Cultivation of Marine Sponges:

From Sea to Cell

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1 Introduction and thesis outline

The seas and oceans occupy approximately 70% of the earth surface (Brown *et al.*, 1989). The inaccessibility of these waters to humans for many centuries resulted in much speculation about fantastic animals that would inhabit the deep oceans (Fig. 1A). The development of submarines, scuba diving and under water cameras revealed that indeed peculiar animals were present at the sea floors, for example sponges (Fig. 1B). Sponges are the most simple and ancient multicellular animals on earth and live attached to the seabed or another substratum. They show a circumpolar distribution and inhabit all seas from Greece to Antarctic and from Indonesia to Norway and from great depths (where they are exposed to high pressure) to coastal waters (Brusca and Brusca, 1990). Some species may reach a height of more than two meters, whereas others are tiny encrusting species with diameters of no more than a few centimetres (Proksch, 1994). The huge diversity with respect to their natural habitat is probably the reason for the estimated number of approximately 15,000 different sponge species (Hooper *et al.*, 2002).
In the introduction of this thesis it is described what sponges do to support life, their general biology and why mankind is interested in sponges. In the last part of the introduction the aim of this thesis is outlined.

Fig. 1A: A hydra was one of the monsters thought to inhabit the seas (Seba, 1734).

Fig. 1B: Vase-shaped sponge: *Niphates digitalis* (picture by R. Osinga).

Mode of Action

To support life, sponges pump huge amounts of seawater (170-72,000 x their own body volume per day) through their bodies (Simpson, 1984) and filtrate it to capture food particles, such as bacteria, micro algae, other unicellular organisms or dead organic

particles (Reiswig, 1971; 1974). The whole sponge body is designed for efficient filtration of the surrounding seawater (Fig. 2A), which is essential because of the low nutrient availability at the sea floor. Water is pumped into the sponge via many small canals that start at the outer surface of the sponge. The water current into the sponge is generated by specialised flagellated cells, called choanocytes, which are clustered in choanocyte chambers in the interior of the sponge (Van Trigt, 1919). The feeding structures have evolved from originally respiratory structures, but their pumping capacity had to increase many-fold to provide the sponge with a sufficient amount of food. Dissolved oxygen is taken up via inefficient diffusion inside the canals and choanocyte chambers (Jørgensen *et al.*, 1986). A more ingenious trap is required to retain nutrients in the processed seawater as their concentration is very low. Choanocytes are sponge cells that are equipped with a collar of microvilli that surrounds the flagellum to withdraw small food particles from the passing seawater. The food particles are stored in food vacuoles of the choanocytes and are passed on to archeocytes. It is generally assumed that archeocytes distribute the nutrients over the rest of the sponge, as they can travel through the sponge (Simpson, 1984).

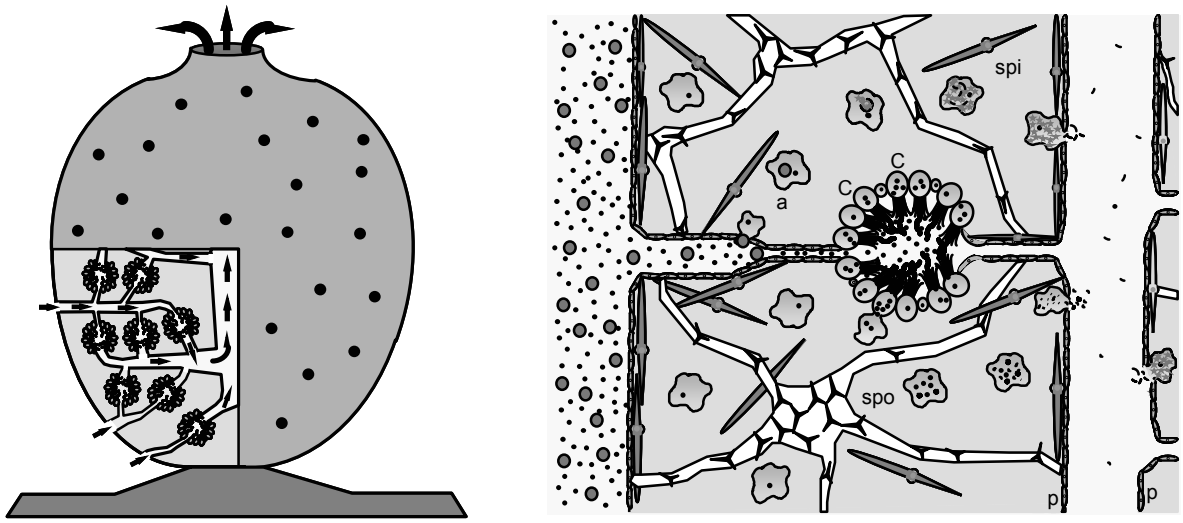

Fig. 2: Morphology and feeding physiology of sponges. **A:** Outer appearance of a simple, vase-shaped sponge with several small inflowing pores (ostia) and one large out flowing opening (osculum). A cross-section shows the aquiferous system (canals and choanocyte chambers). Arrows indicate the water flow through the sponge body. **B:** Detail of a canal and a choanocyte chamber and the uptake of food particles. A number of different cell types and skeletal elements are shown: pinacocytes (p), choanocytes (c), archeocytes (a), spicules (spi) and spongin fibres (spo) (Osinga *et al.*, 1999).

In addition, there are many other ways for ingestion of food particles (Fig. 2B). Especially larger particles are taken up directly by archeocytes from the canals before they reach the choanocyte chambers (Michin, 1900; Simpson, 1984). Furthermore, food particles can be taken up by exo- and endopinacocytes that cover the outside of the sponge and the canals and the surfaces inside the sponge (Pourbaix, 1933). Moreover, it has been suggested that sponges are capable of absorbing dissolved organic nutrient directly from the water (Pütter, 1909; 1914; Reiswig, 1971; Yahel *et al.*, 2003).

Sponge Bauplan

Skeleton and mesohyl

Sponges are currently divided into three different classes (hexactinellida, calcarea and demospongiae) based on the nature of their skeleton (Vacelet, 1985). Calcareous sponges possess a skeleton that is composed entirely of calcite spicules (Jones, 1970), while the hexactinellida, which are primarily deep-water sponges, have a skeleton that is built of six-rayed (hexactinal) siliceous spicules (Reiswig, 1979) Demospongiae form the largest class, comprising approximately 95% of all species, of which the skeleton is composed of siliceous spicules that is often supplemented by organic collagenous fibres (Brien, 1973). The spicules and collagen fibres form a strong network in the mesohyl that comprises the space between the exopinacoderm and the endopinacoderm (Fig. 2B). In addition to collagen fibres the mesohyl comprises galectins, fibronectin-like molecules, dermatopontin and polysaccharides (Schütze *et al.*, 2001). These macromolecules form the extracellular matrix, which provides the platform for specific cell adhesion as well as for signal transduction and cell growth. Because of these functions the extracellular matrix plays vital roles in digestion, gamete production, transport of nutrients and waste products by archeocytes that can move freely through the mesohyl (Müller *et al.*, submitted)

Cell types in sponges

Although many different cell types are present in sponges, only two types of organ-like structures can be defined: *pinacocytes* forming a pinacoderm and *choanocytes* forming choanocyte chambers (Fig. 2B). The other cell types are scattered through the mesohyl (Lévi, 1970).

Archeocytes are the most prominent cells in the mesohyl. Besides transport through the sponge and digestion of nutrients, they have the capacity to differentiate into any other

cell type. They provide a regulatory mechanism establishing and maintaining the equilibrium between different cell types. Some capacity for further development is retained by choanocytes, which can form gametes and by collencytes, which can become pinacocytes or myocytes (Borojevic, 1966).

The sponge skeleton is built by collencytes, spongocytes and sclerocytes. *Collencytes* secrete dispersed fibrillar collagen, while *spongocytes* build a complex supportive collagen matrix (spongin), which is the framework for the sponge. Spicula are often embedded in the collagenous matrix (Fig. 2B) (Bergquist, 1978). The production of spicules occurs inside specialised cells, the *sclerocytes*, where silica or calcite is deposited in an organised way (Garonne, 1969; Bergquist, 1978).

Finally, there are also many cell types containing small granules or vesicles. All these cells types can be grouped as *granulocytes*. A large number of different functions is attributed to the different granulocyte cell types. They have been found to be a depot of certain secondary metabolites (Thompson *et al.*, 1983), unconventional sterols (Lawson *et al.*, 1988), pigments (Liaci, 1963) or glycogen (Bergquist, 1978). In addition some granulocytes release components that comprise the mesohyl, like mucous (Donadey, 1982) or lectins (Bretting *et al.*, 1983). Moreover, there are many more sponge-cell types (e.g. porocytes, lophocytes, myocytes, bacteriocytes, trophocytes or thesocytes). Their more specialised roles in sponge physiology will not be discussed here.

Associated organisms

Other cell types that can be very abundant in sponges are microorganisms, such as bacteria, algae, cyanobacteria, fungi or other unicellular organisms, such as thraustochytrids (Roth *et al.*, 1962; Wilkinson, 1978a; Price *et al.*, 1984; Sponga *et al.*, 1999). Traditionally, the role of symbionts was considered to be related to their ability to recycle nutrients, or in the case of cyanobacteria and other chemoautotrophic bacteria, to supplement the diet of the sponge by fixing carbon and nitrogen, while the sponge provides its guests with a substratum for attachment and with nutrients (Wilkinson and Garonne, 1980). Another role of symbionts in sponges is the production of bioactive compounds, such as antibiotics, antifungal compounds and compounds that prevent predation or fouling to protect the sponge against undesired microorganisms (Osinga *et al.*, 2001). Symbionts are mainly located in the mesohyl, and especially bacteria can be numerous in some sponges, occupying up to 40% of the mesohyl volume (Wilkinson, 1978b). Lectins, which are a major constituent of the mesohyl, mediate sponge-cell attachment to the mesohyl matrix and it has been suggested that they can also play a role in the specific interactions between the sponge and its symbionts in the mesohyl (Müller *et*

al., 1988). *Pseudomonas insoluta*, a bacterium inhabiting the marine sponge *Halichondria panicea*, could only be cultured in the presence of *Halichondria panicea* lectin. Other lectins could not induce growth of the bacterium, while the *Halichondrian* lectin did not support the growth of bacteria isolated from six other marine sponges (Müller *et al.*, 1981). In addition, it was found that different sponge species in the same area contain different bacterial populations (Wilkinson, 1978a). However, more recently, molecular evidence suggested that sponges have a relatively uniform microbial community in the mesohyl (Hentschel *et al.*, 2002). In addition, intracellular bacteria have been found in sponges. They are present within large vacuoles of archeocytes, which are termed bacteriocytes (Vacelet, 1970; Bertrand and Vacelet, 1971). However, there is still only very little information on the nature of these associations.

Reproduction

Sponges are capable of both asexual (Fig. 3) and sexual reproduction (Simpson, 1984). The most simple way of reproduction is fragmentation of a sponge, for example due to heavy wave action. The dispersal of such sponge fragments can lead to reattachment and establishment of new individuals (Simpson, 1984). Budding is a process that is comparable with fragmentation, with the difference that budding is controlled by the sponge. Buds begin as thin filaments, which contain a few spicules in their core, at the exterior of the sponge or in the walls of oscules. These filaments then develop a distal swelling, some 5 mm in diameter. Subsequently, the buds drop off and round up. The current in the sea can transport them to a new location, where they can attach to a substratum and develop into a new functional sponge (Connes, 1967; 1968). The outside of buds is covered by exopinacocytes and numerous collagen-secreting cells. The interior of buds consists mainly of archeocytes, granulocytes and collencytes (Connes, 1967; Boury-Esnault, 1970).

Some sponges form gemmules as survival structures. Gemmules are small spheres, which range in size from about 300 up to 1000 μm. They have an outer spongin coat, which generally contains spicules and an inner mass of yolk-laden cells (thesocytes) (Brien, 1973). The formation of gemmules starts with the aggregation of archeocytes in the mesohyl of the sponge (Rasmont and De Vos, 1974). Archeocytes arriving early develop into thesocytes and contain nutrients to support development into a new sponge. Archeocytes arriving later differentiate into spongocytes and form a spongin sheath on the exterior of the gemmule (Rasmont, 1956). Spicules can be inserted in the spongin

layer in a random fashion (Hartman, 1958). Gemmules are located at the base of the sponge and remain attached to the substrate after the sponge has died (Pourbaix, 1934). Fully formed gemmules are kept in a quiescent state by low temperature (3-4ºC) (Simpson and Gilbert, 1973) or in the presence of the inhibitor gemmulostasin (Rasmont, 1965). Gemmulostasin is produced by the parent tissue and inhibits the germination of these gemmules. However when the parent sponge disintegrates, gemmules can start to germinate. Germination starts with the outflow of the thesocytes through a narrow opening. They spread out on the gemmule coat and the substratum and attach. Within a week after germination, spicules, canals and choanocyte chambers are formed and a functional sponge is developed (Connes, 1975).

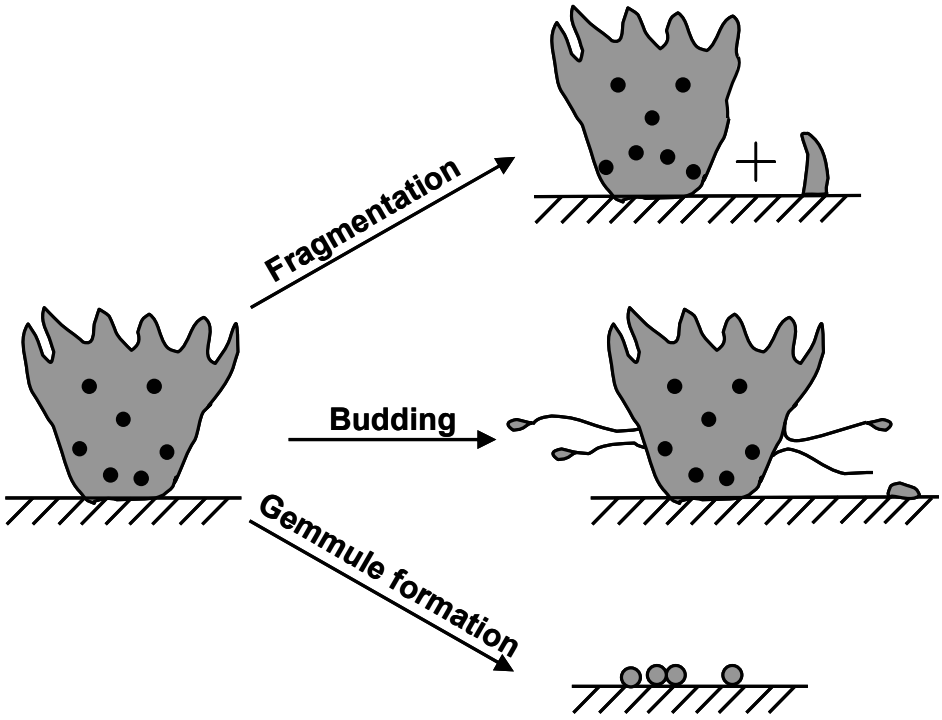

Fig. 3: A schematic presentation of asexual reproduction of sponges.

Although they are fixed to a substratum, a number of different sexual reproductive processes exist in sponges. In general, sponges are hermaphrodite, but produce oocytes and spermatocytes at different times. Spermatocytes are formed by differentiation of choanocytes (Tuzet and Pavans de Ceccatty, 1958), while for oocytes, both archeocytes and choanocytes have been described as stem cells (Leveaux, 1941; Sara, 1974). Oocytes are located in the mesohyl of 'female' sponges and need to be fertilised *in situ*. 'Smoking sponges' emit clouds of sperm from the oscules over periods up to 20 min. Spermatozoa can be taken into the 'female' sponge via the inflowing water. They are captured in the choanocyte chambers and enter the mesohyl to locate the oocytes (Reiswig, 1970).

Subsequently, embryogenesis is initiated and a number of cleavages takes place (Reiswig, 1976). The embryo is transformed into a ciliated mature larva in a few steps (Tuzet, 1970). It is usually considered that larvae are released via the oscules before they swim a 3-48 hours in the sea. Prior to attachment the larvae enter a short creeping phase. After attachment the larvae quickly become functional young sponges (Lévi, 1956).
In addition, the sexual products of some demosponges develop directly into small perfect young sponges without an intervening larval stage, which can also be released via the oscules (Watanabe, 1957). Fertilised oocytes can also be released, before they have developed into larvae. The time between expulsion of the eggs and development into a larva is approximately 24 hours. These larvae crawl for a period up to 20 days before they attach and differentiate into a young sponge (Borojevic, 1967; Reiswig, 1976).

Sponges as a Product

It is not exactly clear when sponges were used by humans for the first time. It goes back at least to the time of Homer, some seven hundred years before Christ. In the Iliad, he describes Hephaestus, the lame god of artists and blacksmiths, using a sponge to wash his face and body after working at the forge. In the Odyssey, he writes that when the suitors of Penelope had dined, white-armed maidens cleared the food and washed down the table with sponges. In the bible, it is described that Jesus Christ asked for some water when he suffered on the cross, but the Roman centurions gave him a sponge soaked in bile and vinegar instead. On the Greek island of Kalymnos, which has been the centre of commercial bath sponge business during the last centuries, the sponge divers used to say that Jesus Christ had cursed that sponge and from that time, sponges were sent to the deepest seas and it was ordained that men would suffer in bringing them to land (Warn, 2000). The first sponges that were obtained were also not taken from the sea floor, but they were sponges that were drifted ashore and collected by the Phoenicians. Greek divers started harvesting sponges from the sea (Hofrichter and Sidri, 2001) and in the 19th and 20th century natural bathing sponge trade became big business (Warn, 2000). Sponges have been used for numerous applications: cleaning, painting, filtration, or as a gas mask (Hofrichter and Sidri, 2001). Women used sponges to absorb menstrual discharge or as contraceptive (Tone, 2001). Classical bootblacks used sponges instead of a piece of cloth. Knights and soldiers used them as pad under their helmets and leg guards in order to reduce the strength of hostile pushes. The Roman emperor Caligula used sponges to sentence people to death by letting them suffocate. Burglars even tied sponges to their

feet to have their steps unheard (Hofrichter and Sidri, 2001). South American and African tribes used fresh-water sponges as additive to clay for making ceramics. The sponge spicules had the function of metal wires in concrete and made ceramics less vulnerable for cracks (Janussen and Hilbert, 2002). One sponge that does not contain spicules, *Chondrosia reniformis*, was eaten raw, roasted or cooked by Dalmatian fisherman (Steuer, 1904; Siewing, 1985). Nowadays most natural sponges have been replaced by synthetic ones or other devices and natural sponges are regarded as a luxury product or even as an oddity or just for decoration.

Sponges and Biotechnology

Currently, sponges have gained renewed interest due to many secondary metabolites with potential pharmaceutical applications that have been discovered. Some of them, such as manoalide and halichondrin B were harvested in large quantities for clinical trials (Kernan *et al.*, 1987; Litaudon *et al.*, 1997). However, the combination of predominantly low concentrations of these molecules 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, a total of 5000 tonnes of sponge would be required. In addition, it was estimated that only approximately 300 tonnes are present in the seas (Munro *et al.*, 1999). The halichondrin B case is not an exceptional example and therefore, other methods to obtain large quantities of sponge metabolites have gained attention in the last decade. Researchers have explored 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.

Production of the sponge metabolites by a genetically modified host is not included in this list, as there is currently only very little information about the genes that are involved in the pathways that lead to the production of the bioactive compounds.

Outline of this Thesis

The aim of this thesis is to develop a biotechnological process for the production of secondary metabolites from sponges, and in the second place, to obtain insight in the feasibility of such a process. In **chapter 2** of this thesis the secondary metabolites that have been discovered in marine sponges and their pharmacological effects are reviewed in order to explore the pharmaceutical potential of these compounds.

A number of different methods were studied for the production of the secondary metabolites by cultivation of marine sponges. The choice of the sponge species that were used was based mostly on availability of the species for experiments. Therefore, most experiments should be regarded as a model for the cultivation of marine sponges in general. The methods that were used to obtain sponge biomass differed in their resemblance with the sea. Simulated seas, in which natural carbon- and nitrogen cycles are mimicked (Fig. 4), were used and further developed for the maintenance and growth of sponges in cooperation with EcoDeco B.V. Development of a suitable growth medium is one of the most important issues to optimise 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.

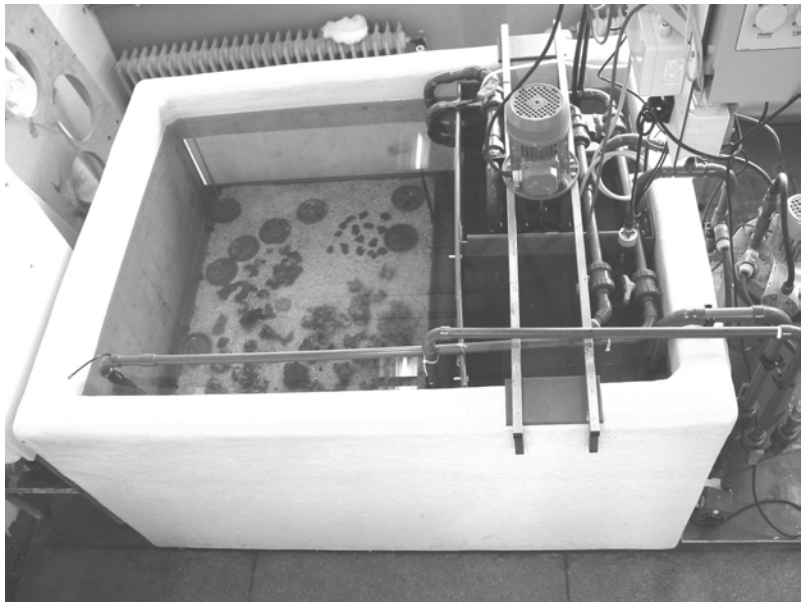

Fig. 4: An upper view of a bioreactor in which the natural cycles in the sea are mimicked (volume ~ 1400 L). Three sponge species are visible as dark spots on the 'seabed' at the bottom of the reactor. Equipment to control the pH, oxygen concentration, redox potential and temperature is connected to the reactor at the right side of the picture.

In **chapter 3** a number of substrates that were different in their particulate organic carbon (POC) and dissolved organic carbon (DOC) content, were offered to sponge explants (cuttings from a parent sponge) of two different species. The growth measurements and data that were retrieved from the literature were used to find a generic mechanistic model that could describe the growth of sponges. Such a model can be used to understand how sponges grow and to predict the yield of a sponge culture.

In **chapter 4** and **chapter 5** the potential of tissue culture to grow sponge biomass is explored. In chapter 4 the formation of primmorphs of seven different sponges is described. Primmorphs are spherical-shaped cell aggregates with a diameter of approximately 1 μm. They are formed from a dissociated cell suspension under gentle agitation. Primmorphs resemble buds and gemmules and may turn out to be artificially induced regeneration bodies. The putative first step of development of new functional sponges from primmorphs is discussed in chapter 5.

A number of basic steps for the development of sponge-cell cultures have been initiated in **chapter 6** and **chapter 7**. Sponge-cell cultures have always been looked at with suspicion, because cell growth was usually related to growth of contaminants, which are often introduced during the preparation of primary sponge-cell cultures. In chapter 6 a genetic identification method for sponge cells in cell cultures is described. With this method, it can be determined whether the cells in culture are truly sponge cells and what fraction of the cultured cells are actually sponge cells. In chapter 7 a method to estimate the viability of sponge cells in culture is described. Cell viability is a crucial parameter for the optimisation of culture conditions, but a reliable method to assess the viability of sponge cells is currently not available. The combined use of the fluorescent dyes fluorescein diacetate and propidium iodide has been applied to monitor the viability of sponge-cell cultures in different conditions.

The conclusion of this thesis is given in **chapter 8**. In this chapter, the technical and economical potential of different production methods of sponge metabolites is assessed. The feasibility of potential sponge-based medicines is compared with currently used pharmaceuticals.

2 Marine sponges as pharmacy

Abstract

Marine sponges have been considered as a gold mine during the past fifty years, with respect to the diversity of their secondary metabolites. The biological effect of new metabolites from sponges has been reported in hundreds of scientific papers and they are reviewed in this paper. It can be stated that sponges could provide potential future drugs against important diseases, such as cancer, a range of viral diseases, malaria and inflammations. While for most metabolites their molecular mode of action is still unclear, for a substantial number of compounds the mechanisms by which they interfere with the pathogenesis of a wide range of diseases has been reported. The latter point is one of the key factors that are required to transform bioactive compounds into medicines. Sponges produce a plethora of chemical compounds with widely varying carbon skeletons, which have been found to interfere with the pathogenesis at many different points. The fact that a particular disease can be fought at different points increases the chance of developing selective drugs for specific targets.

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Introduction

The relationship between sponges and medicines 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. Sponges were soaked with pure wine and put on the left part of the chest in case of heartaches and soaked in urine to treat bites of poisonous animals. Plinius recommended to use sponges against sunstrokes and they were used against all kinds of wounds, bone fractures, dropsy, stomach aches, infectious diseases, and testicle tumours (Hofrichter and Sidri, 2001) or even as implant after breast operations (Arndt, 1938). At least since the 18th century up to now, Russian, Ukrainian and Polish physicians use a fresh-water sponge, they call Badiaga (Fig. 1), for the treatment of patients (Nozeman, 1788). The dry powder of this sponge is rubbed in on the chest or back against lung diseases or a cough, or in case of foot and leg aches (e.g. rheumatism) on the sore places (Schröder, 1942). Oficjalski (1937) discovered that Badiaga is not really one sponge, but different mixtures of several fresh-water sponges depending on the region. In Poland it consisted of powder of *Euspongilla lacustris*, *Ephydatia fluviatilis* and *Meyenia muelleri*, while the Russian Badiaga was a mixture of *Euspongilla lacustris*, *Ephydatia fluviatilis*, *Spongilla fragilis* and *Carterius stepanowi*. He suggested that the high iodine concentration in all sponge species gives rise to the wholesome effect of Badiaga. At present Stodal, syrup containing roasted *Spongia officinalis*, is used for homeopathic treatment of dry and asthmatic cough in the Western world (Stodal, 2003).

Fig. 1: Examples of homeopathic drugs based on sponge extracts that are still used at present (Badiaga and Stodal syrup).

Pharmaceutical interest in sponges was aroused in the early 1950's by the discovery of a number of unknown nucleosides: spongothymidine and spongouridine in the marine sponge *Cryptotethia crypta* (Bergmann and Feeney, 1950; 1951). These nucleosides were the basis 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 currently used in the routine treatment of patients with leukaemia and lymphoma. One of its fluorinated derivatives has also been approved for use in patients with pancreatic, breast, bladder, and lung cancer (Schwartsmann, 2000). At the same time it was revealed that certain lipid components such as fatty acids, sterols and other unsaponifiable compounds occur in lower invertebrates in a diversity far greater than that encountered among animals of higher organisation (Bergmann and Swift, 1951). These early promises have now been substantiated by an overwhelming number of bioactive compounds that have been discovered in marine organisms. More than 15.000 marine products have been described up to now (MarinLit, 1999; Faulkner, 2000; 2001; 2002). Sponges are 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).

Most bioactive compounds from sponges can be classified as antiinflammatory, antitumour, immuno- or neurosurpressive, antiviral, antimalarial, antibiotic or antifouling.

The chemical diversity of sponge products is remarkable. In addition to the unusual nucleosides, bioactive terpenes, sterols, cyclic peptides, alkaloids, fatty acids, peroxides, and amino acid derivatives (which are frequently halogenated) have been described from sponges (Fig. 2).

For this review, we have surveyed the discoveries of marine sponge-derived products up to now, and attempted to show the variety of possible potential medical applications of metabolites from sponges and the mechanisms how they interfere with the pathogenesis of diseases inside the human body. The latter is a prerequisite for the development of a drug from a bioactive compound. For example, many secondary metabolites are inhibitors of growth of cancer cell lines, but this does not imply that they will be suitable as a medicine against cancer, because they may exhibit important side effects. The next sections will summarise compounds per disease type and describe their mode of action, and discuss the reasons why sponges would produce these metabolites. A medical glossary is included at the end of this review.

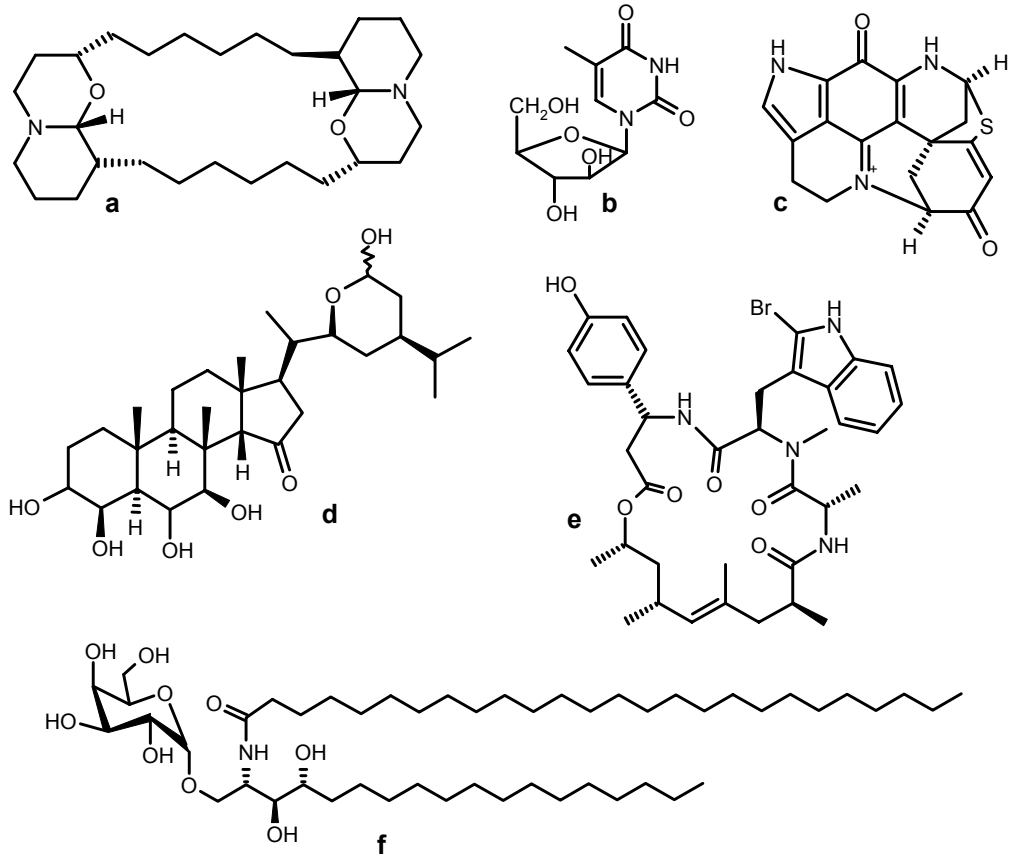

Fig. 2: An illustration of the chemical diversity of sponge-derived molecules: **a** xestospongin C (*Xestospongia* sp. / macrocyclic bis-oxaquinolizidine); **b** spongothymidine (*Cryptotethia crypta* / unusual nucleoside); **c** discorhabdin D (*Latrunculia brevis*; *Prianos* sp. / fused pyrrolophenanthroline alkaloid); **d** contignasterol (*Petrosia contignata* / oxygenated sterol); **e** jaspamide (*Hemiastrella minor* / macrocyclic lactam/lactone); **f** agelasphin (*Agelas mauritianus* / α-galactosylceramide).

Antiinflammatory compounds

Acute inflammations in the human body can occur due to microbial infection, physical damage or chemical agents. The body reacts by changing the blood flow, increasing the permeability of blood vessels and escape of cells from the blood into the tissues (Tan *et al.*, 1999). Chronic inflammation of the skin or joints may lead to severe damage of the body, because it may lead to psoriasis or rheumatic arthritis (Pope *et al.*, 1999). Sponges have been proven to be an interesting source of antiinflammatoric compounds (Table 1).
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 action is based on the irreversible inhibition of the release of

arachidonic acid from membrane phospholipids by preventing the enzyme phospholipase A_2 from binding to the membranes (Glaser *et al.*, 1989). A rise in the intracellular arachidonic acid concentration would lead to upregulation of the synthesis of inflammation mediators as prostaglandins and leukotrienes (Fig. 3). Phospholipase A_2 inhibition has been recorded for many sesterterpenes from sponges of the order Dictyoceratida, but also for bis-indole alkaloids such as topsentin (Jacobs *et al.*, 1994). The mechanism by which they affect the inflammation process is different from commonly used non-steroidal antiinflammatory drugs. Only a few sponge-derived terpenoids have been found to inhibit lipoxygenase, another enzyme that is involved in the inflammatory response (Carroll *et al.*, 2001).

The antiinflammatory sponge products are selective inhibitors of specific enzymes of a range of diseases, like psoriasis or rheumatic arthritis. The currently used non-steroidal antiinflammatory drugs often fail to control the disease and present important side effects such as an increased risk of gastrointestinal bleeding and renal complications (De Rosa, 2002). These are caused by unselective inhibition of cyclooxygenases of which some are also involved in the promotion of the production of the natural mucus which protects the gastrointestinal tract (Bjarnason *et al.*, 1993).

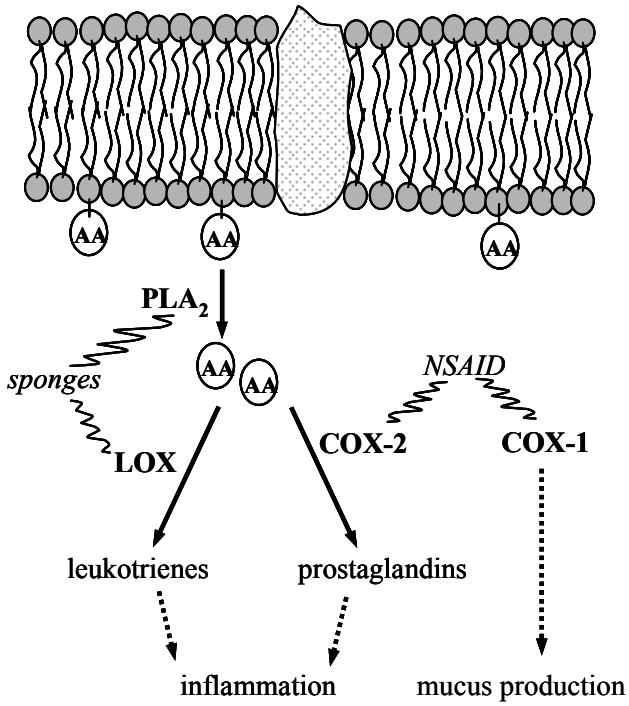

Fig. 3: The inflammatory cascade inside the cell. Phospholipase A_2 (PLA_2) catalyses the release of membrane-bound arachidonic acid (AA) to free arachidonic acid. Arachidonic acid is converted to leukotrienes and prostaglandins by lipoxygenase (LOX) and cyclooxigenase-2 (COX-2) respectively. Sponge-derived antiinflammatory molecules are mainly inhibitors of PLA_2 or LOX, while non-steroidal antiinflammatory drugs inhibit COX-2, but also the constitutive COX-1.

Table 1: Examples of antiinflammatory products from sponges.

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

Table 2: Examples of antitumour products from sponges.

Compound	Compound class	Sponge / order	Mode of Action	reference
BRS1	diamino-dihydroxy polyunsaturated lipid	calcareous sponge / ?	protein kinase C inhibitor[1]	Willis and De Vries, 1997
isoaaptamine	benzonaphthyridine alkaloid	*Aaptos aaptos* / Hadromerida	protein kinase C inhibitor[1]	Fedoreev *et al.*, 1989
debromohymenial-disine	pyrrole-guanidine alkaloid	*Hymeniacidon aldis* / Halichondrida	protein kinase C inhibitor[1]	Kitagawa *et al.*, 1983
	prenylhydroquinone derivative	*Sarcotragus* sp. / Dictyoceratida	A1,3-fucosyltransferase inhibitor	Wakimoto *et al.*, 1999
adociasulfates	triterpenoid hydroquinones	*Haliclona* (aka adocia) sp. / Haplosclerida	kinesin motor protein inhibitors	Blackburn *et al.*, 1999
discodermolide	linear tetraene lactone	*Discodermia dissoluta* / 'Lithistids'	stabilisation of microtubules	Ter Haar *et al.*, 1996
laulimalide	macrocyclic lactone	*Cacospongia mycofijiensis* / Dictyoceratida	stabilisation of microtubules	Mooberry *et al.*, 1999
peloruside A	macrocyclic lactone	*Mycale hentscheli* / Poecilosclerida	stabilisation of microtubules	Hood *et al.*, 2002
hemiasterlin	unusual tripeptide	*Auletta* sp. / Halichondrida	stabilisation of microtubules	Anderson *et al.*, 1997
dictyostatin	macrocyclic lactone	*Corallistidae* sp. / 'Lithistids'	stabilisation of microtubules	Isbrucker *et al.*, 2003
spongiastatin 1	bis(spiroacetal) macrolide	*Spongia* sp. / Dictyoceratida	stabilisation of microtubules	Bai *et al.*, 1993
arenastatin A	macrocyclic lactam/lactone	*Dysidea arenaria* / Dictyoceratida	stabilisation of microtubules	Koiso *et al.*, 1996

[1] has also antiinflammatory activity

Table 2 continued: Examples of antitumour products from sponges.

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
salicylihalamide A	salicylate macrolide	*Haliclona* sp. / Haplosclerida	v-ATPase inhibitor	Erickson *et al.*, 1997
6-hydroximino-4-en-3-one steroids	oximated steroid	*Cinachyrella* sp. / Spirophorida	aromatase inhibitor	Holland *et al.*, 1992
halichondrin B	polyether macrolide	*Halichondria okadai* / Halichondrida	stabilisation of microtubules	Hirata and Uemura, 1986
haligramides A and B	cyclic peptide	*Haliclona nigra* / Haplosclerida	anticancer	Rashid *et al.*, 2000
discorhabdin D	fused pyrrolophenanthroline alkaloid	*Latrunculia brevis* / Poecilosclerida; *Prianos* sp. / Haplosclerida	anticancer	Perry *et al.*, 1988
callystatin A	polyketide	*Callyspongia truncata* / Haplosclerida	anticancer	Kobayashi *et al.*, 1997
tedanolide	macrocyclic lactone	*Tedania ignis* / Poecilosclerida	anticancer	Schmitz *et al.*, 1984
glaciasterols A and B	9,11-secosterol	*Aplysilla glacialis* / Dendroceratida	antileukaemia, anti breast cancer	Pika *et al.*, 1992
crambescidins 1-4	pentacyclic guanidine derivative	*Crambe crambe* / Poecilosclerida	anticancer, antiviral (herpes)	Balconi *et al.*, 1995; Jares-Erijman *et al.*, 1991
axinellins A and B	cyclic peptide	*Axinella carteri* / Halichondrida	antiproliferative	Randazzo *et al.*, 1998b
chondropsin A and B	macrolide lactam	*Chondropsis* sp. / Poecilosclerida	anticancer	Cantrell *et al.*, 2000
incrustasterols A and B	sterol	*Dysidea incrustans* / Dictyoceratida	against many cancers	Casapullu *et al.*, 1995

Antitumour compounds

A number of isolated sponge compounds are inhibitors of protein kinase C (PKC). PKC inhibitors have attracted interest worldwide, as there is evidence that too high levels of PKC enzyme are both involved in the pathogenesis of arthritis and psoriasis (due to regulation of phospholipase A_2 activity), and in tumour development (Bradshaw *et al.*, 1993; Yoshiji *et al.*, 1999). PKC is believed to be the receptor protein of tumour-promoting phorbol esters, and PKC inhibitors prevent binding of carcinosarcoma cells to the endothelium (Liu *et al.*, 1991). Glycosylation of the receptors, and especially the presence of fucose residues, plays an important role in the binding of carcinosarcoma cells and leukocytes to the receptors in the endothelium (Springer and Lasky, 1991). Fucosyltransferase inhibitors, for instance the octa- and nonaprenylhydroquinone sulfates that were isolated from a *Sarcotragus* sp. (Wakimoto *et al.*, 1999), may therefore be promising candidates for controlling inflammatory processes such as arthritis or for combating tumour growth.
In addition to PKC inhibitors and fucosyl transferase inhibitors, numerous anticancer molecules with a different mode of action have been discovered in marine sponges (Table 2). 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).

Many non-specific cell growth inhibitors have been discovered in sponges. They are valuable to treat cancer under certain conditions, but they also affect the division of healthy cells. Therefore, their applications are limited, depending on their specific characteristics. The cytoskeleton is an interesting target for cancer therapy, as the microtubules and microfilaments are involved in cellular organisation during cell division. A number of adociasulfates (triterpenoid hydroquinones) from a *Haliclona* sp. were the first inhibitors of the kinesin motor protein to be discovered. These toxins are believed to inhibit the protein by binding to the microtubule binding site and "locking up" the protein's motor function and thereby blocking cell division (Blackburn *et al.*, 1999). 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), laulimalide (Mooberry *et al.*, 1999), peloruside A (Hood *et al.*, 2002) and dictyostatin (Isbrucker *et al.*, 2003). Other metabolites, such as latrunculin A from

Latrunculia magnifica (Coue *et al.*, 1987) and swinholide A from *Theonella swinhoei* (Bubb *et al.*, 1998), disrupt the polymerisation of actin, which is the key element of the microfilaments, and it can block many cellular processes among which cell division. Spongiacidin B (Inaba *et al.*, 1998) and fascaplysin (Soni *et al.*, 2000) are examples of sponge-derived metabolites that inhibit cell division by inhibition of cyclin-dependent kinase 4, which leads to arrest of cells in the G1 phase. Other metabolites, such as mycalamide (Burres and Clement, 1989) and aragusterol (Fukuoka *et al.*, 2000) disturb cell division by inhibition of protein synthesis. Neoamphimedine (De Guzman *et al.*, 1999) and elenic acid (Juagdan *et al.*, 1995) inhibit the development of tumours by blocking topoisomerase II, the nuclear enzyme which makes transient DNA breaks that are required for replication (Liu and Chen, 1994).

