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Review

Cultivation of Marine Sponges

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Abstract: There is increasing interest in biotechnological production of marine sponge biomass owing to the discovery of many commercially important secondary metabolites in this group of animals. In this article, different approaches to producing sponge biomass are reviewed, and several factors that possibly influence culture success are evaluated. In situ sponge aquacultures, based on old methods for producing commercial bath sponges, are still the easiest and least expensive way to obtain sponge biomass in bulk. However, success of cultivation with this method strongly depends on the unpredictable and often suboptimal natural environment. Hence, a better-defined production system would be desirable. Some progress has been made with culturing sponges in semicontrolled systems, but these still use unfiltered natural seawater. Cultivation of sponges under completely controlled conditions has remained a problem. When designing an in vitro cultivation method, it is important to determine both qualitatively and quantitatively the nutritional demands of the species that is to be cultured. An adequate supply of food seems to be the key to successful sponge culture. Recently, some progress has been made with sponge cell cultures. The advantage of cell cultures is that they are completely controlled and can easily be manipulated for optimal production of the target metabolites. However, this technique is still in its infancy: a continuous cell line has yet to be established. Axenic cultures of sponge aggregates (primmorphs) may provide an alternative to cell culture. Some sponge metabolites are, in fact, produced by endosymbiotic bacteria or algae that live in the sponge tissue. Only a few of these endosymbionts have been cultivated so far. The biotechnology for the production of sponge metabolites needs further development. Research efforts should be continued to enable commercial exploitation of this valuable natural resource in the near future.

Key words: Marine sponge biomass, aquaculture, in vitro culture, cell culture, bioreactor design

Introduction

Sponges (Porifera) are primitive, multicellular animals. They are sessile, filter-feeding organisms that feed unselectively on organic particles within a size range of 0.1 to 50 μm (i.e., phytoplankton, heterotrophic bacteria, heterotrophic eukaryotes, and detritus). Sponges are believed to have an advantage over other suspension feeders in that they can both process large amounts of water and efficiently retain the particles. This enables them to inhabit nutrient-poor environments like tropical reef habitats, where they are the dominant group of suspension feeders (Bergquist, 1978). Approximately 10,000 species of sponges have been described, most of which live in marine waters. Some examples of the diversity within the phylum Porifera are shown in Figure 1.

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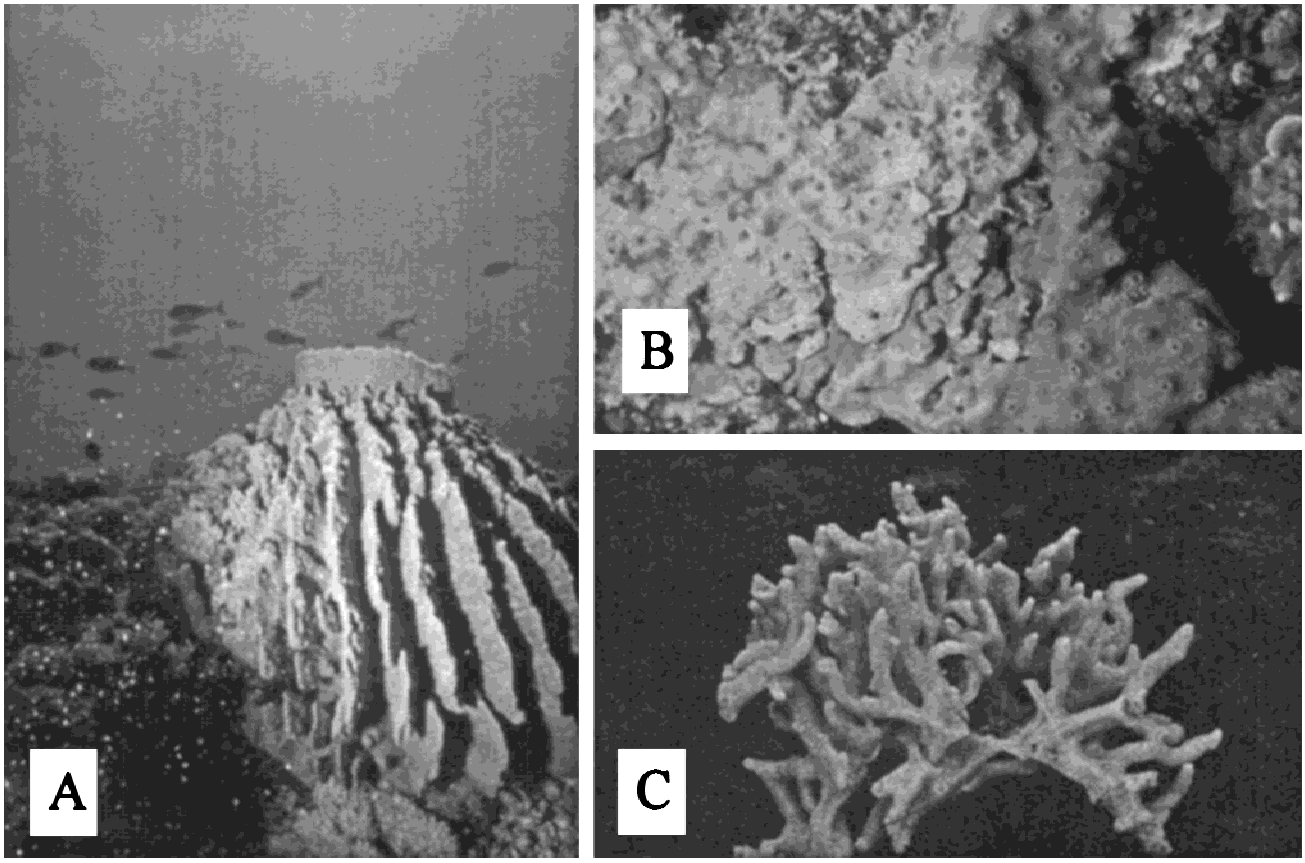


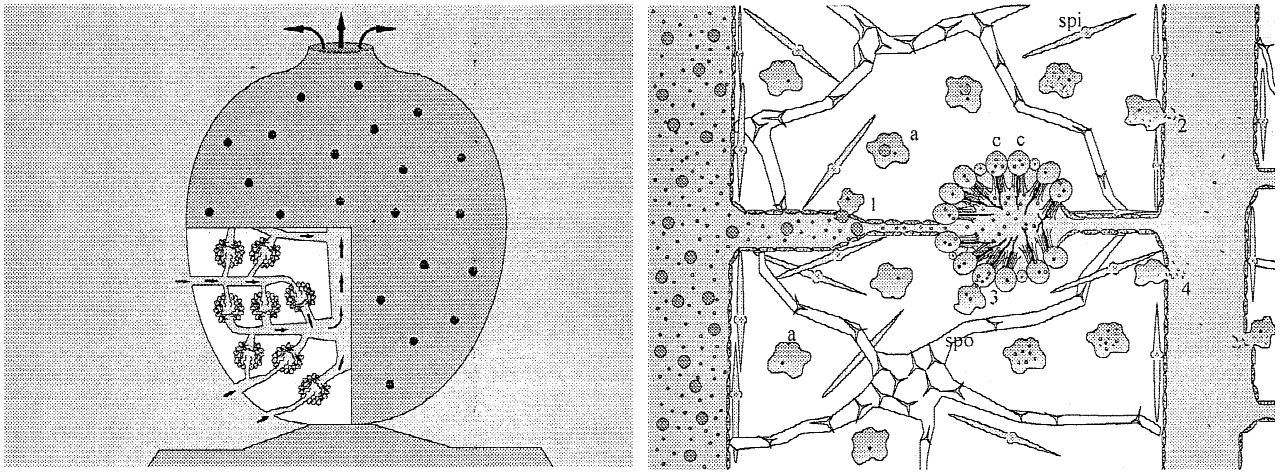
Figure 1. Three examples of the diversity in growth forms within the phylum Porifera. **A:** The giant Indo-Pacific sponge *Xestospongia* sp. **B:** Two encrusting sponge species (*Mycale* sp. and *Tubipora* sp.) **C:** *Halichondria panicea,* a very common sponge with a circumpolar distribution. (*Photographs were provided by R.W.M. van Soest.*)

The organization of a sponge body is simple (Figure 2). Epithelial cells (pinacocytes) line the outer surface and internal system of openings, channels, and chambers, through which water is pumped continuously by flagellated cells called choanocytes. This water current supplies food particles and oxygen and removes metabolic waste products. The major part of the sponge biomass consists of a gelatinous matrix containing free-floating, nondifferentiated cells. This part of the sponge body is called the mesohyl. The mesohyl also contains the skeletal elements of the sponge body: spicules (needle-like structures, made of either silicon or calcium carbonate) and spongin (collagenous fibers).

Some marine sponge species have been of interest to mankind as natural bath sponges for millennia; according to Brusca and Brusca (1990), trade in natural bath sponges was mentioned by Homer and other ancient Greek writers. Hence, there has been interest in methods to produce sponge biomass for ages. The first scientific reports on methods for cultivating sponges were published in the late 19th and early 20th centuries (Smith, 1897; Allemand, 1906; Cotte, 1908). These early studies generally concerned in situ cultivation experiments with species of the family Spongiidae, which were, and still are, of commercial importance as bath sponges.

More recently, sponges have also been discovered as sources of a wide variety of useful natural products like cytotoxins (reviewed by Schmitz, 1994), antifouling agents (e.g., Miki et al., 1996), antibiotics, and anti-inflammatory and antiviral compounds (Munro et al., 1994). In the early 1950s, the first papers on the isolation of bioactive compounds from marine sponges were published (Bergmann and Feeney, 1950, 1951). Since then, research activities on this topic have increased continuously (Munro et al., 1994; Munro et al., 1999). Nowadays, several papers on new bioactive metabolites from sponges are published monthly.

