CHAPTER IV

RESULTS AND DISCUSSION

Results

1. High-performance liquid chromatography (HPLC) analysis of renieramycins

1.1. The suitable HPLC condition for renieramycins

The HPLC analysis was performed with a RP-18e column (LiChrospher® 125 mm × 4 mm, 5 μm) and a UV-visible diode array detector. An isocratic solution of methanol and water (7:3) was used as the mobile phase with the flow rate at 1.0 mL/min and the temperature at 25°C. Renieramycins were detected at a wavelength 270 nm.

1.2. Qualitative determination of renieramycin references

The HPLC chromatogram in Figure 4.1 showed the retention times of four renieramycin references including deangeloylrenieramycin M, renieramycin N, renieramycin O, and renieramycin M at 3.0, 3.9, 6.9, and 8.8 min, respectively. The UV-visible diode array detector was used for qualitative analysis of the compounds obtained. The UV spectra of renieramycins typically showed the maximum absorption at a wavelength 270 nm as shown in Figure 4.2.

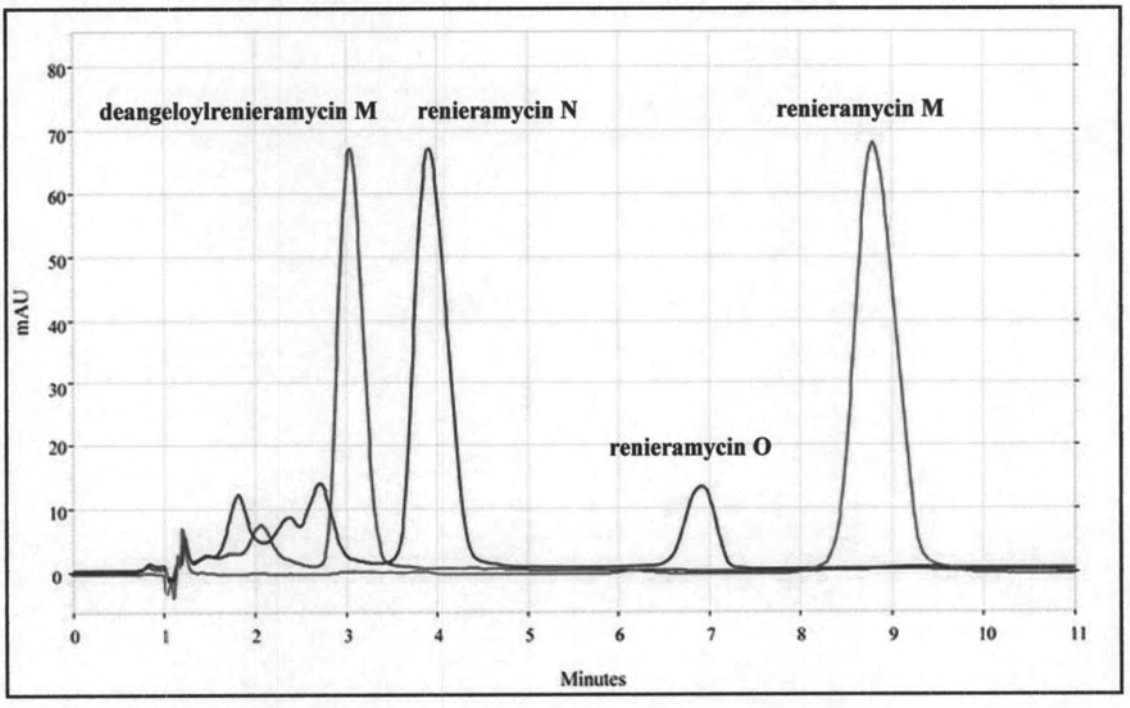

Figure 4.1. The overlaid HPLC chromatograms of renieramycin references.

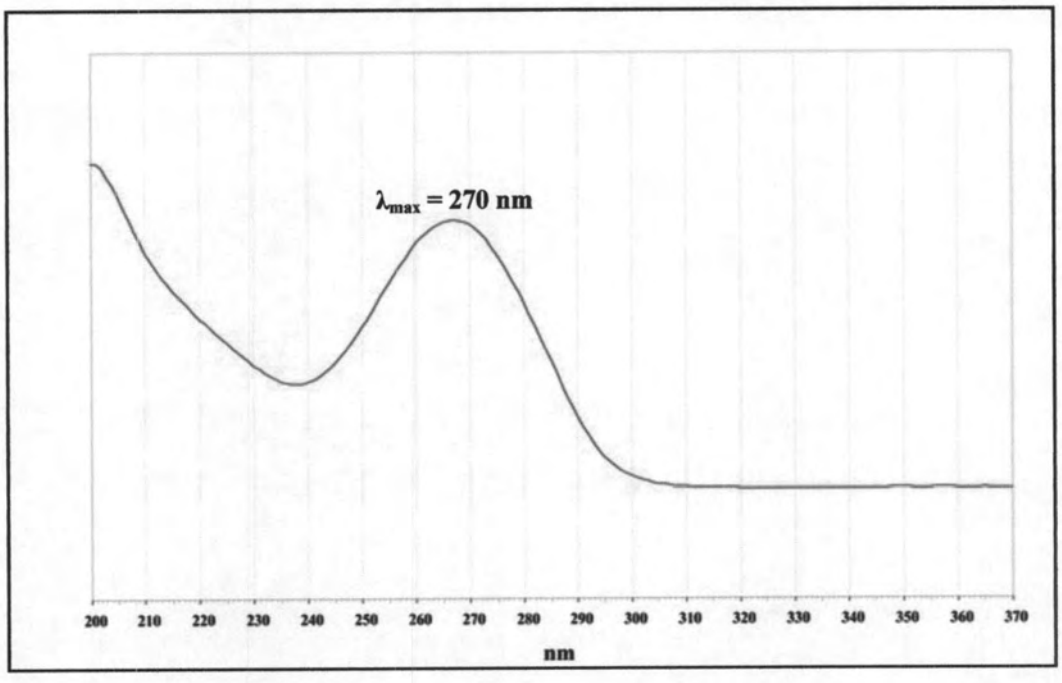

Figure 4.2. The typical UV spectrum of renieramycins.

1.3. Validation of the HPLC method for renieramycins analyses

The purpose of validation of the HPLC method is to generate reliable and accurate data for the qualitative and quantitative analyses. In this experiment, the validation parameters including linearity, accuracy, and precision were studied according to International Conference on Harmonization (ICH) guideline as follow:

1.3.1. Linearity

The linearity of an analytical method is its ability to obtain test results according to the Beer's law which is dependent on the compound analyzed and the detector used. The linear range is directly proportional to the concentration of the analyte in the sample. The working sample concentrations and samples tested for accuracy should be in the linear range. The linearity is commonly reported as the variance of slope of regression line by appropriate statistical methods. In this study, the linearity was analyzed from five renieramycin M concentrations (5, 10, 20, 30, and 35 μg/mL) in the internal standard acenaphthene solution (15 μg/mL, concentration). A linear relationship was observed between the intercept and the slope. The linear equation between the amounts of renieramycin M (ng) and peak area ratios of renieramycin M and acenaphthene was $y = 0.0049x$. The coefficient of determination (R^2) value was 0.9999 (Table 4.1 and Figure 4.3).

Table 4.1. Peak area ratios of renieramycin M and acenaphthene for linearity test of the HPLC method.

Renieramycin M amount (ng)	Peak area ratios of renieramycin M/acenaphthene				SD
	1	2	3	Average	
100	0.49	0.49	0.50	0.49	0.0018
200	1.00	1.00	1.01	1.00	0.0036
400	1.95	1.94	1.96	1.95	0.0079
600	2.94	2.95	2.94	2.94	0.0063
700	3.42	3.43	3.42	3.42	0.0065

