Sponge Culture:
Learning from Biology and Ecology

Sonia de Caralt Bosch - 2007

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Learning from Biology and Ecology

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Sònia de Caralt Bosch

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Learning from Biology and Ecology

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Leren van Biologie and Ecologie

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Chapter I

Introduction and thesis outline

General Introduction

Marine sponges

Marine Sponges (phylum Porifera) inhabit a wide range of ecosystems, from polar to temperate and tropical seas, from shallow waters to the highest depths. Sponges are filter feeders, sessile organisms, which are, from a phylogenetical point of view, at the base of the pluricellular organisms (Metazoans). Sponges reproduce by releasing free-swimming larvae, which do not feed from the plankton but rely on their own reserves for living during their free-swimming phase (lecytotrophic). Larvae settle on the appropriate substrate and metamorphose to become juveniles, which have to compete with other sessile organisms for space to defend against predation, and to maintain their surface clean of fouling organisms. Sponges compete with other organisms thanks to several particularities such as a high plasticity and production of cytotoxic secondary metabolites.

Like in true colonial invertebrates, plasticity allows sponges to change their shape and size, to fuse and divide, and to recover from damage for encompassing biotic and abiotic changes in the sponge surroundings (e.g. Turon *et al.* 1998, Garrabou & Zabala 2001).

Sponges produce secondary metabolites, which play several ecological roles (McClintock & Baker 2001) such us competition for the space (Martí 2005), antifouling activity (Martin & Uriz 1993, Becerro 1994), and deterrence from predators (e.g. Becerro *et al.* 2003). The secondary metabolites produced by sponges have an enormous potential to be used in applications as medical drugs and cosmetics. Most medicines have their origin from natural resources, traditionally from terrestrial plants and, more recently, from marine organisms. Especially, sponges are the origin of a huge number of bioactive compounds, which are chemically very diverse (McClintock & Baker 2001).

Sponge-derived bioactive compounds have different pharmacological activities: anti-inflammatory, anti-tumour, immuno-suppressive, neuro-suppressive,

muscle relaxant, antiviral, antimalarial, antibiotic, fungicidal, and antifouling. Moreover, bioactive compounds can be potential drugs for the treatment of the thrombosis, atherosclerosis, and diabetes (Sipkema *et al.* 2005a). Several of those medicines are in clinical and pre-clinical phases, and only few are in the market (e.g. "Ara A" and the "Ara C", compounds derived from the sponge *Cryptotethya crypta*, with an antiviral and anticancer activity, respectively) (Guyot 2000). The relatively low success in commercializing a medicine from sponges, despite the high number of bioactive metabolites they produce, is in many cases due to the difficulty in obtaining sufficient sponge biomass for research, clinical assays and finally for covering the market needs.

Sponge culture

To supply the market with sufficient amounts of sponge bioactive compounds is a bottleneck currently unsolved. Sponge collection directly from the sea is harmful for the environment (Munro *et al.* 1994). Chemical synthesis of the target compound is a difficult and expensive process due to the complexity of the natural molecules (Pomponi & Willoughby 1994). Genetic approaches to obtain enough sponge biomass or metabolites by transgenic cell cultures is a novel method but the results are still too preliminary to solve the supply problem.

Sponge culture, both at sea and under controlled conditions, is the most assayed method to produce sponge biomass. The origin of sponge culture goes back to the beginning of the last century when bath sponges were cultured at sea (e.g. Cotte 1908). More recently, aquaculture attempts have been done to obtain sponge biomass and thus their bioactive metabolites (e.g. Batershill & Page 1996, Duckworth *et al.* 2004).

The cultivation of intact sponges and fragments at sea prevent the control of the environmental conditions, which may not be the optimal for sponge growth. Thus, to control the external variables, cultures of sponge fragments (explants) have been performed in semi-open and closed systems (e.g. Barthel & Theede 1986, Osinga *et al.* 2003, Sipkema *et al.* 2006). Different conditions have been assayed in these cultures to determine the best parameters for sponge growth and survival. Explant growth under culture was usually variable, slow and species

specific. Those results complicate the sponge supply but are positive enough to merit further research.

Cultures of sponge cells and aggregates (primmorphs) have also been intensely studied although a continuous cell line is still far from being obtained (e.g. Pomponi & Willoughby 1994, Müller *et al.* 1999). Until now, only short-term primary cell cultures have been developed due to contamination problems and low cell division. However, cell cultures are becoming promising due to recent advances, especially in the genetic field (transfected cells) (e.g. Thomson *et al.* 2007).

Sponge symbionts

Microorganisms, mostly heterotrophic bacteria (e.g. Sarà & Vacelet 1973, Wilkinson 1987) but also zooxanthellae (e.g. Rosell & Uriz 1992), cyanobacteria (e.g. Vacelet 1984, Turon *et al.* 2000a, Usher *et al.* 2004b), yeasts (Maldonado *et al.* 2005), and viruses (Lohr *et al.* 2005) live frequently in symbiosis within sponges. The symbiotic microbial community is frequently transmitted from the parental sponges to the progeny (e.g. Enticknap *et al.* 2006, de Caralt *et al.* 2007c submitted). Microsymbionts may have essential functions in sponge biology: serving as food (Wilkinson & Garrone 1980), or producing bioactive metabolites (e.g. Schmidt *et al.* 2000). Several secondary metabolites that, at a first sight, were though to be produced by the sponge, were in fact synthesized by the symbiotic microorganisms (Elyakov *et al.* 1991, Bewley *et al.* 1996, Unson *et al.* 1994). When the target compound is produced by the symbiotic bacteria, culture of the microorganism has been tried as an alternative method for supplying the bioactive compound. However, isolation of those microorganisms from the sponge cells, and especially the low viability of these symbionts outside their host (only less than 0.1% of the total symbiotic bacterial community is amenable to culture, Webster & Hill 2001) make this approach unreliable.

Nevertheless, a high proportion of the bioactive molecules are produced by the sponges themselves (e.g. McClintock & Baker 2001, Faulkner *et al.* 2000). In those cases, the symbionts may have a relevant function in the sponge viability success due to their role in the sponge metabolism. Consequently, the

establishment of an axenic sponge culture, which must pass through the elimination of the microsymbionts, may result in sponge death.

Outline of the thesis

The aim of this thesis was to gain new knowledge on biology and ecology of sponges (particularly of *Corticium candelabrum*) that can be applied to the culture sponges for biotechnological purposes.

In **chapter II,** we aimed to assess the growth capacity of *C. candelabrum* in the field. We monitored monthly survival, growth, shrinkage, regeneration, fusion, and fission of 45 individuals of this species for three years. In parallel, experiments of clearance rates on natural food particles were performed seasonally. The clearance rates of *C. candelabrum* on the different types of food particles available in the water column provided us with information on the preferable food type to be further used for the sponge culture.

In **chapter III**, *in vitro* sponge culture of *C. candelabrum* explants was assayed in part based on the information acquired from **chapter II**. In the first part, we attempted to define a protocol for obtaining healthy sponge explants by examining explant survival under several biological and environmental conditions. In the second part, an explant culture was implemented. The effect of several cultivation conditions: with and without addition of antibiotics, and algae and bacteria as a food, were analyzed to find the best conditions for explants survival and growth. Survival and growth of *C. candelabrum* in culture were compared with those of sponges at sea (**chapter II**).

In **chapter IV,** a new *in vitro* sponge culture technique, based on juveniles from larvae, was investigated. The experimental design was based on the results obtained in **chapters II** and **III**, where a general pattern of slow and variable growth was observed, with the smallest individuals growing at the highest rates at sea. Starting from larvae, we wanted to take benefit of the high growth capacity of juveniles. Moreover, by culturing individuals of the same age (same cohort), we expected to reduce the growth variability among individuals. Due to the difficulty to obtain *C. candelabrum* larvae in sufficient amounts, the experiments were

performed with larvae of *Crambe crambe, Dysidea avara*, *Ircinia oros* and *Hippospongia communis*, which were easier to obtain. After larval settlement in the laboratory, juveniles were cultured under different conditions of flow and food. In addition, juveniles were transplanted into the sea to compare survival and growth in both environments (sea and laboratory). This study showed the potentiality of sponge larvae as a source of sponges to culture.

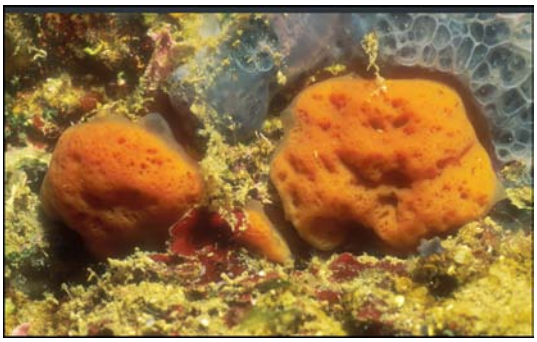
In **chapter V**, we studied, through transmission and scanning electron microscopy, the developmental process involved in the formation of the *C. candelabrum* larva, which is a cinctoblastula type. After the success obtained with the culture of juveniles from larvae of several sponge species (**chapter IV**), a more in deep study on larval development of *C. candelabrum*, which was poorly known, appeared to be necessary as a basis for a massive larval supply in the future. The species selected for this study was *C. candelabrum* because their larvae were poorly known and because this species is a promising species to culture (**chapter III**).

In **chapter VI** the different symbiotic bacteria types in *C. candelabrum* and their transmission to the larvae were described through transmission and scanning electron microscopy for a better understanding of their possible role in the sponge fitness and the consequent repercussions in the success of the sponge culture. We investigated whether all the bacteria types present in an adult were transmitted to the larvae, and whether these bacteria are really used as a food by the sponge (as it had been reported occasionally in the literature).

To conclude, in **chapter VII,** the state of the art on the sponge cell culture is shortly reviewed, and new proposals for future research are outlined. We suggest the use of embryos and larvae instead of adults (archeocytes) as a new source of stem cells due to their greater potential, and to focus on apoptosis to obtain a continuous sponge cell line.

Target species

The Mediterranean species used for this thesis have been selected on the basis of different criteria. The selected species have a biotechnological potential and moreover, the period of larval release of most of them was known, and this facilitated larval collection in sufficient numbers for the experiments. The species were:



Corticium candelabrum Schmidt 1862, (Homosclerophorida, Demospongiae) a common sublittoral species that can undergo both sexual and asexual reproduction, has a high regenerative capacity, harbors symbiotic bacteria, and is bioactive.

Dysidea avara Schmidt 1862, (Dictyoceratida, Demospongiae) which is also a common sublittoral species and produces avarol, a metabolite investigated by pharmaceutical companies due to its anti-tumor and antiviral activities such as *in vitro* inhibition of HIV-1 RT. This species releases huge amounts of larvae, which allowed us to study larval settlement and culture of juveniles.

Ircinia oros (Pallas, 1864), (Dictyoceratida, Demospongiae) is also a common sublittoral species. It produces abundant larvae in a short period of time, and it also produces secondary metabolites with anti-inflammatory properties.

Hippospongia communis (Lamarck 1814), (Dictyoceratida, Demospongiae) is one of the common bath sponges and has antifungal activity.

Crambe crambe Schmidt, 1862, (Poecilosclerida, Demospongiae) is one of the most common and toxic Mediterranean sponges. It produces crambescidines and crambines, metabolites that are being investigated as anti-tumour and antiviral drugs. Larval release is spectacular with thousands of larvae swimming in the water column in summer months.

Chapter II

Growth dynamics of a Mediterranean sponge species. *Corticium candelabrum* (Demospongiae: Homosclerophorida)

de Caralt S, Uriz MJ & Wijffels RH (2007) Growth dynamics of a Mediterranean sponge species. *Corticium candelabrum* (Demospongiae: Homosclerophorida). Marine Ecology Progress Series *(submitted)*

Abstract

The growth dynamics and survival of the sponge *Corticium candelabrum* was studied in the northwestern Mediterranean for more than three years. Growth and regeneration rates, fission and fusion events, and survival were monitored monthly. Moreover, *in situ* punctual clearance experiments were conducted seasonally searching for possible relationships between food uptake and sponge dynamics. The growth rates of *C. candelabrum* were low, variable, and seasonal, with the highest values in summer. The cumulative survival function followed a stepped curve, with several consecutive months without mortality separated by shorter mortality events, which mainly occurred in cold months. However, an event of high mortality took place in the particularly warm summer 2003. Fission events were frequent after previous damage (e.g. partial predation) and only one fusion event was recorded along the study period. The diet of *C. candelabrum* was highly heterogeneous. Differences in clearance rates among prey types with season indicated that the sponge retained with different efficiency the several prey types present in the water. Survival and growth rates were significantly different for small, medium, and large individuals (size-classes I, II, & III), with the small sponges showing the lowest survival and the highest growth rates. As a whole, the results indicate that *C. candelabrum* is an extraordinarily dynamic sponge.

Introduction

Sponges are sessile, surface dependent (filter feeders), modular organisms with a reported plasticity that allows them to change their shape and size to accommodate environmental changes (e.g. Pansini & Pronzato 1990, Garrabou & Zabala 2001). Moreover, sponges are able to regenerate their tissues after damage (e.g. Jackson & Palumbi 1979, Ayling 1983, Leys & Lauzon 1998, Hoppe 1988, Turon *et al.* 1998, Wulff 2006) and thus they are among those modular organisms that can recover from partial mortality (reviewed in Henry & Hart 2005) which is a phenomena that is strongly related to cellular/tissue plasticity.

Sponges inhabit rocky bottoms where the substrate is crowded. As their growth is usually slow (e.g. Ayling 1983, Turon *et al.* 1998), they take benefit of their plasticity to compete for the space with other sessile, faster growing invertebrates and algae (e.g. Sebens 1987, Stocker 1991). The growth dynamics of a sponge species is the result of integrating its life history and its responses to the environmental (biotic and abiotic) changes; and therefore, it represents the sponge life strategy. Sponge dynamics involves growth, shrinkage, fission, and fusion events. Data on the sponge growth dynamics are basic in ecological studies such as those that focused on sponge capability to colonize new substrates, to compete with neighbors and to persist once installed. Furthermore, the knowledge of the sponge dynamics has a collateral, applied interest in the field of sponge culture for supplying sponge biomass to the pharmaceutical companies that search bioactive secondary metabolites (new drugs) from sponges. Sponges under culture have been reported to show chaotic growth, with no general trend but individual, difficult to interpret behavior (high inter-individual growth variability). Thus, understanding the dynamics and the food requirements of wild sponges is an essential, first step to optimize sponge growth in the cultures.

To have a realistic overview of the general growth dynamics of a target sponge species, long-term studies (i.e. more than one biological cycle) should be conducted (e.g. Ayling 1983, Johnston 1979, Duckworth & Batershill 2001, Turon *et al.* 1998, Hoppe 1988, Garrabou & Zabala 2001, Tanaka 2002, Pansini & Pronzato 1990, Leys & Lauzon 1998). Moreover, in sponge monitoring, data

should be recorded at short intervals, and based on a large number of specimens, since studies encompassing low frequency observations and/or performed on few individuals (e.g. Pansini & Pronzato 1990, Leys & Lauzon 1998) may fail to detect changes or general trends due to the fast sponge dynamics and the large inter-individual variability. Previous studies evidenced that sponge growth is extremely variable among individuals, with time, which may be due to differences in individual fitness as a function of the environmental factors (e.g. temperature, food availability, interaction with neighbors).

Regeneration is also decisive for the sponge survival and persistence. The studies on sponge regeneration rates have mainly been addressed by assessing recovery after artificially induced injury (Jackson & Palumbi 1979, Ayling 1983, Hoppe 1988, Leys & Lauzon 1998, Turon *et al.* 1998). In contrast, few studies monitored sponge recovery from natural damage after perturbation or predation (e.g. Wulff 2006) and none of them has been performed in the Mediterranean Sea where the main factors affecting the regeneration processes (e.g. temperature, predator abundance, food and exosomatic energy entries) vary seasonally (Margalef 1985).

One of the main factors, which determine growth rates in sessile invertebrates, is food availability. To know whether the quality and quantity of food exert some influence in the sponge dynamics, *in situ* clearances rates along the year may help to interpret the seasonal growth variations of a Mediterranean sponge.

In this study, we have monitored monthly for three years the main variables involved in the dynamics of the bioactive sponge *Corticium candelabrum* Schmidt, 1862: growth, fusion, fission, and regeneration rates. Moreover, *in situ* clearance experiments have been done seasonally searching for possible relationships between food uptake and sponge dynamics.

Material and methods

Growth dynamics

The target species *Corticium* ca*ndelabrum* is a common bioactive sponge (de Caralt *et al.* 2003) widely distributed in the North-western Mediterranean (Uriz *et al.* 1992). A total of 45 specimens of *C. candelabrum* ranging in area from 0.13 to 18.78 cm^2 were labeled in a sublittoral area of the Blanes locality (North-east Spain, western Mediterranean). All the monitored individuals inhabited a rocky wall (25 m long) facing North, between 10 and 12 m deep, to reduce to a minimum the effects of the different micro-environmental conditions in the sponge dynamics.

After one study year, an event of high mortality occurred (i.e. summer 2003) which reduced considerably the number of specimens monitored. Thus, in December 2003, a new set of individuals, ranging in area from 0.23 cm^2 to 27.7 cm^2, from the same zone were included in the study to replace those that died during the mortality event (total N = 45). Since small individuals showed highest mortality frequency, larger individuals were selected this time among the specimens added to ensure a two-year monitoring. Thus, the average size of the monitored individuals increased, although there was still a good representation of the different sponge sizes in the studied population.

Along the three study years, from September 2002 to December 2005, each labeled individual was photographed monthly and the sponge area was measured by image analysis (NIH Image program). From the pictures, mortality, depredation, fusion, fission, growth and recovery rates were computed.

A specimen was considered to die when it was missing from the sequential pictures since a given month on. Predation was recognized due to the grazing marks and was recorded as a damaged area in a specimen that was healthy in the previous month. Recovery was computed when the damaged area healed. A fission event was computed when a single individual divided into two or more smaller new individuals that remained physically separated. A fusion event was computed when two close individuals became a single individual.

Growth rates (GR) and recover rates (RR) at an interval of time were calculated with the following formula:

$$GR_t = ((area_{t+1} - area_t)/area_t) \cdot time^{-1}$$

Since the measurements were done monthly; the $area_t$ was the area in a given month and the $area_{t+1}$ was the area of the same individual in the next month.

Feeding experiments

Feeding experiments were carried out seasonally in 2003 by incubating sponges *in situ*. Incubation chambers represent closed spaces in which we can control food decrease in the water due to sponge feeding. Picoplankton constitutes the main part of the sponge diet (e.g. Reiswig 1971) since it is retained with the highest efficiency by sponges (Ribes *et al.* 1999). Thus, we analyzed decreases in picoplankton (i.e. heterotrophic bacteria, *Synecococcus* sp., *Prochlorococcus* sp., and picoeukariotes) in the chambers before and after incubation.

For the incubations, several specimens of *Corticium candelabrum,* similar in size, were removed from their natural substrate and attached with a two-component epoxy resin (IVEGOR®) to horizontally placed tiles. The tiles with the attached specimens were placed at 12 m depth in the sponge natural habitat and maintained there for sponge adaptation during one week. Then, the healthiest specimens were selected for the experiment.

Feeding experiments were conducted in individual, 1 l chambers (N=3). Three additional empty chambers were used to control possible increases or decreases in picoplankton due to other factors (e.g. plankton division, mortality, sedimentation, etc.) than sponge grazing (controls). Chambers were sealed to the tiles with epoxy resin (IVEGOR®). 5 ml water samples from the chambers were taken through an upper aperture at time 0 and one hour later. Water samples were immediately fixed in a cocktail of paraformaldehyde and glutaraldehyde (1% and 0.05% respectively, final concentration) and kept cold in the dark. Once in the laboratory, the samples were frozen at -80ºC for posterior flow cytometer analysis. The specimens were removed from the tiles after the experiments, taken

to the laboratory, and placed in an oven at 90ºC for 24 h to determine their dry weight.

The water samples were analyzed for quantification of heterotrophic bacteria, cyanobacteria (i.e. *Prochlorococcus* sp., *Synechococcus* sp.) and picoeukaryotes using flow cytometer (ACSCalibur, Becton Dickinson, 488 nm excitation laser flow cytometer) according to Gasol & Moran (1999).

Just before processing, water samples were divided in two sub-samples: one of 0.5 ml (S1) to quantify the heterotrophic bacteria, and another of 1 ml (S2) to quantify the rest of picoplankton. Then, we added 150 µl and 5 µl to the samples S1 and S2, respectively, of a stock pre-calibrated solution (Becton-Dickinson True-Count beads) containing fluoresbrite yellow-green beads (polysciences). The number of beads counted in each sample run was used to determine the sample volume processed and thus the cell abundance (density).

To separate different cell types, the Logical gating in Becton-Dickinson Cell Quest software was used. The small subsamples (S1) were treated with Syto 13 (Molecular Probes) at 2.5 µmol·l^{-1} for staining the heterotrophic bacteria, and run at a flow rate of approximately 18 l·min^{-1}. Heterotrophic bacteria were detected by their signature in a plot of side scatter *versus* green fluorescence. For quantification of *Prochlorococcus* sp., *Synechococcus* sp., and picoeukaryotes, the large sub-samples (S2) were run at a flow rate of approximately 44 l·min^{-1}. *Synechococcus* sp. was detected by their signature in a plot of orange fluorescence versus red fluorescence. The signature for *Prochlorococcus* sp. was low red fluorescence instead of orange fluorescence, and picoeukaryotes had high red fluorescence and no orange fluorescence.

Sponge clearance rates were calculated from the flow cytometer counts, as decreases in picoplankton concentrations (cells·ml^{-1}) in the water samples of each experimental chamber relative to the control chamber per unit time.

Clearance rate (CR) is the product of the amount of water being filtered per amount of particles being retained from this water. The basic equation modeling filtration is an exponential function of this:

$$CR = (v / DW \cdot t) \ln (C_t/C_0)$$

Where C_t and C_0 are the final and initial particle concentration, respectively, v is the total volume of the incubation chamber (1 l), and the t is the time of the experiment (1 h).

Specific clearance rates (CR) were calculated as the volume of water cleared of each particle type (i.e. heterotrophic bacteria, *Prochlorococcus* sp., *Synecoccocus* sp., picoeukariotes) per unit time and sponge weight (ml swept clear g DW^{-1} h^{-1}), where DW is dry weight.

Clearance rates have also been called *Filtration rates* by several authors (e.g. Petersen & Riisgard 1992) by assuming that the particles in the water are retained with 100% efficiency. However it has been reported that particles are retained by sponges with different efficiency depending on particle size (Turon *et al.* 1997). Thus, in our case, it is more consistent instead to consider that the volume of water passed through the aquiferous system per unit sponge weight and time was constant and that the several prey types were retained with different efficiency (Turon *et al.* 1997, Ribes *et al.* 1999).

The total clearance rate was calculated as the volume of water cleared of all types of particles present in the water in each season.

Statistical methods

To assess weather small and large sponges behaved differently, the target individuals were arbitrarily grouped into three size-classes: I (N =18; from 0.1 to 3 cm^2), II (N =16; from 3.1 to 9 cm^2) and III (N =11; > 9.1 cm^2). Survival and growth during the two last study years (January 2004-December 2005) were analyzed separately for each size-class.

Survival was analyzed using the "life tables" statistics (Fox 1993). Significant differences between size-classes were assessed by "Comparing Survival in Multiple Groups". Then, comparison between each pair of size-classes was performed using the Gehan'n Wilcoxon test (Statistica 6.0 pakage).

Differences in monthly growth rates between size-classes were analyzed by a randomization method based on Mainly (1991) since data did not comply with the circularity and homogeneity of the variance-covariance matrix assumptions (as assessed by the Mauchly's sphericity and Box M-tests, respectively). The method

consisted of a two-stage permutation of the data: first, individuals were randomly reassigned to the three size-classes, and then readings for each individual were randomly rearranged among observation times. The whole series of data was randomised 4999 times (plus the observed one) to approximate the null hypothesis distribution of the sum of squares for each factor and their interaction, and then we examined how extreme were the observed values in this distribution. An effect was judged significant when the observed sum of squares was exceeded by less than 5% of the corresponding values in the randomisation series. A modified version of the Turbopascal program by Turon *et al.* (1998) was applied to perform the permutation tests.

Cross-correlation analysis (using the Pearson coefficient) was performed to check for relationships between monthly growth rates and temperature (Statistica 6.0 pakage).

Significant differences in clearance rates (CR) for each prey type in each season were analyzed by one-way ANOVA. The Tukey test was used for *a posteriori* pair comparisons. Data fulfilled the normality and homogeneity assumptions as tested by Kolmogorov-Smirnov and Bartlett tests, respectively (Statistica 6.0 package).

Results

Growth dynamics

The time course of the monthly growth rate of *Corticium candelabrum* along more than three years (September of 2002 to December of 2005) is depicted in **Fig. 1**. The growth rates are presented separately for 2003 and 2004-2005 due to a massive mortality event occurred after summer 2003 that obligated us to add new individuals in the monitoring. Water temperature at the study site along the monitoring period is also included in the graph.

A seasonal trend in growth rates was observed although growth had a slightly different trend depending on the year. Growth rates picked in June-July 2003 (summer) while the maximal growth rates were recorded October-November

2004 (in autumn) and in May 2005 (spring). In general, growth rates started to decrease at the end of autumn, being the lowest in winter of the three study years. Growth rates presented a significant positive correlation with temperature at time lags of -2, -1, 0, and 1 (Cross correlation analysis) (**Fig. 2**).

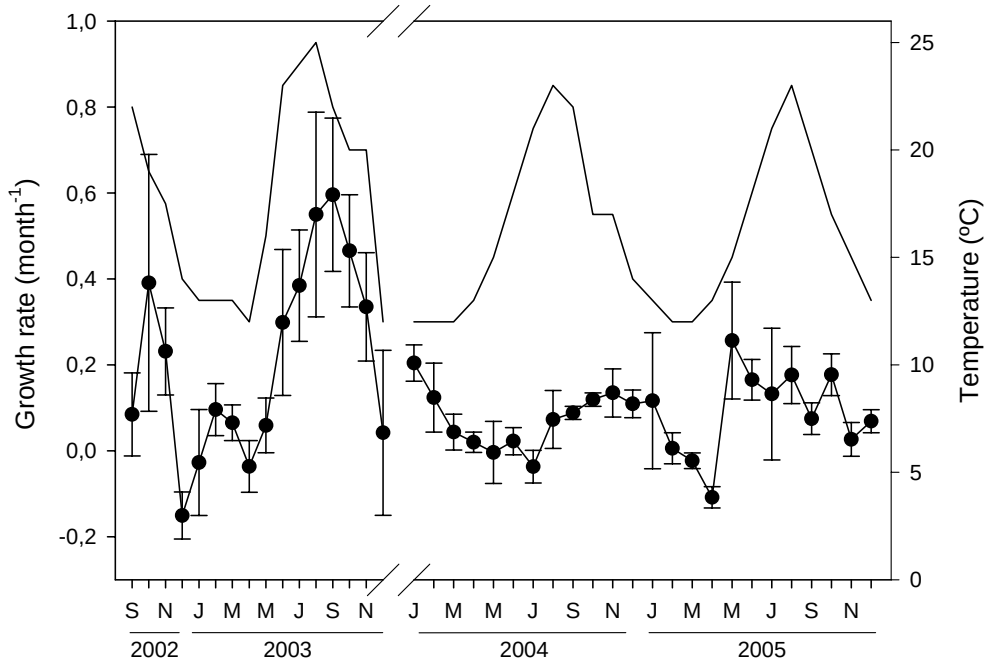


Fig. 1- *Corticium candelabrum.* Time course of the monthly mean growth rate (vertical lines are standard errors) and water temperature (ºC) at the study site. The graph is broken to indicate the inclusion of new individuals to replace those disappeared at the end of 2003 due to a drastic mortality event.

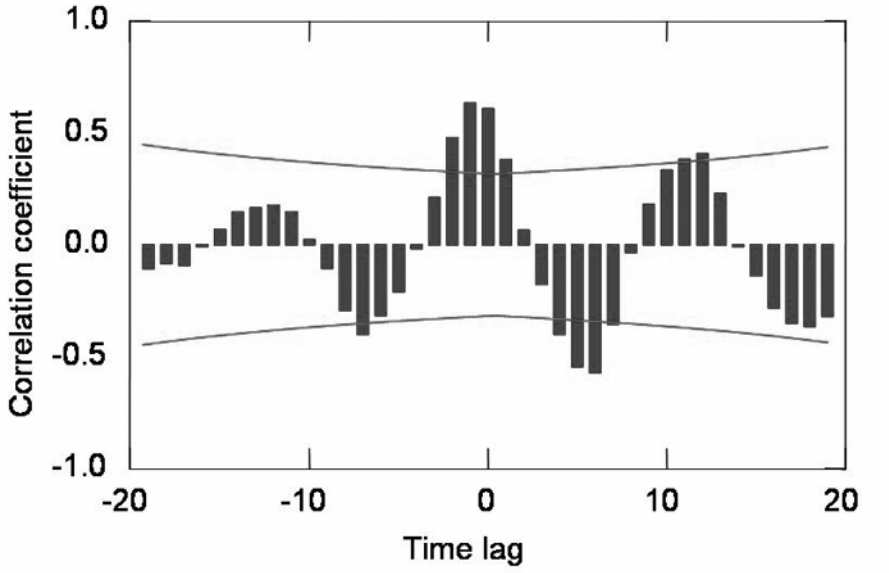


Fig. 2- *Corticium candelabrum.* Cross-correlation analyses of growth rates and temperature. Time lag is in months.

The maximum monthly growth rate computed along the three years was 0.55 ± 0.24 (Mean ± SE) and was recorded in August of 2003 in accordance with the highest temperature. The average growth rate for the three study years was 0.19 ± 0.02 (Mean ± SE).

Negative average growth rates occurred only once or twice per year, generally in winter but also in spring (2003 and 2004). The maximum negative growth rate achieved was -0.15 ± 0.05 (Mean ± SE) and was recorded in December of 2003. The negative mean growth rate for the three study years was -0.06 ± 0.02 (Mean ± SE)

Variability in growth rates was high among individuals likely due to a size effect (i.e. differential growth rates according to the sponge size).

The life dynamics of *C. candelabrum* included fission and fusion events at relatively few frequencies. Fission events were computed to occur in six out of 39 months monitored, mainly in summer and occasionally in winter. Only one or two fission events per month were recorded, except in August 2003 when 12 fission events took place in he same individual. Only one fusion event occurred during the three years (August 2003).

Predation events occurred spread along the year but especially in summer. Sponge predation was observed in nine out of the 38 study months, in which 2-7% of the sponge individuals monitored were predated to some extent. The nudibranchia *Platydoris argo* was the only predator observed but other potential predators cannot be discarded.

Regeneration of damaged individuals was also recorded by observing the recovery of the orange-brown pigmentation typical of the sponge cortex. The 40% of the predated individuals recovered totally their original shape (**Fig. 3**), 40% recovered partially their damaged area with the subsequent decrease in size. On the other hand, in 13 % of the cases, the area damaged was so high that the individual fissioned into several small ones (**Fig. 4**). Finally, 7 % of the predated sponges died. The recovery process usually took ca. one month, and only when the specimens experienced strong damage, the process took two to three months. Regeneration rates were calculated for those individuals that recovered

total or partially (i.e. 80%). Mean regeneration rates were 0.45 ± 0.12 (Mean ± SE), significantly higher than mean growth rates ($p < 0.05$, t-student) (**Fig. 5**).

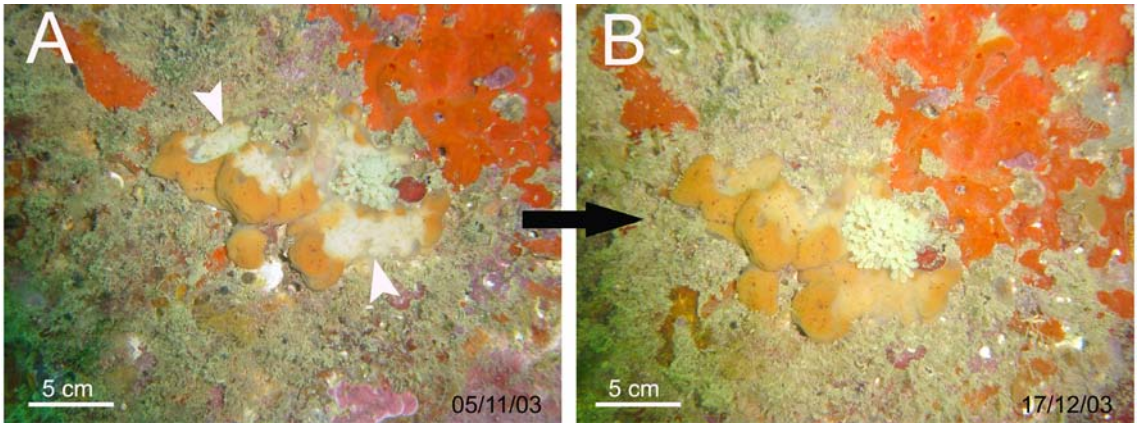


Fig. 3- *Corticium candelabrum.* Instance of regeneration after a month: **A:** individual partially damaged (white in color) in November 2004. **B:** the same individual almost totally recovered (typical orange-brown in color) one month later.

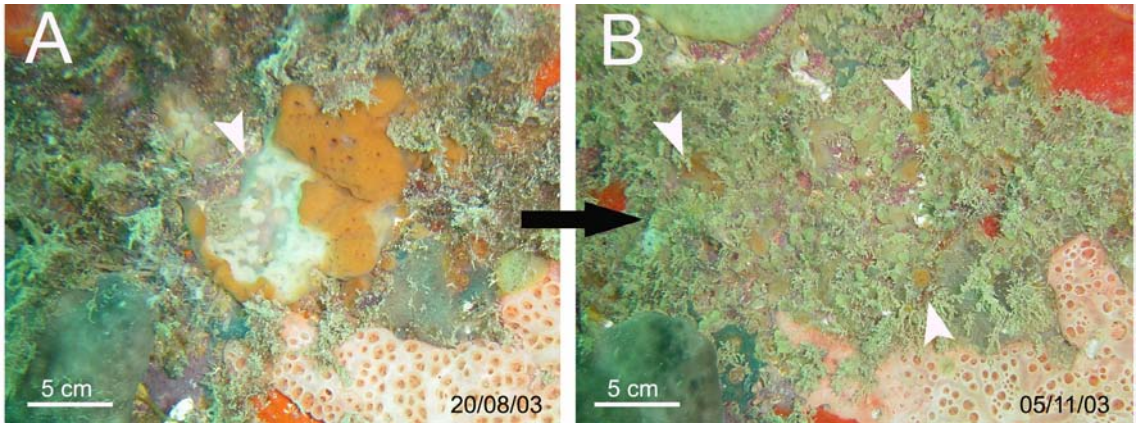


Fig. 4- *Corticium candelabrum.* Fission event in a previously damaged individual: **A:** damaged (white in color area) individual. **B:** Individuals resulted from the fission event, three months later.

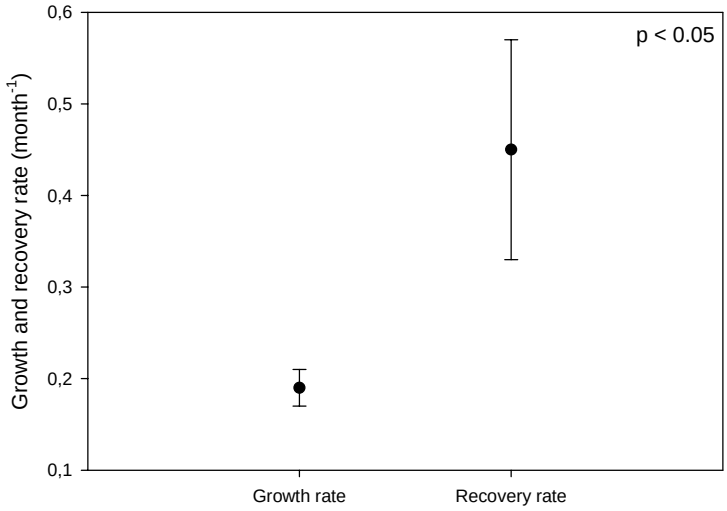


Fig. 5- *Corticium candelabrum.* Mean growth and regeneration rates for the three monitoring years (vertical lines are standard errors).

The cumulative survival function of *Corticium candelabrum* along the study period is represented in **Fig. 6**. The survival curve is presented separately for 2003 and 2004-2005 due to a massive mortality event occurred after summer 2003 that obliged us to include new individuals in the monitoring. During 2003, survival decreased drastically in summer-autumn ranging from 80% in July to ca. 24 % in December.

During 2004-2005 a stepped survival curve was observed, with several consecutive months without mortality separated by shorter mortality events mainly occurring in cold periods (i.e. spring-winter).

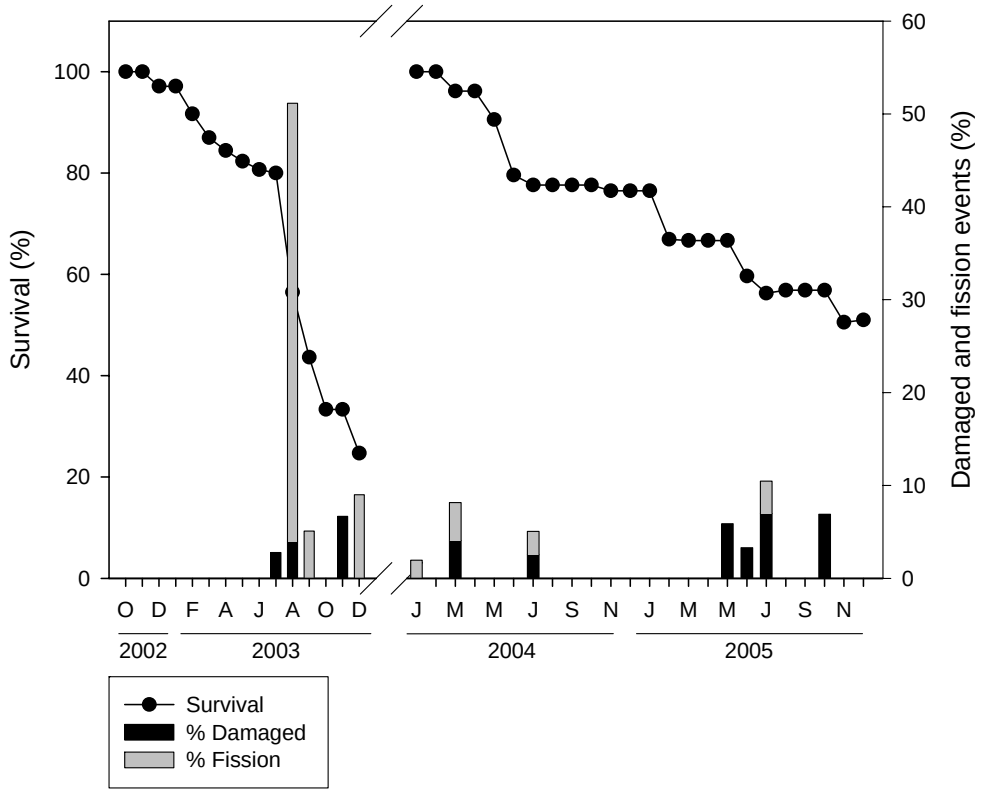


Fig. 6- *Corticium candelabrum*. Cumulative survival curve along the three monitoring years (2002-2005). Stacked bars indicate the percentage of damaged and fissioned individuals at each month. The graphic is broken to indicate the inclusion of new individuals to replace those disappeared at the end of 2003 due to a drastic mortality event.

