

# **Sponge cell culture**

Klaske J. Schippers

# Sponge cell culture

**Klaske J. Schippers**

## **Thesis committee**

### **Promotors**

Prof. dr. ir. R.H. Wijffels  
Professor of Bioprocess Engineering  
Wageningen University

### **Co-promotors**

Prof. dr. S.A. Pomponi  
Professor of Marine Biotechnology, Wageningen University / Professor Harbor  
Branch Oceanographic Institute-Florida Atlantic University, USA

Dr.ir. D.E. Martens  
Assistant professor, Bioprocess Engineering  
Wageningen University

### **Other members**

Prof. dr. J. van der Oost, Wageningen University  
Dr. N.J. de Voogd, Museum Naturalis, Leiden  
Prof. dr. M.J. Uriz, Centro de Estudios Avanzados de Blanes, Spain  
Dr. ir. G.P. Pijlman, Wageningen University

Submitted in fulfillment of the requirements for the degree of doctor  
at Wageningen University  
by the authority of the Rector Magnificus  
Prof. dr. M.J. Kropff,  
in the presence of the  
Thesis Committee appointed by the Academic Board  
to be defended in public  
on Friday 15 February 2013  
at 4 p.m. in the Aula.

This research was conducted under the auspices of the Graduate School VLAG  
(Advances studies in Food Technology, Agrobiotechnology, Nutrition and Health  
Sciences).

Klaske J. Schippers  
**Sponge cell culture**

PhD thesis, Wageningen University, Wageningen, NL, 2013  
With summaries in English and Dutch

ISBN 978-94-6173-441-9

## Table of contents

<b>Chapter 1</b>	Introduction and thesis outline	7
<b>Chapter 2</b>	Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. <i>Advances in Marine Biology 2012. 62: 273-337.</i>	21
<b>Chapter 3</b>	Cell cycle analysis of primary sponge cell cultures. <i>In Vitro Cellular &amp; Developmental Biology – Animal 2011. 47:302-311.</i>	87
<b>Chapter 4</b>	Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures. <i>Submitted for publication.</i>	105
<b>Chapter 5</b>	Methods for insertion and expression of heterologous genes in sponge cells. <i>Submitted for publication.</i>	127
<b>Chapter 6</b>	General discussion: Genomic-based approach for sponge cell culture. <i>Submitted for publication.</i>	149
	<b>Summary</b>	173
	<b>Samenvatting</b>	179
	<b>Dankwoord</b>	185
	<b>Curriculum Vitae</b>	188
	<b>List of Publications</b>	189
	<b>Overview of completed training activities</b>	190

# Chapter 1

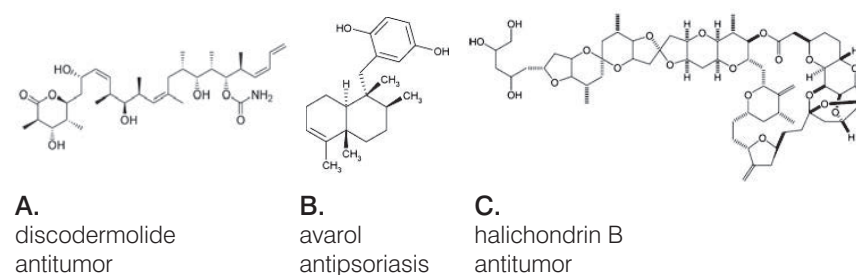
Introduction and thesis outline



## 1 GENERAL INTRODUCTION

### 1.1 Biotechnological potential of sponges

Sponges are simple multicellular animals and historically had commercial value as bath sponges, because of their absorptive properties. Due to the invention of the less expensive synthetic bath sponge, farming of the natural bath sponge decreased (Hogg et al., 2010). Current biotechnological applications of sponges are more directed towards the production of sponge-derived bioactive compounds (Pomponi, 2001). As sponges are benthic and sessile as adults, they have evolved a sophisticated chemical communication and defense system based on secondary metabolites (Müller et al., 2004; Hogg et al., 2010). These secondary metabolites can protect the sponge against fouling (Proksch, 1994), predation (Becerro et al., 1997), or microbial infection (Sipkema et al., 2005a) and accordingly have pharmacological activities, such as antibiotic, antiviral, anti-inflammatory and antitumor activity. Figure 1 shows examples of sponge-derived bioactive compounds with pharmaceutical potential. Consequently, marine sponges are the most prolific source of newly discovered bioactive compounds, with more than 7,000 sponge-derived, novel molecules discovered in 40 years (Blunt et al., 2009).

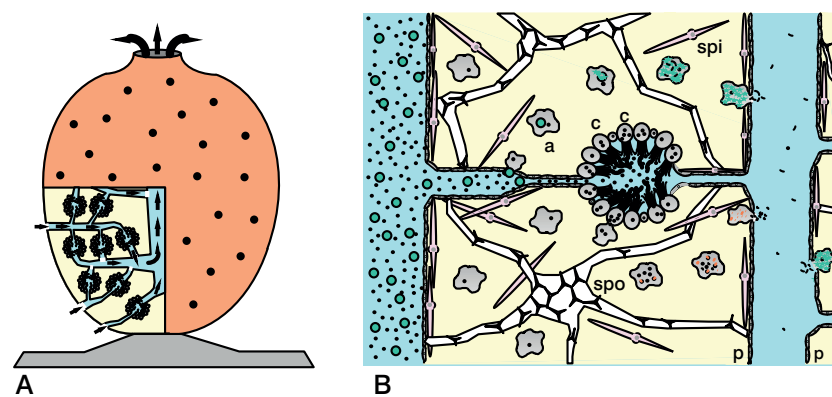


**Figure 1** Examples of sponge-derived bioactive compounds with pharmaceutical potential. A: Discodermolide is found in the sponge *Discodermia dissoluta* and is a potent inhibitor of tumor cell growth (Pomponi, 2001); B: Avarol is found in the sponge *Dysidea avara* and is found to be effective in treating psoriasis (Müller, 1991); C: Halichondrin B has been obtained from *Lissodendoryx* spp. and has a strong anticancer effect (Pomponi, 2001).

### 1.2 What are sponges?

Sponges (phylum Porifera) are sessile benthic animals and are the most simple (they lack true tissue) and ancient multicellular animals on earth (Bergquist, 1978). They inhabit a wide range of ecosystems, varying from marine to freshwater, polar to tropical and shallow to deep waters. Around 8,000 sponge species are described, with an estimated species number of more than 15,000 (Hooper and Soest, 2002).

To support life, sponges pump huge amounts of water through their bodies and filter it to capture particulate organic carbon (POC), such as bacteria, micro algae and dead organic particles (Koopmans et al., 2010). In addition they are also able to absorb dissolved organic carbon (DOC) from the water (de Goeij et al., 2008). The organization of the body of the sponge is designed for efficient filtration (see Fig 2A). Water is pumped through small pores (ostia), which are on the outside of the sponge. The water current inside the sponge is generated by flagellated cells, called choanocytes, which are clustered in so-called choanocyte chambers inside the sponge. The water current leaves the sponge via a large outflowing opening (osculum). Food particles that are trapped by the microvilli of the choanocytes are partly digested by the choanocytes and then transferred to the archaeocytes, which further digest them. The archaeocytes are the most important cellular component of the mesohyl, as they distribute the nutrients over the rest of the sponge and are able to differentiate into any other cell type (totipotent). The mesohyl is the tissue layer between the channels, choanocyte chambers and outer layer of the sponge (colored yellow in Fig 2B) and contains besides cellular components, also non-cellular components, such as spicules, needle like structures made of silica or calcite, sponging or collagen fibers (see Fig 2B). These non-cellular components form the framework of the sponge and are made by sclerocytes, spongocytes and collencytes, respectively. The flat skin cells, which cover the channel walls and the exterior of



**Figure 2** Schematic representation of morphology and feeding physiology of sponges (Osinga et al., 1999). **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 choanocyte chambers). Arrows indicate the water flow through the sponge body. **B:** Detailed overview of canals and choanocyte chamber. A number of different cell types and skeletal elements are shown: choanocytes (c), archaeocytes (a), pinacocytes (p), spicules (spi), spongin fibers (spo).

the sponge, are called pinacocytes. There are many more sponge cell types, such as granulocytes, thesocytes, oocytes or spermatocytes, which are described by Simpson (1984).

Sponges reproduce either sexually or asexually (Bergquist, 1978). When they reproduce sexually the sponge will develop larvae, which consist of a package of stem cells (archaeocytes), and are released via the oscula. The larvae are free-swimming before they attach on a substratum and develop into a juvenile sponge. Asexual reproduction occurs either via fragmentation, budding or gemmule formation. Fragmentation can occur for example after a storm, and the dispersed fragments can reattach and form new individuals. Budding is similar to fragmentation, but then organized by the sponge itself. Some sponges form gemmules to survive. Gemmules are small spheres with a coating, which are located at the base of the sponge. This coating can resist harsh environmental circumstances, such as low temperatures or desiccation. Just like larvae, gemmules contain a package of stem cells (thesocytes), and after hatching on a substratum they can develop into juvenile sponges.

Many sponges live in symbiosis with microorganisms, such as bacteria, algae, cyanobacteria or fungi (Taylor et al., 2007). These symbionts mostly reside in the mesohyl and can contribute up to 40% of the mesohyl volume. They have the ability to recycle nutrients or can fix carbon and nitrogen due to their photosynthetic activity. Another role of the symbiont is the production of bioactive compounds. For many sponge-derived bioactive compounds, it is not clear whether they are produced by the sponge or the symbiont. Metabolites known to be produced by the sponge are for example, avarol, found in *Dysidea avara* (Uriz et al., 1996) and stevensine, found in *Axinella corrugata* (Andrade et al., 1999). On the other hand, the cyanobacterial symbiont *Oscillatoria spongelia*, is known to produce antimicrobial polybrominated biphenyl ethers (Unson et al., 1994).

### 1.3 How to produce sponge-derived bioactive compound?

Despite the enormous biotechnological potential of sponge-derived bioactive compounds, only a few compounds have been successfully developed into products. A major obstacle is the lack of sufficient supply of biological material for preclinical and clinical development (Osinga et al., 1999; Munro et al., 1999). Although controlled chemical synthesis of the bioactive molecules (or their analogues) is the preferred production method for pharmaceutical application, most bioactive natural compounds are chemically complex structures that cannot be synthesized easily. Also, harvesting of sponges with bioactive compounds from nature is neither environmentally nor economically feasible (Pomponi, 1999). Cultivation of the sponge that produces the bioactive compound may overcome the supply issue (Osinga et al., 1998). Depending

on the product of interest, different cultivation approaches may be applied. When product concentrations are high inside the sponge, cultivation of adult sponges (i.e. explants) is at the moment the most feasible option (Sipkema et al., 2005b). Mariculture (sea-based *in situ* culture) has been successfully used for sponge cultivation (Osinga et al., 2010). However the cultivation conditions *in situ* cannot be controlled. *Ex situ* culture (aquaria-based) is a method which allows for a semi-controlled environment, but sponge growth rates in *ex situ* culture are slow (Sipkema et al., 2006). Moreover, many secondary metabolites are present only at low concentrations. To obtain an economically feasible production system, the product concentrations have to be increased. The use of *in vitro* cultures, such as cell or tissue culture, may then be the method of choice as *in vitro* cultivation allows for control of culture conditions to increase product concentrations. If the compound of interest is produced by a microbial symbiont, the production system could consist of only the symbiont, if it is possible to culture the symbionts separately. If this is not possible or if the compound is partly synthesized by the sponge and partly by the symbiont, co-culture may be required. This thesis focusses on the cultivation of sponge cells.

#### 1.4 Development of sponge cell lines

In general, animal cell cultures are obtained by isolating cells from an axenic piece of tissue. These cells are cultured in medium that will support proliferation. This stage is called primary cell culture and most cell types will undergo a limited number of divisions and then enter senescence. Continuous cell lines are generally obtained from spontaneously immortalized primary cell cultures (e.g., random mutagenesis) or are derived from cancerous tissue (Freshney, 2005). Another approach towards developing a continuous cell line is the insertion and expression of immortalizing genes (e.g., SV40LT, hTERT) in primary cells. Immortalizing genes interfere with the regulatory pathways for cell division, and in this way cause unlimited cell division, resulting in a continuous cell line (Freshney, 2005).

In principle, sponges have great potential for cell culture because of the presence of totipotent stem cells (i.e. archaeocytes) and because cells can be easily dissociated from its tissue, due to their loosely cellular organization. However, despite efforts by several research groups, a continuous sponge cell line has not yet been developed, and the number of primary sponge cell cultures developed is very limited (Rinkevich, 2005; Pomponi, 2006; Schippers et al., 2012). This is related to the following aspects:

- Due to the presence of symbionts inside the sponge, it is practically impossible to obtain an axenic piece of sponge tissue. Researchers have used antibiotics and antimycotics to reduce growth of contaminants (Pomponi and Willoughby, 1994), but still contamination occurs (Ilan et al., 1996).
- Also obtaining proliferating starting material remains problematic, since most dissociated sponge cells go into apoptosis and die (Schippers et al., 2011).

However, Pomponi and Willoughby (2000) were able to show sponge cell division when exposed to the mitogen, phytohaemagglutinin. Other options for obtaining proliferating starting material are the use of primmorphs, gemmules or larvae. Primmorphs are reaggregated sponge cells which have the ability to proliferate *in vitro*, although no net growth has been observed (Sipkema et al., 2003). Gemmules and larvae both contain a package of stem cells, and can develop into a juvenile sponge *in vitro*.

- Nutritional requirements of sponge cells are still poorly understood. Most of the sponge cell culture media are based on mammalian cell culture media, but certain components such as fatty acids or growth factors, are expected to be different for sponge cells.

Summarized, the establishment of sponge cell lines is still hampered by three issues: the presence of contaminants in the starting material, the lack of knowledge on nutritional requirements and the lack of proliferating starting material. This thesis focusses on the last item; sponge cell proliferation.

## 2 THESIS OUTLINE

The aim of this thesis was to gain insight in cell proliferation and cell death of sponge cell cultures and to make the first step towards an immortalized sponge cell culture for biotechnological purposes, such as the production of bioactive compounds.

In **chapter 2** "Cultivation of sponges, sponge cells and symbionts: achievements and future prospects", we analyzed the state of the art for cultivation of whole sponges, sponge cells and sponge symbionts for biotechnological purposes and we elaborate on approaches to overcome bottlenecks, including transformation of sponge cells and using media based on yolk.

In **chapter 3** "Cell cycle analysis of primary sponge cell cultures", we discussed the development of a flow cytometric cell cycle analysis method to measure the proliferative state of sponge cells, in combination with a caspase assay to detect apoptosis. These methods allowed for a quick determination of the proliferative status of a sponge cell population. We analyzed the cell cycle distribution of five different species (*Haliclona oculata*, *Haliclona xena*, *Dysidea avara*, *Axinella polypoides* and *Xestospongia muta*) from different locations to compare the proliferative status at the start of the culture. In addition, we measured the cell cycle distribution and caspase activities of cells from *H. oculata* during cultivation to study the change in distribution of cells over time.



Subsequently in **chapter 4** “Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures”, we used the developed methods from chapter 2 to compare culture conditions of two Mediterranean sponges (*D. avara* and *Crambe crambe*) and one Dutch sponge (*H. oculata*). We hypothesized that *in vitro* tissue culture will be a more favorable culture condition than cell culture, because the dissociation process is avoided, meaning the cells are not exposed to shear stress and the cell-cell contacts are retained. As a control we also included tissue culture in the sea (*in situ*) to test, in case of lack of growth, whether this is due to the cutting of the sponge into tissue pieces or to environmental conditions that are different in the sea compared to the *in vitro* culture.

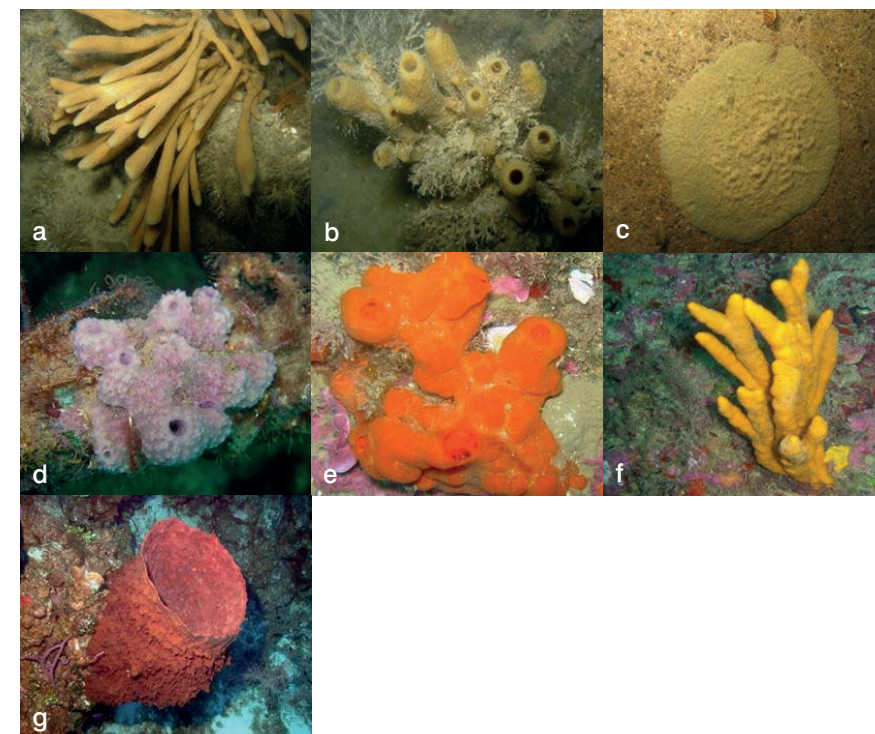
In **chapter 5** “Methods for insertion and expression of heterologous genes in sponge cells”, we made the first step towards immortalization of sponge cells by comparing different methods for the insertion and expression of heterologous genes in sponge. We tested three gene delivery systems, lipofection, particle bombardment and viral transduction, in juveniles of the freshwater sponge *Ephydatia fluviatilis*, and lipofection was tested in primary cell cultures of the marine sponge *H. oculata*. Juveniles of *E. fluviatilis* were chosen as a model sponge for this research, because they are able to proliferate *in vitro*. To visualize transfection efficacy, we used the green fluorescent protein (GFP) reporter gene. To test promoter recognition by sponge RNA polymerase, we tested the cytomegalovirus (CMV) and OplE2 promoter, which are generally used for transfection of mammalian cells and insect cells, respectively.

Finally in **chapter 6** “Genomic based approach for sponge cell culture”, we provided an outlook on how recent developments in the field of genomics and transcriptomics can help us to gain more insight in sponge growth and death and consequently obtain a continuous sponge cell line.

### 3 TARGET SPONGES

The sponge species used for this thesis have been selected based on criteria such as, their availability, the ability to proliferate *in vitro* or the production of a bioactive compound.

*Haliclona oculata* (Pallas 1766, Haplosclerida, Demospongiae) is a branching, compressible sponge and occurs in the North Atlantic and Arctic (Fig 3a). Since this sponge is easily available in the Netherlands, we used this sponge as a model sponge for cell culture (chapter 3 and 4), tissue culture (chapter 4) and transfection experiments (chapter 5).



**Figure 3** Sponges used for this thesis. a. *Haliclona oculata*, b. *Haliclona xena*, c. *Ephydatia fluviatilis*, d. *Dysidea avara*, e. *Crambe crambe*, f. *Axinella polypoides*, g. *Xestospongia muta* [pictures a and b are made by M. Koopmans, picture c is made by R. Timmermans].

*Haliclona xena* (Pallas 1766, Haplosclerida, Demospongiae) is an encrusting sponge with hollow tubes (Fig 3b). This sponge is seasonally present in the Netherlands. No bioactive compounds have been reported for this sponge. We used this sponge for cell cycle analysis in chapter 3.

*Ephydatia fluviatilis* (Linnaeus, 1759, Haplosclerida, Demospongiae) is a thin encrusting freshwater sponge and is worldwide available (Fig 3c). Gemmules of this sponge can be stored and sterilized and have the ability to develop into juvenile sponges *in vitro*. We used these juveniles for transfection experiments in chapter 4.

*Dysidea avara* (Schmidt 1862, Dictyoceratida, Demospongiae) is a massive, conulose, compressible sponge that lacks spicules and occurs abundantly in the Mediterranean (Fig 3d). *D. avara* produces avarol, a sterol with antitumor and antiviral properties. This sponge has the ability to proliferate *in vitro*, either as primmorph or as



larva. We used this sponge for cell cycle analysis in chapter 3 and for cell and tissue culture experiments in chapter 4.

*Crambe crambe* (Schmidt 1862, Poecilosclerida, Demospongiae) is a thin, smooth encrusting sponge and occurs abundantly in the Mediterranean (Fig 3e). *C. crambe* produces the toxin crambescin, which has cytotoxic properties. This sponge was used for cell and tissue culture in chapter 4.

*Axinella polypoides* (Schmidt 1862, Halichondrida, Demospongiae) is branching sponge with firm consistency and smooth surface and is common in the Mediterranean (Fig 3f). No bioactivity has been reported for this species so far. We used this sponge for cell cycle analysis in chapter 3.

*Xestospongia muta* (Schmidt 1870, Haplosclerida, Demospongiae) is a barrel sponge with a hard texture and occurs in the Caribbean sea (Fig 3g). *X. muta* is known to produce a variety of compounds with antimicrobial and cytotoxic properties. We used this sponge for cell cycle analysis in chapter 3.

## REFERENCES

- Andrade, P., Willoughby, R., Pomponi, S.A. and Kerr, R.G. (1999). Biosynthetic studies of the alkaloid, stevensine, in a cell culture of the marine sponge *Teichaxinella morchella*. *Tetrahedron Lett*, 40: 4775-4778.
- Becerro, M.A., Turon, X. and Uriz, M.J. (1997). Multiple functions for secondary metabolites in encrusting marine invertebrates. *J Chem Ecol*, 23: 1527-1547.
- Bergquist, P.R. (1978). *Sponges*, London: Hutchinson and Co Ltd.
- Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H.G., Northcote, P.T. and Prinsep, M.R. (2009). Marine natural products. *Nat Prod Rep*, 26: 170-244.
- De Goeij, J.M., Van Den Berg, H., Van Oostveen, M.M., Epping, E.H.G. and Van Duyl, F.C. (2008). Major bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavity sponges. *Mar Ecol Prog Ser*, 357: 139-151.
- Freshney, R.I. (2005). *Culture of animal cells : a manual of basic technique*, Hoboken, NJ: Wiley-Liss.
- Hogg, M.M., Tendal, O.S., Conway, K.W., Pomponi, S.A., Van Soest, R.W.M., Gutt, J., Krautter, M. and Roberts, J.M. (2010). Deep-sea sponge grounds: Reservoirs of biodiversity. UNEP-WCMC Biodiversity Series No 32, Cambridge, UK.
- Hooper, J.N.A. and Soest, R.V.M. (2002). *Systema Porifera*, Dordrecht: Kluwer Academic Publ.
- Ilan, M., Contini, H., Carmeli, S. and Rinkevich, B. (1996). Progress towards cell cultures from a marine sponge that produces bioactive compounds. *J Mar Biotechnol*, 4: 145-149.
- Koopmans, M., Martens, D. and Wijffels, R.H. (2010). Growth efficiency and carbon balance for the sponge *Haliclona oculata*. *Mar Biotechnol*, 12: 340-349.
- Müller, W.E.G., Schatton, W.F.H., Gudrum, M. (1991). Verwendung von avarol oder dessen derivaten zur bekämpfung von entzündlichen systemischen und dermatologischen erkrankungen. [Usage of avarol or its derivatives to combat inflammatory systemic and dermatological diseases.]. *International Patent Application DE 4137093*.
- Müller, W.E.G., Schröder, H.C., Wiens, M., Perovi -Ottstadt, S., Batel, R. and Müller, I.M. (2004). Traditional and modern biomedical prospecting: Part II—The Benefits, approaches for a sustainable exploitation of biodiversity (secondary metabolites and biomaterials from sponges). *J Evid Based Complementary Alternat Med*, 1: 133-144.
- Munro, M.H.G., Blunt, J.W., Dumdei, E.J., Hickford, S.J.H., Lill, R.E., Li, S., Battershill, C.N. and Duckworth, A.R. (1999). The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol*, 70: 15-25.
- Osinga, R., Sidri, M., Cerig, E., Gokalp, S.Z. and Gokalp, M. (2010). Sponge aquaculture trials in the East-Mediterranean sea: New approaches to earlier ideas. *Open Mar Biol J*, 4: 74 - 81.
- Osinga, R., Tramper, J. and Wijffels, R.H. (1998). Cultivation of marine sponges for metabolite production: Applications for biotechnology? *Trends Biotechnol*, 16: 130-134.
- Osinga, R., Tramper, J. and Wijffels, R.H. (1999). Cultivation of marine sponges. *Mar Biotechnol*, 1: 509-532.
- Pomponi, S.A. (1999). The bioprocess-technological potential of the sea. *J Biotechnol*, 70: 5-13.
- Pomponi, S.A. (2001). The oceans and human health: The discovery and development of marine-derived drugs. *Oceanography*, 14: 28-42.
- Pomponi, S.A. (2006). Biology of the Porifera: Cell culture. *Can J Zool*, 84: 167-174.
- Pomponi, S.A. and Willoughby, R. (1994). Sponge cell culture for the production of bioactive metabolites. IN Van Soest, R., Van Kempen, T.M.G. and Braekman, J.C. (Eds.) *Sponges in time and space*. Rotterdam: A A Balkema, 395– 400.
- Pomponi, S.A. and Willoughby, R. (2000). Development of sponge cell cultures for biomedical applications. IN Austin, B. and Mothersill, C. (Eds.) *Aquatic invertebrate cell culture*. London: Springer, 323-336.
- Proksch, P. (1994). Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. *Toxicon*, 32: 639-655.
- Rinkevich, B. (2005). Marine invertebrate cell cultures: new millennium trends. *Mar Biotechnol*, 7: 429-439.
- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol - Anim*, 47: 302-311.

- Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Adv Mar Biol*, 62: 273-337.
- Simpson, T.L. (1984). *The cell biology of sponges*, New York: Springer-Verlag.
- Sipkema, D., Franssen, M.C.R., Osinga, R., Tramper, J. and Wijffels, R.H. (2005a). Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142-162.
- Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J. and Wijffels, R.H. (2005b). Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201-222.
- Sipkema, D., Van Wielink, R., Van Lammeren, A.a.M., Tramper, J., Osinga, R. and Wijffels, R.H. (2003). Primmorphs from seven marine sponges: formation and structure. *J Biotechnol*, 100: 127-139.
- Sipkema, D., Yosef, N.a.M., Adamczewski, M., Osinga, R., Mendola, D., Tramper, J. and Wijffels, R.H. (2006). Hypothesized kinetic models for describing the growth of globular and encrusting Demosponges. *Mar Biotechnol*, 8: 40-51.
- Taylor, M.W., Radax, R., Steger, D. and Wagner, M. (2007). Sponge-associated microorganisms: Evolution, ecology and biotechnological potential. *Microbiol Mol Biol R*, 71: 295-347.
- Unson, M.D., Holland, N.D. and Faulkner, D.J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar Biol*, 119: 1-11.
- Uriz, M.J., Turon, X., Galera, J. and Tur, J.M. (1996). New light on the cell location of avarol within the sponge *Dysidea avara* (Dendroceratida). *Cell Tissue Res*, 285: 519-527.

## Chapter 2

Cultivation of sponges, sponge cells and symbionts:  
achievements and future prospects



## ABSTRACT

Marine sponges are a rich source of bioactive compounds with pharmaceutical potential. Since biological production is one option to supply materials for early drug development, the main challenge is to establish generic techniques for small-scale production of marine organisms. We analysed the state of the art for cultivation of whole sponges, sponge cells and sponge symbionts. To date, cultivation of whole sponges has been most successful *in situ*; however, optimal conditions are species specific. The establishment of sponge cell lines has been limited by the inability to obtain an axenic inoculum as well as the lack of knowledge on nutritional requirements *in vitro*. Approaches to overcome these bottlenecks, including transformation of sponge cells and using media based on yolk, are elaborated. Although a number of bioactive metabolite-producing microorganisms have been isolated from sponges, and it has been suggested that the source of most sponge-derived bioactive compounds is microbial symbionts, cultivation of sponge-specific microorganisms has had limited success. The current genomics revolution provides novel approaches to cultivate these microorganisms.

### This chapter has been published as:

Schippers KJ\*, Sipkema D\*, Osinga R\*, Smidt H, Pomponi SA, Martens DE, Wijffels RH (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Advances in Marine Biology*, 62: 273-337

\*Authors contributed equally

## 1 INTRODUCTION

The use of sponges dates back many centuries. As early as 700 BC, Homer and Aristotle described the use of sponges in medicine (Voultsiadou, 2007). Until the end of the nineteenth century, sponges had been widely used in surgery (Müller et al., 2004b). For example, sponges soaked with extracts of opium were used to anaesthetize patients prior to surgery, and extracts of *Spongia tosta* were found to be effective in the treatment of scrofula (Müller et al., 2004b). Besides their biomedical application, sponges also had commercial value as bath sponges, because of their absorptive properties. Until 1960, commercial bath sponge farming was a lucrative business spreading from the Mediterranean to the Atlantic and Pacific Ocean (Bernard, 1968; Duckworth, 2009). From 1960, due to the invention of the less expensive synthetic bath sponge, farming of the natural bath sponge decreased and now only occupies a small niche in the market for high-quality applications (Hogg et al., 2010). Current biotechnological applications of sponges are more directed towards the production of marine natural products and biomaterials.

Screening for bioactive compounds in sponges began in the mid-twentieth century. Bergmann and Feeney isolated some of the first nucleosides from the Caribbean sponge *Cryptotethya crypta* in 1951 (Bergmann and Feeney, 1951). These nucleosides appeared to have antiviral properties and synthesis of derivatives of these nucleosides resulted in the production of Ara-A (active against herpes) and Ara-C (effective in leukaemia treatment). As a result, Roche Research Institute of Marine Pharmacology started pioneering research in marine natural product drug discovery in the mid-1970s, and scientists began to explore the chemical diversity found in marine organisms and its potential for drug discovery (Pomponi, 2001). More than 20,000 novel, marine-derived chemicals have been discovered since 1965, of which hundreds have potential pharmaceutical applications (Blunt et al., 2009). Marine sponges have continuously been the most prolific source of newly discovered bioactive compounds, with more than 7000 sponge-derived, novel molecules (Blunt et al., 2009). Table 1 lists sponge-derived metabolites that have advanced to clinical trials.

Sponges are benthic and sessile as adults, and they have evolved a sophisticated chemical communication and defence system based on secondary metabolites (Müller et al., 2004b; Hogg et al., 2010). These compounds protect against fouling (Proksch, 1994), predation (Becerro et al., 1997) and microbial infection (Sipkema et al., 2005a).

For most sponge-derived bioactive compounds, it is not clear whether they are produced by the sponge or the symbiont (which can contribute up to 40% of the

sponge volume). Metabolites known to be produced by the sponge are, for example, avarol, found in *Dysidea avara* (Uriz et al., 1996) and stevensine, found in *Axinella corrugata* (Andrade et al., 1999). On the other hand, it has been demonstrated that *Oscillatoria spongelia*, a cyanobacterial symbiont, produces antimicrobial polybrominated biphenyl ethers and might keep the sponge free of other bacteria (Unson et al., 1994).

Besides pharmaceutical products many other applications of sponge derived biomaterials have been identified. For example, the silicon skeleton of glass sponges may serve as a blueprint for the production of very efficient fibre optics (Sundar et al., 2003) and biosilica-producing enzymes from sponges have been applied in nanotechnology (Schröder et al., 2007). In fact, sponges are the only animal able to polymerize silica to generate massive skeletal elements in a single reaction at ambient temperature and pressure (biosintering) (Müller et al., 2009).

**Table 1** Sponge metabolites resulting from drug discovery, modified from (Pomponi, 2001), (Newman and Cragg, 2004), (Molinski et al., 2009) and (Sashidhara et al., 2009).

Metabolite	Source	Status	Target	Developed by
<b>In clinical use</b>				
E7389 (halichondrin B derivative)	<i>Lissodendoryx</i> sp. and <i>Halichondria okadae</i>	Phase III (cancer)	tubulin	Eisai
HMR-4011A (aka IPL-576,092, contignasterol derivative)	<i>Neopetrosia contignata</i>	Phase II (anti-asthmatic)	histamine	Aventis
Zalypsis (renieramycin derivative)	<i>Xestospongia</i> sp.	Phase I (cancer)	DNA	Pharmamar
E7974 (hemiasterlin derivative)	<i>Cymbastela</i> sp. and <i>Auleta</i> sp.	Phase I (cancer)	tubulin	Eisai
taltobulin (aka HTI-286, hemiasterlin derivative)	<i>Cymbastela</i> sp. and <i>Auleta</i> sp.	Phase I (cancer)	tubulin	Wyeth-Andersen
KRN-7000 (agelasphin derivative)	<i>Agelas mauritianus</i>	Phase I (cancer)	NKT	Koezuka-Kirin
LBH 589 (psammaphin derivative)	<i>Psammaphinaplysilla</i> sp.	Phase I (cancer)	HDAC	Novartis
debromohymenialdisine	<i>Stylotella aurantium</i>	Phase I (Alzheimer's)	kinases	Genzyme Tissue Repair
Peloruside A	<i>Mycale hentscheli</i>	Preclinical (cancer)	tubulin	Reata Pharmaceuticals
<b>Discontinued from clinical trials</b>				
cryptophycin 52 (=arenastatin)	<i>Dysidea arenaria</i>	Phase II (cancer)	tubulin	Lilly, Valeriotte
manoalide	<i>Luffariella variabilis</i>	Phase II (antipsoriatic)	calcium channel	Allergan
discodermalide	<i>Discodermia dissoluta</i>	Phase I (cancer)	tubulin	Novartis, HBOI
LAF 389 (bengamide derivative)	<i>Jaspis</i> sp.	Phase I (cancer)	MetAP	Novartis, Crews
LAQ 824 (psammaphin derivative)	<i>Psammaphinaplysilla</i> sp.	Phase I (cancer)	HDAC	Novartis, Crews
girolline (aka girodazole)	<i>Cymbastela cantharella</i>	Phase I (cancer)	protein synthesis	Potier

Collagen is among the most promising sponge-derived biomaterials. Sponge collagen (e.g. from the marine sponge *Chondrosia reniformis*; Swatschek et al., 2002) offers advantages in medical and cosmetic applications compared to mammalian connective tissue-extracted collagens because it is free of risks associated with Bovine Spongiform Encephalopathy (BSE) (Heinemann et al., 2007).

Despite their enormous biotechnological potential, only a few marine natural products have been successfully developed into products. A major obstacle is the lack of sufficient supply of biological material for preclinical and clinical development. Although controlled chemical synthesis of the bioactive molecules (or their analogues) is the preferred production method for pharmaceutical application, most bioactive natural compounds are chemically complex structures that cannot be synthesized easily. Investments in establishing a synthesis route for complex molecules are usually not done until the potency of the compound has been sufficiently proven (i.e.



after completion of preclinical development studies or early clinical trials). As a result, material obtained from natural sources is often needed for preclinical development. Since many of the marine-derived compounds are found in nature in small quantities tons or thousands of tons of fresh material may be needed to supply sufficient amounts. It is obvious that such large quantities of biomass can never be harvested from nature without risking serious damage to the sponge population or even the extinction of the respective species. Culture of marine organisms that produce the bioactive compounds may overcome the supply issue (e.g. Dumdei et al., 1998; Mendola, 2003; Mendola et al., 2006), but at present, many marine organisms are considered unculturable. This paradox (early drug development being dependent on biological production methods that do not exist), which is often referred to as “The Supply Issue” (e.g. Osinga et al., 1998; Munro et al., 1999; Faulkner, 2000; Fusetani, 2000), emphasizes the need to establish appropriate culture techniques. Since biological production is particularly needed for early drug development, the main challenge for marine biotechnologists is to establish generic techniques for efficient, small- to medium-scale production of large numbers of marine organisms.

Depending on the product of interest, different cultivation approaches may be applied to overcome “The Supply Issue”. When product concentrations are high inside the sponge or if the sponge itself is the product (e.g. bath sponges), cultivation of adult sponges may be the best option (Sipkema et al., 2005b). However, many secondary metabolites are present only at low concentrations. In this case, the amount of sponge biomass required is simply too high, and to obtain an economically feasible production system, the product concentrations have to be increased. The use of *in vitro* cell cultures may then be the method of choice. *In vitro* cultivation allows for control of culture conditions and manipulation to increase product concentrations. If the compound of interest is produced by a microbial symbiont, the production system could consist of only the symbiont, if it is possible to culture the symbionts separately. If this is not possible or if the compound is partly synthesized by the sponge and partly by the symbiont, co-culture may be required. Thus, different molecules will require different production systems, and it is important to have a versatile set of cultivation tools.

In this overview, the state of the art of cultivation of whole sponges, sponge cells and sponge symbionts is analysed, and an outlook is presented for development or integration of different cultivation methods.

## 2 SPONGE AQUACULTURE

In the past 15 years, more than 10 review papers have been published on the production of sponge materials for biotechnological purposes and for the trade in bath sponges (Osinga et al., 1998, 1999; Belarbi et al., 2003; Müller et al., 2004a; Mendola et al., 2006; Sipkema et al., 2005b; De Caralt et al., 2007b; Pronzato and Manconi, 2008; Duckworth, 2009; Koopmans et al., 2009). Two of these reviews integrated over 100 years of sponge aquaculture activities (Pronzato and Manconi, 2008; Duckworth, 2009). It is not the aim of this section to repeat the previously reviewed information. We will limit the current review to recent findings and new developments related to *in situ* and *ex situ* aquaculture of sponges. In addition, design principles for sea-based sponge aquaculture will be outlined and we will look in more detail to metabolite production in aquaculture.

### 2.1 *In situ* culture

Sea-based culture of sponges (also termed mariculture or *in situ* culture) refers to growing sponges in their natural environment on man-made constructions such as longlines with scallop lanterns, vertical ropes, underwater platforms, frames and cages (Duckworth, 2009). To start up a sponge mariculture, donor sponges obtained from the wild are usually fragmented artificially by cutting them into smaller pieces termed explants. The explants are then secured to an artificial substrate, for example, ropes, nylon lines or plastic plates, or are positioned into nets or cages.

The idea to fragment sponges into explants originates from the nineteenth century (see historical perspective by Pronzato and Manconi, 2008). Schmidt (1863) was the first to publish a scientific report on mariculture using fragmented sponges. He described cultures of commercial bath sponges in the Northern Adriatic Sea. Since then, many case studies on sponge mariculture have been published (Duckworth, 2009). Despite this long historical record, there are still many problems associated with the concept. Mariculture is vulnerable to biological factors such as predation and fouling. Other potential biological and non-biological risks include diseases (Webster et al., 2002), and damage to the cultures due to rough weather, vandalism and boats. Some real-life examples of these risks will be described.

Table 2 summarizes recently published data on survival and growth for maricultured sponge species. Growth rates reported range between 157% (i.e. a decrease in size) and 2437% per year, with a record in growth rate still being the 5000% increase per month reported by Battershill and Page (1996) for *Lissodendoryx* sp., which has been included in Table 2 for comparison. Most authors determined growth as the increase in size between the start and the end of the culture trial. This makes it difficult to





**Table 2** Growth rates and survival percentages of sponges in mariculture. The abbreviation n.d. means “not determined”. Estimated specific growth rates (SGR) per day were calculated assuming first order kinetics (Sipkema *et al.*, 2006).

Species	Method	Location	Survival	Growth rate reported	Estimated SGR (d <sup>-1</sup> )	Reference
<i>Discodermia dissoluta</i>	Net pockets	Santa Marta, Colombia, 18m	93%	20-39% in 6 months	0.001-0.00183	(Zea <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Plastic pins	Eastern Mediterranean, under fish farm, 21m	100%	137% in four months	0.0072	(Osinga <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Horizontal ropes	Eastern Mediterranean, under fish farm, 21m	90-100%	100% year <sup>1</sup>	0.0019	(Osinga <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Horizontal ropes	Eastern Mediterranean, 18m	90-100%	0% year <sup>1</sup>	0	(Osinga <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Horizontal ropes	Eastern Mediterranean, 28m	90-100%	0% year <sup>1</sup>	0	(Osinga <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Horizontal ropes	Eastern Mediterranean, 38m	90-100%	20-50 % year <sup>1</sup>	0.0005-0.0011	(Osinga <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Glued to frames	Western Mediterranean	40%	468.9% in 10 months	0.0057	(De Caralt <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Ropes	Western Mediterranean	11%	145.25% in 10 months	0.003	(De Caralt <i>et al.</i> , 2010)
<i>Dysidea avara</i>	Cages	Western Mediterranean	70%	166.75% in 10 months	0.0033	(De Caralt <i>et al.</i> , 2010)
<i>Haliclona oculata</i>	Bound to tiles	Scheldt Estuary, Netherlands	n.d.	0.82 % day <sup>-1</sup>	0.0082	(Koopmans and Wijffels, 2008)
<i>Diacarnus erythraenus</i>	Attached with steel wires to PVC plates on frames	Red Sea, 10m	72%	64.5% year <sup>1</sup>	0.0014	(Bergman <i>et al.</i> , 2011)
<i>Diacarnus erythraenus</i>	Attached with steel wires to PVC plates on frames	Red Sea, 20m	70%	79.3% year <sup>1</sup>	0.0016	(Bergman <i>et al.</i> , 2011)
<i>Lissodendoryx</i> sp.	Scallop lanterns	Wellington Harbour, New Zealand	n.d.	5000% month <sup>-1</sup>	0.131	(Battershill and Page, 1996)
<i>Mycale cecilia</i>	Bound to ceramic tiles	Mexican Pacific, 3-6m	95%	207% in 60 days	0.0187	(Carballo <i>et al.</i> , 2010)
<i>Mycale hentcheli</i>	Horizontal ropes, F0 generation explants	Pelorus Sound, New Zealand, near mussel farm, 5-10m	n.d.	1355-2437 % year <sup>1</sup>	0.0073-0.0089	(Page <i>et al.</i> , 2011)
<i>Mycale hentcheli</i>	Horizontal ropes, F1 generation explants	Pelorus Sound, New Zealand, near mussel farm, 5-10m	n.d.	359% year <sup>1</sup>	0.0042	(Page <i>et al.</i> , 2011)
<i>Mycale hentcheli</i>	Horizontal ropes, F2 generation explants	Pelorus Sound, New Zealand, near mussel farm, 5-10m	n.d.	-157% year <sup>1</sup>	negative	(Page <i>et al.</i> , 2011)

compare the growth rates. We have estimated the specific growth rate (SGR) per day for all experiments listed in Table 2, under the assumption that all sponges grew exponentially following first-order kinetics. The highest SGR reported was 0.0187 day<sup>-1</sup> for *Mycale cecilia* (Carballo *et al.*, 2010). For comparison, the record of 5000% per month reported for *Lissodendoryx* sp. (Battershill and Page, 1996) equals an SGR of 0.131 day<sup>-1</sup>, that is, one order of magnitude higher.

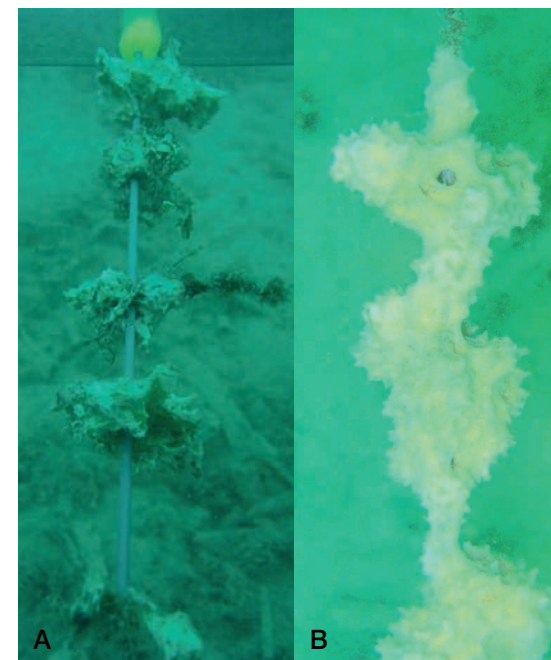
Recently, much effort has been dedicated to develop culture methods for *D. avara* (Schmidt, 1863) in order to produce the bioactive compound avarol. In a joint research

project, *D. avara* was cultured both in the Western Mediterranean (De Caralt *et al.*, 2010) and in the Eastern Mediterranean (Osinga *et al.*, 2010) using comparable methods. Stainless steel frames (height: 50 cm) were positioned on the seafloor and explants of *D. avara* were secured to the frames on horizontal nylon lines (both studies), by gluing (De Caralt *et al.*, 2010) and by putting the explants on plastic pins (Osinga *et al.*, 2010). De Caralt *et al.* (2010) also grew the explants in stainless steel cages. Survival rates were much higher in the Eastern Mediterranean (90–100%) than in the Western Mediterranean (11–70%, depending on the method used). Surprisingly, survival in the Eastern Mediterranean was also nearly 100% for explants that had



been prepared during summer, despite the general consensus in the literature that cold water is better for explant survival after cutting, due to a slower response of infectious microorganisms (Duckworth, 2009). In both studies (de Caralt et al., 2010; Osinga et al., 2010), growth rates depended on the methodology applied and varied from 40% to 800% increase per year. De Caralt et al. (2010) found specific benefits for all three methods that were applied: cage culture displayed the lowest mortality, explants glued to frames showed the highest growth rates and culturing on ropes promoted bioactivity. Osinga et al. (2010) found that growth of *D. avara* explants was highest when plastic pins were applied (Fig. 6.1). This method also proved to be far less labour intensive than establishing rope cultures, and hence, Osinga et al. (2010) concluded that plastic pins provided an economically feasible production method for avarol. Duckworth et al. (2007) had applied a comparable technique termed “spike method” to the bath sponge species *Rhopaloeides odorabile* and *Coscinodermia* sp. and concluded that these spikes could not be recommended to culture these species (growth rates being much lower than for other techniques such as culture in nets and on ropes), confirming once again that optimization of sponge aquaculture is a species-specific process (Duckworth, 2009). The study by Osinga et al. (2010) also demonstrated the vulnerability of sponge mariculture to external factors: the culture frame was found upside down on the seafloor when it was revisited after 1 year.

One of the most comprehensive studies on sponge aquaculture so far was published recently by Page and co-workers (Page et al., 2005, 2011). These authors reviewed 7 years of aquaculture trials on *Mycale hentscheli*, targeting the production of the bioactive compound peloruside A for preclinical research. Their studies included many aspects related to culture success, including culture methods, location selection, broodstock selection, seasonality and metabolite production. Page et al. (2011) were the first to thoroughly document the effects of repeated cloning on culture success. After doing three repeated trials with explants obtained from wild parent sponges (termed F0), two subsequent cultures were made by preparing explants from sponges harvested from the previous culture trials (termed F1 and F2). Although variability between years was considerable for F0 explants, growth rates obtained were at the top end of the range shown in Table 2. Annual growth decreased dramatically for F1 explants and was even negative for F2 explants. The F2 series was confronted with an outbreak of a predatory nudibranch (*Hoplodoris nodulosa*) that consumed a considerable proportion of the explanted biomass. It remains to be studied whether the outbreak of the predator was a coincidence or the result of decreased fitness of the sponges due to repeated cloning, but this case study clearly shows that mariculture remains vulnerable to biological pests.



**Figure 1** Explants of *Dysidea avara* on plastic pins, which were horizontally mounted into a metal frame, approximately 50 cm above the seafloor. (A) Explants at the start of the culture trial. (B) Explants after 4 months in culture. Explants had fused into sponge sausages (cf. Page et al., 2011).

Fouling is another biological threat. According to Page et al. (2011), rope culture is the least vulnerable to fouling. This is presumably due to the fact that when explants are positioned close to each other, they will rapidly cover the entire rope surface (forming structures that the authors termed sponge sausages), thus preventing the settlement of fouling organisms onto the ropes. Similar sponge sausages also formed on the plastic pins used by Osinga et al. (2010) for the culture of *D. avara* (Fig. 6.1B). Explants obtained from the same genetic individual tend to fuse when approaching each other, whereas different genotypes will reject each other (Kruse et al., 1999). Hence, in order to get proper sausage formation, materials from the same genetic individual should be used.

Both Osinga et al. (2010) and Page et al. (2011) positioned their cultures in the vicinity of other aquacultures (fish and mussels, respectively), which was observed to be beneficial for sponge growth. The benefit was most likely due to an increased availability of organic food for the sponges. Osinga et al. (2010) reported a 5- to

10-fold lower visibility at the fish farm site, which is indicative for a higher concentration of particulate organic matter.

In a study on *Diacarnus erythraenus*, Bergman et al. (2011) studied changes in communities of sponge-associated microorganisms between wild and maricultured sponges, another previously neglected aspect of sponge culture. No striking differences were detected. Comparable results were obtained by Webster et al. (2011) for *R. odorabile*. Apparently, maricultured sponges are capable of maintaining a natural bacterial composition within their tissue, which is particularly relevant when the associated microorganisms are involved in the production of the targeted secondary metabolites (see also Section 5).

## 2.2 Design principles for sponge mariculture

Several studies have demonstrated the feasibility of mariculture for the production of sponges on a commercial scale, from which it can be concluded that mariculture is a suitable method for supply. Another conclusion is that successful culture procedures are to a large extent species specific (Duckworth, 2009) and site specific, the latter being nicely illustrated by the conflicting results obtained for *D. avara* at two different parts of the Mediterranean (De Caralt et al., 2010; Osinga et al., 2010). For starting up a sponge mariculture on a new species, it is therefore recommended to first conduct a 1-year trial to evaluate productivity at different locations using different methods. A further point of consideration is seasonality: explant growth may vary considerably throughout the year. Hence, initial aquaculture trials should preferably comprise at least one complete annual cycle. Examples of recent studies describing growth throughout the year include works on *Haliciona oculata* (Koopmans and Wijffels, 2008) *Corticium candelabrum* (De Caralt et al., 2008), *Discodermia dissoluta* (Zea et al., 2010), *D. erythraenus* (Bergman et al., 2011) and *Mycale hentscheli* (Page et al., 2011). Effects of seasonality appear to be limited to temperate and subtropical zones. The growth of temperate and subtropical species (*H. oculata*, *C. candelabrum* and *M. hentscheli*) was positively correlated with temperature, whereas growth of the two tropical species (*D. dissoluta* and *D. erythraenus*) did not show significant seasonal effects.

Surprisingly, not many studies have discussed design principles for sponge aquaculture. A simple design model is represented by

$$Y = N_{exp} \times P_{exp} \quad (1)$$

in which  $Y$  is the yield (the desired harvest, expressed, for example, in kg wet weight),  $N_{exp}$  is the number of explants to be seeded and  $P_{exp}$  is the average harvest per

explant during the production period (kg/explant). From this, it follows that the initial amount of explants to be seeded can be calculated as

$$N_{exp} = Y / P_{exp} \quad (2)$$

In order to determine  $P_{exp}$  for the targeted species, growth rate and survival percentage must be determined from an initial culture trial.  $P_{exp}$  can then be calculated as

$$P_{exp} = (1 - M) E_t \quad (3)$$

in which  $E_t$  is the size of the explant (kg) at end of a culture period with duration  $t$ , and  $M$  is the mortality factor, which is calculated as  $(100 - \text{percentage survival})/100$ . In case of repeated production (i.e. when part of the harvest is used as broodstock for the next production cycle), Eq. (2) can be extended to

$$P'_{exp} = (1 - M) E_t - \left( \frac{Y_2}{Y} \times E^0 \right) \quad (4)$$

where  $Y_2$  represents the desired harvest for the subsequent year and  $P'_{exp}$  is the harvest per explant after subtraction of the part of the harvest that is needed as broodstock to seed the next culture cycle. For example, the targeted sponge grows from an initial explant size of 0.1 kg to a final explant size of 0.8 kg in 1 year, during which time 25% of the explants die. For a repeated annual production of 100 kg,  $P'_{exp}$  equals  $(1 - 0.25)0.8 - (100/100)0.1 = 0.5$  kg. From Eq. (2), it follows that  $N_{exp} = 100/0.5 = 200$ . If these 200 explants are seeded, 150 will survive, representing a total biomass after 1 year of  $150 \times 0.8 = 120$  kg. Since the desired yield is 100 kg, the remaining 20 kg of harvested biomass can be used to seed the next culture cycle. The 20 kg of biomass is enough for another 200 initial explants of 0.1 kg so that the cycle can be repeated. It should be noted here that productivity in subsequent years may decrease due to effects of repetitive cloning (Page et al., 2011).

The calculation principles outlined above can be applied irrespective of the kinetic principles underlying growth but are in that case limited to the duration of the culture period that is chosen for the initial trial (expressed as  $t$  in the equations). Predicted production (yield) should preferably be based upon a good understanding of the growth kinetics of the targeted species (Sipkema et al., 2006). In practice, this means that repeated measurements of size should be done during regular intervals. When growth kinetics are known, it becomes possible to translate previously found growth rates to different culture periods. A commonly made mistake in this respect is that a linear growth model is applied to calculate a dimensionless daily growth rate (% day<sup>-1</sup>) based upon a longer culture interval (e.g. 365% increase per year =  $365/365 =$



1% per day). This incorrect approach suggests an SGR that is much higher than the specific growth would be when based upon an exponential growth model (first-order kinetics; see Sipkema et al., 2006). Linear growth should be expressed in size units per day, for example, cm day<sup>-1</sup>, mg day<sup>-1</sup>, etc.

Koopmans and Wijffels (2008) give a convenient overview of growth rates of sponges in aquaculture and corresponding kinetics. This type of information is crucial for a proper design of commercial sponge maricultures.

### 2.3 Ex situ culture

Keeping sponges in *ex situ* systems such as aquaria remains a challenging task. Although success stories remain scarce (see Table 3 for an overview of growth rates of sponges in aquaria), some progress has been realized in better understanding the cultivation requirements of sponges. Osinga and Kotterman (2007) discovered that continuous addition of ferric iron (Fe<sup>3+</sup>, supplied as ferric citrate) to aquarium water promoted oscule formation and pumping activity in the Mediterranean sponges *C. reniformis* and *Acanthella acuta*. This finding is in agreement with the work of Krasko et al. (2002), who showed that ferric iron upregulates genes involved in channel formation in primmorphs of *Suberites domuncula*. Xue and Zhang (2009) further elaborated on this aspect and found that variations in Fe<sup>3+</sup> concentrations significantly affected culture success of juveniles of *Hymeniacidon perlevis*.

