บทบาททางนิเวศวิทยาของสารเรเนียรามัยซินในฟองน้ำสีน้ำเงิน *Xestospongia* sp.

นายอุดมศักดิ์ ดรุมาศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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ECOLOGICAL ROLES OF RENIERAMYCINS ON THE BLUE SPONGE *XESTOSPONGIA* SP.

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for the Degree of Doctor of Philosophy Program in Marine Science

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อุดมศักดิ์ ดรุมาศ: บทบาททางนิเวศวิทยาของสารเรเนียรามัยซินในฟองน้ำสีน้ำเงิน *Xestospongia* sp. (ECOLOGICAL ROLES OF RENIERAMYCINS ON THE BLUE SPONGE *Xestospongia* sp.) อ.ที่ปรึกษา: ผศ. ดร. สุชนา ชวนิชย์ อ.ที่ปรึกษาร่วม: ดร. คณิต สุวรรณบริรักษ์,. 134 หน้า

การศึกษาลักษณะทางนิเวศวิทยาของฟองน้ำสีน้ำเงิน (*Xestospongia* sp.) ในแนวปะการังต่างๆของอ่าวไทย พบว่าอาศัยอยู่กับปะการังโขด (*Porites lutea*) และพรมทะเล (*Palythoa caesia*) เป็นหลัก โดยฟองน้ำที่มีขนาดใหญ่ที่สุดอาศัยกับพรมทะเล (*Pa. caesia*) และฟองน้ำที่มีขนาดเล็กที่สุดอาศัยอยู่กับสาหร่ายขนาดใหญ่ ฟองน้ำชนิดนี้สามารถผลิตสารเรเนียรามัยซิน (Frincke และ Faulkner 1982) การวิเคราะห์เชิงปริมาณของสารเรเนียรามัยซินในฟองน้ำชนิดนี้จากบริเวณต่างๆ ของอ่าวไทยแสดงให้เห็นว่ามีปริมาณแตกต่างกันตามบริเวณต่างๆ ของอ่าวไทย ผลการวิเคราะห์คาร์บอน ไฮโดรเจน ไนโตรเจน และสารเรเนียรามัยซินเชิงปริมาณ ในบริเวณต่างๆ ของตัวฟองน้ำ (ขอบ, ภายใน และภายนอก) พบว่ามีความแตกต่างอย่างมีนัยสำคัญทางสถิติ การเปรียบเทียบปริมาณคาร์บอน ไฮโดรเจน ไนโตรเจน และสารเรเนียรามัยซิน ณ บริเวณเดียวกันของฟองน้ำ ที่อาศัยอยู่กับสิ่งมีชีวิตต่างชนิดกัน(*Porites* และ *Palythoa*)ไม่แสดงความแตกต่างอย่างมีนัยสำคัญทางสถิติ การศึกษาบทบาททางนิเวศวิทยาของสารเรเนียรามัยซิน พบว่าไม่ทำให้ปะการังโขด (*Porites lutea*) เกิดการฟอกขาวหรือตายและไม่ยับยั้งการเจริญของแบคทีเรียกลุ่มที่ใช้ออกซิเจน(aerobic bacteria)ในน้ำทะเล แต่สารนี้ยับยั้งการลงเกาะของเพรียงหิน (*Semibalanus balaniodes*) การศึกษาผลของการเกิดแผลของฟองน้ำต่อการเปลี่ยนแปลงปริมาณสารเรเนียรามัยซิน พบว่าสารเรเนียรามัยซินมีความเข้มข้นเพิ่มขึ้นอย่างรวดเร็วภายหลังจากการทำให้ฟองน้ำเกิดแผล ผลการศึกษาพบเพิ่มเติมว่าฟองน้ำสีน้ำเงิน (*Xestospongia* sp.) เป็นอาหารเพียงชนิดเดียวของทากเปลือย (*Jorunna funebris*) โดยทากเปลือยมีการกระจายสารเรเนียรามัยซินที่ได้รับจากฟองน้ำไว้ตามอวัยวะต่างๆ เช่น เยื่อแมนเทิล (mantle) และ กล้ามเนื้อเท้า ผลการทดลองยังพบว่าการอดอาหารในทากเปลือยทำให้ปริมาณสารเรเนียรามัยซินในอวัยวะต่างๆดังกล่าวของทากเปลือยลดลง เมื่อนำชิ้นเนื้อจากอวัยวะต่างๆ ของทากเปลือยที่อดอาหารไปทดสอบการต่อต้านการกินโดยปลาชนิดต่างๆเปรียบเทียบกับสารเรเนียรามัยซินพบว่าเนื้อของทากเปลือยให้ผลต่อต้านการกินของปลาชนิดต่างๆ ทั้งในห้องปฏิบัติการและแนวปะการังเช่นเดียวกับสารเรเนียรามัยซิน

การศึกษาครั้งนี้สามารถสรุปได้ว่า ฟองน้ำสีน้ำเงิน (*Xestospongia* sp.) ส่วนใหญ่อาศัยกับปะการังโขด (*Porites lutea*) และพรมทะเล (*Palythoa caesia*) ในแนวปะการัง ฟองน้ำสีน้ำเงินสามารถผลิตสารเรเนียรามัยซินและกระจายไปตามส่วนต่างๆ ของร่างกาย สารเรเนียรามัยซินไม่มีฤทธิ์ในด้าน allelopathic และ anti-microbial แต่มีฤทธิ์ต่อต้านการลงเกาะของตัวอ่อนบางชนิดและต่อต้านการกินของปลาชนิดต่างๆ นอกจากนี้ยังพบสารนี้ในอวัยวะต่างๆ ของทากเปลือย มีความเข้มข้นลดลงเมื่อทากเปลือยอดอาหาร

ภาควิชาวิทยาศาสตร์ทางทะเล ลายมือชื่อนิสิต.....

สาขาวิชาวิทยาศาสตร์ทางทะเล ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา 2550 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

#4573868023 : MAJOR MARINE SCIENCE
KEY WORD: *Xestospongia* sp./ *Jorunna funebris*/ RENIERAMYCIN M/ COEXISTING ORGANISMS/ NUTRIENTS/ ALLELOPATHY/ WOUND/ FEEDING DETERRANCE/ THE GULF OF THAILAND.

ECOLOGICAL ROLES OF RENIERAMYCINS ON THE BLUE SPONGE *Xestospongia* sp.
THESIS ADVISOR: ASST. PROF. SUCHANA CHAVANICH, Ph.D.
THESIS CO-ADVISOR: KHANIT SUWANBORIRUX, Ph.D. 134.pp.

The blue sponge, *Xestospongia* sp., is a coral reef inhabitant occurring in the Gulf of Thailand. The distribution pattern of *Xestospongia* sp. and its association with other organisms were investigated. The results showed that the most frequently coexisting organisms were the massive coral, *Porites lutea*, and the colonial zooanthid, *Palythoa caesia*. However, *Xestospongia* sp. was also found with algal patches and dead coral rubble. The largest individuals of *Xestospongia* sp. were found growing on *Pa. caesia* while the smallest individuals were found on the algal patches. This sponge has been known to produce renieramycins (Frincke and Faulkner 1982). The results also showed that concentrations of renieramycin M, the main alkaloid with highly potent cytotoxicity, extracted from this sponge differed significantly among sites.

Carbon-nutrient contents and renieramycin M concentrations were found to be significantly different between the areas of *Xestospongia* sp. (edge, inner and outer) whereas carbon-nutrient contents and renieramycin M concentration of *Xestospongia* sp. coexisting with different organisms were not significantly different.

In the laboratory renieramycin M did not show any allelopathic effect on its coexisting organisms (*Porites lutea*). Furthermore, it did not inhibit the growth of aerobic bacteria. However, renieramycin M inhibited settlement of acorn barnacle (*Semibalanus balanoides*) but did not inhibit the settlement of pelecypods. In the experiments, renieramycin M concentrations increased immediately after *Xestospongia* sp. was wounded. Renieramycin M was also detected in *Jorunna funebris*, specifically the predatory nudibranch on *Xestospongia* sp. (Saito et al. 2004). This study found that the concentration of renieramycin M was not equally distributed into the body parts of *Jorunna funebris*. The concentration of renieramycin M was decreased by increasing starvation periods. However the feeding deterrent activity was found in tissues of *Jorunna funebris*, as well as, the renieramycin M.

In conclusion, *Xestospongia* sp. is a reef inhabitant that frequently coexists with *Porites lutea* and *Palythoa ceasia*. This sponge produces renieramycin M, the alkaloid substance and distributes it along its body. Although, renieramycin M did not show either allelopathic or anti-microbial effects, it exhibited anti-fouling and anti-feeding effects. *Jorunna funebris* was found to contain renieramycin M and Jorunnamycin in the mantle and foot muscle. The concentrations of these chemicals decreased when *Jorunna funebris* was starved.

Department Marine Science Student's signature Mdomsak Darunas

Field of study Marine Science Advisor's signature

Academic year 2007 Co-advisor's signature Khanit Suwan

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CHAPTER I

DESCRIPTION OF *XESTOSPONGIA* SP.

The blue sponge, *Xestospongia* sp., in our study has unique characteristics which differ from other renieramycin-producing sponges such as *Reniera* sp. (accepted as *Haliclona (Reniera)* sp., Van Soest et al. 2005), *Haliclona cribricutis* (accepted as *Haliclona (Reniera) cribricutis*, Van Soest et al. 2005), *Cribrochalina* sp., *Neopetrosia* spp. (Frincke and Faulkner 1982, Parameswaran et al. 1998, Pettit et al. 2000, Oku et al. 2003, Nakao et al. 2004, Van Soest et al. 2005), and other species in the genus *Xestospongia* that previously have been documented. It is likely that the species is undescribed although a more-rigorous taxonomic investigation of this large genus would be necessary prior to describing and naming it as a new species. This sponge differs from renierid, cribrochalinid, and neopetrosid sponges by its choanosomal skeleton with pauci- to multispicular tracts, and ectosomal skeleton with a tangential disordered network and no specialized structure. Renierid sponges have a unispicular, isotropic reticulation of the choanosomal skeleton and a tangential, unispicular, isotropic reticulation of the ectosomal skeleton (de Weerdt 2002), while cribrochalinid sponges have an ectosomal network consisting of a palisade of spicule brushes covered by a fine membrane (crust) (Desqueyroux-Faúndez and Valentine 2002a). Although this sponge is

very similar to neopetrosid sponges, its oxea megascleres are longer than 200 μm while those of neopetrosid sponges are shorter than 200 μm. In addition, neopetrosid sponges have a secondary subectosomal tangential network not present in our species (Desqueyroux-Faúndez and Valentine 2002b). The blue *Xestospogia* sp. can be distinguished from other previously described species of *Xestospongia* such as the Indo-Pacific species *X. exigua* (accepted as *Neopetrosia exigua*, Van Soest et al. 2005), *X. testudinaria*, and *X. bergquistia*, and the Caribbean species *X. carbonaria* and *X. muta* as follows. *Xestospogia exigua* is sticky to the touch when alive and preserved, and its ectosome adheres to the fingers. Both *X. testudinaria* and *X. bergquistia* are volcano-shaped while the Caribbean *X. muta* is barrel-shaped (Fromont 1991). The major differentiating morphological characteristic from *X. carbonaria* is the hispid blue surface of *Xestospongia* sp. compared to a smooth surface, black live coloration, and volcano-shaped elevation of the oscules (STRI 2006).

Samples of blue *Xestospongia* sp., collected from the coral reefs in the Gulf of Thailand, were found to coexist with other reef organisms (e.g., corals, algae, and other sponges) as well as settling on rock beds and dead coral rubble. This species is thickly encrusting and mostly lobate in growth form; the texture is hard, brittle, and friable; the surface is prominently bulbous, almost digitate-like; the color is light blue externally, yellowish-gray internally when alive and yellowish-brown in ethanol; and the oscules are numerous and mostly

found on the apices of the surface lobes. The ectosomal skeleton forms a tangential disordered network with no specialized spiculation. The choanosomal skeleton exhibits isotropic reticulation of paucispicular to multispicular tracts of oxeas forming tight oval meshes. There are no visible fibers and only a small amount of collagen in the mesohyl. The oxeas are straight or slightly curved at the center, sharply pointed and hastate, and 218, 241, and 257 µm long and 10, 17, and 20 µm wide (Figure. 1.1).

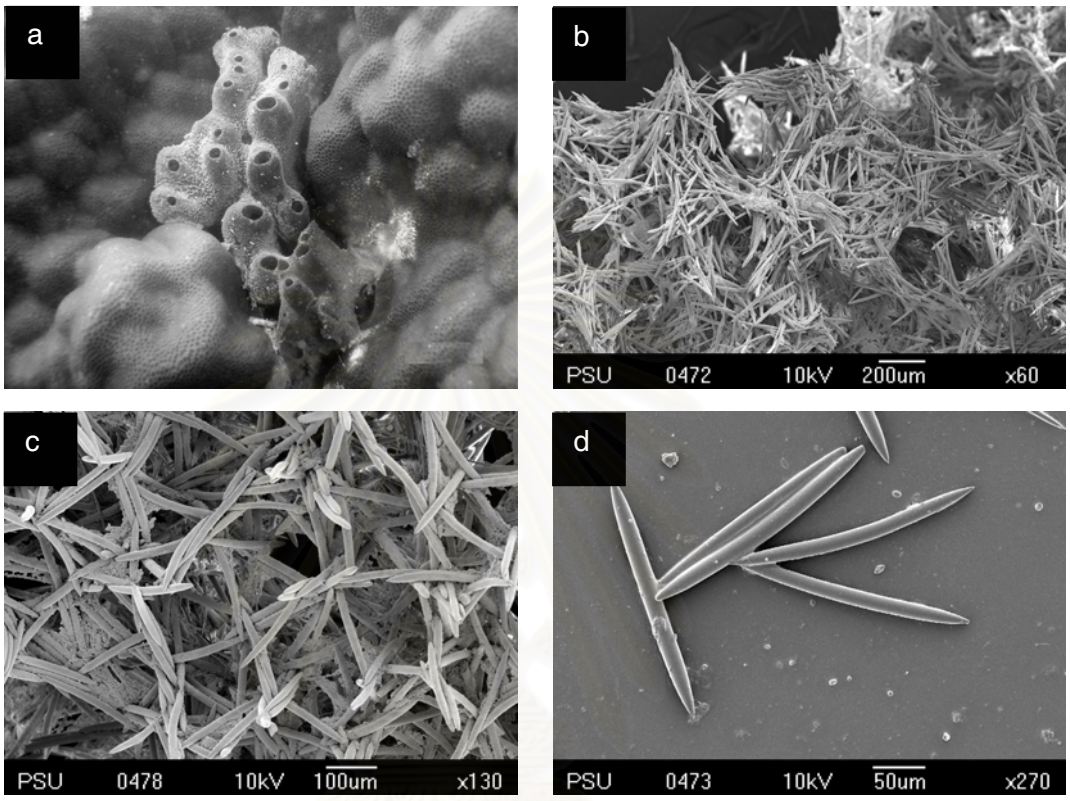

Figure. 1.1 a) The blue sponge *Xestospongia* sp. coexisting with the hard coral, *Porites lutea*. b) Choanosomal skeleton, showing a highly dense network of multispicular tracts. c) Longitudinal section through the surface, showing ectosomal tangential disordered network of spicule brushes. d) Oxeas.

CHAPTER II

INTRODUCTION TO CHEMICAL ECOLOGY AND SCOPE OF THIS STUDY

Chemical ecology has been defined by Paul (1992) who said "Chemical ecology, or ecological biochemistry, examines the roles of naturally occurring compounds in plants and animal interactions". This is a multidisciplinary area of interest which investigates the structures of chemicals produced biosynthetically by organisms, as well as their synthesis, mechanisms of actions and behavioral and developmental responses to these chemical signals. In biology, the details of chemical ecology have been investigated primarily for terrestrial habitats. In marine ecosystems, chemicals play an important role at the individual, the population, and particularly the community levels (Paul 1992). A number of studies of chemical ecology have been conducted at the community level, such as studies on feeding stimulatory affects, feeding inhibitory, spatial and temporal competitions, antimicrobials and larval settlement inductions/ resistances (De Boer et al. 1992, McClintock et al. 1994). Enzymes, hormones, pheromones, chemical signals (intra- and inter-specific communications, prey-predator interaction) and behavioral modifying agents are examples of the ecological role of chemicals produced by organisms (Atema 1992, Tyndale et al. 1994, Murphy and Hadfield

1997). There are some questions regarding the reasons for diversity in chemicals produced by organisms (namely intraspecific and interspecific communication chemicals). Paul (1992) and Wulff (2005) pointed out that the efficiency of chemicals produced by organisms is varied between habitats, as different habitats contain different kinds of competitors and predators. Another explanation was that the spatial variations in biogeographic variation, local variation and habitat variation (Green 1977, Faulkner et al. 1990, Chanas and Pawlik 1997, Duffy and Hay 2000, Kelman et al. 2000, Puglisi et al. 2000, Salmore and Hunter 2001, McGovern and Hellberg 2003) could account for these differences. However, these explanations, particularly the latitudinal gradient, were not sufficient as evidence for chemical ecology in the polar marine environment. High-latitude marine communities contained huge numbers of benthic organisms in a numbers of phyla (Moyer et al. 2003). A lowered suppression of predatory fish was observed because there were less predatory fish species in this region. Many researches agreed that most chemicals produced by organisms were not functional at all, as some chemicals had multifunctional roles and were synergistic with physical defensive structures (Pawlik 1993, Chanas and Pawlik 1996). Moreover, Paul (1992) concluded that slight differences in the chemical structures in compounds isolated from seaweeds probably result in differences in toxicity or deterrence.

Benthic organisms can be varied in terms of life styles, as some were predominately surfacing whereas some were commonly burrowing (Sorokin 1993). These differences in living styles resulted in different biological pressures. For example, epifaunas might be more stressed by fish predators. The infaunas might be stressed from worms or predatory mollusks. Even different body parts of the same individual were affected differently by a diverse range of enemies (Avila et al. 2000, Furrow et al. 2003). In one example, the *Oceanapia* sponge burrowed into its substrate but had the fistule with a capitum protruding into the surrounding water. The fistule and capitum were expected to be more at risk of attack from predatory fishes while the rest of its body was expected to be under pressure from spatial competition and predation by benthic organisms (Schupp et al. 1999). Therefore, it was concluded that species had a diverse range of defensive chemicals and allocated the defensive chemicals unequally in quantity and in quality along their body parts or cell types (Uriz et al. 1990, Schupp et al. 1999, Salomon et al. 2001). In addition, variations were caused by seasonal effects, the quality and quantity of raw materials to produce such chemicals, and symbionts (Reichardt et al. 1991, McGovern and Hellberg 2003). Some defense chemicals were expected to be harmful towards the hosts that produce it as well (Hay and Fenical 1988). How did the hosts deal with the toxicity of the chemicals that they produce? Many studies have shown that organisms stored their defensive chemical in a non-toxic form, but could easily

covert them to a very toxic form and rapidly send them out of their bodies or cells (Paul 1992). When wounded, the concentration of defensive chemicals could immediately rise up to 100 times above the normal concentrations (Ebel et al. 1997). However, benthic organisms also had the potential to regenerate when they were wounded (Duffy and Hay 2000, Henry and Hart 2005). In addition, during the wound healing period, there were fluctuations in the chemicals in their bodies and at their wounds such as antimicrobial and antifouling compounds, to control the infections caused by pathogens and to control the potentially competitive larva of competitors (Lemos et al. 1985, Puyana et al. 2003, Lunetta 2005).

The mechanism of spatial defense in benthic organisms has evolved over time (Engel and Pawlik 2000). Some species mechanically pushed their competitor away, some overgrew their competitors, and some used tentacles to fight for space, whereas others used allelochemicals (Engel and Pawlik 2000, Williams 2004). Studies on allelochemicals found that the allelopathic effect was not only caused by tissue contact between the competitive couples but also via waterborne chemicals which had an allelopathic effect as well (Aerts and Van Soest 1997, Nishiyama and Bakus 1999, Lirman 2001,)

Predatory defensive chemicals were diversely produced and stored in the body of preys (Pohnert 2004). To overcome this problem, a numbers of predators have evolved to be “prey specific” species (Parker et al. 2006). Predators were capable of detoxifying the

defensive chemicals that they received whilst consuming their prey and were capable of tolerating the defensive physical structures of their preys (McClintock 1987, Duffy and Paul 1992, Pereira et al. 2002). In addition, some predators could sequester the defensive chemicals or secondary metabolites of their prey and then biochemically modify to use as their own defensive chemical against higher predators (Becerro et al. 2001, Frick 2003).

Some secondary metabolites and chemical compounds produced by marine organisms have been intensively screened as marine natural products. The compounds that have pharmaceutical potential would be further studied and developed for drugs. For example the marine isoquinoline alkaloid, ecteinascidin (ET-743) derived from a sea squirt (*Ecteinascidia turbinata*), has been developed as a drug for cancer therapy, however is still little known about ecological role. Renieramycins, the closely related marine isoquinoline alkaloid with ecteinascidin, are potential anticancer drugs (Suwanborirux et al. 2003, Saito et al. 2004a,b). In addition, they are known as inhibiters for bacteria and antileishmania (Frincke and Faulkner 1982, Nakao et al. 2004). Following is some background information on renieramycins. Renieramycins are produced by several species of sponge such as *Reniera* sp., *Haliclona cribricutis cribricutis,*, *Cribrochalina* sp. and *Neopetrosia* sp./spp. (Frincke and Faulkner 1982, Parameswaran et al. 1998, Pettit et al. 2000, Oku et al. 2003, Nakao et al. 2004, Van Soest et al. 2005). Structures (Types) of renieramycins have been modified

and documented. However, the natural structure, but easily degraded, was renieramycin E. The hydroxyl function group causes this compound to be unstable. The protocol to make renieramycin E more stable, is to replace the hydroxyl function group with a cyanide functional group, to become renieramycin M. This protocol allowed the high yield extraction of renieramycin (Suwanborirux et al. 2003). But the ecological roles of these compounds are less well understood. I expect that the ecological understandings of the *Xestospongia* sp., predator of *Xestospongia* and renieramycins are capable to supply renieramycins as anticancer substances for pharmaceutical and medical.

