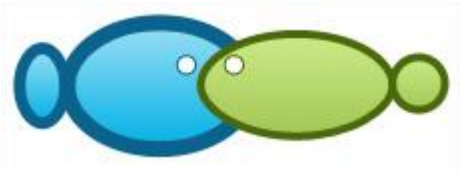

# Impacts of sulfide exposure on juvenile *Tor tambroides*: behavioral responses and mortality

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**Abstract**. Construction of hydroelectric reservoirs had been reported to be the cause of increased sulfide levels resulting from the decomposition of organic matter. As more dams are being built, a better understanding of the impact of sulfide on indigenous species is required. In Sarawak, *Tor tambroides* is a highly valuable and sought after species which is facing declining population. This study aimed to determine the behavioral responses and mortality of juvenile *T. tambroides* exposed to sulfide. The three exposure experiments were gradual sulfide exposure, gradual sulfide exposure under lowering DO and gradual sulfide exposure under lowering pH. A modified flow-through design was used to expose the juveniles in containers to sulfide of different concentrations. Actual total sulfide in containers was determined according to standard method. During the duration of the experiment, behavioral responses, DO and pH were monitored. Experimental results show that negative controls recorded no behavioral response and no mortality was observed in all control experiments. However, under all sulfide exposure experiments, the juveniles displayed at least one behavioral response in the progression of huddling together, aquatic surface respiration, loss of equilibrium and turning upside down except for the gradual sulfide exposure experiment where no response was observed with the lowest total sulfide concentration tested (82 μg $L^{-1}$). For all three exposure experiments, faster responses and mortalities were observed when the concentration of sulfide increased. The $LC_{50}$ at $6^{th}$ hour of exposure was estimated to be 306 μg/L total sulfides (138 μg $L^{-1}$ $H_2S$) at 95% confidence level. Sulfide toxicity was found to be highly related to the decreasing DO and pH levels attributable to intensifying toxicity which led to mortality.
**Key Words**: tolerance, hydrogen sulfide, gradual sulfide exposure, negative controls, toxicity.

**Introduction**. Over the years, anthropogenic activities are said to be one of the major factors in the disruptions of aquatic habitat. Construction of reservoirs for example, fragmented rivers and become an obstacle for longitudinal exchanges such as recycling, water chemistry and migration (Brismar 2004; Mc Cartney 2009). In Sarawak, three major hydroelectric reservoirs had been built for the purpose of energy production over the past 30 years namely Batang Ai Hydroelectric Reservoir (1985), Bakun Hydroelectric Reservoir (2010) and Murum Hydroelectric Reservoir (2014). The alteration of lotic water bodies into lentic could lead to the increase of sulfide and lowering of dissolved oxygen (DO) and pH levels in the water bodies. The increase of sulfide in water bodies had been discussed by several papers such as in Bakun Hydroelectric Reservoir, Malaysia (Nyanti et al 2012), Batang Ai Hydroelectric Reservoir, Malaysia (Ling et al 2012), Danau Maninjau, Indonesia (Henny & Nomosatryo 2012), and the effect of hypoxic condition and low pH levels with sulfide in nature water bodies (Tobler et al 2006).

Hydrogen sulfide emits rotten egg smell and high levels are toxic to both environment and living organisms. It is introduced into aquatic habitat through runoff or the decaying process of organic matters (Guidotti 1996) and can also be found in aquatic environment associated with oil deposits and geothermal activity (Van Dover 2000; Tunnicliffe 1991). Hydrogen sulfide affects fishes by binding to haemoglobin and replacing oxygen (Tobler et al 2006), interacting with essential enzymes (Affonso et al 2002) and disrupting disulfide bonds in macromolecules (Guidotti 1996).

Locally known as Kelah, *Tor tambroides* is one of the most expensive and sought after freshwater species for both game and food fish in the region (Soon et al 2014). It is highly sensitive and is said to only inhabit clean and clear fast flowing waters and feed on riverine fruits. Its population is declining in the wild due to habitat degradation and overfishing (Ingram et al 2005). However, knowledge on the effects of sulfide on the behavior and survival of such indigenous fish is unknown. This calls for better understanding of the response of this indigenous fish to changes in environmental condition such as sulfide so that appropriate conservation efforts could be implemented. Thus, the objective of this study was to determine the tolerance of indigenous species *T. tambroides* to sulfide.

**Material and Method**

***Juveniles' collection and acclimatization***. Juveniles of *T. tambroides* were collected from the Inland Fishery Branch of the Department of Agriculture in Tarat of Serian district. Prior to the transfer from Tarat to the laboratory, the juveniles were not fed to avoid mortality during the transfer. Upon reaching the laboratory, the juveniles were acclimatized by adding 25% of the water volume in the bag holding the samples with water from the holding tanks and were then left to acclimatize for 30 minutes. After 30 minutes of the acclimatization period, the juveniles were transferred into the holding tanks where the juveniles were left for a week prior to experiment to prevent stress and fed twice a week. The average weight was obtained by weighing a total of thirty juveniles by a weighing balance (AND, GH-252).

