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Effect of Phytoplankton Feed on Growth and Renieramycin M Producing of a Blue Marine Sponge (*Xestospongia* Sp.)

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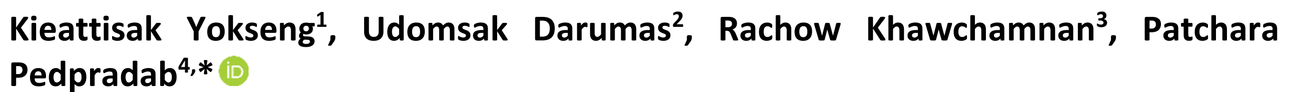

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Abstract

Objective of the study was to determine growth and the accumulation of an anticancer metabolite, renieramycin M (RM) of the sponge *Xestospongia* sp. Two phytoplankton, *Chaetocerose gracilis* and *Nannochloropsis* sp., were used as live feed. Sponges fed by *C. gracilis* weighed significantly more than sponges fed by *Nannochloropsis* sp. and control (2.95 g vs 1.27 and 0.66 g, respectively; P<0.05), respectively. Sponges fed by *Nannochloropsis* sp. showed maximum RM accumulation (0.32 mg/1500 mg tissue), followed by sponges fed by with *C. gracilis* (0.19 mg/1500 mg tissue) and the control (0.16 mg/1500 mg tissue). RM accumulation was not significantly different among treatment groups, suggesting the type of phytoplankton feed affects the growth of *Xestospongia* sp. To confirm the existence of sponge associated cyanobacteria (*Ca. E. renieramycinifaciens*), two renieramycin biosynthetic genes (*renC* and *renJ*) belonging to the endosymbiont were amplified. Quantification, purification and sequencing analysis of recombinant DNA revealed the existence of *renC* and *renJ* in sponge tissue, with similarities of 99.40% and 99.33% to other isolates in the GeneBank suggesting the association of *Ca. E. renieramycinifaciens* with the sponge. It can conclude that *C. gracilis* promotes the growth of a blue sponge, while RM was produced by an associated cyanobacterium.

Introduction

Sponges are filter-feeding metazoans that obtain food by pumping water through their body structure; suspended food particles are captured by flagellated cells, and waste is drained through the osculum. Sponges primarily consume a variety of organic materials suspended in seawater, depending on the type and size of the material. Phytoplankton are a potential food source for marine sponges because they contain carbon and nitrogen, which are necessary for sponge growth and used to synthesize secondary metabolites in various sponge species. Several diatoms have been used as feed for cultured marine sponges; for example, the sponge *Mycale cecilia* reached higher biomass and produced the bioactive compound pyrrole-2-carbaldehyde when fed *Tetraselmis* sp. and *Isochrysis* sp. The marine sponges *Axinella corrugata* and *Hymeniacidon perlevis* similarly grew faster when fed with the diatom *Isochrysis galbana* (Xue and Zhang, 2009), and the sponge *Crambe crambe* achieved a 322% weight gain in 210 days when fed with the diatom *Phaeodactylyl tricornutum* (Belarbi *et al*., 2003). Duckworth and Battershill (2003) reported a higher yield

of the antitumor substance stevensine in *A. corrugata* fed with *I. galbana* and *Thallasiosira weissflogii*. However, the types of phytoplankton that can be used successfully as live feed for individual marine sponge species are still poorly known, especially the effect of phytoplankton feed on bioactive compound production, because sponges are the habitat of diverse associated bacteria, some of which play a role as secondary metabolite producers. Several former secondary metabolites ascribed to production by sponges were later proven to be synthesized by associated bacteria that vary with specific host sponges. (Naim *et al*., 2014; Remya *et al*., 2010; Anteneh *et al*., 2021; Bultel-Ponce V. *et al*., 1999). One of the most interesting groups of marine sponge-associated bacteria synthesizing bioactive metabolites is uncultivable filamentous cyanobacteria, *Cadidatus*, which are associated with specific marine sponges, such as *Cadidatus* Entotheonella hemina associated with the sponge *Theonella swinhoei*, which produces polyketides and peptides, and *Candidatus* Endohalichlona-associated with *Haliclona* sponges, which produce renieramycins (Lackner *et al*., 2017). Due to axenic culture of these cyanobacteria in the laboratory, there are still many obstacles, such as proper media, cultural conditions and genetic stability. Therefore, host sponge cultivation is an alternative way to produce large-scale bioactive metabolites for pharmaceutical purposes.

A blue marine sponge, *Xestospongia* sp. (Figure 1), produces several highly potent anticancer cell metabolites, namely, renieramycins, a bistetrahydroisoquinoline alkaloid group of compounds. Renieramycin M (RM) is a derivative of this group of compounds that is currently being investigated in clinical trials for new anticancer agents. In this work, we report the laboratory culture of the sponge *Xestospongia* sp. to measure growth and the accumulation of the RM when the sponge was fed with these two different species of phytoplankton, the diatom *Chaetocerose gracilis* and the chlorophyte *Nannochloropsis* sp. Both phytoplankton species are widely distributed in the marine environment and are easily cultured in universal media. Their cells contain valuable nutrients that are consumed by the larvae of many marine species, including shrimp, mollusks, and fish. The frustule of *C. gracilis* and the cytoplasm of *Nannochloropsis* sp. contain primarily silica and chlorophyll, but the two phytoplankton species differ with respect to their polyunsaturated fatty acid (PUFA), protein, and carbohydrate content (Gauvin *et al*., 2004). The existence of associated cyanobacteria is also proven in this report.

