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Quality of giant clam (*Tridacna derasa*) juveniles as non-target organisms after exposure to clove oil in concentrations suitable for anaesthetising ornamental fish

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Abstract. The use of clove oil as an alternative to cyanide has proven effective for catching several types of ornamental fish on coral reefs. However, it is very important to evaluate the post-exposure condition of non-target organisms in coral reef ecosystems where ornamental fishing occurs, such as juvenile tridacnid clams. The aim of this study was to analyse the condition of juvenile clams (*Tridacna derasa* juveniles, size class 3-4 cm shell width) after exposure to clove oil at the concentrations found effective for ornamental fish capture. A factorial experimental design was used with 7 clove oil concentrations (control, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm) and 5 post-exposure times (1,5,9,13 and 17 days) with 3 replicates per treatment. The juvenile clams were acclimatized for a week before being exposed to clove oil for 5 minutes, then transferred to a recovery aquarium for 60 seconds before being placed in holding aquaria. The density and mitotic index of the zooxanthellae in the clam mantles were measured for each treatment and time. There was no significant difference ($p > 0.05$) between control clams and those exposed to clove oil with concentrations from 20 - 70 ppm in either the density or the mitotic index of zooxanthellae present. These results indicate that juveniles of the clam *Tridacna derasa* did not experience a significant decline in quality (condition) after a short exposure to clove oil, such as might typically occur if clove oil was used in ornamental fishing on coral reefs.

1. Introduction

The use of cyanide for the capture of reef fish is still widespread, in particular among ornamental fishermen [1–3]. One reason is the lack of less damaging but effective alternatives for catching ornamental fish living in coral reefs. Clove oil is one alternative that has been proposed as a replacement for cyanide [3,4]. Clove oil could have several advantages over cyanide for both ornamental and food fisheries. It is relatively cheaper than cyanide, as well as being safer for fish and for humans who work with the substance or consume the fish caught [5]. Clove oil should be relatively easy to use, and can work in very low concentrations [4,6]. Clove oil contains the active substance eugenol which can be found in the stem, flowers, and leaves of the clove tree (*Syzygium sp.*) [7]. It is a natural substance which should be relatively easy to obtain, as cloves are a major local plantation crop across much of Indonesia.

Clove oil has been produced in Indonesia for centuries, and is commonly used as a topical anaesthetic for minor ailments such as toothache [7,8]. Clove oil is also a highly effective fish anaesthetic, known to cause rapid and calm immobilization [4,8,9]. In addition, fish do not require a



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withdrawal period after exposure [10] and after undergoing anaesthesia, the fish retain their protective mucus layer and do not lose their appetite.

Previous research has shown that the use of clove oil can be effective as a tool for catching ornamental fish on coral reefs, including the angel fish *Centropyge bicolor* [11], blacktail zebra fish, *Dascyllus melanurus* [12] and butterfly fishes [13], at concentrations of 20-60 ppm. The impact of clove oil on several coral species has also been reported, including *Porites lobata* [14]; *Acropora* sp. [15] and *Pocillopora damicornis* [16][17].

Nevertheless, there are still gaps in our knowledge of the effects of the use of clove oil as an alternative to cyanide in fishing, in particular with respect to potential impacts on non-target organisms such as invertebrates living in coral reef ecosystems. These potentially vulnerable organisms include tridacnid clams (genera *Tridacna* and *Hippopus*). These clams are heavily exploited across Indonesia and are recognised as a species group requiring conservation action [18]. This study aimed to evaluate the impact of exposure to clove oil at the concentrations suitable for use in ornamental fishing on the survival and condition of juvenile clams of the species *Tridacna derasa*.