Nitric oxide synthetase inhibitors, such as the imidazole alkaloid Naamine D that was isolated from the calcareous sponge *Leucetta* cf. *chagosensis* (Dunbar *et al.*, 2000), are not involved in growth inhibition of cancer cells, but may prevent events in the early phases of tumourigenesis. Nitric oxide could participate in the tumourigenesis by mediating DNA damage and support tumour progression through the induction of angiogenesis (Lala and Orucevic, 1998). However, inhibition of nitric oxide synthetase may also affect other physiological processes in which nitric oxide is involved, such as intra- or transcellular messaging and it is involved in regulation of the immunogenic respons by T-lymphocytes. Agelasphin (KRN7000) from *Agelas mauritianus* (Kobayashi *et al.*, 1995) has been found to stimulate the immune system by activation of dendritic and natural killer T-cells (NKT). The NKT cells level in the blood is lower in the blood of patients with cancer or autoimmune disease, such as type 1 diabetes (Shimosaka, 2002) and in mice it was shown that tumors could be rejected by stimulation of the immunesystem by agelasphin (Yamaguchi *et al.*, 1996).

The activity of other compounds is more specific towards tumour cells. Multidrug resistance in human carcinoma cells that is caused by overexpression of two kinds of membrane glycoproteins, is reversed by Agosterol A from the marine sponge *Spongia* sp. It has been suggested that an altered cytosolic pH plays a role in drug resistance. Vascular (H^+) ATPase (v-ATPase) is an enzyme that is involved in many cellular processes that are often upregulated in cancer cells, such as acidic vesicular organelle formation, which is a response to radiation injury or manipulation of the pH to decrease entry of chemotherapeutics into the cells (Martínez-Zaguilán *et al.*, 1999). Salicylihamide A was isolated from a *Haliclona* sp. as a selective inhibitor of v-ATPase and has been shown to be 60-fold more cytotoxic towards certain cancer cells than to their normal non-cancerous counterparts (Erickson *et al.*, 1997).

The first natural 6-hydroximino-4-en-3-one steroids were isolated from *Cinachyrella* spp. (Rodriguez *et al.*, 1997) and are examples of molecules that can be deployed 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 (Fig. 4). Blockade of this step allows treatment of hormone-sensitive breast cancer that is dependent on estrogen (Lønning *et al.*, 2003). A peculiar fact about the 6-hydroximino-4-en-3-one steroids is that they were chemically synthetised before they were even discovered in nature.

In addition, many more compounds, that displayed growth inhibition activity of tumour cell lines have been isolated (Table 2), although their exact effects are still unclear. Discorhabdin D (Perry *et al.*, 1988), Chondropsin A and B (Cantrell *et al.*, 2000), Haligramides A and B (Rashid *et al.*, 2000), and Glaciasterols A and B (Pika *et al.*, 1992) are only a few examples of these molecules.

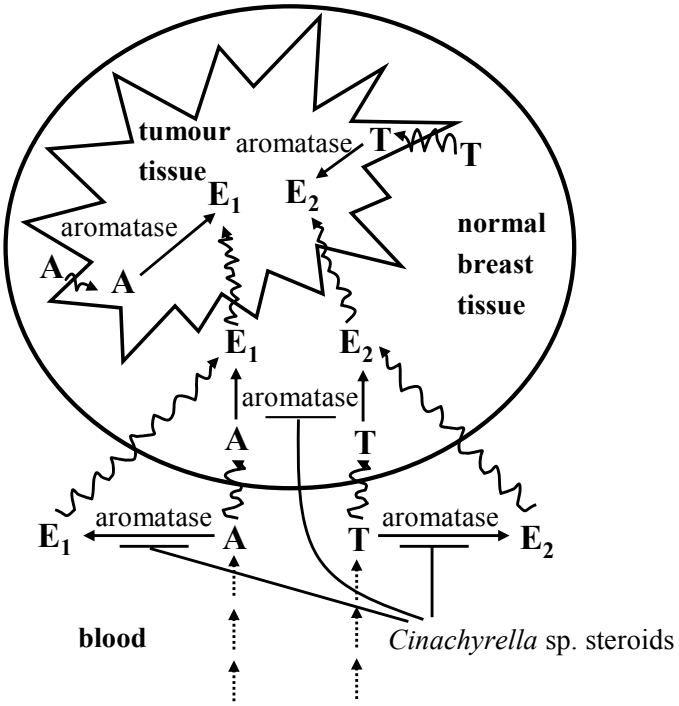

Fig. 4: The inhibition of breast cancer by *Cinachyrella* sp. steroids. Aromatase is the key enzyme in the formation of the estrogens estrone (E_1) and estradiol (E_2). It catalyses the final steps, from androstenedione (A) to estron and from testosterone (T) to estradiol, in the estogen pathway. Estrogen 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. The inhibition of aromatase in the tumour tissue is not included in the picture for the clarity of the picture.

Immunosuppressive activity

In addition to their possibilities for treatment of cancer, 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 (Griffith and Gross, 1996). Immune system suppression is desired in case of hypersensitivity to certain antigens (e.g. allergies) or organ transplantations. Patients who receive a donor organ need life-long medication to prevent rejection by the immune system, and for that reason it is extremely important that these medicines are very specific suppressors. Therefore there is a continuous demand for new immunosuppressives. A number of new molecules with immunosuppressive activity have been discovered in marine sponges, which interfere at different points of the immune response (Table 3; Fig. 5).

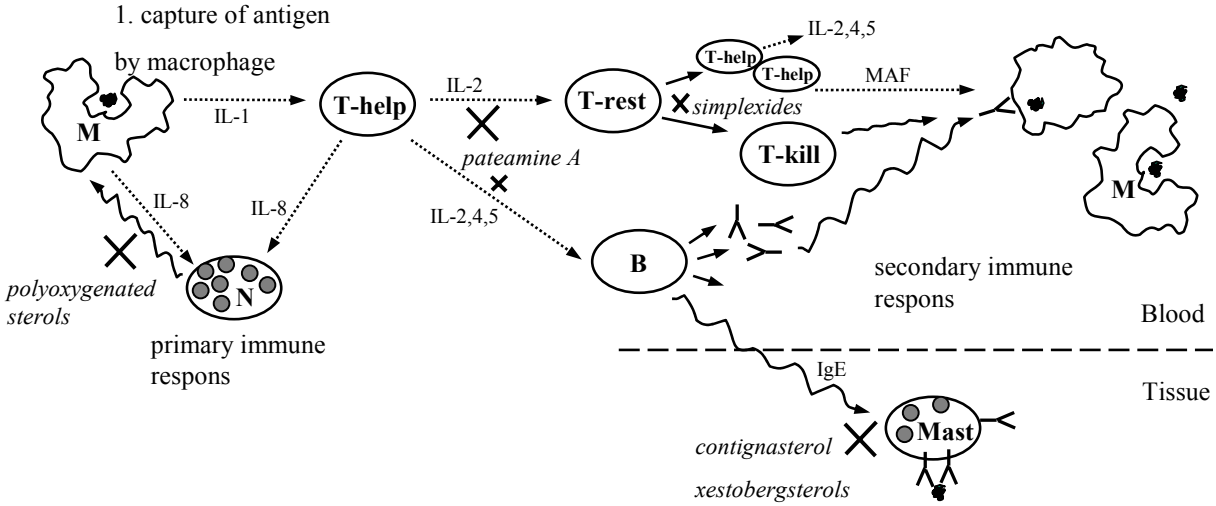

Fig. 5: A simplified representation of the immune respons after capture of an antigen by the macrophages (M). Both macrophages, but especially T-helper cells (T-help) secrete many interleukins (IL-x) or macrophage activation factor (MAF), to trigger the primary immune response via neutrophils (N), or 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 as a response of binding of an antigen to IgE molecules that are present in their cell membranes. The black crosses indicate the position where sponge-derived immunosuppressive compounds interfere with the immune response.

Three polyoxygenated sterols from a *Dysidea* sp. from Northern Australia are selective immunosuppressive compounds that inhibit the binding of interleukin-8 (IL-8), a cytokine that attracts neutrophils into an area of tissue injury, to the IL-8 receptor (Leone *et al.*, 2000). The simplexides from the Caribean sponge *Plakortis simplex* are a group of

immunosuppressive glycolipids that inhibit proliferation of activated T-cells by a non-cytotoxic mechanism (Costantino *et al.*, 1999). Pateamine A, from 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* (Bramley *et al.*, 1995) and the activation of eosinophils into airways in guinea-pigs and could be used to treat asthma (Langlands *et al.*, 1995).

Blood-related diseases

In addition to regulators of the white blood cells, also molecules that interfere with other blood-related diseases as thrombosis, atherosclerosis or diabetes, have been discovered in sponges (Table 4). The process of blood coagulation is triggered by a complex proteolytic cascade that leads to the formation of fibrin. Thrombin is a serine protease that cleaves a peptide fragment from fibrinogen, which then leads to the generation of fibrin, a major component of blood cloths (Shuman *et al.*, 1993). Cyclotheonamide A, isolated from a *Theonella* sp. (Fusetani *et al.*, 1990), represents an unusual class of serine protease inhibitors and is a potential drug for the treatment of thrombosis (Maryanoff *et al.*, 1993). Eryloside F from *Erylus formosus* was found to be a potent thrombin receptor antagonist (Stead *et al.*, 2000). Thrombin receptor activation is not only likely to play a key role in arterial thrombosis but also in atherosclerosis (Chackalamannil, 2001). Atherosclerosis starts with damage to the endothelium and subsequent deposition of fats, cholesterol platelets, cellular waste products, calcium and other substances in the artery wall. These may stimulate endothelial cells to produce a vascular cell adhesion molecule that results in further build-up of cells and shrinkage of the arterial diameter (Zapolska-Downar *et al.*, 2001). Halichlorine from *Halichondria okadai* is an inhibitor of the expression of vascular cell adhesion molecule-1 (Kuramoto *et al.*, 1996) and may thus impede atherogenesis (Arimoto *et al.*, 1998).

Callyspongynic acid that was isolated from *Callyspongia truncata* , is an α-glucosidase inhibitor (Nakao *et al.*, 2002). α-Glucosidase inhibitors interfere with the hydrolysis of carbohydrates, keeping the glucose concentration in the blood at a lower level, and can be used to treat diabetes patients (Lebovitz, 1992).

Neurosuppressive activity

Keramidine, that was isolated from an *Agelas* sp. (Nakamura *et al.*, 1984), is an example of a number of neurosuppressive compounds that have been isolated from marine sponges (Table 5). It is a serotonergic receptor antagonist and blocks the serotonin-mediated neural communication. Several different serotonin receptors have been identified and they are related to:

1. platelet aggregation and may therefore be useful against thrombosis (Ruomei *et al.*, 1996).
2. smooth muscle contraction (Garcia-Colunga and Miledi, 1996).
3. vomiting due to their presence in the gastrointestinal tract (Lang and Marvig, 1989).
4. and most interestingly, serotonergic receptor antagonists function as an antidepressant drug in the brain (Nagayama *et al.*, 1980).

Dysiherbaine from *Dysidea herbacea* (Sakai *et al.*, 1997) is a potent excitatory amino acid that causes seizures by interfering with the L-glutamate based neurotransmitter communication and may provide a lead compound for therapeutical agents of neurological disorders (Sakai *et al.*, 2001).

Muscle relaxants

Disturbances in the neuro-muscular communication by stress is a cause of permanent muscle activation (Lundberg, 1995; Edgar *et al.*, 2002). In addition to the before mentioned centrally acting muscle relaxants, that mediate neuro-muscular communication, peripherally acting muscle relaxant may be used for local muscle relaxation. They are applied for relief of strokes, or during intubations and surgery (Frakes, 2001). 1-Methylguanosine from *Tedania digitata* (Quinn *et al.*, 1980) and xestospongin C that was isolated from a *Xestospongia* sp. (Gafni *et al.*, 1997) are examples of muscle relaxants that have been discovered in sponges (Table 5). Xestospongin C is a potent inhibitor of the inositol 1,4,5-triphosphate (IP_3) receptors and the endoplasmatic-reticulum Ca^{2+} pumps (De Smet *et al.*, 1999) and inhibits IP_3-induced increase in the oscillatory contraction of muscles (Miyamoto *et al.*, 2000). ß-Adrenoreceptor agonists, such as S1319 that was isolated from a *Dysidea* sp. (Suzuki *et al.*, 1999), have utero-relaxant properties, which can be therapeutically used for the preterm delivery of infants (Dennedy *et al.*, 2002) and are widely used as antiasthmatic drugs (Suzuki *et al.*, 1999). However, ß-adrenoreceptor agonists may cause severe side effects as arterial hypertension, corony heart disease and tachycardia due their low selectivity (Borchard, 1998). Therefore, there is a continued interest to find more novel selective ß-adrenoreceptor agonists as S13.

Table 3: Examples of immunosuppressive products from sponges.

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 berquistia* / 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

Table 4: Examples of sponge products that affect blood-related diseases.

Compound	Compound class	Sponge / order	Mode of Action	reference
cyclotheonamide A	cyclic pentapeptide	*Theonella* sp. / 'Lithistids'	Serine protease inhibitor	Maryanoff *et al.*, 1993
eryloside F	penasterol disaccharide	*Erylus formosus* / Astrophorida	thrombin receptor antagonist	Stead *et al.*, 2000
halichlorine	cyclic aza-polyketide	*Halichondria okadai* / Halichondrida	VCAM-1 inhibitor	Arimoto *et al.*, 1998
callyspongynic acid	polyacetylene	*Callyspongia truncata* / Haplosclerida	α-glucosidase inhibitor[1]	Nakao *et al.*, 2002

Table 5: Examples neurosuppressives and muscle relaxants from sponges.

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 relaxtant, 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 relaxtant	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

Antiviral compounds

Sponges are also a rich source of compounds with antiviral properties (Table 6). 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. The strong focus on screening for anti-HIV activity has led to discovery of numerous compounds, but the mechanism of inhibition is still poorly characterised. Papuamides C and D (Ford et al., 1999), haplosamates A and B (Qureshi and Faulkner, 1999) and avarol (Müller *et al.*, 1987), which has also been patented as antipsoriasis (Müller *et al.*, 1991), are examples of HIV-inhibiting compounds from different sponges. Avarol is one of the very few compounds of which the mechanism how it inhibits progression of HIV infection is more or less known. *In vitro* and animal data indicate that avarol combines useful properties of an increased humoral immune response, as IgG and IgM production is significantly increased, and interference with the posttranscriptional processes of viral infection (Müller *et al.*, 1987). Avarol inhibits HIV by almost completely blocking the synthesis of the natural UAG suppressor glutamine tRNA. Synthesis of this tRNA is upregulated after viral infection, and is important for the synthesis of a viral protease, which is necessary for viral proliferation (Müller and Schröder, 1991). Low concentrations of only 0.9 or 0.3 μM avarol resulted in 80 and 50% inhibition of virus release from infected cells respectively (Schröder *et al.*, 1991) while uninfected cells were highly resistant against avarol (Müller *et al.*, 1985; Kuchino *et al.*, 1988). Furthermore, it was shown that the avarol derivatives, 6'-hydroxy avarol and 3'-hydroxy avarone (Fig. 6), were very potent inhibitors of HIV reverse transcriptase. This enzyme has a key role in the early stages of HIV infection and is a specific target for antiviral drugs, as it is responsible for converting the viral genomic RNA into proviral doublestranded DNA which is subsequently integrated into the host chromosomal DNA (Loya and Hizi, 1990).

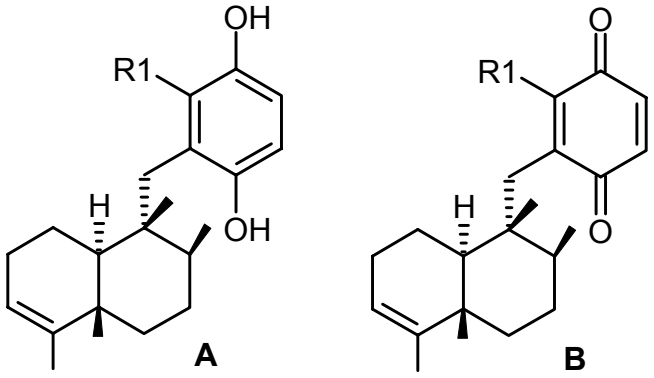

Fig. 6: Molecular structures of avarol (a: R1 = H), 6'-hydroxy avarol (a: R1 = OH), avarone (b: R1 = H) and 3'-hydroxy avarone (b: R1 = OH).

Table 6: Examples of antiviral products from sponges.

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 tarangaensis* / 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 varialosa* / Poecilosclerida	antiviral	Perry *et al.*, 1994
avarol	hydroquinone sesquiterpenoid	*Dysidea avara* / Dictyoceratida	UAG suppressor glutamine tRNA inhibitor[1]	Müller *et al.*, 1987; Müller *et al.*, 1991
2-5A	2',5'-linked oligonucleotide	many sponges	interferon mediator	Kelve *et al.*, 2003
hennoxazole A	bisoxazole	*Polyfibrospongia* sp. / Dictyoceratida	antiviral	Ichiba *et al.*, 1991

[1] has also antiinflammatory and antitumour activity

Table 7: Examples of antimalarial products from sponges.

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

In addition to their applications to treat diabetes, α-glucosidase inhibitors, such as callyspongynic acid, are potentially broad based anti-viral agents. They disturb protein glycosylation and cause some viral envelope proteins to be misfolded, which leads to arrest of these proteins within the endoplasmatic reticulum, where protein folding takes place. It has been demonstrated that alteration of the glycosylation pattern of human immunodeficiency virus (HIV), hepatitis B virus and bovine viral diarrhoea virus by α-glucosidase inhibitors attenuates viral infectivity (Ratner *et al.*, 1991; Mehta *et al.*, 1998).
A very different class of virus inhibitors that has been found in many different sponges are 2'-5'-oligoadenylates (2-5A), which are involved in the interferon-mediated response against a wide range of viruses in mammals. The antiviral action is based on the activation of a latent endoribonuclease that prevents viral replication by degradation its mRNA as well as cellular RNA (Kelve *et al.*, 2003).
From many other antivirals, the mechanism of inhibition is still unclear, but they are active against a range of viruses. Hamigeran B from *Hamigera tarangaensis*, for example, showed 100 % *in vitro* inhibition against both the Herpes and Polio viruses (Wellington *et al.*, 2000), and the weinbersterols A and B from *Petrosia weinbergi* exhibited *in vitro* activity against feline leukaemia virus, mouse influenza virus and mouse corona virus (Sun *et al.*, 1991; Koehn *et al.*, 1991).
In general, antiviral molecules from sponges do not give protection against viruses, but they may result in drugs to treat already infected persons. In addition, broad based antiviral agents such as 2-5A and α-glucosidase inhibitors may be employable in case of sudden outbreaks of (unfamiliar) viruses like SARS and Ebola.

Antimalarial compounds

A number of sponge-derived antimalarial compounds has been discovered during the last decade (Table 7). New antimalarial drugs 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) display selective *in vitro* antimalarial activity against *P. falciparum*. Also a number of free carboxylic acids from *Diacarnus levii* were used as precursors to yield a number of new cyclic norditerpene peroxides after esterification. These epidioxy-substituted norditerpenes and norsesterterpenes displayed selective activity active against both

chloroquine-sensitive and chloroquine resistant *P. falciparum* strains (D' Ambrosio *et al.*, 1998). The Manzamines are the most promising antimalarial compound that have been discovered in a number of sponges (Sakai *et al.*, 1986; Ang *et al.*, 2000; Youssaf *et al.*, 2002). It has been suggested that the antimalarial effect of manzamine A is due to an enhanced immune response (Ang *et al.*, 2001).

Antibiotics and Fungicides

With respect to antibiotics and fungicides, similar multiresistance problems have concerned physicians for a long time. Many new molecules with antibiotic properties are discovered every year, but in marine sponges their ubiquitousness is remarkable (Table 8). An early screening by Burkholder and Ruetzler (1969) revealed that out of 31 sponges tested, 18 showed antimicrobial effects of which some were very strong against a range of Gram positive and Gram-negative bacteria. The added value of some 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). Fungicides that are currently used, are less diverse than antimicrobials and the use of many of them is restricted due to toxic effects to humans, animals and plants (Nakagawa and Moore, 1995; Rahden-Staron, 2002). It remains to be demonstrated whether antifungals like topsentiasterols D and E from *Topsentia* sp. (Fusetani *et al.*, 1994), acanthosterol sulfates I and J from an *Acanthodendrilla* sp. (Tsukamoto *et al.*, 1998) or the macrolide leucascandrolide A from the calcareous sponge *Leucascandra caveolata* (D'Ambrosio *et al.*, 1996) will have different characteristics than the fungicides that are currently used, but the fact that they are produced by an eukaryotic organism (if not produced by a symbiont) may imply that they are less toxic to other non-fungal eukaryotes.

Antifouling compounds

A last class of bioactive compounds from marine sponges are antifouling molecules (Table 9). They are not associated with the development of new drugs, but could be 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; Houghton, 1978).

Long-term use of chemical antifoulants has led to increased concentrations of tributyltin and its present replacements in coastal sediments (Konstantinou and Albanis, 2004) and to mortality and change of sex of non-target organisms (Katranitsas *et al.*, 2003). Natural sponge molecules may provide a less toxic and more specific antifouling activity. They have been found to inhibit the settlement of barnacle larvae (Tsukamoto *et al.*, 1996b), fouling by macroalgae (Hattori *et al.*, 1998) or repellent against the blue mussel *Mytilus edulis galloprovincialis* (Sera *et al.*, 1999).

Ecological role of sponge metabolites

Such an extensive collection of sponge-derived bioactive compounds demands for some explanation why sponges produce so many metabolites that can be useful to treat *our* diseases. The huge number of different secondary metabolites that has been discovered in marine sponges and the complexity of the compounds and their biosynthetic routes (and corresponding kilobases of DNA for the programming of their synthesis) can be regarded as an indication for their importance for survival.

An obvious example of the benefits of their 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 sponge products is high enough to even create a bare zone around the sponge (Thompson, 1985) that is maintained by the emission of a mucus containing the toxins (Sullivan *et al.*, 1981). This allows the conquest of densely populated rocks or corals and the competition with faster growing organisms, but it is striking that the sponge can selectively use its poisons without self-destruction.

Secondary metabolites can protect the organism against predation, which is especially important for physically unprotected sessile organisms like sponges (Becerro *et al.*, 1997). Relatively few 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 represent only a tiny fraction of the animals inhabiting the seas. Secondary metabolites can also protect their producers against bacteria, fungi or parasites (Davies, 1992). In sponges, the role of the chemical constituents is clouded by the complexity of the sponge-symbiont relationship (Dumdei *et al.*, 1998). Many different bacterial species permanently inhabit sponges and

contribute considerably to the total sponge biomass (Wilkinson, 1978b). It has been suggested that the growth of 'useful' microorganisms may be under control of the sponge host and serve as source of food or supply other metabolic products (Müller *et al.*, 1981). However, it has also been found that associated bacteria might be the actual producers of a number of compounds that have been isolated from sponges. *Oscillatoria spongelia*, a cyanobacterial symbiont that can constitute up to 40% of *Dysidea herbacea*, is the producer of antimicrobial polybrominated biphenyl ethers and might keep the sponge free of other bacteria (Unson, 1994).

Although for many products it is not yet know whether they are produced by the sponge or by a symbiont, it is clear that sponges are responsible for the production of a rich arsenal of 'chemical weapons'. Their early appearance in evolution has given them a lot of time for the development of an advanced chemical defence system. It is interesting to note that the synthesis of secondary metabolites is regulated depending on conditions that the sponge experiences. Specimens of *Crambe crambe* grow faster in well illuminated regions, than their counterparts exposed to darker conditions, but the specimens in the dark are better defended as they accumulate higher concentrations of cytotoxic metabolites (Turon *et al.*, 1998). Another example is the production of halichondrin by *Lissodendoryx* sp., which varies seasonally, with depth, and with the condition of the sponge. Halichondrin yields could be enhanced by an order of magnitude during serial cloning, suggesting a defensive response to damage (Battershill *et al.*, 2002). The possibility to stimulate the production of secondary metabolites by sponges is an important fact when one wants to harvest compounds from sponges for the production of potential new medicines.

Conclusion

Marine sponges are the producers of an enormous array of antitumour, antiviral, antiinflammatory, immunosuppressive, antibiotic, and many other bioactive molecules, which can affect the pathogenesis of many human diseases. The relationship between the chemical structures of the secondary metabolites from sponges and the disease(s) they affect is usually not obvious. The overview is obfuscated due to different mechanisms by which different components affect the targeted disease (e.g. microtubule stabilisation or or interaction with DNA to combat tumours). Moreover, inhibitors of transcription may be effective against both cancer and viral diseases. To make things more complex, there are many relations between, for instance, inflammation, cancer and viral infections via the

Table 8: Examples of antibacterial and antifungal products from sponges.

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

Table 9: Examples of antifouling products from sponges.

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

immune system, which plays a key role in certain responses of the body to these diseases. Chronic inflammation of the lungs by cigarette smoke often leads to lung cancer (Ohwada *et al.*, 1995) and cervical or liver cancer can follow chronic inflammation caused by papilloma viruses (Smith-McCune *et al.*, 1996) and hepatitis B and C viruses respectively (Zhu *et al.*, 1997). In addition, limited activity testing (e.g. only on cell growth inhibition and not on antiviral properties) causes an incomplete overview of the actual properties of the metabolites. Finally, for a lot of bioactive molecules from sponges their exact mode of action and their origin (sponge or symbiont) are still unclear.

Most bioactive metabolites from sponges are inhibitors of certain enzymes, which often mediate or produce mediators of intra- or intercellular messengers, that are involved in the pathogenesis of a disease. Since this is usually a cascade of reactions inside the cell or tissue, many enzymes in the cascade are a target for potential therapy. The different enzymes in the cascade can be structurally completely different proteins, and therefore it is not surprising that a wide range of metabolites can be used for the treatment of one disease. This especially applies for a complex disease, such as cancer, which is affected by so many different factors. Furthermore, also antiviral molecules appear to belong to a wide array of chemical structures, such as peptides, lipids, alkaloids, sterols, oligonucleotides, and a phenolic macrolide. A similar diverse pattern is observed for antibacterial and immunosuppressive metabolites. Most compounds that display antiinflammatory activity are sesterterpenoids. Nevertheless, in these cases the activity of the sponge metabolites is concentrated on certain steps, as for instance most antiinflammatory compounds act against phospholipase A_2.

The potency of sponge-derived medicines lies in the fact that each of these thousands of metabolites and their derivatives has its own specific dose-related inhibitory effect, efficacy and potential (diminished) side effects that determine its suitability for medicinal use. In addition, the skeleton or active core of these molecules may be used as a vehicle to develop derivatives with their own specific efficacy and side effects. Therefore, the most important challenge in transforming bioactive molecules into medicines is now to screen the treasure-house of sponge metabolites and select those that display a specific mode of action with the desired characteristics against a disease. An important future question remains how to actually prepare the potential novel drugs on a large scale.

Medical Glossary

angiogenesis: the process of vascularisation of a tissue involving the development of new capillary blood vessels.

atherosclerosis: the progressive narrowing and hardening of the arteries involving fatty acids inside the arterial walls.

arthritis: an inflammatory condition that affects joints.

carcinosarcoma: a malignant tumor that is a mixture of carcinoma (cancer of epithelial tissue) and sarcoma (cancer of connective tissue).

cervical cancer: cancer of the neck of the womb.

corony heart disease: narrowing of the corony, which feeds the heart with blood.

diabetes: relative or absolute lack of insulin leading to uncontrolled carbohydrate metabolism.

ebola: an epidemic viral illness that leads to massive bleeding.

feline leukaemia virus: a retrovirus causing many proliferative and degenerative diseases in domestic cats.

hepatitis: inflammation of the liver caused by a virus or a toxin.

herpes: a number of viral diseases that cause painful blisters on the skin.

leukaemia: an acute or chronic disease, characterised by an abnormal increase in the number of leucocytes in the body tissues.

lymphoma: malignant tumour of lymhpoblasts derived from B lymphocytes.

malaria: an infective disease caused by sporozoan parasites that are transmitted through the bite of an infected *Anopheles* mosquito, marked by sudden chills and fevers.

migraine: a severe recurring vascular headache.

mouse corona virus: a virus that causes hepatitis in mice.

papilloma virus: a genus of viruses of which some are associated with the induction of carcinoma.

polio: an acute viral disease marked by inflammation of nerve cells of the brain, stem and spinal cord.

psoriasis: an immune-mediated, genetic disease manifesting in the skin and/or the joints.

SARS: a disease caused by a corona virus leading to severe respiratory problems.

tachycardia: abnormal fast heart rate.

thrombosis: the formation or presence of a clot of coagulated blood in a blood vessel.

3 Growth kinetics of Demosponges: the effect of size and growth form

Abstract

The marine sponges *Dysidea avara* and *Chondrosia reniformis* were cultured on a diet of viable *Phaeodactylum tricornutum* cells and on a diet of dissolved nutrients (algae- and fish powder). Our sponge growth data were combined with literature data of *Pseudosuberites andrewsi*, *Oscarella lobularis*, *Hemimycale columella* and *Crambe crambe*. The suitability of three growth models was assessed to describe the growth of globose and encrusting sponges: linear growth, exponential growth, and radial accretive growth. Radial accretive growth seemed the best model to describe growth of both encrusting and globose sponges. Average growth rates of 0.051 ± 0.016 and 0.019 ± 0.003 mm/d, calculated as the increase of the radius of the sponge per day, were obtained for *D. avara* and *C. reniformis*, respectively.

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Introduction

The discovery of many secondary metabolites from marine sponges with interesting pharmaceutical properties (e.g. Faulkner 2000; 2001; 2002) has been followed by a number of attempts to culture sponges for their products. Cultivation of sponges *in situ* has been moderately successful. Although many species have been cultured, sponge growth rates are generally low and the mortality of the explants is high (Duckworth *et al.*, 1997, 2003a; Van Treeck *et al.*, 2003). Other researchers have tried to culture sponges in the laboratory, but optimum conditions for sponges in captivity are not known (Barthel and Theede, 1986; de Caralt *et al.*, 2003; Osinga *et al.*, 2003; Belarbi *et al.*, 2003; Mendola, 2003; Duckworth *et al.*, 2003b). Marine sponges appear to be very sensitive to environmental, and therefore also experimental, conditions (De Vos *et al.*, 1991; Nickel *et al.*, 2000). It is striking to see a blooming sponge population at the basalt blocks of a dyke and total absence of sponges just a few hundred metres further, at apparently equal conditions to human perception. It is probably these subtleties, in combination with the slow growth rate of sponges (and thereby long-lasting experiments with a small number of replicates) that have caused the small number of successful *ex situ* sponge cultures and the low reproducibility.

The use of different methods to define or measure sponge growth (e.g. 2-D photography (Garrabou and Zabala, 2001), drip dry weight (Thomassen and Riisgård, 1995), underwater weight (Osinga *et al.*, 2003) or qualitative indications (Nickel, 2001)) and different ways to express growth (e.g. %/day (Belarbi *et al.*, 2003), cm^2/cm border*day (Ayling, 1983), or d^{-1} (Garrabou and Zabala, 2001)), make it impossible to compare data from different sponges and have led to fragmented knowledge about the growth kinetics of sponges.

It is our aim to find a mechanistic model, which appropriately describes the growth of marine sponges, in order to be able to make a sound comparison between growth rates of different sponges. In addition, the model can be used to study the quantitative effect of factors, such as pressure, light, current, age, temperature or the nutrient source or –concentration on the growth rate of sponges. Moreover, it could be valuable to predict sponge growth in maricultures. For this work, we used growth data from experiments with two Mediterranean demosponges, *Dysidea avara* and *Chondrosia reniformis*. In addition, we used literature growth data, from which a relation between sponge size and growth rate could be extracted, in order to obtain insight in the growth kinetics of sponges.

Growth Kinetics

We have assessed the suitability of three mechanistic growth models for the literature- and our own data (Fig. 1):

A. linear growth (0-order kinetics).
B. exponential growth (1st-order kinetics).
C. growth related to the surface/circumference for globose/encrusting sponges, respectively.

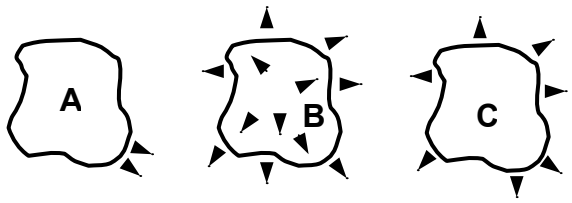

Fig. 1: The different growth models that were assessed for encrusting sponges. A: linear growth, B: exponential growth, and C: circumference-dependent growth of the sponge.

A Linear growth describes a situation where growth occurs at the peripheric end (or a fixed number of growth tips) of the sponge, comparable to growth of the apical meristem of a plant root. This type of growth has been assumed to describe the growth rate of *Crambe crambe* explants in closed systems (Belarbi *et al.*, 2003). Linear growth is described by:

$$\frac{dX}{dt} = k \qquad [\text{mL/d or mm}^2\text{/d}]$$

where X is the volume or area of globose- or encrusting sponges, respectively, and k is the linear growth rate constant. Integration leads to:

$$X_t = X_0 + k \cdot t \qquad [\text{mL or mm}^2] \qquad [1]$$

B Exponential growth would imply that the proliferation rate of cells inside the sponge is independent of their position in the sponge. The mesohyl of demosponges leaves some open spaces inside the sponge that are not filled with cells. Therefore, cell division inside the sponge is physically possible and new cells could move through the mesohyl to their destination. An approximation of exponential growth has been used in a number of publications (e.g. Garrabou and Zabala, 2001; de Caralt *et al.*, 2003). Exponential growth is described by:

$$\frac{dX}{dt} = \mu \cdot X \qquad [\text{mL/d or mm}^2\text{/d}]$$

where μ is the specific growth rate constant. Integration leads to:

$$X_t = X_0 \cdot e^{\mu \cdot t} \quad [\text{mL or mm}^2] \qquad [2]$$

C1 Surface-dependent growth would imply that growth only occurs at the outer surface of a globose sponge. This could be explained by the fact that the influx of nutrient-containing seawater is dependent on the number of ostia, which is correlated to the surface area of the sponge. Surface-dependent growth is described by:

$$\frac{dV_r}{dt} = c \cdot A_r \quad [\text{mL/d}]$$

where V and A are the volume and surface area (that are both dependent on the radius r) of the globose sponge, respectively, and c is the surface-dependent growth rate constant. Integration leads to:

$$V_t = (\sqrt[3]{V_0} + c \cdot (\sqrt[3]{2/3\,\pi}) \cdot t)^3 \quad [\text{mL}] \qquad [3]$$

if it is assumed that the sponge is a hemisphere.

C2 Circumference-dependent growth of encrusting sponges is the 2-dimensional version of surface-dependent growth of globose sponges. Ayling (1983) has used this type of growth to express the growth rate of a number of encrusting sponges. Circumference-dependent growth is described by:

$$\frac{dA_r}{dt} = c \cdot O_r \quad [\text{mm}^2/\text{d}]$$

where O is the circumference of the encrusting sponge (Both A and O are dependent on r). Integration leads to:

$$A_t = (\sqrt{A_0} + c \cdot \sqrt{\pi} \cdot t)^2 \qquad [\text{mm}^2] \qquad [4]$$

if the sponge is assumed to be circular.

The surface- or circumference-dependent growth rate constant c is both for surface-dependent growth of globose sponges and circumference dependent growth of encrusting sponges expressed as:

$$c = \frac{r_t - r_0}{t} \qquad [\text{mm/d}] \qquad [5]$$

where r is the radius of the sponge.
Thus, it can be concluded that both surface-dependent growth and circumference-dependent growth assume radial accretive growth of the sponge.

Materials and Methods

Sponges

Dysidea avara specimens were obtained from Professor Werner Müller, who kept the *D. avara* specimens in an aquarium in Mainz (Germany) at 16 °C. Originally, they were collected by Scuba divers in the northern Adriatic Sea near Rovinj (Croatia). They were transported from Mainz to our laboratory in an isolated vessel at a constant temperature. The seawater in the transport vessel was aerated, without exposing the sponges to air bubbles, and it was purified by means of an active carbon filter. In Wageningen, the sponges were maintained in an aquarium at 16.5 °C. Explants were prepared by cutting pieces of 1-4 cm^3 from the 'parent' sponges by use of a razor-sharp knife. The cuttings were tied on terracotta tiles with tie-raps. The explants attached to the tiles, usually after 2-4 weeks and then the ty-raps were removed. Only attached explants were used for cultivation experiments after they had been attached for several weeks so that possible growth we would observe, would not be the result of wound healing.

Chondrosia reniformis specimens were collected by Scuba diving in the Mediterranean near Kalymnos (Greece) and Blanes (Spain). Transport and preparation of explants was done in the same manner as described for *D. avara* specimens.

Chemicals and Substrates

- Artificial sponge seawater was prepared from 33 $g{\cdot}L^{-1}$ instant ocean reef crystals (Aquarium systems, Sarrebourg, France) and 7.1 $mg{\cdot}L^{-1}$ sodium metasilicate in demineralised water. After preparation, the artificial sponge seawater was aerated for at least one week to stabilise the chemical reactivity.
- Artificial algae seawater was prepared by enrichment of artificial sponge seawater with 0.013 $g{\cdot}L^{-1}$ NaH2PO4*H_2O, 0.101 $g{\cdot}L^{-1}$ KNO_3, $2{\cdot}10^{-5}$ $g{\cdot}L^{-1}$ thiamine*HCl, $4{\cdot}10^{-8}$ $g{\cdot}L^{-1}$ biotin, $8{\cdot}10^{-7}$ $g{\cdot}L^{-1}$ cyanocobalamine, $9.5{\cdot}10^{-4}$ $g{\cdot}L^{-1}$ $FeCl_3$, $9{\cdot}10^{-5}$ $g{\cdot}L^{-1}$ $MnCl_2*4H_2O$ and 1.21 $g{\cdot}L^{-1}$ Tris.
- Fish powder (GVP) was purchased from Snick Ingredients (Beernem, Belgium). It was prepared from the meat of frozen coalfish that was boiled, dehydrated and micro-milled (composition: 73% protein, 4.5% fat, 21% ash and 1% NaCl).
- Algae powder was prepared from two batches of *Phaeodactylum tricornutum* that were cultured in a 70 L bubble column. The 140 L micro algae suspension was concentrated to a volume of 14 L by use of a "super centrifuge" (Sharples, Rueil, France). Subsequently, the centrifuged suspension was mildly spray dried (T inlet ~ 73

°C and T outlet ~ 46 °C) with a laboratory mini spray dryer (Büchi, Flawill, Switzerland) and the algae powder was stored in the refrigerator.

- A stock solution of Complete Nutrient Mix was made up of 2.5 g·L^{-1} fish powder, 25 mL·L^{-1} newborn calf serum (Life Technologies, Paisley, UK) and 12.5 mL·L^{-1} MEM vitamin solution (Sigma).

Cultivation of Dysidea avara

System: A three-step reactor system was built, to realise a constant environment for the cultivation of *D. avara* explants on life food and Complete Nutrient Mix (Fig. 2). The system consisted of a 2.5 L photo bioreactor (22 °C), for the continuous cultivation of the micro alga *Phaeodactylum tricornutum*, which was supplied as nutrient to the sponges. This microalgal strain was selected because previous research showed its suitability (Osinga *et al.*, 2003). The overflow of the photobioreactor was led to an 8.5 L dilution reactor, where the algae suspension was diluted with artificial sponge seawater to the desired concentration. The dilution reactor was protected from light to stop further growth of the micro algae in the dilution reactor. For the same reason, the residence time in the dilution reactor was short (0.3 d) and the temperature suboptimal (16.5 °C). A continuous flow was pumped from the dilution reactor to the three 7 L bioreactors containing the sponges (16.5 °C). The dilution reactor was included in the system only for the practical reason that pumping directly from photo bioreactor to the bioreactors containing the sponges would result in extremely low flows of a dense algae suspension, which leads to clogging of the tubes. The bioreactors containing sponges were mixed with a magnetic stirrer at the bottom of the reactor, to create some current. The *D. avara* explants, which were attached to the terracotta tiles, were placed upon a brick in the bioreactor to prevent contact with the stirrer and to limit sedimentation of detritus on the explants. The bioreactors were covered to obtain a light situation comparable to the natural environment with low light during the day and complete darkness during the night. The overflow of bioreactors 1 and 3 was recycled via a 3-step filtration unit. The seawater was passed through a biological stone filter, a cotton wool filter, and a 0.2 µm filter before it was reused in the dilution reactor. A weighing column, for determination of the underwater-weight of the sponges, was placed in the water circulation to prevent thermo- or salinity shocks for the sponges during the underwater weight determination.

Experimental: Two *D. avara* explants (1 and 2) were cultured in bioreactor 1. They were solely fed with life *P. tricornutum* by a continuous flow (7 L·d^{-1}), containing 1·10^5 cells/mL from the dilution reactor. Explants 3 and 4 were cultured in bioreactor 2 and received an additional inflow of Complete Nutrient Mix stock solution (concentration in the reactor:

0.1 $g \cdot l^{-1}$ fish extract, 1 $mL \cdot L^{-1}$ serum and 0.5 $mL \cdot L^{-1}$ vitamins). At a later stage (after day 40) explants 5 and 6 were cultured in bioreactor 2 under exactly the same conditions as in bioreactor 1. Bioreactor 3 was operated as a control reactor. No sponges were cultured in this reactor, while it received the same continuous flow of algae cells as bioreactor 1.

