Histopathological study on nephropathy caused by oral administration with melamine and cyanuric acid in humpback grouper (*Cromileptes altivelis*)

Ketut Mahardika, Indah Mastuti, Zafran Zafran

Gondol Research Institute for Maricultuire, Bali, Indonesia.
Corresponding author: K. Mahardika, kmahardika@yahoo.com

Abstract. Melamine (2,4,6-triamino-1,3,5-triazine) is a chemical, which is originally used to make durable plastic for dishes and countertops. Melamine contamination incidents had been reported in pet food ingredients or products and caused the deaths of pet animals as a result of kidney failure. The purpose of this study was to know the histopathological-effect of oral administration of melamine and cyanuric acid in humpback grouper, *Cromileptes altivelis*. A total of 200 fishes were given commercial pellet containing melamine and cyanuric acid with dosage as follows: (a) 200 mg of melamine/kg of pellet, (b) 200 mg of cyanuric acid kg^{-1} of pellet, (c) 100 mg of each compound of melamine and cyanuric acid kg^{-1} of pellet, and (d) control without melamine and cyanuric acid Each treatment was repeated in two periods: period I was done with 20 fish per tank and period II with 30 fish per tank. All fishes were fed twice a day at satiation for three months. The result of the experiment has shown the fish fed commercial feed containing both of melamine and cyanuric acid became weak and lost appetite after 20 days. The fish death occurred after 25 days of oral administration. Treatment A resulted in total mortality of 80% in period I and II, treatment B 60% in period I and 55% in period II, treatment C 80% in period I and II. No mortality occurred in control treatment within both experimental periods. Histopathological observation showed renal failure indicated by renal nephropathy and crystal like structure in renal tubules.
Key Words: melamine, cyanuric acid, humpback grouper, renal failure.

Introduction. Melamine (2,4,6-triamino-1,3,5-triazine) is commonly used in manufacturing many industrial products such as cement, paper, paint, glues, fire retardants and so on. It is widely used to laminate and decorate plastic for cooking utensil or other material which is in contact with foodstuff. In 2007, contamination of melamine was found in milk, milk products and pet foods. It is suspected that melamine was deliberately added to increase nitrogen value which is regarded as protein according common chemical protein assay (Ingelfinger 2008). Standard assay such as the Kjeldahl and Dumas estimate protein levels by measuring the nitrogen content and multiply the value by either 5.27 or 6.25, depending on protein content of the food products. The high protein content would increase the economic value of food product through melamine addition. But, there is no approved melamine use in human or animal foods (Afoakwa 2008).

Melamine may be found in food due to migration of melamine from food containers or others into foods. The presence of melamine in food as a result of migration has maximum limit which is legally prescribed in some countries such as 30 mg kg^{-1} in European Union (Commission Directive 2002). In March 2007, several North American manufacturers of pet food voluntarily issued nationwide recall notices for some of their products that were reportedly associated with renal failure in pets (Weis & Purina 2007). The contamination was traced to wheat gluten and rice protein concentrate imported from China. These products were actually poor-quality wheat and rice products, laced with high concentrations of nitrogen-rich melamine and melamine-related s-triazine compounds such as cyanuric acid, which can be added to increase the apparent concentrations of protein in the ingredients. Isolation and analysis of various particle types from the suspect wheat glutens led to the identification of pure melamine, several pure s-triazine compounds, and melamine-cyanurate complex, the latter of which results from the formation of hydrogen bonds between molecules of melamine and cyanuric acid (USFDA 2007).

Melamine was illegally added to food products in order to increase the apparent protein content. Standard tests such as the Kjeldahl and Dumas tests estimate protein levels by measuring the nitrogen content and multiply the value by either 5.27 or 6.25, depending on the food product to obtain the protein content. There is no approved melamine use in direct addition to human or animal foods, and also it is not permitted to be used as a fertilizer anywhere in the world (Afoakwa 2008).