The aims of this study are to determine the distribution and ecological niche of *Xestospongia* related to the concentration of renieramycin at different sites along the Gulf of Thailand. The hypothesis was that distribution patterns of *Xestospongia* and the concentration of renieramycin M in the Gulf of Thailand varied by the reef communities and its coexisting organism(s). In addition renieramycin M may have some ecological roles such as anti-fouling, anti-marine bacterial or allelopathy to other reef organisms. Therefore, the high concentrations of renieramycin M should be allocated in the area of *Xestospongia* connected to organisms. *Xestospongia* may be under the predatory pressure in some habitats. It was expected that *Xestospongia* may respond to predatory pressure by unequally allocate its nutrient contents to the predatory inaccessible areas of its body or produce renieramycins to fight against the feeding activity of its

predator. The response of predator to *Xestospongia* and the behavior of renieramycin M concentration in *Xestospongia* and its predator was also investigated in this study.

Finally, the knowledge from this study would provide the understanding of the ecological roles of renieramycins. With this, *Xestospongia* may be stimulated to produce higher concentrations of renieramycin than that which occurs naturally.

OBJECTIVES

The objectives of this study were to investigate the distribution pattern of the renieramycin-producing sponge *Xestospongia* sp. and its association with other reef organisms in the Gulf of Thailand. Carbon-nutrient contents and allocation of renieramycin M in *Xestospongia* sp., allelopathic effects of renieramycin M, wound effect on renieramycin M concentration in *Xestospongia* sp., predator-prey interaction between *Jorunna funebris* and *Xestospongia* sp., feeding deterrent of renieramycin M on fishes were studied.

CHAPTER III

DISTRIBUTION PATTERN OF THE RENIERAMYCIN-PRODUCING SPONGE *XESTOSPONGIA* SP. AND ITS ASSOCIATION WITH OTHER REEF ORGANISMS IN THE GULF OF THAILAND

INTRODUCTION

The genus *Xestospongia* is distributed worldwide, from the Indo-Pacific to the Caribbean, and is particularly common and diverse in northwestern Australia, the Great Barrier Reef, Papua New Guinea, the Solomon Is., the Palau Archipelago, the West-Central Pacific, the Gulf of Thailand, and the Indo-Malay Peninsula (de Laubenfels 1954, Bergquist 1965, Bergquist and Tizard 1967, Bergquist et al. 1971, Fromont 1991, Amir 1992, Kerr and Borges 1994, Pulitzer-Finali 1996, Kritsanapuntu et al. 2001, Desqueyroux-Faúndez and Valentine. 2002). *Xestospongia* is known to settle and grow on a variety of substrates, such as sand, rock beds, dead coral rubble, and coral heads (Zea 1993, Hooper 1994, Moyer et al. 2003, Bell and Smith 2004, Armstrong et al. 2006). Although *Xestospongia* is found in a range of localities and is associated with a number of different organisms, its morphological heterogeneity has not yet been correlated with microhabitat or geographical factors, although habitat heterogeneity at the micro-scale

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and macro-scales appears to impact variations in the chemical concentrations produced. For example, factors influencing chemical concentrations include the spatial scale, local adaptations, inter- and intra-individual variations, habitat differences, and physical stresses (Chanas and Pawlik 1997, Swearingen and Pawlik 1998, Schupp et al. 1999, Wulff 2005). The chemicals produced by sponges are known to be effective as a feeding deterrent, as being allelopathic to competitors, and as possessing anti-fouling and antimicrobial activities (Frincker and Faulkner 1982, Thacker et al. 1998, Engle and Pawlik 2000). Some of the chemicals produced by sponges are not restricted to a single species. For example, the blue sponge, *Xestospongia* sp., from the Gulf of Thailand, studied herein, produces a similar class of compounds, renieramycins, as does *Reniera* sp. and *Haliclona (Reniera) cribricutis* from the Indian Ocean (Frincker and Faulkner 1982, Parameswaran 1998, Suwanborirux et al. 2003, Amnuaypol et al. 2004, Saito et al. 2004a b). The possibility of either common biosynthetic pathways or associated microorganisms which might be the true producers has been suggested. Renieramycins are a class of bistetrahydroisoquinoline alkaloids which show very potent cytotoxicities against several cancer cell lines. With its promising anticancer properties, our group has attempted to isolate a number of renieramycins in high yields from the Thai blue sponge, *Xestospongia* sp. (Suwanborirux et al. 2003, Amnuoypol et al. 2004).

The objectives of the present study were to investigate the distribution pattern of *Xestospongia* sp. and its association with other organisms, and seek correlations with variations in the amounts of renieramycins produced by individuals among sites in the Gulf of Thailand.

MATERIALS AND METHODS

Study sites

Five reef sites were examined in the Gulf of Thailand, Samui (9°28’05”N, 99°55’52”E), Hin Kob (10°40’33”N, 99°19’54”E), Khlong Wan (11°45’53”N, 99°47’59”E), Chang (12°08’43”N, 102°16’06”E), and Sumpayu (13°11’18”N, 100°47’53”E) (Figure. 3.1).

Sample collection

Surveys were conducted along a 50-m line transect with a 2-m-wide quadrat. Organisms coexisting with *Xestospongia* sp. were recorded and identified to the lowest taxonomic level, and the maximum length and width of individual sponges were measured to estimate sponge coverage. I collected 2-3 g (wet weight) of each sponge individual by hand, and samples were kept in net bags while scuba diving or snorkeling, with a total of 15 replicates taken at each site. To reduce the amount of saltwater, each specimen was cut into 2-cm^2-sized pieces and dried with tissue paper for a minute, twice. The semi-dried specimens were placed in plastic bags in an icebox during transportation to the lab. The specimens were then stored at -20°C until extraction.

Crude extract preparation

Frozen specimens were left in the container for a few minutes until reaching ambient temperature. Each specimen was cut into fine pieces and accurately weighed to the nearest 1500 mg. The sample was macerated with 10 mM potassium cyanide in phosphate buffer solution (6.00 mL, pH 7.0) for 5 h. Then the suspension was extracted with methanol (24.00 mL) for 1 h. After centrifugation at 5000 rpm for 5 min, the supernatant (3.00 mL) was partitioned with ethyl acetate (9.00 mL) and a brine solution (6.00 mL). The ethyl acetate layer (3.00 mL) was evaporated until dry. The dried residue was dissolved in methanol (1.00 mL) containing 300 ng acenapthene as an internal standard. The sample solutions were filtered through 0.45-nm nylon syringe tip filters before the high-performance liquid chromatographic (HPLC) analysis.

Standard calibration solution

Standard renieramycin M derived from Xestospongia sp. in the Gulf of Thailand, were obtained from Dr. Khanit Suwanborirux, Center of Bioactive natural Products from Marine Organisms and Endophytic Fungi (BNPME). A stock standard solution of renieramycin M, at a concentration of 1 mg/mL was prepared in methanol. Calibration solutions containing 2.4, 4.8, 9.0, 180.0, 375.0, 750.0, and 1500.0

ng/mL were prepared by appropriate dilutions of the stock solution with methanol containing 800 ng acetonapthene as an internal standard.

HPLC conditions

A Waters 2690 Controller (Waters, USA) was used with a Shimadzu SPD-10 VP class Absorbance Detector (Shimadzu, Japan) operated at 270 nm. Separation was achieved on a LiChrospher®100RP-18 reversed-phase column (5 µm, spherical, 4.0 x 125 mm (Merck, Germany) with methanol-water (7:3) as the mobile phase at a flow rate of 0.70 mL/min.

RESULTS

I found that *Xestospongia* sp. coexisted with several organisms including algae, bivalves, cnidarians, and other sponges (Figure.3.2). At Samui, the major coexisting organism was algae (with a 71.43% frequency), while at Hin Kob and Chang *Xestospongia* sp. was mainly found coexisting with *Porites lutea* (at 46.78% and 54.84% frequencies, respectively) (Figure. 3.2). *Xestospongia* sp. was most abundant at Sumpayu (86 individuals/100 m^2) and least abundant at Samui (7 individuals/100 m^2) (Figure. 3.3).

Xestospongia sp. at the Sumpayu site had the highest maximum area of cover compared to other sites. The largest individuals were found at Sumpayu (500 cm^2) and Chang (451 cm^2) where they coexisted with *Palythoa caesia* and *Po. lutea* respectively (Figures. 3.3, 3.4). Hin Kob had the lowest maximum cover of *Xestospongia* sp. (96 cm^2) (Figure. 3.3), and overall, the highest average area of cover was at Chang (74 cm^2) (Figure. 3.3). *Xestospongia* sp. coexisting with *Pa. caesia* had the largest range of average area of coverage compared to other coexisting organisms (Figure. 3.4), whereas the smallest range of average cover was found for *Xestospongia* sp. coexisting with the sea anemone, *Heteractis* sp. (Figure. 3.4). There were significant differences in the average percentage of renieramycin M concentrations of *Xestospongia* sp. at different sites (ANOVA, $p < 0.05$) (Figure. 3.5). The highest renieramycin M concentrations were found at Hin Kob and

Chang (Figure. 3.5). However, there was no relationship between renieramycin M concentrations and the latitude of the collection site. There were distinct patterns between the frequency of occurrence of *Xestospongia* sp. associated with other organisms, renieramycin M concentration, and percent area of cover at different sites. The major coexisting organism with the highest frequency of occurrence had the highest average renieramycin M concentration and the highest average area of cover at every site except at Khlong Wan (Tables 3.1, 3.2, Figures. 3.2, 3.4). At Hin Kob and Chang, *Xestospongia* sp. mainly coexisted with *Po. lutea* and had the largest cover (96 and 451 cm^2, respectively), and also the highest renieramycin M concentrations (0.009% and 0.005% w/w, respectively) (Table 3.2). At Samui and Sumpayu there were similar patterns as at Hin Kob and Chang, although the coexisting organisms differed. At Samui, *Xestospongia* sp. coexisting with algae had the highest frequency, highest cover area, and highest renieramycin M concentration, while at Sumpayu, *Xestospongia* sp. coexisting with *Pa. caesia* had the highest frequency, highest cover area, and highest renieramycin M concentration (Table 3.2). Statistical analysis using a 2-factor analysis between sites and coexisting organisms showed that there were significant differences between sites and coexisting organisms, and both had effects on the average cover area and average renieramycin M concentrations (Table 3.3).

DISCUSSION

The abundance of sponges at any particular locality can be influenced by a great number of factors, including the presence of corals and the aggressiveness of each sponge species (Aerts and Van Soest 1997, Ward-Paige et al. 2005). Other biotic and abiotic factors that influence sponge distributions and abundances include coexisting organisms that may either facilitate or be detrimental to the settlement and survival of sponge individuals (Zea, 1993). In this study, *Xestospongia* sp. was found mainly coexisting with *Po. lutea* and *Palythoa* spp. compared to other coral species such as *Lobophyllia hemprichii* and other organisms such as other sponges (*Neopetrosia* sp.), sea anemones (*Heteractis* sp.), and bivalves (*Barbataria belbingia*). Species of *Xestospongia* have been reported in other studies to prefer hard substrates in coral reefs (Asa et al. 2000, Barnes and Bell 2002). In this study, I determined that *Xestospongia* sp. preferred to grow on massive corals (mostly *Po. lutea*), rock beds, algal patches, and dead coral rubble. Coexisting with the coral *Po. lutea*, *Xestospongia* sp. was mostly observed growing over dead areas of the coral, presumably being responsible for killing the living tissues of the coral through smothering or chemical offense, with the sponge having a competitive spatial advantage through its likely faster growth rates than the coral (Aerts and Van Soest 1997, Aerts 1998).

Physical factors such as waves, turbidity, desiccation, and nutrient availability in the water column have been documented as important factors in limiting or promoting the distribution and size of sponges (e.g., de Voogd et al. 1999 and literature cited therein). At Sumpayu, on an isolated rock standing in the ocean and subjected to wave action both from the northeast and southwest monsoons, I recorded the highest abundance of *Xestospongia* sp. compared to other sites. At Hin Kob, directly exposed to the northeast monsoon, 73% of sponge individuals were < 10 cm^2 in area of coverage, while at Khlong Wan, a semi-enclosed area and less susceptible to wave action, only 6% of individuals of < 10 cm^2 in cover were observed. Sponge size can also be influenced by the amount of nutrients in the water column (Ward-Paige et al. 2005). At Sumpayu, where the largest sponge individual (500 cm^2 in cover area) was found, a high ammonium ion concentration (400 µg/L NH_4^+) was detected in the water column (this study, and Pollution Control Department 2005). These nutrient loads are derived from the Bang Pa Kong river mouth, industrial lands, and Leam Chabang deep seaport. At Hin Kob, the largest area of sponge coverage was 96 cm^2 whereas only 200 µg/L NH_4^+ was measured in the water column (this study, and Pollution Control Department 2005). Fresh water discharge is a major input at this site.

Sponge/coral interactions were unique among the coexisting organisms and may be classified into 4 categories: overgrowth, peripheral contact, tissue contact, and non-contact (Aerts and Van

Soest 1997). In this study, I found that interactions between *Xestospongia* sp. and the corals *Po. lutea* and *L. hemprichii* were of the overgrowth type. In the case of *Xestospongia* sp. coexisting with *L. hemprichii*, inter-corallite spaces represent microhabitats for *Xestospongia* sp., and I observed that these corallites were severely affected by overgrowth of the sponge. The overgrown areas were pale, bleached, necrotic, and eroded. Unlike *L. hemprichii*, *Po. lutea* does not have inter-corallite spaces, although ultimately sponge overgrowth had the same effects on the living coral. A combination of interactions among overgrowth, peripheral contact, and tissue contact was observed in the case where *Xestospongia* sp. coexisted with *Palythoa* spp. and another sponge, *Neopetrosia* sp. *Xestospongia* sp. coexisting with *Pa. caesia* was observed originating from the inter-colonial space of the latter. However, neither necrotic scars nor wounds were observed on surface areas of *Pa. caesia* in contact with *Xestospongia* sp. A similar situation was observed with *Xestospongia* sp. coexisting with *Neopetrosia* sp. Moreover, in the intertidal zone of places such as Samui and Hin Kob, I observed no partnership between *Xestospongia* sp. and *Neopetrosia* sp. The volcano-shaped *Xestospongia* spp. were not found in the same habitat as *Xestospongia* sp. either. In addition, *Xestospongia* sp. was frequently observed growing along the margin of *Pa. caesia,* overgrowing and pushing *Palythoa* away, and competing for settlement space. Conversely, no contact interactions were observed

between *Xestospongia* sp. coexisting with algae, hydrozoans, or the anemone *Heteractis* sp.

Published records of variability in chemical defense among sessile marine organisms reveal geographical variations and differences in defense reactions in different habitats (Green 1977, Chanas and Pawlik 1997, Puglisi et al. 2000). Our results found no correlation between the latitude of collection and chemical concentrations, but I did record differences in renieramycin concentrations between the different collection sites (Figure. 3.4), and variations in chemical concentrations appeared to be dependent on partnerships with coexisting species. From this latter result, I suggest that there was a direct correlation between the maximum renieramycin M concentration and the maximum average frequency of organisms coexisting with *Xestospongia* sp. At Samui, HinKob, Chang, and Sumpunyu, the partnerships between *Xestospongia* sp. and the algae, *Po. lutea* and *Pa. caesia*, respectively, showed this pattern (Table 3.2). However, in 2 partnerships (*Xestospongia* sp. with a hydrozoan and *Xestospongia* sp. with *Heteractis* sp.), non-contact interactions were not detectable for any concentration of renieramycin M, while this was not so with tissue contact between *Xestospongia* sp. and *Po. lutea* (Table 3.2). Thus, *Xestospongia* sp. may produce renieramycin M for spatial competition but only via the tissue-contact mode. Although bioassay testing of renieramycins as potential pharmaceuticals has been carried out for decades (Frincker and Faulkner 1982, Suwanborirux et al. 2003,

Amnuoypol et al. 2004, Saito et al. 2004a b), more ecological studies are needed in order to better understand the mechanisms that govern its biosynthesis and the environmental effects on both biosynthetic pathways and its effectiveness in nature as a chemical defensive strategy.

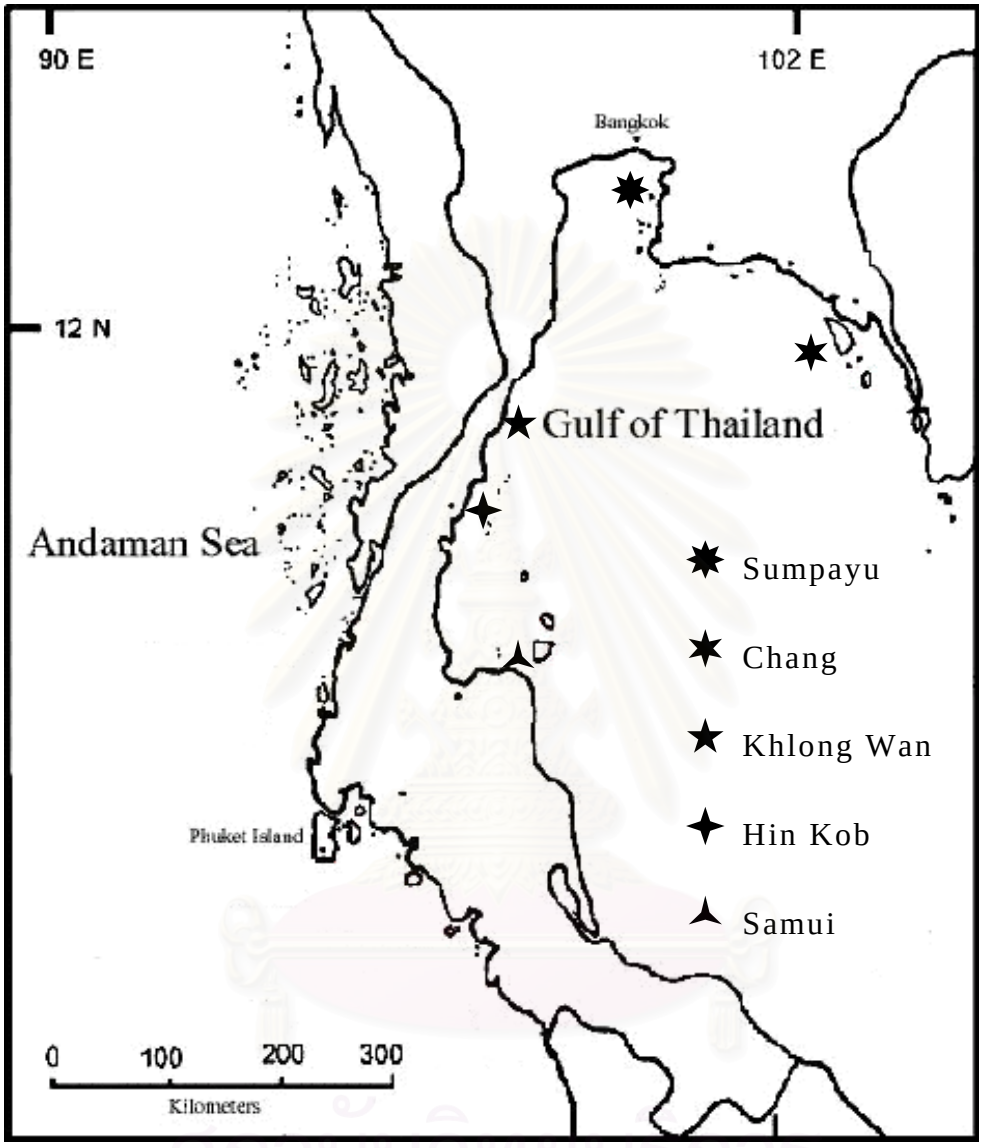


Figure. 3.1. Study sites along the Gulf of Thailand.