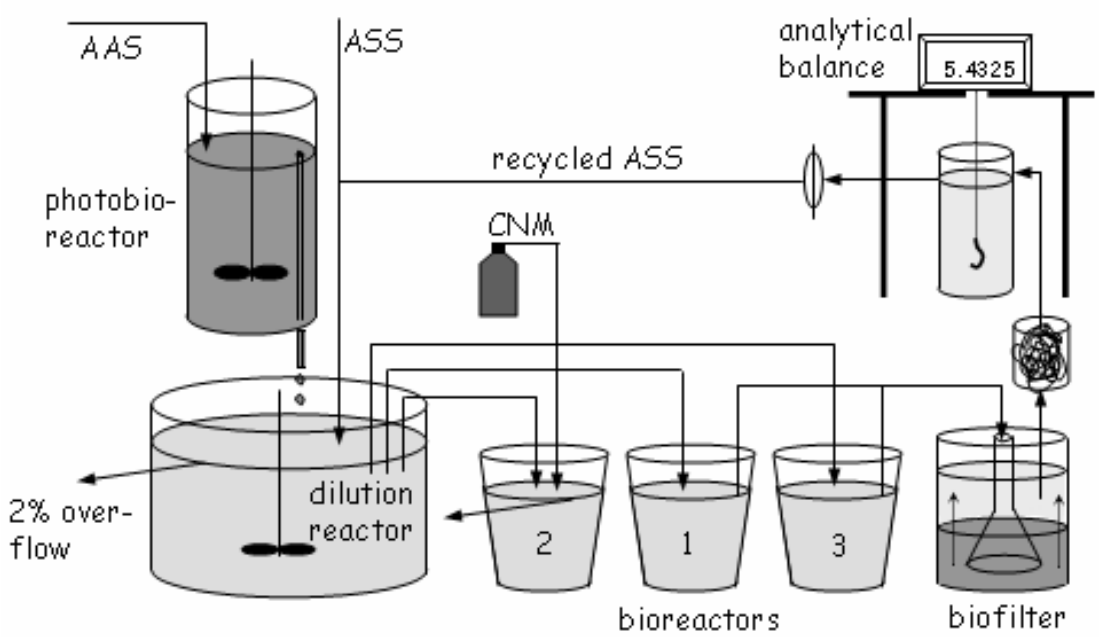

Fig. 2: The *D. avara* continuous cultivation system was composed of a photobioreactor, an algae dilution reactor and three bioreactors for the cultivation of sponges. The out-flowing water from bioreactor 1 and 3 was reused after filtration through 3 filters: a biofilter, a cotton wool filter, and a 0.2 µm bacterial filter. The explants in bioreactor 2 were initially continuously supplied with a complete nutrient mix. AAS = artificial algae seawater; ASS = artificial sponge seawater. CNM = complete nutrient mix.

Chondrosia reniformis cultivation system

C. reniformis explants were cultured in two 7 L bioreactors in a continuous operated system at a temperature of 20 °C. Mixing, light and the positioning of the explants were the same as in the cultivation of *D. avara*. Explants 1, 2, and 3 were placed in bioreactor 1 and received a continuous flow of 7 L artificial sponge seawater per day containing an amount of algae powder equivalent to approximately $2.5 \cdot 10^4$ cells/mL. The overflow of this reactor was recycled via a filtration system and the weighing column, similar to the system used to culture *D. avara*. Explants 4 and 5 were placed in bioreactor 2 and received a continuous flow of 7 $L \cdot d^{-1}$ artificial sponge seawater, containing algae powder (equivalent to $2.5 \cdot 10^4$ cells/mL) and fish powder (0.02 g/L). The overflow of this bioreactor was discarded. Based on total organic carbon (TOC) measurements of the algae- and fish powder, the TOC concentrations in bioreactor 1 and 2 were expected to be approximately 1 and 2 mg/L, respectively. The two vessels containing the seawater

enriched with nutrients were put in the refrigerator and were continuously stirred to prevent sedimentation of the substrates. These vessels were daily cleaned and filled with fresh solutions.

Analyses

Microalgae: the *P. tricornutum* cell concentration in the growth-, dilution-, and sponge reactors was determined daily on a Coulter Multisizer type II automatic particle counter during the *D. avara* cultivation experiment.

Carbon: The total organic carbon (TOC) concentration in the sponge reactors was determined regularly by subjecting samples to wet oxidation, using an OIC 700 Total Organic Carbon analyser. The carbon content of *P. tricornutum* cells was determined by a duplicate measurement of the TOC concentration in artificial seawater containing $7.5 \cdot 10^4$ cells/mL and substracting the TOC concentration of artificial seawater.

Size determination of explants

The size of the explants + the tile was measured as the underwater-weight using an A&D HR300 analytical balance with underweighing device (Fig. 2). The sponge explants were moved from the bioreactors to the weighing column without exposing them to the air. The salinity (3.3 %) and temperature (16.5 or 20 °C) in the weighing column were always the same as in the bioreactors. The underwater-weight of the empy tiles was substracted from the measured weights to obtain the underwater-weight of the explants.

Results & Discussion

Cultivation of Dysidea avara

D. avara explants were cultured in bioreactors for a period of 37-83 days. All explants that were supplied continuously with live *P. tricornutum* solely, displayed growth (Fig. 3). While explant 2 started to grow immediately after its introduction in the bioreactor, the underwater weight of explant 1 decreased during the first 40 days. The relatively fast growth of explant 2 is remarkable if one takes into account that the algae concentration during this period was only a fraction of the desired concentration of $1 \cdot 10^5$ cells/mL. We determined an organic carbon content of 36 pg C/cell for *P. tricornutum*, which implies that the average organic carbon concentration in the bioreactors during the first 20 days was only 0.24 mg C/L. This is much lower than concentrations that are usually found in the Mediterranean Sea, which are ranging from 0.6-3.4 mg/L (Ribes *et al.*, 1999; Seritti *et al.*, 2003; our data: Kos (Greece): 1.29 mg/L, Kalymnos (Greece): 3.43 mg/L, Rovinj

(Croatia): 1.89 mg/L). During this period of nutrient-poor conditions, explant 2 displayed growth while the size of explant 1 decreased, but variability among sponge explants is often observed (de Caralt *et al.*, 2003; Duckworth *et al.*, 2003b). Growth of explant 1 after day 40 may be correlated with the presence of sufficient *P. tricornutum* cells in the bioreactors. The average concentration between day 35 and day 60 was $5.65 \cdot 10^4$ cells/mL, which corresponds to approximately 1 mg C/L. At this time, explants 5 and 6 were introduced in bioreactor 2 and during this period all explants displayed growth. No significant differences between the *P. tricornutum* concentrations in bioreactor 1, 2 and the control bioreactor 3 without sponges were observed, which indicates that algae consumption by the sponges was low compared to the continuous supply of fresh algae. As a control, the underwater weight of an empty tile was monitored, but no significant changes occurred in the weight of the empty dish, indicating that the increase of the underwater weight was truly related to growth of the sponges.

The growth of most explants was highest during the period between day 35 and 60, when there were sufficient microalgae present for consumption, but came to an abrupt end due to failure of the cooling water bath at day 76. This caused the temperature to rise from 16.5 up to 20 °C during the night, and one week after this incident the sponges were brownish instead of pinkish and started to come loose from the dishes.

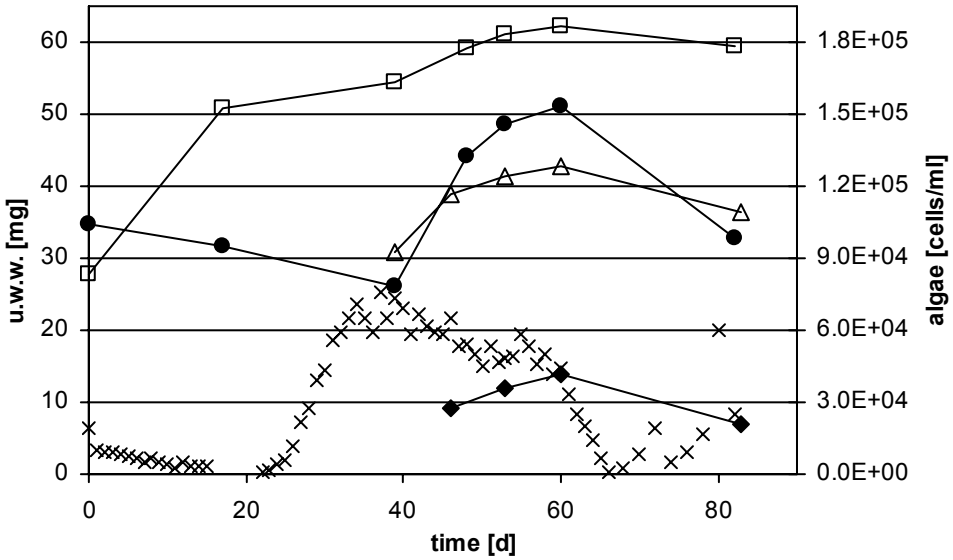

Fig. 3: The underwater weight (uww) of *D. avara* explants 1 (●), 2 (□), 5 (Δ), and 6 (♦) during 83 days of cultivation. The underwater weight of explant 2 must be multiplied by four (real x-y coordinates are: (0-111), (17-203), (39-217), (48-236), (53, 244), (60, 249), (82, 238). The actual data were divided by four to obtain a clearer view in the graph. The *P. tricornutum* cell concentration (×), that was present in the bioreactors as nutrient for the explants is depicted on the secondary y-axis. For clarity of the graph, only the cell concentration in bioreactor 1 is represented, but cell concentrations in the bioreactors were always almost equal.

Explants 3 and 4, which were initially cultured in bioreactor 2, received a continuous extra nutrient flow that was composed of fish powder, serum and vitamins. These explants died after one week, probably due to strong microbial growth and subsequent oxygen depletion. The total organic carbon (TOC) concentration in this bioreactor was 38 mg/L, which is much higher than natural occurring concentrations. The high concentration of easily metabolisable carbon sources was obviously more favourable to bacteria and fungi (which were visible as long slimy threads) than to the sponges and probably disturbed the more or less ecological balance that is present in the sea.

Even though growth of *D. avara* explants on the microalgae *P. tricornutum* was obtained, the joint cultivation of microalgae and sponges is complex to maintain for a long time. Sponges are slow growers and therefore sponge cultivation systems have to be operated for a long time. In our case, 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. In our case, the sponges were exposed to fluctuating algae concentrations, instead of a desired constant concentration of $1 \cdot 10^5$ cells/mL (Fig. 3).

Cultivation of Chondrosia reniformis

Five *C. reniformis* explants were cultured in two bioreactors with a continuous supply of algae powder or a mixture of algae- and fish powder. The underwater weight of all explants increased during the experiment (Fig. 4). In both buckets an empty tile was included to correct for measurement errors due to slight changes in temperature and salinity during the determination of the underwater weight. No significant differences were found between the average growth rates in bioreactor 1 and 2. This may have been the result of comparable average TOC concentrations in bioreactor 1 and 2 (1.43 ± 1.23 and 1.43 ± 1.29 mg C/L, respectively). It was unexpected that the TOC concentrations were the same in both bioreactors, as bioreactor 2 was supplemented with additional fish extract. The TOC concentration in bioreactor 2 may have been lower than expected because some sedimentation of floccules on top of the sponges and on the bottom of the reactor was observed. Therefore, the bioreactors and the sponges were carefully cleaned every week. For most sponges the major part of the increase of underwater weight occurred between day 14 and day 42, except explant 3 that grew constantly since day 0. Growth halted after day 42 and the underwater weight of all explants remained constant for more than one month (not in graph) before the experiment was stopped.

For *C. reniformis*, some growth data in the sea have been obtained and it can be concluded that it is a very slow growing sponge compared to other sponges (Wilkinson and Vacelet,

1979; Garrabou and Zabala, 2001). Garrabou and Zabala found a growth rate of $7.2 \cdot 10^{-4} \pm 6.2 \cdot 10^{-4}$/day, which was calculated via an equation that approximates exponential growth. Wilkinson and Vacelet (1979) tested the effect of different light and current conditions on the growth of *C. reniformis* in the sea. If the exponential model is used to estimate the growth rate for their specimens cultured under optimised conditions with respect to light and current, a growth rate of $1.8 \cdot 10^{-3}$/day is obtained. This is more than a doubling of the growth rate found by Garrabou and Zabala. For the explants that were cultured in our bioreactors an average growth rate of $6.7 \cdot 10^{-3} \pm 1.3 \cdot 10^{-3}$/day is obtained via the exponential model. This is approximately 4-9 times higher than in the sea, and may give an indication of the possibilities of *ex situ* cultivation of marine sponges. However, the *ex situ* growth rate is based on five explants only and as reproducibility is often problematic with the cultivation of marine sponges, the actual growth rate that can be reached in these systems is still unclear.

In addition, the exponential model may not be the best model to describe sponge growth. Therefore a comparison between growth rates derived via the exponential model may result in an incorrect notion about the (im)possibilities of *ex situ* cultivation. For *C. reniformis* it was not possible to compare the growth rate in the sea with our *ex situ* data using the linear or surface-dependent growth model. This comparison could not be made, as the size of only a number of the used specimens is reported in mm^2 (Garrabou and Zabala, 2001) or no size could be deducted (Wilkinson and Vacelet, 1979).

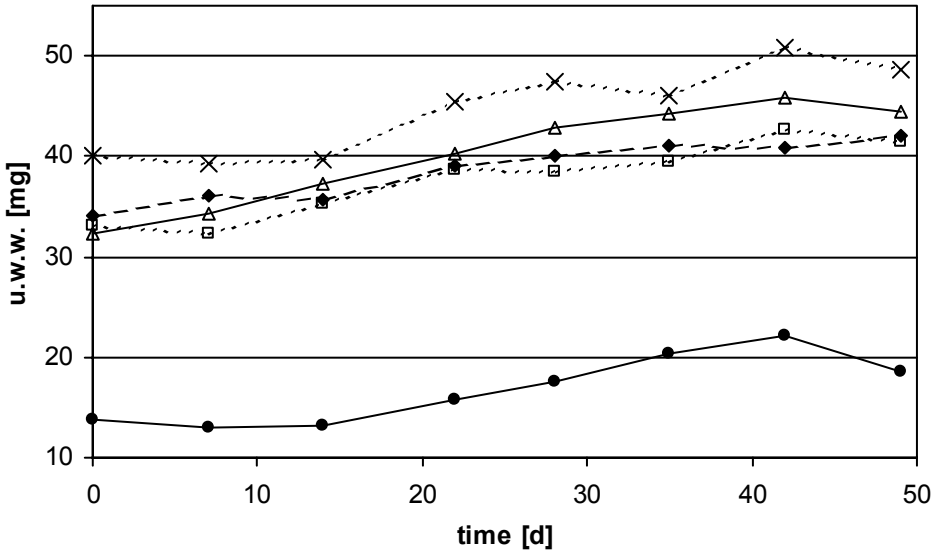

Fig. 4: The underwater weight (uww) of *C. reniformis* explants. Explant 1 (●), 2 (□) and 3 (Δ) were cultured in bioreactor 1 and were supplied solely with algae powder. Explant 4 (♦) and 5 (×) were cultured in bioreactor 2 and were fed with both algae and fish powder.

Growth kinetics

In order to find out what is the best model to describe the growth of sponges, the growth data of the globose sponges *D. avara* and *C. reniformis* growth data were compared to the linear-, exponential- and surface-dependent growth model (equation 1, 2 and 3, respectively). In addition, literature data from the marine sponge *Pseudosuberites andrewsi* (Osinga *et al.*, 2003) were compared to the three models to expand the data set. The growth kinetics of encrusting sponges was studied, based on field data for *Oscarella lobularis*, *Hemimycale columella* (Garrabou and Zabala, 2001) and *Crambe crambe* (Turon *et al.*, 1998; Garrabou and Zabala, 2001). For *O. lobularis* and *C. crambe* that display clearly seasonal growth, only data of the period of growth were used. The model equations (1, 2 and 3 or 4) were used to determine the growth rate constants (k, μ and c) for which the best fits could be obtained (Fig. 5).

It can be seen that especially for very short-term cultivations of approximately 30 days (Fig. 5A) the differences between the three models are smaller than for cultivations of more than 600 days (Fig. 5B). The calculated growth rate constants of all explants according to the three models were related to their initial size. If a model appropriately describes growth of the sponges, there should be no correlation between the growth rate constant and the size of the sponge (Fig. 6). As from Fig. 6, no quantitative information of the best model can be derived, the variance of the normalised calculated growth data in relation to the three models was used, to determine which of the three models describes the data best (Fig. 7).

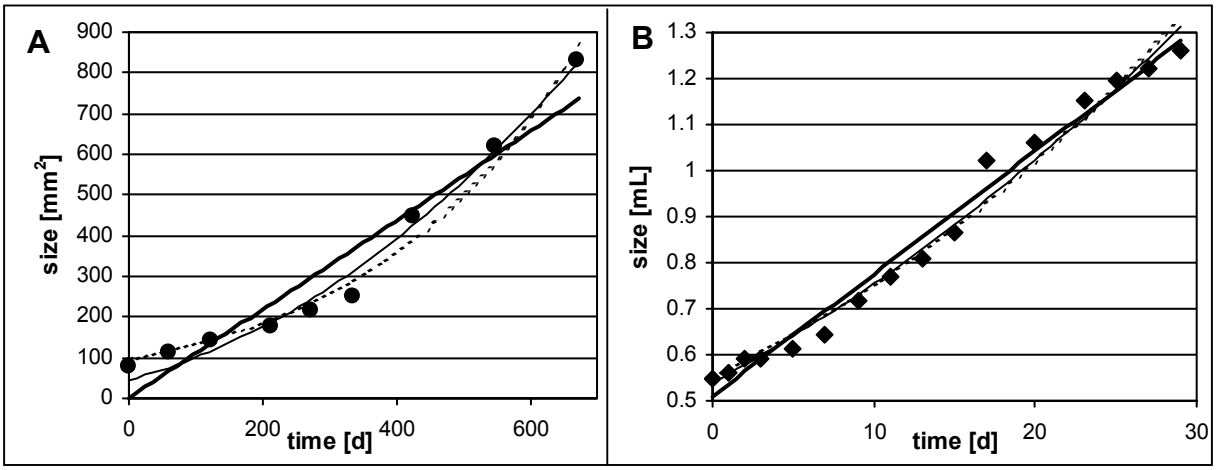

Fig. 5: A: Example of the growth curve of a globose specimen (*P. andrewsi*) with best fits of the linear (▬), exponential (---) and surface-dependent (─) models. B: Example of the growth curve of an encrusting specimen (*H. columella*) with best fits of the linear (▬), exponential (---) and circumference-dependent (─) models.

For the *ex situ* cultivated globose sponges *D. avara*, *C. reniformis* and *P. andrewsi* (Osinga *et al.*, 2003) the surface-dependent model was the best model to describe the growth (Fig. 7). For the encrusting sponges both for *H. columella* and *C. crambe* the circumference model proved to be the best model to describe the growth. For *O. lobularis* the exponential model simulated the growth best. In the latter case, the variance between the model and the calculated data was higher than for the other sponges, which indicates that the reliability of the models is lower than for the other sponges. This was probably because for this species the least data were available.

This means that in general: $r_t = r_0 + c \cdot t$, where r is the radius, is currently the best model to describe growth of both encrusting and globose sponges. The surface-dependent or circumference-dependent growth rates of all sponges that were assessed are summarised in Table 1. The data of Ayling (1983), who expressed growth of a number of encrusting sponges as increase of the area per cm border per day, which is the same as radial accretive growth, are included in the table. It can be seen that *D. avara* and *P. andrewsi* grow faster than *C. reniformis* (*C. reniformis* in the lab), which was characterised as a very slow grower (Garrabou and Zabala, 2001; Nickel and Brümmer, 2003). The radial accretive growth rates of the globose sponges are generally higher than those of the encrusting sponges. However, this difference may be caused by the different conditions to which the sponges were exposed (encrusting sponges in the sea and globose sponges in the laboratory).

Table 1: The calculated surface-dependent and circumference-dependent growth rates (c) and their standard deviations for globose (g) and encrusting (e) species, respectively.

Sponge	c [mm/d]
Pseudosuberites andrewsi (g)	0.068 ± 0.038
Dysidea avara (g)	0.051 ± 0.016
Oscarella lobularis (e)	0.036 ± 0.019
Aplysilla rosea (e)*	0.028 ± 0.019
Stylopus sp. (e)*	0.023 ± 0.009
Chondrosia reniformis (*ex situ*) (g)	0.019 ± 0.003
Hemimycale columella (e)	0.013 ± 0.004
Chondropsis sp. (e)*	0.013 ± 0.009
Crambe crambe (e)	0.011 ± 0.007
Tedania sp. (orange) (e)*	0.008 ± 0.005
Stylopus sp. (e)*	0.008 ± 0.006
Chelonaplysilla sp. (e)*	0.006 ± 0.005
Hymedesmia sp. (red) (e)*	0.005 ± 0.003
Hymedesmia sp. (orange) (e)*	0.002 ± 0.003
Anchinoe sp. (e)*	0.001 ± 0.006
Eurypon sp. (e)*	0.0003 ± 0.031
Microciona sp. (e)*	-0.001 ± 0.0003

* Sponge growth rates determined by Ayling (1983).

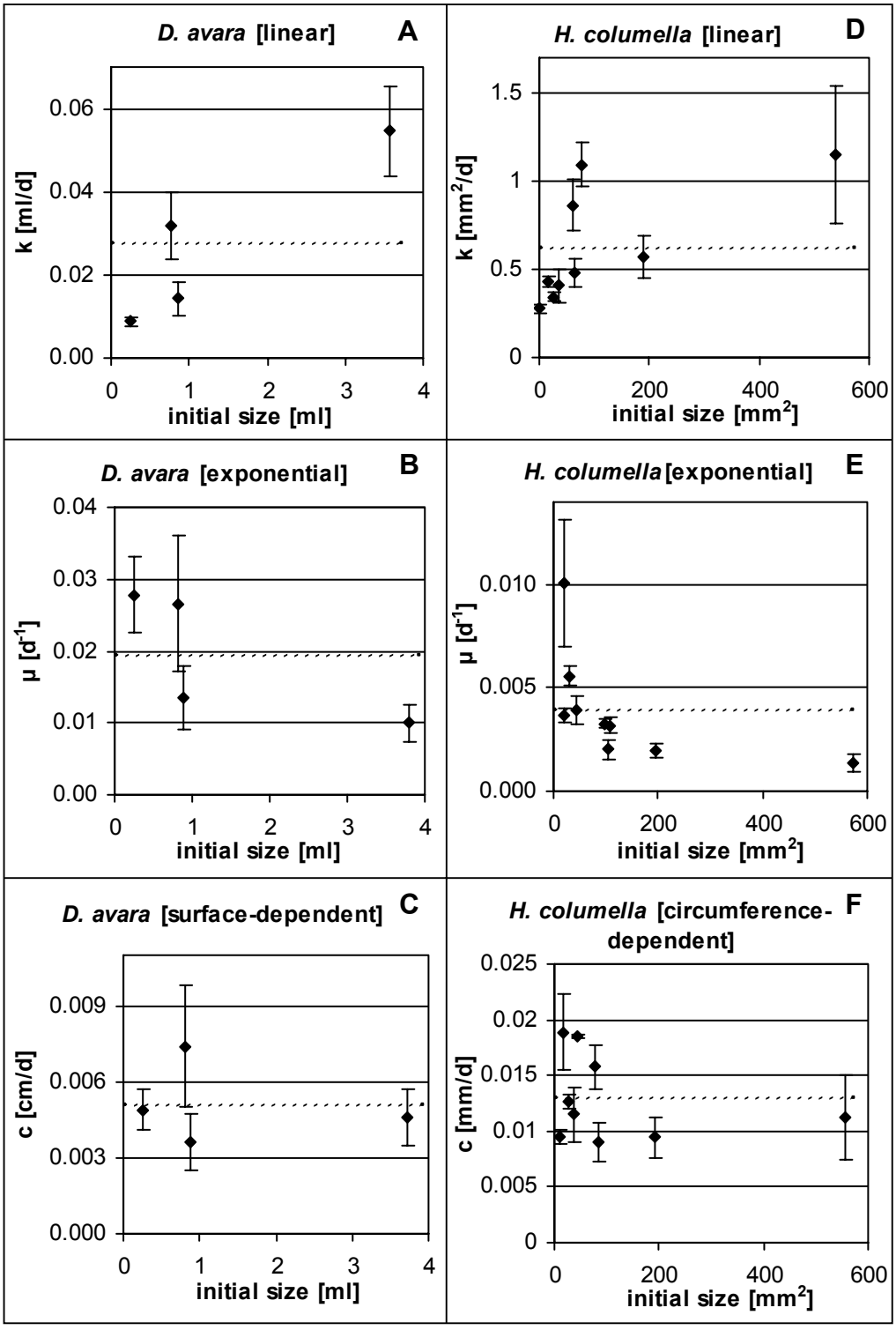

Fig. 6: The relation between the size of the sponge and the growth rate constant according to the three different models for an encrusting and a globose sponge with their standard errors. The linear (A), exponential (B) and surface-dependent (C) describe the growth models for *Dysidea avara* specimens. The linear (D), exponential (E) and circumference-dependent (F) represent the growth models for *Hemimycale columella* specimens. The dotted line represents the constant growth rate with the smallest sum of squares.

It is also concluded that the data that are available in literature up to now and our own data are not sufficient to obtain highly significant differences between the three growth models that were assessed (Fig. 7). Only for *H. columella* the circumference-dependent model was significantly better than the linear model (90% confidence interval with t-test) and for *D. avara* the surface-dependent model was significantly better than the exponential model (98% confidence interval with t-test). However, the significance level, which is based on differences between the normalised calculated growth constants and the model, does not take a trend in the data into account. In Fig. 6 it can be seen that especially for the linear and exponential model there appears to be a correlation between the initial size of the sponge and the growth rate, which is not so obvious for the circumference- or surface-dependent growth. This suggests that the linear and exponential models are not appropriate to describe the growth of sponges.

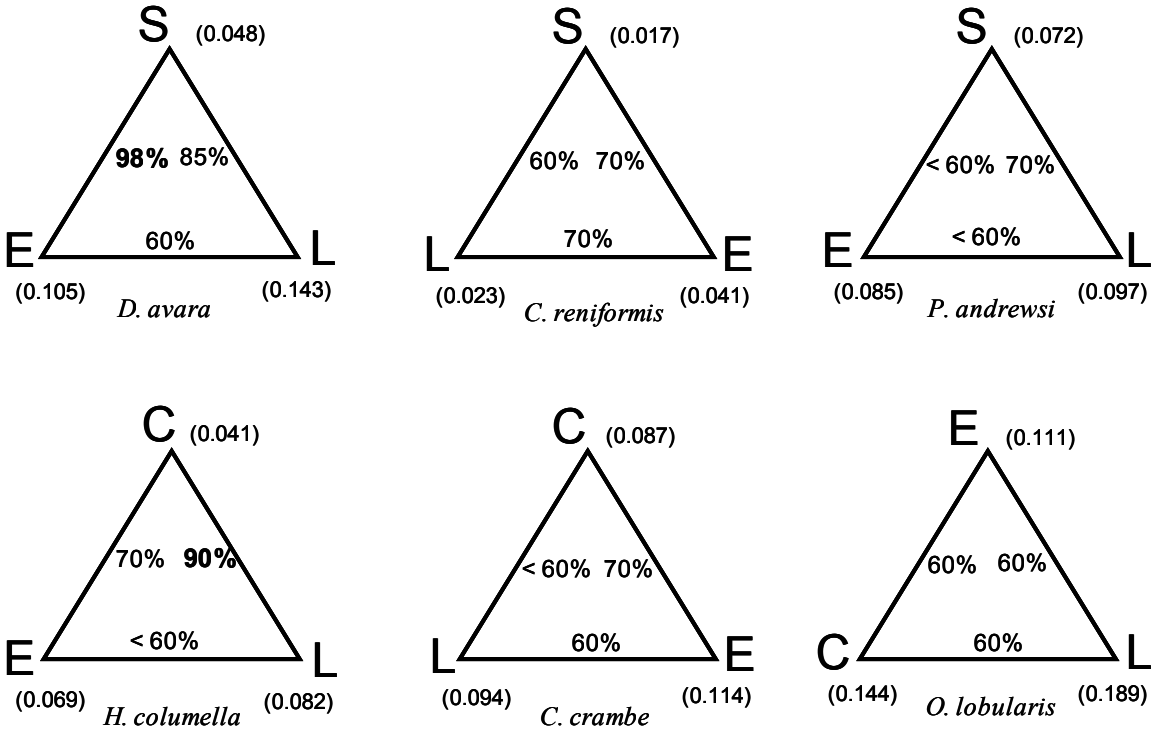

Fig. 7: The variance of the normalised calculated growth constants in relation to the three growth models. The three upper triangles show the result for the globose sponges, while the three lower triangles present the result for the encrusting species. The best model (with the lowest variance) is always depicted at the top of the triangle. The second-best and third-best models are depicted in the left and right corners of the triangles, respectively. L = linear, E = exponential, S = surface-dependent (for globose sponges) and C = circumference-dependent (for encrusting sponges). The variance of the different models is put between brackets. The confidence intervals for significant differences between the models are presented along the ribs of the triangles.

The lack of significant differences between the three growth models may be explained by a number of reasons. In the first place, the data sets were based on a limited number of specimens: *O. lobularis* (4), *H. columella* (9), *C. crambe* (11), *D. avara* (4), *C. reniformis* (5) and *P. andrewsi* (7). A low number of replicates remains a problem, even with incorporation of literature data, as references containing information about both growth rates and size of the sponge are scarce. Secondly, especially the specimens that were cultured in the laboratory were cultured for a relatively short period of time, and due to the slow growth rate of the sponge the differences between the three models are small. In the third place, the globose sponges were modelled as hemispheres for the calculation of the surface-dependent growth rate. Every deviation of this morphology leads to a larger surface, when related to its volume. The *C. reniformis* explants resemble a hemisphere quite well, but *D. avara* and *P. andrewsi* are more erratically shaped (Fig. 8). This means that if the surface area is the major factor that determines sponge growth, a more accurate estimation of the surface of these sponges is required. This also accounts for encrusting sponges that were modelled as circles, but for these sponges it is easier to determine the exact circumference by underwater photography. Fourthly, it is probable that there are more factors, such as pressure, light, nutrient concentration and current, that affect the growth rate of sponges. For example, current profiles around the sponge may cause a nutrient gradient over the sponge. It has been found that these factors can significantly affect growth rate and the morphology of the sponge (McDonald *et al.*, 2003). Kaandorp (1995) has developed elegant morphological models to describe the growth pattern of sponges based on the simulated nutrient profile around the sponge. With these models, the morphological growth pattern of the sponge can be predicted quite well. Combining the morphological models of Kaandorp, with the simple kinetic model described in this paper could lead to a more accurate prediction of the growth rate of sponges under different conditions.

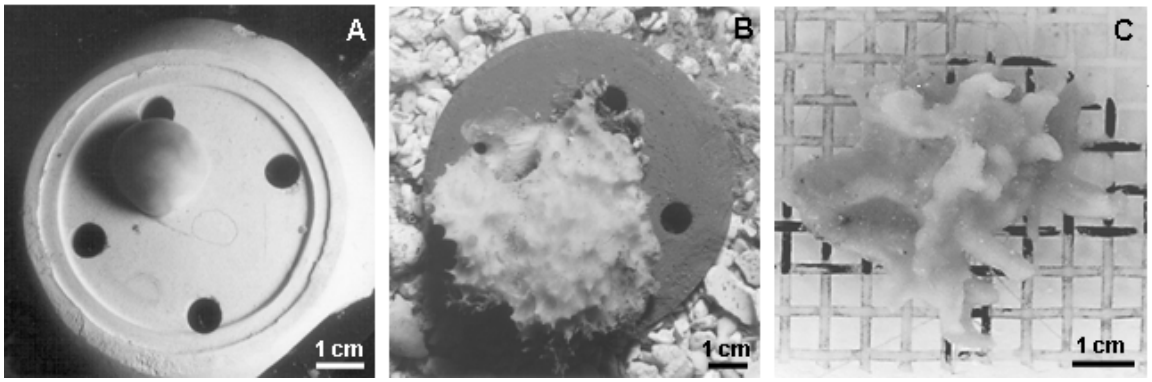

Fig. 8: Differences in the morphology of *C. reniformis* (A), *D. avara* (B), and *P. andrewsi* (C) explants.

It is interesting to have a look at the implications of a growth model for the set-up of mariculture or *ex situ* cultivation of sponges. Assuming radial accretive growth, the highest absolute increase of biomass is supported by the use of small explants, as the surface area/volume and circumference/area ratios are highest for small globose- and encrusting sponges, respectively. However, small explants generally have a higher mortality than their larger counterparts (Duckworth *et al.*, 1997; Turon *et al.*, 1998), and the optimal size of explants is therefore determined by a trade-off between mortality and labor, and the growth rate.

4 Primmorphs from seven Marine Sponges: Formation and Structure

Abstract

Primmorphs were obtained from seven different marine sponges: *Stylissa massa*, *Suberites domuncula*, *Pseudosuberites* aff. *andrewsi*, *Geodia cydonium*, *Axinella polypoides*, *Halichondria panicea* and *Haliclona oculata*. The formation process and the ultra structure of primmorphs were studied. A positive correlation was found between the initial sponge-cell concentration and the size of the primmorphs. By scanning electron microscopy (SEM) it was observed that the primmorphs are very densely packed sphere-shaped aggregates with a continuous pinacoderm (skin cell layer) covered by a smooth, cuticle-like structure. In the presence of amphotericin, or a cocktail of antibiotics (kanamycin, gentamycin, tylosin and tetracyclin), no primmorphs were formed, while gentamycin or a mixture of penicillin and streptomycin did not influence the formation of primmorphs. The addition of penicillin and streptomycin was, in most cases, sufficient to prevent bacterial contamination, while fungal growth was unaffected.

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Introduction

Since Bergmann and Feeney (1950; 1951) isolated bioactive compounds from sponges in the early fifties, many interesting natural products have been discovered in sponges. The presence of cytotoxins (Schmitz, 1994), antifouling agents (Miki *et al.*, 1996), anticancer compounds (Munro *et al.*, 1999), antibiotics and antiviral agents (Munro *et al.*, 1994) has been demonstrated. However, until now, there is a large gap between the demonstrated presence of these compounds, and their further development, due to a lack of a suitable sponge cultivation method (Pomponi, 1999). In sea-based aquacultures, conditions cannot be controlled, and reported growth rates are low. A decrease in weight, a doubling of the weight in 262 days and 4% increase per day have all been found (MacMillan, 1996; Duckworth *et al.*, 1997; Thomassen and Riisgård, 1995). In addition, sponges are notorious for being difficult animals to cultivate in controlled systems like aquaria (Osinga *et al.*, 1999). Sponge-cell culture might be the most promising method for the production of sufficient sponge biomass for pharmaceutical purposes. Growth rates can be increased considerably in animal cell cultures. For insect-cell cultures, doubling times of 1 day are common (Marteijn *et al.*, 2000), and for Chinese hamster ovary cells the doubling time is approximately 25 hours (Avgerinos *et al.*, 1990). Furthermore, the production of compounds for pharmaceutical purposes should preferably be done in axenic cultures, under well-defined and controlled conditions in order to guarantee a product that is free from microbial contamination. This is not possible in aquacultures, but with cell cultures in closed bioreactors these requirements can be established.

During the last decade, several studies have been performed to establish a primary sponge-cell culture and finally a sponge-cell line (Klautau *et al.*, 1993; Klautau *et al.*, 1994; Pomponi and Willoughby, 1994; Ilan *et al.*, 1996; Pomponi *et al.*, 1997; Rinkevich *et al.*, 1998; Rinkevich, 1999; De Rosa *et al.*, 2001). However, to date, sponge-cell cultures have been characterised by a lack of growth, or doubt as to the origin of growing cells, since sponge cells are easily confused with protozoa or thraustochytrids, a group of eukaryotic heterotrophic protists. These are very common in the marine environment, and can be present as a contaminant (Rinkevich, 1999).

Some of these problems may be overcome by using the aggregative properties of sponge cells. In a medium containing high calcium and magnesium concentrations, dissociated sponge cells re-aggregate and dense sphere-shaped aggregates of 1-2 mm in diameter, which are termed primmorphs, are formed. Primmorphs can be easily distinguished from protozoa or thraustochytrids, and it has been shown that they can be maintained in un-supplemented seawater for in excess of 5 months (Custodio *et al.*, 1998). Furthermore, it

was shown that sponge cells in primmorphs retain their telomerase activity, which is the genetic indicator for proliferative capacity, while dissociated sponge cells rapidly lose their telomerase activity (Koziol *et al.*, 1998; Custodio *et al.*, 1998; Müller *et al.*, 1999). This could explain why proliferating cells were detected by BrdU-labeling in primmorphs, and not in a single-cell suspension (Müller *et al.*, 1999).

To date, the formation of primmorphs for the species *Suberites domuncula* (Custodio *et al.*, 1998) and *Dysidea avara* (Müller *et al.*, 2000) has been described. The objective of our research is to discover whether formation of primmorphs is a more generic characteristic of dissociated sponge cells. This is important information to gain insight in the possibilities for general application of the primmorph system for the production of sponge biomass. Therefore, we used dissociated sponge-cell suspensions of seven different marine sponges. We will describe the formation and structure of the primmorphs in detail.

Furthermore, the fight against contamination is an important issue, especially when nutrients are supplemented to seawater to obtain growth of sponge biomass. We tested the efficacy of several antibiotics to prevent bacterial and fungal bloom. In addition, we studied the effects of these antibiotics on the formation of primmorphs.

Materials and Methods

Materials

Sponges

Live specimens of *Stylissa massa* were purchased from De Jong Marinelife (Spijk, The Netherlands). They were collected by SCUBA divers near Bali (Indonesia) and were transported in water bags, which were isolated to keep the temperature constant. In Wageningen, the sponges were maintained in an artificial seawater aquarium at a temperature of approximately 26 ºC, for one week to one month before use. Nutrients were supplied to the aquarium by weekly addition of microalgae (*Chlorella sorokiana* and *Nannochloropsis* sp.) and cyanobacteria (*Synechococcus* sp.).

Specimens of *Suberites domuncula* that were used for our experiments originated from gemmules that were collected by SCUBA diving near Rovinj (Croatia), and developed into functional sponges in our aquarium at a temperature of approximately 17 ºC. Nutrients were supplied weekly by adding a mixture of *Chlorella sorokiana*, *Nannochloropsis* sp. and *Synechococcus* sp.

Species of *Pseudosuberites* aff. *andrewsi* were obtained from the Artis Zoo (Amsterdam, The Netherlands). The sponges were maintained in the same aquarium as our *Stylissa massa* specimens at a temperature of approximately 26 ºC for one week to one month before use.

Live specimens of *Halichondria panicea* and *Haliclona oculata* were collected in the Scheldt Estuary (The Netherlands) by SCUBA diving. They were transported by car to Wageningen in a vessel containing 10 L seawater from the Scheldt Estuary. The sponges were maintained in our aquarium at a temperature of approximately 17 ºC. Within 24 hours after transport they were used to prepare cell suspensions.

Specimens of *Geodia cydonium* were collected by SCUBA diving in the Gulf of Naples (Italy), and kept in flow-through natural seawater tanks during transport to the Institute for the Chemistry of Molecules with Biological Interest (CNR-ICB; Pozzuoli, Italy) where cell suspensions and primmorphs were prepared.

Axinella polypoides specimens were collected by SCUBA diving near Kalymnos (Greece). They were transported by aeroplane in a vessel containing 10 L of Mediterranean seawater, and maintained in an aquarium at a temperature of approximately 17 ºC for one week before use.

Solutions

Artificial seawater (ASW) was prepared from 33 g/L instant ocean reef crystals (Aquarium Systems, Sarrebourg, France) in demineralised water. The pH was adjusted to 8.2 with HCl and the solution was filter sterilised (pore size 0.22 μm; Nalgene, Rochester, USA).

Calcium- and magnesium-free seawater (CMFSW) was prepared by dissolving 0.994 g Na_2SO_4, 0.0168 g $NaHCO_3$, 0.746 g KCl, 31.6 g NaCl and 2.42 g Tris in 1 L demineralised water. The pH was adjusted to 8.2 before filter sterilisation (pore size 0.22 μm).

A sterile 90 % Percoll stock solution was prepared by adding a sterile 10-fold concentrated solution of CMFSW to 100 % Percoll (Sigma, St. Louis, USA). The stock solution was diluted with sterile CMFSW to obtain 10 % and 60 % Percoll solutions.

Several antibiotics were used as a supplement in ASW:

Penicillin-streptomycin was used at a concentration of 10,000 U/mL for both penicillin and streptomycin (Life Technologies, Paisley, UK).

Amphotericin was used at a concentration of 2.5 μg/mL (Life Technologies).

Gentamycine was used at a concentration of 10 μg/mL (Life Technologies).

An antibiotics cocktail was used based on results of De Rosa *et al.* (2001). The cocktail contained 100 μg/mL kanamycin (Life Technologies), 100 μg/mL tylosin (Sigma), 100 μg/mL tetracycline (Merck, Darmstadt, Germany) and 10 μg/mL gentamycin. This cocktail will be referred to as the KTTG-cocktail.

Experimental

Preparation of a sponge-cell suspension

The protocol used to prepare a sponge-cell suspension was based on the method of Custodio *et al.* (1998). A piece of approximately 1 cm^3 sponge tissue was cut out and submerged in artificial seawater (ASW). By means of a razor-sharp knife, the sponge tissue was cut into smaller pieces of 1 mm^3. The pieces were washed once more in ASW and transferred into a 50 mL conical tube (Greiner, Solingen, Germany) containing 25 mL of calcium- and magnesium-free seawater (CMFSW). The tube was placed horizontally in a shaking incubator (type GFL, Salm en Kipp, Breukelen, The Netherlands) at low speed and at a temperature of 16-20 ºC. After 60 minutes the supernatant was discarded and replaced by 25 mL of CMFSW and the tube was returned into the shaking incubator. After 120 minutes the supernatant was filtrated through a sterile 50 μm mesh nylon (supernatant 1) and replaced by 25 mL of CMFSW. The tube containing the sponge pieces was incubated for 120 more minutes before supernatant 2 was harvested by filtrating the cell suspension through a 50 μm mesh nylon. Supernatant 1 and 2 were centrifuged for 10 minutes at 800 x g and the pellets were suspended and pooled in 5 mL CMFSW. The sponge-cell concentration was determined by using a Neubauer improved haemocytometer and an inverted light microscope (Olympus CK2). Subsequently the cell suspension was centrifuged for 10 minutes at 800 x g and the pellet was suspended in ASW to establish a cell concentration of $2 \cdot 10^6$ cells/mL. This primary cell suspension was used as starting material for the production of primmorphs. For *Suberites domuncula* initial cell concentrations of $2 \cdot 10^6$, $3 \cdot 10^6$, $4 \cdot 10^6$ and $5 \cdot 10^6$ cells/mL were applied to study the influence of cell concentration on the final size of primmorphs.

