EFFECT OF COPPER ON HATCHING, EGG DEVELOPMENT AND METABOLISM OF FRESHWATER POND SNAIL *FILOPALUDINA MARTENSI*

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Abstract

Little information is known regarding sublethal of copper that affect a freshwater pond snail *Filopaludina martensi*. The objective of this study was to investigated the effect of copper on metabolism, development and hatching of pond snails *F. martensi* exposed to 4 concentrations of copper (0, 6.1, 61 and 610 µgL^{-1}) during period of 5 weeks.The metabolism of snails such as oxygen consumption and ammonia excretion rates were determined in 20 juvenile snails *F. martensi* (0.10±0.01 g wet weight and 0.50±0.01 cm of shell length) at room temperature (28-31°C). The LC50 value for 96 h was 610 µg/L by Probit method. Oxygen consumption measurement were made by using a respiratory chamber (30 ml) and ammonia-N excretion by using Salicylate method. A decrease oxygen consumption was observed in juvenile with increasing concentration of copper. Total ammonia excretion rate was 25.66-33.75 µg/h and there was no significant effect on ammonia-N excretion rate during the exposure at 6.1 and 61µg/L. Juvenile *F. martensi* exposed to 610 µgL^{-1} died at the 5th week. Modification in O:N ratio of juvenile was 1.7–2.5 and showed the lowest value at 610 µg/L Cu. The development of snails was conducted on 120 adult and detected the abnormalities such as reproductive organs, formation of shell and growth. Copper concentration of 610 µg/L was significantly delay on development and decrease in hatching rate. Copper is plentiful in the environment and essential for the normal growth and metabolism of all living organism, in this study demonstrate that copper become toxic to this species when biological requirement are exceeded. Among sublethal effects were loosed in their metabolism cause to loosed their ability for growth and survival, decreased embryo survivals, delay or failure of hatching.

Keywords: Copper, *Filopaludina martensi*, Metabolism, Hatching

Introduction

A wide range of contaminants are continuously introduced into the aquatic environment mostly associated with industrial, agricultural and domestic wastes run-off (Lima *et al.,* 2008). Among these contaminants, heavy metals constitute one of the main dangerous groups, because they are toxic, persistent and not easily biodegradable. The species and concentrations of metals in water are determined by geochemical processes and large scale releases into the aquatic environment by human activities (anthropogenic activities) (Wittmann, 1979). Copper is an essential trace nutrient that is required in small amounts (5-20 micrograms per gram (μg/g)) by humans, other mammals, fish and shellfish for carbohydrate metabolism and the functioning of more than 30 enzymes, It is also needed for the formation of haemoglobin and haemocyanin, the oxygen-transporting pigments in the blood of vertebrates and shellfish respectively. However, copper concentrations that exceed 20 micrograms per gram (μg/g) can be toxic, as explained by Wright and Welbourn (2002).and Bradl (2005)

Filopaludina martensi is a freshwater gastropod mollusk in the family Viviparidae, lives in canals, ponds and another place especially in the field where are a lot of toxins from agricultural such as molluscicide, algicide or herbicide and these toxins are almost come from copper and possible this species will contaminate, their effect can be directly and indirectly lethal and the use of copper to kill algae, fungi and molluscs demonstrates that it is highly toxic to aquatic organisms. Food ingestion, ammonia excretion and oxygen consumption rates are the governing factors for growth in aquatic animals because they reflect energy utilization. In particular, scope for growth (Sfg) elicited from these physiological parameters is useful for estimating the energy budget of animals under environmental challenge (Beiras *et al.,*1994). And previous study demonstrate that copper can be effected to fish gill (Bradl, 2005), olfaction (sense of smell) in fish (Baldwin *et al.,* 2003), decreased sperm and egg production in sea scallops (Taub, 2004) and a little information is known regarding sublethal of copper that affect a freshwater pond snail *F. martensi*. The objective of this study was to investigate the effect of copper on hatching, egg development and metabolism of *F. martensi*.