The sponges monitored during the two last years were analyzed separately in three size-classes (see Material and methods). Survival was significantly different for the three size-classes ($p < 0.001$, Comparing Multiple Samples) being significantly lower for the smallest individuals (i.e. size-class I) ($p < 0.05$ between size-classes I and II and $p < 0.005$ between size-classes I and III, Gehan's Wilcoxon test) than for medium and large individuals (size-classes II and III), which survived the same ($p = 0.6$, Gehan's Wilcoxon test) (**Fig. 7**).

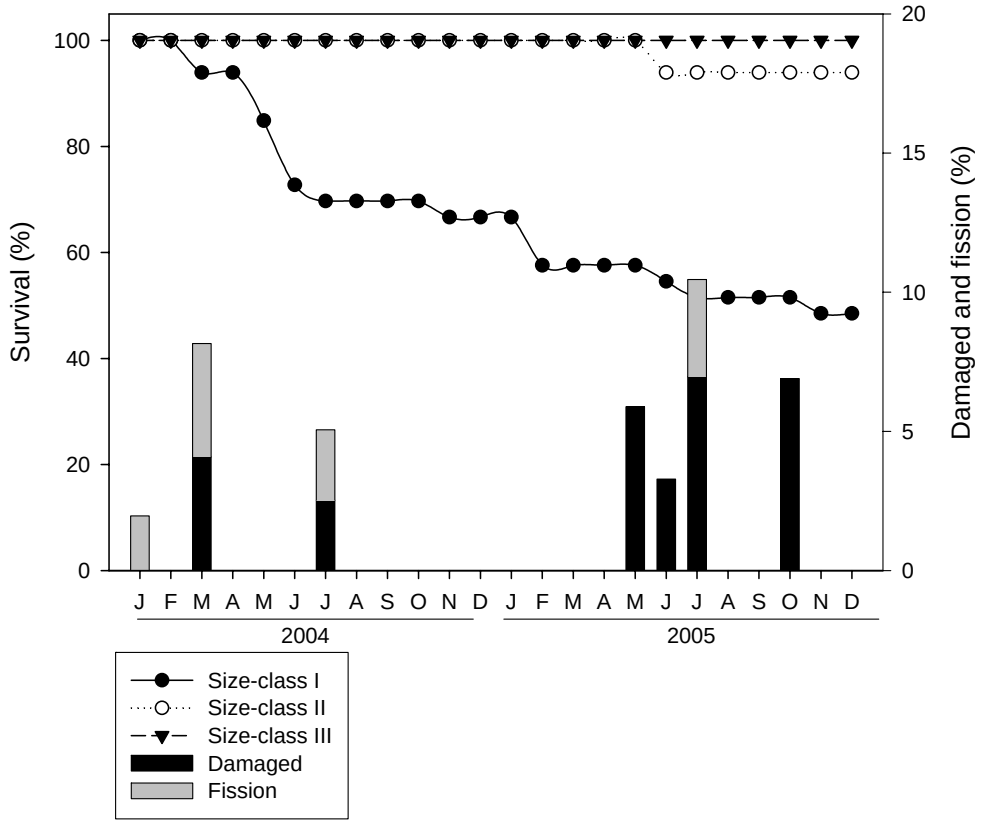


Fig. 7- *Corticium candelabrum.* Cumulative survival curves for each one of the three size-classes since January 2004 on. Stacked bars indicate the percentage of damaged and fissioned individuals at each month.

Time course of the monthly mean area is represented in **Fig. 8** for the three size classes separately. For the size class I, the sponge mean area at the end of the monitoring (2 years) was almost the same than the initial mean area. For the size class II, the final mean area was slightly higher than the initial area and for the size class III, the final mean area was almost twice the initial mean area.

Differences in growth rates among size-classes were significant ($p < 0.01$, permutation test). The *a posteriori* pair comparisons showed (after Bonferroni's correction) that the smaller individuals presented significantly higher growth rates than the other two size-classes ($p < 0.01$ between size-classes I and II and $p < 0.005$ between size-classes I and III, permutation test); no significant differences were found between size-class II and III ($p = 0.8$, permutation test).

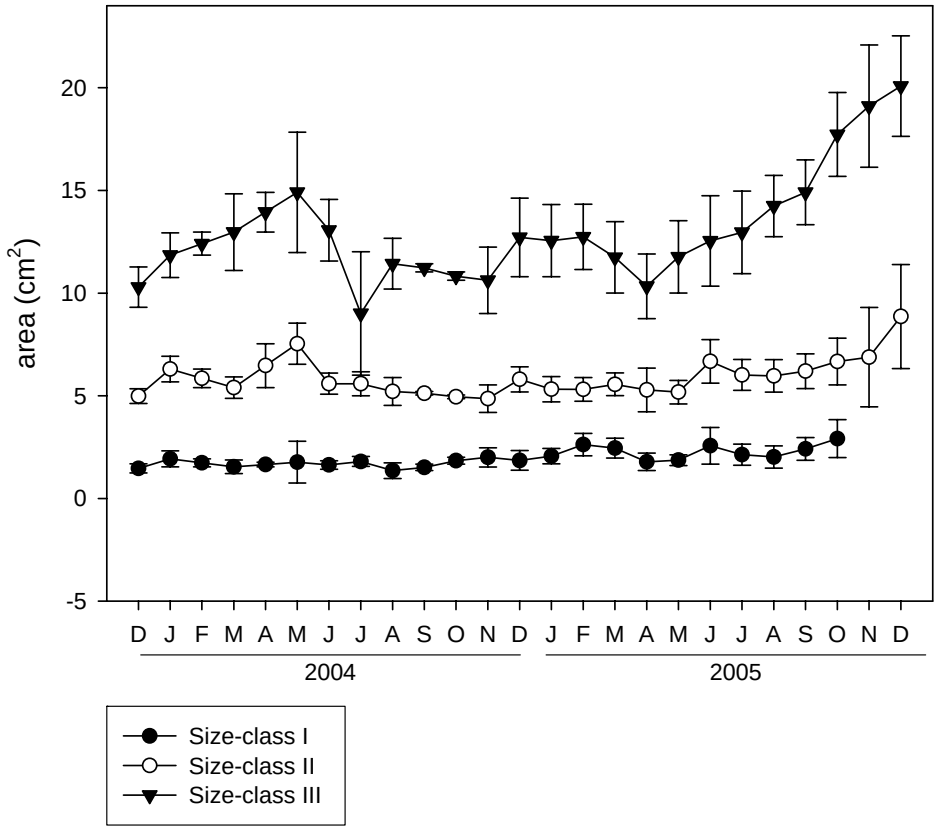


Fig. 8- *Corticium candelabrum*. Time course of the monthly mean area (cm^2) of the three size-classes since January 2004 on (vertical lines are standard errors).

Survival and mean growth rates for the three size-classes are represented in **Fig. 9** where it is shown that the size-class I had the lowest survival and the highest growth rate.

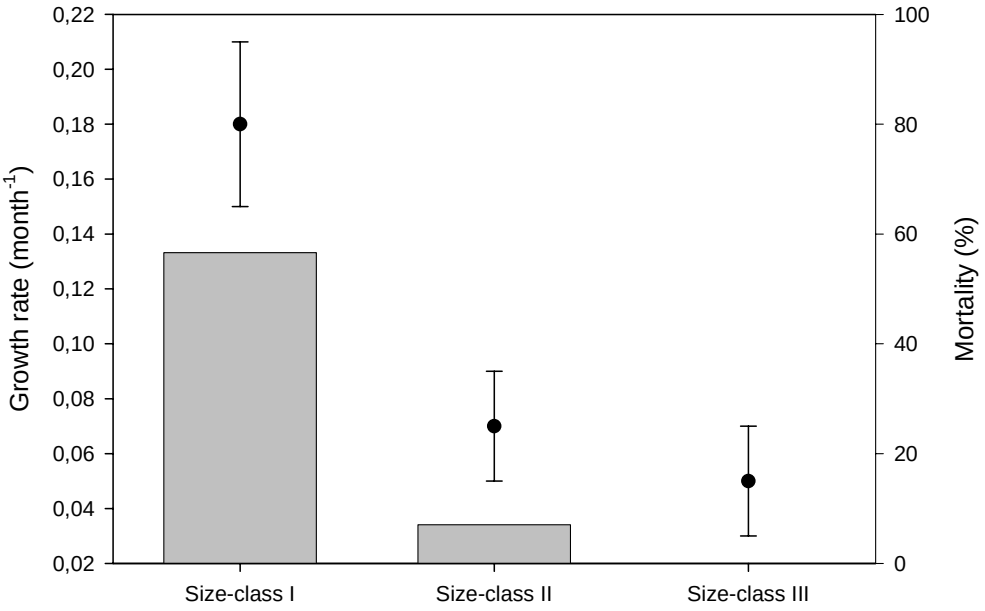


Fig. 9- *Corticium candelabrum*. Two-year (since January 2004 on) growth rate mean for the three size-classes (vertical lines are standard errors). Bars indicate cumulative mortality after the two years for the three size-classes.

Filtration experiments

The potential prey concentration at the study site varied quantitatively and qualitatively along the year. The heterotrophic bacteria and the picoeukaryotes were present all the year round; conversely *Synechococcus sp.* was more abundant in summer, and disappeared in spring; *Prochlorococcus sp.* only was present from autumn to winter.

The diet of *C. candelabrum* was highly heterogeneous according to the seasonal variation of prey abundance in the water. The total clearance rates (all the preys considered) varied significantly with season ($p < 0.05$, ANOVA). Clearance rates were similar in spring and summer, and also in autumn and winter ($p > 0.6$, for both comparisons, Tukey test), being higher in autumn-winter than in spring-summer ($p < 0.05$ for all comparisons, Tukey test) (**Fig. 10**).

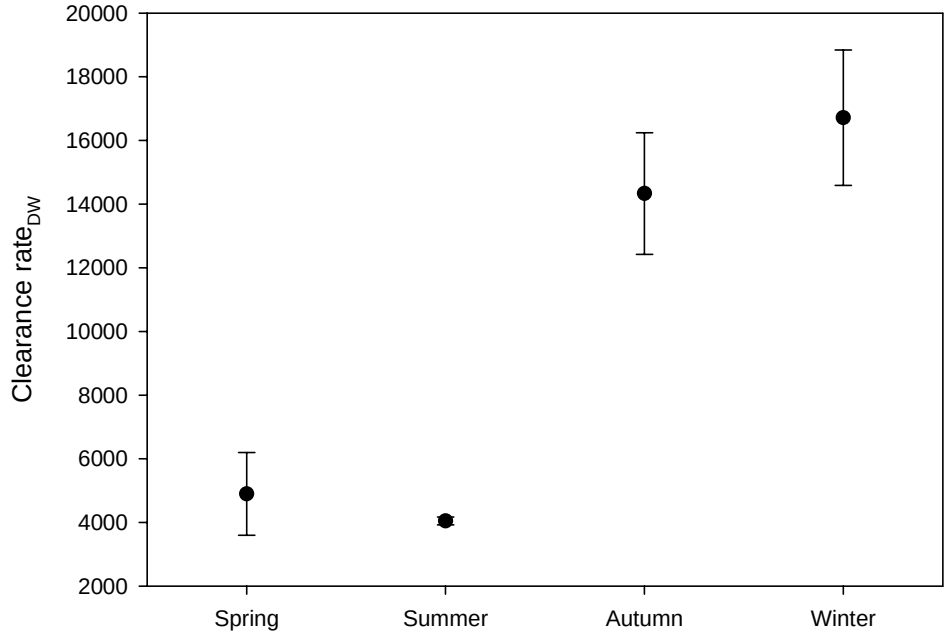


Fig. 10 - *Corticium candelabrum*. Mean clearance rates (ml gDW^{-1} h^{-1}) per season, considering all the prey types (vertical lines are standard errors).

Differences in clearance rates for each prey type with season indicated that the sponge retained with different efficiency the several preys present in the water (assuming that water transport was constant, see Material and methods), especially *Prochlorococcus* (**Fig. 11**). In spring, when picoplankton consisted only of picoeukariotes and heterotrophic bacteria, picoeukariotes were significantly more retained (76%) than bacteria ($p < 0.05$, Tukey test). In summer, when *Synechococcus* was present in the water, no significant differences ($p = 0.75$, ANOVA) in clearance rates were observed for the several preys present in the water. In autumn and winter, when *Prochlorococcus* was present at high densities, the four prey types were differently retained ($p < 0.05$, ANOVA) being *Prochlorococcus* uptake significantly higher than that of the other three prey types (65% of the total preys retained). Conversely, no significant differences were found among the other prey types ($p > 0.05$ for all comparisons, Tukey test).

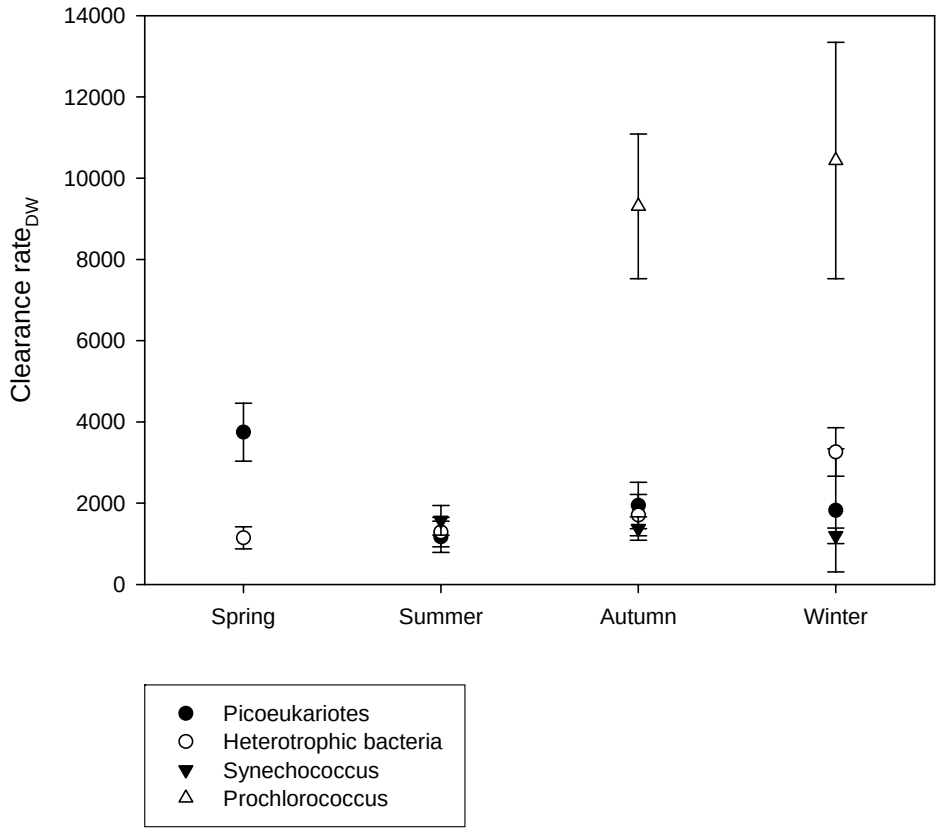


Fig. 11- *Corticium candelabrum*. Mean clearance rate (ml gDW^{-1} h^{-1}) for each prey type per season (vertical lines are standard errors).

Discussion

As more study cases are incorporated to the body of knowledge of the sponge growth in temperate environments, a general pattern becomes more and more evident. The extraordinarily slow growth rates found for *Corticium candelabrum* are in accordance with those reported for other temperate incrusting sponges (i.e. Ayling 1983, Pansini & Pronzato 1985, Turon *et al.* 1998, Garrabou & Zabala 2001, Tanaka 2002).

This slow growth contrasts with the quite fast recovery rates that sponges show after damage. *C. candelabrum* regenerates 2.4 times faster than it grows, a similar ratio to those reported for other species (e.g. Leys & Lauzon 1998, Turon *et al.* 1998). However, the regeneration rates have been reported to be extraordinarily higher in other sponge species (e.g. 10-fold, and up to 2,900 times natural growth rates, in *Neofibularia nolitangere* and *Eurypon sp.* (Hoppe 1988 and Ayling 1983, respectively).

A seasonal growth dynamics similar to that of *C. candelabrum,* with the highest growth in spring-summer, has also been reported for other temperate sponges (e.g. Johnson 1979, Turon *et al.* 1998, Garrabou & Zabala 2001, Tanaka 2002).

When we separated the monitored sponges in three size-classes, the smallest individuals showed the highest growth rates, while no differences between the other two size-classes (medium and large sizes) were found. A different growth rate as a function of the sponge size was also recorded for *Crambe crambe* (Turon *et al.* 1998) and *Halichondria panicea,* with younger individuals growing faster than older individuals (Barthel 1989). Small individuals of *C. candelabrum* did not reproduce, as it has been reported for *C. crambe* (Uriz *et al.* 1995) and *Scopalina lophyropoda* (Blanquer & Uriz 2007, *submitted*) and thus they invest the available energy just in growth. In contrast, medium and large individuals have to divide the available energy between reproduction and somatic growth.

The fission events that have been often reported in sponges (e.g. Johnson 1979, Elvin 1976) may have relevance at a population level by contributing to

clonality in sponge population (Duran *et al.* 2004). In *C. candelabrum,* however, fissions are less frequent than in other sponges (e.g. *S. lophyropoda*, Blanquer & Uriz 2007, *submitted*) and they are mainly due (80.5 % of times) to previous serious damage of the individual. The elimination of the damaged tissue and the regeneration process of the resulting fragments end in several smaller specimens, able to survive and grow, and eventually to fuse. Thus, fissions appear to be a way to overcome negative interactions with competitors or predators and thus they may represent a survival strategy closely related with the reported partial mortality in modular organisms (Turon 1992).

Survival of *C. candelabrum* takes place in a stepped fashion with long periods of stability followed by some mortality in cold months when sponge growth rates were the lowest. When the monitored sponges were separated in three size-classes, it was shown that mortality prevailed among the small individuals. Turon *et al.* (1998) found a significant negative relationship between mortality and size in *C. crambe* as well. Garrabou & Zabala (2001) also indicated that the mortality events observed during their three-year monitoring of *C. crambe,* were exclusively experienced by the smallest specimens. These results altogether suggest that sponges need to reach a given size patch (size refuge) beyond which mortality decreases considerably.

However, although natural mortality in *C. candelabrum* seems to be related with low temperatures, punctual massive events of mortality may take place due to external perturbations, as it occurred at the end of 2003, when 80% of the monitored individuals died after an extremely warm summer. In 2003, water temperatures increased in June from 16ºC to 22ºC, and were maintained abnormally high during all summer (25ºC at 10 m in August). Massive mortality event of sponges and gorgonians, which were attributed to unusual high temperatures related to the western Mediterranean warming (e.g. Bethoux *et al.* 1990) have been repeatedly reported in western Mediterranean (Cerrano *et al.* 2000, Romano *et al.* 2000, Perez *et al.* 2000)

C. candelabrum shows relatively high clearance rates (compared with those by the other Mediterranean species studied (*Dysidea avara*, Turon *et al.* 1997, Ribes *et al.* 1999). In particular, the clearances rates on *Prochlorococcus*

sp. are twice higher than those reported for other Mediterranean species such as *D. avara* (Turon *et al.* 1997, Ribes *et al.* 1999). *Prochlorococcus sp.* is the prey most efficiently retained by *C. candelabrum* in our *in situ* experiments, what might be due to a combination of an optimal prey size (e.g. Turon *et al.* 1998, Ribes *et al.* 1998) and a high nutritional value. *C. candelabrum* filtrates at the lowest rates in late spring-summer, when it showed the highest growth. This at a first sight contradictory relationship may be explained by the reduction of the number of functional pumping units due to the reproduction process, which occurs in spring-summer and involves transformation of choanocyte chambers into spermatogonia (e.g. Gaino *et al.* 1986b). Later on during development, embryos occupy an important part of the sponge choanosome (e.g. Uriz *et al.* 1998), which also may result in a lower pumping capacity.

To summarize, the growth dynamics of *C. candelabrum* is in agreement with that reported for other temperate sponges. Sponges have a reputation as slow growing but dynamic animals, which have experienced great success in benthic ecosystems, since the Palaeozoic. They have maintained their body plan for 700 millions years, (Müller *et al.* 2004) thanks to their enormous plasticity and dynamics, which allowed them to adapt to environmental changes without changing fundamental features (i.e. maintaining their body plan).

Acknowledgements

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Chapter III

Long-term culture of sponge explants: Conditions enhancing survival and growth, and assessment of bioactivity

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

Abstract

Sponges are an important source of secondary metabolites with pharmaceutical interest. This is the main reason for the increasing interest of sponge culture in last recent years. The optimal culture system depends on the species to be cultured: while some species easily produce sponge aggregates after dissociation (primmorphs), others show a great capacity to regenerate after fragmentation (explants). *Corticium candelabrum* is a Mediterranean bacteriosponge that can undergo asexual reproduction. We have taken advantage of this capability and cultured *C. candelabrum* explants in several experimental conditions. To find the best conditions for obtaining functional explants, we assayed a range of conditions, including seasons of collection, culture temperature, filtered *vs.* filtered-sterile seawater, addition of antibiotics and proportion of ectosome. We monitored the changes in shape and ultrastructure during the formation of explants. After 24 h, TEM images showed the aquiferous system disarranged, in particular at the sponge periphery. From 2 to 4 weeks later, the aquiferous system regenerated, and fragments became functional sponges (explants).

Explants were cultured under two regimes: *in vitro* and in a closed aquarium system. Antibiotics were only added to the *in vitro* culture to assess their effect on the symbiotic bacteria, which remained healthy despite the presence of antibiotics. Two food regimens (marine bacteria and green algae) were assayed for their ability to satisfy the metabolic requirements of explants. We monitored explant survival and growth. Explants showed a high long-term survival rate (close to 100 %). Growth rates were higher in the closed aquarium system, without antibiotic addition, and fed with algae. Explant cultures were hardly contaminated because manipulation was reduced to a minimum and we used sterilised seawater. *C. candelabrum* produces bioactive molecules, which may play a defensive role in the sponge and may have pharmaceutical interest. The bioactivity of the explants was similar to that of wild sponges.

Introduction

Sponges represent the lowest metazoan phylum. They are sessile organisms that pump and process large amounts of water through a system of conducts and chambers (aquiferous system), and efficiently retain particles such as phytoplankton, heterotrophic bacteria, heterotrophic eukaryotes and detritus, and also appear to take up dissolved materials (Leys & Reiswig 1998). Sponges have several cell types with different functions. Two zones can be distinguished in sponges: the choanosome or inner region, formed by channels and choanocyte chambers, and the ectosome or peripheral region, which is mainly formed by pseudoepithelial cells (pinacocytes), spherulous cells, collencytes and collagen fibrils (Galera *et al.* 2000). Cell plasticity allows sponges to regenerate and adapt their shape to the environmental conditions (Briens 1973, Bergquist 1978, Simpson 1984).

Sponges usually produce bioactive compounds, which have biological and ecological roles in nature (McClintock & Baker 2001), such as predator deterrence (Becerro *et al.* 2003), antifouling activity (Martin & Uriz 1993, Becerro 1994) or inter-species competition (Martí 2005). Numerous studies have shown the pharmaceutical and commercial interest of these bioactive compounds, with a variety of properties such as cytotoxic, antitumour (Munro 1999), antibiotic, anti-inflammatory and antiviral activities (Munro 1994). Collection of sufficient amounts of bioactive sponges for commercial production of their secondary metabolites may have an adverse impact on the environment (Munro 1994). On the other hand, chemical synthesis of the metabolites is not feasible in all cases (Pomponi & Willoughby 1994). Consequently, sponge culture has been undertaken as a promising alternative. Some authors attempted to culture sponge cells (Pomponi & Willoughby 1994, Ilan *et al.* 1996) and aggregates (Custodio *et al.* 1998, Müller *et al.* 1999, Nickel *et al.* 2001, Sipkema *et al.* 2003c) but problems of microbial and protozoan contamination and poor cell growth (Pomponi & Willoughby 1994) prevented successful culture. Thus alternative systems have also been assayed. *In vitro* sponge cultivation appears promising, since most functional aspects are

maintained and it can be performed under controlled conditions (Osinga *et al.* 1991).

We cultured sponge explants obtained by fragmentation of wild sponges. We used *Corticium candelabrum* Schmidt, 1862, a common Mediterranean species that can undergo asexual reproduction, has symbiotic bacteria, and is bioactive. These characteristics make *C. candelabrum* suitable for collection and providing explants. We monitored the morphological and ultrastructural changes undergone by sponge fragments before they become functional, and measured pre-explant survival and explant growth under several treatments. Antibiotics are necessary to maintain the culture axenic, but they may harm the symbiotic bacteria. To monitor the effect of antibiotics on the symbiotic bacteria, we compared the bacteria populations in wild sponges and explants after the addition of antibiotics, by TEM. Explants bioactivity was also measured to verify that the target compounds were still being produced.

Material and methods

Sponge explant formation

Individuals of *Corticium candelabrum* were collected off the Blanes sublittoral (NE Iberian Peninsula, western Mediterranean), placed in bowls underwater and transported to the laboratory immediately. In the laboratory, we cut the individuals into 3mm^2 fragments with a sterile razor. Cuttings were placed in sterile 6-well plates (1-2 fragments per well) with a sterile pipette. Two 6-well plates were placed in each plastic container filled with 2 l of filtered seawater sterilised by autoclave, so that 12-24 sponge fragments were immersed in 2 l of seawater.

The sponges did not come into contact with the air, manipulation was reduced to a minimum and water temperature was maintained at 14-15ºC.

The water in the containers was changed daily during the first week, and twice a week thereafter. A mixture of antibiotics (100 IU·ml^{-1} of penicillin plus 100 mg·ml^{-1}of streptomycin (Müller *et al.* 1999), was added to the culture at each change of water during the first two weeks.

Morphological and ultrastructural changes during explant formation (pre-explants)

Morphological changes during the first hours were recorded by a video camera connected to a Leika-Wild M-10 stereomicroscope and a Macintosh PC. Subsequent pictures were taken at different time intervals with a Wild camera connected to the stereomicroscope. Pictures were digitalised for analysis.

Ultrastructural changes of both sponge cells and bacteria were monitored on 0h and 24h-old pre-explants, and two-month-old explants. For TEM observation, samples ($2mm^3$) were fixed in a cocktail consisting of 1 % OsO_4 and 2 % glutaraldehyde in 0.45M Sodium acetate buffer (pH 6.4) with 10 % sucrose (Leys & Reiswig 1998), embedded in Spurr resin, thin-sectioned, and stained with uranyl acetate and lead citrate.

Pre-explant survival

We monitored pre-explant survival under different treatments: season of collection of parental sponges (winter-spring *vs.* summer-autumn), culture temperature (14ºC *vs.* 20ºC), addition of antibiotics, filtered *vs.* filtered-sterilised seawater, and several ectosome/choanosome ratio. The same general conditions (filtered-sterilised seawater, 14ºC and antibiotic addition) were maintained in all the experiments except for the treatment that was to be assayed in each experiment.

Pre-explant survival (N = 20-91) from samples collected every month for one year was monitored on days 0, 3, 8, 21, 30, 60, and 240. We compared mean survival of pre-explants from adult sponges collected either during winter-spring months (seawater T, 12-15ºC) or summer-autumn months (seawater T, 19-24ºC).

Fragments of sponges collected in winter were used to assay the best temperature conditions in the cultures for pre-explant survival. Fragments were placed at either 14ºC (N = 20) or 20ºC (N = 12). Survival was monitored at days 0, 6, 9, and 60.

For testing whether, besides adding antibiotics, sterilised seawater improved survival, we placed fragments in filtered (0.7µm pore diameter) seawater

and filtered-sterilised seawater (N = 25). We monitored survival on days 0, 3, 7, 26, and 90.

To assay the effect of antibiotics on pre-explant survival, 100 IU·ml^{-1} of penicillin and 100 mg·ml^{-1} of streptomycin were added to a set of samples (N = 20) while another set (N = 24) did not receive antibiotics. Fragments came from sponges collected in the same season. Survival was monitored on days 0, 3, 9, 21, and 90.

Fragments with a different proportion of ectosome/choanosome: 30-50 %, 10-30 %, and 0-10 % of ectosome were cultured, and survival was monitored on days 0, 3, 7, 26, and 90.

Explants growth

When fragments became functional explants (active, filter-feeding sponges), we monitored explant growth under different treatments: *in vitro* with antibiotics *vs.* in a closed aquarium system without antibiotics, and *in vitro* using bacteria *vs.* algae as a food source.

The *in vitro* system (N = 20) consisted of plastic containers filled with 2 l of filtered sterile seawater to which antibiotics were added (see above). Containers were placed in culture chambers at a constant temperature of 14ºC in the dark to prevent algal contamination. The closed aquarium system consisted of several connected aquaria (30 l each) through which water flowed driven by a pump. Water was filtered mechanically and then sterilised by ozone and ultraviolet light. Water temperature was 14ºC and darkness was applied.

A mixture of heterotrophic Mediterranean bacteria (ca.1 µm in size) grown on marine broth was added as a food to the two systems. Explants were fed regularly twice a week by adding bacteria up to a concentration of 10^6 cells·ml^{-1} in the container (to mimic the natural bacteria concentration in the sea (Sieburth 1976, Isao *et al.* 1990, Kennish 1994). Seawater was changed and antibiotics were added 12 h after food supply. In the closed aquarium system, explants were also fed twice a week with the same bacterial concentration. Water flow was stopped before food supply and then restored 24 h later.

In both systems water chemical parameters were monitored during the culture. Water samples were collected before feeding the explants to analyse nutrients (silicates, phosphates, nitrites and nitrates) and total organic carbon and nitrogen concentrations. Nutrients were analysed by colorimetric techniques (autoanalyser Technicon). For the analysis of particulate organic matter (C:N), a known volume of seawater was passed through a 0.2 µm diameter, GF/F glass fibre filter, previously exposed to hydrochloric acid vapour for 48 hours in order to eliminate any inorganic material. Filters containing the organic matter were dried and analysed with a C:H:N autoanalyser Eager 200 (Cebrian *et al.* 2003).

To test the effect of food on explant growth, *in vitro* explants were placed in containers at a constant temperature of 14ºC. A set of explants (N = 22) was fed with a mixture of marine bacteria at a concentration of 10^6 cells·ml^{-1} and another set (N = 21) was fed with a concentration of 10^5 cells·ml^{-1} of the algae *Chlorella sp.* (3µm in size) grown in F/2 medium. These two concentrations mimic the natural sea concentration of bacteria and phytoplankton, respectively. The explants were fed twice a week. We changed the water 12 h after food supply.

Explant growth under different treatments was monitored by digital camera (SPOT cooled colour), and the explant areas were calculated with an image analysis program (NIH Image, public domain). Since the explants mainly grew in two dimensions, changes in area are a good estimate of changes in biomass (Turon *et al.* 1998, Mariani *et al.* 2000). From the changes in area over time, a growth rate GR_t was computed from the formula:

$$GR_t = (A_t - A_{t-1}) / A_{t-1}$$

Where A_t and A_{t-1} are the areas at time t and at the previous time, respectively. This growth rate is the change in area relative to area at the previous time (Turon *et al.* 1998).

Bioactivity

The toxicity of individuals of *Corticium candelabrum* collected from their natural habitat and explants living for more than six months in the *in vitro* cultures was measured by the standardized Microtox® assay (Becerro *et al.* 1995, Martí *et al.* 2004).

Statistical methods

Survival was assessed as the percentage of fragments that were alive on a given day related to the initial number of fragments. Mean differences in survival between seasons of collection were compared by a *t*-test at each monitored day. Differences in survival for the remaining treatments were analysed by two-sample and multiple-sample (ectosome/choanosome ratio, three levels) Gehan's Wilcoxon tests. When necessary for parametric analysis, normality and homogeneity of data were determined by a K-S and Lilliefors test and Levene's test, respectively

Differences in growth rates between treatments and in toxicity between wild and cultured sponges were analysed by *t*-test. When the two culture systems (*in vitro* and in aquaria closed system) were compared, data were log transformed to meet the assumptions of this parametric test.

Results

Morphological and ultrastructural changes during formation of explants

During the first two hours, cuttings maintained the same morphology as an adult (**Fig. 1A**). Cells then moved towards the sponge periphery and spread on the substrate (**Fig. 1B**). Cell spreading increased pre-explant area but this did not imply pre-explant growth. In parallel, the border of the cuttings started to heal. From one to two weeks later, we could differentiate totally disorganized fragments (**Fig. 1C**) from partially organized fragments (**Fig. 1D**). The latter showed an almost healed central mass, similar in colour and appearance to wild sponges, surrounded by a layer of dispersed cells. Fragments aged 2-4 weeks, which were totally disorganized, died leaving a thin spicule layer (**Fig. 1E**). The partially organized fragments became compact and globular in shape (**Fig. 1F**). These fragments can be considered functional explants since they were similar to adults in shape and showed active water exchange. One- to two-month-old explants

adhered to the substrate by body extensions (**Fig. 1G**). During the process we observed spontaneous formation of primmorphs (**Fig. 1H**).

The ultrastructure of fragments at time 0 (recently collected wild sponges) showed well-structured conducts and choanocyte chambers (**Fig. 2A**). Choanocytes (the basic cell type of sponges, **Fig. 2B**) displayed their typical shape and elements (choana and flagellum, **Fig. 2C**). Several cell types other than choanocytes were also observed: endopinacocytes (pseudoepithelial cells, **Fig. 2D**), spherulous cells (**Fig. 2E**), which may contain the bioactive compounds (Uriz *et al.* 1996a, Turon *et al.* 2000a), and collencytes (cells that segregate collagen) (**Fig. 2F-G**). Spicules with an axial filament (protein filament), which indicates recent spicule formation (Uriz *et al.* 2003), were also present (**Fig. 2H**).

TEM images of 24 h-old fragments showed disorganized conducts and choanocyte chambers, mainly at the periphery (**Fig. 3A**). Choanocytes were rounded and lacked choana and flagellum (**Fig. 3B**). No other cell types could be distinguished.

TEM images of two-month-old explants showed a similar ultrastructural pattern to wild sponges. Conducts and choanocyte chambers were reorganized (**Fig. 4A**). Choanocytes recovered their typical morphology with flagellum and choana (**Fig. 4B**). We also observed collencytes (**Fig. 4C**) and spicules with an axial filament (**Fig. 4D**).

Symbiotic bacteria were abundant in samples from wild sponges. Six different bacterial morphologies could be differentiated (**Fig. 5A**). Symbiotic bacteria degenerated in 24 h-old fragments, giving rise to broken membranes (**Fig. 5B**). Only three morphological types of bacteria could be distinguished and they were less abundant than in wild sponges. TEM images of two-months old explants showed the same bacterial types at similar abundance to wild sponges (**Fig. 5C**) despite antibiotic was added.

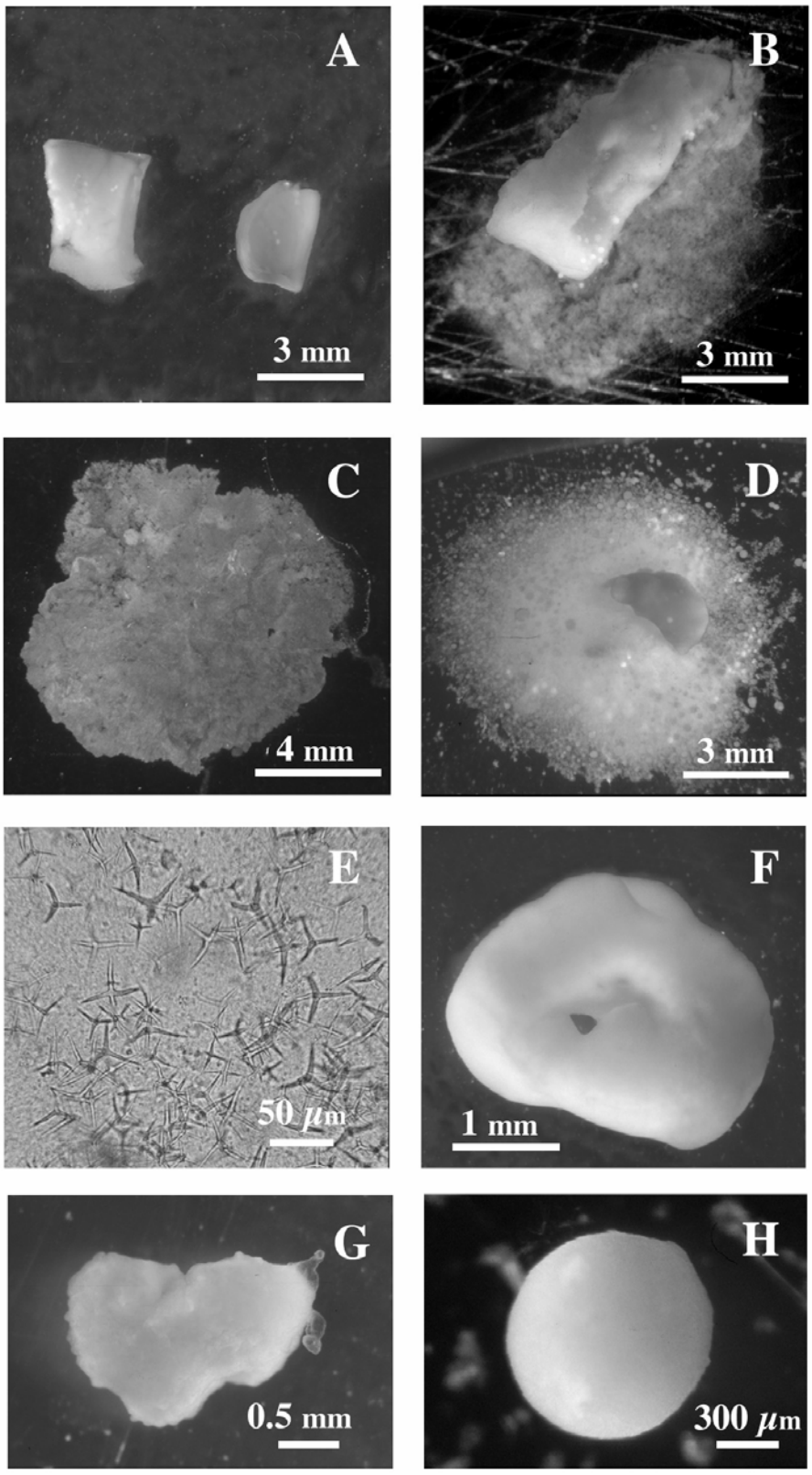

Fig. 1- *Corticium candelabrum.* Morphological changes of fragments during explant formation **A:** Newly cut fragments. **B:** After 2h, peripheral cells spread on the substrate. **C:** Totally disorganised fragments two weeks later. **D:** Partially organised fragments with a healed central mass. **E:** Spicules layer from totally disorganised fragments. **F:** Functional explant 4 weeks later. **G:** Functional explant showing body extensions for adhering to the substrate. **H:** Spontaneously formed primmorph.

