

Toxicity of peracetic acid (PAA) to tomonts of *Ichthyophthirius multifiliis*

T. Meinelt^{1,*}, S. Matzke², A. Stüber¹, M. Pietrock³, A. Wienke⁴, A. J. Mitchell⁵,
D. L. Straus⁵

¹Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Mueggelseedamm 301, 12587 Berlin, Germany

²Humboldt-University of Berlin, Invalidenstr. 42, 10115 Berlin, Germany

³Toxicology Centre, University of Saskatchewan, 44 Campus Drive, Saskatoon, Saskatchewan S7N 5B3, Canada

⁴Martin-Luther-University Halle-Wittenberg Medical Faculty Institute of Medical Epidemiology, Biostatistics and Informatics, Magdeburger Strasse 27, 06097 Halle (Saale), Germany

⁵US Department of Agriculture, Agricultural Research Service, Harry K. Dupree Stuttgart National Aquaculture Research Center, PO Box 1050, Stuttgart, Arkansas 72160, USA

ABSTRACT: The free-living infective theront of *Ichthyophthirius multifiliis* historically has been thought to be the only stage susceptible to treatment. Here we introduce a technique to determine the toxicity of compounds to the newly released tomont, the encysted tomont and the developing tomites within the tomont that emerge as theronts. The toxicity of Wofasteril® E400 (40% peracetic acid, PAA) to free-living forms of *I. multifiliis* was determined shortly after tomonts were physically removed from the surface of the fish and at 2.5 and 24 h after removal. Results indicate that 0.6 to 0.9 mg l⁻¹ PAA killed 39 to 82% of the newly released tomonts within 48 h when treated immediately. In a second experiment, tomonts were allowed to settle for 2.5 h after sampling from the skin and then treated for 12 h; concentrations ≥0.5 mg l⁻¹ PAA produced significantly fewer theronts than the controls. In a third experiment, encysted tomonts that were exposed to PAA 24 h after sampling from the skin and treated for 2 or 4 h produced a variable amount of theronts, but the concentrations tested (0.5 to 3.0 mg l⁻¹) did not halt theront production. This research demonstrates that encysted *I. multifiliis* are less susceptible to chemical treatments.

KEY WORDS: *Ichthyophthirius multifiliis* · Peracetic acid · Toxicity · Wofasteril®

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INTRODUCTION

Ichthyophthirius multifiliis (Fouquet) is the causal agent of white spot disease, or Ich, and is one of the most detrimental parasites in ornamental fish culture and other types of aquaculture around the world. The life cycle of this ciliated protozoan has 3 morphologically distinct stages. Upon entering the skin and gills, the non-feeding (lacking a cytostome), free-swimming theront transforms into a trophont, which feeds on mucus and tissue. The trophont grows within the skin tissue for several days depending on ambient temperatures. The mature trophont leaves the host, attaches to a solid surface and is called a tomont. The encysted tomont undergoes a series of divisions over a short

period of time (dependent on temperature), resulting in tomites that break through the cyst wall to become theronts (Beckert & Allison 1964, Nigrelli et al. 1976, Schäperclaus 1991, Lom & Dyková 1992, Matthews 2005).

In the past, malachite green was used to treat *Ichthyophthirius multifiliis* infestations by killing the infective theronts (Amlacher 1961, Wahli et al. 1993). Amlacher (1961) suggested that malachite green was effective against both the free-living and intradermal stages; however, Wahli et al. (1993) suggested that the compound may irritate the interdermal stage, causing the parasite to exit the fish and then become susceptible to the chemical. Because of its carcinogenic potential, malachite green was banned as a therapeutic in

*Email: meinelt@igb-berlin.de

EU member states, the United States and numerous other countries for the treatment of fish meant for human consumption, but not necessarily for ornamentals. The search for alternative treatments for *I. multifiliis* has been accelerated over the past decade. A method was developed to determine the toxicity of potassium permanganate to *I. multifiliis* theronts (Straus & Griffin 2001) and has also been used to screen other chemicals such as copper sulphate (Goodwin & Straus 2006) and peracetic acid (PAA; Meinelt et al. 2007a, Straus & Meinelt 2009).