2. Materials and Methods

2.1. Experimental set-up

The juvenile clams used in this study were *Tridacna derasa* juveniles with a shell width of 3-4 cm. A total of 90 captive-bred juvenile clams were obtained from the Hatchery of the Faculty of Marine and Fisheries, Universitas Hasanuddin on Barrang Lompo Island, Makassar, South Sulawesi, Indonesia. The juvenile clams were collected from the rearing tanks and placed in a holding tank for a one week adjustment process (acclimatization) before the experiment began. Throughout the acclimatization process, the holding tank was provided with aeration and water circulation.

The experimental units (aquaria) used for the experimental treatments measured 1.0 x 0.5 x 1.0 m and were made of 1.5 mm glass. Each unit was equipped with an adequate aerator. The aquaria were filled with sea water pumped from the sea, and water quality was adjusted to ambient conditions in the field (surrounding reef). Clove oil was dissolved in 95% ethanol at a ratio of 1: 8 [19]. This master solution was then diluted to treatment concentrations selected based on the effective concentration for several types of tropical reef fish (20, 30, 40, 50, 60, 70 ppm). The control treatment concentration was zero (without clove oil).

To analyse the effect of clove oil on juvenile clam condition, the changes in the density of zooxanthellae in juvenile *T. derasa* clams was measured after exposure to clove oil at the treatment concentrations. A factorial experimental design was used with 7 variations in the concentration treatment (control, 20, 30, 40, 50, 60, 70 ppm) and 5 treatment periods (number of days: 1,5,9,13,17) with 3 replicates of each treatment/time combination.

The juvenile clams were placed in an aquarium containing a concentration of clove oil solution corresponding to the relevant treatment (control, 20 ppm, 30 ppm, 40 ppm, 50 ppm, 60 ppm, 70 ppm). Exposure time was 5 minutes. After exposure, the juvenile clams were rinsed with clean sea water for 60 seconds in another aquarium, and then transferred to the treatment unit for recovery. Throughout the recovery time, the experimental units (aquaria) were provided with aeration and water circulation. The density and mitotic index of zooxanthellae in the juvenile clams were observed after a recovery periods of 1 day, 5 days, 7 days, 13 days and 17 days using a haemocytometer and microscope.

2.2. Density and mitotic index of zooxanthellae in juvenile clams

The density of zooxanthellae in the juvenile clams mantle was calculated based on the methods described in [20]. A 1 cm² sample of the mantle surface layer of each juvenile clam was scraped using a scalpel. The material obtained was suspended in 100 ml of filtered seawater. Zooxanthellae were separated from the tissue in the sample and collected using a 25 micrometre size filter, then placed in a sample bottle with a volume of 100 ml. The density of the suspended zooxanthellae was calculated

using a haemocytometer, with five repeats for each sample. The density of zooxanthellae was calculated using the formula in [21] as modified by [22], as follows:

$$\text{Zooxanthellae/cm}^2 = \frac{N \times A_t \times V_t}{A_c \times V_s \times A_s}$$

where:

- N = Number of zooxanthellae counted (cells/cm²)
- A_t = Area of glass cover (mm²)
- V_t = Total volume of initial sample (ml)
- A_c = Area of scraped sample (cm²)
- V_s = Volume of sample used (ml)
- A_s = Area of Micrometre (mm²)

The mitotic index of zooxanthellae in juvenile clams was also observed after recovery periods of 1 day, 5 days, 7 days, 13 days and 17 days. The mitotic index was determined following the methods in [23–27]. This is a homogenization method in which the zooxanthellae are separated from their host by scraping with a scalpel. The material obtained is then suspended in clean sea water. To separate zooxanthellae from other materials, a multilevel filter was used, starting with a 250 micrometre filter, followed by 175 and 50 micrometre filters. The filtrate was then placed in a measuring cup for analysis. The mitotic index was calculated by counting the number of zooxanthellae in the process of dividing (cytokinesis or caryokinesis) which can be seen as a twin cell using a microscope at 400 times magnification. Samples were observed after 03.00, 06.00, 09.00, 12.00, 15.00, 18.00, 21.00, and 24.00 hours with 5 repetitions. The mitotic index (MI) was calculated based on the methods in [22,25] using the formula:

$$MI = \frac{nz}{500} \times 100\%$$

where:

- MI = mitotic index
- nz = number of zooxanthellae cells dividing

2.3. Statistical analysis

To analyse the effect of clove oil on the density and mitotic index of zooxanthellae in juvenile clams, the density and mitotic index were compared between treatments and with the control (clams not exposed to clove oil). Analysis of experimental data was carried out using Excel and SPSS software. Data normality was tested using the Kolmogorov-Smirnov Test and Shapiro-Wilk Test, while uniformity of variance was tested with Levene's Test. The level of significance of the difference between treatments was carried out using a two factor analysis of variance (ANOVA) followed by post hoc testing (Tukey HSD Test) if statistically significant differences (at the 95% confidence level) were found. The results were tabulated.

3. Results

3.1. Changes in the density of zooxanthellae in juvenile clams

The observed densities of zooxanthellae in juvenile clams (Table 1) did not show statistically significant differences in the density of zooxanthellae in juvenile clams between clove oil treatments or between these treatments and the control (no clove oil) (Kruskall Wallis test, P > 0.05).

Table 1. Mean density of zooxanthellae in juvenile clams exposed to clove oil at different concentrations

Clove oil concentration (ppm)	Density of zooxanthellae in juvenile clams (10^6 cells/cm ²)				
	Day 1	Day 5	Day 9	Day 13	Day 17
Control	5.75	6.03	5.83	5.18	5.39
20	5.66	5.98	5.39	5.08	5.89
30	5.30	6.03	5.36	4.80	5.70
40	6.04	5.79	5.68	4.49	5.56
50	5.88	5.95	5.51	5.46	5.09
60	5.58	4.44	4.38	5.03	5.09
70	4.94	5.41	4.66	5.08	5.55

3.2. Mitotic index of zooxanthellae in juvenile clams

The observed mitotic index values for zooxanthellae in juvenile clams are shown in Table 2. The Kolmogorof-Smirnov and Shapiro-Wilk normality test and Levene's variance test indicated that the mitotic index data for zooxanthellae in juvenile clams did not meet the normality or the uniformity of variance requirements necessary for the data to be analysed using parametric statistics. The data were therefore transformed so that the Kruskal-Wallis non-parametric test could be performed. The results of this test showed no significant difference ($P > 0.05$) between the mitotic index of zooxanthellae in juvenile clams with treatment (different concentrations of clove oil) or over time (number of days after exposure).

Table 2. Mean mitotic index of zooxanthellae in juvenile clams exposed to clove oil at different concentrations

Clove oil concentration (ppm)	Mitotic Index of Zooxanthellae in Juvenile clams (%)				
	Day 1	Day 5	Day 9	Day 13	Day 17
Control	0.313	0.325	0.288	0.313	0.300
20	0.325	0.238	0.288	0.313	0.300
30	0.300	0.325	0.288	0.313	0.300
40	0.313	0.338	0.313	0.313	0.288
50	0.325	0.288	0.313	0.288	0.300
60	0.300	0.325	0.300	0.288	0.313
70	0.300	0.338	0.288	0.325	0.313

4. Discussion

4.1. Effect of clove oil on juveniles of the clam *Tridacna derasa*

Tridacnid clams are coral-reef associated marine invertebrates. Juvenile clams are a non-target organism for ornamental fishing fishermen collecting fish, but these animals can experience mortality from fishing practices that are not environmentally friendly, such as the use of cyanide to anaesthetise fishes. This study examined the condition of juvenile clams (*Tridacna derasa*) after exposure to clove oil by measuring the density and mitotic index of zooxanthellae present in these juvenile clams.

The lack of significant difference between the density of zooxanthellae in juvenile clams exposed and not-exposed to clove oil (Table 1) indicates that exposure to clove oil to juvenile clams has little effect on the symbiosis between juvenile clams and their zooxanthellae. As zooxanthellae loss tends to

be caused by stress, in particular stress due to environmental changes [24], the results indicate that exposure to clove oil at the concentrations necessary for use in the ornamental fishery does not seem to cause significant stress for juvenile clams.

