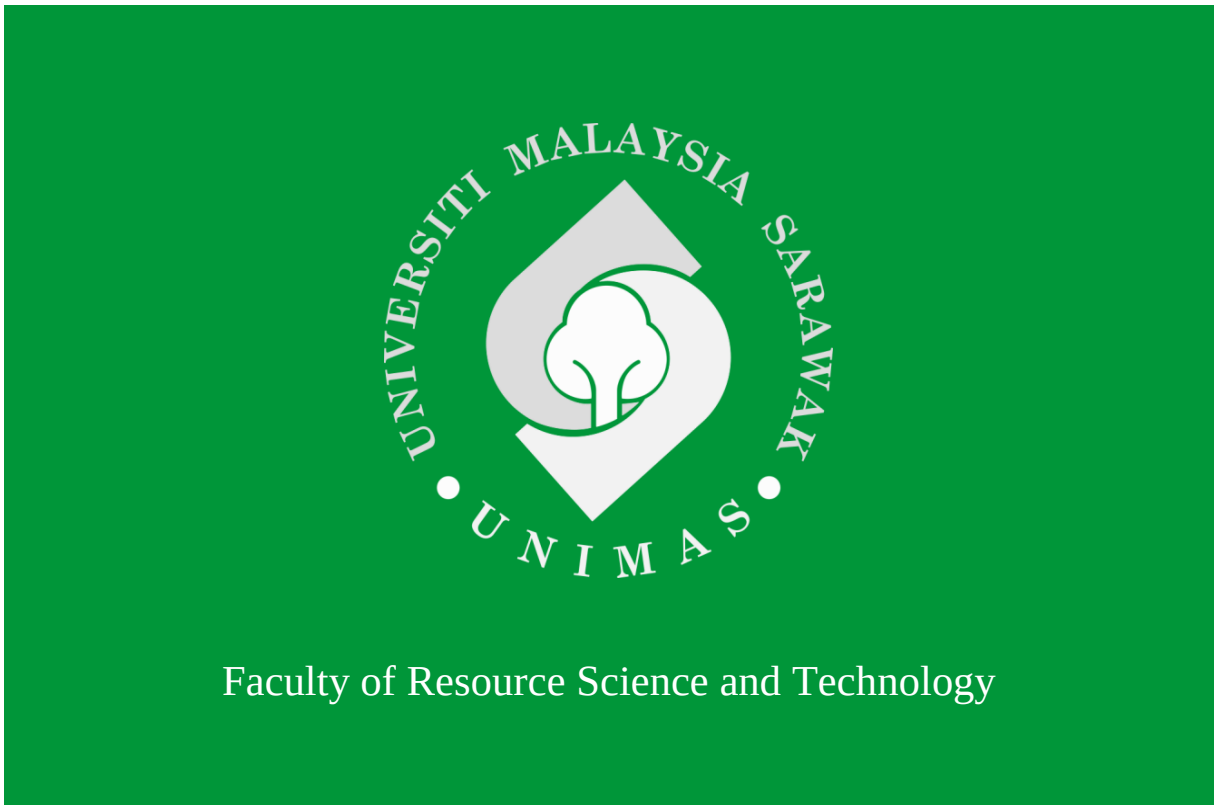

Effects of Selected Water Quality, Sulfide and Mercury on Juveniles *Barbonymus schwanenfeldii* and *Tor tambroides*

Azimah binti Apendi

Master of Science
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Effects of Selected Water Quality, Sulfide and Mercury on Juveniles *Barbonymus schwanenfeldii* and *Tor tambroides*

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The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Alterations of dissolved oxygen (DO), total suspended solids (TSS), temperature and pH could affect the growth and survival of fishes. Hydrogen sulfide and mercury, both under high concentrations are toxic and lethal to the aquatic organisms. In Sarawak, creation of new hydroelectric reservoirs results in changes in DO, TSS, temperature and pH with increasing sulfide and mercury. Freshwater fish species, *Barbonymus schwanenfeldii* and *Tor tambroides* have important economical values and act as a protein source to human. Both species require an optimum condition to survive in the nature and the changes in natural aquatic habitat due to anthropogenic activities lead to the reduced quantity and quality of these two indigenous species. This study aimed to determine the mortality and behavioral responses of both species to changes in water quality parameters, changes in the level of hydrogen sulfide and exposure to mercury in water and feed. In the water quality experiment, both species were exposed to 25 different combinations of temperature, TSS, pH and DO at three different levels, in 30 days' period. Response surface methodology was used to optimize the best set of condition for both species to survive. The results of this study show that avoidance when fed and longer feeding time were observed by both species in experiments with TSS higher than 1000 mg/L. Suspended solids in water reduced the visibility of fishes and affected their feeding activity. *B. schwanenfeldii* was predicted to survive the best under the combination of 27 °C, 0 mg/L TSS, 4.8 mg/L DO and pH 7.04 whereas the best set of condition for *T. tambroides* to survive are 23.9 °C, 0 mg/L TSS, 4.5 mg/L DO and pH 7.02. Sulfide tolerance was determined in 15 L containers with freshwater (100 mL/min) and sulfide stock solutions (5 mL/min) supplied. Methylene blue method was used to analyze the water for total sulfide concentration which was used to plot LC_{50}. Four behavioral responses were observed namely, huddling together, aquatic surface respiration,

loss of equilibrium and turned upside down. The responses were observed earlier for higher sulfide concentration compared to the lower sulfide concentrations. The LC_{50} of *B. schwanenfeldii* at 6 h was found to be 507.8 µg/L at 95% confidence level whereas for *T. tambroides* was 306.1 µg/L at 95% confidence level. Under this condition, the fish reached 100% mortality as early as 6 h at concentration 659 ± 39 µg/L and 50% mortality was earliest at 5 h at the same concentration. Lowering pH and dissolved oxygen levels were proven to aggravate sulfide toxicity. The adsorption of mercury through water was determined by exposing the fishes to mercury-spiked water while for adsorption through feed was done by feeding with mercury-spiked feed. Both experiments were carried out in 30 days. The concentrations of mercury in fish was determined by microwave assisted digestion then analyzed by mercury analyzer. In the mercury toxicity experiment, both *B. schwanenfeldii* and *T. tambroides* exhibited avoidance when fed and time taken to feed was also longer. Exposure of mercury through water exhibited higher survival rate for both species compared to feed exposure. However, the mercury concentration in both species' tissue were higher in water exposure than feed exposure experiment. In general, the experiments carried out show that changes in water quality parameters, hydrogen sulfide levels and exposure to mercury affected the mortality and behavioral responses of both species.

Keywords: Indigenous species, fish survival, behavioral response, sulfide tolerance, mercury toxicity.

***Kesan Kualiti Air Terpilih dan Merkuri terhadap Juvana* Barbonymus Schwanenfeldii *dan* Tor Tambroides**

ABSTRAK

Perubahan oksigen terlarut (DO), jumlah pepejal terampai (TSS), suhu dan pH boleh menjejaskan pertumbuhan dan kemandirian ikan. Kepekatan sulfida dan merkuri yang tinggi adalah toksik dan boleh membawa maut kepada kehidupan akuatik. Di Sarawak, pertambahan bilangan takungan hidroelektrik menyebabkan perubahan dalam aras DO, TSS, suhu dan pH sekaligus meningkatkan sulfida dan merkuri. Ikan air tawar, Barbonymus schwanenfeldii *dan* Tor tambroides *mempunyai nilai ekonomi yang penting dan merupakan sumber protein. Kedua-dua spesies memerlukan keadaan yang optimum untuk terus hidup, namun perubahan habitat akuatik semulajadi disebabkan aktiviti antropogenik telah menyebabkan pengurangan kuantiti dan kualiti spesies ini. Kajian ini bertujuan untuk menentukan tahap kematian dan tindakbalas tingkah laku kedua-dua spesies berdasarkan perubahan parameter kualiti air, perubahan sulfida dan pendedahan kepada merkuri dalam air dan makanan. Dalam eksperimen kualiti air, kedua-dua spesies didedahkan kepada 25 kombinasi suhu, TSS, pH dan DO pada tiga tahap berbeza, dalam tempoh 30 hari. Metodologi permukaan respons digunakan untuk mengoptimumkan keadaan terbaik untuk terus hidup. Keputusan kajian menunjukkan kedua-dua spesies mengelakkan makanan dan mengambil masa yang lebih lama untuk makan bila dalam keadaan TSS melebihi 1000 mg/L. Pepejal terampai dalam air mengurangkan penglihatan ikan dan menjejaskan aktiviti makan mereka.* B. schwanenfeldii *dijangka hidup dengan terbaik dalam kombinasi 27 ° C, 0 mg/L TSS, 4.8 mg/L DO dan pH 7.04 manakala keadaan terbaik untuk* T. tambroides *hidup adalah 23.9 ° C, 0 mg/L TSS, 4.5 mg/L DO dan pH 7.02. Toleransi terhadap sulfida ditentukan di dalam bekas 15 L dengan pengaliran air tawar 100 mL/min dan larutan stok*

sulfida 5 mL/min yang dibekalkan dengan kadar semakin meningkat. Kaedah metilen biru digunakan untuk menganalisis kepekatan jumlah sulfida yang digunakan untuk plot LC_{50}. Empat tindakbalas tingkah laku diperhatikan iaitu, berkumpul bersama, pernafasan permukaan akuatik, ketidakseimbangan dan terbalik. Tindakbalas yang lebih awal telah diperhatikan bagi kepekatan sulfida yang lebih tinggi. LC_{50} untuk B. schwanenfeldii *pada 6 jam berlaku pada kepekatan 507.8 µg/L manakala untuk* T. tambroides *ialah 306.1 µg/L pada tahap keyakinan 95%. Dalam keadaan ini, ikan mencapai kematian 100% seawal 6 jam pada kepekatan 659 ± 39 µg/L dan kematian 50% paling awal pada 5 jam pada kepekatan yang sama. Penurunan pH dan oksigen terlarut terbukti menambahkan ketoksikan sulfida. Pendedahan merkuri melalui air ditentukan dengan mendedahkan ikan kepada air yang ditambahkan merkuri manakala pendedahan melalui makanan dilakukan dengan memberi makanan yang telah dicampurkan merkuri. Kedua-dua eksperimen ini dijalankan dalam tempoh 30 hari. Kepekatan merkuri dalam ikan telah ditentukan oleh pencernaan mikrogelombang diikuti dengan analisa merkuri. Dalam eksperimen ketoksikan merkuri, kedua-dua spesies didapati mempamerkan pengelakan apabila diberi makan dan masa yang diambil untuk makan juga bertambah. Pendedahan kepada merkuri melalui air menghasilkan kadar kemandirian yang lebih tinggi bagi kedua-dua spesies berbanding pendedahan kepada makanan. Kepekatan merkuri di dalam tisu adalah lebih tinggi bagi eksperimen pendedahan kepada merkuri di dalam air daripada pendedahan melalui makanan bagi kedua-dua spesies. Secara amnya, semua eksperimen yang telah dijalankan menunjukkan perubahan parameter kualiti air, aras hidrogen sulfida dan pendedahan kepada merkuri mempengaruhi kematian dan respons tingkah laku kedua-dua spesies.*

***Kata kunci**: Spesies asli, kemandirian ikan, tindakbalas tingkahlaku, toleransi sulfida, ketoksikan merkuri.*

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LIST OF ABBREVIATIONS

%	Percentage
µg/L	Microgram per litre
µM	Micromolar
ANOVA	Analysis of variance
CCD	Central composite design
DO	Dissolved oxygen
FCR	Feed conversion ratio
Hg	Mercury
H_2S	Hydrogen sulfide
LC_{50}	Lethal concentration 50 percent
meHg	Methylmercury
mg/L	Milligram per litre
pH	Potential of hydrogen
PVC	Polyvinyl Chloride
RSM	Response surface methodology
SGR	Specific growth rate
SL	Standard length
SR	Survival rate
TL	Total length
TSS	Total suspended solids
WG	Weight gained

CHAPTER 1

INTRODUCTION

1.1 Research Background

Reservoir construction fragmented the rivers and become an obstacle for longitudinal exchanges such as nutrient recycling, water chemistry and migration (Brismar, 2004; Mc Cartney, 2009). This causes the reservoirs to become lentic thus enabling it to become stratified which can lead to changes in water chemistry, and accumulation of mercury and hydrogen sulfide. Changes in physical and chemical water quality parameters may influence the population of aquatic organisms by affecting the survival rate, growth, distribution and reproduction (Au *et al*., 2004). However, each species may respond differently to changes in water quality at different developmental stages (Ivoke *et al*., 2007).

Water quality parameters such as temperature, total suspended solids, pH and dissolved oxygen play an important role in maintaining an optimum habitat for aquatic organisms. The fluctuation of temperature can be influenced by the removal of trees along the banks, release of chemicals into water column and construction such as reservoir (Lessard and Hayes, 2002). Besides that, eutrophication and decaying process in an aquatic ecosystem will lead to a decline in the dissolved oxygen in the water column. An increase in total suspended solids is associated with the increase of turbidity in water which will affect the feeding rate of fish.

Decomposition of bacteria in the reservoir will convert inorganic mercury into toxic methylmercury. Methylation is carried out by sulfate-reducing bacteria which is usually present in deep and poorly-oxygenated water (Mason *et al*., 2000). Methylmercury is mainly accumulated in fish through the food chain and will harm human who consumes it. Hydrogen

sulfide is toxic to both human and aquatic life. It is introduced into the aquatic system through runoff from terrestrial (sewage treatment plants and manure-handling operations) or from the decaying process of organic materials in a confined place (Guidotti, 1996).

Following the impoundment of Batang Ai Hydroelectric Reservoir in 1985, Sarawak witnessed an increasing number of hydroelectric reservoir constructed namely Bakun Hydroelectric Reservoir in 2010 and Murum Hydroelectric Reservoir in 2014 (Nyanti *et al.,* 2012a; Ling *et al.,* 2013). Reservoirs caused fragmented rivers and become an obstacle for longitudinal exchanges such as nutrient recycling, water chemistry and migration (Brismar, 2004; Mc Cartney, 2009). This causes the reservoirs to become lentic and stratified, enriched with organic matter with depriving oxygen level that leads to production of hydrogen sulfide.

Barbonymus schwanenfeldii and *Tor tambroides* are two indigenous species in Sarawak freshwater (Ingram, 2005; Froese and Pauly, 2015). Both species are economically important as protein source and culturally important to the region (FAO, 2006). It is crucial to understand the behavior of these species including the survival, growth rate and behavioral characteristics under different water quality conditions and different concentrations of hydrogen sulfide and mercury.

Changes in water bodies such as the creation of reservoirs due to impoundment of rivers commonly lead to changes in water quality with the production of hydrogen sulfide and the mobilization of mercury. Some studies have reported on the increase of sulfide and mercury levels in the reservoirs due to the stratification. Increasing the levels of these two parameters could be related to the altered levels of DO, pH, TSS and temperatures in the water column. The deterioration of water quality levels affects fish physiology and growth (Wan Maznah *et al.,* 2014). In general, reservoirs struggle with resource degradation such as fish

assemblages and cause negative impacts on the local communities (Ambak and Jalal, 2006). Thus, the outcome of this study could be beneficial to Sarawak freshwater in both conservation and economic activities such as aquaculture.

What are the effects of these selected parameters on the survival of the juveniles? Were the juveniles able to tolerate altered levels of these parameters? Thus, the aim of this study was to investigate the effect of combined water quality parameters, changing levels of hydrogen sulfide and mercury exposure on the survival and mortality of selected indigenous fish species.

1.2 Specific Objectives

The objectives of this study were to:

i. determine the optimum combination of DO, pH, TSS and temperature for the survival and growth of *B. schwanenfeldii* and *T. tambroides,*
ii. determine the sulfide tolerance of both *B. schwanenfeldii* and *T. tambroides* under different sulfide concentrations with lowering DO and pH, and
iii. determine the mercury accumulation of juveniles *B. schwanenfeldii* and *T. tambroides* through exposure in water and feed.

CHAPTER 2

LITERATURE REVIEW

2.1 Importance of *Barbonymus schwanenfeldii* and *Tor tambroides*

Barbonymus schwanenfeldii or tinfoil barb is known locally as Tengadak. The species is a freshwater fish found in lakes and rivers and is distributed widely in Asia including Borneo, Sumatra and Peninsular Malaysia (Froese and Pauly, 2015). According to Mat Isa *et al*. (2012), *B. schwanenfeldii* breeds twice in 15 months and migrates upstream to release their eggs. The species is economically important as a protein source, and can be found abundantly in lakes, reservoirs, streams, canals and rivers (Mat Isa *et al*., 2012; Froese and Pauly, 2015). The fast reproduction rate of *B. schwanenfeldii* allows it to be cultured for example in Mekong River (FAO, 2006). Apart from that, it is also used as an aquarium fish and occasionally used as baits.

Mahseer *Tor tambroides* (Bleeker) locally known as Empurau or Kelah is culturally significant and possess economic importance in Sarawak (Ingram, 2005). The species is highly priced, valuable and sought after freshwater fish for both game and food fish in the region. It is known to inhabit clear and swift flowing waters with stony, pebbly or rocky bottoms and with availability of riverine fruits from growing trees by the banks (Ingram, 2005; Soon *et al*., 2014). *T. tambroides* has been known to migrate upstream during high flood period to spawn and downstream for feeding during the low-flow period (Ingram, 2005). However, development activities such as deforestation and reservoir construction as well as aquaculture practice and overfishing has threatened the availability of the species (Soon *et al.*, 2014). Such activities will deteriorate the environmental conditions, destroying the habitat of the fish. In Sarawak, its wild population have undergone declines in both abundance and distribution due to degradation of habitat and overfishing. Due to their

importance and value, a breeding program was established for conservation and aquaculture purposes by the Department of Agriculture Sarawak (Ingram *et al.*, 2007).

2.2 Influence of Total Suspended Solids (TSS) on Fish

In a hydroelectric reservoir, the regular fluctuation of large amount of water and reduction in sediment loads resulted in erosion of banks and floodplains where the sediment is deposited into the water (McCartney, 2009). High concentrations of total suspended solids (TSS) can lead to adverse effects such as clogging of gills, reduction in feeding rates, coughing, reduction in tolerance to disease and eventually death (Au *et al.*, 2004). Clogging of gills can lead to reduced oxygen supply as it interrupts with the respiration process. Suspended solids can cause variety of responses from fishes because many attributes of the physical environment are affected. High level of suspended solids lead to increase of turbidity of water thus reducing the visibility of food and reduction of light penetration which affect the primary production (Wood and Armitage, 1997). This explains the reduced feeding behavior in fish. However, some species such as *Alepes djedaba* and *Pranesus ogilbyi* thrive in turbid waters to reduce the risk of predation (Blaber and Blaber, 1980).

2.3 Influence of pH on Fish

Fluctuation of pH in water can lead to a diverse effect in aquatic organisms especially those of low tolerance. pH is important in the modulation of enzyme activity under physiological and pathological conditions in fish (Gorren *et al*., 1998). A study by El-Sherif and El Feky (2009) on the performance of tilapia *Oreochromis niloticus* in relation to pH shows that the decrease in pH caused the decrease in feeding rate thus leading to a fall in body weight. Both the lowest body weight and average feed consumption were recorded at pH 6 while the most optimum pH for the growth of tilapia is pH 8 and 9. The study on *Cyprinus carpio* L. by

Heydarnejad (2012) demonstrated that the species was able to withstand low pH (< 7.5) but the results showed greater increase in weight and length at pH levels 7.5 and 8.0. The species however, were not able to withstand alkaline condition (pH 9.0) likely due to a decrease in ammonia excretion and an increase in ion loss. High pH also has adverse effect on fish such as gill damage, reduced concentration of plasma ion and an increase in plasma ammonia (Lease *et al*., 2003).

2.4 Influence of Temperature on Fish

Impoundment of reservoir can change the physical and chemical properties including water temperature (Lessard and Hayes, 2002). Heat is stored in deep stagnant water of reservoirs leading to stratification in such column (McCartney, 2009). In a study done by Nyanti *et al.* (2012b) on the physico-chemical characteristics in Bakun reservoir, it was shown that the thermocline decreases as it gets further from the reservoir which might be due to inflow from upstream. Freshwater fishes are ectotherms, meaning that their body temperature is determined by the surrounding water temperature (Ficke *et al.,* 2007). Since different species possess different tolerance to ranges of temperature, fluctuation in temperature is a big concern to the ecology.

Temperature is closely related to the feeding rates and metabolism of fish because, an increase in metabolic rate is followed by an increase in feeding rate so as to maintain growth and survival rates (Lessard and Hayes, 2002). The tolerance of fishes toward temperature changes are species-specific in which stenothermal species are tolerant to narrow thermal range whereas eurythermal species are able to withstand wider thermal ranges (Ficke and Myrick, 2007). Alteration in temperature also affects the swimming performance of fish due to the changes in water viscosity (Fuiman and Batty, 1997). Besides that, temperature also

acts as an important factor in sex determination in selected species such as *Dicentrarchus labrax* and *Ictalurus punctatus*, which may produce female-biased sex ratios when in high temperature, and male-biased in low temperature (Baroiller and Cotta, 2001). According to Jobling (1981), the definition of the optimum temperature for growth is the temperature at which growth rate is the highest when the species are reared under conditions of maximum, or excess feeding.

2.5 Influence of Dissolved Oxygen (DO) on Fish

The definition of hypoxia varies among studies. Some authors indicate that hypoxia occurs when the level of DO drops below survival levels, commonly thought to be at 2.0 mg/L or less (Chesney *et al.*, 2000). However, others use hypoxia to describe conditions where DO is higher, including 28% saturation levels (Robb and Abrahams, 2003; Timmerman and Chapman 2004a, 2004b; Hattinnk *et al*., 2005). Hypoxia is a common phenomenon in stagnant water, with absence of currents and convections to introduce oxygenated water, and especially at night, when plants do not photosynthesize (Nilsson and Östlund-Nilsson, 2008). Consequently, hypoxia is regularly encountered by fish living in tropical freshwater habitats, in tide pools and even on coral reefs (Val *et al*., 2006).

