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Cultivation and Optimization of Marine Sponges with Experimental Design Methods

by

Funda ASKAN

August 2013

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SPONGES WITH EXPERIMENTAL DESIGN
METHODS

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Funda ASKAN

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APPROVAL PAGE

This is to certify that I have read this thesis written by Funda ASKAN and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science in Genetics and Bioengineering .

Prof. Dr. Halil Rıdvan OZ
Thesis Supervisor

Assoc. Prof. Dr. Omer Fahrettin DEMIREL
Co-Supervisor

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science in Bioengineering and Genetics.

Prof. Dr. Halil Rıdvan OZ
Head of Department

Examining Committee Members

Halil Rıdvan Öz

Fahri Akbas

Şükrü Okkesim

Haşim Özgür Tabakoğlu

Mustafa Fatih Abasiyanik

It is approved that this thesis has been written in compliance with the formatting rules laid down by the Graduate School of Sciences and Engineering.

(Title and Name)
Director

March 2013

CULTIVATION AND OPTIMIZATION OF MARINE SPONGES WITH EXPERIMENTAL DESIGN METHODS

Funda ASKAN

M.S. Thesis – Genetics and Bioengineering
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Thesis Supervisor: Prof. Dr. Halil Rıdvan OZ

Co-Supervisor: Assoc. Prof. Dr. Omer Fahrettin DEMIREL

ABSTRACT

The aim of this study was to develop a biotechnological process and mechanistic model for the living sponges and optimization of sponge's medium .Such a growth model can be used to study the quantitative effect of factors such as pressure, light, current, temperature, nutrition source, nutrient concentration, or age on the growth rate of sponges. Development of a suitable growth medium is one of the most important issues to optimize the production of biomass. Cultivation of sponges in a bioreactor allows manipulations of the sponge diet that are not possible in the sea, and thus changes of the growth rate.

Keywords: Sponges, Sponge Cultivation, Ex situ Culture, Biotechnology.

DENEYSEL TASARIM YÖNTEMLERİYLE DOĞAL DENİZ SÜNGERLERİNİN KÜLTÜRE EDİLMESİ VE OPTİMUM ŞARTLARININ BELİRLENMESİ

Funda ASKAN

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Mart 2013

Tez Danışmanı: Prof. Dr. Halil Rıdvan ÖZ

Eş Danışman: Doç. Dr. Ömer Fahrettin DEMİREL

ÖZ

Yapılan çalışmanın amacı; deniz süngerlerinin büyümesine olanak sağlayan bir biyosistem geliştirmek ve ortamın optimizasyonunu sağlamaktır. Optimizasyon faktörleri olarak; sünger büyüme hızı üzerinde basınç, ısı, ışık, besin konsantrasyonu gibi kantitatif faktörler kullanılmaktadır. Uygun bir büyüme ortamının geliştirilmesi, deniz süngerlerinin yaşaması ve biyokütle üretimini optimize etmek için gerekli en önemli konulardan biridir. Biyorektördeki sünger kültürü, denizin aksine maniplasyonlara izin vermekte ve bu değişiklikler büyüme oranını etkilemektedir.

Anahtar Kelimeler: Deniz süngerleri, Sünger Kültürü, Ex situ Kültür, Biyoteknoloji

To my parents

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I express my thanks and appreciation to my engaged Semih Kurban and my family for their understanding, motivation and patience. Lastly, but in no sense the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

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SYMBOL/ABBREVIATION	
ASW	Artificial sea water

CHAPTER 1

INTRODUCTION OF SPONGE BIOLOGY

1.1 INTRODUCTION

Sponges are the most simple and ancient animals which live in the sea and attached to substratum for example coral, rock etc. (Brusca and Brusca, 1990). They are benthic invertebrates that include a wide variety of morphological forms (see figure 1.1) (Boury-Esnalt and Rützler, 1997). They live in the all seas from Greece to Antarctic and from Indonesia to Norway (Brusca and Brusca, 1990). Some species may have more than two meters height, some species are a few centimeters (Proksch, 1994). There are approximately 15,000 different sponge species which live in the seas and oceans (Hooper *et al.*, 2002).

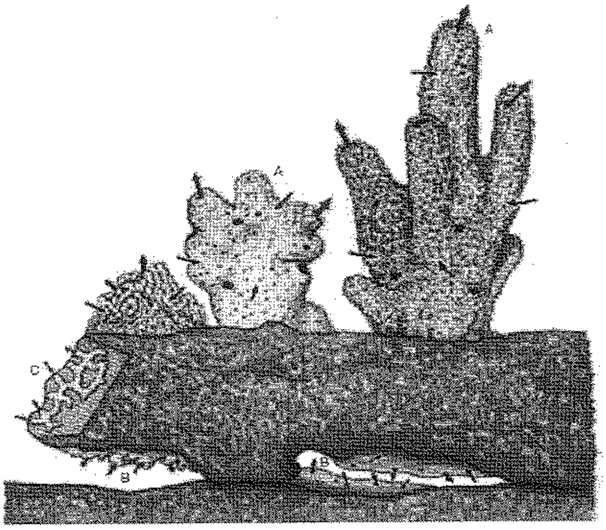


Figure 1.1. Marine sponges are member of Porifera. **A**, Two big sponges on top of a calcareous rock require an exposed surface. **B**, The covering sponges below the rock use much of their surface for attachment. **C**, The sponges on the cutaway vertical rock surface.(Ruppert, Fox and Barnes *Invertebrate Zoology: A Functional Evolutionary Approach*,2003).

1.2 SPONGE BIOLOGY

1.2.1 Form and Basic Structure of Sponges

The filter-feeding body of a sponge is built around one of three anatomical designs: asconoid, syconoid, or leuconoid. The simplest the asconoid design, is a hollow cylinder attached by its base to the substratum. The body surface is covered by a monolayer of flat cells called the pinacoderm. The hollow interior, the atrium or spongocoel, is lined with a monolayer of flagellated collar cells called choanoderm. Many small pores, known as ostia perforate the cylinder wall (Ruppert, 2003).

The sponge body occurs from important parts for efficient filtration of the surrounding seawater. The body of a sponge, shown in below (see Figure 1.2):

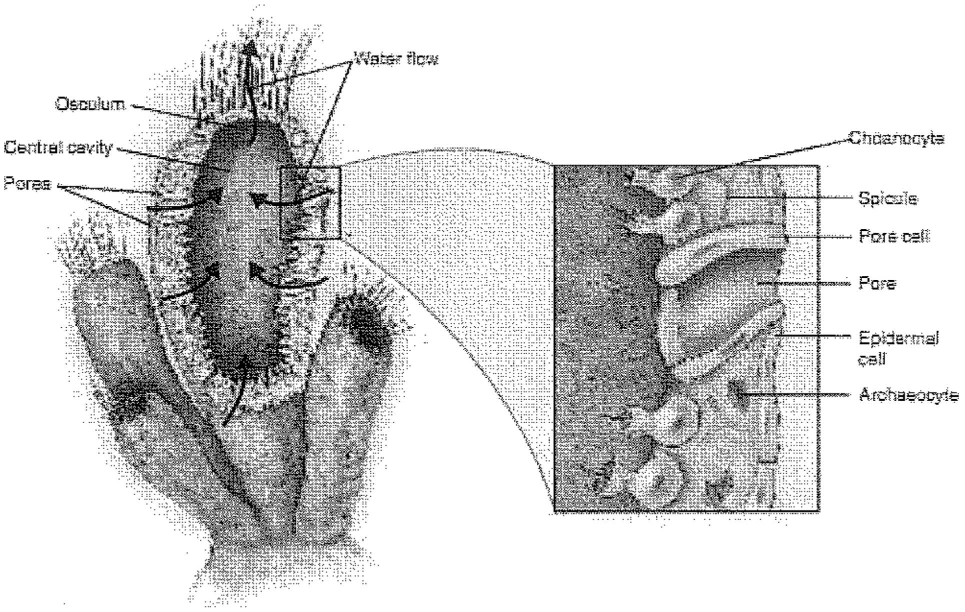


Figure 1.2 Sponges carry out basic functions. For example; feeding and circulation, by moving water through their bodies (Billing West H.S., *Lesson Notes Chapter 26-2*, 2012)

A larger opening, the osculum, is situated at the upper, free end of the body. The flagellated choanoderm creates a unidirectional water flow that enters the ostia, passes over the choanoderm en route to the atrium, and exits through the osculum. This circulatory system of choanoderm, pores, and chambers is called an aquiferous system (Ruppert, 2003). The syconoid design increased surface area and reduced atrial volume by forming alternating inpockets and outpockets of the body wall. The arrangement can be visualized by interdigitating the extended fingers of your two hands and imagining that your fingers are hollow. The body of sponge forms a wall around a large central cavity through which water is circulated continually. The sponge body contains many choanocyte chambers. Choanocytes are specialized cells that use flagella to move a steady current of water through the sponge (see Figure 1.2a). Water is delivered to, and removed from, choanocytes via the aquiferous system, which forms an intricate network throughout the sponge body (Van Trigt, 1919).

The outpockets of the choanoderm are called choanocyte chambers. The inpockets of the pinacoderm are in current canals. Incurrent canals dis-charge into the choanocyte chambers via numerous small openings known as prosopyles At the outer surface of the sponge, water may enter the incurrent canals directly or first pass through a narrow ostium formed by a secondary growth of tissue (Ruppert, 2003).

This water enters through pores located in the body wall. Nutrients with water enter to system by the ostia, and exit via the oscules. Ostia are small and its size from 1 to 50μm in most species, while oscules are generally huge and varying greatly in size from one species to another (Simpson, 1984). The body of the sponge are covered by the exopinacoderm and endopinacoderm cells. Spongin fibres and inorganic siliceous support the sponge by body skeleton consisting of organic collagen.

1.2.1.1 Skeleton of Sponge

Spicules are characteristic for species identification and classification of the phylum. Sponges are divided into three classes. The largest class, of which bath sponges are a member, is the class Demospongiae. The other classes are Hexactinellida and Calcarea (Hooper et al., 2002). Calcareous sponges is composed completely of calcite spicules (Jones, 1970), while the hexactinellida are primarily deep-water sponges, they have a skeleton that is built of hexactinal siliceous spicules (Reiswig, 1979). Demospongiae is the largest class (approximately 95% of all species) which the skeleton is composed of siliceous spicules that is often supplemented by organic collagenous fibres (Brien, 1973). This fibres form a strong network in the mesohyl that includes the space between the exopinacoderm and the endopinacoderm (see Figure 1.2).

Collagen fibres macromolecules occur from the extracellular matrix, which provides the support for specific cell adhesion as well as for signal transduction and cell growth. Because of the extracellular matrix plays important roles in digestion, gamete production, transport of nutrients and waste products by archeocytes that can move freely through the mesohyl (Müller et al., 2000).

The calcifying demosponges (sclerosponges) secrete a massive basal exoskeleton of CaCO3 on which the body rests. This sponges also secrete siliceous spicules in the mesohyl (see Figure 1.3).

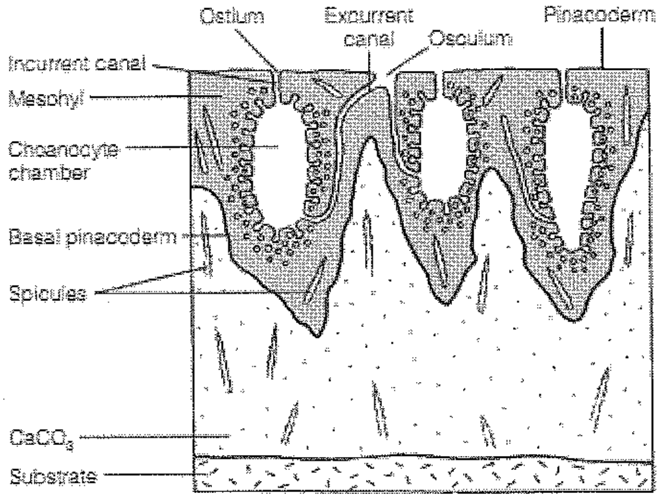


Figure 1.3 Porifera: body wall and skeleton of a calcifying sclerosponge (Demospongiae: Ceratoporellidae). The calcareous exoskeleton is secreted by the basal exopinacoderm and contains embedded siliceous spicules. Siliceous spicules also occur in the mesohyl of the living tissue. (*Modified and redrawn after Hutchinson from* Bergquist, P. R. 1978. Sponges. Hutchinson, London. 268 pp.)

1.2.1.2 Cell Types of Sponges

Sponges have many different cell types, but only two types of organ-like structures: pinacocytes forming a pinacoderm and choanocytes forming choanocyte chambers. The other cell types are delivered through the mesohyl (Lévi, 1970).

Archeocytes are the most important cells in the mesohyl. They have the capacity to differentiate into any other cell type and also have important roles in feeding and in clearing debris that block the ostia (see Figure 1.4). They provide a regulatory mechanism establishing and maintaining the equilibrium between different cell types (Borojevic, 1966).

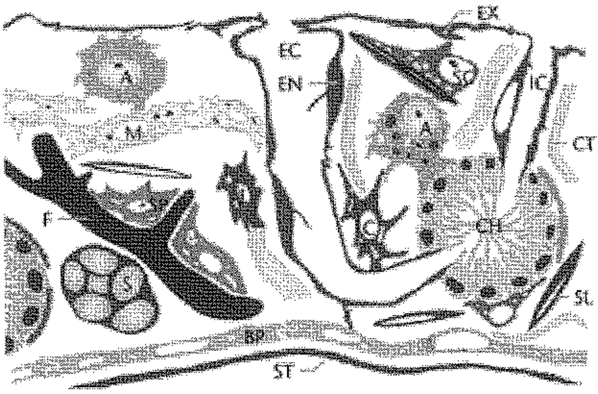


Figure 1.4 The major cell types and their relationships. A, archaeocyte; BP, basopinacocytes; C, collencyte; CH, choanocyte chamber; CT, collagen tract; EC, excurrent canal; EN, endopinacocyte; EX, exopinacocyte; F, fibre; IC, incurrent canal; L, lophocyte; M, myocyte; SC, sclerocyte; S, secretory cell; SL, spicule in fibre; SP, spongocyte; ST, substrate. (After Bergquist PR (1998) Porifera. In: Anderson DT (ed.) Invertebrate Zoology, chap 2, p. 15.Melbourne: Oxford University Press.)

The sponge skeleton is built by collencytes, spongocytes and sclerocytes. Collencytes produce collagen for supporting, while spongocytes secrete a form of collagen that polymerizes into spongin, which is the framework for the sponge. Spicula are often immersed in the collagenous matrix (Bergquist, 1978). Some cells types are occurred a group that called granulocytes. Different granulocyte cell types have different functions. They storage of certain secondary metabolites (Thompson et al., 1983), unconventional sterols (Lawson et al., 1988), pigments (Liaci, 1963) or glycogen (Bergquist, 1978). Also granulocytes release components that like mucous (Donadey, 1982) or lectins (Bretting et al., 1983).

1.2.1.3 Water Flow

Sponges can achieved the water flow by different combinations of closing the osculum and ostia and varying the beat of the flagella, and may shut it down if there is a lot of sand or silt in the water. The flexibility of pinacocytes and choanocytes and remodeling of the mesohyl by lophocytes allow the animals to adjust their shapes to take maximum advantage of local water currents (Ruppert et al., 2004).

The water of flow varies according to the species. In the first type, *Clathria*, water flow is in and out basically through the same tissue mass; thus, the flows are opposing. In the second type, Haliclona, water flow is unidirectional, entering through myriad ostia on the outer surface and exiting through large oscula (chimneys). Interestingly, despite the difference in construction the two types have similar density of choanocyte chambers (1-1.8x107 chambers . cm3) and number of choanocytes per chamber (see Figure 1.5) (Reiswig, 1974).

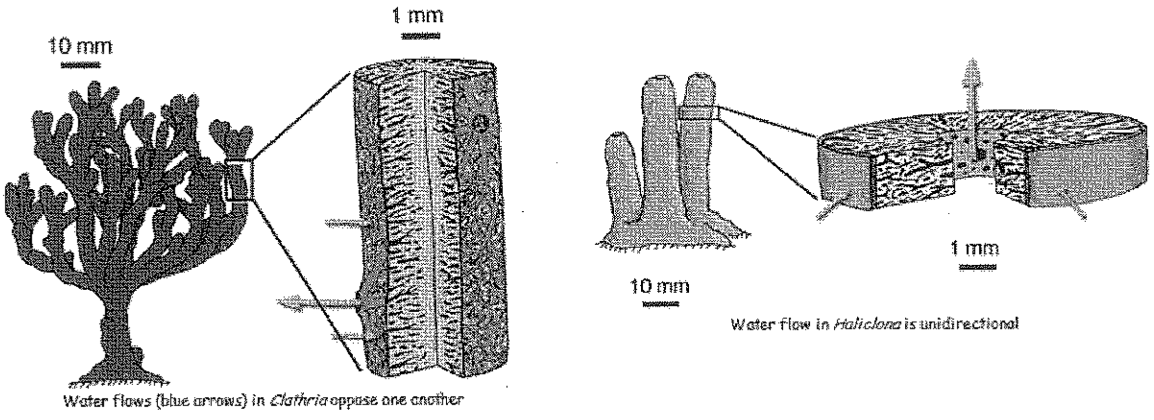


Figure 1.5 Water flows (blue arrows) in Clathria. It opposes one another and Water flow in Haliclona is unidirectional (Reiswig 1975 J. Morph 145: 493).

Asconoid is the simplest body structure of sponges which like tube or vase shape but this severely limits the size of the animal. The body structure is characterized by spongocoel surrounded by a single layer of choanocytes. Asconoid sponges seldom exceed 1 mm (0.039 in) in diameter (Ruppert et al., 2004)

At some of the sponges, flow of water is controlled by flagellated chambers. For example in Leuconia is a small leuconoid sponge.It is about 10 centimetres tall and 1 centimetre in diameter. It get water enters each of more than 80,000 intake canals at 6 cm per minute. (C. Hickman, 2001).

1.2.2 Classes of Sponges

Sponges divided into three classes: Calcarea, Hexactinellida and Demospongiae. However, studies have shown that the Homoscleromorpha, a group thought to belong to the Demospongiae, is actually phylogenetically well separated. (Gazave, 2010)

Table 2.1 Classes of sponges. They identified as the fourth class of sponges (Bergquist, P. R., 1998)

	Types of cells	**Spicules**	**Spongin fibers**	**Massive exoskeleton**	**Body form**
Calcarea	Single nucleus external membrane.	Calcite may be individual or large masses	Never	Common.	Asconoid Syconoid Leuconoid
Hexactinellida	Mostly syncytia in all species	Silica May be individual or fused	Never	Never	Leuconoid
Demospongiae	Single nucleus,external membrane	Silica	In many species	In some species	Leuconoid
Homoscleromorpha	Single nucleus, external membrane	Silica	In many species	Never	Leuconoid

1.2.3 Vital Functions

1.2.3.1 Feeding

Sponges do not have circulatory, respiratory, digestive, and excretory systems. They use the water flow system supports all these functions. They filter food particles out of the water flowing through them (Ruppert et al., 2004). They pump a large volume of water, cycling the net body volume in 7.6 s. The pores, the opening of the inhalant canals to the choanocyte chambers, the tentacles of the choanocyte collar and its mucous net represent a set of sieves in the path of the water current. Particles are extracted with high efficiency, with choanocytes accounting for 80% of organic carbon uptake. Surface and canal-lining pinacocytes can phagocytose particles directly (Bergquist, 1998).

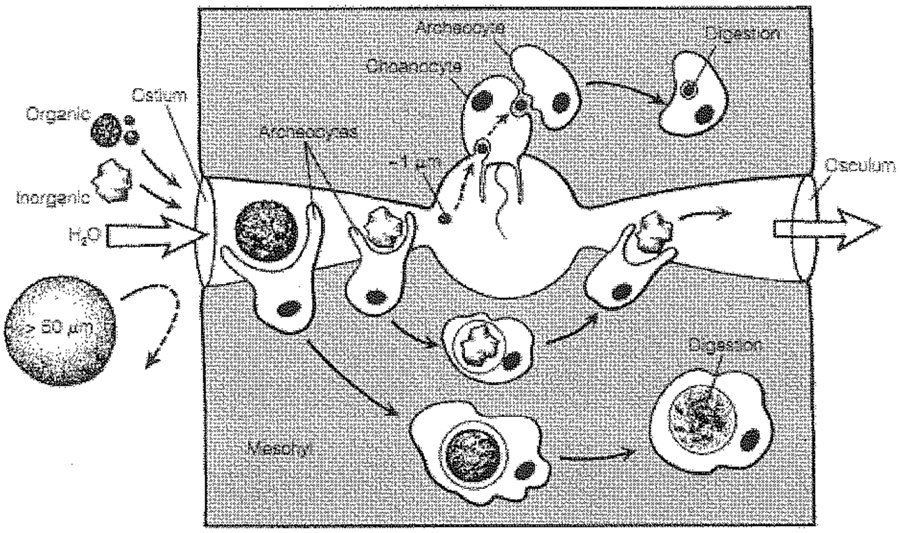


Figure 1.6 Summary of filter feeding, digestion and egestion. (Ruppert, Fox and Barnes *Invertebrate Zoology: A Functional Evolutionary Approach,* 2003)

The ostia cannot get particles which larger than 50 micrometers so pinacocytes expend them by phagocytosis (engulfing and internal digestion). Particles from 0.5 µm to 50 µm are grabbed in the ostia, which shaper from the outer to inner ends. Bacteria-sized particles which smaller than 0.5 micrometers, pass through the ostia and are caught and consumed by choanocytes (Ruppert, 2004).

1.2.3.2 Secretion

Sponges secrete mucus which often contains compounds with strong toxic effects. The most obvious secretory activity is seen in the development of the mineral spicule and fibrous spongin skeletons by 'sclerocytes' and 'spongocytes' (Bergquist, 1998). Excretion of the fibrillar collagen mesohyl matrixis fusiform cells, 'collencytes' and 'lophocytes', and the polysaccharide matrixis put down by heavily granular cells (see Figure 1.7).

The mineral skeleton is either siliceous or calcareous, usually in the form of seperate elements, spicules which have a bewildering range of shapes, sizes and patterns of organization. Megascleres protect the whole form of the sponge but tiny spicules microscleres line surfaces. Fibrous collagenous skeletons occur either separately, as in bath sponges, or investing and binding siliceous spicules (Bergquist, 1998).

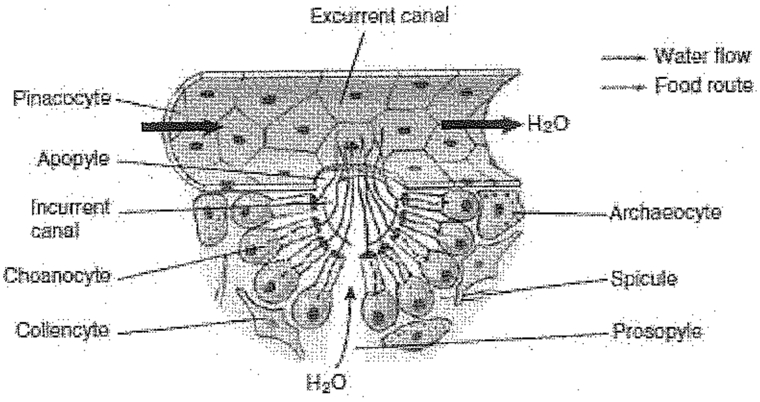


Figure 1.7 Collencytes and lophocytes. They secrete fibrillar collagen but lophocytes different morphologically from collencytes. (Hickman–Roberts–Larson: *Animal Diversity, Chapter 6. Sponges: Phylum Porifera*, 2002)

1.2.3.3 Controls of Activities

The most sponge species control movements that are coordinated whole their bodies, mainly contractions of the pinacocytes, squeezing the water channels and remove excess sediment and other substances that may cause blockages. Some species contract the osculum of the back of the body (Nickel, M. 2004). Cellular sponges have long been known to contract inhalant and exhalent openings (ostia and oscula), and portions of their canal system (Mackie, 1979).

