Ecophysiology of glass sponge reefs

by

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Abstract

Suspension feeders are an important component of carbon exchange between the water column and the seafloor, a process called pelagic-benthic coupling. Sponges (Phylum Porifera) are filter feeders that consume especially small particles. They eat bacteria, which are inaccessible to most other filter feeders but which make up 10-30% of primary productivity. Feeding by sponges in shallow waters may have considerable effects on energy flow through ecosystems, especially in food-poor environments. This dissertation focuses on the ecophysiology of glass sponge (Porifera, Hexactinellida) reefs, one of the densest aggregations of sponges known in deep water, to determine the flow of energy through reefs and its underlying physiological mechanisms from the scale of the cell up to the ecosystem. Combined, the results explore the ecosystem functions of an important habitat on the northeast Pacific continental shelf and lend insight into the ecology of hexactinellids elsewhere in the world and in past oceans including the ancient sponge reefs in the Tethys Sea.

Paired water samples collected *in situ* before and after passing through a sponge showed that reef sponges remove bacterial carbon and release ammonium into the water column. Stable carbon and nitrogen isotope signatures of reef sponges indicate that bacterioplankton came from both terrestrial and oceanic sources to differing degrees at different reefs, and possibly from bacteria associated with sediments. Microscopical investigation showed that reef sponges also released fecal pellets – aggregates that were 100 to 1000 times larger than the particles they consumed – thereby moving microbial food energy to the benthos as well. Glass sponge reefs have the highest grazing rate of any benthic suspension feeding community measured to date because of the high volumes of water they filter, their efficient removal of bacteria, and their sheer density and size. ^{13}C-labeled bacteria fed to the sponges remained in the tissue for at least two weeks, suggesting that sponges retain and sequester carbon as biomass as well. Repeat visits to the same reef sites and same individual sponges over three years showed that reef-forming glass sponges have similar growth rates, recovery after damage, and recruitment rates

to those of shallower water demosponge species. Study of cell and tissue production however showed conservative processes for tissue maintenance. Pieces of the reef species *Aphrocallistes vastus* were collected and newly forming nuclei labelled with the cell proliferation marker EdU. Very little proliferation occurred in mature regions of the body; most labeling occurred in growing (tip) regions of the sponge. Cell turnover rates were similar to those found in non-growing, mature regions of three shallow temperate sponge species (*Sycon coactum*, *Spongilla lacustris*, and *Haliclona mollis*). In general, sponges were found to vary rates of cell turnover depending on season, taxon, and life history stage suggesting an ability to modify energetic investment in tissue maintenance depending on environmental conditions. Most importantly, for demosponges at least, mature choanocytes – the pumping and feeding cell of sponges – were replaced not by direct replication as in colonial flagellates, but by immigration and differentiation of stem cells as in other animals. Light and electron microscopy showed that tissue and skeletal growth was localized to the growing regions at the tips of the glass sponge *Aphrocallistes vastus*. Choanoblasts – founder cells for flagellated chambers – first divided to form clusters, then produced enucleate collar bodies that expanded the flagellated chambers to their full size.

Combined, the results presented here contribute to an understanding of the flow of energy through glass sponge reefs and the energetic requirements of reef sponges. Glass sponge reefs transfer from microbial food energy to the water column and the benthos through pelagic-benthic coupling, with food sources that can sustain their intense feeding and can fuel comparable growth rates to those of shallower species in food-rich habitats. Other glass sponge communities throughout the deep sea may share similar roles of pelagic-benthic coupling and, in their food-poor environment, act as localized oases of food energy. The tissue structure and tissue maintenance of syncytial glass sponges reflects adaptations to a low-food environment, and could also reflect the conditions in which sponges and other early animals evolved.

Preface

Chapter 2 has been published as Kahn, A. S., G. Yahel, J. W. F. Chu, V. Tunnicliffe, and S. P. Leys. 2015. Benthic grazing and carbon sequestration by deep-water glass sponge reefs. Limnology and Oceanography **60**:78-88. GY, VT, and SPL designed the experiment, collected, and analyzed the raw data. Data from JWFC contributed unpublished data for analysis. GY, VT, SPL, and I did data analysis and contributed to the writing of the manuscript.

Chapter 3 has been published as Kahn, A. S., L. J. Vehring, R. R. Brown, and S. P. Leys. 2015. Dynamic change, recruitment, and resilience in reef-forming glass sponges. Journal of the Marine Biological Association of the United Kingdom. **96**(2):429-436. I led this paper's direction and analysis. LJV, RRB, and SPL contributed to data collection. SPL obtained ship time and led the ROV dives to take time-series photos. SPL and I contributed to the writing of the manuscript.

Chapter 4 will be submitted as a coauthored publication with Sally P. Leys (SPL; University of Alberta). SPL and I designed the project's direction and analysis. I developed protocols, laboratory experiments, and statistical analyses. SPL and I contributed to the writing of the manuscript.

Chapter 5 is collaborative work involving A.R. Bramucci, R. Case, J.W.F. Chu, and S.P. Leys (University of Alberta). SPL and I designed the study. ARB, RC, JWFC, SPL, and I collected sponges and isotope samples. JWFC, TR, and I processed samples for isotope analysis. I did all statistical analyses. SPL and I contributed to the writing of the manuscript.

Chapter 6 has been submitted to the journal *Invertebrate Biology* as a coauthored publication (short communication) with S.P. Leys (University of Alberta). SPL and I collected sponge samples. I did all laboratory experiments and sampling processes. SPL and I interpreted the morphology, and we both contributed to writing the manuscript.

Acknowledgments

A wise thesis committee member told me, “You can’t go it alone in science” and it is extremely true. This research would not have developed to fruition without the support of many. Especially, my supervisor Sally Leys dedicated so much of her expertise, knowledge, insight, time, and personal care into my progress that I feel she is one of the few others who are as proud of this final product as I am. Rolf Vinebrooke and Rebecca Case served as excellent advisors as my thesis committee; I appreciate the depth that your diverse expertise brought to my project. I also thoroughly enjoyed my candidacy exam, where Sally, Rolf, Rebecca, Warren Gallin (who even agreed to come back for a thesis defense), and Marianne Douglas grilled me about my knowledge and as a result helped me realize the bigger picture of my research. I also appreciate my external examiner Marta Ribes for agreeing to evaluate my thesis in the midst of moving and for being willing to teleconference all the way from Spain.

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I value the interactions I had with all of my labmates. Pam Windsor Reid stands out as a valued, inspiring labmate and grounded friend. The others helped me get through the highs and lows of graduate school: Nathan Farrar with his love for music from the 90’s and his dismal attitude toward the future of science careers (which was so fun to argue!); Danielle Pendlebury’s incredible work ethic and our naan-eating, row-boating adventures in Bamfield; Rachel Brown’s snarky comments and foodie ways; Jasmine Mah’s empathy and her willingness to roll down grassy hills. I also thank the newbies, Lauren Law and Curtis Dinn, for their entertainment and support. Kristen Kruper helped in so many ways, with long days in Bamfield, long nights of staining and sectioning, and always with a compassionate ear and encouraging words.

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Thank you to the following mentees over the years: Kristen Kruper, Laura Vehring, Matthew Weigel, Stephanie Yu, Afyqah Kamarul-Zaman, Laura Vehring, and Laura Hamonic. I learned a lot from you and I hope you learned a bit from me as well.

Laughter, venting sessions, frustrations, and exaltations as well as unwavering support came from my personal network of favorite people. Clark Pennelly hopped on an airplane for the first time to move to a new country with me. He accepted the snow, the lifestyle, my absences for an average of 1/4 of the year, with surprising ease. Suzanne Kahn rode the highs and lows of the PhD program along with me. She has always supported me to carry on as the non-accountant of the family and her wisdom is what has pushed me to keep asking myself and others for bigger and better goals (what's the worst that could happen?). I hope this makes them both proud.

Table of Contents

List of Tables

List of Figures

Chapter 1. Ecosystem ecology of glass sponge reefs

1.1. Energetics as a driver of ecology and evolution

The amount of energy available to an ecosystem determines several of its properties including number of trophic levels (Odum 1956), primary and secondary productivity (Odum 1968), and the constraints on the energy budgets of an ecosystem's biota (Odum and Odum 1955). For a single organism, the energy available from the food it eats (*C*, consumed food) must be partitioned into various processes, including production of new tissue and chemical storage such as fats and lipids (*P*), maintenance processes and respiration (*R*), excretion of urine/waste (*U*), and excretion as feces (*F*). As a result, an energy balance for an organism is

$$C = P + R + U + F$$

The most important uses of energy for the organism are *P* and *R*. The most important uses of energy for the ecosystem are *U* and *F*, and to an indirect extent *P* (e.g. through predation).

For the organism, production of new tissue or biomass (as either somatic growth or reproduction) and respiration are important uses of the energy that it consumes. Given unlimited energy, an organism could maximize fitness by growing continually, reproducing frequently, and maintaining its tissue continually to minimize accumulation of oxidative stress over time (Pellettieri and Alvarado 2007). However food energy is generally limited, so energy budgets drive adaptations that prioritize different trade-offs, such as a species that grows quickly and with high fecundity or one that grows slowly and with low fecundity but with more investment in parental care. Selective pressure from food-poor conditions drives adaptive radiation, niche partitioning, and other strategic ways to find novel food sources or maximize efficiency (Hutchinson 1957, Bambach et al. 2002, Bambach et al. 2007). For example, in one food-poor environment, the deep sea, some tunicates have traded filter feeding for carnivory, annelids and anglerfish have extreme sexual dimorphism that has reduced male bodies to little more than a neotenous sperm pouch, and annelids, molluscs, and sponges have developed symbiotic relationships with chemoautotrophic bacteria (Pietsch 1976, Vacelet et al. 1996, Okuyama et al. 2002, Rouse et al. 2004, Ogawa et al. 2005, Lösekann et al. 2008, Katz et al. 2011). Energetics can therefore drive the ecology and evolution (adaptations) of a species (reviewed by Futuyma and Moreno 1988).

A highly productive ecosystem is one in which energy is abundant: autotrophs create primary production from sunlight (photoautotrophs) or chemical sources (chemoautotrophs), secondary and higher-level consumers transfer primary production into biomass, and nutrients are either recycled from wastes or imported from other ecosystems. Just as an organism is

constrained by the energy available to it, an ecosystem with low productivity is also constrained: either few trophic levels can be supported, few ecological niches are available, or the value of energy recycling and import from other ecosystems, termed cross-system exchange (Polis and Hurd 2011), increases. In the ocean, a major mechanism of cross-system exchange is the transfer of food energy from the water column to benthic biomass and communities, termed pelagic-benthic coupling.

1.1.1. The role of suspension feeders in pelagic-benthic coupling

Suspension feeders capture plankton and marine snow suspended in the water column (Gili and Coma 1998). Filter feeders, which include bivalves, polychaetes, ascidians, sponges, and even baleen whales, are a subset of suspension feeders that use meshes, pumps, and ciliated appendages to concentrate particles too small to be detected individually (Gili and Coma 1998). Through excretion, suspension feeders connect pelagically-derived food energy with benthic biomass (Gili and Coma 1998, Riisgård and Larsen 2000). Suspension feeders are abundant where productivity is high; their abundance may decrease in areas with lower quality or less available food due to limited resources (Richardson and Mackay 1991).

Suspension feeders on the seafloor can affect their ecosystem by reducing plankton concentrations in nearby water. Zebra mussels and oysters increase water clarity by depleting or controlling phytoplankton concentrations (Ackerman et al. 2001, Coen et al. 2007). Suspension feeding communities in coral reefs affect phytoplankton populations and nitrogen cycling (Genin et al. 2009). Filter feeders can also enhance productivity by excreting nitrogen or carbon compounds that stimulate phytoplankton growth (Arzul et al. 2001).

1.1.2. Sponges: ecologically significant suspension feeders

Sponges (Phylum Porifera) are filter feeders that ingest very small particles (0.5 to 20 μm) (Reiswig 1975b). They can therefore access the 10-30% of primary productivity contributed by bacteria, which is inaccessible to most other filter feeders (Fenchel 1984). Together with *Daphnia*, appendicularians, and others, they therefore bring microbial food energy into the larger trophic web, linking pelagic-benthic coupling with the microbial carbon loop (Legendre and Le Fèvre 1995). Sponges can filter up to one thousand times their body volume each day (Leys et al. 2011) and extract food with up to 95% efficiency (Maldonado et al. 2012). Sponges use flagellated cells called choanocytes to draw water through canals and into choanocyte chambers, where water slows before it is drawn through a fine 45-nm glycocalyx mesh (Reiswig 1975a, Leys et al. 2011). The slowed water presumably gives choanocytes the opportunity to grasp bacteria with pseudopodia and phagocytize them (Leys and Eerkes-Medrano 2006).

Bacteria are later digested and expelled as fecal pellets, dissolved nitrogen, and CO_2 (Wolfrath and Barthel 1989, Hadas et al. 2009).

The ecological effects of sponge feeding have been well studied in shallow habitats. Filtration by freshwater sponges in Lake Baikal enhanced water clarity and depleted bacteria from the water column (Pile et al. 1997), tropical and temperate marine species recycle nitrogen into ammonia, nitrate, nitrite, or dissolved nitrogen (Jimenez Tejero 2011, Maldonado et al. 2012), and species with siliceous skeletons locally act as silica sinks (Maldonado et al. 2011).

Sponges have also been considered as a link between the biological pump and microbial carbon from the microbial loop (Pile and Young 2006, Thurber 2007, De Goeij et al. 2013) (Figure 1.1). The localized effects of sponge feeding may not be important sources of carbon or nitrogen in ecosystems with high productivity, but feeding by sponges may have greater significance in energy-limited habitats. For example, a recent study in a tropical coral reef was able to trace dissolved organic carbon from the water column, through a sponge, and into the tissue of higher-level consumers (De Goeij et al. 2013).

My thesis research focused on the capacity of, and mechanisms behind, benthic-pelagic coupling of a primarily deep-water class of sponges, Hexactinellida.

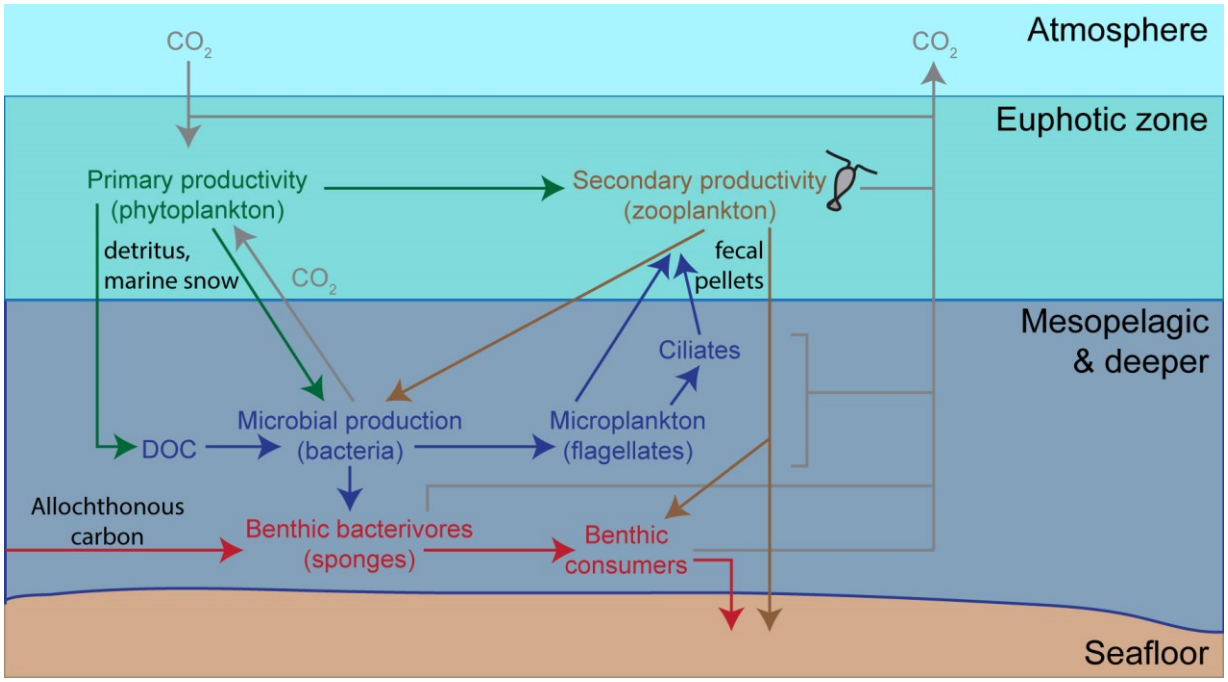

Figure 1.1. Sponges as a link between the microbial loop and the biological pump. Sponges in deep and shallow habitats alter the water they process by connecting the microbial loop (colored in blue) to the biological pump (colored in green, brown, and red). The diagram shows the biological pump for sponges in deep water but sponges serve the same roles in shallow water. Abbreviations: DOC (dissolved organic carbon).

1.1.2.a. *Sponge phylogeny*

The monophyletic phylum Porifera contains four classes: Calcarea, Homoscleromorpha, Demospongiae, and Hexactinellida (**Figure** 1.2). All classes have the sponge body plan: a bacterivorous filter feeder powered by collared, flagellated units (typically choanocytes except in Class Hexactinellida). The only exception is a clade of sponges in the Class Demospongiae, which have secondarily become carnivorous and no longer filter feed (Vacelet and Boury-Esnault 1995)The most abundant and well-studied class of sponges is Demospongiae, which contains about 85% of known species (Van Soest et al. 2012). Class Hexactinellida is sister group to the Demospongiae (Dohrmann et al. 2008). Hexactinellids have a strong fossil record and were recognizable 545 Mya during the Neoproterozoic Era while Demospongiae and Calcarea are not seen in the fossil record until the Lower Cambrian (Mehl et al. 1998, Antcliffe et al. 2014). Due to their early appearance in the fossil record, glass sponges may be informative for the origin of many characters of the sponge body plan.

1.2. Ecology of glass sponges

Glass sponges (Class Hexactinellida) are primarily found in the deep sea, where food from the surface is eaten and recycled to overcome generally food-poor conditions (Smith 1987). Studies of glass sponges show that they grow episodically, possibly in response to pulses of food (Dayton 1979, Leys and Lauzon 1998, Kahn et al. 2012, Dayton et al. 2013, Fillinger et al. 2013). Dense aggregations of glass sponges are found where food , as bacterial productivity, is abundant (Leys et al. 2007). *Sericolophus hawaiicus* is thought to thrive on abundant food from resident plankton communities and arriving by downwelling of surface waters along the continental slope off the island of Hawaii (Pile and Young 2006) while dense stands of *Pheronema carpenteri* coincide with areas where tidal energy imports resuspended or undeposited organic matter on internal waves (Rice et al. 1990).

Hexactinellids are found in all oceans but are poorly studied compared to other sponge groups because they are mostly confined to deep water. However, in four location they reach shallower water the continental shelf in the Southern Ocean (Antarctica), many caves in the Mediterranean, and fjords in New Zealand and western Canada (Barthel and Tendal 1994, Vacelet et al. 1994, Leys et al. 2004, Reiswig and Kelly 2011). These shallow nearshore regions have much greater densities of glass sponges than are found in deep water, with densities as high as 100 individuals per square meter (Vacelet et al. 1994). This leads one to question why dense sponge areas form where they do, and especially in shallower shelf regions. Shelf regions have much more productivity and strong currents compared to the deep sea so food may not be limiting there (Leys et al. 2007).

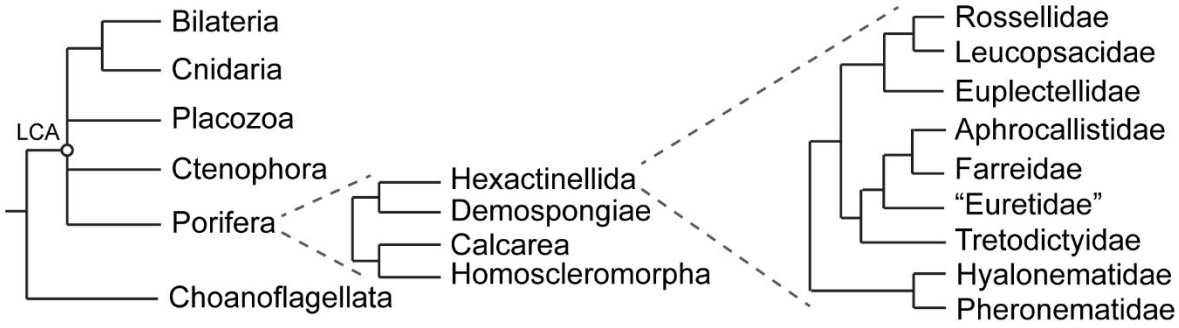

Figure 1.2. The phylogenetic placement of sponges is as one of the first groups of early animals together with ctenophores (Phylum Ctenophora) and placozoans (Phylum Placozoa). These early branching groups may shed light on traits shared with the last common ancestor to animals (LCA). Within monophyletic Porifera, Hexactinellida is sister group to Demospongiae. Within the Hexactinellida, reef-forming species are found in the families Aphrocallistidae and Farreidae. Hexactinellid relationships were reproduced from Dohrmann et al. (2008).

Dense populations of glass sponges have the potential to affect the surrounding environment through their filter feeding activity. Indeed, dense aggregations of glass sponges process large volumes of water – a square meter patch of *Sericolophus hawaiicus* could filter 3,208 L water d^{-1} (calculated using data from Pile and Young 2006). A mixed community of sponges in the Southern Ocean that includes glass sponges consumes so many bacteria from a sewage outfall that lipid tracers of sewage were measured in their tissue (Thurber 2007).

In addition to their role in filtration, live sponges, denuded stalks, and even spicule mats of dead sponges serve ecological functions by providing hard substrate for other animals to colonize in the deep sea (Bett and Rice 1992, Beaulieu 2001b, Beaulieu 2001a) (Figure 1.3). Glass sponges can persist for a long time in the environment and form a substrate for other animals to settle on. Radiocarbon dating the organic matrix sequestered in spicules from a museum specimen of the Antarctic glass sponge *Rossella racovitzae racovitzae* suggests it was approximately 440 years old (Fallon et al. 2010). Spicule mats cover about one third of the seafloor in the Porcupine Seabight (Bett and Rice 1992) and parts of the seafloor in Antarctica can have mats from a few cm to 2 meters thick (Dayton et al. 1974), providing hard substrate for polychaetes and other animal groups even after a sponge dies.

1.3. Glass sponge reefs

In the straits and fjords of British Columbia, hexactinellids form meter-tall reefs in a similar process as hermatypic corals, growing upon dead skeletons of past generations (**Figure** 1.4A) (Conway et al. 2001, Krautter et al. 2006). Several glass sponge species flourish in the waters of British Columbia but three species in particular were able to grow upon the fused skeletons of previous generations to form reefs. *Farrea occa* (Family Farreidae, Order Hexasterophora) is found only in fjords and the reefs north and west of Vancouver Island (Leys et al. 2004) while *Heterochone calyx* and *Aphrocallistes vastus* (Family Aphrocallistidae, Order Hexasterophora) are found in shelf waters along the entire coastline of British Columbia (**Figure** 1.4B-C). Some regions have dense, diverse glass sponge communities growing on hard rock called sponge gardens. The three reef-forming species could cement a scaffolding of spicules that persisted beyond the lifespan of a single generation, allowing juveniles to grow on the skeletons of past generations and form reefs (Krautter et al. 2006).

Sponge reefs were prominent habitats during the Jurassic and Cretaceous periods (Leinfelder et al. 1994). A belt of sponge reefs ~7,000 km long stretched across the Tethys Sea during that time (Ghiold 1991). Upper Jurassic reefs were made of mixed assemblages of hexactinellids (both with fused and unfused skeletons) and lithistid demosponges (Leinfelder et al. 1996). Modern-day coral reefs are known to need food from sunlight, so presumably the

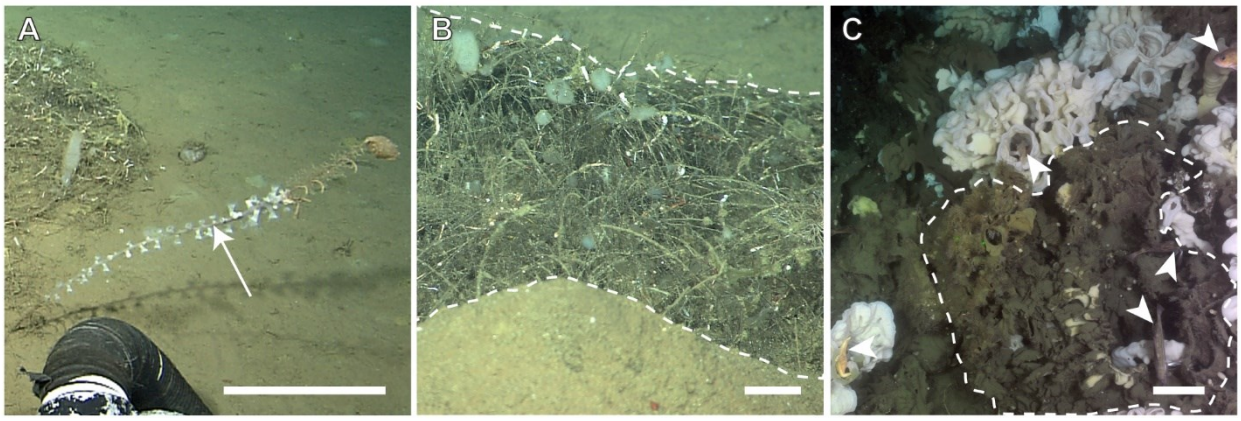

Figure 1.3. Glass sponges provide hard substrate in the deep sea, whether dead or alive. A. Stalks of glass spicules (arrow), used to anchor sponges in soft sediments, become colonized with anemones, echinoderms, and other taxa that need hard substrate (image from the Monterey Bay Aquarium Research Institute). B. The spicule mats left behind after sponges die (outlined) give heterogeneous surfaces and hard substrate throughout the deep sea and an increase macrofaunal diversity (image from the Monterey Bay Aquarium Research Institute). C. Live sponges and the fused skeletons of dead sponges (outlined) in a glass sponge reefs harbor fish and echinoderms (arrowheads). Scale bars: A. 10 cm; B: 1 cm; C: 20 mm.

production of so much biomass required high levels of food and nutrients such as silica. Today's glass sponge reefs likely serve similar habitat-forming and ecosystem functions as Jurassic reefs, but importantly are analogous systems. Only glass sponges with fused (dictyonine) skeletons form modern-day reefs, although other glass sponge species without fused skeletons can live in reefs.

Reef formation occurred following the last glacial maximum as hexactinellid species grew on glacially carved ridges. Reefs grew as the fused spicule skeletons of reef forming sponge species remained after death of the live sponges and eventually became cemented with sediment, producing new hard substrate for recruits to settle and grow on (Krautter et al. 2006). Glass sponge reefs in Hecate Strait have grown up 19 m in height above the glacial furrows and ridges that were originally colonized by sponges 9,000 years before (Conway et al. 2001). Most of that height is buried by sediments, with only 1-2 meters of the reef surface exposed and containing live sponge (Conway et al. 2001). Today, 22 patches of reefs have been found between southern Alaska and the Strait of Georgia in British Columbia with 13 reefs in the Strait of Georgia alone (Conway et al. 2007), with marine protected areas being proposed to protect reef sponges from trawling and other human activity (**Figure** 1.4E-F).

The straits and fjords where sponges are found are high in productivity. Seasonal upwelling brings in productive water from the deep Pacific, surface waters produce strong seasonal plankton blooms, and rivers act as sources of terrestrial productivity (Johannessen et al. 2003, Whitney et al. 2005, Johannessen et al. 2008). Sediments in the reef are Holocene muds rich with organic carbon (>3 weight percent, wt. %, in Queen Charlotte Sound reefs compared to 0.8 wt. % measured from non-reef muds) (Conway et al. 2001) and support high densities of polychaetes that are more diverse than polychaete communities found outside of the reefs (Cook 2005).

Glass sponge reefs support high levels of biodiversity, including for commercially important fish and shellfish species (Krautter et al. 2001, Marliave et al. 2009, Chu and Leys 2010b). Squat lobsters, hairy crabs, shrimp, and rockfish are found in and around oscula of sponges (Chu and Leys 2010b). Octopus, sharks, and halibut are also frequent visitors or residents (personal observation). Nudibranchs, and possibly fish and sea stars, prey upon reef sponges (Chu and Leys 2012, personal observation), so reef-forming sponges are known to be foundation species for their communities like other animals (Jones et al. 1994).

Glass sponge reefs can also locally affect nutrient cycling. So far, only their effect on silica has been studied and it was found that they sequester silica into the seafloor (Chu et al. 2011). It is possible that reefs could play in important role in carbon cycling as well, like that

Figure 1.4. Glass sponge reefs Glass sponge reefs are found along the continental shelf of British Columbia and southeastern Alaska. B. *In situ* experiments and surveys were carried out using the remotely operated vehicle ROPOS. (Image from S.P. Leys.) C. and D. Three species make up the reefs: *Aphrocallistes vastus* (C., Av) and *Heterochone calyx* (D., Hc) are found in all waters while *Farrea occa* (D., Fo) is only found in fjords and northern reefs in Hecate and Queen Charlotte Straits. Their hard fused skeletons provide hard substrate for larval recruits to settle on. (Image D provided courtesy of Department of Fisheries and Oceans, Miriam O.) E. Reefs can be damaged by human activity such as trawling or cable-laying activity. Here sidescan sonar detects a sponge reef and tracks of trawls that ran through the reef. Modified from Conway et al. (2001). F. The Department of Fisheries and Oceans is designing Marine Protected Areas around three glass sponge reefs. Here, the boundaries of the core protection zone (gray box) around one of the three reefs (orange).

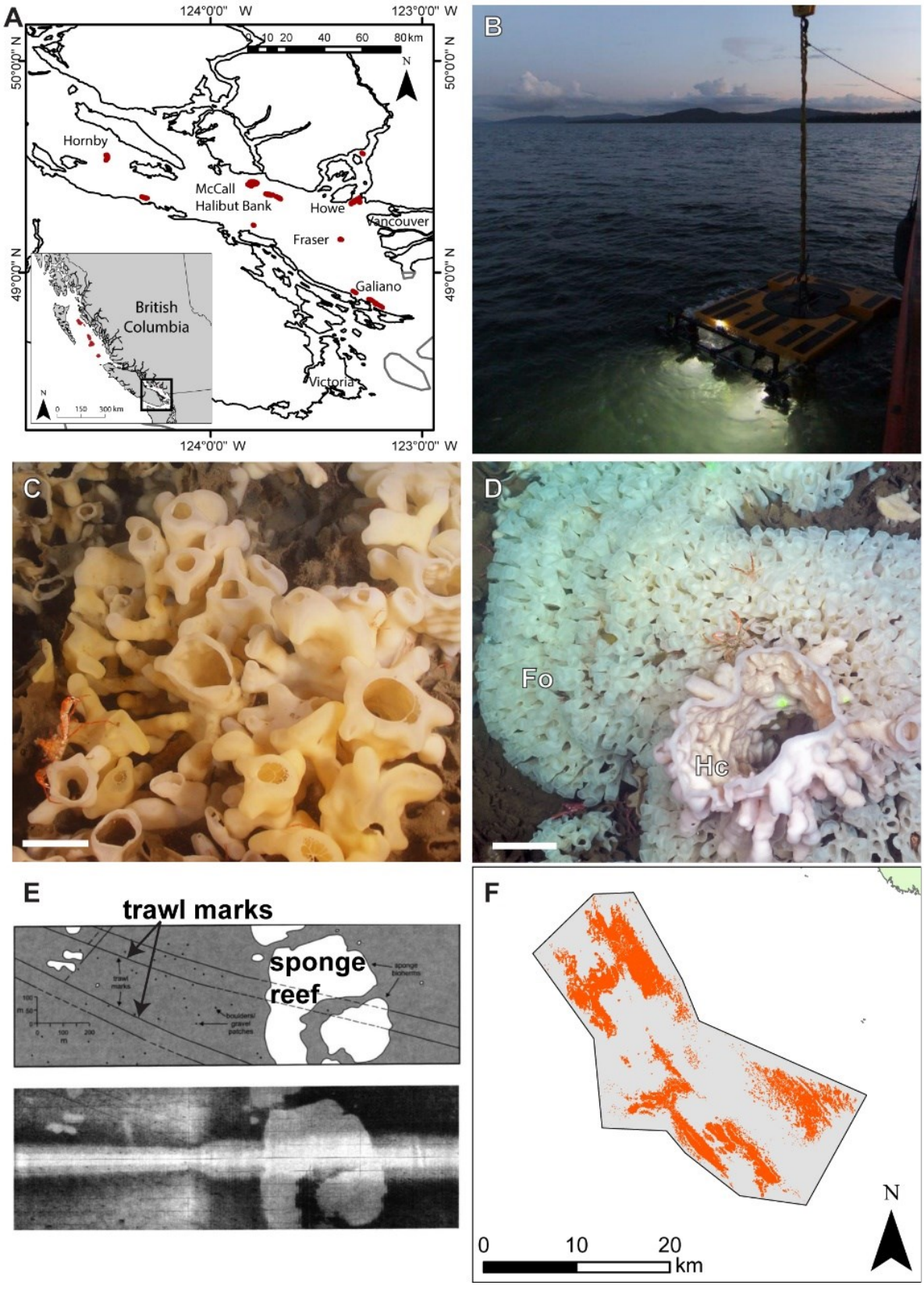

Figure 1.4. Glass sponge reefs

observed for the same species of glass sponges in fjords (Yahel et al. 2007) and shallow sponges (Pile et al. 1997, De Goeij et al. 2013). The study of nutrient cycling by reef sponges along the continental shelf of western Canada would also give insight into the possible effects of feeding by glass sponges in the food-poor deep sea.

1.4. Cell biology of glass sponges

In addition to understanding the ecological energetics of glass sponge reefs, knowledge of the energy flow through glass sponges themselves is important for understanding what may affect their ecology. As with all animals, food consumed by a glass sponge is used for excretion as urine and feces, in the tissue as production (new biomass or reproduction), or for respiration. Respiration includes the cost of filter feeding and other activity (Reiswig 1971a, Reiswig 1974, 1981, Reiswig 1990, Leys et al. 2011), while production encompasses growth, tissue maintenance (Shore 1971, De Goeij et al. 2009), and repair (Bell 2002, Alexander et al. 2014). Little is known about any of these processes in a glass sponge.

1.4.1. Tissue structure

Six-rayed glass spicules form a fused or unfused scaffolding upon which the tissue lies (Reiswig and Mehl 1991). Spicules form as the acidic vacuoles in the syncytial tissue concentrate silicic acid from the environment and deposit nanospheres of amorphous silica around a collagen- and silicatein-rich matrix (Uriz et al. 2003, Leys et al. 2007, Ehrlich et al. 2010). Glass sponge spicules can undergo various degrees of secondary fusion from spot soldering spicules together at the tips (Reid 2003) to full cementation of several anchoring basal spicules for reinforcement in *Euplectella aspergillum* (Weaver et al. 2007). Spicule fusion presumably could be widespread throughout the Hexactinellida, but in *E. aspergillum* it was only to reinforce clusters of spicules while most of the spicules in the sponge body remain unfused (Weaver et al. 2007). Secondary fusion forms a rigid scaffolding in sponges of the Order Hexactinosida, which includes the three species that form reefs (Leys et al. 2007).

Terminology for tissue structure follows that of Mackie and Singla (1983). Trabecular tissues contain open cytoplasmic bridges and closed, perforated plugs that connect to 'cells' (mononucleated but still connected to the rest of the syncytium). Plugged cytoplasmic bridges maintain cytoplasm continuity and allow propagation of electrical signals that coordinate flagellar beating through the body (Mackie et al. 1983, Leys et al. 1999). The glass sponge body as a whole has regions no different from cellular sponges: incurrent and excurrent canals for water to pass through chambers powered by flagellated collar units, and regions that produce spicules (Mackie and Singla 1983). As described by Leys (2003b) the trabecular syncytium

makes up 75% of the tissue; it includes the dermal and atrial surfaces (membranes) of the sponge, makes up the primary and secondary reticula of flagellated chambers, interconnects chambers with cord regions, and contains cytoplasmic streams that transport organelles, vesicles, and phagocytized particles throughout the body (Figure 1.5) (Leys 1995, Wyeth 1999).

Choanoblasts are 'cells' (have a single nucleus) connected to the rest of the multinucleated tissue by cytoplasmic bridges. From them extend collar-flagella units called collar bodies, which beat flagella to draw water through the aquiferous system (Mackie and Singla 1983, Leys 1999). Collar bodies are structurally similar to the choanocytes of cellular sponges by bearing collars of microvilli around a flagellum, but they lack nuclei. They lie embedded in a primary reticulum (part of the trabecular syncytium) with collars of microvilli that project up through a secondary reticulum (also part of the trabecular syncytium) (Figure 1.5). Two types of nuclei are visible in the tissue: the many nuclei of the syncytia, which are small and uniformly distributed throughout the tissue, and the nuclei found in choanoblasts and archaeocytes.

1.4.2. Feeding and energetics

An understanding of how feeding occurs in a glass sponge could be useful since feeding is what drives nutrient flow through sponges. The stages of feeding that have been observed in glass sponges follow a similar pattern to that observed in cellular sponges. Water movement through the glass sponge is caused by the beating of flagellated collar bodies, which pulls water through incurrent canals. Water enters pores on the dermal surface and flows into large vestibules in the subdermal space that lead to incurrent canals and eventually to flagellated chambers. Water enters a chamber through holes in the primary reticulum called prosopyles. The water slows before it is rapidly pulled through a glycocalyx mesh strung across the collars of the collar bodies (Figure 1.5) (Leys et al. 2011). Bacteria appear to be captured from the water in the space between the primary and secondary reticula, then are transported through the cytoplasm in vacuoles (Perez 1996, Wyeth 1999), and wastes are digested and excreted out the excurrent chimney of the sponge, the osculum. Excretion has only been observed from demosponges, which release aggregates of particles in membrane-bound fecal pellets (Wolfrath and Barthel 1989, Gobel 1993). It is not known how and where digestion takes place nor what excreted material looks like in hexactinellids. If fecal matter is packaged into pellets as it is in cellular sponges, then excreted material could provide food for deposit feeders in the surrounding community.

