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da Costa Marchiori, Natalia; Muller Silva, Fabiano; Laterça Martins, Maurício; Amaral Junior, Hilton; Corrêa da Silva, Bruno
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PARASITOLOGY

Hydrogen peroxide and chlorine dioxide against parasite *Ichthyophthirius multifiliis* (Protozoa, Ciliophora) in jundiá fingerlings

Natalia da Costa Marchiori[1*] Fabiano Muller Silva[1] Maurício Laterça Martins[2] Hilton Amaral Junior[1] Bruno Corrêa da Silva[1]

[1]Centro de Desenvolvimento em Aquicultura e Pesca (Cedap), Empresa de Pesquisa Agropecuária e Extensão Rural de Santa Catarina (Epagri), Rodovia Admar Gonzaga, 1188, 88034-901, Florianópolis, SC, Brasil. E-mail: nataliamarchiori@epagri.sc.gov.br. *Corresponding author.
[2]Programa de Pós-graduação em Aquicultura, Universidade Federal de Santa Catarina (UFSC), Florianópolis, SC, Brasil.

ABSTRACT: *Ichthyophthiriasis is a worldwide fish disease with great financial impact on freshwater fish farming due to its associated high mortality rates. Current study assesses the parasiticidal capacity of hydrogen peroxide (H_2O_2) and chlorine dioxide (ClO_2) against the causative agent, Ichthyophthirius multifiliis, in jundiá. Median lethal concentration (LC_{50}, 96h) of each chemical agent was established, as well as the minimum inhibitory concentration of hydrogen peroxide for the parasite's infectious larval phase (theront). Products were tested asynchronously in parasitized fingerlings for short and long baths at the following concentrations and exposure times: 1. Hydrogen peroxide: (T1) continuous bath - 30ppm and (T2) 50ppm; (T3) short bath - 150ppm, during 1h and (T4) 250ppm during 1h; control group (without any chemical agent). 2. Chlorine dioxide: (T1) continuous bath – 4ppm and (T2) 20ppm; (T3) short bath – 200ppm, during 1min; (T4) short bath – 400ppm, during 1min and control group. Data analysis demonstrated a concentration of 82.54ppm of the commercial product (or 24.76ppm of the active chemical agent) as LC_{50}, 96h of H_2O_2 and 38.4ppm product (or 2.68ppm of the active chemical agent) for ClO_2. Hydrogen peroxide concentration causing 100% mortality rate of theronts in 1h was 25ppm (product, or 7.5ppm of the active chemical agent). At the end of the fourth day of curative experiment, 98% of the animals died by ichthyophthiriasis. No treatment was effective against the parasite.*
Key words: *"ich", parasite, Rhamdia quelen drug, treatment.*

O peróxido de hidrogênio e dióxido de cloro no combate ao parasito *Ichthyophthirius multifiliis* (Protozoa, Ciliophora) em alevinos de jundiá

RESUMO: *A ictiofitiríase em peixes tem distribuição mundial e forte impacto econômico na piscicultura de água doce devido às altas taxas de mortalidade associadas. Este estudo avaliou o uso do peróxido de hidrogênio (H_2O_2) e do dióxido e cloro (ClO_2) no controle do parasito protozoário Ichthyophthirius multifiliis em jundiá. A concentração letal mediana (CL_{50}, 96h) de cada agente químico foi estabelecida, assim como a concentração mínima inibitória do peróxido de hidrogênio para a fase larval infecciosa do parasito (teronte). Os agentes foram testados assincronicamente em alevinos parasitados na forma de banhos de curta e longa duração, nas seguintes concentrações e tempos de exposição: 1. Peróxido de hidrogênio: (T1) banho contínuo - 30ppm e (T2) 50ppm; (T3) banho curto - 150ppm com duração de 1h e (T4) 250ppm com duração de 1h, além de um grupo controle (sem a adição do agente químico). 2. Dióxido de cloro: (T1) banho contínuo – 4ppm; (T2) banho contínuo - 20ppm; (T3) banho curto – 200ppm, com duração de 1min; (T4) banho curto – 400ppm, com duração de 1min, além de um grupo controle. A análise dos dados indicou a concentração de 82,54ppm do produto comercial utilizado (ou 24,76ppm do princípio ativo) como a Cl_{50}, 96h de H_2O_2 e de 38,4ppm produto (ou 2,68ppm do princípio ativo) para o ClO_2. A concentração de H_2O_2 que causou 100% de mortalidade dos terontes em 1h foi de 25ppm (do produto, ou 7,5ppm do princípio ativo). Ao final do quarto dia de experimento curativo, 98% dos animais morreram devido à ictiofitiríase. Nenhum dos tratamentos foi efetivo frente à parasitose.*
Palavras-chave: *"ictio", parasito, Rhamdia quelen, fármaco, tratamento.*

