Growth and metabolism of sponges

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Chapter 1

Introduction

Introduction

Sponges

Sponges (phylum Porifera) are multi cellular filter-feeding invertebrate animals living attached to a substratum in mostly marine but also in freshwater habitats. Marine sponges inhabit a large range of marine ecosystems, from polar to tropical seas, and from shallow waters to the deepest oceans. It is estimated that over 15.000 sponge species exist worldwide (Hooper et al 2002). There is an enormous variety of sponge growth forms and colors (for several examples see page 16 and 17), which have evolved since more than 550 million years.

Sponges do not possess true tissue but have different cell types with different functions to carry out normal body functions. The morphology of a sponge is shown in Figure 1. Collagen fibers, sponge fibers and calcium carbonate or silica containing skeleton form the structure of the sponge body (Hooper and van Soest 2002). Many sponge cell types exist and their function is discussed in detail by Bergquist (1978). The two main cell types in a sponge are choanocytes and archeaocytes. The choanocytes are flagellated cells, which are used for pumping an unidirectional water current through the water canal systems that run through the sponge body. The large amount of seawater is filtered to provide the sponges with food and oxygen and to excrete waste products. The archeaocytes are totipotent (able to change into any of the sponge-cells) that are responsible for either transport or digestion of dietary and respiratory products or they can become reproductive cells etcetera. Many sponges have a symbiosis with micro-organisms, which mostly occur in the mesohyl (Lee et al 2001, Richelle-Maurer et al 2003). Symbionts include archaea, bacteria, cyanobacteria, and microalgae. Photosynthetic symbionts provide the sponge with photosynthetic nutrients that are a supplement to the nutrients obtained by their own filter-feeding activities.

Sponges reproduce either sexually or a-sexually. They are hermaphrodite, although oocytes and spermatocytes are generally produced at different times within one organism. Sperm is released by the sponge into the sea and surrounding sponges take these sperm cells up by filtering. Individuals containing oocytes use the taken sperm cells for fertilization. Subsequently, eggs are either excreted immediately or they will develop into larvae inside the sponge body before excretion. Larvae are motile and swim or crawl away from their parent before attaching to the seabed.

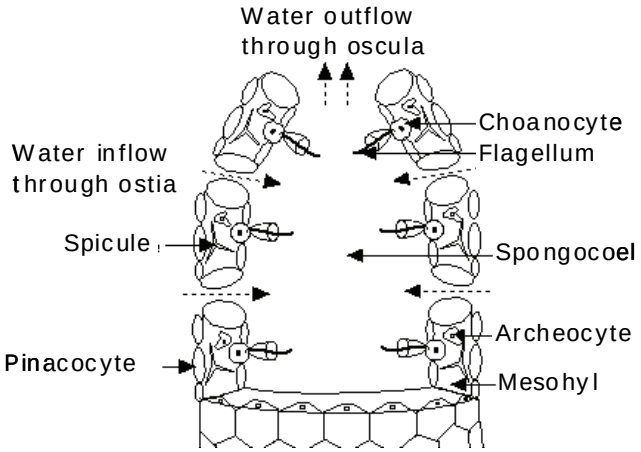


Figure 1: The inhalant and exhalant canals, called the aquiferous system, are shown. Also the different sponge cells are displayed.

The most simple way of reproduction is a-sexual by fragmentation, where the sponge fragments are able to re-attach and form a new individual, for example, after being disrupted due to a storm. Budding is another form of a-sexual reproduction. Buds are formed as a lump of cells containing all necessary cells to form a new sponge individual. They are released and the current can then take them to another location, where they attach and become a new individual. Some sponges are able to form gemmules as a survival mechanism (e.g. Wilson 1907, Fell 1974). Gemmules are formed at the base of the sponge and stay attached to the substratum, they contain mostly archeocytes that can differentiate into spongocytes for skeleton formation or they can contain nutrients. When the parental sponge dies, formed gemmules can survive until more desirable circumstances recur. Figure 2 gives a schematic representation of asexual reproduction.

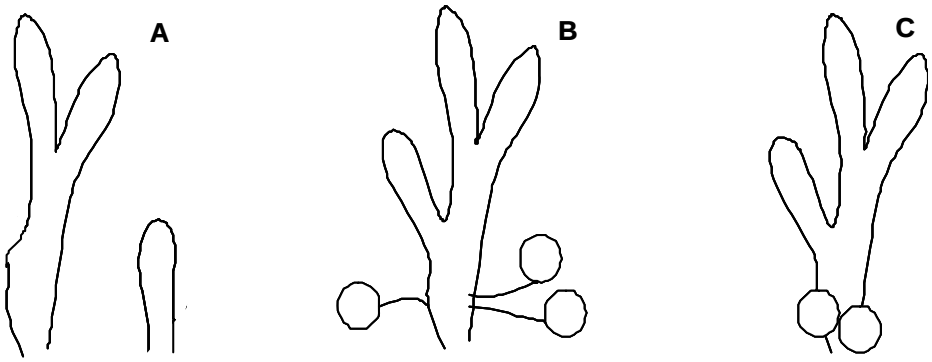


Figure 2: Schematic representation of asexual reproduction. A) fragmentation, B) budding, C) gemmule formation

Bioactive compounds

The interest in marine sponges started to increase since the 1950's, when the first marine-derived pharmaceuticals, the nucleosides spongothymidine and spongouridine, were discovered in the sponge Cryptotethya crypta (Bergmann and Freeney 1950). Many marine natural products originate from sponges and microorganisms that are associated with sponges. These chemicals can potentially be used as e.g. cytotoxic (Uriz et al 1992), antibiotic (Fattorusso et al 1972), anti-inflammatory (Alvi and Crews 1992), anti-fouling (becerro et al 1997), and antiviral (Minale et al 1974) compounds. Most marine natural products commercially available or in clinical trials are obtained through chemical synthesis (Molinski et al 2009). The natural sponge populations are not sufficiently large and/or not accessible for producing commercial quantities of metabolites of interest (Belarbi et al 2003[a]). The time from initial discovery to commercial use of a pharmaceutical is very long due to the long process of testing in clinical trials. Nevertheless, several marine derived compounds are getting close to approval by authorities and thus possible commercial use (Newman and Cragg 2004). Therefore, research is needed to find sustainable production methods for sponge metabolites.

Sponges produce these bioactive compounds as a defense mechanism against fouling, predation and to compete for space (Becerro et al 1997). As they are sessile organisms they cannot escape from predators or protect themselves by any physical movement. Moreover, sponges are mostly slow-growing organisms, which makes them easy victims of faster growing organisms such as ascidians and corals that take up the available space and nutrients. However, sponges have survived for a very long time, thus their defense mechanism must be effective.

Production techniques

Most sponge bioactive compounds are chemically complex, thus chemical synthesis is mostly impossible. To make sponge bioactive compounds commercially available as medicines, a sustainable production method needs to be developed. The concentration of bioactive compounds in the sponge body is generally very low. For example, *Lissodendorix* sp. contains concentrations of about 400µg/kg of halichondrin B. Since approximately 5 kg pure product would be needed annually for treatment, the amount of sponge biomass needed will be very high (Munro et al 1999, Sipkema et al 2005[b]). Therefore, harvesting from the sea is not feasible, since such large amounts of sponge biomass are either not available or at least collection is ecologically harmful. Strategies to produce bioactive compounds from sponges include sponge, sponge cell, sponge symbiont cultures or metagenomic approaches in which large gene fragments responsible for production of the bioactive compounds are identified and transferred into a suitable host. So far, none of the approaches resulted in applicable technologies for production of sponge bioactive compounds despite significant progress.

In order to achieve sufficient production by sponge or sponge cell culture, there are two main subjects to focus on primarily. One is to get the organism (cell or whole sponge) to grow fast and the other is to be able to stimulate production of the bioactive compound in the organism. For most bioactive compounds, however, biosynthetic pathways are unknown. Besides, for most sponges it is still unknown which (sponge) cells or microorganism is/are responsible for the production of the bioactive compound and which factors stimulate production of bioactive compounds. For several sponges (e.g. *Dysidea avara* (Uriz et al 1996[a]) and *Teichaxinella morchella* (Andrade *et al* 1999)) it is shown that the sponge cells are responsible for the metabolite pro-

duction. But in other cases it is expected that the symbionts or a combined synthesis in the symbiont and sponge is responsible for the bioactive compound production (Unson and Faulkner 1993, Dumdei et al 1998). Moreover, the inducer for bioactive compound production in the host is in most cases unknown.

Thesis outline

1

A possibility for sustainable supply of sponge compounds could be *in vitro* sponge cultivation. However, knowledge on how and how fast sponges grow and how to enhance sponge growth is limited. The aim of this thesis was to gain more knowledge about the growth and metabolism of sponges. The focus was especially on the rates of growth and metabolism in the sponge.

In chapter 2 the growth rate was measured for the sponge *Haliclona oculata* during one year in their natural habitat. We monitored the volume of 11 individuals monthly using a non invasive 3d-photogrammetry technique. Simultaneously parameters as temperature, salinity, carbon and nitrogen concentration and other nutrients were monitored in the same region. Using these data we were able to find positive and negative correlations between surrounding parameters and sponge growth rate.

Next, in chapter 3 the growth efficiency and carbon balance for *Haliclona oculata* was studied is described. We have used the growth rate data from chapter 2 and additionally measured respiration rate and filtration rate for the same sponge specimen in the field. Experimental chambers containing magnetic stirrers were developed to be able to measure *in situ* without harming the individuals. All combined data gave us the opportunity to calculate the amount of carbon used by the sponge for respiration and growth.

Chapter 4 describes the fatty acid (FA) composition of different sponges and the surrounding suspended particulate matter. One of the problems in studying sponge metabolism is that food particles reside inside the sponge and that it is unclear which compounds are still present in food particles and which are metabolized and part of the sponge. In order to gain more knowledge about the use of carbon taken up by sponges we searched for compounds that were present inside the sponge and not inside the food particles filtered from the water. Since fatty acids can be uniquely present in organisms, we studied the fatty acid composition of the sponge and the surrounding suspended particulate matter. Five sponge species were studied, *Haliclona oculata*, *Haliclona xena*, *Halichondria panicea*, *Aplysina aerophoba* and *Dysidea avara* from three different habitats. We have identified several unique fatty acid biomarkers in each sponge species.

In chapter 5 we studied the seasonal dynamics of the fatty acids inside the five sponges and in the surrounding as well. Moreover we studied the natural abundance of the stable isotopic signature of carbon (^{13}C) in the specific fatty acids at different seasons. Sponges are heterotrophic organisms and will

use different classes of organisms as food source. Sponges contain many long chain fatty acids, which are elongated from exogenously obtained fatty acids. These exogenous FAs are obtained from algal or bacterial cells. Different organisms like algae and bacteria have different ^{13}C signature and consequently the ^{13}C signature of the sponge fatty acids gives information on the food taken up by the sponge.

Chapter 6 shows the result of a ^{13}C labeling study using two sponges, *Haliclona oculata* and *Dysidea avara*. We studied the rate of conversion inside the sponges. The sponges were fed with a ^{13}C enriched diatom, *Skeletonema costatum* during eight hours and after the pulse labeling, sponge individuals were sampled at different time intervals during three weeks. Within eight hours most fed cells were taken up and after the pulse, the sponges were either fed using natural seawater continuously flowing through the aquaria in the case of *H. oculata* or they were placed back into the sea in the case of *D. avara.* Total amount of ^{13}C label in the sponge individuals was followed in time and ^{13}C label uptake in specific fatty acids was also followed. Sponge metabolic rate was determined using total and specific compound enrichment.

In chapter 7 another ^{13}C labeling study is described where we have used the same two sponge species as in chapter 6, and now the focus was on changing metabolic rate by inflicting damage. We have looked at the place in the sponge where more or ^{13}C less label could be found, and whether damaged tissue resulted in an increased metabolic rate. By studying this we know if regenerative rates are larger than growth rates and if metabolic rate can be influenced by external factors.

Chapter 8 discusses the obtained result and is focused towards possible methods for commercial bioactive compound production in different sponges. An approach is discussed about how to understand biosynthesis, induction and location of metabolite production. Moreover, different possible production techniques are discussed.

Target sponge species

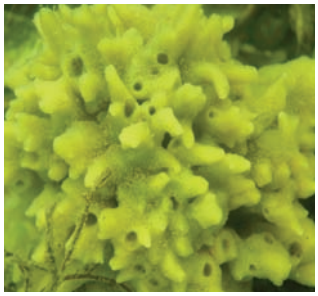
1

Haliclona oculata (Pallas 1766), Haplosclerida

Main sponge specie used in this study. *H. oculata* is abundant in the Oosterschelde, the Netherlands. No bioactive compounds are known from this species, although in several other *Haliclona* species bioactivity was reported.

Haliclona xena (Pallas 1766), Haplosclerida

This species is abundant in both the Oosterschelde and in Lake Veere, the Netherlands. It appears to have a seasonal occurrence as they could not be found at all times. No bioactivity is reported for this species.



Halichondria panicea (Pallas 1766), Halichondrida

H. panicea occurs in many marine habitats and has been studied widely (see Hooper and van Soest 2002). The used species here came from Lake Veere, the Netherlands. *H. panicea* contains symbiotic bacteria and harbors bioactivity.

Aplysina aerophoba (Schmidt 1862), Verongida

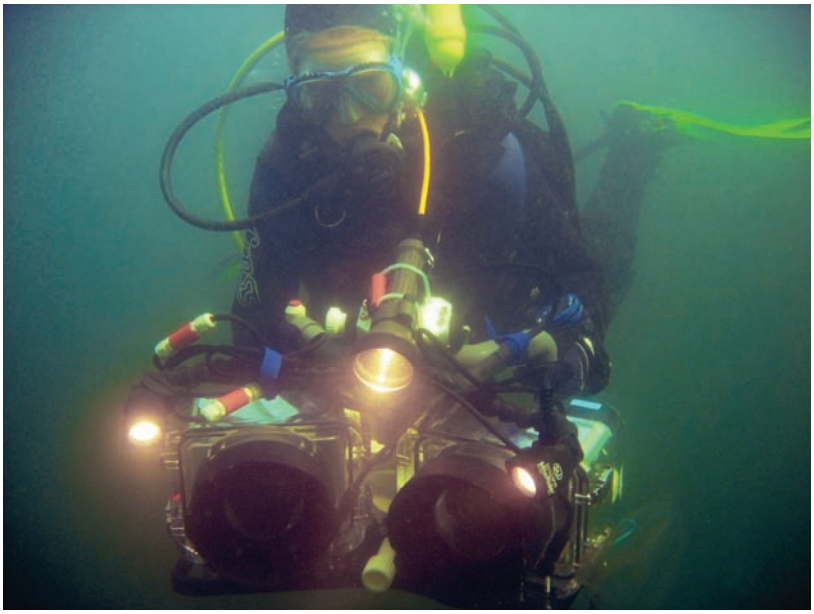
This species is commonly found in the Mediterranean and contains many symbiotic bacteria. Antibiotic and cytotoxic activity has been found in this sponge species (Teeyapant et al 1993)

Dysidea avara (Schmidt 1862), (Dictyoceratida)

A very abundantly found species in the Mediterranean. The bioactive metabolite avarol with antitumor and antiviral activity is produced by this species.

Chapter 2

Seasonal growth rate of *Haliclona oculata*



Koopmans M, Wijffels RH (2008) Seasonal growth rate of the sponge *Haliclona oculata* (Demospongiae: Haplosclerida). Marine Biotechnology 10:502-510.

Abstract

The interest in sponges has increased rapidly since the discovery of pharmaceutical interesting compounds produced by many sponges. A good method to produce these compounds using sponges is not yet available, because there is insufficient knowledge about the nutritional needs of sponges. To gain more insight in the nutritional needs for growth we studied the growth rate of *Haliclona oculata* in its natural environment and monitored environmental parameters in parallel. This study lasted from September 2005 to September 2006. Monthly, stereo pictures were taken and used to measure volumetric changes. Volumetric growth rate of *Haliclona oculata* showed a seasonal trend with the highest average specific growth rate measured in May, 0.012 ± 0.004 day^{-1}. In our study a strong positive correlation ($p<0.01$) was found for growth rate with temperature, algal biomass (measured as chlorophyll *a*) and carbon and nitrogen content in suspended particulate matter. A negative correlation ($p<0.05$) was found for growth rate with salinity, ammonium, nitrate, nitrite and phosphate. No correlation was found with dissolved organic carbon, suggesting that *Haliclona oculata* feeds on particulate organic carbon.

Introduction

Since the discovery of pharmaceutical interesting compounds in sponges many attempts have been made to culture sponges for the production of these metabolites. Sponges are sessile benthic organisms that have shown the ability to change their shape and internal structure to cope with environmental changes (Simpson 1984, Garrabou and Zabala 2001, Mendola et al 2007). Despite the ability of sponges to adjust to changes in their environment still only a low number of successful *ex situ* culture systems have been developed. Several small successes in sponge cultures achieved so far mostly resulted in very slow, variable and chaotic growing sponges (de Caralt et al 2003, Mendola 2003, Duckworth and Pomponi 2005, Sipkema et al 2005[c]). So far sponges cultured in their natural habitat gave the best results especially for longer term survival. However, a disadvantage of this method is that conditions cannot be controlled or optimized.

Many different factors can cause variability in sponge growth such as sponge size, age, damage and environmental conditions (Ayling 1983, Handley et al 2003). How exactly these different factors influence sponge growth and development is still not well understood. By studying sponge growth rates in their natural environment a better understanding can be obtained about factors influencing growth rates. In the field seasonal trends can be found in sponge growth rates. Several researchers (Barthel 1986, Garrabou and Zabala 2001, Page et al 2005[a], de Caralt et al 2008[b]) found a relation between temperature and growth rate. Besides temperature, nutrient availability is affecting growth (Duckworth and Pomponi 2005). Optimal conditions can be translated to laboratory cultures to be able to achieve continuous cultures instead of seasonal growth patterns.

Preferred conditions can be found at highest growth rate when monitoring sponge growth rate, nutrient concentrations, algal biomass and temperature in nature year round. The role of dissolved organic carbon (DOC) as a food source for sponges is still not very well understood. Yahel et al (2003) found that most of the carbon retained by sponges came from the dissolved pool. Particulate organic carbon (POC) was found to be the food source for *Dysidea avara* (Ribes et al 1999). Studying shifts in both particulate organic carbon and dissolved organic carbon will give more insight in the role of these two for the growth of sponges.

Many of the methods used for measuring growth are destructive, such as determining underwater weight, wet weight and ash free dry weight. Photographing and measuring area is non-destructive and gives good results for thinly encrusting sponges (Garrabou and Zabala 2001, de Caralt et al 2008[b]). For globular and finger sponges area determination will not be accurate enough. Abdo et al (2006) developed a method to non-destructively measure volume of sessile benthic organisms using a stereo photogrammetry method. They have shown that the method is accurate and takes short data processing and analysis time. Using this method it is possible to monitor volumetric growth rates for sessile organisms on a long term without disturbing the individuals.

In this study we have monthly monitored growth of the finger sponge *Haliclona oculata* (Pallas 1766) in the Oosterschelde in The Netherlands from September 2005 to September 2006. Growth is followed by determining volume using a stereo photogrammetry technique. In literature no studies can be found on non destructive measuring the volumetric growth rate of sponges using 3d-photogrammetry. In addition monitoring growth rate of sponges in The Netherlands in general has not been done in the past. Apart from the growth rate food availability, temperature and salinity changes during the monitoring period are measured to find possible relationships between these factors and sponge growth rate.

Material and methods

Study area

2

Haliclona oculata was monitored in The Netherlands, Oosterschelde estuary (Lokkersnol, 51°38'58.07"N, 3°53'5.11"E) at 13m depth. *H. oculata* widely occurs in the Oosterschelde and grows attached to oyster shells. According to monitoring data of the Dutch ANEMOON foundation (Gmelig Meyling and de Bruyne 2003) *H. oculata* was the most stable sponge present at the dive site Lokkersnol for at least eight years (Figure 1). GA in figure 1 is a logarithmic value and stands for general abundance of a certain species. GA is in the range of 0 to 3, 1 means that in every dive 1 individual of this species was found, 2 stands for 10 individuals per dive and 3 for 100. *Cliona celata* was stable as well, but this sponge is impossible to monitor monthly in volumetric changes as it is an excavating sponge. Both *Halichondria panicea* and *Halichondria bowerbanki* were decreasing in number throughout the Oosterschelde. *Haliclona xena* was increasing in number in the last year, but has an annual cycle and it disappeared at the start of our monitoring period.

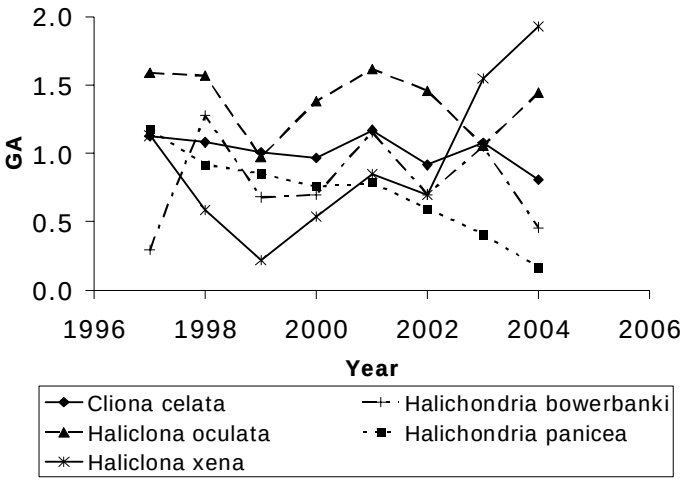


Figure 1. General abundance of different species of sponges at Lokkersnol, Oosterschelde, The Netherlands.

A platform (Figure 2) was placed at the study site and 42 concrete pavement tiles (30cm x 30cm) were placed on top of the platform. On every tile a sponge specimen was attached using an elastic band. Specimens were attaching to the tiles within one or two months. Along one year, from September 2005 to September 2006, sponge growth was monitored.

Figure 2. Platform that was placed at the study site containing 42 tiles, sponges were attached on every tile. Size platform: 2,5m x 1,6m x 1,2m.

Monitoring growth

Monthly, pictures were taken using a stereo camera system (Figure 3) to monitor volume changes in time. The camera system consisted of two Nikon coolpix 5200 five megapixel digital cameras. The two cameras were mechanically connected to make pictures at the same time. Both cameras were set on autofocus, macro, iso 100, no flash and no zoom. A twin set 50W video lights was mounted to the camera system to provide light for the low light conditions. A reference frame was connected to the camera frame to make sure that the pictures were always taken at the same distance. Reference points were present on the frame for calibration.

Calibration of the camera system was done in two steps. The first step of calibration used a photogrammetric bundle adjustment to determine camera calibration properties (focal length, principal point location, radial and tangential distortion). The calibration was performed using imagery of a calibration fixture captured underwater (see also Abdo et al 2006). This first step was only done once for the camera system. As the camera positions at the different monitoring dates were not exactly the same a second step of calibration was necessary.

The second step was to adjust the camera calibration properties according to the coordinated reference frame visible in all stereo images. Using the properties of the first calibration, measurements could be made of the coordinated reference frame. Using these values the camera properties and relative orientation of the cameras were re-computed every time large deviations in the distances between reference points on the frame occurred.

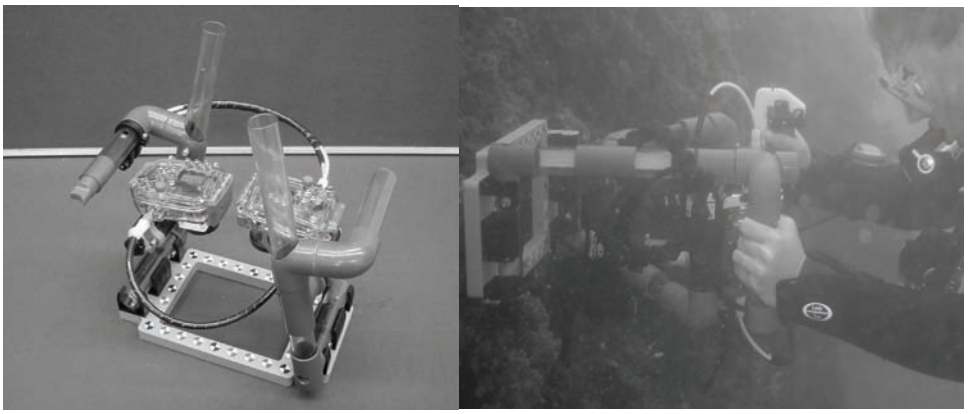


Figure 3. Stereo camera system. The left image shows a top view of the system, and the right image shows the system being used under water.

Measure volume

Haliclona oculata is a branching sponge which makes three dimensional reconstructions almost impossible, due to the fact that branches are overlapping in pictures from different views. Therefore we chose to reconstruct the branches as cylinders. In all stereo images length and width is determined for all branches. Lengths were measured using stereo calibration and measurement software (CAL and PhotoMeasure) created by J. Seager (www.seagis.com.au). In one stereo pair beginning and end of a branch can be indicated and the pro-

gramme uses the determined coordinates to measure the branch length. Figure 4 shows how the lengths and widths of the sponge branches were measured.

Figure 4 Illustration of computed lengths in one image of a stereo pair.

For every specimen and time point minimally three stereo images were made, in these three stereo images all lengths and widths were measured, averages were used in further calculations. Volume was determined using next equation:

$$V = \sum V_n \Rightarrow V_n = 0.25 \cdot \pi \cdot \left(Average\ (W_n)\right)^2 \cdot Average\ (L_n)) \qquad [mm^3]$$

In which V_n, W_n and L_n are volume (mm^3), width (mm) and length (mm) of branch n respectively. We could have measured total volume in every stereo pair and average for the different stereo pairs, but not in every stereo pair it was possible to measure every branch.

Growth rates between two data points are determined exponentially using next equation:

$$\mu_t = \frac{Ln\left(\frac{V_t}{V_{t-1}}\right)}{dt} \qquad [day^{-1}]$$

In which m is specific growth rate (day^{-1}) at time t, V_t is the volume (mm^3) at time t, V_{t-1} is the volume (mm^3) at time t - 1 month and dt is the time between two measurement points in days. Because circumstances change continuously specific growth rate was determined per time interval.

Volume versus ash free dry weight

To determine if the calculated volumes were representative for the amount of biomass 8 individuals were used to make a calibration curve. Different sizes of sponges were used and pictures were taken under water for volume measurement. Afterwards the sponges were dried in a stove overnight at 80°C. Dry weight was determined and then the sponges were burned at 450°C for 4 hrs to determine their ash free dry weight.

Nutrient availability

The Netherlands institute of ecology monitors monthly and from April to September twice a month the nutrient and pigment concentration in the Oosterschelde Estuary (51°37'12.94"N and 3°55'19.85"E). Samples were taken from surface water, which is representative as the Oosterschelde is well mixed due to the regular tide. Analyses of nitrite, nitrate, phosphate and dissolved organic carbon (DOC) are done using a Skalar auto analyser (see also Goosen et al 1995).

Water samples were filtered using a GF/F filter (0.7mm) and suspended particulate matter (SPM) was determined by weighing. Total nitrogen and organic carbon was analysed in SPM in a Carlo-Erba NA-1500 elemental analyser according to Nieuwenhuize et al (1994). Plant pigments were extracted from SPM samples according to the method used by Barranguet et al (1997). Temperature and salinity were monitored during the monthly survey as well.

Nutrients and pigments were not measured on the same days as sponge volume was measured. As we have calculated growth rate from time t-1 to time t, the nutrient and pigment concentration was averaged for the period of time t-1 to time t or the value measured halfway this period was used.

Statistical methods

Significant differences in growth rate between different time intervals were analysed by one-way ANOVA. The Tukey test was used for pair comparisons. Homogeneity assumption is justified; this was tested by calculation of the Levene statistic (SPSS 12.0.1, Inc., 1989-2003).

Pearson correlation coefficient was used to find relationships between water temperature, salinity, nutrient concentration, pigment concentration and growth rate (SPSS 12.0.1).

Results

Growth

All specimens included in this study show an increase in volume for every time interval measured, except for one individual that got partly consumed by a predator during the last measurement interval (Figure 5). 11 of 42 individuals were monitored for a long period and included in this study. The others either did not attach or were damaged from predation or several insufficient pictures of good quality were available. Massive mortality occurred in August - September 2006 and all specimens of the study died. Thus, we were forced to stop the monitoring. *Haliclona oculata* specimens around the platform also died in the same period, thus the high mortality was not related to the experiment.

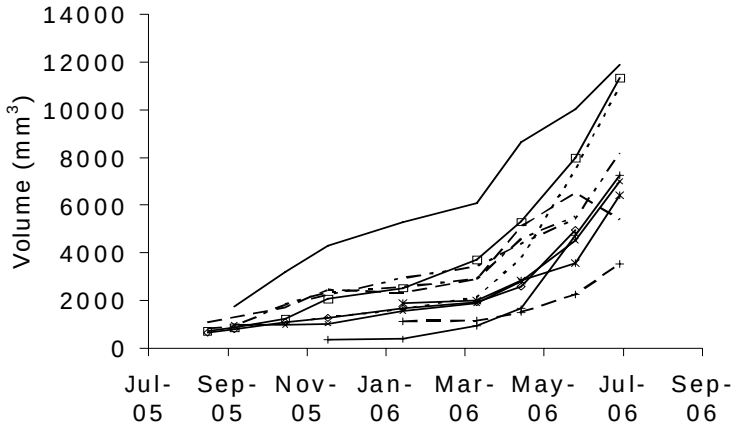


Figure 5. Volume changes of monitored specimen of Haliclona oculata (N=11)

The volumes measured using photomeasure are compared with ash free dry weight (Figure 6). A good linear correlation (r^2 = 0.991) was found between dry weight and the volume measurement demonstrating that the volume measurement as done here is representative for the amount of biomass and thus can be used to calculate growth rate.

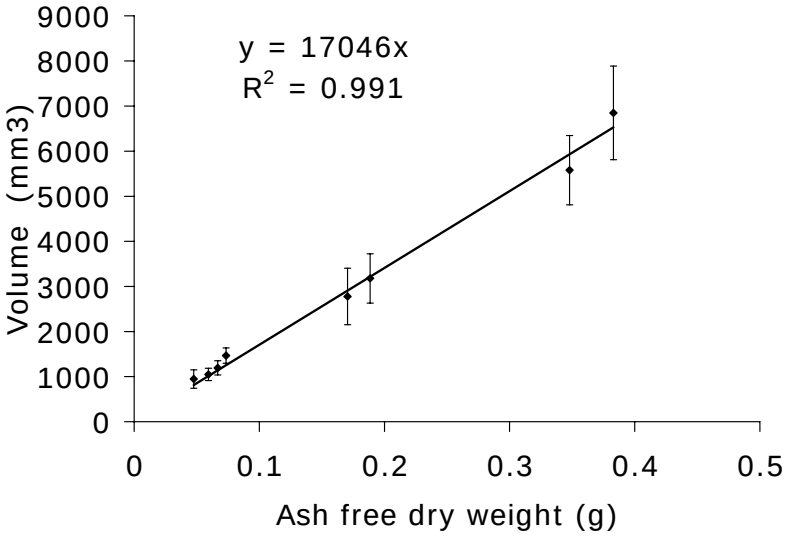


Figure 6. Calibration curve of measured volume and ash free dry weight of H. oculata.

Growth rate

Both total branch length and average branch width increased in time. Therefore, we assume exponential growth instead of linear growth. Figure 7 shows the average growth rate at the different time steps. Growth rate shows a temporal pattern. The maximum average specific growth rate measured was 0.0118 ± 0.0035 day^{-1} (average m ± sd), which was measured in the beginning of May 2006. The lowest average growth rate was recorded on the end of January and was 0.0032 ± 0.0025 day^{-1}. The average growth rate for the whole year was 0.0082 ± 0.0055 day^{-1}.

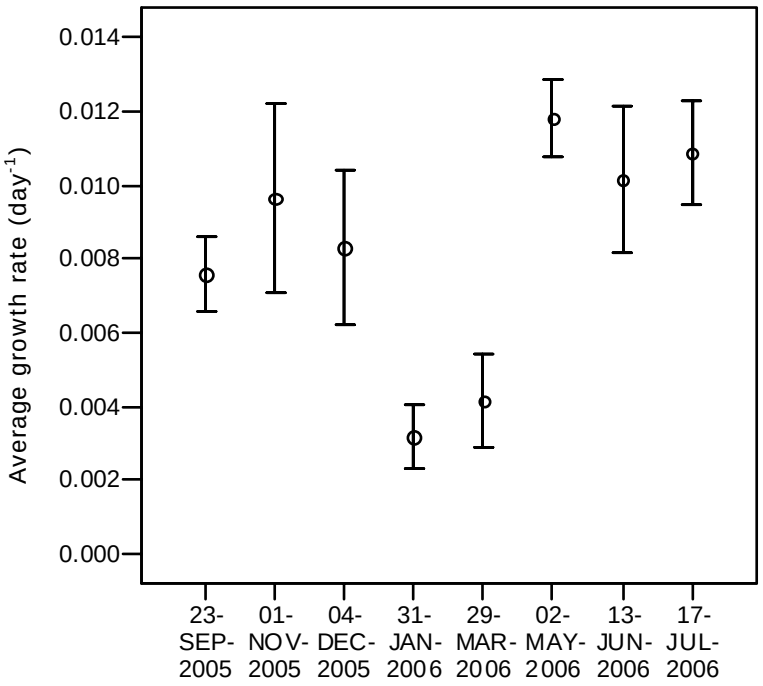


Figure 7. Average growth rate per day ± standard error of Haliclona oculata.

Figure 7 shows that the measured specific growth rate has quite some variation. Therefore, an one-way ANOVA test was used to test if there is a significant difference between the growth rates measured on the different dates. This showed there is a significant difference between at least two groups ($p <$

0.01). Using a Post Hoc Tukey test a significant difference was found between growth rates in winter (January and March) and spring (May, June and July) ($p < 0.01$). This difference might be due to changes in circumstances. Growth rates of September, November and December are close to the overall average growth rate and no significant difference could be found for these months with another month.

Nutrient availability

Temperature, salinity, nutrient and pigment concentration varied in time (Figure 8). Table I shows the correlation coefficients and their significance level between the exponential growth rate and temperature, salinity and the different measured nutrients and pigments. A significant negative correlation with exponential growth rate is found for ammonium, nitrite, nitrate, phosphate and salinity, while a significant positive correlation is found for carbon content in SPM (SPMC), nitrogen content in SPM (SPMN), water temperature and chlorophyll a. No correlation is found between the growth rate and dissolved organic carbon (DOC) although there is a strong correlation between DOC and water temperature ($p < 0.01$) (data not shown). Figure 8 shows that for most parameters a seasonal trend was found.

Table 1. Pearson correlation coefficients and their significance level for exponential growth rate and different nutrients and pigments. N is the number of data points taken into account. Data used from the Netherlands institute of ecology.

Variable	NH_4 (mmol/l)	NO_2 (mmol/l)	NO-3- (mmol/l)	DOC (mmol/l)	PO_4 (mmol/l)
Pearson correlation	-.263(*)	-.354(**)	-.308(*)	.183	-.319(*)
N	64	64	64	64	64
Variable	SPMC (mg/l)	SPMN (mg/l)	Salinity (PSU)	Temperature (°C)	Chla (mg/l)
Pearson correlation	.436(**)	.451(**)	-.381(**)	.398(**)	.349(**)
N	64	64	64	64	64

** Correlation is significant at the 0.05 level (2-tailed).*
*** Correlation is significant at the 0.01 level (2-tailed).*

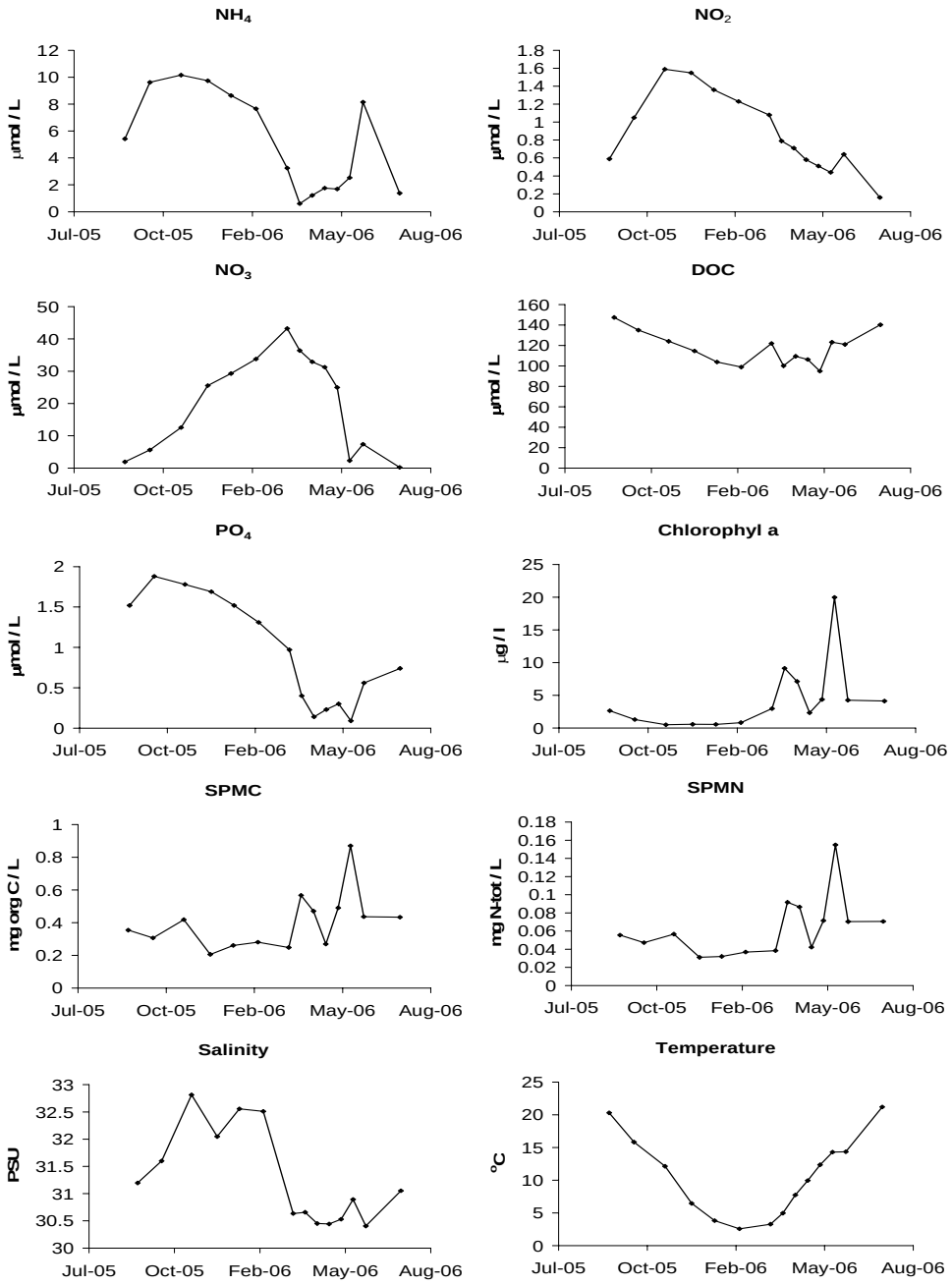


Figure 8. Time series of nutrients, pigments, salinity and temperature. Data obtained from the Netherlands institute of ecology

Discussion

Sponge growth rates have been measured in different ways in literature, which makes it difficult to compare results. The only non-destructive method that has been used so far was 2d photography measuring two-dimensional growth rates in area (eg. Garrabou and Zabala 2001, de Caralt et al 2008[b]). In this study we used a non-destructive three-dimensional 3d photogrammetry method that appeared to be useful for measuring volumetric growth of sponges. Growth rates found for *Haliclona oculata* are in the range found for other sponges (table 2). However, in most studies the linear growth rate is determined, which generates slightly higher values than the exponential growth rate. Therefore, the highest measured linear growth rate based on our data is given between brackets for comparison.