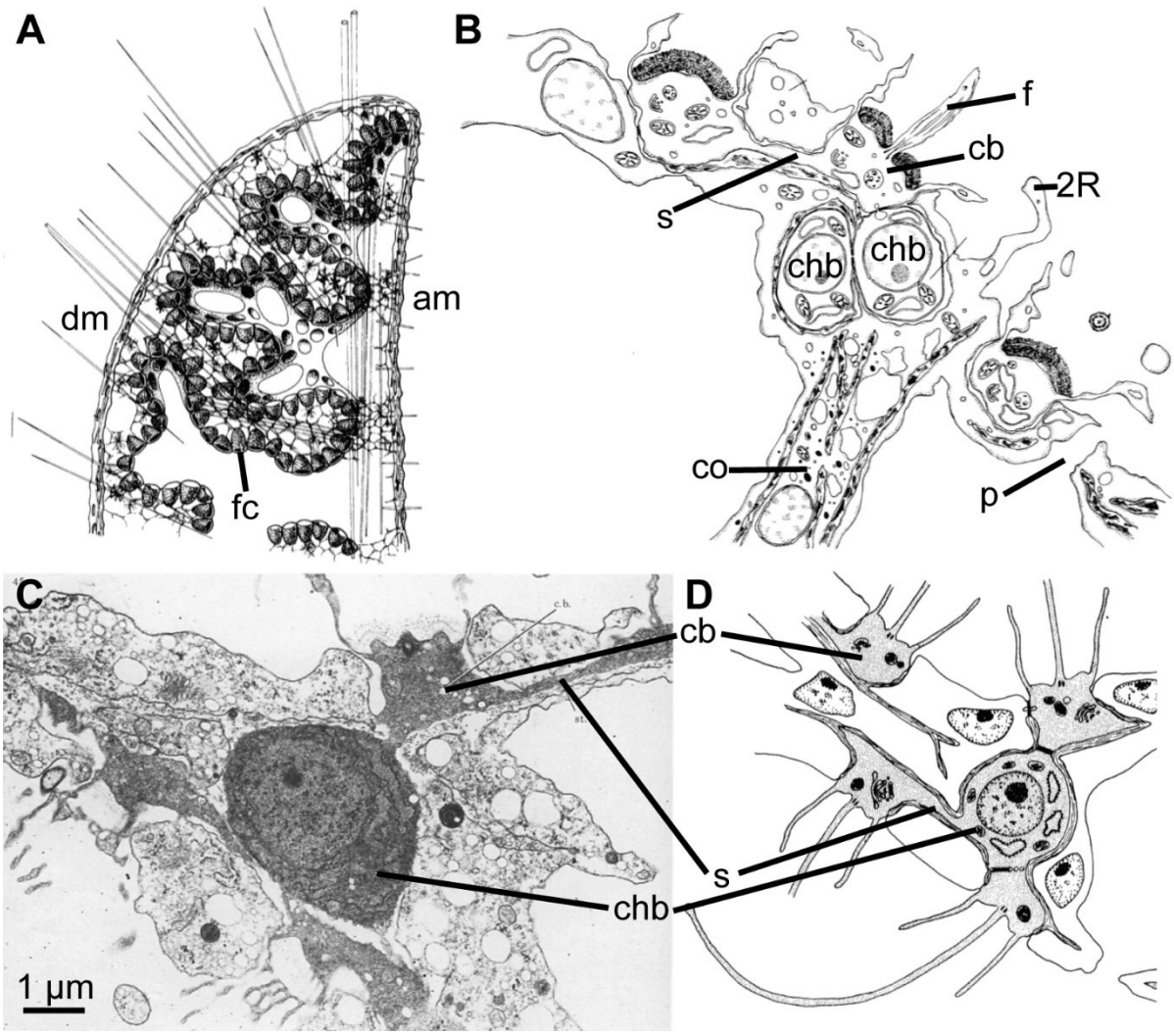

Figure 1.5. Tissue structure of a glass sponge from Mackie and Singla (1983) Water (red arrows) is drawn through the flagellated chamber by the action of enucleate collar bodies (cb) that are connected to each other and to nucleated choanoblasts (chb) by cytoplasmic bridges. Mononucleate choanoblasts (chb) produce branching enucleate collar bodies (cb) connected by stolons (s) to the parent choanoblast.

Also unknown is whether collar bodies and other tissues are shed from the glass sponge as part of tissue renewal. It was recently proposed that rapid rates of cell turnover and shedding by the demosponge *Halisarca caerulea* released enough carbon into the environment to affect other animals (De Goeij et al. 2013). It is impossible to determine from their data whether *Halisarca caerulea* truly sheds its own cells rather than recycles them, but this idea presents an untested additional hypothesis for how sponges can affect other communities: by fecal pellets as described above, or through release of shed tissue. For the glass sponge, Mackie and Singla (1983) suggested that collar bodies may detach and be shed from the body of *Rhabdocalyptus dawsoni* but also cautioned to the possibility that their observations came from an artefact of sample processing. Leys (1999) later found evidence of flagellated chambers being resorbed by tissue outgrowths of the trabecular reticulum. The degree to which cell shedding actually occurs in the glass sponge is not known, and is likely dependent on knowledge of the energy budget for a glass sponge. Several questions arise regarding how tissues are maintained: what tissue is produced and stays put, and what skeletal and organic matter is secreted? Only after these are known can one ask whether enough energy remains for the sponge to be able to discard its tissue, or whether its tissue is a precious, hard-won resource in the body.

1.5. Thesis objectives

Glass sponges are typically found in the deep sea below 1,000 meters, where food is the most common limiting resource (McClain et al. 2012). Food may not be expected to be a limitation for hexactinellids living on the continental shelf where productivity is high, but there and elsewhere the sponges may play a role in pelagic-benthic coupling for other animals. This thesis addresses five aspects of glass sponge ecophysiology that enhance our understanding of the energy balance of the glass sponge reef ecosystem by studying energy flow into, within, and out of the reefs at several scales.

In Chapter 2, I studied data collected by Sally Leys, Gitai Yahel, and Verena Tunnicliffe during a 2005 cruise to the Fraser Ridge reef in the Strait of Georgia near Vancouver, British Columbia. The goal was to estimate whole reef fluxes to understand the flow of carbon and nitrogen into and out of the glass sponge reefs, both what they take in from the pelagic picoplankton (From Equation 1, *Consumption*) and what they release to the water column and benthos (From Equation 1, *Urine*). Paired ambient and exhaled water collected *in situ* from individuals of *Aphrocallistes vastus* were analyzed, then scaled up to whole-reef fluxes of carbon, nitrogen, dissolved nutrients, and oxygen using measurements of average osculum size, live cover and density, and excurrent flow rates from the literature (Chu and Leys 2010b, Leys et al. 2011). Appendix 1 contains a supporting table for Chapter 2.

In Chapter 3, recruitment and resilience of reef sponges were studied using repeat visits to the same reef sites at Fraser Ridge and Galiano Reefs over three years using the remotely operated vehicle ROPOS for precise navigation and field manipulations. An understanding of reef dynamics is important because of the lasting effects that anthropogenic disturbances can have on ecosystem functions if the reefs are slow to recover from damage. Surveys of juvenile density and distribution and a search for reproductive adults were used to determine the frequency of new recruitment. Recovery was studied from two scales of disturbance in the field: small pieces broken off large sponges and complete removal of sponges in a large patch of reef to simulate larger scale disturbance. Appendix 2 contains supplemental figures that accompany Chapter 3.

In Chapter 4, the relative investment of energy into tissue maintenance (from Equation 1, part of *Respiration*) was compared between a primarily deep-sea hexactinellid species (*Aphrocallistes vastus*), other species of sponges, and with other animals by comparing cell or nucleus turnover rates. Chapter 4 also tested whether sponges in general, and reef sponges in particular, may be able to change their investment in 'tissue homeostasis' according to the food or conditions available to them. Appendix 3 contains supplemental tables and figures that accompany Chapter 4.

Chapter 5 investigated the flow of food, as bacteria, through an individual sponge and through the reefs. This chapter begins with a description of feeding by *Aphrocallistes vastus* from uptake through excretion, especially focusing on where uptake and digestion occurred in the syncytial organization of the sponge and the size and form of fecal pellets released by the sponge (from Equation 1, *Feces*). Natural $\delta^{13}C$ and $\delta^{15}N$ stable isotope signatures were used to trace the provenance of food to the reefs and a ^{13}C feeding experiment was used to measure the residence time of food in the tissue of reef sponges. Appendix 4 contains supplemental tables that accompany Chapter 5.

Chapter 6 focuses on growth of the sponges, both how glass sponges built new spicules and new tissue in flagellated chambers. Samples collected by SCUBA and remotely operated vehicle were studied using light, fluorescence, and electron microscopies to assess how, and how quickly, reef sponges grow. Appendix 5 contains a supplemental figure that accompanies Chapter 6.

Finally, Chapter 7 concludes by discussing the role of glass sponge reefs in pelagic-benthic coupling, and addresses the ecological and evolutionary contexts of these findings.

Chapter 2. Benthic grazing and carbon sequestration by deep-water glass sponge reefs[1]

2.1. Introduction

Feeding by large communities of benthic suspension feeders, known as benthic grazing, can greatly affect water column properties and forms an important component of benthic-pelagic coupling in lakes and oceans (Gili and Coma 1998). Benthic grazing rates quantify the mass transfer from the water column to the benthos (Genin et al. 2009) and are used to understand the effect of suspension feeders on the surrounding water. Grazing rates are well quantified for near-shore, shallow-water communities, however the effect of grazing by dense deep-water communities is less well studied. A better understanding of the energetics of suspension-feeding communities is especially needed in light of growing evidence of the removal of these communities by deep-water trawling in the ocean (Heifetz et al. 2009, Puig et al. 2012).

In many regions sponges (Phylum Porifera) dominate benthic communities and, because sponges are particularly effective at removing suspended particulates, they are often implicated in water quality control (Gili and Coma 1998). Where sponges dominate shallow benthic communities their grazing can affect overlying water (Pile et al. 1997). Glass sponges (Class Hexactinellida) are deep-sea animals that occur in large numbers on seamounts, in the Southern Ocean, and on continental slopes and fjords in several oceans (Hogg et al. 2010). They draw water through numerous small pores (ostia) on the dermal surface where it passes via canals to chambers of flagellated collar cells, choanocytes. Particles are sieved by a mesh on the collar and water and wastes are expelled via canals and out of an apical osculum. Glass sponges are abundant on hard substrata throughout the north Pacific (Leys et al. 2004) but also form unusual reefs that cover hectares of seafloor on the continental shelf of the northeast Pacific (Conway et al. 2005b). Over the last two decades, we have built a body of knowledge about glass sponge physiology (Leys and Mackie 1997, Tompkins-MacDonald and Leys 2008), feeding (Yahel et al. 2007), distribution and densities (Chu and Leys 2010b, Du Preez and Tunnicliffe 2011), and their requirements for silica and water flow (Chu et al. 2011, Leys et al. 2011). We now seek to calculate fluxes through entire reefs to understand the magnitude of their grazing from and excretion into overlying water. Glass sponge reefs are built by three species, *Aphrocallistes vastus*, *Heterochone calyx,* and *Farrea occa*, all of which form fused (dictyonine)

[1] A version of this work has been published in *Limnology and Oceanography*: Kahn, A. S., G. Yahel, J. W. F. Chu, V. Tunnicliffe, and S. P. Leys. 2015. Benthic grazing and carbon sequestration by deep-water glass sponge reefs. Limnology and Oceanography **60**:78-88.

skeletons of silica. Over the last 6,000-9,000 years they have constructed bioherms on glacially carved ridges where water flow is high and sediment accumulation low (Conway et al. 2005b) and, over time, the sponge skeletons become cemented together by sediment into a semi-solid substratum. Young sponges settle and grow upon the skeletons of older generations to form the reefs (Conway et al. 2005b).

Glass sponge reefs form one of the densest communities of deep-water suspension feeders known, with up to 40 large oscula (each representing a pumping unit) in a square meter and hundreds of thousands of oscula across a hectare of reef (Chu and Leys 2010b). Like coral reefs, sponge reefs form a habitat for many animals (Du Preez and Tunnicliffe 2011) and, because of their large filtration capacity, they may also have an important role in benthic-pelagic coupling. But the deep habitat of the sponge reefs, well below the photic zone, is typically poor in planktonic cells, and bacteria (10^5 mL^{-1}) are the primary food of the sponges (Yahel et al. 2007). Although bacteria could be enriched in bottom waters by sediment resuspension or internal waves (Clark et al. 2010), the source of sufficient bacteria to sustain the reported growth rates of 1 to 7 cm yr^{-1} (Chapter 3, Leys and Lauzon 1998, Austin et al. 2007) and of such large communities as the sponge reefs is not evident.

One glass sponge reef on Fraser Ridge near Vancouver, British Columbia is well-studied and serves as a model for understanding other dense glass sponge communities, including hundreds of km^2 of known sponge reefs (Conway et al. 2005b) and dense sponge populations in the Antarctic and on continental slopes and seamounts. Reefs in the Strait of Georgia, a marginal sea near Vancouver British Columbia, are nearly monospecific, with the species *Aphrocallistes vastus* (hereafter *Aphrocallistes*) forming approximately 86% of individuals in a reef. Here we combine measurements of individual grazing and excretion rates of *Aphrocallistes* with sponge size and density data to develop reef-wide estimates of the community metabolism and the effect of these animals on overlying waters. Our results indicate that glass sponge reefs have grazing rates and water processing capacity up to an order of magnitude higher than other suspension feeding communities. Our results also provide insight into the conditions needed to sustain such a dense community of suspension feeders. As conservation of cold-water sponge and reef habitats is a pressing issue, we examine the ecosystem functions of these large glass sponge communities in shelf ecosystems, both present and past.

2.2. Methods

2.2.1. Study site

Samples and measurements were collected from Fraser Ridge reef in July 2005. The Fraser Ridge is a relict glacial deposit of boulders and gravel with its base at about 200 m. The ridge lies roughly perpendicular to the prevailing northerly currents in the central Strait of Georgia (SoG), British Columbia (Conway et al. 2005b, Bedard 2011). The Fraser Ridge reef covers about 170,000 m^2 (Conway et al. 2005a) on the northern and western edges of the ridge (49° 9' 15.7" N, 123°23' 3.7" W; Figure 2.1A). The reef ranges from 150 to 180 m depth beneath the outflow of the Fraser River, which is the source of 73% of the freshwater and 64% of particles entering the Strait (Johannessen et al. 2003). Strong southward riverine outflow from the Fraser River creates stratification that limits downwelling of surface waters and induces a northerly flow of water at the bottom (Masson 2002). Current speeds through Fraser Ridge reef are very high, reaching up to 92 cm s^{-1} during flood tides (Leys et al. 2011), but follow a mixed semi-diurnal tide schedule so currents vary throughout each tide cycle. Mean northward currents amplify flood tides and dampen currents during ebb tides (Bedard 2011) so that the flow over the ridge is almost always to the north.

2.2.2. *Water sampling*

The remotely operated vehicle (ROV) *ROPOS* carried out nine dives to gather samples. A pumping conductivity, temperature, depth (CTD) instrument (SBE19plus, Seabird) mounted on the ROV continuously recorded water conditions to produce a vertical profile of salinity, density, temperature, transmissivity, and oxygen concentration (SBE43 dissolved oxygen sensor attached to the CTD). Water samples were collected with precision positioning beside the sponges (zero meters above bottom, mab) and from the sponge excurrent flow using custom designed paired samplers (SIPs) that can be manipulated by the ROV and draw water in at a rate lower than the excurrent velocity, as described by Yahel et al. (2007). Ambient water conditions at and above the reef were determined from samples collected with Niskin bottles. During four separate dives, one Niskin attached to the ROV was triggered at each of 5, 10, and 20 meters above the reef and water from each Niskin was divided for analysis of dissolved nutrients (ammonium, nitrate, dissolved silica, and phosphate), bacteria, and total organic carbon (TOC) concentrations.

Previous work has shown that *Aphrocallistes* pumps continuously unless disturbed by sediment (Tompkins-MacDonald and Leys 2008). To confirm that each sponge was actively filtering prior to sampling with SIPs, fluorescein dye was released beside each sponge to visualize filtration by dye uptake and release from the osculum (Figure 2.1D). We collected a

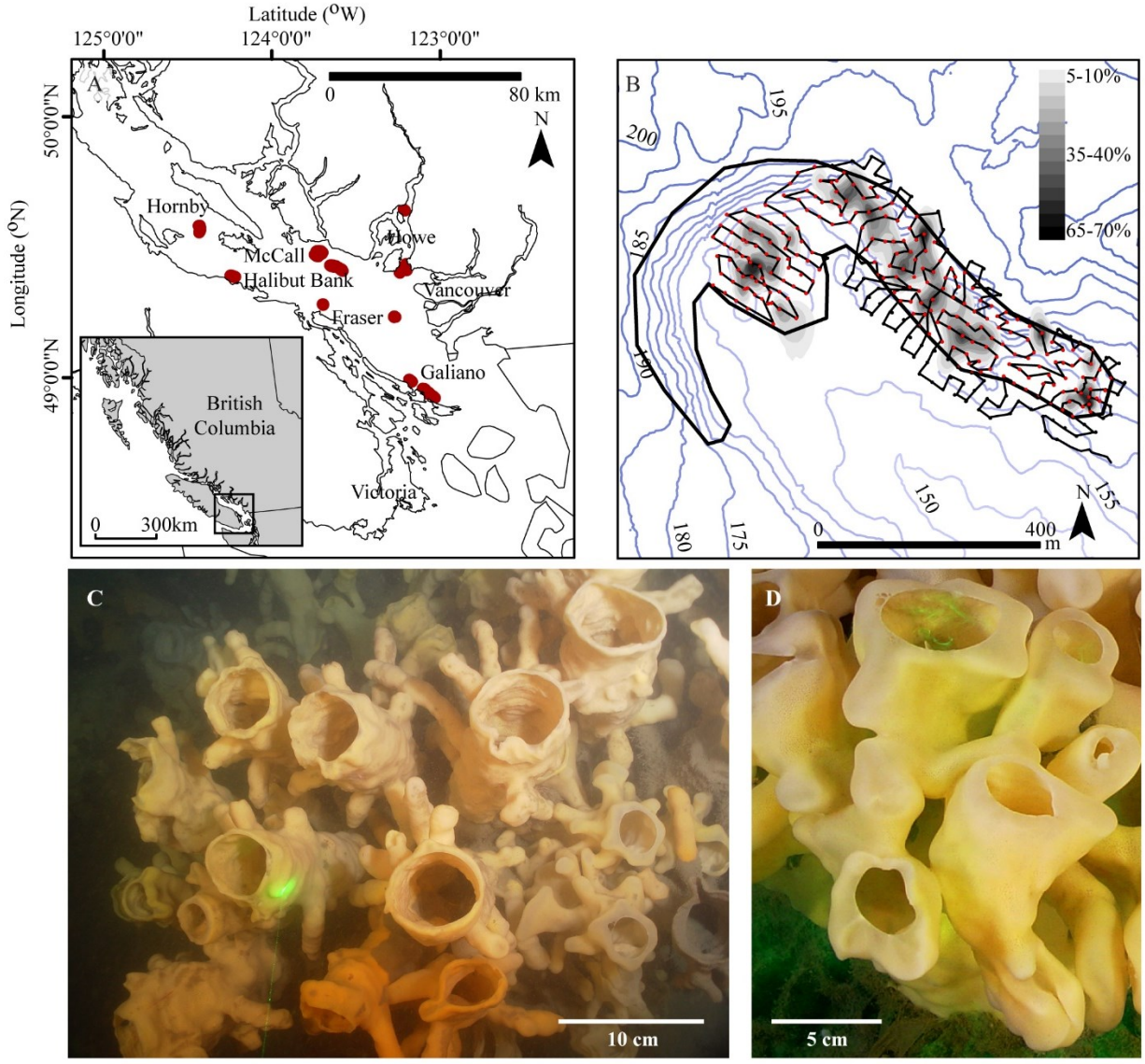

Figure 2.1. Glass sponges form dense reef habitats in the northeastern Pacific (A) Locations of sponge reefs in the Strait of Georgia. Sampling was conducted at Fraser Ridge reef and fluxes were compared with populations from Galiano Ridge and Howe Sound reefs. All three reefs lie on the continental shelf between Vancouver Island and mainland Canada. (B) A map showing the reef at Fraser Ridge and the extent of the surveys in 2005 and 2007 (gray gradients are interpolated reef live percent cover), with survey tracks set to overlap with the area predicted to be reef from multibeam mapping (black outline). Survey points outside (black dots) and inside (red dots) of the predicted reef area. (C, D) Sponge reefs are dense associations of live sponges growing on the skeletons of previous dead generations. The large openings are the excurrent oscula and are approximately 5 to 10 cm across in these photos. (D) Green fluorescein dye was used to verify the sponges were pumping water.

total of 22 paired samples (ambient water [in] and water filtered by the sponge [ex]). When sampling water from a sponge *in situ*, the sampling tube must be well inside the osculum and must not touch the sponge to prevent contamination by ambient water or sponge tissue; we checked video records of each sample collection to confirm SIP samplers did not touch the sponge and were inserted sufficiently into the osculum. Our previous data showed the sponges filter with up to 95% efficiency (Yahel et al. 2007), so samples with less than 25% filtration efficiency (5 of 22 samples collected) were considered contaminated with ambient seawater due to improper position of the sampler.

SIP water samples were processed for nutrient analysis (as above), TOC and total nitrogen (TN), as well as for flow cytometry for bacteria removal following Yahel et al. (2007). Pieces of each sponge from which water was sampled were collected to verify species identification by spicule composition. Of 243 samples collected to date, 86% have been *Aphrocallistes*; all sponges we sampled water from in this study were *Aphrocallistes*.

2.2.3. Sponge respiration

Dissolved oxygen in ambient and excurrent water from 24 individuals was measured using a long tube connected to a pumping CTD with an attached oxygen sensor (SBE 43 dissolved oxygen sensor, Seabird). For each sponge, the tube was positioned inside the osculum and, once the CTD record stabilized, a 2 min time series was recorded at 4 Hz. This procedure was repeated adjacent to the sponge to measure the ambient oxygen concentrations. We took the difference between the two measurements to be an estimate of sponge respiration in μmol O_2 per liter processed. Flow visualization with fluorescein dye was carried out before collecting respiration samples to ensure the sponge was pumping.

2.2.4. Calculating grazing and excretion rates

We estimated whole-reef fluxes using previously published data for the average excurrent velocity (2.8 ± 0.4 cm s^{-1}, mean ± standard error SE, range 0-5.2 cm s^{-1}; (Leys et al. 2011)) and sponge oscula diameter and density (Chu and Leys 2010b) for each of three reefs that occur in the SoG region: Fraser Ridge, Galiano Ridge, and Howe Sound (Figure 2.1A). We propagated error to calculate standard error and 95% confidence intervals using the exact method of Goodman (1962) for grazing rates and excretion rates per m^2. Relative error approximations were used for scaling up calculations beyond 1-m^2 by adding each SE—normalized by its respective mean—for each variable in a product (Taylor 1997). Sponge reefs in British Columbia have been identified by multibeam mapping, providing an outline of past (buried) and present (living and dead) glass sponges (Conway et al. 2005a). In order to calculate fluxes for only live (actively filtering) reef, we first calculated the fluxes through an average one

m^2 of reef using published estimates of osculum density (Chu and Leys 2010a), and then applied that number to the percent cover of live sponges in reefs. Previous work by Chu and Leys (2010b) estimated live sponges to occupy 14% of the area surveyed at Fraser Ridge, but the survey areas in that study extended beyond the boundaries of area predicted to be reef by multibeam mapping (Conway et al. 2005a), therefore some areas surveyed had no reef, and in two instances the reef extended beyond the predicted area. To account for patchiness of the reefs and estimate the live sponge cover more accurately at the Fraser Ridge reef and other reefs in the SoG, we used two data sets: the area predicted to be reef based on multibeam sonar (177,486 m^2, courtesy of K. Conway), and images by ROV that surveyed a grid of 593 1 m^2-plots, of which 98 m^2 or 16.5% showed live reef structure (Figure 2.1B). We extended this approach to determine the area predicted to be reefs by multibeam mapping in the SoG (1.8×10^6 m^2 live cover from 1.1×10^7 m^2 predicted) and from Alaska to southern British Columbia (6.6×10^7 m^2 live cover from 4.0×10^8 m^2 predicted).

2.3. Results

2.3.1. Ambient conditions at Fraser Ridge

Full water column profiles reflected the strong influence of the Fraser River outflow on temperature, salinity, density, and transmissivity at the surface (Figure 2.2A). Closer to the seafloor, measurements made from water collected at 0, 5, 10, and 20 meters above the sponges were variable because replicates were collected across different days, times, and stages of the tide (Table 2.1). On average however, nutrient concentrations (nitrate, phosphate, and dissolved silica) showed a peak 5 meters above the reef rather than among the sponges (Figure 2.2B). Average ammonium concentration was lower among the sponges than at all depths above (Figure 2.2B), and while bacteria concentrations were also reduced among the sponges compared to all other depths, total organic carbon was greater among the sponges than above the reef (Figure 2.2C); there was low variability in bacterial concentration across all Niskin samples.

Ambient water among the sponges (zero mab) was moderately low in oxygen and highly enriched with dissolved silica, nitrate, and phosphate (Table 2.1). Dissolved inorganic nitrogen (particulate nitrogen, NH_4^+, NO_3^-, and NO_2^-) formed nearly 89% (26.1 ± 0.74 μmol L^{-1}, mean ± SE) of the total nitrogen (TN). Dissolved organic carbon (DOC) made up 85% of total organic carbon in the water. The concentration of particulate organic matter (POM) at the sponge reef habitat was low (10.5 ± 4.2 μmol C L^{-1}) and the POM was nitrogen-poor (C:N ratio 8.5 ± 1.9). The POM component consisted partly of picoplankton of which bacteria dominated whereas

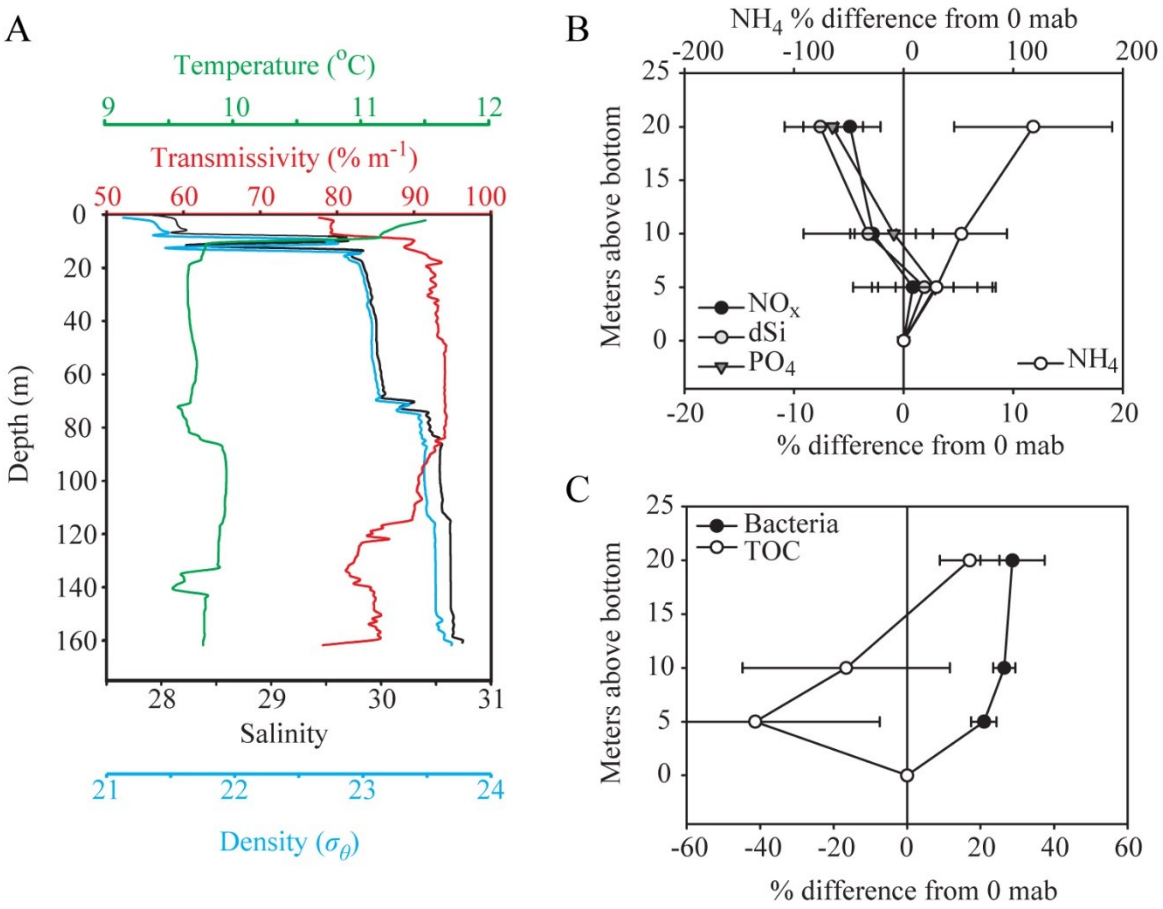

Figure 2.2. Water characteristics above Fraser Ridge reef. (A) Depth profiles of temperature, salinity, density, and transmissivity from July 2005 during one ROV ascent from the reef. Bottom depth was 160 m. (B) Nutrient profiles above the seafloor. Data are presented as the difference from the average value measured among the sponges (n=17). Three Niskin bottles were triggered during each ROV dive, at 5, 10, and 20 meters above the sponges. Niskin samples and SIP samples from water surrounding the sponges were analyzed to compare bottom water conditions. Error bars: SE. (C) Vertical profiles of bacteria and TOC concentration above the reef presented as the average percent difference from their concentration among the sponges. Error bars: SE.

Table 2.1. Concentrations of nutrients in the water column above Fraser Ridge Reef. Ammonium, nitrate+nitrite (NO_x), dissolved silica (dSi), phosphate (PO_4), total organic carbon (TOC), and bacterial concentrations were measured 0, 5, 10, and 20 meters above Fraser Ridge Reef. Mean ± SE.

meters above bottom	Ammonium (nmol L^{-1})	NO_x (μmol L^{-1})	dSi (μmol L^{-1})	PO_4 (μmol L^{-1})	TOC (μmol L^{-1})	Bacteria (cells mL^{-1})
0	571 ± 110	25.8 ± 0.4	49 ± 1	2.24 ± 0.07	65 ± 3.3	$6.7 \times 10^5 \pm 3.5 \times 10^4$
5	625 ± 184	26.0 ± 0.6	49 ± 2	2.31 ± 0.09	69 ± 1.1	$8.3 \times 10^5 \pm 5.0 \times 10^4$
10	708 ± 170	25.1 ± 0.2	47 ± 2	2.22 ± 0.05	73 ± 8.2	$8.7 \times 10^5 \pm 4.5 \times 10^4$
20	993 ± 271	24.6 ± 0.3	45 ± 1	2.10 ± 0.12	75 ± 4.2	$8.8 \times 10^5 \pm 3.9 \times 10^4$

picoeukaryotes were much less abundant than other picoplankton constituents (714 ± 213 cells mL^{-1}, n=17).

2.3.2. Feeding and excretion

Comparisons of water samples before and after filtration by *Aphrocallistes* show removal of bacteria with up to 90% efficiency (78.6 ± 3.20% mean ± SE, paired t-test, t = 14.063, degrees of freedom (df) =14, $p < 0.001$; Figure 2.3A). Sponges also removed small populations of non-photosynthetic larger cells that we isolated through flow cytometry but could not identify unambiguously. Numbers of bacteria removed by individual sponges increased linearly with ambient bacteria concentrations (a Type I functional response) (r^2=0.69, p<0.0005; Figure 2.3B). Ammonia was the major nitrogenous waste product with 0.17 ± 0.02 μmol ammonium per liter of water filtered (paired t-test, t =-7.37, df = 14, $p < 0.001$; max 70% difference from ambient). Oxygen removal was slight but significant, 0.56 ± 0.37 μmol L^{-1} (0.45%; ambient [O_2] 126.39 ± 3.57 μmol L^{-1}, n=24).

Aphrocallistes did not take up or excrete notable amounts of dissolved nutrients (phosphate, silica), or total carbon (Figure 2.3A). It should be noted that the small sample size (n=15 pairs), dictated by the logistics of deep-sea work, resulted in low statistical power (<0.5) of the paired t-tests we used.

2.3.3. Flux through the reef

We calculated the volume processed by each osculum each day for one m^2 of seafloor using flow rate, osculum area, and density on Fraser reef reported in Table 2.2. Each osculum processes 9,140 ± 1750 liters $osculum^{-1}$ d^{-1}, and one square meter processes 210 ± 35 m^3 d^{-1} (95% confidence interval: 132-288 m^3 m^{-2} d^{-1}). Although Howe Sound reef covers a larger area, the sponge density is lower and therefore this reef filters the least water per unit area, 108 ± 19 m^3 m^{-2} d^{-1} (95% CI: 65-150 m^3 m^{-2} d^{-1}). Galiano reef sponges, in contrast, are the most dense and therefore process the most water at 252 ± 42 m^3 m^{-2} d^{-1} (95% CI: 158-345 m^3 m^{-2} d^{-1}) (Table 2.2).

2.3.4. Reef metabolism

Grazing rates (α) reflect the efficiency of removal of particles or nutrients from a volume of water, and can be compared across suspension feeding communities to determine effectiveness of extraction. For Fraser Ridge reef, we calculated that the benthic grazing rate on bacteria, the primary food item extracted by sponges, was 165 ± 29 m^3 m^{-2} d^{-1} (95% CI: 102-228 m^3 m^{-2} d^{-1}) – that is, the volume from which 100% of bacteria are cleared. Since bacteria concentrations in water near the sponges were 6.9 x 10^6 ± 3.0 x 10^4 cells mL^{-1}, this equates to 1.2 x 10^{14} ± 2.36 x 10^{13} bacterial cells m^{-2} d^{-1} removed by 1 m^2 reef sponges. We calculated the total

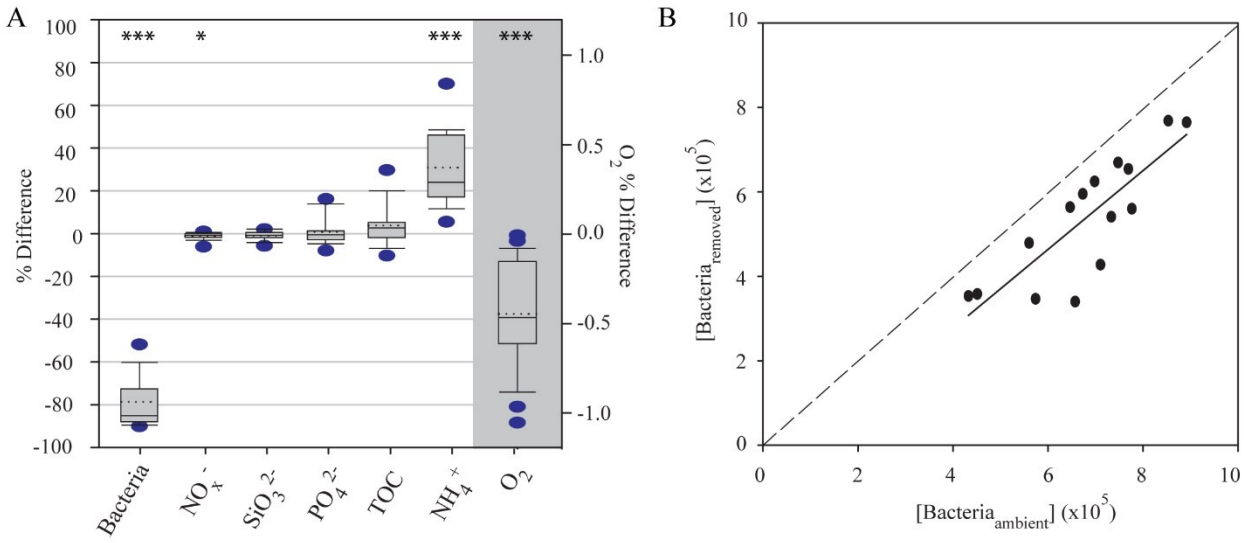

Figure 2.3. Sponge pumping efficiencies and behavior. (A) Percent differences of bacteria, nutrients, total organic carbon, ammonia, and oxygen between ambient water (IN) and the water emerging from the exhalent osculum (EX), calculated as (EX-IN)/IN; samples collected by SIPs as described by Yahel et al. (2007). Boxes encompass 25th and 75th percentiles and contain medians (solid lines) and means (dotted lines); whiskers encompass 10th and 90th percentiles. Outliers are shown as dots. Asterisks indicate statistically significant p-values from paired t-tests of water sampled from ambient and excurrent water (* for $p<0.05$, *** for $p<0.0005$). (B) Removal of bacteria by a sponge is plotted as a function of bacteria availability in ambient water. 'Ambient' refers to water immediately adjacent to the sponge.

Table 2.2. Component fluxes mediated by the sponges on Fraser Ridge, Galiano Ridge, and Howe Sound reefs based on *in situ* measurements of osculum size and density from Chu and Leys (2010*a*, *b*) and excurrent flow speeds from Leys et al. (2011). Mean ± SE. Dashes indicate values that were not measured.