The contamination was not limited to pet foods and human infant formula. By-products from pet food manufactured with adulterated wheat flour had been used in chicken and pig feeds. Melamine was also detected in fish feeds but at concentrations lower than those detected in pet food (USFDA 2007). Since the price of the fish meal depends on the protein content, there is a possibility of adulterating this ingredient of fish fed with melamine to artificially inflate protein levels. Recently, there have been reports of detection of melamine (up to 150 ppm) in fish meal and fish feed in different countries (Karunasagar 2009). Determination and confirmation of melamine residues in fish by liquid chromatography with tandem mass spectrometry suspected that market-ready shrimp, catfish, tilapia, salmon, eel, and other types of fish in the USA (31.4%) had melamine at concentrations above Limit of Detection (LOD). Melamine at levels ranging from 51 to 237 μg kg^{-1} were detected from 10 of 105 samples (9.5%) (Andersen et al 2008).

Groupers are the commercial important fishes in Southeast Asia countries (Muhammadar et al 2012) where humpback grouper, *Cromileptes altivelis* is one of the popular fish target for mariculture in Indonesia. The grouper farmers in Indonesia have used trash fish and the commercial pellets for their cultured groupers (Muhammadar et al 2011). The purpose of this study was to examine the histopathological-effect of oral administration of melamine and cyanuric acid in humpback grouper, *C. altivelis*.

Material and Method. This study was conducted in 2013 at Institute of Marine Research and Development (IMRAD), Gondol-Bali.

Experimental fish. Humpback grouper juveniles used in this study, were obtained from private hatchery around Institute of Marine Research and Development (IMRAD), Gondol-Bali, whose averages of weight and total length were 5.6 g and 7.51 cm respectively. The fishes were acclimated in 1,000 cm^3 fiber tank for 2 weeks until all of the fishes were adapted with pellet feed.

Feed. This study used commercial groupers pellet commonly used in private hatcheries. One kg commercial pellet was added to the melamine and cyanuric acid with dosage as follows: 100 and 200 mg of melamine, and 100 and 200 mg of cyanuric acid.

Experimental design. The fishes were separated on 8 fiber tanks (100 L of volume) with aeration and water circulation. These tanks were kept in 4 cm^3 of oval fiber tank containing a half of the amount of sea water in order to maintain water temperature of 27-31^{o}C. The experiment was divided into 4 groups with 50 fishes per group. This experiment was done in two periods. In period I we used 20 fish per group while in period II we used 30 fish per group. Every group was given commercial pellet containing (a) 200 mg of melamine plus 1 kg pellet; (b) 200 mg of cyanuric acid plus 1 kg pellet; (c) melamine and cyanuric acid could not be mixed homogenously because of a rapid formation of crystal solution (white color). Therefore, fishes in this group were fed with commercial pellets containing 100 mg of each compound of melamine and cyanuric acid plus 1 kg pellet, and it was given to fish separating by time feeding; and (d) 0 mg of melamine and cyanuric acid or commercial pellets only as control. All fishes were fed according to the above design pellets on two times a day (morning and afternoon) *ad libitum* for 3 months.

Histopathology observation. Moribund fishes just before death and healthy fishes at the end of the experiment were sampled and the internal organs (head and posterior kidney, spleen, and liver) were fixed in buffer formalin. The fixed samples were processed for histopathology observation according to Mahardika et al (2004). Briefly, the pieces of internal organs were dehydrated by serial ethanol concentration and then cleared in the xylol. Cleared samples were embedded in Paraplast wax and cut with of 5 µm thickness. The samples were then placed on the glass slide and stained by Harris hematoxylin-eosin. The slide was observed under light microscopy.

Results and Discussion

Effect of melamine-cyanuric acid on humpback grouper. Humpback grouper juveniles were fed with commercial pellets where addition of melamine or cyanuric acid or a combination of feeding of both materials is still showing a good appetite in the initial three weeks of administration. But the appetite of the fish decreases gradually in 25 days. Decreased appetite is indicated by the unwillingness of the fish to rise to the surface of the water and catch the feed given. The fish seemed to dwell at the bottom of the tank with a weak and emaciated condition. The fishes are slowly dying one by one. On the other hand, the fishes of the control group or the fishes fed commercial feed without the addition of melamine and cyanuric acid look healthy with a high appetite and swim vigorously until the end of the study (Figure 1).

