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DEFINIZIONE DEI PARAMETRI OTTIMALI PER L'ALLEVAMENTO IN ACQUARIO DELLA SPUGNA RICCA DI COLLAGENE *CHONDROSIA RENIFORMIS* Nardo, 1847 PER LA PRODUZIONE DI BIOMASSA

SETTING OPTIMAL CONDITION FOR REARING THE COLLAGEN-RICH SPONGE *CHONDROSIA RENIFORMIS* Nardo, 1847 IN AQUARIUM FOR PRODUCTION OF SPONGE BIOMASS

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Riassunto

Chondrosia reniformis Nardo, 1847 è una spugna tipica del Mar Adriatico che negli ultimi anni ha attirato l'attenzione della comunità scientifica per la sua versatilità e soprattutto per la grande quantità di collagene presente al suo interno. Questa proteina, caratterizzata da una bassa immunogenicità, è ampiamente utilizzata non solo in campo medico, ma anche in quello alimentare e cosmetico. Negli ultimi anni la sua richiesta è aumentata notevolmente e nuove fonti più sostenibili sono in continua ricerca. Una di queste è proprio *Chondrosia reniformis*, che si è rivelata essere una spugna capace di adattarsi bene a condizioni di allevamento. Tuttavia, molti aspetti devono essere ancora approfonditi e migliorati per garantire il massimo incremento in biomassa e di conseguenza la produzione di collagene.

Questo lavoro è stato svolto nell'ambito del MedSpon Project, che nasce dalla collaborazione dell'Università Politecnica delle Marche con l'Alfred Wegener Institute in Germania al fine di ottimizzare i processi di allevamento per specie target quali *Chondrosia reniformis* perché fungano da fonte di metaboliti secondari e in particolare di collagene.

Il lavoro si è concentrato sull'ottimizzazione delle tecniche di allevamento di questa spugna in un ambiente controllato (acquari) e sull'individuazione delle sue richieste nutrizionali al fine di creare una dieta idonea che permetta non

solo il suo mantenimento in aquario ma anche la crescita e la successiva estrazione di collagene. Una volta ottimizzato questo processo, i frammenti di spugna potranno anche essere utilizzati come talee per la reintroduzione in mare ed evitare così uno sfruttamento della risorsa.

Dopo un primo momento in cui abbiamo testato varie concentrazioni di fitoplancton per individuare quella ottimale, abbiamo fissato la dose media da somministrare e osservato la crescita o decrescita degli individui.

Oltre alla dieta, sono state indagate le dimensioni ottimali di allevamento, la temperatura ottimale, le preferenze di substrato e l'eventuale aggiunta di integratori che possano andare ad indurre un maggior aumento di biomassa e una maggior produzione di collagene.

Per valutare l'accrescimento, sono stati utilizzati due approcci differenti: una metodologia 2D e una 3D. Per le misurazioni 2D la spugna è stata fotografata dall'alto a intervalli regolari (1 volta a settimana) e l'area è stata misurata tramite il software ImageJ. Per quanto riguarda la metodologia 3D, abbiamo utilizzato la fotogrammetria, una tecnica che permette di ricostruire un modello tridimensionale della spugna e di calcolarne il volume e l'area tramite i software Agisoft Metashape e Recap Pro.

Sono state svolte in tutto 5 sperimentazioni, al fine di valutare come la spugna reagisce a differenti condizioni all'interno degli acquari.

Nella prima sperimentazione abbiamo cercato di valutare le dimensioni ottimali per l'allevamento di *C. reniformis* con la sola somministrazione di fitoplancton. Abbiamo scelto spugne minori 5 cm^2 o maggiori di 30 cm^2 ottenendo ottimi risultati per la classe dimensionale < 5 cm^2, con un incremento medio settimanale del 14%.

Nella seconda sperimentazione abbiamo invece testato la crescita a differenti temperature (15°C e 7°C), che possono andare ad influire positivamente o negativamente su di essa, ottenendo una miglior crescita a temperatura maggiore (15°C).

Nella terza sperimentazione le spugne sono state nutrite con una ridotta quantità di cibo (una volta a settimana), per testare la loro resistenza e vedere come si comportano a due temperature differenti (15°C e 7°C), ottenendo risultati migliori per quanto riguarda quelle allevate alla temperatura più elevata.

Nella quarta sperimentazione abbiamo aggiunto un supplemento di Acido Ascorbico (Vitamina C), che gioca un ruolo fondamentale nella dissoluzione del quarzo con un effetto positivo sulla produzione di collagene. I risultati di questo esperimento riportano una decrescita media delle spugne allevate a 15°C e un lieve incremento medio di quelle tenute e 7°C, unico caso di aumento per quando riguarda le spugne allevate a questa temperatura. Tuttavia, non si hanno

ancora abbastanza dati per poter trarre delle conclusioni riguardo all'utilizzo dell'Acido Ascorbico.

Nella quinta sperimentazione infine abbiamo testato l'utilizzo di sedimenti come supplemento nella dieta. *Chondrosia reniformis* è infatti una spugna nota per la sua capacità di incorporare particelle estranee, in particolare quelle silicee.

Da questo studio è stato possibile ottenere maggiori informazioni riguardo a questa spugna ricca di collagene e dalle elevate potenzialità. L'obbiettivo finale del progetto di ottenere un modello replicabile e standardizzato per una dieta adatta a *Chondrosia reniformis* è stato raggiunto, seppur ci sia ancora bisogno di ulteriori ricerche e approfondimenti per migliorare le metodologie utilizzate e abbassare la mortalità delle spugne.

1. Introduction

1.1. The Adriatic Sea

The Mediterranean Sea is an enclosed basin connected to the Atlantic Ocean by the narrow and shallow Strait of Gibraltar (width ~ 13 km; sill depth ~ 300 m). It is composed of two similar size basins, western and eastern, connected by the Strait of Sicily (width ~ 35 km; sill depth ~ 250 m).

Four sub-basins can be defined in the Eastern Mediterranean: the Ionian, the Levantine, the Adriatic, and the Aegean Seas (Malanotte-Rizzoli, 2001).

The Adriatic Sea is an elongated basin, with its major axis in the northwest–southeast direction, located in the central Mediterranean, between the Italian peninsula and the Balkans. The overall Adriatic basin is subdivided in three areas: the Northern basin, the middle Adriatic and the Southern basin.

Its northern section is very shallow and gently sloping, with an average bottom depth of about 35 m. The middle Adriatic is 140 m deep on average, with the two Pomo Depressions reaching 260 m. The southern section is characterized by a wide depression more than 1200 m deep. The water exchange with the Mediterranean Sea takes place through the Otranto Channel, whose sill is 800 m deep (Artegiani et al., 1997).

The eastern coast is generally high and rocky, whereas the western coast is low and mostly sandy. A large number of rivers discharge into the basin, with significant influence on the circulation, particularly relevant being the Po River in the northern basin, and the ensemble of the Albanian rivers in the southern basin (Artegiani et al., 1997).

The North Adriatic Sea, a sub-basin of the Mediterranean Sea, is a shallow semi-closed sea receiving high nutrients inputs from important rivers. These inputs sustain the highest productive basin of the Mediterranean Sea (Di Camillo & Cerrano, 2015).

In the northern Adriatic the entire water column exhibits an evident seasonal thermal cycle. A well-developed thermocline is present in spring and summer down to 30 m depth, whereas a significant cooling begins close to the surface in autumn when the bottom temperature reaches its maximum value, probably due to increased vertical mixing and intrusion of middle Adriatic waters.

We can recognize a seasonal layer of northern Adriatic surface water (NAdSW), which corresponds to low salinities and relatively high temperatures of the summer, and a northern Adriatic deep water (NAdDW) layer, which is cooled and renewed in winter (Artegiani et al., 1997).

The Italian coasts of the northern Adriatic Sea are generally sandy, and the Conero Promontory represents one of the rare zones characterized by hard bottoms. These, together with scattered substrates of anthropic origin, break the continuity of the Adriatic sandy coast and represent important stepping stones for species dispersal. This zone is characterized by shallow waters, a high sedimentation rate and a wide annual range of temperature (from 7°C in winter to 27°C in summer) (Di Camillo et al., 2012).

The coast of the North Adriatic Sea that include our study area (Passetto beach in Ancona) is characterized by large specimens of *Chondrosia reniformis* that is also the commonest sponge of the area (Di Camillo et al., 2012). Several giant individuals larger than one square meter were observed in the sampling area.

1.2. Chondrosia reniformis

Sponges, phylum Porifera, are the oldest metazoan group still extant on our planet. Sponges are found at all latitudes, living in a wide array of ecosystems varying in temperature and depth (van Soest et al., 2012).

Sponges have a simple level of organization: there are specialized cells for a variety of life functions, but these are not organized into tissues or organs.

Sponges possess an efficient filter system to acquire suspended food particles by pumping large amounts of water through a unique highly vascularized canal system. The water enters the sponge body through small apertures called ostia (normally 20–60 micron) that cover most of the sponge outer surface. Pumped water is drawn into the inhalant canals due to the slightly negative pressure created by the movement of choanocyte flagella gathered in the choanocyte chambers (Asadzadeh et al., 2019). The latter constitute the basic pumping units operating in parallel (Larsen & Riisgård, 1994), and the volume of water pumped is directly correlated to their density (Massaro et al., 2012). The capture of small particles occurs in the choanocyte chambers, while larger particles are trapped in the branching inhalant canals. After passing the chambers, the water leaves through the exhalant canals that merge into the excurrent apertures, called oscula (Morganti et al., 2019).

Nevertheless, the organization of the sponge body is simple. It is divided in different layers. The outer surface (exopinacoderm) and inner surface (endopinacoderm) is formed by epithelial cells (pinacocytes). The mesohyl layer is characterised by a more or less dense aggregation of a small number of differentiated, but partly pluripotent, cell types embedded in a collagenous matrix. Flagellated choanocytes organised in choanocyte chambers (choanoderm) are the ‘filtering units’, removing food particles from water these

cells pump through the pores. On cellular level sponges show a high plasticity due to the high amount of non-differentiated amoeboid cells (archaeocytes) (Nickel et al., 2001).

Although sponges are generally regarded as sessile, reports have been published that these animals can displace themselves bodily across their substrate. Burton (1949) and Bond & Harris (1988) reported sponge locomotion in the wild as well as in aquarium.

Sponges grow in distinct shapes and sizes due to the form of the internal mineral and/or organic skeletons secreted by specialized cells. The skeleton may also be supplemented by exogenous materials, such as sand grains. Skeletons, when present, are constructed of discrete siliceous or calcareous elements (spicules) and/or organic collagenous fibers (spongin), and rarely skeletons may be aspicular massive limestone constructions (van Soest et al., 2012).

Chondrosia reniformis Nardo, 1847 (*Figure 1.1*) is a common marine demosponge that lives in the shallow costs of the Mediterranean Sea and the South-West cost of the Atlantic Ocean (Lazoski et al., 2001). Its typical habitats are shaded rocky cliffs or caves at a depth of 1-50 m.

Figure 1.1 - Specimen of Chondrosia reniformis, Passetto beach (Ancona).

This sponge devoid of inorganic spicules and of spongin fiber, is surrounded by a resistant dermis composed of dense fibrillar bundles and ground substance. A section through the sponge reveals three distinct regions: a cortical zone called ectosome, an internal zone called mesohyl and the internal choanosome, which is of a softer consistency and densely filled with choanocyte chambers (Nickel & Brümmer, 2003). The ectosome is composed of a layer of flattened cells, exopinacocytes, that surround dense interwoven bundles of fibrils of collagen. The mesohyl is characterised by the high amount of collagen fibrils and a lower number of cells. In many circumstances the pinacocyte layer is loose, and the collagen fibrils can be in direct contact with water (Garrone et al., 1975).

Chondrosia reniformis had a conservative strategy, with slow growth but with greater resistance to damage (Garrabou & Zabala, 2001), in fact it is a robust

sponge in comparison to same other species and it can be maintained well in aquariums (Nickel & Brümmer, 2003). This species is also characterized by good regenerative properties (Nickel & Brümmer, 2003) whose molecular mechanisms have recently been described (Pozzolini et al., 2019).

As studied by Garrone et al. (1975) Bavestrello et al. (1998), Bonasoro et al. (2001) and Parma et al. (2007) *C. reniformis* can generate long, attenuated outgrowths which extend from the parental body for up to 3 m (normal sponges being on average 10 cm in length) then detach and form a new sponge (propagule). These outgrowths can extend down wards, as if under the force of gravity, or horizontally.

These dynamic phenomena, called “creeping”, have been interpreted in different ways. For instance they have been regarded as passive responses to environmental changes: the whole sponge body can slowly flatten and slide under compressive stress (Garrone et al., 1975). Other authors, such as Bavestrello et al., 1998, interpreted this phenomenon as a form of asexual reproduction, this type of reproduction by budding occurs in some species and sometimes is accompanied by the development of long retractile filaments which usually do not detach from the parent body (Fell, 1994), this type of reproduction via drop-like propagules occurs all the year around, with a

particularly enhancement during summer (Di Camillo et al., 2012). Finally, Bond & Harris (1988) suggested that these dynamic deformations are a form of localized locomotion by parts of the sponge, possibly preceding asexual reproduction (Bonasoro et al., 2001).

Concerning the reproductive biology of *C. reniformis*, oocytes were recorded throughout the entire period of observation with the maximum density observed in August and the minimal in January (Di Camillo et al., 2012). The reproductive cycle in *C. reniformis* is believed to be governed by temperature. Oogenesis starts soon after the water temperature begins to rise, and the oocytes are released before the temperature peak (Riesgo & Maldonado, 2008).

Chondrosia reniformis grow at the slowest rates due to its greater investment of energy and materials in massive growth. On the other hand, this growth form provided greater resistance to damage from biological and physical disturbances and also to being overgrown by other species (Garrabou & Zabala, 2001).