INTRODUCTION

Ichthyophthiriasis, usually referred to as "ich" or "fish whitespot", is a serious disease caused by the ciliate protozoan parasite *Ichthyophthirius multifiliis* (Fouquet, 1876), which exists worldwide and may infect all freshwater fish (SUDOVÁ et al., 2010), with great financial impact on the culture of fish for human consumption and in aquariums due to high mortality rates typically associated with the disease.

Ichthyophthirius multifiliis is a highly studied species especially with regard to its life cycle, epidemiological aspects, elements involved in the greater susceptibility of some fish species

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and in effectiveness of treatments and prevention. According to the review article offered by PICÓN-CAMACHO et al. (2012), more than 116 compounds have been tested since the 1980s for the laboratory and field control of the parasite. After several developed countries have prohibited certain drugs for therapeutic use due to their high toxicity and potential toxic risks and since these chemicals posit a danger stance to fish stock and farm worker handling them, several researches were developed to identify natural and synthetic compounds, which would be efficacious and environmentally supportable at one or more stages in the parasite development.

Rhamdia quelen (popularly known as jundiá in Brazil) is a South American catfish with promising results in fish farming in Southern Brazil due to its encouraging zootechnical performance. However, it is highly susceptible to the parasite.

Current analysis evaluates the effectiveness of less toxic agents and less impacting negative environmental factors. It tests the curing capacity of the disinfectants hydrogen peroxide (H_2O_2) and chlorine dioxide (ClO_2) in ichthyophthiriasis associated with *R. quelen*. Whereas the former agent is a disinfectant commonly used to clean human wounds and is normally considered safe to the environment (when in contact with water, hydrogen peroxide is naturally dissociated into water and oxygen), the second agent is a chlorine-based oxidative disinfectant commonly employed in the treatment of drinkable water (ANDRADE, 2010).

MATERIALS AND METHODS

Chemical agents

Hydrogen peroxide was manufactured by Dinâmica PA (35%), whereas product Dioxiplus, employed for chlorine dioxide, was manufactured by Dioxide.

Median lethal concentration (LC_{50}, 96h)

Fingerlings of *R. quelen* were used for the two asynchronous assays, between January and April 2015. Further, 420 animals (total length 8.3±0.88cm; weight 3.96±1.25g; n=30) were distributed in 14 experimental units (100L capacity each), with 30 fish per unit for median lethal concentration assays of hydrogen peroxide. The following concentrations of the chemical agent were tested: 0 - 17.5 - 35 - 70 - 122.5 - 175 and 245ppm, in triplicate. Animals were monitored every six hours and dead fish were removed to calculate accumulated mortality for each analyzed concentration.

Thereafter, 630 animals (total length 5.92±0.84cm; weight 1.70±0.68g; n=50) were distributed in 21 experimental units (100L capacity each), with 30 fish per unit, to calculate median lethal concentration of chlorine dioxide. The following concentrations of the chemical agent were tested: 0 - 1 - 1.5 - 2 - 2.5 - 3 and 4ppm, in triplicate. Animals were monitored every six hours and dead fish were removed to calculate accumulated mortality for each analyzed concentration. Animals were not fed during the assays and after 96h mortality data underwent statistical analysis with Trimmed Spearman-Karber method that determined median lethal concentration (CL_{50}, 96h), following HAMILTON et al. (1977).