Production and maintenance of primmorphs

Aliquots of 6 mL of the primary cell suspension were transferred to 60 mm polyethylene petri dishes (Greiner). The petri dishes were incubated under gentle agitation on a rocking plate. Re-aggregation and primmorph formation was monitored by light microscopy (Olympus CK2 inverted microscope) and stereomicroscopy (Zeiss SV-11). After the formation of primmorphs, individual primmorphs were picked from the petri dish with a

sterile spatula and transferred to separate wells of a 24-well plate (Corning, New York, USA). Each well contained 1.5 mL ASW, which was refreshed every week.

Gradient centrifugation

To obtain a *Suberites domuncula* cell suspension, gradient centrifugation was included in the procedure to obtain a cell suspension. The protocol was adapted from Pomponi and Willoughby (1994). Ten mL of a 60 % Percoll solution was pipetted in a sterile conical tube (Greiner) and 10 mL of a 10 % Percoll solution was pipetted on top of the 60 % layer. A maximum volume of 6 mL cell suspension in CMFSW was pipetted carefully on top of the 10 % layer and the tube was centrifuged at 1000 rpm (138 x g) at 4 ºC for 10 minutes. A sterile Pasteur pipette was used to remove the middle layer containing the sponge cells and to transfer it into a sterile tube. A large volume of CMFSW (40-50 mL) was added to the cell suspension and it was centrifuged at 800 x g for 10 minutes. Subsequently the pellet was resuspended in 5 mL CMFSW.

Image analysis

Scion Image for Windows Beta 4.0.2 (Scion corporation) was used to measure the size of primmorphs by determining the area of primmorphs on 2-dimensional digital pictures. The diameter was calculated with the assumption that primmorphs are perfect spheres.

Scanning electron microscopy (SEM)

Primmorphs were put on a small piece of filter paper. Samples were gently dried until the glossy surfaces had disappeared. This arbitrary method worked well to obtain hydrated samples without an excess of water. The filter paper, carrying the primmorphs, was glued on a specimen holder with carbon cement (Leit-C, Neubauer Münster Germany) at room temperature and subsequently frozen in liquid nitrogen. The frozen samples were placed in a cryo-preparation chamber (Oxford CT 1500 HF, Oxford Instruments, High Wycombe, UK) on a cryostage at -90 °C. Some samples were fractured using a cold scalpel (-180 °C), other samples were used unfractured. Samples were freeze-dried for 2 minutes. For morphological analyses samples, were sputter coated with 10 nm platinum and observed at -160 °C at 5 kV in a scanning electron microscope (JSM 6300F Tokyo, Japan). Mass detection in the surface of the samples was obtained by analyses of uncoated sample surfaces with back scatter electron (BSE) detection (AutraDet, Brno Czech Republic) in the scanning electron microscope at -160 °C at 15 kV. Micrographs were recorded digitally.

Results and Discussion

Formation of primmorphs

We obtained primmorphs from seven different sponges: *Stylissa massa*, *Suberites domuncula*, *Pseudosuberites* aff. *andrewsi*, *Halichondria panicea*, *Haliclona oculata*, *Geodia cydonium* and *Axinella polypoides* (Table 1). Primmorph formation usually proceeded as follows: within 15 minutes after inoculation of the dissociated cells in artificial seawater (ASW) small sponge-cell aggregates (Fig. 1B) were formed from dissociated cells (Fig. 1A). After 3 hours these aggregates were visible by eye. Observation by microscope revealed that the aggregates had an undefined morphology while the cell density inside the aggregates had increased (Fig. 1C). During two days, as aggregation continued, dense cell aggregates were transformed to early-stage primmorphs, which were characterised by a more or less spherical shape, a high cell density and a rough surface layer. Early-stage primmorphs reached a size ranging from 0.2-2.0 mm, which did not change during the development into primmorphs. Subsequently, the surface of the early-stage primmorph developed into a smooth "skin layer" surrounding the high-density cell clump (Fig. 1D). The time that was required for this last step in the development into primmorphs was the most variable step for different species that were tested (Table 1). While primmorph formation within one week was observed for *Suberites domuncula*, *Halichondria panicea*, *Stylissa massa*, *Geodia cydonium* and *Pseudosuberites* aff. *andrewsi*, a much longer period was required for primmorph formation of *Axinella polypoides* and *Haliclona oculata*.

Table 1: Primmorph formation from seven marine sponge species. # Primmorphs = the total number of primmorphs that was obtained. # batches = number of separate experiments to obtain primmorphs (between brackets the number of primmorphs for the different batches). Formation time = the period in which new formed primmorphs were detected. Life span = the period in which no changes in outer appearance occurred.

Sponge	**# primmorphs**	**# batches**	**Formation time [days]**	**Lifespan [months]**
Stylissa massa	48	2 (30,18)	7-10	>2
Pseudosuberites aff. *andrewsi*	25	2 (10, 15)	4-8	0.3
Suberites domuncula	83	3 (28, 30, 25)	3-7	4-5
Haliclona oculata	4	2 (0, 4)	18	0.6
Halichondria panicea	44	2 (40, 4)	7-20	>5
Geodia cydonium	10	1 (10)	7-10	>2
Axinella polypoides	2	1 (2)	35-40	>5

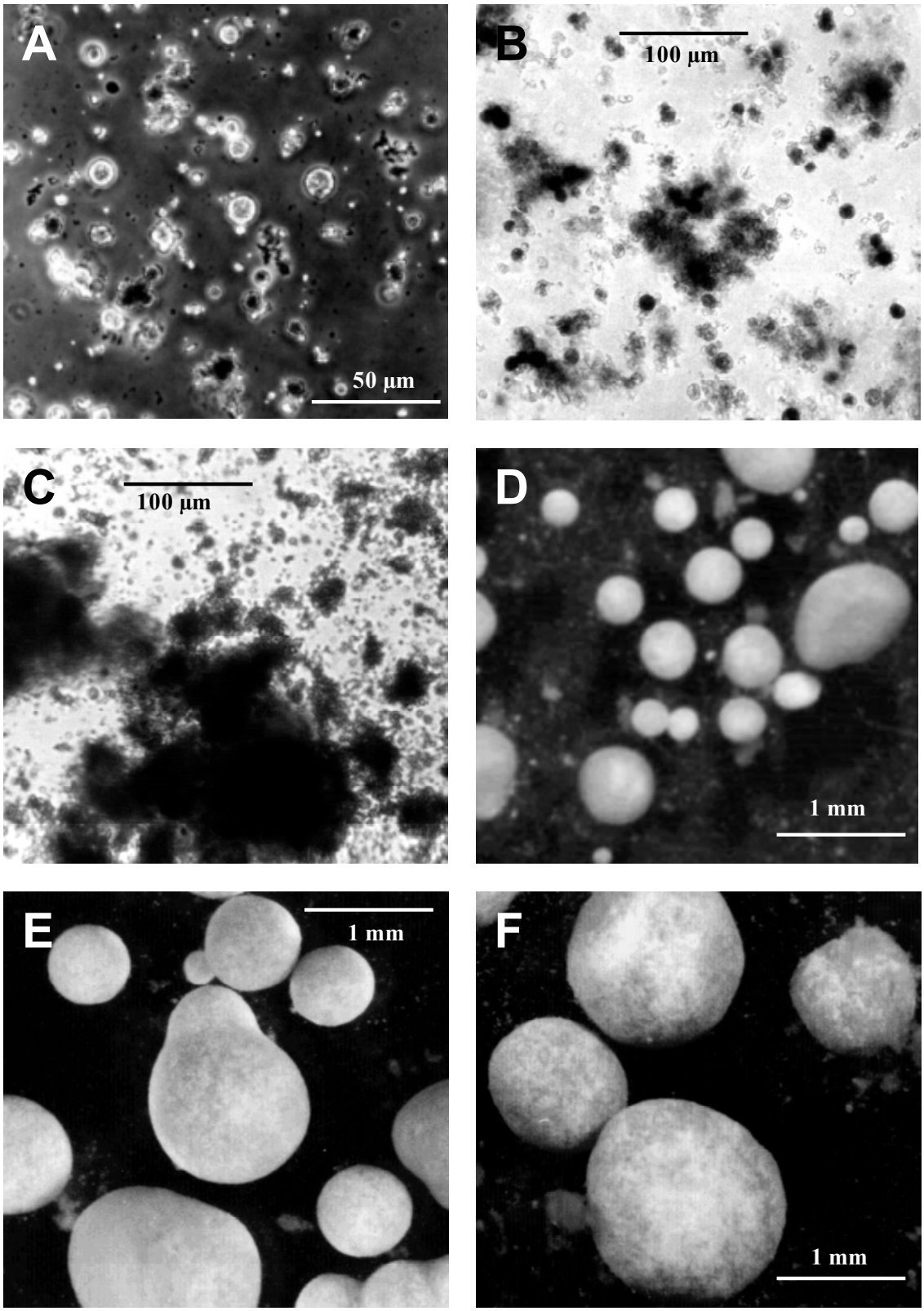

Fig. 1: Formation of primmorphs from a *Stylissa massa* cell suspension. (A) Cell suspension of $2 \cdot 10^6$ cells/mL. (B) Cell aggregates 15 min after incubation. (C) Cell aggregates 3 h after incubation. (D) Small primmorphs at day 7. (E) Fusion of primmorphs at day 11. (F) Primmorphs at day 14.

After development of the smooth skin layer, the primmorphs were almost perfect spheres. However, because of fusion of some primmorphs, the morphology became more diverse (Fig. 1E). When the primmorph fusion stopped, they regained their spherical shape (Fig. 1F) and no further changes in outer appearance were observed. In addition to fusion of primmorphs, splitting of a primmorph in two or more smaller primmorphs was also observed. This occurred when a primmorph was damaged mechanically in a narrow opening of a pipette. The spherical shape and the skin layer of the primmorph were restored within 4 days (Fig 2A-D).

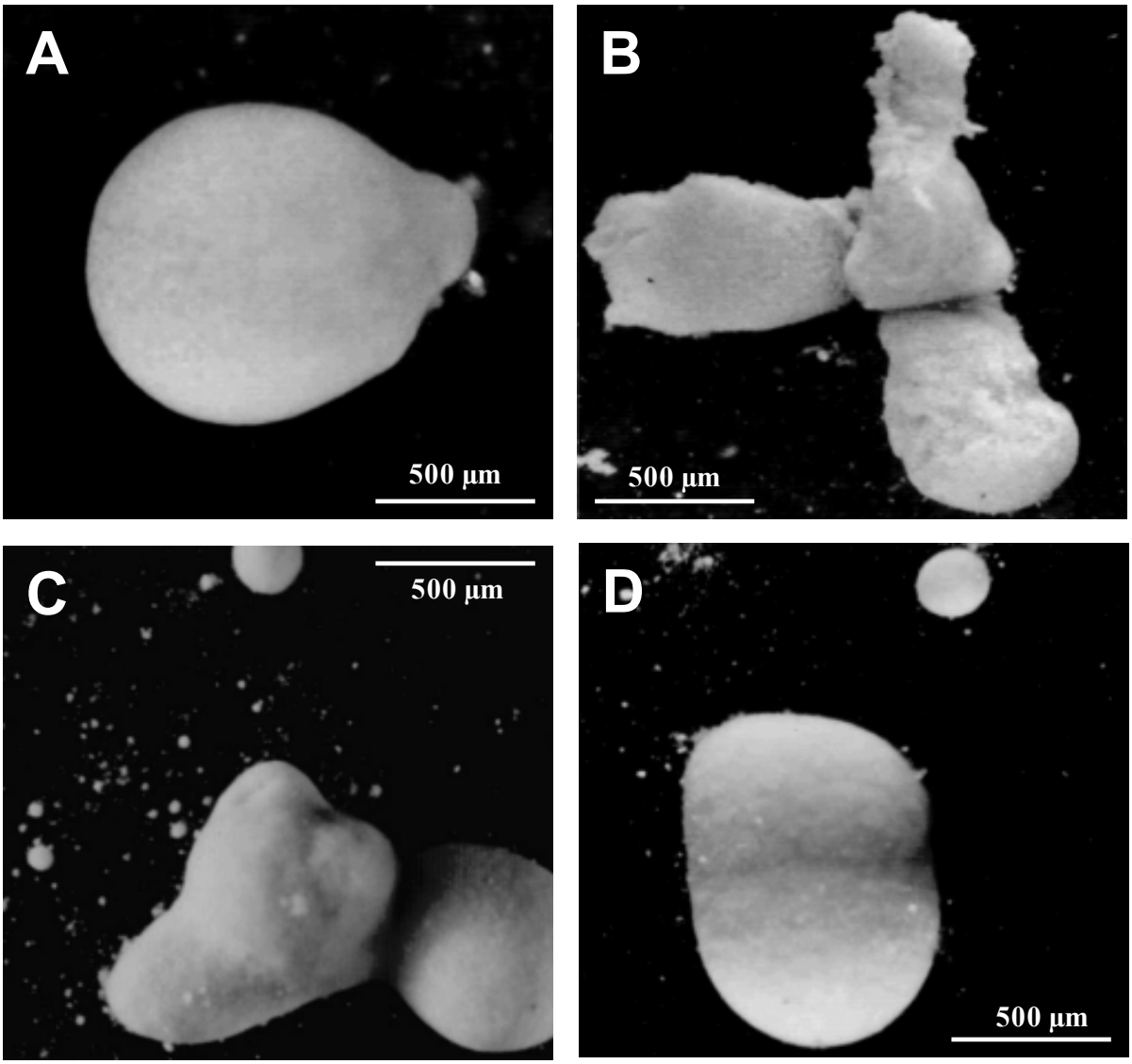

Fig. 2: Restoration of a damaged *Stylissa massa* primmorph. (A) Intact primmorph. (B) Damaged primmorph at t = 0 h. (C) Primmorph at t = 26 h. (D) Primmorph at t = 97 h.

In order to obtain *Suberites domuncula* primmorphs, we had to modify the protocol previously developed by Custodio *et al.* (1998), because no primmorphs were obtained using the original protocol. The specimen that we used for the experiment originated from gemmules that had developed into small functional sponges. They consisted of a thin layer of sponge tissue surrounding a snail's shell. The thin layer of tissue contained a relatively high number of spicules, which were also observed by microscopy in dissociated cell suspensions from these specimens. When the cell suspensions were incubated in petri dishes, sponge-cell aggregates were formed, but they were completely riddled with spicules. Spicules that are present in sponge-cell aggregates are usually excreted by the early-stage primmorphs. However, in this case, the abundance of spicules apparently prevented formation of primmorphs. It seemed that the cells preferred attachment to spicules over cell-cell contact. Therefore, it was decided to separate the spicules from the dissociated cells by gradient centrifugation. Microscopic analysis of the obtained cell suspension revealed that practically all spicules had disappeared and primmorphs were formed. In general, primmorphs could be maintained for a long period (Table 1). The short survival times for *Pseudosuberites* aff. *andrewsi* and *Haliclona oculata* primmorphs that we used for this experiment were caused by fungal infections. For *Suberites domuncula*, we observed that between 4 to 5 months after formation of primmorphs, most of them lost their smooth skin and started to disintegrate. A few spicules were observed in all disintegrated primmorphs, which indicates that active spicule formation had occurred in the presence of 12 μM silicate that was present in the seawater that we used. Since the spicules from the parent sponge were removed by gradient centrifugation, we can be sure that the observed spicules were formed in the primmorphs. This is in agreement with the results of Krasko *et al.* (2000), who observed spicule formation in *Suberites domuncula* primmorphs in the presence of 60 μM silicate and no spicule formation in the absence of silicate.

For the species *Suberites domuncula*, we also studied the effect of the initial cell concentration on primmorph formation (Fig. 3). Higher initial cell concentrations significantly increased the size of the primmorphs that were obtained (T-test; $P < 0.001$). Custodio *et al.* (1998) and Müller *et al.* (1999) used a relatively low initial cell concentration ($1.5\text{-}2.0 \cdot 10^6$ cells/mL), but obtained primmorphs with a diameter between 1 and 2 mm from the same sponge. When we assume a linear relationship between the cell concentration and the primmorph size, a concentration of $1 \cdot 10^7$ cells/mL would be expected to obtain primmorphs with this size. However, these differences can be

explained by differences in the state of the sponges that were used, and the procedure for dissociating the sponge.

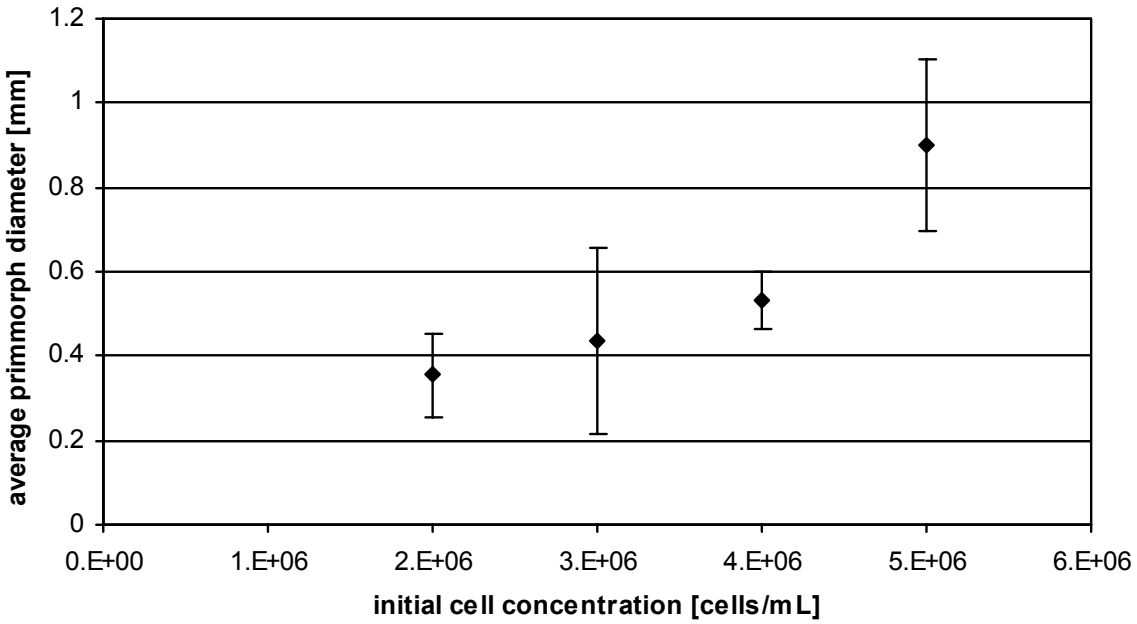

Fig. 3: Relation between initial *Suberites domuncula* cell concentration and size of primmorphs. Datapoints are based on 30 primmorphs ($2 \cdot 10^6$ cells/mL), 14 primmorphs ($3 \cdot 10^6$), 6 primmorphs ($4 \cdot 10^6$) and 12 primmorphs ($5 \cdot 10^6$); Y-error bars are standard deviations.

Ultrastructure of primmorphs

Scanning electron microscopic (SEM) analysis was applied to reveal the ultrastructure of the primmorphs. The development of the skin layer during the formation of primmorphs was monitored closely. In the surface layer of early-stage primmorphs, individual cells are visible (Fig. 4A). The period it takes for early-stage primmorphs to transform into mature primmorphs, which are characterised by a smooth skin (Fig. 4C), was found to be variable, and ranged from 3 to 33 days. During the transformation, an inter-phase was observed. Primmorphs in the inter-phase were characterised by an almost completely smooth skin containing a few rough spots (Fig. 4B). With an inverted light microscope it could be seen that the rough spots were small attached cell aggregates. Since they had the same structure and colour as dead cell aggregates, which were observed after long-term maintenance in seawater, we assumed that these small attached aggregates consisted of dead cells. It is possible that this is secreted dead-cell material that the primmorph drained into the environment. Also, very often, we observed that spicules were transported to the outside of developing primmorphs. The skin layer of primmorphs is composed of pinacocytes (Custodio *et al.*, 1998). However, individual pinacocytes cannot be observed from the outside, due to the formation of the smooth layer covering the surface of the

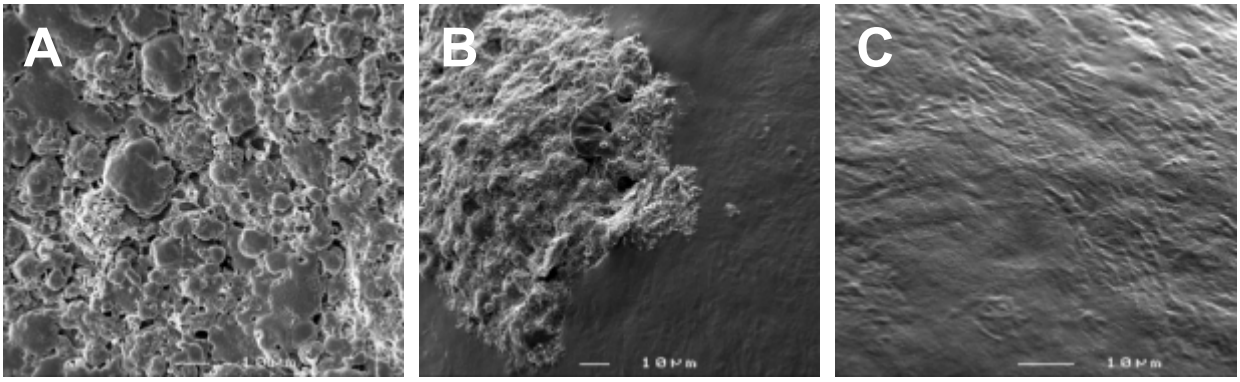

Fig. 4: Electron microscopic scans of development of the skin layer of primmorphs from *Stylissa massa*. (A) Surface layer of early-stage primmorphs after 4 days. (B) Inter-phase (6 days). (C) Skin layer of a primmorph (8 days).

primmorphs. Verdenal and Vacelet (1990), who studied aquaculture of sponges on ropes, found that the entire surface of sponges that did not adhere to the ropes, was covered with a cuticle. These cuticle-covered sponges survived for a long time, but did not grow. De Vos *et al.* (1991) described that the pinacoderm of a sponge may be covered, or replaced, by a layer of coherent collagen in unfavourable physiological conditions, during which the intake of water is stopped. It is conceivable that the smooth layer surrounding the pinacocyte tissue of primmorphs is also composed of collagen fibres.

A cross-section of a smooth primmorph showed that it is densely packed with cells of different types (Fig. 5). The layer of flat pinacocytes can be observed just underneath the skin layer of the primmorph. Deeper inside the primmorph, larger cells and a few intercellular spaces can be seen. These cells can be recognised as archeocytes and granulocytes (Müller *et al.*, 1999).

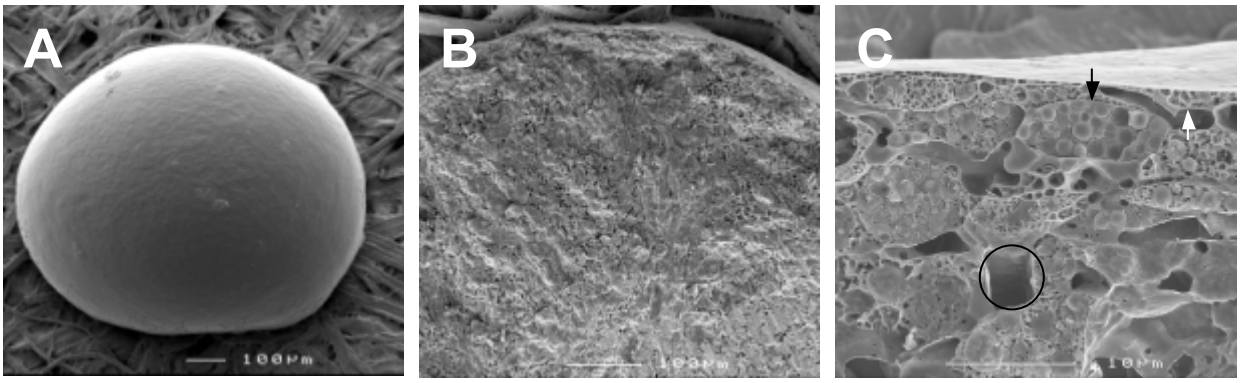

Fig. 5: Electron microscopic scans of a *Stylissa massa* primmorph. (A) Intact primmorph. (B) Cross section of a primmorph showing the high-density cell packing. (C) Close up of cross section; white arrow points at pinacocyte, black arrow indicates granulocyte, intercellular space within circle.

Antibiotics

We tested the efficacy of a number of antibiotics to prevent contamination by bacteria and fungi, and its effects on the formation of primmorphs for several sponges. The results are summarised in Table 2. Primmorphs were only obtained in the absence of antibiotics, or when ASW was supplemented with penicillin-streptomycin or with gentamycin. The formation of primmorphs in seawater without antibiotics resembles the classical experiments of Wilson (1907), who obtained sphere-shaped aggregates from dissociated *Microconia prolifera* cells. Custodio *et al.* (1998) and Müller *et al.* (2000) showed that in the presence of penicillin and streptomycin, cell re-aggregation was not disturbed for *Suberites domuncula* and *Dysidea avara*. Penicillin, streptomycin and also gentamycin act against bacteria, but eukaryotes are generally not affected (Kuhlmann, 1996). This is in agreement with the results of Pomponi and Willoughby (1994) who observed no effects of rifampicine on aggregation of dissociated cells from the sponge *Hymeniacidon heliophila*. Rifampicine also acts specifically against bacteria. However, fungi are not affected by these antibiotics and for all sponges tested, fungal infections occurred frequently in the presence of penicillin-streptomycin and gentamycin, or when no antibiotics were added. Therefore, we supplemented ASW with the fungicide amphotericin or with the KTTG-cocktail. The latter was used successfully by De Rosa *et al.* (2001) to purify a dissociated sponge-cell suspension. However, the addition of these antibiotics inhibited formation of primmorphs for all sponges that were tested.

Table 2: Effectivity of antibiotics to prevent contamination by bacteria and fungi and its effects on the formation of primmorphs after 7 days. 0 = no antibiotics; P-S = penicillin-streptomycin; Amph. = amphotericin; Gent. = gentamycin; KTTG = cocktail of kanamycin, tylosin, tetracyclin and gentamycin. + = primmorph formation; - = no primmorph formation; Clean = no contamination; Cont. = contamination; n.d. = not determined.

	0	P-S	Amph.	Gent.	Cocktail
Stylissa massa	+ / Clean	+ / Clean	- / Clean	+ / Clean	n.d. / n.d.
Pseudosuberites aff. *andrewsi*	+ / Clean	+ / Cont.	- / Cont.	n.d. / n.d.	- / Cont.
Halichondria panicea	+ / Clean	+ / Cont.	n.d. / n.d.	n.d. / n.d.	- / Cont.
Haliclona oculata	+ / Clean	+ / Cont.	n.d. / n.d.	n.d. / n.d.	- / Cont.
Suberites domuncula	+ / Clean	+ / Clean	- / Clean	n.d. / n.d.	- / Clean
Geodia cydonium	n.d. / n.d.	+ / Clean	n.d. / n.d.	n.d. / n.d.	n.d. / n.d.
Axinella polypoides	n.d. / n.d.	+ / Clean	n.d. / n.d.	n.d. / n.d.	- / Clean

Unstructured sponge-cell aggregates were formed but they never developed into primmorphs (Fig. 6). For amphotericin, Armstrong *et al.* (1999) observed a negative effect on the condition of sponge cells, which may have caused the different aggregation pattern that we observed. When the KTTG-cocktail was used, the aggregation process showed a pattern that was similar to the aggregation of cells treated with amphotericin. The total amount of antibiotics in the KTTG-cocktail may be too high to obtain primmorphs. Both tylosin and tetracyclin are present in a concentration that is ten-fold higher than the suggested working concentration (Sigma, 2000-2001). The high concentration of these antibiotics in combination with kanamycin and gentamycin probably inhibits sponge-cell aggregation.

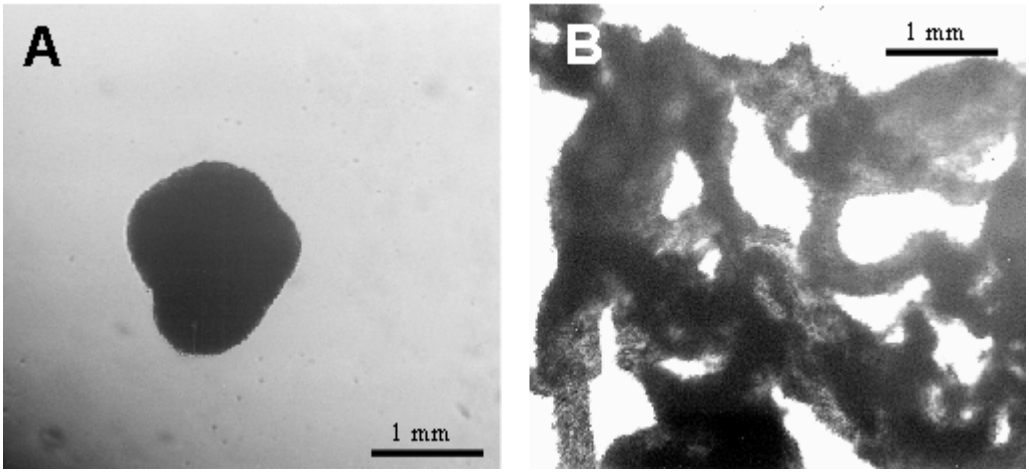

Fig. 6: *Halichondria panicea* aggregation after 7 days: (A) primmorph formation without antibiotics and (B) formation of large open aggregates in the presence of the antibiotics cocktail.

When seawater without additional nutrients was used, contamination was not a major problem. Although fungal contamination occurred now and then under all conditions tested, the occurrence seemed to be dependent on the condition of the dissociated cells (and the parent sponge specimen) that were used to obtain primmorphs. However, physical separation of sponge-cell aggregates and fungi (by transferring aggregates to other petri dishes) was in most cases sufficient to prevent fungal (and also bacterial) contamination. The possibility to physically separate sponge aggregates from fungal biomass is an additional motive for the use of primmorphs to produce sponge biomass. On the other hand, microbial contaminations can also be present inside primmorphs. Müller *et al.* (1999) showed the presence of bacterial RNA in primmorphs from *Suberites domuncula*. However, no sign of bacterial infection was detected in primmorphs when they were maintained in ASW. This could be explained by the hypothesis that certain bacteria live in symbiosis with the sponge, which controls microbial growth (Müller *et al.*, 1981). Currently, we are working on the detection of possible eukaryotic contaminants inside primmorphs. Since no steps are undertaken to separate sponge cells from potential

unicellular eukaryotic contaminants, which are very likely to be present in the parent sponges (Rinkevich, 1999), they may still be present inside primmorphs.

It is remarkable that we never obtained clean sponge-cell aggregates from *Pseudosuberites* aff. *andrewsi*, *Halichondria panicea* and *Haliclona oculata* in the presence of antibiotics, while in ASW without antibiotics, we obtained clean primmorphs. Our hypothesis is that this is the result of extermination of all sponge endogenous bacteria by the use of antibiotics in seawater. For *Pseudosuberites* aff. *andrewsi*, it has been shown that it accommodates 16 strains of bacteria on its surface, of which at least one displays anti-fouling activity (Osinga *et al.*, 2001). For *Halichondria panicea* it was found that bacteria belonging to the genus *Rhodobacter* were dominant in the mesohyl of the sponge (Althoff *et al.*, 1998). These authors discovered that extracts isolated from *Halichondria panicea* displayed toxicity on human leukaemia cells *in vitro* and suggested that this was caused by a toxin produced by *Rhodobacter*. This toxin could also be effective against fungi *in vivo*, which are also eukaryotic cells. *Oscillatoria spongeliae*, the symbiotic cyanobacterium that is present in large amount in the marine sponge *Dysidea herbacea*, was found to be responsible for the production of polybrominated biphenyl ethers, that were found to be active against a wide range of bacteria (Unson *et al.*, 1994). Destruction of the endogenous bacterial population of the sponge by antibiotics could thus lead to higher vulnerability to microbial infections.

Prospects for biotechnology: what is a primmorph?

We have shown here that primmorph-like aggregates can be obtained from a large number of sponge species. The potency to form these aggregates is likely to be general characteristic of demosponges. As such, the primmorph system may be very suitable to start up *in vitro* sponge cultures, since primmorphs can be made on request from many different sponges, and problems associated with transport of living sponges (Ilan *et al.*, 1996) can be circumvented. Furthermore, the primmorphs formation process could serve as a purification step of a dissociated sponge-cell suspension from fungi and non-symbiotic bacteria. Redissociation of primmorphs into single cells could thus lead to an (almost) axenic sponge-cell suspension to facilitate research on sponge-cell culture. However, it remains to be demonstrated to what extent these aggregates can be used to produce sponge biomass. We can only evaluate the biotechnological potential of the primmorph system when we understand why these aggregates are formed, and how they can be stimulated to produce sponge biomass.

The term primmorph was introduced by Custodio *et al.* (1998). Müller (1998) has defined primmorphs as multi-cellular aggregates of sponge cells in which cell proliferation occurs, obtained from dissociated cell suspensions. This implies that primmorphs have regenerative power. However, a substantial increase in biomass due to cell proliferation

has not been demonstrated for primmorphs, probably because cells in primmorphs also undergo apoptosis at a considerable rate (Koziol et *al.*, 1998; Krasko *et al.*, 1999).
It must be noted here that until now, primmorphs have always been produced and maintained under conditions of starvation, i.e. no food is added to the medium. The formation of smooth spherical aggregates covered with a collagen-like skin layer may be the result of starvation. In this respect, it is impossible to overlook the resemblance of primmorphs with natural resting stages such as gemmules and buds, which possess regenerative power. Gemmules and buds are provided with a pinacoderm, that separates the internal cell mass from the environment (Simpson, 1984; Custodio *et al.*, 1998). In gemmules, the pinacoderm is covered by a collagenous coat, which can be armoured with spicules (Bergquist, 1978). Our SEM pictures show that primmorphs possess a coating surrounding the pinacoderm, which is probably also constructed from a collagenous substance. Besides resemblance with natural resting stages, primmorphs seem to be very similar to experimentally induced buds. Bud formation can be experimentally stimulated by exposing sponges to stress conditions. The development of experimentally induced buds into functional sponges is completely comparable to that of naturally formed buds, except that the former are delayed in their development, requiring approximately ten months of dormancy before rapidly forming canals and choanocyte chambers. During the dormant period many cells are phagocytised, a process not apparent during normal bud development. This result can be viewed as suggesting that the proportions of cell types present in the experimentally induced buds must be readjusted before development can proceed (Simpson, 1984). The formation of primmorphs occurs after a sponge has been exposed to severe mechanical and chemical conditions when it is cut into small pieces, which are chemically dissociated. The subsequent formation of primmorphs might be comparable with the formation of experimentally induced buds. An indication that, in primmorphs, redistribution of cell types also has to occur before further development can take place are the earlier mentioned observations that cell proliferation and apoptosis occur simultaneously in primmorphs (Koziol *et al.*, 1998; Krasko *et al.*, 1999). The presence of proliferating cells in primmorphs is an indication for their regenerative power. If the occurrence of apoptosis can be suppressed, this regenerative power may result in the formation of new biomass. In a similar way to buds and gemmules, primmorphs may be able to hatch and form functional sponges. It is interesting to note in this respect that spicule formation has been demonstrated in primmorphs, which can be considered as an early step in morphogenesis. Furthermore, Müller *et al.* (2000) observed canal formation when primmorphs were cultivated in an aquarium for 3 weeks. Hence, our hypothesis for future research is that primmorphs have the capacity to develop into functional sponges.

5 The influence of silicate on *Suberites domuncula* primmorphs

Abstract

Primmorphs of the marine sponge *Suberites domuncula* were maintained in seawater at different silicate concentrations. We observed that in the presence of high silicate concentrations (70 and 148 μM) attachment of primmorphs to a solid matrix was stimulated, while at low concentrations (3.5 and 25 μM) attachment of primmorphs was never observed. In addition, we observed that spicule formation had occurred in primmorphs in the presence of high silicate concentrations.

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Introduction

Since Bergmann and Feeney (1950; 1951) isolated bioactive compounds from sponges in the early fifties, many interesting natural products have been discovered in sponges. However, commercial applications have been hampered by the lack of a suitable production system. Wild harvest, aquaculture and cell culture have not yet resulted in a solution for the supply problem (Osinga *et al.*, 1999). Some of these problems may be overcome by culturing sponges *in vitro* as dense sphere-shaped axenic aggregates of 1-2 mm in size, which are termed primmorphs. Primmorphs can be maintained in unsupplemented seawater for more than 5 months (Custodio *et al.*, 1998) and it was shown that sponge cells in primmorphs retain their telomerase activity, which is the genetic indicator for proliferative capacity, while dissociated sponge cells rapidly lose their telomerase activity (Koziol *et al.*, 1998; Custodio *et al.*, 1998; Müller *et al.*, 1999). The sponge skeleton is important for the internal organisation and development of a growing sponge (Bergquist, 1978). Thus if a primmorph is able to develop into a functional sponge it probably needs silicate, the building block of the spicule skeleton of demosponges. Krasko *et al.* (2000) showed that silicate induced the formation of silicatein, an enzyme which catalyses deposition of inorganic silica under the formation of spicules. They observed that in addition to the formation of silicatein, silicate induces production of myotrophin. Myotrophin from *Suberites domuncula* has the potency to stimulate the production of collagen, an important skeletal element in sponges, in the primmorph system (Schröder *et al.*, 2000). It is assumed that myotrophin is released by sclerocytes, motile secretory cells which produce spicules, and causes the expression of genes involved in the production of collagen-like spongin in exopinacocytes.
Our objective is to study if silicate triggers the further development of primmorphs to develop into functional sponges.

Materials and Methods

Materials

Sponge

Specimens of *Suberites domuncula* that were used for our experiments originated from gemmules that were collected near Rovinj (Croatia) and developed into functional sponges in our aquarium at a temperature of approximately 17 °C.

Solutions

Artificial seawater (ASW) was prepared from 33 g/L instant ocean reef crystals (Aquarium Systems, Sarrebourg, France) in demineralised water and supplemented with 1% penicillin (10,000 U/mL)-streptomycin (10,000 µg/mL) solution (Life Technologies, Paisley, UK). The pH was adjusted to 8.2 with HCl and the solution was filter sterilised (pore size 0.22 µm; Nalgene, Rochester, USA). ASW containing silicate was prepared by addition of sodium metasilicate (Sigma, St. Louis, USA) to ASW before filtration.

Natural seawater (NSW) was purchased from Sigma and supplemented with 1 % penicillin (10,000 U/mL)-streptomycin (10,000 µg/mL) solution (Life Technologies).

Calcium- and magnesium-free seawater (CMFSW) was prepared by dissolving 0.994 g Na_2SO_4, 0.0168 g $NaHCO_3$, 0.746 g KCl, 31.6 g NaCl and 2.42 g Tris in 1 L demineralised water. CMFSW was supplemented with 1 % penicillin (10,000 U/mL)-streptomycin (10,000 µg/mL) solution and pH was adjusted to 8.2 before filter sterilisation (pore size 0.22 µm).

A sterile 90 % Percoll stock solution was prepared by adding a sterile 10-fold concentrated solution of CMFSW to 100 % Percoll (Sigma). The stock solution was diluted with sterile CMFSW to obtain 10 % and 60 % Percoll solutions.

Experimental

Preparation of a sponge-cell suspension and maintaining primmorphs

The protocol used to prepare a sponge-cell suspension was based on the method of Custodio *et al.* (1998). Approximately 1 mL sponge tissue was submersed in artificial seawater (ASW) and cut into pieces of 1 mm^3. The pieces were washed once more in ASW and transferred into a 50 mL conical tube (Greiner, Solingen, Germany) containing 25 mL of calcium- and magnesium-free seawater (CMFSW). The tube was placed horizontally in a shaking incubator (type GFL, Salm en Kipp, Breukelen, The Netherlands) at low speed and at a temperature of 16-20 ºC. After 60 min the supernatant was discarded and replaced by 25 mL of CMFSW and the tube was returned into the shaking incubator. After 120 min the supernatant was filtrated through a sterile 50 µm mesh nylon (supernatant 1) and replaced by 25 mL of CMFSW. The tube containing the sponge pieces was incubated for 120 more min before supernatant 2 was harvested by filtrating the cell suspension through a 50 µm mesh nylon. Supernatant 1 and 2 were centrifuged for 10 min at 800 x g and the pellets were suspended and pooled in 5 mL CMFSW.

Ten mL of a 60 % Percoll solution was pipetted in a sterile conical tube (Greiner) and 10 mL of a 10 % Percoll solution was pipetted on top of the 60 % layer. The cell suspension in CMFSW was pipetted carefully on top of the 10 % layer and the tube was centrifuged at 1000 rpm (138 x g) at 4 ºC for 10 min. A sterile Pasteur pipette was used to remove the middle layer containing the sponge cells and to transfer it into a sterile tube. A large volume of CMFSW (40-50 mL) was added to the cell suspension and it was centrifuged at 800 x g for 10 min. Subsequently the pellet was resuspended in 5 mL of CMFSW. The sponge-cell concentration was determined by using a Neubauer Improved haemocytometer and an inverted light microscope (Olympus CK2, Olympus, Hamburg, Germany). Subsequently, the cell suspension was centrifuged for 10 min at 800 x g and the pellet was dissolved in ASW to establish a cell concentration of $2 \cdot 10^6$ cells/mL. Aliquots of 6 mL cell suspension were transferred to polyethylene 60 mm petri dishes (Greiner). The petri dishes were incubated under gentle agitation on a rocking plate. After 7 days, individual primmorphs with a diameter of at least 1 mm were picked from the petri dish with a sterile spatula and transferred to separate wells of a 24-well plate (Corning, New York, USA). Each well contained 1.5 mL ASW, which was refreshed every week.