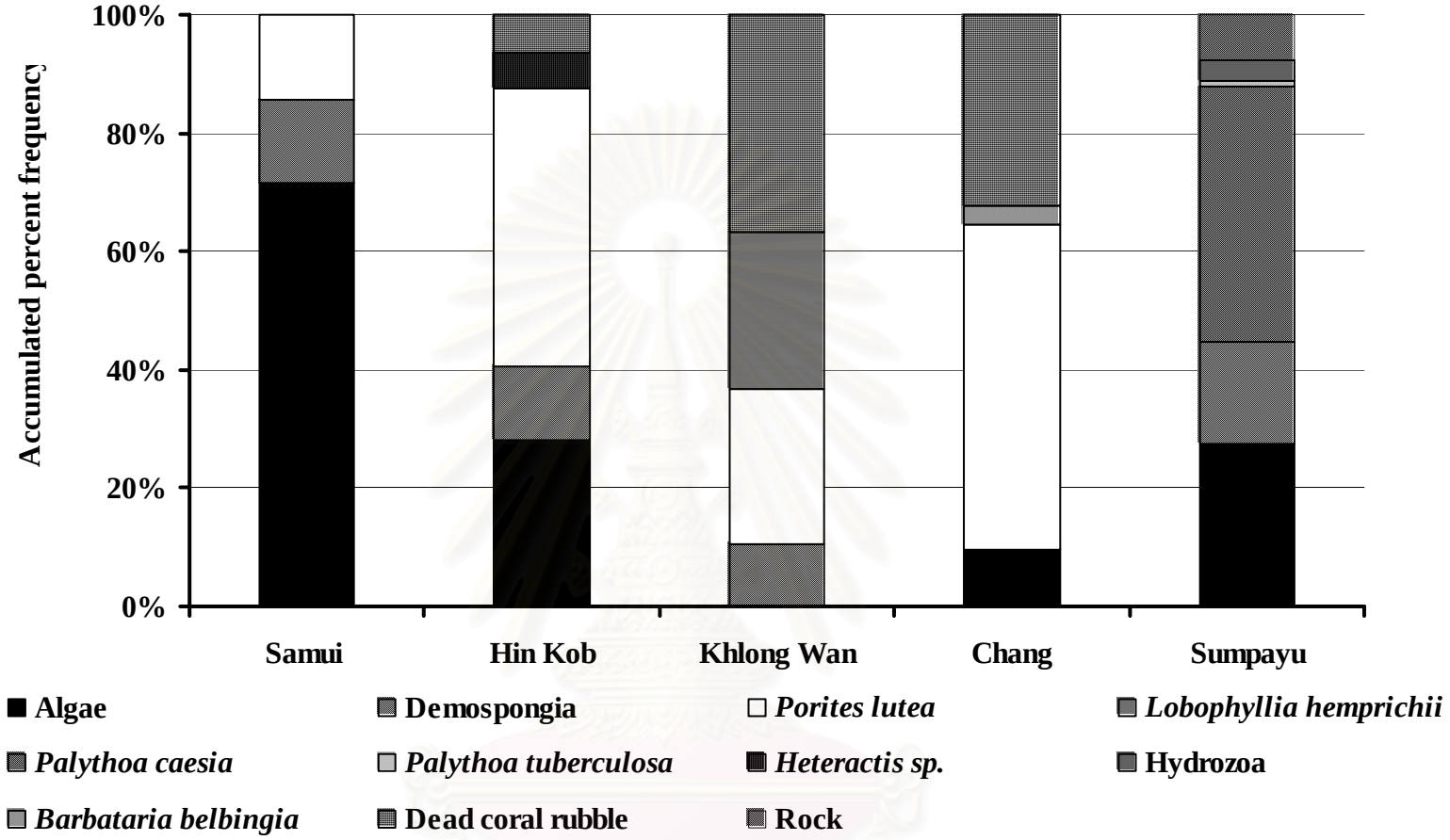


Figure. 3.2. Accumulated percent frequency of organisms and substrata that *Xestospongia* sponge coexisted with or inhabited.

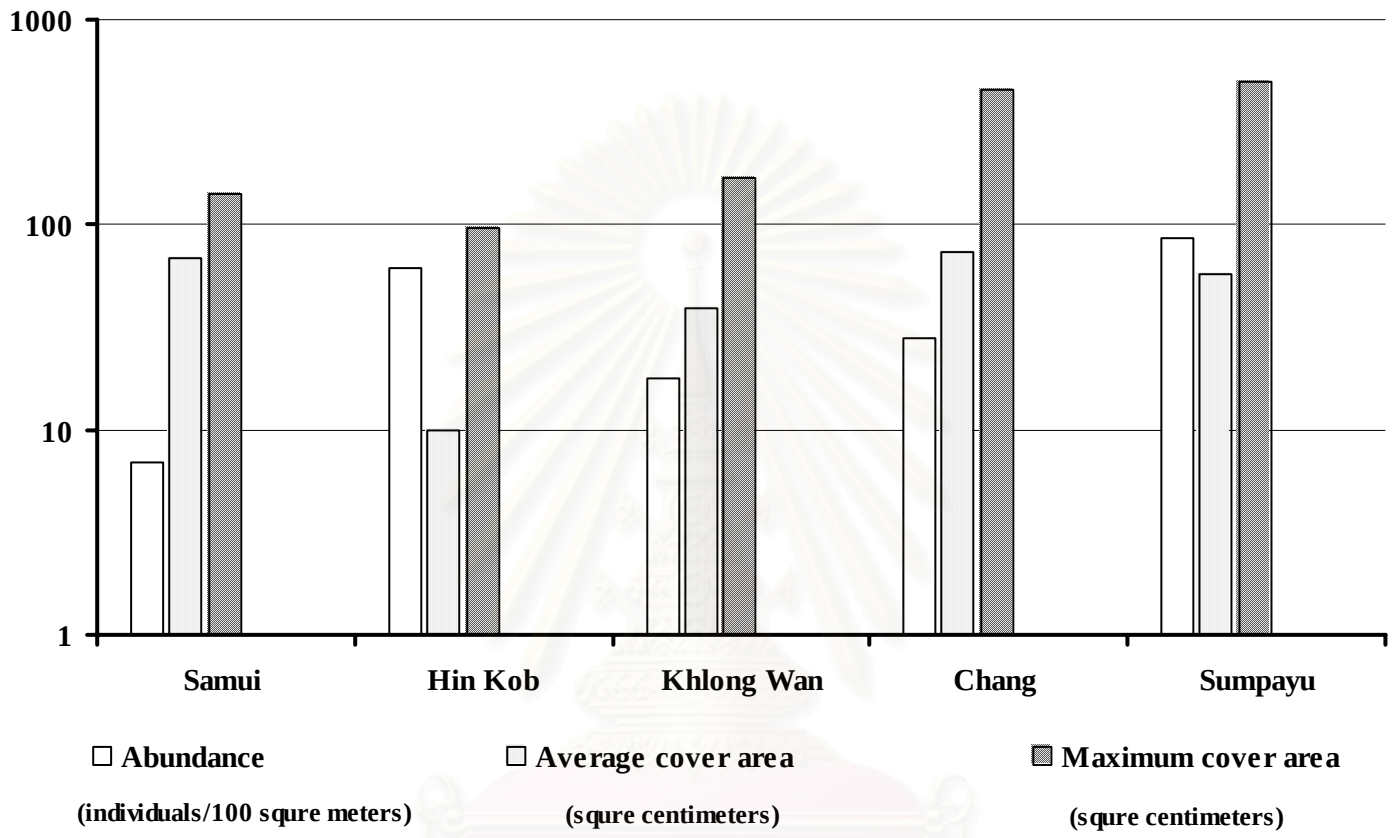


Figure. 3.3. Abundance, average area of coverage, and maximum area of coverage of *Xestospongia* sp. at different sites.

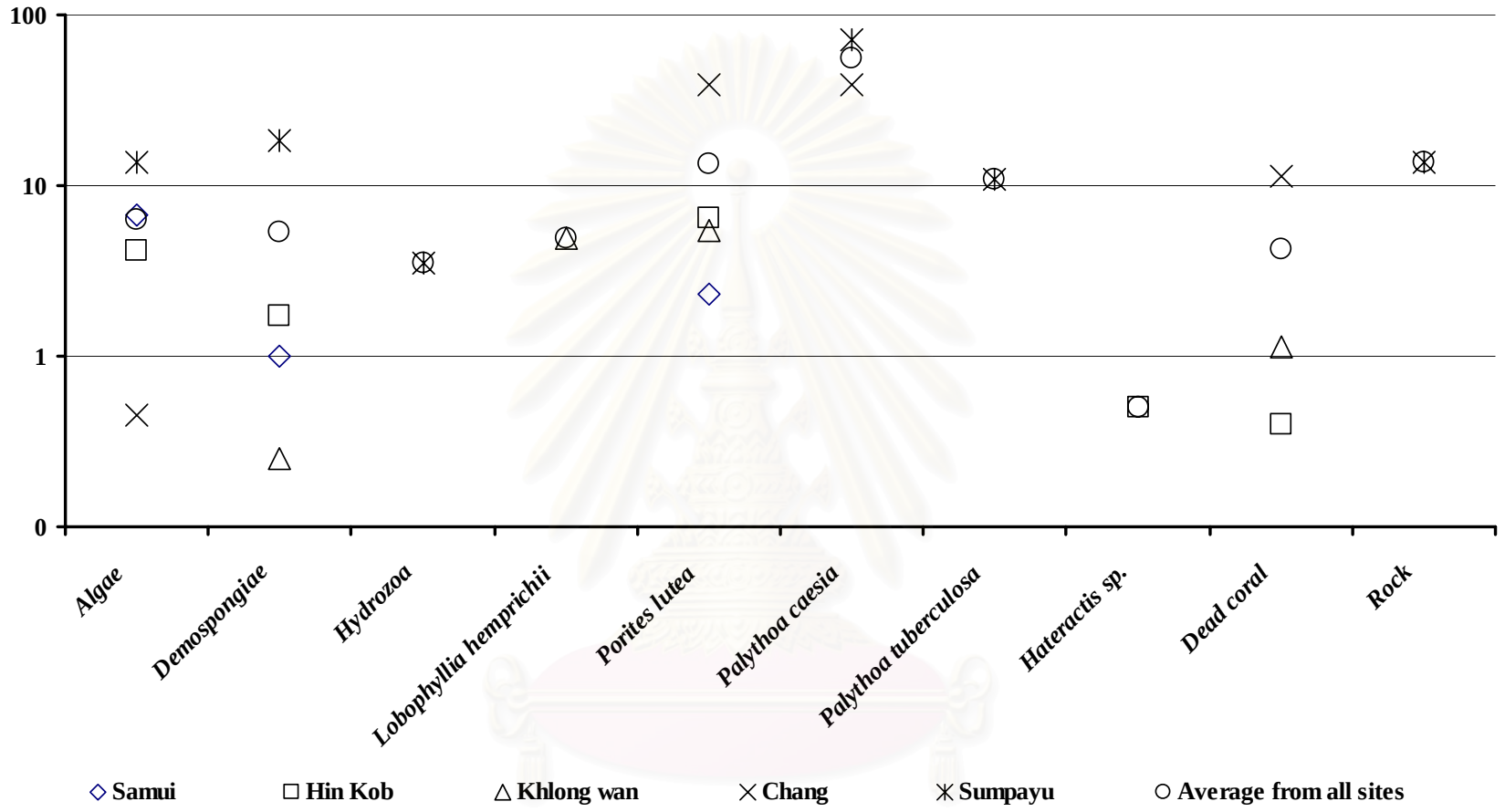


Figure. 3.4. Average cover area of *Xestospongia* sp. coexisting with different organisms and habitats among the different sites.

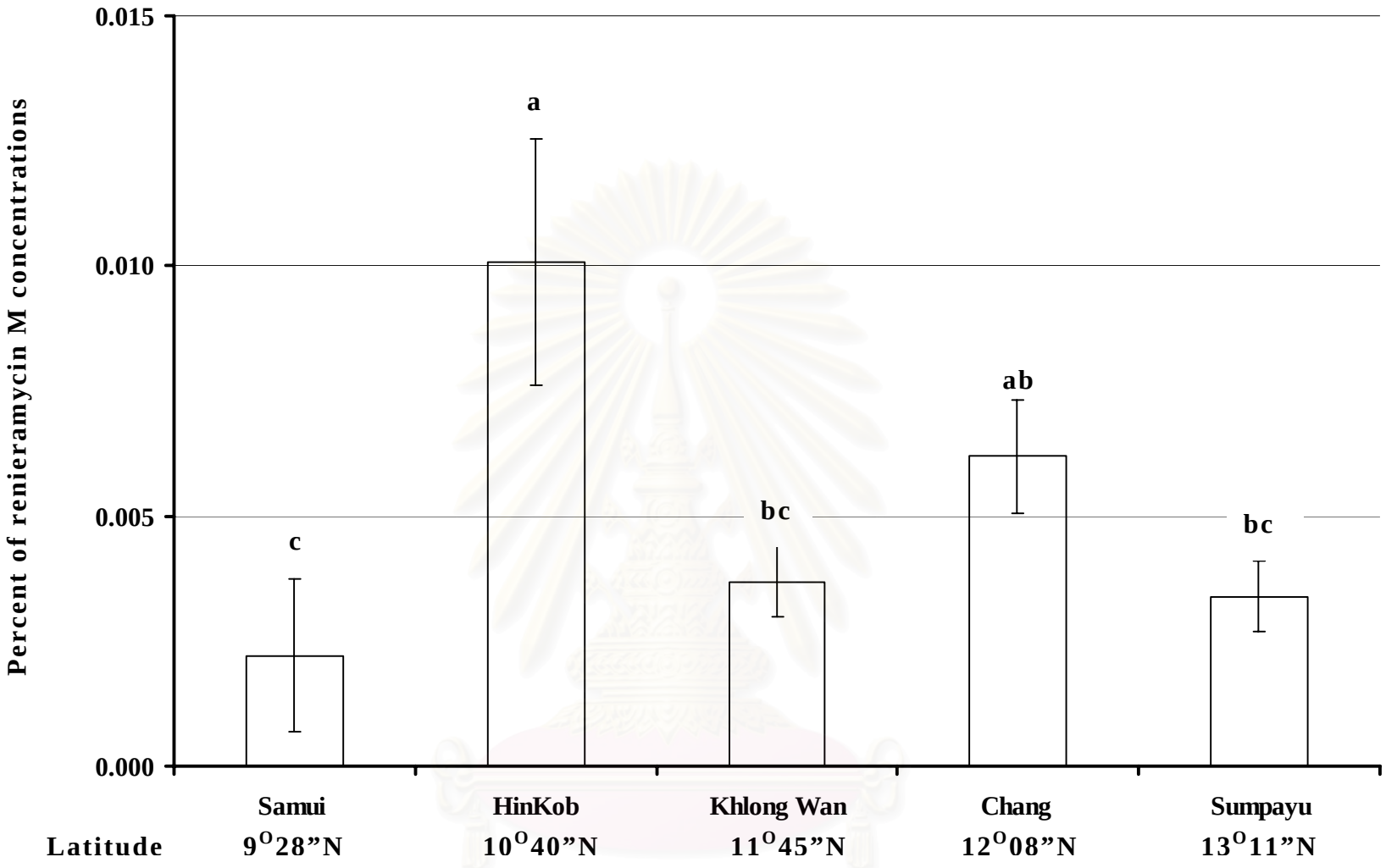


Figure. 3.5. Percent renieramycin M concentrations (mean ± SE) in semi-dried weight of *Xestospongia* sp. The means of groups with the same letter above the columns do not significantly differ (ANOVA and Tukey's test).

Table 3.1. Average percent of renieramycin M concentration of *Xestospongia* among sites with different coexisting organisms and habitats

	Sites				
Coexisting organisms	Samui	Hin Kob	Khlong Wan	Chang	Sumpayu
Algae	0.005	0.004	-	ND	ND
Demospongiae	ND	0.001	0.001	-	ND
Hydrozoan	-	-	-	-	ND
Lobophyllia hemprichii	-	-	0.002	-	-
Porites lutea	ND	0.009	0.001	0.005	-
Palythoa caesia	-	-	-	-	0.003
Palythoa tuberculosa	-	-	-	-	0.001
Heteractis sp.	-	ND	-	-	-
Barbataria belbingia	-	-	-	ND	-
Dead coral rubble	-	ND	0.002	0.003	-
Rock	-	-	-	-	ND

(-, no coexisting organisms occurred at that site; ND, non-detectable concentration of renieramycin M)

Table 3.2. Maximum renieramycin M concentration, maximum percent frequency, and maximum percent area of coverage of coexisting organisms found with *Xestospongia* sp. at different sites

Maximum	Site				
	Samui	Hin Kob	Khlong Wan	Chang	Sumpayu
Renieramycin M concentration	Algae	*Porites lutea*	Algae	*Porites lutea*	*Palythoa caesia*
Average frequency	Algae	*Porites lutea*	*Lobophyllia hemprichii* Dead coral	*Porites lutea*	*Palythoa caesia*
Average cover area	Algae	Algae	*Lobophyllia hemprichii*	*Porites lutea*	*Palythoa caesia*

Table 3.3. *p* values of the 2-factor analysis of coexisting organisms and sites on the average area of coverage and average renieramycin M concentration

Factors	*p-values*	
	Coverage area	Renieramycin M
Site	0.99	<0.001
Coexisting organisms	<0.001	<0.001
Site x Coexisting organisms	<0.001	<0.001

CHAPTER IV

CARBON-NUTRIENT CONTENTS AND ALLOCATION OF RENIERAMYCIN M IN *XESTOSPONGIA*

INTRODUCTION

Predation, competition and pathogen have driven organisms to survive in someway. Some plants, escaping from a predator by allocating the high nutrition value at the predator inaccessible parts (Bryant et al. 1983, Coley et al. 1985, Reichardt et al. 1991). Other defense themselves by an optimal defense by organisms was another reasonable explanation about how organisms respond to the biological interactions (Hamilton et al. 2001). This theory was about the cost and benefit of defense mechanisms in the presence or absence of predators, competitors and pathogens. Karban et al. (1997) elaborated on this hypothesis stating that as “if all plant parts were equally susceptible to loss, parts with the greatest fitness value were expected to be most heavily defended”. This theory has been supported by many studies, which found that organisms produced varieties of defensive chemicals (spatially and temporally) and allocated defensive chemicals differently into body parts. Seedlings of parsnip (Apiaceae: *Pastinaca sativa* L.) produced different species of defensive chemicals with different concentrations between their roots and shoots (Lohman and

McConnauhghay 1998). *Sanguinaria canadensis*, which was pressured by herbivores and pathogens, allocated the defensive alkaloids into the reproductive organ (rhizome) in some cases (Salmore and Hunter 2001). Brown alga (*Dictyota ciliolate*) allocated more lipophilic extracts, an unpalatable chemical, in the older tissues than in the apicals (Cronin and Hay 1996). In marine habitats, the optimal defense theory has been used to explain the individual variation of defensive chemicals in a number of studies. The halogenated sesquiterpenes were allocated in the cells close to the surface and first broken open by herbivores (Hay and Fenical 1992). The level of phlorotannin in the sporophylls was 5-6 times higher than that in the vegetative portion of kelp (*Alaria* sp.) (Hay and Fenical 1988). A study in the Antarctic showed the defensive chemicals were only at the surface of the sponge (*Latrunculia apicalis*) (Furrow et al. 2003). Variations of chemicals in the individual and population scales of brown algae and soft corals were presumably due to the difference in herbivore or competition intensity (Hay and Fenical 1988, Kelman et al. 2000). *Oceanapia* and *Crambe* sponges distributed defensive chemicals into different cell types and body parts (Uriz et al. 1990, Schupp et al. 1999, Salomon et al. 2001). *Xestospongia* was expected to face intensive predatory and competitive pressures because of the habitat in the coral reef community (Chapter 3). *Jorunna* may cause predatory pressure on the surface of *Xestospongia*, whereas *Porites* and *Palythoa* may apply competitive pressure to the edge of *Xestospongia* that contacts with

Porites and *Palythoa*. *Xestospongia* may exhibit lower nutrition values at the surface as it is more at risk to predators than the inner or the edge. The high nutritional value is the high nitrogen content, essential as energy sources and growth. In this case, the low nitrogen content was expected at the surface. Otherwise, they may be higher renieramycin concentrations, which are expected as an allelopathic chemical, at the edge that is in contact with its competitor.

The objectives of this present study were to investigate the nutritional values (carbon, hydrogen and nitrogen) and renieramycin M concentrations in different areas within the individuals of *Xestospongia* coexisting with *Porites* and *Palythoa*.

MATERIALS AND METHODS

Sample collection

Fifteen specimens of *Xestospongia* coexisting with *Porites* (Phylum Cnidaria; Class Anthozoa; Family Poritidae) and fifteen sponge specimens coexisting with *Palythoa* (Phylum Cnidaria; Class Anthozoa; Family Zoanthidae) were collected from Ran Dok Mai Island ($13^{o}09'05.35"N$, $100^{o}50'01.41"E$), by scuba diving. Each sponge specimen was separated into three areas: the outer area (blue color); the inner area (yellowish-grey color), and the edge of the sponge. The definition of each area was the followings: the outer area was about 1 mm thick of the sponge's upper surface which was totally blue in color; the inner area was about 1 mm thick of the sponge's surface attached to the substrate which was totally yellowish-grey; the edge was about 1 mm thick of the sponge's surface attached to the living tissue of the coexisting organisms (herein were *Porites* and *Palythoa*) which was located at the edge of the sponges and mostly were bluish-grey in color (Figure. 4.1). The protocol for saltwater reduction, the specimen collection and preservation were the same as in the previous Chapter 3 (page 16).

Carbon-nutrient contents analysis

The samples were analyzed for carbon-nutrient contents by Dynamic Flash Combustion Technique. The instrument was the CE Instruments Flash 1112 Series EA CHNS-O Analyzer at the Scientific Equipment Center, Prince of Songkla University. The analyzing condition was: furnace temperature: 900 ^{o}C; oven temperature: 65 ^{o}C; carrier flow: 130 mL/min; reference flow: 100 mL/min; oxygen flow: 250 mL/min.

Crude extract preparation

All samples were lyophilized for 12 hours using a freeze dryer. Each sample was accurately weighed at 100.0 mg for extraction. The procedure for crude extraction was the same as that described previously (Chapter 3, page 17).

Standard calibration solution

The procedure was the same as that described previously (Chapter 3, page 17).

HPLC conditions

A Waters 2690 Controller was used with a Waters 996 Photodiode Array (PDA) Detector operating at 270 nm. The separation was performed on a LiChrospher®100RP-18 reversed phase column (5 µm, spherical, 4.0 x 125 mm) with methanol-water (7:3) as the mobile phase at a flow rate of 1.00 mL/min.