Schäperclaus (1991) used Wofasteril® as a disinfectant when working with diseased fish. Wofasteril® is widely used in agriculture and has recently been recommended as an alternative parasiticide to malachite green to treat external parasites (Meinelt et al. 2007b). Wofasteril® E400 contains 40 g PAA, 15 g hydrogen peroxide and 25 g acetic acid in 100 ml solution. In addition to having a broad antimicrobial spectrum, PAA does not contribute to the formation of resistance. Even at low concentrations, Kitis (2004) found antimicrobial effects of PAA. Several studies (Meinelt et al. 2004, 2005, 2006) have documented the effects of PAA against the parasitic water mould *Saprolegnia parasitica* and against various other fungal infections on fish and fish eggs.

Treatment of an *Ichthyophthirius multifiliis* infestation with PAA is effective on theronts that are not protected by the fish mucus and skin. Meinelt et al. (2007a) determined that 0.3 mg l⁻¹ PAA reduced the number of free-living theronts by 24% when compared to the controls 5 min after exposure. Concentrations of 0.5 mg l⁻¹ PAA killed all free-living theronts within 9 min. Rintamäki-Kinnunen et al. (2005a,b) treated *I. multifiliis* in ponds and concrete raceways with mixtures of PAA-products and formalin and reduced the abundance of the parasite in fish. However, nothing is known about the toxicity of PAA to trophonts, tomonts or tomites within the tomont, which are protected from adverse environmental conditions by the cyst wall during development (Ewing et al. 1983).

The purpose of these investigations was to determine the toxicity of PAA to recently released *Ichthyophthirius multifiliis* tomonts and to determine the effect of time after removal from the fish skin until parasite encystment on PAA toxicity.

MATERIALS AND METHODS

The chemical treatment used in this study (Wofasteril® E400) was obtained from KESLA PHARMA WOLFEN. Reconstituted water prepared according to DIN EN ISO 7346-3 (International Organization for

Standardization 1996) was used in all tests and *Ichthyophthirius multifiliis* cultures. The water consisted of 294.0 mg l⁻¹ CaCl₂·2H₂O, 123.3 mg l⁻¹ MgSO₄·7H₂O, 63.0 mg l⁻¹ NaHCO₃ and 5.5 mg l⁻¹ KCl dissolved in deionised water. Consequently, the reconstituted water contained 2 mmol l⁻¹ Ca²⁺, 0.5 mmol l⁻¹ Mg²⁺, 0.077 mmol l⁻¹ K⁺ and 0.77 mmol l⁻¹ Na⁺. Dissolved oxygen was 7.2 ± 0.5 mg l⁻¹ (~74% saturation), pH ranged between 7.5 and 8.0 and water temperature was maintained at 15°C.

Stages of *Ichthyophthirius multifiliis* used in the experiments. *I. multifiliis* was obtained from infected goldfish *Carassius auratus* (L.) sampled at various commercial fish hatcheries outside Berlin, Germany. Fully developed trophonts (as determined by relative size) were gently scraped from the skin of the fish with a spatula into crystallising dishes (Carl Roth C095.1) containing reconstituted water. The tomonts were rinsed several times to remove mucus or tissue in the dishes. The *I. multifiliis* stages that were subjected to treatment included: (1) tomonts removed from fish by scraping and immediately used in experiments; (2) tomonts removed from the fish and allowed to settle for 2.5 h before being used in experiments; (3) tomonts removed from the fish and allowed to settle for 24 h before being used in experiments. Theronts that were produced from the tomonts were used as a measure of tomont survival in Expts 2 & 3.

