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SUBSTRATE PREFERENCES AND EFFECTS OF MEDIUM WITH SOIL EXTRACT ON GROWTH OF GENUS *Coolia* (DINOPHYCEAE)

NORMAWATY MOHAMMAD NOOR[1*], AIMIMULIANI ADAM[1], SHAHBUDIN SAAD[1], FIKRI AKMAL KHODZORI[1] AND SITI RAEHANAH MUHD SHALEH[2]

[1]*Department of Marine Science, Kulliyyah of Science, International Islamic University Malaysia, Jalan Sultan Ahmad Shah Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia,*
[2]*Borneo Marine Research Institute, Universiti Malaysia Sabah, 88899 Kota Kinabalu, Sabah, Malaysia*

**Corresponding author email: normahwaty@iium.edu.my*

Abstract: *Coolia* is a benthic dinoflagellate that lives epiphytically with a substrate. It has been reported that several of the species produce biotoxins. Therefore, this study was done to identify the substrate preferred by *Coolia* and the effect of media with soil extraction on the growth of the species of *C. tropicalis* and *C. malayensis*. Several substrates; the seagrass (*Enhalus*), seaweed (*Sargassum*, *Padina* (Phaeophyta), *Halimeda* (Chlorophyta) and coral rubbles were collected and *Coolia*'s cell density was determined. *C. tropicalis* and *C. malayensis* were grown in ES-DK medium with and without soil extract at similar conditions. The specific growth rate, division per day and generation time were determined for both species. Results showed that *Coolia* flourished in the substrate with a greater amount of thalus from brown macroalgae (Phaeophyta). While the cell density of *C. tropicalis* was significantly higher in medium without soil extract, *C. malayensis* was significantly higher in the medium with soil extract. This indicates that both species have similar basic requirements, but differ in their specific needs. This information on the substrate and medium preferences increased our knowledge of the ecology of *Coolia*.

KEYWORDS: *Coolia* tropicalis, *Coolia malayensis*, ES-DK medium, nutrient, benthic dinoflagellates

Introduction

Genus *Coolia* (Gonyaulacales, Ostreopsidaceae) has a wide geographic distribution (Leaw *et al.,* 2016). Seven species of *Coolia* have been reported, viz. *C. monotis* Meunir, *C. areolata* L. Ten-Hage, J. Turquet, Quod & A. Couté, *C. canariensis* S. Fraga, *C. malayensis* Leaw, P. T. Lim & Usup, *C. tropicalis* M. A. Faust, *C. santacroce* Karafas, Tomas & York and *C. palmyrensis* Karafas, Tomas & York. So far, no Harmful Algal Bloom (HAB) cases have been reported due to *Coolia* spp. However, toxicity studies on these species have revealed that the species produce biotoxins and this raises the possibility that the species could cause problems in the future. A number of toxins have been described from *C. tropicalis*, including cooliatoxin (in *C. monotis*) Mohammad-Noor *et al.* (2013), a yessotoxin by Holmes *et al.* (1995) and several more yessotoxins were identified by Wakeman *et al.* (2015). Other studies reported the toxicological effects of *Coolia* toxin in mice; *C. malayensis* caused low toxicity in mice with adhesion in the peritoneum and enlarged the spleen at a concentration of 900 mg/kg, while at the same dose, toxin from *C. tropicalis* caused the mice to die (Rhodes *et al.,* 2014). Both species tested were isolated from Malaysian coastal waters, referred to Table 1 in Rhodes *et al*. (2014), C. *malayensis* (KO92) and C. *tropicalis* (K1156).

Study of substrate preferences by benthic dinoflagellates have been reported elsewhere. Several reasons have been pointed out on substrate preference, mainly referring to the morphology of the substrates (Accoroni *et al.,* 2015). Substrates that have a wide surface area possess a higher cell density of benthic dinoflagellates due to the greater number of spaces for attachment. However, substrate preferences can be species-specific as has been demonstrated for *Gambierdiscus* spp (Rains & Parsons, 2015). Study on substrate preference by *Coolia* is limited. This species occurrs naturally in the environment, particularly living epiphytically with substrates such as seaweed and play an important role in the benthic ecosystem.

In order to perform research on phytoplankton, which includes *Coolia*, the establishment of viable cultures for the species will provide advantages. For *Coolia*, several media have been used to culture the species, among them were L1 (Fraga *et al.,* 2008), ES-DK (Leaw *et al.*, 2010; Mohammad-Noor *et al.*, 2013), f/2 (Jeong *et al.,* 2012), IMK (Tawong *et al.,* 2014), and modified K medium with f/2 trace metals (Karafas *et al.,* 2015). Notably, these studies focused on other aspects and not on the viability of the medium itself. Tawong *et al.* (2014), on the other hand, compared the suitability of a medium to enhance growth, reporting that f/2 medium was more suitable to culture *Ostreopsis*, a benthic dinoflagellates in comparison to PES medium. However, there is a limited study on the suitability of a medium to enhance the growth rate and the use of enrichment in the media. These are important aspects as unsuccessful culture establishment will lead to a limited study of a particular species. For a detailed study to be performed on a species, the establishment of healthy and high biomass cultures is important, especially for species that have the potential to produce toxins and may harm human health such as *Coolia*.