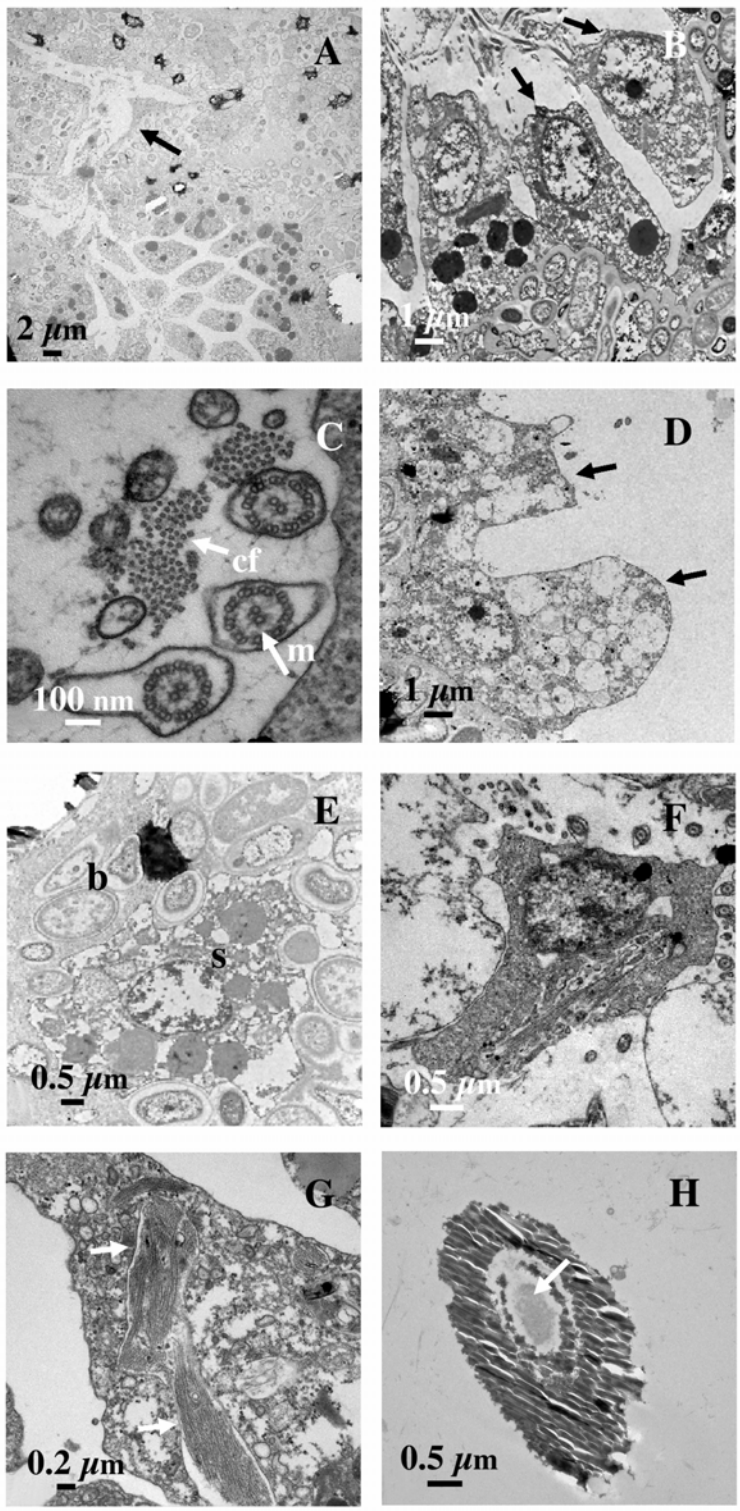

Fig. 2- *Corticium candelabrum.* TEM images of ultrastructural aspects of wild sponges. **A:** Well organised conducts (arrow) and choanocyte chambers (cc); **B:** Typical elongate in shape choanocytes (arrows) with choana (ch) and flagellum (f); **C:** Transversal section of choanocyte flagella (f) showing microtubules triplets (m) joined by glycocalyx and choana (ch); rough collagen fibrils (cf) are also visible; **D:** Endopinacocytes (arrows) lining a conduct (co). **E:** Spherulous cell (s) surrounded by symbiotic bacteria (b); **F:** Collencyte containing collagen fibrils (arrow). **G:** detail of a collencyte containing collagen fibrils (arrows); **H:** Transversal section of a spicule showing an irregular central axial filament (arrow).

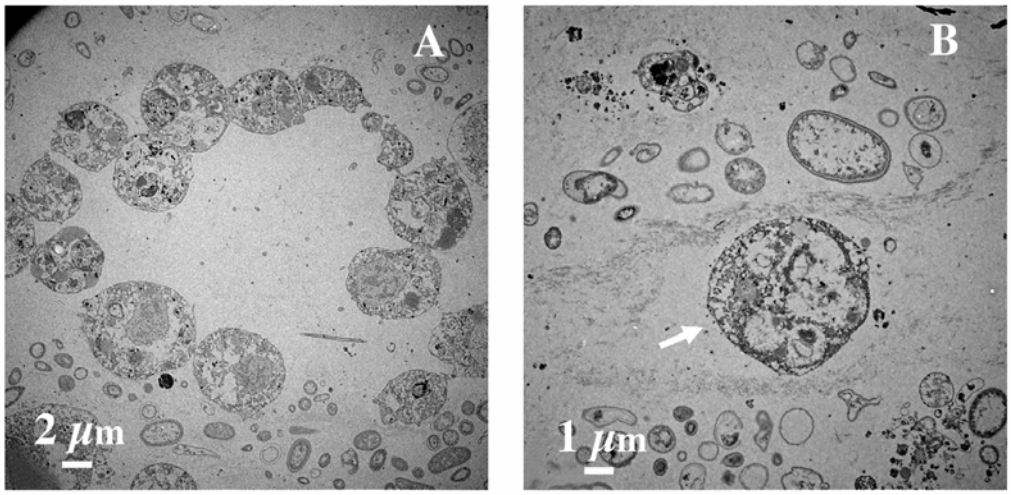

Fig. 3- *Corticium candelabrum.* TEM images of 24h-old fragments. **A:** Disorganised choanocyte chamber **B:** Rounded choanocyte (arrow) without choana or flagellum.

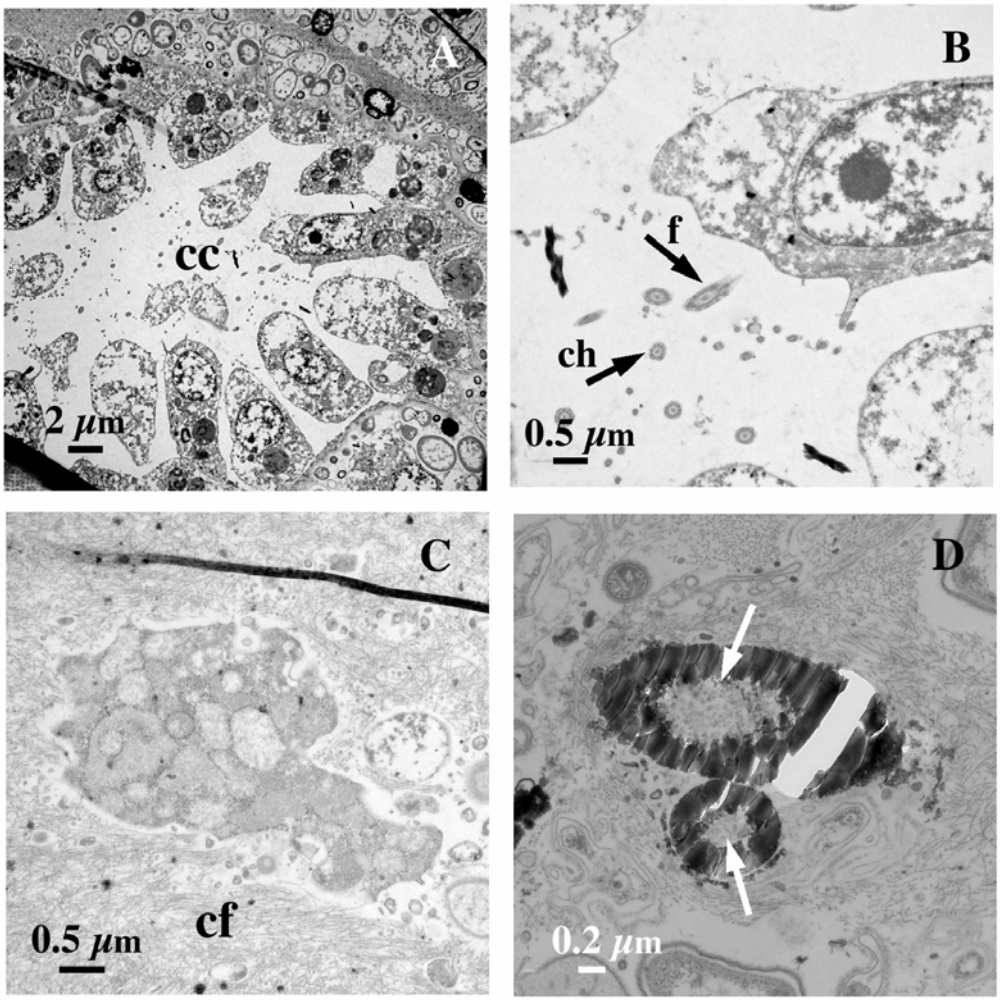

Fig. 4- *Corticium candelabrum.* TEM images of 2 month-old functional explants. **A:** Well-organised choanocyte chamber (cc); **B:** Typical in shape choanocyte with choana (ch) and flagellum (f); **C:** Collencyte surrounded by collagen (cf); **D:** Transverse section of two spicules with axial filament (arrows).

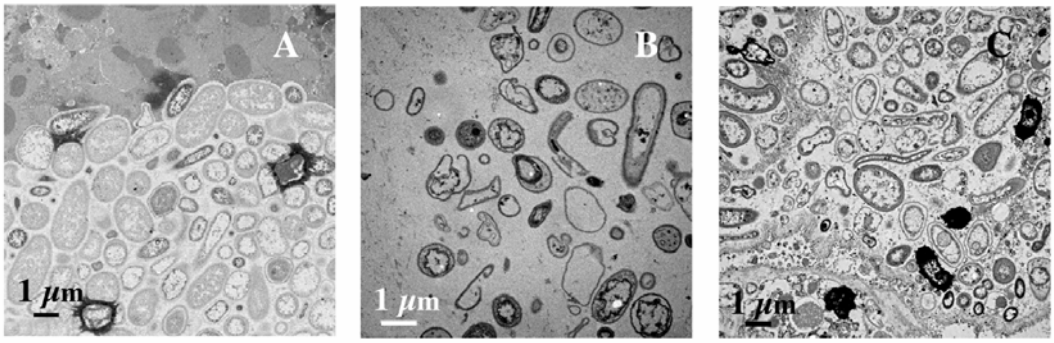

Fig. 5- *Corticium candelabrum.* TEM images of the sponge symbiotic bacteria. **A:** Healthy bacteria in wild sponges; **B:** Broken bacteria in 24h-old fragments; **C:** Healthy bacteria in two-month-old explants.

Pre-explant survival

Pre-explant survival was affected by the different culture conditions assayed: season of collection, water temperature, filtered *vs.* filtered-sterilised seawater, antibiotic addition, and ectosome ratio.

Mean survival during the first days was higher for pre-explants obtained from individuals collected in winter-spring (**Fig. 6**) but differences were not significant until day 21 ($p < 0.05$, *t*-test) (45.85% and 15.66% for pre-explants collected in winter-spring and summer-autumn, respectively). These survival rates were maintained until the end of the experiment (ca. 12 months).

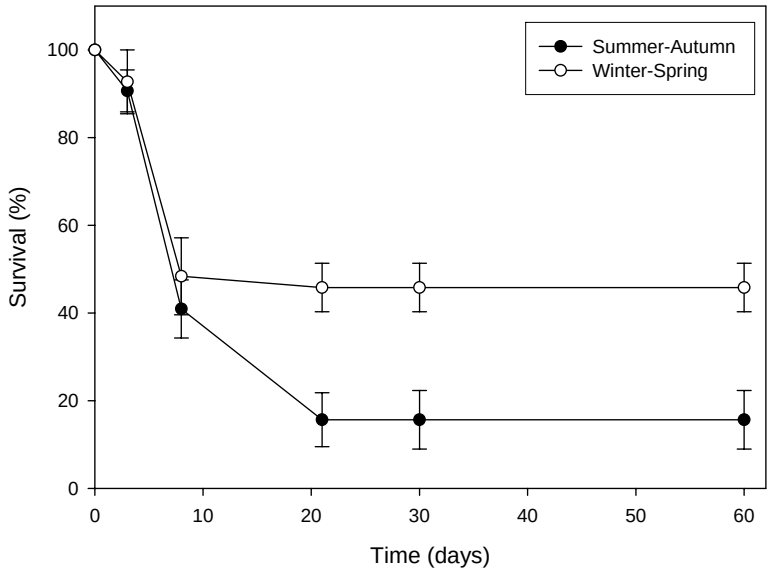

Fig. 6- Time course of pre-explant survival from sponges collected in winter-spring and in summer-autumn.

Water temperature determined differential survival in the cultures (**Fig. 7**). Survival was significantly higher ($p < 0.005$, Gehan's Wilcoxon test) in pre-explants cultured at the lower temperature (14ºC). All fragments cultured at 20ºC died before day 9.

Although survival was slightly higher for fragments cultured in filtered-sterilised seawater (**Fig. 8**), differences with respect to those cultured in only filtered seawater were not significant ($p = 0.36$, Gehan's Wilcoxon test).

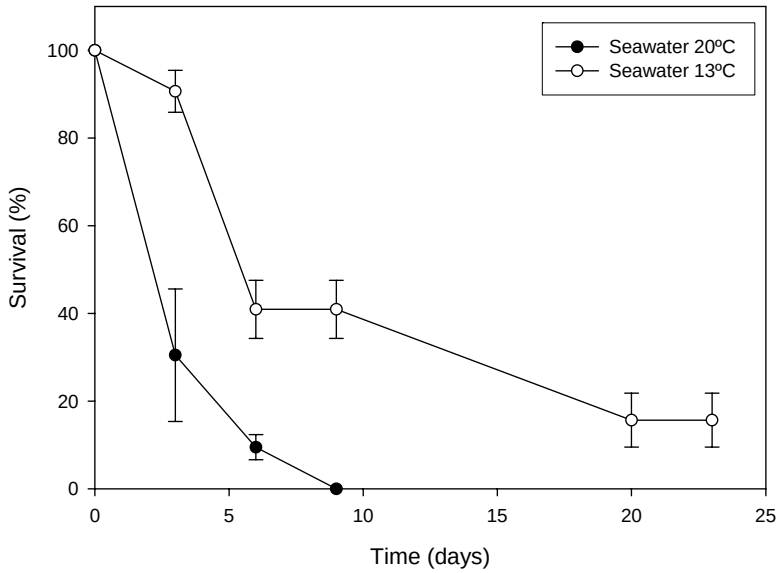

Fig. 7- Time course of pre-explant survival cultured at two different temperatures.

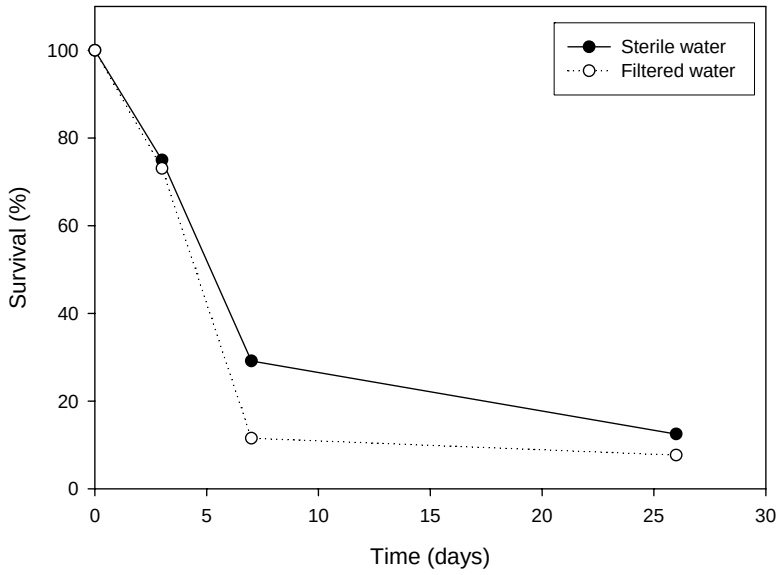

Fig. 8- Time course of pre-explant survival cultured in filtered and filtered-sterilised seawater (both with antibiotic addition).

Antibiotic addition significantly improved pre-explant survival ($p < 0.05$, Gehan's Wilcoxon test). Although differences were detected from day 3 (100 % *vs.* 83.3 % of survival for pre-explants with and without antibiotics, respectively), differences were particularly high after day 9, with a survival of 33.3 % for explants without antibiotics *vs.* 60 % for explants with antibiotics (**Fig. 9**).

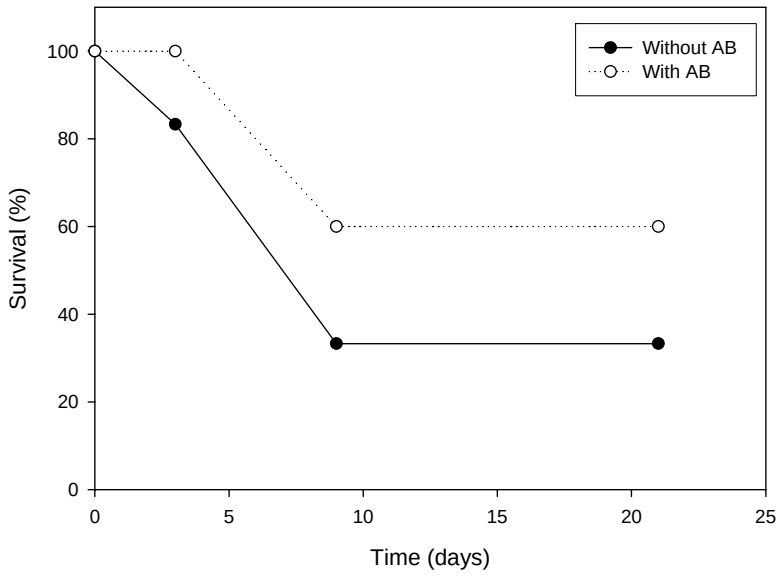

Fig. 9- Time course of pre-explant survival cultured with and without antibiotic addition.

The amount of ectosome relative to the choanosome of the fragments appeared to affect survival: the higher the ectosome proportion, the higher the survival. Fragments with 30 to 50 % of ectosome and with 10-30 % had significantly higher survival ($p < 0.05$, Gehan's Wilcoxon test) than explants with only a 1-10 % of ectosome (**Fig. 10**). Differences became evident on day 3 (only 12% of survival for fragments with 1-10 % of ectosome *vs.* 75% for the other two).

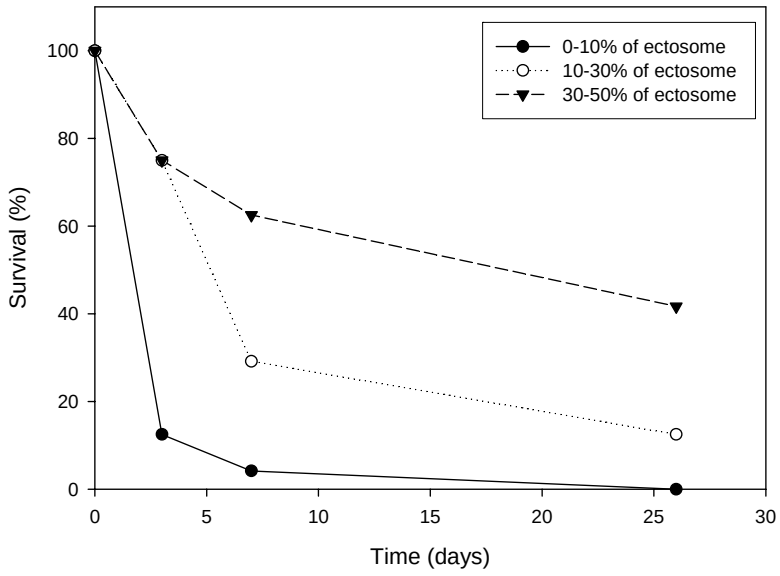

Fig. 10- Time course of pre-explant survival from fragments with different ectosome/choanosome proportions.

Explant growth

Explants in the two culture systems behaved differently ($p < 0.05$, *t*-test). In the closed aquarium system explants grew at a mean rate of 0.058 per month. In contrast, in the *in vitro* system with antibiotics, explants decreased their area at a mean rate of -0.235 for the same period (**Fig. 11**).

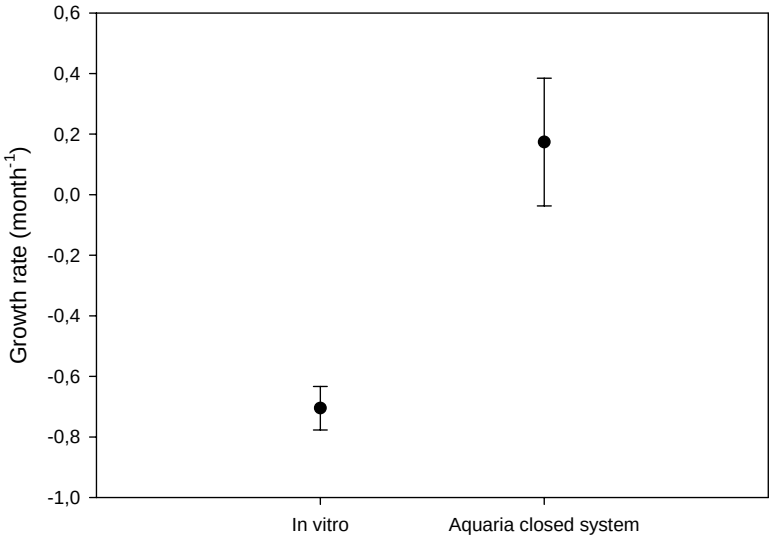

Fig. 11- Explants growth rate per three months *in vitro* and in the closed aquarium system.

Nutrients and total carbon in the water were in general higher in the closed aquarium system than in the *in vitro* system. Silicate, nitrate phosphate and nitrite values ranged between 15.87 and 61.85, 135.75 and 495.5, 4.45 and 11, 0.43 and 3.17 $\mu M \cdot l^{-1}$, respectively in the closed aquarium system, and between 1.83 and 61.7, 14.62 and 76.25, 3.34 and 9.65, 0.40 and 2.73 $\mu M \cdot l^{-1}$, respectively in the *in vitro* system. Total carbon ranged between 1095 and 4935$\mu g \cdot ml^{-1}$ both *in vitro* and in the closed aquarium system.

When different food was assayed, explants fed with algae (*Chlorella sp.*) showed a significantly higher growth rate ($p < 0.05$, *t*-test) than those fed with bacteria. These differences were not significant at month 1 (mean growth rate 0.065 and 0.164 for algae and bacteria food, respectively). However, at month 5 explants fed with *Chlorella sp*. had a higher growth rate ($p < 0.05$, *t*-test) than explants fed with a bacteria mixture (mean growth rate 0.038 and -0.1 per month, respectively) (**Fig. 12**).

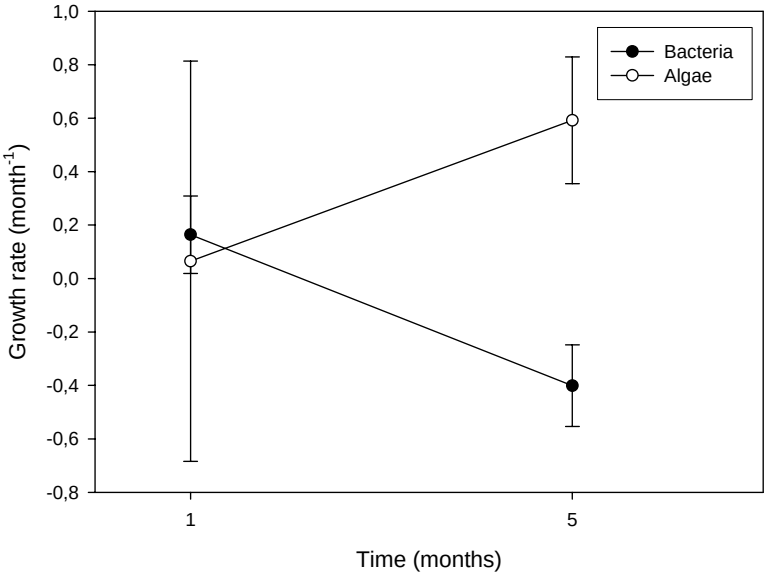

Fig. 12- Explant growth rates fed with bacteria and algae.

Bioactivity

No significant differences ($p > 0.05$, *t*-test) in toxicity (Microtox ®, EC50) were found between cultured explants and wild sponges. This indicates that explants continue to synthesize the bioactive compounds.

Discussion

Sponges are characterised by their plasticity and capacity to regenerate complete animals from fragments (Briens 1973). However, species such as *Corticium candelabrum* regenerate faster than others and even perform asexual reproduction by propagula, thus being particularly suitable for explant production.

The morphological and ultrastructural changes during explant formation from sponge fragments indicate that, after an initial phase of disorganisation and cell transformations, cell differentiation and rearrangement occur within a period of 2-4 weeks. During this period fragments do not grow but lose biomass, since the cells that spread on the substrate die. Dead cells were removed to reduce the risk of contamination.

The sponge symbiotic bacteria underwent a similar process: disintegration followed by recovery, likely by cell division. The healthy appearance of the bacteria in the explants despite the addition of antibiotics was unexpected. Although symbiotic bacteria might be resistant to the wide-spectrum antibiotic used, their healthy state may be due to isolation from the external medium and from the sponge cells by a collagen coat (see **Fig. 2**), which may protect them.

Among the several conditions assayed during explant formation, we found that fragments from sponges collected in winter and spring, cultured at 14ºC, with filtered sterilised seawater, antibiotic addition and a high ectosome/choanosome ratio survived longest.

The higher survival of fragments from individuals collected during the colder months of the year could be related to the biological cycle of *C. candelabrum,* which releases larvae in early summer (pers. obs.) and, as a result, its aquiferous system become disarranged (Rosell 1993). Fragments from sponges with disarranged conducts and chambers may be less able to rearrange into functional explants.

Lower temperature, antibiotic addition and water sterilisation diminish bacterial proliferation. Consequently, all these conditions promote explant survival during the critical first steps of the process in which a certain degree of cell death is inevitable and fragments are most sensitive to infections.

As previously reported for *Chondrosia reniformis* (Nickel & Brümmer 2003), a high ectosome proportion seems to favour healing of the cut faces. The ectosome is mainly formed by pinacocytes, which are pseudoepithelial cells involved in isolating the sponge from the external medium. These cells and the collencytes, which secrete the collagen (structural protein) and are particularly abundant in the ectosomal region, play an important role in healing (Connes 1968).

Growth rates were higher in the aquaria closed system without antibiotics than *in vitro* with antibiotics, although we fed both cultures in the same way. The higher nutrients and total carbon concentrations in the former (see results) and a negative effect of antibiotics on sponge growth in the latter might account for the differences.

Previous authors disagree about the optimal food source for the growth of sponges *in vitro*. Some chose marine algae (Barthel & Theede 1986, Osinga *et al.* 1999), others tried bacteria (Francis *et al.* 1990, Kaimori *et al.* 1998) and others chose a mixture of algae and bacteria (Nickel *et al.* 2000). The few studies dealing with sponge feeding *in situ* reported that sponges predominantly retain small organisms (< 4 µm) such as heterotrophic bacteria, cyanobacteria and prochlorophytes (Reiswig 1971, Pile *et al.* 1996, Ribes *et al.* 1999). Moreover, in semi-enclosed systems, fragments of *Halichondria panicea* (Pallas, 1766) were grown successfully with *Chlorella sp.* (Barthel & Theede 1986). Furthermore, the study by (Turon *et al.* 1997) on *Crambe crambe* and *Dysidea avara* demonstrated that although there were differences in filtering abilities with particle size, time and species, the most efficiently retained particles were smaller than 4 µm. On the basis of these results, we selected heterotrophic bacteria (1 µm) and the alga *Chlorella sp.* (3 µm) as the two alternative food sources. Our study showed that growth was higher in the cultures fed with *Chorella sp.* than with bacteria, possibly due to the higher carbon content of the former.

Although the growth rates found in our cultures appear to be low even when we grew explants with *Chlorella*, the values correspond to the growth rates found for wild populations of *C. candelabrum* in winter (-0.1- 0.1 per month), when sea T is similar to the T in our cultures (ca. 14 ºC). Higher temperatures are expected to enhance explant growth, as occurs for wild sponges in summer (0.3-0.7 per month, authors, current research) and are here proposed for culturing this species once the explants are functional and the risk of bacterial contamination has been reduced.

The characteristic bioactivity of *C. candelabrum* was maintained in explants cultured for more than six months. Some bioactive compounds originally isolated from invertebrates such as sponges have been subsequently localized in microbial associates (Bewley *et al.* 1996). However, we cannot state yet whether the bioactivity of *C. candelabrum* is due to the sponge cells or to the symbiotic bacteria, since both healthy bacteria populations and bioactivity were maintained in the cultured explants. Further studies are required to assess the role of symbiotic bacteria (if any) in the biosynthesis of the bioactive compounds of *C.*

candelabrum. It is also important to establish the relationships between bacteria populations and between bacteria and sponge cells, since competition for resources might determine the production of the toxic metabolites with potential applications.

In this preliminary study, we have fixed some of the favourable conditions for enhancing explant survival and growth of a Mediterranean bacteriosponge, and showed that antibiotics do not affect the symbiotic bacteria. Temperature and food seem to be decisive for explants survival and growth. Thus, we propose summer temperatures and continuous algae supply in a closed aquarium system (bioreactor) to improve culture yield.

Acknowledgements

Our special thanks go to Rene Wijffels and his team for giving us the opportunity to participate at the Marine Biotechnology Conference in Matalascañas. We also thank X. Turon for helping with the electron microscope, E. Cebrian and R. Martí for field work and E. Adell and M. Bardají for laboratory work. This study was partially funded by grants INTERREG III A France/Spain 2000-2006 from the EU and GENECO REN 2001-2312-CO3-01/MAR from the CICYT (Spanish Government).

Chapter IV

Cultivation of sponge larvae: Settlement, survival and growth of juveniles

de Caralt S, Otjens H, Uriz MJ & Wijffels RH (2007) Cultivation of sponge larvae: settlement, survival and growth of juveniles. Marine Biotechnology *(in press)*

Abstract

The aim of this study was to culture sponge juveniles from larvae. Starting from larvae we expected to enhance the survival and growth, and to decrease the variation in these parameters during the sponge cultures. First, settlement success, morphological changes during metamorphosis and survival of *Dysidea avara, Ircinia oros, Hippospongia communis*, under the same culture conditions, were compared. In a second step, we tested the effects of flow and food on survival and growth of juveniles from *D. avara* and *Crambe crambe*. Finally, in a third experiment, we monitored survival and growth of juveniles of *D. avara* and *C. crambe* transplanted to the sea to compare laboratory and field results. The results altogether indicated that sponge culture from larvae is a promising method for sponge supply and that laboratory culture under controlled conditions is preferred over sea cultures in order to prevent biomass losses during these early life stages.

Introduction

Sponges produce secondary metabolites with pharmaceutical interest (e.g. Blunt *et al.* 2005, Sipkema *et al.* 2005a). The supply of these bioactive metabolites in sufficient amounts for the pre-clinical and clinical assays is an unsolved problem (e.g. Fusetani 2000, Proksch *et al.* 2003, Sipkema *et al.* 2005b). Chemical synthesis is too expensive and wild sponge collection has a negative impact on the environment and put in danger the sustainable use of the marine resources. Sponge culture have been assayed since the beginning of the last century (e.g. Moore 1910); more recently, besides mariculture (*in situ* sponge culture) (e.g. Pronzato *et al.* 1999, Duckworth & Battershill 2003, Mendola 2003), three methods for culturing sponges, under controlled conditions have been used: explant culture (e.g. Osinga *et al.* 2001, de Caralt *et al.* 2003, Hoffmann *et al.* 2003, Nickel & Brümmer 2003), primmorphs (Müller *et al.* 1999, Zhang *et al.* 2003), and cells (e.g. Pomponi & Willoughby 1994, Sipkema *et al.* 2003b, De Rosa *et al.* 2003). However, most of these methods have encountered drawbacks as for survival and contamination (especially cell cultures) and/or growth rates (explants and primmorphs), and more research is to be done in order to make them suitable for scaling up the sponge biomass production.

In explant cultures, we make use of the regenerative capacity of sponges (Simpson 1984). It has been shown, however, that explants have very slow and variable grow rates (Osinga *et al.* 1999, de Caralt *et al.* 2003, Sipkema *et al.* 2006). The large variability in growth rates of explants could be due to the different ages of the sponges from which the explants were made of, since slow and variable growth has also been reported for adult sponges in the sea (e.g. Turon *et al.* 1998, Garrabou & Zabala 1999). To optimize and standardize culture conditions, we should prevent this variability by culturing sponges of the same age. However, age-classes cannot be determined for adult sponges since their size is not necessarily related to their age. As a consequence, it is difficult to avoid variation in cultures of explants obtained from adult individuals.

In vitro cultures of sponges have been developed from adult individuals. Long-term continuously proliferating cell cultures have failed so far, and the

maintenance of axenic cell lines (i.e. microbial-free) has proved to be difficult (Rinkevich 2005). Continuous cell-lines require proliferative stem cells. In adult sponges, the cells closer to stem cells are archeocytes, which are able to differentiate in several cell types (e.g. oocytes, sclerocytes, pinacocytes). Some authors have performed cell culture from archeocyte enriched cell fractions, which presented active DNA synthesis, but failed to obtain continuous cell proliferation due to an early cell death (e.g. Pomponi & Willoughby 1994, Zhang *et al.* 2003).

Embryos are a source of totipotent and proliferating cells, and thus embryonic cells could be a good source to start cell culture from. However, only one attempt to culture cells from sponge embryos has been reported. The results were promising as the culture was more resistant to infections by microorganisms, and survived longer than those from adult cells (Rinkevich *et al.* 1998).

By culturing sponges from larvae, we attempt to circumvent the three main constraints that are commonly found in cultures from adults. First, we expect higher growth rates during the juvenile stages of sponges, as this has been reported for small sized individuals (Garrabou & Zabala 1999). Moreover, variability may be reduced, since culturing larvae warrants the same age for all the individuals (cohorts) and a similar behaviour under culture. Second, larval survival in the laboratory is expected to be higher than at sea because of the absence of predators and competitors (e.g. Uriz *et al.* 1996a). Third, larval culture can be a source of more suitable starting material (embryonic cells) for the development of cell culture. Consequently, larval culture offers some advantages with respect to other assayed methods.

The sponge species for our study were: *Dysidea avara, Ircinia oros, Hippospongia communis,* and *Crambe crambe*. These species have been selected because of their interest for pharmaceutical applications and their abundance in western Mediterranean. They produce several bioactive compounds with a commercial value. *D. avara* produces avarol (e.g. Uriz *et al.* 1996b), a sesquiterpenoid antitumor (Müller *et al.* 1985) and antiviral compound (Sarin *et al.* 1987) that is a potent *in vitro* inhibitor of HIV-1 RT. (Loya & Hizi 1990). *I. oros* produces a cyclic sesterpenoid (Cimino *et al.* 1972) with anti-inflammatory

properties. *H. communis* has antifungal activity due to the production of untenospongin B (Rifai *et al.* 2004). Finally, *C. crambe* produces crambines and crambescidins, which are derivates of a pentacyclic guanidine with anticancer and antiviral activities (Jares-Erijman *et al.* 1991, Balconi *et al.* 1995).

The general objective of this study was to culture sponges from larvae, which was never done before, in order to try to improve biomass supply for biotechnological purposes. The study was performed in the laboratory under several food and flow conditions, and at sea. Three experiments with different goals were conducted. The aim of the first experiment was to select the most adequate species for culturing among *D. avara*, *I. oros* and *H. communis*. In this experiment, settling, metamorphosis, and juvenile survival in the laboratory were monitored. In the second experiment, we assayed larval culture of *D. avara,* the most successful species in the previous experiment, and *C. crambe*, already reported to have high survival (Uriz *et al.* 1998) under different environmental conditions. Finally, a third experiment was addressed to compare juvenile survival and growth of settlers of both species (*D. avara* and *C. crambe*) after transplanting them to the sea.

Materials and methods

Collection, maintenance of sponge larvae, and experimental procedures

For the first experiment, ripe individuals of *Ircinia oros, Hippospongia communis,* and *Dysidea avara* were collected from l'Escala (western Mediterranean Sea) and transferred to an open aquarium system (CMIMA-CSIC, Barcelona, Spain) at the beginning of June of 2003. *I. oros* and *H. communis* released larvae in mid July, and *D. avara* released at the end of July (similar periods to those previously reported in sponges at sea, Mariani *et al.* 2005).

Swimming larvae of *I. oros* (N = 224), *H. communis* (N = 30) and *D. avara* (N = 230) were collected with a pipette and transferred to 6-multi-well dishes, which were placed in aquaria filled with filtered (0.7 µm pore diameter) seawater at field temperature (20° C). After 24h, the larvae started to settle spontaneously on

the 6-multi-well dishes bottom and metamorphosed becoming juveniles after 5 - 7 days. We monitored settlement success, morphological changes and survival of settlers in time.

For the second experiment, we selected *D. avara,* the most successful species in the previous experiment, and *C. crambe*, already reported to have high survival (Uriz *et al.* 1998).

Larvae of *D. avara* were taken after release from ripe sponges maintained in the open system aquarium (CMIMA) in July 2004, as in the previous experiment. Conversely, larvae of *C. crambe* were obtained by carefully tearing ripe individuals (Uriz *et al.* 1998) collected from the sea (Blanes, western Mediterranean). Larvae were placed in aquaria filled with filtered (0.7 µm pore diameter) seawater at field temperature (20 ºC). The aquaria bottom was covered by plastic (11 x 8 cm) sheets where larvae settled spontaneously. After settlement, juveniles of *C. crambe* (N = 50) and *D. avara* (N = 30) were submitted to the experimental conditions, in aquaria of Wageningen University (Sipkema *et al.* 2006). The variables measured were survival and growth.

Flow treatment consisted of unidirectional, constant flow of 5 cm/s, and was compared against static condition. In this experiment living algae was used as a food.

Food treatment consisted in feeding the juveniles with $5{\cdot}10^{5}$ cells/ml of living algae *vs.* feeding the juveniles with dried algae (equivalent carbon content). The alga used, as a source of food, was *Phaeodactylum tricornutum* (proven suitable in previous studies, Osinga *et al.* 2003, Sipkema *et al.* 2006). This treatment was conducted under the above-mentioned flow conditions.

The third experiment was also conducted on *D. avara* (N = 164) and *C. crambe* (N = 175). Released larvae from ripe sponges (July 2005), were settled on rigid plastic plates in the laboratory and 1 week later they were transferred to the sea (western Mediterranean). Survival and growth were monitored as a function of time.