Methods and materials

1. Snail collection and culture

Individual of adult *F. martensi* of similar size and weight were carefully collected from non-contaminated artificial snails culture ponds situated at Pattani and transferred to Prince of Songkhla university laboratory. They were maintained in glass aquaria. Snails were acclimatized to laboratory conditions for 2 weeks before experimentation, at temperature of 21±1 $^{\circ}$C and fed daily with freshwater green aquatic plant (*Marsilia* sp.) leaves. The average wet weight of snails was 450 mg (350–550 mg) and shell length of 21 mm (19–25 mm).

2. Test compound and acute toxicity test

A stock solution of copper (1mg/L of Cu) (as $CuSO_4 \cdot 5H_2O$) in double distilled water was prepared and from this solution; a series of log-based Cu concentrations were prepared. An acute (96 h) toxicity test was performed using juvenile (15 day old, 18–45 mg ww) snails. Copper exposures were performed in tap water under flow-through conditions with three replicates of 15 snails tested for each Cu treatment. Nominal test concentrations were 600, 610, 620, 630, 640,650, and 660 μgL^{-1}Cu. Snail survival was monitored daily and water samples were collected at the beginning and end of the experiment for measurement of dissolved Cu concentrations. Snails were not fed during the Cu exposure.

3. Metabolism experiment

Oxygen consumption rate (OCR) of 10 individuals from each treatment and control were determined every week by using bottle water method. Individual snail was place for 1h inside a sealed container (30ml) with Cu solutions. The dissolved oxygen (DO) in container was measured of the start and after 1 h by Winkler method. One container without snail was used as control. Each treatment and control had three replications. Oxygen consumption rate was calculated by the following formulation.

$$OCR = \frac{(DO_0 - DO_t)\, V}{t} \quad (\mu g/h)$$

Where DO_0 is the DO of the water at the start of the experiment (µg/L), DO_t is the DO of the water at the end of the experiment (µg/L), V is the volume of the container (L) and t is the experiment time (h).

The ammonia production was measured individual by similar method of oxygen consumption. Ammonia assays from treatment and control performed using the salicylate method of Solorzano (1969). Ammonia excretion rate (AER) was calculated by the following formulation

$$AER = \frac{(N_0\text{-}N_t)\ V}{t} \quad (\mu g/h)$$

Where N_0 is the ammonia-N concentration of the water at the start of the experiment (µg/L), N_t is the ammonia-N concentration of the water at at the end of the experiment (µg/L), V is the volume of the container (L) and t is the experiment time (h).

O:N ratios were calculated by dividing the amount of oxygen consumed by the nitrogen excreted in each developmental stage (Mayzaud and Conover, 1988). The ratio of oxygen consumption to ammonia – nitrogen excretion in atomic equivalents (O:N) was calculated to determine the proportion of protein relative to carbohydrate and lipid catabolized for energy metabolism, with the following formula (Widdows., 1973)

$$O{:}N = (\text{mg } O_2\ h^{1}/16) / (\text{mg } NH4\ \ Nh^{1}/\ 14)$$

where mg O_2 h ^{1}/16 was the rate of oxygen consumption and mg NH4 Nh ^{1} / 14 was the rate of ammonia–nitrogen excretion for the same snail during the same period.

4. Percentage of hatching

Percentage of hatching was measure by checking the number of early hatching snails or percentage of hatching .The separate adult snails (wet weight of 450 mg and shell length 21 mm) were place in the glass aquaria and exposed to copper concentrations for 5 weeks, fed with freshwater green aquatic plant (*Marsilia* sp.) leaves .Snails survival and the number of hatchings were was monitored daily.

