Acute toxicity of cyanide (KCN) on two types of marine larvae: *Acropora* sp. planulae and D-veliger larvae of *Tridacna squamosa*

Acute toxicity of cyanide (KCN) on two types of marine larvae: *Acropora* sp. planulae and D-veliger larvae of *Tridacna squamosa*

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Abstract. Cyanide fishing has been intensively used to catch aquarium fishes and fish for human consumption around coral reefs in the South East Asian countries, such as Indonesia. There is little known about the lethal effect of cyanide on the early life stages of marine organisms. We assessed mortality and morphological changes of *Acropora* sp. planulae and fluted giant clam (*Tridacna squamosa*) D-veliger larvae exposed to potassium cyanide (KCN) concentrations closed to those used in cyanide fishing. The KCN concentration exposure treatments for coral planulae were 50, 100, 300, 600, and 1000 mg L^{-1}; and those for giant clam D-veliger larvae were 18.875, 37.5, 75, 300, and 600 mg L^{-1}. The 24 hour static-acute toxicity test was used with four replicates for each concentration. The 24h-LC50 was calculated based on Finney's Probit Analysis Method, and the 24 h-LC50 for coral planulae and giant clam veliger were 121.854 and 84.421 mg L^{-1}, respectively. The D-veliger larvae of *Tridacna squamosa* were more sensitive to KCN exposure than *Acropora sp.* planulae. In addition to mortality, we observed that, in both the planulae and D-veliger larvae, morphological abnormalities increased in frequency and severity with increasing KCN concentration, even at the lowest KCN concentrations.

1. Introduction

Cyanide fishing has been intensively used to catch fish in and around coral reefs in many South East Asian countries, such as Indonesia. These fish are destined for the marine ornamental trade and human consumption, with one driver being the high market demand for live reef food fish trade in several Asian countries [1,2,3]. This illegal practice of poison fishing aims to stun the fish in order to capture it alive. When cyanide fishing, fishermen tend to use high concentrations (1.5-120 $g{\cdot}1^{-1}$) of sodium cyanide or potassium cyanide [4], produced by dissolving tablets in seawater. This solution is generally squirted into coral crevices to stun the target fish [5]. The high concentrations of cyanide used are not intended to be fatal to the target fish, but may cause lasting damage to internal organs of the target fish itself and have lethal or sub-lethal effects on other organisms.

Despite the relatively low rate of coral degradation due to cyanide fishing alone compared to some other methods of destructive fishing [6] this poison can cause corals to bleach and kill polyps [7,8,9,10]. Corals can also be damaged when fishermen break them apart to extract the stunned fish [11]. The use of cyanide fishing can also have potentially devastating effects on smaller (non-target) fish and invertebrates in the surrounding coral reef habitat [12]. Giant clams are one invertebrate that

can be impacted by the use of cyanide fishing [13,14,15]. It has also been reported [16] that habitat degradation due to destructive fishing (blast fishing and poison fishing) can have a negative effect on clam population dynamics, hampering the settlement and recruitment of giant clam larvae.

Corals (Ordo: Scleractinia) and giant clams (Family: Tridacnidae) reproduce sexually; the process begins with the spawning of gametes (egg and sperm cells). While some corals are brooders with internal fertilization, for most corals fertilization occurs in the water column, as for giant clams. In both modes of reproduction, the fertilized eggs develop into planktonic larvae. A single pair of broadcast-spawning adult corals can produce thousands of juveniles from one annual reproductive event [17]. Coral larvae at the planula stage and giant clam larvae of the D-veliger stage are both 6-7 days old when they begin to settle on the substrate; this settlement stage is a critical phase in the life-cycle of these benthic invertebrates [18,19]. When fishermen use cyanide to catch coral reef fish, the threat of coral death and of coral recruitment failure is unavoidable [11].

The toxicity of cyanide to corals has mostly been studied in the adult stage. Reported impacts include the inhibition of photosynthesis and calcium in *Acropora cervicornis* [20]; disruption of photosynthesis and loss of zooxanthellae in *Pocillopora damicornis, Porites lichens, Stylophora pistillata, Acropora aspera*, and *Plesiastrea versipora* [7,8,9]; mitochondrial membrane disruption in *Pocillopora damicornis* [21]; and mortality of *Acropora millipora* [22].

