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In situ direct study of filtration and respiration rate of Mediterranean sponges

Estudio *in situ* de la filtración y la respiración de esponjas Mediterráneas

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Tesis presentada per l'obtenció del títol de Doctor per la Universitat Politècnica de Catalunya

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"*In situ* direct study of filtration and respiration
rate of Mediterranean sponges"

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ABSTRACT

Sponges play important roles in the functioning of marine ecosystem in which they are abundant. These roles range from stabilizers of substrate, to acting as major link between benthic and pelagic realms by filtering large quantities of water and retaining the particles with high efficiency. Despite sponges have been the focus of much interest in the past years our knowledge on sponge physiology is still poorly understood. This study focused on ecophysiology of five of the most prominent sponge species dwelling the coralligenous community in the NW Mediterranean Sea, and employed an energetic approach to understanding the complex interactions between physiological constrains and seasonal fluctuations of environmental factors experienced by the organism under natural conditions. In this thesis, we contributed to the still limited knowledge of energetic mechanisms that regulate seasonal dynamics and elucidated divergent metabolic profiles between high microbial (HMA) and low microbial (LMA) abundance species accordingly to their different adaptive life strategies. For this purpose we examined the *in situ* feeding, filtering and respiration activity over annual cycle. Firstly, we started by developing a system for quantifying the particulate and dissolved compounds processed by sponges under natural conditions. In terms of feeding, we observed that all species retained plankton at high efficiency and DOC was the main source of carbon. However, the nitrogen fluxes showed a markedly different trophic niche between the two groups: HMA species mostly rely on dissolved compound as main source of N, while LMA solely rely on particulate fractions. Interestingly, natural variation of sponge pump did not follow natural temperature changes. During the period of maximum temperature the sponge pump did not reach its maximum values, suggesting that intrinsic mechanism as a decrease in choanocytes during reproductive cycle may regulate this metabolic process. On the other hand respiration clearly showed a seasonal pattern following natural temperature fluctuation. Combined, these results allowed us to estimate the overall energy budget, which appeared to be regulated by an increase of energy demand in summer as well as the availability of dissolved organic carbon fraction in the water column. Our result also showed different limited energetic profiles between HMA and LMA species accordingly to their different feeding strategies. LMA species appeared to procure sufficient energy to meet metabolic requirements for

maintenance and growth by filtering suspended particulates, and dissolved fraction represented an additional input of carbon when available. On the contrary the heterotrophic nutrition in the natural environment may be insufficient to meet basal metabolic requirements in HMA species, suggesting other metabolic pathways as relevant for the energy budget of these species.

KEYWORDS: *benthic ecology*, *nutrient fluxes*, *energetic metabolism*, *eco-physiology*, *feeding acgivity*, *pumping and respiration rates*

RESUM

Les esponges juguen un paper important en el funcionament del ecosistemes marins on hi son abundants. Les funcions atribuïdes a les esponges son molt diverses i inclouen entre altres actuar com estabilitzadores del substrat, així com fer de lligam entre la columna d'aigua i el bentos en l'anomenat acoblament bento-pelàgic; aquest procés te lloc mitjançant una captura eficient de partícules i el retorn de compostos modificats per l'activitat metabòlica de les esponges. Tot i que les esponges han estat punt de mira en l'interès científic durant els darrers anys, s'identifica encara una manca de coneixement de la seva fisiologia. Aquesta tesi es centra en l'ecofisiologia de cinc especies d'esponges emblemàtiques del coral·ligen Mediterrani. Utilitzant una aproximació energètica s'ha avaluat les interaccions entre possibles limitacions fisiològiques en les esponges i fluctuacions estacionals en els paràmetres ambientals a les que estan sotmeses en la natura. En aquesta tesi contribuïm al coneixement encara limitat dels mecanismes energètics que regulen la dinàmica estacional de les esponges així com en aportar informació sobre el perfils metabòlics divergents entre les esponges amb alta (HMA) i baixa (LMA) concentració de microbis associats segons les seves diferents estratègies adaptatives. Amb aquest objectiu examinem *in situ* l'alimentació, la filtració i la respiració al llarg d'un cicle anual. En primer lloc comencem desenvolupant un sistema que permeti la quantificació de compostos particulats i dissolts processats per les esponges en el seu habitat natural. En termes d'alimentació hem observat que totes les especies retenen molt eficientment el plàncton i que el carboni orgànic dissolt (DOC) es la seva principal font de carboni. Respecte als fluxos de nitrogen s´ha trobat diferents nínxols tròfics entre especies amb diferent concentració de microbis associats: especies HMA depenen bàsicament de compostos dissolts com a font de nitrogen mentre que les especies LMA depenen de la fracció particulada. Curiosament, la variació en la tassa de filtració al llarg de l'any no segueix el cicle de temperatura. El període de temperatura mes alta no va coincidir amb les tasses de filtració mes elevades suggerint que altres mecanismes intrínsecs a les esponges, com pot ser una disminució en la quantitat de coanòcits durant el cicle de reproducció, podrien regular el metabolisme de les esponges. Per altra banda la respiració ha mostrat un patró estacional seguint les fluctuacions naturals de la temperatura. La combinació d'aquests resultats ens ha permès estimar el balanç

energètic global, aquest sembla estar regulat per un increment en la demanda energètica a l'estiu coincidint amb un increment en la disponibilitat de carboni orgànic dissolt en la columna d'aigua. Els nostres resultats també han mostrat diferencies en el perfil de limitació energètica entre especies HMA i LMA coincidint amb les seves diferents estratègies en la captura de nutrients. Les especies LMA sembla que obtenen suficient energia per cobrir el seu metabolisme a partir de la matèria particulada de l'aigua, essent la fracció dissolta un aportació addicional de carboni. Contràriament en especies HMA la nutrició heterotròfica sembla ser insuficient per cobrir els requeriments metabòlics basals suggerint altres vies metabòliques rellevants per cobrir el balanç energètic.

PARAULES CLAU: *ecologia bentònica, flux de nutrients, metabolisme energètic, eco-fisiologia, alimentació, filtració i taxes respiratòries*

PREFACE

"The relatively short term future of sponges will certainly be modified by humanity. They will have to face disruptions to the ecological equilibrium caused by the proliferation of our species and activities. We can infer from the rate of extinction over the last two centuries that we are facing a major extinction period of the same order of magnitude as the seven or eight recorded in the fossil record. Sponges have survived these major extinction periods, with up to 80% of marine species known from the fossil record disappearing at the end of Permian. It is therefore likely that they will have a chance of being able to cope with this new threat. We fear a rise in sea-level due to global warming. This is not a problem for sponges. They have seemingly failed in their role, discovered by a French humorist, Alphonse Allais, around 1900, who maintained that sponges were placed in the oceans by Providence to prevent overflowing from all the rivers, but this failure may be turned to their advantage, as more seabed surface will become available for colonization. A little more seriously, the colonization or urbanization of the ocean by mankind, as predicted by some visionary architects, could also turn to the advantage of sponges, which generally prefer solid substrates to soft sediments. These futuristic developments could include submarine cities, with house walls covered by brilliantly coloured sponges, cleaning the water by their filtering activity, with equipment for farming genetically modified species producing molecules of exceptional biological interest."

Jean Vacelet 1999 Outlook to the future of sponges, Memoirs of the Queensland Museum 44: 27-32.

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General Introduction

Phylum Porifera

The Phylum Porifera (from Latin *porus* 'pore' and *fero* 'to bear') comprises the most primitive metazoans, characterized by a simple body plan lacking of organs that are commonly associated to animal kingdom (such those of reproductive, muscular, digestive, excretory, nervous and respiratory system) (Schütze et al. 1999). The monophyletic Phylum of Porifera diverged from the ancestors of other metazoans around 1.3 billion years ago (Hedges et al. 2004). Sponges occupy a relevant place in the phylogeny of the metazoan tree, since they have been traditionally placed as the earliest diverging extant metazoan group (Figure 1) (Peterson and Butterfield 2005; Sperling et al. 2007; Erwin et al. 2011). This argument together with their cytological similarities with choanoflagelates and their antiquity of their fossil records (~535 My ago, Antcliffe et al. 2014) supports the hypothesis that a common ancestor possessed a sponge-like body plan (Nichols et al. 2009). However, not all phylogenic studies place sponges as the earliest lineage to all other animals. Recent phylogenies indicated ctenophores rather than sponges as first group to diverge from the last animal ancestors, revisiting new hypothesis about the morphology of ancestral metazoans (Philippe et al. 2009; Wörheide et al .2012; Ryan et al. 2013; Moroz et al. 2014).

Sponges are an ecologically important and highly diverse benthic group, with an estimated 11,000 species worldwide (only 8,500 are considered valid), and with a number of described species increasing at a steady rate (35-87 per year) (Van Soest et al. 2012). On the basis of the chemical composition of the skeleton and/or specific morphology of siliceous spicule the Phylum Porifera is characterized by four classes: Calcarea, Hexactinellida, Demospongiae and Homoscleromorpha. The latter has been considered part of Demospongiae class for long time and only in 2012 was officially recognize as forth class (Gazave et al. 2012). Phylogeny studies place hexactinellids and demosponges together (Philippe et al. 2009), further supported by the evidence of similar siliceous spicules organization around proteic axial filament (Uriz et al. 2003). While Homoscleromorpha is considered as sister group of Calcinospongiae (Dohrmann et al. 2008; Philippe et al. 2009), further supporting their exclusion from Demospongiae class. Among the four classes, Demonspongiae is the largest and most

diverse in terms of number of species (~83%, Van Soest et al. 2012) inhabiting most aquatic and freshwater habitats from tropical to polar regions. This diversity in habitats reflects their taxonomic diversity. On the basis of morphological data this class is organized in 14 orders that comprise 88 families and 500 genera (Systema Porifera 2002, Hooper and Van Soest 2002). Nevertheless due to new insight in molecular data a recent revision of its classification was proposed (Morrow and Cárdenas 2015).

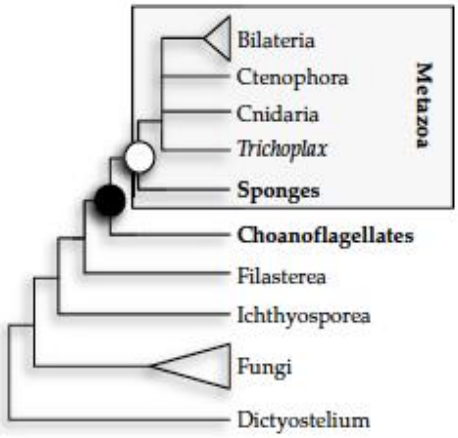


Figure 1. Phylogenetic relationships reveal sponges as the earliest branching metazoan lineage, supporting the close nature of the last common metazoan ancestor (white circle) and sponge-like morphological body. Black circle indicates last unicellular ancestor of Metazoans (from Nichols et al. 2009).

Sponge body plan

In general, sponge body posses three well-defined types of epithelial tissue: pinacoderm, mesohyl and choanoderm (Figure 2). The former is the outer layer, composed by epithelial cells known as pinacocytes, the mesohyl is the connective tissue, comprised by mineral deposits (calcareous or siliceous), supporting collagenous fibers (spongina) and inorganic structures of the skeleton (spicules). The choanoderm is the inner layer composed by flagellated cells (choanocytes) responsible to create a unidirectional water flow throughout sponge body. The Hexactinellida represents one exception from this general body plan, being much of their body (about 75%) represented by syncytial tissues, known as multinucleate mass of cytoplasm that is not separated into cells (Leys et al. 2007). They lack of

epidermal cells is usually replaced by a syncytial net of amebocytes and the few non-syncytial cells are connected each other with syncytial tissue by cytoplasmatic bridges (Pavans de Ceccatty and Mackie, 1982). A weaving of glass spicules supports their entire body.

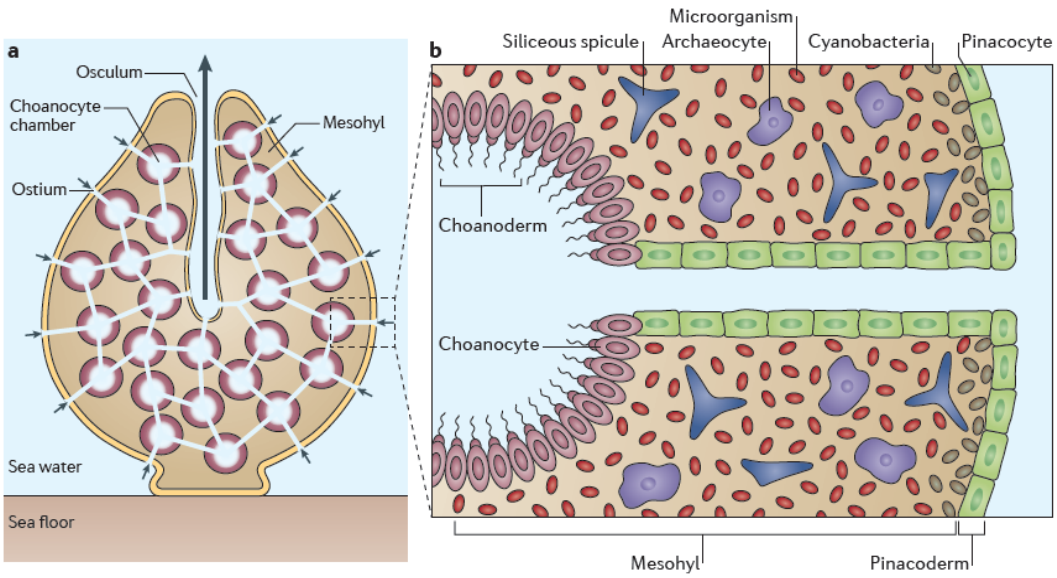


Figure 2. Sponge body plan: (a) a schematic overview of a typical Demospongiae; (b) an enlargement of choanocyte chambers, and structure and cell components in the mesohyl (from Hentschel et al. 2012).

Sponge body is entirely specialized for suspension feeding through a unique and highly vascularized canal system that allows them to obtain nutrients and oxygen from the water they actively pump. The water enters the sponge body through the micrometric ostia into the inhalant canals due to the slight negative pressure created by the movement of choanocyte flagella, gathered in the choanocyte chambers. After passing the chambers it leaves through the exhalant canals that merge into the excurrent apertures (oscula). Sponges have two independent systems of particulate capture: phagocytosis by amebocyte cells capturing directly the particles (> 2µm). This system avoids the occlusion of the aquiferous system by suspension particles, and phagocytosis by choanocyte cells, which create the water flow inside the channel and retain the particles (2µm or nanoparticles) at the microvilli collar (Reiswig 1971a). In Hexactinellida, due to the syncytial nature of the epithelium and mesohyl,

the aquiferous system differs from that of other classes and is characterized by a broad, expansive incurrent and excurrent canals bounded by a network of trabeculae (Leys 1999). The particulate capture occurs almost exclusively at the stage of primary and secondary trabecular reticulum in the flagellated chambers (Wyeth et al. 1996).

Sponge ecology and function

Sponges are crucial for the functioning of marine ecosystems in which they are abundant (*e.g.*, Rützler 2004; Wulff 2006a; Bell 2008), and their ecological importance may be related to some characteristics: (1) their ubiquity (Figure 3): they are abundant throughout tropical, temperate and polar regions, from shallow to deep habitats (Kowalke 2000; Diaz and Rützler 2001; Hooper et al. 2002). (2) Their impact on the substrate and reefs, acting as binders, stabilizers and dismantlers (bioeroders) (Wulff 2001). In the soft sediments of deep sea habitat, glass sponges provide hard substrate for other animals whether dead, with denuded stalks or spicule mats, or alive (Bett and Rice 1992; Beaulieu 2011a,b) and form large sponge reef comparable to coral reef (Krautter et al. 2006). (3) Their heterogeneous diet: they are opportunistic feeders, specialized on filtration of ultra-planktonic cells (< 10 µm, Pile et al. 1996; Coma et al. 2001; Yahel et al. 2007). (4) Their role in benthic-pelagic coupling: sponge's high retention efficiency (>90% efficiency removal of *Synechococcus* sp. and *Prochlorococcus* sp. cells, *e.g.*, Pile et al. 1996; McMurray et al. 2016) coupled with high volume of water they processed (Reiswig 1981; McMurray et al. 2016) and their role in nutrient cycling and energy flux (Maldonado et al. 2012a; de Goeij et al. 2013) make them a significant trophic link between the benthos and the overlying water column (Savarese et al. 1997; Peterson et al. 2006; Pile and Young 2006). Sponges can serve as either a sink and/or source for dissolved carbon (Yahel et al. 2003; Ribes et al. 2012; Mueller et al. 2014), nitrogen (Jiménez and Ribes 2007; Fiore et al. 2010, 2013; Keesing et al. 2013), phosphate (Hatcher 1994; Yahel et al. 2007; Ribes et al. 2012) and silicate (Frøhlich and Barthel 1997; Reincke and Barthel 1997; Maldonado et al. 2012b). Therefore sponges may influence the availability of those dissolved compounds, and exert a major impact on primary and indirectly on secondary production of the overlying water column. Their role in organic matter

cycling has been equated to microbial loop in so called “sponge loop”; by removing dissolved

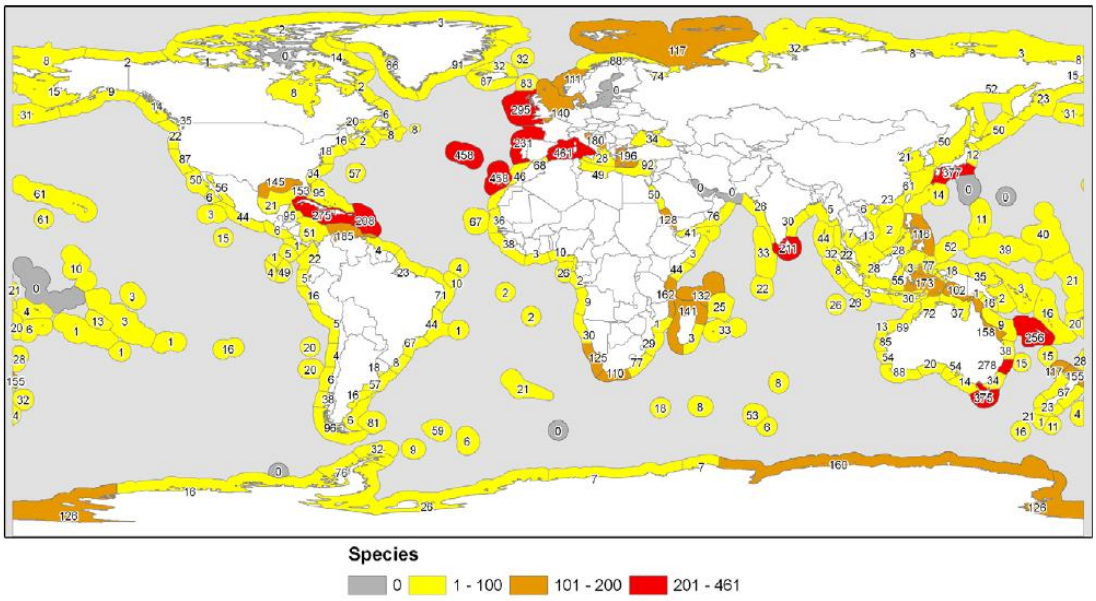


Figure 3. Number of sponge species recorded in each of 232 marine eco-regions of the world extracted form the World Porifera Database (from Van Soest et al. 2012).

organic matter sponges make it available in form of detritus to higher trophic levels (de Goeij et al. 2013). This mechanism is fuelled by continuously cell shedding (de Goeij et al. 2009), though shed cells have not been observed in detrital waste of some sponge species (Alexander et al. 2014; Kahn PhD thesis, 2015). Recently, a feeding study on deep glass sponges has developed new insight of the role of sponges in organic matter cycling by drawing carbon from the bacteria presented in the resuspended bottom sediment and make it available in form of fecal pellet (Kahn PhD Thesis, 2016). (5) Their association with microorganisms: the associations between sponges and prokaryotes have existed for 600 million years, making them one of the most ancient of all symbioses between microbes and metazoan (Wilkinson 1984). Sponges contain by far the highest number of different bacterial symbionts documented (up to 3000 detected) for an invertebrate host and symbiont richness in sponges is comparable to the number of different microbes in the human gut system (Webster et al. 2010). Sponges are generally divided into two large groups according to the microbial density in their tissue: high microbial abundance species (HMA),

defined as sponges containing 10^8 to 10^9 bacteria per g sponge tissue and low microbial abundance species (LMA), defined as sponges containing 10^5 to 10^6 bacteria per g sponge tissue, that is similar to the microbial abundance in seawater (Vacelet and Donadey 1977; Hentschel et al. 2012; Gloeckner et al. 2014).

Despite sponges may be consider the simplest metazoan due to their simple body plan and function, they have also demonstrated versatility in feeding/pumping behavior and an incredible ability to rearrange their body. For instance, sponges may maintain the pumping activity fairly constant, although they may periodically interrupt water transport during storm events, increased sediments in the water, massive sperm release by another species or even randomly through the population and irregularly among individuals without any correlation to environmental parameters (Reiswig 1971b). Moreover sponges are able to continuously rearrange their anatomic structure (Bond 1992), determining that sponge shape is variable over time in relation to biotic and abiotic factors (Becerro et al. 1994a; Mendola et al. 2008). Sponges are also of commercial interest, because they produce biosynthetic compounds with useful cosmetic and pharmaceutical properties, including antitumor, anti-infective and anti-inflammatory properties (Pomponi 2006).

Given the importance of the role of sponges in the functioning of ecosystem, they are still unfortunately under represented in marine science, monitoring and conservation programs and have previously been declared a “neglected-group” (Wulff 2001; Saleuddin and Fenton, 2006; Bell 2008; Schönberg and Fromont, 2012; Fang and Schönberg, 2015). Massive sponge mortalities occurred worldwide in the past (Gaino et al. 1992; Wulff 2006b), and in many cases the impacts of most relevant environmental pressures on sponges were poorly understood, and mostly studied at the level of responses of sponges abundance and richness (Bell et al. 2015). Sponge ecology has been overlooked and their physiology is still poorly understood. Physiological studies represent only 18% of the literature in this phylum (calculated as the average of proportion of articles on sponges in 12 coastal eco-regions, from Bell et al. 2015). The loss of benthic organisms may stand for severe impacts on the survival of benthic population and may have a cascade effects on the neighboring species, especially if the affected species are ecologically important group such sponges. A consequence of a sponge biomass loss few years ago in the Adriatic Sea has

been a reduction of the filtration activity over 60%, with the subsequent implication in the benthic-pelagic coupling processes as well as nutrient and organic matter cycling (Di Camillo and Cerrano 2015). Sponge biomass loss is also caused by the human activity. Fish trawling together with cable-laying are threatening the sponge reef dwelling deep sea of North-East Pacific Coast. These activities damaged up to 67% of sponge population (Freese et al. 1999; Freese 2001) and probably required long time for sponge population to recovery (Kahn et al. 2015a). The impact of fish trawling may reach also shallow water by increasing loads of suspended sediment (Puig et al. 2012) that affects the filtration activity of sponges (Reiswig 1971b; Gerrodette and Flechsig 1979; Leys and Lauzon 1998; Gabrielle et al. 2008).

Understanding the causes of local and global sponge decline are fundamental for increasing the awareness of their consequences at the community and ecosystem levels and will help to develop tools for conservation issues.

The case of study

Coralligenous community is a multispecific assemblage of suspension filter feeders that dominate sublitoral benthic communities of Mediterranean Sea, which prevails on hard substrata in sciaphilic biotopes. Coralligenous is highly diversified community; it constitutes the second most important “hot spot” of species diversity in the Mediterranean, after the *Posidonia oceanica* meadows (Boudouresque 2004). It is composed by a wide variety of organisms, from encrusting calcareous corallines and green algae living in dim light conditions, which are the major contributors of coralligenous framework building, to suspension filter feeders as bryozoans, anthozoans, hydrozoans, sponges, tunicates, serpulids and moluscs (Ballesteros 2006). Among them, sponges are the third most representative taxa in term of species richness (142 species, Ballesteros 2006). The coraligenous community is highly vulnerable, due to longevity and low rate of renewal of the main builder species (Coma et al. 1998; Garrabou et al. 2002). Our knowledge of the functioning of this complex community and the role in the benthic-pelagic coupling is still poor (Gili and Coma 1998; Ballesteros 2006).

In the last decades, suspension filter feeders from the coralligenous community have been affected by several mortality events. Most of these events were concomitant

with anomalous high temperature, suggesting such climatic anomalies as a direct or indirect cause of these phenomena. In western Mediterranean Sea two major mortality events of benthic invertebrate occurred in summer 1999 and 2003. These events had a higher impact with respect to size of geographic area concerned (1000 km of coastline), high diversity of taxa affected (approximately 30 species from 5 phyla) and high mortality rates ever observed in the NW Mediterranean during the last few decades (Cerrano et al. 2000; Pérez et al. 2000; Coma et al. 2006; Garrabou et al. 2009). In both massive mortalities the most affected taxa were corals (from 60% to 100% of colonies with 10 species affected) and sponges (13 affected species) from shallow water up to 40 m depth (reviewed by Lejeusne et al. 2009). The magnitude of these events can be compared with coral mortalities reported from tropical areas, especially from the Caribbean Sea, where the rising of temperature coupled with other environmental stressors has been considered the main cause of mortality (Ware 1997; Berkelmans and Oliver 1999; Stone et al. 1999; Carpenter et al. 2008). In addition to these two major events, other more recent mass mortalities affecting sponge species were reported in Mediterranean Sea (Maldonado et al. 2010; Cebrian et al. 2011; Di Camillo et al 2012a; Stabili et al. 2012; Di Camillo and Cerrano 2015). Although these events occurred locally, a large part of sponge population was affected by mortality (up to 90%, Cebrian et al. 2011) (see table 1 for a list of sponge species affected by mortality events in Mediterranean Sea from 1999 to 2011).

Both cases of mortalities in 1999 and 2003 were characterized by exceptional higher seawater temperature registered during the late summer-early autumn (3-4ºC higher than average), which affected the whole water column down up to 40m depths, and the warm lasted for unusually long time (Romano et al. 2000; Garrabou et al. 2009). These conditions led to an extension of the stability of the water column (Cerrano et al. 2000; Romano et al. 2000; Marullo and Guarracino 2003; Sparnocchia et al. 2006), characterized by stronger and longer summer conditions (higher temperature and food shortage), which likely affected the specimens dwelling above the thermocline (Coma et al. 2009). A positive correlation was observed between mortality rates and the exposure to higher temperatures (Coma et al. 2009), therefore high temperatures have been considered the main cause of mortalities due to: i) the exposure to lethal temperatures causes the loss of molecular function and may lead to

the death of the organisms; ii) the exposure to temperatures close to the upper limit of the critical temperature may also cause acute physiological stress; iii) proliferation of opportunistic pathogens such as fungi or protozoa ciliates promoted by thermal-stress (Gaino et al 1992; Cerrano et al. 2000; Pérez et al. 2000; Romano et al. 2000; Martin et al. 2002; Coma and Ribes 2003; Bally and Garrabou 2007; Torrents et al. 2008; Lejeusne et al. 2009; Stabili et al. 2012).

The causes of mortality events in sponges still remain unclear (Lejeusne et al. 2009; Maldonado et al. 2010). Although some of mortalities were attributed to bacteria disease (Gaino and Pronzato 1989; Vacelet et al. 1994; Stabili et al. 2012), as a consequence of the interaction of an environmental stressor (*e.g.*, high temperature) and opportunistic pathogens (Cerrano et al. 2000), during the 1999-2003 and 2008-2009 events any parasite disease was observed (Pérez et al. 2000; Cebrian et al. 2011). The role of associated cyanobacteria was discussed by Cebrian and colleagues (2011), as high temperatures reduced their photosynthetic efficiency, the authors hypothesized it might likely affect the contribution to the carbon requirements of the sponge host. But since also sponges lacking of autotrophic symbionts were affected by those mortalities, the dysfunction of associated cyanobacteria is unlikely the main cause. Other study demonstrated that during those warmer periods gorgonian might be affected by energy shortage that led to morality (Coma et al. 1998, 2009). Energy constrains result in a reduction of energy intake (starvation) and increase of energy output (respiration). The hypothesis of energy shortage assessed in gorgonians might further explain as the main causes of mortalities in sponges (Coma and Ribes 2003). Therefore sponges may be seriously threatened when long exposed to environmental factors that may cause a reduction of filter activity or an increase of respiration, especially under summer oligotrophic regime, when food availability is scarce. The evidence of the temperature-dependence of respiration rate in the sponge *Dysidea avara* may support this hypothesis (Coma et al. 2000, 2002; Coma and Ribes, 2003).

To test the hypothesis of energy shortage in sponges, it is needed to investigate extensively the energy intake and expenditure over annual cycle (Coma et al. 1998; Coma and Ribes, 2003). We are only beginning to understand the physiology of sponges under natural conditions, even less in a scenario of environmental change. According to Bell and colleagues (2015) the most intensively studied environmental

pressure on sponge was temperature (44 over 202 articles on environmental pressure in marine sponges), but only 3 and 2 articles showed a specific response of temperature on feeding/pumping and respiration, respectively. Research on sponge physiology is urgently required to better understand their physiological responses to their natural environment.

An energetic shortage has been suggested not only as main phenomena that may contribute to understanding the causes of mortalities as explained above, but also as common mechanism that regulates seasonal dynamics (Coma et al. 1998; Coma and Ribes 2003). An energetic approach could provide a more mechanistic understanding of the complex interactions between physiological constrains and environmental factors experienced by organisms under natural conditions. The energetics of sponges is particularly complex because their association with bacteria and their intricate interactions among bacteria-bacteria and bacteria-host. It is generally thought that abundant microbial communities benefit their host, but this assumption has to be taken cautiously since not all bacteria are beneficial to the sponge-host (Freeman et al. 2013). Distinct metabolic profiles and energetic budgets of different sponge species were attributed to the presence of symbionts in sponge tissues (Reiswig 1974, 1981). Several studies reported a mismatch between energy gained (*e.g.,* ingested carbon) and metabolic expenditure (*e.g.,* carbon lost by respiration) in tropical and temperate species (Reiswig 1974, 1981; Thomassen and Riisgård 1995; Yahel et al. 2003; Hadas et al. 2008, 2009). In LMA species the ingestion of particulate carbon was available in excess to meet their respiratory demand (Reiswig 1974, 1981; Koopmans et al. 2010), even though in case of net production of biomass for growth some species might be limited (Thomassen and Riisgård 1995; Hadas et al. 2008, 2009). Overall, the discrepancy between carbon gained and respired is higher in HMA than LMA species (Reiswig 1974, 1981; Yahel et al. 2003; reviewed by Maldonado et al. 2012a). Most of these studies attributed the carbon imbalance to the lack of quantification of the carbon input from the dissolved fraction (Reiswig 1974, 1981) which to our knowledge represents the main fraction of the C ingested by HMA species (Yahel et al. 2003; de Goeij et al. 2008b; Mueller et al. 2014). Whether this discrepancy may be due to specific energetic constrains or lack of quantification of entire carbon pool available for sponges need to be elucidated.

The examination of energy acquisition and expenditure in sponges needs to encompass natural variability on pumping, retention and, respiration to examine seasonal patterns and to elucidate how the different life strategies adapted by HMA and LMA species face seasonal changes. This study will improve the comprehension of several aspects of sponge physiology and energetic, and the effect that some environmental stressors have on them.

Table 1. List of Mediterranean sponges species affected by mortalities from 1999 to 2011. The species studied in this thesis are in bolded.

Species	Year	Reference
Agelas oroides	2003	Garrabou et al. 2009
Aplysina aerophoba	2011	Di Camillo and Cerrano 2015
Aplysina cavernicola	1999	Pérez et al. 2000
Cacospongia mollior	1999-2003	Pérez et al. 2000; Garrabou et al. 2009
Cacospongia scalaris	1999-2003	Cerrano et al. 2000;Pérez et al. 2000; Garrabou et al. 2009
Chondrosia reniformis	2011	Di Camillo and Cerrano 2015
Clathrina clathrus	1999	Pérez et al. 2000
Crambe crambe	1999-2003	Pérez et al. 2000; Garrabou et al. 2009
Haliclona fulva	1999	Pérez et al. 2000
Hippospongia communis	1999-2003	Cerrano et al. 2000;Pérez et al. 2000; Garrabou et al. 2009
Iricinia dendroides	1999-2003	Pérez et al. 2000; Garrabou et al. 2009
Ircinia fasciculata	2008-2009	Maldonado et al. 2010; Cebrian et al. 2011
Ircinia oros	1999-2003	Pérez et al. 2000; Garrabou et al. 2009
Ircinia variabilis	1999-2003-2009-2011	Pérez et al. 2000; Garrabou et al. 2009; Di Camillo et al. 2012a; Stabili et al. 2012; Di Camillo and Cerrano 2015
Petrosia ficiformis	2003	Garrabou et al. 2009
Sarcotragus spinosulus	2009-2011	Di Camillo et al. 2012a; Di Camillo and Cerrano 2015
Spongia agaricina	1999	Pérez et al. 2000
Spongia officinalis	1999-2003-2009-2011	Cerrano et al. 2000;Pérez et al. 2000; Garrabou et al. 2009; Di Camillo et al. 2012a; Di Camillo and Cerrano 2015

Overview and objectives

The main goal of this thesis is to understand the energetic mechanisms that regulate seasonal dynamics of the five species and their relation with the causes of massive mortality events, occurred in northwest Mediterranean Sea in summer 1999 and 2003. This energetic approach will also elucidate different metabolic profiles exhibited by LMA and HMA species. Our working hypothesis is that sponges are energetically limited when exposed to longer and warmer periods, relies on low amount of water filtered by sponges coupled with increase of respiration. In order to address this hypothesis, we investigated the energy acquisition and expenditure on

five of the most prominent desmosponge species in the Mediterranean Sea, *Agelas oroides* (Schmidt, 1864), *Chondrosia reniformis* (Nardo, 1833), *Petrosia ficiformis* (Poiret, 1789), *Dysidea avara* and *Crambe crambe* (Schmidt, 1862) by examining the feeding, pumping and respiration rates under the natural environment fluctuation over annual cycles.

Studied species

All the sponge species studied in this thesis are common species of the Mediterranean coralligenous community and belong to Demonspongiae class. This class is the largest and most diverse in terms of number of species of the phylum Porifera (~83%, Van Soest et al. 2012), it comprises sponges with siliceous spicules and/or with a skeleton of organic fibers or fibrillar collagen. The studied species together with a typical view of corallidenous community at the study site is shown in Figure 4.

***Chondrosia reniformis* (Nardo 1847)** (Order: Chondrosida, Family: Chondrillidae) is a common Mediterranean marine sponge, although it is also present in some areas of the African-Atlantic coast and Canarias Islands (World Porifera Database). This species lacks both endogenous siliceous spicules and sponging fibers (Garrone et al. 1975; Harrison and De Vos, 1991) and uses foreign material such as sand grains and other particles for the construction of its skeleton (Bavestrello et al. 1996). *C. reniformis* has a massive lobate shape, but it is known also to develop long and slender extending form from the parental body (a phenomenon called "creeping"), which according to some authors can be related to asexual reproduction (Fassini et al. 2012). It is generally colored in variables shades of grey and brown-violet. *C. reniformis* has been classified as HMA species (Wehrl 2006), ~ 70% of its tissue is occupied by microbes (Ribes et al. 2012). In summer 2011, this specie has been severely affected by a mortality event in North Adriatic Sea, in which its coverage reduced up to 70% (Di Camillo and Cerrano et al. 2015).

***Agelas oroides* (Schmidt 1864)** (Order: Agelasida, Family: Agelasidae) is present only in Mediterranean Sea (World Porifera Database). It is a massive sponge, forming

irregular lobed and stubby colonies. This species is normally vivid orange colored, shading from yellow to orange-brown. *A. oroides* has been classified as HMA (Vacelet and Donadey 1977), ~ 30% of its tissue volume is occupied by microbes (Ribes et al. 2012). This species is of pharmaceutical interest, because produces several bioactive metabolites with antihistaminic proprieties (Fattorusso and Taglialatela-Scafati 2000). *A. oroides* was affected by mortality in summer 2003 along the Provence coast and Gulf of Genoa (Garrabou et al. 2009).

***Petrosia ficiformis* (Poiret 1789)** (Orden: Haplosclerida, Family: Petrosiidae) has been described in Mediterranean Sea, Eastern Atlantic shelf, from Portugal to Cape Verde Island and Azores and Canarias Island (World Porifera Database). It is a stiff species, also known as the stony sponge, because of its high mineral content. It has a massive lobular shape, although it may have several morphotypes as result of abiotic factors (Bavestrello and Sará 1992; Bavestrello et al. 1993). The surface is rough, and the color is usually purple-violet due to symbiosis with cyanobacteria, but it can also be white in absence of light (in the caves). *P. ficiformis* has been classified as HMA (Gloeckner et al. 2014). *P. ficiformis* was affected by mortality in summer 2003 along the Provence coast, Gulf of Genoa, Corsica and Sardinia Islands (Garrabou et al. 2009).

***Crambe crambe* (Schmidt 1862)** (Orden: Poecilosclerida, Family: Crambeidae) is endemic and widespread sponge species of the Mediterranean Sea (World Porifera Database) dwelling the rocky shore from 5 to 35 m depth. It is a red-orange thinly encrusting form and its endoskeleton is composed by spicules. It is a photophilic species, resulting dominant in algae-dominated communities. This species is known to posses a strong bioactivity to produce diverse toxic metabolites that have several ecological rules as antifouling, antipredatory and space competition mechanisms (Becerro et al 1994b; Uriz et al. 1996). *C. crambe* has been classified as LMA (Wherl 2006). This specie was severely affected over the last years by the massive mortality events in summer 1999 and 2003 along the Provence coast, Corsica and Sardinia Islands (Pérez et al. 2000; Garrabou et al. 2009).

Dysidea avara **(Schmidt 1862)** (Orden: Dictyoceratida, Family: Dysideidae) is distributed in Mediterranean and Black Sea (World Porifera Database). It is a rosy to violet massive, conulose soft sponge. It lacks of spicule, and its skeleton is mainly formed by a reticulation of sponging fibers filled with foreign material as sand grains, shell and spicule debris that form a network providing skeletal support (Galera et al. 2000). *D. avara* has been classified as LMA (Wherl 2006), only 6% of its volume is occupied by microbes (Ribes et al. 2012). To date there is not record of mortalities for this species.

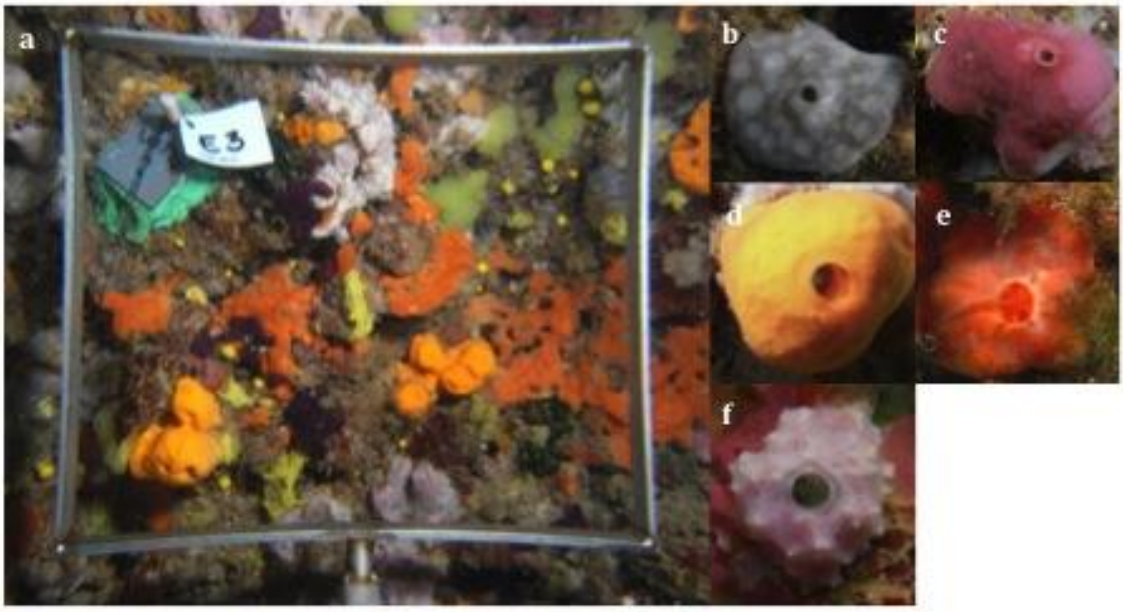


Figure 4. (a) Overview of the sponge species of the Mediterranean coralligenous community at the study site on the Montgrí Catalan coast. The species studied in this study: (b) *Chondrosia reniformis*; (c) *Petrosia ficiformis*; (d) *Agelas oroides*; (e) *Crambe crambe* and (f) *Dysidea avara*.

The thesis is organized in four chapters as follows:

Chapter 1. "VacuSIP", an Improved *InEx* method for *in situ* measurement of particulate and dissolved compounds processed by active suspension feeders.

A key point in experimental biology is the realization of *in situ* devices that allow researchers to test experimental hypotheses without manipulate or disturb the studied organisms. In this chapter we present a device named VacuSIP, which allows sample simultaneously the water inhaled and exhaled by the sponges or other suspension filter feeders *in situ*. This chapter is entirely dedicated to methodology; we present the usage and makeup of the VacuSIP that was used to quantify the ingestion and excretion of particulate and dissolved compounds processed by the studied sponges.

This chapter represents a collaboration with R. Coma, M. Ribes and G. Yahel and it has been accepted for publication in Journal of visualized experiments (Morganti, T., G. Yahel, M. Ribes, R. Coma. (accepted) VacuSIP, an Improved InEx Method for *In Situ* Measurement of Particulate and Dissolved Compounds Processed by Active Suspension Feeders. *J. Vis. Exp.* (), e54221, doi:10.3791/54221 (2016)).

Chapter 2. Sponge feeding: ingestion/excretion of particulate and dissolved compounds processed by the studied species.

High and low microbial abundance (HMA-LMA) species were expected to reflect two different feeding strategies: HMA species may rely on their associated microbes for energy inputs, by feeding on dissolved compounds when particulate fraction is scarce. In contrast, LMA species obtain carbon and nutrients mostly from the particulate fraction. Different patterns (ingestion and/or excretion) of several compounds processed by three Mediterranean sponge species were previously examined in laboratory study with the same technique employed in this work. Unfortunately, the dissolved and particulate compounds available for sponges in laboratory did not reflect the real availability of compounds observed *in situ*. In this context, we aimed to examine whether: i) the rate of uptake and/or excretion of dissolved and particulate carbon and nitrogen differs among the species under natural range of food availability

and whether it could be related to the HMA and LMA dichotomy in the study species; ii) if these rates are related to the availability of compounds.

This chapter represents a collaboration with R. Coma, M. Ribes and G. Yahel and it has been submitted for publication in Limnology and Oceanography Journal (Morganti, T., R. Coma, G. Yahel, M. Ribes. (submitted) Trophic niche separation that facilitates co-existence of high and low microbial abundance sponges is revealed by in situ study of carbon and nitrogen fluxes).

Chapter 3. Seasonality of *in situ* pumping rate

Factors influencing the metabolism are intrinsic (*e.g.*, genetics, age, reproduction) and/or extrinsic (*e.g.*, temperature, food, salinity). Pumping rate has been expected to increase with temperature. However, up to date, only few studies have evaluated the direct effect of temperature on pumping behavior by using indirect technique, and unfortunately only one study examined the natural variation of sponge pump under long measurement *in situ*. A correct quantitative measurements of the volume of water pumped by sponges is a crucial aspect of basic sponge biology for calculation on *in situ* respiration and energy budget, as well as ecologically relevant to estimate the magnitude of sponge-mediated fluxes of energy and nutrients from/to pelagic-benthic realms. In this chapter, we examined the seasonal fluctuation of pumping rate to distinguish to what extend environmental parameters, such as temperature and food availability, regulate the volume of water pumped by the study species *in situ*.

Chapter 4. *In situ* respiration rate and carbon balance over annual cycle.

Energy constrains may arise from a mismatch between energy intake and energy output. In order the test the hypothesis of energy shortage during the warmer periods (*i.e.*, summer months), we examined the energy intake (ingesta) and energy output (respiration expenditure) throughout an annual cycle. Firstly, we investigated the respiration rate *in situ* over annual cycle to assess any seasonal pattern related to the seawater temperature. Secondly, together with the data collected in previous chapters of this thesis, we estimated the carbon balance by comparing the C ingested (availability of food, diet and feeding rate) (chapter 2 and 3) and C consumed by

respiration (chapter 3 and this chapter). We focused on three main goals: (i) to examine the seasonal variation of *in situ* respiration rate; (ii) to asses whether sponges are energetically limited during the warmer period; (iii) to determine to what extend environmental parameters regulate respiration and energetics of study sponges.