***Experimental design***. The experimental design follows a flow-through bioassay by Apendi et al (2018) which was modified from the design of Bagarinao & Lantin-Olaguer (1999). Tested juveniles were kept in a 15 L airtight containers filled with 12 L aerated freshwater during the experiments. Aerated freshwater was supplied into the containers at 100 mL min $L^{-1}$ and sulfide stock solution at 5 mL min $L^{-1}$ (Figure 1).

Figure 1. The experimental setup for testing the effects of sulfide exposure on juveniles of *Tor tambroides* in the laboratory.

To allow the flow of the freshwater and sulfide by gravity, both supplies were elevated higher than the experimental containers and their flow rates were controlled using control

valves. The flow rates were calibrated prior to the start of each experiment. Temperature of water was kept at a range of 26-28°C. All experiments including negative control were carried out for 12 h or until all fish died, whichever comes first.

***Positive control***. To determine the suitable sulfide stock solutions and time period of the experiment, a positive control was firstly carried out. The same modified flow-through design was used with 10 juveniles in each container. However, different sulfide concentrations were tested until suitable result was obtained. A total of six sulfide stock solutions were then decided (6.25, 13.50, 19.75, 27.00, 33.25, 40.50 µg $L^{-1}$) and the experiment was carried out for 12 hours.

***Sulfide solution preparation***. Stock solutions of six different concentrations (6.25, 13.50, 19.75, 27.00, 33.25, 40.50 µg $L^{-1}$) were prepared fresh daily in three replicates each for the experiments. The preparation of sulfide stock solution was done following the standard method (APHA 2005). If any yellow discoloration was present on the crystals, they were thoroughly washed and dried prior to dissolving. 0.1 mg $Na_2S.9H_2O$ crystals in 2 L nitrogen-bubbled water to prepare 6.25 µg $L^{-1}$ of sulfide stock solution. The nitrogen-bubbled water was prepared by flowing nitrogen gas through the water for 30 minutes which reduced the dissolved oxygen level to 1-2 mg $L^{-1}$. Each sulfide stock solution was supplied with nitrogen gas in a balloon to reduce the oxidation of sulfide. For each tested concentration, three containers were used as replicates and filled with 12 L aerated freshwater and stocked with ten juveniles each.

***Experiment 1: Gradual sulfide exposure***. This experiment was carried out from August-September 2015 with the juveniles weighing at an average weight of 0.60±0.07 g. Gradual sulfide exposure experiment started with the flow of aerated freshwater at the rate of 100 mL min $L^{-1}$ and sulfide at the rate of 5 mL min $L^{-1}$ into the containers containing juveniles. Both DO and pH were recorded at 1-hour interval until the end of experiment or until all juveniles died, whichever came first.

***Experiment 2: Gradual sulfide exposure under lowering DO***. Gradual sulfide exposure under lowering DO was carried out in January 2016 with juveniles weighing 0.80±0.06 g. For this experiment, aerated freshwater and aeration was cut off to allow the lowering of DO levels with sulfide being supplied into the containers with juveniles. Prior to the start of the experiment, each container that had been stocked with juveniles was supplied with aerated freshwater (100 mL min $L^{-1}$) and aeration. After 12 hours, both supplies were cut off and sulfide began to be channeled into each container at 5 mL min $L^{-1}$, indicating the start of the experiment. Both DO and pH levels were recorded at one-hour interval and the time of death was also recorded until the end of the experiment. For the negative control, no sulfide was flowed into the containers after the cut-off of aerated freshwater and aeration supplies.

***Experiment 3: Gradual sulfide exposure under lowering pH***. Gradual sulfide exposure under lowering pH was carried out in February 2016 with juveniles weighing 0.83±0.06 g. For this experiment, aerated freshwater, 10% sulfuric acid, and sulfide were flowing at 100 mL min $L^{-1}$, 5 mL min $L^{-1}$, 5 mL min $L^{-1}$ respectively into each of the containers containing the juveniles. Unlike the previous two experiments, both DO and pH were recorded at half an hour interval until all fishes died due to the fast mortality of juveniles in this experiment. The negative control was done with aerated freshwater and sulfuric acid but without the supply of sulfide.

***Behavioral responses***. Throughout all the three experiments, four behavioral responses were monitored and recorded hourly from the start until the end of the experiments namely, huddling together, aquatic surface respiration (ASR), loss of equilibrium and turn upside down.

***Water sampling***. For Experiment 1 and Experiment 2, water samples were taken at the 6$^{th}$ hour after the start of the experiment whereas, the taking of water samples for Experiment 3

was carried out at the 3rd hour of experiment due to the faster mortality response. Water sampling was done at the same spot (height) for all containers to avoid biased results.

***Sample analyses***. Water samples collected were analyzed as soon as possible to avoid oxidation of sulfide in the samples. Methylene blue method (HACH, 2014) was used to analyze the actual sulfide concentrations from the water samples collected. The absorbance value was the read by using a spectrophotometer (HACH, DR 2800). Dissolved oxygen was taken by using EXTECH DO Meter/Datalogger SDL150 and pH was taken by using EXTECH pH/ORP Meter SDL100v.

***Statistical analysis***. Paired *t*-test and one-way ANOVA were used to compare the means of time taken to 50% and 100% mortality and total sulfide concentrations between experimental sets. The $LC_{50}$ of the juveniles at 6th hour in Experiment 1 was plotted at 95% confidence limit. All statistical analyses in this study were carried out by using SPSS version 23.