A strong correlation was found between volumes determined using our approach and ash free dry weight. However, the error bars calculated for growth rate show high variation. Using more individuals will give smaller confidence intervals. Another cause for the large error bars could be the assumption of cylindrical branches, which may be questionable. Due to environmental factors like flow *H. oculata* can have different forms such as flat like branches and thicker, more conical branches (Kaandorp 1995). In this study the sponges are exposed to the same environmental factors and the sponges showed the same growth forms, thus similar errors are made for each individual. Large error bars were also found for the volume measurements. This can be improved by changing the camera system. Cameras could only be used in auto focus. Therefore the parameters found for the cameras after calibration were not completely the same each time. Furthermore, the images were not taken perfectly synchronically, which decreases quality. Nevertheless the correlation with ash free dry weight was so strong (r^2 = 0.991) that it was accurate enough to use here for growth rate measurements.

Many growth studies showed that an increase in growth rate coincided with an increase in water temperature (Page et al 2005[a], de Caralt et al 2008[b], Garrabou and Zabala 2001, Barthel 1986). Here it was found again that growth rate was correlated with temperature. Besides temperature a lot of other factors change in different seasons, a strong positive correlation was found with carbon and nitrogen content in suspended particulate matter and chlorophyll *a*. This is to be expected as more food is available when more algae and bacteria are present in the water.

Table 2. Maximal specific growth rates (μ) measured for several species of sponges found in literature. Values are recalculated to get same units; different measuring techniques are used. Growth form is indicated with l for lobate, e for encrusting, m for massive and b for branching.

Sponge species	Order	Max μ (day^{-1})	Reference
Oscarella lobularis (l)	Homo-sclerophorida	0.030[l]	Garrabou and Zabala 2001
Corticium candelabrum (e)	Homo-sclerophorida	0.018[l]	De Caralt et al 2008[b]
Mycale hentscheli (m)	Poecilosclerida	0.1306[l]	Page et al 2005[a]
Crambe crambe (e)	Poecilosclerida	0.060[l]	Garrabou and Zabala 2001
Hemimycale columella (m)	Poecilosclerida	0.035[l]	Garrabou and Zabala 2001
Iotrochota birotulata (b)*	Poecilosclerida	0.0125[e]	Wulff 1991
Chondrosia reniformis (m)	Chondrosida	0.0027[l]	Garrabou and Zabala 2001
Aplysina fulva (b)*	Verongida	0.017[e]	Wulff 1991
Halichondria melanadocia	Halichondrida	0.006[e]	Duckworth & Pomponi 2005
Halichondria panicea (m)	Halichondrida	0.028[e]	Thomassen & Riisgård 1995
Amphimedon rubens (b)*	Haplosclerida	0.0097[e]	Wulff 1991
Haliclona permollis (e)	Haplosclerida	0.023[l]	Elvin 1976
Haliclona oculata (b)	Haplosclerida	0.012[e] (0.015[l])	This study

** Values determined using graphs in the article*
[e] Exponential growth rate
[l] Linear growth rate

It is striking that DOC does not appear to be correlated to sponge growth rate. Yahel et al 2003 did find a strong uptake rate of DOC by sponges. The sponge species used in that study contains over two-third of its biomass of bacterial symbionts, which may be responsible for the DOC uptake. *H. oculata* does not contain a lot of bacteria, which could explain that here such a relation is not found. This study showed that for *H. oculata* carbon from suspended particulate matter has a correlation with growth rate. This might indicate that a sudden increase of suspended organic matter induces growth. When taking the

carbon requirements for growth into account we found that when growth rate was high, 0.0118 day^{-1}, total organic carbon (SPMC + DOC) was 1.69 $mgC.l^{-1}$ of which only 0.43 mg was SPMC. Duckworth and Pomponi (2005) found that the carbon concentration required for *Halichondria melanadocia* was 0.356 $mgC.l^{-1}$ and Thomassen and Riisgård (1995) found 0.277 $mgC.l^{-1}$ for Halichondria panicea. Growth rates of these sponges are in the same order of magnitude as of *Haliclona oculata*; this indicates that there is enough carbon present SPMC only. More research is necessary to find the relation between particulate and dissolved organic matter for different species of sponges.

Nutrients like nitrate, nitrite, ammonium, phosphate and salinity have a negative correlation with growth rate. Not much can be found in literature about the effect of these nutrients on growth of sponges or other invertebrates. For ammonium it was found that it can be lethal for marine invertebrates at a concentration of only 60 mM (Richardson and Gangolli 1993). Although the concentrations found in this study are somewhat lower they still may have a negative effect on sponge growth. A nitrate concentration below 320 $mmol.l^{-1}$ is considered acceptable for marine invertebrates (Camargo et al 2005). Almost 10-fold lower amounts are found in the Oosterschelde, which indicates this could not influence growth. However, according to Muir et al (1991) early developmental stages of some marine invertebrates well adapted to low nitrate concentrations can be susceptible to concentrations of 16 $mmol.l^{-1}$. Nitrite levels stayed far below toxic levels found for sensitive freshwater invertebrates (Alonso and Camargo 2006).

Although it has been found that high nutrient levels can have negative effects or be lethal to marine animals the values found in the year of our study are well below toxic levels. Nevertheless still little is known about the effects of nutrient concentrations on growth of sponges. In *in situ* studies one can learn which concentrations are acceptable but finding the optimal or lethal values is difficult as many different factors change parallel to each other.

Conclusion

Volumetric growth rate of *Haliclona oculata* could be accurately measured using a novel 3d-photogrammetry technique. *H. oculata* shows a seasonal trend in growth rate with an increase in growth rate when temperature and carbon and nitrogen levels in suspended particulate matter increases. No correlation is found between growth rate and dissolved organic carbon. Ammonium, nitrate, nitrite, phosphate and salinity show a negative correlation with growth rate of *H. oculata*. Growth rates measured are in the same order of magnitude as found for other sponges.

Acknowledgements

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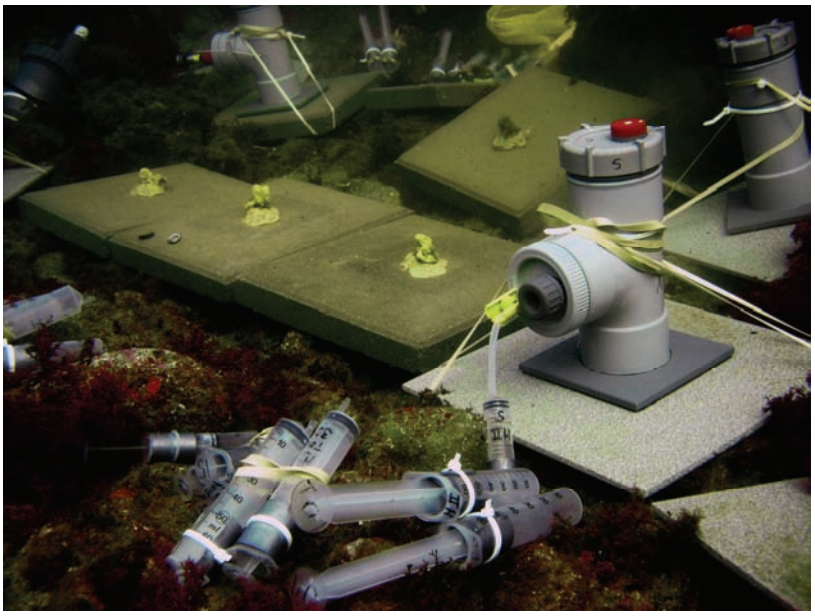
Chapter 3

Growth efficiency and carbon balance for *Haliclona oculata*



Koopmans M, Martens D, Wijffels RH (2009) Growth efficiency and carbon balance for the sponge *Haliclona oculata.* Marine Biotechnology: *accepted for publication*.

Abstract

To obtain more knowledge about carbon requirements for growth by sponges, the growth rate, respiration rate and clearance rate was measured *in situ* for *Haliclona oculata*. We found that only 34% of the particulate carbon pumped through the sponge was used for both respiration and growth. The net growth efficiency, being the ratio of carbon incorporated in biomass and the total carbon used by the sponge for respiration and growth, was found to be 0.099 ± 0.013. Thus, about 10% of the total used carbon was fixed in biomass and over 90% was used to generate energy for growth, maintenance, reproduction and pumping. *H. oculata* had 2.5 µmol C available for every µmol O_2 consumed. The average value for respiratory quotient (RQ in µmol CO_2 µmol O_2^{-1}) reported in literature for different marine invertebrates is 0.75. Thus, carbon was available in access to meet the respiratory demand. Oxygen was found not to be the limiting factor for growth either, since only 3.3% of the oxygen pumped through the sponge body was used. Our results indicate that both oxygen and carbon availability are not limiting. The low growth efficiency agrees with the low growth rates found for many sponges.

Introduction

Sponges are potential producers of pharmaceutical compounds due to their rich variety of bioactive metabolites. In order to obtain sufficient amounts of bioactive compounds a sustainable production technique is required. Culturing sponges *ex situ* would be an interesting option if we can stimulate sponge growth. Many growth studies showed that sponges are slow growing organisms (e.g. Dayton 1979, Garrabou and Zabala 2001, Duckworth and Pomponi 2005, de Caralt et al 2008[b], Chapter 2). Specific growth rates have been measured between 0.006 to 0.05 day^{-1}. In many studies seasonal variation in growth is found (e.g. Barthel 1986, Garrabou and Zabala 2001, Page et al 2005[a], Chapter 2), with lower growth rates generally being measured in winter. Plausible explanations for the lower growth in winter could be the lower availability of food, which results in uptake of less energy and food. And the lower temperature causes a lower metabolic rate. Due to the few successes in *ex situ* sponge cultivation, sustainable production methods have not been attained yet. To improve the growth of sponges, basic knowledge on how they acquire and use their food is necessary. Therefore we studied the carbon balance and growth efficiency for *Haliclona oculata* in the Oosterschelde, The Netherlands.

Sponges mainly feed by filtering particles from the large amounts of water that they pump through their body. Many studies have focused on the filtering capacity of sponges (Riisgård et al 1993, Ribes et al 1999, Lynch and Phlips 2000, Stabili et al 2006, de Goeij et al 2008[a]). It was shown that they can filter up to 72,000 times their body volume per day. Particle retention efficiencies are found to be very high up to 58-99% (Pile et al 1997), 86 % (Duckworth et al 2006) and 96.1% for bacterial uptake in three species of sponges (Reiswig 1971). Besides filtering particles, sponges are also able to obtain food particles through the external epithelia cells, via phagocytosis, and sponges containing symbiotic bacteria can obtain nutrients from these symbionts. In addition, it was shown that some sponges can assimilate dissolved organic material (de Goeij et al 2008[b]).

The retained food is used for two purposes; to supply nutrients for sponge biomass and to supply energy in which case the carbon is converted to carbon-dioxide assuming aerobic metabolism. Sponges extract the necessary oxygen for this from the water pumped through their body. From the oxygen available in the water maximally 10% is taken up by sponges (Jorgensen et al 1986). The amount of carbon needed for respiration can be calculated using

the respiratory quotient (RQ). The respiratory quotient is the ratio between CO_2 produced and O_2 consumed. Depending on which carbon compound is used as a food source for oxidation the ratio varies from 0.7 (lipid oxidation) to 1.0 (carbohydrate oxidation). RQ values vary depending on organism and used food source. Not many studies have focused on the respiratory quotient for marine invertebrates and sponges in general. An average RQ value of 0.75 was found for four different marine invertebrates by Hatcher (1989), namely for a bryozoan, an ascidian, a chiton and an abalone specie. In other comparable studies an RQ value of 0.69 was found for cephalopoda (Boucherrodoni and Boucher 1993), and 0.74 for ophiurid (Migne and Davoult 1997). An RQ of 0.75 means that for every mole of oxygen 0.75 moles of carbon are converted to carbon-dioxide. This indicates that lipids are probably a large fraction of the compounds used for oxidation by marine invertebrates. This agrees with the fact that most invertebrates feed on particulate organic matter including algae (Reiswig 1971, Mohlenberg and Riisgård 1978), which can accumulate lipids up to 50% of their dry weight (Collyer and Fogg 1954, Hu et al 2008).

The obtained energy is used in different processes in the sponge; for pumping, maintenance, reproduction and growth. The fraction of the total amount of metabolized carbon that is incorporated into biomass is called the net growth efficiency (NGE). Thus a higher NGE means that a relatively high amount of carbon is fixed into biomass. Thomassen and Riisgård (1995) found that NGE is positively related to the specific growth rate. At a growth rate of 4% d^{-1} they found a NGE of 30 and at 1% d^{-1} it was about 10. Thus, higher growth rates give a higher net growth efficiency. The total rate with which carbon is metabolized is the sum of the incorporation rate in biomass (growth) and the rate of carbon liberated as carbon dioxide (respiration). The NGE can be calculated using the next equation (adjusted from Thomassen and Riisgård 1995):

$$NGE = \frac{\mu \cdot C^x}{\mu \cdot C^x + RQ \cdot R} \quad (1)$$

In which C^x is the carbon content (µmole C·g dw^{-1}), μ the growth rate (d^{-1}), RQ the respiratory quotient (µmole C· µmole^{-1} O_2) and R is the specific oxygen consumption rate (µmoleO_2·g dw^{-1}·d^{-1}).

The amount of carbon taken up by the sponge can be calculated using the clearance rate multiplied by POC. In chapter 2 we showed that *Haliclona oculata* has a seasonal trend with respect to growth rate. We showed that there is a strong correlation of growth rate with particulate organic carbon (POC) and no correlation with dissolved organic carbon (DOC), suggesting that growth of this sponge is more dependent on available POC.

To obtain more insight in the carbon usage of sponges in relation to energy and biomass increase, the growth rate, respiration rate and clearance rate were measured *in situ* for the sponge *Haliclona oculata*. The growth rate was reported in chapter 2. The combination of these three factors have not been studied much. A carbon balance is made to find if all available carbon is used efficiently by the sponge for growth. Clearance efficiency and net growth efficiency were calculated and compared with other sponges. We want to obtain more insight in how sponges acquire and use their food, which in future may allow improvement of the sponge growth rate.

Materials and methods

Study area

Measurements were done *in situ* in the Netherlands, Oosterschelde estuary (Lokkersnol, 51°38′58.07″N, 3°53′5.11″E) at 13 m depth at low tide. The sponge species used was *Haliclona oculata*. This sponge widely occurs in the Oosterschelde and grows attached to solid surfaces such as oyster shells. A platform was placed at the study site and 42 concrete pavement tiles (30-cm x 30-cm) were placed on top of the platform. On every tile a sponge specimen was attached. For 11 specimens the growth rate was monitored for one year (Chapter 2). Due to dive time limitation only three sponge individuals were used for the results presented here. The respiration and clearance rate experiments were performed in summer 2006.

Respiration rate

Respiration rates were measured both *in situ* for three individuals and in the laboratory for three individuals. Laboratory experiments were done to find out if the *in situ* measured values coincide with values measured using more controllable equipment in the laboratory and were performed in the same period.

For the *in situ* experiments, experimental chambers made of polyvinylchloride (PVC) with 765 mL volume were developed. The chamber had an opening underneath to completely cover the sponge. The chamber was placed over the sponge, and by using elastic bands the chamber could easily be attached to the tiles. Two different chambers were made; one to connect an oxygen probe and one containing a small sampling tube for taking water samples. A magnetic stirrer inside the chamber continuously mixed the water inside to prevent particles from settling and to equalize oxygen distribution. O_2 concentrations were measured using two Oxi 340i handheld oxygen meters (WTW, Weilheim, Germany). The oxygen concentration inside a chamber was logged every 10 seconds. The meter connected to the probe was in a waterproof box and taken underwater. Experiments lasted for 15 minutes, to minimize the effect of oxygen depletion. Five measurements were done for every individual over a period of 4 weeks in July and August 2006. Blank measurements were done simultaneously, to see if oxygen concentration stayed equal, by using an empty tile on the platform.

In the laboratory, glass chambers of 450 mL volume were used for the respiration rate experiments. Three other sponge specimen were collected in the area where the *in situ* experiments were done and transported in a Styrofoam box to the university laboratory, which was a 2 hrs drive. There the sponges were kept in a 1200 liter aquarium for acclimatization. The aquarium contained artificial seawater, using Instant Ocean Reef Crystals artificial sea salt, at a pH of 8.1 and the same temperature and salinity as in the natural environment. One day after collection the sponges were introduced in the experimental glass chambers. Artificial seawater solutions in the chamber were stirred and particles were kept in suspension using a magnet spun at a speed of 300 rpm. Custom made wire baskets prevented the sponges from touching the stirring mechanism. A blank experiment was performed simultaneously, being an empty glass chamber. Per individual 5 measurements were done by re-saturating the seawater with air.

The oxygen concentration inside the chamber decreased linearly, where the slope of this decrease multiplied by the volume of the chamber is equal to the respiration rate (mg O_2 min^{-1}) of the sponge. The specific respiration rate can then be determined by dividing the respiration rate by sponge dry weight, which was measured as described in later section.

Clearance rate

The clearance rate is defined here as the amount of water pumped through the sponge body and cleared from cells. Here we assumed a 100% clearance efficiency to calculate clearance rate. Clearance rates were only measured *in situ* for 2 individuals with 5 repetitive measurements per individual. The third specimen got detached from the platform before we finished the clearance rate experiment. After placement of the experimental chamber for measuring clearance rate, the first water sample of 10 mL was taken immediately using a syringe. Every 3 minutes water samples were taken and the measurement lasted for 15 minutes, which made a total of 6 water samples per clearance rate measurement. 15 minutes was chosen as it showed to have a good and measurable result in both oxygen depletion and particle decrease. From the respiration measurements we could see that there was no effect from oxygen depletion or excretion of waste by the sponge. Immediately after the performed experiments *in situ,* 5 mL of the water samples were fixed with glutaraldehyde (1% final concentration). Fixation lasted for 30 minutes in the dark after which the samples were stored in dry ice.

Particle count was performed with the CyAn ADP flow cytometer (Beckman Coulter, Miami, FL) equipped with a 50-mW solid state laser at 488 nm and a 60 mW diode laser at 642 nm. Autofluorescent emission of the particles in the water sample was measured at different wavelengths ranging from 450 to 750 nm. The clearest population was found at green fluorescence (FL1) at 530 ± 40 nm, and yellow fluorescence (FL2) at 575 ± 25 nm. Calibration prior to each analysis was done by measuring the fluorescent signal of 3.0 µm SpectrAlign beads (Beckman Coulter, Miami, FL) exciting and emitting at 405 nm and 488 nm, respectively, prior to utilization of the flow cytometer. The sample analysis time was 2 min. The flow rate of the system was calibrated using fluorospheres with a diameter of 3 µm, at a concentration of 1.000 particles per microliter. The calibration was performed in triplicate for 2 min at different flow rates. Subsequently, the volume (microliters per minute) of each analyzed sample was calculated. An average flow rate of 65 µL per minute was used as a standard value to calculate the concentration of particles present in each sample in further experiments.

Clearance rates (F) were determined based on the clearance of a single population that was 100% retained by the sponge using the formula:

$$F = \frac{V}{t} * \ln\left(\frac{C_0}{C_t}\right) \quad (2)$$

In which V stands for volume of water (mL), t is time (min) and C_0 and C_t are the concentration of counted particles per ml at time 0 and time t (min), respectively.

POC concentration

Total POC was determined by measuring the total organic carbon content in the suspended organic matter. Water samples were filtered by using a GF/F filter (0.7 µm) and suspended particulate matter (SPM) was determined by weighing. Total organic carbon was analyzed in SPM in a Carlo-Erba NA-1500 elemental analyzer according to Nieuwenhuize et al (1994).

C-content and dry weight

The carbon content of 5 whole sponges was measured after oven drying at 50° C, using a Fisons NA 2500 element analyzer (serial number 991493), with auto sampler and Haysep-Q column 80-100 mesh I.D. 2mm (see also Nieuwenhuize et al 1994). In short, the dried sample was combusted in a oxygen rich environment at 1010°C. After drying the formed CO_2 and N_2 are separated on a GC column and detected using a thermal conductivity detector. For the dry weight measurement the sponges from the laboratory experiment were dried in a stove overnight at 80°C after which dry weight was determined. The average measured carbon content was used in our further calculations.

Carbon assimilation

The carbon assimilation rate was calculated by multiplying the carbon content by the specific growth rate. For the laboratory experiments we used the average growth rate measured in summer to calculate carbon assimilation. The field experiments were always performed with the same individuals and therefore we used the specific growth rate per individual as presented in chapter 2 for our calculations of carbon assimilation for the field experiments.

Results

Available carbon

To study whether the availability of carbon could limit the growth of *H. oculata*, we estimate the uptake of carbon from the POC and the clearance rate. Flow cytometry showed one separate population (Figure 1) that was cleared with 100% retention efficiency. Figure 1 shows that not all particles of this population were retained at the end of the experiment. However, at the time of the measurement not all water was filtered by the sponge yet. Moreover we found that the equation assuming 100% retention efficiency fitted the data significantly better than an equation assuming a lower efficiency. The population seems to be *Synechococcus* cyanobacteria as it shows the same signature as the *Synechococcus* cyanobacteria population shown in Moreira-Turcq and Martin (1998). *Synechococcus* has a size between 0.8 and 1.5µm and is highly retained up to 100% by other species of sponges as well (Ribes et al 1999, Hadas et al 2008). Thus, this population is well suited to be used for clearance rate calculations. Therefore, we have used this population for our clearance rate calculations assuming an exponential decrease (equation 2). We are conscious about the fact that more food particles are available to the sponge and that more will be used by the sponge, however, for calculating clearance rate one population is sufficient. Figure 2 shows the normalized average particle concentration of the specific population shown in Figure 1 for the two used sponges and the blank experiments. The particle concentrations are normalized for the start concentration. The average *Synechococcus* cell concentration at the start of the experiments was 6500 ± 1700 cells mL^{-1}. For all blank experiments no significant change in particle concentration was found. For all sponge experiments a clear decrease in particle concentration was seen. The total carbon taken up by the sponge was calculated by multiplying POC concentration by the clearance rate.

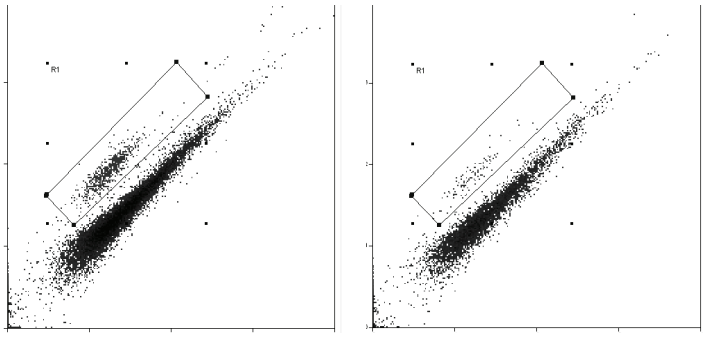


Figure 1. Flow cytometer results of 2 water samples (without addition of calibration particles), yellow (y-axis) versus green (x-axis) fluorescence. Left figure shows cell count at t = 0 and right figure at t = 15 min. The boxes represent the population used for our clearance rate calculations.

A particulate organic carbon (POC) concentration of 0.49 mg C L^{-1} was found (see Chapter 2), which is 40.8 µmol C L^{-1}. The average clearance rate was 5.63 L g dw^{-1} h^{-1}, meaning that 230 µmol C g dw^{-1} h^{-1} was pumped into the sponge. For the two sponges used here we found a constant clearance rate per gram sponge.

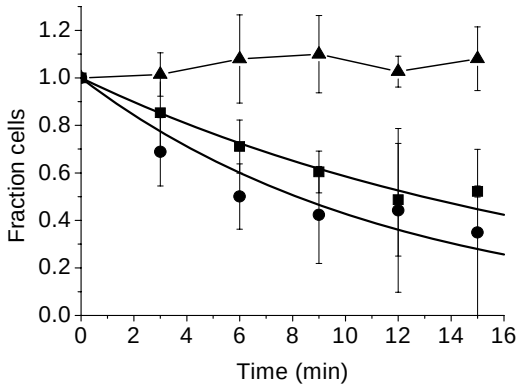


Figure 2: Normalized concentration of counted Synechococcus cells at different time steps for sponge 1 (■) and sponge 2 (●) and a blank measurement (▲). The error bars show the standard deviation calculated from the replicates performed for one individual or blank. The lines show exponential regression lines for both sponges. The concentrations are normalized for the start concentration.

Carbon assimilation rate in biomass

To calculate the rate of carbon incorporation into biomass the growth rate is multiplied by the carbon content of the sponge. The average growth rate in the summer period was 0.011 day^{-1}, this value was used to calculate carbon assimilation in the individuals used in the laboratory. And for the field experiments the specific growth rates per individual reported in chapter 2 were used in the calculations (Table 1). The carbon content of *H. oculata* was measured to be 19.1 ± 2.0% of the dry weight, which was used to determine the carbon assimilation rate in µmol C g dw^{-1} day^{-1}.

Table 1: Dry weight, specific growth rate, carbon assimilation rate (Cgrowth), carbon dioxide production rate (C resp) and the net growth efficiency of 6 individuals of Haliclona oculata. Carbon assimilation and carbon dioxide production are both expressed in µmol C g dw^{-1} h^{-1}.

Sponge	Dry weight (g)	Growth rate (d^{-1})	Cgrowth (µmol g-dw^{-1}.h^{-1})	C resp (µmol g-dw^{-1}.h^{-1})	Net growth efficiency (NGE)
#1	0.42	0.0131	8.67	63.13	0.12
#2	0.87	0.0125	8.28	80.15	0.09
#3	0.77	0.0106	7.02	73.48	0.09
Lab 1	0.64	0.0110[1]	7.28	72.96	0.09
Lab 2	1.10	0.0110[1]	7.28	72.07	0.09
Lab 3	0.86	0.0110[1]	7.28	60.58	0.11

[1] *Average growth rate found for this sponge in summer.*

Carbon dioxide production rate

The carbon dioxide production rates were calculated using the respiration rate and the respiratory quotient (RQ), where an RQ of 0.75 was assumed as described in the introduction. Figure 3 shows an example of a sponge respiration measurement together with a blank measurement. The oxygen concentration during the blank measurements stayed constant, while a decrease was seen when a sponge was inside the chamber. The slope is the respiration rate in mg O_2 $L^{-1}min^{-1}$.

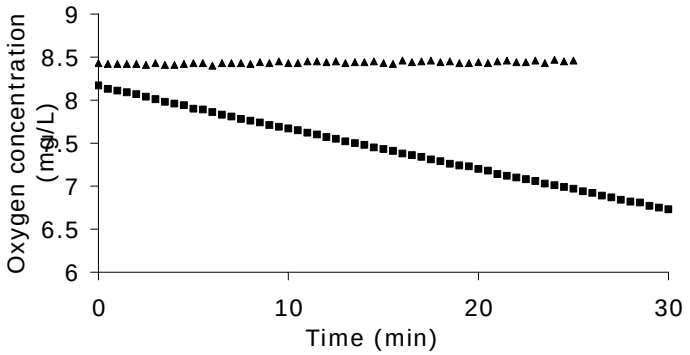


Figure 3. The oxygen concentration inside the experimental chamber as a function of time. A blank measurement (▲) as well as a sponge respiration measurement (■) is shown.

Figure 4 shows the average respiration rates per used sponge individual both *in situ* and in the laboratory. It can be seen that the respiration rate has a clear positive correlation with sponge size (t-test, p=0.000). In this study a linear relation (R = 1.78dw - 0.24, Adj R^2 = 0.768) resulted in the best fit. The results for the carbon dioxide production rates per gram dry weight as calculated from the respiration rates are shown in Table 1.

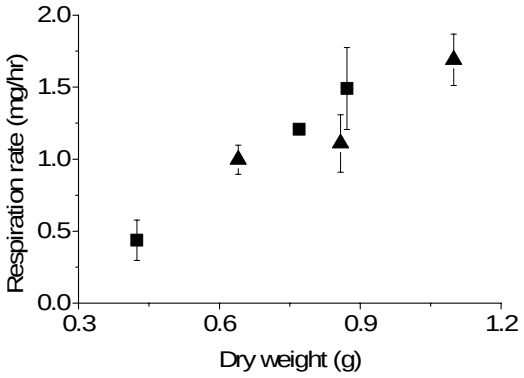


Figure 4. Average respiration rates (±standard deviation) in mg O_2 per hour related to dry weight of the sponge. The laboratory measurement (▲) and the field measurement (■) are shown using different symbols. The line represents the best linear regression for all experiments together.

Carbon balance

Next, it can be calculated how much of the POC present in the water is actually metabolized by the sponge. The amount of POC pumped through the sponge was 230 µmol C g dw^{-1} h^{-1}. The average carbon growth rate was 7.6 µmol C g dw^{-1} h^{-1} and the average carbon dioxide production rate 70.4 µmol C g dw^{-1} h^{-1} (Table 1), leading to a average total carbon demand per hour of 78.0 µmol C. Thus, about 34% of the carbon entering the sponge as POC was used by the sponge.

Growth efficiency

The net growth efficiency is the ratio of carbon fixed in biomass to the total amount of carbon used by the sponge (see equation 1), where the total amount used is the sum of the carbon fixed in biomass and the carbon converted to carbon dioxide. Table 1 shows the net growth efficiency as calculated from equation 1. Net growth efficiencies found for the 6 individuals were in the same range with an average value of 0.099 ± 0.013 (average ± SD). Thus, almost 10% of the assimilated carbon is used for direct incorporation in biomass and over 90% is used for generating energy for growth, maintenance, reproduction and pumping.

Replicate experiments

In our experiments we have used three replicate sponge specimen *in situ*, which is the absolute minimum for experiments. We chose to have more (5) measurements spread over a limited period (4 weeks) for the three individuals, in order to obtain accurate measurements per specimen in a limited time. The variation between the three *in situ* individuals was low and the respiration rate results for the laboratory individuals were close to the *in situ* results (Figure 4). Based on this we believe that using more individuals would not have altered the conclusions of this paper.

Discussion

The respiratory quotient (RQ) has an influence on the total carbon balance and the resulting calculations for both clearance efficiency and net growth efficiency (NGE). The RQ-value can be different for different sponges and different invertebrates, 0.75 is an average value found in literature for different invertebrate species. The RQ is mainly determined by the elemental composition of the food source and the type of metabolism. Keeping in mind the elemental composition of algae ($CH_{1.6}O_{0.4}N_{0.2}$) (Richmond 2004), a value for the RQ for sponges, feeding on algae is theoretically 0.72. Likewise, for bacteria ($CH_{1.8}O_{0.5}N_{0.2}$) (Roels 1983) a value of 0.85 can be calculated. If only fatty acids are used a value of around 0.7 is obtained, which is the minimal value that can be reached. While for growth on only sugars a value of 1.0 is obtained. Since POC contains a lot of algae and sponges feed on algal cells a value of 0.75 of the RQ seems logical. Higher values of RQ lead to a higher percentage of carbon used for respiration. The influence of RQ for the different calculations will be discussed further.

Of the available particulate carbon 34% was used by the sponge *Haliclona oculata*. Possible explanations for the low carbon usage are that carbon is not limiting or that not all carbon in the POC is available for consumption. Ribes et al 1999 found that a large part of the particulate organic matter consisted of detritus and that this was not retained by *Dysidea avara*. Yahel et al (2003) concluded that about 12% of the total organic carbon was removed from the water by the sponge, taking both POC and DOC into account. We have neglected the available dissolved organic carbon (DOC), which in our experiments was ~3 times more than the particulate carbon (Chapter 2). DOC was not taken into account, because the growth rate for *H. oculata* was found to have no correlation with DOC. If we would take the DOC pool into account only 9% of the available carbon is used by the sponge. Moreover, a lower RQ would result in a lower usage of the total carbon pool, for an RQ of 0.7 this would be 32%. And vice versa, a higher RQ would result in a higher carbon fraction used, an RQ of 1.0 leads to 44% carbon usage.

The values for the respiration rate are relatively high for *H. oculata* (Table 2). Osinga et al (1999) made a comparison for respiration rates of different sponges. In this study the one *Haliclona* species present also had higher respiration rates, which were in the same range as found in this study. Moreover, sufficient carbon was available to cope with this high respiration rate. For calculating the carbon uptake we used a retention efficiency of 100% for the *Synechococcus* cells, because we found that the equation assuming 100% re-

tention efficiency fitted the data significantly better than an equation assuming a lower efficiency. Lower retention efficiencies for Synechococcus would result in higher clearance rates and thus in even more carbon entering the sponge. Of course a large part of this carbon is present in other particles than synechococcus and these particles are not all 100% retained meaning not al carbon entering the sponge is retained. This can at least in part explain the low percentage of carbon usage as compared to the amount of carbon flowing through the sponge.

Table 2: Temperature, POC, clearance efficiency, clearance and respiration rates for different sponge species expressed per 1 gram of dry weight unless stated otherwise

Species	Temp (°C)	Conc POC (mg/l)	Clearance rate (L h^{-1})	Respiration rate (µmolO_2 h^{-1})	Clearance efficiency (µmolC µmol O_2^{-1})	Reference
Haliclona oculata	19	0.49[1]	5.67	95.54	2.45	This study
Mycale sp	29[2]	0.064[3]	12.29[2]	25.26[2]	2.60	Reiswig 1974
Verongia gigantea	29[2]	0.064[3]	2.77[2]	27.39[2]	0.54	Reiswig 1974
Tethya crypta	30[2]	0.085[3]	3.72[2]	6.64[2]	3.96	Reiswig 1974
Halichondria panicea	14	0.2	1.70	26.75	1.06	Thomassen 1995
Dysidea avara	17	0.387	0.65	9.25	2.28	Ribes 1998
Negombata magnifica	22.5	0.048[4]	4.20	14.88	1.13	Hadas 2008

[1] Data from chapter 2

[2] Temperature and g DW /g AFDW conversion from Reiswig 1973

[3] Values from Reiswig 1971

[4] Personal communication

Table 2 lists the respiration rate, clearance rate and clearance efficiency per gram dry weight for different sponges. Clearance efficiency gives the ratio between carbon uptake by the sponge and the respiration rate. We were expecting that sponges living in nutrient-rich habitats like in this study would have relatively low clearance rates, but no relation is observed between POC concentration and clearance rate for the different sponges (Table 2). Figure 5 shows the clearance efficiencies for seven sponge species. The line indicates the minimal needed RQ value of 0.75. Clearance efficiencies below this value means that not enough carbon is taken up to account for the carbon dioxide formed through respiration. Clearance efficiencies found for *H. oculata* are within the range found in literature. *Haliclona oculata* has a clearance efficiency of 2.5, which shows that a sufficient amount of particulate carbon is taken up to account for the high respiration rate. The same holds for *Mycale* sp, *T. crypta* and *D. avara.* However, *V. gigantea* has a value of 0.54, which is lower than the limit of 0.75, suggesting insufficient carbon uptake from the particulate carbon pool. Also *H. panicea* and *N. negombata* show relatively low values although still a little bit higher than the value of 0.75. This means that these two species would need very high retention efficiencies to deal with the carbon demand.

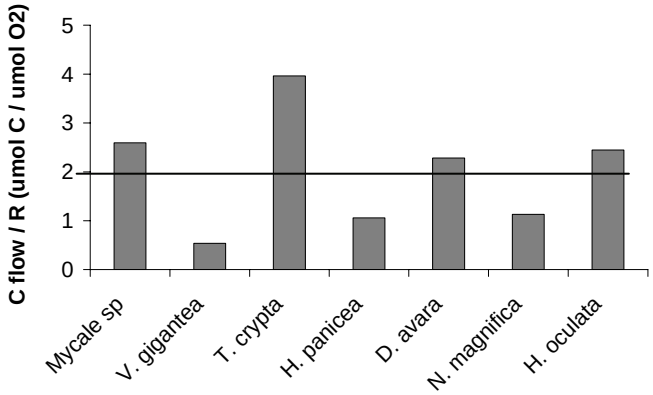


Figure 5: Clearance efficiency for different sponge species. The line indicates the assumed minimal value for respiratory quotient of 0.75 needed for invertebrates.

A possible explanation for the low clearance efficiency of *V. gigantea* is that these sponges obtain carbon from the dissolved carbon (DOC) pool. Reiswig (1973) concluded that the less efficient and symbiotic *V. gigantea* obtains a large part of its food requirements from the dissolved pool, which is probably done by the symbiotic bacteria. Notably, also *Negombata magnifica* and *Halichondria panicea* contain symbiotic bacteria although not in such high amounts as *V. gigantea* (Gillor et al 2000, Althoff et al 1998). To obtain sufficient carbon these sponges would need very high clearance rates for which there is no evidence. Therefore, we suggest that also these sponges that contain bacteria will use the dissolved carbon pool partly. Furthermore, the sponges in Table 2 that show values higher than 0.75 for clearance efficiency are sponges that contain none or few bacteria. Until now it is not clear what the exact role of DOC is for sponges. It is shown that sponges can use the DOC pool (Yahel et al 2003, de Goeij et al 2008[a]). However, it is still debated if the symbionts inside sponges are responsible for the total DOC usage.

The net growth efficiency of *H. oculata* was only 10%. An RQ value lower than the assumed 0.75 would result in less carbon dioxide production and thus in a higher NGE (for example, NGE=10.5% for RQ=0.7). A higher RQ would result in more carbon dioxide production and thus in even lower NGE's (for example NGE=7.6% for RQ=1.0). Carbon compounds from the food source are used to build biomass and generate energy for growth and for maintenance and work. The carbon used to generate energy is converted to carbon dioxide. If we assume that the amount of carbon needed to produce energy for work (pumping), reproduction and maintenance is constant, higher growth rates will result in higher net growth efficiencies. Thomassen and Riisgård (1995) showed that NGE is indeed positively related to specific growth rate. They found for *Halichondria panicea* that the highest growth rate of 0.04 d^{-1} related to a NGE of 30%. At a growth rate of about 0.01 d^{-1} they found a NGE of 10%, similar to what we found. The experiments done here are performed in the summer, where sponge growth was measured to be higher than in other times of the year. Therefore it can be assumed that NGE will be lower in winter and autumn. Although the respiration rate could also be lower at lower temperatures.

