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Assessment of tunicates found in the northern waters of Ambon Island and its rearing requirements in a laboratory setting

Y Tapilatu¹ and T Ginzel¹

¹Center for Deep-Sea Research, Indonesian Institute of Sciences (PPLD LIPI), Jl. Y. Syaranamual Guru-guru Poka Ambon 97233, Indonesia

yosmina.tapilatu@lipi.go.id

Abstract. The Banda Sea region is situated in the Wallacea biogeographical area, known as one of the biodiversity hotspots of the world. The region harbours various types of ecosystems, ranging from mangroves and coral reefs to seaweeds, indicating its potential for harbouring organisms that produce bioactive compounds, such as sea cucumbers, soft corals, and tunicates. While many studies have been carried out on the first two organisms, much lesser is known about the latter one, notably in this region. The assessment is our first attempt to explore the tunicates found in northern coastal waters of Ambon Island, an area that is a part of the Banda Sea. Two types of tunicates were obtained during the survey, namely the violet-green and white-yellow variants of solitary tunicates *Polycarpa aurata* (15 specimens) and social ones of *Didemnum molle* (3 specimens). The *Polycarpa* specimens' heights ranged from 2.5 to 4.8 cm with wet weights from 9.4 to 28.03 g, and the *Didemnum* ones were of 1.2 - 2 cm and wet weights from 1.2 to 5 g. Feeding experiment indicated that low volume (5-10 ml) of pure cultures such as *Dunaliella* sp., *Nannochloropsis* sp. and *Tetraselmis* sp. in small aquarium (40 x 60 cm) resulted in good survival rate for *Polycarpa aurata* collected from northern Ambon Island waters. Rearing experiments in a discontinuous aquarium setting using solitary tunicates indicated the following requirements: thorough cleaning of the tanks, regular feeding intervals, and regular cleaning of each tunicate. The regular cleaning of mucous at the external part of tunicates is indispensable to ensure the wellbeing of the organisms.

Keywords: Banda Sea, *Polycarpa* sp., discontinuous incubation, feeding experiment

1. Introduction

The Banda Sea is situated in the Wallacea biogeographical area, known as one of the biodiversity hotspots of the world. It harbors various types of ecosystems, ranging from mangroves and coral reefs to seaweeds, indicating the Banda Sea potential for harboring organisms that produce bioactive compounds, such as sea cucumbers, soft corals, and tunicates. While many studies have been carried out on the first two organisms, much lesser is known about the latter one, notably in this region. Northern coastal waters of Ambon Island are a part of the Banda Sea. Coral reefs coverage is relatively vast in these waters, but information regarding invertebrates living in them is scarce, if not known at all. This lack of information is not only caused by the limited studies carried out but also due to the lack of infrastructures to do such studies.

Tunicates are soft-bodied invertebrates (Class Ascidiacea) living in all sort of ecosystems, from coral reefs to the deep sea. They are very diverse with around 2,000 different species known to date.



[1] They have various functions ranging from food sources in certain countries (Chile, South Korea, Japan and France) [2], producers of bioactive compounds [3-5] to biomarkers in ecotoxicological studies [6]. However, tunicates are much understudied in Indonesia, particularly in its eastern marine region. Literature search carried out online only yielded limited results on studies about and/or related to tunicates in Indonesian eastern marine region, such as tunicates inventory data from Siboga Expedition in 1899-1900 [7], a recent coral reef organisms inventory in Halmahera (2015) [8], the discovery of new alkaloids from *Polycarpa aurata* collected in north Sulawesi [4] and the identification of antimicrobial compound produced by a bacterial symbiont of *Phallusia julinea*, a tunicate species found in Doreri Gulf of West Papua [9]. None were available about incubation settings for tunicates found in the Banda Sea ecosystem. Moreover, there were articles available online about tunicates incubation in laboratory settings, but they were either too brief (and covered only temperate ones) [10] or only covered the social type of tunicate [11]. None to our knowledge existed on tropical tunicates incubation settings in limited infrastructures.

This assessment is thus our first attempt to explore the tunicates diversity in the Banda Sea. The main objective of this research is to provide the latest information about tunicates found in the said region and to provide initial information on its rearing possibility in a discontinuous aquarium setting for future research activities. The discontinuous setting is optimized for incubation since it is the only option available in a limited infrastructure.