## Results

***Behavioral responses***. Throughout the all the sulfide exposure experiments, four behavioral responses namely huddling together, aquatic surface respiration, loss of equilibrium and turning upside down were observed and recorded for each tested concentrations (Table 1). No responses were observed in all negative controls and gradual sulfide exposure experiment at 82 µg $L^{-1}$ total sulfide. At 144 µg $L^{-1}$ total sulfide in gradual sulfide exposure, only huddling together was displayed by the juveniles. However, all four responses were observed in all other concentrations of all three experiments. For each sulfide exposure experiment, earlier display of responses was seen in higher sulfide concentrations.

Table 1

Time taken from the beginning of the experiment to the occurrence of a behavioral response of *Tor tambroides* in sulfide exposure experiments

| *Supplied sulfide solution (µg $L^{-1}$)* | *Sulfide in container (µg $L^{-1}$)* | *Time taken for detection of response (h)* | | | |
|---|---|---|---|---|---|
| | | *Huddle together* | *ASR* | *Loss of equilibrium* | *Turn upside down* |
| Experiment 1: Gradual sulfide exposure | | | | | |
| 0 | 0 [a] | n.d. | n.d. | n.d. | n.d. |
| 6.75 | 82±10 [b] | n.d | n.d. | n.d. | n.d |
| 13.50 | 144±27 [abc] | 8 | n.d. | n.d. | n.d |
| 20.25 | 210±4 [de] | 6 | 7 | 9 | 11 |
| 27.00 | 347±18 [gh] | 4 | 5 | 6 | 7 |
| 33.75 | 515±28 [i] | 3 | 4 | 5 | 6 |
| 40.50 | 658±6 [j] | 2 | 4 | 5 | 5 |
| Experiment 2: Sulfide exposure under lowering DO | | | | | |
| 0 | 0 [a] | n.d. | n.d. | n.d. | n.d. |
| 6.75 | 107±36 [ab] | 12 | 12 | n.d | n.d |
| 13.50 | 136±38 [abc] | 11 | 12 | n.d | n.d |
| 20.25 | 229±24 [e] | 6 | 7 | 9 | 10 |
| 27.00 | 251±24 [ef] | 6 | 7 | 8 | 9 |
| 33.75 | 321±63 [fg] | 5 | 6 | 6 | 7 |
| 40.50 | 429±36 [h] | 4 | 4 | 5 | 6 |
| Experiment 3: Sulfide exposure under lowering pH | | | | | |
| 0 | 0 [a] | n.d. | n.d. | n.d. | n.d. |
| 13.50 | 84 ± 27 [b] | 2.5 | 3 | 3.5 | 4.5 |
| 20.25 | 123 ± 4 [ab] | 2.5 | 3 | 3.5 | 4 |
| 27.00 | 170 ± 23 [cde] | 1.5 | 2 | 3 | 3.5 |
| 33.75 | 218 ± 24 [de] | 1 | 1.5 | 2 | 2 |
| 40.50 | 333 ± 33 [fg] | 0.5 | 1 | 1 | 1.5 |

n.d. = not detected during the 12-hour experimental duration; The same lowercase superscripts indicate no significant difference (p>0.05) between all containers total sulfide concentrations.

***Gradual sulfide exposure***. Based on Table 2, the negative control showed no mortality throughout the experimental period. Upon the flow of sulfide into the containers, as the concentration in the container increased, the time taken to the occurrence of mortality shortened. At 658 µg $L^{-1}$ total sulfide, it only took 6 hours to observe the occurrence of 50% mortality and 6 hours to observed 100% mortality of the juveniles. However, in lower concentrations of 82-144 µg $L^{-1}$, the juveniles survived the 12 h period of exposure.

Table 2

Time to mortality of *Tor tambroides* juveniles exposed to different sulfide levels and negative control

| *Supplied sulfide solution (µg $L^{-1}$)* | *Sulfide in container (µg $L^{-1}$)* | *Time to mortality (h)* | |
|---|---|---|---|
| | | *50%* | *100%* |
| 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 |

Probit regression of $LC_{50}$ was plotted at the 6$^{th}$ hour of exposure (Figure 2) and estimated the $LC_{50}$ of *T. tambroides* as 306.1 µg $L^{-1}$ at 95% confidence level.