Annexe I. Determination of sponge size

The annexe I is a comprehensive study on the determination of the sponge size *in situ* and in laboratory. We provided the sizes of the individual sponge colonies in several ways: area (cm^2) and volume (cm^3) measured *in situ*, and volume (ml), dry mass (g) and ash-free dried weight (g), carbon and nitrogen organic content (g) measured in the laboratory. We provided conversion factors to estimate dry mass, ash-free dried weight, carbon and nitrogen organic content from sponge colony volume estimated *in situ* by photograph. These conversion factors have been used throughout this thesis and may represent a useful tool to estimate the biomass of sponge colonies based on not destructive sampling methods (photo survey) for future studies on the these five species.

Chapter 1

"VacuSIP", an Improved *InEx* method for *in situ* measurement of particulate and dissolved compounds processed by active suspension feeders.

Introduction

Benthic suspension feeders play essential roles in the functioning of marine ecosystems (Gili and Coma 1998). By filtering large volumes of water (Reiswig 1971b; McMurray et al. 2014), they remove and excrete particulate (plankton and detritus) and dissolved compounds (Gili and Coma 1998) (and references therein) and are an important agent of benthic-pelagic coupling (Pile and Young 2006; Nielsen and Maar 2007) and nutrient cycling (Maldonado et al. 2012a; de Goeij et al. 2013). Accurately measuring the particulate and dissolved compounds removed and excreted by benthic suspension feeders (such as sponges, ascidians, polychaetes, and bivalves) is fundamental to understand their physiology, metabolism, and feeding ecology. Together with pumping rate measurements, it also enables a quantification of the nutrient fluxes mediated by these organisms and their ecological impact on water quality as well as on ecosystem scale processes.

Choosing the appropriate method of measuring removal and production rates of particulate and dissolved compounds in suspension filter feeders is crucial for obtaining reliable data concerning their feeding activity (Riisgård 2001). As pointed out by Riisgård and others, inappropriate methodologies bias results, distort experimental conditions, produce incorrect estimations of ingestion and excretion of certain substances, and can lead to erroneous quantification of the nutrient fluxes processed by these organisms.

The two most frequently employed methods to measure particulate and dissolved nutrient fluxes in filter feeders involve either incubation (indirect techniques) or simultaneous collection of ambient and exhaled water (direct techniques). Incubation techniques are based on measuring the rate of change in the concentration of particulate and dissolved nutrients in the incubated water, and estimating rates of production or removal compared to adequate controls (Riisgård

2001). However, enclosing an organism in an incubation chamber can alter its feeding and pumping behavior due to changes in the natural flow regime, due to a decline in oxygen and/or in food concentration, or due to accumulation of excretion compounds in the incubation water (Reiswig 1974; Maldonado et al. 2012a and references therein). In addition to the effects of confinement and modified water supply, a major bias of incubation techniques stems from re-filtration effects (*e.g.*, see Jiménez and Ribes 2007). Although some of these methodological problems have been overcome by using the right volume and shape of the incubation vessel (Diaz and Ward 1997) or with the introduction of a recirculating bell-jar system *in situ* (Ribes et al. 1999a), this technique often underestimates removal and production rates. Quantifying the metabolism of dissolved compounds such as dissolved organic nitrogen (DON) and carbon (DOC) or inorganic nutrients, has proven to be especially prone to biases caused by incubation techniques (Jiménez and Ribes 2007).

In the late 60s and early 70s, Henry Reiswig (1974) pioneered the application of direct techniques to quantify particle removal by giant Caribbean sponges, by separately sampling the water inhaled and exhaled by the organisms *in situ*. Due to the difficulty to apply Reiswig's technique on smaller suspension feeders and in more challenging underwater conditions, the bulk of research in this field was restricted to the laboratory (*in vitro*) employing mostly indirect incubation techniques (Yahel et al. 2005). Yahel and colleagues refitted Reiswig's direct *in situ* technique to work in smaller-scale conditions. Their method, termed InEx (Yahel et al. 2005) , is based on simultaneous underwater sampling of the water inhaled (In) and exhaled (Ex) by undisturbed organisms. The different concentration of a substance (*e.g.*, bacteria) between a pair of samples (InEx) provides a measure of the retention (or production) of that substance by the animal. The InEx technique employs open-ended tubes and relies on the excurrent jet produced by the pumping activity of the studied organism to passively replace the ambient water in the collecting tube. While Yahel and colleagues have successfully applied this technique in the study of over 15 different suspension feeders taxa (*e.g.*, see Genin et al. 2009), the method is constrained by the high level of practice and experience required, by the minuscule size of the some excurrent orifices, and by sea conditions.

To overcome these obstacles, we developed an alternative technique based on controlled suction of the sampled water through minute tubes (external diameter < 1.6 mm). Our goal was to create a simple, reliable, and inexpensive device that would allow clean and controlled in situ water sampling from a very specific point, such as the excurrent orifice of benthic suspension feeders. To be effective, the method has to be non-intrusive so as not to affect the ambient flow regime or modify the behavior of the studied organisms. The device presented here is termed VacuSIP. It is a simplification of the SIP system developed by Yahel et al. (2007) for ROV-based point sampling in the deep sea. The VacuSIP is considerably cheaper than the original SIP and adapted for SCUBA-based work. The system was designed according to principles presented and tested by Wright and Stephens (1978) and Møhlenberg and Riisgård (1978) for laboratory settings.

Although the VacuSIP system was designed for *in situ* studies of the metabolism of benthic suspension feeders, it can also be used for laboratory studies and wherever a controlled and clean, point-source water sample is required. The system is especially useful when integration over prolonged periods (min-hours) or *in situ* filtrations are required. The VacuSIP has been used successfully at the Yahel lab since 2011, and has also been employed in two recent studies of nutrient fluxes mediated by Caribbean and Mediterranean sponge species (Mueller et al. 2014; Morganti et al. submitted).

The use of specific samplers, the prolonged sampling duration, and the field conditions, in which VacuSIP is applied, entail some deviations from standard oceanographic protocols for collecting, filtering, and storing samples for sensitive analytes. To reduce the risk of contamination by the VacuSIP system or the risk of modification of the sampled water by bacterial activity after collection, we tested various *in situ* filtration and storage procedures. Different filtering devices, collection vessels, and storing procedures were examined in order to achieve the most suitable technique for the analysis of dissolved inorganic (PO_4^{3-}, NO_x^-, NH_4^+, SiO_4) and organic (DOC + DON) compounds, and ultra-plankton (<10 µm) and particulate organic (POC + PON) sampling. To further reduce the risk of contamination, especially under field conditions, the number of handling steps was reduced to the bare minimum. The visual format in which the method is presented is oriented to facilitate reproducibility and to reduce the time required to efficiently apply the technique.

System overview

To sample pumped water from suspension feeders with exhalant orifices as small as 2 mm *in situ*, the pumping activity of each specimen is first visualized by releasing filtered fluorescein dyed seawater next to the inhalant orifice(s) and observing its flow from the excurrent aperture (Yahel et al. 2005) (see also Figure 2B in Yahel et al. 2007). The water inhaled and exhaled by the study specimen (incurrent and excurrent) are then simultaneously sampled with the use of a pair of minute tubes installed on custom-built manipulator or on two of the "arms" of an upside-down flexible portable tripod (Figure 1). The water inhaled by the study organism is collected by carefully positioning the proximal end of one tube inside or near the inhalant aperture of the study organism. An identical tube is then positioned inside

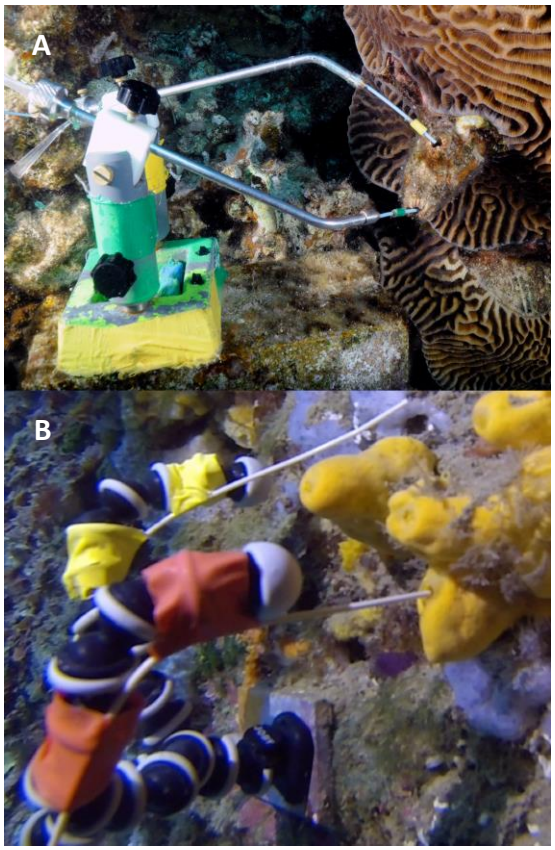


Figure 1. An example of correct installations of the VacuSIP: (a) sampling the ascidian *Polycarpa mytiligera* (Gulf of Aqaba, Red Sea) using a custom-built manipulator with the color code used green for inhaled and yellow for exhaled water samples (photo by Tom Shelizenger and Yuval Yacobi); (b) sampling the sponge *Agelas oroides* (NW Mediterranean Sea) with an osculum width of 3 mm, using the VacuSIP device. The color code used is yellow for inhaled and red for exhaled water samples.

the excurrent orifice. This operation requires good care to avoid contact or disturbance of the animal, *e.g.*, by sediment resuspension. To begin the sampling, a diver pierces a septum in the collecting vessel with a syringe needle attached to the distal end of each tube, allowing the external water pressure to force the sampled water into the vessel through the sampling tube. The suction is initiated by the vacuum previously created in the vials and by the pressure difference between the external water and the evacuated sample container.

To ensure a clean collection of exhaled water and to avoid accidental suction of ambient water (Yahel et al. 2005), the water sampling rate needs to be kept at a significantly lower rate (<10%) than the excurrent flow rate. The suction rate is controlled by the length of the tube and its internal diameter (ID). The small internal diameter also ensures a negligible dead volume (< 50 µL per meter of tubing). Sampling over prolonged periods (minutes to hours) makes it possible to integrate the inherent patchiness of most substances of interest. To ensure that samples are adequately preserved in prolonged underwater sampling sessions as well as for transportation to the lab, an *in situ* filtration is recommended for sensitive analytes. The selection of sampling vessels, filtration assembly, and tubing are dictated by the study organisms and the specific research question. The protocol described below assumes that a full metabolic profile is of interest (for an overview see Figure 2). However, the modular nature of the protocol allows for easy modification to accommodate simpler or even very different sampling schemes. For a full metabolic profile, the sampling protocol should include the flowing steps: (1) Flow visualization; (2) Sampling ultra-plankton feeding (plankton < 10 µm); (3) Sampling inorganic nutrients uptake and excretion (using in-line filters); (4) Sampling dissolved organic uptake and excretion (using in-line filters); (5) Particulate feeding and excretion (using in-line filters); (6) Repeat step 2 (ultra-plankton feeding as quality check); (7) Flow visualization.

When logistically feasible, it is recommended that the metabolic profile measurements are combined with pumping rate (*e.g.,* the dye front speed method, in (Yahel et al. 2005) as well as with respiration measurements. These measurements are best taken at the beginning and end of the sampling session. For respiration measurement, underwater optodes or micro-electrodes are preferable.

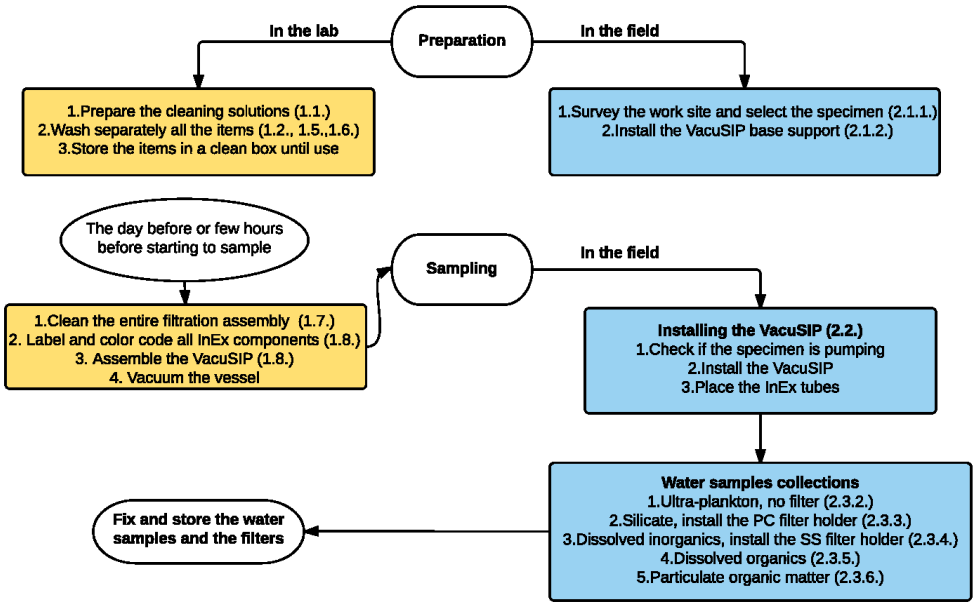


Figure 2. Overview of the VacuSIP technique described in the protocol section. The lab work is represented in yellow boxes, the fieldwork in blue boxes.

Results

Optimization of seawater collection methods

Selection of collector vials and cleaning procedure. VacuSIP-compatible collecting vessels should have a septum that allows sampling to be initiated by piercing with a syringe needle. They should withstand the elevated underwater pressure (2-3 bars at typical scuba working depths), and should hold a vacuum. Many (but not all brands) of vials approved by the EPA for the analysis of volatile organics meet these criteria. Pre-cleaned vials approved for DOC and DON analysis are also available. To test the suitability of these vials for the collection and analysis of nutrients and to optimize cleaning procedures, high quality double distilled water was collected in acid-cleaned polypropylene tubes (PP tubes), newly purchased, in acid-cleaned high-density polyethylene vials (HDPE vials), and in EPA glass vials, all equipped with a polytetrafluoroethylene (PFTE) septum cap. The HDPE vials and polypropylene tubes were cleaned as described in section 1.5.2 above, and the EPA glass vials were cleaned by the manufacturer.

The amount of NH_4^+ found in EPA glass vials was relatively minimal (≤ 0.1 µmol L^{-1}) and depends upon the high quality double distilled water standard quality. In contrast, NH_4^+ concentrations significantly increased (up to 3 and 7 fold, respectively) and exhibited a higher variability in acid-cleaned polypropylene tubes and in high-density polyethylene vials (ANOVA $F_{(5,53)}$=7.183, p<0.001, Figure 3). There was no effect of high quality double distilled water contact with the silicon septum on the ammonium analysis.

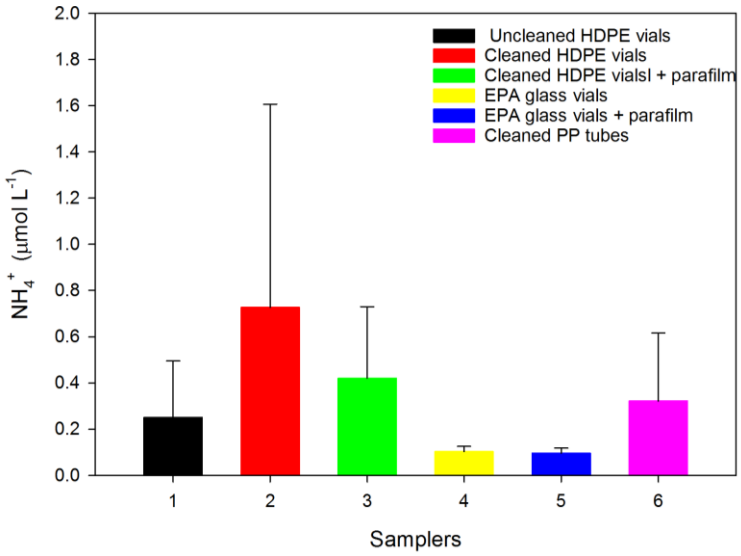


Figure 3. Ammonium concentration (µmol L^{-1}, average ± SD) collected with different vials: (1) Uncleaned HDPE vial; (2) Cleaned HDPE vial; (3) Cleaned HDPE vial + parafilm; (4) EPA glass vial; (5) EPA glass vial + parafilm; (6) Cleaned PP tube. The parafilm was placed to test whether the silicon septum may contaminate the water samples. For each treatment 9 samples of high quality double distilled water were analyzed. The samples were analyzed fresh. Significant differences were found between the four sampling vessels (ANOVA, $F_{(5,53)}$=7.183, p<0.001, power test= 0.992).

Comparison of new glass vials versus cleaned/recycled glass vials. To test whether EPA glass vials could be utilized for nutrient analysis more than once, the NO_x^-, PO_4^{3-}, and NH_4^+ concentrations in seawater samples collected in new EPA glass vials were compared to those collected in used EPA glass vials. The new EPA glass vials were pre-cleaned by the manufacturer, while the recycled glass vials were cleaned as described above (1.7.2). Recycled vials had significantly higher NH_4^+ concentration, up to 1.5 fold the level found in new glass vials (*t* test, p<0.001, n=5).

No significant differences were found in NO_x^- and PO_4^{3-} content between the samples collected in recycled vials and the samples collected in new glass vials (Figure 4).

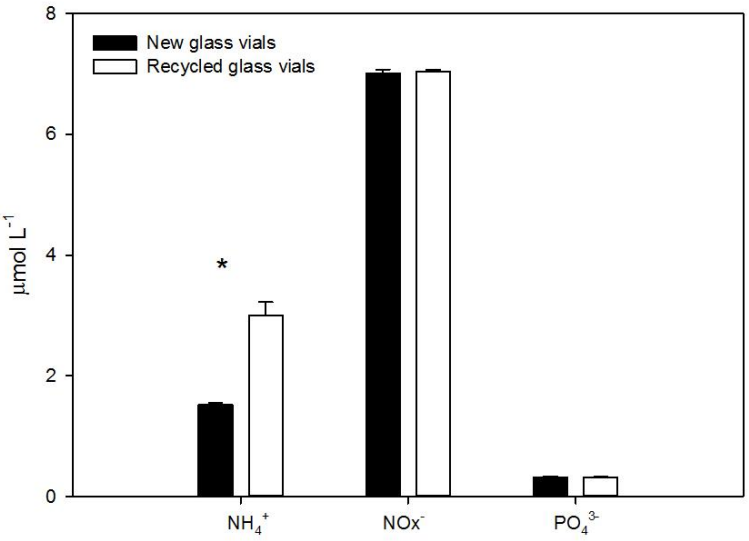


Figure 4. Ammonium (NH_4^+), nitrite + nitrate (NOx^-), and phosphate (PO_4^{3-}) concentrations (µmol L^{-1}, average ± SD) of seawater samples collected in new (dark) and recycled/cleaned (white) EPA glass vials. Seawater was collected at the Experimental Aquaria Zone of the Institute of Marine Science and was filtered with stainless steel filter holder and glass filter. The water samples were analyzed fresh. The asterisk (*) indicates that the difference is significant (*t* test, $p<0.001$, n=5, power test=1).

Silicate collection and storing procedures. To determine the best sampling vessel for the analysis of silicate, high quality double distilled water was collected in non-cleaned and in acid-cleaned polypropylene tubes (PP tubes), in acid-cleaned high-density polyethylene vials (HDPE vials), and in EPA glass vials. The expected silicate concentration was close to zero, so values that deviated from the expected concentration were considered contaminated. The silicate concentration significantly differed between the samples collected in the different vials (ANOVA, $F_{(3,19)}=210.047$, $p<0.001$), showing the lowest SiO_4 concentration in the acid-cleaned HDPE vials. Borosilicate glass vials contaminated the samples, with the final SiO_4 concentration increasing by up to 7 µmol L^{-1} (Figure 5).

Selection of filtration apparatus for dissolved organic matter (DOM) and nutrient analysis. To determine which filter apparatus produces the lowest blank in the analysis of dissolved organic (DOC and DON) and inorganic nutrients (NOx-, NH4+, PO43-), stainless steel filter holders were compared to polycarbonate in-line Swinney filter holders. With each filter holder type we tested both polycarbonate membrane and pre-combusted glass fiber filter. The combination of stainless steel filter holder and combusted glass fiber filter provided the lowest blanks, whereas the polycarbonate Swinney filter holder equipped with polycarbonate membrane clearly contaminated the samples by up to 9 fold. Increasing the wash volumes did not resolve this problem (Figure 6).

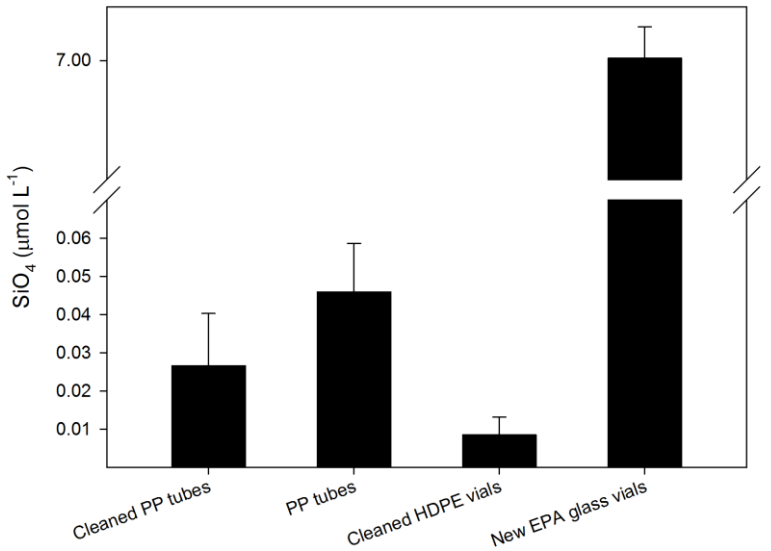


Figure 5. Silicate concentration (µmol L^{-1}, average ± SD) in high quality double distilled water collected in different vials: acid-cleaned PP tubes, PP tubes, acid-cleaned HDPE vials, new EPA glass vials. Significant differences were found between the four sampler materials (ANOVA, $F_{(3,19)}$=210.047, p<0.001, power test=1).

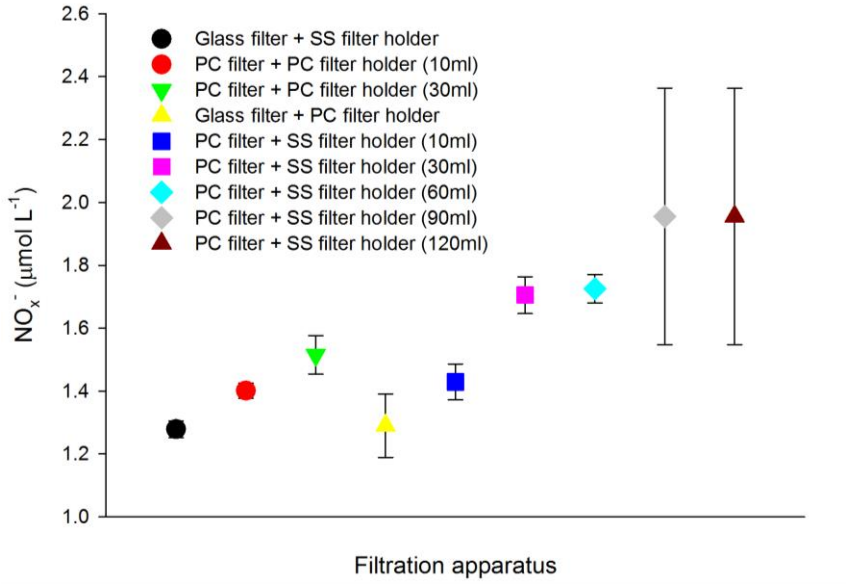


Figure 6. Examining the effect of different filtration assemblies and wash volumes on nitrite + nitrate (NO_x^- µmol L^{-1}) as an example. NO_x^- obtained by filtering the seawater samples with stainless steel (SS filter holder) or polycarbonate in-line Swinney filter holders (PC filter holder) equipped with either a polycarbonate membrane (PC filter) or a pre-combusted glass fiber filters. For the PC filters, different volumes (10, 30, 60, 90 and 120 mL) of 5% HCl and high quality double distilled water were used for washing the filter assembly. Values are expressed as mean ± standard deviation (n=5). Seawater was collected at the Experimental Aquaria Zone of the Institute of Marine Science and the samples were analyzed fresh after the filtration.

Discussion

Preparatory steps

Collector vials for DOM and nutrient analysis. Since collector vessels may interact with dissolved micro-constituents and the sampler walls may be a substrate for bacteria growth (Degobbis 1973; Tupas et al. 1994; Zhang et al. 1999; Yoro et al. 1999; Yoshimura 2013), different vials for DOM and nutrient collection were tested. Borosilicate is not recommended for silica quantification (Zhang et al. 1999; Strickland and Parsons 1968), since glass bottles can increase the initial concentration of silica by up to two fold if the samples are not quickly frozen (Degobbis 1973). The current results demonstrate that in addition to DOM collection, using EPA vials also results in low concentration (*i.e.,* non-detectable) blanks for inorganic nutrients, most notably for ammonium.

DOC filtration and storage. Filtration is a required and, in many cases, is the first analytical step in marine chemistry and microbiology. While it is possible to filter the samples after collection in the lab, this procedure is not recommended for *in situ* work, where samples are collected underwater, often in remote locations, hours or days away from proper laboratory facilities. The use of in-line, *in situ* filtration minimizes sample handling and thus reduces the risk of contamination. *In situ* filtration also removes most of the bacteria and reduces the risk that the sample composition will be altered by bacterial metabolism during the prolonged sampling and transport time. The filtration assembly increases the dead volume of the sampling apparatus and may also be a source of contamination. A selection of the smallest possible filter holders (*e.g.*, in-line Swinney filter holder 13 mm) and minute PEEK tubing (*e.g.,* 254 µm ID, <0.05 mL) reduces the dead volume while decreasing the risk of contamination by ambient water.

If the proper filter is not used or if it is not washed carefully, artifacts and contamination of the water samples are likely (Eaton and Grant 1979; Norrman 1993; Carlson and Ducklow 1996; Yoro et al. 1999). Studies on DOC analysis showed that filters and filter holders made of organic compounds (polycarbonate and PFA-PTFE) may result in severe DOC contamination(Norrman 1993; Yoro et al. 1999), especially when not thoroughly flushed with high quality double distilled water (Carlson and

Ducklow 1996). The present protocol and results follow these guidelines and also indicate that polycarbonate filter holders should be avoided.

In situ work and interoperation

The VacuSIP system is a direct sampling technique that facilitates the study of the metabolism of undisturbed suspension feeders in their natural environment and the quantification of their ecological role in the system. For experienced and equipped divers, the application of the VacuSIP method is simple and requires only short training. InEx VacuSIP experiments are designed for a 'within'-design statistical analysis (*i.e.*, paired or repeated measure analysis), therefore controlling for most analytical artifacts including high blanks. The use of controlled suction ensures slow and adjustable sampling rates, thus preventing accidental contamination of exhaled water with ambient water. Where possible, the selection of work sites with low current and low turbidity is recommended and will ensure cleaner and more accurate results. The prolonged sampling time (minutes to hours) allows integration of the high patchiness that characterizes the benthic boundary layer. All these features ensure that when properly applied the VacuSIP method is highly robust, providing reliable and replicable results even when working with a small number of replicates. An example of typical results obtained from a Mediterranean sponge and Indo-Pacific clam species is shown in Figure 7 and Supplementary Figure 1.

As with any technique, VacuSIP is not free of potential pitfalls. The most common problem is contamination of the exhaled water sample with ambient water. Reasons for these artifacts include high suction rate, tube dislodgments, and animal behavior. Proper selection of the correct sampling rate is dependent on prior estimates of the excurrent flow rate. Such estimates can be obtained by using the dye front speed method (Yahel et al. 2005). Ideally, the suction rate should be kept below 1% of the pumping rate (*e.g.,* 1 mL min^{-1} for a 6 L $hour^{-1}$ pumping rate). To avoid contamination with ambient water, sampling rate should never be greater than 10% of the pumping rate.

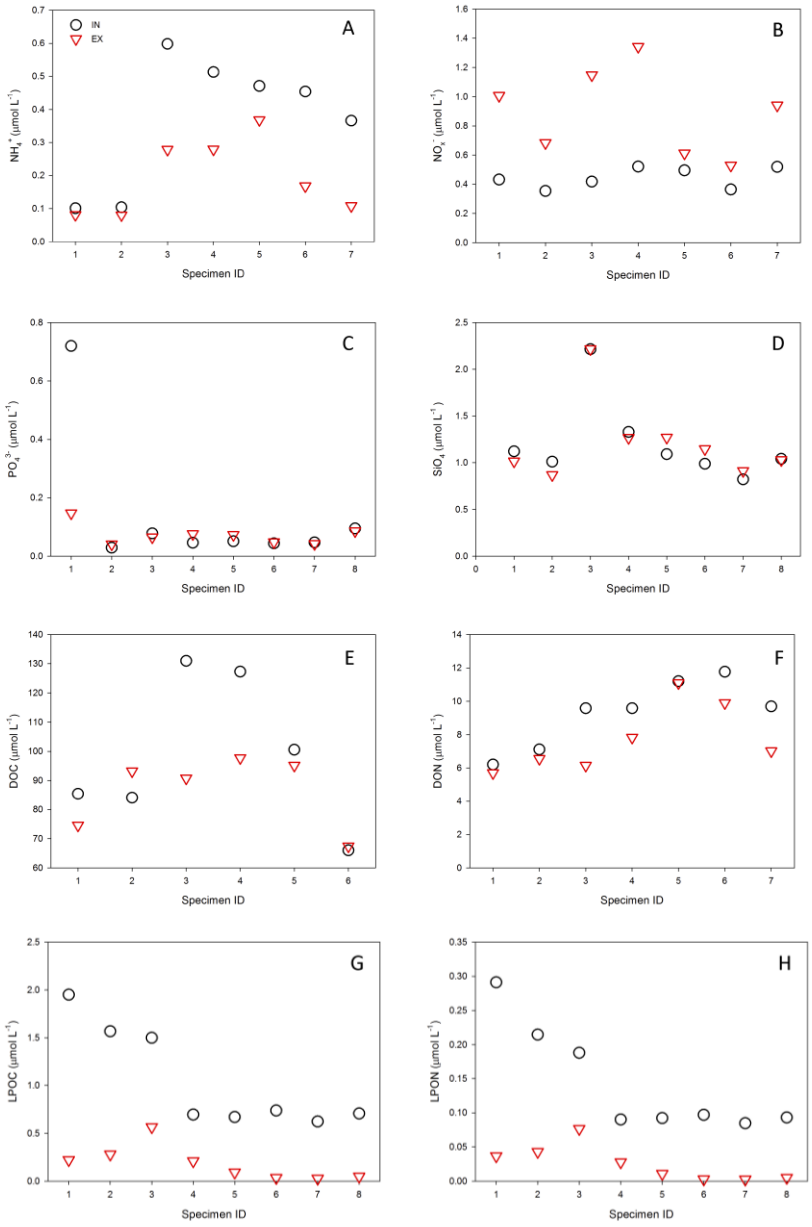


Figure 7. Example of experimental results: inhaled (IN, dark circle) and exhaled (EX, red triangle) paired water sample concentrations (µmol L^{-1}) of different substances processed by the sponge *Chondrosia reniformis* in the Mediterranean Sea: (A) ammonium (NH_4^+); (B) nitrite + nitrate (NOx^-); (C) phosphate (PO_4^{3-}); (D) Silicate (SiO_4); (E) dissolved organic carbon (DOC); (F) dissolved organic nitrogen (DON); (G) planktonic organic carbon (LPOC); (H) planktonic organic nitrogen (LPON).

To control for the sampling rate, the length and internal diameter of the intake tubing should be adjusted according to the planned work depth and water temperature. The Hagen–Poiseuille equation (see section 1.3.1 above) may be used as a guide. However, this equation should be considered as a first order approximation since ΔP and sampling rate decrease with sampling time and in-line filtration adds uncertainties. The use of evacuated containers, sometimes with unknown vacuum pressures, introduces further complications. An example of how sampling rates varies as a function of different evacuated containers with different vacuum, is shown in Table S1.

Reducing the sampling rate is easily achieved by adjusting the tube length and ID, with no technical limitations to this reduction (sampling rates of few microliters per hour are feasible). Nevertheless, experimenters should be aware of the slow sampling rate dictated by this limitation for slow pumpers and for small organisms or specimens. The immediate implication of slow rate is the limited volume of water that can be collected during a single sampling session. This low volume will limit the number of analyses and replicates that can be run with these samples, and will thus also limit the information that can be obtained from these populations.

Tube dislodgement can be easily spotted and sampling can be aborted or restarted, provided that a diver is keeping constant watch. In contrast, cessation of pumping during sampling is not always easy to detect. This is true not only for sponges, but also for tunicates, bivalves, and polychaetes. In fact, contrary to common belief, events in which an ascidian or a bivalve stopped pumping were documented with no visible change in the siphon geometry (Yahel, unpublished data). Moreover, in some cases, tunicates can maintain active pumping with no mesh secretion (that is, no filtration is taking place).

Controlling the sampling rate is critical. In this respect the VacuSIP is better than other methods, especially when the study animals are relatively small or when they pump slowly. Syringes are particularly difficult to control (Reiswig 1971a). For instance, Perea-Blázquez and colleagues (2012a) used a syringe to sample the water exhaled by several temperate sponge species and surprisingly did not find a general pattern of ingestion/excretion of particular nutrients (NO_2^-, NO_3^-, NH_4^+, PO_4^{3-}, SiO_4). The lack of a clear pattern is likely a result of contamination of the exhaled samples

with ambient water due to syringe use. This possibility of contamination is evident from the extremely low retention efficiency of pico plankton reported by Perea-Blázquez and colleagues (2012b) for their sponges: 40 ± 14% of heterotrophic bacteria and 54 ± 18% of *Synechococcus sp*. For comparison, using the VacuSIP, Mueller *et al*. (2014) reported a removal efficiency of heterotrophic bacteria of 72 ± 15% in *Syphonodistyon sp.* and 87 ± 10% in *Cliona delitrix*.

To verify sample quality and ensure that no contamination of the ambient water occurs, we strongly recommend to first analyze the pico and nano plankton samples using flow cytometry. This fast, reliable, and cheap analysis will provide immediate information of sample quality. It is very common for some prey taxa such as *Synechococcus sp*. to be removed at close to 90% efficiency (Reiswig 1971a; Pile et al. 1996) by sponges and ascidian. Significant deviations from this benchmark suggest that contamination might have occurred (Figure 8).

For reliable and clean sampling, make sure the experiment design satisfies seven simple rules: (1) perform a preliminary survey (including pumping rate estimates) and prepare the worksite well; (2) know the studied animals; (3) verify that the studied specimen has a well-defined excurrent aperture and accessible location; (4) verify that the studied specimen is pumping before and after each sample collection; (5) place the tube for the collection of the exhaled water slightly inside the excurrent aperture (Figure 1); (6) use sampling rate < 10% of the excurrent flow rate, 1% is highly recommended; (7) define a quality criterion and omit suspected InEx pairs.

Following these simple rules, the VacuSIP system offers a practical and reliable method of measuring how active suspension feeders process particulate and dissolved compounds in natural conditions, allowing accurate and comparable estimates that can be used to assess the functional role of filter feeders in different ecosystems around the world.

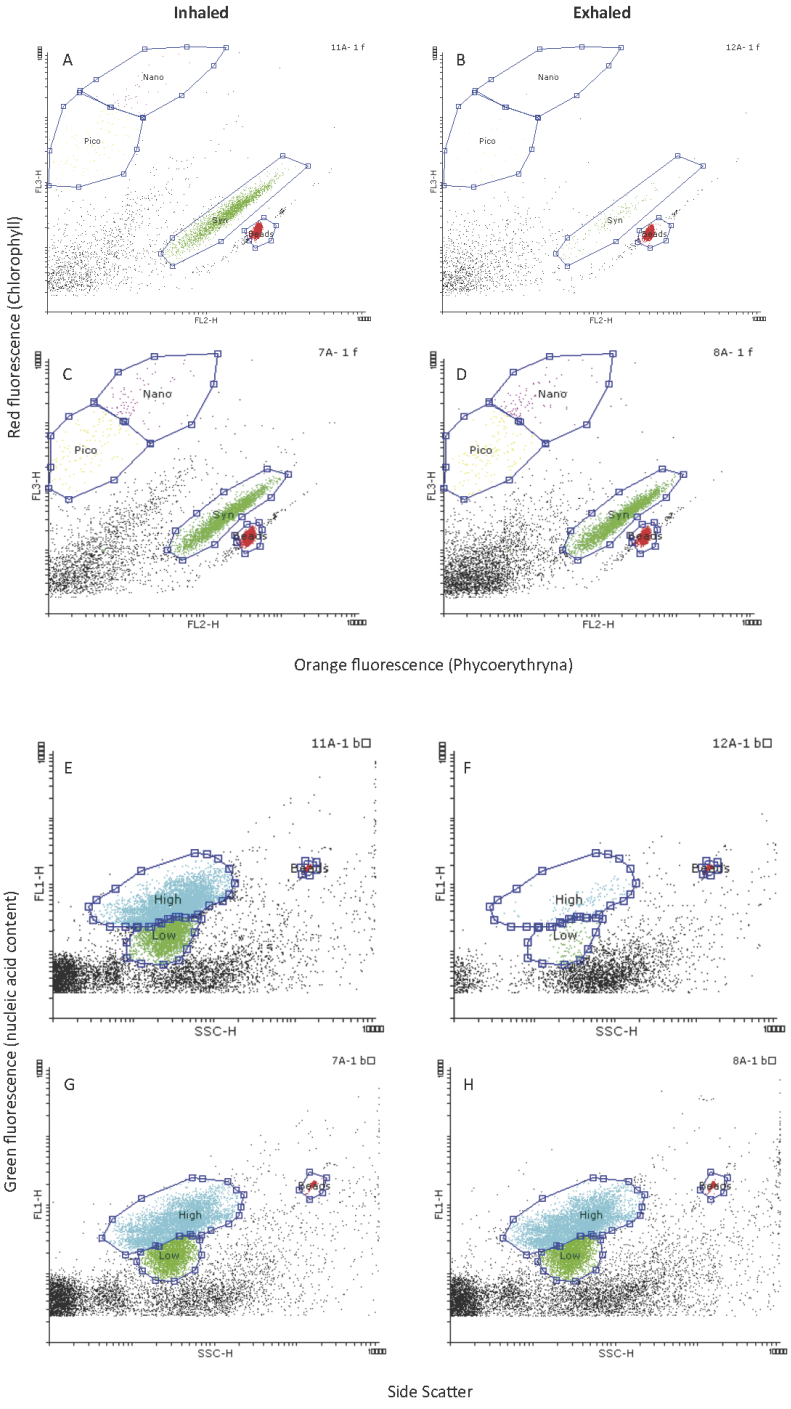


Figure 8. An example of a flow cytometry analysis of paired water samples drawn from the water inhaled (A,C,E,G) and exhaled (B,D,F,H) by the sponge *Chondrosia reniformis*: (A,B,C,D) phytoplankton populations; (E,F,G,H) heterotrophic bacteria. In A-B and E-F the sampling was clean and accurate (all planktonic groups were efficiently retained). C-D and G-H are examples of exhaled water contamination, showing low removal of all planktonic groups. Syn: *Synechococcus sp.*, pico: autotrophic picoeukariotes, nano: autotrophic nanoeukaryotes, high: heterotrophic bacteria with high DNA content, low: heterotrophic bacteria with low DNA content.

Supplementary material

PROTOCOL:

1.1. Cleaning solution

1.1.1. Wear protective gear, a lab coat, and gloves at all times. Carry out these preparatory steps in a clean space free of dust and smoke.

1.1.2. Prepare a 5-10% hydrochloric acid (HCl) solution with fresh, high quality, double distilled water (add 270 mL of 37% HCl to 2 L water to make 5% HCl final concentration).

1.1.3. Prepare a 5% highly soluble basic mix of anionic and non-ionic surfactant solution (See Materials Table) with fresh, high quality, double distilled water.

1.1.4. Store all solutions in clean, acid washed containers.

1.2. Preparatory steps and cleaning procedures (in the lab)

NOTE: If phosphorus compounds are not of interest, the HCl wash can be replaced by high quality phosphoric acid (H_3PO_4) wash (8% H_3PO_4 final concentration).

1.2.1. Wear protective gear, a lab coat, and gloves at all times.

1.2.2. Wash the sampling apparatus (excluding the in-line stainless steel Swinney filter holder) with ample amount of high purity water. Leave the apparatus soaking in 5-10% HCl solution overnight. Rinse the apparatus again with ample high quality double distilled water.

1.2.3. Wash the in-line stainless steel Swinney filter holders with ample amount of high purity water. Leave the filter holders soaking in 5% highly soluble basic mix of anionic and nonionic surfactants solution overnight. Rinse them again with ample high quality double distilled water.

1.2.4. Leave all the sampling apparatus dry and wrap them in aluminum foil in a clean box until use.

1.3. Suction rate control

1.3.1. Control the sampling rate, adjusting the length and internal diameter of the intake tubing according to the planned work depth and water temperature. Use the following equation (derived from the Hagen–Poiseuille equation used for fully

developed laminar pipe flow) as a guide: $F = \frac{\Delta P \cdot \pi \cdot r^4}{8 \cdot K \cdot L \cdot V}$ where F= flow rate ($cm^3\ min^{-1}$), ΔP= differential pressure (bar), r= inlet tubing internal radius (cm), K= 2.417 x 10^{-9} (s^{-2}), L= tube length (cm), V= water viscosity ($g\ cm^{-1}\ s^{-1}$). See Table S1 for more details.

1.3.2. Keep sampling rate below 1% of the pumping rate of the studied animal.

NOTE: Using evacuated containers, sometimes with unknown vacuum poses additional complications. Therefore, a field test is highly recommended. At 10 m depth and ~22°C seawater (40 PSU), a 50 cm inlet tubing with an internal diameter of 250 µm delivers an average suction rate of ~26 $\mu L\ s^{-1}$ (1.56 $mL\ min^{-1}$).

Table S1. The overall average sampling rates (mL min^{-1}) obtained with different containers used for water collections and different vacuum levels: the flasks were not vacuumed (none); EPA glass vials and HDPE vials were vacuumed half of their volume (½ volume); sterile plastic tubes were already vacuumed by the manufacturer. Working at 5-8 m depth, water temperature of 18-22 °C, using PEEK tubes of 79 cm length and of 250 µm internal diameter.

Container	Flask Volume	Vacuum	Volume collected (mL)	Sampling rate (mL min^{-1})
Glass flask	250 ml	None	64.38 ± 33.06	1.13± 0.61
EPA glass vial	40 ml	1/2 volume	17.95 ± 2.81	0.59 ± 0.35
HDPE vial	40 ml	1/2 volume	7.73 ± 3.16	0.46 ± 0.27
Vacuumed sterile plastic tubes	9 ml	from manufacturer	8.78 ± 0.65	0.61 ± 0.32

1.4. Sampling vessels

1.4.1. For small volume samples (3-20 mL, *e.g.,* ultra-plankton for flow cytometry) use pre-vacuumed sterile plastic tubes.

NOTE: Pre-vacuumed sterile plastic tubes are routinely employed for standard blood tests in humans; make sure to use the sterile tubes with no additives. These vacuumed sterile plastic tubes are best sampled with the use of sterile, single-use tube holder with off-center luer. While this is the safest and most efficient sampling apparatus, it has a slightly larger dead volume compared to a simple needle.

1.4.2. For larger water samples such as nutrients and dissolved organics, use 40 or 60 mL glass vials that meet the Environmental Protection Agency (EPA) criteria for volatile organic analyses. These vials include a polypropylene cap with a PTFE-faced silicone septum.

1.4.3. For even larger volumes, use penicillin bottles with rubber stoppers or vacuum flasks.

1.4.4. Use high-density polyethylene vials (HDPE vials) for silica samples.

1.4.5. To increase sample volume and to reduce the risk of the stopper dislodging during the ascent, evacuate (vacuum) items 1.4.2-1.4.4 before the dive with a vacuum pump. Vacuum manually by using a hand vacuum pump or even by sucking the air with a syringe. However, for best results, a good vacuum pump is recommended. Standard lyophilizers provides high vacuum.

NOTE: Pay special attention when using large vacuumed flasks to ensure that the faster initial suction rate would not contaminate the exhaled water samples.

1.5. Vessel cleaning procedures

1.5.1. For dissolved organics and NH_4^+ analysis, use new pre-cleaned EPA vials.

1.5.2. Rinse the vials (glass and HDPE) for analysis of other nutrients as follows:

1.5.2.1. Rinse the vials (glass and HDPE) and the polypropylene caps with high quality double distilled water. Install a new silicon septum.