The soil is commonly used in microalgal cultures to enrich a culture by providing trace elements and vitamins needed for growth and reproduction of certain species or group of species while inhibiting the growth of other species (Lee *et al.,* 2013). Likewise, the soil has been used in a biphasic culture to represent a solid substrate. This culture technique has been used to establish cultures of microalgae especially benthic microlage (Brand *et al.,* 2013). Herein, the soil enriched the medium with nutrients, ions and compounds that are required in trace amounts. Erd-Schreiber medium is one of the earliest mediums that used soil extract in producing unialgal cultures of marine diatoms and flagellates (Provasoli *et al.,* 1957). Nevertheless, this medium is difficult to reproduce precisely because the soil used for making soil extract came from different sources (Provasoli *et al.,* 1957).

In this study, substrate preferences for *Coolia* were studied from two different locations, i.e., coastal and lagoon areas. In addition, the effect of medium enriched with soil extract on the growth of *C. tropicalis* and *C. malayensis* using ES-DK medium was determined. Results of this study can be used for the ecological study of *Coolia* and help to understand the suitability of medium with soil extract to culture *C. tropicalis* and *C. malayensis* for high biomass yields.

Materials and Methods

Location of Field Study

This study was carried out on Dinawan Island, Sabah. Samples were collected at two sampling sites, i.e., coastal area and lagoon area (Figure 1). The depth of the water during neap tide was about 2 to 3 m. The condition of the study sites was explained in Mohammad-Noor *et al.* (2016).

Sample Collection

Two replicates of substrate samples were collected at both sampling sites. Substrates samples collected were seaweed (*Sargassum*, *Padina*, *Halimeda*), seagrass (*Enhalus*) and coral rubble. These substrates were kept in separate plastic bags containing filtered seawater. To dislodge benthic dinoflagellates from the substrates, each of the substrates was shaken vigorously before being filtered through a 12 µm sieve. Then, samples were filtered using a 20 µm sieve and samples collected on the filter were transferred into a urine bottle. Samples were preserved with Lugol's iodine andlabelled with a location, date, and substrate type.

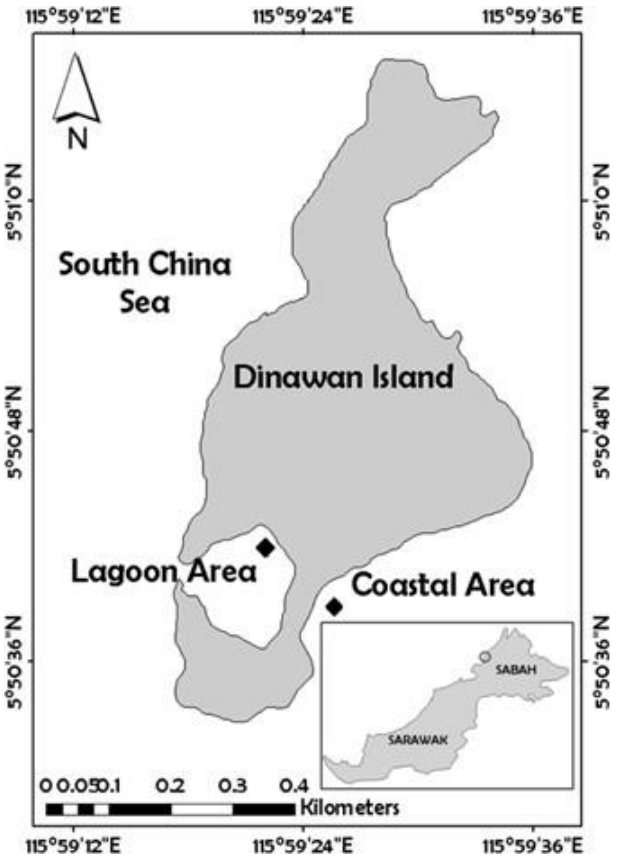

Figure 1: Sampling location in Dinawan Island, Sabah

Physico-chemical parameters such as salinity, pH, temperature and dissolved oxygen were measured *in situ* using multiparameter Hydrolab. Water samples were collected and analysed for nutrients as outlined in Mohammad-Noor *et al.* (2016). Nutrients such as nitrate and phosphate were determined according to Parson *et al.* (1984).

