Acute and sub-chronic toxicity of a trypsin-modulating oostatic factor (TMOF) on the growth, body composition and histopathology of red hybrid tilapia, *Oreochromis* sp. as a non-target organism

Nicholas Romano, Noor Mursyida binti Ayob, Nurul Amin, S.M. and Mohd Salleh Kamarudin

ABSTRACT

Trypsin-modulating oostatic factors (TMOF) have been shown to be an effective larvicide to mosquitos as a means of controlling their populations and spread of diseases, however there is limited information on its effects to fish, a non-target organism. Two experiments were performed to assess the acute and sub-chronic toxicity of a TMOF product to tilapia. For the acute toxicity test, treatments consisted of a control (0), 100, 500, 1000, 1500 and 2000 mg/L TMOF, which were triplicated with 10 fish/replicate according to the static renewal method. For the sub-chronic test, there were four triplicated treatments (50 fish/replicate) consisting of a control, TMOF at 60 mg/L, TMOF treated diets and a combination of these and the test was conducted for 35 days. After 35 days, growth performance, hepatosomatic index (HSI), vicersomatic index (VSI), whole-body proximate composition, and histopathology of the gills, liver and intestine were recorded. No LC_{50} values were obtained, even when using TMOF at saturated levels, within 96 hours. Meanwhile, after 35 days no significant differences in all of the measured parameters were detected among the treatments. Based on these findings, the TMOF product showed no acute or sub-chronic toxicity to tilapia and is safe to this non-target organism.

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INTRODUCTION

Mosquitos are vectors for major diseases such as dengue and malaria. To control mosquito populations, there is a high reliance on organophosphates, crabamates, and pyrethroids. Organophosphates and carbamates are known to inactivate acetylcholinesterase, which hydrolyzes the neurotransmitter acetycholine (O'Brien, 1976). However, these pesticides can also exert toxic effects to non-target organisms. For example, these chemicals were shown to cause histopathological liver damage to *Oreochromis niloticus* (Matos *et al.,* 2007) and *Oncorhynchus mykiss* (Banaee *et al.,* 2013), as well as inducing oxidative stress (Matos *et al.,* 2007; Banaee *et al.,* 2013). Meanwhile, pyrethroids have been shown to be highly toxic, even to a microgram scale, to non-target organisms (Sepici-Dinçel *et al.,* 2009; Karthigayani *et al.,* 2014). Moreover, research is showing increasing resistance of insects to these pesticides (Labbe *et al.,* 2007; Maestre-Serrano *et al.,* 2014), which can require higher amounts that can eventually enter various waterways and pose serious dangers to the environment and human health (Arjamandi *et al.,* 2010). Subsequently, there has been increasing interest in the use of potentially more environmentally friendly methods of controlling mosquito populations such as insect growth inhibitors (to inhibit chitin synthesis and thus molting) (Zaidi and Soltani, 2013) as well as trypsin-modulating oostatic factors (TMOF) (Shen *et al.,* 2009; Kamareddine *et al.,* 2013).

TMOF is a decapeptide hormone found in high concentrations within mosquito ovaries that inhibits trypsin biosynthesis which subsequently leads to starvation and death to mosquito larvae (Borovsky,

2003; Shen *et al.,* 2009). In one study, Borovsky (2003) deteremined the LC_{50} values of TMOF, when absorbed on yeast, to various mosquito specieswhich ranged from 0.38 to 1.05 mmol L^{-1} for *Anopheles quadrimaculatus* and *Culex quinquefasciatus*, respectively.

It was therefore ssuggested that TMOF could be an effective and environmentally friendly method for controlling mosquito populations (Borovsky, 2003).However, it has been reported that TMOF can distrupt the digestive enzymesin the cat flea *Ctenocephalides felis*, stable fly *Stomoxys calcitrans*, housefly *Musca domestica,* midge *Culicoides variipennis* (Borovsky et al., 1990;1993), well as to thefall webworm moth *Hyphantria cunea* and Lesser mulberry pyralid *Glyphodes pyloalis* (Ajamhassani *et al.,* 2011).On the other hand,the EPA (2004) reported that the presence of yeast containing TMOF in the water, at sufficient levels to kill mosquito larvae, did not kill the freshwater invertebrates, daphnids and mysids, or the eggs of the freshwater fathead minnow *Pimephales promelas*.