**Table 3** Growth and survival of sponges in *ex situ* systems. Estimated specific growth rates (SGR) per day were calculated assuming first order kinetics (Sipkema et al., 2006).

Species	Food	Survival	Growth rate reported	Estimated SGR (day <sup>-1</sup> )	Reference
<i>Axinella corrugata</i>	4 bacterial strains, <i>Isochrysis galbana</i> , <i>Thalassiosira weissflogii</i> and <i>S. cerevisiae</i>	100%	22% in 8 weeks	0,0036	(Duckworth et al., 2003)
<i>Chondrosia reniformis</i>	Algae powder from <i>Phaeodactylum tricornutum</i> and fish powder	100%	0,0067 day <sup>-1</sup>	0,0067	(Sipkema et al., 2006)
<i>Crambe crambe</i>	RPMI 1640 commercial medium enriched with amino acids and 20% extract of <i>Octopus vulgaris</i>	n.d	400% in 100 days	0,0161	(Garcia Camacho et al., 2006b)
<i>Dysidea avara</i>	<i>Phaeodactylum tricornutum</i>	100%	0,01 – 0,028 day <sup>-1</sup>	0,01-0,028	(Sipkema et al., 2006)
<i>Halichondria melanadocia</i>	4 bacterial strains: <i>M. halophilus</i> , <i>V. alginolyticus</i> , <i>E. coli</i> , and <i>B. subtilis</i>	50-100%	0.08% day <sup>-1</sup>	0,0008	(Duckworth and Pomponi, 2005)
<i>Halichondria melanadocia</i>	2 bacterial strains, <i>Isochrysis galbana</i> and <i>Saccharomyces cerevisiae</i>	50-100%	0.60% day <sup>-1</sup>	0,006	(Duckworth and Pomponi, 2005)
<i>Hymeniacidon perleve</i> (juveniles)	<i>Isochrysis galbana</i> and photosynthetic bacteria	65%	2900% in 43 days	0,078	(Xue and Zhang, 2009)
<i>Mycale cecilia</i>	<i>Isochrysis</i> sp., <i>Tetraselmis</i> sp., powdered yeast	65%	65% in 60 days	0,0084	(Carballo et al., 2010)
<i>Pseudosuberites andrewsi</i>	<i>Phaeodactylum tricornutum</i> or an extract of <i>P. tricornutum</i>	100%	2% day <sup>-1</sup>	0,02	(Osinga et al., 2003)

Mendola (2008) studied the role of water flow on the pumping behaviour of *D. avara*. This species exhibits the formation of fragile exhalant structures, which are flattened by drag forces caused by flow rates higher than 7.5 cm s<sup>-1</sup>. In nature, *D. avara* was found to grow only at sheltered habitats where the average (oscillating) flow did not exceed 5.9 cm s<sup>-1</sup> (Mendola, 2008). Mendola (2008) concluded that flow rates in tanks designed for the culture of *D. avara* should not exceed 8–10 cm s<sup>-1</sup>. Further evidence for the role of flow was provided by Carballo et al. (2010), who successfully cultured explants of *M. cecilia* in airlift aquaria under continuous flow (the flow rate was not specified by the authors). However, growth of the sponges in the aquarium system was threefold lower than growth in mariculture: 65% and 207% in 60 days for aquarium culture and mariculture, respectively (Carballo et al., 2010).

Xue and Zhang (2009) studied effects of temperature on the growth of juvenile specimen of *H. perlevis*. Increasing the temperature from 18 to 23 °C throughout the incubation resulted in a 29-fold increase in size within 43 days, whereas a decreasing temperature caused the juvenile sponges to cease. Juveniles maintained under a constant temperature showed a moderate growth (6.5-fold increase). Natural fluctuations in temperature are crucial for the initiation of sexual reproduction in temperate sponges (Ereskovsky, 2000) and may also influence juvenile development. It remains to be studied to what extent adult sponges are influenced by changes in temperature.

A persistent question with respect to aquaculture success relates to the dietary requirements of sponges. A short synopsis of developments in our understanding of sponge nutrition and productivity may aid further attempts to design feeding regimes for sponges in aquaria. Sponges were traditionally regarded as particle feeders, a view that is still common among sponge scientists. Evidence is growing that particle feeding alone is often not sufficient to sustain the metabolic requirements of sponges (Osinga et al., 2002). Dissolved organic carbon (DOC) appears to be a major source of food for some sponges (Yahel et al., 2003; de Goeij and van Duyl, 2007), comprising up to 90% of the sponge diet. Other studies show that particle feeding alone can be sufficient to sustain sponge growth, both in nature (Hadas et al., 2009; Koopmans et al., 2010) and in *ex situ* culture (Duckworth et al., 2003; Osinga et al., 2003; Duckworth and Pomponi, 2005; Xue and Zhang, 2009; Carballo et al., 2010), although Duckworth and Pomponi (2005) had to apply five times the natural concentration of planktonic food to obtain positive growth rates. Koopmans et al. (2010) presented a carbon balance for the temperate sponge *H. oculata*, based upon measurements of growth, respiration and particle feeding. *H. oculata* used only 34% of the ingested (particulate) carbon for growth and respiration. It was concluded that this sponge grows inefficiently. A recent paper by De Goeij et al. (2009) sheds a different light on productivity and efficiency of resource utilization in sponges. In the Caribbean sponge *Halisarca caerulea*, these authors found extremely high rates of cell division in choanocytes, and these are the cells responsible for the pumping activity of sponges. However, a large proportion of the cellular material was released into the exhalant channels, a process termed shedding. As such, *H. caerulea* exhibited a high cellular turnover without a corresponding increase in biomass. Observations on rapid somatic growth, such as reported for *Lissodendoryx* sp. (Battershill and Page, 1996), indicate that sometimes, rapid cell proliferation does lead to a rapid increase in biomass.

Further evidence for the potential role of DOC as food for sponges in culture was provided by Garcia Camacho et al. (2006b), who successfully cultured small fragments of *Crambe crambe* on an commercial medium for cell culture enriched with amino acids and a marine extract obtained from *Octopus vulgaris* (see Section 4.3 for a more detailed analysis of this study). Growth rates declined in time (a phenomenon that had also been observed by Duckworth et al., 2003) but remained positive for a 100-day period. The only other successful attempts to grow sponges *ex situ* on DOC were studies by Osinga et al. (2003) and Sipkema et al. (2006), who managed to grow *Pseudosuberites andrewsi* and *C. reniformis* on an extract of the marine diatom *Phaeodactylum tricornutum*.

Since the presence of associated microorganisms in sponges can be of crucial importance for their primary and secondary metabolism, it is of importance to know

whether the microbial community in sponges shifts after transfer of the animals into aquarium systems. Analogous to the *in situ* studies by Bergman et al. (2011) and Webster et al. (2011) described in Section 2.1, Isaacs et al. (2009) and Mohamed et al. (2008a,b) compared the bacterial communities of wild specimen of *Clathria prolifera*, *Mycale laxissima* and *Ircinia strobilina* to *ex situ* cultured specimen of each species and found profound differences in community structure. Webster et al. (2011) also found changes in microbial composition when explants of *R. odorabile* were transferred from the sea to aquarium systems. Although some species do not exhibit such changes upon transfer to aquaria (Gerce et al., 2009), the aquarium effect appears to be a commonly occurring phenomenon among marine animals, since several aquarium corals also show dramatic changes in bacterial composition when compared to their wild conspecifics (Kooperman et al., 2007). Culturing sponges and other invertebrates outside their natural environment may disturb symbiotic relationships and may impede natural product formation, since this may be related to the secondary metabolism of the sponge–symbiont association.

Although commercial *ex situ* sponge culture may not be realized in the near future, we provide a short perspective on future design studies. The design principles outlined in Section 2.2 can be applied to *ex situ* culture systems as well. However, in *ex situ* culture systems, seasonality effects can be reduced or even entirely excluded. Therefore, *ex situ* systems may be operated in a semi-continuous mode (regular seeding and harvesting of a small part of the culture) rather than in a batch mode. This has an advantage in terms of volumetric productivity and makes maintenance routines easier because feeding and maintenance regimes do not have to be adapted to changes in standing stock in the system (see analogy for coral culture by Osinga et al., 2011).

## 2.4 Metabolite production in aquaculture

It is crucial for commercial production of sponge metabolites through aquaculture that the cultured sponges retain their ability to produce the metabolites of interest. Some of the studies described above provide data on the production of secondary metabolites by the cultured sponges. Osinga et al. (2010) and Bergman et al. (2011) found comparable levels of avarol and norsesterterpene peroxide acids in wild and maricultured sponges (*D. avara* and *D. erythraenus*, respectively). Carballo et al. (2010) did a qualitative assay on the presence of mycalazal-type of metabolites in crude extract from wild, maricultured and *ex situ* cultured sponges. The metabolites were present under all circumstances. Klöppel et al. (2008) reported a slight increase in metabolite production in *Aplysina aerophoba* when cultured *ex situ*, but the sponges did not grow under the aquarium settings applied. A very promising result was obtained by Zea et al. (2010), who found a fourfold increase in the discodermolide concentration in specimen of *D. dissoluta* that had been maricultured for 6 months.



Table 4 summarizes recent and earlier data on metabolite production in sponge aquacultures. The results in Table 4 indicate that metabolite production is maintained in cultured sponges, both *in situ* and *ex situ*. Variability was high, reported values ranging from a fourfold increase to a complete absence of bioactivity.

**Table 4** Records of metabolite formation in sponge aquaculture.

Sponge species	Metabolite formation	Reference
<i>Acanthella cavernosa</i>	Similar levels of kalihinols in mariculture and wild type, no effects of stress treatments on metabolite production	(Mendola, 2003)
<i>Aplysina aerophoba</i>	Different alkaloid metabolites were present in similar or slightly higher concentration when compared to wild-type sponges; light slightly inhibited metabolite formation	(Klöppel et al., 2008)
<i>Axinella corrugata</i>	Stevensine levels in aquarium culture were 110-157% of the levels found in wild type	(Duckworth et al., 2003)
<i>Callyspongia biru</i>	Similar levels of amphitoxin in mariculture and wild type; high variability among individuals	(De Voogd, 2007)
<i>Diacarnus erythraenus</i>	Similar levels of norsesterterpene peroxide acids in mariculture and wild type	(Bergman et al., 2011)
<i>Discodermia dissoluta</i>	Fourfold increase in discodermolide in maricultured sponges compared to levels in wild type	(Zea et al., 2010)
<i>Dysidea avara</i>	Similar levels of avarol in mariculture and wild type	(Osinga et al., 2010)
<i>Latrunculia sp./ wellingtonensis</i>	Maricultured sponges exhibited bioactivity, levels were similar or higher than in wild type; toxicity of the sponge tissue varied among the different farming methods applied	(Duckworth and Battershill, 2003a; 2003b)
<i>Lyssodendrix sp.</i>	Halichondrins in mariculture were 25-50% of the levels found in wild type	(Dumdei et al., 1998)
<i>Mycale cecilia</i>	Mycalazals present in wild type, mariculture and aquarium culture (qualitative analysis)	(Carballo et al., 2010)
<i>Mycale hentscheli</i>	Mycalamide A and pateamine were present in similar quantities in donor sponges and cultured sponges, the presence of Peluroside A was strongly dependent on location	(Page et al., 2005)
<i>Negombata magnifica</i>	Concentration of latrunculin B in maricultured sponges was up three times higher than previously reported in literature	(Hadas et al., 2005)

**Table 4** Continued.

<i>Polymastia croceus</i>	Maricultured sponges exhibited bioactivity, levels were similar or higher than in wild type; toxicity of the sponge tissue varied among the different farming methods applied	(Duckworth and Battershill, 2003a; 2003b)
<i>Verongia aerophoba</i>	Production and release of aeroplysinin by <i>ex situ</i> cultured sponge fragments, mainly under influence of light and aeration	(Kreuter et al., 1992)

Koopmans et al. (2009) have recently reviewed factors determining metabolite production in sponges. In many species, concentrations of the targeted metabolites are variable in time and space, and production of the metabolites can be induced by stimuli such as simulated predation (wounding) and changes in temperature. In contrast, manoalide production in *Luffariella variabilis* is not at all affected by season, depth and location, and attempts to induce increased manoalide production were not successful (Ettinger-Epstein et al., 2008).

Similarly, *Acanthella cavernosa* cultured in aquaria showed no change in metabolite production upon exposure to light, tissue damage, squeezing of tissue and combinations thereof (Mendola, 2003). In contrast, additional feeding did promote bioactivity in aquarium cultures of *A. corrugata* (Duckworth et al., 2003). Farming techniques have also been reported to influence metabolite production (Duckworth and Battershill, 2003a,b). Hence, optimizing metabolite production in aquaculture is another species-specific process.

### 3 IN VITRO SPONGE CULTURE

#### 3.1 Monodispersed cells

Cell or tissue culture (both subsequently referred to as *in vitro* culture) is a preferred method because the system can be defined and controlled. Sponges have great potential for cell culture because of the presence of totipotent stem cells and because cells can be easily dissociated from its tissue, due to their loosely cellular organization. Despite efforts by several research groups (see Table 5), a continuous<sup>1</sup> sponge cell line has not yet been developed, and the number of primary<sup>1</sup> sponge cell cultures

<sup>1</sup> Primary cell cultures contain cells which are cultured directly from the tissue and have a limited lifespan. Continuous cell cultures are cells with an unlimited lifespan, are often transformed or are derived from tumours.



**Table 5** Publications about primary sponge cell cultures (1993-2010).

Sponge species	Reference	Proliferation observed?	Comments
<i>Clathrina aurea</i>	(Klautau et al., 1993; 1994)	Yes, but...	Later identified as protozoa (Custodio et al., 1995)
<i>Hymeniacidon heliophila</i>	(Pomponi and Willoughby, 1994)	Yes	PHA stimulated cells doubled after 72 hours
<i>Ephydatia muelleri</i>	(Imsiecke et al., 1995)	Yes	Cells were taken from hatched gemmules
<i>Latrunculia (=Negombata) magnifica</i>	(Ilan et al., 1996; Rinkevich et al., 1998)	n.d.	Eventually, cultures were dominated by thraustochytrids (Ilan et al., 1996)
<i>Teichaxinella morchella = Axinella corrugata</i>	(Pomponi et al., 1997; 1998; Pomponi and Willoughby, 2000)	Yes	PHA stimulated cells doubled after 36 hours
<i>Acanthella acuta</i>	(Nickel et al., 2000)	No	
<i>Dysidea avara</i>	(Nickel et al., 2000; De Rosa et al., 2003)	No (Nickel et al., 2001) n.d. (De Rosa et al., 2003)	
<i>Ircinia muscarum = Sarcotragus foetidus</i>	(De Rosa et al., 2001; 2003)	Yes, but...	No data of sponge cell growth over time are shown
<i>Suberites domuncula</i>	(De Rosa et al., 2003; Zhang et al., 2004)	n.d.	Cell viability was measured using a MTT assay (Zhang et al., 2004)
<i>Xestospongia muta</i>	(Richelle-Maurer et al., 2003)	n.d.	
<i>Hymeniacidon perleve (= perlevis)</i>	(Zhao et al., 2005; Sun et al., 2007)	n.d. (Zhao et al., 2005) Yes (Sun et al., 2007)	Purified archaeocytes doubled after approx. 3 days (Sun et al., 2007)
<i>Haliclona oculata</i>	(Schippers et al., 2011)	No	

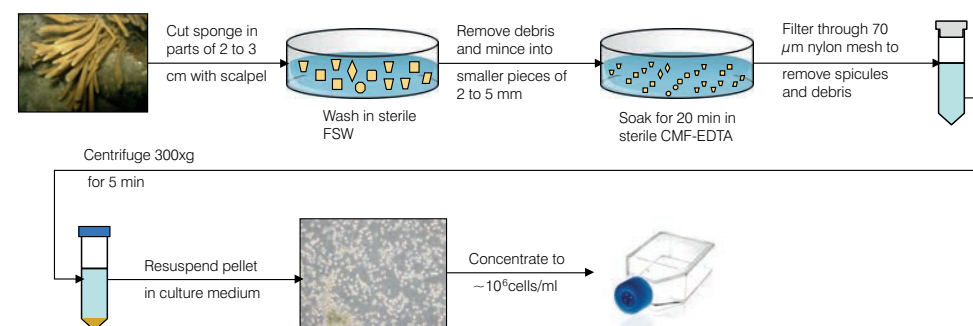
developed is very limited (Rinkevich, 2005). The only report of a continuous sponge cell culture was Klautau et al. (1993, 1994); however, this cell line was subsequently identified as a protozoan (Custodio et al., 1995).

### 3.1.1 Sponge cell dissociation

The first step in obtaining a primary sponge cell culture involves dissociating the cells into a monodisperse suspension. Several dissociation techniques, mechanical, chemical, enzymatic and spontaneous, have been studied (reviewed by Pomponi, 2006). The most successful dissociation method is a combination of mechanical and chemical dissociation (Pomponi and Willoughby, 1994; Rinkevich et al., 1998). If a monodisperse cell suspension is needed, the method described by Pomponi and Willoughby (1994) is generally used (see Fig. 6.2), whereas the method described by Custodio et al. (1998) is applied when re-aggregation of sponge cells (i.e. to form primmorphs) is needed (see Fig. 5).

The dissociation method, described in Fig. 2, will result in a mixture of cell types, for example, archaeocytes, choanocytes, pinacocytes, collencytes. Archaeocytes and choanocytes are considered to be the pluripotent stem cells in sponges. These stem cell-like cells have the capacity to proliferate and to differentiate into other cell types (Funayama, 2010; Funayama et al., 2010). Most cell types in a sponge are expected

to be terminally differentiated and not able to divide. Consequently, selecting and enriching for archaeocytes and choanocytes may result in a proliferating sponge cell culture (reviewed by Pomponi, 2006). Sun et al. (2007) developed a novel four-step protocol for the purification of archaeocytes from the marine sponge *H. perlevis*. By using a process involving differential centrifugation, selective aggregation in low  $\text{Ca}^{2+}$  / $\text{Mg}^{2+}$  seawater, differential adherence in artificial seawater and Ficoll-Urografin



**Figure 2** Dissociation method described by Pomponi and Willoughby (1994). Photograph of *Haliclona oculata* taken by M. Koopmans.

**Table 6** Sponge cell culture media (1994-2011).

Media	Antibiotics	Supplements	Contamination	Sponge species
SW	-	-	No	<i>Xestospongia muta</i> <sup>a</sup> <i>Haliciona oculata</i> <sup>b</sup>
M199 in CMF	rifampicin	5% FBS, 1.5% PHA <sup>*</sup>	No	<i>Hymeniacidon heliophila</i> <sup>c</sup>
M199 in CMF	rifampicin, fungizone	5% FBS, 1.5% PHA <sup>*</sup> ; glutamine, sodium selenite	No	<i>Axinella corrugata</i> <sup>d,e</sup>
Iscoe's MDM	-	epidermal growth factor, bovine pituitary extract <sup>*</sup> , arachidonic acid, insulin, prostaglandin E2, 1.5% PHA <sup>*</sup>	n.d.	<i>Axinella corrugata</i> <sup>d</sup>
Leibowitz L15/Landureau in SW	gentamycin, penicillin, streptomycin, amphotericin, nystatin	10% FBS <sup>g</sup> , [fibroblast growth factor; epidermal growth factor; insulin <sup>*</sup> ; concavalin A, wheat germ agglutinin <sup>*</sup> ; <i>Ulex europaeus</i> agglutinin <sup>*</sup> ; sponge extract] <sup>g</sup>	Yes! n.d. <sup>g</sup>	<i>Negombata magnifica</i> <sup>g</sup>
DMEM in ASW	nystatin, rifampicin, gentamycin, Penicillin, Ciprofloxacin	-	No	<i>Acanthella acuta</i> <sup>b</sup> <i>Dysidea avara</i> <sup>b</sup>
DMEM (half dose) in SW	ampicillin, gentamycin, kanamycin, tylosin, tetracycline (removed after 2 weeks)	cholesterol <sup>h</sup> , [oleic acid, linoleic acid, retinol, cyclodextrin]	No	<i>Ircinia muscarum</i> <sup>i</sup> <i>Dysidea avara</i> <sup>i</sup> <i>Suberites domuncula</i> <sup>i</sup> <i>Suberites domuncula</i> <sup>k</sup>
SW	penicillin, streptomycin	glutamine, pyruvate <sup>*</sup> , iron citrate <sup>*</sup> , silicon, RPMI 1640, Marine Broth 2216	n.d.	
Modified ASW (Zhao's medium <sup>m</sup> )	-	inorganic salts (e.g. ferric iron <sup>*</sup> , NaCl <sup>*</sup> ), amino acids, sugars, vitamin C <sup>*</sup> , DMEM, RPMI 1640, 1.5% PHA	n.d.	<i>Hymeniacidon perlevis</i> <sup>n</sup>
Zhao's medium <sup>l</sup>	gentamycin	2.5% PHA, insulin, transferrin	n.d.	<i>Hymeniacidon perlevis</i> <sup>n</sup>

n.d. not determined; SW = sterile seawater, CMF = calcium and magnesium free seawater, ASW = artificial seawater, DMEM = Dulbecco's Minimum Essential Medium, FBS = foetal bovine serum. <sup>\*</sup> Improved viability of sponge cells; <sup>a</sup> Richelle-Maurer et al. (2003); <sup>b</sup> Schippers et al. (2011); <sup>c</sup> Pomponi and Willoughby (1994); <sup>d</sup> Pomponi et al. (1997); <sup>e</sup> Willoughby and Pomponi (2000); <sup>f</sup> Ilan et al. (1996); <sup>g</sup> Rinkevich et al. (1998); <sup>h</sup> Nickel et al. (2000); <sup>i</sup> De Rosa et al. (2001); <sup>j</sup> De Rosa et al. (2003); <sup>k</sup> Zhang et al. (2004); <sup>l</sup> Zhao et al. (2005); <sup>m</sup> Sun et al. (2007).

density gradient centrifugation, they obtained an archaeocyte-enriched fraction greater than 80%, and proliferation was demonstrated by BrdU incorporation.

### 3.1.2 Sponge cell culture medium

Development of a culture medium is necessary to support the growth of sponge cells. Generally, an axenic inoculum is used to initiate a primary cell culture from animal tissue (Freshney, 2005). It is not possible to isolate such an axenic part from sponges, since both eukaryotic and prokaryotic symbionts are intermingled with the sponge in the tissue. This has made it very difficult to develop nutrient media for sponge cells, since adding nutrients for sponge cell growth will also stimulate growth of contaminants. Therefore, measures have been taken to limit the growth of contaminants, such as bacteria, yeasts, protozoa and thraustochytrids. To prevent growth of these contaminants, antibiotics and antimycotics can be added to the medium, but most of these only temporarily inhibit growth of bacteria and fungi. This may be due to the fact that some bacteria in sponges may have antibiotic resistance. In addition, high concentrations of antibiotics and antimycotics can also have a negative effect on the viability of the sponge cells.

Despite these difficulties, researchers have tried to develop culture media for sponge cells. The most basic culture medium consists of filtered seawater (Richelle-Maurer et al., 2003; Schippers et al., 2011), which provides the correct salinity and pH, and contains some dissolved nutrients and trace elements which are generally present in the sea. Sponge cells could be maintained in filtered seawater for several weeks without contaminants overgrowing the culture. However, since no growth of the sponges cells was observed (Schippers et al., 2011), it is concluded that the dissolved nutrients in seawater are not sufficient to support long-term growth of sponge cells.

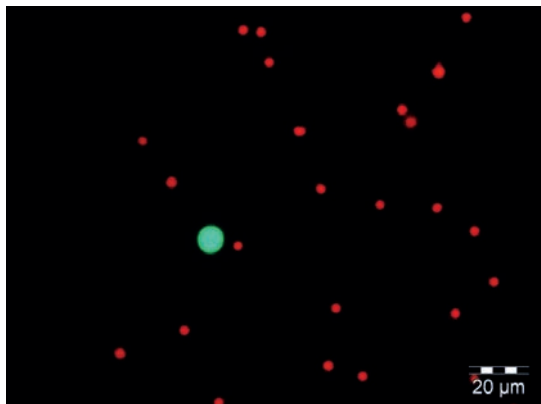
Other sponge cell culture media (reviewed by Pomponi, 2006) have mostly been derived from existing animal cell culture media, which contain amino acids, vitamins and glucose. Since these media are rich in nutrients, the use of antibiotics and antimycotics is also essential. Despite the use of antibiotics and antimycotics, contamination can still occur (Ilan et al., 1996), and the addition of cell culture media alone does not support sponge growth. Growth factors are essential. For example, the addition of PHA (phytohemagglutinin), a mitogenic lectin, stimulated cell division (Pomponi et al., 1997; Sun et al. 2007) and the addition of cholesterol resulted in increased cell density compared to cell cultures without cholesterol (De Rosa et al., 2001, 2003). Evidently, cells *in vitro* are unable to biosynthesize sterols, and the addition of sterols is needed to satisfy membrane requirements. Ferric iron was also found to significantly improve cell viability (Zhang et al., 2004; Zhao et al., 2005). Table 6 provides a more detailed overview.



### 3.1.3 Sponge cell enumeration and viability

Several methods have been used to study sponge cell growth and viability. Counting cells with a haemocytometer has been widely used to measure cell growth (Klautau et al., 1994; Ilan et al., 1996; Pomponi et al., 1997). This method is laborious and time consuming and makes high-throughput screening difficult. Automatic cell counting systems based on image analysis are available but have not been applied to sponge cell culture, most likely because sponge cells have very different sizes and shapes, and contaminants and symbionts need to be discriminated from the sponge cells.

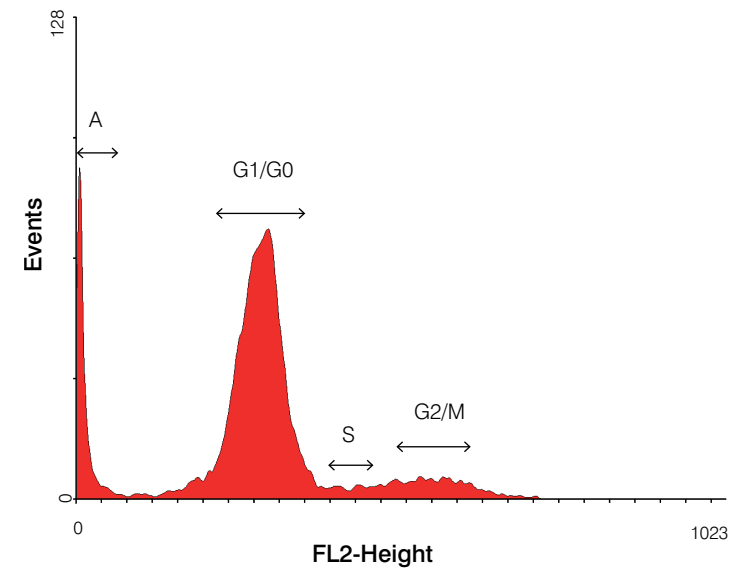
To discriminate between viable and dead sponge cells, trypan blue exclusion from live cells has been used (De Sutter and Van de Vyver, 1977; Pomponi and Willoughby, 1994; Ilan et al., 1996; De Rosa et al., 2003; Richelle-Maurer et al., 2003). However, Sipkema et al. (2004) found that trypan blue diffusion into dead sponge cells can be disturbed, which makes trypan blue an unreliable marker to measure sponge cell viability. A more reliable method is the combined use of fluorescein diacetate (FDA) and propidium iodide (PI), which, respectively, stain viable and dead eukaryotic cells (Sipkema et al., 2004). The dead and viable cells can be visualized by fluorescence microscopy (see Fig. 3) or can be quantified using flow cytometry, as described by Sipkema et al. (2004).



**Figure 3** Viability of cryopreserved *Dysidea avara* cells. Red indicates dead cells and green indicates viable cells.

Another method that has been used to measure viability of sponge cells is the MTT assay (Zhang et al., 2004; Zhao et al., 2005). An advantage of this method is the possibility of using 96-well plates, which makes it possible to screen many samples simultaneously. However, since the cells need to be lysed for the MTT assay, it is no

longer possible to discriminate between sponge cells and contaminants. To determine whether sponge cells are dying through apoptosis, caspase activity, an indicator of early apoptosis, can be measured. Schippers et al. (2011) measured caspase activities of sponge cells before and after dissociation and found a threefold increase of caspase activity of the sponge cells after dissociation, indicating that the dissociation process evidently induced apoptosis.



**Figure 4** Flow cytometric cell cycle analysis, single-parameter histogram of FL2-height (red fluorescence, DNA content) of *Dysidea avara* cells. A, apoptotic cells; G1, cells in gap1; G0, quiescent cells; G2, cells in gap2; M, mitotic cells; S-phase, cells synthesizing DNA (Schippers et al., 2011).

To get more insight into the proliferative state of the sponge cells, it is also possible to measure the different phases of the cell cycle (Schippers et al., 2011). The cell cycle distribution of sponge cells was measured via flow cytometry after staining the DNA with PI. Figure 4 shows an example of a single-parameter histogram of cells from *D. avara*. It is possible to distinguish between the apoptotic, G1/G0 and G2/M fraction. The S-phase fraction is hardly visible, indicating that the cells were not actively dividing. Schippers et al. (2011) used this method in combination with measurement of caspase activity to quickly determine the proliferative status of sponge cell culture inoculum or starting material.

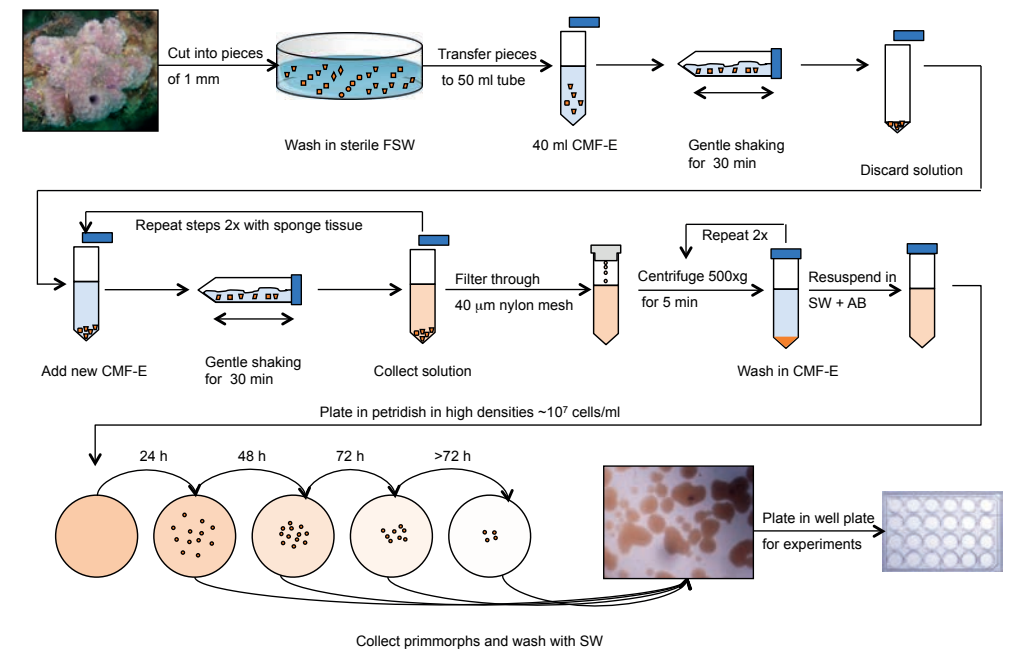
Cell proliferation can also be detected by means of bromodeoxyuridine (BrdU) incorporation. BrdU is an analogue of thymidine that is incorporated in the DNA during DNA synthesis and can be detected with a fluorescent antibody. The antibody can be used on thin sections of the whole sponge or on fixed cells, which has the advantage that one can still discriminate between cell types and in the case of thin sections also between the location in the sponge where proliferation occurs. It can also be used with the above mentioned PI staining of suspended cells and flow cytometry, with the advantage that the S-phase is better separated from the G1 and G2/M phases. BrdU incorporation is stained with a green fluorescent-labelled antibody. Incorporation of BrdU has not been used for dissociated sponge cells but has been used for measuring cell proliferation in primmorphs, gemmules, tissue fragments and adult sponges (Custodio et al., 1998; Nickel and Brummer, 2003; De Goeij et al., 2009; Funayama et al., 2010).

### 3.1.4 Sponge cell-type verification

Contamination of sponge cell cultures remains a problem, which is mainly caused by the fact that it is impossible to obtain an axenic sponge inoculum. Therefore, if growth of sponge cells is observed, it is important to verify if this growth is caused by dividing sponge cells or dividing contaminants, hence the need for cell-type verification. Klautau et al. (1994) performed isoenzyme analysis to confirm that they were working with sponge cells; however, since it appeared later that they were cultivating protozoa, isoenzyme analysis seems unreliable for sponge cell identification. A more reliable technique is to identify the sponge cell culture based on unique DNA sequences. Lopez et al. (2002) were the first to use molecular techniques to identify sponge cell cultures. They applied amplified fragment length polymorphism (AFLP) to compare fingerprints of the internal transcribed tracer (ITS) region of adult sponges and cell cultures of *A. corrugata*. They could successfully detect sponge cells; however, they could not detect the presence of other eukaryotic contaminants. Sipkema et al. (2003a) developed a method based on 18S rDNA, which made it possible to identify sponge cells and eukaryotic contaminants. Another method that could be applied to sponge cell-type verification is fluorescent *in situ* hybridization (FISH). FISH uses fluorescent probes that can bind to specific DNA sequences such as 18S rDNA. By using fluorescent microscopy or flow cytometry, it would be possible to detect if cells are from sponge origin or not. Until now, however, there are no published reports of a FISH-detection method for sponge cells.

### 3.2 Primmorphs

Koziol et al. (1998) found that monodisperse sponge cells lack the capacity to proliferate. A possible explanation could be the loss of cell adhesion factors, such as cell-cell and cell-matrix contact, when cells are dissociated using calcium- and magnesium-free seawater (Koziol et al., 1998). They found that the activity of telomerase, a bio-marker for cell proliferation, in tissue of *S. domuncula* and *Geodia cydonium* was very high, while the telomerase activity of dissociated sponge cells was almost zero. When the dissociated sponge cells re-aggregated, the telomerase activity was recovered, confirming the hypothesis that cell adhesion factors are important for cell proliferation (Custodio et al., 1998). These multicellular aggregates from dissociated sponge cells are termed primmorphs. Primmorphs are dense sphere-shaped aggregates, 1–2 mm in diameter. They differ from aggregates that occur after dissociation of sponge tissue in that they have an external layer of pinacocytes and a central zone with primarily spherulous cells (Custodio et al., 1998; Sipkema et al., 2003c). The formation of primmorphs is based on the discovery of Wilson (1907) that sponge cells have re-aggregative properties and can form a newly functional sponge.



**Figure 5** Method to generate primmorphs, described by Custodio et al. (1998). Abbreviations: FSW, filtered seawater; CMF-E, calcium- and magnesium-free seawater with EDTA; SW, seawater; AB, antibiotics.



**Table 7** Publications about primmorph cultures (1998-2010).

Sponge species	Reference	Proliferation observed?	Lifespan
<i>Suberites domuncula</i>	(Custodio et al., 1998; Muller et al., 1999; Nickel et al., 2001; Sipkema et al., 2003c)	Yes, with BrdU incorporation (Custodio et al., 1998; Muller et al., 1999). n.d. (Nickel et al., 2001; Sipkema et al., 2003c)	> 5 months (Custodio et al., 1998; Muller et al., 1999) > 3 months (Nickel et al., 2001) > 4 months (Sipkema et al., 2003c)
<i>Dysidea avara</i>	(Muller et al., 2000; Valisano et al., 2006b)	Yes, with [3H]dThd incorporation. (Muller et al., 2000) n.d. (Valisano et al., 2006b)	0.8 month (Muller et al., 2000) > 1 month (Valisano et al., 2006b)
<i>Petrosia ficiformis</i>	(Nickel et al., 2001; Pozzolini et al., 2004; Valisano et al., 2006a; 2006b)	n.d.	> 2 months (Nickel et al., 2001, Pozzolini et al., 2004) > 1 month (Valisano et al., 2006a, 2006b)
<i>Axinella verrucosa</i>	(Nickel et al., 2001)	n.d.	> 1 month
<i>Stylissa massa</i>	(Sipkema et al., 2003c)	n.d.	> 2 months
<i>Pseudosuberites aff. andrewsi</i>	(Sipkema et al., 2003c)	n.d.	0.3 months
<i>Geodia cydonium</i>	(Sipkema et al., 2003c)	n.d.	> 2 months
<i>Axinella polypoides</i>	(Sipkema et al., 2003c)	n.d.	> 5 months
<i>Halichondria panicea</i>	(Sipkema et al., 2003c)	n.d.	> 5 months
<i>Stylotella agminata</i>	(Zhang et al., 2003a)	Yes, with cell density	> 7 days
<i>Hymediacidon perlevis</i>	(Zhang et al., 2003b)	Yes, with BrdU incorporation and MTT assay	> 1 month
<i>Spirastrella cunctatrix</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Cliona celata</i>	(Valisano et al., 2006b)	n.d.	0.2 month
<i>Hemimycale columella</i>	(Valisano et al., 2006b)	n.d.	0.5 month
<i>Phorbast fictitius</i>	(Valisano et al., 2006b)	n.d.	0.5 month
<i>Batzella inops</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Axinella damicornis</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Axinyssa aurantiaca</i>	(Valisano et al., 2006b)	n.d.	0.3 month
<i>Acanthella acuta</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Agelas oroides</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Haliclona fulva</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Pleraplysilla spinifera</i>	(Valisano et al., 2006b)	n.d.	> 1 month
<i>Hymeniacidon heliophila</i>	(Vilanova et al., 2010)	n.d.	> 0.3 month
<i>Lubomirskia baikalensis</i>	(Chernogor et al., 2011)	Yes, by measuring optical density	> 10 months

### 3.2.1 Primmorph formation

Custodio et al. (1998) were the first to describe the application of this three-dimensional (3D)-sponge cell culture system for the production of bioactive compounds. They developed a method for creating primmorphs from sponges, and this method has been widely applied by other researchers for generating primmorphs from

various sponge species. Figure 5 shows the procedure for generating primmorphs. The main difference between this method and that of Pomponi and Willoughby (1994) is the high cell concentration during incubation and the presence of calcium and magnesium resulting in re-aggregation of cells.

Primmorph formation depends on several factors. First, not every sponge species has the ability to generate primmorphs. Valisano et al. (2006b) screened 21 Mediterranean sponge species, of which 13 species were able to produce primmorphs. Nickel et al. (2001) screened nine sponge species, of which three produced primmorphs. Sipkema et al. (2003c) screened seven sponge species and all seven were able to generate primmorphs. Table 7 lists all species which have been demonstrated to generate primmorphs. It is not known why certain sponge species are able to produce primmorphs and others are not. Holmes and Blanch (2008) investigated if there is a taxonomic trend in the success of primmorph formation. Although their sample set was small (33 sponges of 9 different orders), they found that sponges from the Poecilosclerida, Suberitidae (Hadromerida), Agelasida and the family Axinellidae I (Clade C) have more success in the formation of primmorphs, while sponges from the Astrophorida, Verongida, Chondrosida, Dendroceratida and the family Axinellidae II show limited or no potential for primmorph formation. These findings, however, are not unambiguous, since contradictory results have been published. For example, Valisano et al. (2006b) could not obtain primmorphs from *Axinella polypoides*, while Sipkema et al. (2003c) could. Also, Nickel et al. (2001) could not obtain primmorphs from *Axinella damicornis* and *A. acuta*, while Valisano et al. (2006b) were able to obtain primmorphs from these sponges.

Another factor related to the success of primmorph formation is seasonal variability. Valisano et al. (2006a) found that for *Petrosia ficiformis*, a Mediterranean sponge, the amount of primmorph biomass formed (size of primmorph x number of primmorphs) is high from April to July, and negligible in the other months. These results indicate that there is not only interspecific variability in the production of primmorphs but also intraspecific variability with respect to the seasonal cycle.

The size of the aggregates is influenced by the inoculum cell density. Both Zhang et al. (2003a) and Sipkema et al. (2003c) found that higher initial cell concentrations resulted in an increased size of the primmorphs. However, Chernogor et al. (2011) found exactly the opposite, that is, a higher cell concentration resulted in smaller primmorphs. A fundamental difference between both experiments is fact that Zhang et al. (2003a) and Sipkema et al. (2003c) used marine sponges and Chernogor et al. (2011) used a freshwater species. These results could indicate that the formation of primmorphs between freshwater and marine sponges is different. However, no decisive conclusions can be drawn, since no other reports of primmorph formation in freshwater sponges have been published.

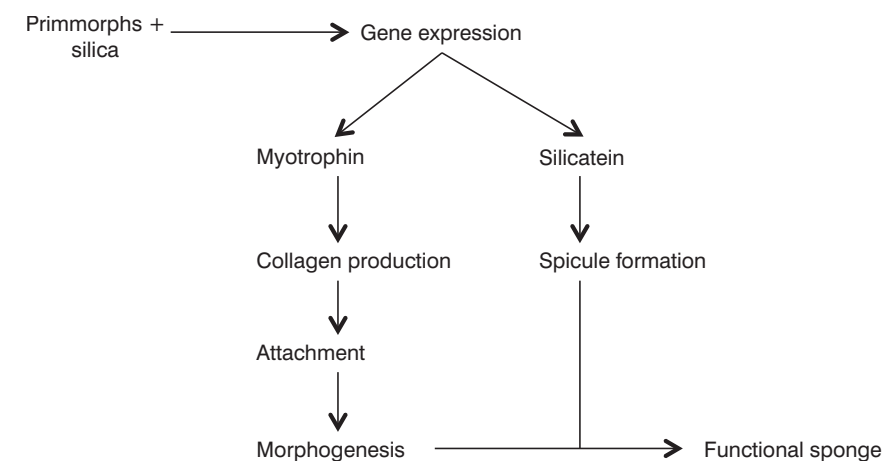
### 3.2.2 Effect of silica and iron

Another factor influencing primmorph formation is the concentration of silica and iron.

By increasing the dissolved silica concentrations from 1 to 60  $\mu\text{M}$ , the formation of silicatein and myotrophin in *S. domuncula* increased (Krasko et al., 2000), resulting in enhanced spiculogenesis and the production of collagen, respectively (see Fig. 6). Subsequently, when silica concentrations increased from 1 to 60  $\mu\text{M}$ , the primmorphs tripled in size from 2 to 6 mm (Le Pennec et al., 2003). Attachment and development of spicules in primmorphs were also induced by the presence of high silica concentrations ( $>70 \mu\text{M}$ ) (Sipkema et al., 2003b). For primmorph formation in *P. ficiformis*, the optimal silica concentration was found to be 120  $\mu\text{M}$ , and these high silica concentrations had a positive effect on primmorph size and spiculogenesis (Valisano et al., 2007). Increased concentrations of iron showed a positive influence on proliferation levels of cells in primmorphs from *S. domuncula* (Krasko et al., 2002). When the concentration of iron was increased to 30  $\mu\text{M}$  in combination with 60  $\mu\text{M}$  silica, the size of the primmorphs almost doubled to 10 mm in diameter. However, for primmorphs from *P. ficiformis*, an increase in iron concentration did not influence the size of the primmorphs, indicating that the influence of iron on primmorph formation is species dependent (Valisano et al., 2007).

### 3.2.3 Effect of antibiotics

To prevent contamination in primmorphs, antibiotics were added to the culture medium in most studies. Penicillin and streptomycin have been commonly used in primmorph cultures (Custodio et al., 1998; Muller et al., 1999; Muller et al., 2000; Zhang et al., 2003a,b). Sipkema et al. (2003c) tested the efficacy and effects of



**Figure 6** Hypothesized influence of silica on morphogenesis and spiculogenesis of primmorphs. Modified scheme from Krasko et al. (2000) and Sipkema et al. (2003b).

several antibiotics on the formation of primmorphs of seven species of marine sponges. The addition of penicillin, streptomycin and gentamycin did not have an effect on the formation of primmorphs, but the addition of amphotericin and KTTG (antibiotic cocktail by De Rosa et al., 2001 containing kanamycin, tylosin, tetracycline and gentamycin) inhibited primmorph formation and is therefore expected to have a negative effect on the viability of the sponge cells. It is remarkable that when no antibiotics were added to the seawater, all primmorph cultures were free of contamination, while almost half of the cultures were contaminated when antibiotics were added. Sipkema et al. (2003c) hypothesize that addition of antibiotics in the seawater kills the endogenous bacterial population of the sponge, which could protect the sponge against microorganisms that are pathogenic to the sponge.

### 3.2.4 Cell proliferation

Cell proliferation has been observed in primmorphs by means of BrdU incorporation (Custodio et al., 1998; Zhang et al., 2003b); however, an increase in total biomass over time has not been observed. Possibly there is an equilibrium in the primmorphs between proliferating and apoptotic cells (Kozioł et al., 1998). A possible explanation for the lack of increase in biomass is the deficiency of nutrients in the media. Primmorphs were generally cultivated in nutrient-poor medium, such as natural seawater or artificial seawater without the addition of any nutrients (Custodio et al., 1998; Nickel et al., 2001; Sipkema et al., 2003c; Zhang et al., 2003a,b; Valisano et al., 2006b). Only Muller et al. (2000) and Krasko et al. (2002) added 0.1% (v/v) marine broth 2216 or 0.2% RPMI 1640, respectively, to their media, but they do not show data for nutrient uptake by the primmorphs. To be able to obtain continuous biomass growth, it is important to supply appropriate nutrients and growth factors.

### 3.3 Tissue fragments

Tissue fragments have been used for cultivating sponges *in situ* and *ex situ* as described in Sections 2.1 and 2.3 but can also be used in an *in vitro* approach (De Caralt et al., 2003; Nickel and Brummer, 2003; Garcia Camacho et al., 2006b; Gunda and Janapala, 2009). The main differences are the sizes of the fragments, which in *in vitro* culture range from 25 to 2500 mm<sup>3</sup>—considerably smaller than the tissue fragments used for *in situ* and *ex situ* cultivation, and the use of a closed system, which allows control of the cultivation parameters. An important advantage of using tissue fragments instead of dissociated cells and primmorphs for *in vitro* culture is the avoidance of cellular stress caused by dissociation and the fact that the cell–cell and cell–matrix contacts are kept intact (Nickel and Brummer, 2003). Although this is a promising method, only five reports of *in vitro* sponge fragment cultivation have been published (see Table 8).

**Table 8** Publications about tissue fragment *in vitro* culture (2003-2009).

Sponge species	Reference	Proliferation observed?	Lifespan
<i>Chondrosia reniformis</i>	(Nickel and Brummer, 2003)	Yes, by using BrdU labelling	> 19 months
<i>Corticium candelabrum</i>	(De Caralt et al., 2003)	Yes ( <i>ex situ</i> ), no ( <i>in vitro</i> ), by measuring the area by digital imaging	> 3 months
<i>Crambe Crambe</i>	(Garcia Camacho et al., 2006a; 2006b)	Yes, by measuring the volume by digital imaging.	> 4 months
<i>Haliclona pigmentifera</i>	(Gunda and Janapala, 2009)	n.d.	> 1 month

Nickel and Brummer (2003) cultured fragments of *C. reniformis* in six-well plates with filter sterilized seawater, supplemented with silicon and gentamycin to prevent bacterial contamination and fed the cultures with lyophilized *Escherichia coli*. During the first weeks, reorganization of tissue took place, and proliferation was detected by BrdU incorporation. However, the amount of proliferating cells was below 1% of the total cell number, indicating a very low proliferation rate. After a couple of months, proliferation could no longer be observed, but the sponge fragments still showed active silica metabolism and fragments could be kept viable for more than 19 months.

De Caralt et al. (2003) cultured fragments of *C. candelabrum* in six-well plates with seawater and monitored explant survival under several experimental conditions. They found that fragments with a high ectosome/choanosome ratio collected from sponges during winter/spring and cultured at a low temperature (14 °C) with antibiotics (penicillin/streptomycin) survived longest. Growth rates were highest when explants were cultured in aquaria without addition of antibiotics. De Caralt et al. (2003) hypothesize that antibiotics are important during the critical first steps when fragments are sensitive for infections but for long-term culture antibiotics have a negative effect on growth. Also, growth rates were higher when explants were fed with algae than with bacteria, which is possibly due to the higher carbon content of algae.

Garcia Camacho et al. (2006b) cultured fragments of *C. crambe* in glass vials with RPMI, enriched with salts, amino acids and *Octopus* extract. Before starting cultivation, they dipped the sponge fragments in 5% sodium hypochlorite and in media with gentamycin, nystatin and penicillin to prevent contamination. The sponge fragments increased 400% in volume in 120 days when 20% (v/v) of *Octopus* extract was added to the medium. If *Octopus* extract was omitted from the medium, no growth took place. This indicates that the *Octopus* extract apparently contains certain





nutrients and/ or growth factors which stimulate growth of the sponge tissue fragments. Also they showed that the sponge fragments were able to take up DOC (dissolved organic matter), since DOC levels decreased rapidly during the first 6 h after adding nutrients.

Gunda and Janapala (2009) cultured fragments of *Haliclona pigmentifera* in flasks with filtered seawater without supplementing nutrients because they only wanted to observe the effect of dissolved oxygen (DO) on the viability of the sponge fragments. Under hypoxia conditions (1.5–2.0 ppm DO), the sponge fragments survived >1 month and the fragments exhibited adhesive growth. Under anoxia conditions (<0.3 ppm DO), the sponge fragments survived only a couple of days. These results indicate that not only nutrients but also DO levels are essential for *in vitro* culture of sponge fragments.

#### 4 CULTIVATION OF SYMBIONTS

Recent technological developments in genomics have changed the way we study genes, species and communities and offer new possibilities for exploiting sponge-derived natural products and sponge–microbial symbioses. The revolution in DNA sequencing with the establishment of pyro- and illumina sequencing has changed the way complex microbial communities, or even whole biotopes can be studied and exploited. Therefore, one might wonder why we would need microbial cultivation after all, especially since the majority of microorganisms seem not capable of growth under artificial conditions. However, there are important reasons why cultivation of target microorganisms is of added value to studying their genes in a (meta)genomic context:

- Isolation of the target microorganisms enables studies of regulation and functionality of (secondary) metabolic pathways in their natural genetic environment. In addition, genomes derived from pure cultures present a simpler data set when compared to complex environmental metagenomes.
- Isolation of the target organism allows preliminary chemical characterization, bioactivity analysis and small-scale production of secondary metabolites with potential pharmaceutical applications.
- Prokaryotic strain collections are (yet) the cornerstone of cellular biodiversity research and conservation.

Marine sponges are host to a very diverse range of microbial symbionts<sup>2</sup> and a source of discovery for many new candidate species, genera and even phyla of micro-

<sup>2</sup> Symbiosis is used in the broad sense according to de Bary's definition as "a close association between organisms of different species" De Bary, A. (1879).

organisms (Fieseler et al., 2004; Sipkema and Blanch, 2010). Although some of these novel microorganisms have been obtained in culture (e.g. Sipkema et al., 2011), most of them are no exception to the rule that the large majority of the microorganisms from environmental samples do not thrive *in vitro*. The relationship of sponges with their microbial symbionts escapes from the classical paradigm of symbiosis that one host and one symbiont perform a complicated dance (Haygood, 2006). Rather, the sponge–microbe conglomerate can be seen as an ecological niche in which complex interactive networks between the host and a variety of partners exist.

A digest of studies on microbial diversity in sponges has yielded the following rules of thumb:

1. Generally, bacteria make up for the bulk of the microorganisms in sponges, and they are often complemented by archaea, fungi, unicellular algae or protozoa (Taylor et al., 2007). In this review, we restrict ourselves to bacteria, archaea and fungi as only a few papers have been published on the cultivation of sponge-associated algae or protozoa.
2. Sponges harbour mostly sponge-specific bacteria and archaea, that is, species belonging to a phylogenetic clade that has no representatives from other environments, such as seawater (Taylor et al., 2007).
3. Two classes of sponges exist with respect to microbial density: high-microbial-abundance sponges, harbouring up to  $10^8$ – $10^{10}$  bacteria per gram sponge, which may account for up to 40% of their body mass and low-microbial-abundance sponges, which typically harbour  $10^5$ – $10^6$  bacteria per gram sponge which is within the range of concentrations in natural seawater (Hentschel et al., 2006).
4. The majority of sponge symbionts are located in the mesohyl, but they are also found on the outer surface of sponges and in a number of cases intracellularly (Hentschel et al., 2006).
5. Sponge symbionts are particularly rich in microorganisms that produce secondary metabolites with potential pharmaceutical applications (e.g. Piel, 2004).

The latter issue has often been the source of inspiration for studies directed towards the isolation of sponge symbionts, and the cumulative number of microbial species that have been isolated from marine sponges in different studies is impressive. In addition, the number of bioactive metabolites that have been detected in these isolates is large and their chemical structures are versatile. However, the number of microbial species that are associated with sponges and has not been obtained in any of these cultivation studies is far more impressive. In addition, none of the sponge-derived lead compounds in clinical trials that are of known or suspected microbial origin, such as halichondrin B and discodermolide, have been obtained via cultivation of their putative symbiotic bacteria.