Effect of silicate

To study the effect of silicate on primmorphs, 24 primmorphs were maintained in seawater containing different silicate concentrations (3.5 µM in NSW and 25 µM, 70 µM and 148 µM in ASW) in separate wells of a 24-well plate. Primmorphs from two *Suberites domuncula* specimen were used for the experiment. Primmorphs from one sponge were used to test silicate concentrations of 3.5 and 148 µM, while primmorphs from another specimen were used to test silicate concentrations of 25 and 70 µM. During the experiment the primmorphs were inspected regularly with an inverted light microscope (Olympus CK2) and a stereomicroscope (SV11, Zeiss, Jena, Germany). The silicate concentrations that were applied were checked by spectrophotometric analysis. The protocol was adapted from Strickland and Parsons (1972). Only plastic materials were used to prevent release of silicate from glassware. A calibration curve was made from 0 to 100 µM. For each sample or standard, 0.25 mL molybdate solution (13.33 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per L double destilated water (dds)) was pipetted into a tube and subsequently, 0.625 mL sample or standard was added. The samples were mixed and after 10 min, 0.375 mL reduction reagent was added (100 mL reduction reagent: 40 mL ascorbic acid solution (28 g ascorbic acid/L dds); 20 mL oxalic acid (saturated solution in dds); 16.7 mL sulphuric acid (90%); 23.3 mL dds). The samples and standards were

incubated in a 50 ºC water bath for 30 min. Subsequently, the extinction of the standards and samples was measured with a spectrophotometer (Spectronic-20 Genesys, Spectronic Instruments, Rochester, USA) at a wavelength of 810 nm. A standard containing 0 μM silicate was used to calibrate the zero value of the spectrophotometer.

Results and Discussion

The influence of silicate on primmorphs was tested by exposing primmorphs to different silicate concentrations. The primmorphs that were maintained at silicate concentrations of 3.5 and 25 μM showed no growth or morphological changes. After two days, fungi were growing on the surface of five of the primmorphs in seawater containing 3.5 μM silicate. The fungal biomass formed a web surrounding the primmorphs, that remained intact. Within 14 days these primmorphs had turned from orange into black and they were discarded. Some of the primmorphs in ASW containing 25 μM silicate started disintegrating 2 weeks after starting the experiment. The smooth skin had disappeared and morphology had changed in such a way that the primmorphs resembled early-stage primmorphs and some spicules could be observed. Some released cell aggregates from the disintegrating primmorphs were attached to the well, but the rough primmorphs itself did not attach. After 55 days all 6 primmorphs were disintegrated and they were discarded.
Within 14 days after the addition of 70 μM silicate to 6 primmorphs, no changes in outer appearance occurred. However, in the period between 14 and 28 days after the addition of silicate 3 out of the 6 primmorphs attached to the bottom of the well. The spherical shape and the smooth skin of the primmorph disappeared and a more or less 2-dimensional tissue-like attached structure was formed in which the presence of spicules was observed (Fig. 1A). The number of spicules in the tissue seemed to increase during time. They were not organised in the sponge tissue, but distributed randomly, which is also the case in tissue of mature *Suberites domuncula*.
The spicules that were present in the sponge tissue must have been produced by the primmorph and the tissue-like aggregate during the experiment. They cannot originate from the parent sponge that was used to prepare primmorphs, because microscopic analysis had revealed that after gradient centrifugation all spicules from the parent sponge had been removed from the sponge-cell suspension. During the period from 4-6 weeks no changes were observed for the attached sponge tissue-like aggregates in the wells. However, after 6 weeks the size of the tissue-like aggregate started decreasing, while spicules that were present in the exterior parts of the tissue were left behind (Fig. 1B).

This was probably caused by a deficiency of nutrients to maintain the sponge tissue. In the period between 6 and 8 weeks after starting the experiment the 3 smooth primmorphs that had not attached started to disintegrate. This occurred 3 months after the preparation of the primmorphs and might be the result of age of the primmorphs.

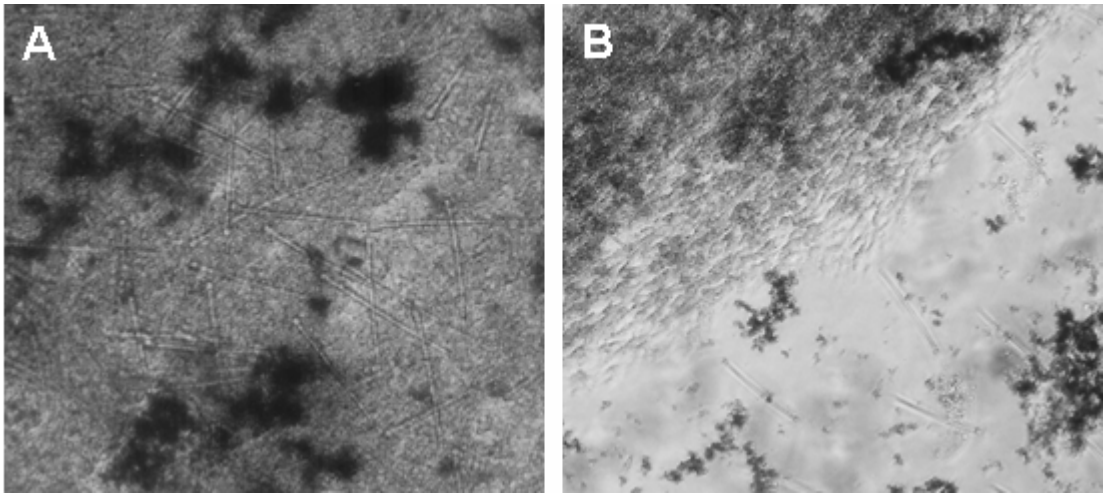

Fig. 1: (A) Part of the tissue of an attached *Suberites domuncula* primmorph containing many spicules, magnification 200x. (B) Spicules that were present in the sponge tissue were left behind when the sponge tissue decreased in size, magnification 100x.

The primmorphs that were incubated in ASW containing 148 μM silicate originated from the same sponge as the primmorphs that were maintained at a silicate concentration of 3.5 μM. Also these primmorphs at 148 μM silicate were covered with a fungal layer within 2 days. However, attachment of primmorphs in the presence of a high silicate concentration was not disturbed. After 6 days, 1 of the 6 primmorphs attached to the bottom of the well and after 8 days 3 primmorphs were attached. Some spicules could be observed, but it was difficult to look into the dense biomass clump. No spreading of the cell mass was observed as in the case with 70 μM silicate. This might be caused by the presence of fungal biomass, which increased clearly during time. The colour of the primmorph changed from orange to black within 14 days, which indicates that the sponge cells were dying.

Although we have not done a quantitative analysis of the number of spicules at different silicate concentrations, it was obvious that more spicules were formed in primmorphs that were incubated in ASW containing 70 μM silicate than at 25 μM silicate. The observation that the number of spicules increased after attachment of the primmorph implies that sclerocytes were active in the attached sponge tissue. It is difficult to compare spicule formation in primmorphs at low and high silicate concentrations due to differences in development and the presence of fungi at the concentrations of 3.5 and 148 μM silicate.

However, these results confirm the general idea that a positive correlation exists between silicate concentration, silicate uptake rate and spiculogenesis (Frølich and Barthel, 1997; Reincke and Barthel, 1997; Mercurio *et al.*, 2000). The enhanced spiculogenesis is explained by the finding that the formation of silicatein increases strongly at concentrations of 60 μM silicate when compared to the formation in the presence of 1 μM silicate (Krasko *et al.*, 2000).

Silicate has also been found to stimulate the biosynthesis of myotrophin, which enhances the production of collagen in *Suberites domuncula* cells *in vitro* (Schröder *et al.*, 2000). It is assumed that myotrophin is released by sclerocytes and causes the formation of collagen-like spongin in exopinacocytes. At a concentration of 60 μM silicate the production of collagen was found to be approximately 700% higher than in seawater containing 1 μM silicate (Krasko *et al.*, 2000). A difference in collagen production in primmorphs in seawater containing a low silicate concentration (3.5 and 25 μM) in comparison to primmorphs in seawater containing a high silicate concentration (70 and 148 μM) is probably the explanation why only primmorphs in seawater containing a high silicate concentration attached to the substratum. It is well known that collagen plays an important role in both the attachment of gemmules to a substratum and their subsequent morphogenesis (Mizoguchi and Watanabe, 1985). The basopinacoderm, which is the first layer of cells to attach to a substratum contains many collagen fibrils (Garonne, 1984). Inhibitors of collagen synthesis such as azetidine-2-carboxylic acid and α,α^1-dipyridyl prevent spreading of the basopinacoderm. At a concentration of 1.0 mM of azetidine-2-carboxylic acid gemmules did not attach to a substratum (Mizoguchi and Watanabe 1985). Shimizu and Yoshizato (1993) discovered that inhibitors of collagen biosynthesis did not affect the reaggregation of dissociated sponge cells, but caused incomplete morphogenesis in the processes of spreading and development. These data combined with our results indicate that the exogenous silicate concentration is a trigger for both attachment and further development of primmorphs (Fig. 2).

Conclusions

Attachment of primmorphs and development of spicules is induced by the presence of high silicate concentrations (> 70 μM) in sea water. This might be the first step in the development of a functional sponge from a primmorph.

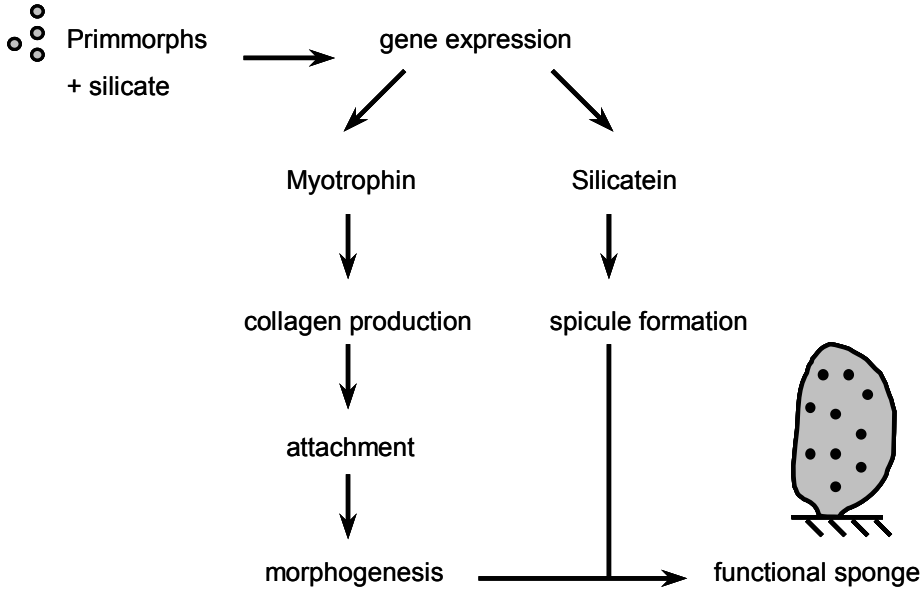

Fig. 2: Postulated formation of a functional sponge triggered by silicate. Modified scheme from Krasko *et al.*, 2000.

6 Sponge-cell culture? A molecular identification method for sponge cells

Abstract

Dissociated sponge cells are easily confused with unicellular organisms. This has been an obstacle in the development of sponge-cell lines. We developed a molecular detection method to identify cells of the sponge *Dysidea avara* in dissociated cell cultures. The 18S rRNA gene from a *Dysidea avara* specimen was sequenced and compared to eukaryotic 18S rDNA sequence(s) that were picked up from a proliferating cell culture that originated from a dissociated *Dysidea avara* specimen. Our method was successful to prove unambiguously, that in this case, the cell culture was no sponge-cell culture. Therefore, it provides a valuable tool for further research on sponge-cell cultures.

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Introduction

Sponges are a very rich source of bioactive compounds (Munro *et al.*, 1999). The presence of cytotoxins (Schmitz, 1994), antifouling agents (Miki *et al.*, 1996), anticancer compounds (Munro *et al.*, 1999), antibiotics and antiviral agents (Munro *et al.*, 1994) has been demonstrated in many different sponges. However, the metabolites of interest are often only produced in trace amounts, which implies that larger quantities of biomass are needed for commercial production of these metabolites, than can be harvested from the sea (Munro *et al.*, 1994; Pomponi and Willoughby, 1994; Ilan *et al.*, 1996; Osinga *et al.*, 1998b). One approach for the production of the required biomass is sponge-cell culture. The most important reason to choose for sponge-cell culture is the possibility to work under well-defined and controlled conditions, which allows optimisation of both growth rate and target product formation. Especially during the last decade, several studies have been performed to establish a primary sponge-cell culture and finally a sponge-cell line (Klautau *et al.*, 1993; Klautau *et al.*, 1994; Pomponi and Willoughby, 1994; Ilan *et al.*, 1996; Pomponi *et al.*, 1997; Rinkevich *et al.*, 1998; Rinkevich, 1999; Nickel *et al.*, 2000; De Rosa *et al.*, 2001). However, so far no immortal sponge-cell line has been obtained.

A major problem with sponge-cell culture is that primary cultures are often contaminated with bacteria, fungi or unicellular eukaryotes. When a primary sponge-cell culture is initiated, a sponge specimen is usually taken from the sea, after which a single cell suspension is obtained by mechanical or chemical dissociation of the sponge (Pomponi *et al.*, 1997; Rinkevich *et al.*, 1998). This means that also bacteria, fungi and protozoa, which are present in and on the sponge, are introduced at the start of the primary sponge-cell culture. Bacteria, and to a lesser extent fungi, can be eliminated when growth media are supplemented with antibiotics. However, these methods are not suitable to eliminate unicellular eukaryotes such as yeast, amoebae, other prokaryotes and fungi. Especially thraustochytrids, a group of eukaryotic heterotrophic protists, which are very common in the marine environment, are frequently occurring contaminants in invertebrate cell cultures (Rinkevich, 1999). A further complicating factor is our inability to discriminate between sponge cells and some contaminants. More than 20 different cell types have been characterised in sponges (Bergquist, 1978; Simpson, 1984), which often show a striking similarity in outer appearance with eukaryotic contaminants. In addition, morphologies of sponge cells *in vitro* culture may differ from those *in vivo* (Rinkevich *et al.*, 1994). This has resulted in publications about sponge-cell cultures (Klautau *et al.*, 1993; Klautau *et al.*, 1994), which were later recognised as protozoa (Custodio *et al.*, 1995). Klautau *et al.* (1994) tested whether their cells were sponge cells by performing isoenzyme analysis for three

enzymes, and concluded that they were indeed culturing sponge cells. This indicates that isoenzyme analysis does not result in 'waterproof' evidence on the origin of the cells. De Rosa *et al.* (2001) observed strong differences in fatty acid and amino acid profiles between the intact sponge and its cell cultures. This suggests either that there are significant differences in metabolism between intact sponges and sponge cells in culture (which can be expected due to different substrates that were available for the intact sponge and the sponge-cell culture) or that the proliferating cells were not sponge cells. Ilan *et al.* (1996) identified their proliferating 'sponge-cell' cultures as thraustochytrids and other publications about sponge-cell growth remain questionable until it has been proven that the growing cells are really sponge cells.

Therefore, it is essential to have the disposal of a method to unambiguously determine the origin of the proliferating cells in sponge-cell culture, as was also suggested by Nickel *et al.* (2000). The work of Lopez *et al.* (2002) and the method that we developed are the first identification methods described for sponge cells that are based on DNA.

We used the sequence of the 18S ribosomal RNA gene as a target for identification of the cells. This method can be applied to all sponges, whilst in this paper we concentrated on the demosponge *Dysidea avara*. We determined the 18S ribosomal rDNA sequence of *Dysidea avara,* and compared it with retrieved sequences of the same gene from a cell culture that was initiated from a suspension of *Dysidea avara* cells.

Materials and Methods

Sponges

Specimens of *Dysidea avara* were collected by SCUBA diving in the Gulf of Naples (Italy). The sponge was dissociated into single cells according to the protocol of Custodio *et al.* (1998). The cells were suspended in filtered natural seawater containing a cocktail of antibiotics (100 mg/L kanamycin (Life Technologies, Paisley, UK), 100 mg/L tylosin (Sigma, Saint Louis (USA), 100 mg/L tetracycline (Merck, Darmstadt, Germany) and 10 mg/L gentamycin (Life Technologies)) (De Rosa *et al.*, 2001). The cells were transported in a 50 mL tube by courier to The Netherlands within 24 – 48 hours. Immediately after arrival, the cells were washed in sterile artificial sea water (33 g/L instant ocean reef crystals (Aquarium Systems, Sarrebourg, France) in demineralised water). A fraction of the cells was pelleted and stored at –80 ºC (for DNA isolation), while the remainder was used for cell culture.

Cell culture

Dissociated cells from a *Dysidea avara* specimen were incubated in artificial sea water (ASW) supplemented with 100 mg/L kanamycin, 100 mg/L tylosin, 100 mg/L tetracycline and 10 mg/L gentamycin (pH 8.2). The ASW was replaced twice during 14 days so that only attached cells remained in culture. Subsequently the cells were harvested by a cell scraper (Sigma) and inoculated in 50% Dulbecco's Modified Eagle Medium (Sigma, product no. D5030) containing 2.47 g/L glucose, 0.292 g/L glutamine, 10 mg/L kanamycin, 10 mg/L tylosin, 10 mg/L tetracycline and 10 mg/L gentamycin (pH 7.6) The osmolarity of the medium was adjusted to the osmolarity of sea water by the addition of NaCl. The medium was replaced every week. The cell cultures were maintained at 18 ºC and they were harvested for genetic identification after 4 weeks.

The procedure that was used to determine the genetic diversity and the genetic origin of the cells in culture is depicted in Fig. 1.

DNA isolation

DNA isolation was performed based on the protocol of Sambrook *et al.* (1989). Approximately 250 mg of the frozen cell material was transferred to a tube containing 500 µl guanidinium buffer (4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.1 M 2-mercaptoethanol, 0.5% sarkosyl). Eight small glass beads (ø = 2.5 mm) were added to the solution and the cells were ground by vortexing the tube for 1 min. Four hundred µL of the ground liquid mixture was transferred to a clean microtube and 200 µL chloroform and 200 µL phenol (equilibrated with TE buffer: 10 mM Tris·Cl, 1 mM EDTA, pH 7.8) were added. The tube was inverted 10 times for mixing, and centrifuged for 15 min at 13,000 x g. Subsequently, the upper aqueous fraction, containing the DNA, was transferred to a clean tube containing 40 µL 3M sodium acetate (pH 4.8) and 1 mL 96% cold ethanol. The tube was inverted 10 times for mixing, and the DNA was precipitated by overnight incubation at -20ºC. The next day, the pellet was obtained by centrifugation (20 min at 13,000 x g), washed with 1 mL 70% cold ethanol, and the DNA was pelleted by centrifugation (10 min at 13,000 x g). Finally, the supernatant was discarded, the pellet air-dried and resuspended in 50 µL double distilled water.

18S ribosomal subunit DNA isolation

The 18S ribosomal subunit gene was amplified from genomic DNA by PCR using universal eukaryotic primers A and B (Collins, 1998). The primer sequences were synthesised by MWG Biotech AG (Ebersberg, Germany) and are listed in Table 1. PCR was performed using a Thermocycler T1 (Whatman Biometra, Göttingen, Germany) with

initial denaturation at 94 ºC for 5 min. This was followed by 35 amplification cycles of 94 ºC for 20 s, 55 ºC for 20 s, 68 ºC for 2 min, and a final extension step at 68 ºC for 5 min. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany).

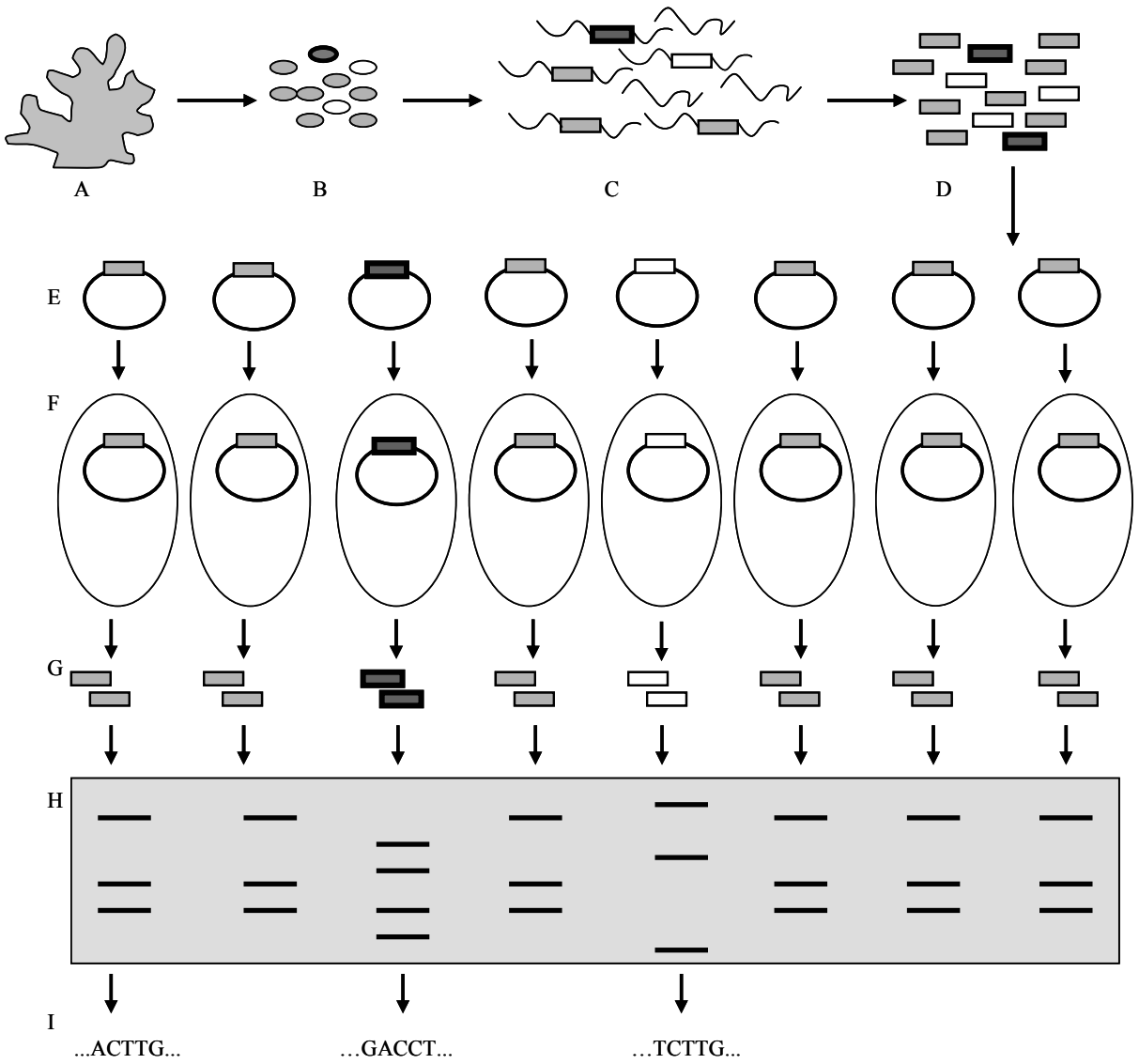

Fig. 1: Procedure for the determination of the genetic diversity and the genetic origin of the cells in culture. A) *Dysidea avara* specimen used to prepare a sponge-cell suspension. B) Cell culture possibly consisting of various eukaryotic cells (different colors represent cells of different origin). C) DNA harvested from the (mixed) cell population. Some chromosomes contained an 18S rRNA gene (▭). D) Eukaryotic 18S rRNA genes were multiplied by PCR. E) The PCR products were cloned into a plasmid and F) introduced in *E. coli*. G) The 18S rDNA from all different clones was purified and multiplied by PCR. H) Restriction analysis on the PCR products was performed and I) from all different restriction patterns one PCR product was partially sequenced.

Cloning of PCR-amplified products

Purified PCR products were cloned into *E. coli* JM109 by using the Promega (Madison, WI, USA) pGEM-T vector system. White colonies, containing the plasmid, were transferred to a microtube containing 20 μL of TE buffer with a sterile toothpick. The cells were lysed by heating them at 95 ºC for 15 min. PCR was performed on the cell-lysates using pGEM-T specific primers T7 and Sp6 (Table 1) to confirm the size of the inserts. The PCR consisted of initial denaturation at 94 ºC for 5 min, and 35 cycles of 94ºC for 30 s, 44 ºC for 30 s, 68 ºC for 90 s, and a final extension for 7 min at 68 ºC.

Restriction analysis

To establish the diversity within the group of clones, amplicons of the correct size were subjected to restriction fragment length polymorphism (RFLP) analysis using the restriction enzyme *CfoI*. Inserts resulting in unique restriction patterns were purified by the QIAprep Spin Miniprep Kit (Qiagen) and were subjected to DNA sequence analysis (Fig. 1).

Sequence analysis

One μg of purified pGEM-T plasmid was used for sequence analysis of the cloned 18S rDNA fragment. Sequencing reactions were performed using the sequenase (T7) sequencing kit (Amersham Life Sciences, Slough, U.K.). In case of the sponge specimen, the whole 18S gene was sequenced using IRD-800 5'-prime end labelled internal primers 1-6, that divide the 18S rRNA gene roughly in thirds in each direction (Collins, 1998). In addition, the 18S rRNA gene was partly sequenced from both ends by using IRD-800 5'-prime labelled T7 and Sp6 primers to confirm the 18S DNA sequence that was obtained with the 6 internal primers. In case of the cell culture, the 18S rRNA gene was only partially sequenced by using IRD-800 5'-prime labelled Sp6 primer. The partial sequence was used to confirm the identity of the cell (*Dysidea avara* or not). Sequences were automatically analysed on a LI-COR DNA Sequencer 4000L (Lincoln, NB, USA), and corrected manually.

Nucleotide sequence accession number

The complete *Dysidea avara* rDNA 18S sequence was deposited with the GenBank database and assigned the accession number: AF456236.

Table 1: Primers used in this study.

Primer name	Primer sequence (5'-3')	Reference
Universal Eukaryotic Primer A	CCG AAT TCG TCG ACA ACC TGG TTG ATC CTG CCA GT	Collins, 1998
Universal Eukaryotic Primer B	CCC GGG ATC CAA GCT TGA TCC TTC TGC AGG TTC ACC TAC	Collins, 1998
Internal primer 1	CTG GTT GAT CCT GCC AG	Collins, 1998
Internal primer 2	GTG CCA GCA GCC GCG G	Collins, 1998
Internal primer 3	GGT GGT GCA TGG CCG	Collins, 1998
Internal primer 4	CTG CAG GTT CAC CTA C	Collins, 1998
Internal primer 5	CGG CCA TGC ACC ACC	Collins, 1998
Internal primer 6	GAA TTA CCG CGG CTG CTG	Collins, 1998
T7	TAA TAC GAC TCA CTA TAG G	Promega
Sp6	GAT TTA GGT GAC ACT ATA G	Promega

Results

Dysidea avara 18S rDNA sequence

From ten *E. coli* transformants that were obtained after transfection with the 18S rDNA that originated from a frozen *Dysidea avara* specimen, only two contained an 1800 bp insert. The 18S rDNA insert from one of these clones (transf.7) was sequenced completely (GenBank accession number: AF456236). The 18S rRNA gene from the other clone (transf.10) was sequenced only partially (400 bp). By sequence comparison it was found that it originated from the same species as transf.7. In addition, identical *CfoI* restriction patterns were obtained for transf.7 and transf. 10 (Fig. 2). Sequence comparison of the complete 18S rRNA gene, using the BLAST program, resulted in the highest similarity with the 18S rRNA genes of the marine sponges *Pleraplysilla spinifera* and *Hippospongia communis* (98 and 97% similarity, respectively).

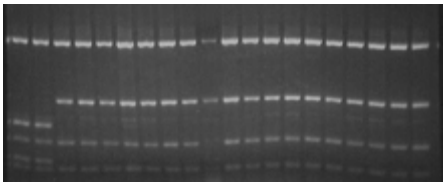

Fig. 2: *CfoI* restriction pattern of the 18S rDNA. From left to right: lane 1 and 2: 18S rRNA gene from *Dysidea avara* specimen (lane 1: transf.7, lane 2: transf.10). Lane 3-20: 18S rRNA gene from 18 different clones derived from cell culture.

Cell culture

Cells that were derived from a dissociated *Dysidea avara* specimen, were cultured for 4 weeks. Initially, after cell dissociation a wide range of cell sizes was observed. However, after cell culture and medium replacement only one cell type remained. These cells were mostly growing in small aggregates, which were very loosely attached to the petri dish

(Fig. 3). They were uniform in their appearance, and the growth rate was approximately 0.13 d^{-1}. Since there is only very little experience with the morphology of proliferating sponge cells *in vitro*, it remained very difficult to confirm by microscope whether these cells were sponge cells or not. Therefore, we compared the inserted 18S rDNA sequences of 18 *E. coli* colonies with the *Dysidea avara* 18S sequence according to the scheme depicted in Fig. 1. From restriction analysis it was concluded that the 18S rDNA from all 18 clones from the cell culture was identical and that it was different from the *Dysidea avara* 18S rDNA (Fig. 2). That implied that our cell culture was no sponge-cell culture, but a cell culture from another eukaryotic organism. The fact that all 18 clones contained the same 18S gene confirmed our microscopic observation that only one cell type was present in our cell culture. The 18S rRNA gene from one of the clones was partially sequenced and sequence comparison with the *Dysidea avara* 18S rRNA gene resulted in a similarity of only 85%. This result proved indisputably that in this case, our cell culture was no sponge-cell culture. In addition, when compared to 18S rDNA sequences deposited in the ribosomal database program, the sequence had 99.4 % similarity to the 18S rRNA gene from the yeast *Candida parapsilosis*.

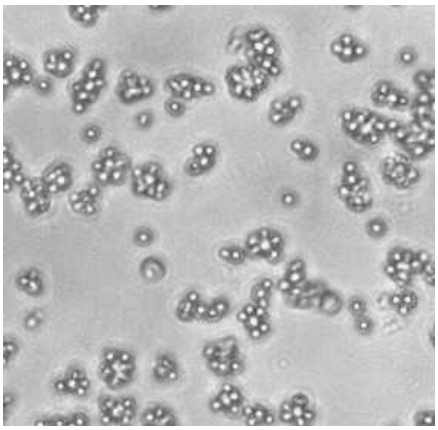

Fig. 3: Cells from the cell culture.

Discussion

Contamination is one of the most prominent concerns in sponge-cell culture (Pomponi and Willoughby, 1994; Ilan *et al.*, 1996; Rinkevich, 1999). Bacterial infections are not a major problem since they can easily be recognised by microscope, and can be inhibited successfully by using a cocktail of antibiotics as suggested by De Rosa *et al.* (2001). However, as long as it is impossible to purify sponge-cell suspensions, contamination of primary sponge-cell cultures by unicellular eukaryotes or fungi is likely to occur. The various possible eukaryotic contaminants cannot easily be eliminated, since sponge cells

also seem to be susceptible to fungicides (Armstrong *et al.*, 1999; Sipkema *et al.*, 2003a). Furthermore, there is the possibility that contamination is introduced during laboratory handling of the cells. Therefore, a clear identification method is essential to verify the origin of proliferating 'sponge-cell' cultures. The method described here, was based on the 18S rRNA gene, which makes it widely applicable for the identification of other sponges of which the 18S rRNA has been sequenced. Furthermore, the availability of PCR primers specific to the eukaryotic 18S rDNA facilitates the detection of possible eukaryotic contaminants. Lopez *et al.* (2002) used amplified fragment length polymorphism (AFLP) to compare fingerprints of the ITS region of *Axinella corrugata* specimens and cell cultures derived from *A. corrugata* cell suspensions. They concluded that total reliance on AFLP patterns for sponge cell verification *in vitro* was not possible. However, a combination of their method based on AFLP patterns with sequence analysis will probably lead to a completely reliable outcome of the test. Therefore, these methods can be regarded as the first tests for sponge-cell identification that result in unambiguous evidence about the origin of the cells in culture. Thus, the origin of all future sponge-cell cultures can be verified, using one of these methods. To simplify our detection method, one could think of the application of quantitative PCR using two sets of primers, one universal eukaryotic set and one sponge-specific set. However, in order to extend this method with the use of sponge-specific primers, one requires more insight into the 18S rDNA sequence variation among sponges and related organisms. Lopez *et al.* (2002) analysed the ITS sequence and discovered a sponge-specific DNA fragment. They successfully detected sponge cells in a cell culture by showing the presence of this DNA fragment. However, this technique can only be applied for the verification of cell cultures in combination with a general genetic marker. This combination is necessary in order to estimate the percentage of sponge cells in culture, since the presence of the sponge-specific fragment can also be confirmed when e.g. only 10% of the cells in culture are sponge cells.

When more specific information on the sponge 18S rRNA sequences becomes available, fluorescent in situ hybridisation (FISH), a method used for a variety of micro-organisms (Amann *et al.*, 1995), would be an option to detect sponge cells and could even be applied for physical separation of sponge cells from other eukaryotic cells. For bacteria, it has been shown that cells can be sorted by flow cytometry based on the fluorescence of a 16S rRNA probe (Snaidr *et al.*, 1999). Purification of sponge-cell suspensions will be the next challenge for setting up a sponge-cell line.

7 The life and death of sponge cells

Abstract

Cell viability is an essential touchstone to study the effect of medium components on cell physiology. We have developed a flow-cytometric assay to determine sponge-cell viability, based on the combined use of fluorescein diacetate (FDA) and propidium iodide (PI). Cell fluorescence measurements based on incubation of cells with FDA or PI, resulted in a good and reproducible estimate of the viability of primary sponge-cell cultures.

We studied the effects of temperature, ammonium and the fungicide amphotericin B on the viability of a primary-cell culture from the marine sponge *Suberites domuncula* using the aforementioned flow-cytometric assay. *S. domuncula* cells die rapidly at a temperature of 22 °C or higher, but they are insensitive to ammonium concentrations up to 25 mM. Amphotericin B, which is frequently used in sponge-cell culture media, was found toxic to *S. domuncula* cells.

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Introduction

Since Bergmann and Feeney (1950; 1951) discovered the first secondary metabolites in marine sponges, thousands of new bioactive compounds have been isolated (MarinLit, 1999; Faulkner, 2000; 2001; 2002). Unfortunately, this rich arsenal of potential drugs against cancer (Munro *et al.*, 1999), viral diseases like AIDS (Tsujii *et al.*, 1988), malaria (Wright *et al.*, 1996) and inflammatory diseases (Cimino *et al.*, 1972) has not yet resulted in commercial available medicines. The limited availability of sponge biomass (Osinga *et al.*, 1999) and the finding that the bioactive compounds are often present in trace amounts have hampered the development of these medicines. For example, the anticancer compound halichondrin B is present in a concentration of only 0.00003 % in *Lissodendoryx* sp. (Munro *et al.*, 1999), and the deep-water sponge *Discodermia dissoluta* contains only 0.002 % of the anticancer compound discodermolide (Ter Haar *et al.*, 1996). In fact, Halichondrin B has successfully passed the first preclinical test phase, but the clinical programme has been arrested until the supply problem is solved (Munro *et al.*, 1999).
Sponges grow very slowly. Therefore, sponge-cell culture in which higher growth rates could be achieved, is an interesting alternative for the production of pharmaceuticals. Moreover, metabolic engineering could direct cell metabolism towards formation of the compound of interest, therewith enhancing the production further. Sponges possess certain features that make them good candidates for developing immortal cell lines. Cells called archeocytes travel through the sponge as single cells *in vivo*. They are totipotent cells that can differentiate into all other sponge-cell types (Simpson, 1984), which makes them comparable to human stem cells. Moreover, sponges have high telomerase activity levels, which might indicate that cell aging due to serial passage *in vitro* is relatively slow (Koziol *et al.*, 1998). However, sponge-cell culture attempts up till now (Klautau *et al.*, 1994; Pomponi and Willoughby, 1994; Ilan *et al.*, 1996; Pomponi *et al.*, 1997; Rinkevich *et al.*, 1998; De Rosa *et al.*, 2001; Richelle-Maurer *et al.*, 2003) have not been very successful mainly for lack of fundamental knowledge about sponge cells in suspension. This is also true for cell cultures from other marine invertebrates (Rinkevich, 1999). Despite the work of numerous groups only primary, and no immortal cell lines have been obtained for oysters, clams (Domart-Coulon *et al.*, 2000), mussels (Odintsova *et al.*, 1999) and tunicates (Kamer and Rinkevich, 2002). Only recently, some promising results with a primary sea urchin cell line were obtained by Bulgakov *et al.* (2002). They succeeded in transferring the transcriptional activator *Gal4* gene from yeast to dissociated embryonic sea urchin cells and obtained considerable cell growth. This approach might also be the key to success for

sponge-cell culture, but some other basic issues deserve attention before genetic modification is attempted.

A method to obtain an axenic sponge-cell suspension, e.g. by the use of sponge-specific antibodies, is a vital tool to launch genetic modification of sponge cells, since sponges are usually not alone but they are inhabited by many bacteria, fungi, and unicellular eukaryotes (Osinga *et al.*, 2001). Some progress in this direction has been made by the development of an unambiguous cellular identification method (Lopez *et al.*, 2002; Sipkema *et al.*, 2003b). In addition to cell purification and identification, monitoring of cell viability is an essential parameter for cell culture. Especially for non-growing cells, which are usually found in sponge-cell cultures, it is important to confirm that they are still viable when cultivation experiments are performed.

Because cell viability is a critical parameter to assess to the success of a cell culture medium, the objective of our research was to find a reliable method to estimate the viability of sponge-cell cultures. Fluorescein diacetate (FDA) and propidium iodide (PI) are commonly used fluorescent molecules to stain life and dead eukaryotic cells, respectively (Widholm, 1972; Pullen *et al.*, 1981). In metabolically active cells, the non-fluorescent FDA molecule is cleaved by esterases into a green fluorescent product, while inactive cells remain non-fluorescent (Dorsey *et al.*, 1989). PI is impermeable to cells with an intact plasma membrane, but complexes with DNA of cells with a damaged membrane, which results in cells with a highly fluorescent nucleus (Vornov *et al.*, 1991).

Yentch and Pomponi (1986) explored the possibilities of flow cytometry to study dissociated sponge cells and described the use of FDA (Yentch and Pomponi, 1994) and PI (Pomponi and Willoughby, 1994). Although the results were promising, this has not led to a commonly used sponge-cell viability assay. In this work, we refined and extended the method to determine sponge-cell viability by the combined use of FDA and PI in such a way that a reproducible assay was acquired. We have used the viability assay to study the effect of a number of relevant parameters on the viability of sponge-cell cultures:

1. Temperature: Sponges are known to be extremely sensitive to temperature shocks (Arndt, 1933; Bachinski *et al.*, 1997), which is especially important to take into account when transporting sponges (Nickel *et al.*, 2001). However, the effect of temperature on sponge-cell viability has not been established.
2. Ammonium: Ammonium, and especially its uncharged form, is toxic to a variety of animal cell lines (Butler and Spier, 1984; Hassell *et al.*, 1991). NH_3 diffuses easily through cell membranes, NH_4^+ much slower, but it can be transported by certain transport proteins (Martinelle and Häggström, 1993). There are strong differences in

sensitivity to ammonium between different animal cells. For example, cell growth of baby hamster kidney cells was reduced by 80% in the presence of 2 mM ammonium, while the growth of Vero cells was unaffected at these concentrations (Hassell *et al.*, 1991). Sponges are exposed to low ammonium concentrations, ranging from 0.5-5 μM, in their natural habitat (Kérouel and Aminot, 1997), but ammonium concentrations in cell cultures may be much higher due to the presence of amino acids in the growth medium.

3. Amphotericin B: Many different antibiotics have been used in sponge-cell culture media (Pomponi and Willoughby, 1994; De Rosa *et al.*, 2001; Sipkema *et al.*, 2003a). Unfortunately, the impact of most antibiotics on sponge cells is unknown, but some antibiotics are thought to be inhibitory or toxic to sponge cells (Sorokin *et al.*, 1993). The use of amphotericin B in sponge cell cultures was reported by Ilan *et al.* (1996) and Willougby and Pomponi (2000). In other publications, amphotericin B was suspected to be harmful to sponge cells (Armstrong *et al.*, 1999; Sipkema *et al.*, 2003a). Therefore, we investigated the effect of amphotericin B on sponge-cell viability.

Materials & Methods

Sponges

Specimens of *Suberites domuncula* were collected by SCUBA diving in the Northern Adriatic near Rovinj (Croatia). They were transported in cool containers at a constant temperature (17 °C), while the water was continuously supplied with oxygen and detoxified with active carbon. The sponges were maintained in aquaria containing artificial sea water (33 g/L instant ocean reef crystals (Aquarium Systems, Sarrebourg, France)) in Wageningen (The Netherlands) at a temperature of approximately 17 ºC.

Media and solutions

Calcium- and magnesium-free seawater (CMFSW) was prepared by dissolving 0.994 g Na_2SO_4, 0.0168 g $NaHCO_3$, 0.746 g KCl, 31.6 g NaCl and 2.42 g Tris in 1 L demineralised water. The pH was adjusted to 8.1 with HCl before filter sterilisation (pore size 0.22 μm).

Ammonium (1, 5 or 25 mM) or amphotericin B (1, 2.5 or 10 μg/mL: Sigma, St. Louis, USA), were added to the CMFSW before pH adjustment. Changes of the ammonium concentration in the culture medium during the experiment were monitored by daily ammonium analysis using the UV-Method (R-Biopharm, Darmstadt, Germany).

A fluorescein diacetate (FDA) stock solution was prepared by dissolving 46 mg FDA (Sigma) in 10 mL acetone (11 μM) and was stored in the dark at –4 ºC. A propidium iodide (PI) stock solution was prepared by dissolving 10 mg PI (Sigma) in 10 mL demineralised water (1.5 μM) and was stored in the dark at +4 ºC.
Two different trypan blue solutions were used: 0.4 % trypan blue (Merck, Darmstadt, Germany) in CMFSW or in a 2.7 % NaCl solution.