It is well known that tilapia *Oreochromis* spp. actively consume mosquito larvae, and have been used as one method to control mosquito populations (Howard *et al.,* 2007; Mbuya and Kateyo, 2014). However, to the best of our knowledge, there is no report on the acute toxicity of TMOF to tilapia or their sub-lethal effects on their histopathology. Acute toxicity experiments are often a first step to obtain lethal concentration $(LC)_{50}$ values, which can be compared with other animal and pollutant species as well as providing important information prior to conduting longer term sub-lethal experiments. Morevoer, by examining organ histopatholgy it may be possible to detect more subtle changes that may not necessarily be found when measuring survival, growth or some body indices.

The aim of this experiment was to determine the LC_{50} values of a TMOF product to tilapia, followed by a sub-chronic toxicity test to assess the effects of TMOF when added to the water, sprayed on diets or a combination of these to the survival, growth, body indices, whole-body proximate composition, and histopathology of the gills, liver and intestine of tilapia after 35 days.

MATERIALS AND METHODS

TMOF product

The TMOF product, called "Mousticide",was supplied by Entogenex Sdn Bhd, Malaysia, which was produced by extracting TMOF from the ovaries of mosquitoes and expressed in yeast cells along with*Bacillus thuringiensisisraliensis* (Bti) serotype H-14, to act as an entomopathogenic bacterium.This wettable powder was confirmed for their toxicity to mosquito larvae two months prior to conducting the experiments. This was done by exposing 25 mosquito, *Aedes aegypti*, third instar larvae at 0.25 and 0.50 mg/L and examining the number of deaths at 5, 10, 15, 30, 60, 120 and 180 min. It was shown that all mosquito died within 30 and 180 min., respectively.

Acute toxicity test

Tilapia fingerlings (3.8 ± 0.1 g) were obtained from the Puchong Aquaculture Experimental Station, UPM, and brought to Wet Laboratory, Department of Aquaculture, Faculty of Agriculture, UPM. The fish were acclimated in a 1,000 L fiberglass tank and fed a commercial tilapia diet for one week. After acclimation, a pilot study was conducted to range find the most appropriate concentrations which included, 0.5, 10, 50, 150, 300, 500 and 700 mg/L. No mortalities occurred within 24 hours and the acute toxicity test was then set up to consiste of higher concentrations.

There was a total of six treatments consisting of a control (0), 100, 500, 1000, 1500 and 2000 mg/L. Each treatment was triplicated with 10 fish (5.2 ± 0.1g) in each treatment according to the "static renewal method" following standard APHA (1985) methods. The tilapia were held in 12 L capacity clear plastic aquaria filled with 10 L of water and each aquarium received gentle aeration and the water source was from the city municipal. Prior to being used, sodium thiosulphate was added to neutralize any residual chlorine. The fish were starved 24 hours prior to conducting the test, and were also not fed throughout. After 96 hours, no fish died and appeared active and healthy. From the same container, the TMOF product was then tested on mosquito larvae and were confirmed to exhibit toxicity within several hours.

Sub-chronic toxicity test

A total of four treatments, which were triplicates, were set up at the Puchong Aquaculture

Experimental Station, UPM. The LC_{50} values were unable to be determine since no mortalities occurred within 96 hours at any of the concentrations, even when saturated, and therefore for the sub-chronic test a concentration of 60 mg/L was used. This value was based on a tenfold higher recommended dose of the TMOF product (6 mg/L) to control mosquito populations. Moreover, since tilapia may be exposed to this product through the water, ingestion of dead mosquito larvae or both, the treatments were set up to reflect these scenarios.

The four treatments consisted of a control (no TMOF), adding the TMOF product directly to the water at 60 mg/L, spraying to commercially available feed, or a combination of both. These treatments will hereafter be referred to as "control", "water", "feed" or "water/feed" treatments. For the feed treatment, a 100 ml solution at 60 mg/L TMOF was sprayed onto one kilogram of commercially available feed, allowed to air dry, and kept at -20°C. A combination of these two protocols were then used for the water/feed treatment, in which tilapia were exposed to TMOF in the water as well as when ingesting the treated diets. Each week, a new batch of feed was prepared.

