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# A novel treatment against the monogenean parasite, *Gyrodactylus turnbulii*, infecting guppies (*Poecilia reticulata*), using a plant-based commercial insecticide Timor C

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**Abstract**

Monogenean infections are a common cause of fish morbidity and mortality in aquaculture. In most cases, treatment relies on the application of toxic or unapproved chemicals. Therefore, alternative treatments against monogenean infections in fish that are safe and environmentally friendly are needed.

In the present study, the efficiency of Timor C, a plant-based commercial insecticide, was investigated against *Gyrodactylus turnbulii,* a monogenean parasite infecting guppies (*Poecilia reticulata*).

Since *G. turnbulii* primarily infects the fins and skin, with a predilection for the caudal fin, tail clips of heavily parasitized guppies were exposed *in vitro* to Timor C at various concentrations and parasite detachment and death were recorded over time. There was a positive correlation between time to detachment and Timor C concentration, with complete detachment within 60 min of exposure at a concentration of 50 ppm. Withdrawal of Timor C afterward did not lead to recovery of the exposed parasites.

Treatment of infected fish for 24 h at 10 and 20 ppm was effective in lab-based trials, reducing the infection prevalence from 100% at time 0 to 22% post-treatment, and the infection rate to an average of less than one parasite per fish, as compared to an average of about five parasites per fish in the control. Repeated weekly treatment application for three consecutive weeks appeared safe for the fish, and did not cause an adverse effect to the biofiltration system, as determined by examining nitrification activity, compared to non-treated controls. Treatment was successfully tested in three separate applications at a commercial closed recirculating guppy farm, where a significant reduction in infection rates was achieved. Timor C was not toxic to *Artemia* at concentrations ranging from 10 to 100 ppm over 24 hours of exposure.

This study demonstrates the effectiveness of Timor C as a treatment against *G. turnbulii* infection in guppies and potentially against similar parasites in other aquacultural species.

**Key Words:**

Monogenean; Gyroductylus turnbulii; treatment; plant; commercial; guppy

## 1. Introduction

Monogenean infections are responsible for significant economic losses in commercially farmed fish (Noga, 2014; Shinn et al., 2015). Gyrodactylids are monopisthocotylean monogeneans, which infect the fins and skin, or gills of fish (Buchmann and Bresciani, 2006). These parasites cause damage to the host fish through their attachment with sclerotinized hooks and their feeding on mucus and epithelial cells. Damage is compounded by the host's inflammatory response to infection (Buchmann and Bresciani, 2006).

Global ornamental fish production is continually growing, with an export value that almost doubled between 2000 and 2014 (Dey, 2016). Guppies (*Poecilia reticulata*) are one of the most widely distributed ornamental fish and one of the most popular aquarium species. This fish species is affected by the gyrodactylid species *Gyrodactylus turnbulii* and *G. bullatarudis* (King et al., 2009), both infecting the fins and skin, with the caudal fin being a major infection site (Buchmann and Bresciani, 2006). Due to their direct life cycle, this parasite establishes itself and propagates easily in intensive aquaculture systems (Bauer, 1991), resulting in substantial morbidity and mortality.

The conventional treatment for monogenean infections is based on chemotherapy, including praziquantel, organophosphates and formalin (Buchmann and Bresciani, 2006; Noga, 2014), yet the application of organophosphates and formalin is increasingly restricted due to these

chemicals' potentially negative effect on public health (Carpenter et al., 2002; Wooster et al., 2005). The risk of human toxicity by treatment chemicals is substantially increased when they are applied in closed system fish production setups (Wooster et al., 2005). Praziquantel is effective against monogeneans in fish (Noga, 2014), although resistance appears to occur after repeated treatments (unpublished observation). As praziquantel is used in human medicine, it is unlikely to be approved for use in aquaculture.

The need for alternative, safe and environmentally friendly treatments for diseases in aquaculture has led to research exploring the use of plants and their extracts (Reverter et al., 2014). Ginger and garlic were found to be effective as a treatment for *G. turnbulii* infection in guppies (*Poecilia reticulata*) by bath and oral applications (Fridman et al., 2014; Levy et al., 2015). However, garlic and ginger-based commercial treatment products are not readily available for use in aquaculture. In addition, relatively high treatment concentrations are required for immersion bath applications (Fridman et al., 2014; Levy et al., 2015). Commercially available plant-based formulations could serve as readily available treatments for cultured fish, if proven effective and safe.

This study examines the use of Timor C, a plant-based commercial product of Stockton-Aquamore Ltd. (Israel), against *G. turnbulii* infection in guppies. The efficacy was examined *in vitro* and *in vivo*, including on-farm trials. The safety of the treatment was evaluated in fish, as well as in the biofiltration system, which is an integral component of recirculating aquaculture systems.

## 2. Materials and methods

### 2.1. Source of animals and parasites

Guppies were obtained from a commercial supplier. Upon arrival, fish were anaesthetized and screened for infection with *Gyrodactylus turnbulli* by direct wet mount microscopic observation of skin and gill scrapes. Species identification was based on Paladini et al. (2009). Fish were stocked in 100-L tanks supplied with aeration and in-tank biological filters. Water temperature was kept at 25 ± 1 °C. Fish were fed daily at 2% of their body weight (Ocean Nutrition, Belgium). Uninfected fish were added, and fish stocking density was maintained at high levels (ca. 400 fish per tank) to encourage propagation of the parasite. Experimental protocols were carried out in compliance with the principles of biomedical research involving animals, obtained from the Ben-Gurion University Committee for the Ethical Care and Use of Animals, Ben-Gurion University of the Negev, Israel. Authorization number: IL-79-10-2012.