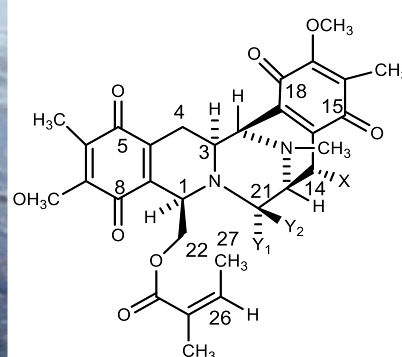
Materials and Methods

Collection of Sponge Samples

Samples of *Xestospongia* sp. were collected by SCUBA divers at Sarai Island, Satun Province, Thailand (6°42'51.67" N; 99°50'35.65" E, Figure 2) at a depth of 5 meters (m). The specimens were kept in water circulation boxes during transport to a marine aquarium and then rehabilitated in a pond (5 m×10 m×5 m) for 7 days. We assessed sponge health after the acclimation period by observing the shape, color, and size of each sponge. Before the samples were used for this experiment, we tested for the presence of RM using liquid chromatography/mass spectrometry (LC/MS). The sponge was previously identified by Darumas *et al*. (2007).

Phytoplankton Feed Preparation

Cultures of the phytoplankton species *Chaetoceros gracilis* and *Nannochloropsis* sp. were obtained from the Research Center of Excellence in Shrimp (Walailak University, Thailand) and cultured in F/2 medium and Conway medium (Guillard and Ryther, 1962; Lananan *et al*., 2013), respectively, at 30 part per thousand (ppt) salinity. Primary stocks were cultured in 250 milliliter (ml) Erlenmeyer flasks containing 250 microliters (µl) of media at a temperature of 27±2 degrees Celsius (°C).



RM: X=Y_2=H, Y_1=CN

Figure 1. The blue marine sponge, *Xestospongia* sp. and the chemical structure of RM.

Flasks were placed under ultraviolet (UV) illumination for 12 hours (h) each day. Phytoplankton growth was measured as direct cell counts obtained using a hemocytometer (HBG, Glessen, Germany) under a binocular microscope; the cellular growth pattern was also recorded.

Mass cultures of both phytoplankton species were performed in 1.5 liter (l) plastic bottles containing 1.5 ml of media. Phytoplankton were inoculated at a density of $5x10^5$ cells/ml and cultured at 27±2°C under a light intensity of 3,000 lux for 12 hours each day. We confirmed that cultures were in stationary phase before they were harvested for use as sponge feed.

Experimental Design and Culture Method

We used a randomized complete block design to operate the experimental unit. The experimental design consisted of three groups with different food sources: two treatments (for the two phytoplankton species) and one control (pure seawater). Three replicates were performed for each group, for a total of nine experimental units. Sponges were cultured using the vertical long line technique in a semicirculating closed system (Figure 3). For this system, small (12 g) pieces of *Xenospongia* sp. tissue was cut from the mother plant and attached to a plastic net with copper wire. Wires

Figure 2. Location of sponge collecting area.

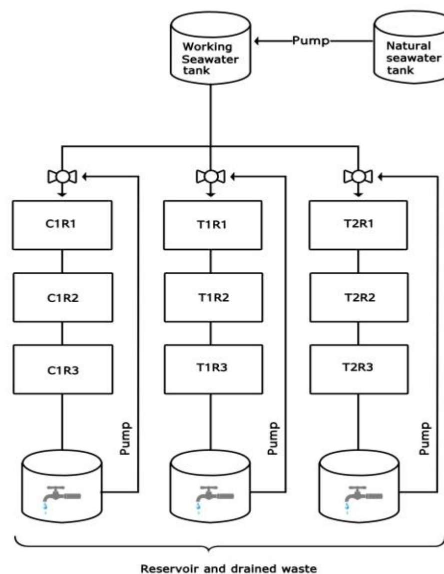


Figure 3. Schematic of the culture system**.** T, R and C are treatments, replications, and controls, respectively.

were connected to form a chain with three pieces of sponge and were then placed in a 40 cm×60 cm×60 cm glass box containing 120,000 cm^3 of seawater. The box contained a total of three ropes.

Each week during the experiment, 30% of the water in the culture tank was replaced to remove detritus and residual particles and to replenish water lost through evaporation. The room temperature in the laboratory was maintained at 27±1°C for the duration of the experiment. Phytoplankton cultures were diluted to $2x10^5$ cells/ml and fed to the sponge samples once per day. Water flow was stopped for 8 h after feeding.

Growth Measurements

Sponge samples were weighed at the end of the experiment, and wet weights were compared against a standard wet-dry curve to obtain the estimated dry weight. The wet-dry curve was obtained by cutting a sponge sample into ten sections that weighed 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 g (wet weight). The sections were then oven-dried at 80°C for 24 h before being weighed again to obtain the dry weight. We performed a linear regression to quantify the relationship between the wet and dry weights and used this regression to calculate the dry weight of our experimental samples.

Quantification of RM Concentrations

RM concentrations in each culture were determined using the method described by Darumas *et al*. (2007). In brief, 150 mg sponge samples were cut into fine pieces, macerated with 10 millimolar (mM) potassium cyanide in a phosphate buffer solution (6.00 ml, pH 7.0) for 5 h, and then treated with 24 ml methanol. After methanol extraction, the samples were centrifuged at 5,000 revolutions per minute (rpm) for 5 minutes (min), and the supernatant (3.00 ml) was partitioned with ethyl acetate (9.00 ml). The ethyl acetate layer was allowed to evaporate completely *in vacuo* before the remaining sample was redissolved with methanol (1.00 ml) containing 300 nanograms (ng) of acenaphthene (internal standard). The resulting solution was filtered through 0.45-nanometer (nm) nylon syringe filters before being used for high-performance liquid chromatography (HPLC; Dionex UltiMate 3000, Thermo Scientific, Waltham, MA, USA). For the HPLC analysis, an AcclAim 120, C18 reversed-phase column (5 µm, 4.6 x 150 mm, Thermo Scientific, Waltham, MA, USA) was used, and the column was eluted with a 7:3 (v/v) mixture of methanol and water at a flow rate of 0.70 ml/min. The photodiode array detector was set to a wavelength of 270 nm. Pure renieramycin M (1 mg/ml) was used as a standard.