5. Egg development

Adult snails wet weight of 450 mg (350–550 mg) and shell length of 21 mm (19–25 mm) were individually place in the glass aquaria and exposed to control, 610, 61 and 6.1 µg/L of copper concentrations for 3 weeks, fed with freshwater green aquatic plant (*Marsilia* sp.) leaves, three replications in each treatments (n = 150). After 3 weeks the 10 adult snails were collected and broke the shell to check their abnormalities of embryo everyday for 15 days. This snail is a hermaphrodite gastropod. Embryogenesis inhibition was defined as embryo development blocked at one stage for more than 3–4 days (Das and Khangarot, 2010*)*. The normal and abnormal development of the embryos was

checked by stereoscopic and the main abnormalities and malformations detected at the various stages of embryogenesis were recorded. The duration of incubation, survival and the number of hatchings were noted for each tested Cu concentration. The following parameters are most feasible and recorded daily or at least at the suggested days of development as described by Das and Khangarot (2010)

6. Calculations and statistics

All result are expressed as mean ±S.D. All data was statically analyzed by two way analysis of variance (ANOVA) to determine the effect of Cu on the physiology response. The effect of Cu on O:N ratio was analyzed by single factor ANOVA and Tukey HSD multiple pair wise comparison of means P ≥ 0.05 was considered significant (SYSTAT version 10). The acute toxicity test was analyzed using Probit analysis (Finney, 1971)

Results

Strong concentration response relationships were observed in 96-h acute (Fig. 1). The estimated 96-h LC50 for *F. martensi* was 610 (600 – 620 95% CI) μg/L Cu. In the acute toxicity test, significant effects on snail survival were observed with 100% mortality occurring in the highest treatments. From the value of 96-h LC50 for *F. martensi*, demonstrate that this snail is very insensitive to copper toxicity test.

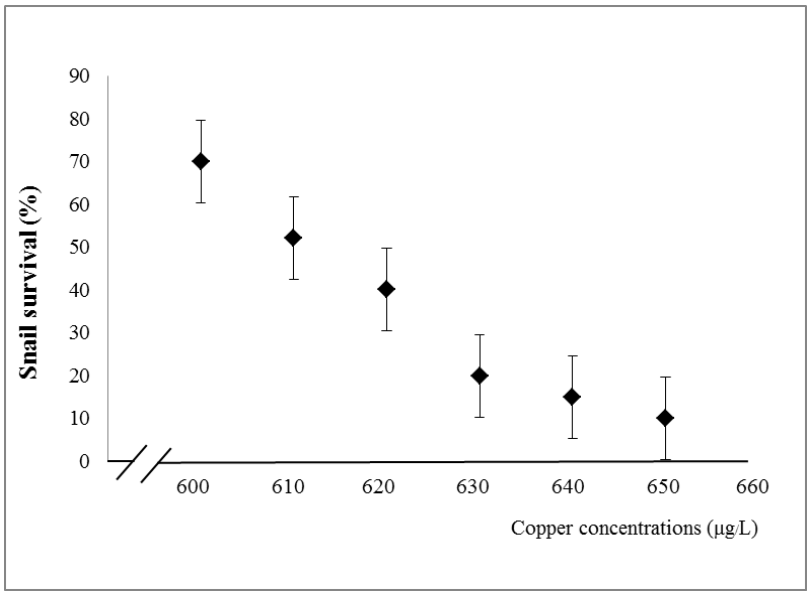

Fig.1 Dose response relationship for 96-h acute toxicity test in various of copper concentrations. Mean±SEM (n=15).

