Recent advances in the mitigation of *Saprolegnia* infections in freshwater fish and their eggs

Shimaa E Ali[1], **Øystein Evensen**[2] and **Ida Skaar**[3]

[1] Department of Hydrobiology, National Research Centre, 33 El Bohouth St. Dokki, P.O. Box 12622, Giza, Egypt
[2] Department of Basic Sciences and Aquatic Medicine, Norwegian University of Life Sciences, P.O. Box 8146, N-0033, Oslo, Norway
[3] Department of Mycology, Norwegian Veterinary Institute, P.O Box 750 Sentrum, N-0106 Oslo, Norway

Saprolegnia infections (saprolegniosis) are commonly referred to as the fungal infection of freshwater fish and their eggs. The disease can cause severe economic losses and significant decline in fish and amphibian populations. Moreover, the infection could persist in the presence of treatment due to biofilms. In the past, the disease was kept under control with malachite green, but due to its suspected carcinogenicity, it was banned for use in fish intended for human consumption. As no other treatments are currently comparable to malachite green in efficiency, the problem with saprolegniosis in fish and their eggs has increased in aquaculture globally. Formalin-based products have been widely used. However, the concern about the environmental impact of formalin and toxicity has led to limited availability and it is even banned for use in aquaculture in some countries. Bronopol is also applied against *Saprolegnia* infections (prophylactically) with moderate success. Recently, the use of boric acid as a potential prophylactic and curative measure against *Saprolegnia* infection in fertilized salmon eggs have been investigated. Also other alternatives have been suggested. This chapter discusses recent advances in the mitigation of *Saprolegnia* infections in aquaculture. The possibilities of using some chemical alternatives, biological control methods and immunization trials are highlighted.

Keywords: *Saprolegnia*; water mould; control; freshwater fish

1. Introduction

Similar to humans and other animals, aquatic vertebrates are susceptible to a wide variety of bacterial, viral, fungal and parasitic diseases. Among the most common and widespread causative agents of freshwater fish diseases is *Saprolegnia*, a genus in a class Oomycetes [1, 2]. Originally, Oomycetes classified as fungi, and the term water mold was traditionally used to define *Saprolegnia* infection [1, 2]. Since Oomycetes share common features with both fungi and algae [3], they were re-classified recently as fungal-like organisms with brown algae and diatoms [4]. Thus, saprolegniosis or fungal-like disease is a common term used to describe *Saprolegnia* spp. infection with fish and their eggs. A wide variety of fish species important to aquaculture and their life stages from eggs to adults are susceptible to infection [5]. Moreover, saprolegniosis has been associated with mortalities recorded in amphibians, crustaceans, insects, diatoms and algae [5, 6, 7]. Among *Saprolegnia* spp., *S. parasitica* is the most economically important fish pathogen, especially for salmon and trout species [8]. Million dollar losses in aquaculture worldwide have been attributed to the devastating fish pathogen *S. parasitica*, especially in Scotland, Scandinavia, Chile, Japan, Canada, and the USA [8, 9, 10]. Fish saprolegniosis is easily recognized as visible, white or gray patches of filamentous mycelium of cotton-like appearance [11, 12] localized in head or fins [13] and can spread over the entire surface of the body.

Death usually occurs in fish, due to the failure in the osmotic balance (haemodilution) following massive destruction of the epidermis by invasive hyphal growth [14]. In fish eggs, the hyphal breaching of the chorionic membrane regulating the embryo osmosis is suggested to be the cause for egg mortalities [15].

The organic dye, malachite green has been used previously as an extremely effective treatment for saprolegniosis in fish and eggs. But due to suspected carcinogenicity and toxicity [16, 17], it was banned for use in fish intended for human consumption [18]. Treatment with formalin-based products is a commonly used alternative, however, it also banned in some countries [19]. Bronopol, hydrogen peroxide and sodium chloride are also used, but still not as effective as malachite green. Accordingly, trials to identify more effective, safe and economic anti-*Saprolegnia* compounds that possess no or a very low environmental impact are conducted.