Thus, 90% of the carbon uptake is used to generate energy for other processes like pumping, reproduction or secondary metabolite production. Several studies have shown that suspension feeding animals are unlikely to be restricted by the energy cost of water pumping (Riisgård et al 1993 and references within). Riisgård et al (1993) found that the energy cost for pumping was only 0.85% of the gained energy by respiration. Up till now not much is

known about the energy demand of sponges for reproduction. It was found that other processes are ceased due to reproductive efforts. *Halichondria panicea* decreases silica uptake during reproduction and therefore spicule production was reduced (Frohlich and Barthel 1997). In which reproductive state the sponges were during our experiments is not known but it was shown that *H. oculata* in the Netherlands contains oocytes and embryos almost year round (Wapstra and van Soest 1987) and thus reproduction seems to be an ongoing process in this sponge. Other studies have shown that sponge explants or smaller sponges show higher growth rates (eg. Page et al 2005[a], de Caralt et al 2008[b]). However, in our growth study the sponge growth rate was about the same at the start and at the end, while at the beginning of the growth experiment the sponges were explants (Chapter 2).

Finally, sponges show high regeneration rates and they continuously reform (Ayling 1983, de Caralt et al 2008[b]). On one side sponges can grow while on the other they may shrink. In accordance with this de Caralt et al (2008[a]) suggests that sponges have a high apoptosis rate as well as a high rate of mitoses resulting in a low net growth rate. This continuous regeneration of sponge tissue will cost a lot of energy while low net growth is obtained.

Benthic filter feeders in general typically show oxygen extraction coefficients up to 10% (Jorgensen et al 1986). For *H. oculata* we found the oxygen extraction coefficient to be 3.3%. Thus, oxygen clearly does not limit sponge growth rate under normal conditions. Carbon availability seems to be sufficient as well, although we do not know exactly what part of the particulate carbon is usable by the sponge. The NGE agrees with the low growth rates found for this sponge species, since at low growth rates a relatively large part of carbon taken up is used to generate energy for maintenance and work.

Conclusion

Respiration rate, clearance rate and growth rate have been measured for the sponge *Haliclona oculata*. We found that only 34% of the available particulate carbon is used by the sponge for both respiration and growth. *H. oculata* appears not to be carbon limited. Sufficient carbon is available in the particulate pool for *H. oculata* as the respiratory carbon requirement can be easily fulfilled with the taken up particulate carbon. The net growth efficiency, being the ratio of carbon assimilated in biomass and the total carbon used by the sponge, was found to be 0.099 ± 0.013 (average ± SD). Thus only about 10% of the assimilated carbon is fixed in biomass and over 90% is used for generating energy for growth maintenance pumping and reproduction. The low NGE agrees with the low growth rates found for this sponge species. Oxygen was found not to be the limiting factor for growth, since only 3.3% of the oxygen pumped through the sponge body was used.

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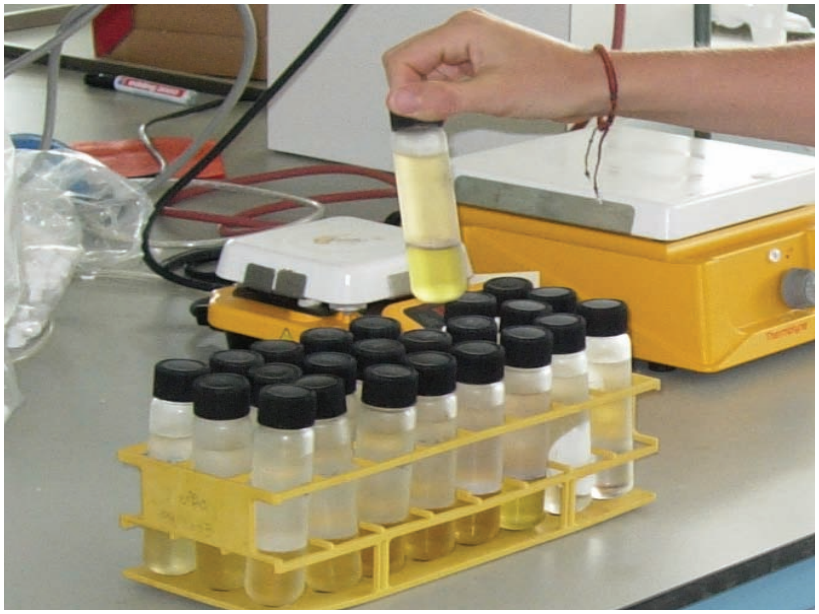
Chapter 4

Fatty acids as biomarkers in sponges



This chapter will be submitted for publication in combination with chapter 5 as:

Koopmans M, van Rijswijk P, Houtekamer M, Boschker HTS, Martens D, Wijffels RH (2009) Seasonal variation of fatty acid biomarkers and carbon isotopes in sponges.

Abstract

Sponges contain a large variety of bioactive compounds that can potentially be used as medicines for human. They are mostly slow growing organisms and in order to produce drugs by culturing sponges their growth must be improved. To improve growth, basic knowledge about how food sources are used by the sponge is needed. To find the exact relation between food retained and food converted to sponge biomass we need to be able to distinguish between feed components and sponge biomass, which means we need biomarkers. The fatty acid (FA) composition of organisms is specific and can therefore be used as biomarkers. In this study we identified and compared fatty acid profiles of five different sponges in three habitats and the corresponding FAs in the suspended particulate matter (SPM) in the surrounding water. *Haliclona oculata* and *Haliclona xena* from the Oosterschelde, *Haliclona xena* and *Halichondria panicea* from Lake Veere, both in the Netherlands and *Dysidea avara* and *Aplysina aerophoba* from the Mediterranean were studied. In the SPM we found comparable FAs to the FAs of sponges up to chain lengths of 28 C-atoms. Different species of sponges showed similarities, but also very different FA profiles while they were collected from the same habitat at the same moment. The biomarkers for diatoms and dinoflagellates were abundantly found in all sponges except *A. aerophoba* as this sponge relies mostly on bacterial food sources based on the many bacterial FAs found in this sponge. In all species, except *A. aerophoba,* C26:3(5,9,19) and C26:2(5,9) were very abundantly present. These FAs were also abundant in the SPM, while it was stated in literature that these compounds are very typical for sponges. Several FA biomarkers were found for the different sponges.

Introduction

Sponges are known for their rich variety of bioactive metabolites (Sipkema et al 2005[a]). These metabolites are potential drugs and in order to obtain sufficient amounts, a sustainable production technique is required. Culturing sponges *ex situ* will be an interesting option if we can stimulate sponge growth. Sponges are known to be slow growing organisms (e.g. Dayton 1979, Chapter 2). Due to the slow growth rate, *ex situ* sponge cultivation and thus sustainable production methods have not been attained this far. To be able to improve the growth of sponges, basic knowledge on how they acquire and use their food is necessary.

Sponges mainly feed by filtering particles from the large amounts of water they pump through their body. Besides filtering particles, sponges are also able to obtain food particles through the external epithelia cells and sponges containing symbiotic bacteria can obtain nutrients from these symbionts. In addition, it was shown that sponges can use dissolved organic material (de Goeij et al 2008). Many studies focused on the filtering capacity of sponges (e.g. Riisgård et al 1993, Ribes et al 1999, Chapter 3). However, in between the filtering of the particles and the actual entering of the particle components in metabolism is a lag time. Thus, when measuring increase in sponge biomass after feeding, a part of the measured increase in biomass will be sponge material, but still part possibly is non-metabolized food. Therefore to understand sponge metabolism it is necessary to be able to distinguish between feed and sponge components. Fatty acids (FAs) are very divers in structure and chain length and some are unique for certain organisms. Consequently, many researchers have used FAs and combination of FAs as biomarkers (eg. Kharlamenko et al 2008, Boschker and Middelburg 2002). See Table 1 for a summary of biomarkers that have been used in literature.

Table 1: Biomarkers known from literature

Source	Biomarker FA	Reference*
Bacteria	i15:0, ai15:0, i17:0, ai17:0	Bergé and Barnathan 2005
Diatoms	14:0, 16:1w7, 20:5 w3	Dunstan et al 1994
Dinoflagellates	22:6w3	Dalsgaard et al 2003

** A representative reference was chosen*

Among aquatic animals, sponges are characterized by the greatest diversity of fatty acids, which have an unusual and sometimes unique structure. Sponges contain large quantities of C_{22}-C_{30} fatty acids with branched and odd-chains and hydroxyl-acids. These very long chain fatty acids are also called 'demospongic acids' (Rod'kina 2005, Nechev et al 2002, Christie et al 1992). Very typical for 'demospongic acids' is the $\Delta^{5,9}$-diene double bond system, although monounsaturation is also found (Dasgupta et al 1986, Hahn et al 1988, Djerassi and Lam 1991). Long chain fatty acids are mostly found in biological membranes and determine the properties of membrane structures of cells.

Fatty acid biosynthesis in sponges relies greatly on short chain exogenous precursors (Djerassi and Lam 1991). Therefore, the composition of sponge FAs is probably related to the composition of FAs in their feed and symbionts. Microalgae and bacteria form an important fraction of the food taken up by sponges. Based on fatty acid biomarkers, bacteria and two large groups of the microalgae, dinoflagellates and diatoms, can be distinguished (see Table 1). These biomarkers can be used to find the origin of food inside the sponges. Apart from the food FAs also the sponge class has influence on its fatty acid composition. Bergquist et al (1984) concluded that different sponge classes could be determined by their FA profile.

The aim of this paper is to find sponge specific fatty acids that can serve as biomarkers for sponges. To find sponge specific fatty acid biomarkers we studied the fatty acid composition of the total lipids of five sponge species living in three habitats and identified biomarkers. In each location we studied the FA profile of two different sponge species and of the suspended particulate matter (SPM). *Haliclona oculata* and *Haliclona xena* from the Oosterschelde, the Netherlands, *Halichondria panicea* and *Haliclona xena* from Lake Veere, the Netherlands, and *Dysidea avara* and *Aplysina aerophoba* from the north-west Mediterranean were studied. Using this approach we compared different species in relation to SPM in the same habitat and the same species (*Haliclona xena)* in different habitats. The most abundant fatty acids in each specie were selected and identified. In future studies these sponge specific fatty acids will be used to study fatty acid metabolism in sponges using ^{13}C labeled algae.

Material and methods

Sponge and SPM collection

The sponge and SPM collection was done in February 2007 at three different locations by scuba diving. All sponges were collected and the fatty acid composition was measured in triplo. *Haliclona oculata* and *Haliclona xena* were collected from the Oosterschelde estuary, the Netherlands (Lokkersnol, 51°38′ 58.07″N, 3°53′5.11″E) at ~ 13 m depth. *Haliclona xena* and *Halichondria panicea* were collected from Lake Veere, the Netherlands (Geersdijk, 51°33′ 16.38″N, 3°45′57.86″E) at ~ 3 m depth. *Aplysina aerophoba* and *Dysidea avara* were collected in the Mediterranean, Spain (Cala Montgo, 42°06′50.33″ N, 3°10′02.10″E) at ~ 8 m depth. The collected specimen were immediately frozen on dry ice, and in this way transported to the laboratory. The sponges were stored at -80°C until freeze drying for 24 hrs. The dry sponges were grinded with a mortar and pestle before further usage.

Water samples were filtered by using a GF/F filter (0.7µm) to obtain suspended particulate matter (SPM). Due to differences in SPM concentration, different amounts of water were filtered for each habitat. In the Oosterschelde 1.5 liter, Lake Veere 1 liter and in the Mediterranean 4 liters per sample. All samples were collected in triplo.

Fatty acid extraction and analysis

The filters with SPM or 100 mg sponge dry weight were used for lipid extraction using an adjusted Bligh and Dyer method (Boschker et al 1999). The total lipid fraction was derivatized by mild alkaline methanolysis to obtain fatty acid methyl esters (FAME). Both C12:0 and C19:0 were used as internal FAME standards. Identification of the fatty acids was done using equivalent chain length data with known standards measured on a non-polar column (see below). Additional identification was done by gas chromatography mass spectrometry (GC-MS) on a Thermo Finnigan mass selective detector (Voyager). Helium was used as a carrier gas (150 kPa) with an apolar column (Hewlett-Packard HP-5MS, 60 m*0.32 mm*0.25µm). The column was kept at 70°C for 2 min, then temperature was programmed from 70 to 150°C at 20°C min^{-1}, subsequently from 150 to 290°C at 3°C min^{-1} and from 290°C to 325 °C at 5 °Cmin^{-1}, temperature is kept at 325 °C for 10 minutes. Fatty acid concentrations were determined by area correction according to the C19:0 internal standard. The remaining un-

known fatty acids were further identified by preparing picolinyl esters. The FAME samples were incubated for 30 minutes at 40ºC with 0.1 ml 1.0 M potassium tert-butoxide in tetrahydrofuran and 0.2 ml 3-pyridylmethanol. Then 2 ml H_2O and 4 ml hexane was added to get phase separation, the hexane containing the picolinyl esters was recovered and measured using GC-MS (see also www.lipidlibrary.co.uk). The column was now heated for a longer period to obtain all picolinyl esters: end temperature 325 °C for 30 minutes.

Data analysis

All measured retention times were adjusted using C12:0 and C19:0 standards to obtain equivalent chain length (ECL) data. Standards were used for the identification of FAs. Additional identification was done using mass spectra of the different compounds, and of the picolinyl esters compared with a NIST library and the lipid library from www.lipidlibrary.co.uk. Further confirmation was done by comparison with literature (Christie et al 1992, Nechev et al 2002, Rod'kina et al 2003).

Many compounds were found in the different samples of which not all could be identified. We have focused on the FAs that were present more than 1% of total FAs in one of the samples. For each sponge and location we took samples in triplo. The obtained data were averaged for these three samples. The relative content was determined for all FAs.

Results and discussion

General results

Three figures are used to show the fatty acid (FA) profile of the SPM and the sponges from the different locations (Figure 1-3), each location is shown in a different figure. For clarification; appendix I shows the values of the relative content of all found FAs in the different sponges. Many compounds inside the sponges were also present in the SPM. The shorter FAs (up to 22 C-atoms) are mostly retained from environmentally or symbiotically present organisms and stored or elongated in the sponge cells. Diatoms, algae and bacteria contain these shorter chain fatty acids and their biomarkers are shown in the graphs (see Table 1 for biomarkers). 49 FAs were found to be over 1w% in at least one sample. These FAs represent 84 – 96% of total FAs in the samples. The major peaks and most of the other peaks could be identified. We did not focus

on identifying new compounds as our final goal is to study feed uptake and metabolism in sponges.

Comparing locations

In all three locations we found similar FA profiles, with some differences in concentration. The fact that the FA profiles were so similar makes it difficult to study the effect of location or food fatty acid composition on sponge fatty acid composition. Dinoflagellates and diatoms were very abundant in the SPM as their biomarkers were abundantly present (see Table 1 for used biomarkers). The bacterial biomarkers were less abundant. We found that FAs up to C28 were also present in the suspended matter, especially C26:3(5,9,19) and C26:2(5,9) were found abundantly in the SPM. This is surprising, since these fatty acids with 5,9 poly unsaturation, so called demospongic acids, are believed to originate from sponges (Hahn et al 1988, Rod'kina 2005) and are not present in bacteria and algae. To our knowledge no record is made before about poly unsaturated very long chain fatty acids in SPM and the origin of these fatty acids in the suspended matter is not clear. The SPM of all three locations show the short chain fatty acids being dominant and always more dominant than for the sponges. C28:3(5,9,21) was most abundantly found in Oosterschelde and much lower values were found in the other locations. The concentration of particles in Lake Veere was found higher than in Oosterschelde and Mediterranean, which resulted in a higher FA content as well.

Comparing sponges

H. xena from both Oosterschelde and Lake Veere show similar FA profiles, where for both locations C28:3(5,9,21) was abundantly present in the sponge despite the fact that less of this compounds was found in the SPM of Lake Veere. The FA profile of *H. oculata* was also very similar to the FA profile of *H. xena*, whereas *H. panicea* showed some differences from the other Dutch sponges. The major compound for *H. panicea* is C26:3(5,9,19), while for the *Haliclona* sponges the major compound was C28:3(5,9,21). Thus, the differences in fatty acid composition between sponges depend at least for a major part on the family relation. The short chain FA biomarkers were more abundant in the sponges in Lake Veere than in the sponges in the Oosterschelde. This is probably a result from the higher particle content in the water and thus more particles will be present in the sponge bodies. The Mediterranean sponges were

found to be very different from the Dutch sponges. In *D. avara* we found more FAs with a concentration larger than 1% compared to the other sponges in this study. And a complete different profile was found for *A. aerophoba* compared with the other sponges.

All sponges except *A. aerophoba* contain the biomarkers for dinoflagellates and diatoms and the demospongic fatty acids C26:3(5,9,19) and C26:2 (5,9) abundantly. Biomarkers of dinoflagellates were more abundantly present than biomarkers of diatoms in the sponges of the *Haliclona* family and *D. avara* as FA C22:6w3 (DHA) was found higher than C20:5w3 (EPA) in these sponges. For *H. panicea* it can be seen that the EPA and DHA content were about the same. Thus it seems that the *Haliclona* sponges preferably filter out dinoflagellates over diatoms, or conversion from EPA to DHA occurs at a fast rate. In *A. aerophoba* these FAs were not abundantly present. Instead, *A. aerophoba* contained bacterial FAs in large amounts, which indicates the presence of a large population of symbiotic bacteria inside the sponge. *A. aerophoba* is known to be a bacterial sponge. They can contain bacteria up to 40% of their biomass (Hentshel et al 2002). Probably *A. aerophoba* mostly relies on its symbiotic bacteria for food supply and less on particle uptake. Bacterial FAs were found in low amounts in all Dutch sponges and in *D. avara*. Low concentrations of these biomarkers in the sponges indicate that they do not contain many symbiotic bacteria.

Table 2. Sum of FA content (%) divided in short chain FAs (C22 and lower) and large chain FAs (C23 and higher). O, V, M means Oosterschelde, Lake Veere and Mediterranean respectively. Values are derived by using the average of three sponge samples.

	O	H. oculata	H. xena O	V	H. xena V	H. panicea	M	A. aerophoba	D. avara
Short chain Fas	74.9 ± 2.5	33.0 ± 2.9	36.0 ± 1.7	71.4 ± 18.6	44.8 ± 3.6	50.2 ± 3.3	68.8 ± 10.0	62.1 ± 9.0	47.2 ± 6.4
Long chain Fas	27.0 ± 2.2	67.0 ± 5.5	64.0 ± 2.0	29.3 ± 5.6	55.2 ± 3.2	49.8 ± 3.2	31.3 ± 7.2	35.9 ± 10.0	52.5 ± 7.5

Biomarkers for the different sponges

The longer chain fatty acids (>C22) represent the 'demospongic acids', and for most sponges the longer chain FAs are dominant (Table 2). *H. panicea* and *A. aerophoba* do not show this difference. As discussed before *A. aerophoba* contains many bacteria, which contain many short chain fatty acids. The high amount of shorter chain FAs in *H. panicea* is probably related to the high content of particulate matter in Lake Veere.

To identify sponge fatty acid biomarkers, the sponge fatty acid composition was compared to that of the SPM FAs. The fatty acids that are present in the sponges and not in SPM can be used as biomarkers. The FAs larger than 28 carbon atoms as found in *A. aerophoba* can clearly be used as biomarkers. Also *D. avara* has several clear biomarkers. For the other sponges the found biomarkers were less obvious, as the found biomarkers were present in low amounts. For the sponges located in Lake Veere we found no long chain biomarkers. Some of the C26 PUFAs and C28 PUFAs were also unexpectedly found in the SPM. Table 3 shows the biomarkers per sponge specie as found in this paper.

Table 3. Found biomarkers for the different sponges used in this study.

Sponge specie	Biomarkers
Haliclona oculata (Netherlands)	C25:2(5,9), C24:1(15)
Haliclona xena OS (Netherlands)	C25:2(5,9), C24:1(15)
Haliclona xena VM (Netherlands)	7MeC16:1(7)
Halichondria panicea (Netherlands)	7MeC16:1(7)
Aplysina aerophoba (Spain)	22MeC28:2(5,9), C30:3(5,9,23), C28:3(5,9,21)
Dysidea avara (Spain)	C22:1(11), C24:1(15), C27:2(5,9)

In all sponges we found both algal and bacterial biomarkers, albeit in different concentrations. Herewith we can conclude that they filter out both substrates. *A. aerophoba* contains more bacterial biomarkers and we also found high amounts of branched long chain FAs, which results from elongation of shorter chain bacterial FAs. In the other sponges no or few of these FAs are found. Therefore *A. aerophoba* probably relies more on bacteria than on algae for their substrate supply, whereas the other sponges probably feed mostly on microalgae and less on bacterial cells.

Conclusion

In this study we identified and compared fatty acid (FA) profiles of five different sponges in three habitats and compared these to the FA profile of the suspended particulate matter (SPM) present in the three habitats. We found that sponges from the same family, *Haliclona oculata* and *Haliclona xena* were very similar in FA profile. *Halichondria panicea* also in the Netherlands showed a similar FA profile to *Haliclona xena* in the shorter chain FAs, but in the sponge specific longer chain FAs differences occurred. *Aplysina aerophoba* and *Dysidea avara* both living in the Mediterranean, show complete different profiles from each other and from the Dutch sponges. All sponges show the 5,9 double bond formation mostly in the longer chain fatty acids.

From the biomarkers present in the sponges we found that an important food source for most sponges, *Haliclona oculata*, *Haliclona xena*, *Halichondria panicea* and *Dysidea avara*, are diatoms and dinoflagellates. *A. aerophoba* contains mostly bacterial fatty acids and hardly algal biomarkers, and thus mostly relies on bacteria as a food source.

In the suspended particulate matter (SPM) in the surrounding water we found similar FA profiles to the sponges even up to the long chain fatty acids containing 28 carbon atoms. These long chain fatty acids in SPM were not reported before and the origin is unclear. Most importantly also fatty acids were found in the sponges that were not found in the surroundings and thus can be used as sponge-biomarkers (Table 3).

Acknowledgements

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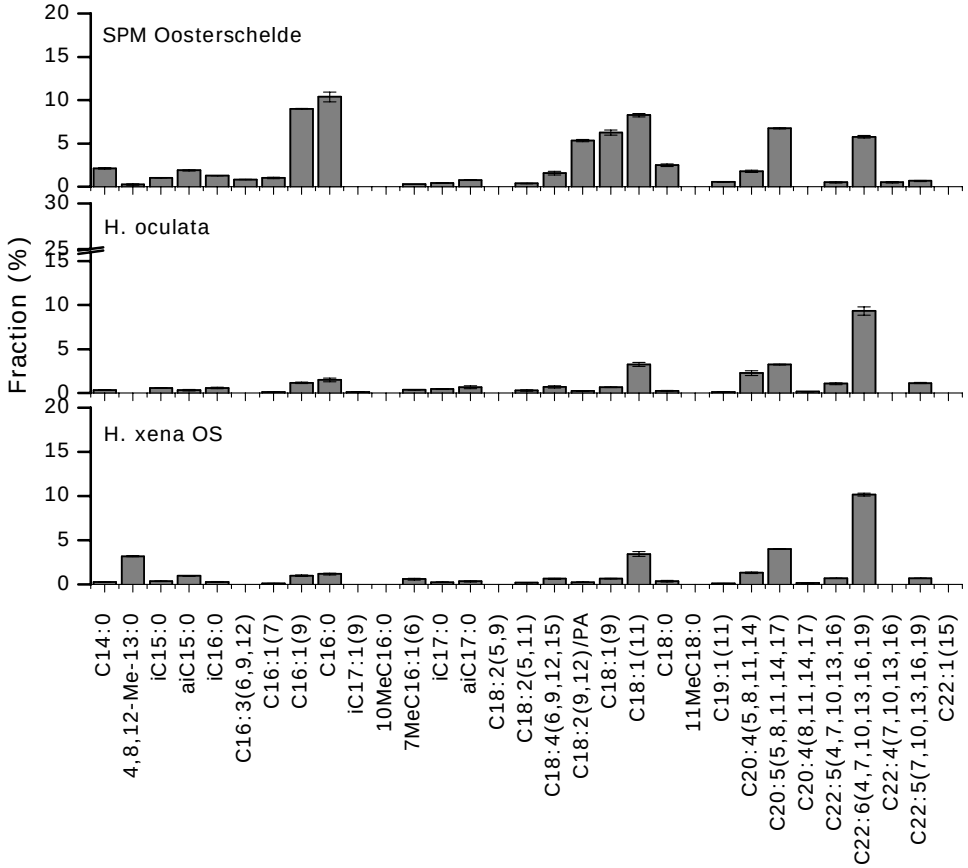

Figure 1A: Small chain fatty acid profile of SPM and *Haliclona oculata* and *Haliclona xena* from the Oosterschelde. PA stands for phytanic acid.

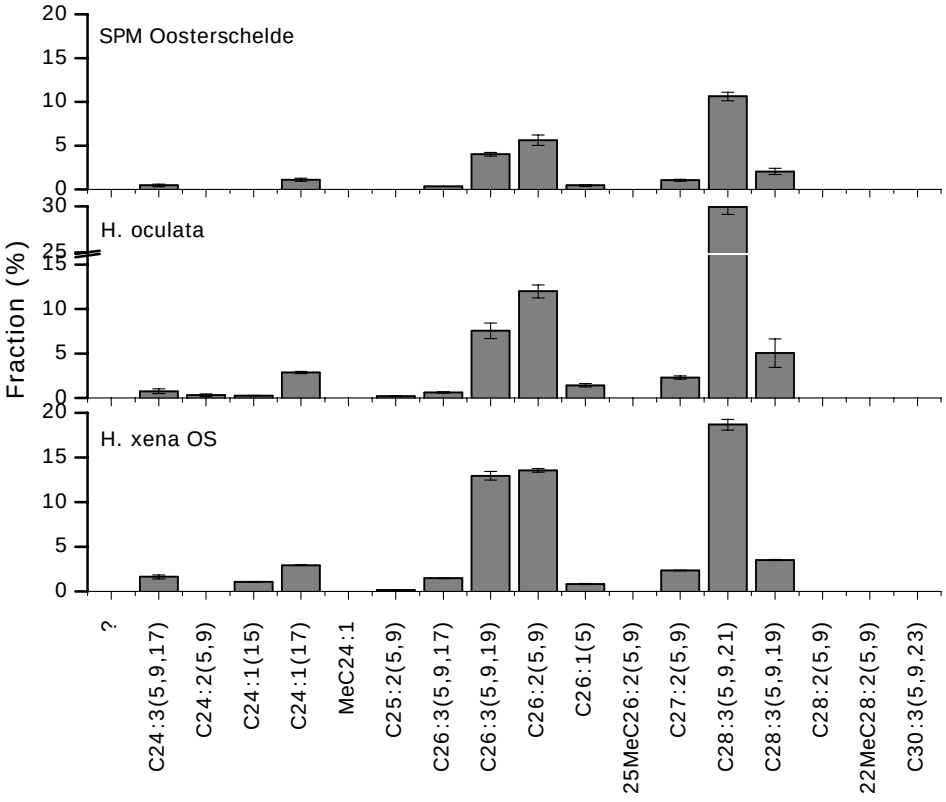

Figure 1B: Long chain fatty acid profile of SPM and *Haliclona oculata* and *Haliclona xena* from the Oosterschelde.

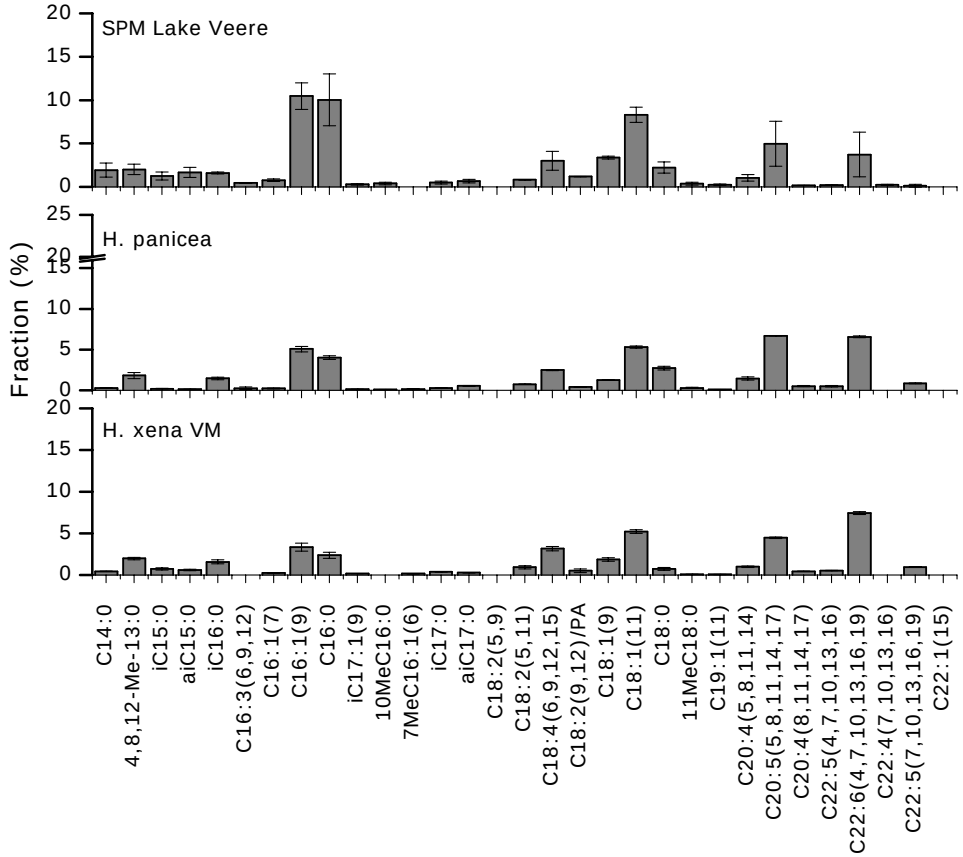

Figure 2A: Small chain fatty acid profile of SPM, *Halichondria panicea* and *Haliclona xena* from Lake Veere. PA stands for phytanic acid.

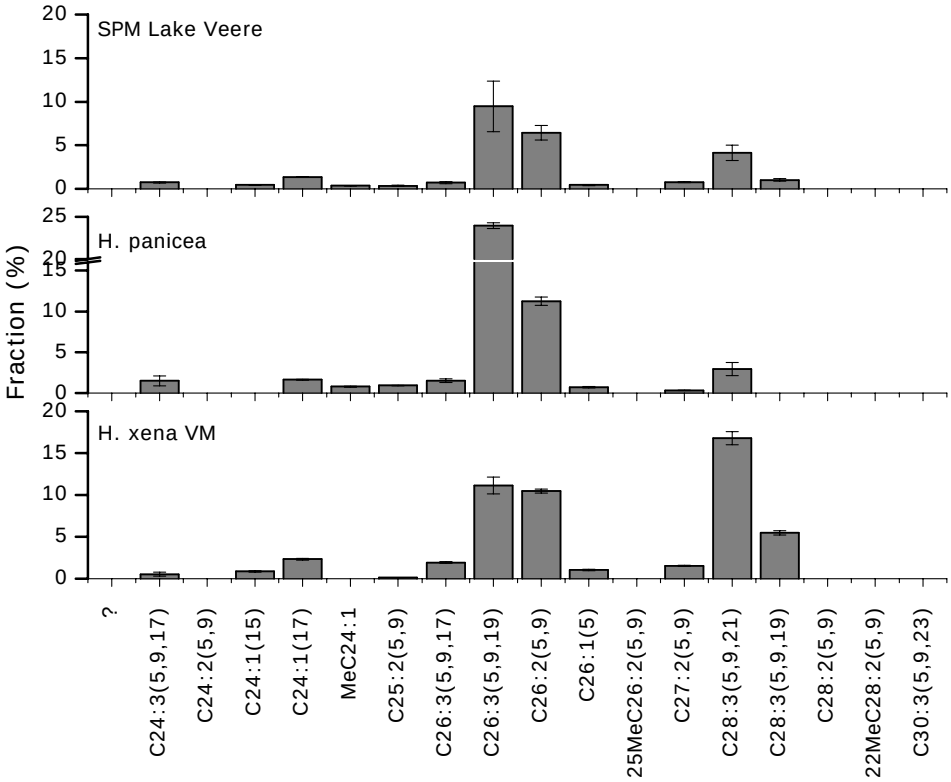

Figure 2B: Long chain fatty acid profile of SPM, *Halichondria panicea* and *Haliclona xena* from Lake Veere.

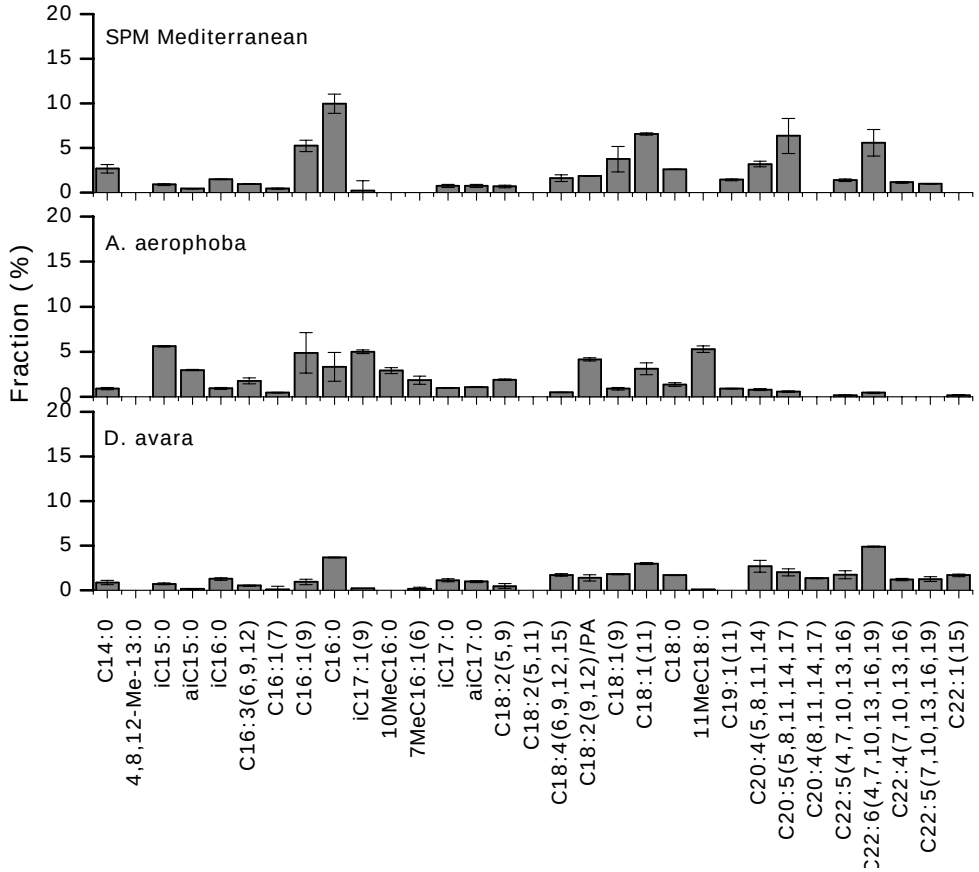

Figure 3A: Small chain fatty acid profile of SPM, *Aplysina aerophoba* and *Dysidea avara* from the Mediterranean. PA stands for phytanic acid, and in these sponges PA is the major compound compare to C18:2(9,12).

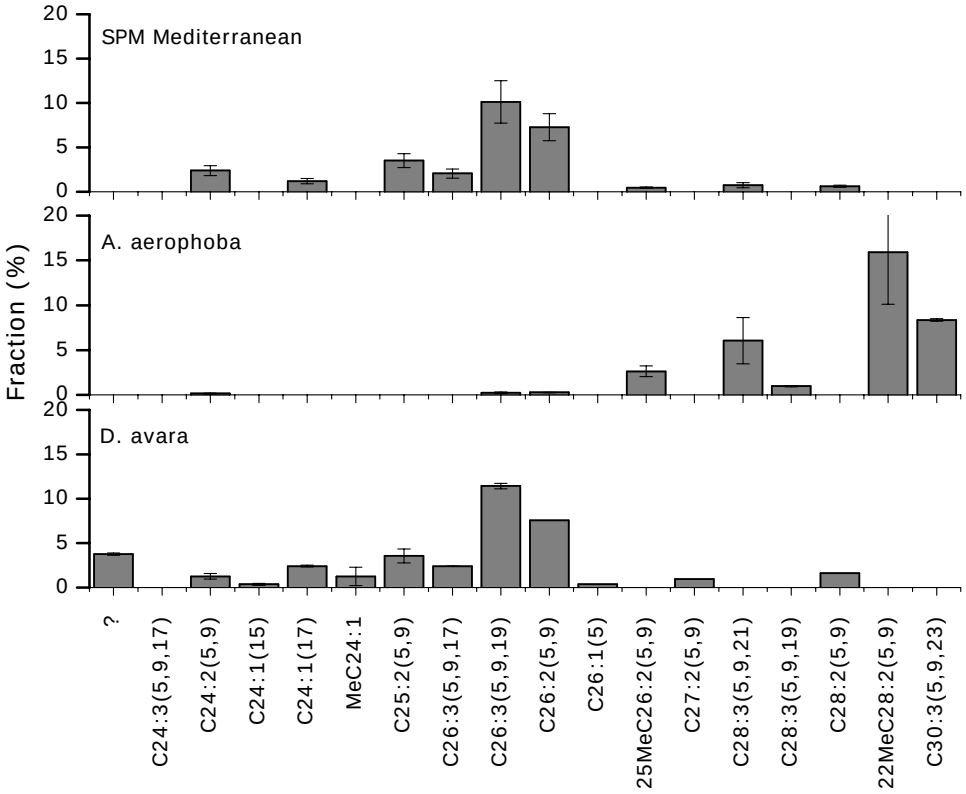

Figure 3B: Long chain fatty acid profile of SPM, *Aplysina aerophoba* and *Dysidea avara* from the Mediterranean. PA stands for phytanic acid, and in these sponges PA is the major compound compare to C18:2(9,12).

Appendix I

Relative content of fatty acids and corresponding equivalent chain length in 5 different sponge species from 3 different habitats. X means that FA is present in trace amounts.