RM Standard Curve Preparation

RM concentration versus absorbance (mAU*s) at 270 nm was plotted as a standard curve for calculating RM in sponge tissue. Standard RM was prepared at concentrations of 0.05, 0.1, 0.3, 0.5, 0.7 and 0.9 mg/ml.

The presence of RM in the samples was additionally confirmed by liquid chromatography/mass spectrometry (LC/MS). LC measurements were performed using a1290 Infinity (Agilent Technologies, Santa Clara, CA, USA). We used a ZORBAX SB-C18 as the separation column (Rapid Resolution HD, 2.1 x 150 mm, 1.8 µm; Agilent Technologies, Santa Clara, CA, USA), and the injection volume was 10 µl. The liquid chromatography system was connected to the Agilent Technologies 6490 Triple Quad electrospray ionization mass spectrometer (ESIMS; Agilent Technologies, Santa Clara, CA, USA). Both negative and positive modes were detected.

Water Quality Measurement

Physicochemical parameters of the culture water, including salinity, temperature, dissolved oxygen, and pH, were recorded throughout the experiment using an autoanalyzer (Global Water Instrumentation, Phoenix, AZ, USA). Dissolved silica, calcium, magnesium, ammonia, nitrite, nitrate, and phosphate were analyzed daily following the methodology of Stickland and Parson (1972).

The Presence of a symbiosis cyanobacteria, *Ca. E. renieramycinifaciens*, in the Sponge Specimen

Amplification of *renC* and *renJ*, cloning and DNA sequencing verify the existence of intracellular bacteria, *Candidatus Endohaliclona renieramycinifaciens*, in a blue marine sponge. *renC* and *renJ* (parts of the renieramycin biosynthesis gene cluster in the symbiont) were also amplified. Each polymerase chain reaction (PCR) volume was 25 µl, containing 0.5 units of Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific™, Waltham, MA), 1× Phusion HF buffer, 200 µM dNTP mix, 250 nM of each primer set (Table 1), and 25 ng of previously isolated DNA. The PCR cycling conditions consisted of 98°C for 2 min, followed by 35 cycles of 98°C for 10 s, 59°C for 30 s, 72°C or 30 s, and finally 72°C for 5 min. The amplified products were electrophoresed using a 0.8% agarose gel and stained

Table 1. Oligonucleotides used as gene specific primers

Primer names	Sequences (5'-3')	amplicon size (bp)
renC-F	GGGGCATATGATTCAACAAGTTGAACTAACG	663
renC-R	GGGGCTCGAGTTAACGTTTCAAAGCCATTG	
renJ-F	GGGGCATATGAAAGATCCAGGGTATG	600
renJ-R	GGGGCTCGAGTCATGATAACTTGTGCTTAAAATG	

with ethidium bromide before visualization. After the excision of two targeted bands from the gel, the DNA was cleaned up by using a Monarch® DNA Gel Extraction Kit (New England Biolabs, Ipswich, MA, USA). Each amplicon was later cloned into the pJET1.2/blunt vector using a CloneJET PCR Cloning Kit (Thermo Fisher Scientific™, Waltham, MA) following the recommended protocol. The recombinant plasmid containing a coding region of either *renC* or *renJ* was transformed into *E. coli* DH5α and subsequently extracted using the Monarch® Plasmid Miniprep Kit (New England Biolabs, Ipswich, MA). The recombinant plasmid was verified by DNA sequencing (Macrogen, Korea). Finally, the obtained nucleotide sequences were analyzed by BioEdit Sequence Alignment Editor version 7.2.5, blasted against the GenBank database (https://blast.ncbi.nlm.nih.gov), and illustrated by using GeneDoc Multiple Sequence Alignment Editor and Shading Utility Version 2.7.

Statistical Analysis

All statistical analyses were conducted using SPSS 20.0 software (IBM Corp., Armonk, NY, USA). We used analysis of variance (ANOVA) and Tukey's *post hoc* tests to compare mean sponge growth and renieramycin M production among our three experimental groups.

Results

The Presence of RM in Sponge Samples

The sponge sample was collected from the sea and then analyzed for the presence of renieramycin M by HPLC and LC/MS before being cultured in a semicirculating closed system. The HPLC chromatograms and LC/MS spectrum (Figure 4) of sponge extracts confirmed the presence of RM in their tissue, as shown in Figure 4. A standard curve used to