In each treatment there was a total of 50 tilapia/tank, (4.71 ± 0.35g), yielding a total of 150 fish/treatment. All tilapia were stocked in 600 L rectangular fiberglass tanks and a biofilter was installed to each tank to minimize nitrogenous waste accumulation and maintain sufficient water quality. All tanks had individual aeration, and a commercial diet, specifically designed for tilapia, was fed to the fish twice/day at 4% body weight. Each week, a 100% water exchange was performed, to ensure sufficient water quality and efficacy of the TMOF product.

After 35 days, all fish were mildly anesthetized using clove oil and all were measured for their lengths and weights. Six fish from each treatment were then dissected and the liver and viscera were weighed to calculate the hepatosomatic index (HSI) and viscerosomatic index (VSI), respectively. The whole-body proximate composition was determined using standard AOAC methods (AOAC, 1997). The gills, liver and intestine (proximal and distal) from three tilapia were then fixed in 10% phosphate buffered formalin (v/v) for one day, and then 70% (v/v) ethanol until processing. The samples were then embedded in paraffin wax, sectioned (6 μM) and stained with haemotoxylin and eosin.

Data analysis

All data (survival, growth, whole-body proximate composition, condition factor, hepatosomatic index and viscerosomatic index) were subjected to a one-way ANOVA after prior confirmation of homogeneity of variance. If any significant differences were detected, a Tukey's post hoc test to determine any differences among treatments. Statistical analysis was performed using SPSS (version 16).

RESULTS AND DISCUSSIONS

Acute toxicity test

No mortalities of tilapia occurred during the range finding pilot test or during the acute toxicity test within 96hours, even when the TMOF product was added at saturated levels (up to 1,500 mg/L) and thus higher concentrations were not possible. Therefore LC_{50} values were unable to be computed.

Table 1. The mean (±SE) survival, growth performance, condition factor, hepatosomatic index (HSI) and viserosomatic index (VSI) of tilapia from the different TMOF treatments after 35 days.

Parameters	Control	Water	Feed	Water/feed
Initial weight (g)	4.41 ± 0.12	4.66 ± 0.07	5.69 ± 0.36	4.07 ± 0.44
Final weight (g)	19.40 ± 1.39	19.46 ± 0.45	21.42 ± 1.03	22.64 ± 1.35
SGR (%/day)	4.22 ± 0.28	4.09 ± 0.06	4.10 ± 0.27	4.98 ± 0.34
CF	1.90 ± 0.05	1.77 ± 0.05	1.89 ± 0.07	1.89 ± 0.11
Survival (%)	79.3 ± 0.6	79.3 ± 0.6	72.0 ± 1.1	74.0 ± 6.0
HSI	2.54 ± 0.16	2.59 ± 0.15	2.36 ± 0.20	2.46 ± 0.31
VSI	12.12 ± 0.39	10.61 ± 0.29	12.37 ± 0.35	11.61 ± 0.74

SGR-specific growth rate = (LN final weight – LN initial weight)/(35 days) × 100

CF-condition factor = (final weight/length^3) × 100

Sub-chronic test

Survival, growth and body indices

The survival of the tilapia ranged between 72 – 79% and no significant differences (df = 3; F = 3.314; P = 0.087) were detected among the treatments. In all treatments, the fish grew over four times in size after 35 days and growth was also not significantly different among treatments (df = 3; F = 0.393; P = 0.092). Similarly, no significant differences were detected for the CF(df = 3; F = 0.908; P = 0.484),HSI (df = 3; F = 0.136; P = 0.936), or VSI (df = 3; F = 1.687; P = 0.246) among the different treatments.