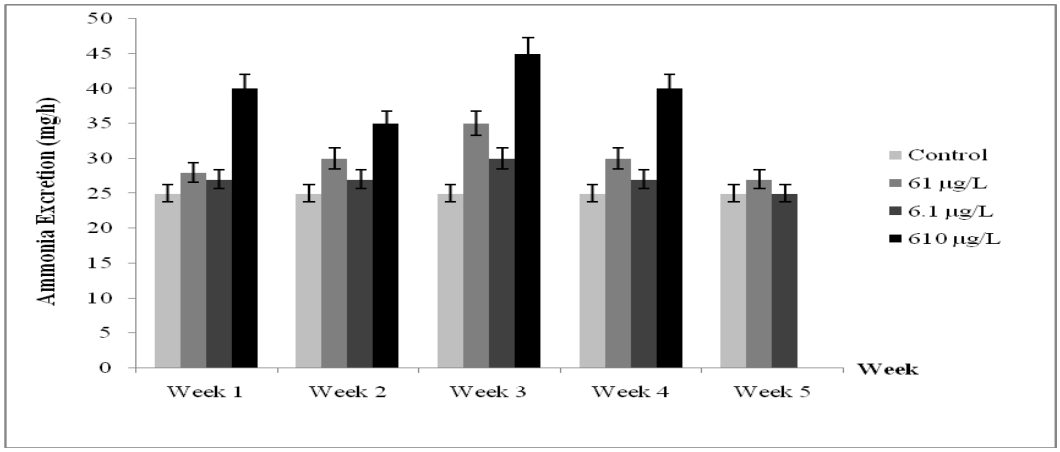

Fig.2 Effects of 5 week of Cu exposure on ammonia excretion rate parameters of juvenile snails *F.martensi* at 6.1, 61, and 610 µg/L Mean±SEM (n=15).

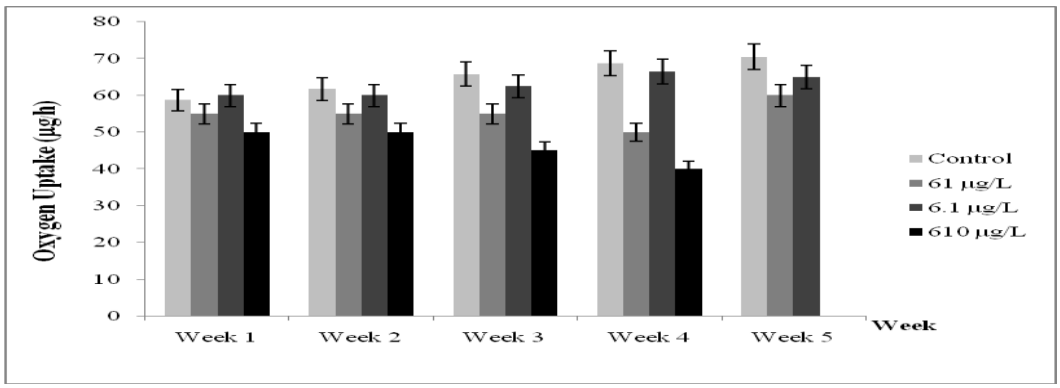

Fig.3 Effects of 5 week of Cu exposure on oxygen consumption rate parameters of juvenile snails *F. martensi* at 6.1, 61, and 610 µg/L Mean±SEM (n=15).