The data of carbon, hydrogen and nitrogen contents and renieramycin M concentrations at different areas within the individuals of *Xestospongia* coexisting with *Porites* or *Palythoa* were analyzed using paired samples for mean (t-test) on Systat 7.0 (Systat 1997).

The significant difference in the carbon-nutrient contents and renieramycin M concentrations of *Xestospongia* coexisting with different species were analyzed by single factor analysis of variance (ANOVA) on Systat 7.0 (Systat 1997).

RESULTS

The nutrition value (C/N ratio) at different areas of *Xestospongia* coexisting with *Porites* and of those coexisting with *Palythoa,* were dissimilar (Figure. 4.2). The highest C/N ratio in *Xestospongia* coexisting with *Porites* was found at the edge area, whereas in *Xestospongia* coexisting with *Palythoa*, the highest ratio was found in the inner area (Figure. 4.2). The carbon, hydrogen and nitrogen contents were significantly different among areas of *Xestospongia* coexisting with *Porites*, but there were no differences in *Xestospongia* coexisting with *Palythoa* (Table 4.1). The significant differences in carbon, hydrogen and nitrogen contents at the same areas (edge, inner and outer) of *Xestospongia*, were not found between *Xestospongia* associated with *Porites* and *Palythoa* (Table 4.2). There were no significant differences in renieramycin M concentration of *Xestospongia* coexisting with Porites and Palythoa between areas (Table 4.3). However, the average concentration of renieramycin M at the edge area of *Xestospongia* coexisting with *Porites* was remarkably higher than the average concentration of renieramycin M at the edge area of *Xestospongia* coexisting with *Palythoa* (Figure. 4.3). In addition, 53% of *Xestospongia* individuals that coexisted with *Porites* had their highest renieramycin M concentrations in the edge area, whereas 33% of *Xestospongia* individuals coexisting with *Palythoa* had

their highest renieramycin M concentrations in the edge area (Figure. 4.4).

DISCUSSION

In this study, the nutritional value (carbon, hydrogen and nitrogen contents) was not uniformly allocated in the body of *Xestospongia* coexisting with *Porites*, even though, there were no remarkable differences between carbon, hydrogen or nitrogen contents in same areas of *Xestospongia* when it coexisted with different potential spatial competitors (*Porites* or *Palythoa*) (Tables 4.1 and 4.2). However, the varieties of nutrition, chemical contents, morphological, and physiological changes were commonly found in plants, sponges, and other sessile organisms (Haukioja et al. 1998, Slaringen and Pawlik 1998, Puglisi et al. 2000, Bruce et al. 2003, Meroz-Fine et al. 2005). For example, plant species in boreal forest, balance their carbon/nutrients to survive in the intensely competitive and herbivory pressured habitats (Bryant et al. 1983). In addition, some nutrient-based chemicals, produced by organisms, were varied by nutrient limited habitats (Bryant et al. 1983).

There were not significant distributions of renieramycin M concentrations along the body parts of *Xestospongia* (Table 4.2). On the other hand, other sponge species, *Oceanapia* and *Crambe* sponges have been proven to significantly distribute their toxicity and secondary metabolites within-specimen such as at fistule, capitum, basal or even different cell types (Uriz et al. 1996, Schupp et al. 1999, Salomon et al. 2001). By comparison, renieramycin M concentrations

at the edges of *Xestospongia* coexisting with *Porites* were higher than that of *Xestospongia* coexisting with *Palythoa*. These results might be due to the allocation of chemicals on different spatial competitors of *Xestospongia*. Although there were a number of studies on the effects of allelochemicals and secondary metabolites of marine sponges on potential spatial competitors, particularly corals, the comparison of chemicals produced by sponge(s) when it/they coexist with different potential spatial competitors were less available (see examples as: Porter and Targett 1988; Aerts and Van Soest 1997; Aerts 1998; Nishiyama and Bakus 1999; Engel and Pawlik 2000; Pawlik et al. 2007).

In conclusion, *Xestospongia* does not unequally distribute its nutritional values for predatory defense. The overall contents of carbon, hydrogen and nitrogen in every area of *Xestospongia* coexisting with *Porites* were not different from those of *Xestospongia* coexisting with *Palythoa*. Although the concentrations of renieramycin M, were not differently distributed within the body and among individuals of *Xestospongia*, different proportions of *Xestospongia*, with the highest concentration of renieramycin M at the edge area, coexisting with *Porites* were higher than that proportion of *Xestospongia* coexisting with *Palythoa*. In addition the average concentration of renieramycin M at the edge area of *Xestospongia* coexisting with *Porites* was higher than that of *Xestospongia* coexisting with *Palythoa*. Therefore

Xestospongia may use renieramycin M as anti-feeding mechanism for predatory fishes and as an allelochemical.

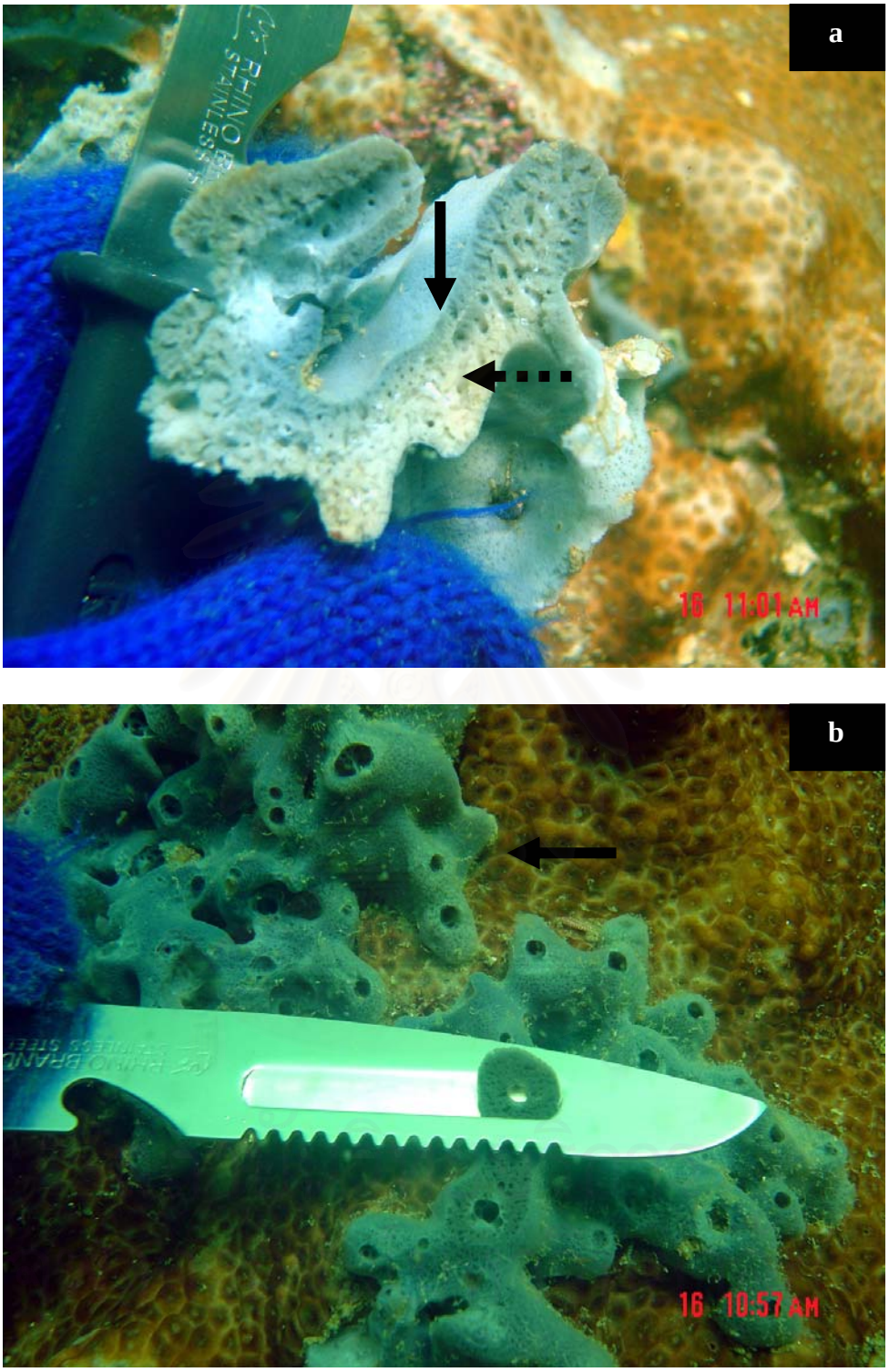


Figure. 4.1. a) The outer area (solid arrow) and the inner area (dot arrow) of *Xestospongia*. b) The edge (solid arrow) of *Xestospongia* and the cutting method.

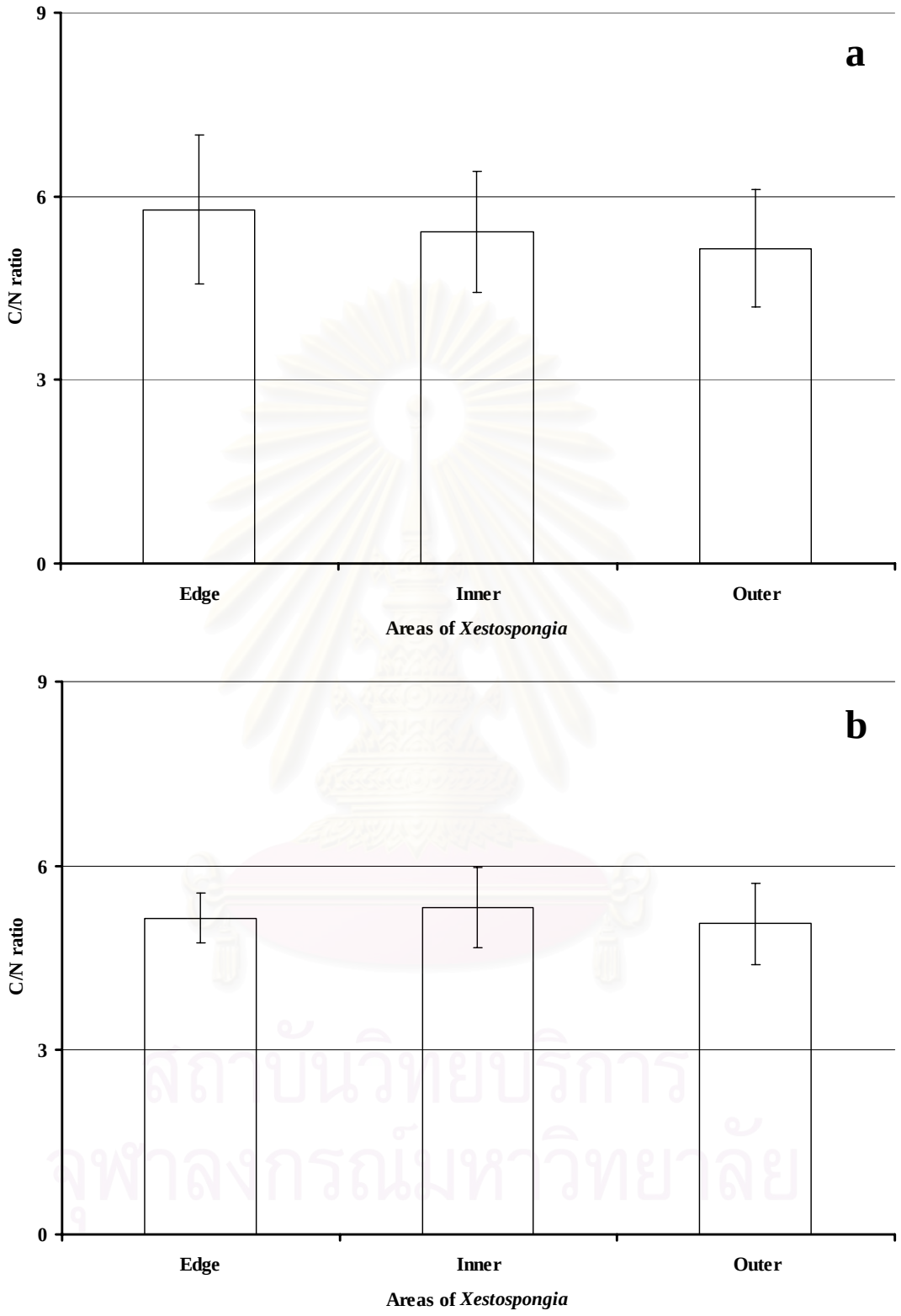


Figure. 4.2. Carbon/nitrogen ratios (mean ± SE) in the edge, inner and outer areas of *Xestospongia* coexisting with (a) *Porites* and (b) *Palythoa*.

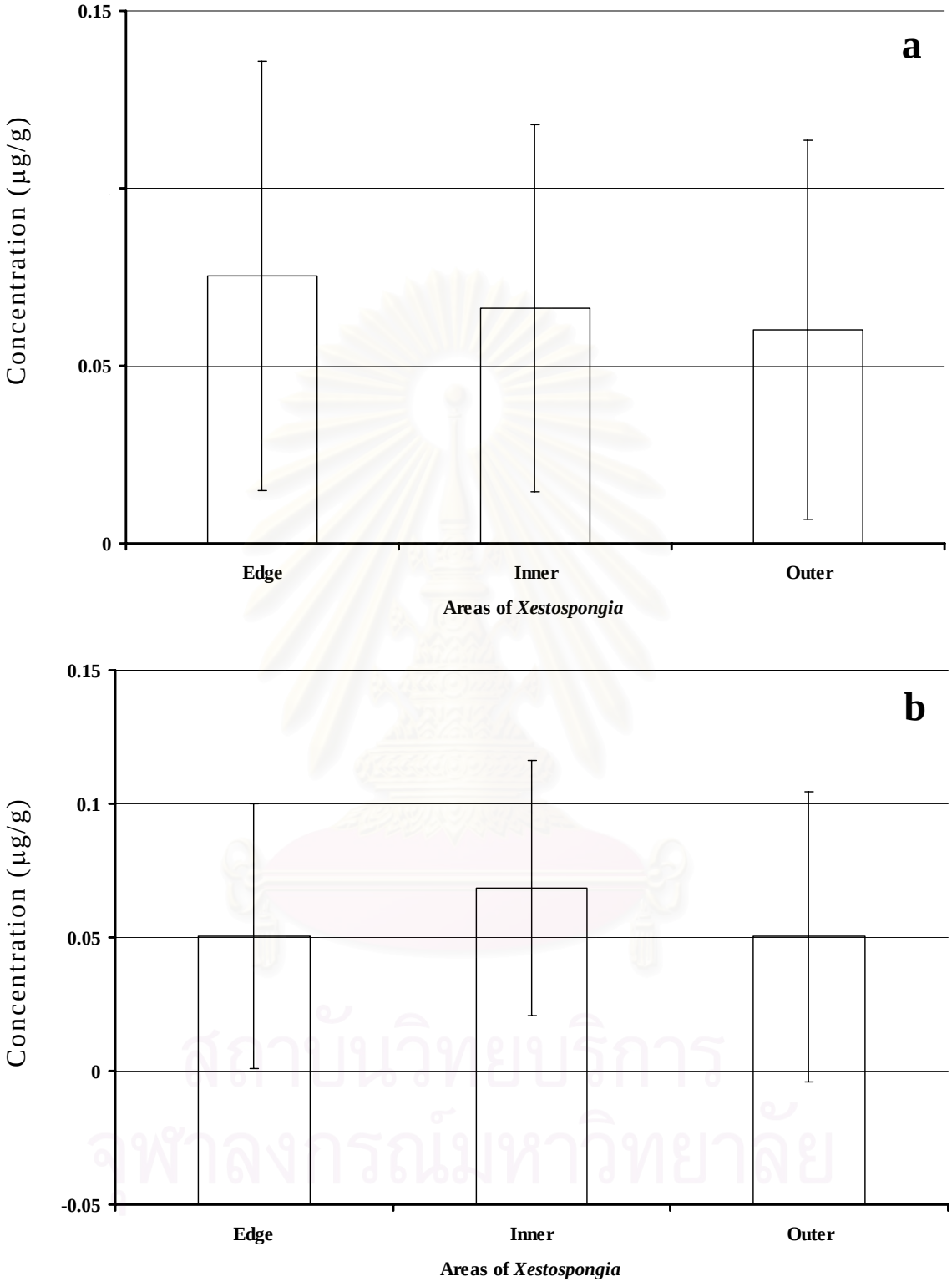


Figure. 4.3. Renieramycin M concentrations (mean ± SE) in the edge, inner and outer areas of *Xestospongia* coexisting with (a) *Porites* and (b) *Palythoa*.

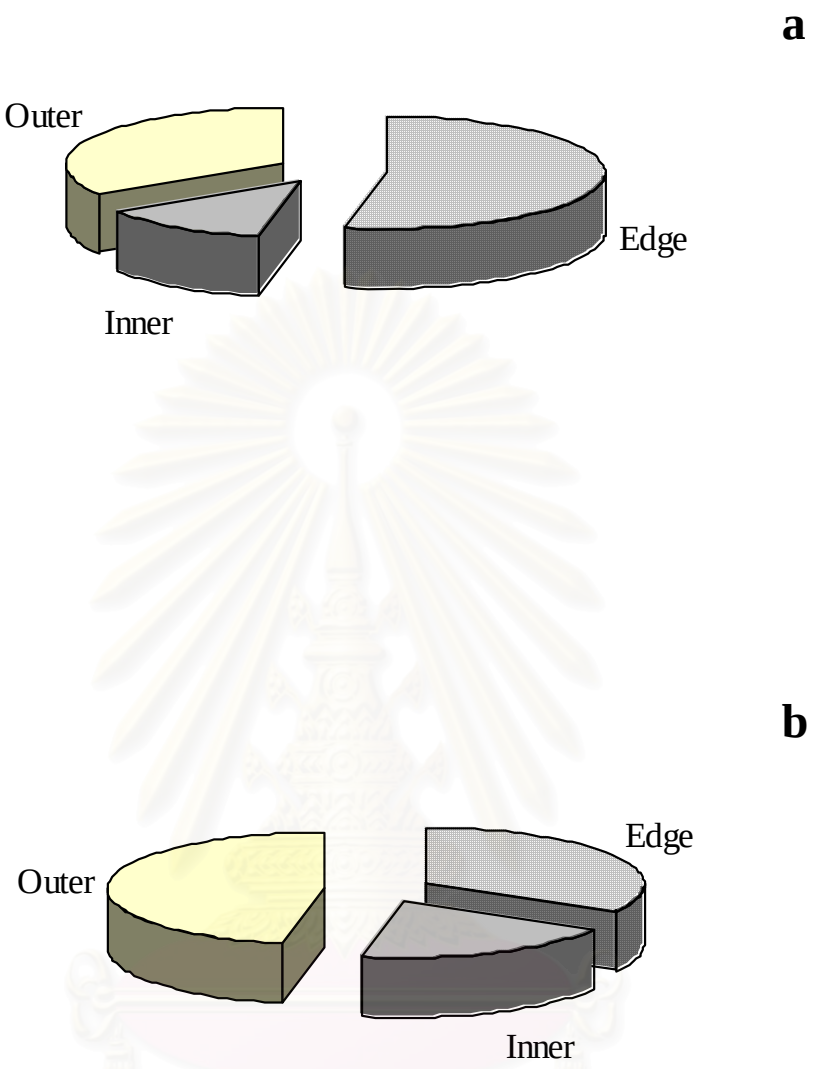


Figure. 4.4. The proportions of *Xestospongia* individuals that contain the highest renieramycin M concentrations at different areas, when it coexists with (a) *Porites*, (b) *Palythoa*.

Table 4.1. The percent (mean ± SE) of carbon, hydrogen, nitrogen contents in different areas of *Xestospongia* coexisting with different organisms.

Coexisting organism	Carbon content	Hydrogen content	Nitrogen content
Porites			
Edge VS Inner	8.64(±1.66)/9.44(±1.99)	1.74(±0.16)/1.94(±0.19)	1.53(±0.36)/1.77(±0.40)*
Edge VS Outer	8.64(±1.66)/9.86(±1.56) *	1.74(±0.16)/2.07(±0.14)**	1.53(±0.36)/1.96(±0.41)**
Inner VS Outer	9.44(±1.99)/ 9.86(±1.56)	1.94(±0.19) /2.07(±0.14)*	1.77(±0.40)/1.96(±0.41)*
Palythoa			
Edge VS Inner	9.88(±0.77)/10.55(±1.04)	1.89(±0.12)/2.02(±0.15)	1.93(±0.25)/2.00(±0.27)
Edge VS Outer	9.88(±0.77)/10.41(±1.17)	1.89(±0.12)/2.07(±0.15)	1.93(±0.25)/2.08(±0.28)
Inner VS Outer	10.55(±1.04) /10.41(±1.17)	2.02(±0.15)/2.07(±0.15)	2.00(±0.27)/2.08(±0.28)

*significantly different ($p \leq 0.05$), **significantly different ($p \leq 0.01$)

Table 4.2. The percents (mean ± SE) of carbon, hydrogen nitrogen contents and renieramycin M concentration in the same areas of *Xestospongia*, coexisting with different organisms (*Porites*/*Palythoa*).

