as a biological control to reduce the infection dynamics of *I. multifiliis*.

The current study, explores the use of *G. gibbiceps* as a possible biological control agent to remove cysts of *I. multifiliis*. In that aim, a series of experiments were performed to examine: 1) the settlement behaviour of tomonts and their preference for different substrates, 2) the relative susceptibility of *G. gibbiceps* to infection by a strain of *I. multifiliis* originating from *Tilapia* sp., and 3) the ability of *G. gibbiceps* to control *I. multifiliis* infections.

## 2. Materials and methods

### 2.1. Infection culture

Zebra tilapia (*Tilapia buttikoferi* Hubrecht, 1881) naturally infected with *I. mutifiliis* were obtained from an ornamental fish retailer and transferred to a temperature-controlled experimental facility within the Institute of Aquaculture (IoA, University of Stirling, U.K.) where they were maintained in two static PVC tanks (66 cm (l) × 24.5 cm (w) × 20 cm (d) ; volume 32 L) until the mature

trophonts could be harvested and used for subsequent rounds of infection to increase the size of the parasite population. Throughout the experimental period and for all trials, the rearing water was dechlorinated using an ELGA C960 carbon filter, aerated and maintained at 25ºC ± 1ºC using three 100 W heaters (Tetratec Aquarium heater, Tetra, U.K.) within a 70 L tank. Approximately 20% of the water in each tank was exchanged daily. Fish were fed at 2% body weight per day using floating trout pellets (Skretting, UK) and solid wastes removed daily by siphon. After each round of infection, the fish were replaced with a naïve stock of *Oreochromis aureus* (body weight = 4.90 ± 0.05 g) obtained from the Institute of Aquaculture (IoA, University of Stirling, U.K.) tropical aquarium facilities to maintain the parasitic infection. When required for experimental use, tomonts naturally exiting the fish were collected from the water column using a Pasteur pipette, placed into a 10 cm diameter Petri dish (Sterilin, UK) containing 20 mL aerated dechlorinated carbon-filtered water at 25ºC ± 1ºC from the experimental water supply. After transfer to the Petri dishes, the tomonts were periodically observed under a dissecting microscope (×4; Olympus SZ40) to confirm their viability that they had encysted and were beginning to undergo binary fission.

### 2.2. Tomont settlement behaviour and their preference for different substrates

Ten Petri dishes measuring 10 cm in diameter, were marked externally into two equal halves. Petri dishes were then submerged in a 20 L PVC aquaria containing aerated dechlorinated carbon-filtered water at 25°C ± 1°C and were subjected to a 12L: 12D light-regime within a temperature controlled room. The tank water was then enriched by the addition of 15 ornamental fish algal wafers (Tetra, UK) to

encourage biofilm growth (Liess & Hillebrand, 2006). The dishes were then observed 1 week later to ensure good biofilm growth. On the day of use, each Petri dish was wiped clean over precisely one half of their surface using paper tissue keeping the biofilm layer intact on the other half of the surface, rinsed and then filled with 20 mL dechlorinated carbon-filtered water at 25°C ± 1°C. Freshly emerged tomonts (10 tomonts per Petri dish) collected from the infection tank were then dropped over the centre line of each Petri dish (*i.e.* the boundary between the biofilm and biofilm-free surface).

Experimental Petri dishes were immediately transferred to a precision environmental chamber incubator (WTB Binder Labortechnik GmbH, Germany) set at 25°C ± 1°C with a light regime of 12L: 12D. They were checked every 2 h and, after all the tomonts had settled, encysted and reached, at minimum, the 8-cell stage (~4 – 8 h following the addition of the tomonts to the dishes), the number of cysts on each biofilm and clean region in each dish was counted and recorded. The procedure was subsequently repeated three times over three consecutive days (triplicate design with a total of 30 Petri dish used to assess the settling behaviour of 300 tomonts).

### 2.3. Fish stock and rearing conditions

The *in-vivo* experiment assessed the susceptibility of *G. gibbiceps* to infection by experimentally exposing them to a strain of *I. multifiliis* theronts originating from ciclid species *T. buttikoferi* and the potential of *G. gibbiceps* as a biological control to reduce the number of cysts developing in an experimental culture system. Prior to the start of the experiment (10 days), six static PVC tanks (34 cm (l) × 19 cm (w) × 16 cm (d)) were filled with 10 L of aerated dechlorinated carbon-filtered water.

The tank water was then enriched by the addition of 15 ornamental fish algal wafers to encourage biofilm growth on the base and sides of the tank, as previously described. Naïve *Oreochromis aureus* obtained from the Institute of Aquaculture (IoA, University of Stirling) tropical aquarium facility and naïve *G. gibbiceps* obtained from a local pet supplier in the Stirling district were transferred and acclimated within the experimental system for a period of 4 days prior to the start of the experiment. A total of 57 *O. aureus* (body weight = 4.90 ± 0.05 g) were allocated to one of six experimental tanks as follows: Three replicate control tanks received 10 *O. aureus* each and three treatment tanks received 9 *O. aureus* each. In addition, two *G. gibbiceps* (n = 2 / tank; body weight = 2.55 ± 0.06 g) were allocated to each treatment tank. The total biomass and density of fish were balanced among the six experimental tanks prior to the start of the experiment. Within the treatment tanks, the experimental design provided a ratio of 1 *G. gibbiceps* to 4.5 *O. aureus* with each *G. gibbiceps* having 1143 $cm^2$ of tank surface. During the acclimation period, both fish species were fed daily by providing algal wafers at a rate of 4g / *G. gibbiceps* / day and commercial floating trout pellets to *O. aureus* (2% body weight / day). Food was withheld on the day of infection and no additional algal wafers were added over the course of the experiment. Only the addition of the floating trout pellets for the *O. aureus* (2% body weight / day) was continued to encourage the *G. gibbiceps* to graze on the biofilm layer present in each tank. The fish were maintained as described above.

### 2.4. Experimental infection and post-mortem examination

The infection was started by adding 30 fresh tomonts to each tank. The experiment was terminated on day 6 post-infection so that the number of trophonts

developing on the experimental fish could be determined before they exited the fish. This time period takes account of the development of *I. multifiliis* maintained at 25-26°C (Matthews, 2005). Upon termination, all the experimental fish were killed by an overdose of the anaesthetic (200ppm 2-phenoxyethanol MERCK, Germany), and then immediately examined under a dissecting microscope (×4; Olympus SZ40) to determine the total number of trophonts present on each fish (gills, fins and skin). No mortalities were observed over the acclimation and experimental period.

**2.5. Statistical analysis**

For the *in vitro* experiment, a Chi-square test for goodness of fit was used to determine statistical differences between the distribution of the cysts on the different zones (biofilm *vs* no biofilm) and the distribution that could have been expected by chance alone (50% tomonts encysting on the biofilm surface and 50% encysting on the biofilm-free surface). Due to the limited number of observations per Petri dish, analyses were performed on each daily set of 10 Petri dishes corresponding to a replicate within the triplicate experimental design. A Chi-square test of independence was used to determine statistical differences in the proportion of cysts observed on the biofilm surface between replicates.

For the *in vivo* experiment, a one-way nested analysis of variance (ANOVA) using replicate tank as the nested factor was applied to determine significant differences in the total biomass per tank between experimental groups, in the number of trophonts per *O. aureus* between experimental groups and in the number of trophonts per fish between fish species (treatment group only). Prior to analysis, datasets were tested for normality (Kolmogorov-Smirnov test) and

homogeneity of variance (Bartlett's test) and data were square-root transformed to respect normality. All the analyses were carried out using the software SPSS version 18.0 (IBM, USA) with a significance level of $p < 0.05$. Data are expressed as mean ± SEM.

## 3. Results

In the *in-vitro* experiment, a total of 275 cysts at the 8-cell+ developmental stage were recovered from a total of 300 tomonts incubated (95% survival rate). There was no significant differences between the three replicate sets of 10 Petri dishes in the distribution of cysts according to the substrate quality (Chi-test, $p>0.05$) (Table1). The proportion of tomonts that settled and encysted on the biofilm surface (50% of the total surface available) was constant across replicates (77.4% to 79.8%) and averaged 78.5% (n = 216) whereas an average of 21.5% (n =59) of the cysts were observed on the biofilm-free raw plastic surface (Table 1). Within each replicate, the proportion of cysts observed on each type of experimental substrate was significantly different to 50% which could have been expected by chance alone. The availability of two substrate qualities (biofilm or biofilm-free raw plastic) had a significant effect on the location where the tomonts settled and encysted.

At the end of the *in-vivo* experiment, the *G. gibbiceps* were infected with a strain of *I. multifiliis* originating from *T. buttikoferi* (Table 2) with trophonts being observed on the skin, fin and gills of *G. gibbiceps*.

**Table 1.** Comparison between numbers of *Ichthyophthirius multifiliis* tomonts settling on biofilm or non-biofilm substrates present in each vessel at a 1:1 ratio. The number of tomonts on the biofilm and non-biofilmed surface were always significantly different from the expected 1:1 distribution that would have been obtained by chance alone (Chi-square test for goodness of fit; $p < 0.05$). The number of tomonts present on the biofilm or non biofilm surfaces was never significantly different between replicates (Chi-square test of independence, $p < 0.05$).

| **Replicate** | **Tomont location (n)** | | **Total tomont (n)** | **Expected distribution (1:1)** | **$\chi^2$ (1:1)** | ***p*** |
|---|---|---|---|---|---|---|
| | **Biofilm** | **Non-biofilm** | | | | |
| 1 | 77 | 21 | 98 | 49 | 32.00 | 0.0001 |
| 2 | 67 | 17 | 84 | 42 | 50.00 | 0.0001 |
| 3 | 72 | 21 | 93 | 46.50 | 27.96 | 0.0001 |
| TOTAL | 216 | 59 | 275 | 137.5 | 89.63 | 0.0001 |

There were no significant differences between replicates in the mean number of trophonts per *O. aureus* in the controls and per *O. aureus* or *G. gibbiceps* in the treatment tanks. Within the treatment tanks, the mean number of trophonts per fish was not statistically different between *G. gibbiceps* (4.3 ± 1.8; n = 6 fish) and *O. aureus* (4.9 ± 2.3; n = 27 fish). However, the mean number of trophonts per *O. aureus* was significantly lower (4.9 ± 2.3; $p < 0.001$) in the treatment tanks than in the control tanks (13.8 ± 4.8). Overall, the infection burden on *O. aureus* reared with *G. gibbiceps* was 62.8% lower than on the *O. aureus* reared in isolation (*i.e.* without *G. gibbiceps*).

**Table 2.** Number of fish, mean body-weight and the total biomass per tank determined at the beginning of the experiment and the mean number of trophonts per fish determined on day 6 post- infection. The experiment assessed the capability of *Glyptoperichthys gibbiceps* to control *I. multifiliis* infection in tanks containing *O. aureus* and the susceptibility of *G. gibbiceps* to the experimental strain of *I. multifiliis* used. Values are expressed as mean ± SE. There was no significant difference in the total biomass per tank between replicates and between experimental groups (ANOVA; $p>0.05$). Asterisks (*) indicates significant differences (ANOVA. $p<0.001$) in the mean number of trophont per *O. aureus* when reared alone or in common with *G. gibbiceps*. Within the treatment group, there was no significant difference in the parasite load between *O. aureus* and *G. gibbiceps* (ANOVA. $p>0.05$).

| | Control group | | | Treatment group | | |
|---|---|---|---|---|---|---|
| **Replicate** | 1 | 2 | 3 | 1 | 2 | 3 |
| **Number (N)** | | | | | | |
| *O.aureus* | 10 | 10 | 10 | 9 | 9 | 9 |
| *G. gibbiceps* | | | | 2 | 2 | 2 |
| **Mean body-weight (g ± SE)** | | | | | | |
| *O.aureus* | 4.84±0.15 | 4.99±0.11 | 4.87±0.14 | 4.90±0.11 | 4.87±0.13 | 4.92±0.16 |
| *G. gibbiceps* | | | | 2.68±0.08 | 2.49±0.13 | 2.48±0.10 |
| **Total biomass (g ± SE)** | | | | | | |
| | 48.44 | 49.85 | 48.73 | 49.43 | 48.83 | 49.23 |
| | | 49.01±0.43 | | | 49.16±0.18 | |
| **Mean number of trophonts (n ± SE)** | | | | | | |
| *O.aureus* | 12.4±1.4 | 15.9±1.0 | 11.4±2.0 | 4.4±0.7 | 4.1±0.5 | 6.2±0.9 |
| | | 13.3±0.9 | | | 4.9±0.4* | |
| *G. gibbiceps* | | | | 4.5±0.5 | 5.0±2.0 | 3.5±1.5 |
| | | | | | 4.3±0.7 | |

## 4. Discussion

This study is the first to document that the tomont stage of *I. multifiliis* preferentially settles and encysts on a biofilm substrate as opposed to a biofilm-free plastic surface. *Glyptoperichthys gibbiceps* was susceptible to an infection of *I. multifiliis* originating from *T. buttikoferi* and were observed to significantly reduce the infection intensity within the experimental rearing tanks. These findings

demonstrate the potential of *G. gibbiceps* as a biological control against *I. multifiliis* infection in warm water aquacultural species. From a broader perspective, the use of a substrate detrivorous / algae feeder is shown for the first time to significantly reduce the prevalence of a parasitic disease by targeting its non-parasitic stage.