It must be stressed that some of the supposed sponge metabolites may actually not be produced by the sponges themselves but by endosymbiotic microorganisms, which



A **B**

Figure 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 outflowing opening (osculum). A cross-section shows the aquiferous system (channels and chambers); arrows indicate the water flow through the sponge body. **B:** Detail of a channel and a chamber. A layer of flat skin cells (pinacocytes) covers the channel walls and the exterior of the body. Flagellated cells called choanocytes (*c*) form the walls of the chambers. These cells generate the water current through the sponge. The tissue layer between the channels is called the mesohyl, which is basically a gelatinous protein matrix containing several cellular and noncellular components. Noncellular components are spicula (*spi*), needle-like structures made of either silica or calcite, and spongin fibers (*spo*), collagenous fibers that often contain many smaller spicula. Both spicula and spongin are skeletal components. Ameboid cells called archaeocytes (*a*) are the most important cellular components of the mesohyl. Archaeocytes are continuously moving through the matrix and play an important role in the feeding process: larger food particles that are trapped in the narrow parts of the channels can be phagocytized by archaeocytes (*1*). The food particle is then digested while the archaeocyte is moving toward the outflowing channel. Finally, the archaeocyte releases the undigestible parts of the particle in the channel (*2*). Smaller particles are trapped in the choanocyte chambers. The choanocytes have a collar of so-called microvilli around their flagellum. This collar acts as a very fine mesh, trapping even very tiny particles such as bacteria and prochlorophytes. The trapped particles are ingested and digested by the choanocytes and partially transferred to archaeocytes (*3*), which further digest these particles and release the undigestible material in the outflowing channel (*4*).

are present in virtually all sponges (Unson et al., 1994). This aspect is discussed below in the section Endosymbionts.

The "Supply Problem"

The metabolites of interest are often produced only in trace amounts by the sponges or their endosymbionts. Several authors have stressed the fact that much more sponge biomass is needed for commercial production of these sponge metabolites than can be harvested from the seas (e.g., Munro et al., 1994; Pomponi and Willoughby, 1994; Ilan et al., 1996; Osinga et al., 1998a). Currently, this "supply problem" still hampers the development of many promising metabolites from sponges and other marine macroorganisms (Munro et al., 1999; Pomponi, 1999). A good example is the case of halichondrin B, a compound that has, among others, been isolated from the sponges *Halichondria okadai* and *Lissodendorix* sp. The first preclinical test phase for this potential anticancer agent has proceeded successfully, but it cannot be studied further until the supply issue has been addressed (Munro et al., 1999).

The supply problem has stimulated research on alternative methods for sponge metabolite production. Chemical synthesis is the most direct way to produce sponge metabolites in large quantities. As soon as the structure and the biosynthetic pathway of a bioactive compound are known, procedures for chemical synthesis can be developed (Garson, 1994). However, developing a chemical production process can be very expensive (especially when complex

molecules or novel classes of compounds are concerned), and is generally not begun before the target compound has completely passed through the long pathway of clinical trials. Biotechnology may provide an easier and less expensive alternative to achieve a sufficient supply of the target compound to continue clinical research. This realization has given new impulse to research on methods for cultivation of sponges and production of sponge biomass. In the case of halichondrin B, biotechnological production of sponge biomass is currently considered the only feasible option to complete the pathway to commercialization of this compound (Munro et al., 1994, 1999).

Biotechnological production of sponge metabolites may even be a permanent alternative to chemical synthesis, as it is not always possible to develop low-cost, large-scale chemical production methods for the often unusual and complex structures of bioactive compounds (Pomponi, 1999). In addition, the yield of the chemical process is sometimes poor. For example, the maximal yield of a total chemical synthesis of latrunculins (cytotoxic compounds isolated from the sponge *Latrunculia magnifica*) was only 2% (Smith et al., 1992; White and Kawasaki, 1992). As this sponge species is not commonly found, and latrunculin concentrations in the sponge tissue are relatively low (up to 0.35% of the dry weight), harvesting from the sea is also not a durable option for the commercial production of these compounds (Ilan et al., 1996). In this case, sponge cultivation may again be the best alternative.

When concentrations of target compounds in the sponge tissue are high, biotechnological production could also be an interesting option. For instance, the antibacterial polybrominated biphenyl ethers in the Indo-Pacific, encrusting sponge *Dysidea herbacea* can constitute up to 12% of the sponge dry weight (Unson et al., 1994). In this case, production of a kilogram of target compound would require only a few cubic meters of sponge volume, an amount that can be obtained with the existing techniques for in situ sponge cultivation.

As was mentioned previously, the possibility of cultivating sponges in situ was already being investigated long before the discovery of sponge metabolites as useful natural products. The methods described in these early studies were based on the strong regenerative capacity of most sponge species: pieces of living sponge tissue are able to grow and regenerate into healthy sponges. Although this observation had already been made by Aristotle in 350 B.C., Schmidt (1863) was the first to suggest that this strong regenerative power of sponges could be the basis of a cultivation method. Since then, many in situ culturing experiments based on this principle have successfully been carried out (see next section for details). However, the in situ conditions of temperature and nutrition are not always optimal for rapid biomass production. In vitro cultivation of sponges may be a useful alternative because it can be done under controlled conditions. It will also enable more efficient downstream processing, especially when the target product is continuously released by the sponges. Unfortunately, long-term in vitro cultivation of marine sponges has been found to be extremely difficult (Arndt, 1933; Kinne, 1977; Fosså and Nilsen, 1996). Mass production of sponge biomass under completely controlled conditions has not been realized so far.

Scientific information on in vitro cultivation of sponges is scarce and often anecdotal. More than 20 years have passed since Kinne (1977) wrote the latest extensive review on this subject. It is the aim of the present review to bring together the older information and the latest developments in the field of sponge cultivation and sponge biomass production. The potentials and problems of the different approaches will be evaluated.

Aquacultures

Until recently, sponge aquaculture focused exclusively on the production of natural bath sponges. The trade in natural bath sponges, which already had a long history, peaked in 1938 with a total production of 2.6 million pounds, of which a substantial part came from aquaculture (Brusca and Brusca, 1990). In 1910, Moore gave a detailed description of a method for cultivation of bath sponges in the sea. The sponges were cut into pieces, which were attached with an aluminum wire to an artificial substratum (concrete disks) and placed into the sea. The basic procedures described by Moore (1910) are still used worldwide to produce commercial bath sponges. Such large-scale bath sponge aquacultures in several parts of the world have been described by Cahn (1948), Moore (1951), Storr (1964), Shubow (1969), Vacelet (1985), Verdenal and Vacelet (1990), Adams et al. (1995), and others. An example of bath-sponge aquaculture is shown in Figure 3.

MacMillan (1996) has written a manual with instructions for setting up a commercial sponge aquaculture farm with sponge cuttings fixed onto nylon ropes. He emphasizes

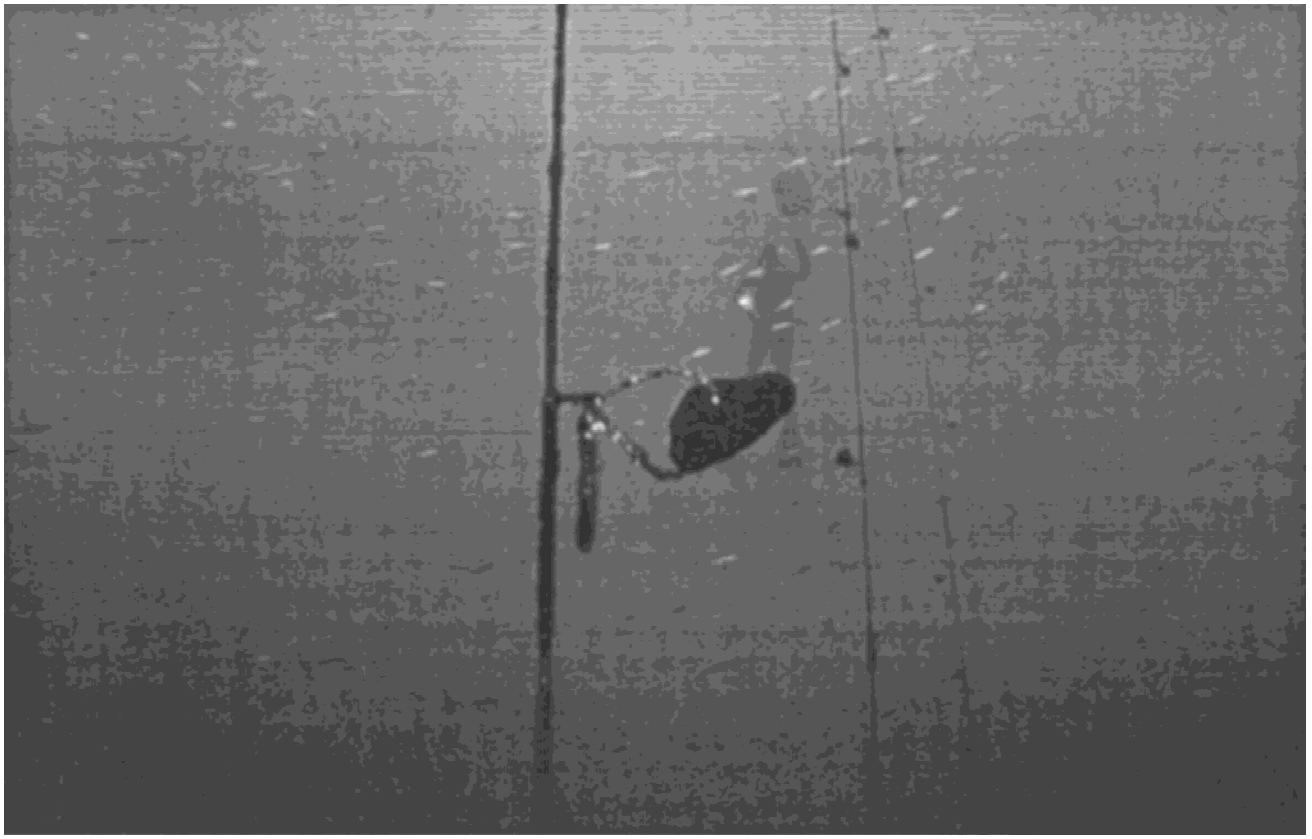


Figure 3. Aquaculture of the natural bath sponge *Spongia officinalis* on vertical ropes in the Mediterranean. (*Photograph provided by J. Vacelet*).

that when making the sponge cuttings, razor-sharp knifes should be used, and that at least one side of the cutting must be covered by skin cells (pinacoderm). The proportion of the cutting that should be covered with pinacoderm depends on the size of the cutting: smaller cuttings should have a larger percentage of the outer surface covered. The sponge cuttings should never be removed from the water (air will then be pumped into the sponge body, and this will block the water circulation). The sponge cuttings must not be squeezed, as this can damage the tissue. Salinity changes (due to the input of fresh water from rivers) can also kil the sponges. When selecting the location for a sponge farm, outflowing areas of rivers should therefore be avoided.