2. Material and Method

2.1. Sample collection

Samples were collected from the northern coastal area of Ambon Island, in particular in three locations namely the waters of Morela, Mamala and Hitu villages (figure 1). These locations were selected due to their relatively preserved area of coral reefs. Tunicates were randomly collected using SCUBA diving equipment after visual assessment of its presence in the surveyed area. Sampling locations positions were recorded using a GPS Garmin 76 Cxi. Samples collected were put in sterile clipped plastic bags containing *in situ* sea water. Once reaching the surface the samples were kept in a cool box (8-10°C) throughout the sampling period until transfer to the aquaculture laboratory.

2.2. Sample identification

Tunicates obtained were identified based on its external appearance (body structure, tunic texture, etc). The identification was done according to guidelines that were available online, i.e. *Sous les Mers* [12], World Register of Marine Species (WoRMS) [13], Ocean Biogeographic Information System (OBIS) [14] and Reef Guide [15].

2.3. Incubation setting

Dimension and weight of samples obtained were measured and noted prior to transfer into the aquarium. Due to the limited infrastructure available at our aquaculture laboratory, we used only one aquarium in this experiment, with the dimensions of 40 x 60 cm. To mimic their original habitat, the tunicates were incubated with three sea cucumbers in the same aquarium. Natural seawater was pumped from nearby Ambon Bay and filtered prior to usage and replaced every two weeks. The bottom materials (sand and gravels) were replaced when algal growth was visible to eliminate the negative impact of organic waste build up and excessive algal growth in discontinuous incubation. Air was provided through a tube with constant pumping. Physico-chemical parameters were monitored at least once every two weeks to observe the water quality during incubation. Samples were observed daily and manual cleaning of mucous enveloping individual tunicates was done if necessary.

2.4. Feeding experiment

Tunicates obtained were fed with phytoplankton culture available at the laboratory, i.e. *Nannochloropsis* sp., *Tetraselmis* sp. and *Dunaliella* sp. The phytoplankton cultures were fed in two

treatments, namely individually (5 and 10 ml each) and the combination of three, 10 ml each (30 ml in total). All cultures were in their stationary phase by the time of feeding. Feeding was done every two weeks, and mortality rate would be recorded 7 days after feeding until cleaning and replacement of the aquarium, the sea water and the sand-gravel materials.