Items	Edge	Inner	Outer
Carbon content	8.64(±1.66) /9.88(±0.77)	8.64(±1.66)/9.88(±0.77)	9.86(±1.56)/10.41(±1.17)
Hydrogen content	1.74(±0.16) /1.89(±0.12)	1.94(±0.19)/2.02(±0.15)	2.07(±0.14)/2.07(±0.15)
Nitrogen content	1.53(±0.36) /1.93(±0.25)	1.77(±0.40)/2.00(±0.27)	1.96(±0.41)/2.08(±0.28)
Renieramycin M concentration	0.075(±0.060) /0.050(±0.049)	0.066(±0.051) /0.068(±0.047)	0.060(±0.053)/0.050(±0.054)

Table 4.3. The percent (mean ± SE) of renieramycin M within individuals of *Xestospongia* coexisting with different organisms.

Coexisting organisms	
Porites	
Edge VS Inner	0.075(±0.060)/0.066(±0.051)
Edge VS Outer	0.075(±0.060)/0.060(±0.053)
Inner VS Outer	0.066(±0.051)/0.060(±0.053)
Palythoa	
Edge VS Inner	0.050(±0.049)/0.068(±0.047)
Edge VS Outer	0.050(±0.049)/0.050(±0.054)
Inner VS Outer	0.068(±0.047)/0.050(±0.054)

CHAPTER V

ALLELOPATHIC EFFECTS OF RENIERAMYCIN M

INTRODUCTION

Allelopathy is the beneficial or harmful effect of one organism on another organism, by the release of chemicals from body part(s) by leaching, root exudation (in plants), volatilization, residue decomposition and other processes (Fueguson and Rathinasabapathi 2003). Allelochemicals have varieties of action sites. Some influence the cell division, whereas some affect nutrient uptake, inhibit photosynthesis and disrupt the function of some specific enzymes (Fueguson and Rathinasabapathi 2003). The allelochemicals could be single chemicals or a mixture of chemicals, usually with greater allelopathic effects (Fueguson and Rathinasabapathi 2003). The effect of allelopathy might more strongly depend on the environmental or physiological stresses. The allelochemical sometimes has multifunctional roles and unequally distributes to different parts of organisms. Some species of isothiocyanates distributed in higher concentrations at the shoot of the plant than other parts (Peterson et al. 2001). Moreover, this compound was the suppressant of seed germination (Caamal-Maldonado et al. 2001). Allelochemicals might enhance some

enzyme activity to somehow reduce the fitness of organisms (Yang et al. 2004). The phenolic compounds increased degradation of chlorophyll in rice. The success of invasive plants by producing harmful chemicals that affect the fitness of native species such as: reduced root biomass, reduced day to emergence of native species, reduced leaf biomass (Orr et al. 2005). In some terrestrial habitats, the environmental conditions (biotic and abiotic) played a significant effect on allelopathy of organisms (Inderfit 2001, Mattner and Parbery 2001). Allelopathy was also common in marine habitats. Some cyanobacteria produced chemicals to inhibit the growth of algae (Suikkanen et al. 2004). The same multifunctional roles of allelopathic chemicals were observed in marine habitats as well (Kubanek et al. 2002). For example, hexanic extracted from soft coral, *Stereonephthya* aff. *curvata* caused serious necrosis on gorgonians and deterred fish feeding (Lages et al. 2006). However, the indirect allelopathy occurred in terrestrial and marine habitats with different strategies. In the terrestrial environment, some plants left allelopathic chemicals in the soil that harmed the new recruits, while in marine habitats the adjacent competitors were affected by waterborne allelochemicals that caused a necrotic affect of the settlement on fouling organisms (Jackson and Buss 1975, Sammarco et al. 1983, Fueguson and Rathinasabapathi 2003, Maida et al. 2006).

The allelochemicals that prevented space from settlement of either intra or interspecific larva were known as antifouling agents. These inhibited larval settlement (pre-settlement allelopathic) or killed the new settlers (post settlement allelopathic) (Sears et al. 1990, Hirota et al. 1998). However, more complex antifouling activity was reported. Some chemicals prevent settlement of specific species, while some species benefit from settlement of others on them, producing the chemicals promotes settlement of specific species (Henrikson and Pawlik 1995). According to the field surveys of Chapter 3, every *Porites* coexisting with *Xestospongia* showed necrotic scars in the area where the two were in contact. Moreover, an average renieramycin M in *Xestospongia* coexisting with *Porites* was highest at the edge area (Chapter 4). It was possible that the necrotic scars, at the area in contact with *Xestospongia*, of *Porites* were caused by renieramycin. In addition, no fouling organisms were observed on the surface of *Xestospongia*. Tissue degradation in *Xestospongia* (expected disease infection) was very rare from my surveys. Is renieramycin the multifunctional allelochemical in *Xestospongia*? The objectives of this study were to investigate whether renieramycin M has a necrotic effect on the *Xestospongia*'s coexisting species, *Porites*. Can renieramycin M inhibit settlement of fouling organisms, and control aerobic bacteria from seawater?

MATERIALS AND METHODS

Bleaching and necrotic effects of renieramycin M on *Porites*

A colony of live massive coral, *Porites*, was collected from the intertidal zone of Sichon (9°00’16”N, 99°55’20”E). It was then broken into small pieces. There were about 10-15 corallites in each piece (nubbins). A nubbin was attached on top of a plastic rod, using a very small amount of ethyl-2-cyanoacrylate glue and then left in the air for several seconds for the hardening of the glue. All coral nubbins were vertically set onto the plastic net (approximate mesh size 5 mm). The plastic net (culture frame) with coral nubbins was hung in the ocean for two months. The survival rate was monitored weekly.

Thirty coral nubbins were collected from the culture frame and then were acclimatized in the lab for a week prior to the experiment. There were five treatments of different renieramycin M concentrations, 0.00 ppm (control), 0.02 ppm, 0.20 ppm (natural concentration), 1.00 ppm and 10.00 ppm. A 100.00 µL of dimethyl sulfoxide (DMSO), that was preliminarily tested and showed no effects on *Porites*, was used as a solvent for each treatment, including the control. The total volume was made to 50.0 mL by 30 ppt of seawater. For each treatment, 5 coral nubbins were submerged in the solution with air bubbles for 8 hours. After that,

coral nubbins from each treatment were separately placed into transparent containers, each of them containing 500 mL seawater. To control the growth of algae during the experiment, every container was left under approximately 40% of natural light. Bleaching and narcosis were daily observed for two weeks.

Antifouling effect of renieramycin M on larval settlement

This method was modified from Maida et al. 2006. Several dead coral rocks of *Porites* sp. with approximately 20 x 30 x 20 cm^3 (W x L x H) were collected from an intertidal zone of Sichon ($9^o00'16"N$, $99^o55'20"E$). They were then, cut into approximately 4 x 4 x 1 cm^3 (W x L x H) cubes. Each coral cube was de-salinated by submerging it in distilled water several times. After that, the coral cubes were dried in an oven at 50 ^oC for two days. In all of the cubes, a hole (6.35 mm in diameter) was made at the center by a drilling machine.

Standard renieramycin M, derived from *Xestospongia* sp. in the Gulf of Thailand, were obtained from Dr. Khanit Suwanborirux, Center of Bioactive Natural Products from Marine Organisms and Endophytic Fungi (BNPME), Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Five experiments were prepared with 0.00 ppm (control), 0.05 ppm (natural concentration), 0.10 ppm, 0.20 ppm and 2.00 ppm

renieramycin M in 500.00 mL methanol. Three cubes were randomly assigned for each experimental unit. The cubes were submerged into the methanolic solutions for 24 hrs. Then they were hung and dried at room temperature (28 ^{O}C). The experiment contained four experimental sets. Each set consisted of a control unit and four treatment units with different renieramycin M concentrations as described above (Figure. 5.1). There were three replicates of each unit. All experimental sets were then, hung in the sea (approximately 1 meter below the low tide level), at the same coral reef habitat, where the dead coral rocks were collected. The first experimental set was collected from the sea, 5 days after the experiment started and the second, third and fourth experimental sets on day 10, day 15, and day 20 respectively. Only the upper and lower surfaces of every cube were investigated for fouling organisms under a binocular stereo scope. The number of sessile organisms were counted and grouped into class taxa level. Species diversity index (H') was used to determine the fouling effect of renieramycin M as well.

Antimicrobial effect of renieramycin M on aerobic bacteria

Fresh sea water was collected from a coral reef habitat from Sichon (9^{o}00'16"N, 99^{o}55'20"E). It was aerated 24 hours prior to the experiment. 1.00 mL of this water was pipeted using a

micropipet and was poured onto an aerobic count plate (petrifilm sheet) for each treatment. A petrifilm sheet was unmoved for a couple of minutes to let the gel solidify. Paper disks (5.0-mm diameter) have either 200.0, 100.0, 10.0, or 1.0 µg of renieramycin M. As a control, paper disks bearing 0.0 µg of renieramycin M were prepared (Figure. 5.2). The petrifilm sheets were placed, and were incubated at 30^{o}C for 24 hours. After 24 hours, the diameter of a zone of inhibition in each paper disk was recorded. The numbers of bacterial colonies growing on the grid areas containing the paper disks were also counted. The numbers of bacterial colonies on paper disks (3 disks for control and 3 disks for treatment) of each treatment petrifilm sheet were statistically compared by using t-test on Systat 7.0 (Systat, 1997).

RESULTS

Bleaching and necrosis effects of renieramycin M on *Porites*

Bleaching and necrosis of coral nubbins were not observed during two weeks in every treatment. Coral nubbins were still brown in color in both control and treatments, and sometimes covered by a mucous sheet. During the night time or low light intensity, corallites of some coral nubbins protruded, waved their trunks and their tentacles into the water column.

Antifouling effect of renieramycin M on larval settlement

Renieramycin M was found to inhibit the settlement of some sessile organism. However, the duration of the antifouling effect was just five days (Figure. 5.3). There were three groups of sessile organisms found on the control plates; pelecypoda, polychaeta and barnacle (*Semibalanus balanoides*), whereas only pelecypoda was found on the treatment plates during the five-day period. After ten days, pelecypods were the major group of sessile organisms on control and treatment plates. Polychaeta was found settled on the treatment plates of 0.05 ppm to 2.0 ppm concentrations (Figure. 5.4). After fifteen days period, *S. balanoides* was found again on the settlement plates (control, 0.05 ppm, 0.1 ppm and 2.0 ppm),

whereas at the five-day period, *S. balanoides* was found only on the control plates (Figure. 5.5). The composition of sessile organisms on every treatment was the same as the composition of sessile organisms on the control after the twenty days (Figure. 5.6). The diversity index also supported the effect on antifouling of renieramycin M (Figure. 5.7). After five days, the highest diversity index was for the control plates, whereas other treatment plates had a zero diversity index. After longer periods (day 15 and 20), the diversity index values were high in all treatments.

Antimicrobial effect of renieramycin M on aerobic bacteria

There was no significant difference in the number of aerobic bacterial colonies, in all contents of renieramycin M (Figure. 5.8). The average number of bacterial colonies on the control paper disks ranged from 30 to 62 colonies per 50 µL, whereas the average number of colonies on treatment paper disks ranged from 45 to 61 colonies per 50 µL (Figure. 5.8). The zone of colonial growth inhibition was not observed on any control or any treatment paper disks.

DISCUSSION

Bleaching and necrosis effects of renieramycin M on *Porites*

The effects of allelochemicals of sponges on corals and on other sponge species were necrotic effects, bleaching and the effect on the photosynthetic ability of coral's symbionts (Potter and Targett. 1988, Engle and Pawlik. 2000, Pawlik et al. 2007). However, renieramycin M exhibited neither bleaching nor necrosis effects on *Porites*.

During my surveys, the necrotic scars or the dead zones of *Porites* were observed only on the tissue that contacted *Xestospongia* (see Chapter 3). Therefore, the dead zones or necrotic areas might be caused by the synergy of physical damage (by spicules) and other allelochemicals. In addition, sessile organisms were known to compete for space. The strategies to compete with their competitors were overgrowth, physical damage, allelochemicals and waterborne substances (Paul 1992, Porter and Targett 1988, Nishiyama and Bakus 1999, Aerts 1998). According to the field surveys of this study (see Chapters 3 and 4), the interactions between *Xestospongia* and the massive coral, *Porites*, were of the single category, sponge overgrowing coral. The sponge/coral interactions were classified into four categories: overgrowth, peripheral contact, tissue contact and non-contact

(Aerts and Van Soest 1997). Their study showed that 46.6% of *Xestospongia* spp. (*X. caminata, X. muta, X. proxima*) were tissue contact to corals and 32.6% were non-contact to corals, while 2.5 % of *Xestospongia* overgrew corals. On the other hand, just 2.2, 14.3 and 33.8 % of massive coral, *Po. astreoides*, were overgrown, peripheral contact and tissue contact by sponges respectively. In addition the major proportion of the interaction (49.7%) of this coral was non-contact to sponges (Aerts and Van Soest 1997). Although, the effect of overgrowth by sponges on corals was not directly documented, one study showed that the overgrowth of sponge on introduced mussels in the Great Lakes (USA) caused mortality or at least loss in the mussels (Ricciardi et al. 1995). Different categories were observed from this study when *Xestospongia* sponge coexisted with *Palythoa*. The interaction was mainly tissue contact between *Xestospongia* and *Palythoa* (43.24 % at Sumpayu, herein Chapter 3).

Antifouling effect of renieramycin M on larval settlement

Renieramycin M had no antifouling effect on pelecypod but on polychaetes and barnacles (*S. balanoides*) during the first five days of the experiments. The antifouling chemicals produced by pre-settlement organisms were not universally against new comers (Pual 1992, Nishiyama and Bakus 1999, Potter and Targett 1988).

Antifouling was the active competitive defense of sessile organisms. This strategy prevented interspecific larva from settling on surfaces of the previous one (Jackson and Buss 1975, Nichitama and Bakus 1999, Kubanek et al. 2002). The antifouling process operates through mechanical and chemical mechanisms. Some soft corals were swept out and were damaged by sweeper tentacles and nematocysts of hard corals (Paul 1992). Some sessile organisms used at least two mechanisms to inhibit interspecific larval settlement (Bak and Borsboom 1984, Coll et al. 1987, Sears et al. 1990, Henrison and Pawlik 1995, Hirota et al. 1998, Nishiyama and Bakus 1999). First was a waterborne substance production, which was released into the water column to affect the competitor's fitness. Second was the allelochemical, which affected the competitors when the tissues were in contact.

Antimicrobial effect of renieramycin M on aerobic bacteria

Renieramycins were tested to inhibit *Staphylococcus aureus*, *Bacillus subtilis* and *Vibrio angularium* but not *Escherichia coli*, *Candida albicans*, *Pseudomonas aeriginosa* and *Enterobacter aerogenes* (Frincke and Faulkner 1982, Kelman et al. 2001). In this study, renieramycin M did not inhibit the growth of aerobic bacteria in any treatment. However, antibiotic chemicals produced by sessile organisms or the symbionts were effective against

specific bacteria rather than a broad spectrum (Frincke and Faulkner 1982, Kelman et al. 2001). A number of studies elaborated more about the interaction between bacteria versus sponge and bacteria versus macro-algae such as anti non-symbiotic species and antifouling (Imhoff and Trüper 1976, Wilkinson 1978, Lemos et al. 1985, Althoff et al. 1998, Armstrong et al. 2001, Ibster and Hill 2001, Thacker and Starnes 2003). Sponges of the genus *Dysidea* were a specific host of cyanobacterium (Thacker and Starnes 2003). Sometimes the chemicals produced by microsymbionts were beneficial to the host (Lemos et al. 1985, Ibster and Hill 2001). Epiphytic bacteria attached to seaweed produced anti-microbial chemicals for the host (Lemos et al. 1985). α-Proteobacteria, surrounding the choanocyte chamber of *Rhopaloeides* sponge, was expected to play the role of nutrient uptake in the host sponge (Webster and Hill 2001). In some cases, microorganisms may be harmful to sessile organisms. This can be inferred by the varieties of antibiotic substances produced by sessile organisms or their symbionts (Frincke and Faulkner 1982, Lemos et al. 1985, Kelman et al. 2001, Kubanek et al. 2002, Müller et al. 2004).

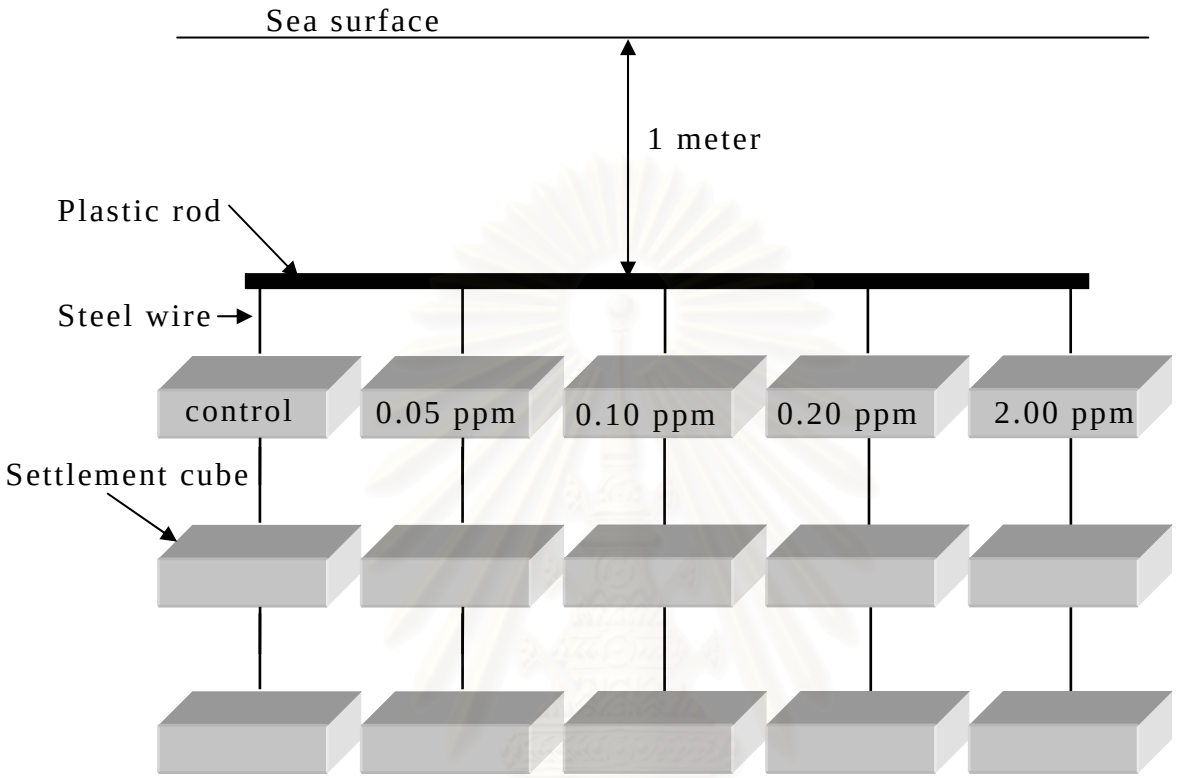


Figure. 5.1. Diagram of the experimental setup of an anti-fouling experiment.

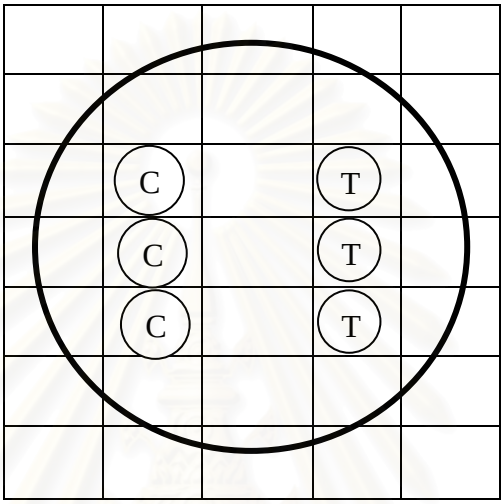


Figure. 5.2. Diagram of the paper disk technique on an aerobic count plate. C stands for the control baring only 3 µL of DMSO. T stands for the treatment bearing renieramycin M either 200.0, 100.0, 10.0, or 1.0 µg with 3 µL of DMSO.

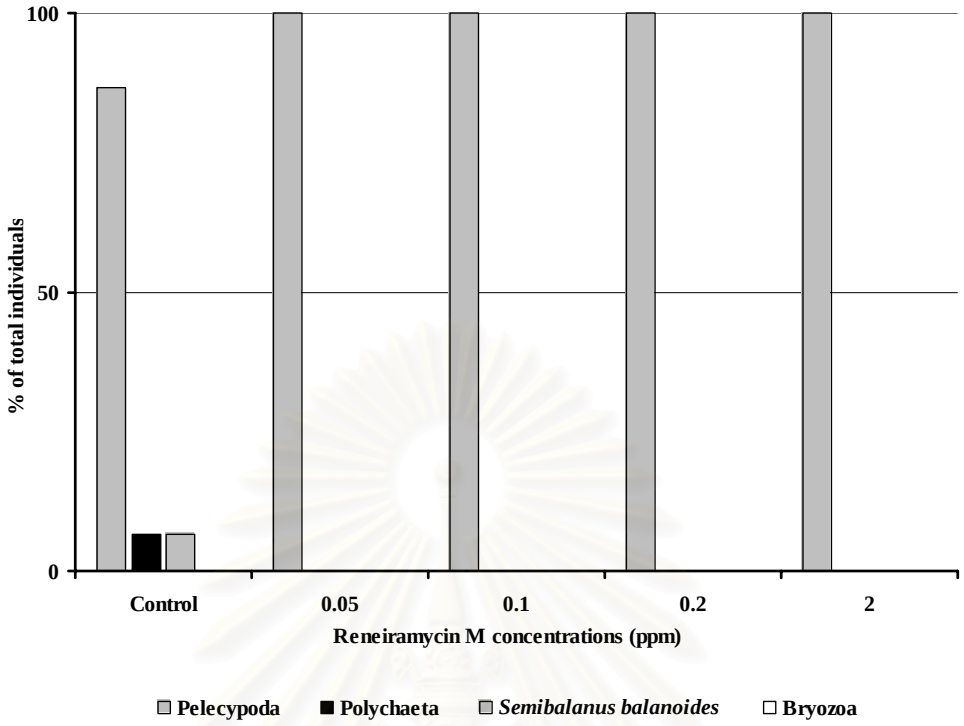


Figure. 5.3. The percentage of total individuals of sessile organisms on different experimental plates after five days.

