Remedy for Combating Ich in Finfish Aquaculture

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The parasite *Ichthyophthirius multifiliis*, often referred to simply as "Ich", causes "white spot disease" in many finfish species in the ornamental fish industry as well as in foodfish aquaculture. Ich is a ciliate protozoan, for which fish is an obligate host.

The Ich parasite has four life stages (Fig. 1): 1) the trophont stage embeds in and feeds on fish skin or gill epidermis, 2) the liberated and free-swimming tomont, which eventually encysts, to dwell at the bottom, 3) the proliferating tomocyst, containing up to 1000 daughter cells and 4) daughter cells that are eventually liberated to seek out new hosts, named theronts. The length of the parasite's life cycle is dependent on water temperature, ranging from days to weeks.

Depending on the fish species, a strong, large and mature fish may survive a mild infection of Ich, but prolonged disease will always be detrimental and fry and small fish will most often succumb. Being a long-known and widespread disease, a number of remedies have historically been used to counter Ich. The most efficient is malachite green, which is the only one affecting the embedded trophont. Unfortunately, malachite green may be a carcinogen and is persistent in nature and so its use is mostly banned.

The stages of the parasite not sitting on the fish host may be targeted by several agents: sodium chloride, copper sulphate, oxidizing agents such as hydrogen peroxide, sodium percarbonate, peracetic acid, potassium permanganate and formalin. Sodium chloride and copper sulphate are impractical to use because the fish needs to be moved to a separate treatment tank. Oxidizing agents work relatively well but need to be applied repeatedly at rather short intervals. Formalin works well but the therapeutic window is narrow, meaning that the concentration needed to kill the Ich parasites is close to concentrations that will harm beneficial water organisms or fish. This potential toxicity means that formalin is banned in many places and only used under an exemption granted by regulatory agencies and, if used, much care needs to be taken.

Some years ago, researchers in the European Union research program ParaFishControl tested many extracts and molecules for their potential against Ich and other fish parasites. In these studies, a molecule extracted from a bacterium of the *Pseudomonas* genus (Liu *et al*. 2015) turned out to be very efficient in rapidly killing the Ich parasite. The molecule is a cyclic lipopeptide (CLP) that, due to its strong surfactant properties, disturbs and destroys certain naked cellular membranes. In a follow-up study (Al-Jubury *et al*. 2018), the molecule, now named "Biokos", killed all three free-living life stages of Ich, most within 5-20 min at low milligrams/L concentrations.

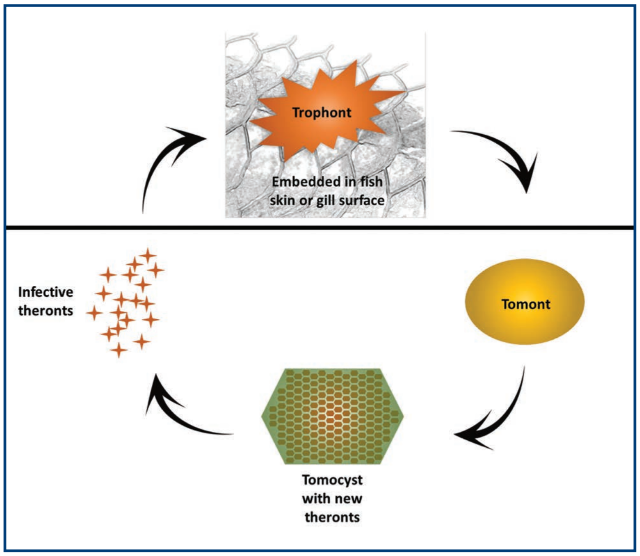

FIGURE 1. *The life cycle of* Ichthyophthirius multifilis.

> A molecule extracted from a bacterium of the Pseudomonas genus turned out to be very efficient in rapidly killing the Ich parasite. The molecule is a cyclic lipopeptide (CLP) that, due to its strong surfactant properties, disturbs and destroys certain naked cellular membranes.

A challenge study with Ich-infected rainbow trout fry demonstrated full protection against Ich infection by Biokos at 10 mg/L (Li *et al*. 2022). Matthiesen *et al*. (2021) tested the propensity of Biokos and a range of currently used Ich biocides (including formalin, hydrogen peroxide and peracetic acid) to raise molecular inflammation markers. Of all the molecules and extracts tested by ParaFishControl, Biokos had, by far, the lowest inducing activity and peracetic acid the highest.