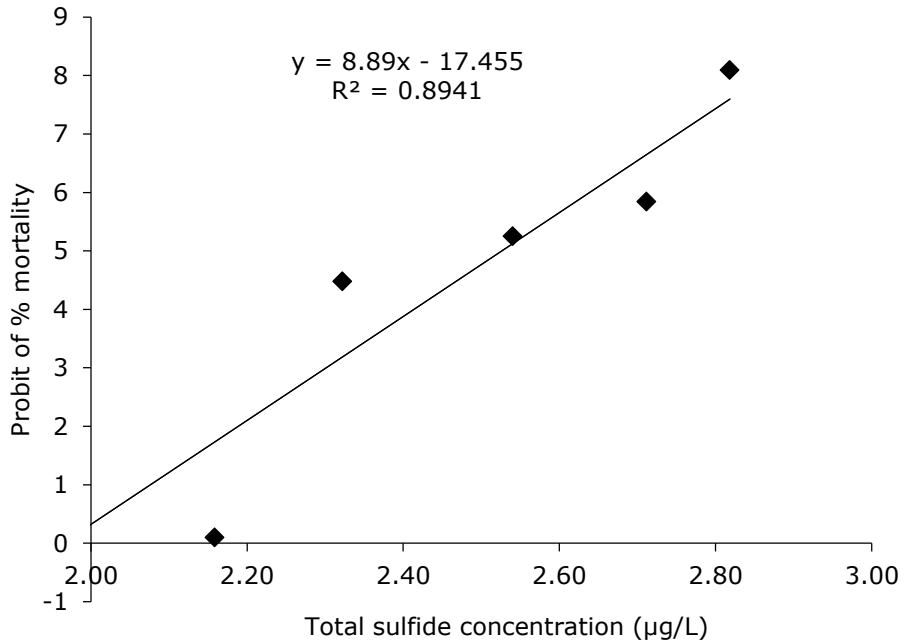


Figure 2. Probit regression of percentage of mortality against log of total sulfide concentration to obtain $LC_{50}$ at 6 h for *Tor tambroides.*

***Sulfide exposure under lowering DO***. In this experiment, the negative control showed decline in DO from 5.80 mg $L^{-1}$ to 3.13 mg $L^{-1}$ and pH from 7.38 to 4.87 from the start to the end of the experiment (Figure 3). No mortality was observed throughout the 12-h period in negative control. The mortalities of gradual sulfide exposure experiment and sulfide exposure under lowering DO experiment were depicted in line graphs and compared based on the accumulated sulfide concentrations that are about the same range in Figure 4. Based on the comparison, no apparent difference was observed in the time of exposure to 100% mortalities between the experiments. However, DO in sulfide

exposure under lowering DO experiment decreased to lower level than the gradual sulfide exposure experiment due to the cut-off of aeration and freshwater supplies.

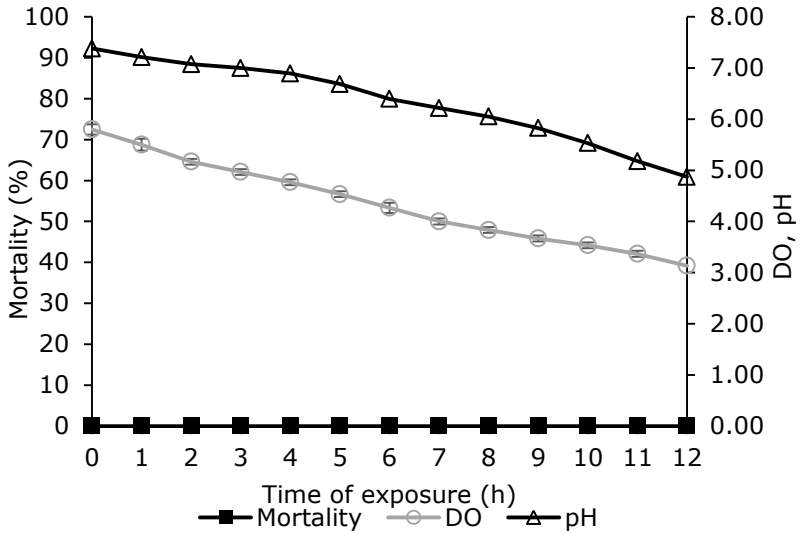


Figure 3. The mortality of juveniles under lowering DO in negative control.