1.5.2.2. Soak the vials (glass and HDPE) in 10% of HCl for at least 3 days and rinse with ample high quality double distilled water.

1.5.2.3. Combust the glass vials at 450 °C for 4 hr and allow to cool in the furnace. Install the cap, and wrap in aluminum foil until use.

1.6. Filters

1.6.1. Use binder-free glass fiber filters for filtration of all dissolved organic samples (*e.g.*, DOC, DON) and for the collection of particulate organics (*e.g.*, POC, PON). Pack each glass filter in a separate aluminum foil envelope. Combust at 400 °C for 2 h to volatilize organic residues and store in a clean and dry vessel until use.

1.6.2. Use either a binder-free glass fiber filters as above, or 0.2 µm polycarbonate membranes for sampling inorganic nutrients (*e.g.,* PO_4^{3-}, NO_x^-, NH_4^+). Clean the latter once installed in the filter holder as explained below (1.7.3).

1.6.3. Use 0.2 µm polycarbonate membranes filters for silica sampling. Clean them once installed in the filter holder as explained below (1.7.3).

1.7. Preparation of filtration assembly

1.7.1. Filter the highly soluble basic mix of anionic and non-ionic surfactant solution and the high quality double distilled water through a 0.2 µm filter before using them to clean the filtration assembly.

1.7.2. Filtration assembly for nutrients and dissolved organics other than silica:

1.7.2.1. Place a combusted binder-free glass fiber filters inside the cleaned in-line stainless steel Swinney filter holder.

1.7.2.2. Use an acid-cleaned syringe to run 100 mL of 5% highly soluble basic mix of anionic and non-ionic surfactant solution and then 100 mL of high quality double distilled water through the entire assembly.

1.7.3. Filtration assembly for SiO_4:

1.7.3.1. Place the polycarbonate filter inside the cleaned polycarbonate filter holder (PC filter holder).

1.7.3.2. Use an acid-cleaned syringe to run 30 mL of 5% HCl and 30 mL of high quality double distilled water through the entire assembly.

1.8. System assembly

1.8.1. Assemble the system for underwater work using PEEK (Polyether Ether Ketone) tubing with an external diameter (OD) of 1.6 mm and an internal diameter (ID) of 254 µm or 177 µm.

1.8.2. Use a sharp knife or PEEK cutter to cut the tubes to the required length.

1.8.3. At its distal end (sample container side), fit each tube with a male luer connector attached to a syringe needle. Make sure you follow the manufacturer's instruction and align the flat side of blue flangeless ferrule with the end of the tube before tightening the green nut.

1.8.4. Attach the PEEK tubing to tripod “arms” or custom-built manipulator by using an insulating tape.

1.8.5. Attach a disposable syringe needle to the male luer connector. Keep the needle with its protective cap to prevent injuries.

1.8.6. Clearly label the sampling gear and color code all inhaled and exhaled components (*e.g.*, green=In, red=Ex).

1.8.7. Similarly color code the sampling vessels with sets of paired sampling vessels sequentially numbered.

2. Working underwater

2.1. *Work site preparation*

2.1.1. Preliminary survey and selection of specimens

NOTE: Due to the complex nature of the underwater sampling protocol, devoting the necessary time for preparation will ensure an efficient sampling dive.

2.1.1.1. Survey the work site and make necessary preparations ahead of time.

2.1.1.2. Select and mark suitable target organisms that can be accessed relatively easily. Since not all organisms may necessarily be active at the time of the sampling dive, prepare more workstations than you expect to sample.

2.1.2. Installation of base supports

2.1.2.1. When working on leveled substrate:

2.1.2.1.2. Mount the flexible portable tripod original quick release clips on 1 kg diving weights and simply position it next to the target animal.

2.1.2.2. When working on vertical walls:

2.1.2.2.1. Mount base support plates for the VacuSIP system, hooks for accessory gear, and hangers for the collecting vessel carrying tray during the work site preparation stage (2.1.1).

2.1.2.2.2. When flexible portable tripods are used, use bolts or two-component epoxy resin to fix 10x10 cm PVC plates next to each target animal. Each plate needs to have a hole to attach the flexible portable tripod quick release clips.

2.1.2.2.3. Once the resin has cured and the base plates are solidly attached to the wall, screw in the quick release clips, serving as a firm attachment point for the flexible portable tripod VacuSIP system.

2.2. *Installing the VacuSIP*

2.2.1. Check whether the specimen is pumping by releasing filtered fluorescein dye next to the inhalant orifice and confirm that the dye is emerging through the exhalent orifice as described in Yahel et al. 2005.

2.2.2. Install the VacuSIP device and place the inhalant (IN) sampling tube within the inhalant orifice or just next to it (within~ 5 mm). Make sure that the inhalant tube is not in the proximity of another exhalant orifice.

2.2.3. Carefully direct the exhalant (EX) sampling tube toward the osculum/exhalant siphon and very gently insert it in, until it is positioned 1-5 millimeters inside the osculum/exhalant siphon (see Figure S1). Take great care not to make contact with or otherwise disturb the sampled organism.

2.2.4. Before and during the sampling double-check the location of both tubes.

2.2.5. After the sampling check whether the specimen is still pumping as described above (2.2.1).

NOTE: Because the movement of one arm of the tripod when manipulating the other might occur, make sure to firstly place the inhalant sampling tube and secondly the exhalant tube, which requires more precise manipulation. Following this order, even if the manipulation of the exhalant sampling tube might cause the movement of the inhalant tube, it will not affect the sampling.

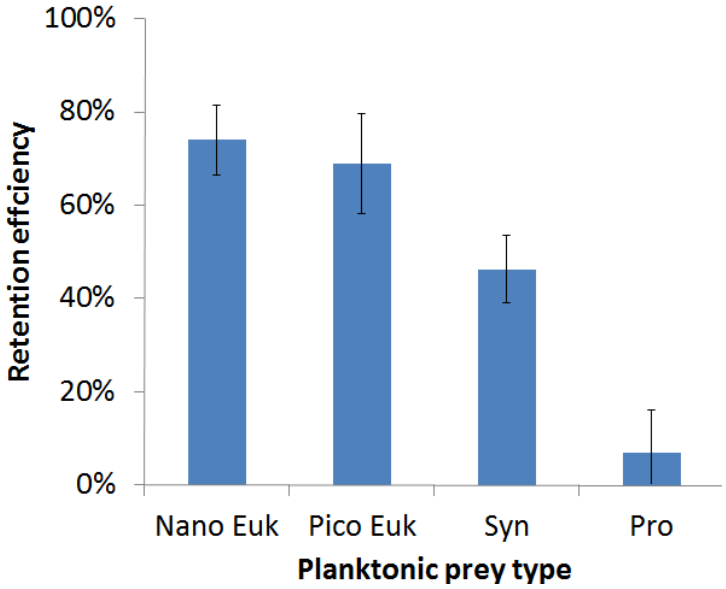


Figure S1. Cell retention efficiency of different planktonic prey by the clam *Chama pacifica*: *Prochlorococcus* sp. (Pro), *Synechococcus* sp. (Syn), pico-eukaryotes (Pico Euk), nano-eukaryotes (Nano Euk). Error bars= 95% CI.

2.3. *Modular underwater sampling procedure*

NOTE: Pending on the research question, each of the experimental steps below can be performed as a stand-alone experiment. The full metabolic profile sampling protocol described below is a lengthy process, requiring up to 8 hours per specimen (for an overview see Figure 2 and Table S2). As diving conditions and regulations differ between sampling sites, regions, and institutions, diving plans are not included in this protocol. Nevertheless, devote extreme care and meticulous planning to the diving plan. Pay special care to avoid saturation and yoyo dive profiles. When possible, it is advisable to conduct these experiments at shallow

depth (< 10 m). Closed circuit rebreathers can be very handy for such prolonged sampling schemes.

2.3.1. Before actual sampling begins, make sure no visible traces of fluorescein residue remain and that suspended sediments have been settled or have wafted away.

Table S2. Overview of the sampling vessel, fixative, in-line filter assembly, storage and analytical methods described in the protocol section. The analyzed compounds are: ultra-plankton abundance (plankton < 10 µm), silicate (SiO_4), phosphate (PO_4^{3-}), nitrite + nitrate (NO_2^-+ NO_3^-), dissolved organic matter (DOM), ammonium (NH_4^+) and particulate organic matter (POM). The vessels used are: pre-vacuum sterile plastic tubes, high-density polyethylene vials (HDPE vials), glass vials that meet EPA criteria for volatile organic analyses either new or re-used after the appropriate cleaning (EPA glass vials) and penicillin bottles (vacuum flasks). All the sampling vessels have silicon septum cap and are vacuumed before sampling. The fixatives are: paraformaldehyde + glutaraldehyde (Glut + Parafor), orthophosphoric acid (H_3PO_4) and hydrochloric acid (HCl). The in-line filter assemblies used are: polycarbonate filter holders and polycarbonate membrane 0.2 µm filters (PC filter holder + PC membrane) and stainless steel filter holders and binder-free glass fiber 0.7 µm filters.
1, Gasol and Moran 1999a; 2, Korolef 1972; 3, Murphy and Riley 1962; 4, Shin 1941; 5, Wood et al. 1967; 6, Sharp 1997, Sharp et al. 2002; 7, Homes et al. 1999.

Compound	Sampling vessel	Fixative	In-line filter assembly	Storage	Analytical method	Protocol
Ultra plankton	Pre-vacuum sterile plastic tube	Glut + Parafor	None	Liquid N (-80 °C)	Flow cytometry [1]	2.3.2
SiO_4	HDPE vials		PC filter holder + PC membrane	- 20°C	Molividate colorimetry [2]	2.3.3
PO_4^{3-}, NO_2^-, NO_3^-	New/recycled EPA glass vials		Stainless steel + 13 mm glass fibers filter	- 20°C	Molividate colorimetry [3] Colorimetry [4] Cadmium redaction [5]	2.3.4
DOM	New EPA glass vials	H_3PO_4 HCl		4°C or -20°C	High temperature combustion [6]	2.3.5
NH_4^+	New EPA glass vials			- 20°C	Fluorometric nanomolar technic [7]	2.3.4
POM	Vacuum flask			-20°C	CHN analyzer	2.3.6

2.3.2. Ultra-plankton, (no filter is installed in this step!)

2.3.2.1. Use the needle to pierce the IN (inhaled) and EX (exhaled) vacuumed sterile plastic tubes septa. Verify that water is dripping in at the planned rate and collect 2-6 mL water samples.

2.3.2.2. On retrieval, keep samples in a cold box on ice. In the lab, preserve with 1% paraformaldehyde + 0.05% EM grade glutaraldehyde (final concentration), or 0.2% EM grade glutaraldehyde. Freeze them in liquid N and store at -80 °C until analysis.

2.3.3. Silicate sampling and storing

2.3.3.1. Install the pre-cleaned in-line stainless PC filter holder containing a 0.2 μm polycarbonate membrane between the needle and the luer male connector at the distal end of the tube.

2.3.3.2. Pierce the septum cap of the pre-cleaned high-density polyethylene vials (HDPE vials) to start sampling. Verify that both samplers are dripping and collect 15 mL of water in each vial.

2.3.3.3. Keep the samples refrigerated (4 °C) until analysis. If analysis cannot begin within two weeks, store at -20 °C. For analysis, make sure that the samples are thawed at 50 °C for at least 50 min to dissolve silica gels.

NOTE: The membrane can be preserved for microscopy or DNA analysis as needed.

2.3.4. Dissolved inorganics (PO_4^{3-}, NO_x^-, NH_4^+)

2.3.4.1. Before sampling, ensure that at least 20 mL of seawater samples were passed through the entire filtration system by using EPA, HDPE vials or other vacuum vessel to start the suction. Measure the volume of the collected water before they are discarded.

2.3.4.2. Replace the PC filter assembly with a pre-cleaned in-line stainless steel filter holder that contains a pre-combusted glass filter.

2.3.4.3. Pierce the septum cap of the appropriate EPA glass vials to start sampling, verify that both samplers are dripping, and collect 25-30 mL for nitrate and phosphate analysis.

2.3.4.4. Switch to new EPA glass vials for ammonia analysis, verify that both samplers are dripping, and collect 20 mL in each vial.

2.3.4.5. Keep the samples in a cold box on ice and store at -20 °C until the analysis.

NOTE: If only the filtrate is of interest, disposable syringe filters can be used for steps 2.3.3 and 2.3.4.

2.3.5. Dissolved organics (DON+DON) sampling and storing

NOTE: Keep the samples as upright as possible throughout handling so that sample water does not come in contact with the silicon septa.

2.3.5.1. Continue using the stainless steel filter assembly and collect 20 mL of seawater samples into new EPA glass vials, as described above.

2.3.5.2. Upon retrieval, keep the samples in a cold box on ice. In the lab, use a pre-combusted glass Pasteur pipette to fix the samples with orthophosphoric acid (add 5-6 drops of 25% trace metal grade acid into a 20 mL sample, final concentration 0.04%) or hydrochloric acid (add 2 drops of trace metal grade concentrated acid into a 20 mL sample, final concentration 0.1%) and keep refrigerated.

2.3.5.3. Keep the samples refrigerated (4 °C) until analysis. If samples are not analyzed within a week of collection, store at -20 °C until the analysis.

2.3.6. Particulate organic matter (POC, PON, POP)

2.3.6.1. Continue using the stainless steel filter assembly and filter at least 500 mL of seawater into an evacuated 250 mL vacuum flask. Replace flasks if necessary.

2.3.6.2. On retrieval, use an air-filled syringe to evacuate all remaining seawater from the filter holder, wrap it in aluminum foil, and store in a cold box on ice. In the lab, remove the filters from the filter holders, freeze them in liquid N and store at -20 °C until the analysis.

Chapter 2

Sponge feeding: ingestion/excretion of particulate and dissolved compounds processed by the studied species.

Introduction

Multispecific assemblages of suspension feeders such as sponges, ascidians, polychaetes, and bivalves are ubiquitous throughout most oceanic regions (Gili and Coma 1998; Hooper et al. 2002; Gili et al. 2006). Their feeding activity can control plankton distribution, generating prey-depleted boundary layers above the bottom (Kimmerer et al. 1994; Yahel et al. 1998; Nielsen and Maar 2007). A scarce prey availability might trigger inter and intra specific competition (Buss and Jackson 1981; Best and Thorpe 1986). However, filter feeders employ different foraging strategies and rely on different preys according to particle size, morphological structure and water movements (Riisgård and Larsen 1995; Coma et al. 2001), facilitating their coexistence in such dense communities.

Sponges host abundant, diverse and specific microbial communities. They are generally divided into two large groups: high microbial abundance species (HMA), defined as sponges containing 10^8 to 10^9 bacteria per gram of sponge tissue and low microbial abundance species (LMA), defined as sponges containing 10^5 to 10^6 bacteria per gram of sponge tissue, that is similar to the microbial abundance in seawater (Vacelet and Donadey 1977; Hentschel et al. 2012; Gloeckner et al. 2014). LMA species may have similar microbial richness to HMA sponges, but the diversity of the microbial community is lower due to the small number of bacteria species that account for the bulk of the population (Erwin et al. 2011; Giles et al. 2012; Poppell et al. 2013). Although LMA microbial composition usually appears to be closer to seawater bacteria composition (Giles et al. 2012; Hentschel 2003; Hentschel et al. 2006; Björk et al. 2013), sponge-specific bacteria have recently been found in the mesohyl of some LMA, just as in HMA species (Gerçe et al. 2011; Blanquer et al. 2013; Moitinho-Silva et al. 2014; Erwin et al. 2015). Vertical transmission appears to be the

main mechanism to maintain the specific lineages of symbionts of the host (Schmitt et al. 2008; Webster et al. 2010; Hentschel et al. 2012).

Besides microbial abundance and diversity, HMA and LMA sponges also differ in their morphology and physiology. HMA species have a denser mesohyl, longer and narrower canals (Weisz et al. 2008) and a lower density of choanocyte chambers than LMA (Poppell et al. 2013). This leads HMA species to exhibit a lower specific pumping rate than LMA (Weisz et al. 2008). These morphological characteristics reflect two different feeding strategies. HMA species may rely on their associated microbes for energy inputs (Weisz et al. 2007; Freeman and Thacker 2011), because they remove high amounts of dissolved organic matter (DOM) that can account for up to 80-90 % of the carbon ingested (Yahel et al. 2003; de Goeij et al. 2008a; Ribes et al. 2012; Mueller et al. 2014). They can also remove NH_4^+ from seawater (as energy source for ammonium oxidizing bacteria and archea) and excrete nitrate and nitrite (hereafter referred to as NO_x^-), which is suggested to be an outcome of nitrification (Corredor et al. 1988; Southwell et al. 2008a; Ribes et al. 2012). In contrast, LMA species obtain carbon and nutrients mainly by filtering particulate organic matter (pico-and nanoplankton and detrital POM) (Ribes et al. 1999a, Schläppy et al. 2010; Freeman and Thacker 2011). As end products of remineralization, LMA excrete dissolved compounds such as NH_4^+, PO_4^{3-} and DOM (Southwell et al. 2008b; Maldonado et al. 2012a; Ribes et al. 2012).

Mediterranean sponges from the coralligenous community differed from the aforementioned pattern of HMA mainly relying on dissolved compounds for energy and nutrients, and LMA relying on particulate sources (E. Jiménez PhD Thesis 2011). Jiménez (2011) reported that DOM uptake accounts for 50% of the carbon and 80-90% of the nitrogen ingested by the examined HMA sponges. But, although the examined LMA species relied mostly on pico-and nanoplankton as a carbon source, >75% of the nitrogen they ingested was gained from dissolved compounds. These results were obtained in a series of laboratory experiments carried out with a running seawater system that commonly suffered from been affected by fouling communities that led to a strong depletion of the planktonic community and to elevation of dissolved inorganic nitrogen (DIN) (see also Alexander et al. 2015). Ambient DIN concentrations during the experiments were up to seven-fold higher than *in situ*

natural concentrations (Jiménez and Ribes 2007; Ribes et al. 2012). Therefore, while Jiménez (2011) provided a nice example of the trophic plasticity that sponges can display, these results cannot be directly attributed to the nitrogen metabolisms of these sponges under natural conditions. The present study examines the in situ metabolism of five common sponge species that cohabit rocky walls in a temperate environment and quantify the relative importance of planktonic and dissolved nutrients in the sponge holobiont, particularly in the context of the HMA and LMA dichotomy and their putative relationships.

A non-intrusive in situ technique was used to quantify the uptake and excretion of N and C of five abundant Mediterranean sponge species that cohabit the coralligenous community by measuring the concentration of dissolved compounds and pico-and nanoplankton in the water that they inhaled and exhaled. We aimed to examine: i) whether the rate of uptake and excretion of dissolved and particulate carbon and nitrogen differ among species and whether it could be related to the HMA and LMA dichotomy; ii) whether these rates are related to the availability of compounds. Our findings suggest the occurrence of a trophic niche separation among the most common and abundant Mediterranean species in which HMA sponges use waste products excreted by LMA sponges, thus facilitating the dense co-existence of sponge species within the concentration boundary layer on the walls of the coralligenous community.

Material and method

Study site

The Mediterranean Sea is a temperate oligotrophic sea in which nitrogen and phosphorous are the main limiting nutrients (*e.g.,* Thingstad et al. 1998). It is characterized by a marked seasonality that contrasts relatively high productive periods in late winter and early spring with low productive periods of water stratification (Estrada 1996). As in other temperate oligotrophic seas, these oligotrophic waters are dominated by pico- and nanoplankton communities (Tremblay and Legendre 1994; Denis et al. 2010). This is also the dominant pattern of plankton composition near the bottom where the coralligenous communities dwell (Ribes et al. 1999b).

The coralligenous community is a multispecific assemblage of active and passive suspension feeders that coexist feeding on a wide spectrum of prey types. The coralligenous community is among the main "hot spots" of species diversity in the Mediterranean. It is inhabited by a wide variety of organisms, from encrusting calcareous corallines and green algae, which are the major contributors of the coralligenous framework, to suspension feeders such as bryozoans, anthozoans, hydrozoans, sponges, tunicates, serpulids and mollusks (Ballesteros 2006).

The study was conducted at "Reserva Natural de Montgrí, Illes Medes i Baix Ter" (NW Spain, northwestern Mediterranean Sea, 42°. 06'N, 3°. 21'E), where the rocky benthic coralligenous community is well developed. The studied Demospongiae species are all common and widespread habitants of the coralligenous community (Uriz et al. 1992) where they normally cohabit the same rocky surfaces and the tightly packed sponges commonly create an intervening mosaic of colors (Figure 1). A survey of species composition of the coralligenous community at the study site showed that these five species covered 26.4% of the seafloor and represented 54% of the area covered by active suspension feeders (Teixidó et al. 2013), suggesting a major role for sponges in general, and for the examined species in this community.

On the basis of electron microscope observations, *Agelas oroides* (Schmidt, 1864), *Petrosia ficiformis* (Poiret, 1789) and *Chondrosia reniformis* (Nardo, 1833) are high microbial abundance species (HMA), and *Crambe crambe* and *Dysidea avara* (Schmidt, 1862) are low microbial abundance species (LMA) (Vacelet and Donadey 1977; Wehrl 2006; Gloeckner et al. 2014 and reference therein).

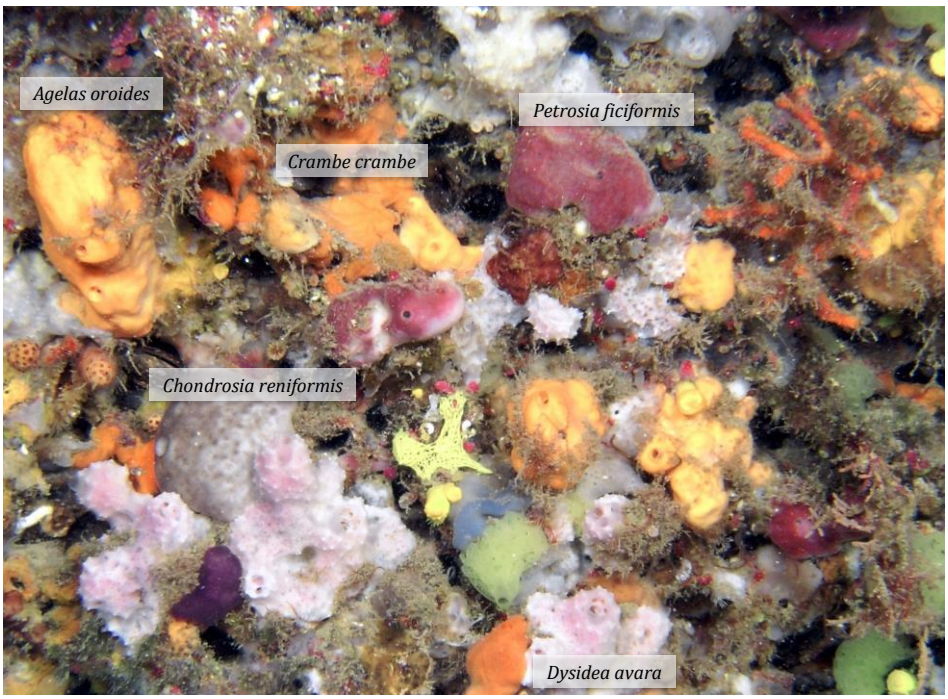


Figure 1. A typical view of a rocky crop of the Mediterranean coralligenous community at the study site on the Montgrí Catalan coast, densely populated by all five studied sponge species (labeled in the picture) among other suspension feeders and coralligenous algae.

Cross-shore transect

To estimate the amount of available plankton in the ambient water, the abundance of pico-and nanoplankton was examined on six cross-shore transects at a north-east facing vertical wall with a well-developed coralligenous community. In order to integrate the inherent variability of plankton distribution, we used mooring lines to position nine sampling stations at increasing distances from the wall (0, 0.1, 0.8, 1.4, 2.9, 4.2, 5.6, 10 and 20 m from the wall). A VacuSIP sampler (Morganti et al. in press) equipped with evacuated EPA glass vials (Cole-Parmer 03756-20) was positioned on each station at a fixed depth of 15 meters. For the initiation of sampling of each cross-shore transect, the suction of the VacuSIPs was triggered by a diver so that the ambient water was slowly sucked (<1 mL min^{-1}) into the collecting vial. Sampling was terminated after 30 minutes. Upon retrieval, samples were stored on ice until they could be aliquoted and preserved for flow cytometry analysis as described below. To characterize the local hydrodynamic regime during the study at the site of the transects, a current meter (Argonaut ADV, Sontek) was positioned at 50 cm from

the wall throughout the experiment. Temperature was recorded continuously during the study with an Onset HOBO Pendant Temperature Data Logger (UA-002-64, Onset Computer Corporation).

In situ sampling

InEx. Sampling of the water inhaled (In) and exhaled (Ex) by the sponges was conducted by SCUBA diving during July 2012 and July 2013. Three weeks before sampling, ten representative specimens from each species were selected during preparatory dives on the basis of ease of access. A support plaque was placed next to each specimen using two-component resin epoxy (IVEGOR, 09257). On the day of the experiment, 5 specimens were checked for pumping, placing diluted and filtered fluorescein dye near the sponge surface. To cleanly collect inhaled and exhaled water, we used the VacuSIP technique. A detailed description of the experimental procedure and step-by-step video clips can be found in Morganti et al. (in press). Briefly, the VacuSIP system allows simultaneous, clean, and controlled collection of the water inhaled and exhaled by the sponge without any contact or interference with the studied animal. The retention or excretion of the different compounds was estimated from the difference in concentration between the inhaled (In) and exhaled (Ex) water (InEx). For each InEx pair, the concentrations of: pico-and nanoplankton, ammonium (NH_4^+), nitrite + nitrate (NO_2^- + NO_3^-), phosphate (PO_4^{3-}), silicate (SiO_4), dissolved organic carbon (DOC) and nitrogen (DON) were measured. Samples for dissolved components were filtered in situ using in-line stainless steel 13 mm filter holders (Pall, 516-9067) and binder-free glass fiber filters (0.7µm, Pall, 516-9126). A strict cleaning protocol was applied to ensure contamination-free sampling (for details, see Morganti et al. in press). Filters were then processed for C:N analysis (elemental organic analyzer Thermo EA 1108, Thermo Scientific). Upon retrieval, samples were stored on ice until they could be frozen and preserved as described below. For more detail, see chapter 1.

Pumping rate. The pumping rate of each sponge species was estimated for randomly selected sponge specimens possessing only a single osculum. We used a modification of the dye front speed method (DS) described by Yahel et al. (2005). For

a detailed description of the dye technique employed in this study see method section chapter 3.

Sample preservation

Upon collection, water samples were immediately fixed on the boat. For pico- and nanoplankton samples, three aliquots of 2 mL were withdrawn and fixed with 1% paraformaldehyde + 0.05% Electron Microscopy grade glutaraldehyde (final concentration, Sigma P6148, and Merck 8.206.031.000, respectively) and placed in ice. Once in the harbor, samples were frozen in liquid nitrogen and stored at -80◦C until flow cytometry analysis was performed. Water samples for dissolved organic compounds (DOC + DON) were filtered in situ as described above and fixed on the boat with 25% orthophosphoric acid (ultrapure, Sigma 79617) and stored in the dark at 4◦C for a maximum of one week before the analysis. Water samples for dissolved inorganic compounds analysis (NH_4^+, NO_x^-, PO_4^{3-}, SiO_4) were also filtered in situ (as described above) and kept in ice on the boat and then frozen at -20°C until the analysis.

Water sample analysis

Flow cytometry for pico-and nanoplankton quantification. *Synechococcus* sp., *Prochlorococcus* sp., non-photosynthetic bacteria, pico-and nanoeukaryotes were quantified with flow cytometer (FACSCalibur, Becton-Dickinson, 488 nm excitation blue laser) following the method of Gasol and Moran (1999). Once the samples were quick-thawed, a Polysciences Fluoresbrite beads (1µm) solution was added as an internal standard from a stock solution pre-calibrated with Becton-Dickinson Truecount High Control Beads (Cat.# 340335). The Becton-Dickinson Cell Quest program was used to separate different cell groups, which were detected on the basis of cell-side scatter and forward scatter (as a proxy of cell size) and orange fluorescence of phycoerythrin, red fluorescence of chlorophyll and green fluorescence of DNA stained, emitted after excitation by blue laser. Phytoplankton cells were analyzed at high flow rate (~ 46 µl min^{-1}) for four minutes. *Synechococcus* sp. (hereafter, *Syn*) was identified based on both orange and red fluorescence emission. *Prochlorococcus* sp. and eukaryotes were identified based on low and high red

fluorescence respectively and the lack of orange fluorescence. The non-photosynthetic bacteria (hereafter *Bact*) were stained with nucleic acid dye (SYBER Green I) and analyzed at a medium flow rate (≈ 19 µl min $^{-1}$) for two minutes. Two different groups of Bact were distinguished based on their DNA content: high- and low – DNA non-photosynthetic bacteria, respectively (Gasol et al. 1999). They were characterized by the lack of both red and orange fluorescence. The Flow cytometry data analysis was performed using Flowing Software (Perttu Terho, Turku Center for Biotechnology, Finland, www.flowingsoftware.com) as depicted in Morganti et al. (in press).

Carbon and nitrogen content was estimated based on conversion factors from the literature. Since a wide range of conversion factors is available in the literature, the most appropriate factors to our study site and season were selected: *Synechococcus* sp. 470 fg C cell^{-1} and 50 fg N cell $^{-1}$ (Campbell et al. 1994), pico-and nanoeukaryotes 1540 fg C cell^{-1} (Zubkov et al. 1998) and 26.1 fg N µm^{-3} (Caron et al. 1995) non-photosynthetic bacteria 20 fg C cell^{-1} (Ducklow et al. 1993) and 5.4 fg N cell^{-1} (Caron et al. 1995).

Analysis of dissolved compounds. Nutrients NO_x^-, NH_4^+, $PO_4^{3-}$and SiO_2 were measured with an Alliance autoanalyzer following the method of Grasshof et al. (1999). DOC was determined by the high-temperature catalytic oxidation method using Schimadzu TOC-V analyzer (Sharp et al. 2002). Total dissolved nitrogen (DN) was analyzed by high-temperature catalytic oxidation using a chemiluminescence detection method with the Schimadzu TOC-V analyzer for the samples collected in July 2012 and by using a Seal Analytical determination after persulfate oxidation for samples collected in July 2013. In both cases DON concentrations were calculated by subtracting the DIN concentration (NH_4^++NO_x^-) from DN.

Data analysis

Net removal and excretion of each compound was calculated by the difference in concentration between the inhaled and exhaled water for each pair of samples (ΔC_{In-Ex}, µmol L pumped^{-1} or cells L pumped^{-1}). Positive values indicate ingestion and negative values indicate excretion. To test for a significant removal or excretion ($\Delta C_{In-Ex} \neq 0$) we used a paired t-test unless the number of available pairs was small or data violated the normality assumption. In those cases, the non- parametric Mann-Whitney

Rank Sum Test was applied. For each species the percentage of pairs showing ingestion or excretion was calculated. Since the studied sponges normally remove *Synechococcus* sp. (Syn) at high efficiency (86 ± 11%), we screened the data set using Syn removal as a tracer. Experiments, showing *Synechococcus* sp. retention efficiency lower than 60%, were interpreted as contaminated with ambient water (tube dislodged from the oscula or the specimen might have stopped pumping) and removed from the analysis. To test for significant differences between retention efficiency of each planktonic group between the studied species we conducted a one-way analysis of variance (ANOVA). Analysis of covariance (one-way ANCOVA) was used to test for differences in the slope of the regression of cell removal over inhaled cell concentration. The analyses were performed with SigmaPlot program (Ver 12, Systat co.). The area of each sponge was measured using size-calibrated images in Image J. An estimate of the mean mass flux mediated by a sponge was generated for each sponge species as the product of mean pumping rate (Chapter 3) and the mean ΔC_{In-Ex}, (µmol oscula^{-1} hr^{-1} or cells oscula^{-1} hr^{-1}). These flux estimates were later normalized to sponge area (cm^2) to produce sponge specific aerial flux estimates and then multiplied by the coverage area of each species in the studied area to obtain areal flux estimates.

Results

Environmental parameters and pico-and nanoplankton distribution along the cross-shore transects.

During the sampling period water temperature ranged between 19.3 and 22.9 ˚C. Pico-and nanoplankton concentrations at the walls, where the studied sponges resided, were at the low end typical to the area and the sampling period (Ribes et al. 1999b) ranging from 1.1 to 5.1 x 10^5 cells mL^{-1} for non-photosynthetic bacteria, 0.5 to 2.6 x 10^4 cells mL^{-1} for *Synechococcus* sp., and 1.0 to 9.2 x 10^2 cells mL^{-1} for pico-and nanoeukaryotes (Table 1). *Prochlorococcus* was not detectable during the experiments, because of its seasonal pattern (Ribes et al. 1999b). Dissolved nutrients were relatively high for surface Mediterranean water, ranging from 0.3 to 1.1 µmol L^{-1} NO_x^-, 30 to 700 nmol L^{-1} PO_4^{3-}, 100 to 700 nmol L^{-1} NH_4^+, and 0.8 to 2.2 µmol L^{-1} SiO_4 (Table 1). Dissolved organic compounds ranged from 4 to 14 µmol L^{-1} DON and from 61 to 131 µmol L^{-1} DOC (Table 1).

Table 1. Mean (± SD) concentration of the sampled water constituents derived from inhaled water during the two experiments performed in July 2012 and July 2013. Bact: non-photosynthetic bacteria; Syn: Synechococcus sp.; Euk: pico-and nano eukaryotic algae; NH_4^+: ammonium; NO_x^-: nitrite + nitrate; DIN: dissolved inorganic nitrogen; DON: dissolved organic nitrogen; DOC: dissolved organic carbon; PO_4^{3-}: phosphate; SiO_4: silicate.

Environmental Parameters	(Averages ± SD)
T(ºC)	21 ± 1.25
Bact (x 10^5 cells mL^{-1})	1.94 ± 0.78
Syn (x 10^4 cells mL^{-1})	1.13 ± 0.53
Euk (x 10^2 cells mL^{-1})	6.19 ± 1.75
NH_4^+ (µmol L^{-1})	0.32 ± 0.15
NOx^- (µmol L^{-1})	0.55 ± 0.17
DIN (µmol L^{-1})	0.87 ± 0.32
DON (µmol L^{-1})	9 ± 2
DOC (µmol L^{-1})	86 ± 18
PO_4^{3-} (µmol L^{-1})	0.10 ± 0.12

The cross-shore transects revealed a clear, sharp and consistent decrease in plankton concentration of both phytoplankton and non-photosynthetic bacteria in proximity to the sponge wall. The thickness of the concentration boundary layer at which the drop of the pico-and nanoplankton concentration could be measured was on the order of 3 m with clear decrease of pico-and nanoplankton concentration toward the wall, reaching the lowest concentrations just above the substrate (Figure 2). Within the depleted boundary layer, phytoplankton (Syn and Euk) and non-photosynthetic bacteria concentrations were 40% and 50% of those observed at the off-shore (20 m away from the wall), respectively. Cell concentrations at the wall were within the range of abundance measured by the inhaled VacuSIP samples (Table 1). The current speed during the period that the cross-shore transects were performed was low (mean value 1.7 ± 0.28 cm s^{-1}) and no correlation was observed between current speed and the magnitude of plankton depletion (data not shown).

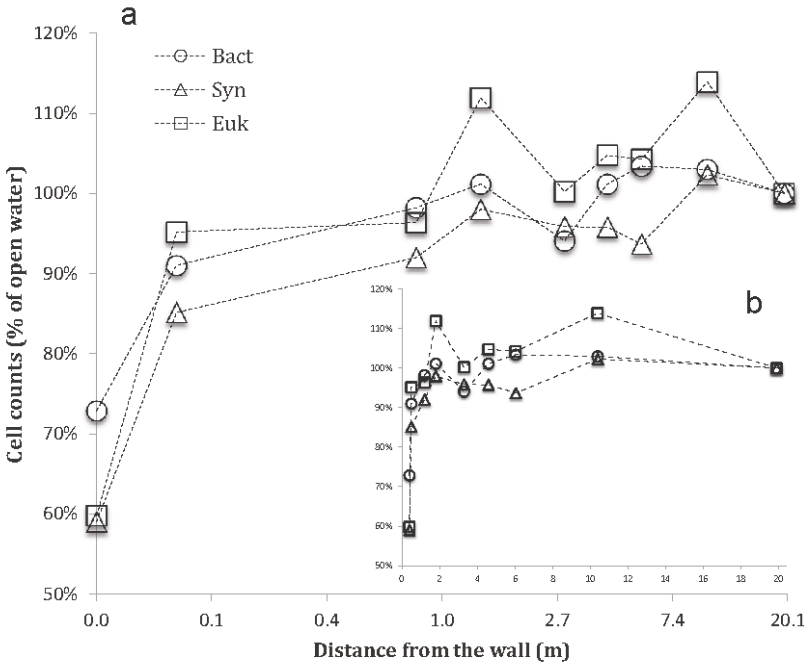


Figure 2. Cross-shore distribution of Synechococcus sp. (Syn), non-photosyhthetic bacteria (Bact), and pico-and nanoeukaryotes (Euk) based on six different transects from the wall (left) to the "open sea" (right). The data are presented as a relative percentage of their concentration in the most distant sample of the transect (20 m from the wall).

Pico-and nanoplankton retention

No significant differences between sponge species were found in the removal efficiency of *Synechococcus* sp. and eukaryotic cells (one way ANOVA, $F_{(4, 36)}$= 1.884, p=0.137). The examined sponge species efficiently removed *Synechococcus* sp. and eukaryotic cells from the ambient water with a grand average (over all five species) removal efficiency of 86 ± 11% and 69 ± 14% (mean ± SD), respectively (Figure 3, Table 2). Nevertheless, due to the relatively small sample size, the statistical power of our experiments was too low (<0.8) to allow robust inference about the differences in retention efficiency between the sponge species. In contrast, the removal efficiency of non-photosynthetic bacteria varied significantly among the sponge species (one way ANOVA, $F_{(4,36)}$=4.146, p=0.008) with *C. crambe* (LMA) and *P. ficiformis* (HMA) removing less than 60% of the bacteria, whereas the other sponges removed non-photosynthetic bacteria (82 ± 4%) with a similar efficiency to that of *Synechococcus* sp. (Figure 3, Table 2).

Table 2. The retention efficiency (mean ± SD) of planktonic cells quantified with a flow cytometer and the regression parameters of the concentration of cells removed over ambient cell concentration for each sponge species. The intercepts were not significantly different from 0 (p>0.05).

	***Synechococcus* sp.**			**Eukaryotic algae**			**Bacteria**		
Species	**Retention efficiency**	**slope**	**R^2**	**Retention efficiency**	**slope**	**R^2**	**Retention efficiency**	**slope**	**R^2**
D. avara	89 ± 10 %	0.85	0.91	68 ± 13 %	0.74	0.94	85 ± 11%	0.81	0.76
C. crambe	84 ± 16 %	0.88	0.93	63 ± 20 %	0.64	0.46	53 ± 10 %	0.85	0.86
A. oroides	86 ± 11 %	0.81	0.87	74 ± 13 %	0.73	0.62	77 ± 17 %	0.71	0.26
C. reniformis	86 ± 12 %	0.84	0.91	76 ± 7 %	0.75	0.83	83 ± 16 %	0.80	0.91
P. ficiformis	86 ± 12 %	0.88	0.88	61 ± 13 %	0.65	0.85	60 ± 9%	0.60	0.85

The examined sponge species removed *Synechococcus* sp., non-photosynthetic bacteria and eukaryotic cells from the water in direct proportion to its ambient concentration with no evidence of a threshold or saturation over the range of concentrations found in this study (Figure 3). Therefore, the sponge species exhibited

a clear Type I functional response (Holling 1965) for all cell types. A significant difference among species was found between the slopes of the regression of non-photosynthetic bacteria removal over their ambient concentrations ($F_{(4,36)}$=5.556, p=0.002, ANCOVA), but not for photosynthetic cells.

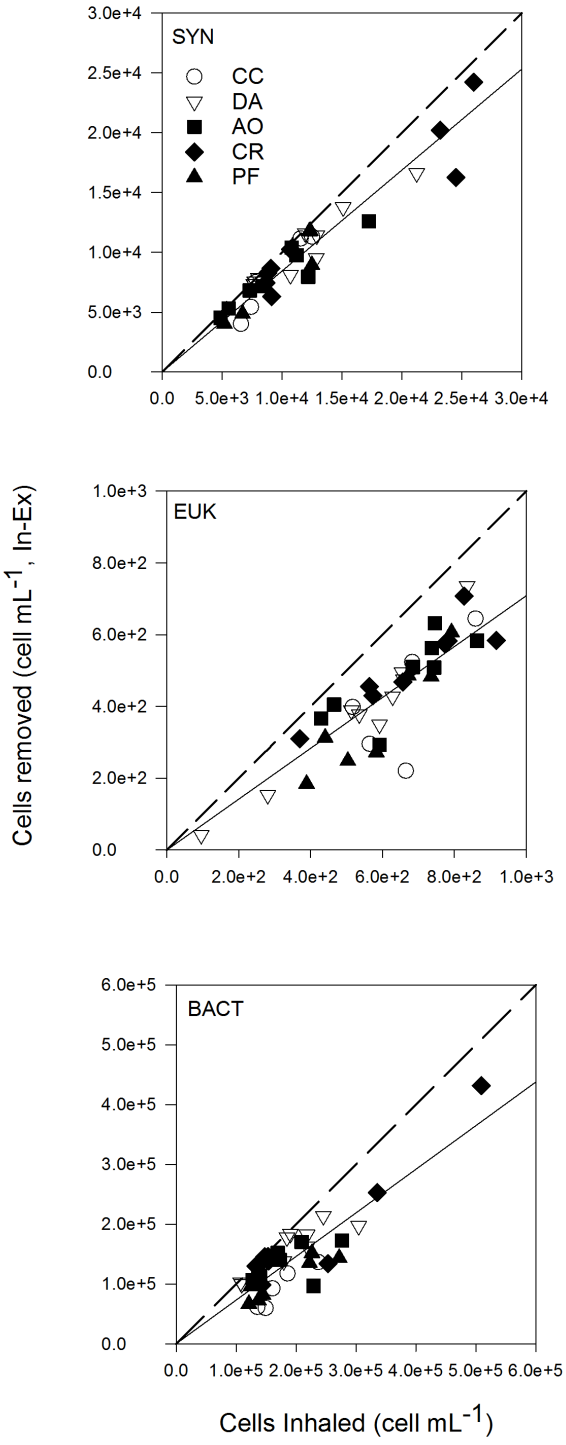


Figure 3. The functional response by the five species to variable prey concentration. Cell retention (In-Ex) is plotted against inhaled water concentrations. *Synechococcus* sp. (SYN), pico-nano eukaryotes (EUK), non-photosynthetic bacteria (BACT). Linear regression parameters are presented in table 2. CC, *Crambe crambe*; D.A, *Dysidea avara*; A.O, *Agelas oroides*; C.R, *Chondrosia reniformis* and P.F, *Petrosia ficiformis*.

Dissolved compounds uptake and excretion

Dissolved nitrogen. HMA and LMA sponges exhibited a markedly different pattern of nitrogenous compound processing. *C. reniformis*, *P. ficiformis* and *A. oroides* (HMA) usually removed considerable amounts of dissolved organic nitrogen (DON, 0.22 – 1.12 µmol L^{-1}) from the water they pumped and excreted NO_x^- (0.5 -1 µmol L^{-1}). HMA species also removed up to 48% of the ambient concentration of ammonium (Table 3). In contrast, *D. avara* and *C. crambe* (LMA) excreted considerable amounts of both DON (0.2 – 1.8 µmol L^{-1}) and ammonium (0.1–0.2 µmol L^{-1}) whereas their NO_x^- excretion was small or null (<0.2 µmol L^{-1}) (Table 3, Figure 4). No correlation was found between the amount of NO_x^- produced and NH_4^+ removed in any of the studied sponges (Figure 4b).