Toxicity of PAA to unsettled tomonts. In the initial experiment, effective concentrations were determined. The concentrations of PAA in this experiment ranged from 0.1 to 0.9 mg l⁻¹ (in 0.1 mg l⁻¹ increments) and a negative control. There were 36 tomonts individually exposed to each concentration of PAA (n = 36). One tomont was transferred along with 100 µl of reconstituted water into each well of a 24-well plate (Cellstar® 662160, Greiner Bio-One) that contained 900 µl of tempered reconstituted water. Next, 1 ml of each PAA stock solution was applied to give the appropriate final concentration. In the controls, 1 ml of reconstituted water without PAA was applied. The solutions were not changed throughout the 48 h experiment. The 24-well plates were stored in an incubator at 15°C. At 48 h, an Olympus IMT-2 inverse-microscope was used to determine if the tomonts had encysted and were dead or alive. Tomonts were considered dead if their nucleus was invisible or not horseshoe-shaped (coagulated) and cilia movement was not detected.

Toxicity of PAA to tomonts allowed to settle for 2.5 h. In the second experiment, *Ichthyophthirius multifiliis* tomonts were obtained from infected goldfish and rinsed as described above. A 10 ml aliquot of a gently stirred suspension was transferred into each of 14 crystallising dishes; the mean concentration of this suspension was 11.2 tomonts ml⁻¹. The tomonts were

allowed to settle for 2.5 h and were then exposed to a final concentration of 0.5, 1, 2 or 3 mg l⁻¹ PAA by adding 10 ml of the respective PAA solution to the test dishes. There were 3 replications of each concentration, and 2 dishes without PAA served as negative controls. The crystallising dishes were incubated at 15°C. At 12 h after exposure, the test solution was replaced by tempered test water.

After a total of 48 h of incubation, any theronts that had emerged from tomonts were fixed and counted. For this purpose, eight 100 µl samples from each dish were transferred to a 96-well plate (BD Primaria™ 353872, Becton Dickinson Labware) and fixed with 100 µl of 4.5% buffered formalin. The fixed theronts were allowed to accumulate at the bottom of the well for 2 h and counted with an inverse microscope.

Toxicity of PAA to tomonts allowed to settle for 24 h. In the third experiment, tomonts were collected as in the second experiment; the mean concentration of this suspension was 5.8 tomonts ml⁻¹. Freshly recovered tomonts were allowed to attach to the bottom of the crystallising dishes for 24 h. After the 24 h settling period, a subset of 3 replicates was exposed to 0.5, 1, 2, 2.5 or 3 mg l⁻¹ PAA for 2 h. A second subset of 3 replicates was exposed to the same concentrations for 4 h with the exception of the 3 mg l⁻¹ treatment; the latter concentration was not used because of lack of tomonts. After a total of 48 h of incubation, any theronts that had emerged from tomonts were fixed and counted.

Statistical analysis. For the test with unsettled *Ichthyophthirius multifiliis* tomonts, the lethal concentration (LC₅₀) and the 95% confidence interval (CI) were calculated by Probit analysis. In the experiment where the tomonts were allowed to settle for 2.5 h, an independent *t*-test was used to determine significance between PAA-exposed groups and control. In the experiment where the tomonts were allowed to settle for 24 h, the numbers of live and dead theronts in each treatment were compared by applying an analysis of variance (ANOVA). When there were overall differences between the groups, pairwise differences were analysed by means of the Dunnett T3 test. Differences were considered significant at *p* < 0.05. All analyses were performed using the Statistical Package for the Social Sciences software (SPSS for Windows, release 14.0)

RESULTS

Toxicity of PAA to unsettled tomonts. Preliminary experiments indicated that 1 mg l⁻¹ PAA resulted in 90% mortality of the tomonts, and concentrations of ≥2 mg l⁻¹ PAA resulted in 100% mortality of the exposed individuals within 48 h. The 0.1 to 0.3 mg l⁻¹

PAA treatments demonstrated little or no toxicity. At 0.4 and 0.5 mg l⁻¹ PAA, there was 21 and 20% mortality by 48 h, respectively (Fig. 1). From 0.6 to 0.9 mg l⁻¹ PAA, *Ichthyophthirius multifiliis* mortality at 48 h increased from 39 to 82%. In the controls, 100% of the tomonts became encysted and had started cleavage by 48 h. The 48 h LC₅₀ was 0.68 mg l⁻¹ PAA (95% CI = 0.59 to 0.79 mg l⁻¹).