Low level of dissolved oxygen is common in aquatic ecosystems with nutrient loadings and is seasonally stratified (Breitburg *et al*., 1997). The construction of reservoirs will lead to the formation of stratification in the water column where the concentration of dissolved oxygen is usually low at the bottom. The presence of thermal stratification lowers the rate of exchange between epilimnion and hypolimnion, thus resulting in the decrease of dissolved oxygen with depth (Ling *et al*., 2013). According to Ficke *et al*. (2007), oxygen solubility is related to changes in water temperature in which oxygen level is higher in lower water

temperature. Since reservoir allows heat storage in the water column, it affects the solubility of oxygen near to the surface of the water column.

Nyanti *et al*. (2012b) reported that around fifteen months after the reservoir had been filled, the dissolved oxygen at subsurface in Bakun reservoir dropped rapidly at 1-4 m depths into anoxic conditions. Besides that, the decomposition of matter such as submerged vegetation at the bottom of the reservoir also depletes the level of dissolved oxygen (Chapman, 1996). Limited level of dissolved oxygen in the water can result in behavioral changes such as gulping for air at the water surface and an increase of gill movement in order to let more water volume pass across gills (Dean and Richardson, 1999). The acceptable dissolved oxygen concentration for most aquatic organisms is 5 mg/L (Stickney, 2000).

Besides stratification in impounded reservoirs, other natural phenomenon such as seasonality (photoperiod and temperature) and anthropogenic input of nutrients and organic matter could also cause low DO or hypoxia in water (Pollock *et al.*, 2007). Schooling, avoidance and changes in reproductive behavior of fishes had been associated with low DO levels (Pollock *et al.*, 2007).

2.6 Influence of Hydrogen Sulfide (H_2S) on Fish

The oxidation of sulfide into sulfuric acid can cause decrease of pH values in reservoirs (Nyanti *et al.*, 2012b). Hydrogen sulfide emits rotten egg smell and is toxic to the environment and living organisms in high concentration. As sulfide is present in low concentrations in the atmosphere, most living organisms are able to withstand it to some extent (Tobler *et al*., 2011). In a lentic reservoir, the level of hydrogen sulfide can go up very high exceeding the tolerance level of certain species and bring adverse effect to it. H_2S binds to haemoglobin by replacing oxygen (Tobler *et al*., 2006), interacts with crucial enzymes

(Affonso *et al*., 2002) and disrupts the disulphide bonds in macromolecules (Guidotti, 1996). Frequently respiratory adaptations are used in surviving sulfidic environment such as enlarged gill surface and aquatic surface respiration (Plath *et al*., 2007). In a study done by Affonso (2000), *Colossoma macropomum* forms a funnel by swelling up its lower lip to allow more oxygenated surface water passing through the gills. This mechanism is similar to the mechanism used by the species under hypoxic condition. This is because supply of oxygen is needed to detoxify hydrogen sulfide, given dissolved oxygen is limited under sulfidic environment due to the reaction between sulfide and oxygen (Tobler *et al*., 2011).

2.7 Influence of Mercury on Fish

Heavy metals usage has increased over the past decades which lead to the rise of metallic substances in the aquatic system (Yang and Rose, 2003). According to Chan *et al*. (2003), methylmercury is the only type of mercury that can be bioaccumulated in aquatic organisms and biomagnified through the trophic chains. According to US Environmental Protection Agency (1997), the recommended safe daily intake level of meHg is 0.1 µg/kg body weight. The level of mercury in piscivorous fish is higher than non piscivorous fish due to bioaccumulation (Da Silva *et al*., 2005). This is supported by a study done by Sim *et al.* (2014) where the average concentration of mercury is recorded highest in *Hemibagrus planiceps*, which is a piscivorous fish that feeds on smaller fishes. The concentration of mercury in fish is dependent on the rate of fish consumption. An example of heavy metal pollution is the Minamata disease which was discovered in 1956. It is one of the serious cases resulting from the discharge of wastewater containing mercury from industrial plant which lead to fish kill (Harada, 1995). This outbreak shows that the poisoning of methylmercury is focused on the nervous system as the primary target (Dolbec *et al*., 2000).

The bioaccumulation of mercury into fishes or aquatic organisms could be magnified as it moved up the trophic levels (Chan *et al.*, 2003).

CHAPTER 3

EFFECTS OF WATER QUALITY PARAMETERS ON JUVENILES *BARBONYMUS SCHWANENFELDII* AND *TOR TAMBROIDES*

3.1 Introduction

Over the years, cases of fish mortality due to alteration in water quality parameters had increased greatly. Changes in water quality parameters are known to affect the inhabitants of the water column including river, sea and lake (Lessard and Hayes, 2002). In this chapter both *B. schwanenfeldii* and *T. tambroides* were tested against four water quality parameters namely, dissolved oxygen (DO), total suspended solids (TSS), temperature and pH.

One of the worst effects of poor water qualities to freshwater fishes is fishkill. The phenomenon was described as sudden death of large amounts of aquatic animals over a short period of time and is not caused by the fishes' natural life cycle or predation (La and Cooke, 2011). Fishkill incidents related to poor water qualities had been reported by several studies such as fishkill in Malacca River, Malaysia due to low oxygen level (Suriyanti *et al.*, 2017) and fish kill in Philippines lakes caused by algal bloom and temperature rise leading to oxygen depletion (Jacinto, 2011). Most of the incidents were caused by alteration in temperature, total suspended solids and pH which lead to depletion of dissolved oxygen. Apart from that, studies on the responses of fish to dissolved oxygen had been studied in a controlled lab experiments (Dean and Richardson, 1999).

Previous studies had been carried out in a controlled laboratory experiment to study the on the effects of DO, pH, temperature and TSS on the survival and growth of *B. schwanenfeldii* (Nyanti *et al.*, 2017; 2018). In those studies, only one factor was considered at a time. In this study, the survival and growth of *B. schwanenfeldii* and *T. tambroides* under the effects of

the four water quality parameters were evaluated based on central composite design, of four factors which are the water quality parameters DO, TSS, temperature and pH.

The present experiment aimed, 1) to determine the optimum dissolved oxygen, pH, total suspended solids and temperature for the survival and growth of juveniles of *B. schwanenfeldii* and *T. tambroides,* and 2) to determine the effect of changes in tested parameters on the behavioral response of juveniles of the two species.

3.2 Materials and Methods

3.2.1 Tank Preparation

Thirty rectangular tanks of equal dimensions were used in this study. Each tank had a volume of 1.67 m^3 with the dimension of 2.10 m long × 1.30 m wide × 0.61 m depth (Figure 3.1). In each tank, three rows of smaller compartments were placed at equal distance from the edge of the tank and among each row. Each row was subdivided into three equal compartments which were made from PVC (frame) and net with length of 0.40 m, width of 0.40 m and depth of 0.61 m. Thus, the volume of each compartment was 0.09 m^3.

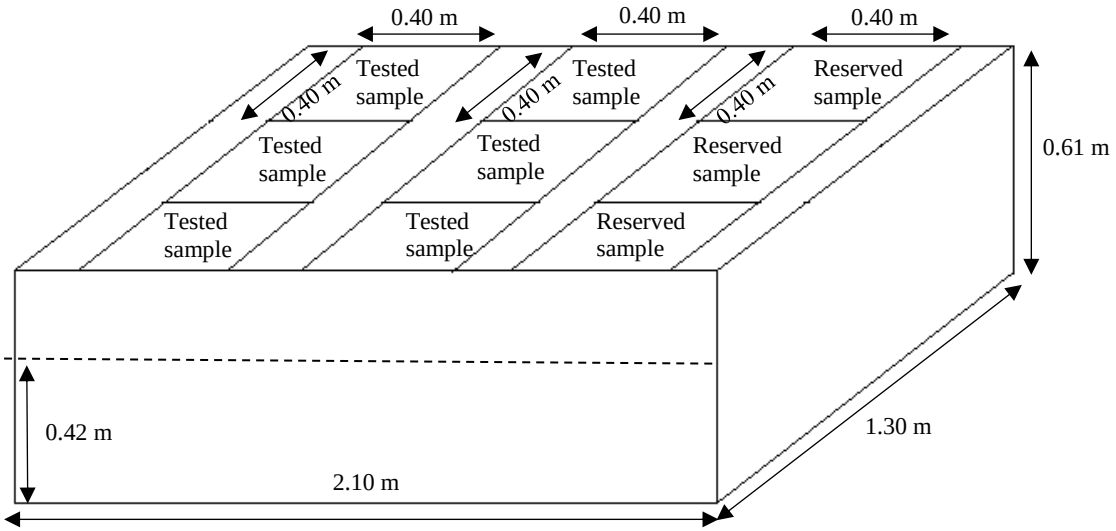

Figure 3.1: The dimensions of a fiberglass tank and set up of the experiment.

3.2.2 Tank Water Preparation and Volume

Tap water was filled into each tank to a depth of 0.42 m. The dimension of the tank was 2.10 m long × 1.30 m wide × 0.42 m depth, which gave a volume of 1.15 m^3 or equivalent to 1150 L. Anti-chlorine solution (NIKA) was then added to each tank with a volume of 0.2 ml per liter of water. The water was aerated and left for a period of 7 days before the start of the experiment.

3.2.3 Acclimatization of Juvenile

Juveniles of *Barbonymus schwanenfeldii* and *Tor tambroides* were obtained from the Inland Fishery Branch of the Department of Agriculture, Tarat, Serian. Prior to placing the juveniles into the tank in the laboratory, 25% of water volume from the tank was added to the plastic bag holding the juveniles and it was left for 30 minutes. Then, the fish was stocked into acclimatization aquaria for a week before the experiment to ensure the fish became active to avoid mortality due to stress during transportation and handling.

3.2.4 Fish Stocking

Thirty juveniles of each species were weighed using a weighing balance (AND, GH-252) whereas, the standard and total length were measured using a digimatic dial caliper (Mitutoyo, CD-12" CP). These were done to obtain the initial average mean weight and average mean standard length and total length of the fish. The average weight of *Barbonymus schwanenfeldii* was 0.353 ± 0.082 g with standard length and total length of 2.6 ± 0.2 cm and 3.8 ± 0.3 cm, respectively. *Tor tambroides* weighed at an average of 1.152 ± 0.360 g, and the standard length and total length were 3.4 ± 0.4 cm and 4.6 ± 0.2 cm, respectively. Feeding was done twice daily at 5% body weight of the fish with floating pelleted fish feed throughout the experimental period and the weight of feed given was recorded daily.

Aeration was facilitated using air stones that were connected to air pumps. Each compartment was stocked with a total of 20 juveniles and three compartments for each species (n=60). They were exposed for a period of 30 days to different levels of DO, pH, temperature and suspended solids content.

3.2.5 Sediment Preparation

Sediment preparation was done according to Dunnivant and Anders (2006). The sediment was weighed and air dried at 105 ºC overnight; the process was repeated until a constant weight is obtained. Then, the sediment was soaked with sodium hexametaphosphate overnight to separate fine and clay particles. Using a 62 µm mesh sieve, the sediment was puddled in the sieve immerse in water and sodium hexametaphosphate. The sand particle will remain in the sieve whereas silt and clay particle will pass through. Pipette method was then performed based on Folk (1980) for silt and clay particle. From the dry sieve analysis, the mass of sediment grain size content in the sediment was recorded. The obtained dried silt and clay were used in the preparation of different concentrations of TSS in tanks.

3.2.6 Response Surface Methodology

Response surface methodology was used to optimize the best set of water quality parameters for the growth of both *B. schwanenfeldii* and *T. tambroides.* The objective of this model was to simultaneously optimize the levels of the selected factors to attain the best result or performance (Bezerra *et al.,* 2008). This study used a 4-factor-3-level circumscribed central composite design (CCD) which includes, 1) a full factorial or fractional factorial design (Experiments 1-16), 2) additional design in which experimental levels are at a distance from its center and (Experiments 25-30), 3) a central point, mid-values of selected levels for each factors (Experiments 17-24) (Bezerra *et al.*,2008). The CCD gave rise to a total of 30

experiments (Table 3.1). Experiments No. 25-30 were replicates and were represented as Experiment 25 throughout this study. The water quality parameters and levels used were: pH (5.0, 6.0 and 7.0); DO (3.0, 4.5 and 6.0 mg/L); TSS (0, 1000, 5000 mg/L) and temperature (24, 27 and 30 ºC). The levels or concentrations for each parameter were chosen because they were in the range that was believed to affect the juveniles' survival and growth rate. Besides that, a few studies on the selected parameters individually had also been studied (Nyanti *et al.,* 2017; 2018). Based on their studies, the levels affecting the survival and growth of fishes were chosen.

3.2.7 Experimental Design

For the water quality parameters combination, 20 juveniles of both *B. schwanenfeldii* and *T. tambroides* were stocked into each fish tank, respectively. The dissolved oxygen, pH and temperature were monitored daily using dissolved oxygen meter (SPER SCIENTIFIC, 850041) and pH meter (HORIBA, D-51E) respectively, to maintain the conditions of each tank whereas total suspended solids (APHA, 1998) was measured weekly. Both ammonia-nitrogen (Method 10031, HACH) and nitrite (Method 8153, HACH) were measured in triplicates weekly to maintain fixed concentrations of less than 0.8 mg/L and 0.03 mg/L, respectively. The silt and clay were kept suspended by using pump and aeration throughout the 30-day experimental period. The dissolved oxygen was adjusted by adding sodium sulfite powder to decrease it and aeration to increase it. The pH was adjusted by adding weak sulfuric acid (10%) into the tank to gain the desired pH level and thermostatic heater (Tianrum, W-500HG) was equipped to adjust the temperature.

3.2.8 Data Collection

Fish mortality was recorded for all tanks throughout the experimental period. In order to maintain the density of fry or juveniles in each compartment, the dead fishes were replaced

by those of equal size from the reserved compartment exposed to the same water quality condition as that of the dead fish. The responses observed in this study include, 1) avoidance during feeding, when the fish scattered upon the presence of human during feeding, and 2) time taken to feed on pellets. At the end of the test, the standard length, total length and weight of each fish in all compartments were measured whereas the growth, mortality and FCR were calculated as described by Hardy (2002) and Ridha (2006). The values were calculated as follows:

i) Weight gain, WG (g) = Final mean body weight - Initial mean body weight

ii) Standard length gains, SL (cm) = Final mean standard length - Initial mean standard length

iii) Total length gains, TL (cm) = Final mean total length - Initial mean total length

iv) Survival rate, SR (%) = [(No. of stocked fish - No. of dead fish) / No. of stocked fish] × 100

v) Feed conversion ratio, FCR = Feed intake (g) / Body weight gain (g)

vi) Specific growth rate. SGR (%) = [(Final body weight - Initial mean body weight) / No. of experiment day] × 100

Table 3.1: Experimental design for each tank in the experiment.

Experiment	Temperature (°C)	Total suspended solids (mg/L)	Dissolved oxygen (mg/L)	pH
1	24	0	3	5
2	24	0	3	7
3	24	0	6	5
4	24	0	6	7
5	24	5000	3	5
6	24	5000	3	7
7	24	5000	6	5
8	24	5000	6	7
9	30	0	3	5
10	30	0	3	7
11	30	0	6	5
12	30	0	6	7
13	30	5000	3	5
14	30	5000	3	7
15	30	5000	6	5
16	30	5000	6	7
17	25.5	1000	4.5	6
18	28.5	1000	4.5	6
19	27	500	4.5	6
20	27	3000	4.5	6
21	27	1000	3.75	6
22	27	1000	5.25	6
23	27	1000	4.5	5.5
24	27	1000	4.5	6.5
25	27	1000	4.5	6
26	27	1000	4.5	6
27	27	1000	4.5	6
28	27	1000	4.5	6
29	27	1000	4.5	6
30	27	1000	4.5	6

3.2.9 Statistical Analysis

Responses surface methodology was used to predict the optimum set of condition for the survival of both species. The adequacy of the CCD was determined by using ANOVA and F-test. Analytical statistics was done by using SPSS version 23 and the response surface plots was plotted by using MATLAB 2009b.

3.3 Results

3.3.1 Behavioral Responses

The behavioral responses are presented in Table 3.2 for *B. schwanenfeldii* and Table 3.3 for *T. tambroides.* Avoidance and time taken to feed were observed among juveniles to determine their behavior in the different combinations of water quality parameters. Avoidance was observed (present) during feeding in which the juveniles dispersed and moved into the lower part of the water column in the presence of a person during feeding. Three descriptions were used to describe the time taken to feed, 1) immediate where fishes feed on the pellet as soon as being fed; 2) moderate where fishes feed on the pellet after 1 minute of being fed; 3) slow where fishes feed on the pellet after 2 minutes of being fed.

In Table 3.2, it was observed that *B. schwanenfeldii* showed avoidance in tanks 1, 5-9, 13-16, 20, 21 and 23-25. These tanks were designed mostly with TSS concentrations of 3000 mg/L and above and DO level of 3.75 mg/L and below. Besides that, *B. schwanenfeldii* of tanks 5-8, 13-18 and 20 showed moderate response to feed compared to other tanks. The similarity these tanks possessed is that the TSS concentrations of all tanks were at 3000 mg/L and above.

Table 3.2: Behavioral responses of *B. schwanenfeldii* in all experiments.

Experiment	Behavioral responses	
	Avoidance	Time taken to feed
1	Dispersed when fed	Immediate
2	Not observed	Immediate
3	Not observed	Immediate
4	Not observed	Immediate
5	Dispersed when fed	Moderate
6	Dispersed when fed	Moderate
7	Dispersed when fed	Moderate
8	Dispersed when fed	Moderate
9	Dispersed when fed	Immediate
10	Not observed	Immediate
11	Not observed	Immediate
12	Not observed	Immediate
13	Dispersed when fed	Moderate
14	Dispersed when fed	Moderate
15	Dispersed when fed	Moderate
16	Dispersed when fed	Moderate
17	Not observed	Moderate
18	Not observed	Moderate
19	Not observed	Immediate
20	Dispersed when fed	Moderate
21	Dispersed when fed	Immediate
22	Not observed	Immediate
23	Dispersed when fed	Immediate
24	Dispersed when fed	Immediate
25	Dispersed when fed	Immediate

Table 3.3: Behavioral responses of *T. tambroides* in all experiments.

Experiment	Behavioral responses	
	Avoidance	Time taken to feed
1	Dispersed when fed	Immediate
2	Not observed	Immediate
3	Dispersed when fed	Immediate
4	Not observed	Immediate
5	Dispersed when fed	Slow
6	Dispersed when fed	Slow
7	Dispersed when fed	Slow
8	Dispersed when fed	Slow
9	Dispersed when fed	Immediate
10	Not observed	Immediate
11	Dispersed when fed	Immediate
12	Not observed	Immediate
13	Dispersed when fed	Slow
14	Dispersed when fed	Slow
15	Dispersed when fed	Slow
16	Dispersed when fed	Slow
17	Dispersed when fed	Moderate
18	Dispersed when fed	Moderate
19	Not observed	Immediate
20	Dispersed when fed	Moderate
21	Dispersed when fed	Slow
22	Not observed	Moderate
23	Dispersed when fed	Moderate
24	Dispersed when fed	Moderate
25	Dispersed when fed	Moderate

Table 3.3 shows avoidance of *T. tambroides* in all tanks except 2, 4, 10, 12, 19 and 22. Avoidance was observed in tanks with TSS concentration of 1000 mg/L and above, DO levels of 3.75 mg/L and lower and pH levels of 6 and below. Response to feed were found to be slower in tanks 5-8, 13-16 and 21, whose tanks had TSS concentrations of 5000 mg/L and DO of 3.75 mg/L and below.

In summary, TSS values of more than 3000 mg/L and 1000 mg/L led to avoidance behavior in *B. schwanenfeldii* and *T. tambroides,* respectively. The DO levels of 3.75 mg/L and lower also lead to avoidance of both species. Such behavior could be related to the increase of vulnerability towards predation. On top of that, feeding time was negatively and greatly affected by high TSS values of more than 3000 mg/L.

3.3.2 Survival, Growth Performance and Growth Rate

The highest and lowest values of each response in relation to the tested water quality parameters were recorded and presented in Table 3.4.

For *B. schwanenfeldii,* the highest weight gain (0.734 g) recorded was at 27 °C, 500 mg/L TSS, 4.5 mg/L DO and pH 6 while the lowest (0.062 g) was at 24 °C, 0 mg/L TSS, 3 mg/L DO and pH 7. Meanwhile, *T. tambroides* recorded the highest weight gain (0.318 g) at 24 °C, 0 mg/L TSS, 6 mg/L DO and pH 7 and lowest (2.339 g) at 24 °C, 5000 mg/L TSS, 3 mg/L DO and pH 5. The lowest weight gain for both species were observed at DO concentration of 3 mg/L.

The highest standard length (2.2 cm) and total length (2.2 cm) increase were recorded at 27 °C, 3000 mg/L TSS, 4.5 mg/L DO and pH 6 while the lowest was (0.2 & 0.1 cm, respectively) at 24 °C, 0 mg/L TSS, 3 mg/L DO and pH 7 for *B. schwanenfeldii.* whereas *T. tambroides* showed the highest standard length (3.1 cm) increase at 30 °C, 5000 mg/L TSS,

3 mg/L DO and pH 5, and the lowest (1.2 cm) at 24/ 28.5 °C, 1000/ 5000 mg/L TSS, 3/ 4.5 mg/L DO and pH 5/6. For total length gained, the highest (2.8 cm) for *T. tambroides* was at 30 °C, 0/ 5000 mg/L TSS, 3 mg/L DO and pH 5/ 7 whereas the lowest (0.9 cm) was at 24/ 28.5/ 30 °C, 0/ 1000/ 5000 mg/L TSS, 3/ 4.5/ 6 mg/L DO and pH 5-7.

B. schwanenfeldii showed the highest survival rate (100%) at 24 °C, 0 mg/L TSS, 6 mg/L DO and pH 7 and the lowest (75%) at 24/ 30 °C, 5000 mg/L TSS, 3/ 6 mg/L DO and pH 5. At 24 °C, 0 mg/L TSS, 3/6 mg/L DO and pH 5/7, *T. tambroides* recorded the highest survival rate (100%) whereas the lowest (75%) was at 24 °C, 5000 mg/L TSS, 6 mg/L DO and pH 5.