Morphology, survival and growth rates measurements

Morphological features, during metamorphosis and juvenile stages, were observed through a stereo-microscope. Survival and growth rates were monitored every 15 days by taking pictures through a stereomicroscope (in the laboratory) and with an underwater camera (in the field). Image analysis was performed to quantify increases in area of juveniles by the NIH Image (public domain) software.

In the first experiment, settlement success was calculated as the percentage of larvae attached to the substratum after one week with respect the total number of swimming larvae.

Survival of juveniles was assessed in the three experiments as the percentage of juveniles that were alive on a given day. Death of juveniles was made evident by changes in colour, shape and size (i.e. paler colour, rounder shape, and small size) ending in sponge disaggregation. To compare two survival curves, we used the Gehan's Wilcoxon Test statistics. For comparing more than two survival curves, we used the analysis Comparing Survival in Multiple Groups (Statistica software).

Growth rates were derived from changes in the sponge area with time (i.e. 15 days) by the equation:

$$GR_t = ((A_t - A_{t-1}) / A_{t-1}) / t$$

Where A_t and A_{t-1} are the sponge areas at time t and at time t-1, respectively (Turon *et al.* 1998, Garrabou & Zabala 1999, Mariani *et al.* 2000). The changes in surface area are an acceptable estimation of growth when organisms mainly grow in two dimensions, as encrusting sponges do (Turon *et al.* 1998). Although, the method did not take into account increases in thickness and thus the measured growth rates could underestimate true growth, the method was suitable here because juveniles were relatively flat and the measures obtained were only used with comparative purposes.

Differences in growth rates between treatments and species were analyzed statistically using a nonparametric version of the repeated-measures

analysis of variance (ANOVAR). This method was used because data did not meet the circularity assumption (Mauchly's sphericity test) required by parametric ANOVAR (Potvin *et al.* 1990, Von Ende 1993). It is based on a permutation test by means of a two-level randomisation method (Turon *et al.* 1998, Manly 1991). The whole series of data was randomised 4999 times (plus the observed one) to approximate the null hypothesis distribution of the sum of squares for each factor and their interaction, and then we examined how extreme were the observed values in this distribution. An effect was judged significant when the observed sum of squares was exceeded by less than 5% of the corresponding values in the randomisation series. A modified version on the Turbopascal program used by Turon *et al.* (1998) was applied to perform the permutation tests.

Differences in growth rates on juveniles fed with either living or dried algae at the last point time of the culture were analysed by a two-way ANOVA. Data normality and homogeneity were determined by a Kolmogorov-Smirnov test and the Levene's test, respectively.

To compare the extent of variation in growth rates in adult individuals (Literature data) and in larvae cultures, the variation coefficients were calculated as a (standard error/ mean) x 100.

Results & Discussion

Larval collection and morphological changes at settling

Sponge larvae were obtained in the laboratory from ripe sponges: 224 larvae from *Ircinia o*ros, 30 from *Hippospongia communis*, and 424 from *Dysidea avara*, and 325 from *Crambe crambe*. This method is the most effective to obtain sufficient larvae for reproducible experiments (i.e. enough replicates per treatment). Alternative methods such as collecting sponge larvae from the field are more complex because of the relatively short period of larval release and the fast larval dilution once released (Mariani *et al.* 2005). In addition, there are few studies about the period of larval release, which is species-specific and may vary depending on the geographical location (water temperature, hydrodynamism).

The larvae of *H. communis, I. oros, D. avara* (Fig. 1A) and *C. crambe* (Fig. 1B), are parenchymella that consist of a pseudostratified layer of flagellated cells surrounding an internal mass of cells (Boury-Esnault & Rützler 1997). Larvae swam during a variable but short period (from 3 to 7 days in the laboratory) before settling. Usually, *D. avara* and *C. crambe* larvae settled sooner than *H. communis* and *I. oros* larvae. Larvae of the four species attached to the substrate through their posterior pole and became hemispherical. Settled larvae flattened as the cells spread on the substrate ("growth marginal layer") and initiated metamorphosis. During metamorphosis, the few cell types present in the larva differentiated into several adult cell types (e.g. Amano & Hori 1996, 2001) and rearranged to develop the aquiferous system. Settled larvae completed metamorphosis in about one week and a prominent exhalant tube was formed. From then on, juvenile sponges were able to filter water and feed. Nevertheless, settlers of *H. communis* and *I. oros* did not show conspicuous inhalant/exhalant orifices even through light microscope. At this state, most juveniles of the two species died before forming the skeletal fibres.

Most juveniles of *D. avara* and *C. crambe* survived allowing morphological changes to be monitored. Two days after settlement a monolayered pseudoepithelium (growth marginal layer) was visible through the

stereomicroscope (**Fig. 1C, D**). In the case of *D. avara*, 1-week old juveniles already showed from 20 to 80 choanocyte chambers and a long exhalant tube (**Fig. 2A**). These early juveniles are called "rhagons". Fifteen days after settlement, some structural elements were formed: rudiments of spongin fibres in *D. avara* (**Fig. 2B, C**) and spicules and collagen in *C. crambe.* Choanocyte chambers increased in number and were densely packed. In that stage, sponge juveniles grew mainly in height. One month after settlement, the choanocyte chambers are connected to a complex network of inhalant and exhalant canals. The completely organised exhalant canals flow into a cloacal exhalant tube that ends in an osculum (**Fig. 2C**). In some of these juveniles with over 200 choanocyte chambers, more than one exhalant tube was present (**Figs. 2C, D**).

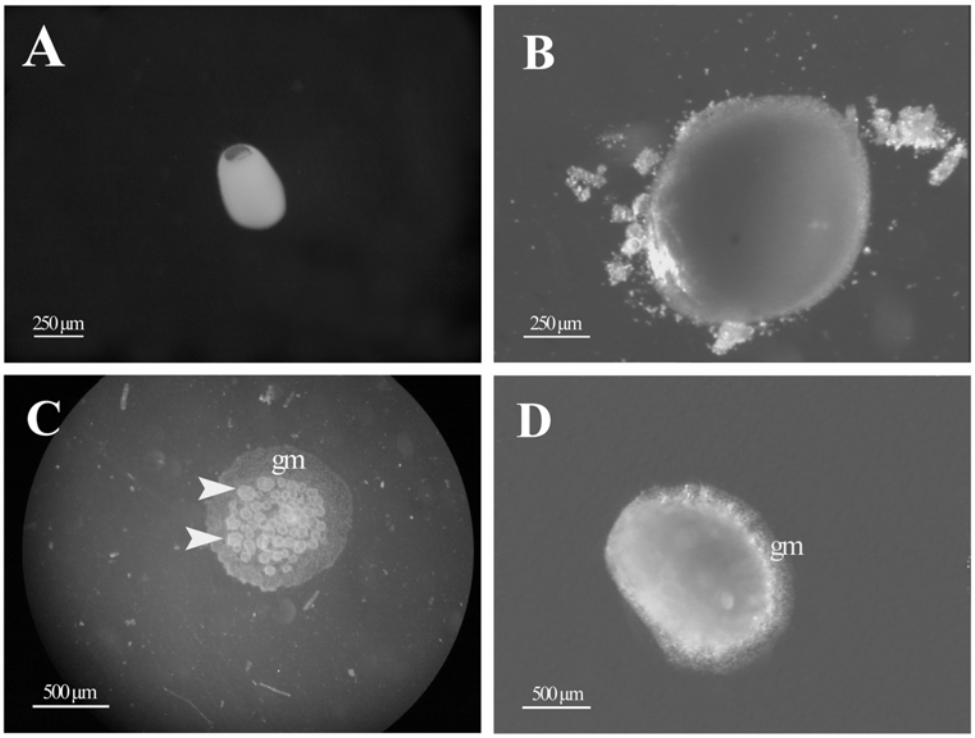

Fig. 1- A: Larva of *D. avara*. **B:** Larva of *C. crambe*. **C:** *D. avara* juvenile after two days from settlement: (arrow heads) choanocyte chambers, (gm) growth marginal layer -the white points correspond to cells. **D:** *C. crambe* juveniles after two days from settlement: (gm) growth marginal layer.

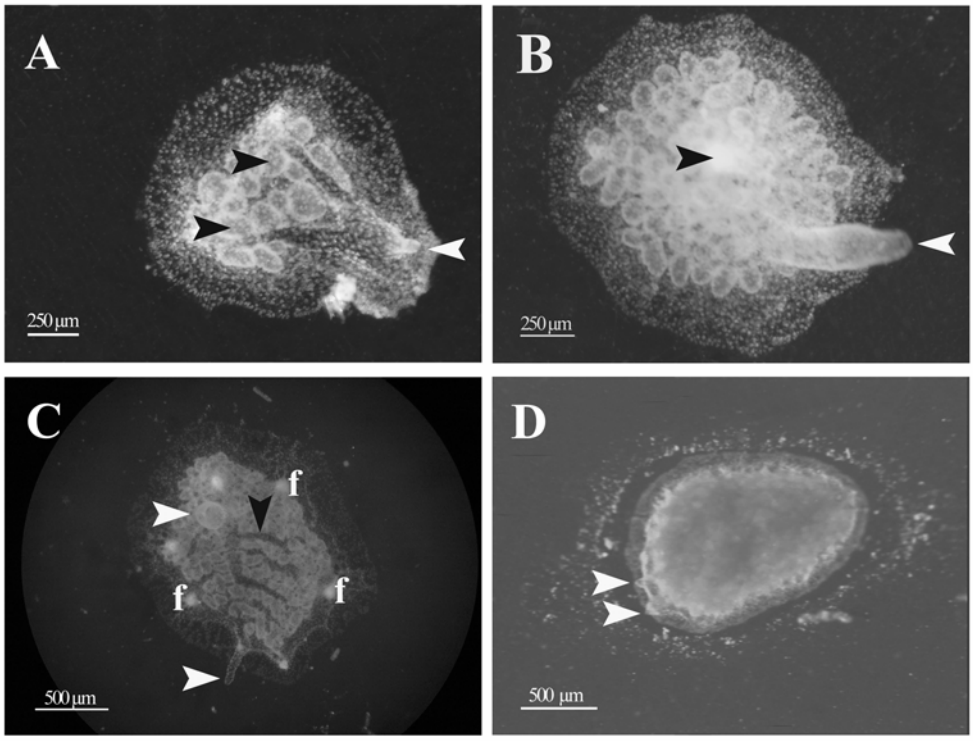

Fig. 2- A: *D. avara,* 1 week-old juveniles: (black arrow heads) few choanocyte chambers, (white arrow head) exhalant tube. B: *D. avara,* 15 days-old juvenile: (black arrow head) skeletal fiber, (white arrow head) exhalant tube. C: *D. avara* one month-old juveniles: (f) skeletal fibers, (black arrow head) canals, (white arrow heads) exhalant tubes. D: *C. crambe*, 15 days-old juveniles: (arrow heads) two exhalant tubes.

Settlement success, survival and growth rates of juveniles

Species comparison

In the first experiment, differences in settlement success and survival were found among *H. communis*, I. oros and D. avara. The three species presented a high settlement success: 66.6 % in H. communis and 99.5 % in D. avara and 94.5 % I. oros (Fig. 3). The different percentage of settlers for the three species under the same laboratory conditions suggests that success in settlement may depend on species-specific environmental requirements and/or particular larval characteristics. Although general acceptable conditions have been found in our cultures since the settlement rates obtained appeared to be higher than those at sea (Uriz *et al.* 1998), optimal culture conditions should be species-specific. The high settlement success obtained in the laboratory may be due to the extremely favourable conditions that larvae encountered there for settlement (i.e. still water and no substrate competition). At sea, larvae do not settle immediately but are

transported by currents and must swim until they find appropriate conditions (e.g. Kaye & Reiswig 1991, Uriz *et al.* 1998). During the swimming period, larvae are spending their reserves, which may become depleted before settling. Depletion of reserves in early life stages may strongly affect the success in the next stages. It has been reported for other invertebrates that adults perform worse when they originate from settlers with lower energy contents (Marshall & Keough 2003). It also has been shown for the demosponge *Sigmadocia caerulea* that juveniles originating from larvae that settled fast survived better, grew faster and were more regular in shape than those originating from long living larvae (Maldonado & Young 1999).

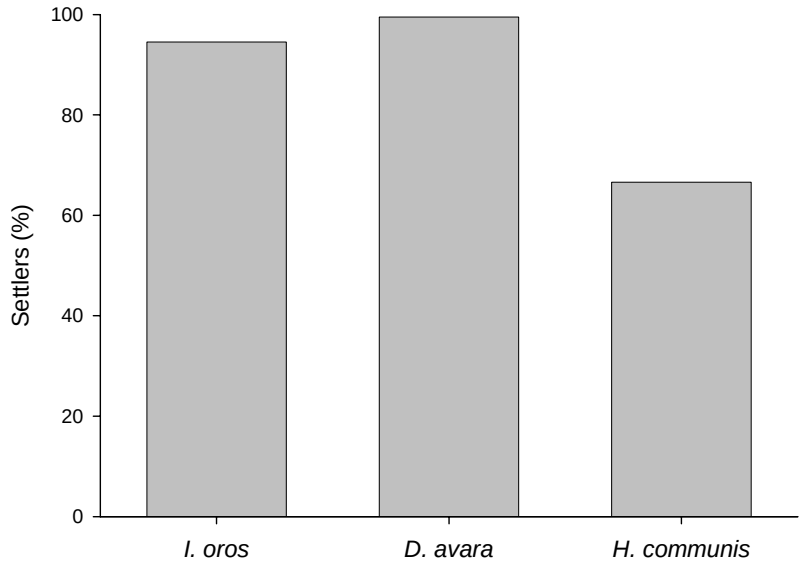

Fig. 3- Settlement success of larvae of *D. avara, H. communis* and *I. oros* under the same laboratory conditions (filtrated sweater at 20 ºC under static conditions).

Survival of juveniles with time was significantly different among the three species ($P < 0.05$, Comparing Survival in Multiple Groups) (**Fig. 4**). *D. avara* was the species with the highest percentage of survivors ($P < 0.05$, in both comparisons) along the experiment. Survival in this species ranged from 100 % (at day 5) to ca. 80 % (at day 80) reaching 62 %, at the end of the experiment (after three months). There were no significant differences ($P = 0.8943$, Gehan's Wilcoxon Test) between *H. communis* and *I. oros*. Only 15% of *H. communis* juveniles survived at the end of the experiment, and no survivors of *I. oros* occurred after day 50, despite settlement success was high (see above).

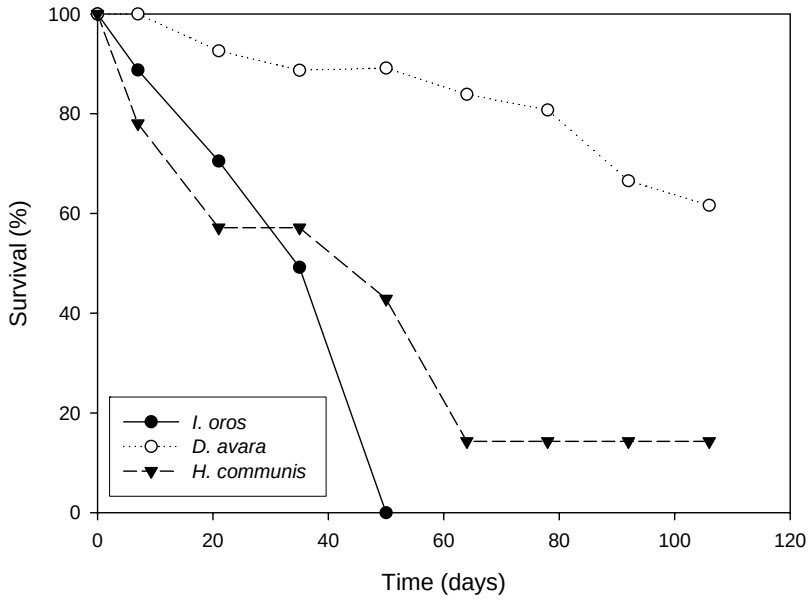

Fig. 4- Survival of *D. avara*, *I. oros* and *H. communis* juveniles with time under the same laboratory conditions (filtrated seawater at 20ºC under static conditions).

In the three species, a maximum increase in area took place at the beginning of the culture: at week 1 in *D. avara* and at week 2 in *H. communis* and *I. oros*. This maximum increase in area did not represent true growth but corresponded to biomass redistribution due to the reorganization processes involved in metamorphosis. After metamorphosis, juveniles displayed sequential phases of growth: first they increased in area by extending the marginal growth layer and then, they formed structural elements, grew in thickness, and decreased in area. Some juveniles that could not grow in thickness due to a physical barrier (sandwich culture) grew exclusively in area (**Fig. 5**), achieving the largest size. Thus, as stated in the methods section, we were aware that the measured growth underestimated real growth but was considered accurate enough for comparative purposes.

Taken into account settlement success and survival rates measured during the experiment, we selected *D. avara* among the three species studied, as a model for the next experiment in which different environmental conditions were assayed. *C. crambe* was selected as a second species because juveniles have been reported to show high survival rates in a previous study (Uriz *et al.* 1998).

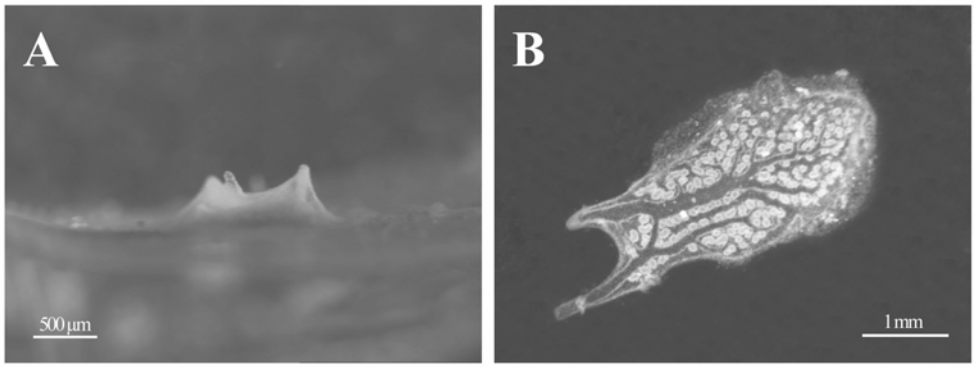

Fig. 5- A: Lateral view of a juvenile of *D. avara* showing its thickness. **B:** *D. avara* juvenile allowed growing only in two dimensions (sandwich culture).

Comparison of culture conditions

In a second experiment, we tested the effects of water flow and food type (living *vs*. dried algae) on the survival and growth of juveniles of *D. avara* and *C. crambe*.

Survival of both species was high in all treatments (between 80 and 100 %) except for *D. avara* under "no flow" conditions, which showed a significantly lower survival (32 %) ($P < 0.001$, Comparing Survival in Multiple Groups) (**Figs. 6** and **7**).

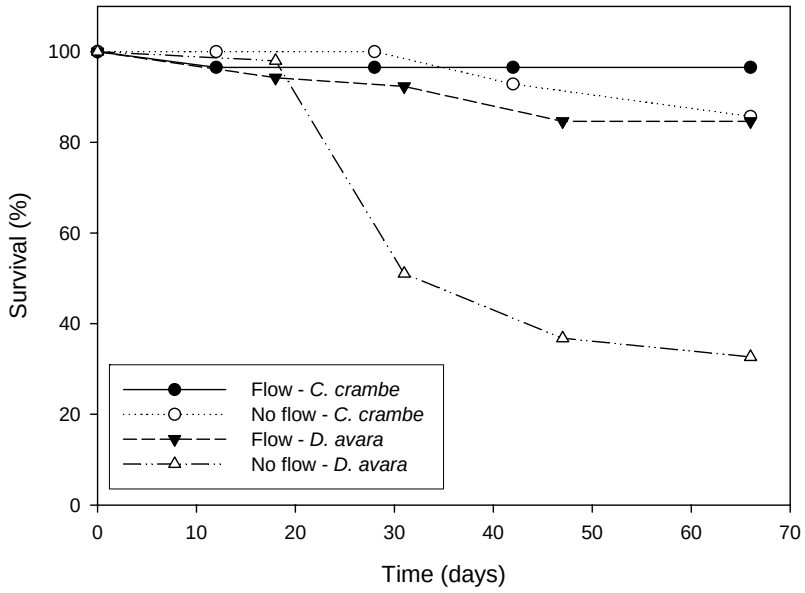

Fig. 6- Survival of *D. avara* and *C. crambe* juveniles with time under flow *vs.* no flow conditions; juveniles were feed with living algae. *D. avara* survival under "no flow" conditions showed the significantly lowest survival ($P < 0.001$).

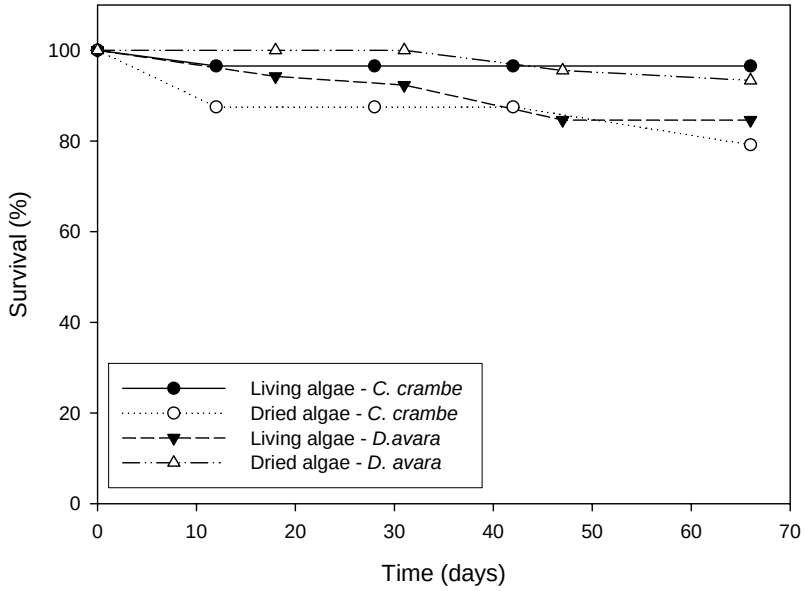

Fig. 7- Survival of *D. avara* and *C. crambe* juveniles with time fed with living algae *vs.* dried algae; juveniles were under "flow" conditions. No significant differences were observed.

As for growth rates, no significant differences (P = 0.504) were observed, between settlers of *D. avara* cultured under "flow" and "no flow", but the interaction term (time x treatment) was significant (P < 0.001), indicating that the trends changed with time. Conversely, *C. crambe* showed a significantly higher growth rate under flow conditions (P < 0.001) than without flow, and also the trend varied with time (P < 0.001, significant interaction term).

Comparing both species, differences were significant under both conditions: *C. crambe* grew more (P < 0.001) than *D. avara* under flow, in particular during the first months, and the interaction term (time x treatment) was also significant (P < 0.001). In contrast, *D. avara* grew more (P < 0.001) than *C. crambe* under no flow and the trends run in parallel with time (P = 0.202, no significant interaction term) (**Fig. 8**).

When the kind of food was tested, no differences between *C. crambe* fed with either dried or living algae were observed (P = 0.138). In contrast, a significantly higher growth rate was recorded when *D. avara* was fed with dried algae (P < 0.001) than with living algae.

Comparing both species, *C. crambe* showed higher growth rates (P < 0.01) than *D. avara* when the sponges were fed with living algae, and the interaction term was significant (P < 0.001). In contrast, no differences were

found when the food consisted of dried algae (P = 0.334) (**Fig. 9**). However, at the last time point of monitoring (after two months), differences in growth rates were significantly higher for both species fed with dried algae (P < 0.01, two-way ANOVA).

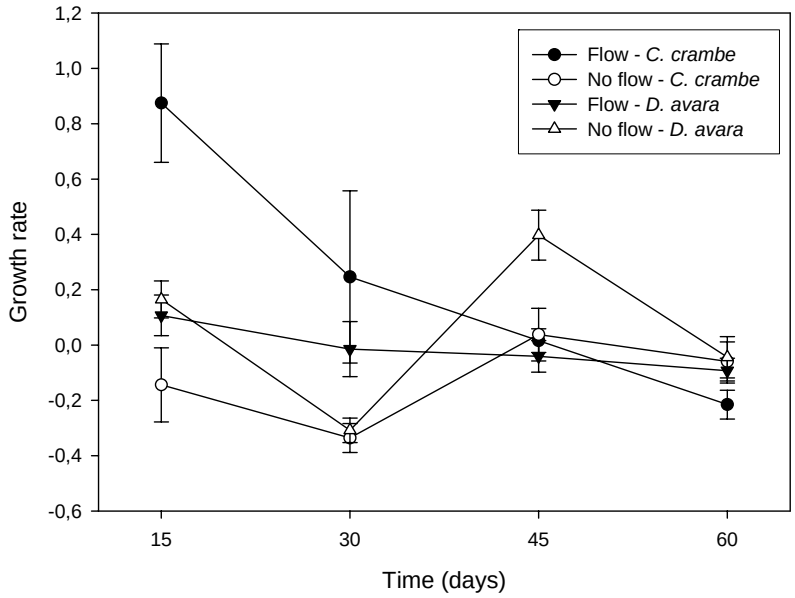

Fig. 8- Time course of the growth rate (15 days^{-1}) of *D. avara* (*N* = 30) and *C. crambe* (*N* = 50) juveniles, under flow *vs.* no flow conditions; juveniles were feed with living algae. Vertical lines are standard errors.

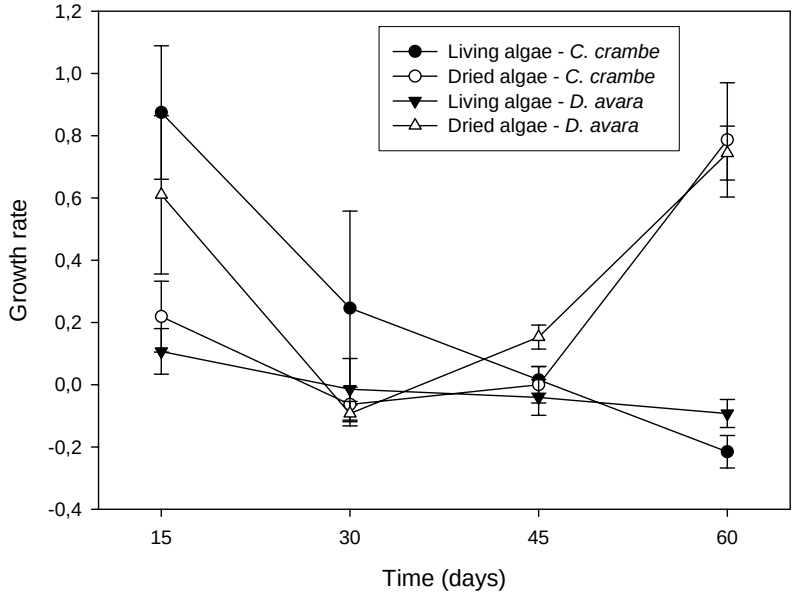

Fig. 9- Time course of the growth rate (15 days^{-1}) of *D. avara* (*N* = 30) and *C. crambe* (*N* = 50) juveniles, fed with living *vs.* dried algae; juveniles were under "flow" conditions. Vertical lines are standard errors.

The results found altogether reflect the particular characteristics of each species. *C. crambe* has a conservative life strategy with slow growth, lower clearance rates (Turon *et al.* 1997) and lower volume of choanocyte chambers (Uriz *et al.* 1995, Galera *et al.* 2000) than *D. avara,* which makes the former species less able to capture food under adverse conditions (i.e. lack of flow). Conversely, *D. avara* has an opportunistic life strategy with higher growth and clearance rates than *C. crambe* and also has a strong filtering activity (Ribes *et al.* 1999), thus it is able to capture food from the water even under no flow conditions (lower particles availability). These differential capabilities may explain why *C. crambe* grew more under flow conditions that can enhance the low sponge capacity for pumping water and facilitate the availability of food to the sponge.

On the other hand, *D. avara* has been reported to be a dynamic species with a fast metabolism (Turon *et al.* 1997) and thus it seems to need relatively high amounts of food for survival. Dried algae were more effective than living algae to feed sponges likely because dried algae consisted of broken cells with a smaller particle size than intact algal cells (from 4 to 8 µm, *P. tricornutum*). In previous studies, the highest clearance rates were obtained with 1 µm particle size for *D. avara* and *C. crambe,* while larger particles (4 µm) were retained with less efficiency (Turon *et al.* 1997). However, although the size of the algae used can enter the sponge ostia in both species (ostia diameter is 11.4 ± 1.2 µm in *C. crambe* and 30.8 ± 2.2 µm in *D. avara*, Galera *et al.* 2000), they may not enter through the smaller orifice (prosopyle) connecting inhalant canals and choanocyte chambers (e.g. de Vos *et al.* 1991), that may complicate algae capture.

If we consider the last observation time (see **Fig. 9**), we can conclude that the two types of food determined the final growth rate for both species. The dry algae appeared to be significantly more effective as a food source than the living algae, probably due to a higher retention rate of the sponge for the dry algae.

Juvenile survival and growth in the field

In a third experiment, juveniles of *D. avara* and *C. crambe* settled in the laboratory were transferred to the sea in order to monitor survival and growth under natural conditions.

Juveniles of *C. crambe* had a significantly higher survival than those of *D. avara* ($P < 0.001$, Gehan's Wilcoxon Test). During the first 20 days of the monitoring, both species experienced a high mortality (about 60 %) and from then on, differences between species became evident: all the *D. avara* juveniles died after day 60 whereas 24 % of *C. crambe* juveniles survived at that day. Moreover, 20 % of *C. crambe* juveniles survived until day 136 (**Fig. 10**). The higher mortality of *D. avara* juveniles in the field may be due to a higher palatability and a more fragile consistency of this species with respect to *C. crambe* (Uriz *et al.* 1996a).

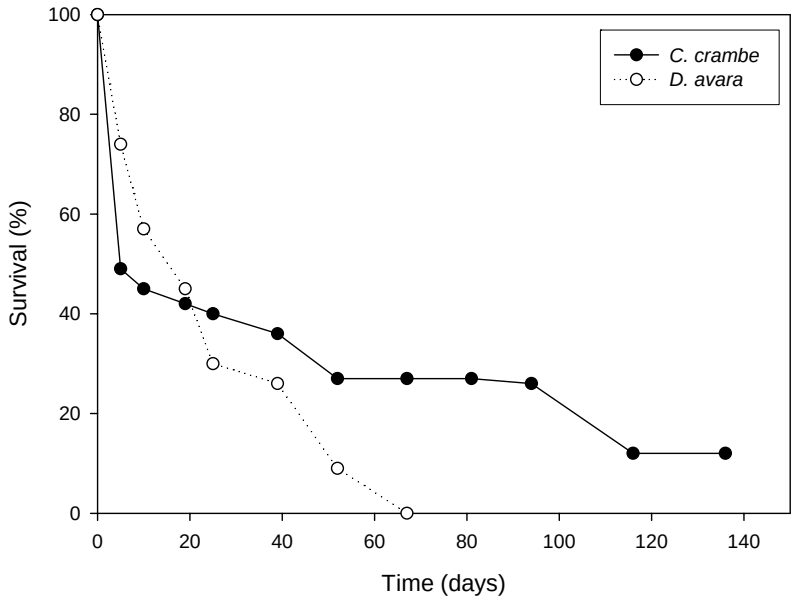

Fig. 10- Survival of *D. avara* and *C. crambe* juveniles with time at sea.

Juveniles cultured in the field survived significantly less than juveniles cultured in the laboratory (previous experiment) for the two species (**Fig. 11**) ($P < 0.001$, comparing survival in multiple groups). In natural conditions, a high mortality is frequently reported for juvenile stages of sponges because of predation, competition for the substrate with other benthic organisms (e.g. Uriz *et al.* 1996a), and physical perturbations. All these drawbacks can be avoided by

culturing larvae in the laboratory, and, thus, culture of juveniles under controlled conditions are recommended for avoiding biomass losses at these early stages.

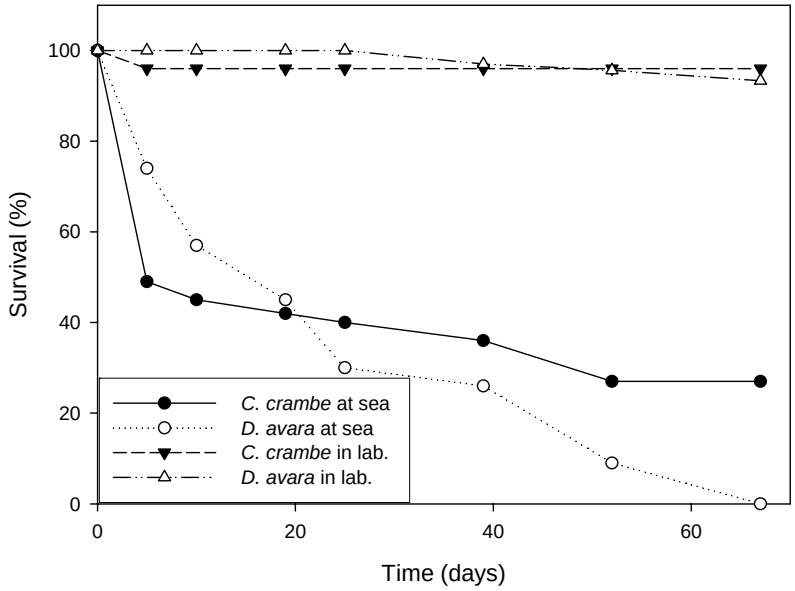

Fig. 11- Survival comparison of *D. avara* and *C. crambe* juveniles with time under the best laboratory conditions (with flow and fed with dried algae), and in the field.

Growth rates at sea were not significantly different between species (**Fig. 12**) (P = 0.101, and interaction term P = 0.235). *D. avara*, growth rates at sea were similar (P = 0.294) to those in the laboratory fed with dried algae. However, under less favourable conditions, juveniles grown in the laboratory grew significantly less that those at sea (P < 0.001). In contrast, *C. crambe* juveniles grew significantly more (P < 0.01) at sea than in the laboratory whatever the experimental conditions were (**Fig. 12**).

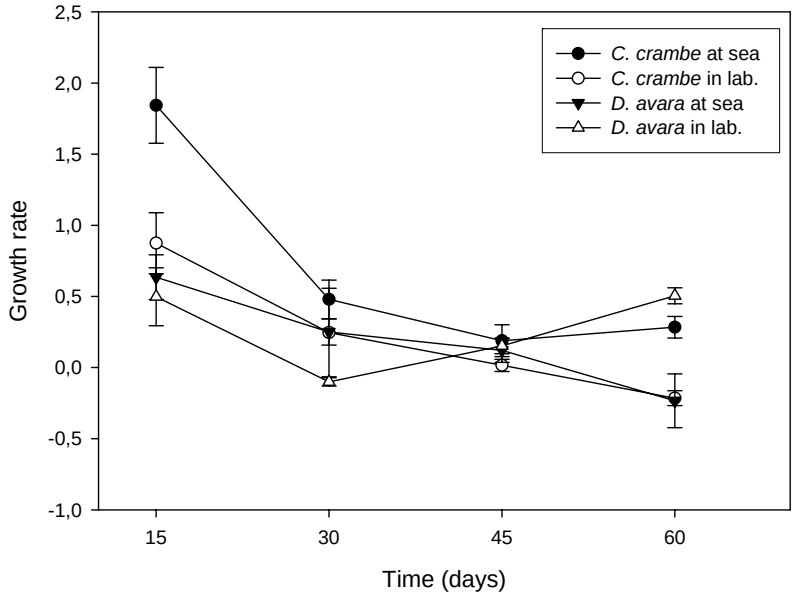

Fig. 12- Growth rates (15 days^{-1}) comparison of *D. avara* (*N* = 30) and *C. crambe* (*N* = 50) juveniles under the best laboratory conditions (with flow and fed with dried algae), and in the field (*D. avara* N= 164, and *C. crambe* N= 175). Vertical lines are standard errors.

In the field site where juveniles were placed, *C. crambe* is much more abundant than *D. avara*, which may indicate better environmental conditions for the former species to grow. Furthermore, settlers of *D. avara* appeared to be more fragile than those of *C. crambe* and thus unable to resist physical perturbations. Predation can also contribute to the differential mortality of both species since *D. avara* has been reported to be grazed by sea urchins (Uriz *et al.* 1996a), whereas no predators are known for *C. crambe*. Furthermore, growth did not differ between juveniles cultured in the laboratory and those transplanted to the sea. Conversely, survival was hugely higher in the laboratory.

Comparing growth rates of juveniles of both species in the laboratory under the best conditions (flow and dried algae as a food) with those of adult individuals monitored during ca. 4 years in the field (Turon *et al.* 1998, 2000b): *D. avara* juveniles presented higher growth rates in our cultures (average 0.44± 0.22 per month) than adults did in the field (average 0.03 ± 0.01 per month, Turon *et al.* 1998). Similarly, considerable differences in growth rates were observed between juveniles (0.46 ± 0.2 per month) and adults of *C. crambe* (average 0.1± 0.02 per month, Turon *et al.* 1998). In contrast, growth variability was higher in juveniles (this study) than in adult sponges for both species. The variation coefficient for

larval growth obtained from this study was 66.6 % and 48.9 % for *D. avara* and *C. crambe* juveniles respectively, while it was 33.3 % and 20 % for *D. avara* and *C. crambe* adults, respectively (Turon *et al.* 2000b). These data reflect the inherent growth variability in sponges since, even eliminating the "age factor", the level of variation is high. However, the comparison between juveniles and adults must be taken with care since the phases of growth in thickness showed by our juveniles were associated with a decrease in area (see above), which inevitably was incorporated in our growth data, contributing to the variability recorded.

Altogether, these results point to sponge larvae as a promising source for the sponge/metabolite supply, since the culture is performed during the period of maximum sponge growth and thus the culture yield can be optimised. The study has also provided interesting information about biological aspects on the sponge life cycle, helping to fill the gap in the literature about juvenile stages, which are difficult to follow in the field because of their small size.

Acknowledgements

This study was partially funded by the project INTERGEN from the Spanish Agency CICYT: CTM2004-05265-C02-02/MAR. We thank M. Ribes, E. Cebrian and A. Blanquer for helping in larval supply and field monitoring, and X. Turon for facilitating the program to perform the permutation tests.