### 2.2. Timor C

Timor C, a natural insecticide, was obtained from the manufacturing company Stockton Aquamor (Stockton Group, Israel) and purchased from Hagarin (Beer Sheva, Israel). The Timor C was stored in a cool dry place according to the manufacturer's instructions.

### 2.3. *In vitro* effect of Timor C on *G. turnbulli* survival

Heavily parasitized guppies from the infection tanks were euthanized by Sedanol (Stockton-Aquamor Ltd., Israel) at a concentration of 250 ppm, followed by brain pithing. Sedanol was shown to have little to no effect on parasite detachment, as compared to the conventionally used clove oil (Supplementary Material S1). Tail fins infected with *G. turnbulli* were excised, and fin

clips with a minimum of three parasites were transferred, using watchmakers' forceps, to a single well in a 12-well plate, containing 1 mL of tank water (Corning Inc., USA). Timor C was prepared at double concentration using filtered tank water, and 1 ml was added to each well to achieve the desired dose. Concentrations tested included 20, 50 and 100 ppm of Timor C. Tank water without Timor C served as a control treatment. As tea tree oil is a major component of Timor C, the effect of pure tea tree oil (Thursday Plantation, Australia) was similarly tested against the parasite at a concentration of 100 ppm. Parasites were observed with an inverted microscope (Zeiss) every 20 min for 3 h; time to detachment and death was recorded, and the percentage of detached and dead parasites was calculated for each time point. A total of at least 10 parasites were analysed for each concentration, in at least three separate replicate wells.

To evaluate parasite recovery from treatment, parasites were exposed to Timor C at a concentration of 60 ppm in 12-well plates for 1, 2 and 3 h, followed by removing 1.8 ml of the Timor C solution and adding 1.8 ml of water. Parasite recovery was observed for 1–3 h.

2.4. *In vivo* bath treatment with Timor C

2.4.1. Fish toxicity tests and effects on biofilter

Trials were performed to determine the concentration of Timor C that could be used in immersion without compromising either the fish health or the biofilter activity. Timor C concentrations at which acute and chronic toxicity occurred in exposed fish and their effects on the biofilter under repeated applications were examined.

To evaluate acute toxicity in exposed fish, trials were performed in 1-L beakers with 800 mL of water and gentle aeration. Beakers were stocked with 10 fish and Timor C was added at 20, 40, 60 and 80 ppm. Fish were observed every hour for 16 h and again after 24 h for signs of stress, characterized by abnormal erratic swimming and/or lethargy, and for mortality. Exposure was terminated at the occurrence of death and surviving fish were returned to freshwater. The time at which stress had started to occur and time of the occurrence of death were recorded.

Based on the acute toxicity trial, a safe concentration of 20 ppm was selected and repeatedly applied in 10-L aquaria, in triplicates. Each 10-L aquarium was stocked with 20 guppies and supplied with a submerged biological filter (0.5 L) containing bio-balls. Timor C was applied at 20 ppm once weekly, for five consecutive weeks. Fish were observed for the occurrence of morbidity, mortality or any change in behaviour.

After the third application, five fish from each aquarium were sampled for histopathological analysis. After the fifth application, 10 ppm of ammonium chloride was added to each aquarium, and water quality was analysed at time 0 and after 1, 4 and 8 d for ammonia, nitrate and nitrate levels using commercial kits (Merck, Darmstadt, Germany).

For the histopathological analysis, whole fish were euthanized in clove oil (250 ppm) and fixed in 10% neutral buffered formalin. Fixed whole fish were cut into ca. 0.4-cm thick transverse sections and processed using routine histological techniques (as described in Sharon, Pimenta-Leibowitz, Chettri, Isakov & Zilberg, 2014). The slides were stained with H&E and examined under a compound microscope. The following organs were analysed: head (gills and brain), thorax (heart and muscles) and abdominal cavity (internal organs and lateral muscles).

2.4.2. Environmental toxicity using the *Artemia* bioassay

To evaluate the potential environmental toxicity of Timor C, we performed an *Artemia* toxicity trial based on Caldwell et al. (2003) and Persoone and Wells (1987) . Briefly, wells of 24 well plate were filled with 400 µl of 30 ppt seawater (red sea salt, Israel) containing 30-60 *Artemia* (*Artemia salina*, Salt Creek Inc., Salt Lake City, UT) per well. Timor C was added at 10, 20, 50 and 100 ppm, at a final volume of 800 µl per well. For negative control water with no Timor C was applied, and for positive control, Timor C was added at 10,000 ppm. *Artemia* was observed for mortality and for changes in swimming behaviour (expressed as lethargy), hourly for the initial 7 hours, and then after 24 hours. Three replicate wells were used for each concentration.

2.4.3. *In vivo* bath treatment

The experiment was designed to allow the number of parasites on each fish before and after treatment application to be tracked. Infected fish were randomly selected from the infection tank and anesthetized for 10 min with 250 ppm Sedanol (Stockton-Aquamor), and the parasite number was determined with a stereo microscope (Zeiss, Stemi 2000 - C). Only fish infected with at least one parasite on the tail were included in the trial. Up to three fish with the same number of parasites were each stocked in a 1-L glass beaker with 800 ml of dechlorinated water and gentle aeration. Timor C was added at 10 and 20 ppm for 18 h, and no added treatment for the control. The number of parasites on each fish was recorded at time 0 and then again at the end of 18 h, by observing the tail fin of anesthetized fish with a stereo microscope. A total of 12 fish per treatment were analysed (n = 12).

