The Multifunctional Role of Marine Sponges in Multi-trophic Mariculture Systems

M. Mert Gökalp

PROPOSITIONS

1- Innovative applications of integrated multitrophic aquaculture are essential in securing a sustainable supply of seafood.
(this thesis)

2- Application of large-scale sponge culture is a solution to reduce eutrophication of coastal waters.
(this thesis)

3- The functional roles of sponges in marine ecosystems still are highly disregarded.

4- Corporation-funded science turns scientists into white collars.

5- To safeguard the marine environment humankind should fully protect 50% of coastal zones globally and stop polluting and illegal fishing and overharvesting of marine resources.

6- Instead of passively waiting for media attention, scientists should actively promote their work via (social) media, books, podcasts and screen appearances.

Propositions belonging to the thesis, entitled

The multifunctional role of marine sponges in multi-trophic mariculture systems

M. Mert Gokalp

Wageningen, June 11th 2021

The multifunctional role of marine sponges in multi-trophic mariculture systems

Towards sustainable use of sponges in polluted marine waters

M. Mert Gökalp

Thesis

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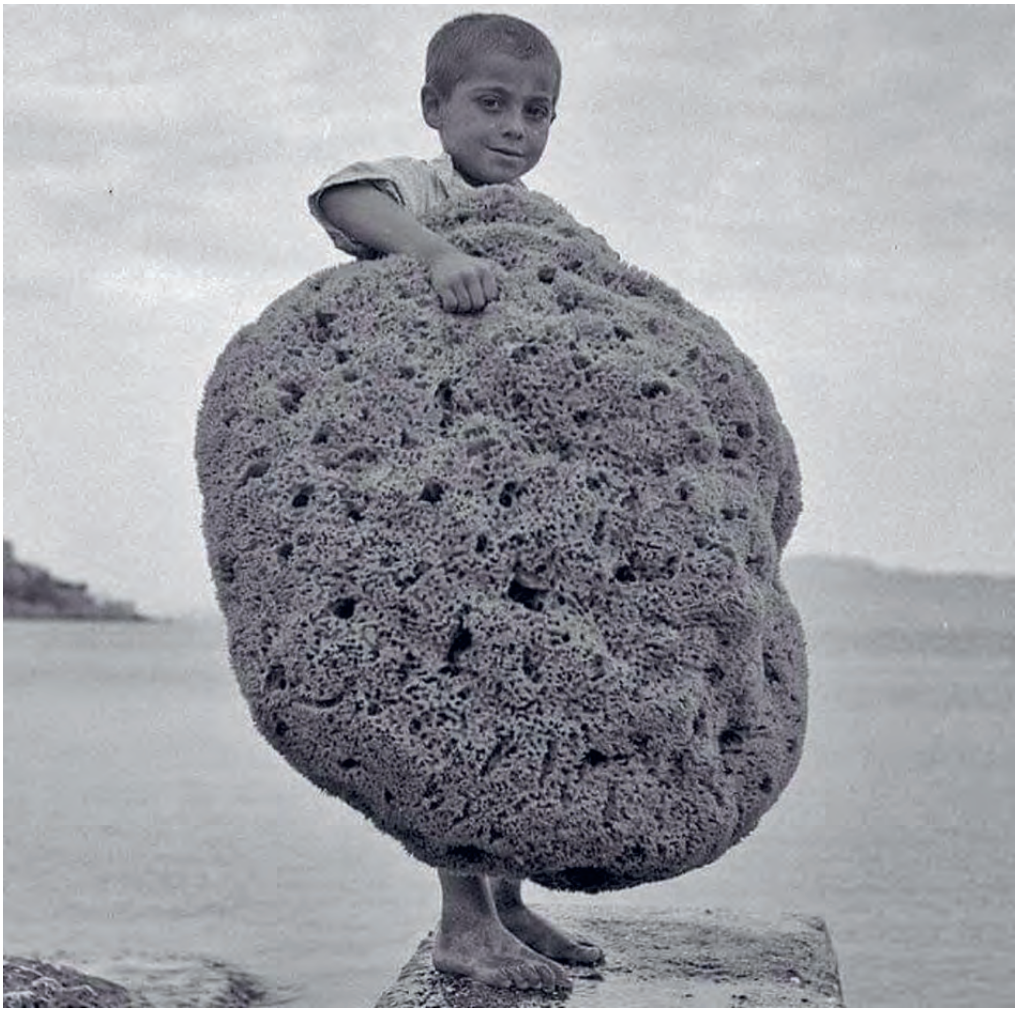

Boy holds a massive commercial bath sponge, Dimitris Harissiadis,

Kalymnos Island, Greece 1950 Benaki Museum, Photographic Archives

"And with a sponge wiped he his face and his two hands withal,

and his mighty neck and shaggy breast"

Homer, The Iliad, Book 18

"No ordeal is worse than sponge hunters, no effort is harder than theirs"

Oppian, Halieutica, Book 5

"At last, quite out of temper with an art, which, in spite of him, would still obtrude itself, he dashed his sponge against the vexatious spot; when behold: the sponge replaced the colours that it had just removed, exactly in accordance with his utmost wishes, and thus did chance represent Nature in a painting."

Pliny the Elder, The Natural History, Book 35

"Of hot applications the most powerful is hot water in a bottle, or bladder, or in a brazen vessel, or in an earthen one; but one must first apply something soft to the side, to prevent pain. A soft large sponge, squeezed out of hot water and applied, forms a good application; but it should be covered up above, for thus the heat will remain the longer, and at the same time the vapor will be prevented from being carried up to the patient's breath, unless when this is thought of use, for sometimes it is the case."

Hippocrates, De diaeta in morbis acutis, Part 7

"Ah state of mortal man! in time of wealth, a line, a shadow! and if ill fate falls, one wet sponge-sweep wipes all our traces away"

Aeschylus, Agamemnon

*In the chambered cavities of **sponges** pinna-guards or parasites are found. And over the chambers there is a kind of spider's web, by the opening and closing of which they catch mute fishes; that is to say, they open the web to let the fish get in, and close it again to entrap them.*

*Of **sponges** there are three species; the first is of loose porous texture, the second is close textured, the third, which is nicknamed 'the **sponge** of Achilles', is exceptionally fine and close-textured and strong. This **sponge** is used as a lining to helmets and greaves, for the purpose of deadening the sound of the blow; and this is a very scarce species. Of the close textured **sponges** such as are particularly hard and rough are nicknamed 'goats'.*

***Sponges** grow spontaneously either attached to a rock or on sea-beaches, and they get their nutriment in slime: a proof of this statement is the fact that when they are first secured they are found to be full of slime. This is characteristic of all living creatures that get their nutriment by close local attachment. And, by the way, the close-textured **sponges** are weaker than the more openly porous ones because their attachment extends over a smaller area.*

*It is said that the **sponge** is sensitive; and as a proof of this statement they say that if the **sponge** is made aware of an attempt being made to pluck it from its place of attachment it draws itself together, and it becomes a difficult task to detach it. It makes a similar contractile movement in windy and boisterous weather, obviously with the object of tightening its hold. Some persons express doubts as to the truth of this assertion; as, for instance, the people of Torone.*

*The **sponge** breeds parasites, worms, and other creatures, on which, if they be detached, the rock-fishes prey, as they prey also on the remaining stumps of the **sponge**; but, if the **sponge** be broken off, it grows again from the remaining stump and the place is soon as well covered as before.*

*The largest of all **sponges** are the loose-textured ones, and these are peculiarly abundant on the coast of Lycia. The softest are the close-textured **sponges**; for, by the way, the so-called **sponges** of Achilles are harder than these. As a general rule, **sponges** that are found in deep calm waters are the softest; for usually windy and stormy weather has a tendency to harden them (as it has to harden all similar growing things), and to arrest their growth. And this accounts for the fact that the **sponges** found in the Hellespont are rough and close-textured; and, as a general rule, **sponges** found beyond or inside Cape Malea are, respectively, comparatively soft or comparatively hard. But, by the way, the habitat of the **sponge** should not be too sheltered and warm, for it has a tendency to decay, like all similar vegetable-like growths. And this accounts for the fact that the **sponge** is at its best when found in deep water close to shore; for owing to the depth of the water they enjoy shelter alike from stormy winds and from excessive heat.*

*There is a particular species that is named the 'aplysia' or the 'unwashable', from the circumstance that it cannot be cleaned. This species has the large open and visible pores, but all the rest of the body is close-textured; and, if it be dissected, it is found to be closer and more glutinous than the ordinary **sponge**, and, in a word, something lung like in consistency. And, on all hands, it is allowed that this species is sensitive and long-lived. They are distinguished in the sea from ordinary **sponges** from the circumstance that the ordinary **sponges** are white while the slime is in them, but that these **sponges** are under any circumstances black.*

*And so much with regard to **sponges** and to generation in the testaceans.*

Aristotle, History of Animals

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CHAPTER 1

General Introduction

GENERAL INTRODUCTION

Sponges are marine animals that have been present in the oceans for at least 580 million years. Around 11.000 species of sponges have been identified today, which are widely distributed in habitats that range from coastal shores to abyssal depths (Van Soest *et al.* 2012). They are filter-feeding organisms often dominating the benthos in terms of abundance and biomass (Reiswig 1971, 1975). Sponges have been used by humans already for several millennia. The earliest use of sponges in the Mediterranean Sea appears on frescoes and vases from the late bronze age, together with plenty of records existing from the classical Greek literature (Voultsiadou, 2007). Sponges are known for their elasticity, softness and water retention abilities (Voultsiadou *et al.* 2011). The Greco–Roman poet Oppian (2nd century B.C.) gives a detailed explanation of bath sponge harvesting by fishermen 'Halieutica – Fishing' in his book (Arndt, 1937). Traditional Mediterranean sponge fishing was practiced till the end of the 20th century, until natural and human-derived stressors brought commercially important "bath sponge" species close to extinction (Pronzato and Manconi, 2008). Declines in natural populations have prompted the idea to culture sponges. Since first attempts by Cavolini (1785) to culture bath sponges in the Mediterranean Sea, additional motives for culturing sponges have evolved as a result of the discovery of an overwhelming array of biologically active metabolites and biomaterials produced in sponges (Sipkema *et al.* 2005).

HISTORY OF SPONGE MARICULTURE

The ability of some sponges species to recover their broken surfaces was well-known during the antique era and thoroughly explained in Aristotle's 'Historia Animalium' (History of Animals, 4th century B.C.), where the sponge can grow back from its damaged parts (Crawshay 1939). Cavolini (1785) was the first researcher to test this regenerative ability of bath sponges (also commonly referred to as "commercial sponges") via small cuttings. Schmidt (1862) repeated Cavolini's observation and suggested to utilize this ability for the systematic culture of bath sponges (Moore 1910b). Following Schmidt's observation, there have been three waves of sponge aquaculture trials in the last 235 years. The first was initiated (1785-1940) partly due to diver casualties, and partly to bring an alternative to the ill-considered harvest of natural specimens and relative collapse of bath sponge fisheries. Researchers from the United States and the Mediterranean area conducted several trials for optimizing sponge culture methods in regions where the commercial sponge fishing business continued (Moore 1910a; Radcliffe 1938; Crawshay 1939; Smith 1941; Storr 1964). Despite these efforts, there had been a gap in sponge cultivation until the bath sponge mass mortalities occurred all around the Mediterranean Sea in 1980's and Verdenal and Vacelet (1990) experimented on bath sponges by applying hanging a wire culture method in Southern France (Pronzato *et al.* 1999; Duckworth 2009). The persistent negative trend in commercial sponge trade was evident (reducing from 350 tones/y in 1935 to 50

tones/y in 2005; Pronzato and Manconi 2011) and motivated researchers to investigate ways to bring back the business that was once very lucrative. Researchers aiming to provide income to local communities commenced studies in the Mediterranean, Caribbean Sea and the Pacific (MacMillan 1996; Müller *et al.* 2004; Kelly *et al.* 2004; Corriero *et al.* 2004; Duckworth *et al.* 2007; Çelik *et al.* 2011). Organized sponge mariculture trials in Turkey started in late 80's due to the bath sponge disease and collapse of the commercial sponge business. The Bodrum Fisheries and Aquaculture Institute was founded in 1987 in Bodrum to support/regenerate the collapsing sponge business in the area (Katagan *et al.* 1991). However, the first culture efforts with bath sponges had already been conducted by Gökalp (1974) in the Northern Aegean Sea where researchers investigated the feasibility of sponge culture by culturing 5 commercial sponges species abundant in Turkish waters.

MARICULTURE OF NON COMMERCIAL SPONGES DUE TO DISCOVERED MATERIALS

The mariculture of non–commercial sponges started with the pioneering work of Wilkinson and Vacelet (1979) who cultured four Mediterranean sponge species under different light and current conditions. Three decades ago (1996-), a second wave of in situ sponge culture commenced due to the sponge–derived bioactive metabolites and biomaterials identified from various species (Battershill and Page, 1996; Munro *et al.* 1999; Müller *et al.* 1999; van Treeck *et al.* 2003; Duckworth and Battershill 2003a; Page *et al.* 2005; de Voogd 2007; Duckworth and Wolff 2007; Carballo *et al.* 2010; de Caralt *et al.* 2010; Padiglia *et al.* 2018; Santiago *et al.* 2019; Gökalp *et al.* 2019). Sponge mariculture was identified as a viable method to supply sustainable quantities of desirable sponge biomaterials (Osinga 1999; Sipkema *et al.* 2005). During the last 30 years, global research efforts increased to find cost-effective, large-scale sponge production methods to supply sponge materials for various applications (reviewed by Sipkema *et al.* 2005; Corriero *et al.* 2004; Duckworth *et al.* 2009; Schippers *et al.* 2012; Gökalp *et al.* 2020). However, despite extensive reviews on sponge cultivation methods and abovementioned in situ experiments with various potential sponges, commercial–scale mariculture for the production of sponge biomaterials did not succeed due to the trace amount of biomaterials within sponges and longevity and costly nature of clinical trials (Pomponi 1999; Belarbi *et al.* 2003; Müller *et al.* 2004; Schippers *et al.* 2012).

BIOLOGICAL POTENTIAL OF SPONGES (BIOREMEDIATION)

Together with bivalves, sponges are considered as the most efficient filter feeders thriving in marine habitats. They actively pump several thousand liters of water per kg sponge per day through their porous body and have a high particle retention efficiency (up to 98%) preferably on small particles (<10 µm), such as bacteria and phytoplankton (Maldonado *et al.* 2010), dissolved organic matter (Yahel *et al.* 2003; De Goeij *et al.* 2008a) and even viruses (Hadas *et al.* 2005; Welsh *et al.* 2020). The efficient and versatile filtering makes sponges key drivers of the uptake,

retention and transfer of energy and nutrients within benthic ecosystems (De Goeij *et al.* 2013) and makes them interesting candidate species for bioremediation of organic pollution, such as waste streams from aquacultures (Pronzato *et al.* 1999; Osinga *et al.* 2010; Gökalp *et al.* 2019) and unpurified urban waste water effluent (Ledda *et al.* 2014; Gökalp *et al.* 2020a). In theory, sponges grown in polluted ecosystems could be used to eliminate fish aquaculture waste and urban waste and in return, sponge growth might benefit from the abundantly available food in the water column.

ORGANIC POLUTION AND MANAGEMENT OF WASTE STREAMS IN COASTAL AREAS

The worldwide growing need for protein has led to an exponential increase in seafood production through aquaculture (Goldburg and Naylor 2005; Jennings *et al.* 2016). Locally, this expansion of the aquaculture industry has caused conflicts of interest with other coastal development sectors (Neofitou and Klaoudatos 2008; Yucel-Gier *et al.* 2013; 2019; Figure 1). In the Aegean Sea, the expanding fish farm sector produces substantial amounts of organic waste in the surrounding waters (Neofitou and Klaoudatos 2008), deteriorating the water quality (Price *et al.* 2015) and accumulation of organic material resulting in an anoxic sediment layer on the seabed beneath the farm cages (Karakassis *et al.* 2000, 2002; Sara *et al.* 2004; Yucel-Gier *et al. 2007*; Sweetman *et al.* 2014).

One of the problems within the Turkish aquaculture is the high concentration of fish farms in specific regions. For example, aquaculture occupies 21% of total water surface area of Gulluk Bay in Southwest Turkey (Figure 1). The fish farmers share the marine environment with the tourist sector, which requires both space and a good water quality (Demirak *et al.* 2006; Basaran *et al.* 2010; Yucel-Gier *et al.* 2013). Effects of aquaculture, such as high turbidity caused by overfeeding and bad waste management, can be devastating in combination with tourism. In the early 2000's, the tourist industry started a successful lobby against the fish farms and the Turkish government has decided to stop providing licenses to fish farms situated directly along the coast and forced the fish farms to offshore platforms in the hope of solving the problem (Figure 1). These off-shore fish farms, however, proved to be costly, needing substantial new investments for the aquaculture sector. Following the relocation of the fish farms, however, urbanization of the coastline (home/resort construction projects) accelerated, which created diversified impact over the coastline due to increase in population and usage of the coastline by various stakeholders (Yucel-Gier *et al.* 2013). Still not solved is the problem of water pollution (Yucel-Gier *et al.* 2013, 2019). Mediterranean aquaculture has grown 7% in 10 years, total production reaching 2 million tons in 2010 (FAO, 2014, 2018). Considering unprecedented growth of suspended cage mariculture and urban development in Gulluk Bay and likely throughout the whole Mediterranean, there will be further conflicts between the mariculture sector and other users in the future (Sanchez-Jerez *et al.* 2016: Yucel-Gier *et al.* 2019). A potential solution could be the integrated multi trophic aquaculture (IMTA) concept, which closes nutrient cycles by combining fish culture

with mariculture of effective filter feeders such as sponges for the cleanup of organically polluted waste water (Gökalp *et al.* 2019; 2020a). Preferably, sponge species of commercial interest should be selected for such IMTA practices.

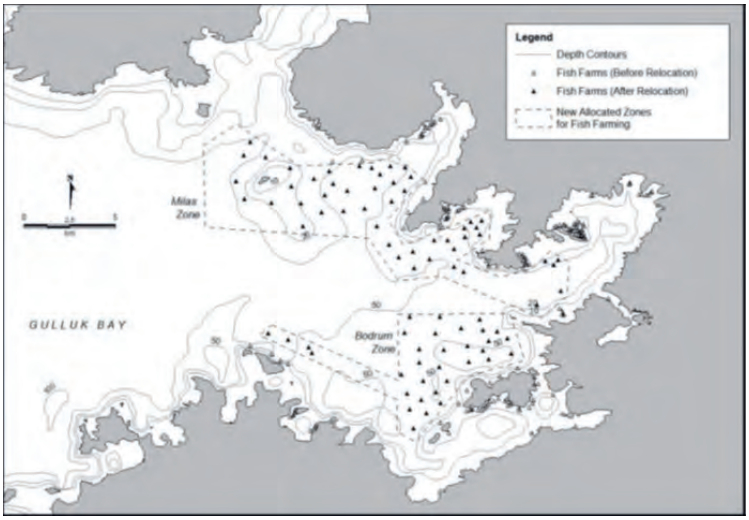

Figure 1. Gulluk Bay before and after the relocation of the fish farms. The aquaculture sector and the tourism-urban development-holiday home businesses clashed and fish farms were moved further offshore in 2007.

THE NEED FOR INTEGRATED MARICULTURE

Albeit the potential for relieving the pressure from global decline in fish stocks and becoming the predominant source of seafood for human nutrition, there is urgent necessity for revising mariculture as it is globally applied nowadays. Applying a single-crop approaches to culture marine resources generates excessive amount of organic material in the water column and associated pathogen outbreaks, deteriorates water quality through nutrient pollution and creates anoxic sediment layers on the seabed (Karakassis *et al.* 2000, 2002; Sara *et al.* 2004; Neofitou and Klaoudatos 2008; Yucel-Gier *et al.* 2013, 2019; Sweetman *et al.* 2014). Insufficient management of mariculture-associated waste streams (e.g. undigested feed, feces and inorganic waste) and conflicts between coastal shareholders are of increasing concern. Anthropogenic disturbances induced by urban water discharge and correlated decline in resilience of marine habitats is another growing concern for fragile coastal ecosystems globally. IMTA has the potential to mitigate negative effects of fed single crop farming and anthropogenic induced disturbances around sewage outlets to some extent (Naylor *et al.* 2000; Buschmann *et al.* 2001; Troell *et al.* 2003; Gifford *et al.* 2007; Chopin *et al.* 2012; Ledda *et al.* 2014; Gökalp *et al.* 2020a). By integrating co-cultured organisms, IMTA could provide additional harvestable products that have commercial values and/or ecological benefits (waste reduction) as compared to monoculture (Chopin *et al.* 2001). In addition, fed aquaculture by itself results in production of dissolved organic matter (DOM) and DOM feeding by sponges provides an additional benefit to

their application as extractive IMTA component (de Goeij *et al.* 2007, 2013; Wang *et al.* 2013; Gökalp *et al.* 2020b).

STUDY AREA AND SPONGE SPECIES OF INTEREST

The studies within this thesis used the Mediterranean sponge species, *Chondrosia reniformis* (Nardo, 1847) and *Dysidea avara* (Schmidt, 1862). *C. reniformis* has potential economic relevance as producer of collagen for applications in human therapy (Swatschek *et al.* 2002; Nickel and Brümmer 2003). More recently, the collagen of *C. reniformis* has been identified as an excellent scaffold material for tissue engineering (T. Silva, pers. com.) and it has been the model species for the collaborative project overarching parts of this thesis (M-ERA.NET II-BiogenInk). *D. avara* with its celebrated secondary metabolite, Avarol (cytotoxic, antiviral, anti-bacterial and anti-psoriasis activity; Tommonaro *et al.* 2015), is another good candidate for a model species to be used in integrated multitrophic aquaculture (IMTA). The current studies were realized in the coastal waters of the Bodrum Peninsula and in the Kas-Kekova Marine Protected Area (Figure 2), where natural populations of *D. avara* and *C. reniformis* are present.

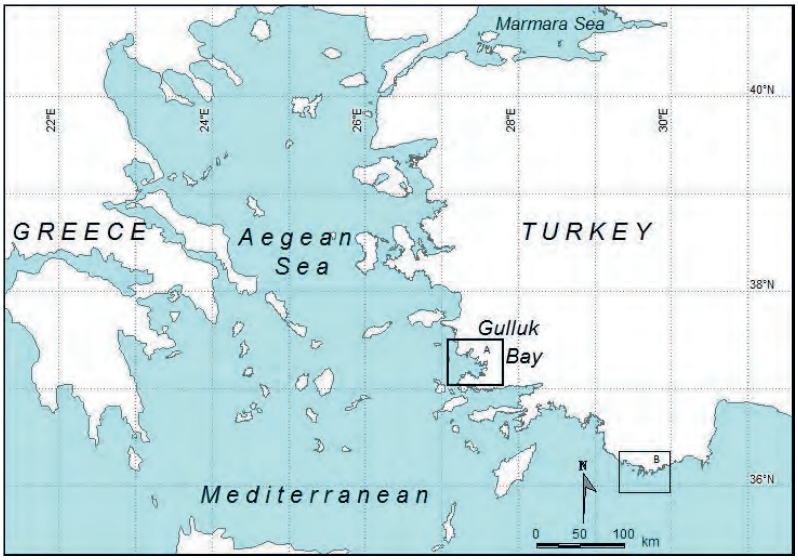

Figure 2. The field study area of this PhD project is located in the Eastern Mediterranean, Turkey. Field work locations: (A) Bodrum Peninsula, (B) Kas-Kekova Marine Protected Area.

AIM, RESEARCH QUESTIONS AND RATIONALE

In this context, this thesis aimed to design a functional IMTA with sponges as an extractive component to close nutrient loops while providing high added-value compounds. The targeted sponge species were the native *D. avara* and *C. reniformis*, because they are of commercial interest for production of a potential drug (avarol) and a potential biomedical product (collagen). Most emphasis was given to the development of an IMTA mariculture with *C. reniformis*, because

it was the primary target species for the recent project BiogenInk. To achieve a functional IMTA, it is essential that the sponges efficiently filter suspended material and that they grow fast. Hence, the studies primarily addressed the following scientific questions: 1) How do sponge pumping and filtering activity vary with location? 2) What do sponges require for optimal growth in mariculture?

Combining the answers to these research questions provides insight in how to produce and maintain sponges with a high filtration potential as biofilter in mariculture. An additional benefit of the use of sponges is the added value of the sponge biomass produced.

APPROACH AND OUTLINE

To gain better insight in culturing sponges, a quantitative approach was used throughout the thesis that relied on underwater observations/applications.

In **Chapter 2**, we evaluated the use of marine sponges in integrated culture systems whether intended to be used for *in situ* bioremediation of sewage discharge or waste produced by fish cages in addition to the produced sponge biomass. We presented an idea to use sponges as an engine to convert DOM into POM that can be consumed by deposit feeders through a chain of processes termed the sponge loop and included a theoretical design to demonstrate an IMTA (with seaweed, sponge and sea cucumber species). In order to become a commercial product for aquaculture, significant amounts of sponge biomass must be produced in a cost-effective manner.

In **Chapter 3**, we conducted aquaculture trials with sponge species with commercial interest under a sea-based aquaculture cages in Gulluk Bay and Bodrum Peninsula (Figures 1,2). *D. avara* explants were cultured on suspended nylon threads and *C. reniformis* explants were grown in stainless steel cages. In this study, we succeeded culturing *D. avara* in both locations and presented a scale-up method (PVC pins) for biomass production of this species. Culture of *C. reniformis*, however, was only successful at the pristine site and failed at the polluted site due to the smothering of explants with fish farm sediment. This result prompted further studies on *C. reniformis* in IMTA settings. We wanted to assess how two important factors for site selection (water depth and organic pollution) affected the growth and filtration capacity of *C. reniformis* (**Chapter 4**) and we wanted to improve culture methodology for *C. reniformis* for application in organically polluted areas (**Chapters 5** and **6**)

In **Chapter 4**, we evaluated the effect of depth on sponge morphology, growth, physiology, and functioning to support the successful application of sponges for water purification and collagen production. Specimens transplanted from 20 to 5 m presented morphological plasticity and altered their morphology to match the control group at 5 m, however, the explants transplanted from 5 to 20 m did not follow this pattern. Eventually, we found that the clearance, respiration, and growth rates were comparable among all the experimental groups which

indicated that depth-related morphological changes do not affect the overall performance of the sponges. Hence, the potential for the growth and bioremediation of *C. reniformis* in mariculture is not likely to change with varying depth.

In **Chapter 5** we tested several methods to culture the collagen-rich sponge *C. reniformis* in the proximity of floating fish cages and succeeded at growing them also at the organically polluted site. In a series of trials, survival and growth of cultured explants were monitored near a polluted fish farm and a pristine site, where we found that both survival and growth were significantly higher at the polluted site than at the pristine site. This finding, two-fold higher sponge growth at the polluted site is promising for culturing sponges in coastal waters with high organic matter loads.

In **Chapter 6**, we replicated the study conducted in **Chapter 5** in order to observe the growth performance of *C. reniformis* when exposed to another type of pollution and to optimize the culturing method. This time, instead fish farm effluents, urban water discharge was targeted. In this final Chapter, the recovery and growth of explants were monitored annually and further optimization of the sponge biomass production methodology was targeted. Three consecutive culture trials revealed consistent growth and high recovery rates in 3 culture seasons both under pristine and polluted conditions. Ultimately, we reported on a successful culture method for a sponge production pipeline and we presented a final design, entitled 'Sponge lantern', which is simple, sustainable, enhances productivity and is adaptable to seawater environments with variable organic particle loads and water currents.

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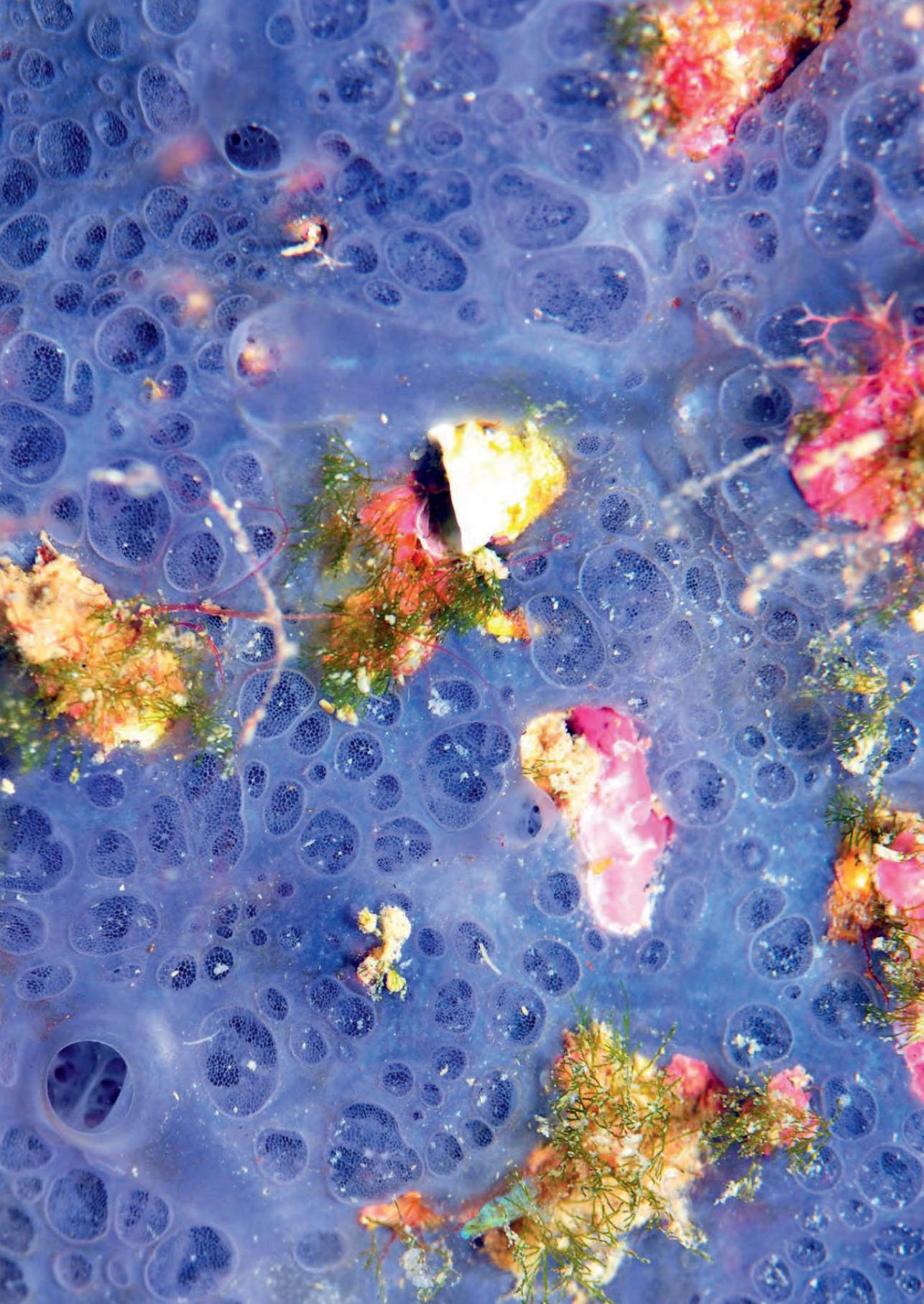

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The potential roles of sponges in integrated mariculture

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ABSTRACT

This mini-review evaluates the use of marine sponges in integrated culture systems, two decades after the idea was first proposed. It was predicted that the concept would provide a double benefit: sponges would grow faster under higher organic loadings, and filtration by sponges would improve water quality. It is promising that the growth of some commercially interesting sponges is indeed faster in organically enriched areas. The applicability of sponges as filters for undesired microorganisms has been confirmed in laboratory studies. However, upscaled farming studies need to be done to demonstrate the value of sponges for in situ bioremediation of sewage discharge or waste produced by fish cages. In addition, a new idea is presented – the use of sponges as an engine to convert dissolved organic matter (DOM) into particulate organic matter (POM) that can be consumed by deposit feeders through a chain of processes termed the sponge loop. A theoretical design of an integrated culture with seaweeds (*Gracilaria sp.*), sponges (*Halisarca caerulea*) and sea cucumbers (*Apostichopus japonica*) shows that 37% of the part of the primary production that is excreted by the seaweeds as DOM can be directly recovered in sponge biomass and a subsequent 12% in sea cucumber biomass after mediation (conversion of DOM to POM) by sponges. Hence, the total recovery of DOM into (sponge and sea cucumber) biomass within this IMTA is 49%.

Key words: sponges, integrated multitrophic aquaculture, sea cucumbers, seaweeds, sponge loop

INTRODUCTION

The concept of combining the aquaculture of species from different trophic niches to achieve an ecologically efficient and profitable production dates back to 2200–2100 B.C., where the Chinese applied this concept for the first time (Chopin 2013). In present days, this aquatic polyculture gained renewed attention. Since aquaculture has become the predominant source of seafood for human nutrition (FAO 2014, 2018), management of its associated waste streams (e.g., undigested feed, faeces and inorganic waste) is of increasing concern. As a more sustainable solution to monoculture, the concept of integrated multitrophic aquaculture (IMTA) has been proposed. IMTA is based on the ecological concept of efficient re-use of organic and inorganic waste streams through the application of different trophic levels of the food web within the culture system. Furthermore, by integrating co-cultured organisms, IMTA will provide additional harvestable products that have commercial values and/or ecological benefits (waste reduction) as compared to monoculture (Chopin *et al.* 2001). Most IMTA designs combine farming of (1) fed species (e.g. finfish) with (2) cultures of suspension and/or deposit feeders (e.g., sponges, bivalves, polychaetes, sea cucumbers, sea urchins) that take up organic nutrients and/or with (3) macroalgae (e.g., kelps, *Gracilaria salicornia*, sea lettuce) that take up inorganic nutrients (Neori *et al.* 2004; Chopin *et al.* 2012; Buck *et al.* 2018). In this way, it is suggested that negative effects of fed 'single crop' farming, such as deterioration of water quality through nutrient pollution, pathogen outbreaks, sedimentation of undigested food and faeces particles, which may cause conflicts between stakeholders (e.g., aquaculture businesses, tourist industry, nature conservation organizations, water quality boards), can be mitigated (Naylor *et al.* 2000). Thus, the sustainability, productivity, profitability, and resilience of aquaculture systems are increased by applying an environmental-friendly, product-diversified, and socially beneficial concept of integrated farming (Buschmann *et al.* 2001; Troell *et al.* 2003; Chopin *et al.* 2012).

In 1998, Pronzato and co-workers were the first to consider including sponges (phylum Porifera) as an extractive component in IMTA (Pronzato *et al.* 1998). Sponges are ubiquitous benthic marine and freshwater animals that can extract a large amount of waterborne organic substances from extensive amounts of water through very fast and efficient filter–feeding (Weisz *et al.* 2008). The sponge is comprised of a maze of channels and chambers harboring specialized cells that function in transport of water and efficiently retain a large variety of suspended particles (Reiswig 1971a,b; 1974; Vogel 1977; Riisgård *et al.* 1993), including (pathogenic) bacteria (Zhang *et al.* 2010; Longo *et al.* 2010) and viruses (Hadas *et al.* 2006; Welsh *et al.* 2020). In addition to the capturing of organic particles, sponges also bioaccumulate environmental pollutants, such as surfactants (Pérez *et al.* 2002), polychlorinated biphenyls (PCBs; Pérez *et al.* 2003), and heavy metals (Patel *et al.* 1985; Olesen and Weeks 1994; Hansen *et al.* 1995; Müller *et al.* 1999; Philp 1999; Pérez *et al.* 2005; Cebrian *et al.* 2007). Since these types of pollutants are not commonly associated with aquaculture, we will not discuss this feature of sponges in the current review. The use of sponges to absorb antibiotics from seawater may be of interest to aquaculture, since the use and release of antibiotics in aquaculture is an issue of growing concern (Cabello *et al.* 2009). However, no data exist to date on the potential of sponges to bioaccumulate this type of compounds.

The efficient filtering of organic particles by sponges coupled with their ability to produce commercially interesting bio-products, such as biomedical agents, bio-silica, bio-sintering, and collagen (Pomponi 1999, 2001; Sipkema *et al.* 2005; Schröder *et al.* 2007; Müller *et al.* 2009; Gökalp *et al.* 2019) have raised interest in the inclusion of these animals in IMTA applications. Sea-based aquaculture of sponges has successfully been studied for multiple purposes (reviewed by Duckworth 2009; Schippers *et al.* 2012), showing that inclusion of this technique in IMTA systems is nowadays technically feasible (see for example Fig. 1). Nevertheless, sponge farming is a complex process with 235 years of research history (Moore 1910; Duckworth 2009), with many unknowns remaining and with sometimes conflicting results (e.g. about farming protocols, effects of generations etc.). Examples of commercial application of sponge farming are still scarce (Duckworth 2009), and the inclusion of sponges as an element in IMTA is still at its infancy.

Within the last two decades, evidence has accumulated that the majority of the organic diet of many sponges consists of dissolved organic matter (DOM), operationally defined by all organic matter passing a 'fine' filter, typically 0.2-0.7 µm (Benner 2002), when compared with their particulate organic intake (see review by de Goeij *et al.* 2017). As DOM is not bioavailable as food source to most other heterotrophic organisms, DOM-feeding by sponges provides an additional benefit as extractive IMTA component. Fed aquaculture by itself results in production of DOM (Wang *et al.* 2013) and this is substantially increased when seaweeds are included as IMTA components. Seaweeds and other marine algae release up to more than 50 % of their photosynthetic products into the surrounding seawater as DOM (Khailov and Burlakova 1969; Haas *et al.* 2011). Hence, a substantial proportion of the primary production of an in situ seaweed aquaculture is lost into the environment as DOM, where it may fuel bacteria, including pathogenic microbes. This, in turn, may add to the proposed 'microbialization' of marine ecosystems (Haas *et al.* 2016). Sponges may potentially buffer against microbialization (de Goeij *et al.* 2017) and use DOM to increase their own productivity within the integrated aquafarm in an IMTA setting . Sponges do not only take up DOM, but can efficiently return the resources stored in DOM into the benthic food chain by converting DOM into particulate organic matter (POM; or referred to as detritus) through a pathway called 'the sponge loop' (de Goeij *et al.* 2013), which may further extend our view on the role of sponges in IMTA. As such, sponges can be net producers of POM instead of being net particle filters. The detritus produced by sponges has been shown to be a food source for detritivores, such as small crustaceans (de Goeij *et al.* 2013, Rix *et al.* 2018). In IMTA systems, sponge detritus production may facilitate the culture and production of commercially attractive detritivores, such as sea cucumbers (Maxwell *et al.* 2009). Sea cucumbers do consume particulate wastes and byproducts generated by other trophic levels, and have a high economic value (Ahlgren 1998; MacDonald *et al.* 2013; Yokoyama 2013; Yu *et al.* 2012, 2014). Certain sea cucumber species are highly desired by Asian sea product wholesalers, as a result of an escalating demand for their nutritional and medicinal use in dietary and pharmaceutical markets (Zamora *et al.* 2018). Moreover, sea cucumbers increase sediment productivity and biodiversity of ambient benthic ecosystems through bioturbation and assimilation of bacterial, fungal and detrital organic matter (MacTavish *et al.* 2012).

Two decades ago, Pronzato *et al.* (1999) predicted a double bonus for integrated farming of sponges: 1) purified water and 2) an enhanced production of high-quality sponge biomass . In

the first part of this review, we will evaluate to what extent this double bonus has been capitalized: to what extent can sponges enhance ambient water quality through bio-filtration and the production of high- quality sponge biomass (e.g. for biotechnological purposes) in an IMTA setting? In the second part of this review, we outline the new potential role of sponges as intermediates between primary producers of dissolved organic matter and consumers of detrital organic matter in an IMTA system, thus capitalizing on the sponge loop. As an example, we present a model calculation of carbon transfer from seaweeds via sponges to sea cucumbers.

Figure 1a,b. a) A specimen of *Chondrosia reniformis* (Nardo, 1847) dropping from the ceiling of a rock crevice in order to reach and grow over a new ground. This species is of commercial interest because it is a rich source of collagen with biomedical potential (Fassini *et al.* 2017; Gökalp *et al.* 2019, 2020a). b) Divers cleaning the sponge lanterns designed for IMTA – *C. reniformis* was subjected to a wide range of mariculture applications and IMTA applications due to its collagen-rich cortex and common appearances throughout the Mediterranean coastline.

The earlier idea: sponges as biofilters for organic particles

The filter-feeding abilities of sponges intrigued several scientists in the last century (Jørgensen, 1949; 1955; Reiswig 1971a,b; 1974; 1975; Riisgård *et al.* 1993). Sponges can have considerable impact on water quality, considering the immense water processing rates of sponges (up to 50 m^3 per dm^3 of sponge tissue per day; Weisz *et al.* 2008), their high particle retention efficiencies (up to 98 % (e.g., Reiswig 1971a; Lesser 2006) and their abundance (in many ecosystems, sponges dominate the benthic community and they have been proposed as potential winners under future climate scenarios; Fabricius *et al.* 2011; Bell *et al.* 2013, 2018; Pawlik *et al.* 2016; de Bakker *et al.* 2018). Sponges are sessile suspension feeders grazing on organic particles within the range of 0.1-50 µm (Reiswig 1971a; Pile *et al.* 1996; Osinga 1999a; Ribes *et al.* 1999; Maldonado *et al.* 2010). Carbon uptake rates of sponges were found to be within the same range or even exceed the uptake rates of other, well-established filter-feeders, such as bivalves (29-1970 mg C m^{-2} d^{-1} for bivalves vs 9–3621 mg C m^{-2} d^{-1} for sponges; Riisgård and Larsen 2000). This prompted the idea to use sponges for the remediation of organic pollution from aquaculture cages and urban environments (e.g. sewage) (Pronzato *et al.* 1999; Cattaneo-Vietti *et al.* 2003; Gifford *et al.* 2006; Stabili *et al.* 2006; Osinga *et al.* 2010; Ledda *et al.* 2014; Gökalp *et al.* 2019). The wide variety of microorganisms taken up by sponges as revealed in laboratory-based experiments, demonstrated the added value of sponges as will be further detailed below.