	Reef		
Component	Fraser	Galiano	Howe
[1]Osculum size (cm^2)	38.2 ± 1.9	22.7 ± 0.8	14.6 ± 0.5
[1]Osculum density (m^{-2})	23.0 ± 1.7	46.3 ± 3.7	30.9 ± 3.0
[2]Excurrent flow speed ($cm\ s^{-1}$)	2.8 ± 0.40	-	-
Volumetric pumping rate ($m^3\ m^{-2}\ d^{-1}$)	210 ± 35	252 ± 42	108 ± 19
Benthic grazing rate ($m^3\ m^{-2}\ d^{-1}$)	165 ± 29	198 ± 34	85 ± 15
Bacteria (cells $m^{-2}\ d^{-1}$)	$1.1x10^{14}$ ± $0.25x10^{14}$	$1.4x10^{14}$ ± $0.29x0^{14}$	$5.8x10^{13}$ ± $1.3x10^{13}$
[3]Bacterial carbon (g C $m^{-2}\ d^{-1}$)	3.4 ± 1.4	4.1 ± 1.6	1.8 ± 0.7
[3]Bacterial nitrogen (g N $m^{-2}\ d^{-1}$)	0.66 ± 0.31	0.79 ± 0.37	0.34 ± 0.16
Oxygen (μmol $m^{-2}\ d^{-1}$)	32.8 ± 13.3	39.3 ± 15.6	16.8 ± 6.99
Ammonia (mmol $m^{-2}\ d^{-1}$)	39 ± 17	47 ± 20	20 ± 9

[1] Data from Chu and Leys (2010b)
[2] Data from Leys et al. (2011). n = 9 sponges, 13 measurements.
[3] Using bacterial carbon and nitrogen values of 30.2 and 5.8 $fg\ cell^{-1}$, respectively. From Fukuda et al. (1998).

bacterial carbon and nitrogen removed by reef sponges based on the amount of bacteria consumed. Assuming 30.2 ± 12.3 fg C $cell^{-1}$ and 5.8 ± 1.5 fg N $cell^{-1}$ for coastal bacteria (Fukuda et al. 1998), each square meter of reef at Fraser Ridge consumes 3.4 ± 1.4 g C m^{-2} d^{-1} and 0.7 ± 0.3 g bacterial N m^{-2} d^{-1} (Table 2.2). The rate of excretion of nitrogenous waste was comparable to the rate of uptake of bacterial nitrogen (0.04 ± 0.02 mol ammonium m^{-2} d^{-1}, or 0.55 ± 0.23 g N m^{-2} d^{-1}).

Not all of the area mapped by remote sensing (multibeam mapping) has exposed reef structure today – some is buried under mud – however, we were able to use the area surveyed by ROV (Chu and Leys 2010a) to estimate how much of the area mapped by multibeam consists of sponge cover today at Fraser Ridge reef. Of the area identified by multibeam mapping at Fraser Ridge (177,486 m^2, data courtesy of K. Conway), 16.5% has live sponge cover today, giving a total filtering reef size of 29,242 m^2. This area alone consumes 1.00 x 10^5 ± 3.99 x 10^4 g C d^{-1} (Table 2.3). Parts of Galiano and Howe reefs have been mapped in detail as well, allowing us to also calculate their grazing rate. The area of reef mapped by multibeam in Howe Sound was larger than Fraser (898,541 m^2, data courtesy of K. Conway) so, although ROV surveys showed it had fewer, larger sponges (11.6% live reef cover, Chu and Leys 2010b), because of its size it is calculated to consume more carbon, 1.83 x 10^5 ± 7.46 x 10^4 g C d^{-1} from 104,231 m^2 of live reef. Galiano reef extends north and south along a long ridge, and multibeam mapping estimates suggest it is as large as Howe (862,799 m^2). It also has greater reef cover (26% live reef cover, Chu and Leys 2010b) and more dense oscula, so live cover is estimated to be greater than Howe (224,328 m^2) and to consume more carbon, 9.20 x 10^5 ± 3.67 x 10^4 kg C d^{-1}.

Multibeam mapping has identified 10 additional reefs in the SoG (Conway et al. 2005a). Although we have not yet surveyed these using ROV and video, by using the estimate of average sponge cover calculated for Fraser Ridge reef (16.5%), we estimate that all live sponges at SoG reefs (including Fraser, Galiano, and Howe) cover 1.84 x 10^6 m^2 and remove 6.3 x 10^6 ± 2.5 x 10^6 g C d^{-1} (Table 2.3).

2.4. Discussion

Glass sponges are effective ecosystem engineers by constructing a three-dimensional habitat for other animals (Beaulieu 2001b, Du Preez and Tunnicliffe 2011). Our work now highlights the important role glass sponges have in filtering enormous volumes of water each day, removing bacteria and oxygen, and releasing ammonium. Our calculations show that the sponge reefs have an unusually high grazing rate in comparison to all known benthic suspension feeding communities due to their density, efficient capture of bacteria, and high water processing rates making them a potential carbon sink in Canadian shelf waters.

Table 2.3. Estimates of bacterial carbon consumption by glass sponge reefs.

Mean ± SE.

Reef	Live cover (m^2)	Bacterial C consumed (g C d^{-1})
Fraser Ridge Reef	2.9×10^4	$1.0 \times 10^5 \pm 0.40 \times 10^5$
All SoG reefs	1.8×10^6	$6.3 \times 10^6 \pm 2.5 \times 10^6$
Reefs known from Alaska to southern British Columbia	6.6×10^7	$2.3 \times 10^8 \pm 0.91 \times 10^8$

2.4.1. Grazing rates and bacterial supply

Within the glass sponge reefs in British Columbia, sponge densities and size are much higher than those seen in other glass sponge communities (Table S1.7.1). At Galiano Ridge reef for example, oscula reach densities up to 46 m^{-2} in dense patches of reef (Chu and Leys 2010b), over 18 times greater than those seen in other glass sponge communities. Because each sponge removes on average >75% of the bacteria from the water as it passed through the aquiferous system, the reef functions as a highly efficient filtration system, and have the highest grazing rates of any other suspension feeding animal or community measured to date (Table 2.4). But perhaps more important is what such a high grazing rate translates to in carbon consumption.

Filtration requires active beating of flagella to pull water through the fine passages in the sponge. The oxygen consumed by the sponge mainly reflects this energy expenditure (Leys et al. 2011), so knowing the oxygen removal per liter of water filtered (0.013 ± 0.0017 mL L^{-1}) and assuming 0.46 mg C = 1 mL O_2 (Hadas et al. 2009) we calculated that each sponge osculum requires 0.0058 ± 0.0008 mg carbon per liter of water filtered. Considering the average bacteria removal of 5.36 x 10^5 cells mL^{-1} (0.016 ± 0.004 mg carbon L^{-1}), there remains about 0.010 ± 0.005 mg carbon L^{-1} available for growth. This suggests that 36 ± 14% of food energy consumed by actively pumping sponges is used toward metabolism, as estimated previously (Leys et al. 2011), and most of this must be used for pumping water through the body for feeding and respiration. The remaining food energy is available for growth (and skeleton production), reproduction, maintenance, or is excreted as fecal pellets and waste (Reiswig 1981).
Each day, a square meter of reef clears bacteria from the equivalent volume of 165 meters of water above it. This high grazing rate implies that large sponge reefs need particular hydrographic conditions to provide enough food for growth and to prevent the formation of a depleted boundary layer above the reef (Genin et al. 2009). Just north of Fraser Ridge, carbon delivery to the seafloor is 0.46 g C m^{-2} d^{-1} (Johannessen et al. 2003) whereas reef sponges extract 3.4 g C m^{-2} d^{-1}, seven times more than total POC delivered by vertical flux. Therefore there is a deficit which must be mitigated by lateral transport.

Organic matter comes from several sources in the highly productive SoG. Similar amounts of organic input arrive from terrigenous and phytoplankton sources, with about 80% of the carbon in dissolved form and so available to microbial production (Johannessen et al. 2008). The Fraser Ridge reef grows on the lee of a ridge. A topographically controlled bottom 'jet' forms over the ridge with velocities up to 70 cm s^{-1} during flood tides, thereby resuspending sediments (Bedard 2011) and associated organic matter. The Fraser sponge reef is located where a strong lee wave advances down-slope as the northward jet forms over the tidal cycle. As most

Table 2.4. Comparison of benthic grazing rate, water processing rate, and carbon consumed by suspension feeding communities (numbers come directly from the references unless indicated otherwise by footnotes).

Habitat	Benthic grazing rate α ($m^3\ m^{-2}\ d^{-1}$)	Water processing rate ($m^3\ m^{-2}\ d^{-1}$)	Carbon consumed (g C $m^{-2}\ d^{-1}$)	Material consumed	Reference
Glass sponge reef	85-198	108-252	1.8-4.1	bacteria	this study
Soft-bottom community, San Francisco Bay	40-60	10.3[†]	0.14-0.16	phytoplankton	Jones et al. 2009
Tropical sponge community	16.5-40	-	-	bacteria	Reiswig 1973; Reiswig 1974
Coral reef in Eilat, Red Sea	10-20	2.6[‡]	0.22	phytoplankton	Genin et al. 2009
Bivalve: Corbicula in tidal lakes, Sacramento-San Joaquin river delta	1-11	-[*]	0.04-0.32	phytoplankton	Lucas et al. 2002
Tropical demosponge: *Aplysina* (*Verongia*) *fistularis*[‡‡]	1.7	0.06	0.0070	POC	Reiswig 1981
Lake Baikal sponges	1.4	1.98	1.12[**]	prokaryotes, eukaryotes	Pile et al. 1997; Savarese et al. 1997

[†]Pumping rates presented in Table 4 (Jones et al. 2009).
[‡]Calculated using the same scale-up approach as used here, but only for conspicuous pumping suspension feeders.
[*]Water processing rates were not measured because grazing and carbon consumption were estimated using plankton biomass.
[‡‡]All values were presented for an individual sponge (Reiswig 1981). Densities were assumed as an average of density of *Mycale lingua* and *Verongia gigantea* (Reiswig 1973). Retention efficiency was only for bacteria (97%). POC consumption (16.7 μg POC L^{-1}) accounted for 14% of metabolic needs while DOC made up 86%.
[**]Includes carbon consumed or removed from the water and carbon released. Chloroplast-containing picoeukaryotes were found expelled from one species (*Baikalospongia intermedia*).

POC in the Strait settles in the southern SoG and is redistributed northward by currents (Johannessen et al. 2005), the reef placement is optimal to receive this material or more likely, the bacteria that thrive on it. This lateral delivery of bacteria to the reef seems to be the principal reason for the location of reefs on major topographic features that accelerate water flow such as canyons, shelf walls, and fjord sills (Conway et al. 2005b).

2.4.2. A carbon sink

The amount of carbon consumed by reefs in the SoG (Table 2.3) is only 0.8 ± 0.3% of the daily vertical flux of primary production to the seafloor in the SoG (7.80×10^8 g C d^{-1}; Johannessen et al. (2003)), so there is not a great effect on the mass balance of carbon in the SoG, which is a highly productive basin. Our calculations however, stem from just a fraction of the area covered by known reefs and other dense stands of glass sponges in the northeast Pacific. If all other reefs mapped by remote sensing – from Hecate Strait, Queen Charlotte Strait, the Strait of Georgia, and south-eastern Alaska (Conway et al. 2005a, Stone et al. 2014) – are included (Table 2.3), the amount of carbon consumed by sponge reefs may be considerably larger. Assuming the same relationship between reef cover and area mapped by multibeam sonar found at Fraser Ridge (16.5%) for all reefs identified by remote sensing (403 km^2), glass sponges cover a total of 66 km^2, process $1.4 \times 10^{10} \pm 2.3 \times 10^9$ m^3 water d^{-1} and consume $2.27 \times 10^8 \pm 9.1 \times 10^7$ g C d^{-1} (although it is not known how much carbon is released by reefs each day). Manned submersible dives since the 1980s have documented that fjord walls and sills within straits also have dense communities of glass sponges (up to 24 m^{-2}; Leys et al. (2004)) and since we do not include those sponges, our calculation of carbon consumption by glass sponges is an underestimate.

Sponges in other classes, especially Demospongiae which can be conspicuous inhabitants in tide pools to freshwater lakes, abyssal seafloors and coral reefs, also consume large amounts of carbon, but still substantially less than glass sponge reefs (0.029 – 1.970 g C m^{-2} d^{-1}) (Maldonado et al. 2012). While we are confident in our estimates of overall fluxes for glass sponges, generalizations beyond the Hexactinellida are difficult for two reasons. First, the Demospongiae may contain symbionts that cause them to feed on and excrete different carbon and nitrogen species (reviewed by Maldonado et al. 2012). Second, it is rare to get the full suite of measurements needed to scale up estimates, although there have been some attempts (reviewed by Maldonado et al. 2012).

2.4.3. Ammonia excretion and ammonium

The sponges not only efficiently remove bacteria from the water, but they excrete wastes as ammonia. Water sampled above the reef had five times more ammonium than water

collected around solitary glass sponges growing in fjords in Barkley Sound (Yahel et al. 2007). The difference could be regional, but the voluminous water processing by the reef likely has a substantial effect. Vertical Niskin profiles show higher ammonium concentrations in water 5, 10, and 20 meters above the reef sponges compared to water among the sponges, however we do not imagine excretion by glass sponge reefs has a large effect on total primary production in this nutrient-rich basin. Reefs cover a small fraction of the total size of the SoG and their contribution to the SoG nitrogen budget is small relative to the dominant source: oceanic water brought in via the bounding Haro Strait (3.0×10^4 Mmol $year^{-1}$; Sutton et al. (2013)). This would not be the case however, for more enclosed or less productive waters. Unlike the nitrate-rich waters in the SoG, ammonium is the favored and dominant nitrogen source for phytoplankton in other regions such as the Weddell Sea, where excretion by krill makes up nearly 80% of the ammonium taken up by phytoplankton for primary production (Whitehouse et al. 2011). Likewise, glass sponges perched on seamounts may contribute to primary production in the photic zone through turbulent mixing and internal waves (Clark et al. 2010), transporting ammonia up to where it can fuel further productivity.

2.4.4. Conservation issues: Implications of water processing by glass sponges

We measured nutrient and particle fluxes from sponges in a reef whose flow dynamics, density, and other features are well studied, resulting in first-order estimates of reef-wide fluxes. Multibeam mapping reveals reef structure, both past and present; therefore, if all areas identified by multibeam mapping to have been reef at some time in the past are considered, the potential total reef cover for all past reefs is 403 km^2, which could have resulted in consumption of $1.38 \times 10^9 \pm 5.5 \times 10^8$ g C d^{-1}. These figures indicate that sponge reefs have been a carbon sink, at least locally, for as long as they have been present.

Modern-day reefs occur from southern Alaska to British Columbia (Conway et al. 2005b, Stone et al. 2014) but we do not know their extent before trawling. Bottom trawling has damaged 21% of sponges in the Aleutian archipelago (Heifetz et al. 2009), with potential for greater damage where densities are high. Bottom trawling is common in Canadian waters, with glass sponges recorded in bycatch records since 1996. Trawl records from 1996 to 2001 find a catch per unit effort for sponges between 0.086 and 6.041 kg min^{-1} during trawls through reefs (Jamieson and Chew 2002). Each sponge damaged is one that is no longer sequestering bacterial carbon into its skeleton or recycling nitrogen. Prior to anthropogenic effects in the Northeast Pacific, dense assemblages of glass sponges may have had a substantial effect on nutrient dynamics in bottom waters.

Glass sponges may have served a similar role in prehistoric oceans and the ammonium they excreted may have been more important to the surrounding community; unlike the highly productive waters that reefs are found in today, ancient reefs in the Tethys Sea lived in oligotrophic waters (Leinfelder et al. 1996). Like modern-day oligotrophic habitats, recycled nutrients were very important in driving productivity in Jurassic oceans. Ancient reefs in the Jurassic were not monocultures (Leinfelder et al. 1996) and did not have fused skeletons, which enhance reef structure, as modern reefs do, but assuming that their filtration was similar to present-day reefs, prehistoric reefs were likely very important cyclers of carbon and nitrogen in ancient waters.

Sponges were predicted to be on the 'winners' side of climate change' after dramatic, episodic growth was observed following breakage of ice shelves in the Southern Ocean (Dayton et al. 2013, Fillinger et al. 2013). If this is true, then sponges may be one of many buffers against climate change as carbon concentrations in the ocean rise and subsequently become sequestered into sponge tissue.

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Chapter 3. Dynamic change, recruitment, and resilience in reef-forming glass sponges[1]

3.1. Introduction

Glass sponges (Class Hexactinellida) are conspicuous members of the deep-sea fauna and are thought to be adapted to the long-term constancy of deep water. Glass sponges are estimated to live up to 400 years (Leys and Lauzon 1998, Fallon et al. 2010), yet yearly monitoring has also revealed rapid responses to changing conditions and seasonal or annual spurts of growth in both shallow and deep-water populations (Leys and Lauzon 1998, Kahn et al. 2012, Dayton et al. 2013, Fillinger et al. 2013).

Since their first discovery during seafloor mapping of the shelf waters in the late 1980s, it is known that 4 large glass sponge reefs exist in Hecate Strait and Queen Charlotte Sound, British Columbia and over 12 more are known in southern waters of the Strait of Georgia. There, larvae settle and grow up to form the next generation (Krautter et al. 2006). In the Strait of Georgia *A. vastus* and *H. calyx* settle and grow upon previous generations forming mounds up to 21m high cemented together by sediment (Conway et al. 2005a).

Glass sponge reefs and sponge gardens are important nursery habitats for commercially important species (Cook 2005, Marliave et al. 2009, Chu and Leys 2010b, Miller et al. 2012), contribute to local silica cycling (Chu et al. 2011), and they are major grazers of plankton in deep water (Chapter 2, Kahn et al. 2015). Glass sponges throughout the northeast Pacific are easily damaged by trawl and other fishing activity (Freese et al. 1999, Heifetz et al. 2009). In western Canadian waters glass sponges have been recorded as bycatch with between 0.086 kg^{-1} and 6.041 kg min^{-1} catch per unit effort in trawls through reefs (Jamieson and Chew 2002); there are also many anecdotal records of damage to sponges by recreational prawn fisheries. What is not known is how resilient glass sponges are to damage, nor generally how dynamic growth and regeneration of individuals and populations are.

We used a remotely operated vehicle with highly accurate GPS navigation underwater to monitor change over time at two sponge reefs in the Strait of Georgia. Our aim was to observe and document reproduction and recruitment events, measure normal growth rates, and to determine whether dense glass sponge communities are resilient to disturbance.

[1] A version of this work has been published in the *Journal of the Marine Biological Association of the United Kingdom*: Kahn, A. S., L. J. Vehring, R. R. Brown, and S. P. Leys. 2015. Dynamic change, recruitment, and resilience in reef-forming glass sponges. Journal of the Marine Biological Association of the United Kingdom. **96**(2):429-436.

3.2. Materials and Methods

Fraser and Galiano sponge Reefs were visited in 2005, 2007, 2009, 2011, 2013, and 2014 during cruises on the *CCGS Vector* and *CCGS Tully* (Fraser Reef: 49° 9' 15.7" N, 123°23' 3.7" W; Galiano Reef: 48° 54' 51.5" N, 123°19'27.7" W). Work was carried out using the remotely operated vehicle (ROV) ROPOS (http://ropos.com) which uses an ultra-short baseline navigation (USBL) Global Acoustic Positioning System (GAPS) with a LOKI Kalman filter that allows positioning within 1 m.

3.2.1. Recruitment and growth

Both Fraser and Galiano sponge reefs were mapped extensively using grids of non-overlapping photos taken 1 m above the seafloor in 2005 and 2007 (Galiano Reef: 214 photos covering 594 m^2; Fraser Reef: 109 photos covering 69 m^2) (Chu and Leys 2010b). From the same set of ROV photos juvenile sponges – those with maximum osculum width of <10 cm and minimal branching – were counted and osculum diameters measured using ImageJ software. No attempt was made to differentiate between the two species because *Heterochone calyx* and *Aphrocallistes vastus* cannot be distinguished from photos alone. The density of juvenile sponges was calculated from the total photo area for each survey grid point, plotted on maps of the reefs using ArcGIS (ArcInfo version 10.2, ESRI), and compared to the distribution of adult sponges documented by Chu and Leys (2010b) using Spearman rank correlation (SYSTAT 12).

To look for evidence of reproduction (whether as spermatocysts, eggs, or embryos in tissue), pieces of *Aphrocallistes vastus* collected by SCUBA and by ROV were preserved in a cocktail fixative of 1% OsO_4, 2% glutaraldehyde, and 0.45 M sodium acetate buffer with 10% sucrose (Harris and Shaw 1984). The fixative was replaced after 30 min and specimens were left at 4°C overnight. Specimens were dehydrated through a graded ethanol series to 70% ethanol, and then desilicified in 4% HF in 70% ethanol. Following desilicification, samples were rinsed twice in 70% ethanol, dehydrated to 100% ethanol and fractured in liquid nitrogen. Fractured pieces were critical point dried (Bal-Tec CPD 030), mounted on aluminum stubs, sputter-coated with gold (Xenosput XE200), and viewed in either a Hitachi S-3500N or a field emission scanning electron microscope at 5 keV (JEOL 6301F).

To monitor growth (changes in size of branches) or death (loss of live tissue) over time, the ROV was positioned at the exact same heading and same GPS position to match the camera's view to photos from previous years. Lasers 10 cm apart fixed to the camera provided a scale.

3.2.2. Sediment accumulation

To determine how much sediment accumulates at the reefs, sediment traps and erosion markers were placed at Fraser and Galiano Reefs between 2007 and 2009. Sediment traps were

PVC tubes 0.5 m long with 45-cm^2 opening attached to stakes that were pushed into the sediment until the bottom of the tube was at the sediment surface. Each tube trap was pre-filled with hypersaline water and capped with a lacrosse ball that was removed by an elastic cord after the trap was positioned. Traps and makers were placed adjacent to and about 2-3 m away from clumps of sponges in 2007. In 2009 sediment traps were re-sealed with lacrosse balls to prevent loss of contents, recovered using the ROV, and frozen at -20°C for transport to the University of Alberta. The sediment was pushed out of the traps while still frozen and the total height of accumulated sediment measured. Sediments were dried in an oven at 60°C until less than 3% change in mass was achieved for two days in a row.

Erosion markers were PVC poles with 2.5-cm wide black and white markings. Not all fourteen erosion poles were found each cruise but for those revisited the number of markings above- and below-ground were counted for each pole.

3.2.3. Recovery after disturbance

Disturbance experiments were carried out in 2011, 2013, and 2014. First a clump of sponges was selected for large-scale disturbance at Galiano Reef using an asymmetrical BACI design, with one treatment patch and two control patches to minimize damage to the reef (Underwood 1994). Two PVC markers, described above, were placed on either side of the clump. Overlapping still images of the entire clump including the marker poles were taken 1 meter above the seafloor as the ROV moved around and across the clump. Then the ROV was lowered onto the sponges to crush an approximately 1.5 x 2 m large area. A second image series was captured after damaging the sponges. Two control sites were surveyed using the same methods. All three sites were revisited in 2013 and 2014 and an identical image survey carried out. Seventy photos were selected from each survey based on clarity, image quality, and coverage of the site and adjusted for optimal contrast using Photoshop. Three-dimensional structure-from-motion reconstructions of each BACI site were created using 123d Catch (Autodesk, www.123dapp.com/catch) to visualize changes to the sites from all angles.

Second, in 2013 four sponges adjacent to PVC erosion markers were selected for small-scale damage experiments at Galiano reef. Images were captured of 'mitten'-like extensions and then the ROV manipulator arm was used to remove 3-10 cm portions from the tips of the sponges. The same sponges were revisited in 2014 using heading and GPS coordinates to match the view from images from 2013. Images were captured of the same view.

3.3. Results

3.3.1. Recruitment of new sponges

The smallest sponges visible on the reef using HD video from the ROV were less than 5 cm in overall height and width with oscula as small as 2 cm in diameter. All juvenile sponges observed were attached to dead skeleton, had centrally placed oscula, and had broad ridges indicating the first growth of mitten-like projections.

The density of juvenile sponges (0.4 ± 0.9 juveniles m^{-2}; mean ± standard deviation, SD) was greater in areas where adult sponge density was highest (Figure 3.1A; Spearman rank correlation, ρ=0.299, p<0.0001) and where dead sponge cover (skeletons) was highest (Spearman rank correlation, ρ=0.272, p<0.0001). Live and dead sponge cover were strongly correlated (Spearman rank correlation, ρ=0.666, p<0.00001). While density did not differ between reefs (Mann-Whitney U-test, U=10,905, df=1, p=0.218), juveniles found at Fraser Reef were significantly smaller (1.6 ± 0.8 cm maximum osculum width, mean ± SD) than juveniles at Galiano (2.6 ± 1.7 cm; Mann-Whitney U-test, U=1,376.5, df=1, p=0.004) and had a narrower size distribution (Figure 3.1B-D).

A total of 36 specimens were examined for evidence of spermatocysts, eggs, or embryos, from July 2004, November 2004, July 2005, October 2007, June/July 2008, October 2009, October/November 2011, December 2012, and February 2013. Adult tissues from one ROV collection in November 2011 had spermatocysts by December of the same year, and tissue collected by SCUBA in a nearby fjord (Saanich Inlet) in December 2012 and February 2013 also contained spermatocysts (Figure 3.1E). Developing embryos were found in a single specimen of *Aphrocallistes vastus* collected by scuba by one of us (Leys) in November 1995 (Figure S2.1).

3.3.2. Growth

Because *Aphrocallistes vastus* and *Heterochone calyx* grow in three dimensions and extend mitten-like projections to increase surface area, it is difficult to find an accurate growth parameter to measure. We compared images captured at the same angle (ROV heading) each year to measure changes in shape and change in either height of an osculum or length of a projection. Changes included one sponge found growing around a pole in 2013 that had been absent in 2011, and was dying in 2014 (Figure 3.2A). Individual oscula grew in diameter in a multicolored clump of sponges in 2011, 2013, and 2014 but a portion of that clump died back between 2013 and 2014 (Figure 3.2B). A juvenile grew an estimated 3 cm yr^{-1} (Figure 3.2C). Three large sponges grew between 1 and 3 cm yr^{-1} taller while projections from oscula grew faster, between 7 and 9 cm yr^{-1} (Figure 3.2D).

Figure 3.1. Recruitment in glass sponge reefs. A. Map of the density of juvenile sponges at Fraser Reef and Galiano Reef. Juvenile density is correlated with adult sponge density (shaded gray). B. Size distributions of juveniles found at Fraser (gray) and Galiano reefs (black). C-D. Juvenile sponges have a maximum osculum width less than 10 cm. Image credit: S.P. Leys. E. Spermatocysts were fixed in December from a specimen of *Aphrocallistes vastus* that was collected in November and kept alive in seawater tables at Bamfield Marine Sciences Centre. Scale bars: C: 5 cm, D: 2 cm, E: 10 μm.

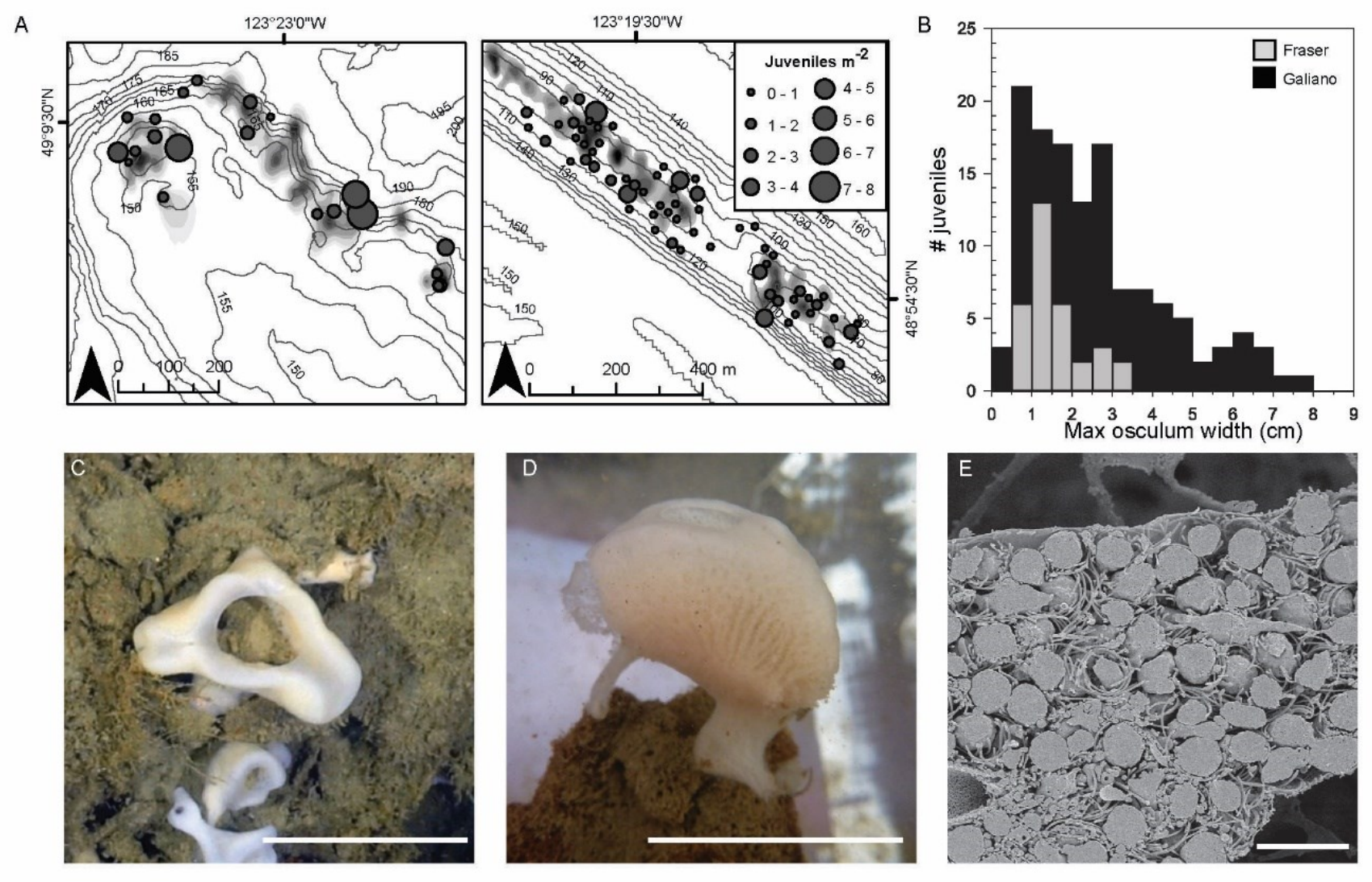

Figure 3.1. Recruitment in glass sponge reefs

Figure 3.2. Growth and change in glass sponge reefs. A. A marker pole planted in open sediments in 2011 (A) had a 20-cm tall sponge (arrow) surrounding it in 2013 (A'), probably dislodged by the ROV. In 2014 (A"), the sponge had died but other sponges had grown up nearby. The photo from 2011 was rotated to provide the same view as in 2013 and 2014. B. A clump of sponges (arrowhead) adjacent to a sediment marker persisted from 2011 (B) to 2013 (B'), but was dead in 2014 (B"). C. Growth of a small sponge (filled arrow) beside a marker. The marker was moved after photographing in 2013, so it now is to the left of the sponge and a juvenile appeared on the settlement plate in 2014 (C: 2011, C':2013, C": 2014). D. Several sponges (filled arrowheads) overgrew a discarded cable we encountered at Fraser Reef. The projections on these sponges grew 7-9 cm yr^{-1} (D: 2011, D': 2013, D": 2014). All scale bars: 50 cm.

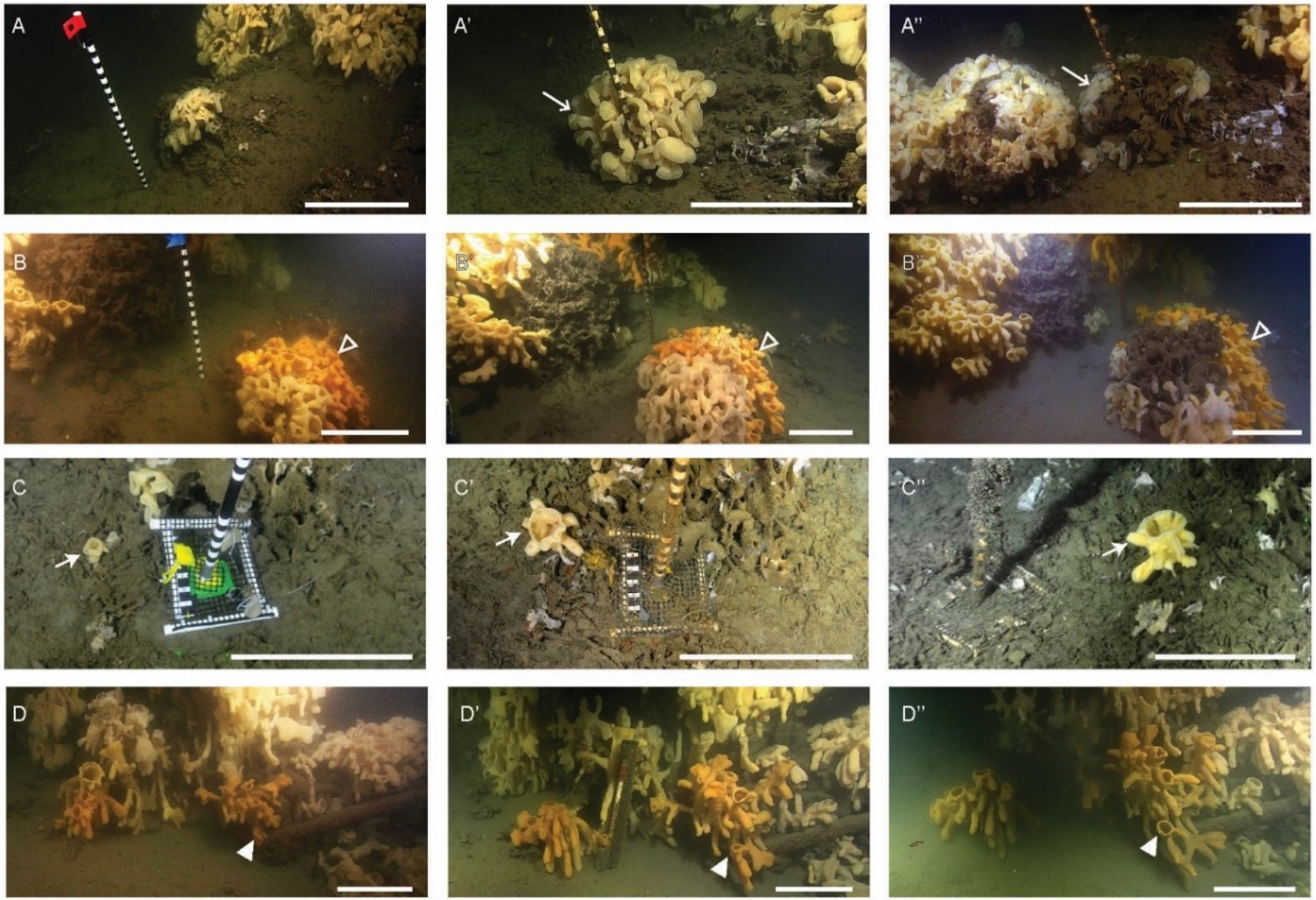

Figure 3.2. Growth and change in glass sponge reefs

3.3.3. Sediment accumulation

Sediment traps showed sedimentation accumulation of 97 mm y^{-1} at Galiano and 137 mm y^{-1} at Fraser reef over 2 years (2007-2009); however, the PVC poles showed both accumulation and erosion with no net overall change over the two years (Figure S2.2). On some poles at Galiano reef the markings at sediment level were erased by scouring caused by high currents.

3.3.4. Recovery after disturbance

3.3.4.a. *Large-scale damage*

The site crushed by the ROV showed no recovery after 3 years (Figure 3.3, Supplemental Material S2.3). Large clumps of sponges immediately adjacent to the damaged site survived through 2014 but did not spread into the space vacated by the dead sponges, nor were new juvenile sponges seen anywhere on the damaged skeletons (Figure 3.3). The two undisturbed control sites showed both new growth and patches of sponges that had died. New growth occurred as overgrowth of skeletons by both glass sponges and by the demosponge *Desmacella austini*.

3.3.4.b. *Small-scale damage*

All four sponge projections that were damaged had repaired completely within one year. In each instance, the sponge had regenerated a soft growing edge to close the damaged area. On one sponge, the damaged projection had grown into what appeared to be an osculum while an undamaged projection of the same sponge had grown 9 cm in length (Figure 3.4).

3.4. Discussion

We took advantage of repeat visits to the glass sponge reefs in the Strait of Georgia British Columbia using a ROV to observe recruitment and changes to individual sponges over time. We found that the changes were similar to those that occur to sponges in shallow-water habitats including growth, death, and resilience to disturbance.

3.4.1. Recruitment of new sponges

The many juvenile sponges observed on the reefs with one distinct size class of osculum diameter (1-3 cm) are indicative of one or more reproductive events per year. We have looked for signs of reproduction in reef sponge tissues during every visit by SCUBA or ROV since the late 1990s. Although putative embryos have only been found in one specimen collected in November 1995 and not in tissues collected in July 2005, October 2007, 2009, 2011 or November 2011, 2013, 2014, nevertheless we found spermatocysts in tissue collected in December 2012 and February 2013. From these three observations of spermatocysts and embryos we conclude that gametes develop asynchronously within a single individual of

Aphrocallistes vastus and that reproduction is asynchronous among individuals, with spawning of sperm and development of embryos by brooding occurring over winter months.

Finding so many very small sponges gives new insight into reproduction and recruitment at the reefs. Given a growth rate of 1-3 cm yr^{-1}, juveniles (2-10 cm) seen on the reefs in November 2013 and 2014 may have settled the previous year. Because larvae recruit to dead sponge skeleton we wondered whether high sedimentation might impede settlement and recruitment. Our two methods of measuring sediment accumulation suggest this is not the case. First, the density of juveniles was independent of sedimentation rates: Fraser reef had double the sediment fallout of Galiano reef, but density of juvenile sponges at both reefs did not differ. Second, the erosion markers showed very little accumulation of sediment even over several years, which explains why reef skeletons remain exposed as a good substrate for recruitment.

The fact that juveniles were found near both adult sponges and dead skeleton suggests that either larvae settle close to their site of release as found by Uriz and colleagues for a demosponge (Uriz et al. 1998), or that larvae settle gregariously based on chemical cues from the material growing on dead skeletons (Ettinger-Epstein et al. 2008). A third possibility is that those skeletons and live sponges provide both good substrate and good growing conditions for sponges. These hypotheses cannot be tested with our data since the density of live sponges and dead skeletons were strongly correlated. In all visits to the reefs, there was no evidence that reef species reproduce asexually by forming tissue drips as suggested by Austin et al. (2007).