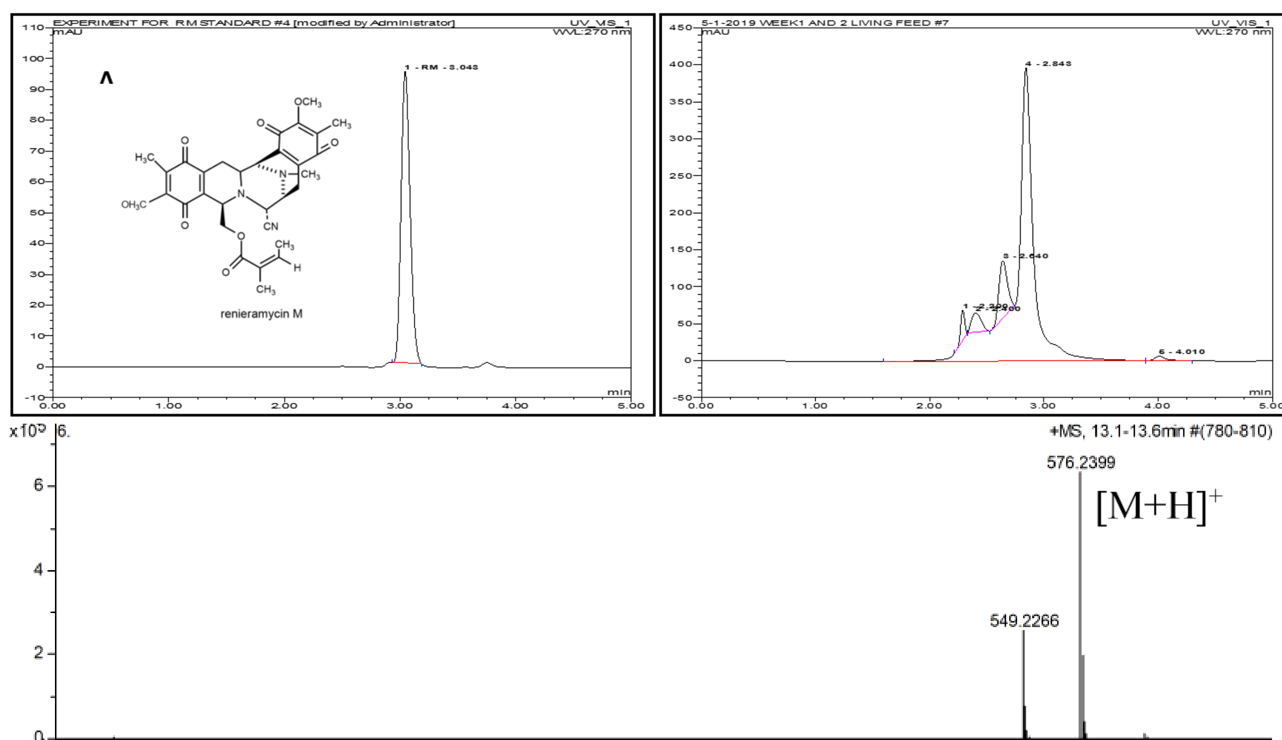


Figure 4. HPLC chromatograms of renieramycin M contain in sponge extract. A=Standard renieramycin M; B=renieramycin M in the extract from the sponge sample.

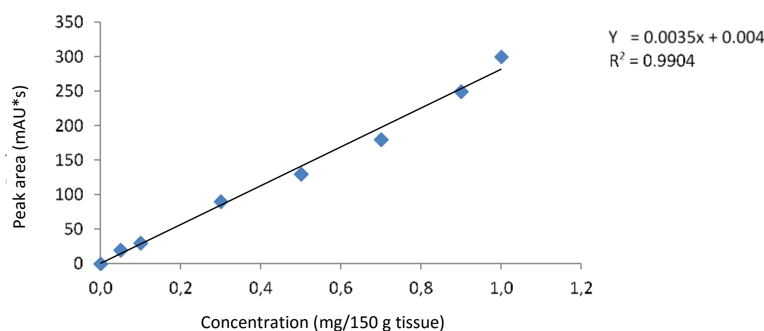


Figure 5. Standard curve for RM quantification.

calculate the RM contained in the sponge samples was also prepared, as illustrated in Figure 5.

Phytoplankton Feed Preparation

The phytoplankton species *C. gracilis* and *Nannochloropsis* sp. were used in separate experiments as the silica and chlorophyll sources for the sponge. Each phytoplankton culture was harvested in the stationary phase and diluted to 6.25x10^6 cells/ml before being fed to the sponge. *C. gracilis* reached the stationary phase after 110 hrs (Figure 6A), whereas *Nannochloropsis* sp. was 264 h (Figure 6B).

Effect of Phytoplankton Feed on RM Accumulation in Sponge Tissue

Sponges were assessed weekly to measure growth and RM concentration. The sponges fed with *Nannochloropsis* sp. reached a maximum concentration of RM in their tissue (0.32 mg/1,500 mg sponge tissue), while the *C. gracilis* and control reached RM concentrations of 0.19 and 0.16 mg/1,500 mg tissue, respectively (Figure 7). There were no statistically significant differences among treatments at the 95% confidence interval.