Chapter V

Embryo development of *Corticium candelabrum* (Demospongiae: Homosclerophorida)

de Caralt S, Uriz MJ, Ereskovsky AV & Wijffels RH (2007) Embryo development of *Corticium candelabrum* (Demospongiae: Homosclerophorida) Invertebrate Biology (*in press)*

Abstract

Corticium candelabrum is a homosclerophorid sponge widespread along the rocky Mediterranean sublittoral. Scanning and transmission electron microscopy were used to describe the gametes and larval development. The species is hermaphroditic. Oocytes and spermatocytes are clearly differentiated in April. Embryos develop from June to July when the larvae are released spontaneously. Spermatic cysts originate from choanocyte chambers and spermatogonia from choanocytes by choanocyte mitosis. Oocytes have a nucleolate nucleus and a cytoplasm filled with yolk granules and some lipids. Embryos are surrounded by firmly interlaced follicular cells from the parental tissue. A thin collagen layer lies below the follicular cells. The blastocoel is formed by migration of blastomeres to the morula periphery. Collagen is spread through the whole blastocoel in the embryo, but is organized in a dense layer (basal lamina) separating cells from the blastocoel in the larva. The larva is a typical cinctoblastula. The pseudostratified larval epithelium is formed by ciliated cells. The basal zone of the ciliated cells contains lipid inclusions and some yolk granules; the intermediate zone is occupied by the nucleus; and the apical zone contains abundant electron-lucent vesicles and gives rise to cilia with a single cross-striated rootlet. Numerous paracrystalline structures are contained in vacuoles within both apical and basal zones of the ciliated cells. Several slightly differentiated cell types are present in different parts of the larva. Most cells are ciliated, and show ultrastructural particularities depending on their location in the larvae (antero-lateral, intermediate and posterior regions). A few smaller cells are non-ciliated. Several features of the *C. candelabrum* larva seem to support the previously proposed paraphyletic position of homoscleromorphs with respect to the other demosponges.

Introduction

Developmental patterns are a fundamental aspect of invertebrate life histories. They were widely used to establish hypotheses about phylogenetic relationships before the advent of molecular techniques, which can rapidly generate phylogenetically useful data on a large number of taxa. However, phylogenies based either on phenotypic, developmental, or genotypic data should be complementary.

In a recent phylogenetic study using molecular markers, the monophyly of the class Demospongiae, as traditionally defined, was questioned because the order Homoscleromorpha appeared as a clade, clearly separated from the remaining Demosponges (Borchiellini *et al.* 2004, Nichols 2005). The separation of Homoscleromorpha in molecular phylogenetic trees is consistent with particular morphological and ultrastructural features previously reported for this order, such as the presence of a basal lamina made of type IV collagen in both adults (Boute *et al.* 1996) and larvae (Boury-Esnault *et al.* 2003) and the presence of an acrosome in spermatozoa of *Oscarella lobularis* and *Pseudocorticium jarrei* (Baccetti *et al.* 1986, Boury-Esnault & Jamieson 1999). Several aspects of embryo development, such as the multipolar egression during formation of the blastula (e.g. Boury-Esnault *et al.* 2003), also seem to support this hypothesis. All these characters, which are potential synapomorphies shared with eumetazoans, point to Homoscleromorpha as a key sponge group for understanding early metazoan evolution (Borchiellini *et al.* 2004). However, more species of this sponge order should be included in phylogenetic molecular studies and more research on adult and larval ultrastructural features should be done to confirm the homogeneity of Homoscleromorpha and its relationships with Eumetazoa.

Several early studies had reported on certain aspects of the reproduction and larvae of Homoscleromorpha sponges (Meewis 1938, Lévi & Porte 1962, Tuzet & Paris 1964, Baccetti *et al.* 1986, Gaino *et al.* 1986a,b). More recently, TEM investigations on larval development of different homoscleromorph species have been performed (Boury-Esnault *et al.* 2003). However, development has been studied for only four of the seven currently known genera of

homoscleromorphans. The most intensive work has been done on members of the genus *Oscarella* (Lévi & Porte 1962, Baccetti *et al.* 1986, Gaino *et al.* 1986, Ereskovsky & Boury-Esnault 2002), which has been often used to describe the distinctive characteristics of the Order (Ereskovsky & Boury-Esnault 2002, Boury-Esnault *et al.* 2003). Development in the genera *Plakina (P. trilopha, P. jani), Corticum (C. candelabrum), and Pseudocorticium (P. jarrei)* has also been described (Boury-Esnault *et al.* 2003), but further work on these genera is required to characterize the range of developmental attributes within the whole Homoscleromorpha.

The present study describes in detail reproductive aspects of *C. candelabrum* from gametogenesis to the cinctoblastula, which were disregarded in previous studies. Our analysis using transmission and scanning electron microscopy highlights several particularities of embryonic development and larval structure, which are not exhibited by other demosponges and are shared exclusively with other species of the Homoscleromorpha.

Materials and methods

Samples of *Corticium candelabrum* Schmidt, 1862 were collected once a month from January to September, 2002 by SCUBA diving in the western Mediterranean Sea (Blanes and Marseilles), at a depth of 7-15 m, and taken to the laboratory for observation by light microscopy. From April to August, when oogonia, spermatogonia, early embryos at different stages of development, and larvae become conspicuous, small subsamples were fixed for observation by transmission and scanning electron microscopy (TEM and SEM).

For TEM, 2 fixation methods were used: (1) 1% OsO_4 and 2% glutaraldehyde in 0.45 M sodium acetate buffer (pH 6.4) with 10% sucrose (1:3) (Leys & Reiswig 1998) - Figs. 1-6, 10, 12, 17-20-, and (2) 2.5% glutaraldehyde in a mixture of 0.4 M cacodylate buffer and seawater (1:4:5; 1120 mOsm) and post-fixation in 2% OsO_4 in seawater (Boury-Esnault *et al.* 1984) - Figs. 9, 21-26- dehydrated in an alcohol series, embedded in Spurr resin and stained with uranyl acetate and lead citrate. Ultrathin sections were observed under a Hitachi H-600

(Microscopy Unit of the Scientific and Technical Services of the University of Barcelona) and a Zeiss-1000 (University of Méditerranée, Marseilles) transmission electron microscopes. For SEM observations, samples were fixed with a mixture of 2% OsO_4 and saturated $HgCl_2$ (6:1) for 90 min (Johnston & Hildemann 1982), dehydrated in an ethanol series, critical-point dried, mounted and sputter-coated with gold-palladium, and examined through a Hitachi-2300 and S570 scanning electron microscopes (Institute of Marine Sciences of Barcelona, CSIC and Centre d'Océanologie de Marseille, respectively).

Results

Gametogenesis

Corticium candelabrum has a relatively long reproductive cycle. We found evidence of gametogenesis (oocytes and spermatogonia) during April and embryos at different stages of development were observed from May to June. Fully developed larvae were present in early July and released in mid July. The species is hermaphroditic since spermatocytes and oocytes were found in the same adult individuals. Mature spermatozoa, although no pictures could be obtained, probably occurred in May, since fertilized oocytes were present in June.

Spermatogonia originated from choanocyte chambers by choanocyte mitosis. During this process, nuclei and cells shrank and some ultrastructural modifications, such as absorption of the choanocyte collar and flagellum were observed (**Fig. 1A**). Spermatocytes have a polygonal pointed nucleus with heterochromatin, empty vesicles and much larger mitochondria than those observed in choanocytes (**Fig. 1B**). A thick layer of collagen surrounds the spermatic cyst. Spermatogenesis inside one spermatocyst is asynchronous.

Oocytes, 100-150 µm in diameter, are filled with abundant yolk granules and some lipids surrounding a nucleolate nucleus of 16 µm in diameter (**Fig. 1C**). The nucleolus can attain 5.3 µm in diameter (**Fig. 1D**).

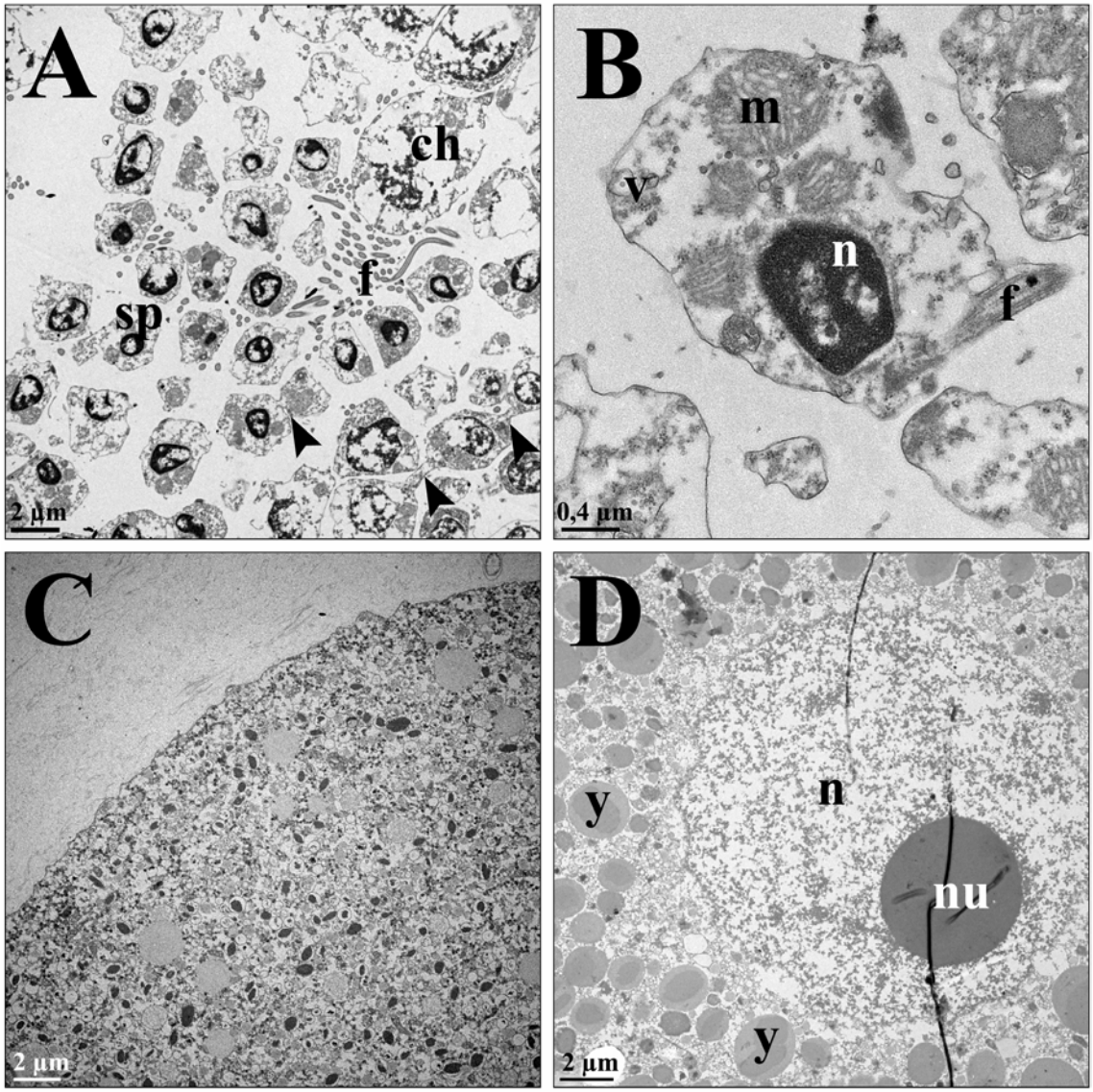

Fig. 1- *Corticium candelabrum.* **A:** TEM image of a choanocyte chamber becoming spermatic cyst: (arrowheads) spermatocytes under division; (ch) choanocytes; (sp) spermatids; (f) flagellae. **B:** TEM image of a detail of a spermatid: (f) flagellum; (m) large mitochondria; (n) polygonal nucleus. **C:** TEM image of early oocyte full of yolk granules (dark) and lipids (light). **D:** TEM image of a detail of an oocyte: (n) nucleus; (nu) nucleolus; (y) yolk granules.

Embryogenesis

Cleavage is holoblastic, equal, and synchronous during the first two divisions. At the third division, cleavage becomes irregular, asynchronous, and results in a solid stereoblastula (morula) of undifferentiated, yolk-rich blastomeres, and a cytoplasm filled with lipids and rhomboidal yolk granules each containing a nucleolate nucleus (Fig. 2A). Follicular cells (pinacocytes) from the parental tissue completely surround the embryo, and are firmly interlaced by an intricate pattern of digitations (Fig. 2B).

Embryos at the late stage of development have a globular shape (Fig. 2C). A collagen layer covered by follicular cells surrounds the embryo, and a network of collagen fibres encases each embryo beneath the follicle cells (Fig. 2D). At this stage, the blastocoel starts to form by centrifugal cell migration (multipolar egression). The blastocoel contains dispersed collagen fibrils and some free symbiotic bacteria. Cells at the basal part of the embryos contain phagocytosed bacteria (Fig. 3A). During development, the larva is surrounded by the follicular cells (Fig. 3B). At the last stages of larval development, the epithelium becomes wrinkled (Fig. 3C) since it cannot be expanded inside the follicle, and the blastocoel is progressively filled by collagen fibrils (Fig. 3C). Through TEM, an invagination of the wrinkled epithelium may wrongly suggest a narrow (8 to15 µm in width) blastocoel, depending on the larva section (Fig. 3D).

Embryos co-existed with larvae and occasionally with oocytes in samples collected in July (Fig. 3A), which indicates asynchronous reproduction.

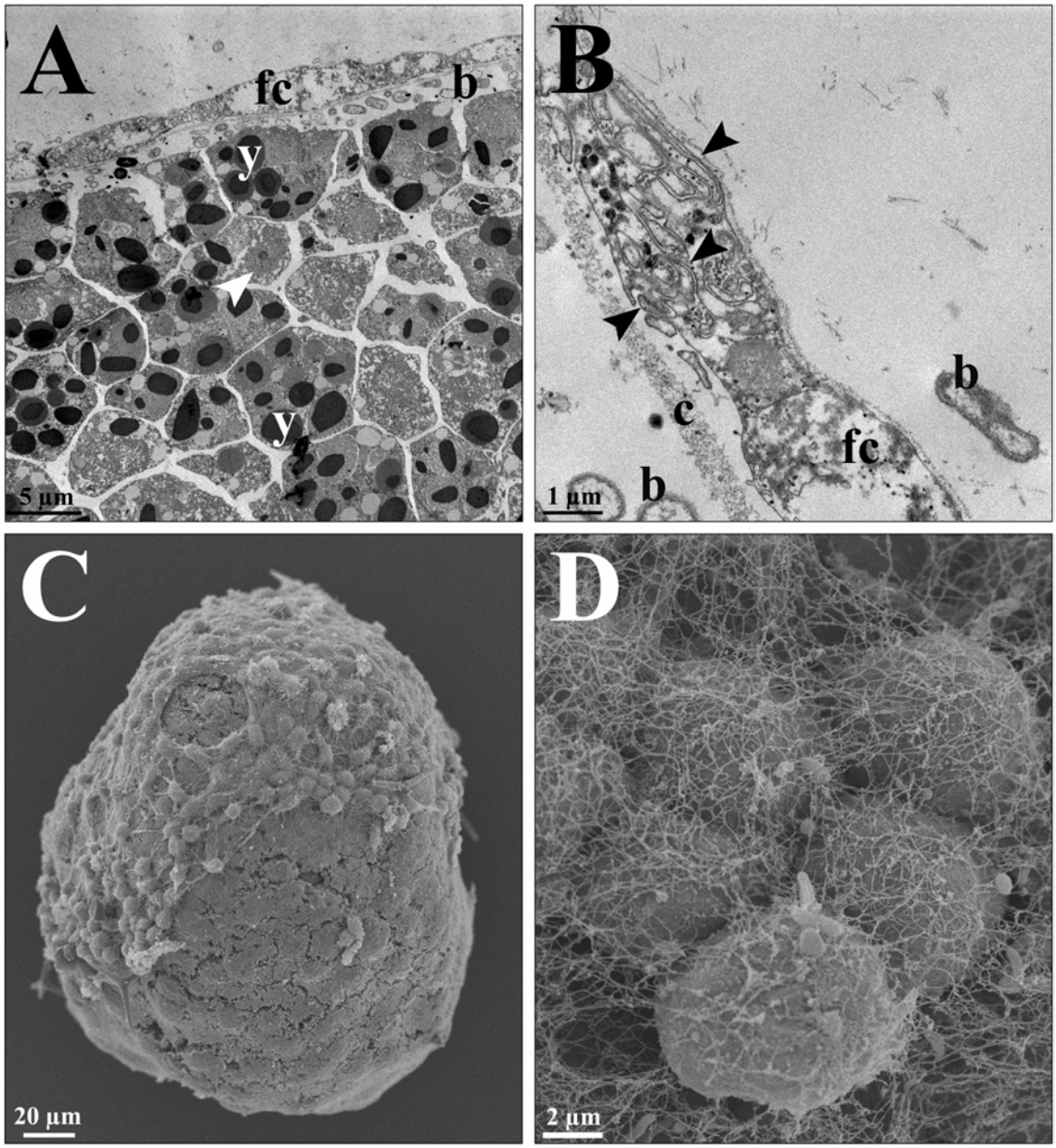

Fig. 2- *Corticium candelabrum.* **A:** TEM image of a morula: (arrowhead) nucleolate nucleus of polygonal blastomeres; (b) symbiotic bacteria; (fc) follicular cells surrounding the embryo; (y) yolk. **B:** TEM image of a detail of follicular cells: (arrowheads) cell folds connecting follicular cells; (c) collagen layer; (fc) follicular cells. **C:** SEM image of a late embryo (removed from the parental sponge): rounded shape with a wider anterior pole and covered by follicular cells in the posterior pole. **D:** SEM image of a detail of follicular cells covered by a dense network of collagen fibrils.

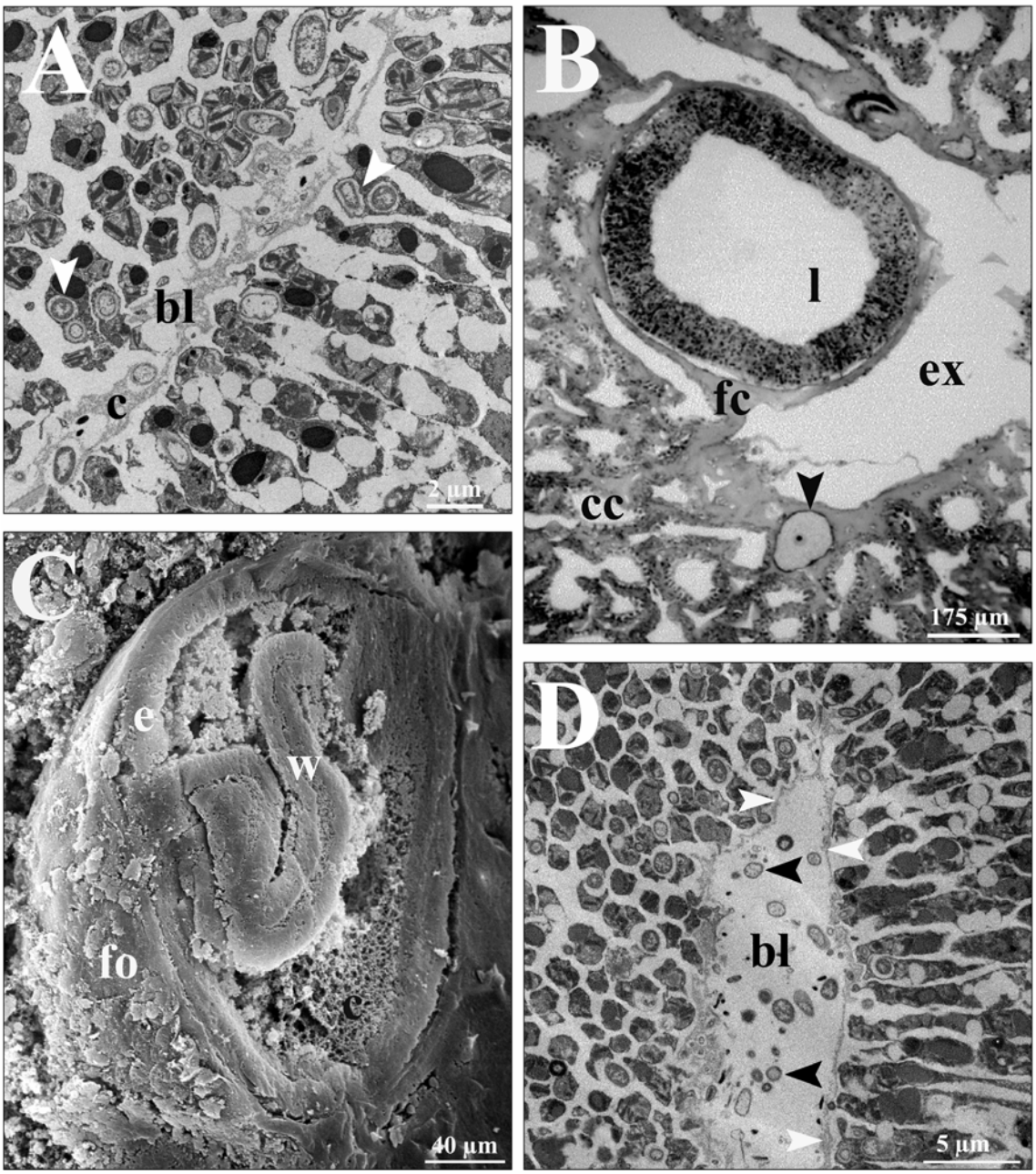

Fig. 3- *Corticium candelabrum.* **A:** TEM image of an early larva: (arrowheads) bacteria engulfed by sponge cells; (bl) early blastocoel; (c) collagen fibrils. **B:** Semi-thin section of an adult (reproductive stage) tissue: (arrowhead) oocyte; (cc) choanocyte chambers; (ex) exhalant conduct; (fc) follicular cells; (l) larva. **C:** SEM image of a wrinkled pre-larva: (e) larval ciliated epithelium; (c) internal collagen matrix; (fo) follicle; (w) wrinkle. **D:** TEM image of a completely formed blastocoel of *C. candelabrum* larva, with a collagen layer (basal lamina) surrounding the cavity. The wrinkled form of the larva within the adult tissue is responsible for the narrow blastocoel in this section. The section is longitudinal to the cells axis on the right side of the picture and transversal on the left side: (black arrowheads) symbiotic bacteria inside the blastocoel; (white arrowheads) basal lamina; (bl) blastocoel.

Larval features

White larvae with a pink-yellow posterior zone are abundant thoroughout the sponge mesohyl in July. They concentrate around the exhalant conducts and at the sponge periphery (**Fig. 4**). The mature cinctoblastulae in *C. candelabrum* are released through the exhalant canals, as reported for other homoscleromorph larvae (Boury-Esnault *et al.* 2003), but also through the sponge surface. Larval release takes place synchronically during a few days at the end of July.

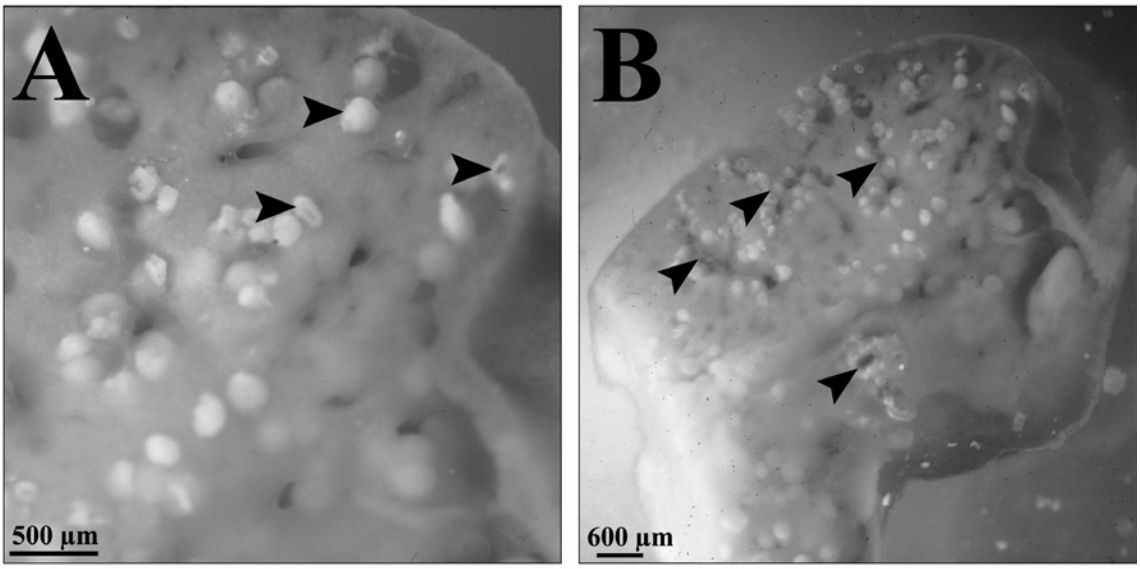

Fig. 4- *Corticium candelabrum.* **A:** Light micrograph of an adult sponge with embryos: (arrowheads) embryos. Some embryos show the central cavity (blastocoel). **B:** Light micrograph of an adult sponge full of ready-for-release embryos concentrated around the exhalant conducts: (arrowheads) exhalant conducts.

Free larvae range from 180 to 260 µm in size, although larvae of 100 µm in diameter, when removed artificially from the parental tissue, could also swim. Larvae are egg shaped with a wider anterior pole, and are uniformly ciliated with cilia measuring 20-25 µm in length (**Figs. 5A** and **5B**). The completely formed blastocoel measures 80-120 µm in width (**Fig. 5B**). Collagen, which was spread through the whole blastocoelic cavity when the blastocoel started to form, is now arranged in a layer that separates cells from the cavity, which immediately beneath the cellular wall of the blastocoel and the blastocoelic cavity is filled with fluid and symbiotic bacteria. This collagen layer has been interpreted as a true basal lamina (Boury-Esnault *et al.* 2003) (see **Fig. 3D**).

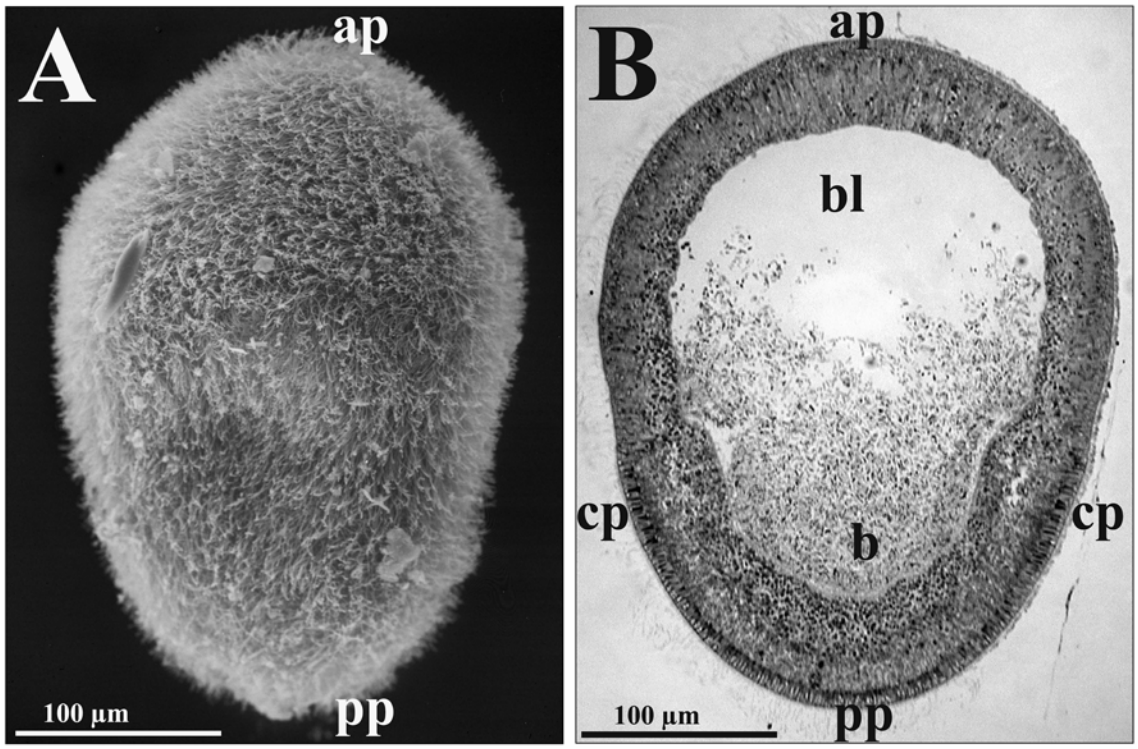

Fig. 5- *Corticium candelabrum.* **A:** SEM image of a cinctoblastula uniformly ciliated: (ap) anterior pole; (pp) posterior pole. **B:** Semi-thin section of the cinctoblastula: (ap) wider anterior pole; (b) symbiotic bacteria near posterior pole; (bl) blastocoel; (cp) belt of cells with intranucleolar paracrystalline inclusion; (pp) narrow posterior pole.

The cinctoblastula of *C. candelabrum* consists of two cell types. Most of cells are ciliated ca. 38 x 3 µm in size, and have some ultrastructural differences depending on their location in the larvae (antero-lateral, intermediate and posterior zones). A very few cells are non-ciliated and smaller (ca. 33 x 9 µm in size). The ciliated larval cells have common features. They are elongated and form a monolayer of pseudostratified epithelium. The basal zone of these cells (adjacent to the blastocoel) contains abundant vacuoles with crystal-like elongated structures, lipid inclusions, and yolk granules. The yolk has drastically diminished compared to that found in embryos (see **Fig. 1D**), which indicates that it has been consumed during larval development. Many instances of phagocytosed bacteria are observed within that basal cell zone (**Fig. 6A**). The intermediate zone of the cells contains the nucleolate nuclei, which almost extends up to the apical zone (**Fig. 6B**). In the apical zone of the cells can be observed cilia arising from shallow pockets in the cell membrane, abundant vesicles with clear contents, and vacuoles with elongate paracrystalline structures, similar to those found at the basal zone of the cells. A Golgi apparatus is also visible close to the vesicular

zone (**Figs. 6C** and **6D**). A specialized junctional complex similar to a *zonula adhaerens* junction develops between cells at the upper lateral zone (**Fig. 7A**). The basal apparatus of the cilia consists of a basal body (ca. 0.38-0.45 µm x 0.32-0.35 µm in size) and, an accessory centriole (ca. 0.41-0.55 µm x 0.3-0.36 µm in size), is always located beneath the basal body. The basal foot (0.27-0.3 µm in length) has a champagne cork shape and originates laterally on the basal body. A single cross-striated rootlet starts from the basal body and surrounds the accessory centriole (**Figs. 7A** and **7B**).

Ciliated cells from different areas of the larva have distinctive features. The antero-lateral ciliated cells form a pseudostratified layer with their nuclei located at different levels of the cytoplasm (**Fig. 8A**). Ciliated cells within the mid-region of the larva have paracrystalline, intranuclear inclusions that are oriented longitudinally within the cells (**Fig. 8B**). They form a 57-70 µm wide belt, which is characteristic of the cinctoblastula larvae. Large, longitudinally arranged vacuoles with heterogeneous content occupy the central part of the cells, below the nuclei. The ciliated cells of the posterior pole are arranged in a palisade with all the nucleolated nuclei placed at same level, in the central part of the cells (**Fig. 8C**). Abundant, clear-to-electron inclusions and lipid droplets fill the cytoplasm.

Non-ciliated stout cells are scattered randomly among the ciliated cells (**Fig. 8D**). They are shorter than the ciliated cells and thus they never reach the base of the ciliated epithelium. The cytoplasm contains lipid droplets, osmiophilic inclusions, and vesicles of different sizes.

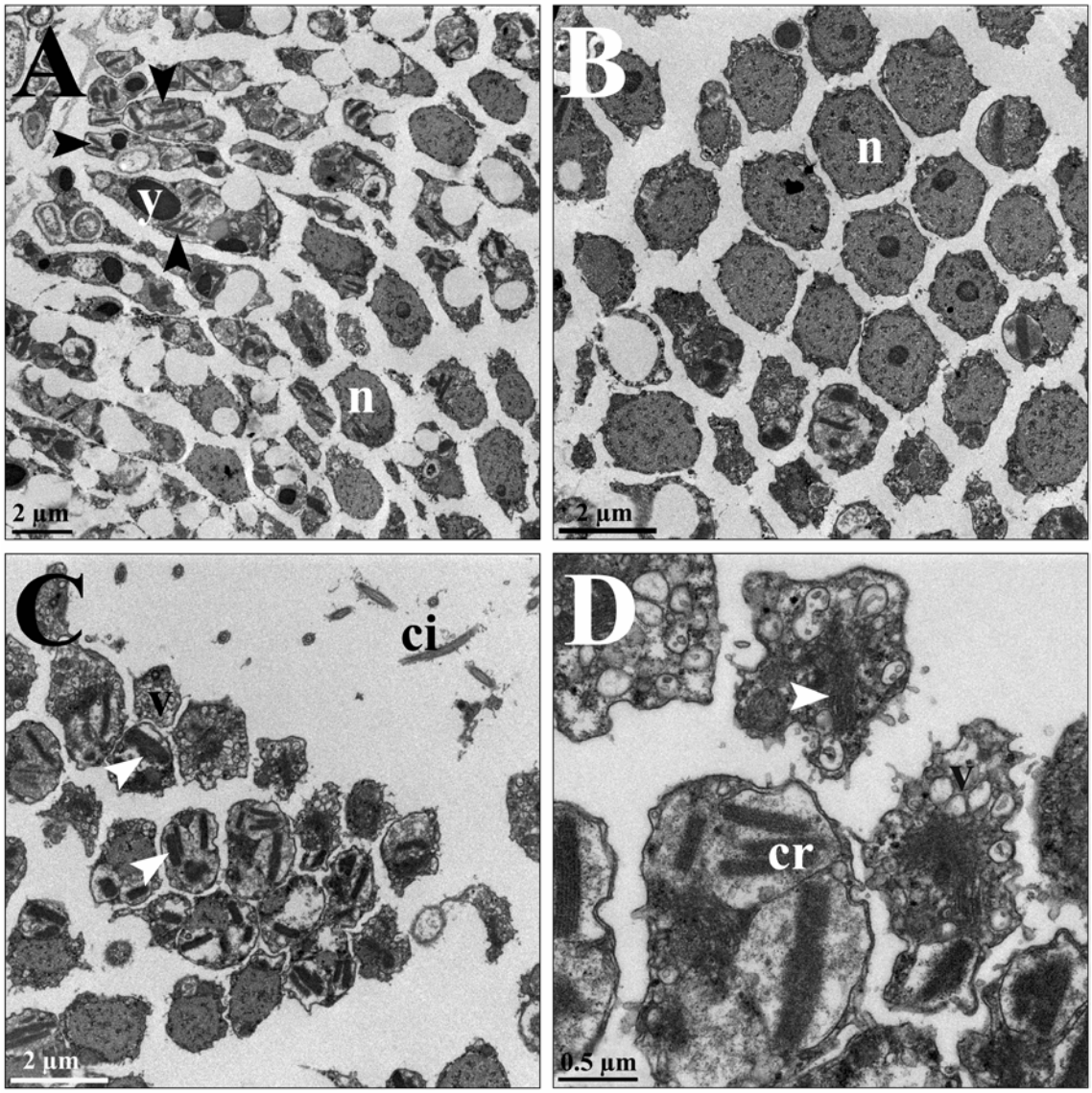

Fig. 6- *Corticium candelabrum*. **A:** TEM image of a longitudinal section of the pseudostratified cell layer of the larva showing the basal zone of the cell with symbiotic bacteria and the intermediate cell zone with lipids and some nuclei: (arrowheads) paracrystalline structures; (n) nucleolate nuclei; (y) yolk granules. **B:** Intermediate zone of cells of the larva containing the nucleolate nuclei (n). **C:** TEM image of the apical zone of the larval cells with high vesicle density: (arrowheads) paracrystalline structures; (ci) cilia; (v) vacuoles. **D:** TEM image of paracrystalline structures inside vacuoles in apical zone of larval cells: (arrowhead) Golgi apparatus; (cr) paracrystalline structures; (v) clear vesicles.

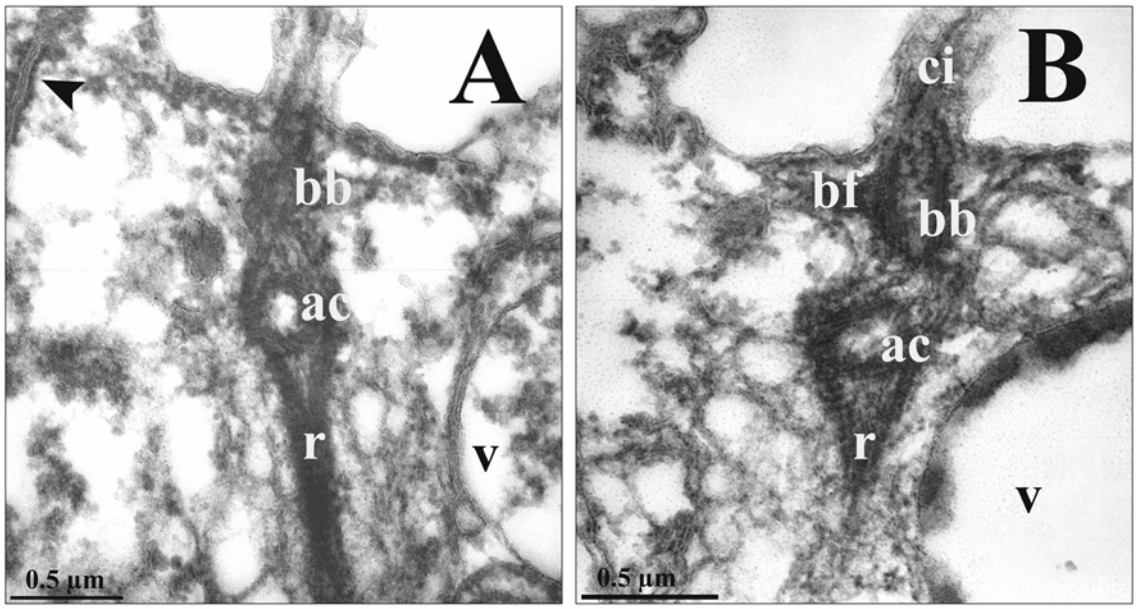

Fig. 7- *Corticium candelabrum.* **A:** TEM image basal ciliary apparatus of a ciliated cell of a cinctoblastula: (arrowhead) junction in the apical part of the cell; (ac) accessory centriole; (bb) basal body perpendicular to the accessory centriole; (r) cross-striated rootlet; (v) vacuole. B: TEM image showing the basal ciliary apparatus of a ciliated cell: (ac) accessory centriole; (bb) basal body; (bf) basal foot; (ci) cilium; (r) cross-striated rootlet; (v) vacuole.