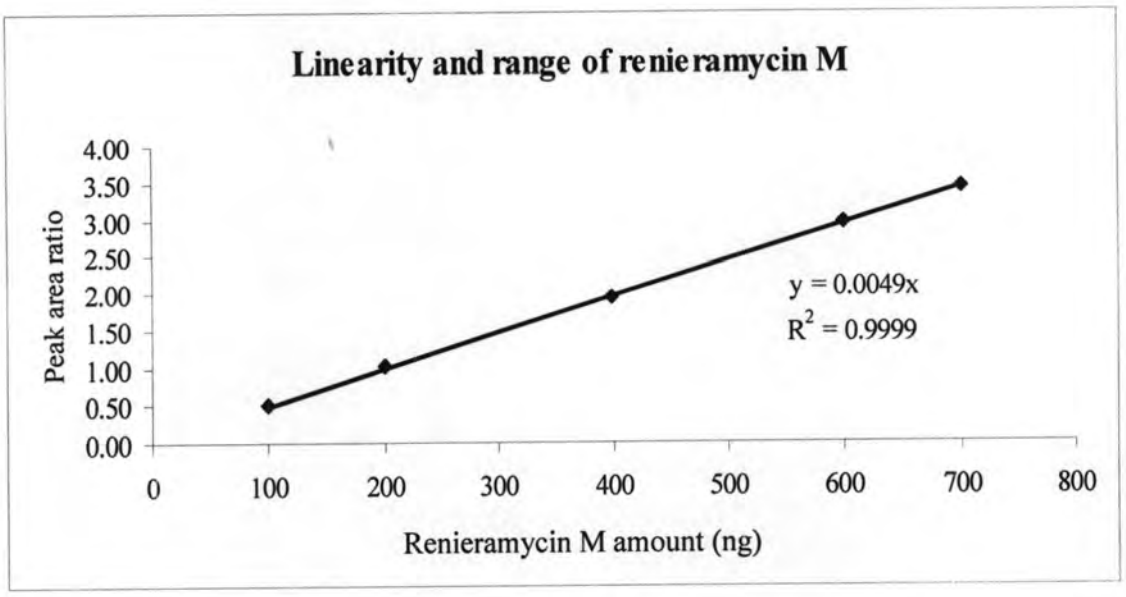

Figure 4.3. Linearity of the HPLC method for renieramycin M analysis.

1.3.2. Accuracy

Accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy assessment can be obtained by comparing the results from an established reference method or by analyzing a sample with known concentrations. Accuracy is validated in terms of percentage recovery (%R) by addition of known quantities of renieramycin M (standard addition). In this study, the experiment was performed by adding known amounts of renieramycin M (50, 150, and 300 ng) in the EtOAc extract samples from the sponge *Xestospongia* sp. The accuracy is then calculated from the test results as the percentage of renieramycin M recovered by the assay. Percent recovery was calculated by using the internal standard quantification method in confidence intervals with the regression equation y = 0.0049x. The results of percent recovery of renieramycin M was in the range of 103.10-107.14% with average of 105.10% as summarized in Table 4.2.

Table 4.2. Percent recovery of renieramycin M for accuracy test.

Added amount (ng)	Items	Number of sample		
		1	2	3
0	peak area of renieramycin M (1)	938061	919298	922781
	peak area of acenaphthene (2)	480001	464325	473750
	peak area ratio (1/2)	1.95	1.98	1.95
	renieramycin M amount (ng)	398.83	404.05	397.51
	average	400.13		
50	peak area of renieramycin M (1)	1058634	1055952	1062104
	peak area of acenaphthene (2)	477757	476690	478152
	peak area ratio (1/2)	2.22	2.22	2.22
	renieramycin M amount (ng)	452	452	453
	%recovery	104.43	104.15	106.64
	average	105.07		
150	peak area of renieramycin M (1)	1359693	1360999	1354651
	peak area of acenaphthene (2)	492395	497508	493414
	peak area ratio (1/2)	2.76	2.74	2.75
	renieramycin M amount (ng)	564	558	560
	%recovery	109.03	105.53	106.87
	average	107.14		
300	peak area of renieramycin M (1)	1689494	1726039	1718132
	peak area of acenaphthene (2)	483102	496797	497265
	peak area ratio (1/2)	3.50	3.47	3.46
	renieramycin M amount (ng)	714	709	705
	%recovery	104.57	103.02	101.71
	average	103.10		
mean of % recovery		105.10		

1.3.3. Precision

The instrument precision of an analytical method expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample. Precision was represented in terms of the relative standard deviation (RSD) or coefficient of variation (CV) in a series of measurements. Precision test was evaluated by analyzing the same sample for six consecutive times. In this experiment, the relative standard deviation (RSD) of peak area ratios and retention times (t_R) was calculated in six replications of three renieramycin M concentrations (10, 25, and 35 μg/mL, concentration) in the same day. The percentage of relative standard deviation (%RSD) of peak area ratio in each renieramycin M solution was in the range of 0.39–0.95% with average of 0.72%. %RSD of retention times of renieramycin M was in range of 0.19–0.82% with average of 0.43% as presented in Tables 4.3 and 4.4.

Table 4.3. Percent RSD of peak area ratios between renieramycin M and acenaphthene for precision test.

Renieramycin M concentration (μg/mL)	Items	Number of sample					
		1	2	3	4	5	6
10	peak area of renieramycin M (1)	465883	471605	459031	462998	462319	462838
	peak area of acenaphthene (2)	478412	488474	480396	483277	485985	487117
	peak area ratio (1/2)	0.97	0.97	0.96	0.96	0.95	0.95
	average	0.96					
	SD	0.009					
	%RSD	0.95					
25	peak area of renieramycin M (1)	1160281	1134125	1143048	1130372	1132942	1145982
	peak area of acenaphthene (2)	485902	485544	482705	482363	482922	487180
	peak area ratio (1/2)	2.39	2.34	2.37	2.34	2.35	2.35
	average	2.36					
	SD	0.019					
	%RSD	0.81					
35	peak area of renieramycin M (1)	1645793	1667889	1660411	1674010	1676235	1667553
	peak area of acenaphthene (2)	484922	492170	489343	489731	492199	487776
	peak area ratio (1/2)	3.39	3.39	3.39	3.42	3.41	3.42
	average	3.40					
	SD	0.013					
	%RSD	0.39					
mean of %RSD		0.72					

Table 4.4. Percent RSD of retention times (t_R) of renieramycin M for precision test.

Renieramycin M concentration (μg/mL)	Items	Number of sample					
		1	2	3	4	5	6
10	t_R of renieramycin M (min)	7.67	7.65	7.64	7.62	7.62	7.61
	average of t_R	7.63					
	SD	0.02					
	%RSD	0.28					
25	t_R of renieramycin M (min)	7.89	7.92	7.92	7.93	7.93	7.92
	average of t_R	7.92					
	SD	0.02					
	%RSD	0.19					
35	t_R of renieramycin M (min)	7.94	7.95	7.95	7.96	8.02	8.11
	average of t_R	7.99					
	SD	0.07					
	%RSD	0.82					
mean of % RSD		0.43					

1.4. Calibration curve of renieramycin M

Five renieramycin M concentrations (5, 10, 15, 20, and 25 μg/mL) were prepared to plot the calibration curve. Each concentration was analyzed in triplicate. 15 μg/mL of acenaphthene concentration was used as the internal standard. The linear equation between the amounts of renieramycin M (ng) and peak area ratios of renieramycin M and acenaphthene was y = 0.0047x. The coefficient of determination (R^2) value was 0.9996 as presented in Table 4.5 and Figure 4.4.

Table 4.5. Peak area ratios of renieramycin M and acenaphthene for calibration curve of renieramycin M.

Renieramycin M amount (ng)	Peak area ratios of renieramycin M/acenaphthene				
	1	2	3	Average	SD
100	0.46	0.46	0.47	0.46	0.0036
200	0.97	0.95	0.95	0.96	0.0108
300	1.38	1.38	1.39	1.38	0.0025
400	1.88	1.88	1.87	1.88	0.0056
500	2.34	2.35	2.34	2.34	0.0028
$y = 0.0047x$, $R^2 = 0.9996$					

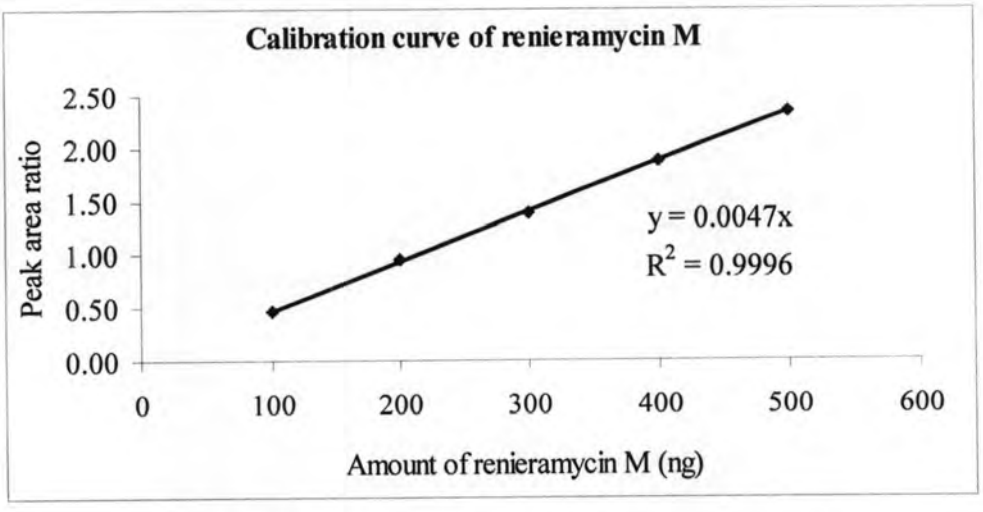

Figure 4.4. The calibration curve of renieramycin M.