Determination of Cell Abundance

Cell abundance was determined by counting 1mL of each sample twice under light microscopy at a total magnification of 100 x. Counting was done using the Sedgwick rafter counting procedure.`

Cultures and Medium Preparation

Cultures of *C. tropicalis* and *C. malayensis* were obtained from the Unit for Harmful Algal Bloom Studies (UHABs) laboratory of Universiti Malaysia Sabah. Cultures were maintained using ES-DK media supplemented with soil extract at a temperature of 26-27 °C, 12:12 light: dark cycle and light intensity of100 µmol m^{-2} s^{-1}. ES-DK was used to maintain both *Coolia* species and for the growth experiments. The ES-DK medium used contained 32.55mg/L $NaNO_3$, 4.65 mg/L Na-glycerophosphate, P_2 stock (Na_2-EDTA, $FeCl_3.6H_20$, H_3BO_3, $MnSO_4.H_2O$, $ZnSO_4.7H_2O$ and $CoSO_4.7H_2O$), Fe stock ($Fe(NH_4)_2(SO_4)_2.6H_20$ and Na_2-EDTA), vitamin B_{12}, biotin and thiamine. The concentration of nitrate and phosphate in the ES-DK medium is presented in Table 1. Soil extract was added to the medium. To prepare the soil extract, 300 g of garden soil was placed in a l L Erlenmeyer flask containing 600 mL of distilled water. The mixture then was autoclaved at 121 °C for 45 min and left to cool to room temperature.

Next, the mixture was filtered through filter paper (Whatmann no. 1). The liquid obtained was centrifuged at 4800 rpm for 15 min. The supernatant was collected and autoclaved at 121 °C for 45 min. Hereafter, the soil extract was kept cool in the refrigerator (4°C) until further use (Guilard, 1975). The soil extracts used to culture and perform the experiments were from the same batch. Nitrate and phosphate concentrations of the soil extract were determined according to the standard method by Parson *et al.* (1984). Other parameters such as trace elements were not determined.

Table 1: Estimated nitrate and phosphate concentrations in the medium and soil extract

	ES-DK Medium (Kokinos and Anderson, 1995)	Soil extract in 1L seawater
Phosphate, µM	14.35	0.018
Nitrate, µM	382.99	3.0×10^{-7}

Growth Study

Growth experiments were conducted using both *Coolia* at a temperature of 30 °C, the salinity of 30 psu, a light intensity of 100 µmol m^{-2} s^{-1} and light and dark cycles of 12:12 hours. The experiments were each done in two different media; one using ES-DK medium containing soil extract and the other using ES-DK medium without soil extract. Initial experiments started with inoculum of 100 ± 45 cells/mL in a 1 L flask of 800 mL medium in three replicates. The experiments were conducted twice. During the experiment, a 3 mL sample was taken every day and preserved with Lugol's iodine. These samples were counted using similar methods as mentioned above and data obtained were used to analyze the growth pattern and the growth rate of each species. The Growth rate was calculated using Equation 1.

Growth rate (K') = ln (N2/N1)/t2-t1,
where N1 and N2 are number of cells at time 1 (t1) and time 2 (t2).
Division per day = K'/Ln2
Generation time = 1/division per day

Statistical Analysis

A test of cell abundance among different substrates and between growth rates of both species using different media was done using the PAST (PAlaeontological STatistics version 2.17b) (Hammer *et al.*, 2001) programme.

Results

Cell Abundance of* *Coolia* *at Coastal Area and Lagoon Area

In the coastal area, the cell density of *Coolia* was higher in *Padina* in comparison to other substrates (Kruskal-Wallis, $p < 0.05$). An average of 149 cells mL^{-1} g^{-1} of *Coolia* was found in *Padina* and the highest cell density was recorded in May (Figure 2). In the lagoon area, the highest cell density of *Coolia* was found in *Sargassum* in April (Kruskal-Wallis, $p < 0.05$) with 71 cells mL^{-1} g^{-1}. For both areas, the cell density of *Coolia* was low in *Enhalus*, coral rubbles and *Halimeda* (Figure 3). Generally, *Coolia* was more abundant in the coastal area compared to the lagoon area.