2.5. Field trial

A field trial was performed in a commercial ornamental fish farm with modular 40-$m^3$ systems, each serviced by a separate biological filter and comprising 20, two-metric-tonne tanks. Each such systems is stocked with many thousands of fish. Treatment was applied in three separate applications following the diagnosis of a *G. turnbulli* outbreak at the farm. In each application a different 40-$m^3$ system was treated. Fish were sampled from the treated system before and after treatment application, 13-22 fish per sampling (as indicated in Table 2) and examined for infection as described in section 2.4.2, with the exception that the total number of parasites on the tail and bilaterally on the body were counted in each fish. Table 1 details the dose of Timor C applied and the number of fish examined in each of the three field trials.

2.6. Statistical analysis

Statistical analyses were performed using the SismaPlot software version 13.0 (Systat Software, Inc., San José, CA). Results of *in vitro* trials (i.e., time to parasite immobilisation) were compared using a one-way ANOVA followed by Dunn's post hoc test. *In vivo* lab trials were analysed by one-way ANOVA to compare parasite abundance between different treatment groups, and by a paired T-test to compare parasite abundance before and after treatment within a treatment group. In the 10 ppm treatment group normality test failed, thus the non-parametric post hoc test, Wilcoxon Signed Rank Test, The data in all other treatment groups had normal distribution. Results of the field trial were analysed by paired T-test. Differences were considered statistically significant at $p < 0.05$.

**3. Results**

3.1. *In vitro* parasite survival

A noticeable antagonistic effect was observed on *G. turnbulli* attached to the fin and tail clips by Timor C at all the tested concentrations within 30 min of exposure. Parasites were observed to make rapid jerking movements, become moribund and undergo violent contractions and twitching followed by detachment. After detachment, the parasites continued to contract with decreasing frequency until death occurred. Time between detachment and death was minutes for the highest concentration of 100 ppm, to hours at the lowest concentrations of 20 ppm (Fig. 1). Complete detachment and death occurred within 40 min post-exposure to 100 ppm Timor C as compared to 60 min to detachment and 90 min to death at 50 ppm (Figs. 2A, B). At the lower concentration of 20 ppm, we observed complete detachment after 210 min and the death of 70% of the parasites within 4 h (Fig. 1). Control groups showed up to 50% detachment 4 h after treatment and 0% mortality (Fig. 1). Differences between treatment groups were significant in parasite's detachment and mortality (Fig. 1). Pure tea tree oil at a concentration of 100 ppm did not affect detachment or mortality of the parasite (Supplementary Material S2)

*In vitro* exposure to 60 ppm Timor C for 1 to 3 h, followed by replacement with clean freshwater, did not result in parasite recovery; moreover, mortality continued to increase. When exposed for 1 h, survival of the parasites reached about 55%. Timor C was then removed, though survival continued to decrease, reaching 20% after 4 h (Fig. 2).

3.2. Fish toxicity test

At 80 ppm, the onset of mortality occurred at 1 h post-exposure, and at 60 and 40 ppm, mortality started to occur after 8 and 18 h, respectively (Fig. 3). Fish showed erratic and impaired swimming, oriented on their side (lopsided) before becoming lethargic, resting on the bottom and dying.

Exposure to a concentration of 20 ppm did not cause any apparent signs of toxicity to the fish after 24 h of exposure in 1-L beakers (Fig. 3). Fish that were repeatedly exposed weekly to 20 ppt of Timor C for three consecutive weeks (treatment was applied for 24 h, followed by 50%c water change), did not show any behavioural changes or any other adverse signs indicating toxic effect of the treatment. A comprehensive histopathological analysis that was performed on these fish revealed no signs of associated pathology in the gills, skin or any of the internal organs and there were no apparent differences between control fish and the fish that were exoposed to Timor C treatment.

3.3. Evaluating the effect on biofilter activity

Ammonium sulfate was added to the control and the 20 ppm Timor C-treated aquaria, to achieve a level of 10 ppm at time 0. Daily monitoring showed no difference in the gradual reduction of ammonia and nitrite levels between the control and the treatment aquaria (Fig. 4a, b). The nitrate increase was similar in the control and treatment aquaria. The nitrate levels over the eight days of analysis remained higher in the control aquaria, although differences were significant only on day 1 (Fig. 4c).

3.4. Evaluating environmental toxicity using the *Artemia* Bioassay

Timor C was not toxic to *Artemia* at concentrations ranging from 10 to 100 ppm over 24 hours of exposure (Table 1). Exposure to excessively high concentration of 10,000 ppm was toxic. Timor C is recommended as a treatment to fish at concentrations of 10-20 ppm.

3.5. Fish treatment trials (laboratory-based trials)

Bath treatment with Timor C at 10 or 20 ppm resulted in a significant reduction in infection rate as compared to the control (Fig. 5). Infection intensity at time 0 averaged about three parasites per fish in all treatment groups. While infection intensity in the untreated control groups increased after 18 h of treatment to an average of over four parasites per fish. Infection intensity in both the 10 ppm and 20 ppm Timor C treatment groups declined to almost zero (Fig. 5). Infection prevalence dropped from 100% at time 0 to 22% after a single treatment application in both the 10 ppm or 20 ppm Timor C treatment groups, as compared to infection prevalence of 90% in the control group (Fig. 5, inserted values).