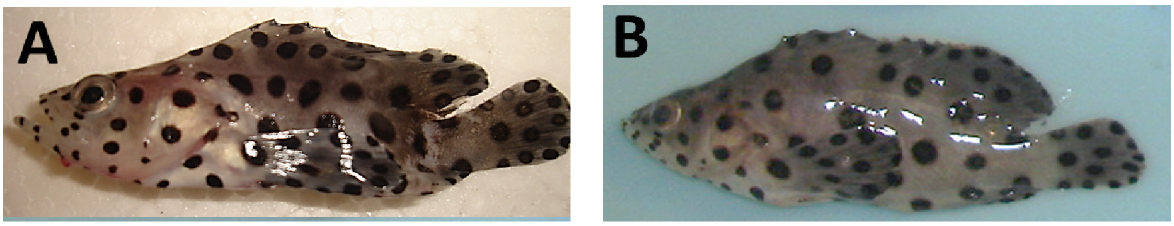

Figure 1. (A). Sick fish in group C shows a thin and pale body, whereas (B). Fish that live in group D shows the plump and healthy body.

The first death was seen in the group of fish fed with the addition of melamine, followed by the group of fish fed with the addition of melamine and cyanuric acid, and the last in the group of fish fed with the addition of cyanuric acid. Cumulative mortality were determine after 3 months of maintenance (percent). Percent of cumulative mortality reached up to 80 percent were occurred in group of fish which were given feed containing melamine (Group A and Group C). This mortality rate achieved within both of experimental periods. Lower mortality rate was occured in group of fish which was given feed containing cyanuric acid without melamine (Group B). In this group, 55% cumulative mortality was occured in period I and 60% in period II (Figure 2). It shows that the addition of melamine and cyanuric acid in feed could cause mortality of grouper.

Histopathological observation. Histopathology of the spleen, liver, and head kidney sections from all treatment groups was unremarkable. Humpback grouper which was fed commercial pellet with added 200 ppm of melamine or cyanuric acid or at 100 pm of both the ingredients, had trunk kidneys, that showed swollen kidney during the histopathological examination. Swollen kidney sections from 200 ppm of melamine, 200 ppm of cyanuric acid, and 100 pm of group of both ingredients had necrosis of renal tubules. Renal calculi consisted of crystal like structures (Figures 3C-3D).

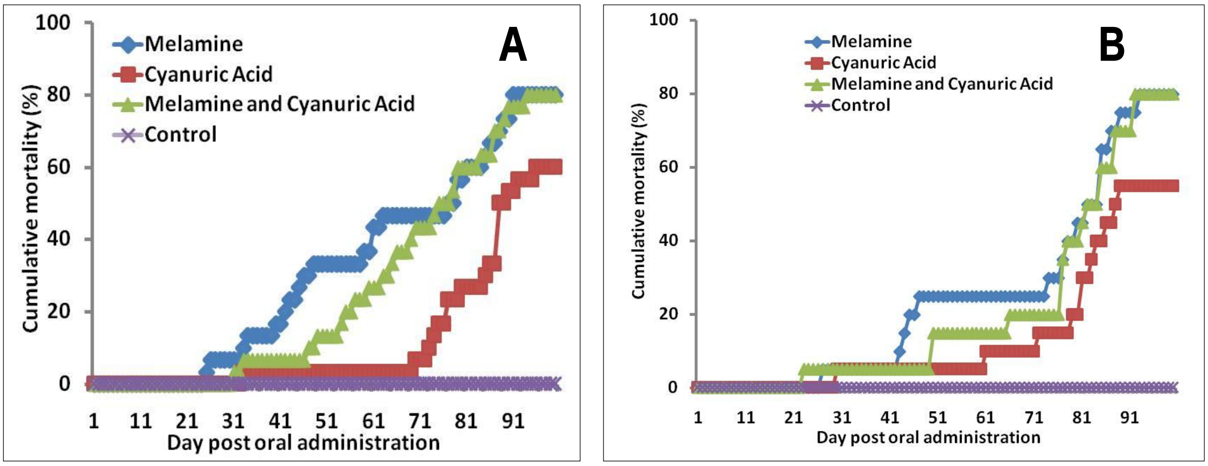

Figure 2. Daily mortality of grouper fish after fed by commercial pellet containing melamine and cyanuric acid for 3 months. (A) Group 1 contained 20 fish every tank, and (B) Group 2 contained 30 fish every tank.