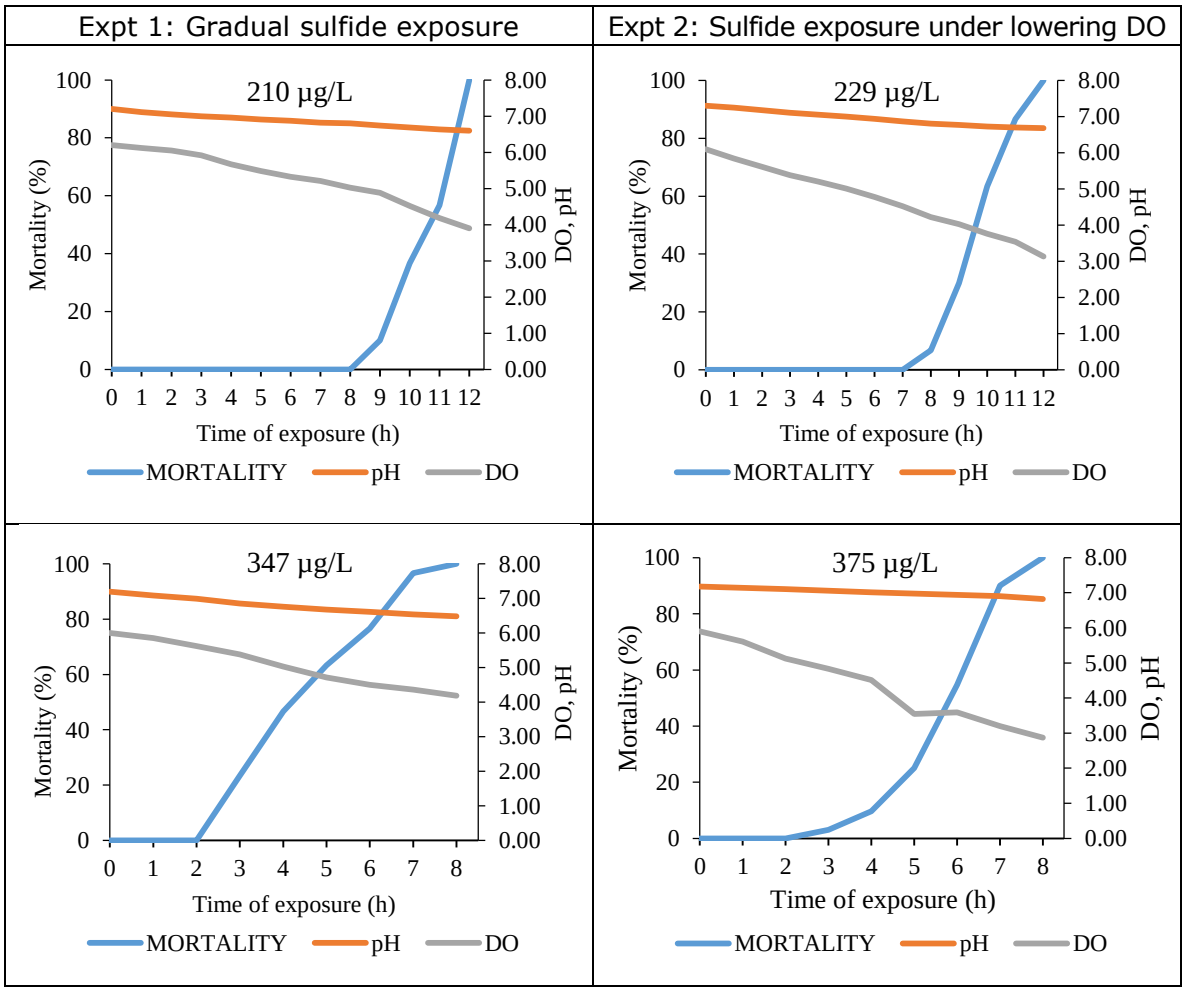


Figure 4. The mortality of juveniles during Experiment 1 (Gradual sulfide exposure) and Experiment 2 (Sulfide exposure under lowering DO) at sulfide concentrations that were not significantly different (210 µg $L^{-1}$ and 229 µg $L^{-1}$ of experiments 1 and 2 respectively; 347 µg $L^{-1}$ and 375 µg $L^{-1}$ of experiments 1 and 2 respectively).

***Sulfide exposure under lowering pH***. In this experiment, both DO and pH in the negative control declined from the start to the end of the experiment at 6.60 to 2.83 mg $L^{-1}$ and 7.32 to 2.87, respectively (Figure 5). First occurrence of mortality took place at the 2$^{nd}$ hour (pH 5.71; DO 3.53 mg $L^{-1}$) and 100% mortality was reached at 5$^{th}$ hour (pH 2.87; DO 2.83). Thus, the juveniles died due to declining pH levels in five hours at pH 2.87. Based on Figure 6, 100% mortality of the juveniles was observed earlier in sulfide exposure under lowering pH experiment than gradual sulfide exposure. 100% mortality was reached 5-9 hours faster in sulfidic waters under lowering pH. Besides that, the mortality of juveniles under sulfide exposure in declining pH was faster than the negative control carried out earlier which could indicate that sulfide toxicity intensified the mortality in the experiments.

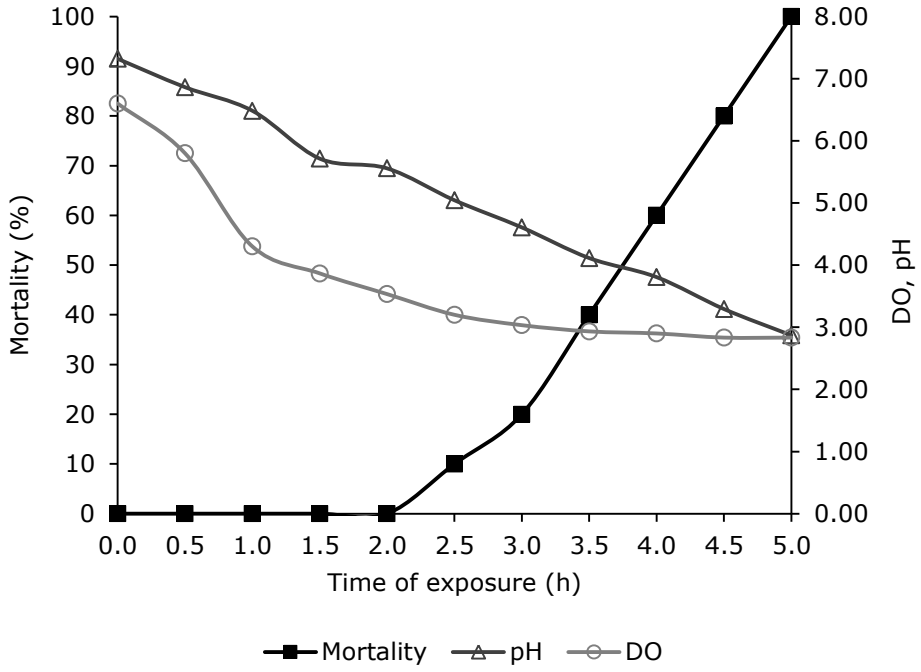


Figure 5. The mortality of juveniles under lowering pH in negative control.