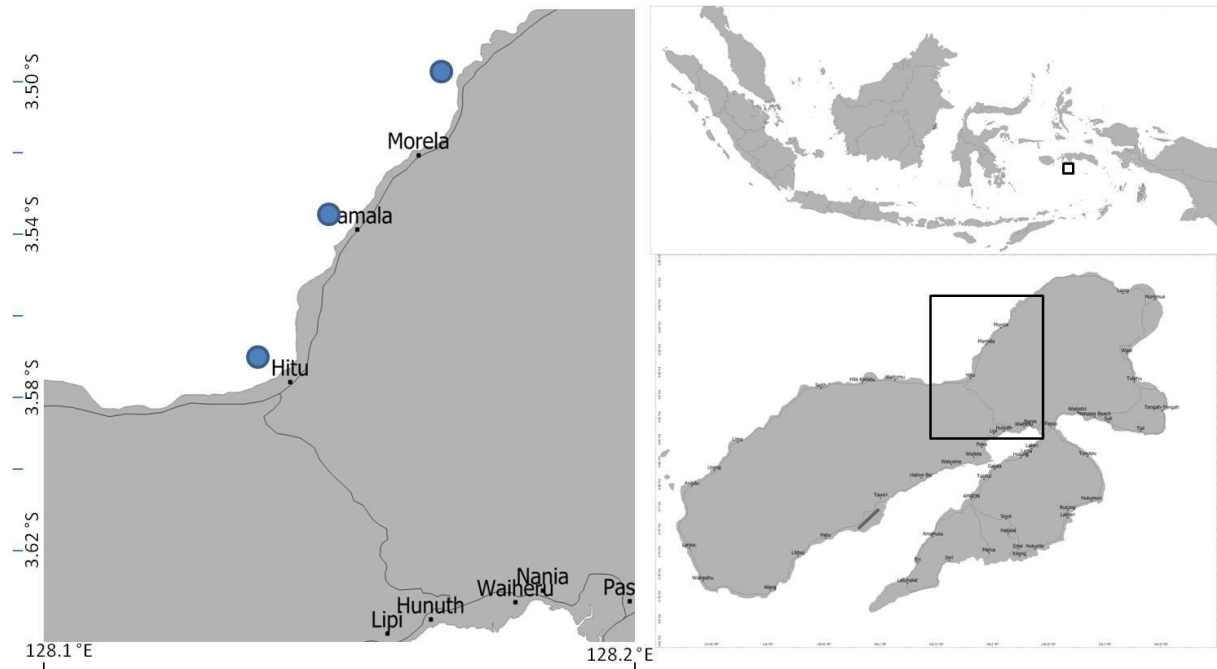


Figure 1. Map of Indonesia (inset, upper right), Ambon Island (inset, lower right) and tunicates sampling location (main, left). Blue dots indicate three locations where samples were collected.

3. Results and Discussion

3.1. Sampling locations profile

Blast fishing was a common practice in Lubang Buaya, Morela and adjacent area around 2005. It was done with the homemade bombs and caused important destruction in these waters. Fortunately, following the local authorities' intervention, the fishermen inhabiting the surrounding village did not continue their usage of this destructive method of fishing. Fragments of branched coral found were mostly due to anchoring and tourism activities. The coral reef condition of Lubang Buaya Morela, in general, was relatively good visually, with water transparency reached more than 75% of water depth (cf. Table 1) during the sampling and only a few floating plastic wastes observed. Overall Morela coral reef ecosystem was in better condition compared to those in Mamala and Hitumessing. The seabed in this coastal area was a shallow shelf from the intertidal area to about 50 meters seawards, continued by a steep slope.

Table 1. Sampling locations profiles.

Description	Morela	Mamala	Hitumessing
Position coordinates (Latitude, Longitude)	3°31'30.8" S, 128°12'47.4" E	3°33'16.5" S, 128°11'26.1" E	3°34'58.2" S, 128°10'21.0" E
Approximate depth (m)	6	4	6
Transparency (m)	6	3	5
Dominant substrate	Coral reef and sand	Sand	Muddy sand (close to river mouth)
Amount of samples collected	10	6	2

3.2. Tunicate morphology and ecology

There were 18 samples collected in total, with 15 of them were solitary tunicates and the other three were social type ones. Ten of fifteen *Polycarpa* sp. were collected from Morela, three from Mamala and two from Hitumessing area. Tunic morphology observation, height and distribution profile indicated solitary ones' resemblance to the violet-green and white-yellow variants of *Polycarpa aurata* (Quoy and Gaimard, 1834), commonly known as the ink-spot sea squirt [15], the ox-heart ascidian or the gold-mouth sea squirt. Their body forms were ovoid, globular, or conical, with the wrinkled leathery test. Their heights ranged from 2.5 to 4.8 cm with wet weights from 9.4 to 28.03 g. Their heights were slightly less compared to the available information in the identification guide (5 to 15 cm) [12]. They were numerous at the steep slope, notably in Morela area (Figure 2a). As suggested by Berrill in 1955, some species of tunicates from the family of Styelidae such as *Polycarpa* are characterized by the brittle sand-embedded test and muscles to withdraw apertures and flatten the body and are adapted to life on the open sea floor [16].

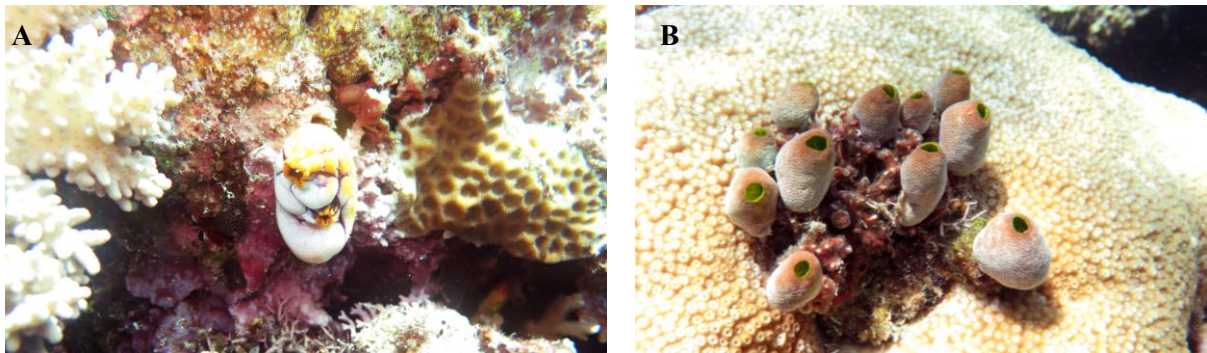


Figure 2. Solitary tunicate morphologically resembled *Polycarpa* sp. variant white-yellow found in the coral reef in Morela (a); a colony of *Didemnum* sp. as observed in Mamala (b). Both pictures were taken in early April 2017. Photo credit: Robert Alik.