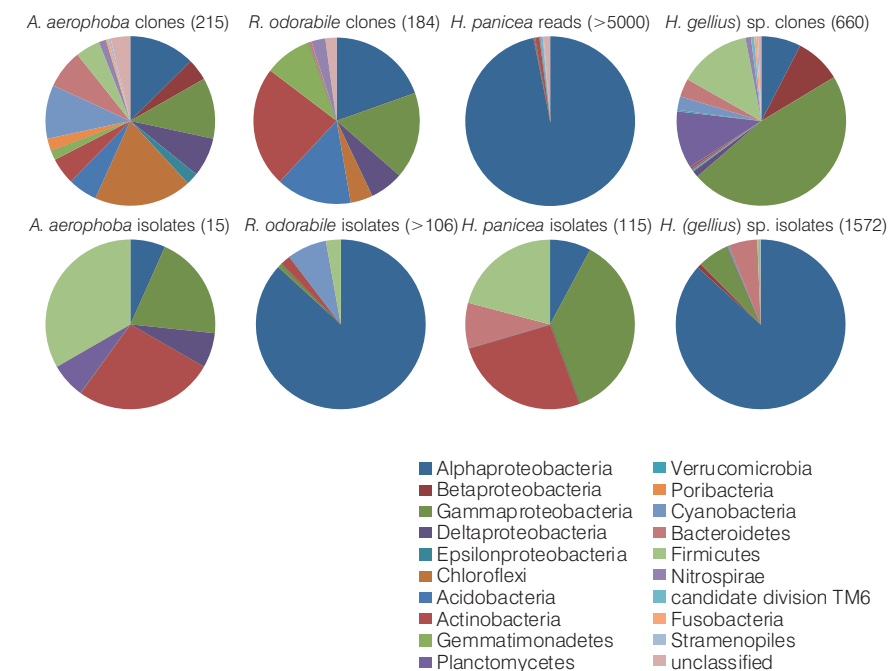
In the next three sections, we will review microorganisms (bacteria, archaea and fungi) that have been isolated from sponges against a background of microorganisms that have been identified in sponges using molecular techniques. It is important to mention that the microbial symbionts from different kingdoms in sponges have currently a different status with respect to the specificity in their association to sponges. For sponge-associated bacteria, it has become clear that most are sponge specific (Hentschel et al., 2002), which is, to a lesser extent, also true for sponge-associated archaea (Holmes and Blanch, 2007). For sponge-associated fungi, no such selective interactions have yet been identified.

#### 4.1 Culturable bacteria from sponges

New papers about bacteria that have been isolated from a marine sponge and are the producer of a previously unknown bioactive compound are being published at a high frequency (e.g. Muscholl-Silberhorn et al., 2008; Schneemann et al., 2010b). However, of these isolated strains, neither the ecological significance of the symbiosis nor their specificity to the sponge as their preferred habitat is generally known. In this review, we illustrate the state of the art in cultivation of sponge-derived bacteria by analysing bacterial isolates from four well-studied sponges for which substantial cultivation- dependent and cultivation-independent data are available: the high-microbial- abundance sponges *A. aerophoba* (Hentschel et al., 2001; Pabel et al., 2003; Pimentel-Elardo et al., 2003, 2010; Ahn et al., 2009; Abdelmohsen et al., 2010) and *R. odorabile* (Webster and Hill, 2001; Webster et al., 2001, 2002) and the low-microbial-abundance sponges *Halichondria panicea* (Perovic et al., 1998; Wichels et al., 2006; Lee et al., 2007, 2009; Schneemann et al., 2010a) and *Haliclona (Gellius)* sp. (Sipkema et al., 2009, 2011). We analysed which part of the bacterial communities could be isolated against the background of cultivation-independent assessments of the bacterial communities from the same species.

At the higher taxonomic level (phylum, but Proteobacteria is analysed at the class level), differences are obvious for all model sponge species when cultivation-dependent and cultivation-independent bacterial profiles are compared (Fig. 7). Firstly, the diversity of phyla/classes obtained via cultivation-independent methods ( $13.3 \pm 5.0$  phyla/classes) is higher than the diversity obtained via cultivation-dependent methods ( $6.5 \pm 2.4$ ) for all four sponge species. Secondly, for none of the four species are there obvious similarities in the dominant phyla between cultivation-independent and cultivation-dependent bacterial profiles. The latter should be no surprise as the “great plate count anomaly” coined by Staley and Konopka (1985) has only been increasing. Despite new developments in cultivation methods of microorganisms, they cannot keep up with the discovery of new phyla by cultivation-independent methods and currently only 30% of the bacterial phyla have cultivated representatives (Achtman and Wagner, 2008).

A more detailed analysis of sponge-associated bacteria (at the species level) via cultivation-independent and cultivation-dependent methods significantly increases the gap between cultivated and uncultivated species (Fig. 8). While for *Haliclona (Gellius)* sp. there is limited overlap between bacterial 16S rRNA gene sequences obtained by cultivation-dependent and cultivation independent methods; for all other model sponges there is not a single isolate derived 16S rRNA gene sequence that



**Figure 7** Cultivation-independent (upper half) and cultivation-dependent (lower half) bacterial profiles from four model sponge species: *Aplysina aerophoba*, *Rhopaloeides odorabile*, *Halichondria panicea* and *Haliclona (Gellius)* sp. per phylum (the phylum Proteobacteria is divided into classes). 16S rRNA gene sequences present in the NCBI GenBank were downloaded at 18 February 2011 using the sponge species name AND “16S” as keywords for the different species. The data were manually corrected for the presence of non-target sponge-derived sequences. In addition, 16S rRNA gene fragments from DGGE bands and diseased *R. odorabile* sponges were excluded from the analysis. As only few 16S rRNA gene sequences from *H. panicea* clones were present in the in the NCBI GenBank, we used the 20 most abundant phylotypes (based on 97% identity) with a length of approximately 350 bp from a 454-pyrosequencing study (Morillo-Perez et al., in progress). The numbers of clones and isolates (or 454-pyrosequencing reads) are in parenthesis for all categories. The number of *R. odorabile* isolates was with a very conservative estimate set at 106, as the exact number of clones could not be derived from the data from Webster and Hill (2001).





matches a sequence obtained via cultivation-independent methods (based on a 97% identity cut-off). These results are not limited to the four model sponges but have been confirmed by other studies where sponge-derived cultivation-dependent and cultivation independent bacterial profiles were compared (Isaacs et al., 2009; Lee et al., 2009; Sun et al., 2010). Although these discrepancies alone are not unusual (see also for comparisons of phylotypes obtained via cultivation-dependent and cultivation-independent methods in gut (Rajilic-Stojanovic et al., 2007) or soil (Zhang et al., 2007)), they underline the alarming inadequacy of our current cultivation methods and the large gap between the discovery of bioactive compounds in sponges and their production in bacterial cultures.

The fact that sponge-derived isolates do not reflect the microbial profiles obtained via cultivation-independent studies implies that although these recovered isolates must be present in the sponge tissue, they are most likely there in low numbers only. We have identified five cultivation clusters from sponge-derived bacteria (i.e. clusters of sponge isolates from at least three of the four model sponges that have no related sequences obtained via cultivation-independent methods) in  $\alpha$ -Proteobacteria,  $\gamma$ -Proteobacteria, Firmicutes and Actinobacteria (Fig. 8). Representatives of these clusters may be present in many sponges, as, for example, NW001 (in CC Alpha), the most common bacterial isolate from *R. odorabile* (Webster and Hill, 2001), relatives of which have also been isolated from many other sponge species worldwide (Enticknap et al., 2006). The only related sponge-derived cultivation-independent sequence that has been obtained (EF092248.1) was present in a very large clone library of *A. corrugata* of 192 clones per sample (Holmes, 2006), which increases the chance of picking up rare bacteria. A similar situation exists for the actinobacterial cultivation cluster (Fig. 8). Representatives have been isolated from a large number of sponges, but they do not coincide with the actinobacterial sponge-specific clades Actinol- III identified by Hentschel et al. (2002). Thus, although Actinobacteria isolated from marine sponges are a very prolific source of new bioactive compounds, these bacteria or their compounds are rarely detected in the sponge itself (Schneemann et al., 2010b; Sun et al., 2010). It is conceivable that many of these isolated bacteria are only present in the canal system when the sponge is collected. Although most researchers rinse the sponge specimens several times with sterile artificial seawater before initiating cultivation experiments, it is very difficult to remove bacteria that are present inside the canal system of the sponge because the sponge most likely ceases pumping upon collection. The presence of more than a million bacteria per millilitre of seawater (Watson et al., 1977) still yields many potential "sponge-derived isolates" selected from a pool of opportunistic passers-by.

When cultivation methods targeting associated bacteria from the four model sponges are assessed, for a substantial number of studies only rich media (e.g. marine agar

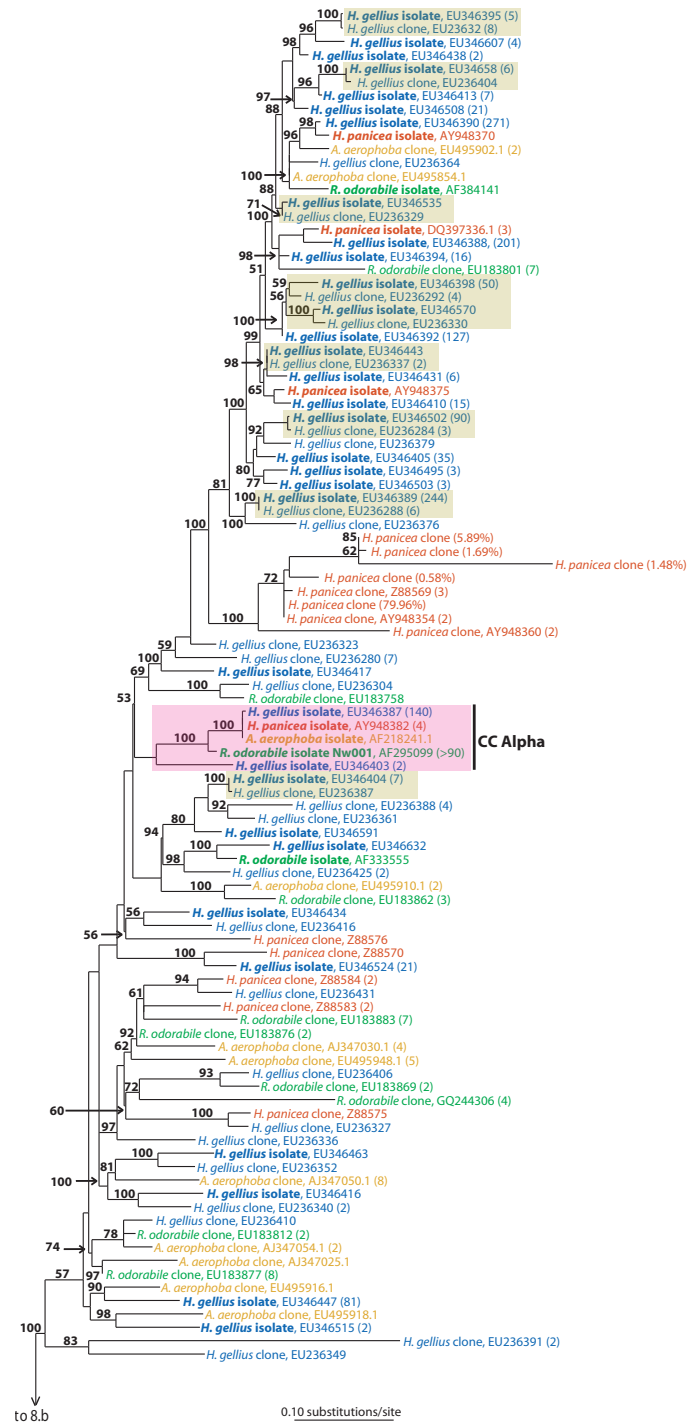
2216) were used (Perovic et al., 1998; Webster et al., 2002; Pabel et al., 2003; Lee et al., 2007). In addition, temperatures significantly above ambient temperatures were used (Lee et al., 2007; Ahn et al., 2009; Abdelmohsen et al., 2010), and in some cases, incubation times were 1 week or less (Perovic et al., 1998; Pabel et al., 2003; Lee et al., 2007; Pimentel-Elardo et al., 2010). These conditions favour isolation of opportunistic bacteria, which may then easily outcompete sponge-specific bacteria.

The only cultivation study of the four model sponge species that resulted in the isolation of bacteria that were confirmed by cultivation-independent data from the same species (*H. (Gellius) sp.*) differed from the other studies in the way that in addition to traditional agar plate cultures also liquid cultures according to the dilution-to-extinction principle and cultivation on floating polycarbonate filters were applied (Sipkema et al., 2011). The use of these "alternative" cultivation methods yielded six isolates that corresponded to cultivation-independently obtained 16S rRNA gene sequences from the same sponge species that were not recovered from agar plates.

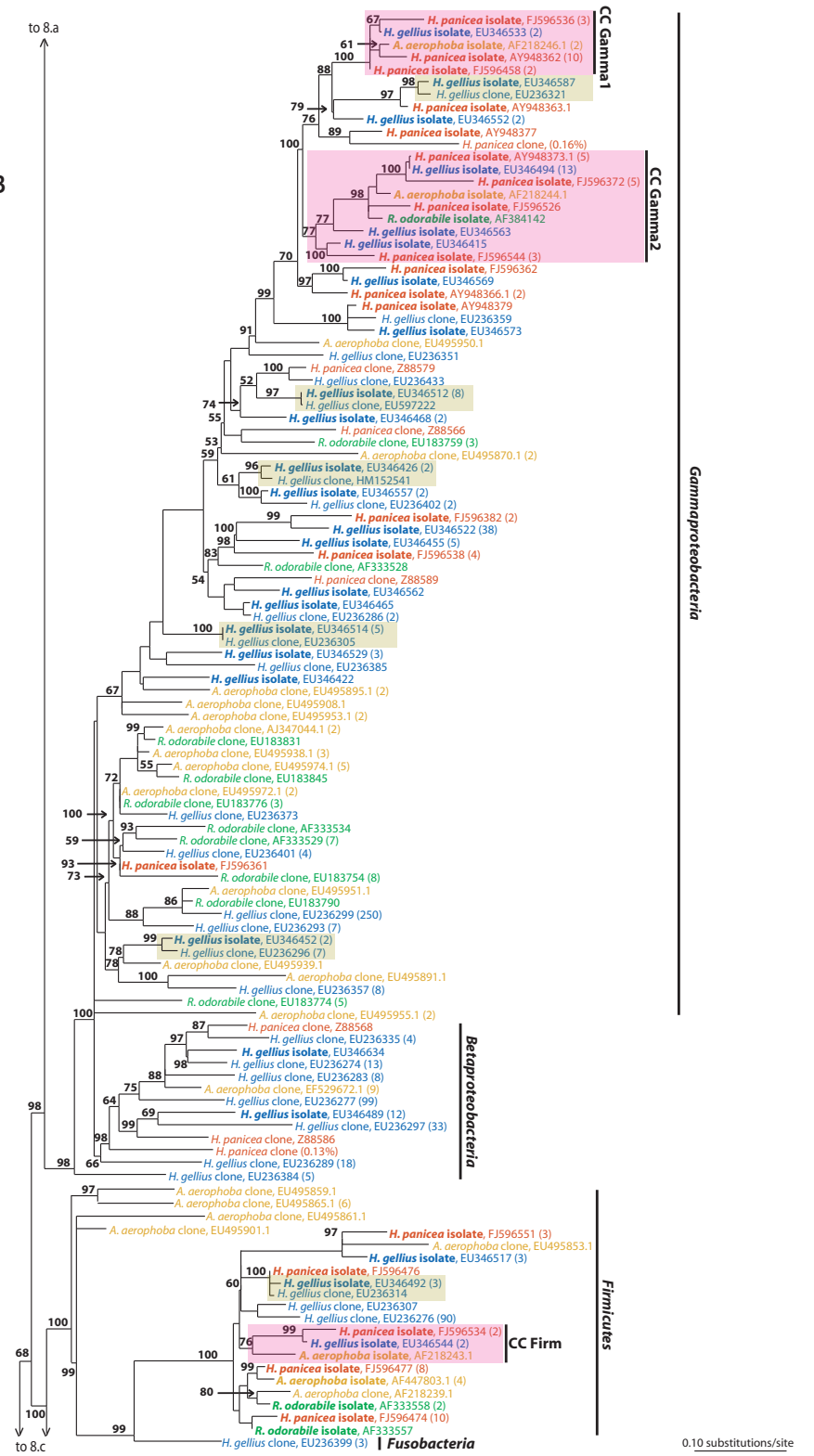
The use of environmental extracts (e.g. sponge extracts) has been frequently advocated as a successful strategy to augment growth of bacteria specific to the particular environment. Sponge extracts have indeed led to the isolation of a different set of microorganisms compared to cultivation conditions without the use of sponge extracts (e.g. Olson et al., 2000; Selvin et al., 2009). Although the latter was also true for the use of sponge extracts to isolate bacteria from the four model sponge species (Webster et al., 2001; Pabel et al., 2003; Wichels et al., 2006; Abdelmohsen et al., 2010; Sipkema et al., 2011), these "extra isolates" were neither sponge-specific nor detected by cultivation-independent techniques in any of the sponge species. This finding may imply that the "dead interactions" supplied with the sponge extract are not sufficient to mimic the natural environment of sponge specific bacteria, but that live interactions are required.

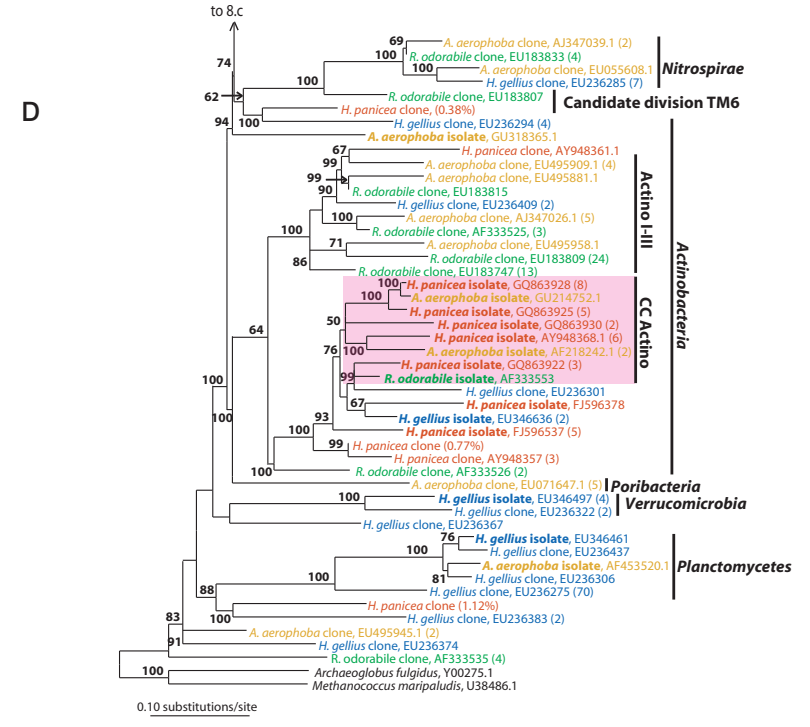
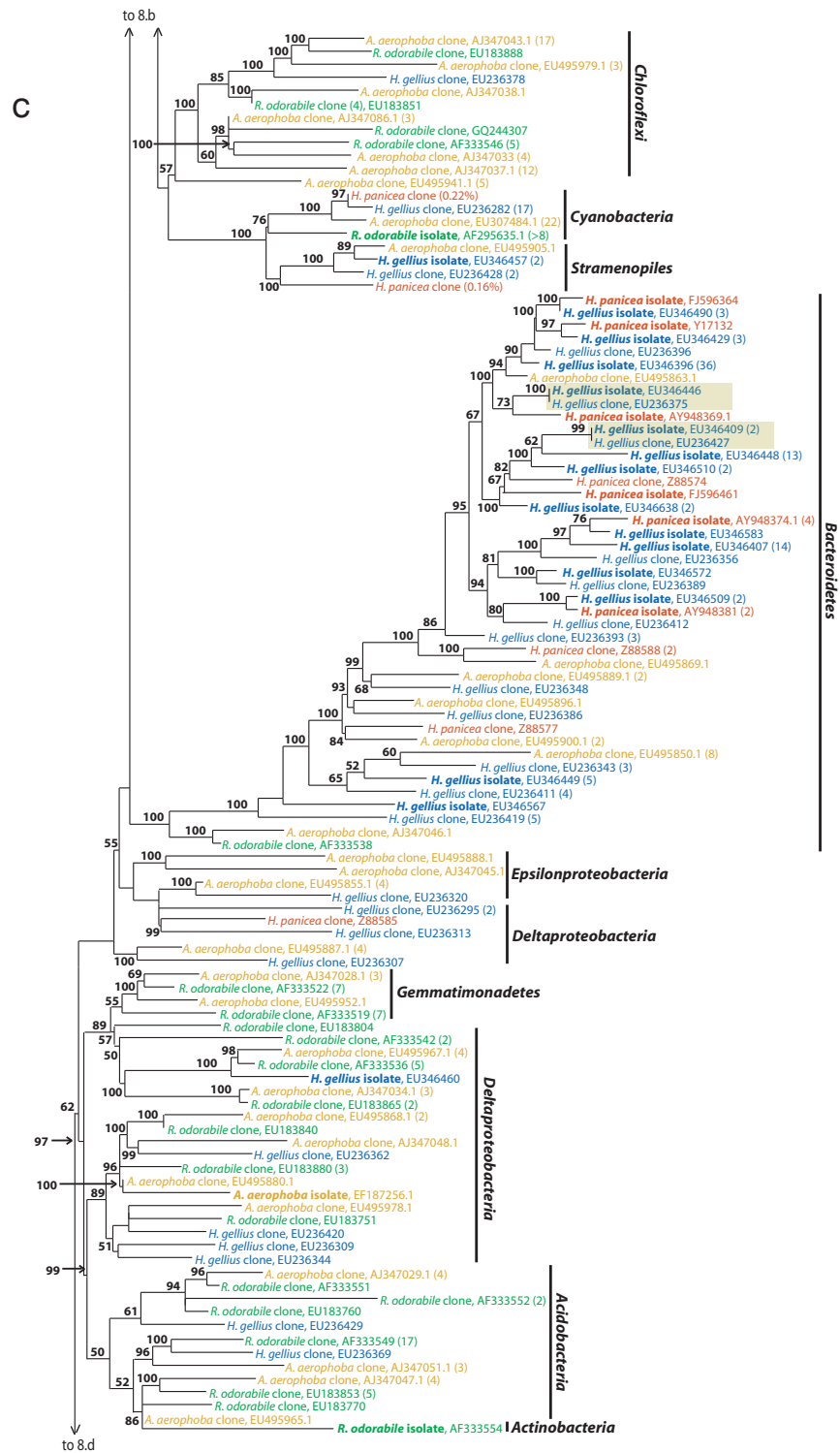
Thus, sponges serve as a very rich reservoir for the isolation of novel bacteria and novel bioactive compounds, but we seem yet incapable in isolating the microorganisms that are important to the sponge.

A



B





**Figure 8** Bayesian phylogram of 16S rRNA gene sequences of all clones and isolates from *A. aerophoba*, *R. odorabile*, *H. panicea* and *H. (gellius)* sp. Isolates are depicted in bold. For the selection of the data, see Fig. 7. If sequences within “isolates” or “clones” from the same sponge species were closer related to each other than to other sequences, they were grouped with the sequence with the longest read length. For these sequences, the number of sequences represented by the clone or isolate is included in parentheses. For the 20 most abundant phylotypes from *H. panicea*, a 454-pyrosequencing study of the 16S rRNA gene the % of the reads within the phylotypes is included in parenthesis. Beige boxes indicate clones and isolates from the same sponge species that are 97% or more than 97% identical. Pink boxes indicate cultivation clusters: a cluster of sequences obtained from isolates from three of the four model sponges without sequences obtained via cultivation-independent techniques. “Actino I–III” are sponge-specific Actinobacteria as defined by Hentschel et al. (2002). The numbers above or below the branches correspond to posterior probability (PP) values of the Bayesian analysis. Nodes with PP values of <50 are not indicated. *Archaeoglobus fulgidus* and *Methanococcus maripaludis* were used as outgroup. Data treatment: all 16S rRNA gene sequences were imported from the Silva database (Pruesse et al., 2007) into the ARB software package (Ludwig et al., 2004) and the alignments were refined manually. Ambiguous regions of the alignment were systematically removed using the program Gblocks v.0.91b (Castresana, 2000). The default programme parameters were used, except allowing a minimum block length of 5 and gaps in 50% of positions. Phylogenetic trees were created by Bayesian analysis, using MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001) at the freely available Bioportal server (www.bioportal.uio.no). All parameters were treated as unknown variables with uniform prior-probability densities at the beginning of each run and their values were estimated from the data during the analysis (Ronquist et al., 2005). All Bayesian analyses were initiated with random starting trees and were run for 1 x 10<sup>7</sup> generations.

## 4.2 Culturable archaea from sponges

No archaea from sponges have been obtained in culture. In contrast, archaea belonging to the recently proposed third archaeal phylum Thaumarchaeota (Brochier-Armanet et al., 2008) have been detected in considerable sized populations in many marine sponges (Holmes and Blanch, 2007). A number of cultivation trials have been done for the sponge-associated Thaumarchaeota, but little information has been published due to the lack of success (e.g. Sipkema et al., 2011). Sponges are not a unique case in that respect as there are only three cases of the successful isolations of thaumarchaeotes (Konneke et al., 2005; Simon et al., 2005; Tourna et al., 2011). They were all a result of long-term experiments with enrichment strategies that lasted for up to 2 years.

The presence of thaumarchaeotes in sponges was first established in *Axinella mexicana* where *Cenarchaeum symbiosum* comprises 65% of the microbial biomass (Preston et al., 1996). A sponge-specific clade of Thaumarchaeota that is predominantly represented by symbionts from the genus *Axinella* has been proposed (Holmes and Blanch, 2007). Genome analysis of *C. symbiosum* has indicated that it can function as a strict autotroph, or as a mixotroph utilizing both carbon dioxide and organic material as carbon sources, while energy is generated by ammonia oxidation (Hallam et al., 2006b). These are important leads for the set-up of cultivation experiments of sponge-associated archaea. At this point, it is important to note that the Thaumarchaeota that were obtained in culture were initially (Konneke et al., 2005; Tourna et al., 2011) or only (Simon et al., 2005) obtained in enrichment cultures. Therefore, successful isolation of sponge-derived Thaumarchaeota may require co-cultivation strategies in addition to a lot of patience and perseverance.

## 4.3. Culturable fungi from sponges

Fungi are at the opposite end of the spectrum with respect to culturability when compared to sponge-associated archaea: hundreds of fungi associated with sponges have been obtained from cultivation studies, while nearly none exist from cultivation-independent studies (Holler et al., 2000; Morrison-Gardiner, 2002; Pivkin et al., 2006; Wang et al., 2008; Baker et al., 2009; Wiese et al., 2011). In addition, sponge-derived fungi are a particularly rich source of bioactive compounds compared to other fungi (Jensen and Fenical, 2002).

It is yet unclear how consistent these sponge–fungus associations are due to limited experimentation. There is some evidence that the species composition of fungal isolates is related to the sponge species (Holler et al., 2000; Pivkin et al., 2006); however, results are not consistent. The sponge *Myxilla incrustans* has been the target of two independent studies and was characterized as very rich (Holler et al.,

2000) or poor (Pivkin et al., 2006) source for the isolation of fungi. This difference may be related to the different geographical origins of the samples (Helgoland and Sakhalin, respectively), but on the other hand, sponge-associated bacterial populations are generally stable across large geographical distances (Hentschel et al., 2006). A second issue is that nearly none of the fungi isolated from sponges are characterized as obligate marine fungi (i.e. fungi that grow and sporulate exclusively in marine or brackish water; Kohlmeyer, 1974), which leaves it unclear whether the isolated strains are consistently associated fungi or passers-by that were trapped in the sponge's filtration system. Fungal spores have a diameter in the order of micrometres and sponges are highly efficient at filtering particles of that size category from the environment (Osinga et al., 2001). Thus, there may be a prolific reservoir of fungi present inside sponges that lead to the isolation of many fungi that are highly related to fungi from terrestrial sources. Terrestrial fungi are generally well able to cope with media containing salt concentrations equal to concentrations in the marine environment, which increases the chance of their isolation from marine sources. Thirdly, when reviewing studies that identify sponge-associated fungi, both by cultivation-dependent and by cultivation-independent methods, cultivated representatives do not reflect data obtained with molecular methods and the paucity of molecular data from sponge-associated fungi leaves it currently unclear whether fungi are present at ecologically relevant concentrations in sponges. Gao et al. (2008) assessed the sponges *Suberites zeteki* and *Mycale armata* by molecular biology-based tools and established the presence of two phyla (Basidiomycota and Ascomycota) and 11 orders of fungi in these sponges, most of them belonging to Malasseziales. The major issue with molecular studies of sponge-associated fungi is that PCR primers used to amplify the 18S rRNA gene or the ITS region generally also amplify the sponge host's 18S rRNA gene or ITS region resulting in only a small fraction of fungal target products. Interestingly, no representatives of Malasseziales were obtained in a cultivation study of fungi derived from the same sponge species (Wang et al., 2008), which is an indication that sponge-associated fungal isolates do not represent the most abundant fungi present in the sponge. Baker et al. (2009) compared fungal isolates from the sponge *Haliclona simulans* with fungal sequences obtained via cultivation-independent methods and found little overlap between fungal species obtained with the two different methods. However, because of the before-mentioned issues with PCR primer specificity, they used primers that predominantly amplify Pezizomycotina and Agaricomycotina, and therefore, they obtained a highly biased molecular profile of *H. simulans*-associated fungi. The scarcity in molecular data of sponge-associated fungi is accompanied by anecdotal microscopic confirmations of the presence of fungi in sponges. The first record is a close association between a coral reef-inhabiting *Koralionastes* species and an unidentified encrusting sponge that grows on top of it (Kohlmeyer and Volkmann-



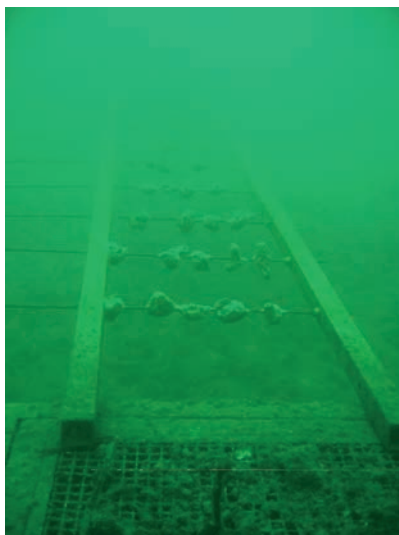
Kohlmeyer, 1990). Another observation of a maternally transmitted endosymbiotic yeast in the marine sponge *Chondrilla* sp. implies a long-standing relationship between the sponge and the yeast (Maldonado et al., 2005).

In summary, there is currently a large discrepancy between fungal species isolated from sponges and fungal species detected in sponges by cultivation independent methods, not in the last place due to very a limited data set on cultivation-independent sponge-associated fungi. The sheer number of different sponge species requires a more robust data set before conclusions on the existence of sponge-specific fungi can be drawn. The rapidly ongoing discovery of novel bioactive compounds from sponge-derived fungi will be a strong driving force that will yield more conclusive information on this issue within the next decade.

## 5 FUTURE DIRECTIONS

### 5.1 Sponge aquaculture

Recent progress in sea-based aquaculture confirms the value of this method for the production of bath sponges and natural compounds. There is renewed interest in multiple use of sponge mariculture as compartments of integrated multitrophic aquaculture systems (see Fig. 9), a trend that has also been recognized by Duckworth



**Figure 9** Mariculture of *Dysidea avara* at 21 m depth underneath a floating-cage sea bass farm in Southwest Turkey. The visibility at the culture site is less than 3 m.

(2009), Osinga et al. (2010) and Page et al. (2011). Targeted sponges grew faster in the vicinity of sea farms growing sea bass (Osinga et al., 2010) and mussels (Page et al., 2011) when compared to pristine sites, where the visibility (a qualitative measure for the availability of organic food) was 5- to 10-fold higher (Osinga et al., 2010). The effect of the sponges on the environment was not evaluated in these studies.

A general overview on zooremediation—the potential use of sponges and other marine animals in water treatment—is provided by Gifford et al. (2007). More specifically, Fu et al. (2006) and Zhang et al. (2010) tested the ability of the sponge *H. perlevis* to remove pathogenic bacteria from seawater. Although the removal potential of the sponges was considerable, removal efficiencies for the different types of pathogens tested were highly variable. Hence, the application of such selective filter feeders in wastewater treatment may lead to competitive advantages for those pathogens that are not effectively being grazed upon (Maldonado et al., 2010).

Despite the good progress that has been made towards controlled sponge culture in aquarium systems (e.g. Garcia Camacho et al., 2006b; Xue and Zhang, 2009; Carballo et al., 2010), *ex situ* culture has currently no competitive advantage over sea-based culture, which is still more productive and cost effective. A further complicating aspect with regard to aquarium culture is the apparent inability of the cultured sponges to maintain a natural composition of associated microorganisms. It remains to be studied to what extent this change in microbiology is reflected in primary and secondary metabolism of the sponge host. In addition, further understanding of the dietary requirements (in particular DOC vs. particles) and the cellular biology of sponges (De Goeij et al., 2009; Koopmans et al., 2010) is likely to turn *ex situ* sponge culture into a feasible technique within the forthcoming years.

### 5.2 *In vitro* sponge culture

#### 5.2.1 Stem cells

Stem cells possess unlimited proliferative capacities *in vivo* and have the ability to differentiate into other cell types, which make them a good starting material for cell culture (Rinkevich, 2011). In adult sponges, certain cell types are present with stem cell properties, such as archaeocytes, which are considered to be pluripotent and to support both sexual and asexual reproduction in sponges. Recent molecular studies also suggest that choanocytes have pluripotent stem cell-like potential (Funayama et al., 2005, 2010; Funayama, 2010). Funayama et al. (2010) found expression of Piwi (genes essential for stem cell self-renewal) homologues in archaeocytes as well as in choanocytes in a freshwater sponge, *Ehydatia fluviatilis*, indicating that both cell types have stem cell properties.

Since adult sponges maintain large pools of adult stem cells, it is possible to isolate



fractions of stem cells (De Sutter and Buscema, 1977; Pomponi and Willoughby, 1994; Sun et al., 2007). Rinkevich (2011) also suggest targeting resting somatic sponge cells and manipulating them to dedifferentiate and become induced pluripotent stem (iPS) cells. These dedifferentiation techniques have already been performed on mammalian cells (Takahashi and Yamanaka, 2006). Besides selecting adult stem cells from sponges, it is also possible to select stem cells from embryos, larvae or gemmules (Imsiecke et al., 1995; Rinkevich et al., 1998; De Caralt et al., 2007b).

### 5.2.2 Immortalization

Most cell types undergo a limited number of divisions and then enter senescence. The establishment of cell cultures with unlimited cell divisions, that is, a transformed or immortalized cell line, has been accomplished in mammalian cells by inserting immortalizing genes by means of transfection or transduction. Immortalizing genes that have been extensively used for mammalian cells are listed in Table 9. To achieve immortalization using immortalizing genes the following prerequisites need to be met: viable primary cells, a proper promoter that is recognized by the RNA polymerase of the sponge, a proper transfection or transduction method, functional immortalizing genes and finally a method to obtain stable insertion of the genes.

Since it is difficult to obtain viable and proliferative sponge cells in primary cell

**Table 9** Genes used for immortalization, modified from (Freshney, 2005).

Gene abbreviation	In full
EBV	Epstein-Bar virus
SV40LT	Simian virus 40 large T
hTERT	Human telomerase reverse transcriptase
HPV	Human papilloma virus
Ad5 E1a	Adenovirus E1a

cultures established from dissociated cells, an alternative approach would be to select other sponge material than dissociated sponge cells for immortalization, for example, gemmules or larvae. Gemmules and larvae both feed on yolk reserves, which provide enough nutrients for the first stages of development into a juvenile sponge. Gemmules have an additional advantage because the outside can be sterilized using 1% H<sub>2</sub>O<sub>2</sub>, which makes contamination less of a problem. It has already been demonstrated that gemmules and larvae can be developed into juvenile sponges *in vitro* (De Caralt et al., 2007a; Funayama et al., 2010).

Successful transfection of freshwater juvenile sponges has already been proven.

Pfannkuchen and Brummer (2009) demonstrated the expression of a red fluorescent protein (DsRed2) under the control of a CMV (cytomegalovirus) promoter in *Spongilla lacustris*, using particle bombardment to insert the plasmid. Pomponi et al. (2007) demonstrated expression of telomerase reverse transcriptase (*tert*) under the control of an MPSV (myeloproliferative sarcoma virus) promoter in primary cell cultures of *A. corrugata*, using lipofection. As transfection has proven to be possible and a promoter that is functional in sponges has been found, the next step towards immortalization is inserting and expressing immortalizing genes.

One candidate gene for sponge cell immortalization is *tert*. Introduction of a human telomerase reverse transcriptase (*htert*) gene into normal human cells showed an extension of the lifespan of the culture, and a portion of the cells became immortal (Bodnar et al., 1998). Telomerase expression has already been demonstrated in adult sponges and primmorphs, as well as primary cultures established from dissociated cells (Pomponi et al. 2007), although expression was not stable and the cells did not become immortalized. It suggests, however, that insertion and expression of a telomerase gene could lead to an immortalized sponge cell line.

### 5.2.3 Growth media

Nutritional requirements of sponge cells are still poorly understood. Since the basic building blocks for biomass (protein, lipids, DNA, RNA) are the same for sponge cells and mammalian cells, mammalian cell culture media may be expected to supply the basic nutrients for these polymers also in sponges. Some sponge-specific compounds, for example, fatty acids, may need to be supplemented. Furthermore, sponge cells may be able to synthesize certain precursors, and some components of mammalian media may not be necessary. Growth factor supplements may be more problematic because it is not likely that mammalian and sponge cells have similar requirements. The use of invertebrate (*Octopus*) serum (Garcia Camacho et al., 2006b) has promoted growth in *C. crambe* tissue fragments. Analysis of the contents of the yolk in gemmules and larvae may also provide insights into improving sponge cell culture medium. Inorganic components, for example, iron and silicate, have promoted cell proliferation Krasko et al. (2002); the effects of other relevant inorganic components should also be analysed.

Genomics, transcriptomics and proteomics are tools that should be used to define culture conditions and to develop rational approaches to immortalization. The genome of one sponge, *Amphimedon queenslandica*, has been sequenced, and the draft genome sequence reveals that it is remarkably similar to other animal genomes in relation to content, structure and organization (Srivastava et al., 2010). Subsequent genome annotation and comparative genomics will allow for the identification of an



extended set of genes and thus reveal the metabolic capabilities and signalling pathways of the sponge. This information can be used to define culture media and provide insights into the regulation of genes related to cell cycling, and perhaps even production of bioactive compounds.

#### 5.2.4 3D culture systems

Normal, untransformed cells from multicellular animals cannot grow in suspension (except for hematopoietic cells) and need to attach to a surface in order to survive and proliferate (Freshney, 2005). Koziol et al. (1998) and Custodio et al. (1998) proved that this is also true for sponge cells in suspension. Isolated sponge cells lacked telomerase activity, while re-aggregated sponge cells (primmorphs) regained telomerase activity. These primmorphs could be kept in culture for several months and were able to hatch and start spreading. Other 3D culture systems could be the cultivation of tissue fragments, gemmules and larvae (Garcia Camacho et al., 2006b; De Caralt et al., 2007a; Funayama et al., 2010). 3D culture systems could be used as starting material for cell culture, since they can be maintained *in vitro* and proliferation has been demonstrated. Ideally, transformation of sponge cells in 3D cultures could lead directly to sponge cell growth in suspension.

#### 5.3 Symbiont culture

The metagenomics revolution will provide an enormously rich resource of genomics, transcriptomics and proteomics information of presently unculturable microorganisms. Practical knowledge on nutrient cycles, amino acid, cofactor and vitamin synthesis potential of individual members of a community is of key importance for the design of tailor-made cultivation conditions for microorganisms that are yet unculturable. For example, based on carbon balances, it has been shown that many sponge species must obtain the major part of their nutrients from DOC rather than from “food bacteria” present in the seawater (Reiswig, 1974; de Goeij et al., 2008). It is expected that a large fraction of DOC in seawater is quite recalcitrant to degradation, but its composition is unknown. Identification of putative enzymes that are involved in the degradation of recalcitrant carbon sources by transcriptome analysis of the sponge community will provide new ideas for growth medium design.

A few recent studies have now resulted in the publication of partial genomes of an unculturable sponge-specific poribacterium (Siegl et al., 2011), an unculturable sponge-specific  $\delta$ -proteobacterium (Liu et al., 2011) and a sponge-specific archaeon (Hallam et al., 2006a). These studies revealed important information about the metabolism of these sponge symbionts: the poribacterium has the genomic repertoire for autotrophic CO<sub>2</sub>-fixation through the Wood–Ljungdahl pathway and is capable of sterol synthesis, which is almost exclusively restricted to eukaryotes. These sterols

most likely play a role in the formation of DNA-containing organelles that are found in Poribacteria (Siegl et al., 2011). Genome analysis of the sponge-specific archaeon has indicated that it can function as a strict autotroph utilizing carbon dioxide as carbon source and ammonia as energy source. The autotrophic nature of these two sponge-specific microorganisms makes them good candidates for isolation in enrichment cultures because highly selective conditions can be applied. The sponge-specific  $\delta$ -proteobacterium was found to possess a glutathione porter that is crucial for growth on glutathione as the sole sulphur source. In addition, its genome encodes a tetracycline resistance protein (Liu et al., 2011). These characteristics are very useful to create specific cultivation conditions for this  $\delta$ -proteobacterium. Moreover, this  $\delta$ -proteobacterium seemed to be specifically associated with (most probably) a cyanobacterium within the sponge tissue and may live off its photosynthetically fixed carbon (Liu et al., 2011). For that reason, co-cultivation with the cyanobacterium may be the best way to obtain this  $\delta$ -proteobacterium in culture. Co-culture with other microorganisms has proven a fruitful strategy for the isolation of “difficult” bacteria and archaea. For example, an organohalide-respiring *Dehalobacter* sp. could only be grown in the presence of a *Sedimentibacter* sp. most likely due to the inability of *Dehalobacter* sp. to synthesize its own vitamins (Van Doesburg et al., 2005). Other bacteria could be grown in pure cultures, but only in diffusion growth chambers, where they were exposed to excreted metabolites from other bacteria (Kaeberlein et al., 2002), and one of the three thaumarchaeotes that could be cultivated was only obtained in a mixed culture (Simon et al., 2005).

The set-up of highly selective cultivation conditions, based on new input by next-generation sequencing technology, is a most promising method to isolate sponge-specific microbes and the microbes that are the putative producers of halichondrin B, discodermolide and many other potent bioactive compounds that have been discovered in marine sponges, but have never been obtained by cultivation of the actual producer.

In addition to genomic information about bacterial and archaeal symbionts from marine sponges, next-generation sequencing will also, perhaps inadvertently, lead to a large number of fungus-derived sequences from sponges that will prove new insights in whether fungi are specifically associated with sponges, and if so, which type of fungi they are.

#### 5.4 Co-cultivation of sponge cells and symbionts

Despite significant efforts, axenic primary sponge cell cultures have not been established and very few sponge-associated microbes that are sponge-specific have been obtained in pure cultures. For most interactions between sponges and



associated microbes, it is currently not clear whether they are commensal or mutualistic. Moreover, it is unclear whether they are obligate or facultative relationships. The mere existence of low-microbial abundance sponges indicates that many sponge species are able to sustain life on their own without the help of microbial symbionts suggesting that the symbiotic relationships may not be obligate, but of a more facultative nature. On the other hand, many symbiotic relationships between sponges and microbes seem to be persistent in time and space (e.g. Friedrich et al., 2001; Lee et al., 2009; Sipkema et al., 2009) and have become very intimate with the presence of bacteria, archaea and yeasts inside the egg cells of sponges (e.g. Maldonado et al., 2005; Sharp et al., 2007; Schmitt et al., 2008). Attempts have been made to study the effect of eradicating sponge-associated bacteria in high-microbial-abundance sponges, but it has been shown that even with an extensive antibiotic treatment under starvation conditions, a large part of the bacteria cannot be cleared (Friedrich et al., 2001; Detmer Sipkema and Sonia de Caralt, unpublished). Therefore, the possibility to cultivate sponges without their associated bacteria could not be tested. For the cultivation of sponge-associated bacteria, sponge extracts have been used as a medium supplement to enhance growth of the bacteria (see Section 4.1); however, these experiments have only been used to somehow supply the bacteria with an artificial sponge host and not the other way around. In addition, only “dead interactions” can be simulated by the use of sponge extracts, while for symbiosis between insects and bacteria, it has been found that many metabolic exchanges exist (Zientz et al., 2004).

Interactions for both primary and secondary metabolism have been hypothesized for sponges and symbionts. Bacteria have been suggested to be involved in the uptake of DOC, which makes up for a large part of the sponge–microbe conglomerate diet (Yahel et al., 2003; de Goeij et al., 2008). Uptake of nitrogen by bacteria and translocation to the sponge host has recently been confirmed (Bayer et al., 2008), and translocation of the photosynthate from endosymbiotic algae to the sponge has also been documented (Wilkinson and Garrone, 1980). Evidence of the exchange of secondary metabolites is scarce, but it has been reported that a sponge accumulates polybrominated biphenyl ethers that are produced by its cyanobacterial endosymbiont (Unson et al., 1994). Due to the mostly uncharacterized interactions between host and microbial symbionts, *in vitro* co-cultivations could provide an intermediate step between the natural environment and axenic cultures. There are many examples of cells that can only be cultivated or indeed be better cultivated in the presence of other cells. For the cultivation of many mammalian cells (e.g. human embryonic stem cells), a layer of feeder cells (e.g. fibroblasts) is used to release nutrients and growth factors into the medium to stimulate growth of the target cells (Unger et al., 2009). For *in vitro* cultivation of lichens, it has been observed that lichen spores germinate but cannot

develop into mycelium without contact with a compatible photobiont (Stocker-Worgotter, 1995).

This does not necessarily have to result in co-cultivation as a final solution, but as an initial step that can be used for further development. Also for *in vitro* cultivation of lichens, it transpired that after initial co-cultivation, the fungus could be cultivated without the photobiont when medium design was aided by information from co-cultivation (Stocker-Worgotter, 1995). For those reasons, co-cultivation of sponge cells and associated microbes is an as yet surprisingly underexplored alternative.

### Acknowledgements

We wish to acknowledge José Morillo-Perez for sharing his preliminary data of 16S rRNA gene pyrosequencing of *H. panicea*.



## REFERENCES

- Abdelmohsen, U. R., Pimentel-Elardo, S. M., Hanora, A., Radwan, M., Abou-El- Ela, S. H., Ahmed, S., and Hentschel, U. (2010). Isolation, phylogenetic analysis and anti-infective activity screening of marine sponge-associated actinomycetes. *Mar Drugs*, 8: 399–412.
- Achtman, M., and Wagner, M. (2008). Microbial diversity and the genetic nature of microbial species. *Nat Rev Microbiol*, 6: 431–440.
- Ahn, Y.B., Kerkhof, L. J., and Haeggblom, M. M. (2009). *Desulfoluna spongiiphila* sp. nov., a dehalogenating bacterium in the *Desulfobacteraceae* from the marine sponge *Aplysina aerophoba*. *Int J Syst Evol Microbiol*, 59: 2133–2139.
- Andrade, P., Willoughby, R., Pomponi, S. A., and Kerr, R. G. (1999). Biosynthetic studies of the alkaloid, stevensine, in a cell culture of the marine sponge *Teichaxinella morchella*. *Tetrahedron Lett*, 40: 4775–4778.
- Baker, P., Kennedy, J., Dobson, A., and Marchesi, J. (2009). Phylogenetic diversity and antimicrobial activities of fungi associated with *Haliclona simulans* isolated from Irish coastal waters. *Mar Biotechnol*, 11: 540–547.
- Battershill, C. N., and Page, M. J. (1996). Sponge aquaculture for drug production. *Aquaculture Update*, 16: 5–6.
- Bayer, K., Schmitt, S., and Hentschel, U. (2008). Physiology, phylogeny and *in situ* evidence for bacterial and archaeal nitrifiers in the marine sponge *Aplysina aerophoba*. *Environ Microbiol*, 10: 2942–2955.
- Becerro, M. A., Turon, X., and Uriz, M. J. (1997). Multiple functions for secondary metabolites in encrusting marine invertebrates. *J Chem Ecol*, 23: 1527–1547.
- Belarbi, E. H., Contreras Gomez, A., Chisti, Y., Garcia Camacho, F., and Molina Grima, E. (2003). Producing drugs from marine sponges. *Biotechnol Adv*, 21: 585–598.
- Bergman, O., Haber, M., Mayzel, B., Anderson, M., Shpigel, M., Hill, R., and Ilan, M. (2011). Marine-based cultivation of Diacarnus sponges and the bacterial community composition of wild and maricultured sponges and their larvae. *Mar Biotechnol*, 6: 1169–1182.
- Bergmann, W., and Feeney, R. J. (1951). Contributions to the study of marine products. XXXII. The nucleosides of sponges. I. *J Org Chem*, 16: 981–987.
- Bernard, H. R. (1968). *Kalymnos: Economic and cultural change on a Greek sponge fishing island*. Urbana-Champaign, USA: University of Illinois Press.
- Blunt, J. W., Copp, B. R., Hu, W. P., Munro, M. H. G., Northcote, P. T., and Prinsep, M. R. (2009). Marine natural products. *Nat Prod Rep*, 26: 170–244.
- Bodnar, A. G., Ouellette, M., Frolkis, M., Holt, S. E., Chiu, C. P., Morin, G. B., Harley, C. B., Shay, J. W., Lichtsteiner, S., and Wright, W. E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science*, 279: 349–352.
- Brochier-Armanet, C., Boussau, B., Gribaldo, S., and Forster, P. (2008). Mesophilic crenarchaeota: Proposal for a third archaeal phylum, the Thaumarchaeota. *Nat Rev Microbiol*, 6: 245–252.
- Carballo, J. L., Yanez, B., Zubia, E., Ortega, M. J., and Vega, C. (2010). Culture of explants from the sponge *Mycale cecilia* to obtain bioactive mycalazal-type metabolites. *Mar Biotechnol*, 12: 516–525.
- Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol*, 17: 540–552.
- Chernogor, L., Denikina, N., Belikov, S., and Ereskovsky, A. (2011). Long-term cultivation of primmorphs from freshwater Baikal sponges; *Lubomirskia baikalensis*. *Mar Biotechnol*, 4: 782–792.
- Custodio, M. R., Imsiecke, G., Borojevic, R., Rinkevich, B., Rogerson, A., and Muller, W. E. (1995). Evolution of cell adhesion systems: Evidence for Arg-Gly-Asp-mediated adhesion in the protozoan *Neoparamoeba aestuarina*. *J Euk Microbiol*, 42: 721–724.
- Custodio, M. R., Prokic, I., Steffen, R., Koziol, C., Borojevic, R., Brummer, F., Nickel, M., and Muller, W. E. G. (1998). Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: A model system for studies of cell proliferation and cell death. *Mech Ageing Dev*, 105: 45–59.
- De Bary, A. (1879). *Die Erscheinung der Symbiose*. Stra burg: Verlag Trubner.
- De Caralt, S., Agell, G., and Uriz, M. J. (2003). Long-term culture of sponge explants: Conditions enhancing survival and growth, and assessment of bioactivity. *Biomol Eng*, 20: 339–347.
- De Caralt, S., Otjens, H., Uriz, M., and Wijffels, R. (2007a). Cultivation of sponge larvae: settlement, survival, and growth of juveniles. *Mar Biotechnol*, 9: 592–605.
- De Caralt, S., Sanchez-Fontenla, J., Uriz, M. J., and Wijffels, R. H. (2010). *In situ* aquaculture methods for *Dysidea avara* (Demospongiae, Porifera) in the Northwestern Mediterranean. *Mar Drugs*, 8: 1731–1742.
- De Caralt, S., Uriz, M. J., and Wijffels, R. H. (2007b). Cell culture from sponges: pluripotency and immortality. *Trends Biotechnol*, 25: 467–471.
- De Caralt, S., Uriz, M. J., and Wijffels, R. H. (2008). Grazing, differential size-class dynamics and survival of the Mediterranean sponge *Corticium candelabrum*. *Mar Ecol Prog Ser*, 360: 97–106.
- De Goeij, J. M., De Kluijver, A., Van Duyl, F. C., Vacelet, J., Wijffels, R. H., De Goeij, A., Cleutjens, J. P. M., and Schutte, B. (2009). Cell kinetics of the marine sponge *Halisarca caerulea* reveal rapid cell turnover and shedding. *J Exp Biol*, 212: 3892–3900.
- De Goeij, J. M., Van Den Berg, H., Van Oostveen, M. M., Epping, E. H. G., and Van Duyl, F. C. (2008). Major bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavity sponges. *Mar Ecol Prog Ser*, 357: 139–151.
- De Goeij, J. M., and Van Duyl, F. C. (2007). Coral cavities are sinks of dissolved organic carbon (DOC). *Limnol Oceanogr*, 52: 2608–2617.
- De Rosa, S., De Caro, S., Iodice, C., Tommonaro, G., Stefanov, K., and Popov, S. (2003). Development in primary cell culture of demosponges. *J Biotechnol*, 100: 119–125.
- De Rosa, S., De Caro, S., Tommonaro, G., Slantchev, K., Stefanov, K., and Popov, S. (2001). Development in a primary cell culture of the marine sponge *Ircinia Muscarum* and analysis of the polar compounds. *Mar Biotechnol*, 3: 281–286.
- De Sutter, D., and Buscema, M. (1977). Isolation of a highly pure archeocyte fraction from the fresh-water sponge *Ephidatia fluviatilis*. *Wilhelm Roux Arch*, 183: 149–153.
- De Sutter, D., and Van De Vyver, G. (1977). Aggregative properties of different cell types of the fresh-water sponge *Ephidatia fluviatilis* isolated on ficoll gradients. *Wilhelm Roux Arch*, 181: 151–161.
- De Voogd, N. J. (2007). The mariculture potential of the Indonesian reef-dwelling sponge *Callyspongia (Euplacella) biru*: Growth, survival and bioactive compounds. *Aquaculture*, 262: 54–64.
- Duckworth, A. (2009). Farming sponges to supply bioactive metabolites and bath sponges: A review. *Mar Biotechnol*, 11: 669–679.
- Duckworth, A. R., and Pomponi, S. A. (2005). Relative importance of bacteria, microalgae and yeast for growth of the sponge *Halichondria melanadocia* (De Laubenfels, 1936): A laboratory study. *J Exp Mar Biol Ecol*, 323: 151–159.
- Duckworth, A. R., Samples, G. A., Wright, A. E., and Pomponi, S. A. (2003). *In vitro* culture of the tropical sponge *Axinella corrugata* (Demospongia): Effect of food cell concentration on growth, clearance rate and biosynthesis of stevensine. *Mar Biotechnol*, 5: 519–527.
- Duckworth, A. R., Wolff, C., and Evans-Illidge, E. (2007). Developing methods for commercially farming bath sponges in tropical Australia. IN Custódio, M.R., Lóbo-Hajdu, G., Hajdu, E., Muricy, G. (Eds.) *Porifera Research- Biodiversity, Innovation and Sustainability*. Rio de Janeiro: Museu Nacional, 297–302.
- Duckworth, A. R., and Battershill, C. N. (2003a). Developing farming structures for production of biologically active sponge metabolites. *Aquaculture*, 217: 139–156.
- Duckworth, A. R., and Battershill, C. N. (2003b). Sponge aquaculture for the production of biologically active metabolites: The influence of farming protocols and environment. *Aquaculture*, 221: 311–329.
- Dumdei, E. J., Blunt, J. W., Munro, M. H. G., Battershill, C. N., and Page, M. J. (1998). The whys and whats of sponge chemistry: Why chemists extract sponges and what problems does this cause? IN Watanabe, Y., Fusetani, N. (Eds.) *Sponge Sciences; Multidisciplinary Perspectives*. Tokyo: Springer-Verlag, 353–364.