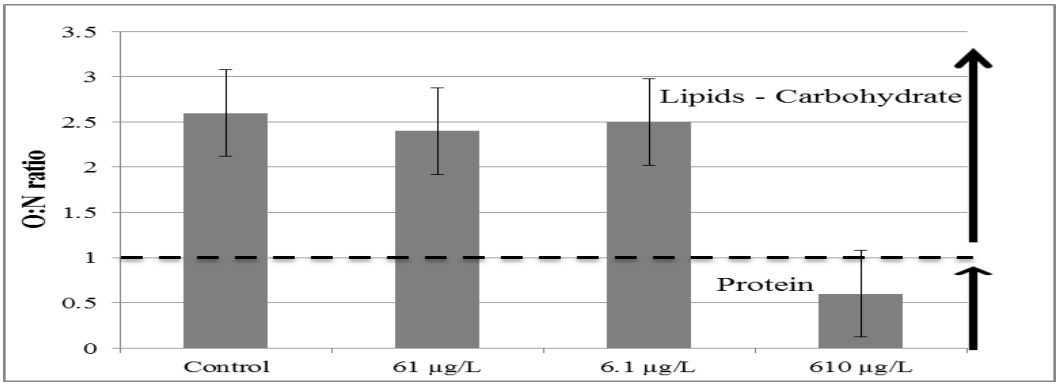

Fig.4 The O:N ratio of *F. martensi* at control and copper concentrations

2. Metabolism test

2.1 Ammonia excretion rate

Results from the metabolism measurements of snails after 5 week exposure to Cu revealed several disturbances in ammonia excretion rate. Comparison of control responses between the highest Cu concentration of 610 μg/L experiments revealed that ammonia excretion were significantly higher ($p \leq 0.05$) than the control. Copper concentrations of 6.1 and 61 μg/L were not significant different effect on ammonia excretions rate compared with to control (Figs. 3)

2.2 Oxygen consumption rate

Results from the metabolism measurements of snails after 5 week exposure to Cu revealed several disturbances in oxygen consumption rate. Comparison of control responses between the highest copper concentration of 610 μg/L experiments revealed that oxygen consumption rate were significantly lower ($p \leq 0.05$) than the control, and another treatment 6.1 and 61 μg /L are no significant different effect on ammonia excretions rate compared to the control

2.3 O:N ratio

The O:N ratio of *F. martensi* of control , 61 and 6.1 μg/L Cu were not significant different with the value of 2.4-2.6 (Fig.4). The lowest O:N (0.7) was observed at 610 μg/L.

3. Development test

The general description of normal developmental stages of freshwater pond snail *F. martensi* hasn't been publish before so in this study we design followed by Khangarot and Das (2010) (a) 2-day-old morula stage appeared in round shape, (b) at 3-day old trochophore larval stage foot and shell formation begins. Shell gland rudiment looks like a small depression, (c) early veliger larva (5–7 days old) showing shell and foot formation, (d) 8-9 day-old late veliger, showing well developed foot, shell, eyes, tentacles and digestive gland, (e) 10-11 day-old hippo stage occupying the whole space in the egg capsule, and (f) newly hatched snail .

An overview of the toxic effects of the copper concentrations on the development of snail eggs at 610 μg/L of Cu is given in Fig. 6A–D. Normal development of *F. martensi* was observed in control groups. Note the normal development of snail embryo in control, 6.1 and 61 (a1–a3, b1-b3, c1-c3), Aberrant snail egg embryo development was observed after 5, 8 and 11days at 610 μg/L of Cu treated groups.

Inhibition of shell growth, eyes and tentacle formation, and shell gland was significantly affected at 610 µg/L of Cu at different periods of exposure (Fig. 6D1–D3). The development eggs was completely inhibited at 610 µg/L of Cu, while abnormal embryonic development, i.e., without shell formation, highly abnormal shell gland and foot was observed after 5, 8 and 11days. Egg development was blocked at trochophore and veliger stages for several days. Individuals were considered dead when, developmental stage, cells were coagulated (i.e., clumped together and whitish in color), or when no movement of developing embryo could be observed or cessation of the heart beat.

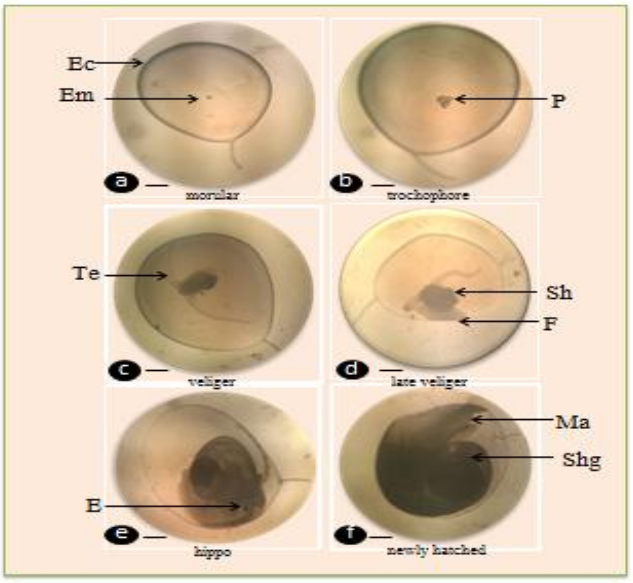

Fig.5 Normal developmental stages of the freshwater pond snail F.martensi. (magnification 10×10). E, eye; F, foot; Ma, mantle; Ec, egg capsule; Sh, shell; Shg, shell gland; P, Prototroch; Te, tentacle. Scale for a–f, 0.1cm