Neurotransmitter molecules have been localized in tissues of calcareous sponges (Lentz, 1966) and demosponge larvae (Weyrer et al., 1999), and many of these chemicals have been shown to affect the contraction of ostia and oscula (Parker 1910, Prosser et al. 1962, Emson 1966, Prosser 1967). Contractions perform after mechanical or chemical stimuli, and kinetics of contractions can be determined by computer assisted image analysis (1 image/10s) (see Figure 1.8).

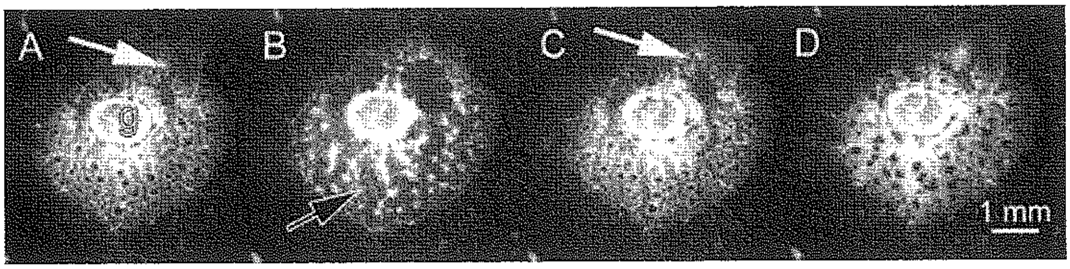


Figure 1.8 Dilation and contraction of the aquiferous system of a 7-day old juvenile *Ephydatia muelleri*. White arrows point to the osculum, which increases in diameter by frame C as the canals dilate in A and B, and contract in C and D. The white regions between the dilated exhalent canals in (B) are the compressed inhalant canals (Elliott and Leys 2007).

Also latest researches have focused on the secreted hormone hypothesis (Ellwanger et al. 2004, Ellwanger and Nickel 2006), it is most likely that several mechanisms interact. For example, a small change in pressure cause activate stretch receptors, which in turn could stimulate the release of a locally acting messenger. Small changes in pressure in the cell membranes at a distant location and causes contraction. This contraction can also be seen throughout a canal and at the same time across canals. While a pressure wave predate the contraction in both areas, evidence that amoeboid cells in the mesohyl intercept slow moving as the contraction passes point to a secreted messenger (see Figure 1.9).

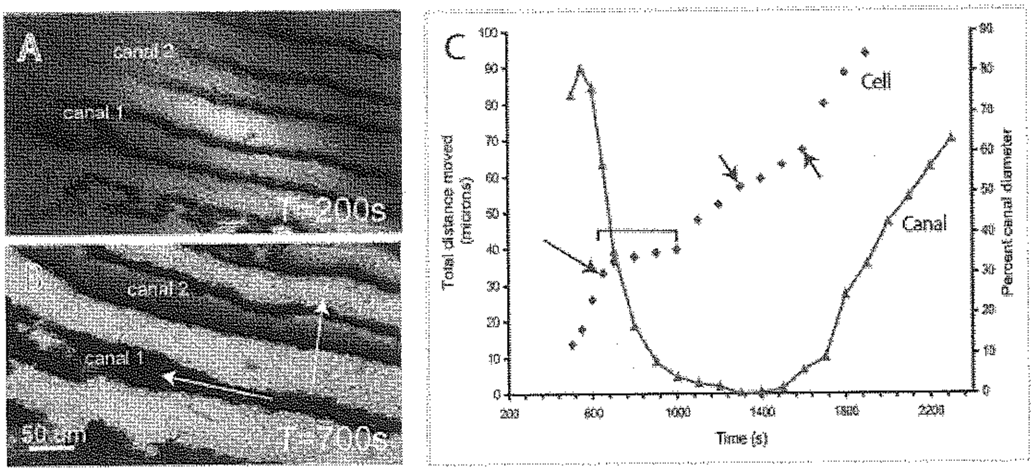


Figure 1.9 Cells crawling through the mesohyl stop when a wave of contraction passes by. A, B. India ink added to a dish with a sandwich preparation is taken into the chambers (black oblique lines). When all chambers are filled a wave of contraction propagates along the canals (white arrow pointing left) and across canals (white arrow pointing across). There is a slight delay before the contraction is seen moving along the second canal (2). C. Plot of the track (diamond) of a single cell crawling in the mesohyl of the first canal during the first contraction. As the canal diameter narrows during a contraction (10 minutes, 600 s after addition of dye), the cell ceases forward movement (long arrow and bar). Crawling commences once the canal is fully contracted (10 minutes later). Forward motion is slowed a second time (small arrows) when the incurrent canal (1) begins to dilate once more (Modified from Elliott and Leys, 2007).

1.3 LIFE STORIES

The sexual and asexual reproductive processes occur in sponges; but because they are not localized in tissues or organs, but difficult to observe (Simpson, 1984).

1.3.1 Clonal (Asexually) Reproduction

Sponges have three methods of clonal reproduction: after fragmentation; by budding; and by producing gemmules (Ruppert, 2004). The most sponges depends primarily on sexual reproduction, a few sponges largely believe on asexual reproduction or a combination of both processes (Dayton, 1979). The current or wave damage cause fragmentation primarily, and perhaps from damage done by grazing carnivores. The removed fragments rely on their remodeling capacity for regeneration. The fragment attaches to the another substratum and reorganizes itself into a functional sponge (Ruppert, 2003). Some sponges produce buds (Simpson, 1984).

Buds are cell which masses growing at the external surface of the body that subsequently separate from the parental body by constriction of the tissue bridges (see Figure 1.10).

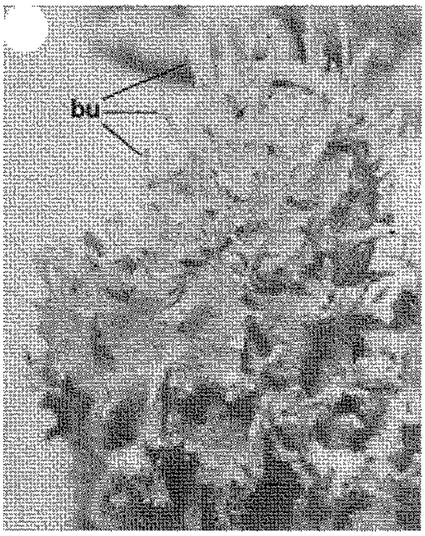


Figure 1.10 Multiple buds (bu) at the body surface of an individual of *Aplysina fistularis*. (Maldonado, *Reproduction in the Phyllum Porifera: A Synoptic Overview* 2008).

Budding is uncommon reproduction method but occur in a few sponges. For example, *Clathrina*, the free ends of the asconoid tubes are said to fell into buds, break free, and then attach and grow into another sponge. Some species of *Tethya* produce handle buds. Species of *Oscarella* and *Aplysilla* produce papillae that self-amputate and grow into new sponges (Ruppert, 2003). After days to months, freed buds attach to the bottom and give rise to a small sponge which seperate by currents and waves. The cytological composition of buds changes largely between species. A dense matrix is the most elements in buds which are of collagen fibrils, totipotent archaeocytes, and cells charged with large inclusions presumably for energy storage . They may also contain skeletal pieces and choanocytes (Maldonado, 2004).

In the fall of the year, many freshwater sponges produce hundreds to thousands of spore like gemmules. The autumn gemmules of freshwater species enter diapause, a state of near metabolic arrest, and require a period of very cold temperature before they are activated, germinate, and differentiate into a new sponge, usually in the spring (Ruppert, 2003). Gemmules also called "survival pods" which a few marine sponges and many freshwater species produce by the thousands when dying. They regularly produce in autumn by mainly freshwater species (see Figure 1.3b) (Ruppert, 2004).

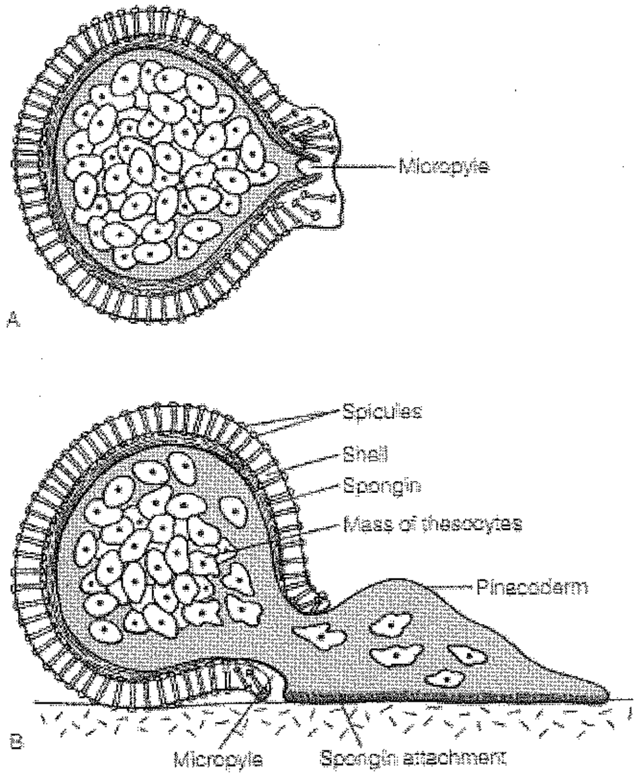


Figure 1.11 Porifera clonal reproduction: gemmules. **A**, A vertical section through a full-grown gemmule. **B**, Hatching of a gemmule. (**A**, *After Evans from Hyman*, L. H. 1940. *The Invertebrates. Vol.1. Protozoa through Ctenophora*. NewYork. 726 pp.; **B**, *Modified and redrawn from Fell*, P. E. 1997. *Poriferans, the sponges*. In Gilbert, S. F., and Raunio, A. M. (Eds.): *Embryology: Constructing the Organism*. Sinauer, Sunderland, MA. pp. 39 – 54.)

The gemmules then become motionless and they survive cold, drying out, lack of oxygen and extreme variations in salinity (Ruppert, 2004), stays cold for a few months and then reaches a near-"normal" level (Smith, 2004). Gemmules are typically produced by sponges in the fresh-water and estuarine habitats, although some marine species also generate them (Fell 1974, 1993 and Simpson, 1984). The gemmules are composed of a dense mass of totipotent archaeocytes and storage cells which surrounded by a thick protective envelope. The structure and thickness of the envelope change in largely between species (Maldonado, 2008).

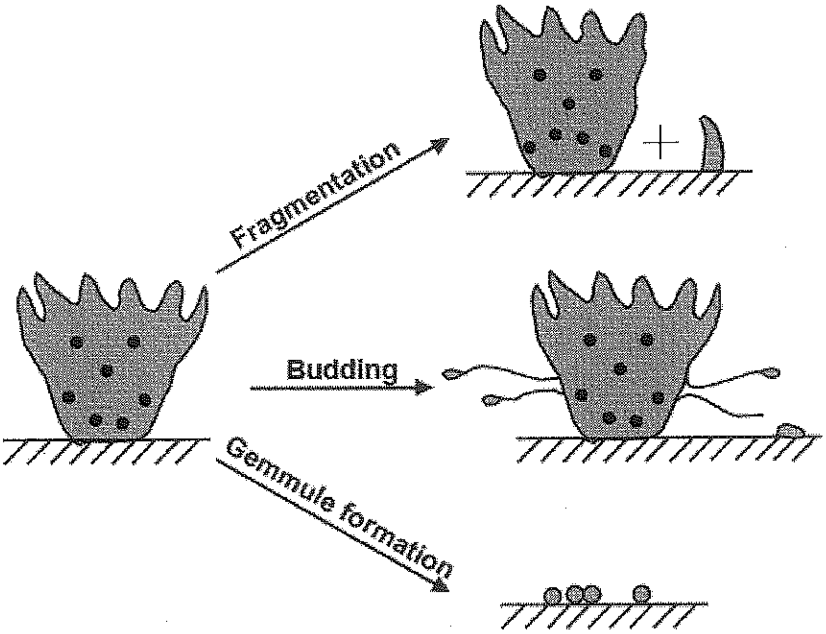


Figure 1.12 A schematic presentation of asexual reproduction of sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Also; storms, waves, animal or human activity cause accidental fragmentation of the sponge body as a mechanism for asexual propagation (see Figure 1.12) (Wulff, 1985, 1991). Interestingly, fragments of some species indicate contain developing embryos of sexual origin . Even small fragments often carry the essentials cells for not only reorganizing as small sponges, but also for nutriting the developing embryos which can successfully complete development and leave fragments as free-swimming larvae (Maldonado and Uriz, 1999).

1.3.2 Sexual Reproduction

Generally, sponges are hermaphrodites although sponges have no gonads. At the appropriate time, sperm are ovulate from one sponge and transported by water currents to another, in which fertilization occurs internally. Gametogenesis in sponges like to other animals same basic sequence of processes. The only important difference is that, in the absence of a predetermined germ line, some lines of somatic cells became gonial cells at the time of gametogenesis. However, this situation has also been shown in other lower invertebrates, such as some cnidarians, acoel flatworms, etc. (Extavour, 2007).

Sperm are produced by choanocytes or entire choanocyte chambers that sink into the mesohyl and form spermatic cysts while eggs are formed by transformation of archeocytes, or of choanocytes in some species. Eggs arise from archeocytes or choanocytes (some calcareous sponges). Each egg generally aggregate its yolk by phagocytosis of adjacent nurse cells. The egg and nurse cells together may be surrounded in a follicle of ensheathing cells. During spawning, sperm breaking off the wall of the spermatic cyst. Then they enter the excurrent canals (or atrium), and are released from the oscula (Ruppert, Fox and Barney, 2004).

The sperm drift into and contact with another sponge, then they are swept into its aquiferous system by the incurrent water flow. They arrive aquiferous system, and are transported to the choanoderm or choanocyte chamber. The choanocyte cell loses its flagellum and collar so becomes ameboid, and transports the sperm head (nucleus) to the egg. The transformed ameboid choanocyte is called a carrier cell. The larvae that develop from embryos are also diverse and are described under the names coeloblastula, amphiblastula, parenchymella, and trichimella larvae (see Figure 1.13) (Ruppert, Fox and Barney, 2004).

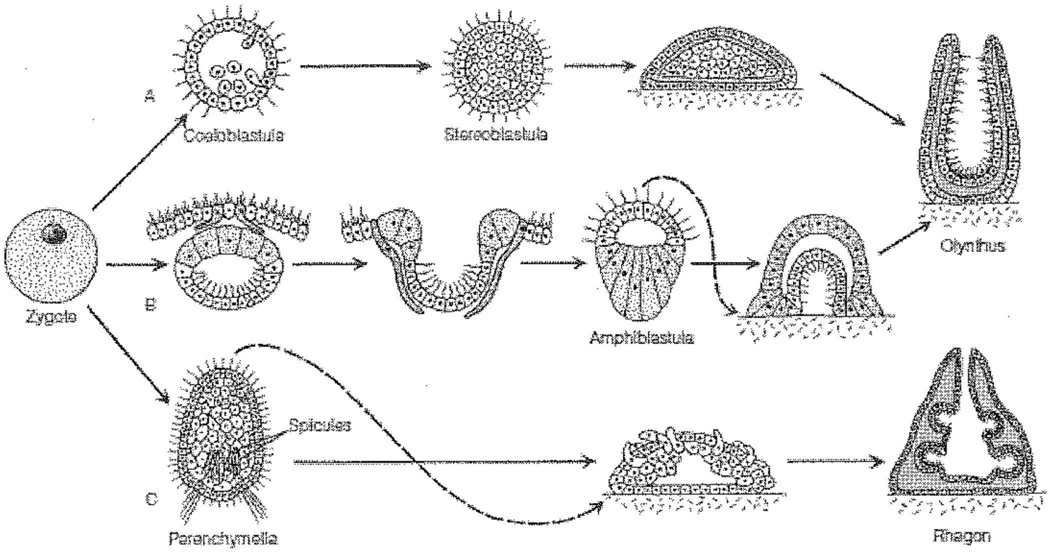


Figure 1.13 Porifera sexual reproduction: larval development and metamorphosis. In **A**, *Clathrina* (Calcarea: Calcinia), **B**, *Sycon* (Calcarea: Calcaronea), **C**, *Haliclona* (Demospongiae) (*Invertebrate Zoology, 7th Ed.* by Ruppert, Fox, & Barnes, 2004).

A coeloblastula larva is produced by calcareous sponges, such as species of Clathrina (see Figure 1.13 A). This larva has a cavity which composed of a single layer of flagellated cells.While in the plankton, some of the surface cells lose their flagella then become ameboid, and enter the blastocoel, eventually obliterating it (Ruppert, Fox and Barney, 2004).

An amphiblastula larva occurs in other calcareous sponges, for example, Grantia, Sycon, and Leucosolenia (Calcaronea, see Figure 1.13 B). After fertilization, the egg (zygote) divides to form an internally flagellated sphere that resembles choanocyte chamber (Ruppert, Fox and Barney, 2004).

In most demosponges, a parenchymella larva is characteristic (see Figure 1.13 C). In this case, the embryo develops directly into asolid mass of cells, forming a stereoblastula. The outer layer is composed of widespread flagellated cells interspersed with occasional vesicle-containing cells that lack flagella. Trichimella larvae typify the glass sponges. These are stereoblastulae that bear a band of flagellated cells around the equator of the larval body (Ruppert, Fox and Barney, 2004).

1.4 ECOLOGY AND DIVERSITY

1.4.1 Habitats

Sponges have lived all aquatic habitats, those with a calcium carbonate skeleton are limited, by factors controlling calcium secretion, to shallower waters; those with fibrous and siliceous skeletons occur from intertidal to abyssal depths. In some habitats sponges are the dominant macrobenthic organisms and so they contributing up to 80% of the biomass. Consequently, their activities effect marine ecological processes at macro- and microlevels (Bergquist, 2001). Most sponges live in quiet and clear waters, because sediment stirred up by waves or currents would block their pores and this cause difficult for them to feed and breathe (Krautter, 1998).

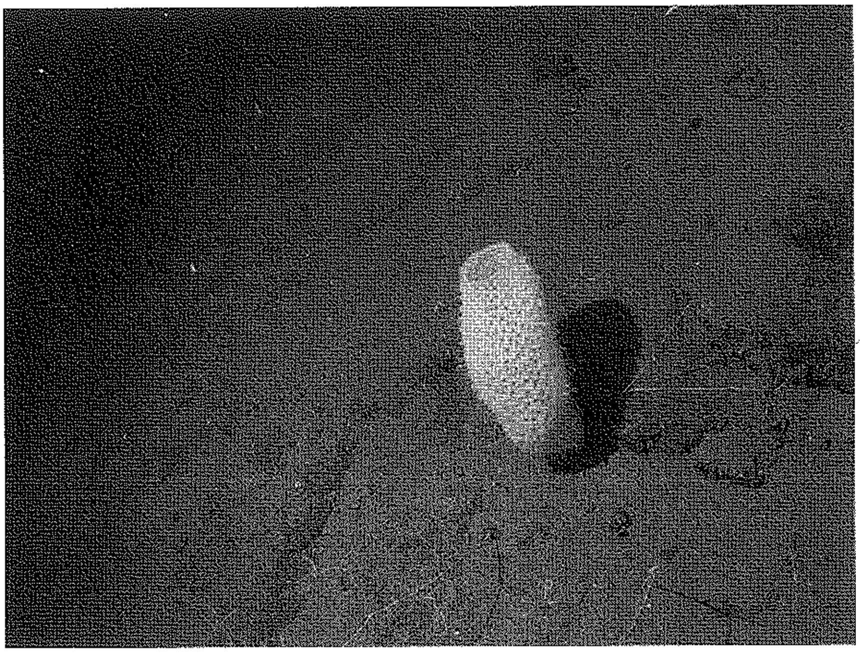


Figure 1.14 *Euplectella aspergillum* at 2572 meters water depth. California, Davidson Seamount (*NOAA Photo Library,NOAA/Monterey Bay Aquarium Research Institute,* 2002).

Some of the largest and longest living sponges (up to 300 years) are in the Southern Polar Regions. Under the ice shelf, communities have been studied directly and the age of individuals determined. In these still waters the siliceous spicules shed over long periods form a mobile mat several meters deep (Bergquist, 2001). Most sponges are generally lived on firm surfaces such as rocks, but some sponges can attach themselves to soft sediment by means of a root-like base (Weaver et al., 2007). Some sponges are attached to the base rock below the ice, but conditions are inappropriate for other organisms, particularly their echinoderm predators. Macrosponges in such habitats structure their immediate environment (Bergquist, 2001). Sponges are more plenty but less diverse in temperate waters than in tropical waters, possibly because organisms hunt sponges that are more abundant in tropical waters (Ruzicka et al., 2008). In all oceans, from tropics to polar regions, bad sponges occur and dig calcareous substrates. In temperate waters they occur commonly bore galleries in mollusc shells and coralline algae. However, in coral reef environments, bioerosion by the many species belonging to the genus Cliona removes 6–7 kg of calcium carbonate from the coral substrate in 100 days. This, depending upon the density of the sponge population, equates to removal of a coral layer 0.1mm to 1.0m deep per year (Bergquist, 2001).

1.4.2 Diversity

It is more difficult to estimate the number of species sponges than other invertebrate taxa. Since sponges do not have defined body regions, symmetry or appendages, deriving a robust, natural classification of the group has been and remains a problem. But best estimates at present are that there are around 9000 described species and an equal number remaining to be describe (Bergquist, 2001).

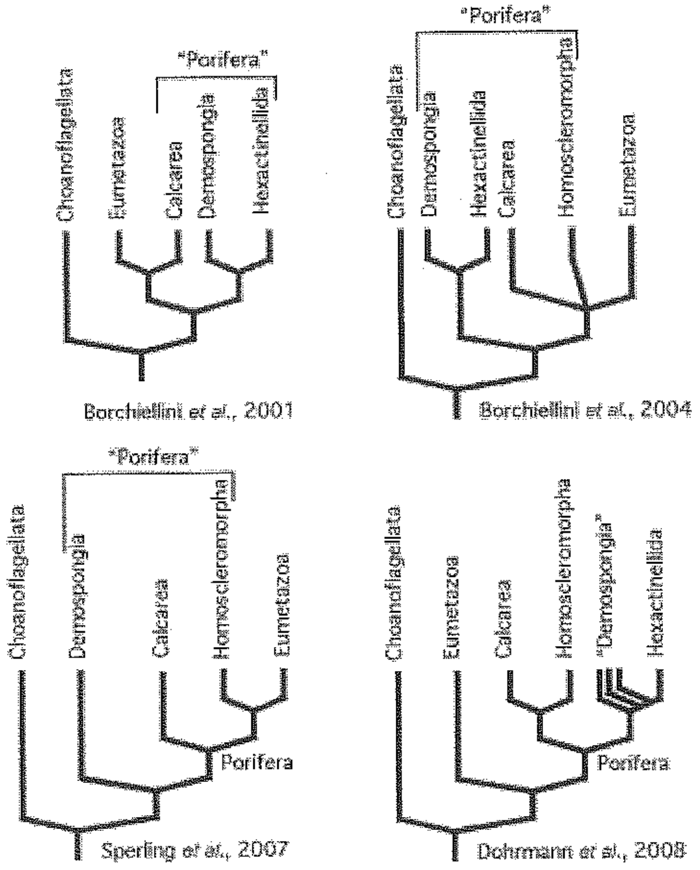


Figure 1.15 Different thesis phylogenesis of Porifera.