These results show, in a similar fashion to those presented by Shinn *et al.* (2009) that the removal of cysts developing on a biofilm by ingestion by *G. gibbiceps* is able to effect a reduction in the number of trophonts that subsequently establish on fish. The present study, however, did not investigate the stomach contents of *G. gibbiceps* to confirm the ingestion of *I. multifiliis* cysts and so it is not known whether the statistical differences observed result from ingestion or through dislodging and interfering with the subsequent development of the cysts. Both mechanisms were likely involved but further research should distinguish the susceptibility of the cyst of *I. multifiliis* to ingestion and physical disruption in order to facilitate the establishment of non-chemical strategies to control the disease. The preferential settlement of tomonts on biofilm layers has not, prior to the current study, been investigated and the findings suggest that this may be selected for the optimal development of the cyst. Although the effect of substrate quality on the development of the reproductive cyst was not assessed in this study, the occurrence of a biofilm-free surface from the feeding activity of *G. gibbiceps* grazing might lower cyst development consequentially leading to a lower number of theronts being released and in turn a lower level of infection in the treatment tanks.

In this study, *G. gibbiceps* appeared to be as susceptible as *O. aureus* to an infection by *I. multifiliis*. This was observed despite the strain of *I.multifiliis* being isolated from the ciclid species *T. buttikoferi* and that *G. gibbiceps* is covered with

dermal body plates. A histological description of the integument of *G. gibbiceps* and of other armoured catfish of the family Loricariidae could not be obtained from the scientific literature. It is likely that a layer of epithelium lies over the dermal bony plates and that this can be penetrated by infective theronts. The effect of *G. gibbiceps* on the dynamics of *I. multifiliis* infection highlights the interest of further assessing the susceptibility of this species to *I. multifiliis*. Importantly, such studies should also determine the sensitivity of *G. gibbiceps* to the osmoregulatory imbalance caused by the departure of trophonts from the epithelia (Hines & Spira, 1973, 1974 a, b; Tumbol *et al*., 2001). Such studies should also extend to other Loricariidae catfish as their ventral sucker-like mouth and detrivorous / algivorous feeding behaviour (Salvador-Jr *et al*., 2009) is expected to provide similar positive effects against *I. multifiliis* while some species could prove more resistant. In any case, it can be argued that the potential of *G. gibbiceps* as a biological control against *I. multifiliis* is not significantly compromised by its susceptibility to the parasite under low levels of infection. In practice, *G. gibbiceps* could reduce the prevalence and intensity of the parasite which, if becoming overwhelming, would require chemical treatment to treat both the biological control and host fish species.

The feasibility of being able to deploy the potentially large quantity of *G. gibbiceps* that could be required in large commercial systems though is key to the commercial viability of such management strategies. This biological strategy relies on the grazing behaviour of the biological control such that the surface of substrate provided per unit of biological control is a more appropriate indicator than the ratio of control to stock fish. In this experiment, the 10 L aquaria provided a substrate surface of 229 $cm^2$ $L^{-1}$ and of 1143 $cm^2$ / biological control. It must be

highlighted that in a commercial raceway of, *e.g.* 6 m × 2 m × 1 m (12 $m^3$), the surface to volume ratio is 28.3 $cm^2$ $L^{-1}$ which is 8-times lower than within our experimental system. Within such a 12 $m^3$ raceway, 297 *G. gibbiceps* would be needed to provide each biological control with the surface of substrate presently tested (1143 $cm^2$ / biological control of 2.55 ± 0.06 g). Clearly, the number of biological control organisms required would be reduced in commercial conditions by using biological controls of a higher body-weight. The number of biological control organisms needed would also vary with the amount of biofilm per surface area within the rearing system. It can be expected that a lower density of biofilm would increase the surface "cleaned" by the biological control assuming a constant feeding rate. Accordingly under commercial operations, maintaining a low level of biofilm by using efficient feeding regimes and an effective self-cleaning system could reduce the number of biological control organisms required and/or increase the efficacy of this biological strategy. This biological strategy is more likely appropriate in intensive artificial aquaculture systems (*e.g.* tanks or raceways) as opposed to more extensive pond systems with a natural substrate. The positive results observed in this trial warrant larger-scale testing of using *G. gibbiceps* as a biological control organism in order to determine the commercial feasibility of this approach and define the most suitable stocking densities.

In the same way that wrasse (corkwing wrasse, *Symphodus melops* L. and goldsinny wrasse, *Ctenolabrus rupestris* L.) have been employed alongside chemical treatments against salmon lice infections on Atlantic salmon (Treasurer, 1996), the use of detrivorous / algal substrate grazer as a biological control within a wider therapeutic strategy against *I. multifiliis* would offer a number of advantages as opposed to strategies relying exclusively on chemical treatments. As

shown by the reduced infection levels observed in this experiment, such a biological control has the potential to avoid or diminish *I. multifiliis* outbreaks negating the administration of chemical treatments. In addition, introducing a biological control at the time of stocking would act as a prophylactic measure affecting the probability of future infection events (Seng, 1997; Bergh, 2007). In addition, maintaining low levels of infection for a longer period of time by stocking *G. gibbiceps* could favour the development of protective immunity against *I. mutifiliis* in the fish population (Subasinghe & Sommerville, 1989; Matthews, 2005; Xu *et al*., 2008a). Chemotherapies are labour intensive, constitute a risk of hazard to the local environment and to the workers alike and can induce a degree of physiological stress compromising fish growth and survival (Noga, 2010). Importantly, a reduction in the frequency of the chemotherapeutic treatments could preserve its efficiency by minimizing the development of resistance in the parasite population as has been observed in the management of *L. salmonis* (Fallang *et al*., 2004; Lees *et al*., 2008). It would also minimize the risk of reaching the maximum discharge consent that often restricts the volume of chemical compounds (*e.g.* formaldehyde and sodium chloride in Scotland, SEPA) available to the farmer. It must be acknowledged that two new chemical treatments of low toxicity have recently been shown to be effective at disrupting normal cyst development: bronopol (Shinn *et al*., 2010; Picón-Camacho *et al*., 2010b) and a peracetic-acid based product (Picón-Camacho *et al*., 2010a). This makes such alternative chemical treatments promising but they require further refinement to optimise their use for the control of *I. multifiliis* infections in commercial-scale aquaculture systems.

The use of biological controls in aquatic systems has, to date, been limited

to the control of ectoparasites using “host-cleaner” fish. Such strategies relying on the direct interaction between the stock and control fish have a number of negative implications as documented when using wrasse species to control salmon lice infections in Atlantic salmon. The wrasse controls were shown to graze on the fouled net despite the occurrence of lice on the salmon stock while, during periods of low infection, it was necessary to provide supplementary artificial food (Kvenseth, 1996; Treasurer, 1996, 2002). In addition, large salmon were shown to attack wrasse (Kvenseth, 1996) while the wrasse were observed attacking the eyes of salmon during periods of low infection (Kent & Poppe, 2002). The use of a substrate detrivorous / algal grazer as biological control holds the four-fold advantage of generating minimal interactions with the cultured stock, of relying on a feed source naturally present within the rearing enclosure and of maintaining a low level of biofilm cover and reducing the level of *I. multifiliis* infection. This could have wider beneficial implications as microorganisms and bacteria are indeed known to concentrate within the biofilm layer developing on submerged surfaces (O’Toole *et al*., 2005) thereby constituting a potential reservoir of pathogens and an additional threat to the health and welfare status of the resident fish population (Ehrlich *et al*., 2004; Flanders & Yildiz, 2004; Bourne *et al*., 2006). Using a biological control organism to target and remove the external proliferative stage of a parasite represents a significant step forward in the control of pathogenic protozoan species like *I. multifiliis*. It is expected that such a biological strategy could prove similarly effective at controlling other parasitic diseases holding a settled multiplicative or protective life-stages. Examples of such parasitic diseases would include both ecto- and endoparasite such as *e.g. Amyloodinium oocelatum, Chilodonella cyprinid*, *Myxobolus cerebralis* and *Piscinoodinium* sp. (Lom & Dyková,

1992; Gilbert & Granath, 2003; Noga, 2010).

The use of an algivorous / detrivorous fish as a biological control organism against the non-parasitic life-stage of *I. multifiliis* was investigated for the first time in this study. *Glyptoperichthys gibbiceps* in cohabitation with *O. aureus* and in a tank system was found to be an effective biological control in reducing the number of trophonts establishing on fish by 63%. This significant reduction of the infections levels is hypothesised to be a consequence of the grazing activities of *Glyptoperichthys gibbiceps* on the biofilm that formed within each tank and upon which the tomonts were shown to preferentially settle to encyst and multiply.

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# CHAPTER 5

# GENETIC BARCODING AND PHYLOGENY

# CHAPTER 5

## PAPER VII

*RESEARCH ARTICLE*

**Examining intra-specific variability in *Ichthyophthirius multifiliis* Fouquet, 1876 using rDNA and mtDNA (COI).**

**Picón-Camacho, S.M.[a],** Sánchez-Fontenla, J.[b], Monroig, O.[a], Bron J.E.[a] & Shinn, A.P.[a]

[a]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, United Kingdom.*

[b]*Institute of Aquaculture of Torre de la Sal, Spanish National Research Council (CSIC), 12595 Ribera de Cabanes, Castellón, Spain.*

**In Preparation**

**Contributions:** The following manuscript was compiled in its entire by the author of this thesis, The co-authors assisted on guidance on molecular work and the proofreading of the manuscript.

**Keywords:** phylogeny, genetic diversity, intra-specific variation, mitochondrial DNA, ribosomal DNA, *Ichthyophthirius multifiliis*.

**Abstract**

*Ichthyophthirius multifiliis* Fouquet, 1876 is a common ciliate protozoan parasite of wild and cultured freshwater fish with a global distribution. The presence of geographical and ecological differences amongst *I. multifiliis* populations has been previously demonstrated, however, to date there have been no suitable markers identified for the detection of intra-specific genetic variation among isolates. In the present study, we compared the use of two molecular markers, the nuclear 18S rDNA region (788 bp region) and the mitochondrial cytochrome c oxidase (COI) gene (986 bp) to discriminate isolates of *I. multifiliis*. The use of non-coding regions of the rDNA, ITS-1 and ITS-2, demonstrated to be too highly conserved to distinguish intra-specific variation. The mtDNA marker (COI), however, provided a higher resolution than the rDNA region marker. COI was able to resolve genetic variation, not only between isolates of *I. multifiliis* but also within populations. The results suggest that COI mtDNA can be used as an effective molecular marker to determine intra-specific variation in *I. multifiliis*. The identification and epidemiological characterisation of isolates of *I. multifiliis* could play an important role on the management of *I. multifiliis* infections in farm systems. Further isolates from a range of other localities, however, are required to confirm the utility of the COI gene as a marker and to explore the extent of variation between them.

## 1. Introduction

*Ichthyophthirius multifiliis* Fouquet, 1876 commonly known as "Ich" or "fish whitespot", is a ciliate protozoan infecting freshwater fish worldwide which during outbreaks can lead to high mortality levels (Matthews, 2005). *Ichthyophthirius multifiliis* belongs to the Phylum Ciliophora, Order Hymenostomatida Delage *et* Herouard, 1896 (Lom & Dyková, 1992). It has a direct and temperature dependant life-cycle with four life-stages: (1) the trophont, a parasitic stage within the fish epithelia, (2) the tomont, a free-swimming stage released from the fish, (3) the cyst, a reproductive stage which divides asexually to produce theronts (4), free-swimming infective stages (Matthews, 2005). Only asexual reproduction by binary division at the cyst stage has been documented in *I. multifiliis* (Lom & Dyková, 1992). However, this parasite presents the nuclear dualism characteristic of other ciliates, which are capable of alternating between sexual and asexual reproduction over their life-cycle (Orias, 1998). This nuclear organisation is composed of two functionally distinct nuclei: a polyploid macronucleous (MAC) containing the "somatic" genome and the diploid micronucleus (MIC) carrying the "germline" genome that is involved in sexual reproduction (Prescott, 1994; Orias, 1998; Riley & Katz, 2001).

The broad distribution of *I. multifiliis,* ranging from cold to tropical water temperatures, has suggested the presence of at least two different strains of *I. multifiliis*: a coldwater (7-11°C) and a warm water (13-16°C) strain (Nigrelli *et al.*, 1976). Extensive research over the last thirty years has demonstrated the close relationship between the source of different isolates of *I. multifiliis* and their life-cycle parameters under different temperature and salinity conditions *e.g.* tomont survival and development rate, and size and number of theronts that are

subsequently produced (McCallum, 1982, 1985; Dickerson *et al*., 1985; Ewing *et al*., 1988; Clayton & Price, 1988; Clark & Dickerson, 1997; Aihua & Buchmann, 2001). As observed in other ciliates *I. multifiliis* appears to show ecophysiological differences between isolates of the same species (Barth *et al*., 2008). Previous studies have demonstrated variations between *I. multifiliis* isolates in terms of pathogenicity (Swennes *et al*., 2007; Ling *et al*., 2009) and sensitivity to chemical treatment (Straus & Meinelt, 2009; Straus *et al*., 2009). Differentiating isolates according to pathogenicity and upon different responses to environmental and toxicological factors has clear implications for establishing more efficient management of *I. multifiliis* infections in farm systems. Five different immobilization serotypes of *I. multifiliis* have been described from eleven geographical isolates by Dickerson *et al*. (1993) and Dickerson & Clark (1998). Interestingly, the serotype variation between isolates of *I. multifiliis* was suggested to relate to different levels of infectivity (Swennes *et al*., 2007). It has been suggested that use of molecular techniques to identify intra-specific variants might provide a key to improve the understanding of isolate virulence/ host immune-response and in the development of vaccines against *I. multifiliis* (Swennes *et al*., 2007).