During integrated mariculture experiments with *C. reniformis*, its culture has been considered to be difficult, to even unsuitable with the methods applied (Pronzato et al., 1999, van Treeck et al., 2003). Wilkinson & Vacelet (1979) reported moderate growth rates of 95% per year (55 weeks doubling time in

volume, measured using volume displacement) when *C. reniformis* was cultured under shaded conditions. Osinga et al. (2010) obtained grow rates of 100 to 200% per year when growing *C. reniformis* on the bottom of metal wire cages under pristine conditions, but this study failed to achieve such results at a fish farm site as the explants cultured were smothered by effluents from the fish farm. Conversely, the study conducted demonstrates that if cultured using an appropriate method, *C. reniformis* will survive and grow (up to 170% in 13 months), even in a fish farm environment with a considerable particle load. These growth rates are considerably higher than those reported for naturally growing specimen. Garrabou & Zabala (2001) reported an *in situ* growth rate of 2.3% per year (deduced from two-dimensional (2D) areal growth) for *C. reniformis*, which was an order of magnitude lower than the growth rate of three other Mediterranean sponge species in their study *Hemimycale Ccolumella* (Bowerbank), *Oscarella lobularis*, and *Crambe Crambe* (Schmidt). They ascribed the slow growth rate of *C. reniformis* to a greater energy investment in tissue production per unit area as a result of its thick collagenous cortex. However, the data found by Osinga et al. (2010) indicate that in aquaculture, *C. reniformis* exhibits growth rates that are nearly two orders of magnitude higher than the *in situ* rates reported by Garrabou & Zabala (2001).

Under optimal circumstances, the production of collagen is apparently not hampered by energy input. The results obtained by Gökalp et al. (2019) show a clear potential for collagen production through the aquaculture of *C. reniformis*. The highly variable growth of *C. reniformis* under different conditions and the high variability within treatments highlight the need for further optimization studies.

It is well known that this sponge is capable of regenerate its tissues (Pozzolini et al., 2019), in fact one of the rearing methods for *Chondrosia reniformis* is by the use of fragments originating by the same individual (Nickel & Brümmer, 2003). Its ability to regenerate itself has been studied at a molecular level by Pozzolini et al. (2019) and during *C. reniformis* tissue regeneration they observed a significant downregulation of fibrillar and non-fibrillar collagen genes combined with a reduction in tropocollagen content. This can be explained because during regeneration, it may be necessary to reduce the energetically expensive fibrogenesis in favor of new exopinacoderm formation.

For the construction of their skeleton, several species of Demospongiae utilise foreign material such as sand grains, sponge spicules, diatom oozes and other particles. As previously seen, this sponge lacks a siliceous skeleton and

reinforces its collagenous ectosome incorporating large amounts of foreign matter (Bavestrello et al., 1996). *Chondrosia* incorporates two kinds of foreign bodies: sponge spicules and sand grains. This uptake can be described as an active choice as is strongly selective and polarized (Bavestrello et al., 1998): the upper surface of the sponge avoids incorporating carbonatic matter, and incorporates siliceous particles only; the lower surface is not selective, being able to attach itself to every kind of mineral. A remarkable aspect of this process is that after quartz particle incorporation the sponge is able to etch and homogenize this material up to a final size of 20-30 μm.

More specifically, it is able to engulf silica sand particles (quartz crystals) in its ectosome, and partially dissolve them through ascorbic acid-driven surface erosion, leading to an increase in the concentration of soluble silicates in the surrounding water (Bavestrello et al., 1995). Indeed, the high concentration of ascorbic acid detected in the sponge body seems to be involved in this peculiar process (Bavestrello et al., 1995), as well as in the biosynthesis of collagen (Ehrlich et al., 2010).

Furthermore, in the work presented by Pozzolini et al. (2017), they have demonstrated for the first time at the molecular level that quartz microcrystal incorporation in *C. reniformis* leads to a beneficial increase of collagen

deposition, which is probably advantageous in terms of strengthening of the sponge.

One of the things that made us choose this sponge for our study is that it is particularly rich in collagen (Fassini et al., 2017) which is the most abundant protein of the extra cellular matrix and can be found in all Phyla (Exposito et al., 2010). For this reason it can be used as a model system to develop feeding strategies and evaluate the biotechnological potential of sponge cultivation (Nickel & Brümmer, 2003).

1.3. Rearing Chondrosia reniformis in aquarium

Sponges are an important source of secondary metabolites with pharmaceutical interest. This is the main reason for the increasing interest of sponge culture in recent years (De Caralt et al., 2003).

Chondrosia reniformis is a robust sponge in comparison with some other Mediterranean species and can be used as a model system to develop feeding strategies and evaluate the biotechnological potential of sponge cultivation (Nickel et al., 2003).

Ex situ cultivation may be preferable to *in situ* mariculture, because it may be possible to switch from seasonal growth to continuous growth during the year

(Sipkema et al., 2004). However, continuous *ex situ* growth of marine sponges has not been established in the laboratory as yet for periods in excess of one full year. *Ex situ* cultivation allows growth parameters to be optimized, such as culture water temperature, light levels and periods, food availability and nutritional balance, dissolved nutrients, and possibly even precursors of the secondary metabolites of interest (if known).

However, marine sponges have not as yet proven to be your typical "lab-rat", but a growing number of researchers have demonstrated small successes in culturing them outside of the sea (Barthel & Theede, 1986; Belarbi et al., 2003; De Caralt et al., 2003; Duckworth & Battershill, 2003; Mendola, 2003; Sipkema et al., 2005).

Attempts of rearing *Chondrosia reniformis* has been made by Nickel et al. in 2001 and 2003, with good results regarding the capacity of breed this sponge in aquariums. In these trials, they tested methodologies with fragments and multicell reaggregate culture, that use the ability of regeneration and healing of this sponge. Both these methods, however, needs to be optimize and further ecological parameters have to be involved to create better culture conditions for this sponge.

Among the various feeding strategy tested during the past studies, we find first of all phytoplankton, such as *Dunaliella sp*., and in addition to this is also used

commercial liquid food for marine invertebrates and Mediterranean bacteria (Nickel et al., 2003).

Other authors (Belarbi et al., 2003; De Caralt et al., 2003) involve in the cultivation of sponges the addition of antibiotics (e.g. penicillin) to control external contamination and prevent the necrosis of tissue.

In particular, as a result of the study conducted by De Caralt et al. (2003) on fragments from the sponge *Corticium candelabrum* Schmidt, 1862, they found that sponges collected in winter and spring, cultured at 14°C, 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 and, as a result, its aquiferous system become disarranged. 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.

Nevertheless, a standardized feeding methodology for rearing sponges in aquarium, especially *C. reniformis*, has yet to be established.

1.4. Collagen

Collagens are the most abundant high molecular weight proteins in both invertebrate and vertebrate organisms, including mammals, that serves multiple functions in both of them (Fassini et al., 2017). It possess mainly a structural role, existing different types according with their specific organization in distinct tissues (Silva et al., 2014).

The characteristic feature of a typical collagen molecule, tropocollagen, is its long, stiff, triple-stranded helix, in which three collagen polypeptide chains are wound around one another in form of a ropelike superhelix. These peptides are extremely rich in proline and glycine, both of which are important for the formation of the collagen-specific helical structure (Swatschek et al., 2002).

This protein has a complex structural and hierarchical organization, with more than 20 types of collagens reported up to now. The different types of collagens are differently distributed in animal tissues (Silva et al., 2014).

This polymer is involved in the formation of complex fibrillar networks, providing the structural integrity of tissues. Its low immunogenicity and mechanical properties make this molecule a biomaterial that is extremely

suitable for tissue engineering and regenerative medicine (TERM) strategies in human health issues (Pozzolini et al., 2018).

Nowadays, collagen has a wide range of applications in the health-related sectors, namely in cosmetics, the pharmaceutical industry and in medical care (including plastic surgery, orthopedics, ophthalmology and dentistry) (Meena et al., 1999). Only considering healthcare industry, the collagen market was over 700 million in 2019, and a more than 8% growth is expected by 2026 (Ahuja & Singh, 2019). In non-health sectors, a noteworthy use of collagen is in the food sector (food processing, as additive and nutraceuticals), but most often as gelatin, i.e., in its denatured form (Gates, 2010).

Industry is constantly searching for new natural sources of collagen and upgraded methodologies for their production. The most common sources are from bovine and porcine origin (about 98%). However, mammalian collagen has been associated with a high pathological risk of transmitting diseases such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and avian/swine influenzas (Swatschek et al., 2002). In addition to this, this source of collagen has other disadvantages related with social and religious constraints as well as extraction and purification processes (Silva et al., 2014). For this reasons, other ways are making their route, such as recombinant production, and also extraction from marine organisms like fish.

Different organisms have been proposed and explored for collagen extraction, allowing the sustainable production of different types of collagens, with properties depending on the kind of organism (and their natural environment) and extraction methodology (Silva et al., 2014).

As demonstrated by Pozzolini et al. (2018), collagen derived from marine sponges is an extremely performant biopolymer that is suitable for biomedical applications, and to optimize the combined use of sponges as producers of natural products and bioremediators, we need to assess the biomass production and associated natural product content and link these to the actual *in situ* filtration capacity of the model species (Gökalp et al., 2020).

In particular, one of the most described collagens in this phylum derives from the demosponge *Chondrosia reniformis*; indeed, in this animal, it displays peculiar physicochemical characteristics and dynamic plasticity (Wilkie et al., 2006).

In recent experimentations attempts have been made for an efficient method of extraction for the collagen. Extraction has been made separately for the two diverse regions of the sponge: ectosome (Ec, the cortical external layer) and choanosome (Ch) that forms the principal component of biomass of the sponge.

Studies on the cortex of *Chondrosia reniformis* showed that the hydroxyproline content approximately corresponds to a 40% of collagen content (Garrone et al., 1975), similar results were obtained by Swatschek et al. (2002).

In *Chondrosia reniformis* the biosynthesis of collagen is favored by the presence of Ascorbic Acid (AA) that is convolved as coenzyme in the hydroxylation of proline and lysin. In addition to this, AA play a key role in the dissolution of quartz that has positive effects in the production of collagen (Ehrlich et al., 2010). The AA might have a role in the stabilization of the ferrous iron (Fe^{2+}) that seem to enhance the formation of oscula in the sponges (Osinga, R., Kotterman, 2007).

Quite recently, some collagen gene sequences, as well as that of a collagen maturation enzyme, have been uncovered in this animal (Pozzolini et al., 2012). Furthermore, *C. reniformis* collagen has also demonstrated its utility as a carrier in form of nanoparticles and as a coating for drug preparations, and its lack of toxicity on human skin has been assessed (Swatschek et al., 2002). Recently, also, the possibility of using it in the form of thin biocompatible membranes for tissue engineering and regenerative medicine purposes has been evaluated (Pozzolini et al., 2018).

Collagens from the demosponge *Chondrosia reniformis* (Nardo 1847) have received attention from researchers since 1970 (Garrone et al., 1975) due to

their diversity (type IV collagen, fibrillar and nonfibrillar collagen) (Pozzolini et al., 2012) and interesting structural, physicochemical, and ecophysiological properties (Heinemann et al., 2007).

For example, as mentioned by Ehrlich et al., 2018, slices of fibrillar collagen incubated with collagenase are not modified even after 48 h of incubation, and do not show any changes in the aspect, consistency, or fine structure of the fibrils. No kind of enzymatic damage was observed by electron microscope on structure of the fibrils.

Recently, special attention has been focused on fibrillar collagens in the mesohyl of *C. reniformis*. This species is the only sponge which has been experimentally proven to contain a dynamic collagenous mesohyl capable of stiffening upon being manipulated (Pozzolini et al., 2012). It was shown that the different physiological states recorded in laboratory experiments are expressions of the mechanical adaptability of the collagenous mesohyl of *C. reniformis*, and suggest that stiffness variability in this sponge is under cellular control (Heinemann et al., 2007).

C. reniformis collagens pose remarkable biotechnology potential: their successful use has been demonstrated in cosmetic preparations, in drug delivery, in biomembrane production and in the production of active peptides with biomedical targets (Ehrlich et al., 2018).

The marine sponge *Chondrosia reniformis* was chosen for this study due to some advantages: this sponge can be found ubiquitously in the Mediterranean Sea and contains a lot of collagen.

1.5. Measurement methods

Measurements of volume, biomass or surface area are particularly important when determining patterns of growth and secondary production by an organism or benthic community (Eleftheriou & McIntyre, 2005). Sponges, like many benthic invertebrates, exhibit a bewildering array of morphologies (Bell & David, 2000) that are highly plastic (Kaandorp, 1999) and this makes obtaining repeatable and accurate measurements of size or biomass difficult.

The biomass and size of sponges have been measured using a different techniques including simple morphometric measurements (Duckworth & Battershill, 2003), as well as traditional measurements of surface area (Elvin, 2012) and volume (Wilkinson & Vacelet, 1979). The problems with these methods are that many of them are destructive, so the size, shape and biomass of sessile organisms have recently been measured through photography and photogrammetry (Eleftheriou & McIntyre, 2005). These methods vary from single camera photographic techniques used for example by Handley et al. (2003), to methods of three-dimensional measurements and reconstructions

using photogrammetry (Bayley & Mogg, 2020; Eleftheriou and McIntyre, 2005; MESH, 2007; Palma et al., 2018; Royer et al., 2018). The latter methods allow more accurate and repeatable measurements of size, shape and biomass without the destruction of the target organisms (Abdo et al., 2006).

For our study and for the determination of sponge growth we initially opted for a 2-dimensional (2D) approach, that can allow us to have an idea of the change in the surface of the sponge. *Chondrosia reniformis* is a relatively flat sponge with a primary horizontal growth so this approach can fit well for this sponge. In addition to this, a 2-dimensional approach is very easy and doesn't require particular computer skills. It has been used in Gokalp et al. (2020) study to define the growth of *Chondrosia reniformis* at different depths.

However, dealing with fragments or irregular individuals, in a second stage we decided to adopt a 3-dimensional approach to better understand the growth of *C. reniformis*.

A 3-dimensional (3D) approach, also called photogrammetry, improves the capacity to accurately measure architectural complexity, topography, rugosity and other structural characteristics, but most important it allow us to measure more precisely the volume (Burns et al., 2015).

Photogrammetry, that is the science or art of obtaining reliable measurements by means of photographs (Thompson, 1966), is a flexible and powerful

technique used in scientific and engineering applications, where the extracted information must comply with a predefined accuracy, reliability and completeness (Förstner & Wrobel, 2016).

Photogrammetry has the advantage of being nondestructive, repeatable, and provides a permanent record of the individual organism (Abdo et al., 2006).