CHAPTER 2

SPONGES AND BIOTECHNOLOGY

2.1 INTRODUCTION

Recently, sponges have achieved renewed interest due to many secondary metabolites with potential pharmaceutical applications that have been discovered. Some of them, for example manoalide and halichondrin B were harvested in large quantities for clinical trials (Kernan et al., 1987; Litaudon et al., 1997). Marine sponges have been considered as a gold mine during the past fifty years, in connection with the diversity of their secondary metabolites. The biological effect of new metabolites which produced from sponges has been reported in hundreds of scientific researchs. In this context, sponges could provide potential future drugs against important diseases, such as cancer, a range of viral diseases, malaria and inflammations (Sipkema et al., 2004).

2.2 BIOACTIVE METABOLITES

Many sponges produce important seconder metabolites which prevent settlement of other organisms on their surfaces or deter grazing predators. Nine out of 16 Antarctic sponges and 27 of 36 Caribbean species were found to be toxic to fish (Ruppert et al., 2004). Between marine invertebrates, sponges appear to be one of the richest phyla in toxicogenic species. In an increasing number of cases it has been demonstrated that this toxicity is associated with the presence of specific secondary metabolites which may act to minimize predation Marine sponges are a rich source of structurally novel and biologically active secondary metabolites and to date many chemically unique compounds with different biological activities have been isolated (Darah et al., 2011).

Secondary metabolites that produced by sponges play defensive role against predation, infections and fouling (Newbold et al., 1999). Marine sponges are shown antibacterial, antifungal, antiplasmodial, antihelminthic, antiamoebitic, antileishmanial (Sipkema, 2004), as well as anti-inflammatory and anticancer (Xue et al., 2004) activities. The sponges produce the largest number and diversity of secondary metabolites, even though the functions of these secondary metabolites are unknown. There are more than 5000 different compounds have been isolated from about 500 species of sponges, and about 800 antibiotic compounds have been isolated from them (Rifai, 2005).

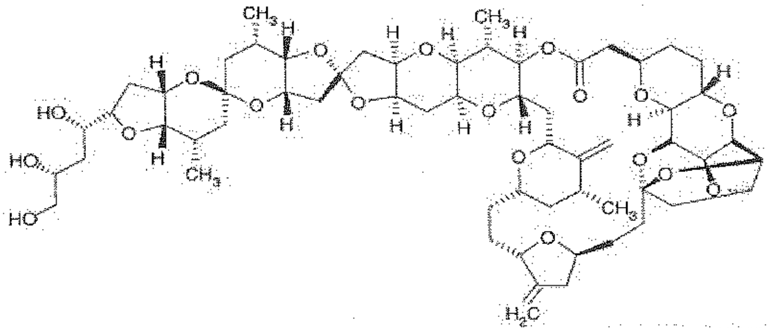

TRENDS in Biotechnology

Figure 2.1 Halichondrin. It is a natural product with anti-tumor activities isolated from the sponge Lissodendoryx sp. (René H. Wijffels, *Potential of sponges and microalgae for marine biotechnology*, 2008).

2.3 MARINE SPONGES AND PHARMACY

Using sponges at pharmacy goes back to Alexandrian physicians and was thoroughly describes by the Roman historian Plinius. Physicians used sponges that were saturated with iodine to stimulate coagulation of the blood, or with bioactive plant extracts to anaesthetise patients (Sipkema, 2004). Plinius recommended to using seconder metabolites from sponges against sunstrokes and they were used against all kinds of wounds, bone fractures, dropsy, stomach aches, infectious diseases, and testicle tumors (Hofrichter and Sidri, 2001) and using after breast operations (Arndt, 1938). At 18th century; Russian, Ukrainian and Polish physicians use a fresh-water sponge, they call Badiaga (see Figure 2.2), for the treatment of patients (Nozeman, 1788).

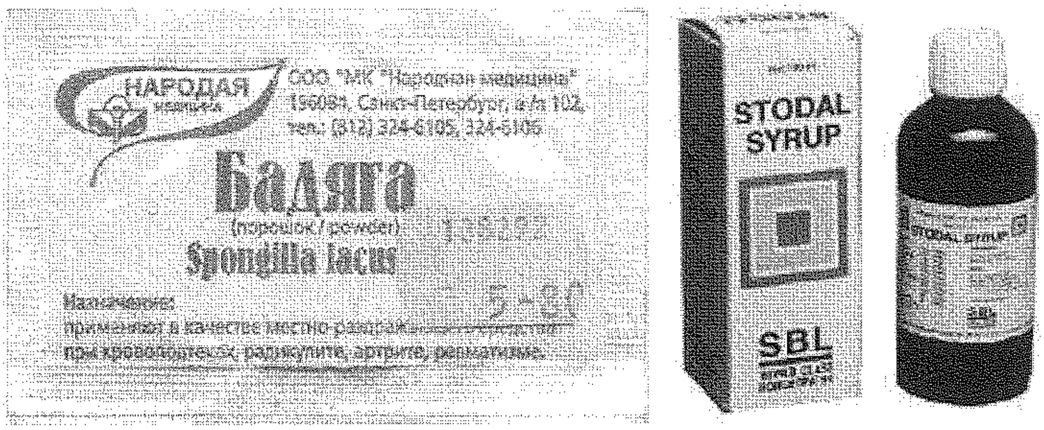


Figure 2.2 Examples of homeopathic drugs based on sponge extracts. That are still used at present (Badiaga and Stodal syrup) (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

The dry powder produced by sponge is scoured in on the chest or back against lung diseases or a cough, or in case of foot and leg aches (e.g. rheumatism) (Schröder, 1991). Badiaga is not really one sponge, but different mixtures of several fresh-water sponges depending on the region (Oficjalski, 1937). Presently, Stodal syrup containing roasted *Spongia officinalis*, is used for homeopathic treatment of dry and asthmatic cough in the Western world (Stodal, 2003). These nucleosides uses for the synthesis of Ara-C, the first marine derived anticancer agent and the antiviral drug Ara-A (Proksch et al., 2002). Ara-C is used in the routine treatment of patients with leukaemia and lymphoma in the present. Sponges are called champion producers, concerning the diversity of products that have been found. They are responsible for more than 5300 different products and every year hundreds of new compounds are being discovered (Faulkner 2000; 2001; 2002).

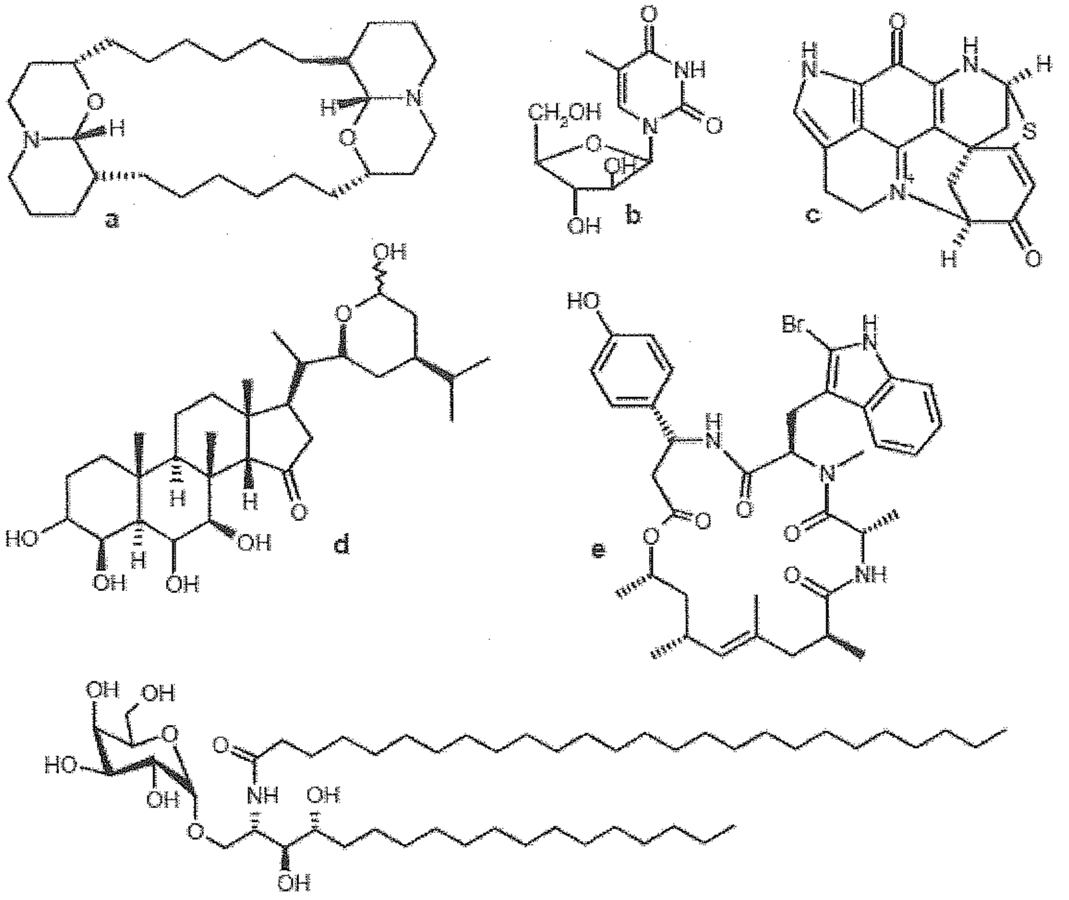
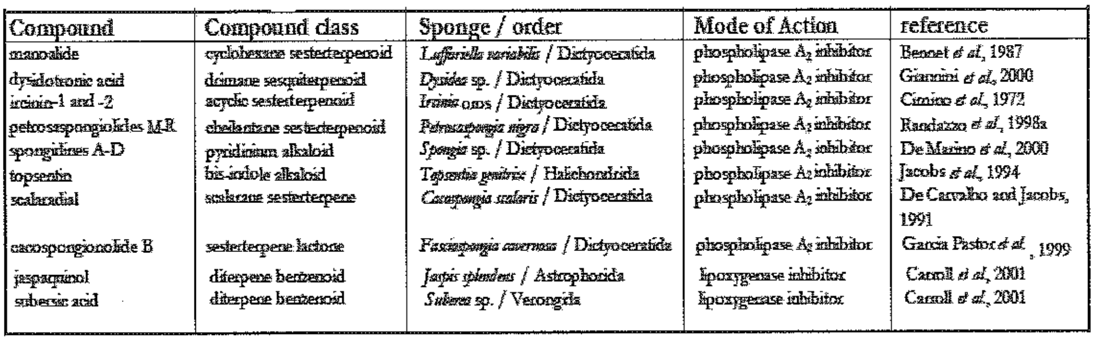


Figure 2.3 Chemical diversity of sponge-derived molecules: a xestospongin C (Xestospongia sp. / macrocyclic bis-oxaquinolizidine); b spongothymidine (Cryptotethia crypta / unusual nucleoside); c discorhabdin D (Latrunculia brevis; Prianos sp. / fused pyrrolophenanthroline alkaloid); d contignasterol (Petrosia contignata / oxygenated sterol); e jaspamide (Hemiastrella minor / macrocyclic lactam/lactone); f agelasphin (Agelas mauritianus / α-galactosylceramide) (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

2.3.1 Anti-inflammatory Compounds

Sponges important source of antiinflammatoric compounds (See Table 2). Manoalide was one of the first sesterterpenoids to be isolated from a marine sponge (Luffariella variabilis) and was found to be an antibiotic (De Silva and Scheuer, 1980) and analgesic (Mayer and Jacobs, 1988) molecule. In addition, it has been studied most extensively with regard to its antiinflammatory properties (Bennet et al., 1987). The antiinflammatory effect is based on the irreversible inhibition of the release of arachidonic acid from membrane phospholipids by preventing the enzyme phospholipase A2 from binding to the membranes (Glaser et al., 1989).

Table 2.1 Examples of antiinflammatory products from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).



Compound	Compound class	Sponge / order	Mode of Action	reference
manoalide	cyclohexane sesterterpenoid	*Luffariella variabilis* / Dictyoceratida	phospholipase A_2 inhibitor	Bennet *et al.*, 1987
dysidotronic acid	drimane sesquiterpenoid	*Dysidea* sp. / Dictyoceratida	phospholipase A_2 inhibitor	Giannini *et al.*, 2000
ircinin-1 and -2	acyclic sesterterpenoid	*Ircinia* oros / Dictyoceratida	phospholipase A_2 inhibitor	Cimino *et al.*, 1972
petrosaspongiolides M-R	cheilantane sesterterpenoid	*Petrosaspongia nigra* / Dictyoceratida	phospholipase A_2 inhibitor	Randazzo *et al.*, 1998a
spongidines A-D	pyridinium alkaloid	*Spongia* sp. / Dictyoceratida	phospholipase A_2 inhibitor	De Marino *et al.*, 2000
topsentin	bis-indole alkaloid	*Topsentia genitrix* / Halichondrida	phospholipase A_2 inhibitor	Jacobs *et al.*, 1994
scalaradial	scalarane sesterterpene	*Cacospongia scalaris* / Dictyoceratida	phospholipase A_2 inhibitor	De Carvalho and Jacobs, 1991
cacospongionolide B	sesterterpene lactone	*Fasciospongia cavernosa* / Dictyoceratida	phospholipase A_2 inhibitor	Garcia Pastor *et al.*, 1999
jaspaquinol	diterpene benzenoid	*Jaspis splendens* / Astrophorida	lipoxygenase inhibitor	Carroll *et al.*, 2001
subersic acid	diterpene benzenoid	*Suberea* sp. / Verongida	lipoxygenase inhibitor	Carroll *et al.*, 2001

Incrise in the intracellular arachidonic acid concentration would allow to upregulation of the synthesis of inflammation mediators as prostaglandins and leukotrienes (see Figure 2.4).

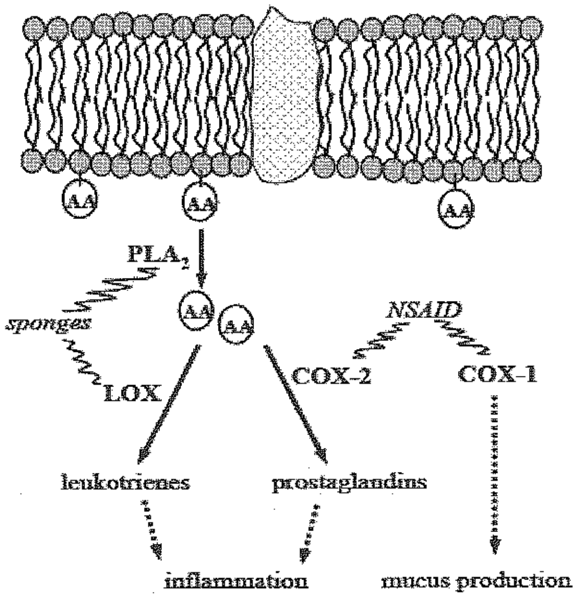


Figure 2.4 The inflammatory cycle inside the cell. Phospholipase A2 (PLA2) catalyses the release of membrane-bound arachidonic acid (AA) to free arachidonic acid. Arachidonic acid is converted to leukotrienes and prostaglandins by lipoxygenase (LOX) and cyclooxigenase-2 (COX-2) respectively. Sponge-derived antiinflammatory molecules are mainly inhibitors of PLA2 or LOX, while non-steroidal antiinflammatory drugs inhibit COX-2, but also the constitutive COX-1. (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

2.3.2 Antitumor Compounds

Some of isolated sponge compounds are inhibitors of protein kinase C (PKC). PKC inhibitors is important and famous compound that evidence that too high levels of PKC enzyme are both involved in the pathogenesis of arthritis and psoriasis (due to regulation of phospholipase A2 activity), and in tumour development (Bradshaw et al., 1993; Yoshiji et al., 1999). PKC is the receptor protein of tumour which promoting phorbol esters, and PKC inhibitors prevent binding of carcinosarcoma cells to the endothelium (Liu et al., 1991). Also PKC inhibitors and fucosyl transferase inhibitors, some anticancer molecules with a different mode of action have been discovered in marine sponges (see Table 3). These compounds can be divided in three classes:

1. non-specific inhibitors of cell growth.
2. specific inhibitors of cancer cells.
3. inhibitors of cancer cells of a certain type of cancer (as the aforementioned PKC inhibitors) (Sipkema, 2004).

Table 3 Examples of antitumour products from sponges (*Cultivation of marine sponge: From sea to cell* (Sipkema, 2004).

Compound	**Compound class**	**Sponge / order**	**Mode of Action**	**reference**
spongiacidin B	pyrrole alkaloid	*Hymeniacidon* sp. / Halichondrida	cyclin-dependent kinase 4 inhibitor	Inaba *et al*, 1998
mycalamide A and B	polyether amide (pederin-like)	*Mycale* sp. / Poecilosclerida	protein synthesis inhibitor	Burres and Clement, 1989
girodazole	imidazole alkaloid	*Cymbastela cantharella* / Halichondrida	protein synthesis inhibitor	Ahond *et al*., 1988; Colson *et al*., 1992
aragusterol A	sterol	*Xestospongia* sp. / Haplosclerida	protein synthesis inhibitor	Iguchi *et al*., 1993; Fukuoka *et al*, 2000
neoamphimedine	pyridoacridine alkaloid	*Xestospongia* cf. *carbonaria* / Haplosclerida	topoisomerase II inhibitor	De Guzman *et al*., 1999
elenic acid	alkylphenol	*Plakinastrella* sp. / Homosclerophorida	topoisomerase II inhibitor	Juagdan *et al*., 1995
naamine D	imidazole alkaloid	*Leucetta* cf. *chagosensis* / Calcinea	nitric oxide synthetase inhibitor^{2}	Dunbar *et al*., 2000
agelasphin (KRN7000)	α-galactosylceramide	*Agelas mauritianus* / Agelasidae	NKT cell activator	Shimosaka, 2002
agosterol A	sterol	*Spongia* sp. / Dictyoceratida	reverses drug resistancy of cancer cells	Aoki *et al*., 1998

Many non-specific cell growth inhibitors have been explored in sponges. They used to treat cancer under certain conditions, but they also affect the division of healthy cells (Sipkema, 2004). In addition to these triterpenoid hydroquinones, a number of potent microtubule-interfering compounds have been discovered in marine sponges, such as discodermolide (Ter Haar et al., 1996).

The first natural 6-hydroximino-4-en-3-one steroids were isolated from Cinachyrella spp. (Rodriguez et al., 1997) and they used against a specific type of cancer. They displayed high affinity to aromatase (Holland et al., 1992), which is the rate-limiting enzyme that catalyses the conversion of androgens to estrogens (see Figure 2.3d)

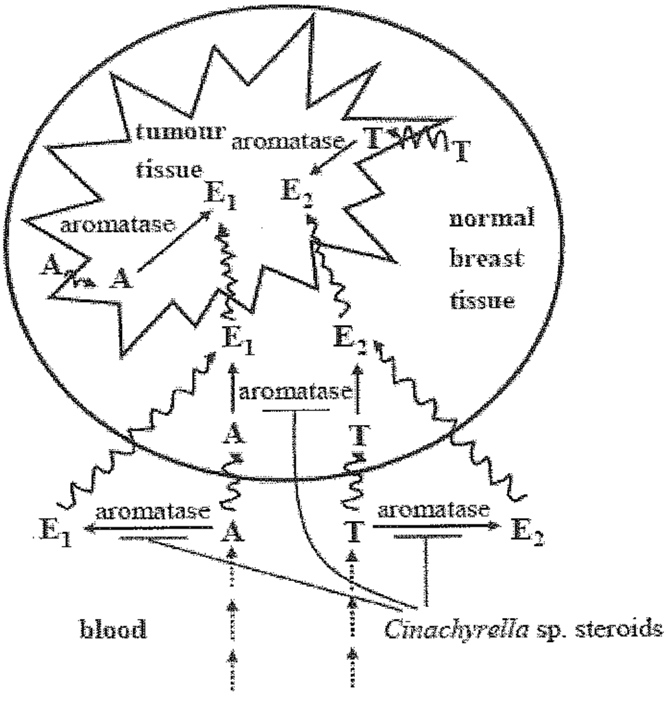


Figure 2.5 The inhibition of breast cancer by *Cinachyrella* sp. steroids. Aromatase is the key enzyme in the formation of the estrogens estrone (E1) and estradiol (E2). It catalyses the final steps, from androstenedione (A) to estron and from testosterone (T) to estradiol, in the estogen pathway. Estrogen conversion can occur in the blood, in normal breast tissue as well as in breast tumour tissue (adapted from Geisler, 2003). The 6-hydroximino-4-en-3-one steroids from Cinachyrella sp. are inhibitors of aromatase. (*Cultivation of marine sponge: From sea to cell*. Sipkema, 2004)

2.3.3 Immunosuppressive Effect

The downregulation of T-cells by nitric oxide synthetase inhibitors are interesting compounds to suppress the immune system, and they diminish the fierceness of migraine attacks and they are used treatment of cancer (Griffith and Gross, 1996). There are also other side-effects, such as hypertension, dyslipidemia, hyperglycemia, peptic ulcers, lipodystrophy, moon face, liver and kidney injury. The immunosuppressive drugs also interact with other medicines and affect their metabolism and action. Actual or suspected immunosuppressive agents can be evaluated in terms of their effects on lymphocyte subpopulations in tissues using immunohistochemistry (see Figure 2.6) (Sipkema, 2004).

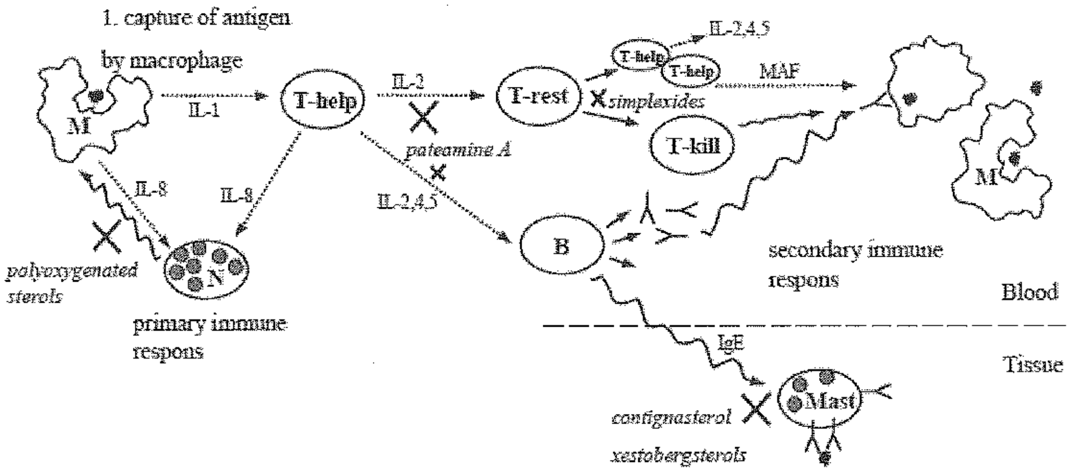


Figure 2.6 Simplified representation of the immune response by the macrophages (M). Both macrophages, but especially T-helper cells (T-help) secrete many interleukins (IL-x) or macrophage activation factor (MAF), to trigger the primary immune response via neutrophils (N), or the secondary immune respons by activating resting T-cells (T-rest) and B cells (B). Activated B cells secrete antibodies that bind to macrophages which have phagocytised an antigen and they are subsequently destroyed by T-killer cells (T-kill). Mast cells (Mast) release histamine. (*Cultivation of marine sponge: From sea to cell*. Sipkema, 2004).

Pateamine A, produced by a Mycale sp., inhibits the production of interleukin-2 (Romo et al., 1998) and thereby the activation of resting T-cells and B-cells to a lesser extent. Contignasterol from Petrosia contignata (Burgoyne and Andersen, 1992) inhibits allergen-induced histamine release from rat mast cells (Takei et al., 1994) and from guinea-pig lung tissue in vitro and the activation of eosinophils into airways in guinea-pigs and could be used to treat asthma (Langlands et al., 1995).