A technique successfully used by Japanese sponge farmers before World War II was described by Cahn (1948): ropes with sponge cuttings were anchored on the seafloor and connected to a float (usually a sealed bottle) to hold them in a vertical position.

In recent times, sponge aquaculture studies have also been directed toward natural products research. In New Zealand, progress is being made with aquaculture of sponges for metabolite production (Battershill and Page, 1996; Munro et al., 1999). Here, not only do sponges have to be grown fast and economically, but they also need to be cultured in a manner that promotes target metabolite synthesis. Recently, five species of marine sponges (*Latrunculia brevis, Lissodendoryx* n.sp., *Mycale murryi, Polymastia croceus,* and *Raspailia agminata*) were grown in sea-based cultures with maintenance of target metabolite synthesis (Battershill et al., 1998; Duckworth et al., 1998; Munro et al., 1999). Munro et al. (1999) estimated that an annual production of a few tons of the sponge *Lissodendorix* n.sp. 1 is needed for the clinical tests with halichondrin B to proceed. Such production figures could be realized in the near future with the existing aquaculture methods (Munro et al., 1999).

For the next 50 years, a commercial farm-scale sea space has been secured in New Zealand for exclusive sponge, invertebrate, and algal aquaculture feasibility research aimed at generating novel natural products of chemotherapeutic interest. This location is used in addition to

more than eight other locations throughout New Zealand, allowing both the examination of optimal growing localities for each sponge species, and the analysis of variability in target metabolite biosynthesis (C.N. Battershill, personal communication).

The physical conditions at the culture locations were found to determine the culture success to a large extent. Duckworth et al. (1997) placed explants of three sponge species (*Psammocinia hawere, Raspailia agminata,* and *Raspailia topsenti*) in both sheltered and exposed locations. Only one of these species was successfully cultured. At the sheltered location, explants of *R. agminata* exhibited growth, but at a rather low rate (25% growth in 262 days). Another recent study by Duckworth et al. (1998) showed that proper selection and use of carrier materials for the explants is another key factor in aquaculture success.

Location influenced not only growth but also target metabolite synthesis. Frequently, sponges growing least well at certain locations were found to elicit the highest concentrations of metabolites of interest (Battershill et al., 1998). Experiments have been carried out suggesting that it will be possible to artificially enhance the yield of target metabolites prior to harvest, thus optimizing yield from most locations (Battershill, 1990; Battershill et al., 1998).

Stevely et al. (1978) evaluated the advantages and disadvantages of commercial sponge aquaculture. One of the disadvantages is that a good location is difficult to find. As was mentioned previously, in situ conditions (temperature, nutritional state) are often suboptimal for growth. Hence, growth rates are usually low. MacMillan (1996) reported that sponge cuttings of 150 to 300 g need 2 years to grow to commercial-size bath sponges (800 g), which means that the doubling rate of these sponges is approximately 1 year. On the other hand, the fastest growth rate ever reported for a sponge (5000% growth within 1 month) also came from an aquaculture study (Battershill and Page, 1996), which nicely illustrates the variability within the natural system.

To overcome the problem of suboptimal in situ conditions, Barthel and Theede (1986) used semi-enclosed, temperature-controlled vessels, through which unfiltered seawater was pumped continuously. In these semi-enclosed systems, cuttings of *Halichondria panicea* could successfully be grown when cultures of the freshwater alga *Chlorella* sp. were regularly added as a food supplement. Although their study was primarily set up to develop a cultivation method for fundamental sponge research, their findings may also have good biotechnological potential.

Short-Term Cultivation

Owing to the low success of long-term cultivation so far, many records on cultivation of sponges in vitro concern short-term, small-scale experiments, of which most were conducted to investigate fundamental sponge biology rather than to produce sponge biomass.

An important pioneer of this in vitro experimental sponge science was H.V. Wilson, who described rearing functional sponges from eggs (Wilson, 1898) and from dissociated cells (Wilson, 1907). In the latter study, tissue of *Microciona prolifera* was squeezed through a thin mesh to dissociate the cells. The released cells reaggregated and differentiated into small sponges with a functioning aquiferous system within 1 week. These sponges could be kept in aquaria for several weeks.

Since the early work of Wilson (1907), the process of dissociation and reaggregation has been studied extensively (see review by Müller, 1982). Generally, two methods are used to dissociate cells artificially: the mechanical method described by Wilson (1907) and chemical dissociation using calcium- and magnesium-free artificial seawater. The latter method is based on the crucial role that Ca^{2+} ions play in the cell-to-cell adhesion process (Müller, 1982). The cell suspensions obtained can be used to produce small sponge aggregates that may form the starting point for an in vitro culture. Artificial sponge cell dissociation, combined with sponge cell separation techniques using density gradients (De Sutter and Van de Vyver, 1977; De Sutter and Tulp, 1981), is also used to produce primary cell lines for sponge cell culture (see Cell Cultures, below).

A frequently used technique to produce short-term, small-scale cultures of freshwater sponges is the so-called sandwich method, introduced by Ankel and Eigenbrodt (1950). Young sponges, obtained from recently hatched gemmules (egg-like reduction bodies, mainly found in freshwater sponges), are grown between a glass slide or plate and a coverslip. In this way, thin, almost two-dimensional sponges are obtained that are very suitable for microscopic observation. Hence, the sandwich method has turned out to be a useful tool for anatomic and physiologic studies (e.g., Kilian, 1952; Weissenfels and Landschoff, 1977).

Langenbruch (1983) was the first to apply this method to a marine species (*Halichondria panicea*). Instead of gemmules, explants of approximately 1 mm^3 were used as a starting point for cultivation. The sandwich cultures obtained were thicker than cultures of freshwater species, but

still suitable for morphogenetic research. Shortly after Langenbruch's publication, Sanchez-Moreno (1984) used the sandwich method to study effects of light exposure on two tropical sponge species (*Desmapsamma anchorata* and *Aplysina fistularis insularis*). However, there is no publication that provides details on the feeding of these sandwich cultures, and there are no data available on long-term maintenance of sandwich cultures in aquaria.

Long-Term in Vitro Cultivation

Many records of successful long-term in vitro cultivation of marine sponges are observations from large public aquaria (reviewed by Arndt, 1933; Kinne, 1977) or incidental observations by private aquarium holders. Much of the latter information has been collected and published by Fosså and Nilsen (1996). Although their book is written from an aquarium holder's point of view, it is a good starting point when selecting a model species for the development of large-scale in vitro cultures.

Aquaria with running, unfiltered seawater seem to be the most successful systems with respect to long-term in vitro cultivation of marine sponges. Although food supply and temperature can be optimized in such systems (see Barthel and Theede, 1986), they are still partially dependent on the quality of the natural seawater that flows through. A completely controlled system would not have this disadvantage, but, as stated by Kinne (1977): "isolation of sponges under controlled nutritional conditions has remained a problem." A solution to this still-existing problem may be found by evaluating the factors that may limit or inhibit in vitro growth of sponges. In the next subsections, we will summarize possible limitations, restrictions, and necessary conditions for controlled in vitro growth of sponges. It must be realized that there will probably never be a standard method for sponge cultivation. Large differences between species may occur, as sponges are a diverse group, inhabiting a wide range of marine environments.

Growth-Limiting Factors

Food

Perhaps the most important question to be addressed when designing an in vitro sponge culture is how to supply adequate food. In the sea, the available amount of unfiltered seawater for the sponges is nearly unlimited, and this water contains a mixed diet of several species of bacteria, algae, heterotrophic eukaryotes, and dead organic material. Most smaller aquaria are closed recirculation systems that are continuously biofiltered to keep the water clean. In these closed, filtered systems, the availability of suitable food particles for sponges is limited. Arndt (1933) already mentioned that starvation was the major cause of death of sponges in small aquaria. Hence, a sufficient supply of suitable organic particles should be established in an in vitro sponge culture. This prerequisite contains both a quantitative aspect (How much is sufficient?) and a qualitative aspect (Which particles are suitable?). Both aspects should be studied for every sponge species that is to be cultured.

Silicon

The skeletons of demosponges (class Demospongia, the largest class within the phylum Porifera) and glass sponges (class Hexactinellida) are partially built of siliceous structures called spicules. These spicules are secreted by specialized cells (sclerocytes). Silicon is taken up by these cells from the water as dissolved silicic acid and deposited around a proteinaceous filament (Bergquist, 1978).

Siliceous spicules can contribute significantly to the total sponge biomass. For instance, spicular silica contents up to 62.3% of the dry weight have been found in species of the order Haplosclerida, and up to 74.9% in species of the order Petrosiida (Desqueyrouz-Faundez, 1990). In some Antarctic species, silica accounted for even more than 90% of the dry weight (Barthel, 1995). This implies that the availability of dissolved silicon in seawater can easily become growth-limiting for demosponges and glass sponges, especially in small, closed systems.

The skeleton of calcareous sponges (class Calcarea) consists of calcium carbonate, which is usually present in sufficient amounts in seawater and therefore not likely to become a growth-limiting factor.