2. Disease mitigation strategies

The most efficacious way to control the Oomycete infections is to prevent them from becoming established. Once the infection is established, fish, especially salmonids, with obvious signs usually will not recover [14]. However, early stages of infection can be treated with some success. Thus, it is crucial to establish prophylactic measures to reduce the risk of infection. Generally, the combination of good management and chemical treatment is the most effective strategy for controlling and preventing fish saprolegniosis [12], typically 2 to 4 days after handling [10]. Meyer (1991) claims that "the reduction of stress appears to be the single greatest factor to help fish to resist the infection" [20]. For ova protection, the components of the immune system were shown to be maternally transferred to the eggs for rainbow

trout, zebrafish and amphibian eggs (21, 22, 23). Several studies have reported that transferred maternal molecules are involved in the early defense against pathogens such as *Saprolegnia* spp. in developing fish embryos [24, 25, 26]. Therefore, it should be ensured that all eggs are of high quality (obtained from un-stressed broodstock). Regular removal of dead eggs or infected dead ones seems to be paramount steps to control the Oomycete infections [14]. Additionally, Rach et al., (1995) [27] postulated that the increase of water flow to 1200 ml/ min with moderate rolling would improve the egg hatchability without causing Oomycete growth.

2.1 Conventional therapy

2.1.1 Malachite green

Malachite green has powerful effect against all infectious stages of *Saprolegnia* in fish aquaculture facilities worldwide [28]. It has been used successfully as a treatment to control fish saprolegniosis and as a prophylactic treatment to protect fish-eggs from infection. Malachite green acts as a respiratory poison, damaging the cell's ability to produce energy required to drive vital metabolic processes [29, 18]. The high efficacy and low cost make this organic dye a popular treatment against saprolegniosis. However, malachite green has been banned for use in fish intended for human consumption due to its carcinogenic properties and toxicological effects [17, 18]. Further to this, malachite green has been reported as potential teratogen, mutagen and tumor promoter in animals [17, 18]. Spinal, head, fin and tail abnormalities were reported in trout fry hatched from eggs treated with high doses of malachite green [17]. Additionally, respiratory distress has been reported in fish exposed excessively to the treatment [30]. Malachite green could be used to control egg saprolegniosis at concentration between 3-5 mg/l for 60 minutes with minimal egg mortalities [31]. For fish with mild lesions, 15 min exposure to 0.25 mg/L at 10 °C would be sufficient to kill the established mycelia on fish [32].

2.1.2 Bronopol (Pyceze®)

Bronopol is a biocide which is widely used as a preservative in medical and pharmaceutical products. Studies carried out at various trout farms showed that bronopol has therapeutic/prophylactic activity against Oomycete infections in fish farming [33] ,but not as good as malachite green. According to Pottinger and Day (1999) [33], a daily bath/flush (15 mg/l) is effectively protecting fish from the infection. At higher concentration (100 mg/l,for 30 min in bath/flush and daily exposure), bronopol is able to protect fertilized rainbow trout eggs from *Saprolegnia* infection [33]. Longer exposure to lower concentrations of bronopol causes acute toxicity in fish and eggs [34]. For example, high mortalities were reported 12 h post immersion of striped catfish exposed to 20 mg/l bronopol. As no toxicological hazards to human [35] or fish were presented [33], bronopol formulated as Pyceze® is licensed to treat Oomycete infections [14]. However, bronopol toxicity against zooplankton and phytoplankton has been demonstrated later [36]. Additionally, bronopol should be diluted before being disposed, which make the usage cost relatively high [36].

2.1.3 Formalin

Formalin is a powerful disinfectant used to kill microorganisms or as a preservative for biological specimens. It has been widely used to control Oomycete infections in aquaculture [37]. It is available as aqueous solution containing approximately 37% formaldehyde by weight [14]. It works by reacting with cell proteins and nucleic acids - altering both structure and function. A concentration of 250 mg/l formalin would be sufficient to prevent Oomycete infections on eggs [38] and rainbow trout following a 60 min exposure [39]. To decrease the manual removal of dead eggs, daily formalin treatments of 1667 mg/l for 15 minutes are recommended [40, 41]. The potential toxicity to handlers, the cost of container disposal and the potential danger to the ecosystem, make formalin still far from ideal alternative to malachite green [42, 31]. Therefore, formalin also has been banned in some countries [19], and is expected to be banned in more countries in the years to follow.