At effective concentrations Biokos is non-toxic to fish (juvenile and embryonic zebrafish), crustacean zooplankton *Daphnia magna*, freshwater cyanobacteria *Phormidium autumnale* and green algae *Raphidocelis subcapitata* (Korbut *et al*. 2022). These results strongly suggest that Biokos could become a convenient and attractive alternative to current remedies used against Ich. In 2019, our company, Sundew ApS, acquired a license to the underlying intellectual property and set out to prove the feasibility of using Biokos to combat Ich disease in commercial fish culture.

Here we report on three studies undertaken: to further prove *in vivo* efficacy of Biokos in a goldfish challenge experiment; to exemplify its lack of toxicity to both fish and beneficial biofilter bacteria; and to assess in detail the propensity of "natural water" to degrade Biokos.

Goldfish Challenge Study

To confirm the published data on efficacy of Biokos to counter white-spot disease on rainbow trout (Li *et al*. 2022) we set out to do

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a similar study with goldfish *Carassius auratus*.

There were four treatment groups, each consisting of two 20-L fish tanks and five fish in each:

A) Negative control, no exposure to Ich, no treatment with Biokos;

B) Infection control, exposure to Ich, no treatment with Biokos;

C) Treatment, "low dose", exposure to Ich, treatment with Biokos at lowest known efficacious concentration; and

D) Treatment, "high dose", exposure to Ich, treatment with Biokos at double the concentration. The fish in treatment groups B, C and D were pre-infected with Ich by co-habitation for five days with Ich-infected goldfish (Fig. 2). Biokos was added every second day, starting at day 0 and 50 percent of the water was exchanged each day, starting at day 1. Fish mortality, behavior and the number of trophonts on skin, fins and gill surfaces was recorded throughout the 20-d study.

All Ich-infected fish not treated with Biokos died before day 14 (Fig. 3). All fish treated with Biokos survived the full length of the study, and much longer. No trophonts were visible on fins, gills, or skin of Biokos-treated fish after day 15. Implicit in these data is that the lower dose of Biokos was sufficient for efficacious treatment. No abnormal behavior was observed in any of the fish in treatment or control groups.

This is the second study showing that low concentrations of Biokos can completely counter/cure Ich infection in fish. The study results add another species of fish that is completely tolerant to efficacious concentrations of Biokos, the others being zebrafish and rainbow trout.

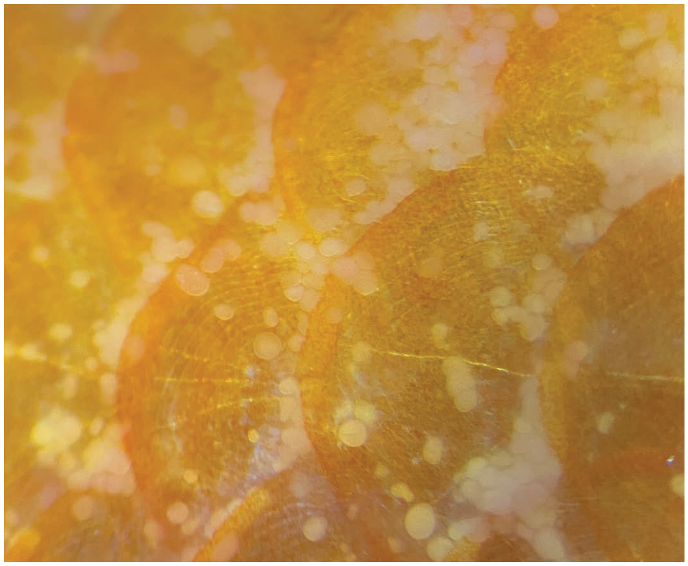

FIGURE 2. *Goldfish scales with embedded Ich trophonts.*

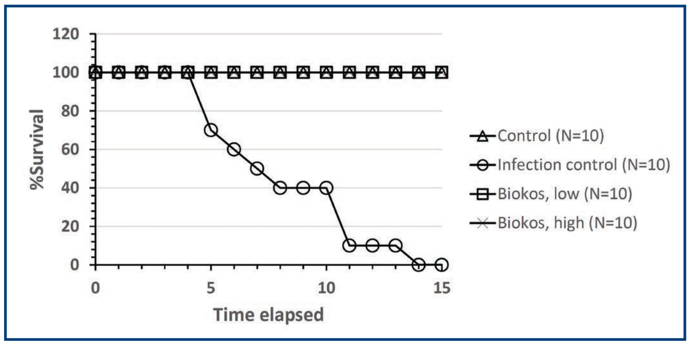

FIGURE 3. *Cumulative mortality of goldfish in challenge study, as percent survival per day since treatment start.*

Biokos is a new natural agent that can fill the need for an effective solution to Ich in aquaculture as well as in ornamental fish. It is a cyclic lipopeptide that kills the Ich parasites by affecting their cellular membrane integrity. Here we have shown that Biokos at very low concentrations can cure goldfish from Ich infection, confirming similar earlier results with rainbow trout. We have also shown that Biokos does not affect the health and functionality of biofilter bacteria needed for conversion of ammonia to nitrate. This means that it can be applied to RAS systems (and ornamental fish tanks) without problems for fish or biofilter health. Finally, we have shown that microorganisms in mature aquaculture and fish tank water will inactivate and degrade Biokos within hours or days into harmless components.