Oxygen

If running the bioreactor is cost-determining, the overall volumetric productivity should be as high as possible, implying a high biomass density. As a consequence, the supply of oxygen often becomes a limiting factor. Sponge respiration rates per cubic centimeter of sponge volume have been found to vary between 0.2 and 25 μmol O_2 h^{-1} (Table 1). These rates are comparable to those for other

Table 1. Oxygen Consumption Rates in Marine Sponges

Species	Temp. (°C)	Oxygen consumption in μmol O_2 h^{-1} per g wet weight	per ml wet volume	Reference
Calcarea				
Grantia sp.	—	4.5–6.7		Hyman (1925)
Sycon ciliatum	12.3	24.7*		Cotter (1978)
Demospongia				
Axinella polycapella	25–28	0.58*	0.54	Osinga et al. (1998b)
Axinella waltonsmithi	25–28		0.72	Osinga et al. (1998b)
Carteriospongia foliascens	—	1.67	1.35*	Wilkinson (1983)
Carteriospongia sp. fp	—	2.36	1.91*	Wilkinson (1983)
Carteriospongia sp. fr	—	2.34	1.90*	Wilkinson (1983)
Cinachyrella apion	25–28	0.56*	0.64	Osinga et al. (1998b)
Halichondria panicea	14		1.97	Thomassen and Riisgård (1995)
	20		2.61	R. Osinga (unpublished data)
	?		1.37	R. Osinga (unpublished data)
	1.4–16.1		0.21–0.98	Barthel (1986, 1988)
Haliclona rubens	—		6.7–24.6	De Laubenfels (1932)
Hippospongia equina	—	0.24		Von Pütter (1914)
Iotrochota birotulata	—		2.99–6.88	De Laubenfels (1932)
Ircinia ramosa	—	0.77	0.62	Wilkinson (1983)
Jaspis stellifera	—	0.85	0.69	Wilkinson (1983)
Mycale sp.	26.5–29.5		1.97	Reiswig (1971, 1974)
Neofibularia iriata	—	1.42	1.15	Wilkinson (1983)
Pericharax heteroraphis	—	0.95	0.77	Wilkinson (1983)
Phyllospongia papyracea	—	2.41	1.95	Wilkinson (1983)
Pseudaxinyssa sp.	—	0.94	0.76	Wilkinson (1983)
Pseudosuberites andrewsi	25–28		1.60	Osinga et al. (1998b)
Spongia sp.	—	0.99	0.80	Wilkinson (1983)
Suberites carnosus	10	1.70–2.56		Cotter (1978)
	6.3	0.19–0.53		Cotter (1978)
Suberites domuncula	—	0.35		Von Pütter (1914)
Suberites massa	22	1.07		Nicol (1970)
Tethya aurantium	—	0.33		Von Pütter (1914)
Tethya crypta	26.5–29.5		0.89	Reiswig (1971, 1974)
Verongia gigantea	26.5–29.5		3.04	Reiswig (1971, 1974)

*These values are not direct measurements, but have been calculated using weight-to-volume conversion factors.

invertebrate groups like crustaceans and mollusks (Nicol, 1970), which can be cultivated at high densities. This suggests that with respect to oxygen supply, sponges may be suitable for high-density cultivation as well. However, some sponge species are sensitive to hypoxic conditions (Barthel and Theede, 1986; Ates, 1991; D. Barthel, personal communication). In cultures of these species, oxygen supply should be in excess of the oxygen consumption in the bioreactor and oxygen mass transfer should be high.

Light

Many tropical sponge species contain photosynthetic endosymbionts (cyanobacteria), comparable to the zooxanthellae of corals. The organic matter produced by these endosymbionts can act as another food source for the sponge (Sarà, 1971). Wilkinson (1983) measured net primary productivity in six common sponge species at the Great Barrier Reef, indicating the potential importance of

this process as a food source for sponges. The distribution of some sponge species is largely dependent on the light intensity (Wilkinson, 1978).

Sponges with a high endosymbiotic photosynthetic activity may be very suitable for in vitro cultivation because light may serve as an energy source, thus decreasing the need to provide an adequate organic food source to the culture. The tropical Indo-Pacific sponge *Collospongia auris,* which contains such endosymbionts, is often found in aquaria and considered to be easy to cultivate when enough light is available (Fosså and Nilsen, 1996).

Growth-Inhibiting Factors

Unuseful Particles

As a consequence of their unselective uptake of particles, sponges also ingest unuseful particles like suspended clay or silt. It was found that sponges significantly reduced their pumping rate when they were exposed to an increasing concentration of clay particles (Gerodette and Flechsig, 1979). A reduced pumping rate implies a lower food uptake rate and, hence, lower metabolic activity and slower growth. This may explain why the growth rate of the bath sponge species *Spongia officinalis* was found to be much lower in turbid, polluted water near a sewage discharge area, than at clear, open-sea stations (Verdenal and Vacelet, 1990).

Air

Sponges should not be exposed to open air or air bubbles. When air enters the canal system, it will damage the choanocyte chambers and block the sponge pump (Fosså and Nilsen, 1996). Many sponges die after only a short exposure to air, for instance, during transport. Sometimes, however, air in the canal system can be removed by firmly shaking the sponge underwater. Sponges inhabiting intertidal areas may be adapted to exposure to air, as was suggested for *Halichondria panicea* (Vethaak et al., 1982) and *Hymeniacidon sanguinea* (Aiello et al., 1993). An underlying physiologic mechanism for this air tolerance was not described by these authors. Possible mechanisms are slime production at the surface to keep the surface layer wet or rapid closing of the ostia or both.

Light

Although light stimulates the growth of sponge species with photosynthetic endosymbionts, it also inhibits the growth of certain sponge species (Wilkinson and Vacelet, 1979; Fosså and Nilsen, 1996). Sensitivity to UV radiation is thought to be a major cause of this photoinhibition (C.R. Wilkinson, personal communication).

Salinity and Temperature

The average salinity of seawater (i.e., the average concentration of dissolved salts) is 35‰ by weight. Marine organisms have adapted themselves to this saline environment, and it is obvious that rapid changes in the salinity will affect the osmoregulation of these organisms. Although species living in shallow or estuarine waters may be used to sudden changes in salinity, most marine organisms, including sponges, will be sensitive to such changes. The sparse information available on effects of salinity on sponges suggests that they are most sensitive to low salinities. MacMillan (1996) states that salinity downshocks are very harmful for tropical bath sponges and advises not to cultivate these sponges in estuarine regions. Storr (1964) described the effects of salinity on the bath sponge *Hippospongia lachne* (commonly known as wool sponge). This species could withstand increased salinities up to 46%, but salinities below 26% were lethal.

In addition to salinity shocks, temperature shocks also can affect the condition of sponges. Most sponges experience only slow (seasonal) changes in temperature in their natural environment and may therefore not be adapted to strong and rapid temperature fluctuations. Arndt (1933) mentioned, in this respect, that temperature downshocks are better tolerated than upshocks. A similar trend was reported by Storr (1964) for wool sponges: the sponges decreased in size, but remained healthy after a temperature downshock. According to F.G. Walton Smith (as cited in Storr, 1964), a temperature upshock was detrimental to commercial sponges.

Water Quality

It is often stated that sponges require "high water quality." This usually means that the water should not contain high concentrations of dissolved organic and inorganic compounds and should have a normal and stable pH. Seawater is slightly alkaline, with a pH varying between 7.8 and 8.4 (Brown et al., 1992). A decrease of pH below 7.8 indicates unusual circumstances like low salinity or hypoxia, conditions that are unfavorable for the cultivation of marine animals. Also, high phosphate concentrations may decrease the pH.

A well-known problem with high-density cultures of aquatic animals is the accumulation of inorganic nitrogenous metabolic waste products in the surrounding water. Ammonia (here defined as both NH_3 and NH_4^+), the most common nitrogenous waste product, can rapidly reach concentrations that reduce the growth rate of the animals or even kill them. Lethal concentrations for 50% of the population (LC_{50}) of 60–100 μM were found for different species of invertebrates and fishes (Richardson and Gangolli, 1993), indicating that very low concentrations may already have considerable effects on animals.

Biological effects of ammonia are influenced by the pH of the seawater, which determines, to a large extent, the ratio between NH_3 and NH_4^+. The ionic form is far less toxic because it is unable to permeate cell membranes. At higher pH, the proportion of NH_3 (and thus the toxicity) increases. The exact relation between pH, NH_3 and NH_4^+ is described by Bower and Bidwell (1978). In addition to a pH effect, the dissolved oxygen concentration also affects ammonia toxicity, which is stronger at lower oxygen concentrations (Kinne, 1976).

Most literature on ammonia toxicity in marine animals concerns commercially important groups like fish and crustaceans (see reviews by Colt and Armstrong, 1981; Meade, 1985; Randall and Wright, 1987). In these animals, ammonia mainly affects the respiratory system (damage of gill tissue and blood cells). The effects of ammonia on lower invertebrates are less well known. We have not found any information on dose-response relations for ammonia and sponges, or on the production and release of this compound by sponges. Both should be studied to estimate the importance of ammonia accumulation in high-density sponge cultures.

Poisons and Contaminants

A toxic compound produced in a closed recirculation system can rapidly accumulate and affect the sponges. Toxins of both artificial and natural origin may occur in bioreactors. Examples of contaminants from artificial sources are softeners from rubber tubes that dissolve into the medium, or metal ions from stainless steel equipment. It is beyond the scope of this review to deal with these contaminants, which are not specific for sponges. An overview of possible chemical contaminants and their control is given by Bernhardt (1977).

Examples of natural toxins are the bioactive compounds produced by many sponge species; these compounds are produced as a chemical defense against predation (Pawlik et al., 1995; Chanas et al., 1996), or to prevent fouling and settlement (competition for space) by other organisms (Porter and Targett, 1988; Davis et al., 1991; Bakus et al., 1994; Beccero et al., 1997). Interspecific chemical interactions between sponge species may cause problems in mixed cultures. It is not yet known whether intraspecific interactions of this kind can occur as well.