The Mediterranean sponge species *Chondrilla nucula* was able to retain up to 70 billion Escherichia coli cells m^{-2} of sponge surface area, hereby clearing 14 L of water h^{-1} in a laboratory experiment (Milanese *et al.* 2003). A similar study reported remediation of the bacteria E. coli and *Vibrio anguillarum* by the sponge *Hymeniacidon perlevis*, filtering up to 8 million E. coli cells g^{-1} fresh sponge h^{-1} (Fu *et al.* 2006). The Mediterranean sponge *Spongia officinalis var. adriatica* (i.e. the bath sponge) had a very high efficiency of removing bacteria (12.3 x 10^4 cells ml^{-1} with a maximum retention efficiency of 61 %) when used in marine environmental bioremediation (Stabili *et al.* 2006). Similarly, the Mediterranean sponge species *Aplysina aerophoba* exhibited high efficiencies in taking up food microorganisms (bacterial isolates) from environmental water in a flow-through system (Wehrl *et al.* 2007). *Hymeniacidon perlevis* was able to remove pathogenic bacteria, achieving a removal of 60.0-90.2 % of faecal coliform bacteria, 37.6-81.6 % of pathogenic *Vibrio spp*. and 45.1-83.9 % of the total bacteria in a 1.5 m^3 turbot (*Scophthalmus maximus*) aquaculture system (Zhang *et al.* 2010). In a similar study, *H. perlevis* was able to accumulate, remediate, and metabolize halophilic Vibrio spp., heterotrophic bacteria, total culturable bacteria, faecal coliforms and faecal *Streptococci* (Longo *et al.* 2010). Algal blooms occurring in central Florida Bay are suggested to be related to the loss of suspension feeders in the system, implying sponges do not only take up unwanted bacteria, but can also be used as a tool for mitigating (harmful) algal blooms (Peterson *et al.* 2006; Wall *et al.* 2012).

Although these studies clearly demonstrate the remediation potential of sponges for (unwanted) bacteria and algae, some considerations must be made. First, the reported removal efficiencies for pathogenic bacteria might be deceptive, as most of these studies were done with non–marine microbes and pathogens (e.g. *E. coli*). Second, selective feeding by sponges on non–pathogenic bacteria may lead to sudden proliferation of opportunistic pathogenic microbes,

such as *V. anguillarum*, thus increasing the problem (Maldonado *et al.* 2010). Third, there is no consensus on the effect of particle concentration on uptake rates by sponges. The grazing rate of the sponge *H. perlevis* was demonstrated to be dependent on the concentration of the microbes (Fu *et al.* 2006; Maldonado *et al.* 2010). In contrast, in several studies, grazing and retention rates by sponges were independent of the concentration of the food source (Reiswig 1971, 1975; Frost 1978, 1980a,b; Wehrl *et al.* 2007). For larger particles (microalgae), sponge-mediated clearance rates were concentration independent up to a particular threshold concentration, above which clearance rates rapidly decreased (Osinga *et al.* 2001). To further complicate the issue, it is often hard to make a comparison between the different studies on sponge feeding efficiency due to the multitude of the sponge size metrics used in scientific literature, which include wet weight (ww), dry weight (dw), ash-free dry weight (afdw), volume, and individual. In conclusion, more systematic studies should be done on the density dependency of grazing by sponges, preferably using standardized size metrics. Unanswered but important questions remain: what are the optimal food particle densities and sizes and is there a threshold in densities/sizes above which grazing efficiency is seriously compromised? The large variability in feeding dynamics among sponge species shows that candidate sponges for IMTA should be carefully selected to find the species that aligns best with the characteristics of the targeted organic and microbial pollution. Before applying a certain sponge species as biofilter for organic pollutants, repetitive feeding tests on microbial targets should be conducted and the possibility of selective feeding on non-pathogenic bacteria should be excluded before a multitrophic integration is first tested (Maldonado *et al.* 2010).

Pilot-scale studies conducted in situ have confirmed that sponges can benefit from the increased availability of food in the vicinity of mariculture farms (co-culture with seabass: Osinga *et al.* 2010; Gökalp *et al.* 2019; co-culture with mussels: Page *et al.* 2011) and in waste-water streams created by urban runoff (Ledda *et al.* 2014). *Dysidea avara* explants (i.e. re-grown clones cut from host sponge) cultured under seabass aquaculture cages in Southwest Turkey, exhibited 100 % survival and doubled in size within four months, whereas explants cultured under pristine conditions showed lower growth (up to 0-50 % per year). *Chondrosia reniformis* explants cultured on suspended PVC plates in the proximity of fish farm effluent (2.4 times difference in TOC levels between the pristine and reference site) achieved a better annual survival and growth rate (86 and 170 %) than explants grown at a pristine site (39 and 79 %), thus corroborating the earlier findings on *D. avara* (Gökalp *et al.* 2019). Mediterranean sponge species *Ircinia variabilis* and *Agelas oroides* showed the highest retention and clearance rates at a polluted site (waste water and urban runoff) during a study conducted in the Ligurian Sea, which suggests that these conditions are beneficial for sponges (Ledda *et al.* 2014). There was substantial growth (exceeding 2000 % per year) of explants of *Mycale hentscheli* in the vicinity of mussel farms, but in this case, growth was not augmented when compared to cultures in the natural reference site (Page *et al.* 2005). This may relate to the nature of mussel cultures, which are considered as a filter-feeding extractive component of organic matter themselves. The examples above demonstrate that culture of sponges in organically polluted areas can enhance the production of valuable sponge biomass. In fact, the primary motivation behind the integrated mariculture trials described above was to produce sponge biomass with high added value, i.e. *D. avara* for the production of Avarol (Osinga *et al.* 2010), *M. hentscheli* for the production of Peloruside A (Page *et al.* 2005; 2011), and *C. reniformis* for the production of collagen (Gökalp *et al.* 2019; 2020a).

In addition to the aforementioned studies on bioremediation, potential caveats in sponge farming procedures need to be addressed as well to fully realize the integration of sponge farming in IMTA systems. Potential caveats include (but are not limited to) effects of seasonality, repeated cloning, fouling, and predation. Growth rate and filtration activity can vary among seasons (Gökalp *et al.* 2019; De Caralt *et al.* 2008), which may lead to temporal imbalances between the sponges and the other IMTA components. Effects of repeated cloning have hardly been studies. Repeated cloning led to reduced growth in *Mycale hentscheli* (Page *et al.* 2011), but the effect was confounded by increased fouling and predation as culture trials proceeded. Predation of cultured sponges sometimes occurs unexpectedly. For example, sea turtles removed the majority of *Chondrosia reniformis* specimen cultured in open boxes in the East Mediterranean (M. Gökalp, personal observations). However, when fully protecting the sponges with mesh, fouling may become an issue of concern. Fouling on culture systems by algae and invertebrates can compromise the functioning of sponges, for example by blocking water current around the sponges, thus impairing the ability of the sponges to take up food.

In conclusion, two decades after the idea was launched, the proposed double bonus of integrating sponges in maricultures (improved control on organic pollution and enhanced growth of sponges) has been partially confirmed. Enhanced growth of sponges in multitrophic aquaculture settings has been demonstrated. Control on organic pollution has only been confirmed on laboratory scale and these studies indicated that selective feeding by sponges on non-pathogenic microorganisms is the main risk associated with this idea. To complement these laboratory scale studies, large-scale studies on the effects of sponge cultures on water quality in in situ settings are needed. Whereas positive effects on ambient water quality have been demonstrated for in situ shellfish cultures (Handå *et al.* 2012), examples from large-scale integrated maricultures with sponges are currently not available. An additional issue of concern is the production of nutrients by sponges. Sponges are known to produce inorganic nutrients, such as phosphate and dissolved inorganic nitrogen (DIN; Diaz and Ward 1997; Jiménez and Ribes 2007; Southwell *et al.* 2008; Bayer *et al.* 2008). Effects of DIN excretion by sponges on water quality should be included in studies exploring the potential of sponges as extractive components in IMTA.

A new idea: the use of sponges to convert DOM into POM

There has been an increased interest in carbon budgets and energetics of marine benthic fauna (Jørgensen 1955; 1976; Reiswig 1971a;b; Ribes *et al.* 1999; Yahel *et al.* 2003; de Goeij *et al.* 2008a). In the past century, particulate organic matter (POM) was considered as the prime food source for sponges, whereas dissolved organic matter (DOM) was only hypothesized to be a potential source of food for some marine invertebrates (Jorgensen 1976). The dogma of sponges being particle feeders was challenged by the suggestion that sponges were not able to balance their demand of organic carbon (i.e. their respiratory demand) by the uptake of POM alone and that uptake of dissolved organic carbon (DOC) could balance their respiratory demand (Reiswig 1981). This nutritional role of DOM in benthic communities was proven only when an accurate methodology to analyze DOC at a sufficient detection limit (μmol L^{-1}) had been established (Sharp 2002). Following this advancement, extensive feeding on bulk DOC—representing > 90 % of their daily organic carbon uptake—by a sponge (*Theonella swinhoei*) was reported (Yahel *et al.* (2003). De Goeij and co-workers ignited a renewed attention on DOM

studies at ecosystem scale. They found that extensive amounts of DOC were disappearing in coral reef cavities (de Goeij and van Duyl 2007), the largest habitat on coral reef ecosystems, where it was taken up and processed (i.e. respired and assimilated) by encrusting sponges (de Goeij *et al.* 2008a), dominating the cavity walls. Again, DOC represented more than 90 % of the daily organic carbon intake, and the uptake rates of DOC by encrusting sponges were found to be in the same order of magnitude as the primary production rates of the entire coral reef ecosystem (de Goeij *et al.* 2008a; 2013). More studies found similar dominance of DOC in the diet of excavating sponges (Mueller *et al.* 2014) and massive sponges (McMurray *et al.* 2016; Hoer *et al.* 2018) (see also review by de Goeij *et al.* 2017). In addition, the encrusting sponges were found to produce large amounts of particulate organic waste (detritus) by massive shedding of predominantly rapidly proliferating filter cells (choanocytes), but also other cellular waste and undigested food into the ambient water (de Goeij *et al.* 2009, 2013, Alexander *et al.* 2014, Maldonado *et al.* 2015, Rix *et al.* 2016). This finding led to the sponge loop hypothesis (de Goeij *et al.* 2013): sponges take up the largest pool of organic energy resources produced on the reef (DOM), they transform the majority of DOM into particulate detritus (i.e. POM), which is then consumed by detritivores and thus returned back into the food web. In this way, sponges play a crucial role in benthic ecosystems, which may explain how productive ecosystems such as coral reefs can thrive in nutrient-poor waters (de Goeij *et al.* 2013). The sponge loop pathway has now been established in a Red Sea coral reef ecosystem (Rix *et al.* 2016, 2018) and even in cold-water deep-sea coral reefs (Rix *et al.* 2016).

Based on the increasing body of aforementioned scientific literature, it can be concluded that DOM uptake is a widespread phenomenon among sponges and that DOM is the main source (> 50 % of their daily intake) of nutrition for many sponge species. Hence, aquaculture systems that produce large amounts of DOM could benefit from sponges to retain a resource that would otherwise be lost. This idea is particularly relevant for aquaculture systems that include seaweed farming. Seaweeds and other algae release a substantial proportion (up to 56 %) of their photosynthetically acquired carbon in the form of DOM (Khailov and Burlakova 1969; Haas *et al.* 2010b; 2011). This DOM is not only considered as a lost resource, it is also known as a factor that can considerably alter the abundance and composition of microbes of marine ecosystems, such as coral reefs (Haas *et al.* 2016). Algal-derived DOM can promote the occurrence of pathogens that have been associated with coral disease (Smith *et al.* 2006). Consumption of algae-derived DOM by sponges could therefore be considered as a process that is beneficial for the health of reef ecosystems. In fact, sponges may even have a preference for algal-derived over coral-derived DOM. For example, Red Sea sponges *Chondrilla sacciformis*, *Hemimycale arabica,* and *Mycale fistulifera* were found to assimilate algal-derived DOM at significantly higher rates than coral-derived DOM (Rix *et al.* 2017). Interestingly, from a fisheries perspective, the sponge loop hypothesis has even been proposed to increase local fish production on coral reefs under a shift from coral to algal biomass dominance (Silveira *et al.* 2015).

There is also an alternative theory that puts the role of DOM consumption by sponges in coral reef ecosystems in a different perspective. This theory states that sponges and macroalgae enforce each other at the expense of space for corals through a positive feedback loop termed the 'vicious circle' (Pawlik *et al.* 2016): algae promote sponge growth by producing DOM, whereas sponges produce dissolved inorganic nitrogen, which in return fertilizes macroalgae. Effects of

such a vicious circle on the environment should be fully understood before implementing integrated seaweed–sponge–fish farms in marine environments.

Also, not all sponges seem to produce detritus at the same quantities as encrusting sponges (approximately 5–25 % of their biomass per day; De Goeij *et al.* 2013; Alexander *et al.* 2014; Rix *et al.* 2016; 2017). Instead of releasing assimilated carbon in the form of detritus, the massive Caribbean sponge species *Xestospongia muta* uses DOM predominantly for somatic growth, thus retaining most of the assimilated DOM as biomass (McMurray *et al.* 2018). The sponge loop can be accomplished not only through the conversion of DOM into POM, but also through predation on sponge biomass, for example by spongivorous fish (McMurray *et al.* 2018). High cell shedding and detritus production may be predominantly associated with encrusting sponges, since these sponges experience high competition for space, which limits their potential to grow (McMurray *et al.* 2018). Kahn and Leys (2016) tested the sponge loop hypothesis on massive, cold-water deep-sea sponge species and did not observe rapid cell cycling and shedding of sponge cells as detritus, which they explained as the consequence of nutrition-limitation in these sponges. However, this study was conducted on small tissue fragments (i.e. not on fully functional sponges) and are therefore difficult to compare to in vivo studies. Another study on massive deep-sea sponges did show extensive deposition of POM (Witte *et al.* 1997). Considering that there are currently more than 9,000 sponge species described and many more expected (World Porifera Database; van Soest *et al.* 2020), it is also expected that sponges have a wide variety of traits and functions, which should be considered when choosing a candidate for an IMTA.

In conclusion, conversion of DOM into edible POM occurs in many, but perhaps not all sponge species, at different rates. Selected sponge species can be used in IMTA to create trophic links between seaweeds and detritivores. Such trophic links have been established for (macro)algae, sponges and detritivores, such as polychaetes, hermit crabs and ophiuroids (de Goeij *et al.* 2013; Rix *et al.* 2018), but not yet for sea cucumbers (Holothurian taxon), which represent another good candidate detritivore for IMTA applications. Grazing of *Holothuria* species on sponge-derived POM is likely: there are reported observations of sponge-ophiuroid/holothuroid associations, where ophiuroids feed on the detritus of sponges (Hendler 1984; Hammond and Wilkinson 1985; Rix *et al.* 2018) and there are unpublished observations of Holothurian species that are directly feeding on deposits of sponges in cryptic habitats (M. Gökalp, personal observation). Hence, there is an urgent necessity for research that confirms this trophic link and thus demonstrates that IMTA systems with seaweeds, sponges and sea cucumbers can be established. The IMTA design presented in the next section remains hypothetical until the trophic link between sponges and sea cucumbers has been confirmed. However, it shows a first step in exploring the potential for this type of IMTA.

A case study: IMTA with seaweeds, sponges and sea cucumbers

In this section, we provide a theoretical design for a tropical integrated mariculture that combines the seaweed *Gracilaria sp.*, the Caribbean sponge *Halisarca caerulea* and the Asian sea cucumber *Apostichopus japonica*. These species were selected based upon availability of relevant data. As such, they serve as model species to calculate carbon fluxes between these potential IMTA components. The genus *Gracilaria* is of commercial interest as a producer of marine agar (Peng *et al.* 2009) and this seaweed species has been long studied as extractive

component in fish–seaweed IMTA's (Troell *et al.* 1997; Halling *et al.* 2005; Hernández *et al. 2005*; Abreu *et al.* 2009). *Apostichopus japonica* is a well–studied, commercially harvested sea cucumber species that has already been tested in IMTA settings (Yokoyama 2013). *H. caerulea* was chosen as a model sponge, because its potential to convert DOM into POM has been well characterized (de Goeij *et al.* 2009; 2013; Alexander *et al.* 2014). Since these individual species occur in distinct geographical areas, we want to stress that this combination of species is purely hypothetical and not intended for implementation in reality.

In the proposed seaweed-sponge-sea cucumber system, the sponges are primarily regarded as an intermediate component, whose role it is to (1) convert organic exudates from seaweed (DOM) into particulate sea cucumber food and to (2) increase production of the seaweed through sponge inorganic nutrient exudates (e.g., phosphate, ammonium, nitrate). Additionally, as positive side effect, (3) the sponge is expected to improve the water quality around the IMTA by reducing the microbial load around the farm. Next to their role as trophic intermediates, sponges in IMTA will also convert part of the ingested DOM and particulate food into sponge biomass. This biomass can be harvested for commercial purposes such as sales as natural bath sponges (Duckworth 2009) or production of collagen (Gökalp *et al.* 2019, 2020a). For example, the extracellular matrix of the chosen model sponge species *H. caerulea* is known to contain large amounts of collagen (de Goeij *et al.* 2009). Collagen is a key component in sponge tissue regeneration that is helping to rapidly cover up an exposed wound (Alexander *et al.* 2015). The sponge collagen may be used to promote the regeneration of human tissue or as scaffold for human bone tissue engineering (Silva *et al.* 2014). Notwithstanding this, the primary aim of this case study is to explore the dimensions of the different IMTA components needed to obtain an effective trophic connection.

The dimensions of the IMTA (Fig. 2) were calculated using literature data on productivity (e.g. growth, DOM production) and conversion factors (e.g. wet weight to dry weight, dry weight to carbon). First, the sponge biomass needed to take up all DOM excreted daily by 1 kg of *Gracilaria* dry weight (DW) was calculated (see Box 1). Daily excretion of DOM by 1 kg DW of *Gracilaria* was estimated at 10.8 g organic carbon (g C; Box 1). To take up 10.8 g C within a day, a *H. caerulea* sponge biomass of 176 g DW would be needed (Box 1). Second, it was assumed that out of the daily DOM excretion of 10.8 g C by *Gracilaria*, *H. caerulea* sponges assimilate 61 % or 6.6 g C into biomass, the other 39 % (4.2 g C) being used for respiration (de Goeij *et al.* 2008a). Based on a DOM to cellular detritus conversion factor for *H. caerulea* of 24 % (de Goeij *et al.* 2013), we calculated that an uptake of 10.8 g C by the sponges would result in a release of 2.6 g C of cellular detritus. Hence, an amount of 2.6 g C d^{-1} would be available for ingestion by sea cucumbers in the form of sponge detritus. According to Gao *et al.* (2011), 1 kg (WW) of *A. japonicus* ingests 8.7 g C d^{-1}. This implies that the available amount of sponge detritus of 2.6 g C would be sufficient to feed 299 g WW or 27 g DW of *A. japonicus* biomass (assuming a 9 % DW : WW ratio; Md *et al.* 2018). We assume an assimilation efficiency for carbon in *A. japonicus* of 50 %, based on the range reported for a related species (*Parastichopus californicus:* 40–60 %; Paltzat *et al.* 2008; Hannah *et al.* 2013), which would result in a daily assimilation in biomass of 1.3 g C, i.e. a recovery of 12 % of the DOM that is excreted daily by the seaweeds into sea cucumber biomass (1.3 g C recovered / 10.8 g C produced * 100 %). An additional 4.0 g C (37% of the seaweed-derived DOM) is stored in sponge biomass daily (6.6 g C assimilated - 2.6 g C

released as detritus). The total recovery of seaweed-derived DOM into (sponge and sea cucumber) biomass within this IMTA thus adds up to 49 % (37 % in sponges + 12 % in sea cucumbers).

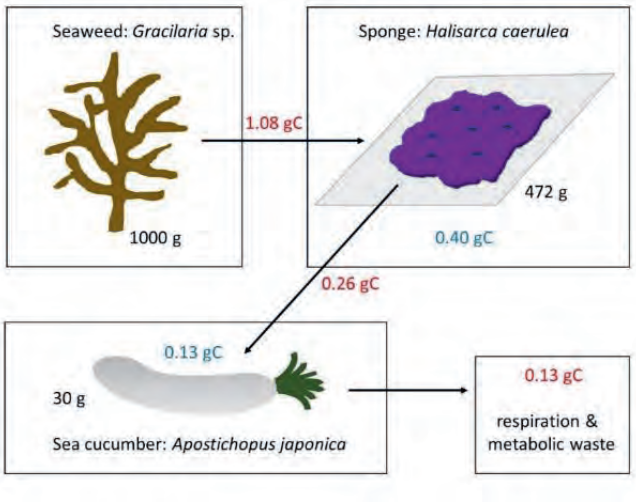

Figure 2. Design of a seaweed-sponge-sea cucumber IMTA. Numbers in black indicate the standing stocks of the three components in g dry weight (DW), numbers in red indicate the daily carbon (C) fluxes between the three components in g C, blue numbers indicate the daily increase in biomass in g C.

This hypothetical design shows that the standing stock masses of the different culture components are within two orders of magnitude (1000 g of seaweed, 176 g of sponge and 27 g of sea cucumber biomass for a balanced IMTA) and that the gain may be substantial: 49 % recovery of a resource that would otherwise have been lost (30 % of the primary production of the seaweeds). These outcomes justify further investigations to test this idea. It should be noted, however, that within any IMTA system, the conversion efficiencies of the waste streams depend on the extent to which the produced waste streams can be retained in the system. Hydrodynamics play a crucial role in this respect: a system placed in the open ocean will have a substantially lower conversion efficiency than a fully closed land-based system (Reid *et al.* 2020).

CONCLUSIONS

The promises of integrated mariculture with sponges (faster growth of sponges and purified water) have been partially fulfilled. Growth and filtration activity of commercially interesting sponges is enhanced when sponges are cultured in the vicinity of organic waste streams from aquafarms and urban discharges (Osinga *et al.* 2010; Ledda *et al.* 2014; Gökalp *et al.* 2019). However, to demonstrate the effects of sponges on seawater quality in in situ systems, scale–up of integrated sponge farming is needed. Since demonstration scale projects are often expensive and logistically challenging, it is recommended to direct efforts on upscaling of sponge farming within integrated mariculture towards sponge species with an established commercial value, such as bath sponges (Duckworth 2009).

Box (1): Excretion of DOM by *Gracilaria sp*. And uptake of DOM by *H. caerulea*:

Five assumptions were used to calculate the daily excretion of DOM per unit of dry weight (DW) of *Gracilaria* and to calculate the amount of *H. caerulea* biomass (DW) needed to take up this amount of DOM:

(1) *Gracilaria* primary production is estimated at 36 g C kg^{-1} DW d^{-1}, which is a conservative estimate within the range 2.7–8.5 g C kg^{-1} WW d^{-1}reported (Orduna-Rojas *et al.* 2013) and assuminga DW : WW ratio of 10% in *Gracilaria spp.* (McLachlan & Bird 1986; Leedham *et al.* 2013).

(2) Gracilaria excretes 30% of its primary C as DOM. This assumption is based on the median of the range 2.8–56.7% of fixed C as reported for other seaweed species (Khailov and Burlakova, 1969, Brylinsky 1977; Haas *et al.* 2011), as no data exist for *Gracilaria sp.*

(3) When actively pumping, DOC uptake by *Halisarca caerulea* is 180 lg C cm^{-3} sponge h^{-1}, which is the median of the range of 157–205 lg C cm^{-3} h^{-1} reported (de Goeij *et al.* 2008a).

(4) The volume : DW ratio of *Halisarca caerulea* is 35 mg DW cm^{-3} (de Goeij *et al.* 2008b; de Goeij *et al.* 2013).

(5) *Halisarca caerulea* is conservatively estimated toactively pump during 12 h d^{-1} (de Goeij *et al.* 2008a). Under the aforementioned assumptions, 1000 g DW of *Gracilaria* would excrete 10.8 g C d^{-1} as DOM (30% of a primary production of 36 g C kg^{-1} DW d^{-1}). To take up this amount of DOM, 5000 cm3 *H. caerulea* tissue would be required (10.8 g C / (0.00018 g C cm^{-3} sponge h^{-1} 9 12 h)). A volume of 5000 cm^3 *H. caerulea* tissue is equivalent to 176 g DW of sponge tissue (5000 cm3/35 mg DW cm^{-3}).

Technology for sponge farming has been established for several species (Duckworth 2009), and some of these techniques have already been applied successfully in IMTA settings (Page *et al.* 2005; Osinga *et al.* 2010). Hence, setting up a large-scale integrated farm can be considered as technically feasible for some of the previously studied species. Nevertheless, because culture success can vary highly among locations (Duckworth 2009), scale-up efforts should always be preceded by small-scale optimisation studies to reconfirm the methodology for the applied settings. Such optimisation studies should include assessments of selective feeding and concentration–dependent feeding by the candidate sponges, as candidate sponges for extractive IMTA applications should have filtration characteristics that optimally suit their proposed role in the IMTA system.

Apart from tailored optimisation of sponge culture procedures, there are many additional factors to consider before the initiation of an integrated algae–sponge–sea cucumber IMTA system (see reviews by Duckworth 2009 for sponges; Chopin *et al.* 2012 for IMTA; Zamorra *et*

al. 2018 for sea cucumbers). Selected sites should have salinity, light and current regimes that are suitable for all IMTA components, and availability of nutrients should be sufficient for each of the components all year around. Safety and suitability of the culture materials and attachment methods must be evaluated with respect to storms, fishing activities, marine traffic, biofouling and predation. The risk for (density-dependent) diseases must be assessed for each of the individual components, and all aspects mentioned must be included in a final estimation of the operational costs.

The ability of sponges to feed on DOM and to convert DOM into POM gives them an added value for use in IMTA, fulfilling complementary functions to other filter feeders commonly applied in IMTA such as shellfish. The sponges can remove potentially harmful particulates (e.g., bacteria, viruses, faecal pellets) and dissolved organic wastes and convert these into food for deposit feeding animals, including commercially interesting species such as sea cucumbers. An IMTA consisting of seaweeds, sponges, and sea cucumbers seems a realistic scenario. To test this idea, the trophic link between sponges and sea cucumbers needs to be confirmed: do sea cucumbers eat sponge-derived detritus? Once established, the proposed seaweed-sponge-sea cucumber IMTA system can provide valuable plant and animal biomass based solely upon the input of sunlight and a natural supply of inorganic nutrients.

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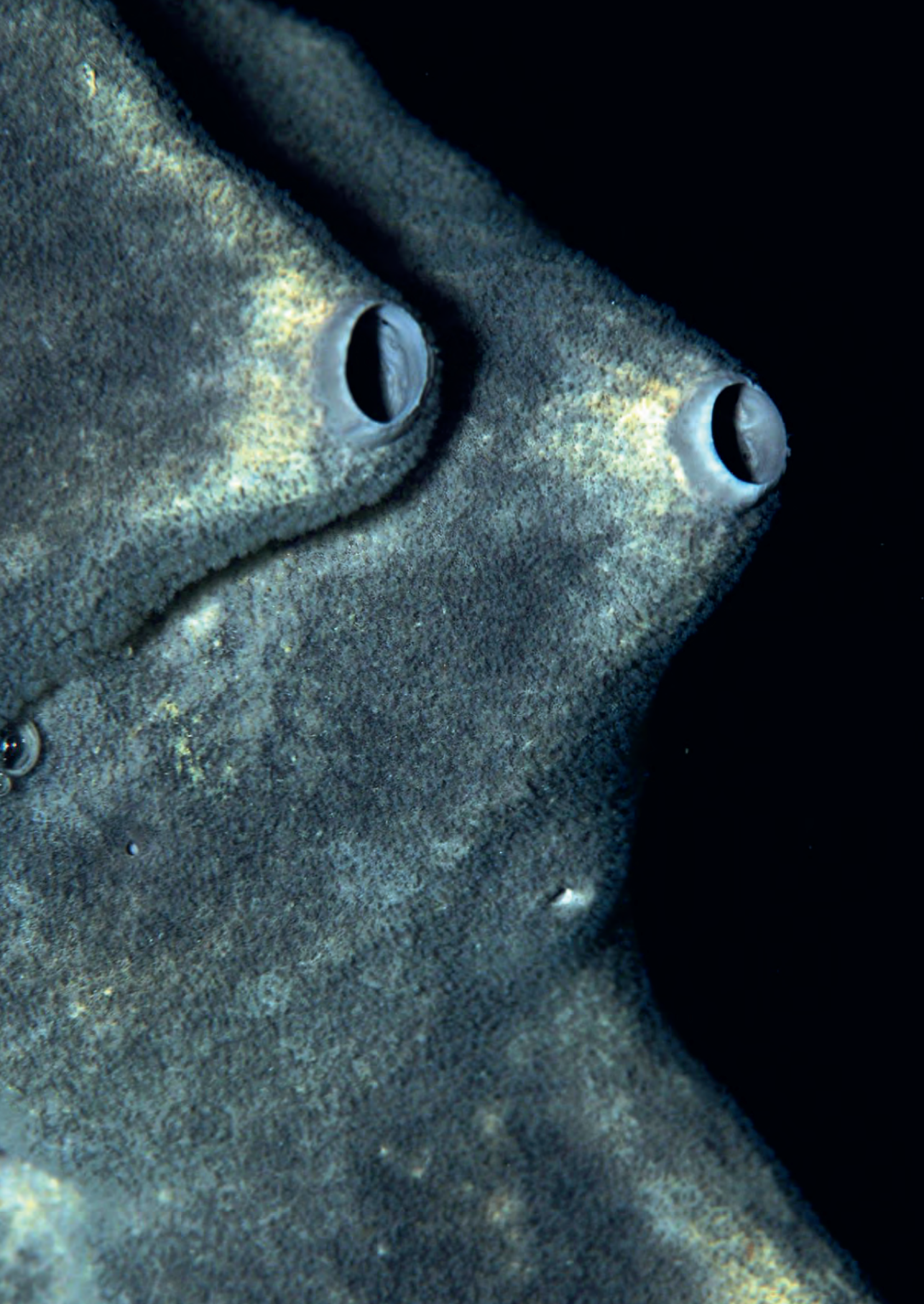

3

Sponge Aquaculture Trials in the East-Mediterranean Sea: New Approaches to Earlier Ideas

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Abstract

Aquaculture trials were conducted in the East Aegean Sea with *Dysidea avara* and *Chondrosia reniformis* to test the possibility of growing these sponges in the vicinity of sea-based fish farms. Culturing sponges in the vicinity of fish farms may have two benefits: the sponges may grow faster due to an increased availability of organic food and the pollution caused by the fish farms is remediated by the filtering activities of the sponges. An initial trial was conducted to compare growth of the two sponge species under floating fish cages to growth in a natural, pristine environment. Explants of *D. avara* were grown suspended on nylon threads, explants of *C. reniformis* were grown in cages constructed of stainless steel. After being one year in culture, nearly 100% of all explants of *D. avara* survived. Growth was highest underneath the fish cages, but growth rates were low compared to earlier studies. For *C. reniformis* survival at the pristine site was 100%, and growth was estimated at 800% per year. All explants cultured underneath the fish cages died due to smothering with sediment. After the initial trial, a new, cost-saving and growth promoting method for *D. avara* was tested at the fish farm location. Explants were grown on PVC pins that were mounted into a metal frame. Growth of the sponges on the pins was eight times faster than that of sponges growing on threads. We conclude that culturing *D. avara* under floating fish cages is feasible when using the new methodology.

Keywords: Sponges, aquaculture, *Dysidea avara*, *chondrosia reniformis*.

INTRODUCTION

Sponge culture in the Mediterranean started already more than two centuries ago with the early work of Cavolini (1785), who made the first attempt to aquaculture natural bath sponges. Since then, many studies have been conducted to develop suitable methods for sponge culture, both in the Mediterranean (e.g. Verdenal Vacelet 1990; Pronzato *et al.* 1999; Van Treek *et al.* 2003; Corriero *et al.* 2004; Pronzato 1999; Pronzato *et al.* 2008] and beyond [e.g. Moore 1910; Dumdei 1998; Duckworth and Battershill 2003a,b; Duckworth *et al.* 2007; Kelly *et al.* 2004). The interest in sponge culture increased due to the discovery of many natural products in sponges: the focus shifted from production of natural bath sponges to sustainable supply of marine natural products.

In the late nineties of the last century, the idea was developed to combine the production of sponges with remediation of pollution, particularly pollution caused by sea-based fish farming (Pronzato *et al.* 1998; Manconi *et al.* 1999). Sponges are very efficient filter feeders. Hence, a large-scale sponge culture may have a profound effect on the water quality in the vicinity of fish farms. Conversely, the organic enrichment originating from the fish may stimulate sponge growth, thus making sponge aquaculture more efficient. Although the idea of integrated sponge/fish aquaculture has been discussed in several papers (Pronzato *et al.* 1999, Fu *et al.* 2006, 2007; Milanese *et al.* 2003; Stabili *et al.* 2006), until now it has not been applied on a commercial scale. Here, we describe preliminary attempts to combine the mariculture of fish (sea bass) with the culture of two Mediterranean Demospongiae species: *Dysidea avara* (Schmidt, 1862) and *Chondrosia reniformis* (Nardo, 1847). Both species have potential commercial interest. *D. avara* is known for its secondary metabolite avarol (Minale *et al.* 1974), which has been in clinical trials as a potential HIV inhibitor (Müller and Schröder 1991). Later, it was discovered that avarol can also be effective against skin diseases such as psoriasis (Müller *et al.* 1991). *C. reniformis* is of interest as a producer of collagen, particularly collagen for cosmetic and biomedical applications (Swatschek *et al.* 2003). Since both avarol and collagen are produced in relatively large quantities by the sponges, aquaculture appears to be a feasible alternative for production of the compounds through chemical synthesis or recombinant production. In this study, the survival and growth of cultured fragments of these two sponge species was measured in the Aegean Sea (East-Mediterranean), hereby comparing an organically polluted fish farm site to different pristine, natural sites. For *D. avara*, the influence of water depth on culture success was studied as well. In the Aegean Sea, *D. avara* is hardly found in shallow water. In deeper waters, the species is more abundant and the individuals are larger. This suggests that the growth of this species is inhibited in shallow water, either by light, or by high water temperatures that occur in the summer season at lower depth.

In addition to comparative studies for site selection, a new, successful technique for the large scale culture of *D. avara* was developed, which may be suitable for the economically feasible production of avarol.

MATERIALS AND METHODOLOGY

Comparison of Sites

Comparative culture trials were executed on four locations in the area around Turgutreis (Bodrum Peninsula, Turkey) with different characteristics: a pristine, rocky site, depth: 38 m; a pristine rocky site, depth 28 m; a pristine site covered with seagreass (Posidonia), depth 18 m; a polluted muddy sediment below a floating cages fish farm, depth 21 m.

Mendola *et al.* (2008) related distribution patterns to flow regimes and argued that *D. avara* preferred sites with average flow velocities below 10 cm s-1. The culture sites used in this study were also characterized by low flow: even during stormy weather conditions, the water around the cultures was nearly stagnant, the average flow velocity being estimated at less than 2 cm s-1 (visual observation).

Figure 1. Stainless steel frame hosting ten explants of *D. avara*.

Table-like stainless steel frames (Fig. 1) were used to culture *D. avara*. *D. avara* explants were held on nylon threads, conforming to earlier studies on this and other species (Pronzato *et al.* 1999, Sipkema *et al.* 2005). One frame was moored on each of the four locations to study both the effect of depth (18-28-38 m) and the effect of fish farm effluent. Cages of stainless steel grids (Fig. 2) were used for *C. reniformis*. For *C. reniformis*, we compared a pristine site (28 m) to the fish farm site. The two cages were moored on these sites and explants of *C. reniformis* were positioned on the cage bottom without artificial attachment. This method was applied because previous studies on *C. reniformis* [Pronzato *et al.* 1999; Van Treek *et al.* 2004) and the related species *Chondrilla nucula* (Pronzato, 2004) indicated that these species cannot be grown suspended on threads or meshes.

For each trial, ten explants of each species were used. To prepare the explants, parent specimens were taken from hard substrate in the vicinity of the deeper culture site (at 28 and 38 m). Some of the materials were transported from the deeper sites to the shallow sites (22 m seagrass meadow and fish farm), where there was no standing stock of this species available.

The four culture sites were revisited two times, in October 2006 and in June 2007 (additional attempts failed due to bad weather conditions and/or limited availability of ship-time). Pictures of the explants were taken while holding a ruler next to the explant as a reference. Growth was determined as the increase in surface area, estimated from the length and width measurements of each explant.

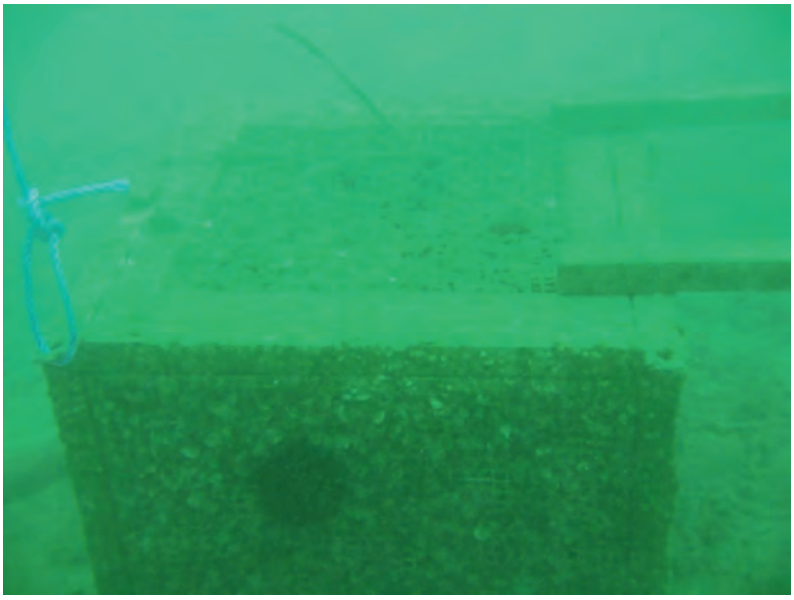

Figure 2. Stainless steel cage hosting explants of *C. reniformis* on the inside.

Scale-Up

In June 2007, i.e. at the end of the first trials, a first scaleup was done for *D. avara*. A new method was designed to promote sponge growth and reduce labor.

The new method uses plastic (PVC) pins as a solid carrier for sponge explants. The pins have a sharp end to accommodate easy positioning of sponge fragments on the pin. After adding the fragments, a rubber stopper is mounted on the sharp end of the pin. A four meter long metal frame was constructed that consisted of two stainless steel bars through which 20 holes were drilled. These bars were mounted in parallel on the two existing culture structures (a frame and a cage; Fig. 3), thus creating a four meter long rack with 20 slots for sponge pins (the rubber stoppers fit into the holes in the steel bars). Because of its resemblance to a local Turkish dish, the new method was termed “Shish Kebab Method” (Fig. 4). Specimens of *D. avara* were harvested from a vertical wall at a depth of 28 m, in a pristine area 30 kms away from the culture site. The sponges were transported immediately to the culture site in cool-containers. Twenty sponge kebabs (each holding 4 to5 sponge fragments) were prepared underwater just below the

sea surface by SCUBA divers and immediately thereafter transported to, and mounted into the culture frame at 21 m depth. The total number of explants was 86.

Pictures of each explant were taken 4 times using a Canon Ixus 750 digital camera with underwater housing: at the day of preparation, after 2 months in culture, after 4 months in culture and after one year. A ruler was held next to the explants as a reference for its size. The horizontal surface of the explants was assessed from the pictures, by multiplying the average length and height of each explant. Volume was estimated by assuming that the average width of an explant was equal to the average length and height measurements.

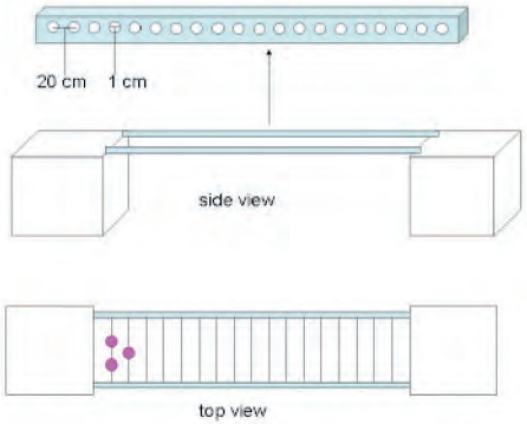

Figure 3. Schematic drawing of the frame used for the Shish Kebab method for *Dysidea avara* aquaculture.