2. Annual variation of renieramycins contents in the blue sponge *Xestospongia* sp. during August 2005 to July 2006

In each month, three sponge colonies were randomly collected in the cages at Sichang Island and the samples were cut into small pieces about one centimeter size and then the seawater in the sponge body was immediately removed by a tissue paper for one minute. The percentage mean of seawater loss from the sponge *Xestospongia* sp. (n=20) was 48.1±4.5% as shown in Table 4.6. The semi-dried weight samples were further extracted of renieramycins contents in the sponge *Xestospongia* sp.

Annual variation in contents of renieramycin M (%w/w) in semi-dried weight sponge *Xestospongia* sp. was calculated according to the regression equation from the calibration curve (y = 0.0047x). The results are shown in Table 4.7. Renieramycin M contents of the sponge *Xestospongia* sp. samples varied from 0.08–0.20 %w/w with mean of 0.13±0.02%. The HPLC chromatogram of sponge extract showed that renieramycin M was normally detected as the only component at retention time 8.9 min and acenaphthene as internal standard at retention time 13.7 min (Figure 4.5). The other renieramycins were found in much smaller amounts and were not quantified. Figure 4.6 shows the means of renieramycin M concentrations in each month (± SD; vertical lines). It should be noted that the amounts of renieramycin M were the greatest in April (0.20% w/w) and in October (0.20% w/w) and the least in January (0.08% w/w) and in July (0.09% w/w).

Table 4.6. The percentage of seawater weight loss from the sponge *Xestospongia* sp.

Number of sample	Wet wt. (g)	Semi-dried wt. (g)	% Seawater loss
1	2.63	1.50	43.1
2	3.37	1.88	44.1
3	3.30	1.74	47.2
4	2.47	1.22	50.7
5	3.12	1.66	46.7
6	2.87	1.61	44.0
7	2.34	1.38	40.9
8	3.02	1.69	43.9
9	1.98	1.11	44.2
10	2.54	1.23	51.8
11	2.67	1.23	54.1
12	1.67	0.76	54.7
13	2.31	1.16	49.8
14	2.29	1.05	53.9
15	3.12	1.66	46.7
16	2.96	1.58	46.7
17	3.74	1.98	47.0
18	4.31	2.00	53.7
19	2.01	0.90	55.2
20	3.03	1.68	44.4
Mean			48.1
SD			4.5

Table 4.7. Renieramycin M contents in the sponge *Xestospongia* sp. during August 2005 to July 2006 at Sichang Island.

Month	Renieramycin M concentration* (%w/w)				SD
	sponge 1	sponge 2	sponge 3	Average	
August	0.13	0.11	0.11	0.12	0.01
September	0.16	0.11	0.12	0.14	0.02
October	0.18	0.24	0.17	0.20	0.04
November	0.15	0.10	0.11	0.12	0.03
December	0.15	0.11	0.16	0.14	0.02
January	0.07	0.07	0.10	0.08	0.02
February	0.13	0.13	0.14	0.13	0.00
March	0.12	0.11	0.11	0.11	0.01
April	0.24	0.20	0.15	0.20	0.05
May	0.15	0.09	0.14	0.12	0.03
June	0.13	0.09	0.12	0.11	0.02
July	0.11	0.06	0.10	0.09	0.02

*Values of renieramycin M is given as percent of semi-dried weight (%w/w)

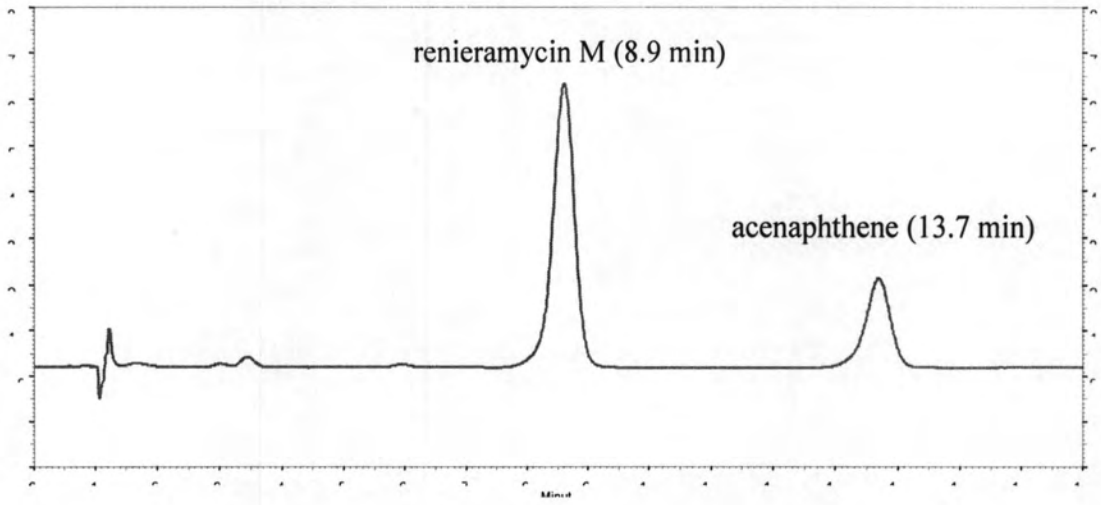

Figure 4.5. A representative HPLC chromatogram of the sponge *Xestospongia* sp. ethyl acetate extract.

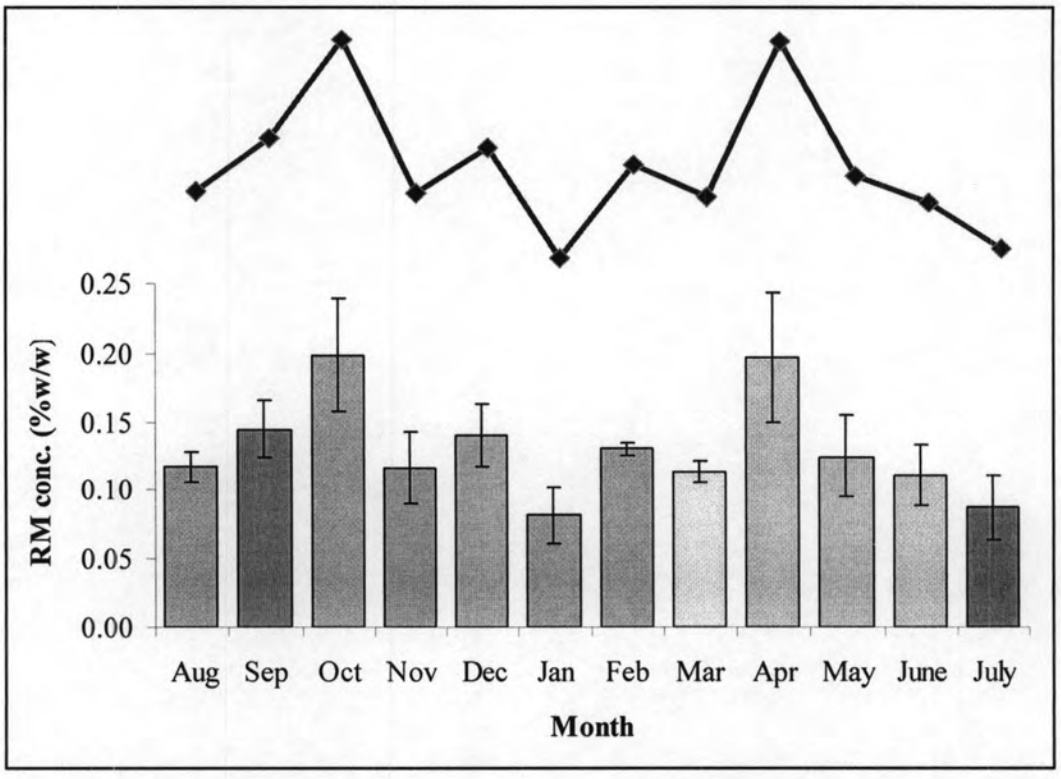

Figure 4.6. Renieramycin M contents in the sponge *Xestospongia* sp. in August 2005 to July 2006 (± SD; vertical lines).

3. Environmental factors measurement

During the study period on August 2005 to July 2006, the seawater samples for environmental factors analyses were also collected together with the sponge samples. The physical factors such as **salinity**, **pH**, and **water temperature** were directly measured by using a hand refractometer (N.O.W), an automatic pH meter (EUTECH), and a thermometer, respectively. For the chemical factors such as **chlorophyll-a**, **total dissolved solids** (TDS) and **total suspended solids** (TSS) were also determined.