Dying or decaying sponges can also pollute the water. A good example of this is the release of acetylene by dying *H. panicea* specimens, which can poison all the other sponges in an aquarium (R. van Soest and H. Baptist, personal communication). Release of the above-mentioned toxins from decaying sponges may have similar effects. Besides, large amounts of ammonia are produced when a sponge is degraded by bacteria. Other toxic microbial waste products, such as reduced inorganic sulfur compounds, may be formed as well, especially during the decay of larger sponges.

Futhermore, problems can be caused by culturing sponges together with other organisms. For instance, free-floating stinging cells released by anemones will enter the sponge body with the incurrent water flow through the ostia and cause damage inside the sponge (De Graaff, 1969).

Diseases

Contagious sponge diseases can have catastrophic effects on natural sponge populations and maricultures (Storr, 1964; Vacelet et al., 1994). In 1938–1939 and 1947–1948, a great deal of the commercial sponges in the Gulf of Mexico were killed by such a disease (Storr, 1964). It was observed that survival in low-density populations was much better than in high-density cultivation sites. Hence, such contagious diseases could be extremely harmful when introduced in an in vitro culture. Osinga et al. (1998b), who attempted to culture four tropical marine demosponges in a closed system, once observed that all specimens of one species declined within 1 week, while the other species remained unaffected. They suggested a species-specific disease to be the cause of this mortality.

Although little is known about the exact causes of and cures of sponge diseases (see reviews by Lauckner, 1980; Peters, 1993), it is assumed that high temperatures increase the susceptibility of sponges to pathogens. The 1986–1990 outbreak of disease in the Mediterranean mainly affected populations in the warmest regions (Vacelet et al., 1994). Peters (1993) suggested a relation between global warming

and the enhanced development of marine diseases including sponge diseases.

Predators

Sponges are generally assumed to be well protected against predation. Their dispersed spicule skeleton makes it unattractive to eat them. Besides, many sponges produce toxic compounds, which are sometimes specific antipredation agents (Pawlik et al., 1995; Chanas et al., 1996). Nevertheless, some natural enemies of sponges do exist. In particular, nudibranches are known to eat sponges. The nudibranches sometimes accumulate the sponge toxins, which also protects them from predation (Cimino and Sodano, 1994). Although accidental introduction of adult nudibranches, sea urchins, or parrotfishes to an in vitro sponge culture is not very likely, the possibility that the young of these animals are cointroduced with the sponges must not be overlooked.

Design of an In Vitro Sponge Culture

When designing a bioreactor system for in vitro sponge cultures, the limiting and inhibiting factors mentioned in the previous sections should be taken into account. Of course, the sensitivity of sponges to environmental factors is largely species dependent. Coral reef sponges come from a relatively unpolluted, oligotrophic, and constant environment and may be much more sensitive than species from eutrophic, temperate waters or intertidal areas. In addition, the food particle composition will vary largely between different environments. The natural environment of a sponge species should be well known before an in vitro culture system is designed for it. In the next section, problems and possible solutions for bioreactor designs will be reviewed and discussed.

Selection of Sponges

The easiest method to obtain sponge material for an in vitro culture is to collect adult individuals from an in situ population. Explants (cuttings) of these parent sponges are a good starting point for an in vitro culture, as they tend to grow faster than intact sponges (Kinne, 1977), although this may hold true primarily for species with an encrusting growth form (Duckworth et al., 1997). Techniques to prepare explants are described by MacMillan (1996) and Simpson (1963).

It is important to realize that the success of a culture largely depends on the condition of the selected parent sponges. To increase the chance of culture success, the parent sponges must have a healthy appearance and should be clean—i.e., free of epibionthic flora and fauna, free of lesions, and free of endosymbiotic macrofauna (D. Barthel, personal communication). Other organisms that may cause problems, such as anemones and predators, can easily be introduced into the culture by accident, along with the sponges. Hence, the sponge material should be examined carefully before it is introduced into a culture vessel. Sponges or explants that start to decay (despite their initially healthy appearance) should be discarded from the culture as soon as possible, in order to prevent poisoning of the other organisms with sponge toxins or (bacterial) degradation products.

Although collecting adult sponges from the sea is the easiest way to obtain material for in vitro culturing, it may be better to raise sponges from early developmental stages like eggs, reduction bodies, or larvae. Sponges grown this way will be adapted to the in vitro conditions from the start and may show better long-term growth and survival. The first to realize this was Wilson (1898), who described a simple method to obtain eggs and raise sponges. Sponges were collected during the breeding season (summer and early autumn), raised near the surface, and placed into a small bucket or aquarium. Within half an hour, several eggs (now called larvae) had spontaneously been released and could be collected from the water. The collected larvae were put in glass dishes that were placed in aquaria with seawater, which was regularly exchanged. After the free-swimming larvae had attached to the walls of the dishes, young sponges began to form, which could be held in aquaria for at least a few weeks.

The breeding season mentioned by Wilson (1898) already suggests a relation between reproduction and temperature. Although some sponge species continuously produce eggs and larvae, formation of reproductive elements is often correlated with an increasing temperature (e.g., Simpson, 1968; Fell and Lewandrowski, 1981; Barthel, 1986). Fry (1971), who studied liberation of larvae of *Ophlitaspongia seriata* in a semi-enclosed system, found that liberation started at a threshold temperature of 18°C. However, the actual liberation process was triggered by exposure to light and a sudden decrease in current velocity. Light-induced oogenesis was also suggested by Elvin (1976). The findings of Fry (1971) may thus hold true for more species and could be the basis of a method for inducing sponge spawning and, thus, producing young sponges for cultivation.

Two other possibilities for obtaining early developmental stages are collection of reduction bodies and artificial creation of early developmental stages by dissociation and reaggregation of sponge cells (Kinne, 1977). The latter technique was described above under Short-Term Cultivation. Reduction bodies (also called buds or gemmules) are mainly formed when sponges encounter unfavorable conditions. The sponge tissue withdraws, and small clumps of cells are formed that can survive long periods of starvation, desiccation, and low temperature. Under more favorable conditions, the reduction bodies will quickly regenerate into functional sponges (Bergquist, 1978).

Size of Sponges in a Bioreactor

In the wild, sizes of sponges vary from 1 mm to 1 m or more. To what extent the size of a sponge determines its metabolic functioning and growth rate is unknown. In sponges, mass transfer takes place both by diffusion and by convective flow through the aquiferous system (pumping, see Figure 1). In larger sponges, mass transfer of oxygen, silicon, or food into the deeper interior of the sponge body may become a limiting factor, thus reducing the overall growth rate of the sponge. It is therefore important to determine internal mass transfer rates in sponges. This will provide information on the maximal size of sponges for optimal volumetric productivity in a bioreactor. Case studies on the effect of convective flow on internal mass transfer in three tropical sponges are presented below.

Case Studies

Sponges consume substrate. The substrate consumed needs to be transported in the sponge. Transport of the substrate is assumed to take place via diffusion and convection (pumping by the sponges). The overall substrate consumption rate can be determined by the rate of conversion or by the rate of transport. In the latter case, a gradient in substrate concentration develops across the support and the process is "transport limited."

Transport limitation can be quantified with an effectiveness factor (η). The effectiveness factor is defined as the observed conversion rate (so under the circumstances of transport limitation) divided by the conversion rate that would be observed if there were no transport limitation. In general, the effectiveness factor is between 0 and 1. In the case $\eta = 1$, there is no transport limitation.

Because of the pumping system of sponges, transport limitation will be minimized. In the case where transport would be determined by diffusion only, the effectiveness factor will be lower than in the case where transport would be determined by both diffusion and convection (pumping). In order to illustrate this, effectiveness factors with only diffusion (η_{diff}) and with both diffusion and convection (η_{conv}) were compared.

The effectiveness factor for sponges with only diffusion limitation was determined on the basis of the Thiele modulus (i.e., the ratio of the conversion rate and the diffusion rate) using the equations given by Van 't Riet and Tramper (1991). The same equations were used for calculation of the effectiveness factor with both diffusion and convection. In the latter case, however, the diffusion coefficient was replaced by a convection term as described by Willaert et al. (1996).

In order to perform the calculations, a few assumptions were made as well. The limiting substrate was assumed to be oxygen, and the oxygen concentration was assumed to be saturating (0.2 mol m^{-3}). It was assumed that oxygen consumption took place according to zero-order kinetics and that the effective diffusion coefficient in the sponge was 2.05×10^{-9} m^2 s^{-1}, which is similar to the one in gel materials we studied earlier (Wijffels et al., 1995).

Calculations were done for three sponge types studied by Reiswig (1973, 1974): *Tethya crypta, Verongia gigantea,* and *Mycale* sp. Reiswig gave data about their maximum oxygen consumption rate (v_{max}), radius (L), shape, and pumping rates. Pumping rates were given as volume of liquid transported per volume of sponge per time unit. The intraparticle liquid velocity (v_{int}) was calculated here by dividing the pumping rate by the specific surface area of the sponge. Results are given in Table 2. The data in Table 2 emphasize the importance of convection for the effectiveness factor in sponges. Because of convection, sponges seem not to suffer at all from diffusion limitation. Hence, if these three species are cultivated in a bioreactor, the volumetric productivity will be independent of the size of the aggregates.

Food

How to supply an adequate food source? First, it should be determined what an adequate food source is in qualitative terms. Second, it should be determined how much of the adequate food source has to be added to enable maximal growth. Finally, a method to supply this optimal amount of food should be chosen.