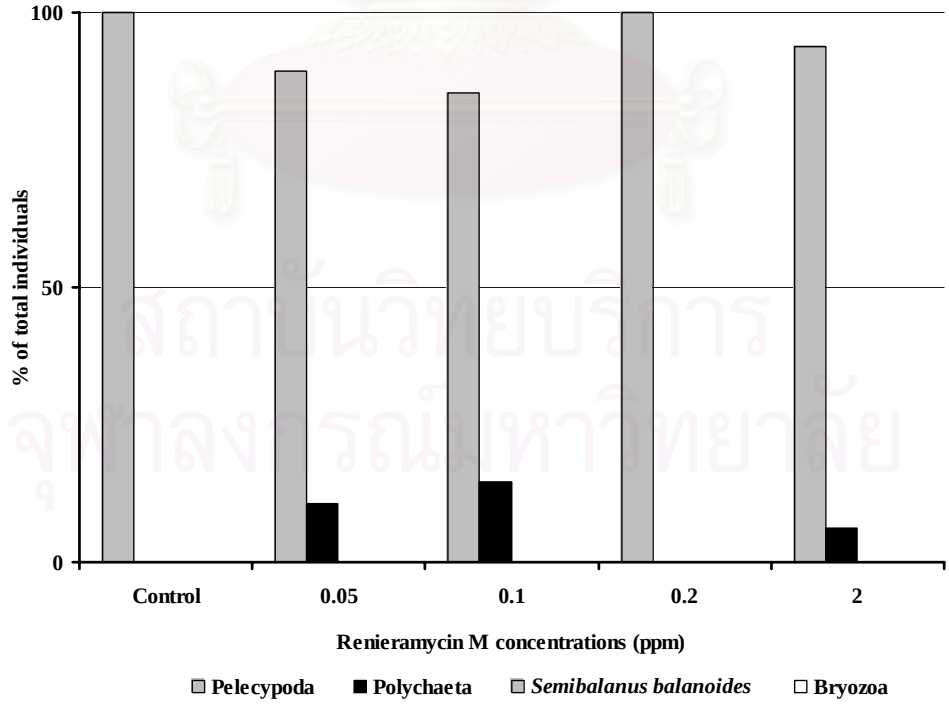


Figure. 5.4. The percentage of total individuals of sessile organisms on different experimental plates after ten days.

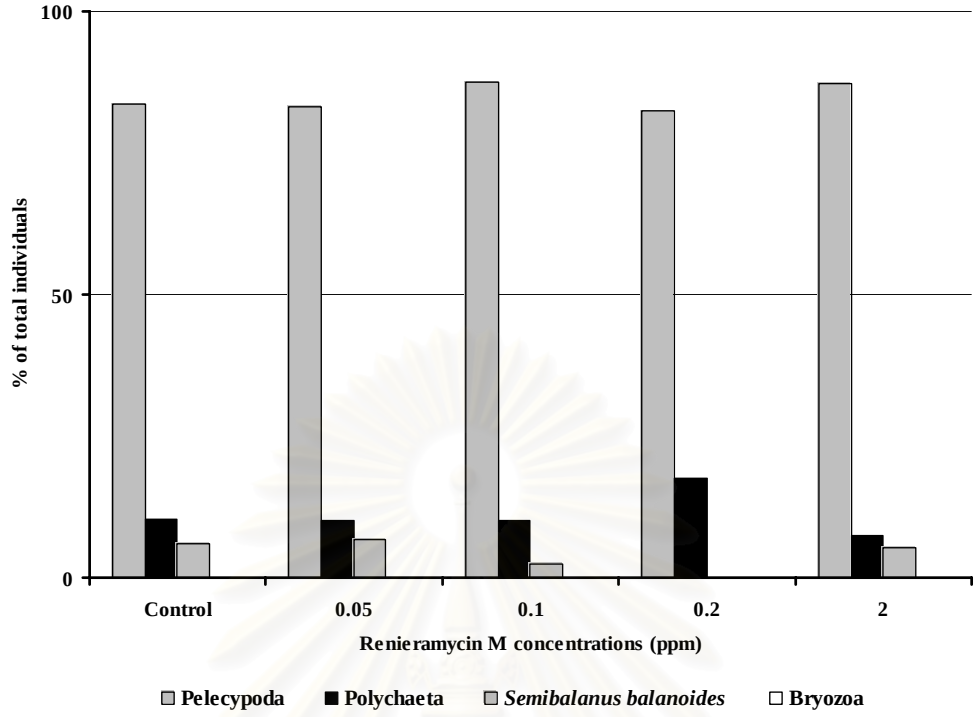


Figure. 5.5. The percentage of total individuals of sessile organisms on different experimental plates after fifteen days.

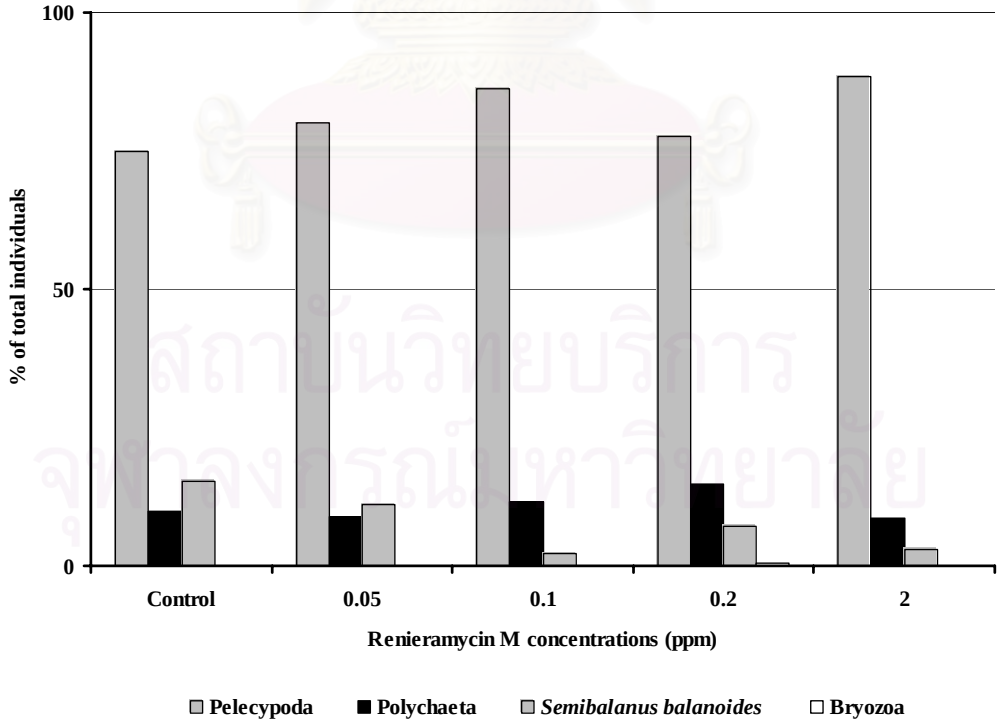


Figure. 5.6. The percentage of total individuals of sessile organisms on different experimental plates after twenty days.

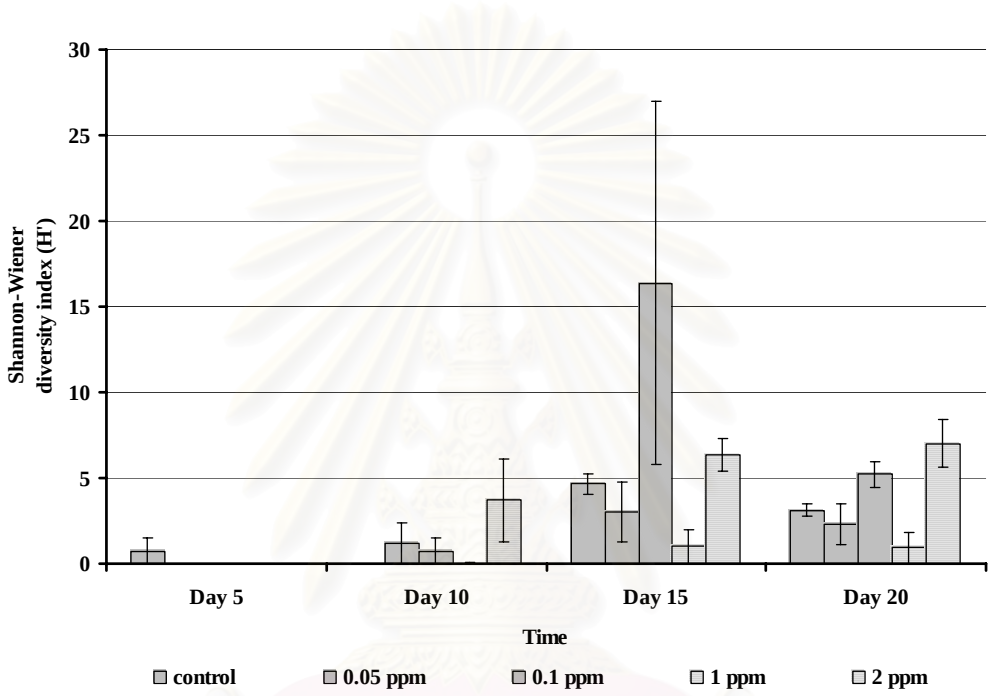


Figure. 5.7. Shannon-Wiener diversity index (H') (mean ± SE) on different experimental plates at different concentrations of renieramycin M and different periods.

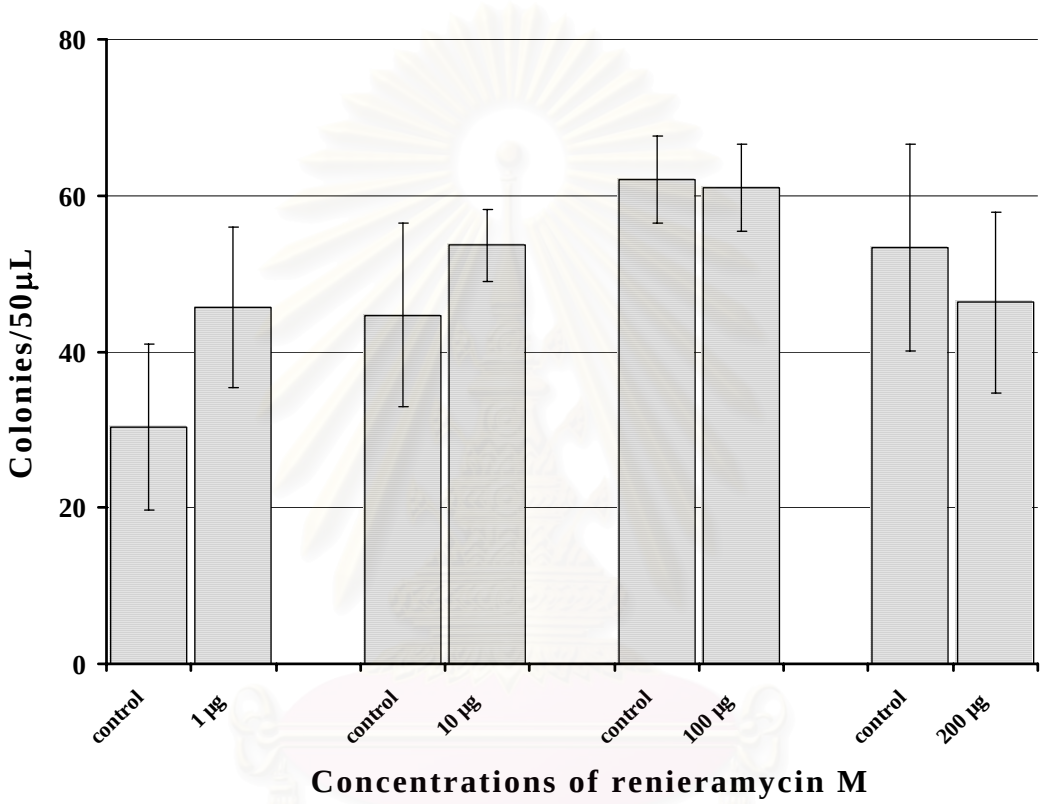


Figure. 5.8. Numbers of colonies (mean ± SE) of aerobic bacteria growing on aerobic count plates.

Table 5.1. Statistical analysis of antimicrobial effects between different concentrations of renieramycin M compared with control.

Concentration	1 µg	10 µg	100 µg	200 µg
p-values	0.146	0.284	0.837	0.529

CHAPTER VI

WOUND EFFECT ON RENIERAMYCIN M CONCENTRATION OF *XESTOSPONGIA*

INTRODUCTION

Wounding organisms make them less fit in some ways. The causes of wounding are mainly predation and competition (Walters and Pawlik 2005). The wound healing process usually begins just following the defensive process. Many organisms build up a wound plug to terminate the leak of body fluid or cytoplasm (Dreher et al. 1982). Then, the regeneration of damaged tissue takes place. However, the regeneration process depends on the degree of that wound and its effect on the organisms overall fitness (Henry and Hart 2005). During the wounding period, organisms are weak and easily get infections by pathogens, and are easily colonized by other sessile organisms (Henry and Hart 2005). Thus, some specific anti-pathogens and/or anti-fouling chemicals were produced during this regeneration period. However, their effectiveness varied depending on environmental factors (Frincke and Faulkner 1982, Ward et al. 2007). In addition, the defensive chemicals were expected to be harmful to all organisms. It follows that organisms have to manage these defensive chemicals in some way (Paul 1992). In seaweeds, corals and sponges,

a number of less toxic or inducible chemicals, that can be rapidly converted into more toxic and effective deterrents were reported (sometimes called “defense on demand”) (Paul 1992, Ebel et al. 1997, Pohnert 2004).

In this study, *Xestospongia* was observed to be seasonally wounded by *Jorunna*. Surprisingly, *Jorunna* was never observed to be feeding in the same area as *Xestospongia* that just previously fed area of *Xestospongia* by other *Jorunna*. *Xestospongia* might use the defense on demand strategy by immediately increasing the concentration of renieramycins, expected to be the defensive chemical, to defendable concentration to prevent feeding in the same area. The objective of this study was to investigate the effect of wounds on renieramycin M concentration in *Xestospongia*.

MATERIALS AND MATHODS

Sample collection and culture of *Xestospongia*

Forty-five individuals of *Xestospongia* were collected from the coral reef of Samui ($9^{o}48'80"E$, $99^{o}92'02"E$). Each sponge was cut into approximately 10 x 10 x 3 (W x L x H) cm^3. Five 1 m^2 culture frames were set up in the ocean. Every culture frame was hung at a depth of 1.8 m. In each frame, nine intersection points were made with carbon fiber rods. The cut sponge individuals were randomly tied to each knob of the intersections by the coated string (Figure. 6.1). They were cultured for two months prior to the experiment. Survival rates were monitored every 5 days.

Wounding method and schedules

The cuttings were made, one on the upper surface of every individual cultured sponge to make the surface flat. The cut piece of the sponge surface of each cultured sponge was labeled (#1-#45) and was kept separately to investigate the initial concentration of renieramycin M. The cutting treatment was then repeated several different times (Table 6.1) on the same surface as the previous cut (see Chapter 4 page 37 for the cutting method). Every cut piece was

lyophilized for 12 hrs using a freeze dryer and kept in a plastic bag at -20 ^{o}C until extraction.

Crude extract preparation

All samples were lyophilized for 12 hours using a freeze dryer. Each sample was accurately weighed at 100.0 mg for extraction. The procedure for crude extraction was the same as in Chapter 3 page 17.

Standard calibration solution

The procedure and source of standard renieramycin M was the same as in Chapter 3 page 17.

HPLC conditions

A Waters 2690 Controller was used with a Waters 996 Photodiode Array (PDA) Detector operated at 270 nm. The separation was performed on a LiChrospher®100RP-18 reversed phase column (5 µm, spherical, 4.0 x 125 mm) with methanol-water (7:3) as the mobile phase at a flow rate of 1.00 mL/min.

The difference in renieramycin M concentrations between the initial cut piece and the treatment cut pieces were analyzed by paired samples of mean (t-test) on Systat 7.0 (Systat 1997).

RESULTS

Most *Xestospongia* showed an increase in the renieramycin M concentration when they got wounded. 93.8% of the total individuals increased renieramycin concentration at the wounded area, whereas 6.2% of *Xestospongia* did not do so (Figure. 6.2). After six hours, the average concentration of renieramycin M at the wounded area of *Xestospongia* was highest (1169.2 $\pm$ 687.4 µg/g DW), whereas the lowest concentration of renieramycin M was an hour after wounding (140.7 $\pm$ 128.9 µg/g DW) (Figure. 6.3). From the second day until the thirty days, the concentrations of renieramycin M showed no obvious pattern, but the concentrations of renieramycin M at the wounded area were still higher than the initial concentrations (Figure. 6.4). However, the concentrations of renieramycin M in every *Xestospongia* were at non-detectable levels ninety days after the *Xestospongia* was wounded (Figure. 6.4).

DISCUSSION

The wounded *Xestospongia* are often found in coral reef habitats (Chapters 3 and 4 in this study). Some individuals were able to regenerate rapidly (several weeks) after grazing by *Jorunna funebris*, while in some individuals regeneration of feeding scars was incomplete even six weeks after being consumed. On the other hand, if the wound was severe, particularly in the case of small *Xestospongia* individuals, very low regeneration rates were observed (i.e. regeneration took months and the size of *Xestospongia* was much reduced). There were documents demonstrated the asexual reproduction of sessile prey might be promoted by body wounded to be fragments and then quick regeneration of the fragments to occupy the substrate (Highsmith 1982, Heyward and Collins 1985). In this study, the fragments of *Xestospongia* previously eaten by *Jorunna funebris* were not observed.

Lesions were commonly found on sessile organisms. They were caused by physical (wave, current, and drifting matters) and biological factors (predation, spatial competition, fouling). Henry and Hart (2005) concluded that the regeneration from an injury in a sponge or coral was influenced by both intrinsic factors (size of wounded individual, age, morphology and genotype) and extrinsic factors (wound characteristic, wound size, wound perimeter, wound depth, wound location, water temperature, food availability, sedimentation and disturbance history).

In the wounded *Xestospongia*, renieramycin M concentration, which was known to inhibit microbes was observed to rise steeply. The concentrations of renieramycin M persisted at high levels for a period of time. Then, the concentrations of renieramycin M slowly declined (Figure. 6.2). Lesions in sessile organisms such as corals, soft corals were caused by the reductions of predatory and competitive defenses. A rapid increase in some chemicals, immediately after wounding in many organisms (plants, animals), were commonly observed (Ebel et al. 1997, Pohnert 2002, Puyana et al. 2003, Pohnert 2004, Lunetta 2005, Walters and Pawlik 2005).

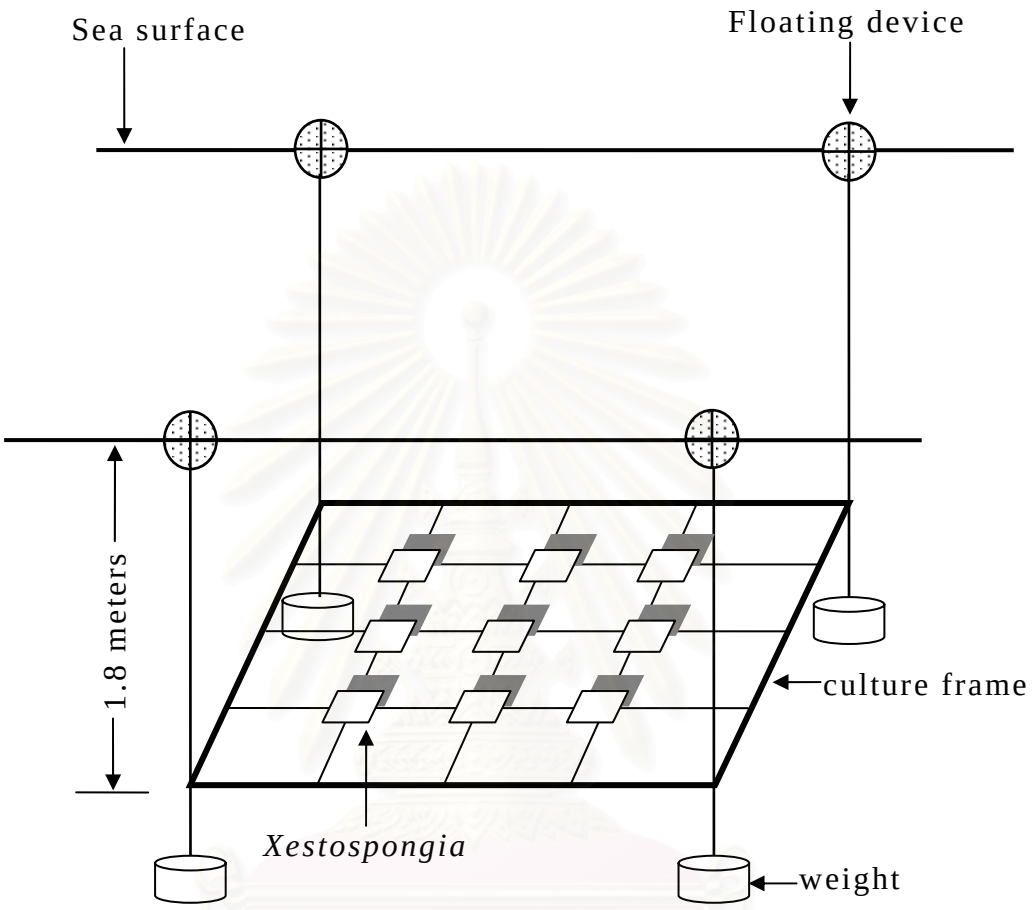


Figure. 6.1. Diagram of a *Xestospongia* culture frame.