Figure 4. Overview (left) and detail (right) of Shish Kebab method for aquaculture of *D. avara*

RESULTS

Comparison of Sites

Survival rates of the two species were good (90 to 100 %), except for *C. reniformis* at the fish farm location. These explants had become very soft and were covered with a layer of deposited organic matter, which caused them to die (smothering). *C. reniformis* grew well in the

cage at 28 m (Fig. 5; growth was estimated to be a 2 to 3 times increase in size per year, i.e. up to 700 % per year, survival was 100 %). There was no difference in growth of explants growing on the walls/floor of the cage and explants growing in small iron boxes positioned on the bottom of the cage. Survival of *D. avara* was high at all locations, although some explants fell of the culture threads. Four months after being fragmented, all explants had recovered well and had several pumping oscula. The sponges did not attach very well to the nylon lines. In particular at the fish farm location, the explants formed big holes around the threads (Fig. 6) instead of attaching themselves to the threads. Despite this lack of attachment, growth of *D. avara* was most pronounced at the fish farm location (Table 1). No increase in size was observed in explants grown at 28 m and 18 m, explants grown at 38 m had increased in size slightly.

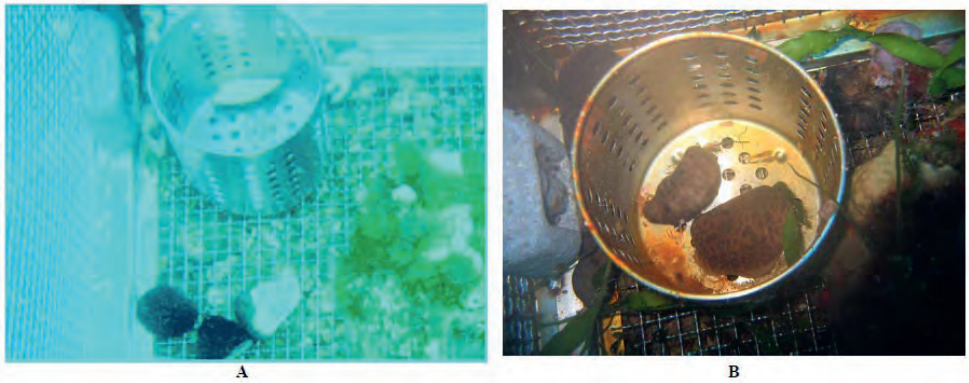

Figure 5. Explants of *C. reniformis* cultured in a cage at 28 m depth (pristine site). A: Initial fragments. B: the same fragments, after 4 months in culture. The tissue completely recovered and the explants had doubled their size.

Scale-Up

Explants of *D. avara* cultured using the Shish Kebab Method showed 100 % survival after being in culture for four months. Recovery was remarkable, given the fact that the explants had been prepared by pulling pieces of sponge from the parent sponges by hand and that they experienced a heat shock during transport and further explanting procedures. Hence, at the start of the culture trial, the explants were in bad condition (Fig. 7a). Within a time frame of two months, all 86 explants had transformed into healthy looking, actively pumping sponges (Fig. 7b).

Table 1. Growth Rates of *D. avara* During First Trial (Expressed as the Percentage of Newly formed Projected Surface Area Related to the Initial Explant Size)

Culture Site	Growth (% increase year-1)
Pristine, rocky site; 38 m	20-50
Pristine, rocky site: 28 m	0
Pristine, Posidonia-covered site: 22 m	0
Floating cages fish farm: 22 m	100

The sponges grew faster than during the initial trial. After four months in culture, the average projected surface area was 1.78 times the initial average projected surface area (Fig. 8). This

increase was significant (paired t-test, n = 86, t = 10.88, p < 0.001). The extrapolated volume was 2.37 times the starting volume, hence biomass more than doubled within a period of four months. Unfortunately, when we revisited after one year, the culture was lost: the frame was disrupted and some of the pins with the explants were found in the sediment around the frame. Therefore, it was not possible to assess the annual growth rate. However, analysis of the data obtained during the first four months (Fig. 8) shows that growth was exponential: in both time intervals measured, the estimated volume had increased approximately 1.5 times. When extrapolating this trend, an annual growth rate of more than 1100 % can be assumed.

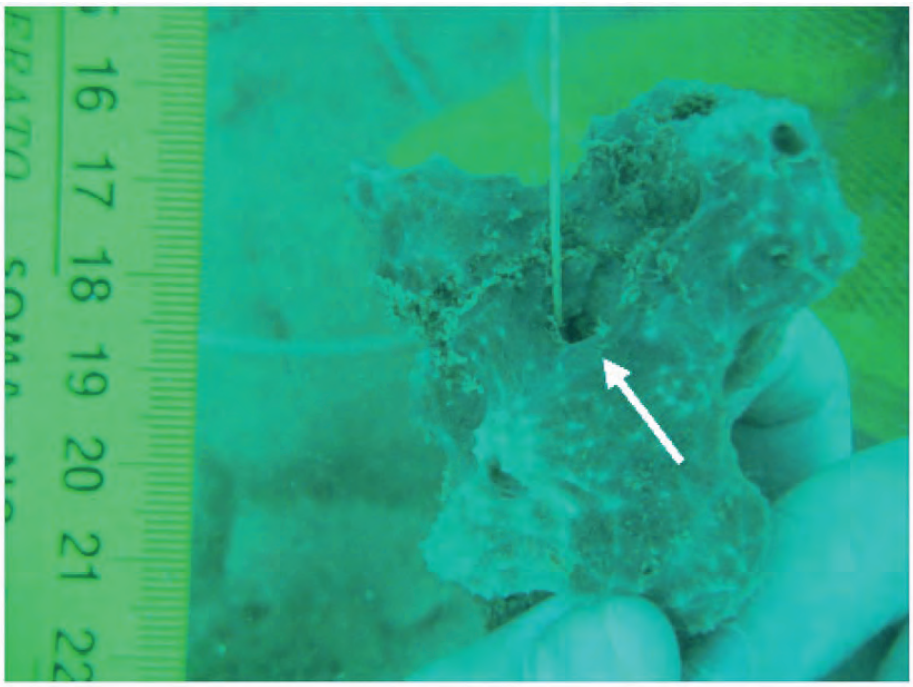

Figure 6. Explant of *D. avara* cultured on a nylon thread at the fish farm site, pictured after being in culture for one year. A hole had developed around the nylon thread (see arrow).

DISCUSSION AND CONCLUSIONS

Our aquaculture trials gave promising results and represent significant progress towards feasible aquaculture of *Dysidea avara* and *Chondrosia reniformis*. The good growth of *C. reniformis* in the cage at 28 m depth supports earlier observations in the Aegean Sea (W. Schatton, personal communication) that this species is easy to grow in clear water environments and that it grows well on solid substrata. Growth rates were higher than those reported in literature for this species (Garrabou and Zabala 2001, Wilkinson and Vacelet 1979). When assuming first order exponential growth kinetics as described by Sipkema *et al.* (2006), a growth rate of 700 % per year equals a daily growth rate of 0.57 %. Garrabou and Zabala (2001) reported a rate of 0.084 % day-1, Sipkema *et al.* (2006) calculated that the highest growth rates reported by Wilkinson and Vacelet (1979) corresponded to a daily growth of 0.18 %, which is three times lower than the growth rate estimated in this study.

The technique applied here to grow *C. reniformis* appeared not to be suitable for turbid environments such as the fish farm location. However, we made an interesting visual observation that may give new clues for designing cultures at such sites. At the polluted site, the downside of the lid of the cage was completely covered with invertebrate life (molluscs, sponges, tunicates, etc.). Growing in this position, the organisms are prevented from being covered by depositions of solids, while maintaining their access to the high suspended food levels. For comparison, the lid of the cage at the pristine location was completely covered, mainly with algae. It is likely that culture of *C. reniformis* will be more successful at the fish farm location when this sponge is grown in a more suspended way. Analogous to this view is the successful culture of corals that has been achieved in the vicinity of fish cages in the Gulf of Eilat (Israel). Corals growing on an open nursery construction that was positioned half way between the surface and the seafloor exhibited higher growth rates than corals growing in pristine areas (Bongiorni *et al.* 2003). Concurrently, reefs on the seafloor in the vicinity of floating fish cages suffer from smothering by high loads of organically rich sediments (Loya *et al.* 2003). Our study sheds new light on the factors determining vertical distribution of *D. avara* in natural waters. During the initial trial, the explants grew fastest at the fish farm location. This site was relatively shallow (22 m) and warm (in summer, the temperature rose above 25 °C). Irradiance was low (considerably lower than at 38 m in clear water) due to the high turbidity at the fish farm site (2-3 meters visibility, compared to 15-25 m at the pristine sites). Hence, light rather than temperature appears to be inhibiting the growth of *D. avara* in shallow water. This is consistent with the distribution patterns of *D. avara* in the Western Mediterranean. Here, *D. avara* is often found in shallow waters, but its presence there is limited to caves and crevices (Mendola *et al.* 2008, Uriz *et al.* 1992).

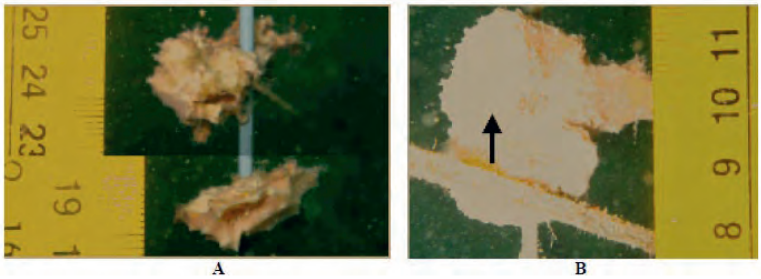

Figure 7. Recovery of explants of *D. avara* cultured using the Shish Kebab method. A: initial fragments. B: the same fragments, 2 months in culture (the explants had fused and developed pumping oscules - see arrow). The scale of the images is equal.

Despite their high survival rate and fast regeneration, growth rates of *D. avara* explants during the initial trial were low (0-100 % per year). Previous estimations of growth of this species made by Sipkema *et al.* (2005) using similar methodologies resulted in much higher growth rates (more than five-fold increase per year). Probably, the low growth was caused by a lack of solid substratum. In contrast to the study by Sipkema *et al.* (2005), we used soft separators (artificial sponges) instead of corks or plastic to separate the individual explants. Hardy separators may act as additional substrates, thus helping to keep the sponges continuously in the same position. *Dysidea avara* was found to reorganize its internal structure as a result of being put into another position ("re-plumbing") (Mendola *et al.* 2007). The sponges in this study failed to attach to the threads and the separators. As a result of this, the explants may face a continuous change of

their position on the thread due to water flow. Better attachment (i.e. more support by solid substratum) will keep the sponge permanently in the same position, so that it will not put too much metabolic energy in processes such as “re-plumbing”. Indeed, the results obtained using the Shish Kebab method (plastic pins) were much better than the results of the nylon line cultures. This is in contrast to a study by Duckworth *et al.* (2007), who did not obtain good results when using a similar technique (termed “spike-method”) to culture the tropical bath sponge species *Rhopaloides odorabile* and *Coscinoderma sp.* This emphasizes that different species require different culture techniques.

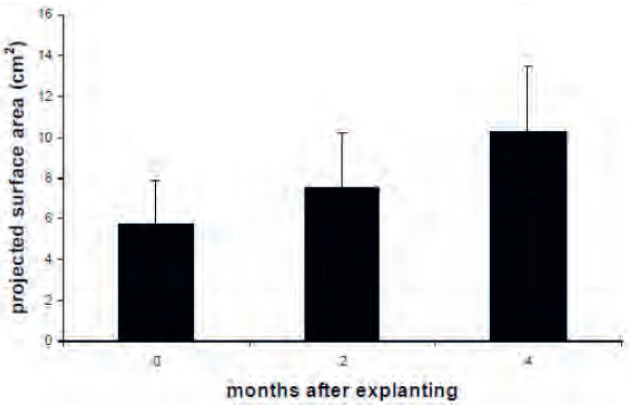

Figure 8. Growth of *D. avara* at the fish farm site using the Shish Kebab method. Error bars indicate standard deviations, n = 86.

The Shish Kebab method was designed to promote growth, but also to reduce costs. Indeed, installing the culture proved to be tenfold faster than the nylon line cultures. During one dive, two divers were able to prepare 20 sponge kebabs, to mount them into the culture frame at 22 meters and to take pictures of each individual explant. Based on this experience, the costs of the materials and the growth rate of the sponges, we estimated a production cost of 36 euro per kg sponge (wet weight). Following the economic analysis made by Sipkema *et al.* (2005), production of *D. avara* becomes profitable below a cost price of 50 euro/kg. An earlier estimation of production costs for *D. avara* (Sipkema *et al.* 2005) was 1.5 times lower (24 euro/kg), but this value was based on a growth rate that was four times higher than the growth rate reported here. This shows that the Shish Kebab method effectively reduced labor costs. It is important to note that the sponges in the culture had an avarol content that did not differ from naturally growing specimens (W. Schatton and R. Osinga, unpublished data). Therefore, it can be concluded that commercial production of avarol is possible using the methodology described in this paper. Using new approaches, the earlier idea of combined fish/sponge culture has come one step closer to reality.

ACKNOWLEDGEMENTS

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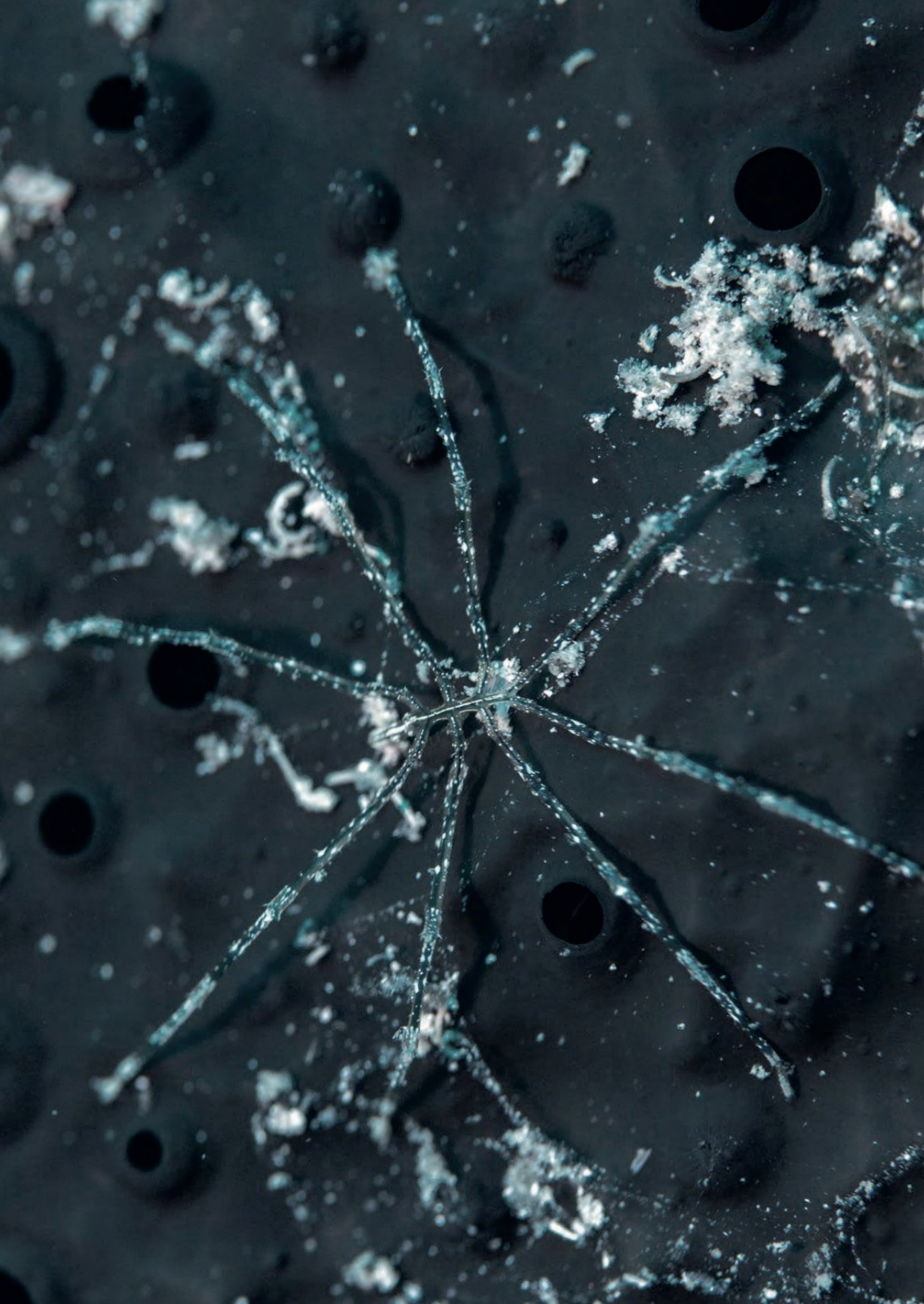

4

The effect of depth on morphology, bacterial clearance and respiration of the Mediterranean sponge *Chondrosia reniformis*

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ABSTRACT

To support successful application sponges for water purification and collagen production, we evaluated the effect of depth on sponge morphology, growth, physiology and functioning. Specimens of Eastern Mediterranean populations of the sponge *Chondrosia reniformis* were reciprocally transplanted between 5 and 20 meters depth within the Kas–Kekova Marine Reserve Area. Control sponges at 5 m had fewer but larger oscula than their conspecifics at 20 m, and a significant inverse relationship between osculum density and size was found in *C. reniformis* specimens growing along a natural depth gradient. Sponges transplanted from 20 to 5 m altered their morphology to match 5 m control sponges, producing fewer but larger oscula, whereas explants transplanted from 5 to 20 m did not show reciprocal morphological plasticity. Despite the changes in morphology, clearance, respiration and growth rates were comparable among all experimental groups. This indicates that depth-induced morphological changes do not affect the overall performance of the sponges. Hence, the potential for growth and bioremediation of *C. reniformis* in mariculture is not likely to change with varying culture depth. The collagen content, however, was higher in shallow water *C. reniformis* compared to deeper–growing sponges, which requires further study to optimize collagen production.

Keywords: Sponge, osculum size, respiration, clearance rate, depth, *Chondrosia reniformis*, collagen, integrated multitrophic aquaculture

INTRODUCTION

Sponges are found at all latitudes, living in a wide array of ecosystems varying in temperature and depth (Hooper & Van Soest 2002; Van Soest *et al.* 2012). They are filter feeding organisms often dominating the benthos in terms of abundance and biomass (Reiswig 1971, 1975; Bell 2008; Ribes *et al.* 2012). Sponges process huge amounts of water daily, 900 times their body volume of water per hour, up to 50,000 liters of seawater per liter sponge volume per day (Vogel 1977; Osinga *et al.* 1999; Weisz *et al.* 2008; Ludeman *et al.* 2017), which is comparable to well–established suspension feeders, such as mussels (Jorgensen 1949, 1954; Riisgård and Larsen 2000). Sponges have a high efficiency and capacity for particle retention (Reiswig 1971, 1975; Ribes *et al.* 1999), preferably small particles (<10 µm) such as bacteria (Reiswig 1975; Milanese *et al.* 2003; Fu *et al.* 2006; Stabili *et. al* 2006; Zhang *et al.* 2009), phytoplankton (Reiswig 1971; Pile *et al.* 1996), viruses (Hadas 2006) and dissolved organic matter (Yahel *et al.* 2003; De Goeij *et al.* 2007; Alexander *et al.* 2014; Mueller *et al.* 2014). This efficient and versatile filtration makes sponges key drivers of the uptake, retention and transfer of energy and nutrients within benthic ecosystems (Gili and Coma 1998; Perez–Blázquez *et al.* 2012; Maldonado *et al.* 2012; De Goeij *et al.* 2013) and makes them interesting candidate species for bioremediation of organic pollution, such as waste streams from aquaculture (Pronzato 1999; Osinga *et al.* 2010; Gökalp *et al.* 2019).

Around sea-based fish cultures, elevated densities of bacteria and reduced oxygen levels caused by excreta of cultured fish and uneaten fish feed negatively impact the seabed and add substantial pressure on the surrounding environment that could even negatively impact the aquaculture and other ecosystem services (Naylor *et al.* 2000; Sara *et al.* 2004; Zhang *et al.* 2009; Nimmo *et al.* 2011; Aguilar-Manjarrez *et al.* 2017). However, the excess amount of bacteria in/around such an organically loaded culture system generates a potential food source for sponges (Pronzato 1999). In our recent study, we discovered that *Chondrosia reniformis* (Nardo, 1847) benefited from mariculture–sourced organic pollution and showed better growth performance in polluted waters compared to a pristine environment (170% versus 79% in 13 months; Gökalp *et al.* 2019). *C. reniformis* has been reported as a rich source of biomedically interesting types of collagen (Silva *et al.* 2016; Fassini *et al.* 2017; Pozzolini *et al.* 2018), which can be used in 3D printing inks for medical applications (i.e. scaffolds for bone and cartilage tissue regeneration, Silva *et al.* 2014; Chattopadhyay *et al.* 2014). This result suggests potential for utilization of this sponge in an IMTA (integrated multi–trophic aquaculture) system where it does not only improve the water quality but also produces collagen.

To optimise the combined use of sponges as producers of natural products–and bioremediators, we need to assess biomass production and associated natural product content and link these to actual in situ clearance rates of the model species. In the literature, different terms are used that relate to the filtration activity by sponges. In this paper, we will refer to the terms pumping, filtration, and clearance as follows: pumping is the volumetric water flow rate through the sponge body generated by the aquiferous system of the sponge; filtration and clearance are defined in the same way and refer to the amount of water per unit of time that is entirely cleared of a specific type of component, e.g., bacteria, microalgae, or dissolved organic matter (DOM). We use the term clearance usually in association with the measurements that are

applied to determine the clearance/filtration rates. Pumping and clearance/filtration relate to each other through the retention efficiency for the specific component, which means that, for example, clearance rates for bacteria can be different from clearance rates for microalgae. Under in situ conditions in open water, the actual particle uptake rate for a sponge can be calculated by multiplying the ambient concentration by the clearance rate. In this case, a constant ambient particle concentration can be assumed because seawater around the sponge is replenished by water movement before the exhalant water can be inhaled for a second time.

To fully comprehend the role of sponges in transfer of nutrients and energy in natural and artificial ecosystems such as IMTAs, it is important to understand how their particle uptake rates are affected by physiological and environmental factors. Some factors that influence sponge clearance have already been identified. For example, sponges hosting large quantities of associated microbes (high microbial abundance, or HMA sponges) including *C. reniformis* usually have lower pumping rates than species with low numbers of associated microbes (Weisz *et al.* 2008). Also elevated amounts of suspended sediment in the surrounding water reduced the sponge pumping activity (Gerrodette & Flechsig 1979). Ambient flow and sponge morphology may influence pumping activity and clearance efficiency of a sponge by reducing the amount of energy that is needed for active pumping (Vogel 1974; Mendola *et al.* 2008) and the efficiency of retaining particulate matter from the filtered water. Conflicting results have been reported on the relationship between temperature and filtration by sponges (Frost *et al.* 1980; Riisgård *et al.* 1993; Ribes *et al.* 1998), and information on effects of seasonality on filtration by sponges is scarce. Very recently, Morganti *et al.* (2019) conducted a comprehensive study spanning over 2 annual cycles to assess the factors that regulate in situ sponge pumping of 5 Mediterranean sponges (including *C. reniformis*). Unexpectedly, these authors reported no significant effect of temperature and particulate organic matter on sponge pumping rates and no clear trend of seasonality. Instead, sponge size was found to be the main predictor of pumping rate.

To date, no studies are available that investigated the role of water depth on sponge pumping rate and bacterial clearance. We investigated the bacterial clearance rates as it both substitutes a good proxy for pumping and it has relevance to the bioremediation potential of the sponges. In maricultures, culture depth may vary between and within locations. Eventual effects of water depth on the sponge pumping activity may influence the choices for sponge culture sites and sponge culture methodology. In an earlier survey, we observed some physiological differences between *C. reniformis* populations from different depth zones (Gökalp *et al.* 2020c). Osculum density, osculum size and the associated osculum outflow rate differed significantly between two depth groups (0-3 m and 20-25 m). Bacterial clearance, however, was not measured so the functional consequences of the observed morphological differences for the filtration/bioremediation capacity of *C. reniformis* remain unknown (Gökalp *et al.* 2020b). Concurrent with potential effects on bacterial clearance and filtration efficiency, water depth may also affect other parameters that are relevant to sponge mariculture, such as sponge growth and the production of secondary metabolites and collagen. Therefore, the aim of this study was to investigate the effect of depth on the filtration capacity (in situ bacterial clearance rates), metabolism (respiration rate as oxygen consumption), morphology (density and size of oscula), growth and collagen/biomass production of *C. reniformis*. Up to this date, there is no scientific work that combines sponge growth performance and related biomaterial content to natural

clearance rates of sponges (except from Cebrian *et al.* 2006, who focused on the effect of copper tolerance on clearance rate). Thus, the current work represents a key step forward in terms of understanding the morphological plasticity and performance of sponges, as well as the application of this knowledge to IMTA for bioremediation and collagen production.

MATERIALS AND METHODS

Study location & seawater parameters

The study was conducted over a period of 79 days, during July–October 2018, at Pina Reef, a location within the Kas-Kekova Special Environmental Protected Area (SEPA), Turkey (Fig.1). Pina Reef is located at the eastern side of Five-Islands, 4.3 kilometers south of Kas, and is exposed to wind and wave action originating from the open sea (west direction). Pina–reef wall is located right next to a 350 m long wall structure located transversely at Northwest–Southeast in between 14 m to 32 m depth. Pina–small reef, located 70 m south of Pina–reef wall, is a reef shoal with a depth ranging from 3.8 m to 22 m, with adjacent *Posidonia* sea grass meadows in south and east directions. Water temperature and salinity during the study period were measured by a multimeter during the clearance rate & respiration analysis (Multi 3620 IDS with TetraCon 959 and FDO 925 sensors, WTW, Germany).

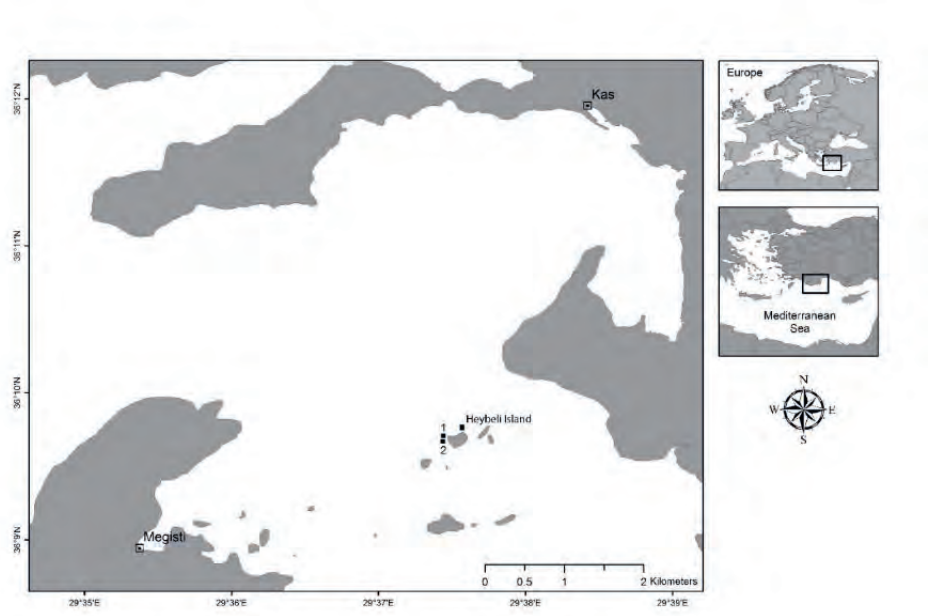


Figure 1. Map of Kas-Kekova Special Environmental Protected Area. Pina Reef diving location is located at the eastern tip of Heybeli Island. The exact locations of experiments; 1) Pina reef wall (20 m depth) 2) Pina small reef (5 m depth) (36°09'42.4"N 29°37'26.5"E; 36°09'40.2"N 29°37'26.3"E; respectively).

Sponge collection, seeding and transplantation

Sponge specimens for experiments were collected from 5 and 20 meters (20 per depth, 40 in total), cut into pieces of 3–4 cm following the method described in Gökalp *et al.* (2019) and

fixed onto grey PVC plates with a rim with a radius of 17 cm and 2.7 cm high. Each PVC plate had 15 cm high cylindrical protective PVC rim (Fig. 2) and was covered with 2 cm–mesh sized chicken wire in order to eliminate predation from larger marine life (e.g. sea turtle intrusion on sponges in culture with boxes lacking protection; personal observation). The protective rim also prevented sponges from migrating off the plate and prevented that they would be carried away by occasional strong water currents (Gökalp *et al.* 2019).

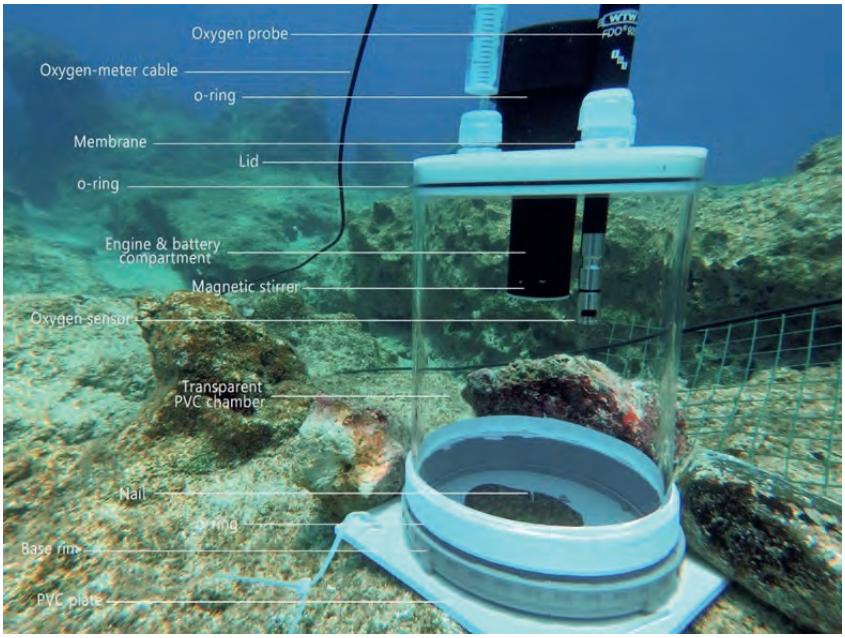


Figure 2. Incubation chambers used in bacterial clearance and respiration experiments. The oxygen probe was connected to a surface multimeter with a Kevlar-protected 20 m cable (WTW, Germany). Four O-rings ensured a water-tight incubation chamber. The first one was located around the black engine and battery compartment, sealing the lid. The second one was in the oxygen sensor lid. A third one was located around the white lid, sealing the transparent acrylic chamber. The final one was located inside the grey base-rim, sealing the chamber. At the end of the black engine and battery compartment, a magnetic stirrer was located.

Each plate carried a chromium nail at the centre with the sharp edge facing upwards (4 cm in height) to secure specimens to the plate. After fixing the explants on pre–labelled PVC plates, they were left at their respective depth of origin (either 5 or 20 m) for 5 days to provide sufficient time to recover and attach to the PVC plates (Cebrian *et al.* 2006). Subsequently, the PVC plates were transplanted by moving 10 plates from 5 to 20m depth and 10 plates from 20 to 5 m depth (Fig. 3; N=10). Following transplantation, sponges were left to acclimatize for seven days before the incubations started.

Determination of sponge size, growth and morphological characteristics

The size of the sponges, the number and size of the oscula were measured following the acclimatization period. Simultaneously, sponges were photographed from top and four sides with a ruler for scale by using a Canon Eos 5D Mark IV camera & Ikelite 5D housing setup. Sponge surface area (SSA) was measured from the projected surface area based on a picture taken from the top by using ImageJ software. The pictures from the sides were used to determine the

average height of the sponge. Multiplying the measured average height with the SSA provided the approximate volume of the sponge (V_{sponge}), assuming a cylindrical shape. At the end of the experimental period, 5 sponges per group were weighed (wet weight, WW) and volume was measured by water displacement to determine the accuracy of the volume calculation. To determine wet weight (WW) to dry weight (DW) ratio, 6 individuals of the same species that were not used for the experiment, were weighed, dried and reweighed (Supplementary Table 1). These size measurements were used 1) to determine growth (using (t1-t0/t0)*100% to calculate % increase in SSA over 8 weeks; Gökalp *et al.* 2019) and 2) to normalise metabolic rates to a biomass parameter (volume). Average osculum size (hereinafter referred to as osculum surface area (OSA), sponge surface area (SSA) and osculum density (OD, the number of oscula per unit of sponge surface) were determined for each of the 40 experimental sponges, hereby using the pictures taken for size measurements. From these parameters, osculum number to sponge size ratio and the ratio of oscular surface area to sponge surface area (OSA/SSA) were calculated. In addition, multiplication of OD with OSA provides the pumping potential of a sponge, by expressing total OSA per SSA. For comparison, the same parameters were determined for a series of *C. reniformis* specimens occurring along a natural depth gradient ranging from 2 to 25 m depth with scuba diving at several sites within 1 km of the experimental locations.

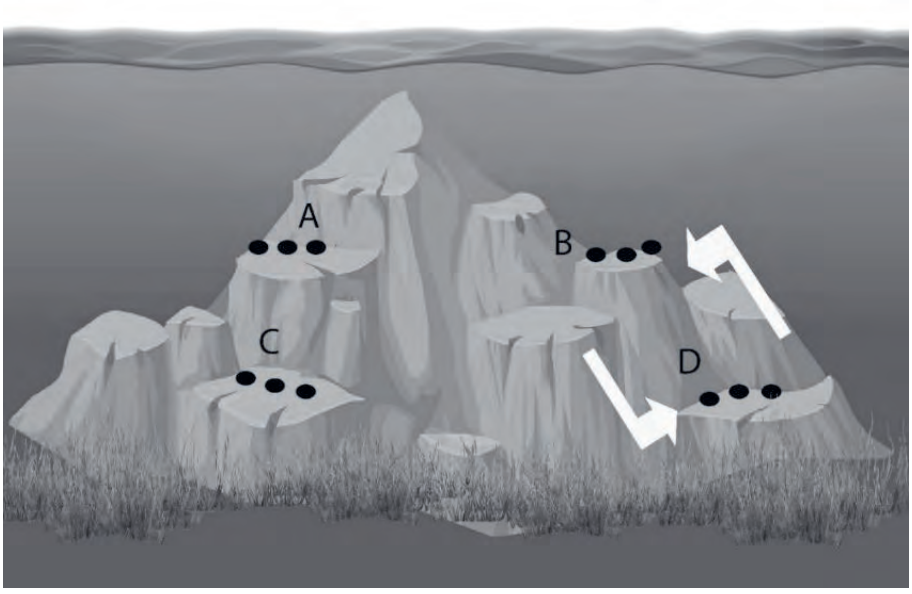


Figure 3. Overall view of transplant and control groups : Group A–5 m control; Group B–5 m transplanted; Group C–20 m control; Group D–20 m transplanted. For the experiment, 10 specimens from 5 m moved to 20 m, and 10 specimens from 20 m moved to 5 m (50 m distance to each other). The other remaining sponges were kept at their depths as control, resulting in four groups of N=10 sponges per group.

Clearance and respiration rates

The circular rims on square PVC plates (Fig. 2), were designed to fit airtight to a transparent PVC chamber with 6.75 L inner volume and specifically developed for in situ clearance and respiration measurements (Wageningen University). The upper side of the cylinder could be closed with a lid in which a magnetic stirrer with battery pack (Jansen Tholen B.V., The

Netherlands) was mounted. The stirrer could create sufficient water circulation and continuous mixing of seawater in order to equalize the oxygen distribution within the cylinder and to prevent particles from settling onto the bottom plate. Each lid also were fitted with two diaphragms for water sampling and an opening for an oxygen probe (WTW, Germany). Together, the PVC plate with rim, cylinder and lid formed a water–tight incubation chamber (Fig. 2). During the incubation experiments the protective cylinder and lid of chicken wire was removed and replaced by the water–tight incubation chamber set up. The chambers were applied for simultaneous in situ measurement of bacterial clearance and respiration of the experimental sponges. Eight weeks after transplantation, all 40 specimens in the experiment were incubated to determine bacterial clearance and respiration rate. 10 empty PVC plates were incubated to determine background activity not associated with the sponges. Prior to the incubations, the chicken wire and the protective rim were carefully removed from the bottom plates, thus minimising stress to the sponge specimens. The deposited sediment and live organisms accumulated on the O–rings and PVC plates were removed with a toothbrush and no organic matter was left on the PVC plate inside the base rim. Then, the cylinders were secured onto the PVC plates and left to acclimatise for 15 minutes before the incubations. After the acclimatisation period, the chamber lid was closed and using a syringe, the first 10 mL water sample was taken directly (t0) and a second 10 mL water sample was taken 15 later (t1). Fifteen minutes was deemed sufficient time to obtain reliable results for both oxygen depletion and bacterioplankton decrease considering the size of the containers and reported bacterial grazing rates and respiration rates from the literature (Turon *et al.* 1997; Milanese *et al.* 2003; Cebrian *et al.* 2006). The collected water samples were labelled, right after surfacing from the dive, immediately fixed with 0.57 mL 35% formaldehyde solution to a final concentration of 2–4% and placed in ice containers. At the end of each incubation day, fixed samples were filtered over white 25 mm diameter 0.2 µm polycarbonate membranes (GE Osmonics, Minneapolis, MN), which were structurally supported by a 0.45 µm GF–F–type membrane (Whatman International Ltd., Maidstone, England). Subsequently, filters were air–dried in the laboratory for at least 15 min and stored at -20˚C in Eppendorf tubes until further use. To determine the total bacterial counts the filters were placed on a microscopic slide and 10 µL DAPI–mix was added to stain bacteria present as described previously in de Goeij *et al.* (2007). Bacterial numbers were determined based on pictures taken under a fluorescence microscope (Leica DM6 B; Leica Microsystems, Germany). Per filter, 10 images were taken using Leica–LasX software and a DFC365 FC camera (Leica Microsystems, Germany). From the pictures average bacterial numbers were determined by counting 10 fields or up to a maximum number of 250 bacteria by using ImageJ. According to Coughlan (1969) the clearance rate is a measure of depletion of microorganisms found in the surrounding water as a function of time. Hence, clearance rates were calculated using the following formula (modified from Coughlan, 1969):

$$CR = V_{chamber} \cdot {V_{sponge}}^{-1} \cdot (rate\ sponge - rate\ blank) \qquad (1)$$

in which $V_{chamber}$ stands for volume of water in the incubation chamber (millilitre), V_{sponge} is the volume of the sponge. Rate of bacterial concentration change for the sponge (rate sponge) and the blank chambers (rate blank) are calculated as:

$$\text{Cell change rate} = \frac{\ln(C0) - \ln(Ct)}{t} \qquad (2)$$

in which C_0 and C_t are the concentration of counted bacteria per millilitre at (t_0) and (t_1) and t is the total incubation time (15 minutes).

To determine respiration rates, oxygen concentrations in the chambers were measured during the incubations with a Multi 3620 IDS (WTW, Germany) connected with 20 m cables to two FDO925 probes (WTW, Germany). During the incubations, oxygen values were logged every 10 seconds from a boat, which was anchored right on top of the divers to release sufficient amount of cable underwater. Respiration rates were only measured for the sponges at 5 m depth due to the limited reach of the WTW cables and prolonged diving times. Linear graphs were fitted to the measurements and respiration rates were calculated with the slope of the graphs, which represents the oxygen decline in mg $O_2 \cdot L^{-1} \cdot min^{-1}$. Respiration rates (RR) were calculated with the following formula:

$$RR = (O_2\ slope\ sponge - O_2\ slope\ blank) \cdot V_{chamber} \cdot {V_{sponge}}^{-1} \qquad (3)$$

Collagen Extraction

After 8 weeks, 3 to 5 sponges from each of the four experimental groups were randomly collected and immediately frozen at -18 °C following the dives. Frozen samples were transported in dry ice containers to the facilities of University of Minho, Portugal, and kept at -20 °C until further analysis. All procedures described below were performed separately with sponges pooled per experimental treatment to obtain enough material for the analysis. Samples were thawed and any exogenous materials on the sponges were removed by rinsing in dH2O. All steps for collagen extraction were carried out at 4 °C. Next, the wet weight was determined and the sponges were cut into small pieces of roughly 1 x 1 x 1 mm. Excess water was poured from the marine sponge samples, 5 sponge volumes of disaggregating solution (50 mM Tris–HCl buffer pH 7.4, 1M NaCl, 50 mM EDTA and 100 mM 2–mercaptoethanol) were added and left under stirring for 4 days. The collagen solutions (CS) were filtered with a nylon mesh to remove remaining undissolved fragments and the solution extensively dialyzed in dialysis tubing cellulose membrane for 7 days with 2 dialyzing buffer changes every per day (CS/dialyzing buffer ratio 1:1000) against d_{H2O} to remove all traces of 2–mercaptoethanol. The suspensions were centrifuged for 10 min at 1200 g to further remove cell debris and sand particles. To collect the collagen from the suspension, another centrifugation followed for 30 min at 12100 g yielding pellets containing collagen, which then were resuspended in dH2O. Collagen re–extraction was performed, by repeating the second centrifugation step. Collagen solutions were stored at 4 °C. Total collagen content was determined by freeze–drying and weighing (dry weight) the extracted material.

Collagen Quantification

Following collagen extraction, the obtained collagen solutions were analyzed regarding its collagen content. To determine the total amount of collagen extracted, each collagen solution was freeze–dried and then weighted (dry weight). The wet collagen extraction yield was determined using Equation (4):

$$Yield\ of\ collagen\ (wet)\ (\%) = \frac{Weight\ of\ collagen\ (mg)}{Wet\ mass\ C.reniformis\ (mg)}) \times\ 100 \qquad (4)$$

Data Analysis

All data were tested for normality and homogeneity of variances. A one–way ANOVA was performed to test for differences between the four treatment groups on sponge morphology, clearance rate and growth. Respiration rates of transplanted and control sponges at 5 m depth were also compared with one–way ANOVA. Planned contrasts with Bonferroni correction (i.e. using a corrected α for significance) were used to follow–up significant ANOVA's. Pearson's product–moment correlations were used to correlate osculum size and density of natural sponges with depth, whereas Spearman's rho for total oscula surface area (SA) per unit of sponge surface area (SA) was used when the data were not normally distributed. Statistics was done using SPSS 25 (IBM, USA), graphs were plotted with Sigmaplot 12 (Systat Software, USA).