Table 2. Effect of Internal Mass Transfer by Convection on Diffusion Limitation in Sponges

Sponge	Shape	Radius (m)	v_{int} (m s^{-1})	v_{max} (mol m^{-3} s^{-1})	η_{diff}	η_{conv}
Tethya crypta	Sphere	0.08	0.0048	2.48×10^{-4}	0.005	1
Verongia gigantea	Cylinder	0.05	0.0025	8.44×10^{-4}	0.008	1
Mycale sp.	Cylinder	0.015	0.0020	5.46×10^{-4}	0.026	1

Qualitative Aspects. Food organisms can be selected by determining the natural diet of the sponge that is to be cultured. Reiswig (1971), Pile et al. (1996), and Pile (1997) did extensive studies on in situ feeding by six different sponge species. These authors found that the sponge species studied predominantly retained small organisms (<2 μm) like heterotrophic bacteria, cyanobacteria (mainly *Synechococcus* sp.), and Prochlorophytes. On the basis of these results, one could decide to provide cultures of *Synechococcus* and *Prochlorococcus* to sponge cultures: these microorganisms have a circumpolar distribution and are abundant in most marine waters (Pile, 1997).

Food microorganisms can also be selected by consulting the literature. There are some reports of sponge cultures using defined food media (summarized in Table 4), and besides, there are many examples of microorganisms that are used to cultivate organisms other than sponges (e.g., Volkman et al., 1989; Duerr et al., 1998).

The risk of taking only the most abundant microorganisms or the most generally applied microorganisms as a food source is that deficiencies in the sponges' metabolism could be created. Two important factors in this respect are the fatty acid composition, especially polyunsaturated fatty acids (PUFAs), and the amino acid composition of the food particles. Both are crucial in the metabolism of all living organisms, but most organisms cannot synthesize the complete spectrum of PUFAs and amino acids that is necessary for their metabolic functioning. Hence, these compounds have to be taken up from the food. By supplying the sponge with a single-species substrate, deficiencies in the supply of these essential acids may easily occur. In order to prevent such deficiencies, the fatty acid metabolism and the amino acid metabolism of both the sponge and its food should be studied.

There is already an extensive literature on both PUFAs and amino acids in algae and on the factors that influence the quantity and the composition of these compounds in algal cultures. However, literature on PUFAs and amino acids in sponges is scarce and, in the case of PUFAs, mainly concerns unusual compounds that are synthesized by the sponges themselves (e.g., Lawson et al., 1990; Barnathan et al., 1992, 1996). Aiello et al. (1993) reported high levels of eicosapentaenoic acid (EPA) in sponges from the lagoon of Venice, which were absent in a specimen of the same species collected in the open sea. They related this difference to differences in the natural diet of these populations, but they did not speculate on the potential benefit of this EPA enrichment to the sponges in the lagoon.

Interesting with respect to amino acids and PUFAs is the potential ability of sponges to take up dissolved organic compounds from the surrounding water. Direct uptake of dissolved amino acids by a sponge (*Cliona celata*) has been demonstrated by Ferguson (1982). Addition of amino acids, PUFAs, and other potentially useful compounds as dissolved food supplements to the medium is therefore an option that should be further studied.

Quantitative Aspects: Case Studies. Once a food source has been chosen, the quantitative aspect should be studied. A good example of such a quantitative study (although it does not concern a sponge) is given by Clausen and Riisgård (1996), who did experiments on the in vitro growth of the mussel *Mytilus edulis* on a single-species substrate (the Cryptophyte microalga *Rhodomonas* sp.). The mussels exhibited maximal growth at a particle density in the surrounding water of more than 4000 cells cm^{-3}.

Based on studies by Riisgård et al. (1993) and Thomassen and Riisgård (1995) and a study by Osinga et al. (1998b), two quantitative calculations of the food demand of sponges are presented below as case studies for the sponges *Halichondria panicea* and *Pseudosuberites andrewsi.*

Optimal particle concentrations for growth of the sponge *Halichondria panicea* (see Figure 1) can be calculated from the data obtained by Thomassen and Riisgård (1995), who determined growth rates and respiration rates of this species. It was found that the specific respiration rate (i.e., milligrams of carbon respired per milligrams of carbon

Table 3. Food-Particle Concentrations that Should Minimally Be Present in the Medium for Cultures of *Halichondria panicea* and *Pseudosuberites andrewsi,* as Calculated with Equation 1 for Five Different Types of Food Particles (Algae)*

Food particle	C-content (pgC cell^{-1})	Cells cm^{-3} Needed by *H. panicea*	Cells cm^{-3} Needed by *P. andrewsi*
Rhodomonas sp.	42	4900	18,000
Dunaliella sp.	19	11,000	40,000
Thalassiosira weissflogii	219	940	3,500
Tetraselmis suecica	59	3500	13,000
Chlorella sorokiniana	6	34,000	130,000

*See text for further explanation.

in sponge tissue per day) at maximal growth was about 0.08 mgC mgC^{-1} d^{-1}. This value can be converted to respiration per unit of sponge volume using a conversion factor of 0.01 cm^3 mgC^{-1} (Thomassen and Riisgård, 1995). Together with the carbon content of the food particles and an average filtration rate of *H. panicea* of 3.9 dm^3 cm^{-3} sponge volume d^{-1} (Riisgård et al., 1993), the minimal particle concentration needed to cope with the sponges' respiratory demands can be calculated using the following equation:

$$[\text{Particles}] = R/(F \times 0.35C_p),$$

in which R is the respiration in mgC cm^{-3} sponge tissue d^{-1}, F is the particle filtration rate in cm^3 cm^{-3} sponge tissue d^{-1}, and C_p s the carbon content of a particle (mg). Algal organic carbon often consists of a fraction that is rapidly biodegradable and a more recalcitrant fraction. Studies on the microbial degradation of different microalgae revealed that the rapid fraction (which is presumably also the fraction available for the sponges) comprised between approximately 35% and 50% of the total algal carbon (Newell et al., 1981; Osinga et al., 1997). Hence, C_p is multiplied by 0.35 to account for the recalcitrant carbon fraction. In Table 3, this exercise has been done for different kinds of food particles (algae). The carbon contents of four of these algae were taken from Koski et al. (1998). The value for *Chlorella sorokiniana* is based on unpublished results from our own laboratory.

Osinga et al. (1998b) measured the respiration and filtration rates of cultured *Pseudosuberites andrewsi* (Figure 4). Their data (1.23 mgO$_2$ cm^{-3} sponge volume d^{-1} and 0.6 dm^3 cm^{-3} sponge volume d^{-1}) have been used in Table 3 to perform similar quantitative calculations on the nutritional needs of this sponge species. The oxygen consumption rate was converted to carbon respiration using a 1:1 molar ratio.

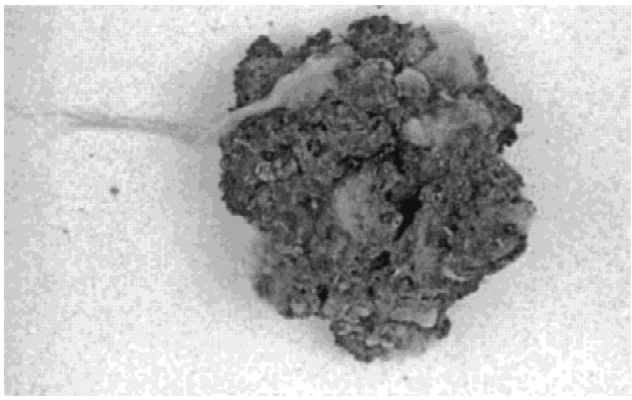


Figure 4. Several small colonies of *Pseudosuberites andrewsi* growing on a stone.

From a process-engineering point of view, it is interesting to know the ratio between the volume of the culture vessel for the food algae and the volume of the cultured sponges. Osinga et al. (1998b) estimated that their 10 dm^3 continuous culture of *Dunaliella* sp. could sustain the respiratory demand of only 0.08 dm^3 tissue of *Pseudosuberites andrewsi.* However, these algae were not cultured under optimal conditions. A similar calculation can be done for *Chlorella sorokiniana* as the food source: 1 dm^3 tissue of *P. andrewsi* respires 0.05 gC h^{-1}, which is equal to approximately 8×10^9 *C. sorokiniana* cells. If a dilution rate of 0.2 h^{-1} is applied, which should be possible with this alga (Janssen et al., 1999), and if a culture density of 2×10^7 cells cm^{-3} is assumed, the volume of the algae bioreactor should at least be 2 dm^3 to maintain the sponges.

Method of Supply. In nature, particularly in tropical waters, sponges will not often be subjected to rapid changes