Table 4 Examples of immunosuppressive products from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Compound	**Compound class**	**Sponge / order**	**Mode of Action**	**reference**
simplexides	glycolipid	*Plakortis simplex* / Homosclerophorida	Inhibitor of T-cell proliferation	Costantino *et al.*, 1999
polyoxygenated sterols	sterol	*Dysidea* sp. / Dictyoceratida	IL-8 inhibitor	Leone *et al.*, 2000
contignasterol	oxygenated sterol	*Petrosia contignata* / Haplosclerida	histamine release inhibitor	Takei *et al.*, 1994; Bramley *et al.*, 1995
xestobergsterols A and B	pentacyclic sterol	*Xestospongia bergquistia* / Haplosclerida	histamine release inhibitor	Shoji *et al.*, 1992
taurodispacamide A	pyrrole-imidazole alkaloid	*Agelas oroides* / Agelasida	IL-2 inhibitor	Fattorusso and Taglialatela-Scafati, 2000
pateamine A	thiazole macrolide	*Mycale* sp. / Poecilosclerida	IL-2 inhibitor	Northcote *et al.*, 1991

2.3.4 Neurosuppressive Effect

Keramidine was isolated from an *Agelas* sp. (Nakamura et al., 1984), it includes neurosuppressive compounds that have been isolated from marine sponges. It is a serotonergic receptor antagonist and they are related to; platelet aggregation and so may be beneficial against thrombosis (Ruomei et al., 1996), smooth muscle contraction, throwing up due to their presence in the gastrointestinal tract (Lang and Marvig, 1989), serotonergic receptor antagonists function as an antidepressant drug in the brain (see Table 5) (Nagayama et al., 1980).

Table 5 Examples neurosuppressives and muscle relaxants from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Compound	**Compound class**	**Sponge / order**	**Mode of Action**	**reference**
dysiherbaine	unusual amino acid	*Dysidea herbacea* / Dictyoceratida	neurotoxin	Sakai *et al.*, 1997
keramadine	pyrrole-guanidine alkaloid	*Agelas* sp. / Agelasida	serotonergic receptor antagonist	Nakamura *et al.*, 1984
1-methylisoguanosine	nucleoside analogue	*Tedania digitata* / Poecilosclerida	muscle relaxant, antiallergic	Quinn *et al.*, 1980
xestospongin C	macrocyclic bis-oxaquinolizidine	*Xestospongia* sp. / Haplosclerida	IP_3-inhibitor	De Smet *et al.*, 1999
okinonellin B	furanosesterterpenoid	*Spongionella* sp. / Dendroceratida	muscle relaxant	Kato *et al.*, 1986
bromotopsentin	bis-indole alkaloid	*Spongosorites* sp. / Halichondrida	α1 adrenic receptor antagonist	Phife *et al.*, 1996
penaresidin A	azetidine alkaloid	*Penares* sp. / Astrophorida	actomyosin ATPase inhibitor	Kobayashi *et al.*, 1991
S1319	benzothiazole derivative	*Dysidea* sp. / Dictyoceratida	antiasthmatic, uterine relaxation	Suzuki *et al.*, 1999

2.3.5 Antiviral Compounds

Sponges are also has antiviral compounds. The high number of HIV-inhibiting compounds that has been discovered does not reflect the huge potential of sponges to fight AIDS compared to other viral diseases, but rather the interest of many researchers (Sipkema, 2004). Papuamides C and D, haplosamates A and B and avarol (Müller et al., 1987), which has also been discovered as antipsoriasis (Müller et al., 1991), are examples of HIV-inhibiting compounds produced from different sponges. Avarol is example of compounds of which the mechanism how it blocked progression of HIV infection (Müller et al., 1987).

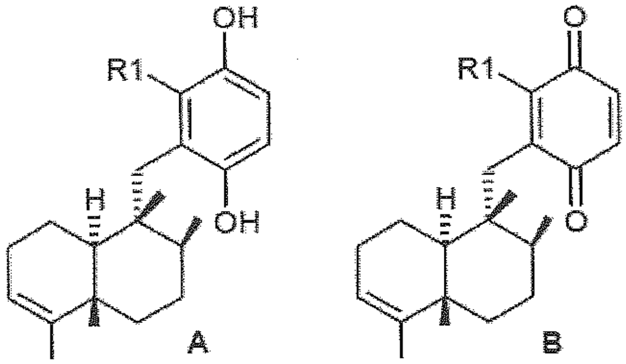
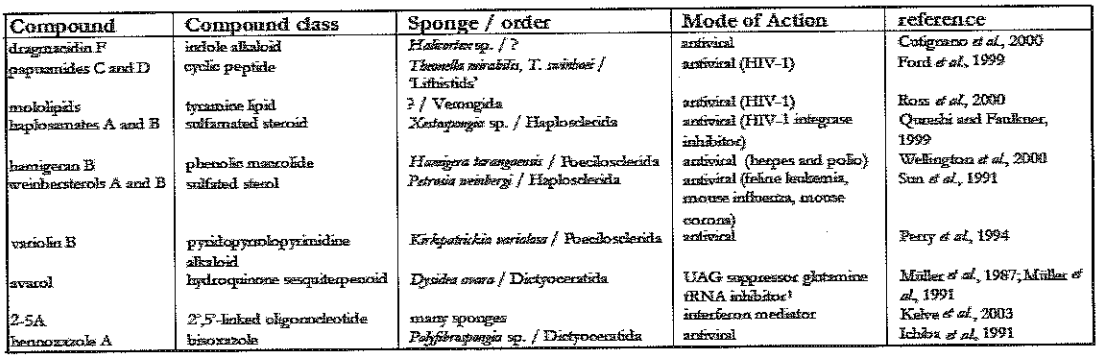


Figure 2.7 Molecular structures of avarol (a: R1 = H), 6'-hydroxy avarol (a: R1 = OH), avarone (b: R1 = H) and 3'-hydroxy avarone (b: R1 = OH) (*Cultivation of marine sponge: From sea to cell*. Sipkema, 2004).

Table 6 Examples of antiviral products from sponges (*Cultivation of marine sponge: From sea to cell*. Sipkema, 2004).



Compound	Compound class	Sponge / order	Mode of Action	reference
dragmacidin F	indole alkaloid	*Halicortex* sp. / ?	antiviral	Cutignano *et al.*, 2000
papuamides C and D	cyclic peptide	*Theonella mirabilis*, *T. swinhoei* / 'Lithistids'	antiviral (HIV-1)	Ford *et al.*, 1999
mololipids	tyramine lipid	? / Verongida	antiviral (HIV-1)	Ross *et al.*, 2000
haplosamates A and B	sulfamated steroid	*Xestospongia* sp. / Haplosclerida	antiviral (HIV-1 integrase inhibitor)	Qureshi and Faulkner, 1999
hamigeran B	phenolic macrolide	*Hamigera taragaensis* / Poecilosclerida	antiviral (herpes and polio)	Wellington *et al.*, 2000
weinbersterols A and B	sulfated sterol	*Petrosia weinbergi* / Haplosclerida	antiviral (feline leukemia, mouse influenza, mouse corona)	Sun *et al.*, 1991
variolin B	pyridopyrrolopyrimidine alkaloid	*Kirkpatrickia variolosa* / Poecilosclerida	antiviral	Perry *et al.*, 1994
avarol	hydroquinone sesquiterpenoid	*Dysidea avara* / Dictyoceratida	UAG suppressor glutamine tRNA inhibitor¹	Müller *et al.*, 1987; Müller *et al.*, 1991
2-5A	2',5'-linked oligonucleotide	many sponges	interferon mediator	Kelve *et al.*, 2003
benzoxazole A	bisoxazole	*Polyfibrospongia* sp. / Dictyoceratida	antiviral	Ichiba *et al.*, 1991

2.3.6 Antimalarial Compounds

New antimalarial compounds produced by sponges are needed to cope with the increasing number of multidrug resistant Plasmodium strains that cause malaria. Plasmodium falciparum has become resistant against chloroquinone, pyrimethamine and sulfadoxine (Bwijo et al., 2003). Kalihinol A from a Acanthella sp. (Miyaoka et al., 1998) and a number of terpenoid isocyanates, isothiocyanates and isonitriles from Cymbastela hooperi (König et al., 1996) shown selective in vitro antimalarial activity against P. falciparum. Many antimalarial compounds which produced by sponge has been discovered during the last decade (see Table 7).

Table 7 Examples of antimalarial products from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Compound	Compound class	Sponge / order	Mode of Action	reference
axisonitrile-3	sesquiterpenoid isocyanide	*Acanthella klethra* / Halichondrida	antimalarial	Angerhofer *et al*, 1992
manzamine A	manzamine alkaloid	e.g. *Haliclona* sp. / Haplosclerida	immune stimulator	Ang *et al*, 2001
	diterpene isocyanates, isothiocyanates and isonitriles	*Cymbastela hooperi* / Halichondrida	antimalarial	König *et al*, 1996
	norditerpenoid and norsesterterpenoid endoperoxides	*Diacarnus levii* / Poecilosclerida	antimalarial	D' Ambrosio *et al*, 1998
kalihinol A	isonitril-containing kalihinane diterpenoid	*Acanthella* sp. / Halichondrida	antimalarial	Miyaoka *et al*, 1998

These epidioxy-substituted norditerpenes and norsesterterpenes shown selective activity active against both chloroquine-sensitive and chloroquine resistant P. falciparum strains (D' Ambrosio et al., 1998). Also the Manzamines recently discovered compounds, antimalarial compound that have been discovered in a number of sponges (Sakai et al., 1986).

2.3.7 Antibiotics and Fungicides

The first detection included 31 sponges tested, 18 showed antimicrobial effects of which some were very strong against a range of Gram positive and Gram-negative bacteria (Burkholder and Ruetzler, 1969). Recently, new sponge-derived antibiotics was shown by the inhibitory effect of arenosclerins A-C from Arenosclera brasiliensis on 12 antibiotic-resistant bacteria that were isolated from a hospital (Torres et al., 2002).

Many new compounds with antibiotic properties are discovered every year, but in marine sponges their ubiquitousness is remarkable (see Table 8).

Table 8 Examples of antibacterial and antifungal products from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Compound	Compound class	Sponge / order	Mode of Action	reference
discodermins B, C, and D	cyclic peptide	*Discodermia kiiensis* / 'Lithistids'	antibacterial	Matsunaga *et al.*, 1985
topsentiasterol sulfates A-E	sulfated sterol	*Topsentia* sp. / Halichondrida	antibacterial/antifungal (D and E)	Fusetani *et al.*, 1994
arenosclerins A, B, and C	alkylpiperidine alkaloid	*Arenosclera brasiliensis* / Haplosclerida	antibacterial	Torres *et al.*, 2002
axinellamines B-D	imidazo-azolo-imidazole alkaloid	*Axinella* sp. / Halichondrida	antibacterial	Urban *et al.*, 1999
acanthosterol I and J	sulfated sterol	*Acanthodendrilla* sp. / Dendroceratida	antifungal	Tsukamoto *et al.*, 1998
oceanapiside	bisaminohydroxylipid glycoside	*Oceanapia phillipensis* / Haplosclerida	antifungal	Nicolas *et al.*, 1999
spongistatin	polyether macrolide lactone	*Hyrtios erecta* / Dictyoceratida	antifungal	Pettit *et al.*, 1998
leucascandrolide A	oxazole-containing polyether macrolide	*Leucascandra caveolata* / Calcaronea	antifungal	D'Ambrosio *et al.*, 1996

Generally used fungicides, are less different than antimicrobials and the use of many of them is limited due to toxic effects to humans, animals and plants (Nakagawa and Moore, 1995).

2.3.8 Antifouling Compounds

The compounds used environmentally friendly substitutes of chemical antifoulants. Biofouling organisms such as blue mussels, barnacles and macroalgae cause serious problems to ship's hulls, cooling systems of power plants and aquaculture materials (Holmes, 1970). Recently discovered of bioactive compounds from marine sponges are antifouling molecules (see Table 9).

Table 9 Examples of antifouling products from sponges (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

Compound	Compound class	Sponge / order	Mode of Action	reference
C_{22} ceramide	ceramide	*Haliclona koremella* / Haplosclerida	antifouling	Hattori et al., 1998
	sterol diperoxide	*Lendenfeldia chondrodes* / Dictyoceratida	antifouling	Sera et al., 1999
ceratinamide A and B	bromotyrosine derivative	*Pseudoceratina purpurea* / Verongida	antifouling	Tsukamoto et al., 1996a
pseudoceratidine	dibromopyrrole-containing spermidine derivative	*Pseudoceratina purpurea* / Verongida	antifouling	Tsukamoto et al., 1996b

2.4 ECOLOGICAL ROLE OF SECONDER METABOLITES

Example of the benefits of secondary metabolites for the sponge itself, is the presence of antifouling products. In order to safeguard the water pumping capacity, sponges cannot tolerate biofilm formation or settlement of barnacles or bryozoans on their surface (Proksch, 1994). The level of cytotoxicity of some seconder sponge products is high enough to even create a bare zone around the sponge that is maintained by the emission of a mucus containing the toxins (Sullivan et al., 1981). Secondary metabolites defend the organism against predation, which is especially important for physically unprotected sessile organisms like sponges (Becerro et al., 1997). Relatively some animals, such as the hawksbill turtle and some highly evolved teleost fishes (Meylan, 1990) are largely dependent on sponges for their diet. Also some nudibranches feed on sponges and they even manage to use the sponge's metabolites for their own chemical defence (Pawlik et al., 1988). However, these spongivores shown only a tiny fraction of the animals inhabiting the seas. Secondary metabolites can also protect their producers against bacteria, fungi or parasites (see Figure 2.8) (Davies, 1992).

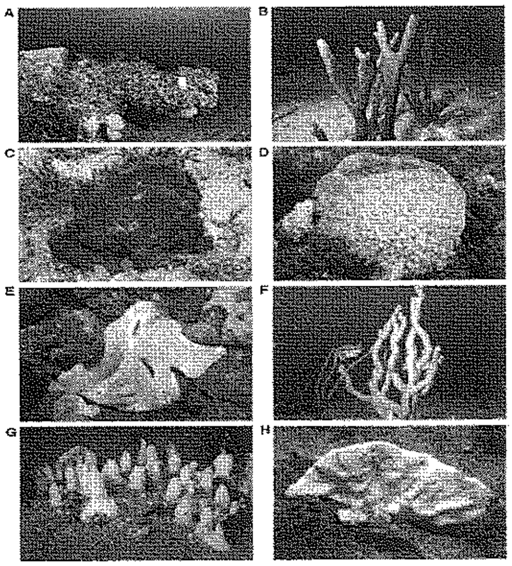


Figure 2.8 Examples of the scarp (A) and plateau (B) habitat on SAB reefs and 3 representative scarp sponge phenotypes: C) Amorphous (I.felix); D) Vase (Ircinia campana); E) Encrusting (C.collectrix); and 3 representative plateau sponge phenotypes: F) Arborescent (P.walpersi); G) Digitate (A.ambrosia); H) Pedunculate (A.waltonsmithi); Photographs by Rob Ruzicka (B, E, F, G, H), Greg McFall (A, C, D) (Ruzicka, Gleason, *Sponge community structure and anti-predator defenses on temperate reefs of the South Atlantic Bight*, 2009).

CHAPTER 3

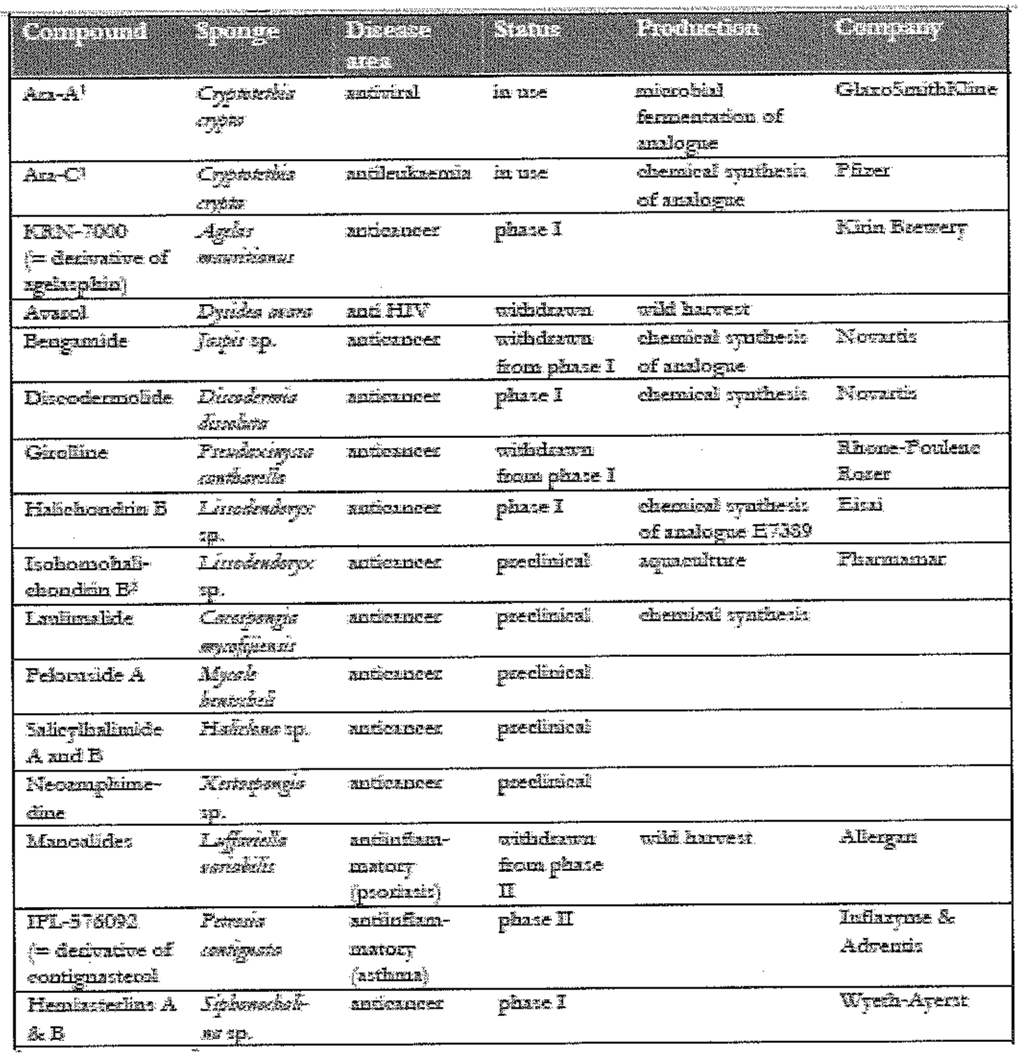
CULTIVATION OF MARINE SPONGES

3.1 INTRODUCTION

Altough seconder metobolites have many advantages, this metabolites produce very in small amounts and the low growth rates of sponges in the sea results in a very slow production of the bioactive compounds. For Lissodendoryx sp., the sponge species containing the highest halichondrin B concentration (400 μg/kg), it was estimated that for the production of a medicine to treat patients with melanoma, require total of 5000 tonnes of sponge. In addition, it was estimated that only approximately 300 tonnes are present in the seas (Munro et al., 1999). So we require production methods for the synthesis drugs. Some methods to obtain large quantities of sponge metabolites have gained attention in the last decade. Researchers have discovered a range of possible ways:

1. Mariculture: cultivation of sponges on designated areas in the sea.
2. Ex situ culture: cultivation of sponges under controlled conditions outside of the sea.
3. Cell- and tissue culture.
4. Chemical synthesis of the metabolites or analogues (Sipkema, 2004).

Some methods, such as mariculture and chemical synthesis have received considerable attention to identfy their potential. Others however, such as cell culture and genetic transfer of the metabolic routes are more futuristic and require more assumptions due to a lack of knowledge about application of these methods to produce sponge metabolites (Sipkema, 2004). Some pharmaceutical companies are preferred these methods for the production of large scale seconder metabolites (see Figure 3.1).



Compound	Sponge	Disease area	Status	Production	Company
Ara-A^1	*Cryptotethia crypta*	antiviral	in use	microbial fermentation of analogue	GlaxoSmithKline
Ara-C^1	*Cryptotethia crypta*	antileukaemia	in use	chemical synthesis of analogue	Pfizer
KRN-7000 (= derivative of agelasphin)	*Agelas mauritianus*	anticancer	phase I		Kirin Brewery
Avarol	*Dysidea avara*	anti HIV	withdrawn	wild harvest	
Bengamide	*Jaspis* sp.	anticancer	withdrawn from phase I	chemical synthesis of analogue	Novartis
Discodermolide	*Discodermia dissoluta*	anticancer	phase I	chemical synthesis	Novartis
Girolline	*Pseudaxinyssa cantharella*	anticancer	withdrawn from phase I		Rhone-Poulenc Rorer
Halichondrin B	*Lissodendoryx* sp.	anticancer	phase I	chemical synthesis of analogue E7389	Eisai
Isohomohalichondrin B^2	*Lissodendoryx* sp.	anticancer	preclinical	aquaculture	Pharmamar
Laulimalide	*Cacospongia mycofijiensis*	anticancer	preclinical	chemical synthesis	
Peloruside A	*Mycale hentscheli*	anticancer	preclinical		
Salicylihalimide A and B	*Haliclona* sp.	anticancer	preclinical		
Neoamphimedine	*Xestospongia* sp.	anticancer	preclinical		
Manoalides	*Luffariella variabilis*	antiinflammatory (psoriasis)	withdrawn from phase II	wild harvest	Allergan
IPL-576092 (= derivative of contignasterol	*Petrosia contignata*	antiinflammatory (asthma)	phase II		Inflazyme & Aventis
Hemiasterlins A & B	*Siphonochalina* sp.	anticancer	phase I		Wyeth-Ayerst

Figure 3.1 Sponge-derived compounds in production and clinical or preclinical trials. Extracted from Newman and Cragg (2004) (1, Pomponi, 1999; 2, Munro et al., 1999).

3.2 CULTIVATION METHODS

3.2.1 Chemical Synthesis

Method of chemical synthesis would be the preferred to produce bioactive compounds that have been discovered in sponges, as there is no longer a dependancy on biological uncertainties. Many secondary metabolites from sponges have resulted in a masterpiece of chemical synthesis for scientific purposes (Faulkner 2000; 2001). However, many different sponge metabolites are highly complex structures and substitution patterns with lots of chiral centres. Synthetic routes usually consist of dozens of individual reaction steps because a lot of protection-deprotection chemistry has to be done. Ara-C is the only successful metabolites produced from sponges, but for most more complex marine natural products, that are rich in centres of asymmetry, no economically feasible strategies exist (Proksch et al., 2002).

For example; Halichondrin B has been successfully synthesised by Kishi and co-workers (Norcross and Paterson, 1995) from relatively simple molecules such as ascorbic acid and L-mannonic γ-lactone. However, total synthesis requires no less than 100 chemical reactions and the overall efficiency is well below 1%. But generally this synthesis occur from 30 steps (Sipkema, 2004). Therefore, large-scale chemical synthesis of halichondrin B has not possible yet. One possible way has found chemical synthesis is the production of simple smaller analogues of halichondrin B (see Figure 3.2) This method has been accomplished by scientists of Eisai and is used for the production of the compound for clinical trials. However, recently the synthesis of bioactive analogues requires still more than 30 steps (Wang et al., 2000).

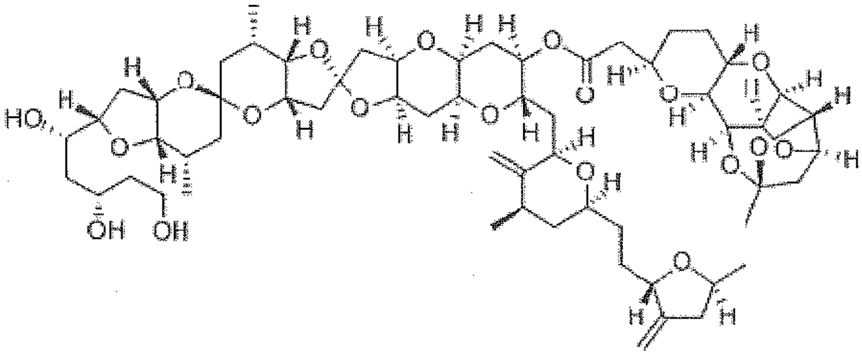


Figure 3.2 Halichondrin-B (http://www.chemicalbook.com)

The another example Avarol's total chemical synthesis has been accomplished by a number of groups (Sarma and Chattopadhyay, 1982). According to the procedure of Ling and co-workers (1999) is connected to the synthesis of the enone (1,2,3,4,5,6,7,8,8a-octahydry-4,8a-dimethyl-3,8-naphtalenedione) (Hiroi and Yamada, 1975), which is the starting substrate used by avarol can be chemically synthesised in 20 steps with a maximum overall efficiency of 2% (Ling et al. 1999).