Preparation of a sponge-cell suspension
A piece of approximately 0.5 cm^3 sponge tissue was cut with a razor-sharp knife and subsequently submerged in calcium- and magnesium-free seawater (CMFSW). It was divided into smaller pieces of 1 mm^3 and transferred into a conical tube (Greiner, Solingen, Germany) containing 25 mL of CMFSW. The tube was closed and placed horizontally in a shaker (type GFL, Salm en Kipp, Breukelen, The Netherlands) at low speed at 18 ºC. After 2 hours, the supernatant was discarded, while the pieces were squeezed through a 50-μm mesh nylon. The dissociated cells were collected in a tube containing 5 mL CMFSW. The optical density of the cell suspension at 530 nm was measured in a spectrophotometer (Spectronic 20 Genesys, Thermo Spectronic, Cambridge, UK) and the cell concentration was estimated using a Neubauer improved haemocytometer and an inverted light microscope (Olympus CK2). Subsequently, the cell suspension was centrifuged for 10 min at 800 x g, and the pellet was re-suspended in the desired medium to establish a cell concentration of approximately $4{\cdot}10^6$ cells/mL (OD_{530} = 0.03). Of the cell suspensions, 30 mL were added to Petri dishes (ø = 8.5 cm, Greiner) that were subsequently incubated at 16 °C (Snijder Scientific, Tilburg, The Netherlands) under gentle agitation to prevent sedimentation of the cells. When the influence of temperature was studied, cells were slowly accustomed to their incubation temperature (12.5, 16, 22, or 28 °C, temperature change < 1 °C per h). Samples were taken every 2 days in such a way that at even days samples were taken from the Petri dishes with different conditions and at odd days from their duplicates. In the temperature experiment, a number of triplicate samples was taken on days overlapping the other samples.

Cell staining
FDA: One milliliter samples of the cell cultures were incubated with 10 μL FDA stock solution at the experimental temperature. The incubation times that were tested were 5, 10, 15, 30, 45, 60, and 75 min.
PI: One milliliter samples of the cell cultures were incubated with 10 μL PI stock solution. The incubation times that were tested were 1, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120

min. Since short incubation of 5 min was preferable (see Results and Discussion), they were incubated at RT.
Cell fluorescence due to FDA- and PI-staining was measured in a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, USA).
Trypan Blue: Hundred microliter sample was mixed with 100 µL trypan blue solution and they were incubated for 5 min at RT before microscopic analysis of the cells. In case trypan blue-NaCl was used, the cells in the sample were centrifuged (10 min at 800 x g) and dissolved in 2.7 % NaCl before the addition of trypan blue-NaCl.

Viability analysis
The viability, based on both FDA and PI measurements, was calculated according to equation 1:

$$V = \frac{f_v}{f_v + f_d} \times 100\% \qquad [1]$$

in which:
V = viability [%]
f_v = fraction of viable cells based on FDA-staining [-]
f_d = fraction of dead cells based on PI-staining [-]

Changes in the viable cell concentration in cell cultures can be described by a first-order equation:

$$C_{v(t)} = C_{v(0)} \cdot e^{(\mu - k_d)\cdot t} \qquad [2]$$

in which:
$C_{v(t)}$ = viable cell concentration at t = t [cells/mL]
$C_{v(0)}$ = viable cell concentration at t = 0 [cells/mL]
μ = growth rate [d^{-1}]
k_d = death rate [d^{-1}]
t = time [d]

If it is assumed that the total cell concentration, C (= C_v + C_d) is constant ($C_{(0)} = C_{(t)}$), the viable cell concentrations in equation 2 can be substituted by the viability (equation 3). This implies that the cell growth rate is zero and that no cell lysis occurs. The assumption

of a zero growth rate is supported by the current status of sponge-cell culture (especially under starvation conditions). Cell lysis cannot be measured by the use of FDA and PI. However, flow cytometric analysis of samples revealed that the number of cells that passed the nozzle of the flow cytometer per sec. was quite constant during the experiment, indicating that no cell loss occurred.

$$V_{(t)} = V_{(0)} \cdot e^{-k_d \cdot t} \qquad [3]$$

in which:
$V_{(t)}$ = viability at t = t [%]
$V_{(0)}$ = viability at t = 0 [%]

The viability data were used to calculate the death rate (k_d), which is a physiological parameter that characterises the conditions that the cells experience, and is a helpful parameter for designing a cell-culture medium. Values for k_d and $V_{(0)}$ were calculated for the first-order equation [equation 3] by minimising the sum of squares of the equation and the data. The results obtained with the first-order model were compared to results obtained with a zero-order model, and a Weibull model (Van Boekel, 2002). The corrected Akaike criterion was used to compare the three models and decide which model should be preferred.

Results & Discussion

Development of the viability assay

Regular microscopic observation of the cell suspensions in the Petri dishes revealed that most cells were present as single cells or in small aggregates (5-50 cells per aggregate), and only very few cells were attached to the bottom. Aggregates were disintegrated by repeated pipetting of the cell suspension before sampling. In that way, it was possible to inject a homogenous cell suspension into the flow cytometer. The sponge-cell suspensions that were thus obtained, were a mixture of many different sponge-cell types, and consequently, a wide distribution in cell size was found. The differences in scattering due to different cell characteristics complicated flow-cytometric analysis, because often no distinct cell populations could be observed. This influenced the accuracy and the unambiguousness of the results that were obtained. Therefore it is important to define the optimal settings for evaluation of cell characteristics by flow-cytometric analysis (Table 1).

Table 1: Optimised flow cytometer settings to determine the viability of *Suberites domuncula* cells. FSC = forward scatter, SSC = side scatter, Fl 1 = fluorescence detector 1, and Fl 2 = fluorescence detector 2.

	voltage	scale	amplifier	threshold
FSC	E+00	linear	3.00	52
SSC	480	linear	1.00	-
Fl 1	400	linear	1.00	-
Fl 2	776	linear	1.00	-

The flow-cytometer plots were divided into regions to define which particles are cells and which are no sponge cells (FSC-SSC plot), and which are viable or dead (FSC-Fl 1 and FSC-Fl 2 plots. The region containing mainly non-cellular particles was defined by flow-cytometric analysis of the supernatant of a centrifuged (10 min at 800 x g) sample (Fig. 1A). Most of these particles could not be stained with either FDA or PI, which confirmed that they were mainly non-cellular particles. In Fig. 1B, it can be seen that not all sponge cells are included in the viability assay, since also small cells (or bacteria) are located in the region containing debris.

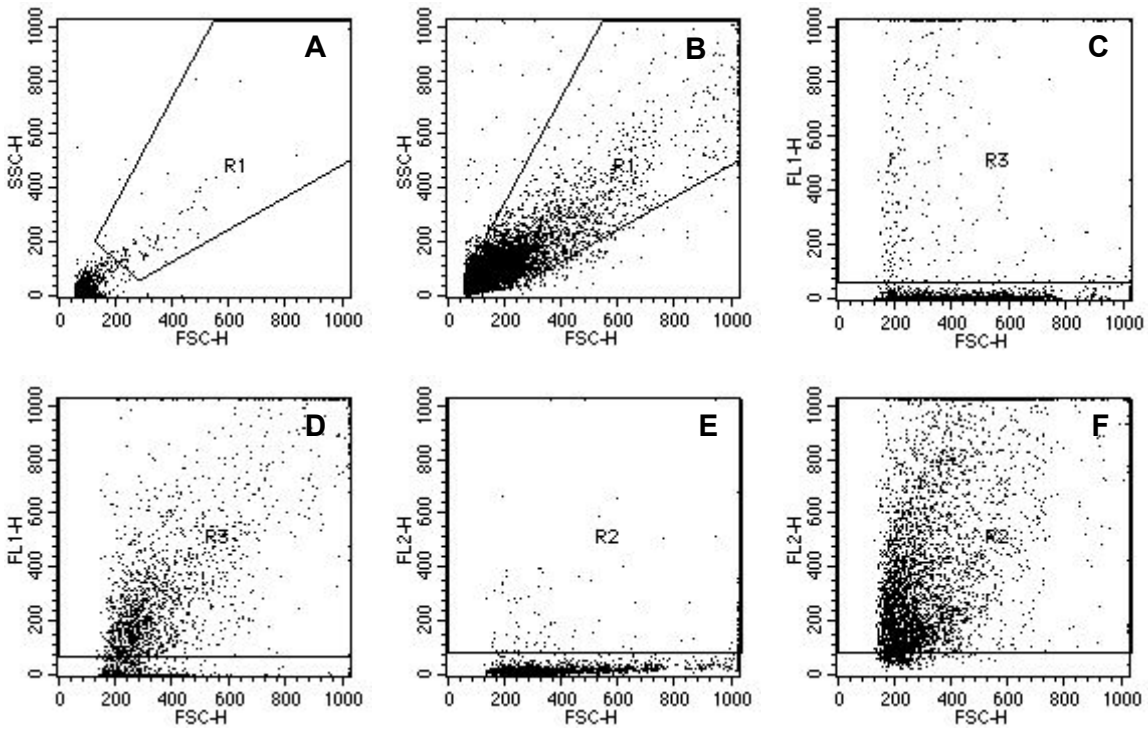

Fig. 1: Optimised settings for the viability assay with dissociated *Suberites domuncula* cells. A: supernatant of centrifuged sponge cells; B: random cell sample; C: sample with mostly dead cells (FDA-stained); D: sample with mostly viable cells (FDA-stained); E: sample with mostly viable cells (PI-stained); F: sample with mostly dead cells (PI-stained). FSC-H = forward scatter, SSC-H = side scatter, FL1-H = fluorescence detector 1, and FL2-H = fluorescence detector 2.

The regions for viable and dead cells were based on a sample with killed cells (incubation for 2 min in 0.1% triton-X-100) and many samples of untreated cells. The regions that were obtained for FDA-stained (Fig 1C and D) and PI-stained cells (Fig. 1E and F) are no absolute borders due to the large variation in scattering caused by different cell types. Therefore, we used fixed gates in the flow-cytometer plots to be able to make a justly comparison between differently treated cell samples and they gave reproducible results.

In principle, it is also possible to locate the position of the different sponge cells in the diagram. De Sutter and Van de Vyver (1977) showed that cells of the fresh-water sponge *Ephydatia fluviatilis* could be separated into three different fractions: an archeocyte-rich fraction, a choanocyte-rich fraction and a pinacocyte-rich fraction. They did not acquire entire separation, but flow-cytometric analysis of separate fractions could give more insight in the viability of different cell types in culture; however, this is beyond the scope of this paper.

The response time of sponge cells to propidium iodide (PI) and fluorescein diacetate (FDA) was unknown, and therefore, we optimised the incubation times of the samples with PI and FDA before flow-cytometric analysis. For PI, it was found that the optimal incubation time was approximately 5 minutes (data not shown). Shorter incubation (1 min) was not sufficient for PI diffusion into dead cells, while incubation for more than 30 min caused cell death, due to PI toxicity. The fraction of FDA-positive cells remained constant after incubation longer than 45 min. (Fig. 2). Therefore, samples were always incubated for 45 min. with FDA before flow-cytometric analysis.

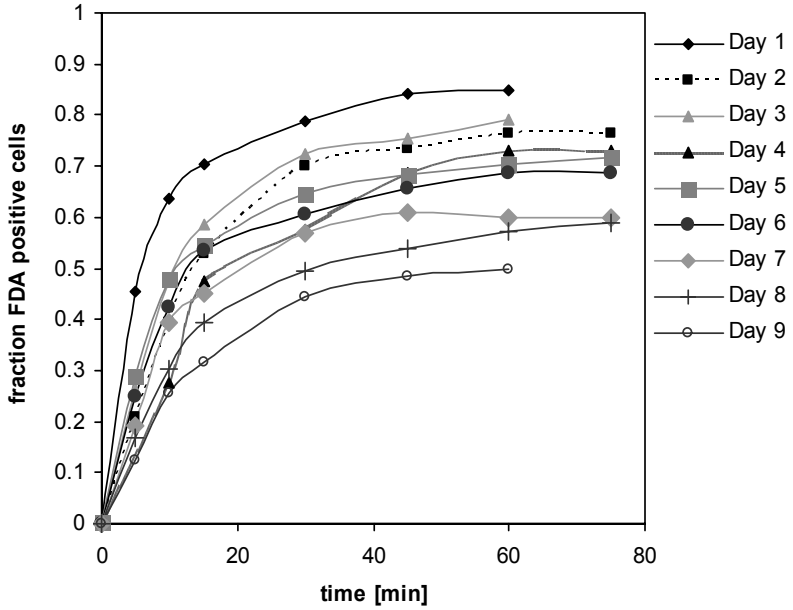

Fig. 2: Relation between incubation time of a sample with FDA and fraction of FDA-positive cells, during nine successive days.

The results that were obtained with samples that were stained with PI or FDA, indicated that they were complementary. When the percentages PI-stained cells and FDA-stained cells were added up, approximately 100 % was obtained and this was a reproducible result. This is illustrated with an example of sponge cells that were incubated for 1 h in phosphate buffered saline (PBS) and a control in which the cells were incubated for 1 h in CMFSW (Fig. 3). It can be seen that most the cells that were incubated in the hypotonic PBS died within 1 h, but that both for cells incubated in PBS and in CMFSW the sum of viable and dead cells is approximately 100 %.

Trypan blue exclusion is another, commonly used, method to estimate the viability in animal cell cultures and it has also been used for primary sponge-cell cultures (De Sutter and Van de Vyver, 1977; Rinkevich *et al.*, 1998; Pomponi and Willoughby, 1994; De Rosa *et al.*, 2003). However, most researchers share the opinion that trypan blue is not a reliable marker to measure sponge-cell viability (pers. com. W.E.G. Müller and S.A. Pomponi). This may be because microscopic analysis of cells that are stained with trypan blue is obscured by the presence of the many different cell types in sponge cultures leading to large measurement errors. It is our impression that trypan blue diffusion into dead sponge cells is disturbed. When sponge cells were exposed to severe conditions (incubation for 2 min. in 0.1 % triton X-100 or 10 % ethanol, or microwave heating for 1 min) we did not observe clearly blue-coloured cells after the addition of trypan blue. From the authors that describe the use of trypan blue, only De Sutter and Van de Vyver (1977) and Rinkevich *et al.* (1998) show quantitative viability results, while Pomponi and Willoughby (1994) and De Rosa *et al.* (2003) only mention that they use trypan blue to estimate viability. The results of Sutter and Van de Vyver differ from the other publications since they use fresh-water sponges instead of marine species. Therefore, for the cells investigated here, we regard estimation of sponge-cell viability by means of FDA and PI the only reliable method.

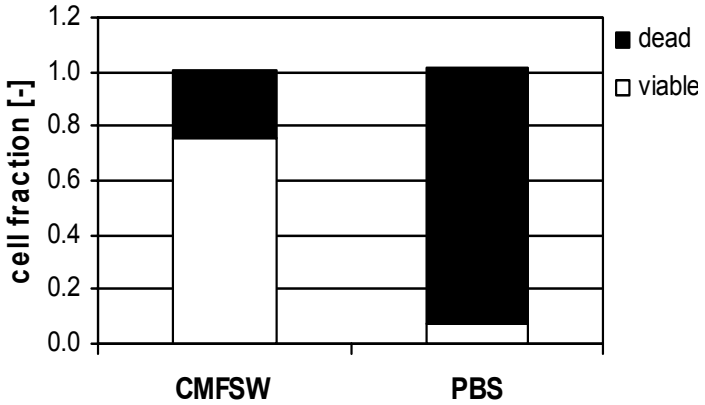

Fig. 3: Fractions viable and dead cells in CMFSW and PBS. Cells that were kept in CMFSW, were centrifuged and dissolved in CMFSW or phosphate buffered saline (PBS). Viability was measured after 1 h. The standard deviations of the viable and dead cell fractions are not displayed in the graph, as they were all less than 0.5% of the corresponding viable and dead cell fractions.

Temperature

Dissociated sponge-cell suspensions were kept at 12.5, 16, 22, and 28 ºC for 11 days. The incubation temperature strongly affects the viability of dissociated sponge cells in culture (Fig. 4). At 12.5 and 16 °C, the viability of the culture decreased steadily and in a comparable way. A steady decrease of the viability was expected, as no nutrients were added to the seawater to diminish the influence of contaminants. At 22 °C and, the viability of the cells decreased more rapidly. This effect was even clearer for 28 °C. The viability measurements for the cultures at 28 °C were stopped after 5 days, as bacterial growth disturbed the analysis. Microscopic analysis showed that a few bacteria were present in the Petri dishes at all temperatures that were tested, but significant multiplication only occurred at 28 °C. It is difficult to distinguish between temperature and bacterial effects at 28 °C. The quick initial decline in viability at 28 °C (when only a few bacteria were present) could indicate that the rapid cell death is mainly caused by the temperature.

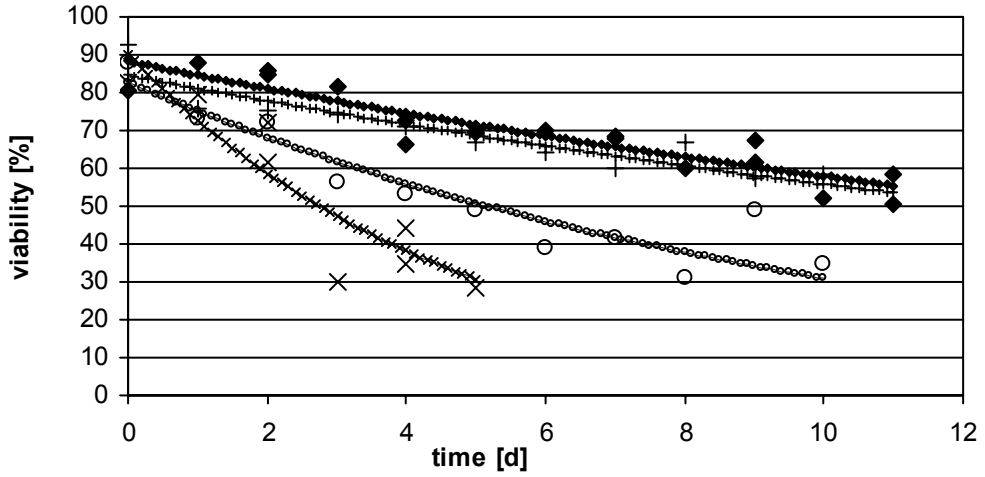

Fig. 4: The viability of dissociated sponge cells at different temperatures (♦: 12.5 °C; +: 16 °C; ○: 22 °C; x: 28 °C and their respective 1st-order kinetic models ♦♦♦♦, ++++, oooo and xxxx). Data from duplicate or triplicate experiments were pooled.

The viability data were fitted to the first-order, zero-order, and Weibull model. By means of the Akaike criterion, the different models were compared and it was concluded that there was no significant difference between the models (-60.9, -60.8, and -61.8 were the Akaike values for the first-order, zero-order and Weibull model respectively) and that all models accurately describe the data. It was decided to use the first-order model, because it is based on an equation with physiological significance. The death rates (k_d) were calculated at different temperatures (Fig. 5). In case of proliferating sponge-cell lines, μ-k_d (equation 2) is the parameter that should be optimised for maximum biomass increase. With the current status of sponge-cell culture, it is not yet possible to establish the

optimal conditions for a high growth rate, but our results clearly indicate that *Suberites domuncula* cell cultures should be kept at a temperature lower than 22 °C to minimise the death rate. The low death rates at 12.5 and 16 °C seem to correspond with the temperatures *Suberites domuncula* experiences in its natural environment. At a depth of 30 m the annual temperature in the Mediterranean varies between approximately 9 and 19 °C (Vatova, 1928). At 22 °C and especially 28 °C there is a strong increase in the death rate, which indicates that sponge cells are very sensitive to higher temperatures. A possible explanation is found in the work of Bachinski *et al.* (1997) who discovered that *Suberites domuncula* pieces failed to increase, and even decreased their intracellular trehalose level when they were exposed to thermal stress. In plants, yeasts and invertebrates, trehalose is a well-known protectant against anhydrobiosis (Panek, 1995) and temperature shocks (Eleutherio *et al.*, 1993). *Saccharomyces cerevisiae* cells that accumulated trehalose during a thermal shock were able to survive heat stress, while a mutant *S. cerevisiae* strain, which was unable to synthesise trehalose, could not cope with temperature stress (Coutinho *et al.*, 1988).

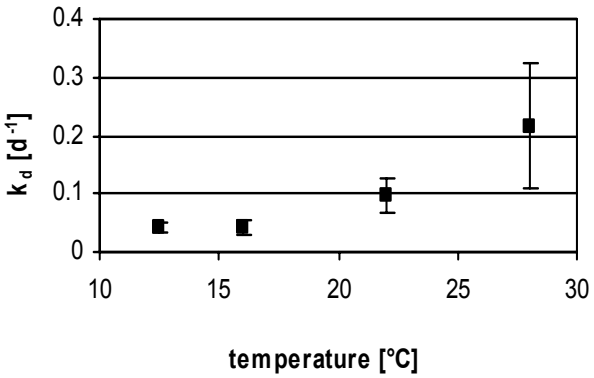

Fig. 5: Sponge-cell death rates between 12.5 and 28 °C with their 95 % confidence interval.

Ammonium

No significant differences were found between cells in the absence or presence of ammonium (Fig. 6), which indicates that ammonia does not affect the viability sponge cells in the concentration range that was investigated here. This does not imply that sponge cells are insensitive to ammonia because the concentration at which cell metabolism is disturbed, is generally lower than the lethal concentration (McQueen and Bailey, 1991). Since we did not perform measurements on cell metabolism, this possibility cannot be excluded. However, for other cell types, considerably lower ammonium concentrations are reported to have lethal effects. For mouse hybridoma cells, lethal ammonium doses of 5-8 mM have been found (McQueen and Bailey, 1991); concentrations of only 0.06 mM and 0.19 mM lead to death of marine invertebrates like the clam *Musculium transversum* and the shrimp *Penaeus chinensis* respectively (Richardson, 1993).

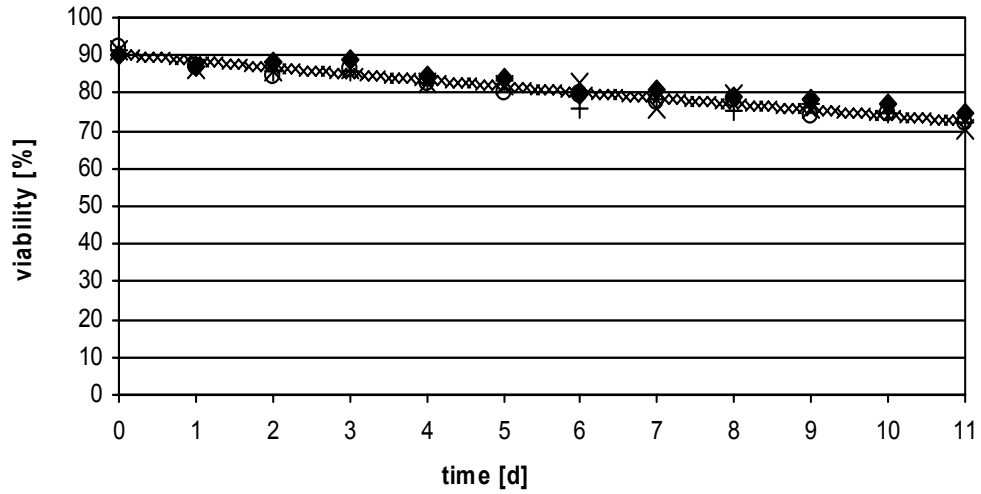

Fig. 6: The viability of dissociated sponge cells at different ammonia concentrations (♦: 0 mM; +: 1 mM; ○: 5 mM; x: 25 mM. For the clarity of the graph, only the 1st-order kinetic model for the concentration of 25 mM is shown (xxxx). Data from duplicate experiments were pooled.

It was tested whether ammonia vaporised during the experiments. A relative large fraction of the ammonium is present in its volatile form due to the high pH of seawater (Bower and Bidwell, 1978) in comparison to most animal cell culture media. Therefore, the total ammonium concentration (NH_3 + NH_4^+) was monitored during the experiment and this confirmed that the set ammonium concentrations were actually reached (data not shown). Some evaporisation occurred in the dishes that were supposed to contain 25 mM ammonium, but still the average concentration during the experiment was 25 mM, as the initial concentration was 34 mM and at day 11 it was 15 mM.

When comparing the ammonium experiments with the temperature experiment at 16 °C, the death rates seem to differ. The sponge cell death rate was not significantly affected by the ammonium concentration (0.017, 0.02, 0.02 and 0.02 d^{-1} for 0, 1, 5, and 25 mM ammonium respectively). These death rates are lower than the death rate that was found in the temperature experiment at 16 °C (k_d = 0.042 d^{-1}). This is most probably due to biological variation of the different cell 'batches' that were used for the temperature and the ammonium experiments. The 'fitness' of the sponges that were used to prepare a cell suspension may have been different. This in combination with the dissociation protocol, which is quite a harsh treatment for the cells (e.g. squeezing the cells through the 50 µm mesh), may have affected the initial cell 'fitness'. Due to the biological variation that is noticed in our experiments, the k_d values that are presented in this work should not be considered as absolute values, but rather as appropriate estimates to compare the results obtained from one cell batch. Therefore, it is not attempted to compare k_d values between the temperature, ammonium, and amphotericin B experiments.

Amphotericin B

The viability was based solely on FDA measurements, which showed that amphotericin B has a detrimental effect on sponge-cell viability (Fig. 7). Quantification of the dead cell fraction by means of propidium iodide was not successful because all cells displayed red fluorescence in the presence of amphotericin, which may be caused by auto fluorescence of amphotericin. The first viability measurement was performed after 2 hours, and a strong decrease of the viability was found. High amphotericin doses (0, 1, 2.5 or 10 mg/L) correlated with more dead cells at 2 hours. The viability of cells exposed to 10 mg/L decreased within 2 hours from 95 % to 67 %, while at concentrations of 1 and 2.5 mg/L the effect was less pronounced. It was assumed that at $t = 0$, the viability was equal for all cultures since they were derived from the same cell suspension. This result might indicate that cells that were already damaged by the dissociation procedure are more sensitive to amphotericin and die instantly.

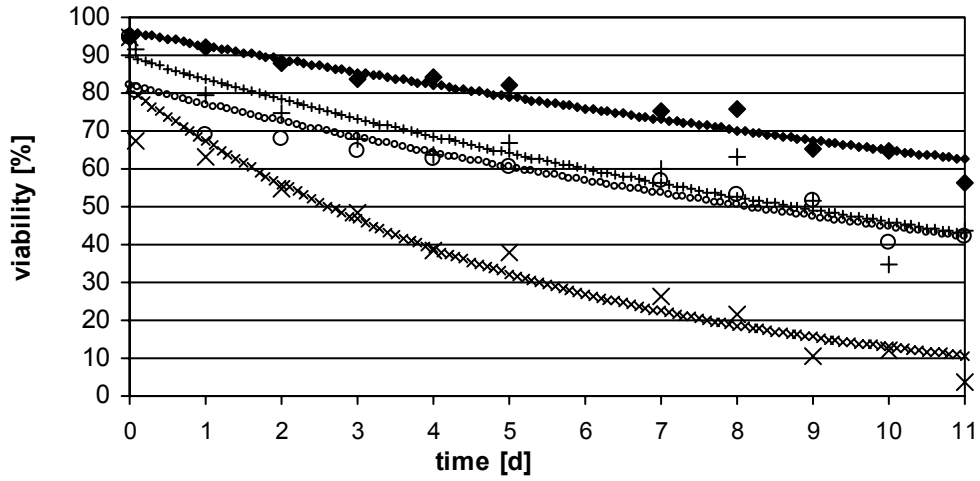

Fig. 7: The viability of dissociated sponge cells at different amphotericin B concentrations (♦: 0 mg/l; +: 1 mg/l; ○: 2.5 mg/l; x: 10 mg/l and their respective 1st-order kinetic models ♦♦♦♦, ++++, oooo and xxxx). Data from duplicate experiments were pooled.

The death rates that were calculated for the tested amphotericin concentrations clearly expressed that amphotericin affects sponge cells (Fig. 8). The death rate rises more than 50 % in the presence of 1 or 2.5 mg/L, when compared to cells incubated without amphotericin. This suggests that the amphotericin concentration that is advised for use by the manufacturer (2.5 mg/L), and which has been used by researchers (Ilan *et al.*, 1996; Willougby and Pomponi, 2000) is already harmful for *Suberites domuncula* cells. The effect is even stronger at an amphotericin concentration of 10 mg/L.

Amphotericin B is a fungicide that disrupts the fungal cell membrane by binding to sterols, preferentially to the primary fungal and yeast cell membrane sterol, ergosterol

(Terrell and Hughes, 1992). Amphotericin B also interacts, to a lesser extent, with cholesterol, the major sterol of mammalian cells and is therefore toxic to animal cell cultures at high concentrations (Medoff *et al.*, 1983). However, its toxic effect on sponge cells, already at low concentrations, is probably explained by the presence of ergosterols in sponge cell membranes. Ergosterol was found to be the major sterol in the cell membrane of the marine sponges *Dysidea avara*, *Ircinia pipetta* (Sica *et al.*, 1987) and *Spongionella gracilis* (Sica and Piccialli, 1985) and was present in *Tethya aurantia* (Sheikh and Djerassi, 1974). It remains to be demonstrated whether ergosterols are present in the membranes of all sponge species. Thus, amphotericin B might be applicable to cell culture of certain sponges, which do not have ergosterols in their membranes, but fungicides with a different mode of action are probably more suitable. This implies that nystatin, another commonly used fungicide which is a structural analog of amphotericin B, is probably also toxic to sponge cells (Hahn *et al.*, 1996). However, also other fungicides as SPA-S-843 (Pessina *et al.*, 1999) and hexachlorobenzene (Salmon *et al.*, 2002) have been found to be toxic to eukaryotic cell cultures and may affect sponge-cell viability. Therefore one should carefully assess the effects of fungicides on cell viability before their application in sponge-cell culture media.

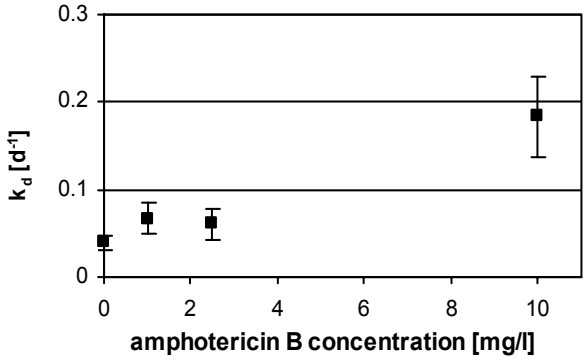

Fig. 8: Sponge-cell death rates for 0, 1, 2.5 and 10 mg amphotericin per liter with their 95 % confidence interval.

8 Large-scale production of pharmaceuticals by marine sponges: sea, cell or synthesis?

Abstract

Marine sponges are known to produce an overwhelming array of secondary metabolites with pharmaceutical potential. The technical and economical potential of using marine sponges for large-scale production of these compounds were assessed for two cases: the anticancer molecule halichondrin B from a *Lissodendoryx* sp., and avarol, which has been patented for antipsoriasis activity, from *Dysidea avara*. An economic and technical analysis was done for three potential production methods: mariculture, *ex situ* culture, and cell culture. We concluded that avarol production by mariculture or *ex situ* culture could become a viable alternative to currently used pharmaceuticals to treat psoriasis. Production of halichondrin B from sponge biomass is not a feasible process, mainly due to its extremely low concentration inside the sponge. The technical feasibility was analysed for five alternatives: chemical synthesis, wild harvest, using primmorphs, genetic modification and semi-synthesis. The latter two approaches may prove to be valuable methods for the production of pharmaceuticals, which are based on the chemical structures of those secondary metabolites that are present in trace amounts in marine sponges.

This chapter has been submitted for publication as: Detmer Sipkema, Ronald Osinga, Wolfgang Schatton, Johannes Tramper, René H. Wijffels. Large-scale production of pharmaceuticals by marine sponges: sea, cell or synthesis.

Introduction

Marine sponges have been called a promise for the development of new medicines since the discovery of antiviral and antileukaemic sponge nucleosides by Bergmann and Feeney in the fifties of the last century (Bergmann and Feeney, 1950; 1951). Thousands of sponge-derived bioactive compounds have been discovered since then (MarinLit, 1999; Faulkner 2000; 2001; 2002) and increased the aura of sponges as high potentials. Talents however, have to prove their real value at a certain moment. Regarding bioactive products from sponges, a number of products has entered preclinical and clinical trials (Table 1), but only Ara-A and Ara-C, which are derivatives of the sponge nucleosides that were discovered by Bergmann and Feeney, have made it to the pharmaceutical market as antiviral and antileukaemic agents, respectively (McConnell *et al.*, 1994). If the time between discovery of the compound and its entrance on the pharmaceutical market would only be a matter of time, it could be expected that hundreds of sponge-derived pharmaceuticals would be commercially available within the next decades. However, this is not likely to occur in large numbers, because the medical effect of a compound is not the only parameter that determines its success. Large-scale availability of the functional secondary metabolites, at a preferably low price, will also determine if companies will even consider compounds to enter preclinical and clinical trials. If the latter condition is not met, natural products from other sources or chemically synthesised molecules with comparable characteristics will shut out bioactive compounds from sponges. Expensive or scarcely available secondary metabolites would only stand a chance if they have a unique pharmacological profile. Therefore, most metabolites from sponges that made it to production or clinical trials were those that could be efficiently chemically synthesised (Table 1).

In this paper we have analysed the status and technical and economical potential of different methods for the production of sponge metabolites. Methods that have been taken into account are:

- Chemical synthesis
- Wild harvest
- Mariculture
- *Ex situ* culture
- Primmorphs
- Cell culture
- Genetic modification
- Semi-synthesis

Table 1: Sponge-derived compounds in production and clinical or preclinical trials. Extracted from Newman and Cragg (2004).

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
Isohomohali-chondrin B[2]	*Lissodendoryx* sp.	anticancer	preclinical	aquaculture	Pharmamar
Laulimalide	*Cacospongia mycofijiensis*	anticancer	preclinical	chemical synthesis	
Peloruside A	*Mycale hentscheli*	anticancer	preclinical		
Salicylhalimide A and B	*Haliclona* sp.	anticancer	preclinical		
Neoamphime-dine	*Xestospongia* sp.	anticancer	preclinical		
Manoalides	*Luffariella variabilis*	antiinflam-matory (psoriasis)	withdrawn from phase II	wild harvest	Allergan
IPL-576092 (= derivative of contignasterol	*Petrosia contignata*	antiinflam-matory (asthma)	phase II		Inflazyme & Adventis
Hemiasterlins A & B	*Siphonochali-na* sp.	anticancer	phase I		Wyeth-Ayerst

[1]Pomponi, 1999; [2]Munro *et al.*, 1999.

Some methods, such as mariculture and chemical synthesis have received considerable attention to assess 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. Finally, we have compared the economical aspects of the production of sponge products with currently used drugs with a similar activity spectrum. This was done by estimation of the production costs and including a profit of 10 % on top of the production costs.

Halichondrin B and Avarol

Halichondrin B and avarol (Fig. 1) are chosen as model products for our study, as quite a lot of effort has been put in the production of these compounds for the pharmaceutical market. With respect to the natural product concentration halichondrin B and avarol are on opposite sides of the range. A deep-water species *Lissodendoryx* sp. that is found near New Zealand contains typically 400 μg halichondrin B per kg wet weight of sponge (Munro *et al.*, 1999), while *Halichondria okadai*, from which halichondrin was first isolated (Hirata and Uemura, 1986), *Raspalia agminata*, *Phakellia carteri* and an *Axinella* sp. from Palau contain even lower concentrations (Hart *et al.*, 2000). Avarol has been isolated from only one sponge. The abundant Mediterranean sponge *Dysidea avara* is the producer of avarol in quantities up to 3 g per kg sponge wet weight (Müller *et al.*, 1985).

Halichondrin B has a strong antitumour effect, in particular for melanomas and leukaemia. Halichondrin B treated mice with B-16 melanoma or P388 leukaemia showed a 300% increase in life expectancy compared to the control group (Hirata and Uemura, 1986). A chemically synthesised halichondrin B analogue is currently in phase I clinical trials by the Japanese company Eisai in conjunction with the American National Cancer Institute (Newmann and Cragg, 2004). Isohomohalichondrin B that has been obtained from wild harvest and mariculture is being developed by the Spanish company PharmaMar. If either halichondrin B or isohomohalichondrin B would successfully proceed through clinical trials an amount of approximately 5 kg pure product would be needed annually to treat 40.000 melanoma patients, based on the potency of halichondrin B in animals *in vivo* (Munro *et al.*, 1999). If we assume that a medicine from halichondrin B would be used by approximately 25 % of the diagnosed melanoma patients world-wide (~16,000 out of approximately 65,000 annual cases (Globocan, 2000)), approximately 2.8 kg halichondrin would have to be obtained.

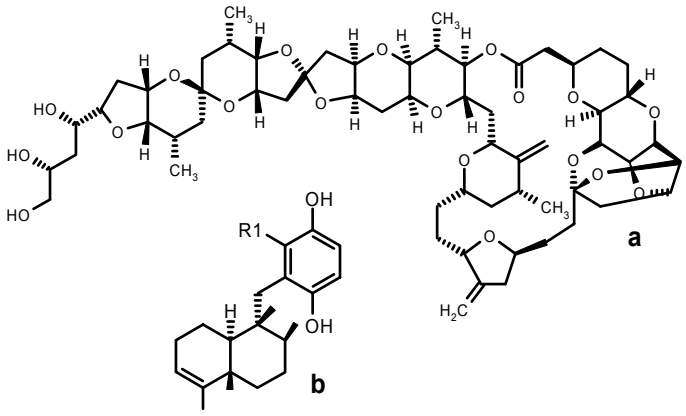

Fig. 1: Molecular structures of halichondrin B (a) and avarol (b).

Avarol was withdrawn from clinical trials although it displayed a strong inhibition of HIV (Müller and Schröder, 1991). It was also patented as an antipsoriatic compound (Müller *et al.*, 1991) and the German company Klinipharm currently uses it in a paramedic medicine against psoriasis and a small mariculture has been sufficient for small-scale production of the ointment up to now (~ 100 g avarol/year). The number of psoriasis patients in Europe and North-America can be estimated at 15 million (Najarian and Gottlieb, 2003). If avarol would reach a market share for therapeutic treatment of 10 % of all psoriasis patients in Europe and North-America, this would lead to a demand for approximately 150 kg avarol on an annual basis (it is assumed that the required avarol dose for therapeutic treatment is ten times higher than for paramedic treatment. This is based on clinical studies in psoriatic patients as well as regulatory considerations).
The estimated market shares of 25 and 10 % for halichondrin B and avarol respectively, are very optimistic. However, the aim of this paper is to assess the requirements and potency of sponge-derived medicines as a blockbuster. The estimated market share of halichondrin B is higher than the market share of avarol, because the competition with other drugs is lower for treatment of melanomas than for treatment of psoriasis.

Chemical synthesis

Chemical synthesis would be the preferred method 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 (reviewed by Aiello *et al.*, 1999; Faulkner 2000; 2001). However, many interesting 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 commercial example from sponges (Table 1), but for most more complex marine natural products, that are rich in centres of asymmetry, no economically feasible strategies exist (Proksch *et al.*, 2002).

Halichondrin B
Halichondrin B has been successfully synthesised by Kishi and co-workers (reviewed in Norcross and Paterson, 1995) from relatively simple molecules as ascorbic acid and L-mannonic γ-lactone. However, total synthesis requires no less than 100 chemical reactions and the overall yield is well below 1%. Generally, it is assumed that 30 steps is the

maximum for an economically feasible process (pers. com. D. Mendola; D.J. Newman). Therefore, large-scale chemical synthesis of halichondrin B is not feasible. One possible way to pursue with chemical synthesis is the production of smaller (and more simple) analogues of halichondrin B. This has been accomplished by scientists of Eisai and is used for the production of the compound for clinical trials. However, at the moment the synthesis of bioactive analogues requires still more than 30 steps (Wang *et al.*, 2000) and therefore large-scale production of halichondrin B or its analogues via chemical synthesis is regarded as not feasible at the moment.

Avarol

Total chemical synthesis of avarol has been accomplished by a number of groups (Sarma and Chattopadhyay, 1982; An and Wiemer, 1996; Locke and Hecht, 1996; Ling *et al.*, 1999). If 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 Ling *et al.* (1999), avarol can be chemically synthesised in 20 steps with a maximum overall yield of 2%. However, one of the substrates that is required for this synthesis, L-proline pyrrolidon amide, will still have to be synthesised. Other substrates that are required are quite complex (and expensive > 2.5-6 €/g (Aldrich, 2003)) molecules but they can be purchased: 2-sulfanyl pyridine N-oxide, 2-methyl-1,3-cyclohexanedione, ethyl vinyl ketone and the more simple compound 1,4 benzoquinone. The combination of a number of expensive substrates, a low yield and more than 20 reaction steps made us decide not to assess the feasibility of chemical synthesis of avarol at this moment.

Wild harvest

Wild harvest of sponge that is the bearer of the desired metabolites would be an easy start to acquire some material of a sponge-derived compound for preclinical and clinical trials. One only needs SCUBA diving equipment and a small boat for a first harvest, and no hassling with investments of setting-up the cultivation of an organism that has been notoriously difficult to be cultured (Osinga *et al.*, 1999). Several drug-leads have proceeded up till clinical phase I using material from wild harvest, for instance manoalide from the sponge *Luffariella variabilis* (Kernan *et al.*, 1987) and bryostatin 1, from the bryozoan *Bugula neritina* (Schaufelberger *et al.*, 1991).

However, the required amount of 2.8 kg of halichondrin B to treat 25% of all melanoma patients would result in a demand of 7000 tonnes of *Lissodendoryx* sp. (containing 400 μg halichondrin B/kg wet weight) and this is simply not available. *Lissodendoryx* sp. is a rare sponge and Dumdei and colleagues (1998) estimated that only 300 tonnes of this species exists in nature.

Things look a little brighter for avarol. If we assume that wild *Dysidea avara* specimens contain on average 2 g avarol per kg wet weight, a total amount of 75 tonnes of sponge would be needed every year to sustain the market demand. *D. avara* is an abundant Mediterranean sponge and the natural sponge population has been large enough to collect enough material for clinical trials. However, annual collection of 75 tonnes of *D. avara* without disturbing the ecological balance in the sea sounds less feasible. It would require a huge number of different spots for the harvest of reasonable amounts of *D. avara* and a nomadic approach of allowing the used spots to recover for a number of years before harvesting again. The logistics and environmental issues that are related to this method to obtain the resources for the production of pharmaceuticals are regarded as too massive to proceed this way.