3.4.2. Growth

Because we could carry out repeated visits to the same sites on the reefs we could see changes to individual sponges over time. Rates we calculate of 1-3 cm yr^{-1} for very young reef sponges are slower than those estimated for overall growth for the hexactinellid *Rhabdocalyptus dawsoni*, which can grow in length by up to 12% per year in small specimens and by 6% per year in larger specimens (Leys and Lauzon 1998). Projections of reef sponges grew more rapidly, at 7-9 cm yr^{-1}, which is in agreement with rates measured by Austin and colleagues (Austin et al. 2007). Generally it seems then that smaller sponges grow relatively quickly or episodically (Leys and Lauzon 1998, Dayton et al. 2013, Fillinger et al. 2013). Appearance and growth of new sponges, and death of patches of sponge, indicate that the population is continually renewing and replacing across annual timescales as described for other glass sponge populations (Kahn et al. 2012). In 2013 we re-visited an erosion pole planted in 2011. A large sponge now lay against it (Figure 3.2A), which suggests that this sponge was dislodged from elsewhere and came to rest against the pole. If the sponge was dislodged in 2011

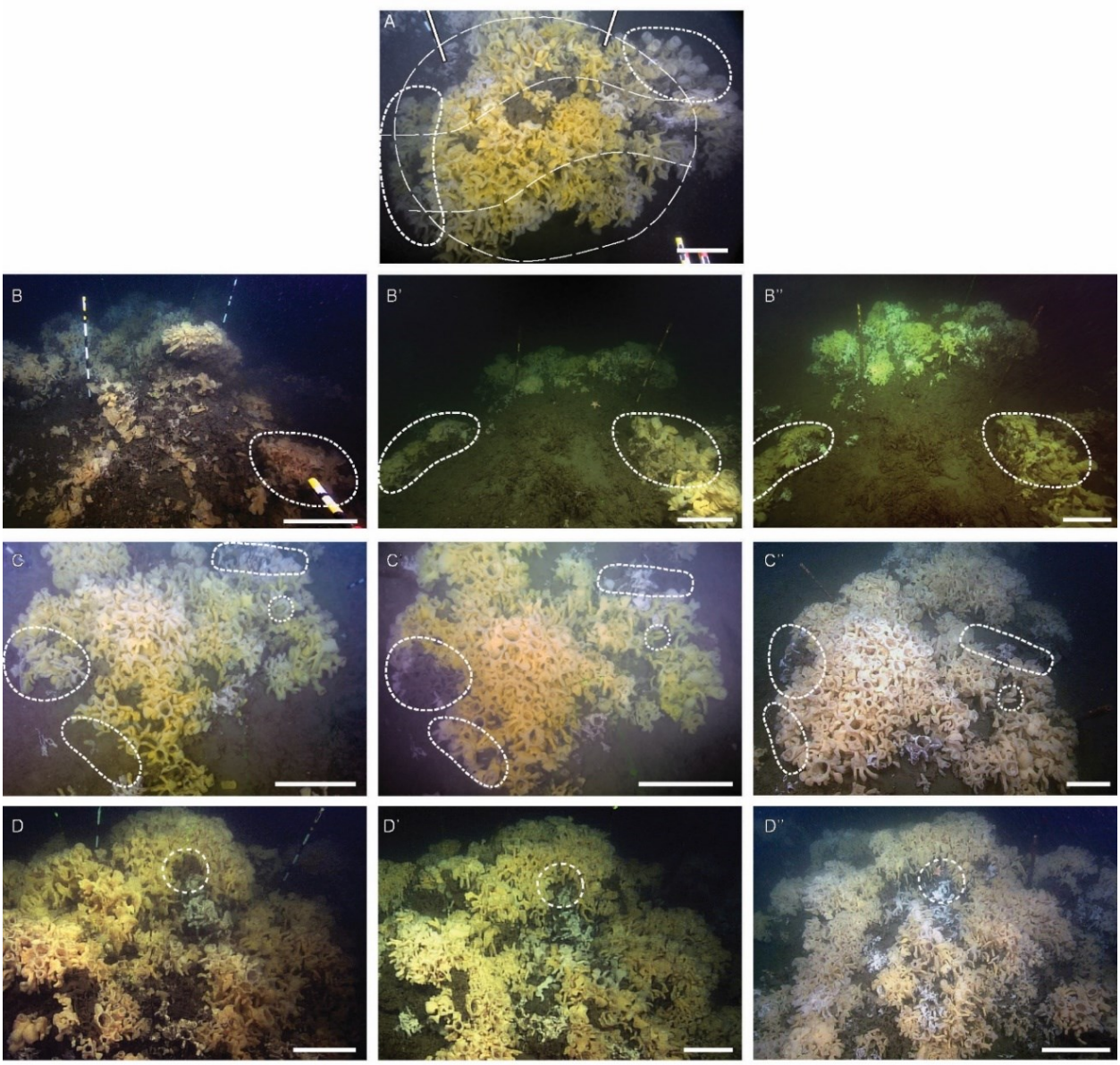

Figure 3.3. Disturbances to the sponge reefs using an asymmetrical BACI design. A. Images of an 'impacted' site in 2011 before physical disturbance with paths for ROV surveys shown by dashed lines. (B, B', B") Image surveys following our experiment in 2011 and showing the state of the site in 2013 and 2014 show little recovery, though adjacent sponges appeared unaffected. Control sites 1 (C: 2011, C': 2013, C": 2014) and 2 (D: 2011, D': 2013, D": 2014) showed overgrowth of sponge tissue, death of other tissue, and colonization by *Desmacella*. Dotted circles indicate regions of growth or death between years. All scale bars: 50 cm.

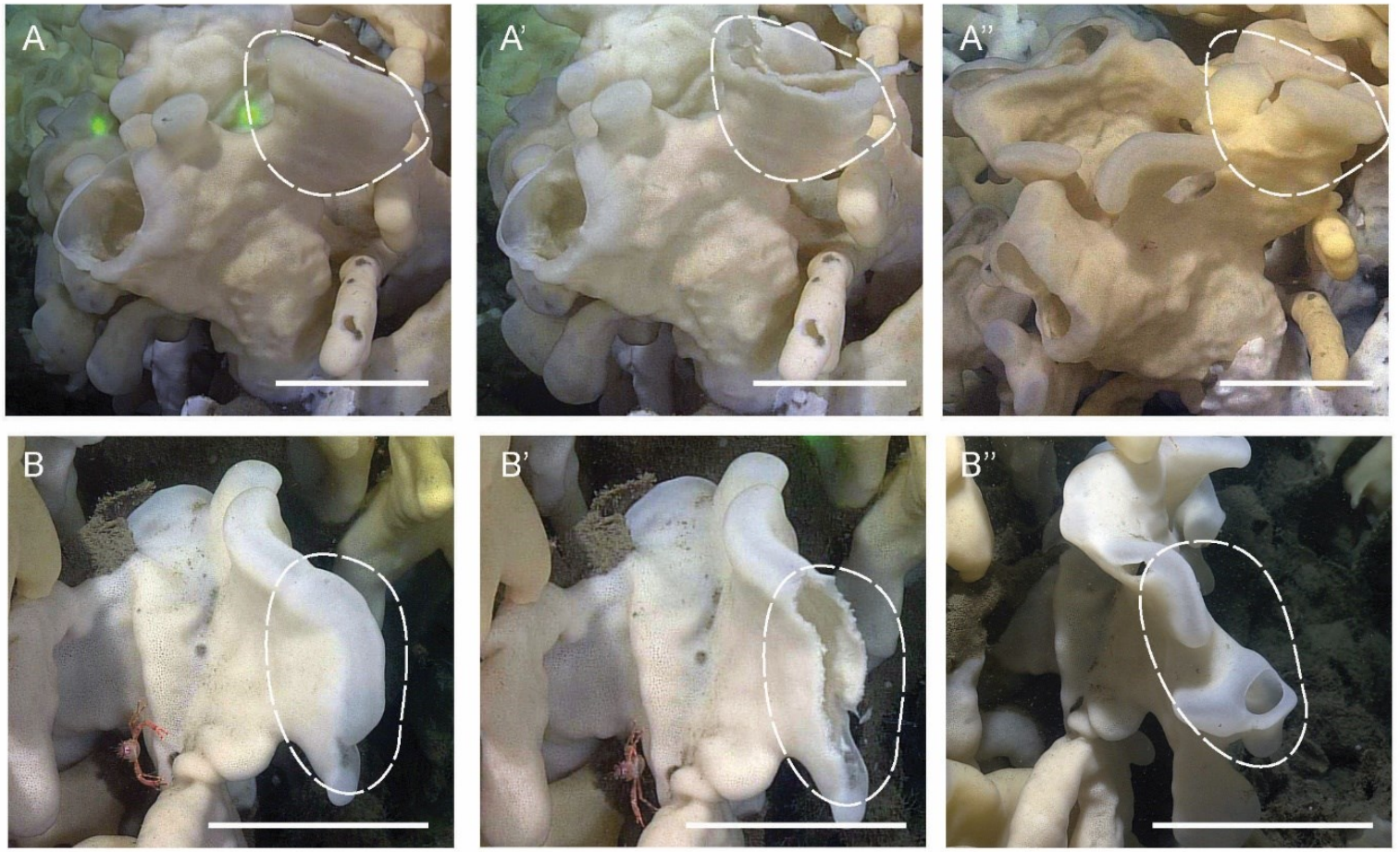

Figure 3.4. Recovery of reef sponges from small-scale damage. All panels show regions with projections of sponges that were damaged in 2013. A, B: 2013 Still images captured from HD video before damage occurred. A', B': 2013 Still images from HD video taken after the projections had been damaged. A", B": 2014 Still images from HD video taken one year later. All scale bars: 10 cm.

and was still alive two years later then glass sponges can reattach following breakage. There is little chance that this sponge settled and grew from a larva to some 30 cm in diameter in 2 years, although that possibility should not be ruled out. This was a single observation however, and further observations are needed to determine whether this sort of reattachment could happen more often in sponge reefs due to disturbance by sharks or high currents.

3.4.3. Recovery after disturbance

Glass sponges are easily broken with trawls and prawn traps (Freese et al. 1999, Wassenberg et al. 2002, Ardron and Jamieson 2006, Heifetz et al. 2009), but the ability of reefs to recover from damage is unknown. We therefore took advantage of the ability to make repeat visits to identical sites to determine the ability of reef forming sponges to regenerate.

Having expected that deep sponge communities change slowly, we were surprised to find that small parts of sponges intentionally damaged by the ROV could recover completely in one year. Similar regeneration rates were found previously in shallower populations of glass sponges wounded experimentally (0.05cm^2 d^{-1} or 18 cm^2 yr^{-1}; Leys and Lauzon (1998)). Naturally broken fragments of sponges and damage to oscula and projections were frequently seen on our ROV dives. This damage was thought to be caused by both fish bites and knocks by fish tails (e.g. dogfish, ratfish) because in 2014 we watched as both the Pacific spiny dogfish (*Squalus acanthius*) and the lingcod (*Ophiodon elongatus*) caused this sort of damage when they caught crustacean prey from among the sponges (Figure 3.5A). Grazing by the nudibranch *Peltodoris lentiginosa* also causes extensive damage to the uppermost projections of sponges (Figure 3.5B).

We were even more surprised to learn that, in contrast to the quick repair of small parts of the sponges, no new sponges grew into or colonized the 'impacted' BACI site even after three years. Because sponges at the edge of the damaged site grew into the area but no new sponges recruited, we suspect that it was the extensive damage to the skeletons, used as substrate by reef sponges, that prevented new recruitment. Trawling has similar effects in removing rugosity and creating flat terrain (Puig et al. 2012), so damage caused by trawling in regions with sponge reefs and gardens is expected to be long lasting.

In addition to anthropogenic disturbance, reef sponges face predation as described above and also by Chu and Leys (Chu and Leys 2012) as well as competition for space. The demosponge *Desmacella austini* grows on the spicule skeletons of reef sponges and may overtake live sponge tissue, although this remains to be verified (Lehnert et al. 2005). Three different color morphs – possibly different species – of *Desmacella* were seen at both reefs. *Desmacella* was most common on dead glass sponge skeleton (Figure 3.5C), but careful study of

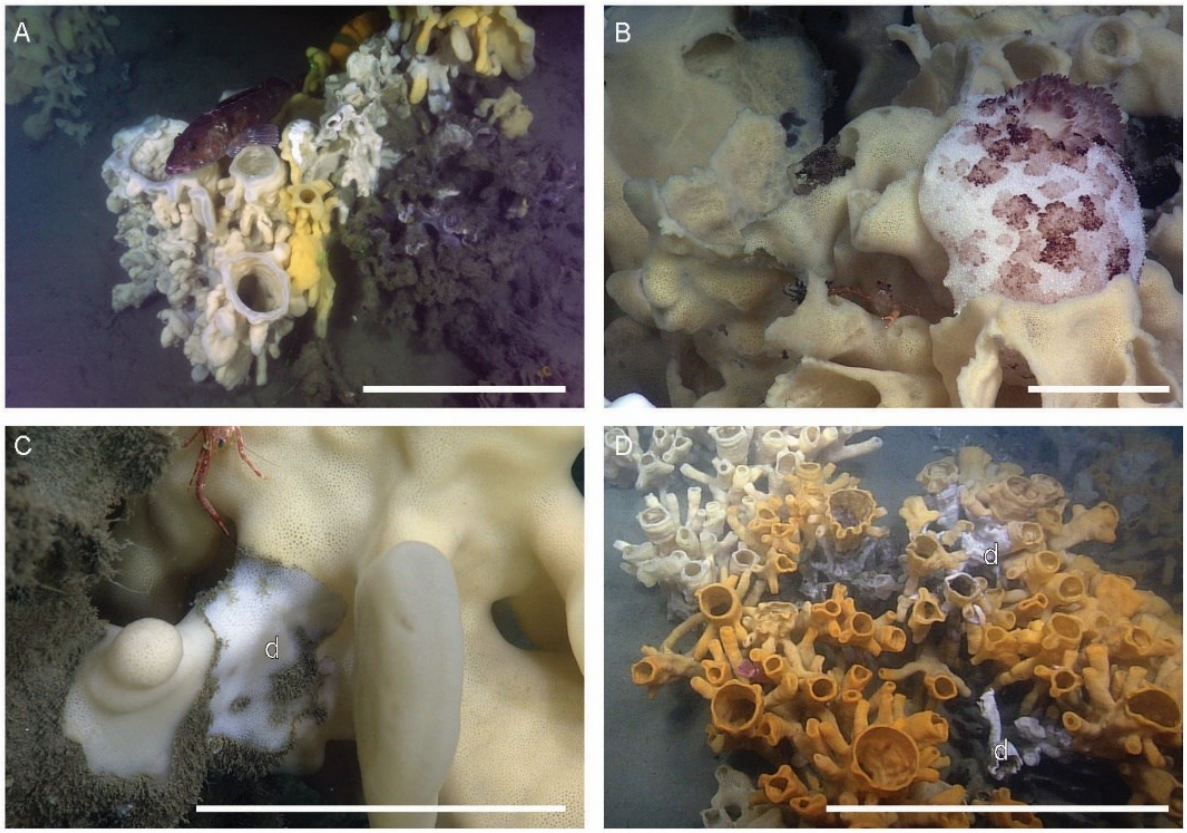

Figure 3.5. Other animals in sponge reefs. A. A lingcod (*Ophiodon elongatus*), one of many fish that frequent the reefs and can cause disturbance from bites and knocks, perches on the sponges. B. The nudibranch *Peltodoris lentiginosa* on a sponge; adjacent oscula showing damage from grazing. C. *Desmacella austini* (d), a demosponge that occupies the skeleton, here at the base of *Aphrocallistes vastus*. D. *Desmacella* was commonly seen at the base of glass sponges in the reefs. Scale bars: A & D: 50 cm, B & C: 5 cm. All scale bars are approximations.

the high definition video showed that *Desmacella* occupied the base of many individuals of glass sponges (Figure 3.5D). Whether *Desmacella* colonizes live glass sponges or takes advantage of areas of skeleton left exposed by regressing tissue as the sponges grow upward is unknown. Its growth on dead skeletons however, may prevent larvae from settling.

3.5. Conclusions

Three years of observations of deep sponge reefs showed that many aspects of reef ecology – recruitment, growth, and resilience to disturbance – were comparable to those of shallower sponges. Recruitment occurs annually with growth rates for the smallest sponges of 1-3 cm yr^{-1} and projections of the larger sponges at up to 7 cm yr^{-1}. New sponges continually grow and replace sponges that have died. Glass sponge reefs are resilient to minor natural disturbance, but showed no signs of recovery from large-scale physical breakage within the timescale of this study.

3.6. Acknowledgments

We thank the captain and crew of *CCGS Vector* and the staff and pilots of the Canadian Scientific Submersible Facility ROPOS. D. Eerkes-Medrano, D. Ludeman, J. Chu, V. Tunnicliffe, and G. Yahel helped with ship support and equipment deployment. We thank Pinnacle Divers of Victoria, B.C. for diving support. Lab space was provided by the School of Earth and Ocean Sciences (University of Victoria) and Bamfield Marine Sciences Centre. V. Marshall, J. Mah, and especially C. Pennelly helped with data management and curation.

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Chapter 4. Cell replacement in sponges sheds light on stem cell origins in Metazoa

4.1. Introduction

It has been proposed that rapid renewal of choanocytes – feeding and water pumping cells – may be responsible for a large amount of the carbon released by sponges (De Goeij et al. 2009, De Goeij et al. 2013, Alexander et al. 2014) as part of their role in pelagic-benthic coupling; however, it remains unclear how much this is due to choanocyte renewal rather than excretion of detritus and mucus. Is tissue homeostasis really so rapid and so uniform across taxa and over time for an individual sponge that gross estimates are possible? Cell turnover and replacement are essential properties of animal tissues and organs, but how and whether the earliest animals regulated cell turnover and achieved tissue homeostasis is unknown.

Tissue homeostasis in animals occurs at different rates for different cell types depending on function, with metazoans and other multicellular organisms typically using stem cells to replenish cell populations (e.g. Newmark and Sánchez Alvarado 2000). In sponges, differentiated cells make up structures that must be maintained, including outer and inner epithelia (pinacoderm) and pumping and feeding units (choanocyte chambers). As an early branch of animals, sponges provide a model to determine whether the last common ancestor to animals needed to regulate cell proliferation on its way to multicellularity.

In relation to other animals, sponges are significant because of their traditional placement as the earliest branch of the metazoan tree (e.g. Philippe et al. 2009). The morphology and activity of sponge choanocytes resembles that of choanoflagellates, the closest protistan relatives to animals. Although the morphological similarity between sponges and choanoflagellates may be convergent (Mah et al. 2014), sponges may provide hints at how the last common ancestor (LCA) to animals transitioned from a unicellular, colonial, choanoflagellate-like individual to a complex animal for whom multicellularity is compulsory. The transition to multicellularity was accompanied by several other major transitions, many of which originated in unicellular ancestors but all of which must be found in multicellular groups (Grosberg and Strathmann 2007). These transitions include a division of labor among differentiated cell types, cell-cell signaling, interdependence between cell types which would lead to differentiation of tissues with organs (Grosberg and Strathmann 2007), and controls to keep various cell populations from growing unchecked (cancer is the result when these controls are disrupted in multicellular life; Pellettieri and Alvarado 2007).

Some recent phylogenomic analyses have introduced doubts about whether sponges or ctenophores (Phylum Ctenophora) were the first group to branch off from the last common ancestor to animals (Dunn et al. 2008, Pisani et al. 2015, Whelan et al. 2015). These analyses have inspired new hypotheses of what the last common ancestor to animals must have been like – ctenophores have complex neural and muscular systems that would have either had to evolve twice independently or have been secondarily lost in sponges. However, despite their possible position as sister group to the metazoans, the rapid radiation and what appears to be independent evolution of the neural and muscular systems (Moroz 2015) makes ctenophores morphologically very divergent from whatever the LCA was like. Sponges remain a more appropriate phylum to study to understand how the LCA made the transition from a unicellular individual to a multicellular animal.

In other animals cell proliferation is anything but constant: proliferation varies between different cell types within the body (Pellettieri and Alvarado 2007), between different species (Otto and Campbell 1977, David 1983, Holstein and David 1990), at different stages of development and growth (Cheng and Leblond 1974, Alexiades and Cepko 1996), following circadian, tidal, and seasonal rhythms (Chisholm et al. 1984, Leibson and Frolova 1994, Zaldibar et al. 2004), and depending on starvation and food availability (Otto and Campbell 1977, Bosch and David 1984). Longer cell lifespans are associated with larger body mass, cell size, and lower temperatures (Gillooly et al. 2001, Gillooly et al. 2012). The proliferation rate of single-celled organisms is also affected by nutrient and light levels (Olson and Chisholm 1986, Zachleder and Van Den Ende 1992, Taroncher-Oldenburg et al. 1999).

Cell turnover rates (i.e. the cell cycle length) estimated for different demosponges range from 5 to 20 h (Shore 1971, Efremova and Efremov 1977, Tanaka and Watanabe 1984, De Goeij et al. 2009). In comparison cells from mouse gut epithelia and *Hydra* epithelial and stem cells are replaced every 4 to 7 d (David 1983, Bosch and David 1984), although new cells can be formed as quickly as every 8 h during development and growth (mouse neural progenitor cells; Al-Kofahi et al. 2006). Choanoflagellates are the sister group to metazoans and considered to feed in a similar way to sponge (Mah et al. 2014). In *Monosiga brevicollis* cells are replaced every 6 h during log phase of growth in a fed culture (King et al. 2003), and in general all cells in a cell culture will have similar cellular kinetics. In contrast, different cell types within an animal are even replaced at different rates depending on their functions. For example, cell turnover in mammary glands and the uterus is tied to cyclical needs for reproduction (reviewed by Pellettieri and Alvarado 2007).

To determine the conditions that affect cell proliferation rates of sponges, choanocyte proliferation rates in four temperate sponge species were measured and factors known to affect cell proliferation in other animals were examined: life history stage, season, and food availability. In situ time-lapse imaging was used to study the formation of choanocyte chambers to understand the mechanics behind choanocyte turnover and replacement. Sponges must use energy to maintain their filter-feeding system, replenish spent cells, grow, and reproduce. These results illustrate the complexity of calculating the ecological effects of choanocyte proliferation and demonstrate that sponges can modify the rate that stem cells replace spent cells at different stages of their life history.

4.2. Methods

The four species we studied came from different taxonomic groups with a range of life histories and habitats within British Columbia, Canada. *Spongilla lacustris* is a freshwater demosponge (Class Demospongiae, Order Spongillida, Family Spongillidae) that grows from overwintering gemmules each year, reproduces in late summer and regresses back to gemmules each winter. *Sycon coactum* is a marine calcareous sponge (Class Calcarea, Order Leucosolenida) that grows, reproduces, and dies within one year. *Haliclona mollis* is a subtidal marine demosponge (Class Demospongiae, Order Haplosclerida, Family Chalinidae) that lives year-round in Barkley Sound. *Aphrocallistes vastus* is a hexactinellid sponge (Class Hexactinellida, Order Hexactinosida, Family Aphrocallistidae) that lives in deep water >1000 meters across the northern Pacific and at 40-200 meters on the continental shelf of western Canada.

Unless otherwise noted, samples were collected in June and July to estimate choanocyte proliferation rates. *Spongilla lacustris* was collected at 1 m depth in Rosseau Lake. *Sycon coactum* was collected at 10 m from docks at the Bamfield Marine Sciences Centre (BMSC). *Haliclona mollis* was collected by SCUBA at 10 m from Wizard Islet. *Aphrocallistes vastus* was collected both by SCUBA at 40 m in Saanich Inlet in December 2012 and February 2013 and by the remotely operated vehicle ROPOS (http://www.ROPOS.com) at 70 m from Galiano Ridge Reef in November 2012 and 2013.

Freshwater sponges were kept in 18°C lake water, refreshed daily. Marine sponges were maintained in flow-through seawater tables (9-10°C) at BMSC or, for the *A. vastus* collected by SCUBA, fragments 1 cm^2 were kept in 0.5 L containers of seawater at 9°C in an incubator at the Marine Technology Centre, University of Victoria, in Sidney, B.C.

4.2.1. EdU incubations

Pieces (5 x 5 x 2 mm) were cut from the central region of each of 3 individuals immediately after collection and allowed to heal overnight. Cuttings were mixed together and randomly assigned to different incubations in EdU (5-ethynyl-2-deoxyuridine; Life Technologies). Natural variability from individuals collected at the same time was found to be negligible (for *S. lacustris*, $t = 0.102$, $df = 4$, $p = 0.92$). *S. lacustris* was incubated in 100 μM EdU in 0.2 μm filtered lake water at a typical lake temperature of 18°C. *S. coactum*, *H. mollis*, and *A. vastus* were incubated in 100 μM EdU in 0.2 μm filtered seawater at 9°C, although the concentration could have been slightly lower because it became diluted as sponges were transferred into the treatment water. Length of incubation in EdU varied for each sponge depending on the time at which the ratio of labeled cells to total cells labeled by Hoechst 33342 no longer changed.

Proliferation rates in *S. lacustris* hatched from gemmules were compared to those of adult sponges. Gemmules were collected from Rosseau Lake, B.C. by SPL in December 2011, kept at 4°C in unfiltered lake water aerated monthly. Gemmules were hatched and cultured in April 2012 as described by Elliott and Leys (Elliott and Leys 2007). Once a full aquiferous system had developed 5 d post-hatching (dph), sponges were incubated in 50 μM EdU in M-medium (Rasmont 1961) for between 4 and 72 h.

To compare choanocyte proliferation in winter with proliferation in summer, *H. mollis* were collected in October 2013 and November 2014. Incubations were carried out as described above. *S. coactum* was collected during senescence in August to compare with growing adults sampled in June.

Water was filtered to prevent EdU uptake by bacteria and to avoid differences in proliferation caused by variable food availability; however to test the effect of feeding activity on choanocyte proliferation, pieces from one individual of *H. mollis* were incubated in EdU in unfiltered (normal) seawater in November 2014 and June 2015. Duplicate water samples were collected from the filtered and unfiltered treatments in June 2015, fixed with 0.15% glutaraldehyde, and frozen at -80°C for quantification of bacteria with a FACSCalibur MACPro flow cytometer at the University of Alberta. Cell proliferation of sponges was measured using incubations of EdU as described above.

4.2.2. Sample processing

Sponges from all experiments were fixed in 4% paraformaldehyde and 0.03% glutaraldehyde in phosphate-buffered saline (PBS) or Bouin's fixative (*S. coactum*) overnight and dehydrated to ethanol. Silica was removed with 4% hydrofluoric acid. Tissues were cleared

in toluene and embedded in paraffin wax (Paraplast). Sections 7-10 μm thick were rehydrated in PBS, washed once in in PBS with 3% bovine serum albumin (wash buffer), permeabilized two min in PBS with 0.1% Triton-X (PBTx), and rinsed three times in wash buffer. Sections were incubated in 250 μl of reaction cocktail (Click-iT EdU AlexaFluor 594® Imaging Kit, Life Technologies, Carlsbad, CA) in the dark for 30 min at room temperature, rinsed once with wash buffer and once with PBS. AlexaFluor 488 was used for *S. lacustris* to avoid overlap with autofluorescence from symbiotic algae. Nuclei were stained with 100 μM Hoechst 33342 for 30 min; slides were rinsed 3 times with PBS and mounted with Mowiol.

At least two slides were prepared from each embedded sponge; several sections from each slide were viewed with a Zeiss Axioskop2 Plus microscope and images captured with a QiCam (QImaging) and Northern Eclipse software (Empix Imaging Inc.). Nuclei labeled with EdU (newly synthesized DNA) and nuclei labeled with Hoechst (all nuclei) were counted using the Cell Counter plug-in for Fiji-ImageJ (Schindelin et al. 2012). Statistics were calculated using SYSTAT 12 and R.

4.2.3. Calculating cell proliferation

Characteristics of choanocyte proliferation assumed to be proliferating at a steady state were determined by plotting the average proportion of EdU-labeled cells at each time point, termed the labeling index (LI; Nowakowski et al. 1989)) (Figure 4.1). The proliferation rate of cells (percent cells hr^{-1}) indicates the rate at which new cells enter S-phase in their progress through the cell cycle, and is the slope of the linear regression of labeling indices. The y-intercept indicates the proportion of cells in S-phase at any given moment (LI_o). The length of S phase (T_s) is the time interval from the x-intercept to the y-axis. As incubations increase in length, more and more cells enter S-phase of the cell cycle until all cells that are replicating are labeled, a proportion called the growing fraction (GF). The length of the cell cycle from S-phase to the next round of S-phase (T_c), also called the turnover rate, was calculated as the time until the GF is reached (T_c-T_s) plus the length of time spent in S-phase (T_s). As a result, estimates of cell proliferation rates require at least three time points for a linear regression; cell proliferation values at a single time point can vary greatly depending on the number of cells initially labeled (cells in S-phase) and when no more cells label (Figure 4.1C).

4.2.4. Microscopy of choanocyte chambers

To understand the mechanism behind cell replacement in a chamber, choanocyte production and replacement was studied in *Spongilla lacustris* grown on glass coverslips as described by Elliott and Leys (Elliott and Leys 2007). Choanocyte chambers were located and images captured using a 40x water immersion lens (Zeiss Achroplan on a Zeiss Axioskop2

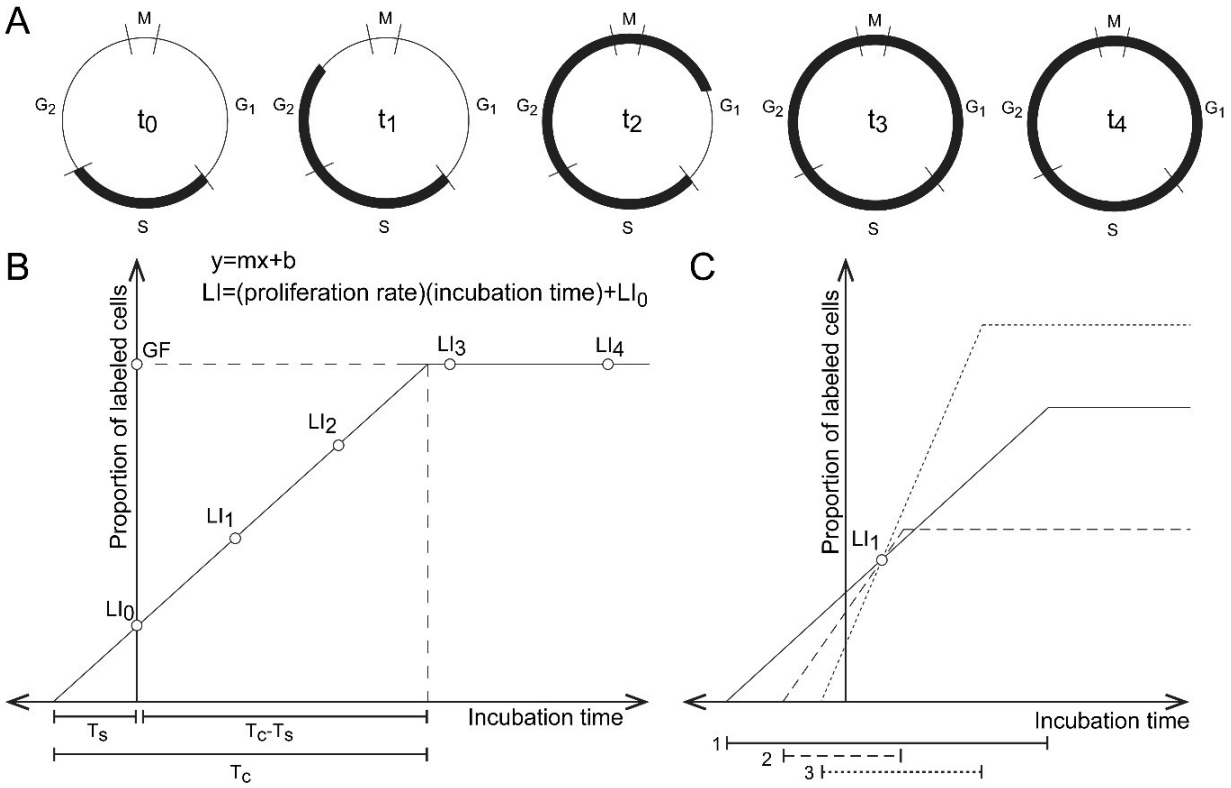

Figure 4.1. Theoretical model for labeling steady state cell populations, after Nowakowski 1989. A. The proportion of EdU-labeled nuclei increases with time as more cells enter S-phase of the cell cycle, until all cells in a population have passed through S-phase. B. EdU incubations of a steady state population for different lengths of time result in labeling indices (LI) along a linear curve that eventually levels off when the growing fraction of cells (GF) has all passed through a complete cell cycle. The y-intercept corresponds with the proportion of cells in S-phase at a given moment and is back-calculated from the regression line. The time from the x-intercept to the ordinate axis is the calculated length of S phase (T_s). The time from the x-intercept to the asymptote is the length of the cell cycle (T_c). C. A single time point (LI) cannot be used to compare cell cycle kinetics between species or treatments because of the different cell cycle lengths and GF that result from different slopes (proliferation rates).

microscope). Images from each recording were imported as an image stack into ImageJ software and converted to time-lapse video at 25 frames sec^{-1} (Schindelin et al. 2012, Schneider et al. 2012).

4.3. Results

4.3.1. Cell cycle lengths and proliferation rates

Choanocytes in *Sycon coactum* had the shortest cell turnover rate (30.7 h) and fastest proliferation rate (Table 4.1, Figure 4.2). Choanocyte turnover and proliferation rates of choanocytes in adult *S. lacustris, H. mollis,* and *A. vastus* were similar to each other, and greater than 4 d (Table 4.1, Figure 4.2).

Cell proliferation also varied within a species depending on life history stage. Proliferation rates were 10 times slower in mature *S. lacustris* than in individuals hatched from gemmules, although the proportion of cells in S phase at any given time (t=0 h) was identical (Table 4.1, Figure 4.2). All individuals of the calcareous sponge *S. coactum* collected were reproductive by June, which presumably affected choanocyte chamber function (Eerkes-Medrano and Leys 2006) and likely turnover rates, but was unavoidable. However, *S. coactum* collected in August were beginning to senesce and consequently had many fewer proliferating cells than those collected in June; 4.5 ± 1.4% of choanocytes in chambers from sponges collected in August had taken up EdU after 30 h compared with 18 ± 4% after 36 h from samples collected in June.

Glass sponges (Class Hexactinellida) differ from other sponge groups in having syncytial tissues. Instead of choanocytes lining flagellated chambers, the collar-flagella units that draw water through chambers are enucleate and are extensions of nucleated choanoblasts which occur in small clusters beside the chambers (Reiswig and Mehl 1991, Leys 1999). Collar bodies are thought to be discarded and renewed by the choanoblasts (Mackie and Singla 1983) but because they are enucleate it is not possible to track their turnover. Instead we studied proliferation of choanoblasts in *A. vastus*. Choanoblast nuclei seldom labeled in mature regions of *A. vastus* and then only after 4 d of incubation in EdU so that there were too few nuclei labeled to estimate rates of proliferation from. Nuclei in the trabecular syncytium never labeled with EdU. A growing region found in the edge, or lip, of the osculum however, contained a band of rosettes of EdU-labeled choanoblasts (Figure 4.3). Although this was a region of new growth, the turnover rate was 170 h and the proliferation rate was equal to that of mature *H. mollis* and *S. lacustris* (Table 4.1, Figure 4.3).

Table 4.1. Cell turnover rates and characteristics, determined assuming a steady state population of cells.

Proportions of cells in S phase at a given time, the rate of proliferation, asymptotic/maximum proportion of proliferating cells in a population (growth fraction, GF), estimated length of S phase, and estimated length of cell turnover.

	Sycon coactum	*Spongilla lacustris* (gemmules)*	*Spongilla lacustris* (adult)	*Haliclona mollis*†	*Aphrocallistes vastus*
Cells in S phase (%)	7.1±1.7	6.6±6.8	17±1.6	27±2.1	12±1.2
Proliferation rate (% cells h^{-1})	0.7±0.1	2.0±0.36	0.19±0.03	0.25±0.04	0.17±0.02
Max labeled (GF; %)	20±1.4	70±9.8	30±1.5	44±1.5	29±0.5
Length of S phase (T_s; h)	10.9	3.2	91	108	72
Cell turnover rate (T_c; h)	30.7	34.6	≥159	176	170

*For full-size, central chambers in gemmules incubated 5 d post-hatching. Smaller peripheral chambers had 16.8±4.6% of cells in S phase, proliferation rate of 2.0±0.3% cells h^{-1}, 73±5.2% of cells as the GF, 8.3-h T_s, and 36.2 h T_c.

†For *H. mollis* in filtered seawater. *H. mollis* incubated in unfiltered seawater had 24.0±3.1% of cells in S phase, proliferation rate of 0.25±0.04% cells h^{-1}, 48±2.3% of cells as the GF, 69.2-h T_s, and 138 h T_c.