There are still very few studies of the toxicity of cyanide on the larval stages of coral and giant clams, despite the reported sensitivity of these early life stages to other pollutants [19,23,24,25]. In particular, there is limited information on the impact of high concentrations of cyanide on coral and giant clam larvae. Therefore, in this study, we aimed to assess the larval mortality and morphological abnormalities of *Acropora sp*. planulae and D-veliger of *Tridacna squamosa* larval exposed to concentrations of potassium cyanide (KCN) close to those generally used in cyanide fishing.

2. Materials and Methods

Experimental animals used were planula stage larvae (6-7 d) of *Acropora* sp. and D-veliger stage (6 d) larvae of *Tridacna squamosal*. The larvae were obtained through captive breeding (induced spawning) in the hatchery of the Universitas Hasanuddin Marine Science Station on Barranglompo Island, Makassar, South Sulawesi, Indonesia. Spawning of *Acropora* sp. was induced through flow-through physical induction in March 2016 [27]. The spawning of *T. Squamosa* was induced through a combination of physical and chemical treatment methods in August 2016 [18]. Prior to the experiment (up to day 6-7 for coral larvae and up to day 6 for clam larvae) the larvae of each species were kept in 1000 L tanks filled with filtered seawater (1µm mesh size) and aerated. Zooxanthellae were added to the clam larvae tank on day 3 but not to the coral larvae tank.

A stock solution of 1000 $mg{\cdot}L^{-1}$ potassium cyanide (KCN, Merck) was prepared according to method 4500-CN in [28]. The stock solution was diluted with filtered natural seawater to provide nominal concentrations of 50, 100, 300, 600, and 1000 $mg{\cdot}L^{-1}$ KCN for the coral planula toxicity test and nominal concentrations of 18.875, 37.5, 75, 150, and 300 $mg{\cdot}L^{-1}$ KCN for the D-veliger giant clam toxicity test. A standard range finding test was not conducted due to the limited number of larvae produced from the induced spawning. Therefore the highest concentration used in the planula toxicity test was decided based on the lowest average concentration used by fishers and the lower concentrations were chosen based on [22]. The highest concentration used for D-veliger test, 300 mg L^{-1}KCN, was based on the results of the planula toxicity test, which was conducted first, and was the level at which 100% planula mortality was observed.

The 24-h acute toxicity test for each type of larvae was prepared according to the protocols in Part 8010 of [28]. There were four replicates for each of the five concentrations plus control, and larvae were randomly distributed between the 24 experimental units. Each experimental unit consisted of 10 newly hatched-larvae placed in a 30 mL glass vial filled with a test solution at the appropriate KCN concentration. Water was not changed during the 24-h exposure tests. Mortality was observed at 6-hour intervals and water quality was monitored at the beginning and the end of the toxicity testing. A coral planula stage larva was considered dead when one or more of the following

diagnostic conditions was observed: no movement was observed; the larva had shrunk; the larva appeared fuzzy as a result of disintegrated or ruptured epidermis [29]. Similarly, a D-veliger stage clam larva was considered dead when one or more of the following diagnostic conditions was observed: no movement was observed; the soft tissue was transparent; the shell was empty because the mantle tissue and zooxanthellae had been expelled. Morphological abnormalities were observed using a microscope with 10x10 magnification and photographed at the end of the experiment. All glassware was initially washed with detergents, soaked in 10% HNO_3 overnight, and rinsed with distilled water.

Median lethal concentration (24 h-LC_{50}) and 95% fiducial confidence interval were calculated using Finney Probit Analysis [30]. Differences in mortality between the treatments and the control were assessed using the Dunnet Test. All results were presented as nominal concentrations.

3. **Results and Discussion**

3.1. Mortality

The water quality parameters recorded during the toxicity testing were: dissolved oxygen 5.9-6.3 mg·L^{-1}; temperature 25.3-25.7°C, salinity 34-35 ppt and pH 8.1-8.5. All water quality parameters during the toxicity testing met the criteria of optimum growth and survival for tropical marine invertebrates [31]. Mortality occurred in all experimental units under all exposure concentrations. However, mortality in the control treatments was less than 10% for both planulae and D-veliger larvae. At the highest concentrations (600 and 1000 mg·L^{-1} KCN), planula mortality reached 100% within the first two (2) hours of exposure; at the lower maximum concentration of 300 mg·L^{-1} KCN, D-veliger mortality had reached 100% by six (6) hours of exposure. The concentration-response relationship showed the average mortality increased with increasing KCN concentrations in both tests (Figure 1).