Describing the genetic variation between sequences obtained from a defined DNA region, or genetic "barcoding", has proven a reliable technique to identify a given species and/or differentiate between strains (Hebert *et al*., 2003a). The different components of the ribosomal DNA (rDNA) region have been widely used as molecular markers to explore inter and intra-specific phylogenetic relationships between different groups of fish parasites such as the Myxozoa and Monogenea (Matějusová *et al*., 2001; Meinilä *et al*., 2004; Fiala, 2006; Whipps &

Kent, 2006). The rDNA region has the advantage of being present in a high number of copies throughout the genome and its different components show different rates of evolution. The coding 18S (small subunit (SSU)), 28S (large subunit (LSU)) and the 5.8 S components are highly conserved and suitable for examining inter-specific variation (Hillis & Dixon, 1991). Among them, the 18S has been frequently used (Clark, 2006), including determination of the phylogenetic relationships between members of the class Oligohymenophorea (Yi *et al*., 2009) and subclass Hymenostomatia (Wright & Lynn, 1995). The non-coding internal transcribed spacer regions (ITS-1 and ITS-2), however, are more variable, hence appropriate to detect genetic variation within a given species (Van de Peer *et al*., 2000). ITS-1 and ITS-2 were, for example, successfully used to identify distinct genetic strains within the parasitic fish ciliate *Cryptocaryon irritans* Brown, 1951, the marine counterpart of *I. multifiliis* (Diggles & Adlard, 1997; Yambot *et al*., 2003; Sun *et al*., 2006). Two isolates of *I. multifiliis* from distant geographic locations showed however, low genetic variation in the ITS-1 and ITS-2 regions (Sun e*t al.*, 2006). Similarly in the free-living ciliates *Paramecium* sp. and *Tetrahymena* sp. isolates from distant geographical origin, ITS-1 and ITS-2 sequences showed no variation levels and were deemed too conserved to characterize intra-specific phylogenetic relationships in both species (Barth *et al*., 2006; Lynn & Strüder-Kypke, 2006).

Mitochondrial DNA (mtDNA) genes such as the cytochrome c oxidase subunit I gene (COI) have been explored as an alternative to nuclear rDNA markers (Rand, 2001; Hebert *et al*., 2003a, Ratnasingham & Hebert, 2007). mtDNA appears more appropriate than nuclear rDNA for use as genetic markers for phylogenetic analysis (Hebert *et al*., 2003b). mtDNA is characterised by its lack of introns and experiences limited recombination due to its haploid mode of inheritance (Hebert

*et al*., 2003 a, b). In addition, COI shows a high occurrence of base substitution of third position nucleotides, allowing the discrimination of intra-specific phylogeographic groups despite a slow rate of change in amino acid sequence (Herbert *et al*., 2003 a; Moritz & Cicero, 2004).

The use of multiple indicators to identify geographical isolates, including ecological, serological and DNA markers of variability, could improve identification and characterisation of *I. multifiliis* isolates, allowing more efficient management of *I. multifiliis* infections in farm systems. Despite their potential, DNA markers have seldom been applied to the investigation of population structuring in parasitic ciliates and have shown discrepancy between species in their capacity to elucidate intra-specific variation. The aim of the present study was to examine and compare the utility of the nuclear rDNA region and the mitochondrial COI gene for the investigation of intra-specific variation between isolates of *I. multifiliis* collected from four different geographical locations.

## 2. Materials and methods

### 2.1 Parasite material collection and DNA extraction.

Tomonts of *I. multifiliis* from each isolate were harvested as they naturally exited the fish host. After collection tomonts were transferred to a clean Eppendorf and washed with distilled water to remove any mucus from the fish host. Tomonts were then fixed in 95% ethanol and stored at 4°C until DNA extraction. For the DNA extraction, pools of 15 tomonts were placed in 300 μL of lysis buffer with 3 μL of Proteinase K (10mg $L^{-1}$) and were incubated at 55 °C for an hour. Protein and DNA precipitation were carried out using the REALPURE DNA extraction kit (REAL laboratories, Spain) according to the manufacturer's instructions. The precipitated

DNA was then eluted in 20 µL of minipure DNAse and RNAse free water and stored at -20 °C. Isolate codes, host species and collection localities are listed in Table 1.

**Table 1.** *Ichthyophthirius multifiliis* isolates with coding employed for the study, host species and collection locality.

| Isolate coding | Host species | Locality |
|---|---|---|
| Ich Ala. | *Ictalurus punctatus* | Alabama, USA |
| Ich Dan. | *Oncorhynchus mykiss* | Jutland, Denmark |
| Ich Sco. | *Oncorhynchus mykiss* | Dumfries, Scotland |
| Ich Sha. | *Carassius auratus* | Shanghai, China |

### 2.2. PCR amplification, primers design and sequencing

Extracted DNA from each isolate was amplified using PCR in a total volume of 25µL. Amplification using Taq DNA polymerase may result in a few mismatched bases per kilobase of DNA, hence amplification and sequencing of each isolate employed three separate DNA extractions (replicates). For the Scottish and Shanghai isolates of *I. multifiliis*, sequences obtained from the replicates appeared to belong to three and two different haplotypes respectively therefore they have been considered separetly.

### 2.3. Amplification and purification of the ribosomal DNA region

To amplify the rDNA region comprising 227 bp of 18S, 143 bp ITS-1, 152 bp 5.8S, 194 bp ITS-2 and 72 bp 28S (Figure 1), the general primers P1 and NC2 (Table 2)(Diggles & Adlard, 1997; Zhu *et al*., 2000, 2002; Yambot *et al*., 2003) were used. Primers were synthesised by MWG-Biotech AG (Germany). For the PCR reaction,

125 ng of extracted DNA was used, mixed with 5 Units Takara Ex TaqTM (Takara Shuzo Co., Japan), Ex TaqTM buffer (3 mM $MgCl_2$), 0.25 mM dNTP and 25 pmol of each oligonucleotide primer. PCR temperature profiles for all reactions were: preheating at 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 50°C for 30 s and 72°C for 1 min 30 sec, followed by a further final elongation step of 7 min at 72°C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, U.K.).

**Table 2.** Base sequence of the primers used to target rDNA region. * Designed in the present study.

| Primer | Sequence |
|---|---|
| P1 | 5'- GTT CCC CTT GAA CGA GGA ATT C -3' |
| NC2 | 5'- TTA GTT TCT TTT CCT CCG CT -3' |
| RibIchF1* | 5'- GAT GAA GAA CGC AGC GAA AT -3' |
| RibIchR1* | 5'- ATT TCG CTG CGT TCT TCA TC -3' |
| RibIchF2* | 5'- ATG ATT GGG TGG TTG GAG AA -3' |
| RibIchR2* | 5'- TTC AGC GGG TAA TCT TGC TT -3' |

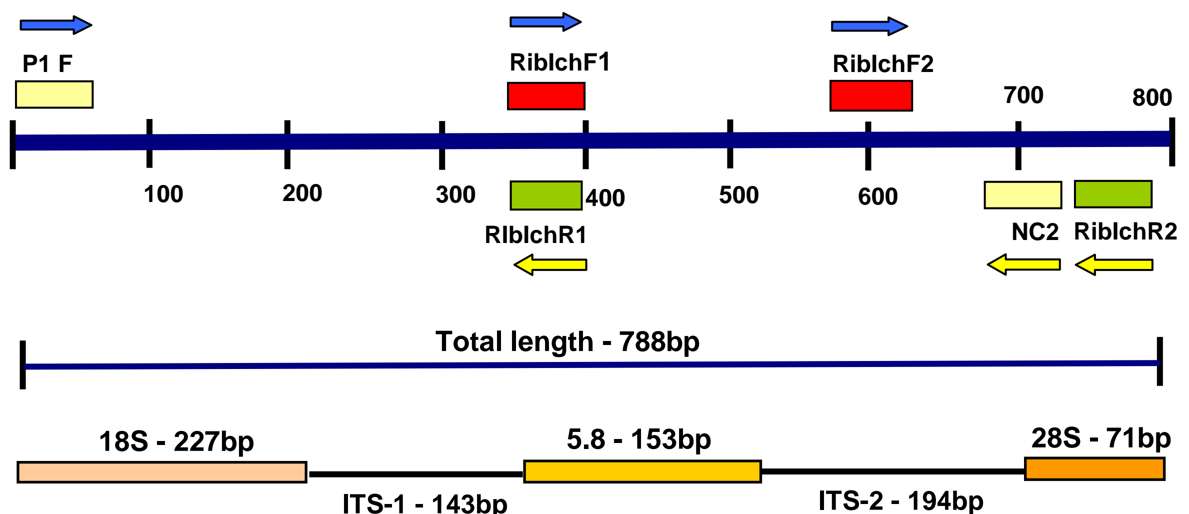


**Figure 1.** Diagram representing the rDNA region targeted in the current study showing the approximate length of each fragment amplified. The localisation of the pairs of primers employed is also shown.

**2.4. Amplification and purification of the mitochondrial DNA fragment**

The amplified target region of 985 nucleotides of the COI gene corresponded to the region 38427 to 39412 of *Tetrahymena thermophila* (GeneBank Asccession no. AF396436). The initial primers 288 and FolB (Table 3)(Folmer *et al*., 1994) were chosen after determining the conserved regions by aligning the sequences of the COI gene from *Tetrahymena pyriformis* (AF 160864.1, L28677, XO6133) and *Plasmodium falciparum* (XM001347640) for the forward sequence and *Tetrahymena thermophila* (AF396436), *Tetrahymena pigmentosa* (DQ927305) and *Paramecium tetraurelia* (DQ912530.1) for the reverse sequence. Primers were obtained from MWG-Biotech AG (Germany).

The PCR was carried out using 125 ng of extracted DNA mixed with 1.25 Units Takara Ex TaqTM (Takara Shuzo Co., Japan), Ex TaqTM buffer (1.5 mM $MgCl_2$), 0.25 mM dNTP and 25 pmol of each oligonucleotide primer. PCR temperature profiles for all reactions were: preheating at 94°C for 5 min of initial denaturation, followed by 40 cycles of 94°C for 1 min, 58°C for 1min and 30 s and 72°C for 1 min, followed by a further final elongation step of 5 min at 72°C. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, U.K.).

**Table 3**. Base sequence of the primers used to target the COI region of *I. multifiliis*.*Designed in the present study.

| **Primer** | **Sequence** |
|---|---|
| 288 | 5’- TCA GGT GCT GCA CTA GC- 3’ |
| FolB | 5’- TAA ACT TCA GGG TGA CCA AAA AAT CA-3’ |
| Co1F1* | 5’- TCA AAA TGT TCT GCC CGT ACT-3’ |
| Co1R1* | 5’- GTA CGG GCA GAA CAT TTT GA-3’ |

### 2.5. Cloning and sequencing

Purified PCR products obtained from the rDNA region and COI were cloned into a pBluescript II KS (-) vector (Stratagene, U.K.). Four colonies from each strain were picked and sequenced using the standard vector primers M13 Forward and M13 Reverse. In addition to these primers, internal primers designed in the current study for the rDNA region of *I. multifliis* named RibIchF1, RibIchR1, RicbIchR2 and RibIchF2 (Table 2) and for the mtDNA region Co1F1 and Co1R1 (Table 3) were employed for sequencing using BigDye™ Terminator v3.0 cycle sequencing ready reaction kit and an ABI 310 automatic gene sequencer (Applied Biosystems Inc., USA). All the primers were synthesised by MWG-Biotech AG (Germany).

### 2.6. Sequence alignment and phylogenetic analyses

Sequences of the ciliates *Tetrahymena thermophila* (GenBank Accession no. X54512.1; Engberg & Nielsen, 1990) and *Tetrahymena vorax* (EF070319.1; Chantangsi *et al*., 2007) and were included in the analyses as outgroups for ribosomal sequence and mitochondrial datasets respectively.

Alignments of the sequences (available upon request) were performed automatically using the ClustalX2 program (Larkin *et al*., 2007) with further manual alignment undertaken using the BioEdit 7.0 program (Hall, 1999). For non-coding rDNA region, a total exclusion of positions presenting gaps resulted in a loss of substantial phylogenetic information (Figure 2). For mtDNA coding sequences, alignment positions with gaps were removed by complete deletion (Figure 3).Thus, only aligned sequence sites comprising more than 70% gaps were removed. Remaining gaps in *Tethrahymena* sequences were treated as 'missing data' for further phylogenetic analyses (Figure 3) (Manuel *et al*., 2003).

Genetic p-distances amongst rDNA region sequences were found to be highly conserved (Table 4). The COI gene genetic p-distances matrix was computed with MEGA 4 (Tamura *et al*., 2007). The pairwise distances were determined using the nucleotide p-distance substitution model (Table 5). Matrices for rDNA region and mtDNA sequence datasets were analyzed independently by maximum parsimony (MP) and neighbour-joining (NJ) inference methods using MEGA 4. All positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). Therefore, a total of 971 positions were considered for the final mtDNA dataset, and a total of 789 positions for the final rDNA region dataset. No outgroups were selected *a priori* for both MP and NJ analyses since the outgroup sequences were treated as a part of the whole sequence dataset.

For the MP approach, data were analysed using the Close-Neighbor-Interchange algorithm with search level 3 (Felsenstein, 1985; Nei & Kumar, 2000), where the initial trees were obtained with the random addition of sequences (10 replicates).

For the NJ approach, the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004). All phylogenetic reconstructions inferred were tested using a Bootstrap test (Felsenstein, 1985) of 1000 replicates, in order to assess the confidence (robustness) of the trees.