3.1. Seasonal variation of environmental parameters

All data were expressed in terms of mean ± standard deviation (SD) and range as shown in Table 4.8 and Figure 4.7. Environmental factors, pH varied from 8.02 to 8.46 with the mean of 8.26±0.13 and salinity oscillated between 28 ppt and 34 ppt with the mean of 32±2 and water temperature ranged from 27 °C to 31°C. Total dissolved solids (TDS) are due to soluble materials whereas total suspended solid (TSS) are discrete particles. They may be divided into organic matter and inorganic matter. The mean values of total dissolved solids (TDS) and total suspended solids were 34.88±2.91 mg/L and 10.72±2.38 mg/L, respectively. Concentration of chlorophyll-a in seawater samples indicated the fluctuation of natural phytoplankton population. Concentration of chlorophyll-a varied from 1.34 to 6.47 mg/m^3.

Table 4.8. Means of environmental factors variation during August 2005 to July 2006

Items	Mean ± SD	Range
Environment factors		
pH	8.26 ± 0.13	(8.02 - 8.46)
Salinity (ppt)	32 ± 2	(28 -34)
Water temperature (°C)	29 ± 2	(27 - 31)
TDS (mg/L)	34.88 ± 2.91	(30.93 - 38.89)
TSS (mg/L)	10.72 ± 2.38	(7.01 - 15.77)
Chlorophyll-a (mg/m^3)	3.59 ± 1.62	(1.34 - 6.47)

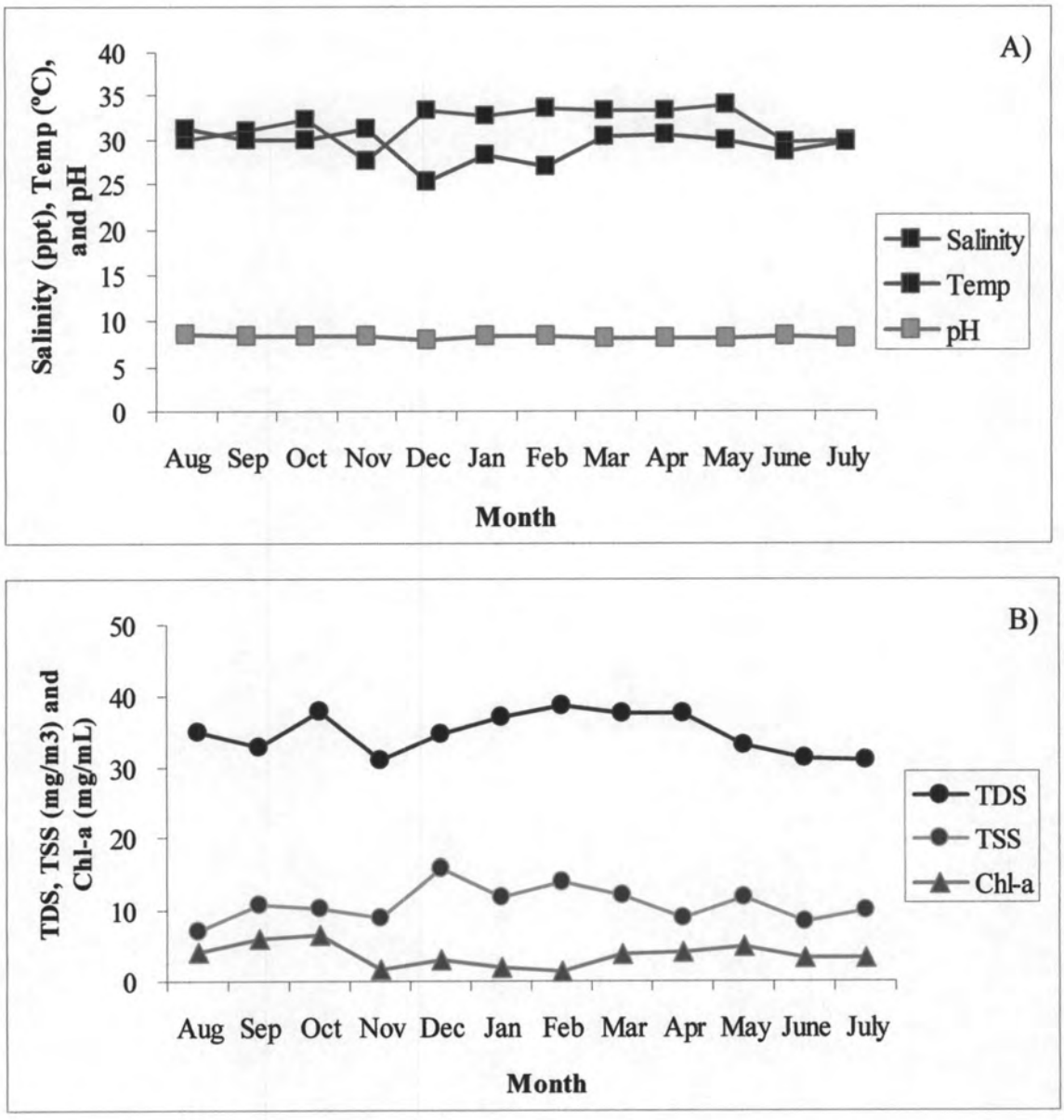

Figure 4.7. Graphs showing annual variation (from August 2005 to July 2006) of environmental parameters:

A) physical factors: salinity (ppt), water temperature (°C), and pH; and

B) chemical factors: TDS (mg/L), TSS (mg/L), chlorophyll-a (mg/m^3).

3.2. Relationship between environmental parameters and renieramycin M

Data concerning the environmental parameters and renieramycin M in the sponge *Xestospongia* sp. are reported in Table 4.9 and Figure 4.8. Our study period, the relationships can be computed between renieramycin M and environment parameters by using the Pearson correlation coefficient (r) as shown in Table 4.10 and Figure 4.9. Renieramycin M annual variation was found to have the positive correlations with both total suspended solids, TDS ($r = 0.34$, $p<0.05$) and chlorophyll-a ($r = 0.44$, $p<0.01$).

Table 4.9. Annual variation between renieramycin M contents (%w/w) and environmental parameters during August 2005 to July 2006

Month	pH	Salinity (ppt)	Temp. (°C)	TDS (mg/L)	TSS (mg/L)	Chl-a (mg/m^3)	RM (%w/w)
Aug	8.46	30	31	34.92	7.01	4.06	0.12
Sep	8.30	31	30	32.93	10.69	5.98	0.15
Oct	8.38	32	30	38.04	10.03	6.47	0.20
Nov	8.32	28	31	31.00	8.93	1.66	0.12
Dec	8.02	33	25	34.89	15.77	2.87	0.14
Jan	8.40	33	28	37.28	11.73	1.97	0.08
Feb	8.38	34	27	38.89	13.80	1.34	0.13
Mar	8.10	33	30	37.74	11.90	3.78	0.11
Apr	8.12	33	31	37.68	8.80	4.02	0.20
May	8.22	34	30	33.12	11.70	4.75	0.12
June	8.28	30	29	31.19	8.30	3.08	0.11
July	8.20	30	30	30.93	10.01	3.14	0.09
Mean	8.27	32	29	34.88	10.72	3.59	0.13
SD	0.14	2.02	1.78	3.00	2.45	1.60	0.02