Effect on Biofilter Functionality

Biofilters in fish tanks and some fish farms with recirculation systems (RAS) have the crucial function of turning ammonia from the fish into less toxic compounds. *Nitrosomonas* bacteria turns ammonia into nitrite and *Nitrobacter* forms less harmful nitrate from nitrite. In a fish tank, nitrate can be used for growth by any plants present and/or removed by regular water exchange. In RAS aquaculture systems nitrate may be reduced to molecular nitrogen by denitrification bacteria or removed in other ways. Obviously, any remedy for parasites must not be toxic to these bacteria.

To study this, we combined surviving fish from the goldfish challenge study described above and assessed total ammonia nitrogen (TAN), nitrite and nitrate for 15 days, using water quality test strips. The rationale for the study was that, if Biokos had harmed the bacteria that facilitates biofiltration, we would see this in this follow-up study as peaks of TAN or nitrite or both with continued feeding.

There were six treatments in this study: A) No fish, no biofilter; B) No fish, biofilter, no Biokos; C) Fish (n=6), "low" Biokos, biofilter; D) No fish, "low" Biokos", biofilter; E) Fish (n=6), "high" Biokos, biofilter; and F) No fish, "high" Biokos, biofilter. Feed was added every second day, and no water exchange was performed during the study.

All goldfish from treatments C and E survived to the end of the study, indicating that water quality had been good. Biofilters remained fully functional, as indicated by measured concentrations of TAN, nitrite and nitrate (Fig. 4). The TAN assay has a sensitivity of 0.25 mg/L and only in two cases was 0.25 mg/L and in one case 0.5 mg/L measured. These are safe values for fish at the pH levels measured, between 6.8 and 7.8.

For nitrite, only in two cases was anything measured above the sensitivity of the assay (1 mg/L), both in group A with neither fish nor biofilter.

For nitrate, values up to 100 mg/L were measured (group F, no fish, "high" Biokos) and an outlier at 250 mg/L (group B, no fish, biofilter, no Biokos). For all groups with fish, values did not exceed 50 mg/L. This is a value in the high end for fish safety but is to be expected in the absence of growing plants in the tanks and absence of water exchange.

Summarizing these data, there was no apparent difference in biofilter performance with or without Biokos treatment.

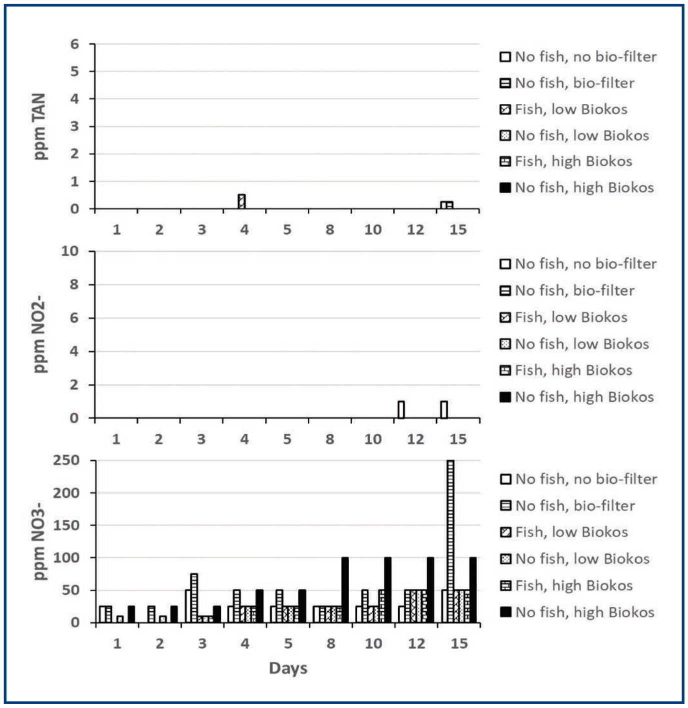

FIGURE 4. *Concentration of (a) total ammonia nitrogen (TAN) (b) nitrite and (c) nitrate during the 15 days after the goldfish challenge.*