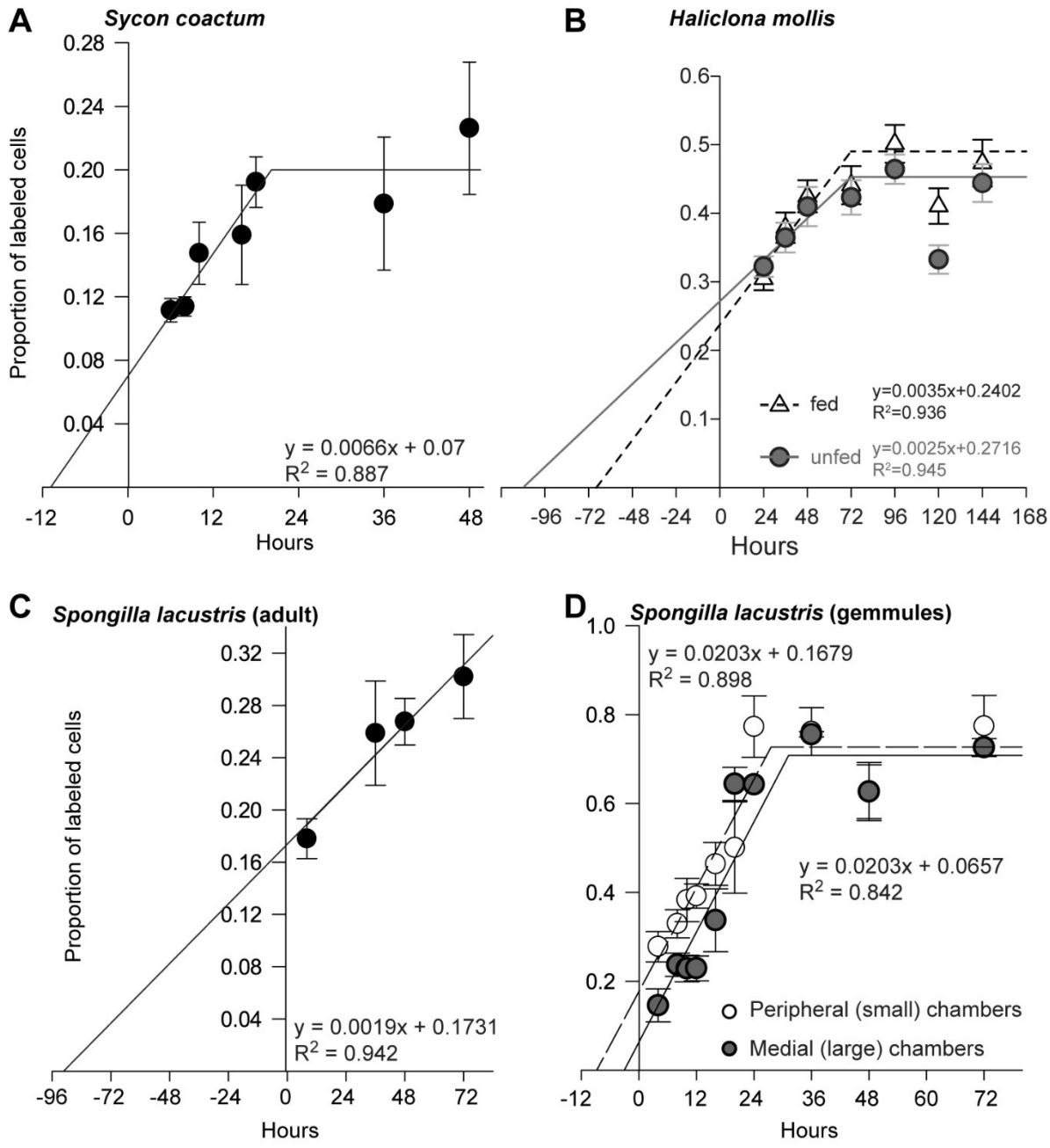

Figure 4.2. Proportions of EdU-labeled choanocytes with calculations assuming a steady state choanocyte population, after Nowakowski 1989. A. *Sycon coactum*; B. *Haliclona mollis* in unfiltered water and in filtered water; C. *Spongilla lacustris* sampled from adult tissue; D. *Spongilla lacustris* hatched from gemmules. Error bars indicate standard error.

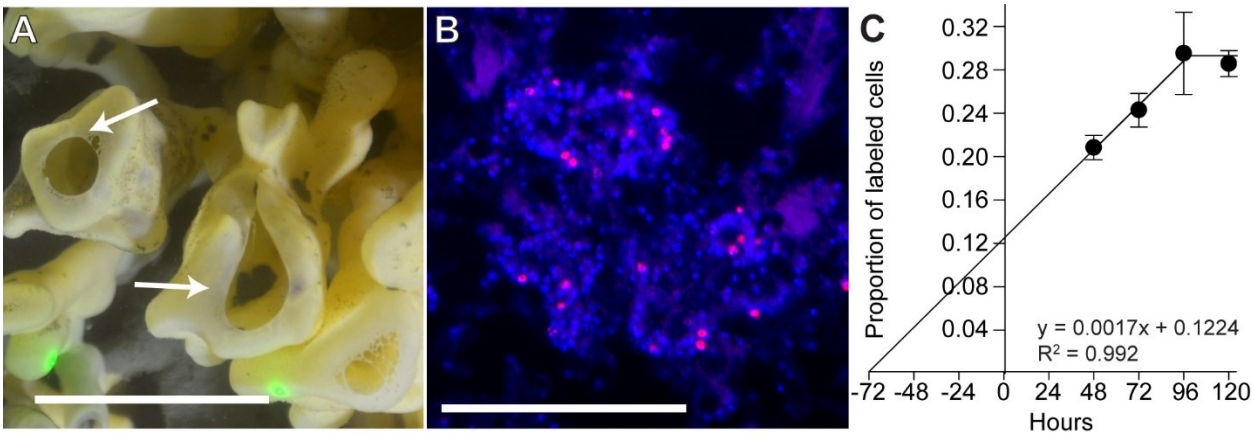

Figure 4.3. EdU uptake in nuclei of the syncytial choanoblasts of *Aphrocallistes vastus*. A. Few nuclei labeled with EdU in the main body of the sponge but several choanoblasts took up the EdU label in the unfused region of the osculum lip (arrows). B. EdU-labeled choanoblasts were arranged in rosettes in the growing region of the sponge. Nuclei labeled with Hoechst 33342 are blue and nuclei labeled with EdU are red. C. Proportions of EdU-labeled choanoblasts with calculations assuming a steady state population. Scale bars: A: 10 cm; B: 100 μm.

4.3.2. Cell proliferation by cell type and region

Cells labeled with EdU were in the choanosome, mesohyl, and pinacoderm of all sponges, but some had higher turnover rates than others (Figure 4.4). For example, choanocytes and mesohyl cells labeled with equal frequency in *Haliclona mollis* whereas 2-5 times more choanocytes than mesohyl cells labeled in *Spongilla lacustris*. Pinacocytes were labeled in *S. lacustris* and *H. mollis* after 72 h but were never labeled in *S. coactum*.

For *Spongilla lacustris* more choanocytes labeled in small chambers near the periphery than in full-sized chambers near the center of the sponge for each incubation interval, yet the overall proliferation rate was identical (2 ± 0.31% h^{-1} for peripheral chambers, 2 ± 0.36% h^{-1} for larger central chambers; slope ± SE of slope) (Table 4.1, Figure 4.2). Consequently, even though the peripheral chambers were about half the diameter of central chambers and were still growing, the cell turnover rate of choanocytes was very similar (36.2 h and 34.6 h, respectively).

4.3.3. Effect of season and feeding activity on cell proliferation

There were seasonal differences in choanocyte proliferation in *Haliclona mollis,* with many choanocytes labeled in summer, and very few in the fall (October/November). Seasonal differences were also noted in choanocyte proliferation as *Sycon coactum* senesced in August.

To determine whether feeding activity had an effect on choanocyte turnover, proliferation rates and cell turnover were compared in sponges incubated in filtered and unfiltered seawater. It proved impossible to remove all bacteria from the water suggesting that the sponge carried with it through each water change a number of bacteria which reproduced. Nevertheless, the filtered water produced very low bacterial concentrations (filtered water: 2.0 x 10^4 cells ml^{-1}) compared to normal seawater (2.9 x 10^5 cells ml^{-1}). Interestingly, while proliferation rates of choanocytes in *Haliclona mollis* (Table 4.1) were not significantly different between the two treatments (proliferation rate: ANCOVA, $F = 1.0367$, $p = 0.309$; proportions of labeled cells: ANOVA, $F = 0.687$, $p = 0.407$), sponges in water with more bacteria had a shorter estimated cell turnover (138 h in normal seawater compared to 176 h in filtered seawater) (Table 4.1, Figure 4.2), suggesting that feeding activity may affect turnover rates. In contrast however, choanocytes in sterile culture of gemmule-hatched *S. lacustris*, showed a much higher proliferation rate than sponges grown in non-sterile lake water (Table 4.1). These contrasting observations suggest turnover rate is more complex than simple exhaustion and replacement of cells due to feeding activity.

Figure 4.4. EdU labeling in choanocyte chambers from all species of sponges studied. Mesohyl cells labeled with EdU are visible in the images of *Spongilla lacustris* and *Haliclona mollis*. A. *Spongilla lacustris*, a shallow freshwater demosponge (A': Hoechst label; A": EdU label; A"': Hoechst (blue) and EdU (red) overlaid onto a DIC image of a choanocyte chamber; other columns follow this labeling scheme). B. *Sycon coactum*, a shallow marine calcareous sponge. C. *Haliclona mollis*, a shallow marine demosponge that persists across several years. D. *Aphrocallistes vastus*, a deep-water marine hexactinellid sponge. Scale bars: all are 25 μm unless listed here. A: 1 cm; B: 1 cm; C: 1 cm; D: 10 cm. Images A and B credit: S.P. Leys.

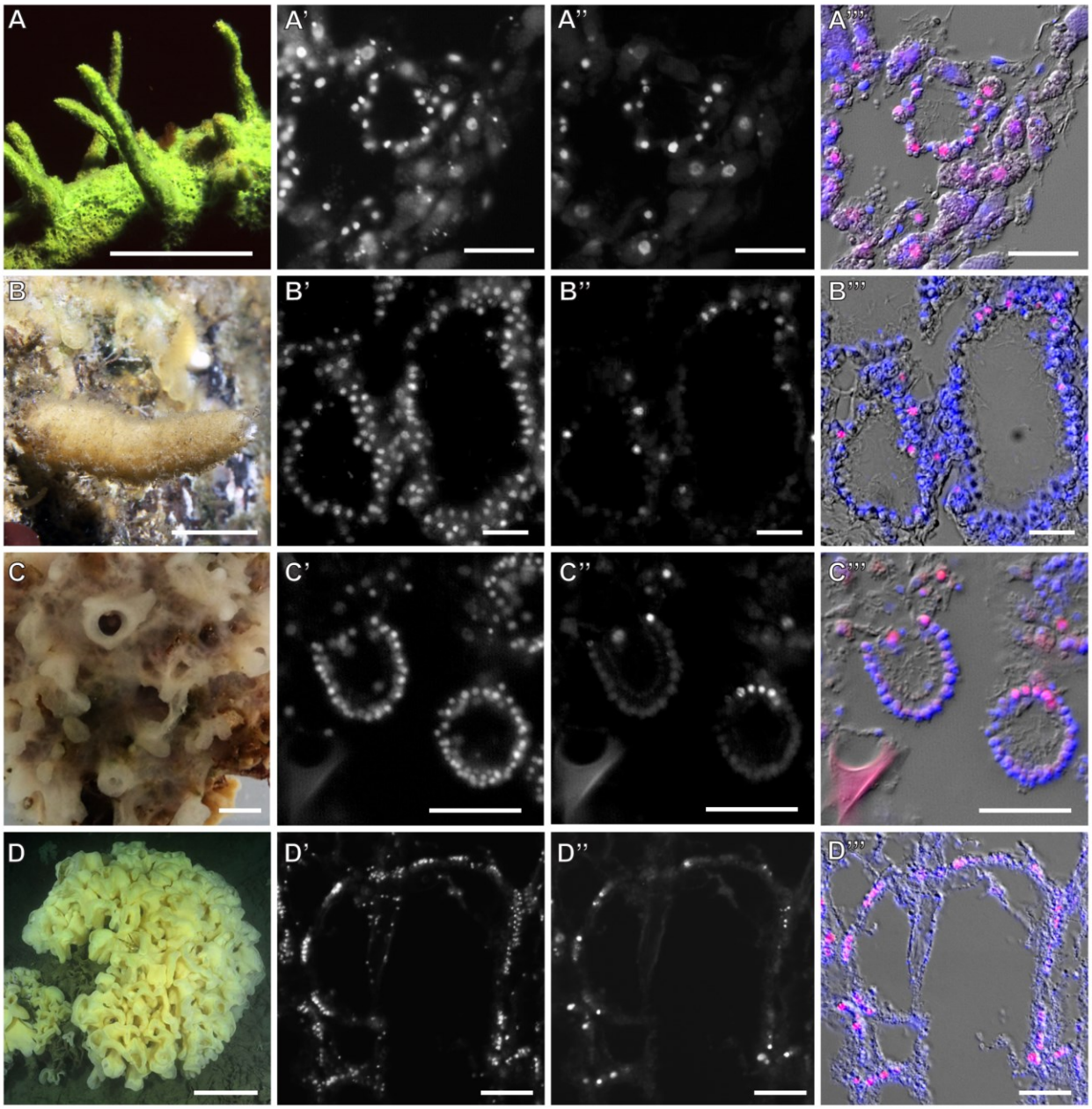

Figure 4.4. EdU labeling in choanocyte chambers from all species of sponges studied

4.3.4. Video microscopy of choanocyte chambers

What remains unclear is how choanocytes proliferate and are replaced in a chamber. Small and large choanocyte chambers were filmed *in situ* in the thin tissue of recently hatched freshwater sponges. At least 4 types of amoeboid cells moved through the mesohyl, each of unique size and activity. Remarkably, chambers moved freely through the mesohyl as a unit until plumbed into a canal and in two separate instances a whole chamber was seen being tugged by a mesohyl cell connected to it by two pseudopodia (Figure S3.1, Supplementary video S3.1). Newly forming chambers (2-6 cells) had replicated by mitosis, but an unusual finding was that larger chambers replaced choanocytes in a different way. Small amoeboid cells moving through the mesohyl, approximately the size of a choanocyte, stopped near large choanocyte chambers and integrated into the chambers, becoming choanocytes (Figure 4.5, Supplementary video S3.2). These 'immigrant' choanocytes represent a previously unknown and yet seemingly common way that choanocytes form and are replaced in mature chambers of cellular sponges.

4.4. Discussion

The discovery of immigrant choanocytes suggests that mesohyl cells are pluripotent stem cells in sponges and that choanocytes in mature chambers are not replaced by mitosis. This finding highlights the complexity of cell-cell interactions in sponge tissues and importantly shows that replacement rates of metazoan tissues cannot be readily compared to the steady states found in cell cultures. Choanocyte turnover rates were not uniform across sponge species and varied from 31 h to over a week, which falls more within the range of cells of other multicellular animals than of single celled protists or cell cultures (Figure 4.6, Table S3.1). Furthermore, our results suggest that cell replacement rates vary among sponge species, with life history stage, and seasonally, but are not immediately affected by feeding activity.

4.4.1. Sources of choanocytes in mature choanocyte chambers

There is surprisingly very little literature providing morphological evidence of whether and how choanocytes divide. For newly forming chambers, choanocytes arise from several divisions of a single archaeocyte (Brien 1976, Tanaka and Watanabe 1984) or from transdifferentiation of larval ciliated epithelial cells into choanocytes, possibly with an intermediate step as archaeocytes (Amano and Hori 1996, Leys and Degnan 2002). Clearly early choanocytes form by mitosis, but there is no evidence that this happens in mature chambers given their small sizes, limited ability to divide because of machinery tied into the flagellum (King 2004), low nucleic acid content, and no visible evidence

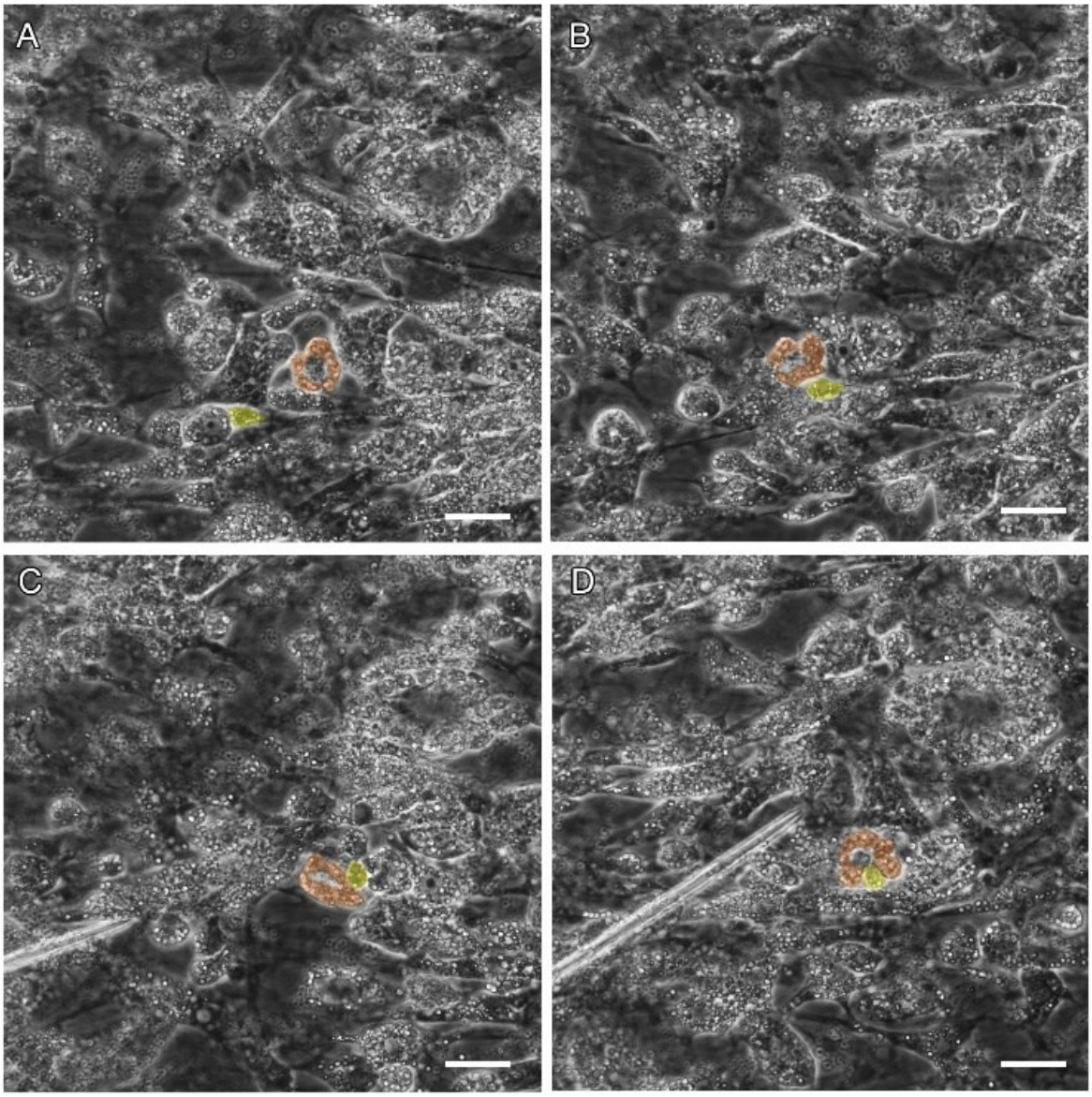

Figure 4.5. Immigration and differentiation of mesohyl cells into mature choanocyte chambers in *Spongilla lacustris*, imaged using *in situ* time-lapse microscopy. A. An amoeboid cell moved through the mesohyl with pseudopodia (pseudocolored yellow). B. The cell slowed and changed directions as it passed two small choanocyte chambers (one pseudocolored orange). C. The cell moved against one of the choanocyte chambers and stopped. D. The cell squeezed between the choanocytes integrating itself into the choanocyte chamber and, as it did so, changed shape to resemble that of other choanocytes in the chamber. This new 'immigrant choanocyte' remained as part of the growing flagellated chamber. Scale bars: 25 cm.

of mitosis despite plenty of cells labeled by EdU. Choanocyte populations are therefore maintained by a stem cell population of mesohyl cells that migrate into chambers to take the place of spent cells. Pinacocytes also likely originated from cells in the mesohyl that migrated to the pinacoderm and then differentiated into pinacocytes (Brien 1976, Simpson 1984). Rates of replacement of choanocyte and pinacocyte populations are therefore at least partially driven by the rate that stem cells from the mesohyl immigrate rather than by direct proliferation.

4.4.2. Variability of cell turnover rates

The variability of cell proliferation rates has significant implications for the extent of ecological effects of sponges on their surroundings. More effort is allocated toward growth during actively growing phases of a sponge's life (Elvin 1976), which shows that cell proliferation is a good indicator of energetic investment in growth and maintenance. Choanocytes were replaced faster in young, growing tissue than in mature tissue, and slower in senescing sponges or in winter months. Choanoblast proliferation in *A. vastus* was also much faster in a growing edge than in mature regions.

Proliferative activity was seasonal in *H. mollis* and varied with life history for other species. Sponges with short life histories (1 year for *S. coactum, S. lacustris* which hatches from yearly overwintering gemmules, and *Hymeniacidon sinapium* (Shore 1971, Eerkes-Medrano and Leys 2006, Cao et al. 2012) had comparable replacement rates of 20-40 h. The relatively rapid cell turnover of choanocytes in these species with a short life history may contribute to their success as seasonal or invasive species (Cao et al. 2012) and may optimize the active season for freshwater sponges (Frost 1982). Temperature may also have an effect – the tropical marine sponge *Halisarca caerulea* had a cell replacement rate of 5.4 h which is much faster than any of the rates we measured for temperate species (De Goeij et al. 2009), although the faster rate may also stem from differences in methodology such as the use of BrdU, which can be teratogenic, mutagenic, and a trigger of cell death (reviewed by Taupin 2007), or by keeping the sponges in food-rich aquaria for a week before beginning incubations.

The construction of glass sponges is very different from other sponges. Collar bodies branching out from choanoblasts may be a way to reduce energetic costs because the collar bodies contain no nuclei and few organelles (Mackie and Singla 1983, Leys 1999); and indeed, very little choanoblast proliferation, a more costly process, was visible in mature tissue and even in the actively growing region. While enucleate collar bodies can be shed, choanoblasts may not need to divide as often in *A. vastus* as in other sponges, reducing the energetic need for DNA synthesis to produce water pumping structures.

Calculating turnover rates from labeled cells assumes that cells in sponge flagellated chambers are in a steady state of cell proliferation and cell death because chambers reach a terminal size (Tanaka and Watanabe 1984). But this assumption could also easily be violated during growth or repair (Simpson 1984). Growing chambers occur when a sponge is first forming but also during rearrangements of the choanosome seasonally and during reproduction (Harrison 1974, Simpson 1984, Gilis et al. 2011). For sponges, it is more appropriate to compare proliferation rate rather than cell cycle length because proliferation rates are independent of any assumptions of a steady state population (Nowakowski et al. 1989). Incubations using a pulse-chase labelling approach could allow one to follow labelled cells over time and learn the relative contribution of stem cell immigration and mitosis by choanocytes.

4.4.3. Choanocyte proliferation rates and feeding activity

Different concentrations of bacteria in the water did not affect the proliferation rate of choanocytes, although the calculated cell turnover rate was slightly shorter in sponges grown in high concentrations of bacteria. Sponges grown in water supplemented with $2x10^6$ cells ml^{-1} heat-killed bacteria produced a cloud of waste material around the osculum (Figure S3.2); but showed no change in cell proliferation so waste material seen here is mostly fecal pellets (Wolfrath and Barthel 1989), mucus, and detritus – not shed cells from rapid cell replacement. The same may be the case in previous studies (De Goeij et al. 2009, Alexander et al. 2014), which could affect estimates of the role of sponges in the 'sponge loop' that were based on those values (De Goeij et al. 2013, Rix et al. 2016).

4.4.4. Ecological implications of variable cell turnover

Variability in cell proliferation between species and within an individual leads us to question the effect sponges have on carbon transfer through cell shedding (De Goeij et al. 2013). Using de Goeij's estimates of cell turnover rate for *Halisarca caerulea* (*Halisarca caerulea*; De Goeij et al. 2009) and Reiswig's standardized dimensions of a choanocyte chamber (57 cells per chamber; Reiswig 1975a), each choanocyte chamber would be completely replaced an astonishing 3 times daily compared to every three days for rates matching *Sycon*, the fastest from our study. EdU-labeled cells or cellular material were not visible in excurrent canals as has been suggested for some species (Donadey 1978, De Goeij et al. 2009, Alexander et al. 2014, Maldonado 2015) and even for those, shed cells do not make up 100% of the detritus. A more likely scenario is that excretion of waste drives the connection sponges make between microbes in the water column and benthic biomass (Wolfrath and Barthel 1989).

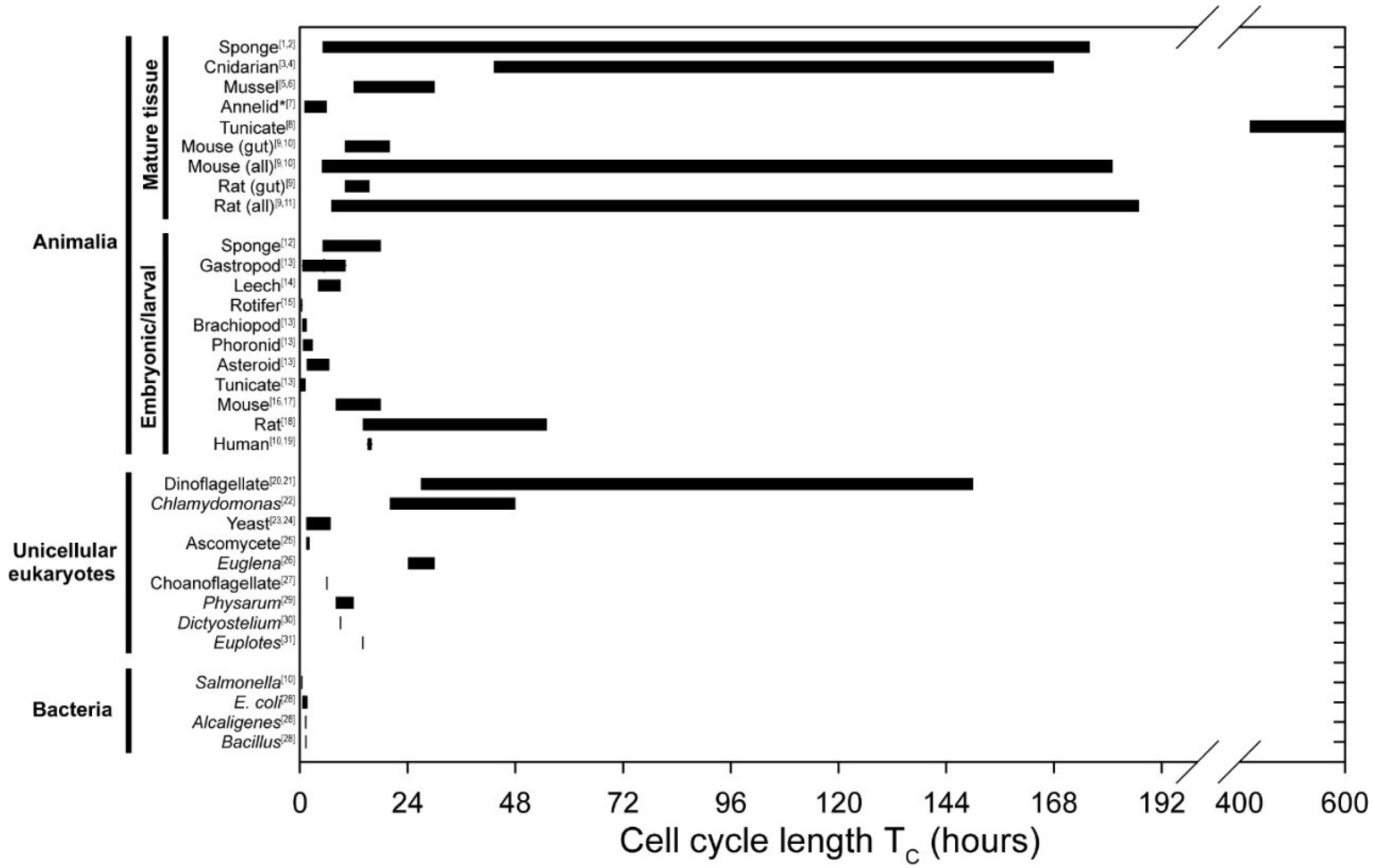

Figure 4.6. Cell turnover rates (T_c) for cell types in unicells and metazoans. Bars show the range reported in hours. Mature animal tissue had a wide range of cell turnover rates but were typically several days longer than turnover rates in unicellular eukaryotes and prokaryotes. A notable exception is embryonic and larval tissue, which has much shorter cell turnover rate. (Reference values are provided in Appendix 3).

Changes in cell proliferation rates reflect a physiological plasticity to changing conditions – a sponge may increase choanocyte proliferation rates during food-rich seasons. This physiological plasticity is known in both single-celled protists (Olson and Chisholm 1986, Zachleder and Van Den Ende 1992) and cells of other metazoans (e.g. Otto and Campbell 1977), presumably to allow energy to be available for other processes of the body.

4.5. Summary

The cells of a sponge function as a cooperative unit to grow, feed, reproduce, and senesce. Results presented here show that choanocytes are not actually stem cells in sponges; instead, cells from the mesohyl immigrate into chambers, and importantly immigration drives rates of replacement of choanocyte and pinacocyte populations. Short term feeding activity has little effect on cell loss or replacement but season (growth and metabolic activity seasonally), life history stage, and possibly water temperature (warm water versus temperate habitats) have the greatest effects on cell replacement rates. Our results demonstrate the complexity of estimating the ecological effects of choanocyte proliferation. If proliferation rates are not constant, nor as highly prolific in some species compared to others, then the recent quantification of the 'sponge loop' needs reconsideration. Variability in proliferation and evidence of stem cells for choanocytes suggest that tissue homeostasis has been an important factor to multicellularity for as long as animals were multicellular.

4.6. Acknowledgements

We thank the director and staff at the Bamfield Marine Sciences Centre, A. Oatway, and G. Huang for assistance with microscopy and flow cytometry, and K. Juniper for access to the MTC in Sidney BC. Pinnacle Divers of Victoria, the pilots of the ROV ROPOS, K. Anderson, S. Anthony, K. Kruper, D. Ludeman, N. Trieu, and N. Webster provided field and lab assistance. Funding for this research came from NSERC Discovery and Ship Time grants to SPL; NSERC Vanier Canada Graduate Scholarship to ASK; Bank of Montreal Graduate Student Scholarship, WCUMSS, and Donald M. Ross Scholarships to ASK.

Chapter 5. Feeding and excretion by the glass sponge *Aphrocallistes vastus*

5.1. Introduction

In Chapter 2 it was found that sponges contribute to nutrient cycling by bringing microbial food energy from the microbial loop into the larger trophic web (Gili and Coma 1998). Glass sponges in the deep water sponge reefs of western Canada have the highest benthic grazing rate of any suspension feeding community measured to date, affecting bacteria concentrations in the overlying water but also removing seven times more carbon than can be provided by vertical flux alone (Chapter 2, Kahn et al. 2015). Trophic subsidies must provide the remainder of the carbon needed but where these subsidies come from is not known. How, and from where, do glass sponge reefs get enough bacteria to sustain such high densities? Some demosponges supplement their diet with different food sources, using symbionts that take up DOC (De Goeij et al. 2008b) or that photosynthesize (Frost and Williamson 1980), but reef species do not.

Feeding by glass sponge reefs nevertheless can affect the overlying water column, with sponges reducing bacteria concentrations up to 5 meters above the reef through their feeding (Chapter 2, Kahn et al. 2015). Water currents accelerate over the reef (Bedard 2011), which may enhance feeding through use of current-induced flow (Leys et al. 2011) and make trophic subsidies from lateral currents possible. The intense grazing by glass sponge reefs may also have downstream effects on local communities if their feeding activity packages bacterial carbon into larger waste (Wolfrath and Barthel 1989). Food energy could also be incorporated into skeletal and tissue biomass that can build reef and provide food for predators of sponges (McClintock 1987, McClintock et al. 2005, Chu and Leys 2012).

Sponge reefs lie on the continental shelf so they may receive bacteria from various sources including fallout from surface plankton blooms, tidally driven currents, or resuspension of sediments. Tidal currents bring deep water known to fuel upwelling-driven phytoplankton blooms from the Strait of Juan de Fuca (Yin et al. 1997, Masson 2002, Masson 2006). Local phytoplankton blooms can be fueled by nutrients from the Fraser River, which provides 68% of freshwater to the SoG and discharges terrestrially derived sediments and organic carbon seasonally (Johannessen et al. 2003, 2005, 2008) and mixes with ocean water to produce bacterioplankton blooms (Albright 1983). Considerable bacterial productivity also occurs in sediments, which can have bacteria concentrations 100 or 1000 times greater than in the water

column (Kuwae and Hosokawa 1999) and which has been suspected to feed other dense deep-water sponge communities (Rice et al. 1990).

We studied uptake and excretion of food by reef sponges (*Aphrocallistes vastus*) to determine the source of food, where food is captured and processed by the sponge, and to characterize material excreted from the sponge as a potential food source for other animals. Understanding the feeding behavior and sources of food may help explain why reefs are found where they are, and may lead to predictions of where other sponge beds may live globally and why those are good sponge habitats.

5.2. Methods

5.2.1. Particle uptake and excretion

Pieces of sponge from individuals of *Aphrocallistes vastus* were collected during research cruises on the Canadian Coast Guard Ship Vector in 2011, 2013, and 2014 using the remotely operated vehicle ROPOS (http://www.ropos.com/). Sponges were transferred to Bamfield Marine Sciences Centre without removal from seawater (at 9°C) and once there, maintained in flow-through aquaria that were continually refreshed with water from 30 meters depth.

To identify particle capture and feeding, 2 x 5 cm pieces of sponges were placed into a 2 L container of seawater and either latex beads (0.1, 1.0, and 3.0-µm diameter; Fluoresbrite microspheres in red, Polysciences, Inc.) or heat-killed *Roseobacter* bacteria were added to a concentration of 2 x 10^6 particles ml^{-1}. Containers were kept in a 9°C incubator during feeding incubations to maintain constant temperature. After 15 min, 1 h, 8 h, and 24 h, several small pieces were preserved for electron microscopy.

To collect excreted material, we tried several methods. Initially, large branches of sponges (approximately 10x10x2 cm and each with an osculum) were incubated in 2 x 10^6 of a mixture of 0.1- and 0.5-µm beads ml^{-1} overnight, then rinsed in filtered seawater and placed in 3-L containers of filtered seawater for 24 h. The water in the containers was concentrated on a 0.2-µm Durapore filter and examined under a fluorescent microscope. Although the sponge tissue appeared the pink or yellow color of fluorescent beads used, very few beads were captured on the filter. Because the sponges did not filter in still water of closed containers, we then placed bead-fed sponges in flowing water oriented with oscula facing down into funnels lined with filters. Because of their large size and asymmetrical shape these individuals often fell off of the tubes or tissues abraded into the funnels. Finally, filters pinned across the osculum allowed

sponges to be oriented any direction in the flowing seawater but either had too large a pore size to capture the fluorescent beads (Nitex mesh) or had such small pore sizes that too much pressure was needed to push water through them (0.2-µm or 0.45-µm Durapore filters). Fine filters may also have impeded pumping and flow by the sponges. The method which we report data from followed that of Wolfrath and Barthel (1989) and used smaller fragments of sponge. Pieces of sponge were immersed in a mixture of 0.1 and 0.5 µm Fluoresbrite fluorescent beads at a concentration of 2×10^6 beads ml^{-1} for 2 h. They were then rinsed in seawater and inverted over 50 ml tubes to collect excreted material. Pieces of sponge were left for 24 h suspended above the tubes. The contents – material released by the sponge – were allowed to settle for 1 h. Water was carefully siphoned off until 2 ml of seawater and any excreta it contained was fixed with 1% osmium tetroxide. After 30 min, the fixative was changed to a cocktail of 1% osmium tetroxide and 2% glutaraldehyde in 0.45 M sodium acetate (pH 6.4) buffered with 10% sucrose (Harris and Shaw 1984).

Small pieces of tissue were preserved for electron microscopy in the cocktail fixative described above. The fixative was refreshed after 30 min, then left overnight at 4°C. Tissue was dehydrated to 70% ethanol, desilicified in 4% hydrofluoric acid overnight, and stored in 70% ethanol until further processing. Tissue was dehydrated to 100% ethanol, freeze-fractured in liquid nitrogen, and critical point dried using a Bal-Tec CPD 030 critical point dryer in the Scanning Electron Microscope Laboratory at the University of Alberta. Fractured, dried tissue fragments were mounted on metal stubs, sputter coated with gold (Nanotek SEMprep 2 sputter coater), and viewed using a JEOL 6301F field emission scanning electron microscope. Excreted material was processed following the same method except it was not desilicified or freeze fractured.

5.2.2. Particle and carbon transport

To further track carbon uptake and transport, a sponge was immersed in seawater supplemented with heat-killed bacteria that had been grown in ^{13}C D-glucose as its food source kindly provided by Rebecca J. Case (RJC) and Anna R. Bramucci (ARB). In brief, Rhodobacteraceae bacteria *Ruegeria* sp. R11 were grown in a shaking incubator (30°C at 160 rpm) to early log phase in ½ marine broth (24 h) then re-cultured at a 1:10 inoculum into ½ marine broth supplemented with ^{13}C-labeled D-glucose (1 g L^{-1}) as a sugar source. Bacteria were cleaned into sterile marine media, concentrated, and heat killed in a 55°C water bath for 1 h. Dead cultures were verified by plating the cultures and incubating at 37°C overnight to ensure that no colonies grew on the plates.

A large piece of sponge (10 x 20 cm) was incubated in 2 x 10^6 cells ml^{-1} ^{13}C-labeled bacteria heat-killed bacteria for 8 h. Three pieces from different regions of the sponge were also rinsed in distilled water and frozen as pre-treatment controls. Triplicate pieces were collected at 1, 2, 3, 4, 5, 10, and 14 d post-labeling (dpl) and unfed control pieces were collected at the conclusion of the experiment to identify any drift in the isotopic signature of the sponge. Each of the samples was rinsed in distilled water, wrapped in foil, and frozen (-80°C).