The photogrammetric process consists, in brief, of the three-dimensional reconstruction of the shape and location of an object starting from a set of images of that object (e.g., still frames extracted from videos, as in the case of our transects, or photographs). The result of this process is the production of digital and graphical outputs (i.e. the 3D model with its derived geometric elements) with the purpose of deriving accurate and reliable 3D measurements of the object from images (Granshaw, 2016).

The recent development of new technologies to record physical structures digitally, alongside rapid increases in computing power have allowed these traditional methods to be substantially improved upon. 'Structure from Motion' (SfM) photogrammetry (Westoby et al., 2012) now allows us to create a detailed non-destructive 3D digital model of the physical environment from overlapping camera images and are perhaps the most successful example within the context of benthic community mapping (Figueira et al., 2015; Palma et al., 2017).

SfM is a topographic survey technique for the reconstruction of real scale three dimensional models of subjects or scenarios, using imagery collected at unknown camera positions (James & Robson, 2012). It enables non-destructive, repeatable measurements and facilitates rapid sampling (Figueira et al., 2015). Applications of SfM surveying methods in terrestrial and seascape environments have provided accuracies and resolutions comparable to more sophisticated technologies (e.g., laser scanning) (Palma et al., 2018).

Models can be morphometrically analysed in a range of ways, and can be archived for future analysis and comparison by multiple observers (Anderson et al., 2019), in our study we overlapped older models with newer ones in order to visualize the growth of the sponge.

As this technology expands its use into underwater survey and research (from a largely terrestrial starting point), a range of methodologies are developing for creating and analysing 3D reef models, primarily over a small scale. However, there is still uncertainty for researchers new to this field over how to create their own models given the range of options available, and therefore a barrier to its standardised use in this setting from the initial training hurdles (Bayley & Mogg, 2020).

In marine biology, photogrammetry is now increasingly being used to rebuild the 3D complexity of the underwater habitats and it is an important tool for a

number of studies that have employed automatic photogrammetric techniques to analyse the complexity of coral reefs and seafloor (Bayley & Mogg, 2020; MESH, 2007; Royer et al., 2018), gorgonian forests (Palma et al., 2018), mid depth shipwrecks (Aragón et al., 2018), benthic ecosystems in Antarctica (Piazza et al., 2019) or for an high resolution survey of a coral reef (Vogler, 2019). In addition to this, underwater photogrammetry could be a tool for citizen science (Raoult et al., 2016) thanks to the proliferation of cheaper high-megapixel cameras, affordable unmanned aerial vehicles, and more powerful personal computers have made SfM/Photogrammetry more widely accessible. In our study photogrammetry will be applied in laboratory for the determination of the volume of *Chondrosia reniformis* and this will help us successively in the quantification of the content of collagen, of which this sponge is rich.

2. Purpose of the thesis

This study is part of the MedSpon Project, in collaboration with the Alfred Wegener Institute in Germany. The objective of the project is to optimize an aquaculture processes for the target species by successfully breeding sponge fragments to serve as a source of secondary metabolites.

In recent years, the interest in sponge cultivation increased, due to the growing number of sponge secondary metabolites of economic value (Nickel et al., 2001). One of these metabolites is collagen, the use of which is nowadays evolved until the application in cell therapy, biomedicine, cosmetics and the food industry (Ehrlich et al., 2018). Collagen market demand for healthcare is expected to reach 622.2 kilotons by 2025 (Global Collagen Report, 2020).

As seen previously *C. reniformis* is a marine sponge rich in collagen that can be used as a model system to develop feeding strategies and evaluate the biotechnological potential of sponge in vitro cultivation (M. Nickel & Brümmer, 2003).

First aim of my thesis is to set a standardized method to rear and feed in aquarium specimens of *C. reniformis*. The optimal rearing conditions will be found by testing several factors to maximize production of sponge biomass. This will give the possibility to obtain further sponge cuts to be reintroduced in

the natural environment and have the possibility to extract collagen without depleting the sponge source.

Previous studies have been focusing on functional fragment culture and multicell reaggregate culture (see Nickel, 2001 for more details), but we can assume that another good way to produce biomass could include the use of integer specimens of *C. reniformis* collected from the sea and kept in aquariums. Similar studies have been made by Pozzolini et al. (2018) and Gokalp et al. (2020), where sponges were kept respectively in aquariums for the first study and *in situ* for the second one. The increase of interest in this versatile sponge pushed us to explore its potential to be raised in a controlled system for the maximization of its growth rate.

One of the positive aspects of rearing *C. reniformis* in aquariums is that, once find the right conditions and feeding strategy for this sponge, we can eliminate the seasonal variability that we have in mariculture and so we can maximize its growth.

In *Table 2.1* are summarized all our objectives and research questions of this study.

Table 2.1 - Scheme of the Objectives and Research questions of the study.

Issues	Objectives	Research questions	Hypotesis	N.	Methods
Chondrosia reniformis is a common marine sponge of the Adriatic Sea and is particulary rich in collagen. This sponge could be a good model for the rearing in aquariums and for the production of sponge biomass associated with the extraction of collagen. This protein has recently become more and more requested by the industries because it can be used in different sectors, from medical applications to cosmetical. To accomplish this, we need to optimize its conditions in aquarium and create an optimal diet for the sponge.	To set optimal condition for rearing *Chondrosia reniformis.*	Which are the best aquarium conditions for rearing *Chondrosia reniformis* ?	*Chondrosia reniformis* need optimal aquarium conditions to maximize the production of biomass.	1	Measure of the parameters of the aquariums: salinity, temperature, pH, Nitrates and hydrodimanism.
	To find the optimal diet for *Chondrosia reniformis* with conventional source of food.	Which is the ideal concentration of phytoplanckton for *Chondrosia reniformis* ?	Sponges are filter feeders and a diet based on phytoplankton could ensure the source of amminoacids that are at the base of the production of collagen.	2	Somministration of different amount of phytoplankton and control of the health of *Chondrosia reniformis.*
		How can we measure the concentration of phytoplanckton administered to *Chondrosia reniformis* ?	We need to know how much phytoplankton is contained in the daily dose of food administered to the sponges.	3	Counting of the quantity of phytoplankton thanks to the Neubauer chamber.
	Once found the ideal concentration of food for *Chondrosia reniformis* , test different supplements to administrate in addition to the main source of nourishment.	Does *Chondrosia reniformis* need other nutrients in addition to phytoplankton?	A proteic source could be a good supplement to increase the biomass of the sponge as a source of aminoacids.	4	Somministration of a known concentration of mussels' mixture (proteic source) and measure of the surface over time.
			Ascorbic acid is involved, as reducing agent, in the intensive biosynthesis of collagen, the major component of this sponge. We hypotize that adding it at the sponges' diet could increase their biomass.	5	Somministration of a known concentration of Ascorbic acid and measure of the surface over time.
			Chondrosia reniformis is famous for the capacity of incorporate forein materials, in particular silica sand particles. So, thay can be a good supplement for the production of biomass.	6	Somministration of sediments on the surface of the sponge and measure of the surface over time.
	To measure the growth of *Chondrosia reniformis.*	Which methods are more appropriate to measure the growth of *Chondrosia reniformis* ?	2D photos can allow us to have an idea of the growth of the sponges measuring the surface of them.	7	2D photos of the sponges from above were made in order to measuring the their surface. Photos were analyzed using the software ImageJ.
			3D photogrammetry can help us to have a more comprehensive idea on how much and in which way *Chondrosia reniformis* grows.	8	3D photogrammetry was made using a Gopro and photos were analyzed with two software: Metashape and Recap Pro.
	To evaluate a mean cost of the aquarium systems in order to compare it with maricolture systems' costs.	How much does an aquarium system cost monthly? Is it better than maricolture of *Chondrosia reniformis* ?	Aquariums systems for *Chondrosia reniformis* are a good solution in order to rearing this sponge.	9	Evaluation of all the factors and esteem of an average cost of the system.

3. Materials and methods

3.1. Study site and samplings

The sampling area (*Figure 3.1*) is part of the Conero Riviera, more precisely the area nearby the Passetto beach (43°37'02.4"N 13°32'06.9"E). Samplings were made during September, October and November 2020.

This area is characterized by a rocky shore, shallow waters, a high sedimentation rate and a wide annual range of temperature (from 7°C in winter to 27°C in summer) where we can find big individuals of *C. reniformis* (Di Camillo et al., 2012). During the sampling period the temperature of the water variate from 24.9°C of September to 17.2°C of the beginning of November (https://www.mareografico.it).

We choose this area because of the large number of specimens of *C. reniformis*, that are easily to found and tag for subsequent diving sampling.

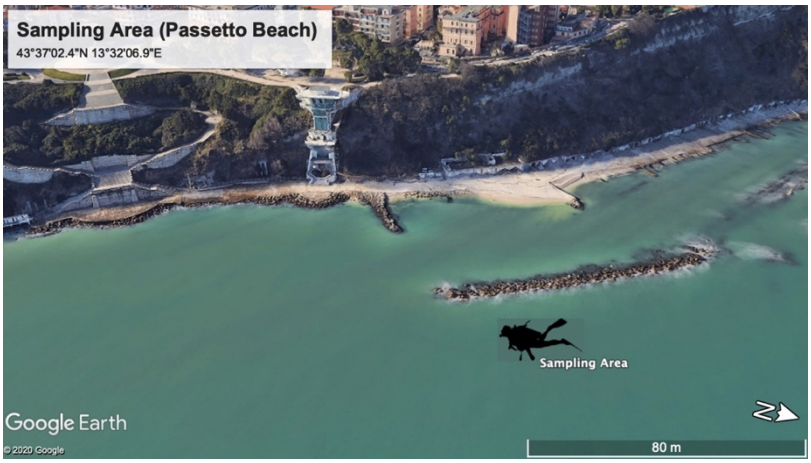

Figure 3.1 - Sampling area Passetto beach (Ancona).

3.2. Collection, transport and placement in the aquariums

Specimens of *C. reniformis* were collected by scuba divers at Passetto beach (Ancona) at 6-7 meters deep and transported in the laboratory thanks to bags filled with sea water and maintained in a cooler to prevent mortality.

As seen by Nickel & Brümmer, 2003, *C. reniformis* is a robust sponge in comparison to some other species. Nevertheless, collection and transport have to be done very carefully. Air contact and temperature shifts were avoided.

Specimens of *Chondrosia* were preferably choose by a single small individual and not cut from a bigger one in order to avoid the risk of necrosis of the tissue. Sponges from which we had collected our fragments were tagged in order to find them in the following dives and monitor the healing *in situ*.

Once arrived at the laboratory sponges were attached at different substrates in order to verify the affinity of the species with the different types of materials. We choose three substrates: PVC (polyvinyl chloride), glass and porcelain stoneware (gres). Sponges were fixed at these different materials with the help of a nylon thread positioned around them.

Thereafter we used a commercial glue (ATI Easy Glue), also used for the attachment of fragments of corals during the transplant. This glue was placed on the substrates and the sponge was set over it, after 30-40 seconds the glue was dry, and the sponge was positioned in the aquarium. In this way we avoided

the risk of stress for the sponge due to the compression caused by the nylon thread.

3.3. Aquariums

Aquariums system was built in order to guarantee stable condition inside the tanks. We built 6 tanks of 27 liters each (*Figure 3.2*) with a single filtering system composed by mechanical, chemical and biological filter. In addition to this is fundamental a skimmer that purified the water from harmful compounds derived from the degradation of organic matter (unconsummated nourishment, excrement, dead organisms), an UV lamp that help the sterilization of the water and a refrigerator that can maintain the temperature of the water constant.

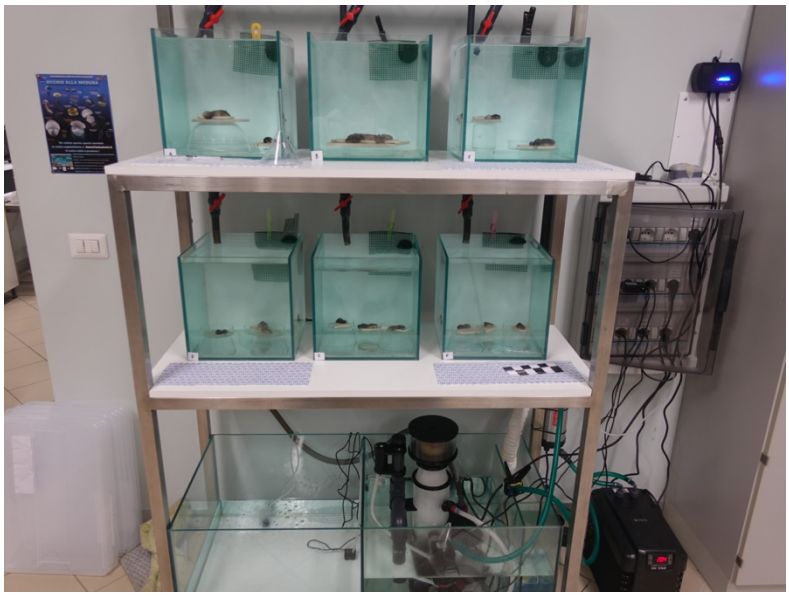

Figure 3.2 - Aquarium system composed by 6 tanks (27 liter) and a filtering system below them.

Aquariums were filled with Artificial Sea Water (ASW). We added 38,2 g of Red Sea salt per 1 liter of distilled water, in order to obtain a salinity of 35 psu. Salinity was measured using an Hand Held Refractometer, that allowed us to easily measure it and maintain it constant.

Around 1/3 of the seawater was changed at irregular intervals every two weeks or earlier if necessary, for example, when nitrates were beyond the threshold.

The water temperature was kept at 15°C, an artificial light/dark cycle of 8-12 h light:16-12 h darkness was applied.

Simultaneously we set up other two aquariums (about 90 liter) with different physical condition, the temperature was 7°C and the salinity 35 psu. These aquariums were provided with a filtering system and a pump to guarantee a good quality of the water.

Chemical parameters of the water were measured regularly in order to guarantee the good quality of the water. Nitrates were measured using a dedicated kit (Nitrat-Test) capable of measuring their concentration inside the tanks. This could be always under 25 mg/l in order to avoid risks for the health of the sponges.

Another parameter measured was the pH of the aquariums, using a precision pH meter (inoLab pH 720).