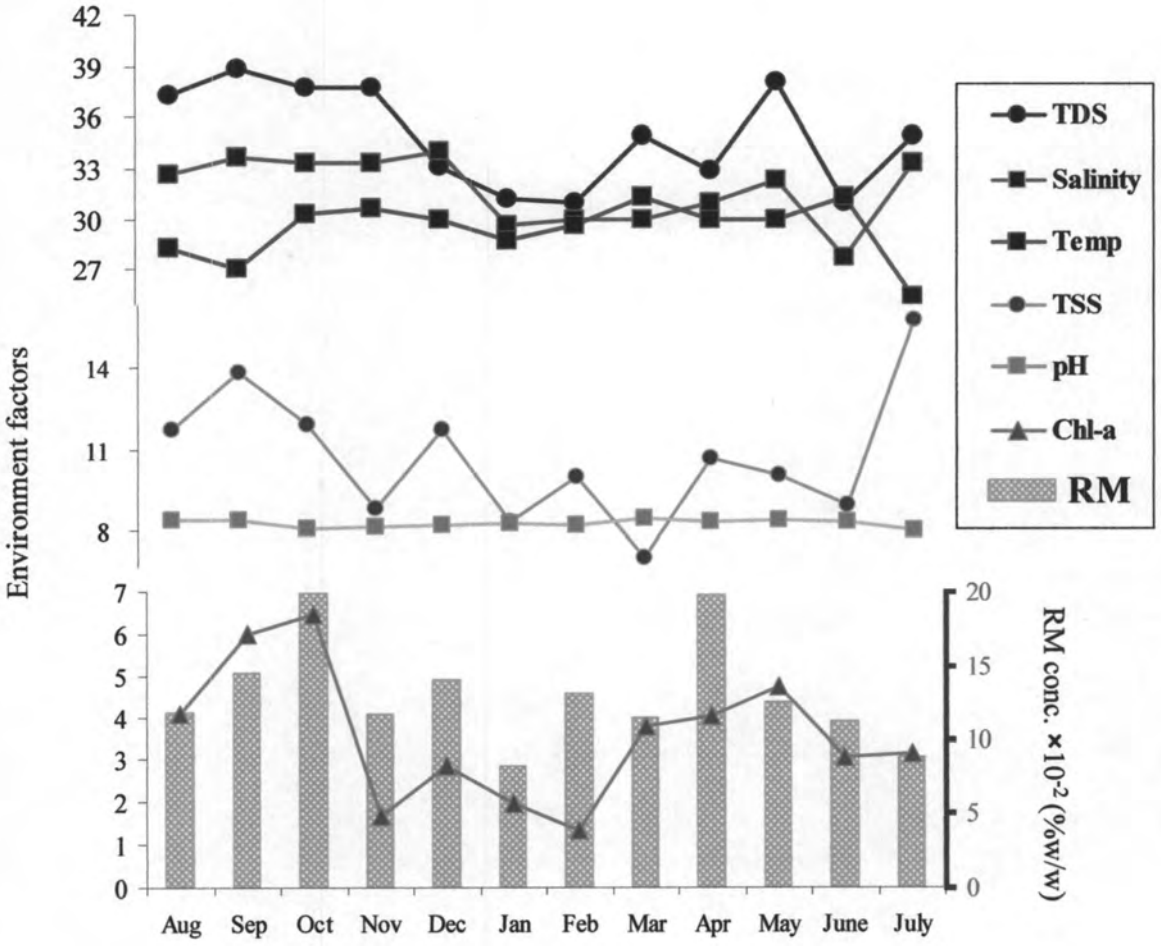

Figure 4.8. Annual variation between renieramycin M contents ($\times 10^{-2}$ %w/w) and environmental parameters as salinity (ppt), water temperature (°C), pH, total dissolved solids (TDS; mg/L), total suspended solids (TSS; mg/L), and chlorophyll-a (mg/m^3).

Table 4.10. Correlation coefficients (n = 36) between renieramycin M of *Xestospongia* sp. and environmental parameters during August 2005 to July 2006.

	Pearson Correlation coefficients (*r*)					
	pH	Salinity	Temp.	TDS	TSS	Chl-a
Renieramycin M	-0.16	0.25	-0.10	0.34*	0.05	0.44**

** Significant ($p < 0.01$)
* Significant ($p < 0.05$)

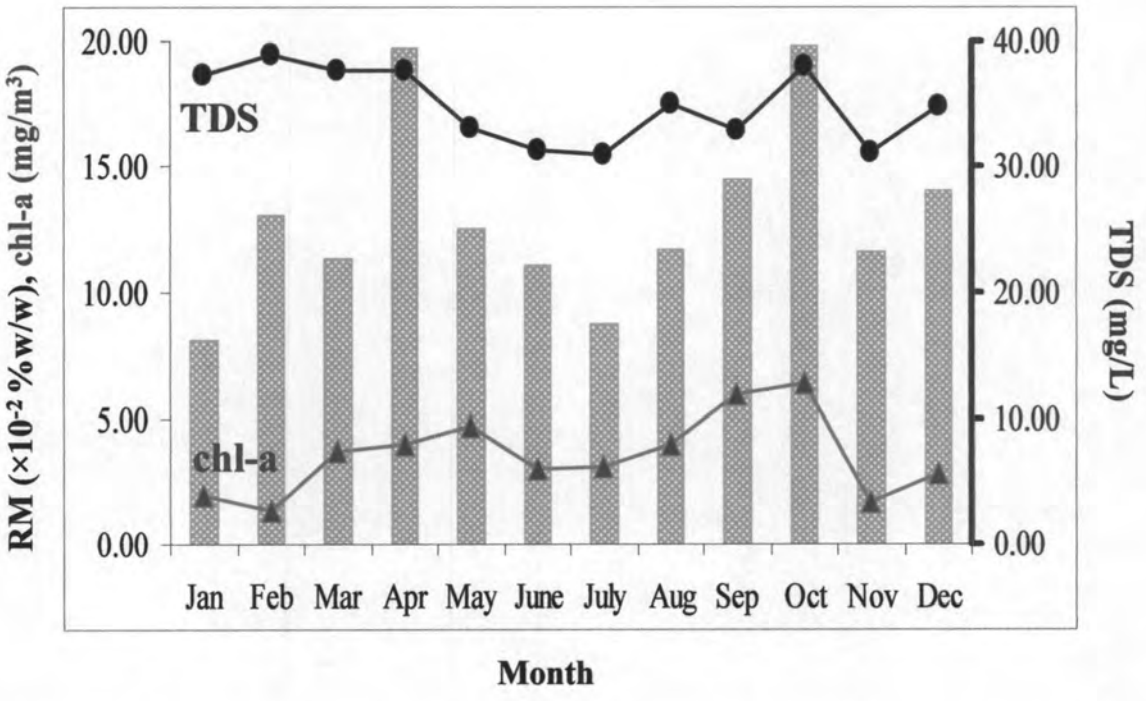

Figure 4.9. Annual variation between renieramycin M contents ($\times 10^{-2}$ %w/w) and both environmental parameters as total dissolved solids, TDS (mg/L) and chlorophyll-a (mg/m^3).

4. Cellular source of renieramycin M in the blue sponge *Xestospongia* sp.

The freshly collected sponges *Xestospongia* sp. for cell fractionation were sampled and stored in 10 mM potassium cyanide in 0.05 M phosphate buffer solution in artificial seawater (pH 7.0). A part of the collected sponge was immediately fixed with 2.5% glutaraldehyde in artificial seawater for sponge cell observation using a scanning electron microscope.

4.1. Scanning electron microscopic (SEM) observation in whole-sponge tissue

The sponge tissue was characterized using a scanning electron microscope (SEM). Thick sections of intact sponge tissue are necessary to study the skeletal structure of the sponge. In our study, cross section of the sponge *Xestospongia* sp. tissue contained the highly dense network of numerous spicules which are straight or slightly curved at the center, sharply pointed and hastate (approximately 200 μm long) as shown in Figures 4.10A and B). The open tissue structure contained different types of sponge cells which consist mainly of choanocyte chambers in contact with large sponge cells, archaeocytes (Figures 4.10C-E). Each size of the sponge cell types was approximately 2–3 μm in diameter of choanocytes and 6–10 μm in diameter of archaeocytes. The size of other small sponge cells was only 1 μm in diameter (Figure 4.10F). The SEM images in Figure 4.11 show some bacteria which was approximately less than 1 μm in diameter, and the SEM images on surface area and inside the sponge tissue displayed morphological features of the bacteria including bacilli and cocci forms.