Whole-body proximate composition

The whole-body proximate composition of tilapia exposed to different TMOF treatments are presented in Table 2. No significant differences were detected among the treatments for crude protein (df = 3; F = 1.269; P = 0.349), crude lipid (df = 3; F = 0.691; P = 0.583), crude fiber (df = 3; F = 1.219; P = 0.364), crude ash (df = 3; F = 0.675; P = 0.594) or moisture (df = 3; F = 0.376; P = 0.773). No significant differences were detected among the treatments.

Table 2. The mean (±SE) of the whole-body proximate composition (% dry weight) of tilapia from the different TMOF treatments after 35 days.

	Control	Water	Feed	Water/feed
Protein	63.59 ± 3.02	62.26 ± 3.07	56.03 ± 4.89	65.83 ± 3.61
Lipid	18.65 ± 0.44	18.28 ± 1.22	19.30 ± 0.57	19.85 ± 0.89
Ash	15.72 ± 0.16	16.59 ± 0.76	15.72 ± 0.29	15.29 ± 1.41
Fiber	0.34 ± 0.02	0.31 ± 0.13	0.41 ± 0.02	0.55 ± 0.15
Moisture	95.59 ± 0.12	95.34 ± 0.43	95.13 ± 0.59	95.73 ± 0.28

Histopathology

The histopathology of the gills, liver and intestine appeared similar among all TMOF treatments. The gills were characterized by numerous secondary lamellae with occasional pillar cells, epithelial cells and a thin epithelium. For the liver, the predominate cells were hepatocytes which were circular or oval in shape and no abnormally shaped cells were noticed. Meanwhile, there was no evidence of water accumulation and the overall structure and tissue were intact. There were occasional blood vessels that generally appeared clear and uncongested with no evidence of any immunological response or concentration of blood cells. Finally, the intestine was characterized by smooth tissue attached to numerous intestinal folds. On the intestinal folds, there were numerous villi and globlet cells and no damage was noticed among any of the treatments.

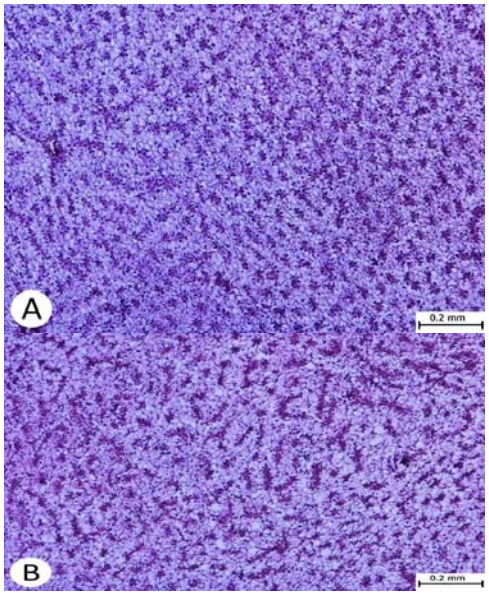

Fig. 1. Histopathology of liver from tilapia in the **(A)** control treatment and **(B)** "water/feed" treatment showing normal structure, no evidence of an immunological response, necrosis, excessive water accumulation or basophilic cells (H&E; × 10 magnification).

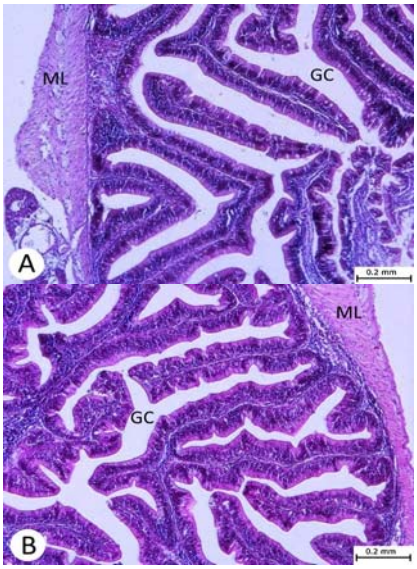

Fig. 2. Histopathological section of the proximal intestine from tilapia in the **(A)** control treatment and **(B)** "water/feed" treatment showing normal and intact mucous layer on the villi (arrowhead), presence of normal globlet cells (GC) and intake smooth muscle layer (ML) (H&E; × 10 magnification).