3.2.2 Mariculture of Sponge

The mariculture term is used agriculture in the sea. Many marine sponges have been tried for farming in the sea. In the 20th century bathing sponges were cultured on designated places in the sea on concrete disks (Moore, 1910). Mariculture had become interesting at that time due to over-fishing on bathing sponges (Manconi et al., 1998). Because of the remarkable almost plant-like regeneration characteristics of sponges, the sea make for them very suitable for multiplication and transplantation (Simpson, 1984).

There is a big advantage of cultivation in the sea is enough of it. However, it is very important to find the right spot and equipment in the sea to start-up a mariculture (see Figure 3.3) Depth, current, light and the carrier material have been shown to strongly influence the mortality, growth and production of the bioactive compounds by the sponge (Wilkinson and Vacelet, 1979).

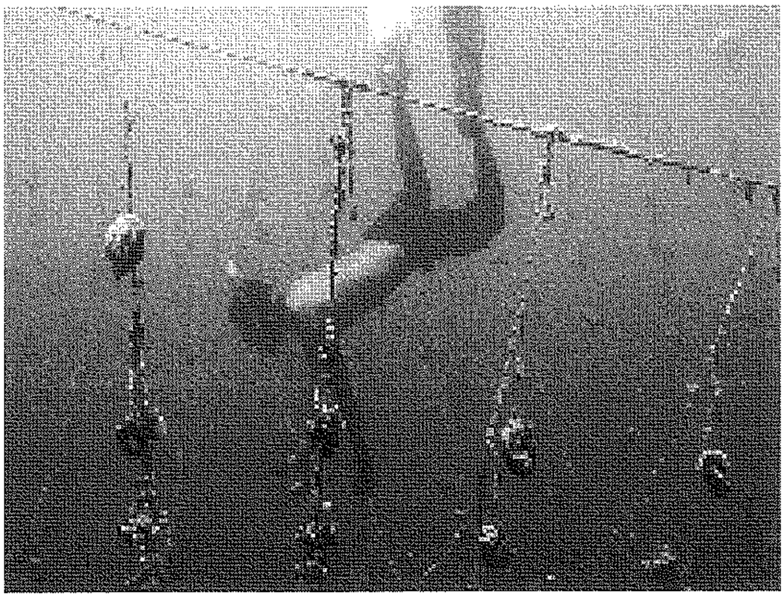


Figure 3.3 Example of mariculture (Simon Ellis, *Alternative Income Generation for MPA Communities in Pohnpei, FSM through Small-scale Mariculture ventures*, 2008)

Sponge mariculture is an industry that has started in the Western Pacific for many years. AT first developed by the Japanese over 60 years ago, only at Pohnpei in the Federated States of Micronesia has it been developed as a serious industry (Croft, 1995). There are some difficulties in controlling the environment after selection of a suitable spot are a drawback of mariculture. Growth of sponges is often strongly associated with a certain season and is thus suboptimal during most time of the year. Also, nutrient supply and disease control are difficult to attain in the sea. (Turon et al., 1998; Duckworth and Battershill, 2003). Nevertheless, mariculture has been successful for a large number of sponges with a usual annual increase in biomass of 100-700 % (Van Treeck et al., 2003; Duckworth and Battershill, 2003).

3.2.2.1 About and Analyses of the Method

a) The mariculture would lead to nutrient depletion of the surrounding seawater, the consumption by the sponges, cause exceeds the plankton growth and refreshment rate of the 'local' seawater. For the mariculture, required distance of 2.5 m between the lines carrying the sponges is far enough to prevent nutrient depletion of the water (Sipkema, 2004).

b) Altough many different mariculture systems used, but for the growth of Lissodendoryx sp. it has been found that growth was highest in bags that were connected to long-lines (Munro et al., 1999). For D. avara the growth data from Klinipharm's mariculture in the Mediterranean Sea were used (see Figure 3.4).

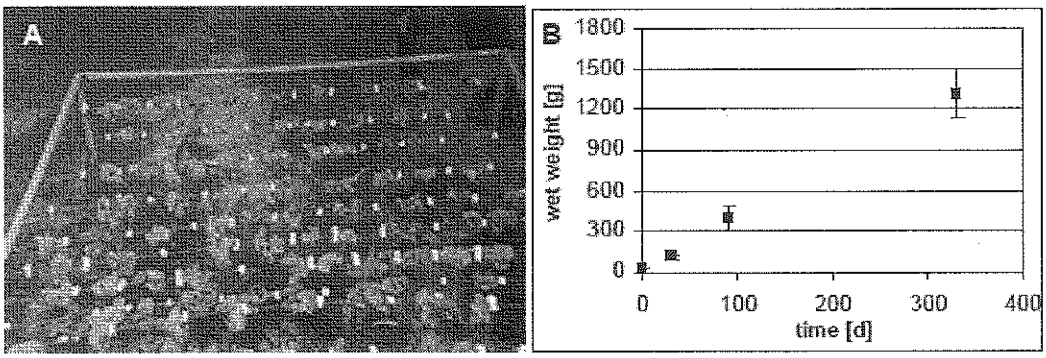


Figure 3.4 Mariculture of D. avara specimens in the Mediterranean Sea by Klinipharm GmbH. Sponges are separated by corks (A). The mariculture was started in April and growth was measured by underwater photography. The diameter of the sponges was used to calculate the volume (of a spherical sponge) and the wet-weight of the sponge (weight = 1.039 x volume). The error bars are the standard deviation of the measured sponge explants (N = 20, 26, 24 and 21 for time = 30, 60, 120 and 360, respectively) (B). (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

c) Several experiments have been set up to identify a simple, low cost and low management input system for mariculture of sponges, and to determine which produced the best shapes. Three basic suspension structures have been used, vertical lines, horizontal lines and frames (see Figure 3.2d).

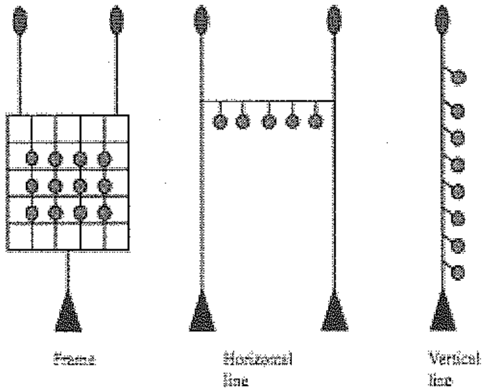
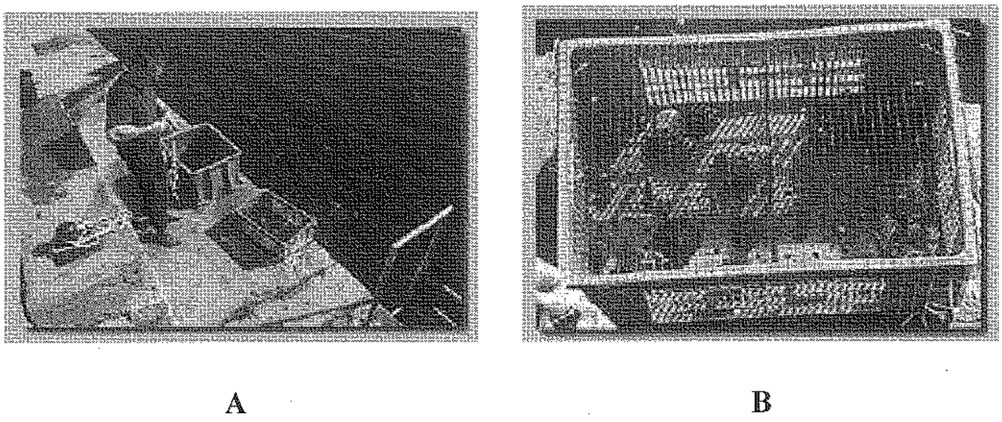


Figure 3.5 Basic arrangements used for culturing cuttings. Red ovals indicate buoys, black triangles, anchors (bags of coral rock) and grey circles, sponge cuttings. (Ian Hawes & Cletus Pita Oengpepa, *Village Scale Sponge Aquaculture in the Solomon Islands*).

d) The mortality (% of sponges that dies during the cultivation) for both species in maricultures is estimated to be approximately 50 % (Munro et al., 1999).

e)The method generally occur this steps (see Figure 3.6)



A

B

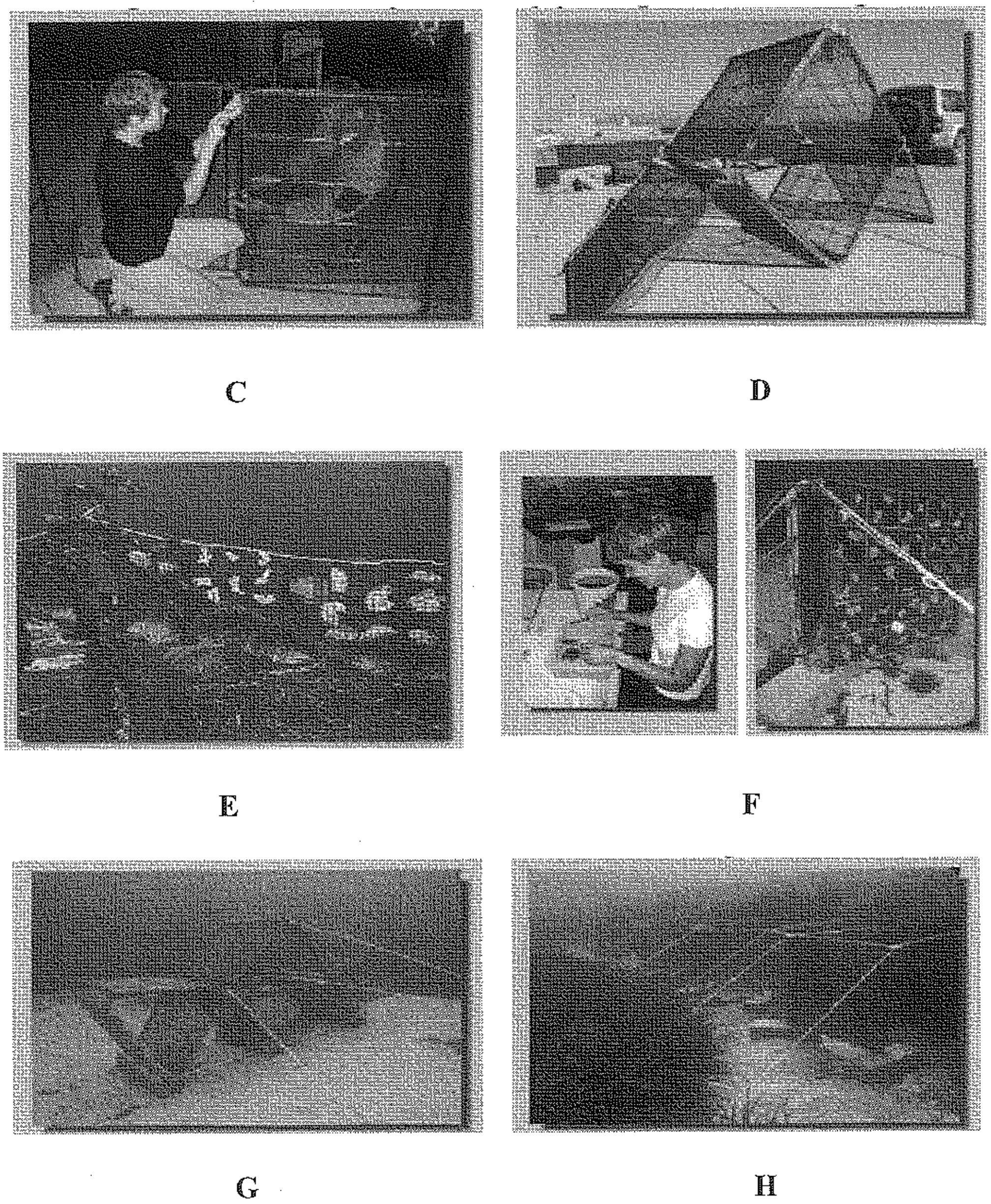


Figure 3.6 Mariculture cultivation of marine sponge at Limski. **A**. Preparation of sponge cages. **B**. Sponge cages which include various sponge **C**. Preparation mariculture units **D**. Unit is waiting to be placed on the sea floor **E**. Types of network which at live sponges **F**. 1) Preparation of sponges for transplantation 2) Completed sponge unit. **G**, **H**. Placed aquaculture unit (15m) (Gokalp, *Investigation of Eastern Mediterranean-Aegean selected sponge (Porifera) species and study of the existance of collajen type II, IV and integrin 1 b proteins in these animals with immunohistochemical and molecular genetic methods,* 2006).

3.2.3 Cell culture, Primmorphs

Primmorphs is multi-cellular aggregates obtained from dissociated sponge cell suspensions in which cell proliferation occurs (Müller, 1999). The seperated cells are allowed to re-aggregate in the presence of antibiotics and sphere-shaped aggregates covered with a skin-like tissue are obtained (Custodio et al., 1998; Sipkema et al., 2003). Cell division of primmorphs has been measured by BrdU incorporation, but there are few reports about biomass increase of primmorphs. This is probably explained by the occurrence of apoptosis that counterbalances cell division (Koziol et al., 1998).

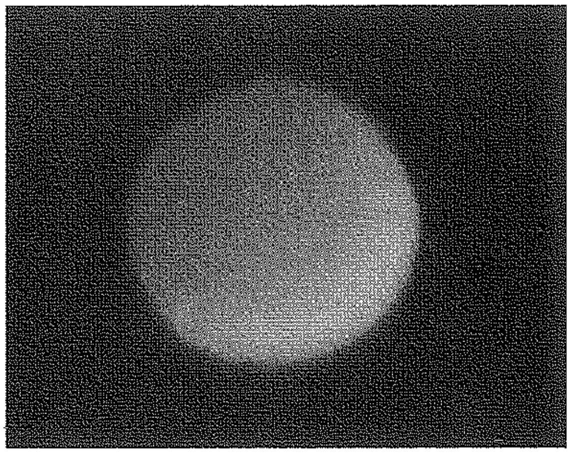


Figure 3.7 A *Suberites domuncula* primmorph (*Cultivation of marine sponge: From sea to cell.* Sipkema, 2004).

The cells which generate the scaly epithelium of the primmorphs are pinacocytes as evaluate from, their flattened, fusiform extensions and their prominent nucleus; the size of the cells ranges from 15 to 20 pm (Simpson 1984). There are cells in the primmorphs which are called primarily spherulous cells. They have a diameter of 40 to 45 pm and are characterized by large round vacuoles which keep most of the space in the cells. The other cells (-55 to 60 pm) may be called amoebocytes and archaeocytes (Bergquist 1978).

It has also been researched whether primmorphs are able to grow, or if they will develop into functional sponges before growth can occur (Sipkema et al., 2003).

Development of functional sponges from sphere-shaped aggregates that were formed from seperated cells would be in line (Wilson, 1907). Proving that some of the findings, as for example spicule formation (Krasko et al., 2000) and attachment and flattening of primmorphs to a solid matrix (Sipkema et al., 2003).. Therefore, they may be able to tolerate an extraction fluid that could be used to 'milk' the product from the primmorphs (Sipkema, 2004).

A doubling of the metabolite concentration in primmorphs that is present in wild sponges is possible within 3 days. For D. avara this has been measured for primmorphs between day 3 and day 6 after their formation (Müller et al., 2000). Primmorph generally live for 6 months. Various sponges the life span of their primmorphs has been determined. Generally, they can be maintained for at least half a year (Sipkema et al., 2003, 2004). 1 ‰ of the sponge cells which is used to prepare primmorphs actually ends up in a primmorph. The number and size of primmorphs that were obtained from a dissociated cell suspension (Zhang et al., 2003)

The procedure is given which obtain of primmorph (see Figure 3.8).

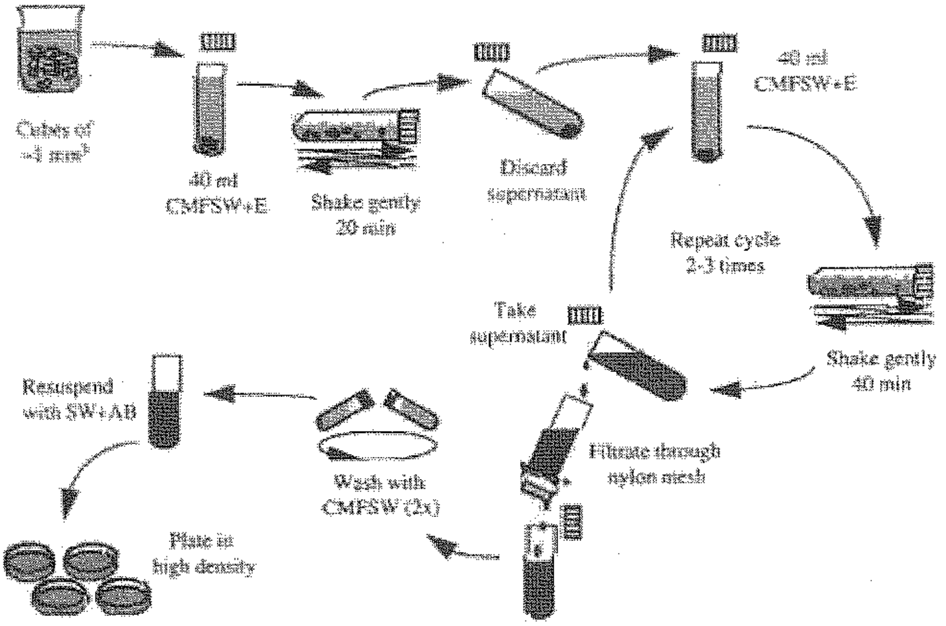


Figure 3.8 Generation of primmorphs from seperated cells of *Subentes domuncula*.

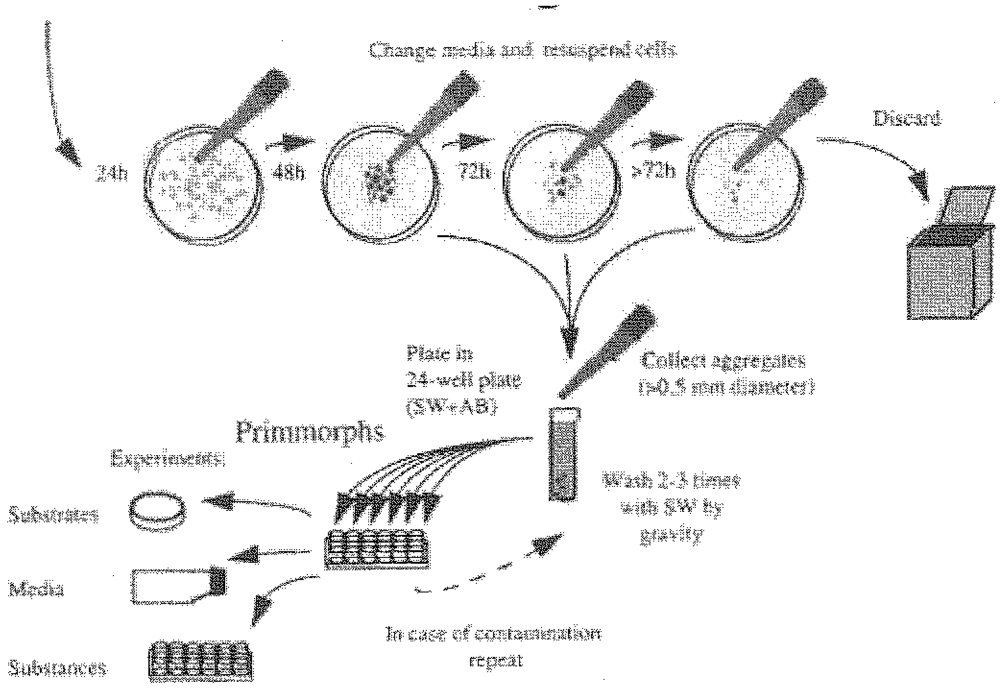


Figure 3.8 (Cont.)

3.2.4 Ex-Situ Culture of Sponges

The ex situ culture is cultivation of functional sponges outside the sea. With ex situ cultivation, may be possible to switch from seasonal growth to continuous growth during the year. However, continuous ex situ growth of marine sponges for more than a year has not been established in the laboratory yet (see Figure 3.9) (Sipkema, 2004).

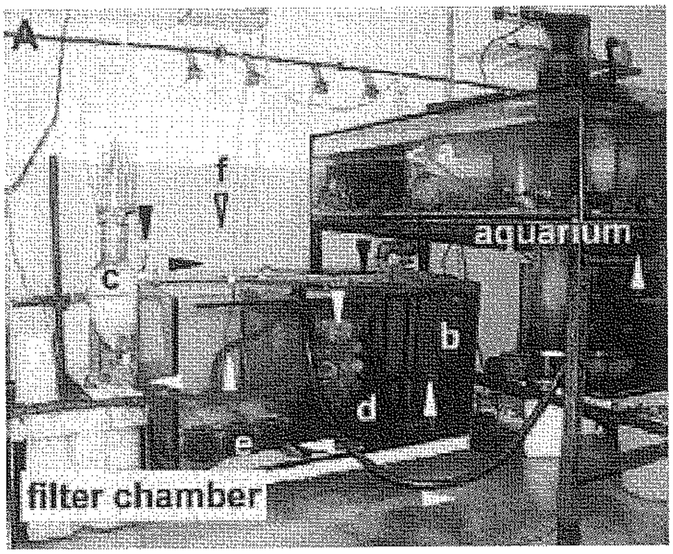


Figure 3.9 Example of two ex situ cultivation tanks. These system can be continuously measured and controlled (Osinga et al., 1999).

Development of a suitable growth medium is one of the most important problems to optimise the production of biomass. Cultivation of sponges in a bioreactor allows manipulations of the sponge medium but it is not possible in the sea, and thus changes of the growth rate (Sipkema, 2004). Ex situ cultivation allows controlled of nutrients and other parametres of the secondary metabolites of interest so growth and production rates could be enhanced. Marine sponges have proven to be no laboratory rats yet, but a growing number of researchers has small successes in culturing in the laboratory (see Figure 3.10) (Osinga et al., 2003; Belarbi et al., 2003; Duckworth et al., 2003; Sipkema et al., 2004). Chondrosia reniformis and Crambe crambe both growth in the sea and in the laboratory are available.

The growth rate of C. reniformis was estimated to be 4-9 times higher than laboratory under suboptimal conditions (Sipkema et al., 2004). For C. crambe, it was more difficult to compare growth in the laboratory (Belarbi et al., 2003) and in the sea (Turon et al., 1998; Garrabou and Zabala, 2001) because of used different methodsdifferent methods that were used to measure growth. But, a seasonal growth sample was obvious in the sea, while was not present for ex situ cultivation (Sipkema, 2004).