Feed conversion ratio estimates the efficiency of conversion of feed into the desired output, be it body weight or length. The highest value (19.46) was recorded at 24 °C, 0 mg/L TSS, 3 mg/L DO and pH 7 and the lowest (1.63) was at 27 °C, 500 mg/L TSS, 4.5 mg/L DO and pH 6 for *B. schwanenfeldii*. For *T. tambroides* the highest FCR (7.86) was observed at 24 °C, 5000 mg/L TSS, 3 mg/L DO and pH 5 while the lowest value (1.07) was at 24 °C, 0 mg/L TSS, 6 mg/L DO and pH 7.

Besides that, the highest value of SGR (2.385%) for *B. schwanenfeldii* was recorded at 27 °C, 1000 mg/L TSS, 3.75 mg/L DO and pH 6 and the lowest value (0.206%) at 24 °C, 0 mg/L TSS, 3 mg/L DO and pH 7. For *T. tambroides*, the highest SGR (7.796%) was recorded at 24 °C, 0 mg/L TSS, 6 mg/L DO and pH 7 whereas the lowest (1.060%) was found at 24 °C, 5000 mg/L TSS, 3 mg/L DO and pH 5.

Table 3.4: The highest and lowest values for each recorded response from juveniles of both *B. schwanenfeldii* and *T. tambroides*.

Fish species	Growth Performance	Highest					Lowest				
		Values	°C	TSS	DO	pH	Values	°C	TSS	DO	pH
B. schwanenfeldii	WG (g)	0.734	27	500	4.5	6	0.062	24	0	3	7
	SL (cm)	2.2	27	3000	4.5	6	0.2	24	0	3	7
	TL (cm)	2.2	27	3000	4.5	6	0.1	24	0	3	7
	SR (%)	100	24	0	6	7	75	24/30	5000	3/6	5
	FCR	19.459	24	0	3	7	1.634	27	500	4.5	6
	SGR (%)	2.385	27	1000	3.75	6	0.206	24	0	3	7
T. tambroides	WG (g)	2.339	24	0	6	7	0.318	24	5000	3	5
	SL (cm)	3.1	30	5000	3	5	1.2	24/ 28.5	1000/ 5000	3/ 4.5	5/ 6
	TL (cm)	2.8	30	0/ 5000	3	5/ 7	0.9	24/28.5 /30	0/1000/ 5000	3/ 4.5/ 6	5/ 6/ 7
	SR (%)	100	24	0	3/ 6	5/ 7	75	24	5000	6	5
	FCR	7.862	24	5000	3	5	1.069	24	0	6	7
	SGR (%)	7.796	24	0	6	7	1.060	24	5000	3	5

3.3.3 Response Surface Methodology

Response surface methodology was used to predict the best water quality parameters for the survival of both species based on the results obtained earlier. Based on Table 3.5, only survival rate of *B. schwanenfeldii* was significantly different among the experiments with $p < 0.05$. However, *T. tambroides* was not significantly different among the experiments with $p<0.05$.

Table 3.5: The *p*-value of survival, growth performance and growth rates of both *B. schwanenfeldii* and *T. tambroides*.

Species	WG	SL	TL	SR	FCR	SGR
B. schwanenfldii	0.689	0.563	0.564	0.043*	0.538	0.685
T. tambroides	0.929	0.352	0.412	0.090	0.625	0.929

* Significantly different (F-test, $p < 0.05$)

Figure 3.2 and Figure 3.3 show the predicted values of the four water quality parameters based on the growth performance and growth rate of both species. It was predicted that at temperature of 27.0 ºC, TSS of 0 mg/L, DO at 4.8 mg/L and pH 7.04 is the best set of condition for the survival of *B. schwanenfeldii* (Figure 3.2). Meanwhile, *T. tambroides* was predicted to survive the best under the condition of 23.9 ºC, 0 mg/L TSS, 4.5 mg/L DO and pH 7.02 (Figure 3.3).

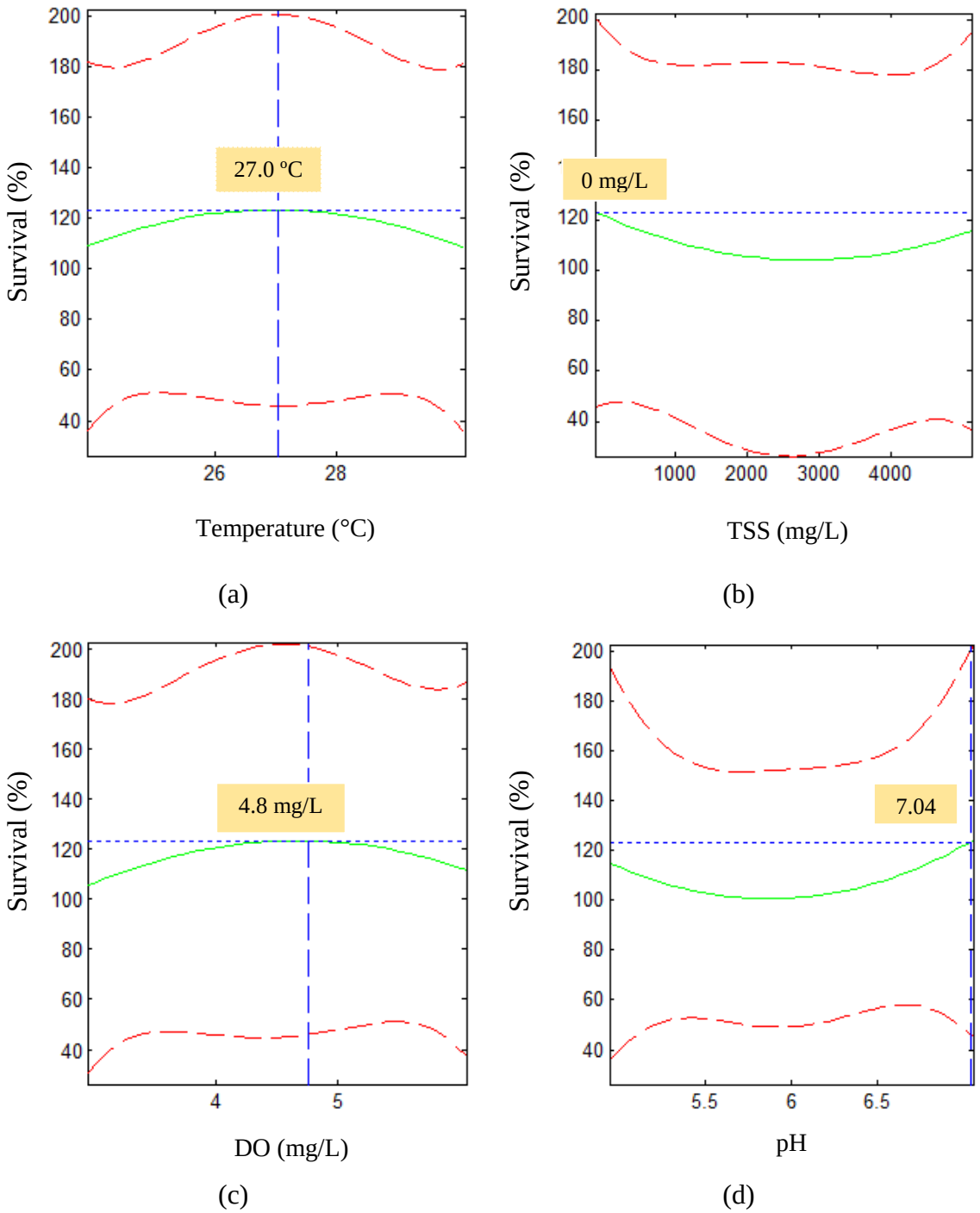

Figure 3.2: Prediction plot of full quadratic model on the effect of a) temperature; b) TSS; c) DO and d) pH on the survival of *B. schwanenfeldii.*

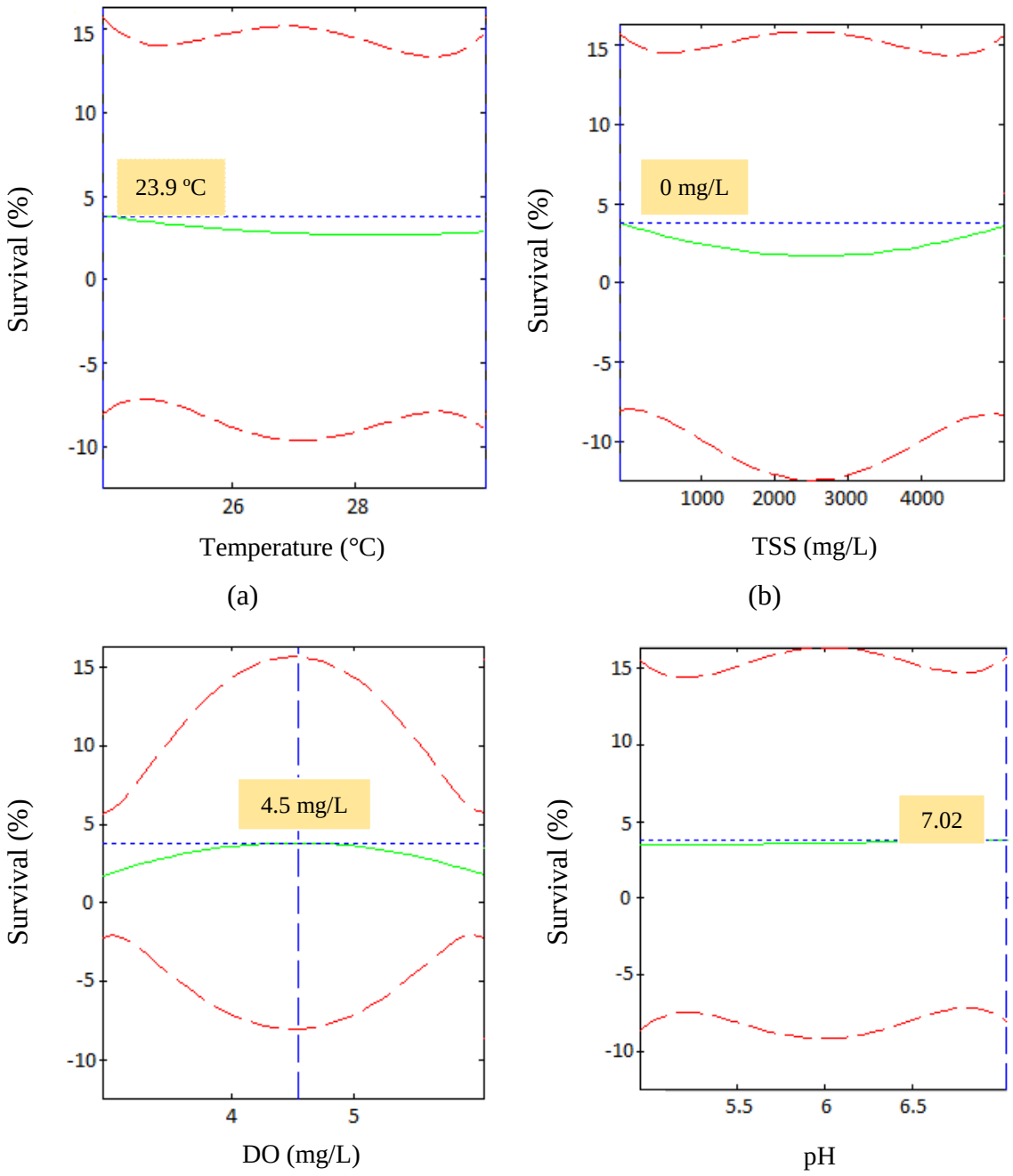

Figure 3.3: Prediction plot of full quadratic model on the effect of a) temperature; b) TSS; c) DO and d) pH on the survival of *T. tambroides.*

3.4 Discussion

3.4.1 Behavioral Responses

When encountered with stressors such as changes in water quality parameters, fishes often responded by altering their behavior. Based on the observations, both species depicted a delayed feeding time (to eat the feeds) when being fed. Such response was observed mostly among experiments with more than 1000 mg/L of TSS. Increased suspended solids in aquatic environments limits the visibility of aquatic livings by, 1) decreasing light penetration thus impaired vision (Kirk, 1985), and 2) degrading apparent contrast in the water by reducing the difference between an object and its background (Lythgoe, 1979). According to Hecht (1992), the increase in turbidity affected piscivorous predators in which the particles of suspended solids may reduce horizontal visibility under turbid waters. For fishes that rely on their visions, turbid water is a disturbance to their feeding habit. Reduced visibility affects the ability of predator to detect prey and vice versa in aquatic ecosystems (Utne-Palm, 2002), thus reducing the effectiveness of feeding or preying. This explained the longer time taken for the juveniles to react to feed in experiments with higher TSS values.

A study done by Rowe and Dean (1998) on the effect of turbidity on feeding of New Zealand freshwater species reported that four species, *Galaxias fasciatus, Retropinna retropinna, Galaxias, maculatur* and *Gobiomorphus cottidianus* showed reduced feeding rates upon an increase in suspended solids. Unlike the experiment done on both *B. schwanenfeldii* and *T. tambroides* which was of longer duration (30 days), the experiment on the four mentioned species was only 30 minutes upon feeding. However, even with the relatively short period of experiment, it was reported that the amount of feed ingested by the four species were relatively affected by increased suspended solids in the water. Piscivorous fish feeding was more sensitive to increased turbid waters as they rely on their vision to prey (Robertis *et al.*,

2003). This was the opposite of planktivorous fishes and other fishes which can depend on non-visual senses to prey of feed, whose feeding rate seems unaffected by suspended solids in water (Rowe and Dean, 1998).

Besides TSS, DO level lower than 3.75 mg/L was also found to cause avoidance and delayed feeding time in both species. In the natural habitat, low DO or hypoxic condition demonstrated avoidance in fish through distribution. Piscivorous fish were found to prefer oxygenated water column or area whereas planktivorous fish was distributed in lower DO levels (Ludsin *et al.*, 2009). This is to avoid being predated by their predators. However, avoidance was also exhibited in juveniles of piscivorous fish in which the juveniles were forced into less desirable area to avoid predation (Werner and Hall, 1988). A laboratory experiment to relate mass of fish with DO levels showed that small-mass fish were able to tolerate wider range of DO levels compared to large-mass fish (Burleson *et al.*, 2001). The bigger fish were found to only stay at oxygenated water column while smaller fish swam between oxygenated and less oxygenated waters repeatedly. However, juveniles of both species in this study were observed to move into lower water column in the presence of human during feeding. This indicates avoidance behavior of juveniles in the presence of possible predator.

3.4.2 Survival, Growth Performance, Growth Rate and Response Surface Methodology

Based on the results of response surface methodology, at the temperature of 27 ºC, TSS at 0 mg/L, DO at 4.8 mg/L and pH 7.04, *B. schwanenfeldii* was predicted to achieve the highest survival. This is supported by the results of this study in which the highest survival rate of *B. schwanenfeldii* was recorded from Experiment 4 with temperature of 24 ^{o}C, TSS at 0

mg/L, DO at 6 mg/L and pH 7. On the other hand, it was predicted that *T. tambroides* survive the best under temperature of 23.9 ºC, TSS at 0 mg/L, DO at 4.5 mg/L and pH 7.02. Based on the experimental results obtained, the highest survival rates were recorded in Experiment 3 (24 ºC, TSS at 0 mg/L, DO at 6 mg/L, pH 5), 4 and 8 (24 ºC, TSS at 5000 mg/L, DO at 6 mg/L, pH 7). Both species recorded 100% survival rate in Experiment 4. Apart from temperature, the values of TSS, DO and pH in Experiment 4 were all above the predicted values for *B. schwanenfeldii* while temperature, TSS and pH levels were the closest to predicted values for *T. tambroides*. However, the predicted DO levels for both species were close to the minimum DO required for sensitive aquatic organisms which is 5-7 mg/L (DOE, 2009).

Besides that, results show that the lowest survival of both species were recorded at pH 5 which is the lowest level in this experiment. The survival of fishes against pH levels may be dependent on the species of fish, in which different species can tolerate different pH levels. For instance, at pH ranged of 7.59-9.94, it is considered as optimal for freshwater fishes (Boyd, 1990; Harith and Hassan, 2011) and 6.5-7.0 specifically for *B. schwanenfeldii* (Mansour *et al.*, 2017). Mortality of fish under exposure to low pH is caused by circulatory failure due to the rapid loss of ions which also caused fluid volumes disturbances (Wood, 1989; Baldisserotto, 2011).

Based on the results, the highest values of standard and total lengths in both species were recorded at a higher temperature level. Increase in temperature may lead to increase of feeding rate or intake among fishes. Most fishes are exothermic, unable to regulate their own body temperature through physiological means (Moyle and Joseph, 2004) in which the surrounding waters will affect their body temperature, growth rate, food consumption and

feed conversion (Franklin *et al.,* 1995). The regulatory mechanism behind the relationship of growth rate and temperature are likely due to the enzymatic modulation of metabolic processes (Buentello *et al.,* 2000). Increasing temperature could lead to increase metabolic rate which in turn will increase the feeding rate of the fish (Jobling, 1981), which means that in 30 ºC water temperature, metabolic rate of fishes was increased which in turns increased their feed conversions. Low FCR values indicate successful or efficient conversion of feed intake into output or in this case, body length. The lowest FCR values for both species were recorded in experiments with higher temperature level. This is due to the temperature-dependent relationship of feeding intake or rate of fishes which had been discussed earlier.

Both species recorded the lowest survival rate (75%) at 24 °C, 5000 mg/L TSS, 6 mg/L DO and pH 5. In such condition, the TSS is at the highest concentration in the experimental design. *T. tambroides* was known to inhabit fast flowing, clear, pebbled water (Ingram, 2005). As mentioned earlier (Section 3.4.1), high TSS value lower the visibility of fishes to feed. This affected their feeding behavior and might affect the survival due to privations of feed. Aside from that, high levels of TSS can lead to clogging of gills in fishes (Au *et al.,* 2004). Once the gills had been clogged, it could interrupt the respiration process due to the reduced supply of oxygen.

Apart from that, *B. schwanenfeldii* also recorded the lowest survival rate at 30 ºC, TSS at 5000 mg/L, DO at 3 mg/L and pH 5. The DO level was lower than 5 mg/L which is the optimum level for most freshwater fishes (Stickney, 2000). Although temperature had a positive effect to the weight gain, it is highly associated with DO in water column. The metabolic demand for oxygen increased and the solubility of oxygen in water reduced under high temperature (Chapman and Liem, 1995; Dean and Richardson, 1999) and prolong

hypoxic condition, which may lead to mortality in certain species of fishes. At condition of 24 °C, 0 mg/L TSS, 6 mg/L DO and pH 7, 100% survival was recorded for both species. Thus, it can be said that such combination of water quality levels was the most suitable for fish survival among the other combinations.

3.5 Conclusions

In conclusion, the behavioral responses to changes in levels of DO, TSS, pH and temperature were species dependent. Most of the results show that high TSS values and low DO concentrations led to negative behavioral responses (avoidance and feeding time) of both *B. schwanenfeldii* and *T. tambroides.* The best levels of DO, TSS, temperature and pH differ for the two species in relation to the growth rate and performance of the species. Response surface methodology was applied to predict the best combination of DO, TSS, temperature and pH for the survival of each species. However, the modelling predictions agreed with observations in parameters of TSS and pH.

CHAPTER 4

RESPONSES OF NATIVE FISH SPECIES *BARBONYMUS SCHWANENFELDII* AND *TOR TAMBROIDES* TO CHANGES IN HYDROGEN SULFIDE CONCENTRATIONS

4.1 Introduction

Sulfide is a substance that is produced naturally and has been around since the beginning of life (Bagarinao, 1992). Sulfide also can be found in other aquatic habitats such as salt marshes, stagnant basins, coastal lagoons, mangrove and anoxic fjords and from natural land resources such as volcanoes (Bagarinao, 1992). According to Weiner (2010), the decomposition of organic matter released sulfur in the form of hydrogen sulfide (H_2S).

Aqueous hydrogen sulfide can dissociate into hydrosulfide anion (HS^-) and bisulfide anion (S^{2-}), depending on the pH of water or medium. At pH 5, H_2S is available at 99 %; pH 7, sulfide exists as H_2S and HS^- at a ratio of 1:1 whereas at pH 9 it will dissociate to HS^- at 99 % (Hughes *et al.,* 2009; Kabil and Banerjee, 2010). Meanwhile, S^{2-} exists only at pH 12. The reversible equation is as follows (Weiner, 2010),

$$S^{2-} + 2\ H_2O \longleftrightarrow OH- + HS^- + H_2O \longleftrightarrow H_2S\ (g) + 2\ OH^-.$$

Throughout this chapter, sulfide refers to the sum of all three species unless stated otherwise.

Most living organisms are able to withstand sulfide to some level, however few are able to withstand continuous exposure to higher concentration in which can be found in aquatic habitat with occasional sulfide concentration peak (Tobler *et al.,* 2011). Several fish kill cases due to high sulfide concentrations had been reported (Brinkman and Santos, 1974; Luther III *et al.,* 2004). These cases occur in a water canal and lakes where the water is prone to stratification.

The toxicity of hydrogen sulfide is mainly due to the inhibition of cytochrome *c* oxidase in aerobic respiration and interference with crucial enzymes (Affonso *et al.,* 2002; Tobler *et al.,* 2006). Effects on fish under both anoxic and high sulfide condition are mostly similar such as gasping for air and loss of equilibrium, causing sulfide toxicity to be mistaken as the former (Bagarinao, 1992; Bagarinao and Lantin-Olaguer, 1999).

The objective of this study was to determine the tolerance level and behavioral response of juvenile *Barbonymus schwanenfeldii* and *Tor tambroides* under different concentrations of hydrogen sulfide exposure under lowering DO and pH levels.