- Enticknap, J. J., Kelly, M., Peraud, O., and Hill, R. T. (2006). Characterization of a culturable alphaproteobacterial symbiont common to many marine sponges and evidence for vertical transmission via sponge larvae. *Appl Environ Microbiol*, 72: 3724–3732.
- Ereskovsky, A. V. (2000). Reproduction cycles and strategies of the cold-water sponges *Halisarca dujardini* (Demospongiae, Halisarcida), *Myxilla incrustans* and *Lophin piceus* (Demospongiae, Poecilosclerida) from the White Sea. *Biol Bull*, 198: 77–87.
- Ettlinger-Epstein, P., Tapiolas, D. M., Motti, C. A., Wright, A. D., Battershill, C. N., and De Nys, R. (2008). Production of monoalide and its analogues by the sponge *Luffariella variabilis* is hardwired. *Marine Biotechnology* 10, 64–74.
- Faulkner, D. J. (2000). Marine pharmacology. *Antonie van Leeuwenhoek*, 77: 135–145.
- Fieseler, L., Horn, M., Wagner, M., and Hentschel, U. (2004). Discovery of the novel candidate phylum "Poribacteria" in marine sponges. *Appl Environ Microbiol*, 70: 3724–3732.
- Freshney, R. I. (2005). *Culture of Animal Cells: A Manual of Basic Technique*. Hoboken, NJ: Wiley-Liss.
- Friedrich, A. B., Fischer, I., Proksch, P., Hacker, J., and Hentschel, U. (2001). Temporal variation of the microbial community associated with the Mediterranean sponge *Aplysina aerophoba*. *FEMS Microbiol Ecol*, 38: 105–115.
- Fu, W. T., Sun, L. M., Zhang, X. C., and Zhang, W. (2006). Potential of the marine sponge *Hymeniacidon perleve* as a bioremediator of pathogenic bacteria in integrated aquaculture ecosystems. *Biotechnol Bioeng*, 93: 1112–1122.
- Funayama, N. (2010). The stem cell system in demosponges: Insights into the origin of somatic stem cells. *Dev Growth Differ*, 52: 1–14.
- Funayama, N., Nakatsukasa, M., Hayashi, T., and Agata, K. (2005). Isolation of the choanocyte in the fresh water sponge, *Ephydatia fluviatilis* and its lineage marker, Ef annexin. *Dev Growth Differ*, 47: 243–253.
- Funayama, N., Nakatsukasa, M., Mohri, K., Masuda, Y., and Agata, K. (2010). Piwi expression in archeocytes and choanocytes in demosponges: Insights into the stem cell system in demosponges. *Evol Dev*, 12: 275–287.
- Fusetani, N. (2000). *Drugs from the Sea*. Basel, Switzerland: Karger.
- Gao, Z., Li, B. L., Zheng, C. C., and Wang, G. Y. (2008). Molecular detection of fungal communities in the Hawaiian marine sponges *Suberites zeteki* and *Mycale armata*. *Appl Environ Microbiol*, 74: 6091–6101.
- García Camacho, F., Chileh, T., Ceron García, M. C., Sanchez Miron, A., Belarbi, E. H., Chisti, Y., and Molina Grima, E. (2006a). A bioreaction-diffusion model for growth of marine sponge explants in bioreactors. *Appl Microbiol Biotechnol*, 73: 525–532.
- García Camacho, F., Chileh, T., Ceron García, M. C., Sanchez Mirón, A., Belarbi, E. H., Contreras Gómez, A., and Molina Grima, E. (2006b). Sustained growth of explants from Mediterranean sponge *Crambe crambe* cultured *in vitro* with enriched RPMI 1640. *Biotechnol Prog*, 22: 781–790.
- Gerce, B., Schwartz, T., Voigt, M., Ruhle, S., Kirchen, S., Putz, A., Proksch, P., Obst, U., Syltatk, C., and Hausmann, R. (2009). Morphological, bacterial, and secondary metabolite changes of *Aplysina aerophoba* upon long-term maintenance under artificial conditions. *Microb Ecol*, 58: 865–878.
- Gifford, S., Dunstan, R. H., O'connor, W., Koller, C. E., and Macfarlane, G. R. (2007). Aquatic zooremediation: Deploying animals to remediate contaminated aquatic environments. *Trends Biotechnol*, 25: 60–65.
- Gunda, V., and Janapala, V. (2009). Effects of dissolved oxygen levels on survival and growth *in vitro* of *Haliclona pigmentifera* (Demospongiae). *Cell Tissue Res*, 337: 527–535.
- Hadas, E., Shpigel, M., and Ilan, M. (2005). Sea ranching of the marine sponge (Demospongiae, Latrunculiidae) as a first step for latrunculin B mass production. *Aquaculture*, 244: 159–169.
- Hadas, E., Shpigel, M., and Ilan, M. (2009). Particulate organic matter as a food source for a coral reef sponge. *J Exp Biol*, 212: 3643–3650.
- Hallam, S. J., Konstantinidis, K. T., Putnam, N., Schleper, C., Watanabe, Y., Sugahara, J., Preston, C., De La Torre, J., Richardson, P. M., and Delong, E. F. (2006a). Genomic analysis of the uncultivated marine crenarchaeote *Cenarchaeum symbiosum*. *Proc Natl Acad Sci USA*, 103: 18296–18301.
- Hallam, S. J., Mincer, T. J., Schleper, C., Preston, C. M., Roberts, K., Richardson, P. M., and Delong, E. F. (2006b). Pathways of carbon assimilation and ammonia oxidation suggested by environmental genomic analyses of marine *Crenarchaeota*. *PLoS Biol*, 4: 520–536.
- Haygood, M. G. (2006). Identification of a vertical transmitted microbial assemblage in the tropical sponge *Corticium candelabrum*. IN *7th International Sponge Symposium*. Buzios, Brazil.
- Heinemann, S., Ehrlich, H., Douglas, T., Heinemann, C., Worch, H., Schatton, W., and Hanke, T. (2007). Ultrastructural studies on the collagen of the marine sponge *Chondrosia reniformis* Nardo. *Biomacromolecules*, 8: 3452–3457.
- Hentschel, U., Hopke, J., Horn, M., Friedrich, A. B., Wagner, M., Hacker, J., and Moore, B. S. (2002). Molecular evidence for a uniform microbial community in sponges from different oceans. *Appl Environ Microbiol*, 68: 4431–4440.
- Hentschel, U., Schmid, M., Wagner, M., Fieseler, L., Gernert, C., and Hacker, J. (2001). Isolation and phylogenetic analysis of bacteria with antimicrobial activities from the Mediterranean sponges *Aplysina aerophoba* and *Aplysina cavemicola*. *FEMS Microbiol Ecol*, 35: 305–312.
- Hentschel, U., Usher, K. M., and Taylor, M. W. (2006). Marine sponges as microbial fermenters. *FEMS Microbiol Ecol*, 55: 167–177.
- Hogg, M. M., Tendal, O. S., Conway, K. W., Pomponi, S. A., Van Soest, R. W. M., Gutt, J., Krautter, M., and Roberts, J. M. (2010). Deep-sea sponge grounds: Reservoirs of biodiversity. UNEP-WCMC Biodiversity Series No 32, Cambridge, UK.
- Holler, U., Wright, A. D., Matthee, G. F., König, G. M., Draeger, S., Aust, H. J., and Schulz, B. (2000). Fungi from marine sponges: diversity, biological activity and secondary metabolites. *Mycol Res*, 104: 1354–1365.
- Holmes, B., and Blanch, H. (2007). Genus-specific associations of marine sponges with group I crenarchaeotes. *Mar Biol*, 150: 759–772.
- Holmes, B., and Blanch, H. (2008). Possible taxonomic trends in the success of primary aggregate formation in marine sponge cell cultures. *Mar Biotechnol*, 10: 99–109.
- Holmes, B. M. (2006). *Marine sponges: Systematics, symbiosis and cell culture* [PhD thesis]. UC Berkeley, Berkeley, USA.
- Huelsenbeck, J. P., and Ronquist, F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, 17: 754–755.
- Ilan, M., Contini, H., Carmeli, S., and Rinkevich, B. (1996). Progress towards cell cultures from a marine sponge that produces bioactive compounds. *J Mar Biotechnol*, 4: 145–149.
- Imsiecke, G., Steffen, R., Custodio, M. R., Borojevic, R., and Muller, I. M. (1995). Formation of spicules by sclerocytes from the freshwater sponge *Ephydatia muelleri* in short-term cultures *in vitro*. *In Vitro Cell Dev Biol- Anim*, 31: 528–535.
- Isaacs, L. T., Kan, J. J., Nguyen, L., Videau, P., Anderson, M. A., Wright, T. L., and Hill, R. T. (2009). Comparison of the bacterial communities of wild and captive sponge *Clathria prolifera* from the Chesapeake Bay. *Mar Biotechnol*, 11: 758–770.
- Jensen, P. R., and Fenical, W. (2002). Secondary metabolites from marine fungi. IN *Fungi in Marine Environments*. Hong Kong: Fungal Diversity Press Ctr Res Fungal Diversity, 293–315.
- Kaeberlein, T., Lewis, K., and Epstein, S. S. (2002). Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment. *Science*, 296: 1127–1129.
- Klautau, M., Custodio, M. R., and Borojevic, R. (1993). Cell cultures of sponges *Clathrina* and *Polymastia*. *In Vitro Cell Dev Biol*, 29A: 97–99.
- Klautau, M., Custodio, M. R., and Borojevic, R. (1994). *In vitro* culture of primary cell lines from marine sponges. IN Van Soest, R., Van Kempen, T. M. G., Braekman, J. C (Eds.) *Sponges in Time and Space*. Rotterdam: A A Balkema, 401–406.
- Klöppel, A., Pfannkuchen, M., Putz, A., Proksch, P., and Brümmer, F. (2008). *Ex situ* cultivation of *Aplysina aerophoba* close to *in situ* conditions: Ecological, biochemical and histological aspects. *Mar Ecol*, 29: 259–272.

- Kohlmeyer, J. (1974). On the definition and taxonomy of higher marine fungi. *Veröffentlichungen des Instituts für Meeresforschung in Bremerhaven*, 5, 263–286.
- Kohlmeyer, J., and Volkmann-Kohlmeyer, B. (1990). New species of *Koralionastes* (Ascomycotina) from the Caribbean and Australia. *Can J Bot*, 68: 1554–1559.
- Konneke, M., Bernhard, A. E., De La Torre, J. R., Walker, C. B., Waterbury, J. B., and Stahl, D. A. (2005). Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature*, 437: 543–546.
- Kooperman, N., Ben-Dov, E., Kramarsky-Winter, E., Barak, Z., and Kushmaro, A. (2007). Coral mucus-associated bacterial communities from natural and aquarium environments. *FEMS Microbiol Lett*, 276: 106–113.
- Koopmans, M., Martens, D., and Wijffels, R. H. (2009). Towards commercial production of sponge medicines. *Mar Drugs*, 7: 787–802.
- Koopmans, M., Martens, D., and Wijffels, R. H. (2010). Growth efficiency and carbon balance for the sponge *Haliclona oculata*. *Mar Biotechnol*, 12: 340–349.
- Koopmans, M., and Wijffels, R. H. (2008). Seasonal growth rate of the sponge *Haliclona oculata* (demospongiae: Haplosclerida). *Mar Biotechnol*, 10: 502–510.
- Kozioł, C., Borojevic, R., Steffen, R., and Müller, W. E. G. (1998). Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: The telomerase activity in somatic cells. *Mech Ageing Dev*, 100: 107–120.
- Krasko, A., Lorenz, B., Batel, R., Schroder, H. C., Muller, I. M., and Muller, W. E. G. (2000). Expression of silicatein and collagen genes in the marine sponge *Suberites domuncula* is controlled by silicate and myotrophin. *Eur J Biochem*, 267: 1–11.
- Krasko, A., Schroder, H. C., Batel, R., Grebenjuk, V. A., Steffen, R., Muller, I. M., and Muller, W. E. G. (2002). Iron induces proliferation and morphogenesis in primmorphs from the marine sponge *Suberites domuncula*. *DNA Cell Biol*, 21: 67–80.
- Kreuter, M. H., Robitzki, A., Chang, S., Steffen, R., Michaelis, M., Kljajic, Z., Bachmann, M., Schröder, H. C., and Müller, W. E. G. (1992). Production of the cytostatic agent aeropylsinin by the sponge *Verongia aerophoba* in *in vitro* culture. *Comp Biochem Physiol C*, 101: 183–187.
- Kruse, M., Steffen, R., Batel, R., Muller, I. M., and Muller, W. E. (1999). Differential expression of allograft inflammatory factor 1 and of glutathione peroxidase during auto and allograft response in marine sponges. *J Cell Sci*, 112: 4305–4313.
- Le Pennec, G., Perovic, S., Ammar, M. S. A., Grebenjuk, V. A., Steffen, R., Brummer, F., and Muller, I. M. (2003). Cultivation of primmorphs from the marine sponge *Suberites domuncula*: Morphogenetic potential of silicon and iron. *J Biotechnol*, 100: 93–108.
- Lee, O. O., Tsoi, M. M. Y., Li, X., Wong, P.-K., and Qian, P.-Y. (2007). *Thalassococcus halodurans* gen. nov., sp. nov., a novel halotolerant member of the *Roseobacter* clade isolated from the marine sponge *Halichondria panicea* at Friday Harbor, USA. *Int J Syst Evol Microbiol*, 57: 1919–1924.
- Lee, O. O., Wong, Y. H., and Qian, P.-Y. (2009). Inter- and intraspecific variations of bacterial communities associated with marine sponges from San Juan Island, Washington. *Appl Environ Microbiol*, 75: 3513–3521.
- Liu, M. Y., Kjelleberg, S., and Thomas, T. (2011). Functional genomic analysis of an uncultured [delta]-proteobacterium in the sponge *Cymbastela concentrica*. *ISME J*, 5: 427–435.
- Lopez, J. V., Peterson, C. L., Willoughby, R., Wright, A. E., Enright, E., Zoladz, S., Reed, J. K., and Pomponi, S. A. (2002). Characterization of genetic markers for *in vitro* cell line identification of the marine sponge *Axinella corrugata*. *J Hered*, 93: 27–36.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, B. A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S. et al. (2004). ARB: A software environment for sequence data. *Nucleic Acids Res*, 32: 1363–1371.
- Maldonado, M., Cortadellas, N., Trillas, M. I., and Rutzler, K. (2005). Endosymbiotic yeast maternally transmitted in a marine sponge. *Biol Bull*, 209: 94–106.
- Maldonado, M., Zhang, X. C., Cao, X. P., Xue, L. Y., Cao, H., and Zhang, W. (2010). Selective feeding by sponges on pathogenic microbes: a reassessment of potential for abatement of microbial pollution. *Mar Ecol Prog Ser*, 403: 75–89.
- Mendola, D. (2003). Aquaculture of three phyla of marine invertebrates to yield bioactive metabolites: Process developments and economics. *Biomol Eng*, 20: 441–458.
- Mendola, D. (2008). The importance of water flow for culture of *Dysidea avara* sponges [PhD thesis]. Bioprocess Engineering Wageningen University, Wageningen.
- Mendola, D., Naranjo Lozano, S. A., Duckworth, A. R., and Osinga, R. (2006). The promise of aquaculture for delivering sustainable supplies of new drugs from the sea: Examples from in-sea, and tank-based invertebrate culture projects from around the world. IN Proksch, P., Muller W.E.G. (Eds.) *Frontiers in Marine Biotechnology*. Norfolk, UK: Horizon Science Press, 21–72.
- Mohamed, N. M., Enticknap, J. J., Lohr, J. E., McIntosh, S. M., and Hill, R. T. (2008a). Changes in bacterial communities of the marine sponge *Mycale laxissima* on transfer into aquaculture. *Appl Environ Microbiol*, 74: 1209–1222.
- Mohamed, N. M., Rao, V., Hamann, M. T., Kelly, M., and Hill, R. T. (2008b). Monitoring bacterial diversity of the marine sponge *Ircinia strobilina* upon transfer into aquaculture. *Appl Environ Microbiol*, 74: 4133–4143.
- Molinski, T. F., Dalisay, D. S., Lievens, S. L., and Saludes, J. P. (2009). Drug development from marine natural products. *Nat Rev Drug Discov*, 8: 69–85.
- Morrison-Gardiner, S. (2002). Dominant fungi from Australian coral reefs. *Fungal Divers*, 9: 105–121.
- Muller, W. E. G., Bohm, M., Batel, R., De Rosa, S., Tommonaro, G., Muller, I. M., and Schroder, H. C. (2000). Application of cell culture for the production of bioactive compounds from sponges: Synthesis of avarol by primmorphs from *Dysidea avara*. *J Nat Prod*, 63: 1077–1081.
- Müller, W. E. G., Grebenjuk, V. A., Le Pennec, G., Schröder, H.-C., Brümmer, F., Hentschel, U., Müller, I. M., and Breter, H.-J. (2004a). Sustainable production of bioactive compounds by sponges - cell culture and gene cluster approach: A review. *Mar Biotechnol*, 6: 105–117.
- Müller, W. E. G., Schröder, H. C., Wiens, M., Perovic-Ottstadt, S., Batel, R., and Müller, I. M. (2004b). Traditional and modern biomedical prospecting: Part II—The benefits, approaches for a sustainable exploitation of biodiversity (secondary metabolites and biomaterials from sponges). *Evid Based Complement Alternat Med*, 1: 133–144.
- Müller, W. E. G., Wang, X., Cui, F.-Z., Jochum, K., Tremel, W., Bill, J., Schröder, H., Natalio, F., Schloßmacher, U., and Wiens, M. (2009). Sponge spicules as blueprints for the biofabrication of inorganic-organic composites and biomaterials. *Appl Microbiol Biotechnol*, 83: 397–413.
- Muller, W. E. G., Wiens, M., Batel, R., Steffen, R., Borojevic, R., and Custodio, M. R. (1999). Establishment of a primary cell culture from a sponge: Primmorphs from *Suberites domuncula*. *Mar Ecol Prog Ser*, 178: 205–219.
- Munro, M. H. G., Blunt, J. W., Dumdei, E. J., Hickford, S. J. H., Lill, R. E., Li, S., Battershill, C. N., and Duckworth, A. R. (1999). The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol*, 70: 15–25.
- Muscholl-Silberhorn, A., Thiel, V., and Imhoff, J. F. (2008). Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microb Ecol*, 55: 94–106.
- Newman, D. J., and Cragg, G. M. (2004). Marine natural products and related compounds in clinical and advanced preclinical trials. *J Nat Prod*, 67: 1216–1238.
- Nickel, M., and Brummer, F. (2003). *In vitro* sponge fragment culture of *Chondrosia reniformis* (Nardo, 1847). *J Biotechnol*, 100: 147–159.
- Nickel, M., Leininger, S., Proll, G., and Brummer, F. (2001). Comparative studies on two potential methods for the biotechnological production of sponge biomass. *J Biotechnol*, 92: 169–178.
- Nickel, M., Proll, G., and Brummer, F. (2000). Natural products of marine sponges—From ecology to biomass. IN Bruner H. (Ed.) *Proceedings of the 4th International Congress on Biochemical Engineering*, Stuttgart : Fraunhofer IRB Verlag, 194–198.





- Olson, J. B., Lord, C. C., and McCarthy, P. J. (2000). Improved recoverability of microbial colonies from marine sponge samples. *Microb Ecol*, 40: 139–147.
- Osinga, R., Belarbi, E. H., Molina Grima, E., Tramper, J., and Wijffels, R. H. (2003). Progress towards a controlled culture of the marine sponge *Pseudosuberites andrewsi* in a bioreactor. *J Biotechnol*, 100: 141–146.
- Osinga, R., Belarbi, E. H., and Wijffels, R. H. (2002). Feeding sponges in bioreactors: What do they eat? *Boll Mus Ist Biol Univ Genova*, 66–67: 147.
- Osinga, R., Kleijn, R., Groenendijk, E., Niesink, P., Tramper, J., and Wijffels, R. H. (2001). Development of *in vivo* sponge cultures: Particle feeding by the tropical sponge *Pseudosuberites aff. andrewsi*. *Mar Biotechnol*, 3: 544–554.
- Osinga, R., and Kotterman, M. (2007). Ferric iron promotes the formation of oscules: Observations on sponges in aquaria. IN Custodio, M.R., Lôbo-Hajdu, G., Hajdu, E., Muricy, G. (Eds.) *Porifera Research-Biodiversity, Innovation and Sustainability*. Rio de Janeiro: Museo Nacional, 497–502.
- Osinga, R., Schutter, M., Griffioen, B., Wijffels, R., Verreth, J., Shafir, S., Henard, S., Taruffi, M., Gili, C., and Lavorano, S. (2011). The biology and economics of coral growth. *Mar Biotechnol*, 13: 658–671.
- Osinga, R., Sidri, M., Cerig, E., Gokalp, S. Z., and Gokalp, M. (2010). Sponge aquaculture trials in the East-Mediterranean sea: New approaches to earlier ideas. *Open Mar Biol J*, 4: 74–81.
- Osinga, R., Tramper, J., and Wijffels, R. H. (1998). Cultivation of marine sponges for metabolite production: Applications for biotechnology? *Trends Biotechnol*, 16: 130–134.
- Osinga, R., Tramper, J., and Wijffels, R. H. (1999). Cultivation of marine sponges. *Mar Biotechnol*, 1: 509–532.
- Pabel, C. T., Vater, J., Wilde, C., Franke, P., Hofemeister, J., Adler, B., Bringmann, G., Hacker, J., and Hentschel, U. (2003). Antimicrobial activities and matrix-assisted laser desorption/ionization mass spectrometry of *Bacillus* isolates from the marine sponge *Aplysina aerophoba*. *Mar Biotechnol*, 5: 424–434.
- Page, M. J., Handley, S. J., Northcote, P. T., Cairney, D., and Willan, R. C. (2011). Successes and pitfalls of the aquaculture of the sponge *Mycale hentscheli*. *Aquaculture*, 312: 52–61.
- Page, M. J., Northcote, P. T., Webb, V. L., Mackey, S., and Handley, S. J. (2005). Aquaculture trials for the production of biologically active metabolites in the New Zealand sponge *Mycale hentscheli* (Demospongiae: Poecilosclerida). *Aquaculture*, 250: 256–269.
- Perovic, S., Wichels, A., Schutt, C., Gerds, G., Pahler, S., Steffen, R., and Muller, W. E. G. (1998). Neuroactive compounds produced by bacteria from the marine sponge *Halichondria panicea*: Activation of the neuronal NMDA receptor. *Environ Toxicol Phar*, 6: 125–133.
- Pfannkuchen, M., and Brummer, F. (2009). Heterologous expression of DsRed2 in young sponges (Porifera). *Int J Dev Biol*, 53: 1113–1117.
- Piel, J. (2004). Metabolites from symbiotic bacteria. *Nat Prod Rep*, 21: 519–538.
- Pimentel-Elardo, S., Wehrl, M., Friedrich, A. B., Jensen, P. R., and Hentschel, U. (2003). Isolation of planctomycetes from *Aplysina* sponges. *Aquat Microb Ecol*, 33: 239–245.
- Pimentel-Elardo, S. M., Kozytska, S., Bugni, T. S., Ireland, C. M., Moll, H., and Hentschel, U. (2010). Anti-parasitic compounds from *Streptomyces* sp. strains isolated from Mediterranean sponges. *Mar Drugs*, 8: 373–380.
- Pivkin, M., Aleshko, S., Krasokhin, V., and Khudyakova, Y. (2006). Fungal assemblages associated with sponges of the southern coast of Sakhalin Island. *Russ J Mar Biol*, 32: 207–213.
- Pomponi, S. A. (2001). The oceans and human health: The discovery and development of marine-derived drugs. *Oceanography*, 14: 28–42.
- Pomponi, S. A. (2006). Biology of the Porifera: Cell culture. *Can J Zool*, 84: 167–174.
- Pomponi, S.A. and Willoughby, R. (1994). Sponge cell culture for the production of bioactive metabolites. IN Van Soest, R., Van Kempen, T.M.G. and Braekman, J.C. (Eds.) *Sponges in time and space*. Rotterdam: A A Balkema, 395–400.
- Pomponi, S.A. and Willoughby, R. (2000). Development of sponge cell cultures for biomedical applications. IN Austin, B. and Mothersill, C. (Eds.) *Aquatic invertebrate cell culture*. London: Springer, 323–336.
- Pomponi, S. A.; Willoughby, R.; Kaighn, M. E.; Wright, A. E. (1997) Development of techniques for *in vitro* production of bioactive natural products from marine sponges. IN Maramorosch, K.; Mitsuhashi, J. (Eds.) *Invertebrate cell culture. Novel directions and biotechnology applications*. Enfield: Science Publishers, 231–237.
- Pomponi, S. A., Willoughby, R., and Kelly-Borges, M. (1998). Sponge cell culture. IN Cooksey, K.E. (Ed.) *Molecular approaches to the study of the ocean*. pp. London: Chapman and Hall, 423–429.
- Pomponi, S. A., Thompson, J., Bottesch, J. H., and Sennett, S. H. (2007). Methods for extending the life span of marine invertebrate cells. *US patent number: 20070254367*.
- Pozzolini, M., Sturla, L., Cerrano, C., Bavestrello, G., Camardella, L., Parodi, A. M., Raheli, F., Benatti, U., Muller, W. E. G., and Giovine, M. (2004). Molecular cloning of silicatein gene from marine sponge *Petrosia ficiformis* (Porifera, Demospongiae) and development of primmorphs as a model for biosilicification studies. *Mar Biotechnol*, 6: 594–603.
- Preston, C. M., Wu, K. Y., Molinski, T. F., and Delong, E. F. (1996). A psychrophilic crenarchaeon inhabits a marine sponge: *Cenarchaeum symbiosum* gen. nov., sp. nov. *Proc Natl Acad Sci USA*, 93: 6241–6246.
- Proksch, P. (1994). Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. *Toxicon*, 32: 639–655.
- Pronzato, R., and Manconi, R. (2008). Mediterranean commercial sponges: Over 5000 years of natural history and cultural heritage. *Mar Ecol*, 29: 146–166.
- Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J., and Glöckner, F. O. (2007). SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res*, 35: 7188–7196.
- Rajilic-Stojanovic, M., Smidt, H., and De Vos, W. M. (2007). Diversity of the human gastrointestinal tract microbiota revisited. *Environ Microbiol*, 9: 2125–2136.
- Reiswig, H. W. (1974). Water transport, respiration and energetics of three tropical marine sponges. *J Exp Mar Biol Ecol*, 14: 231–249.
- Richelle-Maurer, E., Gomez, R., Braekman, J. C., Van De Vyver, G., Soest, R. V. M., and Devijver, C. (2003). Primary cultures from the marine sponge *Xestospongia muta* (Petrosiidae, Haplosclerida). *J Biotechnol*, 100: 169–176.
- Rinkevich, B. (2005). Marine invertebrate cell cultures: New millennium trends. *Mar Biotechnol*, 7: 429–439.
- Rinkevich, B. (2011). Cell cultures from marine invertebrates: New insights for capturing endless stemness. *Mar Biotechnol*, 13: 345–354.
- Rinkevich, B., Blisko, R., and Ilan, M. (1998). Further steps in the initiation of cell cultures from embryos and adult sponge colonies. *In Vitro Cell Dev Biol- Anim*, 34: 753–756.
- Ronquist, F., Huelsenbeck, J. P., and Van Der Mark, O. (2005). MrBayes 3.1 Manual. School of Computational Science. Florida State University, USA.
- Sashidhara, K. V., White, K. N., and Crews, P. (2009). A selective account of effective paradigms and significant outcomes in the discovery of inspirational marine natural products. *J Nat Prod*, 72: 588–603.
- Schippers, K. J., Martens, D. E., Pomponi, S. A., and Wijffels, R. H. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol- Anim*, 47: 302–311.
- Schmidt, O. (1863). Auszug aus dem Berichte des Professor Dr. O. Schmidt über die im Auftrage der Triester Handels- und Gewerbekammer in Dalmatien angestellten Versuche über die künstliche Schwammzucht. *Zeitschrift für Akklimatisation*, 1: 290–295.
- Schmitt, S., Angermeier, H., Schiller, R., Lindquist, N., and Hentschel, U. (2008). Molecular microbial diversity survey of sponge reproductive stages and mechanistic insights into vertical transmission of microbial symbionts. *Appl Environ Microbiol*, 74: 7694–7708.
- Schneemann, I., Nagel, K., Kajahn, I., Labes, A., Wiese, J., and Imhoff, J. F. (2010a). Comprehensive investigation of marine Actinobacteria associated with the sponge *Halichondria panicea*. *Appl Environ Microbiol*, 76: 3702–3714.
- Schneemann, I., Ohlendorf, B., Zinecker, H., Nagel, K., Wiese, J., and Imhoff, J. F. (2010b). Nocapyrones AD, g-pyrone from a Nocardiosis strain isolated from the marine sponge *Halichondria panicea*. *J Nat Prod*, 73: 1444–1447.



- Schröder, H., Brandt, D., Schloßmacher, U., Wang, X., Tahir, M., Tremel, W., Belikov, S., and Müller, W. (2007). Enzymatic production of biosilica glass using enzymes from sponges: Basic aspects and application in nanobiotechnology (material sciences and medicine). *Naturwissenschaften*, 94: 339–359.
- Selvin, J., Gandhimathi, R., Kiran, G. S., Priya, S. S., Ravji, T. R., and Hema, T. A. (2009). Culturable heterotrophic bacteria from the marine sponge *Dendrilla nigra*: Isolation and phylogenetic diversity of actinobacteria. *Helgoland Mar Res*, 63: 239–247.
- Sharp, K. H., Eam, B., Faulkner, D. J., and Haygood, M. G. (2007). Vertical transmission of diverse microbes in the tropical sponge *Corticium* sp. *Appl Environ Microbiol*, 73: 622–629.
- Siegl, A., Kamke, J., Hochmuth, T., Piel, J., Richter, M., Liang, C. G., Dandekar, T., and Hentschel, U. (2011). Single-cell genomics reveals the lifestyle of *Poribacteria*, a candidate phylum symbiotically associated with marine sponges. *ISME J*, 5: 61–70.
- Simon, H. M., Jahn, C. E., Bergerud, L. T., Sliwinski, M. K., Weimer, P. J., Willis, D. K., and Goodman, R. M. (2005). Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. *Appl Environ Microbiol*, 71: 4751–4760.
- Sipkema, D., and Blanch, H. (2010). Spatial distribution of bacteria associated with the marine sponge *Tethya californiana*. *Mar Biol*, 157: 627–638.
- Sipkema, D., Franssen, M. C. R., Osinga, R., Tramper, J., and Wijffels, R. H. (2005a). Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142–162.
- Sipkema, D., Heilig, H. G. H. J., Akkermans, A. D. L., Osinga, R., Tramper, J., and Wijffels, R. H. (2003a). Sponge cell culture? A molecular identification method for sponge cells. *Mar Biotechnol*, 5: 443–449.
- Sipkema, D., Holmes, B., Nichols, S., and Blanch, H. (2009). Biological characterisation of *Haliclona* (?gellius) sp.: Sponge and associated microorganisms. *Microb Ecol*, 58: 903–920.
- Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J., and Wijffels, R. H. (2005b). Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201–222.
- Sipkema, D., Schippers, K., Maalcke, W. J., Yang, Y., Salim, S., and Blanch, H. W. (2011). Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona* (gellius) sp. *Appl Environ Microbiol*, 77: 2130–2140.
- Sipkema, D., Snijders, A. P. L., Schroen, C. G. P. H., Osinga, R., and Wijffels, R. H. (2004). The life and death of sponge cells. *Biotechnol Bioeng*, 85: 239–247.
- Sipkema, D., Tramper, J., Osinga, R., and Wijffels, R. H. (2003). The influence of silicate on *Suberites domuncula* primmorphs. IN Collic-Jouault, S., Bergé, J.P., Guézennec, J., Fleurence, J., Le Gal, Y., Roy, P. (Eds.). *Marine biotechnology: an overview of leading fields*, Plouzane, France : Ifremer, 56–64.
- Sipkema, D., Van Wielink, R., Van Lammeren, A. A. M., Tramper, J., Osinga, R., and Wijffels, R. H. (2003c). Primmorphs from seven marine sponges: Formation and structure. *J Biotechnol*, 100: 127–139.
- Sipkema, D., Yosef, N. A. M., Adamczewski, M., Osinga, R., Mendola, D., Tramper, J., and Wijffels, R. H. (2006). Hypothesized kinetic models for describing the growth of globular and encrusting Demosponges. *Mar Biotechnol*, 8: 40–51.
- Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E. A., Mitros, T., Richards, G. S., Conaco, C., Dacre, M., Hellsten, U., Larroux, C., Putnam, N. H. et al. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature*, 466: 720–726.
- Staley, J. T., and Konopka, A. (1985). Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Ann Rev Microbiol*, 39: 321–346.
- Stocker-Worgotter, E. (1995). Experimental cultivation of lichens and lichen symbionts. *Can J Bot*, 73: 579–589.
- Sun, L. M., Song, Y. F., Qu, Y., Yu, X. J., and Zhang, W. (2007). Purification and *in vitro* cultivation of archaeocytes (stem cells) of the marine sponge *Hymeniacidon perleve* (Demospongiae). *Cell Tissue Res*, 328: 223–237.
- Sun, W., Dai, S., Jiang, S., Wang, G., Liu, G., Wu, H., and Li, X. (2010). Culture dependent and culture-independent diversity of Actinobacteria associated with the marine sponge *Hymeniacidon perleve* from the South China Sea. *Antonie van Leeuwenhoek*, 98: 65–75.
- Sundar, V. C., Yablou, A. D., Grazul, J. L., Ilan, M., and Aizenberg, J. (2003). Fibre-optical features of a glass sponge. *Nature*, 424: 899–900.
- Swatschek, D., Schatton, W., Kellerman, J., Muller, I. M., and Kreuter, J. (2002). Marine sponge collagen: Isolation, characterization and effects on the skin parameters surface-pH, moisture and sebum. *Eur J Pharm Biopharm*, 53: 107–113.
- Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*, 126: 663–676.
- Taylor, M. W., Radax, R., Steger, D., and Wagner, M. (2007). Sponge-associated microorganisms: Evolution, ecology and biotechnological potential. *Microbiol Mol Biol Rev*, 71: 295–347.
- Tourna, M., Stieglmeier, M., Spang, A., Könneke, M., Schintlmeister, A., Ulrich, T., Engel, M., Schloter, M., Wagner, M., Richter, A., and Schleper, C. (2011). *Nitrososphaera viennensis*, an ammonia oxidizing archaeon from soil. *Proc Natl Acad Sci USA*, 108 8420–8425.
- Unger, C., Gao, S., Cohen, M., Jaconi, M., Bergstrom, R., Holm, F., Galan, A., Sanchez, E., Irion, O., Dubuisson, J. B., Giry-Laterriere, M., Salmon, P., Simon, C., Hovatta, O., and Feki, A. (2009). Immortalized human skin fibroblast feeder cells support growth and maintenance of both human embryonic and induced pluripotent stem cells. *Hum Reprod*, 24: 2567–2581.
- Unson, M. D., Holland, N. D., and Faulkner, D. J. (1994). A brominated secondary metabolite synthesized by the cyanobacterial symbiont of a marine sponge and accumulation of the crystalline metabolite in the sponge tissue. *Mar Biol*, 119: 1–11.
- Uriz, M. J., Turon, X., Galera, J., and Tur, J. M. (1996). New light on the cell location of avarol within the sponge *Dysidea avara* (Dendroceratida). *Cell Tissue Res*, 285: 519–527.
- Valisano, L., Bavestrello, G., Giovine, M., Arillo, A., and Cerrano, C. (2006a). Seasonal production of primmorphs from the marine sponge *Petrosia ficiformis* (Poiret, 1789) and new culturing approaches. *J Exp Mar Biol Ecol*, 337: 171–177.
- Valisano, L., Bavestrello, G., Giovine, M., Arillo, A., and Cerrano, C. (2007). Effect of iron and dissolved silica on primmorphs of *Petrosia ficiformis* (Poiret, 1789). *Chem Ecol*, 23: 233–241.
- Valisano, L., Bavestrello, G., Giovine, M., and Cerrano, C. (2006b). Primmorphs formation dynamics: A screening among Mediterranean sponges. *Mar Biol*, 149: 1037–1046.
- Van Doesburg, W., Van Eekert, M. H. A., Middeldorp, P. J. M., Balk, M., Schraa, G., and Stams, A. J. M. (2005). Reductive dechlorination of [beta]-hexachlorocyclohexane ([beta]-HCH) by a *Dehalobacter* species in coculture with a *Sedimentibacter* sp. *FEMS Microbiol Ecol*, 54: 87–95.
- Vilanova, E., Coutinho, C., Maia, G., and Mourao, P. A. S. (2010). Sulfated polysaccharides from marine sponges: Conspicuous distribution among different cell types and involvement on formation of *in vitro* cell aggregates. *Cell Tissue Res*, 340: 523–531.
- Voultziadou, E. (2007). Sponges: An historical survey of their knowledge in Greek antiquity. *J Mar Biol Assoc UK*, 87: 1757–1763.
- Wang, G., Li, Q., and Zhu, P. (2008). Phylogenetic diversity of culturable fungi associated with the Hawaiian sponges *Suberites zeteki* and *Gelliodes fibrosa*. *Antonie van Leeuwenhoek*, 93: 163–174.
- Watson, S. W., Novitsky, T. J., Quinby, H. L., and Valois, F. W. (1977). Determination of bacterial number and biomass in the marine environment. *Appl Environ Microbiol*, 33: 940–946.
- Webster, N., Cobb, R., Soo, R., Anthony, S., Battershill, C., Whalan, S., and Evans- Illidge, E. (2011). Bacterial community dynamics in the marine sponge *Rhopaloeides odorabile* under *in situ* and *ex situ* cultivation. *Mar Biotechnol*, 13: 296–304.
- Webster, N. S., and Hill, R. T. (2001). The culturable microbial community of the Great Barrier Reef sponge *Rhopaloeides odorabile* is dominated by an alpha-proteobacterium. *Mar Biol*, 138: 843–851.
- Webster, N. S., Negri, A. P., Webb, R. I., and Hill, R. T. (2002). A spongin-boring alphaproteobacterium is the etiological agent of disease in the Great Barrier Reef sponge *Rhopaloeides odorabile*. *Mar Ecol Prog Ser*, 232: 305–309.
- Webster, N. S., Wilson, K. J., Blackall, L. L., and Hill, R. T. (2001). Phylogenetic diversity of bacteria associated with the marine sponge *Rhopaloeides odorabile*. *Appl Environ Microbiol*, 67: 434–444.



- Wichels, A., Würtz, S., Döpke, H., Schütt, C., and Gerdtz, G. (2006). Bacterial diversity in the breadcrumb sponge *Halichondria panicea* (Pallas). *FEMS Microbiol Ecol*, 56: 102–118.
- Wiese, J., Ohlendorf, B., Blümel, M., Schmaljohann, R., and Imhoff, J. F. (2011). Phylogenetic identification of fungi isolated from the marine sponge *Tethya aurantium* and identification of their secondary metabolites. *Mar Drugs*, 9: 561–585.
- Wilkinson, C. R., and Garrone, R. (1980). Nutrition of marine sponges. Involvement of symbiotic bacteria in the uptake of dissolved carbon. IN Smith, D.C., Tiffon, Y. (Eds.) *Nutrition in the Lower Metazoa*. Oxford: Pergamon, 157–161.
- Willoughby, R., and Pomponi, S. A. (2000). Quantitative assessment of marine sponge cells *in vitro*: Development of improved growth medium. *In Vitro Cell Dev Biol- Anim*, 36: 194–200.
- Wilson, H. V. (1907). On some phenomena of coalescence and regeneration in sponges. *J Exp Zool*, 5: 245–258.
- Xue, L., and Zhang, W. (2009). Growth and survival of early juveniles of the marine sponge *Hymeniacidon perlevis* (Demospongiae) under controlled conditions. *Mar Biotechnol*, 11: 640–649.
- Yahel, G., Sharp, J. H., Marie, D., Hase, C., and Genin, A. (2003). *In situ* feeding and element removal in the symbiont-bearing sponge *Theonella swinhoei*: Bulk DOC is the major source for carbon. *Limnol Oceanogr*, 48: 141–149.
- Zea, S., Castellanos, L., Valderrama, K., Puentes, C. A., Gomez-Leon, J., and Pomponi, S. A. (2010). Potential of antitumoral (b)-discodermalide production by the Caribbean sponge *Discodermia dissoluta*. IN VIII World Sponge Conference, Girona, Spain.
- Zhang, H.-B., Yang, M.-X., Shi, W., Zheng, Y., Sha, T., and Zhao, Z.-W. (2007). Bacterial diversity in mine tailings compared by cultivation and cultivation-independent methods and their resistance to lead and cadmium. *Microb Ecol*, 54: 705–712.
- Zhang, W., Zhang, X., Cao, X., Xu, J., Zhao, Q., Yu, X., Jin, M., and Deng, M. (2003a). Optimizing the formation of *in vitro* sponge primmorphs from the Chinese sponge *Stylotella agminata* (Ridley). *J Biotechnol*, 100: 161–168.
- Zhang, X., Cao, X., Zhang, W., Yu, X., and Jin, M. (2003b). Primmorphs from archaeocytes- dominant cell population of the sponge *Hymeniacidon perleve*: Improved cell proliferation and spiculogenesis. *Biotechnol Bioeng*, 84: 583–590.
- Zhang, X., Zhang, W., Xue, L., Zhang, B., Jin, M., and Fu, W. (2010). Bioremediation of bacteria pollution using the marine sponge *Hymeniacidon perlevis* in the intensive mariculture water system of turbot *Scophthalmus maximus*. *Biotechnol Bioeng*, 105: 59–68.
- Zhang, X. Y., Le Pennec, G., Steffen, R., Muller, W. E. G., and Zhang, W. (2004). Application of a MTT assay for screening nutritional factors in growth media of primary sponge cell culture. *Biotechnol Prog*, 20: 151–155.
- Zhao, Q. Y., Zhang, W., Jin, M. F., Yu, X. J., and Deng, M. C. (2005). Formulation of a basal medium for primary cell culture of the marine sponge *Hymeniacidon perleve*. *Biotechnol Prog*, 21: 1008–1012.
- Zientz, E., Dandekar, T., and Gross, R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiol Mol Biol Rev*, 68: 745–770.



# Chapter 3

Cell cycle analysis of primary sponge cell cultures



## ABSTRACT

Proliferation of sponge cells is generally measured via cell counts or viability assays. However, more insight into the proliferative state of a sponge cell population can be obtained from the distribution of the cells over the different phases of the cell cycle. Cell cycle distribution of sponge cells was measured via flow cytometry after staining the DNA with propidium iodide. The five sponges studied in this paper all showed a large fraction of cells in G1/G0 compared to G2/M and S, indicating that cells were not actively dividing. In addition, some sponges also showed a large apoptotic fraction, indicating cell death. Additional apoptosis measurements, based on caspase activity, showed that harvesting and dissociation of sponge tissue to initiate a primary cell culture was directly correlated with an increase in apoptotic cells. This indicates that for the development of cell cultures, more attention should be given to harvesting, dissociation, and quality of starting material. Finally, cultivation conditions used were ineffective for proliferation, since after 2 d of cultivating *Haliclona oculata* cells, most cells shifted towards the apoptotic fraction, indicating that cells were dying. For development of *in vitro* sponge cell cultures, flow cytometric cell cycle analysis is a useful method to assess the proliferative state of a sponge cell culture and can be used to validate improvements in harvesting and dissociation, to select sponges with good proliferative capacities and to study the influence of culture conditions for stimulating cell growth.

### This chapter has been published as:

Schippers KJ, Martens DE, Pomponi SA, Wijffels RH. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cellular & Developmental Biology - Animal*, **47**: 302-311.

## 1 INTRODUCTION

Marine sponges are a rich source of bioactive compounds, which have the potential to provide future medicines, such as new antibiotics, anticancer drugs, and antiviral compounds (Munro et al., 1999; Sipkema et al., 2005a). However, the limited availability of sponge biomass hampers the development of these potential drugs into commercial products (Pomponi, 1999; Sipkema et al., 2005b). An approach to increase sponge biomass availability can be *in vitro* sponge cell culture (Pomponi, 2006; De Caralt et al., 2008; Koopmans et al., 2009). Despite efforts by different research groups, a continuous sponge cell line has not yet been developed and the number of primary sponge cell cultures developed is very limited (Rinkevich, 2005). Apart from inappropriate culture conditions for growth, one possible explanation is that the sponge cells are already in a non-proliferative state at the start of the culture, either because they were taken from a sponge in a resting non-proliferative state or as a consequence of the dissociation process.

Proliferation of sponge cells is usually measured by cell counts and viability assays (e.g., trypan blue, FDA, and PI (Sipkema et al., 2004)). However, counting cells is time consuming and inaccurate due to the large variety of cell sizes in a sponge, which makes it difficult to discriminate between sponge cells and certain contaminants, like yeast and protozoa. In addition, cell counts may not always correctly represent what is happening, because they do not give information about the status of the cells, but only discriminate between viable and dead cells. For example, cells in early apoptosis do not have a leaky membrane and will therefore not be detected by trypan blue or the FDA/PI method.

Information on the proliferative state of a cell population can be obtained from the distribution of the cells over the different phases of the cell cycle. Cell populations which are actively dividing have a large fraction of cells in the S phase, while cell populations that are not actively dividing have a large fraction in the G1/G0 phase (most mammalian cell lines) or in the G2/M phase (insect cells) (Fertig et al., 1990). The cell cycle distribution can be measured with flow cytometry after staining the DNA in the cells with propidium iodide. Also apoptotic populations can be detected, since apoptotic cells fragment their DNA, which results in a sub-G1 peak (Pozarowski and Darzynkiewicz, 2004). Apoptosis can also be detected by measuring caspase activity. Caspases are a family of cysteine proteases and play an essential role in apoptosis and can therefore be used as indicators for apoptosis (Nicholson and Thornberry, 1997; Wiens and Muller, 2006). An important advantage of these methods is that they give information about the condition of the cells already at the start of the culture while for cell counting it takes a few days to see whether there is cell growth or death.



The aim of this research was to evaluate the potential of flow cytometric cell cycle analysis to measure the proliferative state of sponge cells.

The cell cycle distribution of five different species obtained from different locations was analyzed: *Haliclona oculata* and *Haliclona xena* from Lake Grevelingen in The Netherlands, *Dysidea avara* and *Axinella polypoides* from the Mediterranean at the Costa Brava in Spain, and *Xestospongia muta* from Dania Beach in Florida, USA. Next to this we also measured the cell cycle distribution and caspase activities of cells from *H. oculata* during a 2- and 10-d cultivation to study the change in distribution of cells over time.

## 2 MATERIAL AND METHODS

### 2.1 Specimen collection and transportation

Specimens of the sponges (see Table 1.) were collected by scuba diving. *H. oculata* and *H. xena* were collected from Lake Grevelingen (Dreischor: Frans Kok reef) in the Netherlands at a depth of approximately 8 m. Specimens of *D. avara* and *A. polypoides* were collected from the Mediterranean (Cala Montgo) in Spain at a depth of approximately 8–10 m. *X. muta* was collected from Florida (Dania Beach) in the USA at a depth of approximately 10 m. The sponges were transported in coolers to maintain the temperature the same as in the sea and were constantly aerated. Cells from *D. avara* and *X. muta* were cryopreserved, stored, and thawed based on the method of Pomponi et al. (1997).

**Table 1** Sponge collection.

Sponge	Location of harvest	Date of harvest	Storage
<i>Haliclona oculata</i>	Lake Grevelingen, Dreischor: Frans Kok reef	16-11-2009 and 11-1-2010	70% EtOH, -20 °C
<i>Haliclona xena</i>	Lake Grevelingen, Dreischor: Frans Kok reef	16-11-2009	70% EtOH, -20 °C
<i>Dysidea avara</i>	Mediterranean, Cala Montgo, l'Escala	22-1-2008	cryopreserved
<i>Axinella polypoides</i>	Mediterranean, Cala Montgo, l'Escala	17-6-2009	70% EtOH, -20 °C
<i>Xestospongia muta</i>	Dania Beach, Florida	18-9-2007	cryopreserved

### 2.2 Media and solutions

Calcium- and magnesium-free seawater, CMF-EDTA (10 mM), was prepared by dissolving 32.1 g NaCl, 1.1 g Na<sub>2</sub>SO<sub>4</sub>, 0.9 g KCl, 10 ml of Trizma (0.5 M, pH 8.0), and 20 ml of 0.5 M EDTA stock solution in 1 L of demineralized water. The pH was adjusted to 8.0 with HCl and salinity was set to 960 mOsm/kg before filter sterilization (pore size 0.22 μm).

Filtered seawater (FSW) was prepared by filter sterilizing (pore size 0.22 μm) fresh seawater collected from Lake Grevelingen. Salinity was 960 mOsm/kg and the pH was 8.0.

The propidium iodide (PI) staining solution (3.8 mM sodium citrate, 40 μg/ml PI in phosphate-buffered saline (PBS)) was prepared by dissolving 98.1 mg sodium citrate and 4 mg of PI in 100 ml of PBS and was stored at 4°C in the dark.

The RNase A stock solution (10 μg/ml) was prepared by dissolving 1 mg of RNase A (Roche Diagnostics, Almere, Netherlands; Cat.#10109142001) in 100 ml demineralized water, and was boiled for 5 min, aliquoted and stored at -20°C.

Wash buffer (PBS+0.1% BSA) was prepared by dissolving 0.1 g of bovine serum albumin (BSA) in 100 ml PBS.

The passive lysis buffer (Promega, Fitchburg, WI; Cat.# E1941) was prepared by diluting the buffer five times with demineralized water.

### 2.3 Sponge cell dissociation

The protocol used to prepare a sponge cell suspension was based on the method of Pomponi and Willoughby (1994). The sponge was rinsed in FSW and cut into small pieces of 1 to 2 cm. The sponge parts were transferred to a Petri dish containing CMF-EDTA (10:1, CMF-EDTA volume/sponge volume). After soaking the sponge in CMF-EDTA and squeezing it through a sterile gauze, cells were easily released and the cell suspension was filtered using a 70-μm cell strainer (BD Falcon™, BD Biosciences, Breda, Netherlands; Cat.#352350) to remove cell aggregates and spicules. The crude cell suspension was centrifuged (Heraeus Primo centrifuge, Thermo Scientific, Breda, Netherlands) at 300×g for 5 min to enrich for sponge cells, which were in the pellet, and remove most of the bacteria, which were in the supernatant. The supernatant was discarded and the cell pellet was resuspended in CMF-EDTA to be able to determine the cell concentration by counting with a disposable haemocytometer (C-Chip DHC-N01, Incyto, Orange Medical, Tilburg, Netherlands) using a microscope (Inverted fluorescence microscope IX-71, Olympus, Zoeterwoude, Netherlands).

### 2.4 Cultivation experiment with *H. oculata*

Two cultivation experiments were done with *H. oculata*, in December 2009 and January 2010. The sponge cells were dissociated as described in Section 2.3. For

each experiment, the cell concentration was set to  $\sim 10^6$  cells/ml in FSW. To resuspend the cells in FSW, a certain volume of cells in CMF-EDTA was centrifuged at  $300\times g$  for 5 min and the cells in the pellet were resuspended in a certain amount of FSW to acquire  $\sim 10^6$  cells/ml. The cells were cultured in T25 flasks, 5 ml per flask, and incubated at the same temperature of Lake Grevelingen, which was at that time  $2^\circ\text{C}$ . During the first experiment, one sponge was dissociated and the cells were incubated for 2 d. At days 0 and 2, samples were taken for flow cytometry analysis (see Section 2.5) and for caspase measurements and total protein content (see Section 2.6). Also, at day 1, part of the T25 flasks with sponge cells were exposed to 0.5 h of UV light to induce apoptosis. These cells were harvested at day 2. During the second experiment three sponges were dissociated, and the cells were incubated for 10 d. Every day cells were harvested for all three analyses and cell counting. Again at day 1, part of the T25 flasks with sponge cells were exposed to 0.5 h of UV light and these flasks were harvested at day 2.

### 2.5 DNA staining and cell cycle analysis using flow cytometry

The cells were harvested and a single cell suspension was prepared by spinning down 5 ml (T25 flask) of cells ( $300\times g$ , 5 min) and resuspending the pellet in 1 ml of CMF-EDTA. If aggregates were still present, the suspension was vortexed briefly. To  $300\ \mu\text{l}$  of cell suspension,  $900\ \mu\text{l}$  of ice-cold absolute ethanol was added dropwise while vortexing. Samples were stored at  $-20^\circ\text{C}$  for at least 1 h and could be stored at  $-20^\circ\text{C}$  for several weeks. After fixation, cells were washed twice at room temperature with 1 ml of wash buffer (PBS+0.1% BSA) by centrifuging at maximum speed (13,000 rpm) for 1 min and resuspending the pellet in the wash buffer. Finally, the pellet was resuspended in 1 ml of propidium iodide staining solution by pipetting up and down and briefly vortexing. Also  $50\ \mu\text{l}$  of RNase A stock solution was added and the fixed cells were incubated for 3 h at  $4^\circ\text{C}$  (Crissman and Steinkamp 1973; Krishan 1975). Fluorescence due to PI staining was measured in a flow cytometer (FACScalibur, Becton Dickinson, Franklin Lakes, NJ). The instrument settings for each sponge species are given in Table 2.