Mariculture

The term mariculture stands for agriculture in the sea. Many sponge species have been tried for farming in the sea. Already early 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). The discovery of potential medicines in sponges that were merely beautiful in former days, renewed the interest in mariculture of sponges. The extraordinary almost plant-like regeneration characteristics of sponges (reviewed in Simpson, 1984) make them very suitable for multiplication and transplantation.

A big advantage of cultivation in the sea is that there is enough of it. However, it is important to find the right spot and equipment in the sea to start-up a mariculture. 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; Turon *et al.*, 1998; Battershill *et al.*, 2002; Van Treeck *et al.*, 2003; Duckworth and Battershill, 2003c). These findings lead to sometimes conflicting considerations for setting-up a sponge mariculture. In general, sponges grow best at exposed places, where the current is strong enough to supply a sufficient amount of food

(Duckworth *et al.*, 1997; Duckworth and Battershill, 2003c), but exposed places are often more vulnerable to storms that can cause severe damage to maricultures. The difficulties in controlling the environment after selection of a suitable spot are a drawback of mariculture. Growth of sponges is often strongly correlated with a certain season (Turon *et al.*, 1998; Duckworth and Battershill, 2003c) and is thus suboptimal during most time of the year. Furthermore, nutrient supply and disease control are difficult to attain in the sea. However, the undefinedness of the sea may be its success as long as it is not really known which defined conditions are best for the sponge. 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, 2003b).

Analysis of the method

A number of assumptions has been made for the evaluation of the feasibility of mariculture:

1. Mass mariculture could lead to nutrient depletion of the surrounding seawater if consumption by the sponges exceeds the plankton growth and refreshment rate of the 'local' seawater. For the mariculture, we assume that a distance of 2.5 m between the lines carrying the sponges is far enough to prevent nutrient depletion of the water.
2. Many different mariculture systems could be 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 (Fig. 2).
3. The cultivated *Lissodendoryx* sp. display growth during one half of the year and remain constant in size during the other half (Dumdei *et al.*, 1998). For *D. avara*, we assumed growth during the whole year as our data do not indicate that growth is related to a certain season, although many sponges in temperate waters display seasonal growth (Turon *et al.*, 1998; Duckworth and Battershill, 2003b).
4. The mortality (% of sponges that dies during the cultivation) for both species in maricultures is estimated to be approximately 50 % (Munro *et al.*, 1999).
5. The product concentration in the cultured sponges equals the concentration in wild specimens. The first aquaculture trials with *Lissodendoryx* sp. resulted in total halichondrin concentrations that were significantly lower (25-50 % of the concentration in wild sponges), but it is assumed that it is possible to reach concentrations comparable to wild sponges (Dumdei *et al.*, 1998).

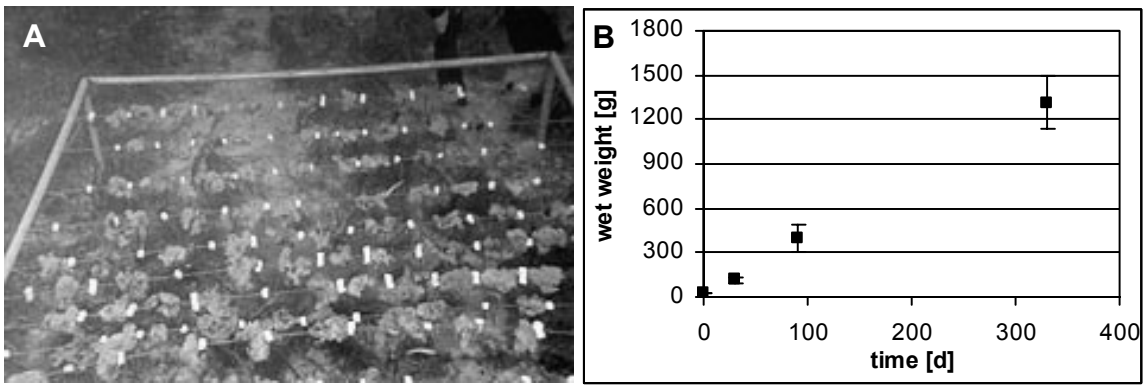

Fig. 2: Mariculture of *D. avara* specimens in the Mediterranean Sea by Klinipharm GmbH. Sponges are separated by corks (A). The mariculture was started in April and growth was measured by underwater photography. The diameter of the sponges was used to calculate the volume (of a spherical sponge) and 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).

Halichondrin B

Much work on mariculture has been done by research groups in New Zealand (Battershill and Page, 1996; Dumdei *et al.*, 1998; Munro *et al.*, 1999). Maximum growth rates up to 5000 % in one month have been found for cultured *Lissodendoryx* sp. explants (Dumdei *et al.*, 1998), but average growth in the bag-type of culture, which proved to be the best culture system, was from 8 cm^3 (~8.3 g wet weight) to 52 cm^3 in 80 days (Munro *et al.*, 1999). When radial accretive growth is assumed (Sipkema *et al.*, submitted), extrapolation of these results to a half year period leads to a sponge volume of 199 cm^3 (~207 g wet weight). This implies that each explant that survives has reached approximately 25 times its initial weight within half a year. Thus, it can be calculated that a number of 73.6 million explants is needed to produce 7,000 tonnes of *Lissodendoryx* sp. in case of a mortality of 50% (Table 2). A hundred meter long-line can bear around 2.5 tonnes of sponge (Dumdei *et al.*, 1998), which would lead to a requirement of approximately 3044 long-lines for the cultivation of 7,000 tonnes of sponge.

The economic analysis of mariculture of *Lissodendoryx* sp. and the purification of halichondrin B is shown in Table 3. The downstream processing is the cost-determining factor, although most employees are needed for the mariculture (for harvesting and cleaning the sponges). The purification of halichondrin B from the sponge was done according to the method that was used by Litaudon and colleagues (1997). An annual turnover of approximately 200 million € can be calculated, which would lead to a production price of 71 €/mg halichondrin B or approximately 12,000 €/patient·year.

Table 2: Analysis of the size of maricultures that would be required for the production of 2.8 kg halichondrin B or 150 kg avarol.

starting figures for	**Halichondrin B**	**and Avarol**	[unit]
required amount:	2.8	150	kg/year
natural concentration:	$4 \cdot 10^{-4}$	2	g/kg
required amount of sponge:	7,000	75	tonnes
initial size explant	8.3	28	g
final size explant	207	1316	g
mortality	50	50	%
results			
# of explants:	$73.6 \cdot 10^{6}$	$1.19 \cdot 10^{5}$	explants
# of long lines:	3044	31.3	long-lines

Avarol

Dysidea avara has been grown in maricultures in the Mediterranean Sea by Klinipharm GmbH (Fig. 2). Sponge explants increased from 27 cm^3 (~28 g wet weight) to 1267 cm^3 (~1316 g wet weight) in approximately a year. Thus, it can be calculated that a number of 119,000 explants is needed to produce 75,000 kg of *D. avara*, which corresponds to 32 long-lines (Table 2). This implies a 47-fold increase of the biomass during the cultivation period. However, the faster growth of *D. avara*, when compared to *Lissodendoryx* sp., is not of major importance for the calculated size of the mariculture.

The size of the mariculture is mainly determined by the product concentration in the sponge. The avarol concentration is also advantageous for downstream processing, as the amount of solvents for the extraction of avarol can be reduced. For purification of avarol from crude sponges, the method from Schatton *et al.* (2002) for large-scale recovery of avarol from *D. avara* was applied. The economic analysis of mariculture of *D. avara* and the extraction of avarol is included in Table 3 and it can be seen that the downstream processing is the cost-determining factor, mainly due to large volumes of ethanol and petrol ether that are required to extract the avarol. Production of 150 kg avarol would lead to an annual turnover of approximately 22 million € per year, and a production price of 0.14 €/mg or 14 €/patient·year.

Other data of *D. avara* growth in the natural environment indicate that growth of this species is highest in the summer and is very small in the winter (Turon *et al.*, 2000).

However, if growth would only occur during half of the year, this has no major influence on the production price of avarol. In the scenario with seasonal growth, the production price would increase from 0.14 to 0.15 €/mg (not in table). The increase is only small because the cultivation of the sponges represents only a small part of the total costs.

There is a number of issues that are not visible in the cost calculation, but that can play an important role in the success or failure of the mariculture. The sea is a cheap location for business, but obtaining permission to start a mariculture often leads to long and costly procedures with respect to the environmental impact of the mariculture (pers. com. D. Mendola; E. Luiten). Furthermore, mariculture is a typical seasonal business with high peaks in the working load at the harvesting time. In case of *Lissodendoryx* sp. it would imply that 4,400 tonnes of sponge would have to be harvested within approximately one month. On the other hand, the sponge explant size could still be optimised in order to minimise labour, and maximise the harvest per long-line.

Table 3: Economic analysis of *Lissodendoryx* sp. and *D. avara* maricultures.

Economic analysis for	**Halichondrin B**	**and**	**Avarol**	[unit]
Fixed costs				
investment	$4.9 \cdot 10^7$		$1.0 \cdot 10^6$	€
annual costs	$8.5 \cdot 10^6$		$1.8 \cdot 10^5$	€
Variable costs				
personel	$3.7 \cdot 10^7$ (1012)[a]		$1.7 \cdot 10^6$ (45)[a]	€
mariculture	$8.5 \cdot 10^6$		$8.7 \cdot 10^4$	€
DSP	$1.1 \cdot 10^8$		$1.6 \cdot 10^7$	€
insurance	$2.5 \cdot 10^6$		$2.7 \cdot 10^5$	€
overhead	$1.2 \cdot 10^7$		$1.4 \cdot 10^6$	€
profit	$1.7 \cdot 10^7$		$1.8 \cdot 10^6$	€
annual turnover	$2.0 \cdot 10^8$		$2.2 \cdot 10^7$	
price of product	71		0.14	€/mg
treatment costs	$1.2 \cdot 10^4$		14.4	€/patient*year

[a]Between brackets: the number of employees needed.

A detailed description of the calculation of the costs is provided in the appendix.

Ex situ culture

The term *ex situ* culture refers to cultivation of functional sponges outside the sea (Fig. 3). *Ex situ* cultivation may be preferable because it 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. *Ex situ* cultivation allows controlled supply of nutrients and precursors of the secondary metabolites of interest (if known) and in that manner growth and production rates could be enhanced. Marine sponges have proven to be no laboratory rats yet, but a growing number of researchers has demonstrated small successes in culturing them outside of the sea (Barthel and Theede, 1986; De Caralt *et al.*, 2003; Osinga *et al.*, 2003; Belarbi *et al.*, 2003; Mendola, 2003; Duckworth *et al.*, 2003b; Sipkema *et al.*, submitted). For the species *Chondrosia reniformis* and *Crambe crambe* both data from growth in the sea and in the laboratory are available. The growth rate of *C. reniformis* was estimated to be 4-9 times higher in the laboratory under suboptimal conditions (Sipkema *et al.*, submitted). 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 different methods that were used to measure growth. However, a seasonal growth pattern was obvious in the sea, while such a trend was not present for *ex situ* cultivation.

Fig. 3: Example of two *ex situ* cultivation tanks (1.3 m^3), in which water quality can be continuously measured and controlled, at Wageningen University.

Analysis of the method

A number of assumptions has been made for the evaluation of *ex situ* culture of sponges for the production of halichondrin B and avarol:

1. Natural seawater with additional nutrients (fish powder) is used to supply to the sponges.
2. The conversion ratio from nutrients to sponge biomass is 50:1. This is a rough estimation based on measured uptake rates at different substrate concentrations (unpublished data) and biomass increase of the sponge *Chondrosia reniformis* (Sipkema *et al.*, submitted).
3. The mortality of the sponges is 25 %, but due to immediate replacement of explants that died with new explants, only 10 % of the *ex situ* cultivation tanks is occupied by dead sponges.
4. The maximum sponge hold-up in the tanks is 3 %. This estimation is based on oxygen consumption by sponges (Osinga *et al.*, 1998a) in the chosen reactor set-up (flow-through tanks of 5 m^3).
5. The cultivation systems are set-up in such a way that 50 % of the tank is filled with new explants when the other 50 % has been cultured for half of the time that is needed for the explants to reach their harvesting size. This is done in order to use the space of the tanks more efficiently.
6. The growth rate of the sponges is 7 times higher than in the natural environment and growth occurs all the year round (Sipkema *et al.*, submitted).

Halichondrin B & Avarol

The calculation of the number of explants required to produce the desired amount of halichondrin B or avarol is identical as for mariculture, as the initial and final weights of the explants are copied from the experimental mariculture data. The difference in the number of *Lissodendoryx* sp. or *D. avara* explants that can be cultured per cultivation tank is caused by the difference in explant sizes that are used (Table 4). The initial and final weights of the *Lissodendoryx* sp. and *D. avara* explants are copied from the original data, as no growth data are available from explants with different sizes. Smaller explants are related to larger relative biomass increase (Sipkema *et al.*, submitted), which implies that the actual growth of *D. avara* would slightly increase compared to the *Lissodendoryx* sp. explants, when the *D. avara* explants would be cut to an initial size of 8.3 g. The growth rate is assumed to be seven times higher than in maricultures and therefore the time that is needed for the sponges to reach their final weight is approximately 26 days (instead of half a year) and 47 days (instead of 330 days), for *Lissodendoryx* sp. and *D. avara*, respectively. Production of 2.8 kg halichondrin B or 150 kg avarol would require approximately 1521 or 45 cultivation tanks, respectively (5 m^3/tank).

Table 4: Analysis of the required production size for *ex situ* cultures of *Lissodendoryx* sp. and *D. avara* to yield 2.8 kg halichondrin B or 150 kg avarol, respectively.

starting figures for	**Halichondrin B**	**and Avarol**	[unit]
required amount:	2.8	150	kg/year
natural concentration:	$4 \cdot 10^{-4}$	2	g/kg
required *Lissodendoryx* sp.:	7,000	75	tonnes
initial size explant	8.3	28	g
final size explant	207	1316	g
mortality	25	25	%
size cultivation tank	5	5	m^3
results			
cultivation time	25.7	47.1	day
# of explants per cultivation tank:	1042	169	
# of cultivation tanks	2420	45	

The economic potential of *ex situ* cultivation of *D. avara* is comparable to mariculture (Table 5). This is caused by the costs of downstream-processing that remain the same as for mariculture, and determine the major part of the production costs. For the production of halichondrin B, the production costs are more or less doubled when compared to mariculture (Table 5). The higher price is caused by the costs of nutrients used to feed the *Lissodendoryx* explants. An important difference with mariculture is that *ex situ* cultivation might allow an increase of the specific production rate of the secondary metabolites if suitable precursors are supplied to the sponge explants, or if the conditions in the cultivation tanks are changed in favour of secondary metabolism. If it is assumed that the halichondrin B and avarol concentration in the sponges can be increased 4- and 2-fold , respectively, the production costs are almost inversely linear correlated with the product concentration. This is the result of a strong decrease in the costs for downstream processing. It was assumed that the avarol concentration could only be increased with a factor 2 as the natural concentration is already relatively high. With this scenario, the estimated production costs are decreased to 45 and 0.09 €/mg or 7,800 and 8.8 €/patient·year for halichondrin B and avarol, respectively (not in table).

These calculations, make clear that *ex situ* culture can compete with mariculture for the production of secondary metabolites from sponges. Very few data are available to confirm the assumption that the *ex situ* growth rate of the sponges is seven times higher than in the sea. In a worst-case scenario, that the *ex situ* growth rate equals the growth rate

in the sea the *ex situ* production costs would be 232 and 0.19 €/mg or 41,000 and 19 €/patient·year for halichondrin B and avarol, respectively (without an increased product concentration). This means that, especially in the case of avarol, the growth rate of the sponge has no major influence on the production costs.

Table 5: Economic analysis of *Lissodendoryx* sp. and *D. avara ex situ* cultures.

Economic analysis for	**Halichondrin B and**	**Avarol**	[unit]
Fixed costs			
investment	$1.1 \cdot 10^8$	$1.9 \cdot 10^6$	€
annual costs	$1.9 \cdot 10^7$	$3.3 \cdot 10^5$	€
Variable costs			
personel	$9.6 \cdot 10^6$ (263)[a]	$1.3 \cdot 10^6$ (35)[a]	€
ex situ culture	$2.2 \cdot 10^8$	$2.6 \cdot 10^6$	€
DSP	$1.1 \cdot 10^8$	$1.6 \cdot 10^7$	€
insurance	$5.5 \cdot 10^6$	$3.1 \cdot 10^5$	€
overhead	$2.6 \cdot 10^7$	$1.5 \cdot 10^6$	€
profit	$3.7 \cdot 10^7$	$2.0 \cdot 10^6$	€
annual turnover	$4.3 \cdot 10^8$	$2.4 \cdot 10^7$	
price of product	155	0.16	€/mg
treatment costs	$2.7 \cdot 10^4$	16.2	€/patient*year

[a]Between brackets: the number of employees needed.
A detailed description of the calculation of the costs is provided in the appendix.

Primmorphs

Primmorphs (Fig. 4) have been defined as multi-cellular aggregates obtained from dissociated sponge cell suspensions in which cell proliferation occurs (Müller, 1998). The dissociated 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.*, 2003a; Zhang *et al.*, 2003a). Cell division in primmorphs has been measured by BrdU incorporation, but reports about biomass increase of primmorphs have been lacking up to now. This is probably explained by the occurrence of apoptosis that counterbalances cell division (Koziol *et al.*, 1998). The only exception to this is rapid

growth that was recorded for primmorphs of the Chinese sponge *Hymeniacidon perleve* (Zhang *et al.*, 2003b). These authors measured a 9-fold increase of the cell mass within 7 days, but observed death of the primmorphs after 10 days, which is peculiar as primmorphs are known for their long survival times (Custodio *et al.*, 1998; Sipkema *et al.*, 2003a).

It has also been questioned whether primmorphs are able to grow, or if they will develop into functional sponges before growth can occur (Sipkema *et al.*, 2003a). Development of functional sponges from sphere-shaped aggregates that were formed from dissociated cells would be in line with Wilson's classical experiments (1907). Some signs point in this direction, as for example spicule formation (Krasko *et al.*, (2000) and attachment and flattening of primmorphs to a solid matrix (Sipkema *et al.*, 2003c), have been observed.

Another way of obtaining the bioactive compounds from sponges would be continuous production and selective extraction of the products. These processes have been developed for other organisms, such as plant cells, hybridoma cells and microalgae (Brodelius *et al.*, 1979; Zijlstra *et al.*, 1996; Hejazi and Wijffels, 2004). Compared to other forms of sponge biomass, primmorphs are remarkably resistant to physical stress and they can easily be kept for longer times. Therefore, they may be able to tolerate an extraction fluid that could be used to 'milk' the product from the primmorphs. Some knowledge about the secondary metabolic routes is required in this case, as specific substrates involved in the production of the bioactive compound have to be supplied and taken up by the primmorphs. Small molecules like BrdU have been shown to be taken up by primmorphs (Custodio *et al.*, 1998) but it is not known to what extent larger secondary metabolites can be transported across the skin-like coating of the primmorphs. The idea of milking primmorphs is put forward as Müller and colleagues (2000) measured production of avarol in non-growing *D. avara* primmorphs.

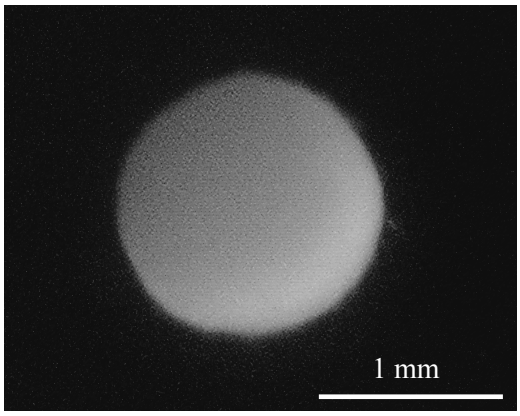

Fig. 4: A *Suberites domuncula* primmorph.

Analysis of the method

A number of assumptions is required in order to make calculations to assess the feasibility of using non-growing primmorphs for the production of bioactive compounds from sponges:

1. A doubling of the product concentration in primmorphs to 50 % of the concentration 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). The controls, which consisted of freshly dissociated cells contained no detectable levels of avarol at day 0, 1 and 3. The latter point is somehow remarkable, as avarol was present in the sponge that was used to obtain the dissociated cells for the preparation of primmorphs and the controls.
2. Primmorph have a life span of 6 months. For a number of sponges the life span of their primmorphs has been determined (Sipkema *et al.*, 2003a). In general, they can be maintained for at least half a year. No longer life span is assumed, as contaminations and other incidents will occur.
3. 1 ‰ of the sponge cells that is used to prepare primmorphs actually ends up in a primmorph. Zhang and colleagues (2003a) studied the average number and size of primmorphs that were obtained from a dissociated cell suspension. From the initial cell concentration and average cell size of *Stylotella agminata* cells, the fraction of cells that ends up in a primmorph can be calculated. It is assumed that this holds also for *Lissodendoryx* sp. and *D. avara.*

Halichondrin B & Avarol

Although there are no reports about the formation of primmorphs from *Lissodendoryx* sp., the formation of primmorphs from many sponges has been established and it was concluded that primmorph formation is probably a universal characteristic of demosponges (Sipkema *et al.*, 2003a). Therefore, we assume that it is possible to obtain primmorphs from *Lissodendoryx* sp. In Table 6 the result of the calculations for the production of halichondrin B with *Lissodendoryx* sp. primmorphs are presented. The assumed specific production (and extraction) rate of 33 μg/kg·d would be difficult to reach with increase of biomass in maricultures or closed systems, as an average growth rate of 8 % per day would be required, to produce 33 μg halichondrin B per kg *Lissodendoryx* sp. per day. However, the low efficiency of primmorph formation causes an annual requirement for sponge biomass of 461,000 tonnes, which is even higher than the amount that is needed for direct extraction of halichondrin B. As the primmorph formation efficiency is assumed to be the same for all sponges, the results for *D. avara* are

similar to those obtained for *Lissodendoryx* sp. Because this assumption plays such an important role in the assessment of the suitability of using primmorphs for the production of secondary metabolites, we made an estimation of the fraction of dissociated *Suberites domuncula* cells that ends up in primmorphs (Sipkema *et al.*, 2003a). It is a rough estimation, since the number of primmorphs that was formed per ml cell suspension is not exactly known. This estimation gives a more optimistic result, as approximately 1.4 % of the cells ends up in a primmorph. However, also with these figures the biomass required for the formation of primmorphs is larger than the biomass for direct extraction of halichondrin B or avarol (data not shown). Based on these calculations and the uncertainties regarding the possibilities of simultaneous extraction and production of secondary metabolites in primmorphs, it is concluded that non-growing primmorphs are currently not suitable for large-scale production of sponge pharmaceuticals.

Table 6: required amount of sponge biomass for the production of sufficient halichondrin B.

starting figures for	**halichondrin B**	[unit]
required amount:	2.8	kg/year
	7.7	g/day
natural concentration:	400	μg/kg
production:	33.3	μg/kg·d
(is increase from 100 to 200 μg/kg in 3 days)		
results		
primmorphs:	$2.30 \cdot 10^5$	kg/6 months
	$4.61 \cdot 10^5$	kg/year
adult sponge:	$4.6 \cdot 10^5$	tonnes/year

Sponge-cell culture

Sponge-cell culture has attracted scientists already for a decade, as cell lines are associated with faster growth rates and controlled conditions. However, the preparation of immortal cell lines from sponges has not been achieved up till now. A few small successes have been noted (Pomponi and Willoughby, 1994; Pomponi *et al.*, 1997), but they could not be further expanded. Most attempts have been characterised by a lack of growth or doubts about the true sponge origin of the proliferating cells (Ilan *et al.*, 1996; Rinkevich, 1999). At first sight, sponges appear to be promising animals for obtaining cell lines. Their

loosely organised cellular structure and the presence of totipotent cells, the archeocytes, seem to favour the initiation of sponge-cell lines. However, the presence of sponge-associated organisms, like bacteria, algae, fungi or unicellular organisms that can make up more than 40 % of the sponge biomass (Wilkinson, 1978c), complicates the preparation of axenic primary sponge-cell lines. A first step in the development of an unambiguous method to determine quantitatively the actual fraction of sponge cells in 'sponge'-cell cultures has been made (Sipkema *et al.*, 2003b). A next logical step would be selective enrichment of primary cell cultures to obtain pure sponge-cell cultures. This is a difficult task as it requires a highly specific selection target. One could think of making use of specific fatty acids that are present in the cell membranes of sponges (Barnathan *et al.*, 1996) or the variable regions of species specific receptors in the cell membrane. By means of labelling sponge cells with selective antibodies or the much smaller iMabs (Catchmabs, 2003) against the specific targets and cell sorting on a flow cytometer, an axenic solution may be obtained. This is no guarantee for subsequent cell growth, but the high occurrence of contaminated cultures could be drastically lowered. Perhaps it is necessary to mimic the micro-environment of the sponge mesohyl instead of developing media that are based on seawater, as has usually been attempted up to now to obtain sponge-cell growth (Ilan *et al.*, 1996; Pomponi *et al.*, 1997; Sipkema *et al.*, 2003b). The local micro-environment in the mesohyl can deviate significantly from the surrounding seawater with respect to oxygen gradients (Hoffmann, 2003), but this may also account for other parameters such as the pH. Somatic sponge-cell culture media may initially have to be supplemented with sponge-specific growth factors of which a number have been characterised by Müller and colleagues (2003). The creation of hybridoma cells from sponges would be a breakthrough for sponge-cell culture, but this has not yet been established.

Analysis of the method

In order to determine whether sponge-cell culture might be a feasible way to produce bioactive compounds from sponges in the future, we assume that all required development steps described before have successfully been accomplished. Sponge-cell culture can then be compared to mammalian cell culture, except that products from mammalian cell cultures are often secretory proteins (e.g. antibodies). For secondary metabolites from sponges, this will probably not be the case, as they would be lost in the sea. In addition, the material of the cultivation reactors needs to be resistant against the high salt concentrations that are normally present in the sea. Some animal cells, such as baby hamster kidney (BHK) cells, require a microenvironment similar to the tissue, which

can be mimicked by providing the cell surface interactions that are present in the tissue on a solid substratum. We assume that sponge cells also need a matrix, comparable to the mesohyl, to attach before growth can occur. For a large-scale cultivation system, one can think of cultivation in a stirred vessel on microcarriers or cultivation in a hollow fibre reactor. We have chosen to simulate sponge-cell culture in a hollow fibre reactor due to some structural similarity between the reactor and the canal system in the sponge. For a cautious estimation of the total amount of biomass that is needed to produce the required amount of product, we assumed that the product concentration in the biomass is the same as in nature. This parameter could be improved in cell cultures with knowledge about the secondary metabolic routes and supply of the proper substrates. However, we chose to keep the product concentration at its natural level because of the high cytotoxicity of for instance halichondrin. As halichondrin B binds the tubulin proteins that are involved in cell division (Kitagawa and Kobayashi, 1990), higher concentrations may also interfere with sponge-cell division. Nevertheless, some observations indicate that it may be possible to increase the product concentration without growth inhibition of the sponge, as it has been found for avarol that concentrations that inhibit DNA synthesis in bacteria and mammalian cells, do not affect DNA synthesis in *D. avara* (Müller *et al.*, 2000).

Halichondrin B & Avarol

It is questionable whether halichondrin B is really produced by the sponge and not by its symbionts. Halichondrin B was detected in five different sponge species (Hart *et al.*, 2000), which may point to production by a symbiotic bacterium, which is present in all five sponges. However, for this study it is assumed that halichondrin B is produced by *Lissodendoryx* sp. cells. Avarol has been found to be located inside the sponge choanocytes and has therefore not been subject of speculation about the origin of the producer (Uriz *et al.*, 1996).

The cell growth rate that is used for the calculation (0.0054 h^{-1}) is derived from the fastest growth of a *Lissodendoryx* sp. specimen that was ever recorded: 5000 % in one month (Dumdei *et al.*, 1998). This is still fairly low compared to other animal cells, which have typical growth rates of approximately 0.03 h^{-1} (Avgerinos *et al.*, 1990; Marteijn *et al.*, 2000). For *D. avara* we have assumed the same growth rate as for *Lissodendoryx*, because it is not known whether differences in natural growth rates will also lead to different growth rates in cell cultures. Inoculation- and final cell densities have been adapted from cultures of baby hamster kidney (BHK) cells (Gramer and Poeschl, 2000), which have a diameter (~10 μm) (Ludwig *et al.*, 1992) that can be compared with sponge cells. The number of

cells that is equal to the amount of sponge that is needed to obtain the required amount of product, was calculated for cells with a diameter of 10 μm and a density of the sponge of approximately 1.03 g/mL (unpublished data for *Chondrosia reniformis* and *Dysidea avara*).

Table 7: required number of hollow fibre bioreactors for the production of sufficient halichondrin B and avarol.

starting figures for	**Halichondrin B**	**and**	**avarol**	[unit]
required amount:	2.8		150	kg/year
cell concentration in sponge:	$1{\cdot}85.10^{9}$		$1{\cdot}85.10^{9}$	cells/g
natural concentration:	$4{\cdot}10^{-4a}/16{\cdot}10^{-4b}$		$2^{a}/4^{b}$	g/kg
growth rate:	$0.0054^{a}/0.03^{b}$		$0.0054^{a}/0.03^{b}$	h-1
final cell concentration	$3{\cdot}10^{8}$		$3{\cdot}10^{8}$	cells/mL
initial cell concentration	$5{\cdot}10^{5}$		$5{\cdot}10^{5}$	cells/mL
volume hollow fiber reactor:	150		150	mL
results				
[a] # required hollow fiber reactors:	$2.88{\cdot}10^{8}$		$3.09{\cdot}10^{6}$	reactors
[b] # required hollow fiber reactors:	$7.20{\cdot}10^{7}$		$1.54{\cdot}10^{6}$	reactors
[a] cultivation time (incl. down time)	51		51	d
[b] cultivation time (incl. down time)	10.9		10.9	d

It was calculated that a huge number of $2.9{\cdot}10^{8}$ hollow fibre bioreactors would be needed to produce the required amount of cells for a harvest of 2.8 kg halichondrin B (Table 7, scenario a). Due to the higher product concentration in *D. avara* cells, 'only' $3.1{\cdot}10^{6}$ hollow fibre bioreactors would be needed for the production of the required amount of avarol. The enormous number of hollow fibre reactors that is required, is partly caused by the relatively small operation volume of 150 mL and because they are disposable. This size of hollow fibre reactors is very limited because of substrate gradients that result from the very high cell concentrations that can be reached. The possibility in cell cultures to increase the intracellular concentrations of halichondrin B or avarol is the most promising approach to decrease the number of reactors, as they are inversely linearly correlated (Table 7, scenario b). The assumed product concentrations are probably underestimated, as the presence of spicules, collagen fibres and symbiotic organisms decrease the product concentration in functional sponges. Therefore, this calculation probably overestimates the sponge biomass that is required to produce 2.8 kg halichondrin B and 150 kg avarol.

The calculated product prices for halichondrin B and avarol are largely determined by the cell culture costs, in which the disposable hollow fibre reactors are responsible for the major part of the costs (Table 8). They cause prices of 780,000 and 16 €/mg, respectively for halichondrin B and avarol.

Table 8: Economic analysis of *Lissodendoryx* sp. and *D. avara* cell cultures.

Economic analysis for	**Halichondrin B and**	**Avarol**	[unit]
Fixed costs			
investment	$1.4 \cdot 10^9$	$1.1 \cdot 10^8$	€
annual costs	$2.4 \cdot 10^8$	$1.5 \cdot 10^7$	€
Variable costs			
personel	$9.6 \cdot 10^7$ (2609)[a]	$2.1 \cdot 10^6$ (57)[a]	€
cell culture	$1.8 \cdot 10^{11}$	$2.0 \cdot 10^9$	€
DSP	$1.1 \cdot 10^8$	$1.6 \cdot 10^7$	€
insurance	$2.7 \cdot 10^9$	$3.0 \cdot 10^7$	€
overhead	$1.4 \cdot 10^{10}$	$1.5 \cdot 10^8$	€
profit	$1.8 \cdot 10^{10}$	$2.0 \cdot 10^8$	€
annual turnover	$2.2 \cdot 10^{11}$	$2.4 \cdot 10^9$	
price of product	$7.8 \cdot 10^4$	16	€/mg
treatment costs	$1.4 \cdot 10^7$	$1.6 \cdot 10^3$	€/patient*year

[a]Between brackets: the number of employees needed.
A detailed description of the calculation of the costs is provided in the appendix.

In order to estimate the influence of the cell growth rate, the product concentration and the reactor type, a number of scenarios was analysed (Table 9). It shows that the growth rate is a relatively unimportant factor with respect to the final product price, when compared to the impact of the product concentration. If sponge cells could be cultured in commonly used stirred tanks, the price of cultivation would be roughly four times lower than in hollow fibre bioreactors and the culture medium would become the most expensive component.

Table 9: Product prices for different growth rates, product concentrations and reactor types.

	growth rate [h^{-1}]	product conc. [-]	price [€/mg] halichondrin B	avarol
hollow fiber	0.0054	1	$7.8 \cdot 10^4$	15.7
	0.03	1	$7.8 \cdot 10^4$	15.7
	0.03	4/2[a]	$1.9 \cdot 10^4$	7.8
stirred vessel	0.0054	1	$2.4 \cdot 10^4$	5
	0.03	1	$2.1 \cdot 10^4$	4.3
	0.03	4/2[a]	$0.52 \cdot 10^4$	2.2

[a]product concentration normalised to the natural concentration (=1). Product concentration multiplied by 4 and 2, respectively for halichondrin B and avarol.

Genetic modification

Transfer of the DNA involved in the production of the secondary metabolites to a fast-growing host that overexpresses these genes may result in high product concentrations and for this reason this could be a valuable approach. The problem is that the bioactive compounds from sponges are usually not proteins, for which often transfer of only one gene is sufficient for expression in the host. They are metabolites that are the result of a complex, mostly unknown, metabolic pathway. The enzymes that are involved in the first steps of the biosynthesis of avarol are probably common enzymes of the terpenoid biosynthesis that lead to the formation of the intermediate farnesyl diphospate. However, the subsequent reaction steps in the sesquiterpenoid biosynthesis and the enzymes that catalyse these reactions that lead to the formation of avarol are not known. Therefore, it is not yet possible to construct a heterologous host for the production of avarol. Another issue to take into account in case of heterologous expression is the toxicity of the product. Avarol has been shown to be a strong cell growth inhibitor for eukaryotic cells and bacteria (Seibert *et al.*, 1985; Müller *et al.*, 1985) and may therefore be harmful for the host. If genes involved in the production of the desired metabolites are located within one cluster (e.g. for many polyketides), genetic transfer of the pathway to a host is possible (Pfeifer and Khoshla, 2001). This has been established for a number of bacterial and fungal polyketides, such as erythromycin (Kao *et al.*, 1994). Fortunately, self-resistance genes against the polyketide are often present in the same gene cluster and therefore the

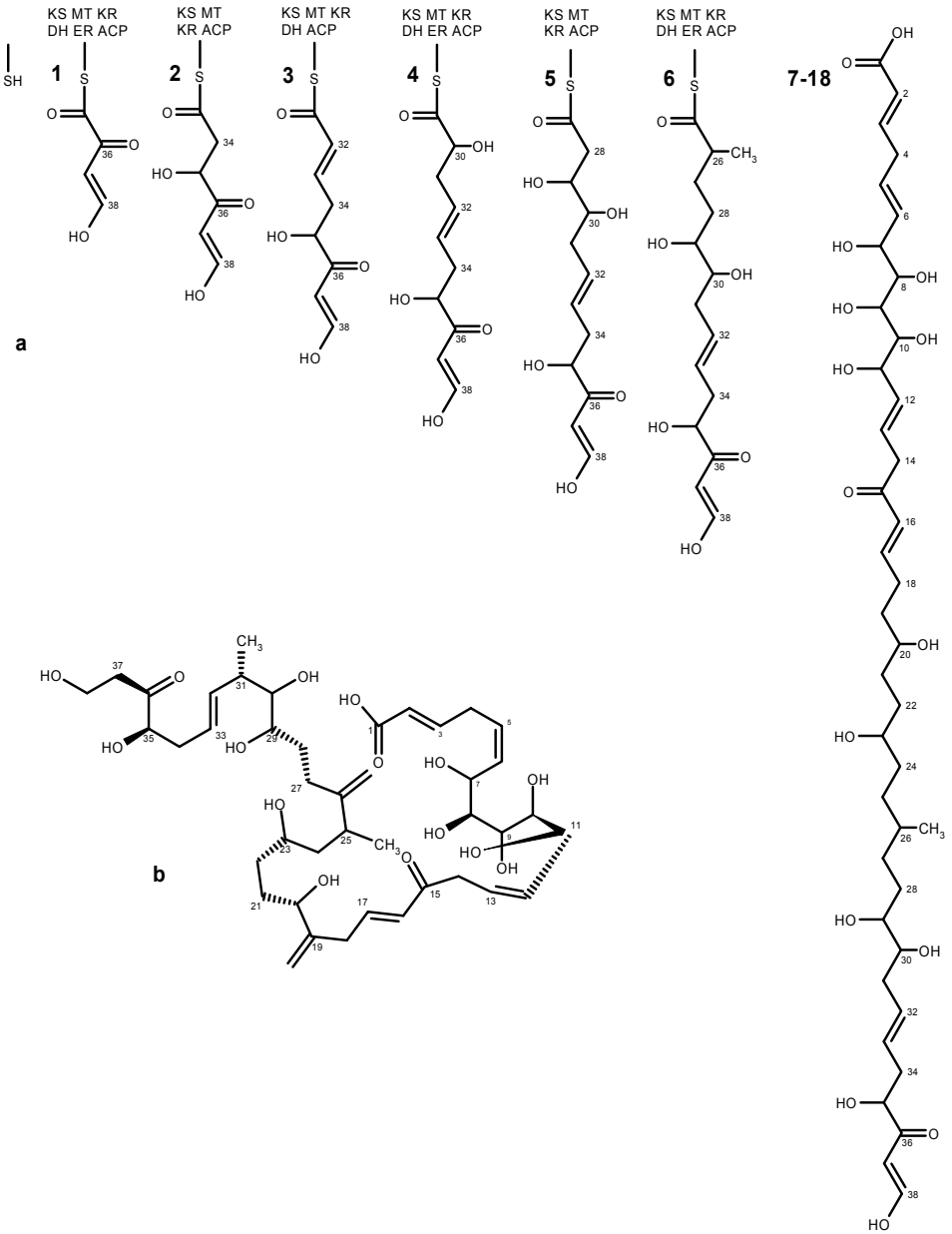

Fig. 5: The hypothesised biosynthesis of the C_1-C_{38} active core of halichondrin B (a). The complete linear polyketide could be synthesised in 18 steps by step-wise addition of malonyl-CoA (m-CoA), hydroxymalonyl-CoA (m-OH-CoA) or methylmalonyl-CoA (M-CH_3-CoA). The specific polyketide synthase module required for each reaction step is connected to the "growing" polyketide: KS = ketosynthase; MT = malonyl transferase; KR = ketoreductase; DH = dehydratase; EH = enoyl reductase; ACP = acyl carrier protein. Step 1: oxalo acetate (substrate) + KS MT KR DH ER ACP (enzyme complex); step 2: m-CoA + KS MT KR ACP; step 3: m-CoA + KS MT KR DH ACP; step 4: m-OH-CoA + KS MT KR DH ER ACP; step 5: m-CoA + KS MT KR ACP; step 6: m-CH_3-CoA + KS MT KR ACP; step 7: m-CoA + KS MT KR DH ER ACP; step 8: m-CoA + KS MT KR ACP; step 9: m-OH-CoA + KS MT KR DH ER ACP; step 10: m-CoA + KS MT KR DH ER ACP; step 11: m-CoA + KS MT KR DH ACP; step 12: KS MT ACP; step 13: m-CoA + KS MT KR DH ACP; step 14: m-OH-CoA + KS MT KR ACP; step 15: m-OH-CoA + KS MT KR ACP; step 16: m-CoA + KS MT KR ACP; step 17: m-CoA + KS MT KR DH ACP; step 18: m-CoA + KS MT KR DH ACP followed by hydrolysis from the enzyme. By methylation and oxidation at C_{19}, methylation at C_{25}, oxidation of the methyl-group at C_{26} and methylation at C_{31} and folding the structure presented in (b) can be obtained. After ring-closing the C_1-C_{38} active core of halichondrin B is formed (compare with Fig. 1).

toxicity problem for the host may be circumvented. However, the putative polyketide gene cluster for halichondrin B has not yet been identified in the sponge or a symbiotic organism. Identification and isolation of this cluster would offer promising new perspectives for large-scale production of halichondrin B. Recombining cloned polyketide synthase genes could be another strategy to create the halichondrin B gene cluster. This approach is currently investigated for the marine sponge compound discodermolide by researchers of Kosan Biosciences (Kosan, 2004). The hypothetical biosynthesis of the active core of halichondrin B can be deduced (Fig. 5), based on knowledge of general polyketide synthesis (Pfeifer and Khosla, 2001; Leadlay, 2004). Nowadays, recombination techniques make it possible to delete, swap, or modify individual domains of a polyketide synthase with alternative domains, taken from the same or foreign polyketide synthase proteins (Ranganathan *et al.*, 1999; Cane and Walsh, 1999). However, construction of the whole putative biosynthesis gene cluster and insertion into a heterologous host is no common practice yet.

Isolation of the halichondrin B synthase genes from the sponge would be an important establishment to facilitate progress in this direction. The total amount of DNA that is required for complete synthesis of the active core of halichondrin B may be too large to be accepted by one host because of the large number of reaction steps (Fig. 5). If the cluster is too big, enzymes may be produced by different hosts and isolated to be used for instance in multi-step biocatalysis in microreactors (Lee *et al.*, 2003). Genetic modification could become an important tool to obtain secondary metabolites from marine sponges (or their symbionts), but improvements of the-state-of-the-art are required before this could be realised.