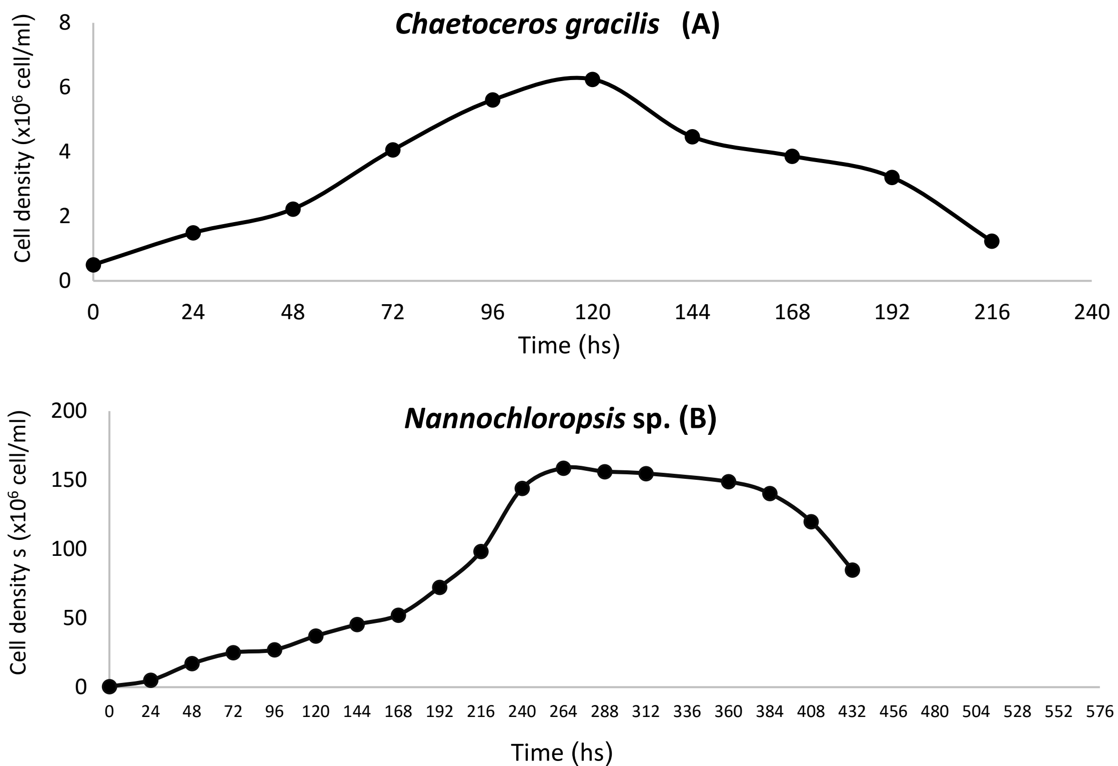


Figure 6. Growth curves of *C. gracilis* and *Nannochloropsis* sp.

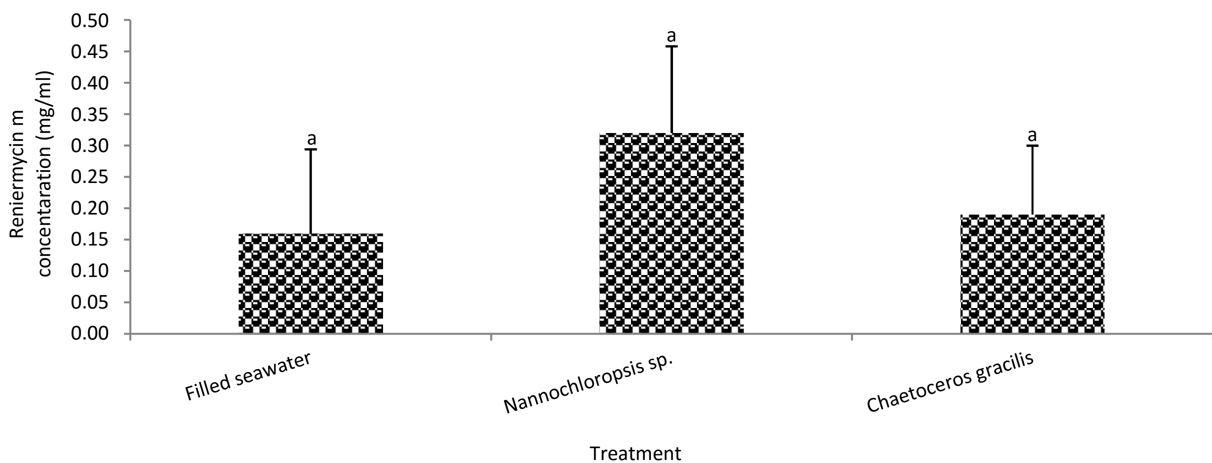


Figure 7. Renieramycin M concentrations in blue *Xestospongia* sp. after being fed with different phytoplankton species.

Effect of Phytoplankton Feed on Sponge Growth

Sponges fed *C. gracilis* weighed more (2.95 g) than sponges fed *Nannochloropsis* sp. (1.27 g) and control sponges provided with pure water (0.66 g) (Figure 8).

Water Quality Measurement in Cultural Systems

We measured water quality in the culture tanks weekly. The values of various physicochemical parameters, including temperature, salinity, dissolved oxygen, pH, phosphate, ammonia, nitrite, nitrate, silicate, calcium and magnesium, were 26.34-26.83°C, 33.87-34.50 ppt, 4.12-5.71 mg/L, 8.20-8.33, 0.03-0.08 mg/L, 0.00-1.150 mg/L, 0.00-0.88 mg/L, 0.00-12.50 mg/L, 0.01-0.25 mg/L, 367.50-370.00 mg/L, and 1140.00-1200.00 mg/L, respectively (Figure 9).

The Existence of Symbiosis Cyanobacteria (*Ca. E. renieramycinifaciens*) in Cultured Sponges

To identify an association of bacteria with *Xestospongia* spp., the extraction and purification of DNA from the sponge tissue was carried out. The DNA quantity was measured, and a known concentration was used as a template for 16S rDNA gene amplification with a specific primer set. The results showed an approximate band size of 1,500 bp (Figure 10). The presence of the 16S rDNA amplicon was detected in the PCR containing 25 ng DNA, while the presence of the 16S