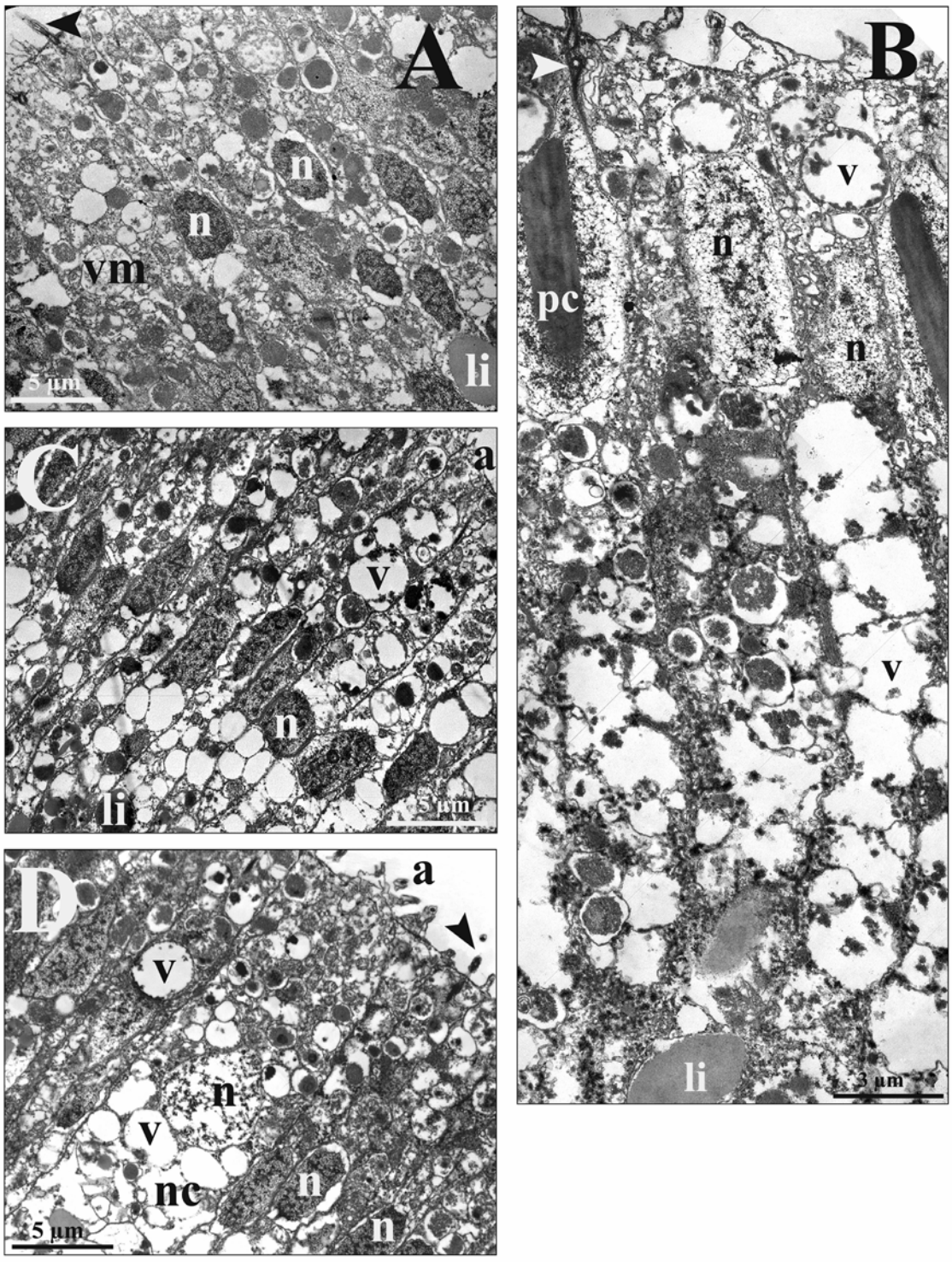

Fig. 8- *Corticium candelabrum.* **A:** TEM image of antero-lateral ciliated cells of the larva: (arrowhead) cilium; (li) lipid; (n) nuclei; (vm) vacuoles with mucus. **B:** TEM image of intermediate ciliated cells of the larva: (arrowhead) ciliary rootlet; (li) lipids; (n) nuclei; (pc) paracrystalline structures; (v) vacuoles. **C:** TEM image of posterior pole ciliated cells of the larva: (a) apical part of the cell; (li) lipid; (n) nuclei; (v) vacuoles. **D:** TEM image of non-ciliated cell of the larva: (a) apical part of the cell; (arrowhead) cilia; (n) nuclei; (nc) non-ciliated cell; (v) vacuole.

Discussion

Gametogenesis of *Corticium candelabrum* is typical of Homoscleromorpha (Gaino *et al.* 1986a,b) and different from that of other demosponges. The asynchronous spermatogenesis, with several development stages coexisting within each spermatocyst, is in agreement with the wave-like maturation pattern of male germ cells reported for other species of Homoscleromorpha such as *Oscarella* spp. (Efremova *et al.* 2006). Cleavage of *C. candelabrum* is holoblastic, and also asynchronous, as in other homosclerophorids (Schulze 1877, 1880, 1881, Meewis 1938, Ereskovsky & Boury-Esnault 2002, Ereskovsky 2005). *C. candelabrum* harbors oocytes and embryos at different stages of development, simultaneously, which would suggest a gradual larval release. However, only one massive release event per year has been observed at the study site although other weak release events might have been overlooked. If larval release occurs in this sponge several times, as a given larval pool becomes mature, it may increase the chance of finding favourable conditions for larvae to settle.

As for morphological aspects, several ultrastructural characteristics observed in *C. candelabrum* are shared with other genera of the same order and may represent particular features of Homoscleromorpha. All the embryonic cell types (this paper) as well as the cells of adult individuals of *C. candelabrum* (de Caralt *et al.* 2003) have a nucleolated nucleus. Although this cell characteristic has been reported in embryos of other demosponges (e.g. Weissenfels 1989, Sizova & Ereskovsky 1997, Ereskovsky & Gonobobleva 2000, Ereskovsky & Boury-Esnault 2002, Leys & Degnan 2002, Usher & Ereskovsky 2005), its presence in all the cell types of adults and larvae (also reported in *Oscarella*, Lévi & Porte 1962) seems to be a distinctive feature of Homoscleromorpha.

In addition, larval cells contain, besides intranuclear crystals (Boury-Esnault *et al.* 2003), numerous paracrystalline inclusions inside cytoplasmic vacuoles within both the basal and apical zones of the cells. This seems to be a particular feature of *C. candelabrum.* Paracrystalline structures have been reported within spherulous and vacuolar cells from adults of *Hamigera hamigera* and *Acanthella acuta*, respectively (Boury-Esnault 2006), and in the intermediate ciliated larval

cells of eight species of Homoscleromorpha (Boury-Esnault *et al.* 2003), were always intranuclear.

Larvae of *C. candelabrum* and those of other homoscleromorphs show several characters typical of the Eumetazoa: a collagen layer, which separates the ciliated epithelium from the blastocoel (considered a primitive basal lamina, Boury-Esnault *et al.* 2003, Ereskovsky 2005), larval desmosomes, and cell polarization (Boury-Esnault *et al.* 2003). Furthermore, blastocoel formation by multipolar egression, observed in *C. candelabrum,* and previously reported in eight homoscleromorph species (Ereskovsky & Boury-Esnault 2002, Boury-Esnault *et al.* 2003, Ereskovsky 2005), together with no differentiation between megascleres and microscleres (Muricy & Diaz 2002), and the absence of a well-delimited axial filament in spicules (Uriz *et al.* 2003), confirms the uniformity within the Homoscleromorpha. These characters support the separation of the Homoscleromorpha from the rest of demosponges and confirms that these sponges are close to the Eumetazoa.

Acknowledgements

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Chapter VI

Vertical transmission and successive location of symbiotic bacteria during embryo development and larval formation in *Corticium candelabrum* (Porifera: Demospongiae)

de Caralt S, Uriz MJ & Wijffels RH (2007) Vertical transmission and successive location of symbiotic bacteria during embryo development and larval formation in *Corticium candelabrum* (Porifera: Demospongiae). Journal of Marine Biology Association of the United Kingdom *(submitted)*

Abstract

This study reports on the transfer of heterotrophic bacteria from parental tissue to oocytes in the Mediterranean bacteriosponge *Corticium candelabrum* (Homosclerophorida) and the description of the successive locations of the microsymbionts during embryo development through transmission and scanning electron microscopy. Eight different types of symbiotic bacteria are described morphologically. These eight bacteria morphotypes are found in both adult individuals and larvae. Symbiotic bacteria are transferred to oocytes, but not to spermatocytes. Bacteria are first located at the oocyte periphery below a thick collagen layer and then they migrate to the oocyte cytoplasm, forming spherical clusters. After cleavage, the bacteria can be found in the free space between blastomeres but mainly accumulate at the embryo periphery below the follicular cells that surround the embryo. Once the blastocoel is formed, the symbiotic bacteria move to this central cavity where they actively divide by bipartition, increasing their number considerably. Many examples of phagocyted bacteria in the proximal zone of the larval cells are observed at this stage. Consequently, bacteria may represent a complementary source of energy for free larvae and settlers before they are able to capture food from the surrounding water.

Introduction

Microbial symbionts are plentiful in many sponge species (e.g. Sarà & Vacelet, 1973, Wilkinson 1987) in such a way that it has been speculated that remineralization of the organic matter in the water column would be the main sponges' role in benthic ecosystems, thanks to their symbiotic populations. Sponge microsymbionts belong to different taxonomical entities: zooxanthellae (e.g. Rosell & Uriz 1992), cyanobacteria (e.g. Vacelet 1984, Turon *et al.* 2000a, Usher *et al.* 2004b), and heterotrophic bacteria (e.g. Vacelet 1975, Vacelet & Donadey 1977, de Caralt *et al.* 2003), and can represent a significant part of the sponge volume (e.g. up to 30-50%, Vacelet 1975, Wilkinson 1987). Yeasts (e.g. Maldonado *et al.* 2005), and viruses (e.g. Lohr *et al.* 2005) have also been reported to live within sponges but whether these microorganisms are parasites or symbionts has not been elucidated yet.

Due to their abundance, microsymbionts are assumed to be important for the biology and ecology of the sponge, although their functions are unknown in most cases. It has been reported that photosynthetic microsymbionts have a role in sponge nutrition (e.g. Wilkinson & Vacelet 1979, Rosell & Uriz 1992, Sarà 1971). Wilkinson & Vacelet (1979) showed that cyanobacteria transfer glycerol and sugar phosphate to the host sponges. Moreover, Rosell & Uriz (1992) recorded significantly higher growth rates in clionid sponges cultured under a light regime than in those maintained in the dark, and attributed these differences to the zooxanthellae primary production. Sarà (1971) suggested that sponges benefit from these microorganisms by phagocytosis of excess symbionts. On the other hand, the bacterial symbionts can benefit from the nitrogen-rich medium provided by the host sponge in the form of ammonia (e.g. Hentschel *et al.* 2006).

Microbial symbionts may also be responsible for the production of bioactive compounds (e.g. Elyakov *et al.* 1991, Bewley *et al.* 1996, Faulkner *et al.* 2000). Phototrophic bacteria associated with the sponges *Dysidea* sp. and *D. herbacea* (Keller 1889) produced brominated diphenyl ethers (Elyakov *et al.* 1991, Unson & Faulkner 1993, Unson *et al.* 1994). Two different classes of compounds from the sponge *Theonella swinhoei* are produced by two different bacterial symbionts

present in the sponge (Bewley *et al.* 1996). Antimicrobial compounds from the sponge *Hyatella* sp. are actually produced by an associated *Vibrio* sp. (Oclarit *et al.* 1994). Additionally, it has been shown that polyketides and non-ribosomally synthesized peptides of sponges, tunicates, and bryozoans were produced by their bacterial symbionts (Piel 2006).

Microbial symbionts also occur in sponge larvae. Sponge larvae have been reported to harbour bacteria and zooxanthellae (e.g. Mariani *et al.* 2001, Uriz *et al.* 2002). Thus, as in adults, symbiotic microorganisms might represent a non-negligible source of food for larvae, complementary to (or involved in) the reported incorporation of dissolved organic molecules (Jaeckle 1995).

One remarkable case is that of *Crambe crambe* (Schmidt 1862), in which symbiotic bacteria are reported to be present in the larva (Uriz *et al.* 2001) but not in the adults (Galera *et al.* 2000). Adults of *C. crambe* contain spherulous cells with antibacterial properties, which may prevent the presence of symbiotic bacteria. In contrast, spherulous cells are absent in the larvae and, consequently, larvae can harbour bacteria. As a result, *C. crambe* larvae may benefit from their symbionts during their pre-feeding stage (Uriz *et al.* 2001).

Sponge microsymbionts can be intra- and extracellular. Zooxanthellae are found inside the archaeocytes (Mariani *et al.* 2000), and specialized cells called bacteriocytes harbour heterotrophic bacteria (Vacelet 1970). Moreover, cyanobacteria and heterotrophic bacteria can coexist in the same species, spread through the sponge mesohyl (e.g. Vacelet & Donadey 1977, Turon *et al.* 2000a, Usher *et al.* 2001, 2004a).

The larvae of bacteriosponges also contain abundant bacteria (e.g. Ereskosky & Tokina 2004, Uriz *et al.* 2002), which can be acquired by two different ways: vertical transmission from adults and "*de novo*" acquisition from the environment. No evidence of "*de novo*" acquisition has been documented in sponges, although the finding of similar bacteria in taxonomically distant sponges inhabiting the same cave has been interpreted as indirect proof of "*de novo*" acquisition (Muricy *et al.* 1999). Moreover, a recent study presents some evidence of similar microbial types in different sponges, which pose the question about the mechanism of their acquisition (Enticknap *et al.* 2006). In contrast,

several instances of vertical transmission have been reported in recent years (Kaye & Reiswig 1991, Usher *et al.* 2001, 2005, Ereskovsky *et al.* 2005).

Homosclerophorids are a particularly suitable sponge group for studying bacterial transmission because all the species of this group examined so far, are bacteria-rich (e. g. Ereskovsky & Boury-Esnault 2002). Moreover, larvae of various genera of homosclerophorids have been reported to contain symbiotic bacteria (Boury-Esnault *et al.* 2003), but transfer of bacteria from parents to larvae has only been documented in *Oscarella* spp. (Ereskovsky & Boury-Esnault 2002). The present study reports on the transfer of heterotrophic bacteria from parental tissue to oocytes in the bacteriosponge *Corticium candelabrum* (Homosclerophorida), describing the successive locations of bacteria during embryo and larval development. We also describe and compare the several morphotypes of bacteria present in adults and larvae, through electron microscopy.

Material and methods

Samples of *Corticium candelabrum* Schmidt, 1862 were collected during the reproductive period (Spring-Summer, 2002) by Scuba diving in the western Mediterranean (Blanes), at a depth of 7-12 m. Once in the laboratory subsamples of about $3mm^3$ in size were fixed for TEM and SEM observations.

Samples for TEM were fixed in a mixture of 1% OsO_4 and 2% glutaraldehyde in 0.45 M sodium acetate buffer (pH 6.4) with 10% sucrose (1/3) (Leys & Reiswig 1998), dehydrated in an alcohol series and embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate and then observed under a Hitachi H-600 transmission electron microscope (Microscopy Unit of the Scientific and Technical Services of the University of Barcelona). For SEM observations, samples were fixed with a mixture of 2% OsO_4 and saturated $HgCl_2$ (6/1) for 90 min (Johnston & Hildemann 1982), dehydrated in an ethanol series, critical-point dried, mounted and sputter-coated with gold-palladium, and examined through a Hitachi-2300 scanning electron microscope (Institute of Marine Sciences of Barcelona, CSIC).

Bacteria morphotypes (from both adults and embryos) were characterized through TEM. The main phenotypic characters considered were size and general shape, the bacteria wall, the presence or absence of periplasm, the number of membranes, the nuclear region, and the cytoplasm density.

Results

Adults

Symbiotic bacteria represent about 50% of the sponge volume in *Corticium candelabrum*. The symbiotic bacterial population, which is extracellular, is often embedded in collagen (**Figs. 1**). Symbiotic bacteria are isolated from the choanocyte chambers by a thick collagen layer. Eight different phenotypic bacterial types can be recognized in the adult tissue (**Fig. 2**). Bacteria types B, C, D, F, G and H have complex cell walls, typical of gram-negative bacteria.

Type A) Large (to a maximum of ca. 3.1 µm in length, and ca. 1.2 µm in width), cylindrical to ovoid bacteria. The cell is surrounded by two close membranes (ca. 30 - 40 nm thick altogether), the inner one (cytoplasm membrane) poorly differentiated from the cell cytoplasm. The cytoplasm is dense to electrons, without a distinct nuclear region. This bacteria type is relatively abundant and the largest one recorded in the adult sponge tissue.

Type B) Ca. 0.7 - 1.3 µm in length and ca. 0.6 - 0.8 µm in width, bacteria. The cell has a double membrane separated by a relatively wide (ca. 60 nm), clear to electrons, periplasm. The external membrane (cell wall), ca. 10 nm in thickness, is dark and well delimited. The cytoplasm is lax at the central zone and denser at the periphery of the cell. The nucleoid (zones clear to electrons) is spread across the cytoplasm.

Type C) Medium-sized (ca. 1.3 - 1.5 µm in length and ca. 0.9 µm in width), variable in shape (from triangular to circular in transverse sections) bacteria with a very wide (up to 180 nm) cover consisting of two layers separated by a well developed, periplasm clear to electrons. The internal matrix is granulated, with one

highly electro-dense spot (not always visible in the sections). The nuclear region is large and occupies almost the whole cell.

Type D) Cylindrical, ca. 1 µm in length and ca. 0.7 µm in width, bacteria. Thick cover consisting of a cytoplasmic membrane, a very wide (ca. 0.2 µm) medium electro-dense (grey) periplasm, which is wider at the two cell poles, and an electro-dense membrane (cell wall). The cytoplasmic membrane projects several folds towards the periplasm (vesicular aspect). The nuclear region is large, mainly located at the centre of the cell, surrounded by a granular cytoplasm dense to electrons.

Type E) Cylindrical, slightly irregular in cross-section (ca. 1.4 - 0.7 µm in diameters) bacteria with the cytoplasmic membrane and the cell wall intimately in contact (without periplasm). The nucleoid is large and occupies a central position while the cytoplasm is mainly relegated to the periphery with granules dense to electrons in close contact with the cytoplasmatic membrane.

Type F) Cylindrical (ca 1.1 µm in length and ca. 0.5 µm in width) bacteria with two membranes separated by a poorly developed periplasm (ca. 20 nm). The cell wall is finely undulated. The internal matrix is very dense and granular without a conspicuous nuclear region.

Type G) Cylindrical (ca 1.2 µm x 0.8 µm) bacteria with a complex cover consisting of three conspicuous membranes. The two internal ones are close to each other, separated from the most external and darker membrane by a periplasm clear to electrons, ca. 70 nm width. The cytoplasm is very dense and uniformly granulated. No nuclear region is visible.

Type H) Small (ca. 0.8 µm x 0.2 µm), irregularly elongated bacteria, with a uniform, very dense to electrons, cytoplasm (completely black in our pictures) and without a conspicuous nuclear region. A periplasm clear to electrons separates the "black" area from the external membrane (cell wall), which is clearer to electrons than the cytoplasm.

Taking into consideration the many sections observed, we can state that most of the bacteria types are similarly frequent in the adult tissue. Only types A and H appear to be more abundant (two and three times more frequent than the

other types, respectively). No instances of phagocytosis have been seen in the many pictures of adult tissue observed.

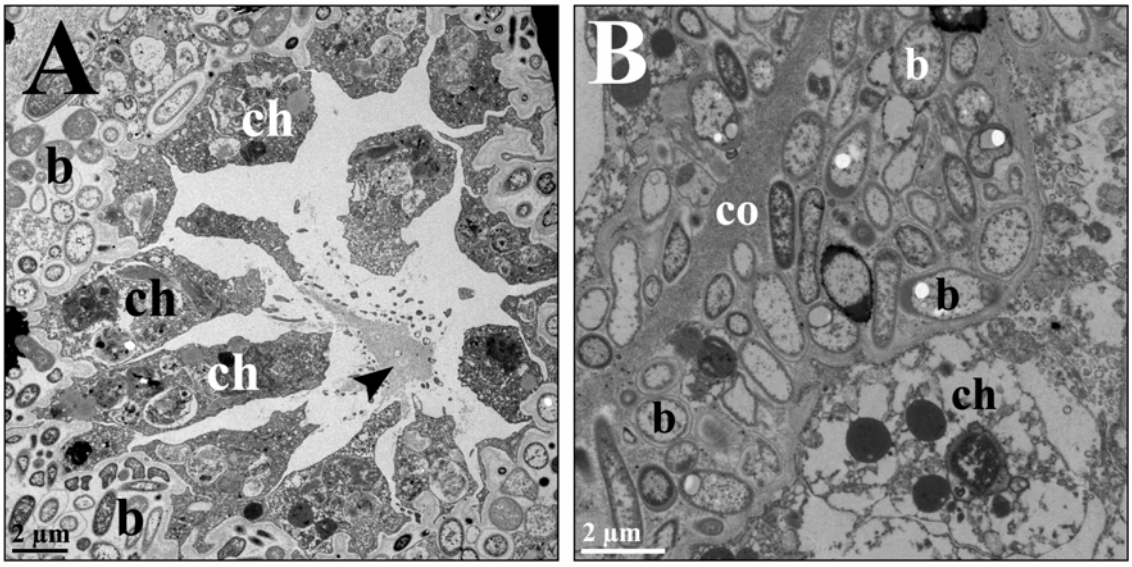

Fig. 1- *Corticium candelabrum*. **A:** TEM picture of symbiotic bacteria in adult tissue: (ch) choanocytes forming a chamber; (b) high amounts of bacteria belonging to several morphotypes; (arrowhead) collagen mass in the chamber light. **B:** TEM picture of a detail of symbiotic bacteria in adult tissue: (b) Extracellular bacteria; (co) thick collagen layer surrounding the bacteria; (ch) choanocyte separated from the bacteria by the collagen layer.

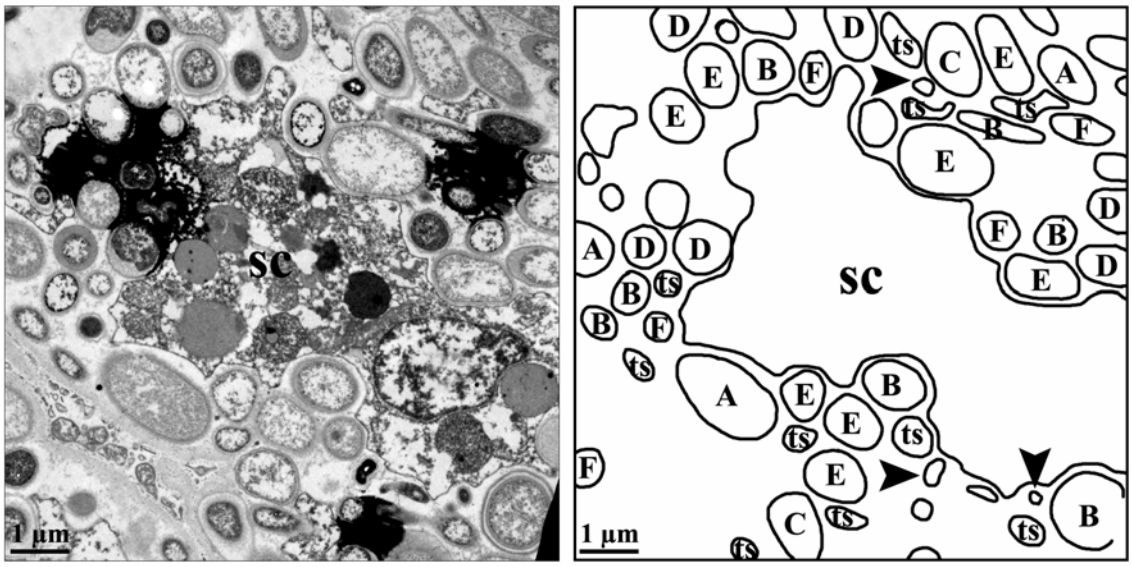

Fig. 2 - *Corticium candelabrum*. TEM picture showing the eight types of symbiotic bacteria (from A to H in the scheme) observed in adult tissue (see text for description of the characteristic of each type): (sc) sponge cell.

Gametes

Although *C. candelabrum* is a hermaphrodite species, symbiotic bacteria are only transferred to oocytes while spermatogonia are bacteria-free.

Since spermatogonia originate from choanocyte chambers, the thick collagen layer that surrounds the functional choanocyte chambers is maintained during spermatogonia formation operating as a barrier that prevents the entrance of symbiotic bacteria (**Fig. 3A**).

In contrast, the oocytes, which are originated from archeocytes, are in direct contact with the parental mesohyl. Thus, all types of symbiotic bacteria seem to be transferred directly during oocyte growth, together with the nurse cells from the adult. After fertilization, a collagen layer is formed surrounding the egg (**Fig. 3B**).

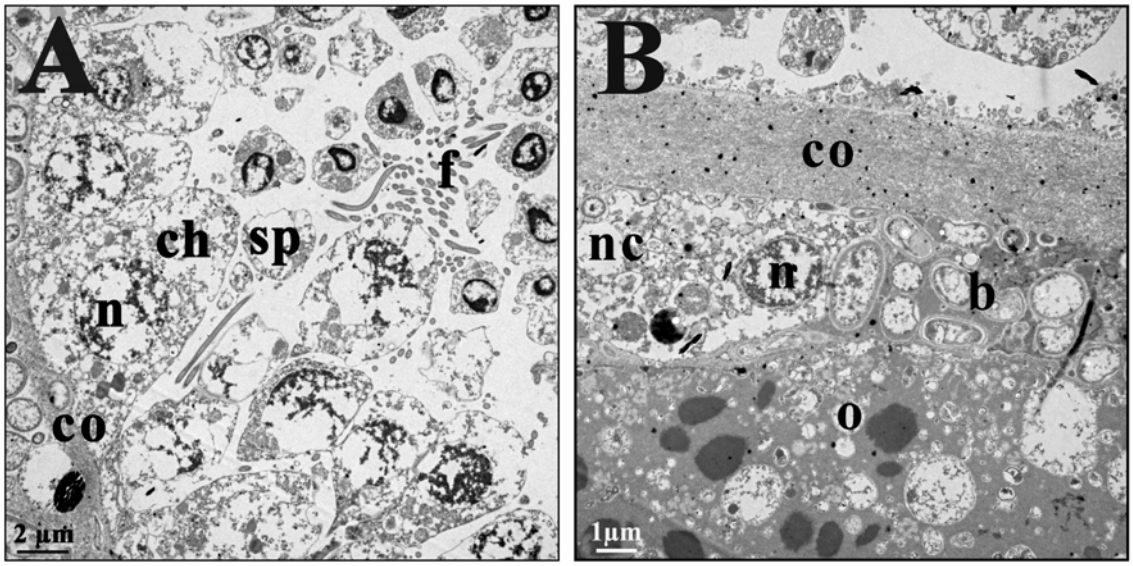

Fig. 3 - *Corticium candelabrum.* **A:** TEM picture of an early stage of spermatogonia (note the absence of bacteria): (ch) choanocyte; (n) choanocyte nucleus; (sp) spermatid; (f) flagella; (co) collagen layer surrounding the spermatogonia. **B:** TEM picture of a later (fertilised?) oocyte surrounded by bacteria: (o) oocyte; (nc) nurse cells surrounding the oocyte; (n) nucleus of the nurse cells; (b) bacteria at the oocyte periphery; (co) thick collagen layer surrounding the nurse cells.

Mature (100 - 150 µm in diameter) oocytes have symbiotic bacteria inside the cytoplasm. At this stage, in which the oocyte cytoplasm is filled with yolk granules and some lipids, the symbiotic bacteria are concentrated in spherical clusters resulting from bacteria that migrated from the oocyte periphery (**Fig. 4**).

Bacterial clusters are in direct contact with the oocyte cytoplasm without any surrounding collagen layer. The eight different morphotypes present in the adults are found with a similar relative abundance in the oocyte (**Fig. 5A**). The types A and H, as in adults, are the most abundant bacteria in both the oocyte and embryo. Bacteria clusters in the oocyte increase in size by bacterial bipartition. The similarity between adult and embryo bacteria suggests that all the bacterial types are transmitted directly from parental tissue to oocytes (vertical transmission).

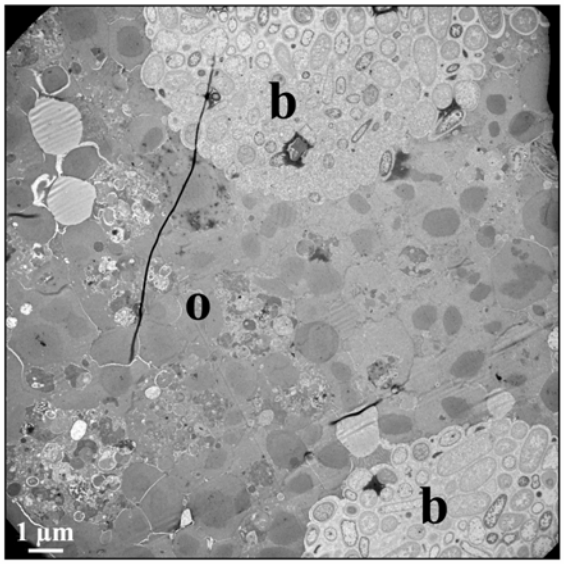

Fig. 4 - *Corticium candelabrum.* TEM picture of an oocyte with bacteria concentrated in two spherical clusters: (o) oocyte; (b) bacteria.

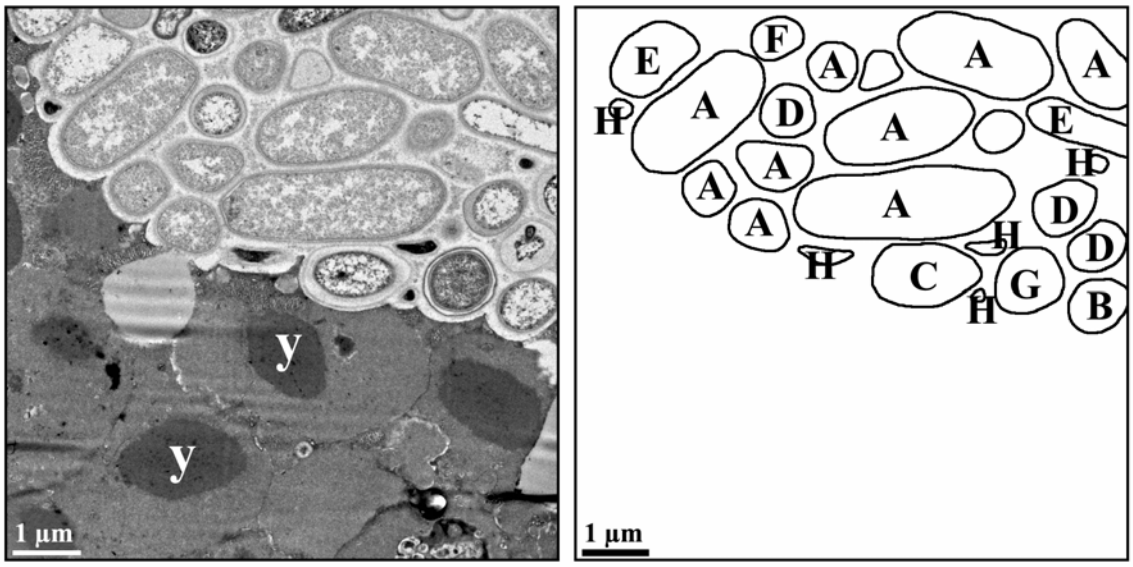

Fig. 5 - *Corticium candelabrum.* TEM picture of the bacteria types in an oocyte (see text for description of the characteristic of each type; letters correspond to the same type as in the adult): (y) yolk granules of the oocyte.

Embryo

During cleavage, follicular cells and a thin collagen layer surround the embryo. When the blastomeres start to form (initial cleavage phase), the spherical clusters of bacteria disaggregate and most bacteria are pushed to the embryo periphery although some of them can be found in the free space among blastomeres (**Fig. 6**).

When the internal cavity (blastocoel) starts to form by centrifugal cell migration (i.e. from the centre of the embryo to the periphery), the symbiotic bacteria migrate from the embryo periphery to this internal cavity, which also contains dispersed collagen fibrils.

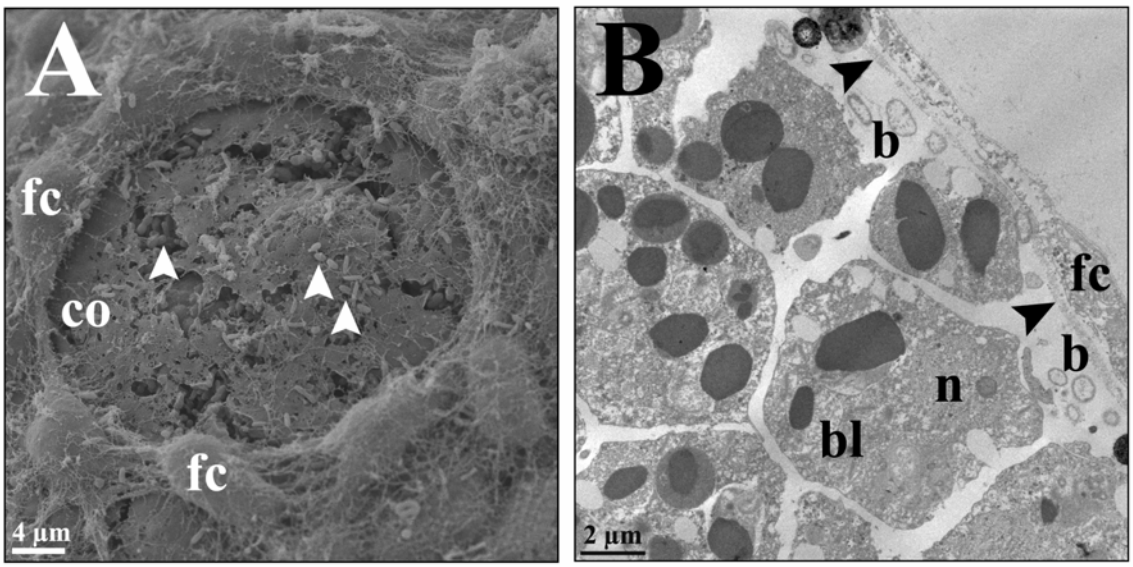

Fig. 6 - *Corticium candelabrum.* **A:** SEM picture of the surface of an embryo covered by the follicular layer. Some follicular cells were removed to show the collagen layer surrounding the embryo: (fc) follicular cells; (co) collagen layer; (arrowheads) bacteria. **B:** TEM picture of an embryo: (bl) blastomeres; (n) nucleus of the blastomeres; (b) bacteria at the embryo periphery; (co) collagen layer surrounding the embryo; (fc) follicular cells.

Larvae

Bacteria within the internal cavity of the mature embryo increase in number by bipartition as the cell cavity also increased in size (blastocoel). The bacterial population of the completely formed larva consists of the same morphotypes found in the adults (**Figs. 7A**, **7B** and **7C**). A discrete collagen layer, considered corresponding to a basement membrane (Boury-Esnault *et al.* 2003), separates the pseudostratified layer of larval cells from the bacterial population inside the blastocoel (**Fig. 7D**).

Many instances of phagocyted bacteria are observed at the proximal zone of the larval cells, close to the blastocoel (**Fig. 7D**). In contrast, the distal zone of the cells is bacteria free. Phagocytosis of bacteria seems to be a particular feature of larval cells since no instances of engulfed bacteria have been found in adult tissues.

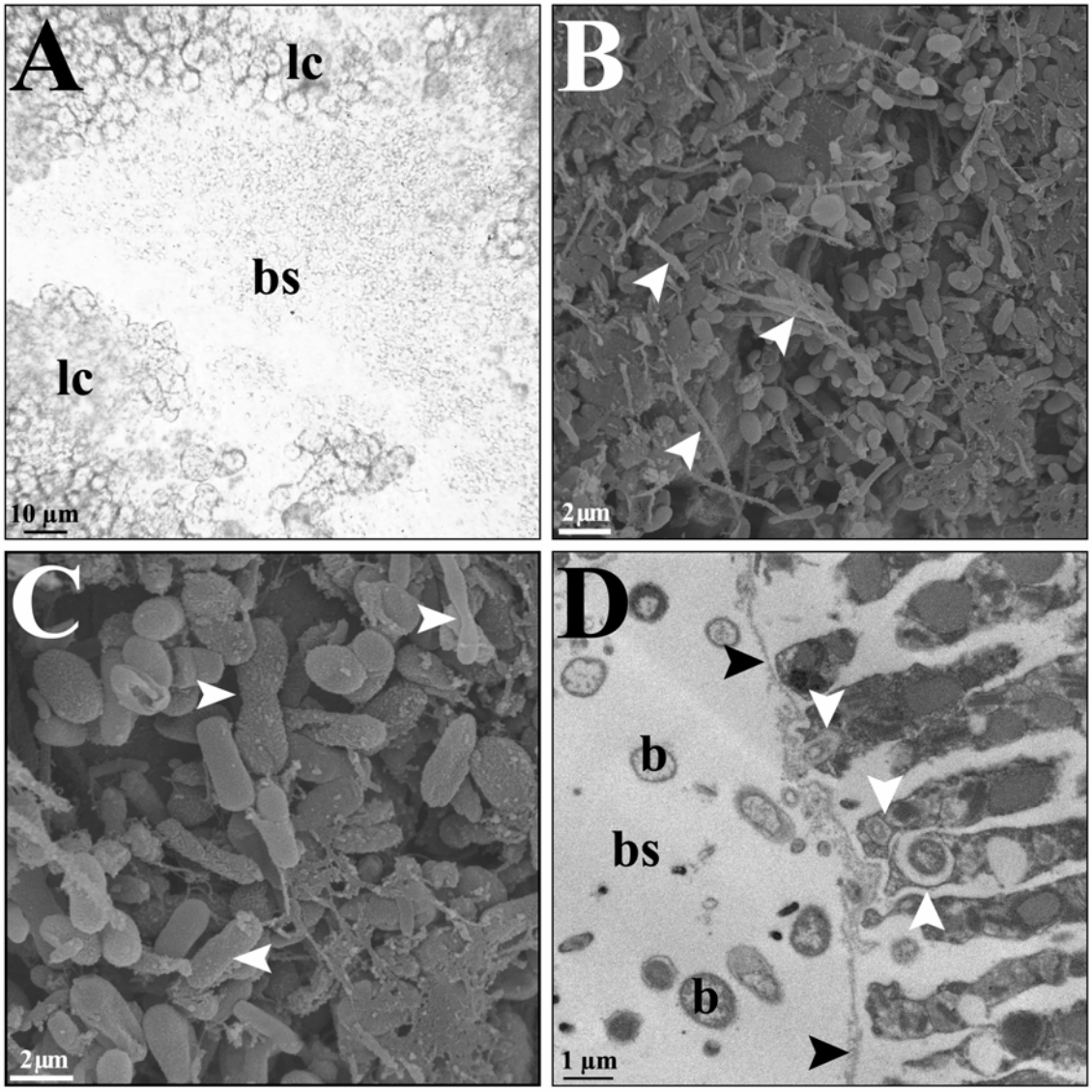

Fig. 7- *Corticium candelabrum.* **A:** Light microscopy picture of a larva: (bs) blastocoel full of bacteria; (lc) larval cells. **B:** SEM image of a broken larva releasing the inner bacteria: (arrowheads) larva cilia. **C:** SEM image of several bacterial morphotypes: (arrowheads) detail of a bacteria bipartition. **D:** TEM image of a *Corticium candelabrum* larva: (bs) completely formed blastocoel; (black arrowheads) collagen layer (i.e. basement membrane) at the base of the pseudostratified larval cells; (b) free bacteria in the blastocoel; (white arrowheads) engulfed bacteria in the proximal zone of larval cells.