### 2.6 Caspase 3/7 measurements and total protein content

For the caspase measurements, 5 ml (T25 flask) cells were harvested by spinning down the cells ( $300\times g$ , 5 min) and removing the supernatant. Subsequently, 1 ml of passive lysis buffer was added to the pellet, and the cells were dissolved by pipetting. The cells were incubated 20 min at room temperature to lyse all cells. The cell debris was spun down by centrifuging at maximum speed (13,000 rpm) for 5 min. The supernatant was aliquoted per  $100\ \mu\text{l}$  and stored at  $-20^\circ\text{C}$  for further analysis. To measure caspase activities, we applied the caspase glo<sup>®</sup> 3/7 assay from Promega (Promega, Fitchburg, WI; Cat.#G8090), which is a luminescent assay that can

**Table 2** Flow cytometer settings.

<i>Haliclona oculata</i> & <i>Haliclona xena</i>	Voltage	Amplifier	Scale
<b>FSC</b>	E+00	5.00	Linear
<b>SSC</b>	450	1.00	Linear
<b>FL 2</b>	750	1.00	Linear
<i>Dysidea avara</i>			
<b>FSC</b>	E+00	4.00	Linear
<b>SSC</b>	450	1.00	Linear
<b>FL 2</b>	600	1.00	Linear
<i>Axinella polypoides</i>			
<b>FSC</b>	E+00	5.00	Linear
<b>SSC</b>	450	1.00	Linear
<b>FL 2</b>	675	1.00	Linear
<i>Xestospongia muta</i>			
<b>FSC</b>	E+00	5.00	Linear
<b>SSC</b>	600	1.00	Linear
<b>FL 2</b>	675	1.00	Linear

FSC, forward scatter; SSC, side scatter; FL 2, fluorescence detector 2.

measure caspase 3 and 7 activities of cell suspensions. Measurements were performed in a luminometer (LUMIstar OPTIMA, BMG Labtech, Offen burg, Germany) using 96 white-walled well plates. To  $25\ \mu\text{l}$  of stored supernatant,  $25\ \mu\text{l}$  of caspase glo reagent was added and incubated for minimally 30 min and maximally 3 h at room temperature. The blank was made of  $25\ \mu\text{l}$  passive lysis buffer and  $25\ \mu\text{l}$  caspase glo reagent. SF21 insect cells incubated with Actinomycin D were used as a positive control and SF21 cells were included as a negative control.

To measure total protein content of the lysed cells, we applied the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Veenendaal, Netherlands; Cat.#500-0002), which is based on the method of Bradford. Measurements were performed in a spectrophotometer (LUMIstar OPTIMA, BMG Labtech, Offen burg, Germany) set to 595 nm, using 96-well transparent ELISA plates. BSA (0.5, 0.25, 0.125, and 0.065 mg/ml) was used as the protein standard. To  $10\ \mu\text{l}$  of stored supernatant,  $200\ \mu\text{l}$  of  $1\times$  Dye Reagent was added and incubated for  $\sim 10$  min at room temperature. Since the linear range of

the microtiter plate assay is only 0.05 mg/ml to approximately 0.5 mg/ml, we also measured a 10× dilution of each sample. During the first cultivation experiment, measurements were performed in duplicate, for the second cultivation experiment all measurements were performed in triplicate.

### 3 RESULTS

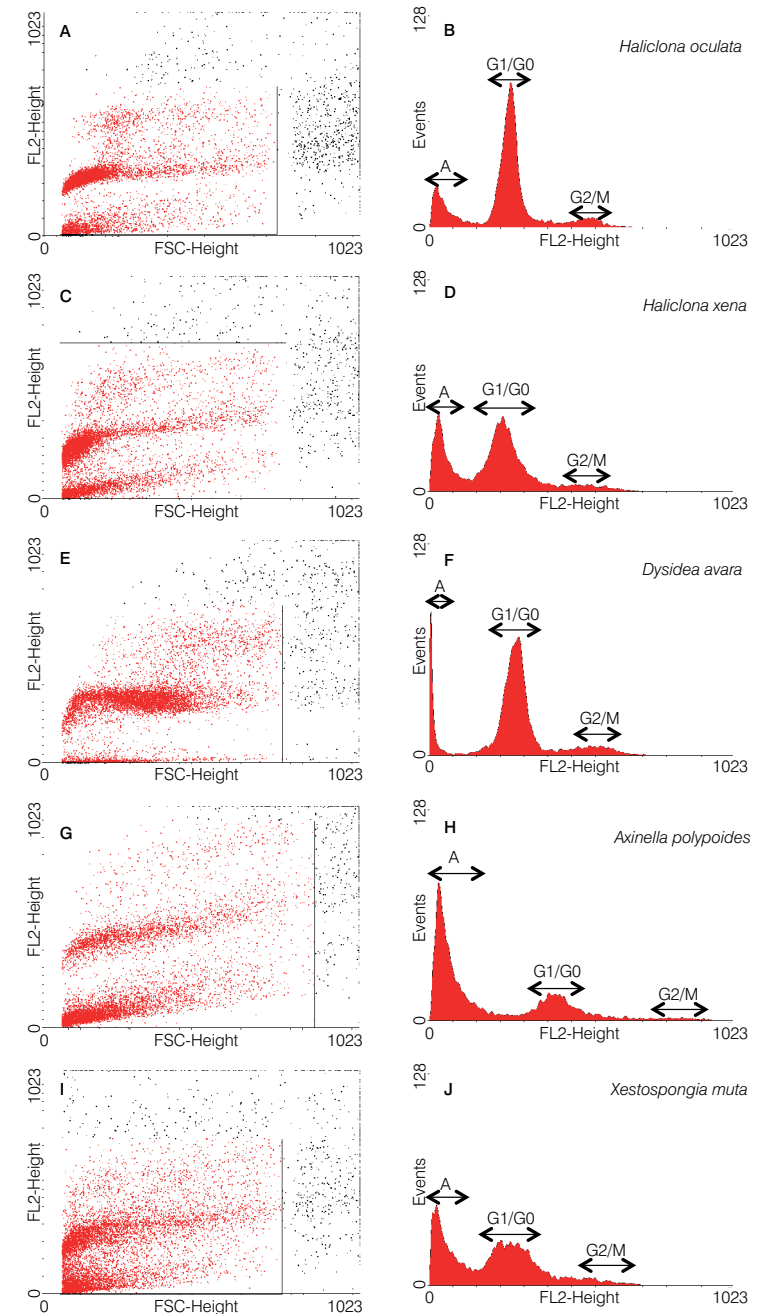
#### 3.1. Cell cycle analysis of five different species

Cell cycle distribution was measured for five different sponge species, obtained from different locations. Figure 1 represents the cell cycle distribution of these five sponges. The figures on the left show scatter plots of forward scatter (FSC-Height), which is a relative measure for cell size, versus red fluorescence (FL2-Height), which is a relative measure for DNA content. The figures on the right show single parameter histograms of red fluorescence (DNA content). For all five species the cell populations have a broad range of FSC values, which means that cells with the same DNA content cover a wide range of cell sizes. This is as expected, because sponges contain many different cell types with different sizes (~10 μm choanocytes compared to ~50 μm archaeocytes) and we did not select for a certain cell type. All scatter plots show three populations of cells: the middle population represents cells residing in G1/G0 phase, the upper population, located at a red fluorescence of 1.8–2.0 times that of G1/G0 population, represents cells in the G2/M phase, and the lower population represents apoptotic cells. The S phase cells are located between the G1/G0 and G2/M populations and are hardly or not present here. A single gate was placed around these three populations of cells as shown by the squares in Fig. 1 and cells inside the gate were plotted in the single-parameter histograms of red fluorescence (DNA content). This provided data for estimations of distribution of cells existing in G0/G1 (2N DNA), in S (2–4N DNA), in G2/M (4N DNA), and in the apoptotic population (<2N DNA).

#### 3.2 Cultivation experiments *Haliclona oculata*

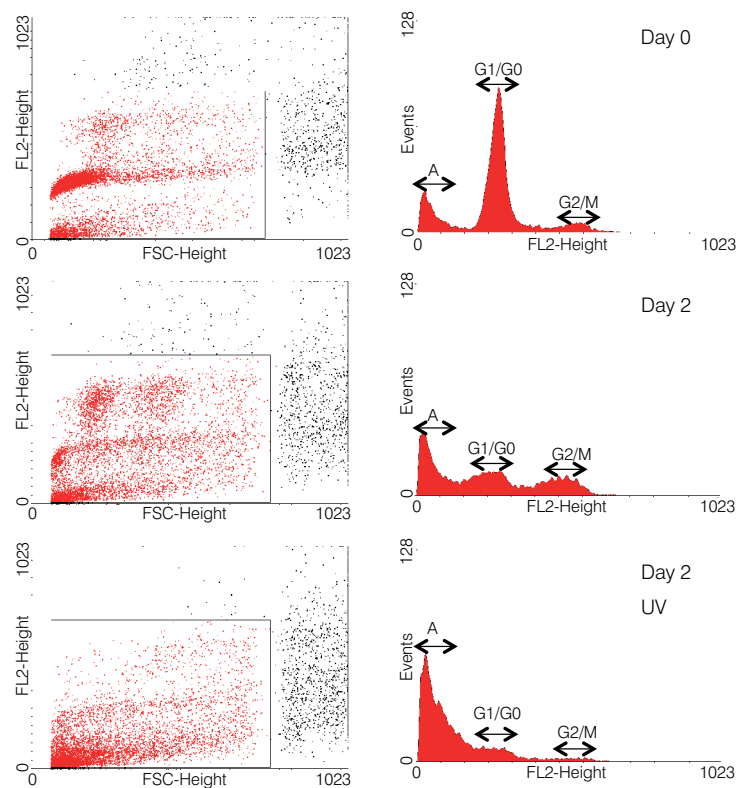
##### 3.2.1. Cell cycle analysis with flow cytometry

Figure 2 shows the scatter plots and single-parameter histograms of *H. oculata* from the first cultivation experiment. The first cultivation lasted for 2 d, and cells were harvested at day 0 (right after dissociation) and at day 2. To confirm that apoptosis is indeed occurring and that the population with the low red fluorescence consists of apoptotic cells or their remnants, a control sample was exposed on day 1 to 0.5 h of UV light, which is a known inducer of apoptosis and analyzed on day 2 (day 2 UV).



**Figure 1** Left scatter plots of FSC-Height (cell size) versus FL2-Height (red fluorescence DNA content). Right single parameter histograms of FL2-Height (red fluorescence DNA content). a, b: *H. oculata*, collected at 16 November 2009; c, d: *H. xena*; e, f: *D. avara*; g, h: *A. polypoides*; i, j: *X. muta*.

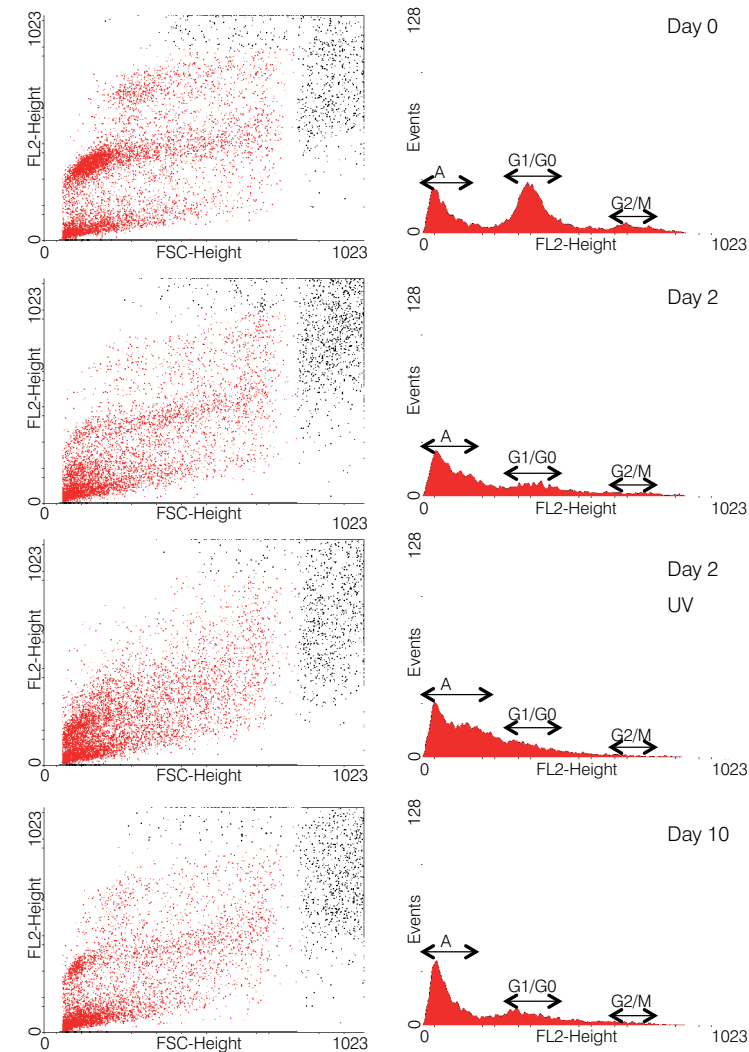




**Figure 1** First cultivation experiment with *H. oculata*, collected at 16 November 2009. *Left* scatter plots of FSC-Height (cell size) versus FL2-Height (red fluorescence DNA content). *Right* single-parameter histograms of FL2-Height (red fluorescence DNA content).

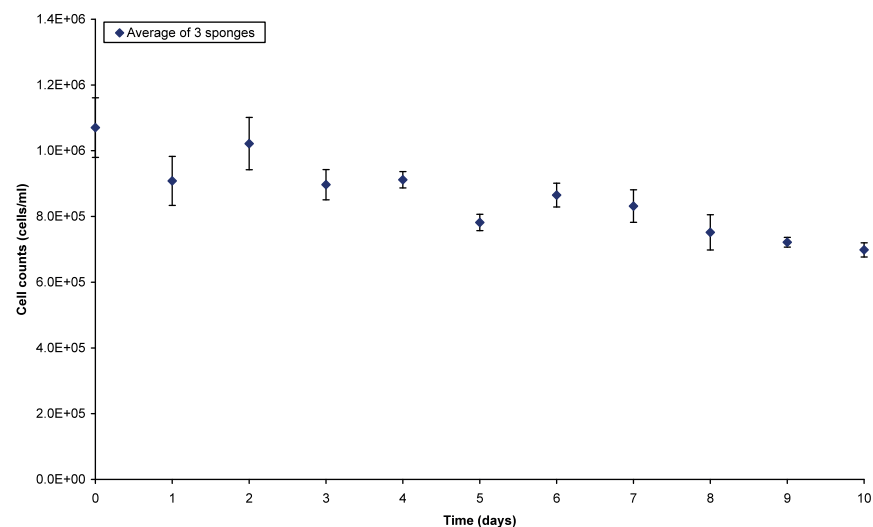
For the second experiment some adjustments were made compared to the first experiment. Cells from three different sponges, instead of one sponge, were used, and cells were cultivated for 10 d instead of 2 d. In addition to cell cycle analysis, cell concentrations were determined during the second experiment. Figure 3 shows the scatter plots and single-parameter histograms of *H. oculata* of the second cultivation experiment. Only the results of one sponge are shown, since the other two sponges gave similar results. Figure 3 also does not show results of day 3 till day 9, since the results did not change much between days 2 and 10.

Figure 4 shows the cell concentrations (with standard deviation) of the second cultivation experiment of all three sponges. The cell concentration at the start of the experiment was 1.1 million cells per milliliter and after 10 days of cultivation the cell



**Figure 3** Second cultivation experiment with *H. oculata*, collected at 11 January 2010. *Left* scatter plots of FSC-Height (cell size) versus FL2-Height (red fluorescence DNA content). *Right* single-parameter histograms of FL2-Height (red fluorescence DNA content).

concentration was 0.7 million cells per milliliter, which indicates that approximately one third of the cells died and lysed after 10 d of cultivation.

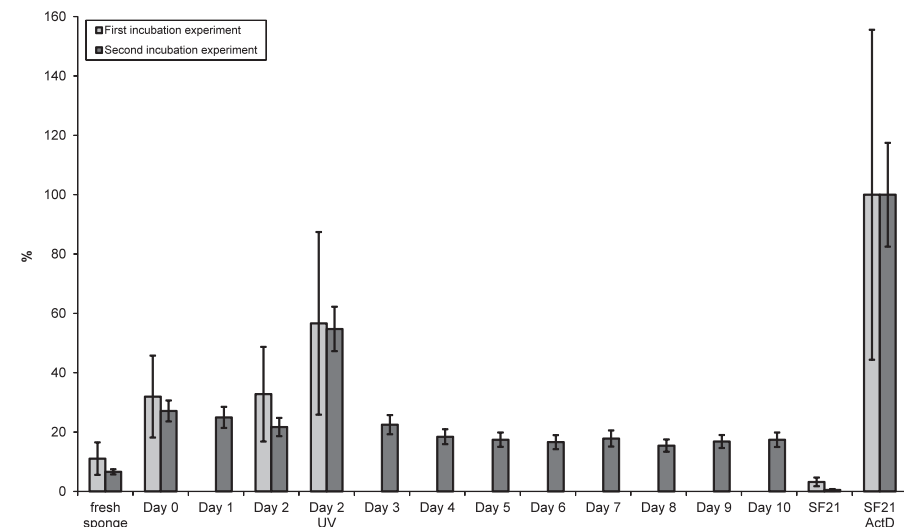


**Figure 4** Cell density during time of second cultivation experiment with *H. oculata*, collected at 11 January 2010, with standard deviation.

### 3.2.2 Caspase activity per total protein content

To confirm that the lowest population of cells in the scatter plots were really apoptotic cells, an additional assay, based on measurements of caspase 3/7 activity, was used. To be able to compare the results of each sample, we divided the caspase activities by the total amount of protein, assuming that there is a relation between total protein content and cell numbers. Since cells do not have to be monodisperse for this method, we could also use a sample of fresh, not dissociated sponge material, which made it possible to observe the effect of the dissociation process on apoptosis. For the cell cycle analysis method this is not possible, because sponge tissue has to be dissociated before it can be fixed and stained with PI. The positive control consisted of insect cells (SF21 ActD) incubated with actinomycin D, which inhibits transcription and interferes with DNA replication, causing cells to go into apoptosis (Clem and Miller, 1994). The negative control consisted of continuously dividing insect cells (SF21), which are expected to show no apoptosis.

To be able to compare measurements of both experiments, which were measured at different gains, all results were normalized in relation to SF21 ActD, since this sample was the same in both experiments.



**Figure 5** Caspase activity per total protein, normalized in relation to the positive control, insect cells incubated with actinomycin D (SF21 ActD), with standard deviation.

Figure 5 shows the results of the caspase activity per total amount of protein. The results for the negative and the positive control are as expected. Exponentially growing SF21 cells show a negligible caspase activity in comparison to SF21 cells treated with ActD.

## 4. DISCUSSION

### 4.1 Cell cycle distribution

For five different sponge species the cell cycle distribution was measured (Fig. 1) and all scatter plots show three populations of cells. The middle population represents cells in the G1/G0 phase. The upper population, located at a red fluorescence 1.8–2.0 times that of the G1/G0 population, represents cells in the G2/M phase. This ratio of 1.8–2.0 is in general found for different types of cells and confirms that these populations are indeed G1/G0 and G2/M. For example, the ratio between the middle population (G1/G0) and the lowest population is much higher. The cells between the middle and upper population are considered to reside in the S phase. The lowest population of cells is most likely the apoptotic population, which represents cells with fragmented DNA, but can also contain some cell debris, like apoptotic bodies and possibly some bacterial contamination. However, the bacterial contamination is

negligible, since most of the bacteria were removed from the cell suspension by differential centrifugation. Thus, most likely this population represents cells with fragmented DNA and can therefore be considered to be the apoptotic population.

To confirm that the lowest population consists of apoptotic cells, for both cultivation experiments a sample was exposed to UV light, which is a known inducer of apoptosis. Figures 2 and 3 illustrate that 1 d after UV exposure, almost all cells are in the lowest population, indicating that indeed this population contains apoptotic cells.

In addition to flow cytometric cell cycle analysis, caspase activity of the cells was measured for both cultivation experiments. Caspase activity is an indicator of early apoptosis. When comparing the results of day 2 with results of day 2 after UV exposure (Fig. 5), for both experiments, an increase in caspase was observed, indicating that indeed UV light induces apoptosis. This increase in caspase activity correlates with the increase in the size of the lowest population as observed by flow cytometry, confirming that the lowest population is indeed an apoptotic population.

#### 4.2 Different species

The scatter plots of all five sponges in Fig. 1 show three populations of cells, although for *H. oculata*, *H. xena*, and *D. avara* these three populations can be seen more clearly than for *A. polypoides* and *X. muta*. For all five sponges the G1/G0 fraction is much larger than the G2/M fraction and the S-phase fraction is hardly visible. This indicates that most of the cells were not actively dividing or were dividing very slowly. There is also an apoptotic population which is largest for *A. polypoides* and lowest for *D. avara*. The size of this apoptotic population may represent the viability of the sponge. At the time of harvest, *D. avara* looked very healthy and was present abundantly, which may explain the low apoptotic fraction and the clear cell cycle distribution. On the other hand, apoptosis may also be due to harvesting and treatment of the sponges which in this case would indicate that *D. avara* is less sensitive to this treatment than the other four sponges.

#### 4.3 Cultivation experiments *Haliclona oculata*

Two cultivation experiments were executed with *H. oculata*. During both experiments, the cell cycle distribution (Figs. 2 and 3) and the caspase activity (Fig. 5) were measured and for the second experiment also the cell density (Fig. 4) was determined. Figure 5 shows that there is approximately a threefold increase of caspase activity for both experiments between the fresh sponge sample and the day 0 sample after harvesting and dissociation, which indicates that harvesting, transporting, and dissociating the sponge induced apoptosis. By dissociating the sponge, cells are removed from their extracellular matrix and will lose cell–cell contact and adhesion. It is known for primary cells that these factors are important for survival (Freshney,

2005). Also filtering, centrifuging, and resuspending the cells will cause shear stress, which is another potential cause of cell death (Laken and Leonard, 2001). To circumvent these apoptosis inducing factors, tissue or explant culture, for which dissociation into cells is not required, could be a solution (Garcia Camacho et al., 2006).

When comparing the cell cycle distributions at day 0 of both experiments, we can see that for the first cultivation experiment (Fig. 2), most cells were in the G1/G0 phase and the apoptotic population was relatively small in comparison with the G1/G0 population. For the second cultivation experiment (Fig. 3) most cells were also in the G0/G1 phase, but there is also a large apoptotic fraction, which is clearly larger than the apoptotic fraction in the first experiment. In other words, the cell population at the start of the second experiment was less viable as compared to the first experiment. This suggests that the selected sponges for the second experiment were either less viable or more sensitive to the dissociation process. However, there is no significant difference in caspase activity between the first and second cultivation experiment (Fig. 5).

When comparing the cell cycle distributions at days 0 and 2 for both cultivation experiments, a decline in the fraction of cells in G1/G0 and an increase in the apoptotic fraction can be observed (Figs. 2 and 3), implying that most cells died or were dying. However, when observing the cell counts in Fig. 4, we can see that there was no significant decrease of cell numbers between days 0 and 2. This shows the added value of measuring the different subpopulations, showing that although there was no decrease in cell number, the majority of the cells were, in fact, dying. Still, at day 2 of the first experiment, a small increase of the G2/M fraction can be observed. A possible explanation is that the absolute number of cells in G2/M remained constant, while the total number decreased, which automatically means that the fraction of G2/M increases. However, it is also possible that part of the G1 cells were actively dividing and moved to the G2/M population.

Between days 3 and 10 of the second experiment, the caspase activities do not increase anymore (Fig. 5). This could be explained by the fact that the sponges were harvested during winter and that water temperatures were 2°C. Therefore, also the cultivation temperature was set to 2°C and at this temperature all biological processes slow down including apoptosis (Koopmans and Wijffels, 2008). Another explanation could be that the rate of cells going from the G1/G0 fraction towards the apoptotic fraction, is just as large as the rate of apoptotic cells lysing, keeping the fraction of apoptotic cells constant.

The cell cycle distribution at day 10 (Fig. 3) shows that most cells were in the apoptotic phase. However, we can also still see a clear population of cells in the G1/G0 phase, and a very small population in the G2/M phase, which means that not all cells have become apoptotic. This could imply that from the cell population at the start, only a small subpopulation of cells is proliferating, while the majority of cells is dying. If this is the case, it may be necessary to culture for longer periods of time in order to get rid of the non-proliferative cells, because they will die and fall apart and thus select for the few cells that are able to proliferate.

## 5 CONCLUSIONS

Flow cytometric cell cycle analysis allows for a quick determination of the proliferative status of a cell population for sponge cells. This method makes it possible to distinguish between apoptotic, G1/G0, S, and G2/M phase cells. Apoptosis was confirmed using a caspase assay. Sponge cells obtained from different sponges all showed a large fraction of cells in G1/G0 compared to G2/M and S, which was hardly present. Some sponges also showed a large apoptotic fraction. This indicates that cells were not actively dividing. Apoptosis measurements using an enzymatic caspase assay show that the large apoptotic fraction is at least in part due to harvesting and dissociation of the sponge. Cultivation of the sponge cells resulted in a further increase in the apoptotic cell fraction indicating that either the cultivation conditions do not support proliferation or that the collected sponges itself may be in a non-proliferative state. In future, this flow cytometric cell cycle analysis method in combination with caspase measurements can be used to validate improvements in harvesting and dissociation and to study the influence of culture conditions on cell proliferation, in order to develop an *in vitro* sponge cell culture.

## Acknowledgements

This research was supported by IPOP Coast and Sea, Graduate School VLAG and the Florida Sea Grant (R/LR-MB-25). We thank M. Koopmans for collecting *D. avara* and R. Baumberger for collecting *X. muta*. Also, we would like to thank G. Pijlman, C. Geertsema, and S. Metz from the Laboratory of Virology at Wageningen University for their help with the caspase and Bradford protein assays and providing the SF21 cells.

## REFERENCES

- Garcia Camacho, F.; Chileh, T.; Ceron García, M. C.; Sanchez Mirón, A.; Belarbi, E. H.; Contreras Gómez, A.; Molina Grima, E. (2006) Sustained growth of explants from Mediterranean sponge *Crambe crambe* cultured *in vitro* with enriched RPMI 1640. *Biotechnol Prog*, 22: 781–790.
- Clem, R. J.; Miller, L. K. (1994) Control of programmed cell death by the baculovirus genes p35 and iap. *Mol Cell Biol*, 14: 5212–5222.
- Crissman, H. A.; Steinkamp, J. A. (1973) Rapid, simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. *J Cell Biol*, 59: 766–771.
- De Caralt, S.; Uriz, M. J.; Wijffels, R. H. (2008) Cell culture from sponges: pluripotency and immortality. *Trends Biotechnol*, 25: 467–471.
- Fertig, G.; Klöppinger, M.; Miltenburger, H. G. (1990) Cell cycle kinetics of insect cell cultures compared to mammalian cell cultures. *Exp Cell Res*, 189: 208–212.
- Freshney, R. I. (2005) *Culture of animal cells: a manual of basic technique*. Hoboken NJ: Wiley-Liss.
- Koopmans, M.; Martens, D.; Wijffels, R. H. (2009) Towards commercial production of sponge medicines. *Mar Drugs*, 7: 787–802.
- Koopmans, M.; Wijffels, R. H. (2008) Seasonal growth rate of the sponge *Haliclona oculata* (demospongiae: Haplosclerida). *Mar Biotechnol*, 10: 502–510.
- Krishan, A. (1975) Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *Journal Cell Biol*, 66: 188–193.
- Laken, H. A.; Leonard, M. W. (2001) Understanding and modulating apoptosis in industrial cell culture. *Curr Opin Biotechnol*, 12: 175–179.
- Munro, M. H. G.; Blunt, J. W.; Dumdei, E. J.; Hickford, S. J. H.; Lill, R. E.; Li, S.; Battershill, C. N.; Duckworth, A. R. (1999) The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol*, 70: 15–25.
- Nicholson, D. W.; Thornberry, N. A. Caspases: killer proteases. (1997) *Trends Biochem Sci*, 22: 299–300.
- Pomponi, S. A. (1999) The bioprocess-technological potential of the sea. *J Biotechnol*, 70: 5–13.
- Pomponi, S. A. (2006) Biology of the porifera: Cell culture. *Can J Zool*, 84: 167–174.
- Pomponi, S. A.; Willoughby, R. (1994) Sponge cell culture for the production of bioactive metabolites. IN Van Soest, R.; Van Kempen, T. M. G.; Braekman, J. C. (Eds.) *Sponges in time and space*. Rotterdam: A A Balkema, 395–400.
- Pomponi, S. A.; Willoughby, R.; Kaighn, M. E.; Wright, A. E. (1997) Development of techniques for *in vitro* production of bioactive natural products from marine sponges. IN Maramorosch, K.; Mitsuhashi, J. (Eds.) *Invertebrate cell culture. Novel directions and biotechnology applications*. Enfield: Science Publishers, 231–237.
- Pozarowski, P.; Darzynkiewicz, Z. (2004) Analysis of cell cycle by flow cytometry. *Methods Mol Biol*, 281: 301–311.
- Rinkevich, B. (2005) Marine invertebrate cell cultures: new millennium trends. *Mar Biotechnol*, 7: 429–439.
- Sipkema, D.; Franssen, M. C. R.; Osinga, R.; Tramper, J.; Wijffels, R. H. (2005a) Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142–162.
- Sipkema, D.; Osinga, R.; Schatton, W.; Mendola, D.; Tramper, J.; Wijffels, R. H. (2005b) Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201–222.
- Sipkema, D.; Snijders, A. P. L.; Schroen, C. G. P. H.; Osinga, R.; Wijffels, R. H. (2004) The life and death of sponge cells. *Biotechnol Bioeng*, 85: 239–247.
- Wiens, M.; Muller, W. E. G. (2006) Cell death in Porifera: molecular players in the game of apoptotic cell death in living fossils. *Can J Zool*, 84: 307–321.

## Chapter 4

Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures





## ABSTRACT

In general, primary sponge cell cultures lack proliferative capacity. Possible explanations are the cellular stress caused by the dissociation process and the loss of cell-cell and cell-extracellular matrix contact. *In vitro* tissue culture is a potential alternative approach that would eliminate stressors associated with cell dissociation.

In this study, we compared tissue culture with primary cell culture for three species of marine sponges (*Haliclona oculata*, *Dysidea avara* and *Crambe crambe*) to determine if maintaining a three-dimensional structure results in better cell proliferation and viability. We also compared proliferation capacity of tissue fragments cultured in the sea (*in situ* tissue culture) with fragments cultured in the lab (*in vitro* tissue culture). Cell proliferation and cell death were measured by cell counts, cell cycle distribution, total protein content and caspase activity.

Our results demonstrated that the success of cell or tissue culture is species dependent, and *in vitro* tissue culture does not result in better cell proliferation and viability than cell culture. *In situ* tissue culture was more favorable than *in vitro* tissue culture (for *D. avara*), which is probably caused by the constant supply of nutrients, dissolved oxygen and flushing away of waste products. Although no net growth was observed during cell culture, cell cycle data of *H. oculata* cell cultures indicate that a subpopulation of cells, possibly archaeocytes or spherulous cells, remained intact and did not become apoptotic, and cell cultures of *D. avara* showed evidence of dividing cells. This holds potential for development of primary cell cultures from these sponges.

### This chapter has been submitted for publication as:

Schippers KJ, Martens DE, Pomponi SA, Wijffels RH. Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures.

## 1 INTRODUCTION

Marine sponges are the most prolific producers of newly discovered bioactive compounds (Blunt et al., 2009; Hu et al., 2011). These sponge-derived compounds have pharmaceutical potential (Sipkema et al., 2005a), but due to insufficient supply of biological material, the development of these potential drugs into commercial products is hampered (Osinga et al., 1998). To increase the supply of sponge biomass, several cultivation techniques have been investigated, such as mariculture, *ex situ* culture and *in vitro* sponge cell culture (Sipkema et al., 2005b; Schippers et al., 2012). For mariculture (sea-based) and *ex situ* culture (in aquaria), sponges are cut into pieces, termed explants, and these explants can grow into adult individuals due to the strong regenerative ability of sponges (Osinga et al., 1998). For *in vitro* sponge cell culture, the cells are dissociated from the adult sponge and the cells can be cultivated in suspension (Pomponi and Willoughby, 2000; Sun et al., 2007) or as spherical aggregates, termed primmorphs (Custodio et al., 1998; Valisano et al., 2006; Sipkema et al., 2003; Muller et al., 2000). Although cell proliferation has been observed, continuous cell growth has not been realized yet (Pomponi, 2006; Schippers et al., 2012). Consequently, mariculture is the most feasible method for economic production of sponge biomass (Sipkema et al., 2005b). Nevertheless for pharmaceutical production, *in vitro* cultivation is more desirable, since it allows for control of culture conditions and manipulation of these to increase yield.

Possible reasons for the lack of proliferating sponge cells *in vitro* are the cellular stress caused by the dissociation process and the loss of contact between neighboring cells or between a cell and the extracellular matrix of the sponge. The extracellular matrix contains substances produced and excreted by the cells, acting as a scaffold to hold tissues together. For the sponge this is e.g. spicules, collagen, lectin. Our previous study demonstrated that dissociation of sponge cells resulted in increased caspase activity (Schippers et al., 2011), which is an indicator for apoptosis, and decreased telomerase activity (Kozioł et al., 1998), which is a bio-marker for cell proliferation. Most primary cells are adherent and need to attach to a surface in order to survive and proliferate (Freshney, 2005). One type of adherent culture is organotypic culture, which involves growing cells in a 3-dimensional environment to mimic the situation *in vivo* (Blow, 2009). The collagenous skeleton of the sponge itself provides an excellent 3-dimensional culture environment, as demonstrated by Green et al. (2003), who used natural sponge skeleton as a scaffold for the cultivation of human bone marrow cells.

We hypothesize that tissue culture will provide a more favorable culture condition than cell culture, because the dissociation process is avoided, the cells are not exposed to shear, the cell-cell contacts are retained, and the cells remain in a



3-dimensional culture environment. The aim of this study was to compare tissue culture with cell culture to test the hypothesis that keeping cells in their natural 3-dimensional environment results in better cell proliferation and viability. As a control for tissue cultured in the lab (*in vitro* tissue culture) we also compared proliferative capacity of tissue fragments cultured in the sea (*in situ*).

Three species were studied: *Haliclona oculata*, *Dysidea avara* and *Crambe crambe*. *Haliclona oculata* is a branching, compressible sponge and occurs in the North Atlantic and Arctic. Recent studies showed that explants of *H. oculata* were able to grow into new functional adult sponges when cultivated in the sea (Koopmans and Wijffels, 2008), but attempts with cell cultures of this sponge were unsuccessful (Schippers et al., 2011). The sponges *D. avara* and *C. crambe* are Mediterranean species. *D. avara* is a massive, conulose, compressible sponge that lacks spicules and is known for its avarol production (Uriz et al., 1996b). This sponge has been used in mariculture and *ex situ* culture studies (Osinga et al., 2010; De Caralt et al., 2010; Sipkema et al., 2006; Mendola et al., 2008). *C. crambe* is a thin, smooth encrusting sponge and produces the toxin crambescin (Uriz et al., 1996a). Tissue fragments of *C. crambe* have already been successfully cultivated *in vitro* (Garcia Camacho et al., 2006).

Cell culture and tissue culture experiments were performed for all three sponges. For *D. avara* and *C. crambe* tissue fragments were also cultured in the sea (*in situ* tissue culture) to compare proliferative capacity of fragments maintained in filtered seawater versus their natural environment. Cell proliferation and cell death of the sponge cells and tissue fragments were measured by cell counts (for cell cultures), measuring the cell cycle distribution, total protein content and caspase activity, an indicator for apoptosis.

## 2 MATERIAL AND METHODS

### 2.1 Specimen collection and transportation

Sponge specimens were collected by scuba diving. Three *H. oculata* individuals were collected from the Easter Scheldt (Lokkersnol) in the Netherlands at a depth of approximately 12 meters. The sponges were transported in coolers to maintain the temperature the same as in the sea and were constantly aerated. Three individuals of *D. avara* and *C. crambe* were collected from the Mediterranean (Cala Montgo) in Spain at a depth of approximately 8-10 meters. Part of the mother sponge was left intact on the reef for later sampling and the other fragments were processed immediately.

### 2.2 Media and solutions

Calcium- and magnesium-free seawater (CMF-EDTA), 10mM, was prepared by dissolving 32.1 g NaCl, 1.1 g Na<sub>2</sub>SO<sub>4</sub>, 0.9 g KCl, 10 ml of Trizma (0.5M, pH 8.0) and 20 ml of 0.5M EDTA stock solution in 1 L of demineralized water. The pH was adjusted to 8.0 with HCl, and salinity was set to 960 mOsm/kg (for *H. oculata*) and to 1070 mOsm/kg (for *D. avara* and *C. crambe*) before filter sterilization (pore size 0.22 μm).

Filtered seawater (FSW) was prepared by filter sterilizing (pore size 0.22 μm) fresh seawater collected from the Easter Scheldt and the Mediterranean. Salinity was 960 mOsm/kg and 1070 mOsm/kg, respectively, and the pH was 8.0.

Propidium iodide staining solution (3.8 mM sodium citrate, 40 μg/ml PI in PBS) was prepared by dissolving 98.1 mg sodium citrate and 4 mg of propidium iodide in 100 ml of phosphate buffered saline (PBS) and was stored at 4 °C in the dark.

RNase A stock solution (10 μg/ml) was prepared by dissolving 1 mg of RNase A in 100 ml demineralized water, and was boiled for 5 min, aliquoted and stored at -20 °C. Wash buffer (PBS + 0.1 % BSA) was prepared by dissolving 0.1 g of bovine serum albumin (BSA) in 100 ml phosphate buffered saline (PBS).

The passive lysis buffer (PLB, Promega, Cat.# E1941) was prepared by diluting the buffer 5 x with demineralized water.

### 2.3 Sponge cell culture

The sponge specimens were divided in several parts. One part was used for cell culture and the other part was used for tissue culture.

The sponge fragments used for cell culture were dissociated as described by Schippers et al. (2011). For each experiment the cell concentration was set to ~10<sup>6</sup> cells/ml in FSW. To resuspend the cells in FSW, a certain volume of cells (~ 5-30 ml, depending on cell yield) in CMF-EDTA was centrifuged at 300g for 5 min and the cells in the pellet were resuspended in FSW to obtain a final concentration of ~10<sup>6</sup> cells/ml. The cells were cultured in 6 well plates, 5 ml per well and incubated at the same temperature as of the Easter Scheldt and the Mediterranean at the time of collection, which was 8 °C and 12 °C, respectively.

Primary cell cultures of all three sponges were incubated in FSW medium. The medium was not refreshed during culture. Since the cells were in suspension and not firmly adherent, refreshing the FSW medium would involve centrifugation and expose the sponge cells to additional shear stress. To prevent microbial contamination, the FSW was not supplemented with extra nutrients. Although FSW alone will not support long-term growth, it does contain nutrients, such as dissolved organic matter, which can be taken up by the sponge cells and can be used during the first days of culture. Cells were incubated for 8 days. Each day the cell concentration was determined, and samples were taken for cell cycle analysis and measurement of caspase activity and total protein content (see sections 2.5 and 2.6).



## 2.4 Sponge tissue culture

The sponge fragments used for tissue culture (*in vitro* and *in situ*) were rinsed with FSW and cut into pieces of ~ 15-20 mm<sup>3</sup>. The pieces used for *in vitro* cultivation were placed in 6 well plates and incubated at the temperature of the Easter Scheldt and the Mediterranean at the time of collection, which was 8 °C and 12 °C, respectively. Each well contained one tissue fragment with 8 ml FSW that was refreshed daily to prevent accumulation of waste products.

The pieces used for *in situ* cultivation (only from *D. avara* and *C. crambe*) were also placed in 6 well plates. These plates were covered with nylon screen (mesh size Ø 1 mm) and attached to tiles with elastic bands. Due to the large mesh size, the water could easily flow through the culture chambers without resistance. The tiles were placed in close proximity to the mother sponges, to ensure that environmental conditions of the *in situ* fragments, such as nutrient supply, dissolved oxygen, pressure and water flow, were the same as the mother sponge.

Tissue fragments were cultivated for 8 days. Each day tissue fragments from the lab and the sea were harvested. Half of each tissue fragment was used for cell cycle analysis and the other half was used for measurement of caspase activity and total protein content (see sections 2.5 and 2.6).

## 2.5 DNA staining and cell cycle analysis

Sponge cells from the cell culture experiments were harvested and a monodisperse cell suspension was prepared by centrifuging 5 ml of cells (300g, 5 min) and resuspending the pellet in 1 ml of CMF-EDTA. A small volume (~10 µl) was used for cell counting with a disposable haemocytometer (C-Chip DHC-N01, Incyto) using a microscope (IX-71, Olympus). Tissue fragments from the *in situ* and *in vitro* cultivation experiment were harvested and a single cell suspension was prepared by squeezing sponge fragments in 1 ml CMF-EDTA to release cells from the extracellular matrix. If aggregates were still present, the suspension was vortexed briefly. To 300 µl of cell suspension, 900 µl of ice-cold absolute ethanol was added drop wise while vortexing. Samples were stored at -20 °C for at least 1 hour and fixed cells were analyzed within two weeks. After fixation, cells were washed twice at room temperature with 1 ml of wash buffer (PBS + 0.1% BSA) by centrifuging at maximum speed (13,000 rpm) for 1 min and resuspending the pellet in the wash buffer. The pellet was resuspended in 1 ml of propidium iodide staining solution by pipetting several times and briefly vortexing. Finally, 50 µl of RNase A stock solution were added and the fixed cells were incubated for 3 hours at 4°C. Fluorescence due to PI staining was measured with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ). The instrument settings used for *H. oculata* were similar as described by Schippers et al. (2011) (Forward scatter: Voltage: E+00, Amplifier: 3.50; Side scatter: Voltage: 450, Amplifier: 1.00; Fluorescence detector 2: Voltage: 450, Amplifier: 1.00. All linear scale).

## 2.6 Caspase 3/7 measurements and total protein content

Sponge cells from the cell culture experiment were harvested by centrifuging 5 ml of cells (300g, 5 min) and removing the supernatant. Subsequently 1 ml of passive lysis buffer was added to the pellet, and the cells were lysed by pipetting. Tissue fragments, both *in vitro* and *in situ*, were squeezed in 1 ml passive lysis buffer to release all the cells. Samples were incubated 20 min at room temperature to lyse all cells. Cell debris was spun down by centrifuging at maximum speed (13,000 rpm) for 5 min. Supernatant was aliquoted per 200 µl and stored at -20 °C for further analysis. Caspase activity was measured using caspase-glo<sup>®</sup> 3/7 assay (Promega Cat.# G8090), a luminescent assay that measures caspase 3 and 7 activities of cell suspensions. Measurements were performed with a luminometer (LUMIstar OPTIMA, BMG Labtech) using 96 white-walled well plates. To 25 µl of stored supernatant, 25 µl of caspase-glo reagent was added and incubated for minimally 30 min and maximally 3 hrs at room temperature. The blank was made of 25 µl passive lysis buffer and 25 µl caspase-glo reagent. The positive control consisted of insect cells incubated with actinomycin D (Sf21 ActD), which inhibits transcription and interferes with DNA replication, causing cells to go into apoptosis. The negative control consisted of continuously dividing insect cells (Sf21 cells), which are expected to lack apoptosis. Total protein content of the lysed cells was measured using the Bio-Rad Protein Assay Kit II (Bio-Rad Laboratories, Cat. # 500-0002), which is based on the Bradford method. Measurements were performed in a spectrophotometer (LUMIstar OPTIMA, BMG Labtech) set to 595 nm, using 96 well transparent ELISA plates. BSA (0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.065 mg/ml) was used as the protein standard. To 10 µl of stored supernatant, 200 µl of 1x Dye Reagent was added and incubated for ~10 min at room temperature. Since the linear range of the microtiter plate assay is only 0.05 mg/ml to approximately 0.5 mg/ml, we also measured a 10x dilution of each sample. All measurements were performed in triplicate.

## 3 RESULTS

Table 1 shows an overview of all measurements of each sponge species and cultivation method. For all three sponges cell concentrations were determined for the primary cell culture experiments. Cell counts for the tissue culture experiments were not done, since not all cells can be removed from the tissue fragments. Cell cycle analysis was only conducted on *H. oculata*, since the quality of the samples of *D. avara* and *C. crambe* was affected during transportation. Caspase activities and total protein content were only measured for *H. oculata* (cell culture and tissue *in vitro*), *D. avara* (tissue *in vitro* and *in situ*) and *C. crambe* (tissue *in vitro* and *in situ*) (see Table 1). The protein content of the cell culture samples of *D. avara* and *C. crambe* were too low for analysis.

**Table 1** Overview of measurements of each sponge species and cultivation method. Explanation of symbols: ✓ measured, - not measured, n/a not applicable, \* only 4 time points available, # protein content of samples was too low, ‡ quality of sample was affected during transportation.

		Cell counts	Cell cycle	Caspase activity	Total protein
<i>H. oculata</i>	Cell culture	✓	✓	✓	✓
	Tissue <i>in vitro</i>	n/a	✓*	✓*	✓*
<i>D. avara</i>	Cell culture	✓	-‡	-#	-#
	Tissue <i>in vitro</i>	n/a	-‡	✓	✓
	Tissue <i>in situ</i>	n/a	-‡	✓	✓
<i>C. crambe</i>	Cell culture	✓	-‡	-#	-#
	Tissue <i>in vitro</i>	n/a	-‡	✓	✓
	Tissue <i>in situ</i>	n/a	-‡	✓	✓

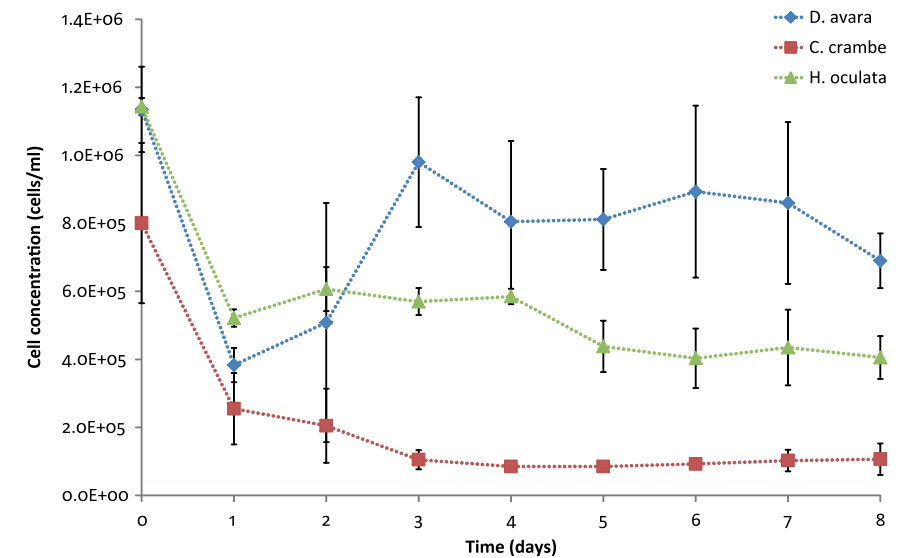
### 3.1 Sponge cell culture: cell counts

During the 8-day cell culture experiment, cell concentrations were determined for *D. avara*, *C. crambe* and *H. oculata* (See Fig 1). The initial cell concentrations of all three sponges were  $\sim 10^6$  cells/ml. After 1 day of cultivation, the cell numbers of all three species declined by a factor 2 to 3. After the first day, cell concentrations varied by species. The cell numbers of *C. crambe* continued to decrease. The cell numbers of *D. avara* more than doubled after the first day and remained mostly constant afterwards. The cell numbers of *H. oculata* initially declined, but remained mostly constant after the first day with a minor decreasing trend. After 8 days of cultivation, the cell numbers of *C. crambe*, *D. avara*, and *H. oculata* declined respectively by  $\sim 90\%$ ,  $40\%$ , and  $65\%$  with respect to the initial cell concentration.

### 3.2 Cell versus tissue culture (*in vitro*) of *H. oculata*

During the cell and tissue culture (*in vitro*) experiment of *H. oculata*, the cell cycle distribution was measured (see Fig 2). Only the results from one individual are shown. Cell cycle distributions from the other two individuals were similar. For *in vitro* tissue cultivation of *H. oculata* only two time points are shown (day 0 and 3), since the cultivation had to be stopped after 3 days due to an experimental error (see Fig 2b). As described by Schippers et al. (2011), the cell cycle data provide estimates of distribution of cells in G0/G1 (2N DNA), in S (2-4N DNA), in G2/M (4N DNA), and in the apoptotic population (<2N DNA).

At day 0, for both cell culture and *in vitro* tissue culture, the apoptotic and the G1/G0 fraction are clearly visible, although for the *in vitro* tissue culture the peaks are sharper.

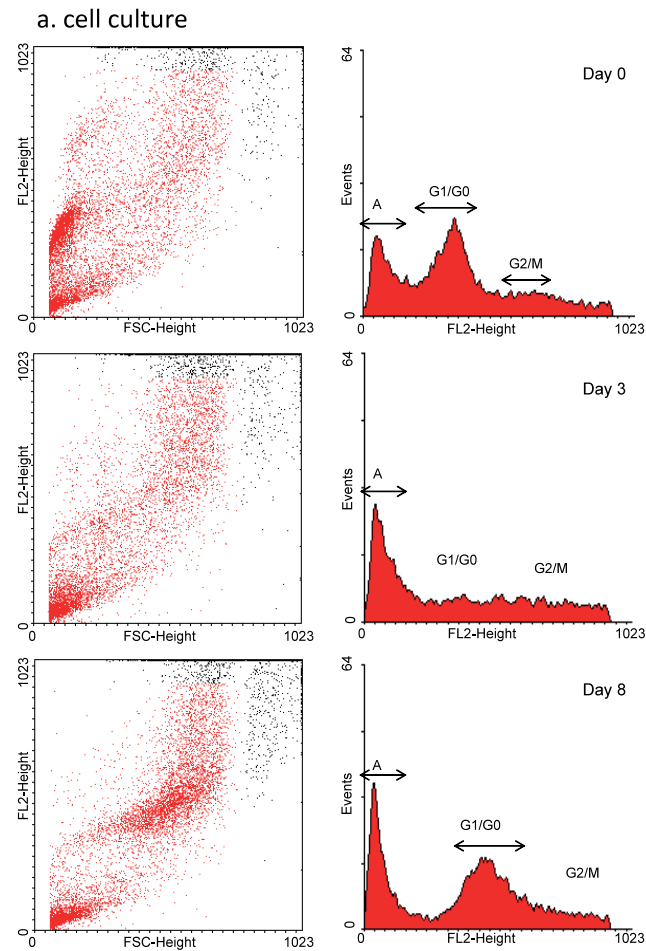


**Figure 1** Cell concentration of *D. avara*, *C. crambe* and *H. oculata* varied during 8-days of cultivation. Error bars indicate standard deviation of triplicate measurements (three cultures).

For both cultivation methods, the G2/M and the S fraction are hardly visible. At day 3 during cell culture, the majority of the cells are apoptotic and the G1/G0 and G2/M fractions cannot be distinguished from each other anymore, whereas at day 3 during *in vitro* tissue culture a G1/G0 fraction is still clearly visible. At day 8 during cell culture, a new peak can again be distinguished at a larger cell size (higher FSC) and slightly higher red fluorescence.

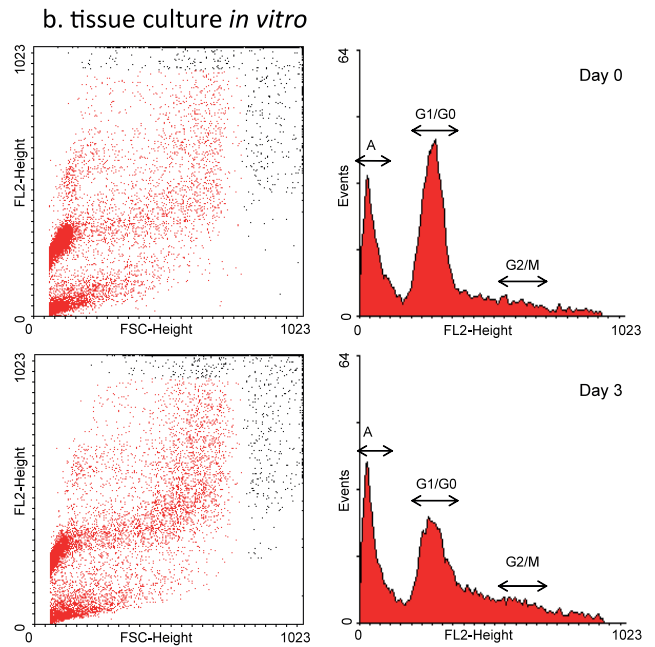
In addition to the cell cycle distribution, the caspase activity during tissue (*in vitro*) and cell culture of *H. oculata* were also measured (see Fig 3). To compare the results for each sample, caspase activity is expressed per total protein, assuming that there is a relation between total protein content and cell number. All results were normalized in relation to the positive control (Sf21 ActD, 100%). The results for the negative and the positive control are as expected; exponentially growing Sf21 cells show a negligible caspase activity in comparison to Sf21 cells treated with ActD. Again, for the *in vitro* tissue cultivation of *H. oculata* only values until day 3 are shown, since the cultivation was stopped after 3 days.