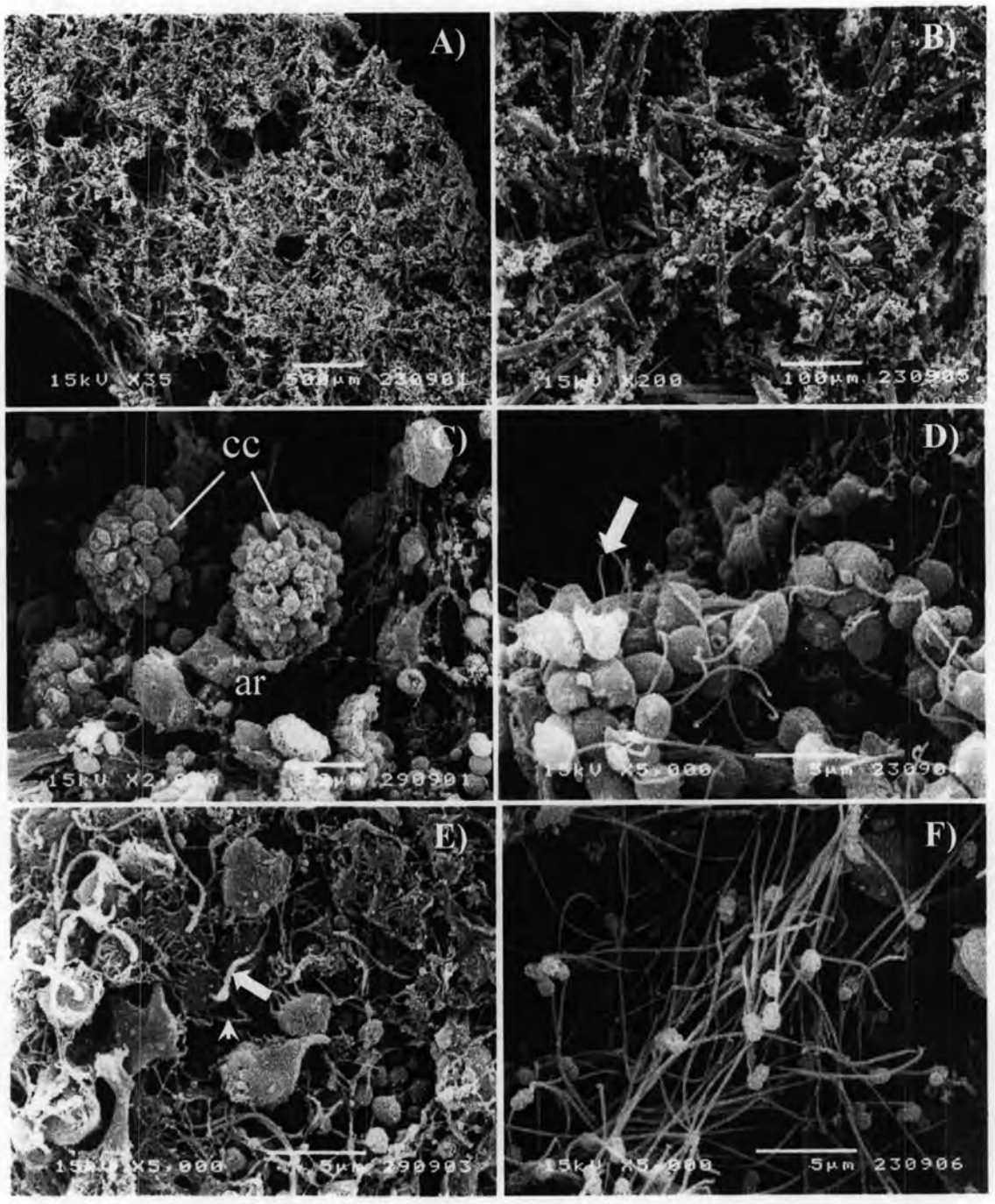

Figure 4.10. Representative SEM images from cross section of the sponge *Xestospongia* sp.: A-B) cross section of the sponge tissue containing numerous spicules, scale bar 500 µm and 100 µm, respectively;

C) large archaeocytes (ar) surrounding choanocyte chamber (cc), scale bar 10 µm;

D-E) the flagella (arrow) and collars (head arrow) from choanocytes; scale bar 5 µm; and

F) small choanocytes containing long flagella, scale bar 5 µm.

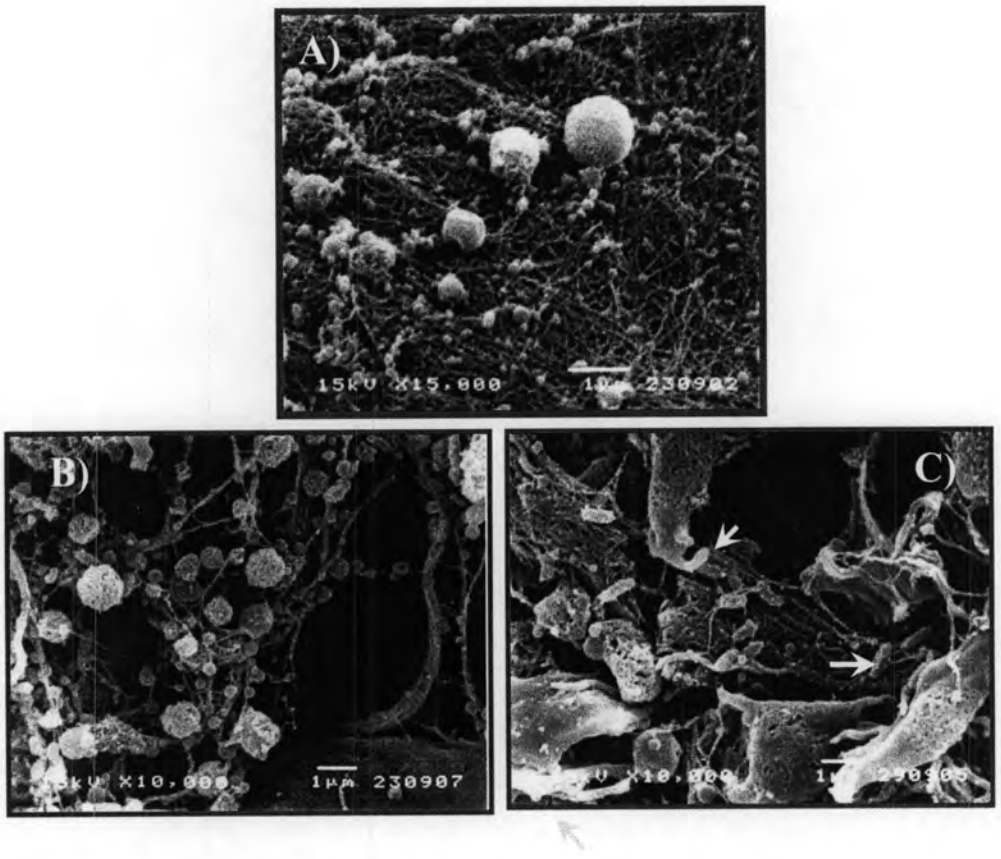

Figure 4.11. Representative SEM images of bacteria in the sponge *Xestospongia* sp.
A) cocci bacteria on outer surface of the sponge colony, scale bar 1 μm;
B) cocci bacteria in the sponge *Xestospongia* sp., scale bar 1 μm; and
C) bacilli bacteria (arrow) in the sponge *Xestospongia* sp., scale bar 1 μm.

4.2. Cell separation and identification

The cell fractionations were performed by differential centrifugation and Ficoll density gradient centrifugation. The cell fractions including **Fa**, **Fb**, and **Fc** was further observed under scanning electron microscopy as shown in Figure 4.12 and the sponge *Xestospongia* sp. cell types are summarized in Table 4.11. The results showed that fraction **Fa**, the pellet from differential centrifugation at higher speed (4,000 g) of the supernatant, was characterized by numerous bacteria and small sponge cells (approximately $\leq$1 μm in diameter size). On the other hand, the sponge cell suspension can be fractionated according to density on discontinuous Ficoll gradients (6%-12%-20%-25%-30%). After centrifugation, there were two bands of sponge cells, fractions **Fb** and **Fc**. A sharp band of sponge cells was observed at the 12/20% Ficoll interface as fraction **Fb**, whereas a large band of sponge cells was observed at the 20/25% Ficoll interface as fraction **Fc**. Cell concentrations of both fractions **Fb** and **Fc** were measured using a haemocytometer as shown in Table 4.12. Cell concentration of fraction **Fc** (3.30×10^8 cells/mL) was higher than that of fraction **Fb** (1.27×10^8 cells/mL) approximately three folds.

Table 4.11. Fractionation of the sponge *Xestospongia* sp. cell types by SEM

Cell fraction	Position	SEM analysis
Fa	pellet (high speed 4,000g)	bacteria and small sponge cells
Fb	interface 12-20%	mainly choanocytes (some archaeocytes)
Fc	interface 20-25%	mainly choanocytes and archaeocytes