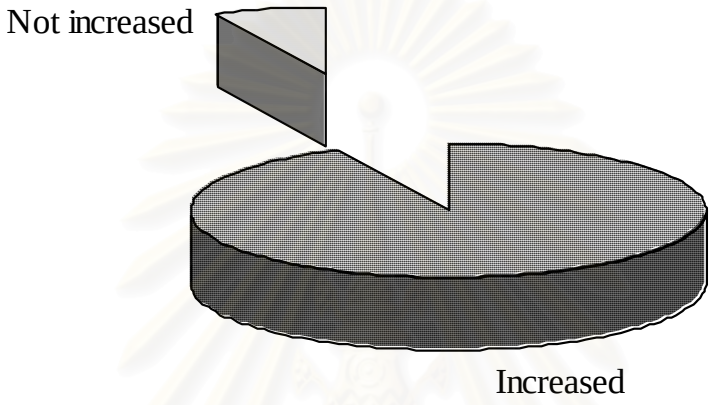


Figure. 6.2. The pie chart of the proportion of *Xestospongia* in which the renieramycin M concentrations changed after it was wounded.

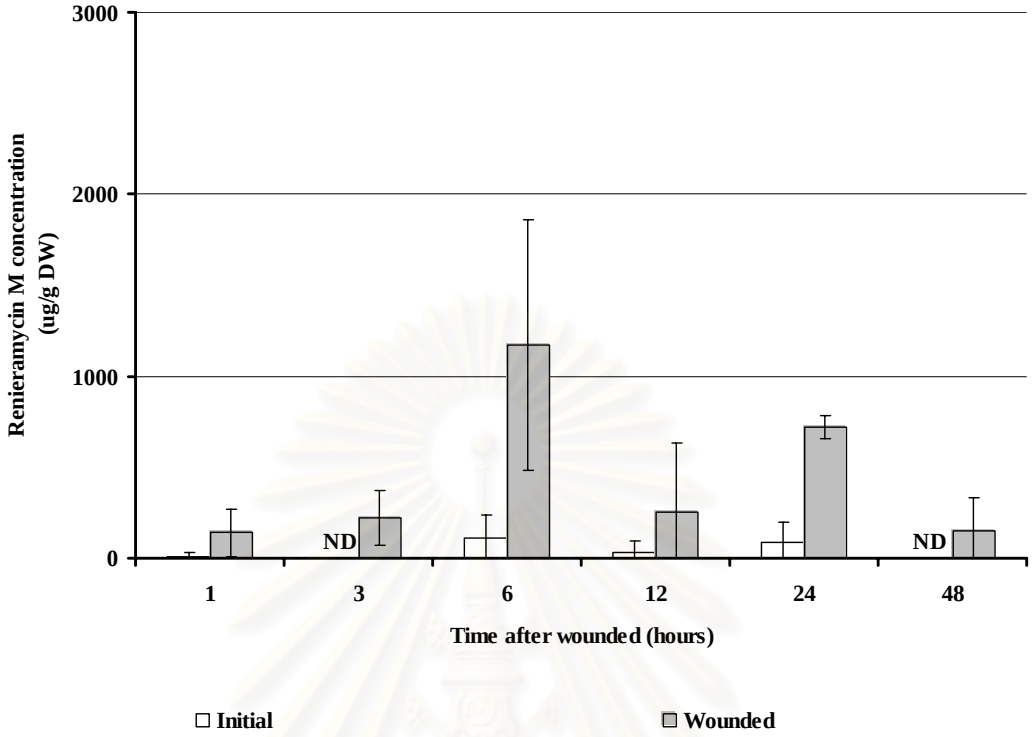


Figure. 6.3. The renieramycin M concentrations in *Xestospongia* after wounding at different times. (nd = non-detectable concentration).

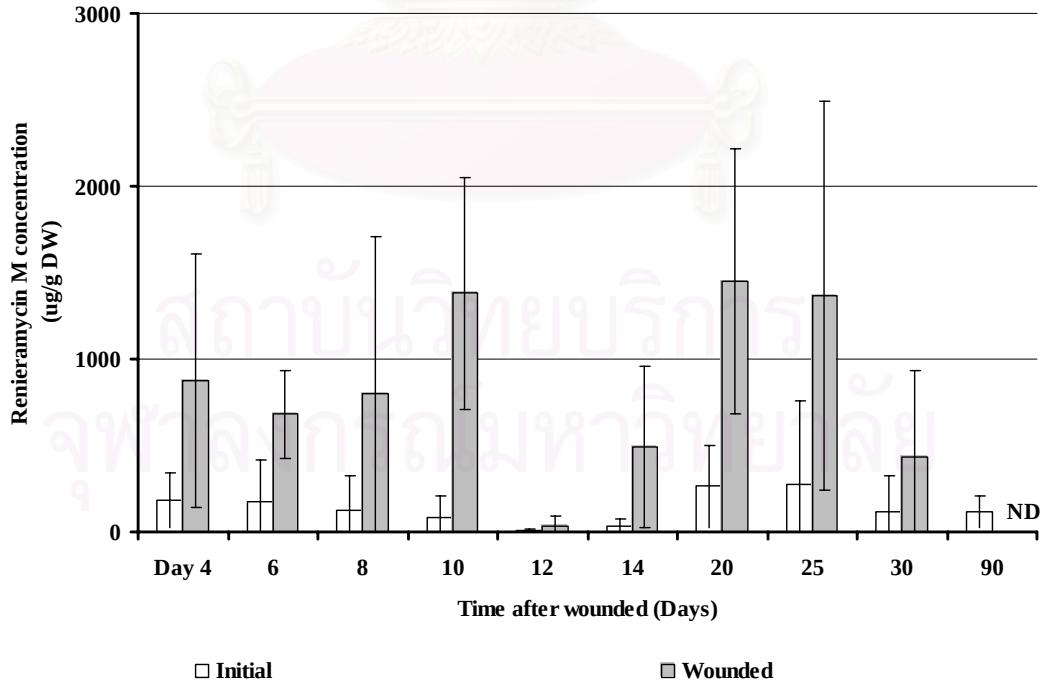


Figure. 6.4. The renieramycin M concentrations in *Xestospongia* after wounding at different times. (nd = non-detectable concentration).

Table 6.1. The cutting schedule of the *Xestospongia* surface.

Treatment cutting	
Time (hours)	**Number**
Initial cutting	#1-#45
Hour 1	#1-#3
Hour 3	#4-#6
Hour 6	#7-#9
Hour 12	#10-#12
Hour 24	#13-#15
Hour 48	#16-#18
Day 4	#19-#21
Day 6	#22-#24
Day 8	#25-#27
Day 10	#28-#30
Day 12	#31-#33
Day 14	#34-#36
Day 20	#37-#39
Day 25	#40-#42
Day 30	#43-#45
Day 90	#1-#45

CHAPTER VII

PREDATORY-PREY INTERACTION: CHEMICAL SEQUESTERING FROM PREY

INTRODUCTION

Prey defended themselves from being consumed by predators using a variety of strategies. Seaweeds diminish losses to herbivores by escaping from herbivores (spatial, associational and temporal refuges), decreasing their attractiveness to herbivores (chemical defenses), morphological/structure defenses, lower nutritional quality to herbivores and the synergies of defensive strategy (Littler and Littler 1980, Duffy and Hay 1990, Jones et al. 2005).

Why do predators still consume the prey that is capable of defending themselves? First, if consuming the defensive prey does not lower the predator's fitness, predators still consume the defensive prey (Duffy and Hay 2001). Second, not every prey is well defended, therefore, some predators have evolved to be the prey-specific (Duffy and Hay 2001, Termonia et al. 2001). Third, synthesis and storage of defensive chemicals might very costly to some consumers. Some consumers have the capability to sequester secondary metabolites from their prey then modify/ detoxify and store it as their own defensive chemicals (Cimino et al. 1985, Pawlik 1993, Avila and Paul 1997,

Becerro et al. 2001, McPhail et al. 2001, Iken et al. 2002). In addition, some have the ability to extract defensive cells from prey, and then move these cells to body parts and use it as their own defense (Cimino et al. 1982, Slattery et al. 1998, Cimino and Ghiselin 1999). The most advantageous co-evolution between predator and prey may be the commensalisms and symbiosis. Aeolid nudibranch consumes zooxanthallae and zoochlorellae, then extracts the chloroplast from plant prey, moving it to the light exposed part of its body to allow the chloroplast to get enough light energy to produce chemical energy that may supply most of the energy required by the herbivore (McFerland and Muller-Parker 1993). However, the defense of some consumers depends on the secondary metabolites from prey. Starvation and nutrient depletion may be worse for consumers because of lower defensibility (Gochfeld and Aeby 1997). In this study, the funeral nudibranch was frequency observed consuming only the blue sponge, *Xestospongia*. *Jorunna* was expected the prey specific nudibranch. It might able to sequester renieramycin from *Xestospongia* and use as its own defensive chemical. If so, the concentration of renieramycin M in the body of *Jorunna* would be expected to decrease when *Jorunna* was starved. If it is true, renieramycin might be unpalatable to fish.

The objectives of this study were to investigate whether *Jorunna* was prey-specific and if there was any preference on body part of prey in *Jorunna*. In addition the concentrations of renieramycin in the body

of *Jorunna* while it was starved were investigated; and determined whether the renieramycin M was unpalatable to fish?

MATERIALS AND METHODS

Prey preference in *Jorunna*

Five individuals of *Jorunna* were collected from Ran Dok Mai Island (13^{o} 09’ N, 100^{o} 49’ E). They were acclimatized in a container of two hundred liters of running seawater for 48 hrs with no food provided. Four species of sponge that sympatric in habitat of *Jorunna*, including *Gelliodes petrosioides*; *Xestospongia*; *Clathria (Thalysias) reinwardti* and *Callyspongia (Cladochalina) diffusa* were chosen. All the sympatric species had never been reported to contain to renieramycins, except *Xestospongia*. However, their hardness of texture was able to be arranged in order. The hardest texture was *Gelliodes petrosioides*, which was stony but brittle in texture. Megascleres were composed of oxeas, strongylote, and stylote with sigma microscleres (Desqueyroux-Faúndeź and Valentine 2002a). The second order was *Xestospongia* containing only oxeas as its magascleres and without microscleres, rare spongin presented in its texture. The third order was *Clathria (Thalysias) reinwardti*, containing both megascleres (strongylotes) and microscleres (isochelae) with moderate spongin (Hooper 2002). Its texture was soft and moderately easy to tear off. *Callyspongia (Cladochalina) diffusa*, the last one, contains the same kind of magascleres as *Xestospongia*, but the texture of *Callyspongia* sponge was soft and hard to tear.

Because, there was much more spongin present in its skeleton (Desqueyroux-Faúndeź and Valentine 2002c). Each sponge sample was cut into 3 x 4 cm^2 and placed into experiment trays. An individual of *Jorunna* was placed in to each experiment tray (Figure. 7.1). The sponge species that were eaten by *Jorunna* within 2 hours were recorded.

Feeding preference of *Jorunna* on *Xestospongia*

Xestospongia was cut into two areas, the surface area (bluish area) and the lower area (yellowish area). The experiment was performed as shown in Figure. 7.2. One at a time *Jorunna* were placed into the experiment. The feeding of *Jorunna* on *Xestospongia* parts was recorded within 2 hours with fifteen replicates.

Renieramycin M concentration and feeding deterrent of the meat of *Jorunna* starved for 0, 4, 7 days

Individuals of *Jorunna* were collected from reefs at Si Chang Island (13^o 11' N, 100^o 47' E) and the adjacent reefs by scuba diving at about 3-5 meters depth. The animals were then acclimatized in a 500-liter tank with air-jet circulated sea water. The acclimatization period was 48 hours. During this period, excess *Xestospongia* was fed to them as their prey. The normal activities such as feeding, creeping,

copulating and egg lying were observed. About one tenth of fresh sea water was replaced into the tank every day throughout the experimental period.

After acclimatization, each individual was caged in a transparent flow-through plastic bottle. Each cage was hung in the 500 L tank with air-jet circulated sea water. The mucus and fecal pellets were cleaned out from each cage every day. The feeding was terminated immediately when the experiment started. Five individuals were randomly sampled from the tank every day, from day 0 (for an initial reading), until day 7 of starvation. Each chosen animal was weighed, and then, the mantle and the foot muscle were dissected. The dissected parts of each individual were lyophilized for 12 hours using a freeze dryer. The dry weight of each dissected part of each individual was recorded. Each sample was separated into two parts. The first part was extracted and then the concentration of renieramycin M was measured using the procedure described below. The second part was tested for the feeding deterrent for reef fishes in the laboratory.

REMARK: according to the preliminary study of this Chapter, *Jorunna* did not accept any artificial foods as follows:

1. Dried *Xestospongia* + sodium alginate
2. Dried *Xestospongia* + fresh water extract of *Xestospongia* + sodium alginate
3. Fresh water extract of *Xestospongia* + sodium algenate

4. Dried *Xestospongia* + fresh meat of mussel (*Perna viridis*) + sodium alginate
5. Dried *Xestospongia* + fresh meat of mussel (*Perna viridis*) + Fresh water extract of *Xestospongia* + sodium alginate
6. Dried *Xestospongia* + fresh meat of fish (*Trichiurus lepturus*) + Fresh water extract of *Xestospongia* + sodium algenate
7. Fresh *Xestospongia* preserved in freezer.

Crude extract preparation

All samples were lyophilized for 12 hours using a freeze dryer. Each sample was accurately weighed at 100.0 mg for extraction. The procedure of crude extraction was the same as in Chapter 3 page 17.

Standard calibration solution

The procedure was the same as in Chapter 3 page 17.

HPLC conditions

A Waters 2690 Controller was used with a Waters 996 Photodiode Array (PDA) Detector operated at 270 nm. The separation was performed on a LiChrospher®100RP-18 reversed phase column (5

µm, spherical, 4.0 x 125 mm) with methanol-water (7:3) as the mobile phase at a flow rate of 1.00 mL/min.

The concentrations of renieramycin M and jorunnamycin A in *Jorunna* starved at different periods in the day were tested for difference using a t-test. The concentrations of renieramycin M and jorunnamycin A between the foot muscle and mantle tissue in starved *Jorunna* at different time periods were tested for difference using t-tests on Systat 7.0 (Systat 1997).

Laboratory experiment of feeding deterrent of *Jorunna* meat on Nile tilapia (*Oreochromis niloticus*)

In this study I chose the Nile tilapia because I expected that Nile tilapic did not co-evolve with marine organisms or marine chemicals. In the laboratory, thirty Nile Tilapia fish (*Oreochromis niloticus*) were fed with excess dry cockle (*Anadara granosa*) once a day for a week for acclimatization. A day prior to the experiment, the fish were starved. Then the fish was randomly put into the experiment containers, one at a time during the experiment. Each dried external organ (mantle and foot) of each individual of *Jorunna* from experiment 6.3 was cut into 1 x 2 mm^2 and was fed to the experiment fish. The dry meat of a cockle was used as a control (n = 5). The feeding behavior of fish and the handling time of fish were noted. The duration of each experiment was 10 minutes.

Experiment of feeding deterrent of *Jorunna* meat on coral reef fishes

Feeding deterrent of the meat of *Jorunna* starved for 0, 4, 7 days was performed in the coral reef habitat at Tan Island ($9^{o}23'18"$ N, $99^{o}56'34"$E). The gonads of the Diadema urchins (Phylum: Echinodermata; Class: Echinoidea: Family: Diadematidae: *Diadema setosum*) were collected, and put into 30 mL syringes. During the experiment a diver brought the syringes under water to the coral reef habitats. One syringe contained sea urchins gonads, whereas the others contained the meat of *Jorunna* starved for 0, 4 and 7 days from experiment 6.3. To make fishes familiar with the diver, the diver took 15 minutes keening and minimizing motion. First the gonads of sea urchins were expelled from the syringe to aggregate the fishes. Three species of reef fishes were observed to rapidly aggregate around the gonads of sea urchins from the syringe. These three species included *Abudefduf bengalensis*, *A. sexfasciatus* and *Halichoeres chloropterus*. Then the meat of *Jorunna* was presented to the reef fishes. The feeding behavior of the fish was recorded.

Feeding deterrent of Renieramycin M in Nile Tilapia (*Oreochromis niloticus*)

In the laboratory, thirty Nile Tilapia fish (*O. niloticus*) were fed artificial food pellets twice a day for a week during acclimatization. A day prior to the experiment, the fish were starved. Then the fish were randomly put into an experiment container, one at a time. The artificial food pellets were mixed with different concentrations of renieramycin M solution at the following concentrations: 0.0 ppm (control), 2 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500 ppm. Five artificial pellets were fed to each fish for each replicate in every treatment. The experiments were terminated after 10 minutes for each replicate. The time it took for the fish to eat the artificial pellets was recorded. The feeding deterrent of renieramycin concentrations was statistically analyzed by a single factor analysis of variance (ANOVA) then honestly on Systat 7.0 (Systat 1997).

RESULTS

Prey preference in *Jorunna*

Jorunna was a prey-specific nudibranch. In this study, *Jorunna* fed only on *Xestospongia* while other sympatric sponge species, were ignored (Table 7.1). During the experiment *Jorunna* crept on other sponges in experimental chambers. However, no feeding activity or feeding scars were observed on those sponge species.

Feeding preference of *Jorunna* on *Xestospongia*

Jorunna fed on the areas of *Xestospongia* which it approached. According to this experiment, *Jorunna* did not have preference for either the surface area (bluish area) or lower area (yellowish area) (Table 7.2).

Renieramycin M concentration and feeding deterrent of the meat of *Jorunna* starved for 0, 4, 7 days

The renieramycin M and jorunnamycin A contents in *Jorunna* did not significantly decrease when the period of starvation was increased. However, the proportions of jorunnamycin A to renieramycin M were close to 1.85 times throughout the experiment (Figures 6.3 and 6.4).

The contents of renieramycin M in the foot muscle of *Jorunna* decreased significantly from day 0 to day 7 of starvation (p = 0.034) (Figure. 7.5). In addition, a significantly decline in the average renieramycin M concentration in the mantle tissue of *Jorunna* was not observed (Figure. 7.6). Fifty-three percent of the *Jorunna* had higher renieramycin M concentration in the mantle tissues than in the foot muscles.

Laboratory experiment of feeding deterrent of *Jorunna* meat on Nile Tilapia (*Oreochromis niloticus*)

The meat of *Jorunna* deterred feeding of the fish (Table 7.3). In the laboratory, *O. niloticus* ejected the pieces of *Jorunna* meat from its mouth immediately after the first approach, and several approaches afterward by hungry fish. This pattern was observed in all experiments using 0, 4 and 7 days starved *Jorunna* meat.

Experiment of feeding deterrent of *Jorunna* meat on coral reef fishes

The meat of *Jorunna* starved for 0, 4 and 7 days was rejected by all species of reef fishes (*Abudefduf bengalensis, A. sexfasciatus* and *Halichoeres chloropterus*) (Table 7.3).

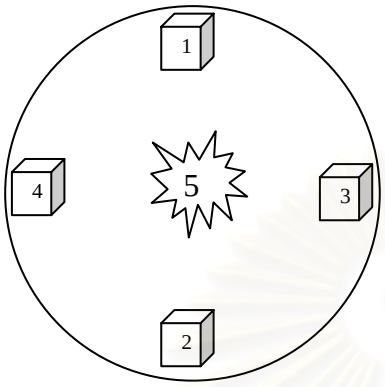
Feeding deterrent of Renieramycin M in Nile Tilapia, (*Oreochromis niloticus*)

The feeding deterrent test, using *O. niloticus*, showed an anti-feeding effect. Renieramycin M at a concentration of 100 ppm and higher significantly deterred the feeding activity of *O. niloticus* ($p < 0.001$) (Figure. 7.7).