One of the first steps when assessing the toxicity of compounds or chemical is conducting acute toxicity tests, since this standardized methodology by APHA (1985) provides LC_{50} values that can be compared with other chemical or animal species as well as at different environmental conditions (Romano and Zeng, 2013). In the current experiment, it was not possible to calculate a LC_{50} point since no tilapia died within 96 hours, even when the TMOF product was added at saturated levels. Moreover, the tilapia appeared active and healthy throughout, despite the high turbidity of the treated water. This is unusual since most compounds and chemicals can become toxic at high levels, particularly when saturated. Nevertheless, the TMOF product was confirmed to being toxic to mosquito larvae prior to this test, as well as in our laboratory after the acute toxicity test was conducted.

Although a LC_{50} value was not obtained in the current experiment, a sub-chronic test was conducted to simulate possible modes of exposure that tilapia could encounter when TMOF is applied in the environment, namely in the water, being ingested or both. Moreover, the levels used were deliberately tenfold higher than the recommended dose to increase the chances of observing toxic effects. In addition, several sensitive stressor endpoints were measured including growth, condition factor, body indices, whole-body proximate composition and histopathology of various organs as potential parameters that would presumably become affected if the TMOF disrupted tilapia digestion.

However, in the current study, none of these parameters were affected by the different modes of TMOF delivery after 35 days indicating that the mode of action of TMOF was not specific to tilapia. For example, if the TMOF product disrupted digestion, the whole-body proximate composition would have likely changed, such as decreased protein levels. Meanwhile, over the course of this study, it was noticed that mosquito larvae were only present in the control and feed treatments, indicating that the TMOF product in the water and feed/water treatments were at sufficiently high concentrations to kill mosquito larvae. To the best of our knowledge, there is only one report on the toxicity of TMOF to fish, which is in agreement with this study. It was found that the hatching rate of the freshwater fathead minnow *Pimephales promelas* eggs were unaffected when TMOF containing yeast cells were added to the water at 1.038×10^6 cells/mL for 33 days (EPA, 2004).

The gills, liver and intestine of fish perform essential physiological functions and their histopathological examination have been suggested to be accurate bioindiators of pollutants and their associated toxic effects (Osman *et al.,* 2010).In the context of toxicology, the gills are particularly sensitive since they are delicate structures that are in constant contact with the water, while the liver is responsible detoxification and finally the intestine for the absorption of nutrients(Vicentini *et al.,* 2005). For example, exposure to as low as 0.25 to 0.5 mg/Lof diazinon or carbryl, caused histopathological liver damage and oxidative stress to Nile tilapia *Oreochromis niloticus* (Matos *et al.,* 2007) and rainbow trout *Oncorhynchus mykiss* (Banaee *et al.,* 2013), respectively. Some of histopathological alterations included necrosis (Matos *et al.,* 2007), hepatocyte hyperplasia and swelling (Banae *et al.,* 2013). Meanwhile, the pyrethroid pesticide, cypermethrin, at 0.008 ppm has been shown to cause histopathological damage to the liver and intestines of tilapia which included hepatocyte vacuolization, increased globlet cells and eventual disintegration of the hepatocytes and intestinal serosa, respectively (Karthigayani *et al.,* 2014). However, in the current study, there were no observable differences among the TMOF treatments, regardless if present in the water, ingested or both at tenfold the recommended levels.

To the best of our knowledge, this is the first experiment on the potential acute and sub-chronic toxicity of a TMOF product to fish. The results showed no acute toxicity of the TMOF product to tilapia, even when present at saturated levels. Similarly, various sensitive stressor endpoints during the sub-chronic test were unaffected including growth, body indices, whole-body proximate composition or histopathology of the gills, liver or intestine. Based on these results from the current study, the TMOF product was safe to tilapia if present in the water, ingested or both.

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Nicholas Romano, Noor Mursyida binti Ayob, S.M. Nurul Amin, and Mohd Salleh Kamarudin*
Department of Aquaculture, Faculty of Agriculture, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
*Corresponding author:
Tel.: +60 3 8947 4817; fax: +60 3 8947 4949
Email: msalleh@agri.upm.edu.my