All tissue pieces were lyophilized for 24 h in a Virtis Freezemobile FM25XL lyophilizer and crushed to a homogeneous powder with an agate mortar and pestle. Each tissue powder sample was weighed (6 ± 0.001 mg) into tinfoil discs and compacted. Samples were analyzed for $\delta^{13}C$ in the Stable Isotope Facility in the Natural Resources Analytical Laboratory at the University of Alberta following internal protocols. In brief, samples were combusted under oxygen, separated chromatographically, and then analyzed using a Continuous Flow Isotope Ratio Mass Spectrometer (CF-IRMS, ThermoFinnigan Delta+ Advantage). Quality control standards from NIST were run for each isotope (for ^{13}C: NBS22, LSVEC, and NBS19; for ^{15}N: IAEA-N1, IAEA-N2, and IAEA-N3). $\delta^{13}C$ and $\delta^{15}N$ values (ppt, ‰) are presented as ratios of the heavy to light isotope of the standard compared to the Pee-Dee Belemnite for $\delta^{13}C$ and air for $\delta^{15}N$ (Equation 1).

$$\delta^n x\ (‰) = \frac{(\delta^n x_{sample} - \delta^n x_{std})}{\delta^n x_{std}} \times 1000 \qquad \text{Equation 1}$$

5.2.3. Carbon sources for the reefs

To trace carbon before and after it passed through reef sponges in the Strait of Georgia (SoG), particulate organic matter (POM) from water, sponge tissue, and sediments were collected between 2007 and 2014 during research cruises on the CCGS Vector using the remotely operated vehicle ROPOS from glass sponge reefs (Figure 5.1). Values from 2007 and 2009 were obtained from the thesis of JWF Chu (Chu 2010). Samples were collected in different locations and in different years (Table 5.1). Water was collected from within the reef using a suction sampler and Niskin bottles mounted on the ROV. Sponge tissue was collected using a suction sampler mounted on ROPOS. Sediments were collected from among the sponges using push cores deployed by the ROV.

Four liters of each water sample were filtered through pre-weighed, pre-combusted (500°C for 12 h) glass fiber filters (GF/F, nominal pore size: 0.7 µm). Filters were rinsed with distilled water, folded into pre-combusted foil packets, and flash frozen in liquid nitrogen. Sponge samples were rinsed three times with distilled water, packed in tinfoil, and flash frozen in liquid nitrogen. Surface sediments from the top of each push core were scraped into two 2-ml

cryovials and flash frozen in liquid nitrogen. All samples were transported to the University of Alberta on dry ice and stored in a -80°C freezer until further processing.

Tissue and filters were lyophilized for 24 h, sediments for 48 h in a Virtis Freezemobile FM25XL lyophilizer. Once dried, all samples from 2007 and 2009 were processed as described by Chu (2010). Samples collected in 2011 and 2014 were ground to a fine powder using an agate mortar and pestle, then loaded into tin capsules (tissue: 6±0.001 mg, sediments: 40 mg) and delivered to the Natural Resources Analytical Laboratory for isotope analysis as described above.

5.3. Results

5.3.1. Particle uptake

Particle uptake by the sponges was visibly evident as the water changed from cloudy to clear during the 1- and 8-h incubations (Figure 5.1A). Sponge tissue also turned a mottled pink color during incubations in pink beads – some regions of the tissue were brilliantly colored while others were still the original yellow color of the sponge. More chambers containing 0.1 μm beads were found than chambers containing 1.0 μm beads. The largest size beads, 3.0 μm, were not found in any SEM samples, though a few were visible in sponge tissue embedded in JB-4 plastic resin.

Beads and bacteria were found in various structures in and around the flagellated chambers, beginning with canal spaces outside of the chambers and the entrances into the chambers (prosopyles) in the shortest incubations (Figure 5.2A-B). Beads were found in extensions of the primary reticulum (Figure 5.2C). Both bacteria and 0.1- and 1.0-μm beads were found in both the primary and secondary reticula and in reticula that bridged the two (Figure 5.2B-D).

5.3.2. Particle and carbon transport

Particles were found in the primary and secondary reticula of flagellated chambers within 15 min of incubation. Phagocytic vesicles were still visible in the primary and secondary reticula after 8 h (Figure 5.3A-B) but vesicles were also found more distant from chambers, in the trabecular syncytium (Figure 5.3B-D). Bacteria were still whole and undigested in vesicles 8 h after incubations began (Figure 5.3C-D).

The sponge tissue fed ^{13}C-enriched bacteria showed a similar pattern of transport. There was a large spike in δ^{13}C after the initial feeding (Figure 5.4). Different regions of tissue showed high variability in δ^{13}C for the first 5 d post feeding. After that, δ^{13}C values had lower variability after 10 and 14 d (Levene's test, F=0.118, p=0.051) and were unchanged from the initial spike

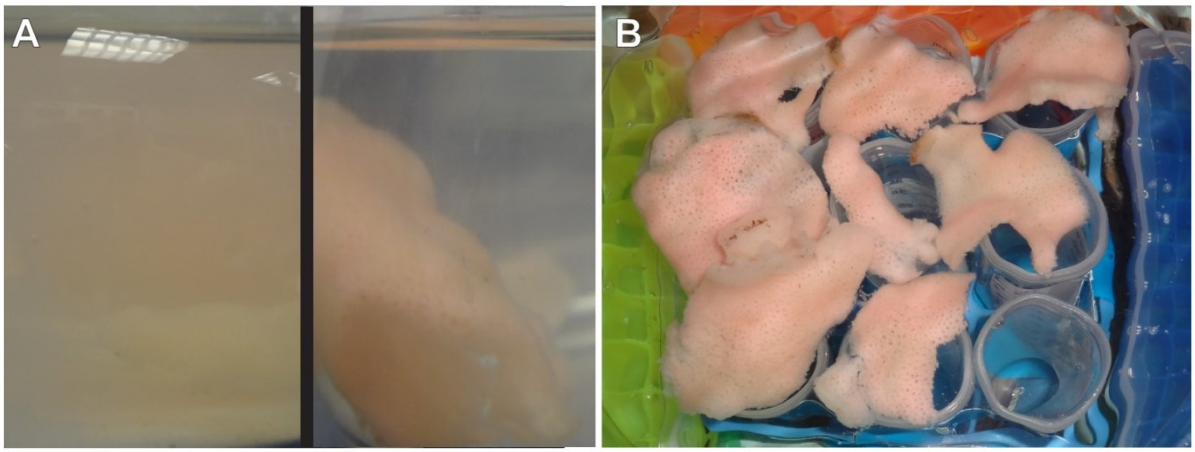

Figure 5.1. Experimental setup for observing particle feeding and excretion by *Aphrocallistes vastus*. A. Sponges were incubated in bacteria, 0.1-, 1.0-, and 3.0-µm latex beads. Water was initially cloudy (left) but became less cloudy as beads were taken up into the tissue (right). B. Bright pink sponges, colored from eating red fluorescent beads, were left suspended over 50-ml tubes overnight to expel any waste material. A negative control is visible for the tube in the lower right.

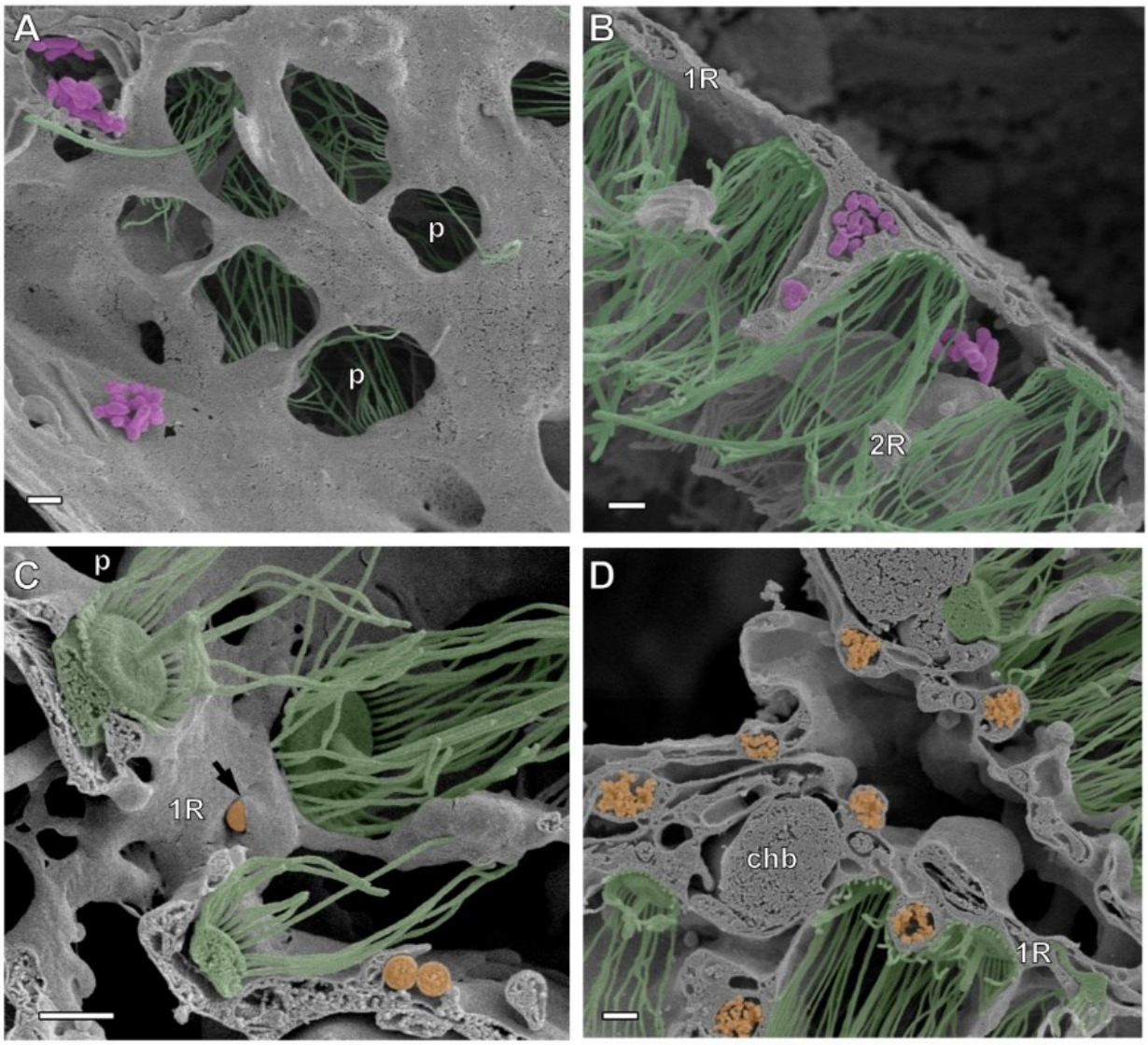

Figure 5.2. A morphological study of particle uptake in the primary and secondary reticula of *Aphrocallistes vastus*. A. Bacteria (pseudocolored pink) were visible outside of prosopyles (p) 10 min post-feeding and B. in the spaces between the primary (1R) and secondary (2R) reticulum at all times post-feeding. Sponge fixed 8 h post-feeding. C. Beads (pseudocolored orange) could be seen being enveloped by small extensions of tissue (arrows) between the collar bodies (pseudocolored green) after 10 min. D. Occurrences of phagocytic vesicles increased as incubations grew longer. Sponge fixed 8 h post-feeding. Also shown: choanoblasts (chb), collar bodies (cb). All scale bars: 1 μm.

Figure 5.3. Late stages of particle capture by *Aphrocallistes vastus*. A., B. 0.1-µm beads (pseudocolored orange) were found in phagocytic vesicles below the primary reticulum. Sponges preserved 1 h post-feeding. C. Over time, phagocytic vesicles filled with undigested bacteria (pseudocolored pink) were visible slightly away from the flagellated chambers behind choanoblasts (chb) and far from the water pumping collar bodies (pseudocolored green). Sponge preserved 8 h post-feeding. D. Eventually, packets of undigested bacteria were moved through the trabecular syncytium (t). Sponge preserved 8 h post-feeding. E. and F. Mucus- or membrane-bound clusters of beads gave evidence of fecal pellets from the sponge that were 100 to 1000 times greater volume than each bacterial particle consumed. Scale bars: for A, B, C, D: 5 µm. For E, F: 1 µm.

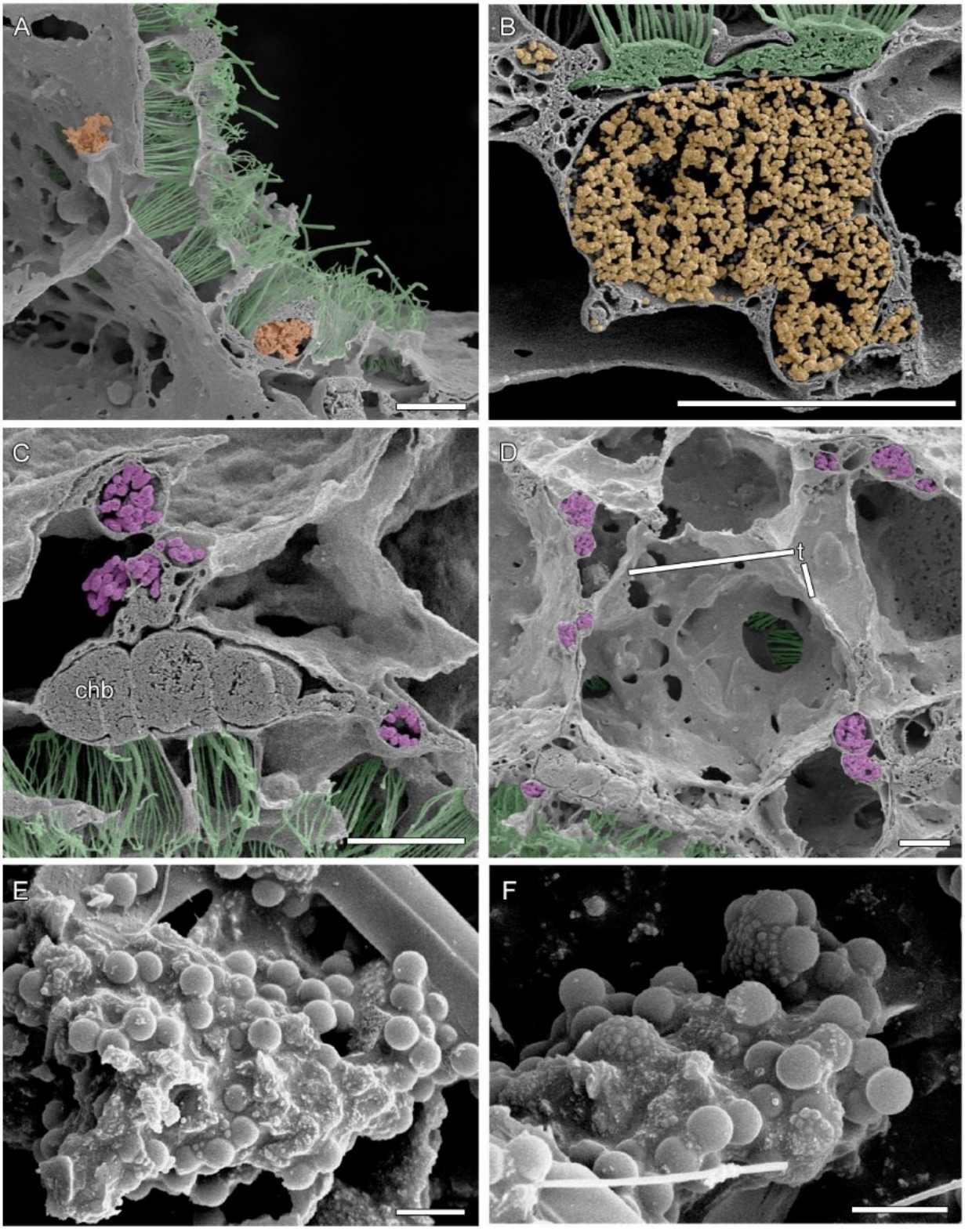

Figure 5.3. Late stages of particle capture by *Aphrocallistes vastus*

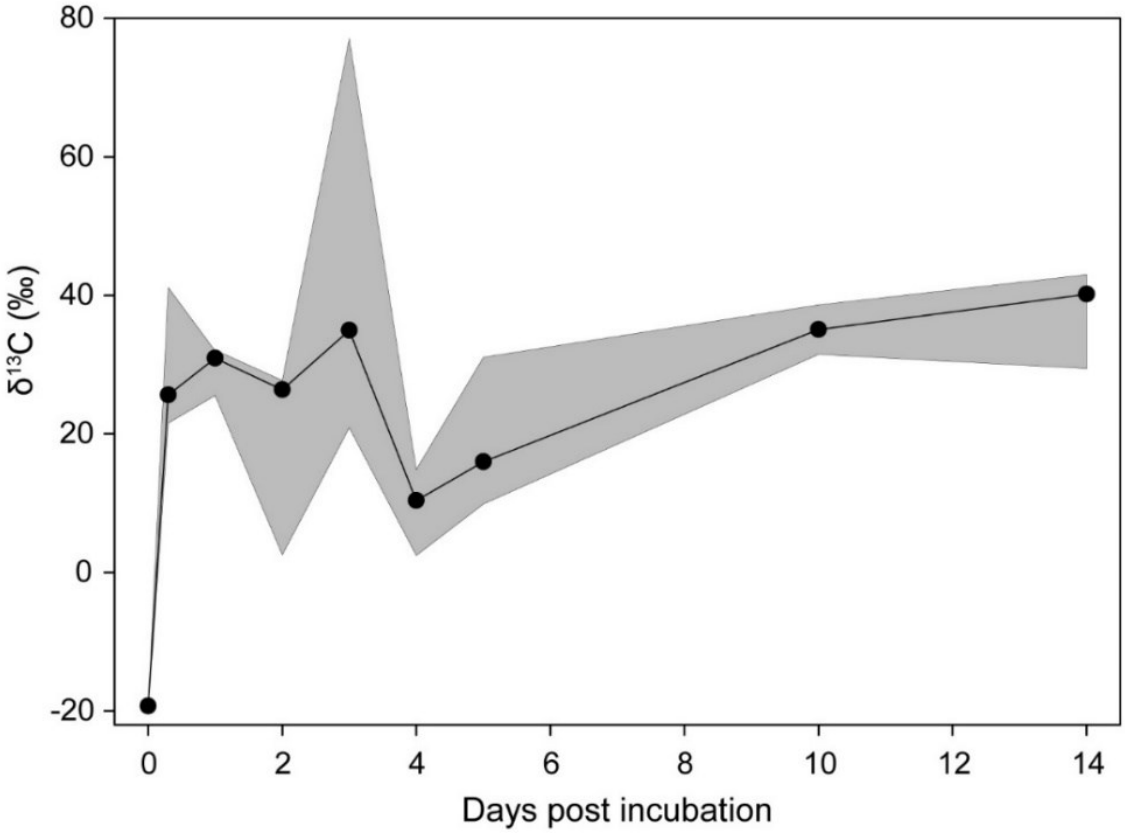

Figure 5.4. Changes in carbon-13 isotope signatures in tissue of *Aphrocallistes vastus* incubated in water supplemented with ^{13}C-labeled bacteria. Sponges at zero d post-incubation were control sponges that had not been fed ^{13}C-labeled bacteria. Subsequent days post-incubation followed sponge tissue that had been fed ^{13}C-enriched bacteria. Black points indicate the mean; gray shading indicate the minimum and maximum δ^{13}C values measured for that time point.

($F_{7,15}$=2.096, p=0.109). There was no decrease in $\delta^{13}C$ over the 14-d experiment (linear regression, slope=0.78±0.94), so the carbon that was assimilated from the bacteria was not excreted or discarded during that time.

5.3.3. Waste excretion

Excreted material collected from sponge pieces suspended over tubes consisted of membrane-bound or mucus-coated clusters of latex beads (Figure 5.3E-F); no similar material was found in control tubes without sponge pieces. Mucus-coated waste packets were ellipsoid, 1031±1178 μm^3 in volume (mean±95% confidence interval (CI); n=10) and were found in larger mucous clusters containing shards of diatom frustules, radiolarian tests, and other unidentifiable detritus. No discarded collar-flagella units or any other recognizable tissues from the sponge were visible.

5.3.4. Food sources to the glass sponge reefs

Isotope signatures in sponge tissue differed between years and locations sampled (Table 5.1, Table S4.1). Only Fraser and Galiano reefs were compared across years for four separate years; Howe reef was only sampled twice and the non-SoG sponges, once. There was no consistent pattern in the changes between years between Fraser and Galiano reefs (Table S4.1). Sponges from Fraser and Howe reefs had significantly lower $\delta^{13}C$ and $\delta^{15}N$ ratios than sponges from Galiano reef and those collected outside of the SoG (Figure 5.5).

The variability seen between years and between reefs was less than the difference of the three sample types: sponge tissue, sediments, and POM. Post hoc Bonferroni comparisons indicated that the mean $\delta^{13}C$ for sponge tissue (range: -21.31 to -19.25‰) was more similar to sediment (range: -23.80 to -19.73‰; p=0.034) than it was to POM in the water filtered by the sponges (range: -25.22 to -22.49‰; p<0.005). In contrast, $\delta^{15}N$ rations in sponge tissue (range: 14.35 to 17.52‰) were more different from those in sediment (range: 4.34 to 10.12‰; p<0.0005) than POM (range: 5.33 to 11.97‰; p=0.042). Large variability meant there was no statistically significant difference between $\delta^{15}N$ values for POM and sediment, but there was a difference in the $\delta^{13}C$ values (p<0.0005).

The fractionation and trophic position of the sponges could not be calculated because $\delta^{13}C$ and $\delta^{15}N$ ratios for the sponges did not fall between the ratios of sediments and POM (Post 2002). POM was the most depleted of ^{13}C followed by sediment and then sponge tissue.

Table 5.1. $\delta^{13}C$ and $\delta^{15}N$ values (‰) measured from Fraser, Galiano, and Howe Reefs, and from outside of the Strait of Georgia. *Aphrocallistes vastus* were sampled between 2007 and 2014 to assess spatial and temporal differences in isotopic signatures. POM was collected as a potential food source for the reefs in 2009, and sediments were sampled in 2011 and 2014 to assess whether sponge-derived material (fecal pellets) were detectable.

				$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)		
Location	Latitude	Longitude	Year	Mean	SD	Mean	SD	n
Sponge								
Fraser	49°09.485'N	123°23.081'W	2007	-21.19	0.08	14.74	0.31	5
			2009	-20.26	0.11	14.74	0.30	10
			2011	-20.45	0.40	15.64	0.45	9
			2014	-20.23	0.13	15.31	0.19	5
Galiano	48°54.477'N	123°19.168'W	2007	-19.80	0.22	14.96	0.30	5
			2009	-19.55	0.14	17.01	0.33	10
			2011	-19.86	0.15	16.43	0.53	11
			2014	-19.32	0.06	16.11	0.25	5
Howe	49°19.901'N	123°17.647'W	2009	-20.28	0.19	15.21	0.50	10
Outside SoG*			2008	-19.68	0.13	16.22	0.44	6
POM								
Fraser			2009	-23.84	1.08	9.63	2.08	9
Galiano			2009	-23.09	0.69	8.46	0.97	9
Howe			2009	-23.51	0.70	10.23	0.96	10
Sediments								
Fraser			2011	-21.42	0.58	7.29	1.80	5
			2014	-21.69	1.18	4.80	0.32	5
Galiano			2011	-20.66	0.63	6.84	2.29	5
			2014	-21.69	1.18	4.80	0.32	5

*Two locations were sampled outside of the Strait of Georgia: Barkley Sound (48°54.262'N, 123°02.637'W) and offshore of the Washington Coast (48°22.676'N, 123°03.056'W).

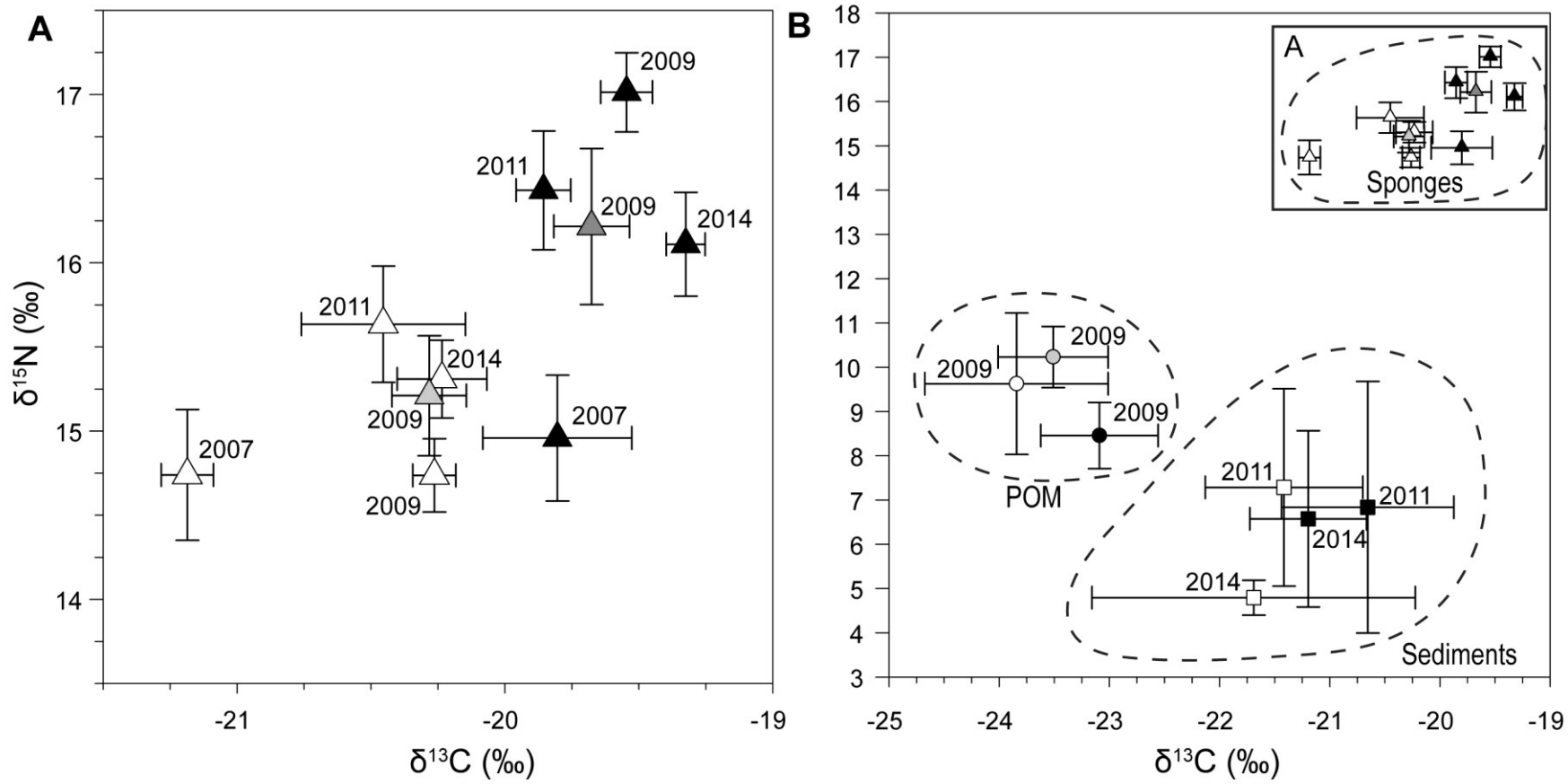

Figure 5.5. Carbon and nitrogen isotopic signatures from several glass sponge reefs over four years. A. Sponge $\delta^{13}C$ and $\delta^{15}N$ isotopic signatures (‰) from Fraser Reef (white), Howe Reef (pale gray), Galiano Reef (black), and Non-Strait of Georgia sponges (dark gray). The year each sample was collected is indicated. B. Particulate organic matter (POM, ●), sponges (▲), and sediments (■) from the same reefs. A rectangle outlines the region of the plot that is magnified in A. Data from 2007 and 2009 come from Chu (2010).

5.4. Discussion

The process of feeding, from particle capture to excretion, in *Aphrocallistes vastus* was studied to understand how food was captured and altered by sponge digestion. Stable isotopes were also used to identify sources of food, which may help explain how glass sponges can form dense reefs. Particle capture occurred on the primary and secondary reticula as has been observed in other glass sponges (Perez 1996, Wyeth et al. 1996, Wyeth 1999) but two new elements were observed: 1) food eaten by the glass sponge was transported elsewhere in the tissue before being digested, and 2) the carbon assimilated from food was detectable in the tissue up to two weeks later. Digested material was released as fecal pellets that were several times larger than the particles consumed. Efficient particle capture and processing likely couple with trophic subsidies to support high densities in the reefs. Stable isotope data suggest that the food ingested by the sponges may come from both terrestrial and oceanic sources, with different contributions of each to different reefs.

5.4.1. Feeding and excretion

The smallest structure that water must pass through in the aquiferous system is consistent across many sponge taxa and is 20-70 nm glycocalyx mesh between the microvilli of collars on choanocytes, the water pumping and feeding cells, (*Spongilla lacustris*, Fjerdingstad 1961, *Aphrocallistes vastus*, Leys et al. 2011). It is therefore not surprising that the smallest particles fed to the sponges in this study, 0.1 μm diameter, were captured. Sponges from the classes Calcarea and Demospongiae have also been known to capture particles of that size (Gobel 1993, Leys and Eerkes-Medrano 2006). That they were not seen captured in the glass sponge *Oopsacas minuta* is more likely a sign that they were missed rather than that the species could not capture the particles (Perez 1996). Particles appeared to be taken up by extensions of the syncytial tissue, similar to the way choanocytes were interpreted to engulf particles in *Sycon coactum* (Leys and Eerkes-Medrano 2006)

In sponges incubated in beads or bacteria for 1 h or longer, particles were found in non-feeding parts of the tissue, if transported they were likely moved there by cytoplasmic streaming through the trabecular syncytium, which allows movement of cytoplasm, organelles, and phagocytized particles through the tissue along microtubule tracts (Leys 1995). In cellular demosponges of the genus *Aplysina*, similar transport was accomplished by migratory cells moving through special strands in the tissue (Leys and Reiswig 1998). The static images suggest that feeding by the glass sponge appears to be partitioned; particle capture (uptake) occurs in the primary and secondary reticula while digestion involves transport of phagocytic vesicles to other regions of the body.

Beads released by the sponge resembled fecal pellets found from demosponges (Wolfrath and Barthel 1989, Gobel 1993); they were the same size as those of *Halichondria panicea* and appeared to have a similar mucous or membrane-bound coating. Many attempts to capture fecal pellets released by the sponge have led to the conclusion that it is not possible to quantify for several reasons. Glass sponges would not pump, and so release fecal pellets, in chambers of water left overnight. They required flowing seawater, which was impossible to filter and remove other particulates from the water at such high volumes. Copepod fecal pellets, and likely excreta from other animals, were recognizable in negative controls of the samples that were collected for electron microscopy. The fecal pellets of other animals did not have beads in them because they had not been incubated in beads like the sponges had, but nevertheless any attempt to quantify carbon content in excreted material would capture that excreted material, along with whatever else is released by the sponges (for example, mucus, spicules or tissue that had been abraded off of the sponge).

The volume of an average-sized fecal pellet collected from *A. vastus* was 1031 ± 730 um^3 (mean±95% CI). The same volume of 1-um^3 bacteria from small coastal bacterial communities with relatively low C content (30.2±12.3 fg C $cell^{-1}$; (Fukuda et al. 1998)) could hold 31 μg of carbon. Assuming that only 10% of carbon from those bacteria are released as waste, each fecal pellet could have housed up to 3.1 μg of carbon derived from microbes. This calculation is an underestimate because the bacteria would likely not be 1 um^3 upon excretion, but it highlights the potential for sponge excretion as a means of transporting carbon from the microbial loop into the benthos. Fecal pellets are 100 to 1000 times the volume of the food they derive from, with importance to pelagic-benthic coupling similar to that of zooplankton fecal pellets on the biological pump (Turner 2015). Producing fecal pellets involves extruding particulates through membrane-bound vacuoles, which requires production of cellular material (Willenz and van de Vyver 1986) that might further increase the carbon output from sponge excretion and use energy. Waste excretion, with its agglomerated food waste and extruded membranes of fecal pellets, is therefore a possible way that sponges transfer carbon to benthic biota.

5.4.2. Carbon assimilation into sponge tissue

The sponge incubated in water supplemented with ^{13}C-labeled bacteria showed a variable but sustained increase in $\delta^{13}C$ after feeding with no decrease of $\delta^{13}C$ over the course of the 14-d experiment. The high variability in $\delta^{13}C$ values from the same time point indicate that not all areas of the sponge were feeding at the same time or rate. This was also evident from the patchy color changes caused by the pink beads for *Aphrocallistes vastus* and *Rhabdocalyptus dawsoni*, and for pigmented algae in *Oopsacas minuta* (Perez 1996, Wyeth et al. 1996, Wyeth 1999). That

some chambers captured particles while others did not may mean that not all chambers were feeding at the same time or that the solution was not well mixed. Over time, however, the variability of $\delta^{13}C$ values decreased, likely as new carbon was distributed throughout the tissue by cytoplasmic streaming (Leys 1995).

Isotope ratios in tissue are affected by the turnover rate in that tissue. For example, red blood cells reflect isotopic signatures from food eaten over several days while bones show the signature of food ingested over a year or more (Gannes et al. 1997). Sponge tissue showed retention of ^{13}C from labeled bacteria for a full 14 d, despite the release of fecal pellets after 24 h in other feeding incubations. It is possible that the nondigestible latex beads were expelled more quickly as something akin to pseudofeces whereas the bacteria were assimilated into tissue as usable carbon. Unchanged ^{13}C values also mean that isotope values measured from glass sponges in the field did not reflect their diet from the past few days, but instead reflected a longer timescale of feeding.

5.4.3. Food sources to different reefs

Differences in carbon isotope signatures between reefs reflected the oceanography of the region. Fraser and Howe reefs had similar $\delta^{13}C$ values that were ^{13}C-depleted relative to Galiano Reef and the non-SoG locations. Depleted $\delta^{13}C$ values suggest a more terrestrial food source at Fraser and Howe than at Galiano and non-SoG locations and indeed, freshwater discharges from cities and rivers flow directly over Fraser and Howe reefs (Johannessen et al. 2005) while Galiano lies further from any terrestrial discharges, therefore receiving a greater proportion of its carbon from oceanic plankton.

Nitrogen isotope signatures of sponges were 5-8 ‰ $\delta^{15}N$ higher than those of POM from the water, and higher still than $\delta^{15}N$ isotope signatures of sediments. Hexactinellid sponges in the Porcupine Abyssal Plain (PAP) also had a very high $\delta^{15}N$ – the highest of many taxa investigated (Iken et al. 2001). The copepod *Metridia pacifica* from the Strait of Georgia had a similar 6 ‰ gap between themselves and POM, which was attributed to its omnivorous diet (El-Sabaawi et al. 2010). The high trophic level of the sponges here and in the PAP could also be partly reflective of their diet, which is preferentially selective on heterotrophic bacteria (Yahel et al. 2006). If bacteria could have been separated from the rest of POM, it is possible that bacteria may have filled the 5-8 ‰ gap between POM and the sponges, although other factors can also raise $\delta^{15}N$ values, such as nitrogen from agricultural runoff (McClelland et al. 1997), coprophagy (Fenolio et al. 2006), and starvation (Hobson et al. 1993, Cherel et al. 2005).

The closest carbon isotope signature to sponge tissue was found in sediment. We had originally thought that isotope signatures of sediments might reflect sponge excretion but the

$\delta^{15}N$ could indicate the opposite: that sponges were a higher trophic level than the material in the sediments. Alternatively, sediments could reflect POM deposition or other confounding factors as well, so further investigation is needed. Still, could sponges feed on bacteria in resuspended sediment? $\delta^{13}C$ values between POM, sponges, and sediment support the hypothesis that sediments may contribute to the diet of the sponges, turning the 'sponge loop' upside down and underground. If sponges do eat bacteria from the sediments, they may draw carbon from the seafloor back up into the water column through resuspension. The reefs may even facilitate sediment resuspension by disrupting laminar tidal flows and causing turbulent mixing. Some sponge reefs in the Strait of Georgia, including Fraser Ridge Reef, are more murky than their surroundings – so much so that decreased visibility was one indication used to know the ROV was approaching the reefs during dives. A suite of instruments mounted to the remotely operated vehicle ROPOS during a survey across Fraser Ridge Reef demonstrated this, with lower transmittance and higher fluorescence in water overlying sponges than in an adjacent sponge-free patch (Figure 5.6). Resuspension has been implicated as an important food source for animals in other systems (Yahel et al. 2008) and even for dense glass sponge aggregations in the northern Atlantic (Rice et al. 1990). Sediment-borne bacteria outnumber bacteria in the water column by 100 to 1,000 times (Whitman et al. 1998) so it will be important to determine the relative contributions of sediment-borne bacteria and pelagic bacteria in the future.

Results presented here suggest that glass sponges in the reefs receive plenty of food from the microbial loop to sustain their high densities in coastal areas, where strong currents import productive water from terrestrial and oceanic sources to the reefs, and possibly from resuspension of sediment-borne bacteria as well. The mechanisms behind the nutrient cycling observed in Chapter 2 were identified here: food is either sequestered into tissue and held there for two weeks or longer, or it is released as fecal pellets that are larger than the food particles eaten by the sponges.