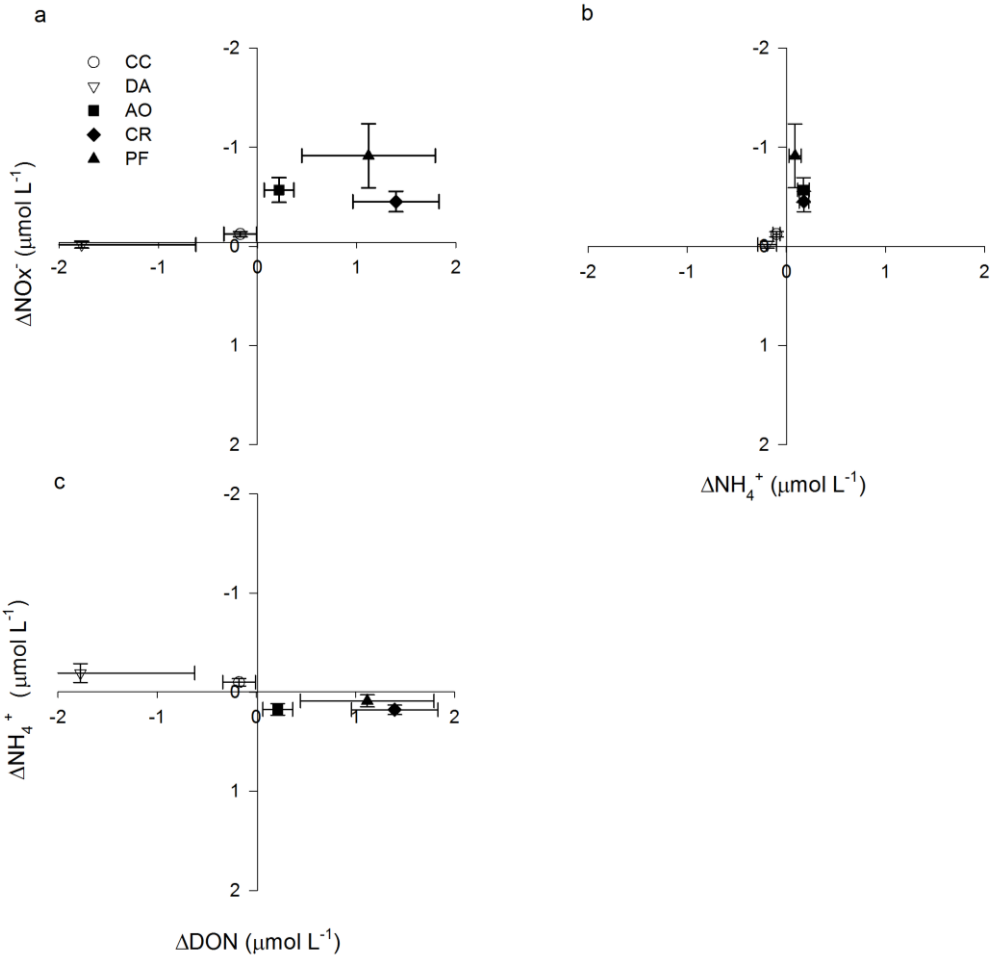


Figure 4. Relationships between net ingestion and/or excretion of different nitrogen compounds calculated as the difference between inhaled and exhaled concentration (ΔIn-Ex). Positive values denote ingestion and negative values denote excretion. Averages ± SE. Note that the Y axis (ΔDIN) is reversed so that excretion can be visually correlated with uptake.

Dissolved organic carbon (DOC). DOC was removed in two-thirds of the InEx pairs (Table 3) showing a net mean retention of 5 - 13 µmol L pumped^{-1} for all sponges but *C. crambe,* that showed net excretion. The amount of DOC removed from the surrounding water linearly increased with the increase of ambient DOC concentration from 80 to 130 µmol L^{-1} with no evidence of an upper threshold or saturation. However, the 80 µmol L^{-1} appeared as a threshold below which most specimens showed DOC excretion (Figure 5).

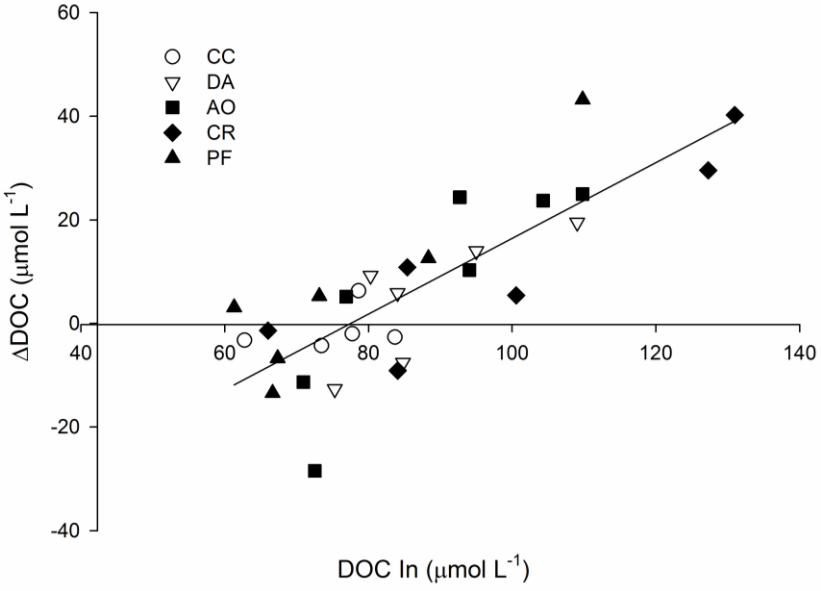


Figure 5. The net ingestion or excretion of dissolved organic carbon (DOC) plotted against DOC concentration in the inhaled (ambient) water in the five species. Linear regression y=0.73 x – 56.69; R^2= 0.66; p<0.001; CC, *Crambe crambe*; DA, *Dysidea avara*; AO, *Agelas oroides*; CR, *Chondrosia reniformis* and PF, *Petrosia ficiformis*.

Particulate N and C

Per liter pumped, the contribution of plankton removal to the N and C budget (estimated using published conversion factors) was similar for all species studied. A grand average over all five species yielded 0.09 ± 0.02 µmol N L^{-1} and 0.65 ± 0.15 µmol C L^{-1} (Table 3). The planktonic groups differently contributed to the live C and N ingested, with *Synechococcus* sp. contributing 56 ± 7% of the planktonic carbon and 38 ± 6% of planktonic nitrogen, whereas non-photosynthetic bacteria contributed 35 ± 5% of the planktonic carbon and 60 ± 6% of the planktonic nitrogen ingested. In contrast, removal of eukaryotes algae contributed <10% of either C or N.

N and C budget

Carbon fluxes showed differences between species but without any pattern for HMA and LMA species. The carbon removed was higher than the C excreted in all studied species except in *C. crambe,* which showed a negative carbon balance (Figure 6). DOC was the major source of carbon, accounting for > 85% of the carbon gained by *D. avara*, *A. oroides*, *C. reniformis* and *P. ficiformis*. *C. crambe* was the only species showing DOC excretion. Despite the high removal efficiency of the examined species on pico-and nanoplankton groups, the particulate live fraction accounted for ≤ 13% of the measured carbon ingestion by the different sponge species.

The balance of nitrogen differed markedly between HMA and LMA sponge species, being positive in HMA and negative in LMA species. HMA species removed nitrogen mostly as dissolved compounds (94%, 93% and 83% in *P. ficiformis, C. reniformis*, and *A. oroides* respectively) with DON constituting > 55% of the total N removed and NO_x^- being the major nitrogenous waste product. On the contrary, 100% of nitrogen ingested by LMA species was as particulate nitrogen (PKPL), and the N output mainly consisted of DON (89% and 44% of the total dissolved nitrogen excreted by *D. avara* and *C. crambe* respectively, Figure 6).

Pumping rates and flux comparison

The average pumping rates (pooled for July 2012 and July 2013) were 3.1 ± 1.1 and 7.1 ± 4.2 mL min^{-1} (cm sponge)$^{-2}$, for the LMA sponges *C. crambe* and *D. avara* and 2.2 ± 0.3, 4.7 ± 0.5 and 5.2 ± 1.2 mL min^{-1} (cm sponge)$^{-2}$ for the HMA sponges *P. ficiformis, C. reniformis* and *A. oroides,* respectively. Using these pumping rates, we could estimate the N and C fluxes, flow rate per sponge area (cm^2). At the study site, LMA species excreted 0.8 ± 1.4 and 0.1 ± 0.2 μmol h^{-1} (cm sponge)$^{-2}$ of dissolved N (DON and DIN, respectively). HMA species excreted 0.4 ± 0.3 μmol h^{-1} (cm sponge)$^{-2}$ of NO_x^- and ingested 0.1 ± 0.1 and 0.6 ± 0.7 μmol h^{-1} (cm sponge)$^{-2}$ of NH_4^+ and DON, respectively. Normalized to sponge area, the NH_4^+ and DON excretion rates of the two LMA species (0.1 ± 0.1 μmol h^{-1} (sponge cm)$^{-2}$ and 0.8 ± 1.4 μmol h^{-1} (cm sponge)$^{-2}$, respectively) were comparable to the ingestion rates of the three co-inhabitant HMA species (see Figure 6b).

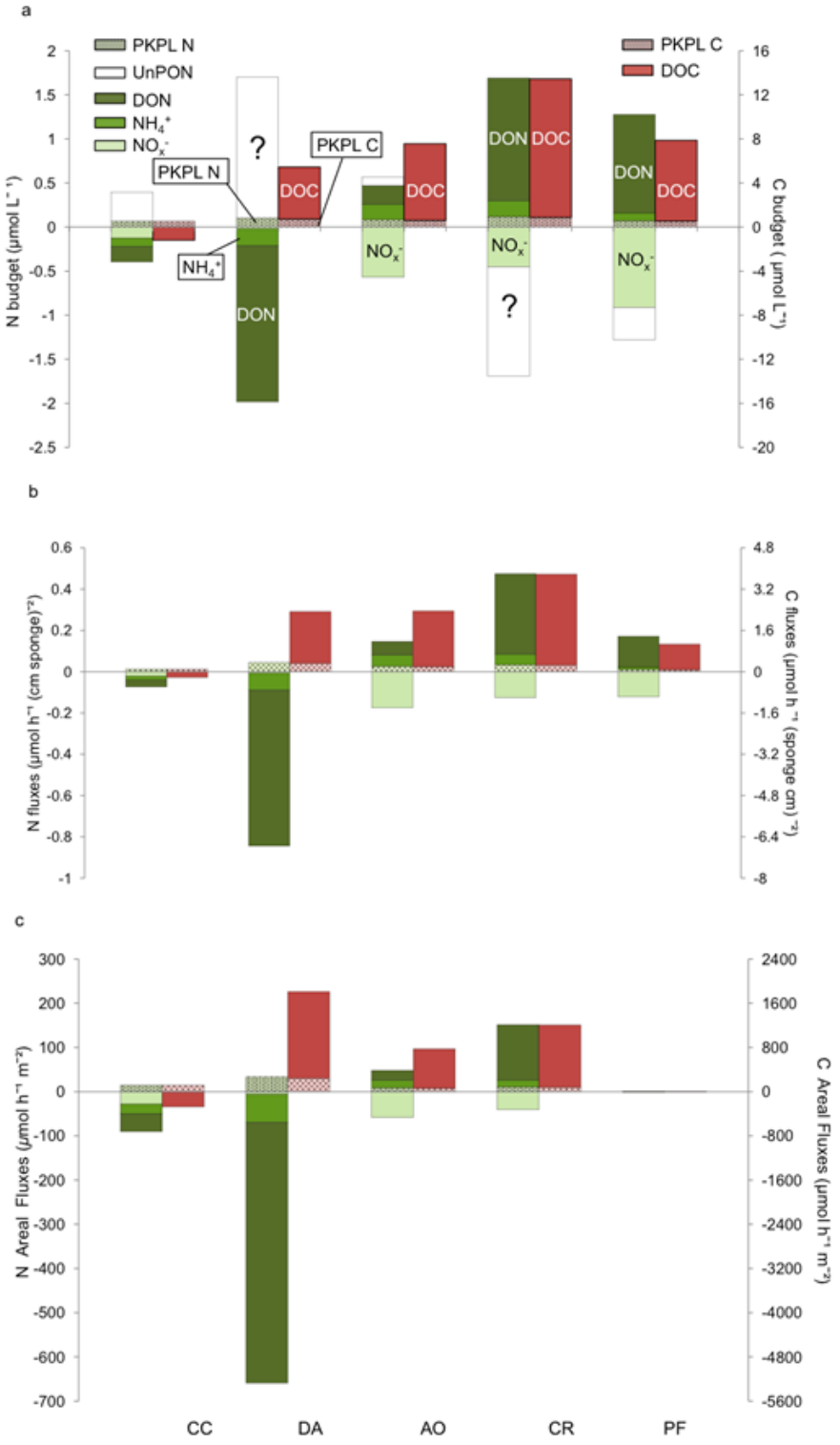


Figure 6. The average contribution of different C and N pools gained and excreted by the five studied species. (a) C and N budget (µmol L^{-1}); (b) C and N fluxes (µmol h^{-1} (cm sponge)$^{-2}$); (c) C and N areal fluxes (µmol h^{-1} m^{-2}). UnPON is the unresolved organic matter that can potentially explain the missing N in the budget.

The areal fluxes mediated by the studied sponge were calculated based on published cover data (*C. crambe* 12%, *D. avara* 7.8%, *A. oroides* 3.3%, *C. reniformis* 3.2, and *P. ficiformis* 0%, Teixidó et al. 2013). While Teixidó et al. (2013) estimated *P. ficiformis* % cover as null, this species is commonly abundant in most sciaphilic environment, such as in caves (M. A. Bibilioni unpubl.). Due to the heterogeneous habitat in which this study was performed, we conservatively considered that *P. ficiformis* coverage area was 0.1%. Using these data, we estimated the N excreted by LMA sponges as 0.7 ± 1.1 mmol DON h^{-1} m^{-2} and 0.1 ± 0.1 mmol DIN h^{-1} m^{-2}. The estimated N uptake by HMA sponges was 0.15 ± 0.15 mmol $NH_4^+ h^{-1}$ m^{-2} and 33.8 ± 29.3 µmol DON h^{-1} m^{-2} and their N excretion was estimated as 99.9 ± 61.1 µmol $NO_x^- h^{-1}$ m^{-2} (see Figure 6c). Altogether, the studied species ingested 3 ± 8 mmol h^{-1} m^{-2} of DOC.

Table 3. Average net difference and average concentration (± SD) of different components in the water inhaled and exhaled by each sponge species. N, number of In-Ex pairs. %I, percentage of In-Ex pairs for which ingestion was recorded. %E, percentage of In-Ex pairs for which excretion was recorded. Positive values indicate ingestion; negative values indicate excretion (bolded). The asterisk (*) indicates significant difference between inhaled and exhaled concentrations (P<0.05, with either t Test or Mann-Whitney Rank Sum Test).

Species	Nutrient	Inhaled	Exhaled	Net difference (In-Ex)	N	%I	%E
D. avara	DOC (µmol L^{-1})	88 ± 12	83 ± 8	5 ± 12	6	67	33
	NH_4^+ (µmol L^{-1})	0.26 ± 0.07	0.45 ± 0.29	**-0.19 ± 0.27***	8	0	100
	NOx^- (µmol L^{-1})	0.57 ± 0.18	0.58 ± 0.12	**-0.02± 0.08**	6	33	67
	DON (µmol L^{-1})	8.17 ± 2.08	9.94 ± 3.02	**- 1.77 ± 3.05**	7	43	57
	PO_4^{3-} (µmol L^{-1})	0.06 ± 0.02	0.15 ± 0.14	**-0.09 ± 0.14***	8	13	87
	SiO_4 (µmol L^{-1})	0.95 ± 0.15	0.94 ± 0.16	0.01 ± 0.15	8	62	38
	Syn (x 10^3 cells mL^{-1})	12 ± 4.35	1.58 ± 1.62	10.4 ± 3.2*	9	100	0
	Euk (x 10^2 cells mL^{-1})	5.32 ± 2.21	1.5 ± 0.56	3.83 ± 1.99*	9	100	0
	Bact (x 10^5 cells mL^{-1})	1.95 ± 0.62	0.33 ± 0.33	1.62 ± 0.4*	9	100	0
	PKPL N (µmol L^{-1})	0.12 ± 0.04	0.02 ± 0.02	0.10 ± 0.03*	9	100	0
	PKPL C (µmol L^{-1})	0.86 ± 0.29	0.14 ± 0.12	0.73 ± 0.2*	9	100	0
C. reniformis	DOC (µmol L^{-1})	99 ± 26	86 ± 12	13 ± 19	6	67	33

	NH_4^+ (µmol L^{-1})	0.37 ± 0.20	0.19 ± 0.11	0.18 ± 0.13	7	100	0
	NOx^- (µmol L^{-1})	0.44 ± 0.07	0.89 ± 0.30	**-0.45 ± 0.27***	7	0	100
	DON (µmol L^{-1})	8.76 ± 2.42	7.37 ± 2.16	1.4 ± 1.23	8	100	0
	PO_4^{3-} (µmol L^{-1})	0.14 ± 0.24	0.07 ± 0.03	0.07 ± 0.21	8	50	50
	SiO_4 (µmol L^{-1})	1.2 ± 0.43	1.22 ± 0.43	**-0.01 ± 0.12**	8	50	50
	Syn (x 10^3 cells mL^{-1})	15 ± 7.98	2.31 ± 2.61	12.7 ± 6.68*	8	100	0
	Euk (x 10^2 cells mL^{-1})	6.83 ± 1.77	1.69 ± 0.83	5.14 ± 1.22*	8	100	0
	Bact (x 10^5 cells mL^{-1})	2.29 ± 1.34	0.44 ± 0.45	1.85 ± 1.09*	8	100	0
	PKPL N (µmol L^{-1})	0.14 ± 0.08	0.03 ± 0.03	0.12 ± 0.06*	8	100	0
	PKPL C (µmol L^{-1})	1.06 ± 0.53	0.19 ± 0.18	0.87 ± 0.43*	8	100	0
A. oroides	DOC (µmol L^{-1})	89 ± 15	82 ± 10	7 ± 20	7	71	29
	NH_4^+ (µmol L^{-1})	0.31 ± 0.16	0.14 ± 0.07	0.17 ± 0.17*	8	100	0
	NOx^- (µmol L^{-1})	0.55 ± 0.09	1.12 ± 0.4	**-0.57 ± 0.35***	8	13	87
	DON (µmol L^{-1})	9.85 ± 0.54	9.64 ± 0.38	0.22 ± 0.34	5	80	20
	PO_4^{3-} (µmol L^{-1})	0.12 ± 0.10	0.11 ± 0.08	0.01 ± 0.05	7	29	71
	SiO_4 (µmol L^{-1})	1.04 ± 0.16	1.09 ± 0.16	**- 0.05 ± 0.15**	8	38	62
	Syn (x 10^3 cells mL^{-1})	9.69 ± 4.05	1.62 ± 1.80	8.07 ± 2.70*	8	100	0
	Euk (x 10^2 cells mL^{-1})	6.57 ± 1.50	1.75 ± 9.22	4.83 ± 1.17*	8	100	0
	Bact (x 10^5 cells mL^{-1})	1.82 ± 5.21	0.48 ± 0.45	1.35 ± 2.88*	8	100	0
	PKPL N (µmol L^{-1})	0.17 ± 0.16	0.03 ± 0.03	0.08 ± 0.02*	8	100	0
	PKPL C (µmol L^{-1})	0.77 ± 0.25	0.17 ± 0.15	0.60 ± 0.15*	8	100	0
C. crambe	DOC (µmol L^{-1})	75 ± 8	76 ± 8	**-1 ± 4**	5	20	80
	NH_4^+ (µmol L^{-1})	0.41 ± 0.24	0.51 ± 0.2	**-0.10 ± 0.07**	4	0	100
	NOx^- (µmol L^{-1})	0.48 ± 0.08	0.60 ± 0.08	**-0.12 ± 0.06***	5	0	100
	DON (µmol L^{-1})	8.98 ± 1.43	9.15 ± 1.17	**- 0.17 ± 0.33**	4	50	50
	PO_4^{3-} (µmol L^{-1})	0.08 ± 0.03	0.09 ± 0.02	**-0.01 ± 0.01**	4	25	75
	SiO_4 (µmol L^{-1})	1.08 ± 0.12	1.04 ± 0.07	0.04 ± 0.1	5	40	60
	Syn (x 10^3 cells mL^{-1})	9.17 ± 2.67	1.28 ± 0.98	7.89 ± 3.30*	5	100	0

	Euk (x 10^2 cells mL^{-1})	6.45 ± 1.32	2.41 ± 1.27	4.16 ± 1.71*	5	100	0
	Bact (x 10^5 cells mL^{-1})	1.73 ± 0.4	0.79 ± 0.14	0.94 ± 0.34*	5	100	0
	PKPL N (µmol L^{-1})	0.10 ± 0.02	0.04 ± 0.01	0.07 ± 0.03*	5	100	0
	PKPL C (µmol L^{-1})	0.73 ± 0.18	0.21 ± 0.06	0.52 ± 0.20*	5	100	0
P. ficiformis	DOC (µmol L^{-1})	78 ± 18	70 ± 8	8 ± 20	6	67	33
	NH_4^+ (µmol L^{-1})	0.28 ± 0.08	0.20 ± 0.12	0.09 ± 0.16	7	71	29
	NOx^- (µmol L^{-1})	0.73 ± 0.22	1.64 ± 1.01	**-0.91± 0.79***	6	0	100
	DON (µmol L^{-1})	11.07 ± 1.48	9.95 ± 0.88	1.12 ± 1.78	7	71	29
	PO_4^{3-} (µmol L^{-1})	0.09 ± 0.04	0.14 ± 0.07	**-0.05 ± 0.07**	7	14	86
	SiO_4 (µmol L^{-1})	1 ± 0.15	0.95 ± 0.17	0.05 ± 0.11	6	67	33
	Syn (x 10^3 cells mL^{-1})	9.33 ± 3.41	1.18 ± 1.18	8.15 ± 3.4*	7	100	0
	Euk (x 10^2 cells mL^{-1})	5.88 ± 1.52	2.17 ± 0.6	3.71 ± 1.55*	7	100	0
	Bact (x 10^5 cells mL^{-1})	1.78 ± 0.61	0.71 ± 0.31	1.07 ± 0.36*	7	100	0
	PKPL N (µmol L^{-1})	0.10 ± 0.03	0.03 ± 0.02	0.07 ± 0.02*	7	100	0
	PKPL C (µmol L^{-1})	0.74 ± 0.22	0.19 ± 0.10	0.55 ± 0.18*	7	100	0

Discussion

In this study, we used in situ techniques to directly measure the diet, ingestion, excretion, and geochemical fluxes mediated by five of the most prominent sponge species of the coralligenous community in the NW Mediterranean Sea. Comparison of the differences in pico-and nanoplankton concentration and dissolved compounds between the water inhaled and exhaled by the sponges in situ provided a partial N and C budget for each species (Figure 6a).

The examined Demospongiae species efficiently removed pico-and nanoplanktonic cells (*Synechococcus* sp., non-photosynthetic bacteria, and eukaryotes) in agreement with previous reports (*e.g.,* Pile et al. 1997; Ribes et al. 1999a; Yahel et al. 2003). The average removal efficiency obtained in this study is in accord with the removal efficiency observed in tropical demosponges and deep glass sponges (Yahel et al. 2003, 2006, 2007; Mueller et al. 2014; Kahn et al. 2015b).

DOC accounted for ~90 % of the carbon removed by the sponges from the examined sources (detritus feeding was not quantified in this study due to technical problems). A higher DOC uptake was observed at higher ambient DOC concentrations with an apparent threshold at ~80 µmol L^{-1} for all five species (Figure 5). Unfortunately, for *Crambe crambe* all experiments were conducted at an ambient DOC concentration of ~80 µmol L^{-1} or lower. It is therefore unclear if the lack of DOC removal by *C. crambe* was due to the low DOC concentration during the experiments or an intrinsic inability to utilize DOC. The removal of DOC by the species *Chondrosia reniformis*, *Petrosia ficiformis*, *Agelas oroides* and *Dysidea avara* (5-13 µmol L^{-1}) resembled reported values for coral reef species (6-13 µmol L^{-1}, Yahel et al. 2003; de Goeij et al. 2008b; Mueller et al. 2014) and our laboratory studies on *C. reniformis* and *A. oroides* (8-10 µmol L^{-1}, Ribes et al. 2012). The complex and variable mixture of molecules that composed the DOC pool can be functionally divided into labile, semi-labile, and refractory fractions (Carlson 2002, and reference therein). Refractory DOC commonly accounts for the main share of the bulk of DOC, even in productive surface waters (Hansell et al. 2009; Santinelli et al. 2010). Our results suggest that ~80 µmol L^{-1} is a threshold of the labile DOC that the examined species can use. This result is consistent with the observed lack of DOC removal below this threshold detected during the recent examination of three Caribbean sponge species (Mueller et al. 2014; McMurray et al. 2016), and in other tropical species (G. Yahel, unpubl.).

Clearly, all studied sponges are well adapted to remove pico-and nanoplanktonic cells, irrespective of their microbial abundance and composition. However, the contribution of planktonic cells to the carbon and nitrogen budget varied between HMA and LMA sponges. HMA sponges mostly relied on dissolved compounds as the main source of reduced C and N, whereas LMA sponges relied mainly on the dissolved fraction as the main source of reduced C, but on the particulate fraction as the main N source. This assertion should be considered cautiously because we did not measure CO_2 or N_2 fixation and could not examine the contribution of particulate detritus to the sponge C and N budget.

The nitrogen budget was neutral or positive for all three HMA species (Figure 6a). Dissolved compounds (NH_4^+ + DON) accounted for ≥ 83% of the total nitrogen ingested by these HMA sponges. These results indicated that for the HMA sponges we

studied, DON uptake is the main nitrogen source. These provide the first evidence of DON removal by Mediterranean sponges. The in situ DON uptake reported here is in contrast with our previous laboratory study (Ribes et al. 2012) where no DON removal was detected by *A. oroides* and DON was excreted by *C. reniformis.* This discrepancy between the field and laboratory results appears to be related to the low DON (a factor of 2 lower) and high ammonium concentration (10-fold higher) present in the water supply of the laboratory system we used back in 2012. The contrast of our former lab results with the present field data reveals the large feeding plasticity of these sponge species and confirms that sponge feeding behavior can change as result of food availability (McMurray et al. 2016).

For the two LMA species, the nitrogen budget was negative (Figure 6a), with pico- and nanoplankton being the sole source of measured nitrogen intake. Excretion of dissolved compounds (DIN+DON) resulted in a mismatch between ingested and excreted nitrogen. In a previous laboratory study, DIN accounted for up to 76% of ingested nitrogen (Ribes et al. 2012). This contrasting result appears to be related to differences in initial concentration of these compounds and the potential occurrence of threshold levels as observed in DOC uptake. The unresolved nitrogen fraction may come from alternative sources of N such as the capture of particulate detritus. Detritus accounted for 40% of total N ingested by *D. avara* in laboratory experiments (E. Jiménez 2011), but was insignificant in *in situ* measurements with a recirculating flume (Ribes et al. 1999a). In contrast, nitrogen fixation has been proved to represent a minimal contribution to the nitrogen needs of the sponges (Wilkilson et al. 1999; Ribes et al. 2015).

All InEx pairs from LMA sponges showed excretion of ammonium, suggesting it is a common metabolic end-product released by the sponges. *D. avara* excreted insignificant amounts of NO_x^- (<0.1 μmol L^{-1}) whereas *C. crambe* excreted some NO_x^-, but this excretion was five times lower than the nearby HMA sponges, suggesting that nitrification is not a relevant process in the examined LMA species under field conditions. Reports of LMA sponge nitrification in a lab experiment (Schläppy et al. 2010) should be regarded with caution due to the method employed (incubation chambers) and to the high ammonium concentration (10 times higher than natural

concentration) present in the water supply, which would be a further indication for the metabolic plasticity of the sponges.

In oligotrophic and prey-depleted waters, HMA species rely on their symbiotic microbiota to provide for the holobiont N needs by using dissolved sources (such NH_4^+ and DON) that most other metazoans are incapable of using. Ammonium oxidizers can use the NH_4^+ as energy source and excrete NO_x^- (*e.g.,* Fiore et al. 2013 and reference therein). Nevertheless, the increase in ammonium ingestion is not correlated with similar increase in NO_x^- excretion. In fact, the ammonium flux accounts for only a small fraction of the total NO_x^- flux, suggesting that DON oxidation and not ammonium is the source of most of the NO_x^- production.

The nitrogen fluxes measured in this study are in accord with previous reports (*e.g.,* Southwell et al. 2008b; Fiore et al. 2013). The rate of nitrate production in our temperate species (155 ± 84 µmol NO_x^- h^{-1} (L sponge)$^{-1}$) resembled reported values for coral reef species, ranging from 97 ± 23 NO_x^- µmol h^{-1} (L sponge)$^{-1}$ excreted by *Aplysina archei* (Southwell et al. 2008b) and up to 445 ± 470 µmol NO_x^- h^{-1} (L sponge)$^{-1}$ excreted by the giant *Xestospongia muta* (Fiore et al. 2013). Unlike our HMA sponges, *X. muta* was revealed to be a big source of NH_4^+, excreting 450 ± 328 µmol NH_4^+ h^{-1} (L sponge)$^{-1}$ (Fiore et al. 2013). The observed ammonium fluxes reported here (uptake of 45 ± 28 and excretion of 199 ± 12 µmol NH_4^+ h^{-1} (L sponge)$^{-1}$ by HMA and LMA, respectively) are also comparable to reports from tropical species (uptake of 49 ± 39 and excretion of 86 ± 112 µmol NH_4^+ h^{-1} (L sponge)$^{-1}$, Southwell et al. 2008b).

Functional response

All sponges exhibited a linear increase in removal rate of food sources with increased concentration of both the particulate food (Syn, Euk, Bact) and the DOC, with no apparent saturation level within the range of examined natural concentrations, suggesting a Type I functional response (i.e., linear increase of food consumption up to a threshold level above which it remains constant). This feeding behavior has been documented in both temperate and tropical species, suggesting it as a typical pattern of sponge feeding on live particulate (Ribes et al. 1999a; Yahel et al. 2003, 2006, 2007; Perea-Blázquez et al. 2013; Kahn et al. 2015) and potentially also on dissolved inorganic nutrients (such as NH_4^+ and NO_x^-, see supplementary figures 1

and 2) and organic carbon above a threshold (Mueller et al. 2014; McMurray et al. 2016). The feeding behavior of an organism is closely related to the optimization of energy intake. The functional response is a manifestation of this relationship between the resource abundance and intake (Holling 1965). A type I functional response has so far only been observed in filter-feeders, but the majority of filter feeders do not display a Type I response (Jeschke et al. 2004). As demonstrated here, sponges seem to be perfectly designed for a Type I response due to their specialization on the smallest and most abundant water constituents. Feeding on the dissolved compounds, pico-and nanoplankton results in a negligibly small handling time and allows the sponges to spend most of the time foraging, thus maximizing intake rate.

Co-existence of HMA and LMA in the coralligenous community

The dense packing of both HMA and LMA sponges in many pinnacles and overhangs of the coralligenous community (see for example Figure 1) suggests that competition for space and access to unfiltered water should be harsh among these closely related suspension feeders (Zabala and Ballesteros 1989). However, signs for interspecific aggression in the coralligenous community are rare (Ballesteros 2006) in comparison to those observed in coral reef communities (Lang and Chornesky 1990; Wulff 2006a). In summer, the oligotrophic conditions of the Mediterranean become accentuated (Coma et al. 2000, 2009). The food scarcity is expected to intensify inter and intra specific competition (Buss and Jackson 1981; Best and Thorpe 1986) among the densely packed sponges as depicted in the cross shore transects (Figure 2) that show a developed concentration boundary layer with near-bottom plankton depletion. Differences in competitive ability, together with differences in niche, are key processes contributing to stabilizing species coexistence (Chesson 2000; Mayfield and Levine 2010).

Data reported here suggest that the dissolved waste products of LMA sponges, that process large volumes of water and feed mostly on particulate food, are consumed by their neighboring HMA sponges (Figure 7). LMA sponges excrete DON and NH_4^+, whereas HMA sponges consume DON and NH_4^+ and excrete NO_x^-.

To estimate the benthic fluxes mediated by the sponge community (Figure 6c) we used the product of the sponge areal cover and their areal pumping rate. The

estimated flux of DON excreted by LMA sponges in the study site (630 ± 1088 µmol h^{-1} m^{-2}) accounts for the flux of DON removed by HMA sponges (149 ± 147µmol h^{-1} m^{-2}), and the flux of NH_4^+ excreted by LMA (86.3 ± 105.6 µmol h^{-1} m^{-2}) accounted for the flux of NH_4^+ removed by HMA (33.8 ± 29.3 µmol h^{-1} m^{-2}). It should be noted that not all DON is necessarily labile and that there are others potential consumers of NH_4^+ (*e.g.,* the coralline algae) and DON (*e.g.,* water born bacteria). These estimates suggest that N released by LMA sponges may be an important source of recycled N (Keesing et al. 2013) not only for primary producers (Feng and Wild-Allen 2010) but also for other sponge species.

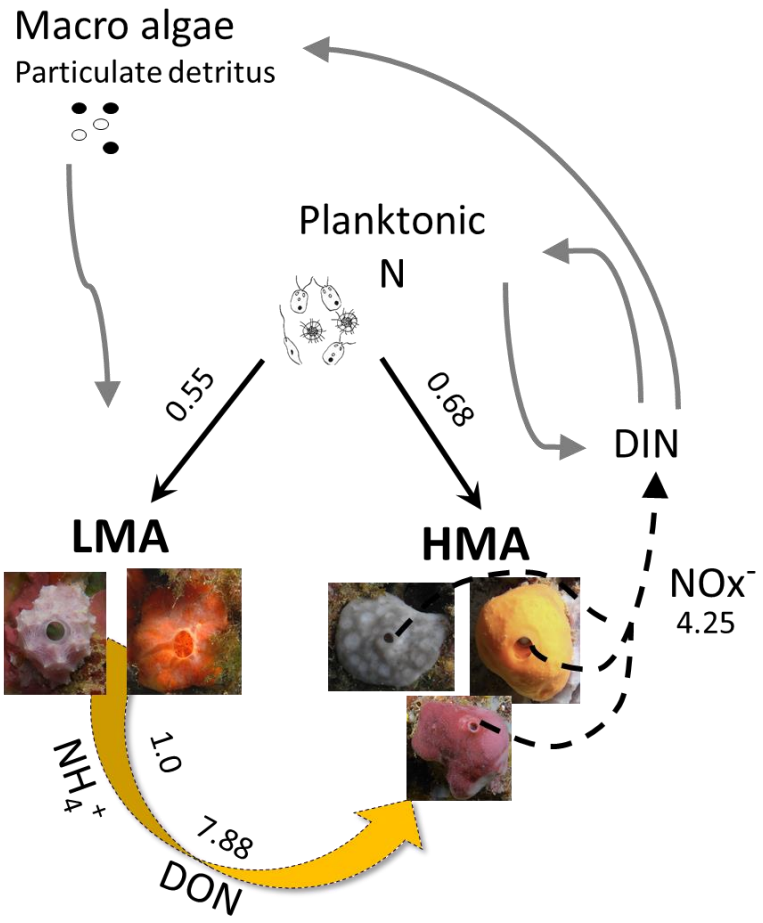


Figure 7. Schematic draft of the trophic niche separation concept and putative nitrogen and carbon fluxes. Nitrogen fluxes are provided per unit of sponge surface (mmol h^{-1} (m sponge) $^{-2}$)). Black arrows indicate uptake, dash arrows indicate excretion, yellow arrow indicates the dissolved nitrogen compounds excreted by LMA, which are suggested to serve as the primary source of N for HMA species. Gray arrows are putative N fluxes not quantified in this study.

Clearly, pico-and nanoplankton accounts for only a small portion of the N ingested by the sponges. The sources of the DON and particulate detritus that account for the bulk of the nitrogen diet of the sponges in our study site are yet unknown, but the prime suspect source is the large algal beds that normally cover the upper portions of the same rocks. Similarly, the fate of the excreted NO_x^- is yet unknown. However, it is very likely that a significant portion of the recycled nitrogen will return to the benthic algae or will be taken up by the planktonic community (Figure 7).

In conclusion, we provided evidence for different feeding strategies in Mediterranean sponges, with HMA having a broader spectrum of food sources, mostly represented by dissolved components that are potentially derived from the LMA species (and other animals) excretions. The differential capability of the different sponge strategies in using resources indicates a trophic niche separation related to the HMA-LMA dichotomy as a mechanism facilitating their coexistence. Our findings suggest that a mixed assemblage of sponges (and their associated bacteria) is able to utilize the suspended particulate and dissolved material more efficiently than a single species population. Niche separation between HMA and LMA and their putative symbiotic relationships may also provide an explanation for the phenomena of deep sponge gardens (*e.g.,* Schönberg and Fromont, 2011) and contribute to the survival, stability and diversity of dense sponge assemblages in oligotrophic habitats.

Chapter 3

Seasonality of *in situ* pumping rate

Introduction

Benthic suspension feeders play essential roles in the functioning of marine ecosystems (Gili and Coma 1998). By filtering large volumes of water (Reiswig 1971; McMurray et al. 2014) and processing large quantities of particulate and dissolved compounds (Gili and Coma 1998 and references therein), they influence the primary and secondary production in littoral food web (Asmus and Asmus 1991; Sullivan et al. 1991; Kimmerer et al.1994; Gili and Coma 1998). Among filter feeders, sponges comprise a major portion of the animal biomass in coral and polar reefs (Dayton et al. 1974; Díaz and Rützler 2001), and are ubiquitous throughout most oceanic regions from shallow and deep benthic habitats (Hooper et al. 2002). Sponges are opportunistic feeders, they can pump over a half liter of water s^{-1} kg^{-1} sponge dry mass (Weisz et al. 2008) removing ultra-planktonic cells with retention efficiency between 72 to 99% (< 10 μm, Pile et al. 1996; Coma et al. 2001; Yahel et al. 2007). Sponges contribute considerably to nutrient cycling; they act as both a sink and/or a source of major dissolved compounds such as DOM, NH_4^+, NO_x^- preventing energy and nutrient losses in the open ocean (Maldonado et al. 2012a and reference therein). More recently it has been proposed that sponges may play a role in organic matter cycling similar to that of the microbial loop; by removing dissolved organic matter from the water column and making it available in form of detritus to higher trophic levels (deGoeij et al. 2013) and simultaneously feed on bacteria. Therefore, the assessment of the rate of water pumped by sponges in nature is crucial to estimate the magnitude of sponge-mediated fluxes of energy and nutrients from/to pelagic-benthic realms, and to better understand their ecological role in geochemical cycles and ecosystem scale process.

Sponge body is entirely specialized in suspension feeding through a unique and highly vascularized canal system that allows them to obtain nutrients and oxygen from the water they actively pump. The water enters the sponge body through the micrometric ostia into the inhalant canals due to the slightly negative

pressure created by the movement of choanocyte flagella, gathered in the choanocyte chambers. After passing the chambers it leaves through the exhalant canals that merge into the excurrent apertures (oscula). This active component is complemented by a passive one generated from the ambient current flowing over the sponge and inducing a gradient of pressure across the sponge wall (Vogel 1974, 1977). The particle capture occurs in the choanocyte chambers, which constitute the basic pumping units operating in parallel (Larsen and Riisgård 1994) and the volume of water pumped is directly correlated to their density (Massaro et al. 2012). Although the efficiency of passive flow depends on morphology of the specimens, it is still a question of debate whether passive flow should be considered as irrelevant, due to the low-energetic cost of pumping (Riisgård et al. 1993; Riisgård and Larsen 1995, 2010), or sponges living in high flow environment may benefit from the current-induced flow due to the expensive process of pumping (Hadas et al. 2008; Leys et al. 2011). Thus sponge pump is not only ecologically relevant to provide central information for energy and organic matter fluxes and nutrient element cycling from/to ecosystem, but it is also a crucial aspect of basic sponge biology for calculation on *in situ* respiration and energy budget.

Given the importance of understanding sponge pump from biological and ecological perspectives, knowledge of natural pumping fluctuation under natural conditions is scarce. The most comprehensive study of *in situ* sponge pump using a direct technique is from Reiswig in 1971b. He was a pioneer in demonstrating changes of pumping activity in Demospongiae, by describing different pumping behaviors of Caribbean species over long periods of measurement. For the first time changes in pump activity were related to temperature changes, storm season and behavioral patterns (*i.e.,* intrinsically generated or day-night cycles). Later on, the majority of studies have measured indirectly the volume of water filtered by the animal in laboratory, by enclosing the organisms in close chambers (Gerrodette and Flechsig 1979; Frost 1980; Riisgård et al. 1993; Thomassen and Riisgård 1995; Turon et al. 1997; Kowalke 2000). These studies estimated the clearance rate, defined as the volume of water cleared of suspended particles per unit of time due to the feeding activity of the organism. Assuming 100% efficiency of particle retention by the animal's filter, clearance rate is equivalent to filtration

rate. This technique, although generally employed, may underestimate the volume of water filtered by the animal, since some particles may overpass the filter system. In addition enclosing a sponge in an incubation chamber causes a reduction of its pumping rate (Parker 1910; Jørgesen 1955; Reiswig 1974; Harrison and Cowden 1976). In the '70s, heated thermistor was the most common *in situ* technique used by Reiswig (1971a,b, 1974, 1981) on several studies on sponge diet, pump and energy requirements, and by Vogel (1977) to test the effect of ambient current on pumping behavior. More recently several studies employed an acustic velocimeter to directly measure the excurrent water velocity *in situ* in depth glass sponges and Caribbean Demospongiae (Leys et al 2011; McMurray et al. 2014; Lewis and Finelli 2015). Another common method is the dye-tracking technique, which consists of videotaping the dye fronts from the excurrent aperture. The fluorescein dye has been applied differently: by injection of dye just below the outer pinacoderm with a syringe (Savarese et al. 1997), by placing a small amount of dyed seawater inside one end of a transparent cylindrical tube placed just above the oscula (*i.e.,* dye front speed, Yahel et al. 2005), by releasing the dyed seawater around the specimen to let the sponge inhale the dyed seawater (Gabrielle et al. 2008; Pfannkuchen et al. 2009) and by releasing concentrated dye solutions directly into the excurrent jet (Weisz et al. 2008). These different techniques were not compared, therefore, there is a critical need for rigorous *in situ* measurements of sponge pump to assess whether the method employed might be the source of the variability recorded in pumping rates.

Sponge pumping behavior is highly variable among species and individuals. Volume flow through sponges may vary from lower value 0.002 L s^{-1} L^{-1} of sponge tissue in *Aplysina lacunosa* (Gerrodette and Flechsig 1979) to 0.58 L s^{-1} L^{-1} of sponge tissue in *Niphates digitalis* (Lewis and Finelli 2015). While some species may maintain the pumping activity fairly constant, periodical interruptions of water transport may occur randomly through the population and irregularly among individuals. Pumping behavior may therefore represent a normal intrinsically generated characteristic of the species over long period of measurements (Reiswig 1971b; Bell et al. 1999). However, even if some pump behaviors were not correlated with any environmental or technical stimuli, the volume of water pumped may depend on external factors, such as temperature and

ambient particulate concentrations. Only few studies have dealt with the effects of temperature on sponge pump, showing a linearly increase of pumping rate with temperature (Reiswig 1971b; Frost 1980; Riisgård et al. 1993). Unfortunately, among them, only one study was performed *in situ*, following the natural temperature fluctuations (Reiswig 1971b), while the others undergo the sponge to temperature changes under laboratory conditions (Frost 1980; Riisgård et al. 1993). Filtration rate was negatively correlated with food concentrations (Frost 1980; Stuart and Klummp 1984; Huysecom et al. 1988) and with suspended sediment (Reiswig 1971b; Gerrodette and Flechsig 1979; Leys and Lauzon 1998; Gabrielle et al. 2008). Again, most of these studies were performed in laboratory where sponges were subjected to elevated bacteria concentrations (from 10^6 up to 10^9 cells mL^{-1}), which rarely occurred in natural environment. Light has been demonstrated to control pumping rate in day-night cycles in *Tethya crypta* (Reiswig 1971b). In addition, endosymbiotic bivalves affect positively the sponge pump in *Spongia* sp. (Tsubaki and Kato 2014), while episymbiotic zoanthids induce a reduction of pumping rate up to 75% in colonized specimens of *Xestospongia muta* and *Niphates digitalis* (Lewis and Finelli 2015). Although the increasing number of literature on pumping activity, the lack of studies on natural fluctuation of sponge pump under natural condition determines necessary to estimate the volume of water pumped by sponges throughout year, to better understand at what extend environmental conditions (*i.e.,* natural fluctuation of temperature and food availability) regulate the pumping rate.

To contribute to the understanding of the physiological responses of sponges under their natural environment, we evaluated activity patterns of water pumped by five sponge species of Mediterranean Sea over two annual cycles. A dye technique was used to quantify the water pumped by these species on a seasonal basis in order to cover the natural range of food abundance and temperature fluctuation. The aim of this study was to examine seasonal variation of *in situ* pumping rate and to distinguish to what extent environmental parameters as food concentration and temperature regulate the water pumped by these species under natural condition. Additionally we validated the dye technique employed in this study by comparing it with a different dye technique previously employed in other studies.