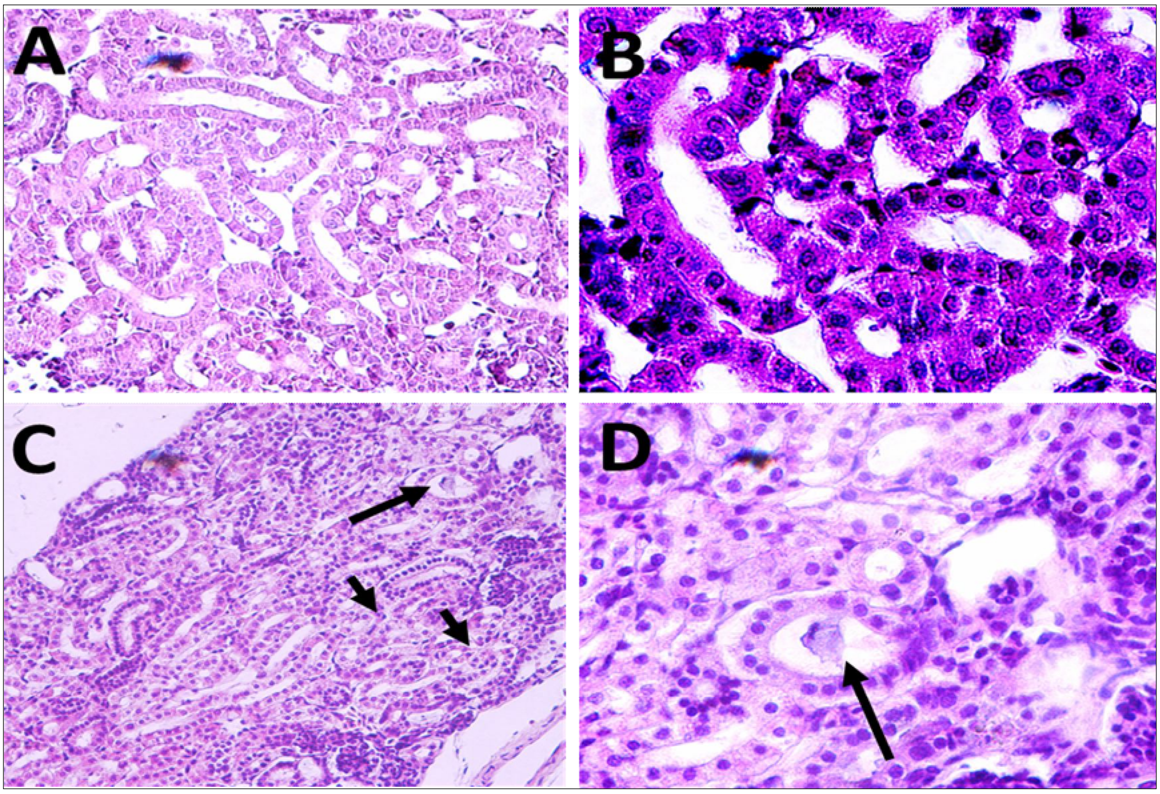

Figure 3. A-B: show the histological features of trunk kidney of live fish in Group D. (A) Trunk kidney consists of renal tubules with normal epithelial cells and tubule-lumen (X400), (B) high power view of renal tubules in Figure A shows normal epithelial cells with empty lumen without any crystal like structure (X1000), and (C-D) they show the histological features of trunk kidney of live fish in Group A: (C) trunk kidney shows necrosis of tubules (short arrows) with some of them containing crystal like structure (long arrow) (x 400) and (D) high power view of renal tubule consists of crystal like structure (x 1000).

Trunk kidney from fish treated with 200 ppm of cyanuric acid and 100 pm of both the ingredients (melamine and cyanuric acid) had severe necrosis of tubule cells indicated by necrotic nuclei and degeneration. Renal calculi also contained amorphous crystal like structure which fulfilled all of the tubules lumens, whereas interstitial cells are replaced by the melano-macrophage center (MMC) that comprises the amorphous structure (Figure 4). In contrast, trunk kidney from control group appeared normal which consisted of formation of renal tubules with normal epithelial cells and empty lumen. It also showed normal glomerulus (Figures 3A-3B).