**PART 1**

```
18S
                       10        20        30        40        50
Ich Sha.       -----GTTCCCCTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCAGCT   45
Ich Ala.       ------TTCCCCTTGAACGAGGAATTCCTAGTAAGTGCAAGTCRTCAGCT   44
Ich Dan.       -----GTTCCCCTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCAGCT   45
Ich Sco.       CGATTGTTCCCCTTGAACGAGGAATTCCTAGTAAGTGCAAGTCATCAGCT   50
T.thermophila  GAATTGTGGATCTTGAACGAGGAATTTCTAGTAAGTGCAAGTCATCAGCT   50

18S
                       60        70        80        90       100
Ich Sha.       TGCGTTGATTATGTCCCTGCCGTTTGTACACACCGCCCGTCGCTTGTAGT   95
Ich Ala.       TGCGTTGATTATGTCCCTGCCGTTTGTACACACCGCCCGTCGCTTGTAGT   94
Ich Dan.       TGCGTTGATTATGTCCCTGCCGTTTGTACACACCGCCCGTCGCCTGTAGT   95
Ich Sco.       TGCGTTGATTATGTCCCTGCCGTTTGTACACACCGCCCGTCGCTTGTAGT   100
T.thermophila  TGCGTTGATTATGTCCCTGCCGTTTGTACACACCGCCCGTCGCTTGTAGT   100

18S ——►◄—— ITS-1
                      210       220       230       _40       250
Ich Sha.       TGTAGGTGAACCTGCAGATGGATCATTAACACAATTAACAAACCTTAATT   245
Ich Ala.       TGTAGGTGAACCTGCAGATGGATCATTAACACAATTAACAAACCWTRATT   244
Ich Dan.       TGTAGGTGAACCTGCAGATGGATCATTAACACAATTAACAAACCTTAATT   245
Ich Sco.       TGTAGGTGAACCTGCAGATGGATCATTAACACAATTAACAAACCTTAATT   250
T.thermophila  TGTAGGTGAACCTGCAGATGGATCATTAACACAATTAACAAACCTTAACT   250

ITS-1
                      260       270       280       290       300
Ich Sha.       AATGTACTTTATTTAGGAGGAGGACTTTTTAAGTTCTCCTCTTAAATTCT   295
Ich Ala.       AATGTACTTTATTTAGGAGGAGGACTTTTTAAGTTCTCCTCTTAAATTCT   294
Ich Dan.       AATGTACTTTATTTAGGAGGAGGACTTTTTAAGTTCTCCTCTTAAATYCT   295
Ich Sco.       AATGTACTTTATTTAGGAGGAGGACTTTTTAAGTTCTCCTCTTAAATTCT   300
T.thermophila  TATGTACTTTC-----GAAGAGAACTTC---GGTTTTCTTC-GAGGTTTT   291

ITS-1
                      310       320       330       340       350
Ich Sha.       ATACACACCTTGTGTAAATAARATACCTTCATATGTCTAAGATCTGGATA   345
Ich Ala.       ATACACACCTTGTGTAAATWAAATACCTTCATATGTCTAAGATCTGGATA   344
Ich Dan.       ATACACACCTTGTGTAAATAAAATACCTTCATATGTCTAAGATCTGGATA   345
Ich Sco.       ATACACACCTTGTGTAAATAAAATACCTTCATATGTCTAAGATCTGGATA   350
T.thermophila  ATTCACACCTAGTGTGAATAAAAATTTTTCATATGTCTAAGATCTGGATA   341

ITS-1 ——►◄—— 5.8S
                      360       370       380       390       400
Ich Sha.       TATCATCCAAAACAAAATTAGAAAATTTTCAACGGTGGATATCTAGGTTC   395
Ich Ala.       TATCATCCAAARCAAAATTAGAAAATTTTCAACGGTGGATATCTAGGTTC   394
Ich Dan.       TATCATCCAAAACAAAATTAGAAAATTTTCAACGGTGGATATCTAGGTTC   395
Ich Sco.       TATCATCCAAAACAAAATTAGAAAATTTTCAACGGTGGATATCTAGGTTC   400
T.thermophila  A--CATCCAAAACGAAA--AGAAAACTTTCAACGGTGGATATCTAGGTTC   387

5.8S
                      410       420       430       440       450
Ich Sha.       CCGTGACGATGAAGAACGCAGCGAAATGCGATACGCAATGCGAATTGCAG   445
Ich Ala.       CCGTGACGATGAAGAACGCAGCGAAATGCGATACGCAATGCGAATTGCAG   444
Ich Dan.       CCGTGACGATGAAGAACGCAGCGAAATGCGATACGCAATGCGAATTGCAG   445
Ich Sco.       CCGTGACGATGAAGAACGCAGCGAAATGCGATACGCAATGCGAATTGCAG   450
T.thermophila  CCGTGACGATGAAGAACGCAGCGAAATGCGATACGCAATGCGAATTGCAG   437

5.8S ——►◄—— ITS-2
                      510       520       530       540       550
Ich Sha.       TCATGTTTGTATCAGTGTGGAAAGGAATCATGTATCTTAATGCGATTGAA   545
Ich Ala.       TCATGTTTGTATCAGTGTGGAAAGGAATCATGTATCTTAATGCGATTGAA   544
Ich Dan.       TCATGTTTGTATCAGTGTGGAAAGGAATCATGTATCTTAATGCGATTGAA   545
Ich Sco.       TCATGTTTGTATCAGTGTGGAAAGGAATCATGTATCTTAATGCGATTGAA   550
T.thermophila  TCATGTTTGTTTCAGTGTGGAAAGGAATCACGCATCTTAATGCGATTGAA   536
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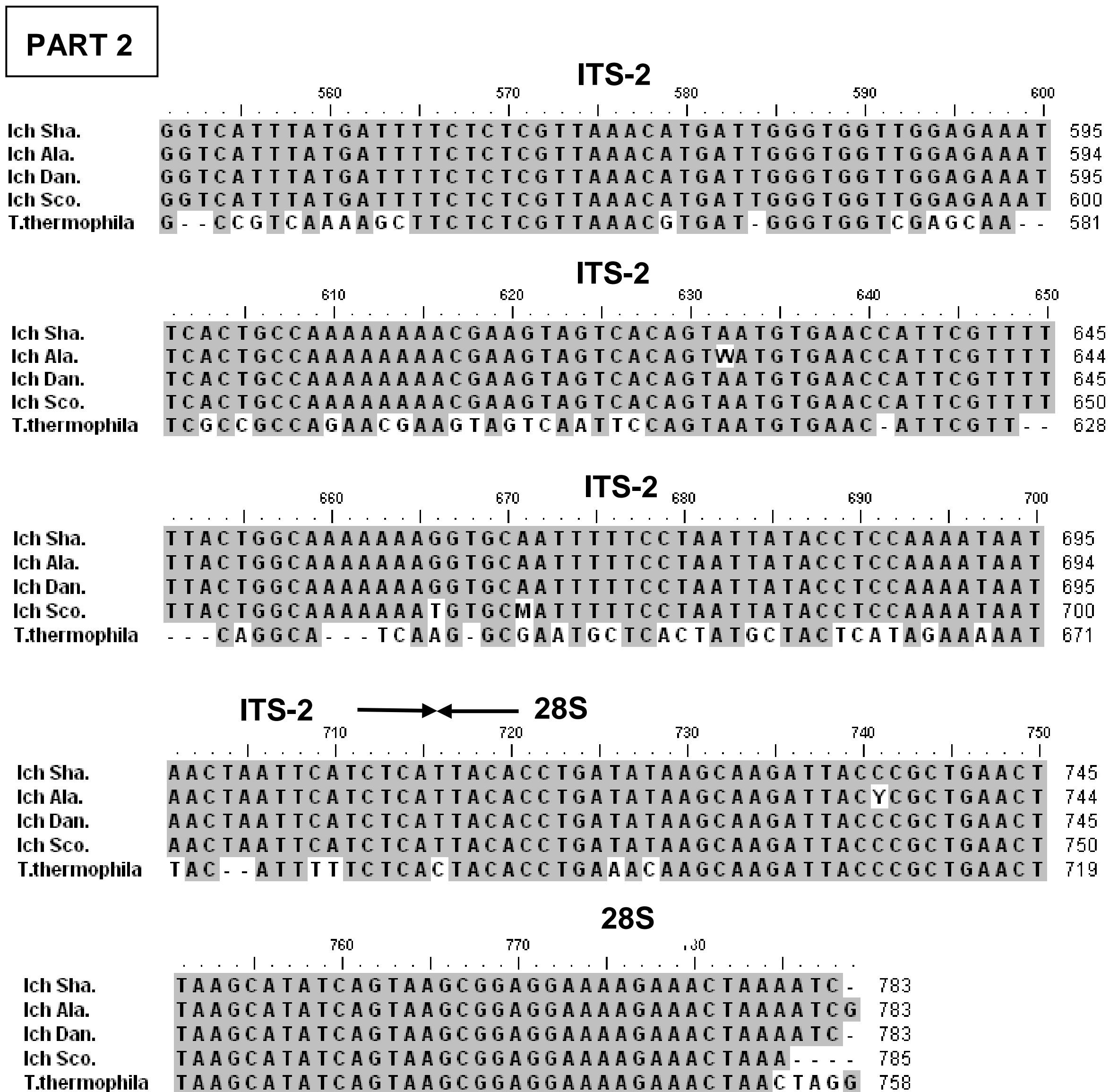


**Figure 2.** The nucleotide sequence alignment of the end 5'-end of the 18S, ITS-1, 5.8S and part of the ITS-2 sequences (Part 1) and ITS-2 and part of the 28S sequences (Part 2) of the four isolates of *I. multifiliis* obtained in the present study. *Tetrahymena thermophila* sequence was used as outgroup.

**PART 1**

```
                 10        20        30        40        50
Ich Sco.Rep1     TACTATGATAAGGGTTGGAATTATC-TCACCCCGGTAGTCCTTTTTTTAA  49
Ich Sco.Rep2     -----------------------------CCCCGGTAGTCCTTTATTTAA  21
Ich Sco.Rep3     ------------------AATTATCGTCACCCCGGTAGTCCTTTTTTTAA  32
Ich Sha.Rep1     --------------------------------TGGTAGTCCTTTTTTTAA  18
Ich Sha.Repl.3   --------------------------------------------------  1
Ich Ala.         ---------------------------CATCCTGGTACTCCTTTATTTAA  23
Ich Dan.         ----------------------------------------CTTTTTTTAA  10
T.vorax          GCAACTATGATAAGAATGGAACTTGCTCATCCCGGTAGTCCGTTTTTCAA  50

                 60        70        80        90        100
Ich Sco.Rep1     AGGTGATGCCCTTAGATATCTTCAGGTTATAACAGCACATGGTTTAATTA  99
Ich Sco.Rep2     AGGTGATGCCCTTAGATATCTTCAGGTTATAACAGCACATGGTTTAATTA  71
Ich Sco.Rep3     AGGTGATGCCCTTAGATATCTTCAGGTTATAACAGCACATGGTTTAATTA  82
Ich Sha.Rep1     AGGTGATGCCCTTACATATCTTCAGGTTATAACAGCACATGGTTTAATTA  68
Ich Sha.Repl.3   --------CCCTTACATATCTYCAGGTTATAACAGCACATGGTTTAATTA  42
Ich Ala.         AGGTGATGCCCTTAGATATCTTCAGGTTATAACAGCACATGGTTTAATTA  73
Ich Dan.         AGGTGATGCCCTTAGATGACTTCAGGTTATAACAGCACATGGTTTAATTA  60
T.vorax          AGGAGACTCTTTAAGATATTTACAAGTTGTAACTGCACATGGTCTAATCA  100

                 110       120       130       140       150
Ich Sco.Rep1     TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  149
Ich Sco.Rep2     TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  121
Ich Sco.Rep3     TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  132
Ich Sha.Rep1     TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  118
Ich Sha.Repl.3   TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  92
Ich Ala.         TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  123
Ich Dan.         TGGTATTTTTTGTGGTTGTTCCATTACTATTTGGTGGTTTTGCTAATTTT  110
T.vorax          TGGTGTTTTTTGTTGTAGTACCTATTTTATTTGGAGGATTTGCTAATTTT  150

                 160       170       180       190       200
Ich Sco.Rep1     TTAATACCATATCACGTAGGTTCTAAAGATGTTGCTTACCCTAGATTAAA  199
Ich Sco.Rep2     TTAATACCATATCACGTAGGTTCTAAAGATGTTGCTTACCCTASATTAAA  171
Ich Sco.Rep3     TTAATACCATATCACGTAGGTTCTAAAGATGTTGCTTACCCTAGATTAAA  182
Ich Sha.Rep1     TTAATACCATATCATGTAGGTTCTAAAGATGTTGCTTATCCTAGATTAAA  168
Ich Sha.Repl.3   TTAATACCATATCATGTAGGTTCTAAAGATGTTGCTTATCCTAGATTAAA  142
Ich Ala.         TTAATACCATATCATGTAGGTTCTAAAGATGTTGCTTATCCTAGATTAAA  173
Ich Dan.         TTAATACCATATCATGTAGGTTCTAAAGATGTTGCTTATCCTAGATTAAA  160
T.vorax          TTAATTCCTTACCATGTAGGTTCTAAAGATGTTGCATACCCAAGATTGAA  200

                 210       220       230       240       250
Ich Sco.Rep1     TAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  249
Ich Sco.Rep2     TAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  221
Ich Sco.Rep3     TAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  232
Ich Sha.Rep1     CAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  218
Ich Sha.Repl.3   CAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  192
Ich Ala.         CAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  223
Ich Dan.         CAGTATTGGTTTTTGAATACAACCTTGTGGTTTTATTTTACTTGCTAAAA  210
T.vorax          TAGTATTGGTTTTTGAATTCAACCATGTGGTTATATTTTATTAGCAAAAA  250

                 260       270       280       290       300
Ich Sco.Rep1     TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  299
Ich Sco.Rep2     TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  271
Ich Sco.Rep3     TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  282
Ich Sha.Rep1     TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  268
Ich Sha.Repl.3   TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  242
Ich Ala.         TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  273
Ich Dan.         TTGGTTTTTTAAGACCTCAATTTTGAAGATATTATGATAAGACTGCTTTT  260
T.vorax          TTGGATTTTTAAGACCTCAATTTTGAAGATATTACGATAAAACTTCTTTT  300
```

**PART 2**

```
                      360       370       380       390       400
Ich Sco.Rep1     TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  399
Ich Sco.Rep2     TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  371
Ich Sco.Rep3     TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  382
Ich Sha.Rep1     TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  368
Ich Sha.Repl.3   TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  342
Ich Ala.         TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  373
Ich Dan.         TAATGATATGTTATTGTATGTAAATTATTTAAAAGATACAACAATTAACA  360
T.vorax          AAATGATTATTTATTTTATTTAGACTTTTTAAAAAAAGAAATTTCTGATG  400