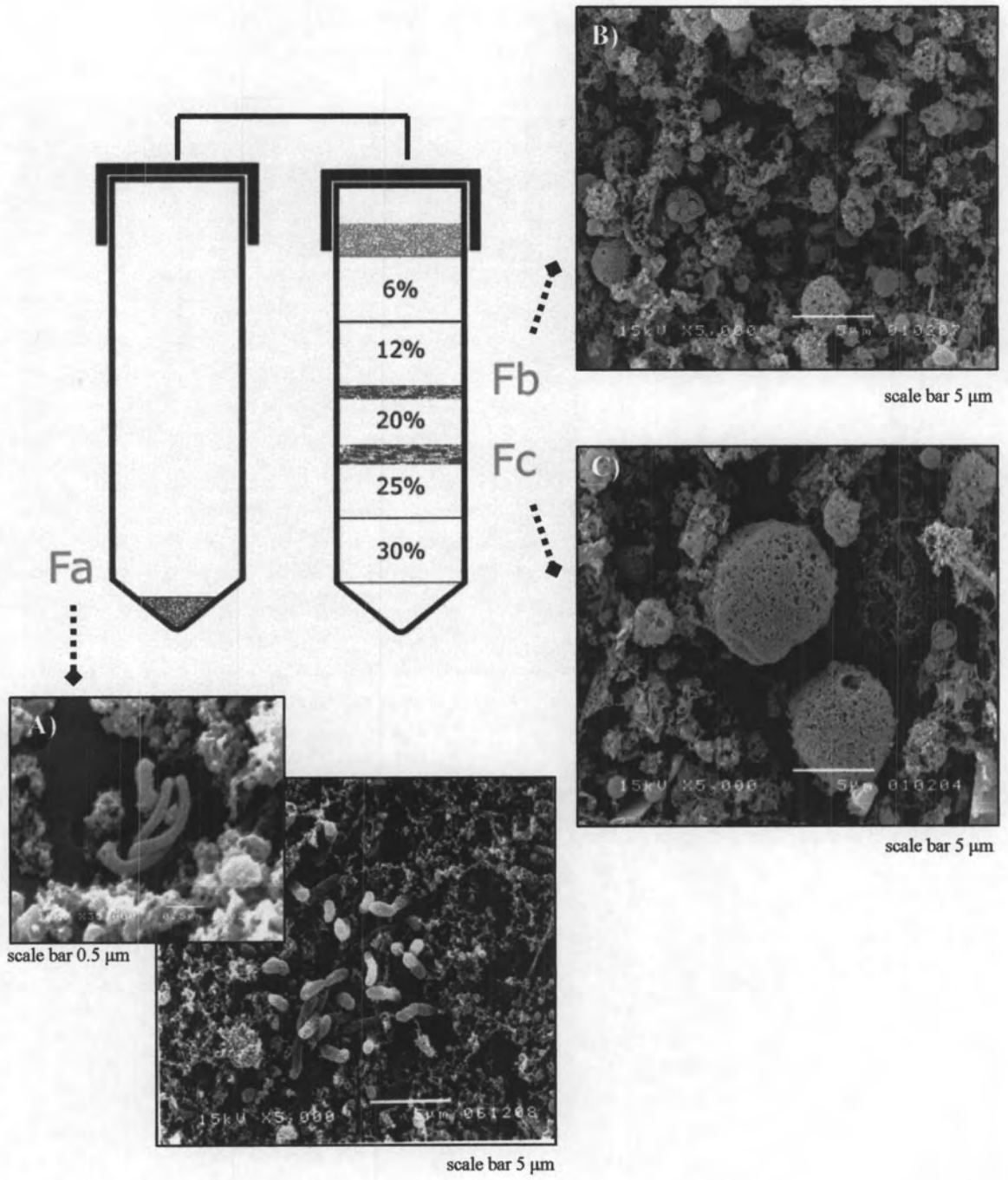

Figure 4.12. Representative SEM images of sponge cell fractions obtained from centrifugation:

A) bacilli bacteria in fraction **Fa**;

B) choanocytes in fraction **Fb**; and

C) choanocytes and archaeocytes in fraction **Fc**.

4.3. Distribution and cellular location of renieramycin M in cell fractions

HPLC analyses revealed the presence of renieramycin M amount (%w/w, wet weight) in cell fractions **Fa**, **Fb**, and **Fc** as shown in Table 4.12.

In sponge cell fractions, there were greater amounts of renieramycin M in fractions **Fb** and **Fc** with concentrations of 0.08±0.04% and 0.18±0.05% w/w, respectively. On the other hand, in fraction **Fa** which contained bacterial and small sponge cells, lower quantity of renieramycin M was found with a mean of 0.04±0.01% w/w (approximately two- and five-folds of **Fb** and **Fc**, respectively).

Table 4.12. Distribution of renieramycin M in cell fractions Fa, Fb, and Fc.

Items	Fraction	Sample		
		1	2	Average
Cell count (cell/mL)	Fb	1.28×10^8	1.25×10^8	$1.27\pm0.01 \times 10^8$
	Fc	3.50×10^8	3.10×10^8	$3.30\pm0.01 \times 10^8$
	Fc/Fb	2.7	2.5	2.6
Renieramycin M content (%w/w)	Fa	0.03	0.04	0.04±0.01
	Fb	0.05	0.10	0.08±0.04
	Fc	0.14	0.21	0.18±0.05
	Fc/Fb	2.8	2.1	2.5

Discussion

In this study, renieramycin M was detected as the main renieramycins from the sea-cultured sponge *Xestospongia* sp. at Sichang Island, Cholburi Province. This result is consistent with the previous reports of the isolation of a mixture of stabilized renieramycins from the Thai sponge *Xestospongia* sp. (Suwanborirux *et al.*, 2003; Amnuoypol *et al.*, 2004; Saito *et al.*, 2004). The newly developed HPLC analysis is a suitable and sensitive method for measuring the renieramycins annual quantity in the marine sponge *Xestospongia* sp. The optimization of HPLC conditions was performed with a LiChrosphere® RP-18e column (125×4 mm, 5 μm) as the stationary phase at 25 °C, an isocratic solution of methanol/water (7:3) as the mobile phase at flow rate 1.0 mL/min, a photodiode array detector at wavelength 270 nm and acenaphthene as the internal standard. The validation of HPLC method was studied with three parameters (linearity, accuracy, and precision) and determined based on the ICH guideline. They all showed good linearity in the range of 5-35 μg/mL renieramycin M concentrations with R^2 value of 0.9999. Accuracy test was validated in terms of percent recovery of renieramycin M in the range of 103.10-107.14% (Table 4.2). Precision of the HPLC method was measured by analyzing the same sample six times (Tables 4.3 and 4.4). The relative standard deviation (RSD) of peak area and retention time of each renieramycin M concentration was less than 1.0%.

The crude EtOAc extracts prepared from the sponge *Xestospongia* sp. pretreated with potassium cyanide were determined by HPLC analyses and their renieramycin M contents were calculated according to the regression equation (y=0.0047x) from calibration curve of renieramycin M. In most cases, renieramycin M was the only component detected by the HPLC method while other renieramycins amounts were too small to be quantified. Annual variation of renieramycin M quantity (%w/w) showed significant difference (ANOVA, $p<0.05$) with the mean of 0.13±0.02%. The highest renieramycin M content was found in April (0.20%) and in October (0.20%) and the lowest in January (0.08%) and in July (0.07%). Those fluctuations can be explained by variation of environmental conditions such as salinity, pH, water temperature, chlorophyll-a, total dissolve solids (TDS) and total suspended solids (TSS) during August 2005 to July 2006 as shown in Table 4.9 and Figure 4.8. The relationships can be computed between renieramycin M contents and environment parameters by using the Pearson correlation coefficient (r). The positive correlations were found between renieramycin M annual content and environmental factors as total dissolved solids, TDS ($r = 0.34$, $p<0.05$) and chlorophyll-a ($r = 0.44$, $p<0.01$). Both factors may refer

to nutrition matters dissolved in seawater that are considered to be beneficial for the sponge. Most of the environment parameters vary according to season, and the changes in ecological conditions can stimulate or inhibit the biosynthesis of several nutrients (Marinho-Soriano *et al.*, 2006). However, the nutritional properties of the sponge are poorly known and the chemical compositions of the sponge such as proteins, carbohydrates, lipids, fiber, ash, nitrogen, etc. should be further investigated. The variation in growth rate of the sponge studied was related inversely to variation in defenses against predators. The chemical variation of secondary metabolites is also related to several influencing factors including habitat differences, local adaptation, and physical stresses (Wulff, 2005). Therefore, the chemical ecological studies of the secondary metabolites from the sponge presumably used for protection against predators, overgrowing space competitors, invading microorganism, etc. should be considered.

During the collection period, we observed that the sexual reproduction of the sponge *Xestospongia* sp. occurred in April and November. Some sponge colonies contained a number of the whitish blue parenchymella larva inside. A high temperature normally stimulates sexual reproduction in sponges (Belarbi *et al.,* 2003). Figure 4.13 shows the parenchymella larvae in the sponge *Xestospongia* sp. by SEM. The size of parenchymella larva was approximately 250-350 μm in diameter. The differences of renieramycins in the blue sponge *Xestospongia* sp. were compared between the normal and reproductive colonies as shown in Figure 4.14. The sponge colonies containing parenchymella larva were found to have higher content of deangeloylrenieramycin M than the normal sponge colonies, suggesting that the presence of deangeloylrenieramycin M may play an important mechanism during the season of reproductive process and/ or in defense mechanisms of the parenchymella larva in the blue sponge *Xestospongia* sp.