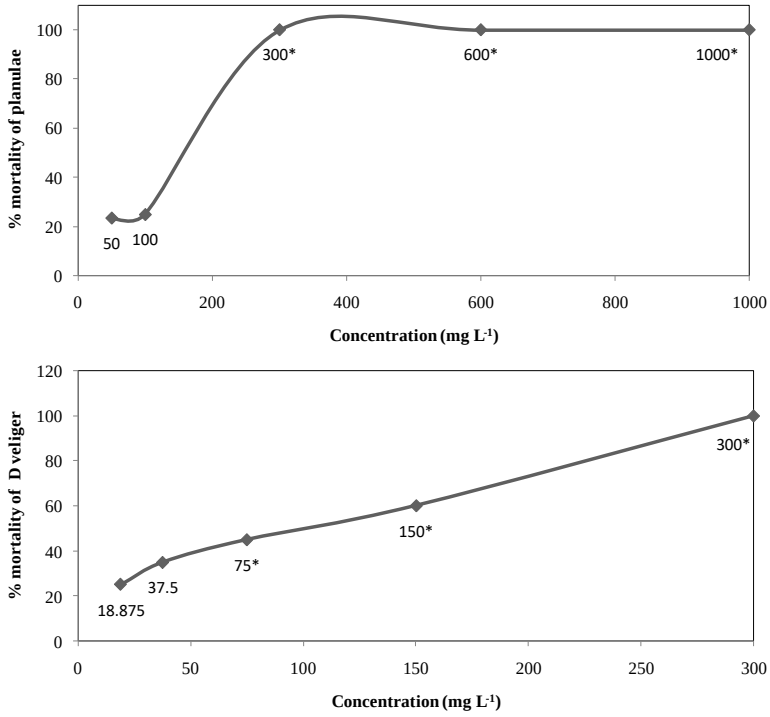

Figure.1. Concentration-response relationship after 24 h exposure (*= significant differences, Dunnett Test, 2-sided, $p<0.05$).

The Dunnett test results showed that the mortality of planulae and D-veliger larvae was significantly higher than the control at 300 mg·L^{-1} KCN and above, and 75 mg·L^{-1} KCN and above,

respectively. The median lethal KCN concentrations (24 h-LC50) were 121.854 mg·L^{-1} for planulae and 84.421 mg·L^{-1} for D-veliger larvae (Table 1). Based on the value of 24 h-LC50, the D-veliger larvae *Tridacna Squamosa* were more sensitive to cyanide than the planulae of *Acropora sp*.

Table 1. Median 24h lethal concentration (24 h-LC50) of KCN to *Acropora sp*. planulae and *Tridacna squamosa* D-veliger stage larvae

Test	24 h - LC50 (mg L^{-1})	95% Fiducial Confidence Interval	
		Lower	Upper
Planulae (*Acropora* sp.)	121.854	80.058	185.471
D-veliger (*Tridacna squamosa*)	84.421	51.637	138.021

3.2. Morphological abnormality

Morphological abnormalities in larvae of *Acropora sp*. and *Tridacna squamosa* exposed to cyanide were photographed. A representative selection is shown in Figure 2. Normal elongated-shape planulae were observed in the control and 50 mg·L^{-1} KCN treatments. After exposure to concentrations of 100 and 300 mg·L^{-1} KCN, the planulae started to exhibit mortality during the first six hours of exposure, indicated by cell deformation and rupture. Oily substances began to be released from the planula cell at 300 mg·L^{-1} KCN; the onset of this phenomenon coincided with a statistically significant increase mortality as defined by the Dunnett Test. At the higher concentrations of KCN (600 and 1000 mg·L^{-1} KCN), all planulae died within two hours of exposure; the planulae cells began to disintegrate and form an organic stock (Figure 2 A.).

In contrast to the coral planulae, there were no morphological abnormalities (shell deformations) detected in D-veliger *Tridacna squamosa* exposed to KCN. However, some behavioural abnormalities and lower zooxanthellate abundance were observed (Figure 2 B). All D-veliger larvae were actively moving in the control; the larvae were slightly less active in 18.875 mg L^{-1}·KCN, but the algal symbiont (zooxanthellae) were fully present in both control and 18.875 mg L^{-1}·KCN treatments. At higher concentrations, 37.5 and 75 mg·L^{-1} KCN, there was less movement and fewer zooxanthellae were observed inside the translucent shells of the larvae. At the 150 and 300 mg·L^{-1} KCN concentrations, all zooxanthellae were released from the tissues of the D-veliger larvae. Broken shells were also observed in the highest exposure (300 mg·L^{-1} KCN) treatment.