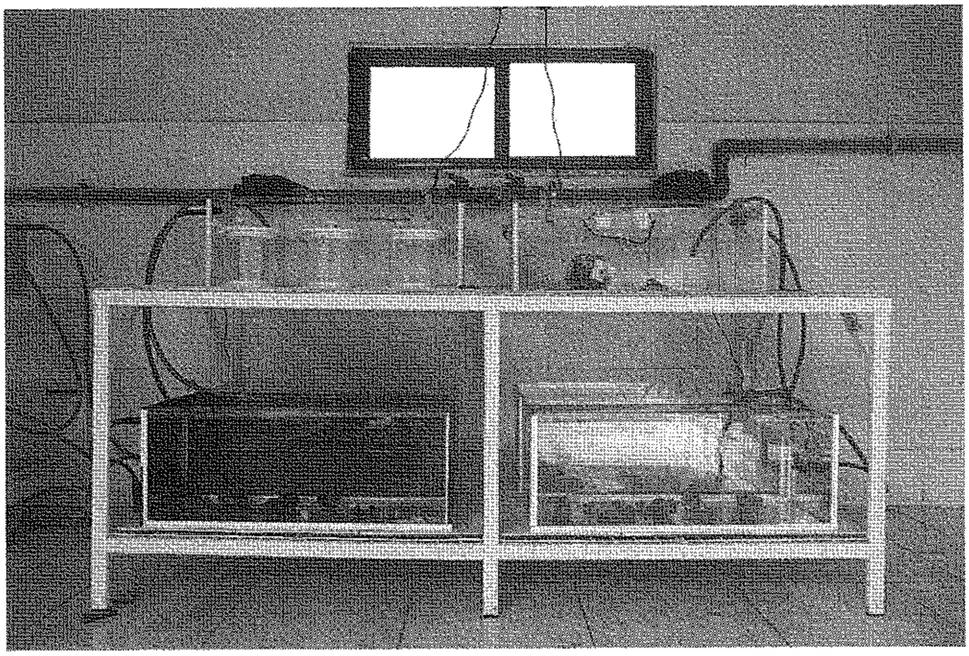


Figure 3.10 An example of ex-situ cultivation of sponges (Aqaba, Jordanien, 9.1.13 – 25.4.13)

Among the advantages of this method which are used for ex situ culture of sponges, avoid problems such as sea storms encountered, sponge diseases, seasonal yield loss, controlled nutrients and bioactive metabolites that are required for the production in a controlled manner and sponge realizing the growth and development of living. Continuously ex-situ sponge culture system has not been achieved more than 1 year yet (see Figure 3.11) (Sipkema, 2004).

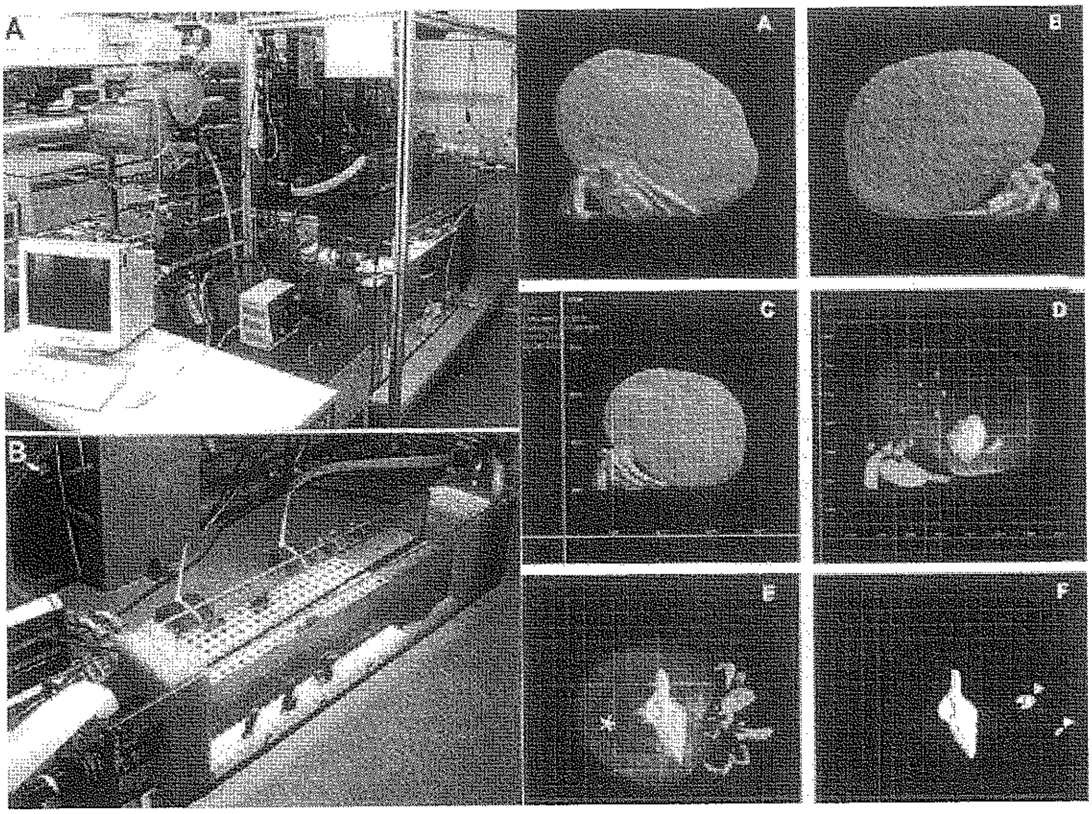


Figure 3.11 Closed culture system where all the growth conditions are controlled, the Institute of Biochemical Eng. / Stuttgart University, on the left photograph *Suberites domuncula* sponge species monitoring of the development of the 3D computer tomography method (Nickel, 2001, 2003).

Sipkema et al. studied that whole sponge culture is the most promising option for large scale production of bioactive metabolites like avarol, which is produced up to 3 g per kg sponge wet weight in *Dysidea avara.* (Müller et al., 1985, Sipkema et al., 2003). Some research results have shown that growth rates are generally low and mortality is high (Duckworth et al., 1997; Duckworth and Battershill, 2003). Other researchers have tried to culture sponges, but optimum conditions for maintaining sponges in the laboratory have not been completely achieved to date (Barthel and Theede, 1986; Belarbi et al., 2003; Duckworth et al., 2003; Osinga et al., 2003). In this study, we use ex situ cultivation and develop valuable methods for ex situ cultivation to living sponge.

CHAPTER 4

EX-SITU CULTIVATION OF *HALICLONA* SP.

4.1 INTRODUCTION

Marine sponges have been called as a gold mine during the past fifty years, with respect to the diversity of their secondary metabolites (Sipkema, 2004). We may prove to be valuable methods for the living marine sponge *Haliclona* sp. in the laboratory and production this seconder metabolites. Development of a suitable growth medium is one of the most important themes to optimise the production of marine sponge. Cultivation of sponges in a bioreactor allows manipulations of the sponge medium but that are not possible in the sea, and thus changes of the growth rate. The aim of this project; develop a biotechnological process and mechanistic model for the living sponges as *Haliclona* sp. and optimization of this sponge's medium, optimization of normal sea conditions, compare growth rates between different factors, may be later production of seconder metobolites of marine sponges. Such a this growth model can be used to study the quantitative effect of factors such as pressure, light, current, temperature, nutrition source, nutrient concentration, or age on the growth rate of *Haliclona*. Also, it could prove valuable as a tool to predict sponge growth in maricultures.

4.2 MATERIALS AND METHODS

4.2.1 Design of Biosystem

Firstly; we designed a biosystem specifically for grow marine sponges in the laboratory which provide the marine environment in order to live a long time of sponges (see Figure 4.1).

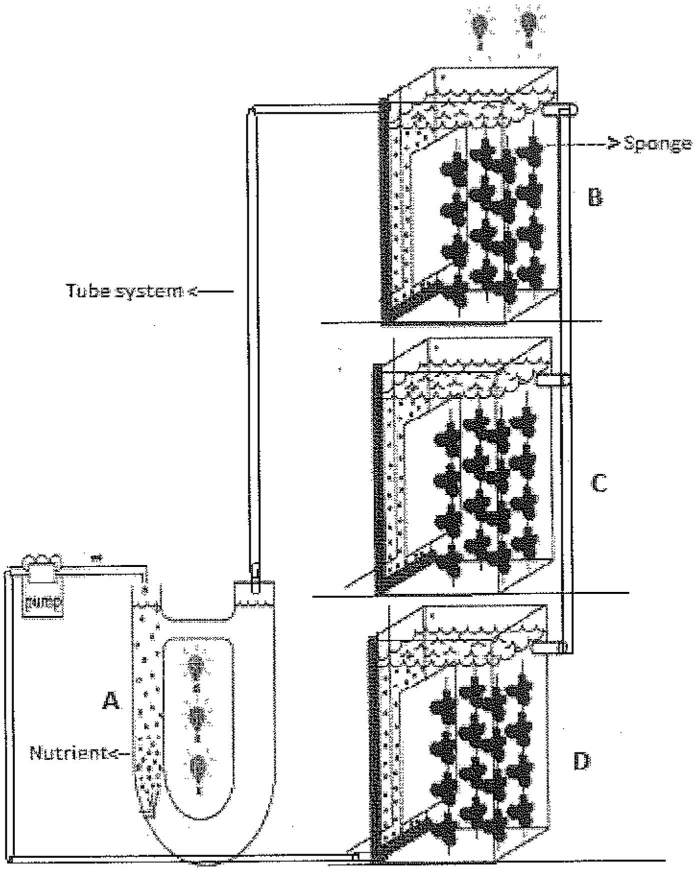


Figure 4.1 The illustration of our ex-situ cultivation system, **A.** Photobiorector include nutrient, **B, C, D.** Bioreactors are sponge tanks which included sponges.

The system was designed three bioreactors which included sponges and a photobioreactor that included nutrients and some devices which provide water circulation linked with each other by tube system (see Figure 4.2 and 4.3). The suspension from photobioreactor reach some rates to bioreactors by tube system. The bioreactors were covered to light system like to the natural sea environment. All parameters are optimally used to create an effective biosystem (see Table 9).

Table 10 Used parametres for prepared optimum medium. For growth sponges in laboratory.

Parameters	**Optimum Conditions**
1)Temperature	16-20 C
2)Salinity	21 ppt – 36 ppt
3)pH	7.8 – 8.4
4)Light	10000-20000 Kelvin
5)Ammonia	Low concentration
6)Nitrate	< 25 mg/ L
7)Oxygen	0.2 - 25 µmol O_2 h^{-1}

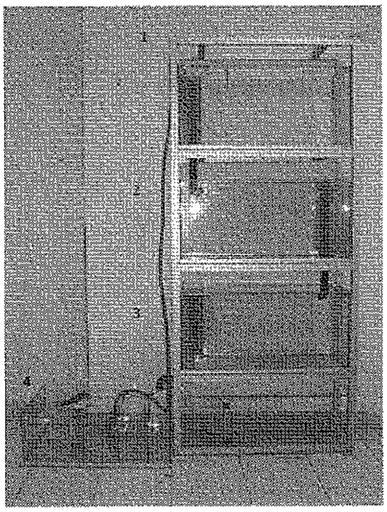


Figure 4.2 System design: 1,2,3: bioreactor tanks; 4: photobioreactors, tube system connected to the photobioreactor and the bioreactors with each other.

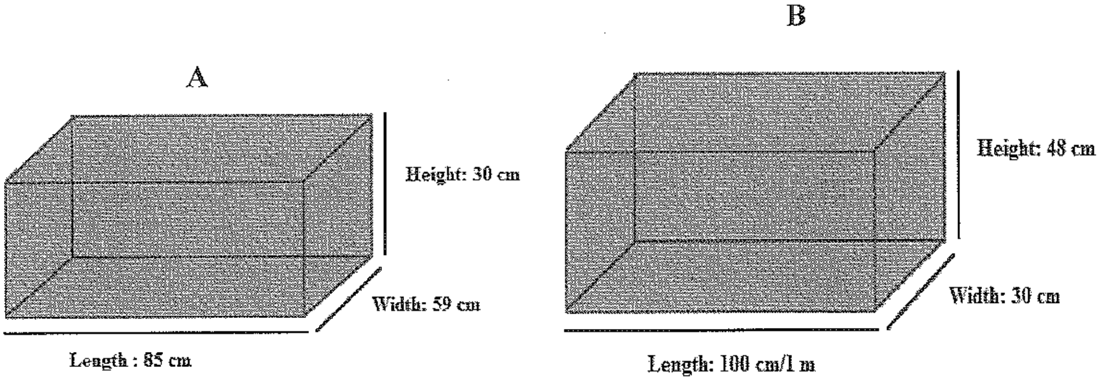


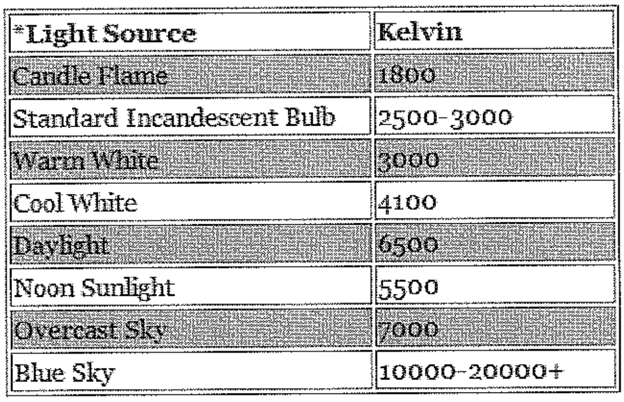
Figure 4.3 Tanks volume: **A.** Photobioreactor is 150 L, **B.** Bioreactor is 144 L in the system

4.2.2 Parametres

4.2.2.1 Light System

The light is life for marine animals so it is very important to living. Special lamps used for create natural marine environment. Fluorescent lighting has been successfully used by reef aquarists for years. Two common forms of standard fluorescent light bulbs, normal output bulbs and very high output are used. Sponges look best and grow best under light with a more blue coloration. Many tropical sponges require light for survival. Compounds produced by these symbionts provide nutrients for the sponge (Sara, 1971). Accordingly, the net primary productivity of some sponges is dependent on the availability of light and light intensity can strongly influence the geographic distribution of sponge species (Wilkinson, 1983).

Light color that a lamp produces is measured in degrees Kelvin, abbreviated K. The commonly used unit to measure the spectrum or "temperature" of light is called degrees Kelvin (K). Kelvin is a scale that has been developed to describe the color of light. At 0 degrees Kelvin (equivalent to -273° Celsius), no light is emitted. Kelvin ratings do not relate to light intensity directly, a higher K number does not represent more intensity, in other words a 20,000K lamp is not more intense than a 10,000K lamp (Drs. Foster & Smith Educational Staff) (see Figure 4.4).



*Light Source	Kelvin
Candle Flame	1800
Standard Incandescent Bulb	2500-3000
Warm White	3000
Cool White	4100
Daylight	6500
Noon Sunlight	5500
Overcast Sky	7000
Blue Sky	10000-20000+

Figure 4.4 Color temperatures and kelvin values. Color temperatures below 5500 Kelvin are not commonly used in reef systems.

The type of light provided for a saltwater reef systems is very important due to the fact that live in the system rely on light for a major portion of their nutritional needs.. Kelvin temperature of the saltwaters which live corals and sponges changed from 5000 to 10,000 degrees. 10,000 to 20,000 degrees Kelvin lamps using saltwater systems, while at freshwater systems prefer 5000 kelvin lamps are sufficient (Drs. Foster & Smith Educational Staff). We need very high output lights for system such as it has 10000 Kelvin values. Very high output lamps have been used for many years by researchers keep all types of corals successfully. No other lamp can produce glowing colors in corals like the very high output lamp (see Figure 4.5).

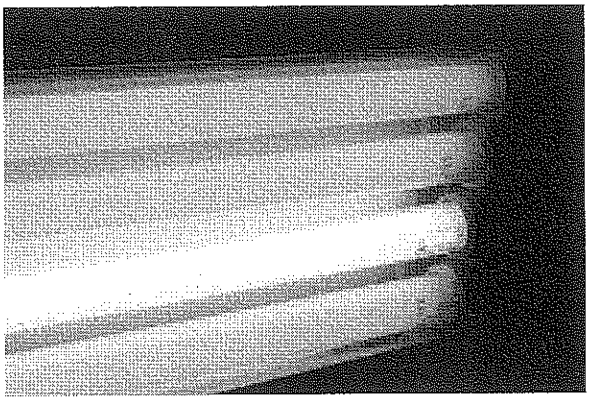


Figure 4.5 The very high output light. It used our biosystem for living marine sponges.

As a result, these lamps emit blue light can go down much deeper than other light colors and feel of the natural environment, and we use 2 lamps for each bioreactors and 6 lamps total (see Figure 4.6).

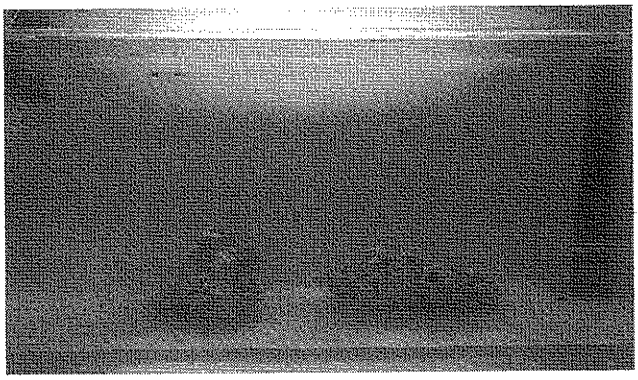


Figure 4.6 The our light system in the laboratory which 10000 Kelvin degrees. The system provide natural sea environment for sponges.

4.2.2.2 Salinity, pH, and Temperature

Marine sponges should be cultured at the salinity of seawater 21 ppt – 35 ppt because of normal sea conditions (Belarbi et al., 2003, Osinga et.al, 1999). A hypersaline environment tends to dehydrate the sponge cells whereas a lower than normal salinity could lead to dilution of the intracellular content. Salinities of up to 46 ppt are tolerated by species such as *Hippospongia lache* but salinities of less than 26 ppt can be lethal (Osinga et al., 1999). We prefer 21-25 ppt rates because of Haliclona sp tolerate and live between these rates.

Temperature is important for sponges. Normally, most sponges show only a slow seasonal change in temperature and are not adapted for too big a change in ambient temperature. A decrease in temperature is generally better tolerated than a temperature rise (Osinga et al., 1999). If temperature is too high, would crashes the culture. Massive dies of the Mediterranean sponge *Crambe crambe* were recorded in France and Italy during the summer of 1999 after a prolonged increase in temperature of up to 24 C°. A high temperature may be stimulates sexual reproduction in sponges. To prevent diversion of metabolic energy from biomass generation to sexual reproduction, the culture environment should maintain a temperature slightly lower than the summer temperature of the nature habitat (Belarbi et al., 2003). We prefer at this project 24-28 C° because of *Haliclona* sp. lives Pasific Ocean at 24-28 C° (World Porifera Data).

The normal cultivation pH for marine sponges would between pH 7.8 and 8.4, because it is normal pH of seawater at Pasific Ocean (Brown et al., 1992).

4.2.2.3 Remove Waste Products

In closed culture systems, metabolic wastes primarily ammonia accumulate rapidly. Metabolically produced by sponges ammonia is toxic to most aquatic animals even if in very low concentrations. Ammonia concentrations even if as low as 60 μM can kill half the exposed of many marine invertebrates. So, ammonia must be continuously removed from a closed culture system by biofilters (Belarbi et al., 2003).

Some sponges may produce bioactive and cytotoxic waste metabolites in aquaculture system which may rapidly build up and inhibit further sponge growth (Taylor et al., 2007). Toxicity of ammonia is pH dependent. The ammonium ion ($NH4^+$) does not readily permeate the cell and is therefore much less toxic than NH3. (Belarbi et al., 2003).

4.2.2.4 Dissolved Oxygen

Sponges require oxygen for live. Oxygen is absorbed from the water flowing through the aquiferous system in the sponge species. Oxygen consumption ranges from 0.2 to 25 μmol O^2 h^{-1} per cubic centimeter of sponge volume (Osinga et al., 1999). Oxygen is generally provided by aerating the water by bubbling before it is fed to the aquarium or bubbling air within a confined volume of the aquarium (Belarbi et al., 2003). Demosponges maintained under laboratory conditions can also tolerate hypoxic conditions could reflect their adaptability to dissolved oxygen (Gunda et al., 2009).

4.2.3 Sponges

Haliclona (Sigmadocia) caerulea is called Blue Caribbean sponge (Phylum Porifera, Class Demospongiae, Subclass Ceractinomorpha, Order Haplosclerida, Family Chalinidae) (Hechtel, 1965). They have thickly massive and irregular sponge with raised thick-walled volcano-shaped oscules. Exterior colour is blue, interior colour is yellow (see Figure 4.2g).

Figure 4.7 Blue sponge, *Haliclona (Sigmadocia) caerulea* (LHSVirtualZoo).

Haliclone ranges at Indian Ocean, Indo-Pacific Ocean. They inhabits coral reef flats and encrusts hard substrates and often found in well lighted areas. Like all marine sponges, Haliclona is a filter feeder, continuously circulating water through their bodies. Microscopic food particles are removed from water by specialized collar cells. Their digestion is intracellular. Haliclona has living temperature range of 75 to 83°F (24 - 28°C). Does best in a moderately 'lighted' area and should be placed where the current is quickly. They feed plankton and suspended detritus, and require numerous feedings per day of live preserved commercial phytoplankton products or that of animal and plant powders that produce suspended products in the bulk water. For Haliclona live healty, nitrate-nitrogen levels should be kept below 15 mg/l.

For this project, *Haliclona* was obtained Indonesia, Pacific Ocean. They were transported from our laboratory in an isolated vessel at a constant temperature of 24 °C and 21 ppt salinty rate. The seawater in the transport vessel was aerated without exposing the sponges to air bubbles. *Haliclona* put into bioreactors without contact oxygen (see figure 4.8).

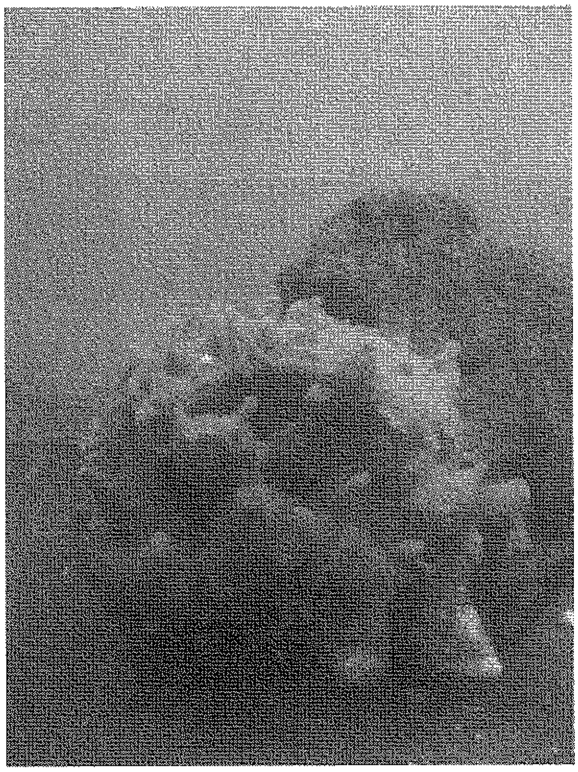


Figure 4.8 *Haliclona* put into bioreactors without contact oxygen in our laboratory.

4.2.4 Special devices for cultivation

4.2.4.1 Protein Skimmer

A protein skimmer is also called foam fractionator is a device used mostly too remove organic compounds from the water before they break down into nitrogenous waste at closed culture system (Saltaquarium). Protein skimmer devices removes certain waste organic compounds, including proteins and amino acids, by using the polarity of the protein itself. Commercial protein skimmers work by generating a large air/water interface, specifically by injecting large numbers of bubbles into the water column. In general, the smaller the bubbles the more effective the protein skimming is because the surface area of small bubbles occupying the same volume is much greater than the same volume of larger bubbles (see Figure 4.9) (Escobal, 2000).