5.5. Acknowledgements

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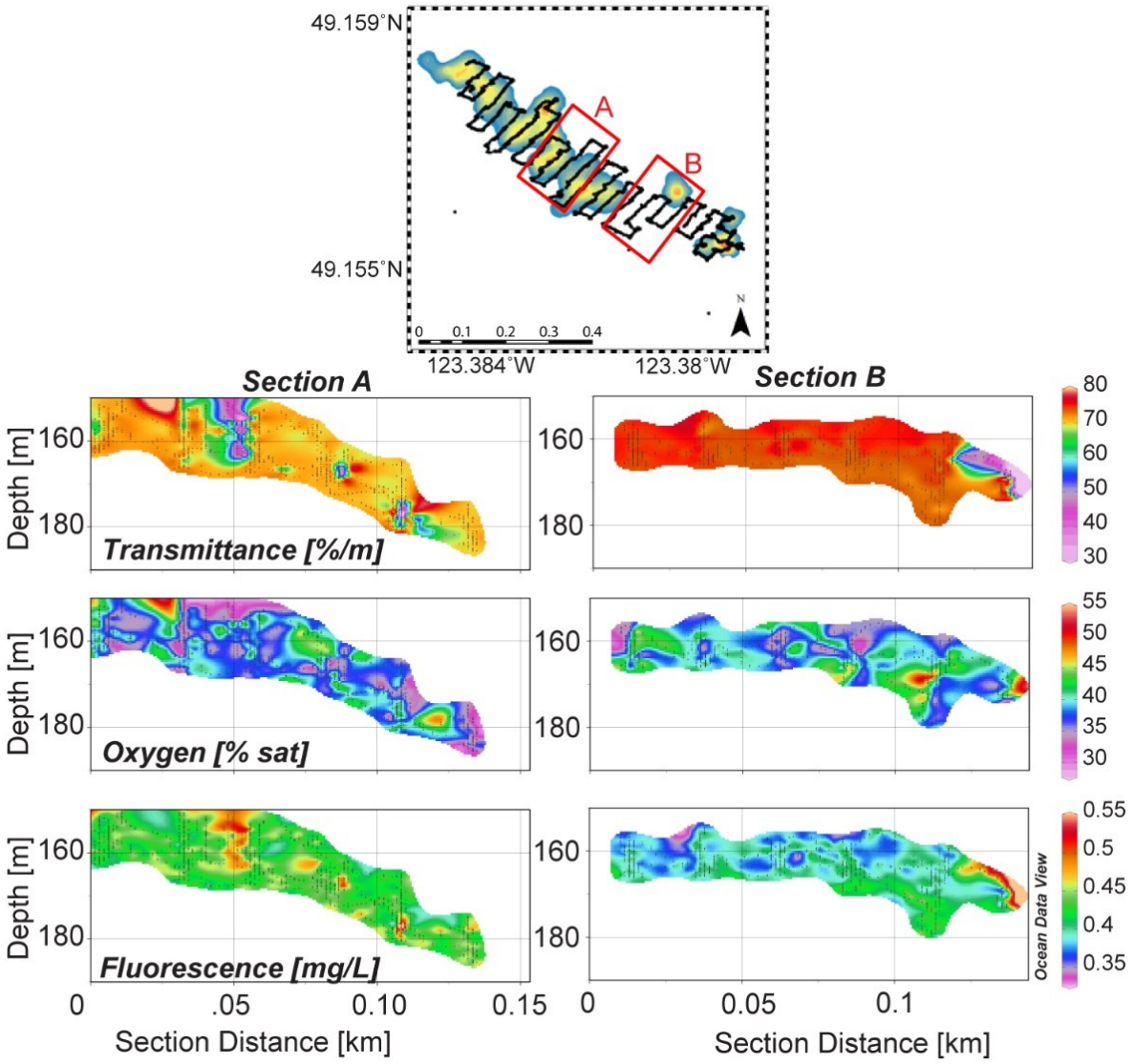

Figure 5.6. Transmissivity, oxygen, and fluorescence overlying reef and non-reef regions at Fraser Ridge, a sponge reef in the Strait of Georgia. A SeaBird CTD mounted with a transmissometer, oxygen sensor, and fluorometer was mounted on the remotely operated vehicle ROPOS during a gridded survey across Fraser Ridge Reef in 2005. Survey data was integrated in 150-m sections and visualized using Ocean Data View software. Sponge-filled regions are denoted in Section A and open sediments in Section B.

Chapter 6. Spicule and flagellated chamber formation in a growth zone of *Aphrocallistes vastus*

6.1. Introduction

Glass sponges colonized glacially carved ridges after the last glacial maximum and reefs have grown almost 20 m vertically in the 9,000 years since. Despite this impressively long time forming, only 1-2 meters of glass sponge skeleton - both living and dead - projects out of the sediments (Krautter et al. 2001). Growing away from the sediments can be seen as a race against burial by sediment; in addition, growing upwards might allow reef sponges to reach stronger ambient currents and presumably would enhance feeding as is known to be important for corals (Genin et al. 1986). The sponge consists of a single large tube, usually with several side branches, each of which can function as an effective excurrent vent; the central tube is the sponge osculum. New tissue forms at the tips of each of these extensions but how this occurs in a syncytial animal, and how the tubes expand laterally as the sponge gets larger, are both unknown. Glass sponges are syncytial, and so exactly how they form new tissue and the skeletal scaffolding upon which it lies, with multinucleate tissues and a shared cytoplasm is puzzling.

The body organization of glass sponges has been well-described (Mackie and Singla 1983, Reiswig and Mehl 1991, Leys 1995, 1999, reviewed in Leys et al. 2007). It is almost entirely formed by a continuous, multinucleate syncytium called the trabecular reticulum which can be either fused together into a rigid framework or lie individually (Leys et al. 2007). The trabecular reticulum includes the dermal and atrial surfaces and runs continuously through the choanosome. At the flagellated chambers, branches of the trabecular reticulum called the primary and secondary reticula support the collar-flagella pump units (Figure 6.1B-D) (Mackie and Singla 1983, Reiswig and Mehl 1991, Leys 1999). There is a very thin collagenous mesohyl, and no mobile cells. Instead transport of materials for feeding, growth and repair, occurs via cytoplasmic streaming along microtubule tracts, through the trabecular reticulum (Leys 1995). Dense transport strands have previously been called 'cord syncytia' (Reiswig and Mehl 1991). The sponge is a mixture of these multinucleated tissues and regions that function as cells but are tethered to the rest of the tissue by cytoplasmic bridges (Mackie 1981, Mackie and Singla 1983). At the flagellated chambers groups of nucleated 'cells' called choanoblasts give rise via cytoplasmic extensions to collar-flagella units – each unit has a beating flagellum surrounded by a ring of microvilli, but these arise from an enucleate base (Mackie and Singla 1983, Leys 1999).

Figure 6.1. Structure of chambers of *Aphrocallistes vastus*. A. The fused spicule skeleton of a dead sponge (brown) persists after the live tissue (yellow) has died. B. The trabecular syncytium (ts) makes up most of the tissue of the sponge, including the atrial and dermal (d) membranes. Dermal spicules project from the dermal membrane, part of the trabecular syncytium, down through a large subdermal space (v). Beneath the subdermal space lie flagellated chambers (c). White boxes outline regions that are magnified in C and D. C. Chambers are lined with two branches of the trabecular reticulum: a primary reticulum (1R) perforated with prosopyles (p) through which water enters the chamber, and a secondary reticulum (2R). Collar bodies (cb) draw water through the flagellated chambers and canals. Collar bodies lie embedded in the primary reticulum with collars of microvilli projecting through holes in the secondary reticulum. D. Collar bodies (cb) are produced by mononucleate choanoblasts (chb). Scale bars: A: 10 cm, B: 100 μm, C: 10 μm, D: 10 μm.

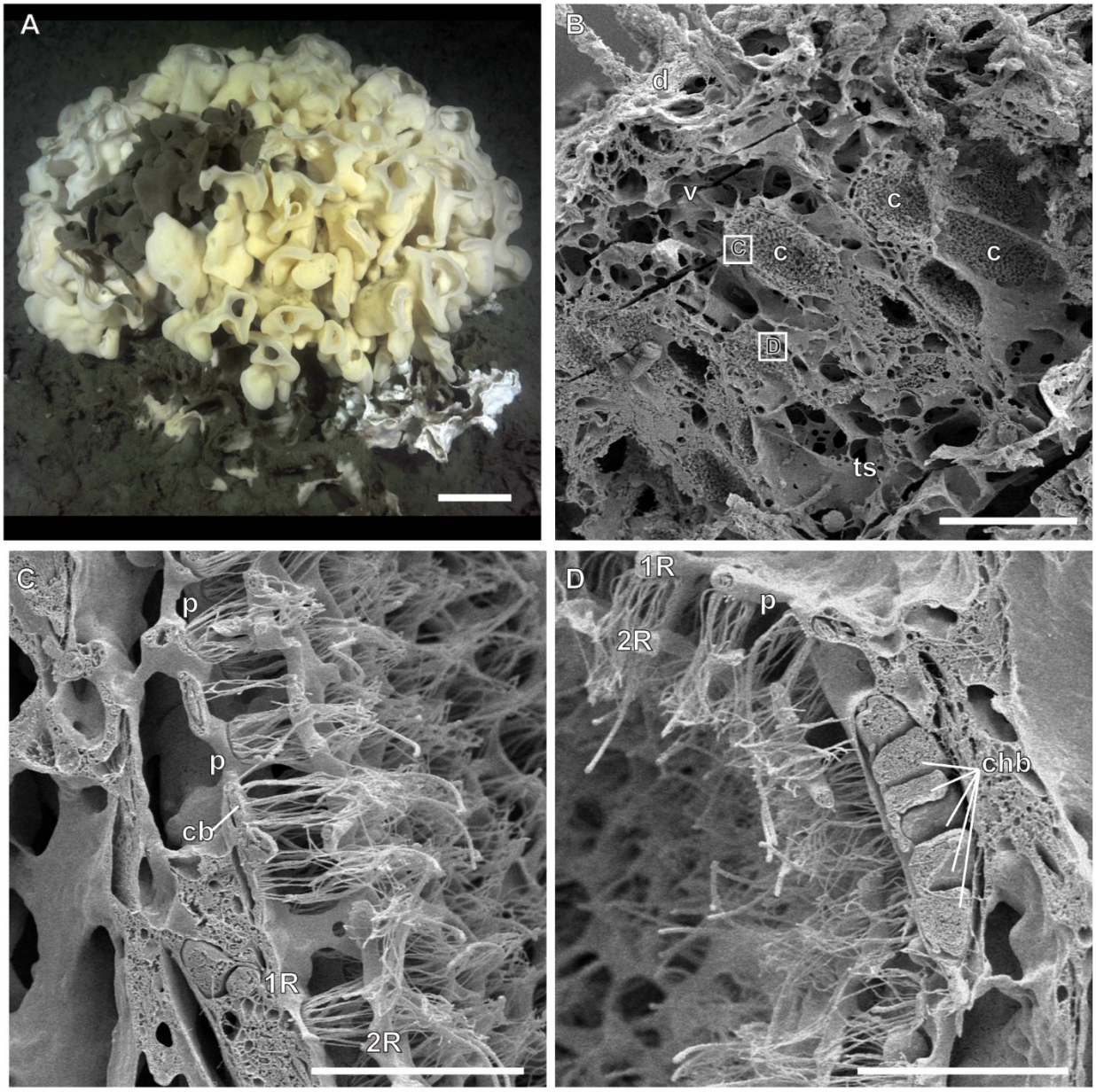

Figure 6.1. Structure of chambers of *Aphrocallistes vastus*.

Spicules are conspicuous and well-studied structured in sponges. In glass sponges spicules are secreted intrasyncytially but not in the trabecular reticulum. It was at first believed that distinctly separated 'scleroblasts' were needed because of the particular microenvironment needed for silica deposition (Mackie and Singla 1983) but it is now known that spicule-secreting regions are multinucleate, may be very large, but are as with all the sponges' tissues connected to all other tissues by cytoplasmic bridges (Leys 2003a). Studies of freshwater sponges have suggested that siliceous spicules grow along their surface area (Elvin 1971) with silicic acid deposited around an organic membrane – the silicalamella – that surrounds the entire spicule (Simpson and Vaccaro 1974). In the glass sponges *Caulophacus* sp. and *Euplectella aspergillum*, it was inferred that spicules form as amorphous silica is deposited around a hydroxylated collagen substrate, and this later fuses into the spicule structure using a silica-calcite composite (Ehrlich et al. 2010, Ehrlich et al. 2011).

To understand where spicules form in a growing sponge, and how new syncytial and cellular regions of tissue are made we studied regions of new growth in the reef-forming, dictyonine glass sponge *Aphrocallistes vastus* Schulze, 1886 (Order Hexactinosida; Family Aphrocallistidae). We used a ratiometric dye that labels newly deposited silica as well as fluorescence, scanning, and transmission electron microscopy to understand the morphology and processes involved in growth of new tissue and skeleton.

6.2. Methods

6.2.1. Specimen collection

Pieces of *Aphrocallistes vastus* Schulze, 1886 (10 x 10 cm) were collected either by SCUBA from Saanich Inlet, British Columbia (40 meters depth) or from the Galiano Ridge sponge reef using the remotely operated vehicle ROPOS with the Canadian Coast Guard Ship *Vector*. Sponges were maintained in an incubator at 9°C or in flow-through seawater tanks at the Bamfield Marine Sciences Centre.

6.2.2. Spicule growth

Three 2 x 8 cm strips were cut from the main body up to the osculum lip and incubated in 250 ml of 0.5 µM 2-(4-pyridyl)-5-((4-(2-dimethylaminoethylaminocarbamoyl)methoxy)phenyl)oxazole (PDMPO; RatioWorks™) in seawater for 2 d. Seawater was filtered (0.22 µm) to remove diatoms, which could take up the PDMPO. Sponges were fixed in Bouin's fixative overnight, rinsed twice briefly in seawater and dehydrated to 70% ethanol for long-term storage. Thin strips of sponge tissues were mounted and viewed under epifluorescence on a Zeiss Axioskop2 Plus using a long-pass DAPI filter.

6.2.3. EdU labeling of cells

Tissue pieces 3 x 5 mm were cut from the main body of the sponge (where the skeleton was fused) and from the soft osculum lip region where the skeleton was not fused. Tissue pieces were allowed to regenerate cut membranes in seawater for 24 h in an incubator (at 9°C), prior to being immersed in 5 ml (1 tissue piece) or 20 ml (2 tissue pieces) of 100 μM 5-ethynyl-2'-deoxyuridine (EdU) in 0.2-μm filtered seawater for 1 to 6 d. Seawater and EdU were refreshed daily. Using a pulse-chase protocol, tissue pieces were incubated in EdU for 3 d, and transferred to filtered seawater without EdU for 1, 2, or 3 d before fixation. Sponges were fixed in 8% paraformaldehyde and 0.03% glutaraldehyde for up to 24 h. Following fixation, pieces were rinsed twice in phosphate buffered saline, dehydrated to 70% ethanol, and desilicified in 4% hydrofluoric acid in 70% ethanol for 1-2 d. Desilicified sponge tissue was dehydrated and embedded in paraffin. Sections 7 μm thick were collected from at least 30 μm medial to the cut face of the tissue to avoid regenerative or repairing tissue. To fluorescently tag the EdU, sections were dewaxed and labeled with Alexa Fluor 594-azide using click chemistry following the standard protocol for the Click-iT EdU whole animal imaging kit (Life Technologies). Nuclei in the same sections were labeled with 100 μM Hoechst 33342 (Molecular Probes™) for 30 min, then sections were rinsed three times with PBS and mounted in Mowiol.

6.2.4. Thick sections and electron microscopy

Sponge pieces (2 x 2 cm) were preserved for electron microscopy in a cocktail fixative consisting of 1% OsO_4, 1% glutaraldehyde, and 0.45 M sodium acetate buffer with 10% sucrose (Leys 1995) except twice the concentration of osmium was used in the first 30 min to account for any dilution of the first mixture by seawater in the sponge tissues. The fixative was replaced after 30 min and samples were left overnight at 4°C. Specimens were rinsed once briefly in freshwater to remove salts, dehydrated through a graded series to 70% ethanol, and desilicified in 4% hydrofluoric acid in 70% ethanol for 1-2 d. Once desilicified, separate pieces were prepared for scanning and transmission electron microscopy (SEM, TEM).

Samples prepared for SEM were dehydrated to 100% ethanol, fractured in liquid nitrogen, and critical point dried (Bal-Tec CPD 030). Dried specimens were mounted onto aluminum stubs, sputter-coated with gold (Xenosput XE200) and viewed in a JEOL 6301F field emission scanning electron microscope. Samples prepared for TEM were dehydrated to 100% ethanol and embedded in epoxy resin (TAAB 812, Electron Microscopy Sciences). Thick sections (1 μm) were cut on a Leica Ultracut T and stained with Richardson's stain (Richardson et al. 1960). Thin sections (60 nm) were mounted onto copper grids, stained with lead citrate and uranyl acetate, and viewed in a Philips/FEI (Morgagni) transmission electron microscope.

6.3. Results

6.3.1. Spicule growth

A survey of the tissue from all parts of the sponge showed newly forming spicules throughout the body, but different spicule types formed in the fused and unfused regions (Figure 6.2A-B). In all cases, when spicules were labeled with PDMPO the whole spicule was fluorescent – not just a tip or segment (Figure 6.2C).

The only new spicules labeled in the lower regions of the sponge where spicules were fused into a scaffold were microscleres. In contrast all spicule types (both microscleres and megascleres) were labeled at the growing edge of the osculum (hereafter called the osculum lip) (Figure 6.2D, D'). Furthermore, secondary silica deposition that locked the loose spicules into a rigid framework was also seen between the fused and unfused parts of the skeleton (Figure 6.2E, E').

6.3.2. Growth of flagellated chambers

Nuclei found scattered throughout the trabecular syncytium never took up the EdU label. Cells adjacent to flagellated chambers did take up EdU, but only after incubation for 4 d; whether these were archaeocytes or choanoblasts could not be determined.

More activity was visible in the osculum lip region. There, choanoblasts took up EdU after 24 h, initially labeling as tight clusters of typically 5 or more (Figure 6.3A), but later becoming arranged in single-file rows of 4 or more (Figure 6.4A). Clumps of microvilli were visible near the clusters of choanoblasts with collar bodies already produced but not inserted into a larger chamber structure (Figure 6.3B-D). Images captured by all methods (SEM, thick sections, and TEM) showed that once choanoblasts were arranged in single-file rows the collar bodies had also moved apart to form a mature flagellated chamber with regularly spaced collar bodies surrounded by the primary and secondary reticula (Figure 6.4E). Naturally from static views it could not be determined when the chambers became functional. Chambers were also found that were intermediate between the early 'clustered choanoblast' stage and the more mature 'single-file choanoblast' stage (Figure 6.3E-F).

The transition from clusters of collar bodies to the regular spacing observed in mature chambers could not be tracked with EdU but several observations from the thick sections and scanning and transmission electron micrographs suggest different stages. Beginning with the lowest degree of organization, microvilli and flagella of the collar bodies were sometimes found surrounded by tissue that extended up into the chamber (Figure 6.5A-C). In other chambers tissue surrounded each collar body but provided more space around each flagellum (Figure 6.5D). In yet other chambers, collar bodies were regularly spaced and embedded in a primary

Figure 6.2. Spicule production in the growing edge of a glass sponge. A and B. Whole sponge (A) and cross section (B) showing how strips were cut from the part of the body with fused (f) spicules to the osculum lip, where spicules were not fused (u). C. Microscleres and megascleres labeled with PDMPO. Clockwise from top left: oxyhexaster (microsclere), pinular hexactin (megasclere), scopule (microsclere). D and D'. The unfused region of the skeleton, showing microscleres and megascleres labeled with the fluorescent dye PDMPO (D: PDMPO label. D': PDMPO label overlaid onto a bright-field view of all spicules.) E and E'. PDMPO labelling of fusion on the dictyonine skeleton in the transitional area between the fused and unfused skeleton. (E: PDMPO label. E.': PDMPO label overlaid onto a bright-field view of all spicules.) Scale bars: A. 5 cm; B. 5 mm; C. 250 μm; D. and D.' 1 mm; E. and E'. 500 μm.

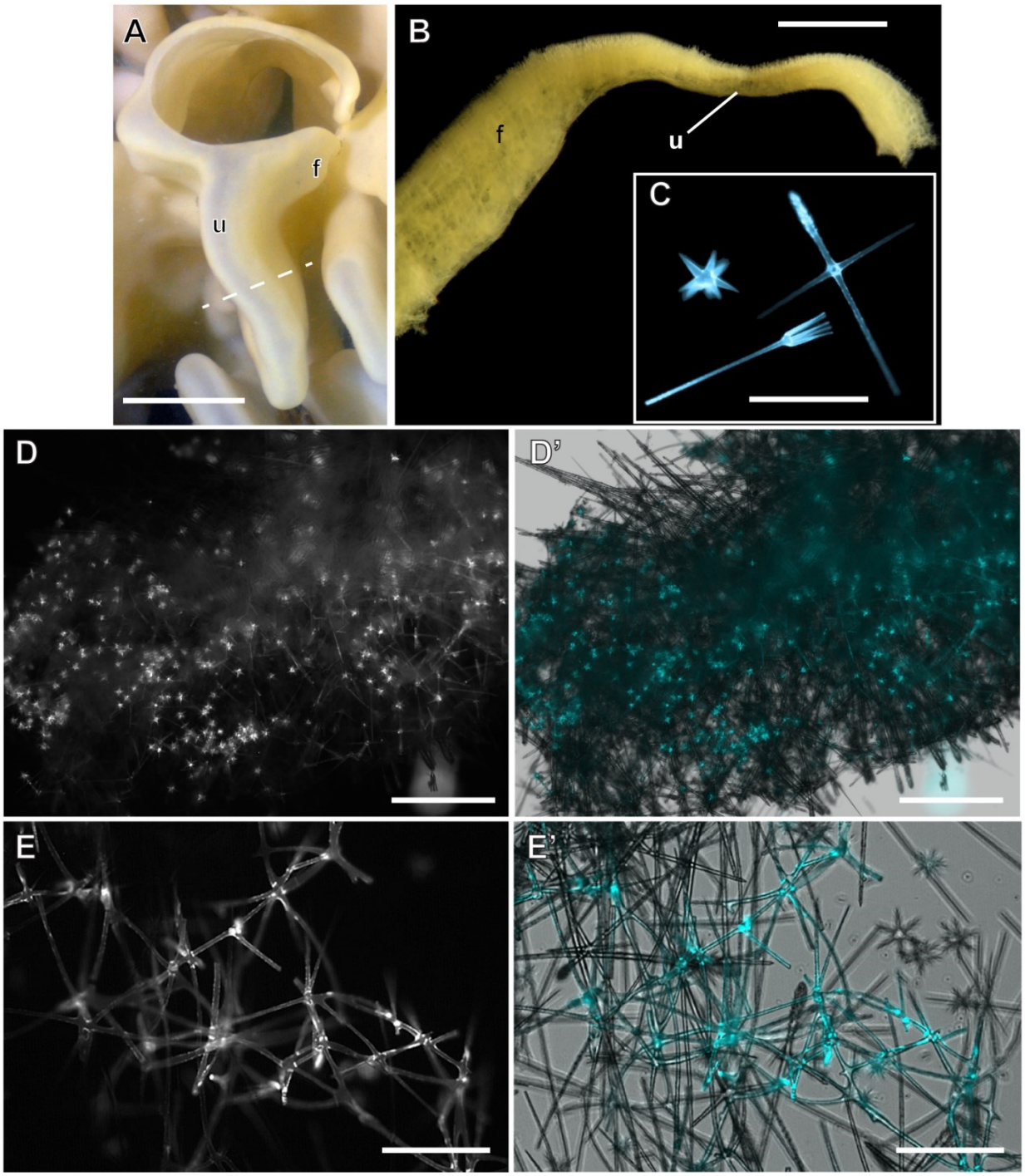

Figure 6.2. Spicule production in the growing edge of a glass sponge.

Figure 6.3. Proposed steps in formation of flagellated chambers. A. Clusters of choanoblasts in the unfused region of the sponge showed signs of replication (new nuclei labeled with EdU, pink) in a band through the tissue after incubation in EdU for 3 d. Nuclei were counterstained with Hoechst 33342 (blue). B. and C. Similar clusters of choanoblasts (pseudocolored purple in B, otherwise labeled *chb*) that resembled the clusters observed with EdU labelling were visible using SEM (B) and thick sections (C). Clusters of microvilli (arrows) were visible interspersed among choanoblasts. D. Clusters of microvilli and flagella (arrowheads) were found interspersed between choanoblasts. E. Slightly larger chambers still contained clusters of choanoblasts but with visible secondary reticulum (*2R*). F. Magnified view of a cluster of choanoblasts and one collar body (*cb*) connected to the cluster. Scale bars: A. 100 μm; B. 10 μm; C. 10 μm; D. 1 μm; E. 25 μm; F. 2 μm.

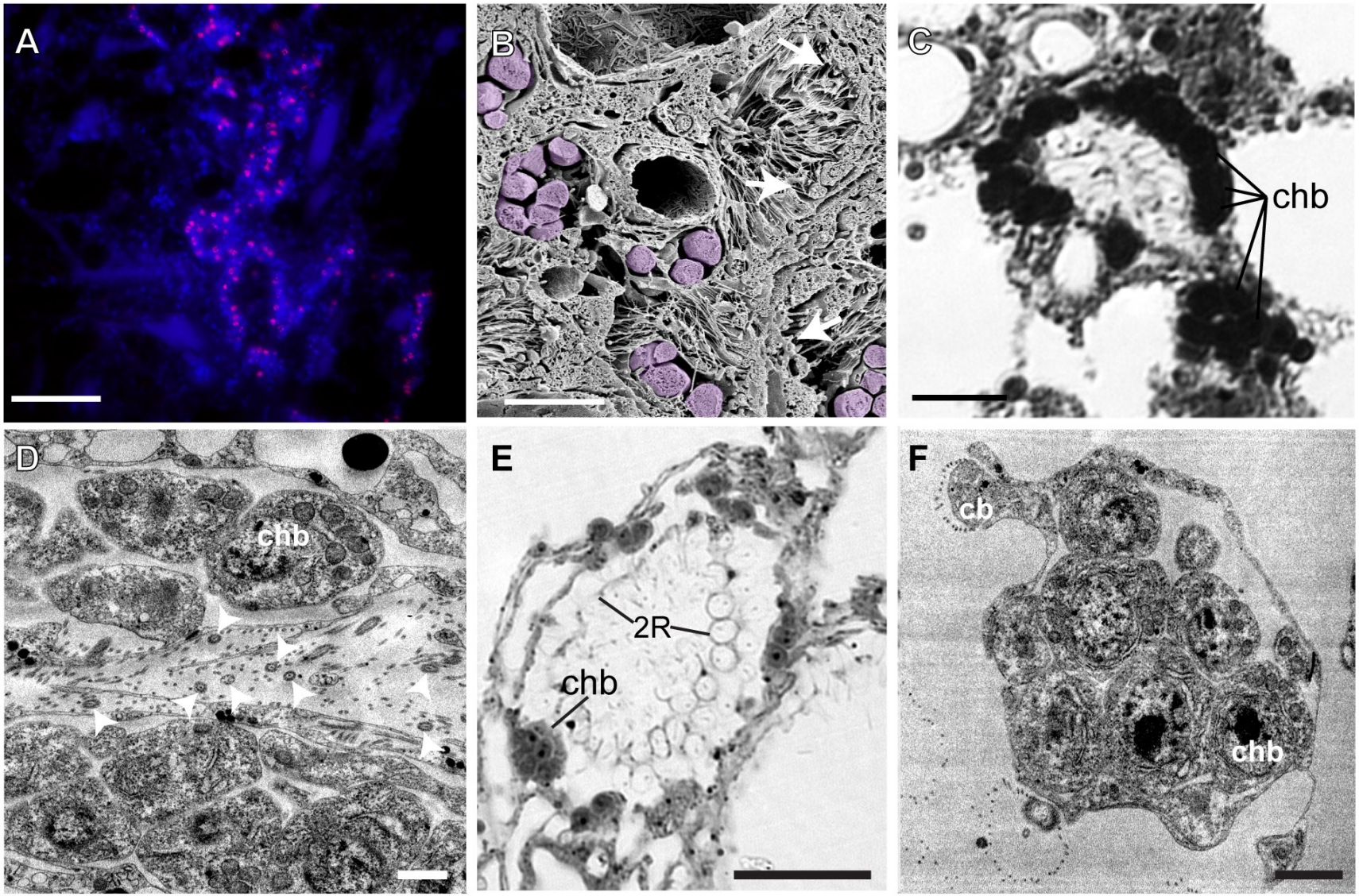

Figure 6.3. Proposed steps in formation of flagellated chambers

Figure 6.4. Proposed stages of enlargement of flagellated chambers. A. Newly produced choanoblasts (Labeled with EdU, pink) were arranged lining the sides of large, mature chambers after a 3 d pulse, 1 d chase procedure. Nuclei were counterstained with Hoechst 33342 (blue). B. and C. Similar rows of choanoblasts (pseudocolored purple in B, otherwise labeled chb) that resembled the rows observed with EdU labelling were visible using SEM (B) and thick sections (C). D. Choanoblasts had a regular arrangement of collars and flagella above them. E. The secondary reticulum (2R) was well formed by the time choanoblasts were in rows around the edges of the chamber. Other labels: cb, collar body. Scale bars: A. 100 μm; B. 20 μm; C. 10 μm; D. 25 μm; E. 2 μm.

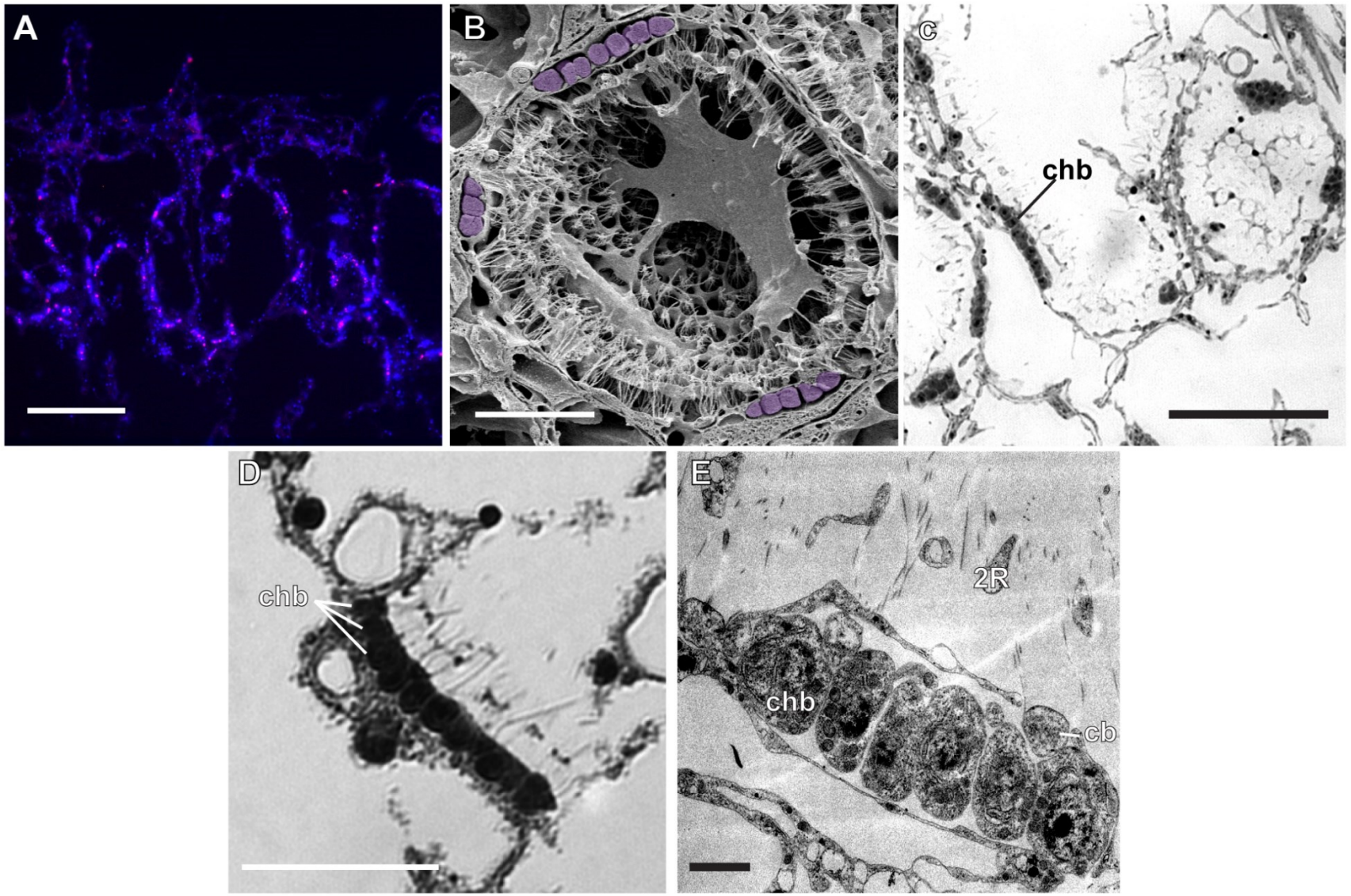

Figure 6.4. Proposed stages of enlargement of flagellated chambers.

Figure 6.5. Proposed steps of formation of the reticula around the microvilli and flagella. All images examined are from the tip area of the sponge, taken at the same time point. A. and B. Tissue projecting down among the collars of microvilli and around the flagella (f). C. A top-down view of a similar area showing tissue surrounding the flagellum and the collars. D. Wider spaces around the flagella while collars of microvilli were still surrounded by tissue. E. All tissue surrounding the flagella is gone but the collars of microvilli are still surrounded by tissue. F. The secondary reticulum (2R) above the primary reticulum (1R) with only a thin tissue layer above the collars remaining. Other labels: chb, choanoblasts; cb, collar body. Scale bars: 1 μm.

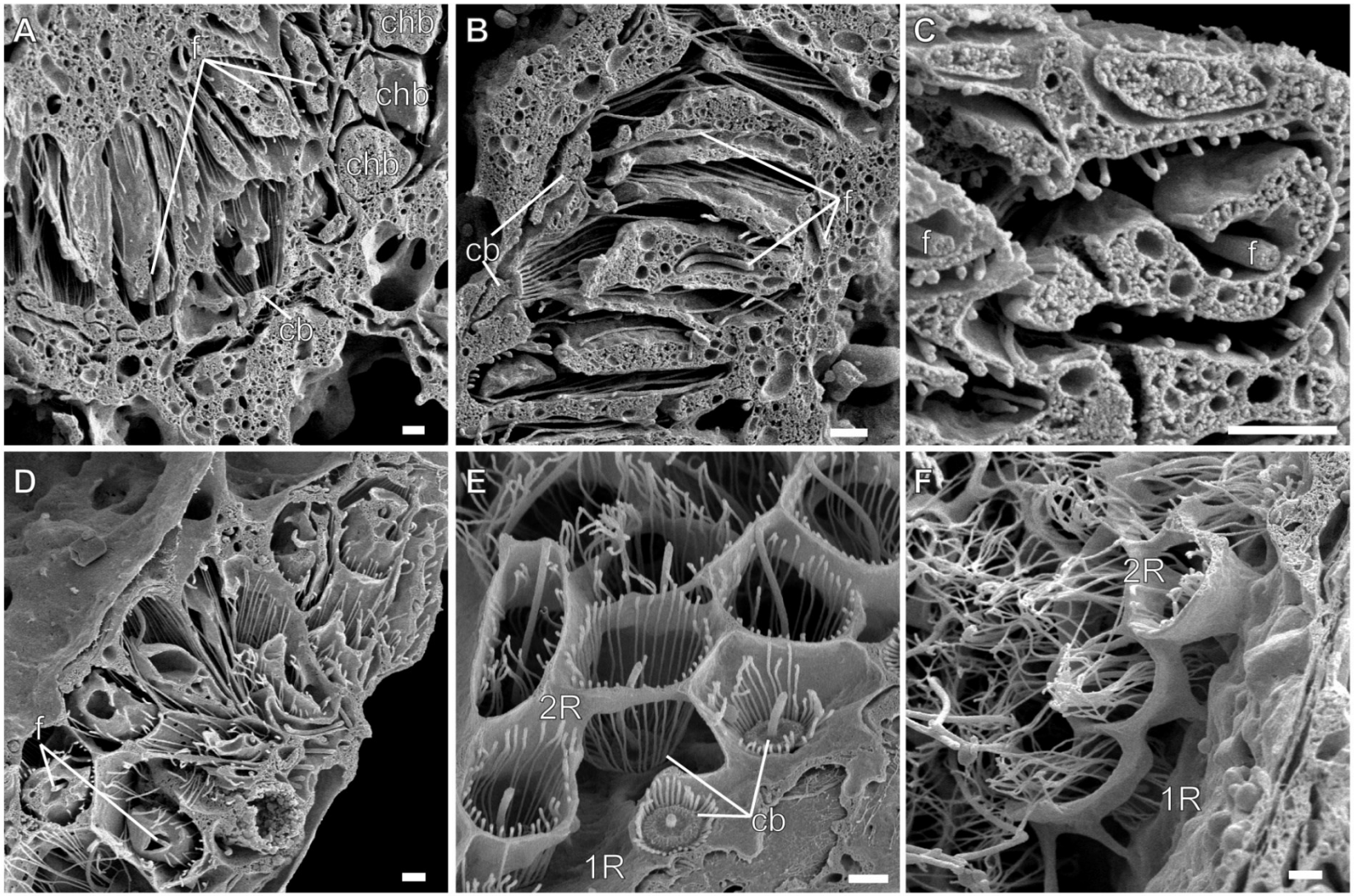

Figure 6.5. Proposed steps of formation of the reticula around the microvilli and flagella.

reticulum with tissue near either collar microvilli or flagellum (Figure 6.5E). In these only the secondary reticulum surrounded the microvilli (Figure 6.5F).

6.4. Discussion

Tissue and spicule production in the glass sponge *Aphrocallistes vastus* both occur at the uppermost edges – the tips of the oscula – of the mature sponge. Spicules are formed throughout the sponge, but megascleres only form and secondarily fuse near the growing tips of the sponge. Flagellated chambers appear to form by division of choanoblasts first into clusters, then those choanoblasts are arranged into a string, and then expansion of the chamber and separation of the choanoblasts to lie around it through production of collar bodies and the primary and secondary reticula. It is still unclear how new syncytial tissues form elsewhere in the body and how the smaller nuclei in the syncytium form. It would appear that no chambers form as the sponge tube widens, however that should be confirmed with additional studies of regions closer to the middle and base of the sponge.

6.4.1. Spicule growth

The silicification process is thought to be the same between mega- and microscleres (Uriz 2006) so both types were expected to take up PDMPO label if they were depositing new silica, and indeed new silica deposition was seen on megascleres and microscleres. Complete labeling of spicules by PDMPO during a short incubation period supports the prevailing model of silicification, that siliceous spicules grow in layers around an axial filament (reviewed by Uriz 2006, Schröder et al. 2007, Wang et al. 2011). This differs from calcareous sponges in which spicules grow from a seed nucleus either from the center to the tips or from discrete calcification sites (Ilan et al. 1996, Sethmann and Wörheide 2008).