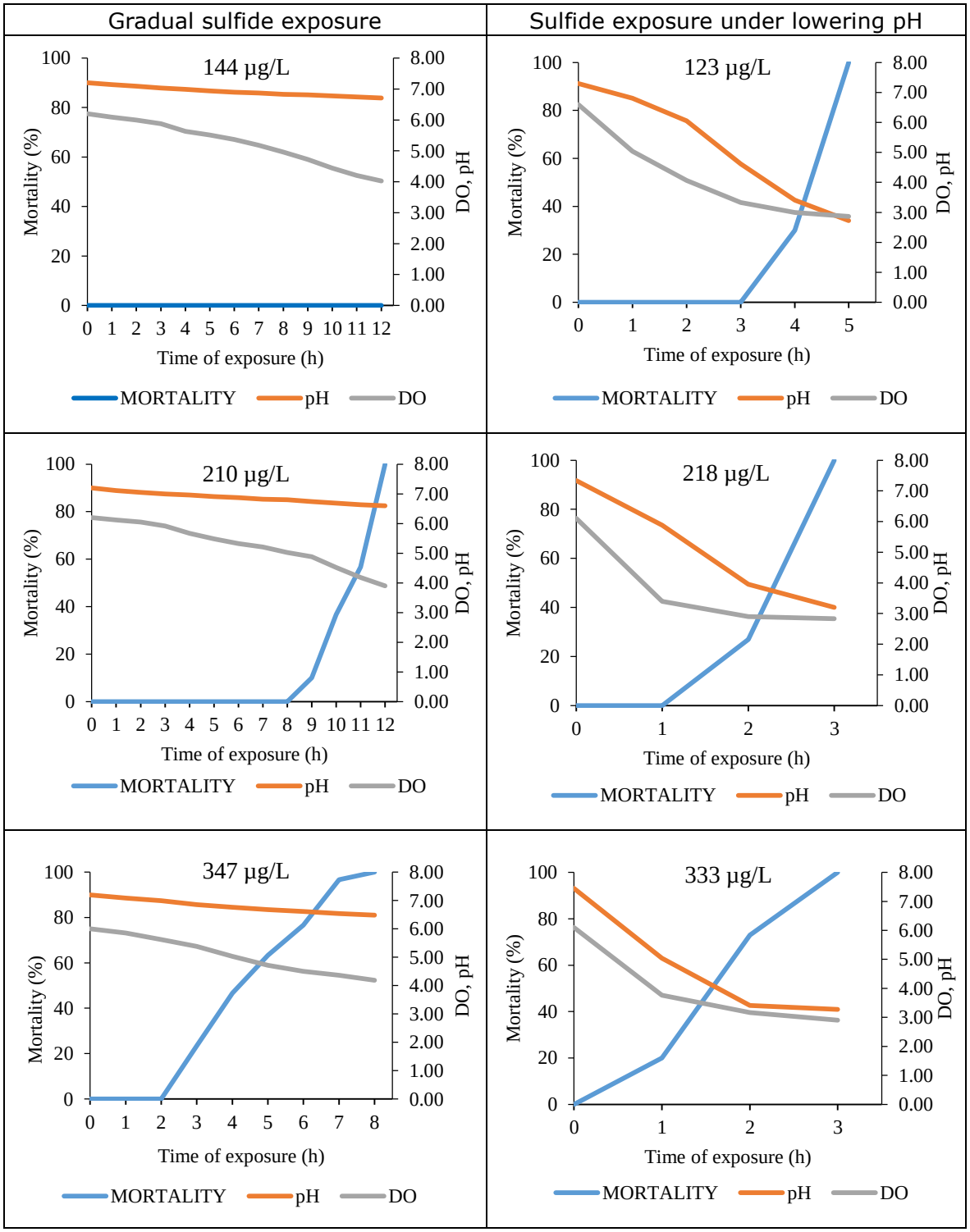


Figure 6. The mortality of juveniles during Experiment 1 (Gradual sulfide exposure) and Experiment 3 (Sulfide exposure under lowering pH) at sulfide concentrations that were not significantly different (144 µg $L^{-1}$ and 123 µg $L^{-1}$ of experiments 1 and 3 respectively; 210 µg $L^{-1}$ and 218 µg $L^{-1}$ of experiments 1 and 3 respectively; 347 µg $L^{-1}$ and 333 µg $L^{-1}$ of experiments 1 and 3 respectively).