3.6. Field trials

Field treatment trials were performed in a commercial ornamental fish farm growing guppies. Three separate trials were performed in individual culture modules on the farm. In all three trials, infection prevalence and intensity were significantly lower following a single treatment application with either 10 ppm or 15 ppm of Timor C (Table 2). For example, in trial 1, infection intensity dropped from an average of 8.8 parasites per fish to one parasite per fish, and infection prevalence dropped from 36% to 9.1%. Similar results were obtained in subsequent trials (Table 2). Statistical analysis revealed significant differences in both infection prevalence and intensity across all three farm trials.

Table 1: Death and lethargy in *Artemia* following exposure to different concentrations of Timor C after 7 and 24 hours (n=3 replicate wells, *ca.* 50 *Artemia* per well).

| Timor C (ppm) | Dead (%) | |
|---|---|---|
| | 7 hours | 24 hours |
| 0 (control) | 0 | 0 |
| 10 | 0 | 0 |
| 20 | 0 | 0 |
| 50 | 0 | 0 |
| 100 | 0 | 0 |
| 10,000* | 17.2 | 100 |

* This concentration is 500-1,000 folds higher than the suggested treatment concentration of 10 to 20 ppm.

Table2: Summary of three separate field trials of Timor C treatment in a commercial ornamental fish farm. Statistical analysis (using paired T-test) revealed significant differences ($p < 0.05$) in infection rates before and after treatment in the three treatment trials.

| Trial no. | Timor C (ppm) | Fish examined[1] | Before treatment | | | After treatment | | |
|---|---|---|---|---|---|---|---|---|
| | | | Infection prevalence (%) | infection intensity[2] | | infection prevalence (%) | infection intensity[2] | |
| | | | | Mean | Range[3] | | Mean | Range[3] |
| 1 | 10 | 22 | 36 | 8.8 | 1-40 | 9.1 | 1 | 1 |
| 2 | 15 | 25 | 76 | 14.7 | 1-100 | 32 | 2.9 | 1-11 |
| 3 | 15 | 13 | 62 | 2.6 | 1-6 | 38.5 | 1 | 1 |

[1] The same number of fish was examined before and after treatment

[2] Average number of parasites per fish

[3] Infection range among fish that were found to be infected

## 4. Discussion

The search for natural treatments against parasitic diseases of fish had been the focus of a growing number of studies, as the adverse effects of available conventional treatments are realised and their use is being banned in an increasing number of countries. Studies exploring natural alternative solutions against monogenean parasites had demonstrated the effective application of caprylic acid (Hirazawa et al., 2000), tea tree oil (Steverding et al., 2005), garlic (Fridman et al., 2014; Militz et al., 2013, 2014), ginger (Levy et al., 2015) and humic substances (Yamin et al., 2017).

In the present study, Timor C, a plant-based commercial insecticide, was shown to be effective against *G. turnbulii* infestation in guppies by bath treatment at 10 ppm to 20 ppm. Timor C was found to be safe for fish at 10 to 20 ppm, and did not adversely affect biofilter activity. Monogenean infections are a significant burden in recirculating aquaculture systems, where the host and parasite are held in a confined environment, often at high fish densities (Thoney and Hargis, 1991). As such, *Gyrodactylus turnbulii* is a major cause of morbidity and mortality in guppies that are grown in intensive recirculating production systems (unpublished data). Recirculating culture systems rely on the activity of biological filters to maintain adequate water quality. Therefore, applying a therapeutant that does not impair biofilter activity is particularly important. Formalin, which is still approved for use in some countries against monogenean parasites of fish (U. S. Food and Drug Administration, 2018), is known to adversely affect biofilter activity, affecting primarily nitrite oxidation, which was shown to be significantly

impaired following exposure to formalin (Keck & Blanc 2002). To evaluate potential environmental toxicity of Timor C, the *Artemia* bioassay was used, an accepted approach to evaluate potential toxic environmental impact of water-borne chemicals (Caldwell et al., 2003; Persoone and Wells, 1987). Results revealed that Timor C in non-toxic to *Artemia* at concentration as high as 100 ppt, which is at least 5 folds higher than the highest suggested safe and effective treatment for fish. In this study we evaluated the short term toxicity of Timor C and used a single, though well acceptable model organism. The potential long term environmental effect of Timor C is yet to be evaluated, especially if this chemical is applied in farms of which effluents are released to the natural environment. This issue is of lesser concern in closed recirculating farms that release their effluents to sewage treatment plants.

Based on the manufacturer's information, the active ingredients in Timor C include tea tree oil, *Sophora* sp. *Melaleuca alternifolia* and natural pyrethrin from the *Pyrethrum* plant. All of these are known bio-active compounds with different properties, including anti-helminthic effects. Tea tree oil, which constitutes 16.5% of Timor C, was previously reported to be effective against *Gyrodactylus* spp. when applied at 3 to 30 ppm, along with Tween 20 (Steverding et al., 2005). Interestingly pure tea tree oil was not effective against *G. turnbulii* in this study. Alkaloids from *Sophora flavescens* were reported to be effective against parasitic helminths (Terada et al., 1982). Feeding helminth-infected sheep with pyrethrins originating from *Pyrethrum cinerariifolium* was reported to be effective against sheep gastrointestinal nematodes (Mbaria et al., 1998). In the present study, we have demonstrated that removal of Timor C did not lead to parasite recovery, and moreover, parasites that were alive at the time of Timor C removal

continued to die (Fig. 2). It is likely that the active ingredients of Timor C that entered the parasite continued their detrimental effects.