RESULTS

General Observations

Water temperatures and salinity values recorded at the site during the transplantation and incubation experiments ranged between 26–28 °C and 38.5–38.8 g L^{-1}, respectively. The *C. reniformis* explants exhibited a swift recovery. Open surfaces had completely healed five days after sampling and cutting (Supplementary Fig. 1). The visible distinctive inner layers of the sponges were observed to change as early as from day one. A white layer was formed in and around the open surfaces, cut marks completely disappeared and the large gaps observed in the mesohyl of some explants were filled completely after 5 days. The explants rapidly reshaped their aquiferous structure, reorganizing the collagen–rich ectosome and mesohyl while regenerating cut surfaces.

Natural Sponge Morphology

In sponges along a natural depth gradient, there was a significant decrease in average osculum size with depth (Pearson's r=-0.245, p=0.044; Fig. 4a) and a significant increase in OD with depth (Pearson's r=0.444, p<0.001; Fig. 4c). No relationship between depth and total OSA per sponge area was found for natural sponges (Spearman's rho=0.117, p=0.335, Fig. 4e). Hence, on average, deep water sponges have more, yet smaller oscula than shallow water sponges, but the total osculum surface per SSA remains the same with increasing depth.

Effect of Depth and Transplantation on Morphology

For OSA, significant differences between the four experimental groups were found (Table 1). Sponges transplanted from 5 m to 20 m did not reduce their osculum size and had significantly larger oscula compared to the 20 m control explants (Table 1). In contrast, sponges transplanted from 20 m to 5 m did increase their osculum size, resulting in no significant difference with the 5 m control group (Fig 4b; Table 1). For OD, a similar pattern was found, as sponges transplanted from 5 m to 20 m did not alter their morphology and thus had significantly different OD from the 20 m control sponges (Fig. 4d; Table 1). Sponges transplanted from 20 m to 5 m matched the OD of 5 m control sponges, with no significant difference found between these groups (Fig 4d; Table 1). One observation to note is the effect of depth on non-transplanted control sponges. Although not significant here (Table 1), there seems to be a considerable osculum size difference between the 5 m and 20 m control groups (0.9 cm^2 vs 0.3 cm^2, respectively, Fig 4b). In addition,

there is a highly significant difference (Table 1) in OD between the aforementioned groups (0.10 oscula cm^{-2} vs 0.24 oscula cm^{-2}, respectively, Fig 4d). Thus, osculum size is about 3 times larger at 5 m, whereas OD is approximately 2 times lower, which matches with the observations on the natural sponges (Fig 4a, c). For total OSA per sponge area, no significant differences were found between the experimental groups, again in line with natural sponges (Fig 4e,f; Table 1).

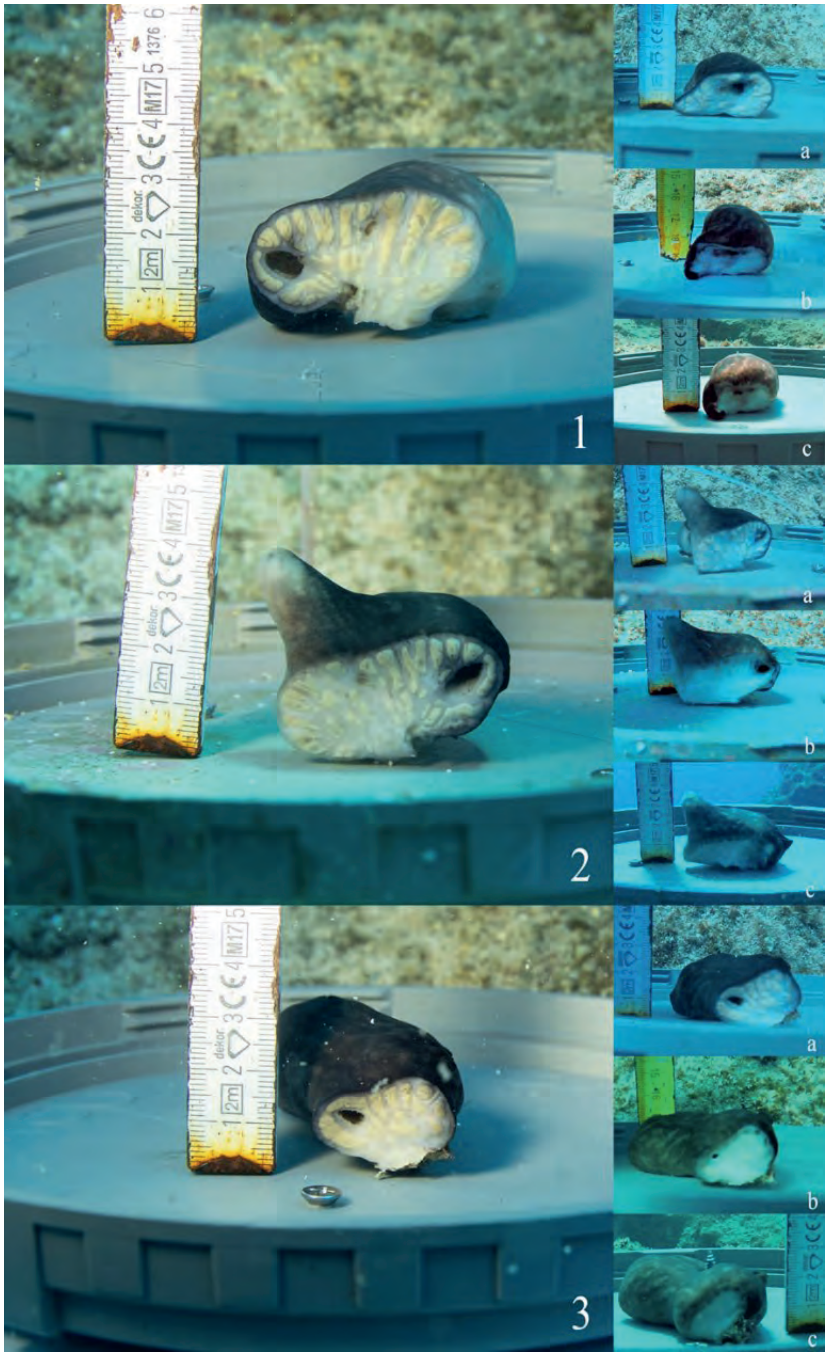
Table 1. One-way ANOVA testing all four experimental groups for differences in oscule surface area, oscule density, % sponge growth in 68 days of culture and clearance rate, and one-way ANOVA testing for the effect of transplantation on respiration rates (N = 8-10).

Variable	F/t	df	error	*p*
Oscule surface area (OSA)				
	3.391	3	30	0.031*
Planned contrasts with Bonferroni correction				
5 m control versus 20 m control	1.817	1	30	0.079
5 m control versus 5 m transplanted	0.811	1	30	0.424
20 m control versus 20 m transplanted	3.052	1	30	0.005**
Oscule density (OD)				
	7.322	3	32	0.001**
Planned contrasts with Bonferroni correction				
5 m control versus 20 m control	-3.557	1	32	0.001**
5 m control versus 5 m transplanted	-1.012	1	32	0.319
20 m control versus 20 m transplanted	-4.464	1	32	0.000***
Total oscule surface area per unit of sponge surface area				
	0.821	3	31	0.492
Clearance rate				
	0.199	3	30	0.896
*Respiration rate***				
	0.143	1	15	0.711
% growth				
	1.447	3	32	0.248

*p<0.050, **p<0.010, ***p<0.001, ****respiration rate measured at 5 m only.

Supplementary Table 1. Volumes, WW and DW from 6 randomly collected *C. reniformis* specimen.

Sponge Specimen	V calculated (cm^3)	V measured (cm^3)	WW (g)	DW (g)	WW:DW	Vm:DW	Vc:DW
1	17	20	21	4	5.25	5.0	4.32
2	24	25	28	4	7.0	6.25	6.10
3	32	25	33	5	6.6	5.0	6.31
4	40	30	42	8	5.25	3.75	5.03
5	39	38	43	8	5.38	4.75	4.84
6	21	17	23	5	4.6	3.4	4.25
mean					**5.68**	4.69	5.14
St.dev.					0.92	1.02	0.88



Supplementary Figure 1. Healing phases of *C. reniformis* specimens following the sampling/cut. The larger background pictures were taken at Day 1 (specimen 1, 2, 3), right after the incision. The smaller pictures were taken at Day 2, Day 3 & Day 5 (top to bottom; a, b, c) respectively. The explants open surfaces observed to be completely healed five days after being cut. The damages caused from incision are covered promptly, the holes are minimized and ectosome layer of the sponge observed to cover the damaged areas.

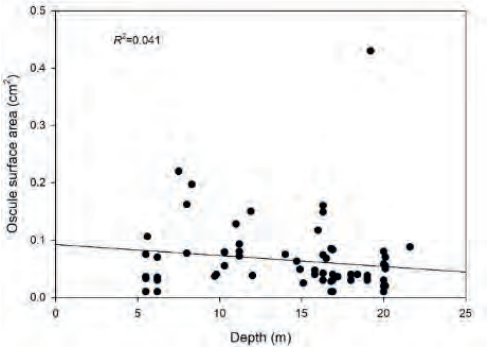


Figure 4a. Natural sponges. Significant decrease of average oscule surface area over depth (Pearson's r=-0.245, *p=0.044*). Dots represent individual sponges.

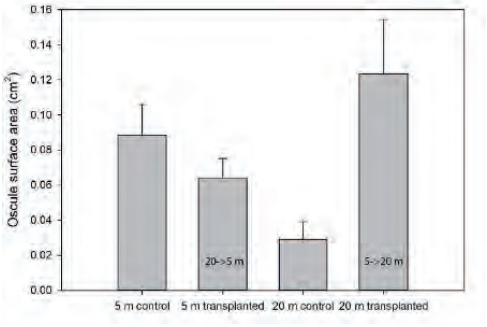


Figure 4b. Experimental sponges. Oscule surface area (cm^2) measured 8 weeks after transplantation. Values are means + SEM, N=8-10 per group (***20 m control vs 20 m transplanted F = 3.052, p = 0.005*).

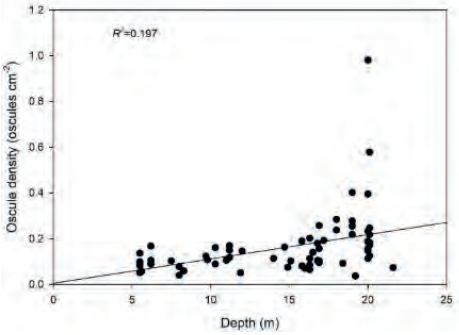


Figure 4c. Natural sponges. Significant increase of oscule density over depth (Pearson's r=0.444, *p<0.001*). Dots represent individual sponges.

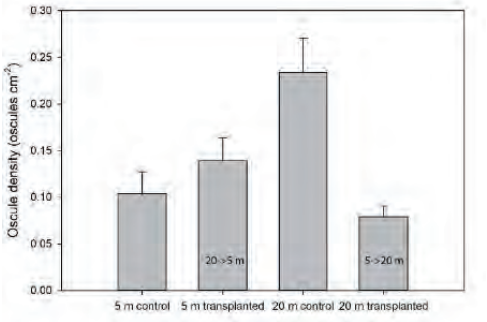


Figure 4d. Transplanted sponges. Oscule density measured 8 weeks after transplantation. Values are means + SEM, N=8-10 per group (***5 m control vs 20 m control, F = 3.052, p = 0.001*; ****20 m control vs 20 m transplanted, F = 3.052, p = 0.000*).

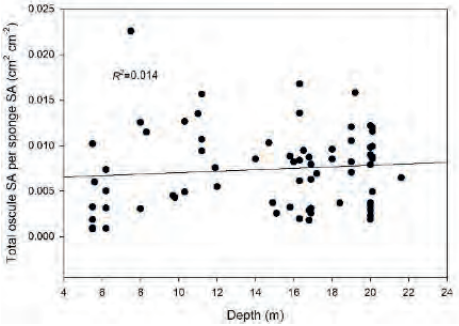


Figure 4e. Natural sponges. Total oscule surface area (OSA) per unit of sponge surface area (SSA) of natural sponges. (Spearman's rho=0.117, *p=0.335*). Dots represent individual sponges.

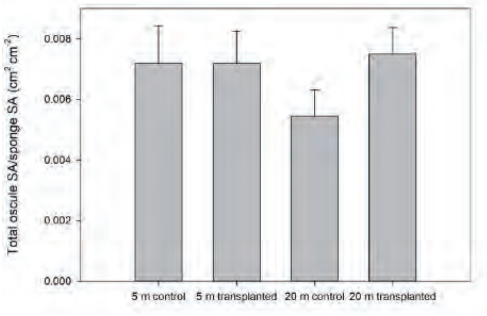


Figure 4f. Experimental sponges. Total oscule surface area (OSA) per unit of sponge surface (SSA) measured 8 weeks after transplantation. Values are means + SEM, N=8-10 per group.

Clearance and Respiration Rates

Mean clearance rates of the experimental sponges were 136.3 ± 21.09 mL cm^{-3} h^{-1} (Table 2). No significant effects of depth and transplantation on clearance rates were found (Table 1). Respiration rates, only measured at 5 m due to technical limitations, were 0.07 ± 0.01 mg O2 cm^{-3} h^{-1} (Table 2) did not differ between transplanted and control sponges (Table 1).

Survival and Growth Rates

Both the control and transplanted sponges readily adapted to their new habitat on the incubation plates. Two sponges from the 5 m control group were lost, they disappeared due to unknown causes, but the rest of the explants (N=38) remained at the centre of the plates, where they were initially attached and 95% survival was achieved (Table 2). Overall growth rate was 63.6 ± 5.4%, after 8 weeks in culture (Table 2) without significant effects of depth and transplantation (Table 1).

Collagen Quantification

Collagen yields of control and transplantation groups are shown in Table 3. The highest extraction yield was observed for the 5 m control group (35.5% of wet mass), whereas the 20=>5 m transplanted group had the lowest collagen content (14.5%). The 20 m control and 5=>20 m transplanted groups revealed similar collagen yields (18.4% and 21.6%, respectively).

Table 2. Survival, growth, clearance and respiration rates (%) of experimental sponges measured after 8 weeks in culture. Values are means + SEM.

Transplantation Experiment	Pooled	5 m control N = 8	5 m transplant N = 10	20 m control N = 10	20 m transplant N = 10
(8 weeks in culture)					
Survival Rates (%)	95	80	100	100	100
Growth Rates (%)	63.6 ± 5.4	83.0 ± 13.2	52.5 ± 8.3	60.3 ± 11.1	70.2 ± 10.4
Clearance Rate (mL cm^{-3} h^{-1})	136.3 ± 21.1	164.8 ± 43.9	120.8 ± 53.3	131.4 ± 32.9	136.5 ± 43.9
Respiration Rate (mg $O^2cm^{-3}h^{-1}$) *	0.07 ± 0.00	0.06 ± 0.00	0.06 ± 0.01		

* Due to the cable reach only measured at 5 m depth.

Table 3. Total collagen content (% ww) of control and transplanted sponges after 64 weeks (N=1 per group).

Treatment	**Collagen Yield (%)**
5 m control	35.5
5 m transplanted	14.5
20 m control	18.4
20 m transplanted	21.6

DISCUSSION

The aim of this study was to investigate the effect of depth on bacterial clearance, morphology, respiration, growth and collagen production of *C. reniformis*. Our data show that osculum morphology is depth–dependent and collagen content probably too, whereas bacterial clearance rate, respiration and growth are not.

Morphology and bacterial clearance rates in relation to depth

Along a natural depth transect, both OSA and OD significantly correlated with depth in a reciprocal way, with deeper living sponges having more, but smaller oscula. These trends are in line with our earlier survey (Gökalp *et al.* 2020c) and with measurements on non–transplanted experimental sponges in the current study. In the earlier survey, average OSA of sponges in shallow water (0–3 m) was 0.71 cm^2 compared to 0.05 cm^2 in deeper water (20–25 m), so 14-fold smaller. In the current study, average OSA of the non-transplanted sponges was 0.09 cm^2 at 5 m depth versus 0.03 cm^2 at 20 m depth, so 3-fold smaller. The most prominent difference with our previous study was the almost 8 times smaller OSA which may relate to the difference in depth of the "shallow" sponges (0–3 m versus 5 m). We hypothesize that in more shallow water effects of wave action and the concurrent resuspension of also larger particles (e.g. course sand, small pebbles) is much greater than in deeper waters. In order to prevent clogging of their oscula by these particles, sponges in shallow waters need stronger pumping forces to remove these particles out of their oscula, which is favoured by fewer but larger oscula. This effect may become most explicit in the surf zone (0–3 m), where wave forces are maximal.

In the current study, the total OSA:SSA ratio remained constant over depth, both in sponges growing along a natural depth gradient and in the non–transplanted experimental sponges at two depths. Hence, the sponges apparently compensate for the increase in OSA by making less oscula, thus maintaining a similar total pumping capacity. We conclude that *C. reniformis* shows morphological plasticity over depth that leads to a depth–independent capacity to filter bacteria from the surrounding water.

Interestingly, the morphological response of *C. reniformis* to transplantation differed between the two transplantation treatments. In agreement with sponges growing along a natural depth gradient, we found that transplantation of sponges from 20 to 5 m resulted in morphological changes, with an increase in OSA and a decrease in OD. Thus, deeper–growing sponges transplanted from 20 to 5 m adapted their morphology to match that of shallow–water individuals. However, sponges transplanted from 5 to 20 m retained their original morphology. This suggests that the conditions in the wave action area immediately trigger the sponges to adapt their morphology as described above. Also the higher collagen content of the shallow sponges (Table 2), which makes them more robust, suggests an adaptation to the greater forces imposed on them. Another explanation for the lack of morphological adaption in the deeper sponges is that 8 weeks was insufficient time for shallow–water sponges to reorganise their aquiferous system. Our own observations on fast healing after the cutting of explants, (Figure S1) and previous findings on morphological plasticity do not support this explanation. In *Dysidea avara*, closure of oscula and formation of new ones occurred within days to weeks (Mendola *et al.* 2007). Moreover, many sponges, including *C. reniformis*, show very fast cell cycling (Alexander *et al.* 2014) and

rapid wound healing (Alexander *et al.* 2015). Indeed, we observed a fast healing process after cutting of explants (Supplementary Fig. 1), suggesting a high potential in *C. reniformis* for fast modification of the aquiferous system. An alternative explanation for the lack of response of sponges transplanted to 20 m relates to the hypothesis outlined above, which suggests that the observed morphological plasticity relates to wave action and corresponding resuspension of larger particles. Since sponges at 5 and 20 m depth showed a similar capacity to clear bacteria, there may be no immediate trigger for replumbing of the aquiferous system in sponges transplanted from 5 to 20 m, whereas sponges transplanted from 20 to 5 m would have to modify their aquiferous system to cope with higher coarse sediment loadings. It remains to be investigated whether sponges transplanted to deeper waters will increase their OD and decrease average OSA over a longer period of time. To investigate whether and how the internal aquiferous system is affected by depth, histology is recommended for future experiments.

Divergent habitats have different requirements for active pumping, depending on stimuli such as ambient current, food availability, storms and sedimentation of particulate matter, which is reflected by sponge morphology (Reiswig 1975a; Gerrodette and Flechsig 1979; Bell and Barnes 2000; Ludeman *et al.* 2017). The sponges living in low current regimes show distinctive morphology compared to the ones living in high current conditions (Bell and Barnes 2000). Sponges also can acutely adapt their pumping activity to ambient conditions. For example, several species of tropical demosponge responded to storm events by temporarily reducing filtration (Reiswig, 1975a). Also *Aplysina lacunosa* (Lamarck, 1814) was shown to regulate its pumping in response to the sediment levels in the water (Gerrodette and Flechsig 1979). A sediment load in the water as low as 11.1 mg l^{-1} was sufficient to cause a significant reduction in pumping and a sediment level of 95 mg l^{-1} caused a continuing decline in pumping rate of the sponge *A. lacunosa*. Sponges have active control over the volume of water they process as an adaptation to reduce the energetic cost of filtration in times of high stress. They might be responding behaviourally to increased particulate matter exposure from strong currents and/or storms by reducing the their particle intake via reducing the volume of water filtered (Ludeman *et al.* 2017).

This study is the first to report in situ bacterial clearance rates and respiration rates for *C. reniformis* at different depths. Therefore, we cannot compare our data with available literature data on clearance rate of other Mediterranean sponge species. There are, however, data on pumping rate, although they greatly differ in method applied, such as ex situ or in situ measurement, sponge size, incubation period and volume and type of the incubation. As indirect methods cannot differentiate pumping (filtration rate) activity and retention efficiency (clearance rate) some studies only provide filtration rates instead of clearance rates, assuming an efficiency in removal of 100%, and therefore the volume of water pumped through the sponge is used as measure of clearance rate (ref: Riisgård and Larsen 2000). This is then expressed as ml of water pumped per g of sponge dry tissue per hour, or as ml per ml of sponge per hour (Table 4). We provided *C. reniformis* pumping rates measured in this study in both units to allow comparison with other Mediterranean species (Table 4). The pumping rates of our *C. reniformis* explants are within one order of magnitude with those reported from both ex situ and in situ studies on other Mediterranean sponges. Compared to our study, nearly all the other experiments featured longer sponge incubations (4–16x) and used chambers varying in size (25L, 1L vs this study; 6L) (Table

4). Perhaps most relevant for comparison with our results are the studies applied in situ. Unfortunately, Cebrian *et al.* (2006) did not provide the unit of filtration in their study, as the clearance rate they report seems to be very comparable to our results. The mean respiration rate of 2.0 ± 0.1 µmol O2 cm^{-3} h^{-1} measured in our study falls in the middle of the range of respiration rates for various sponge species (Osinga, 1999; Morganti, 2015).

Table 4. Clearance rates for Mediterranean sponges in literature. DM = Dry Mass.

Species	Method (Incubation)	Sponge Size#	CR Rate	Reference
Ex situ				
Crambe crambe	0.25 h	5 cm^2	6.6-39.9 mL g^{-1} DW min^{-1}	Turon et al. 1997
Dysida avara	0.25h	5 cm^2	23-63.4 mL g^{-1}DW min^{-1}	Turon et al. 1997
Chondrilla nucula		25 cm^3	0.2& 1.4 mL cm^{-3} h^{-1}	Milanese et al. 2003
Chondrilla nucula	4 h, 1 or 25 L	4 cm^3	740-2596 mL g^{-1} DW h^{-1}	Peterson et al. 2006
Spongia officinalis	4 h, 1 L	91.4 cm^3	34-210 g^{-1} mL DW h^{-1}	Stabili et al. 2006
Corticium candelabrum	1 h, 1 L,	0.13-18.8 cm^2	1.6-4000 mL g^{-1} DW h^{-1}	de Caralt et al. 2008
In situ				
Dysida avara	1 or 3L, 4 h	25 cm^3	104-2046 mL g^{-1} DW h^{-1}	Ribes et al. 1999
Chondrosia reniformis	4 L, 1 h		50-340	Cebrian et al. 2006 *
Ircinia variabilis	1.5 h, 1 L	50 cm^3	15.96 mL cm^{-3} h^{-1}	Ledda et al. 2014
Agelas oroides	1.5 h, 1 L	35 cm^3	20.0 mL cm^{-3} h^{-1}	Ledda et al. 2014
Chondrosia reniformis	0.25 h, 6 L	9-49 cm^2	117-164 mL cm^{-3} h^{-1} 585 mL g^{-1} DW h^{-1}	This study

* Unit not specified in the study; # sponge size is expressed either as volume (m3) or as projected surface area (m2).

Survival, growth and collagen content

Culturing *C. reniformis* explants has always been problematic as a result of the extreme plasticity of the sponge making them 'escape' from the intended location (Wilkinson and Vacelet, 1979; Pronzato et al 1999; Garrabou and Zabala 2001; Van Treeck *et al.* 2003; Osinga et al 2010; Gökalp *et al.* 2019). However, the custom design incubation plates with protective PVC rim preventing them from escaping and protective chicken wire lid to prevent predation as applied in this study proved to be successful. The setup provided easy handling for clearance rate experiments and growth measurements. The main challenge was the pinning of explants onto the nails during out planting, due to contraction of the sponges following initial disturbance (sampling and cutting) making their tissue very hard. When this succeeded, attachment was successful and explants remained in place. The survival rate we achieved of 95% after 8 weeks of culture)is considerably higher than survival in our previous study (39–86% after 56 weeks; Gökalp *et al.* 2019) or 55% in a study by Van Treeck *et al.* (2003). In the study reported by Osinga *et al.* (2010) the protective stainless steel cage around explants of *C. reniformis* had resulted in comparable survival rates for explants cultured under pristine conditions.

In line with the similar pumping capacity, clearance rates and respiration rates across treatment groups, we also did not observe growth differences. This has implications from the perspective of integrated multitrophic aquaculture (IMTA) and bioremediation, as it does not seem to matter at what depth *C. reniformis* explants are placed in terms of performance. The growth

rates observed in this study (52–83%, in two months) are in line with our previous results 70–114% in 6 months; Gökalp *et al.* 2019). In spite of the variable growth rates and peculiarities (growing protrusions, dripping through meshes, etc.) reported by earlier studies (Wilkinson and Vacelet 1979; Pronzato *et al.* 1999; Van Treeck *et al.* 2008), our current and previous studies show that *C. reniformis* remains a good candidate for mariculture (Osinga *et al.* 2010; Gökalp *et al.* 2019). Although the growth rate of this species is not as high as reported for other species such as: *Mycale hentcheli* (359–2437% year^{-1}, Page *et al.* 2011) or *Lissodendoryx sp.* (5000% month-1, Battershill and Page 1996), the high survival rates and reproducible growth rates obtained when applying appropriate methods will enable controlled production of *C. reniformis* biomass through sea–based aquaculture.

Clearly, *C. reniformis* is a potential source of collagen for biomedical applications in tissue engineering and regenerative medicine because of its unique physicochemical properties and high collagen content (Garrone *et al.* 1975; Swatschek *et al.* 2002; Wilkie *et al.* 2006; Fassini *et al.* 2014). Swatschek *et al.* (2002) determined a collagen content of 30% of the freeze-dried mass of *C. reniformis*. Considering a wet mass to dry mass ratio of 5.68 (Supplementary Table 1), this would translate into a collagen yield of 5.3% of the wet mass. Our yield of 14.5-35.5 % ww (Table 3) is much higher. Possibly the fact that we removed excess water during the homogenisation of the sponge, before weighing, may have resulted in a relatively lower wet weight so a relatively higher ww based collagen content. The results show, however, that the cultured materials and the methodology to extract collagen are suitable to collagen for pilot-scale processes. Also, the developed approach enables further collagen yield optimization, e.g. via further investigation of genotype specific yields, the effects of specific depths and of other environmental variables on collagen content.

In conclusion, filtration capacity, metabolism and biomass production of *C. reniformis* are not affected by depth, in contrast to morphology and collagen content. Osculum morphology clearly is depth–dependent, where sponges transplanted from 5 m to 20 m reflect the conspecifics at their origin (do not adapt), whereas sponges transplanted from 20 m to 5 m reflect the conspecifics at their destination (adaptation). This adaptation to shallow water might relate to wave action and sediment loading. The sponges maintain their filtering capacity by presumably re–shaping their aquiferous system depending on their needs. This maintained filtering capacity is very promising for potential future multifunctional application of *C. reniformis* to improve the water quality by filtering out several types of organic pollution, including faeces and unused feed from fish farms and pollution and microorganisms from urban sewage outlets.

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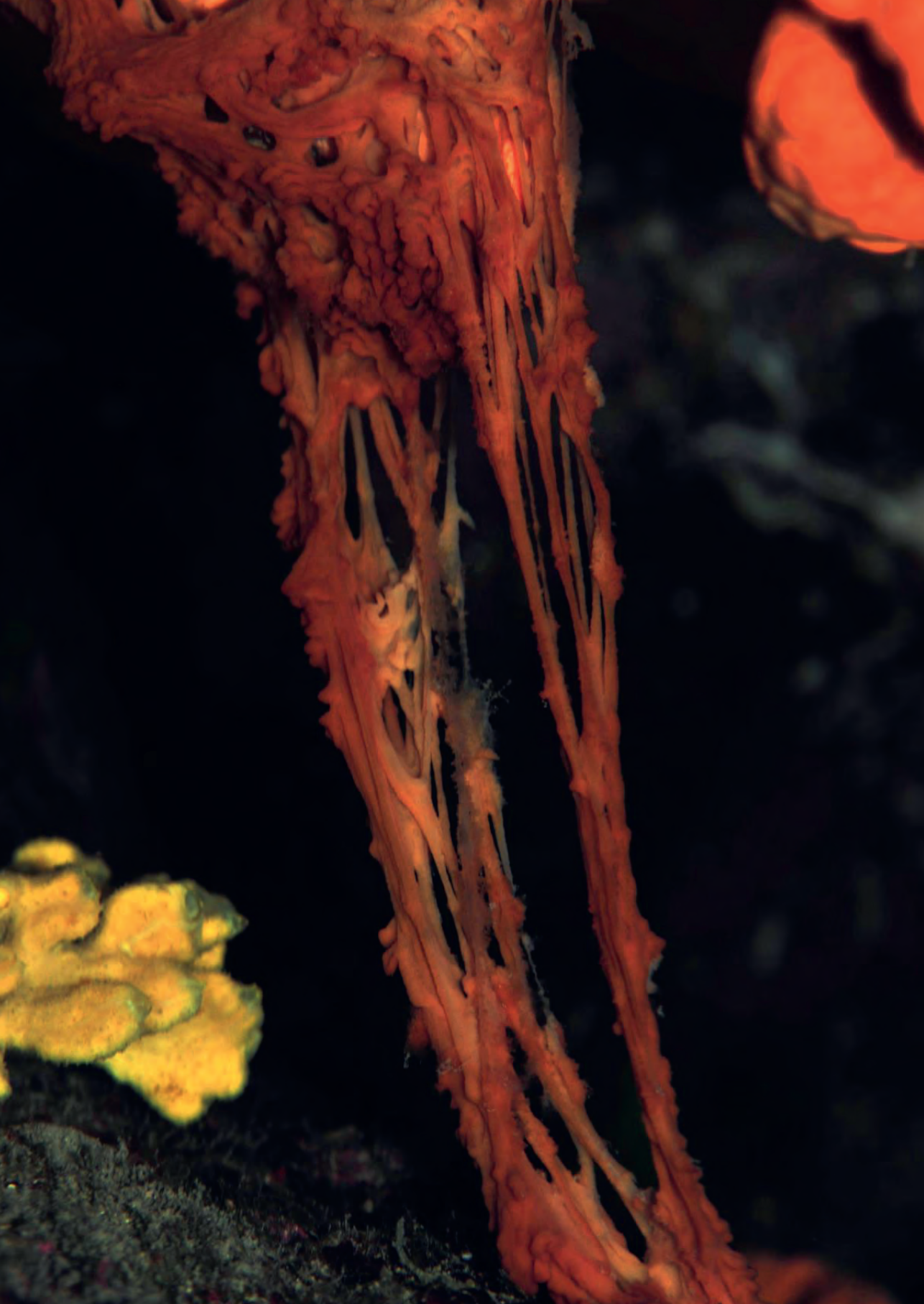

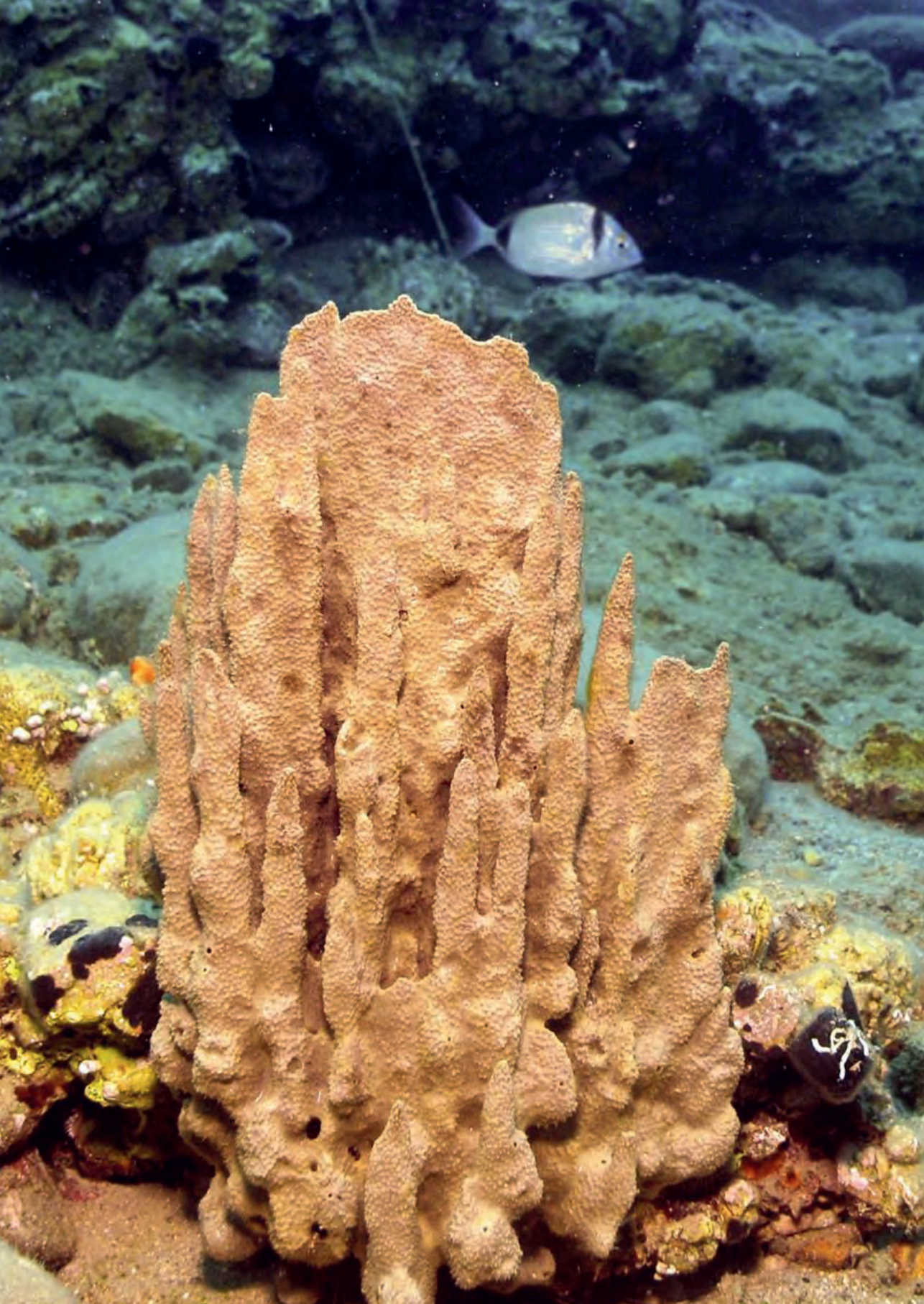

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Development of an Integrated Mariculture for the Collagen-Rich Sponge *Chondrosia reniformis*

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Abstract

In this study, novel methods were tested to culture the collagen-rich sponge *Chondrosia reniformis* Nardo, 1847 (Demospongiae, Chondrosiida, Chondrosiidae) in the proximity of floating fish cages. In a trial series, survival and growth of cultured explants were monitored near a polluted fish farm and a pristine control site. Attachment methods, plate materials, and plate orientation were compared. In a first trial, chicken wire-covered polyvinyl chloride (PVC) was found to be the most suitable substrate for *C. reniformis* (100% survival). During a second trial, survival on chicken wire-covered PVC, after six months, was 79% and 63% for polluted and pristine environments, respectively. Net growth was obtained only on culture plates that were oriented away from direct sunlight (39% increase in six months), whereas sponges decreased in size when sun-exposed. Chicken wire caused pressure on explants and it resulted in unwanted epibiont growth and was therefore considered to be unsuitable for long-term culture. In a final trial, sponges were glued to PVC plates and cultured for 13 months oriented away from direct sunlight. Both survival and growth were higher at the polluted site (86% survival and 170% growth) than at the pristine site (39% survival and 79% growth). These results represent a first successful step towards production of sponge collagen in integrated aquacultures.

Keywords: mariculture; sponge; *Chondrosia reniformis*; fishfarm; integrated multitrophic aquaculture

INTRODUCTION

The first attempts to farm sponges date back to the 19th century, presumably as a consequence of periodical depletion of "bath-sponge" stocks (Storr, 1964; Manconi *et al.* 1999), or—in more recent times—in pursuit of a safer and economically more attractive alternative to wild collection (Pronzato and Manconi 2008; Voultsiadou *et al.* 2008). Overfishing and repeated outbreaks of mass mortality events halted the ancient tradition of Mediterranean fishing of commercially important "bath sponge" species, such as *Spongia officinalis* (Linnaeus) and *Hippospongia communis* (Lamarck) (Pronzato *et al.* 1999; Milanese *et al.* 2003). Sponge mariculture has received increased attention over the last two decades (e.g., Müller *et al.* 1999; Van Treeck *et al.* 2004; De Voogd *et al.* 2007); see also reviews or comparative studies by (Duckworth 2009; Schippers *et al.* 2012; Kelly *et al.* 2010), particularly driven by the discovery of biologically active metabolites in many sponges (e.g., [Pomponi, 2001; Sipkema *et al.* 2005]). Sponge mariculture could potentially provide for a sustainable supply of sponge-derived bioactive compounds and biomaterials.

Sponges can be co-cultured with other organisms in so-called integrated mariculture systems, in which sponges take up metabolic wastes from other system components, including bacterioplankton growing on these metabolic wastes (Milanese *et al.* 2003; Fu *et al.* 2006; Zhang *et al.* 2009; Ledda *et al.* 2014; Longo *et al.* 2016). This way, sponges can effectively reduce waste streams from fish farms (Manconi *et al.* 1999; Pronzato *et al.* 1999; Pronzato *et al.* 1998), since they have been shown to exhibit retention efficiencies of up to 99% for nano- and picoplankton (e.g., [Reiswig 1971; Pile and Whitman 1996; McMurray *et al.* 2016]), while processing large volumes of water, up to 0.6 cm3 cm^{-3} sponge s^{-1} (e.g., [Vogel 1977; Savarese *et al.* 1997; Weisz *et al.* 2008]). Hence, a large-scale sponge culture facility that is constructed near a fish farm may positively affect the quality of the surrounding water. Conversely, the additional nutrition originating from the farmed fish may enhance the growth of the sponges in culture, thus providing a more efficient and profitable business.

In 2006–-2007, an integrated mariculture approach using sponges was tested in the coastal waters around the Bodrum Peninsula, Turkey (Osinga *et al.* 2010). Two Mediterranean demosponge species with possible commercial interest, *Dysidea avara* (Schmidt, 1862) and *Chondrosia reniformis* Nardo, 1847 (*Demospongiae, Chondrosiida, Chondrosiidae*), were cultured at a pristine site (i.e., no fish farms within the nearest 30 km) and an organically polluted fish farm site, the latter sponges being directly cultured underneath an open cage fish farm. *D. avara* was chosen since it produces the bioactive compound avarol, a potential anti–-psoriasis agent (Sipkema *et al.* 2005; Tommonaro *et al.* 2014). *Chondrosia reniformis* synthesizes large amounts of collagen, which is suitable for cosmetic and medical applications (Swatschek *et al.* 2002; Nickel and Brummer 2003; Silva *et al.* 2014). Type I & IV mammalian-like collagens can be effectively extracted from *C. reniformis* (Dos Reis, 2015; Silva *et al.* 2016) and they can be used to promote the regeneration of human tissue and bone tissue engineering scaffolds (Silva *et al.* 2014). *C. reniformis* showed better growth and survival rates at the pristine site, whereas *D. avara* grew and survived better at the polluted site (Osinga *et al.* 2010). The low survival rates of *C. reniformis* at the polluted site were largely due to the farming protocol used. *C. reniformis* is a highly plastic sponge, being able to de–attach and move around (Bavestrello *et al.* 1998), a

phenomenon that was frequently encountered using common culturing structures, such as pins, lines, plaques or metal/net grids (Pronzato *et al.* 1999, Van Treeck *et al.* 2004; Wilkinson and Vacelet 1979, Garrabou and Zabala 2001). To avoid displacement, explants of *C. reniformis* were put inside cages on the seafloor (Osinga *et al.* 2010). However, due to the high particle load in the water around the fish farm, the explants in these cages were suffocated by sediment.

This study describes progress towards the development of a raw collagen production pipeline using the sponge *C. reniformis* in an integrated multi–trophic aquaculture approach, i.e., by culturing the sponges in the vicinity of offshore floating fish cages. Using thin plastic plates as substratum, a series of consecutive trials were executed, aimed at developing an optimal, species–specific culture method. We monitored survival and growth rates of cultured explants of *C. reniformis*, thereby comparing a polluted fish farm site to a pristine site. Variables studied included methods for attaching explants to plates, plate materials and plate orientation. The culture methods (glue, cable-ties on plaques, net/mesh cover) were applied previously on other sponge species by several authors; for detailed information, see review by Duckworth *et al.* (2009).