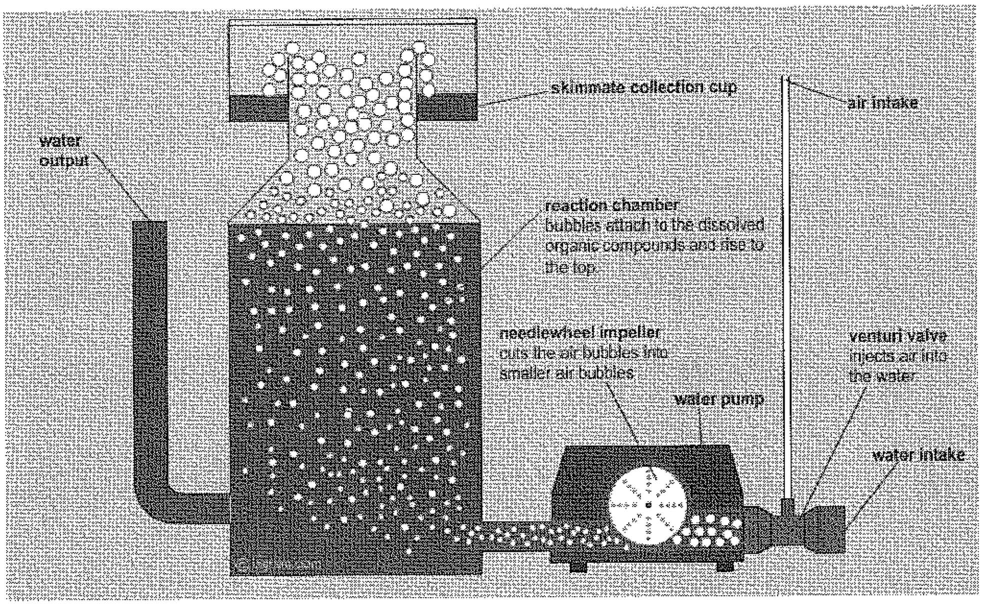


Figure 4.9 Protein skimmer design (Reef Builders)

In addition to the proteins removed by skimming which organic and inorganic molecules that are typically removed. It occurs a variety of fats, fatty acids, carbohydrates, metals such as copper and trace elements such as iodine. Particulates and other waste products is also removed, along with phytoplankton and bacteria. There is at least one published study that provides a detailed list of the export products found in protein skimmer skimmate (Shimak, 2002). We use for this project protein skimmer device which in the photobioreactor (see Figure 4.10).

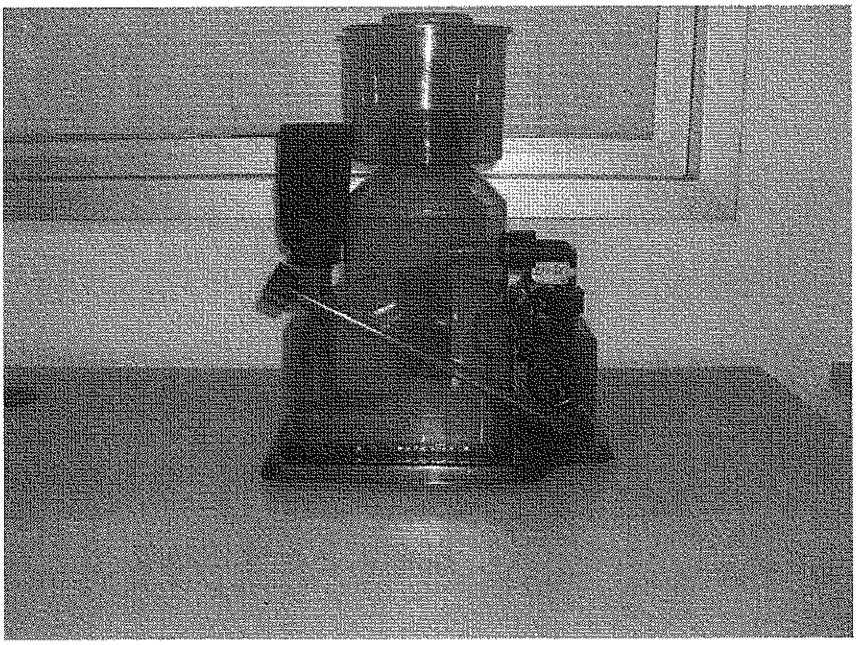


Figure 4.10 Protein skimmer is that used to our project before installation phase.

For a skimmer to function maximally, the optimal protein skimmer design would;

1. A large amount of air/water interface must be generated.
2. Organic molecules must be allowed to collect at the air/water interface.
3. The bubbles forming this air/water interface must come together to form a foam.
4. The water in the foam must partially drain without the bubbles popping prematurely.
5. The drained foam must be separated from the bulk water and discarded (Randy Holmes-Farley, 2006).

4.2.4.2 Water Circulation Machine

A mechanical mixer is designed for prevent collapse in photobioreactor which provide continuously flow of water. For prevent collapse nutrients in photobioreactors, used in a mechanical mixer. Also, for the same reason used small mixers and motors in bioreactors (see Figure 4.11).

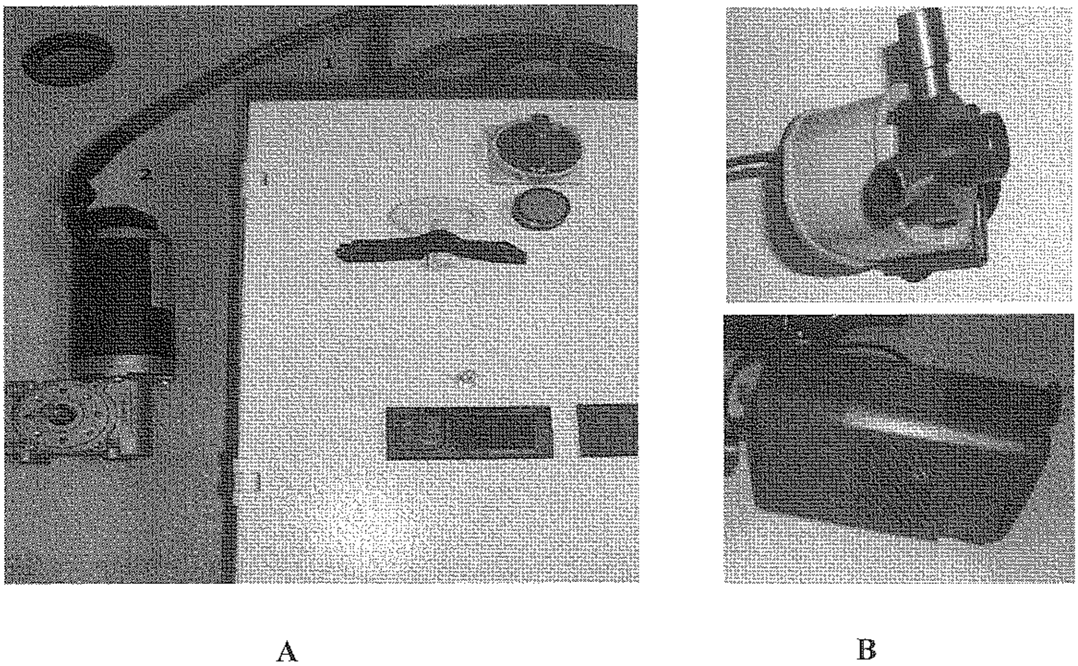


Figure 4.11 A. A mechanical mixer is designed prevent collapse in photobioreactor. 1.Motor, 2. Mixer **B.** Small mixers and motors for bioreactors that include sponges.

4.2.5 Chemicals and Substrates

Artificial sponge seawater was prepared from:

Ferrion: It includes highly-concentrated and stable iron solution for supplementing aquaria containing hermatypic organisms (i.e.hotosynthetic or zooxanthellate corals, clams, and their allies), macroalgae, coralline algae, mangroves, marsh grasses, and other desirable marine algae and plants. Ferrion was added to 5 ml to photobioreactor of each day during the experimental period (see Figure 4.12.1).

İodide and bromide: It includes potassium iodide and potassium bromide which provides sustain water quality and promoting brighter color in sponges. This chemicals use 10 ml/190 L, our photobioreactor 150 L, so we added 7,5 ml once a week (see Figure 4.12.2).

Chelated iron: It includes; ferrous sulfate, thiamine (B-1), cyanocobalamin (B-12) which help maintain the reef water to a proper pH balance. This chemicals use 10 ml/190 L, our photobioreactor 150 L, so we added 7,5 ml once a week (see Figure 4.12.3)

Trace elements: It includes Boron, Cobalt, Copper, Manganese, Molybdenum, Zinc, Rubidium, Nickel, Vanadium that prevents metal ion poisoning caused by fluctuations in pH and these elements vital for healthy coral growth. It is used 2 capful (10 mL) for every 100 L twice a week. So we used 15 ml our photobioreactor (150 L) twice a week (see Figure 4.12 4).

Bacteria feeder: It includes a variety of foods of the beneficial bacteria survived by consume nitrate and phosphate, for growth of quickly. This chemicals use 2 ml/100 L, our photobioreactor 150 L, so we added 3 ml twice a week (see Figure 4.12 5).

Phosguard: It rapidly removes phosphate and silicate from marine and freshwater system. This chemicals use 2 ml/100 L, our photobioreactor 150 L, so we added 3 ml twice a week (see Figure 4.12.6).

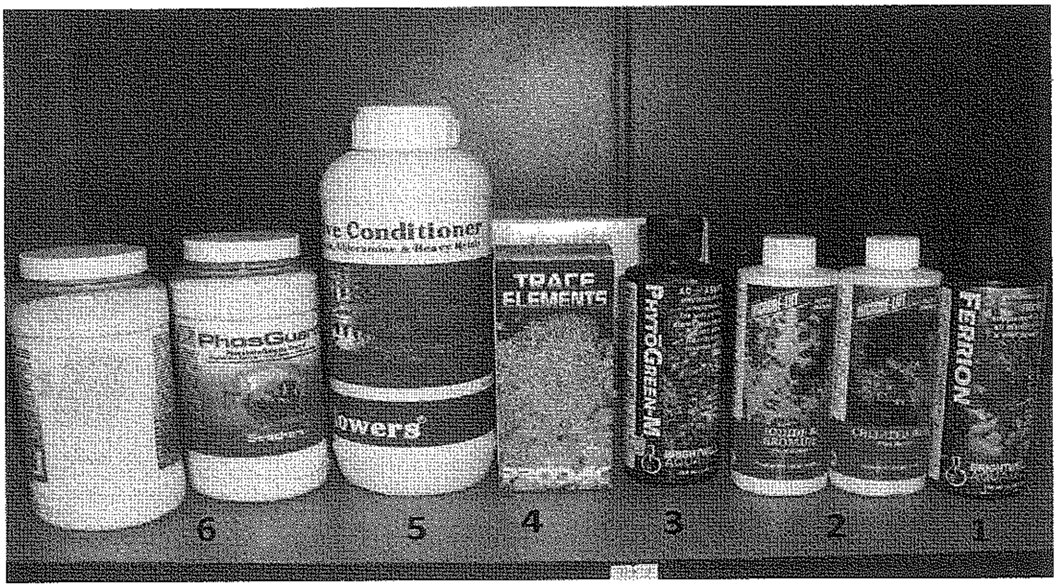


Figure 4.12 Artificial sponge seawater was prepared from this chemicals and stabilizers. 1. Ferrion, 2. İodide and bromide, 3. Chelated iron, 4. Trace elements, 5. Bacteria feeder 6.Phosguard.

Also we used some chemicals for artificial seawater, it was prepared by 0.013 g/L^{-1} NaH2PO4*H2O, 0.101 g/L^{-1} KNO3, 2.10^{-5} g/L^{-1} thiamine*HCl, 4.10-8 g/L^{-1} biotin, 8.10^{-7} g/L-1 cyanocobalamine, 9.5.10^{-4} g/L^{-1} FeCl3, 9.10^{-5} g/L-1 MnCl2*4H2O and 1.21 g/L^{-1} Tris (Sipkema, 2004).

Also; we add *live rock* for growth of bacteria in the artificial sea water. It protects the nitrogen cycle (see Figure 4.13).

Figure 4.13 The live rock in the our bioreactors.

Live rock becomes the main biological nitrification base or biological filter of a saltwater system. Since live rock in the bioreactors contains various types of bacteria, algae and corals, waste products such as ammonia, nitrate and phosphate can have a number of fates. Ammonia, nitrate and phosphate are readily assimilated by algae and photosynthetic corals growing on and in the rock. Ammonia can also be quickly converted into nitrate by the bacteria on and in the rock. This nitrate can be either absorbed by the algae and corals, or it can be denitrified by bacteria in close proximity to the nitrate producing bacteria (see Figure 4.14) (Delbeek, 1994).

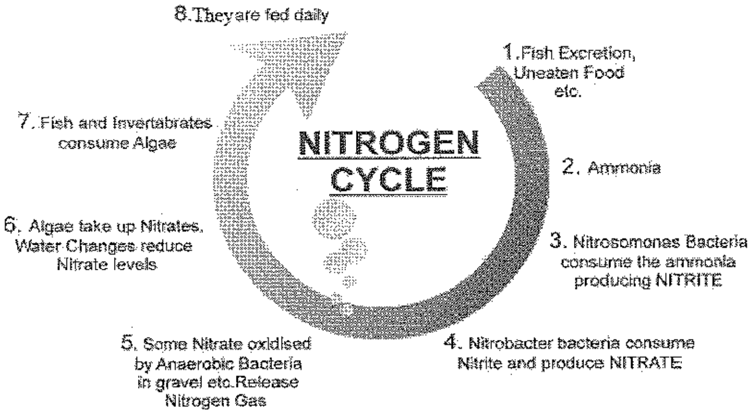


Figure 4.14 The nitrogen cycle at saltwater system (Ez-Saltwater).

Finally; *Instant Ocean Reef Crystals* add to photobioreactor and bioreactors for like to sea environment. They are special salt like to normal sea salt. Normally, it uses 33g/L so we used it 5 kg to each bioreactor and photobioreactor, at totally we used 15 kg reef crystals equally distributed.

4.3 EXPERIMENTAL SETUP

A three-step reactor system was built to provide a natural environment for the cultivation of *Haliclona* sp. explants. The system consisted of three 144 L of bioreactors and a 150 L photobioreactor. At the three-step biosystems installation was used glass tanks because of to live are more possible in glass The reactors were filled with water and devices were controlled. Firstly; a photobioractor is installed which important for biosystem (see figure 4.15).

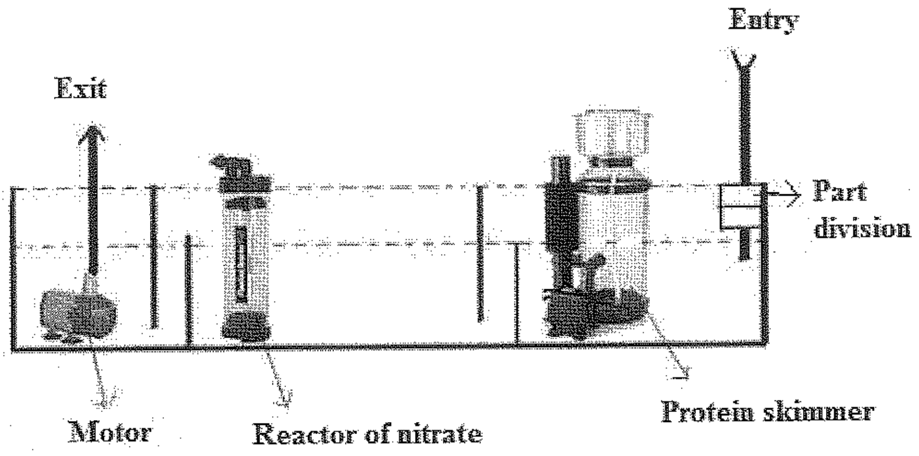


Figure 4.15 Illustration of the photobioreactor.

The filtration unit is made for biosystem that overflow of bioreactors recycled via a three-step filtration unit. The seawater was first passed through a biological stone filter, then a cotton wool filter, and finally through a 0.2-mm filter in the photobioreactor's part division. The motor is that carries water circulation, the protein skimmer, nitrate reactor and filtration unit that provides the vitality were placed in photobioreactor. And the biosystem is filled with water (see Figure 4.16 and 4.17).

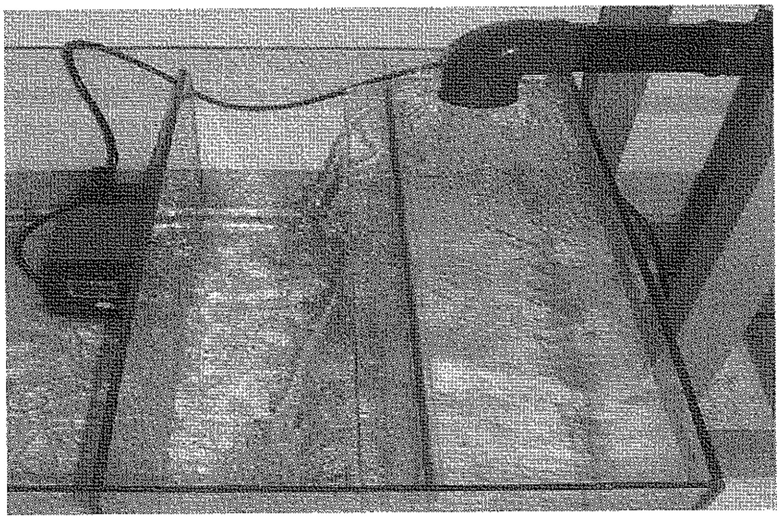


Figure 4.16 Installation phase of photobioreactor.

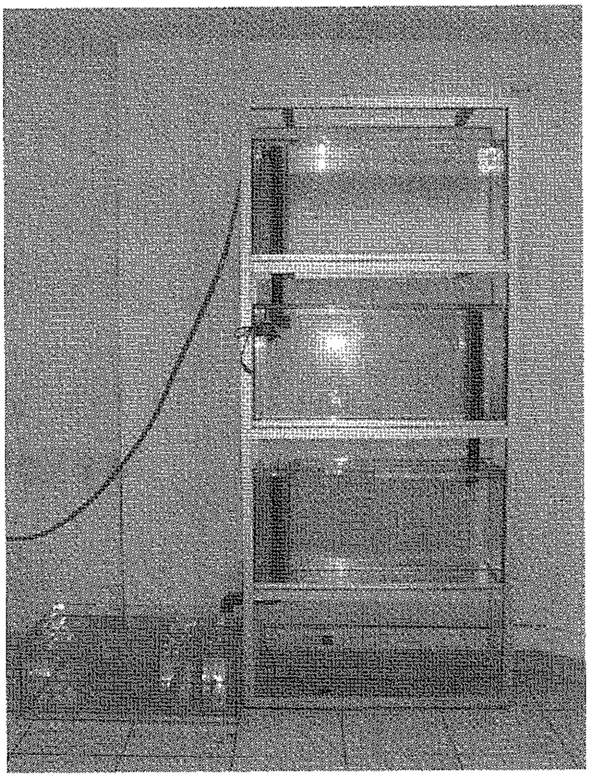


Figure 4.17 The biosystem is filled the water at first phase.

15 kg of The Instant Ocean Reef Crystals add to reef crystals equally distributed photobioreactor and bioreactors for like to sea environment (33g/L) and waited for dissolution for a month. During this month, twice a week added bacteria culture that contains active marine bacteria that aid in the nitrification and denitrification and to promote the decomposition of organic wastes in biosystem. The bacteria culture was prepared to biosystem for *Haliclona*. After three days, we put live rock into bioreactors. The devices were switched on and waited for a month (see Figure 4.18)

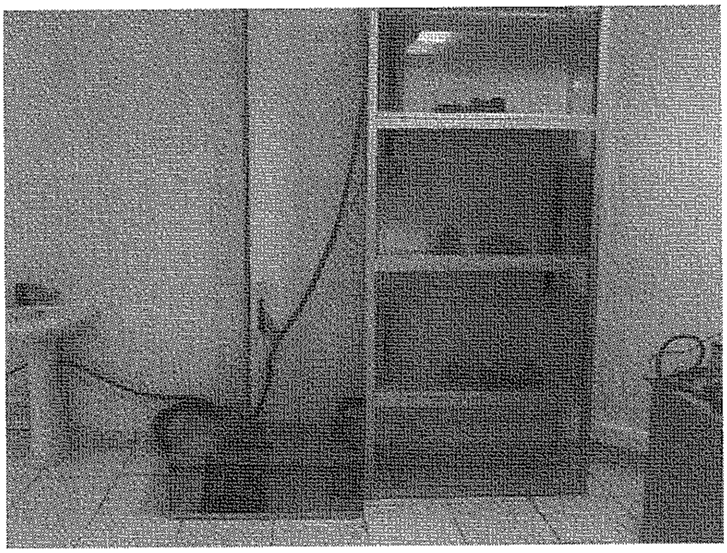


Figure 4.18 The waiting phase after put live rock.

After waited a month, *Haliclona* was obtained Indonesia, Pacific Ocean. They were transported from our laboratory in an isolated vessel at a constant temperature of 24 °C and 21 ppt salinity rate. The seawater in the transport vessel was aerated without exposing the sponges to air bubbles. *Haliclona* put into bioreactors without contact oxygen (see Figure 4.19). We have controlled artificial sea water of salinity, temperature, nitrate and phosphate rates in biosystem by special test devices and indicators. We have provided a suitable environment for our sponge *Haliclona*. We optimized these rates for cultivation of sponge (see Figure 4.20 and 4.21).

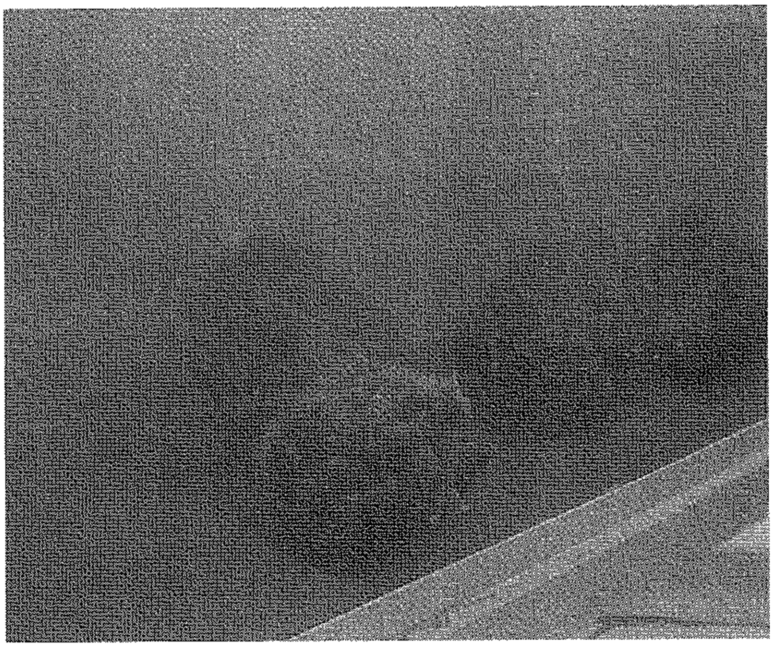


Figure 4.19 *Haliclona* sp. was obtained from Indonesia for our biosystem.