4.2 Materials and Methods

4.2.1 Experimental Set up

A modified flow-through bioassay design (Figure 4.1) based on Bagarinao and Lantin-Olaguer (1999) study was used. It consisted of 15 L airtight plastic containers, freshwater reservoir and stock sulfide solution. For each concentration, three 15 L plastic containers were connected to an aerated freshwater reservoir and three sulfide stock solution bottles. Aerated freshwater was supplied from the reservoir into each plastic containers continuously at a flow rate of 100 mL/min. Sulfide solution was also supplied from the stock solution bottle into each container at a flow rate of 5 mL/min. Both freshwater reservoir and sulfide stock solution bottles were placed higher than the plastic containers to ensure the flow of liquid by gravitational pull whereas the flow rate was controlled by using flow rate control valve. Flow rates of both freshwater and sulfide were calibrated at the beginning of each experiment.

The stock solution was prepared by dissolving the thoroughly washed and dried $Na_2S.9H_2O$ crystals [0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 g] in 2 L nitrogen-bubbled water to make six different concentrations [6.75, 13.50, 20.25, 27.00, 33.75 and 40.50 µg/L]. To prepare the nitrogen-bubbled water, the water was bubbled with nitrogen gas for 30 minutes to decrease the dissolved oxygen level to 1-2 mg/L. Hydrogen sulfide in aqueous state are easily oxidized and lost to the atmosphere. Thus, each sulfide stock bottle was provided with nitrogen (in a balloon) in order to prevent the oxidation of sulfide. The stock solution was prepared fresh for each experiment.

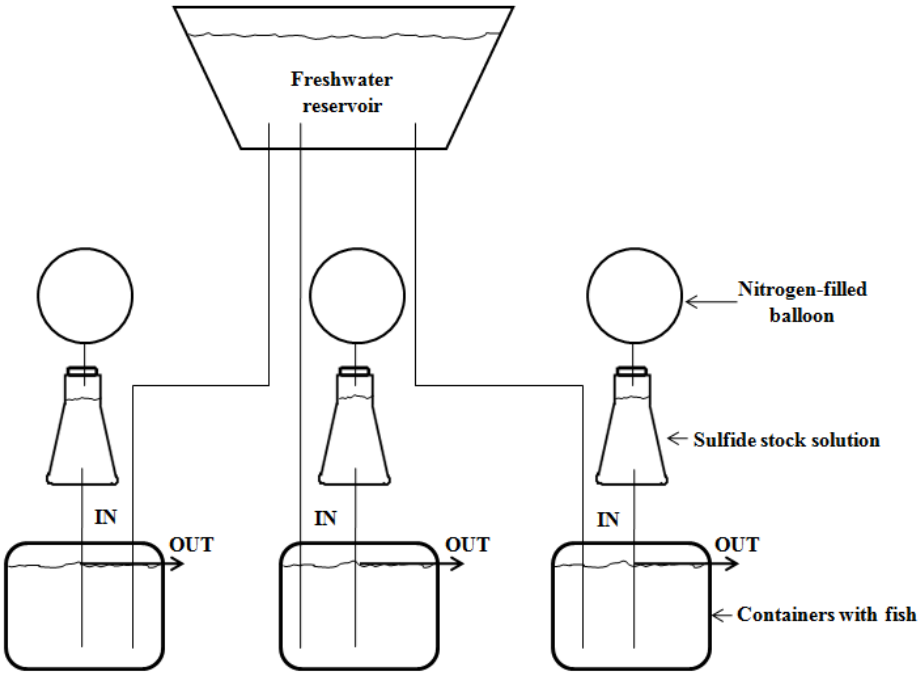

Figure 4.1: The figure illustrates the flow-through design for sulfide exposure test. Freshwater reservoir was connected to all containers. Three sulfide stock solutions equipped with nitrogen-filled balloons were supplied each to the three containers.

4.2.2 Fish Acclimatization

Both juveniles of *B. schwanenfeldii* and *T. tambroides* were obtained from the Inland Fishery Branch of the Department of Agriculture, Tarat, Serian. Prior to placing the juveniles into the containers, 25% of water volume from the tank was added to the plastic bag holding the juveniles and left for 30 minutes. After that, the juveniles were stocked into acclimatization tank for a week before experiment to prevent mortality due to stress during transportation and handling. Thirty juveniles were taken to measure their weights by a weighing balance (AND, GH-252) and the average weight of *B. schwanenfeldii* was 1.15 ± 0.20 g and *T. tambroides* was 0.75 ± 0.01 g.

4.2.3 Positive Control

A positive control was done to determine the most suitable concentrations for the sulfide stock solutions and the time period of the experiment. The control was carried out by using the same modified flow-through design with 10 juveniles in each container but with different sets of concentrations. After numerous trials have been done, six different concentrations of sulfide stock solutions were determined and the time period of the experiments was decided as 12 hours. The 12 h time period was chosen due to the limitations of aerated freshwater supply and stock solution which would need renewals every 4-6 hours.

4.2.4 Experimental Designs

4.2.4.1 Gradual Sulfide Exposure

For this experiment, exposure on *B. schwanenfeldii* (1.28 ± 0.41g) was carried out from August-September 2015 while *T. tambroides* (0.60 ± 0.07g) was done from November-December 2015. Ten juveniles were stocked into each container with three replicates for each concentration. Sulfide stock solution was prepared fresh and supplied into each containers. The aerated freshwater with DO level at an average of 6.12 ± 0.429 mg/L, pH of 7.26 ± 0.487 and temperature at 26-28 °C, was also channeled into each container. The levels of these parameters were maintained at optimum levels to ensure that no other factors were affecting the responses during sulfide exposure experiment. Both sulfide solution and freshwater were supplied until the end of experiment (12 hours) or until all juveniles died. The experiment was carried out at six different concentrations of sulfide stock solution (6.75, 13.50, 20.25, 27.00, 33.75 and 40.50 µg/L) and one negative control (without sulfide supply), each with three replicates. Water samples were taken from all the containers at the 6 h and analyzed for actual sulfide concentrations. The time for water sampling was decided at 6^{th} h because it was half of the experimental time period and to allow for sulfide

accumulation. Water samples were analyzed as soon as possible for actual sulfide concentration to prevent the loss of sulfide.

Behavioral responses (Bagarinao and Lantin-Olaguer, 1999), mortality (No. of samples died/ No. of samples stocked × 100%) and time of death were observed hourly from the start until end of the experiment. The DO and pH level were read by using EXTECH DO Meter/Datalogger SDL150 and EXTECH pH/ORP Meter SDL100 respectively. Both parameters were recorded at the start and the end of the experiment.

4.2.4.2 Sulfide Exposure under Lowering DO Level

Sulfide exposure under lowering oxygen level experiment for both *B. schwanenfeldii* (1.06 ± 0.07 g) and *T. tambroides* (0.80 ± 0.06 g) were carried out in January 2016. In this experiment, the DO level were allowed to go into progressive decline in concentrations until the experiment ends. The same flow-through system was used in this experiment. Prior to the start of the experiment, each container was equipped with aeration and freshwater was pumped in at 100 mL/min and left to adjust for 12 h. Both aeration and aerated freshwater supply was cut off and sulfide were supplied into each container (6.75, 13.50, 20.25, 27.00, 33.75 and 40.50 µg/L) at 5 mL/min, indicating the start of the experiment. DO was recorded at 60 minutes intervals until the end of the experiment (12 h). The time of death was recorded and actual sulfide concentrations were measured. Sampling of water was done at the 6^{th} h after the start of experiment. A negative control was also conducted without the sulfide supply.

4.2.4.3 Sulfide Exposure under Lowering pH

This experiment was carried out in February 2016 for both *B. schwanenfeldii* (1.11 ± 0.07 g) and *T. tambroides* (0.83 ± 0.06 g). Lowering pH levels were done by the supply of weak

sulfuric acid at 10% to allow the pH to decline progressively throughout the experiment. The flow-through system was used for this experiment. Ten juveniles were stocked each into three containers with aerated freshwater channeled into each container at 100 mL/min. An addition of 10% sulfuric acid was flowed into each of the three containers along with sulfide stock solution (6.75, 13.50, 20.25, 27.00, 33.75 and 40.50 µg/L), both at 5 mL/min to ensure the lowering of pH. The pH and DO were monitored and recorded at 30 minutes interval until all fish died. The time of death for all juveniles was recorded and water samples were taken at the 3rd h due to faster responses and mortality of the juveniles. A negative control was done without the sulfide supply.

4.2.5 Methylene Blue Method

All water samples collected were analyzed by using Methylene Blue Method (HACH, 2014) for total sulfide concentrations. A total of 10 mL water sample was pipetted into a sample cell and 0.5 mL of Sulfide 1 Reagent was added into the cell and swirled to mix the solution. Then Sulfide 2 Reagent was added at 5 mL and the sample cell was closed and inverted to mix. A five minutes reaction time was allowed for the reactions to take place. While waiting, a blank was prepared by filling 10 mL deionized water into sample cell. Spectrophotometer DR 2800 and program 690 Sulfide were used to read the total sulfide concentration. The blank was firstly inserted for zeroing followed by the samples for concentrations reading. The results were shown in µg/L.

4.2.6 Statistical Analysis

Paired *t*-test and one way ANOVA were used to compare the means of times to 50% and 100% mortality and total sulfide concentrations between experimental sets. For this study, the LC_{50} at 95% confidence limit was plotted at 6 h of exposure due to the fast-acting

characteristics of hydrogen sulfide. All statistical analyses were carried out by using SPSS software version 23 and tables and graphs were plotted by using Microsoft Excel 2013.

4.3 Results

4.3.1 Behavioral Responses

Four responses were observed and recorded throughout all experimental sets which were, huddling together, aquatic surface respiration (ASR), loss of equilibrium (spins while swimming) and turning upside down (Table 4.1 and Table 4.2).

4.3.1.1 Gradual Sulfide Exposure

In the gradual sulfide exposure experiment, *B. schwanenfeldii* displayed huddling together at 2-8 h for sulfide concentrations ranging from 160 to 659 µg/L; whereas ASR was observed at 5-6 h, loss of equilibrium at 5-7 h and turning upside down at 5-7 h of exposure for sulfide concentrations ranging 350 to 659 µg/L. For sulfide concentrations ranging 160 to 196 µg/L, only huddling together was observed. In the same experimental design using *T. tambroides,* huddling together was recorded at 2-8 h upon exposure of sulfide concentrations ranging from 144 to 658 µg/L. ASR was observed at 4-7 h, loss of equilibrium at 5-9 h and turning upside down at 5-11 h at the exposure of sulfide concentrations ranging 210 to 658 µg/L. At sulfide concentration of 144 µg/L, only huddling together was observed while no behavioral response was recorded for sulfide concentration of 82 µg/L.

4.3.1.2 Sulfide Exposure under Lowering DO

For sulfide exposure under low DO experiment, the behavioral responses in accordance to time of exposure did not vary much from the gradual sulfide experiment. Upon the removal of aeration, DO level dropped from 5.90 to 2.50 mg/L. *B. schwanenfeldii* showed huddling together at 1-7 h, ASR at 2-8 h, loss of equilibrium at 3-9 h and turning upside down at 4-10

h of exposure for sulfide concentrations ranging 203 to 946 µg/L. *T. tambroides* on the other hand displayed huddling together at 4-12 h and ASR at 4-12 h upon exposure for sulfide concentrations of 107 to 429 µg/L. Loss of equilibrium was observed after 5-9 h of exposure while turning upside down at 6-10 h, both at sulfide concentrations ranging from 229 to 429 µg/L. Both of the responses were not observed for sulfide concentrations ranging from 107 to 136 µg/L.

In the negative control of lowering DO, there was no display of behavioral responses from the juveniles throughout the experiment. The decline of DO for both negative control and sulfide exposure experiment showed similar decline and average values. This indicates that the behavioral responses displayed by the juveniles upon sulfide exposure was related to sulfide toxicity.

4.3.1.3 Sulfide Exposure under Lowering pH

In the sulfide exposure under lowering pH experiment, there is a clear difference in the behavioral response to sulfide exposure against time in comparison to both of the earlier experiments. The pH declined from 7.64 to 2.87 due to the continuous supply of 10% sulfuric acid. Both species displayed behavioral responses earlier when exposed to sulfide under low pH level. *B. schwanenfeldii* showed huddling together as early as 0.5-3 h, ASR at 1-3.5 h, loss of equilibrium at 1.5-5.5 h and turning upside down at 2-6.5 h after sulfide exposure at concentrations ranging 76 to 326 µg/L. For *T. tambroides,* huddling together was recorded at 0.5-2.5 h, ASR at 1-3 h, loss of equilibrium at 1-3.5 h and turning upside down at 1.5-4.5 h upon sulfide exposure at concentrations ranging 84 to 333 µg/L.

Table 4.1: Behavioral responses of *B. schwanenfeldii* exposed to different experimental sets and different total sulfide concentrations in relation to hour of exposure.

Sulfide stock solution (µg/L)	Actual total sulfide concentration (µg/L) **	Behavioral Responses			
		Huddle together	ASR	Loss of equilibrium	Turn upside down
Gradual sulfide exposure					
0	0 [b]	n.d.	n.d.	n.d.	n.d.
6.75	160 ± 27 [b]	8 h	n.d.	n.d.	n.d
13.50	196 ± 35 [b]	5 h	n.d.	n.d.	n.d
20.25	350 ± 52 [b]	4 h	6 h	7 h	7 h
27.00	474 ± 35 [b]	3 h	5 h	6 h	7 h
33.75	548 ± 46 [b]	2 h	5 h	6 h	6 h
40.50	659 ± 39 [b]	2 h	5 h	5 h	5 h
Sulfide exposure under lowering DO					
0	0 [b]	n.d.	n.d.	n.d.	n.d.
6.75	203 ± 38 [b]	7 h	8 h	9 h	10 h
13.50	340 ± 28 [b]	5 h	5 h	7 h	8 h
20.25	540 ± 31 [b]	3 h	4 h	6 h	6 h
27.00	641 ± 22 [b]	2 h	3 h	4 h	6 h
33.75	734 ± 35 [b]	2 h	2 h	3 h	4 h
40.50	946 ± 45 [b]	1 h	2 h	3 h	4 h
Sulfide exposure under lowering pH					
0	0 [a]	n.d.	n.d.	n.d.	n.d.
6.75	76 ± 10 [a]	3	3.5 h	5.5 h	6.5 h
13.50	127 ± 5 [a]	2.5	3 h	3.5 h	4 h
20.25	166 ± 22 [a]	2	2.5 h	3 h	4 h
27.00	208 ± 36 [a]	1.5 h	2 h	2 h	2 h
33.75	233 ± 7 [a]	1 h	1.5 h	2 h	2 h
40.50	326 ± 5 [a]	0.5 h	1 h	1.5 h	2 h

* n.d., not detected during the 12 hour experimental duration.

** For each experimental sets, different letters indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$.

Table 4.2: Behavioral responses of *T. tambroides* exposed to different experimental sets and different total sulfide concentrations in relation to hour of exposure.

Sulfide stock solution (µg/L)	Actual total sulfide concentration (µg/L) **	Behavioral Responses			
		Huddle together	ASR	Loss of equilibrium	Turn upside down
Gradual sulfide exposure					
0	0 [a]	n.d.	n.d.	n.d.	n.d.
6.75	82 ± 10 [a]	n.d	n.d.	n.d.	n.d
13.50	144 ± 27 [a]	8 h	n.d.	n.d.	n.d
20.25	210 ± 4 [a]	6 h	7 h	9 h	11 h
27.00	347 ± 18 [a]	4 h	5 h	6 h	7 h
33.75	515 ± 28 [a]	3 h	4 h	5 h	6 h
40.50	658 ± 6 [a]	2 h	4 h	5 h	5 h
Sulfide exposure under lowering DO					
0	0 [a]	n.d.	n.d.	n.d.	n.d.
6.75	107 ± 36 [a]	12 h	12 h	n.d	n.d
13.50	136 ± 38 [a]	11 h	12 h	n.d	n.d
20.25	229 ± 24 [a]	6 h	7 h	9 h	10 h
27.00	251 ± 24 [a]	6 h	7 h	8 h	9 h
33.75	321 ± 63 [a]	5 h	6 h	6 h	7 h
40.50	429 ± 36 [a]	4 h	4 h	5 h	6 h
Sulfide exposure under lowering pH					
0	0 [a]	n.d.	n.d.	n.d.	n.d.
13.50	84 ± 27 [a]	2.5 h	3 h	3.5 h	4.5
20.25	123 ± 4 [a]	2.5 h	3 h	3.5 h	4 h
27.00	170 ± 23 [a]	1.5 h	2 h	3 h	3.5 h
33.75	218 ± 24 [a]	1 h	1.5 h	2 h	2 h
40.50	333 ± 33 [a]	0.5 h	1 h	1 h	1.5 h

* n.d., not detected during the 12 hour experimental duration.

** For each experimental set, different letters indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$.

A negative control experiment was also done under lowering pH levels. Based on the results, no behavioral responses were observed until the end of experiment whereas under sulfide exposure, the responses were observed at all tested concentrations. On top of that, both experiments showed declining pH levels. This shows that the responses displayed were due to sulfide toxicity and not declining pH levels.

4.3.2 Sulfide Exposure Experiments

4.3.2.1 Gradual Sulfide Exposure

When sulfide solution was delivered, 100% mortality of fish was obtained as early as 6 h with concentration of 659 µg/L for *B. schwanenfeldii* and 658 µg/L for *T. tambroides* (Table 4.3). *B. schwanenfeldii* was able to survive to more than 12 h under sulfide exposure for concentrations ranging 160 to 196 µg/L while *T. tambroides* survived more than 12 h when exposed to sulfide concentrations ranging 82 to 144 µg/L.

Since the mortality of both species is 100% in less than 12 h, probit regression LC_{50} for both species were plotted at 6 h of exposure (Figure 4.2 and Figure 4.3). The probit regression estimated the LC_{50} at 6 h as 507.8 µg/L for *B. schwanenfeldii* and 306.1 µg/L for *T. tambroides* at 95% confidence level. This shows that *T. tambroides* had lower sulfide tolerance compared to *B. schwanenfeldii.*

During the exposure experiment, DO at the start was 5.85 ± 0.05 mg/L but reduced to 2.78 ± 0.23 mg/L at the end of 12 h. In addition, the pH of the water was 7.00 ± 0.11 at the start but ended with 5.54 ± 0.10, whereas the dissolved oxygen and pH for the negative control at the end of experiment were 5.57 ± 0.02 mg/L and 2.55 ± 0.21, respectively. Temperature on the other hand was maintained at 26-28 °C. This study observed that fish in negative control undergo the same oxygen and pH decline as the fish supplied with sulfide stock. The

fish in negative control however, did not show signs of low oxygen stress throughout the experiment and survived to more than 12 h.

Table 4.3: The time to 50 % and 100 % mortality of juveniles *B. schwanenfeldiii* and *T. tambroides* exposed to six different sulfide concentrations and one negative control.

Species, experiment, (body weight)	Sulfide stock solution (µg/L)	Actual total sulfide mean ± SD (µg/L)	Time to mortality (h)**	
			50 %	100 %
B. schwanenfeldii (1.28 ± 0.41 g)	0*	0*	>12	>12
	6.75	160 ± 27	>12	>12
	13.50	196 ± 35	>12	>12
	20.25	350 ± 52	10 ± 1	11 ± 1
	27.00	474 ± 35	7 ± 1	9 ± 0
	33.75	548 ± 46	6 ± 1	7 ± 1
	40.50	659 ± 39	5 ± 0	6 ± 0
T. tambroides (0.60 ± 0.07 g)	0*	0*	>12	>12
	6.75	82 ± 10	>12	>12
	13.50	144 ± 27	>12	>12
	20.25	210 ± 4	11 ± 1	12 ± 0
	27.00	347 ± 18	6 ± 1	8 ± 1
	33.75	515 ± 28	5 ± 0	7 ± 1
	40.50	658 ± 6	4 ± 0	6 ± 0

* Negative control, no sulfide was supplied to this set.