MATERIALS AND METHODS

Mariculture Sites and Monitoring of Water Quality

All of the studies were carried out in the coastal waters around the Bodrum Peninsula, Southwest Turkey (Figure 1). Meteoroloji Bay (Figure 1 Pr.1), a shallow area with an abundant population of *C. reniformis* was selected as a pre-–culture and initial testing site (Trial 1). Based on water visibility (Secchi disk, cf. [Hannah *et al.* 2013]) and organic loading (total organic carbon (TOC) measurements), two additional sites were selected for subsequent testing (Trials 2 and 3): Kargi Island (Figure 1 Pr. 2) at the Southern side of the Bodrum Peninsula was selected as a pristine site, whereas Guvercinlik Bay (Figure 1; Po. 1), located at the Northern side of the peninsula, was selected as a polluted site.

Water temperature (Uwatec Aladin Air X Nitrox dive computer, calibrated with mercury thermometer) and visibility were measured 17 times during periodic visits at the polluted site throughout the experimental period from April 2011 to December 2013. To determine organic loading, three replicate water samples (50 mL) were taken within 10 m from the culture platforms by SCUBA diving from each location for TOC analysis using the wet oxidation method [Menzel *et al.* 2003]). TOC samples were stored in pre-combusted (450 °C for 6 h) 50 mL glass bottles with glass caps at -20 °C until analysis. Prior to analysis, sulphuric acid was added to the samples (end concentration 2 mmol L^{-1}) to remove dissolved inorganic carbon species. The acidified samples were supplemented with sodium tetraborate and potassium persulphate and processed using segmented flow analysis (SFA) on a Continuous Flow Analyser (Skalar, Breda, The Netherlands). In SFA, TOC is first oxidized using UV light and then measured as CO2 while using infrared detection. The TOC detection limit of the method, as intercalibrated with other labs, is 25 µmol L^{-1}, the average TOC variation among replicate measurements is 10 µmol L^{-1}. The internal standards used were 3.3 µmol L^{-1} EDTA, 3.3 µmol L^{-1} of a humic acid, and 3.6 µmol L^{-1} phenylalanine.

Sponge Collection and Seeding

Sponge specimens were collected by SCUBA at 5-10 m water depth in the Bay of Meteoroloji (Figure 1 Pr.1). Explants were cut with sharp razor blades and detached from rock surfaces with a spatula (Van Treeck *et al.* 2004; Page *et al.* 2011), leaving the majority (at least 75%) of the donor sponge unaffected. The explants received maximally two cut surfaces and more than 50% of their surface was always covered with intact pinacoderm (i.e., the sponge outer tissue layer). The explant size ranged between 10–15 cm2 with an average thickness of 2 cm, and all of the explants had at least one osculum (i.e., outflow opening). Explants were stored in perforated plastic containers that allowed water flow and they were left underwater until the seeding operations started. To enable sponges to attach and acclimatize after seeding, the seeding plates with the explants were left horizontally next to the culture platforms for three days before the plates were secured to their spots on the culture frame (Sipkema *et al.* 2005).

Figure 1. Map of the Bodrum Peninsula. Small white circle (Pr.1: Pre-culture site—Meteoroloji Bay). Large white circles (Pr.2: Pristine—Kargi Island) and large black circles (Po.1: Polluted—Guvercinlik Bay) circles point the approximate locations of the sites used for growing sponge explants in this study. GPS coordinates 36.9444444, 27.27611111; 36.95166667, 27.30694444; 36.96861111, 27.45083333, respectively. (Source: Google Earth, 2018).

Mariculture Trials

Within the period between April 2011 and December 2013, three subsequent mariculture trials were executed in order to develop and improve a culture method for *C. reniformis*.

1st mariculture trial, April 2011–June 2011: testing materials and attachment procedures

Sponge explants (n = 5 specimens per plate) were attached to four types of supports (autoclaved aerated concrete, white polyvinyl chloride (PVC), black PVC, and cemented PVC) using six different combinations of attachment methods and substrates (Table 1 (a)). The cementation of plates may improve attachment of the sponges to the support and enhance growth, since quartz and silica are found to promote collagen formation in sponges [34,40].

Accordingly, coarse sea–-sand was used to make cement to cover the cemented–PVC supports. All of the supports were positioned in Meteoroloji Bay (Figure 1 Pr. 1) at 2–3 m water depth under overhangs or in crevices (i.e., not in direct sunlight) and fixed with diving weights.

2nd mariculture trial, June 2011–June 2012: testing culture plate orientation and site

Based on the results of the first trial, PVC plates were chosen for the second mariculture experiment. Explants (250 in total per site) were positioned on both sides of five 50 × 50 cm plates, with 25 explants on each side of every plate. In order to find the optimal positioning of the sponges, explants were cultured at nine different angles, under light (exposed side of plate) or dark (underside of plate) conditions, resulting in 10 different conditions (n = 1 plate per treatment) (0°, 30°, 45°, 60°, 90° light, 90°, 120°, 135°, 150°, and 180° dark; Supplementary Table S1 , Figure 2a,b). In order to keep the sponges attached to the plates until natural attachment took place, the sponges were covered with chicken wire and left on the seabed for two days. After the attachment of the explants to the plates, the plates were mounted on a metal frame. Frames were installed in July 2011 at the two selected sites (Kargi Island (Figure 1; Pr. 2)—pristine and Guvercinlik Bay (Figure 1; Po. 1)—polluted) at a water depth of 10 m.

Table S1. Experimental design of aquaculture trials 2 and 3. In Trial 3, 10 explants were taken per parent sponge, five explants were attached to each plate.

Trial	Sponge Species	Culture Site	PVC Plates	Explants per plate	Explants per angle	Total Explant Number
2	*Chondrosia reniformis*	Pristine	5	50	25	250
2	*Chondrosia reniformis*	Polluted	5	50	25	250
3	*Chondrosia reniformis*	Pristine	20	5		100
3		Polluted	20	5		100

3rd mariculture trial, June 2012–July 2013: assessment of growth at the optimal culture orientation

Based upon the observations of the 2nd mariculture trial, it was decided to choose an angle of 90° for primary upscaling of the cultures. Two new frames were installed in early June 2012, one at the pristine site and one at the polluted site, each carrying 20 white PVC plates of 25 × 25 cm with a total of 100 explants of *C. reniformis* (5 per plate), attached using gel-based polyacrylate superglue. A gel-based polyacrylate superglue method was preferred over chicken wire to improve the handling time and reduce the weight and cost of the culture materials. In addition, horizontal blue polypropylene PP plates were placed underneath the 90° PVC plates to provide extra surface for the explants to attach and grow, should they fall (Figure 2c,d). Explants were grown for 13 months, photographed, and measured in June, July, August, September, October, and November 2012 (both sites), in March and May 2013 (polluted site only, due to weather restrictions), and in July 2013 (both sites).

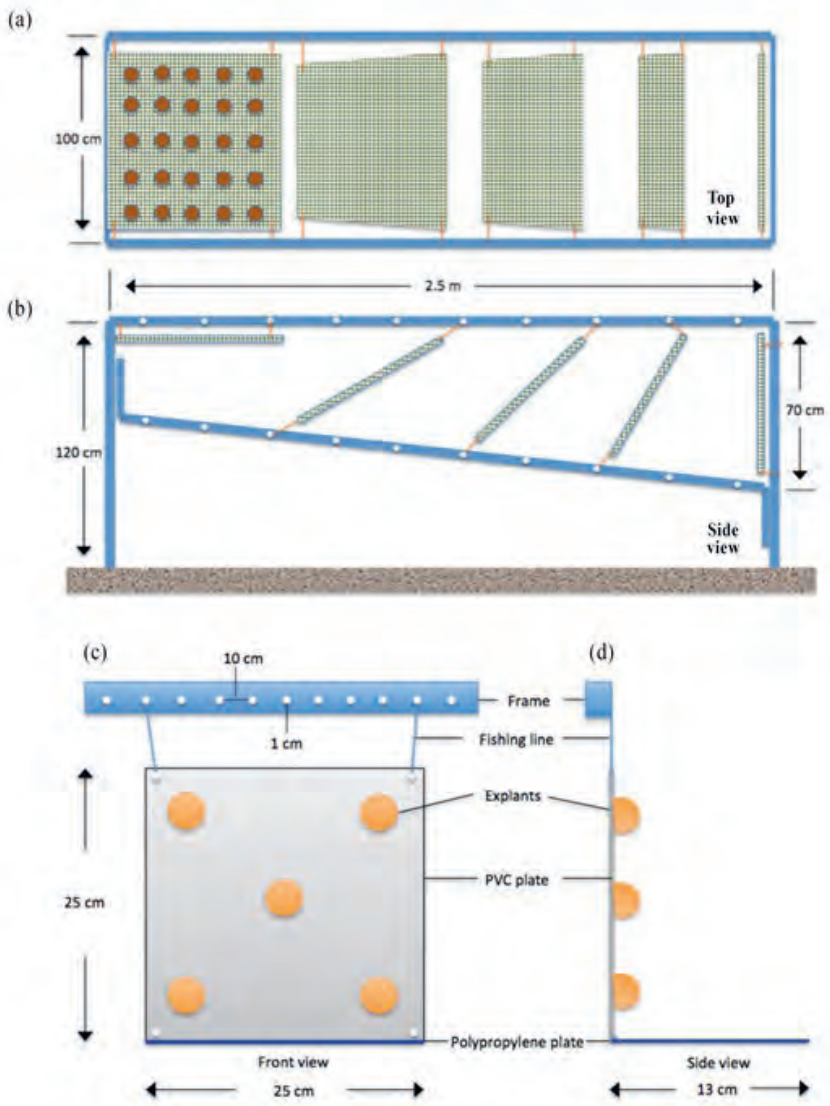


Figure 2. Schematic drawings of the culture platform designs. (a) 2nd trial—Top view ; aluminum culture frame and attached 50 × 50 cm polyvinyl chloride (PVC) plates covered with chicken wire, each carrying 25 sponge explants on one side, totaling 50 sponges per plate. (b) 2nd trial—Side view; plates positioned at 9 different testing angles 0°, 30°, 45°, 60°, 90°, 120°, 135°, 150°, 180°. 3rd trial, (c) 3rd trial—front view, and (d) 3rd trial—side view showing positioning of glued *C. reniformis* explants on 25 × 25 cm PVC plates. PVC plates were secured tightly to the aluminum frame from four corners with 6-mm thick fishing lines, blue PP plates were attached to the bottom sides of the PVC plates.

Table 1. Overview of the results of the three culture trials executed in between May 2011 and November 2013. (a) 1st trial, material test and attachment procedures, pristine site (b) 2nd trial, testing culture orientation and site (c) 3rd trial, assessment of productivity at the optimal culture orientation.

	Material	Attachment Method	Advantage	Disadvantage	Result
a. 1st trial	Air-concrete	Iron screw		No attachment	Not suitable
Material test and attachment procedures	PVC—white	Cable-ties	Survival (80%)	Dispersion of explants	Not selected for 2nd trial
		Superglue	Ease of operation	Lower survival (60%)	Selected for 3rd trial
		Chicken wire	High survival (100%)	handling time	Selected for 2nd trial
	PVC—black	Cable-ties	Survival (80%)	Dispersion of explants	No preference; the color of the plate did not affect the result
	Cemented PVC	Cable-ties	High survival (100%)	Cost, handling time and weight	Not selected

	Site	Material	Disadvantage	Survival Rate	Average Growth	Result	Orientation (°)
b. 2nd trial	Pristine	PVC chicken wire	Squeezed explants, resulted in unwanted epibiont growth, time & cost	63% survival after 6 months of culture	Culture frame demolished by an anchor	Chicken wire method is not suitable	90° was selected for the next trial
Testing culture orientation and site	Polluted			79% survival after 6 months & 1 year of culture	39.2 ± 36.2% in 12 months for dark angles		
					-40.9 ± 37.7% in 12 months for light angles		

	Site	Species	Survival Rate	Average Growth	Growth per Culture Interval		Range of Growth for Individuals
					0-6 Months	7-13 Months	
c. 3rd trial Assessment of productivity at the optimal culture orientation	Pristine	*C. reniformis* (n = 15 plates)	39%	79.0 ± 37.4% in 13 months	69.8 ± 33.6%	5.4 ± 5.7%	−3.6-135.6%
	Polluted	*C. reniformis* (n = 16 plates)	86%	170.4 ± 109.1% in 13 months	114.0 ± 94.6%	30.1 ± 27.9%	0.8-322.9%

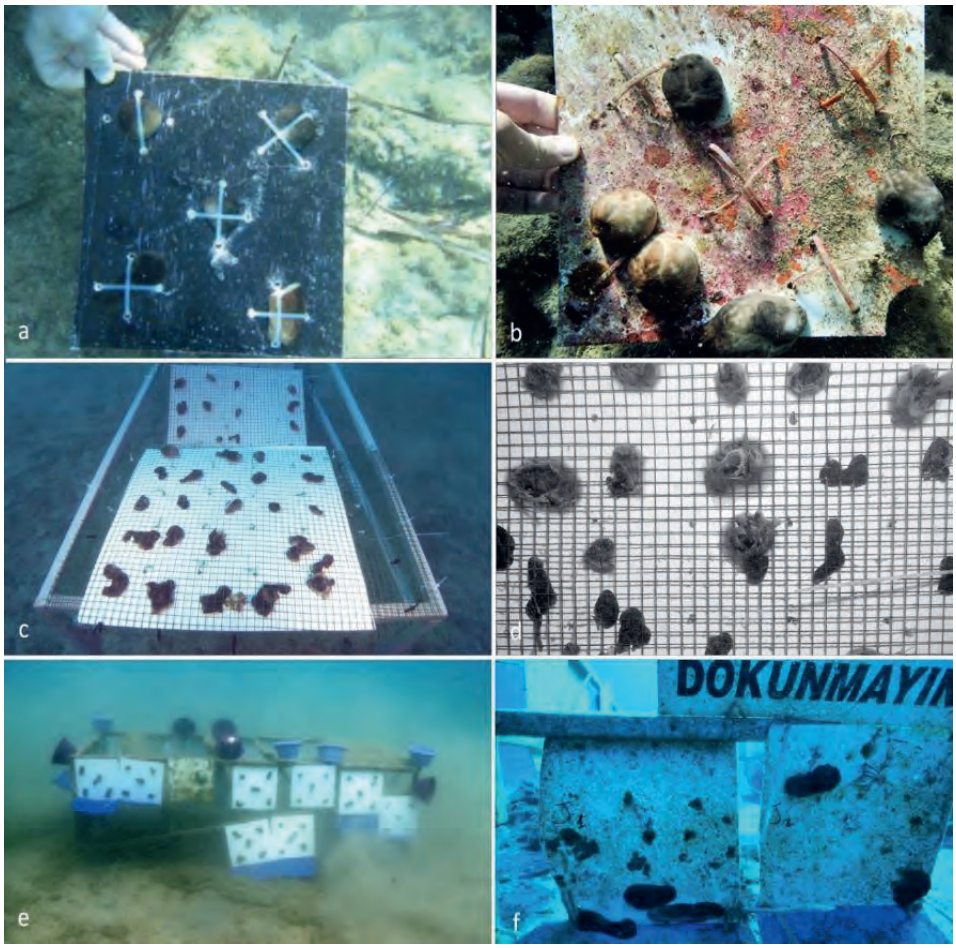


Figure S1. Overview of culture methods; a) 1st trial; explants tie–wrapped to black PVC, experiment start b) 1st trial; sponges tie–wrapped to white PVC, 1 year later explants splitting into two fragment and relocating position. c) 2nd trial, polluted site; image of 50x50 cm plates, sponge explants secured with chicken wire (0° – 30° plates can be seen, 25 additional explants are on the other side of each plate). d) 2nd trial, pristine site; infected explants shortly after seeding. e) 3rd trial, polluted site; first upscaling of aquaculture of *C. reniformis* on vertical PVC plates. Explants are attached with gel–based polyacrylate superglue to PVC plates (10 plates per site) f) 3rd trial, pristine site; explants after 4 months of culture. Some of the explants tend to travel towards the polypropylene plate and grow on it, but others prefer to stay on the PVC plates

Survival Rate Analysis and Sponge Explant Growth

Survival rates in Trial 1 were assessed by visual observation. For Trials 2 and 3, survival was monitored by underwater photography. Explants were photographed using a Nikon D300s digital single lens reflex camera (Nikon Corporation, Tokyo, Japan) and a Sigma 10–20 mm wide-angle lens set (Sigma Corporation, Ronkonkoma, NY, USA), coupled with dedicated Ikelite housing and an Ikelite DS160 substrobe (Ikelite, Indianapolis, IN, USA). Survival was calculated from the initial and final number (N) of explants residing on the PVC and/or PP plates, as follows:

$$\text{Survival} = (N_{final}/N_{initial}) \times 100 \qquad (1)$$

For Trial 3, as described above, blue PP plates were used to collect detached sponges. To calculate survival data, sponges that had fallen onto the PP plates were pooled with the sponges that remained on the PVC plates. However, fallen sponges were excluded from the growth analysis, as PP may affect sponge growth differently from PVC.

For Trial 2, explant growth rates were calculated by measuring wet weights by using a scale (Sinbo SKS 4514) at the start and end of the experiment. To reduce measurement error, the sponges were briefly wiped with clean paper to remove seawater for a duration of approximately 10 s. Growth was calculated from initial and final explant wet weights (WW) as follows:

$$\text{Growth (\%)} = ((WW_{final} - WW_{initial})/WW_{initial}) \times 100 \quad (2)$$

For Trial 3, growth was monitored by underwater photography using the same setup, as described above, for the monitoring of survival. Following each dive, recorded images were transferred to Photoshop CS5 software (Adobe Systems Incorporated, San Jose, CA, USA) and lens distortion was corrected using a Camera Raw 6.7.1 plug-in (Adobe Systems Incorporated, San Jose, CA, USA). The images were calibrated using known plate dimensions, peripheries of explants were marked, and surface area was calculated from pixel counts of the marked areas (Page *et al.* 2011]). Growth was expressed as the increase in the number of pixels, calculated with the pixel counter function of the image editing software. At each time point, the growth in percentage increase in projected surface area was calculated from initial (at start of the time point) and final explant surface areas (A) as follows:

$$\text{Growth (\%)} = (A_{final} - A_{initial})/A_{initial}) \times 100 \quad (3)$$

To assess the correlation between surface area growth to both biomass and volumetric growth, an additional 20 sponges of random size were collected from a neighbouring site. For all 20 sponges, the wet weights were determined, as described above. In addition, volume was determined by measuring displaced seawater in a graduated cylinder. Finally, surface area was determined by using photography and a ruler as a reference, and photographs were processed, as described above.

Statistical Analysis

The normality of data was tested by plotting the residuals of each dataset versus the predicted values, and by performing a Shapiro-Wilk test. Homogeneity of variances was determined using Levene's test. All data were found to be normally distributed and showed homogeneity of variance after a log10 transformation ($p > 0.05$). Student's independent t-test was used to determine growth differences between the light and dark group in the second trial, with n = 5 plates for both groups. A two-way mixed factorial ANOVA was used to determine the main and interactive effects of culture site and time on *C. reniformis* growth in the third trial, with culture site as a between-subjects factor, and time as a within-subjects factor. In all analyses, the culture plate was considered as an experimental unit, i.e., data of explants growing on a single plate was pooled. To correlate the surface area to mass and volume, Pearson's product-moment correlation was used. A *p*-value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed with SPSS Statistics 22.0 (IBM, Somers, NY, USA). Graphs were plotted with SigmaPlot 12 (Systat software, San Jose, CA, USA).

RESULTS

Polluted versus Pristine Site: Water Temperature, Visibility and TOC

Visibility was on average 3.8 times lower at the polluted site (6.5 ± 1.7 m; mean ± SD throughout text unless stated otherwise) when compared with pristine site (25 ± 1.1 m; Figure 3). The water temperatures that were recorded at the polluted site ranged between 19–26 °C (Figure 3). During summer periods, especially in August, as a result of intensive fish feeding activities, Secchi disk water depth dropped to 4–6 m at the polluted site (Figure 3). TOC levels at the polluted site (280 ± 0.07 µmol L^{-1}) were 2.4 times higher than the TOC levels at the pristine site (110 ± 0.01 µmol L^{-1}).

1st Mariculture Trial: Testing Materials and Attachment Procedures

The air concrete material was found to be unsuitable for further experimentation, as none of the explants attached to it (Table 1 (a)). In addition, the material was positively buoyant in seawater, which hampered easy handling. There was no difference in preference between white and black PVC (80% survival for both plates), as sponges attached equally well to both substrates without showing any signs of disparity (Supplementary Figure S1a,b , Table 1 (a)). Cable–ties gave a better recovery percentage than super glue (80% vs. 60%), but, in addition to increasing handling time, cable-–ties also triggered the dispersion of C. renifomis explants into two parts for both black and white PVC's (fission; Supplementary Figure S1b). The combinations PVC/chicken wire and cemented PVC/cable–tie were the most successful methods in terms of recovery percentage (all sponges survived on plate). However, the cost of material, plate weight, and handling time were factors favoring the PVC/chicken wire method (Table 1 (a)). Accordingly, PVC/chicken wire method was selected for the 2nd trial.

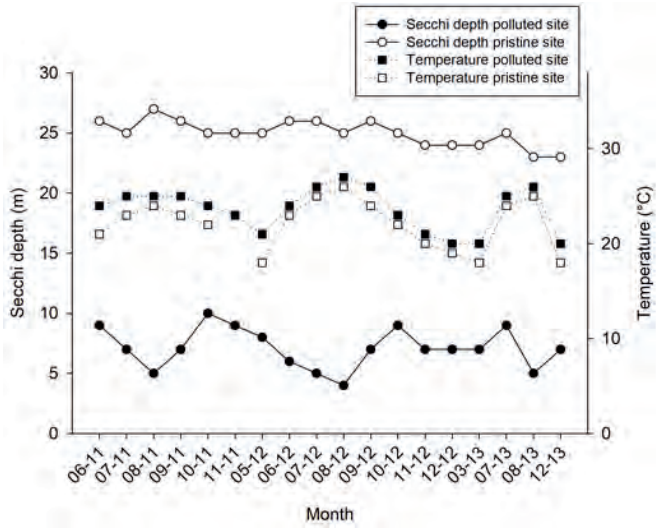


Figure 3. Water temperature (in °C, black squares with dottled line) and Secchi water depth (in m, black and white circles with continuous line) measurements for the pristine and polluted site over a 31-–month time frame (June 2011-–December 2013).

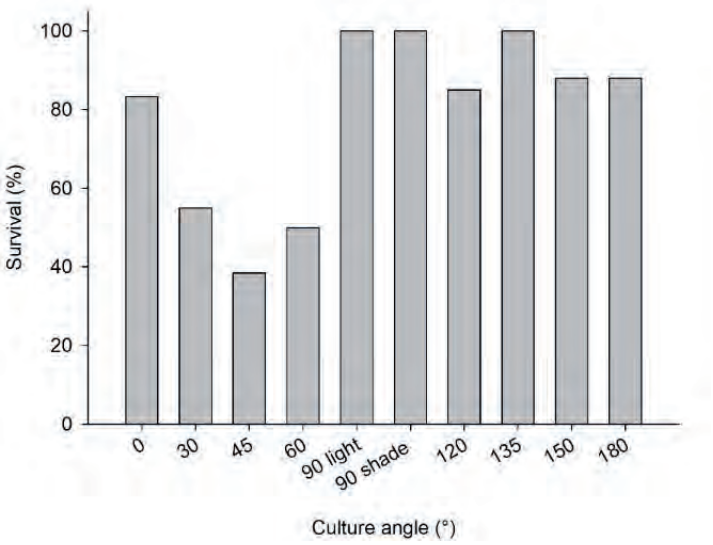


Figure 4. 2nd mariculture trial, polluted site, June 2011-June 2012; survival percentage of *C. reniformis* explants on PVC plates with various angles (0-90° light represents PVC plates with greater light exposure and 90° shade −180° plates receiving less light exposure).

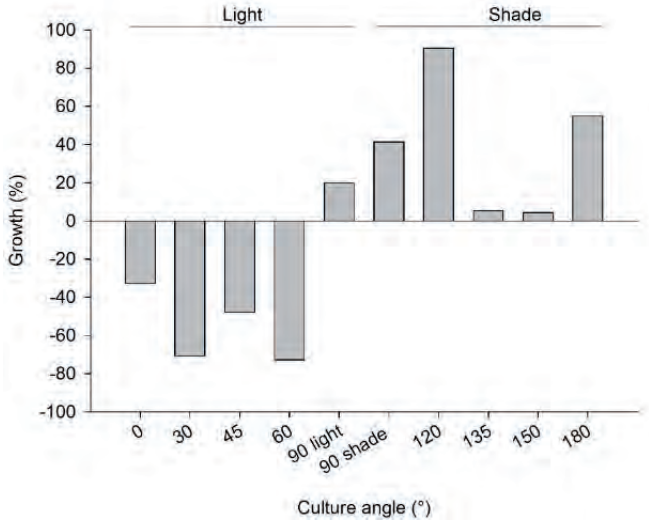


Figure 5. 2nd mariculture trial, polluted site June 2011-June 2012; growth rate as percentage wet weight increase of *C. reniformis* explants on PVC plates with various angles (0-90 light represents PVC plates with greater light exposure and 90 shade −180 plates receiving less light exposure; 25 explants for each plate).

2nd Mariculture Trial: Testing Culture Plate Orientation and Site

The explants at the polluted site (Supplementary Figure S1c) and pristine site (Supplementary Figure S1d) showed signs of bacterial infections and decay within a week after initiation of the cultures, causing initial losses at both sites (4.8% and 2% of explants deteriorated

in the polluted and pristine sites, respectively). Among the explants that survived the initial deterioration, overall survival after six months was slightly better at the polluted site (79% of 238 explants survived at the polluted site and 63% of 245 explants survived at the pristine site; Table 1 (b)). The culture frame at the pristine site was found to be demolished, when revisited in May 2012. It was found 50 m away from the culture site. As a consequence, it was not possible to deduce annual survival and growth rates for the culture at the pristine site. At the polluted site, survival was highest among sponges that were put at an angle of 90° or higher (Figure 4). Growth rates were highly variable among treatments (Figure 5), but the average growth at "light" angles of 0–90° (-41 ± 38%; negative values points to loss of WW biomass) was significantly lower than the average growth at "shade" angles of 90–180° (39 ± 36%; Student's t-Test $z = -3.4$, $p < 0.01$, $n = 5$). The 90° plate was selected as the preferred culture orientation in Trial 3, based on the survival rate and the ease of operation (photography, measurements, and handling; see Table 1 (b) for details). Photographic measurement of growth was found to be impossible with the PVC–chicken wire method as a result of continuous movement, splitting, and fusing of *C. reniformis* explants, and epibiont growth. In addition, chicken wire compressed the explants, which may not be beneficial for their development. Also, installing the large 50 × 50 cm PVC plates was time consuming. Therefore, smaller (25 × 25 cm) PVC plates were used in Trial 3 and superglue was selected as the attachment method.

3rd Mariculture Trial: Assessment of Sponge Culture Productivity Polluted vs. Pristine Site

During the first week of the experiment, 69 (polluted) and 70 (pristine) out of 200 explants dropped off the PVC plates. Fortunately, the PP plates that were placed under the PVC plates were able to catch 55 (at polluted site) and nine (at pristine site) of these explants, which attached onto the PP plates and continued to increase their surface area. Because they could not be related anymore to their original size, explants that were attached on the PP plates were left out of the surface area increase analysis. However, they were included in calculation of survival rates, which were highly different between the pristine and polluted sites (39–86%, respectively—Table 1 (c)). A total of 61 explants survived on the vertical PVC plates (30 explants on 15 plates at the pristine site, 31 explants on 16 plates at the polluted site), which were used for surface area increase analysis. The average increase in surface area over time of these *C. reniformis* explants is presented in Figure 6a. After being cultured for 13 months, the average surface area increase was 79.0 ± 37.4% at the pristine site and 170.4 ± 109.1% at the polluted site (Table 1 (c)). Both culture site and time had a significant main effect on sponge surface area increase rates (Table 2). At both sites, explant surface area increased significantly, but it slowed down after the first six months at both sites (two-way factorial ANOVA, $F_{1,27} = 55.550$; $p < 0.001$), and for the pristine site even stalled after six months. Surface increase was significantly higher at the polluted site as compared to the pristine site (two-way factorial ANOVA, $F1,27 = 14.439$; $p = 0.001$), irrespective of time.

For *C. reniformis*, a highly significant correlation between surface area and wet weight was found (Pearson correlation, $r = .92$, $n = 20$, $p = 0.000$, two-tailed, Supplementary Figure S2), as well as between surface area and volume ($r = .92$, $n = 20$, $p = 0.000$, two-tailed, Supplementary Figure S3). The relationships are size-independent, leading to fixed conversion factors of 1.2 g wet mass per cm^2 of surface area and 1.1 cm^3 sponge volume per cm^2 surface area, respectively.

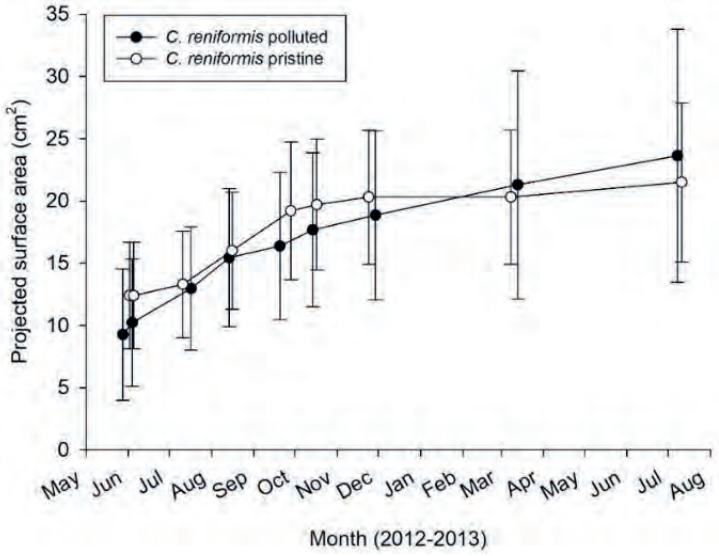
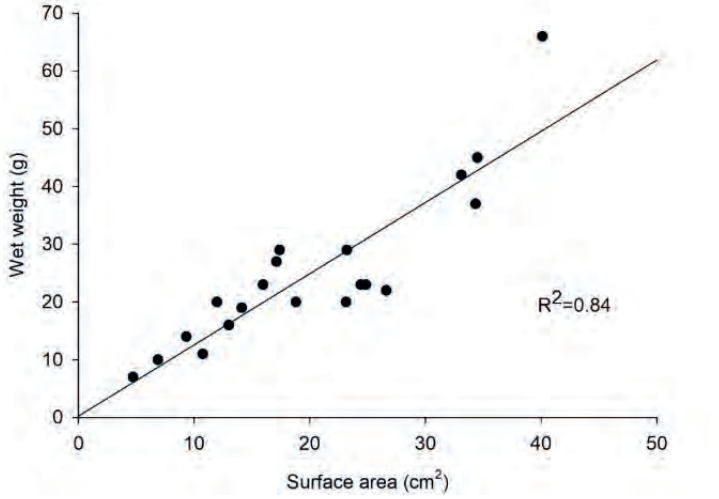


Figure 6. 3rd mariculture trial. Annual growth rate as surface area increase for *C. reniformis* (*n* = 15–16 plates) explants in polluted and pristine sites.

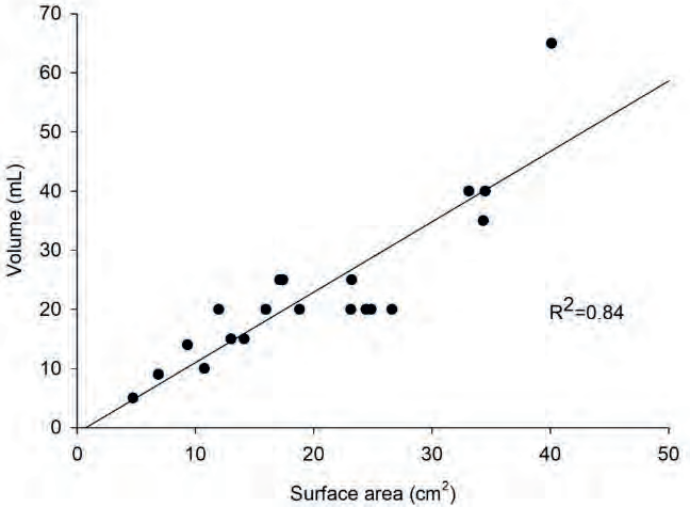


Supplementary Figure S2. Correlation between surface area (cm^2) and wet weight (g) for *C. reniformis* (Pearson correlation, r=.92, *n*=20, p=0.000, two-tailed).

Table 2. Two-way mixed factorial ANOVA, demonstrating main and interactive effects of culture site and time on *C. reniformis* growth rates (*n* = 15-16).

Factor	F	df	Error	*p*
Culture site	14.439	1	27	0.001 **
Time	55.550	1	27	0.000 **
Culture site x Time	2.686	1	27	0.113

** Indicates significant effect ($p < 0.01$).



Supplementary Figure S3. Correlation between surface area (cm^2) and volume (mL) for *C. reniformis* ((Pearson correlation , r=.92, *n*=20, p=0.000, two-tailed).

DISCUSSION

This study explored the feasibility to integrate fish culture with a biomedically promising Mediterranean sponge species, *C. reniformis*. The main aim of the study was to derive the best mariculture practices of *C. reniformis* from a series of subsequent culture trials.

Explant Survival Rates

Survival of explants can be compromised by detachment and by disease. In terms of initial survival, cable-ties and chicken wire were the most effective means of attaching explants onto PVC substrates, with glue giving a slightly lower survival. In the long term, however, the use of chicken wire (mesh culture) gave ambiguous results. Sandwiched mesh structures were designed to promote the explants to grow out of the pocket and to ease harvesting [8]. Mesh culture that is used in turbid waters might reduce water flow and subsequently decrease available food for the explants if mesh size is too small (Duckworth 2009). Although the mesh size used in the second trial was sufficiently large (5 × 5 cm) —as recommended in (Duckworth *et al.* 1997), after some time the space between meshes and the PVC plate was covered by epibionts, and the mesh did not prevent some explants from moving or even dropping themselves off the plate. Despite these drawbacks, the survival rate at the polluted site after one year was 79%, which is higher than in the study by (Van Treeck *et al.* 2003), who reported 55% survival after seven months and who lost entire *C. reniformis* explants with the sandwiched mesh method. However, the mesh method is labor intensive, especially when considering that increased cleaning of biofouling on the mesh is recommended. By attaching the explants with glue in the third trial, it was anticipated to reduce both handling time and fouling. Despite the predicted improvements regarding initiation time (May vs. June) and culture angle (all at 90°), the third trial showed low

survival for the pristine site. This was probably due to occasional strong currents that prevail at this site, which may make the explants more prone to dropping of the plates and physical removal from the site. During the whole month of September 2013, flow velocities above 20 cm/s were recorded at this site by analyzing the velocity of neutrally buoyant particles (video clips of laterally moving natural particles, data not shown). *C. reniformis* inhabits both nearly stagnant to occasional high flow waters (M. Gökalp; personal observation), however the attachment of explants to PVC plates is probably less firm than attachment to natural substrates, especially during the acclimatization time after wounding them to explant the parent sponges. At the polluted site, the use of glue instead of chicken wire did slightly improve long-term survival rate, which shows that gluing is a suitable method to attach explants of *C. reniformis*. It is also the fastest and easiest method. A future recommendation is to perform the initial acclimatization (of 7–10 days; see (Alexander *et al.* 2015) at a more secluded site, after which the attached explants are placed at the study site.

During culture Trial 2, initial sponge survival was compromised by disease-like phenomena. Bacterial infections, which were possibly due to late seeding of explants in Mid-June with relatively higher water temperatures, might have been responsible for the initial losses at both sites. High water temperatures in summer have been reported to be a risk for sponge mariculture in temperate and subtropical climates (Schippers *et al.* 2012; Page *et al.* 2011; Duckworth *et al.* 1997; Maldonado *et al.* 2010), as it makes cuttings more vulnerable to bacterial attack, although such increased vulnerability had not been observed in our earlier studies on this species in this area (Osinga *et al.* 2010).

Culture angle directly affected explant survival, mainly in association with prevailing light levels. Lower light levels at the more turbid polluted site may, therefore, also explain the higher explant survival at the light-exposed angles at the polluted site. These results corroborate the findings of (Wilkinson and Vacelet 1979), which purport *C. reniformis* prefers shaded habitats.

Explant Growth

Since surface area of *C. reniformis* showed a size-independent relationship with wet weight and volume, surface area can be used as a proxy for growth. This enables a direct comparison of growth data obtained for this species using different methods.

Culture of *C. reniformis* has been considered to be difficult, to even unsuitable with the methods applied (Pronzato *et al.* 1999; Van Treeck *et al.* 2003). Wilkinson and 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. Ref. (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 current study 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 and 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] and those from the current study 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 and Zabala (2001). Under optimal circumstances, the production of collagen is apparently not hampered by energy input. The current results 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.

During Trial 3, *C. reniformis* surface area increase rates were significantly different between culture sites, with an approximate two-fold higher growth at the polluted site. This may relate to the higher food availability—i.e., higher TOC concentration as a result of fish farm activities—and, as mentioned earlier, correspondingly lower light levels at the polluted culture site. Hence, the combination with fish farming is potentially beneficial for culture success of this sponge species. The surface area increase of *C. reniformis* was clearly higher in the first six months after initiation of the cultures, regardless of culture site. Although this may partially be explained by seasonal effects (growth might cease in autumn and winter, [Schippers *et al.* 2012; Corriero *et al.* 2004), it is possible that the sponges exhibit lower specific growth rates when being in culture for a longer period (Page *et al.* 2011). This could be due to initial enhanced surface area increase due to explant cutting (Wulff 2005), which could hamper growth at later stages, due to high costs of wound healing and regeneration (Alexander *et al.* 2015). Fast initial surface area increase was also found in a side experiment where the explants were cultured starting in autumn 2013 (data not shown), pointing towards a wound healing and regeneration effect rather than a seasonal effect, but this observation needs to be further investigated.

Culture of *Chondrosia reniformis*—Best Practices

As stated in Schippers *et al.* (2012), initial mariculture trials should span a complete annual cycle in order to perceive effects of seasonality, substrate preference, and growth physiology of the sponge, and possible external impacts to the culture site, such as the occurrence of fouling and specific sponge predators, boat traffic and anchoring, and the presence of fishermen and divers. Accordingly, in this study, valuable information was acquired regarding the preferences for attachment, survival, and growth of *C. reniformis* during the first two trials. *C. reniformis* explants attached to PVC plates tend to move on the plate, thus obfuscating multiple genotypic comparisons on one plate (our study was initially designed to investigate genotype effects, but this part of the study could not be completed due to random movement of the explants over the plates). In addition, fusion and fission of explants makes the proper assessment of survival difficult. Even though attachment to the PVC plates has succeeded, *some C. reniformis* still found ways to divide their body into several parts, moved around the PVC plate (possibly in pursuit of shaded areas), or dropped themselves to the ground possibly in search for better living conditions. Survival and growth is best at culture angles of 90° and above, where explants are not being exposed to direct sunlight, as *C. reniformis* performs better at low illumination levels.

In experiments 2 & 3, the initial losses and/or droppings of explants were slightly high and unpredictable, despite the variety of methods applied. Once attached for a longer time, the explants would remain attached. Therefore, initial losses and/or droppings are the main problem to be solved to secure better culture performance. Restraining bacterial attack on freshly cut explants by initiation of cultures early in the season (spring) and preventing exposure to high currents during the first months should be practiced together with the best performing methods regarding attachment.

Based upon the three mariculture trials described above, the following best practices have been deduced for culturing *C. reniformis* in sea-–based aquaculture under turbid conditions:

Culture method : Sponge explants cut from parent sponges are glued to PVC plates using gel-based polyacrylate superglue. PVC plates are best positioned vertically onto frames and they should be extended with a basket on the bottom site to recover explants falling off the plates. Chicken wire may be applied during the first few weeks after explanting to prevent early losses but should be removed once the attachment is stable. Prolonged use of chicken wire cover tends to hold sediments and promotes epibiont growth and hence undesired space competition with the cultured sponge.

Site selection: Sites should not be prone to strong fluctuations in weather. The area should be secured and should be clear of boat traffic and anchoring (Duckworth 2009; Schippers *et al.* 2012). Sites should be carefully assessed for (e.g., seasonal) strong currents. High water turbidity and increased load of organic content associated with the presence of fish farms does not appear to hamper growth of *C. reniformis* on vertical plates, making this sponge an interesting candidate for integrated multitrophic mariculture. Daily fish feeding activities and occasional net replacing hinders the use of culture platforms inside the fish farm area. Thus, sponge culture platforms have to be placed outside boat traffic area. To eliminate this problem, one method that we consider for future applications is using layered scallop lanterns placed in between an anchor and a submerged buoy system (just outside the fish farm culture area), a method that was successfully applied by both Duckworth *et al.* (2004) and Kelly *et al.* (2010), for *Latrunculia wellingtonensis*, *Polymastia croceus*, and (*Heterofibria*) *manipulatus*, respectively.

Seasonality: Initiating a culture of *C. reniformis* in the Mediterranean is best done in either spring (April-May) or autumn (October-November) to prevent bacterial infections following cutting of explants from parent sponges.