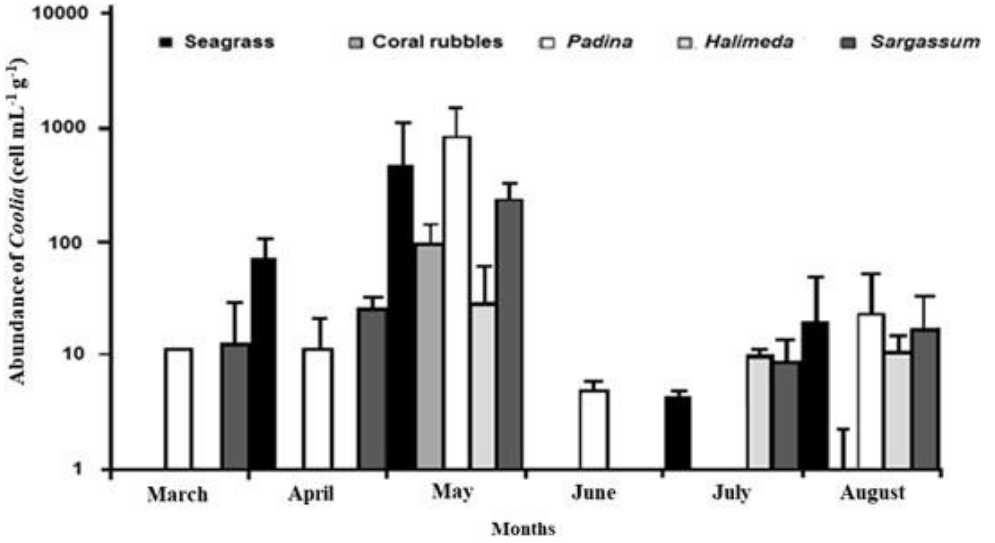

Figure 2: Abundance of *Coolia* (cell mL^{-1} g^{-1}) on different substrates along the coastal area of Dinawan Island from March to August

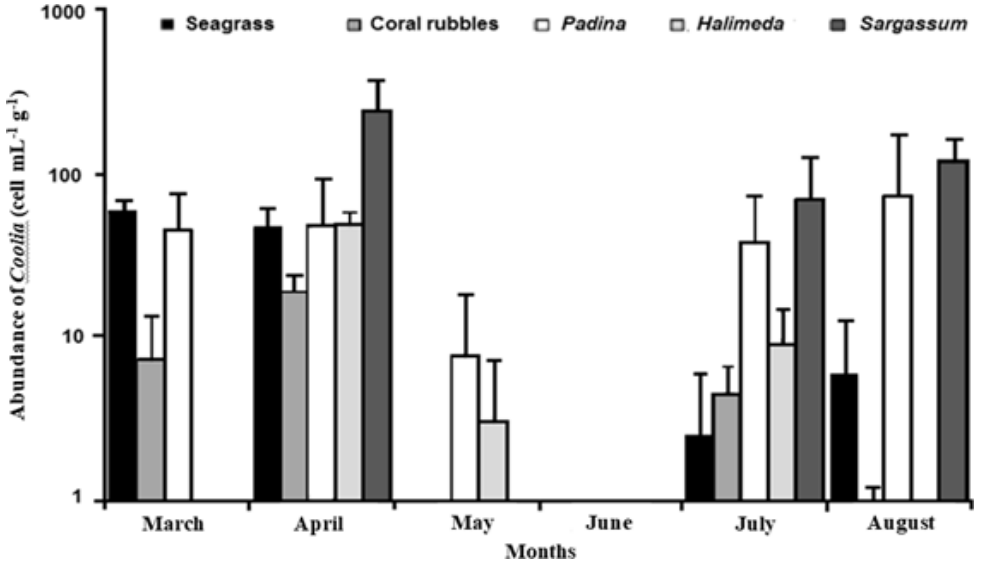

Figure 3: Abundance of *Coolia* (cell mL^{-1} g^{-1}) on different substrates in the lagoon of Dinawan Island from March to August

The physicochemical data recorded in both areas were very similar (Table 2). Regarding nutrient concentrations, a higher nitrate concentration was detected in the coastal area (4.2 ± 2.0 µM) compared to the lagoon area (2.6 ± 1.5 µM). However, the phosphate concentration was higher in the lagoon area (4.3 ± 1.2 µM) compared to the coastal area (1.1 ± 0.4 µM; Table 2).

Table 2: Physico-chemical parameters and nutrient concentration recorded during sample collection (Mohammad-Noor *et al.*, 2016)

Parameter	Coastal	Lagoon
Salinity	30.4 ± 1.9	30.5 ±2.4
pH	8.2 ± 0.1	8.2 ± 0.1
Temperature, °C	30.9 ± 0.1	30.9 ± 1.4
Dissolved oxygen, mg/L	6.4 ± 1.4	5.8 ± 1.2
Nitrate, µM	4.2 ± 2.0	2.6 ± 1.5
Phosphate, µM	1.1 ± 0.4	4.3 ± 1.2

Effects of Soil Extract on the Growth of C. malayensis and C. tropicalis

C. malayensis showed higher growth in medium containing soil extract in comparison to *C. tropicalis* (Figures 4 and 5). However, significant differences (ANOVA, $p < 0.05$) were found between medium containing soil extract and no soil extract for both *Coolia* species in terms of growth performance. The specific growth rate of *C. malayensis* with the presence of soil extract was $0.63K^{-1}$ and without soil extract was $0.32K^{-1}$. For *C. tropicalis*, the specific growth rate with the presence of soil extract was $0.36K^{-1}$ and without soil extract was $0.51K^{-1}$. The division per day and generation time for *C. malayensis* with soil extract were 0.91 and 1.10, whereas without soil extract this amounted to 0.46 and 2.17, respectively. For *C. tropicalis*, division per day and generation time were 0.52 and 1.93 with soil extract and 0.74 and 1.36 without soil extract (Table 3). In 1 L of seawater (medium), the nitrate and phosphate concentration of soil extract were 3.0×10^{-7} and 0.018 µM, respectively (Table 1).