Minimum inhibitory concentration

Trophonts of an *I. multifiliis* strain (the mature phase in the parasite´s life cycle) were isolated from three naturally diseased *R. quelen*. Three fish were anaesthetized with eugenol (75mg L^{-1}) and then euthanized by medular section.

The mature trophonts were scrapped from the body surface and gills, transferred to a petri dish, incubated for 14h and kept in an artificially illuminated room. After the hatching of the tomonts and the release of theronts (infectious stage of *I. multifiliis*), they were collected with a micro-pipette. They were then counted by optic microscope in three 1ml samples in a Sedgewick-Rafter counting chamber. Concentration was adjusted to 503 theronts ml^{-1}. They were then used in the *in vitro* immobilization assay (serial dilution in 96-well flat micro-plates Corning Costar.

Each plate contained 50µl of distilled water, to which were added 100µl of hydrogen peroxide (concentrations started at 400ppm). Factor 2 serial dilution followed up untill the 12^{th} well. Further, 50µl of the solution with theronts were added to each well. In the case of positive control, 50µl of the theronts solution were added in wells with sterile saline solution (0.65%). Minimum inhibitory concentration was determined as the last dilution of the product with total theront inhibition in 1h.

Therapeutic baths

Infection

So that jundiá fingerlings infected by *I. multifiliis* could be obtained, and fish were collected from the fingerling pond and transferred to another one of the same size in which they remained in a 1x1x1.20m net tank for 72 hours. Confinement and management stress under high temperature (approximately 29.5°C) were enough to trigger protozoan infection in the specimens. Data on

water quality in the tank, which included the net tank in which the fish were infected also comprised transparency at 8cm; pH 7; total ammonia 0.25mg L^{-1}; nitrite 0mg L^{-1}; dissolved oxygen 7.14mg L^{-1} and alkalinity 30mg L^{-1} $CaCO_3$.

In order to carry out the proposed trials, infected animals with approximately 106 trophonts (between 57 and 202 parasites per animal; count was done immediately before the start of the experiments in 15 specimens) on the surface of the body (gills were not assessed) were used. According to XU & KLESIUS (2004), the above amount is considered high infestation (above 100 trophonts per fish). Immediately after the appearance of white spots on the body surface, perceived by naked eye, the fish were transferred to the laboratory and kept in a tank with constant water renewal and aeration for 24h before the onset of the assays.

Hydrogen peroxide (H_2O_2)
Assay structure

Four hundred fingerlings of *R. quelen* (total length 7.6±0.74cm; weight 3.6±1.25g) were distributed into twenty 100L experimental units, in quadruplicate (80 animals per treatment, 20 per experimental unit), and received the following treatments: (T1) continuous bath with 30ppm hydrogen peroxide in the water; (T2) continuous bath with 50ppm of H_2O_2 in the water; (B1) therapeutic bath at a concentration of 150ppm of H_2O_2 for 1h; (B2) therapeutic bath at a concentration of 250ppm of H2O2 for 1h; control group (without chemical agent). Concentrations above refer to the product and not to the active chemical agent.

Product was added only once to the experimental units, immediately prior to the start of the assays. In the case of assays with therapeutic baths, the product was added in separate units in which the animals were kept during the period so that they could be afterwards transferred to the experimental units.

Chlorine dioxide (ClO_2)
Assay structure

Two hundred animals (length 5.08±0.54; weight 0.90±0.31g) were distributed into twenty 100L experimental units, in quadruplicate (40 animals per treatment, 10 per experimental unit), with the following treatments: (T1) continuous bath with 4ppm of chlorine dioxide in the water; (T2) continuous bath with 20ppm of chlorine dioxide in the water; (B1) therapeutic bath at a concentration of 200ppm chlorine dioxide for 1min; (B2) therapeutic bath at a concentration of 400ppm of chlorine dioxide for 1min; control group (without any chemical agent). Concentrations refer to the product and not to the active chemical agent.

Therapeutic baths occurred in separate units in which the animals were kept during the period so that they could be afterwards transferred to the experimental units.