Recommendations for Future Research

Culture success can be further improved by optimizing the period of culture. Optimal culture time can be determined by observing sponge growth rates over a period of two or more subsequent years. Page *et al.* (2011), found reduced growth rates for *Mycale (Carmia) hentscheli* (Bergquist & Fromont) (Lerner and Hadju 2002) over time. The growth rates in their study dropped from 2437% $year^{-1}$ to 1355% $year^{-1}$ from the first to the third culture period. Moreover, the growth rates of cloned sponges harvested from cultured explants should also be followed, as Page *et al.* (2001), found reduced growth rates and even negative growth through repeated cloning (F0 to F2).

Other important aspects to include in future studies are seasonality (e.g., is the fast initial growth observed in this study season-influenced or a wound-healing response that is irrespective of season) and genotypic variability.

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Conceptualization, Mert Gokalp, Antonio Sara, Jasper de Goeij and Ronald Osinga; Data curation, Mert Gokalp; Formal analysis, Mert Gokalp, Tim Wijgerde and Ronald Osinga; Funding acquisition, Jasper de Goeij and Ronald Osinga; Investigation, Mert Gokalp; Methodology, Mert Gokalp, Antonio Sara, Jasper de Goeij and Ronald Osinga; Resources, Ronald Osinga; Supervision, Ronald Osinga; Validation, Mert Gokalp, Tim Wijgerde and Ronald Osinga; Visualization, Mert Gokalp and Tim Wijgerde; Writing – original draft, Mert Gokalp; Writing – review & editing, Tim Wijgerde, Antonio Sara, Jasper de Goeij and Ronald Osinga.

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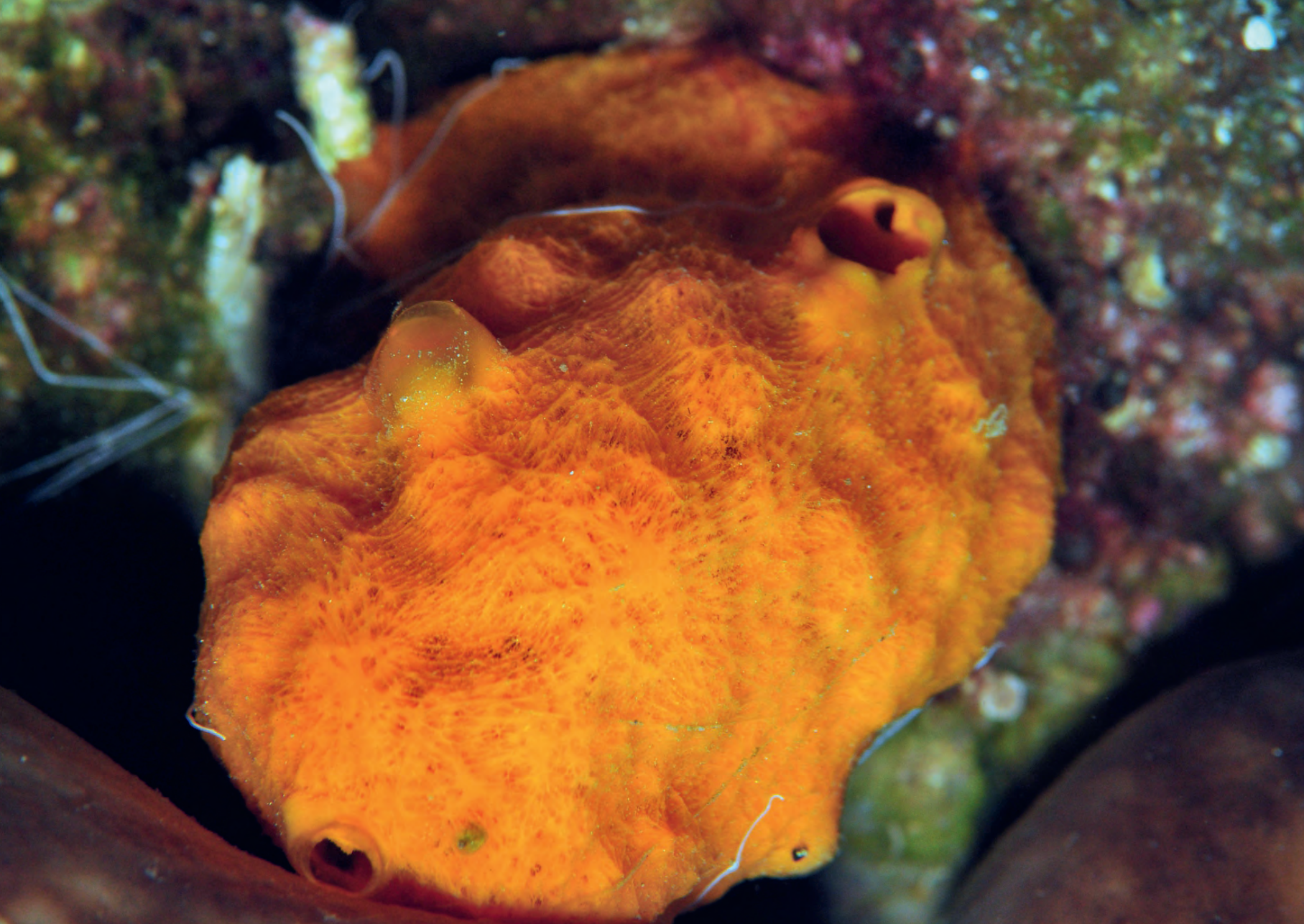

6

Design for large-scale maricultures of the Mediterranean sponge *Chondrosia reniformis* for collagen production

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Submitted to Aquaculture...

ABSTRACT

To support the use of sponges for collagen production in integrated culture settings, we investigated a low–cost, easily applicable and sustainable production method for the culture of *Chondrosia reniformis* Nardo, 1847 (*Demospongiae, Chondrosiida, Chondrosiidae*). Novel methods were tested to culture the collagen-rich sponge in three consecutive trials, during which survival and growth rates of explants were monitored cultured within a polluted site (urban water discharge) and a pristine site. During Trial 1, orientation had a significant main effect on growth rates of glued explants, with vertically mounted sponges showing faster growth compared to horizontal ones (range 63 ± 46% to 116 ± 54%). However, vertically glued explants detached more compared to horizontal ones (30% versus 7%), which was not the case for nailed sponges where only 3% detached regardless of orientation. Thus, horizontally nailed sponges yielded highest survival rates. Based on to these high survival rates with nails, elevated growth rates with PP, and practicality reasons related to drags forces of the currents with vertical placement, PP/nail/horizontal combination was ultimately selected for scale up (Trial 3). An interactive effect of substrate and attachment method on sponge growth rates was also found: glued sponges growing faster on PVC and polypropylene (PP) as compared to iron as substrate, for nailed sponges no growth differences were found between substrates. During Trial 2, no growth differences between sites or orientation were found, although similar to Trial 1, vertically cultured explants showed higher detachment rates (40–52%) and lower survival. Trial 3 revealed survival rates of 75–92% for sponge explants and 83–100% after 467 days of culture. Growth rates were found to be similar between the polluted and pristine sites for both explants facing up/down (range 126 ± 99% to 218 ± 139%) and colonies (range 128 ± 60% to 226 ± 325%). These consecutive culture trials spanning 2 years revealed consistent growth rates and high survival rates in over 1 year of culture, both under pristine and polluted conditions. Ultimately, we now report on a successful culture method for a collagen production pipeline using *C. reniformis*. The final design, entitled 'Sponge lantern', is simple, sustainable, enhances productivity and is adaptable to seawater environments with variable organic particle load.

Keywords: Sponge, Integrated mariculture, *chondrosia reniformis*, collagen, sponge lantern, organic pollution, integrated multitrophic aquaculture

INTRODUCTION

Three decades ago, a wave of in situ and ex situ sponge culture commenced due to the many sponge–derived bioactive metabolites isolated and biomaterials identified from species other than bath sponges (Battershill and Page, 1996; Munro *et al.* 1999; Müller *et al.* 1999; van Treeck *et al.* 2003; Duckworth and Battershill 2003a,b; Page *et al.* 2005; de Voogd 2007; Duckworth and Wolff 2007; Carballo *et al.* 2010; de Caralt *et al.* 2010; Schiefenhövel and Kunzmann *et al.* 2012; Perez–Lopez *et al.* 2014; Ternon *et al.* 2017; Padiglia *et al.* 2018; Santiago *et al.* 2019; Gökalp *et al.* 2019, 2020a). Sponge mariculture was identified to be one of the best applicable methods to supply sustainable quantities of desirable sponge biomaterials such as avarol, collagen, halichondrin B, isohomohalichondrin B, peloruside A, prenylhydroquinones and renieramycins (Sipkema *et al.* 2005; Osinga *et al.* 2010; Perez–Lopez *et al.* 2016; Santiago *et al.* 2019). However, despite extensive reviews on sponge cultivation methods (Osinga 1999; Pomponi 1999; Belarbi *et al.* 2003; Müller *et al.* 2004a; Sipkema *et al.* 2005a,b; Mendola *et al.* 2006; Koopmans *et al.* 2009; Duckworth 2009; Schippers *et al.* 2012; Gökalp *et al.* 2020b) and abovementioned in situ experiments with various potential sponges, commercial–scale mariculture for the production of sponge biomaterials has not yet succeeded. As bioactive compounds are present within sponges in trace amounts only, inflating production costs and hampering economic feasibility (except for the early phases of drug development, Sipkema *et al.* 2005a). Secondly, therapeutic drug development is a time–consuming process, as the clinical phases involved in the process are sumptuous (Pomponi 1999; Müller *et al.* 2004a; Sipkema *et al.* 2005a).

The capability of sponges to process large amounts of water combined with high efficiency in retaining a wide size range of suspended organic particles (Jørgensen, 1949, 1955; Reiswig 1971a; 1974; Vogel 1974, 1977; Riisgard *et al.* 1993) has led to promising clearance experiments with sponges with a wide range of microorganisms (Milanese *et al.* 2003; Hadas *et al.* 2006; Fu *et al.* 2006; Stabili *et al.* 2006; Wehrl *et al.* 2007; Zhang *et al.* 2010; Longo *et al.* 2010; Maldonado *et al.* 2010). There has been a renewed interest in farming of sponges in situ due to the outstanding filtering abilities of sponges and to replenish natural populations of bath sponge stocks (Pronzato *et al.* 1998, 1999; Manconi *et al.* 1999). The ecosystem services provided by sponges in diverse marine habitats, together with their ability to contain and accumulate biomaterials have raised interest in the inclusion of these animals in integrated multitrophic aquaculture (IMTA) applications and in use of treatment of waste water streams (Cebrian *et al.* 2003; Page *et al.* 2005, 2011; Osinga *et al.* 2010; Ledda *et al.* 2014; Longo *et al.* 2016; Gokalp *et al.* 2019, 2020a; Giangrande *et al.* 2020). In this integrated farming approach, sponges consume dissolved and particulate nutrients available in the water column without the need for additional feeding providing a triplet benefit: 1) enhanced production of sponge biomass, 2) prevention of overexploitation from natural stocks and 3) purified water, whether the organic particle load is from suspended fish farm activities or urban runoff (Osinga *et al.* 2010; Ledda *et al.* 2014; Gökalp *et al.* 2019, 2020a).

The success of sponge mariculture is found to be influenced by abiotic factors including light and current conditions of the farming site (Wilkinson and Vacelet 1979). In addition, culture depth, farm location, seawater temperature, and nutrient levels were shown to affect the success of

sponge farming (Verdenal and Vacelet 1990; Osinga *et al.* 1999; Duckworth *et al.* 1997, 2004; Garabou and Zabala 2001; Kelly *et al.* 2004; Corriero *et al.* 2004; Louden *et al.* 2007; Duckworth and Wolff, 2007; Gökalp *et al.* 2019). To achieve maximum growth and survival rates for sponge mariculture, a variety of culture methods (horizontal thread/pin, mesh array, concrete disk, steel cage, suspended rope, tray, PVC plate and lantern) were investigated by researchers, and optimal culture methods were found to be strictly species–specific (reviewed by Duckworth 2009; Schippers *et al.* 2012). A series of long–lasting culture trials revealed several factors that determine the prosperity and sustainability of the culture. These include biotic factors such as predation by marine organisms, diseases and fouling, species-related factors such as broodstock selection and repeated cloning, as well as abiotic factors including water temperature, seasonality/time of seeding and external factors such as storms, wave action, marine traffic and vandalism (Barthel and Theede 1986; Duckworth and Battershill 2003; Duckworth 2009; Osinga *et al.* 2010; Page *et al.* 2005, 2011; Gökalp *et al.* 2019, 2020; Giangrande *et al.* 2020).

Since the 1970's, several attempts have been performed to culture commercial sponges found along the Turkish coastline. An institute was founded in Bodrum to support and regenerate the collapsing commercial sponge business in the region (Gökalp 1974; Katagan and Kocatas 1991; Celik *et al.* 2011). We started farming trials in Turkey with the Mediterranean sponge species *Chondrosia reniformis* (Nardo, 1847) started in 2006, for the particular purpose of production of avarol and collagen for cosmetic and biomedical applications (Osinga *et al.* 2010). Later, we investigated optimal culture conditions and tested in situ bacterial clearance rates by *C. reniformis* in order to secure a raw collagen production pipeline combined with an IMTA method for *C. reniformis* around suspended fish aquaculture cages (Gökalp *et al.* 2019, 2020a). We found that *C. reniformis* benefited from mariculture–sourced organic pollution and showed better growth performance in polluted waters compared to a control site (170% versus 79% in 13 months, Gökalp *et al.* 2019). Sponge aquaculture therefore might benefit from an excess amount of organic matter surrounding urban sewage outlets and grow faster (Ledda *et al.* 2014; Gökalp *et al.* 2020a). Despite these advancements, commercial mariculture of *C. reniformis* remained problematic as because of its morphological plasticity (Bavestrello *et al.* 1995, 1998; Bonasoro *et al.* 2001; Wilkie *et al.* 2006; Fassini *et al.* 2012), culture methods suitable for other sponge species do not apply to this sponge. Therefore, we designed culture plates and special platforms to culture this species. Although the sponges performed best when grown on vertically positioned plates (Gökalp *et al.* 2019), a persisting problem is the loss of many sponge explants from the vertical plates due to active detachment. Furthermore, the vertically oriented plates mounted on seafloor-based culture frames were prone to disrupting drag forces under occasional conditions of high water flow.

The first aim of this study was to test whether losses of sponges in culture due to detachment could be reduced. The use of nails to attach sponges to plates was compared to the previously applied method of attachment by acrylate glue. In addition, we compared the suitability of two alternative plate materials, polypropylene (PP) and iron, to the PVC plates used in our earlier study (Gökalp *et al.* 2019). The choice for PP was based upon observations during the earlier study (Gökalp *et al.* 2019), in which we noted that sponges that had detached from vertical PVC plates often continued to grow on horizontally oriented PP support plates mounted underneath the vertically oriented PVC plates. The choice for iron plates was based on observations by

Osinga and Kotterman (2007), who reported that continuous addition of ferric iron (Fe3+) promotes the formation and activity of the sponge aquiferous system of several species including *C. reniformis*. The current study started with a multifactorial experiment to test the suitability of the three selected plate materials in combination with the new method to attach the sponges by nails (Culture Trial 1). To corroborate our earlier findings that sponges on vertically oriented plates outperform sponges oriented horizontally and that shaded sponges grow faster than sponges exposed to direct sunlight (Gökalp *et al.* 2019), these factors were also included in this first culture trial.

The second aim of this study was to explore whether mariculture of *C. reniformis* can be combined with purification of municipal wastewater. The successful enhancement of growth of *C. reniformis* by placing the cultures in the vicinity of fish farms (Gökalp *et al.* 2019) suggests that the sponge cultures may benefit from sources of organic pollution. In the current study, we experimented in the vicinity of a municipality wastewater discharge point (Culture Trial 2) in order to compare growth performances of *C. reniformis* to: 1) a pristine control site and 2) our earlier study carried out in the vicinity of fish farms (Gökalp *et al.* 2019)

The third aim of this study was to design and implement a robust, scalable culture system that integrates best practices and that reduces vulnerability and operational costs, in order to enable economically feasible, commercial-scale production of raw sponge material for the extraction of collagen (Culture Trial 3). Specifically, Culture Trial 3 aimed to: 1) quantify long-term in situ survival and growth of *C. reniformis*, 2) compare the growth of cut sponge fragments to the growth of non-fragmented colonies, and 3) implement a low–cost, easily applicable, sustainable and profitable production method for the culture of *C. reniformis*, that is able to handle a range of sea conditions and applicable in environments with varying current, depth, turbidity and organic particle load.

MATERIALS AND METHODS

Study Location and Seawater Parameters

Sponge culture experiments were performed in the Levantine Sea within the Kas–Kekova Special Environmental Protected Area (SEPA, protection status since 2014) of Antalya (Figure 1). The marine environment of Kas is characterized by a rocky shoreline dropping rapidly down to about 50 m depth. Frequently structured submerged rocks and diversely settled shoals are scattered around the Posidonia oceanica meadows surrounding the islands and the indented shoreline (Figure 1). The trials for the mariculture of sponges were set on naturally formed sand/gravel patches within the seagrass meadows/submerged rocks at depths between 14 m and 26 m. Two major sites were selected for the sponge culture experiments, based on water visibility (Secchi disk, cf.) and organic loading (total organic carbon (TOC) measurements). The pristine site lies 4.3 kilometers south of Kas (Figure 1), located within the western border of the marine protected area, exposed to westerly wave and wind action. Being sheltered by Çukurbag Peninsula, the polluted site is at 100 meters distance to the shoreline and 2000 meters west of Kas harbor, located right next to the municipal wastewater treatment system (Figure 1). Kas harbor and the treatment facility are located right next to the SEPA area. The harbor is seasonally affected by wastewater, urban runoff and marine recreational activities (boating, tourism and

vessel maintenance). The deep discharge pipeline (DDP) of the wastewater facility has three outlets located in between 20-28 m depths, releasing a plume of treated effluent upwards directly into the seawater. The polluted site was chosen as an alternative source of organic pollution to allow for comparison to the previous study that we conducted in the close vicinity of suspended mariculture cages (Gökalp *et al.* 2019). To determine organic loading, three replicate water samples (50 mL) were taken within 10 m of the culture platforms from each location by scuba diving for TOC analysis using the wet oxidation method, similar to our previous study (Gökalp *et al.* 2019).

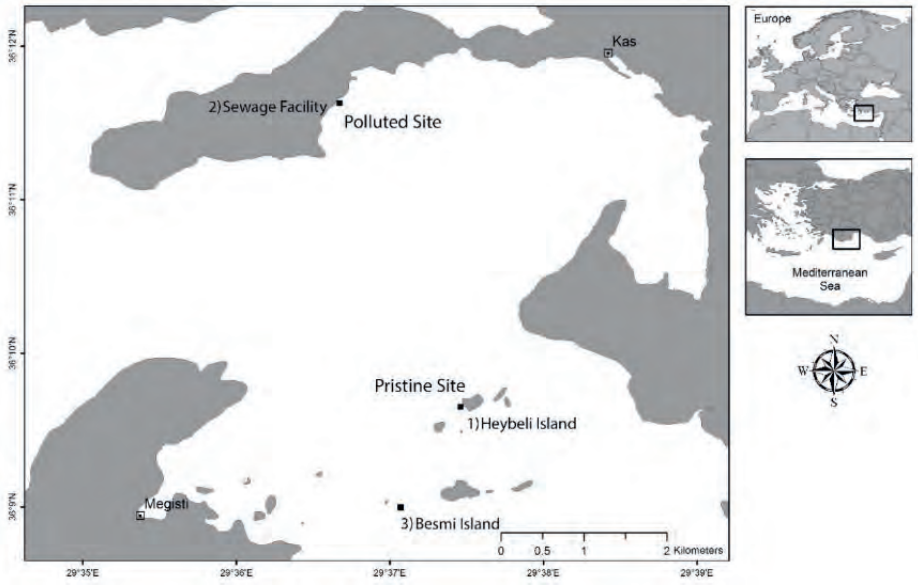

Figure 1. Kas–Kekova Special Environmental Protected Area and Kas and Castellorizo (Megisti) harbors. 1) Pristine site is located at Pina Reef diving location, which is at the eastern tip of Heybeli Island and the western border of Kas SEPA. 2) Polluted site is located right next to the deep discharge pipeline of the Kas municipality water treatment center which is located at the eastern shore of the Çukurbag Peninsula. 3) The sponges were collected at Besmi Island. The exact locations of experiments: 1) Pina–reef (pristine, 36°09'42.4"N 29°37'26.5"E) 2) Deep discharge pipeline (polluted, 36°11'36.2"N 29°36'42.0"E) and the sponge collection site 3) Besmi Island (36°09'02.7"N 29°36'58.2"E).

Sponge Collection and Seeding

Sponge specimens for the three mariculture trials were collected from naturally occurring sponges at Besmi Island (36°09'02.7"N 29°36'58.2"E) in between 15–25 meters depth using Scuba diving (Figure 1, #3) as large populations of *C. reniformis* cover the sea bed, less than an hour boat distance to both culture sites. Following collection, the sponges were then transported to the culture sites, cut into pieces following the method described in Gökalp *et al.* (2019) and seeded onto the designated plates. We left the explants horizontally next to the culture platforms for 4–7 days before the plates were secured to their spots on the culture platforms in order to enable sponges to attach and acclimatize after seeding (Sipkema *et al.* 2005a; Gökalp et al 2019). Following the acclimatization period, plates were installed on their designated culture systems according to the experimental design (see below for details).

Table 1. Summary of the methods tested for the culture of *C. reniformis.* One culture plate was considered the unit of replication.

Culture Experiment	Culture Substrate	Orientation	Attachment	Pollution	Light Exposure	Specimens per culture plate	Depth (m)
Culture Trial 1 *PP Boxes*	PVC PP Iron Plates	Horizontal Vertical	Glue Nail	Pristine	Facing sun Facing shadow	2 explants	14
Culture Trial 2 *PP Boxes*	PVC Plates	Horizontal Vertical	Glue	Pristine Polluted	NA	1 explant	14
Culture Trial 3 *Sponge lanterns*	PP Plates	Horizontal	Nail	Pristine Polluted	Facing up Facing down	1 explant 1 colony	22–26

Culture Trials, June 2018 – June 2020

The three mariculture trials were executed between June 2018 and June 2020 (Table 1). Culture Trial 1 was designed to disclose the best combination of culture materials and positioning of the sponges (Subsection 2.3.1). Culture Trial 2 (Subsection 2.3.2) aimed to test effects of the culture environment (pristine versus organically enriched). In Culture Trial 3, best practises obtained from Culture Trials 1 and 2 were integrated into a first upscaling towards commercial-size mariculture (Subsection 2.3.3). For Trials 1 and 2, polypropylene (PP) boxes with the following dimensions were used as culture platform: height 28 cm, width 40 cm, length 60 cm (Figure 2a,b). All boxes were covered with chicken wire to eliminate possible predation from surrounding marine life, and to prevent specimens migrating off the plate or being carried away by occasional strong seawater currents (Gökalp *et al.* 2019, 2020a). The PP boxes were selected to facilitate a cheap, lightweight and environmentally safe hard substratum for the *C. reniformis* explants that enabled easy transport and culture orientation combinations for the trials (Figure 2a,b). For Culture Trial 3, 12 sponge lanterns (height 400 cm, radius 60 cm, modified from Battershill and Page, 1996 and Kelly *et al.* 2004 were used. These were surrounded by fishing nets as a protection from the factors described above (Figure 2c). As stated by Duckworth and Battershill (2003a), mesh size can influence sponge growth. The mesh size (4 cm) of the lanterns was kept large enough to allow sufficient nutrition carried by water flow to the explants. The lanterns were held vertically in the water column by buoys and anchors where PP plates provided shade for the layer below. Holes were made in the net to allow easy access to the cultured sponges for maintenance and photography.

Culture Trial 1 (Initial Trial) - June 2018–October 2018

In Trial 1, sponge explants (240 in total) were attached to 3 different types of culture plates (PVC, PP and Iron) in 2 different orientation types (vertical vs horizontal), using 2 different attachment methods (glue vs nail) and 2 different light exposure conditions (facing sunlight vs facing shadow; see Figure 2a,3a and Table 1 for details). Twenty polypropylene boxes were used for the experiment, with ten boxes facing south (exposed to sunlight) and ten boxes facing north (shaded). Each box hosted 6 plates with 2 explants per plate, i.e. 12 sponge explants per box. For statistical analysis (see below), a plate was considered the experimental unit (i.e. two explants

on one plate were averaged, n=10). All boxes were placed at 14 m depth, vertically on their long sides, 5 meters apart from each other and secured with 2 stone weights (weighing 4 kg each) on each side. The boxes facing south/sunlight were positioned towards a sand/gravel opening with a Posidonia seagrass meadow 10–20 m beyond this opening whilst the ones facing north/shadow were positioned towards the outskirts of the Pina small reef that was 2 meters away from these boxes.

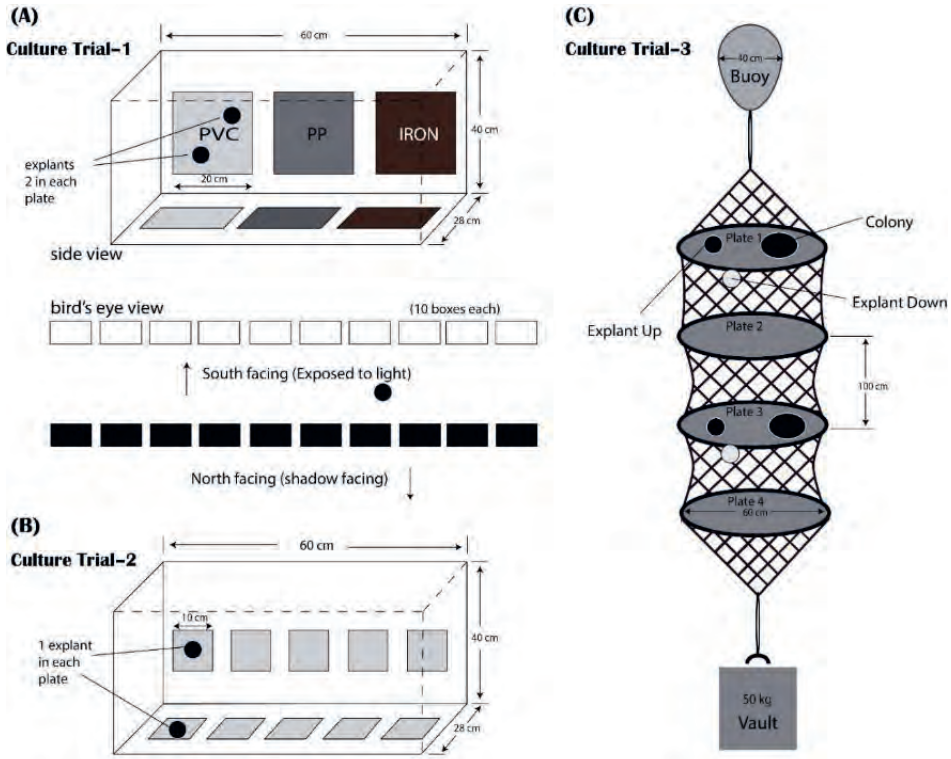

Figure 2. Schematic overview of sponge platforms and plate types tested in Culture Trials 1–3. (A) *Culture trial 1 Side view* and *bird's eye view*: PP boxes were used for both Culture Trial 1 and 2. (B) *Culture trial 2* (C) Placement of explants and colonies in the lantern system (Pictures are shown in figure 3). The metal rings used for stretching the net also carry the PP plates with explants that could be inserted via vertical slots (not shown) in the netting. Plates 2 and 4 in the lantern were left empty. The sponge lanterns used for Culture Trial 3 were constructed by combining the design and principles of scallop lanterns used for sponge mariculture (Battershill and Page 1996; Kelly *et al.* 2004) and European eel nets normally applied horizontally in freshwater creeks.

Culture Trial 2 (Initial trial), June 2018 – October 2018

We designed culture trial 2 to study the effects of the organic pollution from the deep discharge pipeline (DDP) on sponge growth rates based on the results of our previous study, where fish farm effluents had a positive effect on sponge growth rates (Gökalp *et al.* 2019). Sponge explants (1 explant per plate, n=10 per box; 100 explants in total) were glued to PVC plates in 2 different orientation types (vertical vs horizontal; n=5 per box) in 2 different turbidity conditions (pristine vs polluted; n=50 per site; see Figure 2b,3b and Table 1 for details). The culture systems (n=5) were placed at 14 m depth and 5 m apart from each other for both sites.

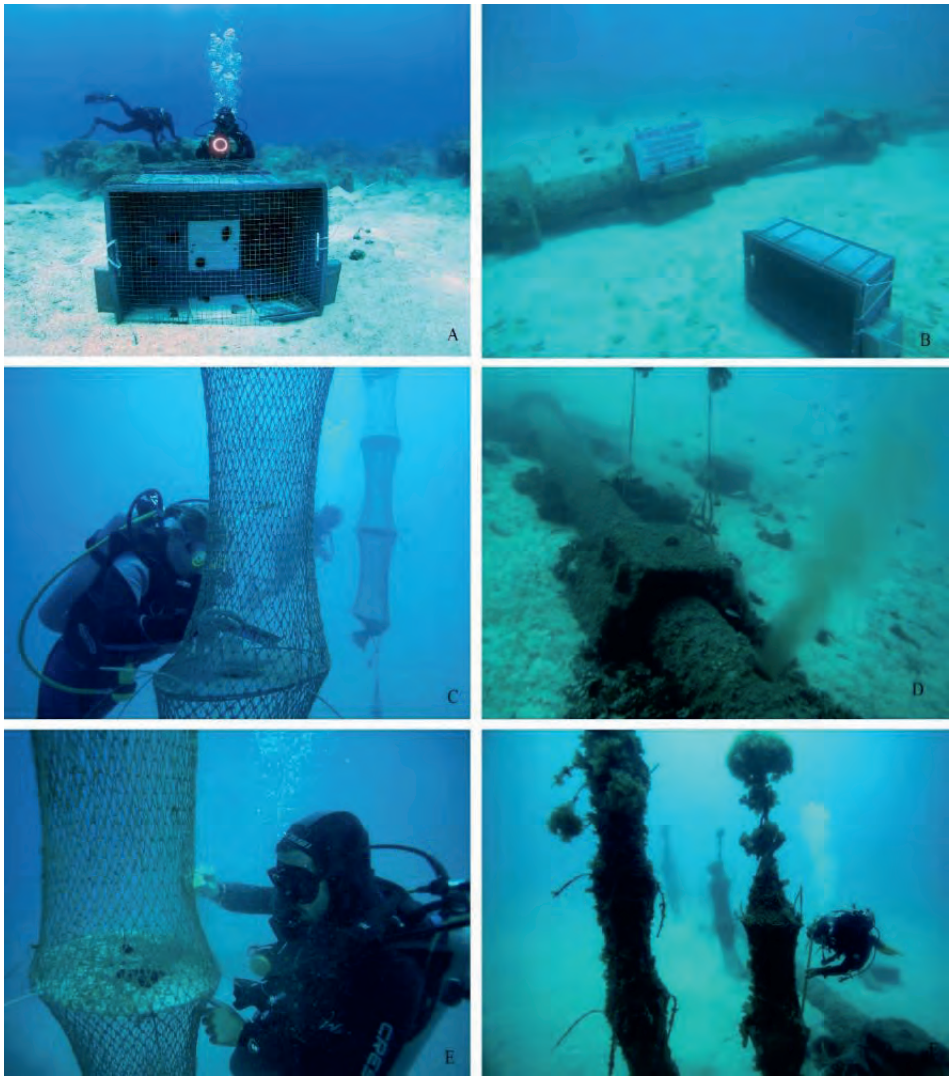

Figure 3. a) Culture trial 1, pristine site; divers working on PP boxes and taking initial pictures of explants b) Culture trial 2, polluted site; PP boxes located on both sides of the deep discharge municipality pipe at 14 m depth c) Culture trial 3, pristine site - divers checking the cultured specimens on PP plates inside the sponge lanterns d) Plume ejecting directly out of the pipeline from one of the free outlets of the deep discharge pipe at 25 m e) Pristine site –Diver cleaning the protective net of the sponge lantern; Kas economy is driven by tourism and there had been multiple complaints on the quality of the seawater (around the deep discharge pipe) both from the diving centres/recreational facilities and from the public for 2 decades and in the course of the current study. f) Polluted site – Major epibiont growth over the system following the unattended winter months. Diver cleaning the algal growth over the nets, buoys and ropes of the sponge lantern system. The weight of the epibionts effected the buoyancy and caused lower plate to touch the bottom for 2 lanterns, however without negative effects on the sponges.

Culture Trial 3 (Scale–up), June 2019 – June 2020

The sponge lantern system (Battershill and Page, 1996), which is basically a modified eel net with PP plates inside, was designed to provide a flexible method of sponge culture for varying

oceanographic conditions (see Figure 2C and Figure 3C for details of the method). The lanterns at the pristine site were deployed on a sand/gravel opening, 50 m southwest of the Pina–reef wall and 30 m northwest of the Pina small reef (Figure 1). The lanterns at the polluted site were placed next to the DDP (Figure 1, 3D). At each site, the culture systems (n=6, per site) were installed at 25–28 m depth, approximately 5 m apart from each other. Each culture unit was anchored by a cement weight (50kg) and buoyed, so that the system would be held vertically. This subsurface float (radius=40 cm) remained at 13–16 m depth (Figure 2C).

Based upon a combination of results obtained during Culture Trial 1 and practical considerations (which will be further explained in the discussion), it was decided to apply horizontally mounted PP plates in Culture Trial 3, and to apply nails as the method for attachment. Each lantern hosted 4 circular PP plates (diameter=60 cm; width=2 cm) that were placed at 1 m depth intervals. To study the effect of cutting on sponge growth/survival rates, we collected *C. reniformis* colonies and left some of these intact for the experiment (Duckworth *et al.* 1997). Intact colonies (n=2 per lantern; width=5–10 cm) were placed on the 1st and 3rd PP plates (top to bottom). To compare explant performance cultured in a more shaded versus a more lit environment, cut explants (n=4 per lantern: width=2–3 cm) were nailed on both on top (n=2 per lantern) and underside (n=2 per lantern, on a smaller rectangular PP plate nailed to the larger plate) of the 1st and 3rd PP plates (Figure 2C; Table 1). The 2nd and 4th plate of each lantern were left empty, to prevent cross-over of replicates. Again, similar to Trials 1 and 2, one PP plate was considered as the experimental unit. We should note here that the downward orientation of the sponges mounted onto the underside of the plates in the 3rd trial differs from Trials 1 and 2, during which explants were mounted vertically. An upside down orientation also occurs in naturally growing populations of *C. reniformis* colonies. *C. reniformis* orients itself over the rocks (facing up, facing down, perpendicular, et cetera) depending on depth, current, light and the location where it settled (Wilkinson and Vacelet 1979; Bavestrello *et al.* 1998; Nickel and Brummer 2003; Cebrian *et al.* 2006).

Sponge Growth Rates

Sponge growth rates were determined from the photographs taken during the periodical visits to the culture locations. Due to the tight space available in the culture frames, explants were photographed with a GoPro Hero 5 Black digital camera next to a ruler (GoPro. Inc. San Mateo, CA, USA). Following each dive, recorded images were transferred to Photoshop CS7 software (Adobe Systems Incorporated, San Jose, CA, USA) and lens distortion was corrected. The images were calibrated using known plate dimensions, peripheries of explants were marked and sponge surface areas were calculated from pixel counts of the marked areas by using ImageJ software (LOCI, University of Wisconsin). Growth was expressed as the increase in the number of pixels, calculated with the pixel counter function of the image editing software and sponge survival and growth rates were calculated according to the method described in Gökalp *et al.* (2019).

Data Analysis

To analyze main and interactive effects of orientation (horizontal vs vertical), substrate (PP, PVC and iron) and attachment (glue vs nail) on growth rates for Trial 1, a three-way factorial

ANOVA was performed. Light exposure condition (facing sunlight vs facing shadow) were discarded from this analysis as no significant differences were found in between these groups (North and South were pooled to substantiate the three-way factorial Anova). To analyze effects of culture site and orientation on growth rates for Trial 2, a two-way factorial ANOVA was used. To determine the effect of orientation on sponge detachment rates for Trials 1 and 2, Fisher's Exact Test was used. For Trial 3, since this design was not fully factorial (i.e. no colonies in down position tested, only up), we performed two separate three-way factorial mixed ANOVA's: one testing for effects of time, site and orientation using the data for explants up and down, and another testing for effects of time, site and sponge type using the data for explants up and colonies up. All data were found to be normally distributed after a square root transformation as indicated by a Shapiro-Wilk test ($p>.050$) and histograms. For Trial 3, the data violated the assumption of sphericity (Mauchly's test, $p<.050$), thus a Greenhouse-Geisser correction was used for testing main and interactive effects of time. Bonferroni corrections in cases of multiple testing were used by inflating p-values by the number of tests. P-values below $\alpha=.05$ were considered statistically significant. Statistical analyses including graph plotting were performed using SPSS v 25 (IBM Corporation, USA).

Productivity and feasibility

To compare methods, data on growth and survival from Culture Trial 1 were used in combination to calculate an overall production factor (P, calculated as the percentage increase in sponge biomass in relation to the initial amount of sponge biomass seeded) for each of the 12 combinations of plate type (PP, PVC, Iron), attachment method (glue, nail) and orientation (horizontal, vertical):

P = ((Final size – Initial size)/Initial size) x 100 x (Nr of survivors)/(Initial nr of sponges) (1)

To assess economic feasibility for the upscaled culture system (Culture Trial 3), productivity was calculated as above and converted into productivity in kg sponge per sponge lantern per year using conversion factor of 1.03 g cm-3 to convert surface area into wet mass (Sipkema *et al.* 2005a). This productivity was then divided by the estimated costs for constructing, deploying and maintaining a sponge lantern to obtain a cost per kg wet mass of the raw sponge material needed for collagen production.

RESULTS

General Observations

The surface temperatures at the study sites exhibited seasonal variations typical of the Eastern Mediterranean Sea with an average annual value of 21.9 ± 3.8 °C (n=12), while the salinity was uniform for both experimental sites, approximately 38.6 PPT, and almost uniform over the year at 20 meters depth. During the span of the trials, the temperature ranged from 17.3 to 28.1 °C for both sites (next to the lanterns at 22 m). From June to September (2018–2020), when the population of Kas–Kekova dramatically increased, the visibility of the seawater declined at the polluted site (10.8 ± 1.6 m), in contrast to the pristine site (19.5 ± 1.9 m; Secchi disk, see above) and there was considerable benthic algal growth at the polluted site. Especially during the season peak (July–August), the visibility of the polluted site occasionally dropped further to 5

meters. TOC levels at the polluted site (161.0 ± 0.057 μmol L^{-1}; N=5) were 1.4 times higher than those of the pristine site (113.8 ± 0.024 μmol L^{-1}; N=5).

In May 2018, a first attempt to start the field experiments had been unsuccessful: 14 days after seeding, most of the explants had disappeared from the boxes during a routine control. It later became clear that sea turtles were most likely responsible for this unfortunate start. Locally habiting *Caretta caretta* sea turtles are occasional feeders of *C. reniformis* sponges and they probably predated on exposed sponge explants (Karaa *et al.* 2018; Haywood *et al.* 2020). This behavior was not directly documented on site, but occasional sea turtle bite marks on both natural *C. reniformis* specimens and chicken wire covers were spotted around the culture site. As a consequence of this predation, the experiments were restarted in June 2018. New explants were collected, and chicken wires covering the open part of the PP boxes successfully prevented the sea turtles from feeding on explants.

Initially, the polluted site was assumed to be calmer than the pristine site, as the latter faces the winter storms directly and is exposed to powerful wave action. However, this proved to be wrong after the disappearance of 2 sponge lanterns following a heavy storm in July 2019 (Trial 3). The lantern systems were recovered undamaged, perfectly erect, but located 60 meters away from the pipeline and 12 meters deeper than the rest of the system. In order to protect the systems during the winter season and occasional heavy storms (Kelly *et al.* 2004), each lantern was secured to the pipeline with a rope. In addition, the pristine sponge lanterns were held together by ropes at the bottom. Finally, after the summer period, in October 2019, we observed several invasive marine organisms taking advantage of our free-floating culture platforms as a result of lack of cleaning during the winter period (6 months). The epibionts were cleaned over a period of 2–3 days at each culture site by using hard brushes, and no negative effects to cultured sponge specimens were observed. After one year of being submerged in the sea, collected PP plates showed no sign of unwanted marine growth on their surfaces. Any material on them was easily discarded (This applies to Trials 1 and 2).

Culture Trial 1

During the 1st trial (83 days), in spite of the acclimation period of 4 days, initial losses were observed mainly for the glued and vertically grown explants. Highest retention rates were found for nailed and horizontally mounted sponges (Table 2). Overall, 168 of the 240 explants (70%) were retained in the experiment. Of the 72 explants lost during this period, 49 were found to be growing on the lower joint part of the PP boxes where they were firmly attached. If these fallen explants are added to the count, the overall survival rate is 87%. However, since they could not be related anymore to their original size, explants that were attached on the PP boxes were left out of the growth analysis. Orientation had a significant effect on detachment rate of glued explants (Table 2), where vertically placed sponge explants detached more compared to the horizontally placed explants. For nailed explants, orientation had a significant effect on survival and overall, horizontally placed explants performed 1.2 times better than vertically placed explants (Table 2). From each group, only 1 explant detached out of 60 (Table 3). Regardless of plate material, some of the vertically nailed explants left on plates were found to be developing a circular ring around the nails as if avoiding contact and de-attach their bodies, as if trying to reach a possible ground to spread. Explants cultured on Iron plates remained slightly better than those

cultured on PVC and PP, horizontally cultured explants retained better compared to their vertical counterparts, nailed explants survived better than glued explants and of shadow facing explants slightly more were recovered than from south facing ones (Table 2).