                      410       420       430       440       450
Ich Sco.Rep1     ATAATTATATAATTTGAAAGGCTAGAAAGAATTTATCAGATTTAAGCTAT  449
Ich Sco.Rep2     ATAATTATATAATTTGAAAGGCTAGAAAGAATTTATCAGATTTAAGCTAT  421
Ich Sco.Rep3     ATAATTATATAATTTGAAAGGCTAGAAAGAATTTATCAGATTTAAGCTAT  432
Ich Sha.Rep1     ATAATTATATAATTTGAAAGGCTAGAAAGAATYTATCAGATTTAAGTTAT  418
Ich Sha.Repl.3   ATAATTATATAATTTGAAAGGCTAGAAAGAATTTATCAGATTTAAGTTAT  392
Ich Ala.         ATAATTATATAATTTGAAAGGCTAGWAAGAATTTATCAGATTTAAGTTAT  423
Ich Dan.         ATAATTATATAATTTGAAAGGCTAGAAAGAATTTATCAGATTTAAGTTAT  410
T.vorax          ATCACTCTTTTTTTTGAAAAGCTAGAA--AAGTTATAAAATTACCACAAT  448

                      510       520       530       540       550
Ich Sco.Rep1     AAACTACCCAGAATCATTTTGATATGCTTCTAGTAGAGTTTTAAAATCAA  549
Ich Sco.Rep2     AAACTACCCAGAATCATTTTGATATGCTTCTAGTAGAGTTTTAAA-TCAA  520
Ich Sco.Rep3     AATCTACCCAGAATCATTTTGATATGCTTCTAGTAGAGTTTTAAA-TCAA  531
Ich Sha.Rep1     AAACTACCCAGAATCATTTTGATATGSTT-TAGTAGAGTTTTAAAATCAA  517
Ich Sha.Repl.3   AAACTWCCCAGAATCATTTTGATATGCTTSTAGTAGAGTTTTAAAATCAA  492
Ich Ala.         AAACTACCCAGAATCATTTTGATATGCTTCTAGTAGAGTTTTAAAATCAA  523
Ich Dan.         AAACTACCCAGAATCATTTTGATATGCTTCTAGTAGAGTTTTAAA-TCAA  509
T.vorax          TAATTATCCAGAATCTTTTTGATATGCTGCTAGTAGGGTTGTACAATCTA  548

                      560       570       580       590       600
Ich Sco.Rep1     GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  599
Ich Sco.Rep2     GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  570
Ich Sco.Rep3     GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  581
Ich Sha.Rep1     GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  567
Ich Sha.Repl.3   GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  542
Ich Ala.         GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  573
Ich Dan.         GAAGAAAAAAAATATTTGTATCAAAATGTTCTGCCCGTACTTTAGTTACC  559
T.vorax          GAAGAAAAAAAAGTTTTGTTACTAAATGTTCTGCTAGAACTTTAACAACT  598

                      610       620       630       640       650
Ich Sco.Rep1     GCAGGTTGAACTTTTATTACACCATTTAGTTCTAATMTAAAATATACTGG  649
Ich Sco.Rep2     GCAGGTTGAACTTTTATTACACCATTTAGTTTTAATTTAAAATATACTGG  620
Ich Sco.Rep3     GCAGGTTGAACTTTTATTACACCATTTAGTTCTAATTTAAAATATACTGG  631
Ich Sha.Rep1     GCAGGTTGAACTTTTATTACACCATTTAGTTCTAATTTAAAATATACTGG  617
Ich Sha.Repl.3   GCAGGTTGAACTTTTATKACACCATTTAGTTCTAATTTAAAATATACTGG  592
Ich Ala.         GCAGGTTGAACTTTTATTACACCATTTAGTTTTAATTTAAAATATACTGG  623
Ich Dan.         GCAGGTTGAACTTTTATTACACCATTTAGTTYTAATTTAAAATATACTGG  609
T.vorax          GCTGGTTGAACTTTTATAACACCTTTTAGTTCTAATATAAAATACACAGG  648