At day 0, the caspase activity of 'fresh' sponge material (Fig 3, day 0 of *in vitro* tissue) was almost nil, whereas the caspase activity of dissociated cells (Fig 3, day 0 of suspension cells) was high. The caspase activity during cell culture remains high for 4 days and then slowly declines, although it remains high in comparison to 'fresh'

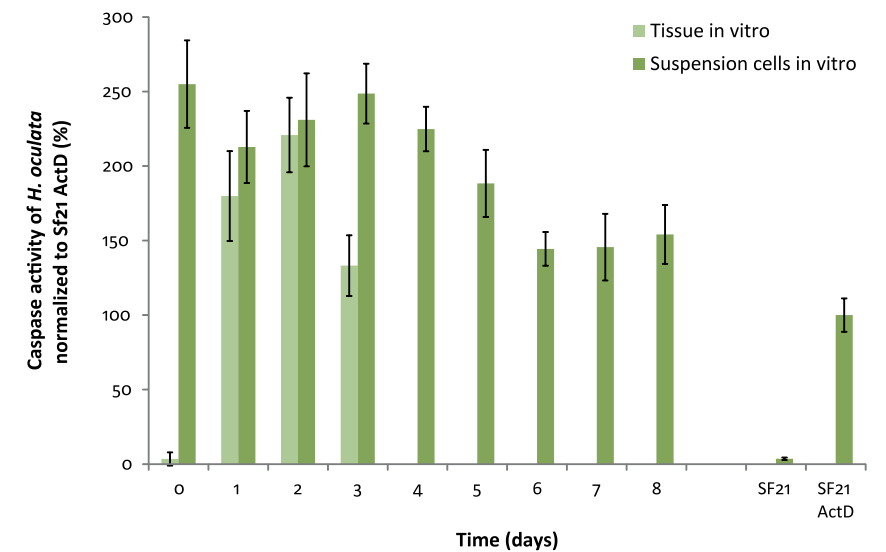


**Figure 2** Cell cycle analysis of cell cultures (a) and *in vitro* tissue culture (b) of *H. oculata*. Left: scatter plots of FSC (cell size) versus FL2 (DNA content). Right: single-parameter histograms of FL2 (DNA content).

sponge material. The caspase activity of *in vitro* tissue fragments after 1 day in culture also increased drastically with values comparable to those of cell culture. However at day 3, the caspase activities of the *in vitro* tissue fragments were lower in comparison to the caspase activities of the cell cultures at day 3.



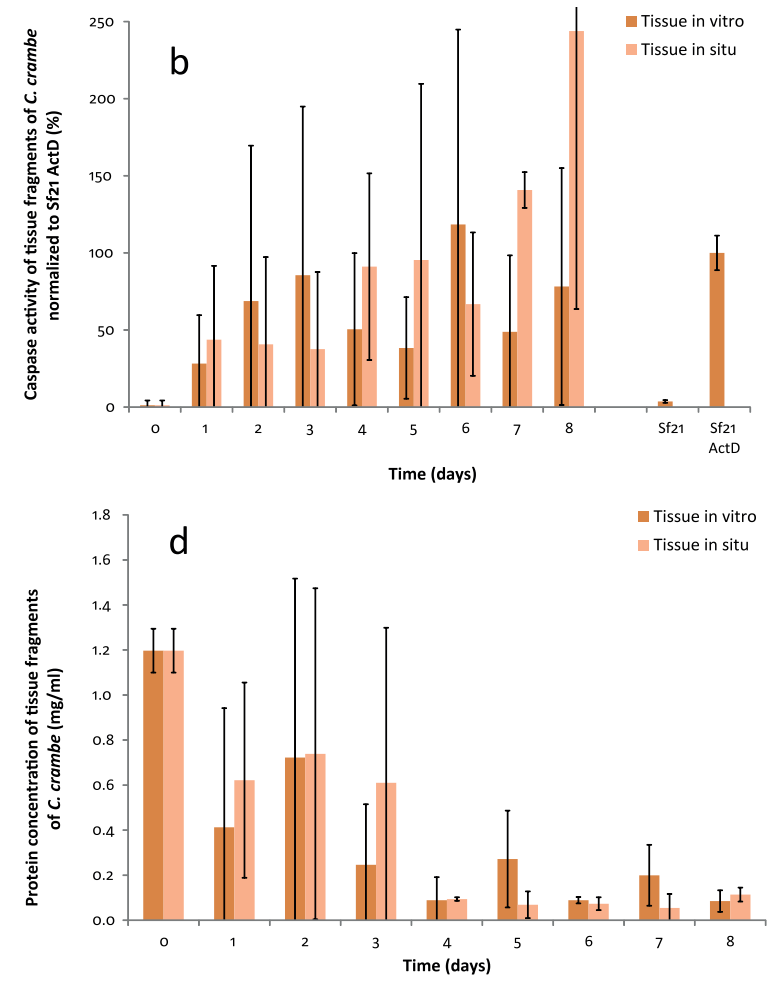
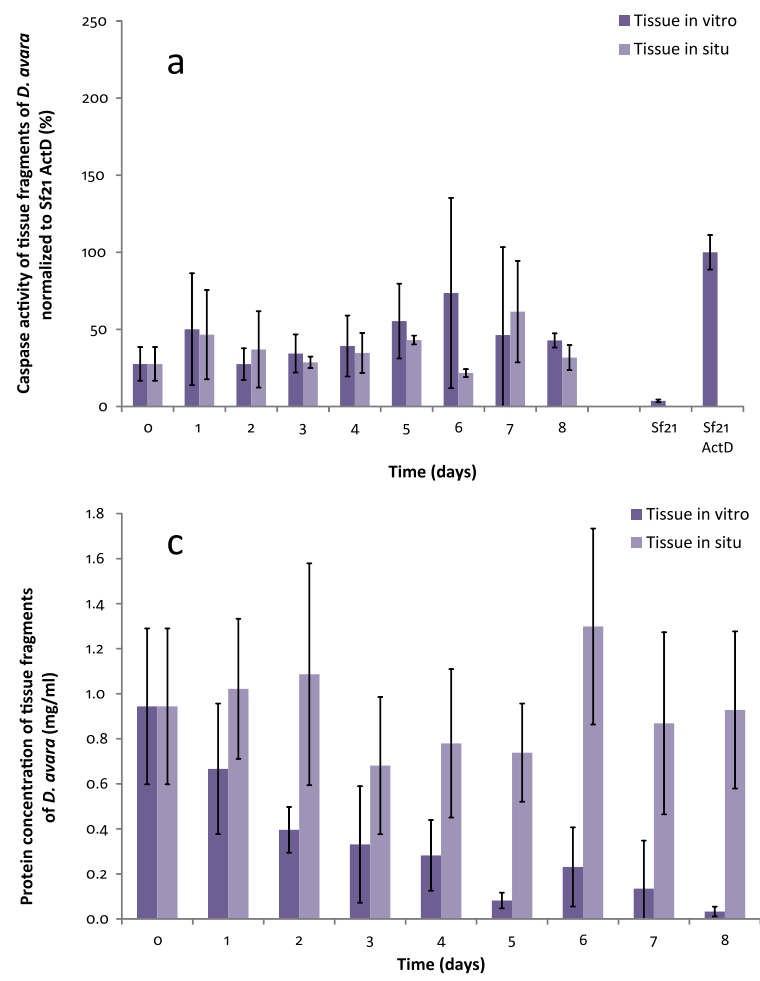
**Figure 2** Continued.



**Figure 3** Caspase activity per total protein content normalized with respect to ActD treated Sf21 cells during tissue (*in vitro*) and cell culture (*in vitro*) of *H. oculata*; error bars indicate standard deviation of triplicate measurements (three cultures).







**Figure 4** Caspase activity per total protein content normalized with respect to ActD treated Sf21 cells (a and b) and total protein content (c and d) of tissue fragments (*in vitro* and *in situ*) culture of *D. avara* (a and c) and *C. crambe* (b and d). Error bars indicate standard deviation of triplicate measurements (three cultures).

### 3.2 Sponge tissue culture (*in vitro* versus *in situ*) of *D. avara* and *C. crambe*

Caspase activities of tissue fragments from *D. avara* and *C. crambe* cultivated *in situ* (in the sea) and *in vitro* (in the lab) were determined (see Fig 4a and b). Since cell counts of tissue fragments are not accurate, the total protein content of the tissue fragments was measured over time (see Fig 4c and d) as an indicator of changes in

**Figure 4** Continued.

biomass. We assume a positive correlation between total protein content and cell numbers. Noticeable are the high standard deviations of the measurements, especially for samples from *C. crambe*, caused by the high variability between samples, not by the measurements.

The caspase activities of 'fresh' *D. avara* (see Fig 4a, day 0) were high in comparison to the caspase activities of 'fresh' *C. crambe* (see Fig 4b, day 0). During time, the

caspase activities of *D. avara* for both *in situ* and *in vitro* tissue fragments remained mostly constant (Fig 4a), whereas the caspase activities of *C. crambe* (*in situ* and *in vitro*) increased drastically in comparison to the starting material of *C. crambe* (Fig 4b). For both *D. avara* and *C. crambe* there was no significant difference in caspase activity per protein between *in situ* and *in vitro* culture. For *C. crambe* the total protein content declined both during *in vitro* and *in situ* cultivation (Fig 4d). However, for *D. avara* the total protein content during *in situ* cultivation remained constant, while it declined for *in vitro* cultivation (Fig 4c).

## 4 DISCUSSION

### 4.1 Sponge cell culture

For all three studied sponge species, dissociation had a negative effect on the viability of sponge cells, as demonstrated in our previous study (Schippers et al. 2011). Sponge fragments taken directly from a healthy *H. oculata* specimen (Fig 3, day 0 of *in vitro* tissue) exhibited a negligible caspase activity, which was comparable to healthy Sf21 cells. However, when *H. oculata* fragments were dissociated to create a monodisperse cell culture, the caspase activity drastically increased (Fig 3, day 0 of suspension cells versus day 0 of *in vitro* tissue). This increased caspase activity is an indicator of cell death, which was confirmed by the decreased cell concentration after one day of cultivation (Fig 1). We hypothesize that removing cells from their extracellular matrix (Frisch and Francis, 1994) and shear stress (Laken and Leonard, 2001) caused by centrifugation and resuspension are the main reasons for this increased cell death.

The cell numbers of *H. oculata* initially declined during cell culture, but remained constant after the first day with a minor negative trend (see Fig 1). The cell cycle distribution of *H. oculata* cell culture also showed a change in distribution of cells over time (see Fig 2a). At day 0, most cells reside in G1/G0 and the apoptotic fraction, but at day 3, the G1/G0 fraction is hardly visible and the apoptotic fraction is most dominant. However, at day 8 a new peak is observed with an increased cell size as based on the higher forward scatter, and a slightly higher red fluorescence. We did not observe contamination in the sponge cell cultures, which excludes that the peak is a population of microorganisms. Sponges contain different cell types with different sizes, for example, the small choanocytes and the larger archaeocytes and spherulous cells. Thus the peak observed at day 8 may be the G1/G0 peak of another population of cells with a larger size. It is not likely that it is the G2/M peak of the subpopulation, since for that the red fluorescence is too low and also G2/M fractions of this size are generally not found in sponge cells. The changes in peaks observed between day 3 and day 8 may be caused by small cells differentiating into larger

cells. However, it is more likely that the small sponge cells, such as choanocytes, were dying and disappeared from the culture, and that the larger sponge cells, such as archaeocytes and spherulous cells, remained intact. This last option agrees with the results obtained by De Goeij et al. (2009), who found that choanocytes have a very high cell turnover, meaning a high proliferation and death (apoptosis) rate. In our case no proliferation occurs and thus the choanocytes rapidly die, thus enriching the culture for the other cells like archaeocytes and spherulous cells.

Remarkably, cells from *D. avara* recovered after one day of cultivation, and cell numbers doubled in less than two days (see Fig 1). After day 3, the cell density remained constant within the margins of variation, which was as expected, since we did not add any extra nutrients to support cell growth. These results suggest that cells from *D. avara* have the potential to proliferate at a relatively high rate making this sponge a good candidate for further research on sponge cell culture.

The cell concentration of *C. crambe* continued to decrease during cell culture, indicating a massive mortality (see Fig 1). Possibly this mortality is caused by the release of toxins by *C. crambe* itself. The toxic compounds in *C. crambe* are encapsulated within spherules in specialized cells, thus preventing self-toxicity (Uriz et al., 1996a). However, due to shear stress during the dissociation process some cells break apart, possibly releasing the toxins in the culture media. We cannot explain why cytotoxic compounds that have been discovered in *D. avara* did not cause mortality in the cultures. It is possible that *D. avara* cells have self-protection mechanisms, or that the compounds are not toxic to sponge cells.

### 4.2 Sponge tissue culture: *in situ* versus *in vitro* for *D. avara* and *C. crambe*

To test the influence of *in vitro* conditions on tissue culture, we compared caspase activities and total protein content of *D. avara* and *C. crambe* tissue fragments during both *in vitro* and *in situ* cultivation conditions (Fig 4). We can conclude that there is a difference in starting material between both species. The caspase activity of 'fresh' *C. crambe* (Fig 4b, day 0) was almost nil, whereas 'fresh' *D. avara* (Fig 4a, day 0) already had significant caspase activity. This could indicate that *D. avara* was less healthy than *C. crambe* at time of harvest or that *D. avara* has by its nature a higher cell turnover and therefore also has higher caspase activities. We think that the latter option is the most plausible explanation, because both *D. avara* and *C. crambe* looked viable and healthy at the time of harvest. It is unlikely that timing of the reproductive cycle is a factor since *D. avara* releases larvae in July and *C. crambe* in August and sponge specimens were harvest in February.

During cultivation, the caspase activities of *D. avara* remained constant and comparable in both *in vitro* and *in situ* tissue culture (Fig 4a), which would indicate that both cultivation methods have the same impact on *D. avara*. However, visual



observations revealed that *D. avara* tissue fragments in *in vitro* culture were falling apart and deteriorating, whereas *D. avara* tissue fragments in *in situ* culture were still intact and healthy looking. Probably during *in vitro* tissue culture the majority of apoptotic cells were washed away when seawater was refreshed. This is confirmed by the results of the total protein content, which is an indication of the amount of biomass (see Fig 4c). The total protein content of *D. avara* tissue fragments *in vitro* decreased during time, while that of *D. avara* tissue fragments *in situ* remained constant during time (see Fig 4c). Since the tissue fragments at the start of the experiment were all similar in size, this indicates that in contrast to *in vitro* there was no loss of biomass *in situ*. A plausible explanation is that during *in situ* culture, tissue fragments were supplied with an unlimited amount of unfiltered seawater containing food particles and waste products were constantly flushed away. During *in vitro* culture, particulate food was not present and although we refreshed seawater daily, the dissolved components may have been rapidly depleted and waste products may have accumulated, resulting in degeneration of the fragments. In addition, the dissolved oxygen could have been limiting during *in vitro* cultivation. Gunda and Janapala (2009) observed that the levels of dissolved oxygen are important for the viability of sponge fragments. Calculations (data not shown) showed that the oxygen consumption rate of the sponge fragment (based on Thomassen and Riisgard, 1995) was in the same order of magnitude as the oxygen diffusion rate (from gas to liquid). Even though the size of the sponge fragment used was small (~15-20 mm<sup>3</sup>) compared to the culture volume (8000 mm<sup>3</sup>), the calculations indicate that dissolved oxygen could have been limiting when the sponge fragments would have actively pumped water. Previous studies have demonstrated that *D. avara* is a good candidate for *in situ* cultivation, due to its high regenerative capacity (De Caralt et al., 2010; Osinga et al., 2010). This study confirms the relative robustness of *D. avara* tissue culture *in situ*, since the biomass (measured as total protein content) did not decrease and the caspase activity did not increase during cultivation.

Tissue fragments of *C. crambe*, both *in vitro* and *in situ*, increased in caspase activity after 1 day cultivation (see Fig 4b). A possible explanation of this drastic increase in caspase activity could be the release of toxins by *C. crambe* due to disintegration of the spherulous cells caused by cutting of the sponge. Also, the total protein content for both *in vitro* and *in situ* tissue culture of *C. crambe* decreased during time (see Fig 4d), indicating a loss of biomass. Therefore, the cultivation conditions for *C. crambe* during *in vitro* as well as *in situ* cultivation were not favorable. This result is in contrast with the results obtained by Garcia Camacho et al. (2006) who demonstrated a 400% increase in biomass of *C. crambe* tissue fragments cultivated *in vitro*. However, *in vitro* culture conditions were not similar, since Garcia Camacho et al. (2006) used nutrient rich media (RPMI + *Octopus* extract), while we did not add any extra nutrients to support growth.

#### 4.3 Tissue versus cell culture

It is not possible to make a simple comparison between tissue and cell culture, since the results differ by species. Besides, cell culture data for *D. avara* and *C. crambe* are limited, since we only have cell concentration data of these species.

For *C. crambe* we can conclude that neither cell culture nor tissue culture was successful. During the cell culture experiment, *C. crambe* cells underwent a massive mortality (Fig 1) and during tissue culture (*in vitro* and *in situ*) the biomass also declined rapidly (Fig 4d).

For *H. oculata* we can make a more precise comparison, because cell cycle and caspase data of the cell culture experiment are available. When comparing caspase activities (Fig 3) of *H. oculata* during cell and tissue (*in vitro*) cultivation, we can conclude that after 1 day of cultivation the caspase activity of the tissue fragments (*in vitro*) increased from almost no caspase activity to a high caspase activity, comparable to that of the cell culture. While the caspase activities of the cell cultures remained high, the caspase activities of the tissue fragments (*in vitro*) showed a decline at day 3. This difference in caspase activity is supported by the cell cycle data (see Fig 2). The cell cycle distribution at day 3 during cell culture (Fig 2a, day 3) showed that the majority of the cells became apoptotic and the G1/G0 and G2/M fractions cannot be distinguished from each other anymore, whereas at day 3 during *in vitro* tissue culture (Fig 2b, day 3) still a G1/G0 fraction is clearly visible. These results suggest that during the first three days, tissue culture of *H. oculata* is more favorable than cell culture. However, this is only based on one time point and we should keep in mind that by refreshing the seawater during the tissue culture, apoptotic cells were washed away and new dissolved nutrients were added, which can give a false positive result. Therefore, based on this we cannot conclude that *in vitro* tissue culture is better than cell culture.

Cell cultures of *H. oculata* and *D. avara* are to a certain point quite comparable. After the first day there is a sharp decline in cell concentration after which *H. oculata* remains more or less constant and *D. avara* even increases again (Fig 1), whereas the biomass of *D. avara* tissue fragments cultivated *in vitro* only declined during time (Fig 4c). Initially, a population of cells dies rapidly during cell culture. Remaining cells can stay alive for longer times and even divide. This is supported by the appearance of a subpopulation of cells in *H. oculata*. These results show that in our experimental setup *D. avara in vitro* tissue culture is less favorable than cell culture. Furthermore, these results suggest that it may be worthwhile to focus on cell culture of enriched fractions of dissociated archaeocytes instead of a mixture of cells (Sun et al., 2007; Rinkevich, 2011; Pomponi and Willoughby, 1994).



## 5 CONCLUSIONS

The success of cell culture or tissue culture is species-dependent. Cell cultures and tissue cultures (*in vitro* and *in situ*) of *C. crambe* were all unsuccessful, probably caused by the release of toxins, while tissue culture (*in situ*) of *D. avara* was successful. For the studied sponges *in vitro* tissue culture did not result in better cell proliferation and viability than cells in primary suspension culture, as was hypothesized. For *D. avara in situ* tissue culture was more favorable than *in vitro* tissue culture, which is probably caused by the constant supply of nutrients, dissolved oxygen and flushing away of waste products.

Although no net growth was observed during cell culture, cell cycle data of *H. oculata* cell cultures indicate that a subpopulation of cells, possibly archaeocytes or spherulous cells, remained intact and did not become apoptotic, and cell cultures of *D. avara* showed evidence of dividing cells. This holds potential for development of primary cell cultures from these sponges.

Cell cycle data of *H. oculata* cell cultures indicate that a subpopulation of cells, possibly archaeocytes or spherulous cells, remained intact and did not become apoptotic, and there was evidence of dividing cells in primary cell cultures of *D. avara*. For future directions, we suggest to continue with *D. avara*, since this sponge has demonstrated to have proliferative capacities in cell culture and *in situ* tissue culture. To proceed with *in vitro* culture (cell or tissue), it will be important to first understand the factors that can explain the difference between growth *in situ* and the absence of growth and occurrence of cell death *in vitro*.

### Acknowledgements

This research was supported by IPOP Coast and Sea, Graduate School VLAG and Florida Sea Grant (R/LR-MB-25). We thank Corinne Geertsema and Gorben Pijlman from the Laboratory of Virology at Wageningen University for their help with the caspase and Bradford protein assays and providing the Sf21 cells. Also we would like to thank Iosune Uriz and Sonia De Caralt from Centre d'Estudis Avançats de Blanes for helping us with the collection of Mediterranean sponges.

## REFERENCES

- Blow, N., (2009) Cell culture: building a better matrix. *Nat Methods*, 6: 619-622.
- Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H.G., Northcote, P.T. and Prinsep, M.R. (2009) Marine natural products. *Nat Prod Rep*, 26: 170-244.
- Custodio, M.R., Prokic, I., Steffen, R., Koziol, C., Borojevic, R., Brummer, F., Nickel, M. and Muller, W.E.G. (1998) Primmorphs generated from dissociated cells of the sponge *Suberites domuncula*: a model system for studies of cell proliferation and cell death. *Mech Ageing Dev*, 105: 45-59.
- De Caralt, S., Sanchez-Fontenla, J., Uriz, M.J. and Wijffels, R.H. (2010) *In situ* aquaculture methods for *Dysidea avara* (Demospongiae, Porifera) in the northwestern Mediterranean. *Mar Drugs*, 8: 1731-1742.
- De Goeij, J.M., De Kluijver, A., Van Duyl, F.C., Vacelet, J., Wijffels, R.H., De Goeij, A., Cleutjens, J.P.M. and Schutte, B. (2009) Cell kinetics of the marine sponge *Halisarca caerulea* reveal rapid cell turnover and shedding. *J Exp Biol*, 212: 3892-3900.
- Freshney, R.I. (2005) *Culture of animal cells : a manual of basic technique*. Hoboken NJ: Wiley-Liss.
- Frisch, S.M. and Francis, H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol*, 124: 619-626.
- Garcia Camacho, F., Chileh, T., Ceron García, M.C., Sanchez Mirón, A., Belarbi, E.H., Contreras Gómez, A. and Molina Grima, E. (2006) Sustained growth of explants from Mediterranean sponge *Crambe crambe* cultured *in vitro* with enriched RPMI 1640. *Biotechnol Prog*, 22: 781-790.
- Gunda, V. and Janapala, V. (2009) Effects of dissolved oxygen levels on survival and growth *in vitro* of *Haliclona pigmentifera* (Demospongiae). *Cell Tissue Res*, 337: 527-535.
- Green, D., Howard, D., Yang, X., Kelly, M. and Oreffo R.O.C. (2003) Natural marine sponge fiber skeleton: a biomimetic scaffold for human osteoprogenitor cell attachment, growth, and differentiation. *Tissue Eng*, 9: 1159-1166.
- Hu, G.P., Yuan, J., Sun, L., She, Z.G., Wu, J.H., Lan, X.J., Zhu, X., Lin, Y.C. and Chen, S.P. (2011) Statistical research on marine natural products based on data obtained between 1985 and 2008. *Mar Drugs*, 9: 514-525.
- Koopmans, M. and Wijffels, R.H. (2008) Seasonal growth rate of the sponge *Haliclona oculata* (Demospongiae: Haplosclerida) *Mar Biotechnol*, 10: 502-510.
- Koziol, C., Borojevic, R., Steffen, R. and Muller, W.E.G. (1998) Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. *Mech Ageing Dev*, 100: 107-120.
- Laken, H.A. and Leonard, M.W. (2001) Understanding and modulating apoptosis in industrial cell culture. *Curr Opin Biotechnol*, 12: 175-179.
- Mendola, D., De Caralt, S., Uriz, M.J., Van Den End, F., Van Leeuwen, J.L. and Wijffels, R.H. (2008) Environmental flow regimes for *Dysidea avara* sponges. *Mar Biotechnol*, 10: 622-630.
- Muller, W.E.G., Bohm, M., Batel, R., De Rosa, S., Tommonaro, G., Muller, I.M. and Schroder, H.C. (2000) Application of cell culture for the production of bioactive compounds from sponges: synthesis of avarol by primmorphs from *Dysidea avara*. *J Nat Prod*, 63: 1077-1081.
- Osinga, R., Sidri, M., Cerig, E., Gokalp, S.Z. and Gokalp, M. (2010) Sponge aquaculture trials in the East-Mediterranean sea: new approaches to earlier ideas. *Open Mar Biol J*, 4: 74 – 81.
- Osinga, R., Tramper, J. and Wijffels, R.H. (1998) Cultivation of marine sponges for metabolite production: applications for biotechnology? *Trends Biotechnol*, 16: 130-134.
- Pomponi, S.A. (2006) Biology of the Porifera: cell culture. *Can J Zool*, 84: 167-174.
- Pomponi, S.A. and Willoughby, R. (1994). Sponge cell culture for the production of bioactive metabolites. IN Van Soest, R., Van Kempen, T.M.G., Braekman, J.C. (Eds.). *Sponges in time and space*. Rotterdam: A A Balkema, 395– 400.
- Pomponi, S.A. and Willoughby, R. (2000). Development of sponge cell cultures for biomedical applications. IN Austin, B., Mothersill, C. (Eds.) *Aquatic invertebrate cell culture*. London: Springer, 323-336.
- Rinkevich, B. (2011) Cell cultures from marine invertebrates: New insights for capturing endless stemness. *Mar Biotechnol*, 13: 345-354.

- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. (2011) Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol- Anim*, 47: 302-311.
- Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. (2012) Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Adv Mar Biol*, 62: 273-337.
- Sipkema, D., Franssen, M.C.R., Osinga, R., Tramper, J. and Wijffels RH (2005a) Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142-162.
- Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J. and Wijffels, R.H. (2005b) Large-scale production of pharmaceuticals by marine sponges: sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201-222.
- Sipkema, D., Van Wielink, R., Van Lammeren, A.A.M., Tramper, J., Osinga, R. and Wijffels, R.H. (2003) Primmorphs from seven marine sponges: formation and structure. *J Biotechnol*, 100: 127-139.
- Sipkema, D., Yosef, N.A.M., Adamczewski, M., Osinga, R., Mendola, D., Tramper, J. and Wijffels, R.H. (2006) Hypothesized kinetic models for describing the growth of globular and encrusting Demosponges. *Mar Biotechnol*, 8: 40-51.
- Sun, L.M., Song, Y.F., Qu, Y., Yu, X.J. and Zhang, W. (2007) Purification and *in vitro* cultivation of archaeocytes (stem cells) of the marine sponge *Hymeniacidon perleve* (Demospongiae). *Cell Tissue Res*, 328: 223-237.
- Thomassen, S. and Riisgard, H.U. (1995) Growth and energetics of the sponge *Halichondria panacea*. *Mar Ecol Prog Ser*, 128: 239-246.
- Uriz, M.J., Becerro, M.A., Tur, J.M. and Turon, X. (1996a) Location of toxicity within the Mediterranean sponge; *Crambe crambe*; (Demospongiae: Poecilosclerida). *Mar Biol*, 124: 583-590.
- Uriz, M.J., Turon, X., Galera, J. and Tur, J.M. (1996b) New light on the cell location of avarol within the sponge *Dysidea avara* (Dendroceratida). *Cell Tissue Res*, 285: 519-527.
- Valisano, L., Bavestrello, G., Giovine, M., Arillo, A. and Cerrano, C. (2006) Seasonal production of primmorphs from the marine sponge *Petrosia ficiformis* (Poiret, 1789) and new culturing approaches. *J Exp Mar Biol Ecol*, 337: 171-177.



# Chapter 5

Methods for insertion and expression of heterologous genes in sponge cells



## ABSTRACT

Three gene delivery systems, lipofection, particle bombardment and viral transduction, were tested in juveniles of the freshwater sponge *Ephydatia fluviatilis*, and lipofection was tested in primary cell cultures of the marine sponge *Haliclona oculata*. The green fluorescent protein (GFP) gene, under control of a cytomegalovirus (CMV) promoter, was used as reporter gene.

Neither viral transduction (lentivirus) nor particle bombardment resulted in heterologous gene expression. Lipofection, on the other hand, resulted in high expression levels of the heterologous gene in juveniles of *Ephydatia fluviatilis*, which indicates that the RNA polymerase of some freshwater sponges is able to recognize the CMV promoter. However, results were difficult to reproduce. The lipofection reagent was not toxic at the used concentrations and able to consistently deliver large amounts of a cell-impermeable fluorescent dye into sponge cells, suggesting that the lipofection reagent may also be able to deliver other macromolecules, such as DNA or RNA, into the cell.

**This chapter has been submitted for publication as:**

Schippers KJ, Geertsema C, Pijlman GP, Pomponi SA, Martens DE, Wijffels RH. Methods for insertion and expression of heterologous genes in sponge cells.

## 1 INTRODUCTION

Sponges are known for their production of bioactive compounds with pharmaceutical potential (Munro et al., 1999; Sipkema et al., 2005a; Blunt et al., 2010). However, application of these compounds as pharmaceuticals is hampered by the limited availability of sponge biomass and the lack of a well-defined and controllable production system (Osinga et al., 1999; Sipkema et al., 2005b). The development of *in vitro* sponge cell cultures for production of these bioactive compounds could solve this problem. However, until now all attempts to obtain a continuous sponge cell line have been unsuccessful (Pomponi, 2006; Rinkevich, 2005; Schippers et al., 2012).

Generally, continuous cell lines are obtained from spontaneously immortalisation in primary cell cultures (e.g., due to random mutations) or are derived directly from cancerous tissue (Freshney, 2005). However, spontaneous immortalization or cancerous tissue has not been observed with sponges. Two other approaches remain. One approach is the cultivation of sponge stem cells, which have unlimited proliferation capacity, by isolating these cells from larvae (De Caralt et al., 2007b), adults (e.g., archaeocytes), or by manipulating somatic cells to become induced pluripotent stem cells (Rinkevich, 2011).

The other approach towards developing a continuous cell line is the insertion and expression of immortalizing genes (e.g., SV40LT, hTERT) in sponge cells (Pomponi et al., 2007). Immortalizing genes interfere with the regulatory pathways for cell division and in this way cause unlimited cell division, resulting in a continuous cell line (Freshney, 2005). To successfully introduce and express heterologous genes (i.e., transfection) in sponge cells, the following requirements must be met: (i) metabolically active primary cells (preferably proliferating cells), (ii) a promoter that is recognized by the RNA polymerase of the sponge, (iii) an effective method to insert genes, and finally (iv) a method to obtain stable insertion of the genes in the sponge genome.

Obtaining metabolically active and proliferating cells is problematic, since, as demonstrated in previous studies, sponge cells *in vitro* generally lack the capacity for proliferation (Nickel et al., 2001; Schippers et al., 2011). Exceptions are freshwater sponge gemmules and marine sponge larvae, of which the cells can proliferate *in vitro* and grow into a juvenile sponge (Poirrier et al., 1981; De Caralt et al., 2007a). In this study, we used the proliferating cells from freshwater gemmules. These cells, termed thesocytes, have stem cell properties. After initiation of "hatching", the thesocytes become metabolically active, migrate out of the gemmule, and start to divide (Funayama et al., 2010; Loomis, 2010). These metabolically active cells are a good target for transfection. There are additional advantages to using gemmules as

source material for transfection. Microbial contamination, a persistent problem in sponge cell cultures (Sipkema et al., 2003), can be prevented or reduced, because gemmules have an outer coating, consisting of collagen layers with specialized spicules, that is resistant to weak solutions of disinfectant. Furthermore, thesocytes contain yolk platelets, which provide nutrition during the first stages of development. Thus, the addition of nutrients, which can also stimulate growth of contaminants, is initially not required (Loomis, 2010).

Insertion of genes can be accomplished by means of lipofection, electroporation, particle bombardment or by transduction (i.e., using viruses as a carrier). There are only two published reports of successful transfection of sponge cells: a patent application on methods for lipofection of primary cultures of marine sponges (Pomponi et al., 2007) and a publication about particle bombardment of a freshwater juvenile sponge (Pfannkuchen and Brummer, 2009). Thus, information on transfection of sponges is very limited.

The aim of this research was to compare different methods for the insertion and expression of heterologous genes in sponge cells as a first step towards development of an immortalized sponge cell line.

We tested three gene delivery systems, lipofection, particle bombardment and viral transduction, in juveniles of the freshwater sponge *Ephydatia fluviatilis*. In addition, lipofection was tested in primary cell cultures of the marine sponge *Haliclona oculata*. To visualize transfection efficacy, we used the green fluorescent protein (GFP) reporter gene. Two promoters were tested being the CMV (cytomegalovirus) and OplE2 (*Orgyia pseudotsugata* immediate-early 2) promoter, which are generally used for transfection of mammalian cells and insect cells, respectively.

Lipofection was chosen, since this method is known for its high efficiency and ability to transfect a wide range of cell types (Felgner et al., 1987) and because it was successfully used for insertion of immortalizing genes in cells of *Axinella corrugata* (Pomponi et al., 2007). Particle bombardment was chosen, since Pfannkuchen and Brummer (2009) successfully used this method to transfect juveniles of *Spongilla lacustris*. Transduction with a lentivirus was chosen, since a lentivirus is able to stably introduce a heterologous gene into a host cell's genome and is able to replicate in non-dividing cells, but has not yet been applied in sponge cells (Vigna and Naldini, 2000). Electroporation is also a technique which is highly efficient in mammalian cells, but due to practical limitations, such as compatibility with high salt concentrations (in case of *H. oculata*) or the use of adherent cells (in case of *E. fluviatilis*), we were not able to test this method on sponge cells.

## 2 MATERIAL AND METHODS

### 2.1 Juveniles of the freshwater sponge *Ephydatia fluviatilis*

Specimens of *E. fluviatilis*, which contained gemmules, were collected by scuba diving from Lake de Kuilen (near Mill) in The Netherlands and were transported in 50 ml Falcon tubes. The gemmules could be stored for several months at 4 °C in the dark. After storage, the viability was not affected, because gemmules always successfully hatched and developed into juvenile sponges. The gemmules were easily removed from the sponge tissue by gently squeezing the tissue. The outside of the gemmules was sterilized by soaking in 1% H<sub>2</sub>O<sub>2</sub> for 5 min. To remove all H<sub>2</sub>O<sub>2</sub>, the gemmules were washed several times with cold distilled water (Funayama et al., 2005). To start cultivation, the gemmules were transferred to M-medium (1mM CaCl<sub>2</sub>, 0.5 mM MgSO<sub>4</sub>, 0.5 mM NaHCO<sub>3</sub>, 0.05 mM KCl and 0.25 mM Na<sub>2</sub>SiO<sub>3</sub> (Rasmont, 1961)), placed in a 24-well plate and incubated at 21 °C. Each well contained ~ 10 gemmules in 1 ml M-medium. After approximately 5 days, the gemmules started "hatching" and cells were released and developed into juvenile sponges. After hatching, the medium was refreshed daily, without exposing the juveniles to air. Juvenile sponges were not fed during the experiments. Two days after hatching, transfection experiments were performed.

### 2.2 Primary sponge cell cultures of the marine sponge *Haliclona oculata*

Specimens of *H. oculata* were collected by scuba diving from the Easter Scheldt (Lokkersnol, Zierikzee) in The Netherlands and were transported in coolers, submerged in seawater that was constantly aerated. Upon arrival in the laboratory, specimens were immediately prepared for cell culture. Specimens of *H. oculata* were dissociated according to the protocol described by Schippers et al. (2011). The cell concentration was set to 10<sup>6</sup> cells/ml and the cells were cultured in 24-well plates (0.5 ml per well) and incubated at 8 °C, the same temperature at which they were collected. After one day incubation, transfection experiments were performed.

### 2.3 HEK293T and Sf21 cells

Human embryonic kidney 293T (HEK293T) adherent cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin in T25 cm<sup>2</sup> tissue culture flasks at 37 °C with 5% CO<sub>2</sub>. *Spodoptera frugiperda* (Sf21) cells were grown in Grace's insect culture medium supplemented with 10% FBS in T25 cm<sup>2</sup> tissue culture flasks at 27 °C. Both cell lines were sub-cultured every 3 days. For transfection experiments, cells were sub-cultured in 24 well plates (0.5 ml per well). HEK293T cells were cultured without antibiotics and Sf21 cells were cultured without FBS. When cells achieved a confluency of 50-90%, transfection was performed.

## 2.4 Particle bombardment

For the particle bombardment, gemmules of *E. fluviatilis* were kept in small petri dishes (Ø 60 mm) in M-medium at 21 °C. Two days after hatching, particle bombardment was performed in a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad, USA). As a positive control, we also applied particle bombardment on HEK293T cells grown in a petri dish (Ø 60 mm). Culture conditions of HEK293T cells are described in section 2.3. The distance of the bombardment was approximately 5 cm, and we used 450 psi rupture disks and macrocarriers from Bio-Rad (Pfannkuchen and Brummer, 2009). Before covering the 1 µm gold particles with plasmid, they were washed three times in abs EtOH and dissolved in sterile ddH<sub>2</sub>O. Next, 50 µl of the washed particles (60 mg/ml) were covered with pEGFP-N1 plasmid (Clontech, a vector with enhanced green fluorescent protein (EGFP) under control of a CMV promoter), by first adding 5 µl of plasmid (1 µg/µl), followed by the addition of 50 µl CaCl<sub>2</sub> (2.5 M) and finally by adding 20 µl of spermidine (0.1 M) (Sigma-Aldrich). In between the additions, the solution was vortexed. Before transferring the gold particles to the macrocarrier, they were washed with abs EtOH. We used approximately 0.3 mg of covered gold particles for each bombardment and each petri dish was bombarded three times. To visualize the presence of gold particles in the sponge tissue we used an inverted fluorescence microscope (Olympus IX-71). To observe the presence of the green fluorescent protein, we used the same microscope, but with a U-MWIBA3 mirror unit.

## 2.5 Viral transduction

Juveniles of *E. fluviatilis* were infected with a lentivirus expressing a GFP reporter gene. As a positive control, we also transduced HEK293T cells. To 500 µl of juveniles or cells, 100 µl lentivirus was added. The presence of the green fluorescent protein was visualized using a U-MWIBA3 mirror unit in an inverted fluorescence microscope (Olympus IX-71). To examine if the lentivirus integrated its cDNA into the genome of the host, we performed PCR on genomic DNA of *E. fluviatilis* and HEK293T cells with primers (FPLV15'-GGACTTGAAAGCGAAAGGG-3' and RPLV15'-CATCTCTCTCCTC-TAGCCTCCG-3') specific for lentiviral cDNA (Sastri et al., 2002). Genomic DNA was extracted using the FastDNA Spin Kit for Soil (Q-Biogene). For PCR analysis, 1 µl (~10 ng) of genomic DNA was mixed with 19 µl of PCR master mix, consisting of 4 µl Go Taq buffer (5x), 1.25 c MgCl<sub>2</sub> (25 mM), 0.25 µl of dNTP's (10mM), 0.1 µl of forward primer (50 µM), 0.1 µl of reverse primer (50 µM) and 0.05 µl of Go-Taq DNA polymerase. Reactions were performed using an initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 50 s, annealing at 60°C for 50 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were analyzed by electrophoresis at 90 V for 20 min in a 1% agarose gel in TAE buffer.

## 2.6 Lipofection

### 2.6.1 Plasmid DNA delivery with Lipofectamine2000

Plasmids used were pEGFP-N1 (Clontech), a vector with enhanced green fluorescent protein (EGFP) under control of a CMV promoter, and pIZ-GFP, a vector with green fluorescent protein (GFP) under control of an OplE2 promoter (Theilmann and Stewart, 1992). Transfection with these plasmids was tested in juveniles of *E. fluviatilis*, primary cell cultures of *H. oculata*, HEK293T cells and Sf21 cells. A few hours before transfection, the medium was refreshed. To transfect 500 µl of cells or juveniles, 800 ng of DNA plasmid and 2 µl of Lipofectamine2000 was needed. First, one Eppendorf vial with 800 ng of DNA plasmid and 50 ml Optimem (Invitrogen) and another Eppendorf vial with 2 µl of Lipofectamine2000 and 50 µl Optimem were incubated for 5 min at room temperature. After 5 min, the content of the two vials were combined and gently mixed, and this DNA-Lipofectamine2000 mix was incubated for 20 min at room temperature. Next, the DNA-Lipofectamine2000 mix was added drop wise to the cells or juveniles. The presence of the green fluorescent protein was visualized using a U-MWIBA3 mirror unit in an inverted fluorescence microscope (Olympus IX-71).

### 2.6.2 Toxicity test: Lipofectamine2000

To test the toxicity effect of Lipofectamine2000 (Invitrogen) on sponge cells, we exposed juveniles (~ 10 gemmules in 500 µl) to a concentration range of Lipofectamine2000 (0, 0.4, 1, 2, 4, 8 µl) and measured the viability of the juveniles after 3, 24 and 48 hours. Viability was measured by adding 5 µl of fluorescein diacetate (FDA, 11 µM), which is an indicator of metabolic activity, and 5 µl propidium iodide (PI, 1.5 µM), which stains DNA of dead cells with a permeable membrane, to 500 µl of juveniles (Sipkema et al., 2004). FDA and PI were visualized using a U-MWIBA3 and a U-MWIG3 mirror unit respectively in an inverted fluorescence microscope (Olympus IX-71).

### 2.6.3 Delivery of cascade blue with Lipofectamine2000

The cell-impermeable fluorescent dye, cascade blue (Invitrogen) was used to investigate the fusion of Lipofectamine2000 with the cells of juveniles of *E. fluviatilis*. To optimize the visualization, juveniles were cultivated in 24 well glass-bottom microplates (Greiner Bio-One, Cat.# 662892). The lipofection protocol required 5 µl of cascade blue (20 mM) and 2 µl of Lipofectamine2000. The rest of the protocol was similar to plasmid DNA transfection as described in section 2.6.1. As a negative control, we also incubated juveniles with only cascade blue. After 3 hours of incubation with the cascade blue-Lipofectamine2000 mix and only the cascade blue, the juveniles were washed thoroughly with M-medium. The insertion of cascade blue into sponge cells was first visualized using a DAPI filter in an inverted fluorescence

microscope (Olympus IX-71). To obtain a more detailed image, visualization with a Zeiss Confocal LSM (excitation 405 nm) was performed.

#### 2.6.4 Delivery of mRNA with Lipofectamine2000

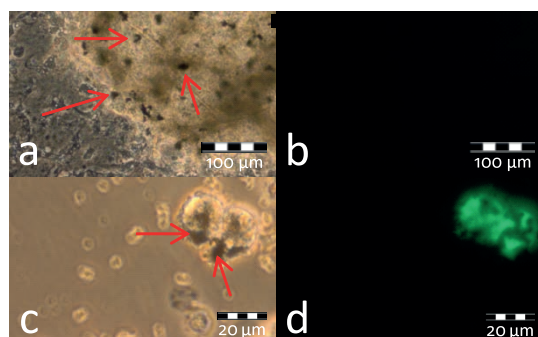
Stemgent eGFP mRNA, (Miltenyi Biotec, Leiden), which encodes green fluorescent protein, was used as a positive control for transfection of juveniles of *E. fluviatilis* and HEK293T cells to determine if cells are able to take up mRNA and translate this mRNA into a protein. A few hours prior to transfection, the medium was refreshed. To transfect 500  $\mu$ l of juveniles or HEK293T cells, 250 ng of eGFP mRNA and 1  $\mu$ l of Lipofectamine2000 was needed. The rest of the protocol was similar to plasmid DNA transfection as described in section 2.6.1.

### 3. RESULTS

#### 3.1 Particle bombardment

Particle bombardment was originally designed for plant transformation, but there is also potential for use in animal tissue and cells (Yang et al., 1990, Pfannkuchen and Brummer, 2009). Gold particles covered with DNA enter the cells by force, caused by high pressure helium gas in the Particle Delivery System. We tested this method on juveniles of *E. fluviatilis* and used gold particles coated with pGFP-N1 (GFP under control of CMV promoter). Gold particles were present inside the sponge tissue (Fig. 1a), but expression of GFP was not observed (Fig 1b).

To confirm that our method was being performed correctly, we also applied particle bombardment to HEK293T cells (Fig 1c-d). Although the method was quite harmful

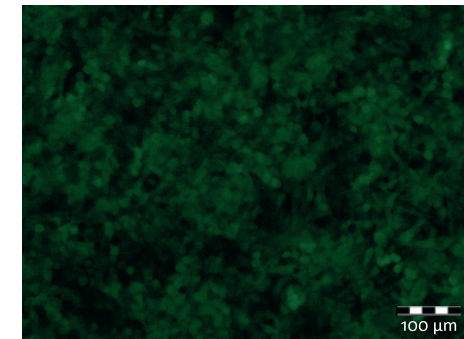


**Figure 1** Particle bombardment of juveniles of *E. fluviatilis* (a and b) and HEK293T cells (c and d) with gold particles (red arrows). A and c light micrograph; b and d fluorescence micrograph.

for HEK293T cells (and resulted in a lot of cell debris), there was expression of GFP (Fig 1d), indicating that, in principle, the method works.

#### 3.2 Viral transduction with lentivirus

To test viral transduction in sponge cells, we infected juveniles of *E. fluviatilis* with a lentivirus expressing the fluorescent marker. As a positive control we also transduced HEK293T cells. After 3 days, HEK293T cells expressed GFP (Fig 2), while juveniles of *E. fluviatilis* did not express GFP, even after 10 days (data not shown).



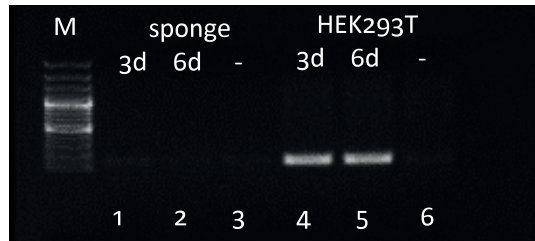
**Figure 2** Fluorescence micrograph of positive control HEK293T cells expressing GFP after 3 days of transduction with lentivirus.

For expression of the GFP, the viral vector must be inserted in the genome. To examine the presence of the viral genome in the host DNA, we performed PCR analysis on genomic DNA with primers specific for the lentiviral genome. Samples of *E. fluviatilis* and HEK293T cells were taken after 3 and 6 days of transduction. There will be a PCR product only if the viral genome is inserted in the host DNA. There was a PCR product for the lentiviral genome in HEK293T cells (Fig 3), but not for sponge cells, indicating that the lentiviral genome is not inserted in the sponge genome.

#### 3.3 Lipofection with DNA plasmid

The transfection efficacy of two plasmid vectors was tested in HEK293T cells, Sf21 cells, primary cells of *H. oculata* and juveniles of *E. fluviatilis*. Plasmids used were pEGFP-N1, a vector with enhanced green fluorescent protein (EGFP) under the control of a CMV promoter, and pIZ-GFP, a vector with green fluorescent protein (GFP) under control of an OpIE2 promoter. Table 1 summarizes the transfection results.





**Figure 3** PCR results of lentivirus transduction. 1: sponge juvenile incubated with lentivirus for 3 days, 2: juvenile incubated for 6 days, 3: juvenile without lentivirus, 4: HEK293T incubated with lentivirus for 3 days, 5: HEK293T incubated for 6 days, 6: HEK293T without lentivirus. M is 100 bp DNA ladder.

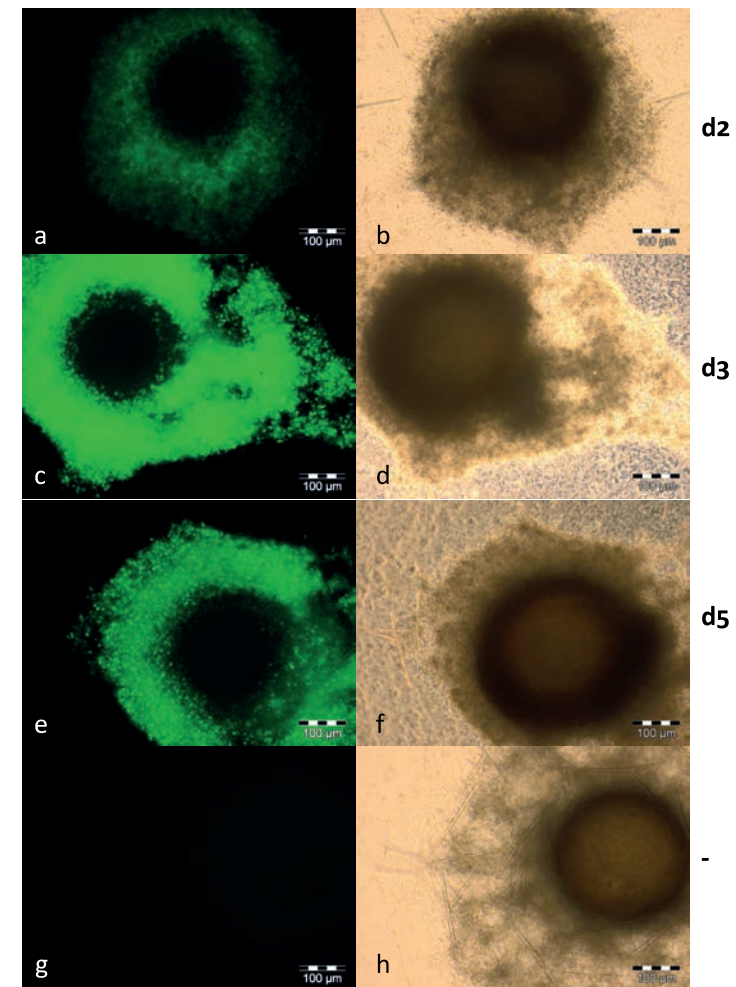
**Table 1** Transfection efficacy results of lipofection with two different plasmids. (+++ 80-100%, ++ ~50%, + ~5%, +/- inconsistent, - 0%, transfection efficacy).

Plasmid	Promoter	Reporter gene	Transfection efficacy:			
			HEK293T	Sf21	<i>H. oculata</i>	<i>E. fluviatilis</i>
pIZ-GFP	OpIE2	GFP	+	++	-	-
pEGFP-N1	CMV	EGFP	+++	-	-	+/-

The OpIE2 promoter is derived from a baculovirus and allows protein expression in a wide range of insect cell lines (Pfeifer et al., 1997). We tested this promoter in both insect cells (Sf21) and mammalian cells (HEK293T). The GFP expression in Sf21 cells was significantly higher than in HEK293T cells, but there was also some expression of GFP in HEK293T cells, which suggests that the OpIE2 promoter has universal eukaryotic properties. Nevertheless, the OpIE2 promoter did not result in GFP expression in juveniles of *E. fluviatilis*.

The CMV promoter is derived from a herpes virus and allows for high-level protein expression in a wide variety of mammalian cell lines (Foecking and Hofstetter, 1986). We tested this promoter in both HEK293T and Sf21 cells. Whereas in HEK293T cells the expression levels of GFP were extremely high (nearly 100% of the cells showed fluorescence), there was no expression in Sf21 cells, suggesting that the CMV promoter is only operational in mammalian cell lines. However, also high levels of GFP expression were observed using the CMV promoter in juveniles of *E. fluviatilis* (see Fig 5). Three days after transfection, the juveniles expressed high levels of GFP and even after 5 days, the expression levels were still high. These results indicate that it is possible to insert DNA into sponge cells using lipofection and that sponge-RNA

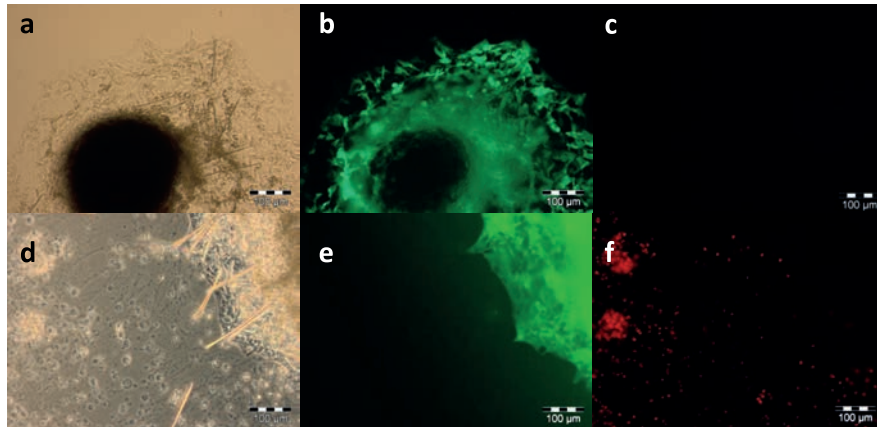
polymerase is able to recognize the CMV promoter, resulting in high expression levels of the reporter gene, GFP. However, results have been inconsistent: we have been able to demonstrate transfection in juveniles in only 2 out of 20 attempts. The plasmids with CMV and OpIE2 promoter have also been tested in primary cell cultures from *H. oculata*, but transfection was unsuccessful.



**Figure 4** Transfection of juvenile *E. fluviatilis* with GFP under control of CMV promoter. Pictures on the left show green fluorescence of the GFP expressed by the juvenile sponge. Pictures on the right are the same image, but visualized with phase contrast light microscopy. 4a and 4b show day 2 of transfection; 4c and 4d, day 3; 4e and 4f, day 5; 4g and 4h, negative control, non-transfected juvenile sponges.

### 3.4 Toxicity of Lipofectamine2000 in juveniles of *E. fluviatilis*

We tested the toxicity of Lipofectamine2000 on juveniles of *E. fluviatilis* by observing the morphology and measuring the viability by using FDA and PI. Green fluorescence of the FDA is an indicator of metabolic activity of viable cells, and red fluorescence of PI-stained DNA indicates that the cell membranes are permeable and, thus, not viable (Sipkema et al., 2004). Juveniles were exposed to a concentration range of Lipofectamine2000 (0, 0.4, 1, 2, 4, 8  $\mu$ l) and viability was measured after 3, 24 and 48 hours. We only measured a toxic effect on the juveniles, which were exposed to 8  $\mu$ l Lipofectamine2000. These juveniles showed some deterioration after 48 hours (see Fig 5d,e,f). Figure 1d shows that cells withdrew from the growth marginal layer (e.g., compare with Fig 5a) and accordingly Figure 6f shows dead cells stained with PI in the area from which the sponge cells. However, remaining cells in the interior of the sponge were still metabolically active (see Fig 5e).



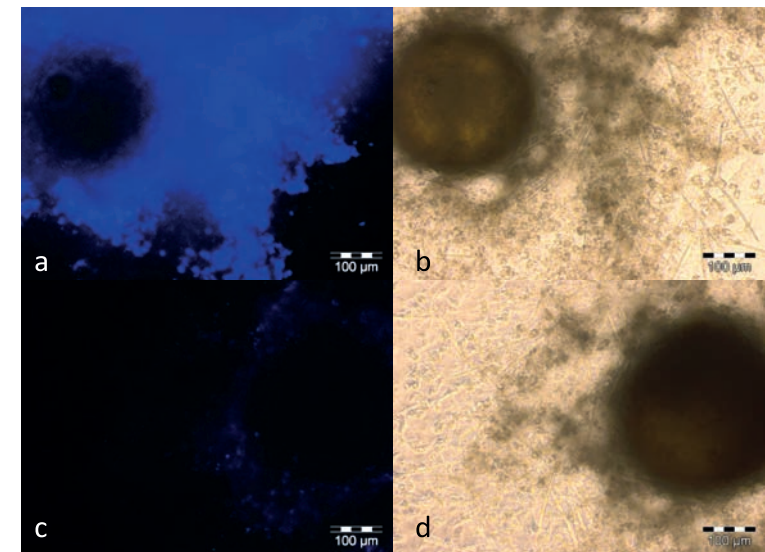
**Figure 5** Effect of Lipofectamine2000 on morphology and viability of juvenile of *E. fluviatilis*. 5a, 5b and 5c show the effect of 2  $\mu$ l Lipofectamine2000 after 48 hours. 5d, 5e and 5f show the effect of 8  $\mu$ l Lipofectamine2000 after 48 hours. 5a and 5d show the light micrograph, 5b and 5e show metabolically active cells stained with FDA and 5c and 5f show dead cells stained with PI.