					Found in sponge:					
					Oosterchelde		Lake Veere		Mediterranean	
RT	ECL	Fatty acid	# C-atoms	M+	H. ocu-lata	H. xena	H. xena	H. panicea	A. aerophoba	D. avara
10.50	11.29	??	?	167	0.96	0.81	0.55	0.4	0.19	
12.40	12	C12:0	12	214	0.15	0.28	0.16	0.12	0.1	0.1
16.25	13.44	iC14:0	14	242	X	X	X	X	0.31	
16.73	13.62	C14:1w5	14	240			0.21	0.17		X
17.25	13.82	C14:0	14	242	0.35	0.27	0.45	0.26	0.9	0.88
18.65	14.35	4,8,12M13:0	16	270	X	3.2	1.98	1.81	X	
19.02	14.48	iC15:0	15	256	0.58	0.37	0.75	0.19	5.6	0.72
19.25	14.57	aiC15:0	15	256	0.34	0.97	0.61	0.12	2.96	0.17
20.08	14.88	C15:0	15	256	0.17	0.13	0.16	0.24	0.72	0.16
21.84	15.54	C16:4w3	16	262	X		X	X	X	
21.95	15.58	iC16:0	16	270	0.58	0.29	1.58	1.45	0.93	1.72
22.09	15.64	C16:3w4	16	264				0.23	1.74	0.52
22.29	15.71	C16:1w9	16	268	X	X	0.26	0.22	0.45	X
22.44	15.77	C16:1w7	16	268	1.17	0.98	3.35	5.07	4.86	0.95
22.53	15.8	C16:1w7	16	268	X	X	X	X		X
22.71	15.87	C16:1w5	16	268	X	X	0.12	0.15	1	0.12
23.06	16	C16:0	16	270	1.51	1.19	2.38	4.03	3.3	3.68
23.21	16.05	?MeC16:0	17	284					0.38	
23.55	16.19	C17:2(5,9)	17	280					0.24	
24.20	16.38	iC17:1w7	17	282	X	X	0.17	0.12	4.99	0.2
24.40	16.44	10MeC16:0	17	284					2.89	
24.48	16.47	aiC17:1w7	17	282	X	X	0.19	0.18		x
24.73	16.55	7MeC16:1(6)	17	282	0.37	0.6	0.16	0.15	1.84	0.17
24.97	16.63	iC17:0	17	284	0.46	0.25	0.4	0.28	0.98	1.1
25.24	16.72	aiC17:0	17	284	0.67	0.35	0.29	0.55	1.08	0.97
25.38	16.77	C17:1w9	17	282			X	X	0.33	X
25.65	16.86	9,10CyC16:0	17	284	X	X	0.17	X	0.58	0.15
26.09	17.01	C17:0	17	284	0.22	0.22	0.15	0.23	0.21	0.28
27.77	17.56	C18:2(5,9)	18	294					1.88	0.47
27.78	17.57	C18:2(5,11)	18	294	0.3	0.21	0.94	0.73		
27.86	17.59	C18:4w3/ iC18:0	18	292	0.71	0.65	3.17	2.49	0.47	1.71
28.18	17.7	C18:2w6/PA	18/20	294/326	0.23	0.26	0.52	0.38	4.14	1.39
28.35	17.76	C18:1w9	18	296	0.67	0.68	1.87	1.26	0.89	1.8
25.52	16.82	C18:1w7	18	296	3.25	3.45	5.21	5.3	3.1	2.99
29.12	18.01	C18:0	18	298	0.23	0.36	0.73	2.72	1.34	1.68
30.30	18.4	11MeC18:0	19	312			X	0.29	0.52	
30.84	18.58	15MeC18:0	19	312			X	X	0.5	
31.25	18.72	aiC19:0	19	312	0.47	X	X	X		0.33
31.69	18.86	C19:1(11)	19	310	0.13	0.11	X	X	0.91	X
32.10	19	C19:0	19	312	X	X	X	X	X	X
33.01	19.3	C20:4w6	20	318	2.27	1.34	0.99	1.44	0.8	2.68
33.23	19.38	C20:5w3	20	316	3.24	3.99	4.49	6.69	0.58	2.01
33.77	19.55	C20:4w3	20	318	0.15	0.15	0.45	X		1.35

					Found in sponge:					
					Oosterchelde		Lake Veere		Mediterranean	
RT	ECL	Fatty acid	# C-atoms	M+	H. ocu-lata	H. xena	H. xena	H. panicea	A. aerophoba	D. avara
33.90	19.6	iC20:0	20	326				0.28		
34.15	19.68	C20:1w7	20	324	0.15	0.25	0.44	0.23	0.12	0.61
34.30	19.73	C20:3w7	20	320	0.16	0.41	0.43	0.37		
34.44	19.78	C20:1w9	20	324	0.15	0.25	0.23	0.31	0.14	0.4
34.98	19.96	C20:0	20	326			X	0.16	0.12	0.38
35.33	20.07	14MeC20:0	21	340					x	X
36.12	20.33	C21:5w3	21	?	0.19	0.16	0.2	0.37	0.22	0.37
33.90	20.55	aiC21:0	21	340				X	0.31	
37.03	20.64	13MeC20:0	21	340	X		X	0.79	0.7	X
37.80	20.89	C21:0	21	340				X		X
38.27	21.05	C22:5w6	22	344	1.07	0.47	0.54	0.49	0.15	1.73
38.53	21.13	C22:6w3	22	342	9.34	12.25	7.45	6.57	0.44	4.89
38.67	21.18	C22:4w6	22	346			X	X		1.19
38.87	21.25	C22:5w3	22	344	1.14	1.28	0.97	0.84	X	1.25
39.29	21.39	C22:2(5,9)	22	350	0.57	0.7	0.39	X	X	X
40.04	21.63	C22:1w7	22	352	0.2	0.77	0.46	X	0.17	1.68
40.12	21.66	C22:1W11	22	352						1.02
40.53	21.8	C22:0	22	354	X		X	0.63		X
41.26	22.04	C23:2(5,9)	23	364					0.21	
42.43	22.43	?								3.76
42.45	22.43	aiC23:0	23	368					X	
43.16	22.67	C23:0	23	368	X	X	X	X	X	0.29
43.75	22.87	C24:6w3	24	370	X	X	0.21			X
44.19	23.01	C24:3(5,9,17)	24	370	0.76	1.63	0.54	1.51		0.26
44.64	23.16	C24:2(5,9)	24	378	0.31			X	0.15	1.26
45.13	23.32	C24:1w9	24	380	0.26	1.08	0.87	X		0.35
45.35	23.4	C24:1w7	24	380	2.87	2.95	2.33	1.63		2.41
45.71	23.52	C24:0	24	382	X	X		X		0.45
46.06	23.63	iC25:1	25	394			X	0.8		1.24
46.71	23.85	C24:2?	24?	378	0.44	0.42	X	X		
46.83	23.89	C25:2	25	392				0.6		
46.35	23.73	C25:3	25	390	X					
47.16	24	C25:2(5,9)	25	392	0.22	0.18	0.15	0.92		3.54
48.13	24.32	C26:?	26		0.24	0.12			0.64	1.85
49.08	24.63	C26:3(5,9,17)	26	404	0.61	1.48	1.93	1.5		2.4
49.32	24.71	C26:3(5,9,19)	26	404	7.57	12.95	11.1	24	0.23	11.4
49.72	24.85	C26:2(5,9)	26	406	12	13.55	10.5	11.3	0.27	7.57
49.91	24.91	C26:1(17)	26	408	0.32	0.71	0.53	X		
50.07	24.96	C26:1(19)	26	408	0.84	0.62	0.53	0.95		0.61
50.20	25.01	C26:1(5)	26	408	1.43	0.84	1.05	0.7		0.39
50.50	25.11	25MeC26:2(5,9)	27	420					2.64	
50.72	25.18	BranchedC27:1	27	422	X	X	X	0.49		
51.54	25.45	C27:3(5,9,20)	27	418	X	X	X	0.63	0.66	0.44
51.65	25.49	C27:3	27	418	X	X	X	X		X
51.97	25.59	C27:2(5,9)	27	420	2.29	2.36	1.53	0.33		0.94
53.01	25.94	BrC27:2(5,9)	28	434					0.83	
53.98	26.26	C28:3(5,9,21)	28	432	29.9	18.66	16.8	2.94	6.06	X
54.15	26.32	C28:3(5,9,19)	28	432	5.05	3.53	5.47		0.98	
54.38	26.39	C28:2(5,9)	28	434	X		X			1.59
55.13	26.64	22MeC28:2(5,9)	29	446					15.92	
57.94	27.58	C30:3(5,9,23)	30	454	X				8.36	

Chapter 5

Seasonal variation of bio-markers and carbon isotopes in sponges

This chapter will be submitted for publication in combination with chapter 4 as:

Koopmans M, van Rijswijk P, Houtekamer M, Boschker HTS, Martens D, Wijffels RH (2009) Seasonal variation of fatty acid biomarkers and carbon isotopes in sponges.

Abstract

The fatty acid (FA) composition and ^{13}C natural abundance of sponges and of suspended particulate matter (SPM) from seawater was studied in different seasons at three locations. *Haliclona oculata* and *Haliclona xena* from the Oosterschelde, the Netherlands, *Halichondria panicea* and *Haliclona xena* from lake Veere, the Netherlands, and *Aplysina aerophoba* and *Dysidea avara* from the Mediterranean, Spain, were studied. The FA concentration variation in sponges was related to changes in fatty acid concentration in SPM. $\delta^{13}C$ in sponge specific FAs showed very limited seasonal variation at all sites. Algal FAs in sponges were mainly acquired from the SPM through active filtration in all seasons. Sponge specific FAs had similar $\delta^{13}C$ ratios as algal FAs in May at the two Dutch sites, suggesting that sponges were mainly growing during spring and probably summer. During autumn and winter, they were still actively filtering, but the food collected during this period had little effect on sponge $\delta^{13}C$ values suggesting limited growth. The bacterial sponge *A. aerophoba* relies mostly on the symbiotic bacteria. In all sponges we found that the ω7 longer chain FAs, C24:1(17) and C26:3(5,9,19) could be traced back to be of bacterial origin. Thus, fatty acid composition can be used to analyze the food source of sponges.

Introduction

Up till now, no sustainable production techniques have been developed to obtain sufficient quantities of bioactive metabolites discovered in sponges. In order to maximize sponge productivity we need to understand their feeding patterns and metabolic capabilities. Sponges pump large amounts of water through their body from which particles are filtered. Many studies focused on the filtering capacity of sponges (e.g. Riisgård et al 1993, Ribes et al 1999, Chapter 3). However, there is a time-lag between the filtering of the particles and the actual entering of the particle components in sponge metabolism. Thus, when measuring sponge biomass increase after feeding, part of the biomass may consist of non-metabolized food. Therefore, to understand sponge metabolism it is necessary to be able to distinguish between food and sponge components. Fatty acids (FAs) are very divers in structure and chain length and some are unique for different organisms. Consequently, specific FAs can be used as biomarkers for sponges and their food sources (Chapter 4).

A broad range of FAs have been found in sponges (e.g. Bergquist et al 1984, Christie et al 1992, Joh et al 1997, Nechev et al 2002, Rod'kina et al 2003, Chapter 4). Fatty acids are a source of metabolic energy and essential materials for the formation of cell membranes (Bergé and Barnathan 2005). Sponges contain large quantities of very long chain fatty acids of C_{22}-C_{30} (Hahn et al 1988, Rod'kina 2005). Within the long chain FAs, odd-numbered, branched and hydroxyl acids were found. The biosynthesis of these fatty acids is done via elongation of monoenoic or branched precursors followed by desaturation (Hahn et al 1988). Very typical for 'demospongic acids' is the $\Delta^{5,9}$-diene double bond system, although monounsaturation is also found (Dasgupta et al 1986, Hahn et al 1988, Djerassi and Lam 1991). Long chain fatty acids are mostly found in biological membranes and determine the properties of membrane structures in sponge cells.

The composition of FAs is to some extent determined by the available food sources. Sponges are filter feeders and are unable to synthesize all necessary FAs themselves. Therefore they take up FAs from suspended particulate matter (SPM) and store and elongate these food FAs (Djerassi and Lam 1991 and references within). Besides, many sponges contain symbiotic bacteria, which can also serve as a source of FAs (Rod'kina 2005). The fatty acid concentration can vary due to different food sources and thus the FA composition can vary seasonally. The contents of small chain FAs considerably differed for

H. panicea at different times, which may be due to changes in associated bacteria or in food source availability (Rod'kina et al 2003). Thus, by measuring FA composition in sponges one can study food sources utilized in the field. Biomarkers used in this study to determine food sources are listed in Table 1.

Table 1: Fatty acid biomarkers for different groups of organisms based on literature.

Source	**Fatty acid**	**Reference[a]**
Diatoms	C20:5(5,8,11,14,17)	Dunstan 1994
Dinoflagellates	C18:4(6,9,12,15), C22:6 (4,7,10,13,16,19)	Kharlamenko 2008
Bacteria	Odd and branched C15 and C17, C16:1(9), C18:1(11)	Kharlamenko 2008
Sponges (*Haliclona* and *Halichondria panicea*)	C24:1(15), C25:2(5,9), C26:2 (5,9)[b] and C26:3(5,9,19)[b]	Chapter 4
Sponge (*Dysidea avara*)	C22:1(11), C24:1(15), C26:2 (5,9)[b], C26:3(5,9,19)[b] and C27:2 (5,9)	Chapter 4
Sponge (*Aplysina aerophoba)*	22MeC28:2(5,9), C30:3(5,9,23)	Chapter 4

[a] A representative reference is shown.

[b] These FAs were not assigned as biomarkers previously, however, in other studies they were found to be sponge specific (Readerstorff et al 1987, Hahn et al 1988) and they are very abundantly present in these sponges.

In nature differences can be found in isotopic ratios (Peterson and Fry 1987). These differences are caused by the preference of ^{12}C over ^{13}C in many biological and chemical processes. Bacteria and algae have variation in $^{13}C/^{12}C$ ratio due to differences in inorganic substrate, fixation pathways or environmental and physiological conditions (Boschker and Middelburg 2002). Both fatty acid biomarkers and isotope ratios can be used separately to study food chains. The use of biomarkers in combination with stable isotope analysis is a rather new approach to study origin of organic matter utilized by microorganisms and higher organisms in complex ecosystems (Boschker and Middelburg 2002). Via fatty acid biomarkers in combination with natural abundance stable isotope analysis, the type of food sources used by the sponge can be determined (Abraham et al 1998, Thurber 2007).

In a previous study we have described the FA composition of five different sponges in three locations including the surrounding FAs (Chapter 4). To find the type of food used by different sponges and the ability to adjust to

changes we studied the variation in FA content in different seasons and the corresponding specific isotope ratios of FAs. The same five sponges are used as in the previous study, namely: *Haliclona oculata* and *Haliclona xena* from the Oosterschelde, the Netherlands, *Haliclona xena* and *Halichondria panicea* from Lake Veere, the Netherlands and *Aplysina aerophoba* and *Dysidea avara* from the Mediterranean, Spain. FA concentrations and ^{13}C content were analyzed to determine the food sources.

Material and methods

Sponge and SPM collection

The collections of sponges and SPM were done at three different locations by scuba diving in February, May and October 2007. All sponges were collected and analyzed in triplo. *Haliclona oculata* and *Haliclona xena* were collected from the Oosterschelde estuary, the Netherlands (Lokkersnol, 51°38′58.07″N, 3°53′ 5.11″E) at ~ 13 m depth. *Haliclona xena* and *Halichondria panicea* were collected from Lake Veere, the Netherlands (Geersdijk, 51°33′16.38″N, 3°45′ 57.86″E) at ~ 3 m depth. *Aplysina aerophoba* and *Dysidea avara* were collected in the Mediterranean, Spain (Cala Montgo, 42°06′50.33″N, 3°10′02.10″ E) at ~ 8 m depth. The collected specimen were immediately frozen on dry ice and transported to the laboratory. The sponges were stored at -80°C until freeze drying for 24 hrs. The dry sponges were grinded with a mortar and pestle before further usage.

Water samples were filtered by using precombusted GF/F filters (nominal poresize 0.7µm) to obtain suspended particulate matter (SPM). Due to differences in SPM concentration, different amounts of water were filtered for each habitat: the Oosterschelde 1.5 liter, Lake Veere 1 liter and the Mediterranean 4 liters per sample. All samples were collected and analyzed in triplo, and immediately stored in extraction fluid after filtration.

Fatty acid and $\delta^{13}C$ analyses

The filters with SPM and 100 mg sponge dry weight were used for lipid extraction using an adapted Bligh and Dyer method (See also Boschker et al 1999). The total lipid fraction was derivatized by mild alkaline methanolysis to obtain fatty acid methyl esters (FAME). Both C12:0 and C19:0 were used as internal FAME standards. Identification of the fatty acids was done using equivalent

chain length data with known standards measured on an a-polar column (see below). Additional identification was done by GC-MS as described in Chapter 4. Carbon isotopic composition of individual FAME was determined on a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS) consisting of a HP G1530 GC (Hewlett Packard) connected to Delta-plus IRMS via a type-III combustion interface from Thermo Finnigan (Bremen). An a-polar column (Hewlett-Packard HP-5MS, 60 m*0.32 mm*0.25µm) was used with helium as a carrier gas (150 kPa). The column was kept at 70°C for 2 min, then temperature was programmed from 70 to 150°C at 20°C min^{-1}, subsequently from 150 to 290°C at 3°C min^{-1} and from 290°C to 325 °C at 5 °Cmin^{-1}, and finally temperature was kept at 325 °C for 11 minutes. Fatty acid concentrations were determined from peak areas calibrated against the C19:0 internal standard. Stable carbon isotope ratios ($^{13}C/^{12}C$) for individual FA were calculated from FAME data by correcting for the one carbon atom in the methyl group that was added during derivatization. High-precision isotope ratios are usually given in the δ-notation defined as the relative difference in parts per thousand against the international standard for carbon of Vienna Pee Dee Belemnite (VPDB) ($\delta^{13}C$ = 0 ‰, R_{VPDB}= $^{13}C/^{12}C$-ratio= 0.0111797) (Peterson and Fry 1987; Boschker and Middelburg 2002). $\delta^{13}C$ can be calculated using the following equation:

$$\delta^{13}C\ (‰) = (R_{sample}/R_{VPDB} - 1) * 10^3 \quad (1)$$

Statistics

Statistics were performed on the original dataset. One-way ANOVA analysis was done to find differences between location, time, organisms and fatty acids. Tukey test was used for pair comparison when homogeneity assumption was justified as tested by calculation of the Levene statistics (SPSS® 12.0.1, Inc., 1989–2003).

Results and discussion

Sponges are dependent on food sources to supply compounds like fatty acids (FAs). They take up essential FAs from these food sources, which are used to build cell membranes or to obtain the very long chain sponge-specific FAs by elongation, also used to build cell membranes (Morales and Litchfield 1977, Readerstorff et al 1987, Hahn et al 1988). Thus when food sources change, the FA concentration and composition in the sponges may change as well.

Seasonal variation in total fatty acid concentration

For the Dutch waters in general the highest total fatty acid concentration in SPM as well as in the sponges were found in May, except for *H. panicea* (Table 2). The lowest concentration was always found in October. Especially in Lake Veere, The Netherlands, large differences in FA concentration were detected between sampling periods. This can be explained by the algal bloom that occurs during spring. In our previous study we found that the chlorophyll *a* and carbon content in the suspended particulate matter (SPM) also peaked in May (Chapter 2). In the Mediterranean the fatty acid concentration in the SPM was low and more or less constant (Table 2). For both sponges the fatty acid concentrations increased towards October where for *D. avara* the highest value was reached in May.

Table 2: Total concentration of fatty acids (average ± SD) at different time steps in the suspended particulate matter of three different locations in µg/l and in six sponge species in µg/g dry weight.

	February 2007 (µg/g or µg/l)	May 2007 (µg/g or µg/l)	October 2007 (µg/g or µg/l)
Oosterschelde SPM	6.9 (1.0)	11.4 (0.5)	3.5
Haliclona oculata	4511 (654)	7996 (1384)	2606 (307)
Haliclona xena OS	8540 (823)	nd[a]	4390 (418)
Lake Veere SPM	28.8 (8.7)	75.5 (8.1)	18.8 (7.0)
Haliclona xena VM	10382 (2053)	11810 (3183)	6604 (1502)
Halichondria panicea	9133 (2213)	6780 (1565)	4995 (688)
Mediterranean SPM	5.0 (1.0)	3.0	4.4
Aplysina aerophoba	8583 (2593)	10535 (1280)	12722 (2299)
Dysidea avara	7956 (1801)	14247 (2958)	10605 (1329)

[a] Not determined: In May, Haliclona xena could not be found in the Oosterschelde

Seasonal variation in specific fatty acid concentrations

The diatom biomarker C20:5(5,8,11,14,17) (EPA) and the dinoflagellate biomarker C22:6(4,7,10,13,16,19) (DHA) are used to detect changes in the contribution from these two groups in the SPM and the sponges (see Table 1 for biomarkers used). Concentrations of both biomarkers, EPA and DHA, increased in the SPM of Oosterschelde and Lake Veere in May (Figure 1). Simultaneously, the biomarker content increased in the sponges *H. oculata* and *H. xena* VM (no specimen of *H. xena* were found OS in May), this increase is not shown by the relative content (Figure 1). However absolute concentrations (data not shown) did substantially increase in May as a result of the much higher total sponge FA content. Thus the increase in total FA concentration was the result from algal growth in the water, this was reflected in both the SPM as the sponges except for *H. panicea. H. panicea* in contrast showed a decrease of EPA and DHA as well as in total FA in May. The sponges from the Mediterranean did not show clear differences and fractions found for EPA and DHA were lower than for the Dutch sponges. This suggests that the sponges of the family *Haliclona* are more responsive to changes in suspended matter composition than *H. panicea* and that *Haliclona* feeds mostly on algal cells and *Halichondria* relies also on its symbionts. And less algal material is found in the Mediterranean.

Consumption of bacteria by sponges is indicated by a high proportion of branched and odd numbered FAs as well as a lack of PUFAs (Thurber 2007). The bacterial FA profile (Figure 1) shows that *A. aerophoba* contains many bacterial FAs (about 30% of the total FAs) and almost no EPA and DHA. The abundance of bacterial FAs in *A. aerophoba* is mainly caused by high contents of bacterial symbionts (Hentschel et al 2002). The other sponges all contain about 8% of bacterial FAs and the biomarkers EPA and DHA are abundantly present, which indicates that these sponges are dependent on larger particles. As was concluded in chapter 4, we found ‘demospongic acids’ in the SPM at all sites. For the SPM samples we found that the very abundant C26:2(5,9) and C26:3 (5,9,19) were mainly present in February, although in Lake Veere they were also abundant in May (Figure 1). In October these FAs were almost non existent in the SPM samples, while they still occurred abundantly in the sponge samples. The long chian FAs in the SPM implies that either these demospongic acids are not unique or that sponge detritus contributes substantially to suspended particle organic matter pools.

In February, the 'demospongic' FA C26:3(5,9,19) was more abundant in the Dutch sponges than C26:2(5,9) and the opposite was found in May. This is in agreement with Hahn et al (1988) who also found larger contents of C26:3 in winter. In general the large polyunsaturated FAs were most abundantly present in February. There are two reasons for changing FA composition due to temperature in sponges. One is active regulation by the sponge. For example at lower temperature more PUFAs are present in the membranes to maintain constant flexibility (Delong and Yayanos 1986). The other reason is the availability of food, which is also influenced by temperature (Cliff 1982, Chapter 2).

Seasonal differences in stable carbon isotope ratios

Figure 2 shows the average $\delta^{13}C$ value at the three different time steps of the bacterial FAs, the algal biomarkers EPA and DHA and the sponge compounds C26:2(5,9) and C26:3(5,9,19) from all samples. $\delta^{13}C$ in sponge specific FAs showed very limited seasonal variation at all sites (Figure 2). The data from Lake Veere are more depleted than at the other sites. This is probably due to the high freshwater inputs into this saline lake. Freshwater dissolved inorganic carbon (DIC) usually has a more depleted ^{13}C signal which is transferred into the local food-web. Salinity differences causes variation in DIC isotopic ratio (Hellings et al 1999). This DIC is used by algae and bacteria as a primary carbon source. In the sponge samples, bacterial FAs closely tracked the sponge FAs suggesting that sponges and associated bacteria depended on the same food sources. However, $\delta^{13}C$ ratios of bacterial FAs in the SPM samples of the two Dutch sites were very different and generally more enriched than bacterial FAs in sponges, suggesting that sponge bacteria were not originating from the SPM through filtration but were probably growing in the sponges on sponge derived organic matter.

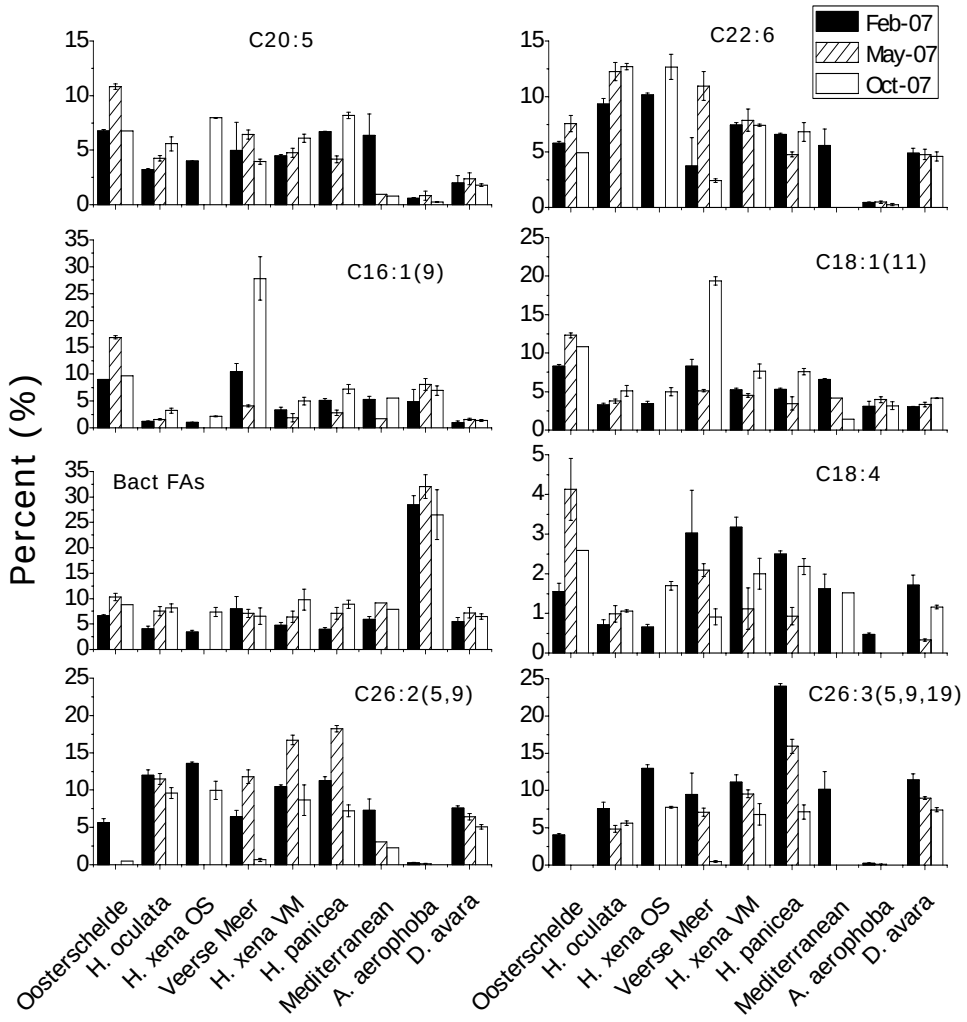

Figure 1: Percentage of C20:5(5,8,11,14,17) (EPA) and C22:6(4,7,10,13,16,19) (DHA), C16:1(9), C18:1(11), bacterial FAs, C18:4(6,9,12,15), C26:2(5,9) and C26:3(5,9,19) in all samples. The fractions of C15, C17 and the branched FAs are included in the bacterial FAs.

The open symbols in Figure 2 show the food compounds. Algal biomarker FAs showed considerable variation between seasons at the two Dutch sites, and absolute $\delta^{13}C$ values and seasonal changes were similar in both SPM and sponges. This suggests that algal FAs in sponges were mainly acquired from the SPM through active filtration in all seasons. This was true for the first two sampling points in February and May, in October algal FAs in sponges were more depleted than in the SPM, which may be due to slower filtration and actual incorporation into the sponge biomass. Sponge specific FAs had only in May similar $\delta^{13}C$ ratios as algal FAs at the two Dutch sites, together with the rather invariant sponge FAs isotope data this suggests that sponges were mainly growing during spring and probably summer. During autumn and winter, they were still actively filtering, although probably slower, based on the algal FA concentration data, but the food collected during this period had little effect on sponge $\delta^{13}C$ values suggesting limited growth.

Results from the Mediterranean are not really conclusive, as mentioned earlier not enough water was sampled from the Mediterranean to get sufficient material for SPM measurements, therefore only results from February are shown, which are also variable, thus no conclusions can be drawn from these data. *Aplysina aerophoba* shows minimal results, Figure 3 shows the high concentrated long chain FAs of *A. aerophoba*, and a seasonal variation was also found (Figure 3). The bacterial biomarkers were somewhat enriched in ^{13}C compared to other FAs (Figure 2). This was also found in the larger branched FAs like in 22MeC28:2(5,9) highly present in *A. aerophoba*, thus this FA has a bacterial precursor. *Dysidea avara* does not show as much seasonal variation as shown in the Dutch samples, which could be the result from low variation in SPM.

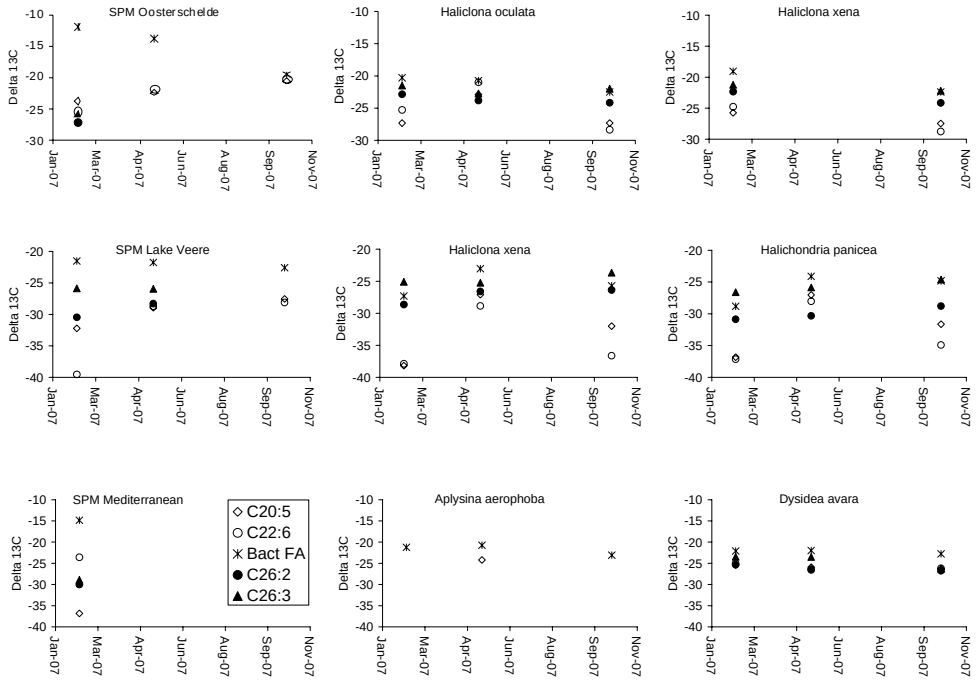

Figure 2: Delta ^{13}C at different time steps for the biomarkers of diatoms (C20:5 (5,8,11,14,17) ◇), dinoflagellates (C22:6(4,7,10,13,16,19) ○), bacteria (bact FAs) x, and the sponge compounds C26:2(5,9) ● and C26:3(5,9,19) ▲ are shown of all SPM and sponge samples

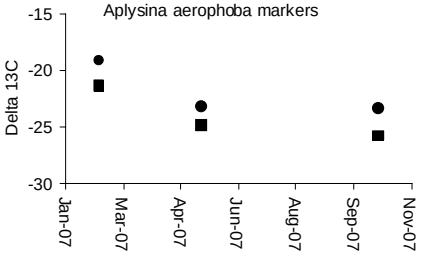

Figure 3: Delta ^{13}C at different time steps for the biomarkers of Aplysina aerophoba (22MeC29:2(5,9)● and C30:3(5,9,23)■)

Specific fatty acid differences

As mentioned earlier sponges generally use food FAs and some of these are elongated and desaturate further to demospongic' FA . Because different compounds have different $\delta^{13}C$ signatures we compared specific compounds within the in individual sponge species. The difference in $\delta^{13}C$ can be used to determine origin of specific compounds inside the sponges (Figure 4). Figure 4 shows the comparison of some biomarkers and longer chain FAs in *Haliclona oculata*, *Haliclona xena* and *Halichondria panicea* at the different time steps. The position of the double bond on the omega (ω) end stays the same when elongating. Hahn et al (1988) showed that C14:0 is elongated to C26:0 and then desaturated to form C26:2(5,9). Here we do not see that C26:2(5,9) shows the same $\delta^{13}C$ signature as C14:0 and as C16:0, although it could still be precursors as in May similar values are found for the two *Haliclona* species. The variation found for the smaller FAs is not shown in C26:2(5,9). For the C26:3(5,9,19) we found a significant higher $\delta^{13}C$ value (Figure 4). The $\delta^{13}C$ values of both C24:1(17) and C26:3(5,9,19) correspond to C16:1(9), and also C18:1(11). C18:1(11) is used as a bacterial biomarker (Kharlamenko et al 2008). C16:1(9) has been used as a biomarker for several groups including bacteria but usually for diatoms. Mostly this FA is combined with data from other FAs to determine its origin. Here the $\delta^{13}C$ value resembles that of the bacterial FAs and it is significantly different from both EPA and DHA. Therefore, we can conclude that C16:1(9) has primarily a bacterial origin. In literature it was already found that an exogenous source of C16:1(9) is used to elongate until C26:1(19) and then further desaturated (Morales and Litchfield 1977, Hahn et al 1988). The FA C26:3(5,9,17) has a similar $\delta^{13}C$ value as the C26:2 (5,9), which means they do not have bacterial origin, but most likely algal origin. Not at all time steps the similarity between the corresponding shorter and longer chain FAs are that clear, however the general trend of higher $\delta^{13}C$ value for C24:1(17) and C26:3(5,9,19) is clear for all samples. The other sponges are not shown because the sponges from the Mediterranean do not show these differences clearly. For *A. aerophoba* it is found that bacterial cells are the main source of food. *Haliclona xena* from the Oosterschelde is not shown because no samples were taken in May, however the samples taken in February and October do show the same trend in $\delta^{13}C$ as the other *Haliclona* sponges.

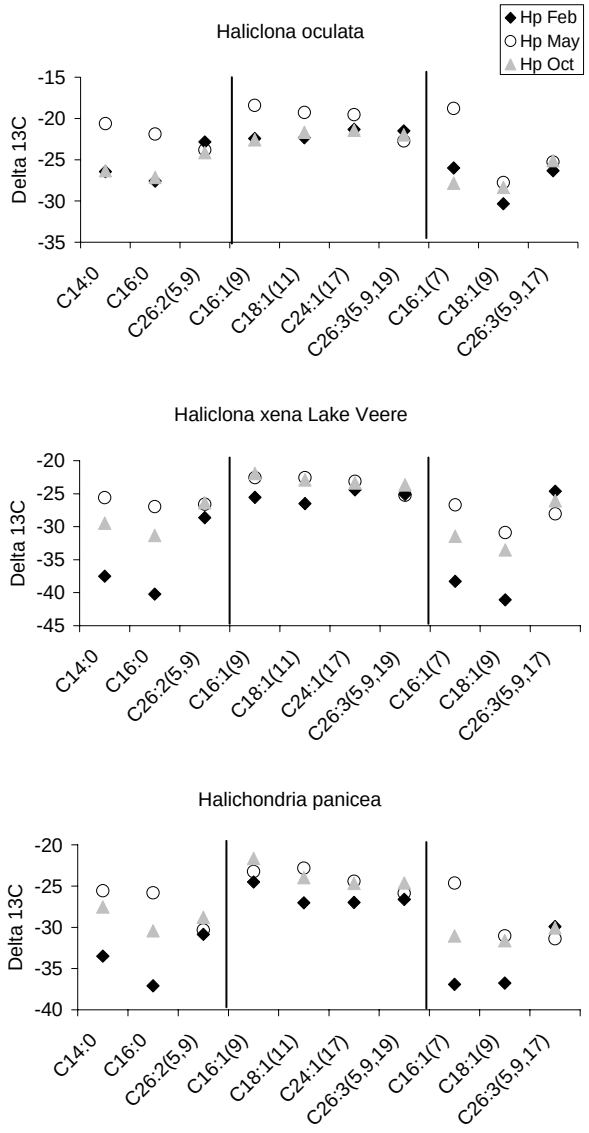

Figure 4: $\delta^{13}C$ values for specific fatty acids in Haliclona oculata, Haliclona xena and Halichondria panicea, the different sections show the corresponding FAs in the same route of biosynthesis.

Conclusion

Fatty acid (FA) concentration in sponges varies with season. This variation corresponds to the variation in suspended particulate matter (SPM) present. Thus the sponges feed on the algal cells in the SPM. The Dutch sponges showed only similarity in $\delta^{13}C$ in both the large sponge FAs as the smaller food FAs in May, suggesting that growth rate is higher in this period. In all Dutch sponges we found that the ω7 long chain FAs, C24:1(17) and C26:3(5,9,19), could be traced back to be of bacterial origin. In the Mediterranean we did not find these variations. The bacterial sponge *Aplysina aerophoba* relies mostly on its symbiotic bacteria.

Acknowledgements

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Chapter 6

Carbon conversion in two marine sponges

Koopmans M, Martens D, van Rijswijk P, Middelburg JJ, Wijffels RH (2009) Carbon conversion in two marine sponges. *To be submitted for publication*.

Abstract

The carbon metabolism of two marine sponges, *Haliclona oculata* from the Oosterschelde (The Netherlands) and *Dysidea avara* from the Mediterrenean (Spain), has been studied using a ^{13}C pulse-chase approach. The sponges were fed ^{13}C labeled diatoms (*Skeletonema costatum)* for 8 hours in a closed system and they took up between 75 and 85 % of the diatoms added. At different times sponges were sampled for total ^{13}C enrichment, and fatty acid (FA) composition and ^{13}C enrichment. During the first day the level of ^{13}C label inside the sponges stayed the same after which the ^{13}C label was metabolized and excreted. Algal biomarkers present in the sponges were highly labeled after feeding but their labeling levels decreased from the second day until none was left 10 days after enrichment. The sponge specific long chain C26 FAs incorporated ^{13}C label already during the first day and the amount of ^{13}C label inside these FAs kept increasing until 3 weeks after labeling. Thus the algae fed to the sponges were taken up by the sponges within 8 hrs, conversion started during the first day and total conversion lasted until at least 3 weeks after feeding.