We also tried to assess the current speed inside the tanks using its relationship with the flow, enunciated by the Continuity Equation.

$$q = Sv$$

Where q is the flow, S is the transversal section and v is the speed. With the inverse formula we can derive the speed of the water inside the aquariums. This parameter affects not only the turnover of the water inside the tanks but also the health of the sponge.

3.4. Feeding strategy

3.4.1. Primary source of food

Specimens of *Chondrosia reniformis* were fed regularly with commercial marine phytoplankton gel in a mineral suspension (Easybooster 25). This mixture is composed by *Nannocloropsis sp.* (31%), *Isochrysis sp.* (33%), *Tetraselmis sp.* (18%) and *Phaeodactylum sp.* (18%).

The marine phytoplankton gel has been diluted before being administered to the sponges: 1 ml of gel in 100 ml of marine water (dilution factor 101).

After some days of acclimatation we started with the feeding strategy.

Food was dosed with a syringe, that allow us to withdraw the right quantity of phytoplankton.

To avoid loss of the mixture during the administration, we set the water current at minimum and we cover every individual with a plastic bell provided of a hole to allow the entrance of the food (*Figure 3.3*).

During our study we tried different concentration of food in order to optimize the administration of it and find the right quantity of phytoplankton that *Chondrosia* needs.

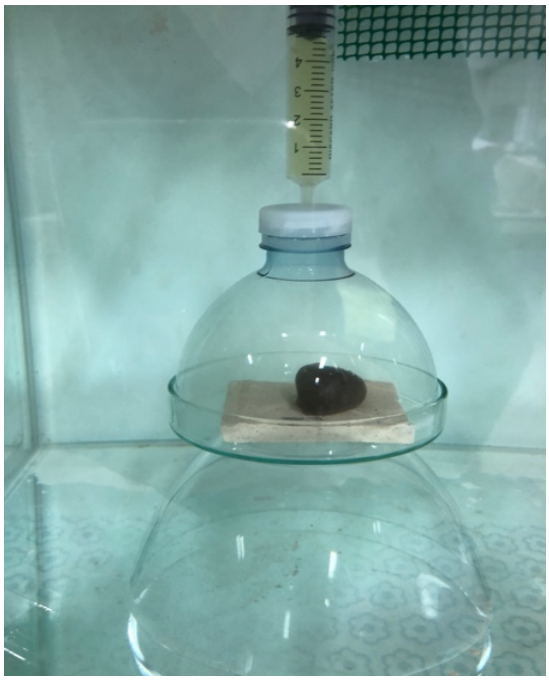

Figure 3.3 - Method for the administration of food. A plastic bell was positioned over the sponge and food was administrated with a syringe.

3.4.2. Supplements to administrate in addition to the main source of nourishment

In addition to the main source of food (phytoplankton), we tested different supplements that could be needed by *Chondrosia reniformis* to grow and mainly to produce more collagen.

Ascorbic acid

In the sponge *Chondrosia reniformis* ascorbic acid (Vitamin C) is involved, as reducing agent, in the intensive biosynthesis of collagen, the major component of this sponge (Garrone et al., 1975). We assumed that adding ascorbic acid to the sponge will help in the production of collagen and consequently of biomass. In addition, AA is extremely important in protecting membrane phospholipids, particularly in reproduction and disease resistance, from oxidative damage.

For the administration of ascorbic acid, we decided to feed the sponges with 0,025 g of it, mixed with the phytoplankton and administrated at the same time.

Protein source

During our experiment we tried also if an enriched diet in protein would fit with the growth of *Chondrosia*. To do this we prepared a mixture of phytoplankton diluted with mashed and filtered mussel, that contain an elevated amount of

protein and it can be easily found in our study area. We decided to administrate this mixture to just one sponge in order to assess the positive or negative effect on it.

Sediments

As is well known, *C. reniformis* is capable of incorporate foreign particles, in particular siliceous one (Bavestrello et al., 1996, Bavestrello et al., 1998). We administrated a small number of sediments from Falconara beach with a diameter of 250 μm. Sediments were positioned on the ectosome of the sponge and we assumed that this could help the production of biomass of the sponge.

3.5. Pre-trial

Since there isn't a standardize protocol for rearing *Chondrosia reniformis* in aquarium, we initially started with the knowledge of this sponge and of its behavior after the relocation in the tanks.

Before starting with the actual trials, we tested different concentration of phytoplankton to understand the nutritional requirements of this species. We increase the dosage of the mixture every week and looking at the effects on the specimens and on the aquarium parameters.

We started with the administration of 2,5 ml/sponge per day for the first week. In the following weeks we doubled the dosage and subsequently we've also increased administration to twice a day, we continue with these dosages for other two weeks. Finally, we increased again the dosage to 10 ml/sponge twice a day.

During the fourth week we started with the administration of a protein source to just one specimen of *C. reniformis* in order to verify the effect of this addition on the sponge.

In this pre-trial we also tested different substrate for the sponges: PVC (polyvinyl chloride), glass and porcelain stoneware (gres), in order to understand their preference and see if this can affect the growth.

3.6. Trials

Once understood the right amount of food needed by *Chondrosia* we started with the trials. In order to better understand the optimal conditions to rear this sponge in aquarium, we set different trials and observed the behavior of the sponge over the weeks. In the *Table 3.1* we outlined all the trials done through the study. During the feeding period we stopped the water current and we positioned the bells over the sponge so we can exclude the current speed as a variable of our results.

Table 3.1 - Scheme of the trials.

TRIAL		ID SPONGE	SPONGE SIZE	TYPE OF FOOD	QUANTITY	INTERVAL	SUPPLEMENTS	TEMPERATURE	SALINITY	CURRENT SPEED	WATER REFILL
I Size	**a**	E3, F3, D1, F1, A2, D3	area <5 cm^2	Phytoplankton	5 ml/sponge per day	6 weeks	No supplements	15°C	35 psu	0,89 m/s	1/3 of water every 15 days
	b	B1, A1, C2	area >30 cm^2	Phytoplankton	5 ml/sponge per day	6 weeks	No supplements	15°C	35 psu	0,89 m/s	1/3 of water every 15 days
II Temperature /High food conc.	**a**	A12, A7, A5, A11, A9 a, A10, A8, A3, A13, A14, A15, A9 b, A6	area <5 cm^2	Phytoplankton	5 ml/sponge per day	7 weeks	No supplements	15°C	35 psu	1,5 m/s	1/3 of water every 15 days
	b	A5, A10, A8, A13, A15, A9 b, A6	area <5 cm^2	Phytoplankton	5 ml/sponge per day	4 weeks (ongoing)	No supplements	7°C	35 psu	1,5 m/s	1/3 of water every 15 days
III Temperature /Low food conc.	**a**	B4, B9, B7, A3	area <5 cm^2	Phytoplankton	5 ml/sponge once a week	3 weeks	No supplements	15°C	35 psu	1,5 m/s	1/3 of water every 15 days
	b	C7, C8, C4, C6, C5	area <5 cm^2	Phytoplankton	5 ml/sponge once a week	3 weeks	No supplements	7°C	35 psu	0,89 m/s	1/3 of water every 15 days
IV Temperature /AA	**a**	B4, C4, A4 a, A4 b, E4, A9, F4	area <5 cm^2	Phytoplankton	5 ml/sponge per day	7 weeks (ongoing)	Ascorbic Acid	15°C	35 psu	0,89 m/s	1/3 of water every 15 days
	b	C7, C8, C6, C5	area <5 cm^2	Phytoplankton	5 ml/sponge per day	4 weeks (ongoing)	Ascorbic acid	7°C	35 psu	1,5 m/s	1/3 of water every 15 days
V Sediments		B4, B9, B7	area <5 cm^2	Phytoplankton	5 ml/sponge per day	4 weeks (ongoing)	Sediments	15°C	35 psu	0,89 m/s	1/3 of water every 15 days

CONTROL	ID SPONGE	SPONGE SIZE	TYPE OF FOOD	QUANTITY	INTERVAL	SUPPLEMENTS	TEMPERATURE	SALINITY	CURRENT SPEED	WATER REFILL
Trial IV, V	D4	area <5 cm^2	Phytoplankton	5 ml/sponge per day	7 weeks (ongoing)	No supplements	15°C	35 psu	0,89 m/s	1/3 of water every 15 days
	C4	area <5 cm^3	Phytoplankton	5 ml/sponge per day	4 weeks (ongoing)	No supplements	15°C	35 psu	1,5 m/s	1/3 of water every 15 days
	A3	area <5 cm^4	Phytoplankton	5 ml/sponge per day	4 weeks (ongoing)	No supplements	15°C	35 psu	0,89 m/s	1/3 of water every 15 days

3.6.1. First trial

During our first trial, we tried to assess the ideal sponge size that can grow easier in aquarium condition. In order to do this, we choose small specimens (surface < 5 cm^2) of sponges and bigger one (surface > 30 cm^2) that were fed differently. To do this, we set two parallel experiments: Trial I a (small sponges) and Trial I b (big sponges).

We administrated 5 ml of phytoplankton per sponge once a day. Temperature was fixed at 15°C and the salinity was 35 psu. No supplements were administrated during this trial.

3.6.2. Second trial

For our second trial we decided to test if the temperature can affect the growth of the sponges. Temperature was fixed respectively at 15°C (Trial II a) and 7°C (Trial II b).

We administrated 5 ml of phytoplankton per sponge once a day and we didn't add any type of supplements at the specimens. This trial was characterized by a high hydrodynamics due to the water pump of the aquarium.

3.6.3. Third trial

During the third trial we tested if the sponges could resist with a reduction in the quantity of food. To do this, we administrated 5 ml of phytoplankton per sponge once a week. We tested this at two different temperature: 15°C (Trial III a) and 7°C (Trial III b).

3.6.4. Fourth trial

In the fourth trial we set two parallel experiments to compare the addition of ascorbic acid at two different temperature: 15°C (Trial III a) and 7°C (Trial III b). In fact, as well as the phytoplankton, we weighed 0,025 g of Vitamin C to administrate at each sponge. Ascorbic acid was added to the mixture of phytoplankton and administrated at the same time.

3.6.5. Fifth trial

In our fifth trial we tested if a supplement of sediments can help the growth of the sponges. Sediments were positioned on the ectosome of the sponge and relocated when we noticed that they were taken away by the water current.

3.7. Determination of food concentration

In order to quantify the amount of food supplied to the sponges we used the Hemocytometer (Neubauer's chamber). This technique is used to count cells or other particles in suspensions under a microscope and remains the most common method used for cell counting around the world.

Neubauer's chamber is a thick glass plate with the size of a glass slide (30x70x4mm). The counting region consists of two square shaped ruled areas. There are depressions on the moats on either side or in between the areas on which the squares are marked thus giving an "H" shape.

The ruled area is divided into 9 large squares each with a 1 mm^2 area. The large central square is divided into 25 medium squares with double or triple lines.

Each of these 25 squares are again divided into 16 small squares with single lines, so that each of the smallest squares has an area of 0,0025 mm^2.

The glass cover is a squared glass of width 22 mm and is placed on the top of the Neubauer's chamber, covering the central area. The ruled area is 0.2 mm lower than the rest of the chamber. So that when a cover slip is kept on the counting region, there is a gap of 0.2 mm between the cover slip and the ruled area.

For our sample we diluted 1 ml of our commercial marine phytoplankton in 100 ml of distilled water and with a pipette we withdraw a small amount of

mixture and place it against the edge of the cover glass and slowly expel the liquid until the counting chamber is full.

We placed the Neubauer chamber on the microscope stage and we used the 20X objective to count every phytoplankton cell in five random small squares. Once counted every cell and obtained the mean value we applied the formula to calculate the concentration of cells (number of cells per ml).

$$Concentration\ \left(\frac{cells}{ml}\right) in\ the\ initial\ volume = \frac{mean\ of\ counted\ cells\ \times dilution\ factor}{volume\ of\ a\ small\ quadrat}$$

This result will be helpful in the quantification of the food given to the sponges and therefore to standardize it for future studies.

3.8. Determination of sponge size growth

The size of the sponges was measured periodically, following the acclimatization period. The sponges were photographed from above and with a ruler for scale (*Figure 3.4*) using a camera equipped with an underwater cage.

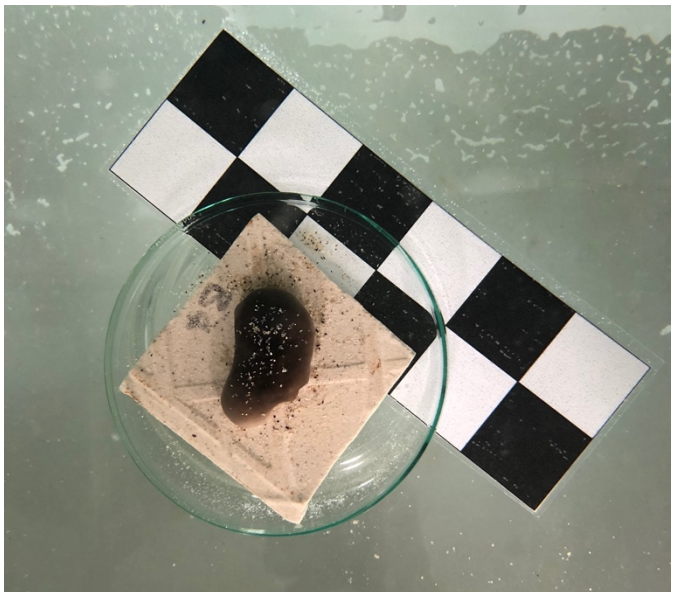

Figure 2.4 - Example of the 2-dimensional method for the measure of the surface.

The sponge surface area (SSA) was measured from the projected surface area based on a picture taken from the top using the ImageJ software. After regular period of time, we repeated the photos to be able to compare them through the time.

ImageJ is a software that allow us to measure the surface of the sponge using a known distance as reference. So, every photo had to include a reference scale in order to provide a known distance that we can use to set up the software and calculate the surface of the sponges.

As a result, we obtained the surface of the top of the sponge and this value can be compared with older ones over time.