Juveniles exposed to 2  $\mu$ l of Lipofectamine2000 were still viable after 48 hours and no dead cells were detected (see Fig 5a,b,c). Our results indicate that toxic effects of Lipofectamine2000 only occurred at the highest concentration (i.e., 8  $\mu$ l) and not at the concentration we selected for transfection (i.e., 2  $\mu$ l).

### 3.2 Confirmation of lipofection with cascade blue

The cell-impermeable fluorescent dye, cascade blue, was used to investigate the fusion of Lipofectamine2000 with the cells of juveniles of *E. fluviatilis*. Cascade blue

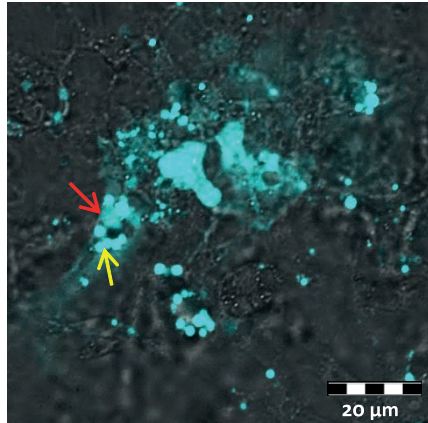
will only be taken up by the cells if the membranes become permeabilized (i.e., if the cells are dead) or if complexed with liposomes that will fuse with the cell membrane (i.e. lipofection). Therefore we used this dye to trace if Lipofectamine2000 was able to deliver macromolecules into sponge cells and thus in principle could be used to insert DNA or RNA into sponge cells. Figure 6a shows that most sponge cells fused with the Lipofectamine2000-cascade blue complex, whereas Figure 6c shows that without addition of Lipofectamine2000 (negative control), some cells took up cascade blue, but this is negligible when compared to the results for the Lipofectamine2000-cascade blue complex (Fig 6a).



**Figure 6** Lipofection of juvenile sponge with cascade blue, a cell-impermeable fluorescent dye. 6a and 6b show juvenile exposed to Lipofectamine2000-cascade blue complex; 6c and 6d is the negative control, juvenile exposed to cascade blue without Lipofectamine2000. 6a and 6c show blue fluorescence of the cascade blue taken up by the sponge. 6b and 6d are the same image as 6a and 6b, but visualized with phase contrast light microscopy.

To confirm that the cascade blue was inside the cell and not just attached to the outside, we also visualized up-take of cascade blue with confocal laser scanning microscopy (CLSM). The CLSM acquires a high-resolution image at various depths ("z-stack") of the specimen, allowing us to visualize if the cascade blue is inside or on the outside of the cell. Figure 7 shows a detailed image of a few sponge cells which have spread on the bottom of the well plate. The sponge cells contain several inclusion bodies with cascade blue, indicating that the cascade blue is inside the cell

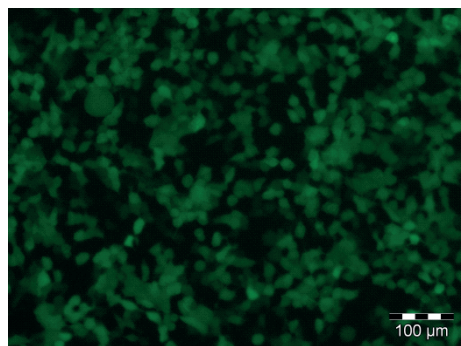
and not on the outside. These results confirm that Lipofectamine2000 is able to deliver macromolecules into sponge cells.



**Figure 7** CLSM image of sponge cells with cascade blue inside the cell. Red arrow indicates a cell membrane and yellow arrow indicates inclusion bodies with cascade blue.

### 3.4. Lipofection with mRNA eGFP

To answer whether problems with reproducibility of DNA transfection were a result of promoter recognition issues (transcription) or expression of mRNA (translation), we transfected juveniles of *E. fluviatilis* with eGFP mRNA. As a positive control, we also transfected HEK293T cells. When transfecting with mRNA instead of DNA, the



**Figure 8** Fluorescence micrograph of positive control HEK293T cells expressing GFP after 1 day of transfection with eGFP mRNA.

transcription step (DNA->mRNA) is avoided and only translation (mRNA-> protein) has to take place in order to express GFP. For HEK293T cells the expression levels of GFP were very high (see Fig 8), however for juveniles of *E. fluviatilis* no expression of GFP could be observed (data not shown).

## 4 DISCUSSION

We have conducted a comprehensive comparison of methods for insertion and expression of heterologous genes in both freshwater and marine sponges as a first step towards development of an immortalized sponge cell line.

### 4.1 Lipofection in primary cells from marine sponges

First, we tested lipofection with plasmid DNA (pEGFP-N1 and pIZ-GFP) in primary cell cultures of *H. oculata*, which was unsuccessful. One possible explanation is that Lipofectamine2000 does not deliver the DNA plasmid into the marine sponge cells due to the high amount of salts in the seawater. The salts could have interfered with the negatively charged DNA-Liposome complex and prevent fusion of the complex with the cell membrane. However, Pomponi et al. (2007) successfully applied lipofection to express heterologous genes in a primary cultures of a marine sponge, which was also in salt water. Another possibility could be that the primary cell culture from *H. oculata* was not viable. Generally, the higher the viability of the culture, the better the transfection efficiency will be. From previous results, we know that *H. oculata* cells in culture were non-proliferating and became apoptotic (Schippers et al., 2011). This lack of proliferation was a reason to change target species and test gene delivery methods in juveniles of *E. fluviatilis*, which are adherent and have the capacity to proliferate *in vitro*.

### 4.2 Comparing three gene delivery methods in freshwater sponges

Subsequently, we tested three gene delivery methods (particle bombardment, viral transduction and lipofection) in juveniles of *E. fluviatilis*.

To test particle bombardment in juveniles of *E. fluviatilis*, we used the same method as Pfannkuchen and Brummer (2009). They were able to successfully apply this method for juveniles of *S. lacustris* and were able to demonstrate expression of DsRedN1 under control of a CMV promoter. We were also able to deliver gold particles coated with DNA (pEGFP-N1) into the cells of *E. fluviatilis* (see Fig 1a). However expression of GFP was not observed. A possible explanation for this lack of GFP expression could be that the CMV promoter is not recognized by *E. fluviatilis*. However, results obtained with lipofection in this paper rebut this hypothesis (see Fig 4).



Another possibility could be that particle bombardment, which is a harsh method, decreased the viability of the juveniles and, with that, the expression of the gene.

To test viral transduction in sponge cells, we infected juveniles of *E. fluviatilis* with a lentivirus expressing the fluorescent marker GFP. This method was expected to be promising, since lentivirus is able to stably introduce a heterologous gene into a host cell's genome and is able to replicate in non-dividing cells (Vigna and Naldini, 2000). However, we found that lentivirus was not able to integrate its genome into the genome of the sponge cells (see Fig 3). Therefore, we consider lentiviral transduction not a feasible gene delivery method for sponge cells.

The most successful gene delivery method tested in *E. fluviatilis* was lipofection. Transfection with plasmid DNA (pEGFP-N1), by means of lipofection, gave a positive result in *E. fluviatilis* and the reporter gene GFP was highly expressed under control of a CMV promoter (see Fig 4). This is in agreement with the results obtained by Pfannkuchen and Brummer (2009), who were able to express DsRed2 under control of a CMV promoter in juveniles of *S. lacustris*. However, our results have been difficult to reproduce, with only two successful attempts out of a total of 20. To determine the reason for this lack of reproducibility, we first tested the possible toxicity of Lipofectamine2000 on sponge cells. Lipofectamine2000 did not affect the viability of sponge cells within the concentration range we used for lipofection (see Fig 5). Another explanation could be that Lipofectamine2000 not always efficiently delivered DNA into the sponge cells. However, Lipofectamine2000 was able to reproducibly deliver large amounts of the cell-impermeable fluorescent dye, cascade blue, into cells from *E. fluviatilis* (see Fig 6 and 7), which suggests that Lipofectamine2000 is also able to deliver other macromolecules (such as RNA and DNA) into sponge cells. Since lipofection with cascade blue always gave a positive result, the inconsistent transfection results are likely not related to the lipofection method. Another possibility is that the CMV promoter was not always recognized by the sponge RNA polymerase. To test this hypothesis, we transfected juveniles with mRNA eGFP. When the mRNA is delivered into the cell by lipofection, the mRNA only has to be translated by the ribosomes to produce the desired protein. If this would lead to expression of GFP, it would confirm our hypothesis that the CMV promoter is not always recognized. However, transfection with mRNA eGFP in *E. fluviatilis* did not result in GFP expression. Therefore, it is still unclear what the exact bottleneck is. Nevertheless, the two positive transfection results (Fig 4) indicate that it is possible to transfect sponge cells, but that there are other factors involved that we do not understand yet.

### 4.3 Concluding remarks

By comparing these three gene delivery methods in freshwater sponge cells we conclude that lipofection is the most effective gene delivery method. Although

particle bombardment is also able to deliver DNA into sponge cells, the number of cells expressing GFP in *E. fluviatilis* obtained with lipofection were significantly higher than the number of cells expressing DsRed2 in *S. lacustris* as demonstrated by Pfannkuchen and Brummer (2009). However, this does not preclude exploring other gene delivery methods, especially for marine sponges. Other alternatives to lipofection could be the use of Poly-APS, which is a pore forming salt (extracted from a marine sponge), which has been used to deliver macromolecules into cells (McLaggan et al., 2006), or the use of supercharged proteins, which are able to penetrate a variety of mammalian cell lines, including cell lines that are resistant to lipofection (McNaughton et al., 2009). With respect to transduction, baculoviruses may be a viable alternative, since baculoviruses can transduce and deliver DNA into both insect cells and mammalian cells (Hu, 2005). It may also be possible to insert foreign DNA by simply feeding the juveniles with bacteria containing the DNA plasmid. Rivera et al. (2011) recently demonstrated silencing of the actin gene in juveniles of *Ephydatia muelleri* by feeding the sponge dsRNA expressing bacteria.

Besides an effective gene delivery method, metabolically active cells are also important for successful transfection. We found that juveniles of freshwater sponges are a good target for transfection experiments, due to their proliferative capacity *in vitro* and the ability of the sponge cells to fuse with the lipofection reagent in order to take up macromolecules, such as DNA or RNA.

Another important aspect of a successful transfection is a strong promoter which is recognized by the sponge. Results in this and one other study (Pfannkuchen and Brummer, 2009) indicate that freshwater sponges (*E. fluviatilis* and *S. lacustris*) are able to recognize the CMV promoter. However, the use of a specific sponge promoter may lead to better expression of the heterologous genes.

Other improvements may come from insights in codon usage in sponge cells and codon optimization of the gene of interest, based on the recently sequenced genome of *Amphimedon queenslandica* (Srivastava et al., 2010). This could also result in the discovery of specific sponge promoters, which may lead to better expression of genes, although our results indicate that the CMV promoter is effective in freshwater juvenile sponges.

Although we do not yet understand all factors that are affecting the transfection results, based on our data, we can now identify suitable target sponges (i.e., juveniles of freshwater sponges), gene delivery method (i.e., lipofection), and promoter (i.e., cytomegalovirus) for continued research on development of a transformed sponge cell line. When expression of heterologous genes in sponges is possible, many steps

will still need to be taken to obtain an immortalized, continuously dividing, sponge cell line, e.g. selecting immortalizing genes, obtaining stable expression, and optimizing culture conditions (including appropriate nutrient media).

### Acknowledgements

This research was supported by IPOP Coast and Sea, Graduate School VLAG and Florida Sea Grant (R/LR-MB-25). We thank Esther Schnettler for her help with transfection of HEK293T cells, Nico Taverne for his assistance during the collection of freshwater gemmules, Julia Eekels for her help with the lentivirus transduction, Bernadette van Kronenburg for her help with the particle bombardment and Norbert de Ruijter for taking the CLSM images.

### REFERENCES

- Blunt, J.W., Copp, B.R., Munro, M.H.G., Northcote, P.T. and Prinsep, M.R. (2010). Marine natural products. *Nat Prod Rep*, 27: 165-237.
- De Caralt, S., Otjens, H., Uriz, M. and Wijffels, R. (2007a). Cultivation of sponge larvae: settlement, survival, and growth of juveniles. *Mar Biotechnol*, 9: 592-605.
- De Caralt, S., Uriz, M.J. and Wijffels, R.H. (2007b). Cell culture from sponges: pluripotency and immortality. *Trends Biotechnol*, 25: 467-471.
- Felgner, P.L., Gadek, T.R., Holm, M., Roman, R., Chan, H.W., Wenz, M., Northrop, J.P., Ringold, G.M. and Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA*, 84: 7413-7417.
- Foecking, M.K. and Hofstetter, H. (1986). Powerful and versatile enhancer-promoter unit for mammalian expression vectors. *Gene*, 45: 101-105.
- Freshney, R.I. (2005). *Culture of animal cells : a manual of basic technique*, Hoboken, NJ: Wiley-Liss.
- Funayama, N., Nakatsukasa, M., Hayashi, T. and Agata, K. (2005). Isolation of the choanocyte in the fresh water sponge, *Ephydatia fluviatilis* and its lineage marker, Ef annexin. *Dev Growth Diff*, 47: 243-253.
- Funayama, N., Nakatsukasa, M., Mohri, K., Masuda, Y. and Agata, K. (2010). Piwi expression in archeocytes and choanocytes in demosponges: insights into the stem cell system in demosponges. *Evol Dev*, 12: 275-287.
- Hu, Y.C. (2005). Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol Sin*, 26: 405-416.
- Loomis, S.H. (2010). Diapause and estivation in sponges. *Prog Mol Subcell Biol*, 49: 231-43.
- Mclaggan, D., Adjimatera, N., Sepcic, K., Jaspars, M., Macewan, D., Blagbrough, I. and Scott, R. (2006). Pore forming polyalkylpyridinium salts from marine sponges versus synthetic lipofection systems: distinct tools for intracellular delivery of cDNA and siRNA. *BMC Biotechnol*, 6: 6.
- Mcnaughton, B.R., Cronican, J.J., Thompson, D.B. and Liu, D.R. (2009). Mammalian cell penetration, siRNA transfection, and DNA transfection by supercharged proteins. *Proc Natl Acad Sci USA*, 106: 6111-6116.
- Munro, M.H.G., Blunt, J.W., Dumdei, E.J., Hickford, S.J.H., Lill, R.E., Li, S., Battershill, C.N. and Duckworth, A.R. (1999). The discovery and development of marine compounds with pharmaceutical potential. *J Biotechnol*, 70: 15-25.
- Nickel, M., Leininger, S., Proll, G. and Brummer, F. (2001). Comparative studies on two potential methods for the biotechnological production of sponge biomass. *J Biotechnol*, 92: 169-178.
- Osinga, R., Tramper, J. and Wijffels, R.H. (1999). Cultivation of marine sponges. *Mar Biotechnol*, 1: 509-532.
- Pfannkuchen, M. and Brummer, F. (2009). Heterologous expression of DsRed2 in young sponges (Porifera). *Int J Dev Biol*, 53: 1113-1117.
- Pfeifer, T.A., Hegedus, D.D., Grigliatti, T.A. and Theilmann, D.A. (1997). Baculovirus immediate-early promoter-mediated expression of the Zeocin™ resistance gene for use as a dominant selectable marker in Dipteran and Lepidopteran insect cell lines. *Gene*, 188: 183-190.
- Poirrier, M.A., Francis, J.C. and Labiche, R.A. (1981). A continuous-flow system for growing fresh-water sponges in the laboratory. *Hydrobiologia*, 79: 255-259.
- Pomponi, S.A. (2006). Biology of the Porifera: Cell culture. *Can J Zool*, 84: 167-174.
- Pomponi, S.A., Thompson, J., Bottesch, J.H. and Sennett, S.H. (2007). Methods for extending the life span of marine invertebrate cells. *US patent number 20070254367*.
- Rasmont, R. (1961). Une technique de culture des éponges d'eau douce en milieu contrôlé. *Ann Soc R Zool Belg*, 91: 147-156.
- Rinkevich, B. (2005). Marine invertebrate cell cultures: new millennium trends. *Mar Biotechnol*, 7: 429-439.
- Rinkevich, B. (2011). Cell cultures from marine invertebrates: New insights for capturing endless stemness. *Mar Biotechnol*, 13: 345-354.
- Rivera, A., Hammel, J., Haen, K., Danka, E., Cieniewicz, B., Winters, I., Posfai, D., Worheide, G., Lavrov, D., Knight, S., Hill, M., Hill, A. and Nickel, M. (2011). RNA interference in marine and freshwater sponges: actin knockdown in *Tethya wilhelma* and *Ephydatia muelleri* by ingested dsRNA expressing bacteria. *BMC Biotechnol*, 11: 67.



- Sastry, L., Johnson, T., Hobson, M.J., Smucker, B. and Cornetta, K. (2002). Titering lentiviral vectors: comparison of DNA, RNA and marker expression methods. *Gene Ther*, 9: 1155-1162.
- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol- Anim*, 47: 302-311.
- Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Adv Mar Biol*, 62: 273-337.
- Sipkema, D., Franssen, M.C.R., Osinga, R., Tramper, J. and Wijffels, R.H. (2005a). Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142-162.
- Sipkema, D., Heilig, H.G.H.J., Akkermans, A.D.L., Osinga, R., Tramper, J. and Wijffels, R.H. (2003). Sponge cell culture? A molecular identification method for sponge cells. *Mar Biotechnol*, 5: 443-449.
- Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J. and Wijffels, R.H. (2005b). Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201-222.
- Sipkema, D., Snijders, A.P.L., Schroen, C.G.P.H., Osinga, R. and Wijffels, R.H. (2004). The life and death of sponge cells. *Biotechnol Bioeng*, 85: 239-247.
- Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M.E.A., Mitros, T., Richards, G.S., Conaco, C., Dacre, M., Hellsten, U., Larroux, C., Putnam, N.H., Stanke, M., Adamska, M., Darling, A., Degnan, S.M., Oakley, T.H., Plachetzki, D.C., Zhai, Y., Adamski, M., Calcino, A., Cummins, S.F., Goodstein, D.M., Harris, C., Jackson, D.J., Leys, S.P., Shu, S., Woodcroft, B.J., Vervoort, M., Kosik, K.S., Manning, G., Degnan, B.M. and Rokhsar, D.S. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature*, 466: 720-726.
- Theilmann, D.A. and Stewart, S. (1992). Molecular analysis of the trans-activating IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology*, 187: 84-96.
- Vigna, E. and Naldini, L. (2000). Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med*, 2: 308-316.
- Yang, N.S., Burkholder, J., Roberts, B., Martinell, B. and McCabe, D. (1990). In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. *Proc Natl Acad Sci USA*, 87: 9568-9572.

# Chapter 6

General discussion:  
Genomic-based approach for sponge cell culture



## ABSTRACT

Sponge cell cultures are studied for the production of bioactive compounds as novel biomedicines as found in nature in sponges. Recently, the first genome of a sponge has been sequenced, and information about genes specific for control of cell proliferation, cell death and cell adhesion were revealed. This paper focusses on how we can use genomic and transcriptomic approaches to get more insight in the function of these genes in sponges and how we can use this knowledge to develop a continuous sponge cell line. First of all, we need to identify a model sponge for the development of a sponge cell line. By using only a few model species, researchers can build upon each other's results. This model sponge should be able to proliferate *in vitro*, be easily accessible for researchers and preferably produce a bioactive compound. Good candidates would be *Ephydatia* spp (gemmules), *Axinella corrugata* (primary cells stimulated with PHA) and *Dysidea avara* (larvae and primmorphs). Next, we recommend to sequence and annotate the genome of the model sponge to identify genes of interest and possibly find new ones. We recommend to focus initially on tumor suppressor genes (e.g. p53 and pRb), proto-oncogenes (e.g.  $\beta$ -catenin, c-Myc, Ras), telomerase (TERT) and cell adhesion genes (cadherin). Conserved homologs of these genes have already been found in sponges, but not yet in our proposed model sponges. Finally, we discuss methods (e.g. transfection and RNAi) to up or down regulate gene expression levels in sponges, based on our own research as well as other studies.

### **This chapter has been submitted for publication as:**

Schippers KJ, Martens DE, Pomponi SA, Wijffels RH. Genomic- based approach for sponge cell culture.

## 1 INTRODUCTION

Sponges are a rich source of bioactive compounds which have the potential to provide future medicines, such as new antibiotics and anticancer drugs (Blunt et al., 2009; Sipkema et al., 2005a). However, the limited availability of sponge biomass hampers the development of these potential drugs into commercial products. The use of *in vitro* sponge cell cultures is a potential alternative for biological supply of sponge-derived products, but until now a continuous sponge cell line has not been developed (Pomponi, 2006; De Caralt et al., 2007b; Schippers et al., 2012). This is primarily due to significant gaps in our understanding of sponge cell proliferation and death (chapter 3 and 4 of this thesis), nutritional requirements and cultivation conditions. Recent developments in the field of genomics and transcriptomics can help us to get more insight in sponge growth and death as well as nutritional requirements. Recently, the first genome of a sponge has been sequenced, and information about genes specific for multicellularity, such as control of cell proliferation and death, were revealed (Srivastava et al., 2010). These genes play a significant role in regulating cell growth, which is crucial for multicellular animals, since unrestricted growth of cells (= cancer) is detrimental for the other cells and the organism as a whole. For the development of a continuous cell line, the possibility to attain unrestricted growth is needed, per definition. Accordingly, most mammalian cell lines are derived from cancerous tissue or are actively immortalized, e.g., by inserting viral genes. The occurrence of cancer in an organism shows that cells of that organism can, in principle, be immortalized and the tumors themselves can serve as a source of immortal cells. Although many genes involved in the development of cancer are also present in sponges, cancerous growth in sponges has not been observed yet, neither *in situ* nor *in vitro*.

This paper focusses on how we can use genomic and transcriptomic approaches to get more insight in genes involved in cell proliferation and death in sponges and how we can use this knowledge to develop a continuous sponge cell line. The genes discussed in this paper are all related to multicellularity, such as regulation of cell proliferation, apoptosis and cell adhesion. These genes are crucial for homeostasis in multicellular animals and are expected to be quite generic for all sponges such that results can be translated to other species as well. In addition, we identified a few model sponges for the development of a sponge cell line, based on their worldwide availability, their ability to proliferate *in vitro* and their production of a bioactive compound. Finally, we provide some recommendations for approaches to study and manipulate gene expression in sponges, based on our own research (chapter 5 of this thesis) as well as other studies.

## 2 MODEL SPONGES

Researchers have used many different sponge species for cell culture studies. The fact that for none of these sponges a continuous cell line could be developed indicates that this may be a universal problem for sponges and not related to one specific species. Furthermore, the large diversity of sponges used makes it hard to compare results between the different studies and thus slows down progress in this field. Hence, there is a need to identify a model sponge (see Table 1) for the development of a sponge cell line, so researchers can build upon each other's results. The most important criterion for the identification of a model sponge is that the sponge is able to proliferate *in vitro*. This makes it possible to manipulate culture conditions and gene expression *in vitro*. Secondly the model sponge should be easily available for researchers worldwide. Ideally, the model sponge produces a bioactive compound, because the developed sponge cell line could immediately be applied to future research on the production of the sponge-derived compound. Finally, the availability of a sequenced genome would be a clear advantage for a model sponge. However in case all the other criteria are met, this issue can of course be solved by sequencing the genome of the model sponge.

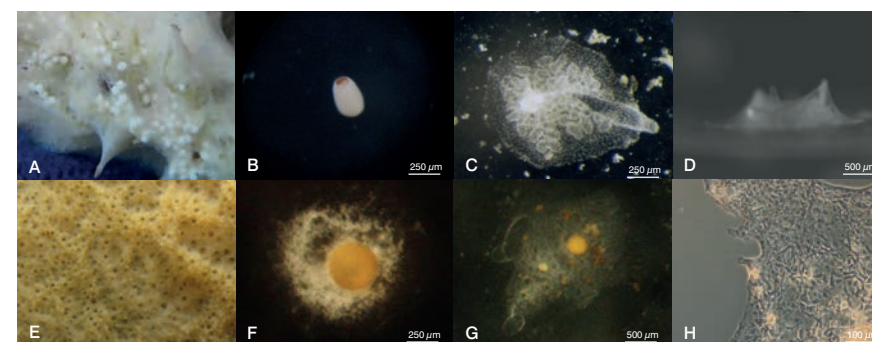
**Table 1** Proposed model species for the development of a sponge cell line.

Model sponge	Worldwide availability	Proliferation <i>in vitro</i>	Sequenced genome	Production of bioactive compound
<i>Ephydatia</i> spp.	++ Worldwide	++ Gemmules	+/- In process	-
<i>Axinella corrugata</i>	+ Caribbean sea and Western North Atlantic	+ Primary cells stimulated with PHA	-	+ Stevensine
<i>Dysidea avara</i>	+ Mediterranean	++ Larvae and primmorphs	-	+ Avarol

### 2.1 *Ephydatia* spp.

Good model organisms are freshwater sponges of the genus *Ephydatia* (Demospongiae, Haplosclerida). The genus has a worldwide distribution; therefore, many researchers would have access to a species of *Ephydatia*. Furthermore, freshwater sponges produce gemmules, which are asexual reproduction bodies comprised of thesocytes surrounded by a collagen and spicule coat. This coating enables gemmules to be treated with mild sterilizing solutions (e.g., H<sub>2</sub>O<sub>2</sub>) prior to

cultivation to prevent contamination. Thesocytes have stem cell properties, i.e. they are undifferentiated cells with unlimited proliferative capacity, and would be the ideal target for a cell culture (Rinkevich, 2011). In addition, the thesocyte cells themselves contain yolk reserves, so adding nutrients in the first stages of development is not necessary. Gemmules can be easily grown into juvenile sponges *in vitro* (see Fig. 1 E-H), which makes it possible to compare different juveniles of the same age. Besides, gemmules can be easily distributed among labs, enabling comparison of results. The genome sequence of this sponge is not yet available in GenBank, but it is in the process of being sequenced and annotated (Dr. Sally Leys, personal communication). This model sponge has one disadvantage, as it does not produce a bioactive compound, therefore the methods would still need to be translated to another sponge species. Alternatively, an *Ephydatia* cell line could be used for development of methods for heterologous expression of genes from bioactive sponges.



**Figure 1** Development of *Dysidea avara* larvae (A-D) and *Ephydatia fluviatilis* gemmules (E-H) into juvenile sponges. A, presence of larvae in sponge tissue. B, free swimming larva. C, 15 day old juvenile with developed oscula. D, lateral view of juvenile. E, tissue of *E. fluviatilis* F, gemmule after 5 days of hatching. G, 10 day old juvenile with developed osculum. H, growth marginal layer of hatched gemmule. [B-D are from (De Caralt et al., 2007a) and E taken by R. Timmerman.]

### 2.2 *Axinella*, *Dysidea* and *Amphimedon*

A promising marine sponge candidate as a model organism is *Axinella corrugata* (Demospongiae, Halichondrida), which is the source of stevensine, an alkaloid metabolite with antitumor properties (Wright et al., 1991). Cell cultures of this sponge which were enriched for archaeocytes (totipotent stem cells) were able to proliferate *in vitro* in the presence of the mitogenic lectin PHA (phytohaemagglutinin) (Pomponi et al., 1997; Willoughby and Pomponi, 2000). During cell culture, the sponge cells

were able to retain the ability to produce stevensine (Pomponi et al., 1997), and feeding the cultures radiolabelled precursors of stevensine resulted in production of radiolabelled stevensine, indicating that stevensine is produced by the sponge itself and not by any symbionts (Andrade et al., 1999). Two disadvantages of this sponge are its limited availability, since it is only found in the Caribbean Sea and some parts of the western North Atlantic (World Porifera Database, <http://www.marinespecies.org/porifera/>) and the lack of genomic information.

Another potential model organism is *Dysidea avara* (Demospongiae, Dictyoceratida). This sponge produces avarol, a terpenoid metabolite with anti-psoriasis and anti-viral activity (Sipkema et al., 2005a). Avarol is found in the choanocytes of the sponge, suggesting that avarol is produced by the sponge itself (Uriz et al., 1996). Also, the avarol content in *D. avara* is high, about 2 gram/kg wet sponge, which is favorable for upstream and downstream processing (Sipkema et al., 2005b). Furthermore, cells from *D. avara* have the ability to proliferate *in vitro*. Primmorphs from *D. avara* were able to synthesize DNA and continued to produce avarol (Muller et al., 2000). Recent studies in our lab demonstrated the potential of cell proliferation in primary cell cultures of *D. avara* (chapter 4 of this thesis). Another advantage of this sponge is the abundance of larval release in summer (Mariani et al., 2005). These larvae can be grown into juvenile sponges *in vitro* (De Caralt et al., 2007a) and have relatively high quantities of stem cells, making them a promising source for cell culture (De Caralt et al., 2007b). One disadvantage of using larvae is their limited availability, since *D. avara* larvae are only present in summer during a short period of time and the larvae cannot be stored. In addition, adult *D. avara* has limited availability, since it is mainly found in the Mediterranean (World Porifera Database, <http://www.marinespecies.org/porifera/>), and genomic information is not available for this sponge.

The sponge *Amphimedon queenslandica* (Demospongiae, Haplosclerida) has a sequenced genome available (Srivastava et al., 2010), which is a clear advantage. However, this sponge is only found on the Great Barrier Reef, which makes it difficult to obtain. Also, there is no information available about *in vitro* cell culture of this sponge. Therefore we consider this sponge not a good model for sponge cell culture research. Nevertheless, we could use the genomic information of this sponge for exploring genes of interest in the proposed model sponges.

### 3 GENES OF INTEREST FOR CREATING AN IMMORTALIZED SPONGE CELL LINE

The genes of interest discussed in this paper focus on genes involved in cell proliferation, cell death and cell adhesion, since these genes play a significant role in maintaining homeostasis in multicellular animals. Deregulation of these genes can cause unlimited growth of cells (i.e. stimulation of cell proliferation in combination with prevention of apoptosis), which is essential for the development of a continuous cell line.

#### 3.1 Tumor suppressor genes

Tumor suppressor genes function as guardians of the genome. Activation of these genes often results in cell cycle arrest, for example, to stop genetically damaged cells from dividing, followed by either repair of the damaged DNA or removing the cell by activating apoptosis (Sherr, 2004). When such genes are no longer working properly, there will be an accumulation of genetic changes, which can result in uncontrolled cell growth. In many animal species this leads to the development of cancer (Weinberg, 1991), and in the case of primary animal cell cultures, this can lead to an immortalized cell line (Dohn et al., 2003). The first tumor suppressor discovered was the retinoblastoma protein (pRb); inactivation of the corresponding gene on both alleles results in the development of retinoblastoma cancer (Murphree and Benedict, 1984). Another important tumor suppressor is the p53 transcription factor, which is inactivated in most cancers (Hollstein et al., 1991). These tumor suppressors, pRb and p53, are frequently inactivated to create immortalized mammalian cell lines from primary cell cultures. Inactivation of these genes can be caused by the binding of viral proteins of, e.g., simian virus 40 (SV40) or human papillomavirus (HPV) (Peters et al., 1996; Ozer et al., 1996). Conserved homologs of these tumor suppressor genes have been found in the sponge *A. queenslandica* (Srivastava et al., 2010) and we could use these genome data to search for similar genes in the proposed model sponges. It will be of value to study the function of these and other tumor suppressor genes in sponges and explore if inactivation of these genes will also lead to the immortalization of sponge cells. Section 4.2 and 5.3 elaborate further on this.

#### 3.2 Proto-oncogenes

Proto-oncogenes are generally involved in stimulation of cell division or inhibition of cell differentiation. A proto-oncogene can become an oncogene when it is mutated or overexpressed. Examples of proto-oncogenes are  $\beta$ -catenin, Ras and c-Myc. Conserved homologs of these proto-oncogenes have also been found in sponges (Lapébie et al., 2009; Srivastava et al., 2010; Schröder et al., 1988). The proto-oncogene -catenin plays an important role in the Wnt signaling pathway (see Fig 2) and



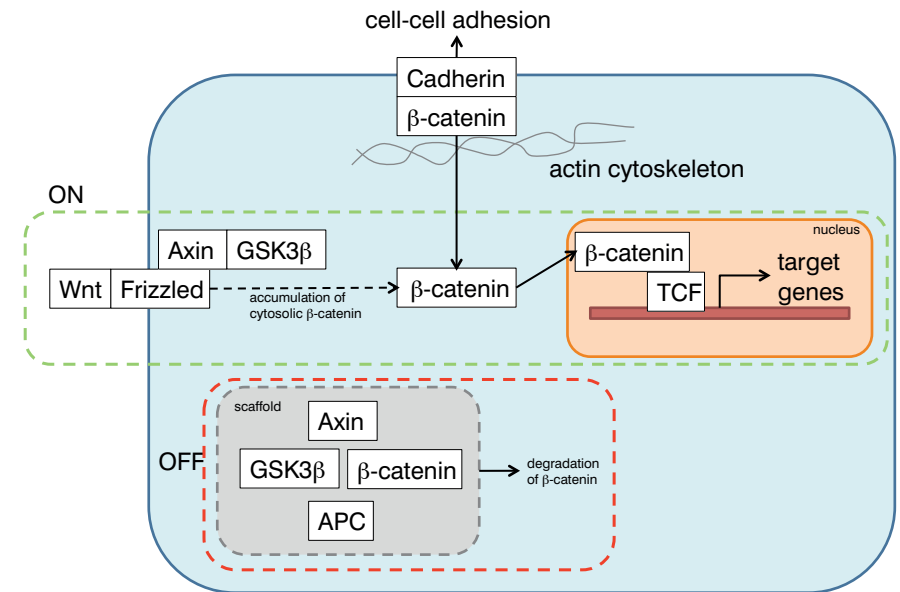
deregulation of this pathway is associated with colon cancer (Polakis, 2000). When the Wnt pathway is activated,  $\beta$ -catenin interacts with TCF (T-cell factor = transcription factor), which leads to the transcriptional activation of target genes involved in cell proliferation (e.g. c-Myc and cyclinD1). Without activation of the Wnt pathway,  $\beta$ -catenin will complex with axin, APC and GSK3 and will be phosphorylated and degraded to prevent accumulation of  $\beta$ -catenin. If the Wnt pathway is deregulated (e.g., mutation of APC or axin), high levels of  $\beta$ -catenin build up, which leads to abnormal expression of genes regulated by the TCF/ $\beta$ -catenin complex, resulting in cancer. Conserved homologs of components involved in the Wnt signaling pathway have been found in sponges (Adamska et al., 2010; Lapébie et al., 2009; Windsor and Leys, 2010), but until now no studies have been performed on the effect on cell proliferation in sponge cells by up-regulating levels of  $\beta$ -catenin. Approaches to up-regulate  $\beta$ -catenin levels are discussed in section 5.

### 3.3 Telomerase reverse transcriptase (TERT)

Telomerase reverse transcriptase is a catalytic subunit of the telomerase enzyme. Normally, telomerase is only expressed in germ cells, stem cells and tumors, and not in somatic cells. In somatic cells, the telomeres will become shorter after each cell division, and after a certain point this will cause chromosome instability and eventually will lead to apoptosis (a.k.a. Hayflick limit<sup>1</sup>). When telomeric shortening is prevented by expression of telomerase, which repairs the telomeres, apoptosis can be prevented, leading to extended life span (bypassing the Hayflick limit). This theory was proven by Bodnar et al. (1998), who introduced a human telomerase reverse transcriptase (hTERT) gene into normal human cells, which resulted in an extension of the life-span of the culture. In most cancer cells telomerase is expressed, allowing them to divide infinitely. However, telomerase is not an oncogene, since telomerase itself does not cause growth deregulation (Harley, 2002).

Adult sponges were found to have high expression levels of telomerase activity, which corresponds to the high plasticity and regeneration capacity of sponges (Koziol et al., 1998). This agrees with results obtained by De Goeij and coworkers (2009), who found that sponges have a high cell turnover. However, when cells were dissociated to create a primary cell culture, the cells lost their telomerase activity and eventually became apoptotic (Koziol et al., 1998). In order to increase the life span of sponge cells in culture, Pomponi and coworkers (2007) inserted hTERT in cells of *A. corrugata*. The cells were able to transiently express hTERT (see Fig 3), but the cells did not become immortalized. In addition, the negative control –untransformed *A. corrugata* cells (see Fig 3.2)- also showed a modest staining, suggesting that native

<sup>1</sup> Hayflick limit is the number of cell divisions of a normal cell population until the cells become apoptotic, due to shortening of the telomeres to a critical length.

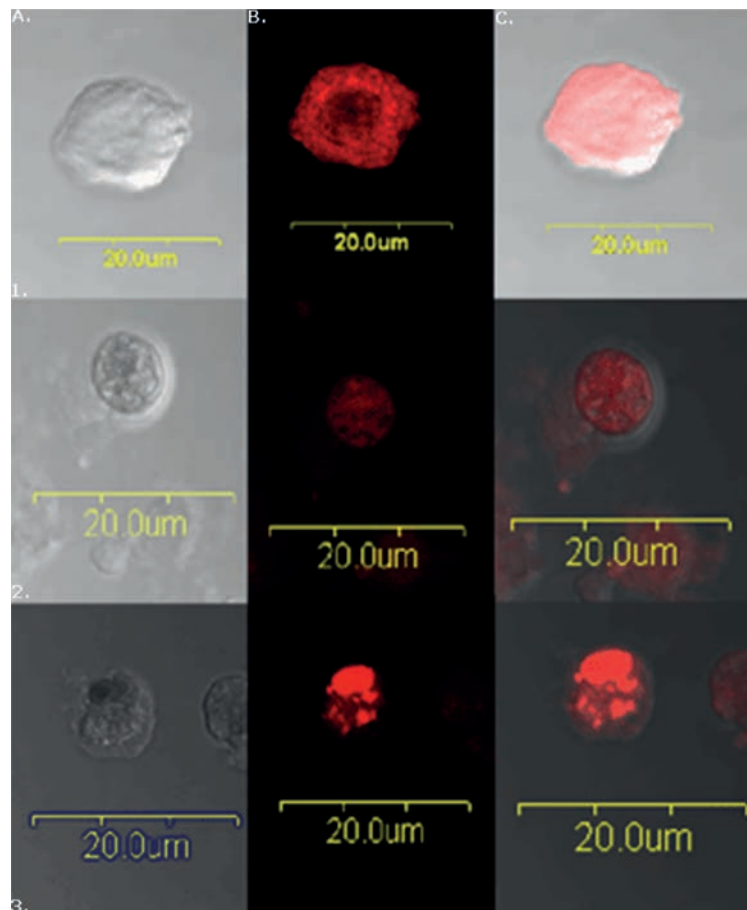


**Figure 2** Simplified overview of role of  $\beta$ -catenin in the Wnt signaling pathway and cell-cell adhesion. When the Wnt pathway is activated (ON), due to the presence of a Wnt ligand,  $\beta$ -catenin will accumulate in the cytosol and will interact with TCF, which leads to the transcriptional activation of target genes involved in cell proliferation (e.g. c-Myc and cyclinD1). Without the presence of the Wnt ligand (OFF),  $\beta$ -catenin will complex with axin, APC and GSK3 $\beta$  and will be phosphorylated and degraded to prevent accumulation of  $\beta$ -catenin. Cadherin can also regulate  $\beta$ -catenin levels. A loss of cadherin will not only result in a loss of cell-cell adhesion, but will also result in more cytoplasmic  $\beta$ -catenin, which will result in increased transcriptional activation of target genes.

sponge TERT is stained with the TERT antibody. Assuming that the presence of native sponge TERT can be confirmed, an alternative to insertion of foreign TERT would be to increase levels of native TERT to develop an immortalized sponge cell line. However, possibly it will be necessary to combine overexpression of TERT with the inhibition of tumor suppressor genes or overexpression of proto-oncogenes. Section 5 elaborates further on this approach.

### 3.4 Cell adhesion genes

Cell adhesion molecules (CAM) are located on the cell membrane and are involved in cell-cell and cell-matrix adhesion and communication, which is important for homeostasis in multicellular animals. One major family of cell adhesion molecules is cadherin. The classical cadherin is the most widely expressed cadherin, and



**Figure 3** Immunocytochemistry results after transfecting *A. corrugata* archaeocytes with hTERT, under control of MPSV promoter, by using Lipofectamine2000 (Invitrogen). From left to right: A. differential interference contrast; B. fluorescence contrast; C. overlay. From top to bottom: 1. Positive control: human breast cancer cell MCF7; 2. Negative control: untransformed *A. corrugata* archaeocytes; 3. Transformed *A. corrugata* archaeocytes. (Pomponi et al., 2007).

conserved homologs of this protein were also found in sponges (Nichols et al., 2006). One function of cadherin is the mediation of cell-cell adhesion in adherens junctions. The extracellular cadherin domain binds to cadherin domains of other cells to produce tight intercellular adhesion. The cytosolic domain of cadherin is linked to the actin cytoskeleton by adapter proteins. One of the adapter proteins is  $\beta$ -catenin, which also plays a key role in the Wnt signaling pathway. Therefore, cadherin can also regulate  $\beta$ -catenin levels available for the Wnt signaling pathway (see Fig 2). A loss of

cadherin will not only result in a loss of cell-cell adhesion, but will also result in more cytoplasmic  $\beta$ -catenin available for the Wnt pathway, which will result in increased transcriptional activation of target genes (Jeanes et al., 2008). Generally the loss of cell-cell adhesion results in an apoptotic response, known as anoikis, which prevents metastasis. However, the loss of the cadherin protein results in anoikis resistance and cells can become metastatic (Onder et al., 2008). It will be of value to study the function of cadherin in sponges and explore if inactivation of this gene will also lead to loss of cell-cell adhesion (valuable for monodisperse cell cultures) and increased proliferation. An approach to down-regulate levels of cadherin is discussed in section 5.2.

## 4 FINDING RELEVANT GENES IN SPONGES

### 4.1 Confirming presence of genes of interest in model sponges

Conserved homologs of all genes discussed in section 3 have been found in sponges (Srivastava et al., 2010; Koziol et al., 1998; Nichols et al., 2006; Lapébie et al., 2009), but have not yet been identified in our proposed model sponge species. To confirm the presence of these genes of interest in the proposed model sponge species, we propose to sequence and annotate the genome of these sponges. The sequenced genome will not only provide us with information about the presence of the genes of interest, but will also provide us information about other genes important for the optimization of sponge cell cultures, such as genes involved in metabolism. Nonetheless, sequencing of a sponge genome can be costly and laborious, even more if symbionts are present. The genome of *Ephydatia muelleri* is currently in the process of being sequenced and annotated (Dr. Sally Leys, personal communication). For *A. corrugata* and *D. avara*, genome sequencing is not yet in the pipeline. To identify the gene homologs in model sponges without an annotated genome, one could construct an EST (expressed sequence tag) library and sequence the cDNA and compare with cDNA in the NCBI GenBank for identification. This method is less laborious than sequencing, but also less complete, since only expressed genes will be identified. Another option is to perform degenerate PCR, using primer pairs designed from an alignment of conserved regions from a collection of genes isolated from other animals (e.g., from sequenced genome of *A. queenslandica*). This is a very targeted approach, but is only applicable if searching for specific genes. Additional relevant genes will not be identified with this method.

### 4.2 Confirming role of gene of interest and find other relevant genes

Most sponge cultivation studies observe the change of biomass (e.g., volume, weight, cell numbers, protein content) over time and do not measure expression of certain genes and their related products. We suggest to study gene expression (tools

to study gene expression are mentioned in box 1) in proliferating and non-proliferating conditions to obtain insight in the role of known genes involved in cell proliferation and cell death and find other genes of interest. Following conditions could be compared:

- **Larvae and gemmules vs. adult sponge.** Juveniles of larvae and gemmules have the potential to proliferate *in vitro*, whereas an adult sponge in general lacks this capacity. A plausible explanation for this could be the large number of stem cells present in larvae and gemmules. Using sponge stem cells for cell culture has already been proposed by De Caralt et al. (2007b) and Rinkevich (2011). Since juveniles of larvae and gemmules are growing and developing into a sponge (see Fig 1), genes related to cell proliferation are expected to be more actively expressed than in an adult sponge which is in homeostasis. By comparing gene expression profiles of juveniles with adult sponges, we can confirm if specific genes involved in cell proliferation are more actively expressed.
- **Primary sponge cell cultures vs. adult sponge.** In general, primary sponge cell cultures lack proliferative capacity. Previous studies already showed that dissociation of sponge cells resulted in increased caspase activity (Schippers et al., 2011 and chapter 3 and 4 of this thesis), which is an indicator for apoptosis, and decreased telomerase activity (Koziol et al., 1998) which is a bio-marker for cell proliferation. Nevertheless, Pomponi et al. (1997) were able to demonstrate sponge cell growth when PHA was added to the growth medium. Subsequently Willoughby (2002) compared gene expression levels of sponge cells cultivated with and without PHA. She found that PHA effects expression levels of proliferative and anti-apoptotic genes in marine sponge cells. This research has not been followed up, but it would be of value to study the expression of the genes mentioned in section 3 in primary sponge cell cultures stimulated with mitogens like PHA and to compare with gene expression in the adult sponge.

**Box 1** Tools to study gene expression.

- **qRT-PCR** (Real Time PCR combined with reverse transcription) can be used to quantify mRNA of specific genes of interest. Only the DNA sequence of the gene of interest needs to be known.
- **DNA microarray** can be used to measure the expression levels of large numbers of genes at the same time. The availability of an annotated genome makes it possible to build custom microarrays specific to sponge cells. In case genomic information is not available to build a sponge specific microarray, it is also possible to employ a cross

species technique as demonstrated by Willoughby (2002). She hybridized sponge cDNA of *A. corrugata* to commercially available microarrays of human genome sequences and could measure changes in gene expression of primary sponge cell culture of *A. corrugata* in response to PHA stimulation.

- **Transcriptome analysis (RNA sequencing)** provides information about genes that are being actively expressed. Transcriptome analysis of proliferating and non-proliferating sponge cells can confirm the role of genes of interest and possibly find new ones.

## 5 UP- AND DOWN-REGULATION OF GENES OF INTEREST

By up- or down-regulating the genes of interest, we can get more insight in their role in sponges. In addition, it can also lead to increased proliferation and reduced cell death which could result in the development of an immortalized sponge cell line. Manipulating gene expression levels should be performed *in vitro*, since it is not desirable to expose genetically modified sponges to the environment. In addition, we suggest using species or life history stages that can proliferate or are at least metabolically active *in vitro*, such as juveniles which arose from gemmules, larvae or buds (De Caralt et al., 2007b) or primary cell cultures stimulated with PHA (Pomponi et al., 1997). Table 2 gives an overview of a possible strategy in relation to up or down-regulation of genes in order to increase proliferation and reduce cell death.

**Table 2** Overview of strategy in relation to up- or down-regulation of genes or proteins of interest.

Gene or protein of interest	Up- or down-regulate	Strategy
Tumor suppressor genes: p53 and pRb	Down	<ul style="list-style-type: none"> <li>• Silence p53 and pRb with RNAi.</li> <li>• Inactivate by inserting immortalizing genes.</li> </ul>
Proto-oncogene: $\beta$ -catenin	Up	<ul style="list-style-type: none"> <li>• Insert and overexpress <math>\beta</math>-catenin.</li> <li>• Activate Wnt signaling pathway by exposing sponges to small molecules/peptides that increase <math>\beta</math>-catenin levels.</li> <li>• Silence APC and axin with RNAi.</li> </ul>
Telomerase: TERT	Up	<ul style="list-style-type: none"> <li>• Insert and overexpress (h)TERT.</li> </ul>
Cell adhesion molecule: cadherin	Down	<ul style="list-style-type: none"> <li>• Silence cadherin with RNAi</li> </ul>

Possible approaches to up- or down-regulate gene expression levels are over-expressing the gene of interest by inserting an expression vector (section 5.1) or by silencing the gene of interest by inserting its complementary dsRNA (section 5.2). Both strategies require a method to insert DNA or RNA into the sponge cells and/or nuclei.

Recent results showed that lipofection was effective in delivering plasmid DNA into primary cells from *A. corrugata* (Pomponi et al., 2007) and into juveniles of *Ephydatia fluviatilis* (chapter 5 of this thesis). Furthermore, the lipofection reagent was not toxic at the used concentrations and was able to consistently deliver large amounts of a cell-impermeable fluorescent dye into sponge cells, suggesting that the lipofection reagent may also be able to deliver other macromolecules, such as DNA or RNA, into the cell (chapter 5 of this thesis). Particle bombardment was effective in delivering plasmid DNA into juveniles of *S. lacustris* (Pfannkuchen and Brummer, 2009). However, the number of cells which were targeted with particle bombardment was significantly less than with lipofection (chapter 5 of this thesis). An effective method to insert dsRNA into sponge cells was recently demonstrated by Rivera et al. (2011). They fed juveniles dsRNA expressing bacteria, which resulted in reduction of actin transcript levels and phenotypic changes. Soaking juveniles of *E. muelleri* in dsRNA also resulted in knockdown, but soaking juveniles of *T. wilhelma* did not result in knockdown due to instability of RNA in seawater. Other methods which would be worthwhile exploring are electroporation (Andreason and Evans, 1988), Poly-APS (McLaggan et al., 2006), supercharged proteins (McNaughton et al., 2009) or transduction with a baculovirus (Hu, 2005).

### 5.1 Overexpressing genes of interest

We suggest to overexpress proto-oncogenes, such as  $\beta$ -catenin, which will lead to increased transcriptional activation of target genes involved in cell proliferation (see Fig 2) or to overexpress telomerase (TERT), which will repair the telomeres and accordingly will prevent apoptosis. Overexpression involves cloning the cDNA of the specific gene into an expression vector with a strong promoter and inserting this expression vector in the sponge cell. Insertion and expression of these genes (i.e. transfection) in sponge cells will require an effective DNA delivery method and a promoter that is recognized by the RNA polymerase of the sponge (chapter 5 of this thesis). A specific sponge promoter has not been discovered yet, but recent results indicate that the CMV (cytomegalovirus) promoter, which is widely used in mammalian cells, is also effective in the freshwater sponges *E. fluviatilis* (chapter 5 of this thesis) and *S. lacustris* (Pfannkuchen and Brummer, 2009) and that the MPSV (myeloproliferative sarcoma virus) promoter is effective in the marine sponge *A. corrugata* (Pomponi et al., 2007). Nevertheless, the discovery of specific sponge promoters may lead to higher expression levels of genes.

In most cases, transfection will be transient. To select for stably transfected cells, i.e., cells where the gene of interest is integrated into the genome, a selectable marker gene (e.g., neo or hygB) will be needed to resist exposure to a selection agent (e.g., G418 or hygromycin). An appropriate selectable marker for sponges is not known yet and more research is needed along with more intensive research on expression of heterologous genes in sponge cells.

### 5.2 RNAi

A powerful tool to down-regulate gene expression is RNA interference (RNAi) (Shan, 2010). We suggest to silence tumor suppressor genes (e.g. p53 and pRb) to prevent cells from going into apoptosis and/or cell cycle arrest, to silence components of the Wnt pathway (e.g. axin or APC) in order to increase  $\beta$ -catenin levels which will lead to increased transcriptional activation of target genes involved in cell proliferation (see Fig 2) or to silence cadherin gene expression to prevent cell-cell adhesion and additionally increase levels of  $\beta$ -catenin (see Fig 2).

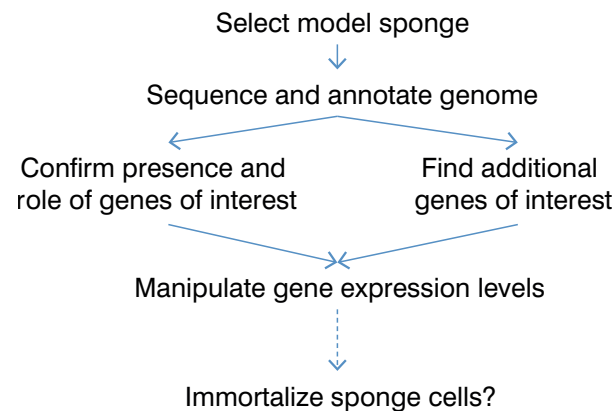
By introducing synthesized dsRNA which is complementary to the gene of interest into the cell, it is possible to suppress specific genes (a.k.a. knockdown). Knocking down a specific gene can provide information about the function of that specific gene in cellular processes, such as cell death, proliferation or differentiation. RNAi has recently been demonstrated in freshwater and marine sponges (Rivera et al., 2011). Rivera and coworkers were able to knockdown cytoplasmic actin mRNA levels in juvenile specimens of *E. muelleri* and *Tethya wilhelma*. These results indicate that the RNAi pathway is able to function in both marine and freshwater sponges. This method can be applicable to other genes in sponges involved in cell proliferation and adhesion, such as tumor suppressor genes and cell adhesion genes. Besides bacterial feeding and soaking also other methods could be explored to insert dsRNA into sponges or sponge cells, such as lipofection (chapter 5 of this thesis).

### 5.3 Others

In most cases RNAi is reversible, and when the cells are no longer exposed to dsRNA, the mRNA levels will increase. To achieve inactivation or knockout of a gene a different strategy is needed. For the inactivation of the tumor suppressor genes, p53 and pRb, a possible strategy is to insert immortalizing genes into the cell. Examples of immortalizing genes are SV40LT or HPV E6 and E7, which are from viral origin. Expression of these viral genes causes inactivation of the tumor suppressor genes due to binding of the viral proteins. These immortalizing genes have been widely used for creating immortalized mammalian cell lines (Mayne et al., 1996; Peters et al., 1996; Bodnar et al., 1998; Liu et al., 2008). A worthwhile approach will be to co-transfect sponge cells with hTERT (or TERT from sponge origin) together with an immortalizing gene from viral origin, such as SV40LT (Liu et al., 2008).