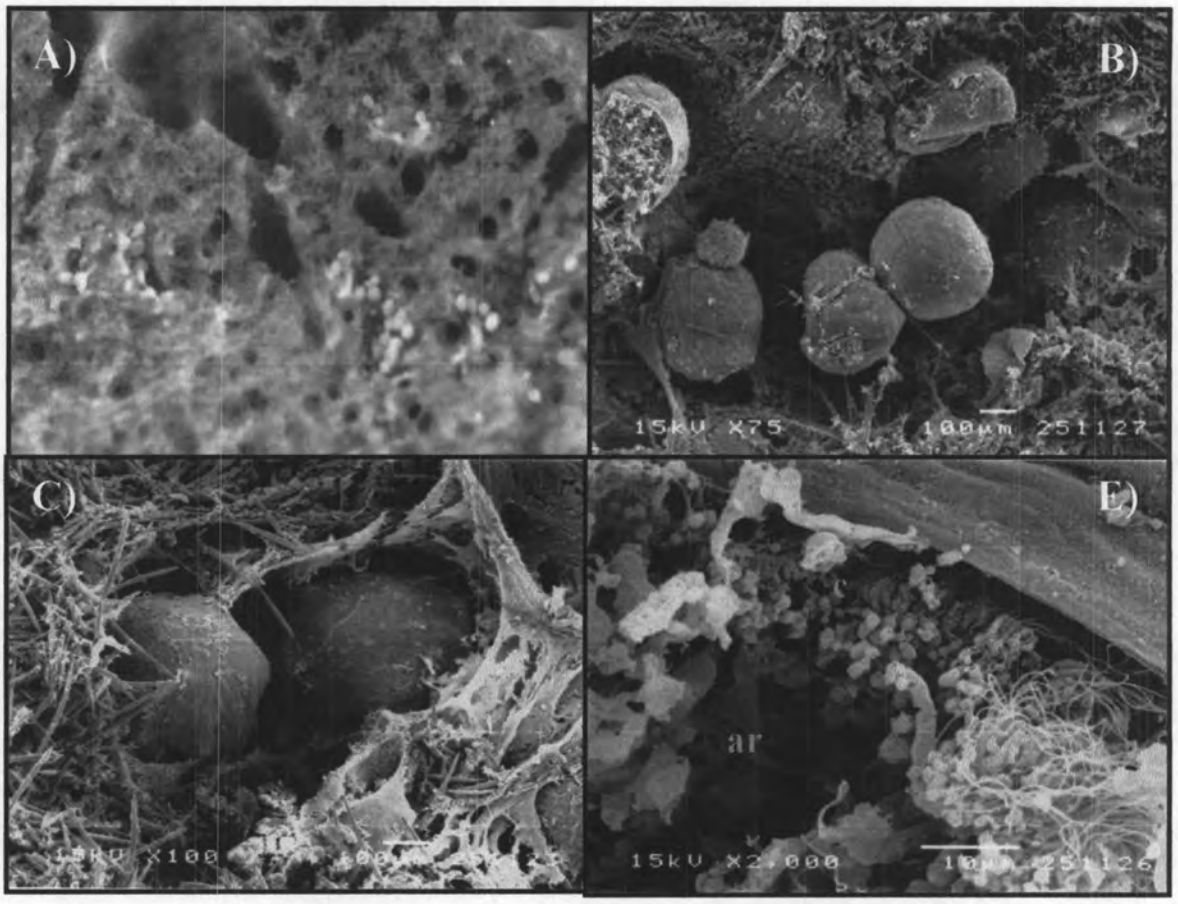

Figure 4.13. Parenchymella larva of the blue sponge *Xestospongia* sp.

A) the whitish blue larvae inside the sponge colony;

B-C) SEM images of parenchymella larva, scale bar 100 µm; and

D) SEM image of larval epithelium showing columnar ciliated cells (**c**)

and large amoeboid archaeocytes (**ar**) placed under the epithelium, scale bar 10 µm.

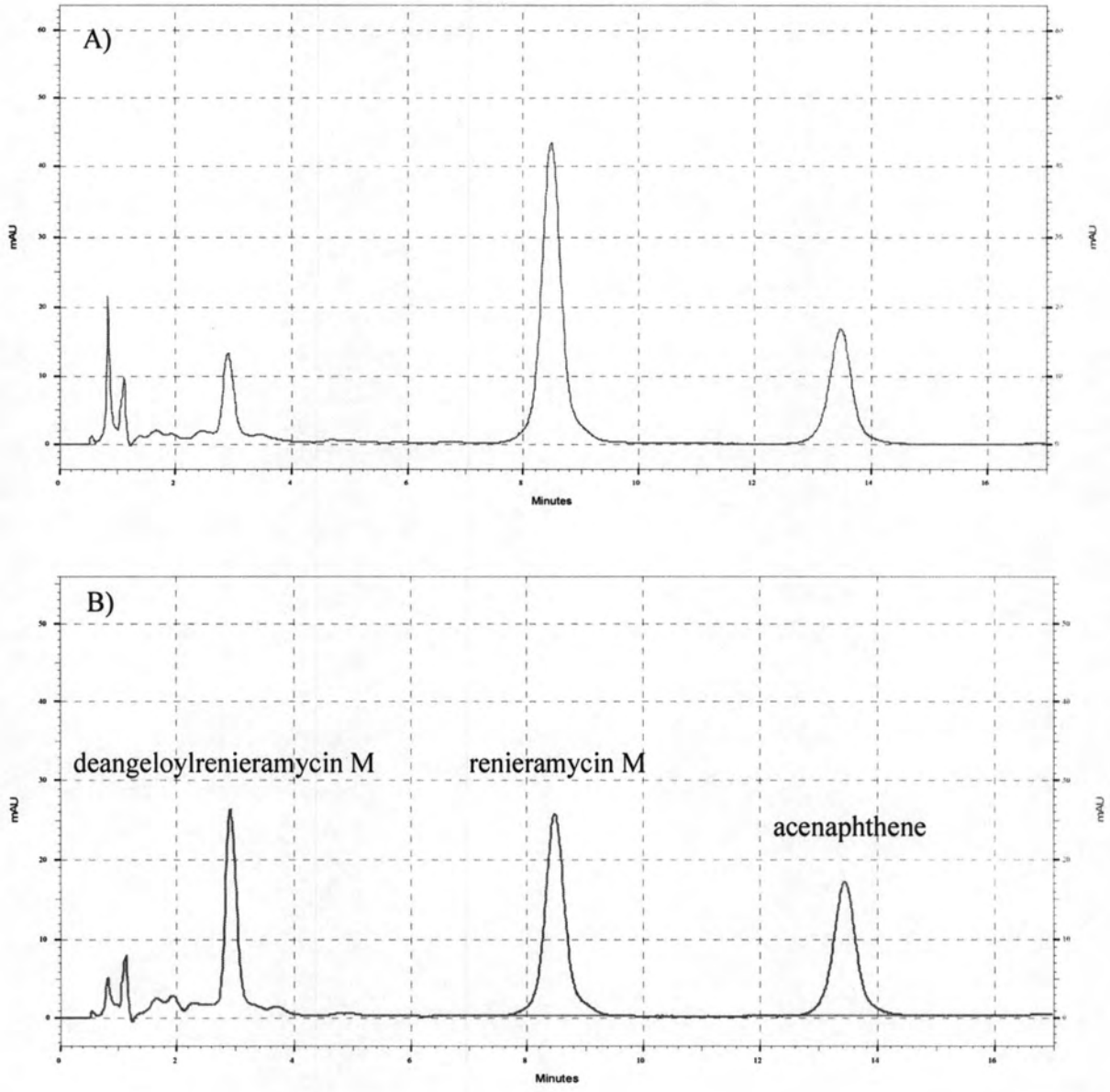

Figure 4.14. HPLC chromatograms of the ethyl acetate extracts in November 2005 of A) normal sponge colony; and B) sponge colony containing parenchymella larva.

SEM studies of sponge tissue revealed that choanocytes (approximately 2-3 μm in diameter) and archaeocytes (approximately 6-10 μm in diameter) are the major sponge cell types and a number of cocci and bacilli bacteria (approximately <1 μm in diameter) were also associated with the sponge. Differential centrifugation of the sponge cell suspension succeeded in the separation of the bacterial cells from the choanocytes and archaeocytes. The HPLC analysis revealed that the renieramycin M content in the bacterial fraction was much lower than the sponge cell fractions. Further Ficoll density gradient centrifugation resulted in the fractions of the choanocytes (Fb) and the mixture of choanocytes and archaeocytes (Fc). The cell concentration of fraction Fb was about 2.6 times higher than that of fraction Fc. In contrast, the HPLC analysis demonstrated that renieramycin M concentration of fraction Fb was about 2.5 times lower than that of fraction Fc. The results can be concluded that renieramycin M was associated with choanocytes and archaeocytes but not with bacteria. This finding is in agreement with several studies showing that sponge cells are the producers of the bioactive metabolites (Garson et al., 1998; Belarbi et al., 2003; Richelle-Maurer et al., 2001, 2003).