At the effective Timor C concentrations of 10 ppm and 20 ppm, there were no adverse effects in exposed fish with up to five repeated treatment applications. Our results suggest that repeated weekly treatment application for at least three consecutive weeks does not adversely affect biofiltration. Unlike the viviparous gyrodactylids, oviparous monogenean species require repeated applications due to the presence of resistant eggs in the life cycle. Therefore, Timor C could be a good candidate for the treatment of oviparous monogenean species.

Farm-based trials in this study provided substantial support for the effective application of Timor C to control monogenean infection in guppies in large-scale commercial settings, as a single treatment resulted in a marked reduction in parasite numbers in exposed fish. Infection prevalence, despite significant reduction post-treatment, remained substantial (Table 1). Repeated treatments have often been shown to be necessary in the control of monogenean parasite infestations (Farmer et al., 2013). Appearing as safe to fish and to the biofiltration system in intensive fish production operations, Timor C warrants further research into its efficacy against additional monogenean parasites, as well as other groups of disease-causing agents in fish.

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Supplementary data

Supplementary material

Supplementary material

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Figure 1. *In vitro* exposure of *G. turnbulli* on tail fin clips to Timor C at 20, 50 and 100 ppm and 0 ppm (water) control. Parasite detachment (A) and death (B) are recorded over time. The letters a, b, c, and d denote significant differences ($p < 0.05$) between treatment groups.

Figure 2. Continued mortality of *G. turnbulli* following treatment with 60 ppm of Timor C for 1 h, 2 h and 3 h followed by the treatment's removal. Arrows indicate time of replacement of the Timor C solution with water the differently exposed groups. Water was used for negative control. ($n$ >10)

Figure 3. Time to onset of signs of toxicity and death in adult guppies, exposed to different concentrations of Timor C by immersion. At 0 and 20 ppm, there was no evident stress or death within 24 h (marked with upwards pointing arrows). (n = 10)

Figure 4. Changes in ammonia (a), nitrite (b) and nitrate (c) levels in aquaria that were subjected to weekly applications of 20 ppm of Timor C over five weeks, as compared to the control. At time 0, 10 ppm of ammonium chloride was added to each aquarium, and water quality was analysed 1, 4 and 8 days after application. Results are presented as averages ± SE. * denotes

significant differences ($p < 0.05$) between treatment and control aquarium at a specific time point.

Figure 5. The effect of treatment with Timor C on infection with *G. turnbulli* in guppies. A laboratory trial was performed in 1 L beakers. Treatment was applied at 10 and 20 ppm for 24 h. The numbers of parasites were counted on the caudal tail fin of each individual fish before and after treatment application. † indicates significant differences in parasite abundance before and after treatment application, within treatment groups; * indicates significant differences between treatment groups ($p < 0.05$). Inserted values in percentages indicate infection prevalence (i.e., % of infected fish) for the respective boxplot. ($n = 12$ fish per treatment group)

**Highlights**

- Timor C, a plant-based commercial insecticide was effective against *Gyrodactylus turnbulii* infection in guppies by bath treatment at concentrations ranging between 10 ppm and 20 ppm.
- *In vitro* studies revealed detachment of the parasite from the tail followed by its mortality.
- Timor C was found to be safe for fish at the treatment concentrations, as toxicity was evident only from 40 ppm, and did not adversely affect biofilter activity.
- A successful field trial using Timor C at a commercial ornamental fish farm is described.