Growth was expressed as the increase in the surface area calculated with the software ImageJ. The growth in percentage increase was calculated from initial (at start of the time point) and final surface areas (A) as follows:

$$Growth\ (\%) = (A_{final} - A_{initial})/A_{inital})\ x\ 100$$

Once calculated the total percentage growth, in order to standardize it, we divided the result for the weeks of survival and obtained the percentage growth per week.

$$Weekly\ growth\ (\%) = \frac{Total\ growth\ (\%)}{weeks\ of\ survival}$$

In addition to this 2-dimensional methodology, to analyze more precisely the growth of *C. reniformis* in aquarium condition we used 3D photogrammetry in order to visually assess the variation in dimension of the species.

Recently, 3D photogrammetry is becoming more and more used, in fact it allowed us to have a more precise value of the volume of the sponge and thanks

to software that can align different 3D models we can compare them and see where the sponge has grown more and where it has grown less.

3D photogrammetry represents a non-destructive, cost-effective tool for a wide field of research, especially coral reef monitoring (Prado et al., 2019).

This technology involves the reconstruction of a real-scale three-dimensional (3D) model of a given object or scenario from a series of overlapping photographs, taken from multiple perspectives. This method only requires a consumer-grade digital camera and basic training to ensure overlap of photos (McCarthy & Benjamin, 2014).

We realized the photos thanks to a GoPro Hero 4; every individual was recorded in a video from every point of view and once we had extrapolated the photos from the videos (approximately 100-150 photos), we used the software Agisoft Metashape Professional to analyze them and recreate the 3D models. It's important that the distance between the camera and the object (sponge) is maintained as much constant as possible (between 10 and 15 cm) and that we include a reference near the subject of study which can be easily measured in order to calibrate the model once it is done.

Agisoft Metashape is a paid software very effective, that is able to produce high quality models thanks to a very simple and intuitive workflow articulated in 4 phases:

1. Photo Alignment: in this phase, the uploaded photos are processed by the program, which determine their position and orientation. In every image are defined the *Tie points*, that are characteristic points clearly identifiable in at least two photos. Subsequentially, the alignment continues with the confrontation of different images and with the research of the tie points in the several photos. The nearest are the photos, the more tie point they have in common, so is very important that photos have a good percentage of overlapping. Once finished the alignment, the software creates the sparse point cloud (*Figure 3.5-1*).
2. Build Dense Point Cloud: on the basis of the sparse point cloud the software calculates the depth maps, that contained information about the distance between the points compared to the point of view, and so a dataset of points in space (*Figure 3.5-2*). During this phase the software can also evaluate the level of confidence of the model, with a scale of colors that represent how precise the dense cloud is.
3. Build Mesh: the points of the dense cloud are used as vertices for the creation of triangles. Together, the triangles form the tridimensional polygonal mesh (Mesh), which represent the reconstruction of the surface of the object (*Figure 3.5-3*).

4. Build Texture: in this phase the software fills the triangles of the mesh with pixel which fall in that area, captured by the photos with the points of view which look in that direction (*Figure 3.5-4*).

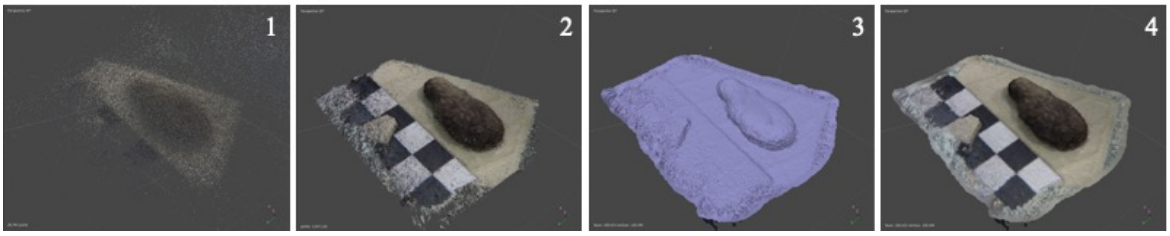

Figure 3.5 - Scheme of the process for the reconstruction of a 3D model of a sponge. (1) Points alignment, (2)dense point cloud, (3)mesh and (4) texture.

In order to have a reliable reconstruction, the steps previously descripted were elaborated using the importations at the highest quality.

We tried to repeat this workflow for every sponge once a week in order to obtain different 3D models to compare through time.

Once created the models we exported them in another software called Recap photo. This software allows us not only to calibrate them, but also to overlap different models and compare them. In this way, overlapping two different models of the same sponge created in subsequent time we can highlight difference in the growth of the species. We can visualize thanks to different colors the areas where the sponge has grown and the areas where has regressed.

3.9. Copepods

During our first trial we found several specimens of copepods on the surface of a sponge. In order to identify them, individuals of this small crustacea were fixed in glutaraldehyde 2.5% (pH 7.8) and dehydrated with a graded ethanol series following the protocol to analyze them at the SEM (Scanning Electron Microscopy).

3.10. Power consumption of the system

In order to assess the environmental impact of the system and its cost, we tried to calculate its power consumption and see if this could be considered a good method for the rearing of *Chondrosia reniformis* and could be increased on a larger scale. To do this, we calculated the consumption of electricity from the watt of the electrical device and to have an idea of the cost of the system we multiplicated the total kWh per day for the mean cost of a kWh which is 0,0625 €/kWh (https://www.arera.it). To obtain a correct estimation of the actual cost of the system we need to include not only all the electrical device of the aquariums (pump, skimmer, refrigerator, aerator, UV lamp and the refill pump) but also the supplements that we add, such as food (phytoplankton) and Artificial Sea Water.

In addition to this we esteemed the CO_2 production of the system thanks to the conversion factor which is 0,256 kg/kWh (Mas et al., 2015).

The results of this estimation are to be related to an aquarium system of about 170-200 liter that can support approximately 10-12 small specimens of *Chondrosia reniformis.*

4. Results

4.1. Collection, transport and placement in the aquariums

Collection and transport of sponges are critical parts in obtaining material for cultivation experiments. *C. reniformis* is an encrusting species and this can cause difficulties in the collection phase so it must be done very carefully. Even if *C. reniformis* is well known for its robustness, specimens damaged during sampling and transport showed signs of necrosis. In some cases, after the removal of the necrotic part, they showed a good recovery and the healing of the tissue.

Regarding the methods of attachment on the substrate, we noticed that the nylon thread seemed to have two different effects on the sponges. For the majority of them, the thread was incorporated during the growth and didn't seem to create any problem at the sponge. In some cases, especially for the smaller individuals, the compression caused by the thread leaded to the division of the sponge in two different parts, that created two different individuals.

In a second time, with the application of the glue, we deleted these risks, and we didn't observe any negative effect on the sponges. By the way, some of the

sponges fixed with the glue feel out from the substrate and needed more time for the total attachment.

4.2. Physical and chemical conditions in the aquariums

Conditions in the aquariums resulted to be optimal for the sponges even if we observed some sign of stress in some individuals after the establishment in the tanks. In fact, after few days the sponges showed some signs of sufferance, we found a gelatinous film is some areas of the sponges. Thanks to a microscopical investigation we found out that was a microbial biofilm composed by ciliates and prokaryotes. In our case was probably due to the low hydrodynamics and the position on the bottom of the tanks. Once removed the microbial biofilm and raised the sponges with supports, they show a good recovery rate and none of them presented the biofilm in the following days. Probably, the upper position and the closeness to the current have favored the removal of the biofilm and prevented the reformation of it.

Hydrodynamics resulted to be essential for the sponge. Thanks to the current the surface of the sponge can be cleaned from stress factor such as bacterial biofilm, algae, sediments and copepods that during our study have been a crucial role and caused the death of some specimens of *C. reniformis*. In addition to this, if the current inside the tanks is low, it can cause the stagnation

of the water inside the tanks, that in turn can lead to the proliferation of bacteria and the alteration of chemical parameters, for example the quantity of nitrate or the pH of the tanks.

At first, we set the temperature of one battery of aquariums at 15°C and another one at 7°C in order to verify any difference or preference for the sponges. Both the systems seemed to be good for the sponge even if the specimens in the tanks with a lower temperature showed a lower growth and a lower rate of mortality. Salinity was fixed at 35 psu, similar to the salinity of the area of study (Passetto beach, Ancona) because this can cause less stress at the sponges after the transport in the laboratory and the placement in the aquariums.

Another important value that was controlled in the aquariums was pH, that resulted to be 8. This value is similar to the one that we can find in the area of study (https://www.arpa.marche.it).

Thanks to Nitrat-Test we kept under control the concentration of nitrates in the water. As expected, with the increase of food administration we noticed a consequent increase in the nitrates inside the aquariums. This has involved more frequent changes of water, to avoid stress for the sponges. Nevertheless, this sponge seemed to tolerate quite good a high concentration of nitrates (> 25 mg/l).

All these parameters turn out to be suitable for the rearing of *Chondrosia reniformis*.

4.3. Substrate preference and locomotion

One of our aims was to verify if *Chondrosia reniformis* has some preference regarding the substrate to which it is attached.

After about 2 weeks we noticed the first signs of attachment in the sponges fixed on the substrate thanks to the nylon thread, especially for the ones positioned on the porcelain stoneware. Same results were obtained for sponges attached with the glue.

As reported by Burton (1949) and Bond & Harris (1988) *Chondrosia* is capable of active locomotion across solid substrate. We observed the same capacity as some specimens of this sponge seemed to move over the substrate where we attach them. Especially one sponge, attached on the PVC. Another sponge where we observed locomotion was specimen A3 (*Figure 4.1*). This sponge was cut in a portion due to a necrotic area, and after that we observed a good healing and a change in the shape as the specimen that was trying to elongate itself until it started to grow along the edge of the substrate (*Figure 4.1-F*).

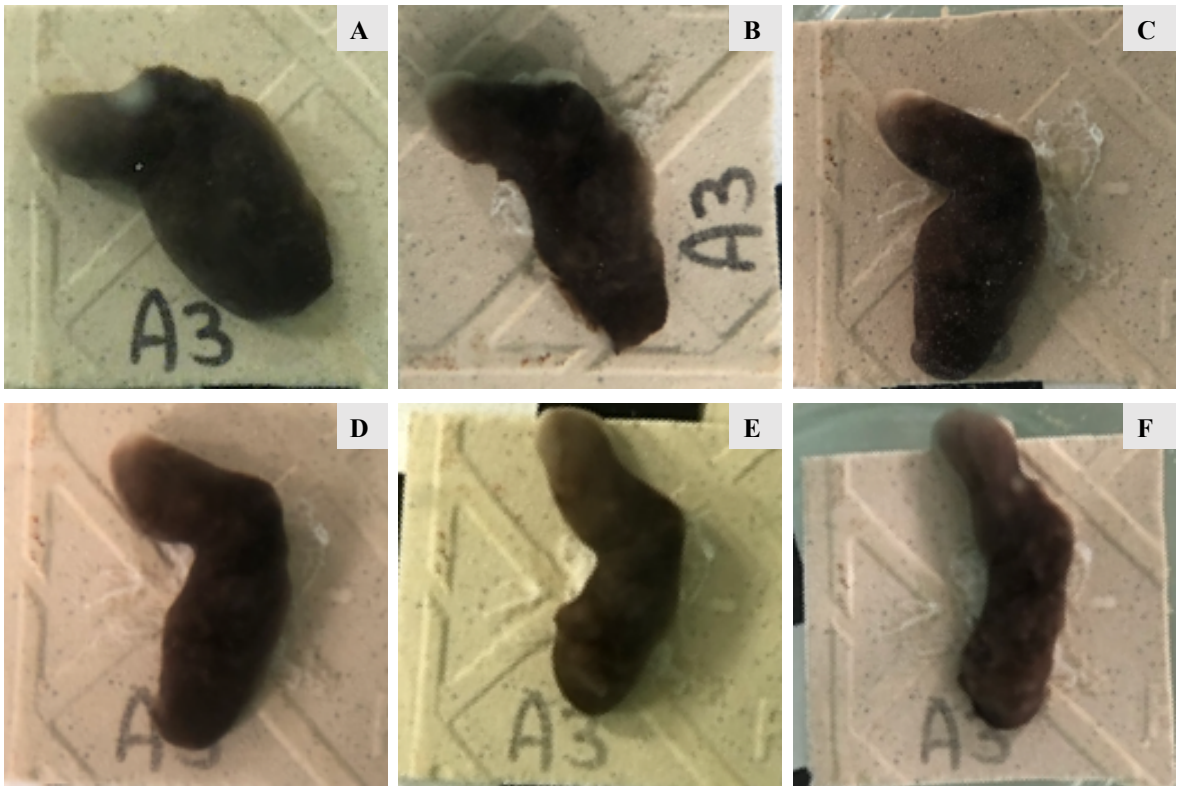

Figure 4.1 - Photos of specimen A3 photographed trough the weeks. (A) Sponge before the cut; (B) sponge after the cut; (C) sponge after one week; (D) sponge after two weeks; (E) sponge after three weeks; (F) sponge after four weeks, we can notice that the sponge is starting to grow on the edge of the substrate.

An important observation was that in aquariums with a lower temperature the sponges seemed to attach to the substrate with a greater difficulty. Nevertheless, we didn't have any consequences on the growth of the sponge, that was present even on the specimens that weren't sticked to the substrate.

During our first trial we noticed the formation of long, attenuated outgrowths which extend from the parental body. The filaments were attached to the substrate in correspondence of the glue.

4.4. Feeding methodology

During our study we tried to obtain the perfect diet for *Chondrosia reniformis*, administrating different concentration of commercial phytoplankton.

We noticed that *C. reniformis* doesn't need food every day and is capable of resists even for weeks with a reduced supply of food, which by the way led to a decrease in the sponge size.

Commercial phytoplankton turn out to be a good and economic source of food and it's easy to administrate to the sponges.

During the pre-trial we started with a small amount of food (2,5 ml of diluted phytoplankton per sponge) and we noticed that, increasing the quantity of it, the phytoplankton went to burden on the quality of the water and seem to promote the proliferation of copepods that could be seen on the surface of the sponges especially during the feeding time. So, we set the dosage under the critical point, that turned out to be over 5 ml/sponge per day.

The only administration of phytoplankton brought to the growth of nearly all the specimens, even if some species showed fluctuation that can be due to the plasticity and the ability of this sponge to change its body shape and expand it to grow.

The use of plastic bells and syringe resulted to be a good methodology in order to standardize and decrease the loss of mixture.

Regarding the addition of supplements to the diet, we obtained good results from the ascorbic acid and sediments, while for the protein source we interrupted the administration after two weeks because the sponge treated with it had become of a darker color with an unhealthy-looking.