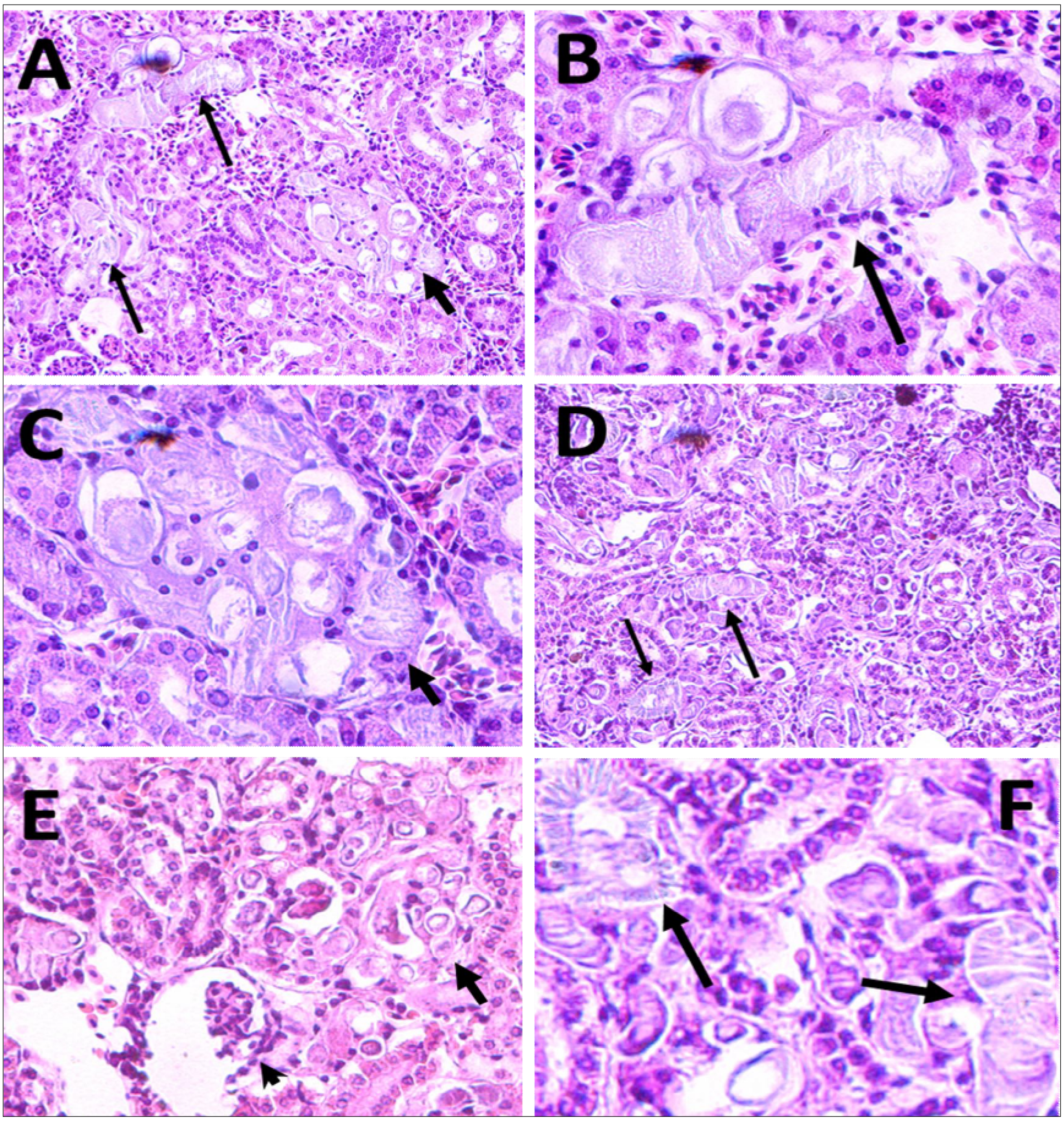

Figure 4. A-C: show the histological features of trunk kidney of moribund fish in Group B which were fed pellets with cyanuric acid. (A) Trunk kidney shows necrosis of the renal tubules (long arrows) and the melano-macrophage center (MMC) like structure in the interstitial cells (short arrow) (x 400), (B) high power view of Figure A showed renal tubule that consisted of crystal like structure (x 1000), (C). High power view of Figure A shows MMC consisting of the amorphous structure at interstitial cells (x 1000), and (D-F) they show moribund fish in Group C which were fed pellets with melamine and cyanuric acid (x 400), (D) trunk kidney shows necrosis of the renal tubules that consisted of crystal like structure (long arrows) (x 400), (E) detail of trunk kidney showed glomerular damage (arrow head) and MMC like structure (x 800), and (F) details of renal calculi of Figure D show necrosis and crystal like structure (arrows) (x 800).