Water quality

The following water quality parameters were assessed once a day throughout the experimental period (LC50, 96h and therapeutic baths): pH and temperature with a multiparameter probe YSI Professional Plus, and dissolved oxygen with a digital oxymeter. Total ammonia was calculated by indophenol blue method and alkalinity by the neutralization titrating method at the start and finish of the experiment.

RESULTS AND DISCUSSION

Median lethal concentration

Water quality parameters for the hydrogen peroxide assay were temperature 21.6±0.74°C; alkalinity 20mg L^{-1} $CaCO_3$; dissolved oxygen: 9.37±0.66mg L^{-1}; total ammonia 0.05mg L^{-1} $N\text{-}NH_3$ and pH 7.65±0. Figure 1 shows results of the median lethal concentration. Data analysis indicated a concentration of 24.76ppm of the active chemical agent (82.54ppm of the product) given as LC_{50}, 96h of hydrogen peroxide in *R. quelen* fingerlings (Figure 1A). In the case of chlorine dioxide, results on water quality parameters were temperature 25.5±0.46°C; alkalinity 40.0mg L^{-1} $CaCO_3$; total ammonia 0.50mg L^{-1} $N\text{-}NH_3$; dissolved oxygen 7.8±0.58mg L^{-1}; pH 7±0. Concentration 2.68ppm of the chemical agent (38.4ppm of commercial chlorine dioxide 7%) was given as $LC_{50,}$ 96h (Figure 1B).

Minimum Inhibitory Concentration

The concentration of hydrogen peroxide added to the water to cause total mortality rate of theronts in 1h was 25ppm (of the product or 7.5ppm of the chemical agent).

Therapeutic baths

Data on water quality for hydrogen peroxide during the experimental period comprised pH at 7.44; dissolved oxygen in water at 7.01±1.24mg L-1; temperature 24.4±0.79°C and total ammonia 0.25mg L-1 N-NH3. Data for chlorine dioxide were pH 7.25; dissolved oxygen in water at 6.87±1.04mg L-1; temperature 25±0.46°C and total ammonia at 0.25mg L-1 N-NH3.