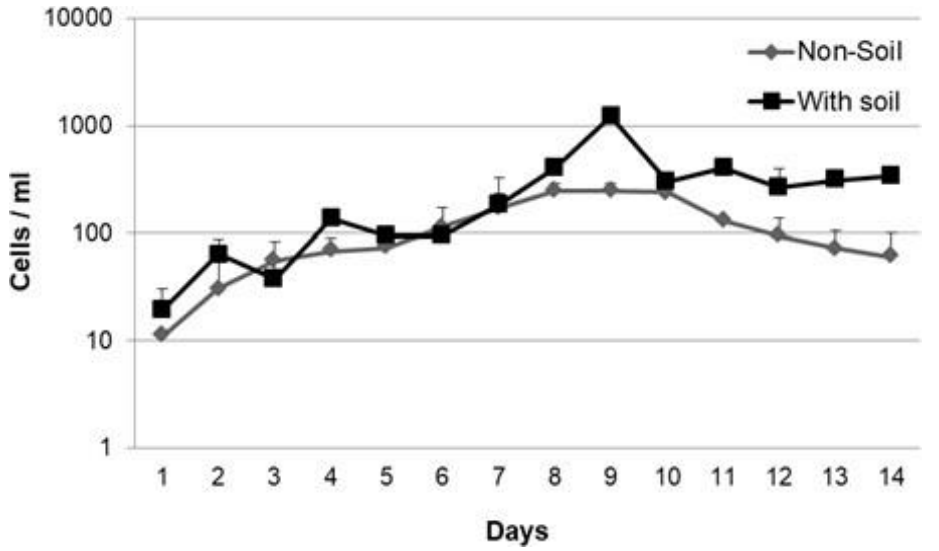

Figure 4: Comparison of growth curves for *C. malayensis* cultured in medium with and without soil extracts

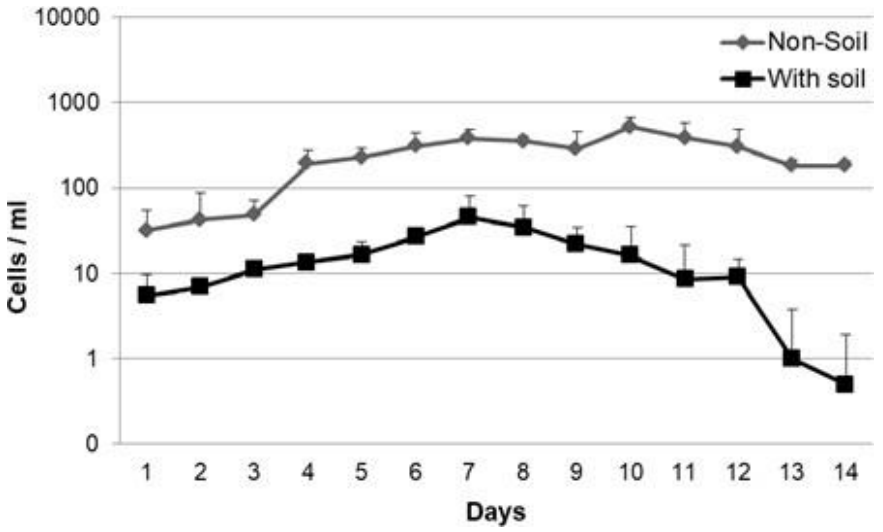

Figure 5: Comparison of growth curves for *C. tropicalis* cultured in medium with and without soil extracts

Table 3: Specific growth rate, division per day and generation time for *C. malayensis* and *C. tropicalis*

	C. malayensis		*C. tropicalis*	
	Soil Extract	No Soil Extract	Soil Extract	No Soil Extract
Specific growth rate K^{-1}	0.63	0.32	0.36	0.51
Division per day	0.91	0.46	0.52	0.74
Generation time	1.10	2.17	1.93	1.36

Discussion

The results showed that *Coolia* species preferred brown macroalgae: identified as *Sargassum* and *Padina*, as substrates in both locations, i.e., coastal and lagoon areas. Several studies on benthic dinoflagellates have reported that brown macroalgae and red macroalgae are preferred as substrates compared to green macroalgae (Morton & Faust, 1997). Substrate preference by benthic dinoflagellates was suggested to be species specific due to their epiphytic behavior (growth and attachment), (Rains & Parson, 2015). Nevertheless, a number of factors such as the morphology of the seaweed (Totti *et al.*, 2010; Selina *et al.*, 2014) and allelopathy effect (Accoroni *et al.*, 2015) play a crucial role in the selection of the substrates by benthic dinoflagellates. In this study, *Sargassum* and *Padina* provided wider areas for attachment and this is believed to be the reason for the higher number of cells found in these substrates compared to other substrates. However, in the literature, *Coolia* have been isolated from various substrates, including macroalgae (Mohammad-Noor *et al.*, 2013, Momigliono *et al.*, 2013, Tawong *et al.*, 2014) floating macroalgae (Rhodes *et al.*, 2014), turf algae (Wakeman *et al.*, 2015), seagrasses (Leaw *etal.*, 2010; Rhodes *et al.*, 2014), dead corals (Leaw *et al.*, 2010; Leung *et al.*, 2017), rock surfaces (Leung *et al.*, 2017), sand (Faust, 1995; Leaw *et al.*, 2010), tide pools (Faust, 1995) and plastic screens (Faust, 1995; Karafas *et al.*, 2015).