** Time to 50 % and 100 % mortality (under 12 h exposure) are significantly different (paired *t*-test, $p < 0.05$).

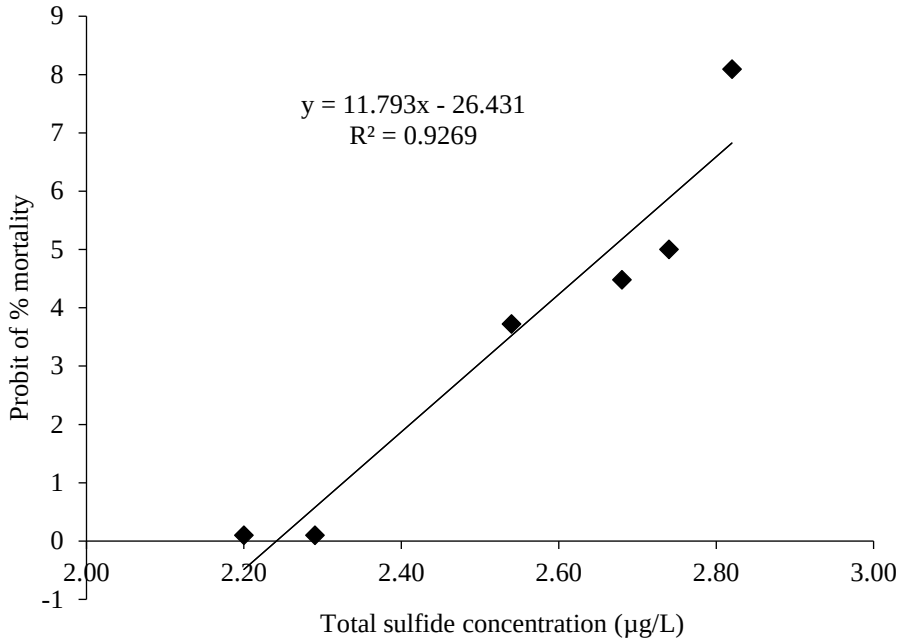

Figure 4.2: Probit regressions of percentage of mortality against log of total sulfide concentration to obtain LC_{50} at 6 h for *B. schwanenfeldii.*

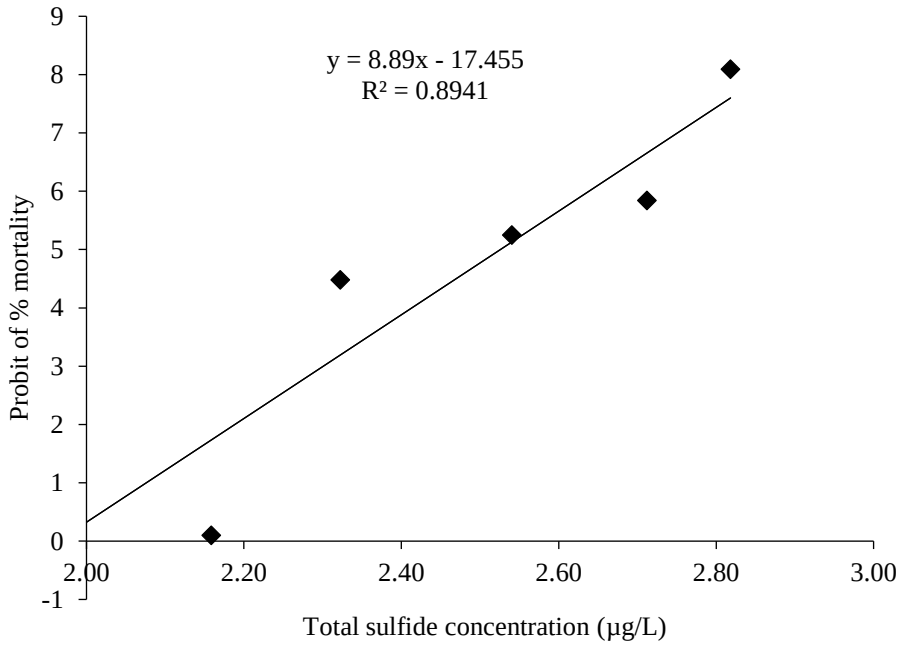

Figure 4.3: Probit regressions of percentage of mortality against log of total sulfide concentration to obtain LC_{50} at 6 h for *T. tambroides.*

4.3.2.2 Sulfide Exposure under Lowering DO

In the negative control, upon the aeration and flow-through aerated freshwater being turned off, the dissolved oxygen in the containers of the two fish species fell from the 5.90 mg/L to 2.50 mg/L in Figure 4.4 and 5.80 mg/L to 3.13 mg/L in Figure 4.5, all under 12 h. The pH level dropped from 7.35 to 5.78 in Figure 4.4 whereas in Figure 4.5, the pH level declined from 7.38 to 4.87. For the negative control, no mortality was recorded for both species, and only aquatic surface respiration was observed (Figure 4.4 and Figure 4.5).

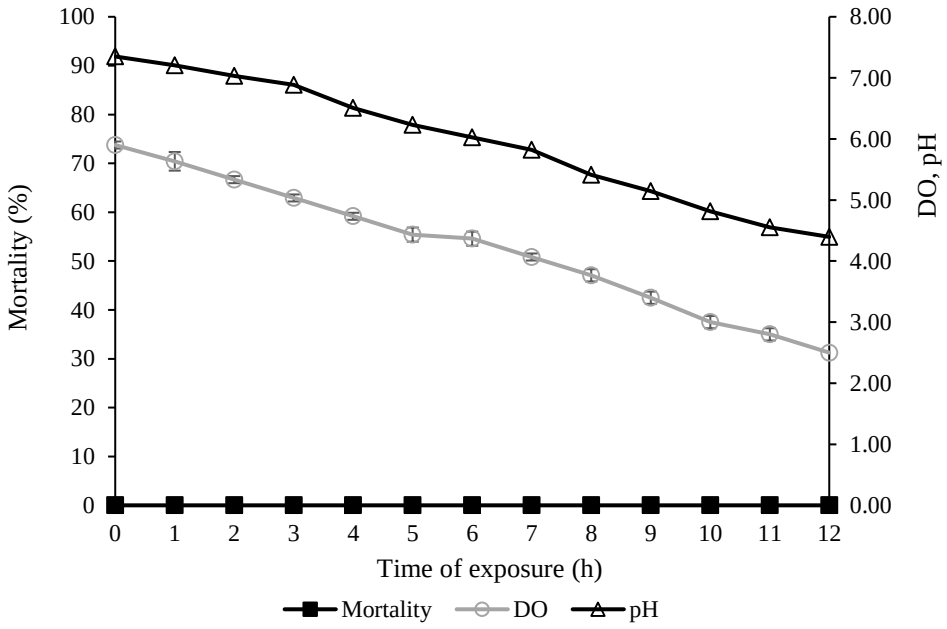

Figure 4.4: The mortality of *B. schwanenfeldii* during negative control experiment, lowering of DO in the absence of sulfide.

Meanwhile, under the condition of low oxygen level with sulfide being pumped at 5 mL/min, *B. schwanenfeldii* recorded 100 % mortality at 6-11 h of exposure at concentrations ranged 340-946 µg/L and *T. tambroides* showed 100 % mortality at 8-12 h of exposure at concentrations ranged 229-429 µg/L. Under the exposure of sulfide at concentration 203 µg/L, *B. schwanenfeldii* did not reach 100 % mortality, however 50 % mortality was

observed at 11 h of exposure. *T. tambroides* under sulfide exposure at concentrations 107-136 µg/L showed 100 % survival throughout 12 h period.

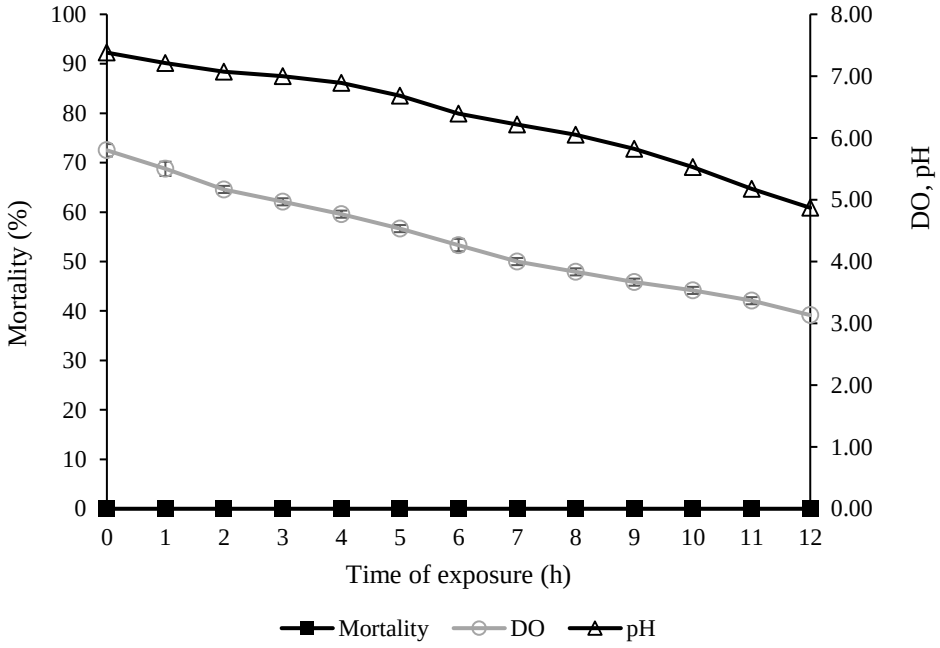

Figure 4.5: The mortality of *T. tambroides* during negative control experiment, lowering of DO in the absence of sulfide.

To compare between gradual sulfide exposure experiment and sulfide exposure under low oxygen experiment, line graphs depicting the pattern of mortality against hour of sulfide exposure were plotted for both species as shown in Figure 4.6 and Figure 4.7. Based on Figure 4.6, *B. schwanenfeldii* maintained zero mortality for few hours before a sudden increase in mortality until it reached 100% mortality. Both experiments showed that 100% mortality was reached after 3-4 h of the first occurrence of death in all concentrations.

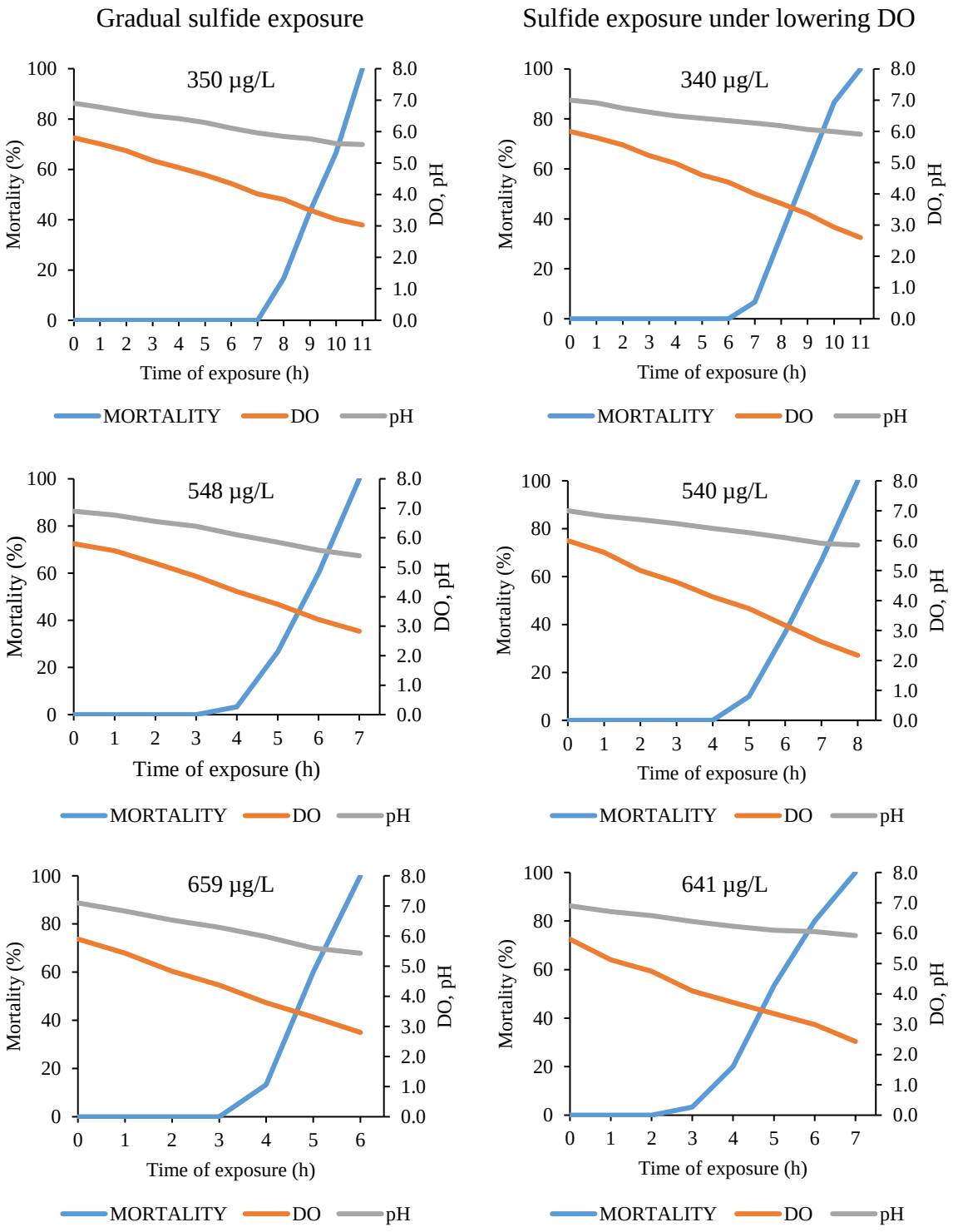

Figure 4.6: Mortality of *B. schwanenfeldii* during gradual sulfide only (350, 548 and 659 µg/L) and sulfide exposure under lowering DO (340, 540 and 641 µg/L).

The same pattern was also observed in *T. tambroides* as shown in Figure 4.7 where it started with zero mortality and then an increase in mortality until 100%. Unlike *B. schwanenfeldii*, *T. tambroides* took 5 h to reach 100% mortality upon the onset of first death for both experimental sets and in concentrations ranging from 210 to 347 µg/L. This indicates that sulfide toxicity is fast acting and fatal to fish in a short time under continuous supply.

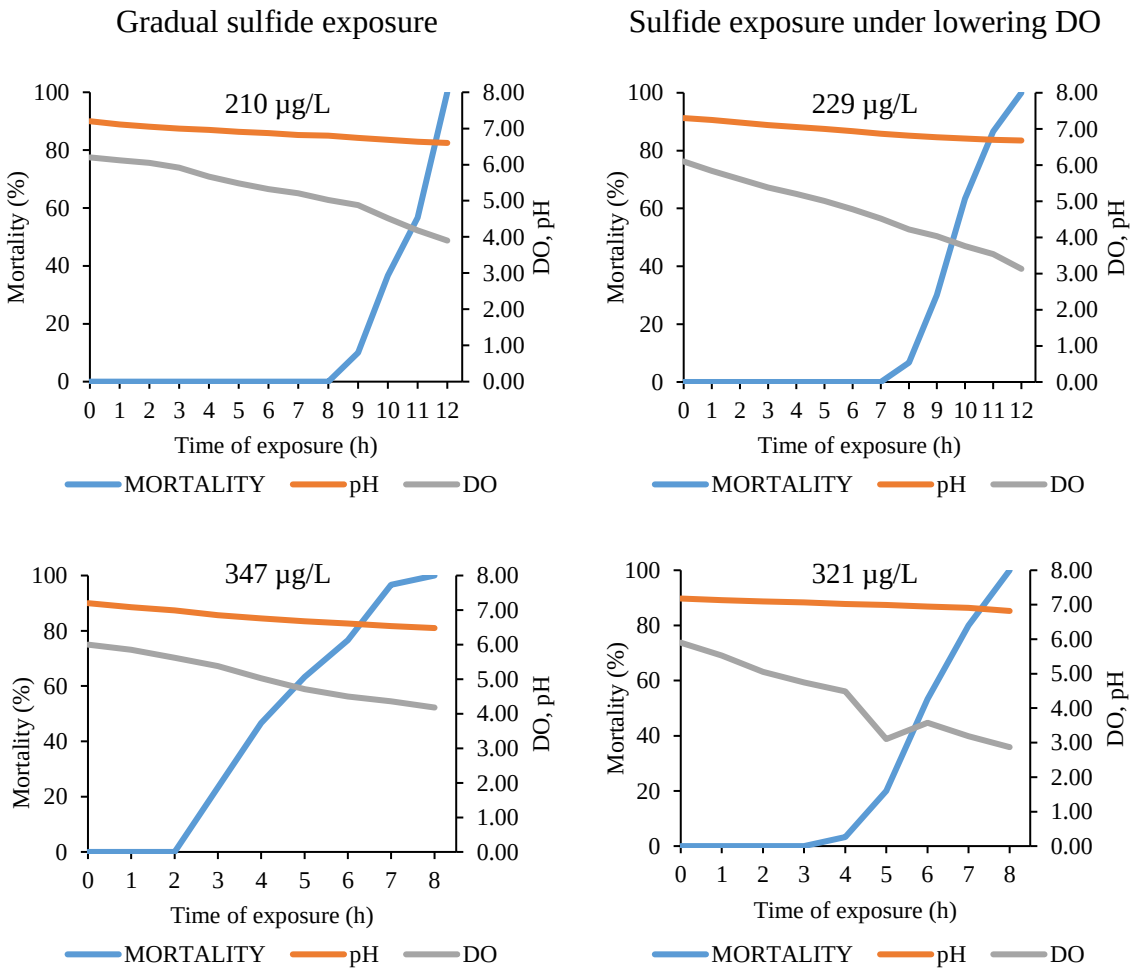

Figure 4.7: Mortality of *T. tambroides* during gradual sulfide only (210 and 347 µg/L) and sulfide exposure under lowering DO (229 and 321 µg/L).

Based on the results of negative control (Figure 4.4 and Figure 4.5) and sulfide exposure under lowering DO (Figure 4.6 and Figure 4.7), both negative control and sulfide exposure tests undergo progressive decline of DO until the end of the period. However, mortalities were only cbserved when sulfide was supplied into the containers. This showed that the

mortality was due to sulfide toxicity, not due to lowering DO concentrations in the duration of the experiment.

4.3.2.3 Sulfide Exposure under Lowering pH

Based on the negative controls in which sulfuric acid was supplied into the containers of both fish species without sulfide, pH level dropped gradually from 7.64 ± 0.14 to 3.23 ± 0.02 in Figure 4.8, and from 7.32 ± 0.00 to 2.87 ± 0.04 in Figure 4.9. Figure 4.8 shows the lowering of pH level led to the mortality of *B. schwanenfeldii* at 2 h (pH 6.08, DO at 3.57 ± 0.12) of exposure and reached 100 % mortality at 5.5 h (pH 3.23, DO at 2.83) of exposure while Figure 4.9 shows the mortality of *T. tambroides* began at 2 h (pH 5.71, DO at 3.53 ± 0.38) and reached 100% mortality at 5 h of exposure (pH 2.87, DO at 2.83).

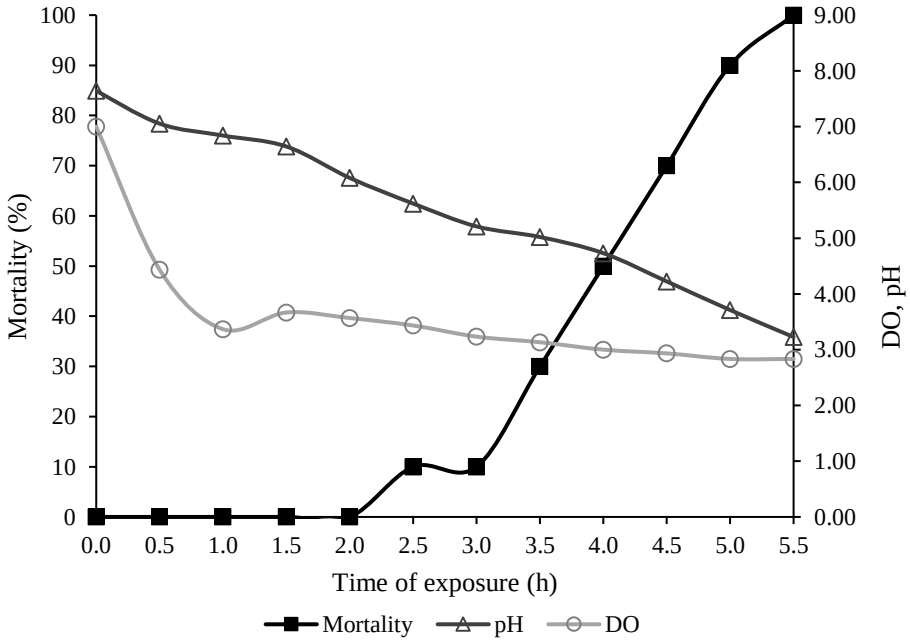

Figure 4.8: The mortality of *B. schwanenfeldii* during negative control experiment, lowering of pH and DO in the absence of sulfide.

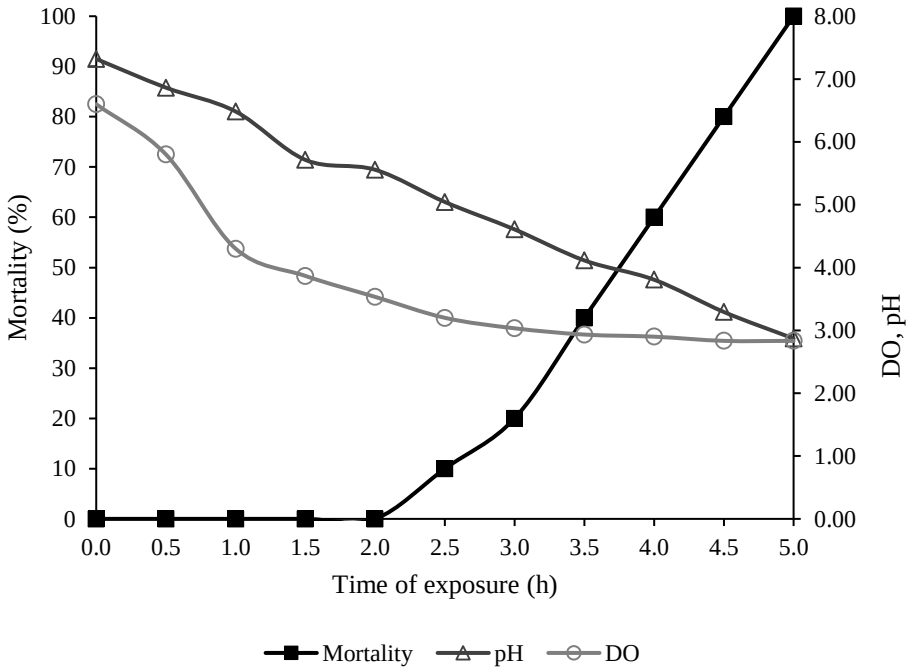

Figure 4.9: The mortality of *T. tambroides* during negative control experiment, lowering of pH and DO in the absence of sulfide.

When both acid and sulfide were pumped into the containers, 100 % mortality was reduced to 4-5 h at 166-326 µg/L sulfide concentrations for *B. schwanenfeldii* (Figure 4.10). On the other hand, *T. tambroides* reached 100 % mortality within 3-4 h at 123-333 µg/L sulfide concentrations (Figure 4.11). This proved that lowering of pH led to increase sulfide toxicity leading to higher and faster mortality of both species.

B. schwanenfeldii when exposed to both sulfide and lowering pH showed faster mortality when compared to exposure to sulfide only (Figure 4.10). For instance, when exposed to sulfide at concentration of 326 µg/L with lowering pH it took 4 h to reach 100 % mortality while it only took 11 h to reach 100 % when exposed to sulfide only at concentration 350 µg/L. *T. tambroides* also show similar results as *B. schwanenfeldii* where the mortality increased under sulfide exposure and lowering pH condition. This can be seen in Figure 4.11, where 100 % mortality is reached after 3 h of exposure with sulfide at concentration

347 µg/L with lowering pH while for sulfide exposure experiment only, it took 8 h to 100 % mortality at sulfide concentration 333 µg/L.