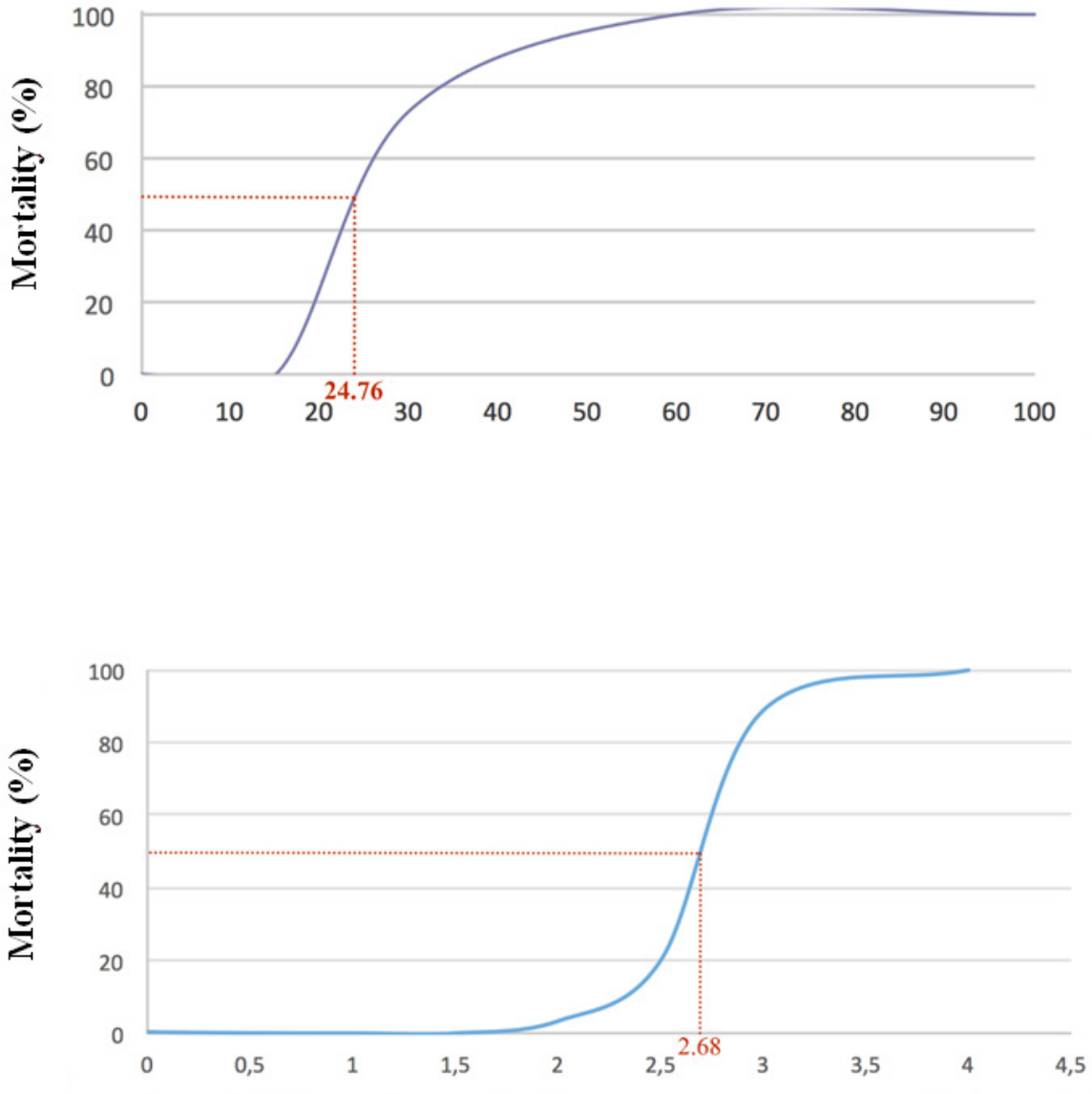

Figure 1 - A. Mortality (%) of juvenile *Rhamdia quelen* after 96h exposure to hydrogen peroxide concentrations. B. Mortality (%) of juvenile *Rhamdia quelen* after 96h exposure to chlorine dioxide concentrations.

Figure 2 shows accumulated mortality rate for each treatment.

At the end of the fourth day of the experiment, 98% of the animals had died due to ichthyophthiriasis (Figures 2A and B). No treatment, either with hydrogen peroxide or with chlorine dioxide, was effective against the parasitosis under lab conditions.

For now, the most frequent method employed to control *I. multifiliis* infections in farm systems is the use of in-bath chemical treatments (PICÓN-CAMACHO et al., 2012). These can be undertaken either in short (e.g. seconds to hours in tanks and flow-through systems) or long (e.g. 7-15 days in ponds) exposure times and target the free-swimming stages of the parasite (the other stages are rarely susceptible to this sort of treatment). Its success; however, depends on a bunch of variables, sometimes difficult to control. It is known, for example, that the toxicity of a drug can be directly related to abiotic environmental conditions (hardness, pH, alkalinity and organic matter, for example). In addition, host´s