Table 2. Culture Trial 1,2 and 3. Survival rates (%) of the explant at the end of the culture period (June 2018 to October 2018) for Culture Trials 1&2 and survival rates of explants and colonies cultured in sponge lanterns (June 2019 – October 2020) for Culture Trial 3.

<table>
<tr><td></td><td>Culture Material</td><td>Orientation</td><td>Attachment</td><td>Light Exposure</td></tr>
<tr><td colspan="5">Culture Trial 1 (Pristine site)</td></tr>
<tr><td>Survival Rates</td><td>PVC 63%
PP 71%
IRON 76%</td><td>Horizontal 86%
Vertical 54%</td><td>Glue 53%
Nail 88%</td><td>South – Light 65%
North – Shadow 75%</td></tr>
<tr><td colspan="5">Culture Trial 2 (Pristine & Polluted site)</td></tr>
<tr><td></td><td colspan="2">Site</td><td colspan="2">Orientation</td></tr>
<tr><td>Survival Rates</td><td colspan="2">Pristine: 78%*
Polluted: 64%**

*if detached explants included: 92%
**if detached explants included: 82%</td><td colspan="2">Horizontal: 88%*
Vertical: 54%**

*if detached explants included: 88%
**if detached explants included: 87%</td></tr>
<tr><td colspan="5">Culture Trial 3 (Pristine & Polluted site)</td></tr>
<tr><td>385 Days in culture</td><td colspan="2">Pristine</td><td colspan="2">Polluted</td></tr>
<tr><td></td><td>Colony</td><td>Explant</td><td>Colony</td><td>Explant</td></tr>
<tr><td rowspan="2">Survival Rates</td><td rowspan="2">100%</td><td>Explant-1
Facing Up 92%</td><td rowspan="2">100%</td><td>Explant-1
Facing Up 83%</td></tr>
<tr><td>Explant-2
Facing Down 83%</td><td>Explant-2
Facing Down 75%</td></tr>
<tr><td>467 Days in culture</td><td colspan="2">Pristine</td><td colspan="2">Polluted</td></tr>
<tr><td></td><td>Colony</td><td>Explant</td><td>Colony</td><td>Explant</td></tr>
<tr><td rowspan="2">Survival Rates</td><td rowspan="2">100%</td><td>Explant-1
Facing Up 92%</td><td rowspan="2">83%</td><td>Explant-1
Facing Up 75%</td></tr>
<tr><td>Explant-2
Facing Down 75%</td><td>Explant-2
Facing Down 75%</td></tr>
</table>

As direction (North versus South facing explants) had no significant effect on growth (ANOVA $p>0.05$), data for North and South were pooled. Subsequently, a three-way ANOVA was was performed. Substrate, attachment method and orientation all had a significant main effect on sponge growth rates as measured by surface area increase (Supplementary Table 1; Fig. 4). Explants grew significantly faster when mounted vertically (116 ± 54%) as compared to horizontal ones (63 ± 46%), irrespective of substrate and attachment method (Figure 4). An interactive effect of attachment method and substrate on explant growth was found (Supplementary Table 1). This was due to the fact that substrate had an effect on growth of glued sponges only (Supplementary Table 1), where glued sponges grew faster on PVC and PP as compared to iron ($p=.020$ and $p=.015$, respectively, simple effects contrast). Nailed sponges did not show any growth differences between substrates ($p>.050$). Vice versa, glued sponges grew faster than nailed sponges on PVC ($p=.024$), which was not the case on PP ($p=.260$) or iron ($p=.904$).

Table 3. Frequencies of detached sponge explants for trial 1 and 2. For trial 1, explants were split into two groups; mounted using either glue or nails. Direction (North versus South) and substrate (PVC, PP and Iron) did not yield significant results and are therefore not shown. Explant was used as the experimental unit. For trial 2, explants were split into the pristine and polluted culture site. P-values for Fisher's Exact test, showing the effect of orientation (horizontal versus vertical) are included.

Culture Trial–1

			Detached			
Attachment			no	yes	Total	*P (two-sided)*
Glue	Orientation	horizontal	46	14	60	0.000***
		vertical	17	43	60	
Nail	Orientation	horizontal	57	3	60	0.025*
		vertical	48	12	60	
	Total		168	72	240	

Culture Trial 2

			Detached			
Site			no	yes	Total	*P (two-sided)*
Pristine	Orientation	horizontal	24	1	25	0.005**
		vertical	15	10	25	
Polluted	Orientation	horizontal	20	5	25	0.038*
		vertical	12	13	25	
	Total		71	29	100	

*p<0.05, **p<0.01, ***p<0.001

Supplementary Table 1. Culture Trial 1 & 2, statistical analysis. Three-way ANOVA testing for main and interactive effects of orientation, substrate and attachment on growth rates for Culture Trial 1 and two-way ANOVA testing for main and interactive effects of culture site and orientation on growth rates for Culture Trial 2 after 83 days. Both culture directions (i.e. North and South) were averaged as they were not statistically different for Culture Trial 1.

Culture Trial 1	**Factor**	**F**	**df**	**Error**	**p**
N=10 plates per treatment combination	Orientation	40.916	1	85	.000 ***
	Substrate	10.664	2	85	.000 ***
	Attachment	11.152	1	85	.001 **
	Orientation * Substrate	.896	2	85	.412
	Orientation * Attachment	.505	1	85	.479
	Substrate * Attachment	3.128	2	85	.049 *
	Orientation * Substrate * Attachment	.489	2	85	.615
*Simple effects contrast for Substrate * Attachment*					
N=10 plates per treatment combination	**Substrate**	**F**	**df**	**Error**	**p**
	Glue	5.577	2	91	.005**
	Nail	2.124	2	91	.125
Culture Trial 2	**Factor**	**F**	**df**	**Error**	**p**
N=25 plates per treatment combination	Culture Site	.239	1	67	.627
	Orientation	.823	1	67	.368
	Culture Site * Orientation	.220	1	67	.641

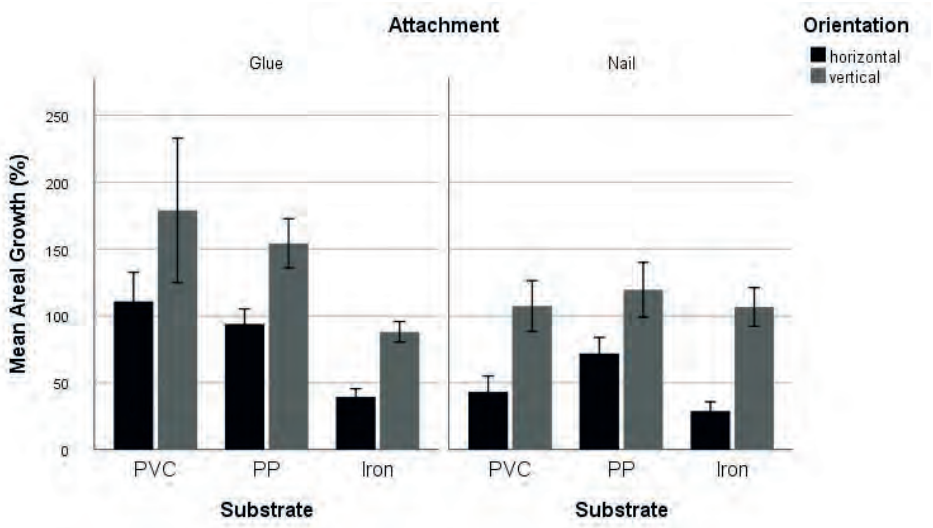

Figure 4. Culture Trial 1, June to October 2019 (83 days). Mean areal growth rate of explants (N=10 plates per treatment combination) grown on 3 different plates (PVC, PP, Iron), attached by two different methods (glue, nail) and placed in two various orientations (horizontal/vertical).

The most spectacular growth performances were recorded for explants vertically cultured on PVC and PP plates that were attached by gluing (179 ± 76% and 154 ± 41%, respectively, Figure 4). Nevertheless, these plates provided the highest loss (45% and 55%), which reduces the overall productivity (P) for this method. Productivity calculations (Equation 1) show that PP plates outcompete PVC and iron (Table 4). When nails are used, vertically oriented cultures are more productive than horizontal cultures, when glue is used, horizontal plates overall are most productive. It should be noted that on PP plates, the use of nails on horizontally oriented plates resulted in a productivity that was almost equal to the productivity of glued sponges on horizontal plates.

Horizontally nailed sponges resulted in the highest survival rate and a good growth performance for all culture materials tested, and therefore was the selected method of attachment and orientation for Trial 3 (Table 4). Because of the low growth rates, high weight of the material, and difficulty of use under water we excluded Iron from trials 2 and 3. For Culture Trial 3 we decided to continue with PP because of its superior production performance (71.5%) compared to PVC and Iron (55.3%, 46.7%; respectively) along with higher survival rates (Table 4). The reasoning for selection of PP/nail/horizontal combination is as follows:

- Nails overall outperform glue, so that is the safest choice to keep sponges on plate.
- PP overall shows the highest growth rates.
- Horizontal orientation of the plates may prevent drag forces. However, if the current pulls the lanterns into a more horizontal position (which was observed sometimes), they become vertically oriented and increase the drag. On the other hand, this movement of the lanterns in the current makes the difference between horizontal and vertical less pronounced: if there is current, the sponges will be oriented vertically, if there is no current, they will be oriented horizontally.

- ❖ The earlier observed difference between shade and light is not confirmed, so this is apparently not as relevant as we thought.

Table 4. Culture Trial 1–2–3, production calculations.

Production parameters		**Vertical**		**Horizontal**		
Culture Trial–1		Nail	Glue	Nail	Glue	
Retaining Rates (%)	PVC	75	15	95	65	
	PP	80	30	95	75	
	IRON	85	40	95	85	
Growth Rates (%)	PVC	107	179	44	111	
	PP	120	154	72	94	
	IRON	107	88	29	39	
(Growth x Survival)/100	PVC	80.3	26.9	41.8	72.2	55.3
	PP	96.0	46.2	68.4	75.2	**71.5**
	IRON	91.0	35.2	27.6	33.2	46.7
		89.1	36.1	45.9	60.2	**Average**

Culture Trial–2	**Pristine Horizontal**	**Polluted Horizontal**	**Pristine Vertical**	**Polluted Vertical**
Growth (%)	132.0	133.0	139.0	149.0
Retention (%)	96.0	80.0	60.0	48.0
(Growth x survival/100)	126.7	106.4	83.4	71.5

Culture Trial–3	**Pristine Explant**	**Polluted Explant**	**Pristine Colony**	**Polluted Colony**
Growth (%)	150.0	182.0	103.0	152.8
Retention (%)	92	75	100	100
(Growth x survival/100)	138.0	136.7	103.0	152.8

Culture Trial 2

For both the pristine and the polluted site, orientation had a significant effect on detachment rate, where horizontally cultured explants performed better than their vertical counterparts (Table 2,3). In general, the pristine site survived slightly better (1.2 times) compared to the polluted site (Table 2). If explants dropped inside their boxes are included in the analysis, survival rates for both sites were 92% and 82%. Explants cultured at the pristine site grew at a similar rate compared to the polluted site in 83 days of culture (135 ± 63 % vs 142 ± 57%; Figure 5), with no statistical differences between sites or orientation (Supplementary Table 1), the latter being in contrast to the results obtained in Culture Trial 1, where growth of sponges glued on vertical PVC plates was significantly faster than growth of sponges glued on horizontal PVC plates. Productivity for horizontally oriented plates at the pristine site was 127% (Table 4), which is considerably higher than productivity on comparable plates (PVC, horizontal, glued sponges) in Culture Trial 1, which was only 72% (Table 4).

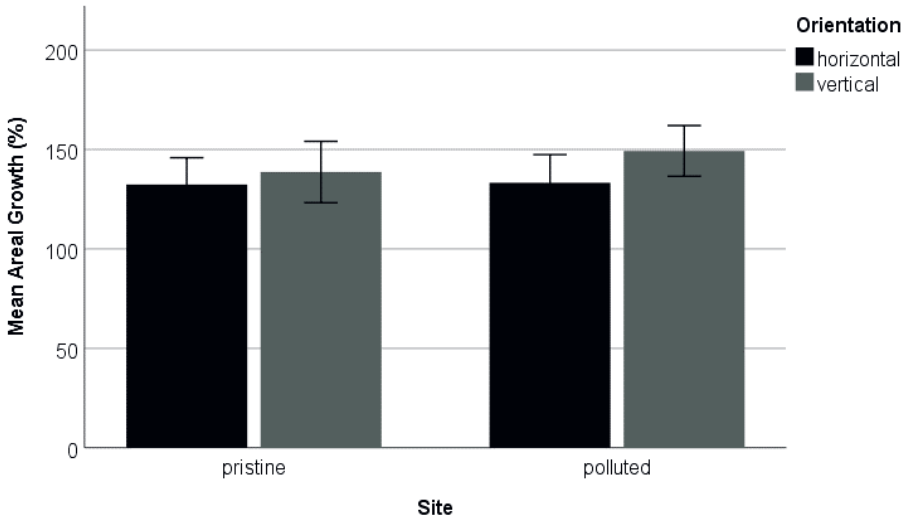

Figure 5. Culture Trial 2, June to October 2019 (83 days). Mean areal growth rate of explants (N=25 plates per treatment combination) grown on PVC plates attached by gluing, at two different culture sites (pristine and polluted).

Culture Trial 3

Just over one year (385 days) after the sponge lanterns were installed, there was a 100% recovery rate for the cultured colonies at both the pristine and the polluted site (Table 2). Recovery of explants facing up and facing down were almost identical for both sites during this period (Table 2). After 467 days however, 2 colonies were lost from polluted site due to unknown reasons, while pristine colonies retained their numbers. Up facing explants at the pristine site showed better recovery (%92) compared to the explants facing down (pristine) and up and down facing explants of polluted site (%75; Table 2). After being cultured for 467 days, average surface area increase for colonies was found to be 128 ± 60% at the pristine site and 225 ± 325% at the polluted site (Figure 6). In contrast, the average surface area increase for explants facing up at the pristine site was around 1.2 times more compared to the polluted site. Whereas average surface area increase for explants facing down at the pristine site was 1.4 less compared to the polluted site (Figure 6). A significant main effect of time on growth rates for explants as well as colonies was found (Supplementary Table 2). When corrected for interval size (i.e. using daily growth rate), explants grew faster during the first summer as compared to the winter and second summer intervals (p=.001 and p=.000, respectively; Figure 7). No growth difference was found between the winter and second summer interval for explants (p=.892). For colonies, we observed a similar pattern, with fast growth during the first summer (on average 0.8 %/day) followed by a reduction to a consistent average growth of 0.25%/day after that during the winter and second summer (p=.000 and p=.000, respectively). Again, no daily growth difference was found between the winter and second summer (p=.786). No effects of sponge type, orientation or site were found (Supplementary Table 2). Productivity for the polluted colonies (153%) outscored the explants at both sites (138%, 137%; respectively) and the pristine colonies (103%; Table 4).

In this current study, the system was left unattended intentionally during the winter period for 6 months. Hence, the sponge lantern systems were slightly fouled and attracted several Lessepsians that were recently described in the Eastern Mediterranean Sea. The list of fouling organisms (some considered as invasive) at the pristine site included long spine sea urchins growing on the plates (*Diadema setosum*, Leske 1778) which is an invasive echinoderm first spotted in the Mediterranean in 2006, the frond oyster *Dendostrea frons* (Linnaeus 1758) attached to the plates, the invasive tunicates *Herdmania momus* (Savigny 1866, first spotted in the Mediterranean in 2008) and *Phallusia nigra* (Savigny 1816) growing over the net/plate junctions (Shenkar and Loya 2009), and large shools of juvenile Indian Ocean two spot cardinal fish *Cheilodipterus quinquelineatus* (Cuvier 1828). Apart from the invasive list, we spotted occasional *Eudendrium* sp. hydroids growing over the nets

and nudibranch species *Flabellina affinis* (Gmelin 1791) and *Coryphellina rubrolineata* (O'Donoghue 1929), residing over these hydroid branches. As for the polluted site, there was massive algal cover *Stypopodium schimperi* (Kützing, Verlaque and Boudouresque 1991) over the nets, *H. momus* and *P. nigra* over the net/plate junctions, schools of *C. quinquelineatus* almost in every part of the system, and a respectable amount of *Pteragogus pelycus* (Randall 1981) individuals as a result of macro algae habitat.

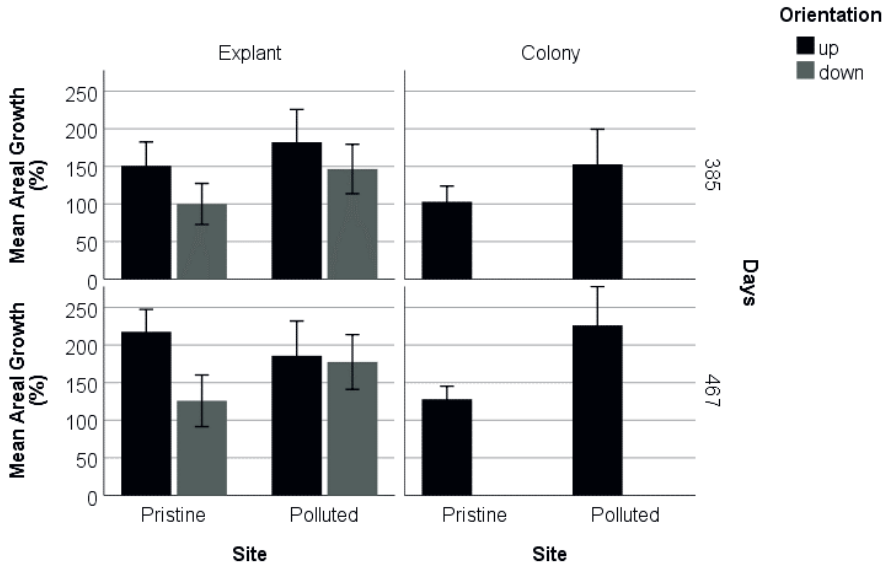

Figure 6. Culture Trial 3, Pristine and Polluted Sites after 385 days and 467 days in culture: Mean areal growth rate of explants and colonies attached by nails. Up: explants facing upwards on PP plates, down: explants facing downwards on PP plates. N=12 plates per treatment combination.

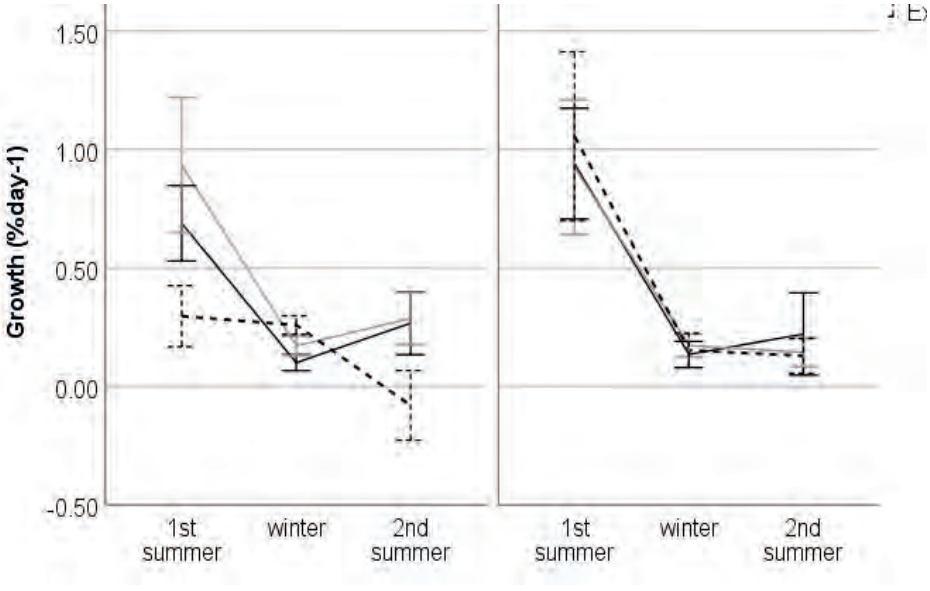

Figure 7. Culture Trial–3: Growth rates of colonies (up only) and explants (up & down) for three intervals; the first summer (83 days), the winter period (302 days) and the second summer (65 days).

Supplementary Table 2. Culture Trial–3, statistical analysis: Three-way factorial ANOVA's testing for effects of time, site, sponge time and orientation on growth rates of explants and/or colonies after 83, 385 and 467 days (N=12 plates per treatment combination).

Sponge Lantern/Explants up & Explants down (Three–way factorial ANOVA)					
	Factor	**F**	**df**	**Error**	**p**
	Time	17.093	1.284	39.812	.000***
	Orientation	1.921	1	31	.176
	Site	1.446	1	31	0.238
	Time * Site	1.554	1.284	39.812	.224
	Site * Orientation	2.919	1	31	.098
	Time * Orientation	.689	1.284	39.812	.446
	Time * Site * Orientation	1.450	1.284	39.812	.242
Sponge Lantern/Explants up & Colonies up (Three–way factorial ANOVA)					
	Time	22.336	1.369	49.277	.000***
	Sponge type	.319	1	36	.576
	Site	.034	1	36	.855
	Time * Site	.414	1.369	49.277	.586
	Site * Sponge type	.616	1	36	.438
	Time * Sponge type	.183	1.369	49.277	.748
	Time * Site * Sponge type	.120	1.369	49.277	.808

Productivity and Feasibility analysis

The sponge lantern culture system was designed to contain 4 plates in each lantern unit, where upper and lower parts of the plates are used. We left sufficient space (100 cm) in between each plate for photographic analysis. However, in a commercial scale farm scenario, these empty spaces could be used for placing more plates. This way, it was calculated that each sponge lantern unit can harbour 12 plates and a single plate can contain either 20 explants or 5 colonies and in total there will be 240 explants and 60 colonies per lantern. The explant and colony mass (in grams) were calculated by multiplying the volume (based on height, width and length) of the explants/colonies with the density of *C. reniformis* (Sipkema *et al.* 2005b). For the feasibility analysis, the pristine and polluted sites were pooled as no significant differences were found between these groups (see Box 1). The calculations in Box Table 2 show that the price per kg sponge dramatically drops from 28.30 Euro to 1.64 Euro with increasing number of lanterns deployed from 1 to1000 lanterns. With a collagen yield of 5.3% wet weight of the sponge (Gokalp *et al.* 2020a), the price per kg collagen drops from 566.04 Euro (1 lantern) to 32.83 Euro when the system is upscaled to 1000 lanterns (culturing 53 tonnes of sponge material, Box Table 2b). Silva *et al.* (2014) indicated that the target standard cost of marine collagens is <83 Euro per kilogram for food applications and 4–40 Euro per kilogram for healthcare products based on the

Box 1 Production calculations according to the lantern design and availability of space

Box Table 1.	**Pristine-explant**	**Polluted-explant**	**Pristine-colony**	**Polluted-colony**
Growth (%)	150.0	182.0	103.0	152.8
Survival (%)	92	75	100	100
Production (growth x survival/100)	138.0	136.7	103.0	152.8
Average sponge mass (g)	27.2	27.2	349.8	349.8
Harvest per sponge (g)	37.5	37.2	359.4	533.2
Sponges per lantern	240	240	60	60
Production per lantern (kg)	9.3	9.0	22.2	33
Lanterns deployed	0.02 (1) lantern for 1kg	2 lanterns for 100 kg	19 lanterns for 1000 kg sponge	94 lanterns for 5000 kg sponge

The initial weight of the explants and colonies found to be in average 27.2 g and 349 g, respectively. By using the survival and growth data from the current study following the 1 year in culture period, the produced amount of sponge per lantern was found to be 9.3 kg for explants and 27.5 kg for colonies (Box Table1). By selecting the optimal growth rates of the colonies and average sponge mass from Table 1, the harvest per sponge (882g) and production per lantern (53 kg) and number of lanterns necessary for culturing 100kg, 1 ton and 5 tones of sponge material (Table 2) were calculated.

Box Table 2. a) Harvest per sponge and production per lantern b) Calculation of the mariculture expenses: price per kg wet sponge mass and price per kg sponge raw material.

a				
Production (growth x survival/100)	153			
average sponge mass (g)	349			
harvest per sponge (g)	883			
sponges per lantern	60			
production per lantern (kg)	53			
b	**1 lantern**	**100 lanterns**	**1000 lanterns**	**2000 lanterns**
Materials	100	1000	8000	60000
Deployment (boat+ manpower)	500	500	900	5400
Filing/harvesting (boat+ manpower)	600	600	1800	10800
Maintenance (boat+ manpower)	300	300	1800	10800
Total cost per lantern	1500	240	125	87
Price per kg sponge (wet mass)	28.30	4.53	2.36	1.64
Price per kg sponge (raw material)	566.04	90.57	47.17	32.83

By using the results of Box Table 1, we can calculate the mariculture costs for the production of desired amount of sponge material, where total cost per lantern is calculated per growing demand of sponge material (1, 10, 100, 1000 lanterns). For instance, 2 lanterns are necessary to harvest 100 kg and 94 lanterns to harvest 5 ton of sponge material where this number grows in relation to the sponge material demanded (Box Table 2a). The price per kg sponge (wet mass) is then calculated by dividing the total cost per lantern (Euro) with the production per lantern (kg) which was calculated earlier in Box Table 1 (53 kg).

average market prices of collagen-based products. With further upscaling, culture of *C. reniformis* specimens in sponge lanterns would be a viable method for the production of collagen, although expenses for investment, insurance, overhead et cetera are not yet included. explants/colonies with the density of *C. reniformis* (Sipkema *et al.* 2005b). For the feasibility analysis, the pristine and polluted sites were pooled as no differences were found in between these groups (see Box 1). Analysing the results given in the box calculation, we can see that, the price per kg sponge

dramatically drops from 28.30 Euro to 1.64 Euro as the number of lanterns deployed increases (1 lantern to1000 lantern). In other terms, the cost per kilogram of sponge material falls dramatically when the system is upscaled (Box Table 2b). Lastly, the collagen yield was calculated by us earlier as 5.3% wet weight of the sponge and using this amount, we could find the price per kg collagen (Gökalp *et al.* 2020a). Similar to the cost of sponge material, cost per kg collagen drops from 566.04 Euro (1 lantern) to 32.83 Euro when 1000 lanterns are deployed to culture 53 tones of sponge material (Box Table 2b). Silva *et al.* (2014) reported earlier that the target standard cost of marine collagens is believed to be in the <83 Euro per kilogram range for food applications and 4–40 Euro range per gram for healthcare products based on the average selling prices of collagen-based products. This suggests that with further upscaling, culture of *C. reniformis* specimens in sponge lanterns would be a feasible method for the production of collagen, although expenses such as investment, insurance, overhead et cetera are not included.

DISCUSSION

The overall aim of this study was to develop a viable method for the culture of *C. reniformis* sponges for collagen production in integrated mariculture applications. Following a series of subsequent culture trials, we were able to develop a robust system which enhances productivity for the production of raw sponge material while removing organic pollution from the water column.

Culture Trial 1

We observed differences in behavior of explants cultured on plates. Glued explants oriented vertically were lost more often but grew better compared to horizontally oriented counterparts. The high morphological plasticity of *C. reniformis* and escaping behaviour observed in this study (see section 3.1), were reported before in earlier experiments and natural specimens (Wilkinson and Vacelet, 1979; Bavestrello et el. 1998; Garrabou and Zabala 2001; Van Treeck *et al.* 2003; Gökalp *et al.* 2019; 2020). Interestingly, this behaviour was not observed for explants that were cultured (nailed or glued) horizontally. The high detachment rate of vertically glued sponges (72% of the explants were lost), resulting in the lowest survival rate, confirms our earlier study, where only 61% of the explants detached (Gökalp *et al.* 2019). Although there was a slight morphological disturbance of the sponge avoiding the nails, nailing explants proved more successful in retaining them on the plate. As expected, explants that dropped from the plates started to grow over the end parts of the PP boxes (cf. Gökalp *et al.* 2019). The glue/vertical/PVC and PP combinations provided the highest growth rates, although retaining rates of these groups were lowest. The elevated growth rates for these combinations could be explained by the lack of sample size as a result of low survival rates. The low growth of the horizontally mounted explants on iron was unexpected, as a boost in growth was predicted due to ferric iron promotion observed earlier by Osinga and Kotterman (2007).

Culture Trial 2

In Culture Trial 2, horizontal explants were retained better compared to their vertical counterparts at both sites. Similar to Culture Trial 1, explants dropped from the plates and continued to grow inside the PP boxes, although in less numbers. The PVC/Glue combination in Culture Trial 2 was far more successful (1.7 times more productive) than Culture Trial 1, despite

the fact that both experiments were exactly similar. This shows the variability of this type of study, probably related to high individual variability among *C. reniformis* genotypes/explants: a few fast growers can totally change the result. In our earlier study (Gökalp *et al.* 2019) we used similar plate material (PVC), attachment method and design of the experiments (Pristine versus Polluted sites), but now we found higher sponge recovery at the pristine site (78% vs 39%, respectively) but lower at the polluted site (64% vs 86%). In our previous study, however, we included dropped explants which were found to continue growing on PP plates into the survival calculations. But in the current study they were left out. The growth rates achieved in the current study for both sites (132–150%, pristine and polluted, respectively) were much higher during 4 months of culture compared to our earlier work in the first 6 months of culture (70–114%, pristine and polluted, respectively) in the same season (summer 2013 vs summer 2018). The protective nature of the polypropylene boxes may have caused the recent improvement in growth rates for this study in contrast to the earlier one where the explants were prone to occasional currents. In contrast to our earlier study, we observed no significant growth differences between sites, which could be explained by the only 1.4 times higher TOC concentration at the polluted site as compared to the pristine site. The recorded visibility was 1.8 times higher and the difference in pollution was visible.

Culture Trial 3

The Culture Trials 1 and 2 provided hints to design a best practise method for Culture Trial 3: 1) Nailing sponges instead of gluing helps to prevent losses, in particular on vertically oriented cultures; 2) PP plates overall showed the highest productivity; 3) *Chondrosia reniformis* can be grown in the vicinity of sewage outlets. These findings prompted us to design a scalable trial that combined best practises, low costs, robustness and ease of operation under different environmental conditions. In Culture Trial 3, the performance of colonies has shown promising results at both locations, and no specimens were lost during the first year. However, right after the second summer season, two specimens were lost at the polluted site due to unknown reasons, reducing the recovery rate to 83%, and no specimens were lost from the pristine site. For both sites, all explants were retained during the experiment so we can conclude that the sponge lantern system was successful at keeping colonies and explants healthy and in place. Occasional currents were a problem in our previous study at the pristine location where the culture system was prone to such conditions (Gökalp *et al.* 2019). It is evident that, by providing initial care during the first week of culture and by including a more secure attachment method, together with the advantages (open flow environment) of sponge lanterns, the system created a noteworthy improvement in survival rates for both sponge colonies and explants (Gökalp *et al.* 2019).

In Culture Trial 2, growth rates did not differ between the two sites, suggesting that the degree of organic enrichment at the polluted site was insufficient to enhance growth of *C. reniformis*. In our previous study the TOC levels at the polluted site around fish farms were 2.4 times higher than the TOC level at the pristine level site (Gökalp *et al.* 2019). It cannot be excluded that not only the greater TOC level but also the type of organic pollution (originating from the fish farm instead of municipal waste) were more suitable to stimulate sponge growth.

Interestingly, one colony performed consistently higher and massively increased its size (1105%) after 15 months in culture. This was the highest growth rate observed in this study and greater than the maximum values reported in our previous studies: up to 700% a year by Osinga *et al.* (2010) and 401% after 13 months in culture by Gökalp *et al.* (2019). We do not know what triggered this massive size increase in size for this colony, but it is possible that genetics played a role. This implies that brood stock selection might be a promising tool to increase intrinsic growth rates from such specimens. In future studies, these high performers should be subcloned, in order to assess whether the clones continue to grow with these high rates. Interestingly, a similar extreme growth rate was observed for the explant grown on the same plate (470%), so it can also not be excluded that some environmental variable stimulated this growth. If taken out from the analysis, the mean growth rates of both sites even nearly equalized (157% for pristine polluted, 151% for polluted). No effect on explant growth rates was observed from sponge orientation.

The colony and explant growth rates displayed a clear difference over the three time intervals monitored (first summer, winter and second summer). All groups, except for the down–facing explants at the pristine site, had significantly reduced growth rate after the first summer period (figure 7). As colonies perform highly similarly to the up–facing explants, at both sites, there is no indication that cutting sponges will influence their initial growth. The reduced growth during winter (for all but the down–facing explants at the pristine site) could be due to decreasing temperatures, reducing metabolism and therefore growth (Verdenal and Vacelet 1990; Duckworth and Battershill 2003b; Page *et al.* 2005; Duckworth 2009). However, during the second summer, we did not observe a growth increase for any of the experimental groups. There is a strong indication, that what we observe here, is a starting effect initiated by the disturbance of being removed from the original location and transplanted to a new environment. In previous experiments, we observed an initial growth boost, also for explants that were cultured from fall to winter (unpublished results). Such a temporary growth boost could be taken advantage of by cutting and outplanting *C. reniformis* every 3–4 months. This way, several times a year, boosted growth and subsequent elevated harvest could be achieved. Our results show that the reduction in (stabilisation of) growth rate later on is independent of sponge size. The production table (Table 4) shows us that colonies at the polluted site had the highest overall production at the end of 467 days, and for the explants there was no difference.

Evaluation of the Sponge Lantern Method

It was reported earlier that certain sponge species grow fastest and reach their largest size at sites with high water movement, as flow might promote sponge growth through increased food availability or by increasing internal flow through the sponge aquiferous system (Vogel 1974; Wilkinson and Vacelet 1979; Duckworth *et al.* 200; Mendola *et al.* 2008). We observed occasional moderate to heavy currents, reaching velocities up to 25 cm/s recorded during the summer periods. At depths where the lanterns were located we did not measure currents, but at times all sponge lanterns angled at 20 to 30° from their original upheld positions (personal observations). The water flow threshold for feeding of *C. reniformis* was not documented in the current study, but considering our experiences, we anticipate that the lanterns could be placed in high flow areas that occur i.e. around offshore fish farms without any problem. The lanterns being covered by nets proves to be advantageous during these heavy flow periods, as it prevents loss of sponges.

However, for the system to work effectively, it needs periodic cleaning every 3-6 months so that the net openings stay open and the epibiont growth also cannot cause too much drag on the system (Giangrande *et al.* 2020). Both Kelly *et al.* (2004) and Duckworth *et al.* (2004) documented several biofouling organisms such as tunicates, anemones, algae, tubeworms and bryozoans in and outside the lanterns. Duckworth and Battershill (2003a) reported more severe biofouling organisms compared to mesh begs applied in their study, adding that the larger size of lanterns provides more surface area for the attachment of fouling organisms. They eventually concluded lanterns to be cumbersome and unsuitable for farming sponges commercially. Kelly *et al.* (2004) reported heavy fouling at certain sites and recommended maintenance of lanterns on a semi-annual basis. Previously shown for coral mariculture, the occurrence of fouling also depends on the presence of a healthy fish community that might eat away certain portion of fouling (Knoester *et al.* 2019). We anticipate that the fouling experienced in Duckworth and Battershill (2003a) in New Zealand might be more prominent than in the Mediterranean Sea. As we had no problems whatsoever while deploying, cleaning and making photographic analysis on sponge lanterns and considering the consistency of the system observed in our study, we report that the sponge lanterns could be suitable for commercial sponge culture. We also included in our sponge lantern system design that we had to retain *C. reniformis*, which is a specimens notorious for escaping from their designated spots on the plates (Gökalp *et al.* 2019). Also by placing them horizontally (facing up or down) on the plate, and we avoided the drop–off problem that we encountered before associated with occasional currents. Furthermore, by using nails we increased survival rates compared to our earlier study. The thus developed sponge lantern system has proven its stability and robustness after being submerged underwater for two summers.

In conclusion, the here developed and validated sponge lantern system provides a secure system that delivers consistent survival for *C. reniformis* along with high growth rates, boosted during the first months after placement, that is suitable to deploy in forthcoming innovative approaches to IMTA systems. The estimated minimum requirements for economically viable sponge culture of 90% survival and growth of 100% per annum (as proposed for bath sponges by Verdenal and Vacelet (1990) were easily met in this study. Future perspectives are brood stock selection, searching for those individuals that thrive in the specific culturing method as we always observed fast growers and no-growers. In addition, synergy with feeding fish mariculture can be further developed, to optimize the feeding removal of organic particles (including potential pathogens) from the water column by the sponges.

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CHAPTER 7

Discussion: Necessity for a revolution in aquaculture

There lies little novelty in the claim that coastal ecosystems are experiencing large-scale ecological degradation due to increased anthropogenic impact and overharvesting of marine resources. Fish stocks are depleting globally and uncontrolled expansion of aquaculture farms to provide food security for the growing human population further compromises the health and resilience of marine environments (Naylor *et al.* 2000). Thus, there is urgent necessity to reduce human impact on marine habitats by applying environmental-friendly, product-diversified and socially beneficial concepts of integrated farming and coastal management (Buschmann *et al.* 2001; Troell *et al.* 2003; Chopin *et al.* 2012). Since sponges feed on suspended and dissolved organic matter, it has often been suggested to apply sponge culture to remediate marine organic pollution, such as the effluent from sea-based fish cages and unpurified urban wastewater discharge (Milanese *et al.* 2003; Longo *et al.* 2010; Ledda *et al.* 2014). Large-scale sponge culture may help reduce eutrophication of coastal waters and its concomitant disruptive effect on local ecology and biodiversity (Pronzato *et al.* 1999; Gifford *et al.* 2007). Sponge culture can also locally improve water quality around fish farms, which benefits the cultured fish. This thesis assessed the performance of a Mediterranean sponge species under different eutrophication and depth conditions. By using a multifactorial approach, we investigated the aquaculture potential, in situ filtration activity and pollution remediation efficiency of the selected species at pristine sites and organically polluted sites. Species-specific culture methods were optimised, ultimately achieving a novel integrated fish-sponge farm model, which is self-cleaning and could maximise production of high quality raw sponge material.

Chapter 2 evaluated the use of marine sponges in integrated culture systems. Pronzato (1999) suggested integrating sponges in culture systems that would lead to a double benefit. In such a setting, sponges would grow faster under higher organic loading, and filtration by sponges would improve water quality. Optimized culture and improved growth achieved at the polluted site (**Chapter 5**) suggested a possibility for successful IMTA with *C. reniformis* and other commercially interesting sponges. Hence, **Chapter 2** presented a new idea– the use of sponges to convert dissolved organic matter (DOM) into particulate organic matter (POM) that can be consumed by deposit feeders as hypothesised by de Goeij *et al.* (2013) earlier. We developed a theoretical design of an integrated culture with seaweeds, sponges and sea cucumbers to demonstrate the idea. The total recovery of DOM (i.e. primary production excreted by the seaweeds) into sponge and sea cucumber biomass within this theoretical IMTA was found to be 49%, of which 37% was recovered in sponge biomass and a subsequent 12% in sea cucumber biomass after conversion of DOM to POM by sponges.

During the initial phase of this thesis, we cultured two Mediterranean sponge species (*Dysidea avara* and *Chondrosia reniformis*) with biomedical potential under pristine and polluted environments (**Chapter 3**). We failed at culturing *C. reniformis* at the polluted site due to smothering of particles falling from the fish farm (~25 m distance). However, culture of *D. avara* was successful at both locations where we developed a scale-up method for the possible IMTA application of this sponge species. Nevertheless, clinical trials on the compounds obtained from *D. avara* were abandoned shortly thereafter, and the discovery of a simple, feasible chemical synthesis method for Avarol further minimized the interest in aquaculture of this sponge species (Sakurai *et al.* 2008).

In **Chapter 4**, we continued with the collagen-rich model sponge *C. reniformis* and evaluated the effect of depth on sponge morphology, growth, physiology and investigated the *in situ* filtration activity of *C. reniformis* to apply sponges for water purification and collagen production. Sponges transplanted from 20 m to 5 m depth adjusted their morphology to match the 5 m sponges, producing fewer but larger oscula, whereas this morphological plasticity was not observed for the opposite. This trend was observed previously in Gökalp *et al.* (2020c) in a more profound way, where we documented less but 'giant' oscula of 1–2 cm in diameter at 3 m instead of smaller oscula of 0.01–0.1 cm at 20 m (Figure 1).

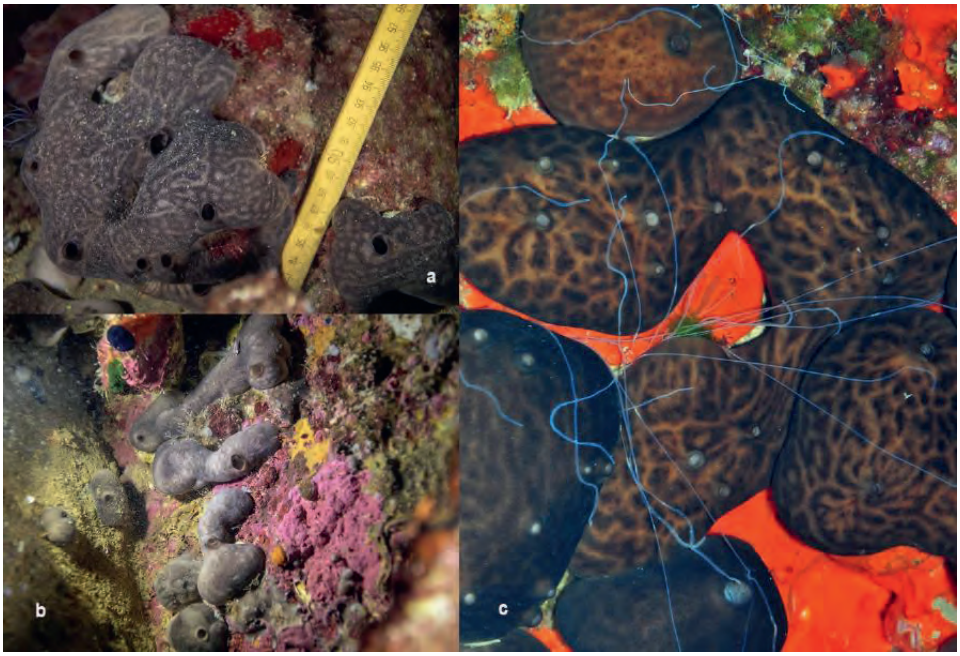

Figure 1. a) Specimens of *Chondrosia reniformis* a) with giant oscula encountered in Kas-Kekova Marine Protected Area at 3 m depth. b) Similar sponge colonies with such oscula were observed in Bodrum at same depth (Gökalp *et al.* 2020. c) Example of a *C. reniformis* colony with much smaller oscula at 20 m depth.