Table 4: Origin, substrates, culture medium of *C. malayensis* and *C. tropicalis*

Species	Origin	Substrate	Medium	References
C. malayensis	Port Dickson, Kota Kinabalu, Langkawi, Malaysia	Seaweed, coral fragments, sea grass, sand	ES-DK	Leaw *et al.*, (2010) Leaw *et al.*, (2016)
	Kota Kinabalu, Malaysia	*Sargassum*, *Padina*	ES-DK	Mohammad-Noor *et al.*, (2013)
	Lundu, Sarawak, Malaysia	nd	ES-DK	Leaw *et al.*, (2016)
	Andaman Sea, Thailand	*Sargassum*, *Padina*	IMK/2	Tawong *et al.*, (2014)
	Jeju Island, Korea	Gelidium amansii	f/2	Jeong *et al.*, (2012)
	Okinawa, Japan	Turf algae	IMK	Wakeman *et al.*, (2015)
	Northland, New Zealand	Floating macroalgae, coraline turf, eelgrasses	f/2, f/2:seawater (1:1)	Rhodes *et al.*, (2014)
	Great Barrier Reef, Autralia	Macroalgae	L1	Momigliono *et al.*, (2013)
	Hong Kong	Rock and dead corals	L1	Leung *et al.*, (2017)
	Guangxi, China	nd	L1	Leaw *et al.*, (2016)
	Florida, USA	nd	L1	Leaw *et al.*, (2016)
	Carrie Bow Caye, Belize	nd	L1	Leaw *et al.*, (2016)
	Florida	nd	L1	Leaw *et al.*, (2016)
C. tropicalis	Kota Kinabalu, Sabah, Malaysia	*Sargassum*, *Padina*	ES-DK	Mohammad-Noor *et al.*, (2013)
	Manado, Indonesia	*Ectocarpus*	L1	Mohammad-Noor *et al.*, (2013)
	Platypus Bay, Queensland, Australia	nd	f_{10k}	Holmes *et al.*, (1991) Mohammad-Noor *et al.*, (2013)
	Pallarenda, Great Barrier Reef, Australia	Macrolagae	L1	Momigliono *et al.*, (2013)
	KohRaed, Chonburi, Thailand	*Sargassum*, *Padina*	IMK/2	Tawong *et al.*, (2014)
	Hong Kong	Rock and dead corals	L1	Leung *et al.*, (2017)
	Carrie Bow Caye, Belize	nd	L1	Leaw *et al.*, (2016)

nd=not determine

Other than the choice of substrate, the environmental parameters were also important in determining the presence of the *Coolia*. Notably, in the current study, the salinity, temperature, pH and dissolved oxygen recorded show similar readings in both areas. However, nitrate and phosphate concentrations in both areas showed a different trend; the coastal area had a higher nitrate concentration in comparison to the lagoon area. Correlatively, *Coolia* showed higher abundance in the coastal area compared to the lagoon area. The effects of nutrient on the growth of benthic dinoflagellates has been reported elsewhere (Vidyarathna & Granéli, 2013). However, data collected in this study are very limited to draw into any conclusion on the effect of physico-chemical parameters on *Coolia* abundance. Growth studies indicated that *C. malayensis* has higher cell abundance in enriched medium with soil extract compared to *C. tropicalis*. This can be seen as the specific growth rate (K^{-1}) of *C. malayensis* increased from 0.32 to 0.63 when grown in medium with soil extract. Results from nutrient analyses (nitrate and phosphate) indicated that soil extract provided a small number of nutrients to the medium prepared. Moreover, it has been reported that soil extract contributes to trace elements and vitamins to the medium (Provasoli *et al.*, 1957; Brand *et al.*, 2013). This indicates that the medium with soil extract enhances the growth of *C. malayensis*.

Unfortunately, the condition is quite the opposite for *C. tropicalis*, whereby the species preferred a medium without soil extract to grow in abundance. This shows that *C. tropicalis* preferred 'common' medium to grow in abundance. This is similar to the findings of Mohammad-Noor *et al.* (2013) which used low nutrient medium to culture *C. tropicalis.* They used medium f_{10K} which contain 0.1 concentrations of the f medium nutrient and enriched with selenium (Holmes *et al.,* 1991). In culture, it is well known that using a full strength medium sometimes caused the cells of certain species to be distorted and not to grow well in comparison to a diluted medium (Fraga *et al.,* 2012). A literature survey showed that many media have been used to culture the *C. malayensis* and *C. tropicalis*. The most common media used were L1 and followed by ES-DK medium (Table 4). Nevertheless, the preparation of the medium depends very much on the source of the seawater as each contains different chemical concentration and biological flora. Thus, it is best to use seawater collected from the same place where the samples are collected from for making the medium.