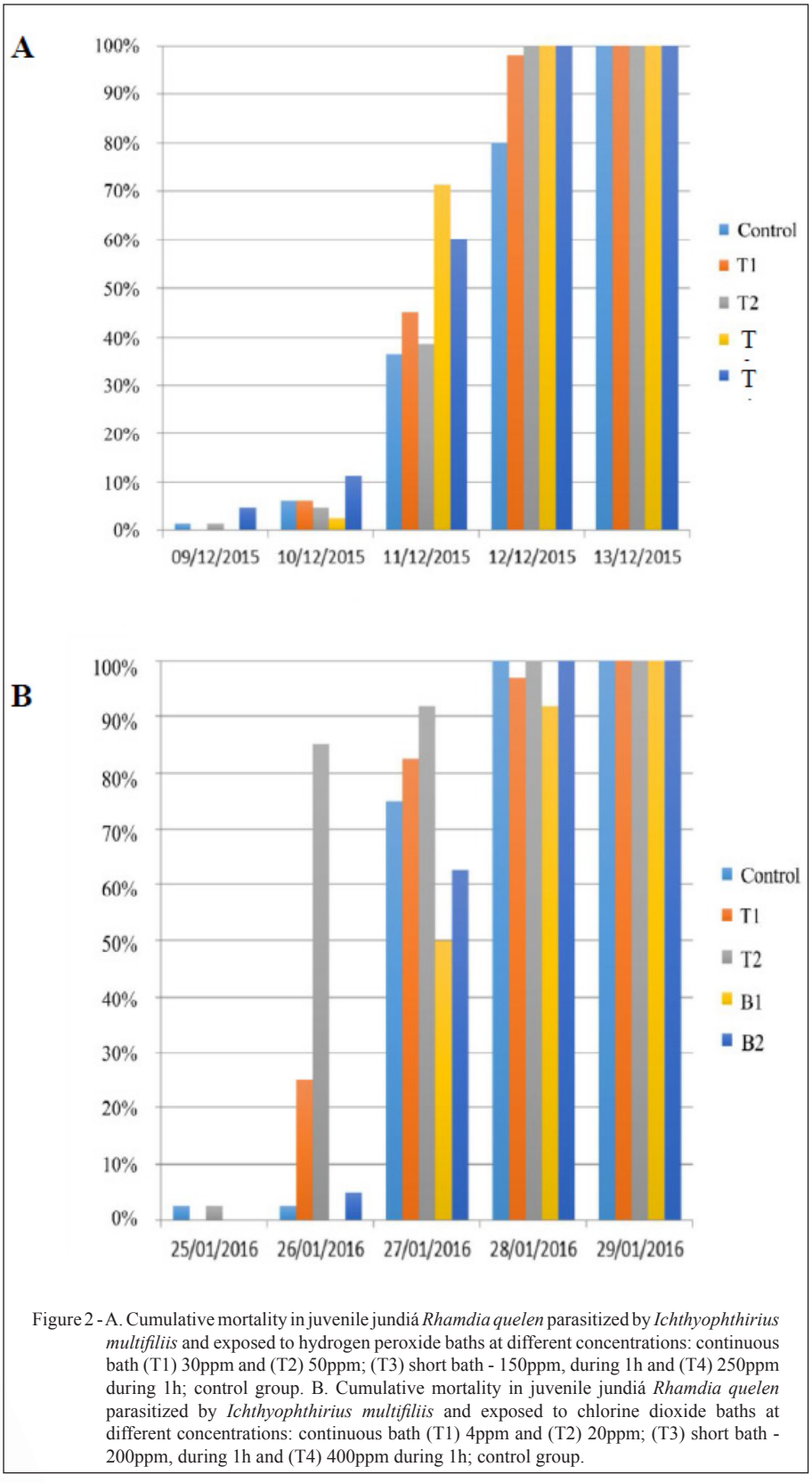

Figure 2 - A. Cumulative mortality in juvenile jundiá *Rhamdia quelen* parasitized by *Ichthyophthirius multifiliis* and exposed to hydrogen peroxide baths at different concentrations: continuous bath (T1) 30ppm and (T2) 50ppm; (T3) short bath - 150ppm, during 1h and (T4) 250ppm during 1h; control group. B. Cumulative mortality in juvenile jundiá *Rhamdia quelen* parasitized by *Ichthyophthirius multifiliis* and exposed to chlorine dioxide baths at different concentrations: continuous bath (T1) 4ppm and (T2) 20ppm; (T3) short bath - 200ppm, during 1h and (T4) 400ppm during 1h; control group.

susceptibility and the optimal time to begin treatment also influences on how successful the therapy will be. In this study, therapeutic baths were performed 24 hours after the appearance of white spots visible to naked eye. Due to the parasite´s aggressiveness, it is possible that fish were, at the time of the onset of baths, already severely damaged compromising thus their efficaciousness.