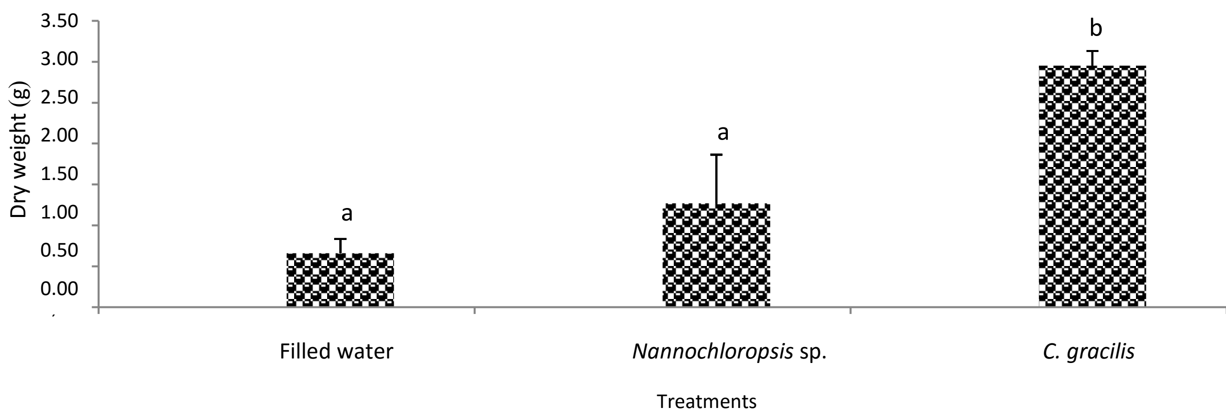


Figure 8. Dry weight of *Xestospongia* sp. after being fed with different phytoplankton species. Different superscript letters indicate statistically significant differences ($p<0.05$) between experimental groups.

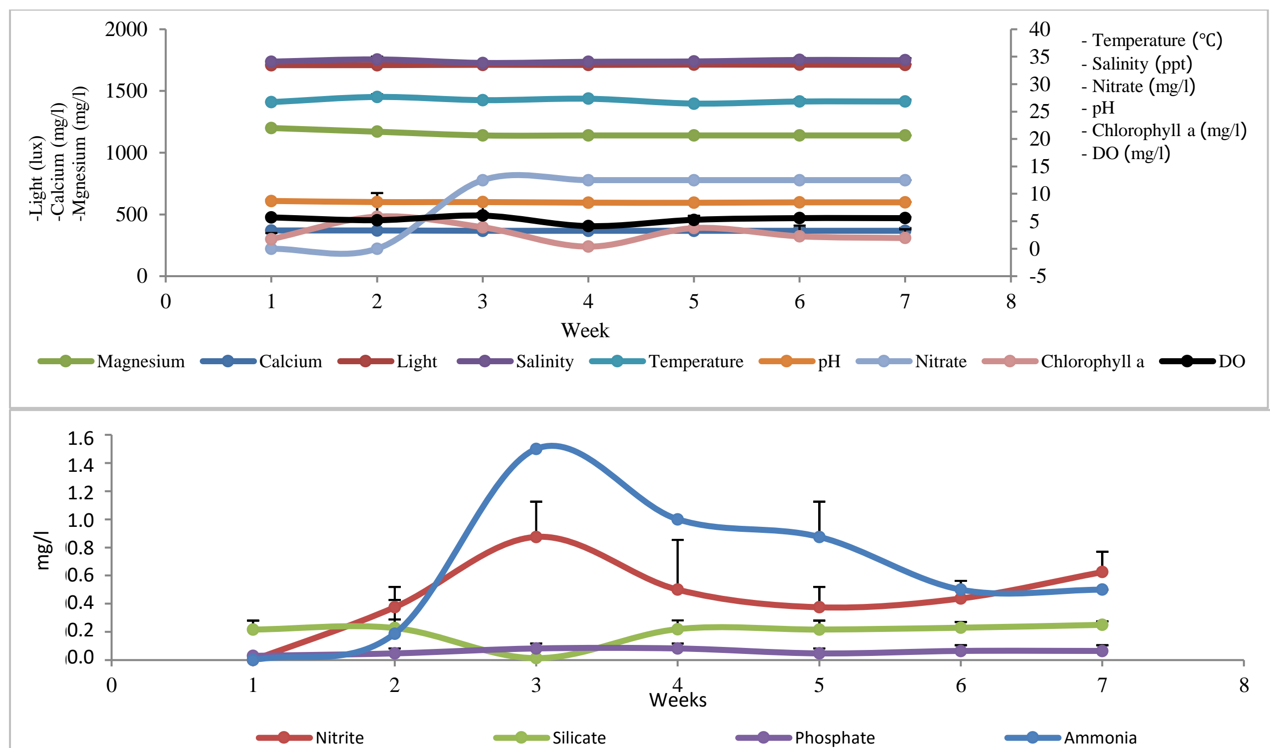


Figure 9. Water quality data during the culture period.

rDNA amplicon was not obvious in the PCR containing 10 and 100 ng DNA. Hence, 25 ng DNA was later used to confirm the presence of the endosymbiont *Ca. Erenieramycinifaciens* in the sponge tissue. Two of the renieramycin biosynthetic genes, *renC* and *renJ,* belonging to the endosymbiont were amplified. Following the PCR of each target gene, agarose electrophoresis showed amplified products of approximately 700 and 600 bp for *renC* and *renJ*, respectively, from triplicate samples (Figure 11). After the ligation of the product with a vector, transformation into *E. coli* cells and plasmid extraction, verification of the insert by DNA sequencing showed 663 nucleotides of the open reading frame (ORF) of *renC* and 600 nucleotides of *renJ*'s ORF. A multiple-sequence alignment of an insert with *renC* or *renJ* of other isolates showed the highest similarity with isolate MK748465.1, MK748463.1, MK748462.1 at 99.40%; (*renC*) and MK748465.1, MK748464.1, MK748463.1 at 99.33% (*renJ*). This result suggested the association of *Ca. E. rosenamycinaciens* with cultured *Xestospongia* sp.