2.1.4 Hydrogen peroxide

Hydrogen peroxide has been identified as an effective antifungal, antibacterial and antiviral compound and is potentially also an important oomyceticide with low environmental impact [14, 31, 43, 44]. It has been used effectively to control saprolegniosis on eggs of rainbow trout and chinook salmon [37, 45, 46]. A concentration of 250–500 ml/l hydrogen peroxide (based on 100% active ingredient) for 15 min could be efficient as prophylactic treatment for eggs [45]. Both control of *Saprolegnia* infection of eggs and increase of hatching rate were observed when hydrogen peroxide was applied at the concentration of 1000 ml/l [38, 45, 47]. Hydrogen peroxide has also been used to control Oomycete infections on adult Chinook salmon, using a flow-through dose of 25 mg /l [14]. According to the Food and Drug Administration (FDA), hydrogen peroxide is classified as a low regulatory priority (LRP) for the control of Oomycetes on all species and life stages of fish [14, 44]. However, the efficacy of hydrogen peroxide might be influenced by water quality, temperature and population levels of *Saprolegnia* spp. [48]. Additionally, its margin of safety is rather narrow [42].

2.1.5 Sodium / calcium chloride

Salt mixture has been suggested as a prophylactic mean against saprolegniosis. Egg mortalities were reduced when a mixture of sodium and calcium chloride (26:1) was applied at 20 g/l for 1 h three times a week [49]. Improvement of hatching rate with decrease in *Saprolegnia* infection was observed on trout eggs exposed to NaCl bath at 30 g/l [38, 44]. However, using this salt mix of such high concentration might be impractical in the intensive hatcheries.

2.1.6 Sea water flush

Sea water flush is reported to be a successive and inexpensive method to limit oomycete growth in aquaculture facilities [50]. *Saprolegnia diclina* on salmon eggs was effectively controlled by sea water flush for 2–3 h for 5 out of 7 days over 10 weeks [14].

2.1.7 Iodophores

Buffered bicarbonate iodophores are usually applied as a disinfectant for eyed ova 1 h following fertilization to destroy the infectious agents including Oomycetes on the egg surface [14]. It has been used also as oomyceticide for the eggs of trout [38] and channel catfish [37]. Iodophores are commonly used in fish farming as effective treatment at a concentration of 100 mg/l available iodine for 30 min [16] or as a flush twice daily at concentration of 50, 100 or 200 mg/l to increase the hatchability of channel.

2.1.8 Ozone

Ozone was tested as an alternative to prevent egg saprolegniosis by Forneris and colleges [51]. They concluded that ozone shows a similar effectiveness as of formaldehyde in the prevention of saprolegniosis in brown trout eggs at dose from 0.01-0.2 ppm with hatching rate from 42.6% to 49.1%. However, a dose of 0.3 ppm applied every second day seems to represent the threshold of toxicity [14].

2.1.9 Ultraviolet irradiation (UV)

A combination of UV light, ozone and hydrogen peroxide was used successively to control egg saprolegniosis in a Finnish trial [52]. However, UV alone is not an effective treatment against *Saprolegnia* hyphae under the laboratory conditions [53].

2.2 New strategies for the control of Saprolegniosis

2.2.1 Bacterial antagonist

The introduction of protective microbiota have been suggested recently as potential strategy to reduce the risk of emerging diseases in fish and amphibians [15, 54, 55, 56]. It has been shown that specific bacteria can play a role in the protection of fish and amphibians against fungal and oomycete pathogens. *Janthinobacterium lividum* for example, a bacterium that was isolated from frog skin could contribute in the protection against *B. dendrobatidis* [57]. For *Saprolegnia* in particular, the antagonistic effect of some probiotics has been identified by several authors [14, 58, 59]. The bacterial inhibition was attributed to the production of an antibiotic by the bacteria [60]. However, many reports suggested that the inhibition might depend on the secretion of a chemical or nutrient acting between the bacteria and the *Saprolegnia* [58, 61].

Actinobacteria, prolific producers of an array of antimicrobial compounds, have been suggested as probiotic agents in aquaculture [62, 63,64]. A recent study showed that genus *Frondihabitans* (Microbacteriaceae) effectively inhibits the attachment of *Saprolegnia* to salmon eggs [15]. Additionally, Saprolmycins A-E, isolated from culture broth of *Streptomyces* spp., was suggested as an antibiotic against *Saprolegnia parasitica* [36].