Material and method

Environmental conditions

During each sampling, seawater was collected to estimate total particulate organic carbon (total POC= live + detrital) and pico-and nanoplankton abundances in the water column. *Synechococcus* sp., *Prochlorococcus* sp., autotrophic pico-and nanoeukaryotes and non-photosynthetic bacteria were quantified with flow cytometer (FACSCalibur, Becton-Dickinson, 488 nm excitation blue laser) following the method of Gasol and Moran (1999). Carbon content was then estimated from literature conversion factors as follows: *Synechococcus* sp. 470 fg C $cell^{-1}$(Campbell et al. 1994), pico-and nanoeukaryotes 1540 fg C $cell^{-1}$ (Zubkov et al. 1998), non-photosynthetic bacteria 20 fg C $cell^{-1}$ (Ducklow et al. 1993). Total POC was measured by filtering 500 mL of seawater samples on pre-combusted GF/F glass fiber filters. Filters were then frozen in liquid nitrogen and kept at -80ºC until analysis. Prior to the analysis filters were quick-thawed and folded with vanadium oxide in sterile plaques and left in 37% HCl steam bath for 48 hr and subsequently dried at 60ºC for 24 hr. Organic carbon content was determined with C:H:N autoanalyser (EA 1108 CHNS-O Carlo Erba Instruments). Detrital organic carbon, hereafter detrital POC, was estimated as the difference between the total POC measured on filter analysis and the live carbon estimated from cell counts. Temperature at the study area was continuously recorded by means of using HOBO Pendant Temperature Data Loggers over the study period. The logger was shaded to avoid heating by direct sunlight.

Measurement of seasonal pumping rate

The study was conducted at "Reserva Natural de Montgrí, Illes Medes i Baix Ter" (NW Spain, northwestern Mediterranean Sea, 42°. 06'N, 3°. 21'E). *In situ* pumping rates were measured for five Mediterranean sponge species every 3-degree of natural temperate change (between 13 and 23ºC) from July 2012 to July 2014. The studied Demospongiae species were: *Agelas oroides* (Schmidt, 1864), *Petrosia ficiformis* (Poiret, 1789), *Chondrosia reniformis* (Nardo, 1833), *Crambe crambe* and *Dysidea avara* (Schmidt, 1862). These species were selected because they are all common and widespread habitants of the coralligenous community (Uriz et al. 1992) and represent the most abundant sponge species at the studied

area (Teixidó et al. 2013). Pumping rate was measured using a modification of the dye front speed method (DF) described by Yahel et al. (2005). Briefly, a transparent tube was positioned as close as possible above the sponge osculum and the movement of the dye inside the tube was recorded by a diver using a video camera. To allow normalization of the measured oscular flow to sponge volume and biomass, pumping rate was estimated for haphazardly selected sponge specimens that were visually healthy and possessed only a single osculum. The physical dimensions of each specimens were measured *in situ* to estimate gross sponge volume. Unlike Yahel et al. (2005) the dye was not applied inside the tube, instead the sponges were allowed to inhale small amounts of fluorescein dyed seawater that was released next to the sponge ostia just before the sampling. To insure minimal interference with animals behavior and to avoid deviations from ambient water density, the sodium fluorescein powder was mixed with ambient water drown into the syringe just before sampling. A disposable syringe filter (25 mm, 0.2 μm) was installed on the syringe to avoid the release of dye particles. A frame-by-frame analysis was used to measure the speed of the dye front inside the tube. The rate of water flow from the oscula, hereafter referred to as osculum flow rate (OFR, mL min^{-1}) was calculated following Yahel et al. (2005) as the product of tube cross-section area and the dye front speed, or, in cases where the tube was smaller than the oscula, as the product of dye front speed and oscula area. Three to five replicates were conducted per each specimen at each sampling session. The average value was then divided by sponge ash-free dry weight (AFDW, g) to normalize the rates for comparative analyses (see sponge size section below).

Method comparison

To assess whether the dye speed method (DS) developed in this study might provide different pumping rate estimation from the established dye front speed (DF) method (Yahel et al. 2005), the pumping rate of the five studied species was estimated using both methods. Only specimens with one osculum were selected to allow the flow of the entire sponge to be measured from a single excurrent aperture. At least 17 different specimens, haphazardly selected, were tested for each sponge species with a minimum of 3 replicates per each method employed. To test if the pumping rate significantly differed between the two

methods, a paired t-test was used and the data were log or square root transformed to satisfy the normality assumption and/or heteroscedasticity assumptions. The two methods did not significantly differ in any of the studied species (t-test, $p>0.05$) (Table 1).

Table 1. Dye front speed (DF) and dye speed (DS) measurements (mL min^{-1} cm^{-3} sponge) for each species. Data are expressed as mean ± standard deviation. n= number of sampled specimens; p value obtained by t-test.

Species	n	DF	DS	P
C. reniformis	19	4.46 ± 2.00	4.36 ± 2.01	0.828
P. ficiformis	20	2.41 ± 2.08	2.14 ± 2.57	0.488
D. avara	21	14.42 ± 9.48	13.60 ± 7.64	0.813
A. oroides	22	2.48 ± 2.19	2.01 ± 1.85	0.413
C. crambe	17	25.13 ± 20.53	17.08 ± 14.77	0.107

Determination of sponge size

For at least 30 individuals of each species, the whole sponge size was estimated *in situ* and in lab. The area was estimated *in situ* by taking a picture of the individual with a ruler placed close to it. The area was calculated with the Imaje J program by drawing the perimeter of each individual from the picture. Gross specimen volume was determined by approximating regular geometric shapes to external dimensions gained by direct measurement (height) and by photography (area). Sponge volume was calculated by multiplying the area with height, based on the approximated single or combined geometrical shapes that characterized the five species. Afterwards the specimens were collected, placed individually in plastic bags and transported to the laboratory for size estimations. The sizes of the individuals were expressed in several ways: volume displacement (mL of displaced water in a graduated cylinder); dry weight (DW, 100ºC, 24h); ash dry weight (6h ashing of DW samples in a muffle furnace at 500ºC). The ash-free dry weight (AFDW, g) was calculated by subtracting the ash weight from the sponge dry weight and it represents the estimation of organic matter (OM). At the end of the size laboratory measurements, the relations between sponge volume

measured *in situ* and DW and AFDW were determined in order to relate these variables of body size with the pumping rate estimates conducted in the present study. A detailed description of the experimental procedure and analysis for estimates of sponge biomass can be found in annexe I.

Statistical analysis

Multiple regression analysis was used to establish the percentage variance of the estimated mass-specific pumping rate (PR, mL min^{-1} sponge $mass^{-1}$) and independent factors monitored during each sampling: water temperature (ºC), pico-and nanoplankton abundances (cell mL^{-1}), detrital POC (µg L^{-1}) and sponge size (g, AFDW). The percentage of the variance in PR that was accounted by variation in each environmental variable was estimated using the partial R^2 of the multiple regression. Variables were square root transformed and log-transformed to satisfy the normality and /or heteroscedasticity assumptions and complete residuals analysis was performed to validate the robustness of the resulting model. Statistical analyses were made with SigmaPlot.

Temperature is considered as key factor controlling physiological processes of poikilotherms, and the variation in a rate over 10ºC temperature increase (Q_{10}) is used as a reference. When temperature differed by <10ºC, the temperature coefficient Q_{10} values were estimated by applying Van't Hoff's formula (Clarke 1983):

$Q_{10}= [PR_{T2}/PR_{T1}]^{[10/T_2-T_1]}$

Where PR_{T1} and PR_{T2} are the pumping rates (mL min^{-1} g $AFDW^{-1}$) at temperatures T_1 and T_2.

Sample size

The standard error (SE)-sample size function was used to determine the minimum sample size needed to examine pumping rate. A total of 54 specimens of *C. reniformis* were haphazardly selected. The recorded values were recombined into sample groups of increasing size from 1 to 18. The SE as a proportion of the mean of the specific sponge pumping rate decreased quickly with the increasing of the sample size (Figure 1) and their stabilization was obtained at a sample size of

8, where the variance became approximately about 5-10%. These results allowed us to determine the sample size needed to represent the specific pumping rate of the five species at each sampling time. On the basis of these results we tried to sample at least 12 different specimens for each sponge species with a minimum of 3 replicates per specimen. Due to the difficulty of sampling during the winter months and the occurrence of arrest of pumping in some specimens (*i.e.,* the osculum was closed) a minimum of 8 specimens were sampled in some cases.

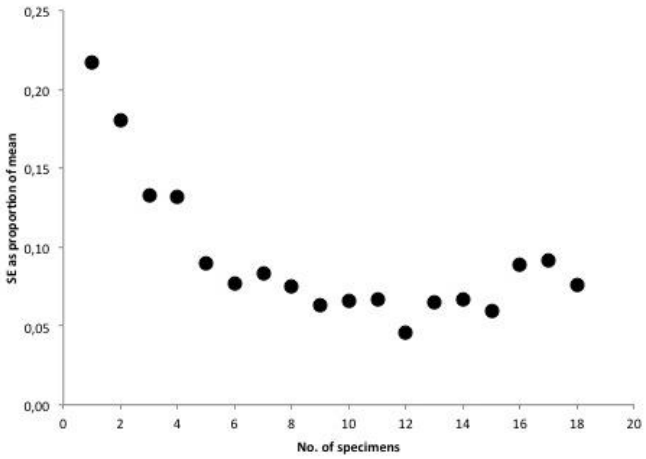


Figure 1. Variation in SE as a proportion of mean with sample size (No. of specimen) to determine minimum sample size for the species pumping rate in a particular session.

Results

Environmental conditions

Mean annual seawater temperature at 5 m depth did not vary among the sampling years (mean ± SD: 17.18 ± 0.81ºC). Temperature exhibited a seasonal pattern, with minimal values (12-14ºC) from December to March and maximal values (20-23ºC) during the summer months from June to September. The potentially particulate food resources for the study species at the study site were quantified as detrital organic carbon (µg L^{-1}) and pico-and nanoplankton abundances (cell mL^{-1}) (Figure 2). Detrital POC represented the main organic C fraction and it was about an order of magnitude higher than plankton C units (data not shown). Suspended detrital POC exhibited marked seasonal pattern with high values in spring and low levels in winter (Figure 2a). The live particles represented

about 10% of the particulate organic C fraction and the different planktonic groups varied in abundances and seasonal patterns. Non-photosynthetic bacteria were the most abundant throughout the sampling period (mean ± SD: 2.25 ± 0.90 x 10^5 cell mL^{-1}). They followed a marked seasonal pattern with high values in spring and early summer, with a maximum registered in May 2013 (mean ± SD: 4.39 ± 0.46 x 10^5) and they rapidly decreased in late summer-autumn (Figure 2b). *Synechococcus* sp. was the second most abundant group (mean ± SD: 1.45 ± 0.7 x 10^4 cell mL^{-1}) and, similarly to non-photosynthetic bacteria, it was more abundant in spring-summer, with lower values in winter (Figure 2b). *Prochlorococcus* sp. (mean ± SD: 3.45 ± 3.83 x 10^3 cell mL^{-1}) exhibited a different seasonal pattern, with high values in autumn-winter and almost absent in summer. This is in accordance to its seasonal pattern (Ribes et al. 1999b) (Figure 2b). Autotrophic pico-and nanoplankton represented the lowest fraction in terms of abundances among pico- and nanoplankton communities (mean ± SD: 1.6 ± 1.04 x 10^3 cell mL^{-1}). Pico-eukaryotes followed a marked seasonal pattern, with high values in winter and low levels in summer (Figure 2c). In contrast, nano-eukaryotes exhibited little seasonal variation, with only a pick observed in spring 2013 (Figure 2c).

Sponge size

Sponge volume determined from sponge dimensions measured *in situ* showed a high degree of correlation (R^2 >0.70) with their lab-measured volume (see annexe I). The relationship between sponge volume estimated *in situ* as independent variable and their dry weight and ash-free dry weight as the dependent variables were best fitted by a power function yielding R^2 ≥0.84, indicating that sponge volume measured *in situ* accurately predicted sponge biomass. Ash-free dry weight (AFDW, g) was estimated according to the relation found with sponge volume (V, cm^3) measured *in situ* as follow: AFDW= 0.31 $V^{0.77}$ (R^2=0.84, n=40, range 2 to 218 cm^3) in *C. reniformis*; AFDW= 0.15 $V^{0.79}$ (R^2=0.89, n=30, range 4 to 395 cm^3) in *A. oroides*; AFDW= 0.22 $V^{0.78}$ (R^2=0.94, n=30, range 1 to 358 cm^3) in *P. ficiformis*; AFDW=0.06 $V^{0.70}$ (R^2=0.84, n=31, range 0.4 to 19 cm^3) in *C. crambe*; and AFDW=0.004 $V^{1.35}$ (R^2=0.93, range 3 to 145 cm^3) in *D. avara*.

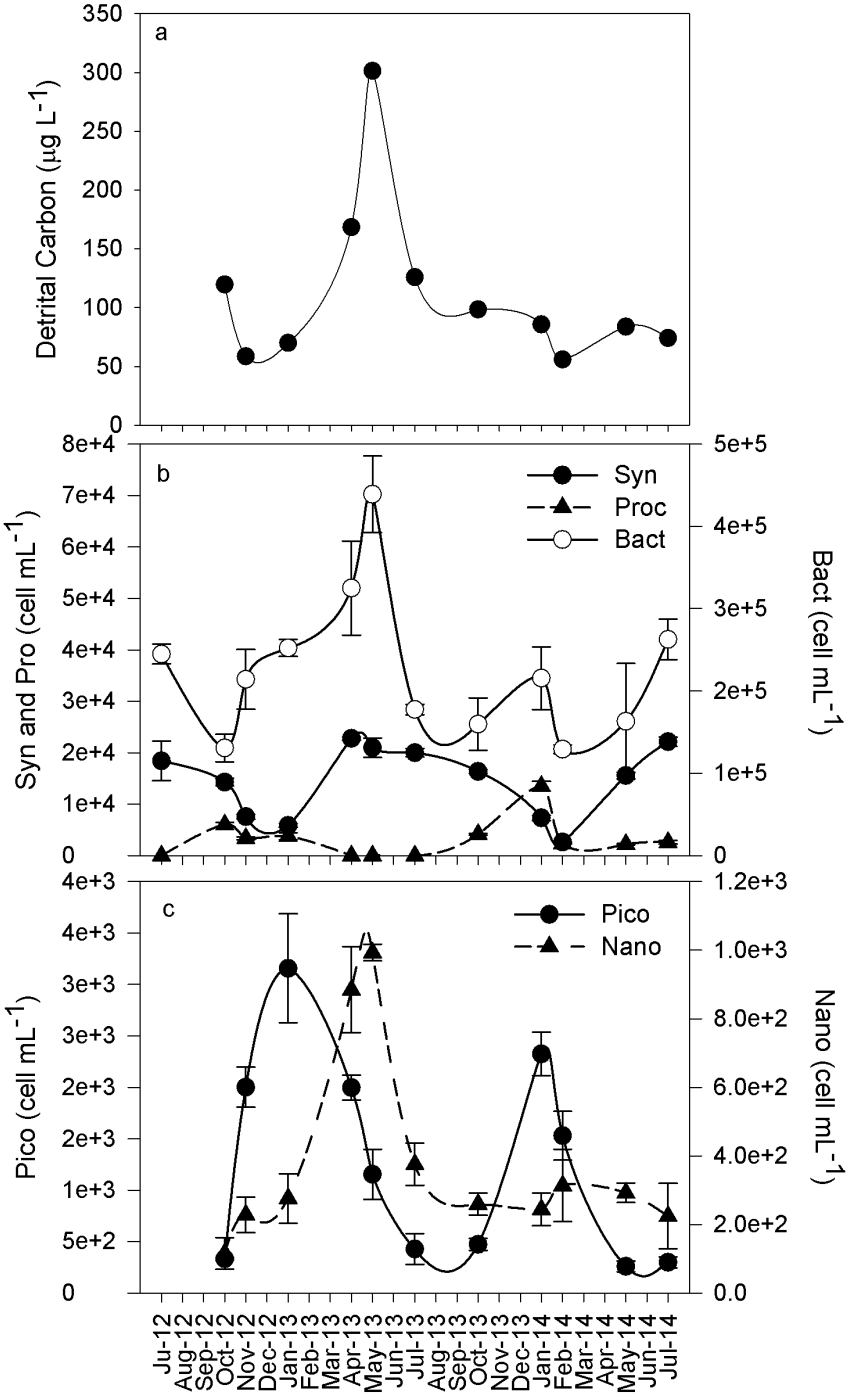


Figure 2. Seston composition over the sampling period (from July 2012 to July 2014) in the study area and its seasonal variation in the water column: a) detrital particulate organic carbon (detrital POC, µg L^{-1}), b) *Synechococcus* sp. (Syn), *Prochlorococcus* (Proc) and non-photosynthetic bacteria (Bact) cell concentration (cell mL^{-1}), c) pico-eukaryotes algae (Pico) and nano-eukaryotes algae (Nano) cell concentration (cell mL^{-1}). Data expressed as mean ± SD.

Sponge pumping rate

Within the natural ranges, this study allowed us to examine to what extend food abundance, temperature and sponge size control the variation in mass-specific pumping rate (PR, mL water pumped min^{-1} g $AFDW^{-1}$). For all species, sponge size explained a significant portion of the variance in PR (Table 2), exhibiting a clear pattern of decrease in PR with sponge size that was best fitted by the allometric function ($Y=aM^b$) (Table 3). This effect was highly pronounced for sponges below 10 cm^3 in *A. oroides*, *C. reniformis*, *P. ficiformis*, *D. avara*, and below 2 cm^3 in *C. crambe*. It is notable that the exponents (*b*) for all five species ranged from -0.54 to -0.69 (Table 3).

In general, the variation of PR along the year correlated positively with water temperature and planktonic abundances, and negatively with detrital particles. However, PR did not show any significant relationship with detrital particles and planktonic abundances in *C. reniformis* and *D. avara,* and PR did not show any significant relationship with temperature in *C. reniformis*, *D. avara* and *C. crambe* (Table 2). Although the effect of temperature was significant in two out of five of the studied species (multiple regression analysis, $p<0.01$, Table 2), PR did not increase linearly with temperature within the temperature range at the study site (Figure 3). The Q_{10} estimated over the natural temperature range (12ºC to 23ºC) for the studied species was below 2 (ranging from 1 to 1.98). The overall averaged pumping rates and sponge sizes for the five studied species are listed and expressed in different units in Table 4.

Table 2. (a) Multiple regression analysis to estimate the variance of specific pumping rate (mL min^{-1} g $AFDW^{-1}$) explained by temperature (ºC), AFDW ln-transformed (g), pico-and nanoplankton concentrations (cell mL^{-1}), detrital POC (µg L^{-1}). Slopes (±SE) for the different species, intercept (A), R squared (R^2) and adjusted R squared (Adj R^2). (b) Standardized regression coefficient.

(a) Variable	***A. oroides***	***C. reniformis***	***P. ficiformis***	***D. avara***	***C. crambe***
Temperature (ºC)	0.12 ± 0.03***	ns	0.08 ± 0.02**	ns	ns
ln AFDW (g)	-1.46 ± 0.11***	-0.85 ± 0.11***	-0.55 ± 0.10***	-0.33 ± 0.02***	-0.19 ± 0.04***
LPKPL (cell mL^{-1})	4.33 ± 1.36E-06**	ns	5.42 ± 1.32E-06***	ns	0.78 ± 0.25E-06**
Detrital POC (µg L^{-1})	-7.57 ± 1.55E-03***	ns	-8.76 ± 1.65E-03***	ns	-1.09 ± 0.32E-03***
A	2.23 ± 0.58***	4.86 ± 0.52***	1.44 ± 0.50**	1.74 ± 0.13***	1.72 ± 0.15***
R^2	0.58***	0.30***	0.33***	0.80***	0.18***
Adj R^2	0.57	0.28	0.31	0.79	0.16

(b) Variable	***A. oroides***	***C. reniformis***	***P. ficiformis***	***D. avara***	***C. crambe***
Temperature (ºC)	0.27***	ns	0.25**	ns	ns
ln AFDW (g)	-0.69***	-0.46***	-0.40***	-0.89***	-0.36***
LPKPL (cell mL^{-1})	0.27**	ns	0.50***	ns	0.36**
Detrital POC (µg L^{-1})	-0.40***	ns	-0.61***	ns	-0.37**

***p<0.001; **p<0.01;*p<0.05 ; not significant (ns).

Table 3. Relationship between specific pumping rate (PR_V mL min^{-1} cm^{-3} sponge) and sponge size (V, cm^3). n: number of sampled specimens over the entire sampling period.

Species	Power function	R^2	p	n
A. oroides	$PR_V = 9.44 \times V^{-0.69}$	0.59	<0.0001	158
C. reniformis	$PR_V = 13.42 \times V^{-0.54}$	0.54	<0.0001	221
P. ficiformis	$PR_V = 4.35 \times V^{-0.58}$	0.47	<0.0001	147
D. avara	$PR_V = 13.96 \times V^{-0.56}$	0.53	<0.0001	144
C. crambe	$PR_V = 15.66 \times V^{-0.66}$	0.41	<0.0001	181

Table 4. Sponge size expressed as volume (V_s), dry weight (DW), ash-free-dry weight (AFDW) and pumping rates of the five species. Data expressed as mean ± SE.

Species	Vs	DW	AFDW	Pumping rate		
#	(mL)	(g)	(g)	mL of water pumped per min, per		
				mL sponge	g DW	g AFDW
A. oroides	6.2 ± 5.4	1.92 ± 1.31	1.30 ± 0.89	5.25 ± 3.91	13.96 ± 8.52	20.58 ± 12.56
C. reniformis	6.9 ± 4.77	3.16 ± 1.63	2.48 ± 1.31	8.5 ± 5	15.94 ± 7.02	20.45 ± 9.17
P. ficiformis	5.2 ± 5.15	3.47 ± 2.63	1.04 ± 0.78	2.95 ± 2.26	3.5 ± 2.18	11.74 ± 7.3
D. avara	1.44 ± 1.69	0.1 ± 0.18	0.04 ± 0.07	26.42 ± 19.99	657.8 ± 666.31	1739.64 ± 1773.22
C. crambe	0.43 ± 0.27	0.10 ± 0.04	0.07 ± 0.03	46.39 ± 26.83	183.94 ± 85.54	260.81 ± 119.86

Because temperature-dependence in viscosity has been documented as a crucial factor affecting the volume of water pumped by ciliary filter feeders (Riisgård and Larsen 2010), we estimated the pumping rates at different kinematic viscosity according to the formula provided by Larsen and Riisgård (2009). Assuming that pumping rate versus the change in kinematic viscosity due to temperature follows a power-law regression with a "b coefficient" = -2 (average of b values reported in Table 1 by Larsen and Riisgård 2009), the estimated pumping rates at different viscosities were calculated and are shown together with those measured in field (Figure 4). The predicted effect of increase in pumping rate with viscosity decrease due to its temperature-dependence, did not match with the observed data.

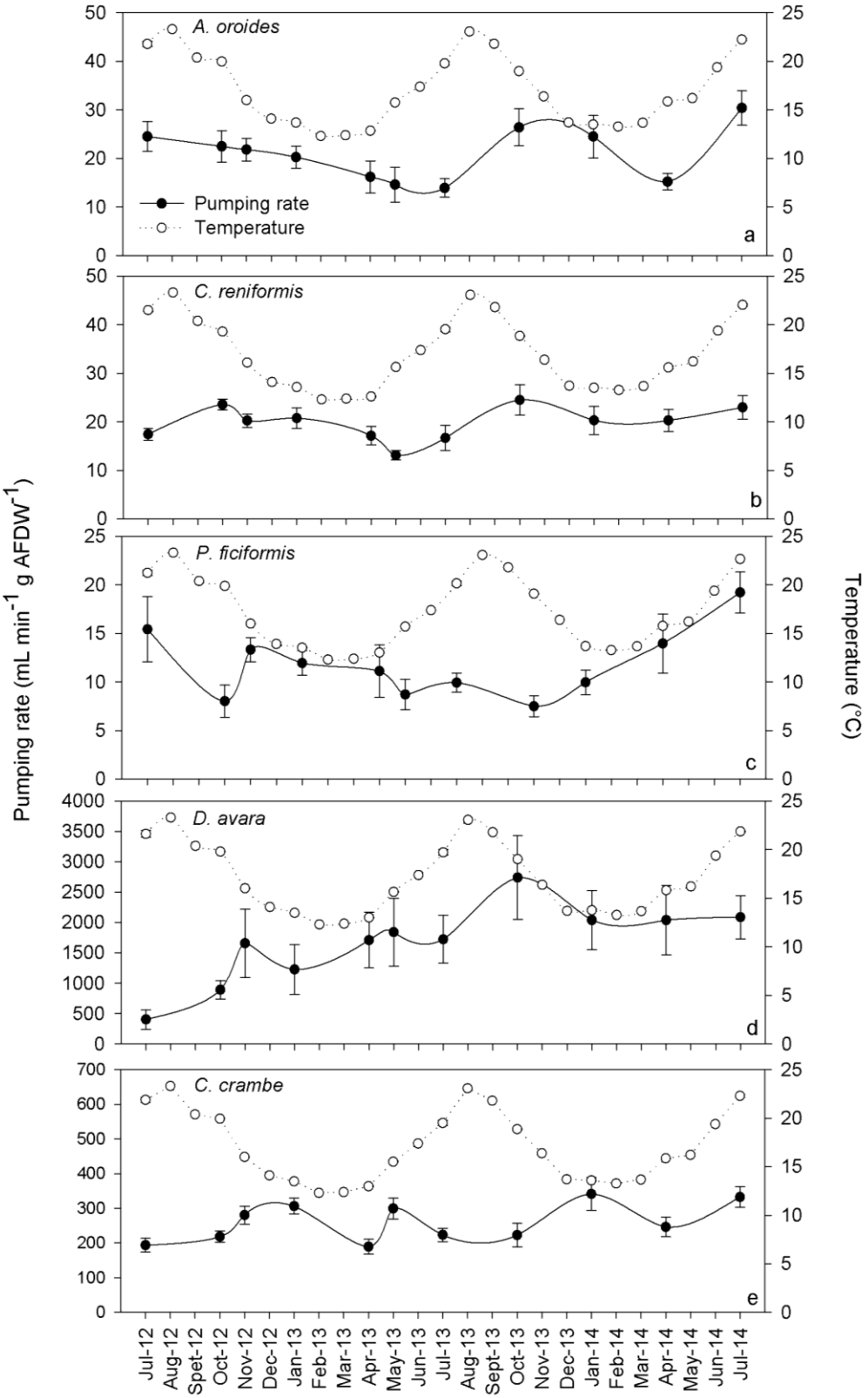


Figure 3. Variation of mass-specific pumping rate (mL min^{-1} g $AFDW^{-1}$) throughout the years. (a) *A. oroides*, (b) *C. reniformis*, (c) *P. ficiformis*, (d) *D. avara*, (e) *C. crambe*. The temperature variation over the annual cycles at the study site is included in each species graph. Data expressed as mean ± SE.

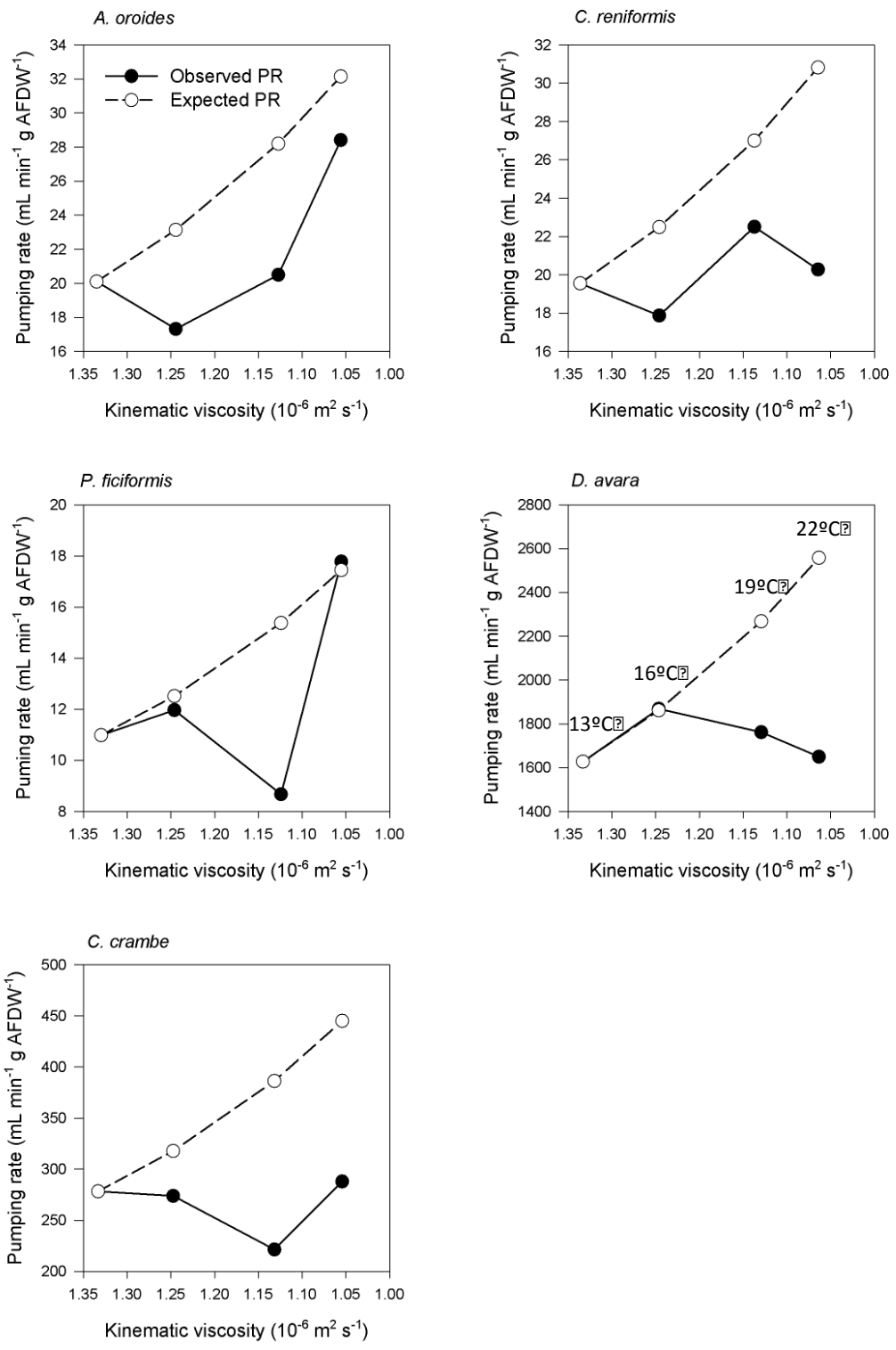


Figure 4. Estimated (empty circle) versus observed (full circle) mass-specific pumping rate (mL min^{-1} g $AFDW^{-1}$) at different kinematic viscosity for the five study species. Expected pumping rate was calculated assuming that pumping rate-viscosity dependence follows a power-law regression with scaling coefficient = -2 (average of coefficients reported in table 1 by Larsen and Riisgård, 2009). Kinematic viscosity was calculated according to the equation expressed by Larsen and Riisgård (2009). Temperatures are indicated at different viscosity.

Discussion

Our knowledge of sponge pump has greatly increased in the last years, however the most comprehensive study of *in situ* sponge pump is dated back from the '70s. Reiswig (1971b) was a pioneer in describing the natural variation of sponge pump of Caribbean species over long periods of measurements. His study demonstrated that sponge-pumping behavior was more complex than previously thought and might be affected by abiotic (*e.g.,* storms, sedimentation, temperature) as well as by intrinsically generated factors (*e.g.,* daily cycles, behavioral patter). Since then other studies investigated the effect of environmental parameters on the sponge pump under natural conditions, but few examined temporal variation of pumping rate over long-term measurements. In this study, we used a dye technique to estimate the volume of water pumped by five of the most prominent sponge species of the coralligenous community in the NW Mediterranean Sea every 3-degree of temperature changes over two annual cycles. By measuring the natural variation of sponge pump under the natural range of conditions to which the organisms are subjected through annual cycles, we obtained the real picture of sponge pump activity likely regulated by the combination of several factors.

Overall, the mean pumping rate values observed in this study were within the range of those reported in the literature for temperate and tropical species. Sponge pump estimated with dye technique varied from 0.02 L s^{-1} (L sponge)$^{-1}$ by *Spongia* sp. (Tsubaki and Kato 2014) to 2.76 L s^{-1} (L sponge)$^{-1}$ by *Xestospongia muta* (Fiore et al. 2013). In this study *D. avara* exhibited higher pumping rate than what previously observed by Ribes et al. (1999a) (0.44 ± 0.33 L s^{-1} (L sponge)$^{-1}$ and 0.02 ± 0.001 L s^{-1} (L sponge)$^{-1}$, respectively). This contrasting result appears to be related to differences in size of sampled specimens, (from 0.01 to 0.6 g AFDW in this study and from 0.3 to 2.3 g AFDW in Ribes et al. 1999a) and to the different method employed (dye speed in this study and clearance rate in Ribes et al. 1999a).

Our study confirms pumping rate as distinctive feature of two different phenotypes: high (HMA) and low (LMA) microbial abundance species. On the basis of electron microscope observations the species studied in this study were classified as HMA (*C. reniformis*, *P. ficiformis*, *A. oroides*) and LMA (*D. avara* and *C. crambe*) (Vacelet

and Donadey 1977; Gloeckner et al. 2014 and references therein). Pumping rate estimated for the three examined HMA ranged from 0.05-0.14 L s^{-1} (L sponge)$^{-1}$, while for the two LMA species ranged from 0.44 to 0.77 L s^{-1} (L sponge)$^{-1}$, and the average annual values indicate that HMA species pumped per unit of volume 85% less water than LMA did. These values are in resemblance with those reported in previous studies that employed a dye technique (0.04-2.76 L s^{-1} (L sponge)$^{-1}$and 0.02-0.58 L s^{-1} (L sponge)$^{-1}$) in HMA and LMA, respectively; Weisz et al. 2008; Fiore et al. 2013). Also Weisz and colleagues (2008) observed that HMA species pumped 52-92% less volume of water than LMA do, and this discrepancy was attributed to different morphological characteristics of the aquiferous system: HMA species are characterized by a denser mesohyl, with narrower and shorter canals, while LMA species are characterized by a more developed acquiferous system with wider and longer canals (Weisz et al. 2008).

Temperature, light intensity, water flow, water viscosity, food concentration, sediment, size have been suggested as factors that affect sponge pump (*e.g.*, Reiswig 1971b; Vogel 1974; Frost 1980; Riisgård et al. 1993; Riisgård and Larsen 1995; Tompkins-McDonald and Leys 2008). High rate of sedimentation (>7 mg L^{-1}) has been documented to determine a reduction of sponge pump and even complete cessation, due to a clogging of the canal system (Reiswig 1971b; Gerrodette and Flechsig 1979; Leys and Lauzon 1998; Yahel et al. 2007; Tompkins-McDonald and Leys 2008). Our study was conducted in low sediment oligotrophic area, and suspended detrital POM was always <0.3 mg L^{-1}. Despite this, the abundance of detrital particles (>0.7μm) was observed to negatively affect the pumping rate of three examined species, which may be a response to avoid clogging of canal system.

Both normal (from 2 to 10 cm s^{-1} up to 50 cm s^{-1} in depth habitat) and artificially intensified (from 2 to 30 cm s^{-1}) ambient flow has been shown to have a positive effect on sponge pump (Vogel 1977; Leys et al. 2011). During short episodes of high ambient current (*i.e.*, >15 cm s^{-1}), flow through the oscula could increase up to 7 folds and sponges could filter up two-thirds of the total volume they processed daily (Leys et al. 2011). Mean water flow around the sponges at our study area is ~ 4 cm s^{-1}, with flow >15 cm s^{-1} only occurring on occasional storms (Mendola et al. 2008). Then, although the ambient flow might increase the volume of water filtered by sponges, in our data we excluded that it could have interfered with pumping rate measurements,

because the dye speed technique employed requires to sample at low flow conditions to accurately estimate the dye speed inside the tube. Therefore we examined whether variation in pumping rate was related to food abundance, temperature and sponge size.

Although food availability has been demonstrated to affect the filtration/pumping rate of sponges and other suspension filter feeders such as polychaetes, bivalves and ascidian (Winter 1973; Frost 1980; Navarro and Winter 1982; Robbins 1983; Stuart and Klumpp 1984; Riisgård and Ivarsson 1990; Riisgård 1991; Petersen and Riisgård 1992; Petersen et al. 1999), we did not observe any specific pattern on the study species within the natural range of food availability. The pattern of decrease of pumping rate with the increase of food concentration observed in previous studies was attributed to a regulation of food uptake, in such a way that the amount of particles filtered remains constant as a protection against overloading of the feeding system (Winter 1973; Navarro and Winter 1982; Robbins 1983; Riisgård and Ivarsson 1990; Riisgård 1991; Petersen and Riisgård 1992; Petersen et al. 1999). In sponges the effect of food concentration on feeding rate was tested only in two laboratory studies in which the specimens were subjected to high bacteria or yest concentrations (Frost 1980; Stuart and Klumpp 1984). The reduction of pumping at high food concentration was attributed to a contraction of some flagellated chambers (Stuart and Klumpp 1984), a blockage of the aquiferous system or a complete contraction of sponges (Huysecom et al. 1988). The former studies, worked at high bacteria and yeast concentration (from 10^6 up to 10^9 bacteria cells mL^{-1}), which was not observed to occur in natural conditions during the two examined annual cycles, nor was previously observed in a previous detailed study of pico- and nanoplankton abundance (Ribes et al. 1999b). Therefore, a relevant effect of food concentration on pumping seems unlikely due to the current natural concentration of pico-and nanoplankton (Ribes et al. 1999b, this study).

Despite the study focus on sampling similar small size specimens to overcome the effect of sponge body on pumping rate (Ribes et al. 1999a), sponge size explained a large portion of the variance in pumping rate. Mass-specific pumping rate decreased with the increase of sponge size following allometric function ($y=M^b$, where y is the mass-specific pumping rate, M is the sponge mass and b is the scaling exponent). In

our results the exponents ranged between -0.54 and -0.66, indicating a large attenuation of pumping with increase in sponge size. Then, according to general model for metabolic scaling (Enquist et al. 2003; Brown et al. 2004) in which the exponent b for mass-specific metabolic rates is about -1/4, our observed values point to an slightly more pronounced effect of pumping rate attenuation with increase in size than predicted. The effect of body size on metabolic processes in sponges is still a subject of debate. Although several authors have also observed a strong negative correlation between pumping and sponge body (Reiswig 1974, 1981; Frost 1980; Riisgård et al. 1993; Ribes et al. 1999a; Yahel et al. 2003), it is largely believed that the power function allometry usually found in metazoans may not be valid in sponges, in which isometric is expected due to their modular construction (Riisgård et al. 1993; Thomassen and Riisgård 1995; McMurray et al. 2014; Lewis and Finelli 2015). Dependence of pumping rate on body size might be a result of reduced surface–volume ratio (Frost 1980) and of a fewer living choanocytes per unit of colony volume in larger sponges (Riisgård et al. 1993). The dependence of pumping rate on body size observed also in the encrusting *C. crambe*, which was supposed to have higher surface-volume ratio and therefore not to be size dependent (de Goeij et al. 2008b), might further suggest that the proportion of the number of chonaocyte chambers on sponge volume might decrease with increase of sponge biomass, resulting in a reduction of number of chonaocyte chambers-volume ratio in large specimens. This hypothesis is supported by the experimental work that demonstrated a positive correlation between pumping rates and choanocyte chamber density (Massaro et al. 2012).

Laboratory experiments and theoretical considerations indicated that pumping rate should be positively correlated with temperature (Riisgård et al. 1993). High Q_{10} reflected an acute response of temperature on clearance rate in *Halichondria panicea* and it was suggested that it could be related to the constriction/dilation of inhalant canals and/or choanocyte chambers (Riisgård et al. 1993). However, in our study, the estimated Q_{10} over the natural temperature range (12ºC to 23ºC) for the studied species was below 2 (ranging from 1 to 1.98) which are below the general values indicated for biological processes (between 2 and 3) and below the values obtained by previous studies on sponge pump (ranged from 2.88 to 25) (Reiswig 1971b; Riisgård

et al. 1993). In other filter feeders the positive correlation between temperature and filtration rate has been attributed to temperature-dependent changes in viscosity (Riisgård and Ivarsson, 1990; Jørgensen et al. 1990; Riisgård and Larsen 2007; Larsen and Riisgård 2009). However, the predicted physical effect of increase in pumping rate with viscosity decrease due to its temperature-dependence, did not match with the observed data. Then, although temperature contributed to some of the variation in pumping rate, it does not appear as a major factor affecting it and suggests that intrinsic factors of the species, such as reproduction, may be involved.

The fact that gametogenesis develops in the pumping units (the choanocyte chambers), converting them into reproductive cells (gametocytes, Bergquist 1978; Simpson 1984) is at the basis of the interference of the reproductive cycle (gametogenesis and spawning) with the rate of pumping. Different studies have reported a disruption of regular sponge histology, called mesohyil disruption, which consists in the disorganization of the canal system due to occupation of choanocyte chambers by reproductive cells during the gametogenesis (Tanaka-Ichihara and Watanabe 1990; Tsurumi and Reiswig 1997; Ereskovsky and Gonobobleva 2000; Riesgo and Maldonando 2008). The density of sexual elements in some sponges can occupy from 6 to 20% of parental volume (Reiswig 1973; Elvin 1976; Di Camillo et al. 2012b). This phenomenon has been documented to cause a reduction of the number of choanocytes chambers in *C. reniformis* (Riesgo and Maldonado 2008). Then, because pumping rate is positively correlated with the number of choanocytes (Massaro et al. 2012), a reduction of their density during the gametogenesis could lead to a decrease of the volume of water pumped by sponges. This hypothesis is supported by the evidence of drastic reduction in pumping activity during the reproductive cycle in the temperate *C. crambe* and tropical *Svenzea zeai* species, attributed to a disorganization of the aquiferous system after larvae release (Turon et al. 1999; Lopez-Legentil and Turon 2010).

The reproductive investment and spawning in most Mediterranean sponges occurs between spring and fall (Coma et al. 2000; Mariani et al. 2005). *Chondrosia reniformis*, *Dysidea avara* and *Crambe crambe* reproduce from June to August (Mariani et al 2005; Riesgo and Maldonado 2008, de Caralt unpublished data), *Agelas oroides* from June to July (Liaci and Sciscioli 1975) and the onset of oogenesis in *Petrosia*

ficiformis has been observed in May although spawning occurs in late autumn-early winter (November-December, Maldonado and Riesgo 2009). Although we did not have any histological evidence of the mesohyl disruption in the studied species during the sampling, the fact that pumping rates are related to the number of choanocyte chambers, a reduction of their density during gametogenesis and spawning could lead to a decrease in pumping rate of the studied species as has been documented in a tropical sponge (López-Legentil and Turon 2010).

In the natural environment organisms are simultaneously exposed to a variety of extrinsic and intrinsic factors. Our results on the estimated pumping in the field appear as the outcome of two counteracting factors: the physical effect of water viscosity and the biological phenomenon of reproductive process. The decrease of water viscosity with temperature increase should theoretically increase pumping, while the reproductive process causing a reduction of the choanocyte chambers decreases pumping. The overlap of the period of higher temperature with that of reproduction in most of the studied species makes it difficult to distinguish the effects of temperature and reproduction on pumping rate in the field. Their opposite effect is conducive to a overall constant pumping rate of the species throughout the year.

Chapter 4

In situ respiration rate and carbon balance over annual cycle

Introduction

Metabolic rates are used to calculate animal's energy requirements and can be measured as heat released per unit of time, oxygen consumption and carbon dioxide production (Randall et al. 2001). In aerobic organisms, respiration is the catabolic process by which organic molecules are oxidized with releasing energy required for several processes as basal metabolism, locomotion activity and secondary production (*i.e.*, synthesis of new tissue and reproduction). Therefore, fluctuation in respiration might be a complex response to heterogeneous nature of respiratory demand (*e.g.*, cost of different energy demanding processes) and/or external factors (*e.g.*, temperature, food, salinity) (Clarke 1987, 1991). Despite sponges have been the focus of much interest in the past years for their importance in the functioning of ecosystem (Bell 2008; Maldonado et al. 2012a; de Goeij et al. 2013), our knowledge on sponge physiology is still poorly understood (Bell et al. 2015). This might be due to the technical challenges in measuring most physiological processes in vivo and due to complex interaction among host and associated bacteria that makes metabolic processes of the entire holobiont more complex to understand (*i.e.,* oxic and anoxic processes).