3.3. Discussion

High concentrations of potassium cyanide (KCN) or sodium cyanide (NaCN) has been squirting out into the coral reefs area during the cyanide fishing. In this study, we were assessing high concentrations of KCN exposed to larval stage of *Acropora* sp. planulae and *Tridacna squamosa* D-veliger. We found median lethal concentration (24 h-LC50) of KCN exposed to D-veliger (84.421 mg L^{-1}) was lower than those exposed to planulae (121.854 mg L^{-1}), indicating high concentrations of KCN was more toxic to D-veliger larval stage of *T. squamosa* than to planulae of *Acropora* sp. While no information could be found on cyanide toxicity to giant clam, there have been several studies on corals. Table 2 presents key findings from a number of studies on the acute effects of cyanide exposure on corals and other aquatic organisms.

There was only one unpublished (grey literature) study found on the acute effect of sodium cyanide (NaCN) to the planula stage of coral *Stylophora pistillata* (https://repository.seafdec.org.ph/bit stream/handle/10862/2147/2147-Mingoa-LicuananSS2007-AEM37.pdf) and there were none done on giant clam larvae and adult stages. Lethal and sub-lethal effects of cyanide to coral ranged from loss of zooxanthellae and coral bleaching to mortality. An early study in the 1970's [20] revealed calcification inhibition and photosynthesis impairment occurred when colonies of the corals *Acropora cervicornis* and *Acropora formosa* were incubated for 1-2 h in > 4.8 mg·L^{-1} NaCN. Research using high concentrations of cyanide in the 1990's [9]

observed loss of zooxanthellae, bleaching and mortality of *Pocillopora damicornis* and *Porites lichens* coral fragments at 5203 mg·L^{-1} CN^{-1}.