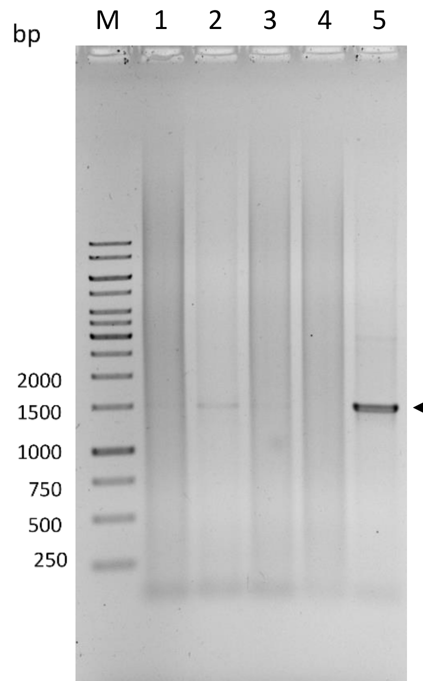


Figure 10. 16S rDNA gene amplification. Agarose electrophoresis of 16S rDNA amplicon from the PCR containing 10, 25 and 100 ng (lane 1-3), DNA compared to negative (lane 4) and positive controls (lane 5), Lane M: GeneRuler 1 kb DNA Ladder.

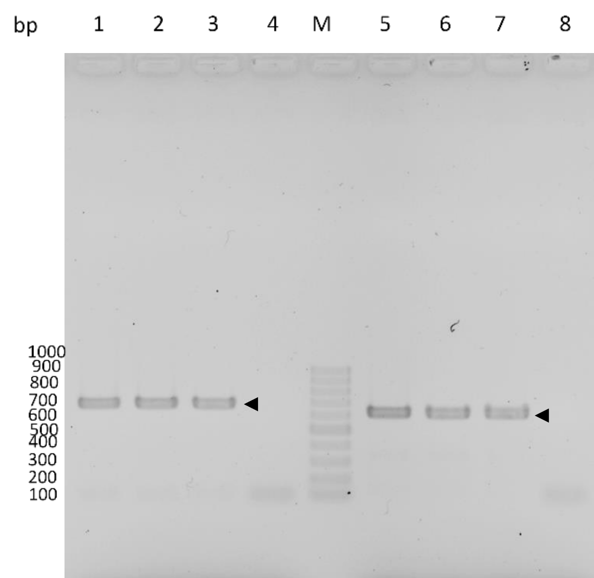


Figure 11. The amplified product of *renC*'s and *renJ*'s ORFs. The gel shows the PCR product of *renC* (Lane 1-3) and *renJ* (Lane 5-7) amplified from purified DNA of *Xestspongia* spp and PCR negative controls (Lane 4 and 8). Lane M is a GeneRuler 100 bp DNA ladder.

Discussion

Influence of Phytoplankton Feed on RM Synthesis

In natural environments, marine sponges filter different sizes of suspended food particles depending upon the species of sponge and types of foods. Many species of marine phytoplankton contain nutrients that can promote growth and secondary metabolite synthesis in marine sponges. In this study, we used two phytoplankton species, *C. gracilis* and *Nannochloropsis* sp. The sponge was collected from the sea and was assessed for the presence of RM before culturing in a hatchery. The HPLC chromatograms for standard RM showed a retention time (Rt) of 3.04 min (Figure 4), whereas the chromatograph of the extract from sponge samples showed an Rt of 2.84 min. This slight shift can be attributed to: (i) the noise caused by other renieramycin derivatives in the solution, and (ii) the stability of the chemical reaction in the extract during the derivatization process. The RM contained in the samples was further confirmed by LC/MS before sponges were cultured. The positive electrospray ionization mass spectrometry (ESIMS) spectrum confirmed the presence of RM at a molecular ion peak at $[M+H]^+$ 576.3000 (Figure 4), which was calculated for the molecular Formula $C_{31}H_{33}N_3O_8$. In general, marine sponges increase secondary metabolite synthesis when they are influenced by nutrient efficiency, stress or defensive processes; this increase in synthesis has been reported for several marine sponges, including *Aplysina* spp., *Isodictya erinacea*, *Pseudoceratina* sp., *Callyspongia* sp., and *Haliclona atra* (Carballo *et al*., 2010; Thoms *et al*., 2006; Helber *et al.,* 2018). The effects of phytoplankton feed on secondary metabolite synthesis in marine sponges are unpredictable and can vary with both the species of sponge and the type of phytoplankton feed. For example, feeding a marine sponge *A. corrugata* with the phytoplankton *Isochrysis galbana* and *Thalassiosira weissflogii* results in the production of the alkaloid metabolite stevensine (Duckworth and Battershill, 2003), whereas feeding a sponge *Mycale cecilia* with the phytoplankton *Isochrysis* sp. and *Tetraselmis* sp. results in the production of bioactive mycalazal-type metabolites (Jose et al., 2010). These studies indicated that the appropriate phytoplankton feed can vary with the type of sponge, type of nutrients and concentration contained in phytoplankton. However, our experimental data suggest that the two phytoplankton species we tested did not affect RM synthesis in the blue sponge *Xestospongia* sp. The molecular structure of RM is similar to the structure of a cytotoxic compound, saframycin A (Figure 1), which was produced by the actinomycetes *Streptomyces lavendula*. Similar to saframycin A, Tianero et al. (2018) reported that RM was produced by a filamentous cyanobacterium, *Candidatu*s *Endohaliclona renieramycinifaciens* (*Ca*. *E. renieramycinifaciens*) associated with the *Halichlona* sponge. We clarified the presence of such bacteria in the sponge *Xestospongia* sp. by searching for the reniermycin biosynthesis genes *RenC* and *RenJ* belonging to *Ca*. *E. renieramycinifaciens*. The existence of *Ca*. *E. renieramycinifaciens* sponge, as shown by the gel electrophoresis profile (Figure 11) and oligonucleotide sequence of the target genes was established, indicating that RM was produced by a microorganism with sponge symbiosis. Therefore, the RM concentration is not involved in species of phytoplankton feed but should be some trace metabolites produced by a sponge to supply the symbiotic cyanobacteria.