The density of zooxanthellae in juvenile clams exposed to clove oil and the control clams show temporal fluctuations within each treatment. However, throughout the study period the density of zooxanthellae in all treatments (0 to 70 ppm clove oil) remained within what would appear to be normal limits. The highest density of zooxanthellae in juvenile clams was 6.04×10^6 cells/cm² (recorded in the 40 ppm clove oil treatment on day 1) while the lowest was 4.38×10^6 Cells/cm² (recorded in the 60 ppm clove oil treatment on day 9). Throughout the study, the density of zooxanthellae in juvenile clams remained higher than the 3.4×10^6 cells/cm² reported by [20] or 1.13×10^6 cells/cm² in [22].

Observation of the average mitotic index of zooxanthellae in the mantle of juvenile clams after exposure to clove oil within a concentration range of 20 ppm to 70 ppm over a 17 day post-exposure period showed no significant difference between exposed and control *T. derasa* juveniles. In each experimental treatment, including the control replicates, the mitotic index fluctuated over the 17 day experimental period, but remained within a similar range (Table 2). This indicates that the exposure to clove oil with concentrations of 20-70 ppm did not change the normal rate of division in zooxanthellae living in symbiosis with the juvenile clams.

4.2. Application of clove oil as a fishing aid

Clove oil contains anaesthetic compounds (specifically eugenol) which can be used *inter alia* to anaesthetise fish. The efficacy of clove oil is well-known and compares favourably to that of other potential fish anaesthetics. However, effectiveness in its intended use is only one of several criteria that must be met when evaluating the use of clove oil as an anaesthetic fit to be released into the aquatic environment [28]. Previous studies have shown that many types of fish can be anesthetized quickly on exposure to clove oil prepared in concentrations of 20-60 ppm [4,8,9,28]. The US Food and Drug Administration (FDA) has categorized clove oil as a generally recognized as safe (GRAS) ingredient for use in dental cement (fillings) and as a food additive. Clove oil has long been used in several countries, including in Indonesia, as a basic anaesthetic compound in local analgesics and anaesthetic substances used in dentistry [7,8]. Clove oil is considered to be safe as a food additive in small quantities (<1500 ppm) [29], so that allowable concentrations for humans are considerably higher than those required for anaesthetising many fishes. Thus, unlike cyanide, the use of clove oil should not pose any risk to the health of fishers and their families.

In this study clove oil at a concentration of 20-70 ppm did not appear to cause damage to juvenile tridacnid clams (specifically *Tridacna derasa*), one of the ecologically and economically valuable non-target organisms which could potentially be affected. These results can be taken into consideration in the application of clove oil as an environmentally friendly (or at the very least a less damaging) alternative to destructive cyanide fishing methods. However, further research on other tridacnid clam size classes and a wider range of invertebrate taxa is needed to confirm whether clove oil can indeed be considered as a truly environmentally friendly fishing aid.

5. Conclusion

Exposure of juveniles of the tridacnid clam *Tridacna derasa* to clove oil in concentrations from 20 ppm to 70 ppm did not result in any significant deterioration in the two indicators of juvenile clam condition measured. The density and mitotic index of zooxanthellae within the clam mantle did not differ significantly between exposed clams and clams not exposed to clove oil. While other tridacnid clam species (or earlier life stages) might differ in their sensitivity to clove oil exposure, these results indicate that the use of clove oil within this concentration range as an aid to ornamental fisheries seems unlikely to impact the growth and survival of juvenile tridacnid clams in a 3-4 cm shell width (or larger) size class. However, the effects of exposure to clove oil on tridacnid clam larvae (including metamorphosis and settlement) and smaller, recently settled tridacnid clam juveniles remain unknown.

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