The other three were *Didemnum molle* (Herdmann, 1886), a social type that was collected from Mamala area (Figure 2b), and commonly known as the green barrel sea squirt. The observed green and brown colors were from *Phrochlorons* sp., the cyanobacterial symbionts of *Didemnum* sp. [12]. The social type tunicates were visibly smaller and lighter, with height ranged from 1.2 to 2 cm and wet weights from 1.2 to 5.0 g only. The size of *Didemnum* sp. encountered in Mamala was smaller than reported elsewhere in Indonesia. In the Flores Sea, for example, the adult ones could reach 3 to 10 cm in height [12]. It is thus possible that the samples collected from Mamala were only young tunicates, or probably the surrounding environment did not facilitate the optimal growth of this tunicate, as was indicated by their limited occurrence in the three sampling areas.

Both tunicate types are commonly found in eastern Indonesian waters. They attach to hard substrates such as reefs and stones. *Polycarpa aurata* and *Didemnum molle* are found in the tropical eastern Indian Ocean and the western Pacific Ocean, including the Philippines, Indonesia and northern Australia. Their habitat depth ranges are 5 to 50 m (*Polycarpa*) and 5-30 m (*Didemnum*) [12]. A recent inventory of coral reef organisms recorded species of *Polycarpa* and *Didemnum* among ascidians found off western Halmahera [8], an island situated in the north Moluccas. However, further identification is necessary, either by dissection or genetically to confirm the affiliation of all samples collected in our study.

3.3. Incubation in the discontinuous setting trial

Incubation in the discontinuous setting of 40 x 60 cm aquarium (figure 3a) indicated solitary tunicates higher survival rate compared to the social type ones. In other parts of the world, certain species of *Didemnum* are included in invasive species list ([8] and references therein), due to its rapid growth and aggressive competing capability. However, the mortality rate of *Didemnum* collected was 100% by the

third day of incubation in this setting. *Didemnum molle* was reported to live in the following range of natural environmental conditions: 19.13-28.95°C (temperature), 34.07-35.13 PSU (salinity) and at 3.56-4.78 ml l⁻¹ (DO) [17].

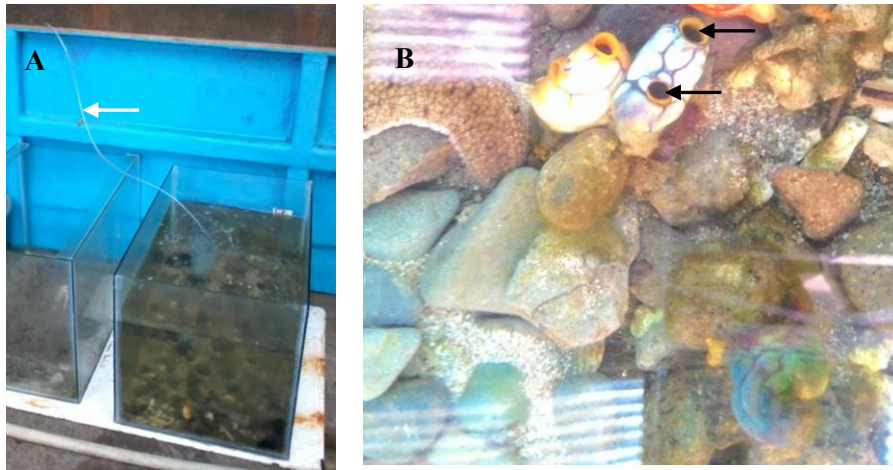


Figure 3. Incubation setting in a 40 x 60 cm aquarium (a) and solitary tunicates in the aquarium, week 9 of incubation (b). *Polycarpa* sp. variant white-yellow (upper-center), violet-green (left-bottom) and sea cucumber (upper-right). The white arrow indicates the plastic pipe that was used to deliver air to the aquarium. The black arrows indicate opened siphons of a tunicate already adapted with the aquarium condition.