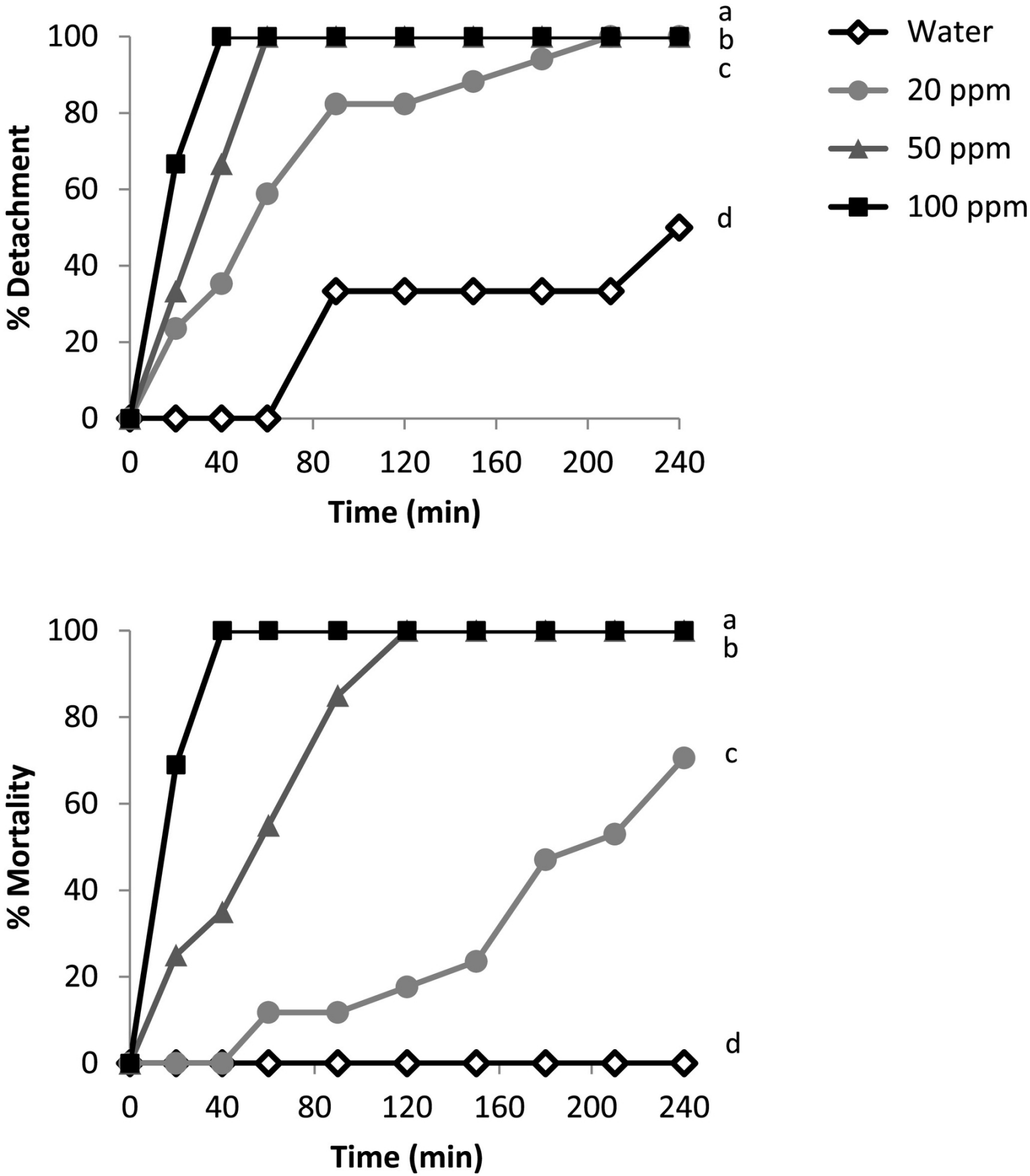

% Detachment
100
80
60
40
20
0
0
40
80
120
160
200
240
Time (min)
a
b
c
d
Water
20 ppm
50 ppm
100 ppm
% Mortality
100
80
60
40
20
0
0
40
80
120
160
200
240
Time (min)
a
b
c
d

Figure 1

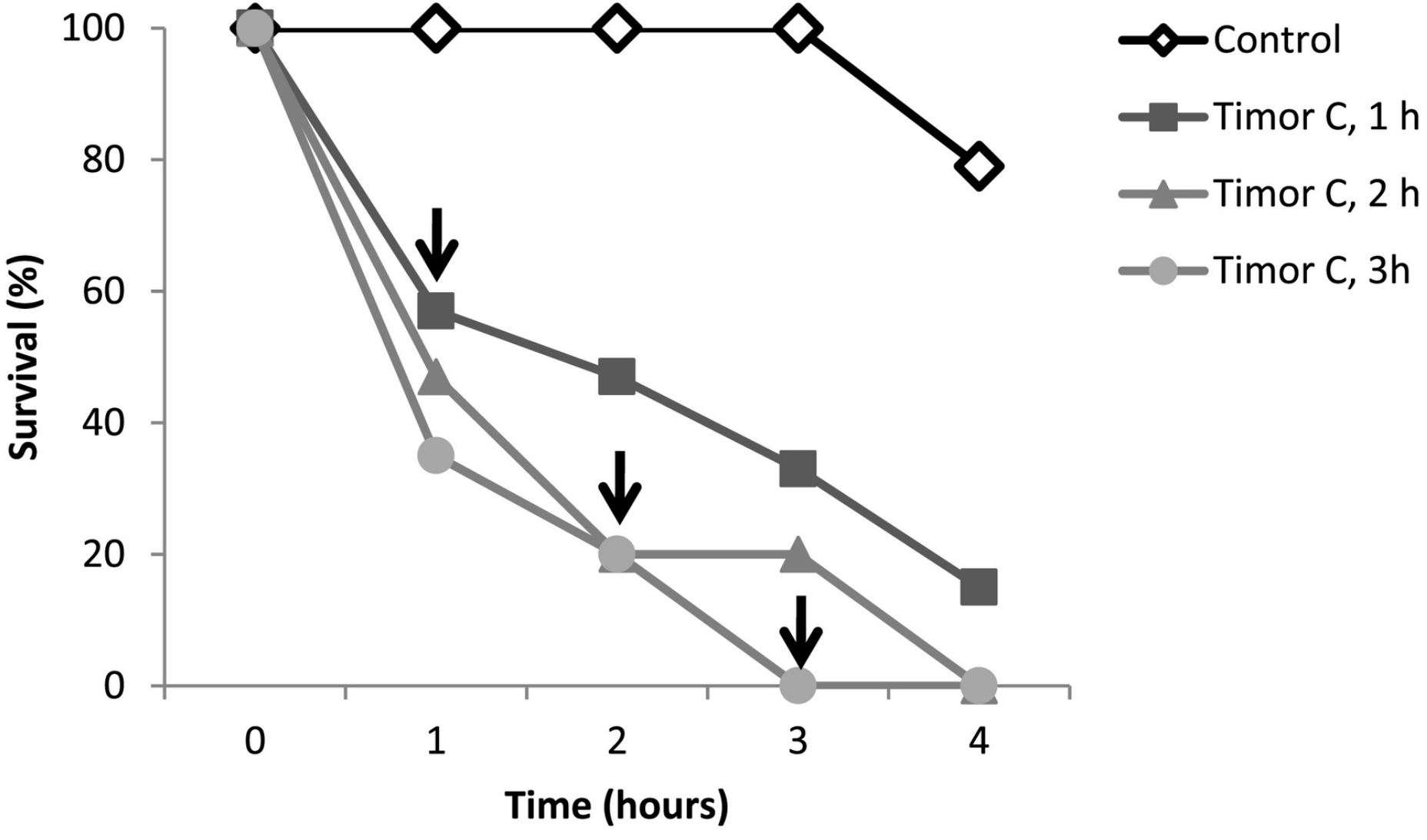

100
80
60
40
20
0
Survival (%)
0
1
2
3
4
Time (hours)
Control
Timor C, 1 h
Timor C, 2 h
Timor C, 3h