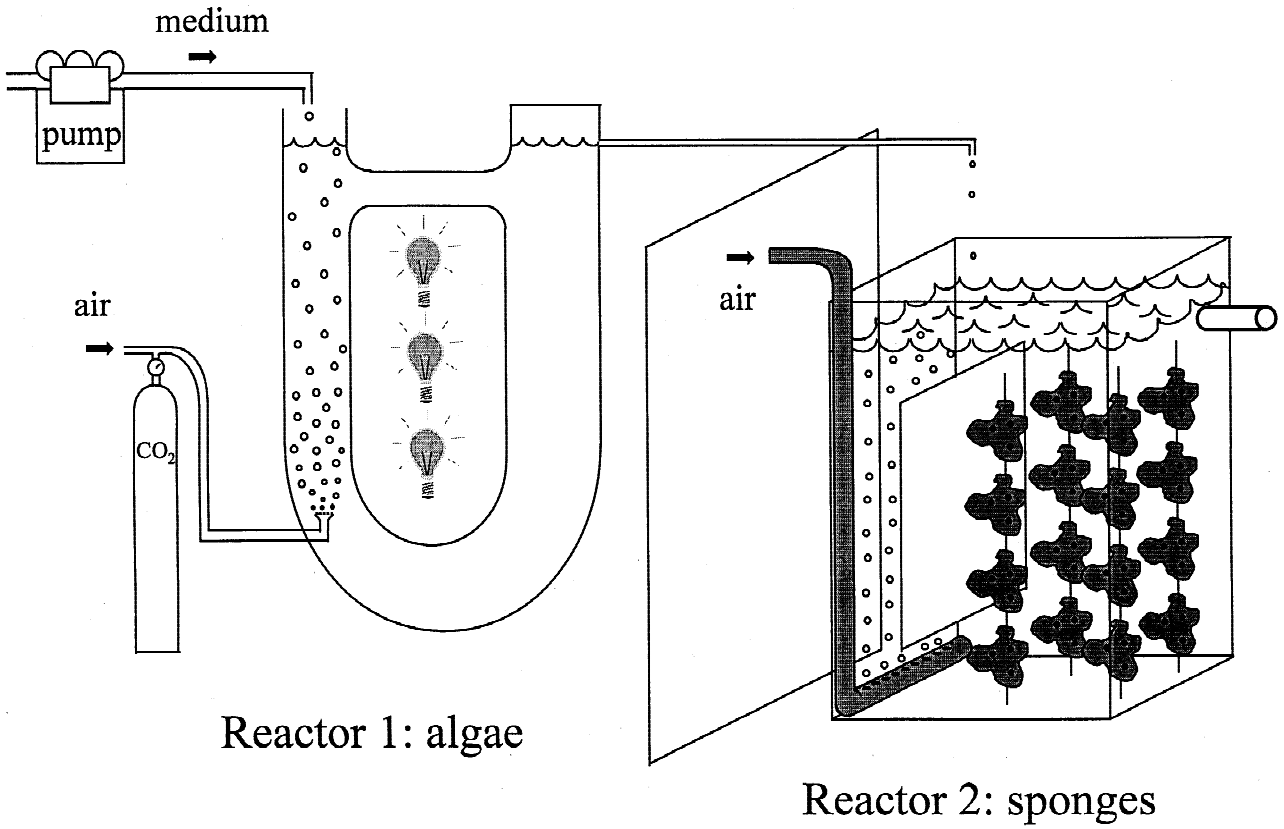


Figure 5. Design for an in vitro sponge culture that consists of two airlift bioreactors. In the first reactor, algae are grown in continuous culture. The overflow of this reactor is feeding the second reactor, which contains the sponges.

in the food particle concentration of the surrounding water. In controlled systems, such a constant availability of food may best be mimicked by using continuous cultures of algae or bacteria to feed the sponges (Figure 5). Examples of such continuously fed sponge cultures are given in the case studies described above (Thomassen and Riisgård, 1995; Osinga et al., 1998b). However, most of the sponge cultures described so far were fed by adding batches of living or dead cultures of algae or bacteria (Table 4). Osinga et al. (1998b) used a combination of a continuous culture of *Dunaliella* sp. and batch cultures of *Chlorella vulgaris* to cultivate four tropical demosponge species. In such a way, a mixed diet of bulk food and specific food supplements can be provided without the need of operating many different continuous cultures.

Thomassen and Riisgård (1995) cultured colonies of the sponge *Halichondria panicea* using *Rhodomonas* sp. as a single food source. Although the maximum growth rate in their experiment (0.016 d^{-1}) was lower than the maximal rate measured in the field (0.043 d^{-1}), they apparently succeeded in growing this species under controlled conditions. The biotechnological applicability of this cultivation method was not indicated by the authors, as their work was aimed to study fundamental aspects of sponge physiology.

Silicon

Silicon can simply be added as sodium metasilicate (Na_2SiO_3; Osinga et al., 1998b, 1999) or as Na_2SiF_6 (Reincke and Barthel, 1997) to the (artificial) seawater used in a sponge bioreactor. The amount of silicon that must be added should be deduced from silicon uptake rates from the cultured sponge species. So far, the only quantitative studies on silicon uptake rates by sponges were carried out on the temperate sponge species *Halichondria panicea* (Frøhlich and Barthel, 1997; Reincke and Barthel, 1997) using natural seawater and Na_2SiF_6-enriched natural seawater. These studies revealed that silicon uptake followed Michaelis-Menten kinetics and was strongly dependent on the nutritional state of the sponge. Silicon uptake was minimized during periods of starvation. It was therefore assumed by Frøhlich and Barthel (1997) that the formation of siliceous structures is an expensive process in terms of energy.

Table 4. Food Regimens Applied to Sponge Cultures

Sponge species	Food	Method of supply	Reference
Cliona celata	Unfiltered seawater	Continuous	Warburton (1958)
Cliona celata	*Chlorella vulgaris*	Batch	R. Osinga, unpublished
Ephydatia fluviatilis/Spongilla alba	*Escherichia coli*	Batch	Poirrier et al. (1981)
Halichondria panicea	Liquifry marine*	Batch	Langenbruch (1983)
	Liquizell*	Batch	
	Dried phytoplankton	Batch	
Halichondria panicea	Unfiltered seawater	Continuous	Barthel and Theede (1986)
	Chlorella vulgaris	Batch	
Halichondria panicea	*Rhodomonas* sp.	Continuous	Thomassen and Riisgård (1995)
Microciona prolifera	*Dunaliella euchlora*	Batch	Simpson (1968)
	Isochrysis galbana	Batch	
Ophlitaspongia seriata	*Isochrysis galbana*	Batch	Fry (1971)
	Micromonas squamata	Batch	
	Monochrysis Lutheri	Batch	
	Tetraselmis suecica	Batch	
	Dead bacteria (dominated by *Pseudomonas* and *Arthrobacter*)	Batch	
Pseudosuberites andrewsi	*Dunaliella* sp.	Continuous	Osinga et al. (1998b)
	Chlorella vulgaris	Batch	

*These are commercial aquarium food products.

Reincke and Barthel (1997) did not find the optimal silicon concentration for *Halichondria panicea.* Even at the highest concentration applied by these authors (200 μM), silicon uptake had not yet reached a maximal rate, indicating that for this species, the silicon concentration to be applied in a bioreactor should be higher than 200 μM. Osinga et al. (1998b, 1999) applied a silicon concentration of 250 μM in their sponge bioreactor, which appeared to be appropriate to maintain small colonies of *Pseudosuberites andrewsi.*

To the best of our knowledge, it is still unknown whether demosponges and glass sponges can also use the silica skeletons of ingested diatoms to build up their own skeleton. If they can, feeding a sponge culture with diatoms may be an elegant way to provide both food and silicon.

Mixing and Oxygen Supply

A sponge bioreactor must be well mixed in order to generate a constant distribution of food and oxygen, but also to avoid sedimentation of organic material, which may lead to undesirable anaerobic microbial degradation processes. In addition, the generated water current will take away the metabolic wastes produced by the sponges, which are excreted through the oscula. Although sometimes mentioned, facilitation of the sponge pumping by the water current is probably not of significant importance for the energy budget of sponges. According to Riisgård et al. (1993), pumping accounts for less than 1% of the total energy used by a sponge.

Airlift loop bioreactors as shown in Figure 5 may be suitable systems for in vitro sponge cultures because these are very effective in both generating a current and providing oxygen. To prevent oxygen limitation, the maximal biomass density that can be supported by the applied oxygen supply system must be calculated from the oxygen demand of the sponges and the mass transfer rate of oxygen from the gas phase into the liquid phase. Therefore, these parameters must be determined before the culture is set up. A case study for the sponge *Verongia gigantea* is presented below.

Case Study

Bioreactors are designed in such a way that costs are minimized. In many cases, the overall volumetric produc-

tivity (i.e., the production capacity per unit volume and time) of the bioreactor needs to be maximized. This can be done by increasing the biomass concentration in the bioreactor until physical constraints such as mass and heat transfer limitations are met (Van 't Riet and Tramper, 1991). In natural circumstances, the amount of sponge per volume of seawater is so low that volumetric productivity is very low. One of the first ways to increase volumetric productivity in a bioreactor is by increasing the biomass concentration while taking the mass transfer properties into account. In many processes oxygen transfer is the rate-limiting step. Therefore, we made a simple calculation to judge whether oxygen transfer from the gas to the liquid phase in an airlift loop reactor would be sufficient at a high biomass concentration in the reactor. For this we used the following balance:

$$\text{Oxygen uptake rate} = \text{Oxygen transfer rate}$$

$$r_s' \cdot \epsilon = k_l A(C_{ox}^{\star} - C_{ox})$$

In which r_s' is the volumetric oxygen consumption rate of the sponge (mol m^{-3} s^{-1}), ϵ is the volume of sponges per volume of bioreactor, k_lA is the mass transfer coefficient (s^{-1}) in which the specific surface area of the gas bubble is expressed as m^2 of gas bubble surface per m^3 of liquid, $C_{ox}^{\star}$ is the oxygen concentration in saturation with the gas phase (mol m^{-3}), and C_{ox} is the oxygen concentration in the liquid phase (mol m^{-3}). In this case we assume a bioreactor in which 50% of the volume is the sponge *Verongia gigantea* ($\epsilon = 0.5$) and the volumetric oxygen consumption rate is equal to the maximum volumetric consumption rate as given in Table 2 ($r_s' = 8.44 \times 10^{-4}$ mol m^{-3} s^{-1}). The saturated oxygen concentration, $C_{ox}^{\star} = 0.2$ mol m^{-3}, and the actual concentration in the liquid phase was assumed to be 20% of that value $C_{ox} = 0.04$ mol m^{-3}). The k_la value necessary to provide sufficient oxygen in that bioreactor can be calculated with the balance equation, resulting in a value of 2.64×10^{-3} s^{-1}. This value is very low and can already be reached at very low superficial gas velocities of approximately 0.001 m s^{-1} (Van 't Riet and Tramper, 1991). Airlift loop reactors normally can easily be operated at superficial air velocities of 0.15 m s^{-1}, indicating that oxygen transfer presents no problem for a bioreactor containing large amounts of this sponge species. It should be emphasized here again that sponges could be sensitive to hypoxic conditions. To what extent the respiration rate of the sponge (and hence its metabolism) is affected by a low C_{ox} must always be tested.

By placing the sponges in the downcomer, contact with the air bubbles (which may be harmful to sponges) can be avoided. Hence, to enable a high volumetric productivity of sponge biomass, the volume of the riser should be as small as possible compared with the volume of the downcomer. The efficiency of oxygen mass transfer will limit this volumetric ratio of riser to downcomer.