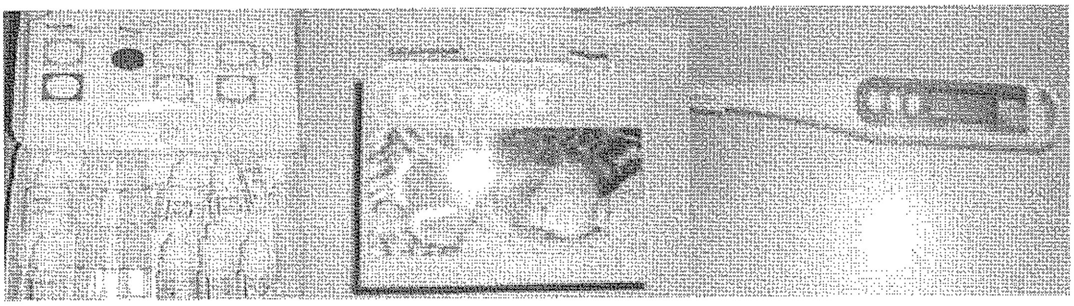
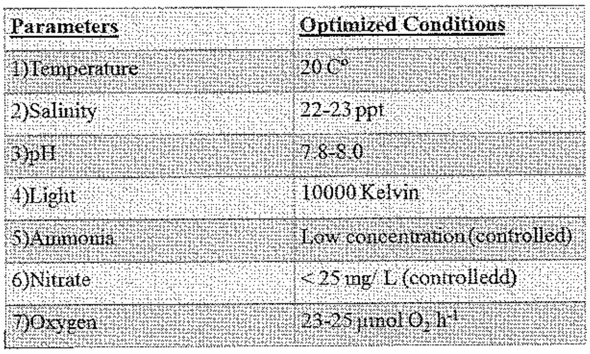


Figure 4.20 The test kits. They are used to measure oxygen, nitrate, nitrite, ammonium, ammonia, pH, salinity in the bioreactors.



Parameters	**Optimized Conditions**
1)Temperature	20 C°
2)Salinity	22-23 ppt
3)pH	7.8-8.0
4)Light	10000 Kelvin
5)Ammonia	Low concentration (controlled)
6)Nitrate	< 25 mg/ L (controlledd)
7)Oxygen	23-25 μmol O_2 h^{-1}

Figure 4.21 The optimized parameters at our cultivation methods.

Later optimized the mainly parameters, we add particular measures of chemicals, substrates and stabilizers every day. At the same time, the light system was established in our biosystem. The bioreactors were covered to obtain a light level comparable to that of the natural environment. (see Figure 4.22).

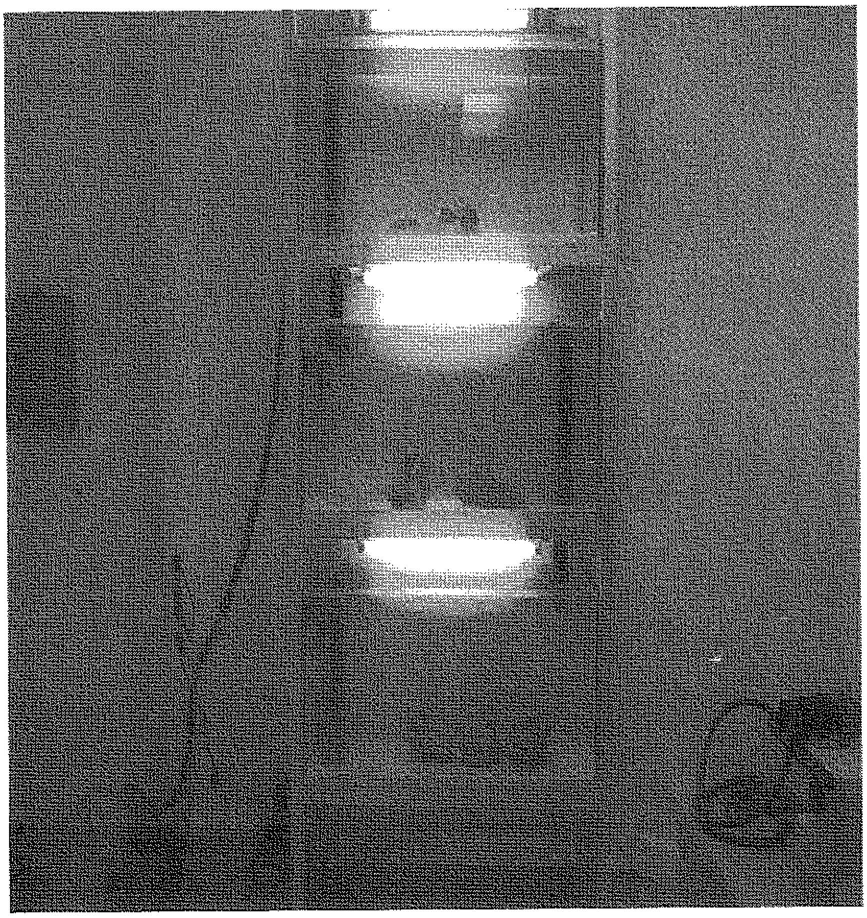


Figure 4.22 The light system in the biosystem

With regular controls the parameters, the sponge has lived by add chemicals, substrates and stabilizers.

CHAPTER 5

RESULTS AND DISCUSSION

Haliclona explants were cultured in bioreactors for a period of 67 days. All explants supplied solely and continuously with live special nutrients displayed growth. While December month, explants have lived very healthy and colorful after added to whole chemicals, and stabilizers at opened light system (see Chapter 4, Chemicals and substrates rates). At this time, explants in bioreactor displayed growth (see Figure 5.1).

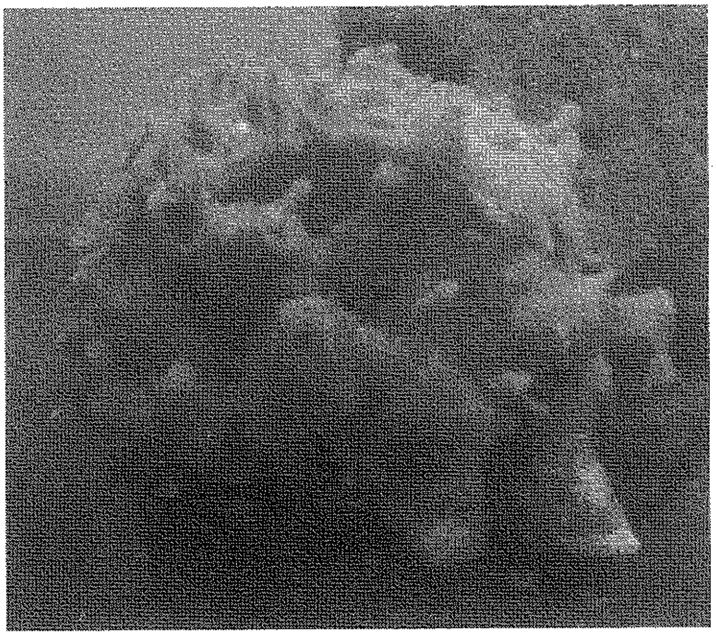


Figure 5.1 *Haliclona* sp. reached maximum vitality during day 25 and day 45.

But in January, we closed light system at nights and we decreased salinity rate, for try another parameters. And the sponge's living is decreased during this period of nutrient-poor conditions, size of explant decreased, but variability among sponge explants is often observed. We measures to vitality and growth every day. *Haliclona* sp. normally is blue and it has a lot of branches, so we have observed to blue branches, color turns from blue to white and decrease branches, it means *Haliclona* loses its vitality. We determined the salinity rate of Pacific Ocean at 15 m depth 21-23 ppt but our salt concentration in the bioreactors during the first 20 days was only 18 ppt max. This is much lower than concentrations that are usually found in the Pacific Ocean. Growth of explant during December may be correlated with the presence of regular nutrients and correct rate of salinity in the bioreactors. The average concentration salinity between day 20 and day 45 was 22 ppt which optimal rates for living sponges. No significant differences vitality of sponge between day 45 and day 50.

Later day 45, increase consumption nutrient and continuous opened high output lights caused the temperature to rise from 20 °C up to 25 and one week after this incident the sponges color were started white instead of blue and started to come loose vitality. Loss of vitality of explants, probably due to strong microbial growth and subsequent oxygen depletion. The total organic carbon (TOC) concentration in this bioreactor was 35 mg/L, which is much higher than natural occurring concentrations (see Graphic 1).

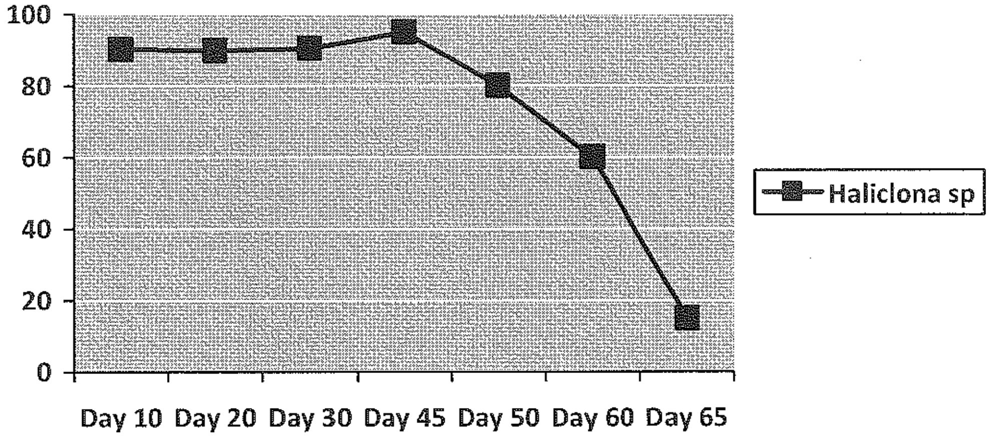


Table 5.1 The vitality rate of *Haliclona* sp. during 65 days of ex-situ cultivation. In the early days, due to transporting the observed decrease in vitality. Reached the maximum level of vitality between 20-45 days. Loss of vitality of the explants after 45 days, probably due to strong microbial growth and subsequent oxygen depletion.

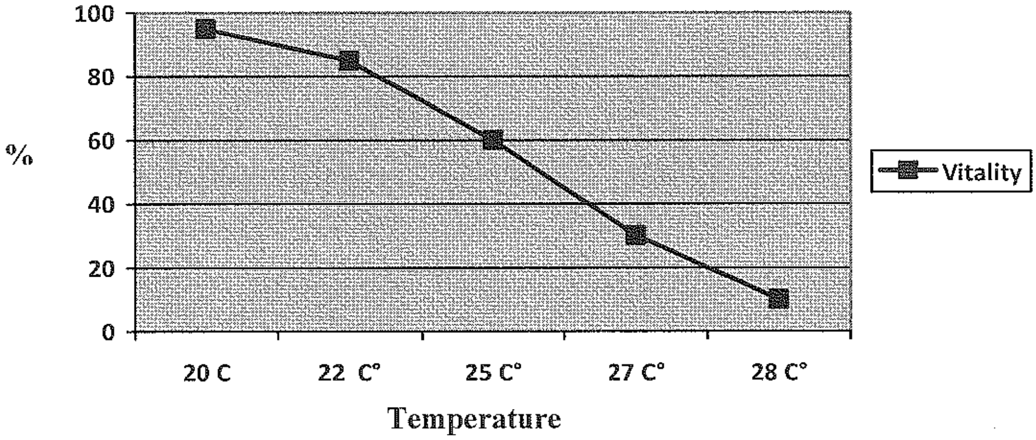


Table 5.2 The vitality rate of Haliclona sp explants depending temperature of system. The average temperature of between day 10 and 20 was 20 C° .The optimal temperature was for our biosystem and the vitality of our sponges reached maximum level. Later day 20, the temperature was 22 – 23 C° because of continuous opened high output lights and vitality of our sponges decreased. Between day 30- 40, the temperature was 24 C°, and our sponges of vitality decreased and the sponges of color were strated white instead of blue. Later day 45 , increase consumption nutrient caused the temperature to rise from 24 C° up to 28° and later our sponges vitality come loose.

The high concentration of carbon sources was obviously more suitable to bacteria and fungi than to the sponges and probably disrupted the more or less ecological balance that is present in the sea Sponges are very slow grows and so sponge cultivation systems have to be operated for a long time. The dependency of the nutrient concentration in the bioreactors on the algae concentration in the photobioreactor is risky if a steady state situation is to be maintained for several months (Sipkema, 2003). Accuracy in determining the actual circumference of encrusting sponges modeled as circles is likewise important, it is probable that additional factors such as pressure, light, nutrition concentration, and current speed affect the growth rate of sponges, for which terms are not included in the simplistic equations derived in this study. Also, ambient current profiles may cause a nutritional gradient around and over the sponge. In recent research it has been found that such factors can significantly affect growth rate and the morphology of the sponge (McDonald et al., 2003).

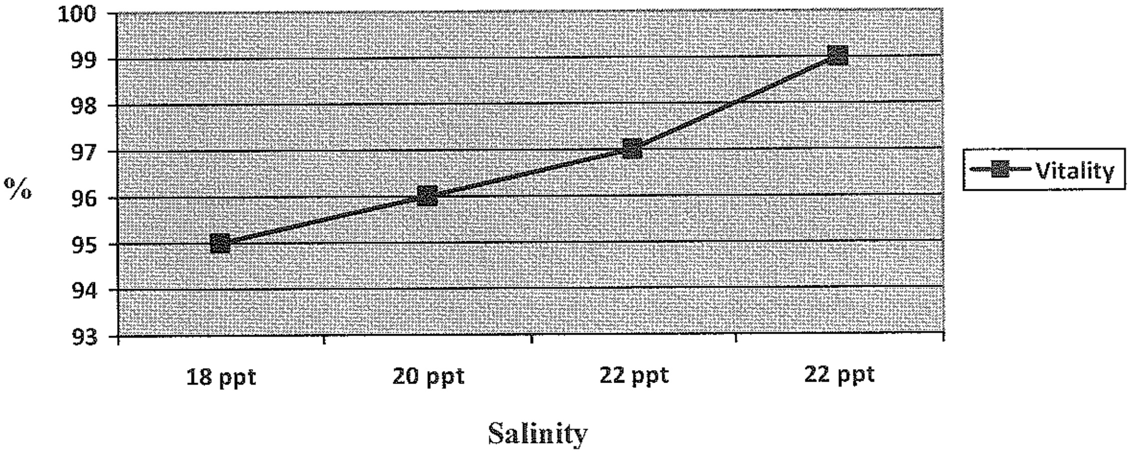


Table 5.3 The vitality rate of Haliclona sp explants depending salinity of system in the first 20 days. The salinity rate of our biosystem was 18 ppt during the first 10 days. This was much lower than concentration that are usually found in the pacific Ocean. Growth of explant during December may be correlated with the presence of regular nutrients and correct rate of salinity in the bioreactors. The average concentration salinity between day 20 and day 45 was 22 ppt which optimal rates for living sponges. No significant differences vitality of sponge between day 45 and day 50.

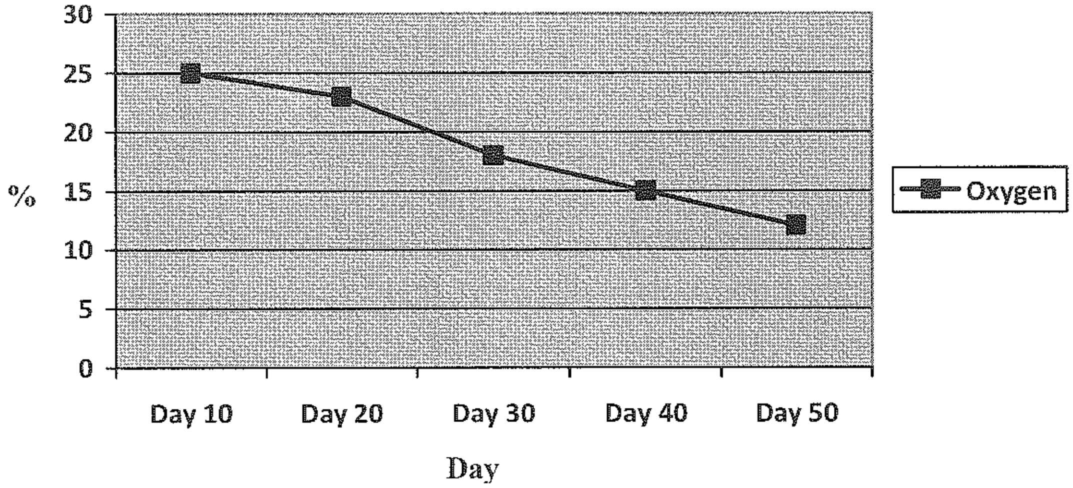


Table 5.4 The oxygen rate in the biosystem depending on increase consumption nutrient and continuous opened high output lights. At first days, the oxygen concentration was 25 µmol $O_{2\,h}^{-1}$ and it was optimum degree for biosystem. Later 20 - 30 day, the oxygen concentration decreased because of increased consumption nutrient and the sponges color were started white instead of blue and started to come loose vitality. Later day 40, the oxygen concentration was reached 15 µmol $O_{2\,h}^{-1}$ and it was so harmful for our sponges. Loss of vitality of explants, probably due to strong microbial growth and subsequent oxygen depletion.

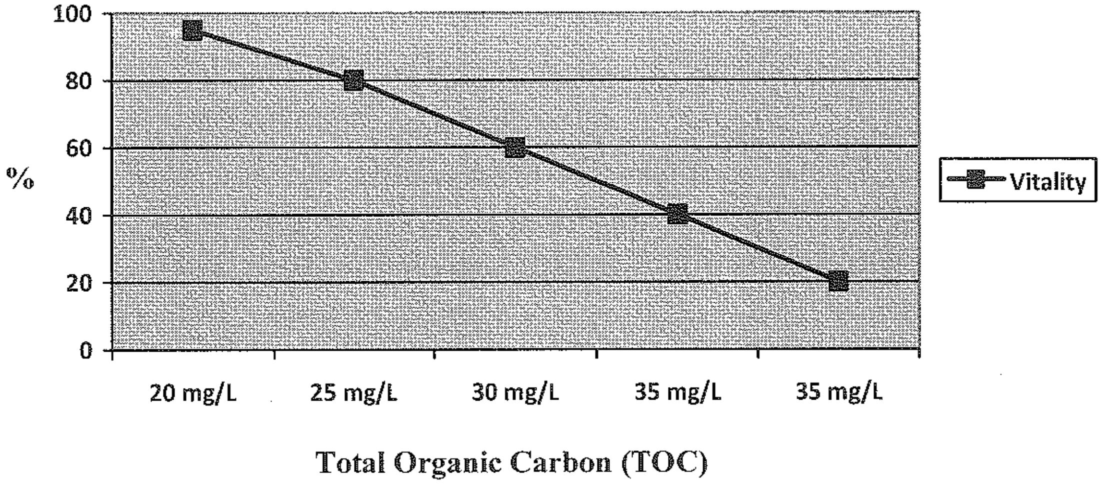


Table 5.5 The vitality rate of our sponges depending on total organic carbon (TOC) concentration. Loss of vitality of explants, probably due to strong microbial growth and subsequent oxygen depletion. Between day 10-20 the total organic carbon (TOC) concentration in the bioreactor was 20-25 mg/L.It was optimal concentration for our sponge explants. Between day 20- 30 the total organic carbon (TOC) concentration in this bioreactor was 30 mg/L and the vitality of our sponges were decreased. Later day 30, the total organic carbon (TOC) concentration in this bioreactor was 35 mg/L, which is much higher than natural occurring concentrations.

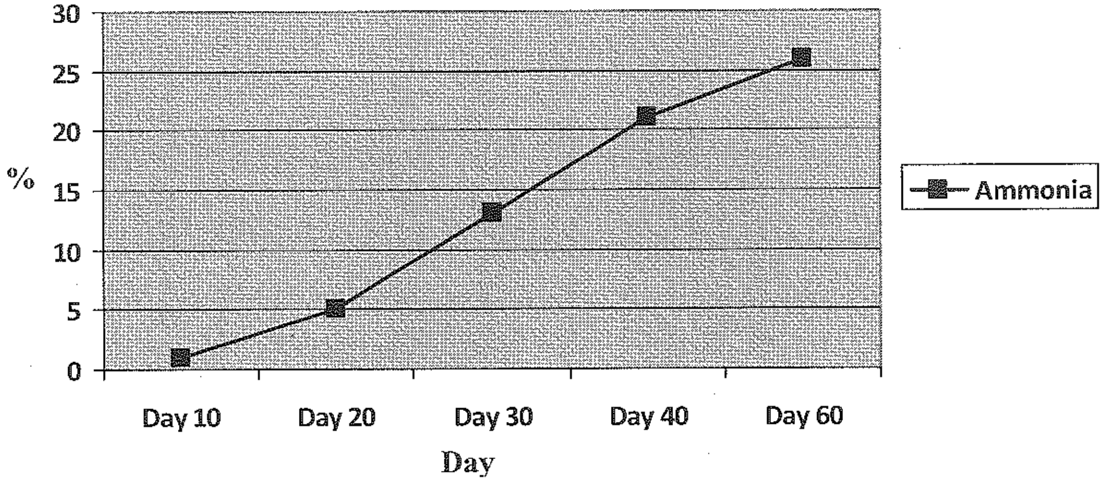


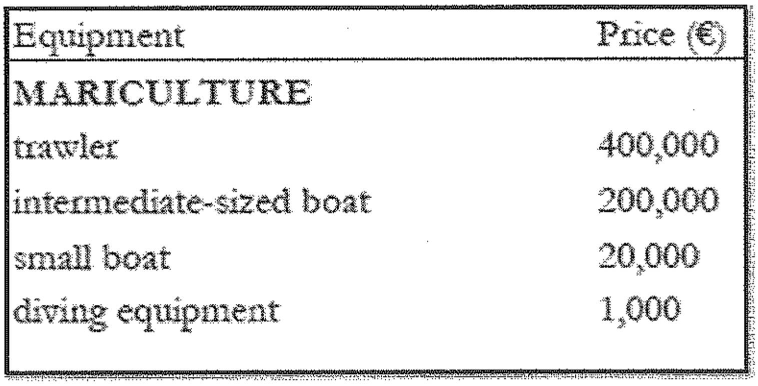
Table 5.6 The vitality rate of our sponges depending on total ammonia concentration . The viability of dissociated sponges at different ammonia concentrations. Metabolically produced by sponges ammonia is toxic to most aquatic animals even if in very low concentrations. At first days, the ammonia concentration was very low but later the concentration increased dissociated with increase consumption nutrient and increased temperature.

According to many studies, ex-situ cultivation of marine sponges has not been successful for a long time, some of them died after 1-2 weeks, some sponges 80-90 days lived maximum (Sipkema, 2004; Osinga, 1999). However, small explants generally have a higher mortality than larger explants (Duckworth et al., 1997; Turon et al., 1998). So the optimal size and number of explants to be used to start and do mariculture or an ex situ tank culture operation will be a trade-off between an acceptable mortality and a predicted growth rate for the species of choice. These choices will in turn be managed by the prevailing environmental and laboratory conditions and the desired results of the project, based on desired yields and acceptable economics (Sipkema, 2004).

CHAPTER 6

CONCLUSION

Marine sponge can produce many unique metabolites of potential commercial value. Using biotechnology these metabolites produced from sponges would require large quantities of sponge biomass that cannot be sustainably harvested from natural populations. Production of cultivated sponge metabolites from sea-based farms is feasible, but productivity is variable but metabolites production in controlled environments of aquariums has the potential to provide consistent yields, but many aspects of aquarium cultivation remain unknown for most sponges (Belarbi, 2003). According to researches, ex- situ cultivation has not been established in the laboratory for more than 2-3 months yet (Osinga, 1999; Belarbi, 2003; Sipkema, 2004). In our 67 days system; we have proved that it is more economical than mariculture, the cost is about 700.000€ in the mariculture, but we don't need this equipments in the ex-situ culture (see Figure 6) (Sipkema, 2004).



Equipment	Price (€)
MARICULTURE	
trawler	400,000
intermediate-sized boat	200,000
small boat	20,000
diving equipment	1,000

Figure 6 Cost of equipment in the mariculture (Sipkema, 2005).

Major questions is the production of sponge-sourced metabolites: can methods be developed for culturing healthy sponge without its metabolites? Studies are needed of sponge nutrition and how nutrition can influence growth and metabolite production. What can be the influence of precursor feeding? All these questions are answered, a healthy culture can be performed. As a result, if achieved optimization of ex-situ culture, will produce larger quantaties less expensive, desired size and quality sponges so their metobolites for use biotechnology, pharmcy and researchs. Also; with the widespread of sponge culture; sponge hunting is reduced and natural sponge population is improvement.

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