***Sulfide exposure under lowering DO and pH***. Figure 7 shows the mortality of juveniles in sulfide exposure under lowering DO and pH. The mortality of juveniles from these two experiments was compared based on the concentrations of accumulated sulfide in the container within a close range. It was observed that, mortality is faster in sulfide exposure under lowering pH levels. Under lowering pH, 100% mortality was observed at

3-5 hours upon exposure for container sulfide concentrations ranging 123-333 µg $L^{-1}$. Meanwhile under lowering DO condition, 100% mortality was recorded at 8-12 hours of exposure in container sulfide concentrations ranged 136-321 µg $L^{-1}$. This showed that under lowering pH, the toxicity of sulfide is more lethal to the juveniles even at low sulfide concentrations.

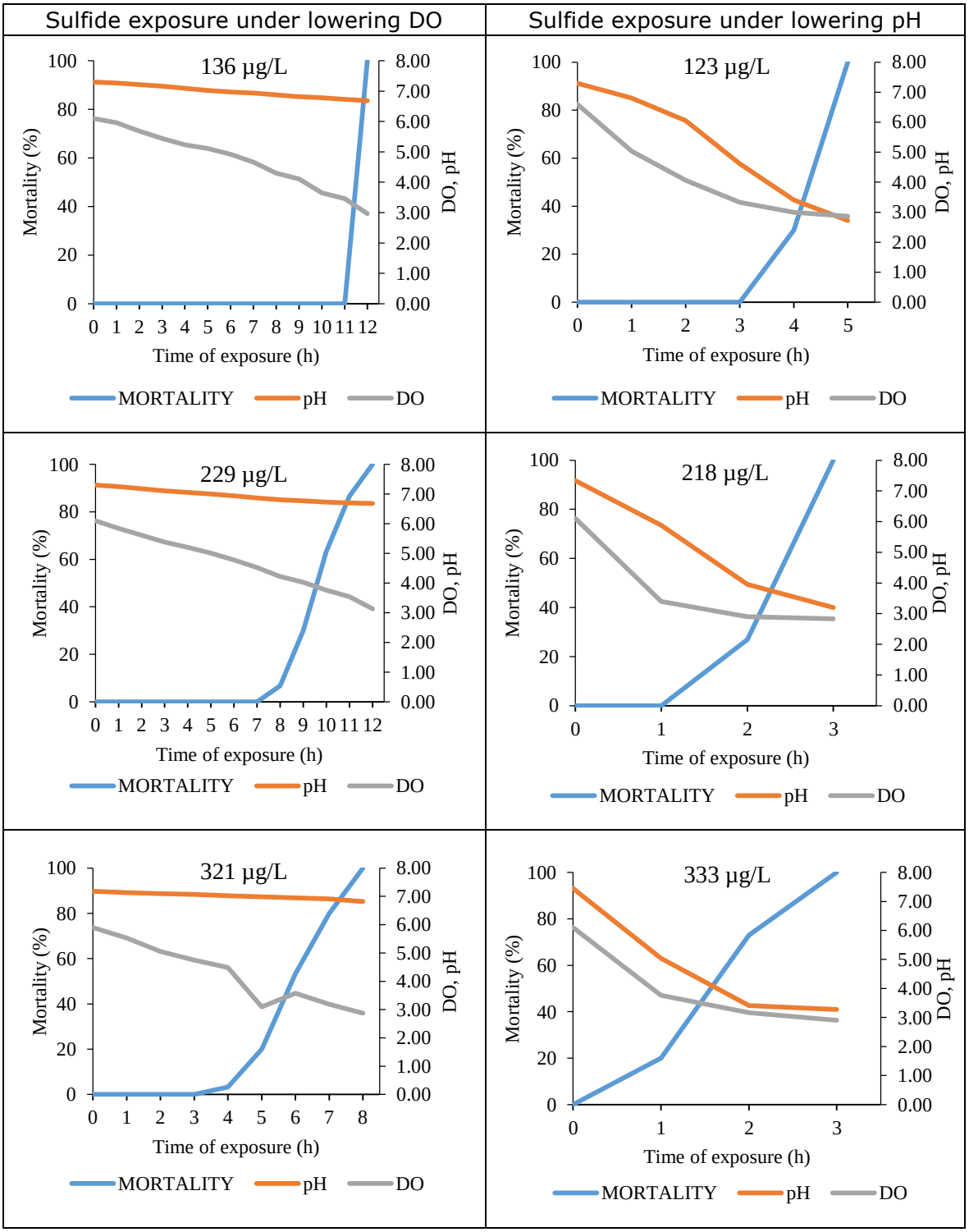


Figure 7. The mortality of juveniles during Experiment 2 (Sulfide exposure under lowering DO) and Experiment 3 (Sulfide exposure under lowering pH) at sulfide concentrations that were not significantly different (136 µg $L^{-1}$ and 123 µg $L^{-1}$ of experiments 2 and 3 respectively; 229 µg $L^{-1}$ and 218 µg $L^{-1}$ of experiments 2 and 3 respectively; 321 µg $L^{-1}$ and 333 µg $L^{-1}$ of experiments 2 and 3 respectively).

**Discussion**. In this study, the behavioral responses of *T. tambroides* juveniles under sulfide toxicity were observed. Sulfide exposure of 210-658 µg/L and sulfide exposure under lowering DO (107-429 µg $L^{-1}$) and pH (84-333 µg $L^{-1}$) led to the display of juveniles huddling together, ASR, loss of equilibrium and turning upside down.