Although the exposure lasted 48 h, lethal effects of PAA could be observed after 20 min. During microscopic examination, dead tomonts were typically darker than live ones due to the initiation of necrotic coagulation (Fig. 2). Additionally, dead tomonts had lysed cell membranes, no signs of division and no cilia movement. In contrast, most live tomonts had become encysted, underwent division or had released theronts.

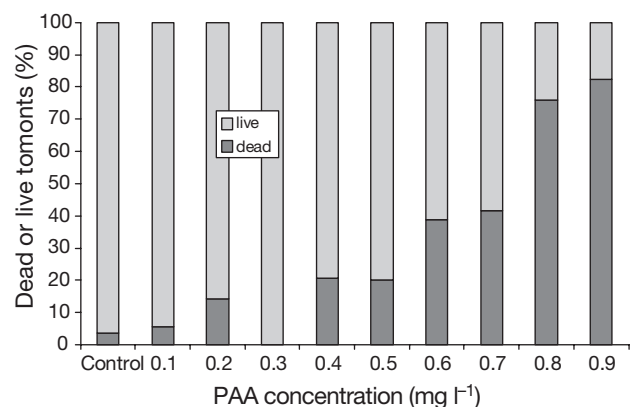


Fig. 1. *Ichthyophthirius multifiliis*. Dose-response relationship between percent of dead and live trophonts or tomonts in the detached trophont toxicity experiment after 48 h exposure to peracetic acid (PAA)

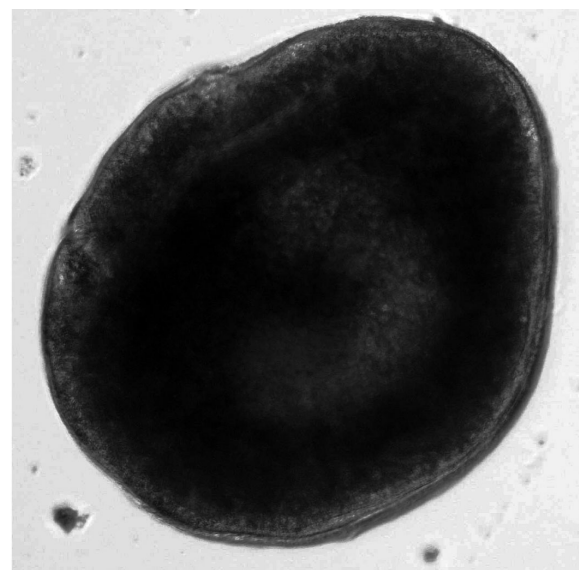


Fig. 2. *Ichthyophthirius multifiliis*. Coagulating tomont after peracetic acid (PAA)-treatment (400× magnification)

Non-coagulated tomonts were counted as vital, even if they neither encysted nor produced any live theronts.

Toxicity of PAA to tomonts allowed to settle for 2.5 h. After 48 h of incubation, the control produced 580 ± 190 theronts ml^{-1} (mean \pm SD). The application of 0.5 mg l^{-1} PAA significantly ($p < 0.05$) reduced the mean theront number to 58% of the control. Exposure to 1 mg l^{-1} PAA reduced the mean theront number to 25% of the control, while exposure to 2 mg l^{-1} PAA reduced the mean theront number to 2% of the control (Fig. 3). Applying 3 mg l^{-1} PAA resulted in >99% mortality of the exposed tomonts (only 1 theront in 1 well survived).

Toxicity of PAA to tomonts allowed to settle for 24 h. Significant differences were observed between controls and *Ichthyophthirius multifiliis* tomonts exposed to PAA for 2 or 4 h ($p < 0.05$), respectively, except for the exposure to 0.5 mg l^{-1} for 4 h ($p = 0.157$). However, differences were not correlated to PAA concentration or to duration of application (Fig. 4). After 48 h incubations, all treatments showed live theronts. The theront number differed in all treatments independent of PAA-concentration and duration of the application. Tomont cell division and appearance of the protective cyst wall were evident at 24 h.