Conclusion

Coolia preferred brown macroalgae substrates as high cell density was found with *Sargassum* and *Padina* compared to other substrates. *C. malayensis* and *C. tropicalis* have similar basic requirements for growth as both species were able to survive in media without soil extract. Nevertheless, *C. malayensis* showed better performance in both specific growth rate and division rate when cultured in medium with soil extract whereas *C. tropicalis* preferred medium without soil extract. This indicates that each species has its own optimum condition for the best growth requirement which reflects their niche biome. Knowledge on the growth requirements and substrate preference of these species is important because it can be used as a guideline to establish a culture or for high biomass production for a variety of studies such as toxicity, ecology, life cycle and therefore increase our understanding of their important role in the marine ecosystem.

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References

Accoroni, S., Percopo, I., Cerino, F., Romagnoli, T., Pichierri, S., Perrone, C., & Totti, C. (2015). Allelopathic interactions between the HAB dinoflagellate *Ostreopsis cf. ovata* and macroalgae. *Harmful Algae*, 49: 147-155.

Brand, J. J., Andersen, R. A. & Nobles Jr, D. R. (2013). Maintenance of microalgae in culture collections. *Handbook of Microalgal Culture: Applied Phycology and Biotechnology* (pp. 80-89). John Wiley and Sons. https://doi.org/10.1002/9781118567166.ch5.

Fraga, S., Penna, A., Bianconi, I., Paz, B. & Zapata, M. (2008). *Coolia canarensis* sp. nov. (Dinophyceae) a new nontoxic epiphytic benthic dinoflagellate from the Canary Islands. *Journal of Phycology, 44*(4), 1060-1070. doi:10.1111/j.1529-8817.2008.00555.x.

Fraga, S., Rodríguez, F., Bravo, I., Zapata, M. & Marañón, E. (2012). Review of the main ecological features affecting benthic dinoflagellate blooms. *Cryptogamie, Algologie, 33*(2), 171-179. https://doi.org/10.7872/crya.v33.iss2.2011.171.

Faust, M. A. (1995). Observation of sand-dwelling toxic dinoflagellates (Dinophyceae) from widely differing sites, including two new species. *Journal of Phycology, 31*(6), 996-1003. https://doi.org/10.1111/j.0022-3646.1995.00996.x.

Guillard, R. R. (1975). Culture of phytoplankton for feeding marine invertebrates. In Smith, W. L. & Chanley, M. H., Edition: *Culture of Marine Invertebrate Animals,* Plenum

Press, New York, 29-60. http://dx.doi.org/10.1007/978-1-4615-8714-9_3.

Hammer, Ø., Harper, D. A. T. & Ryan, P. D. (2001). PAST: Palaeontology Statistical software package for education and data analysis. *Palaeontologia Electronica*, 4: 19.

Holmes, M. J., Lewis, R. J., Poli, M. A. & Gillespie, N. C. (1991). Strain dependent production of ciguatoxin precursors (Gambiertoxins) by *Gambierdiscus* toxicus (Dinophyceae) in culture. *Toxicon, 29*(6), 761-775.

Holmes, M. J., Lewis, R. J., Jones, A. & Hoy, A. W. W. (1995). Cooliatoxin, the first toxin from *Coolia monotis* (Dinophyceae). *Natural Toxins, 3*(5), 355-362.

Jeong, H. J., Yih, W., Kang, N. S., Lee, S. Y., Yoon, E. Y., Yoo, Y. D., Kim, H. S. & Kim, J. H. (2012). First report of the epiphytic benthic dinoflagellates *Coolia canariensis* and *Coolia malayensis* in the waters off Jeju Island, Korea: Morphology and rDNA sequences. *Journal of Eukaryotic Microbiology, 59*(2), 114-133. doi:10.1111/j.1550-7408.2012.00610.x.

Karafas, S., York, R. & Tomas, C. (2015). Morphological and genetic analysis of the *Coolia* monotis species with the introduction of two new species, *Coolia santacrose* sp. nov. and *Coolia palmyrensis* sp. nov. (Dinophyceae). *Harmful Algae*, 46: 18-33. https://doi.org/10.1016/j.hal.2015.05.002.

Leaw, C. P., Lim, P. T., Cheng, K. W., Ng, B. K. & Usup, G. (2010). Morphology and molecular characterization of a new species of thecate benthic dinoflagellate, *Coolia malayensis* sp. nov. (Dinophyceae). *Journal of Phycology, 46*(1), 162-171. https://doi.org/10.1111/j.1529-8817.2009.00778.x.