T.vorax          TGTAGGTTCTCAAGATGTTTTAATTTTATCAGTTGTTTTTGCTGGTATTA  698

                      710       720       730       740       750
Ich Sco.Rep1     GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATRT  749
Ich Sco.Rep2     GTACAACTATGACATTTACAAATTTATTAATAACAAGGAGAACTTTATYT  720
Ich Sco.Rep3     GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATCT  731
Ich Sha.Rep1     GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATCT  717
Ich Sha.Repl.3   GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATCT  692
Ich Ala.         GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATCT  723
Ich Dan.         GTACAACTATATCATTTACAAATTTATTAATAACAAGGAGAACTTTATYT  709
T.vorax          GTACCACTATATCATTTACAAATCTATTAATAACAAGAAGAACTTTAGCT  748
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                        810       820       830       840       850
                   ....|....|....|....|....|....|....|....|....|....|
Ich Sco.Rep1       TTTATTTTTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  849
Ich Sco.Rep2       TTTATTTTTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  820
Ich Sco.Rep3       TTTATTTTTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  831
Ich Sha.Rep1       TTTATTTCTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  817
Ich Sha.Repl.3     TTTATTTCTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  792
Ich Ala.           TTTATTTCTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  823
Ich Dan.           TTTATTTCTAGCATTAAGAATGTTAGCTATAATTACACCAGTATTAGCTG  809
T.vorax            TATATTCTTAACTTTAAGAATGTTGGCTACAATAACACCTGTTTTAGGTG  848
                        860       870       880       890       900
                   ....|....|....|....|....|....|....|....|....|....|
Ich Sco.Rep1       CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  899
Ich Sco.Rep2       CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  870
Ich Sco.Rep3       CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  881
Ich Sha.Rep1       CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  867
Ich Sha.Repl.3     CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  842
Ich Ala.           CTGCGATGATAATGATGATTTTAGATAGACATTMACWAACTACATTTTTT  873
Ich Dan.           CTGCGATGATAATGATGATTTTAGATAGACATTGACAAACTACATTTTTT  859
T.vorax            CAGCTGTTATTATGATGGCTTTTGATAGACATTGACAAACAACCTTTTTT  898
                        910       920       930       940       950
                   ....|....|....|....|....|....|....|....|....|....|
Ich Sco.Rep1       GAATACGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  949
Ich Sco.Rep2       GAATACGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  920
Ich Sco.Rep3       GAATACGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  931
Ich Sha.Rep1       GAATATGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  917
Ich Sha.Repl.3     GAATATGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  892
Ich Ala.           GAATATGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  923
Ich Dan.           GAATATGCTTATGGTGGAGATCCTATATTATCACAACATTTATTTTGATT  909
T.vorax            GAATATGCCTACGGTGGTGATCCTATATTATCTCAACACTTATTTTGATT  948
                        960       970
                   ....|....|....|....|.
Ich Sco.Rep1       TTTTGGTCACCCTGAAGTTTA  970
Ich Sco.Rep2       TTTTGGTCACCCTGAAGTTTA  941
Ich Sco.Rep3       TTTTGGTCACCCTGAAGTTTA  952
Ich Sha.Rep1       TTTTGGTCACCCTGAAGTTTA  938
Ich Sha.Repl.3     TTTTGGTCACCCTGAAGTTTA  913
Ich Ala.           TTTTGGTCACCCTGAAGTTTA  944
Ich Dan.           TTTTGGTCACCCTGAAGTTTA  930
T.vorax            TTTTGGTCACCC-GAAGTATA  968
```

**Figure 3.** The nucleotide sequence alignment of the end 5' of the cytochrome c oxidase 1 (COI) of the four isolates of *I. multifiliis* obtained in the present study. *Tetrahymena vorax* sequence was used as the outgroup. Y = Pyrimidine (thymine or cytosine); R = Purine (adenine or guanine); W = adenine or thymine; S= guanine or cytosine; M = adenine or cytosine.

## 3. Results

### 3.1. Genetic distances of the rDNA region

Sequences between replicates obtained from the same isolate were identical in the four geographical isolates of *I. multifiliis* considered in the present study. Genetic p-distances amongst rDNA region sequences were found to be highly conserved (Figure 2). The p-distances within the isolates of *I. multifiliis* ranged from 0.13-0.24% (Table 4). The p-distances of the rDNA region ranged 12.87-13.00% compared to the outgroup *Tetrahymena thermophila* (Table 4).

**Table 4.** rDNA region p-distances matrix (data computed under pairwise distance calculation, nucleotide p-distance substitution model applied; gaps and missing data were completely removed; all codon and non-coding sites, transition and transversion substitutions included; rates among sites set as uniform; data are given in percentage of dissimilarity).

| | Ich Sco. | Ich Sha. | Ich Ala. | Ich Dan. |
|---|---|---|---|---|
| **Ich Sco.** | - | | | |
| **Ich Sha.** | 0.14% | - | | |
| **Ich Ala.** | 0.14% | 0.00% | - | |
| **Ich Dan.** | 0.27% | 0.14% | 0.13% | - |
| ***T. thermophila*** | 12.87% | 12.87% | 12.87% | 13.00% |

### 3.2. Genetic distances of the COI mtDNA

Sequences obtained from the replicates from USA and Denmark isolates had identical sequences. Sequences however obtained from the replicates from the Scottish and Chinese isolates revealed the presence of three distinct mitochondrial haplotypes in the Scottish isolates and two in the Chinese isolate. Pairwise genetic p-distances among ingroup taxa ranged from 0-2.02%. When compared with outgroup, p-distances ranged 0-21.95% (Table 5, Figure 3). Shanghai replicates

**Table 5.** COI mtDNA p-distances matrix (data computed under pairwise distance calculation, nucleotide p-distance substitution model applied; gaps and missing data were completely removed; all codon and non-coding sites, transition and transversion substitutions included; rates among sites set as uniform; data are given in percentage of dissimilarity).

| | Ich Sco.Rep.1 | Ich Sco.Rep.2 | Ich Sco.Rep3 | Ich Sha.Rep1 | Ich Sha.Rep2 | Ich Ala.Rep1 | Ich Ala.Rep2 | Ich Dan.Rep1 | Ich Dan.Rep2 |
|---|---|---|---|---|---|---|---|---|---|
| **Ich Sco.Rep.1** | - | | | | | | | | |
| **Ich Sco.Rep2** | 0.89% | - | | | | | | | |
| **Ich Sco.Rep3** | 0.00% | 0.89% | - | | | | | | |
| **Ich Sha.Rep1** | 1.23% | 2.02% | 1.23% | - | | | | | |
| **Ich Sha.Rep2** | 1.23% | 2.02% | 1.23% | 0.00% | - | | | | |
| **Ich Ala.Rep1** | 0.78% | 1.68% | 0.78% | 0.45% | 0.45% | - | | | |
| **Ich Ala.Rep2** | 0.78% | 1.68% | 0.78% | 0.45% | 0.45% | 0.00% | - | | |
| **Ich Dan.Rep1** | 0.78% | 1.68% | 0.78% | 0.45% | 0.45% | 0.00% | 0.00% | - | |
| **Ich Dan.Rep2** | 0.78% | 1.68% | 0.78% | 0.45% | 0.45% | 0.00% | 0.00% | 0.00% | - |
| ***T. vorax*** | 21.39% | 21.72% | 21.39% | 21.95% | 21.95% | 21.73% | 21.73% | 21.73% | 21.73% |

showed the greatest distances among the remaining isolates, whereas isolates from USA and Denmark shared full genetic similarity.

### 3.3. Phylogenetic reconstructions

Preliminary trees obtained with the different methods applied to both and rDNA region and COI mtDNA datasets present the same topology (Figures 4 and 5). For rDNA region dataset analyses, only the optimal tree under NJ inference method is shown, because only 102 variable characters detected were parsimony-informative (Figure 4). For mtDNA parsimony analyses, 217 characters (positions) are variable in the dataset, out of which 13 were parsimony informative. The most parsimonious tree (length=243) out of 9 most parsimonious trees (MP) and the optimal tree (NJ) are represented in one single tree (Figure 5).

In all cases, isolates of the species *I. multifiliis* cluster in a single clade separate from the outgroup species *T. thermophila* (for rDNA dataset) and *Tetrahymena vorax* (for mtDNA dataset) indicating the monophyly of the isolates considered for the present study.

#### 3.3.1 rDNA region: partial 18S, ITS1, 5.8S, ITS2 and partial 28S rDNA

Only the optimal tree under NJ inference method is presented when dealing with rDNA region sequences, given that no parsimony-informative characters are available in any sequence position (Figure 2). NJ reconstruction presents a well-supported monophyletic clade comprising isolates from Denmark, USA and China (clade 1, bootstrap 87%; Figure 5) while the Scotland strand clusters separately as clade 2. (bootstrap 82%; Figure 5).

The topology of the tree matches the mtDNA tree with clade 1 sub-divided into

two sub-clades, with the Chinese isolate comprising one sub-clade and the American and Danish isolates the other. The second clade comprises the Scottish isolate (Figure 4).

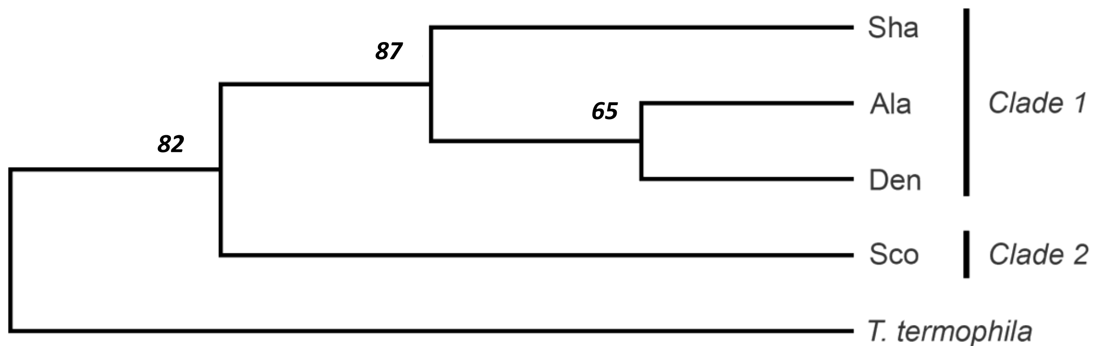


**Figure 4.** Phylogenetic analysis inferred from rDNA dataset under Neighbor-Joining method. The optimal tree with the sum of branch length = 0.185 is shown (only topology). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

**3.3.2. mtDNA: cytochrome c oxidase subunit I (COI)**

The mtDNA sequences obtained revealed the presence of three different haplotypes in the Scottish isolate (named replicate 1, 2 & 3), two haplotypes in the Chinese isolate (named replicate 1 & 2) and a single haplotype in the Danish and USA isolates.

The mtDNA dataset analyzed shows Denmark, USA and China isolates clustered together, whereas Scotland isolate replicates stand alone in a different cluster (designated clade 1 and clade 2, respectively). Both clades are strongly supported under both inference methods, though MP bootstrap values are higher (98%, 95% respectively) than those for NJ (68%, 86%) (Figure 5).

Clade 1 also forms two subclades: 1) China isolate replicates, which found high support under MP (73%) and a support below 50% under NJ; and 2) USA and Denmark isolates, presenting weak (28%) or moderate support (67%) under MP and NJ, respectively.

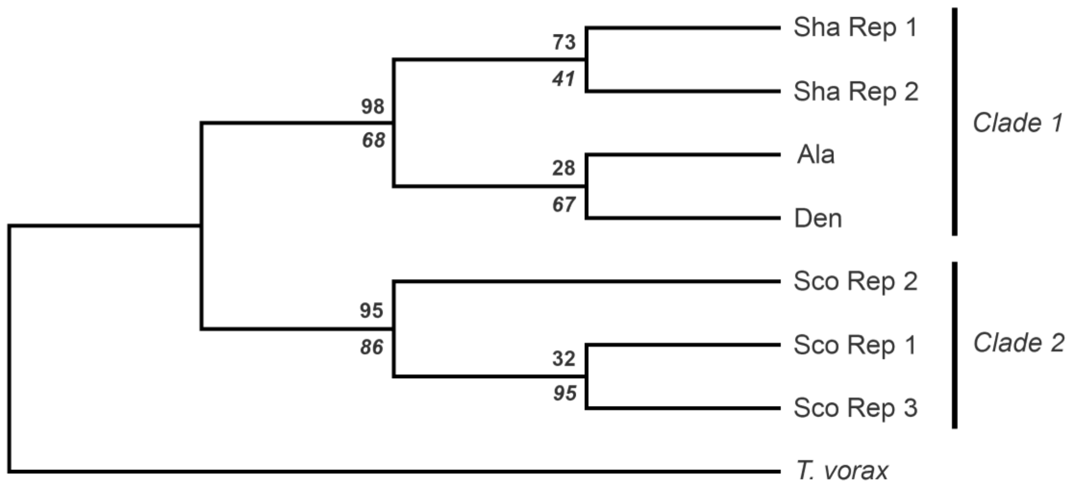


**Figure 5.** Phylogenetic analyses inferred from COI mtDNA dataset under Maximum Parsimony and Neighbour-Joining methods. Maximum Parsimony: Tree 1 out of 9 most parsimonious trees (length = 243) is shown (only topology). The consistency index is (0.857), the retention index is (0.916), and the composite index is 0.908 (0.785) for all sites and parsimony-informative sites, (in parentheses); Neighbor-Joining: The optimal tree with the sum of branch length = 0.269 is shown (only topology). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to each branch for MP above and NJ in italics below.

**4. Discussion**

Molecular-based phylogenetic reconstruction using mitochondrial and nuclear DNA sequences have shown enough resolution to confirm the occurrence of discrete isolates of *Ichthyophthirius multifiliis*. The COI marker showed a higher resolution than the rDNA region marker, which seemed to be highly conserved and gave no further phylogenetic information. This work supports the monophyly of the species, as well as major relationships between isolates. The two main clades identified are consistent for both rDNA and COI markers according to the bootstrap values provided using two inference methods for the reconstructions. Thus, isolates of *I. multifiliis* obtained from China, USA and Denmark are closely related, whereas the Scottish isolate has diverged substantially. Isolates from USA and Denmark seemed to be closely related (as is also shown by genetic mtDNA p-distances), whilst the Chinese strand clusters alone, as shown by the same topology in all reconstructions. The latter groupings are not, however, highly supported if only mtDNA analyses are considered. Meanwhile, rDNA region reconstruction under NJ method supports this relationship.

In the present study, there were distinguishable differences between the two molecular markers used (rDNA region and mtDNA), when considering the estimated genetic distances (or average number of nucleotide substitutions per site) (Thompson & Lymbery, 1990) obtained from the different isolates of *I. multifiliis*.

In the rDNA region, there was no genetic variation among the isolates of *I. multifiliis*. The ITS-1 and ITS-2 regions in *I. multifiliis* appear to be highly conserved to detect intra-specific variation such as previously observed in other ciliate species like *Paramecium* sp. and *Tetrahymena* sp. (Barth *et al*., 2006; Lynn &

Strüder-Kypke, 2006). The low variability found in the ITS-1 and ITS-2 regions has been suggested to relate to the homogenising effect of sexual reproduction by conjugation observed in other ciliate species (Prescott, 1994; Orias, 1998). In *I. multifiliis* the presence of sexual reproduction has been also hypothesised to occur, specifically during the trophont stage as it develops in the host epithelium (Matthews 2005). In this context, it has been observed that the number of trophonts within a specific region of the fish epithelium increased throughout the infection process (Ewing *et al*., 1988). Matthews *et al*. (1996) disovered that theronts were able to fuse with trophonts already established on the fish skin, suggesting the possibility of trophonts acting as macrogamonts and theronts as microgamonts in the conjugation process. The absence of sexual reproduction in laboratory-maintained isolates of *I. multifiliis* could explain the senescence of these cultures after 2 years (Ekless & Matthews, 1993; Xu & Klesius 2004), as sexual reproduction is considered essential for cell rejuvenation (Ewing *et al*., 1988; Noe & Dickerson, 1995). The presence of different mating types is well recognised in *Tetrahymena* sp., a free-living ciliate closely related to *I. multifiliis* (Prescott, 1994). It is therefore most likely that *I. multifiliis* undergoes sexual reproduction, with the theront stage acting as the mating type. How and exactly when this happens, remains unknown and is still under investigation.

The mtDNA region, however, showed higher genetic variation when compared to rDNA region (up to 2.02 % *versus* 0.24% rDNA). A recent study by Kher *et al*. (2010) explored the efficacy of COI as a molecular marker to identify *Tetrahymena* species. In addition to *Tetrahymena* sp., the COI from seven isolates of *I. multifiliis* were sequenced exhibiting intra-specific sequence divergence (<1%) in line with the results obtained in the present study. This variation in the mtDNA

of *I. multifiliis* was found not only between isolates but also within replicates from the same isolate: three haplotypes in the Scottish isolate and two haplotypes in the Chinese isolate. The changes observed in the mtDNA sequence of the different haplotypes of the Scottish isolate and Chinese isolate did not translate into any changes on the amino acid sequence of the protein. This intra-population genetic variation is also present in other ciliate species as the free-living *Paramecium* sp. and *Coleps* sp. (Barth *et al*., 2006; Barth *et al*. 2008).

The presence of polymorphisms or haplotypes within an isolate cannot be explained through sexual reproduction. It has been hyphotesied in other ciliate species that after undergoing sexual reproduction, a specific mtDNA haplotype should remain the same (Barth *et al*., 2008).

The high haplotype variation within an isolate of *I. multifiliis* obtained in the present study could be related to the high evolutionary rate present in mtDNA. The mtDNA mutation rate in ciliates is unknown but considering that the mutation rate of mitochondrial genes is approximately ten times higher than in the ITS regions (0.5-1% per million years in dinoflagellates (LaJeunesse, 2005), it could be estimated to be approximately 10% every million years (Brown *et al.*, 1979). Hence, the mtDNA haplotype variation observed in the present study on the Scottish and Chinese isolate of *I. multifiliis* would correlate to a necessary life span of 100000 years. This is unlikely given the fact that *I. multifiliis* was first recorded in China before AD 1126 (Dashu & Lieng-Siang, 1960) and probably did not spread into the rest of Europe (*e.g.* United Kingdom) until the Middle Ages (Hoffman, 1970). Another possible explanation would be the colonisation of *I. multfiliis* from other habitats. The Chinese isolate of *I. multifiliis* used in the present study was obtained from a pet shop retailer. The import of ornamental fish coming from very

distant localities and the fact that different isolates of *I. multifiliis* could be encountered while fish are held prior to sale could explain the presence of different haplotypes within the same population.

In natural habitats, free-living ciliates such as *Paramecium* sp. similarly exhibit intra-population genetic variation of mtDNA. This variation in COI has been proposed to be a valuable tool at identifying possible sygens or mating types for this species (Barth *et al*., 2006) suggesting that *I. multifiliis* populations in a local habitat might consist on more than one haplotype and that sexual reproduction might play an important role in the life-cycle of *I. multifiliis*.