Influence of Phytoplankton on the Growth of Sponges

C. gracilis cells contain high levels of valuable nutrients, including polyunsaturated fatty acids (PUFAs), proteins and silica (Gauvin *et al*., 2004). Silica is involved in several metabolic processes in marine sponges, including spicule formation and energy consumption (López *et al*., 2018). The silica uptake rates of marine sponges are influenced by ambient silica concentrations in the environment; basically, sponges can take up silica at a rate of 5.27 micromolar (µM) silica per hour, with some variation due to sponge size and species (Leys *et al*., 2011). Under natural conditions, the size and features of sponge spicules are related to fluctuations in seawater silica concentrations. For example, the spicules of the sponge *Hymeniacidon perleve* were positively correlated with *in situ* silica concentrations (Stone, 1970). The spicules of *Haliclona canaliculata* were larger in winter due to higher silica concentrations in the surrounding water (Nickel and Brummer, 2003). Increasing ambient silica concentrations also affected spicule size, while silica depletion can cause low energy consumption in marine sponges, as found in a sponge, *Halichondria paniceapanacea* (López *et al*., 2018; Hartman, 1981). PUFAs and protein in diatoms can stimulate the growth of sponges; for example, feeding the diatom *Phaodactylum tricornutum* to the sponge *Crambe crambe* resulted in a 322% weight gain over 210 days (Xue and Zhang 2009; Duckworth and Battershill, 2003, Ferrante *et al*., 2018) because both PUFAs and proteins are involved in some metabolic function in marine sponges. PUFAs play an important role in yolk formation (It assumed that PUFAS play a role as part of vitellogenin accumulation as found in a deep sea sponge, *Geodia* sp.), while proteins are involved in energy metabolism, whose activation precedes embryonic development and is a counterpart in the metamorphosis process of sponge larvae during transformation to adulthood (Koutsouveli *et al*., 2020; Borisenko *et al*., 2022; Koutsouveli *et al*., 2022). Several green phytoplankton species are also valuable carbon sources for marine sponges: *Chlorella* sp. has been fed to the marine sponge *Pseudosuberites* aff. *andrewsi*, and the cyanobacterium *Oscillatoria spongeliae* has been fed to *X. walentinae* (Osinga *et al*., 2001; Nickel and

Brummer, 2003). The chlorophyte *Nannochloropsis* sp. contains various nutrients, including PUFAs (8-14%), proteins (33-38%), and lipids (22-60%), although the main component of this species is chlorophyll a (Lubián *et al*., 2000); thus, the chlorophyte can also be used as a carbon source for a blue sponge, *Xestospongia* sp. Based on the experimental results, Figure 8 shows that the growth of *Xestospongia* sp. fed with *Nannochloropsis* sp. was significantly lower than the growth of sponges fed *C. gracilis* but was not different from the growth of the controls, indicating that the nutrient concentration and chlorophyll content in *Nannochloropsis* sp. don't affect the growth of *Xestospongia* sp. in comparison to feed by *C. gracilis*. The weight of sponges in the control group decreased slightly after the third week of our experimental period, and by the fifth week, all the control sponges had died. However, sponges fed by phytoplankton survived past the end of the experiment, although we observed a few dead colonies during the fifth week. These results indicate nutrient starvation in the control group and the accumulation of waste in the culture system (Carballo *et al*., 2010). Based on these results, sponge death was likely caused by the accumulation of nitrogenous waste (Figure 9), including ammonia and nitrite, as well as by other toxic metabolites in the culture system. The biological effects of ammonia in seawater are controlled by pH and dissolved oxygen; ammonia is particularly toxic at low oxygen concentrations and high pH because these conditions increase diffusion through the cell membrane of sessile marine invertebrates (Nemoy *et al*., 2021).

Conclusions

The type of phytoplankton provided as a feed source for cultured marine sponges affects sponge growth and secondary metabolite synthesis, which vary by species. In this experiment, *C. gracilis* and *Nannochloropsis* sp. used silica and chlorophyll as major sources for a blue marine sponge (*Xestospongia* sp.). The results showed that *C. gracilis* promotes sponge growth but does not affect RM synthesis, whereas *Nannochloropsis* sp. was not associated with either growth or RM synthesis, suggesting that the nutrient composition of the live feed used for sponge culture should vary depending on the sponge species. In this case, the spicules of *Xestospongia* sp. contain silica, so the appropriate feed should be diatoms. The discovery of *Ca. E. renieramycinifaciens* in sponge confirmed that RM was synthesized by associated bacteria. The accumulation of nitrogenous waste while sponges are being cultured must still be addressed because high concentrations of this waste can cause mass mortality.

Ethical Statement

No vertebrate animals were used in this experiment.

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Author Contribution

Kieattisak Yokseng: Methodology and Formal analysis; Udomsak Darumas: Resources and Formal analysis; Rachow Khawchamnan, Kitipong Angsujinda and Wanchai Assavalapsakul: Formal analysis, investigation and resources, Patchara Pedpradab: Project administration, Conceptualization, Data Curation, Writing-Original draft Preparation and Writing-Review & Editing.

Conflict of Interest

All authors have declared that no conflict of interest in any parts of the research.

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