The whole process of the vertical transmission of symbiotic bacteria in *C. candelabrum* is schematically illustrated in **Fig. 8**: bacteria are transferred from parental tissue during early stages of oogenesis. The transferred bacteria replicate inside the oocyte, forming bacteria clusters. Embryogenesis involves bacteria proliferation at the embryo periphery below the collagen layer and, in less abundance, in the space between blastomeres. Once the blastocoel is formed,

bacteria accumulate in this fluid-filled cavity. Thus, the free larvae consist of a layer of ciliated cells surrounding a bacteria-filled blastocoel.

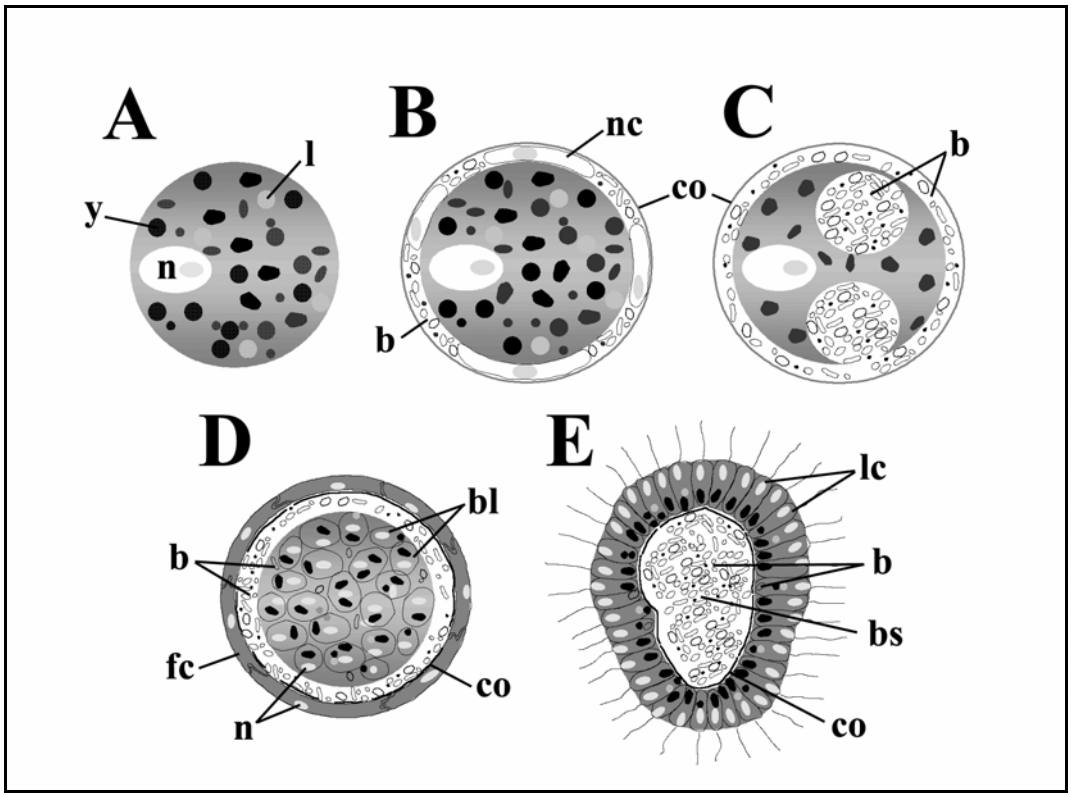

Fig. 8 - Schematic representation of the successive location of bacteria from oocyte to larva in *Corticium candelabrum*. **A:** Oocyte: (n) nucleus; (y) yolk granules; (l) lipids. **B:** Fertilized oocyte: (nc) nurse cells; (b) bacteria; (co) collagen layer. **C:** Oocyte before cleavage with spherical clusters of bacteria: (co) collagen layer; (b) bacteria. **D:** Embryo: (co) collagen layer; (fc) follicular cells; (b) bacteria mainly located at the periphery and also in the free space between blastomeres; (bl) blastomeres; (n) nucleus. **E:** Completely formed cinctoblastula: (lc) larval cells; (co) collagen layer/basement membrane; (bs) blastocoel; (b) bacteria inside the blastocoel and also phagocyted by larval cells.

Discussion

Bacteriosponges are found in most orders of Demospongiae but are particularly abundant among the Dictyoceratida, Dendroceratida and Homosclerophorida. The way by which bacteria of bacteriosponges are acquired by the progeny is an issue repeatedly dealt with in the literature. Vertical transmission is the most often reported mechanism (e.g. Kaye 1991, Sciscioli *et al.* 1994, Usher *et al.* 2001, 2005, Ereskovsky *et al.* 2005, Enticknap *et al.* 2006) and favours mutualism to evolve (Ewald 1987).

In *Corticium candelabrum,* vertical transmission of microsymbionts occurs before oocyte fertilization and cleavage take place, as has been reported for other homosclerophorids (Ereskovsky & Boury-Esnault 2002). With the information at hand, transmission before cleavage seems to be a rule for sponges with cinctoblastula larvae (Homosclerophorida) and for oviparous sponges (e.g. Sciscioli *et al.* 1991, Gallissian & Vacelet 1976, Warburton 1961, authors pers. obs.). In contrast, bacterial transmission seems to take place during cleavage in most larviparous sponges that produce solid larvae (parenchymella) (Sizova & Ereskovysky 1997, Ereskosky & Gonobobleva 2000, Kaye & Reiswig 1991). In oviparous sponges, oocytes surrounded by a chorion are released to the environment before cleavage takes place (Mariani *et al.* 2001) and, thus, only during oocyte formation can microsymbionts be acquired from the sponge parental tissue. Similarly, in *C. candelabrum*, bacteria are transferred to the oocyte when it is in contact with the parental tissue (i.e. before fertilisation), whereas at later stages (fertilised oocyte), a thick collagen layer (**Fig. 3B**) isolates the egg from the parental tissue (i.e. no bacteria can be transferred anymore).

The cinctoblastula of *C. candelabrum* has the same morphotypes of symbiotic bacteria as the adults, which confirms that all bacterial types are transferred parentally. These observations point to a species-specificity of the symbiotic bacteria of *C. candelabrum* as has been reported for other homosclerophorids (Boury-Esnault *et al.* 1995, Muricy *et al.* 1999, Ereskovsky & Boury-Esnault 2002).

The role of symbiotic bacteria in sponges has been largely speculated (e.g. Wilkinson & Vacelet 1979, Faulkner *et al.* 2000). Phagocytosis of bacteria has not been observed in adults of *C. candelabrum* probably due to the presence of a dense collagen layer, which isolates bacteria from the sponge tissue (de Caralt *et al.* 2003). This seems to be a particular feature of *C. candelabrum* (and maybe of Homosclerophorida) since no collagen layer has been observed separating the symbiotic bacteria from the sponge tissue in larvae or adults of any other bacteriosponge examined to date (e.g. Kaye 1991, Kaye & Reiswig 1991, Ereskovsky & Tokina 2004, Uriz *et al.* 2002).

This is the first time that significant phagocytosis of bacteria has been recorded in larvae of a homosclerophorid. Several possible roles could put forward for the observed phagocyted bacteria by larval cells such as housekeeping, culled of foreign bacteria or a nutritive function. We suggest a nutritional role of these microorganisms as the most likely function in *C. candelabrum* during larval stages, when bacteria may represent a supplementary source of nutrients. This extra-food may contribute to enlarging the competence period of free larvae (Uriz *et al.* 2002), and to increase the success of settlers before their feeding system (aquiferous system) is developed and the filtering activity provides the sponge with external food. On the other hand, although phagocytosis of bacteria has not been previously reported in larvae of homosclerophorids, it has been repeatedly documented in parenchymellae of Dictyoceratida (e.g. Uriz *et al.* 2002, Ereskovsky & Tokina 2004) and thus it seems to represent a non-negligible source of nutrients for larvae of bacteriosponges in general during their free-swimming period.

Acknowledgements

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Chapter VII

Cell culture from sponges: Pluripotency and immortality

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Abstract

Sponges have an enormous potential for the development of new medical drugs. Methods of sponge cell culture for production of these bioactive compounds are shortly reviewed, and new approaches are examined to overcome the main bottlenecks. The use of embryos is proposed as a new source of sponge material for cell culture. Embryonic stem cells are more versatile and resistant to infections than adult stem cells (archeocytes). Moreover, stem cells are in higher amounts in embryos than in adult sponges. The optimization of the method for obtaining and cryopreserving larvae, as well as the culture of juveniles under laboratory conditions may provide embryonic cells along the year. Additionally, genetic engineering and cellular research on mitotic and apoptotic mechanisms are new promising fields that may help to stimulate cell division and improve cell survival. In particular, one of the topics for future research should focus on how to reduce apoptosis, which appears to be very high in sponge cells. Finally, the molecular research should be combined with studies on basic aspects of the biology, ecology, physiology of the target sponges for a more complete understanding of all requirements of sponge cells under culture. Only after such studies, culture technologies could be standardized to be applied to any target sponge species.

Introduction

Sponges produce bioactive secondary metabolites, which play several ecological roles (defence against predators, deterrence, and antifouling, e.g. Becerro *et al.* 2003). These metabolites are potentially interesting as new pharmaceuticals or cosmetics (e.g. Newman *et al.* 2004, Sipkema *et al.* 2005a) and thus have an enormous prospect. The bottleneck to produce new medicines from sponges is the supply of sufficient sponge biomass for both research and market needs.

Sponges have been cultured with different purposes over the last century. Traditionally, sponge mariculture was performed for commercial production of bath sponges (Moore 1910). At present, culture of sponge explants, or cells under controlled conditions is investigating with the aim of supplying the pharmaceutical industry with sponge biomass

The target bioactive metabolites obtained from sponges can be produced either by the sponges themselves or by their symbiotic microorganisms (Faulkner *et al.* 2000), which are abundant in a large number of species (e.g. Sarà & Vacelet 1973). However, in most cases, the sponges are direct responsible for the production of the bioactive compounds (e.g. Thompson *et al.* 1983, Uriz *et al.* 1996a,b, Pomponi *et al.* 1997) and only in a few cases it has been unequivocally demonstrated that they are produced by the symbionts (e.g. Elyakov *et al.* 1991, Bewley *et al.* 1996).

Obtaining sponge biomass by harvesting the sponges directly from the sea is not feasible in most cases. Even when the sponge species is very abundant, a massive extraction of these "slow-growing" animals would result in a harmful impact for the environment.

On the other hand, the chemical synthesis of the target metabolites is not feasible either, in many cases due to the complexity of the chemical structures of these compounds, which make their synthesis impossible or too expensive. Transfection of the genes involved in the production of some bioactive substances to a cultivable microorganism such as *E. coli* has also been explored. However, the large number of genes that express the enzymes involved in the metabolic

pathways greatly complicate this process and the current results are not conclusive yet (Schmidt 2005).

As an alternative, several sponge culture methods have been used with variable success:

1. Mariculture: based on the previous experience on bath sponge mariculture, sponges have been cultured in their natural habitat (e.g. Battershill & Page 1996).
2. Cultivation of sponge explants under controlled or semi-controlled environmental conditions (closed or semi-open systems) (e.g. Duckworth *et al.* 1997).
3. *In vitro* cultures, under totally controlled conditions, of explants, primmorphs or dissociated cells (e.g. Osinga *et al.* 1999, Pomponi & Willoughby 1994, Muller *et al.* 1999)

For *in vitro* cultures, intact individuals are not appropriate, but large numbers of small sponge units are required. Thus, sponge explants have extensively been used for *in vitro* sponge culture (e.g. de Caralt *et al.* 2003, Osinga *et al.* 2003, Sipkema *et al.* 2006). The research on this type of culture focused on two main issues: **(i)** to determine the optimal conditions to obtain healthy explants (e.g. de Caralt *et al.* 2003) and **(ii)** to monitor the explant survival and growth under different treatments (e.g. Belarbi *et al.* 2003, de Caralt *et al.* 2003, Osinga *et al.* 2003). Although long-term survival of explants has been obtained, explant growth was, at best, comparable to the usually variable and low sponge growth in the field (e.g. Turon *et al.* 1998, de Caralt *et al.* 2003, de Caralt *et al.* 2007d submitted). With the aim of reducing the variability and the slow growth of cultured explants, the culture of juveniles from larvae instead of from adult sponges, was assayed. The use of juveniles allowed us to perform experiments based on many replicates, (i.e. thousands). Moreover, juveniles showed a higher growth capacity than explants from adults (de Caralt *et al.* 2007a submitted).

In this review we focus on the culture of sponge cells as a direct source of bioactive substances. In those cases, a prerequisite for their biotechnological production is to obtain a clear defined system for culturing dissociated sponge

cells or tissue free of bacteria, yeast, and protozoa contaminants. At the cell level, most sponge culture methods have been based on suspended sponge cells (e.g. Pomponi & Willoughby 1994) or cell aggregates derived from cell suspensions, which were called primmorphs (Müller *et al.* 1999). In these studies, primary cell cultures were obtained successfully, but continuously dividing cell lines could not be maintained. The main problems encountered were the difficulties to keep the cultures axenic, to stimulate cells to divide continuously, to reduce cell mortality, and to obtain pluripotent cells. Studies about cell culture focused on these three main issues: **(i)** to assay different antibiotic cocktails and methods for sponge cell isolation **(ii)** to develop optimal growth media with addition of growth precursors (e.g. Willoughby & Pomponi 2000, Zhao *et al.* 2005), and **(iii)** to use archeocytes for the cultures because of their pluripotency.

In this review, we propose a new approach that may help to solve some of the difficulties that are encountered in cell culture studies and may open new possibilities: to use the embryos and larvae as a new source of sponge material to culture.

Cell culture from adult sponges

Primary cell lines have been developed from 6 phyla of marine invertebrates, (Porifera, Cnidaria, Crustacea, Mollusca, Echinodermata and Urochordata). For sponges, several studies have described protocols to get a primary cell culture. The maintenance of the living sponge material in good conditions during their collection and shipment is important to start a viable cell culture. Rinkevich *et al.* (1994), Ilan *et al.* (1996) and Pomponi *et al.* (1997) showed that, once in the laboratory, the most efficient method to disaggregate sponge cells was spontaneous dissociation followed by mechanical dissociation (Ilan *et al.* 1996). The use of a sponge without cortex demonstrated good results in the disaggregation process (Ilan *et al.* 1996). Moreover, the type of substratum offered to cells for attachment also had an effect on the cell culture viability (Gaino & Magnino 1994, Leys 1997).

However, no continuous cell lines have been obtained from sponges (Rinkevich 2005), in part due to the lack of essential information on the biology and physiology of the species under culture. There is insufficient knowledge about: **(i)** the requirements for growth and the biochemical mechanisms involved in cell growth **(ii)** the procedures to reduce contamination to a minimum and **(iii)** the methods to obtain viable pluripotent cells.

(i) *Culture medium.* Once the cells are dissociated, primary cells should be grown and so the composition of the growth media, including specific growth factors or particular molecules could improve the culture success. In several studies a basal medium for animal cell cultures (Freshney 1994) was used supplemented with new constituents, which are supposed to be critical for the sponge cells (e.g. amino acids, vitamins, salts, glucose, organic supplements, hormones and growth factors) (reviewed in Pomponi 2006). For instance, addition of pyruvate, vitamine C, and NaCl has been reported to increase significantly cell viability (e.g. Zhang *et al.* 2004, Zhao *et al.* 2005). Other studies modified the concentrations of the constituents to find empirically the optimal medium. Reducing glutamine and selenium concentrations in the standard medium appeared to result in greater DNA, protein, and esterase activity signals (Willoughby & Pomponi 2000).

Furthermore, several studies demonstrated the positive effect of adding growth factors on cell viability and growth. Phytohemagglutinin, a conventional mammalian mitogen, stimulated cell division in *Hymeniacidon heliophila* (Pomponi & Willoughby 1994). Insulin, a wheat germ agglutinin, and the *Ulex europaeus* agglutinin improved cell viability in cultures compared with the controls (Rinkevich *et al.* 1998). However, none of those studies succeeded in providing an ideal medium in which sponge cells could grow continuously.

(ii) *Contamination*. An unsolved problem in cell culture from sponges and other marine invertebrates is the contamination by protozoans and bacteria (Rinkevich 1999), which shortens the culture duration and thus prevents a continuous cell line to be maintained (e.g. in mollusk: Chen & Wen 1999, Barik *et al.* 2004). Removing microorganisms from the sponges is particularly difficult because of the wide presence of bacteria in the sponge tissues. In many cases,

bacteria are sponge symbionts, while in other cases they are incorporated from the environment as a source of food. Physical separation of the sponge cells from microorganisms before setting up the cell culture, and the use of antibiotics during cultivation are the best methods commonly used to prevent culture contamination (e.g. Pomponi & Willoughby 1994).

(iii) *Stem cells.* For a continuous cell line, cells need to be "immortal" (continuously dividing) and pluripotent (able to differentiate in several specialized cells). Stem cells have unlimited cell division capacity, to self-renew and to produce differentiated progeny (Blau *et al.* 2001). In sponges, molecular evidences for the existence of stem cells have been provided (Müller *et al.* 2003). Pluripotent cell types in sponges are archeocytes in adult sponges, thesocytes in resistance gemmules, and blastomers in embryos (**Fig. 1**). Archeocytes are pluripotent cells that are present along the whole development process, being capable to differentiate in any other cell type (Simpson 1984, De Sutter & Buscema 1977, Pomponi & Willoughby 1994).

Several attempts to obtain sponge cell lines from pure fractions of archeocytes have been done. De Sutter & Van der Vyver (1977, 1979) reported on a method for obtaining a pure sample of archeocytes from the fresh-water sponge *Ephydatia fluviatilis*. However, a mixture of archeocytes and several differentiated cells (e.g. choanocytes, pinacocytes, sclerocytes, etc.) has been more commonly obtained after dissociation and fractionation of cell types by density gradient methods (e.g. Pomponi & Willoughby 1994, Uriz *et al.* 1996a, Müller *et al.* 2003). Primmorphs obtained from cell suspensions enriched in archeocytes performed better (more active DNA synthesis and subsequent cell division) than those originating from a non-enriched in archeocytes cell mixture (Zhang *et al.* 2003). Nevertheless, continuous cell lines from archeocyte-enriched cell cultures were not obtained: only a few cell divisions followed by an early cell death were observed (e.g. De Rosa *et al.* 2003, Zhang *et al.* 2003). Maybe the drawbacks encountered were related to the impossibility of obtaining a pure cell fraction of adult stem cells, which might be solved by culturing sponge cells from embryonic stem cells.

Cell culture from sponge embryos and larvae

Sponge embryos and larvae could be a promising source of cells for continuous cultures since they contain a higher proportion of stem cells than adults and are present in a large number in ripe sponges (**Fig. 1**). Consequently, obtaining a pure cell fraction of stem cells may be easier from embryos and larvae than from adult sponges. For instance, Uriz *et al.* (1998) measured a 72.2 ± 12.5 (mean ± SE) embryos per cm^2 of sponge tissue in *Crambe crambe*. If we consider a hypothetical adult about 80 cm^2 in size, this individual could brood ca. 6000 embryos. Embryos are formed mainly by blastomeres (embryonic stem cells), and thus all their cells are suitable to be cultured. Sponge larvae have relatively high quantities of stem cells as well. In fact, larvae are mature embryos, in which some cell differentiation has occurred (e.g. Uriz *et al.* 2001). The few cell types that can be distinguished in a larva are in fact transient cell types that can still differentiate in most adult cell types (e.g. Amano & Hori 2001). Thus, although an embryo probably will be the optimal source of stem cells, larvae also represent a promising source.

Invertebrate embryonic cells are reported to be more versatile and less vulnerable to infections from microorganisms than the adult stem cells (Rinkevich & Rabinowitz 1994) and seem to be suitable to establish long-term cell cultures of marine invertebrates (Rinkevich *et al.* 1994, 1998, Rinkevich & Ravinowitz 1994, Rinkevich 1999). Embryonic stem cells have been used to develop cell cultures mainly in molluscs and echinoderms (e.g. Ellis & Bishop 1989, Boulo *et al.* 2000, and Ermak & Odintsova 1996, Hwang *et al.* 1999, respectively), less often in other marine invertebrates (e.g. crustaceans: Fraser & Hall 1999, Owens & Smith 1999, ascidians: Rinkevitch & Rabinowitz 1994), and rarely in lower metazoans such as cnidarians (Frank *et al.* 1994) and sponges (Rinkevich *et al.* 1998).

Rinkevich *et al.* (1998) showed the suitability of the embryonic sponge cells. Primary cultures developed from embryonic cells of *Negombata magnifica* were maintained over a longer period of time (284 days) than the cultures obtained from adult individuals (130 days). This study showed that mechanical dissociation was the best method to obtain cells from embryos, according to the

number of viable cells (ca. more than 10^4 cells/embryo), the relatively high proportion of blastomeres (60%), and a longer survival of the cells. Although this result is promising as it shows that cultures from embryonic cells are more viable, this work was not followed up.

The two main difficulties found in the above-mentioned study were to discriminate between the embryonic cells (stem cells) and contaminant protozoans and the problem of obtaining a continuous supply of embryos along the year due to the seasonal reproduction of most species (Rinkevich *et al.* 1998, Rinkevich 1999).

The difficulty in identifying embryonic cells is solved with the advent of molecular techniques. Molecular markers allow us to distinguishing between sponge and contaminant cells (e.g. Müller *at al.* 1999, Lopez *et al.* 2002, Sipkema *et al.* 2003a). Moreover, the identification of specific cell types (e.g. pinacocytes, sclerocytes and collencytes) by genetic markers in sponges is in development (Müller 2006). In addition, studies about larval development have proliferated in the last decade, providing key aspects of the morphological and ultrastructural characteristics of embryonic and larval cells (e.g. Amano & Hori 2001, Uriz *et al.* 2001, Leys & Degnan 2002, Boury-Esnault *et al.* 2003, de Caralt *et al.* 2007b *in press*).

The supply of embryonic cells is unresolved so far. Several solutions have been proposed that should be investigated in depth. Crypopreservation of sponge embryos may provide a continuous supply of material for experimentation (Rinkevich *et al.* 1998; Rinkevich & Shafir 2000). It was reported that the cryopreservation of larvae did not affect the initial cell viability, although later on, during the culture more bacteria contamination was observed (Rinkevich *et al.* 1998). More recently cryopreserved cell stocks of adult sponges have been used for research purposes with viability comparable to that obtained with cryopreserved mammalian cells (Willoughby & Pomponi 2000, Pomponi & Willoughby 2001). Thus, this technique can be proposed for cell storage.

Although embryos seem to be the optimal source of stem cells, larvae also contain stem cells in high numbers and are easier to be obtained. We have recently explored how to obtain larvae from ripe adult individuals that were kept in

aquaria under controlled conditions. Those ripe individuals released hundreds of larvae spontaneously (de Caralt *et al.* 2007a, submitted), which proved to be in good conditions since they settled and developed into healthy juveniles. Obtaining large amounts of larvae in the laboratory from ripe sponges may warrant a stock of stem cells to develop cell cultures along the year. The higher survival rates of larvae under laboratory conditions, in contrast to the extraordinarily high mortality of larvae at sea (Cebrian *et al.* 2007, de Caralt *et al.* 2007a, submitted), makes the first method preferable.

Massive extraction of embryos from ripe individuals may also be considered. Separation of the sponge tissue containing embryos could be performed by hand under a stereomicroscope. After cell dissociation, the stem embryonic cells (blastomers) could be sorted from the remaining cells by flow cytometry, provided some embryonic characteristic (e.g. yolk abundance) or a fluorescent labeling allows blastomer discrimination.

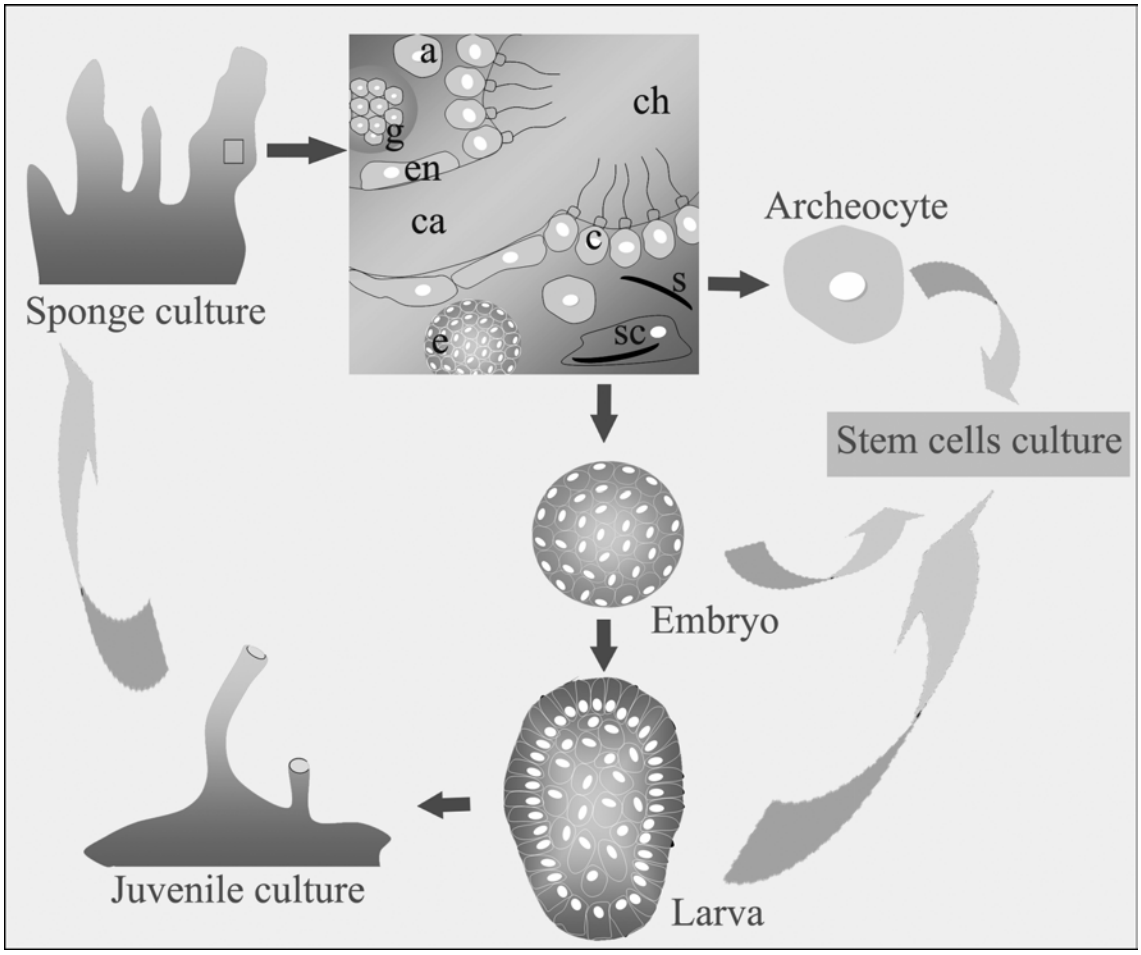

Fig. 1- Schematic representation of different sponge material for stem cell cultures. Adult sponges have differentiated cells (e.g. choanocytes, pinacocytes, sclerocytes) and stem cells (i.e. archeocytes). Ripe sponges also harbour embryos in high numbers, which are composed of blastomeres (stem cells) and thus, the number of stem cells increases notably per sponge unit during the later stages of the reproduction cycle. The embryos are released as larvae and their culture can be use in two directions: as a source of (i) stem cells and (ii) sponge juveniles for obtaining sponge biomass. (a) archeocytes; (c) choanocytes; (ca) canal; (ch) choanocyte chamber;(e) embryo; (en) endopinacocyte; (g) gemmule; (s) spicules; (sc) sclerocyte

Immortal sponge cells

The use of sponge stem cells that are grown in an optimum medium will increase the chances of establishing a continuously proliferating cell line. However, also in that case a mechanism to ensure continuous cell division and control of cell differentiation has to be implemented.

Animal cell lines from insects and mammals usually are transformed cells with an unlimited proliferative capacity (immortal cells). In mammalians, transformed cells can be acquired from tumourous tissues, or induced artificially, either by hybridization of normal cells with tumourous cells (e.g. hybridoma's) or by submitting the cells to mutagenic agents such as carcinogenic compounds, viruses, radioactivity or by tranfecting the cells with oncogenes (e.g. E1 gene, PER.C6®). Occasionally, immortal cells evolve spontaneously by mutation of normal cells growing in rich media. In analogy with animal cell cultures, in sponges it has been common to use growth factors to stimulate cell division.

A transgenic sponge culture is at present a utopia, which can be transformed soon into a reality. Culture of transgenic cells has been done in other marine invertebrates. Embryos of sea urchins were treated with plasmid DNA containing the Gal4 gene, which is transcriptional activator gene found in yeast. Cells from transfected embryos were held *in vitro* for up 2 months, showing up to 4.6 times increase in number (Bulgakov *et al.* 2002, Odinstova *et al.* 2003). A similar transfection of sponge embryos with Gal4 or other similar genes may be a way to enhance cell division. However, a successful immortalization of sponge cells has never been reported.

On the other hand, we propose not only to focus on stimulation of cell division, which has been the main aim of cell culture research, but on prevention of cell death. Several observations suggest that sponge cells divide and die at high rates. In natural conditions, sponges continuously change in shape (e.g. Garrabou & Zabala 2001), and experience fission on fusion events (e.g. de Caralt *et al.* 2007d, Turon *et al.* 1998). The same individual is capable to grow at one side and to decrease at another side at the same time. This high dynamism and plasticity, is not necessarily resulting in absolute growth, since sponges grow

slowly, but in biomass rearrangement and regeneration, which may require a high rate of mitosis and apoptosis in the same individual sponge.

Fast regeneration is a feature that is commonly observed in sponges (Simpson 1984, Wulff 2006). *In situ* experiments to study the regenerative capacity of sponges showed that perforated sponges were able to repair their wound at faster rates than their growth rates (e.g. Turon *et al.* 1998, de Caralt *et al.* 2007d); small holes experimentally made in the sponge tissue were healed within two days (Jackson & Palumbi 1979). In the laboratory, this phenomenon was also observed in fragmented sponges, where tissue regeneration started some hours after cutting (Osinga *et al.* 2003, de Caralt *et al.* 2003, Belarbi *et al.* 2003). Furthermore, specimens of *D. avara* can change the oscula emplacement in a few days when the flow conditions change, by rearranging the exhaling canals to favour water circulation through the sponge (Mendola *et al.* 2007, submitted). As a whole, the sponge plasticity showed under natural and artificial perturbations (e.g. damage and hydrodynamics changes) indirectly indicate a rapid cell division.

Müller *et al.* (1999) looked to cell division in primmorphs and observed DNA replication by BrdU labeling. However, no net growth was recorded, which induced Müller *et al.* (1999) to suggest that cells in primmorphs were dying just as fast as they were growing. A rapid cell division was also recorded for *Halisarca caerulea* at sea (de Goeij *et al.* 2007 *submitted*). Their experiments showed DNA replication (positive BrdU staining) by a high percentage of the cells around the choanocyte chambers, already after 30 minutes of incubation.

This unusual cell behavior may be one of the reasons of the sponge success across time and space (Simpson 1984, Wiens & Müller 2006). Fast cell division and death may result in an organism mainly composed of young cells, which will not show malfunctioning due to ageing. However, further research is needed to confirm that both cell division and cell death occur at high rates in a sponge individual.

Perspectives

Thus, to develop continuous sponge cell lines, we should not only use the strong capability of sponge cells to divide but also concentrate on reducing the high apoptosis activity of sponge cells.

The studies on the apoptotic process in sponges are in progress (e.g. Wiens *et al.* 2003, Tepsuporn *et al.* 2003). Several genes involved have been identified and the same apoptotic molecules that have been described in mammals (members of the Bcl-2 family, members of the TNF family, caspases, transcription factors and various proteins) have been identified in sponges (reviewed in Wiens & Müller 2006).

Previous research on mammals showed that many intracellular proteins are involved in both apoptosis activation and inhibition, and some methods to control apoptosis have been developed. They consist of addition of nutritional or chemical elements to the media, and the use of genetic engineering to modify the intracellular biochemistry of the cell (Arden & Betenbaugh 2004). In sponges, a decisive first step for obtaining transfected cells has been made by Thompson *et al.* (2006). They introduced an immortalizing agent (human telomerase reverse transcriptase (hTERT) into *Axinella corrugata* cells. Telomerase stimulates the restoration of the telomeric ends of the chromosomes after mitosis, which is assumed to be a crucial process for cells to continue their division cycle. The hTERT agent elevated the telomerase activity and thus, it reduced the rate of apoptosis. In this study, DNA and RNA isolation revealed the presence and expression of the vector in the cell sponge suspensions. This represents a promising research line, which should be followed for obtaining a continuous sponge cell culture.

To summarize, although continuously proliferating cultures from immortalized sponge stem cells have not been developed yet, the information accumulated from sponge biology, cell requirements and genetic techniques offer good perspectives for future developments in this field.

However, once a continuous sponge cell culture is obtained, the question remains as whether the cultured cells will continue to produce the bioactive

molecules, which are necessary for the wanted new medicines. There are some evidences that cells in suspension of a bioactive sponge still produce the secondary metabolite: stevensine was detected in the primary cell culture of *Axinella corrugata* (Pomponi & Willoughby 2001).

Sponges have an enormous potential for the development of new medical drugs. Thus, although several bottlenecks in the whole process remain to be solved, efforts to develop a technology for continuous cell cultures, is worthwhile and may become successful at a medium term.

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Summary

Introduction

Marine sponges are an important source of secondary metabolites with pharmaceutical interest. To supply the market with the sufficient amounts of sponge bioactive compounds is the main reason for the increasing interest of sponge culture in recent years. Sponge collection directly from the sea is harmful for the environment. Chemical synthesis of the target compound is a difficult and expensive process due to the complexity of the natural molecules. Sponge cell cultures and aggregates (primmorphs) have been developed although attempts to obtain continuous cell lines have failed so far. Transgenic cell cultures are new promising methods to solve the supply problem, but they still are in their infancy. Many attempts have been undertaken to culture whole sponge individuals and explants in closed, semi-closed and open systems to obtain sponge biomass without much success. Basic research on the biology, ecology and physiology of sponges is essential to define the culture conditions for enhancing the production of sponge biomass and bioactive compounds. The aim of this thesis has been to improve the knowledge on the biology and ecology of sponges, particularly of *Corticium candelabrum*, in order to incorporate the biological-ecological information acquired to overcome the current drawbacks in the field of the sponge culture with biotechnological applications. The target sponges for the various aspects dealt with in this study were *Corticium candelabrum* (Homosclerophorida), *Crambe crambe* (Poecilosclerida), *Dysidea avara*, *Ircinia oros* and *Hippospongia communis* (Dictyoceratida). These species are widely distributed along the rocky Mediterranean sublittoral and have potential biotechnological interest in the pharmacology and cosmetic fields.

Field study of growth dynamics of *C. candelabrum*

Growth dynamics of the sponge *Corticium candelabrum* was studied in the northwestern Mediterranean during more than three years. Growth and regeneration rates, fission and fusion events, and survival were monitored

monthly. In addition, *in situ* punctual clearance experiments were conducted seasonally searching for possible relationships between food uptake and sponge dynamics. The growth rates of *C. candelabrum* were low, variable, and seasonal, with the highest values in summer. The cumulative survival function followed a stepped curve, with several consecutive months without mortality separated by shorter mortality events, which mainly occurred in cold months. However, an event of high mortality took place in the particularly warm summer 2003. Fission events were frequent after previous damage (e.g. predation) and only one fusion event was recorded along the study period. The diet of *C. candelabrum* was highly heterogeneous. Differences in clearance rates among prey types with season indicated that the sponge retained with different efficiency the several prey types present in the water. Survival and growth rates were significantly different for small, medium, and large individuals, with the small sponges showing the lowest survival and the highest growth rates. As a whole, the results indicate *C. candelabrum* is an extraordinarily dynamic sponge that grows faster at high temperatures and feeds preferably on small picoplancton.

Explant culture

We have taken advantage of the high regenerative capability of *Corticium candelabrum* and cultured explants in several experimental conditions. To find the best conditions for obtaining functional explants, we assayed a range of conditions, including season of collection, culture temperature, filtered *vs.* filtered-sterile seawater, addition of antibiotics, and proportion of ectosome. We monitored the cellular and ultrastructural changes during the explants formation. After 24 h, TEM images showed the aquiferous system disarranged, in particular at the sponge periphery. From 2 to 4 weeks later, the aquiferous system regenerated, and fragments became functional sponges (explants).

Explants were cultured under two regimes: *in vitro* and in a closed aquarium system. Antibiotics were only added to the *in vitro* culture to assess their effect on the symbiotic bacteria, which remained healthy despite the presence of antibiotics. Two food regimens (marine bacteria and green algae) were assayed based on the results of the previous field filtration experiments. We monitored

explant survival and growth. Explants showed a high long-term survival rate (close to 100 %). Explants growth rates were higher in the closed aquarium system, without antibiotic addition, and fed with algae. The mean growth rates per month of the explants under culture were equivalent to those of wild sponges in winter (see chapter II), when sea temperature was similar to those in our cultures (ca. 14ºC). Higher temperatures are expected to enhance explant growth, as occurred for wild sponges in summer, and are here proposed for culturing this species once the explants are functional and the risk of bacterial contamination has been reduced. Explant cultures were hardly contaminated because manipulation was reduced to a minimum and we used sterilized seawater. *C. candelabrum* produces bioactive molecules, which may play a defensive role in the sponge and may have pharmaceutical interest. The bioactivity of the explants was similar to that of wild sponges.

Juvenile culture from larvae

Monitoring of *Corticium candelabrum* (chapter II) and the explant culture (chapter III) revealed a general pattern of slow and variable sponge growth. Taking into consideration these previous results, an *in vitro* sponge culture, based on juveniles from larvae, was studied. Starting from larvae, we expected to decrease the growth variability due to the different age of the individuals in conventional cultures since all the resulting juveniles belonged to the same cohort. Moreover, juvenile growth was also expected to be high because growth rates were shown to be the highest in small individuals during the field monitoring (chapter II). Larvae from several sponge species with different morphological and biological features were used. Unfortunately, larvae of *C. candelabrum* could not be obtained in sufficient numbers for the experiments due to the cryptic small size, and low abundance. First, settlement success, morphological changes during metamorphosis and survival of *Dysidea avara, Ircinia oros, Hippospongia communis*, under the same culture conditions, were compared. In a second step, we tested the effects of flow and food on survival and growth of juveniles from *D. avara* and *Crambe crambe*. Dried algae as a food increased juvenile growth of both species, whereas the presence of flow only favoured *C. crambe* growth.

Finally, in a third experiment, we monitored survival and growth of juveniles of *D. avara* and *C. crambe* transplanted to the sea to compare laboratory and field results. Survival of juveniles in laboratory conditions was higher than in the field. As for growth, the two species showed contrasting results: *C. crambe* juveniles grew more in the field than in the laboratory cultures while the pattern was the opposite for *D. a*vara. The results altogether reflected different requirements depending on the species. To conclude, sponge culture from larvae appeared to be a potentially good method for sponge supply and laboratory culture under controlled conditions is preferred over sea culture in order to prevent biomass losses during these early life stages.