Leaw, C. P., Tan, T. H., Lim, H. C., Teng, S. T., Yong, H. L., Smith, K. F., Rhodes, L., Wolf, M., Holland, W. C., Vandersea, M. W., Litaker, R. W., Tester, P. A., Gu, H., Usup, G. & Lim, P. T. (2016). New scenario for speciation in the benthic dinoflagellate genus *Coolia* (Dinophyceae). *Harmful Algae*, 55: 137-149. doi:10.1016/j.hal.2016.02.010.

Lee, Y. K., Chen, W., Shen, H., Han, D., Li, Y., Jones, H. D. T., Timlin, J. A. & Hu, Q. (2013). Basic culturing and analytical measurement techniques. *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, 2nd Edition, Wiley Blackwell. 37-68 pp.

Leung, P. T. Y., Yan, M., Yiu, S. K. F., Lam, V. T. T., Ip, J. C. H., Au, M. W. Y., Chen, C. Y., Wai, T. C. & Lam, P. K. S. (2017). Molecular phylogeny and toxicity of harmful benthic dinoflagellates *Coolia* (Ostreopsidaceae, Dinophyceae) in a sub-tropical marine ecosystem: The first record from Hong Kong. *Marine Pollution Bulletin*, 124(2), 878-889. https://doi.org/10.1016/j.marpolbul.2017.01.017.

Mohammad-Noor, N., Al-Has, A., Shahbudin, S. & Than, A. (2016). Comparison on the cell abundance of benthic dinoflagellates in macrophytes and water column collected from open coastal waters and semi-enclosed lagoon. *Sains Malaysiana, 45*(4), 595-599.

Mohammad-Noor, N., Moestrup, Ø., Lundholm, N., Fraga, S., Adam, A., Holmes, M. & Ejria, S. (2013). Autecology andphylogeny of *Coolia* tropicalis and *Coolia malayensis* (Dinophyceae), with emphasis ontaxonomy of *C. tropicalis* based on light microscopy, scanning electron microscopy and LSU rDNA1. Journal of Phycology, 49(3). 536-545.

Momigliono, P., Sparrow, L., Blair, D. & Heirman, K. (2013). The diversity of *Coolia* spp. (Dinophyceae, Ostreopsidaceae) in the Central Great Barrier Reef Region. *PloS one, 8*(10), e79278. https://doi.org/10.1371/journal.pone.0079278.

Morton, S. L. & Faust, M. A. (1997). Survey of toxic epiphytic dinoflagellates from the Belizean barrier reef ecosystem. *Bulletin of*

Marine Science, 61(3), 899-906.

Parson, T. R., Maita, Y. & Lalli, C. M. (1984). *A Manual of Chemical and Biological Methods for Seawater Analysis.* Oxford: Pergamon Press.

Provasoli, L., McLaughlin, J. J. A. & Droop, M. R. (1957). The development of artificial media for marine algae. *Archiv für Mikrobiologie, 25*(4), 392-428.

Rains, L. K. & Parsons, M. L. (2015). *Gambierdiscus* species exhibiy different epiphytic behaviors toward a variety of macroalgal hosts. *Harmful Algae, 49*, 29-39. https://doi.org/10.1016/j.hal.2015.08.005.

Rhodes, L., Smith, K., Papiol, G. G., Adamson, J., Harwood, T. & Munday, R. (2014). Epiphytic dinoflagellates in sub-tropical New Zealand, in particular genus *Coolia* Meunier. *Harmful Algae*, 34: 36-41. https://doi.org/10.1016/j.hal.2014.02.004.

Selina, M. S., Morozova, T. V., Vyshvartsev, D. I. & Orlova, T. Y. (2014). Seasonal dynamics and spatial distribution of epiphytic dinoflagellates in Peter the Great Bay (Sea of Japan) with special emphasis on *Ostreopsis species. Harmful Algae,* 32: 1-10.

Tawong, W., Nishimura, T., Sakanari, H., Sato, S., Yamaguchi, H. & Adachi, M. (2014). Characterization of *Gambierdiscus* and *Coolia* (Dinophyceae) isolates from Thailand based on morphology and phylogeny. *Phycological Research, 63*(2), 125-133. https://doi.org/10.1111/pre.12074.

Totti, C., Accoroni, S., Cerino, F., Cucchiari, E. & Romagnoli, T. (2010). *Ostreopsis* ovata bloom along the Conero Riviera (northern Adriatic Sea): relationships with environmental conditions and substrata. *Harmful Algae, 9*(2), 233-239. https://doi.org/10.1016/j.hal.2009.10.006.

Vidyarathna, N. K. & Granéli, E. (2013). Physiological response of *Ostreopsis ovata* to changes in N and P availability and temperature increase. H*armful Algae, 21-22*: 54-63.

Wakeman, K. C., Yamaguchi, A., Roy, M. C. & Jenke-Kodama, H. (2015). Morphology, phylogeny and novel chemical compounds from *Coolia malayensis* (Dinophyceae) from Okinawa, Japan. *Harmful Algae,* 44: 8-19. https://doi.org/10.1016/j.hal.2015.02.009.