Most of the studies in sponges use oxygen uptake as a measure of metabolic rate. The most comprehensive study on metabolism in sponges using a direct *in situ* technique is dated back from Reiswig in 1974. He was pioneer in characterizing the variation of oxygen removal by three tropical Demospongiae with differences in body form, habitat type, and presence of symbionts. Later on, the majority of the studies on respiration in sponges come from measurement of oxygen removal under laboratory condition and, commonly, in metabolic chambers (Cotter 1978; Thomassen and Riisgård 1995; Kowalke 2000; Lohrer et al. 2006; Hadas et al. 2008; Hoffman et al. 2008; Koopmans et al. 2011; Bannister et al. 2012; Tjensvoll et al. 2013; Mills et al. 2014; Kutti et al. 2015). This method, although generally employed may alter animal activity; which may result in several-fold changes in metabolic rate as a reduction of

specific rate of oxygen consumption (*e.g.,* Newell and Northcroft, 1967; Reiswig 1974; Hadas et al. 2008). Experimental chambers have been also occasionally used *in situ* (Coma et al. 2002; de Goeij et al. 2008a; Koopmans et al. 2010). Other studies have measured dissolved oxygen concentration in the sponge tissue using microsensors (Gatti et al. 2002; Hoffmann et al. 2005, 2008; Schläppy et al. 2007, 2010), but only one was carried on *in situ* (Schläppy et al. 2010; Lavy et al. submitted). After Reiswig's work few studies have directly measured *in situ* the oxygen removal by comparing the dissolved oxygen concentration in the incurrent and excurrent water of individual sponges (Yahel et al. 2003; Leys et al. 2011; Khan et al. 2015; Ludeman Master Thesis, 2015). Most of those studies had as main purpose to estimate the carbon demand associated to specific oxygen removal, but they did not examine how environmental parameters might affect respiration rate under natural conditions. Therefore there is a critical need for rigorous *in situ* measurements of respiration to better understand its natural variation and to what extend environmental factors might regulate this metabolic process in sponges.

Sponges obtain continuous nutrient and oxygen from the water by active pumping, however, because diffusive flux is the only mode at which oxygen is made available during inactive periods, sponge tissues can reach suboxic or anoxic condition (Hoffmann et al. 2005, 2008; Schläppy et al. 2007, 2010). Therefore pumping activity is fundamental to supply oxygen to entire sponge body and a major portion of sponge respiratory demand may be probably linked with water transport (Hadas et al. 2008; Hoffmann et al. 2008; Ludeman Master Thesis, 2015). Unfortunately the cause-effect relations between these two processes still remain unclear. To date, whether pumping rate is an energetically costly process is still a question of debate (Riisgård et al. 1993, 1995; Thomassen and Riisgård 1995; Hadas et al. 2008; Leys et al. 2011; Ludeman Master Thesis 2015), and therefore whether an increase of volume of processed water would require an increase of respiratory demand still needs to be elucidated.

Oxygen consumption of sponges shows great variability among species within the same habitat and it can range as an example from 0.71 μmol O_2 h^{-1} per sponge dry tissue in *Tethya californiana* (Luderman Master Thesis 2015) to 95 μmol O_2 h^{-1} per sponge dry tissue in *Haliclona oculata* (Koopmans et al. 2010). Physical factors, as temperature, sediment, ambient flow, light, as well as intrinsic factors, as sponge size,

filtration activity and secondary production, have all been observed to affect respiration of sponges (*e.g.,* Coma et al. 2002; Hoffmann et al. 2008; Schläppy et al. 2010; Koopmans et al. 2011; Tjensvolt et al. 2013). Although temperature usually plays a relevant role in physiological processes of poikilotherms, only two studies have dealt with the effects of temperature on respiration in sponges, showing a linearly increase of respiration (Barthel and Theede 1986; Coma et al. 2002). Unfortunately only one study was performed *in situ*, following the natural temperature fluctuations (Coma et al. 2002), while the other undergo the sponge to temperature changes under laboratory conditions (Barthel and Theede 1986). Sedimentation induces different response in the respiratory activity of the sponges, which can be related to differences in the observed physiological response (*e.g.,* mucus production or decrease of pumping activity) (Lohrer et al. 2006; Bannister et al. 2012; Tjensvoll et al. 2013; Kutti et al. 2015). A drastic reduction of ambient water flow may determine a reduction of oxygenation in sponges, which may be regulated by active compensatory mechanism through increase of pumping (Schläppy et al. 2010). Some species are sensitive of hypoxic conditions (Barthel and Theede 1986), while others can stand at low oxygen levels (Mills et al. 2014). In addition, light may influence the oxygen removal, likely due to changes in photosymbiont activity in sponge tissue (Yahel et al. 2003). In fact, respiration in sponges includes both symbiont and sponge oxygen requirements, therefore different metabolic patterns (*i.e.,* photoautotrophic and heterotrophic) as well as density of associated bacteria in sponge tissue may influence the oxygen removal (Reiswig 1974, 1981). It is therefore expected that HMA species removed with higher efficiency the oxygen from the water than LMA species, likely due to metabolic activities of associated heterotrophic bacteria in sponge tissue (Reiswig 1974, 1981). Overall, these studies have provided an understanding of the physiological and environmental factors that affect respiration in sponges under laboratory conditions. However, under natural conditions the physiological response of an organism is driven by a combined effect of several factors that may likely coupled differently, especially in response to seasonality.

The dynamics of Mediterranean benthic suspension feeders are marked by strict seasonality, in which temperature and food availability have been identified as

crucial environmental factors (Coma et al. 2000; Coma and Ribes 2003). An energetic shortage has been suggested as common mechanism that regulates such seasonal dynamics, as well as main phenomenon that may contribute to understanding of massive mortality events, which affected in particular sponges and gorgonians in NW Mediterranean Sea in last decades (Coma et al. 1998; Coma and Ribes 2003). Summer period results unfavorable season for many Mediterranean benthic feeders, due to high temperature and stratification of water column, which reduces food concentration above the thermocline (Coma et al. 2000; Coma et al. 2009). Such characteristics, especially if prolonged, may lead the organisms to energy constrain as a result of reduction in energy intake (*i.e.,* food shortage) and/or an increase in energy output (*i.e.,* respiration expenditure). Sponges appear to be limited during the summer due to an increase of energy expenditure for respiration (Coma and Ribes, 2003). The strong temperature-dependence of respiration rate observed in *Dysidea avara* (Coma et al. 2002) might lead to energetic limitation during exceptional warmer and longer periods. Previous studies observed that a relatively large part of carbon gained by sponges is used for respiration (40-90%) (Barthel 1988; de Goeij et al. 2008b; Koopmans et al. 2010, 2011). Therefore an increase of this metabolic process during warmer months might affect the energy budget of sponges by reducing the energy available for secondary production (*i.e.,* growth and reproduction), which generally occurs during the spring-summer (Liaci and Sciscioli 1975; Mariani et al 2005; Riesgo and Maldonado 2008). The respiratory cost associated with net growth in sponge may be high (139%) (Thomassen and Riisgård 1995) and therefore the low level of energy that would be potentially available for growth might be a limiting factor. In this sense, the carbon gained may not meet the specific oxygen demand for maintenance, pumping and growth as observed in some laboratory and *in situ* studies (Reiswig 1974, 1981; Thomassen and Riisgård 1995; Yahel et al. 2003; Hadas et al. 2009). Hence, the variation of respiration rate may have an important consequence for energy expenditure associated to this metabolic process and together with feeding rate may provide a preliminary energy budget that might contribute to the understanding of the seasonal dynamics of sponge species and likely elucidate the causes of mortalities.

To date, the mismatch between carbon ingested from the particulate fraction and respiration tends to be larger in HMA than LMA species (reviewed by Maldonado et al. 2012a). This discrepancy may be due to specific energetic constrained or lack of quantification of entire carbon pool available for sponges. The heterogeneous diet (Ribes et al. 1999a) together with their feeding plasticity (McMurray et al. 2016; Chapter 2) may represent an advantage for sponge, because they can attenuate the effects of seasonal fluctuation in relation to food availability (*e.g.,* planktonic community). Nevertheless the different metabolic profiles observed in HMA (*e.g.,* low rate of filtration, main nutrient input from dissolved fraction) and LMA species (*e.g.,* high rate of filtration, main nutrient input from particulate fraction) can reflect two different limited energetic profiles as a consequence of alternative adaptive strategies to the same habitat.

Given the importance of understanding respiration in sponges from physiological and energetic perspectives, and given the lack of knowledge of natural fluctuation of respiration rate under natural conditions; a detailed *in situ* seasonal study of respiration rate throughout the year determines necessary to contribute to the understanding of the physiological responses of sponges and seasonal energetic dynamics in relation to environmental fluctuations.

The aim of this study was to understand the energetic dynamics of five of the most prominent sponge species of NW Mediterranean Sea and to assess whether different limited energetic profiles are related to HMA and LMA dichotomy. To address the hypothesis of energetic constraints during the warmer period as main mechanism driving seasonal dynamics, we examined the energy ingestion and output through the year. The present work deals with a quantitative analysis of respiration and synthesis of previous feeding rate results (Chapter 2 and 3) to derive a partial energy budget of the studied species through the year.

Material and method

Respiration rate measurement

The study was conducted at "Reserva Natural de Montgrí, Illes Medes i Baix Ter" (NW Spain, northwestern Mediterranean Sea, 42°. 06'N, 3°. 21'E). *In situ* respiration rates were measured for the five studied Mediterranean sponge species

every 3-degree of natural temperate change (between 13 and 22ºC) from July 2013 to July 2014. Respiration rate was estimated for haphazardly selected sponges specimens with a single osculum to allow estimating the oxygen removal of the entire sponge to be measured from a single excurrent aperture. The oxygen removal was measured following the InEx method designed by Yahel et al. (2005), which consists on a simultaneous collection of paired water samples from the water inhaled and exhaled by the studied organism. Water samples were collected by scuba-divers in two open-ended tubes with special matched plastic stoppers at the extremes of each tube and with a dissolved oxygen spot-sensor (Presens.de) in one of them. Samples of exhaled water were collected passively, letting the exhaled water jet to flush and replace the ambient water within the collecting tube. The tube was held aligned with the exhalent jet and as close as possible (<2mm) to the excurrent aperture (osculum), avoiding contact or disturbance of the animal. Inhaled water samples were drawn slowly into an identical tube with an attached syringe and held next to the sponge inhalant apertures. The tubes were transported to the boat submerged in ambient water to keep constant temperature. Oxygen concentration (µmol L^{-1}) of the tubes was measured by an optical oxygen Meter (Fibox 4, Presens.de). For each sampling at least 12 individuals for each species were sampled. Three replicates of InEx pairs of water samples were carried out on each specimen. The different oxygen concentration between the InEx pair of water samples provided a measure of oxygen removal, which was determined as the mean value of the three replicates for each individual. Only the mean values that showed oxygen removals were used for the analysis, therefore the number of analyzed specimens were occasionally reduced. Estimates of volume of seawater processed by sponges with dye technique were carried out after collecting the InEx samples. Respiration rate was then estimated by multiplying the oxygen removal (µmol O_2 L^{-1}) with the volume of water pumped (ml min^{-1}) (Chapter 3). Sponge physical dimensions were measured *in situ* to estimate their gross volume and ash-free dry weight was estimated by conversion factor provided in the annexe I.

Food availability

The particulate (POC) and dissolved (DOC) organic carbon were examined as potentially available food source for the sponge species and were used for the

estimates of carbon budget. During each sampling, seawater was collected to estimate total particulate organic carbon (total POC= live + detrital) and pico-and nanoplankton abundances in the water column. *Synechococcus* sp., *Prochlorococcus* sp., autotrophic pico-and nanoeukaryotes and non-photosyntetic bacteria were quantified with flow cytometer (FACSCalibur, Becton-Dickinson, 488 nm excitation blue laser) following the method of Gasol and Moran (1999). Carbon content were then estimated from literature conversion factors as follows: *Synechococcus* sp. 470 fg C $cell^{-1}$ (Campbell et al. 1994), pico-and nanoeukaryotes 1540 fg C $cell^{-1}$ (Zubkov et al. 1998), non-photosynthetic bacteria 20 fg C $cell^{-1}$ (Ducklow et al. 1993). Total POC was measured by filtering 500 mL of seawater samples on pre-combusted GF/F glass fiber filters. Filters were then frozen in liquid nitrogen and kept at -80ºC until analysis. Prior to the analysis filters were quick-thawed and folded with vanadium oxide in sterile plaques and left in 37% HCl steam bath for 48 hr and subsequently dried at 60ºC for 24 hr. Organic carbon content was determined with C:H:N autoanalyser (EA 1108 CHNS-O Carlo Erba Instruments). Detrital organic carbon, hereafter detrital POC, was estimated as the difference between the total POC measured on filter analysis and the live carbon estimated from cell counts. DOC concentrations were obtained from Aparicio and colleagues (in prep.) from the Estartit Oceanographic Station (EOS).

Data analysis

Multiple regression analysis was used to establish the percentage of variance in the respiration rate (µmol O_2 h^{-1} sponge $mass^{-1}$) through the year that could be explained by independent factors monitored during each sampling: water temperature (ºC, recorded using an Onset HOBO Pendant Temperature Data Logger, UA-002-64, Onset Computer Corporation), sponge size (g, AFDW) and volume of pumped water (mL g $AFDW^{-1}$ h^{-1}). Variables were log or square root transformed to satisfy the normality and/or heterosdacity assumptions.

The temperature coefficient Q_{10}, which determines the rate of change of a biological or chemical process as consequences of increasing the temperature by 10ºC, was calculated by applying Van't Hoff's formula when temperature differed by <10ºC, (Clarke 1983):

$Q_{10}= [R_{T2}/R_{T1}]^{[10/T_2-T_1]}$

Where R_{T1} and R_{T2} are the respiration rates (μmol O_2 h^{-1} sponge $mass^{-1}$) at temperatures T_1 and T_2 (from 13 to 22ºC).

The rate of oxygen consumed per sponge mass (hereafter refers as mass-specific respiration rate, μmol O_2 h^{-1} sponge $mass^{-1}$), was expressed in terms of carbon by considering respiratory quotient (RQ) of 0.9 (Muscatine et al. 1981; Fang et al. 2014) and converted to carbon equivalent (0.375). The respiratory quotient of 0.9 has been used as conservative value among those reported in the literature and accordingly to the mixed nature of sponge diet (algae, bacteria, protein and fatty acid) (1.4, Leys et al. 2011; 1, Maldonado et al. 2012a and deGoeij et al. 2008b; 0.9 Fang et al. 2014; 0.75 Koopmans et al. 2010, 2011).

F/R ratio (L $(mLO_2)^{-1}$, liter of water filtered per mL of oxygen consumed) was calculated to examine the adaptation of filter feeders to their environment with different plankton availability (Thomassen and Riisgård 1995; Riisgård and Larsen 2000; Riisgård et al. submitted). F/R ratio was used to estimate the food content of suspended particles that should be present in the water filtered by the sponges to cover a consumption of 1 mL O_2 (equivalent to 0.46 mg C, Jørgensen 1955). Assuming 100% particle retention efficiency, the particulate organic carbon content should be above 0.46/liters of water filtered (Riisgård et al. submitted)

The I/R index, calculated as the ratio between energy gain (I: estimated as ingested carbon in μmol C) and the cost of respiration (R: estimated as oxygen removed in μmol O_2), was examined to assess the energetic dynamics of the species over the year (Maldonado et al. 2012a). Assuming a respiratory quotient of 0.9, I/R index smaller than 0.9 may indicate carbon shortage for maintenance and growth, while I/R index above 0.9 indicate a positive energy surplus, during which specimens can invest in somatic growth, reproduction and energy storing. Retention rate data for each species come from previous studies (Chapter 2) and was combined with pumping rate data (Chapter 3) to calculate the carbon ingestion rates over the annual cycle according to the food availability in the water column.

Results

Respiration rate measurements

The amount of dissolved oxygen removed by the five sponges during a single transit of water through the canal system was 3.91 ± 0.36 μmol O_2 L^{-1} (overall five species annual average ± SD). Sponges extracted dissolved oxygen per unit of water transport with same efficiency (about 1.5%) irrespective of the microbial composition in their tissue. In contrast, the annual average respiration rate was 6 times higher in LMA than in HMA species (8.80 ± 4.99 μmol O_2 h^{-1} (mL sponge)$^{-1}$ and 1.45 ± 0.83 μmol O_2 h^{-1} (mL sponge)$^{-1}$, respectively). Among the studied species, *C. crambe* (LMA) showed the maximum annual average value (12.33 ± 18.37 μmol O_2 h^{-1} (mL sponge)$^{-1}$), while *P. ficiformis* (HMA) showed lowest respiration rate (0.67 ± 1.07 μmol O_2 h^{-1} (mL sponge)$^{-1}$), removing three times less oxygen per mL sponge than other HMA did. The averaged annual mass-specific respiration rates observed in this study are shown in different units in Table S1.

Respiration rates significantly varied with temperature (multiple regression analysis $p<0.001$, Table 1) in all studied species, following a marked seasonal pattern (Figure 1). Respiration rates increased linearly with temperature within the temperature range at the study site (Figure S1). From 13ºC to 22ºC, a Q_{10} of 24.3, 14.7, 9.9, 5.8 and 3.4 was estimated for *D. avara*, *P. ficiformis*, *A. oroides*, *C. crambe* and *C. reniformis*, respectively. The volume of water processed by *C. reniformis*, *P. ficiformis* and *D. avara* explained a significant portion of the variance in respiration (multiple regression analysis $p<0.05$, Table 1), a correlation that is not observed in *C. crambe* and *A. oroides* (multiple regression analysis $p>0.05$, Table 1). In the latter two species, a linear regression analysis performed at the different sampling time indicated that pumping rate and respiration rate were significantly correlated only on July 2013 (19.94 ± 0.97°C) for *A. orodies* (linear regression analysis $R^2=0.46$, $p=0.015$) and on October 2013 (18.85 ± 0.21°C) for *C. crambe* (linear regression analysis $R^2=0.85$, $p<0.001$). In none of the five studied species, sponge size explained a significant portion of the variance in respiration (multiple regression analysis $p>0.05$, Table 1), nevertheless a general patter of decrease in respiration with sponge size was observed (Figure 2). In *C. reniformis* and *D. avara*, this general pattern was best fitted by the allometric function although the % of variable explicated was low ($R^2 \leq 0.30$).

Table 1. (a) Multiple regression analysis to estimate the variance of respiration rate (µmol O_2 g AFDW^{-1} h^{-1}) explained by temperature (ºC), AFDW ln-transformed (g), and volume of water pumped expressed as mass-specific pumping rate (mL g AFDW^{-1} h^{-1}). Slopes (±SE) for the different species, intercept (A), R squared (R^2) and adjusted squared (Adj R^2). (b) Standardized regression coefficient (each variable can be standardized by subtracting its mean from each of its values and then dividing these new values by the standard deviation of the variable). ***p<0.001; **p<0.01;*p<0.05 ; not significant (ns).

(a)

Variable	***A. oroides***	***C. reniformis***	***P. ficiformis***	***D. avara***	***C. crambe***
Temperature (ºC)	0.21 ± 0.05***	0.15 ± 0.04***	0.22 ± 0.06***	0.47 ± 0.07***	0.30 ± 0.07***
ln (g AFDW)	ns	ns	ns	ns	ns
PR (mL h^{-1} g AFDW^{-1})	ns	8.3 ± 2.0E-04***	1.05 ± 0.30 E-03**	8.4 ± 3.5 E-06*	ns
A	- 3.36 ± 0.79***	-2.42 ± 0.72**	-4.70 ± 1.06***	- 3.99 ± 1.76*	ns
Rsqr	0.38***	0.50***	0.41***	0.55***	0.28**
Adj Rsqr	0.35	0.47	0.37	0.52	0.24

(b)

Variable	***A. oroides***	***C. reniformis***	***P. ficiformis***	***D. avara***	***C. crambe***
Temperature (ºC)	0.54***	0.42***	0.46***	0.68***	0.55***
ln (g AFDW)	ns	ns	ns	ns	ns
PR (mL h^{-1} g AFDW^{-1})	ns	0.49***	0.48**	0.42*	ns

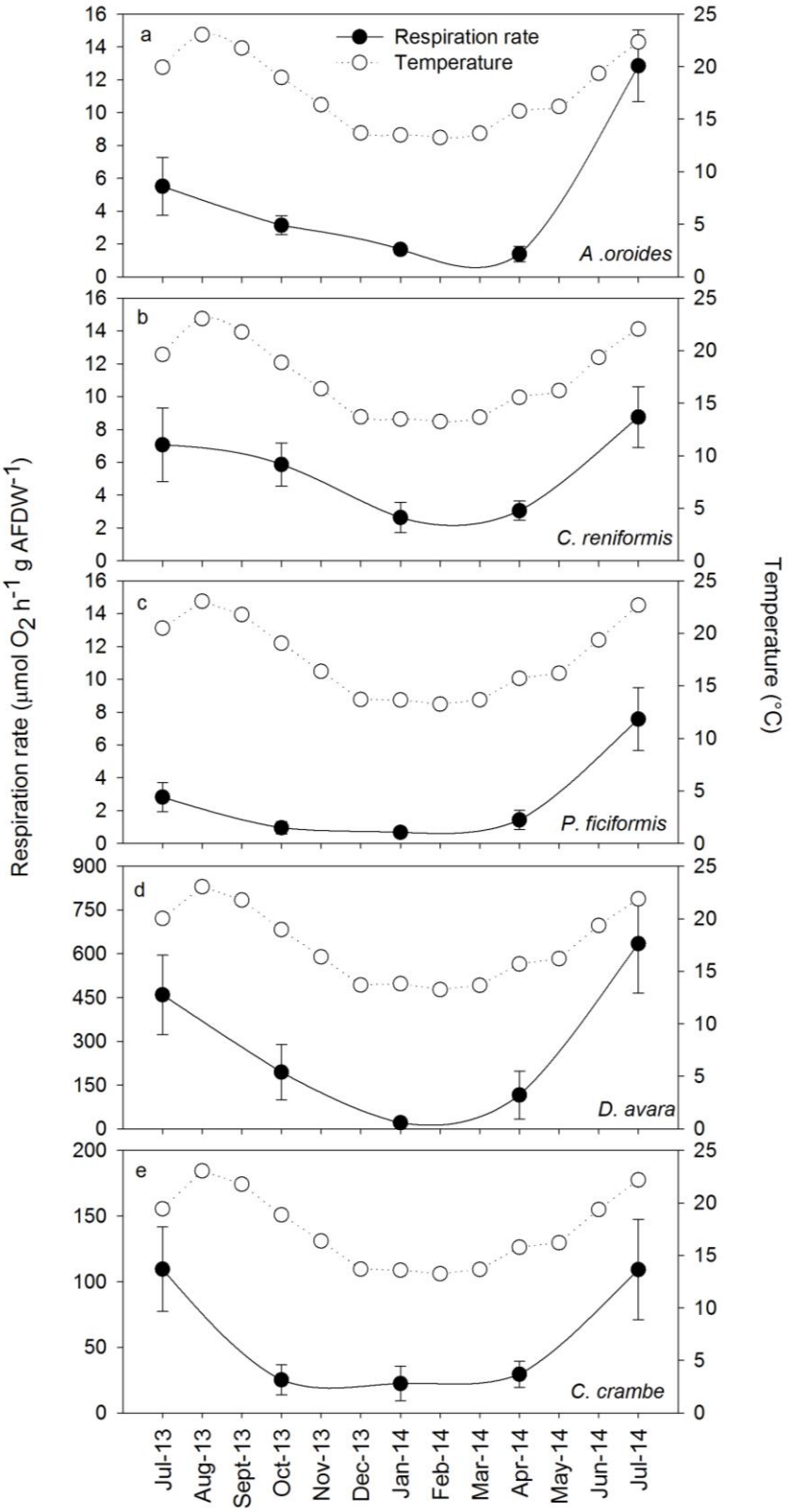


Figure 1. Variation of respiration rate (µmol O_2 g AFDW^{-1} h^{-1}) throughout the year. (a) *A. oroides*, (b) *C. reniformis*, (c) *P. ficiformis*, (d) *D. avara*, and (e) *C. crambe*. The temperature variation is included in each species graph. Data are expressed as mean ± SE.

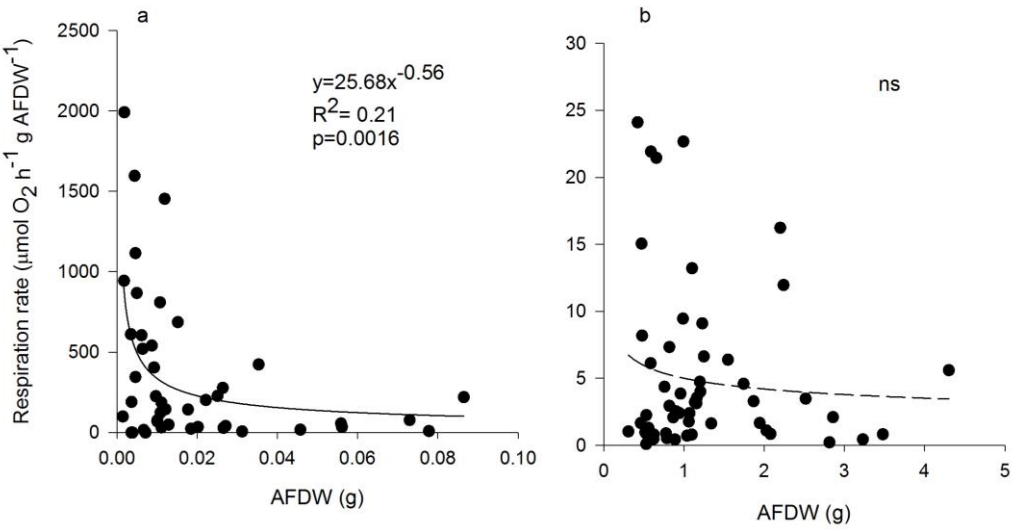


Figure 2. The relationship between size-specific respiration rate (µmol O_2 h^{-1} g $AFDW^{-1}$) and specimen size (g, AFDW): (a) the pattern of decrease of respiration with sponge size was significantly fitted by power function in *D. avara* (the equation is reported in the plot), (b) any significant function was observed in *P. ficiformis*, but caution must be exercised in interpretation.

Energetics

The total (live + detritus) particulate organic carbon available for the sponges through the year was 0.10 ± 0.02 mg L^{-1} (annual average ± SD) and the live fraction represented solely 12 ± 3% of the total POC. Live C followed a marked seasonal pattern, with high values in early summer (0.015 ± 0.002 mg C L^{-1}) and rapidly decreased in late summer-autumn reaching low concentration in winter (<0.001 mg C L^{-1}). For a detailed description of seasonal fluctuation of different planktonic groups that represented the pool of live C at the study site see result section chapter 3.

The annual average F/R ratio of 86.68, 71.10, 48.75, 39.18, 21.74 was estimated for *C. crambe*, *D. avara*, *P. ficiformis*, *A. oroides* and *C. reniformis*, respectively (Table 2). Over the annual cycle the F/R ratio showed a maximum during the winter in correspondence of both lower planktonic concentration and lower water temperature, and low values during the summer in the five studied species (Figure 3).

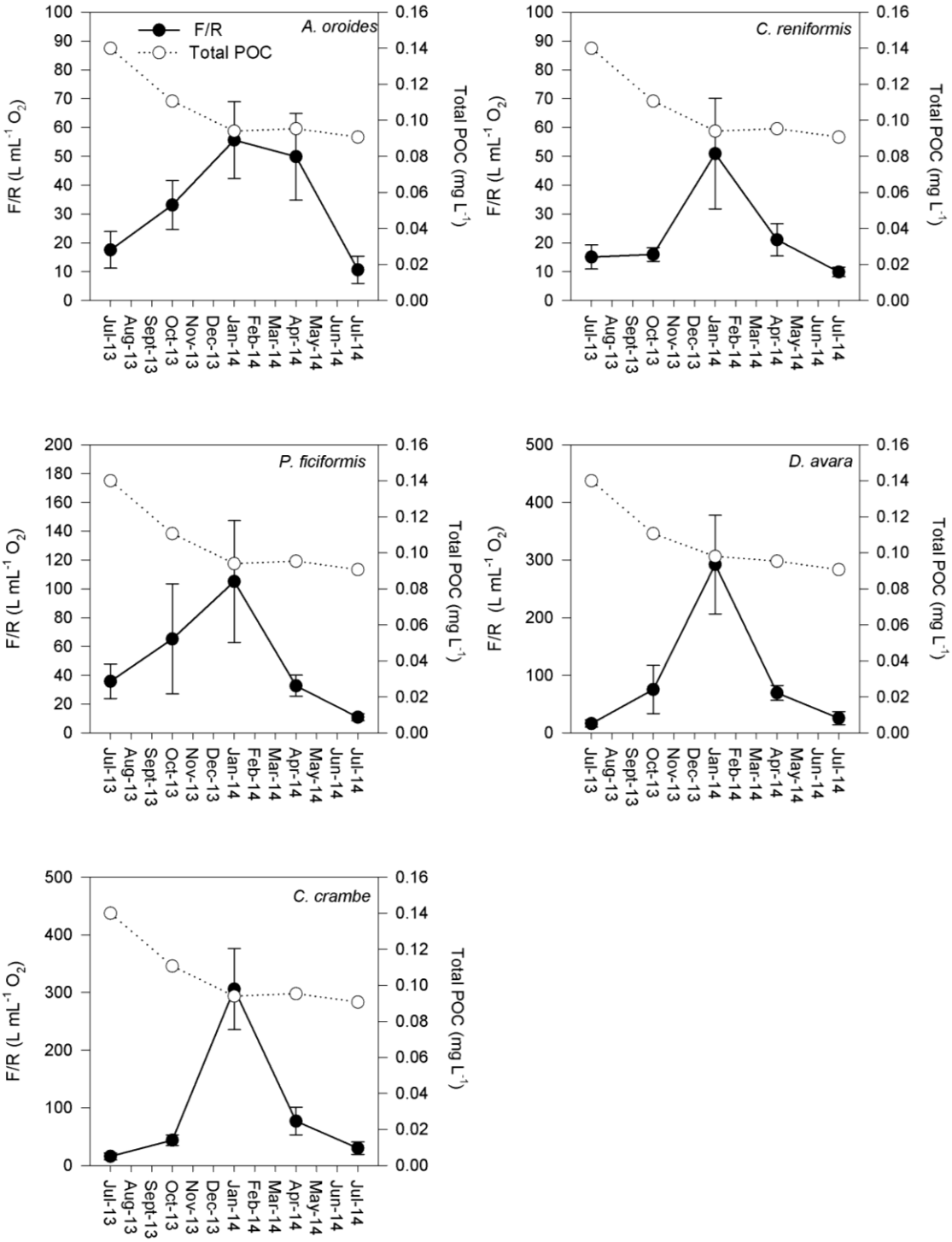


Figure 3 Variation of F/R ratio (liters of water filtered per mL of oxygen removed, L mL^{-1} O_2) throughout the year for the five studied sponge species. The total POC (live + detrital carbon) is included in each species graph. Data are expressed as mean ± SE.

Figure 4 shows the I/R index for the five studied species. Assuming RQ= 0.9, I/R index below 0.9 indicated that not enough carbon is taken up to account for the carbon dioxide formed through respiration. All sponges appeared to be energetically limited in July 2014 (I/R <0.9), but not the same pattern was observed in previous summer, which corresponded to a favorable period for *C. reniformis*, *A. oroides* and *P. ficiformis*. In general winter and spring appeared to be favorable periods for all sponge species, except for *C. reniformis* that showed an evident imbalance between carbon ingestion and respiration through the year, especially accentuated in early autumn and summer (only July 2014). During the summer 2013 DOC represented about 72 ± 22% of C ingested by the studied species, while for the rest of the year it was not available (*i.e.,* low ambient water concentration and/or origin and composition of molecules) for all the species except for *P. ficiformis* (Figure 5).

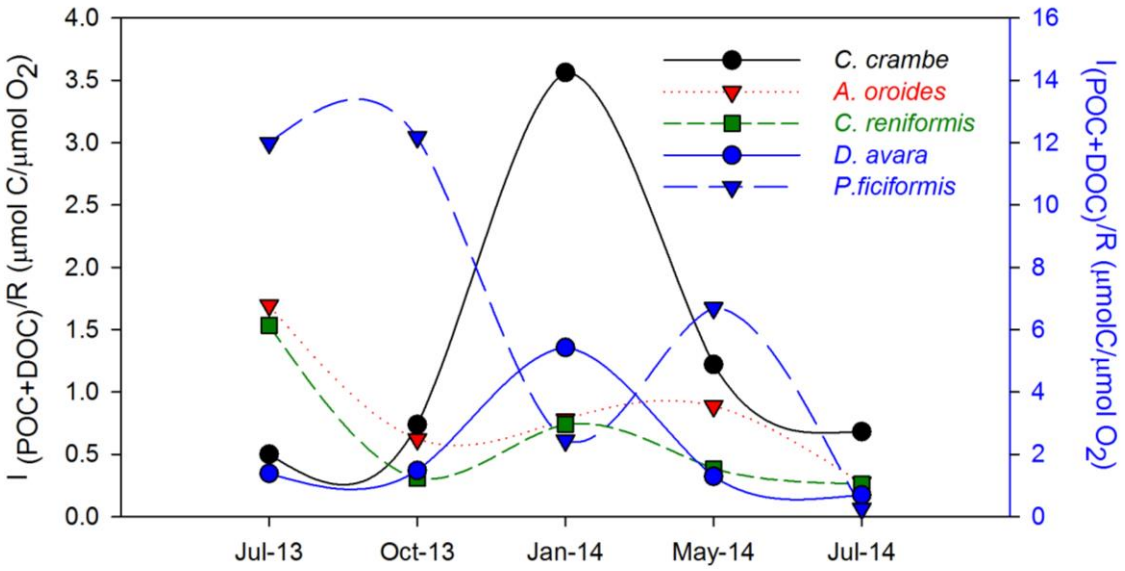


Figure 4. Variation of the I/R index (i.e. ratio between ingested carbon from particulate and dissolved fraction and oxygen removed) through the year for the five sponges species. *C. crambe*, *A. oroides* and *C. reniformis* refer to left axis (black), *D. avara* and *P. ficiformis* refer to right axis (blue).

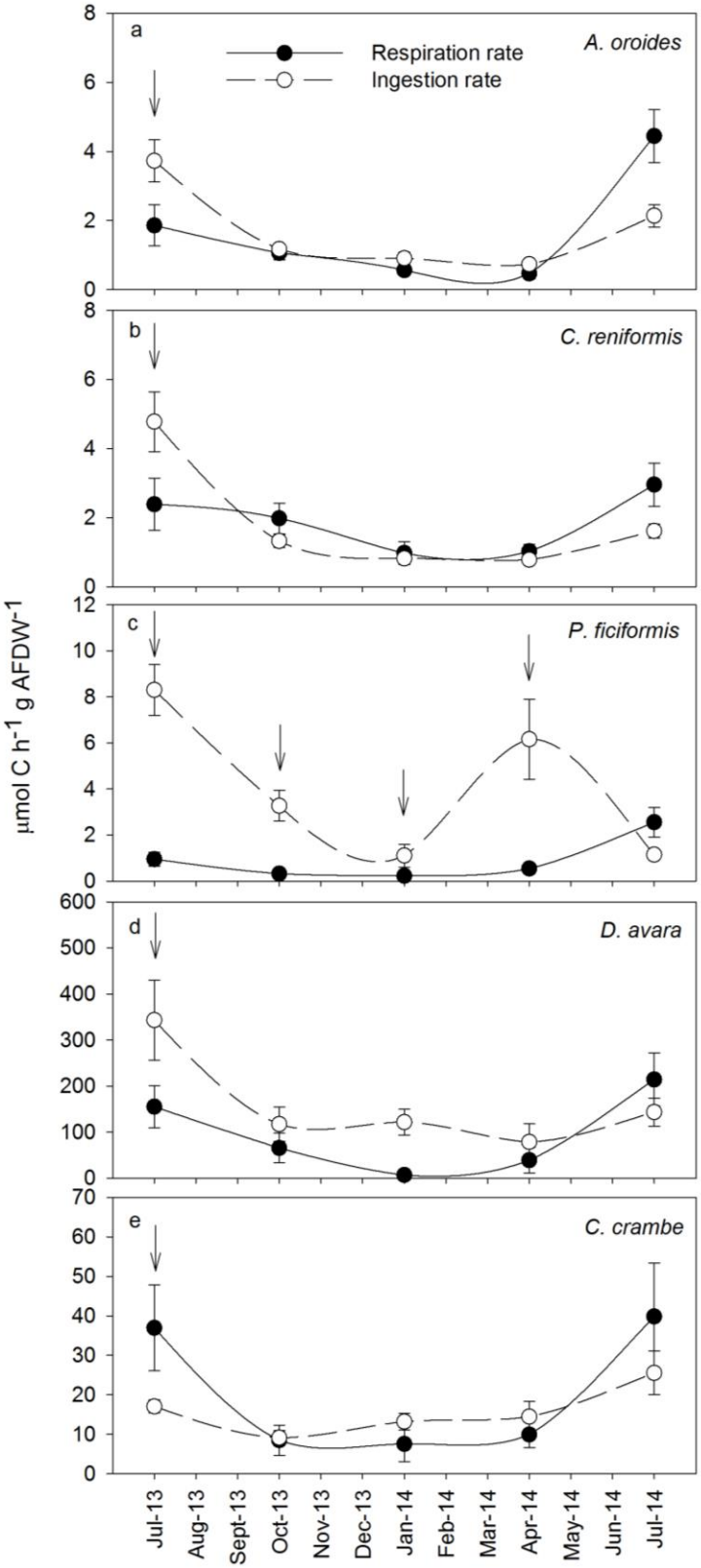


Figure 5 Variation of respiration and ingestion rates (µmol C g AFDW^{-1} h^{-1}) throughout the year. (a) *A. oroides*, (b) *C. reniformis*, (c) *P. ficiformis*, (d) *D. avara*, and (e) *C. crambe*. Data are expressed as mean ± SE. Arrows indicate when sponges ingested DOC.

Discussion

Seasonal respiration rates

The mean respiration rate values reported in this study for the five species were within the range of values reported in the literature for temperate and tropical species (Table S1). Mass-specific respiration rate from the literature ranged between 0.71 μmol O_2 h^{-1} (g DW sponge)$^{-1}$ by *Tethya californiana* (Luderman Master Thesis 2015) and 95.54 μmol O_2 h^{-1} (g DW sponge)$^{-1}$ by *Haliclona oculata* (Koopmans et al. 2010). Within this range, the mean mass-specific raspiration rates in *Agelas oroides*, *Chondrosia reniformis* and *Petrosia ficiformis* were in resemblance with those observed in temperate species *Tethya californiana*, *Haliclona mollis* and *Neopetrosia problematica* (0.71 ± 0.12, 2.53 ± 0.63, 3.08 ± 0.63 μmol O_2 h^{-1} (g DW sponge)$^{-1}$, respectively) (Luderman Master Thesis 2015). While mass-specific respiration rate observed in *Crambe crambe* was similar to value reported for tropical specie *Callispongia vaginalis* (49.72 ± 15.87 μmol O_2 hr^{-1} (g DW sponge)$^{-1}$, Luderman Master thesis 2015). In this study *D. avara* exhibited higher mass-specific respiration rate than what previously observed by Coma et al. (2002) (133.82 ± 175.88 μmol O_2 h^{-1} (g DW sponge)$^{-1}$ and 9.25 ± 4.02 μmol O_2 h^{-1} (g DW sponge)$^{-1}$, respectively). This contrasting result appears to be related to differences in size of sampled specimens, (0.05 ± 0.06 g DW in this study and 0.6 ± 0.09 g DW in Coma et al. 2002) and to the different method employed (*in situ* direct and indirect technique). The contrast of Coma's work with the present data might be a consequence of the highly pronounced effect of size in small individuals and/or the effect of metabolic chambers by reducing the oxygen removal of the studied organism (Newell and Northcroft, 1967; Reiswig 1974; Hadas et al. 2008).

It is expected that respiration of HMA species is higher in comparison with LMA species, likely attributed to respiration of bacteria in sponge tissue (Reiswig 1974, 1981). This trend has been observed also in octocoral species with symbionts in comparison to that of octocoral species without symbionts (Coma et al. 2002). Here, both HMA and LMA species removed oxygen with same efficiency (1.6 ± 0.2% and 1.4 ± 0.1%, respectively), but, contrary to what observed before (Reiswig 1974, 1981), HMA species consumed per unit of sponge volume 78% less oxygen than LMA did. This difference is likely related to different volume of water processed by these

species, accordingly with their morphological characteristics (HMA species pumped per unit of volume 85% less water than LMA did, see Chapter 3).

Respiration rate might be affected by several factors in sponges as temperature, pumping activity, ambient flow, ambient oxygen concentration, sediment, size (*e.g.,* Barthel and Theede 1986; Coma et al. 2002; Hadas et al. 2008; Schläppy et al. 2010; Kutti et al. 2015). The ambient oxygen concentration was always fully saturated or slightly supersaturated during the experiments, which unlikely affected the respiration measurement (Barthel and Theede 1986; Mills et al. 2014). High rate of sedimentation (from 30 to 500 mg L^{-1}) has been shown to induce different responses in the respiratory activity of the sponges (Lohrer et al. 2006; Bannister et al. 2012; Tjensvoll et al. 2013; Kutti et al. 2015). A reduction of respiration rate (up to 86%) was attributed to decrease of pumping activity in high sediment regime (Tjensvoll et al. 2013; Kutti et al. 2015). Conversely, an increase of respiration activity (up to 40%) was attributed to the cost associated to active response (*e.g.,* mucus production) generated to face high rate of sedimentation (Bannister et al. 2012). Our study was conducted in low sediment oligotrophic area, and suspended POM was always <0.3 mg L^{-1}. We therefore excluded that it could have interfered with respiration rate measurements. Absence of ambient flow (~ 0 cm s^{-1}) has been shown to have a negative effect on the oxygen supply in the sponges (Gatti et al. 2002; Schläppy et al. 2010). Nevertheless, ambient flow above 1 cm s^{-1} did not substantially influence the oxygenation in sponge tissue (Schläppy et al. 2010). The sponges we studied are unlikely to be subject to stagnant waters and the mean water flow at our study area is ~ 4 cm s^{-1} (Mendola et al. 2008). Then, although the absence of ambient flow might decrease the oxygen supply in the sponges, in our data we excluded that it could have interfered with respiration rate measurement, because sponges were undergone to natural ambient flow quite far from stagnant conditions. Therefore we examined whether variation in respiration rate was related to pumping activity, temperature and sponge size.

The use of specimen of similar size reduced the variability of respiration rate explained by size throughout the annual cycles. Nevertheless mass-specific respiration rate appeared to be negatively correlated with body size as observed in previous studies (Reiswig 1981; Gatti et al. 2002). A fundamental problem based on how

exchange surfaces and shapes change with body size: an isometric relationship is expected when both dimensions scale up by the same multiplier, while allometric is expected when the proportion is not maintained with changes in body size. Our data showed that the pattern of decrease of respiration activity with sponge size was best fitted by allometric function ($y=M^b$, where y is the mass-specific respiration rate, M is the sponge mass and b is the scaling exponent; by means size-related changes in shape are in response of the size changes) in two studied species (*Dysidea avara* and *Chondrosia reniformis*). However, whether mass-specific respiration rate in sponges should follow the theoretical allometric scaling is still a question of debate. It is largely believed that the power function allometry usually found in metazoans may not be valid in sponges, in which isometric is expected due to their modular construction (Thomassen and Riisgård 1995; Hadas et al. 2008; Koopmans et al. 2010) or to porous character of tissue organization (Cotter 1978). However, the pattern of decrease of size-specific respiration rate with sponge size might be a function of higher size-specific pumping rate in smaller individuals, rather than an increased efficiency of oxygen removal (Reiswig 1981). Although sponge size affects the metabolic process, the use of specimen of similar size in this study reduced the variability of respiration rate explained by size throughout the annual cycles, and temperature and pumping activity appeared to be the main factors regulating the seasonal fluctuation of respiration.