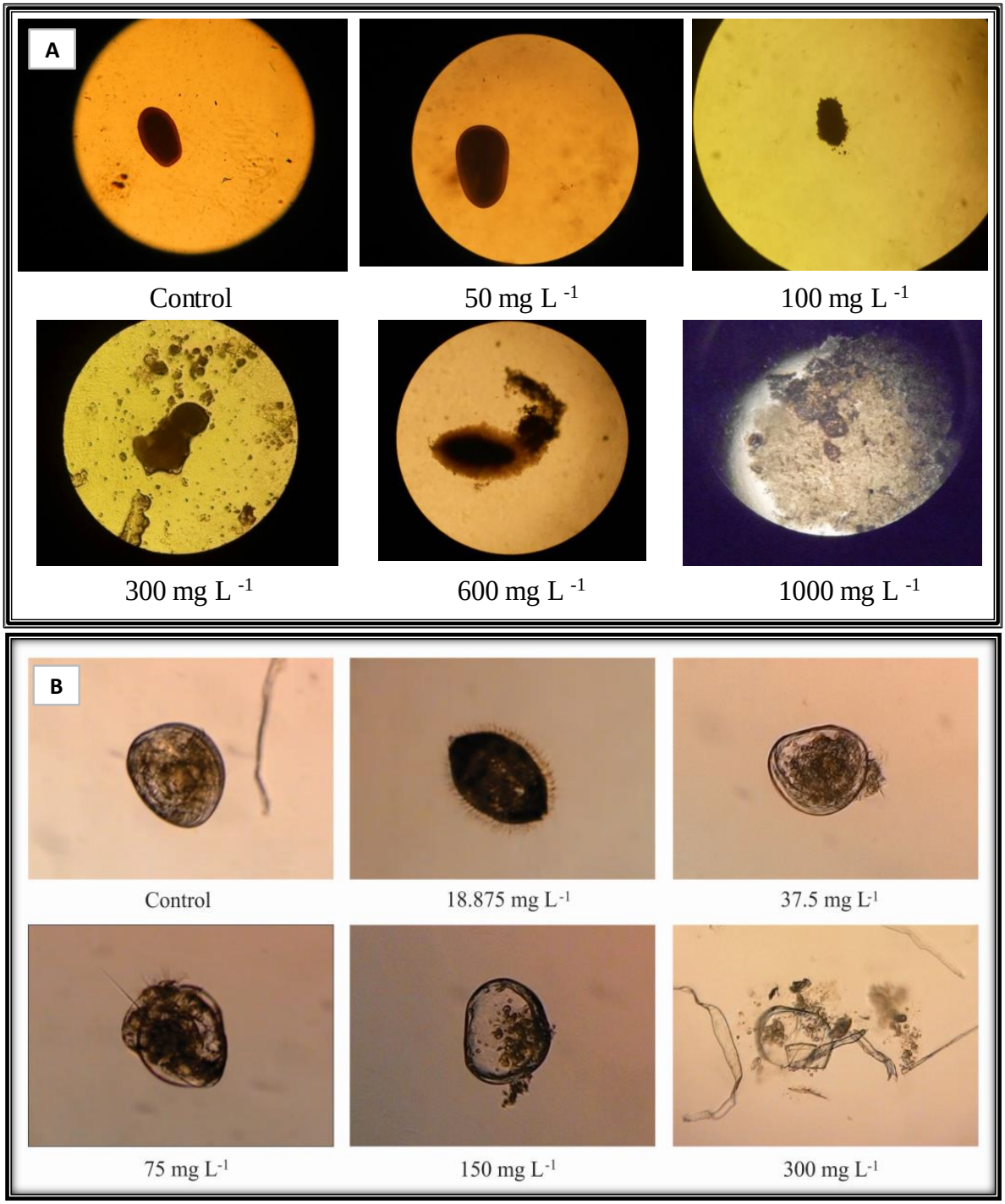

Figure. 2. Morphological abnormalities in larvae exposed to KCN: (A) *Acropora sp*. planulae and (B) *Tridacna squamosa* D- veliger stage larvae

Table 2. Toxicity of Cyanide to coral species, bivalves, and other marine organisms

Test species	Life stage	Test chemical/ duration	Effect measured	Concentration	Reference
Coral, *Acropora* spp.	Planula	KCN, 24 h, static test	LC50, morphological abnormality	121.854 mg L^{-1}	present study
Giant clam, *Tridacna squamosa*	D-veliger	KCN, 24 h, static test	LC50, morphological abnormality	84.421 mg L^{-1}	present study
Mussel, *Mytilus galloprovincialis*	Veliger	CN, 48 h, static test	LC50	154 µg L^{-1}	[32]
Coral, *Stylophora pistillata*	Planula	NaCN, 24 h	dead and grossly deformed	5 mM (245.036 mg L^{-1})	http://www.haereticus-lab.org/wp-content/uploads/2016/10/ecotox-coral-planula-tox.pdf
Coral fragment, *Pocillopora damicornis*	Adult	CN^{-1}, 1-30 min (incubation), observations 12 h and 24 h after incubation	Zooxanthellae loss, discolouration, mortality	$2x10^{-1}$ M (5203.48 mg L^{-1})	[9]
Coral fragment, *Porites lichens*	Adult	CN^{-1}, 1-30 min (incubation), observations during incubation time	all colonies died and discoloured	$2x10^{-1}$ M (5203.48mg L^{-1})	[9]
Coral colony, *Acropora cervicornis* and *A. formosa*	Adult	NaCN, 1-2 h incubation	Photosynthesis and calcification inhibited	$>1x10^{-4}$ M (>4.9 mg L^{-1})	[20]
Coral fragment, *Acropora millipora*	Adult	NaCN, exposed to cyanide for 60 or 120 s by directly dipping fragments into cyanide solution	mortality within 3 weeks at lower concentrations and 1 week at highest concentration; mild to severe bleaching, tissue detachment, swollen tissue	50, 100, 300, or 600 mg L^{-1}	[22]
Coral tissue, *Pocillopora damicornis*	Adult	KCN, 4 h	EC50- 3 h mitochondrial membrane potential LC50-3 h	253,6 µg L^{-1} 5.1 mg L^{-1}	[21]
Coral fragment, *Stylophora pistillata* and *Acropora aspera*	Adult	NaCN, 24 h	Photoinhibition and photosynthesis electron transport in zooxanthellae ceased	$2x10^{-1}$ M (9801.4 mg L^{-1})	[8]
Coral fragment, *Plesiastrea versipora*	Adult	NaCN, Incubation for 3 h	Photosynthesis disruption, coral discolouration and bleaching	$>1x10^{-5}$ M (>0.49mg L^{-1})	[7]
Coral fish, *Chromis viridis*	Adult	KCN, 96 h, static-renewal	LC50	0.0413 mg L^{-1}	[4]