DISCUSSION

Preliminary investigations were designed to determine the toxic PAA concentration to *Ichthyophthirius multifiliis* tomonts. Results demonstrated that the PAA product tested in this study (Wofasteril® E400) is acutely toxic, with concentrations $\geq 2 \text{ mg l}^{-1}$ resulting in 100% mortality of the tomonts within 48 h. Although the exposure lasted 48 h, lethal effects of PAA could be observed after 20 min.

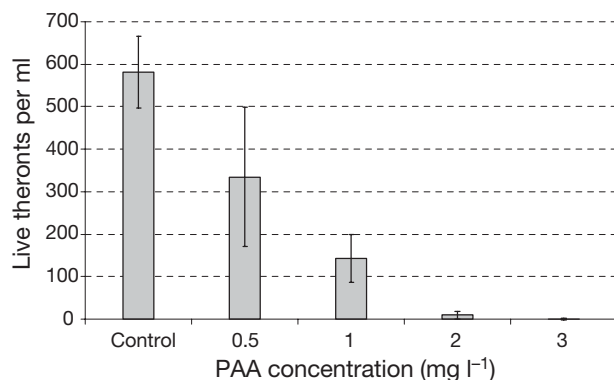


Fig. 3. *Ichthyophthirius multifiliis*. Mean number of live theronts (\pm SD) produced from trophonts allowed to settle 2.5 h and then exposed to 0, 0.5, 1, 2 or 3 mg l^{-1} peracetic acid (PAA) for 12 h. Total incubation time was 48 h. All exposed groups are significantly different from the control ($p < 0.05$)

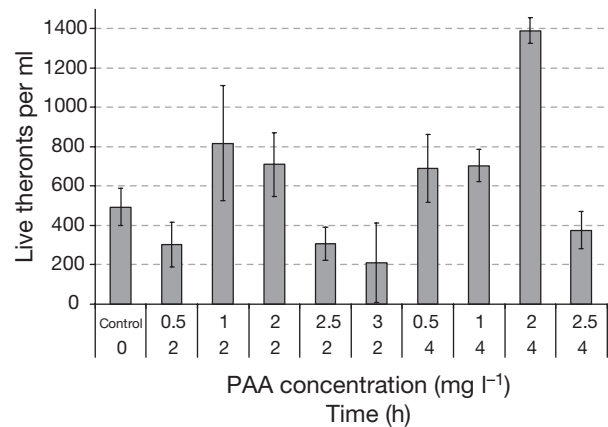


Fig. 4. *Ichthyophthirius multifiliis*. Mean number of live theronts (\pm SD) produced from trophonts allowed to settle 24 h and then exposed to 0, 0.5, 1, 2, 2.5 or 3 mg l^{-1} peracetic acid (PAA) for 2 or 4 h. Total incubation time was 48 h

In the first experiment, the range of PAA concentrations (Fig. 1) demonstrated a distinct dose-response relationship. At the end of the tomont experiment, the only evaluations made were whether the tomonts had encysted, whether they had survived exposure or whether they had died and were not intact. Due to the test design, it was not possible to draw reliable conclusions about the vitality and/or infectivity of theronts produced by surviving tomonts. Therefore, the number of theronts produced, as well as their vitality, was included in the test design of the following experiments.

MacLennan (1937) described a time interval of 2.5 h at 26°C for tomonts leaving the host until the beginning of encystment. According to Matthews (2005), *Ichthyophthirius multifiliis* tomonts encyst within a period of 15 min to 6 h after leaving the host. In the second experiment of the present study, PAA exposure was initiated 2.5 h after scraping the trophonts from the fish at 15°C . During this period, most of the tomonts had already settled in the crystallising dishes and were possibly starting to encyst. A 12 h exposure to 0.5 mg l^{-1} PAA reduced the mean number of theronts to 58% of the control (Fig. 3). Exposure to concentrations ranging from 0.5 to 2 mg l^{-1} PAA reduced the vitality of the tomonts and impaired theront development. Similar to the previous experiment of the present study, a dose-response relationship was observed. The effect of PAA is particularly evident before the trophonts finish developing the cyst wall. However, some of the literature suggests that the development of vital theronts is not necessarily linked with the development of a cyst wall (Ewing et al. 1983, Aihua & Buchmann 2001). The present study was not designed to differentiate between PAA preventing the tomonts from reaching the encysted stage and PAA reducing survival rate of the tomonts or tomites within.