Another approach to up-regulate the proto-oncogene  $\beta$ -catenin is to manipulate the Wnt signaling pathway by exposing the sponge or sponge cells to small molecules such as LiCl, soluble Wnt protein or norrin (Hedgepeth et al., 1997; Clevers, 2004; Willert et al., 2003). These molecules will activate the Wnt pathway and subsequently the binding of  $\beta$ -catenin to TCF and the transcriptional activation of target genes (see Fig 2).

Another alternative is exposure of sponge cells to carcinogens or UV-radiation. Carcinogens are substances which can induce cancer, which is in most cases related to mutagenesis. Exposure to carcinogens can for example lead to increased oncogene expression and can therefore also be applied to transform cells. Examples of carcinogens which lead to immortal transformation in mammalian cells are UV-radiation (Wazer et al., 1994), heavy metals (Hamilton et al., 1998) and benzo(a) pyrene (Stampfer and Bartley, 1985). By exposing sponge cells to these substances, we can alter the activity of e.g. tumor suppressor genes and proto-oncogenes and possibly immortalize sponge cells.



**Figure 4** Proposed strategy of a genomic-based approach towards a sponge cell line.

## 6 CONCLUSIONS

This paper proposes a genomic-based approach for the development of a sponge cell line. Figure 4 shows a schematic overview of the steps to be taken.

First of all, we recommend to select a model sponge for further research on sponge cell line development, which has the ability to proliferate *in vitro* and is easily accessible for researchers. *Ephydatia* spp., *D. avara* and *A. corrugata* have such properties.

We recommend to sequence and annotate the genome of the model sponge to provide information about genes important for optimization of sponge cell cultures, such as genes involved in cell proliferation, death and metabolism.

We recommend to focus initially on genes involved in cell proliferation and death. Conserved homologs of tumor suppressor genes (e.g. p53 and pRb), proto-oncogenes (e.g.  $\beta$ -catenin, c-Myc, Ras), telomerase and cell adhesion genes (cadherin) have already been found in sponges. It will be of value to confirm the presence and function of these genes in sponges and explore if up- or down-regulating (over-expression of genes or RNAi) of these genes results in increased cell proliferation/life span, reduced cell death or resistance to anoikis.

In addition, we suggest to search for additional genes in sponges important for regulating cell proliferation and death by studying the transcriptome of proliferating sponge cells (e.g. larvae, gemmules, PHA stimulated sponge cells) in comparison with non-proliferating sponge cells (e.g. adult sponge, non-PHA stimulated sponge cells).

Finally, a promising method to develop a continuous sponge cell line is to insert immortalizing genes, such as SV40 LT together with TERT. To develop a continuous sponge cell line using immortalizing genes, the following prerequisites need to be met: metabolically active primary cells, a promoter that is recognized by the RNA polymerase of the sponge, effective DNA delivery method, functional immortalizing genes, and a method to obtain stable insertion of the genes (e.g. appropriate selectable marker for sponges).

## Acknowledgements

This research was supported by IPOP Coast and Sea, Graduate School VLAG and Florida Sea Grant (R/LR-MB-25). We acknowledge Jane Thompson and André Uitterdijk for sharing their immunocytochemistry results of the transfected *A. corrugata* cells.



## REFERENCES

- Adamska, M., Larroux, C., Adamski, M., Green, K., Lovas, E., Koop, D., Richards, G., Zwafink, C. and Degnan, B. (2010). Structure and expression of conserved Wnt pathway components in the demosponge *Amphimedon queenslandica*. *Evol Dev*, 12: 494 - 518.
- Andrade, P., Willoughby, R., Pomponi, S.A. and Kerr, R.G. (1999). Biosynthetic studies of the alkaloid, stevensine, in a cell culture of the marine sponge *Teichaxinella morchella*. *Tetrahedron Lett*, 40: 4775-4778.
- Andreason, G.L. and Evans, G.A. (1988). Introduction and expression of DNA molecules in eukaryotic cells by electroporation. *Biotechniques*, 6: 650-660.
- Blunt, J.W., Copp, B.R., Hu, W.P., Munro, M.H.G., Northcote, P.T. and Prinsep, M.R. (2009). Marine natural products. *Nat Prod Rep*, 26: 170-244.
- Bodnar, A.G., Ouellette, M., Frolkis, M., Holt, S.E., Chiu, C.P., Morin, G.B., Harley, C.B., Shay, J.W., Lichtsteiner, S. and Wright, W.E. (1998). Extension of life-span by introduction of telomerase into normal human cells. *Science*, 279: 349-352.
- Clevers, H. (2004). Wnt signaling: Ig-norin the dogma. *Curr Biol*, 14: R436-R437.
- De Caralt, S., Otiens, H., Uriz, M. and Wijffels, R. (2007a). Cultivation of sponge larvae: settlement, survival, and growth of juveniles. *Mar Biotechnol*, 9: 592-605.
- De Caralt, S., Uriz, M.J. and Wijffels, R.H. (2007b). Cell culture from sponges: pluripotency and immortality. *Trends Biotechnol*, 25: 467-471.
- De Goeij, J.M., De Kluijver, A., Van Duyl, F.C., Vacelet, J., Wijffels, R.H., De Goeij, A., Cleutjens, J.P.M. and Schutte, B. (2009). Cell kinetics of the marine sponge *Halisarca caerulea* reveal rapid cell turnover and shedding. *J Exp Biol*, 212: 3892-3900.
- Dohn, M., Nozell, S., Willis, A. and Chen, X. (2003). Tumor suppressor gene-inducible cell lines. *Meth Mol Biol*, 223: 221-235.
- Hamilton, J.W., Kaltreider, R.C., Bajenova, O.V., Ihnat, M.A., Mccaffrey, J., Turpie, B.W., Rowell, E.E., Oh, J., Nemeth, M.J., Pesce, C.A. and Lariviere, J.P. (1998). Molecular basis for effects of carcinogenic heavy metals on inducible gene expression. *Environ Health Persp*, 106: 1005-1015.
- Harley, C.B. (2002). Telomerase is not an oncogene. *Oncogene*, 21: 494-502.
- Hedgepeth, C.M., Conrad, L.J., Zhang, J., Huang, H.C., Lee, V.M.Y. and Klein, P.S. (1997). Activation of the Wnt signaling pathway: a molecular mechanism for lithium action. *Dev Biol*, 185: 82-91.
- Hollstein, M., Sidransky, D., Vogelstein, B. and Harris, C. (1991). p53 mutations in human cancers. *Science*, 253: 49-53.
- Hu, Y.C. (2005). Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol Sin*, 26: 405-416.
- Jeanes, A., Gottardi, C.J. and Yap, A.S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression? *Oncogene*, 27: 6920-6929.
- Koziol, C., Borojevic, R., Steffen, R. and Müller, W.E.G. (1998). Sponges (Porifera) model systems to study the shift from immortal to senescent somatic cells: the telomerase activity in somatic cells. *Mech Ageing Dev*, 100: 107-120.
- Lapébie, P., Gazave, E., Ereskovsky, A., Derelle, R., Bézac, C., Renard, E., Houliston, E. and Borchiellini, C. (2009). Wnt/ -catenin signalling and epithelial patterning in the Homoscleromorph sponge *Oscarella*. *PLoS ONE*, 4: e5823.
- Liu, X.S., Song, B., Zhang, S.F., Wang, C.Y., Cai, X.H. and Xu, Y.M. (2008). Transfection of human bone marrow mesenchymal stem cells with hTERT and SV40LT genes. *Life Sci J*, 5: 30-34.
- Mariani, S., Uriz, M.J. and Turon, X. (2005). The dynamics of sponge larvae assemblages from northwestern Mediterranean nearshore bottoms. *J Plankton Res*, 27: 249-262.
- Mayne, L.V., Price, T.N.C., Moorwood, K. and Burke, J.F. (1996). Development of immortal human fibroblast cell lines. IN Freshney, R.I., Freshney, M.G. (Eds.) *Culture of immortalized cell*. New York: Wiley-Liss, 77-93.
- Mclaggan, D., Adjimatera, N., Sepcic, K., Jaspars, M., Macewan, D., Blagbrough, I. and Scott, R. (2006). Pore forming polyalkylpyridinium salts from marine sponges versus synthetic lipofection systems: distinct tools for intracellular delivery of cDNA and siRNA. *BMC Biotechnol*, 6: 6.
- Mcnaughton, B.R., Cronican, J.J., Thompson, D.B. and Liu, D.R. (2009). Mammalian cell penetration, siRNA transfection, and DNA transfection by supercharged proteins. *Proc Natl Acad Sci USA*, 106: 6111-6116.
- Muller, W.E.G., Bohm, M., Batel, R., De Rosa, S., Tommonaro, G., Muller, I.M. and Schroder, H.C. (2000). Application of cell culture for the production of bioactive compounds from sponges: synthesis of avarol by primmorphs from *Dysidea avara*. *J Nat Prod*, 63: 1077-1081.
- Murphree, A.L. and Benedict, W.F. (1984). Retinoblastoma: clues to human oncogenesis. *Science*, 223: 1028-1033.
- Nichols, S.A., Dirks, W., Pearse, J.S. and King, N. (2006). Early evolution of animal cell signaling and adhesion genes. *Proc Natl Acad Sci USA*, 103: 12451-12456.
- Onder, T.T., Gupta, P.B., Mani, S.A., Yang, J., Lander, E.S. and Weinberg, R.A. (2008). Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res*, 68: 3645-3654.
- Ozer, H.L., Banga, S.S., Dasgupta, T., Houghton, J., Hubbard, K., Jha, K.K., Kim, S.H., Lenahan, M., Pang, Z., Pardinas, J.R. and Patsalis, P.C. (1996). SV40-mediated immortalization of human fibroblasts. *Exp Gerontol*, 31: 303-310.
- Peters, D.M., Dowd, N., Brandt, C. and Compton, T. (1996). Human papilloma virus E6/E7 genes can expand the lifespan of human corneal fibroblasts. *In Vitro Cell Dev Biol- Ani*, 32: 279-284.
- Pfannkuchen, M. and Brummer, F. (2009). Heterologous expression of DsRed2 in young sponges (Porifera). *Int J Dev Biol*, 53: 1113-1117.
- Polakis, P. (2000). Wnt signaling and cancer. *Genes Dev*, 14: 1837-1851.
- Pomponi, S.A. (2006). Biology of the Porifera: cell culture. *Can J Zool*, 84: 167-174.
- Pomponi, S.A., Thompson, J., Bottesch, J.H. and Sennett, S.H. (2007). Methods for extending the life span of marine invertebrate cells. *US patent number 20070254367*.
- Pomponi, S.A., Willoughby, R., Kaighn, M.E. and Wright, A.E. (1997). Development of techniques for *in vitro* production of bioactive natural products from marine sponges. IN Maramorosch, K., Mitsuhashi, J. (Eds.) *Invertebrate Cell Culture: Novel Directions and Biotechnology Applications*. Enfield: Science Publishers, 231-237.
- Rinkevich, B. (2011). Cell cultures from marine invertebrates: New insights for capturing endless stemness. *Mar Biotechnol*, 13: 345-354.
- Rivera, A., Hammel, J., Haen, K., Danka, E., Cieniewicz, B., Winters, I., Posfai, D., Worheide, G., Lavrov, D., Knight, S., Hill, M., Hill, A. and Nickel, M. (2011). RNA interference in marine and freshwater sponges: actin knockdown in *Tethya wilhelma* and *Ephydatia muelleri* by ingested dsRNA expressing bacteria. *BMC Biotechnol*, 11: 67.
- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol- Anim*, 47: 302-311.
- Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Adv Mar Biol*, 62: 273-337.
- Schröder, H.C., Kuchino, Y., Gramzow, M., Kurelec, B., Friese, U., Uhlenbruck, G. and Müller, W.E. (1988). Induction of ras gene expression by homologous aggregation factor in cells from the sponge *Geodia cydonium*. *J Biol Chem*, 263: 16334-40.
- Shan, G. (2010). RNA interference as a gene knockdown technique. *Int J Biochem Cell Biol*, 42: 1243-1251.
- Sherr, C.J. (2004). Principles of tumor suppression. *Cell*, 116: 235-246.
- Sipkema, D., Franssen, M.C.R., Osinga, R., Tramper, J. and Wijffels, R.H. (2005a). Marine sponges as pharmacy. *Mar Biotechnol*, 7: 142-162.
- Sipkema, D., Osinga, R., Schatton, W., Mendola, D., Tramper, J. and Wijffels, R.H. (2005b). Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? *Biotechnol Bioeng*, 90: 201-222.
- Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M.E.A., Mitros, T., Richards, G.S., Conaco, C., Dacre, M., Hellsten, U., Larroux, C., Putnam, N.H., Stanke, M., Adamska, M., Darling, A., Degnan, S.M.,

- Oakley, T.H., Plachetzki, D.C., Zhai, Y., Adamski, M., Calcino, A., Cummins, S.F., Goodstein, D.M., Harris, C., Jackson, D.J., Leys, S.P., Shu, S., Woodcroft, B.J., Vervoort, M., Kosik, K.S., Manning, G., Degnan, B.M. and Rokhsar, D.S. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature*, 466: 720-726.
- Stampfer, M.R. and Bartley, J.C. (1985). Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. *Proc Natl Acad Sci USA*, 82: 2394-2398.
- Uriz, M.J., Turon, X., Galera, J. and Tur, J.M. (1996). New light on the cell location of avarol within the sponge *Dysidea avara* (Dendroceratida). *Cell Tissue Res*, 285: 519-527.
- Wazer, D.E., Chu, Q., Liu, X.L., Gao, Q., Safaii, H. and Band, V. (1994). Loss of p53 protein during radiation transformation of primary human mammary epithelial cells. *Mol Cell Biol*, 14: 2468-2478.
- Weinberg, R.A. (1991). Tumor suppressor genes. *Science*, 254: 1138-1146.
- Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R. and Nusse, R. (2003). Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*, 423: 448-452.
- Willoughby, R. (2002). In vitro gene expression in marine sponge cells stimulated by phytohemagglutinin [PhD Thesis]. Department of Biological Sciences, Florida Institute of Technology, Melbourne, Florida, USA.
- Willoughby, R. and Pomponi, S.A. (2000). Quantitative assessment of marine sponge cells *in vitro*: development of improved growth medium. *In Vitro Cell Dev Biol- Anim*, 36: 194-200.
- Windsor, P. and Leys, S. (2010). Wnt signaling and induction in the sponge aquiferous system: evidence for an ancient origin of the organizer. *Evol Dev*, 12: 484 - 493.
- Wright, A.E., Chiles, S.A. and Cross, S.S. (1991). 3-amino-1-(2-aminoimidazolyl)-prop-1-ene from the marine sponges *Teichaxinalla morchella* and *Ptilocaulis walpersi*. *J Nat Prod*, 54: 1684-1686.

Summary  
Samenvatting  
Dankwoord  
Curriculum Vitae  
List of publications  
Overview of completed training activities



## SUMMARY

Marine sponges are a rich source of bioactive compounds with pharmaceutical potential and are the most prolific source of newly discovered bioactive compounds with more than 7,000 novel molecules discovered in 40 years. Despite its enormous potential, only a few sponge-derived bioactive compounds have been successfully developed into products. A major obstacle is the lack of sufficient supply of biological material for preclinical and clinical development. An option to supply materials for early drug development is production by the sponge, sponge tissue, sponge cells and/or the sponge symbionts. The main challenge with this is to establish generic techniques for small-scale production of sponge biomass.

In **chapter 2** we analyzed the state-of-the-art for cultivation of whole sponges, sponge cells and sponge symbionts and an outlook is presented for development or integration of different cultivation methods. Depending on the product of interest, different cultivation approaches may be applied. When product concentrations are high inside the sponge, cultivation of whole sponges *in situ* is the most successful approach. However, optimal conditions are species-specific and cultivation parameters cannot be controlled *in situ*. Moreover, many secondary metabolites are present only at low concentrations and to obtain an economically feasible production system, the product concentrations have to be increased. The use of *in vitro* cultures, such as cell or tissue culture, may then be the method of choice. *In vitro* cultivation allows for setting and controlling of specific culture conditions to increase product concentrations. Until now, the establishment of sponge cell lines has been unsuccessful mainly because of the inability to obtain an axenic inoculum as well as the lack of knowledge on factors that influence proliferation and death of sponge cells *in vitro* among with their nutritional requirements. Approaches to overcome these bottlenecks, including transformation of sponge cells and using media based on yolk, are elaborated. If the compound of interest is produced by a microbial symbiont, the production could be done with only the symbiont. Several bioactive metabolite-producing microorganisms have already been isolated from sponges, but cultivation of sponge-specific microorganisms has had limited success so far. Co-culture of the symbionts and sponge cells may be required, if the bioactive compound is partly synthesized by the sponge and partly by the symbiont. The current genomics revolution will provide novel approaches for cultivation of sponge cells and symbionts.

This thesis focuses on gaining insight in cell proliferation and cell death of sponge cell cultures and making the first step towards a continuous sponge cell culture.

### Cell proliferation and apoptosis in primary sponge cell cultures

A possible reason for the limited number of primary sponge cell cultures, is that the sponge cells are already in a non-proliferative state at the start of the culture, either because they were taken from a sponge in a resting non-proliferative state or as a consequence of the dissociation process and taking the cells out of their multicellular environment.

In **chapter 3**, we evaluated the potential of flow cytometric cell cycle analysis (by staining DNA with propidium iodide) to gain more insight in the proliferative state of sponge cells. Flow cytometric cell cycle analysis allows for a quick determination of the proliferative status of a cell population. This method makes it possible to distinguish between apoptotic, G1/G0, S, and G2/M phase cells.

The five sponges studied (*Haliclona oculata*, *Haliclona xena*, *Dysidea avara*, *Axinella polypoides* and *Xestospongia muta*) all showed a large fraction of cells in G1/G0 compared to G2/M and S, indicating that cells were not actively dividing. In addition, some sponges also showed a large apoptotic fraction, indicating cell death. Additional apoptosis measurements, based on caspase activity, showed that harvesting and dissociation of sponge tissue to initiate a primary cell culture was directly correlated with an increase in apoptotic cells. This indicates that for the development of cell cultures, more attention should be given to harvesting, dissociation and the quality of the starting material.

In addition, we measured the cell cycle distribution and caspase activities of cells from *H. oculata* during cell cultivation to study the change in distribution of cells over time. We found that cultivation conditions used were ineffective for proliferation, since after two days of cultivating *H. oculata* cells, most cells shifted towards the apoptotic fraction, indicating that cells were dying.

For development of *in vitro* sponge cell cultures, flow cytometric cell cycle analysis is a useful method to assess the proliferative state of a sponge cell culture and can be used to validate improvements in harvesting and dissociation, to select sponges with good proliferative capacities and to study the influence of culture conditions for stimulating cell growth.

### Sponge cell versus tissue culture

In **chapter 3**, we showed that harvesting and dissociation of sponge tissue to initiate a primary cell culture was directly correlated with an increase in apoptotic cells. A possible explanation is the cellular stress caused by the dissociation process and the loss of cell-cell and cell-matrix contact. To prevent these stress factors, *in vitro* tissue culture could be a solution.

In **chapter 4**, we compared *in vitro* tissue culture with cell culture for three species of marine sponges (*D. avara*, *Crambe crambe* and *H. oculata*) to determine if keeping cells in their natural 3-dimensional environment results in better cell proliferation and viability. We also compared proliferation capacity of tissue fragments cultured in the sea (*in situ* tissue culture) with fragments cultured in the lab (*in vitro* tissue culture). We used the developed methods from **chapter 3** to measure cell proliferation and cell death.

Our results demonstrated that the success of cell or tissue culture is first of all species dependent. Cell cultures and tissue cultures (*in vitro* and *in situ*) of *C. crambe* were all unsuccessful, probably caused by the release of toxins, while tissue culture (*in situ*) of *D. avara* was successful.

For all three studied sponges *in vitro* tissue culture did not result in better cell proliferation and viability than cell culture, which is in contradiction with our hypothesis. For *D. avara in situ* tissue culture was more favorable than *in vitro* tissue culture, which is probably caused by the constant supply of nutrients and flushing away of waste products.

Finally, results demonstrated that sponge cells in culture have the potential for the development of a sponge cell line. Cell cycle data of *H. oculata* cell cultures indicated that a subpopulation of cells, possibly archaeocytes or spherulous cells, remained intact and did not become apoptotic, and cell cultures of *D. avara* showed evidence of dividing cells.

For future directions, we suggest to continue research with sponges which have proliferative capacities *in vitro*. In addition, it will be important to first understand the factors that can explain the difference between growth *in situ* and the absence of growth and occurrence of cell death *in vitro*. The current genomics revolution can contribute in gaining more insight in cell proliferation and death in sponge cultures as is described further in **chapter 6**.

### Towards immortalization of sponge cells

Generally, continuous cell lines are obtained from spontaneously immortalized primary cell cultures (e.g., random mutagenesis) or are derived from cancerous tissue. However, spontaneous immortalization requires the possibility to have many primary cultures, which on this moment is not possible. Also cancerous tissue has not been observed in sponges. Another approach towards developing a continuous cell line is the insertion and expression of immortalizing genes (e.g., SV40LT, hTERT) in sponge cells. Immortalizing genes interfere with the regulatory pathways for cell



division, and in this way may cause unlimited cell division, resulting in a continuous cell line. To successfully introduce and express heterologous genes (i.e., transfection) in sponge cells, the following requirements must be met: (i) metabolically active primary cells (preferably proliferating cells), (ii) culture medium that supports cell growth (iii) a promoter that is recognized by the RNA polymerase of the sponge, (iv) an effective method to insert genes, and finally (v) a method to obtain stable insertion of the genes in the sponge genome.

In **chapter 5**, we compared different methods for the insertion and expression of heterologous genes in sponge cells as a first step towards development of an immortalized sponge cell line.

Obtaining metabolically active and proliferating sponge cells can be problematic, since, as demonstrated in **chapter 3** and **4**, sponge cells *in vitro* generally lack the capacity for proliferation. Exceptions are freshwater sponge gemmules and marine sponge larvae, of which the cells can proliferate *in vitro* and grow into a juvenile sponge. The cells from larvae and gemmules have stem cell properties and are metabolically active cells and are therefore a good target for transfection. Hence, in **chapter 5** we used the proliferating cells from freshwater gemmules next to primary cell cultures of a marine sponge.

We tested three gene delivery systems, lipofection, particle bombardment and viral transduction, in juveniles of the freshwater sponge *Ephydatia fluviatilis*, and lipofection was tested in primary cell cultures of the marine sponge *H. oculata*. To visualize transfection efficacy, we used the green fluorescent protein (GFP) reporter gene. To test promoter recognition by sponge RNA polymerase, we tested the cytomegalovirus (CMV) and OpIE2 promoter, which are generally used for transfection of mammalian cells and insect cells, respectively.

Lipofection in primary cell cultures of *H. oculata* was unsuccessful. From **chapter 3**, we learned that *H. oculata* cells in culture were non-proliferating and became apoptotic. We expect that is also the reason for the unsuccessful lipofection.

Neither viral transduction (lentivirus) nor particle bombardment resulted in heterologous gene expression in *E. fluviatilis*. Lentivirus was not able to integrate its genome into the genome of the sponge cells. We were able to deliver gold particles coated with DNA into the cells, but expression of GFP was not observed, probably due to the decreased viability of the juveniles, caused by the “harsh” method.

Lipofection, on the other hand, resulted in high expression levels of the heterologous gene in juveniles of *E. fluviatilis*, which indicates that the RNA polymerase of some freshwater sponges is able to recognize the CMV promoter. However, our results were difficult to reproduce, with only two successful attempts out of a total of 20. These inconsistent transfection results were likely not related to the lipofection method, since the lipofection reagent was able to consistently deliver large amounts of a cell-impermeable probe into sponge cells, without being toxic to the cells.

Although we do not yet understand what other factors are affecting transfection results, our comprehensive comparison of methods for insertion and expression of heterologous genes in both freshwater and marine sponges forms the basis for continued research on development of a transformed cell line, using suitable target sponges (i.e., juveniles of freshwater sponges), gene delivery method (i.e., lipofection), and promoter (i.e., CMV).

### Genomics revolution and sponge cell culture

Recently, the first genome of a sponge has been sequenced, and information about genes specific for control of cell proliferation and death were revealed. These genes play a significant role in regulating cell growth, which is crucial for multicellular animals, since unlimited growth of cells (= cancer) is detrimental for the other cells and the organism as a whole. For the development of a continuous cell line, the possibility to attain unlimited growth is per definition needed. Although many genes involved in the development of cancer are also present in sponges, cancerous growth in sponges has not been observed yet, neither *in situ* nor *in vitro*. In **chapter 6**, we focus on how we can use genomic and transcriptomic approaches to get more insight in genes involved in cell proliferation and cell death and the opportunities for developing a continuous cell line.

First of all, we need to identify a few model sponges for the development of a sponge cell line. By using only a few model species, researchers can build upon each other's result, without repeating the same experiments for other sponge species. In addition, we recommend to focus initially on genes involved in cell proliferation and death (such as p53, pRb,  $\beta$ -catenin, APC, TERT and cadherin), since these genes are crucial for homeostasis in multicellular animals and are expected to be quite generic for all sponges, meaning that results can be translated to other species as well. Finally, we provide some recommendations for approaches to study and manipulate gene expression (such as overexpression and RNAi) in sponges, based on our own research as well as other studies.

## SAMENVATTING

Mariene sponzen zijn een rijke bron van bioactieve stoffen met potentiële farmaceutische toepassingen. De laatste 40 jaar zijn meer dan 7000 nieuwe bioactieve stoffen uit sponzen ontdekt en daarmee zijn ze de meest productieve bron van nieuw ontdekte bioactieve stoffen. Ondanks het potentieel van deze stoffen uit sponzen, zijn slechts een aantal met succes ontwikkeld tot een product. Een belangrijk obstakel is het gebrek aan voldoende aanbod van biologisch materiaal voor de preklinische en klinische fase van medicijnontwikkeling. Een mogelijkheid om materialen te leveren voor de eerste stappen in medicijnontwikkeling is de productie van spons, sponsweefsel, sponscellen en/of spons-symbionten. De belangrijkste uitdaging hierbij is om algemene technieken voor kleinschalige productie van sponsbiomassa te ontwikkelen.

In **hoofdstuk 2** analyseren we de “state-of-the-art” met betrekking tot de kweek van hele sponzen, sponscellen en spons-symbionten. Tevens presenteren we de vooruitzichten van de ontwikkeling en integratie van de verschillende kweektechnieken. Afhankelijk van het product kunnen verschillende kweektechnieken toegepast worden. Indien de concentratie van het product hoog is in de spons zelf, dan zal *in situ* kweek van gehele sponzen de meest succesvolle aanpak zijn. Echter, optimale kweekcondities zijn soortspecifiek en gedurende *in situ* kweek kunnen kweekcondities niet worden gecontroleerd. Bovendien zijn veel secundaire metabolieten alleen aanwezig in lage concentraties en om een economisch haalbaar productieplatform te verkrijgen, zullen product concentraties verhoogd moeten worden. Een alternatieve keuze kan dan zijn om *in vitro* kweek, zoals cel- of weefselkweek, toe te passen. *In vitro* kweek maakt het mogelijk om specifieke kweekomstandigheden in te stellen en te controleren om zo product concentraties te kunnen verhogen. Tot nu toe is de ontwikkeling van een sponscellijn niet succesvol gebleken. Dit wordt vooral veroorzaakt doordat we niet in staat zijn axenisch sponsmateriaal te verkrijgen en mede door gebrek aan kennis over factoren die proliferatie en dood van sponscellen *in vitro* kunnen beïnvloeden evenals gebrek aan kennis over hun voedingsbehoeften. Benaderingen om deze knelpunten te ondervangen, inclusief transformatie van sponscellen en ontwikkeling van media gebaseerd op ‘yolk’, worden beschreven. Indien het gewenste product wordt geproduceerd door een microbiële symbiont, dan zou de productie door alleen de symbiont gedaan kunnen worden. Verscheidene metabolietproducerende micro-organismen zijn al succesvol geïsoleerd uit sponzen, maar vooralsnog heeft de kweek van deze sponsspecifieke micro-organismen beperkt succes. Co-cultuur van de symbionten en sponscellen kan nodig zijn wanneer de bioactieve stof gedeeltelijk wordt gesynthetiseerd door de spons en gedeeltelijk door de symbiont. De huidige genomics-revolutie zal nieuwe benaderingen voor de kweek van sponscellen en symbionten bieden.

Dit proefschrift richt zich op het verkrijgen van inzicht in de celproliferatie en celdood van sponscellen gedurende *in vitro* kweek en het maken van de eerste stap op weg naar een continue sponscellijn.

### Celproliferatie en apoptose in primaire sponscelkweken

Een mogelijke reden voor het beperkte aantal ontwikkelde primaire sponscelkweken, is dat de sponscellen reeds in een niet-proliferatieve staat zijn aan het begin van de kweek. Hetzij omdat de cellen geïsoleerd zijn uit een spons die in een rustende, niet proliferatieve staat was of als gevolg van het dissociatie proces waarbij de cellen uit hun meercellige omgeving worden gehaald.

In **hoofdstuk 3** hebben we de mogelijkheden van flowcytometrische celcyclus analyse (door kleuring van DNA met propidiumjodide) geëvalueerd om meer inzicht te krijgen in de proliferatieve staat van sponscellen. Flowcytometrische celcyclus analyse maakt het mogelijk om snel de proliferatieve status van een celpopulatie te bepalen. Deze methode maakt het mogelijk om onderscheid te maken tussen cellen in de apoptotische, G1/G0, S en G2/M fase.

De vijf onderzochte sponzen (*Haliclona oculata*, *Haliclona xena*, *Dysidea avara*, *Axinella polypoides* en *Xestospongia muta*) hadden allen een groot deel van hun cellen in de G1/G0 fase ten opzichte van G2/M en S, wat aantoont dat de cellen niet actief aan het delen waren. Bovendien hadden een aantal sponzen ook een grote fractie van hun cellen in de apoptotische fase, wat celdood impliceert. Additionele apoptose metingen, gebaseerd op caspase activiteit, lieten zien dat het oogsten en dissociëren van sponsweefsel om een primaire sponscel kweek te verkrijgen, direct gerelateerd waren aan de toename van apoptotische cellen. Dit geeft aan dat bij de ontwikkeling van sponscellijnen, meer aandacht besteed moet worden aan het oogsten, dissociëren en de kwaliteit van het uitgangsmateriaal.

Tevens hebben we de celcyclusverdeling en de caspase activiteit van *H. oculata* cellen tijdens celkweek gemeten om de verandering in de celcyclusverdeling gedurende de tijd te bestuderen. We konden vaststellen dat de toegepaste kweekomstandigheden ineffectief waren voor proliferatie, aangezien na twee dagen kweken van *H. oculata* cellen, de meeste cellen verschoven naar de apoptotische fractie, wat aangeeft dat de cellen aan het sterven waren.

Voor de ontwikkeling van *in vitro* sponscelkweken is de flowcytometrische celcyclus analyse een bruikbare methode om de proliferatieve staat van een sponscelkweek snel vast te stellen. Tevens kan de methode gebruikt worden om verbeteringen te valideren met betrekking tot het oogsten en dissociëren, om sponzen met goede

proliferatieve capaciteit te selecteren en om de invloed van de kweekomstandigheden met betrekking tot het stimuleren van celgroei te bestuderen.

### Sponscel versus sponsweefselkweek

In **hoofdstuk 3** is reeds aangetoond dat het oogsten en dissociëren van sponsweefsel om een primaire celkweek te initiëren direct gerelateerd was aan een toename van apoptotische cellen. Een mogelijke verklaring is de cellulaire stress die veroorzaakt wordt door het dissociatie proces en het verlies van cel-cel en cel-matrix contact. Om deze stressfactoren te vermijden zou *in vitro* weefselkweek een oplossing zijn.

In **hoofdstuk 4** vergeleken we daarom *in vitro* weefselkweek met *in vitro* celkweek voor drie verschillende mariene sponzen (*D. avara*, *Crambe crambe* en *H. oculata*) om vast te stellen of het behouden van de cellen in hun 3-dimensionale omgeving resulteert in betere celproliferatie en viabiliteit. Tevens vergeleken we de proliferatie capaciteit van sponsweefsel gekweekt in de zee (*in situ* weefselkweek) met sponsweefsel gekweekt in het lab (*in vitro* weefselkweek). De methodes ontwikkeld in **hoofdstuk 3** werden gebruikt om celproliferatie en celdood te meten.

Onze resultaten lieten zien dat het succes van cel- of weefselkweek in eerste instantie soortspecifiek is. Cel- en weefselkweken (*in vitro* en *in situ*) van *C. crambe* waren beiden niet succesvol, waarschijnlijk veroorzaakt door aanwezige toxines, terwijl weefselkweek (*in situ*) van *D. avara* succesvol was.

Voor alle drie onderzochte sponzen resulteerde *in vitro* weefselkweek niet in betere celproliferatie en viabiliteit dan *in vitro* celkweek, wat in tegenspraak is met onze hypothese. De *in situ* weefselkweek van *D. avara* was gunstiger dan de *in vitro* weefselkweek, waarschijnlijk veroorzaakt door de constante toevoer van voedingsstoffen en het wegspoelen van afvalstoffen gedurende *in situ* kweek.

Tot slot lieten onze resultaten zien dat sponscellen *in vitro* potentie hebben om te ontwikkelen tot een sponscellijn. Celcyclus data van *H. oculata* celkweken duiden aan dat een subpopulatie van cellen, mogelijk archaeocyten of "spherulous" cellen, intact bleven en niet apoptotisch werden. Tevens toonden celtellingen van *in vitro* celkweken van *D. avara* aan dat cellen kunnen delen.

Voor toekomstig onderzoek raden wij aan om sponzen te gebruiken die proliferatieve capaciteit gedurende *in vitro* kweek hebben. Tevens zal het belangrijk zijn om eerst te begrijpen welke factoren het verschil tussen groei gedurende *in situ* kweek en de afwezigheid van groei in combinatie met celdood gedurende *in vitro* kweek kunnen verklaren. De huidige genomics-revolutie kan bijdragen in het verkrijgen van meer

inzicht in celproliferatie en celdood in sponscelkweken, zoals wordt beschreven in **hoofdstuk 6**.

### Op weg naar immortalisatie van sponscellen

In het algemeen worden continue cellijnen verkregen vanuit spontaan geïmmortaliseerde primaire celkweken (bijv. “random mutagenesis”) of worden geïsoleerd uit kankerweefsel. Echter, spontane immortalisatie vereist de mogelijkheid om veel primaire celkweken te kunnen verkrijgen, wat op dit moment nog niet mogelijk is voor sponscellen. Ook kankerweefsel is nog niet waargenomen bij sponzen. Een andere aanpak om een continue cellijn te ontwikkelen is het inbrengen en het tot expressie krijgen van immortalisatie genen (bijv. SV40LT, hTERT) in sponscellen. Immortalisatie genen beïnvloeden de regulatie van celdeling, wat kan leiden tot ongelimiteerde celdeling, en vervolgens kan resulteren in een continue cellijn. Om succesvol heterologe genen in sponscellen te brengen en tot expressie te krijgen (bijv. transfectie), zal aan de volgende eisen moeten worden voldoen: (i) metabool actieve cellen (bij voorkeur delende cellen), (ii) kweekmedium dat celgroei ondersteunt, (iii) een promotor dat wordt herkend door RNA polymerase van de spons, (iv) een effectieve methode om genen in te brengen, en tot slot (v) een methode om de genen in het sponsgenoom te brengen voor stabiele expressie.

In **hoofdstuk 5** vergeleken we verschillende methoden om heterologe genen in sponscellen te introduceren en tot expressie te krijgen om zo een eerste stap naar de ontwikkeling van een geïmmortaliseerde sponscellijn te maken.

Het verkrijgen van metabool actieve en prolifererende sponscellen kan problematisch zijn, aangezien, zoals in **hoofdstuk 3** en **4** is aangetoond, *in vitro* sponscellen doorgaans niet het vermogen tot proliferatie hebben. Uitzonderingen zijn gemmulae van zoetwatersponzen en larven van mariene sponzen, waarvan de cellen *in vitro* kunnen prolifereren en uitgroeien tot een juveniele spons. De cellen van larven en gemmulae hebben stamcel eigenschappen en zijn metabool actief en zijn daarom een goed doelwit voor transfectie. Vandaar dat we in **hoofdstuk 5** naast primaire celkweken van mariene sponzen ook de delende cellen van zoetwater gemmulae hebben gebruikt.

We hebben drie gen introductiesystemen, lipofectie, “particle bombardment” en virale transductie, getest in juvenielen van de zoetwaterspons *Ephydatia fluviatilis* en alleen lipofectie was getest in primaire celkweken van de mariene spons *H. oculata*. Om de transfectie efficiëntie te testen, gebruikten we het “green fluorescent protein” (GFP) reporter gen. Om te beoordelen of de promotor herkend werd door het spons RNA polymerase, hebben we de cytomegalovirus (CMV) en de OpIE2 promotor

getest, welke doorgaans gebruikt worden voor transfectie van respectievelijk zoogdiercellen en insectencellen.

Lipofectie in primaire celkweken van *H. oculata* was niet succesvol. **Hoofdstuk 3** liet zien dat *H. oculata* cellen in kweek niet-prolifererend waren en apoptotisch werden. We verwachten dat dit ook de reden is voor de niet succesvolle lipofectie.

Noch virale transductie (m.b.v. lentivirus), noch “particle bombardment” resulteerde in heterologe genexpressie in *E. fluviatilis*. Lentivirus was niet in staat zijn genoom te integreren in het genoom van de sponscellen. We waren in staat om gouddeeltjes met een laagje van DNA te introduceren in de cellen, maar expressie van GFP werd niet waargenomen, waarschijnlijk door een verminderde viabiliteit van de juvenielen veroorzaakt door de ruwe methode.

Lipofectie resulteerde daarentegen tot hoge expressieniveaus van het heterologe gen in juvenielen van *E. fluviatilis*, wat aangeeft dat het RNA polymerase van zoetwater sponzen de CMV promotor kan herkennen. Echter, de resultaten waren moeilijk te reproduceren met slechts twee succesvolle pogingen op een totaal van 20. Deze inconsistente transfectie resultaten zijn waarschijnlijk niet gerelateerd aan de lipofectie methode, omdat het lipofectiereagens steevast grote hoeveelheden van een cel-on-doorlaatbare probe in de sponscellen kon introduceren zonder toxisch te zijn voor de cellen.

Hoewel we nog niet begrijpen welke andere factoren onze transfectie resultaten beïnvloeden, vormt onze uitgebreide vergelijking van methoden voor het introduceren en tot expressie krijgen van heterologe genen in zowel zoet- als mariene sponzen de basis voor verder onderzoek naar de ontwikkeling van een getransformeerde cellijn, gebruik makende van geschikte sponzen (bijv. juvenielen van zoetwatersponzen), gen introductiesystemen (bijv. lipofectie) en promotoren (bijv. CMV).

### Genomics-revolutie en sponscelkweken

Recent is het eerste genoom van een spons gesequenced en werd informatie over genen die specifiek zijn voor de controle van celproliferatie en dood verheld. Deze genen spelen een belangrijke rol bij het reguleren van celgroei, wat cruciaal is voor meercellige dieren, aangezien onbeperkte celgroei (= kanker) nadelig is voor de andere cellen en het organisme als geheel. Voor de ontwikkeling van een continue cellijn, is de mogelijkheid om onbeperkte groei te bereiken noodzakelijk. Hoewel veel genen die betrokken zijn bij de ontwikkeling van kanker ook zijn gevonden in sponzen, is nog geen kankerweefsel in sponzen waargenomen, noch *in situ* noch *in vitro*. In **hoofdstuk 6**, richten we ons op de vraag hoe we genomische en transcriptomische

benaderingen kunnen gebruiken om meer inzicht te krijgen in genen die betrokken zijn bij celproliferatie en celdood en de mogelijkheden voor het ontwikkelen van een continue cellijn.

Allereerst is het belangrijk om enkele modelsponzen te identificeren voor de ontwikkeling van een sponscellijn. Door het gebruik van slechts een paar modelsponzen, kunnen onderzoekers bouwen op elkaars resultaat, zonder herhaling van hetzelfde experiment voor andere sponssoorten. Daarnaast raden we aan om in eerste instantie te focussen op genen betrokken bij celproliferatie en dood (zoals p53, pRb,  $\beta$ -catenin, APC, TERT en cadherin), omdat deze genen cruciaal zijn voor homeostase in meercellige dieren en naar verwachting behoorlijk generiek zijn voor alle sponzen, waardoor de resultaten vertaald kunnen worden naar andere sponssoorten.

Tot slot geven we een aantal aanbevelingen voor benaderingen om genexpressie (zoals overexpressie en RNAi) in sponzen te bestuderen en te manipuleren op basis van eigen onderzoek en studies van anderen.

## DANKWOORD

Eindelijk is het zover, het boekje is gereed en ligt voor je klaar om gelezen te worden! Alleen was me dit natuurlijk nooit gelukt en dus is het de hoogste tijd om eenieder die direct of indirect een bijdrage heeft geleverd hartelijk te bedanken.

Ten eerste wil ik natuurlijk mijn drie begeleiders bedanken. **René**, bedankt voor het mogelijk maken van dit project, niet alleen op inhoudelijk en financieel vlak, maar ook qua logistiek. Je was altijd bereid om mijn duikbuddy te zijn als ik weer eens sponzen nodig had uit de Oosterschelde of uit de Middellandse Zee. Soms werd er wel een beetje tegengesputterd als we weer eens voor dag en dauw op pad waren om op het getij in de Oosterschelde te kunnen duiken, maar toch ging je altijd trouw mee. Bedankt daarvoor! Ook zal ik de gezelligheid (top500 popquiz in de auto op weg naar Spanje), de duiktradities (vissoep eten na afloop van de duik) en het gezamenlijk afzien (bij 0 graden duiken in de Grevelingen) niet vergeten! Ook wil ik **Dirk** bedanken voor zijn essentiële bijdrage. Als dagelijks begeleider stond je altijd voor me klaar en wist je er altijd voor te zorgen dat er meer structuur in mijn experimenten en papers kwam. Ik vond je een bijzonder prettig persoon om mee samen te werken en ik heb veel van je werkwijze kunnen leren. And last but definitely not least, I would like to thank **Shirley** for her contribution. Thank you for teaching me the principles of sponge cell culture and your everlasting enthusiasm and support concerning this project. I hope we will stay in touch and will collaborate in the future again!

Naast het werk dat ik bij Bioproceskunde heb gedaan, was ik ook veel bij Virologie te gast om daar het moleculair werk uit te voeren. **Gorben** en **Corinne**, ontzettend bedankt dat jullie me altijd zo goed geholpen hebben. Dankzij jullie is hoofdstuk 5 een feit! Daarnaast wil ik ook **Mia**, **Stefan**, **Jelke** en **Esther** bedanken voor de gezelligheid bij Virologie. Dankzij jullie ging ik altijd met veel plezier naar Virologie om mijn monsters te analyseren.

Het eerste jaar tijdens mijn project heb ik ook samengewerkt met Microbiologie. Ik wil graag **John van der Oost** bedanken voor zijn bijdrage gedurende de eerste project meetings, **Ans Geerlings** bedanken voor het helpen met de transformaties en **Hans Heilig** bedanken voor zijn hulp bij het DGGE werk.

During this project we also worked with Mediterranean sponges. To make this possible, we always got a lot of help from **Iosune Uriz** and **Sònia de Caralt**. I would like to thank you both for all the help you offered in the lab, in the sea and for the nice lunches and dinners we had together. It was always great to visit you in Blanes!



De laatste twee jaar werkte ik ook met gemmulae van zoetwatersponzen. Dankzij **Nico Taverne** had ik toegang tot deze sponzen. Bedankt Nico dat je al die keren met mij bent wezen duiken in de plas bij Mill om gemmulae te verzamelen. Het was altijd erg gezellig!

Een persoon die ook een belangrijke rol heeft gespeeld in mijn "sponscarrière" is **Detmer**. Dankzij mijn stage onder begeleiding van jou in Berkeley, is mijn fascinatie voor dit interessante dier ontstaan. Zelfs na die tijd stond je nog steeds voor me klaar als ik feedback nodig had over mijn onderzoek. Bedankt daarvoor!

De vakgroep Proceskunde voelde altijd als een tweede thuis. Velen waren niet alleen collega's maar ook goede vrienden. **Dorinde**, we zijn als kamergenootjes begonnen, en zijn uiteindelijk goede vriendinnen geworden. Samen met Sina en Koen hebben we vele vakgroep-borrels georganiseerd. Daarnaast was onze wandelreis in Turkije (Antalya of Ankara? ☺) ook een fantastische ervaring. Ook al zijn we nu geen collega's meer, ik hoop dat we nog veel leuke dingen samen zullen blijven doen. **Sina**, je bleef me maar achtervolgen, eerst Berkeley, toen de vakgroep en toen zelfs op de Hoogstraat. Maar dat was helemaal niet erg, want alle keren hebben we een erg leuke tijd gehad. Je bent een waardevolle vriend! **Pieter**, ik heb jou pas echt leren kennen tijdens de laatste jaren van mijn promotie. Ik moet altijd erg lachen om je quasi gemopper. Bedankt voor de gezellige pannenkoek en sushi avondjes! **Maria C**, you were not only my colleague, but also my flatmate and a very good friend. Together with **Angel**, you showed me the good things from Spain: nice food, hospitality and good parties. I hope we will stay in touch! **Miranda**, ik denk niet dat een vakgroep zich een fijnere secretaresse kan wensen dan jij. Je bent altijd hulpvaardig, goedgehumt en erg sociaal. Ik zal onze kletsuurtjes (euh, kwartiertjes) op de vakgroep missen! **Rik**, bedankt dat je samen met mij, **Sina** en **Miranda** de PhD-trip naar Colorado en San Diego hebt willen organiseren. Het was een hele klus om alles in goede banen te leiden, maar met z'n vieren waren we een goed team en wisten we dit klusje te klaren! Ik heb erg genoten van onze samenwerking. **Fred**, **Wendy** en **Sebas**, bedankt voor jullie hulpvaardigheid in het lab en voor het doen van al die rare bestellingen van mij. Ook wil ik het BPE-gelegenheidsfietsclubje, **Mathieu**, **Marjon**, **Anne**, **Lenny** en **Ward**, bedanken. Dankzij jullie bleef ik een beetje fit. Ik hoop dat ik in het nieuwe fietsseizoen nog steeds mee mag, ook al ben ik geen BPE-er meer. Ook een bijzondere vermelding voor **Lenneke**, **Jeroen** en **Jacqueline**, die de borrelcommissie zo goed draaiende hebben gehouden, chapeau! Daarnaast wil ik natuurlijk alle **Bio's** en **Foodies** bedanken voor alle gezelligheid tijdens de koffie- en lunchpauzes, de PhD-trip en uiteraard de vrijdagmiddag borrels. Ik stel voor dat we het laatste gewoon blijven doen!

En dan mijn studievriendjes waar ik gelukkig nog altijd een erg goed contact mee heb. **Julia**, bedankt dat je zo'n lieverd en gezelligerd bent! Wij hebben altijd leuke feestjes samen, maar we kunnen ook de belangrijke dingen bespreken. **Joeri**, buurman, wat een toeval dat je een paar deuren verderop kwam wonen. Je bent nu niet alleen een goede vriend, maar ook de beste buurman die ik me maar kan wensen! **Ronald**, jij maakt ons clubje compleet. Dankzij jou praten we niet alleen maar over DNA-dingetjes ☺, bedankt daarvoor!

**Margo**, we hebben elkaar leren kennen in de interliner tijdens ons eerste studiejaar (blijft een leuk verhaal ☺), en zijn sindsdien altijd goede vriendinnen gebleven. Ik hoop dat dat nog heel lang zo blijft! **Aafke**, **Silvia** en **Linette**, het is heerlijk om met jullie "bloedblaren en ander damesleed" te bespreken! En dan mijn trouwe thuis-thuis vriendinnetjes, **Marieke**, **Ellen** en **Yvonne**. Na al die jaren (18 jaar!) nog steeds goed bevriend, dat is toch wel bijzonder.

**Marieke**, alias **Coby**, wat hebben wij veel samen gedaan de eerste jaren tijdens mijn promotie! Samen spons-aio zijn, samen volleyballen, samen op duikvakantie en zelfs samenwonen. Wat een fantastische tijd was dat! Helaas kan ik je nu niet meer zo vaak zien nu je in Mexico woont, maar ik twijfel er geen moment aan dat onze vriendschap zal blijven bestaan.

Toen ik in Wageningen begon met mijn promotie, heb ik ook **Linette** leren kennen en dat had ik niet willen missen. We hebben samen veel leuke dingen gedaan, wandelen in Lapland, fietsen naar Basel en door Mexico toeren samen met **Marieke**. Er valt altijd wat te beleven met jou en zonder jou is Wageningen niet compleet! Bedankt dat je nu ook weer dit avontuur met mij wilt aangaan door mijn paranimf te zijn.

En dan is er natuurlijk mijn familie. **Henny**, grote zus, super dat je met mij op het podium wilt staan als paranimf. Je bent niet alleen mijn grote zus, maar ook een erg goede vriendin. **Paps** en **mams**, bedankt voor jullie steun en vertrouwen en dat jullie me altijd hebben gestimuleerd om verder te studeren. Ik denk dat ik nu wel op eigen benen kan staan, maar ik zal nog altijd graag thuis komen om me te laten vertroetelen.

**Rick**, jij bent degene waar ik afgelopen jaren lief en leed mee gedeeld heb. We hebben samen al veel avonturen meegemaakt, vooral leuke, en soms ook wat minder leuk. Bedankt dat je altijd voor me klaar stond. Ik hoop dat we in de toekomst nog veel avonturen samen mogen beleven! Ik heb er in ieder geval zin in.

## CURRICULUM VITAE

Klaske Janneke Schippers was born in Lelystad, the Netherlands on 20 June, 1982. In 1994 she started secondary school in Emmeloord at the Emelwerda College, where she received her VWO diploma in 2000. In the same year, she started studying Biotechnology, BSc and MSc, at Wageningen University. She finalized her BSc with a minor in Microbiology performed at Environmental Biotechnology at TU Delft. Her MSc thesis focused on optimizing the biomass yield of green algae on light energy, which was performed at Food and Bioprocess Engineering at Wageningen University. She finalized her MSc with an internship at Chemical Engineering at the University of California Berkeley. During this internship she developed novel methods to cultivate unculturable symbiotic bacteria from marine sponges and got acquainted with the wonderful world of marine sponges. In June 2007 she received her MSc degree with specialization Process Technology. In the same year, she started her PhD entitled "Sponge cell culture". After her PhD she took a short break and cycled three months through Canada and Alaska. Currently she is working at Synthon Biopharmaceuticals in Nijmegen, as cell culture researcher.

## LIST OF PUBLICATIONS

- Zijffers, J.F., Schippers, K.J., Ke Zheng, Janssen, M.G.J., Tramper, J. and Wijffels, R.H. (2010). Maximum photosynthetic yield of green microalgae in photobioreactors. *Mar Biotechnol*, 12: 708–718.
- Sipkema, D., Schippers, K., Maalcke, W. J., Yang, Y., Salim, S., and Blanch, H. W. (2011). Multiple approaches to enhance the cultivability of bacteria associated with the marine sponge *Haliclona (gellius)* sp. *Appl Environ Microbiol*, 77: 2130–2140.
- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. (2011). Cell cycle analysis of primary sponge cell cultures. *In Vitro Cell Dev Biol- Anim*, 47: 302-311.
- Schippers, K.J., Sipkema, D., Osinga, R., Smidt, H., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. (2012). Cultivation of sponges, sponge cells and symbionts: achievements and future prospects. *Adv Mar Biol*, 62: 273-337.
- Schippers, K.J., Martens, D.E., Pomponi, S.A. and Wijffels, R.H. Toward development of a sponge cell line: comparison of cell proliferation in sponge cell and tissue cultures. *Submitted for publication*.
- Schippers, K.J., Geertsema, C., Pijlman, G.P., Pomponi, S.A., Martens, D.E. and Wijffels, R.H. Methods for insertion and expression of heterologous genes in sponge cells. *Submitted for publication*.
- Schippers, K.J., Martens, D.E., Pomponi, S.A., and Wijffels, R.H. Genomic-based approach for sponge cell culture. *Submitted for publication*.

## OVERVIEW OF COMPLETED TRAINING ACTIVITIES

### *Discipline specific activities*

HBOI summer internship (Fort Pierce, USA, 2007)  
Sponge minisymposium<sup>1</sup> (Wageningen, The Netherlands, 2008)  
Workshop IPOP Kust en Zee<sup>1</sup> (Wageningen, The Netherlands, 2009)  
Molecular biology techniques course (Groningen, The Netherlands, 2009)  
Olympus microscopy course (Zoeterwoude, The Netherlands, 2009)  
8<sup>th</sup> World sponge conference<sup>1</sup> (Girona, Spain, 2010)  
NBV Animal cell culture minisymposium<sup>1</sup> (Wageningen, The Netherlands, 2010)

### *General courses*

PhD week VLAG (Bergeijk, The Netherlands, 2008)  
Teaching and supervising thesis students (Wageningen, The Netherlands, 2009)  
Project and time management (Wageningen, The Netherlands, 2010)  
Career orientation (Wageningen, The Netherlands, 2011)  
Writing grant proposals (Wageningen, The Netherlands, 2011)

### *Optionals*

BSDL 3 symposium<sup>2</sup> (Delft, The Netherlands, 2007)  
12<sup>th</sup> Netherlands Biotechnology Conference (Ede, The Netherlands, 2008)  
Symposium "Microspectroscopy in Biology" (Wageningen, The Netherlands, 2008)  
Brainstormday Bioprocess Engineering (2008, 2009, 2010, 2011, 2012<sup>1</sup>)  
PhD domestic excursion Intervet<sup>1,3</sup> (Boxmeer, The Netherlands, 2008)  
PhD domestic excursion Synthon (Nijmegen, The Netherlands, 2012)  
PhD foreign excursion to Japan (2008)  
PhD foreign excursion to USA<sup>3</sup> (2010)

<sup>1</sup> Presentation

<sup>2</sup> Poster

<sup>3</sup> Organization

layout: In Zicht Grafisch Ontwerp, Arnhem  
Print: Wohrmann Print Services, Zutphen