4.5. Determination of food concentration

As a result of the count of the phytoplankton, we obtained that the concentration of the commercial marine gel was $4{,}7x10^{10}$ cells/ml. Since we didn't administrate the pure phytoplankton, but we diluted it, it was important to know the concentration in our mixture. To obtain this, we need to divide the results by the dilution factor (101). This meant that in one ml of our diluted mixture we had approximately $4{,}6x10^{8}$ cells.

4.6. Sponge growth measured with 2D approach

During all our trials we observed a fluctuation of the sponges' sizes through the weeks. A 2-dimensional approach turned out to be simple and easy to replicate even if can give us only an approximation of the sponge biomass.

Below, the results of the measurements for every trial done during this study.

4.6.1. First trial

Table 4.1 - Trial I.

		ID Sponge	Initial Area (cm2)	Final Area (cm2)	Weeks of survival	Total Growth %	Growth/week %
I Size	**Trial I a <5 cm2 (15°C)**	E3	4,105	10,128	6	147%	29%
		F3	3,477	7,026	6	102%	20%
		D1	4,143	7,279	6	76%	15%
		F1	4,964	8,543	6	72%	14%
		D3	4,842	7,373	6	52%	10%
		A2	4,811	5,128	6	7%	2%
	Trial I b >30 cm2 (15°C)	**ID Sponge**	**Initial Area (cm2)**	**Final Area (cm2)**	**Weeks of survival**	**Total Growth %**	**Growth/week %**
		B1	83,766	89,014	4	6%	2%
		A1	36,744	31,361	4	-15%	-5%
		C2	40,946	30	4	-27%	-9%

During the first trial (*Table 4.1*) we tested the growth of the sponges belonging to different size classes: < 5cm^2 and > 30 cm^2. We observed an overall growth of the smaller specimens while the bigger ones seemed to decrease in size. Although, we noticed a fluctuation during the measurements of the sizes of the sponges. Unfortunately, during this experiment we encountered an obstacle that caused the death of all the sponge after six weeks from the beginning of this trial. In fact, several individual of copepods were found on the surface of one sponge.

In the trial I a the mean percentage growth per week resulted to be 14% with a standard deviation of 8%. While for the trial I b we had a lower mean percentage growth per week (-4%) with a standard deviation of 6%.

4.6.2. Second trial

Table 4.2 - Trial II.

		ID Sponge	Initial Area (cm2)	Final Area (cm2)	Weeks of survival	Total Growth %	Growth/week %
II Temperature/ High food conc.	**Trial II a T=15°C**	A12	3,178	5,634	3	77%	26%
		A7	5,95	10,495	3	76%	25%
		A5	3,344	5,857	6	75%	13%
		A11	2,36	3,458	3	47%	16%
		A9 a	5,341	7,16	2	34%	17%
		A10	16,538	24,81	6	50%	8%
		A8	3,244	5,252	6	62%	10%
		A3	8,294	10,304	3	24%	8%
		A13	3,292	4,623	6	40%	7%
		A14	16,88	20,188	5	20%	3%
		A15	2,159	2,176	6	1%	0%
		A9 b	7,863	7,889	5	0%	0%
		A6	3,886	3,689	6	-5%	-1%
	Trial II b T=7°C	**ID Sponge**	**Initial Area (cm2)**	**Final Area (cm2)**	**Weeks of survival**	**Total Growth %**	**Growth/week %**
		A9 b	7,889	7,245	6	-8%	-1%
		A6	3,689	3,322	6	-10%	-2%
		A13	4,623	4,419	6	-4%	-1%
		A10	24,81	22,347	5	-10%	-2%
		A5	5,857	5,043	6	-14%	-2%
		A8	5,252	4,295	6	-18%	-3%
		A15	2,176	0,963	6	-56%	-9%

In this second trial (*Table 4.2*) we kept constant the amount of food (5 ml/sponge per day) and fixed the water at two different temperature. While for the first trial we had the issue with the copepods, during the second trial we didn't detect the presence of this small crustacea. The higher hydrodynamics seemed to guarantee a better health of the specimens and a good recovery for the once with some damages.

Sponges kept at 15 °C (Trial II a) showed a higher growth compared to the ones kept at 7°C. The mean percentage growth of the Trial II a was 10% with a

standard deviation of 9%, while for the Trial II b we had a mean percentage growth of -3% with a standard deviation of 3%.

During Trial II a we had a good survival rate of the specimens, in fact the differences in the weeks of survival are due to the relocation of some sponges for other trials or experiments and just one died (Sponge A9 a). In fact, the missing data in the table are due to the relocation of some individuals of *C. reniformis*.

Another observation is that we can see a higher growth during the first three weeks and a stabilization of the body size in the following weeks.

4.6.3. Third trial

Table 4.3 - Trial III.

III Temperature/ Low food conc.	**Trial III a T=15°C**	**ID Sponge**	**Initial Area (cm2)**	**Final Area (cm2)**	**Weeks of survival**	**Total Growth %**	**Growth/week %**
		B4	6,162	7,000	3	14%	5%
		B7	3,708	3,900	3	5%	2%
		A3	7,564	7,124	3	-6%	-2%
		B9	8,844	8,275	3	-6%	-2%
	Trial III b T=7°C	**ID Sponge**	**Initial Area (cm2)**	**Final Area (cm2)**	**Weeks of survival**	**Total Growth %**	**Growth/week %**
		C7	8,160	6,900	3	-15%	-5%
		C5	8,103	6,821	3	-16%	-5%
		C4	4,585	3,772	3	-18%	-6%
		C8	5,208	3,990	3	-23%	-8%
		C6	6,031	4,340	3	-28%	-9%

In the third trial (*Table 4.3*) we tested the resistance of the sponge with a low food concentration. All sponges survived three weeks with this diet, but the majority of them showed a decrease in the size.

In trial III a we had a mean percentage growth of 1% with a standard deviation of 3%, while in trial III b we obtained a mean percentage growth of -7% with a standard deviation of 2%.

While all the sponges kept at 7°C showed a reduction in the surface area, in the sponges kept at 15°C we had the growth of two sponges out of the four.

4.6.4. Fourth trial

Table 4.4 - Trial IV.

IV Temperature/ AA	Trial IV a T=15°C	ID Sponge	Initial Area (cm2)	Final Area (cm2)	Weeks of survival	Total Growth %	Growth/week %
		B4	5,883	4,912	2	-17%	-8%
		A4 a	1,664	1,545	4	-7%	-2%
		A4 b	2,255	-	1	0%	0%
		E4	7,555	6,292	8	-17%	-2%
		A9	5,512	-	1	0%	0%
		F4	1,959	2,627	9	34%	4%
	Trial IV b T=7 °C	**ID Sponge**	**Initial Area (cm2)**	**Final Area (cm2)**	**Weeks of survival**	**Total Growth %**	**Growth/week %**
		C7	6,900	8,232	4	19%	5%
		C8	3,990	-	1	0%	0%
		C6	4,340	5,123	2	18%	9%
		C5	6,821	7,151	4	5%	1%

During the fourth trial (*Table 4.4*) we tested the addition of ascorbic acid at different temperature. We noticed a slight decrease in trial IV a (15°C), in fact

the mean growth resulted to be -1% with a standard deviation of 4%. In trial IV b (7°C) we had a mean percentage growth of 4% with a standard deviation of 4%.

4.6.5. Fifth trial

Table 4.5 - Trial V.

V Sediments	Trial V T=15°C	ID Sponge	Initial Area (cm2)	Final Area (cm2)	Weeks of survival	Total Growth %	Growth/week %
		B4	7,000	7,899	5	13%	3%
		B9	8,275	9,976	5	21%	4%
		B7	3,900	4,373	5	12%	2%

In the fifth trial (*Table 4.5*) we added sediments at the normal diet (phytoplankton), and we obtained the growth of all the sponges. The mean percentage growth resulted to be 3% with a standard deviation of 1%. During this trial the health of the sponges seemed to be very good and we are continuing to apply this diet because sponges are all still alive.

We didn't observe the incorporation of the sediments, however we noticed that the grains of sediments were adhered to the surface of the sponges and weren't taken away from the current.

4.6.6. Control

Table 4.6 - Control.

Control Trial IV,V T=15°C	ID Sponge	Initial Area (cm2)	Final Area (cm2)	Weeks of survival	Total Growth %	Growth/week %
	D4	7,533	8,136	8	8%	1%
	C4	3,772	3,482	4	-8%	-2%
	A3	7,124	8,136	4	14%	4%

Control sponges for trials IV and V were fed with phytoplankton only and obtained a percentage growth per week of 1% with a standard deviation of 3%.

4.7. Trials comparison from 2D area measurements

In the *Figure 4.2* is reported a summary of the several trials done during this study. We can see that the first trial obtained the best results, with a mean percentage growth per week of 14% with a standard deviation of 8%. Instead, the worst trial was the III b where we administrated a reduced quantity of food (once a week) at 7°C. The majority of the trials conducted at 7°C led to a decrease of the sponges' sizes, except for Trial IV b, where we added ascorbic acid to the diet.

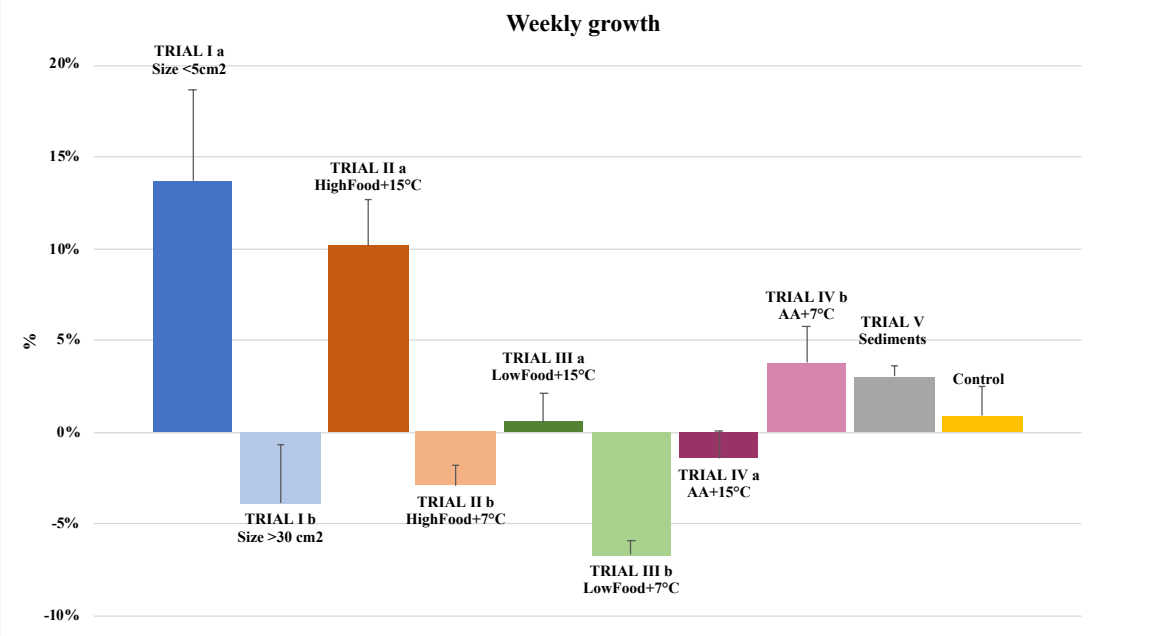

Figure 4.2 - Summary graph of the mean percentage growth per week of our trials.

4.8. Comparison of 2D and 3D measurements

Thanks to the application of two methods for the measurements of the surfaces and volumes of the sponges it was possible to compare the results in term of weekly percentage growth regarding the area measured by 2D approach, the volume measured by photogrammetry and the surface also measured through this approach. These comparisons weren't possible for the first trial, where photogrammetry was not yet started. Furthermore, photogrammetry wasn't applicable to some sponges due to their death before the measurements.

4.8.1. Second trial comparison

Table 4.7 - Trial II.

		ID Sponge	Area(2D)_WeeklyRate	Vol(3D)_WeeklyRate	Area(3D)_WeeklyRate
II Temperature /High food conc.	**Trial II a T=15°C**	A12	25,8%	-	-
		A7	25,5%	-	-
		A5	12,5%	-6,2%	0,0%
		A11	15,5%	-	-
		A9 a	17,0%	-	-
		A10	8,3%	-0,2%	-4,1%
		A8	10,3%	-8,5%	-6,0%
		A3	8,1%		-
		A13	6,7%	0,3%	-4,4%
		A14	3,3%		-
		A15	0,1%	-5,7%	-8,0%
		A9 b	0,1%	14,1%	-1,4%
		A6	-0,8%	-0,8%	-3,1%
	Trial II b T=7°C	**ID Sponge**	**Area(2D)_WeeklyRate**	**Vol(3D)_WeeklyRate**	**Area(3D)_WeeklyRate**
		A9 b	-1,4%	13,0%	-2,9%
		A6	-1,7%	20,0%	-2,5%
		A13	-0,7%	7,0%	-4,4%
		A10	-2,0%	5,0%	-3,3%
		A5	-2,3%	-6,0%	0,0%
		A8	-3,0%	6,0%	-2,0%
		A15	-9,3%	-3,0%	-4,0%

4.8.2. Third trial comparison

Table 4.8 - Trial III.

		ID Sponge	Area(2D)_WeeklyRate	Vol(3D)_WeeklyRate	Area(3D)_WeeklyRate
III Temperature /Low food conc.	**Trial III a T=15°C**	B4	4,5%	16,7%	2,4%
		A3	-2,0%	12,1%	-2,4%
		B7	1,7%	-0,3%	3,7%
		B9	-2,1%	12,2%	-1,8%
	Trial III b T=7°C	**ID Sponge**	**Area(2D)_WeeklyRate**	**Vol(3D)_WeeklyRate**	**Area(3D)_WeeklyRate**
		C7	-5,1%	-10,6%	-3,9%
		C5	-5,3%	-16,2%	-5,9%
		C4	-5,9%	2,4%	-6,7%
		C8	-7,8%	6,2%	-4,8%
		C6	-9,3%	-0,2%	-5,1%

4.8.3. Fourth trial comparison

Table 4.9 - Trial IV.