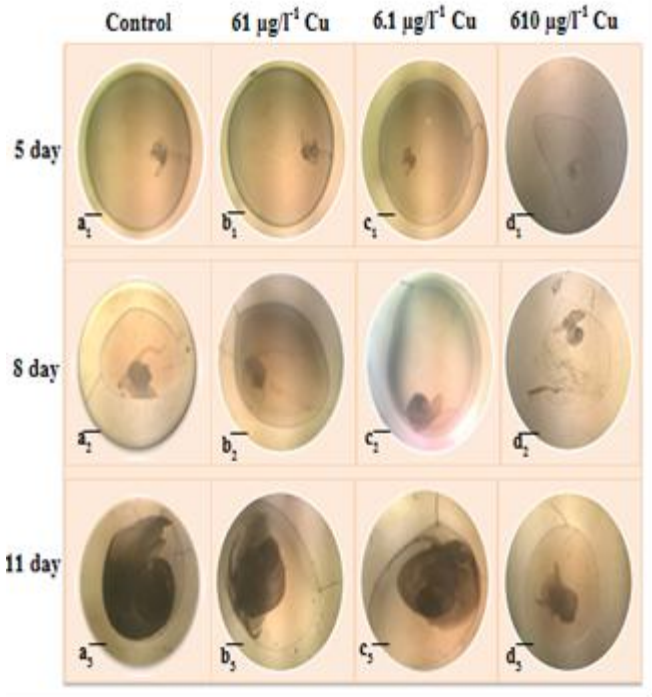

Fig.6 The delayed and incomplete development of *F. martensi* embryos over time day 5, 8, 11 exposed to control, 6.1, 61 and 610 µg/L of Cu, abnormal and malformed embryos noticed in exposed to 610 µg/L of Cu after 5, 8 and 11 days (d1–d3). Retardation of developmental stages occurred after exposure to to 610 µg/L of Cu after 5, 8 and 11 days (d1–d3) Scale bar = 0.66mm.

3.4 Percentage of hatching

The complete hatching failure was observed at 610 µg/L with the value of 36.66% (Fig.7). Hatching rate of *F. martensi* at copper concentrations of 6.1and 61 µg/L were no significant difference with the value of 91.66, 85 and 83.33% respectively.

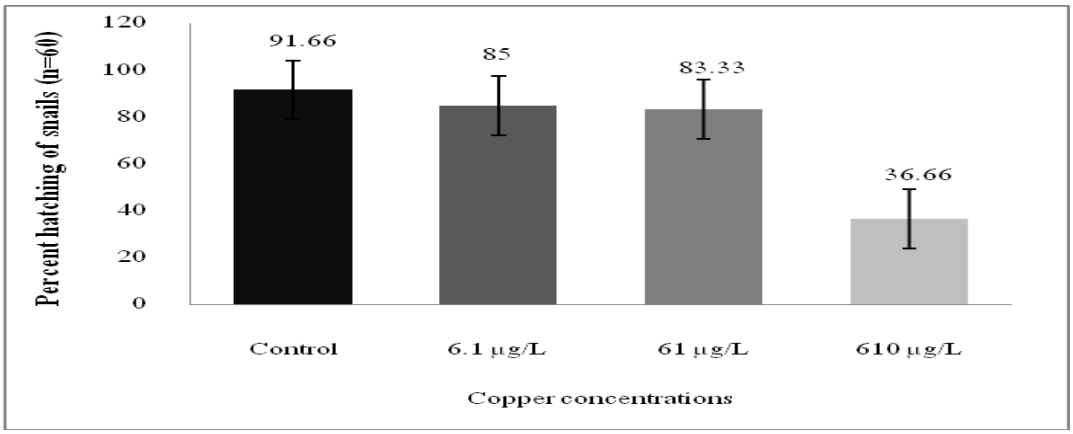

Fig. 7 Effects of 5 week of Cu exposure on hatching parameters of juvenile snails *F. martensi* at 6.1, 61, and 610 µg/L