A positive relationship between pumping activity and oxygen consumption has been observed in sponges (Hadas et al. 2008; Hoffmann et al. 2008; Ludeman Master Thesis 2015) as well as between ventilation and oxygen consumption in some bivalves (Hamwi and Haskin 1969; JØrgensen et al. 1986). JØrgensen and colleagues (1986) proposed that the cause-effect relations between these two processes were ascribed to physical conditions of viscous flow and diffusive boundary layers rather than physiological cost of respiration or filtration regulation according to oxygen requirement. Our data reflected positive relations between oxygen removal and volume of water flow, and large part of the variance of respiration rate was explained by pumping activity in three out of two of the studied species. The combined effect of physical and physiological factors that regulate these metabolic processes might have not determined a clear relation between them in *Agelas oroides* and *Crambe crambe*

over annual cycle. Presumably in oligotrophic coastal waters filter feeders have to process higher amount of water to reach the food requirement (>10L for each mL of oxygen removed), therefore filtration might be not a prominent factor that regulates oxygen removal (Jørgesen 1975; Jørgesen et al. 1986; Riisgård and Larsen 2010). However, whether pumping rate is an energetically costly process, and therefore an increase of volume of processed water would require an increase of respiration, is still a question of debate. (Riisgård et al. 1993; Thomassen and Riisgård 1995; Hadas et al. 2008; Riisgård and Larsen 2010; Leys et al. 2011; Luderman Master Thesis 2015). Early studies estimated the cost of water transport in sponges by calculating the resistance, or head loss, through each structure of aquiferous system. Riisgård and colleagues (1993; 1995; Thomassen and Riisgård 1995) suggested that the cost of pumping represents a small fraction of entire metabolism (<1%), which is in agreement with the hypothesis of low energetic cost of water processing in benthic filter feeders as an adaptation to live in oligotrophic habitats (Jørgesen 1975; Jørgensen et al. 1986). Other studies suggested that pumping represented higher energetically cost for the metabolism (~25%) (Hadas et al. 2008; Leys et al. 2011) reaching up to 40% of metabolic cost, (Luderman Master Thesis 2015). The different cost of pumping activity between species was attributed to different volume of water flow processed by different species (Luderman Master Thesis 2015). Our data demonstrated that pumping activity explained large portion of the variance in respiration rates, but we cannot determine the cause-effect relations between these two metabolic processes.

The respiration rates of the five studied species showed a marked seasonal pattern, closely following the annual pattern of water temperature changes. The respiration rate raised as temperature increased as observed in previous laboratory and *in situ* studies (Barthel and Theede 1986; Coma et al. 2002). The acute temperature effect in sponge is reflected by high Q_{10} values estimated over the natural temperature range (13ºC to 22ºC), which were above the general values indicated for biological processes (between 2 and 3) and above the values obtained by previous study on respiration in the temperate species *D. avara* (2.3 from 12 to 21ºC, Coma et al. 2002) and in North Sea species *Halichondria panicea* (4.6 from 6 to 10ºC, Barthel and Theede 1986). The high Q_{10} values obtained in this study might also reflect a

response of metabolic demand of sponges for secondary production, rather than solely temperature-dependence of respiration. In summer periods respiration rates increased by 2 up to 30 folds the rates observed in winter. Such increase of metabolic process (>40%) might be induced by pattern of secondary production (Parry 1983; Coma et al. 2002). In most of the studied species the period of maximal secondary activity coincides with the maximum temperature (Turon et al.1998; Mariani et al 2005; Riesgo and Maldonado 2008; de Caralt unpublished data), which make difficult to distinguish the temperature and reproduction effect on respiration pattern if respiration rate is measured in the field. Since seasonal patter of respiration in benthic invertebrate may be explained also by patter of secondary production (Parry 1983; Coma et al. 2002) and the cost of growing in sponges is high (139%, Thomassend and Riisgård 1995), we suggest that the seasonal respiration rates observed in this study may be a response of temperature-dependence in respiration and respiratory cost associated with somatic growth and/or reproduction.

F/R ratio

The F/R ratio (liters of water filtered per mL of oxygen respired) represents a useful tool to assess the adaptation of filter feeders to their environment with different planktonic availability (Riisgård and Larsen 2000; Riisgård et al. submitted). The overall averaged F/R ratios observed in this study were > 10, indicating that Mediterranean sponges are adapted to low plankton availability (Riisgård and Larsen 2000). F/R ratios of HMA species were within the range of values observed in previous studies (Table 2) and they were lower than the F/R ratio of LMA sponges which were similar to values observed in depth glass sponge *Aphrocallistes vastus* (LMA) (Table 2, Leys et al. 2011; Khan et al. 2015b). The high F/R ratios observed in this study together with *A. vastus* reflect the adaptation to pronounced oligotrophic conditions ($1.85 \pm 0.47 \times 10^5$ and $6.7 \pm 0.35 \times 10^5$ bacteria cells mL^{-1}, respectively). The higher F/R ratio in LMA compare to HMA species, indicated that they filter more volume of water to meet their energetic requirement by grazing mainly on particulate matter. This result is consistent with the different feeding strategy observed between these two groups (Chapter 2) related to the more efficient use of dissolved compounds by HMA. The emerging view from our results is that F/R ratio in LMA species is

generally higher than HMA species, however further reliable *in situ* studies on physiological performance of undisturbed organisms are needed.

Although the adjustment of F/R ratio in the face of changes in environmental conditions may indicate the ability of suspension-feeding organisms to maintain optimal filtration efficiency (Newell and Branch 1980), the variation of F/R ratios observed through the year in this study was likely related to fluctuation of respiration. The generally higher F/R ratio found in winter was mainly due to a lower specific respiration rate, rather than an adjustment of sponges to different environmental conditions. Our data suggest that F/R ratio is a useful tool to compare the level of adaptation between different species from different habitats, but it has to be use cautiously because this ratio is not constant through the year. For a correct use of the F/R ratio is therefore important to know the physiological response of the studied organism under different laboratory or *in situ* conditions. In this sense, the F/R ratio has been widely used to estimate the food content of suspended particles in seawater that should be present to cover a consumption of 1 mL O_2 (Thomassen and Riisgård 1995; Kowalke 2000; Hadas et al.2009; Khan et al. 2015b; Riisgård et al. submitted). Most of those studies worked on LMA species, for which the main food source comes from the particulate fraction. Although we showed that sponges can ingest DOC from the water column, we did not include the dissolved fraction in this calculations because it was not always available for the sponges (see paragraph below for inclusion of dissolved fraction into the C balance). As 0.46 mg C corresponds to 1 mL O_2, a minimum of 0.029 ± 0.011 mg C L^{-1} and 0.005 ± 0.004 mg C L^{-1} must be available to fulfill the maintenance requirements for the five sponges in summer and winter, respectively. The mean live C observed in the study area was 0.015 ± 0.0004 mg C L^{-1} in summer and 0.0123± 0.001 mg C L^{-1} in winter. This implies that sponges may be not able to cover their carbon requirement on sole diet of pico-and nanoplankton in summer. The amount of carbon represented by detrital particles, which ranged between 0.074 and 0.126 mg C L^{-1} in summer may reach the C requirement, but since it represents a insignificant fraction of sponge diet at least for some species (Ribes et al. 1999a); it is likely that the dissolved fraction may fulfilled the carbon requirement.

Table 2. Volume of water filtered per mL O_2 consumed (F/R, L $mL^{-1}O_2$) and ingestion-respiration ratio (I/R, µmol C µmol O_2 $^{-1}$) of different sponge species. Asterisks (*) refer to annual average ± SD measured in this study.

Species	Host type	F/R	$I_{(poc)}/R$	$I_{(poc+doc)}/R$	Source
Mycale sp.	LMA	19.6	2.59		Reiswig 1974
Verongia gigantea	HMA	4.1	0.54		Reiswig 1974
Aplysina fistularis	HMA	9.7	0.12		Reiswig 1974, 1981
Tethya crypta	LMA	22.8	3.97		Reiswig 1974
Halichodnria panicea	LMA	2.7	1.06		Thomassen and Riisgård 1995
	LMA	15.5			Riisgård et al. (submitted)
	LMA	11			Riisgård et al. 1993
Mycale acerata	LMA	2.1			Kowalke 2000
Isodictya kerguelensis		6.3			Kowalke 2000
Aplysina aerophoba	HMA		0.0026		Wehrl et al. 2007
Halisarca caerulea	L/HMA		0.52	6.85	De Goeij et al. 2008a
Dysidea avara	LMA	2.36	2.28		Ribes et al. 1999a; Coma et al.. 2002
Negombata magnifica	LMA	12.7	1.13		Hadas et al. 2008,2009
Haliclona oculata	LMA	2.65	2.45		Koopmans et al. 2010
Theonella swinhoei	HMA	5.05	0.18	1.29	Yahel et al. 2003; De Goeij et al. 2008a
Aphrocallistes vastus	LMA	84			Leys et al. 2011
	LMA	76.92	2.29		Khan et al. 2015
Agelas oroides	HMA	32.58 ± 36.46*	0.57	0.86	This study
Chondrosia reniformis	HMA	21.74 ± 30.53*	0.40	0.66	This study
Petrosia ficiformis	HMA	48.75 ± 83.36*	0.76	6.67	This study
Dysidea avara	LMA	71.10 ± 122.02*	1.41	1.64	This study
Crambe crambe	LMA	86.68 ± 139.33*	1.22	1.26	This study

I/R ratio and carbon balance

Energetic shortage has been suggested to regulate the seasonal dynamics of Mediterranean benthic suspension feeders (Coma and Ribes 2003). Of prime importance in this context are the processes associated with energy gain from the environment and energy lost through metabolism and excretion. Carbon ingested (I) by the organism is partitioned in various processes as biomass production (P) (as either somatic growth or reproduction), energy production through respiration (R) and excretion of waste or fecal pellets (E). In this study it was possible to calculate a minimum energetic demand for the studied species by quantifying the net carbon ingestion as energy gain and respiration rate, in search of pattern of energy expenditure. In this sense $I_{(poc+doc)}/R$ (organic carbon ingested from live particulate and dissolved fraction and respiration) was used as a measure of metabolic efficiency (Maldonado et al. 2012a) and allowed us to examine the energetic dynamics of the studied Mediterranean sponge species through the year. Additionally, we estimated the scope for growth, defined as the surplus of energy available for growth beyond that required for maintenance. When there is a net gain from the environment, the organisms possess energy for production of new tissue or biomass. By using the growth rate form the literature (Galera et al. 2000; Garrabou and Zabal 2001; Ferretti et al. 2008) we calculated the rate of carbon incorporation into biomass by multiplying the growth rate with the carbon content in the sponge tissue (Reiswig 1981; Koompans et al. 2010).

Distinct differences in the energetic budgets between species were associated to different associated bacteria community in sponge tissues (Reiswig 1981; Maldonado et al. 2012). If we look at the annual average carbon budget, LMA species appeared to procure sufficient energy to balance growth and respiration by filtering suspended particulates. The average annual $I_{(poc)}/R$ ratio observed in the studied LMA species is in resemblance with previous works in which only the particulate carbon ingestion was quantified (Table 2. Reiswig 1974; Thomassen and Riisgård 1995; Ribes et al. 199a; Coma et al. 2002; Hadas et al. 2008, 2009; Koopmans et al. 2010), indicating that particulate organic carbon is a relevant contributor to energy gained. Adding the DOC ingested by the studied LMA species the $I_{(poc+doc)}/ R$ index further increases, indicating higher gain of energy for growth and reproduction (Table 2). On

the contrary, the $I_{(poc)}$/ index is smaller than 0.9 in the HMA studied sponges, indicating carbon shortage for maintenance and growth assuming a respiratory quotient of 0.9. Imbalance of carbon budgets has been reported for several HMA species (Reiswig 1974, 1981; Yahel et al. 2003; de Goeij et al. 2008a). Most of these studies attributed the carbon imbalance to underestimation of carbon ingestion and suggested DOC as main source of C (about 80%). Later, this assumption was demonstrated by evidence of DOC removal by HMA species (Yahel et al. 2003; de Goeij et al.2008b; Mueller et al. 2014 and this study) and the dissolved fraction comes to balance the carbon budget of *Halisarca careulea* and *Theonella swinhoei* (Maldonado et al. 2012a and reference therein). Although DOC may represent an important source of carbon gained by HMA species in this study, adding the consumed DOC the average annual $I_{(poc+doc)}$/R index exceeds 0.9 only in *P. ficiformis* and comes into balanced condition, but not for the other two HMA species (Table 2). Other pathways may potentially contribute to the total carbon acquisition in sponges, most likely CO_2 fixation by associated phototrophic bacteria (Wilkilson 1983; Wilkilson and Cheshire 1990; Thacker 2005; Usher 2008). Contribution of cyanobacteria to the carbon assimilation accounts for up to 80% of the carbon budget in tropical species (Wilkilson 1983; Wilkilson and Cheshire 1990; Thacker 2005). To date the amount of photosynthates that the studied species could potentially obtain from their associated cyanobacteria is unknown and needs to be examined.

The estimated $I_{(poc+doc)}$/R ratio exhibited clear seasonal patterns, which appeared regulated by temperature-dependence of respiration and the ability of sponge to remove DOC from the water column. Surprisingly, summer periods may represent energetically unfavorable periods, due to the increase of respiration rate, as well as favorable periods due to increase of carbon gained from the dissolved pool. As discussed above, pico-and nanoplankton abundance was not enough to fulfill the increase of metabolic demand of sponges during the summer and sponges may compensate the energy requirement with consumption of dissolved organic matter, when available. Our data showed that in July 2013 *D. avara*, *C. reniformis*, *A. oroides* and *P. ficiformis* could meet their energy requirement due to uptake of dissolved organic carbon, which comprised about 70% of ingested carbon. This scenario did not occur the next summer, in which all the species showed a net carbon imbalance, likely

due to the lack of DOC ingestion coupled with increase of respiration. Here, we assumed that sponges remove DOC accordingly to its ambient water concentration and remove primarily the labile fraction. DOC is expected to vary over both time and space. Although in the NW Mediterranean DOC generally follows seasonal pattern with maximum values registered during the summer-autummn (120-177 μmol L^{-1}) and minimum values during the winter (60-80 μmol L^{-1}) (Lucea et al. 2005; Alonso-Saez et al. 2008; Via Reixach et al. 2012; Romero-Castillo et al. 2013; Sánchez-Pérez PhD Thesis 2015), at the study site DOC varied greatly between years and did not follow the common seasonal pattern (Aparicio et al. in prep). When available, DOC represents the main source of carbon ingested by HMA (80 ± 16%) and it may contribute to large portion also for LMA species (54 ± 27%). Therefore the availability of DOC in the water column may represent an important factor regulating the energetic budget of these species.

LMA species might be energetically limited during the summer, while the rest of the year appear to procure sufficient energy to balance maintenance and growth by filtering suspended particulates (except autumn for *C. crambe*) (Table 3). Among HMA species, *P. ficiformis* was the most efficient in removing DOC from the water during the year and showed a positive carbon budget enough to procure sufficient energy to growth in spring, winter, autumn and summer (averaged carbon gained ± SD, 1.25 ± 0.86 μmol C h^{-1} (g DW sponge)$^{-1}$) (Table 3). Contrarily, the C gained by *A. oroides* was slightly higher than its C lost by respiration throughout the year (averaged carbon gained ± SD, 0.43 ± 0.56 μmol C h^{-1} (g DW sponge)$^{-1}$), and result insufficient for procure sufficient energy for growth. Similarly, *C. reniformis* showed imbalance of C budget during most of the year (averaged carbon lost ± SD, -0.46 ± 0.42 μmol C h^{-1} (g DW sponge)$^{-1}$), implying that all the ingested carbon was not sufficient for maintenance energy requirement. Alternative source of carbon may be gained autotrophically by the latters species. The tissue of *C. reniformis* and *A oroides* is occupied by ~ 70% and 30% of bacteria respectively, among them phototrophs cyanobacteria (Ribes et al. 2012). This suggests that autotrophic rather than heterotrophic processes not quantified in this study as carbon fixation may contribute to the carbon assimilated by these sponges (Wilkilson 1983; Wilkilson and Cheshire 1990; Thacker 2005; Usher 2008). Our data suggest that summer may represent

favorable period to invest into secondary production for both HMA and LMA species, as long as DOC is available in water column. The rest of the year the emerging view is that the extent of mismatch between ingestion of carbon and respiration tends to be larger in HMA than in LMA species. While LMA meet their energy requirement by mainly grazing on plankton, HMA species depend on the availability of dissolved fraction to fulfill their energy requirements.

During the last decades benthic community of NW Mediterranean suffered massive mortalities, in which the most affected taxa were sponges and gorgonian. These events occurred in late summer-early fall and were linked to an extension and strengthening of summer conditions (*e.g,* high temperature and stability of water column) (Coma et al. 2009; Calvo et al. 2011). Energy shortage during the summer periods was suggested as main mechanism to understanding such mortalities (Coma et al. 1998; Coma and Ribes 2003). Our study on energetic dynamic of sponges may be related to these mortalities by having shown that during summer sponges may be energetically limited due to increase of metabolic demand as previously suggested by Coma and Ribes in 2003. Nevertheless, the ability of sponges to remove dissolved fraction may compensate the increase of metabolic demand during the summer and procure sufficient energy to balance maintenance and energy to growth.

Here we confirm the energetic approach as important mechanism that regulates the seasonal dynamics in benthic filter feeders. Temperature-dependence of respiration as well as availability of dissolved organic fraction are the main factors that regulate such energetic dynamic in the studied species. LMA and HMA life strategies revealed two different limited energetic profiles that procure new insights into the intrigue alternative adaptations on the same habitat.

Table 3. Scope for growth calculated as difference between the ingested C and lost C by respiration (µmol C h^{-1} (g DW sponge)$^{-1}$), and C assimilation rate for growth calculated by multiplying the carbon content in the sponge tissue (see annexe I) with the growth rates reported from the literature. Seasonal growth rate was measured only in *C. crambe* from Garrabou and Zabala 2001, for the other species was used annual growth rate (Galera et al. 2000; Ferretti et al. 2008). Surplus of carbon represents the carbon available beyond the maintenance and growth. The imbalance of C budget is in bolded.

Species	Sampling	Scope for growth (µmol C h^{-1} g DW^{-1})	C assimilation rate for (µmol C h^{-1} g DW^{-1})	Surplus of Carbon (µmol C h^{-1} g DW^{-1})
D. avara	Jul-13	71.24 ± 92.40	1.99	69.25
	Oct-13	19.44 ± 21.28	1.99	17.45
	Jan-14	40.09 ± 24.37	1.99	38.10
	Apr-14	14.97 ± 12.26	1.99	12.97
	Jul-14	**-26.74 ± 55.26**	1.99	**-28.74**
C. crambe	Jul-13	**-14.04 ± 23.92**	5.72	**-19.76**
	Oct-13	0.44 ± 4.97	2.19	**-1.75**
	Jan-14	5.55 ± 11.33	1.52	4.02
	Apr-14	3.18 ± 7.04	2.86	0.32
	Jul-14	**-9.94 ± 36.51**	5.72	**-15.66**
A. oroides	Jul-13	1.27 ± 1.14	0.51	0.76
	Oct-13	0.08 ± 0.43	0.51	**-0.43**
	Jan-14	0.23 ± 0.54	0.51	**-0.27**
	Apr-14	0.15 ± 0.35	0.51	**-0.36**
	Jul-14	**-1.53 ± 1.72**	0.51	**-2.03**
C. reniformis	Jul-13	1.91 ± 1.55	0.87	1.04
	Oct-13	**-0.51 ± 0.99**	0.87	**-1.37**
	Jan-14	**-0.12 ± 0.53**	0.87	**-0.98**
	Apr-14	**-0.19 ± 0.37**	0.87	**-1.06**
	Jul-14	**-1.03 ± 1.52**	0.87	**-1.90**
P. ficiformis	Jul-13	2.19 ± 0.90	0.11	2.08
	Oct-13	0.88 ± 0.58	0.11	0.77
	Jan-14	0.26 ± 0.43	0.11	0.15
	Apr-14	1.67 ± 1.31	0.11	1.56
	Jul-14	**-0.42 ± 0.64**	0.11	**-0.53**

Supplementary material

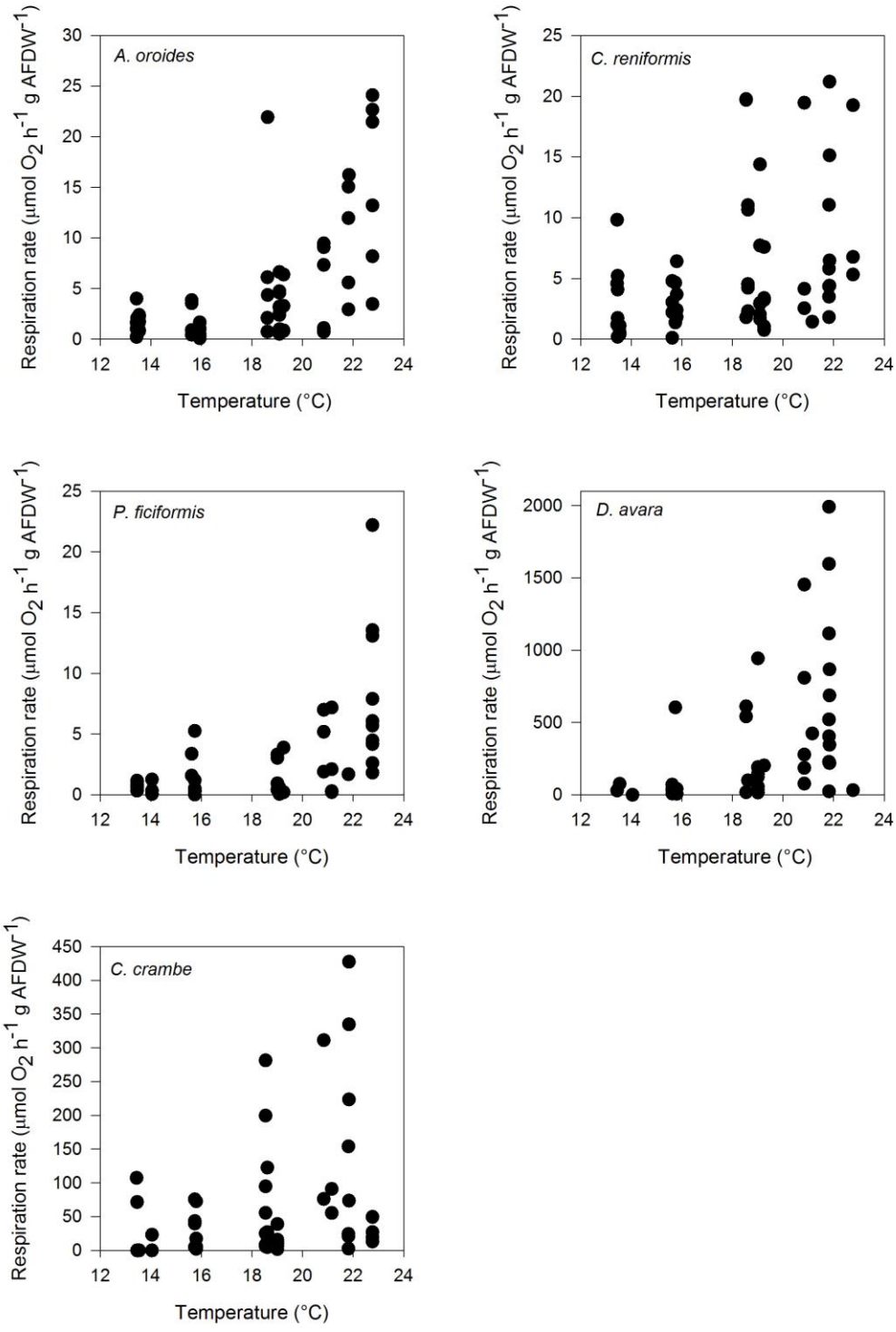


Figure S1. Respiration rate (µmol O_2 g $AFDW^{-1}$ h^{-1}) plotted against seawater temperature encountered at the study site. Each points represent each sampled specimen (*A. oroides* n=56; *C. reniformis* n=55; *P. ficiformis* n=49; *D. avara* n=44; *C. crambe* n=45).

Table S1. Respiration rates for several marine sponges species worldwide, mean ± SD.

Source	Species	Host type	Area	Lab or in situ	µmol O_2 h^{-1} mL^{-1}	µmol O_2 h^{-1} g DW^{-1}	µmol O_2 h^{-1} g $AFDW^{-1}$
Reiswig 1974	*Mycale sp.*	LMA	Tropical	In situ InEx	1.965	25.26	66.56
Reiswig 1974	*Verongia gigantea*	HMA	Tropical	In situ InEx	3.036	27.39	48.12
Reiswig 1974	*Tethya crypta*	LMA	Tropical	In situ InEx	0.893	6.64	19.69
Reiswig 1981	*Aplysina fistularis*	HMA	Tropical	In situ InEx	4.736	33.64	
Cotter 1978	*Suberites carnosus*		Ireland (cold temperate area)	Lab		35.31	
Cotter 1978	*Sycon ciliatum*		Ireland (cold temperate area)	Lab		24.6	
Barthel 1986,1988	*Halichondria panicea*	LMA	Baltic Sea, North Sea (cold temperate area)	Lab	0.21-0.98	20-30	
Thomassena and Riisgård 1995	*Halichondria panicea*	LMA	Baltic Sea (cold temperate area)	Lab	1.936	26.75	82.19
Witte and Graf 1996	*Thenea abyssorum*		Greenland and Norwegian Seas	heat production			22.18 ± 7.19
Witte and Graf 1996	*Thenea muricata*		Greenland and Norwegian Seas	heat production			18.75 ± 11.25
Witte and Graf 1996	*Tetilla cranium*		Greenland and Norwegian Seas	heat production			18.75 ± 3.12
Coma *et al.* 2002	*Dysidea avara*	LMA	Mediterranean sea	in situ (chambers)		9.25 ± 4.02	29.06 ± 11.25
Gatti et al. 2002	*Cinachyra antartica*		Polar areas	Lab			4.8-16.92
Gatti et al. 2002	*Stylocordyla borealis*		Polar areas	Lab			1.95 - 10.71
Yahel et al. 2003	*Theonella swinhoei*	HMA	Red Sea	In situ (InEx)	1.38		
Hoffmann et al. 2008	*Aplysina aerophoba*	HMA	Mediterranean sea	Lab			
Hadas et al.2008	*Negombata magnifica*	LMA	Red Sea	Lab	2.22	14.88	18.6
Kowalke 2000	*Mycale acerata*		Antartic	Lab			3.93 (3.08-5.58)
Kowalke 2000	*Isodictya kerguelensis*		Antartic	Lab			1.56 (1.2-1.8)
Koopmans et al. 2010	*Haliclona oculata*		Netherlands (cold temperate area)	In situ (chamber) and Lab		95.54	
Koopmans et al. 2011	*Haliclona oculata*		Netherlands (cold temperate area)	lab		86.9	
Leys et al. 2011	*Aprochallistes vastus*	LMA	Temperate	In situ (InEx)	0.697		

Ludeman Master thesis 2015	*Cliona delitrix*	HMA	Tropical	In situ (InEx)	0.83 ± 0.38	9.32 ± 4.29	
Ludeman Master thesis 2015	*Callispongia vaginalis*	LMA	Tropical	In situ (InEx)	3.12 ± 0.99	49.72 ± 15.87	
Ludeman Master thesis 2015	*Tethya californiana*	LMA	British Columbia- Temperate	Lab (InEX)	0.23 ± 0.04	0.71 ± 0.12	
Ludeman Master thesis 2015	*Haliclona mollis*	LMA	British Columbia- Temperate	Lab (InEX)	0.31 ± 0.08	2.53 ± 0.63	
Ludeman Master thesis 2015	*Neopetrosia problemática*		British Columbia- Temperate	Lab (InEX)	0.38 ± 0.07	3.08 ± 0.63	
This study	*Agelas oroides*	HMA	Mediterranean sea	In situ (InEx)	1.36 ± 1.85	3.41 ± 4.19	5.02 ± 6.17
This study	*Chondrosia reniformis*	HMA	Mediterranean sea	In situ (InEx)	2.44 ± 2.59	4.44 ± 4.36	5.70 ± 5.63
This study	*Petrosia ficiformis*	HMA	Mediterranean sea	In situ (InEx)	0.67 ± 1.05	0.85 ± 1.26	2.85 ± 4.21
This study	*Dysidea avara*	LMA	Mediterranean sea	In situ (InEx)	5.27 ± 6.19	133.82 ± 175.88	352.62 ± 466.60
This study	*Crambe crambe*	LMA	Mediterranean sea	In situ (InEx)	12.33 ± 18.37	45.35 ± 67.14	64.31 ± 95.20

Conclusion

1. Methodological approach (chapter 1 and 3). In their natural habitats, the organisms are exposed to combined effect of several environmental factors. To better understand the metabolic response of the organisms under natural environmental changes, it is crucial to employ direct *in situ* techniques that allow to test experimental hypothesis without manipulate or disturb the organisms of interest. The system device presented in chapter 1, so called VacuSIP, offers a practical and reliable method of measuring how active suspension feeders process particulate and dissolved compounds under natural conditions. In addition, the dye technique employed in chapter 3 has been corroborated to be effective, non-disturbing and free of artifacts method to estimate the volume of water pumped by the studied species *in situ*. The conjunction use of these techniques allow realistic estimates of the organism's performance under natural conditions, and provides accurate and comparable estimates on sponge-mediated fluxes of energy and nutrients that can be used to assess the functional role of sponges in different ecosystems.

2. Trophic niche separation facilitates co-existence of high and low microbial abundance sponges (chapter 2). The studied sponges are important component of coralligenous community at the study site and are often packed in dens and multispecies assemblages that cover many pinnacles and overhangs. We provided evidence for different feeding strategies in Mediterranean sponges, with HMA species having a broader spectrum of food sources, mostly represented by dissolved components that are potentially derived from the LMA species (and other organisms) excretions. The differential capability of the different sponge strategies in using resources indicates a trophic niche separation related to the HMA-LMA dichotomy as a mechanism facilitating their coexistence. Our findings suggest that a mixed assemblage of sponges (and their associated microbes) is able to use the suspended particulate and dissolved material more efficiently than a single species population. Niche separation between HMA and LMA species and their putative symbiotic relationships may contribute to the survival, stability and diversity of dense sponge assemblages in oligotrophic habitats.

3. **A decrease in choanocyte chambers during the reproductive period may be compensating for the viscosity-temperature effect on sponge pump (chapter 3).** In the natural environment, organisms are simultaneously exposed to a variety of extrinsic and intrinsic factors. Theoretical considerations and laboratory experiments predicted that sponge pump should be correlated with temperature. Interestingly, this study demonstrated that the natural variation of sponge pump throughout the year appears to be mainly driven by two counteracting factors: the physical effect of water viscosity and the reproductive process. The physical effect of decrease in water viscosity with temperature increase should theoretically increase the volume of water processed by sponges, while the biological phenomenon of the reproductive process causing a reduction of the choanocyte chambers determines a reduction of the volume of processed water. The overlap of the period of higher temperature with that of reproduction in most of the study species makes it difficult to distinguish the effects of temperature and reproduction when pumping rate is recorded in the field. Their opposite effect is conducive a rather constant pumping rates of the species throughout the year.

4. Temperature-dependence of respiration together with dissolved organic carbon availability in water column regulate the energetic dynamics of sponges (chapter 4). Temperature likely regulates the metabolic activity of poikiloterm organisms. This study demonstrated that respiration rates of the five species showed a marked seasonal pattern closely following the annual pattern of water temperature changes. The high response of respiration to temperature changes further suggest that other process, such as secondary production, might affect the respiratory demand during the periods of maximum temperature.

An energetic approach could provide a more mechanistic understanding of the complex interactions between physiological constrains and environmental factors experienced by the organism under natural conditions. The physiological processes described in this study contribute to understanding the energetic dynamics of studied sponges, as well as supported the hypothesis of energetic constrains as mechanism to understanding the massive mortalities of sponges occurred in the last decades. This study demonstrated that summer may represent an energetically unfavorable as well

as favorable periods in which sponges faced the increase of energetic demand by ingesting dissolved fraction, when available. The availability of DOC appeared to be a key factor in regulating the dynamics of the studied species, especially for HMA sponges. In addition we demonstrated that HMA and LMA species showed different limited energetic profiles accordingly with their different feeding strategy: LMA species appeared to procure sufficient energy to meet metabolic requirements for maintenance and growth by filtering suspended particulates, and dissolved fraction represents an additional input of carbon gained that can be used for secondary production or energy storing. On the contrary particulate resources are insufficient to balance the energetic demand of HMA species through the year and their energetic dynamics appeared to be dependent on the availability of dissolved fraction. We suggested that the carbon imbalance observed in two out of three HMA species might be fulfill autotrophically.

Future perspectives and open questions

Results presented in this thesis added important information to seasonal dynamics of five of the most prominent sponge species dwelling the coralligenous community of the NW Mediterranean Sea, as well as corroborated the divergence between the two evolutionary life strategies employed by HMA and LMA species.

The seasonal energetic approach used in this study aims to quantify the role of physiological processes in the ecological relations of sponges in their natural habitat. As a result, the assessment of sponge feeding, pumping and respiration in nature are crucial to understand seasonal energetic of undisturbed animals. The overlap of the period of maximum temperature with that of secondary production (as either somatic growth or reproduction) in most of the study species made it difficult to distinguish the effects of temperature and secondary production on physiological processes measured in this study. In this framework the role of reproductive cycle in the regulation of pumping rate in sponges needs to be addressed by future histological studies to assess the variation of choanocyte chambers density through the year. Moreover the cost associated to somatic growth and reproduction on regulation of variation on respiratory demand and seasonal energetic balance needs to be

addressed by future studies monitoring the pattern of production of the species. Data on seasonal patter of biochemical composition will further help to elucidate the seasonal dynamic of the studied species.

The energetics of sponges is particularly complex because their association with bacteria and their intricate interactions among symbionts and host. The nutrient fluxes, pumping rates and carbon balances estimated in this study clearly reflected the different adaptive life strategies employed by HMA and LMA species. A deeper understating of the energy gained from autotrophic pathways, especially in HMA species, is needed to accurately estimate the carbon balance of phototrophic sponge species through the year.

Annexe I

Biomass of aquatic organisms can be expressed in several units based on the purpose of the investigation (*e.g.*, to estimate the population abundance, growth rates and standing crop). Biomass is usually reported in terms of length, volume and weight, but it can be expressed also as the portion of living organism. Metabolic rates are generally expressed per unit of biomass to be comparable between species and phyla. Physiological studies generally report the activity rates (*e.g.*, respiration and pumping rate) in terms of organic content, while are reported in term of volumetric biomass for ecological purpose (Reiswig 1971). Those parts of the animal that are non-living, such spicule and foreign material in the mesohyl of sponges, are generally excluded in studies on sponge physiology. It is therefore important to notify those parts of the biomass of the organism that are removed, so that valid comparison can be made between species. The ash-free dry weight is considered a good measure of the living substance. An alternative is to express the biomass in terms of organic content by analyzing the organic nitrogen and carbon in the tissue. Most of these measurements must be carried out in laboratory, and therefore require to remove the organisms of interest from their natural habitat.

Although any sponges from Mediterranean Sea either worldwide is reported on the International Union for Conservation of Nature Red List (accessed April 2016), the recurrent removal of several specimens of interest for scientific purpose might alter local sponge population especially if already affected by environmental factors. Furthermore sponges are important component of Mediterranean coralligenous community not only as major contributors of coralligenous framework building, but they are also highly appreciated for diving industry. In this study we estimated the sizes of the individual sponge colonies *in situ* and in laboratory expressed in different units: area (cm^2) and volume (cm^3) measured *in situ*, and volume (ml), dry mass (g) and ash-free dry weight (g), carbon and nitrogen organic content (g) measured in the laboratory. Firstly, we provided the relationships between biomass estimations measured *in situ* with those measured in the lab. Secondly, we extensively investigated whether the amount of organic content varied over different sponge sizes.

The aim of this study was to provide conversion factors to estimate dry weight, ash-free dry weight, carbon and nitrogen organic content from sponge colony volume estimated *in situ* by photograph. These conversion factors have been used throughout this thesis and may represent an useful tool to estimate the biomass of sponge colonies based on not destructive sampling methods (photo survey) for future studies on these representative species of Mediterranean coralligenous.

Material and Method

Biomass estimation

The individuals for estimates of biomass were collected in Blanes and Cap de Creus (NW Mediterranean Sea) from September 2014 to January 2015. For at least 30 individuals of each species, the area (cm^2) and volume (cm^3) were estimated *in situ*. The same individuals were collected, placed individually in plastic bags and transported in seawater for lab-measured volume (ml), dry weight (g, DW), ash-free dry weight (g, AFDW) and organic C and N (g) content measurements. The estimates of biomass were carried out to a range of sponge size representative of sponge population at the study area.

The area was estimated by taking a picture of the individual with a ruler placed close to it. The area was calculated with the Imaje J program by drawing the perimeter of each individual from the picture. Gross specimen volume was determined by approximating single or combined regular geometric shapes to external dimensions gained by direct measurement (height) and by photography. *Chondrosia reniformis* and *Petrosia ficiformis* have a globular shape and their volume was estimated by multiplying the area with the maximum height. *Crambe crambe* is an encrusting species and its volume was estimated by multiplying the area with a standard height (0.25 cm) for all the individuals. *Dysidea avara* is characterized by several truncated cone shapes and its volume was calculated by multiplying the area with the average truncate cones heights. *Agelas oroides* commonly has two different shapes: (i) cylindrical or (ii) it is composed by several tubes. In the first case only the maximum height was measured and its volume was calculated as in *C. reniformis* and *P. ficiformis*, while in the second case different heights were measured for each tubes and the volume was calculated as the sum of separated cylindrical shapes.

The lab-volume of the whole sponges was measured by displacement of water in a graduated cylinder. Some big individuals were cut into sections and their volume was calculated as the sum of all sections. Sponges dry weight was determined by drying at 100ºC for 24 h. One series of these dry pieces was used to measure the ash content. Ash was determined by placing the dry pieces of each specimen into a muffle furnace at 500ºC for 6 h. The ash-free dry weight was calculated by subtracting the ash weight from the sponge dry weight and it represents the estimation of organic matter (OM). Carbon and nitrogen organic content was analyzed by C:H:N autoanalyzer (EA 1108 CHNS-O Carlo Erba Instrument) after adding two drops of 10% HCl in small amount of homogenized dried tissue to remove all carbonate in the samples. Only the smallest and biggest individuals (maximum 10 individuals per species) were used for the former analysis.

Prior to lab-measured volume, associated macrofauna and remains of substrate were removed from the sponge. The elimination of foreign material (*e.g.,* calcified endolithic cyanophyceae, tubes of Polychaeta, shells, and pieces of substrate) was particularly difficult in *C. crambe* and *D. avara*, because the former species has a laminar thin shape and most of sand grains, piece of substrate remain within the sponge tissue and the latter species encloses the foreign material in the fibers during its growth (Teragawa 1985). Therefore the foreign material in *D. avara* was cautiously separated from sponge tissue after drying and was then subtracted from the total DW. A second series of sampling was carried out for optimizing the DW estimation in *C. crambe*. To be able to correctly remove most of the foreign material, a small piece was carefully cut from 20 specimens and placed in individually plastic bags and transported to laboratory in seawater. For each piece was estimated the area, DW and AFDW as explained above.

Biomass estimations measured *in situ* (area and volume) were plotted against those estimated in the laboratory (DW and AFDW) to provide several conversion factors. To asses if the relative amount of AFDW per unit of DW, volume and area (%AFW/DW, mg AFDW cm^{-3}, mg AFDW cm^{-2}) varied over the sponge size, these parameters were plotted against respectively estimates of sponge size. If the relative amount of AFDW was size dependent, the fit curve equations were reported and the averages of relative amount of AFDW were provided for different size classes.

Otherwise the overall averages were reported. Since C and N organic content was quantified only for small and big individuals, these data were statistically analyzed using a nested analysis of variance, with size as the main factor (2 levels) and specimens as nested factor. The former is a fixed factor, since the 2 sizes (expressed as area, volume and DW) were not randomly selected but specifically chosen from the collected sponges, and the second factor must be consider random. The nested ANOVA allowed us to test the effect of sponge size once the variance associated with sponge individuals has been partialled out. The percentage of organic C and N was arc tanged transformed for the analysis. The data were log transformed when the normality assumption was no satisfied. If the log-transformed data did not satisfied the normality assumption, no parametric test Kruskall-Wallis was performed, testing only the effect of size among individual. The statistical analyses were performed with R (R Studio).

Results

Biomass estimation

The relationships of the area (cm^2), volume *in situ* (cm^3) and lab-measured volume (ml) as independent variables and dry mass (g) and ash-free dry weight (g) as dependent variables were significantly ($p<0.001$) expressed by power functions yielding R^2 values for each function > 0.80 for the five studied species (Table 1, Figure 1-5). Although it was noticed that in lab-measured volumes sub estimated the gross volume because the water was absorbed into the sponge tissue filling the canal system, sponges volume determined from water displacement measurement showed a high degree of correlation ($R^2> 0.80$) with their sponge dimensions measured *in situ* (Table 1, Figure 6). The relationship between the *in situ* and lab-measured volume was used to estimate the % of water content (porosity) in each sponge species (table 1). The relationship between DW and AFDW was significantly ($p<0.001$) expressed by linear function yielding R^2 values for each function ≥0.96 for the five studied species (Figure 7). The amount of OM expressed as %AFDW/DW represented 79±5%, 71±7%, 69±6%, 42±10% and 33±5% in *C. reniformis*, *C. crambe*, *A. oroides*, *D. avara* and *P. ficiformis,* respectively. The %AFDW per DW unit increased from smaller (77±6%,

from 0.5 to 5 g DW) to bigger individuals (81±4%, from 5 to 26 g DW) in *C. reniformis*, while decreased from smaller (36±5%, from 0.3 to 10 g DW) to bigger individuals (30±2%, from 11-73 g) in *P. ficiformis* (Figure 7). In contrast %AFDW/DW did not vary over the range of sampled size in *A. oroides* and *D. avara* (Figure 7). The %AFDW per DW unit over the range of sampled size was not tested in *C. crambe*, because DW was estimated from small piece of several specimens and it was considered constant in all individuals.

The AFDW per surface unit decreased from 242.41 ± 172.40 mg cm^{-2} in the smaller individuals (≤ 13cm^2) to 174.51 ± 53.12 mg cm^{-2} in the larger individuals (14-100 cm^2) in *A. oroides*. Similarly, decreased from 10.98 ± 7.95 mg cm^{-2} in the smaller individuals (≤ 21 cm^2) to 7.95 ± 3.17 mg cm^{-2} in the larger individuals (22-75 cm^2) in *C. crambe.* The pattern of decreasing was expressed by a power function, although the % of variable explicated is low (R^2 ≤ 0.18) (table 2, Figure 8). In contrast the AFDW per surface unit did not vary over the range of sampled area in *C. reniformis*, *P. ficiformis*, *D. avara*, representing 173.86 ± 51.44, 148.72 ± 71.16 and 17.75 ± 6.87 mg cm^{-2}, respectively (table 2, Figure 8).

The AFDW per volume unit in the smaller individuals of *A. oroides*, *P. ficiformis* and *C. crambe* was twice the amount of the larger individuals. The pattern of decreasing was expressed by a power function, although the % of variable explicated is low (R^2 ≤ 0.35) (table 2, Figure 9). In contrast AFDW per volume unit did not vary over the range of sampled volume in *C. reniformis* and *D. avara*, representing 125.13 ± 42.98 and 17.07 ± 6.60 mg cm^{-3}, respectively (table 2, Figure 9).

The relative amount of organic C and N, expressed as C and N content per unit surface and volume, significantly differed among sponge size class in the five studied species (nested anova or Kruskall-Wallis, $p<0.05$ for the size) (table 3). While the specimen (nested) factor proved significant in most of % C and N, indicating a marked interindividual variability. Table 3 shows the overall averages of each parameter studied for each species (mg C and N cm^{-2}, mg C and N cm^{-3}, %C and N) if any significant difference between sponge size classes was found, while the averages per each size class are provided when significantly different. The area (cm^2), volume (cm^3) and DW (g) range of the specimens sampled in this study as well as the size classes (Big-Small) are reported in table 4.

Remarks

This study provides comprehensive tools for correctly estimates the biomass of the five studied species in different units, according to the scope of each investigation. However, these estimates are not free of limitations. The gross volume estimated *in situ* overestimated the size of the individual sponge colonies due to the approximation of irregular sponge shapes to regular geometric forms. Nevertheless sponge volume determined from sponge dimensions measured *in situ* showed high degree of correlation with their lab-measured volume, DW and AFDW estimated in laboratory.

Although Coma et al. (2002) did not observed any significant difference between the AFDW/DW ratio over annual cycle in *D. avara*, we cannot assume that the portion of organic content reported in this study might maintain constant over the year for the other studied species. Since Mediterranean sponges are subject to seasonality in somatic growth and reproduction investments (Coma et al. 2000), the relative fraction of organic content and structural elements in the sponge tissue might vary following seasonal pattern. Moreover the amount of organic content might be affected by some environmental factor as light in *C. crambe* (Uriz et al. 1995). However, investigate the effect of environmental parameters on the content of organic matter in sponge tissue as well as any putative variability due to seasonal patterns was out of the scope of this work.

Before sampling, we surveyed sponge population to designate different size classes representative of sponge population at the study area to be equally sampled. We cannot ensure that the relationships reported here might be reliable for specimens out of the size range sampled in this study. We therefore recommend to further estimate the biomass of individuals that are bigger than those included in this study.