Semi-synthesis

A middle course for the production of sponge metabolites would be biotechnological production of a precursor by a (genetically modified) bacterial strain, followed by a limited number of chemical steps to obtain the final product. The microbially synthesised Ara-A is an analogue of a sponge-derived nucleic acid (McConell *et al.*, 1994), and one of the very few examples of products from sponges that made it to the pharmaceutical market. In the case of Ara-A no additional chemical steps were needed, as the product of the microorganism already has the required bioactivity, but in other cases a number of chemical steps were sufficient to transform an easily obtained microbial product into the bioactive product of an organism that is difficult to culture. This approach has, for

example, been successful for the production of ET-743, an antitumour agent that has been isolated from a marine tunicate (Rinehart *et al.*, 1990). ET-743 and an analogue have been completely chemically synthesised (Corey *et al.*, 1996; Martinez and Corey, 2000), but it requires many chemical steps and very expensive precursors. Cuevas and colleagues (2000) found that the number of chemical reactions could be markedly reduced if the antibiotic cyanosafracin B, which could be obtained much cheaper via bacterial fermentation of its producer *Pseudomonas fluorescens*, was used as starting point for the chemical synthesis. In this case, toxicity of the product for the producing strain was circumvented and genetic modification was not required.

A similar strategy might prove valuable for avarol and halichondrin B, but suitable biologically produced precursors are currently unknown. For avarol, one could think of derivatives of building blocks of the sesquiterpenoid biosynthesis. As the polyketide halichondrin is suspected to be of bacterial origin, other metabolites sharing some structural similarities with halichondrin, such as okadaic acid (Fig. 6), may present an interesting starting point for further chemical synthesis of halichondrin B. Okadaic acid is a polyketide produced by dinoflagellates (Murakami, 1982), but was first discovered in *Halichondria okadai* (Tachibana *et al.*, 1981), the first sponge that was found to contain halichondrin B (Hirata and Uemura, 1986). However, no intermediates in the biosynthesis of halichondrin B or avarol are currently known and therefore it is difficult to assess the feasibility of this method to produce large amounts of secondary metabolites from marine sponges.

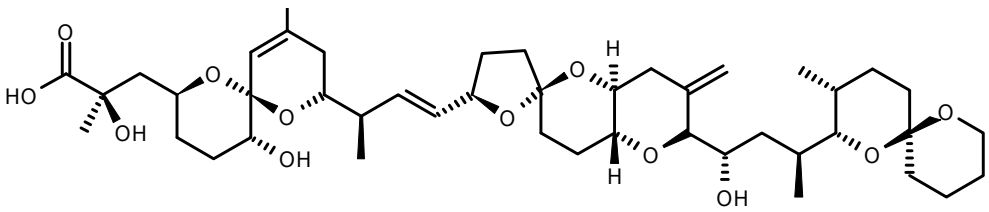

Fig. 6: Molecular structure of okadaic acid

Discussion

A quick comparison between mariculture, *ex situ* culture and cell culture (for which an economic comparison could be made) shows that mariculture is currently the most feasible method for the production of sponge metabolites, if a suitable area for cultivation is available. Halichondrin B and avarol obtained by mariculture lead to production prices of approximately 12,400 and 14.4 €/patient·year, respectively. These prices are lower than

those for *ex situ* culture, which are around 27,000 and 16.2 €/patient·year for halichondrin B and avarol, respectively. However, *ex situ* culture of sponges has an important advantage over mariculture. Many process conditions in *ex situ* cultures can be controlled and they are not as vulnerable to disasters, such as storms or oil spills from tankers, and diseases as maricultures. If more information about the biosynthetic routes of the sponge metabolites becomes available, the conditions in *ex situ* cultures could be manipulated in such a way that the metabolism of the sponge (or its symbiont) is shifted towards the secondary metabolite of interest. The increment of the halichondrin B and avarol concentrations to four and two times their natural concentration, would result in significantly lower production costs of 7,800 and 8.8 €/patient·year, respectively. Increases of the concentration of secondary metabolites with this order of magnitude have been established for plants and corals without genetic modification (Zhong, 2002; Fleury *et al.*, 2004). Both for mariculture and *ex situ* culture, the downstream processing costs represent a major part of the production costs of halichondrin B and avarol. This is caused by high prices of 'pharmaceutical grade' extraction fluids. If it is possible to partially recycle these solvents, a considerable reduction of the production costs could be attained.

Our calculations show that a future development of immortal sponge-cell lines would not automatically provide the preferred method for the production of sponge metabolites. In the worst-case scenario for sponge-cell culture, halichondrin B and avarol lead to production prices of $1.4 \cdot 10^7$ and 1600 €/patient·year, respectively. Even in our best-case scenario the production costs of $9.2 \cdot 10^5$ (=5200 €/mg) and 216 (=2.2 €/mg) €/patient·year, for halichondrin B and avarol respectively, are considerably higher than the production costs with mariculture or *ex situ* culture. The cost-determining factor in our best-case scenario is the cell culture medium, which is much more expensive than the nutrient-enriched seawater that was assumed for *ex situ* cultivation. However, this latter point is a general issue with animal cell cultures (Riese *et al.*, 1994). Commercial products from animal cell cultures, such as monoclonal antibodies and interferon, are generally present in concentrations in the range of 10-50 mg/g cells (Wen *et al.*, 2000; Sakai *et al.*, 2002), which is approximately a ten-fold higher than our calculated avarol concentration. If the avarol concentration could be increased to this level, a production price of approximately 0.21 €/mg would be obtained, which is comparable with the price of avarol from mariculture and *ex situ* culture. For halichondrin B, it would require an increment of 250,000 times the natural product concentration to reach this product level, which is rather inconceivable. Therefore, sponge-cell culture for the production of bioactive compounds is regarded as not feasible, as product concentrations are (still) too low.

To obtain insight in the economic potential of avarol and halichondrin B, the production costs have been compared with cost price of wholesale medicine businesses (Table 10). Wholesale businesses have to pay some margin to the manufacturer of the medicine, which makes comparison of the price not completely sound. However, we included a profit of 10 % in the production price of avarol and halichondrin B in order to be able to make a rough comparison of prices. Production of avarol by mariculture or *ex situ* culture leads to treatment costs that are comparable with betamethasone-valerate, betamethasone-dipropionate and hydrocortisone, the active substances of the commercial antipsoriasis products Celestoderm, Diprolene and Mildisone, respectively. The treatment costs were calculated based on the concentration of the active substance, the recommended dose for use and the cost price per mg of product (Bufa, 2004). This implies that mariculture and *ex situ* culture of *Dysidea avara* for the production of avarol is a feasible alternative to the currently used pharmaceuticals. Treatment of melanoma with halichondrin B is compared with the treatment with paclitaxel, which is a blockbuster drug for treatment of lung- and ovarian cancer (Francis *et al.,* 1995; Trimble *et al.*, 1994; Goodman and Walsh, 2001). Cancer treatment costs with paclitaxel were calculated by using a treatment protocol (B.C. Cancer Agency, 2003) and the cost price per mg of paclitaxel (21CEC Pharm, 2004). Treatment with halichondrin B is at least one order of magnitude more expensive than treatment with taxol and therefore, production of halichondrin B by *Lissodendoryx* sp. will probably not become a feasible process for the development of anticancer drugs.

Table 10: Patient treatment costs based on cost price of active compound. For avarol and halichondrin B the prices by mariculture / *ex situ* culture have been included. The *ex situ* culture price is the scenario with an increased specific production of a factor 4 and 2 for halichondrin B and avarol, respectively.

	treatment costs [€/patient·year]
avarol	14.4 / 8.8
betamethasone-valerate	7.5
betamethasone-dipropionate	4.9
hydrocortisone	12.4
halichondrin B	12,000 / 7800
paclitaxel	520

The crucial parameter that determines if production of secondary metabolites from sponge biomass is feasible or not, is the product concentration inside the sponge. In the quite exceptional case of avarol, mariculture and *ex situ* culture could provide a feasible method for the production of the secondary metabolites. However, the bioactive molecules are usually present in trace amounts in sponges (Pomponi, 1999), which indicates that large-scale production of sponge metabolites by generation of biomass is not probable in many cases. The most important feature of these molecules from sponges is that they can serve as a source of inspiration for the development of analogues, which can be synthesised by different means. Therefore, combined approaches of (genetically modified) bacterial fermentation (to produce a precursor molecule) followed by a number of chemical steps to produce molecules that are derived from sponge chemicals are probably the most successful methods for actually making medicines based on sponge metabolites. The combination of biological and chemical synthesis is a commonly used strategy for pharmaceuticals and is, for example, used for the synthesis of betamethasone-valerate, betamethasone-dipropionate, hydrocortisone and paclitaxel (Techno-preneur, 2004; Ménard Szczebara *et al.*, 2003; Guéritte-Voegelein *et al.*, 1986). The only sponge-derived pharmaceuticals that have been introduced to the market up to now (the relatively simple structures: Ara-A and Ara-C) are synthesised by microbial fermentation and chemical synthesis, respectively (McConnel *et al.*, 1994). However, more complex molecules, such as halichondrin B, may require a combination of these methods.

Appendix

Explanation of cost calculations (the sources that were used are listed at the end of the appendix):

Investment

- Investment is built-up of the costs for building a factory and permanent equipment.
- The space required for laboratory and storage was estimated. The office size was calculated via the same equation for all production methods, reserving 3.5 m^2 for every employee. The equation that was used to calculate the space required for production was based on the production process and was different for every production method that was assessed.
- The price of process equipment was multiplied by 1.9 to adjust for engineering costs, paint, piping, isolation, etc[15].
- The annual costs were calculated by adding the depriciation of the investment costs to the annual maintenance costs.

  - The investment was depreciated in ten years with a constant sum every year, while an interest rate of 7 % was assumed[15].

  - It was assumed that annual maintenance accounts for 3 % of the total investment[15]. The annual costs were calculated by the sum of the annual budget for depreciation and maintenance.

Mariculture:

Halichondrin B:

Factory:
- 1050 m^2 laboratory
- production space = number of freeze dryers (20 kg working space) * 2 m^2 = 1917 m^2
- 2100 m^2 storage house (large due to seasonal peak)
- 3543 m^2 office

Equipment:
- trawler
- 2 small boats
- diving equipment: depreciation was assumed to be 2 years, 1 diving set per 5 mariculture employees.
- freeze dryer: for a processing time of 24 hours per 20 kg sponge biomass batch, 959 freeze dryers would be required to process 7,000,000 kg of sponge.
- 3 LH-20 columns (150 L)
- 3 ODS columns (4.7 L)

Avarol:

Factory:
- 100 m^2 laboratory
- production space = 100 m^2 (estimation).
- 500 m^2 storage house
- 158 m^2 office

Equipment:
- intermediate-sized boat
- small boat
- diving equipment: Depreciation was assumed to be 2 years, 1 diving set per mariculture employee.
- 4 liquid-liquid extraction tanks (2 m^3)

Ex situ culture:

Halichondrin B:

Factory:
- 1500 m^2 laboratory
- production space = number of freeze dryers * 2 m^2 + number of cultivation tanks * 8 m^2 = 21,276 m^2
- 1500 m^2 storage house
- 919 m^2 office

Equipment:
- 2,420 cultivation tanks (5 m^3)
- 2 carbon heat exchangers (150 m^2)
- For downstream-processing (DSP) the same equipment as with mariculture.

Avarol:

Factory:
- 100 m^2 laboratory
- production space = number of extraction tanks * 2 m^2 + number of cultivation tanks * 8 m^2 = 366 m^2
- 700 m^2 storage house
- 123 m^2 office

Equipment:
- 45 cultivation tanks (5 m^3)
- 2 carbon heat exchangers (3 m^2)
- For DSP the same equipment as with mariculture.

Cell culture

Halichondrin B:

Factory: - 1500 m^2 laboratory
- production space = number of freeze dryers * 2 m^2 + number of simultaneously operated hollow fibre reactors * 1/8 m^2 = 707,000 m^2
- 1500 m^2 storage house
- 9130 m^2 office

Equipment: - One 8-channel feed pump per 8 simultaneously operated hollow fibre reactors.
- For downstream-processing (DSP) the same equipment as with mariculture.

Avarol:

Factory: - 700 m^2 laboratory
- production space = number of extraction tanks * 2 m^2 + number of simultaneously operated hollow fibre reactors * 1/8 m^2 = 54,008 m^2
- 700 m^2 storage house
- 199 m^2 office

Equipment: - One 8-channel feed pump per 8 simultaneously operated hollow fibre reactors.
- For DSP the same equipment as with mariculture.

Variable costs

Mariculture

Halichondrin B:

Personnel: - 0.3 employee/long-line based on 231 working days/year[6], 64 employees for DSP and 50 employees for other posts.

Cultivation: - sea (leased) = 100*number of long-lines [m] * 2.5 m (distance between lines) = 761,202 m^2
- number of long-lines: 2500 kg of sponge biomass per long line (Dumdei *et al.*, 1998), including a mortality of 50 %. It was assumed that long-lines can be used for 2 years.

DSP: - The volumes of organic extraction fluids that are required, were extrapolated from Litaudon *et al.* (1997), using their yields in the subsequent DSP steps. This resulted in: ~$6.92 \cdot 10^6$ L CH_2Cl_2, $4.45 \cdot 10^6$ L methanol and $2.74 \cdot 10^5$ L heptane.
- 450 L superdex 200 beads for the LH-20 column, assuming a life time of 0.5 year and a porosity in the column of 0.5 [10].
- 15 L source 30 RPC beads for ODS column, assuming a life time of 0.5 year and a porosity in the column of 0.5 [10].

Avarol:

Personnel: - 0.3 employee/long-line based on 231 working days/year[6], 15 employees for DSP and 15 employees for other posts.

Cultivation: - sea (leased) = 100*number of long-lines [m] * 2.5 m (distance between lines) = 7834 m^2
- number of long-lines: as for halichondrin B.

DSP: - The amount of chemicals that are required, were based on Schatton *et al.* (2002). This resulted in: ~$1.25 \cdot 10^6$ L ethanol, $6.25 \cdot 10^5$ L petrol ether, and $1.25 \cdot 10^4$ kg sodium sulphide.

Ex situ culture:

Halichondrin B:

Personnel: - 1 employee per harvested cultivation tank per day based on 231 working days/year, 64 employees for DSP and 50 employees for other posts.

Cultivation: - $3.5 \cdot 10^8$ kg nutrients (fish powder) based on 2 % efficiency in converting substrate to biomass.

- energy for heat exchanger: 0.43 kW/m^2.°C for 1 hour heating[14].

Required energy = 0.43 * 225 m^2 * 4 °C * 24 h * 365 d = $3.4 \cdot 10^6$ kWh/y.

DSP: - as for mariculture.

Avarol:

Personnel: - 1 employee per harvested cultivation tank per day based on 231 working days/year, 15 employees for DSP and 15 employees for other posts.

Cultivation: - $3.7 \cdot 10^6$ kg nutrients (fish powder) based on 2% efficiency in converting substrate to biomass.

- energy = 0.43 * 4.2 m^2 * 4 °C * 24 h * 365 d = 62,656 kWh/y.

DSP: - as for mariculture.

Cell culture:

Halichondrin B:

Personnel: - 1 employee per 500 harvested hollow fibre reactors based on 231 working days/year, 64 employees for DSP and 50 employees for other posts.

Cultivation: - Hollow fibre bioreactors: based on calculations of table 7.

- 2 L cell culture medium per hollow fibre reactor.

DSP: - as for mariculture.

Avarol:

Personnel: - 1 employee per 500 harvested hollow fibre reactors based on 231 working days/year, 15 employees for DSP and 15 employees for other posts.

Cultivation: - Hollow fibre bioreactors: based on calculations of table 7.

- 2 L cell culture medium per hollow fibre reactor.

DSP: - as for mariculture.

Insurance[15]: 1.5 % of the (annual costs of investment + variable costs).

Overhead[15]: 7.5 % of the variable costs.

Profit[15]: 10 % of the (annual costs of investment + variable costs).

Price list

Factory[1]:

- laboratory: 2000 €/m²
- production space: 800 €/m²
- storage house: 400 €/m²
- office: 1200 €/m²

Employee[7]: 36672 €/year

Equipment	Price (€)	Variable costs	Price (€)
MARICULTURE		**MARICULTURE**	
trawler	400,000	sea[8]	0.1 $m^{-2} \cdot y^{-1}$
intermediate-sized boat	200,000	long-line[6] (100 m)	5,500
small boat	20,000		
diving equipment	1,000	***EX SITU* CULTURE**	
		fish powder[12]	0.58 kg^{-1}
***EX SITU* CULTURE**		energy for heat exchanger[13]	6 kWh^{-1}
cultivation tank[5] (5 m³)	10,000	precursor of halichondrin B	$4.63 \cdot 10^6$ kg^{-1}
heat exchanger[1] (3 m²)	11,000	precursor of avarol (e.g. cholesterol[11])	4636 kg^{-1}
heat exchanger[1] (150 m²)	140,000		
		CELL CULTURE	
CELL CULTURE		hollow fibre reactor (150 mL)[17]	612
8-channel pump[16]	1,100	cell culture medium[18]	10 L^{-1}
stirred tank (2 m³)[19]	450,000		
		DSP	
DSP		CH_2Cl_2[9]	9.51 L^{-1}
freeze dryer[2] (20 kg working volume)	21,600	methanol[9]	8.15 L^{-1}
LH-20 chromatography collumn[3] (150 L)	45,000	heptane[9]	36.7 L^{-1}
ODS-silica column[3] (4.7 L)	13,360	ethanol[9]	4.40 L^{-1}
liquid-liquid extraction tank[4] (2 m³)	21,000	petrol ether[9]	16.5 L^{-1}
		sodium sulfide[9]	34.87 kg^{-1}
		superdex 200 beads[3]	1,660 L^{-1}
		source 30 RPC beads[3]	1,928 L^{-1}

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Summary

Marine sponges are one of the richest natural sources of secondary metabolites with a potential pharmaceutical application. A plethora of chemical compounds, with widely varying carbon skeletons, possessing among other anticancer, antiviral, antibiotic, antiinflammatory and antimalaria activity has been discovered. While for most metabolites their molecular mode of action is still unclear, for a substantial number of compounds the mechanisms by which they interfere with the pathogenesis of a wide range of diseases has been reported. Knowledge on the mode of action is one of the key factors required to transform bioactive compounds into medicines. The rich diversity in bioactive compounds from sponges has provided molecules that interfere with the pathogenesis of a disease at many different points, which increases the chance of developing selective drugs against specific targets (Chapter 2).

Unfortunately, these secondary metabolites are usually present in trace amounts, and natural stocks are too small to sustain the development of widely available medicines. The development of ways to obtain large quantities of the secondary metabolites is therefore currently the most important quest. A number of biotechnological methods could potentially provide the required amount of bioactive substances. Three methods were studied in this thesis:

1. *Ex situ* culture

The term *ex situ* culture refers to cultivation of functional sponges outside of the sea. One of the crucial issues for the *ex situ* cultivation of sponges is the design of a suitable growth medium. Generally sponges are regarded as particle feeders (bacteria and algae), but they are also capable of the uptake of (partly) dissolved organic carbon sources. The use of powdered substrates can be beneficial for the *ex situ* culture of sponges under controlled conditions, because an optimal mix of nutrients can be developed and a constant quality can be guaranteed. The *ex situ* growth rates of sponges cultured on these substrates could be improved, when compared to the sea, but they remain low and resulted in long-term experiments. In order to optimise the growth rate of sponges, it is important to have insight in the way that sponges grow. The suitability of three different models (linear, exponential and radial accretive growth) to describe the growth of both globose and encrusting sponges was assessed. For both morphological appearances, radial accretive growth was the preferred model to simulate the growth. The model can be a valuable tool to make a sound comparison between growth rates of different sponges. In addition, it

can be used to study the quantitative effect of factors, such as pressure, light, current, age, temperature or the nutrient source or –concentration on the growth rate of sponges (Chapter 3).

2. Primmorphs

Primmorphs are spherical-shaped sponge-cell aggregates with a diameter of approximately 1 mm. They are formed from a dissociated cell suspension under gentle agitation and resemble buds and gemmules, which are the naturally produced asexual regeneration bodies. Primmorph formation seems to be a universal characteristic of marine sponges, as they were obtained from seven different species. By scanning electron microscopy (SEM) it was observed that the primmorphs are very densely packed sphere-shaped aggregates with a continuous pinacoderm (skin cell layer) covered by a smooth, cuticle-like structure. The latter characteristic is probably the reason why primmorphs are more robust than functional sponges and can be easily maintained for a long time. Incubation of primmorphs in a rich medium to attempt cultivation of the aggregates frequently resulted in the growth of bacterial, fungal and eukaryotic unicellular contaminants, which prevented a growth study of primmorphs. The addition of gentamycin or a mixture of penicillin and streptomycin could usually avoid bacterial contaminants, but eukaryotic contaminants were persistent. The addition of the fungicide amphotericin B or a cocktail of antibiotics (kanamycin, gentamycin, tylosin and tetracyclin) prevented the formation of primmorphs (Chapter 4).

If primmorphs are actually a kind of experimentally induced regeneration bodies, they could develop into functional sponges. When primmorphs were maintained in seawater enriched with silicate (70 or 150 μM) it was observed that they indeed produced spicules (silica-based skeletal elements) and attached to the bottom of the culture dish, which never occurred at lower silicate concentrations (4 or 25 μM). These results may be explained by available knowledge on the molecular level. Silicate is known to induce the expression of silicatein, the enzyme involved in the production of spicules, at concentrations higher than 60 μM. In addition, silicate has been found to stimulate the biosynthesis of myotrophin, which enhances the production of collagen. Collagen is well known to play an important role in both the attachment of gemmules to a substratum and their subsequent morphogenesis (Chapter 5).

3. Sponge-cell culture

Sponge-cell culture may be the tool to overcome the low growth rate, and the corresponding low production rate of the bioactive metabolites of functional sponges.

However, the presence of large numbers of associated bacteria, fungi and unicellular organisms inside sponges has been a major obstacle in the development of sponge-cell lines. They have prevented the formation of axenic sponge-cell suspensions, and proliferating sponge cells in cell cultures were therefore looked at with suspicion.
For that reason two of prerequisites for the cultivation of sponge cells were developed:

a. A method to distinguish sponge cells in culture from contaminants.
b. A method to assess the viability of cells in culture.

The 18S rRNA gene is a suitable marker to identify the origin of eukaryotic cells and a genetic detection method based on this gene was developed for the sponge *Dysidea avara*. The 18S rRNA gene from a *Dysidea avara* specimen was sequenced and compared to eukaryotic 18S rDNA sequence(s) that were picked up from a proliferating cell culture that originated from a dissociated *Dysidea avara* specimen. This method proved to be successful to unambiguously detect whether the cells in culture were actually sponge cells or contaminants (Chapter 6).
Cell viability is an essential tool to study the effect of medium components on cell physiology. Especially in case of primary sponge-cell lines it is important to know whether slow growth is caused by a low specific growth rate or by a low viability of the cells. Trypan blue exclusion is a commonly used method to estimate the viability of cell cultures, but for unknown reasons this does not work properly with sponge cells. Therefore, a flow-cytometric viability assay, based on the combined use of fluorescein diacetate (FDA) and propidium iodide (PI) was developed. The effects of temperature, ammonium and the fungicide amphotericin B on the viability of a primary cell culture were studied as examples to assess the suitability of the test. Cell fluorescence measurements based on incubation of cells with FDA or PI, resulted in a good and reproducible estimate of the viability of primary sponge-cell cultures. It was found that the cells rapidly die at a temperature of 22 °C or higher, but that they are insensitive to ammonium concentrations up to 25 mM. Amphotericin B was found to be toxic to the cells (Chapter 7) and this could explain why no primmorphs were formed in the presence of this antibiotic.

The current technical status of different methods to produce sponge metabolites was used to study the feasibility of pharmaceuticals from sponges at a large-scale. The production of the metabolites halichondrin B and avarol by chemical synthesis, wild harvest, mariculture, *ex situ* culture, primmorphs, sponge-cell culture, genetic modification and semi-synthesis were compared on a technical and economical basis, as far as possible. Halichondrin B from a *Lissodendoryx* sp. and avarol from *Dysidea avara* were used as model

compounds as their products are opposites with respect to their natural concentration inside the sponge. It is concluded that for avarol, which is present in a relatively high concentration, mariculture and *ex situ* culture could offer feasible methods to compete with currently used medicines against psoriasis. For halichondrin B, the low concentration is a bottleneck for sponge biomass-based production of the compound. A combined approach of (genetically modified) bacterial fermentation (to produce a precursor molecule) followed by a limited number of chemical steps to produce molecules that are derived from sponge chemicals will probably be the most successful method to develop medicines from sponge metabolites that are present in low concentrations (Chapter 8).

Samenvatting

Zeesponzen behoren tot de rijkste natuurlijke bronnen van secundaire metabolieten met potentiële medicinale toepassingen. Een overweldigende hoeveelheid moleculen met sterk verschillende molecuul structuren, die onder andere kanker, virussen, ontstekingen, groei van bacteriën of schimmels en malaria remmen, is ontdekt in zeesponzen. Voor vele van deze verbindingen is de precieze werking (hoe ze ingrijpen in het ziekteproces) onbekend, maar voor een toenemend aantal moleculen is dit mechanisme wel opgehelderd. Deze kennis is een belangrijke voorwaarde voor de ontwikkeling van nieuwe medicijnen uit verbindingen waarvan is aangetoond dat ze, bijvoorbeeld, celgroei remmen. De enorme diversiteit aan bioactieve metabolieten in sponzen heeft geresulteerd in de ontdekking van moleculen die op verschillende plaatsen kunnen ingrijpen in het ziekteproces. Dit vergroot de kansen op de ontwikkeling van een selectief geneesmiddel dat gericht is op de remming van een specifiek enzym of een specifieke receptor (Hoofdstuk 2).

Helaas, zijn deze secundaire metabolieten gewoonlijk aanwezig in minieme concentraties. Bovendien groeien sponzen in de zee over het algemeen tergend langzaam. Daardoor zijn de natuurlijke sponspopulaties te klein om in de behoefte van deze moleculen te voorzien. De grootste uitdaging is daarom op dit moment ook om methoden te ontwikkelen om grote hoeveelheden secundaire metabolieten te verkrijgen. Een aantal biotechnologische methoden zou in deze behoefte kunnen voorzien. Voor dit proefschrift zijn drie methoden onderzocht:

1. <u>Kweek van functionele sponzen in bioreactoren</u>

Het belangrijkste voordeel van de kweek van sponzen buiten de zee is de mogelijkheid om de condities rond de sponzen the reguleren. Eén van de meest essentiële aandachtspunten voor het kweken van sponzen in een bioreactor, is de ontwikkeling van een goed groeimedium. Over het algemeen worden sponzen beschouwd als 'deeltjes eters' (bacteriën en algen), maar ze zijn ook in staat om opgeloste organische koolstof op te nemen. Het gebruik van nutriëntenpoeders is erg aantrekkelijk omdat de exacte samenstelling veel gemakkelijker is te variëren en te optimaliseren dan met hapklare brokjes zoals algen en bacteriën. De groeisnelheden van sponzen in bioreactoren, die werden gevoerd met nutriëntenpoeders, konden worden verhoogd wanneer ze worden vergeleken met de groeisnelheden in de zee, maar geduld blijft erg belangrijk. Om de groeisnelheid van sponzen te optimaliseren is het van belang om te weten *hoe* sponzen groeien. Drie groeimodellen (lineair, exponentieel of een constante toename van de

diameter van de spons) werden gebruikt om de groei van zowel bolvormige als korstvormende sponzen te modelleren. Voor beide verschijningsvormen was groei met een constante toename van de diameter het model dat groei van sponzen het best benaderde. Inzicht in de manier waarop sponzen groeien is belangrijk om groeisnelheden van verschillende soorten te vergelijken. Bovendien kan het gebruikt worden om de kwantitatieve invloed van factoren zoals druk, licht, stroming, leeftijd en voedingsbron op de groeisnelheid te onderzoeken (Hoofdstuk 3).

2. Primmorphen

Primmorphen zijn bolvormige sponscelagregaten met een diameter van ongeveer 1 milimeter. Ze worden gevormd uit een celsuspensie die bestaat uit losse sponscellen en wordt verkregen na het slachten van een spons en filtratie door een heel fijn gaasje. Wanneer deze losse cellen rustig gemengd worden ontstaan de bolvormige structuren die lijken op 'buds' en gemmula, de natuurlijke aseksuele reproductie- en overlevingsstructuren van sponzen. Van zeven verschillende sponssoorten werden primmorphen verkregen en daarmee lijkt de vorming van primmorphen een universele eigenschap van sponzen. Met behulp van elektronenmicroscopie kon worden aangetoond dat primmorphen erg dicht bepakt zijn met cellen en omgeven zijn door een dunne laag huidcellen met daaroverheen een soort waslaag. Dat laatste is waarschijnlijk de reden dat primmorphen robuuster zijn dan functionele sponzen en gemakkelijk voor lange tijd bewaard kunnen worden zonder ogenschijnlijke veranderingen aan de primmorphen. Het blootstellen van primmorphen aan een rijk nutriëntenmedium, met als doel om ze te laten groeien, zorgde er over het algemeen voor dat er van alles groeide, maar geen spons. Bacteriën, schimmels en eencellige eukaryote organismen profiteerde volop van deze nutriënten en overgroeiden de primmorphen. Met de toevoeging van antibiotica kon de groei van bacteriën over het algemeen worden tegengegaan, maar schimmels en andere eukaryote verontreinigingen bleken hardnekkiger. De toevoeging van antischimmelmiddelen blokkeerde de vorming van primmorphen (Hoofdstuk 4).

Als primmorphen werkelijk een soort experimentele reproductielichamen zijn, zouden ze zich kunnen ontwikkelen tot functionele sponzen. Wanneer primmorphen werden geïncubeerd in Petri schalen met zeewater met 70 of 150 μM silicaat (een bouwsteen voor het skelet van de meeste sponzen) bleek dat ze inderdaad skeletnaaldjes maakten en dat ze zich bovendien hechtten aan en zich uitspreidden over de bodem, terwijl dat niet gebeurde bij lagere silicaatconcentraties (4 of 25 μM). Deze resultaten kunnen worden verklaard met kennis op het moleculaire niveau. Van silicaat is bekend dat het de expressie van silicateïne, een enzym dat betrokken is bij de productie van skeletnaaldjes,

induceert als de silicaatconcentratie hoger is dan 60 μM. Bovendien is bekend dat silicaat de biosynthese van myotrophine stimuleert. Myotrophine versterkt op zijn beurt weer de productie van collageen, waarvan bekend is dat het een rol speelt bij de hechting van gemmula aan de bodem en de daaropvolgende morfologische veranderingen (Hoofdstuk 5).

3. Spons celkweek

Spons celkweek zou *de* methode kunnen zijn om de lage natuurlijke groeisnelheid en de daarbij behorende lage productiesnelheid van bioactieve metabolieten op te voeren. Het grote aantal verschillende bacteriën, schimmels en ééncellige organismen die de spons als woning gebruiken, maken het echter moeilijk om alleen *spons*cellen te kweken. Dat is ook de reden waarom publicaties over spons celkweken altijd met enige argwaan worden bekeken.

Daarom werden twee methoden die de status van de cellen in een 'spons' celkweek kunnen bepalen ontwikkeld:

a. Een methode om sponscellen te onderscheiden van verontreinigingen
b. Een methode om te bepalen of sponscellen levend of dood zijn.

Het 18S ribosomaal RNA gen is een geschikt baken om de oorsprong van eukaryote cellen te identificeren. Een genetische detectiemethode op basis van dit gen werd ontwikkeld voor de spons *Dysidea avara*. De genetische code van het 18S rRNA gen van een *D. avara* monster werd opgehelderd en vergeleken met eukaryote 18S rDNA sequenties die opgepikt werden uit een groeiende celkweek van cellen die uit een *D.* avara spons afkomstig waren. Deze methode was succesvol om ondubbelzinnig aan te tonen of de cellen in de celkweek werkelijk sponscellen waren of verontreinigingen (Hoofdstuk 6).

Een hoge levensvatbaarheid van cellen is essentieel wanneer je cellen wilt kweken en de invloed van mediumcomponenten wilt bestuderen. Vooral in het geval van langzaam groeiende cellen is het van belang om te weten of die langzame groei wordt veroorzaakt door een lage specifieke groeisnelheid of omdat de meeste cellen dood zijn. Trypaan blauw is een kleurstof die veel wordt gebruikt om het percentage levende cellen in een celkweek te schatten, maar om onbekende redenen dringt deze kleurstof sponscellen niet binnen. Daarom werd een andere levensvatbaarheidtest ontwikkeld die gebaseerd is op het gebruik van een flowcytometer en twee fluorescerende stofjes: propidium jodide en fluorescine diacetaat, waarmee respectievelijk dode en levende cellen kunnen worden gekleurd. De effecten van de temperatuur, ammonium en het antischimmelmiddel amphotericine B op de levensvatbaarheid van sponscellen werden getest om de betrouwbaarheid van de methode te bestuderen. Celfluorescentie metingen op basis van

propidium jodide en fluorescine diacetaat gaven een reproduceerbare schatting van de levensvatbaarheid van de cellen in primaire spons celkweken. Het werd duidelijk dat sponscellen niet houden van temperaturen boven de 22 °C, maar dat ze ongevoelig zijn voor ammonium. Amphotericine B bleek toxisch te zijn voor sponscellen (Hoofdstuk 7) en dit zou de verklaring kunnen zijn waarom geen primmorphen ontstonden in aanwezigheid van dit antischimmelmiddel.

De huidige technische status van verschillende manieren om sponsmetabolieten te produceren werd geanalyseerd om de haalbaarheid voor de productie van deze metabolieten op grote schaal te bestuderen. De productie van de antikankerstof halichondrine B en de antipsoriasisstof avarol door middel van chemische synthese, verzamelen in de natuur, kweek in zee, kweek in een fabriek, primmorphen, spons celkweek, genetische modificatie en semi-synthese werden vergeleken op basis van hun technische mogelijkheden en economische haalbaarheid, voor zover mogelijk. Halichondrine B en avarol werden gekozen omdat hun natuurlijke concentraties in de spons aan weerszijden van de schaal liggen. Geconcludeerd werd dat avarol, dat in een hoge concentratie aanwezig is, wanneer het door middel van kweek van sponzen in zee of in een fabriek geproduceerd wordt, zou kunnen concurreren met medicijnen die op dit moment worden gebruikt voor de behandeling van psoriasis. Voor halichondrine B is de lage concentratie het knelpunt voor productie door middel van het kweken van sponzen. Een combinatie van bacteriële fermentatie (al dan niet genetisch gemodificeerd) van een bouwsteen van halichondrine met daaropvolgend een beperkt aantal chemische stappen zal waarschijnlijk de meest succesvolle methode zijn om medicijnen te ontwikkelen uit sponsmetabolieten die in lage concentraties aanwezig zijn (Hoofdstuk 8).

Nawoord

Het wetenschappelijk ei is gelegd!
En nu mag er nog even lekker onwetenschappelijk gebrabbeld worden, is beeldspraak een verrijking en zijn stijlfiguren geen modderfiguren meer. Olifantskerkhoven, phylogenetische bossen, paddestoelen plukken, alles mag nu!
Wel volgens goed wetenschappelijk gebruik is de inhoud van het proefschrift niet die, waarop ruim vier jaar geleden werd ingezet (of gehoopt), maar juist die hoop en verbeelding waren nodig om een proefschrift op te bouwen. Verder is het gebouwd op plezier. "De leukste baan van de wereld" zoals ik het vaak heb genoemd. En waarom zou je iets anders doen, als je ook iets heel leuks kunt doen?
En leuk was het niet alleen omdat sponzen nu van die alleraardigste huisdieren zijn, het was het vooral omdat er altijd collega's waren die nooit te beroerd waren om uit gemeende interesse, uit medelijden, met ironie, of uit vriendschap te vragen (al dan niet retorisch) hoe het met de sponzen ging.
Ja **René**, jij vroeg het ook nog wel eens uit wetenschappelijke interesse en bezorgdheid. Zoveel vertrouwen in je mensen en zoveel vrijheid om ideeën aan te passen wanneer sommige oorspronkelijke plannen op sagen gebasseerd leken te te zijn, zoveel mogelijkheden om nieuwe dingen te doen (daarom leeft m'n motto dat alles mogelijk is nog steeds), zoveel herrie op de squashbaan, zoveel humor, zoveel moed (om toch de 8-baan in Disney World in te gaan), en zoveel macht (als belangrijke Zeeuw). OK, genoeg hè? Ik ben blij dat ik je van je afkeer van phylogenetische bomen heb kunnen verlossen. Voor welke baas kan ik nu nog werken?
Ronald, jij hebt me de eerste kneepjes van het vak geleerd en bent erg behulpzaam geweest bij alle kleine en grote dagelijkse (al dan niet biologische) vraagstukken. Het spijt me dat ik zo'n grote aanslag heb gepleegd op je feestquotum (sorry Astrid). Respect voor al je nachtelijke fietstochten naar Lunteren. De vlag mag uitgehangen worden.
Hans, ik ben erg blij dat je me tijdens een communicatie op de valreep wees op de vacature voor het onderzoeksproject waar dit boekje over gaat. Eén week later en iemand anders had dit boekje geschreven.
En dan waren er de andere mariene biotechnologen. **Rouke**, luidruchtige buurman, fotograaf van het WB, schilder van mijn bank, je bent me er één. **Maria**, je hebt mij wat 'zuidelijker' gemaakt en wat van een andere wereld laten zien. Muito obrigado voor het kritisch lezen van (bijna!) mijn hele proefschrift. **Marcel**, **Sebastiaan**, **Mohammad**, **Jan-Willem**, **Wim, Hassan**, en **Eira** bedankt voor jullie hulp en plezier. Dan waren er een hele rits (oud) kamergenoten. **Martijn**, jij was het meest resistent tegen humor van

Ronald en mij alhoewel je wel erg vaak naar het ATO vlucht. **Carlos**, you already left one month after I arrived, but it was a warm welcome. **Jaap**, **Hans** Bosman, **Jan-Willem**, **Simon**, **Daniël**, **René** van den Einde en **Bram** waren allemaal kort of iets langer te gast bij Ronald en mij. Bram, we hebben Moldavië dan weliswaar niet gehaald, maar de Balkan was een mooi avontuur en bedankt voor je hulp met de flowcytometer. **Suzan**, **Ruben**, **Yves**, **Marcin**, **Nejla**, **Vincent**, en **Annette**, jullie hebben een risico-afstudeervak gedaan, maar ik ben erg blij met jullie hulp aan mijn onderzoek. Ik denk dat ik niet alle medewerkers en studenten van proceskunde moet gaan noemen, maar volgens mij heb ik gebruik kunnen maken van de volle breedte van de vakgroep. Het lijkt wel alsof sponzen en extrusie weinig met elkaar te maken hebben, maar daar bleek niets van. Volgens mij is er niemand die ik nooit om raad heb gevraagd. Bovendien maken de vakgroepsbrede teamgeest en de korte lijnen naar iedereen het erg gemakkelijk om vanalles op te zetten. **Fred**, speciale dank voor je hulp bij het op orde brengen en leren begrijpen van alle (bekende en onbekende) processen in onze ecoreactoren. **Karin**, mijn artikelen zijn statistisch beter door jouw hulp. **Pieter**, ik heb altijd erg genoten van jouw meest opruiende opmerkingen. **Tim**, je bent als zendeling in Wageningen neergestreken en je bent er zowaar in geslaagd om mij te doen geloven dat Eindhoven werkelijk mooi is, al zal ik dat natuurlijk nooit in het openbaar toegeven. **Marieke**, las últimas (toch wel zware) páginas de mi tesis kwamen met veel minder zelfmedelijden op papier omdat er in de laatste mooiweer weekenden tenminste iemand nog harder zat te werken dan ik. **Jan**, **André**, **Hans**, **Eric**, **Evert**, **Reinoud** en **Hans**, bedankt voor het vriendelijk in vervulling brengen van al mijn plannen om weer een nieuwe reactor te bouwen. **Boudewijn**, bedankt voor je hulp bij maken van sponzenfilmpjes, de 3-D fotografie (die het proefschrift helaas niet gehaald heeft). **Maurice** Franssen, jouw enthousiasme voor sponzen en hun producten maakte het me wel erg gemakkelijk om je iedere keer weer een naam te laten bedenken voor nieuwe ingewikkelde structuren. **Hans** Heilig, zonder jouw hulp had ik nooit kunnen aantonen dat ik soms heel wat anders kweekte dan sponzen. De sfeer bij microbio maakte het dat ik het helemaal niet erg vond om weer eens microlitertjes te pipetteren en gelletjes te gieten.

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Detmer

PS: Als allerlaatste, SpongeBob bedankt voor het baanbrekende werk ter bevordering van wereldwijde interesse voor sponzen.

Curriculum Vitae

Op 20 augustus 1975 werd Detmer Sipkema geboren in Groningen. Na een hele jeugd in Leek doorgebracht te hebben, werd daar in 1993 het VWO diploma behaald aan het Nienoord College. Vanaf dat moment begon hij aan een studie bioprocestechnologie aan Wageningen Universiteit. Na een korte stop van een jaar om plaats te nemen in de universiteits- en studentenraad vervolgde hij deze in 1998 met een stage aan de Universiteit van Lund in Zweden. Na nog een stage bij DSM-Biologics in Groningen studeerde hij in 1999 af in de cellulair-moleculaire en de technologische richting. De eerste echte baan was als onderzoeker DSM-Biologics. In maart 2000 begon hij aan een promotieonderzoek bij de vakgroep proceskunde aan Wageningen Universiteit.

VLAG Training and supervision plan

Discipline specific activities

Courses

PADI Diving course 2002

Meetings

Nederlands Biotechnologie Congres Ede 2000
Nederlands Biotechnologie Congres Ede 2002
Wageningse kennisdag 2000
Sponge Congress Wageningen 2001
Sponge Congress Rovinj, Croatia 2002
Sponge Congress Rapallo, Italia 2002
Marine Biotechnology Congress Nantes, France 2002
Marine Biotechnology Congress Matalascanas, Spain 2003
Biotechnology and Bioengineering Congress Tampere, Finland 2003
World Congress on Biotechnology Orlando, USA 2004
EU-project meetings (7x) 2000-2003
PhD trip Process Engineering Poland 2000
PhD trip Process Engineering South-Africa 2002

General courses

Hoorcollege geven (OWU course) 2001
Advanced statistics (PE) 2002
Critical reflection on science/technology (Mansholt Institute) 2003

Optionals

Preparation PhD research proposal
Meetings Food and Bioprocess Engineering group, 1999-2003, Wageningen

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