Frequently, species adaptations are related to the ability to survive in extreme environment, in this case, sulfidic environment. ASR was one of the methods commonly used and observed in fishes in high sulfide or oxygen deficient environment. Species inhabiting sulfidic habitat were usually found with alternative respiratory strategies to allow facilitation of oxygen in hypoxic waters (Greenway et al 2014). Synbranchids have aerial-respiratory surfaces in their mouth and branchial chambers which enabled direct extraction of oxygen from atmospheric air (Graham 1997) whereas cyprinodontiforms alternate to aquatic surface respiration, where fishes skim the oxygenated surface water for oxygen (Kramer & McClure 1982). ASR drained the energy and basically costs the time budget of individuals when used (Kramer 1983) due to the frequent trips to the surface to reflect increasing sulfide or decreasing oxygen in the water column (Plath et al 2007). ASR is a form of air breathing that is frequently proposed to facilitate the survival of some species in temporarily sulfidic environments (Brauner et al 1995). Six species were said to inhabit and endemic to sulfide springs which are *Cyprinodon bobmilleri, Aphanius ginaonis*, *Gambusia eurystoma*, *Limia sulphurophila*, *Poecilia sulphuraria* and *Poecilia sphenops* (Palacios et al 2013; Lozano-Vilano & Conteras-Balderas 1999; Coad 1980; Rivas 1980; Miller 1975).

Negative controls were carried out prior to the start of both sulfide exposure experiments under lowering DO and lowering pH. This is to determine whether the responses and mortality of juveniles in these experiments were due to sulfide toxicity rather than the declining DO and pH levels. The results showed earlier and faster mortality when sulfide was supplied. This shows that sulfide toxicity lead to such responses and mortalities displayed by the juveniles.

Low DO had been commonly related to several fish kills in aquatic habitat. Under prolonged low oxygen level, aquatic species are found to be more susceptible to diseases and death (Wannamaker & Rice 2000). Several studies on effects of hypoxia in waters to sulfide levels showed that the two factors are related to each other. In addition, hypoxic tolerance is highly related to sulfidic tolerance because the toxicity of sulfide is exaggerated by low oxygen level in water column (Bagarinao & Lantin-Olaguer 1999). However, Vaquer-Sunyer & Duarte (2010) reported that sulfide toxicity aggravates the hypoxia-driven mortality. Based on these studies, it is clear that high sulfide and low oxygen levels are highly related to each other and could lead to mortality of fishes. In the wild, hypoxic condition in the sediment provide suitable condition for sulfate-reducing bacteria to oxidize sulfide into toxic hydrogen sulfide (Kodama & Horiguchi 2011). As sulfide increase in hypoxic waters, mortalities of aquatic organisms exposed to such condition may be a consequence of both sulfide toxicity and low oxygen levels. Besides that, sulfide also affects the respiratory system of fishes by inhibiting cytochrome c oxidase and blocks electron transport in aerobic respiration (Greenway et al 2014). This explains the behavioral adaptations observed which were mostly aimed at facilitating more supply of oxygen for respiration.

Aside from hypoxic condition, low pH also plays crucial relationship with sulfide toxicity. As seen in the results obtained, earlier mortality was observed when pH level was lowered in sulfidic water. The intensity of sulfide toxicity is greater under acidic water due to the increase of toxic $H_2S$ in the water (Bagarinao & Lantin-Olaguer 1999). The distribution of sulfide elements is dependent on the pH of the water and in water with pH below 7, toxic $H_2S$ is dominant (APHA 2005). In this experiment, even without the supply of weak sulfuric acid, the pH level declined to below 7 due to the acidic stock sulfide itself. This condition allows for the dominant distribution of $H_2S$ in the container. Besides that, acidic water leads to low feeding rates and low hemoglobin concentration (Heydarnejad 2012).

Numerous studies have reported the effects of low levels of DO and pH in sulfidic environment such as in Mexican cave (Tobler et al 2006), Delaware Inland Bays (Luther III et al 2004) and saline lake, Salton Sea (Marti-Cardona et al 2008) and laboratory

experiments (Mann et al 2004; Völkel & Berenbrink 2000). They reported that sulfide concentration rose under low oxygen and pH levels. Eutrophication process in lentic water bodies led to an increase in organic matter, low oxygen and pH levels and in turn leads to sulfide production (Marti-Cardona et al 2008; Luther III et al 2004). Lowering both DO and pH to critical levels (DO at 1-2 mg $L^{-1}$; pH at 2-3) increased the toxicity of sulfide in the water body.

**Conclusions**. Under sulfide exposure, *T. tambroides* showed huddling together, aquatic surface respiration, loss of equilibrium and turning upside down at total sulfide concentrations ranging from 144 to 658 µg $L^{-1}$. No behavioral responses were observed in the negative controls of all three experiments indicating the responses were due to sulfide exposure. The $LC_{50}$ at $6^{th}$ hour of exposure was estimated as 306.10 µg $L^{-1}$ total sulfide, estimated at 138 µg $L^{-1}$ $H_2S$ at pH 6.98 and DO 4.29 mg $L^{-1}$. Low DO and pH levels were highly related to sulfide toxicity and both behavioral responses and mortality worsened as they were lowered.

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