The use of the bacterial antagonists in the commercial level is restricted, as some of these bacteria might contribute to certain diseases in the future. An example is *Pseudomonas* sp. (*P. fluorescens* in particular), which could be used as strong inhibitors to the radial growth of *S. parasitica* under *in vitro* conditions [58, 65]. On the other hand these bacteria are also recognized as a causative agent of bacterial hemorrhagic septicemia in fish.

2.2.2 Proposed chemical alternatives

2.2.2.1 Boric acid (BA)

In aquaculture, immunization and chemical control are among the preferred approaches to mitigate diseases [14, 15,66]. For the chemical control, the ability of boric acid (BA) to control *Saprolegnia* infection in salmon eggs and yolk sac fry was investigated recently [67]. Under *in vitro* conditions, boric acid was able to decrease *Saprolegnia* spore activity and mycelial growth in all tested concentrations above 0.2 g/l, while complete inhibition of germination and growth was

observed at a concentration of 0.8 g/l. In *in vivo* experiments using Atlantic salmon eyed eggs, saprolegniosis was controlled by BA at concentrations ranging from 0.2–1.4 g/l during continuous exposure, and at 1.0–4.0 g/l during intermittent exposure (Fig. 1). The study has also shown that BA (0.5 g/l) was efficient at reducing yolk sac fry mortality during a water-borne infection with *Saprolegnia* (wet lab studies) which would indicate that BA can also be used for treatment of ongoing infections. The high hatchability and survival rates recorded after the treatment suggest that BA is safe for use in salmon eggs and yolk sac fry.

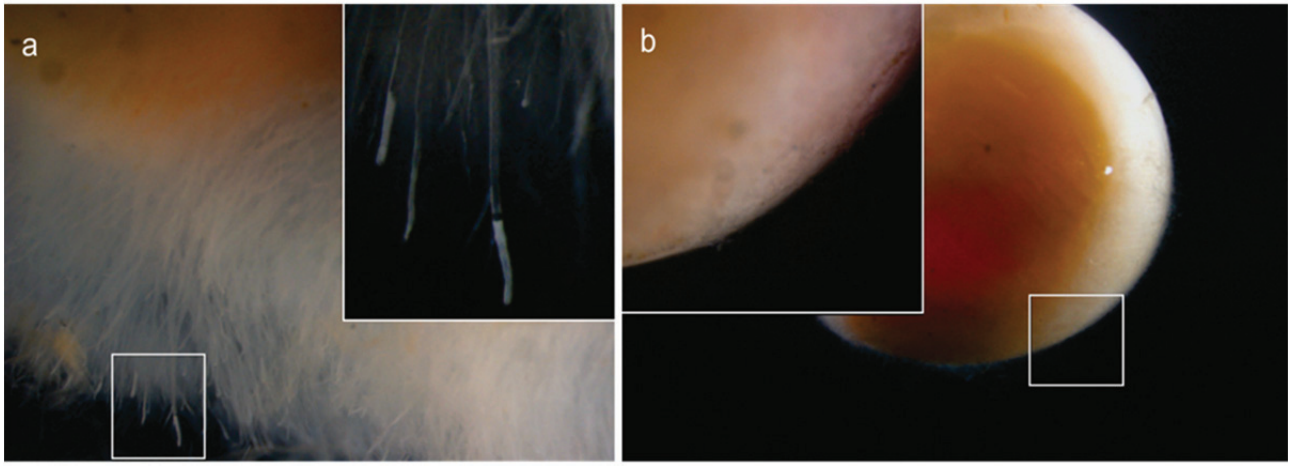

Fig. 1 Effect of continuous boric acid treatment on salmon eggs used as infection source. Microscopical examination of infected dead eggs used as a source of infection after the termination of the continuous exposure experiment. Mature *Saprolegnia* zoosporangium (a) in the non-treated control group compared to treated one exposed continuously to boric acid treatment (0.6 g/l) (b)

2.2.2.2 Peracetic acid (PAA)

Recently, peracetic acid (PAA) has been suggested as a potential antimicrobial candidate in aquaculture facilities [68]. PAA is produced as a mixture of acetic acid and hydrogen peroxide and has similar antimicrobial effect to formalin but more degradable [68]. A study conducted recently showed that PAA could reduce the growth of two fish pathogens, *Flavobacterium columnare* and *Saprolegnia parasitica* under the *in vitro* conditions [69], however, more studies should be performed to confirm its feasibility for use against *Saprolegnia* infections.