Figure 2

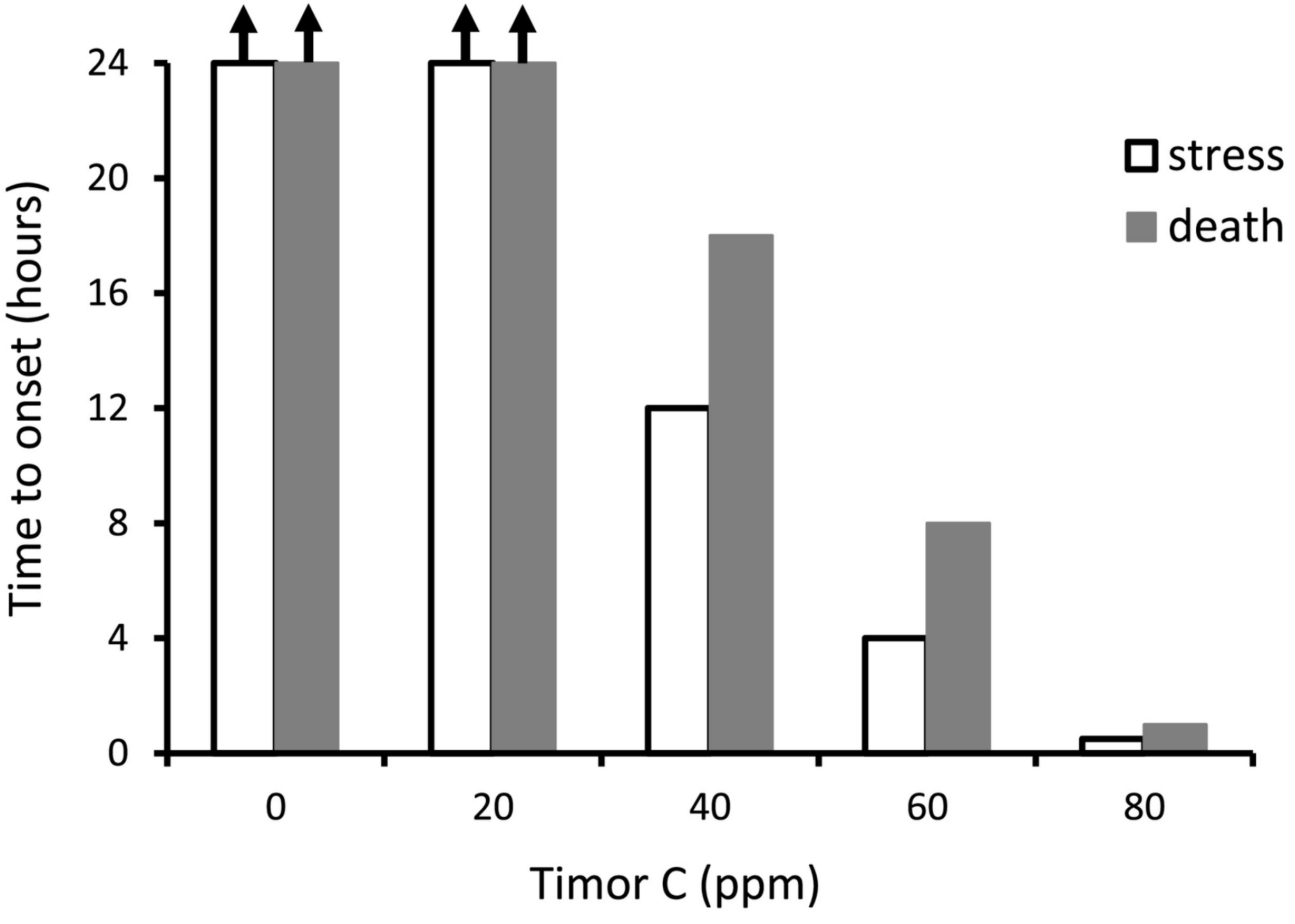
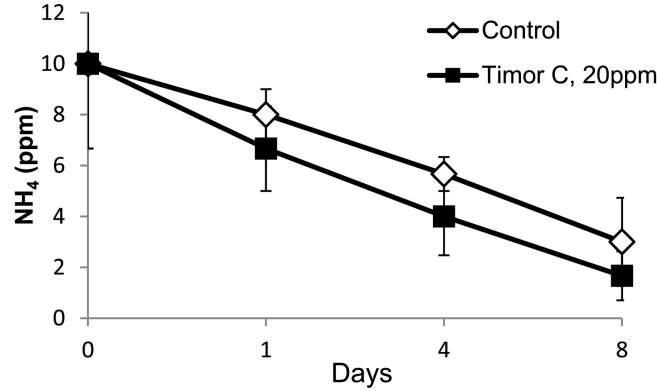
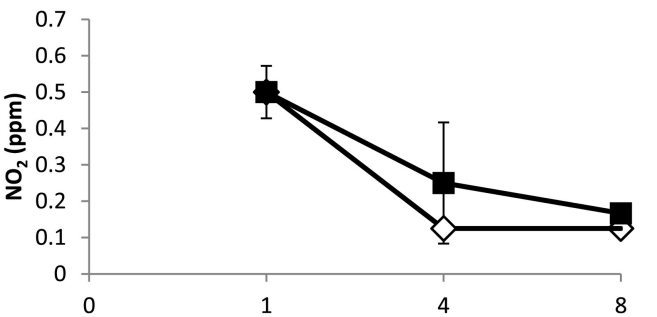