The small aquarium could be the cause of this high mortality rate, as the size did not permit incubation of too many tunicates, along with the sea cucumber. Other than that, the rapid growth of existing algae in the aquarium seawater resulted in the increase of water pH (table 2). This probably created a higher stress level to the social tunicates compared to the solitary ones and thus did not allow their optimal survival. The high fluctuation of salinity values could be due to the evaporation or probably because the equipment used during the measurement was not properly calibrated.

Table 2. Water quality parameters observed in the aquarium. The values presented were obtained after six measurements during 13 weeks of incubation. Standard values were based on environmental conditions information of *Polycarpa aurata* according to the references cited.

Parameter	Measurements	Standard values for <i>Polycarpa aurata</i>	Standard reference
pH	8.24 ± 0.40	7.5-9	[10]
Salinity (PSU)	35.58 ± 4.25	34.98-35.13	[14]
Temperature (°C)	27.02 ± 0.50	26.22-26.8	[14]
DO (ml l ⁻¹)	6.6 ± 0.65	4.57-4.68	[14]

3.4. Feeding experiment

The feeding experiment carried out using *Polycarpa* sp. indicated that the highest mortality rate (40%) was observed when the tunicates were fed with 10 ml *Nannochloropsis* sp. (Table 3). The second highest mortality rate was observed when they were fed with 30 ml of combined three phytoplankton cultures, as it provoked rapid growth of algae at the aquarium wall and thus reduced the water quality. The combination also provoked rapid mucous envelopment of tunicates that led to mortality. Manual

cleaning of enveloping mucous should be done at least once every two days to prevent mass mortality of tunicates.

Berrill [10] suggested the pure culture of *Nitzschia* sp. as the nutritional source, but mentioned that mixed assortment of small diatoms and algae could also be satisfactory. However, in our incubation settings, mixed culture tended to provoke a rapid increase of algal mass that occupied aquarium walls and thus increase pH values (cf. Table 2) that could provoke mortality. The lower dose (5-10 ml) of pure cultures such as *Dunaliella* sp., *Nannochloropsis* sp. and *Tetraselmis* sp. indicated good survival rate for *Polycarpa aurata* collected from northern Ambon Island waters and incubated in small aquarium. Nevertheless, this should be confirmed in the future with further experiments where the aquarium is kept in more sheltered place for incubation setting optimization.

Table 3. Feeding experiment and mortality rate of tunicates (*Polycarpa* sp.) from northern Ambon Island waters. The incubation was carried out for 7 days before the sea water was replaced with the clean one.

No.	Species	Feeding experiment Volume per species (ml)	Total volume fed	Mortality rate (%)
1	<i>Dunaliella</i> sp.	5	5	0
2	<i>Dunaliella</i> sp.	10	10	29
3	<i>Nannochloropsis</i> sp.	5	5	0
4	<i>Nannochloropsis</i> sp.	10	10	40
5	<i>Tetraselmis</i> sp.	5	5	22
6	<i>Tetraselmis</i> sp.	10	10	0
7	<i>Dunaliella</i> sp.	10		
	<i>Tetraselmis</i> sp.	10	30	33
	<i>Nannochloropsis</i> sp.	10		

4. Conclusions

The assessment is our first attempt to explore the tunicates found in northern coastal waters of Ambon Island, an area that is a part of the Banda Sea. Two types of tunicates were obtained during the survey, namely the violet-green and white-yellow variants of solitary tunicates *Polycarpa aurata* and social ones of *Didemnum molle*. Rearing experiments in a discontinuous aquarium setting using solitary tunicates indicated the following requirements: thorough cleaning of the tanks, regular feeding intervals, and regular cleaning of each tunicate. The regular cleaning of mucus at the external part of tunicates is indispensable to ensure the well-being of the organisms.

Future experiments should include incubation in a more sheltered setting, with only a certain amount of tunicates (8 to 10 solitary ones) in the same dimension of the aquarium. Further identification to confirm samples' identity would be necessary. More importantly, bioprospecting effort could be initiated to examine whether *Polycarpa aurata* growing in Ambon island waters contain similar bioactive compounds as those sampled in Manado in a prior report.

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