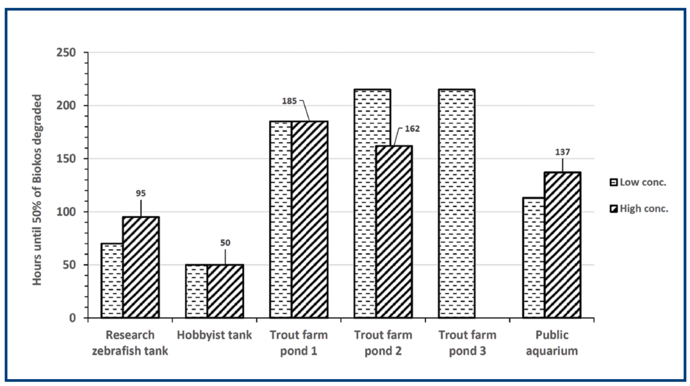

FIGURE 5. *The time required (in hours) for more than 50 percent of Biokos to be degraded in different types of water.*

Degradation Study

Presumed to be a defense system, some bacteria have developed enzymatic systems for their cleavage and thereby functional inactivation (Nielsen and Sørensen 2003, D'Costa *et al*. 2020). While this means one must carefully avoid contamination with foreign microorganisms during production of Biokos, it is convenient when it comes to using this product in the field. Such inactivation systems are widely distributed in nature, so all applied Biokos will eventually be cleaved and degraded further to harmless constituents.

This study was designed to obtain an understanding of the degradation kinetics of Biokos in different types of "natural water" and at different concentrations. We tested four types of water:

1) Fresh water from zebrafish tanks at a research facility at the University of Copenhagen,

2) Fresh water from a mature private ornamental fish tank,

3) Fresh water from three different rainbow trout farms in Denmark, and

4) Salt water from a public aquarium, National Aquarium Denmark.

We chose different temperature settings for the different types of water, in all cases corresponding to the temperature of the type of water at sampling. Concentrations of Biokos used were therapeutic ("low") and double ("high") dosing. The remaining Biokos was measured using HPLC analysis at least every 24 hr and until all (or the majority) had been degraded (Fig. 5). Water from a zebrafish laboratory was expected to be the cleanest and thus show the slowest Biokos degradation. All the other types were from "mature" culture systems, i.e., long-established fish tanks or trout ponds.

In zebrafish water at 27 C, half of the Biokos was degraded at 70 and 95 hr, respectively, depending on concentration, whereas in mature water from a private fish tank at 27 C, this happened faster, after 50 hr at both concentrations and in line with expectations.

The water from Danish trout farms was collected during winter and therefore we performed this degradation study at 4 C. In the first sample, half of the Biokos was gone after 185 hr and in the second sample this happened after 215 and 162 hr. In the third, things happened more slowly: 50 percent of Biokos added at the "low" concentration was gone after 215 hr while this point was not reached for the "high" concentration within the timeframe of the experiment, although some degradation was measured.

Finally, for saltwater in a public aquarium at 25 C, half the Biokos was gone after 113 and 137 hr. Two things should be emphasized: 1) sampling during the experiment was done at 24-hr intervals, meaning that the 50 percent degradation point in many cases would be significantly earlier than the time points mentioned above; and 2) Biokos would most likely have been degraded much faster in the trout pond water samples at a higher temperature, which is when trout farmers most often encounter Ich infestations.

These data indicate Biokos will be degraded within a few days in any relevant type of water, which is ideal because Biokos kills the Ich parasites within hours.

Conclusion and Summary

Efficacious and inexpensive solutions are needed to control pests and diseases in an environmentally sustainable manner. Current remedies are either somewhat ineffective and/or difficult to use

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FIGURE 6. *The Biokos product formulated into dosing tablets.*

because of their toxicity. Biokos is a new natural agent that can fill the need for an effective solution to Ich in aquaculture as well as in ornamental fish. It is a cyclic lipopeptide that kills the Ich parasites by affecting their cellular membrane integrity. Here we have shown that Biokos at very low concentrations can cure goldfish from Ich infection, confirming similar earlier results with rainbow trout.

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Finally, we have shown that microorganisms in mature aquaculture and fish tank water will inactivate and degrade Biokos within hours or days into harmless components.

Application of Biokos is expected to be as solid formulation tablets (Fig. 6). These studies demonstrate that Biokos can be an efficient and effective remedy for large-scale use in foodfish aquaculture and in the ornamental fish industry.

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Notes

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