Test species	Life stage	Test chemical/ duration test	Effect measured	Concentration	Reference
Freshwater fish, *Catla catla* and *Cirrhinus mrigala*	Adult	NaCN, 96 h	LC50	0.280 - 0.458 mg L^{-1}	[33]

Another study from the 1990's [8] concluded that the concentrations of cyanide (9801 mg·L^{-1} NaCN) squirted from bottles directly on to coral by fishermen will cause bleaching (leading to mortality) as a result of disruption of the photosynthetic electron flow in the symbiotic algae, causing the expulsion of zooxanthellae from the tissue of *Stylophora pistillata* and *Acropora aspera*. A more recent study using a high concentration of cyanide (50-600 mg·L^{-1} CN^{-1}) showed that *in situ* and *in vitro* experiments resulted in the mortality of *Acropora millipora* fragments due to cellular damage [22]. It seemed that the cellular damage in coral tissue resulted in a loss of membrane integrity causing the release of zooxanthellae from the gastrodermal cells. This pathway was consonant with our results at higher concentrations (300, 600, and 1000 mg L^{-1} KCN) leading to cell membrane rupture in *Acropora* sp. planulae. The experiments in [22] also found that *Acropora* sp. was the most sensitive to cyanide exposure of all coral genera studied *(Goniopora* sp., *Favites abdita, Trachyphyllia geoffrio, Plerogyra* sp., *Heliofungia actinformis, Euphyllia divisa,* and *Sarcophyton* sp.), as indicated by more rapid signs of stress and loss of zooxanthellae.

A recent study [21] using *in vitro* toxicity tests on tissue of the coral *Pocillopora damicornis*, found that a fairly high cyanide concentration (3h-LC50 = 5.1 mg·L^{-1} KCN) was required to killed 50% of coral cells within 3 hours of exposure. Compared to [21], our 24-h LC50 of 121.9 mg L^{-1} KCN for the exposure of *Acropora* sp. planulae was quite high, indicating that coral planulae might be somewhat more resistant to cyanide exposure than adult stages. However the methods used and the species were different; furthermore, it might be inappropriate to make a comparison between adult coral tissue and live coral planulae.

No previous publications on the acute toxicity of cyanide to giant clams could be found. However, there are some studies on other molluscs. For example, a study on the effect of cyanide to bivalve, mussel (*Mytilus galloprovincialis*) [32]. This study found that the cyanide concentration that killed 50% of the mussel veliger larvae within 48 hours (48 h-LC50) was 154 µg·L^{-1}. Compared to this figure, the 24h-LC50 of KCN to D-veliger *T squamosa* (84.421 mg L^{-1}) in our study was higher. This might indicated that veliger larvae of *T squamosa* are more resistant to cyanide than mussel veliger larvae; the difference could also be related to the longer exposure time (48 hours compared to 24 hours) in [32].

One result of our study was that KCN appeared to be more toxic to the D-veliger larval stage of *T. squamosa* than to the planula stage larvae of *Acropora* sp. This may be due to the timing of the acquisition of algal symbionts (zooxanthellae) and the level of dependence on symbiosis with zooxanthellae. *Acropora* larvae are known to be lecithotrophs, larvae that can go through metamorphosis without relying on any exogenous feeding [34]. On the other hand, giant clam veliger larvae are planktotrophs, which means that nutrition from the algal symbiont is required to enable the metamorphosis to subsequent life stages [35,36]. Since the main action pathway of cyanide toxicity appeared to be through the impairment and disruption of the symbiotic algae, it would seem logical that the D-veliger of *T. squamosa* would be more sensitive than the planulae of *Acropora* sp.

4. Conclusion

This study indicated that, although both were affected at levels below common concentrations used in cyanide fishing, veliger phase of *Tridacna squamosa* larvae were more sensitive than *Acropora* planulae to cyanide toxicity, based on the respective LC50 values. Morphological abnormalities were only observed in coral planulae, while in giant clam D-veliger stage larvae the shells retained their

shape at all concentrations, and remained intact until rupturing when mortality occurred. Mortality and cell deformation of *Acropora* planulae were significant at KCN concentrations of 300 ppm and higher. Mortality and loss of zooxanthellae in D-veliger stage *T squamosa* larvae was significant at KCN concentrations of 75 ppm and above. These results indicate that cyanide fishing could significantly impact larval survival and quality and thus have a negative impact on the recruitment of reef-building corals and reef-associated benthic invertebrates such as giant clams.

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