Discussion. Fish which received melamine-containing feeds grew less, utilized feeds less efficiently and performed poorly, besides exhibiting defects such as fin erosion, anorexia, sluggish swimming behavior, paling/darkening of skin and low survival. Melamine concentration in the fish reflected its inclusion level in the feeds, and the content was higher in the viscera than in the fish fillet or whole fish. Histopathological alterations were evident in the kidney, liver, and gills of fish subjected to melamine treatment; the severity of lesions corresponded to its dosage. Enlargement of renal tubules was observed in the kidney of fish fed with ≥ 10 g melamine kg^{-1} of feed (Phromkunthong et al 2013). Melamine and cyanuric acid are known to form an insoluble complex in the kidneys, which may lead to renal failure. Melamine residues were found in edible tissues from catfish, trout, tilapia, and salmon with concentrations ranging from 0.011 to 210 mg kg^{-1} (ppm) (Andersen et al 2008).

All animals fed the combination of melamine and cyanuric acid developed gold brown renal crystals arranged in radial spheres (spherulites), similar to those detected in the cat. Spectral analyses of crystals from the cat, pigs, and fish were consistent with melamine-cyanurate complex crystals (Reimschuessel et al 2008). The crystals are a lattice of six molecules, three of melamine and three of cyanuric acid, held together by hydrogen bonds (Afoakwa 2008). The subsequent renal failure may be similar to acute uric acid nephropathy in humans, in which crystal spherulites obstruct renal tubules (Reimschuessel et al 2008).

In the present result, the crystal like structure in humpback grouper kidneys had the same appearance as those detected in renal tubules of the other fishes with renal failure. Fish given an oral dose of a mixture of melamine and cyanuric acid (400 mg kg^{-1} body weight each) for 3 days developed extensive crystals in their renal tubules, but animals treated with the same dose of melamine or cyanuric acid separately did not develop crystals. Crystals have also been reported to form in trout given melamine first (20 mg kg^{-1} body weight per day for 3 days), followed by a single dose of cyanuric acid (20 mg kg^{-1} body weight) 6 days later (Reimschuessel et al 2008). Moreover, the trunk kidney of these fishes displayed renal calculi consisting of the melamine-cyanurate crystal within the renal tubules, followed by granuloma replacement when its fishes were given both melamine and cyanuric acid.

The crystal-induced nephropathy seen in animals exposed to melamine and cyanuric acid appears to be similar to uric acid nephropathy, in that it is a mechanical obstruction that results in renal damage, rather than a systemic toxic effect. Crystals are composed of melamine cyanurate precipitate in renal tubules following ingestion of both of these triazines simultaneously by cats, rats, pigs and fish (Puschner et al 2007). Crystals in fish kidneys were distributed throughout the renal tubules and collecting duct system. Fishes have mesonephric kidneys, and fishes such as goldfish have nephrons comprised of glomeruli, neck segment, proximal tubules, and distal tubules. The nephrons empty into small and then large collecting ducts. Tubular dilatation and epithelial necrosis took place in close association with the crystals (Nelson et al 1999).

Conclusions. This study indicated that oral administration of melamine and cyanuric acid caused renal nephropathy resulting in mortality of humpback grouper fish. Melamine administration either single or in combination with cyanuric acid generating percent of cumulative mortality is higher than that cyanuric acid. Either melamine or cyanuric acid produced necrosis and formation of crystal like structure within renal tubules. Provision of melamine and cyanuric acid in combination produced more damage than single administration.

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Authors:
Ketut Mahardika, Gondol Research Institute for Mariculture, Gondol, Singaraja 81101, Bali, Indonesia, e-mail: kmahardika@yahoo.com
Indah Mastuti, Gondol Research Institute for Mariculture, Gondol, Singaraja 81101, Bali, Indonesia, e-mail: mastuti_indah@yahoo.com
Zafran, Gondol Research Institute for Mariculture, Gondol, Singaraja 81101, Bali, Indonesia, e-mail: zafran16@yahoo.com

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