		ID Sponge	Area(2D)_WeeklyRate	Vol(3D)_WeeklyRate	Area(3D)_WeeklyRate
IV Temperature /AA	**Trial IV a T=15°C**	B4	-8,3%	-	-
		A4 a	-1,8%	-7,8%	-4,2%
		A4 b	0,0%	-	-
		E4	-2,1%	-0,6%	-6,2%
		A9	0,0%	-	-
		F4	3,8%	-8,3%	0,0%
		ID Sponge	**Area(2D)_WeeklyRate**	**Vol(3D)_WeeklyRate**	**Area(3D)_WeeklyRate**
	Trial IV b T=7 °C	C7	4,8%	23,8%	4,4%
		B8	0,0%	-	-
		C1	9,0%	2,0%	0,0%
		C5	1,2%	24,5%	4,8%

4.8.4. Fifth trial comparison

Table 4.10 - Trial V.

		ID Sponge	Area(2D)_WeeklyRate	Vol(3D)_WeeklyRate	Area(3D)_WeeklyRate
V Sediments	**Trial V T=15°C**	B4	0,8%	-5,3%	-4,4%
		B9	1,7%	-11,6%	1,9%
		B7	2,9%	8,1%	0,0%

4.8.5. Control comparison

Table 4.11 - Control.

	ID Sponge	Area(2D)_WeeklyRate	Vol(3D)_WeeklyRate	Area(3D)_WeeklyRate
Control	D4	1,0%	-3,2%	-5,6%
	C4	-1,9%	-2,4%	25,0%
	A3	3,6%	28,8%	2,6%

4.8.6. Graph of the areas/volume comparison

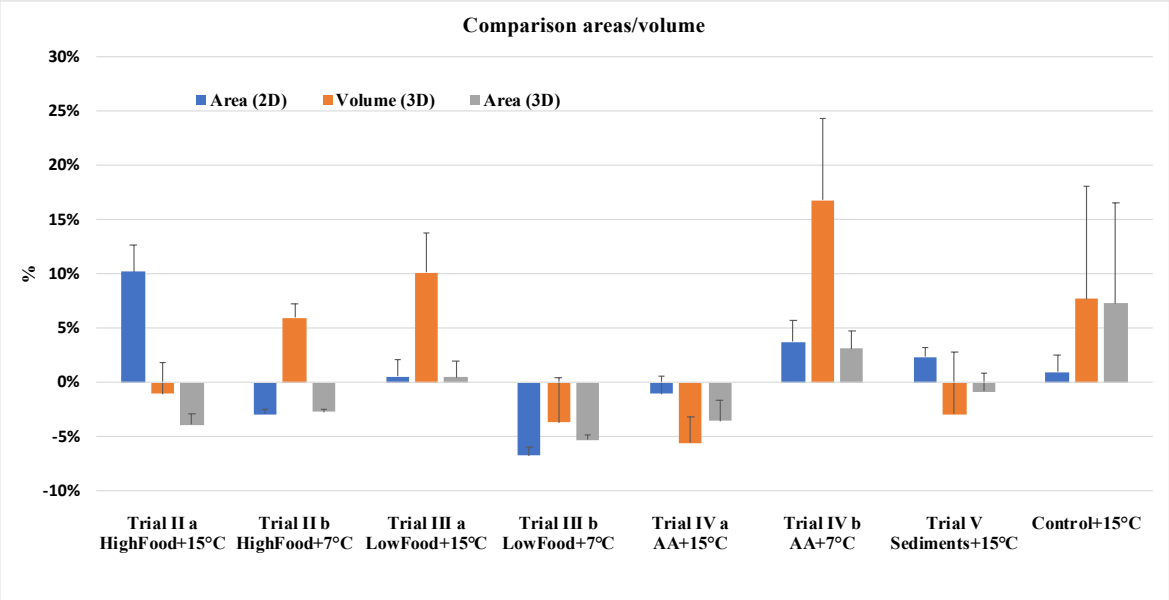

Figure 4.3 - Graph of comparison between the mean percentage growth per week measured using the 2D and 3D approach (photogrammetry).

In *Figure 4.3* is reported the graph of comparison between areas and volume that summarize *Tables 4.7-4.11*. We can observe several discrepancies in the data regarding the mean percentage growth per week measured with the 2D approach and the 3D approach. For example, in Trial II we can see a reverse trend for the area calculated with 2D and the area and volume calculated with the 3D photogrammetry. Instead, for trials III b and IV a we obtained similar results. If we compare only the areas calculated with the two methods, we notice a higher similarity on the results, which are always in agreement, except for trials II a and V. Areas calculated with 3D photogrammetry resulted to be higher than the ones calculated in 2D, because this techniques can measure all

the surface of the sponge. Nevertheless, this doesn't affect the mean percentage growth per week, that take into account the variations over time.

4.9. Comparison of the models

Thanks to the program Recap Pro, we were able to overlap models of the same sponge created as the weeks pass. In this way, we can visualize how the sponge grows and especially if there are differences in body parts.

Figure 4.4 shows how the sponges change through time, all the comparison were made for models with a time distance of one month.

Figure 4.4-A represents sponge A6 from trial II a (high food, 15°C). This sponge was initially elongated and narrow and we noticed that tended to close on itself. In this figure we notice areas where the sponges grow colored in yellow and orange and areas where decrease in blue. In this case, the sponge decreases more on the upper part of the body. On the contrary, in *Figure 4.4-B* we have sponge A10 always from trial II a, where we notice that the grow is focused on the upper part of it, especially in the more articulated zones. In *Figure 4.4-C* we have a sponge from Trial III b (low food, 7°C) and we notice a reduction on the top of it, while is growing on the edges. In *Figure 4.4-D*, from trial IV a (AA, 15°C), we notice that the sponge is narrowing at the extremities, while it is growing in the central part of the body. *Figure 4.4-E* is

a sponge from trial V (sediments) where we can see a growth area in correspondence of the presence of the grains of sediments administered to the specimen during the trial (see *Figure 5.1* for a comparison). Finally, in *Figure 4.4-F* we have a sponge from the control trial. In this case, on the contrary of sponge E4 (*Figure 4.4-D*), we have the extension of the sponge at the vertices and the contraction in the middle body. These are just some example that make us realize how differently this sponge can grow.

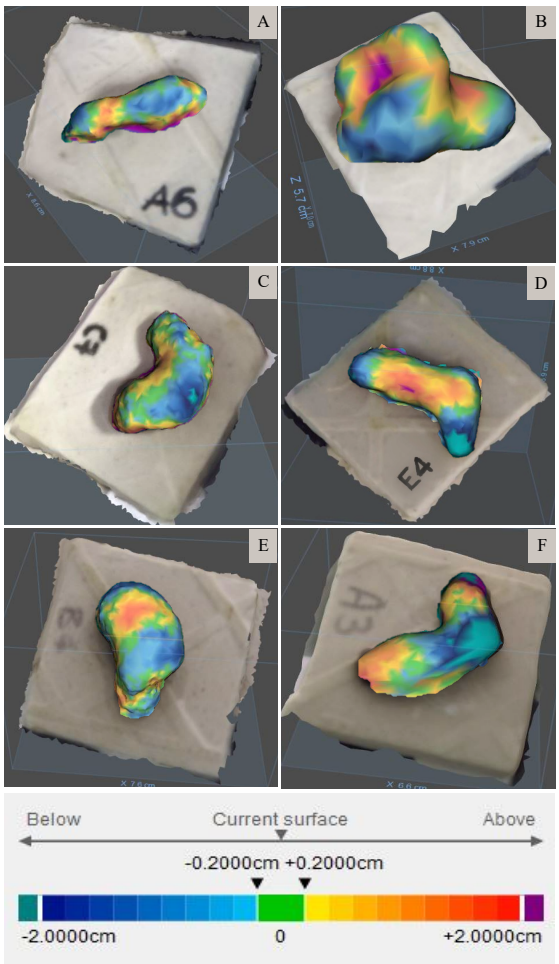

Figure 4.4 - Models comparison after one month of rearing. (A) Sponge A6 from trial II a; (B) sponge A10 from trial II a; (C) sponge C7 from trial III b; (D) sponge E4 from trial IV a; (E) sponge B4 from trial V; (F) sponge A3 from control trial.

4.10. Copepods

After one month from the beginning of the first trial, we found a specimen of *C. reniformis* (IDSponge: C2) covered by copepods (*Figure 4.5*), especially during feeding time. For several days we controlled this cohabitation and we didn't see any type of negative effect on the sponge. For few weeks this was the only specimen with this type of condition, even if there was another sponge in the same aquarium.

After an accurate observation of their behavior under the stereomicroscope, we observed a large number of individuals, both males and females with their ovigerous sac, from all the size classes. An interesting point was the presence of several cysts, with a diameter of 100-125 μm, not only on the substratum, but also on the surface of the sponge, where they were present singularly or in aggregates.

Figure 4.5 - On the left we can see specimens of copepods collected from the surface of the sponge C2 (in the figure on the right).

While at first sight copepods seemed to coexist without any problems for the sponge, after three weeks it starts to show signs of suffering. In particular, we observed that the external layer of the sponge (ectosome) was degraded and the layer of tissue beneath was visible (choanosome). Another observation was the presence on the remaining ectosome of filamentous cyanobacteria, which on the contrary weren't present on healthy sponges. Furthermore, the consistency of the sponge was a lot softer compared to healthy ones. Due to the bad conditions of the individual we decided to remove it from the aquarium even if we noticed that copepods were presents also in the other tanks.

After few days, the other sponges started to show signs of sufferance as the sponge C2: the ectosome began to deteriorate and filamentous cyanobacteria started to appear on the surface of the sponge, even these specimens exhibited a reduction in the strength of the tissues.

In order to decrease the number of copepods and remove them from the tanks we tried different approach. First of all, we changed around 1/3 of the seawater trying to eliminate the majority of the copepods and their cysts. We also tried to remove these crustaceans aspiring them from the surface of the specimens using a small tube. We finally choose to introduce a predator that could eat them without damaging the sponges. We decided to try with *Palaemon elegans*

(Crustacea, Decapoda), that is a very common shrimp easily found in the shallow water of the coast of Ancona (*Figure 4.6*). Once introduce it in the tanks we noticed that during the day it positioned himself over the sponges and seemed eat the copepods, in fact after just one day we observed a reduction in the concentration of copepods over the sponges.

Finally, we decided to remove all the specimens left in the tanks and start a new trial trying to eliminate all the copepods that were still present in the aquariums.

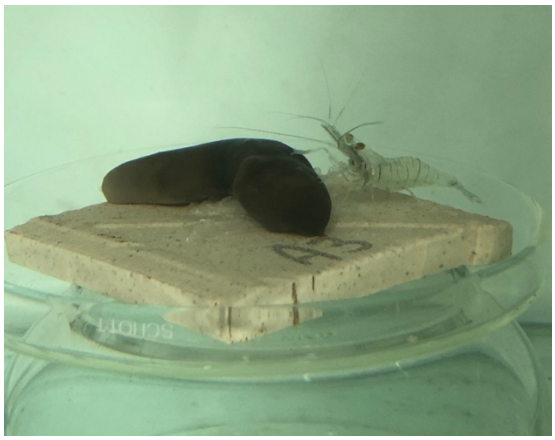

Figure 4.6 - Specimen of Palaemon elegans.

During the analysis at the microscopes, we noticed that the copepods seemed to feed on the surface of the sponge especially in the areas most degraded. Adults were approximately 500-800 μm long and female had only one ovigerous sac carried ventrally. We tried to measure and identify every part of the copepods in order to find information about this species.

From the photos obtained from the SEM examination (*Figure 4.7*), we can observe the details of this copepod, in the *Figure 4.7-A* is present a female with its ovigerous sac and an interesting point is that we can see the naupliar stages present on it.

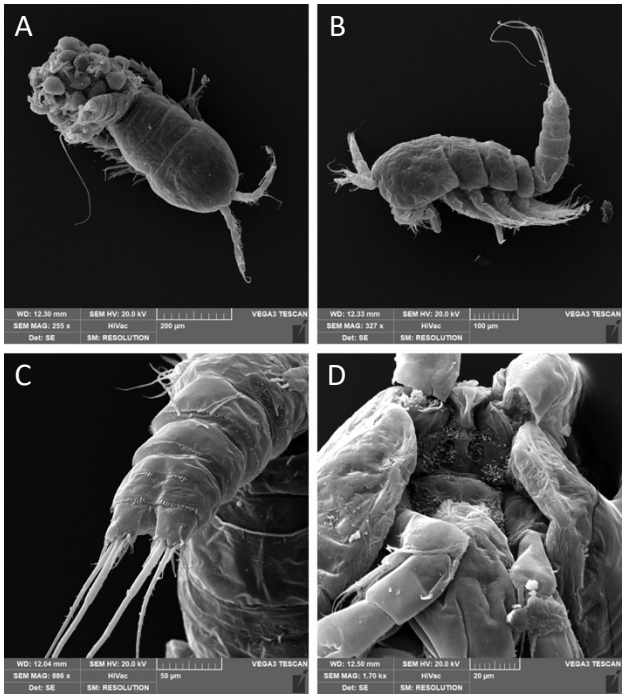

Figure 4.7 - Details of copepods from the SEM examination.

After a detailed analysis of the copepods and a careful comparison of the key characteristics of these individuals, such as antennule, rostrum, pereopods and caudal setae we can assume that they belong all at the same genus, identify as *Tisbe*, most probably of the species *Tisbe cfr. Furcata* (Baird, 1837). This genus belongs to the order of Harpacticoida and the family of Tisbinae.

4.11. Power consumption of the system

After an analysis of the diverse electrical components of the system, we estimated an overall consumption of 4,56 kWh (*Table 4.12*). With this value we can calculate the cost of the system thanks to the mean cost of a kWh (0,0625 €/kWh). In the *Table 4.13* we can see that this system will cost approximately 8,55 €/month. At this value we need to add the other costs, such as the salt for Artificial Sea Water, that in our case costed monthly 16,5 € (considering change of 1/3 of the water every two weeks). For the nourishment (phytoplankton) we estimated a cost of 3,9 €/month, to which must be added the costs of the supplements, if required.

This led to a total cost of about 28,95 €/month.

Table 4.12 - Scheme of the electrical consumption of the system.