2.2.3 Vaccines

Vaccination of fish presents an attractive alternative to the other control methods [70]. However, no vaccines are available for saprolegniosis or other Oomycete infections of fish to date [14]. Beakes et al. (1994) [11] suggested that the specific surface glycoprotein on the cyst-coat spines of *Saprolegnia* could be used as an interesting target that antibodies might be directed towards to reduce the spore stickiness and subsequent attachment to the host. However, the antibodies raised against secondary cyst coat matrix components were not specific. They were able to react with *Saprolegnia* genera but also *A. astaci* and *Achlya* sp. [14, 71]. To develop vaccine against *Saprolegnia*, it might be interesting to consider induction of immune responses at mucosal surfaces [14]. But due to the wide spread of *Saprolegnia* spp., the majority of fish have been exposed naturally to this pathogen [72] which could explain the presence of antibodies against *S. parastica* in the serum of healthy and infected wild brown trout (*Salmo trutta L.*) [70,73]. Specific antibodies were detected in the serum of brown trout following injection with antigenic protein extracts from *S. parasitica* [74]. A single secreted protein, SpSsp1, of 481 amino acid residues, containing a subtilisin domain was identified in *Saprolegnia parasitica* [70]. Expression analysis demonstrated that SpSsp1 is highly expressed in all tested mycelial stages of *S. parasitica*. Additionally, several fish without visible saprolegniosis showed an antibody response towards SpSsp1 [70]. These findings could bring up more possibilities for vaccine generation [14] and suggest that SpSsp1 might be a useful candidate for future vaccination trial experiments [70].

2.2.4 Glucans

Recently, the effects of 1,3;1,6-β-D-glucans on the development and susceptibility of chum salmon *Oncorhynchus keta* (Walbaum) eggs to *Saprolegnia* infection were investigated. Up to 2.5-fold increase in survival of embryos and juveniles and their resistance to *Saprolegnia* infection was reported following the exposure of chum salmon eggs to 1,3;1,6-β-D-glucans with a molecular mass of more than 2 kDa. Additionally, 40–55% weight gain was recorded in treated juveniles compared with the control. The best stimulative effect was observed with 1,3;1,6-β-D-glucans with molecular mass of 6–8 kDa and used at a at concentration of 0.5 mg ml−1 [75].

2.2.5 Essential oils

The antifungal activity of essential oils (EOs) of *Zataria multiflora*, *Geranium herbarium*, and *Eucalyptus camaldolensis* in treating *Saprolegnia parasitica*-infected rainbow trout eggs was evaluated by Khosravi et al. (2012) [76].

At concentrations of 25, 50, and 100 ppm, EOs of *Zataria multiflor*a and *E. camaldolensis* had significant differences in comparison with negative control ($p<0.05$). The highest number of final eyed eggs was recorded in malachite green (postive control), followed by *Z. multiflora*, *E. camaldolensis*, and *G. herbarium* treated eggs.

The anti-Oomycete activity of EOs of the tree *Laureliopsis philippianna* against *Saprolegnia parasitica* and *S. australis* was investigated recently [77]. The results revealed a high susceptibility of *Saprolegnia* spp. strains to *L. philippianna* EOs. For the majority of the tested isolates, bark EO was most efficient and even more than bronopol. A replacement of synthetic compounds with natural products such as EOs, might reduce operating costs, could be safer for employees and relieve some of the environmental pressure on the fish farm industry [77].

2.2.6 Genetic selection for disease resistence

Genetic components play an important role in susceptibility to disease. For salmonids, very few reports are available regarding the relationship between genetic variation and disease resistance [14]. Resistance to *Saprolegnia* infection in Arctic char was improved by selective breeding [14, 78]. Therefore, improvement of disease resistance through genetic selection could be an adjunct to current protocols aimed at constraining losses due to saprolegniosis in aquaculture.

3. Concluding remarks

Existing prophylactic measures and treatment programs against *Saprolegnia* infections are insufficient to constrain losses in aquaculture. Accordingly, more efforts should be conducted to develop effective, safe and economic anti-*Saprolegnia* regimes for fish and their eggs that possess no or a very low environmental impact. Also the avilability of the genomic sequence of *S. parastica* and the recent data on the immune response of fish against *Saprolegnia* infections could open possibilities to develop a vaccine.

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