Introduction

Sponges are known for their capacity to produce bioactive secondary metabolites. However, no sustainable production techniques have been developed yet to obtain sufficient quantities of these potential pharmaceuticals. In order to develop such techniques and to maximize productivity we need to obtain a better understanding of feeding and metabolic rates in sponges. Sponges are heterotrophic organisms and pump large amounts of water through their body from which particles are filtered (Bergquist 1978). How and at which rate these particles are metabolized in sponges has not been studied widely (Schmidt 1970, Hahn et al 1988). One of the problems in studying sponge metabolism is that particles filtered out of the water are not immediately metabolized. To understand sponge metabolism it is necessary to be able to distinguish whether a compound is part of the food added and taken up or of the sponge.

This problem can be solved by looking at the metabolism of biomarkers, which are compounds only present in the sponge and not in the food. In a previous study we found several fatty acids that could serve as a biomarker for sponges (Chapter 4). Among aquatic animals, sponges are specified by the greatest diversity of sterols and fatty acids (FAs), which have an unusual and sometimes unique structure (Litchfield et al 1976, Thiel et al 1999, Rod'kina 2005). Sponges contain large quantities of C_{22}-C_{30} fatty acids with branched and odd-chains and hydroxyl-acids (Bergquist et al 1984, Christie et al 1992, Thiel et al 1999, Nechev et al 2002, Rod'kina et al 2003). These very long chain fatty acids are sometimes called 'demospongic acids' (Rod'kina 2005). Fatty acids are very diverse in structure and chain length and some are unique for certain organisms. Consequently, many researchers have used FAs and combinations of FAs as biomarkers for algal or bacterial biomass (e.g. Dunstan et al 1994, Boschker and Middelburg 2002, Kharlamenko et al 2008). One of the problems we encountered in our previous study (Chapter 4) is that some of the demospongic acids were also found in suspended particles, implying either that these demospongic acids are not unique or that sponge detritus contributes substantially to suspended particle organic matter pools. This non-uniqueness of putative biomarker is a common problem in organic geochemistry and microbial ecology (Boschker and Middelburg 2002). However, this can be solved by using isotopically labeled single algae species as a food source. ^{13}C label appearing in sponge specific fatty acids (not present in algae added) can then be attributed to metabolism of the sponge.

Sponges possess an active homologation enzyme system using short chain fatty acid precursors largely derived from exogenous sources as substrate (Djerassi and Lam 1991). Therefore, the composition of sponge FAs is probably related to the composition of food FAs and symbionts. Biosynthesis of fatty acids has been studied before in different sponges by using ^{14}C-label (Readerstorff et al 1987, Hahn et al 1988). In *Aplysina fistularis* it was found that 22MeC28:2(5,9) was elongated from 10MeC16:0 to 22MeC28:0 and then further desaturated (Readerstorff et al 1987). Hahn et al (1988) elucidated the biosynthesis of two common long chain 'demospongic acids' in the sponge *Microciona prolifera*. They found that C14:0 was first elongated to C26:0, which was then further desaturated to form C26:2(5,9). Moreover, Hahn et al (1988) showed that C26:3(5,9,19) had an exogenous palmitoleic acid (C16:1(9)) as precursor. Recently, de Goeij et al (2008[b]) used a ^{13}C labeling approach to study sponge carbon uptake from different food sources in a bacterial sponge in its natural habitat. Fast uptake and conversion of food sources was found in the sponge and its symbionts. However, how long it takes for ^{13}C label to be incorporated into the longer chain FAs was not studied by de Goeij et al (2008[b]).

The aim of this paper is to get a better understanding of food uptake and metabolism by two sponges (*Haliclona oculata* from the Oosterschelde, the Netherlands and *Dysidea avara* from the Mediterranean, Spain). Sponges were fed ^{13}C labeled diatomea and label appearance and eventual loss in (sponge) fatty acids were followed over time.

Material and Methods

Sponge collection

On 13 August 2007 27 sponge specimen of *Haliclona oculata* were collected by scuba diving in the Oosterschelde, a tidal inlet in the Netherlands (Lokkersnol, 51°38′58.07″N, 3°53′5.11″E) at ~ 13 m depth, water-temperature 20°C. Sponges were transported to the laboratory in aerated boxes in their natural seawater and placed within 2 hours in three aquaria (water volume approximately 80 liter) with continuous flowing natural seawater directly pumped from the Oosterschelde. On 21 January 2008 18 sponge specimen of *Dysidea avara* were collected in the Mediterranean, Spain (Cala Montgo, 42°06′50.33″N, 3° 10′02.10″E) at ~ 8 m depth, water-temperature 14°C. Sponges were transported to two aquaria (water volume approximately 100 liter) containing natural seawater close to the sea within 1 hour after collection.

Feeding experiment

Haliclona oculata specimens were kept for 1 week in the new environment prior to the pulse labeling for acclimation. After 1 week the sponges were fed with 300 ml of live ^{13}C labeled *Skeletonema costatum* per aquarium. The diatom *S. costatum* was cultured at 16°C in F2 medium in 6 liter artificial seawater containing 25% ^{13}C labeled $NaHCO_3$ and 10% ^{15}N labeled $NaNO_3$. The culture was centrifuged at 1000*G and washed 3 times with filtered unlabeled seawater to remove any remaining ^{13}C label after which they were diluted to 17000 cells per ml to feed the sponges. Three samples of 1 ml were taken from the *S. costatum* culture to analyze algal fatty acids and ^{13}C content. During labeling the continuous flowing seawater was stopped and pumps were used to mix the seawater in the aquaria to prevent cells from settling on the bottom. After the pulse of ^{13}C labeled cells we counted the cells every hour to determine sponge filtration rate. After 8 hrs most cells were filtered out and the continuously flowing seawater was turned on again. Whole sponges were sampled from each aquarium at day 0 (before feeding), after 8 hours (directly after labeling), and at days, 1, 2, 3, 7, 10, 14 and 21. The sampled sponges were frozen in liquid nitrogen and stored at -80 ^{o}C until freeze drying for 24 hrs.

Dysidea avara specimens collected from the field were immediately fed with 300 ml *Skeletonema costatum* culture per aquarium. Sponge filtration was assessed by sampling particulate matter after 1 hour and at the end of the

feeding by analysis of ^{13}C content. Sponge feeding in the aquaria lasted for 8 hrs and afterwards the sponges were placed back into the sea at the site of collection. At day 0 (before feeding), after 8 hours (directly after feeding), and at days 1, 2, 4 and 8 three sponge specimen were sampled for further analysis. The collected specimen were frozen on dry ice, and in this way transported to the laboratory. The sponges were stored at -80°C until freeze drying for 24 hrs.

Total carbon and ^{13}C content analysis

The freeze-dried sponges were grinded with mortar and pestle before further usage. The carbon content of all sponge individuals was measured using a Fisons NA 2500 element analyzer with auto sampler and Haysep-Q column 80-100 mesh I.D. 2mm (Nieuwenhuize et al 1994). In short, the dried sample was flash combusted in an oxygen rich environment and the generated CO_2 and N_2 were separated on a GC column and detected using a thermal conductivity detector. Moreover, the CO_2 released was then transferred via a Conflo interface to and isotope ratio mass spectrometry to measure the carbon isotopic composition (EA-IRMS).

Fatty acid and ^{13}C analysis

100 mg sponge dry weight was used for lipid extraction using an adjusted Bligh and Dyer method (see also Boschker et al 1999). The total lipid fraction was derivatized by mild alkaline methanolysis to obtain fatty acid methyl esters (FAME). Both C12:0 and C19:0 were used as internal FAME standards. Identification of the fatty acids was done using equivalent chain length data with known standards measured on a non-polar column (see below). Additional identification was done using GC-MS (Chapter 4). Carbon isotopic composition of individual FAME was determined with a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS); a HP G1530 GC (Hewlett Packard) connected to Delta-plus IRMS via a type-III combustion interface from Thermo Finnigan (Bremen). Helium was used as a carrier gas (150 kPa) with an apolar column (Hewlett-Packard HP-5MS, 60 m*0.32 mm*0.25µm). The column was kept at 70°C for 2 min, then temperature was programmed from 70 to 150°C at 20°C min^{-1}, subsequently from 150 to 290°C at 3°C min^{-1} and from 290°C to 325 °C at 5 °C min^{-1}, temperature was kept at 325 °C for 11 minutes. Fatty acid concentrations were determined by area

correction according to the C19:0 internal standard. Stable carbon isotope ratios ($^{13}C/^{12}C$) for individual PLFA were calculated from FAME data by correcting for the one carbon atom in the methyl group that was added during derivatization.

Enrichment calculation

High-precision isotope ratios were determined as the relative difference in $^{13}C/^{12}C$ of a sample and the international standard for carbon of Vienna PeeDeeBelemnite (PDB). Isotope measurements are usually given in the δ-notation (in part per thousand) calculated using the following equation:

$$\delta^{13}C\ (‰) = (R_{sample}/R_{PDB} - 1) * 1000 \qquad \text{(equation 1)}$$

The absolute amounts of ^{13}C-label (µg $^{13}C \cdot g\ dw^{-1}$) in FA can be calculated from the concentration µg FA·g dw^{-1} (C) and the increase in the fraction ^{13}C after labeling (F^{13}) relative to the control (F^{13}_c):

$$^{13}C = (F^{13} - F^{13}_c) * C \qquad \text{(equation 2)}$$

where $F^{13} = R/(R+1)$ and $R = (\delta^{13}C/1000+1)*R_{PDB}$

Cell count

A Beckman coulter counter was used to count the cells during the pulse feeding of *Haliclona oculata*. Samples of 20 ml were taken after 0, 0.5, 1, 2, 3, 4, 5, 6 and 8 hours from each aquarium. Only cells larger than 4 micron were counted.

Respiration rate

Respiration rates were measured for *H. oculata* at days -4, -3, -2, -1, 0, 1, 2, 7 and 14. Respiration experiments were performed in polyvinylchloride (PVC) experimental chambers with a volume of 765 ml. and that had an opening underneath to completely cover the sponge. Chambers were placed over the sponge, and thus the sponge was not affected. An oxygen probe was connected to each chamber. A magnetic stirrer inside chambers continuously mixed the water inside to prevent particles from settling and to homogenize oxygen distribution. O_2 concentrations were measured and logged every 10

seconds using two Oxi 340i handheld oxygen meters (WTW, Weilheim, Germany). Experiments lasted for 15 minutes, to minimize the effect of oxygen depletion on respiration rates.

Results and discussion

Total enrichment

Both marine sponge species were fed using uniformly ^{13}C labeled living diatoms (4 atom% ^{13}C) and carbon uptake, assimilation and conversion were followed over time. *Dysidea avara* from the Mediterranean, Spain, retained less ^{13}C than *Haliclona oculata* from the Oosterschelde, the Netherlands, which may be partly the result of different feeding strategies and experimental procedures for both sponges. *H. oculata* was given one week to readjust from field to laboratory conditions. Labeling took place using actively growing *Skeletonema costatum* cells and the experiment lasted for 3 weeks. For *D. avara* the experiment only lasted 1 week, and the feeding with living but for 3 days stored diatoms, took place immediately after collection in a stirred but non flushed aquarium. After the 8 hour pulse labeling period *D. avara* sponges were placed back in their natural habitat. We will focus our discussion on the results for *H. oculata* because this experiment lasted longer, more ^{13}C uptake occurred and the diatoms provided were actively growing.

During labeling, water samples were taken to follow diatom uptake. For the *H. oculata* experiment we were able to immediately analyze the water samples by counting cells (Figure 1). This allowed us to optimize experimental procedures and in particular the feeding period; more than 75 % of the diatoms were filtered by the sponges within 8 hours. Moreover, it also allowed estimating a filtration rate (F; l h^{-1} $sponge^{-1}$):

$$C(t) = C(0)e^{-\frac{F}{V}t} \qquad \text{(equation 3)}$$

where C represent the concentration of diatoms, V is the volume of water per sponge (l $sponge^{-1}$) and t is time (h). The filtration rate based on the uptake of *S. costatum* cells as found for *H. oculata* was 1.7 l h^{-1} $sponge^{-1}$. This is somewhat lower than the value of 3.4 l h^{-1} $sponge^{-1}$ found for *H. oculata* in its natural habitat (Chapter 3).

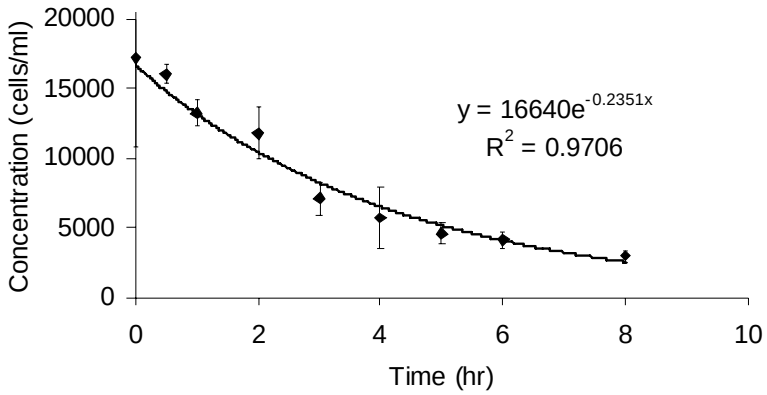

Figure 1: Skeletonema costatum cell decrease during pulse label feeding of Haliclona oculata. Average cell count ± standard deviation of triplo measurements of all three aquaria shown.

Haliclona oculata

Total ^{13}C enrichment of the sponge specimen was followed in time (Figure 2). Most enrichment was found immediately after labeling and up to day 1, after that ^{13}C enrichment decreased gradually. Variability was very high during the initial period (till day 1), which may be due to the difference in activity by sponge individuals. Three different sponges were collected at each time step, thus when a sponge did or did not filter a lot of material during the 8 h feeding period had major consequences for ^{13}C acquisition. After the first day the ^{13}C content decreased and thus *H. oculata* started to excrete or respire this. This decrease was initially rapid and then more gradual. This initial rapid decrease may be due to diatoms cell loss. In the early seventies Schmidt (1970) already demonstrated that ingested fluorescent bacterial cells could not be traced back after 48 hours. They showed that bacterial cells were transported to the choanocytes within 3 hours, that fluorescence was located throughout the sponges and that bacterial cells started to change shape after 4 to 5 hours. The subsequent gradual decrease of ^{13}C label in the sponge was expected as we found in our previous work that about 10% of carbon acquired was used for biomass assimilation and over 90% was used for respiration (Chapter 3). After 3 weeks

still 22.3% of the present carbon after 8 hours was inside *H. oculata*. Thus we can distinguish between an initial phase with rapid ^{13}C loss of diatoms and second phase in which ^{13}C label was assimilated and respired. Tracing ^{13}C in FA will be instructive to further elucidate this (see below).

For the *H. oculata* sponges we also measured respiration rate several times. We found a slight decrease in respiration rate during the first week after collection (during acclimatization period) from 96.2 to 86.9 $\mu molO_2 \cdot hr^{-1} \cdot g\ dw^{-1}$ and 2 weeks after the feeding the respiration rate was 82.5 $\mu molO_2 \cdot hr^{-1} \cdot g\ dw^{-1}$. These values are within the range found for this species in its natural habitat (Chapter 3), indicating that they were performing well in the aquaria. A respiration rate of 86.9 $\mu molO_2 \cdot hr^{-1} \cdot g\ dw^{-1}$ corresponds to a carbon usage for respiration of 65.2 $\mu molC \cdot hr^{-1} \cdot g\ dw^{-1}$, assuming a respiratory quotient of 0.75 $\mu molC/\mu molO_2$ (Hatcher, 1989). Thus per day 18.8 $mgC \cdot g\ dw^{-1}$ was respired, which explains the large decrease of ^{13}C.

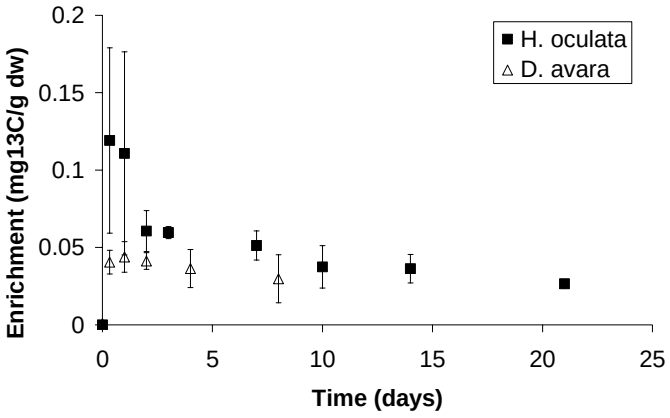

Figure 2: Total ^{13}C enrichment in the whole sponge for both Haliclona oculata and Dysidea avara.

Fatty acid enrichment

The enrichment of ^{13}C in individual fatty acids of sponges was measured and total quantity of label in FA is shown in Figure 3. Note that Figure 3' y-axis is in µg ^{13}C per gram dry weight and in Figure 2 this is in milligram ^{13}C per gram dry weight. Thus only small fractions of the incorporated ^{13}C is incorporated in FAs. After three weeks about 7.6% of the labeled FAs taken up were used for conversion to other FAs (Figure 3). This is about 3 times less than we found for total carbon (Figure 2). Thus fatty acids were either excreted faster or respired more than other organic compounds to obtain energy.

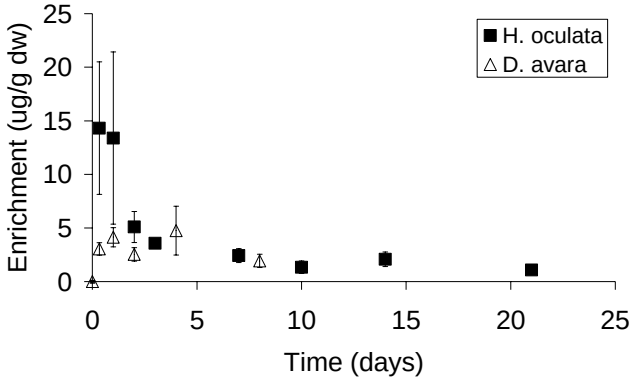

Figure 3: Total fatty acid ^{13}C enrichment for both Haliclona oculata and Dysidea avara.

The ^{13}C content of specific fatty acids can be used to assess the fate of algal material and the metabolism of sponge fatty acids. Table 1 shows the percentage of the major fatty acids in the diatom used as food and the two sponges. For *H. oculata* we found some large sterol peaks at the end of the chromatogram, the major one could be attributed to campesterol ((24)-ergost-5-en-3beta-ol). The FAs C14:0, C16:1ω7, C16:0, C:20:5ω3 have high concentrations in the diatom cultured and fed to the sponges and have rather low concentrations in the sponges. These FAs with high concentrations in diatoms and low concentrations in sponges can be used to follow how fast diatom carbon was metabolized by the sponges. Similarly, a number of FA were not found in diatom material added and were present in the sponges and these can be used to trace formation and transformation of FA by sponges. However, some of these sponge FA may in fact reflect prokaryotic symbionts (branched FA such as iC17:0 and aiC17:0.

Table 1: Major fatty acids present in the diatom used for feeding and the sponges used.

Fatty acid	Mass% in *Skeletonema costatum* (diatom)	Mass% in *Haliclona oculata* (sponge)	Mass% in *Dysidea avara* (sponge)
C14:0	15.72	0.28	0.60
iC16:0	3.21	0.55	1.32
C16:3ω4	7.76	0.05	0.79
C16:1ω7c	26.64	1.61	0.93
C16:0	15.18	3.08	2.84
iC17:0		0.52	1.19
aiC17:0		0.51	1.17
C18:4ω3	3.83	0.24	1.20
C18:1w7	0.38	2.40	2.90
C20:4w6		2.11	3.00
C20:5ω3	6.55	2.30	1.47
C20:1(13)		0.11	5.27
C22:6w3	1.51	6.41	3.49
C22:1(15)	0.41		8.21
aiC23:0			5.69
C24:1(17)		2.53	2.19
C25:2(5,9)			2.98
C26:3(5,9,19)		5.66	9.51
C26:2(5,9)		10.18	6.68
C28:3(5,9,21)		11.41	
Campesterol		17.15	

Figure 4 shows the absolute ^{13}C content for the feed biomarkers per 1 ml *S. costatum* culture (Figure 4). It is shown that most ^{13}C is present in C16:1ω7, which is related to the high concentration of this FA in the diatom *S. costatum* (Table 1). When the diatom cells are present inside the sponge as intact cells, the ^{13}C enrichment of C16:1ω7 will be higher inside the sponge as well. Figure 5 shows that after 8 hrs of feeding indeed the ^{13}C enrichment resembled that of the signature of *S. costatum*. Within the first day FA enrichment levels did not change drastically, but a rapid decrease of ^{13}C excess in diatom biomarkers was observed during the second day. Thus the sponges either started to excrete the cells or to use the retained molecules for their own metabolism. These results confirm the results found by Schmidt (1970) as he found that feed particles were removed from the sponge body after 48 hours. In our study we were able to detect ^{13}C enrichment in diatom FAs up to 10 days, indicating that it takes in the order of a week for sponges to process algal material after capture.

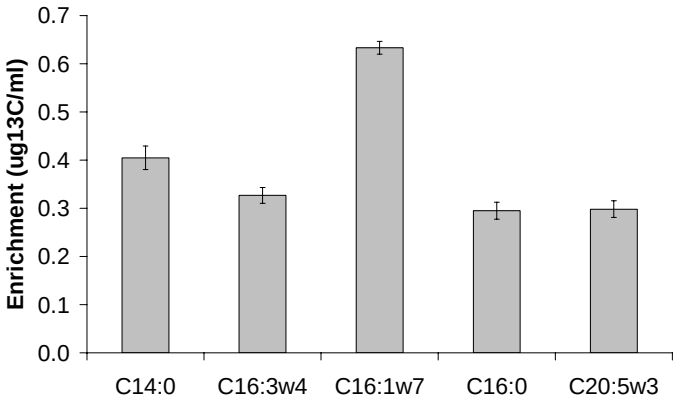

Figure 4: ^{13}C Enrichment of fatty acids in Skeletonema costatum feed

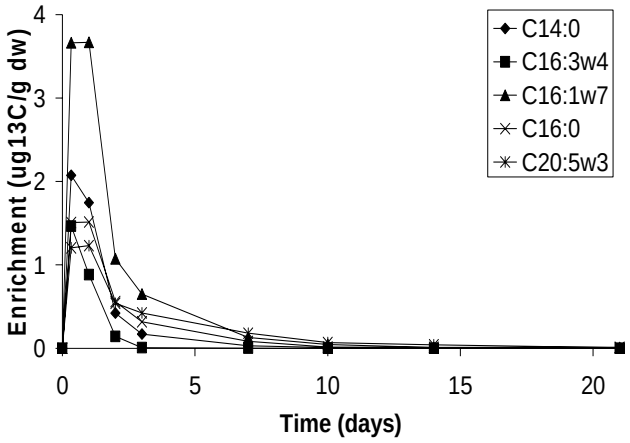

Figure 5: ^{13}C Enrichment of feed fatty acids in Haliclona oculata expressed in µg 13C in the specific FA per gram dry weight of sponge.

Several long chain FAs were abundant in *H. oculata (Table 1)*, although these long chain fatty acids are not sponge specific biomarkers, they were not present in S. costatum. Therefore, label incorporation in these fatty acids is due to sponge metabolism. Figure 6 shows the incorporation of ^{13}C into these long chain FAs. It is clear that ^{13}C incorporation in C24:1(17) started immediately, reached maximum levels at day one and then decreased gradually. This decrease likely caused by the transformation of this compound to a longer chain FAs, like C26:3(5,9,19) and C28:3(5,9,21), which showed increase in label up to the end of the experiment. Especially for C26:2(5,9) and C28:3(5,9,21) ^{13}C enrichment was still increasing 3 weeks after labeling (Figure 6).

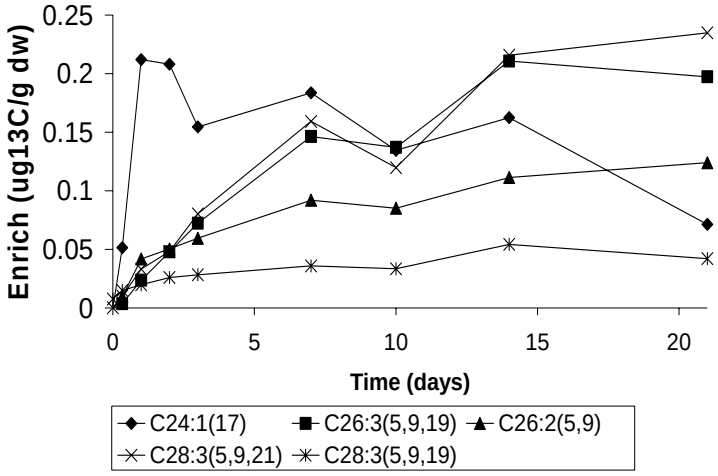

Figure 6: ^{13}C Enrichment in high concentrated long chain fatty acids in Haliclona oculata.

Dysidea avara

During the feeding of *D. avara* we could not count the cell decrease in the water because of logistic restrictions, but we measured ^{13}C of particulate organic carbon in the water, which decreased from 9.4µg/l after 1 hr feeding to 5.2µg/l after 8 hr feeding. $\delta^{13}C$ values decreased from 383 to 262 ‰ at the same time interval, which means that the labeled cells were preferentially filtered from the water. After 8 hours more than half of the added particles were still left, indicating that *D. avara* was less efficient than *H. oculata* in capturing particles,, probably because *D. avara* filtered less water than *H. oculata*. The filtration rate found for *D. avara* was 0.85 l h^{-1} $sponge^{-1}$, this is somewhat low but still in the range as found by Ribes et al (1999). The somewhat low filtration rate was most likely caused by the stress after immediate collection.

Figures 2 and 3 show the total ^{13}C and the total fatty acid ^{13}C enrichment for both sponge species respectively. Although ^{13}C acquisition was lower during the feeding period, retention of ^{13}C was higher in *D. avara* than in *H. oculata.* After 8 days, 67.7% of total ^{13}C excess and 46.7 % of FA ^{13}C excess was still present inside *D. avara.* *D. avara* appears to keep the acquired carbon longer in its body than *H. oculata,* both in terms of total ^{13}C and FA ^{13}C.

During the experiment with *D. avara* some unexpected results appeared. Figure 7 shows that the highest ^{13}C enrichment of diatom biomarkers was found after 1 day for *D. avara*. After incubations the sponge with label in the laboratory for 8 hrs, the were placed back in the sea, but this could not be done immediately. As a consequence, label acquisition continued beyond 8 hours. Following an expected decrease during the second day and a sudden and unexpected increase occurred after 4 days. The increase can only be explained by cross-contamination from another *in situ* labeling experiment done by us nearby. After 8 days the enrichment levels of the diatom biomarkers decreased again but did not approach zero as expected. Another striking result is the low enrichment of two diatom FAs (Figure 7), as if during the culturing of the labeled cells these FAs were less labeled.

In *D. avara* less label was found in the long chain sponge FAs (Figure 8), which is a result of the lower ^{13}C uptake (Figure 2). Enrichment level of C24 and C26 were similar, however C24:1(17) showed a similar trend as the diatom biomarkers, namely the decrease after 2 days with an increase again after 4 days (Figure 8). C24:1(17) was apparently metabolized faster than the other long-chain FA. After 8 days label incorporation in these long chain sponge FA was stabilizing or even decreasing, meaning that *D. avara* has a faster turn over time than *H. oculata*.

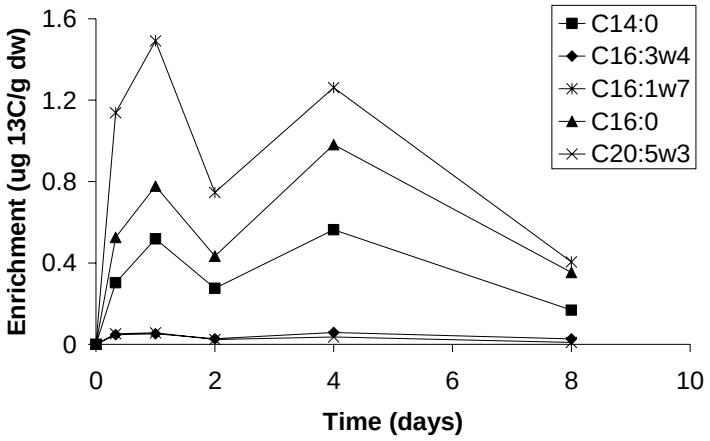

Figure 7: ^{13}C Enrichment of feed fatty acids in Dysidea avara.

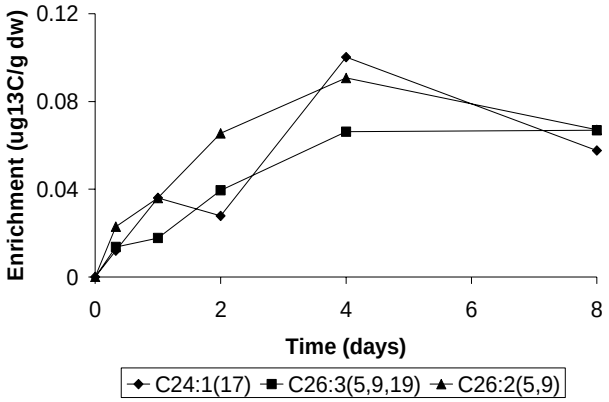

Figure 8: ^{13}C Enrichment in high concentrated long chain fatty acids in Dysidea avara.

Conclusion

Two sponge species were fed with ^{13}C labeled diatomea, *Skeletonema costatum*. Both *Haliclona oculata* and *Dysidea avara* took up the labeled cells. After the increase of ^{13}C inside the sponges we found a large decrease of label inside *Haliclona oculata*, this decrease was also found but much lower in *Dysidea avara*. 10 Days after labeling we did not find label in food biomarkers, meaning that all diatom cells were either excreted or converted. Three weeks after labeling we found ^{13}C levels still increasing in the longer chain fatty acids (FAs) in *Haliclona oculata*. Thus food particles taken up at a certain moment are still being processed three weeks later in the fatty acid conversion. Moreover, fatty acid synthesis does not seem to be the rate limiting step for growth as FAs were turned over faster than other compounds.

Acknowledgements

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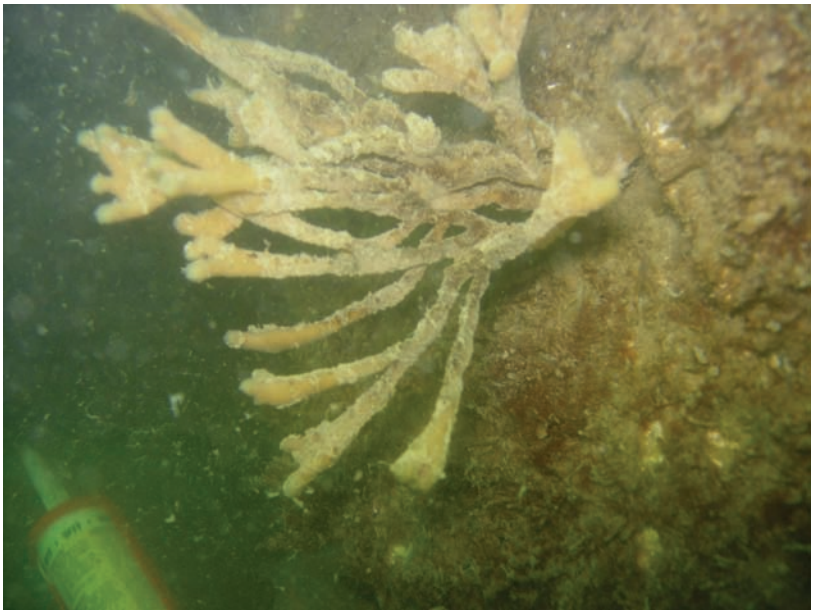
Chapter 7

Increased metabolic rate in damaged sponge tissue



Koopmans M, van Rijswijk P, Martens D, Egorova-Zachernyuk TA, Wijffels RH (2009) Increased metabolic rate in damaged sponge tissue. *Submitted for publication*.

Abstract

Sponges are aquatic animals that are known to produce secondary metabolites with pharmaceutical potential. However, no sustainable production method for these sponge derived medicines has been developed yet. In order to obtain sustainable production techniques it is essential to get more understanding of the biology, growth and metabolism of the sponge. In this study we have examined the change of carbon metabolic rate of fatty acid synthesis due to mechanical damage of sponge tissue. Metabolic studies were performed by feeding sponges with ^{13}C labeled biomass of diatom, *Pheaodactylum tricornutum*, either after or before damaging and tracing back the ^{13}C content in the damaged and healthy tissue. We used two different types of sponges, the finger sponge *Haliclona oculata* from the Netherlands and the temperate sponge *Dysidea avara* from the Mediterranean, Spain. The filtration and respiration in both sponges responded quickly to damage. The rate of respiration in *H. oculata* was reduced immediately after damage. Six hours after damage the filtration rate increased to a level that was higher than the starting value, while the respiration rate returned to the initial value before damage. For the encrusting sponge *D. avara* the filtration rate also decreased directly after damage, but in this case it did not return to the value before damage after one day. The ^{13}C data revealed that *H. oculata* has a higher metabolic rate in the tips where growth occurs compared to the rest of the tissue and that the metabolic rate is increased after damage of the tissue. For *D. avara* no differences were found between damaged and non damaged tissue.

Introduction

Since the 1950s the interest in sponges increased due to the discovery of bioactive secondary metabolites (Bergmann and Feeney 1950). Despite the increased attention in sponge metabolites, no sustainable production techniques have been developed yet to obtain sufficient quantities of these potential pharmaceuticals. Moreover, we still do not fully understand why and how these metabolites are produced within the sponge bodies. Several studies have shown that they are often used as a defense mechanism against predators (Proksch 1994, Pawlik et al 1995, Uriz et al 1996[b], Paul and Puglisi 2004). What exactly induces the production of secondary metabolite and if it is specifically allocated in parts of the sponge body is not completely understood and different results have been obtained so far (see review of Thoms and Schupp 2007).

Many sponges were found to be slow growing organisms (e.g. Dayton 1979, Ayling 1983, Garrabou and Zabala 2001, Duckworth and Pomponi 2005, de Caralt et al 2008[b], Chapter 2). However, damaged sponge tissue was found to regenerate at higher rates than the corresponding growth rates of the same sponge species (Ayling 1983, de Caralt et al 2008[b]). Damaged tissue was also found to induce bioactive metabolite production (Thoms et al 2006, Thoms and Schupp 2008). Due to wounded tissue, converting enzymes can come into contact with less active metabolites and are able to convert these into defense metabolites, a mechanism which in plants has been called activated defense (Saunders et al 1977, Poulton 1988). Only in the sponge genus, *Aplysina*, activated defense was reported. The precursor in *Aplysina* possesses a repellent effect against potential fish predators (Thoms et al 2004). When the sponge gets damaged it was found that these compounds are enzymatically converted to antimicrobial compounds, which probably serve as protection of the wounded tissue against microbial pathogens (Thoms et al 2006).

Thus, so far it has been found that a sponge can chemically respond to mechanical damage (Thoms et al 2006) and that the regeneration rates found can be higher than the growth rates (Ayling 1983, Duckworth 2003, de Caralt et al 2008[b]). However the rate of metabolism associated with regeneration and whether or not more energy is allocated towards the damaged tissue is not known. Stable isotopes, like ^{13}C, occur in the environment and have various applications for metabolic studies and structure biology (Egorova-Zachernyuk et al 2008). It has been shown that by feeding sponges with stable isotope ^{13}C labeled compounds, this label can be traced back into sponge specific FAs (Chapter 6) and thus changes of metabolic rate can in principle be measured.

The aim of this paper is to find the effect of damage on sponge metabolism and growth. For this we fed two sponge species with a uniformly ^{13}C labeled diatom, *P. tricornutum*, in their natural habitat. By means of the ^{13}C label we studied the conversion of carbon into sponge compounds. The sponge *H. oculata* from the Oosterschelde, the Netherlands, was damaged by cutting off the tips, and *D.* from the Mediterranean, Spain was damaged by scraping of the outer tissue of half of the sponge. The collected sponges were separated in damaged and non damaged sponge tissue, which were analyzed separately.

Material and Methods

Area and sponge species

The sponge species used were *Haliclona oculata and Dysidea avara. H. oculata* occurs widely in the Oosterschelde and grows attached to solid surfaces such as oyster shells. ^{13}C labeling experiments with *H. oculata* were done *in situ* in the Netherlands, Oosterschelde estuary (Lokkersnol, 51°38′58.07″N, 3°53′ 5.11″E) at 13 m depth at low tide on 26 September 2007, water temperature 17°C. A platform was placed at the study site and 42 concrete pavement tiles (30-cm x 30-cm) were placed on top of the platform (see Chapter 2). On every tile a sponge specimen was attached. 12 Specimen were used in this experiment, these specimen had been attached to the tiles on the platform already for several months. *Dysidea avara* experiments were performed *in situ* in the Mediterranean, Spain (Cala Montgo, 42°06′50.33″N, 3°10′02.10″E) at ~ 8 m depth on 22 January 2008 with a water-temperature of 14°C. On 21 January 12 *D. avara* specimen were collected with their rocky substrate. The substrate was glued to tiles located close to the place of collection.

Feeding experiment

The uniformly ^{13}C (I.E. 99.5%) labeled biomass of *Pheaodactylum tricornutum* in lyophilized form was purchased from Protein Labelling Innovation (PLI), the Netherlands. The sponges on the platform were fed with this ^{13}C labeled biomass of *P. tricornutum*. A suspension containing 22.5 mg *P. tricornutum* in 15 ml of seawater was fed to the sponges for 25 minutes. For the *in situ* experiments special experimental chambers, made of polyvinylchloride (PVC) with 3800 ml volume were developed. The chamber had an opening underneath to

completely cover the sponge. The chamber was placed over the sponge, and by using elastic bands the chamber could easily be attached to the tiles. The chamber had two connections, one to connect an oxygen probe and a small sampling tube for adding the *P. tricornutum* solution. A magnetic stirrer inside the chamber continuously mixed the water inside to prevent particles from settling and to equalize oxygen distribution. Oxygen concentrations were measured using an Oxi 340i handheld oxygen meters (WTW, Weilheim, Germany). The oxygen concentration inside a chamber was logged every 10 seconds. The meter connected to the probe was in a waterproof box and taken underwater. Feeding experiments lasted for 25 minutes, to minimize the effect of oxygen depletion. In total we used 3 experimental chambers. Since the maximal dive-time was 60 minutes, we could feed six sponges during one dive.

The finger sponge *H. oculata* was damaged using scissors and all tips of the sponge were cut off. *D. avara* was damaged by scraping of sponge tissue on one side of the sponge, keeping more than half of the sponge undamaged. To find differences between sponges that were damaged and not damaged we used 12 sponge individuals, which were divided into four groups that were treated as described in Table 1. Thus, for each strategy 3 sponge specimens were used. In this way we could measure differences in uptake rate due to damage. One week after feeding, the sponges were collected. After collection we separated the damaged tissue and the healthy part of the sponge body to be able to find differences in amount of ^{13}C label in the different parts of the sponge. The collected specimen were frozen on dry ice, and in this way transported to the laboratory in 2 hours. The sponges were stored at -80°C until freeze drying for 24 hrs. The dry sponges were grinded with mortar and pestle before further usage.