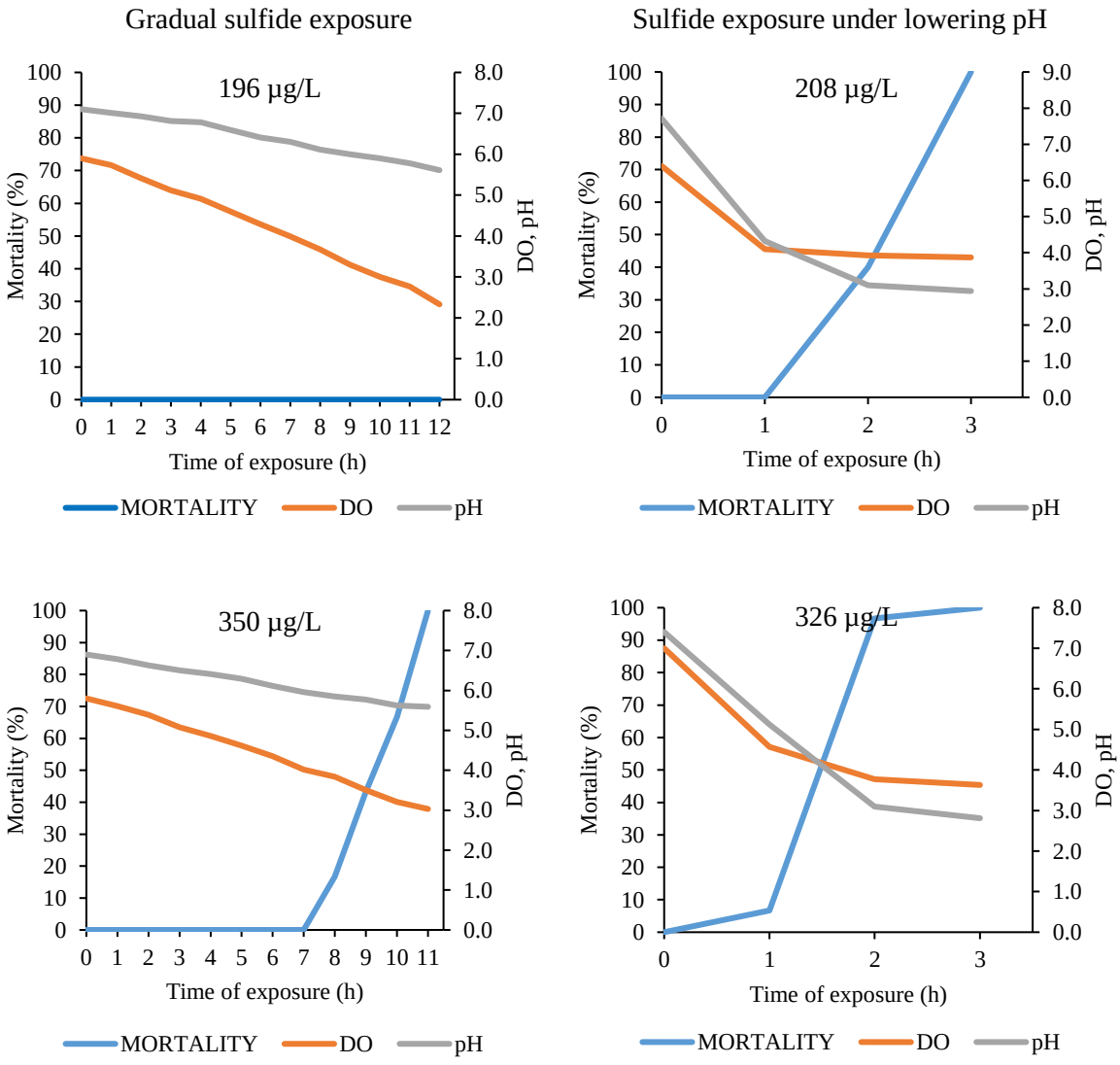

Figure 4.10: Mortality of *B. schwanenfeldii* during gradual sulfide only (196 and 350 µg/L) and gradual sulfide under lowering pH (208 and 326 µg/L).

Both negative controls and sulfide exposure showed mortalities under lowering pH for both species (Figure 4.8, Figure 4.9, Figure 4.10 and Figure 4.11). However, faster mortalities were observed in sulfide exposure experiment with a difference of up to an hour. This

indicates that the death occurring in sulfide exposure under lowering pH were accelerated due to sulfide toxicity.

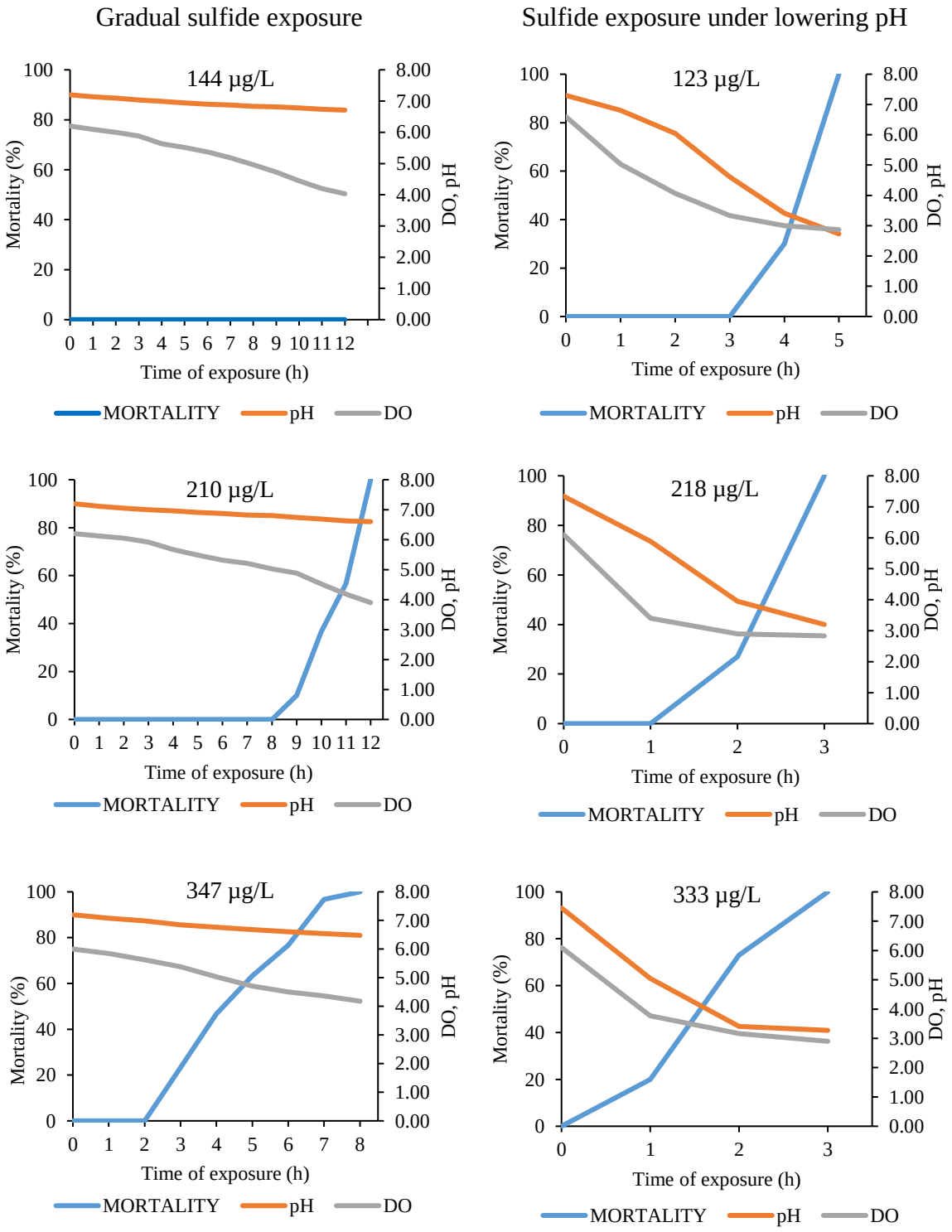

Figure 4.11: Mortality of *T. tambroides* during gradual sulfide only (144, 210 and 347 µg/L) and sulfide exposure under lowering pH (123, 218 and 333 µg/L).

4.3.3 Low DO, Low pH and Sulfidic Environment

Both pH and oxygen effects on sulfide toxicity had been carried out and presented previously. To determine whether both DO and pH affected the intensity of sulfide toxicity, Figure 4.12 and Figure 4.13 show the mortality of both species by comparing low oxygen and low pH in sulfidic water experiments. It is observed that the mortality of both species is earlier in low pH experiments than low DO experiments. *B. schwanenfeldii* recorded 100 % mortality as early as 3 h in low pH with sulfide concentrations 203 and 340 µg/L and 11-12 h under low oxygen with sulfide concentrations 208 and 326 µg/L. For sulfide toxicity under low pH, *T. tambroides* reached 100% mortality at 3-5 h at sulfide concentrations ranged 136-321 µg/L and 7-12 h under low oxygen with sulfide concentrations ranged 123-333 µg/L.

During the experiment, both pH and dissolved oxygen were recorded at the start and end of all experiments. The results obtained showed that for low oxygen in sulfide experiment, the dissolved oxygen and pH dropped gradually in under 12 h from 5.95 ± 0.07 mg/L to 2.71 ± 0.52 mg/L and from 7.10 ± 0.07 to 6.29 ± 0.55, respectively. However, for the low pH in sulfide condition experiment, both dissolved oxygen and pH declined sharply in 12 h. The pH declined from 7.59 ± 0.33 to 3.13 ± 0.18 and even with the supply of aerated freshwater into the containers the oxygen level in the containers also dropped from 6.42 ± 0.28 mg/L at the start to 3.17 ± 0.39 mg/L at the end of the experiment.

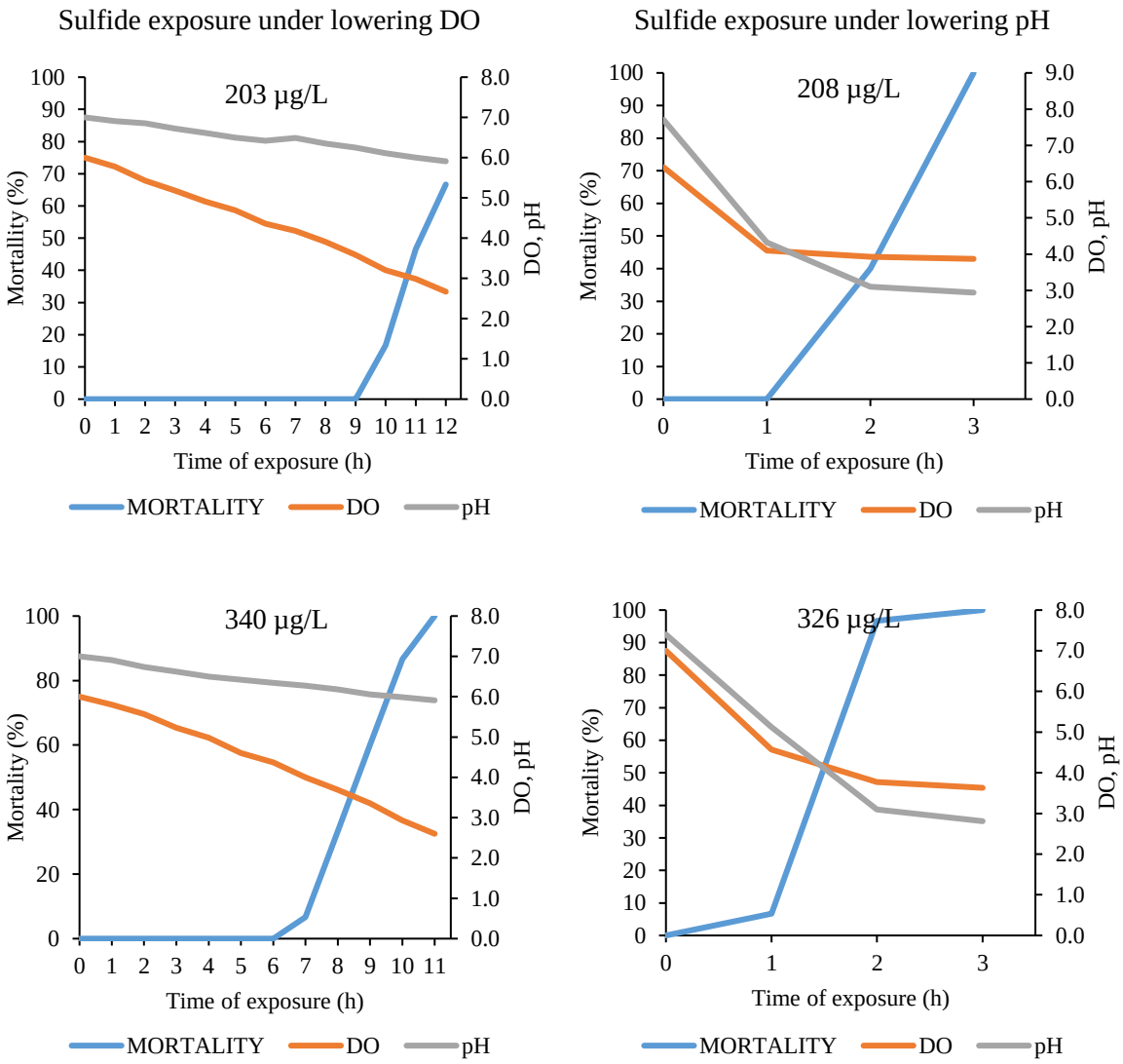

Figure 4.12: The mortality of *B. schwanenfeldii* with the lowering of oxygen under sulfidic water and lowering pH under sulfidic water.

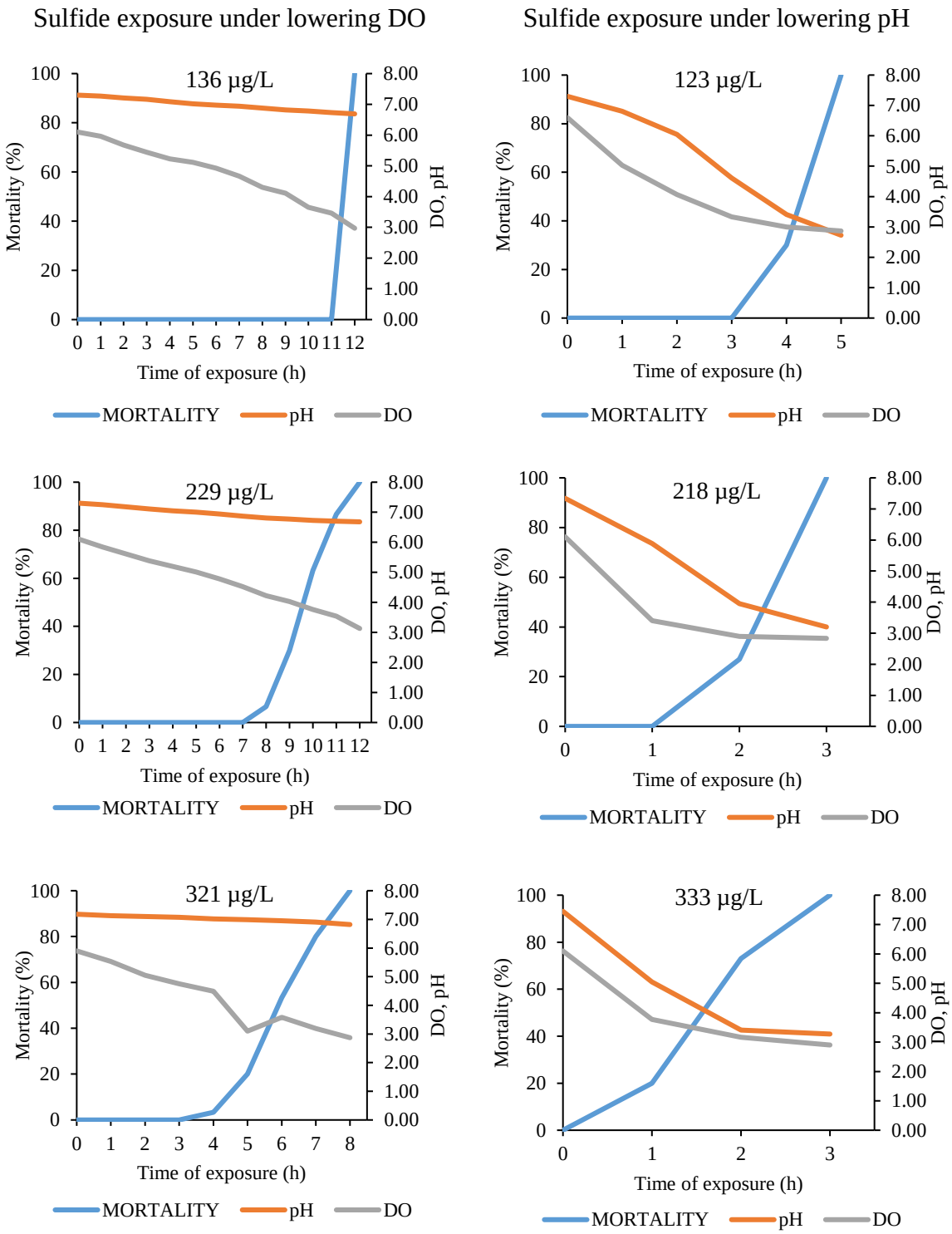

Figure 4.13: The mortality of *T. tambroides* with the lowering of oxygen under sulfidic water and lowering pH under sulfidic water.

4.4 Discussion

4.4.1 Gradual Sulfide Exposure

Based on the results obtained, the juvenile of both tested species had low sulfide tolerance. It was estimated that LC_{50} of *B. schwanenfeldii* at 6 h obtained in freshwater at pH 5.54 was 508 µg/L total sulfide with 490 µg/L H_2S, while for *T. tambroides* LC_{50} was estimated as 306 µg/L total sulfide with 220 µg/L H_2S in freshwater of pH 6.98. In comparison, Bagarinao and Lantin-Olaguer (1999) reported that the LC_{50} of brackishwater juvenile milkfish and tilapia at 4-8 h in freshwater at pH 8.3 were 163 µM (5216 µg/L) total sulfide, with 9.8 µM (313 µg/L) H_2S. The H_2S LC_{50} of both milkfish and tilapia were higher than that of *T. tambroides* but lower than that of *B. schwanenfeldii*. This shows that *B. schwanenfeldii* can tolerate higher sulfide concentrations whereas *T. tambroides* has a lower sulfide tolerance level.

Both studies were carried out by using similar flow-through experiments. However, this study was carried out in a smaller set-up (15 L fish containers) and with faster flow rate of sulfide solution at 5 mL/min while the experiment by Bagarinao and Lantin-Olaguer (1999) used a 25 L aquaria and sulfide solution flow rate of 1 ml/min.

The nature of *T. tambroides* should also be considered as it is known to be a highly sensitive indigenous species inhabiting fast flowing headwaters of river systems (Ingram, 2005; Nguyen *et al.,* 2006). The species has a low tolerance level to changes in both physical and chemical parameters and disruptions of their habitat had been reported to cause the depletion of natural stocks (Nguyen *et al.,* 2006). A study done by Soon *et al.* (2014) reported that the average pH and dissolved oxygen readings in *T. tambroides* aquaculture ponds were 7.59 and 5.4 mg/L, respectively.

Body mass is a crucial factor to sulfide tolerance level. The mean weight of *B. schwanenfeldii* and *T. tambroides* used in this study were relatively small with mean body weight of 1.28 ± 0.41 g and 0.60 ± 0.07 g, respectively. According to a study done by Tobler *et al.* (2011) on fish inhabiting sulfidic springs, smaller sized fish are able to withstand higher sulfide concentrations compared to large sized fish. However, in the study of milkfish and tilapia by Bagarinao and Lantin-Olaguer (1999), smaller sized fish were observed to tolerate lower concentrations of sulfide than larger sized fish with LC_{50} at 8 h for tilapia (5.7 ± 0.7 g) approximately 5134 µg/L and 7106 µg/L for tilapia with mean weight 7.2 ± 0.8 g. It can be said that the tolerance of species to sulfide in relation to body mass is dependent on the species itself.

4.4.2 Sulfide Exposure under Lowering DO

For this experimental set, the similar flow-through experiment design as a gradual sulfide exposure experiment was used. However, the juveniles of this experiment were supplied with continuous sulfide into their containers with no supply of aerated freshwater (DO at 6.12 ± 0.429 mg/L; pH of 7.26 ± 0.487 mg/L) and no aeration unlike gradual sulfide exposure experiment in which continuous supply of sulfide and aerated freshwater was flowed into the containers until the end of the experiment.

Based on the negative control conducted, the mean DO level dropped from 5.85 ± 0.07 mg/L to 2.83 ± 0.42 mg/L under 12 h. However, Bagarinao and Lantin-Olaguer (1999) reported that the oxygen level dropped to ~1.0 mg/L in experiment of milkfish exposed to sulfide under hypoxic condition which resulted in 100% mortality as early as 3.8 h at sulfide concentration of 960 µg/L (30 µM). Both *B. schwanenfeldii* and *T. tambroides* were observed to survive to more than 12 h whereas milkfish reached 100% mortality at 6.6 h

(Bagarinao and Lantin-Olaguer, 1999). The longer survival time may be due to the low dissolved oxygen level in the water as the time of exposure increased. As the oxygen lowered, the fish increased the rate of water passing through its gills to obtain oxygen thus increasing the sulfide to be taken in (Luther III *et al.*, 2004).

Besides that, mortalities were only observed in sulfide exposure under lowering DO and no mortality was recorded in the negative control. This minimized the cause of mortalities to sulfide toxicity. Aquatic species are more susceptible to diseases and death under the prolong exposure to low oxygen concentration (Wannamaker and Rice, 2000; Luther III *et al.*, 2004). However, hypoxic tolerance is highly related to sulfidic tolerance because sulfide toxicity is exaggerated by low oxygen level in water column (Bagarinao and Lantin-Olaguer, 1999; Tobler *et al.*, 2006). A study in dead-end canals was carried out by Luther III *et al.* (2004) to determine the roles of anoxia and hydrogen sulfide in relation to fish kills. They reported that the most common cause of anoxia and stratification were eutrophication due to high temperature and nutrient concentrations. Eutrophication leads to the increase of organic matter which in turn was decomposed by sulfate reducing bacteria, producing H_2S. Thus, it can be stated that sulfide toxicity aggravates mortality of fish with low oxygen concentrations. The more tolerant the species to hypoxic condition, the more it is believed to tolerate sulfide.