Beside the limitations stated above, the overall conversion factors provided in this study represent good estimates of sponge biomass of the five study species expressed in different ways. Our data allow future studies to correctly estimate the sponge size of the most prominent Mediterranean coralligenous species based on not destructive sampling methods (photo survey).

Table 1. Equations of the fit curves obtained from dry weight (g, DW) and ash-free dry weight (g, AFDW) as dependent variables plotted against area (cm^2), volume *in situ* (cm^3) and volume measured by displacement (ml) as independent variables. The relationship was best expressed by a power function $y=ax^b$. Relationship between volume *in situ* and volume measured by displacement (ml), expressed by regression line y=ax+b, with b=0. Percentage of water content in the canal systems as proxy of porosity, calculated from the relationship between volume estimated *in situ* and lab-volume estimated by water displacement. *** p<0.0001

			C. reniformis (n=40)				*A. oroides* (n=30)				*P. ficiformis* (n=30)				*C. crambe* (n=31)				*D. avara* (n=30)			
			a	b	R^2	p	a	b	R^2	p	a	b	R^2	p	a	b	R^2	p	a	b	R^2	p
DW (g)	vs.	Area (cm^2)	0.3594	0.8729	0.87	***	0.5733	0.8088	0.81	***	0.8639	0.8647	0.89	***	0.0329	0.7040	0.84	***	0.012	1. 3467	0.92	***
		Volume *in situ* (cm^3)	0.4084	0.7514	0.85	***	0.2251	0.7933	0.87	***	0.7447	0.7787	0.93	***	0.0872	0.7040	0.84	***	0.0114	1. 3467	0.92	***
		Volume in lab (ml)	0.2583	0.9937	0.98	***	0.0915	1,1238	0.99	***	0.3383	0.9857	0.99	***	0.1656	0.7254	0.92	***	0.2061	0.9632	0.94	***
AFDW (g)	vs.	Area (cm^2)	0.2723	0.8896	0.86	***	0.4117	0.7947	0.81	***	0.2870	0.8404	0.88	***	0.0232	0.7040	0.84	***	0.0045	1,3543	0.93	***
		Volume *in situ* (cm^3)	0.3050	0.7693	0.84	***	0.1525	0.7939	0.89	***	0.2221	0.7790	0.94	***	0.0615	0.7040	0.84	***	0.0043	1,3543	0.93	***
		Volume in lab (ml)	0.1817	1. 0302	0.98	***	0.070	10.916	0.98	***	0.1154	0.9584	0.98	***	0.1167	0.7254	0.92	***	0.0737	0.9876	0.97	***
		Volume *in situ* (cm^3) vs. Volume in lab (ml)	0.43		0.71	***	0.39		0.81	***	0.66		0.88	***	0.36		0.82	***	0.33		0.90	***
		% Water content (porosity)		47%				56%				28%				56%				64%		

Table 2. For each species the power curve equations of AFDW/area (mg cm^{-2}) and volume (mg cm^{-3}) plotted against area and volume respectively are provided when significant, and the averages of relative amount of AFDW/area and volume were provided for different size classes. When not significant, only the overall average is provided.
* p<0.05, ** p<0.001, *** p<0.0001; n.s, no significant

Species	n	AFDW/area vs. area					AFDW/volume vs. volume				
		R^2	p	Eq	Small	Big	R^2	p	Eq	Small	Big
C. reniformis	40	-	n.s	-	173.86 ± 51.44		-	n.s	-	125.13 ± 42.98	
A. oroides	30	0.18	*	$y=0.4841x^{-0.2584}$	242.41 ± 172.40	174.51 ± 53.12	0.33	**	$y=0.1806x^{-0.2361}$	89.21 ± 45.02	51.41 ± 13.14
P. ficiformis	30	-	n.s	-	148.72 ± 71.16		0.34	**	$y=0.1767x^{-0.1550}$	142.79 ± 55.69	75.54 ± 28.97
C. crambe	31	0.14	*	$y=0.0148x^{-0.1583}$	10.98 ± 4.95	7.95 ± 3.17	0.14	*	$y=0.0475x^{-0.1583}$	43.92 ± 19.79	32.36 ± 13.16
D. avara	30	-	n.s	-	17.75 ± 6.87		-	n.s	-	17.07 ± 6.60	

Table 3. Organic carbon (a) and nitrogen (b) content expressed per unit of surface, volume and % of DW for each studied species. When C and N content significantly varied between sponge size classes (p_{size} <0.05) the relative amount of C and N content for each size class is indicated. If not significant (n.s) the overall average is provided. p<0.05, ** p<0.001, *** p<0.0001; n.s, no significant; [a] from no parametric Kruskall-Wallis test

(a)	mg C/cm²			mg C/cm³			%C/DW		
Species	**Small**	**Big**	**p (Size)**	**Small**	**Big**	**p (Size)**	**Small**	**Big**	**p (Size)**
C. reniformis	72.54 ± 14.12	61.22 ± 6.04	***	65.42 ± 9.53	42.49 ± 10.80	***	34.20 ± 2.34 %		n.s
A. oroides	145.99 ± 117.54	78.30 ± 41.50	**	42.08 ± 24.71	16.20 ± 4.98	***	29.13 ± 2.55 %	30.31 ± 2.19%	*
P. ficiformis	47.89 ± 23.57	79.75 ± 11.94	** [a]	59.61 ± 28.77	36.42 ± 17.32	***	13.52 ± 0.95 %		n.s
C. crambe	5.48 ± 2.21	3.43 ± 2.06	***	21.92 ± 8.84	13.71 ± 8.22	** [a]	27.47 ± 2.54 %		n.s
D. avara	8.57 ± 4.72	11.87 ± 3.46	***	8.24 ± 4.54	11.41 ± 3.32	***	19.15 ± 2.86 %		n.s
(b)	mg N/cm²			mg N/cm³			%N/DW		
Species	**Small**	**Big**	**p (Size)**	**Small**	**Big**	**p (Size)**	**Small**	**Big**	**p (Size)**
C. reniformis	22.14 ± 4.23	18.79 ± 1.90	***	19.99 ± 3.09	13.03 ± 3.25	***	10.47 ± 0.73 %		n.s
A. oroides	44.61 ± 34.63	24.08 ± 12.82	***	12.92 ± 7.11	4.97 ± 1.57	***	9.15 ± 0.75 %		n.s
P. ficiformis	11.35 ± 6.11	19.40 ± 2.90	* [a]	14.09 ± 7.54	8.90 ± 4.35	***	3.07 ± 0.13%	3.35 ± 0.26%	***
C. crambe	1.09 ± 0.46	0.68 ± 0.48	* [a]	4.37 ± 1.85	2.72 ± 1.91	** [a]	5.39 ± 0.82%		n.s
D. avara	1.22 ± 0.62	1.62 ± 0.44	***	1.17 ± 0.60	1.56 ± 0.42	***	2.74 ± 0.57%	2.61 ± 0.47%	***

Table 4. The area (cm^2), volume *in situ* (cm^3) and DW (g) range of the sampled specimens in this study. Size classes (small and big) expressed in area (cm^2), volume (cm^3) and DW (g) used for the analysis of organic C and N content

Species	Area range (cm^2)	Volume range (cm^3)	DW range (g)	Area (cm^2)		Volume (cm^3)		DW (g)	
				Small	Big	Small	Big	Small	Big
C. reniformis	3 - 120	3 - 220	0.5 - 26	5 - 15	50 - 120	5 - 17	60 - 220	1 - 5	9 - 26
A. oroides	2.5 - 100	4 - 395	0.8 - 26	3 -13	30 - 70	4- 45	173 - 293	0.5 - 7	8 - 15
P. ficiformis	2.5 - 145	1 - 360	0.3 - 73	2 - 11	32 - 100	1 -15	55 - 360	0.4 - 7	13 - 65
C. crambe	1 - 75	0.4 - 19	0.02 - 0.7	1 - 21	22 - 70	0.4 - 5	6 - 19	0.05 – 0.3	0.5 – 0.7
D. avara	1 - 140	1 - 155	0.04 - 12	1 - 9	37 - 140	1 - 10	40 - 145	0.1 – 0.5	2 - 12

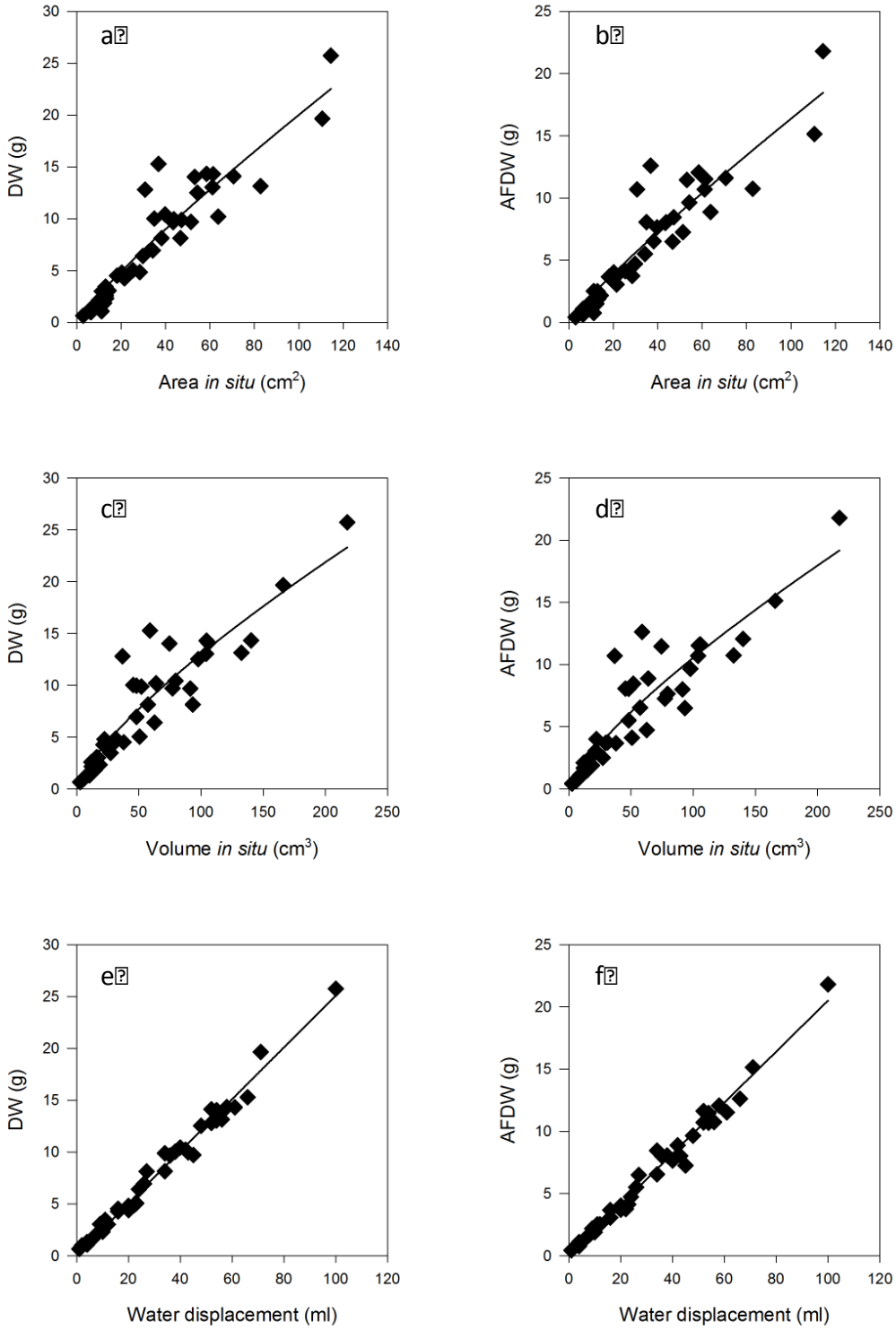


Figure 1 Relationship between (a-b) area *in situ* (cm^2), (c-d) volume *in situ* (cm^3), (e-f) lab-measured volume by water displacement (ml) with DW (g) and AFDW (g) in *C. reniformis*. The power functions are presented in table.1

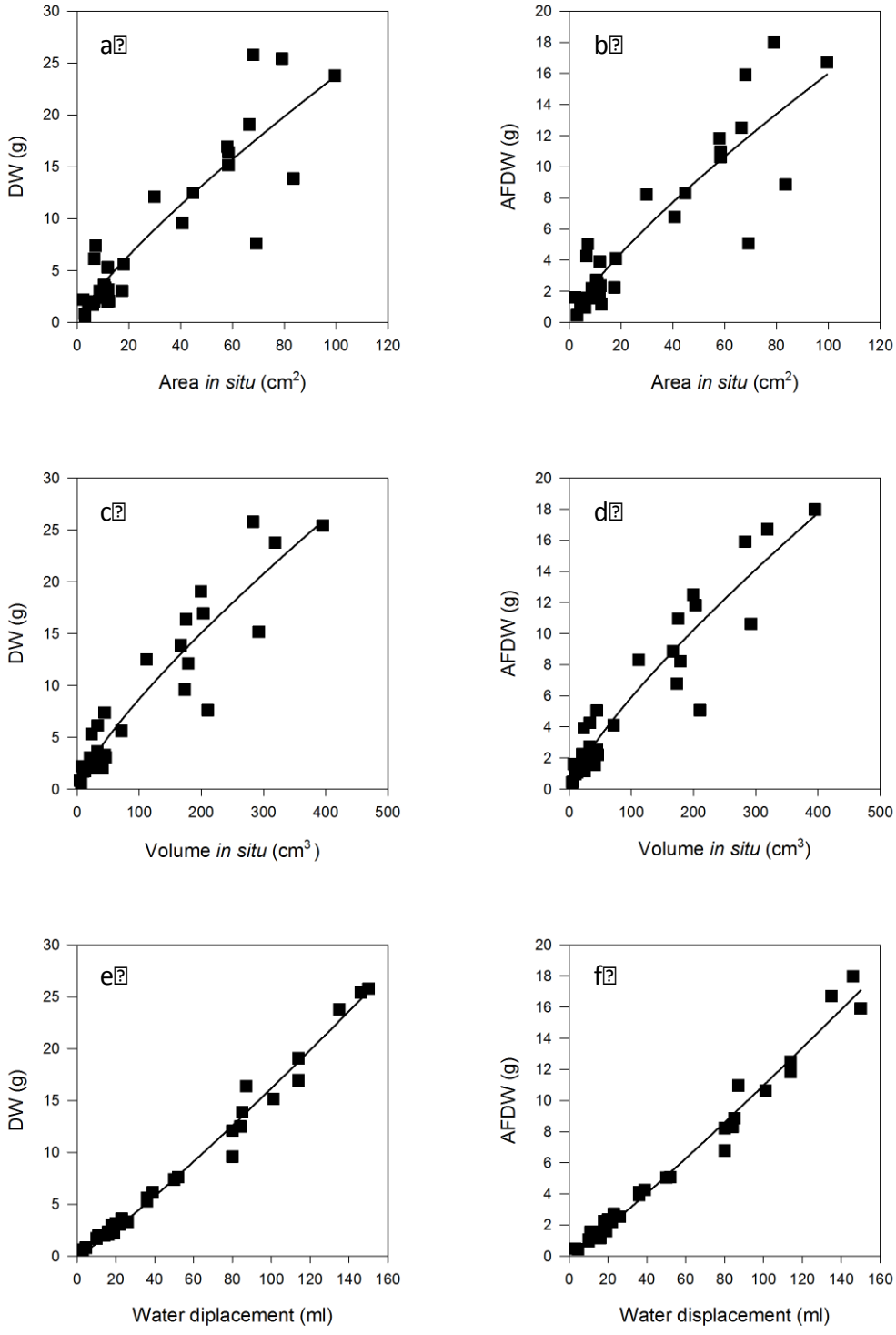


Figure 2 Relationship between (a-b) area *in situ* (cm^2), (c-d) volume *in situ* (cm^3), (e-f) lab-measured volume by water displacement (ml) with DW (g) and AFDW (g) in *A. oroides*. The power functions are presented in table.1

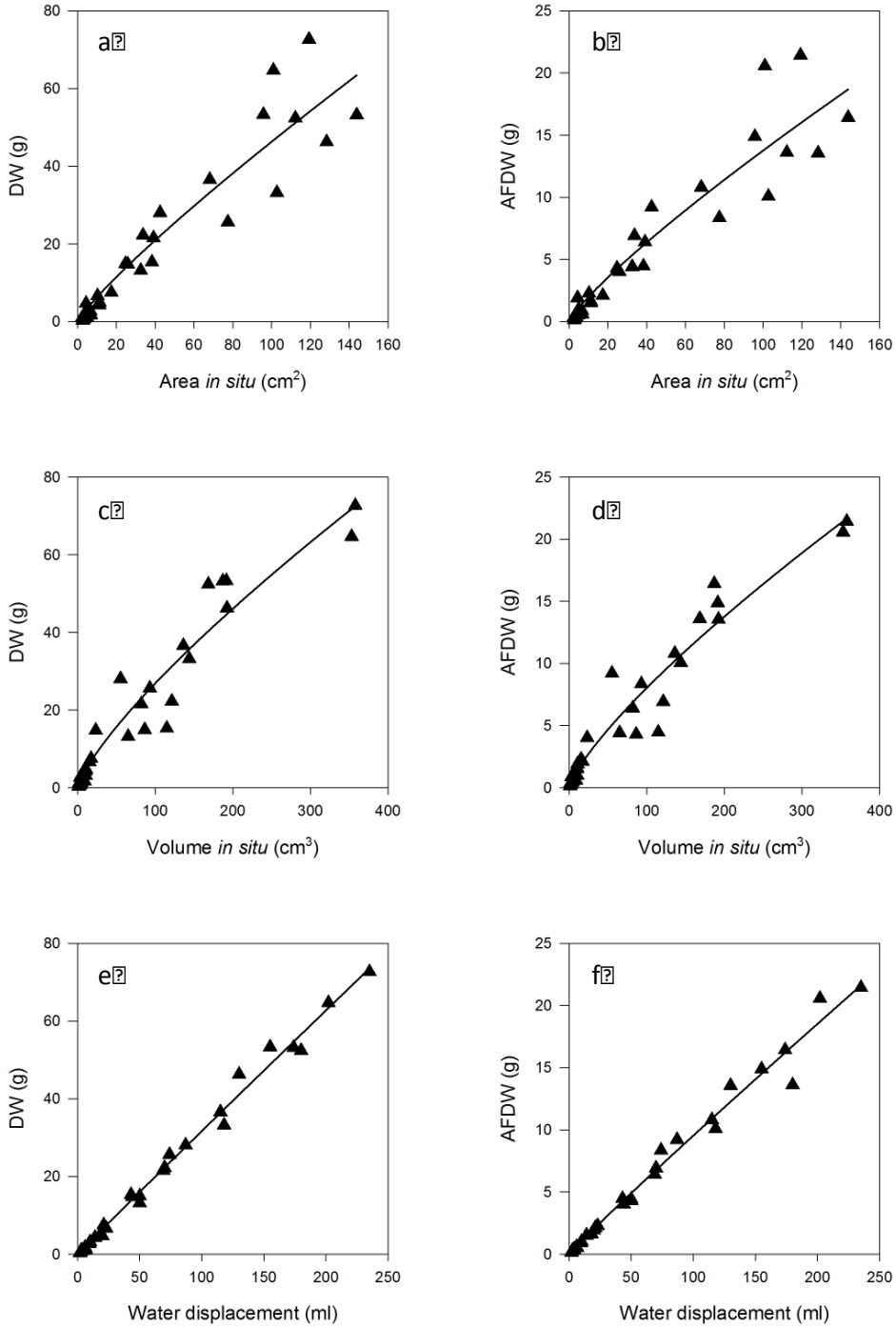


Figure 3 Relationship between (a-b) area *in situ* (cm^2), (c-d) volume *in situ* (cm^3), (e-f) lab-measured volume by water displacement (ml) with DW (g) and AFDW (g) in *P. ficiformis* The power functions are presented in table.1

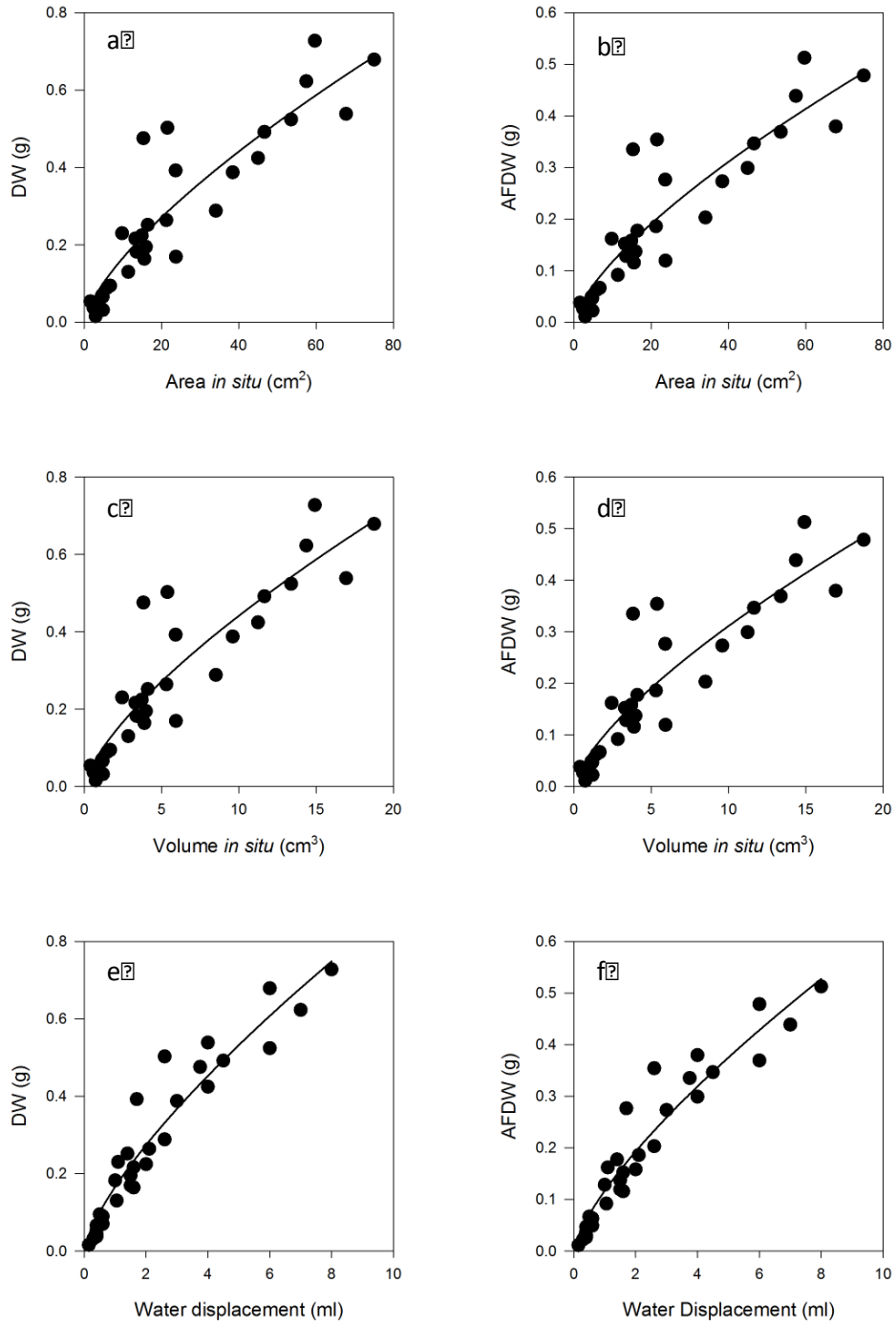


Figure 4 Relationship between (a-b) area *in situ* (cm²), (c-d) volume *in situ* (cm³), (e-f) lab-measured volume by water displacement (ml) with DW (g) and AFDW (g) in *C. crambe*. The power functions are presented in table.1

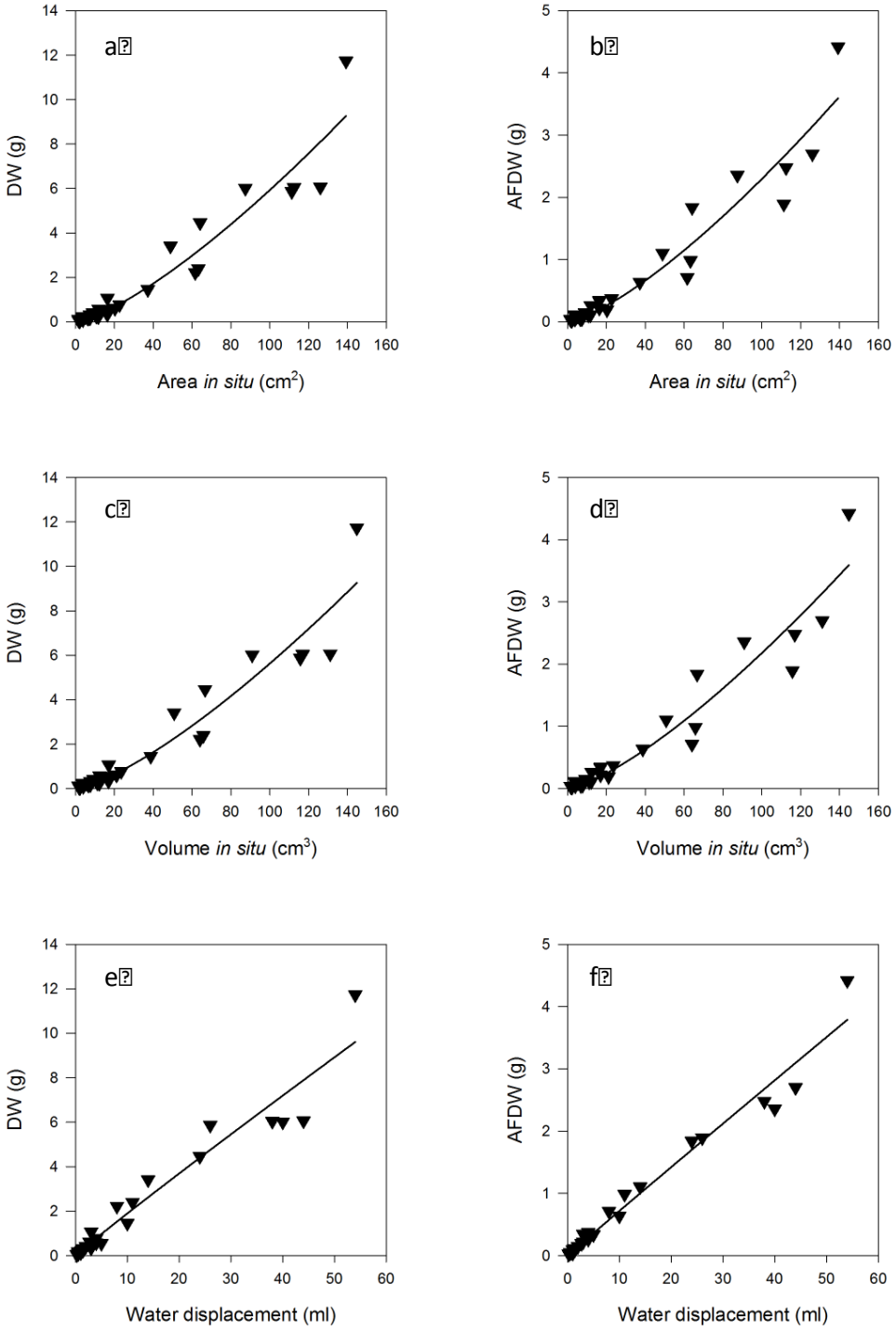


Figure 5 Relationship between (a-b) Area *in situ* (cm²), (c-d) volume *in situ* (cm³), (e-f) lab-measured volume by water displacement (ml) with DW (g) and AFDW (g) in *D. avara*. The power functions are presented in table.1

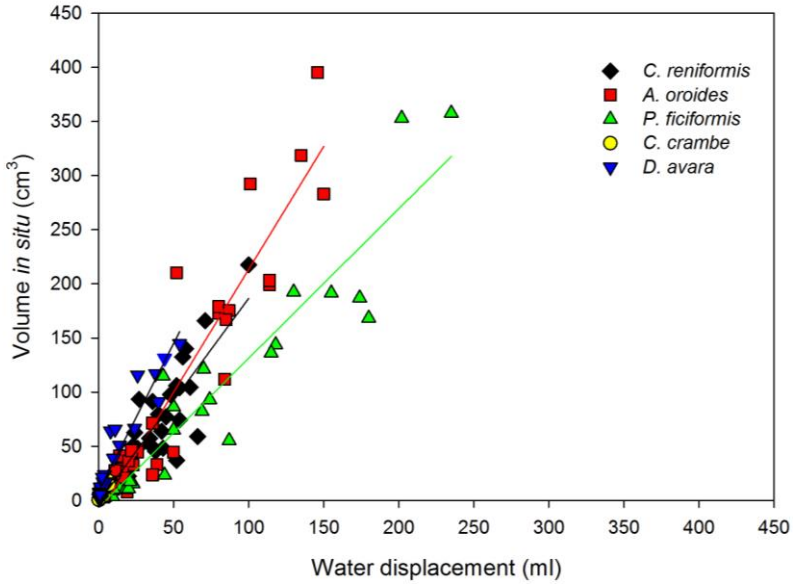
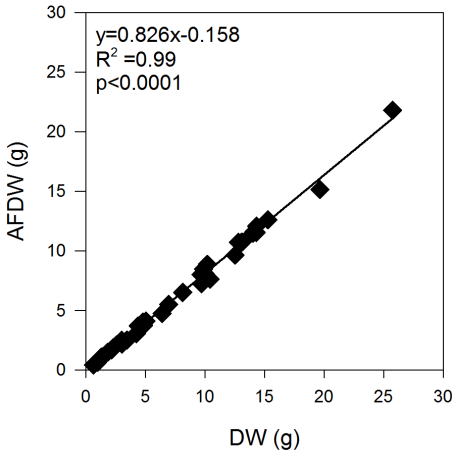
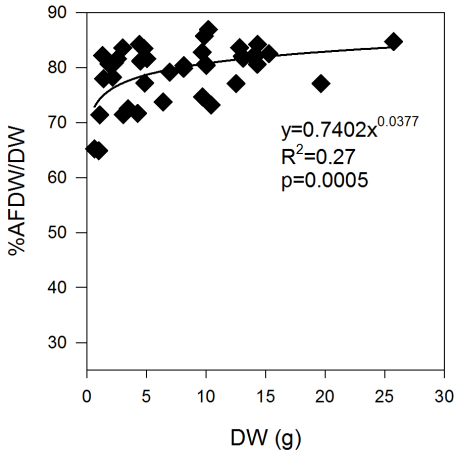


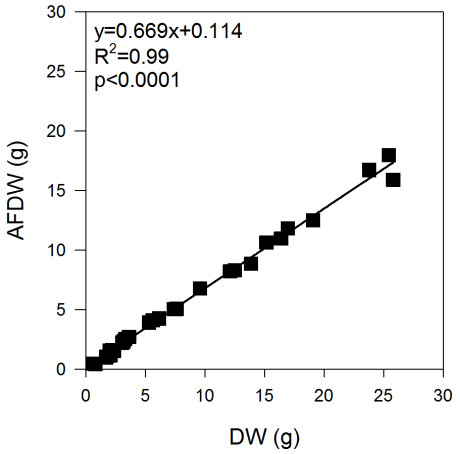
Figure 6 Relationship between the lab-measured volume by water displacement (ml) and the volume estimated *in situ* (cm^3) in the five studied species. The linear regressions are presented in table 1.

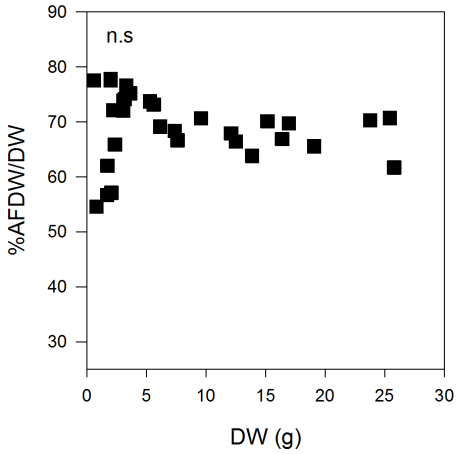
C. reniformis



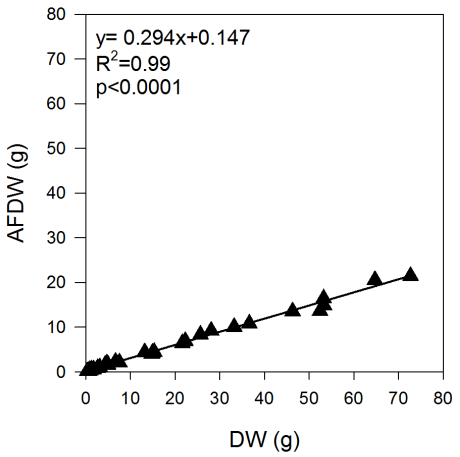


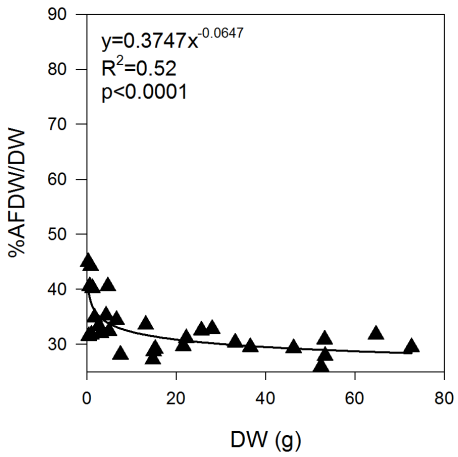
A. oroides





P. ficiformis





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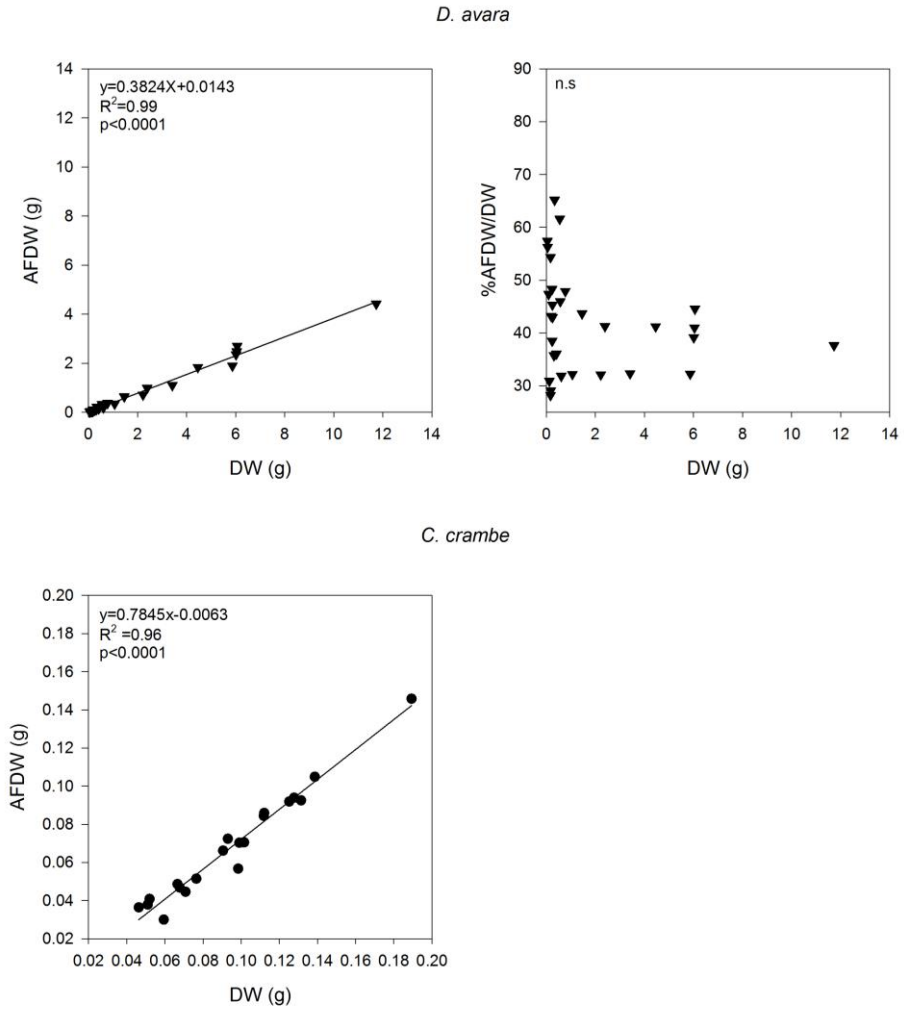

Figure 7 Relationship between DW (g) and AFDW (g) on the left panel and %AFDW/DW on the right panel for the five studied species. The fit curve is presented when significant ($p<0.05$) and the equation is provided. n.s, any significant relationship was found between the two variables. In *C. crambe* the relationship between DW and AFDW was calculated in small pieces of several specimens and %AFDW/DW was omitted because the DW was considered constant in all individuals.

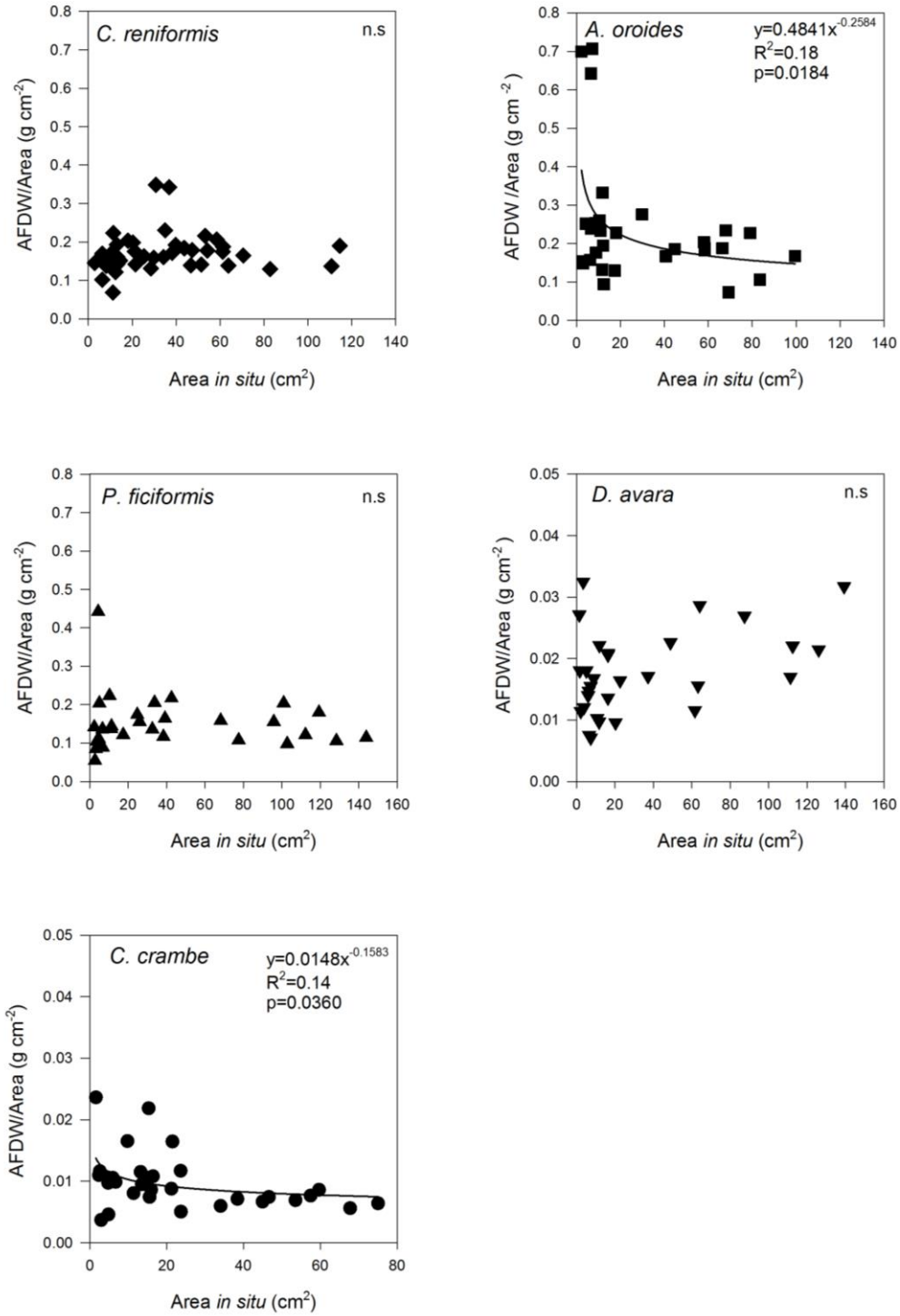

Figure 8 Relationship between AFDW/Area (g cm^{-2}) and area (cm^2) in the five studied species. The fit curve is presented when significant ($p<0.05$). n.s, any significant relationship was found between the two variables.

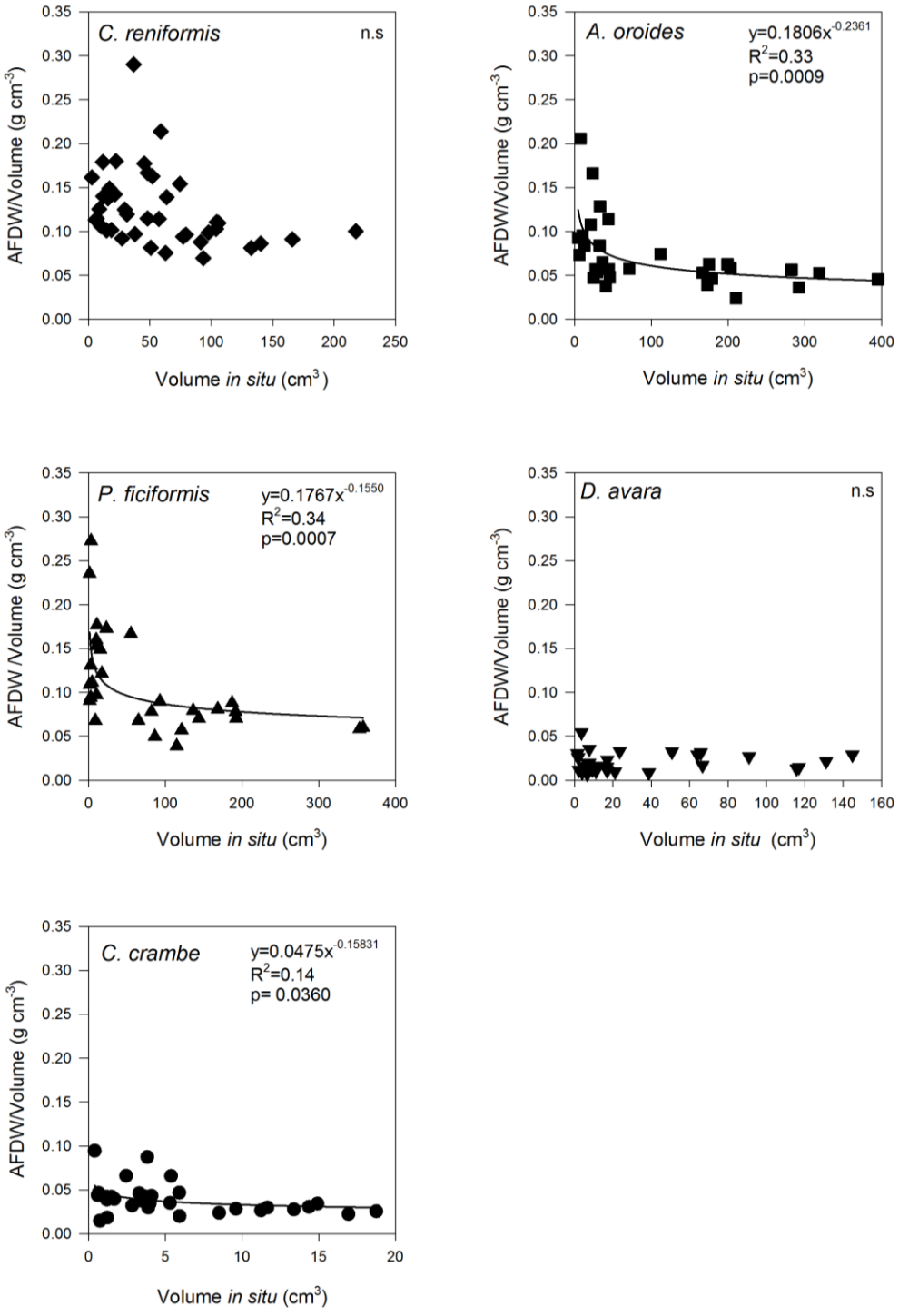

Figure 9 Relationship between AFDW/Volume (g cm^{-3}) and volume (cm^3) in the five studied species. The fit curve is presented when significant ($p<0.05$). n.s, any significant relationship was found between the two variables.

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