24
20
16
12
8
4
0
Time to onset (hours)
stress
death
0
20
40
60
80
Timor C (ppm)

Figure 3


Control
Timor C, 20ppm
NH4 (ppm)
12
10
8
6
4
2
0
0
1
4
8
Days


NO2 (ppm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
4
8

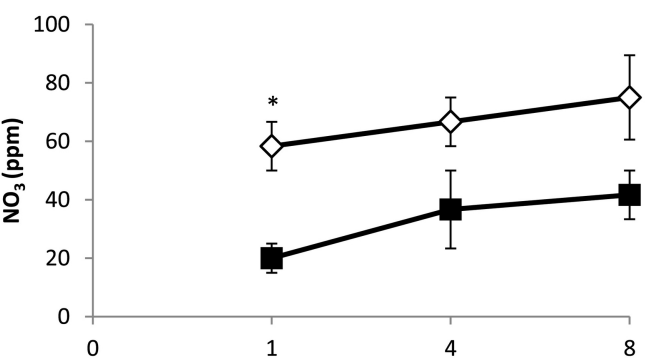

NO3 (ppm)
100
80
60
40
20
0
*
0
1
4
8

Figure 4

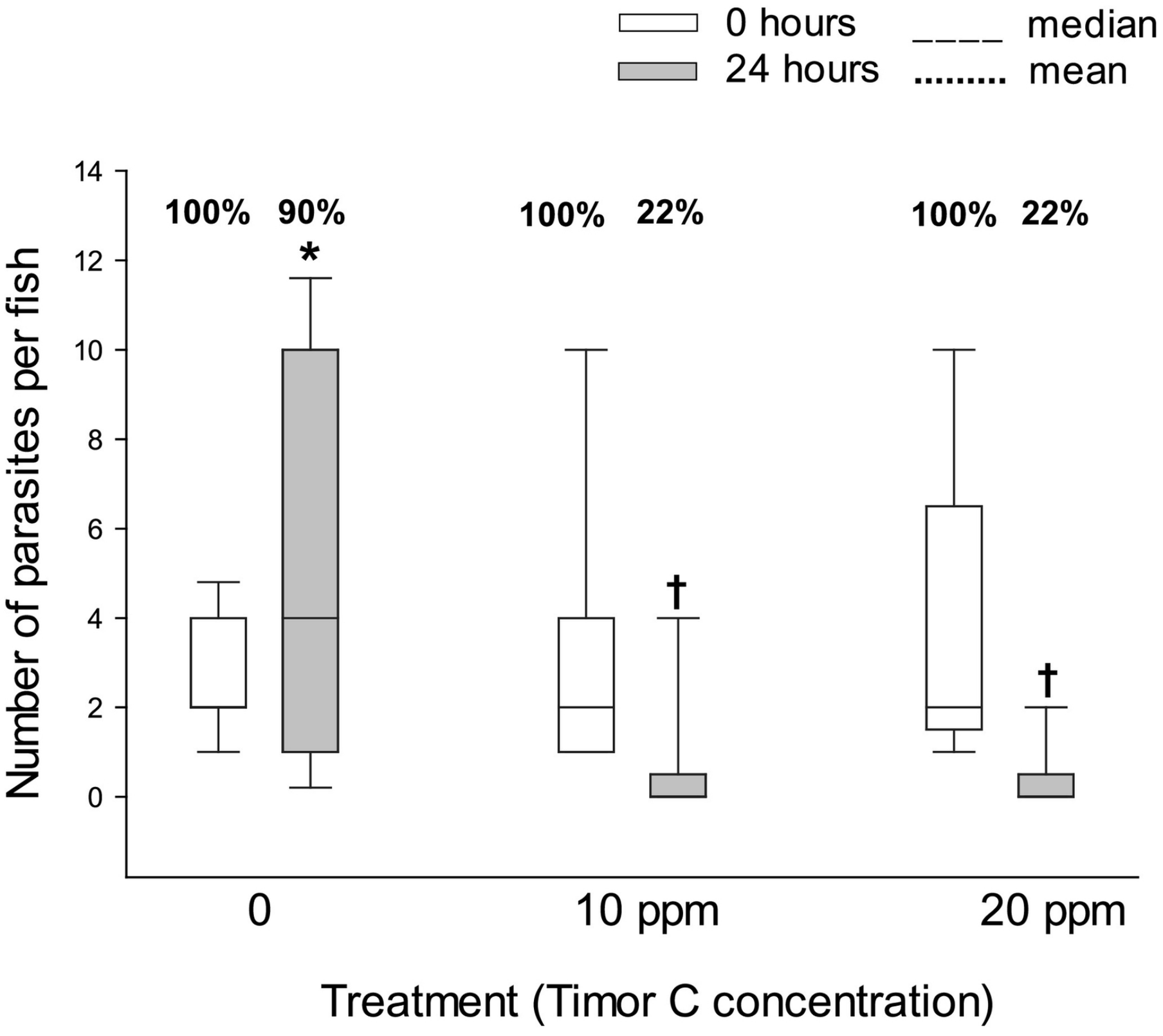

0 hours
24 hours
median
mean
Number of parasites per fish
14
12
10
8
6
4
2
0
100%
90%
*
100%
22%
†
100%
22%
†
0
10 ppm
20 ppm
Treatment (Timor C concentration)

Figure 5