Detoxifying of H_2S cost a lot of energy thus may lead to most of their resources being used in the process. Several studies reported that respiratory adaptations are used by some species in surviving sulfidic environment such as enlargement of gill surface, aquatic surface respiration and switching to anaerobic metabolism (Levitt and Arp, 1991; Bagarinao and Vetter, 1993; Johns *et al.*, 1997; Affonso *et al.*, 2002; Plath *et al.*, 2007). Tobler *et al.* (2009)

reported that species of sulfidic habitat possess respiratory adaptations for both respiration and detoxification of H_2S. Aquatic surface respiration which was also observed in both species in this study, was mostly observed in fishes when exposed to sulfidic habitat to facilitate efficient oxygen uptake. However, for species which are not of sulfidic habitat, this compensatory behavior takes up a lot of energy.

4.4.3 Sulfide Exposure under Lowering pH

Based on the results obtained from sulfide exposure with low pH, it is shown that low pH caused faster and higher mortality rate in both species in comparison to low oxygen. As stated previously, fish exposed to sulfide under low oxygen showed a slight difference in mortality in comparison to fish under sulfide exposure only whereas the difference in fish mortality under low pH is markedly different from exposure to sulfide only.

The mortality of *B. schwanenfeldii* was faster in sulfidic water with lowering pH of concentrations ranging 166-326 µg/L in comparison to sulfidic water only of concentration that ranged from 196 to 350 µg/L. This is also seen in *T. tambroides* where the mortality between sulfidic water with lowering pH of concentrations range 123-333 µg/L was faster than sulfidic water only of concentrations range 144-347 µg/L. A similar observation was reported in a study by Bagarinao and Lantin-Olaguer (1999) and Tobler *et al.* (2006), in which lower pH and sulfidic condition lead to the mortality and reduced diversity of fishes. This was also reported by Luther III *et al.* (2004) that pH was usually recorded lower than 7 due to high concentrations of H_2S in the water and it could go as low as 6.5 which was mainly caused by the H_2S first dissociation constant.

The addition of pH as a manipulated factor in this experiment could question whether the mortalities were due to sulfide or low pH levels itself. However, a negative control had been

carried out prior to the start of sulfide exposure experiment to determine whether the response (mortality) was due to sulfide. The results showed earlier and faster mortality when sulfide was supplied. The relation of sulfide with pH levels is that intensity of sulfide poisoning is greater in acidic water due to the increase of toxic H_2S in the water (Bagarinao and Lantin-Olaguer, 1999). This is because the distribution of H_2S is dependent on the pH of the water. The toxic H_2S is dominant in water with pH below 7 and HS^- is dominant in pH higher than 7 (APHA, 2005; Hughes *et al.,* 2009). Under lower pH levels, the concentrations of H_2S was higher even with lower total sulfide concentrations. This proves that sulfide poisoning is stronger under acidic or lower pH environment as the hazardous H_2S are dominant. Aside from that, acidic water leads to low feeding rates and low hemoglobin concentration (El-Sherif and El-Feky, 2009; Heydarnejad, 2012).

4.4.4 Low DO, Low pH and Sulfidic Environment

By comparing the decline in oxygen and pH throughout both experiments, it can be seen that the decrease of both parameters from start to the end is faster in low pH experiment. As discussed earlier, acidic water allows the presence of toxic H_2S and the stability of sulfide is greatest at acidic pH (Hughes *et al.,* 2009). The lowering of pH value also lead to the decrease in oxygen as oxygen is susceptible in acidic water which can be seen in the results of sulfide exposure under low pH experiment. When oxygen level was low, it gives the perfect condition for sulfate-reducing bacteria to produce sulfide.

The effects of low levels of DO and pH on the intensity of sulfide toxicity had been reported over the years, in natural waters such as in Danau Maninjau (Henny and Nomosatryo, 2012), Mexican cave (Tobler *et al.,* 2006), Delaware Inland Bays (Luther III *et al.,* 2004) and saline lake, Salton Sea (Marti-Cardona *et al.,* 2008; Reese *et al.,* 2008) and laboratory experiments

(Völkel and Berenbrink, 2000; Mann *et al.*, 2004). In all those papers, the sulfide concentration rises under low oxygen and pH concentrations. The eutrophication of lentic water bodies led to an increase in organic matter, low oxygen and pH level thus leading to sulfide production (Luther III *et al.*, 2004; Marti-Cardona *et al.*, 2008; Reese *et al.*, 2008). This further shows that lowering both oxygen and pH at the same time to a critical concentration (DO; 1-2 mg/L, pH 2-3) will increase sulfide toxicity of aquatic life.

4.4.5 Effects of Other Factors in Sulfidic Environment

Besides that, the various habitats the test organisms originate also play a role. In general, sulfide concentration in freshwater is lower than in marine habitat (Völkel and Berenbrink, 2000). The tolerance on sulfide was higher in species of sulfidic habitats compared to species of nonsulfidic habitat. Invertebrates are mostly known inhabiting sulfidic habitats, and they cope with the harsh environment by avoidance of microhabitat with high sulfide levels, substituting to anaerobic metabolism, and denying sulfide from sensitive tissues or oxidizing sulfide to non-toxic forms (Grieshaber and Völkel, 1998; McMullin *et al.*, 2000).

Aside from invertebrates, fishes of sulfidic habitats also exhibit high sulfide tolerance. Several freshwater fishes such as species of family Poeciliidae, however, are able to tolerate high concentrations of sulfide (300 µM) (Tobler *et al.*, 2011). Such species was reported to be inhabiting sulfidic springs in the caves of southern Mexico, and was able to tolerate the harsh sulfidic environment due to their ability to switch to anaerobic metabolism and they also showed morphological differences such as bigger lower lip to aid in aquatic surface respiration (Tobler *et al.*, 2006). *Megalopus atlanticus* and *Hoplosternum littorale* are able to withstand concentrations at approximately 7820 µg/L and 2958 µg/L, respectively (Geiger

et al., 2000; Affonso and Rantin, 2005). This explains why most species of nonsulfidic habitat were unable to survive after continued exposure or abrupt exposure to sulfide.

4.5 Conclusions

Both *Barbonymus schwanenfeldii* and *Tor tambroides* displayed four behavioral responses which were; huddling together, aquatic surface respiration, loss of equilibrium and turn upside down under sulfide toxicity. The LC_{50} at 6 h of *B. schwanenfeldii* was recorded as 507.80 µg/L total sulfide at 95% confidence level with LC_{50} at 485.96 µg/L H_2S at pH 5.54 and DO 2.78 mg/L whereas *T. tambroides* was at 306.10 µg/L total sulfide with 137.75 µg/L H_2S at pH 6.98 and DO 4.29 mg/L. Low oxygen level and low pH level were found to aggravate the toxicity of hydrogen sulfide and led to earlier mortality.

CHAPTER 5

MERCURY ADSORPTION IN *BARBONYMUS SCHWANENFELDII* AND *TOR TAMBROIDES* THROUGH FEED AND WATER

5.1 Introduction

Mercury (Hg) is the only metal that exists as liquid in room temperature and is highly toxic to living organisms (Morel *et al.,* 1998). Hg exists in the environment naturally or is introduced from anthropogenic activities namely smelting of metal ores, burning of fossil fuels and waste incineration (Boening, 2000; Mason *et al.,* 2000; Wang *et al.,* 2004). Hg possesses carcinogenic, mutagenic and teratogenic characteristics. It promotes tyrosinemia causing impairment of pulmonary and kidney function, chest pain and dyspnousea (Berglund and Bertin, 1969). Hg elevation had led to deadly events such as Minamata disease which took place in 1956 in Japan. The outbreak led to the death of aquatic animals, birds, cats and eventually human (Harada, 1995).

Several studies had been done to determine the toxic effects of Hg on fishes (Shah and Altindag, 2005; Kumar and Gupta, 2006; Ishikawa *et al.,* 2007). Although a few studies was done to examine the uptake of Hg through feed and water (Webber and Haines, 2003), the studies on the juvenile stage of fish are still limited.

According to Zillioux *et al.* (1993), wetlands are effective at trapping and releasing Hg; a disturbed wetland showed higher concentration of Hg than undisturbed wetlands. Reports on the rising of mercury concentrations in fish upon reservoir impoundment is alarming. When a reservoir is flooded, the organic-matter is associated with Hg resulting in transformation of inorganic Hg to methylmercury (MeHg), which is highly bioavailable

(Boening, 2000). According to Hylander *et al.* (2006), it could take 20-30 years for the Hg accumulated in fish to return to pre-impoundment concentrations.

Impoundment of rivers created new reservoirs in Sarawak which potentially increases Hg concentrations. The fluctuations of water in reservoirs were associated with hydropower production or flood control which altered the rates of Hg methylation (Driscoll *et al.*, 2007). Besides that, land disturbances such as forest harvesting in reservoirs has been reported to increase total Hg and meHg (Porvari *et al.,* 2003). Both dietary and aqueous exposures are pathways of Hg bioaccumulation or uptake among fishes. Thus, it is crucial to determine which of the two types of exposure would exhibit higher accumulation or uptake. Both *Barbonymus schwanenfeldii* and *Tor tambroides* were selected since they are native species with important economical and domestic value. The objectives of this study were 1) to determine the Hg accumulation in juvenile *B. schwanenfeldii* and *T. tambroides* through the exposure in water and feed, and 2) to record the behavior and survival of both species exposed to Hg through feed and water.

5.2 Materials and Methods

5.2.1 Experimental Design

In this experiment, a plastic tank of 40 cm long × 18 cm wide × 28 cm deep with a total volume of 20 L was used. Compartment made of 1 mm size plastic mesh consisting of three columns was placed in each plastic tank to separate the juveniles into three replicates (Figure 5.1). The tank was filled with 15 L of water and equipped with filtration system. A clear plastic book wrapper in dimensions 50 cm long × 18 cm was placed on top of the tank to reduce the loss of Hg through evaporation.

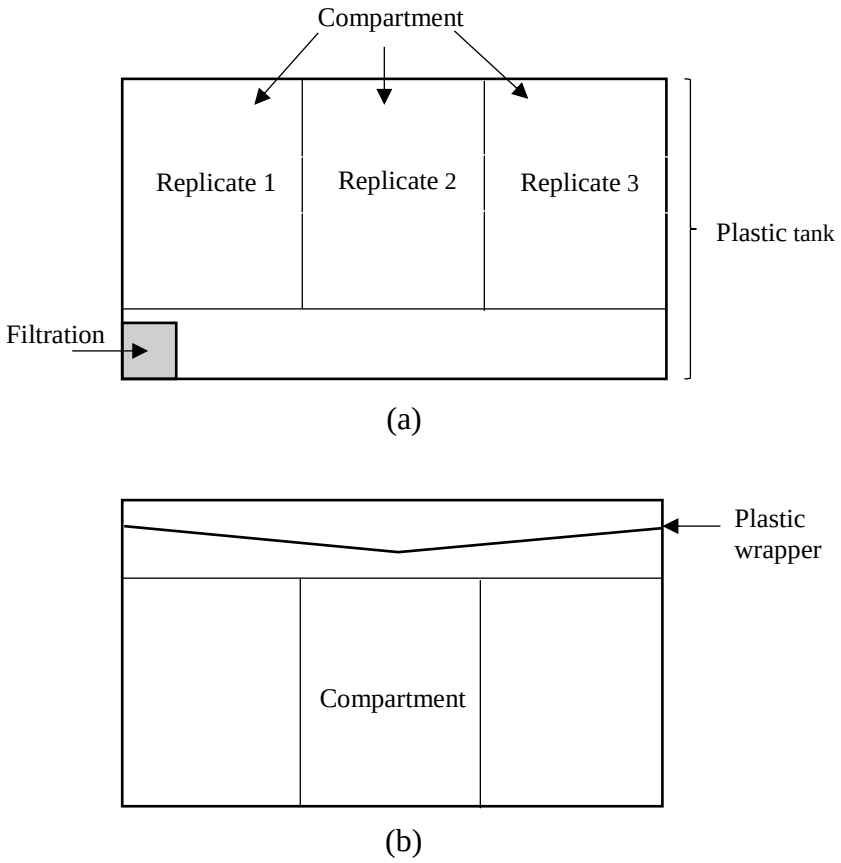

Figure 5.1: (a) Top view of the experimental design, (b) Front view of the experimental design.

A dry run was conducted to determine the possibility of Hg loss through evaporation. The experiment was carried out for 30 days at Hg concentrations of 0.5 and 1.0 mg/L. The concentration of Hg in the water was measured once in every three days.

5.2.2 Mercury Stock Solution Preparation

A stock solution of 50 mg/L Hg was prepared following standard method (APHA, 2014). A total of 0.0677 g of hydrous mercury (II) chloride (Hg(II)Cl) was weighed and dissolved in 1 L of distilled water. The solution was kept in the refrigerator. The stock solution was diluted to the desired concentrations. The stock solution was used to spike the feed to be tested for concentrations later and to be used in the experiment.

5.2.3 Exposure to Hg through Water

A study on exposures of Hg through water was also done by using Hg spiked water in a tank. Two tanks with the same design as shown in Figure 5.1 were prepared, and one tank was used for each species. In this experiment, the water was spiked with Hg to attain concentrations of 0.5 and 1.0 mg/L. Tap water was aerated for 24 hours to remove chlorine. Then, 150 mL and 300 mL of stock solution was added and the water was marked up to 15 L using the aerated tap water.

Ten juveniles of each species *Barbonymus schwanenfeldii* and *Tor tambroides* were placed in each compartment giving a total of 30 juveniles per tank for each concentration level (0.5 and 1.0 mg/L). A set of negative control was also prepared. The concentration of mercury was checked once every three days to ensure the level was maintained whereas feeding was done twice a day at 5% body weight. The feed was analyzed for Hg prior to the experiment. After 30 days, each fish was analyzed for Hg concentration. The body weight, total length

and standard length were also measured. During the experiment, if there is mortality, the dead fish was removed and analyzed for its Hg concentration.

5.2.4 Exposure to Hg through Feed

Few studies of Hg concentrations in feed had been carried out by feeding fish samples with spiked feeds (Berntssen, 2004) or testing commercialized feed for Hg concentrations (Choi and Cech, 1998). The feed in this experiment was spiked with mercury at two concentrations according to Berntssen (2004) where concentrations of 0.5 and 1.0 mg/kg were used. 150 g of feed was weighed and spiked with 150 mL distilled water of 0.5 mg/L mercury concentration. To spike 1.0 mg/kg feed, 150 g of weighed feed was spiked with 150 mL distilled water with 1.0 mg/L mercury concentration. The spiked feed was then kept in the oven at 65 ºC overnight for it to dry completely.

Ten juveniles of each species *B. schwanenfeldii* and *T. tambroides* were stocked into each compartment of a tank with dissolved oxygen fixed at 7.0 mg/L, pH 7 and temperature 27 ºC. The approximate total weight of feed given to the fish was calculated based on the weight of an individual pellet. For each species, one tank was fed with 0.5 mg/L mercury concentration feed, another with 1.0 mg/L mercury concentration feed and another as the negative control which feed untreated. Feeding was done twice daily at 5% body weight and the uneaten pellets were removed immediately. After 30 days, the juveniles were taken for Hg analysis and any dead fish throughout the experiment was removed and analyzed.

5.2.5 Microwave Assisted Digestion

For organic samples such as fish and feed samples, acid digestion was carried out before the analysis for its Hg content. The sample was weighed and the data was recorded. Each sample was mixed with 1 mL of concentrated hydrochloric acid of 37% and 6 mL concentrated nitric

acid of 65% inside Teflon tube. It was then left for 5-10 minutes for the reaction to take place, and the color of the acid had turned brown. Each sample was then digested by using microwave digester (CEM MARS6) to a temperature of 180 °C for 40 minutes. The digested sample was filtered and diluted to 50 mL with deionized water.

Meanwhile for the analysis of water, the samples were filtered with membrane paper (0.45 µm) prior mercury analysis to remove larger debris or sediments. This was to prevent the blockage in the tube during mercury analysis.

5.2.6 Mercury Analysis

The analysis for mercury content in water and digested samples were carried out by using mercury analyzer (FIMS 400). All samples were filtered prior to analyzing in order to prevent blockage in the tube of the machine from any residue in the samples. Calibrations were done with standard solutions of 10, 20 and 30 µg/L (correlation coefficient > 0.99). Then, the samples were analyzed and the results obtained were expressed in ppb. If the samples' concentrations were higher than the calibration curve, the samples were diluted and calculated after analysis. All mercury content data were documented in mg/L for water samples and mg/kg for solid samples.

5.2.7 Disposal of Hg

All the waste water from the experiment was collected and treated before discharge. The wastewater was filtered and flowed through activated carbon. The activated carbon was replaced every three days and disposed in a hazardous waste bag. Prior to discharge of the treated water, the concentration of Hg was analyzed to ensure that it is below the safe levels of 0.5 mg/L. Once the concentration was below the safe level (0.5 mg/L), the treated water

was released. However, if the concentration was higher than the safe level, it was left to be treated until the safe level was reached.

5.2.8 Statistical Analysis

Paired *t*-test and one way ANOVA were used to compare means of water quality parameters in the tanks for all experiment sets. One way ANOVA was also used to compare means of both species' mortality percentage and mercury concentration in both water and fish tissue. The statistical analysis was done by using SPSS software 23 and tables and graphs were plotted by using Microsoft Excel 2013.

5.3 Results

Table 5.1: Water quality parameters in the tank for all experimental sets.

Species (mean ± SD, g)	Experiment sets	Hg levels (ppm)	DO (mg/L)	pH
B. schwanenfeldii (0.59 ± 0.27 g)	Control	0	$6.67 \pm 0.06_{a}$	$6.85 \pm 0.01_{a}$
	Water exposure	0.5	$6.77 \pm 0.06_{a}$	$6.87 \pm 0.03_{ab}$
		1.0	$6.70 \pm 0.20_{a}$	$6.91 \pm 0.02_{b}$
	Feed exposure	0.5	$6.77 \pm 0.06_{a}$	$6.87 \pm 0.02_{ab}$
		1.0	$6.80 \pm 0.10_{a}$	$6.91 \pm 0.03_{b}$
T. tambroides (0.16 ± 0.06 g)	Control	0	$6.63 \pm 0.06_{a}$	$6.89 \pm 0.01_{ab}$
	Water exposure	0.5	$6.63 \pm 0.15_{a}$	$6.92 \pm 0.02_{b}$
		1.0	$6.70 \pm 0.10_{a}$	$6.90 \pm 0.03_{ab}$
	Feed exposure	0.5	$6.63 \pm 0.15_{a}$	$6.88 \pm 0.02_{ab}$
		1.0	$6.73 \pm 0.06_{a}$	$6.89 \pm 0.01_{ab}$

* For each water quality parameter, different letters indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).

A dry run was carried out to ensure that the experimental design reduced the loss of mercury through evaporation. Based on the dry run, the concentrations of Hg in the water remained

the same and no loss was detected. Thus, the experimental design was used to carry out the exposure of Hg to both *B. schwanenfeldii* and *T. tambroides* through water and feed.

Throughout all the experiments, dissolved oxygen and pH levels were maintained to an optimum level at an average of 6.70 ± 0.06 mg/L and 6.89 ± 0.02, respectively for both species (Table 5.1).

5.3.1 Behavioral Responses

Table 5.2: Behavioral responses of *B. schwanenfeldii* and *T. tambroides* during Hg experiments.

Species	Experiment	Hg	Avoidance	Time taken to feed
B. schwanenfeldii	Control	0.0 mg/L	Not observed	Immediate
	Water exposure	0.5 mg/L	Not observed	Immediate
		1.0 mg/L	Dispersed when fed	Moderate
	Feed exposure	0.5 mg/kg	Not observed	Moderate
		1.0 mg/kg	Not observed	Moderate
T. tambroides	Control	0.0 mg/L	Not observed	Moderate
	Water exposure	0.5 mg/L	Dispersed when fed	Moderate
		1.0 mg/L	Dispersed when fed	Slow
	Feed exposure	0.5 mg/kg	Not observed	Moderate
		1.0 mg/kg	Not observed	Moderate

In this experiment, behavioral responses were observed during feeding (Table 5.2). The results presented were in terms of 1) avoidance during feeding, and 2) time taken to feed on the pellet. The time taken to feed were described in three ways, 1) immediate, fish feed as soon as being fed; 2) fish feed after 1 minute of being fed, and 3) fish feed after 2 minutes of being fed. In terms of avoidance, both species were observed to disperse during feeding especially in the experiment of Hg exposure through water at 1 mg/L. In the experiments of

control and Hg exposure through feed, no avoidance was observed in both species during feeding.

5.3.2 Survival Percentage

The survival of the juveniles of both species were recorded at the end of the experimental period and presented in percentage in Figure 5.2 and Figure 5.3. Both species recorded significantly higher percentage of survival in experiments of Hg exposure through water than feed ($p < 0.05$). For *B. schwanenfeldii*, 67% survival was recorded in the control, 63% in experiment of Hg exposure through water at 0.5 mg/L and 53% for that at 1.0 mg/L. However, Hg exposure through feed recorded 57% and 47% survival at exposure level of 0.5 and 1.0 mg/kg Hg, respectively. The lowest survival percentage for *B. schwanenfeldii* was recorded when under feed exposure of 1.0 mg/kg Hg, while the highest survival recorded was the control.