This larger size can facilitate ejaculation of e.g. sand particles that would be more present in subtidal high energy areas. The volumetric pumping rates remained the same, less but larger oscula that each process more water per cm^2 of osculum surface. This observation stresses the high morphological plasticity (Bavestrello *et al.* 1998) and adaptation capability of the sponges to changing environmental and oceanographic conditions (de Goeij *et al.* 2017; Bell *et al.* 2018). At two different geographical locations we found that deep water specimens of *C. reniformis* had significantly smaller oscula than shallow water sponges, and at least partially compensated for this lower pumping potential by a higher osculum density. Indeed, the particle clearance rate, respiration and growth rates were comparable among all the experimental groups that were reciprocally transplanted in between 5 and 20 meters. We observed that depth-induced morphological changes do not affect the overall performance of the sponges. Therefore, the potential for the growth and bioremediation of *C. reniformis* in mariculture is not likely to change with varying culture depth, which provides much flexibility to the aquaculturist. While creating a big scale application, there will be an initial need for great amount of sponge material and being

able to collect from various depths have several advantages: 1) less impact ion sponge colonies by avoiding collection from a specific depth 2) being able to provide sufficient amount of sponge material around the cultivation zone, without a need to bring sponges from other locations 3) reduction in time and resources spent on collection of mother sponges.

In Chapter 5, we investigated the possibility of culturing *C. reniformis* around fish aquaculture cages at 10 m depth. We hypothesized that the excessive amount of suspended organic matter in the water column (i.e. fish feed, faeces, marine bacteria) would stimulate sponge growth. Indeed, we succeeded farming *C. reniformis* for 13 months, and observed a more than twofold difference in growth rates between the polluted and pristine sites. This difference in growth may relate to the 2.4 times higher availability of elevated dissolved organic carbon (DOC) and particulate organic carbon (POC) measured as total organic carbon (TOC). As especially DOC is known to be a major food source for sponges (Yahel *et al.* 2003; de Goeij & van Duyl 2007; de Goeij *et al.* 2008a; Alexander *et al.* 2014; Rix *et al.* 2016, 2018) this should be measured separately in future studies in combination with bacterial clearances. The pollution-bacterial clearance-DOM-growth relationship should be further elucidated to be able to optimize the advantages of sponge culture in IMTA systems.

Based on the findings of **Chapters 3 and 4**, in **Chapter 6** *C. reniformis* culture trials were further elaborated culminating in a novel sponge IMTA method termed as 'Sponge lantern', which maximizes production of high quality raw sponge material. Following 15 months of culture, the sponge lantern provided a secure system that was able to protect and retain the specimens in culture while enabling prospective growth rates up to 1105% in 15 months. Overall, the system satisfied all necessary aims and preset criteria for a profitable sponge culture and proved its stability and robustness over two summers and a winter period (Chapter 6).

BEST PRACTISES

Initiated with the prime trials of Cavolini (1785) on commercial bath sponges, mariculture of sponges has a long history encompassing more than two centuries (Pronzato and Manconi 2008; Voultsiadou *et al.* 2011). Most sponges are easy to culture, and if cut cleanly and without exposure, they quickly heal cut surfaces and grow rapidly (as fast as 2437% within a year (*Mycale hentcheli*, Page *et al.* 2011). The impact of environmental factors and methods on culture success vary profoundly among species, season and culture location (Duckworth *et al.* 2003a). Successful methods have been developed for both temperate and tropical sponge species (reviewed by Duckworth 2009; Schippers *et al.* 2012). Likewise, within the span of this study, we were able to achieve consistent growth rates with the two sponge species *D. avara* and *C. reniformis*, in pristine as well as polluted conditions (**Chapter 3,5**). Scale up methods called Shish kebab (**Chapter 3**) and Sponge lantern (**Chapter 6**) were designed for both species, developed and successfully implemented for prolonged periods submerged underwater. Moreover, the sponge lanterns, that is designed for a sponge collagen pipeline with *C. reniformis,* can be adjusted to suit other commercially interesting sponges. A lantern provides multiple pigeonholes that could provide a proper culture substrate for various type of sponge explants (encrusting, globular and bath sponges). It is designed to maximize sponge retaining and growth, allowing sufficient water currents and thus nutrition and oxygen to reach the explants. The lantern minimizes culture efforts needed and loss of sponges (and culture systems) from waves, storms, predator intrusion,

biofouling and operations related to mariculture activities. The growth performance of the sponge and the durability of the system were tested in an open flow environment during a farm application. Overall, the sponge lantern system fulfilled all necessary aims and preset criteria defined by us earlier (**Chapter 6**) and proved its stability and robustness being submerged underwater for a prolonged time. The method also met the minimum requirements of at least 90% survival and 100% growth per annum defined earlier by Verdenal and Vacelet (1990) for a successful commercial sponge business. The fouling experienced by Duckworth and Battershill (2003a) could be avoided by periodical cleaning (every 3-6 months). This way, the net openings remain devoid of epibiont growth, while excess drag on the system and subsequent storm damage are prevented (Kelly *et al.* 2004). Interesting is the variable performance of *C. reniformis* in our experiments. The growth rates had very high standard deviations, and individual characteristics of the original colonies chosen for the experiments very much determined the culture success. As it seems that genotype matters, broodstock selection and genomics characterization is a promising way forward to enhance productivity.

FEASIBILITY OF IMTA WITH SPONGE LANTERNS

In order to evaluate the potential of the sponge culture method developed in **Chapter 6**, I would like to extend the conducted feasibility study for *C. reniformis* to other Mediterranean sponges with potential commercial interest. **In Chapter 6**, we showed that if 100 kilograms of sponge material would be harvested from the sponge lanterns, the mariculture production cost per kg of *C. reniformis* is 30 Euro. This price drops dramatically to 3.6 Euros/kg if scaled up to 1 tonne of sponge biomass and 2.4 Euros/kg if 5 tonnes of sponge is harvested. Considering the potential collagen yield from *C. reniformis* of 5.3%, this would yield 72 Euros/kg of collagen if 1 tonne of sponge is harvested, dropping to 47 Euros/kg at 5 tonnes harvested (Table 1). Other sponges could be cultured in sponge lanterns with basic adjustments in the system both locally and globally. For instance, *D. avara* could hypothetically be cultured by applying the novel method developed in **Chapter 3** by using plastic pins (the ‘‘Shish Kebab method’’) placed in between the plates. In **Chapter 3** we showed that usage of plastic pins in scale-up improved explant growth rates as it provided an attachment surface for growth that prevents the need for continuous replumbing of the sponges which requires metabolic energy (Mendola *et al.* 2008). The production cost for *D. avara* would be 66 Euros/kg when 100 kg sponge is harvested, and drops to 6 Euros/kg if 1 tonne is targeted, and 10 Euros/kg at 4 tonnes. Despite *D. avara*’s high annual growth rates (1100%; Osinga *et al.* 2010), the reason for the higher price per kg in comparison to *C. reniformis* is the need for additional farming pins and more sponge lanterns. *D. avara* is an encrusting sponge, with initial explant size 5 times smaller (in weight) compared to *C. reniformis*.. Considering the low yield of Avarol (2 gr/kg sponge; Sipkema *et al.* 2005) this relates to 32750 Euros/kg Avarol of mariculture cost if 100 kg of sponge is harvested, 2800 Euros/kg at 1 tonne and 200 Euros/kg at 5 tonnes (Table 1). The current market price of Avarol is 140,000 Euro/kg, which means that sponge lanterns could produce *D. avara* and Avarol in a sustainable and profitable fashion. Unfortunately, the production of Avarol (as a psoriasis or anti-cancer agent; Osinga *et al.* 2010) necessary for clinical trials failed partially due to sponge supply problems. Subsequently, a chemical synthesis method was developed as described by Sakurai *et al.* (2008). The application of this sponge in the natural product origin pharma market therefore failed, as

was the case for many other sponges with secondary metabolites (Duckworth 2009; Schippers *et al.* 2012).

Table 1. Cost of mariculture per kg for cultivating 5 tonnes of raw sponge in order to produce collagen, Avarol and bath sponge from the Mediterranean sponges *C.reniformis*, *D. avara* and *S. officinalis,* respectively.

Sponge Species	Produced Material	Sponge Product Yield	Cost of Sponge per kg	Cost of Sponge Material per kg	Reference
Chondrosia reniformis	Collagen * 4–40 Euro per kg	Collagen (5.3% wet weight)	~ 100 lantern 2.4 Euro per kg	~ 100 lantern 47 Euro per kg	Silva *et al.* 2014; Chapter 3 & 7
Dysidea avara	Avarol * 140.000 Euros per kg	Avarol (2 gr/kg)	~ 174 lantern 4 Euro per kg	~ 174 lantern 2009 Euro per kg	Sipkema *et al.* 2005
Spongia officinalis	Bath sponge; 20–25 cm in size * 30 Euros per piece	2 years to reach market size of 200 gr	~ 100 lanterns 4.3 Euro per kg	~ 100 lanterns 8.4 Euro per kg	Verdenal and Vacelet 1990; Pronzato and Manconi 2009; Corriero *et al.* 2004; Celik *et al.* 2011

My cost-benefit calculations (table 1) could be applied to still profitable scenario by selecting commercial bath sponge species from the Mediterranean that still have ongoing commercial value. Bath sponges could be farmed in sponge lanterns with species-specific adaptations such as ropes or plastic pins placed over the plates (Corriero *et al.* 2004; Kelly *et al.* 2004; Duckworth *et al.* 2007; Duckworth and Wolff 2007). The commercial sponge business was interrupted in mid-80's due to overharvesting and an unknown sponge disease affecting the stocks, however, high quality bath sponge *Spongia officinalis,* still is sold at the market for around 30 Euros per piece (20–25 cm in diameter; Pronzato and Manconi 2008). Like other bath sponges, it takes approximately two years of farming to grow *S. officinalis* from explant size (3-5 cm) to market size (20 centimeters in diameter, Verdenal and Vacelet 1990; Pronzato *et al.* 1999; Corriero *et al.* 2004; Celik *et al.* 2011). In contrast to *D. avara*'s inevitable lower weight, the initial weight of *S. officinalis* is similar to *C. reniformis* and fewer sponge lanterns are necessary to produce sufficient numbers of sponge explant material. Of course, the expenses related to preparation of the bath sponges for the market will slightly increase the cost per kg (Pronzato and Manconi 2008). For bath sponge, the price is 63 Euros/kg if 100 kg of sponge harvested, 7 Euros if 1 tonne is targeted and 4 Euros if 5 tonnes are targeted (Table 1). Considering the sponge processing cost following the harvest (chemicals, workmanship), the cost per kg of bath sponge will be 8 Euros for 5 tonnes of sponge. As a marketable-size bath sponge (20-25 cm in diameter) will weigh about 200 g, 1 kg of sponge is worth around 150 euros. This means that the calculated price per kg maricultured *S. officinalis* is indeed profitable when using the sponge lantern method. Of course, in addition to the mariculture costs also company investments, site rental fees, overhead and insurance expenses and—in the case of Avarol from *D. avara*—extraction costs should be included in the business case evaluation. Nevertheless, there is still sufficient margin of profit for our sponge lantern method, especially taking into account the added value of cleaning the water column from organic pollution. Further, considering the great natural growth rates of tropical sponges (Duckworth 2009; Schippers *et al.* 2012), cultivation of tropical sponges with sponge lanterns could also be attractive as long as biofouling and storm damage are taken care of by sustained maintenance (Kelly *et al.* 2004).

SUSTAINING WATER QUALITY

The aim of this thesis, an integrated mariculture with commercially interesting sponges and demonstration of faster growth in environments polluted with organic compounds while purifying water, was partially fulfilled. When sponges are cultured in the vicinity of organic waste streams from aquafarms and urban discharges, growth and filtration activity of sponges are enhanced (Osinga *et al.* 2010; Ledda *et al.* 2014; **Chapter 5**). The next step to take is to showcase large scale *in situ* effects of sponge filtration on seawater quality in an IMTA setting. This is challenging, due to high costs of IMTA projects, logistical challenges involved and reluctance of aquaculture establishments who don't seem to see their gain in an IMTA approach. Until such an experiment is performed, we can only make theoretical predictions of the collective grazing effect of sponges. As an example, the data on *C. reniformis* filtration rate obtained in **Chapters 3 & 6** can be used to estimate the amount of sponge biomass needed to take up the organic C waste produced by one standard fish pen for the culture of sea bass (*Dicentrarchus labrax*). This C waste can be calculated as:

C waste = (g food per fish per day) x (number of fish in pen) x (fraction of C in feed) x (fraction of feed wasted)

First, the food uptake by the fish must be estimated. Since the average starting size of the fish in outdoor pens is 50 g and harvest size is 350 g, an average fish size in the pen of 200g can be assumed. Hossu *et al.* (2005) reported that at temperatures between 14 and 26 ^{o}C, sea bass can be fed at 1.1-3.9% of their body weight per day, depending on size of the fish and temperature. To simplify the calculation, we used the median value of 2.0%, which is 4.0 g feed per fish per day.

Second, the number of fish in a pen must be calculated. A common fish pen for sea bass with a radius of 30 m and a depth of 10 m has a volume of 7069 m^3. Organic farming of sea bass and sea bream in open water systems commonly used in the Mediterranean, usually applies a stocking density of 10–20 kg m^{-3} (Mente *et al.* 2012; Di Marco *et al.* 2017). If we assume a stocking density of 15 kg m^{-3}, we could have a maximal number of 353450 fish of harvestable size (350 gr) inside our pen.

Wang *et al.* (2013) gives the wasted fraction of fish feed as 62% and Chatvijitkul *et al.* (2018) gives the % C in fish feed as ~40%. Using these numbers, the organic C waste production by a fish pen would be:

C waste = 4 g food $fish^{-1}$ d^{-1} x (353450 fish) x 40% x 62%

= 351 kg C per day

Earlier, in **Chapter 4** we calculated the filtration rate of *C. reniformis* as 639 ml g DM^{-1} h^{-1}. For this order of magnitude calculation, we assume this filtration is equal to the pumping rate, because bacteria are filtered with almost 100% efficiency (Reiswig 1971a; Pile *et al.* 1996; Ribes *et al.* 1999; Wehrl *et al.* 2007). In **Chapter 5**, we measured the TOC concentration in Gulluk Bay as 3 mg C L^{-1} and the retention efficiency of TOC by sponges was calculated by Mueller *et al.* (2014) as 15%. This means that in one passage water column through the sponge, 15% of TOC

is taken out. Multiplying the filtration rate of *C. reniformis* with TOC uptake by sponges gives us 0.29 mg C g DM^{-1} h^{-1}, which is 7 mg C g DM^{-1} d^{-1}. By using this value, we can calculate the number of sponges needed to take up 351 kg C fish waste per day. In order to do that, we first divide the fish waste by the sponge uptake (351 kg C / 7 mg C g DM^{-1} d^{-1}) and convert dry mass to wet mass by using a conversion factor of 0.18 (g DM g WM^{-1}; Gokalp *et al.* 2019), which gives approximately 280,000 kg WM sponge.

However, to understand the relevance of this calculation we should take the hydrodynamics of the bay that the fish farm is located into consideration, in particular the residence time of the water column inside this bay. For instance: - if the fish culture area is a full enclosure (no exchange with the rest of the ocean), the sponge farm will take up an equivalent of all nutrients produced by the fish and thus prevent effects of the fish farm on the water quality -if the fish culture area is in a plug flow environment (or in the open ocean), the residence time of the water in the sponge farm is very short, and the effect of both the sponges and the fish farm on the water quality will be minimal. In practise, the effect will be somewhere in between, depending on the residence time of the water in the bay (equivalent to the % of exchange with the ocean per unit of time). The *in situ* bacterial clearance and respiration rates of *C. reniformis* that was presented in **Chapter 3** were determined in a pristine environment, not at a polluted site with fish farm effluents or urban sewage. The retention efficiency in organically rich waters may be lower compared to the pristine waters, and the pumping rates reduced when there are sufficient levels of organic matter in the water column (Gökalp *et al.* 2020c). On the other hand, the enhanced sponge growth in the first summer period after outplanting was ~4x greater than after reaching a stable growth rate (Figure 7, **Chapter 6**). This could mean that the sponges 'eat' more than now estimated. In addition to the % removal, it also is important what specific part of the organic C is removed, possibly the DOC what other filter feeders do not easily remove, and pathogens from the fish farm. It is very important to test the grazing impact of cultured explants in a larger scale *in situ* clearance experiment, quantifying DOC, POC and TOC and pathogens, measure local oxygen levels and compare sites with and without large numbers of sponge lanterns, determine sponge growth, fish health and accumulation of organic matter at the seafloor under and around the fish pen. This will provide an important basis for further designing IMTA scenario's.

MODEST HOPE FOR ACHIEVING FAIR MARICULTURE AND COASTAL USE

The availability of clean water and seafood security is a key issue for society and coastal ecosystems (Chopin *et al.* 2001; Gifford *et al.* 2007). Integrated multi-trophic aquaculture applications provides the opportunity to bio-mitigate and diversify fed mono-aquaculture practices by combining them with extractive aquaculture in order to realize direct and indirect environmental, economic and societal benefits (Buschmann *et al.* 2001; Troel *et al.* 2003; Chopin *et al.* 2013, Gökalp *et al.* 2019; Giangrande *et al.* 2020). The knowledge obtained in this study contributes to this further developing this opportunity and adds the option for profitable sponge production (**Chapter 3**, **Chapter 5**) and the proof of principle to apply a natural filter principle (sponges; **Chapter 4**) into wastewater bio-filtration solutions (**Chapter 2**). Sponge-mediated biofiltration may be applied to aquaculture, but also to close nutrient cycles for other sources of organic pollution (nutrients, micro-organisms and viruses) that could otherwise cause eutrophication and disease risk. Another potential role of sponges could be as intermediates between primary producers of DOM and consumers of detrital organic matter in an IMTA system

(carbon transfer from seaweeds via sponges to sea cucumbers), which capitalizes on the sponge loop (**Chapter 2**). The aquaculture sector is increasingly important as an alternative to fisheries, but currently mostly produces uncontrolled waste streams of increasing concern (Karakassis *et al.* 2000, 2002; Sara *et al.* 2004; Neofitou and Klaoudatos 2008; Yucel-Gier *et al.* 2013). The use of sponges may provide a nature-based solution, in particular for sea-based aquaculture activities (floating cages) in coastal areas, which already experience water quality problems globally. Sponges can combine a high purification efficiency (**Chapter 3**) with a high added value extra product (sponge biomass and dedicated compounds; **Chapter 2,4,6**). By coupling sponge culture to the fish farms, production of fish can become more sustainable with healthier fish when cultured in better water quality (**Chapter 2,4**) and less oxygen depletion by organic pollution accumulating at the seafloor. Especially when sustainably cultured products in the IMTA system could be certified, their market value would increase. For local tourism cleaner water also will increase profits and marketing tourist appreciation.

Additional users of the sponge culture approach developed in this study could be found among zoos and public aquaria and aquaculture hatcheries. The fish hatcheries located at the coastal areas generally use running sea water circulation systems that provide fresh seawater to the fish pools where the effluents (feed and feces) of the fish are released into the sea. The sponge lanterns deployed at the outlet of such facilities would improve the water clarity and quality by the sponges collectively filtering the excess amount of organic matter. The purification efficiency of sponges should be optimized by balancing the volume of water to be purified with the sponge biomass. In addition to these applications, sponge filters may be used to treat industrial wastewater, for example waste produced during marine food processing. When the waste water does not contain toxic compounds such as from and urban waste water streams, a combination with e.g. shell fish could be made that will provide edible biomass.

The sponge-fish farm systems that were experimented and discussed in this study are a sneak peak of what is forthcoming. As discussed earlier, it is clear that there is an urgency for solving the chronic problems and facilitating the improvement/development of new generation mariculture systems. As shown in the case study mentioned in **Chapter 2**, IMTA with seaweeds-sponges-sea cucumbers (Gökalp *et al.* 2020b) or polychaetas-sponges-macroalgae (Giangrande *et al.* 2020) is possible. Likewise, elaborately- designed innovative approaches to IMTA systems could answer today's problems and satisfy the necessities related to availability of clean water and seafood security that would be intensified by the increasing pressure in the coastal areas due to climate change and human population increase.

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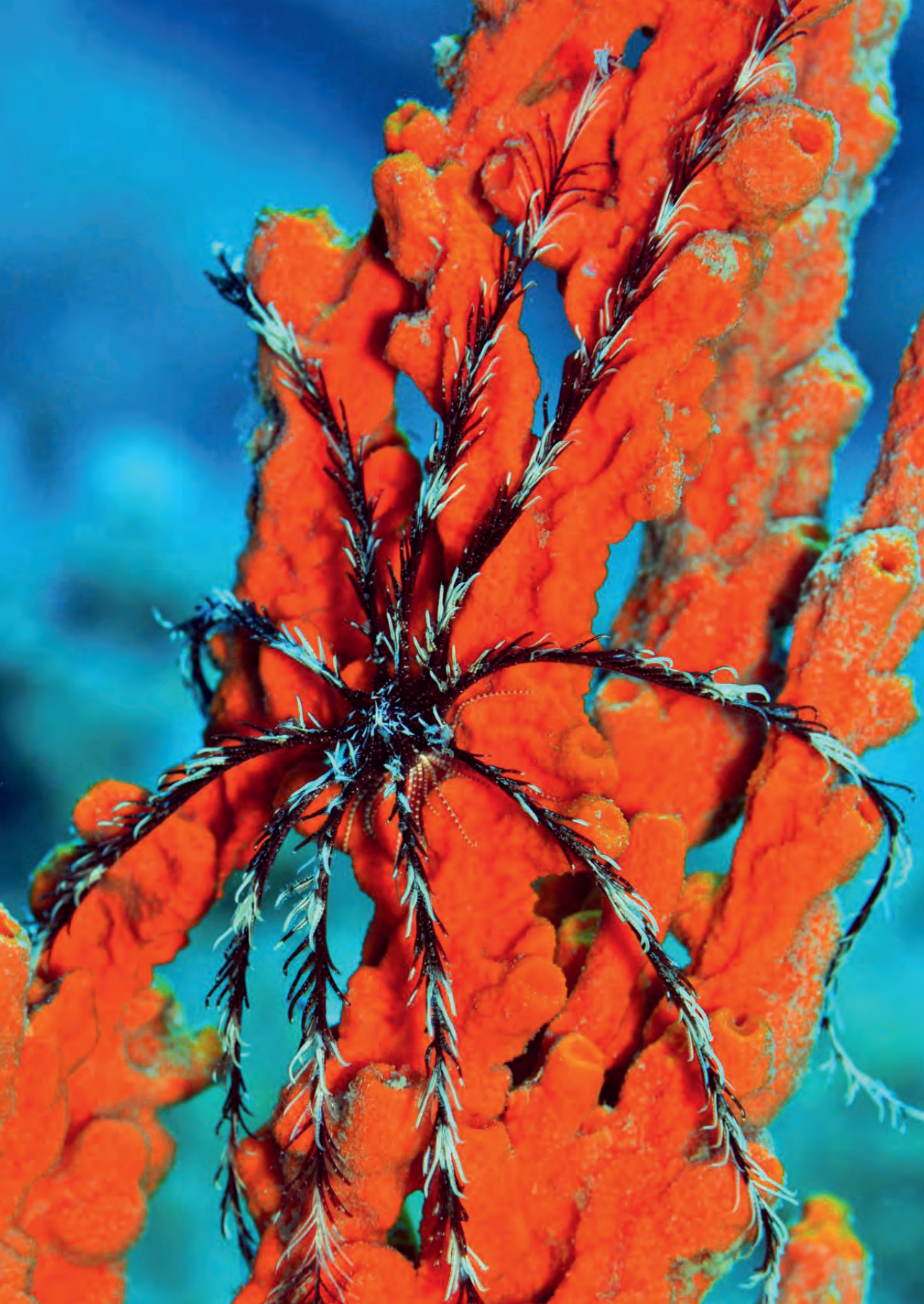

SUMMARY

Sponges are found at all latitudes, living in a wide array of ecosystems varying in temperature and depth. Sponges have important ecological roles, including that of biological filter. They extract and accumulate various organic and inorganic compounds and microorganisms from the water, thus improving the water quality of marine and freshwater systems. Additionally, marine sponges are known to harbor new bio products that have remarkable potential for development as pharmaceutical drugs or biomedical materials. This PhD project focuses on the production of raw sponge materials by mariculturing sponge species that produce potential biomaterials. Since sponges feed on suspended and dissolved organic matter, it has often been suggested to apply sponge culture to remediate marine organic pollution, such as the effluent from sea-based fish cages and unpurified urban wastewater discharge. Large-scale sponge culture may help reduce eutrophication of coastal waters and its associated disruptive effects on local ecology and biodiversity. Sponge culture can also locally improve the water quality around fish farms, which benefits cultured fish health.

Chapter 1

This Chapter serves as an introduction explaining the importance of integrated multitrophic aquaculture and focusses on the main threats that these delicate coastal ecosystems face today due to the organic pollution from fish farms and anthropogenic discharge from municipal sewage. Specifically, it emphases the history and current status of 235 years of sponge mariculture. The main research topics of this thesis are presented, which revolve around the use of sponges for the purpose of water purification and enhanced production of high-quality sponge biomass for sponge bioproducts.

Chapter 2

The concept IMTA with sponges predicts a double benefit: sponges would grow faster under higher organic loadings, and filtration by sponges would improve water quality. The growth of some commercially important sponges were succeeded and the potential of sponges as filters for undesired microorganisms has been confirmed in laboratory studies. However, upscaled farming remains to be demonstrated in large scale where in situ sponges bioremediation of sewage discharge or waste produced by fish cages yet to be performed. In this regard, Chapter 5, evaluated the use of sponges in integrated culture systems by providing a historical perspective of sponge mariculture and summarizing the earlier idea of use of sponges as biofilters for organic particles. In this Chapter a new idea is presented, the use of sponges as an engine to convert dissolved organic matter (DOM) into particulate organic matter (POM) that can be consumed by deposit feeders through 'Sponge Loop'. A theoretical design of an integrated culture with seaweeds, sponges and, sea cucumbers were evaluated to demonstrate the possibility for such utilization. In this model study, the total recovery of DOM into (sponge and sea cucumber) biomass within this IMTA were found to be 49% where, 37% of the primary production that is excreted by the seaweeds (DOM) can be directly recovered in sponge biomass and a subsequent 12% in sea cucumber biomass after conversion of DOM to POM by sponges.

Chapter 3

In this Chapter, the effect of depth on sponge morphology, growth, physiology, and functioning. Specimens of Eastern Mediterranean populations of *C. reniformis* were evaluated for the purpose of successful application of sponges for water purification and collagen production. Explants were reciprocally transplanted between 5 and 20 m depth where, a significant inverse relationship between the osculum density and size was found in *C. reniformis* specimens growing along a natural depth gradient. Control sponges at 5 m had fewer but larger oscula than their conspecifics at 20 m. Sponges transplanted from 20 to 5 m altered their morphology to match the 5 m control sponges, producing fewer but larger oscula, whereas explants transplanted from 5 to 20 m didn't alter their morphology. In spite of the changes in morphology, the clearance, respiration, and growth rates were comparable among all the experimental groups. The overall performance of the sponges were not affected by the depth-induced morphological changes. Hence, the potential for the growth and bioremediation of *C. reniformis* in mariculture is not likely to change with varying culture depth.

Chapter 4

This Chapter described novel methods to test the culture of *C. reniformis* in the proximity of floating fish cages, where, the performance of cultured explants were monitored near a polluted fish farm and a pristine control site. During these trials, attachment methods, plate materials, and plate orientation were compared in order to develop a successful culture method for *C. reniformis* in an organically polluted site (where we failed in **Chapter 2**). Both survival and growth were higher at the polluted site (86% survival and 170% growth) than at the pristine site (39% survival and 79% growth). These results represented a first successful step towards production of sponge collagen in integrated aquacultures. And following these series of culture trials spanning for 2 years culture methods, best practises (culture method, site selection, seasonality) were reported upon the experiences gained from a prolonged culture study.

Chapter 5

Chapter 2 describes the initial mariculture trials conducted with two Mediterranean sponges with biological potential. The sponges were cultured in a comparison study where *D. avara* and *C. reniformis* explants were cultured in the vicinity of fish farms and a pristine site by using various methods to investigate the success of species-specific culture methods. *D. avara* explants were successfully cultured at both sites and a new production method 'Shish kebap', was developed. Whereas culture of *C. reniformis* was only successful at the pristine site. All explants cultured underneath the fish cages died due to smothering with sediment.

Chapter 6

Chapter 6 investigated the gaps in the optimal culture of *C. reniformis* and aimed at perfecting the design for large-scale mariculture of *C. reniformis* for collagen production pipeline. The first aim of this study was to test whether losses of sponges in culture due to detachment could be reduced. The second aim of this study was to explore whether mariculture of *C. reniformis* can be combined with the treatment of municipal wastewater. And the third aim of this study was to design and implement a robust, scalable culture system that integrates best practices and that reduces vulnerability and operational costs, in order to enable economically feasible, commercial-scale production of raw sponge materials for the extraction of collagen.

These research questions were addressed in a series of culture trials where, orientation, attachment, substrate, location, light condition were investigated. These consecutive culture trials spanning 2 years revealed consistent growth rates and high survival rates in over 1 year of culture, both under pristine and polluted conditions. And finally, we reported a successful culture method for a collagen production pipeline using *C. reniformis*, entitled 'Sponge lantern', that is simple, sustainable, enhances productivity and is adaptable to seawater environments with variable organic particle load.

Chapter 7

In the final Chapter, the overall summary of efforts on mariculture potential of bioactive sponges were provided. The long lasting efforts for optimal production of collagen *with C. reniformis* was summarized with a valuable best practises section, where critical knowledge and experience gathered during trials conducted over 6 summers. The Chapter ends with a positive demonstration for mariculture, namely evaluation of potential with sponge lantern method by providing a feasibility study and sustaining water quality part. The feasibility of mariculture were substantiated for three commercially potential sponges, by extrapolating the study conducted for *C. reniformis* (Chapter 6) to *D. avara* and *S. officinalis* for the production of Avarol and for bath sponges, respectively. And the water quality sustaining part were theoretically demonstrated in this Chapter by making use of Chapters 3&4 and two separate studies by Adriaanse (2020) and Banas *et al.* (2007).

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About the Author

Mert Gökalp is a marine-biologist and underwater documentary specialist born in Ankara, Turkey. He graduated from Middle East Technical University, studied MSc. in applied marine physics department of Miami University Rosenstiel School of Marine Sciences (RSMAS) and completed his MSc. study as a marine biotechnologist at Ankara University Biotechnology Institute. He started working on sponges during his MSc study where he met with Dr. Ronald Osinga. He, collaborated with Dr. Ronald Osinga where they worked together in two European Union Projects (5th frame) Sponges & (6th frame) Special on the subject of sustainable use of marine resources. He is currently continuing his PhD (BiogenINK Project) in the Wageningen University of Netherlands, in the Marine Animal Ecology Department. Sponge collagen is the main target of the European project BiogenINK, which overarches his PhD project. BiogenInk aims the development of bio-inspired inks for 3D printing of scaffolds for tissue engineering based on sponge collagen. The project involves field experiments in the Eastern Mediterranean Sea, southern coast of Turkey, to determine bacterial clearance rates & filtration efficiencies of sponges in different depths and pollution conditions for the purpose of eliminating fish farm & urban waste. The project also aims to assess the biological performances of two Mediterranean sponge species under different eutrophication and depth conditions to investigate their aquaculture potential and pollution remediation efficiency at pristine and organically polluted sites. Species-specific culture methods are optimized ultimately achieving a novel integrated fish-sponge farm model, which is self-cleaning and could maximize production of high quality raw sponge material. His speciality is in field science, research involving complex underwater experiments.

Mert also worked in various advertisement projects & NGO promotional as a director, photographer, underwater cameraman and director of photographer. In 2012, he published a guidebook for marine species of Mediterranean Sea ‘Turkiye Deniz Canlilari Rehberi’ (Gökalp 2012) and produced a Marine Nature Guide, a smart phone application for marine species of Mediterranean Sea. He has 4 individual & 1 combined photography exhibitions. He is working as a freelance writer & nature photographer in MAGMA magazine since 2014. He directed award winning short film IRME (2014) & featured documentaries -Bluefish (2017) and -Dusky Grouper ‘The Lord of the Reef’ (2020). He is now working on two featured length documentaries first on coral reef restoration & climate change and second about the invasive fish species in the Mediterranean both will be released in 2021.

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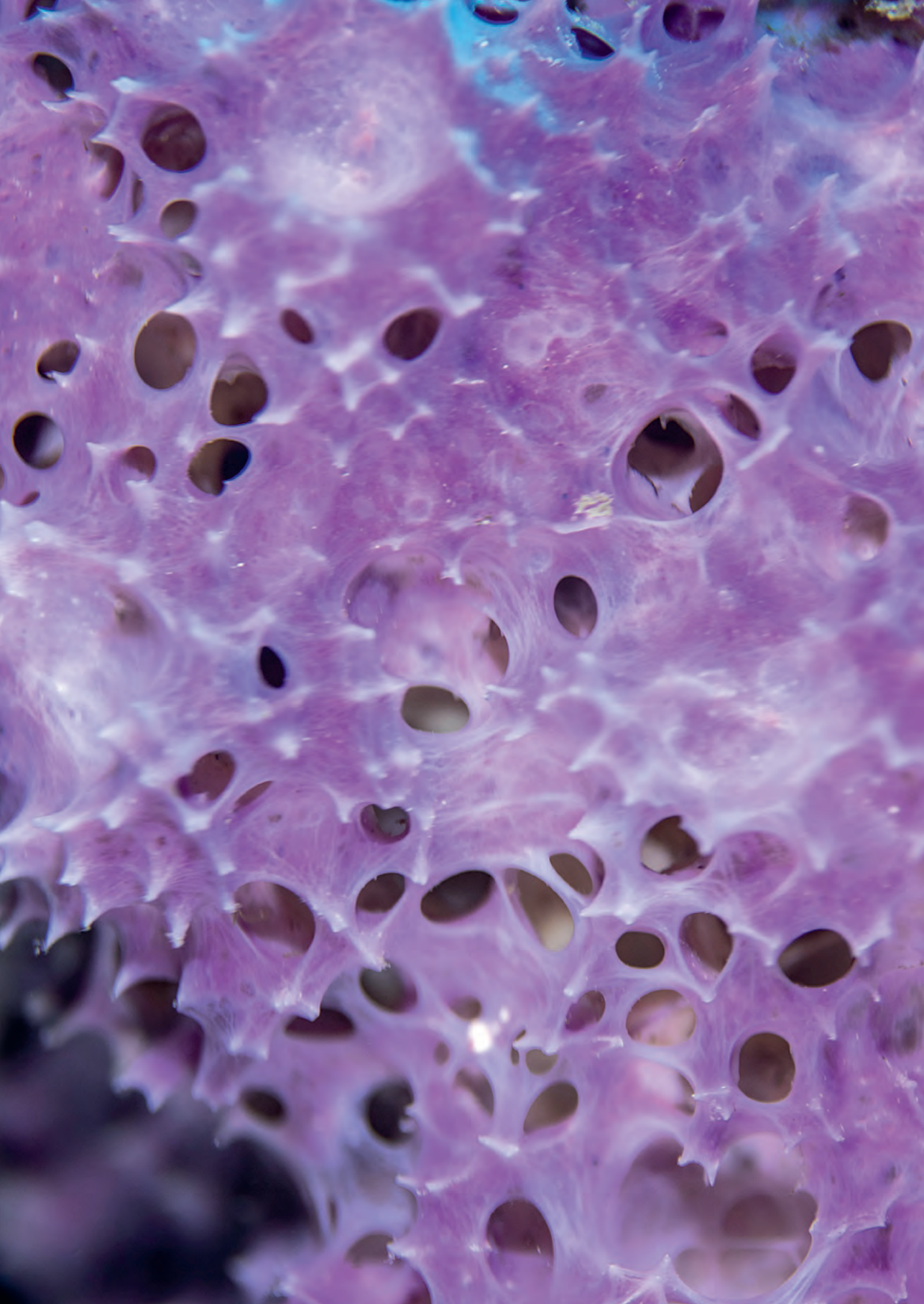

Photo specifications

Page 8: Mediterranean Sea cavern habitat and colonies of *Agelas oroides (Schmidt, 1864)* sponges at the ceiling of the cavern

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Page 10: *Chondrosia reniformis* Nardo, 1847 colony, in the process of stretching from the ceiling of a cavern

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Page 169: Haliclona fistulosa ?? (Bowerbank, 1866) and *Agelas oroides* sponges

Page 173: Ircinia variabilis (Schmidt, 1862) brown lobular form

Page 175: *Axinella cannabina* (Esper, 1794; left) *Axinella polypoides* Schmidt, 1862 (right)

Page 177: *Close up of Dysidea avara (Schmidt, 1862)*

Page 179: *Chondrosia reniformis inside a cavern wall performing an incredible elongating feat* (+3 m; I couldn't fit it in the picture)

Training and Supervision Plan (TSP)

GENERAL INFORMATION	
Name PHD candidate	M Mert Gokalp
Project title	The multifunctional role of marine sponges in multi-trophic mariculture systems
Group	Marine Animal Ecology
Supervising team:	
Promotors	Prof. Dr A.J. Murk, Dr R (Ronald) Osinga, Dr THM (Tim) Wijgerde
Project term	from 2017 until 2020

EDUCATION & TRAINING

A. The Basic Package		year	credits *
WIAS Introduction Day	(mandatory)	2020	0.3
Scientific Integrity	(mandatory)	2020	0.6
Ethics and Animal Sciences	(mandatory)	2020	0.8
Subtotal Basic Package			**1.7**

B. Disciplinary Competences	year	credits *
Research Proposal (Review publication approved by WIAS) https://doi.org/10.1111/raq.12516	2020	6.0
University of Miami/RSMAS-Scientific Diving	2003	1.0
PADI Scuba Diving Instructor Course	2003	1.0
External 1 month long experiment on oil tolerance of sponges Rovinj Croatia	2014	0.5
ROV (Remote Controlled Vehicle) 1 month long piloting for Sponge Collection / Azores / Eilat	2013	0.5
WIAS/PE&RC advanced statistics course Design of Experiments	2019	0.8
Subtotal Disciplinary Competences		**10**

C. Professional Competences	year	credits *
Eastern Mediterranean Marine Animals Guide Book	2013	1.0
6th Frame EU Project Progress Meeting-SPONGES (Organization)	2008	1.0
7th Frame EU Project Progress Meeting-SPECIAL (Organization)	2012	1.0
Final Touch/WIAS Course, Writing General Introduction and Discussion	2018	0.6
Scientific Writing	2019	1.8
Scientific Artwork with Photoshop and Illustrator	2019	0.5
Subtotal Disciplinary Competences		**6**

D. Societal Relevance (recommended)	year	credits *
Bluefish - Feature Length Documentary on Sustainable Fisheries (Shown in WUR - 100 YEARS)	2017	1.0
Dusky Grouper Awarded Feature Length Scientific Documentary on Marine Protected Areas	2021	1.0
Radio Interview TRT	2017	0.5
TV Program Guest NTV	2013	0.5
Interview Resource of Wageningen University	2019	0.5
Interview & Publishing Cover Journal Marine Drugs (MDPI) in National Newspaper Milliyet	2019	0.5
WIAS course Societal impact of your research	2019	1.5
Subtotal Disciplinary Competences		**6.0**

E. Presentation Skills	year	credits *
TED-X-RESET ISTANBUL Conference talk	2011	1.0
Genoa Open Day- Oral Presentation (EU Project SPECIAL)	2013	1.0
ISSR Coral Conference Wageningen-Poster Commitee/Exhibition	2012	1.0
24th WIAS Annual Conference Poster Presentation	2019	1.0
Subtotal Disciplinary Competences		**4.0**

F. Teaching competences	year	credits *
Field Supervision - Msc Student Holger Kuehnhold	2013	0.5
Co-supervising MAE Practical Course	2018	0.5
Msc. Field & Thesis Supervision - Tjitske Kooistra	2018	1.5
Msc. Field & Thesis Supervision - Marretje Adriaanse	2018	1.5
Msc. Field Supervision - Ellen van Marrewijk	2019	1.0
Msc. Field & Thesis Supervision Short Research Project - Anne Top	2019	0.5
Msc. Field & Thesis Supervision Short Research Project - Marlin ter Huurne	2019	0.5
Subtotal Disciplinary Competences		**6**

Education and Training Total	**32.9**

**One ECTS credit equals a studyload of approximately 28 hours*

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