	WATT (W)	**HOURS OF USE (H/DAY)**	**KWH/DAY**
PUMP (SELTZ D 6000)	60	24	1,44
SKIMMER (PERFORMER 150/800)	26	24	0,62
REFILL PUMP	4	1	0
REFRIGERATOR	190	12	2,28
AERATOR	2	24	0,05
UV LAMP (EHEIM REEFLEX UV 350)	7	24	0,17
TOTAL KWH/DAY			**4,56**

Table 4.13 - Scheme of the estimation of the system.

Mean cost €/kWh	Total kWh/day	Total day cost (€)	Total monthly cost (€)	Totale annual cost (€)
0,0625	4,56	0,29	8,55	104,03

The production of CO_2 resulted to be 1,17 kg per day. This value was calculated according to the conversion factor in Mas et al. (2015), which differs on the basis of the energetic sources of a considered country. Presuming the use of the system for a whole year we can assume a total production of 427,05 kg of CO_2 during this period of time.

5. Discussion

Overview

The results of this study will help to better understand the requirements and behaviors of *Chondrosia reniformis* in aquarium system.

During our study we did several trials in order to comprehend the necessity of the sponge *Chondrosia reniformis* in rearing conditions. This sponge turned out to be a very robust sponge with a slow growth rate, as supported by other authors (Nickel & Brümmer, 2003), that shows a good recovery rate even if some of them died during the trials.

Chondrosia reniformis can be considered a good choice for aquariums culture because it doesn't require lots of treatments if the conditions in the tanks are optimal for the sponge.

Regarding the creation of biofilm over the specimens, similar results were observed by Nickel & Brümmer, 2003 where *C. reniformis* was covered with a bacterial and fungal biofilm within 48 h after placement in the aquarium, probably for the long transport in small volumes of seawater or insufficient cooling that decreased recovery.

Chondrosia reniformis turned out to be a shape-shifter, its body shape can change quickly (*Figure 5.1*), and this can affect the measurements of the surface and the volume, changing also their relationship. The fluctuation of sponge size encountered during the measurements could be due to the modification and reorganization of the sponge's body, especially for the fragments of sponge or the ones with an articulated body. In addition to this the 2D approach doesn't take into account of the orthotropic growth of *C. reniformis*, that even if it's less extent respect the plagiotropic one, it was observed anyway. This is one of the reasons that inspired us to try with a 3-dimensional approach and use photogrammetry as tool for our measurements.

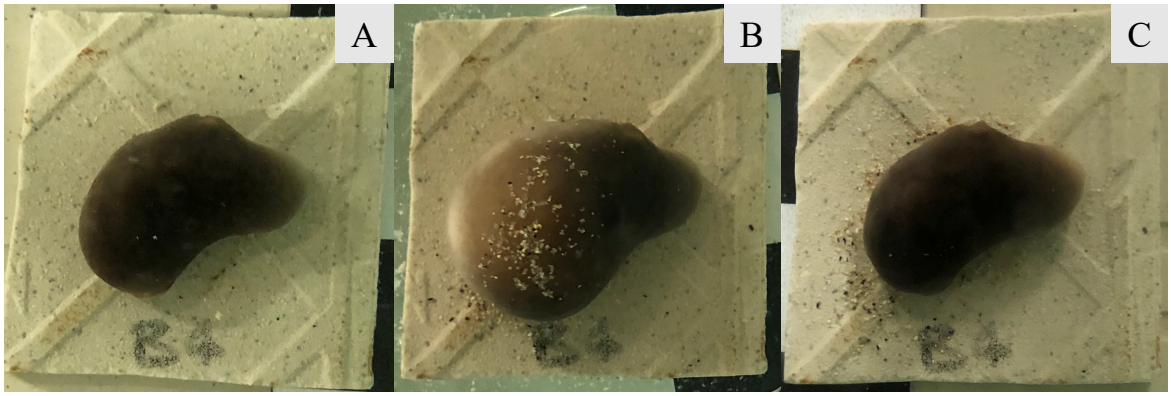

Figure 5.1 - Example of change in body shape of specimen B4 (Trial V). Photos were taken at 48h apart from each other.

Feeding strategy

The quantity of phytoplankton that *C. reniformis* needs to grow was one of our main goal in order to standardize it for future studies. As reported previously

we tried different concentration of food to see how *Chondrosia reniformis* reacts and also how were the conditions of the water. During the pre-trial we noticed that increasing the concentration of phytoplankton involved a decline in the quality of the water.

It's interesting to establish that the feeding strategy used during the trials was suitable for *Chondrosia reniformis*, even though the capacity of the aquariums need to be increased because the parameters of the water (especially nitrates) seemed to deviate from the optimal range. This implied frequent change of water to avoid the damaging of the sponges and a great effort to manage it.

During the third trial we were forced to have a period of starvation for the specimens, where the sponges were fed just once a week. This turned out to be a good test case to see if the sponge can resist without food. It turned out that all the sponges survived three weeks with a reduction in the food supplement, but almost all of them were reduced in size compared to the beginning of the period of starvation, according to 2D measurements. This demonstrates that this sponge, if the parameters inside the aquariums are suitable, doesn't require lots of treatment, as reported also by Nickel et al. (2003).

Growth

The growth of *Chondrosia reniformis* seemed to be faster during the first weeks, with a deceleration as the weeks pass. We noticed a stabilization in the size of the sponges, that can be due to different reasons. We also tried to increase the quantity of food, but this led to a worsening of the parameter of the water.

Regarding the growth of the sponges at different temperature, we obtained better results at higher temperature (15°C), while at 7°C we almost didn't obtain any growth. We can conclude that at higher temperature the sponge can grow faster, probably for the higher metabolic activity.

During the study we had to cut some specimens that presented the formation of necrotic areas. Once removed, nearly all the specimens showed a good recovery and the subsequent increase in size.

With the comparison of 2D and 3D approach we noticed some disagreements between the results, that can be due to different reasons. As seen previously, this sponge is able to change its body shape and volume during the day and this can affect the final result of the measurement. Better results could be obtained over longer periods of time, where we can reduce the error caused by these fluctuations.

With the overlapping of different 3D models, as seen in the results (*Figure 4.4*), we visualized how differently *Chondrosia* can grow. This is absolutely an important result that must be deepened in future studies.

Death of the sponges

During our several trials we had countless deaths that we tried to explain in order to avoid them in future studies. Sometimes this process was very rapid and led to a fast death of the sponge. One of the parameters that can affect the health of the sponge and that we found easily altered in the aquarium is the concentration of nitrates, which increase with the increasing quantity of food administrated to the sponges. This maybe led to a worsening in the conditions of the specimens and required a more frequent water change. As previously seen in the results, during the first trial we ran into the copepods issue, that's probably been aggravated by a suffering condition of the sponges. This led to the death of all the sponges of the first trial.

Sponge's death is a critical point of this study, unfortunately is very difficult to evaluate the causes of the death. These could also depend on the previous conditions of the sponges, that might have been in a suffering state before the placement in the aquariums.

We can conclude that of all the sponges reared during this study, the 63% died for various reasons. This number appears to be significant because we included in the count sponges from pre-trials, when we had not yet optimized our methodology. Regarding the dead specimens, about 46% was attributable to copepods invasion while the other 54% was due to the creation on necrotic areas and deterioration with the presence of cyanobacteria and other bacteria.
In the course of the study, with a better understanding of the requests of this species, we have lowered the incidence of mortality with a more frequent change of water and the right amount of food.

Copepods

The recognition of copepods of the genus *Tisbe* has several difficulties for the lack of information about this taxon (Bergmans, 1981), that contains about 60 described species and occurs worldwide especially in shallow marine waters (Schminke & Pottek, 2001). It is probable that individuals of this harpacticoid copepod were present inside the sponge during the sampling and remained once the sponge was positioned inside the aquarium.

As reported by Marcotte (1984), adults of *Tisbe* are about 500 μm long. They usually live on and above the substrate-sea water interface. Some species live

in muddy habitats. Others are epiphytic and/or epizoic. Many live in estuaries and brackish-water environments. Most live in shallow-water habitats with physically unpredictable, even polluted, environments. Most described species are zoogeographically centered in temperate or subtropical latitudes (Fava & Volkmann, 1975).

Tisbe are raptorial feeders and omnivores, this leads us to believe that they could be the reason of the deterioration of the sponges. Their food is determined by its three-dimensional shape and size. These copepods can glean bacteria and diatoms from spherical floccules of organic debris and clay minerals (Marcotte, 1984). The reproductive potential of *Tisbe* is great (Battaglia, 1970; Muus, 1967) and the presence of many sibling species in *Tisbe* implies that the genus is morphologically conservative. The lack of morphological diversity in *Tisbe* implies that the ecological adaptations, which permit potentially competing sibling species to coexist, must be mediated by something other than morphology (Marcotte, 1984).

During the study conducted in the Great Barrier Reef (Australia) by Hanna et al. (2016), where the focus of the work was to study bioeroding sponges that live in calcium carbonate substrates, in particular *Cliothosa aurivillii* (Lindgren, 1898), they found the same copepods belonging to the genus *Tisbe*.

Opening the erosion chambers during sampling they noticed that the obviously parasitic copepods thus obtained access to the softer and possibly less well chemically defended endolithic tissue of *C. aurivillii*, fed and multiplied, and eventually reversed the healing process by reopening the sponges' newly established ectosome, preventing proper scarring, and finally also allowing the disease to infect the sponge tissue.

Probably, the same happened for our sponge, in fact we noticed that the copepods tended to accumulate under the sponge body and seemed to run away from the excessive light.

Copepods have contributed to the death of the sponges in the first trial that were supposedly already in a state of sufferance. This has played a crucial role in the ending of the first trial that was giving optimal results.

The presence of naupliar stages on the ovigerous sac of a female as seen previously in *Figure 4.6-A* led us to consider the cysts present in the aquariums as a form of resistance cysts, probably formed for the exponential growth which characterizes them. In fact, copepods are r-selected species, also called r-strategist, distinguished by an exponential population growth with a very rapid generational turnover.

Comparison of 2D-3D approach

Thanks to the application of two different techniques for the measurements of the sponges, we now have an idea of the strengths and the weaknesses of both approaches.

The measurements of the sponges' surface with a 2-dimensional approach are undoubtedly easier and faster respect the creation of a 3D model, which by the way contain more information and allow us to have a better understanding of the growth of *Chondrosia reniformis*. In addition to this, during our study we noticed that specimens of this sponge went through a reorganization in the body shape that was not appreciable from a 2D photo. On the contrary, the model reconstructed by the software is able to recreate even the texture of the sponge, which will affect the final measurement. Moreover, the possibility to have both volume and surface of the sponge allow us to compare them and have an idea on how change their relationship with the increase or decrease of the body size.

Regarding the negative aspects of the 3D reconstruction, we initially found challenging the reconstruction of the model because we choose to photograph the subjects from every point of view with a time-lapse function of the camera that didn't allow us to have a reliable reconstruction of it. In a second time we decided to extract the photos from a video that we recorded. We found this method easier and more reliable and the models obtained were all measurable.

Energy consumption

More work needs to be done to optimize controlled environment tank culture systems, since in the end land-based systems present the lowest overall risk, the greatest possibility for controlled production, and the lowest environmental impact when compared with open-sea systems which are all subject to the unpredictable vagaries of nature.

This system resulted to be an economical tool for the development of a sustainable methodology for rearing *Chondrosia reniformis*.

Regarding the production of CO_2, we can estimate an annual emission of 427,05 kg of CO_2, which is comparable to that of household appliance (a fridge or a TV, an air conditioner for domestic use and an electric oven produce approximately 189, 490 and 769 kg of CO_2 per year, respectively) (https://greenstarsproject.org).

What's next?

Once understood the methods for rearing this sponge, we will focus on the extraction of collagen from *Chondrosia reniformis*. Nowadays there are several protocols for the extraction of this protein, which seek to improve the percentage of collagen that can be extracted from this sponge. Thanks to the collaboration with the Alfred Wegener Institute in Germany we now know that

the wet weight of a sponge whose surface is 30 cm^2 can be approximated to 5 g. From this value we can estimate the dry weight that is the 20% of the wet one. Finally, from the dry weight we can say that around 50% of it is the percentage of collagen. This is still a work in progress and these relationships need to be explored more deeply. An important point is that these calculations were made from wild sponges, so it could be interesting to evaluate if the collagen content presents in our reared sponges changes in a positive or negative way. This is one of the key points on which the project will focus in the following months.

Another observation is that, as previously seen, the actual industrial demand of collagen is increased becoming more than 326.000 tons (Silva et al., 2016). Sustainable sources of this protein are in continuous research, based not only on the development of novel processes but also on the use of alternative sources of raw materials, which decrease dependence from fossil fuel resources. In this sense, the sea provides a plentiful resource of potential new products for society including biomaterials (Reis et al., 2015).

Marine sponges are a promising resource, but sponge collagen is not available in large quantities because of the lack of efficient extraction methodologies.

Chondrosia is a sponge with a relatively low growth rate that would require a large scale production in order to sustain, although partly, this request. The risk

is the overexploitation of this species, as already seen for other sponges rich in second metabolites such as *Aplysina spp*. which is now under the protection of the Barcelona Convention (SPA/BD Protocol, Annex II). To date, *Chondrosia* is not protected by any list of conservation but it is not excluded that thanks to its potential applications in future will be needed benchmarks to evaluate its ecological state. One of the positive aspects of our study is that will give the possibility to obtain further sponge cuts to be reintroduced in the natural environment, and therefore have the possibility to extract collagen without depleting the sponge source.

6. Conclusions

It can be generally concluded from these results that *Chondrosia reniformis* can grow in controlled environment aquarium systems. This sponge turned out to be a good choice for this type of study even if these are preliminary results and more in-depth analysis need to be done, especially on the diet's supplements.

Highest growths were obtained during the first trial with no addition of supplements, but we think that with further information we can still improve the protocol for rearing this sponge in a controlled environment.

Both 2D and 3D approach reviled to have strengths and weaknesses that can be attenuated with measurements on longer period of time, due to the fluctuations of *Chondrosia reniformis*.

Undoubtedly there is still much to understand on this sponge, especially on its mortality, which could be reduced with the addiction of antibiotics to the diet. Once improved this, we will focus on the estimation of sponge biomass and the extraction of collagen.

However, we can conclude that our aim to obtain a standardize and replicable method is achieved, and it can be applied for future studies.

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