DISCUSSION

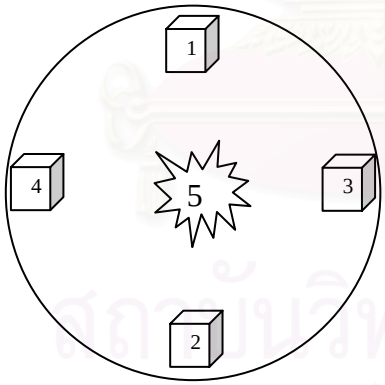
In the coral reef habitat, *Jorunna* was observed to locate its sponge prey by using the rhinophores. It stopped crawling frequently and waved the rhinophores into the water column. The function of the chemoreceptor (rhinophores) and the fluid dynamic flow also helped to understand how animals reached their prey from a remote distance (Harris 1973, Murphy and Hadfield 1997, Iissburg 2000, Wyeth and Dennis-Willows 2006). *Jorunna* was prey specific to *Xestospongia*. There was no difference in feeding selection on the bluish and yellowish areas of *Xestospongia* by *Jorunna*. The explanations about the predators preference for some prey species were varied, such as the morphology of feeding organs of predators, the quality of the prey's symbionts, prey nutrition quality, the stimulatory and inhibitory chemicals of the prey, and the quality of the prey's secondary metabolites (Kimmerer and potter 1987, Lambert 1991, De Boer et al. 1992, Duffy and Paul 1992, McClontock et al. 1994, Augustine and Muller-Parker 1998, Termonia et al. 2001, Yang et al. 2003, Fukasawa et al. 2005, Nimis and Skert 2006). Nudibranch mobilized the defensive chemicals into different body parts (Faulkner 1992, Avila and Paul 1997). In this study, it was found that *Jorunna* distributed renieramycin M and jorunnamycin A in quantitatively significant amounts in the mantle tissue. However, *Jorunna* could not stabilize these chemicals into its body. The concentrations of both chemicals

decreased when the starvation period was increased (Figures. 7.3, 7.4 and 7.5). A number of studies documented that predators were able to sequester the prey's secondary metabolites or other defensive mechanisms (nematocyst) to add to its own defensive chemicals (Ginsburg and Paul 2001, Newman and Schupp 2002, Frick 2003, Thoms et al. 2003). Moreover, some studies showed that predators were able to manipulate the sequestered chemical into a non-toxic form and then store it in their bodies (Faulkner 1992). In sea hares, the defensive metabolites were sequestered from their algal diets (Faulkner 1992). In contrast, nudibranch who is able to synthesize its own defensive chemical such as *Doriopsilla areolata*, was not expected to be at risk from predation (Gavagnin et al. 2001). In this study *Jorunna* was not expected to synthesize its own defensive chemical (renieramycin M). But the concentrations retained in the starved individuals on day 7 were 10^3 above the deterrent concentrations that deterred fish from artificial food in this experiment (Figures 7.3, 7.4, 7.5 and 7.7).



1. *Gelliodes petrosioides*
2. *Clathria (Thalysias) reinwardti*
3. *Callyspongia (Cladochalina) diffusa*
4. *Xestospongia sp*
5. *Jorunna funebris*

Figure. 7.1. Diagram of the experimental design for studying the food preference of *Jorunna funebris* on four sympatric sponges.



1. *Upper part of Xestospongia sponge*
2. *Upper part of Xestospongia sponge*
3. *Lower part of Xestospongia*
4. *Lower part of Xestospongia*
5. *Jorunna funebris*

Figure. 7.2. Diagram of the experimental design for studying the preference of *Jorunna funebris* on different body parts of *Xestospongia* sponge.

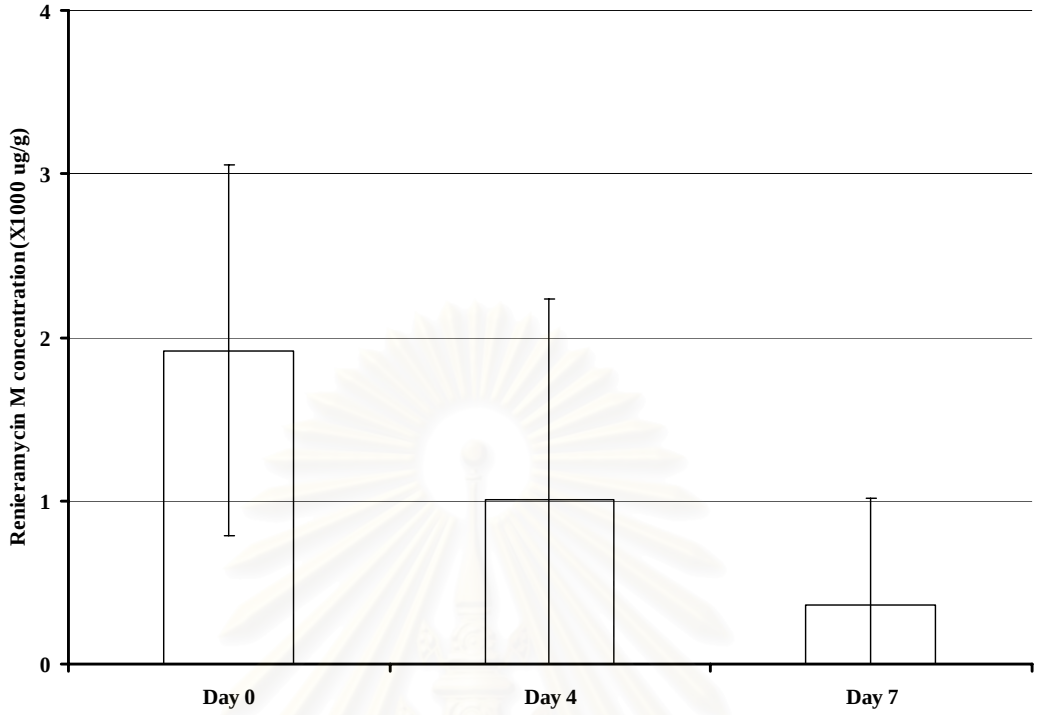


Figure. 7.3. Renieramycin M concentration (mean ± SE) in *Jorunna* starved for 0, 4 and 7 days.

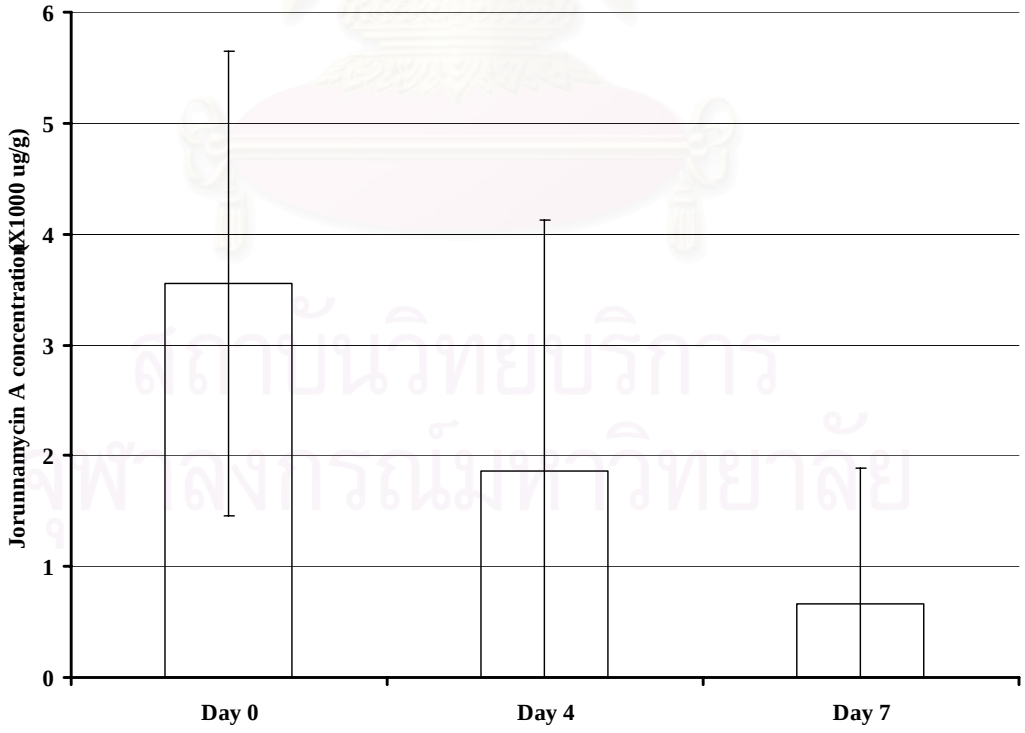


Figure. 7.4. Jorunnamycin A concentration (mean ± SE) in *Jorunna* starved for 0, 4 and 7 days.

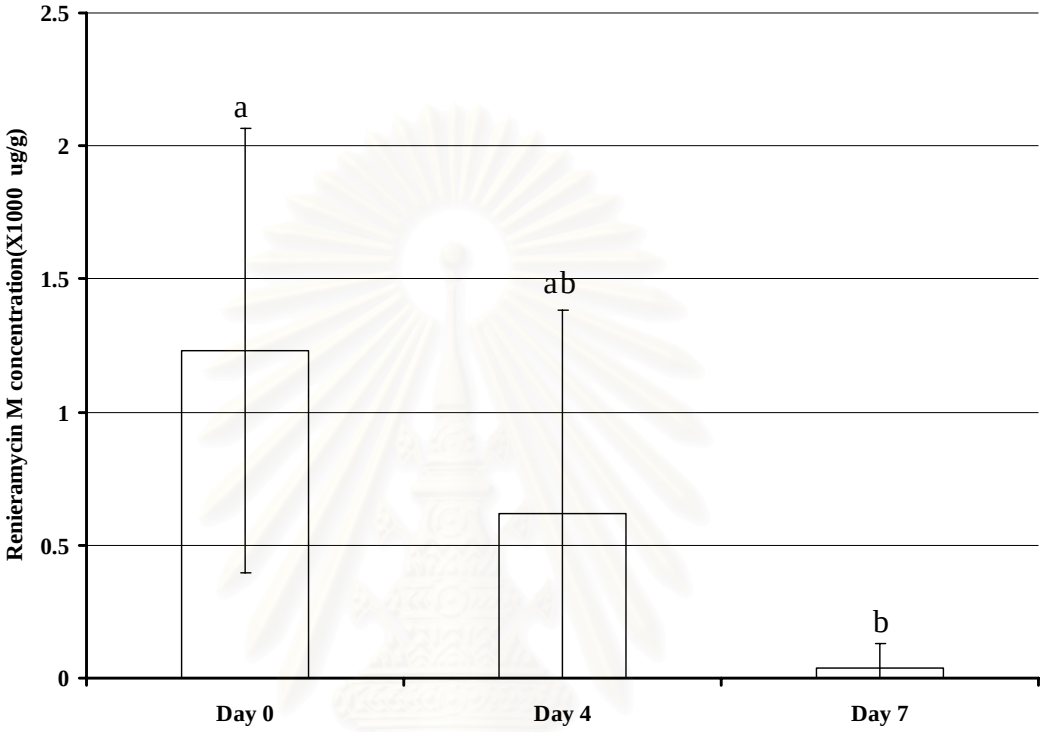


Figure. 7.5. Renieramycin M concentration (mean ± SE) in the foot muscle of *Jorunna* starved for 0, 4 and 7 days. Alphabetic groups above of columns for which means were not significantly different (Honestly significant difference test).

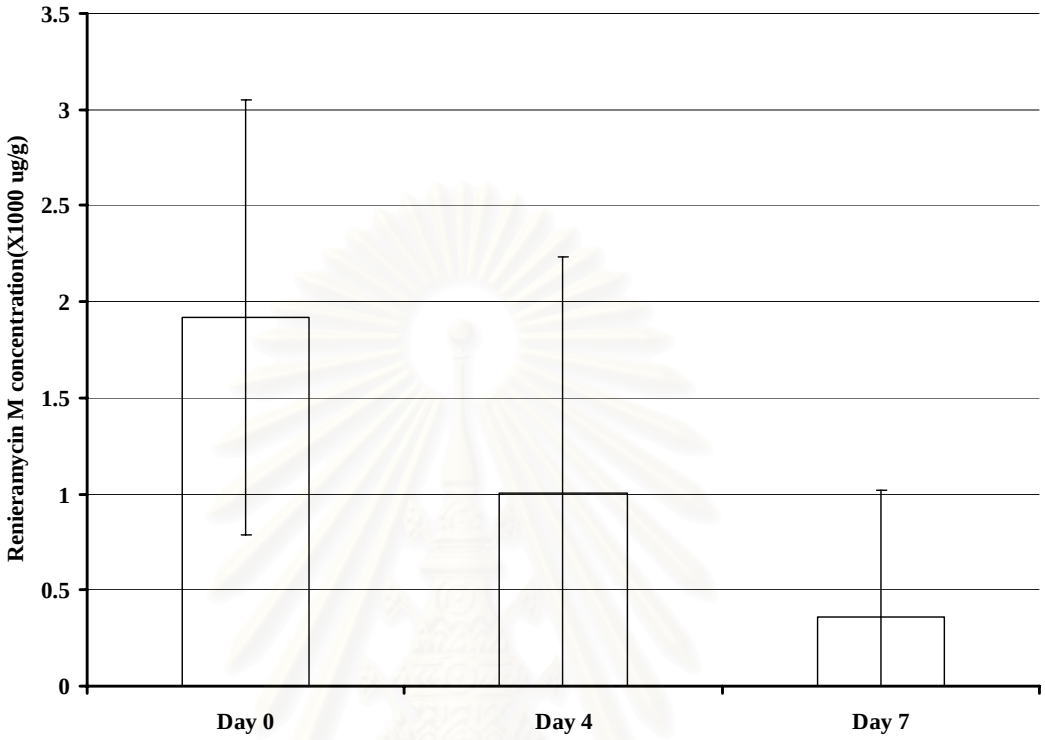


Figure. 7.6. Renieramycin M concentration (mean ± SE) in the mantle of *Jorunna* starved for 0, 4 and 7 days.

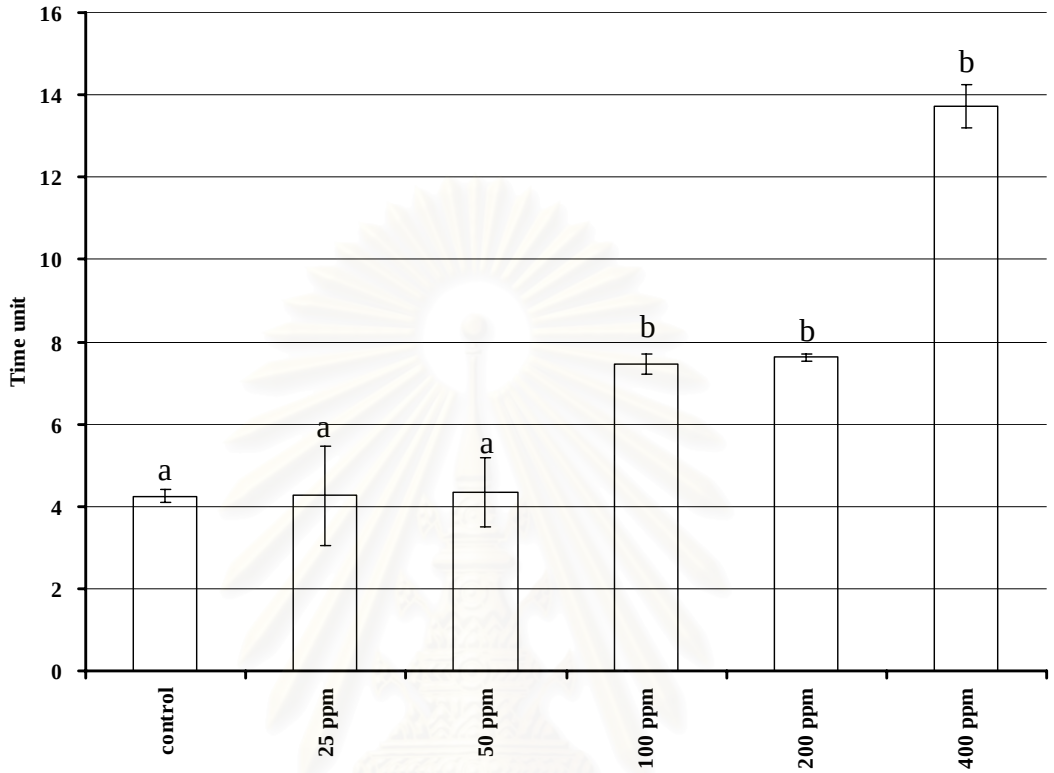


Figure. 7.7. Handling time of food pellets (mean ± SE) containing different concentrations of renieramycin M by Tilapia fish (*Oreochromis niloticus*). Y axis is transformed handling time.

Table 7.1. The preference of *Jorunna* on its sympatric sponges.

Prey species	**Accept**	**Reject**
Gelliodes petrosiodes		√
Calthria (Thalysias) reinwardti		√
Callyspongia (Cladochalina) diffusa		√
Xestospongia sp.	√	

Table 7.2. Body parts of *Xestospongia* consumed by *Jorunna*.

Areas of test	**Surface**	**Lower**
Number of areas eaten	9	8

Table 7.3. The feeding response of 4 species of fishes on the straved *Jorunna*.

Fish species	***J. funebris* starved for**		
	0 day	**4 days**	**7 days**
Oreochromis niloticus	rejected	rejected	rejected
Abudefduf bengalensis	rejected	rejected	rejected
A. sexfasciatus	rejected	rejected	rejected
Halichoeres chloropterus	rejected	rejected	rejected

CHAPTER VIII

CONCLUSIONS

Xestospongia was commonly found distributed in coral reefs in the Gulf of Thailand. It was clearly different from other closely related sponges in the genus *Xestospongia* and externally was bright blue when alive. Megascleres were composed of a single-sided class of oxea without microscleres. Ectosomal skeletal architecture was a tangential disorder of networks. Choanosomal was pauci to multispicular tracts (Figure. 1.1). *Xestospongia* sponges occupy a variety of abiotic and biotic substrates. They were observed growing on rock beds and dead coral rubbles and were found to coexist with the hard corals (*Porites lutea, Lobophillia hemprichii*), sea anemone (*Hateractis* sp.), zoanthids (*Palythoa caesia* and *Pa. tuberculosa*) hydrozoa, bivalve (*Barbataria belbingia*), other sponge species and some macro algae (Figure. 3.2). In addition, the area covered by *Xestospongia* was found to be related to the habitat and other coexisting organisms (Figure. 3.3).

The concentrations of renieramycin M, tetrahydroisoquinoline alkaloids, produced by this sponge were found to vary significantly among the study sites and among the coexisting organisms (Figure. 3.5). In addition, the pattern of maximum renieramycin M, maximum average frequency and maximum cover area found in *Xestospongia* that

coexist with the same species of organism. Two examples of this pattern were at Samui in *Xestospongia* that coexisted with algae and *Xestospongia* that coexisted with *Porites lutea* at Chang (Table 3.2).

The pattern of carbon/nitrogen ratios and renieramycin M concentrations within an individual (edge, inner and outer) of *Xestospongia* coexisting with *Palythoa caesia* were not similar to those of *Xestospongia* coexisting with *Porites lutea* (Figures. 4.2 and 4.3). *Xestospongia* coexisting with *Porites lutea* showed a significant difference in carbon, nitrogen and hydrogen content among areas of an individual, whereas there was no difference within *Xestospongia* coexisting with *Palythoa* (Table 4.1). The carbon, nitrogen and hydrogen contents and renieramycin M concentrations at the same area of *Xestospongia* coexisting with *Palythoa* and *Xestospongia* coexisting with *Porites* were not significantly different (Tables 4.2 and 4.3).

According to the surveys, *Xestospongia* was frequently found inhabiting the massive *Porites* by tissue contact. The area of tissue of *Porites lutea* in contact with the tissue of *Xestospongia* was bleached of color and the exoskeleton of the corallite was eroded. It was expected that this phenomena was caused by renieramycin M. Moreover, renieramycin were investigated that inhibited microbial environments (Frincke and Faulkner 1982). Experiments on the allelopathic effects of renieramycin M showed that renieramycin M neither caused necrosis on *Porites* nor anti-microbial activity (Table

5.1). On the other hand, it was found to be an anti-fouling compound (Figure. 5.7).

In coral reef habitats, *Xestospongia* was observed to be consumed by the nudibranch, *Jorunna*. In some cases, the feeding trail left severe scars on *Xestospongia*. Renieramycin M was expected to respond to the wound on *Xestospongia* in some way. Renieramycin M concentrations in the wounded area of *Xestospongia* were immediately increased from the initial levels (Figure. 6.2).

Jorunna was observed feeding only on *Xestospongia*. The prey preference experiment of *Jorunna* supported this expectation (Table 7.1). In addition, the preference on which parts of *Jorunna* that prey attacked was not different between the surface and the lower area of *Xestospongia* (Table 7.2). The major chemical compounds contained in *Jorunna* were jorunnamycin A and renieramycin M. Both of these active compounds were negatively related to the starvation time when food was withheld from *Jorunna* (Figures. 7.5 and 7.6). *Jorunna* unequally allocated renieramycin M between the mantle tissue and foot muscle (Figures. 7.5 and 7.6). Although the concentrations of renieramycin M decreased with an increase in the starvation period, the meat tissues of *Jorunna* still showed a feeding deterrent to coral reef fishes and fish in the laboratory (Table 7.3). Similarly, renieramycin M concentrations at 100 ppm in artificial food pellets were able to deter feeding of experimental fish. Moreover, the starved fish that

consumed the artificial food pellets that contained renieramycin M at 400 ppm died off within 12 hours after the experiment (Figure. 7.7).

In conclusion, renieramycin M is an anti-fouling and feeding deterrent chemicals produced by the coral reef sponge, *Xestospongia*. Renieramycin M was distributed along the body of *Xestospongia*. The concentration of renieramycin M increased immediately after the *Xestospongia* was wounded. The increase in the concentration of renieramycin M was maintained above a normal level for a period of time. This might be the defense mechanism of *Xestospongia*. However, *Jorunna* was observed to strictly feed on *Xestospongia*. The negative relationship between the concentrations of renieramycin M in the body of *Jorunna* and the starvation period of *Jorunna* could be inferred that *Jorunna* sequestered renieramycin M from its prey. It used renieramycin M as its own defensive chemical.

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Dear Dr. Darumas,

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Title: Distribution pattern of the renieramycin-producing sponge *Xestospongia* sp. and its association with other reef organisms in the Gulf of Thailand

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