Table 1: Feeding strategies for both Haliclona oculata and Dysidea avara

Damage	**Feeding / Labeling**	**Remark**
No	Without damage	Reference samples
Yes	Before damage	
Yes	Immediate after damage	
Yes	6 or 24 hrs after damage	6 hours for *H. oculata*, 24 hours for *D. avara*

Total carbon and $^{13}C/^{12}C$ ratio analysis

The carbon content of all sponge individuals was measured after oven drying at 50°C, using a Fisons NA 2500 element analyzer (serial number 991493), with auto sampler and Haysep-Q column 80-100 mesh I.D. 2mm (see also Nieuwenhuize et al 1994). In short, the dried sample was combusted in an oxygen rich environment at 1010°C. After drying all formed CO_2 and N_2 are separated on a GC column and detected using a thermal conductivity detector. The formed CO_2 is then measured on the Isotope Ratio Mass Spectrometer (IRMS) to determine the isotope ratio $^{13}C/^{12}C$ (R).

^{13}C analysis for fatty acids

Per sample 100 mg sponge dry weight was used for lipid extraction using an adjusted Bligh and Dyer method (see also Boschker et al 1999). The total lipid fraction was derivatized by mild alkaline methanolysis to obtain fatty acid methyl esters (FAME). Both C12:0 and C19:0 were used as internal FAME standards. Identification of the fatty acids was done using equivalent chain length data with known standards measured on a non-polar column (see below). Additional identification was done using GC-MS as in Chapter 4. Carbon isotopic composition of individual FAME was determined with a gas-chromatograph combustion-interface isotope-ratio mass spectrometer (GC-c-IRMS); a HP G1530 GC (Hewlett Packard) connected to Delta-plus IRMS via a type-III combustion interface from Thermo Finnigan (Bremen). Helium was used as a carrier gas (150 kPa) with an apolar column (Hewlett-Packard HP-5MS, 60 m*0.32 mm*0.25µm). The column was kept at 70°C for 2 min, then temperature was programmed from 70 to 150°C at 20°C min^{-1}, subsequently from 150 to 290°C at 3°C min^{-1} and from 290°C to 325 °C at 5 °Cmin^{-1}, temperature is kept at 325 °C for 11 minutes. Fatty acid concentrations were determined by area correction according to the C19:0 internal standard. Stable carbon isotope ratios ($^{13}C/^{12}C$) for individual PLFA were calculated from FAME data by correcting for the one carbon atom in the methyl group that was added during derivatization.

Enrichment calculation

High-precision isotope ratios were defined as $^{13}C/^{12}C$ (R) (mol.mol^{-1}). In tracer studies it is more convenient to express enrichment as absolute ^{13}C build up. For this we need the atom fraction (F^{13}). R_{sample} was measured, and then F^{13} were calculated from the $^{13}C/^{12}C$-ratios (R):

$F^{13} = R_{sample}/(R_{sample}+1)$ (equation 1)

The absolute amounts of incorporated ^{13}C-label in µg $^{13}C \cdot g\ dw^{-1}$ can be calculated from the sample concentration µg FA·g dw^{-1} (C), which was first corrected with the sample mass and chloroform layer, and the increase in the fraction ^{13}C after labeling (F^{13}) relative to the non labeled control (F^{13}_c):

$$^{13}C = 13 * (F^{13} - F_c^{13}) \frac{C}{M_{FA}}$$ (equation 2)

Results and discussion

7

Filtration and respiration rate

Two very different sponge species were used to determine the influence of mechanical damage. *Haliclona oculata* occurs abundantly in the Oosterschelde, the Netherlands in temperatures ranging from 2 – 23°C. It is a finger sponge attached at one base, containing many spicules to build their strong skeleton. *Dysidea avara* is a widely distributed sponge species in the Mediterranean (Uriz et al 1992), where temperature varies between 12 – 25°C. It is an encrusting sponge that can be found on rocky substrata. Also lobulated masses can be found. The skeleton is a meshwork of sponging fibers without spicules (Ribes et al 1999), which makes it a more fragile sponge than *H. oculata*. Due to the difference in sponge structure also different damaging strategies were used. For *H. oculata* we used scissors to cut off all tips of each sponge specimen used. For *D. avara* we scraped off part of the tissue of one half of a sponge, leaving the other half intact. After the experiment we collected the damaged and non damaged parts of each sponge specimen separately and analyzed them on ^{13}C content. For *H. oculata* this was easier as the tips and rest of the body could easily be separated. For *D. avara* this was less easy, because due to the colonized organization it sometimes seemed that the damaged and healthy part

started to act as two separate sponges. Consequently, it is difficult to compare the difference in damaged and healthy part of one sponge individual.

All sponges were fed with the same amount of ^{13}C labeled biomass *P. tricornutum*. For each sponge we took a sample of water at the end of feeding from inside the experimental chamber, which was next tested on ^{13}C content to determine the amount of ^{13}C taken up by the sponge. We compared filter capacities of damaged sponges to the non damaged sponges (Figure 1). Figure 1 shows the ^{13}C fraction in atom% in the SPM of the water inside the experimental chamber after 25 minutes feeding and at the start. The starting value was the same for all experiments. A decrease in ^{13}C fraction means that the added diatoms are filtered preferentially over other carbon containing particles. Only for *D. avara* the total carbon content was measured and actual filtration rates could be measured. This confirmed that a lower ^{13}C fraction in the water at the end of the experiment means a higher filtration rate. The decrease in enrichment after 25 minutes is the same for the non-damage and feed-first experiment as was expected because both treatments mean they are fed when not being damaged.

Directly after damage *H. oculata* does not change filtration rate while *D. avara* immediately decreases filtration rate. After 6 hours filtration rate for *H. oculata* increased to a higher value than the original value. We were forced to use different time intervals for late feeding due to bad weather conditions in the case of *D. avara*. The different sponges differed in size, this is also shown in Figure 1. Apparently the response of *H. oculata* after damage is to increase food uptake for wound healing. *D. avara* responded faster than *H. oculata* to damage. And a reduction of food uptake took place instead of an increase. Most likely the reduction of food uptake by *D. avara* is caused by killing part of the sponge tissue, leaving only half of the sponge alive for filtering.

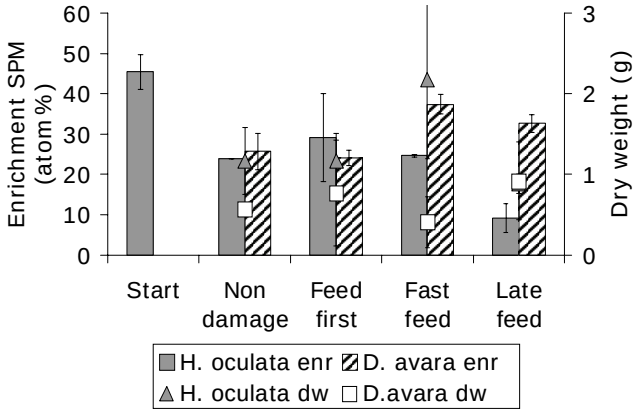


Figure 1: Fraction (± sd) of ^{13}C atoms (%) in the SPM surrounding the sponges before (start) and after feeding. Non damage and Feed first are non damaged sponges while feeding, Fast feed means immediate feeding after damage and late feed means that after damage a lag time was taken into account of 6 hours (H. oculata) or 24 hours (D. avara). On the right y-axis the average dry weight of the used sponges per category is shown.

Respiration rate was also measured during feeding for *H. oculata*. Respiration rate immediately decreased after damage but was restored 6 hours after damage (Figure 2). Apparently the damaged sponges respond fast by reducing both filtration and respiration rate (Figure 1). The large increase in filtration rate 6 hours after damage coincided with an increase of the respiration rate to the original level. The respiration rate measurements failed during the experiment with *D. avara* due to technical problems with the oxygen meters.

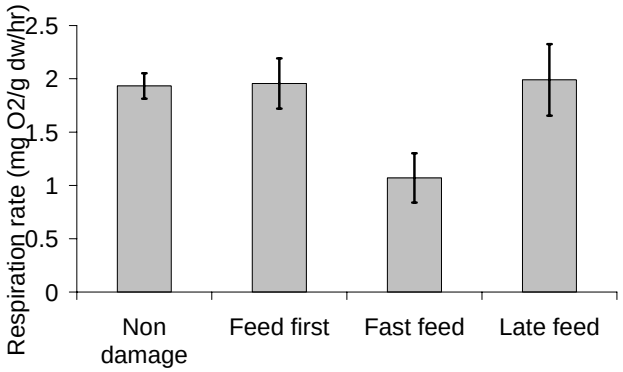


Figure 2: Average respiration rate of H. oculata after different treatments.

^{13}C label in the sponges

Despite clear differences in ^{13}C label uptake between damaged and non-damaged sponges, no significant differences in levels of ^{13}C label inside the sponges were found one week after feeding (Figure 3 and 4), this is also confirmed by our previous results (Chapter 6). From the sponge individuals we collected the damaged and non damaged tissue separately. For *H. oculata* this was easier as we damaged the tips of the sponge-fingers simply by cutting them off. For *D. avara* we split all sponges in half, where one half was damaged and the other half was left intact. *H. oculata* contained higher concentrations of ^{13}C label in the tips, both in the case of damaged and non damaged sponges (Figure 3). The damaged sponges had slightly more ^{13}C in the tips than the non damaged sponges, however the difference was not significant. This indicates that the food uptake in the tips is higher than in the rest of the body and that in damaged tips the concentration of the ^{13}C label is probably higher than in non damaged sponges. Most probably the metabolic activity in the tips is higher than in the rest of the tissue. *H. oculata* has two outliers in ^{13}C uptake, the first fast feed sponge shows higher ^{13}C content than the other sponges and the first late feed sponge shows a lower ^{13}C content. The ^{13}C fraction for *D. avara* showed large variations between the three individuals. No systematic difference was found between damaged and non-damaged parts for *D. avara* (Figure 4) Apparently *D. avara* lives more like colonies and when part of the sponge is damaged it seems to be left to die and the intact part is the part growing on.

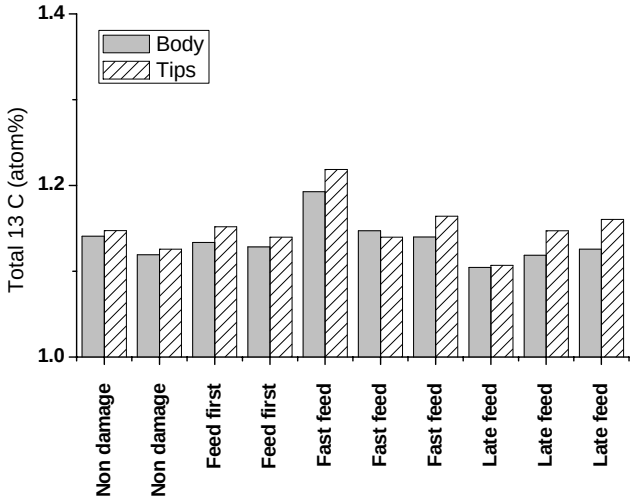


Figure 3: ^{13}C content in each sponge separated for the damaged tips and non damaged body for H. oculata

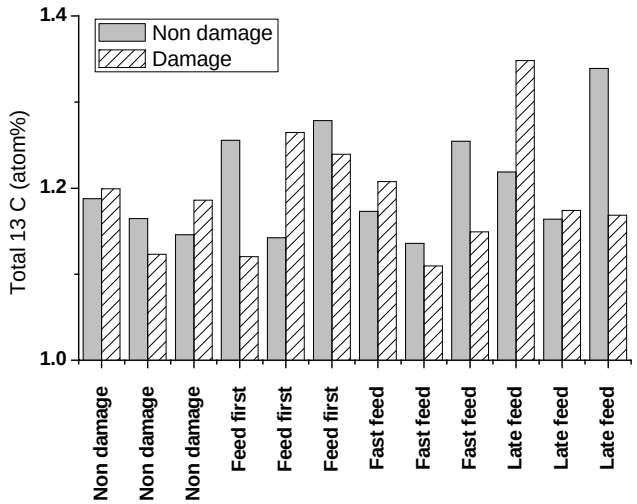


Figure 4: ^{13}C content in each sponge separated for the damaged and non damaged part for D. avara

^{13}C label in specific fatty acids

Whether ^{13}C label is present as algal cells or metabolized algal biomass can be seen in the specific fatty acid content. The fatty acid (FA) C16:0 is a biomarker for diatoms (Dunstan et al 1994). This biomarker was still present in the sponge body after 7 days (Figure 5). For *H. oculata* more ^{13}C label was present in this FA in the tips of the damaged sponges, in contrast to the non-damaged sponges where this was not the case. This confirms the finding that damaged tips took up more ^{13}C label (Figure 3). The FA C26:2(5,9) is very abundant in both sponges and non existent in *P. tricornutum* (data not shown). In the tips of both damaged and non damaged *H. oculata* more enrichment in sponge biomarkers was observed than in the rest of the body (Figure 5). The difference between tips and body was larger for damaged sponges with the exception of one individual at the late feed. Apparently more ^{13}C label was converted in the damaged tips as compared to non-damaged tips. This is in accordance with the fact that *H. oculata* grows at the tips (Kaandorp 1991) which leads to more ^{13}C label in the tips. Regeneration is mostly found to be a faster process in sponges than normal growth (de Caralt et al 2008[b], Ayling 1983, de Caralt et al 2003, Duckworth 2003), which is confirmed here by the higher enrichment found in the tips of damaged sponges. A lower amount of ^{13}C label after one week could also be due to a faster turnover of compounds. However, in our previous work we found that *Haliclona oculata* still converts ^{13}C label three weeks after uptake into the larger compounds and thus the amount of ^{13}C label in the larger sponge FAs was still increasing up to 3 weeks after labeling (Chapter 6). For *D. avara* the variation in ^{13}C fraction is again very high, which makes it impossible to see significant differences between damaged and non damaged parts ot the sponges and between damaged and non-damaged sponges. *D. avara* shows the same trend in the food and sponge FAs as for the total amount of ^{13}C in the sponges (Figure 5). Remarkable for *D. avara* was that the food compounds were still highly enriched after one week, while the sponge components were not that high enriched. For *H. oculata* this was just the other way around, where higher levels of enrichment were found for the sponge component and not for the food components. The latter was also found in chapter 6, where less ^{13}C label was found in the larger FAs in *D. avara* than in *H. oculata*. Although the absolute values of enrichment did not differ too much in the sponge FAs for both *H. oculata* and *D. avara*. Apparently the food FAs are metabolized slower or stored in *D. avara*, and probably normal respiration rate is lower for *D. avara* than for *H. oculata*.

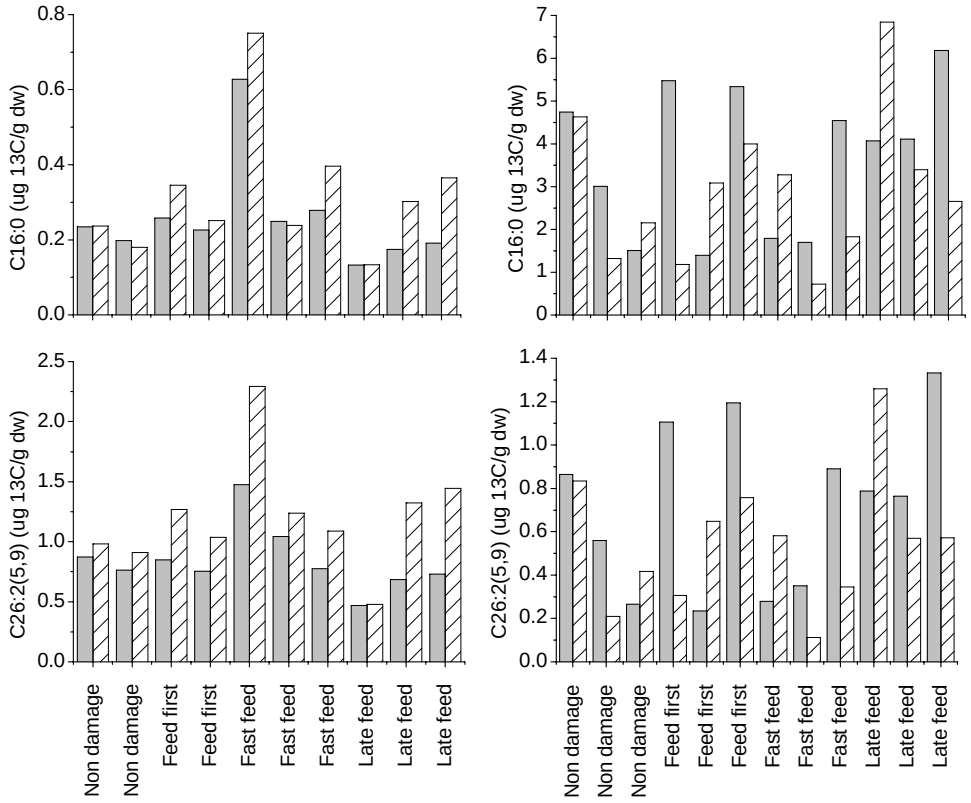


Figure 5: ^{13}C enrichment in C16:0 (diatom biomarker), and in C26:2(5,9) (sponge biomarker) for the damaged tips and non damaged body for H. oculata (left) and for the damaged and non damaged part for D. avara (right).

Conclusion

H. oculata was damaged by cutting off all tips and *D. avara* was damaged by scraping off part of its 'skin' using a knife. Both sponge species used responded quickly to damage. Immediately after damage the finger-sponge *H. oculata* respired less, but respiration rate recovered to the original rates within 6 hours. Filtration rate increased to a higher level within 6 hours after damage. The encrusting sponge *D. avara* immediately decreased filtration rate after damage, which was partly but not fully restored one day later. Relatively less ^{13}C labeled algal biomarkers was found in *H. oculata* compared to ^{13}C label in sponge biomarkers one week after labeling. The other way around was found for *D. avara*, indicating lower metabolic rate in *D. avara*.

Damaged and non damaged tissue was collected separately. In *H. oculata* a higher fraction of ^{13}C label was found in the tips of both damaged and non damaged sponges, thus growth rate is higher in the tips. The sponge biomarkers were higher enriched in the tips of the damaged sponges compared to the non-damaged sponges. Thus *H. oculata* has a higher metabolic rate in the tips and this rate increases after tissue damage. For *D. avara* large variation in levels of ^{13}C label were found in healthy and damaged tissue and significant differences were not found.

The found differences between the sponges can be explained by the different growth forms of the two sponge species. *H. oculata* is a finger sponge and grows mostly at the tips. *D. avara* is an encrusting sponge that lives more like colonies and when part of the sponge is damaged it seems to be left to die and the intact part is the part that continues to grow.

Acknowledgements

This study was funded by NWO-ALW (814.02.010). We thank the Waterboard Zeeuwse Eilanden and the province of Zeeland for issuing the necessary permits for collecting sponges in the Netherlands. And to Maria Uriz for providing us with the permits to collect sponge specimens in name of CSIC-CEAB. We are grateful to A. Kliphuis, J. Lindenbergh, B. van Vark, E. van Vark, P. Lamers and A. Bal for helping with placing the platform and the monitoring. Thanks to the Netherlands institute for ecology for providing equipment for the extraction and analysis of the fatty acids and M. Houtekamer and P. van Breugel for their analytical assistance.

Chapter 8

General discussion

Towards commercial production of sponge medicine

Koopmans M, Martens D, Wijffels RH (2009) Towards commercial production of sponge medicine. *Submitted for publication*.

Abstract

Sponges can provide potential drugs against many major world-wide occuring diseases. Despite the high potential of sponge derived drugs no sustainable production method has been developed. Thus far it is not fully understood why, when, where and how these metabolites are produced in sponges. For the near future sea-based sponge culture seems to be the best production method. However, for controlled production in a defined system it is better to develop *in vitro* production methods, like *in vitro* sponge culture or even better sponge cell culture, culture methods for symbionts or the transfer of production routes into another host. We still have insufficient information about the background of metabolite production in sponges. Before production methods are developed we should first focus on factors that can induce metabolite production. This could be done in the natural habitat by studying the relation between stress factors (such as predation) and the production of bioactive metabolites. The location of production within the sponge should be identified in order to choose between sponge cell culture or symbiont culture. Alternatively the biosynthetic pathways could be introduced into hosts that can be cultured. For this the biosynthetic pathway of metabolite production should be unraveled, as well as the genes involved. This review discusses the current state of sponge metabolite production and the steps that need to be taken to develop commercial production techniques. The different possible production techniques are also discussed.

Introduction

Marine sponges are a 'gold mine' with respect to the diversity of their secondary metabolites discovered during the past fifty years. Sponges can provide potential drugs against many major world-wide occurring diseases. Of the 18,000 marine natural products described over 30% are from sponges and of the antitumor natural product patent registrations in recent years over 75% are from sponges (MarinLit 1999, Faulkner 2002, Sipkema et al 2005[a]). An overview of marine natural products and their status in clinical trials can be found at: www.marinebiotech.org/pipeline.html. Many of these products originate from sponges.

Sponges (phylum Porifera) appear to be very stable, long-living animals, with growth rates that vary enormously between different groups. They are multi-cellular filter-feeding invertebrate animals living attached to a substratum in mostly marine but also in freshwater habitats. Sponges do not possess true tissue, but have different cell types with different functions, which together carry out normal body functions. To provide the sponges with food and oxygen and to excrete waste products a large amount of seawater is filtered. Many sponges have a symbiosis with micro-organisms. Symbionts include archaea, bacteria, fungi, cyanobacteria, and microalgae. Photosynthetic symbionts provide the sponge with photosynthetic nutrients, which they do not obtain by their own filter-feeding activities.

For the development of a sustainable production method of sponge bioactive metabolites, more knowledge is necessary about the biology and the needs of the sponge in nature for both growth and metabolite production. So far there was a lot of attention for the discovery of new bioactive metabolites. To develop these bioactive metabolites into medicines they need to be tested in clinical trials, for which substantial amounts of these compounds are needed. The concentration of bioactive compounds in the sponge tissue is generally very low. For example, *Lissodendorix* sp. contains concentrations of about 400µg/kg of halichondrin B. This means that there is a need for a sustainable production method. To develop a sustainable production technique it must be known whether the sponge or its symbionts or both are responsible for the production of the bioactive compound and in the case the sponge is the producer, which cells are responsible for the production. Because of the low concentrations inside the sponge the amount of sponge biomass needed will be very high (Dumdei et al 1998, Sipkema et al 2005[b]). To be able to increase the production per sponge, more knowledge is needed on the biosynthetic pathways and

their regulation, which includes factors that induce production of the metabolites (Hoover et al 2007).

There are several possible strategies to produce sponge bioactive compounds. Wild harvest is ecologically undesirable, due to the large amounts of sponge biomass necessary for commercial applications. We believe that potential strategies to produce bioactive compounds from sponges include sponge or sponge cell cultures and genetic modification approaches in which large gene fragments responsible for production of the bioactive compounds are identified and transferred into a suitable host. So far, none of the approaches resulted in applicable technologies for production of sponge bioactive compounds despite significant progress.

In this paper we will discuss the two major bottlenecks for sponge metabolite production, namely: Understanding metabolite production in the sponge, and choosing and improving culture systems. We propose a strategy how to develop a sponge metabolite production process in the following order:

1. To understand metabolite production following steps are needed
    - Identification of induction factors of metabolite production
    - Identifying biosynthetic pathways of secondary metabolites
    - Identification of the location of bioactive compound production

2. Choosing and improving one of the following culture systems
    - Whole sponge culture
    - Sponge cell culture
    - Symbiont culture
    - Genetic modification

Understanding metabolite production in the sponge

Induction of sponge metabolite production

It is generally assumed that sponges produce secondary metabolites because they have to compete for space with other organisms, they have to prevent fouling by other organisms and they have to keep predators away (Wulff 2006, Figure 1). The highest frequency of toxic or deterrent metabolites is found in high competing environments. Bioactive compounds are for example often discovered in coral reefs with an enormous biodiversity. Furthermore, sponges found in exposed areas that are vulnerable to fish predators are usually more

toxic than those growing unexposed (Proksch 1994). For example, chemical deterrence of fish predators was found significantly higher for extracts obtained from the tropical sponge community as compared to the temperate community where less predation occurs (Ruzicka and Gleason 2008). Predation pressure thus increases investment into chemical defense and sponges have evolved in higher or lower bioactive compound producing specimen due to possible predation.

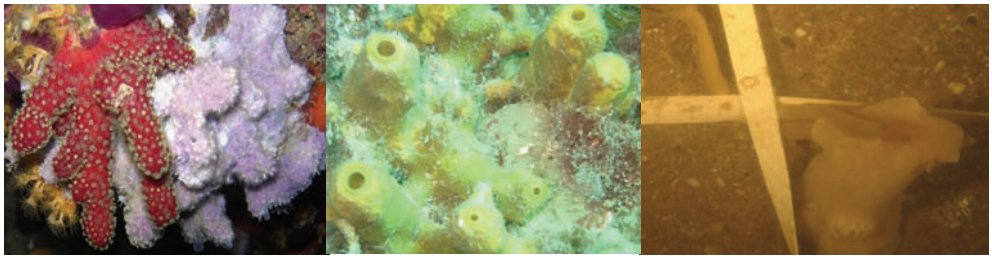


Figure 1: Sponges competing: A) Dysidea avara (right) competing for space with a soft leather coral, B) Aplysina aerophoba fouled with hydrozoa,C) Haliclona oculata predated by a nudibranch.

Sponge bioactive metabolite concentrations were found to be variable corresponding to several environmental factors. The season and variation in temperature, location and illumination all have significant effect on bioactive metabolite concentration. A positive correlation between temperature and metabolite concentration was found for salicylihalmide A in *Haliclona* sp. (Abdo et al 2007), and mycalamide A in *Mycale hentscheli* (Page et al 2005[b]). *Crambe crambe* from the Mediterranean appears to be less toxic in well illuminated habitats compared to shaded habitats (Becerro et al 1995). Also for other invertebrates variations were found. For example, the bioactive compound ascididemin of the ascidian *Cystodytes* sp. shows a seasonal trend and a positive correlation with temperature (Lȯpez-Legentil et al 2006).

In Figure 1 the sponge *Dysidea avara* competes for space with a leather coral. It is expected that under such circumstances the sponge produces more bioactive compounds than in absence of space competing organisms. There are only a limited number of such cases that have been studied, but they all support this hypothesis (Turon et al 1996, Thacker et al 1998). Several studies have shown that sponges and sponge extracts are deterrent towards predators like fishes (Pawlik et al 1995, Schupp et al 1999, Becerro et al 2003, Thoms et al 2004). It is often suggested that sponges are induced to produce more bioactive compounds when predated (Wulff 2006). In terrestrial plants this

mechanism has been well studied. The most common inducer of secondary metabolites in plants is herbivory by insects (Baldwin 1988). Also in gorgonians it has been shown that the natural product content could be increased significantly in the presence of predator snails (Thornton and Kerr 2002).

There are several examples that support the theory of possible induction of metabolite production due to stress factors. Transplantation, infection and different diets showed to induce metabolite production. Individuals transplanted from depth to near surface substantially increased the production of the natural product diterpenes in harvested sponge tissue of *Rhopaloeides odorabile* (Thompson et al 1987). *Aplysina aerophoba* showed to increase aplysinimine production in regions of the sponge close to infected sponge tissue, suggesting a defense response to the denser microbial community in the diseased part of the sponge (Webster et al 2008). Stevensine (an alkaloid metabolite) levels were significantly elevated in the experimental group of sponge explants cultured *in vitro* that were given a mixed diet of food particles at 3 times the natural environment particle concentration compared to sponges fed normal particle concentration (Duckworth et al 2003).

Another possibility to induce metabolite production is called activated chemical defense, which is induced by wounding. The probable ecological relevance of this mechanism is protection of the damaged sponge tissue from invasion of bacterial pathogens (Thoms et al 2006). Wound activation of protoxins results in a pronounced increase of fish deterrent activity of *A. aerophoba* (Ebel et al 1997). In *Aplysina* it was shown that wound-activated chemical defense occurs most likely by enzymatic conversion (Thoms and Schupp 2008). Due to wounding brominated isoxazoline alkaloids were converted into the monocyclic nitrogenous compounds aeroplysinin-1 and dienone by enzymatic cleavage (Thoms et al 2006). Wound regeneration was found to be slower in sponges with chemical defense than in sponges without chemical defense (Walters and Pawlik 2005). The difference in regeneration rate is suggested to be the result of two different strategies where the sponge either invests in a high regeneration rate or in producing compounds for chemical defense.

Despite the differences found in bioactive metabolite concentrations at different environmental circumstances, for most sponges it is still unknown which factors induce production of bioactive compounds. As sponges react to predation, wounding and stress in general, there are probably specific inducers that trigger these responses. No studies have been performed to find which specific compounds induce secondary metabolite production inside the sponge. Finding such inducers would be of great value, since it would allow the induc-

tion of chemical defense and the associated metabolite production without the negative effects of for example wounding. If, for example, studies *in situ* reveal that production is induced due to the presence of predators. Extracts of the predator can be analyzed and gradually purified and tested on the sponges with the goal to identify the bioactive compound inducing chemical defense.

Biosynthesis of secondary metabolites

Inducible production of bioactive compounds can also be helpful in identifying the genes involved. Information on the genes involved in metabolite production can then be obtained by comparing the gene expression before and after inducing bioactive metabolite production. A sequenced genome or *a priori* knowledge of enzymes involved greatly facilitate this approach.

The complex interaction between sponges and symbionts is one of the reasons that relatively little genome research is done on sponges. Technically, it is difficult to separate the DNA of the sponge from that of the other organisms. Recently, the first sponge genome project was launched by the University of Queensland, Australia. The only sponge of which the genome is sequenced and published is *Amphimedon queenslandica* (www.compagen.org). In order to make faster progress in drug development a method is necessary with which it is possible to screen complex genetic material. Metagenomics has developed to be valuable as a tool for studying complex communities (Fortman and Sherman 2005).

Sponges can be seen as complex communities due to the presence of many symbionts. Developments in metagenomics have provided new insights in sponge metabolite production (Grozdanov and Hentschel 2007, Kennedy et al 2008). It can be used to find if certain pathways are of bacterial or sponge origin. For example, polyketide synthase (PKS) enzymes are involved in the synthesis of many natural products (Hutchinson 2003) and were found to be of bacterial origin inside several sponges (Piel et al 2004, Kennedy et al 2008). Identifying gene fragments involved in bioactive metabolite production becomes more difficult when more complex metazoan genomes are studied for isolation of specific biosynthetic genes (Piel 2004). A targeted approach should be used to begin this complex study. Many marine natural products are terpenes (Blunt et al 2004) and thus a good start to investigate gene expression in sponges would be to focus on the mevalonate pathway, which is a known common terpene biosynthesis pathway (Hoover et al 2007).

Another technique to get more insight in sponge metabolism is to study

the metabolome, i.e. the complete set of metabolites. Techniques to study the metabolome have developed fast in the recent years (Moco 2007). The metabolome is the final product of gene expression in a cell and thus represents the interaction of all biochemical processes. Metabolome studies in sponges on bioactive compounds have not been done yet, but it is done in sponges for fatty acid (FA) metabolism. Biosynthesis of fatty acids has been studied in different sponges by labeling techniques (Readerstorff et al 1987, Hahn et al 1988). ^{14}C-label was used by Hahn et al (1988) to elucidate the biosynthesis of two long chain 'demospongic acids' in the sponge *Microciona prolifera*. Synthesis of these fatty acids was found to be done by elongating exogenous short chain FAs. Carbon isotopic labeling can be used to study intermediates of different compounds, or labeled intermediates can be fed to study further conversion pathways (Byrne et al 2002). Multigene expression analysis in combination with metabolic analysis can be used to study production of bioactive compounds under stress conditions. This enables monitoring of expression levels of genes involved in the biosynthetic route of the bioactive compound.

Location of secondary metabolite production in the sponge

To be able to choose a production system it is necessary to know where the metabolites are produced in the sponge. Very often it is not known whether the sponge or the sponge symbiont is responsible for the production of the bioactive compound. Even in cases this is known, it is not known if the sponge-symbiont relation is important for the production of the bioactive compound. It is often suggested, but has never been demonstrated, that in case the bioactive compound is produced by the sponge cell, the precursor for that compound might have been produced by an associated microorganism. Even in cases the compound is present in the sponge cell it may have been produced by a symbiont and stored in the sponge cell. It is often suggested that sponge symbionts are so tightly coupled that the microbial genome size is reduced, making the symbiont for nutrition dependent of the sponge or even that the genomes of sponge and symbiont are integrated (Sara et al 1998, Taylor et al 2007, Wijffels 2008).

In different studies it was shown that sponge bioactive compounds are stored in specific parts of the sponge, such as the outer layer, which is most exposed to predators. The secondary metabolite desacetylscalaradial was found in higher concentrations in the tips than in the base of the branching sponge *Cacospongia* sp. (Becerro et al 1998). In *Rhopaloeides odorabile*, diterpene

concentrations were found highest in the surface tissue (Thompson et al 1987). Similar results were found by Kubanek et al (2002) for *Ectoplasia ferox* as larger concentrations of triterpene glycosides were found in the top layer of the sponge tissue. However, the same article presents the opposite for *Erylus formosus*, where higher concentrations of formoside were found in the inner part of the sponge. Thus several studies support that highest concentrations are produced in the most vulnerable parts of the sponge.

Several methods have been used to identify the location of the bioactive compound in the different cell types of the sponge and its symbionts. For this the different cell fractions from sponges were isolated by flow sorting (e.g. Unson and Faulkner 1993, Figure 2) and Ficoll or Percoll density gradient centrifugation (Uriz et al 1996[a], Garson et al 1998). These cell fractions can then be analyzed for bioactive metabolites and intermediates of the biosynthetic route. An example of a bioactive compound produced by the sponge is stevensine from *Axinella corrugata*. Stevensine was also produced in a primary sponge cell culture (Pomponi and Willoughby 1994). Unson and Faulkner (1993) found that cyanobacteria are responsible for the production of chlorinated metabolites, however the sequiterpenoids were only found in the sponge cells. In the Haplosclerid sponge *Haliclona* sp. the cytotoxic alkaloids were located in the sponge cells (Garson et al 1998). This is also the case for avarol from *Dysidea avara*, which is located in the choanocytes (Uriz et al 1996[a]).

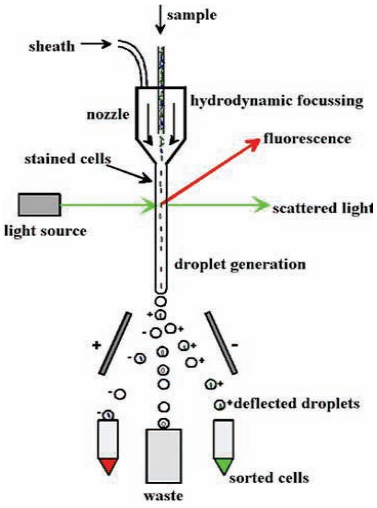


Figure 2: Flow sorting using flow cytometry. Cells can be charged based on difference in fluorescence or size.
http://missinglink.ucsf.edu/lm/molecularmethods/flow.htm

In some cases, the symbionts and not the sponge cells are the likely source of the secondary metabolites of interest (Lee et al 2001, Proksch et al 2002). For example, the polybrominated biphenyl ether antibiotics isolated from the sponge *Dysidea herbacea* are really produced by the endosymbiotic cyanobacterium *Oscillatoria spongeliae* (Unson and Faulkner 1993). Manzamine in the sponge *Acanthostrongylophora* is produced by the associated microorganism from *Micromonospora* sp (Hill et al 2005). Another example is the production of dysiherbaine in the endosymbiotic cyanobacterial cells of the genus *Synechocystis* in the sponge *Lendenfeldia chondrodes* (Sakai et al 2008). Fungi associated with marine sponges are also known to produce many bioactive agents (Holler et al 2000). Work on isolation and cultivation of sponge symbionts and the nature of symbiotic relationships have been reviewed elsewhere (Lee et al 2001, Newman and Hill 2006).

Cell types that can be isolated to study the location of metabolite production are bacteria, microalgae and different types of sponge cells in which presence of bioactive compounds is expected. Sponge cell types of interest in this respect are the archeaocytes, choanocytes and pinacocytes. It is mostly suggested that archeaocytes are responsible for the production of secondary metabolites. Since one of the reasons to make bioactive compounds is protection against predators, one would expect these compounds to be present in the pinacocytes, which form the outer layer of the sponge. However, so far it is not shown that pinacocytes contain bioactive compounds. When compounds are found in specific cells it can still be that intermediates or precursors may have been produced by associated microorganisms or other cells. The debate about this is mainly based on speculations and we have to conclude that at present it is unknown in which cell types the bioactive compounds and their intermediates are produced.

Culture systems

Sponge culture

Sipkema et al 2005[b] concluded that whole sponge culture is the most promising option for large scale production of higher concentrated metabolites like avarol, which is produced up to 3 g per kg sponge wet weight (Muller et al 1985) in *Dysidea avara*. Especially for faster growing sponges containing large amounts of metabolites whole sponge culture is potentially interesting. It was determined that sea-based culture is economically cheaper than land-based culture

(Sipkema et al 2005[b]). The major drawback of culturing sponges is that many sponges were found to be slow or variable growing organisms both in the sea (Dayton 1979, Garrabou and Zabala 2001, Duckworth and Pomponi 2005, de Voogd et al 2007, de Caralt et al 2008[b], Chapter 2) as well as in the laboratory (Barthel and Theede 1986, de Caralt et al 2003, Osinga et al 2003, Belarbi et al 2003[b], Mendola 2003, Duckworth et al 2003, Sipkema et al 2005[c]). Studies have demonstrated that production of metabolites in cultured sponges was sometimes lower (de Voogd et al 2007), but on the other side also higher production rates were found (Hadas et al 2005, Page et al 2005) than in sponges harvested from the sea. Apart from the costs, sea-based culture is less desirable than land-based culture as the conditions cannot be controlled in the sea, and the sponges are vulnerable to diseases and parasites. Several attempts have been made to culture sponges on land. Most of the research focused on food requirements, which is thought to be the key to success (Osinga et al 1999). Sponge cultures in the sea can be done using different structures for attaching sponges (Figure 3), and harvesting could be done partially thus leaving explants behind to re-grow. Sea-based cultivation is still the method where the largest growth rates have been obtained. Apparently, the artificial environment we construct for the sponges still cannot replace the complexity of the sea. A good alternative for this is to culture sponges on land but still use natural seawater flowing through the aquaria containing the sponges. From our own experiments we learned that the sponge *Haliclona oculata* is very difficult to maintain in the laboratory, but specimens survived longer (6 weeks) in aquaria with continuously flowing natural seawater than in aquaria without continuously flowing seawater (3 weeks). Since even in aquaria placed near the sea with a continuous flowing seawater eventually resulted in sponge death, factors other than sea water composition, like pressure or illumination, may play an important role in sponge growth and survival. Despite several growth studies still very limited understanding is present about the exact needs of sponges to improve growth. Therefore, before sponge culture systems can be realized more understanding is necessary about food, attachment surfaces and other requirements that stimulate sponge growth.