To determine the real extent of genetic variation within a population of *I. multifiliis*, it would be necessary to carry out a single cell-DNA extraction. In the present study, the authors did not manage to extract DNA from a single tomont. Recently, Lynn & Pinheiro (2009) have revised the different methods used to successfully extract DNA for single-cell polymerase chain reaction (SC-PCR) from protists. The utilisation of SC-PCR on *I. multifiliis* would avoid the ambiguous sequence reads obtained from multi-cell DNA extractions and allow determination of the degree of intra-individual genetic variability.

The existence of different strains or genotypes in *I. multifiliis* according to water temperature has been hypothesised for a long time (Nigrelli *et al.*, 1976; Price & Clayton, 1998). Strains of *I. multifiliis* obtained from a given fish species have been shown to have significantly different infectivity rates when infecting a new host species (Elsayed *et al.*, 2006). Differences in virulence between different *I. multifiliis* isolates infecting the same fish host have also been reported (Swennes *et al*., 2007). In terms of chemical treatments to control *I. multifiliis*, it has been demonstrated very recently that different strains of *I. multifiliis* can exhibit diverse

levels of sensitivity against a given chemical treatment. When theronts from two different strains of *I. multifiliis* were exposed to copper sulphate, one of them appeared to be more resistant in both high and low alkalinity waters (Straus *et al*., 2009). Straus & Meinelt (2009) also showed differences in resistance between two geographically separated isolates of *I. multifiliis* exposed to peracetic acid. This discrepancy in chemotherapeutant toxicity between geographical isolates of *I. multifiliis* may explain in some extent the variation in effectiveness of treatments.

In the present study, we have demonstrated the utility of COI to genetically discriminate isolates of *I. multifiliis* from different geographical regions at both intra-specific and intra-population level. The identification of different genotypic strains of *I. multifiliis* using a COI molecular marker could serve as a practical tool for controlling infections in culture systems (Riley, 2009). The correlation of a given genotype of *I. multifiliis* with ecological (*e.g*. water temperature and salinity range) and epidemiological factors (*e.g*. host range, infectivity rate and sensitivity/toxicity to chemical treatments) would ultimately assist fish farmers to optimise treatments and other control strategies according to specific strain of *I. multifiliis*.

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# CHAPTER 6

# GENERAL DISCUSSION

The general aim of this research project was to investigate different approaches to improve the management of *I. multifiliis* infections towards more comprehensive, environmentally friendly and sustainable therapeutic strategies in the freshwater fish-food aquaculture (see appendix 1 for summary of findings). Two main approaches were taken to provide practical and commercially acceptable solutions. The first approach focused on the use of novel chemotherapeutants to target the free-living and parasitic stages of *I. multifiliis* (Chapters 3, Papers II, III & IV). The second approach investigated the feasibility and efficacy of removing the reproductive cyst stage of *I. multifiliis* from the culture system (Chapter 4, Papers V & VI). In addition, the possibility of discriminating between genetically different isolates of *I. multifiliis* using suitable molecular markers was assessed (Chapter 5, Paper VII) with a view to provide a tool for establishing strain-specific management strategies.

## 1. Testing of novel chemical treatments

Extensive research has been conducted in the last 30 years to identify suitable chemical replacements to malachite green for controlling infections of *I. multifiliis* in fish destined for human consumption (see Chapter 2, Paper I).

Trials performed *in vitro* demonstrated that the administration of bronopol and peracetic acid-based compounds over a short period of exposure (≤1 h) at high concentrations (bronopol-based product) and low concentrations (peracetic-acid based product) can significantly reduce the survival of the free-living stages (tomonts, cysts and theronts) (Chapter 3, Papers II & IV). In these *in vitro* trials, the cytotoxic effect of bronopol and peracetic acid-based compounds on the parasitic trophonts of *I. multifliis* could not be tested due to its reliance on host tissue to

survive. Interestingly, Nielsen & Buchmann (2000) achieved *in vitro* cultivation of the parasitic stage of *I. multifiliis* using EPC (Epithelioma Papulosum Cyprini) cells. The use of artificial epithelia could be used to maintain the trophont stage *in vitro* and test its relative susceptibility to the chemical compound under investigation under controlled conditions.

Our *in vitro* results demonstrated the potential of both compounds when administered as short duration bath treatments. However in practice, the asynchronous life-cycle of *I. multifiliis* (free living life-stages are constantly being released into the water column) (Lom & Dyková, 1992; Matthews, 2005) restricts the efficacy of a chemical treatment if applied over a short period of time. Repeated treatments of short duration are therefore required to significantly disrupt the propagation of *I. multifiliis* as is typically performed using, *e.g.*, formaldehyde (Wahli *et al.*, 1993; Tieman & Goodwin, 2001; Rintamäki- Kinnunen *et al*., 2005a; Lahnsteiner & Weismann, 2007; see Chapter II, Paper I), leading to, typically, high volumes of chemical being required.

The results of one of the *in vitro* trials showed that low concentrations of bronopol (*e.g.* 1 mg $L^{-1}$) administered over longer time periods *i.e.* 12 h-48 h, were effective at reducing the survival of the theront stage and in disrupting the normal development of the multiplicative (cyst) stage of *I. multifiliis* (Chapter 3, Paper III). This was particularly interesting as continuous-low concentration chemotherapy could target successive generations of the parasite in a cost-effective manner. Together, these results and the theoretical advantage of continuous treatments motivated the choice of administration protocols for *in vivo* testing using rainbow trout fingerlings (Chapter 3, Paper III). *In vivo*, continuous (*e.g*. 27 days) bath administration of low concentrations of bronopol (*i.e.* 2 and 5 mg $L^{-1}$) were also

successful at reducing *I. multifliis* infections when compared to the control groups (*i.e.* a 46-83% reduction in the number of trophonts establishing on fish in the second cycle of infection) (Chapter 3, Paper III).

Although continuous application holds a strong theoretical advantage and commercial potential if low concentrations are effective, such an administration strategy might also lead to high volumes being discharged (Schlenk *et al.*, 1998; Straus & Griffin, 2001, 2002; Mifsud & Rowland, 2008; Rowland *et al.*, 2009; Chapter 2, Paper I). Together, the choice of the administration protocol is a trade-off including a range of practical and financial factors such as volume, cost and discharge consent, toxicity to fish and withdraw period required (Noga, 2010).

In addition, our results demonstrated the higher efficacy of bronopol, if applied before and at the time of the experimental infection with theronts (*i.e.* as a preventative measure), as opposed to a treatment being applied 9-days post-infection (*i.e.* at the time of the first trophont exiting the host; as a remedial measure) (Chapter 3, Paper IV). The observed reduction in the infection level is likely due to the effective removal of the infective stage from the water column, as observed *in vitro* (Chapter 3, Paper II) rather than as a prophylactic effect on the fish *per se*. In order to test for a possible prophylactic potential, the administration of bronopol prior to infection and then stopped on day of infection should also have been tested. In practice, our results highlight that the industry would greatly benefit from the preventive deployment of bronopol and peracetic acid-based products during periods when there is a high risk of *I. multifiliis* infection as opposed to a remedial course of action following the detection of heavy *I. multifiliis* burdens or at the time of an outbreak. Under commercial conditions, it has been suggested that infections arise from only a few theronts entering farm systems

(Shinn *et al*., 2005) and that infections in commercial systems result primarily from propagation of the parasite within the culture system. Considering that *I. multifiliis* outbreaks have a strong seasonal component associated with increases in water temperature (Duarte *et al*., 1993; Valtonen & Koskivaara, 1994; Dickerson & Dawe, 1995; Chapter 4, Paper V), timing to initiate the preventive treatment in a farm system can be predicted by monitoring the water temperature profile and by referring to historical patterns of infection incidence. In our *in vivo* trial, bronopol administered continuously at a concentration of 5 mg $L^{-1}$ resulted in a low level of infection on fish compared to lower concentrations of bronopol, and was shown to be non-toxic when deployed for 30 days (Picón-Camacho *et al*., unpublished). In addition, the administration of 5 mg $L^{-1}$ of bronopol results in a low level of infection which, if below a threshold would allow the development of certain level of immunity in the cultured fish stock (Hines & Spira, 1974c; Burkart *et al*., 1990; Matthews, 1994; Matthews, 2005).

## 2. Testing of novel non-chemical treatments

To date, treatments against *I. multifiliis* infections have focused on chemical treatments targeting the free-swimming stages (tomonts and theronts) considered as the susceptible stages of the parasite (Wahli *et al*., 1993; Shinn *et al*., 2005; Goodwin & Straus, 2006; Lahnsteiner & Weismann, 2007; Heinecke & Buchmann, 2009). The cyst, protected by a wall coat (Ewing *et al*., 1983), is not considered as a potential target to control *I. multifiliis* infections due to its proven resistance to most chemical treatments (Wahli *et al*., 1993; Jensen *et al*., 2001; Meinelt *et al*., 2009). Not previously tested, our results show that the physical removal or disruption of the cyst stage of *I. multifiliis* using a mechanical device in flow-

through raceways in a commercial-scale rainbow trout facility (Chapter 4, Paper V) or an appropriate biological control in static experimental tanks (Chapter 4, Paper VI) was found to have a marked impact on the population dynamics of the parasite, as shown by a significant reduction in the number of trophonts establishing on the fish.

The tomont settlement and development into a multiplicative cyst is particularly significant in the life-cycle and propagation of *I. multifiliis* as a single cyst can produce up to 3,000 infective-stages (at 25°C; Wagner, 1960) that, if succesful, can develop into the parasitic stage. Overall, this research project provided a number of new insights on the tomont settlement behaviour and cyst development that is to date comparatively little studied. Our data revealed that the tomont stage preferentially encysts at the bottom of the rearing system (75% on the horizontal surface and 25% on the vertical sides in close proximity to the bottom) (Chapter 4, Paper V) and on bio-film covered substrates (Chapter 4, Paper VI). In addition, the material composition of the substrate also affects the capability of the tomont to encyst and to survive. Finally, the substrate texture plays an important role on the capacity to dislodge the settled cyst and control *I. multifliis* populations (Chapter 4, Paper V).

To date, the tomont stage has been demonstrated to exhibit a preferential settlement and encystment on light-coloured substrates under yellow and white lighting conditions which was suggested as being a camouflage behaviour (Nickell & Ewing, 1989). In addition, theronts have been shown to exhibit a range of host-finding swimming behaviours that can be stimulated by specific chemical host cues (Haas *et al*., 1999). In the same way that algal chemical cues have been shown to stimulate the settlement of green-lipped mussel, *Perna canaliculus*, larvae (Alfaro

*et al*., 2006), a biofilm surface could elicit a degree of chemo-attraction and provide a more appropriate environment for *I. multifiliis* tomonts to settle on and develop into cysts. As supported by the preferential settlement that was demonstrated, a bio-film substrate could have a beneficial effect on tomont survival and multiplication, although a comparison of the subsequent number of theronts released from cysts on each of the substrate types was not determined in the current study. As compared to our experimental conditions, it must be acknowledged that completely clean biofilm-free submerged substrates do not exist in fish farms and so it would be interesting to assess whether biofilm quality and quantity affects tomont settlement behavior and preferences. Together, the simple maintenance of clean, largely biofilm-free or biofilm low environment is likely to prove beneficial against *I. multifiliis*. This raises the question of whether manipulation of the hydrodynamic conditions and cleaning practices on farms could be optimised to allowing self-cleaning rearing systems.

Besides the *in vitro* results on the effect of substrate quality, on-farm results demonstrate that the utilization of a non-porous surface (plastic-based lining) along with daily brushing of the raceway lowered the level of *I. multifiliis* infection by facilitating the dislodgement of cysts from the substrate (Chapter 4, Paper V). The infection level was further reduced when this non-chemical strategy was used in combination with a suction head. Unfortunately, the positive effect of using a suction head could not be tested on its own due to the commercial nature of the trial. It can be foreseen that using the suction head alone as compared to brushing alone would be more effective in removing, disrupting or dislodging a higher proportion of cysts. Although demonstrated for the first time as a highly effective management strategy under commercial conditions, broad acceptance of this

strategy by the industry is restricted by its feasibility and the investment required. Although plastic-based lining was used in this study to provide a smooth surface, painting the inner surface of the raceways with a non-toxic sealant / paint also produces a smooth surface, blocking pores in the brickwork / concrete that raceways are commonly constructed from and would otherwise provide refuge for developing *I. multifiliis* cysts, bacteria and fungal spores (Shinn *et al.*, 2005). Together, the use of a slow-release antifouling paint with a sealant-based matrix and an active compound toxic to the cyst stage could have the three-fold advantage of reducing biofilm growth, eliminating the concrete cavity and providing chemotherapy (Araño Puig, 2004) thereby eliminating the need for daily brushing or vacuuming. Despite the reluctance to use the suction head, many British farms now seal the inner surface of their raceways to reduce the surface area available for *I. mutlfiliis* cysts to settle on and the area that requires brushing.

The use of the bottom algal grazer the leopard pleco (*G. gibbiceps*) in the current study was shown for the first time to be an effective biological means of controlling *I. multifiliis* infections. This approach as a possible alternative to the mechanical removal of cysts could present a number of advantages. One advantage is the likely positive correlation between the feeding rate of the biocontrol agent (Lovel, 1998), the algal/biofilm growth (Goldman & Carpenter, 1974; Fransolet *et al*., 1985; Rao, 2010) and the propagation of the *I. multifiliis* infection in the fish tank/system (Rintamäki-Kinnunen & Valtonen, 1997; Rintamäki-Kinnunen *et al*., 2005a; Rintamäki-Kinnunen *et al*., 2005b) as each of these individual parameters are known to positively correlate with water temperature. It can be expected, therefore, that the grazing activity of the biological control agent (*i.e.* the leopard pleco) might increase with raised water temperature and metabolic activity

proportionally to a level that is able to control the level of *I. multifiliis* cysts in the system and the biofilm growth.