Temperature and Water Quality: Biofiltration Systems

Once the optimal temperature, pH, and salinity for the growth of a sponge species have been determined, it will be relatively easy to maintain these conditions in the bioreactor. More difficult to control is the accumulation of ammonia and other waste products in the system. There are two options to prevent such a buildup of waste products: continuous replacement of the culture medium or recirculation of the medium through biofiltration systems. Biofiltration systems primarily consist of a matrix onto which bacteria that degrade the accumulating waste products can attach. In this way, an immobilized bacterial biomass is formed that keeps the system clean.

Recirculation and filtration of the medium is often cheaper than flowthrough, and is therefore frequently applied in the culture of aquatic animals. Hence, there is an extensive literature on types and operation of recirculation/biofiltration systems, both for use in aquaria (e.g., Adey and Loveland, 1998) and for use in large-scale, high-density cultures (e.g., Paller and Lewis, 1988; Heinsbroek and Kamstra, 1990).

A specific advantage of recirculation over flowthrough for the cultivation of filter-feeding animals such as sponges is that the filtration system, if properly designed, keeps the food particles inside the system. For instance, the system used by Osinga et al. (1998b), a tube filled with clay balls (diameter approx. 1 cm), captured less than 20% of the food particles when operated at the flow rate that was applied in the experiments (R. Osinga, unpublished results).

Removal of ammonia is often the most important function of biofilters. To design an optimal filtration system for ammonia, the production of ammonia by sponges should be known, as well as the capacity of the filters to remove ammonia, which is dependent on the surface area of the filter material (i.e., the available space for bacterial biofilms to develop).

Cell Cultures

Sponge Cells

Sponge cell culture may be an appropriate way to produce sponge biomass under completely controlled conditions. So far, however, no continuous cell lines of sponges are available, due to the lack of suitable methodology (Rinkevich, 1999). Establishing cell cultures is a long process, requiring a lot of fundamental knowledge about the sponge cell's nutritional, biochemical, and immunologic requirements and properties.

In an attempt to produce a continuous sponge cell line of the sponge *Hymeniacidon heliophila,* Pomponi and Willoughby (1994) describe the following steps: First, primary cultures are established by dissociation of sponge cells (either mechanically, chemically, or enzymatically). Second, selective cell enrichment is achieved by Ficoll density gradient centrifugation and unit gravity sedimentation. This step is carried out to select for the cell types that produce the target compound. Third, antibiotics are used to produce axenic sponge cell cultures. Pomponi and Willoughby (1994) stressed that a dose-response relation should be determined for each combination of antibiotics and sponge species. Treatment with antibiotics should be continued in the final cell culture because endosymbiotic bacteria may be released from dying sponge cells.

Pomponi and Willoughby (1994) developed a basal sponge cell culture medium, in which their primary sponge cell cultures could be maintained. This medium consisted of a commercial medium, calcium- and magnesium-free artificial seawater, phosphate buffer (pH 7.0), and 5% fetal bovine serum. Calcium- and magnesium-free seawater was used because these metal ions play a role in the reaggregation process of sponge cells, which is a process not desired in cell cultures.

Ilan et al. (1996) describe a similar method to produce a cell line of the sponge *Latrunculia magnifica.* They also used a combination of artificial seawater, commercial media, and fetal calf serum. It turned out to be important to maintain the primary cultures in a diluted version of the medium, as it was difficult to control microbial contamination in rich media, despite the use of high concentrations of antibiotics.

Ilan et al. (1996) and Pomponi and Willoughby (1994) did not succeed in producing a continuous cell line. Besides, in both cases, cell proliferation had to be stimulated with mitogenic agents, despite the use of very rich media. More recently, Pomponi et al. (1997) described new attempts to produce a continuous cell line with cells of the sponge *Teichaxinella morchella.* Cell division was successfully stimulated with different growth factors, but a continuous cell line could still not be established.

An important recent finding by Koziol et al. (1998) may explain the lack of success in producing continuous sponge cell lines. These authors showed that tissues of *Suberites domuncula* and *Geodia cydonium* have a high telomerase activity, indicating a strong proliferative capacity, which is in agreement with the undifferentiated, totipotent nature of the sponge cells. However, after being dissociated, the cells of *G. cydonium* rapidly lost their telomerase activity. Apparently, the sponge cells need the cell-to-cell contact and the contact with extracellular adhesion factors to retain the ability to proliferate (Koziol et al., 1998). On the basis of these findings, Custodio et al. (1998) described axenic cultures of multicellular aggregates of *Suberites domuncula,* formed from dissociated sponges cells. Within a period of 5 days, the aggregates developed into structured spheres of 1 to 2 mm in size, which were called primmorphs. These primmorphs could be kept in culture for more than 5 months in unsupplemented seawater. If an appropriate growth medium for primmorphs can be developed, this system may have promising biotechnological potential.

A potential risk of using sponge cell or tissue cultures for metabolite production is that the sponge cells may lose the ability to produce the target compound when grown in axenic culture. Pomponi et al. (1997) showed that in their culture of *T. morchella,* the cells were still able to produce the target metabolite, even after the artificially induced cell division.

Another problem is that sponge cells are difficult to identify. They can easily be confused with other cell types. Protozoan cells have abusively been cultivated as sponge cells by Klautau et al. (1994), as was recognized by Custodio et al. (1995). Thraustochytrids (heterotrophic protists), in particular, have a bad reputation as contaminants of marine invertebrate cell cultures (Rinkevich, 1999). These problems may be prevented by regularly checking the authenticity of the cultured cells, either by using ribosomal DNA sequences or by monitoring the presence of species-specific metabolites (Pomponi et al., 1997).

Endosymbionts

Some "sponge natural products" are in fact produced by endosymbiotic organisms (Stierle et al., 1988; Unson et

al., 1994). For production of these endosymbiotic metabolites, cultivation of the endosymbionts without their hosts may be easier than cultivation of the sponge (cells). So far, the only successful attempts to cultivate photosynthetic sponge endosymbionts have been in studies by Hinde et al. (1994) on the cyanobacterium *Oscillatoria spongeliae,* which was isolated from the sponge *Dysidea herbacea,* and by Price et al. (1984) on the red macroalga *Ceratodictyon spongiosum* from the sponge *Sigmadocia symbiotica.* Other reports of cultured sponge endosymbionts came from Stierle et al. (1988), who cultured a *Micrococcus* species from the sponge *Tedania ignis,* and from Elyakov et al. (1991), who cultured an unidentified bacterium from the sponge *Dysidea* sp.

A problem to overcome while culturing sponge endosymbionts is the unusual, specific environment in which they live. Hinde et al. (1994) stressed that the culture medium should resemble the osmolarity of the sponge mesohyl. Moreover, contact with the cell contents of disrupted sponge cells during the isolation of the endosymbionts should be avoided as much as possible because of the possible occurrence of intracellular toxins in the sponge cells.

Another potential drawback of endosymbiont cultures for metabolite production is the uncertainty about whether or not the endosymbionts will continue to produce the target compound in the absence of the sponge host (Unson et al., 1994). In addition, expression of the genes responsible for the synthesis of the target compound may be inhibited in monospecific cultures.

Discussion

At present, cultivation of sponges can still be considered difficult, although some progress has been made since Kinne's 1977 review. From a biotechnological point of view, in situ culture is still the most reliable and least expensive method to produce sponge biomass. MacMillan (1996) estimated that an investment of less than $1000 (U.S.) could yield at least 8000 kg wet weight of bath sponge, with a market value of $10,000. Recent developments in New Zealand have made it clear that in situ culture also is suitable and can be optimized for large-scale production of natural substances.

In situ growth rates of sponges are relatively low. For bath sponges, annual growth rates of 60% to 500% have been reported (Storr, 1964; Verdenal and Vacelet, 1990). According to Table 2 in the paper of Thomassen and Riisgård (1995), growth rates measured for some other sponges varied between 1.0% and 5.8% per day. Increasing these low specific growth rates in sponge bioreactors is a major challenge for the marine bioprocess engineers that want to work on sponge metabolites.

However, in vitro cultivation of sponges remains a process that is surrounded by questions and uncertainties. Nevertheless, a few successful attempts to culture sponges under controlled conditions have been described (Langenbruch, 1983; Thomassen and Riisgård, 1995; Osinga et al., 1998b), and this should encourage sponge scientists and biotechnologists to continue their research along the lines pointed out above in the section Design of an In Vitro Sponge Culture. Such a systematic scientific approach to the problems may result in successful development of large-scale in vitro sponge biomass production in the near future. In some cases, it may not even be necessary to produce biomass: if a sponge population that continuously excretes a target metabolite can be maintained in a bioreactor, a continuous biological production system has already been established. Therefore, bioprocess technological studies that are directed to the production of a specific compound should always be preceded by ecophysiologic research about the biological background of the metabolite production.

Sponge cell culture is also in progress. Primary cell cultures can be established and maintained for a limited period. This offers the possibility to apply genetic engineering. Sponge cell cultures can provide very pure (axenic) sponge material, which may serve as a starting point for localization and isolation of the genes encoding production of the target metabolites (Pomponi et al., 1997). Transferring these genes into more easily culturable hosts, such as *Escherichia coli* or yeasts, may be an interesting future option for biotechnological production of sponge metabolites.

The establishment of a continuous cell line is still hampered by the inability of the dissociated sponge cells to proliferate. This problem may be overcome by culturing axenic primmorphs and by enriching the growth medium with marine-based food supplements.

Another method with good biotechnological potential that certainly deserves further development is the cultivation of sponges in semi-enclosed systems (see Poirrier et al., 1981; Barthel and Theede, 1986), in which the advantages of controlled temperature and salinity and controlled addition of food are combined with the advantages of using unfiltered, running seawater.

In conclusion, it can be stated that the enormous biochemical potential of the phylum Porifera justifies an increased research effort, to enable sustainable use of this

valuable marine natural resource in the near future. In other words, we should not have to wait another 22 years for the next review on sponge cultivation to be published.

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