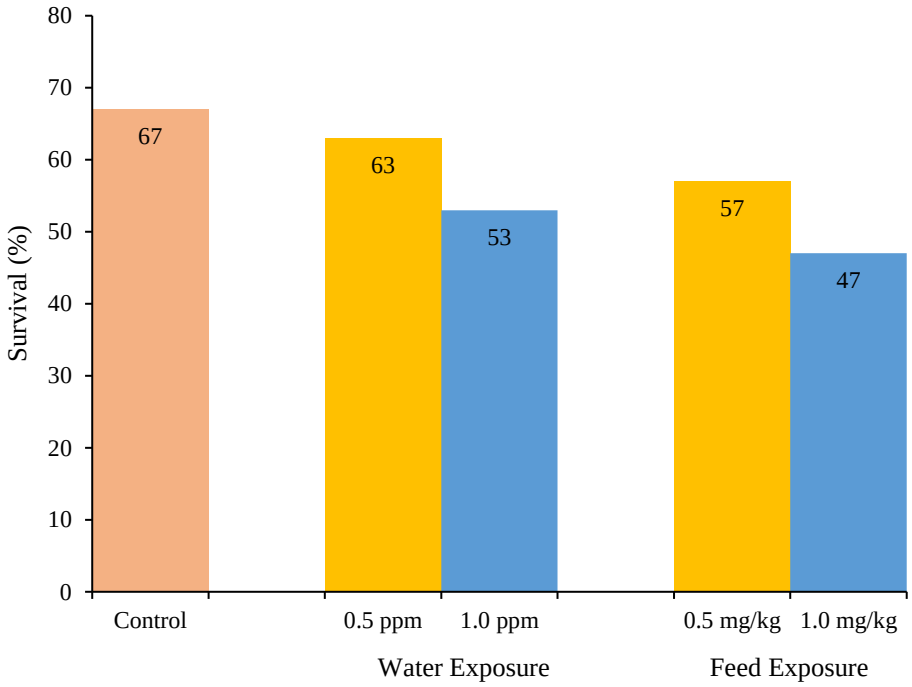

Figure 5.2: Percentage of survival for *B. schwanenfeldii* juveniles in control, water exposure and feed exposure sets ($p < 0.05$).

T. tambroides on the other hand recorded 90% survival for the control. In experiments of Hg exposure through water at 0.5 and 1.0 mg/L, 33% and 90% of survival were recorded, respectively. For the feed exposure, 0.5 mg/kg Hg concentration recorded 60% mortality and 57% at 1.0 mg/kg mercury concentration. For *T. tambroides*, the highest survival percentage was during exposure of juveniles to feed of 1.0 mg/kg Hg and the lowest was at exposure through feed at 0.5 mg/kg Hg.

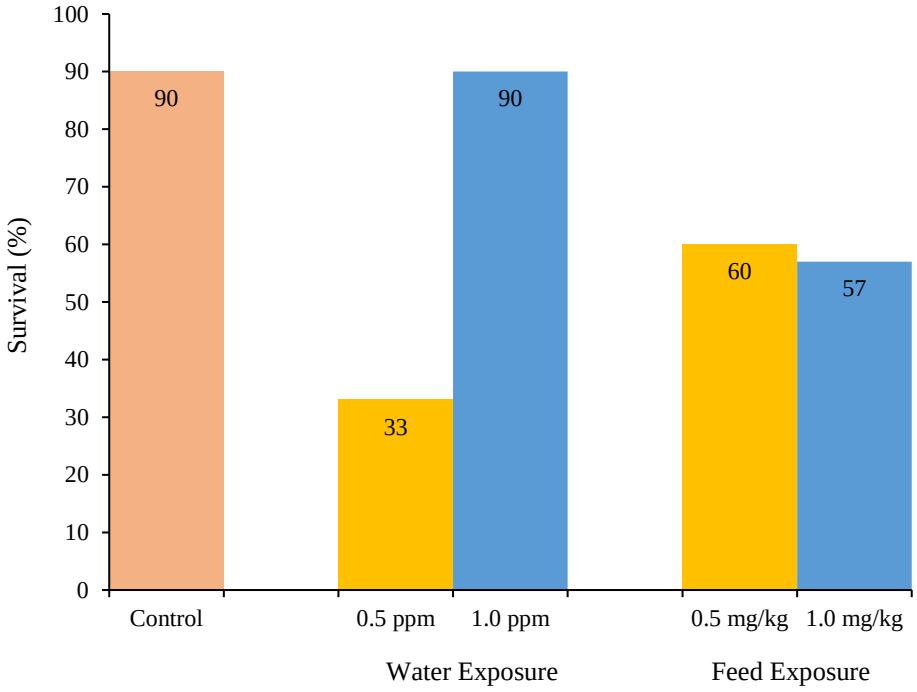

Figure 5.3: Percentage of survival for *T. tambroides* juveniles in control, water exposure and feed exposure sets ($p < 0.05$).

5.3.3 Bioaccumulation of Mercury (Hg)

A dry run was carried out prior to the onset of the experiments to observe the volatility of Hg whether there is any significant loss of Hg through evaporation. Based on the results, the concentration of Hg in the water was maintained throughout the 30 days suggesting no significant loss of Hg.

The mercury concentrations in the juveniles were analyzed to provide insights on the accumulation potential. In *B. schwanenfeldii*, an average of 0.234 ± 0.063 mg/kg was detected in samples exposed to 0.5 mg/L of Hg in water. At higher concentration of 1.0 mg/L, 0.457 ± 0.246 mg/kg of Hg was found in the juveniles (Figure 5.4). Meanwhile, *T. tambroides* accumulated 0.422 ± 0.248 mg/kg and 0.700 ± 0.475 mg/kg of Hg when they are exposed to water containing 0.5 and 1.0 mg/L Hg, respectively (Figure 5.5). Based on the results in Figure 5.4 and Figure 5.5, it can be observed that the Hg accumulated in both species increased according to the level they are exposed to.

For Hg exposure through feed at 0.5 and 1.0 mg/kg, *B. schwanenfeldii* was observed to accumulate 0.054 ± 0.037 mg/kg and 0.017 ± 0.009 mg/kg, respectively. *T. tambroides* on the other hand accumulated 0.100 ± 0.042 mg/kg and 0.109 ± 0.061 mg/kg of Hg when they are fed with feed containing 0.5 and 1.0 mg/kg Hg. Unlike the previous experiment of Hg exposure through water, there is no apparent rise in the Hg accumulated despite the increase in Hg concentration in feed for both species.

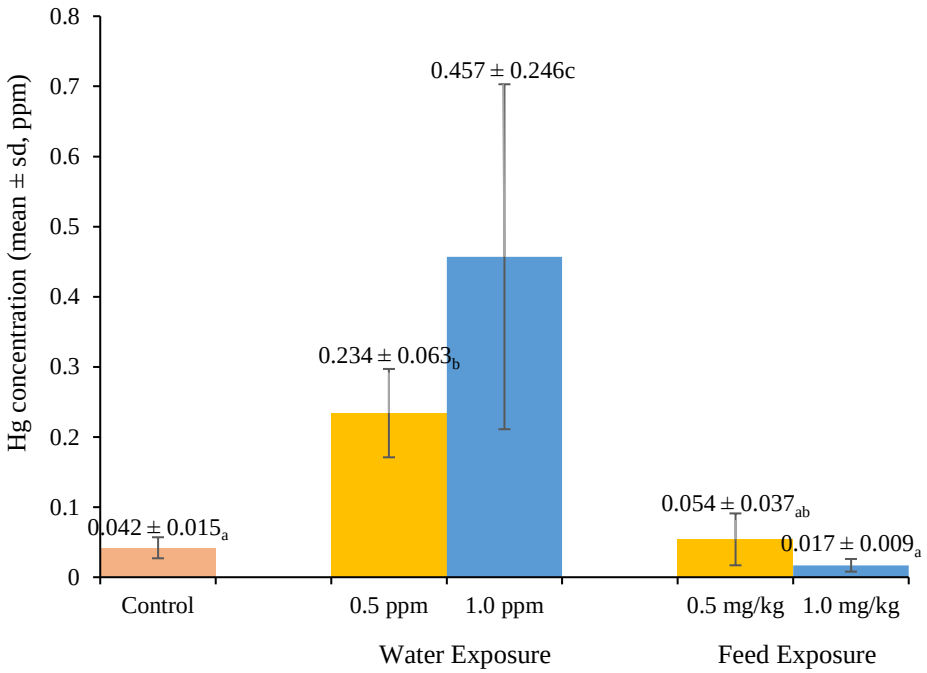

Figure 5.4: Mercury concentrations of *B. schwanenfeldii* juveniles in control, water exposure and feed exposure sets ($p < 0.05$).

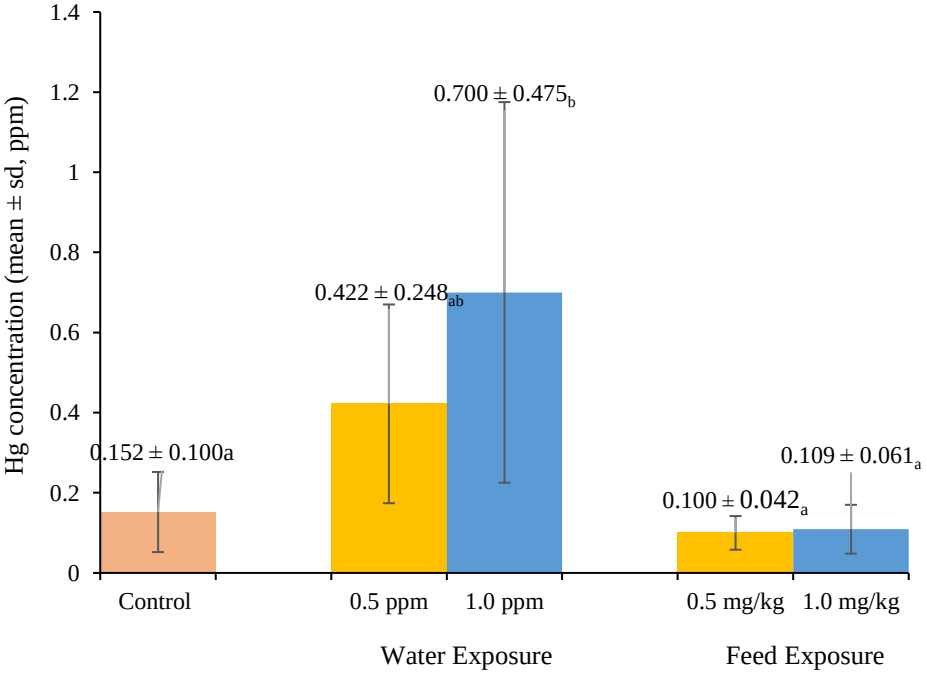

Figure 5.5: Mercury concentrations of *T. tambroides* juveniles in control, water exposure and feed exposure sets ($p < 0.05$).

5.4 Discussion

5.4.1 Behavioral Responses

Both species reared in water with 1.0 mg/L Hg were observed to disperse when fed (presence of human during feeding). However, no particular distinct responses were observed in experiments of Hg exposure through feed. The behavioral response of dispersion was supported by a study on the effect of mercury by Webber and Haines (2003). The behavior of predator-avoidance was similarly reported in golden shiner (*Notemigonus crysoleucas*) exposed to water spiked with mercury. The golden shiners were observed to disperse upon released of predator, suggesting increase in the vulnerability towards predation.

However, a latter study done on freshwater fish (*Brycon amazonicus)* under the exposure of inorganic $HgCl_2$ in water found hyperactivity and aggressiveness, loss of equilibrium and scales as well as pigmentation (Monteiro *et al.*, 2010). The differences in behavioral response between this study and that reported by Monteiro *et al.* (2010) could be due to the exposure period, exposure level and the way the experiment is managed. Monteiro *et al*. (2010) carried out a sublethal short term experiment of 96 h under the exposure concentrations ranged from 0.57-1.20 mg/L where the fish samples were not fed 24 h prior the start of experiment and during the experiment. This showed that exposure of Hg through water could lead to distinct behavioral responses in fishes resulting in vulnerability to predation.

5.4.2 Survival Percentage

Based on the results of the survival percentage, the survival of both species were higher in experiments of Hg exposure through water. Monteiro *et al.* (2010) reported LC_{50} of 0.71 mg/L at 95% confidence limit for *Brycon amazonicus* exposed to Hg in a static bioassay

system for 96 h. *B. schwanenfeldii* recorded 53% survival under 1.0 mg/L Hg exposure in water which suggests that *B. schwanenfeldii* has a higher tolerance to Hg than *Brycon amazonicus*. Batang Ai hydroelectric reservoir recorded 0.451 ± 0.503 µg/L Hg in the water on average which is suitable for the survival of both species (Sim *et al.,* 2014).

It was found that higher survival percentage occurred in water exposure experiment, and higher accumulation of mercury was also recorded in water exposure experiment compares to feed exposure experiment. Accumulation or uptake of Hg into fishes had been studied and documented over the years (Chan *et al.,* 2003; Da Silva *et al.,* 2005; Hylander *et al.*, 2006). When exposed to HgCl(II), fathead minnows showed 52% survival at an average concentration of 4.51 µg/L HgCL(II) (Snarski and Olson, 1982). In this experiment, the highest concentration was recorded at water exposure of 1.0 mg/L in *T. tambroides* with a value of 0.700 mg/L. Rather than mortalities, Hg was often reported to affect reproduction, growth and immune system (Snarski and Olson, 1982; Friedmann *et al.,* 1996).

5.4.3 Bioaccumulation of Mercury (Hg)

Exposure of both *B. schwanenfeldii* and *T. tambroides* to Hg causes an increase of Hg concentration in the juveniles. Due to their smaller size as juveniles, the entire fish was digested for its Hg concentration.

Following the results obtained in Figure 5.4 and Figure 5.5, Hg concentration in fish tissue were found to be lower in feed exposure experiment for both species. The amount of Hg is greatly dependent on the trophic status of the fishes (Kidd *et al.,* 1995; Mason *et al.,* 2000) and the bioaccumulation of trace elements into primary producers is highly influenced by water chemistry as it is the basis of the food chain thus in turns determines the accumulated concentrations in the higher trophic organisms (Mason *et al.,* 2000). This implies that the

higher the trophic status of the fish, the higher the amount of Hg bioaccumulated. In the feed exposure experiment, the primary producers are described as the spiked feed and the juveniles themselves as the first consumer.

It is reported in a study that dietary uptake dominates mercury accumulation at the higher hierarchy whilst aqueous uptake dominates Hg accumulation in the lower hierarchy (Wang and Wong, 2003). The dietary ingestion is strongly related to the type of prey ingested and the feeding rate of individual fish. Besides, the loss of dietary mercury (Hg(II)) was found faster than the aqueous Hg(II) due to the process of digestion. This might be due to the effective barrier of the intestinal walls towards mercury chloride in which mercury chloride was filtered and excreted, thus inhibiting successful uptake into the fish (World Health Organization, 1989). This explained the low Hg concentration of the juveniles of both *B, schwanenfeldii* and *T. tambroides* in the feed exposure experiment.

Another possible cause of lower Hg concentration in fish tissue for feed exposure experiment is the type of Hg used. Berntssen *et al.* (2004) whom studied MeHg and inorganic Hg in fishes found that the assimilation rate of MeHg is higher than that of inorganic Hg. In this study, inorganic Hg was used throughout the Hg exposure experiment. This may explain the low Hg concentration in both species tissue in feed exposure experiment compared to water exposure experiment. The explanation was also supported by a study on rainbow trout (*Oncorhynchus mykiss*), that the intestine forms a barrier for oral uptake of inorganic Hg while oral MeHg is readily transported over the intestinal tract (Handy and Penrice, 1993).

The earlier result showed higher concentration of Hg in fish tissue in water exposure experiment as compared to feed exposure experiment. A study done by Bebianno *et al.* (2007) on *Aphanopus carbo* showed that Hg present in gills was quickly removed to the

circulation system, then into the liver and kidney where it could be retained for a longer time. This pathway of mercury from gills to liver was also proposed by McGeer *et al.* (2000) where increased metallothionein levels was detected in the liver. This may explain the higher concentration of Hg in fish tissue due to the removal of Hg from gills to fish organs and tissue. Other factors affecting the concentration of Hg in fishes includes age, gender and pH levels (Boening, 2000). As the fish gets older, the size and length increased thus increasing the muscle available for accumulation of Hg. According to World Health Organization (1989), the males accumulate higher Hg compared to females in some species. Trudel and Rasmussen (1997) on the other hand summarized that the elimination rate of Hg was highly dependent on temperature. This is caused by the higher metabolic rate in aquatic organisms of larger size or that lived in water bodies of higher temperature. The increase in metabolic rate lead to increase of elimination process of mercury in the organisms. Besides that, chemicals toxicity is highly correlated with temperature (Heath, 1994), which means that toxicity of mercury increased in a higher water temperature.

The bioaccumulation of Hg in *B. schwanenfeldii* were found to be lower than *T. tambroides* in Hg exposure through water. Few studies relate weight or mass of fish to the accumulation of Hg (Braune, 1987; Bosch *et al.*, 2016). However, those studies involved piscivorous adult species in which biomagnification could occur in them which resulted in higher mercury concentrations. This study used juveniles and the fishes were at a low trophic level (primary consumer) in which biomagnification did not occur as there is no food chain but only primary uptake. Thus, the concentration in the tissue was not high.

Higher accumulation in water exposure than feed exposure could be due to the respiration where aqueous Hg passing through the gills into the blood stream. Lower concentration

accumulated in *B. schwanenfeldii* in comparison to *T. tambroides* was likely due to the size of the fish whereby *B. schwanenfeldii* is bigger than *T. tambroides.* Smaller sized fish were said to have higher respiration rate in order for faster growth (Kaufmann, 1990). An increase in respiration rate allowed more water to pass through the gills to allow more oxygen uptake (Randall, 1982; Sollid *et al.,* 2003). However, since the water had been spiked with Hg, it could also lead to increased aqueous Hg passing through the gills into the blood stream and thus higher accumulation was detected in the tissue.

5.5 Conclusions

Upon exposure to mercury in water, both *Barbonymus schwanenfeldii* and *Tor tambroides* were observed to exhibit avoidance when fed and the time taken to feed was also longer. The survival of both species in water exposure was higher than in feed exposure. The mercury concentrations in fish tissue was also higher in water exposure experiment than feed exposure. The adsorption of inorganic mercury in the spiked feed into fish tissue was low possibly due to the fish selective barrier in their intestine to filter inorganic mercury.

CHAPTER 6

GENERAL CONCLUSION AND RECOMMENDATIONS

6.1 General Conclusion

Overall, it was found that alterations in concentrations of DO, TSS, pH, temperature, sulfide and mercury lead to behavioral response and affected both the survival and/or the growth of *B. schwanenfeldii* and *T. tambroides*. However, the tolerance levels or concentrations of the tested factors or parameters were species dependent. For instance, *B. schwanenfeldii* could survive in wider range of TSS levels while *T. tambroides* could survive in limited range of TSS levels in water. Factors such as temperature could lead to larger length increase in fishes but could also affects their survival rate negatively. Combinations of tested parameters at different levels should allow for the considerations that these factors may react and affect each other. For example, low DO levels provide optimal condition for production of sulfide and could lead to earlier responses from fishes.

Response surface methodology was used to estimate the best combination of DO, TSS, temperature and pH but this modeling technique could give different results from observation. *B. schwanenfeldii* was predicted to survive the best in 27 ºC, 0 mg/L TSS, 4.8 mg/L DO and pH 7.04 whereas *T. tambroides* would survive the best in 23.9 ºC, 0 mg/L TSS, 4.5 mg/L DO and pH 7.02. Gradual sulfide exposure allowed for estimation of LC_{50}. At 6 h, LC_{50} of *B. schwanenfeldii* was recorded as 507.8 µg/L sulfide at 95% confidence level with LC_{50} at 490.15 µg/L H_2S at pH 5.54 whereas *T. tambroides* was at 306.1 µg/L sulfide with 220.4 µg/L H_2S at pH 6.98. Exposure of mercury through water was found to affect primary consumer fishes evidenced with higher bioaccumulation in tissue than the exposure through spiked feed.

Avoidance and delayed feeding time were displayed by the fishes when under stress of TSS, low DO and mercury exposure. Besides that, four behavioral responses were also observed in the tested species when under sulfide exposure.

6.2 Recommendations

This study allowed us to document the mortality and behavioral responses of both species to changes in water quality and elevations of hydrogen sulfide and exposure to mercury. However, there are some recommendations for future research.

It is recommended that the water quality experiment to be carried out for a longer duration. This will give a more refined and better results on growth of fish between the experiments. For both hydrogen sulfide and mercury experiments, adult fishes could be used instead of juveniles. By doing so, the samples could be assessed based on specific tissues such as muscle, stomach and gills, and even blood.

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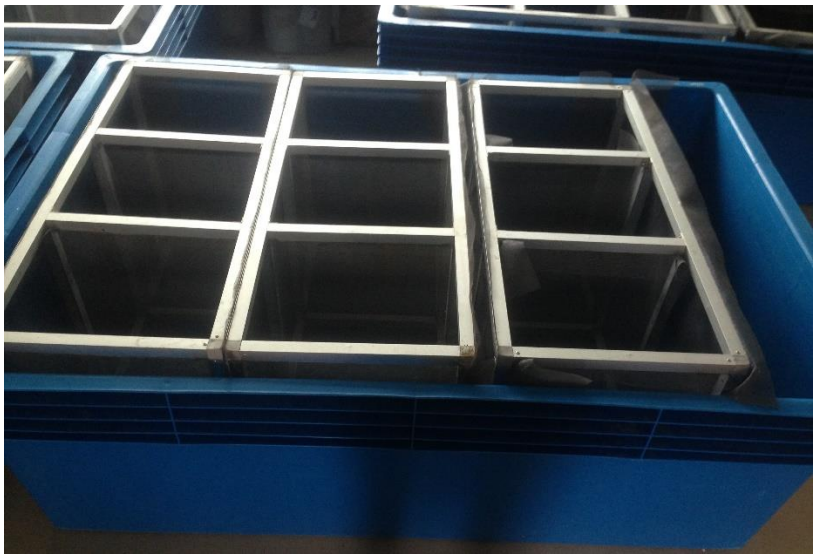
APPENDICES

Water quality study cage design.



Water quality study tank design.

Experimental container in hydrogen sulfide experiment.

Water reservoir tank in hydrogen sulfide experiment.

Hydrogen sulfide experimental design.

Mercury exposure experiment.

Mercury exposure experiment.