Hydrogen peroxide is an oxidizing and disinfectant agent commonly used for the treatment of diseases caused by infectious agents in fish farms, such as fungus in certain freshwater fish, bacteria, protozoan *Ambiphrya* and monogenoid *Gyrodactylus* sp. (RACH et al., 2000). Its toxicity is directly related to the concentration employed. According to SCHMIDT et al. (2006), fish and their eggs are relatively tolerant to the chemical agent, and exposure time to concentrations between 50 and 100ppm (fish) are generally considered safe to the sick animal when dispensed for a short period (less than h). However, its efficiency against the target pathogen seems to be conditioned to several factors such as concentration, water quality, manner of application (several applications may be required), infection degree of the animals and tolerance of the host species to the chemical agent.

RACH et al. (2013) showed that hydrogen peroxide concentrations between 170 and 280mg L^{-1} applied in a 30-min static bath on juvenile rainbow trout *Oncorhynchus mykiss* were efficient in the control of protozoans and monogenoids (Monogenea). Similarly, MONTGOMERY-BROCK et al. (2001) reported that single treatments at concentrations of 75 or 150mg L^{-1} successfully eliminated *Amyloodinium* sp. trophonts without causing any fish loss.

Conversely, TIEMAN & GOODWIN (2001) tested hydrogen peroxide at a concentration of 25mg L^{-1} (daily) against ichs in *Ictalurus punctatus* without any satisfying results, similar to *in vitro* trials (<50mg L^{-1} for 10h) targeting the trophont stage, provided by LAHNSTEINER & WEISMANN (2007) and in current study.

Contrastingly several authors evaluated the efficaciousness of products containing hydrogen peroxide, such as sodium percarbonate (BUCHMANN et al., 2003) and peracetic acid (STRAUS & MEINELT, 2009): both are toxic against the parasite´s infectious form (theront) and are promising alternatives to current chemotherapies for the control of *I. multifiliis* infections (even though their efficacy has been shown to be closely linked to a range of water quality parameters (HEINECKE & BUCHMANN, 2009; PICÓN-CAMACHO et al., 2012).

The oxidant chlorine dioxide (a disinfectant commonly employed for the treatment of drinkable water) has been indicated for several appliances in aquaculture, such as sanitation during fish processing and in the treatment of certain etiological agents such as viruses, algae, fungi and protozoa (JUNLI et al., 1997). Due to its high long-term biocide capacity and low toxicity in the environment (it does not produce nitrogenated toxic wastes (ex., chloramines and it is more effective than chlorine in the control of pathogenic organisms (ANDRADE, 2010), several tests have been performed to prove its applicability in the control of fish parasites.

YAMABE et al. (1990) showed that a continuous bath of chlorine dioxide at a concentration of 15ppm against *I. multifiliis* in *Carassius auratus* was effective (the authors registered release of trophonts after two days of exposure to the chemical agent). Further, POWELL & CLARK (2004) assessed the efficiency of freshwater baths with certain additives (such as chlorine dioxide and hydrogen peroxide) in the Atlantic salmon infested by the parasite *Neoparamoeba pemaquidensis* and concluded that after a 3h exposure bath, chlorine dioxide decreased 50% of parasite infection in concentrations between 25 and 50ppm. However, results were inconclusive in the case of hydrogen peroxide. Further, the authors concluded that the efficaciousness of ClO_2 was modified according to water hardness (the greater the hardness, the higher is the toxicity of the chemical agent) and amount of suspended organic matter in the water column. Although efficient, hydrogen peroxide was reported to be highly toxic at the concentration tested (100µL L^{-1}) by the above-mentioned authors.

According to RACH et al. (2013), two variables may be employed to assess the efficaciousness of a chemical treatment in the control of parasite infestations: percentage of fish mortality and decrease in the number of parasites. Following this thinking, treatments in the current study were not successful under currently defined experimental conditions.

CONCLUSION

Results showed that short and long baths with hydrogen peroxide and chlorine dioxide were not effective against ichthyophthiriasis, one of the most impacting diseases in the breeding of freshwater fish, including *R quelen* (jundiá) in the state of Santa Catarina, Brazil.

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BIOETHICS AND BIOSSECURITY COMMITEE APPROVAL

Animal procedures were approved by the Ethic Committee of Universidade Federal de Santa Catarina (CEUA/ UFSC/ PP00928), Santa Catarina state, Brazil.

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