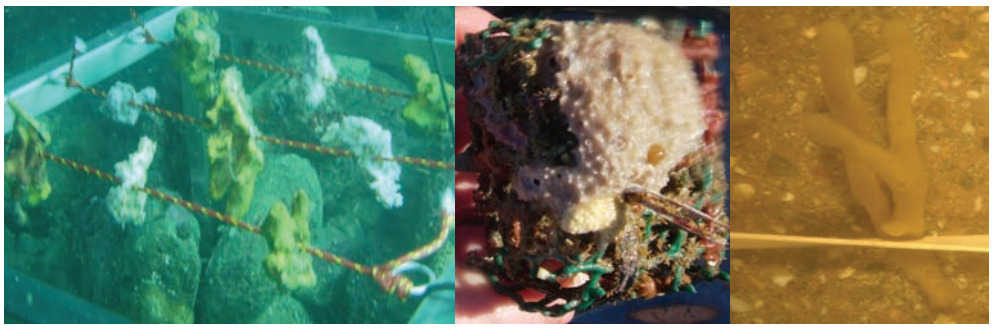


Figure 3: Different attachment forms for sea-based culture. Sponges on A) threads, B) in cages, C) on tiles, attached using a plastic band, where sponges attach to the tile themselves within one month.

In this thesis we have focused on the biology and needs of sponges for growth. To compare growth rates of cultured sponges, reference growth rates occurring in nature are necessary. Therefore we have monitored the volumetric growth rate of *Haliclona oculata* and the variation in nutrients in their natural habitat without harming the sponges (Chapter 2, Figure 4). Low seasonal growth rates were found with an annual average of 0.008 day^{-1} with the highest rate found in May (0.012 day^{-1}) when temperature was increasing and algal blooming occurred. Thus, nutritive values and also temperature was positively related to sponge growth rate. Probably feed components are limiting for growth. However, we found that *Haliclona oculata* has a net growth efficiency (NGE) of 10% (Chapter 3), which means that only 10% of all used carbon (growth and respiration) was used for growth and, thus 90% was being respired. In the seawater there was sufficient particulate carbon available to the sponges, where the particulate carbon flow through the sponge was 3 times larger than the amount of carbon used for either respiration or growth. Also oxygen appeared not to be limiting as only 3.3% of the oxygen pumped through the sponge body was used for respiration. Thus *H. oculata* has a very low biomass yield on food and uses the main part of its carbon for energy generation.

To measure how food is used by the sponge we need to be able to distinguish between food compounds and sponge compounds in the sponge and thus biomarkers are needed. Sponges are characterized by the largest diversity of fatty acids (FAs). We have identified fatty acids in 5 sponge species from three different habitats (Chapter 4). For all 5 sponge species several bio-

markers were identified. For some different sponges the same biomarkers were found by comparing sponge FAs with FAs in the surrounding suspended particulate matter (SPM). Based on fatty acid composition in combination with ^{13}C natural abundance of the FAs it is possible to analyze the food source of sponges (Boschker and Middelburg 2002). The seasonal variation of FA concentration in the 5 studied sponges was related to changes in FA concentration in SPM (Chapter 5). ^{13}C natural abundance values of large sponge FAs did not vary much with season, although ^{13}C values of food FAs did vary. Only in May similar ^{13}C values were found in the large sponge FAs and the food FAs, indicating higher growth rates. Higher ^{13}C values were found in the FA biomarkers of the bacterial sponge *A. aerophoba*, corresponding to higher ^{13}C values found in bacterial FAs. The short chain food FAs are elongated to the larger sponge FAs, and when higher growth rates occur similar ^{13}C values can be found in small and long chain FAs.

Figure 4: The left picture shows the platform placed in the Oosterschelde containing 42 tiles on which sponges were attached. The right picture shows the 3d camera frame used in our study to measure sponge volume.

To get more knowledge about metabolism and requirements in the sponge we studied carbon metabolic rate in two different sponge species from different habitats by feeding with ^{13}C labeled diatoms (Chapter 6). Both sponge species, *Haliclona oculata* and *Dysidea avara*, took up the ^{13}C labeled cells in 8 hours and in both sponges conversion of the fatty acids in the cells started within the first day. Three weeks after labeling we still found increasing levels of ^{13}C in the larger sponge FAs. The larger sponge FAs were metabolized at a higher rate than the growth rate, indicating turnover of FAs. (Chapter 6). We

measured the immediate response to damage by measuring filtration rate and respiration rate, moreover, we fed the sponge with ^{13}C labeled diatoms and traced the ^{13}C label in both damaged and non damaged tissue (Chapter 7). Both sponges responded quickly to damage. *Haliclona oculata* immediately reduced the rate of respiration and filtration after damage. The filtration rate increased to values higher than the initial value and the respiration rate returned back to normal levels 6 hours after damage. The encrusting sponge *Dysidea avara* immediately decreased filtration rate after damage and it was not restored after one day. *H. oculata* had a higher metabolic rate in the tips where growth occurs then in the rest of the tissue and this metabolic rate is increased after damage of the tissue. For *D. avara* no differences were found between damaged and non-damaged tissue (Chapter 7).

Food particles were used fast in the sponge metabolism (Chapter 5), although usage of certain compounds could be found up till 3 weeks (Chapter 6). Apparently sponges are able to use a food source rapidly and build it into biomass but at the same time also loose biomass, resulting in a lower growth rate. Not only food source and availability but also other, physical, factors influence growth and metabolite production of sponges. Although some studies showed small successes of land-based sponge culture, there are still many barriers to overcome. Best successes thus far have been obtained with sea-based culture showing that we are still unable to copy the sea environment and important factors are still unknown.

Sponge cell culture

In the cases that the sponge cells are responsible for the bioactive metabolite production (Thompson et al 1983, Uriz et al 1996, Pomponi et al 1997), sponge cell culture would be an obvious method to use. *In vitro* culture of sponges as dissociated sponge cells or tissue would provide a clean and defined system for the production of sponge metabolites. However, attempts to develop continuously proliferating cell lines from sponges have failed so far. When achieving a continuously dividing sponge cell culture, cells can be grown in controlled bioreactors and controlling circumstances and stimulating production inside the cells will be much easier than in whole sponges. In addition, the use of these bioreactors makes scale up also easier (Figure 5). The difficulty with obtaining sponge cell lines is that cells do not continuously divide, because it is not known what sponge cells need in order to grow (Pomponi and Willoughby 1994, Muller et al 1999). However, phytohemaglutin promoted cell division of

sponge cells in culture (Pomponi and Willoughby 1994). Another problem encountered in these studies is getting the culture axenic, as sponges themselves are not axenic. Using undifferentiated cells may improve cell adjustment to the new environment. Thus far archeaocytes are the most promising cells in sponges to use for cell culture due to their pluripotency. However, using embryonic cells as starting material for cell culture may improve the chance in obtaining continuously dividing cells significantly (de Caralt et al 2008[a]).

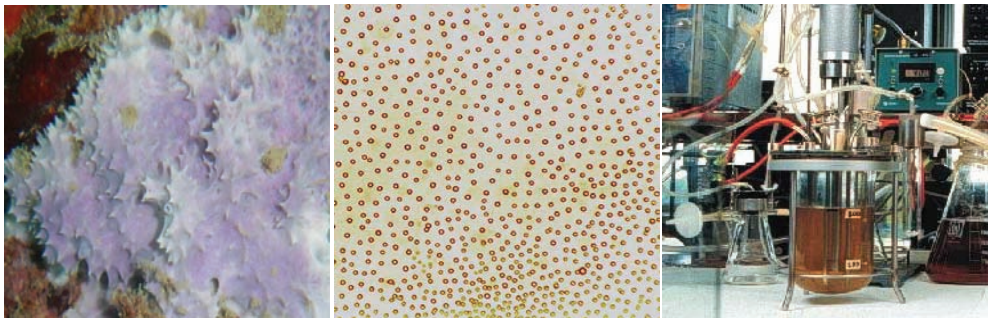


Figure 5: Development of sponge cell-lines. A) Dysidea avara B) cells isolated from Dysidea avara C) bioreactor to culture animal cells.

There are a number of observations that showed that besides stimulating cell proliferation also prevention of cell death may be important for the development of continuous cell lines. These observations are slow growth rates of sponges (de Caralt et al 2008[b], chapter 2), in combination with fast healing of wounded tissue (de Caralt et al 2003, chapter 7), reorganization of sponge tissue (Mendola 2008), DNA replication and no net growth of primmorphs (Sipkema et al 2003) and fast DNA replication and probably apoptosis in whole sponge specimens (see De Caralt et al 2008[a]). We hypothesize that sponges may grow fast and die fast at the same time. This could be the reason that sponges are successful animals during evolution. Cells will not show any malfunctioning due to ageing because of the high turn over of cells and the organism as a whole responds to attacks simply by giving up that part and building new cells. If this is true than sponges are very dynamic organisms that show a very slow net growth as a result of fast cell division and at the same time a high rate of apoptosis. For the development of continuous growing cell lines the strong capability of sponge cells to divide should be used and apoptosis should be prevented. A final advantage of sponge cell culture is that when understanding metabolic pathways and inducing factors for metabolite production the medium and culture conditions can be easily adjusted to improve production rates.

Symbiont culture

If symbiotic micro-organisms inside the sponge are responsible for the production of the bioactive metabolites then of course culturing these symbionts would be the best option. Thus far few publication have focused on culturing symbionts and first successes have been obtained (Li et al 2007, Muscholl-Silberhorn et al 2007). To culture sponge-associated symbiont remains very difficult because of the difficulty in isolating and pure cultivation. Some micro-organisms will not grow in pure culture but can form colonies in the presence of other microorganisms (Kaeberlein et al 2006). Isolation of sponge-associated bacteria was best when using oligotrophic media (Muscholl-Silberhorn et al 2007). Addition of sponge extract seems to have a positive effect on cultivability (Li et al 2007). From different sponge species isolated morphotypes were mostly affiliated to the *Alphaproteobacteria*, although also members of the genus *Bacillus* were found regularly. One alphaproteobacterium was found to dominate the culture, which has antimicrobial activity although weak and unstable and the cells lost its activity during culture (Muscholl-Silberhorn et al 2007). Thus, symbiotic bacteria cultivation did work, but keeping bioactivity is also difficult. However Kennedy et al (2009) showed that 50% of the isolated bacteria from *Haliclona simulans* possessed antimicrobial activity. Bioactive metabolite producing symbionts in culture are still very difficult to obtain. However, more efforts are made towards development of sponge symbiont culture nowadays.

Genetic modification

Metagenomics is the genetic analysis of a complex microbial mixture that can be used to analyze sponge microbial associations. Metagenomic approaches have also been used in which large gene fragments important for production of the bioactive compounds are identified, which could then be transferred to a suitable host (Piel 2006). This is a very promising technique, but a complex biosynthesis pathway means involvement of many genes, all of which need to be identified and all need to be transferred to the other host. Metabolic engineering is under development and the number of successes in introducing combinations of many genes is increasing lately (Engels et al 2008, Ro et al 2008, Anthony et al 2009).

For plant natural products recent advancements were made in the production of terpenoids, phenylpropanoids and alkaloids by using different techniques including protein engineering, codon optimization and combinatorial biosynthesis (Chemler and Koffas 2008). Paclitaxel (Taxol) is a classical example of a plant natural product produced in *Taxus brevifolia*. Taxol is a complex terpenoid that can be used for the treatment of breast cancer. Recently, a step forward was made in the production of Taxol in the yeast *Saccharomyces cerecisiae*. Heterologous genes were introduced encoding biosynthetic enzymes of the beginning of the taxoid biosynthetic pathway, as well as a regulatory factor inhibiting competitive pathways (Engels et al 2008). Especially the combination of combinatorial biosynthesis and elements to inhibit competitive pathways made it possible to increase the production of the intermediate taxadiene in yeast 40-fold to 8.7 mg/L.

The group of Keasling of the university of California, Berkeley has made major progress in expressing multiple genes in both yeast (*Saccharomyces cerevisiae*) and bacteria (*Escherichia coli)*. For example, genes encoding for the anti-malarial drug artemisinin, a sesquiterpene, have been expressed in *S. cerevisiae*. A single plasmid was used to express a combination of three plant genes and 84% of the plasmids were stable in the cells producing the intermediate amorphadiene, whereas poor plasmid stability was found in cells synthesizing artemisinic acid (Ro et al 2008). Also production of the intermediate amorphadiene has been employed in *E. coli* (Anthony et al 2009). A simple cloning system for expressing the whole pathway enabled identification of rate limiting enzymes, which then could be over expressed to increase production seven-fold (Anthony et al 2009). Thus development of expressing multiple genes in host organisms is in full speed and when biosynthetic pathways are identified in sponges this road should be open for sponge drug development as well. However, difficulties are still to be overcome to be able to produce the toxic end products in host organisms, as the bioactive compounds can have toxic effects to the host organism itself.

Concluding summary

For the development of sponge derived drugs still major breakthroughs are necessary. The best method to produce the different compounds depends on various factors. First focus of sponge derived drug development should be on why, when, how, and where the compound is produced. When being able to answer all these questions the best production method can be chosen. Then, focus should be put on development of the production method. Thus far the most promising method is whole sponge production in the sea. This is due to the fact that we do not understand the needs of the sponge for both growth and production. In the sea all elements are available to the sponge for survival, growth and metabolite production. For clean and defined systems it is better to develop cell culture methods, either sponge cell culture, symbiont culture or other host organisms. Before proper production methods for sponge metabolites are developed still a lot of research is necessary.

References

Abdo DA, Seager JW, Harvey ES, McDonald JI, Kendrick GA, Shortis MR (2006) Efficiently measuring complex sessile epibethic organisms using a novel photogrammetric technique. J.Exp.Mar.Biol.Ecol.

Abdo DA, Motti CA, Battershill CN, Harvey ES (2007) Temperature and spatiotemporal variability of salicylihalamide A in the sponge *Haliclona* sp. J.Chem.Ecol. 33:1635-1645

Abraham WR, Hesse C, Pelz O (1998) Ratios of carbon isotopes in microbial lipids as indicator of substrate usage. Appl.Environ.Microbiol. 64:4202-4209

Alonso A, Camargo JA (2006) Toxicity of Nitrite to Three Species of Freshwater Invertebrates . Environmental toxicology 21:90-94

Althoff K, Schutt C, Steffen R, Batel R, Muller WEG (1998) Evidence for a symbiosis between bacteria of the genus *Rhodobacter* and the marine sponge *Halichondria panicea*: harbor also for putatively toxic bacteria? Mar.Biol. 130:529-536

Alvi KA, Crews P (1992) Homoscalarane Sesterterpenes from Lendenfeldia frondosa. J.Nat.Prod. 55:859-865

Andrade P, Willoughby R, Pomponi SA, Kerr RG (1999) Biosynthetic studies of the alkaloid, stevensine, in a cell culture of the marine sponge Teichaxinella morchella. Tetrahedron Lett. 40:4775-4778

Anthony JR, Anthony LC, Nowroozi F, Kwon G, Newman JD, Keasling JD (2009) Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug presursor amorpha-4,11-diene. Metabolic Engineering 11:13-19

Ayling AL (1983) Growth and regeneration rates in thinly encrusting demospongiae from temperate waters. Biol.Bull. 165:343-352

Baldwin IT (1988) The alkaloidal responses of wild tobacco to real and simulated herbivory. Oecologia 77:378 381

Barranguet C, Herman PMJ, Sinke JJ (1997) Microphytobenthos biomass and community composition studied by pigment biomarkers: importance and fate in the carbon cycle of a tidal flat. Journal of sea research, 38: p. 59-70

Barthel D (1986) On the ecophysiology of the sponge *Halichondria panicea* in Kiel Bight. I. Substrate specificity, growth and reproduction. Mar.Ecol.Prog.Ser. 32:291-298

Barthel D, Theede H (1986) A new method for the culture of marine sponges and its application for experimental studies. Ophelia 25, 2:75-82

Becerro MA, Turon X, Uriz MJ (1995) Natural variation of toxicity in encrusting sponge *Crambe crambe* (Schidt) in relation to size and environment. J.Chem.Ecol. 21:1931-1946

Becerro MA, Turon X, Uriz MJ (1997) Multiple functions for secondary metabolites in encrusting marine invertebrates. J.Chem.Ecol. 23:1527-1547

Becerro MA, Paul VJ, Starmer J (1998) Intracolonial variation in chemical defenses of the sponge *Cacospongia* sp. and its consequences on generalist fish predators and the specialist nudibranch predator *Glossodoris pallida*. Mar.Ecol.Prog.Ser. 168:187-196

Becerro MA, Thacker RW, Turon X, Uriz MJ, Paul VJ (2003) Biogeography of sponge chemical ecology: comparisons of tropical an temperate defenses. Oecologia 135:91-101

Belarbi EH, Contreras Gomez A, Chisti Y, Garcia Camacho F, Molina Grima E (2003[a]) Producing drugs from marine sponges. Biotechnol.Adv. 21:585-598

Belarbi EH, Ramirez Dominguez M, Ceron Garcia MC, Contreras Gomez A, Garcia Camacho F, Molina Grima E (2003[b]) Cultivation of explants of the marine sponge *Crambe crambe* in closed systems. Biomol.Eng. 20:333-337

Berge JP, Barnathan G (2005) Fatty acids from lipids of marine organisms: molecular biodiversity, roles as biomarkers, biologically active compounds, and economical aspects. p 49-125

Bergmann W, Feeney RJ (1950) The isolation of a new thymine pentoside from sponges. J.Am.Chem.Soc. 72:2809-2810

Bergquist PR (1978) Sponges. Hutchinson & Co Ltd, London

Bergquist PR, Lawson MP, Lavis A, Cambie RC (1984) Fatty acid composition and the classification of the porifera. Biochem.Syst.Ecol. 12:63-84

Blunt JW, Copp BR, Hu WP, Munro MHG, Northcote PT, Prinsep MR (2008) Marine natural products. Nat.Prod.Rep. 26:170-244

Boschker HTS, de Brouwer JFC, Cappenberg TE (1999) The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: Stable carbon isotope analysis of microbial biomarkers. Limnol.Oceanogr. 44:309-319

Boschker HTS, Middelburg JJ (2002) Stable isotopes and biomarkers in microbial ecology. FEMS microbiology 40:85-95

Boschker HTS, Kromkamp JC, Middelburg JJ (2005) Biomarker and carbon isotopic constraints on bacterial and algal community structure and functioning in a turbid, tidal estuary. Limnol.Oceanogr. 50:70-80

Boucher-Rodoni R, Boucher G (1993) Respiratory quotient and calcification of *Nautilus macromphalus* (Cephalopoda: Nautiloidea). Mar.Biol. 117:629-633

Byrne KM, Smith SK, Ondeyka JG (2002) Biosynthesis of Nodulisporic Acid A: Precursor Studies. J. Am. Chem. Soc. 124(24)

Collyer DM, Fogg GE (1954) Studies on fat accumulation by algae. Journal of Experimental Botany 6(17):256-275.

Camargo JA, Alonso A, Salamanca A (2005) Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58:1255-1267

Chemler JA, Koffas MAG (2008) Metabolic engineering for plant natural product biosynthesis in microbes. Current Opinion in Biotechnology 19:597-605

Christie WW, Brechany EY, Stefanov K, Popov S (1992) The fatty acids of the sponge *Dysidea fragilis* from the Black Sea. Lipids 27:640-644

Cliff G (1982) Seasonal variation in the contribution of phytoplankton, bacteria, detritus and inorganic nutrients to a rocky shore ecosystem. Trans.Roy.Soc.S.Afr. 44:523-538

Collyer DM, Fogg GE (1954) Studies on fat accumulation by algae . Journal of Experimental Botany 6:256-275

Dalsgaard J, St.John M, Kattner G, Muller-Navara D, Hagen W (2003) Fatty Acid Trophic Markers in the Pelagic Marine Environment. Adv.Mar.Biol. 46:225-340

Dasgupta A, Ayanoglu E, Wegmann-szente A, Tomer KB, Djerassi C (1986) Mass spectral behavior and HPLC of some unusual molecular phospholipid species. Chemistry and physics of lipids 41:335-347

Dayton PK (1979) Observations of growth, dispersal and population dynamics of some sponges in McMurdo Sound, Antarctica. *Colloques Internationaux du C.N.R.S.* 291:271–282.

De Caralt S, Agell G, Uriz MJ (2003) Long-term culture of sponge explants: conditions enhancing survival and growth, and assessment of bioactivity. Biomol.Eng. 20:339-347

De Caralt S, Uriz MJ, Wijffels RH (2008[a]) Cell culture from sponges: pluripotency and immortality. Trends in biotechnology 25:467-471

De Caralt S, Uriz MJ, Wijffels RH (2008[b]) Grazing, differential size-class dynamics and survival of the Mediterranean sponge *Corticium candelabrum*. Mar.Ecol.Prog.Ser. 360:97-106

De Goeij JM, van den Berg H, van Oostveen MM, Epping EHG, van Duyl FC (2008[a]) Major Bulk dissolved organic carbon (DOC) removal by encrusting coral reef cavities sponges. Mar Eco Prog Ser 357:139-151

De Goey JM, Moodley L, Houtekamer M, Carballeira NM, van Duyl FC (2008[b]) Tracing 13C-enriched dissolved and particulate organic carbon in the bacteriacontaining coral reef sponge Halisarca caerulea: Evidence for DOM feeding. Limnol.Oceanogr. 53:1376-1386

De Voogd N (2007) The mariculture potential of the indonesian reef-dweeling sponge *Callyspongia* (*Euplacella*) *biru*: GRowth, survival and bioactive compounds. Aquaculture 262:54-64

DeLong EF, Yayanos AA (1986) Biochemical Function and Ecological Significance of Novel Bacterial Lipids in Deep-Sea Procaryotes. Appl.Environ.Microbiol. 51:730-737

Djerassi C, Lam W (1991) Sponge phospholipids. Acc.Chem.Res. 24:69-75

Duckworth AR (2003) Effect of wound size on the growth and regeneration of two temperate subtidal sponges. J.Exp.Mar.Biol.Ecol. 287:139-153

Duckworth AR, Samples GA, Wright AE, Pomponi SA (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 AR, Pomponi SA (2005) Relative importance of bacteria, microalgae and yeast for growth of the demospongiae *Halichondria melanadocia*. J.Exp.Mar.Biol.Ecol. 323:151-159

Duckworth AR, Bruck WM, Janda KE, Pitts TP, McCarthy PJ (2006) Retention efficiencies of the coral reef sponges *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* determined by Coulter counter and plate culture analysis. Marine Biology Research 2 :243-248

Dumdei EJ, Blunt JW, Munro MHG, Battershill CN, Page MJ (1998) The whys and whats of sponge chemistry: why chemists extract sponges and what problems does this cause?

Dunstan GA, Volkman JK, Barrett SM, Leroi JM, Jeffrey SW (1994) Essential polyunsaturated fatty acids from 14 species of diatom (Bacillariophyceae). Phytochemistry 35:155-161

Ebel R, Brenzinger M, Kunze A, Gross HJ , Proksch P (1997) Wound activation of protoxins in marine sponge *Aplysina aerophoba*. J.Chem.Ecol. 23:1451-1462

Egorova-Zachernyuk TA, van Rossum B, Erkelens K, de Groot H (2008) Characterization of unformly ^{13}C, ^{15}N enriched bacteriochlorophyll a and bacteriochlorophyll b in solution and in solid state: assignment of the ^{13}C, ^{1}H and ^{15}N chemical shifts. Magnetic Resonance in Chemistry. 46: 1074-1083

Elvin DW (1976) Seasonal growth and reproduction of an intertidal sponge, *Haliclona permollis* (Bowerbank). Biol.Bull. 151:108-125

Engels B, Dahm P, Jennewein S (2008) Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. Metabolic Engineering 10:201-206

Fattorusso E, Minale L, Sodano G (1972) Aeroplysinin-I, an Antibacterial Bromocompound from the Sponge *Verongia aerophoba*. JCS Perkin I 16-18.

Faulkner DJ (2002) Marine natural products. Nat.Prod.Rep. 19:1-48

Fell PE (1974) Dipause in the gemmules of the marine sponge *Haliclona loosanoffi*, with a note on the gemmules of *Haliclona oculata*. Biol.Bull. 147:333-351

Frohlich H, Barthel D (1997) Silica uptake of the marine sponge Halichondria panicea in Kiel Bight. Mar.Biol. 128:115-125

Fortman JL, Sherman DH (2005) Utilizing the power of microbial genetics to bridge the gap between the promise and the application of matine natural products. ChemBioChem 6:960-978

Garrabou J, Zabala M (2001) Growth dynamics in four Mediterranean Demosponges. Estuarine, Coastal and Shelf Science 52:293-303

Garson MJ, Flowers AE, Webb RI, Charan RD, McCaffrey EJ (1998) A sponge / dinoflagellate association in the haplosclerid sponge *Haliclona* sp.: cellular origin of cytotoxic alkaloids by Percoll density gradient fractionation. Cell Tissue Res. 293:365-373

Gillor O, Carmeli S, rahamim Y, Fishelson Z, Ilan M (2000) Immunolocalization of the Toxin Latrunculin B within the Red Sea Sponge Negombata magnifica (Demospongiae, Latrunculiidae). Mar.Biotechnol. 2:213-223

Gmelig Meyling AW, de Bruyne RH (2003) Het Duiken Gebruiken 2. Monitoringsproject Onderwater Oever

Goosen NK, van Rijswijk P, Brockmann U (1995) Comparison of heterotrophic bacterial production rates in early spring in the turbid estuaries of the Scheldt and the Elbe. Hydrobiologia, 311: p. 31-42.

Grozdanov L, Hentschel U (2007) An environmental genomics perspective on the diversity and function of marine sponge-associated microbiota. Current Opinion in Microbiology 10:215-220

Hadas E, Shpigel M, Ilan M (2005) Sea ranching of the marine sponge Negombata magnifica (Demospongiae, Latrunculiidae) as a first step for latrunculin B mass production. Aquaculture 244:159-169

Hadas E, Ilan M, Shpigel M (2008) Oxygen consumption by a coral reef sponge. Journal of Experimental Biology 211:2185-2190

Hahn S, Stoilov TB, Ha TBT, Readerstorff D, Doss GA, Li H, Djerassi C (1988) Biosynthetic studies of marine lipids. 17. The course of chain elongation and desaturation in long-chain fatty acids. J.Am.Chem.Soc. 110:8117-8124

Handley S, Kelly S, Kelly M (2003) Non-destructive video image analysis method for measuring growth in sponge farming; preliminary results from the New Zealand bath-sponge *Spongia* (*Heterofibria*) *manipulatus*. New Zealand Journal of Marine and Freshwater Research 37:613-621

Hatcher A (1989) RQ of benthic marine invertebrates. Mar.Biol. 102:445-452

Hellings LF, Dehairs M, Tackx M, Keppens E, Baeyens W (1999) Origin and fate of organic carbon in the freshwater part of the Scheldt Estuary as traced by stable carbon isotope composition. Biogeochemistry 47:167–186

Hentschel U, Hopke J, Horn M, Friedrich AB, Wagner M, Hacker J, Moore BS (2002) Molecular evidence for a uniform microbial community in sponges from different oceans. Appl.Environ.Microbiol. 68:4431-4440

Hill RT 2005 Manzamine-producing actinomycetes. US patent, 20050244938

Holler U, Wright AD, Matthee GF, Konig GM, Draeger S, Aust HJ, Schulz B (2000) Fungi from marine sponges: diversity, biological activity and secondary metabolites. *Mycol. Res*. 104(11):1354-1365

Hooper J, Ekins M, Schlacher-Hoenlinger M, Mather P (2002) Sessile Marine invertebrates.
www.qm.qld.gov.au/organisation/sections/SessileMarineInvertebrates/index.asp

Hooper and van Soest (2002) Systema Porifera

Hoover CA, Slattery M, Marsh AG (2007) A functional approach to transcriptome profiling: linking gene expression patterns to metabolites that matter. Mar.Biotechnol. 9:411-419

Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. The Plant Journal 54:621-639

Hutchinson CR (2003) Polyketide and Non-Ribosomal Peptide Synthases: Falling Together by Coming Apart. Proceedings of the National Academy of Sciences of the United States of America. 100(6):3010-3012

Joh Y, Elenkov IJ, Stefanov KL, Popov SS, Dobson G, Christie WW (1997) Novel Di-, Tri-, and teraenoic fatty acids with *bis*-Methylene-interrupted double-bond systems from the sponge *Haliclona cenerea*. Lipids 32:13-17

Jorgensen CB, Mohlenberg F, Sten-Knudsen O (1986) Nature of relation between ventilation and oxygen consumption in filter feeders. Mar.Ecol.Prog.Ser. 29:73-88

Kaandorp JA (1991) Modelling growth forms of the sponge *Haliclona oculata* (Porifera, Demospongiae) using fractal techniques. Marine Biology 110:203-215

Kaandorp JA (1995) Analysis and synthesis of radiate accretive growth in three dimensions. J.Theor.Biol. 175:39-55

Kaeberlein T, Lewis K, Epstein SS (2006) Isolating "uncultivable" microorganisms in pure culture in a simulated natural environment Science 296(5570):1127-1129

Kennedy J, Marchesi JR, Dobson ADW (2008) Marine metagenomics: strategies for the discovery of novel enzymes with biotechnological applications from marine environments. Microbial Cell Factories 7(27)

Kennedy J, Baker P, Piper C, Cotter PD, Walsh M, Mooij MJ, Bourke MB, Rea MC, O'Connor PM, Ross, RP, Gill C, O'Gara F, Machesi JR, Dobson ADW (2009) Isolation and analysis of bacteria with antimicrobial activities from the marine sponge *Haliclona simulans* collected from Irish waters. Mar Biotechnol 11:384–396

Kharlamenko VI, Kiyashko SI, Rod'kina SA, Imbs AB (2008) Determination of food sources of marine invertebrates from a subtidal sand communtiy using analyses of fatty acids and stable isotopes. Russian Journal of Marine Biology 34:101-109

Kubanek J, Whalen KE, Engel S, Kelly SR , Henkel TP, Fenical W, Pawlik JR (2002) Multiple defensive roles for triterpene glycosides from two Caribbean sponges. Oecologia 131:125-136

Lee KL, Lee JH, Lee HK (2001) Microbial symbiosis in marine sponges. J.Microbiol. 39:254-264

Li Z, He L, MiaoX (2007) Cultivable bacterial community from south China sea sponge as revealed by DGGE fingerprinting and 16S rDNA phylogenetic analysis. Curr.Microbiol. 55:465-472.

Litchfield C, Greenberg AJ, Noto G, Morales RW (1976) Unusually high levels of C24-C30 fatty acids in sponges o f the class demospongiae. Lipids 11:567-570

Litchfield C, Morales RW (1976) Are demospongiae membranes unique among living organisms? Aspects of sponge biology183-200

Lopez-Legentil S, Bontemps-Subielos N, Turon X, Banaigs B (2007) Secondary metabolite and inorganic contents in *Cystodytes* sp. (Ascidiacea): temporal patterns and association with reproduction and growth. Mar.Biol. 151, 293-299.

Lynch TC, Phlips EJ (2000) Filtration of the bloom-forming cyanobacteria *Synechococcus* by three sponge species from florida bay, U.S.A. Bulletin of marine science 67:923-936

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, van den Boogaart JGM, van Leeuwen JL, Wijffels RH (2007) Replumbing in a Mediterranean sponge. Biol.Lett. 3(6):595-598

Mendola D (2008) The importance of water flow for culture of *Dysidea avara* sponges. PhD dissertation. ISBN:978-90-8504-908-1

Migne A, Davoult D (1997) Carbon-dioxide production and metabolic parameters in the ophiurid Ophiothrix fragilis. Mar.Biol. 127:699-704

Minale L, Rissio R, Sodano G (1974) Avarol, a novel sequiternoid hydroquinon skeleton from the sponge *Dysidea avara*. Tetrahedron Lett. 38 :3401-3404

Moco S (2007) Metabolomics technologies applied to the identification of compounds in plants. PhD dissertation. ISBN:978-90-8504-742-1.

Mohlenberg F, Riisgård HU (1978) Efficiency of particle retention in 13 species of suspension feeding bivalves. Ophelia 17:293-246

Molinski TF, Dalisay DS, Lievens SL, Saludes JP (2009) Drug development from marine natural products. Nat.Rev. Drug discovery 8:69-85

Morales RW, Litchfield C (1977) Incorporation of 1-14C-Acetat e into C26 Fatty Acids of the Marine Sponge *Microciona prolifera*. Lipids 12:570-576

Moreira-Turcq PF, Martin JM (1998) Characterisation of fine particles by flow cytometry in estuarine and coastal Arctic waters. Journal of sea research 39:217-226

Muir PR, Sutton DC, Owens L (1991) Nitrate toxicity to *Penaeus monodon* protozoea. Mar.Biol. 108:67-71

Muller WEG, Wiens M, Batel R, Steffen R , Borojevic R, Custodio MR (1999) Establishment of a primary cell culture from a sponge: primmorphs from suberites domuncula. Mar.Ecol.Prog.Ser. 178:205-219

Muller WEG, Zahn RK, Gasic MJ, Dogovi N, Maidhof PA, Becker C, Diehl-Seifert B, Eich E (1985) Avarol, a cytostatically active compound from the marin sponge *Dysidea avara*. Comp. biochem. *Physiol.* 8OC(I):47-52

Munro MHG, Blunt JW, Dumdei EJ, Hickford SJH, Lill RE, Li S, Battershill CN, Duckworth AR (1999) The discovery and development of marine compounds with pharmaceutical potential. J.Biotechnol. 70:15-25

Muscholl-Silberhorn A, Thiel V, Imhoff JF (2007) Abundance and bioactivity of sultured sponge-associated bacteria from the Mediterranean Sea. Microbial Ecol. 55:94-106.

Nieuwenhuize J, Maas YEM, Middelburg JJ (1994) Rapid analysis of organic carbon and nitrogen in particulate materials. Mar Chem 45:217-224

Nechev J, Christie WW, Robaina R, de Diego F, Popov S, Stefanov K (2002) Lipid composition of the sponge *verongida aerophoba* from the Canary Islands. Eur.J.Lipid Sci.Technol. 104:800-807

Newman DJ, Cragg GM (2004) Marine natural prodcuts and related compounds in clinical and advanced preclinical trials. J.Nat.Prod. 67:1216-1238

Newman DJ, Hill RT (2006) New drugs from marine microbes: the tide is turning. J.Ind.Microbiol.Biotechnol. 33:539-544

Osinga R, Tramper J, Wijffels RH (1999) Cultivation of marine sponges. Mar.Biotechnol. 1:509-532

Osinga R, Belarbi EH, Molina Grima E, Tramper J, Wijffels RH (2003) Progress towards a controlled culture of the marine sponge *Pseadosuberites andrewsi* in a bioreactor. J.Biotechnol. 100:141-146

Page MJ, Northcote PT, Webb VL, Mackey S, Handley SJ (2005[a]) Aquaculture trials for the production of biologically active metabolites in the New Zealand sponge *Mycale hentscheli* (Demospongiae: Poecilosclerida). Aquaculture 250:256-269

Page MJ, West L, Northcote PT, Battershill CN, Kelly M (2005[b]) Spatial and temporal variability of cytotoxic metabolites in populations of the New Zealand sponge *Mycale hentscheli*. J.Chem.Ecol. 31:1161-1174

Paul VJ, Puglisi MP (2004) Chemical mediation of interactions among marine organisms. Nat.Prod.Rep. 21:189-209

Pawlik JR, Chanas B, Toonen RJ, Fenical W (1995) Defenses of Caribean sponges against predatory reef fish. I. Chemical deterrence. Mar.Ecol.Prog.Ser. 127:183-194

Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Ann.Rev.Ecol.Syst. 18:293-320

Piel J (2004) Metabolites from symbiotic bacteria. Nat.Prod.Rep. 21:519-538

Piel J, Hui D, Wen G, Butzke D, Platzer M, Fusetani N, Matsunaga S (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. PNAS 101:16222-16227

Piel J (2006) Bacterial symbionts: Prospects for the sustainable production of invertebrate-derived pharmaceuticals. Current Medicinal Chemistry 13:39-50

Pile AJ, Patterson MR, Savarese M, Chernykh VI, Fialkov VA (1997) Trophic effects of sponge feeding within Lake Baikal's littoral zone. 2. Sponge abundance, diet, feeding efficiency, and carbon flux. Limnol.Oceanogr. 42:178-184

Pomponi SA, Willoughby R (1994) Sponge cell culture for the production of bioactive metabolites. In Van Soest R, Van Kempen TMG, Braekman JC (Sponges in time and space), 395-400. Rotterdam, Balkema.

Pomponi SA, Willoughby R, Kaighn ME, Wright AE (1997) Development of techniques for in vitro production of bioactive natural products from marine sponges. In. Maramorosch, K. and Mitsuhashi, J. Novel directions and biotechnology applications, 231-237. U.S.A., Science Publishers. Invertebrate cell culture.

Poulton JE (1988) Localization and catabolism of cyanogenic glycosides. In: Evered D, Harnett S (eds). Cyanide compounds in biology. John Wiley & Sons, Chichester 67-91

Proksch P (1994) Defensive roles for secondary metabolites from marine sponges and sponge-feeding nudibranchs. Toxicon 32:639-655

Proksch P, Edrada RA, Ebel R (2002) Drugs from the seas - current status and microbiological implications. Appl.Microbiol.Biotechnol. 59:125-134

Raederstorff D, Shu AYL, Thompson JE, Djerassi C (1987) Biosyntethic studies of marine lipids. 11[1] Synthesis, biosynthesis, and absolute configuration of the internally branched demospongic acid 22-Methyl-5,9-octacosadienoic acid. J.Org.Chem. 52:2337-2346

Reiswig HW (1971) Particle feeding in natural populations of three marine demosponges. Biol.Bull. 141:568-591

Reiswig HW (1973) Population dynamics of three jamaican demospongiae. Bulletin of marine science 23:191-226

Reiswig HW (1974) Water transport, respiration and energetics of three tropical marine sponges. J.Exp.Mar.Biol.Ecol. 14:231-249

Ribes M (1998) Feeding activity and diet of benthic suspension feeders related to metabolic requirements and seston composition. PhD dissertation, University of Barcelona, Barcelona, Spain.

Ribes M, Coma R, Gili J-M (1999) Natural diet and grazing rate of the temperate sponge Dysidea avara (Demospongiae, Dendroceratida) throughout an annual cycle. Mar.Ecol.Prog.Ser. 176:179-190

Richardson ML, Gangolli S (1993) The dictionary of substances and their effects. Royal society of chemistry. Cambridge. p277.

Richelle-Maurer E, Gomez R, Braekman JC, Van de Vyver G, Soest RVM, Devijver C (2003) Primary cultures from the marine sponge *Xestospongia muta* (Petrosiidae, Haposclerida). J.Biotechnol. 100:169-176

Richmond A (2004) Handbook of microalgal culture. Biotechnology and Applied Phycology. ISBN 0-632-05953-2.