Although our results demonstrate the potential of such a biological approach, further, commercial-scale experimentation should be performed to confirm our results obtained in the laboratory experimental scale used in the current study. Some basic answers would need to be answered such as the relationship between the size and feeding/grazing rate of *G. gibbiceps* as well their susceptibility and resistance to infection by *I. multifiliis*. It can be foreseen that application of this approach on a commercial scale would need to use a larger sized biological agent than the leopard plecos used in the current study to be of benefit to the main species by controlling infections. Of note is the complex periphyton/algal fauna present in pond systems which could be altered by the differential grazing preferences of the biological control agent, as has been seen with the use of *Plecoglossus altivelis* Temminck et Schlegel in a natural river system (Abe *et al*., 2006), which in turn could affect the performance of the principal cultured fish stock species.

Overall, both the mechanical and biocontrol trials consistently demonstrated that targeting the multiplicative cyst stage effectively reduces the size of the parasite population. The deployment of a non-chemical strategy is facilitated by the fact that the settled cyst is concentrated within a 2-dimensional environment (as compared to the water column) and on a static physical surface (as compared to the fish host). This clearly warrants the need to undertake further research on the settlement behaviour of the tomont and on the biology and disruption of the cyst stage.

## 3. Genetic barcoding and phylogeny

To date, the extent of genetic variation between distant geographical isolates of *I. multifiliis* has not been fully elucidated (Matthews, 2005; Dickerson, 2006). This is the first study investigating the utility of rDNA region (ITS-1 and ITS-2) and mtDNA (COI) as molecular markers for *I. multifiliis*. Our results have highlighted the inadequacy of the rDNA region (ITS-1 and ITS-2) and the potential of the mtDNA (COI) to genetically identify different geographical isolates of *I. multifiliis*.

The results of the present study however, have been restricted by a series of experimental difficulties. Firstly, the initial problem of extracting a minimum amount of DNA sufficient to perform PCR reactions and ultimately to sequence the target genes from pools of tomonts using traditional DNA extraction protocols (*e.g*. phenol-chloroform or glass beads). Secondly, despite being optimized, the protocol for DNA extraction remained inadequate to perform a single-tomont PCR reaction which ideally is required to establish the real extent of intra-population variation within an isolate of *I. multifiliis*. With this in mind, the future optimization of the extraction method should consider the recent research published on single-cell PCR protocols for protists (Lynn & Pinheiro, 2009).

This study would have clearly benefited from a larger set of samples from a wider range of geographical regions to determine the major strains of *I. multifiliis* but an appropriate method for genetic "barcoding" was identified. In the future, the identification of these major strains of *I. multifiliis* and the characterization of their relationship to abiotic parameters (*e.g.* salinity, pH and water temperature), host species and susceptibility to external conditions and chemotherapeutants in particular would ultimately allow the most suitable treatment regimes, according

to the particular strain infecting a farm system, to be deployed.

## 4. Towards an integrated management strategy

The most effective management strategy to use against *I. multifiliis*, however, may be site/farm specific and require a combination of the different treatments considered in the current study while also considering a number of other components.

Shinn *et al.* (2005) suggested that infections within farms result from a low theronts entering a farm system by the inlet water which subsequently proliferate, through successive rounds of infection within the farm culture systems. Recurrent *I. multifiliis* infections in farm systems are, therefore, highly likely and present the possibility of an over-wintering population of parasites establishing in the farm stock (Shinn *et al.*, 2005)(*e.g.* trophont development at 4°C can take up to 3 months) (Matthews, 2005). Certain sites, therefore, are prone to recurrent infection with sites needing only a few parasites to start an infection, which once established is almost impossible to eradicate unless the complete transfer of stock or the draining and cleaning of the whole system is possible.

*Ichthyophthirius multifiliis* infections exhibit a very seasonal pattern associated with the increase in water temperature (Duarte *et al.*, 1993; Valtonen & Koskivaara, 1994; Dickerson & Dawe, 1995). For instance, the increase in water temperature in a rainbow trout hatchery in southern Scotland over a two-week period during the summer was sufficient to produce an exponential increase in the number of parasites which rose from 13 to 320 trophonts per fish (see Chapter 4, Paper V). This clearly shows that the seasonality of the parasite needs to be factored in when designing effective programmes of control.

The application of a non-porous surface for lining or painting the bottom of farm culture systems and the use of non-chemical strategies (*i.e.* a mechanical device or a biological control agent) throughout the year, and at least during high risk periods, would decrease the size of the resident *I. multifiliis* population reducing or delaying the risk of an outbreak following a rise in water temperature. Importantly, the identification of critical period when the resident population is likely to initiate and undergo exponential multiplication would allow such preventive treatments to be implemented before the occurrence of an outbreak. While different strains of *I. multifiliis* can exhibit different levels of sensitivity to chemical treatments (Straus *et al.*, 2009; Straus & Meinelt, 2009), the physical methods described are arguably as effective across all strains of *I. multifliis*. Where these latter interventions are not applicable/possible, then the application of effective and environmentally friendly programmes of chemotherapy (*e.g.* bronopol and peracetic acid-based products) have been shown to effectively decrease the size of infections by removing not only a proportion of the cysts but also all the free-living stages of the parasite in the water column.

Notwithstanding the effectiveness of the methods already discussed, additional practices could enhance the prevention of *I. multifiliis*. For example, increasing the water flow and water turnover rate in a farm system has been demonstrated to successfully reduce *I. multifiliis* infections by flushing away the free-swimming stages (*i.e.* tomonts and theronts) from the system (Bodensteiner *et al.*, 2000). However, *I. multifiliis* infections are primarily a problem over the spring-summer months which are typically drier which limits the possibility of increasing the water flow rate through farm systems. Rearing systems designed to optimize the flow-rate and the water velocity moving through culture units,

therefore, could facilitate the flush removal of free-living stages of *I. multifiliis*.

Given the rising awareness by governmental agencies and the public regarding fish welfare, the use of physical methods and the licensing of environmentally friendly chemotherapeutants to control parasite infections in food fish farms is expected to be more favourably received than alternative options as there is also an ever increasing move towards complete organic production. According to the Soil Association in 2008, the organic salmon production was approximately 4% of the total UK salmon production with an expected increase in 2009 (Soil Association, 2010). The Norwegian organic certification body (Debio, 2009) stated that organic production should provide routine hygienic measure and that non-chemical (biological and mechanical) methods should be given priority where possible and effective. In addition, new chemotherapeutic compounds should have lower toxicity to animal stocks, humans and the environment. The UK is the leading organic producer in Europe (Hepburn, 2010) and the present research provides, in particular, practical solutions to support this emerging market.

*Ichthyophthirius multifiliis* or "white spot" is a widespread ciliate parasitic protozoan that is commonly encountered in most cultured, freshwater finfish species. The results and methods presented in this thesis are expected to offer a range of practical management options for use in commercial farms systems not only for the control of *I. multifiliis* infections but also of other freshwater and marine protozoan diseases, such as *Chilodonella* sp., *Amyloodynium ocellatum* and *Cryptocaryon irritans* which have similar life-cycles (Lom & Dyková, 1992).

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# PUBLICATIONS

# AND CONFERENCES ATTENDED

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**Picón Camacho, S.M**., Bawden, R., Taylor, N.G.H. & Shinn, A.P. The use of a mechanical device to reduce infections of *Ichthyophthirius multifilis* (Ciliophora) in raceways on a commercial rainbow trout, *Oncorhynchus mykiss*, farm. Interantional Congress of Parasitology (XI ICOPA), Glasgow (Scotland) 6th-11th August 2006. **Poster presentation**.

**Picón-Camacho, S. M**., Taylor, N.G. H., Yoon G.H., Bron, J.E., Hunter, R., Guo, F.C. & Shinn, A. P. Efficacy of continuous exposure to low doses of Bronopol (Pyceze™) on the infection dynamics of *Ichthyophthirius multifiliis* (Ciliophora), infecting rainbow trout *Oncorhynchus mykiss* (Walbaum). PhD Research Conference, Institute of Aquaculture, University of Stirling, Stirling (UK). 29th October 2008. **Poster presentation.** First price poster award.

**Picón-Camacho, S.M**., Bron, J.E., Taylor, N.G. H. & Shinn, A.P. Management of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora) infections in a commercial rainbow trout hatchery. 14th International Conference on Diseases of Fish and Shellfish (14th EAFP), Prague (Czech Republic) 14th-19th September 2009. **Oral presentation**.

**Picón-Camacho, S.M.**, Bron, J.E. & Shinn. A.P. Nuevos métodos de control del protozoario ciliado de agua dulce *Ichthyophthirius multifiliis* Fouquet, 1876. Congreso Nacional de Acuicultura (Spanish National Congress of Aquaculture), Madrid (Spain) 24th-26th November 2009. **Oral presentation**.

**Picón-Camacho, S.M.,** Bron, J.E. & Shinn. A.P. The administration of a bronopol-based chemical treatment to control *Ichthyophthirius multifiliis* (Ciliophora) infections. 6th International Symposium on Aquatic Animal Health (ISAAH), Tampa (Florida, USA), 5th- 9th September 2010. **Oral presentation**.

# APPENDIX 1

# SUMMARY OF FINDINGS

In this section, the main findings of each research manuscript are summarized and each study is schematically presented along with the life-cycle of *Ichthyophthirius multifiliis* (Figure 1).

**PAPER II The anti-protozoal activity of bronopol on the key life-stages of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora)**

- The administration of a high concentration of bronopol (*e.g.* 20, 50 and 100 mg $L^{-1}$) during a short period of exposure (*e.g.* 30 min) significantly reduces the survival of tomonts, cysts and theronts and delays the development of tomont and cysts of *I. multifiliis.*
- Prolonged low concentrations of bronopol (*e.g.* 1 mg $L^{-1}$) greatly reduce the survival rate of infective theronts although can not diminish their capability to infect fish hosts.
- Bronopol is to date one of the few biocide chemical compounds licensed for use in aquaculture described as disrupting the development of the cyst stage of *I. multifiliis*.
- The strong biocidal/cytotoxic effect of bronopol against all free-living stages of *I. multifiliis* (*e.g*. tomonts, cysts and theronts) suggests that this chemical compound could also be effective against the parasitic trophont stage developing within fish epithelia.

**PAPER III Effects of continuous exposure to low doses of bronopol on the infection dynamics of *Ichthyophthirius multifiliis* (Ciliophora), parasitising rainbow trout (*Oncorhynchus mykiss* Walbaum).**

- Continuous prolonged exposure (*e.g.* 27 days) of low concentrations of bronopol (*e.g.* 2 and 5 mg $L^{-1}$) has a significant impact on the population dynamics of *I. multifiliis* as shown by a significant reduction in the number of trophonts developing within fish host in successive waves of infection.
- The colonisation success of *I. multifiliis* establishing on naïve fish is significantly reduced by administrating low concentrations of bronopol (*e.g.* 2 mg $L^{-1}$) prior, over and after the time of infection.
- The present *in-vivo* study demonstrates the efficacy of administrating low doses of bronopol for longer periods of exposure is able to reduce infections by diminishing the free swimming stages (*e.g.* tomonts, cysts and theronts) and the parasitic stage (*e.g.* trophonts) of *I. multifiliis*.

**PAPER IV *In vitro* assessment of the chemotherapeutic action of a specific hydrogen peroxide, peracetic, acetic and peroctanoic acid based formulation against the free-living stages of *Ichthyophthirius multifiliis* (Ciliophora)**

- Peracetic acid administered at low concentrations (*e.g.* 8, 12 and 15 mg $L^{-1}$) for periods of 1 h has a strong biocidal effect on all free-living stages of *I. multifiliis* (*e.g.* tomonts, cysts and theronts).
- Peracetic acid is one of the few manufactured chemical compounds described as disrupting at low concentrations the development of the cyst stage of *I. multifiliis*.
- The demonstrated efficacy of the peracetic acid-based products suggests they have a potential to control *I. multifiliis* infections in commercial aquacultural systems.

**PAPER V Mechanical control of *Ichthyophthirius multifiliis* Fouquet, 1876 (Ciliophora) in a rainbow trout hatchery**

- The tomont stage preferentially settles and encysts at the base of culture systems.
- The survival rate of the tomont stage is affected by the composition of the substrate upon which it settles and is significantly lower on polypropylene-based plastic.
- The lining with a low-adhesion polymer creates a smooth surface at the base of the rearing system that facilitates the dislodgement and elimination of the cyst stage of *I. multifilis* by natural flushing or brushing.
- The physical removal of the cyst stage alone can significantly reduce the propagation of *I. multifiliis* by reducing infection to a low level without the need of deploying an additional chemical treatment.
- This study demonstrates that the multiplicative cyst stage is key in the dynamic of *I. multifiliis* population and its removal from the farm system could constitute a simple means of assisting farmers in the management of *I. multifiliis* infections.

**PAPER VI An examination of the utility of the leopard pleco (*Glyptoperichthys gibbiceps*, Kner, 1854) in the control of *Ichthyophthirius multifiliis* Fouquet, 1876**

- The tomont stage exhibits preferential / selective settlement behaviour towards biofilm–covered substrates.
- The substrate grazer, the leopard pleco, was found to be an effective biological control against the propagation of *I. multifiliis* in a static rearing system, which

effected a 63% reduction in the trophont burdens establishing on fish following the first infection wave.

- The efficacy of a substrate detrivorous/algae feeder as biological control targeting the external, multiplicative (settled) life-stage of an aquatic disease is demonstrated for the first time. This biological approach reduces the potential negative effects of using a chemical intervention. This biological approach reduces the negative interactions between the cultured and biocontrol stocks and could theoretically be used against aquatic parasites with a settled multiplicative life-stage.

**PAPER VII Intra-specific genetic variation in *Ichthyophthirius multifiliis* Fouquet, 1876 using the molecular markers: rDNA region and mtDNA (COI)**

- The ribosomal DNA regions ITS-1 and ITS-2 are too conserved to discriminate geographical isolates of *I. multifiliis.*
- The mtDNA (COI) presents sufficient intra-specific genetic variation to discriminate between isolates of *I. multifiliis.*
- Genetic "barcoding" using mtDNA is likely to be the most effective method to identify *I. multifiliis* isolates. Genetic "barcoding" should be performed when characterizing the ecophysiology, pathogenicity and sensitivity of *I. multifiliis* geographical isolates in order to ultimately manage *I. multifiliis* infections according to the specific genetic isolates that are encountered.

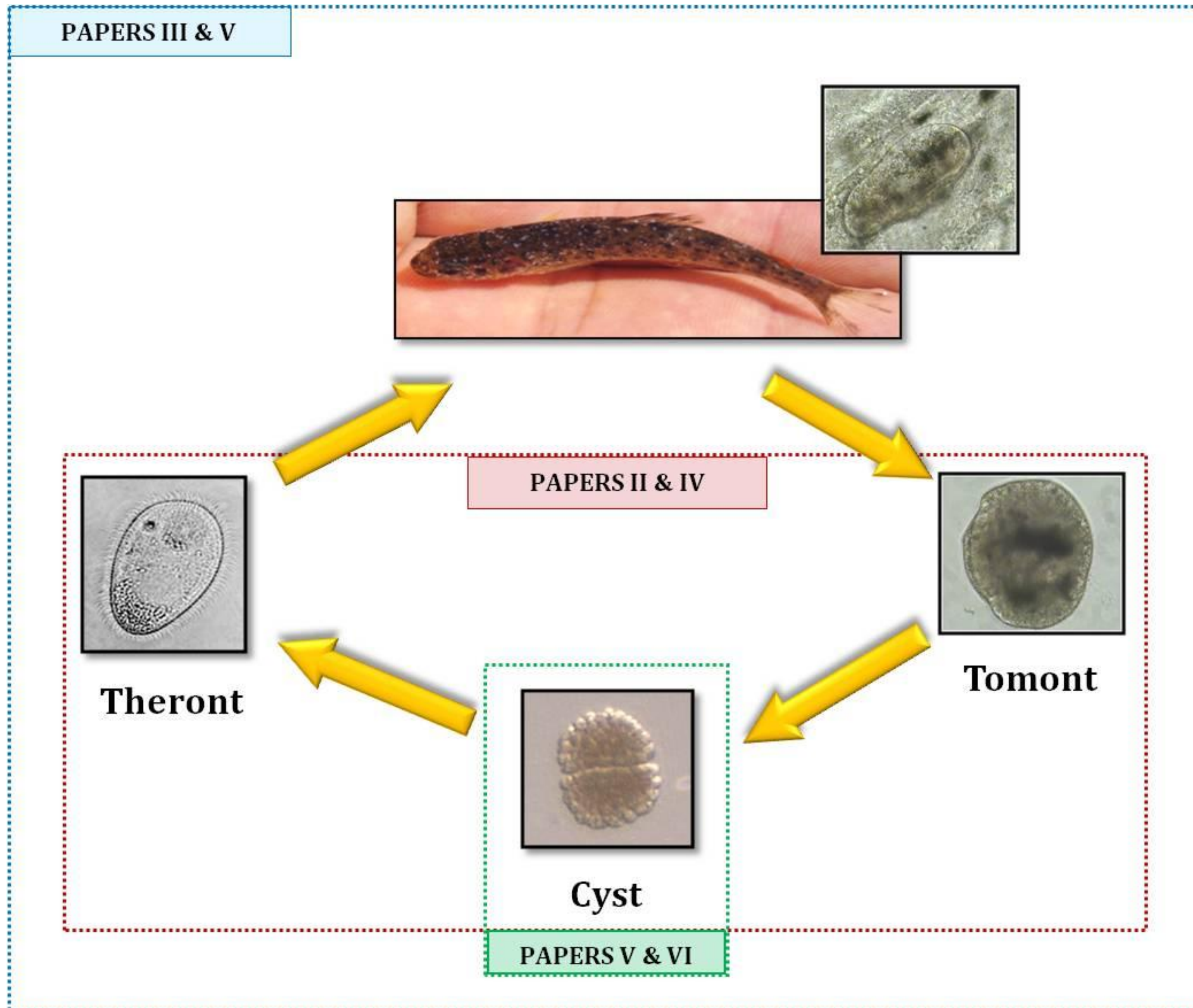


**Figure 1.** Schematic presentation of the studies performed on the control strategies on the life-cycle of *Ichthyophthirius multifiliis* Fouquet, 1876.