Larval development in *C. candelabrum*

Due to the difficulty in obtaining larvae from *Corticium candelabrum*, an in depth study on embryo development and larvae features of this sponge were performed. Scanning and transmission electron microscopy were used to describe the gametes and larval development. The species is hermaphroditic. Oocytes and spermatocytes were clearly differentiated in April. Embryos develop from June to July when the larvae were released spontaneously. Spermatic cysts originated from choanocyte chambers and spermatogonia from choanocytes by choanocyte mitosis. Oocytes had a nucleolate nucleus and a cytoplasm filled with yolk granules and some lipids. Embryos were surrounded by firmly interlaced follicular cells from the parental tissue. A thin collagen layer lied below the follicular cells. The blastocoel was formed by migration of blastomeres to the morula periphery. Collagen was spread through the whole blastocoel in the embryo, but was organized in a dense layer (basal lamina) separating cells from the blastocoel in the larva. The larva was a typical cinctoblastula. The pseudostratified larval epithelium was formed by ciliated cells. The basal zone of the ciliated cells contained lipid inclusions and some yolk granules; the intermediate zone was occupied by the nucleus; and the apical zone contained abundant electron-lucent vesicles and gave rise to cilia with a single cross-striated rootlet. Numerous paracrystalline structures were contained in vacuoles within both apical and basal zones of the ciliated cells. Several slightly differentiated cell types were present in

different parts of the larva. Most cells were ciliated, and show ultrastructural particularities depending on their location in the larvae (antero-lateral, intermediate and posterior regions). A few smaller cells were non-ciliated. Several features of the *C. candelabrum* larva seem to support the previously proposed paraphyletic position of homoscleromorphs with respect to the other demosponges. The cryptic aspect of this larvae and the short and unpredictable period of larval release indicates that it is necessary to implement a highly controlled system around the ripe sponges to ensure their collection in sufficient numbers.

Vertical transmission of symbionts in *C. candelabrum*

Corticium candelabrum is a bacteriosponge, thus it contains huge amounts of symbiotic bacteria in its mesohyl. Bacteriosponges are widespread in several orders of Demospongiae including Homosclerophorida. Our previous studies on explant formation (chapter III) suggest a relevant role of the symbiotic bacteria in the biology of this sponge. Thus, we studied the transfer of heterotrophic bacteria from parental tissue to oocytes in *C. candelabrum* and described the successive locations of the microsymbionts during embryo development through transmission and scanning electron microscopy. Eight different types of symbiotic bacteria were described morphologically. These eight bacteria morphotypes were found in both adult individuals and larvae. Although in *C. candelabrum,* spermatogonia and oocytes coexisted in the same individuals, symbiotic bacteria were only transferred to oocytes. Bacteria were first located at the oocyte periphery below a thick collagen layer and then they migrate to the oocyte cytoplasm, forming spherical clusters. After cleavage, the bacteria could be found in the free space between blastomeres but mainly accumulated at the embryo periphery below the follicular cells that surrounded the embryo. Once the blastocoel was formed, the symbiotic bacteria moved to this central cavity where they actively divided, increasing their number considerably. Many examples of phagocyted bacteria in the proximal zone of the larval cells were observed at this stage. Consequently, bacteria may represent a complementary source of energy for free larvae and settlers before they are able to capture food from the surrounding water.

Cell culture from sponges: pluripotency and immortality

We propose the use of embryos and larvae as a new source of sponge material for cell culture because they contain higher amounts of stem cells than adults (archeocytes), which besides are more versatile and less susceptible to microbial contamination. The optimization of the method for obtaining and cryopreserving larvae, as well as the culture of juveniles under laboratory conditions may provide embryonic cells along the year. Additionally, genetic engineering and cellular research on mitotic and apoptotic mechanisms are new promising fields that may help to stimulate cell division and improve cell survival. In particular, future research should focus on how to reduce apoptosis, which appears to be very high in sponge cells. Moreover, the molecular research should be combined with studies on basic aspects of the biology, ecology, physiology of the target sponges for a more complete understanding of the whole requirements of sponge cells under culture.

Samenvatting

Nederlands

Inleiding

Mariene sponzen zijn een belangrijke bron van secundaire metabolieten met farmaceutische waarde. De belangrijkste reden voor belangstelling voor het kweken van sponzen in de afgelopen jaren is om de markt te kunnen voorzien van voldoende bioactieve stoffen uit sponzen. Verzamelen van sponzen direct uit zee is schadelijk voor het milieu. Chemische synthese van deze stoffen is moeilijk en duur vanwege de complexe structuur van deze natuurlijke componenten. Er zijn celculturen en aggregaten (explants en primmorphen) ontwikkeld, echter alle pogingen continue cellijnen te verkrijgen zijn mislukt. Transgene celculturen zijn veelbelovende methoden om het aanvoerprobleem van bioactieve stoffen op te lossen; echter de ontwikkeling staat nog in de kinderschoenen. Er zijn vele pogingen ondernomen om hele sponzen of stukken sponzen te kweken in gesloten, half open en open systemen. Ook hiermee zijn weinig successen bereikt. Basaal onderzoek aan de biologie, ecologie en fysiologie aan sponzen is nodig om sponsculturen te kunnen opzetten en de kweekomstandigheden te definiëren en bioactieve stoffen te kunnen produceren. De doelstelling van het onderzoek beschreven in dit proefschrift is de kennis van biologie en ecologie van sponzen in het algemeen te verbeteren, en meer specifiek van *Corticium candelabrum*, om gebruik te kunnen maken van deze informatie om sponzen te kunnen kweken voor biotechnologische toepassingen. De sponzen die bestudeerd zijn in dit onderzoek zijn *Corticium candelabrum* (Homosclerophorida), *Crambe crambe* (Poecilosclerida), *Dysidea avara*, *Ircinia oros* en *Hippospongia communis* (Dictyoceratida). Deze soorten komen wijdverspreid voor langs de rotsachtige Middellandse Zeekust en zijn mogelijk interessant om toegepast te worden in de farmacie en cosmetica.

Veldstudie van groeidynamica van *C. candelabrum*

De groeidynamiek van de spons *Corticium candelabrum* werd bestudeerd in het noordwestelijke Middellandse Zeegebied gedurende een periode van meer dan 3 jaar. Groei- en regeneratiesnelheden, splitsing en fusie en overleving werden maandelijks gemeten. Tevens werd in de verschillende seizoenen de *in situ* opnamesnelheid van deeltjes door de sponzen gemeten om mogelijke relaties tussen voedselopname en sponsdynamiek te kunnen leggen. De gemeten groeisnelheden van *C. candelabrum* waren laag, variabel en per seizoen verschillend. De hoogste groeisnelheid werd gemeten in de zomer. Sponzen overleefden gedurende verschillende aaneengesloten maanden. Er waren enkele perioden waarin een aantal sponzen dood gingen. Sponssterfte werd met name in koude periodes waargenomen. Daarnaast was er veel sponssterfte in de warme zomer van 2003. Splitsing van sponzen werd regelmatig waargenomen nadat sponzen beschadigd waren (bijvoorbeeld ten gevolg van predatie) en slechts een waarneming van fusie van sponzen werd waargenomen gedurende deze studie.
Het dieet van *Corticium candelabrum* was zeer heterogeen. Verschillen in opnamesnelheden tussen de verschillende voedseldeeltjes (afhankelijk van het seizoen) toonden aan dat sponzen deeltjes opnamen met verschillende efficiëntie. Overleving en groeisnelheid van de sponzen was significant verschillend voor kleine, gemiddelde en grote sponzen. Bij kleine sponzen was sterfte het laagst en de groeisnelheid het hoogst. Het algemene beeld was dat *C. candelabrum* een extreme dynamische spons is die sneller groeit bij hogere temperatuur en zich bij voorkeur voeden met picoplankton.

Explant cultuur

Wij hebben gebruik gemaakt van de capaciteit van *Corticium candelabrum* snel te kunnen regenereren door explants te maken en deze te kweken onder verschillende experimentele omstandigheden. Om de beste omstandigheden te vinden om functionele explants te krijgen zijn er verschillende condities getest, zoals het seizoen van oogsten, de temperatuur waarbij gekweekt wordt, het gebruik van gefilterd en gesteriliseerd zeewater, toevoeging van antibiotica en de

fractie van ectosoom van de explant. Veranderingen in cellulaire- en ultrastructuur gedurende de ontwikkeling van de explants werd gemeten. Na 24 uur, lieten TEM foto's zien dat het kanaalsysteem in de sponzen anders georganiseerd werd, met name in het gedeelte aan de buitenkant van het organisme. Tussen week 2 en 4 was het kanaalsysteem volledig geregenereerd en waren de explants ontwikkeld in nieuwe functionele sponsjes.

De explants warden gekweekt onder 2 omstandigheden: met en zonder toevoeging van antibiotica en voeding met mariene bacteriën en groene algen.

Het effect van antibiotica om symbiontische bacteriën werd bestudeerd. Bacteriën bleven aanwezig ondanks de toevoeging van antibiotica.

Een tweetal voedingsregimes werd getest (mariene bacteriën en groene algen) op basis van de resultaten van eerder filtratieexperimenten in het veld. Overleving en groei van de explants werd gemeten. De explants bleken goed te overleven (bijna 100 %). De groeisnelheid van de explants was groter in het gesloten aquariumsysteem zonder toevoeging van antibiotica en gevoed met groene algen. De gemiddelde groeisnelheid van de explants onder de kweekomstandigheden waren vergelijkbaar met die van sponzen in de natuur in de winter (zie hoofdstuk II), toen de temperatuur vergelijkbaar was met de temperatuur in onze cultuur (ca. 14°C). Wij verwachten dat hogere temperaturen zouden leiden tot hogere groeisnelheden zoals ook waargenomen wordt bij sponzen in de natuur in de zomer. Voorgesteld wordt de kweek dan ook uit te voeren bij hogere temperaturen zo gauw de explants ontwikkeld zijn tot functionele sponsjes en het risico van bacteriële contaminatie verminderd is. De explant cultures waren nauwelijks geïnfecteerd, omdat handelingen aan de culturen zoveel mogelijk voorkomen werden en doordat gebruik gemaakt werd van gesteriliseerd zeewater. *C. candelabrum* produceert bioactieve moleculen, welke een verdedigende rol spelen in sponzen en mogelijk interessant zijn voor de famacie. De bioactiviteit van de explants was vergelijkbaar aan die van sponzen in de natuur.

Kweken van sponzen startende vanuit larven

Monitoring van de groei van *Corticium candelabrum* (hoofdstuk II) en het kweken van explants van deze spons (hoofdstuk III) lieten ons zien dat de groei variabel en langzaam was. Om deze reden zijn wij ons gaan richten op de kweek van jonge sponzen uit larven. Wij verwachtten dat indien we de kweek zouden starten vanuit sponslarven we minder variatie in groei tussen de verschillende individuen zouden observeren omdat de jonge sponzen allen even oud zijn. Bovendien verwachtten we een hogere groeisnelheid van jonge sponzen omdat we in hoofstuk II gezien hadden dat kleinere sponzen sneller groeien dan grotere sponzen. Er werd gebruik gemaakt van verschillende sponssoorten met verschillende morfologie en biologische eigenschappen. Het bleek niet mogelijk te zijn voldoende larven van *C. candelabrum* te verkrijgen. Larven van *C. candelabrum* zijn moeilijk te zien en het is onvoorspelbaar wanneer zij door de moederspons losgelaten worden. In eerste instantie werd daarom onderzoek gedaan naar de hechting, morfologische veranderingen gedurende metamorfose en overleving onder vergelijkbare kweekcondities aan *Dysidea avara, Ircinia oros* en *Hippospongia communis.* In een tweede stap is het effect van stroming en voedsel op overleving en groei van *Dysidea avara* en *Crambe crambe* bestudeerd. Gedroogde algen als voedsel bleek de groei van beide soorten te stimuleren en stroming had alleen een positief effect op groei van *C. crambe*. Tot slot werd in een derde experiment de overleving en groei van jonge *D. avara* en *C. crambe* in de zee vergeleken met sponzen in het laboratorium. Overleving van sponzen in het laboratorium was hoger dan in het veld. In het geval van groei bleek dat *C. crambe* sneller groeide in de zee, en *D. A*vara groeide sneller onder laboratorium omstandigheden. Resultaten lieten dus zien dat de kweekcondities verschillend waren per soort. Kweken van sponzen uit larven bleek echter een veelbelovende methode. Het verlies van larven onder laboratoriumcondities is veel minder dan onder de natuurlijke omstandigheden in de zee.

Ontwikkeling van larven in *C. candelabrum*

Omdat het zo lasting was larven te verkrijgen van *Corticium candelabrum*, zijn wij de ontwikkeling van embryo's en larven in deze spons in meer detail gaan bestuderen. Scanning en transmissie electronenmicroscopie werden gebruikt om de ontwikkeling van gameten en larven te kunnen volgen. De sponssoort is hermafrodiet. Oocyten en spermatocyten zijn duidelijk gedifferentieerd in april. Embryo's ontwikkelden zich van juni tot juli. In juli werden de larven spontaan losgelaten door de ouderspons. Spermacysten ontstonden vanuit de choanocytkamers en de spermatogonia vanuit de choanocyten via choanocytmitose. Oocyten bevatten een nucleolaatkern en een cytoplasma gevuld met dooiergranulen en vetten. Embryo's waren omgeven door sterk gelaagde folliculaire cellen van het ouderweefsel. Een dunnen laag collageen lag onder de folliculaire cellen. De blastocoel werd gevormd door migratie van blastomeren naar de buitenkant van de morula. Collageen was verspreid over het gehele blastocoel in het embryo, maar was georganiseerd in een dichte laag (basale lamina) die de cellen van de blastocoel scheidden in de larven. De larven waren typische cinctoblastula. Het pseudogestratificeerde larvale epithelium was gevormd door de ciliaatcellen. De basale zone van de ciliaatcellen bevatte vetinclusies en dooiergranulen; de intermediaire zone bevatte een nucleus en een apicale zone welke vele transparante zakjes waarin ciliaten met enkele gekruist gestreepte wortels gevormd werden. De vacuolen bevatten vele kristallijne structuren met zowel apicale als basale zones met ciliaten. Verschillende enigszins gedifferentieerde cellen waren aanwezig in de verschillende delen van de larven. De meeste cellen waren ciliaten en hadden ultrastructurele deeltjes afhankelijk van hun locatie in de larve (antero-laterale intermediaire en posteriaire regio's). Enkele kleinere cellen bevatten geen ciliaten. Verschillende eigenschappen van de larven van *C. candelabrum* lijken de eerder voorgestelde parafyletische positie van homoscleromorfen in relatie tot andere demosponzen te ondersteunen. Het feit dat deze larven enigszins verborgen zijn en de onvoorspelbaarheid van het moment van vrijkomen van deze larve leidt ertoe dat er een goed gecontroleerde strategie nodig is om een groot aantal larven uit rijpe sponzen te kunnen winnen.

Vertical transmissie van of symbionten in *C. candelabrum*

Corticium candelabrum is een bacteriospons. Dit betekent dat het mesohyl van de spons grote hoeveelheden symbiotische bacteriën bevat.. Bacteriosponzen zijn in grote mate vertwegenwoordigd in verschillende orden van Demospongiae, inclusief de Homosclerophorida. Onze eerdere studies naar de vorming van explants (Hoofdstuk III) lieten de belangrijke rol van symbionten in de biologie van deze spons zien. Om deze reden zijn wij de verticale transmissie van heterotrofe bacteriën van het ouderweefsel naar de oöcyten in *C. candelabrum* gaan bestuderen en wij hebben de verschillende locaties van de symbionten in de embryo's tijdens de ontwikkeling ervan gevolgd met behulp van transmissie en scanning electronenmicroscopie. Er werden 8 morfologisch verschillende typen symbiotische bacteriën beschreven. Deze 8 verschillende typen werden zowel gevonden in volwassen sponzen als in larven. Alhoewel in *C. candelabrum* zowel spermatogonia als oöcyten voorkomen in dezelfde individuën werden symbiotische bacteriën alleen doorgegeven aan de oöcyten. In eerste instantie waren bacteriën aanwezig aan de buitenkant van de oöcyten onder een dikke laag collageen om vervolgens te migreren naar het cytoplasma van de oöcyten, waar bolvormige clusters met bacteriën gevormd werden. Na deling konden de bacteriën waargenomen worden in de vrije ruimte tussen de blastomeren, maar voornamelijk accumuleerden bacteriën aan het embryo oppervlak onder de folliculaire cellen die de embryo's omgeven. Zo gauw de blastocoel gevormd was gingen de bacteriën naar deze centrale ruimte waar zij actief gingen delen en dus aanzienlijk in aantal toenamen. In dit stadium werden vervolgens veel door larven gefagociteerde bacteriën waargenomen. Waarschijnlijk vormen deze verticaal getransmitteerde bacteriën een extra voedselbron voor de vrije larven voordat ze hechten en zelfstandig voedsel uit zeewater kunnen invangen.

Celcultures van sponzen: pluripotentie en onsterfelijkheid

Wij stellen voor gebruik te maken van embryo's en larven als een nieuwe bron van weefsel om celculturen uit te ontwikkelen. Embryo's en larven bevatten relatief veel stamcellen ten opzichte van volwassen sponzen (de archeocieten). Optimalisatie van cryopreserveringsmethoden van embryo's larven en methoden om jonge sponzen te kunnen kweken zullen resulteren in de beschikbaarheid van stamcellen gedurende het gehele jaar. Ontwikkeling van methoden om sponzen genetisch te verbeteren en celbiologisch onderzoek naar mitose en apoptose zijn belangrijke nieuwe gebieden die kunnen leiden tot technieken waarmee celdeling gestimuleerd en celdood voorkomen kan worden. Toekomstig onderzoek zal zich moeten richten op de reductie van apoptosesnelheid welke zeer groot lijkt te zijn in sponzen. Moleculair onderzoek zal gecombineerd moeten worden met studies naar de fundamentele aspecten van de biologie, ecologie en fysiologie van sponzen om de behoeften van een sponscelcultuur beter te begrijpen.

Resum

Introducció

Les esponges marines són una important font de metabolits secundaris amb interès farmacològic. Els compostos bioactius produïts per les esponges en condicions naturals, juguen diversos papers ecològics com ara en la competència per l'espai, la inhibició del fouling o la defensa en front els depredadors. D'altra banda, els mateixos metabolits que serveixen per a la defensa de les esponges en el seu ambient, presenten diferents activitats biològiques com ara anti-inflamatòria, anti-tumoral, antibiòtica, antiviral, etc, que troben aplicacion en el camp de la farmacologia. En aquest sentit, els metabolits secundaris produïts per les esponges tenen un enorme potencial com a medicaments i cosmètics. Tot i aquest gran potencial, la comercialització de medicaments derivats d'esponges ha tingut un èxit relativament baix, sobretot degut a la dificultat d'obtenció de biomassa d'esponja o dels seus metabolits bioactius en quantitats suficient per dur a terme la recerca, els posteriors assajos clínics i, finalment, quan el medicament ja esta desenvolupat, per cobrir les necessitats del mercat.

Per intentar resoldre el coll d'ampolla que hi ha en el procés d'investigació i comercialització d'un nou fàrmac procedent d'esponges, degut a la impossibilitat de producció de metabolits bioactius en grans quantitats, s'han estudiat, amb més o menys èxit, diferents mètodes per l'obtenció de biomassa d'esponja. La recol·lecció directa de les esponges del mar acabaria amb el recurs i, en qualsevol cas, suposaria un impacte negatiu en els ecosistemes marins. La síntesi química del compost d'interès és, en general, un procés difícil i car degut a la complexitat de les molècules naturals.

Degut a l'escàs èxit dels anteriors mètodes, el cultiu d'esponges ha estat el mètode més estudiat recentment, tant en el mar com en condicions controlades. S'han fet diversos intents cultivant individus sencers i fragments (explants) d'esponges en sistemes tancats, semi-tancats i oberts, aplicant diferents condicions en els cultius per intentar determinar les condicions que augmentin la supervivència i el creixement de les esponges cultivades. Tot i que aquests estudis mostren que el creixement de les esponges cultivades és

variable, lent i específic per a cada espècie, els resultats són prou positius per seguir investigant en aquest camp.

El cultiu cel·lular i d'agregats (primmorphs) d'esponges també ha estat estudiat intensament, encara que no s'ha obtingut mai una línia cel·lular contínua d'esponja. Fins ara, només s'han pogut desenvolupar cultius cel·lulars primaris i de curta durada, degut a problemes de contaminació i poca divisió cel·lular. Tot i això, el cultiu cel·lular d'esponges és un camp cada cop més prometedor especialment degut als avanços en el camp de la genètica (cèl·lules transfectades). En tots aquests estudis, ha quedat palès que el coneixement de la biologia, ecologia i fisiologia de les esponges d'interès és essencial per tal de definir les condicions òptimes dels cultius a fí de millorar la producció de biomassa d'esponja i del seu del compost bioactiu. Per tant es necessita molta mès recerca bàsica sobre les espècies que es pretenen cultivar, a fi de garantir l'èxit del cultiu.

L'objectiu d'aquesta tesi ha estat progressar en el coneixement de la biologia i ecologia de les esponges, en particular de *Corticium candelabrum*, per així aplicar al cultiu d'esponges la informació biològica i ecològica adquirida. Les espècies tractades en aquesta tesi han estat: *Corticium candelabrum* (Homosclerophorida), *Crambe crambe* (Poecilosclerida), *Dysidea avara* (Dyctyoceratida), *Ircinia oros* (Dyctyoceratida), i *Hippospongia communis* (Dictyoceratida). Aquestes espècies es troben àmpliament distribuïdes al llarg del sublitoral rocós Mediterrani i tenen potencial interès biotecnològic en el camp de la farmacologia i la cosmètica.

Dinàmica del creixement de *C. candelabrum* en el mar

La dinàmica del creixement de l'esponja *Corticium candelabrum* va ser estudiada en el Mediterrani Nordoccidental durant més de tres anys. Es van examinar mensualment les taxes de creixement i regeneració, les fusions i les fissions, i la supervivència de 45 individus. Addicionalment, a cada estació de l' any, es van dur a terme experiments de filtració *in situ* per tal d'establir possibles relacions entre l'aliment capturat i el creixement de l'esponja.

Les taxes de creixement de *C. candelabrum* van ser baixes, variables y amb un component estacional, presentant els màxims valors a l'estiu. La funció

acumulativa de la supervivència va seguir una corba esglaonada, amb mesos consecutius sense mortalitat, separats per períodes curts amb episodis de mortalitat, els quals majoritàriament es van donar durant els mesos freds. No obstant, un període de gran mortalitat va ser enregistrat a l'estiu de 2003, el qual va ser especialment calorós. Freqüentment, després d'haver patit danys (p.e. predació) els individuus es fissionaven. Al contrari, només es va observar un episodi de fusió al llarg de tot l'estudi. La dieta de *C. candelabrum* va ser altament heterogènia. Les diferents taxes de filtració entre els tipus de presa a cada estació, indicaren que l'esponja retè amb diferent eficiència els diferents tipus de presa presents en la columna d'aigua.

El tamny de l'esponja té a veure amb el seu éxit. Les taxes de supervivència i creixement van ser significativament diferents per als individus petits, mitjans i grans, essent les esponges més petites les que van presentar la supervivència més baixa i les taxes de creixement més altes. Els resultats van indicar que *C. candelabrum* és una esponja extraordinàriament dinàmica, que creix més ràpid quan les temperatures són més elevades i que s'alimenta preferiblement de petit picoplàncton.

Cultiu d'explants

Aprofitant els avantatges que presentava *Corticium candelabrum* degut a la seva alta capacitat regenerativa, vam cultivar explants d'aquesta espècie sota diferents condicions experimentals. Per trobar les millors condicions per a l'obtenció d'explants funcionals, vam assajar un rang de variables, com ara l'estació de recol·lecció (primavera-hivern, tardor-estiu), la temperatura durant el cultiu (14 ºC i 20 ºC), l'aigua de mar filtrada *vs.* l'aigua de mar filtrada i esterilitzada, l'ús d'antibiòtics (amb i sense) i la proporció d'ectosoma (0-10%, 10-30% i 30-50%) dels explants. Durant el procés de formació dels explants, vam estudiar els canvis a nivell cel·lular i ultraestructural. 24 h després de tallar l'esponja, imatges del microscopi electrònic de transmissió van mostrar el sistema aqüífer desorganitzat, especialment a la perifèria de l'esponja. Després de 2 a 4 setmanes, el sistema aqüífer es va regenerar, i els fragments van esdevenir esponges funcionals (explants).

Els explants van ser cultivats sota dos règims: *in vitro* i en aquaris de sistema tancat. En el cultiu *in vitro* es van afegir antibiòtics per veure l'efecte que tenien sobre els bacteris simbionts, els quals van persistir saludables tot i la presència d'antibiòtics. Basant-nos en els resultats dels experiments de filtració fets prèviament en el camp, es van comparar dos règims d'alimentació (bacteris marins i algues verdes) en els cultius *in vitro*, mesurant la supervivència dels explants i el seu creixement.

Els explants van presentar taxes de supervivència altes (a prop del 100%) en ambdos casos. Les taxes de creixement van ser més altes en els explants cultivats en els aquaris de sistema tancat sense antibiòtics, que en els sistemes *in vitro* amb antibiòtics. Fins i tot els explants cultivats en els sistemes *in vitro* van presentar taxes de creixement més elevades quan van ser alimentats amb algues (*Chlorella* sp.) que amb bacteris. La taxa mitjana de creixement per mes dels explants en cultiu va ser equivalent a la de les esponges en condicions naturals a l'hivern (veure el capítol II), quan la temperatura del mar era igual a la temperatura en els nostres cultius (aprox. 14ºC). S'espera que temperatures més elevades potenciïn el creixement dels explants, com passa a les esponges en condicions naturals a l'estiu, i proposem aquestes temperatures més altes per cultivar aquesta espècie un cop els explants siguin funcionals i el risc de contaminació bacteriana s'hagi reduït. En els nostres cultius d'explants, la contaminació va ser minimitzada perquè la manipulació va ser reduïda al mínim, i es va fer servir aigua de mar esterilitzada. A més, les molècules bioactives produïdes per *C. candelabrum*, les quals potser tenien una funció defensiva en l'esponja i probablement són d'interès farmacològic, van seguir sent produïdes per les esponges cultivades, ja que la bioactivitat dels explants cultivats mesurada amb el microtox® va ser similar a la bioactivitat mesurada en les esponges en el mar.

Cultiu de juvenils a partir de larves

El seguiment de *Corticium candelabrum* (capítol II) i el cultiu d'explants (capítol III) van revelar un patró general de creixement lent i variable. Tenint en consideració aquests resultats previs, vam realitzar un cultiu d'esponges *in vitro* basat en juvenils d'esponja obtinguts a partir de larves. Començant a partir de larves, esperàvem reduir la variabilitat del creixement entre individus, la qual en els cultius convencionals pot ser deguda a les diferents edats dels individus cultivats. En canvi, els juvenils provinents de les larves pertanyien a la mateixa cohort. Addicionalment, tenint en compte que l'estudi del creixement de *C. candelabrum* al mar (capítol II) va mostrar que els individus més petits presentaven les taxes de creixement més altes, esperàvem que el creixement dels juvenils fos més ràpid que l'obtingut fins al moment en d'altres cultius. En aquest estudi, van ser utilitzades larves de diferents espècies d'esponja, amb característiques morfològiques i biològiques diferents. Malauradament, no vam obtenir larves de *C. candelabrum* en quantitat suficient per dur a terme els experiments, degut al seva petita i críptica mida i al baix nombre de larves alliberades al laboratori.

En un primer experiment, vam cultivar larves de *Dysidea avara, Ircinia oros, Hippospongia communis*, sota les mateixes condicions i vam analitzar l'èxit d'assentament, els canvis morfològics durant la metamorfosi i la supervivència en totes tres espècies. En un segon experiment, vam examinar els efectes del corrent (amb i sense) i de l'aliment (alga viva i alga en pols), mesurant la supervivència i el creixement de *D. avara* i *Crambe crambe*. L'alga en pols com a font d'alimentació va augmentar el creixement dels juvenils de les dues espècies, mentre que la presència de corrent només va afavorir el creixement de *C. crambe*. Finalment, en un tercer experiment, vam estudiar la supervivència i el creixement de juvenils de *D. avara* i *C. crambe* transplantats al mar per comparar els resultats obtinguts al laboratori i els obtinguts en condicions naturals. La supervivència de juvenils en condicions de laboratori va ser molt més elevada que en el mar. En canvi, fixant-nos en el creixement, les dues espècies van mostrar resultats contraris: els juvenils de *C. crambe* van créixer més en el mar que en el laboratori, en canvi els juvenils de *D. a*vara van mostrar el patró contrari.

Tots aquests resultats, mostren que les esponges cultivades presenten requeriments diferents segons l'espècie. Podem concloure que el cultiu d'esponges a partir de larves sembla ser potencialment un bon mètode per la producció de biomasa d'esponja, i que el cultiu de juvenils en el laboratori sota condicions controlades és preferible al cultiu a mar obert per prevenir la pèrdua d'esponges per mortalitat que es molt important en el mar durant aquestes fases del cicle de vida.

Desenvolupament de la larva de *C. candelabrum*

Degut a la dificultat d'obtenir larves de *Corticium candelabrum* vam realitzar un estudi exstensiu sobre el desenvolupament embrionari i les característiques de la larva d'aquesta espècie. Per descriure els gàmetes i el desenvolupament de la larva es van analitzar individus de *C. candelabrum* recol·lectats mensualment, des de gener fins a septembre de 2002, amb microscopis electrònics de rastreig i transmissió. *C. candelabrum* va demostrar ser hermafrodita. Els oòcits i els espermatòcits es van diferenciar clarament a l'abril. Els embrions es van desenvolupar des de juny fins al juliol, moment en el qual les larves es van alliberar espontàniament. Els cist espermàtids s'originen a partir de càmares coanocitàries i les espermatogònies dels coanòcits per mitosis d'aquests. Els oòcits tenen el nucli nucleolat i el citoplasma ple de grànuls de yolk i lípids. Cèl·lules fol·liculars del teixit parental envolten als embrions entrellaçant-se estretament entre elles. Situada sota aquestes cèl·lules fol·liculars, hi ha una capa prima de col·lagen. El blastocel (cavitat interna) es forma per migració dels blastòmers cap a la perifèria de la mòrula. Un cop formada la cavitat interna de l'embrió, el col·lagen s'estén per tot el blastocel. En canvi, a la larva, aquest col·lagen s'organitza en una capa densa (làmina basal) separant les cèl·lules embrionàries del blastocel.

La larva és una típica cinctoblàstula. L'epiteli de la larva és pseudoestratificat i està format de cèl·lules ciliades. La zona basal de les cèl·lules ciliades contenen inclusions lipídiques i alguns grànuls de yolk; la zona intermèdia d'aquestes cèl·lules està ocupada pel nucli; i la zona apical conté abundants vesícules clares als electrons. De la zona apical de les cèl·lules ciliades surt el cili

amb una sola arrel estriada creuada. Tant a la part apical com a la part basal de les cèl·lules ciliades, es troben abundants estructures paracristal·lines a dins de vacuoles.

En diferents zones de la larva, es van observar tipus cel·lulars diversos (amb diferències mínimes). La majoria de les cèl·lules són ciliades, i presenten particularitats a nivell ultraestructural depenent de la seva localització en la larva (regió antero-lateral, intermèdia i posterior). Unes poques cèl·lules, més petites, no presenten cilis.

Diverses característiques de la larva de *C. candelabrum* semblen donar suport a la proposta suggerida prèviament en la literatura de la posició parafilètica dels homoscleròfòrids respecte a la resta de demosponges.

D'altra banda, l'aspecte críptic d'aquesta larva i el curt i imprevisible període d'alliberament larvari, ens indica que és necessari el desenvolupament d'un sistema altament controlat en el qual es puguin mantenir els adults d'esponja madurs, i així assegurar la recol·lecció de les larves en quantitat suficient per dur a terme futurs experiments.

Transmissió vertical de simbionts en *C. candelabrum*

Corticium candelabrum és una bacterioesponja, per tant conté gran quantitat de bacteris simbionts en la seva mesoglea. Les bacterioesponges es troben ben representades en diferents ordres de demosponges, incloent l'ordre Homosclerophorida. Els anteriors estudis sobre la formació d'explants (capítol III) ens suggeria un paper important dels bacteris simbionts en la biologia de *C. candelabrum*. Per tant, vam estudiar la transferència dels bacteris heterotròfics de *C. candelabrum* des del teixit parental fins a l'oòcit, i vam descriure les successives localitzacions d'aquests microsimbionts durant el desenvolupament embrionari, mitjançant el microscopi electrònic de rastreig i de transmissió. Vam descriure morfològicament vuit tipus diferents de bacteris simbionts, els quals tant són presents tant en els individus adults com a les larves. Tot i que en *C. candelabrum* les espermatogònies i els oòcits coexisteixen en els mateixos individus, els bacteris simbionts es transfereixen només als oòcits. En un primer estadi, els bacteris es localitzen a la perifèria de l'oòcit a sota d'una capa de

col·lagen gruixuda, després els bacteris van migrar cap al citoplasma de l'oòcit, formant grups esfèrics. Un cop fecundat l'òocit es comença a dividir. En aquest moment, els bacteris ocupen els pocs espais lliures entre els blastòmers, però sobretot, s'acumulen a la perifèria de l'embrió, sota les cèl·lules fol·liculars que l'envolten. Un cop es forma la cavitat interna (blastocel), els bacteris simbionts es mouen cap aquesta cavitat central i allà es divideixen activament, incrementant considerablement el seu nombre. En aquest estadi, en la zona basal de les cèl·lules de la larva, s'observen molts exemples de bacteris fogositats. Conseqüentment, podem pensar que els bacteris representen una font d'energia complementària per la larva lliure i per aquelles que s'acaben d'assentar, les quals encara no són capaces per elles mateixes de capturar l'aliment del mar.

Cultiu cel·lular d'esponges: pluripotència i immortalitat

Proposem l'ús d'embrions i larves com a una nova font de material d'esponja pel cultiu cel·lular, ja que els embrions i les larves contenen més proporció de cèl·lules mare que els adults (arqueòcits). Fins i tot, aquestes cèl·lules mare embrionàries són més versàtils i menys susceptibles de patir contaminació bacteriana que les cèl·lules mares dels adults. L'optimització dels mètodes per a l'obtenció i després la cryopreservació de la larva, així com el cultiu de juvenils en condicions de laboratori es proposen com a sistema per proveir cèl·lules embrionàries per a l'experimentació durant tot l'any. Addicionalment, l'enginyeria genètica i la investigació cel·lular dels mecanismes de la mitosi i l'apoptosi són nous camps de recerca molt prometedors que potser ajuden a estimular la divisió i la supervivència cel·lular.

En un futur, la recerca s'hauria de centrar especialment en com reduir l'activitat apoptòtica en les cèl·lules de les esponges, ja que hi ha indicis que suggereixen que és molt alta. Addicionalment, la recerca molecular s'hauria de combinar amb estudis d'aspectes bàsics de la biologia, ecologia i fisiologia de les esponges d'interès per tenir una visió més completa i entendre millor tots els requeriments de les cèl·lules en els cultius.

Curriculum Vitae

Curriculum Vitae

Sònia de Caralt Bosch was born the 22nd November 1976 in Barcelona. In 1999 she obtained her BSc in Biology, with a specialization in Organisms and Systems at University of Barcelona (Spain). In 2001, she obtained the MSc in Experimental Biology with a dissertation about "Contrasting biological traits and accumulation of heavy metals in *Clavelina lepadiformis* (Ascidiacea) populations from inside and outside harbours in the Catalan littoral", in the Department of Animal Biology, at the University of Barcelona. In 2005 she obtained the Research Sufficiency at the University of Barcelona. From 2002 until 2007 she has been working on her PhD thesis in the Food and Bioprocess Engineering Group at Wageningen University (The Netherlands), and the Centre for Advanced Studies in Blanes (Spanish Council for Scientific Research). Presently she is contracted as researcher in a European Research project on cultivation of sponges by the Centre for Advanced Studies in Blanes (Spanish Council for Scientific Research).

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de Caralt S, Uriz MJ, Ereskovsky AV & Wijffels RH (2007) Embryo development of *Corticium candelabrum* (Demospongiae: Homosclerophorida). Invertebrate Biology *(in press)*

de Caralt S, Otjens H, Uriz MJ & Wijffels RH. (2007) Cultivation of sponge larvae: Settlement, survival and growth of juveniles. Marine Biotechnology *(in press)*

de Caralt S, Uriz MJ & Wijffels RH (2007) Vertical transmission and successive location of symbiotic bacteria during embryo development and larva formation in *Corticium candelabrum* (Porifera: Demospongiae). Journal of Marine Biology Association of the United Kingdom *(submitted)*

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de Caralt S, Uriz MJ & Wijffels RH (2007) Cell culture from sponges: pluripotency and immortality. Trends in Biotechnology *(submitted)*

Cardona L, López D, Sales M, de Caralt S & Díez I (2007) Effects of recreational fishing on three fish species from the *Posidonia oceanica* meadows of Minorca (Balearic archipelago, western Mediterranean). Scientia Marina *(submitted)*

Oral presentations at conferences

Mendola D, de Caralt S, Uriz MJ, vanden Boogaart JGM, van Leeuwen J & Wijffels RH. Importance of water flow for culture of marine sponges. 8th International Marine Biotechnology Conference. Eilat, Israel. March 2007

Wijffels RH, Koopmans M & de Caralt S. Growth and development of sponges – learning from nature. 8th International Marine Biotechnology Conference. Eilat, Israel. March 2007

de Caralt S, Uriz MJ & Wijffels RH. Cultivation of sponge larvae: settlement, survival and growth of juveniles. XIV Simposio Ibérico de Estudios de Biología Marina. Barcelona, Spain. September 2006

de Caralt S, Uriz MJ & Wijffels RH. Embryo development and the transfer of symbiotic bacteria from adult to larvae in *Corticium candelabrum* (Porifera: Demospongiae). 7th. International Sponge Symposium. Buzios, Brasil. May 2006

de Caralt S, Otjens H, Uriz MJ & Wijffels RH. Sponge larval culture. 7th International Marine Biotechnology Conference. St. Johns, Canada. June 2005

de Caralt S, Agell G & Uriz MJ. Long-term culture of sponge explants: conditions enhancing sponge growth and bioactivity. Marine Biotechnology: Basics and Applications. Matalascañas, Spain. February - March 2003

de Caralt S, Agell G & Uriz MJ. Obtención de explantes de esponjas. Supervivencia y aspectos morfogenéticos. XII Simposio Ibérico de Estudios del Bentos Marino. Gibraltar- La Línea, Spain. October 2002

Overview of completed training activities

Discipline specific activities

Biodiversity of invertebrates, Univerisity of Barcelona, 2003
Reproductive startegies, Univerisity of Barcelona, 2003
Interstitial Edafic Fauna, Univerisity of Barcelona, 2003
Biological conservation, Univerisity of Barcelona, 2003
Management of marine fauna, Univerisity of Barcelona, 2003

General courses

Research study (DEA), University of Barcelona, 2004
Techniques for writing and presenting a scientific paper, Wageningen University, 2005

Optionals

Symposium of Marine Biotechnology, Spain, 2003
International Marine Biotechnology Conference, Canada 2005
Symposium Iberico de estudios de biologia marina
International Sponge Symposium, Brasil 2006

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