Riisgård HU, Thomassen S, Jakobsen H, Weeks JM, Larsen PS (1993) Suspension feeding in marine sponges *Halichondria panicea* and *Haliclona urceolus*: effects of temperature on filtration rate and energy cost of pumping. Mar.Ecol.Prog.Ser. 96:177-188

Ro DK, Ouellet M, Paradise EM, Burd H, Eng D, Paddon CJ, Newman JD, Keasling JD (2008) Induction of multiple pleiotropic drug resistance genes in yeast engineered to produce an increased level of anti-malarial drug precursor, artemisinic acid. BMC Biotechnology 8:

Rod'kina SA, Latyshev NA, Imbs AB (2003) Fatty Acids from the Sea of Japan Sponge *Halichondria panicea*. Russian Journal of Bioorganic Chemistry 29:382-386

Rod'kina SA (2005) Fatty acids and other lipids of Marine sponges. Russian Journal of Marine Biology 31:S49-S60

Roels JA (1983) Energetics and kinetics in biotechnology. Elsevier Biomedical press Amsterdam.

Ruzicka R, Gleason DF (2008) Latitudinal variation in spongivorous fishes and the effectiveness of sponge chemical defenses. Oecologia 154:785-794

Sakai R, Yoshida K, Kimura A, Koike K, Jimbo M, Koike K, Kobiyama A, Kamiya H (2008) Cellular origin of dysiherbaine, an excitory amino acid derived from a marine sponge. ChemBioChem 9:543-551

Sara M, Bavestrello G, Cattanea-vietti R, Cerrano C (1998) Endosymbiosis in sponges: Relevance for epigenesist and evolution. Symbiosis 25(1-3):57-70

Saunders JA, Conn EE, Lin CH, Stocking CR (1977) Subcellular Localization of the Cyanogenic Glucoside of Sorghum by Autoradiography. Plant physiology 59:647-652

Schmidt I (1970) Phagoeytosis and Pinocytosis in Spongillids. Z.vergl.Physiologie 66:398-420

Schupp P, Eder C, Pail C, Proksch P (1999) Distribition of secondary metabolites in the sponge *Oceanapia* sp. and its ecological implications. Mar. Biol. 135:573-580

Simpson TL (1984) The cell biology of sponges. Springer-Verlag, New York. P. 517-523.

Sipkema D, Van Wielink R, Van Lammeren AAM, Tramper J, Osinga R, Wijffels RH (2003) Primmorphs from seven marine sponges: formation and structure. J.Biotechnol. 100:127-139

Sipkema D, Franssen MCR, Osinga R, Tramper J, Wijffels RH (2005[a]) Marine sponges as pharmacy. Mar.Biotechnol. 7:142-162

Sipkema D, Osinga R, Schatton W, Mendola D, Tramper J, Wijffels RH (2005[b]) Large-scale production of pharmaceuticals by marine sponges: Sea, cell, or synthesis? Biotechnology and Bioengineering 90:201-222

Sipkema D, Yosef NAM, Adamczewski M, Osinga R, Mendola D, Tramper J, Wijffels RH (2005[c]) Hypothesized Kinetic Models for Describing the Growth of Globular and Encrusting Demosponges. Mar.Biotechnol. 8:40-51

Stabili L, Licciano M, Giangrande A, Longo C, Mercurio M, Marcano CN, Corriero G (2006) Filtering activity of Spongia officinalis var. adriatica (Schmidt) (Porifera, Demospongiae) on bacterioplankton: Implications for bioremediation of polluted seawater. Water Research 40:3083-3090

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: Evolution, Ecology and Biotechnological potential. Microbiol.Mol.Biol.Rev. 71:295-347

Teeyapant R, Woerdenbag HJ, Kreis P, Hacker J, Wray V, Witte L, Proksch P (1993) Antibiotic and cytotoxic activity of brominated compounds from the marine sponge *verongia aerophoba*. Z.Naturforsch 48c:939-945

Thacker RW, Becerro MA, Lumbang WA, Paul VJ (1998) Allelopathic interaction between sponges on a tropical reef. Ecology 79:1740-1750

Thiel V, Jenisch A, Wörheide G, Löwenberg A, Reitner J, Mechaelis W (1999) Mid-chain branched alkanoic acids from “living fossil” demosponges: a link to ancient sedimentary lipids? Organic Geochemistry 30:1-14

Thomassen S, Riisgård HU (1995) Growth and energetics of the sponge Halichondria panicea. Mar.Ecol.Prog.Ser. 128:239-246

Thompson JE, Barrow KD, Faulkner DJ (1983) Localization of two brominated metabolites, aerothionen and homoaerothionin, in spherulous cells of the marine sponge *Aplysina fistularis*. Acto Zool Stockh 64:199-210

Thompson JE, Murphy PT, Bergquist PR, Evans EA (1987) Environmetally induced variation in diterpene composition of the marine sponge *Rhopaloeides odorabile*. Biochem.Syst.Ecol. 15:595-606

Thoms C, Wolff M, Padmakumar K, Ebel R, Proksch P (2004) Chemical defense of Mediterranean sponges *Aplysina cavernicola* and *Aplysina aerophoba*. Z.Naturforsch 59c:113-122

Thoms C, Ebel R, Proksch P (2006) Activated chemical defense in *Aplysina* sponges revisited. J.Chem.Ecol. 32:97-123

Thoms C, Schupp PJ (2007) Chemical defense strategies in sponge: a review. In. Custodio MR, Lobo-Hajdu G, Hajdu E, Muricy G. Porifera research – Biodiversity, Innovation & Sustainability p627-637

Thoms C, Schupp PJ (2008) Activated chemical defense in marine sponges - a case study on *Aplysinella rhax*. J.Chem.Ecol. 34:1242-1252

Thornton RG, Kerr RG (2002) Induction of pseuopterosin biosynthesis in the gorgonian *Pseudopterogorgia elisabethae*. J. Chem. Ecol.,28(10):2083-2090

Thurber A (2007) Diets of antartic sponges: links between the pelagic microbial loop and benthic metazoan food web. Mar.Ecol.Prog.Ser. 351:77-89

Turon X, Becerro MA, Uriz MJ, Llopis J (1996) Small-scale association measures in epibenthic communities as a clue for allelochemical interactions. Oecologia 108:351-360

Unson MD, Faulkner DJ (1993) Cyanobacterial symbiont biosynthesis of chlorinated metabolites from Dysidea herbacea (Porifera). Experientia 49:349-353

Uriz MJ, Rosell D, Martin D (1992) The sponge population of the Cabrera archipelago (Balearic islands): characteristics, distribution, and abundance of the most representative species. Marine Ecology 13:101-117

Uriz MJ, Turon X, Galera J, Tur JM (1996) New light on the cell location of avarol within the sponge Dysidea avara (Dendroceratida). Cell Tissue Res. 285:519-527

Uriz MJ, Turon X, Becerro MA, Galera J (1996) Feeding deterrence in sponges. The role of toxicity, physical defenses, energetic contents, and life-history stage. J.Exp.Mar.Biol.Ecol. 205:187-204

Walters KD, Pawlik JR (2005) Is there a trade-off between wound-healing and chemical defenses among caribbean reef sponges? Integr.Comp.Biol. 45:352-358

Wapstra M, Van Soest RWM (1987) Sexual reproduction, larval morphology and behaviour in demosponges from the southwest of the Netherlands. NATO ASI Series G13:281-307

Webster NS, Xavier JR, Freckelton M, Motti CA, Cobb R (2008) Shifts in microbial and chemical patterns within the marine sponge *Aplysina aerophoba* during a disease outbreak. Environ.Microbiol. 10:3366-3376

Wijffels RH (2008) Potential of sponges and microalgae for marine biotechnology. Trends in biotechnology 26:26-31

Wilson HV (1907) On some phenomena of coalescence and regeneration in sponges. J.Exp.Zool. 5:245-258

Wulff JL (1991) Asexual fragmentation, genotype success, and population dynamics of erect branching sponges. Journal of experimental biology and ecology, 149: p. 227-247

Wulff JL (2006) Ecological interactions of marine sponges. Can J Zool 84: 146-166.

Yahel G, Sharp JH, Marie D, Hase C, 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

Summary

Sponges (phylum Porifera) are multi cellular filter-feeding invertebrate animals living attached to a substratum in mostly marine but also in freshwater habitats. The interest in sponges has increased rapidly since the discovery of potential new pharmaceutical compounds produced by many sponges. An enormous amount of different chemical structures have been found. Thus far no sustainable production technique has been developed for these marine natural products, because not sufficient knowledge is present about the needs of sponges for both growth and bioactive compound production. The aim of this thesis was to get a better understanding of the growth and metabolism of sponges and of their nutritional needs.

Aquaculture is thus far the best method to produce these compounds, although also this technique is not fully developed. To gain more insight in the nutritional needs for growth, we studied the growth rate of *Haliclona oculata* in its natural environment, Oosterschelde, the Netherlands, and monitored environmental parameters in parallel (**Chapter 2**). A stereo photogrammetry approach was used for measuring growth rates. Stereo pictures were taken and used to measure volumetric changes monthly during 1 year. The volumetric growth rate of *Haliclona oculata* showed a seasonal trend with the highest average specific growth rate measured in May: 0.012 ± 0.004 day^{-1}. In our study a strong positive correlation ($p<0.01$) was found for growth rate with temperature, algal biomass (measured as chlorophyll a), and carbon and nitrogen content in suspended particulate matter. Thus growth rate seems to be dependent on these factors. No correlation was found with dissolved organic carbon, suggesting that *Haliclona oculata* is more dependent on particulate organic carbon.

To obtain more knowledge about the carbon requirements for growth by sponges, respiration rate and clearance rate were measured *in situ* in *Haliclona oculata* and compared to the earlier measured growth rate (**Chapter 3**). The net growth efficiency, being the ratio of carbon incorporated in biomass and the total carbon used by the sponge for respiration and growth, was found to be 0.10 ± 0.013. Thus, about 10% of the total used carbon was fixed in biomass and over 90% was used for generating energy for growth, maintenance, reproduction and pumping. *H. oculata* had 2.5 µmol C available for every µmol O_2 consumed. A value of 0.75 for the respiratory quotient (RQ in µmol CO_2 µmol O_2^{-1}) is the average value reported in literature for different marine invertebrates. Thus, carbon was available in excess to meet the respiratory demand. We found that only 34% of the particulate carbon pumped through the sponge was used for both respiration and growth. Oxygen was not the limiting factor for growth, since only 3.3% of the oxygen pumped through the sponge body

was used. Our results indicate that both oxygen and carbon availability are not limiting. The low growth efficiency agrees with the low growth rates found for many sponges.

In order to produce drugs by culturing sponges their growth must be improved. To improve growth, basic knowledge about how food sources are used by the sponge is needed. To find the exact relation between food retained and food converted to sponge biomass we need to be able to distinguish between feed components and sponge biomass, which means we need biomarkers for the feed and for the sponge. The fatty acid (FA) composition of organisms is specific and can therefore be used as biomarkers. We identified and compared fatty acid profiles of five different sponges in three habitats with those in the suspended particulate matter (SPM) in the surrounding water (**Chapter 4**). *Haliclona oculata* and *Haliclona xena* from the Oosterschelde, *Haliclona xena* and *Halichondria panicea* from Lake Veere, both in The Netherlands and *Dysidea avara* and *Aplysina aerophoba* from the Mediterranean were studied. In the SPM we found comparable FAs to the FAs of sponges up to chain lengths of 28 C-atoms. Different species of sponges showed similarities, but also very different FA profiles, while they were collected from the same habitat at the same moment. The biomarkers for diatoms and dinoflagellates were abundantly found in all sponges except *A. aerophoba* as this sponge relies mostly on bacterial food sources based on the many bacterial FAs found in this sponge. In all species, except *A. aerophoba,* C26:3(5,9,19) and C26:2(5,9) were very abundantly present. These FAs were also abundant in the SPM, while it was stated in literature that these compounds are very typical for sponges. Several FA biomarkers were found for the different sponges.

Fatty acid composition is dependent on different factors like food availability and temperature and thus the composition will change in the different seasons. We have studied fatty acid composition and stable isotope ^{13}C natural abundance of suspended particulate matter (SPM) from seawater and sponges in different seasons in the same locations as in chapter 4 (**Chapter 5**). ^{13}C natural abundance can be used to find the origin of compounds, as the ^{13}C values of compounds are similar to the values from their original producers. The FA concentration variation in sponges was related to changes in fatty acid concentration in SPM. ^{13}C natural abundance in sponge specific FAs showed very limited seasonal variation at all sites. Algal FAs in sponges were mainly acquired from the SPM through active filtration in all seasons. Sponge specific FAs had similar ^{13}C ratios as algal FAs in May at the two Dutch sites, suggesting that sponges were mainly growing during spring and probably summer.

During autumn and winter, they were still actively filtering, but the food collected during this period had little effect on sponge ^{13}C values suggesting limited growth. The bacterial sponge *A. aerophoba* relies mostly on the symbiotic bacteria. In all sponges we found that the ω7 longer chain FAs, C24:1(17) and C26:3(5,9,19) could be traced back to be of bacterial origin.

Using a ^{13}C pulse-chase approach metabolic rate can be studied inside organisms. The carbon metabolism of two marine sponges, *Haliclona oculata* from the Oosterschelde (The Netherlands) and *Dysidea avara* from the Mediterranean (Spain), has been studied (**Chapter 6**). The sponges were fed ^{13}C labeled diatom (*Skeletonema costatum)* for 8 hours in a closed system during which they took up between 75 and 85 % of the diatoms added. At different times whole sponges were sampled for total ^{13}C enrichment, fatty acid composition and ^{13}C enrichment in these fatty acids. During the first day the level of ^{13}C label inside the sponges stayed the same after which the ^{13}C label was metabolized and excreted. Algal biomarkers present in the sponges were highly labeled after feeding and their labeling levels decreased from the second day until no label was left 10 days after enrichment. The sponge specific long chain C26 fatty acids incorporated ^{13}C label already during the first day and the amount of ^{13}C label inside these FAs kept increasing until 3 weeks after labeling. Thus, the algae fed to the sponges were taken up by the sponges within 8 hrs and first conversion started during the first day. Conversion of label occurred at least until at least 3 weeks after feeding.

In different studies it was shown that sponges grow slow, but are able to regenerate damaged tissue fast. Moreover, it has been found that damaged tissue coincides with higher secondary metabolite production. Therefore, we were interested in carbon metabolic rate changes after damaging sponge tissue. We have examined the change of carbon metabolic rate of fatty acid synthesis due to mechanical damage of sponge tissue in *Haliclona oculata* and *Dysidea avara* (**Chapter 7**). Metabolic studies were performed by feeding sponges with ^{13}C labeled biomass of diatom, *Pheaodactylum tricornutum*, either after or before damaging and tracing back the ^{13}C content in the damaged and healthy tissue. Filtration and respiration rate in both sponges responded quickly to damage. For the finger-sponge *H. oculata* the rate of respiration was reduced immediately after damage. 6 Hours after damage the filtration rate increased to a level that was higher than the starting value, while the respiration rate returned to the initial value before damage. For the encrusting sponge *D. avara* the filtration rate also decreased directly after damage, but in this case it did not return to the value before damage after one day. Respiration was not

measured for *D. avara*. The ^{13}C data revealed that *H. oculata* has a higher metabolic rate in the tips where growth occurs compared to the rest of the tissue and that the metabolic rate is increased after damage of the tissue. For *D. avara* no differences were found between damaged and non damaged tissue.

Thus far it is still not fully understood why, when, where and how bioactive metabolites are produced in sponges. For the near future sea-based sponge culture seems to be the best production method. However, for controlled production in a defined system it is better to develop *in vitro* production methods. This could be in vitro sponge culture or sponge cell culture, culture methods for symbionts or transfer production routes into another host. We still have insufficient information about the background of metabolite production in sponges. Before culture methods are developed we should focus on factors that induce metabolite production, which could be done in the natural habitat by studying the relation between stress factors (such as predation) and the production of bioactive metabolites. Next, the biosynthetic pathway of metabolite production should be unraveled, as well as the genes involved. The location of production within the sponge should be identified in order to choose between sponge cell culture and symbiont culture. Alternatively the biosynthetic pathways could be introduced into hosts that can be easily cultured in bioreactors. **Chapter 8** discusses the current state of sponge metabolite production and the steps that need to be taken to develop commercial production techniques. The different possible production techniques are also discussed.

Samenvatting

Sponzen (Stam Porifera) zijn meercellige filtrerende invertebraten die groeien aan een vast substraat in veelal mariene maar ook in zoetwater habitats. Sponzen hebben veel meer aandacht gekregen sinds de ontdekking van potentiële nieuwe farmaceutische stoffen geproduceerd door vele verschillende sponzen. Een enorme hoeveelheid verschillende chemische structuren zijn ontdekt in verschillende sponzen. Voor de productie van deze natuurlijke producten is tot nu toe nog geen duurzame productie techniek ontwikkeld, omdat er onvoldoende kennis aanwezig is over de behoeftes van sponzen voor zowel groei als productie van de bioactieve stoffen. De doelstelling van het onderzoek beschreven in dit proefschrift is meer kennis te krijgen van de groei en het metabolisme in sponzen en de voeding die sponsen nodig hebben om te groeien.

Aquacultuur is tot nu toe nog de beste optie voor spons cultuur, hoewel deze techniek ook nog niet volledig ontwikkeld is. Om meer inzicht te krijgen in de voeding en groei van sponzen hebben we groeisnelheden gemeten in de natuur van de spons *Haliclona oculata* die leeft in de Oosterschelde, Nederland. Gelijktijdig hebben we parameters gemeten zoals temperatuur en voedingswaarden in de Oosterschelde (**Hoofdstuk 2**). We hebben gebruik gemaakt van stereo fotografie om volumes te kunnen bepalen van de spons individuen. Gedurende 1 jaar zijn er elke maand stereo foto's gemaakt aan de hand waarvan de groeisnelheid is bepaald. De gevonden groeisnelheid bleek een seizoenstrend te laten zien met de hoogste groeisnelheid in mei: 0.012 ± 0.004 per dag. Een sterke positieve correlatie bleek te bestaan tussen groeisnelheid en temperatuur en de algen biomassa, koolstof en stikstof concentratie in de deeltjes in het water. Dus groei blijkt afhankelijk te zijn van deze factoren. Voor opgeloste koolstof werd geen correlatie gevonden met groeisnelheid, waaruit blijkt dat deze spons meer gebruik maakt van koolstof uit deeltjes zoals algen dan uit opgelost koolstof.

Om de koolstof behoeften van de spons *Haliclona oculata* voor groei beter te begrijpen hebben we de respiratie (ademhaling) en filtratie (deeltjes opname) snelheid bepaald en vergeleken met de eerder gemeten groeisnelheid (**Hoofdstuk 3**). De groei efficiëntie (groei / (groei +respiratie)) was 0.10 ± 0.013. Dus ongeveer 10% van al het gebruikte koolstof was opgeslagen als biomassa en 90% werd gebruikt voor respiratie om energie te genereren voor zowel groei als onderhoud, als ook voor filteren en voortplanting. *H. oculata* had 2.5 µmol C (koolstof) beschikbaar per µmol O_2 (zuurstof). Aangezien hij maar 0.75 mol C nodig heeft, is dit dus ruim voldoende koolstof om aan de respiratie behoefte te voldoen. Maar 34% van de koolstof in de deeltjes die door de spons was gepompt werd gebruikt voor respiratie en groei. Ook werk

maar 3.3% van de beschikbare zuurstof opgenomen door de spons. Dus zowel koolstof als zuurstof is niet limiterend. De lage efficiënties komen overeen met de lage groeisnelheden die gevonden zijn voor vele sponzen.

Om voldoende spons biomassa voor medicijn productie te krijgen is het nodig om de groei te kunnen stimuleren. Om dit te kunnen doen is het nodig beter te begrijpen hoe de sponzen hun opgenomen voeding gebruiken en omzetten. Aangezien sponzen continu deeltjes filteren uit het water zijn deze deeltjes ook nog intact aanwezig in een spons wanneer sponzen bemonsterd worden. Om toch onderscheid te kunnen maken tussen voedingsdeeltjes en spons biomassa is het nodig om specifieke componenten te vinden voor zowel de voeding als de spons. Deze specifieke componenten worden biomarkers genoemd. De vetzuur samenstelling in organismen is heel divers en blijkt uniek te zijn voor verschillende organismen. Wij hebben gezocht naar vetzuur biomarkers door de vetzuursamenstelling van verschillende sponssoorten uit verschillende omgevingen te vergelijken met de bijbehorende vetzuur samenstelling in de deeltjes in het water (**Hoofdstuk 4**). *Haliclona oculata* en *Haliclona xena* uit de Oosterschelde, Nederland, *Haliclona xena* en *Halichondria panicea* uit het Veerse Meer, Nederland, en *Aplysina aerophoba* en *Dysidea avara* uit de Middellandse Zee, Spanje zijn bestudeerd. Zowel in de deeltjes als in de sponzen zijn dezelfde vetzuren gevonden. Dit was zelfs het geval voor de lange ketens (C28) welke als spons specifiek werden beschouwd. Maar er zijn ook unieke vetzuren gevonden in de verschillende sponzen. *Aplysina aerophoba* bevat veel bacteriën die in symbiose leven en dit geeft dan ook een hele andere vetzuursamenstelling. Verschillende biomarkers voor verschillende algengroepen in het water werden in overvloed gevonden in alle sponzen behalve *Aplysina aerophoba*. Dit betekend dat *A. aerophoba* meest afhankelijk is van een bacteriële voedingsbron en dat de andere sponzen gebruik maken van de algen uit het water als voedingsbron. Voor alle sponzen zijn een aantal specifieke vetzuren gevonden die gebruikt kunnen worden als biomarker.

Verschillende factoren hebben invloed op de vetzuursamenstelling zoals beschikbare voedingsbronnen en temperatuur. Dus zal de vetzuursamenstelling afhankelijk zijn van het seizoen. We hebben de vetzuur samenstelling en abundantie van de stabiele isotoop ^{13}C gemeten in verschillende seizoenen van de deeltjes en sponzen uit dezelfde locaties als in hoofdstuk 4 (**Hoofdstuk 5**). ^{13}C natuurlijke abundantie kan gebruikt worden om de oorsprong van componenten in een organisme te bepalen, want de ^{13}C waarden van bepaalde componenten komen overeen met de ^{13}C waarden van de originele producent van deze component. De variatie van vetzuur concentratie in de sponzen kwam

overeen met de variatie in de vetzuurconcentratie in de deeltjes. ^{13}C waarden in sponsen gaven weinig seizoensvariatie. Algen vetzuren werden veelal verkregen door continue filtratie van deeltjes het hele jaar door. Sponzen vetzuren hadden dezelfde ^{13}C waarden als algen vetzuren in mei, wat erop duidt dat de sponzen in deze tijd harder groeiden. De bacteriële spons *Aplysina aerophoba* is voornamelijk afhankelijk van de bacteriën in de spons. In alle sponzen konden de lange sponzen vetzuren C24:1(17) en C26:3(5,9,19) terug gekoppeld worden aan bacteriële herkomst.

Met behulp van ^{13}C gelabelde voedingsbronnen kan de metabole snelheid in een organisme bepaald worden. In **hoofdstuk 6** is het koolstof metabolisme in vetzuren beschreven in de sponzen *Haliclona oculata* (Oosterschelde, Nederland) en *Dysidea avara* (Middellandse Zee, Spanje). De sponzen zijn gevoerd met ^{13}C gelabelde diatomeeën (*Skeletonema costatum*) gedurende 8 uur waarbij tussen de 75 en 85% van de diatomeeën is opgenomen. Op verschillende tijdstippen zijn hele sponzen bemonsterd, waarvan de totale ^{13}C en de ^{13}C in vetzuren gemeten is. Tijdens de eerste dag bleef de hoeveelheid ^{13}C label in de spons constant waarna de label gemetaboliseerd en uitgescheiden werd. Algen biomarkers in de spons waren zeer hoog gelabeld na het voeden, maar deze hoeveelheid begon vanaf de tweede dag af te nemen en er was geen ^{13}C label meer aanwezig in algen biomarkers na 10 dagen. De sponzen biomarkers, C26 vetzuren bevatten al ^{13}C label op de eerste dag en de hoeveelheid label bleef toenemen tot 3 weken na voeden. Dus de gevoede diatomeeën zijn opgenomen door de spons binnen 8 uur, waarbij de eerste omzetting al begon tijdens de eerste dag en omzetting bleef doorgaan tot minimaal 3 weken na voeding.

In verschillende studies is aangetoond dat groei van sponzen erg langzaam is, in diezelfde sponzen is ook geconstateerd dat beschadigd sponzen tissue veel sneller kan regenereren dan de spons kan groeien. Bovendien is gevonden dat beschadigd weefsel verhoging van bioactieve stoffen in de spons met zich meebracht. Hierdoor raakten we geïnteresseerd in de metabole snelheid van beschadigd weefsel. In dezelfde twee sponzen als in hoofdstuk 6 hebben we verandering van de metabole snelheid van vetzuur synthese gemeten na beschadiging (**Hoofdstuk 7**). Ook hier is gebruik gemaakt van ^{13}C gelabelde diatomeeën, alleen nu *Pheaodactylum tricornutum*. Voeding werd gegeven zowel voor als na beschadiging om het effect op filtratie en respiratie te zien. Beide sponssoorten reageerden snel op de beschadiging. De vingerspons *H. oculata* verlaagde de respiratie snelheid meteen na de beschadiging en 6 uur erna was deze snelheid volledig hersteld naar de originele waarde. Filtratie snelheid was verhoogd 6 uur na beschadiging. De korstspons *D. avara* verlaag-

de onmiddellijk de filtratie snelheid na beschadigen, welke nog niet volledig hersteld was na 1 dag. De ^{13}C data lieten zien dat *H. oculata* een hogere metabole snelheid had in de uiteinden vergeleken met de rest van de spons en na beschadiging was deze snelheid verder verhoogd. *D. avara* liet geen verschillen zien tussen beschadigd en onbeschadigd weefsel aangezien erg variabele data verkregen werden.

Eigenlijk begrijpen we nog steeds niet goed waarom, wanneer, waar, en hoe de bioactieve componenten geproduceerd worden. Voor de nabije toekomst is kweek van sponzen in de natuur nog de beste optie. Een gecontroleerd systeem voor de productie zou echter veel beter zijn en dus zijn de kweek van sponsen in laboratoria, of nog beter spons cel cultuur, symbiont cultuur of genetisch gemodificeerde micro-organismen de methoden die verder ontwikkeld worden. De metaboliet productie in de spons is nog steeds niet volledig begrepen en voordat er een productie methode gekozen kan worden dient bekend te zijn hoe de productie in de spons geïnduceerd wordt. Dit kan in de natuur bestudeerd worden, aangezien stress factoren zoals predatie waarschijnlijk leiden tot een hogere productie van secundaire metabolieten. Ook de biosynthese route van de metabolieten inclusief de betrokken genen zal opgehelderd moeten worden. Om te kunnen kiezen tussen spons cel cultuur of symbiont cultuur dient bekent te zijn waar de productie in de spons plaats vindt. In **Hoofdstuk 8** wordt de huidige status bediscussieerd van metaboliet productie in sponsen en de stappen die nog genomen dienen te worden om een commerciële productie methode te kunnen realiseren. Ook worden de verschillende productie methoden besproken.

Dankwoord

Uiteraard heb ik dit werk niet in mijn eentje kunnen doen, gelukkig is dat ook niet nodig geweest, vandaar dat ik hier mijn welgemeende dank wil betuigen aan alle mensen die mij geholpen hebben in de afgelopen 4 jaar.

Rene, zonder jouw inzet, toewijding, interesse en vertrouwen had ik nooit in de buurt kunnen komen van een ingevuld proefschrift. Onze dagjes naar het strand om te duiken waren altijd leuk en nuttig, weer of geen weer. Het was erg fijn om voor, maar vooral met, je te werken. Om na anderhalf jaar als extra begeleider bij een project komen is in het begin misschien een beetje onwennig, maar ***Dirk***, dankzij jouw kritische blik en focus kon je mijn chaotische manier van werken in goede banen leiden. Ik heb het als zeer waardevol ervaren hoe je elk hoofdstuk hebt opgekrikt naar logische en duidelijke verhalen.

Het *rek*, de basis voor bijna alles in dit proefschrift, is met een heel team te water gelaten en in een tweede poging nog eens recht gezet. Zonder de duikervaring en kunde van ***Jan Lindenberg***, ***Berry van Vark***, ***Ella van Vark*** en ***Adrienne Bal***, was hij nooit op zijn plek gekomen. Daarnaast hebben ***Annette***, ***Packo*** en ***Fam*** ofwel boven ofwel onder water (allen net zo nat) nog een bijzonder nuttige bijdrage geleverd. Voordat het rek te water kon, is het eerst in elkaar gezet in de werkplaats; ***Hans, Hans, Reinout, Jan en Hennie*** bedankt dat jullie altijd voor mij klaarstonden om mee te denken hoe bijvoorbeeld 3d-fotografie, filtratie of zuurstofmetingen ook weer onderwater mogelijk konden worden.

Het grootste deel wat ik geschreven heb heeft zijn roots in het prachtige Zeeland, voornamelijk uit de Oosterschelde, en de verdere analyses zijn veelal in Yerseke gedaan. ***Pieter van Rijswijk*** en ***Marco Houtekamer*** hebben mij erg veel geholpen en bijgestaan in het lab en bij de data-analyse van de ontelbare hoeveelheden vetzuren die in sponzen voorkomen. ***Leon Moodley***, dank voor het in contact brengen met ***Jack Middelburg*** zodat ik gebruik heb kunnen maken van jullie extractie en analyse methodes en laboratoria. Jack, dank voor de gastvrijheid.

Gelukkig was ik niet helemaal alleen in de sponzenwereld bij proceskunde. Luckily I wasn't all alone in the sponge world of Bioprocess Engineering. ***Sonia***, although you worked in Spain I always felt close to you, thanks for your friendship and help with all the diving in Cala Montgó! ***Dominick*** came quite fast after my arrival, and left way before me with his diploma, DM, thank you very

much for your interest en positiveness both professionally and socially, I learned a lot from you. ***Marzia***, as a spongemate, a travelmate and a roommate, it was always nice with you, thanks for calming me down before my presentation in Brasil. ***André***, ***Robert*** and ***Vicky***, you all dared to do a thesis with sponges, were all very motivated and always stayed positive, thanks for that and your interest and input in my work.

Aio-kamers zijn niet echt ruim opgezet en een kleine ruimte heb ik dan ook met 3 andere mensen gedeeld, gelukkig ben ik altijd erg goed bedeeld geweest met mijn kamergenoten. Allereerst de harde kern ***Koen*** en ***Daniël***, met jullie heb ik in mijn aiotijd lief en leed gedeeld. Ik heb erg veel gelachen en alles was bespreekbaar, bedankt voor de gezellige tijd. ***Martijn*** en later ***Martijntje*** jullie vulden ons goed aan en wisselden elkaar goed af, jammer genoeg stond de telefoon wat ver weg voor jullie waardoor ik nogal eens verstrikt in draden kon raken ;-).

Bij proceskunde heb ik me altijd erg thuis gevoeld omdat de sfeer er zo goed is. Vele mensen zijn hiervoor verantwoordelijk en daarvoor wil ik iedereen bij proceskunde dan ook hartelijk bedanken. En een paar mensen specifiek. ***Fred***, jij bent altijd ontzettend behulpzaam, bedankt voor de verzorging van het aquarium en het up to date houden van de temperatuur. ***Sebastiaan***, nog zo'n behulpzaam type, dank voor de hulp met de oxi-meters en het uitleesprogramma op de laptop, ik heb er erg veel gebruikt van gemaakt. De rumoerige ***Rouke***, ook jij bent altijd benaderbaar voor hulp op het lab of iets dergelijks, dank voor je dynamiek! ***Dorinde***, het enthousiasme zelve, dankjewel daarvoor, al ken ik ook je minder enthousiaste kant zodra er weer hardgelopen 'moest' worden, hoe moet ik dat nou volhouden zonder jou? ***Annette***, jij fungeerde meerdere malen als invaller voor Rene, het is fijn duiken met je! ***Gerrit***, altijd gezellig met jou, bedankt voor je hulp bij al mijn computervragen. ***Martin***, je hebt nog een poging gedaan om de niet al te mooie onderwaterfoto's te analyseren, dit bleek jammer genoeg onmogelijk. En uiteraard zijn er velen meer die de koffiepauzes en lunches tot een gedenkwaardig geheel brachten, ik koester mijn tijd bij proceskunde, THANKS. ***Floor***, jij hoort ook bij proceskunde, al werk je bij A&F, je deed altijd je best om aanwezig te zijn bij borrels en uitjes. Voor mij ben je toch vooral de mede-vakantie/reis-genoot; Egypte, Spanje, Honduras, Japan, Denemarken en Zweden, bedankt voor onze mooie reizen. ***Maria***, you gave me and Floor a home when we visited you in Spain, it was a great trip. The least I could do was offer you a room when you came for three

months to Wageningen, it was really nice and fun to live with you. You are always welcome in my house. ¡Mi casa es su casa!

Buiten Yerseke heb ik op meerdere, en samen met andere, laboratoria gewerkt. Bij PRI kon ik terecht bij ***Jan Bergervoet*** voor de flowcytometrie metingen van hoofdstuk 3. Bij Biochemie heb ik samen met Andre en Robert vele uren hulp gehad van ***Pieter de Waard*** en ***Cor Dijkema*** met de NMR, jammer dat er uiteindelijk geen eenduidige resultaten uit voort gekomen zijn. Met behulp van ***Henk Kieft*** bij plantenfysiologie heb ik nog een poging gewaagd om de voortplanting van sponzen te leren begrijpen, ook dit is niet terug gekomen in mijn proefschrift, maar was zeker leerzaam. In Spain I could always count on the cooperation of ***Iosune Uriz*** with permits from the CEAB in Blanes, thanks Iosune for your cooperation and fun evenings. ***Dave Abdo*** and ***Jim Seager*** introduced me their work with 3d-photogrammetry, thanks guys for the nice time and cooperation in Perth! Jim, thanks for all the extra calibrations you did.

Naast promoveren is er natuurlijk nog meer in het leven. Een paar specifieke mensen wil ik er graag nog uit lichten. Een van de eerste echte vriendschappen in Wageningen is nog altijd in de buurt, zelfs op het podium. ***Simon***, bedankt voor je vriendschap, je rust en het zijn van een fijne huisgenoot. Fijn dat je me wilt bijstaan in de aula. ***Aafke***, ook jij was er vanaf het begin in Wageningen, en je bent gelukkig nog in mijn leven, zo puur als jij bent zijn er maar weinig, ik kan nog veel van je leren, bedankt! ***Marcel***, altijd in voor een feestje, activiteit of vakantie, super dat je ook weer op dit feest aanwezig bent. ***Tim***, jij hebt zelfs nog een bijdrage geleverd aan experimenten onder water, maar de allerbelangrijkste bijdrage die je hebt geleverd is de geweldige mooie kaft van dit proefschrift, SUPER-bedankt! ***Olga*** en ***Sarina***, ook al zien we elkaar nu minder; ik hoop van harte dat wij zullen blijven waarmaken wat we jaren geleden meerdere malen hebben afgesproken: Wij blijven vriendinnetjes tot de dood ons scheidt!

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Curriculum Vitae

Curriculum vitae

Marieke Koopmans was born in Reimerswaal, Zeeland, the Netherlands on the 18th of April 1981. After the primary school in Rilland she went to 't Goese Lyceum in Goes where she received her HAVO diploma in 1998. From 1998 to 2002 she studied Chemical Engineering at the polytechnique (Hogere Technische School), Hogeschool Brabant, in Breda. Two internships were done in companies, the first was done in 2000 at Cargill, where she focused on optimizing the wheat process. The second was performed at Nedalco bv in 2002 focussing on recycling process water into the production process. Another internship was performed in 2001 at Massy University, New Zealand, where she worked on determining the strength of tablets. In 2002 she graduated. In the same year she started the study Biotechnology at Wageningen University. Her MSc thesis was conducted at the Food and Bioprocess Engineering group of Wageningen university working on cellulase production using the fungus *Aspergillus niger*. In 2004 she received her MSc degree. At the department of Food and Bioprocess Engineering she worked on optimizing a practical course concerning algal growth for three months before she started in 2005 on her PhD entitled 'Growth and metabolism of sponges'. Currently she is working at Friesland-Campina DMV in Veghel, the Netherlands, as process technologist.

Publications

Koopmans M, Wijffels RH (2008) Seasonal growth rate of the sponge *Haliclona oculata* (Demospongiae: Haplosclerida). Marine Biotechnology 10:502-510.

Koopmans M, Martens D, Wijffels RH (2009) Growth efficiency and carbon balance for the sponge *Haliclona oculata.* Marine Biotechnology: *accepted for publication*.

Koopmans M, van Rijswijk P, Houtekamer M, Boschker HTS, Martens D, Wijffels RH (2009) Seasonal variation of fatty acid biomarkers and carbon isotopes in sponges. *To be submitted for publication*.

Koopmans M, Martens D, van Rijswijk P, Middelburg JJ, Wijffels RH (2009) Carbon conversion in two marine sponges. *To be submitted for publication*.

Koopmans M, van Rijswijk P, Martens D, Egorova-Zachernyuk TA, Wijffels RH (2009) Increased metabolic rate in damaged sponge tissue. *Submitted for publication*.

Koopmans M, Martens D, Wijffels RH (2009) Towards commercial production of sponge medicine. *Submitted for publication.*

Overview of completed training activities

Courses

Discipline specific
Professional diving course (Dunoon, Scotland, 2007)
Training course on Porifera (Marseille, France, 2005)
3d-photogrammetry (Perth, Australia, 2006)
Underwater photography (Roosendaal, the Netherlands, 2005)
Marine Biology (Burgh Haamstede, the Netherlands, 2005)
In vivo NMR (Utrecht, the Netherlands, 2005)
Biochemical thermodynamics (Heidelberg, Germany, 2008)

General courses
Statistics (Wageningen, the Netherlands, 2005)
Time planning and project management (Wageningen, the Netherlands, 2005)
Clearly thinking and writing(Wageningen, the Netherlands, 2008)
Scientific writing (Wageningen, the Netherlands, 2006)
Supervising Msc thesis work (Wageningen, the Netherlands, 2006)
VLAG PhD week (Bilthoven, the Netherlands, 2005)
Career perspectives (Wageningen, the Netherlands, 2008)

Conferences

7th Sponge Symposium (Buzios, Brazil, 2006)
NMR symposium (Wageningen, the Netherlands, 2006)
11th Netherlands Biotechnology conference (Ede, the Netherlands, 2006)
12th Netherlands Biotechnology conference (Ede, the Netherlands, 2008)

Optionals

Organizing Process Engineering study tour to Denmark and Sweden (2006)
Process Engineering PhD study tour to Denmark and Sweden (2006)
Process Engineering PhD study tour to Japan (2008)

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