The third experiment was designed to determine the effect of PAA toxicity on age of tomonts and on exposure time. Age of the parasites clearly had an influence on PAA toxicity because of the development of the cyst wall. In contrast to the experiment where concentrations of 0.5 to 3.0 mg l⁻¹ PAA were given at 2.5 h and showed distinct toxic effects (Fig. 3), the same concentrations were ineffective on all tomonts (presumably encysted) treated 24 h after removing them from the fish (Fig. 4). Extending the exposure time to 4 h had no significant effect on the amount of theronts produced by the tomonts. Microscopic examination indicated that 24 h after the start of the experiment, tomonts had developed a cyst wall, which probably protected the parasites from PAA exposure in concentrations up to 3 mg l⁻¹.

The number of released theronts was not reduced by the PAA exposure when tomonts were protected by encystment. Considerably higher concentrations or extended exposure times will be necessary to kill encysted *Ichthyophthirius multifiliis*. However, high PAA concentrations are particularly toxic to juvenile fish (Meinelt et al. 2007a,b), and such concentrations would not be practical for treating diseased fish; therefore, higher PAA concentrations were not investigated. It is possible that some fish species (and/or life stages) are more resistant to PAA than those tested.

The effects of PAA on *Ichthyophthirius multifiliis* tomont survival may be more severe if constant concentrations could be ensured. However, PAA degrades quickly in relation to water parameters. Reports on the effects of water parameters such as temperature, organic load or pH on PAA concentrations vary. Harakeh (1984) stated that temperature, organic matter and suspended solids had negligible effects on the virucidal efficacy of PAA. Kitis (2004) emphasised the instability of PAA; a 40 % PAA solution loses 1 to 2 % of its active ingredient per month. Furthermore, Kitis (2004) described higher instability of diluted PAA solutions; for example, a 1 % solution loses half its strength through hydrolysis within 6 d. Unpublished data from our lab indicate that 2 mg l⁻¹ degraded to below detection limits in 2 to 4 h depending on water temperature and aeration. In addition to having toxic effects against *I. multifiliis*, PAA has also been shown to be effective against other external parasites. Weitkamp et al. (2007) were able to eliminate external parasites including *Chilodonella* sp., *Trichodina* sp., *Gyrodactylus* sp. and *Epistylis* sp. by applying 2.6 mg l⁻¹ PAA d⁻¹ for 3 d.

Rintamäki-Kinnunen et al. (2005a,b) used PAA-containing products (Per Aqua and Desirox; both were reported to be combinations of 13 % peracetic acid, 20 % acetic acid and 20 % hydrogen peroxide) as single substances and/or in combination with formalin to treat *Ichthyophthirius multifiliis*. They found a reduced

parasite burden and a reduction of losses in salmon and trout stocks. They suggested that fish surviving the *I. multifiliis*-infestation had time to develop immunity against further infestations (Rintamäki-Kinnunen et al. 2005a,b).

The results of the present study suggest that treating *Ichthyophthirius multifiliis* with PAA will be effective on all free, non-encysted stages of the parasite prior to development of a cyst wall; toxicity to these mature stages will be evident in the number of newly released theronts. Based on the results of the present experiments, we suggest treating with concentrations between 2 and 3 mg l⁻¹ PAA. Because the hatch of tomonts and theronts is not synchronous between all *I. multifiliis* on a fish, a constant concentration PAA-concentration on a long-term scale must be ensured while treating this parasite in practice. Treating fish with PAA should be carried out by applying the chemical under dynamic conditions to ensure reasonably constant exposure concentrations. Although PAA concentrations <2 mg l⁻¹ are highly effective to treat theronts (Meinelt et al. 2007a) and tomonts (present study), PAA should be applied at nominal concentrations of at least 2 mg l⁻¹ because PAA is known to be unstable.

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