Discussion

1. The effect of copper on development

The result of present study suggested that as copper concentrations and exposure time increase; the embryo mortality, malformation and development time increase. Among sublethal effects were decreased embryo survivals, delayed or failure of hatching, malformation of foot, eyes, digestive gland, shell, developmental arrest, and thinness of shell and growth retardation at various developmental stages. This study is difficult to study because this snail is self-fertilization. So we had to break the shell to observe the embryo. This study found that the highest copper concentration (610 µg/L) caused malformation and development of snail *F. martensi.* The study about interaction between the effect of copper on development of snail *F. martensi* still unknown. Copper is an essential part of hemocyanin, the oxygen carrying molecule of mollusk and arthropods. The excess of copper cause disorders and mortality. It is possible that copper cause a dysfunction of egg embryos by influencing the neuroendocrine regulation of developing snails.

2. The effect of copper on metabolism and survival

The physiology of copper metabolism in snail and other invertebrate has been well study (Nor *et al*.,1987). Mechanism of biotoxicity of copper to aquatic organisms and its distribution in the environmental were reviewed earlier (Demayo *et al.,*1982). The result suggested that as copper concentrations and exposure time increase; their metabolism such as when the atomic ratio is very low demonstrate that the availability of energy store and the utilization of body protein are abnormal. The ratio of oxygen consumption to nitrogen excretion by atomic equivalent produce an index of the relative amount of protein as compare to carbohydrate and lipid catabolized by the organisms. This study found that the O:N of *F. martensi* was about 2.5. This suggested that this animal used lipid and carbohydrate as energy source. On the other hand the O:N value (0.7) observed at 610 µg/L could be this animal use protein as energy source. Mayzuad and Conover (1988) suggested that O:N ratio could be viable , rising or falling in response to starvation, depending on the biochemical composition of the organism. This study denoted that at highest of copper concentration (610 µg/L) may change in utilization of protein to lipid or carbohydrate store (Bayne et al., 1985)

3. The effect of copper on hatching

At the highest copper concentration 610 µg/L cause to reduce in percentage of hatching is around 36.66 % when compare with the control and other treatment. Hatching is often the result of a joint effort of chemical, osmotic and mechanical mechanism (Yamagani, 1988). Therefore the effect of copper on hatching may result from effect not only one mechanism but from a combination of them. The result showed that the embryo can react to copper at concentration, which are sometime below those that affect egg embryo survival.

Conclusion

Our results showed that reproductive and feeding activities and abnormal embryo development were sensitive indicators of Cu toxicity, copper is deleterious to snail eggs at very low concentrations, causing acute effects at 610 µg/L .Their effects were decreased embryo survivals, delay or failure of hatching, malformation of foot, eyes, digestive gland, shell, developmental arrest, and thinness of shell and growth retardation at various developmental stages. The O:N ratio at 610 µg/L is very low

demonstrated that this animal used protein major component. It is possible that copper cause to abnormal metabolism because generally carbohydrate is first or main for energy metabolism. Copper also cause to reduce in percentage of hatching because of excess of copper cause a dysfunction of egg embryos by influencing the neuroendocrine regulation of developing snails. This snail is very insensitive with the toxicity of copper because of its high LC50 value. Aquatic molluses particulary snails accumulate persistent heavy metals to a great extent than other organism, so could be serve as excellent bio indicator species for biological monitoring of toxic heavy metal pollutant for improving the water quality standard for diverse uses.

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