That different regions of the sponge produce different spicule complements demonstrates regionalization across the body. Sponges with fused (dictyonine) skeletons cannot change their shape as other sponges can so growth would be expected to occur only in regions without fused skeletons. One interpretation of the stages of spicule production is spicules are produced first in the unfused tip of the sponge and then, as the sponge grows, they are then fused together to form the scaffold, now further away from the growing lip. If this were the case, most spicule-forming activity would occur in tissue that lies at the unfused tips of the sponges, with both microscleres and megascleres being produced. Once they have grown for some time and reached terminal size, megascleres would then be fused into the rigid scaffolding (as previously described in Leys et al. 2007). In this case, it is expected that more mature regions of the sponge already have the megascleres needed for structure so only microscleres would be

produced. Not all microscleres labeled, so in the future one may be able to quantify the amount of spicules deposited, and surface area of spicule deposition, by incubating the sponge in PDMPO for different lengths of time.

6.4.2. Growth of flagellated chambers

Flagellated chambers were also found forming in the unfused tips of the sponge, where they formed a surprisingly discrete band of new growth. That growth is directed and confined to one area suggests ontogenetic processes which merit further study. In the growth zone, chambers began as clusters of choanoblasts that first replicated and then produced collar bodies to expand into a fully sized chamber. The organization and arrangement of collar bodies and the primary and secondary reticula may occur in the order described here; however, it is possible that the stages of reticulum formation are reversed, such that what we describe is in fact the degradation and loss of a chamber over time. In earlier work on the glass sponge *Rhabdocalyptus dawsoni,* flagellated chambers were thought to be resorbed after being kept in a tank for several days (Leys 1999); while that may be possible here too, all of our current observations came from the growth zone of the sponge and were never observed elsewhere in the tissue. Flagellated chambers may be lost over time, either because they are resorbed back into the body or because that region of the body has a different function, such as when archaeocyte congeries adjacent to the chambers produce spermatocysts (Figure S5.1) (Boury-Esnault et al. 1999, reviewed by Leys et al. 2007).

Flagellated chambers that form by expansion of enucleate collar bodies is unique to glass sponges. Other sponges produce chambers through transdifferentiation of larval ciliated cells with a possible archaeocyte intermediate (Amano and Hori 1996, Leys and Degnan 2002), by immigration of stem cells from the mesohyl (Chapter 4), or by 4-5 mitotic divisions of a founding archaeocyte into smaller and smaller cells until all choanocytes are made (Tanaka and Watanabe 1984). The rosettes of choanoblasts that appear in early stages of chamber formation in glass sponges match closely to the small, new chambers described from cellular sponges. What is interesting is that while flagellated chambers of cellular sponges expand to full size through mitosis, transdifferentiation, or stem cell immigration, the flagellated chambers of glass sponges expand without producing any more choanoblasts at all by creating collar bodies instead. Using a limited number of choanoblasts to produce a chamber may be an energy-efficient strategy of living in food-poor deep water.

Different growth forms can also confer energetic advantages depending on flow conditions. The hydrozoan *Millepora alcicornis*, scleractinian coral *Pocillopora damicornis*, and the sponges *Haliclona oculata* and *Halichondria panicea* all have thin, branching

morphologies in areas with low flow and are more compact in areas with higher flow (Palumbi 1984, 1986, Kaandorp 1999). Mathematical models of accretive growth in a sponge or stony coral show that a branching, upward growth becomes a dominant morphology for suspension feeders in areas with flow and high nutrients (Kaandorp and Sloot 2001). High flow and high nutrient conditions are what reef sponges are exposed to and so might explain the directional growth observed for both their tissue and their spicule skeletons.

6.5. Acknowledgements

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Chapter 7. General Discussion: Insights into the ecophysiology of glass sponge reefs

In the ocean pelagic-benthic coupling has long been known to connect food energy from the water column to benthic biota, but details of its functions and additional complexity continue to be discovered. Sponge reefs have already been considered as important foundation species that produce three-dimensional habitat for other animals and sequester silica, but their role in pelagic-benthic coupling and carbon flow to the seafloor was not known.

The research in this thesis addressed how glass sponges affect the surrounding ecosystem. The five research chapters each investigated different aspects and different scales of food flow into, within, and out of individuals and the reefs themselves, and all five combine to offer insights into the ecology of glass sponge reefs. My coauthors and I found that sponge reefs altered the surrounding water by consuming microbial carbon and excreting microbial nitrogen as ammonium, with the strongest grazing rates of any suspension feeding community measured to date (Chapter 2). Strong feeding by the reefs was supported by food from both terrestrial and oceanic sources in the Strait of Georgia, with fecal pellets released by the sponges as aggregates of the particles that were consumed (Chapter 5). Fecal pellets may be a way that sponges concentrate bacterial carbon into a usable form for other animals. Repeat visits to the same sites across three years of ship cruises revealed dynamic, changing populations, with sponges growing and dying over the course of a few years at rates similar to those of shallower sponge species (Chapter 3). Given the many ecosystem functions that sponge reefs perform, the chapters that followed focused the mechanisms behind those fluxes at several scales. Measurements of cell turnover rates of four species of sponges showed that sponges controlled and limited the rate of cell proliferation depending on species and conditions (season and life history stage, but not feeding activity) (Chapter 4). We found that the reef sponges grow both new spicules and new flagellated chambers in specific growth zones (Chapter 6), and that choanoblast turnover rate in the growing region of the glass sponge was comparable to choanocyte turnover in non-growing regions of cellular sponges (Chapter 4). Growth in general contributed to the ecosystem functions of sponges identified elsewhere in this thesis, as new spicules provided habitat and substrate for other animals and as tissue sequestered carbon and contributed to nutrient cycling through feeding activity.

7.1. Importance of marine technology in advancing deep-sea biology

Studying biology in deep water presents more technical challenges in accessibility than with other marine habitats. Advances in knowledge of deep-water biology are therefore tightly

linked to advances in technology. Ijima (1901) and other early sponge scientists characterized the basic morphology and taxonomy of hexactinellids by opportunistically using specimens collected by fishermen through trawls, dredges, tangles, and 'dabo-lines' (longlines), as "objects perhaps of great value to naturalists but which to their [the fishermen's] eyes are all unwelcome 'filths,' 'weeds' or 'useless cottons.'" However, Ijima (1901) noted the limitations of trawling, stating that "specimens obtained…were always in a sorely mutilated condition, caused partly by the manner in which they were rooted out but more especially by their having been dragged along in the bag with so many other things." Free vehicles, deployed with a weight and then recovered by dropping that weight to become positively buoyant, were the first technological advance that moved beyond trawling and allowed specimens to be collected undisturbed along with the sediments upon which they rested (Smith et al. 1979b). They were also used in pioneering *in situ* studies of ecophysiology and benthic-pelagic coupling (Smith et al. 1976, Smith et al. 1979a).

The emergence of human-operated and remotely operated vehicles (ROVs) resulted in a radiation of the types of *in situ* studies that could be done (Robison 1999). The *in situ* experiments undertaken in the course of this dissertation were only possible because of precision navigation systems that allowed us to return to the same sites across several days and years and a powerful ROV that could hold its position in strong currents but still had the dexterity to serve as both eyes and hands underwater.

Just as research progressed with technological advances in the past, future work will benefit from other advances in instrumentation, coupling different deep submergence technologies (Fornari et al. 1997), and especially combining the fine dexterity and long working times of remotely operated vehicles with the long-term deployment and abilities of autonomous underwater vehicles (Robison 1999). Time is the biggest limit for data collection during ROV deployments on our trips, and AUVs might help relieve that time limitation by doing necessary but time-consuming menial tasks such as mapping and measuring water properties.

7.2. Ecology of glass sponge reefs

7.2.1. Conditions needed to form reefs

In general, glass sponges need low light, cold temperatures (<12°C for optimal propagation of action potentials, Leys et al. 1999), high concentrations of silica, low levels of sedimentation (<35 mg/L, Tompkins-MacDonald and Leys 2008), and hard substrates to settle on to grow (Leys et al. 2004). There are many fjords in other parts of the world and even within western Canada that satisfy these requirements, yet reefs do not form there. Glass sponge reefs

have the largest grazing rate of any suspension feeding community measured to date (Chapter 2), so surplus food energy may be an additional requirement for sponges to form reefs. We hypothesized that the extra food energy is brought in by strong water currents and resuspended sediments (Chapter 5).

Water flow is of course important to any suspension feeder – it has been shown to induce morphological changes in demosponges and corals (Palumbi 1984, 1986, Kaandorp and Sloot 2001) – but this need is amplified in sponge reefs because they live in such high densities. Like deep-water corals (Genin et al. 1986, Davies et al. 2009), glass sponge reefs in Hecate Strait, Queen Charlotte Sound, and the Strait of Georgia form on bumps and ridges that accelerate flow over them (Bedard 2011). Growth was directional in *A. vastus* (Chapter 6), which may suggest that sponges were in a race to outgrow their neighbors to project farther out of the boundary layer. Growing upward out of the boundary layer would expose sponges to stronger currents, and therefore more food, where greater volumes of water can wash over them than in areas of weaker currents. Another possible advantage might be that when higher, the sponges could take advantage of current-induced flow to take up more food energy without working harder during feeding (suggested by Leys et al. 2011).

Glass sponges grow in fjords throughout the west coast of British Columbia so whereas sediment is needed for reef formation (Conway et al. 2005a), outside of the reef setting the distribution of *Aphrocallistes vastus* was inversely related to sedimentation load (Leys et al. 2004). Skeletons are the settlement substrate for later generations of recruits (Chapter 3, Krautter et al. 2006). Moderate levels of sediment are necessary to cement the spicule skeletons in the reefs (Krautter et al. 2006). However, too much sediment in the water can cause glass sponges to arrest pumping (Tompkins-MacDonald and Leys 2008) so there are clear minimum *and* maximum concentrations of sediments needed for reef formation and continued health. The effect of sediment was the topic of a Canadian Scientific Secretariat report (Leys 2013) and the narrow window of sedimentation required by the reefs is something that requires further study.

Most interesting to me was another effect of sediments: that they may be a source of food, in the form of attached bacteria, for the reef sponges. Based on estimates of particulate organic carbon (POC) flux from the sea surface (Johannessen et al. 2003) that were seven times smaller than the amount of bacterial carbon consumed by a 1-m^2 patch of sponge reef, the reefs would be in a food deficit if fed by POC flux alone (Chapter 2). Lateral currents are likely partly responsible for balancing that deficit, but bacterial concentrations in sediments (10^8 and 10^9 cells ml^{-1}) are also much higher than in the water column (10^6 cells ml^{-1}) (Kuwae and Hosokawa

1999). Sediment resuspension has also been implicated for causing dense aggregations of the sponge *Pheronema carpenteri* near the Porcupine Seabight (Rice et al. 1990, Barthel et al. 1996). Resuspension resulting from fish activity has been found to be important for carbon remineralization in shallow habitats (Yahel et al. 2008). The degree to which bacteria from the water column and bacteria from sediments contribute to the diet of reef sponges is not known and is an important next step to understanding the role of sponges in carbon cycling on the seafloor.

7.2.2. Pelagic-benthic coupling by glass sponge reefs

Glass sponge reefs are already known to be foundation species that create habitat for fish and invertebrates (Cook 2005, Cook et al. 2008, Marliave et al. 2009). They are now also known to serve ecosystem functions through their feeding activity (Chapter 2) like shallower sponge species (Jimenez and Ribes 2007). As part of the biological pump, sponges have an analogous role to that of zooplankton in surface waters: their fecal pellets concentrate bacterial carbon into larger aggregates (Chapter 5). These aggregates could sink to the seafloor and be sequestered into the benthos, or they may become large enough to be edible by deposit feeders. The role of sponges in connecting the microbial loop with the biological pump, which was reviewed in Chapter 1, is revised here to include their suggested roles in pelagic-benthic coupling (Figure 7.1).

The three species that make reefs on the continental shelf are also found throughout the waters of British Columbia, sometimes at densities as great as those found in the reefs (Leys et al. 2004) and throughout the Northern Pacific below 1000 m depth at lower densities. Food availability in general is lower in the deep sea (prokaryote concentrations are 5×10^4 cells ml^{-1} compared to 5×10^5 cells ml^{-1} in ocean depths <200 m) (Whitman et al. 1998) and that could be the reason why dense aggregations do not form there. Other deep regions where there are dense aggregations of suspension feeders have a food supply that is thought to be enhanced by allochthonous inputs (food imported from elsewhere). Deep-water coral reefs of *Lophelia pertusa* (Cnidaria) in the Sea of Hebrides experience intense bouts of downwelling and rapidly imported currents from other deep areas (Davies et al. 2009), beds of *Sericolophus hawaiicus* (Porifera) in Hawaii are suggested to be fed by downwelling along the slope of the island (Pile and Young 2006), and *Pheronema carpenteri* (Porifera) populations on the slope of the Porcupine Seabight are thought to be supported by sediment resuspension through internal waves (Rice et al. 1990). Nevertheless, the average individual of *Aphrocallistes vastus* removes bacteria from over 9,000 L water d^{-1} (Chapter 2), so its feeding activity can have the same ecosystem functions in deep water as in the reefs through excretion of ammonia and fecal

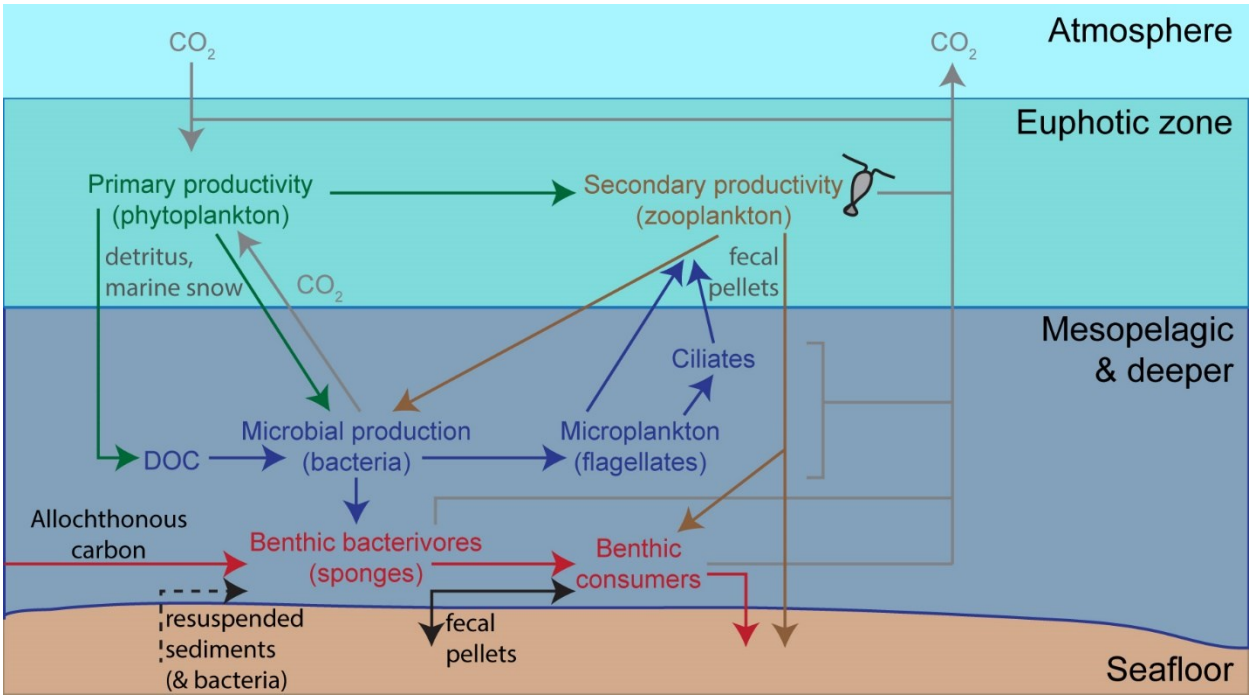

Figure 7.1. The biological pump, revisited. Proposed effects of sponges on the seafloor via pelagic-benthic coupling. Sponges, whether in deep and shallow water, take up carbon from the water column via the microbial loop. Food comes from within the system (autochthonous) and is imported from other ecosystems (allochthonous inputs, mainly terrestrial and oceanic carbon). Microbial productivity in sediments is high from the arrival of marine snow and fecal pellets from the water column, and wastes from benthic consumers. Sediment resuspension from strong currents and baffling by reef sponges moves bacteria bound to sediment grains into the water column, where they partly make up the diet of sponges. Abbreviations: DOC (dissolved organic carbon).

pellets but at a smaller scale. The research presented here suggests that deep-sea sponges may be important contributors to closing the 'food deficit' for other benthic animals when insufficient marine snow and aggregates rain from above (Smith 1987, Smith et al. 2001, Druffel and Robison 2007).

A clear next step is to measure the nutrient fluxes from other abundant sponge species and other suspension feeders in deep water to estimate their impact on nutrient cycling and further study into whether they can ameliorate food-limited conditions for other animals in the deep sea. For that work, studies could focus on other deep water sponges common in the northeastern Pacific such as *Heterochone calyx* and *Farrea occa*, and *Rhabdocalyptus dawsoni*; cold-water corals such as *Primnoa* sp. and *Lophelia pertusa*; glass sponges in Antarctica, such as *Rossella racovitzae* and *Rossella nuda*; demosponge species from the north Atlantic shelf such as *Geodia* spp., *Mycale* spp., and *Vazella pourtalesii*; and those species found in other deep-sea monitoring sites, *Hyalonema* spp. and *Pheronema carpenteri*. In those locations, sponge abundance and sizes can be surveyed in detail to allow measurement of fluxes per m^2, as was done for the glass sponge reefs

7.2.3. Waste excretion as a mechanism behind the sponge loop

Excretion of mucus and other waste products is a common mechanism identified for how other filter feeders affect their surroundings. Mucus and material trapped in it was found to be a major pathway of organic carbon release from warm- and cold-water corals and has been suggested to fuel bacterioplankton blooms (Wild et al. 2004, Wild et al. 2008). Carbon and nitrogen from the feces and pseudofeces released by bivalves are thought to remineralize and enrich the water column and sediments (Smaal and Prins 1993). Discarded mucus houses of larvaceans are major contributors to deep-sea carbon cycling (Alldredge 1972, Robison et al. 2005). Excretion of mucus and waste is a very likely mechanism for sponges too given the considerable detritus (Reiswig 1971b, Alexander et al. 2014) and fecal pellets they produce (Chapter 5, Wolfrath and Barthel 1989), and the low rates of cell proliferation observed (Chapter 4).

7.2.4. Conservation considerations

Glass sponge reefs are being considered for protection through Canada's Oceans Act and Fisheries Act because of their role as habitat-forming communities for commercially important fisheries (Jamieson and Chew 2002). Current protections proposed by the Department of Fisheries and Oceans place core protection zones (CPZ) around the reefs themselves but allow fishing activity in adaptive management zones (AMZ) surrounding the CPZ as long as the fishing activity "is not likely to result in the damage, destruction or removal of any part of the glass

sponge reefs" and is carried out following regulations of various fisheries acts (Department of Fisheries and Oceans 2015).

Reef sponges can withstand grazing by their natural predators, which include nudibranchs (Chu and Leys 2012), sea stars, and many fish (personal observation). They cannot, however, recover from large-scale damage such as trawling (Chapter 3). Sponges did not grow back following removal down to the sediments after three years, highlighting the destructive impact of trawling activity on sponge reefs. Trawl scars have been found throughout reefs in Hecate Strait and fisheries have been known to fish through reef areas with large amounts of sponge bycatch (Krautter et al. 2001, Ardron and Jamieson 2006, Ardron et al. 2007). Not only would reef sponges be removed by trawls, but also the substrate that juveniles settle upon would disappear so sponges would not be able to regrow (Chapter 3). Sponge habitats elsewhere in the world have shown negligible recovery from trawling as well (Van Dolah et al. 1987, Freese et al. 1999, Freese 2001, Wassenberg et al. 2002, Heifetz et al. 2009) and field manipulations of sponges living in fjords suggest that reef species are equally unlikely to recover from large-scale damage, given growth rates of 1-7 cm $year^{-1}$ (Austin et al. 2007). The delicate glass foundations of glass sponge reefs can easily be damaged, and although they have some capacity for recovery - biannual transects across sponge reefs damaged by newly laid submarine power transmission cables showed some recruitment and regrowth – they did not recover to the abundance they had before disturbance, even after four years (Dunham et al. 2015).

Trawl fishing activity does occur in areas around the reefs and the sediment that is kicked up from the trawls has long been known to have lethal and sublethal effects on sponges (Tompkins-MacDonald and Leys 2008) with projected negative effects on reefs (Leys 2013). The distance of the AMZ in some areas is less than 1 km from the borders of the reefs themselves (Figure 1.4) so trawl fishing nearby could potentially raise levels of sediment above a tolerable threshold. Reefs require a narrow range of sediments for a variety of needs: they contain a source of food (Chapter 5), cement skeletons for more reef to form (Krautter et al. 2006), and must be finely balanced between deposition and erosion (Chapter 3). Sediments can also cause glass sponges to stop flagellar beating (Leys et al. 1999), which means sponges could spend less time feeding. The effect can be substantial – the pumping rate for the tropical demosponge *Tethya crypta* was effectively reduced to 51% of its maximum in response to disturbance from winter storms (Reiswig 1971a). A change in sediment exposure from nearby trawling could move levels outside of an acceptable range and could affect pelagic-benthic coupling and other ecosystem functions by the reefs.

7.3. Cell turnover: methodological considerations

The effect of filter feeding by sponges on their surroundings is undeniable. The 'sponge loop' was originally presented as a model to explain how dissolved organic carbon is moved into the food web by sponges (with the help of symbionts) in oligotrophic coral reefs (De Goeij et al. 2013), and can apply to other forms of carbon that are unusable by other animals as well (bacterioplankton in the case of many other sponges including those in the sponge reefs). However, the original mechanism suggested to power the sponge loop, rapid cell shedding (De Goeij et al. 2009, Alexander et al. 2014), has been continually assumed without being critically evaluated and is not well supported. The core assumption that cell proliferation equates to cell shedding has never been confirmed. Detritus has been found in canals of *H. caerulea* and others (De Goeij et al. 2009, Alexander et al. 2014, Maldonado 2015) but it has never been ascertained whether choanocytes make up a large part of it. Autophagy and recycling of cellular contents is a more common mechanism used in tissues to limit cell populations in *Hydra* (Bosch and David 1984) and was suggested previously for sponges (Reiswig 1971b).

Even if cells are shed, three main criticisms of this mechanism arise: 1) quantification of the sponge loop was calculated based on the cell turnover rate of a single species at one moment in time, 2) there are several concerns with the methodology involved in measuring cell turnover, and 3) the energetics of frequently replacing cells are untenable for a sponge.

7.3.1. Variable cell proliferation between and within species

Halisarca caerulea is a tropical sponge that grows in crevices of coral reefs where flow and space are limited (Richter et al. 2001, De Goeij et al. 2008a, De Goeij et al. 2008b), yet its cell turnover rate (5.4 h) was thought to be constant and representative of other sponges and was used to justify cell shedding as the mechanism behind the sponge loop. Cell turnover rates were much slower for five other species studied (Chapter 4, Shore 1971) and were even slower in a developing sponge (Efremova and Efremov 1977). Repeat measurements of proliferation in *H. caerulea* suggested differences in proliferation even occurred within that species in separate years (De Goeij et al. 2009, Alexander et al. 2014). Turnover rates differed again when *H. caerulea* had been wounded and was regenerating tissue (Alexander et al. 2015). A standardized approach to measuring cell turnover, reproducible rates by another researcher, and the range of possible turnover rates within and between species, is needed before any larger assumptions can be made from those data, including being incorporated into quantification of the sponge loop.

7.3.2. Methodological considerations

Approach and methodology can affect cell turnover. The uridine analog BrdU was used to measure cell turnover in *H. caerulea* instead of EdU, but it is known to have toxic effects which include being teratogenic, mutagenic, and a trigger of cell death (reviewed by Taupin 2007). It is possible that incubation in BrdU might kill cells and result in a rapid increase in cell proliferation; a comparison between BrdU and EdU labeling in sponges needs to be tested.

Recovery time after preparing tissue pieces for incubation could also affect cell proliferation. For *H. caerulea*, which typically lives in flow-restricted crevices, the move to aquaria for a week could have triggered new growth after being moved to a large aquarium with plenty of food and water flow from intake pipes. We chose to incubate sponges in EdU 24 h after cutting pieces to minimize the time elapsed between sponge collection and measurement of cell turnover rates so that estimates would reflect turnover rates in the field rather than whatever changes might have occurred from keeping them in the laboratory. There was no way to know whether the choanocyte populations in a sponge were still growing or had reached a steady state except in the case where growth was known to be occurring: for example, from the gemmules of *Spongilla lacustris* and the growing region of *Aphrocallistes vastus* (Chapter 4). Whether the 5.4-h turnover rate of *H. caerulea* really reflected steady state populations or a growing sponge cannot be known. Assumption of a population of cells that is in a steady state is a difficult one to satisfy in general for a sponge; I propose using proliferation rates for comparison between sponge species, since they are unaffected by whether the choanocyte chamber is growing or in a steady state. Other methods than continuous incubations, such as pulse-chase labelling or using multiple labels (EdU, BrdU, IdU) should also be considered to determine the relative contribution of stem cell immigration and direct proliferation by choanocytes in maintaining choanocyte chambers.

Several time points must be collected to calculate and compare cell turnover rates between species. An attempt to broaden sampling beyond *H. caerulea* in the tropics unfortunately assumed turnover rates could be compared between a single time point (6 h, Alexander et al. 2014), but as outlined in Figure 4.1 cell populations with different turnover rates could have the same proportion of cells label at a single time point. Future study of cell turnover is certainly warranted but measurements will require a standardized method of cutting sponges, allowing them to recover, and selecting the number of time points necessary to measure cell turnover rates (at least three).

7.3.3. Energetic costs of rapid cell shedding

Cells are energetically expensive and shedding that occurs as quickly as proposed for *H. caerulea* would involve replacing about 1/3 of its body per day. A study testing whether coral mucus is a source of carbon into the 'sponge loop' cited a similar number, that sponges are "effectively turning over up to 35% of their body C per day" (Rix et al. 2016). This by itself is an expensive proposition, especially since roughly 28% of food energy consumed by a sponge is put toward filter feeding (Leys et al. 2011). If 35% of energy is also put toward choanocyte turnover as they suggest (assuming that energy is equal to the amount of carbon consumed, which is likely a substantial underestimate), then only 37% of the total energy eaten by the sponge remains for all other processes including growth, reproduction, producing spicules, and myriad others. Cells in any phase of the mitotic cycle are effectively removed from the cell population for the moment. "…When the cell is no longer quite one individual nor yet quite two, it might be confused as well as preoccupied" and so may not carry out its needed functions while undergoing mitosis (Mazia 1961). For choanocytes this could mean time spent not feeding, and for archaeocyte stem cells this would pull them away from their many other functions, including as food transporters and being able to differentiate into other cell types.

7.4. Fine control of turnover rates in sponges

It is clear that sponges are sensitive animals that respond to changes in their environment to optimize their energetics. This was recently shown with their ability to sense water flow in their environment (Ludeman et al. 2014), their behavioral response to increase pumping rates to maximize feeding in stronger currents (Ludeman 2015), and now with their ability to modify their energetic investment in cell proliferation (Chapter 4). I hypothesize that more or less effort could be put into cell proliferation as feeding conditions become less favorable in winter or as reproduction or growth takes precedence over maintenance. The next logical step is performing manipulative experiments that change conditions such as temperature, oxygen concentrations, or crowding with other sponges. This will allow us eventually to understand the various factors that cause sponges to change their investment in choanocyte proliferation.

The ability to regulate cell proliferation is shared with unicellular protists and with all multicellular life. The dinoflagellates *Alexandrium fundyense* and *Amphidinium carteri* were both found to change their rates of proliferation in different light, phosphorus, or nitrogen conditions (Chisholm et al. 1984, Olson and Chisholm 1986, Taroncher-Oldenburg et al. 1999). What differs in multicellular life is a finer control of proliferation for different cell populations. While a single-celled culture could grow unchecked and likely eventually overshoot the carrying

capacity of environmental conditions, cell populations in a multicellular organism must be tightly regulated so that no one population outgrows the others, otherwise it would hurt the fitness of the whole organism (Grosberg and Strathmann 2007). It is interesting, then, that choanocytes proliferated both by mitosis and through differentiation of stem cells from the mesohyl. It would be informative to determine whether rates of both processes change in response to conditions or if one is constant and the other is the rate limiting, or rate modifying, process.

7.5. Economy in glass sponge tissue structure

The tissue of glass sponges appears to be centered around economy –tissue makes up only 10-20% of dry weight (Chu et al. 2011) and cell turnover rates of choanoblasts were one of the slowest rates measured among choanoblasts and choanocytes (Chapter 4). Since one choanoblast can create several collar bodies (Chapter 6), they may not need to be replenished as frequently as choanocytes do in other sponges, further reducing the energetic cost for building chambers by not undergoing DNA synthesis. Growth is also localized into a growing zone (at least for *Aphrocallistes vastus*), which could also involve lower investment in energy than radial growth (Sipkema et al. 2006). Further investigation into the production of new structures will help in understanding how syncytium form and function differs from that of analogous structures in cellular tissue and may indicate what drove the transition from clusters of cells to syncytial tissues in the glass sponges.

7.5.1. Archaeocytes in cellular and glass sponges

Archaeocytes in cellular sponges are active cells that move, differentiate into other cell types, and transport phagocytized food to other cells in the body (Simpson 1984). In contrast, archaeocytes (including choanoblasts) in glass sponges are thought to be nonmotile (Mackie and Singla 1983), an idea supported by observations from this research. Choanoblasts were not observed undergoing any of the same tasks that archaeocytes perform in cellular sponges except for mitosis. Choanoblasts had a characteristic round shape with no pseudopodia stretched out (Chapter 6) unlike archaeocytes which are highly mobile and have pseudopodia stretched in many directions for movement (Chapter 4, Simpson 1984).

The lack of mobile archaeocytes in glass sponges may explain several differences in the way a glass sponge functions compared to a cellular sponge, originally put forth by Mackie and Singla (1983), and Leys (1995). Phagocytized food appeared to be transported by the trabecular syncytium (Chapter 5) via cytoplasmic streaming instead of by archaeocytes, as also observed by Leys (1995). Archaeocyte congeries may differentiate into spermatic cysts or oocytes (Ijima

1901, Boury-Esnault et al. 1999) and possibly cystencytes (Ijima 1901) but they otherwise do not work in the same capacity as mobile, active stem cells that can migrate to regions for repair (Alexander et al. 2015). Being syncytial may ameliorate this lack of migratory stem cells because stem cells would not be needed to produce new syncytium through mitosis. Archaeocytes are full of rough endoplasmic reticulum and so likely generate plenty of new tissue (Mackie and Singla 1983) – for instance the way that choanoblasts produce collar bodies – but they do so by producing new organelles and cell membrane rather than moving to a region and dividing.

Smaller nuclei that were scattered throughout the trabecular reticulum never took up the EdU label (Chapter 4), confirming the hypothesis of Reiswig and Mehl (1991) and Leys (2003b) that they were not proliferative and so do not have stem cell properties like archaeocytes. The function of these small nuclei is not known; perhaps they are necessary for specialization of the syncytium through localized transcription and/or translation, as has been hypothesized for coenocytes of green algae (Coneva and Chitwood 2015 and references therein). Localization of RNA expression through *in situ* hybridization would be one way to test that hypothesis, although complications have arisen from such attempts in other sponges, especially nonspecific labeling (Windsor 2014), so this is not a straightforward task.

An interesting puzzle that arises from the differences between archaeocytes of cellular and syncytial sponges is what the stem cells were like in the ancestor to all sponges (given the cellular ancestry of hexactinellid sponges; Leys 2006), or in the last common ancestor to animals. Choanocytes that made up the chambers were thought to be pluripotent (Funayama 2010, 2013), but the finding that stem cells from the mesohyl replenish choanocyte chambers suggests they are not (Chapter 4). Indeed, recent transcriptome analyses comparing choanocyte populations with archaeocyte populations in the sponge *Ephydatia fluviatilis* further support that archaeocytes, but not choanocytes, are stem cells in sponges. Archaeocytes had 9.5-fold greater expression over choanocytes of 180 orthology groups associated with stem cells of the cnidarian *Hydra vulgaris* and the flatworm *Schmidtea mediterranea* (Alié et al. 2015).

7.5.2. Flagellated chambers

Choanocyte chambers form in diverse ways in all cellular sponges. Chambers can arise following dedifferentiation of ciliated cells from the larval surface (Leys and Degnan 2002), from a founding archaeocyte that divides 4-5 times to produce all the cells in a chamber (Tanaka and Watanabe 1984), through fusion of separate chambers (Tanaka and Watanabe 1984), or in mature chambers, from stem cells migrating into chambers, which we found occurring in *Spongilla lacustris* (Chapter 4). The initial stages of flagellated chamber formation in the glass sponge, with choanoblasts replicating to form clusters and rosettes, resembled that of a

founding archaeocyte dividing several times to produce cells of a chamber in a cellular sponge. In the glass sponge, the chambers then expanded to their full size in a different way from cellular sponges, through production of many collar bodies (Chapter 6).

The many different ways that flagellated chambers could develop hints at the importance of conservation of form in chambers. Filter feeding requires precise hydrodynamics to enable efficient particle capture (Jørgensen 1955). The origin of flagellated chambers and their construction differs between syncytial and cellular sponges but is constrained by the same needs to maintain proper hydrodynamics in the aquiferous system. What could not be determined from the morphological investigation of chamber formation investigated here were the molecular underpinnings: did the collars and flagella arise using the same molecular mechanisms, even though their origin and the construction of chambers differed? A very precise method such as single-cell RNA-Seq, or targeted antibody labeling using *Ef annexin*, which has been shown to label choanocytes (Funayama et al. 2005, Funayama et al. 2010), may be a way to begin investigating the similarities and differences between how cellular and syncytial chambers are constructed.

The other logical next step to understanding how glass sponge tissue works, and how it evolved from a cellular ancestor, is to understand how syncytial tissue undergoes repair. EdU labeling of proliferating nuclei primarily occurred in the growing region of *Aphrocallistes vastus* (Chapter 4). Whether archaeocytes elsewhere in the tissue can be activated to proliferate and rebuild chambers would be important to understand the potential plasticity of these stem cells. Do choanoblasts only produce new collar bodies in mature chambers or are they latent stem cells, awaiting a cue to become proliferative? EdU labeling on wounded *Aphrocallistes vastus* would be a good way to test this, especially if done using sandwich cultures that could be followed under a microscope in real-time (Wyeth et al. 1996).

7.6. General conclusions

Knowing the processes that feed energy into and out of both an organism and an ecosystem is vital to understanding their function. These studies of the glass sponge reefs demonstrate their important ecosystem functions as filter feeders but also the precise combination of conditions necessary for a reef to form. The ecosystem functions of glass sponge reefs are driven by their efficient particle capture and feeding and their excretion of wastes. The anatomy and evolution of syncytial glass sponges reflects adaptations to a low-food environment that could also reflect the conditions in which sponges and other early animals evolved.

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Appendix 1

This supplemental table accompanies Chapter 2. Benthic grazing and carbon sequestration by deep-water glass sponge reefs.

Table S1.7.1. Geographical regions with high densities of glass sponges.

Taxa	Location	Density (sponges m^{-2})	Source
Glass sponge reefs	NE Pacific	23-46	(Conway et al. 2005a)
Fjord glass sponges	NE Pacific fjords	24	(Leys et al. 2004)
Antarctic demosponges	Weddell Sea	2	(Barthel and Gutt 1992)
Pheronema carpenteri	Porcupine Seabight	1.5	(Rice et al. 1990)
Various glass sponges	Pribilof Canyon, Bering Sea	0.4	(Miller et al. 2012)
Rossella racovitzae	Weddell Sea	0.23	(Barthel and Tendal 1994)
Hyalonema sp.	Station M	0.02-0.03	(Beaulieu 2001b)
Various glass sponges	Eastern Gulf of Alaska	0-0.12	(Miller et al. 2012)

Appendix 2

These figures accompany Chapter 3. Dynamic change, recruitment, and resilience in reef-forming glass sponges.

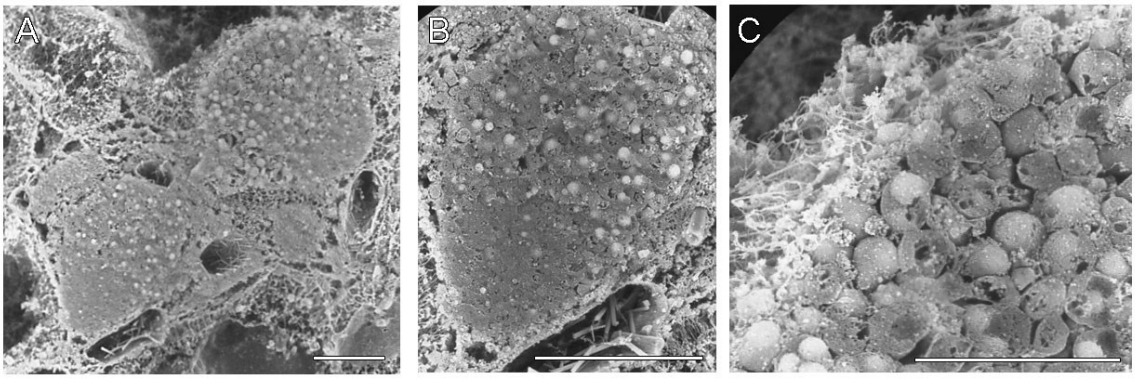

Figure S2.1. Two putative embryos found in tissue of a single specimen of *Aphrocallistes vastus* collected in November 1995. A. Two putative embryos amongst flagellated chambers of the sponge. B. Closer view of one of the possible embryos, showing a pointed anterior end and a yolk-filled posterior end. C. Cilia lining the outside of the embryos. Scale bars: A: 10 cm, B: 10 cm, C: 5 µm.

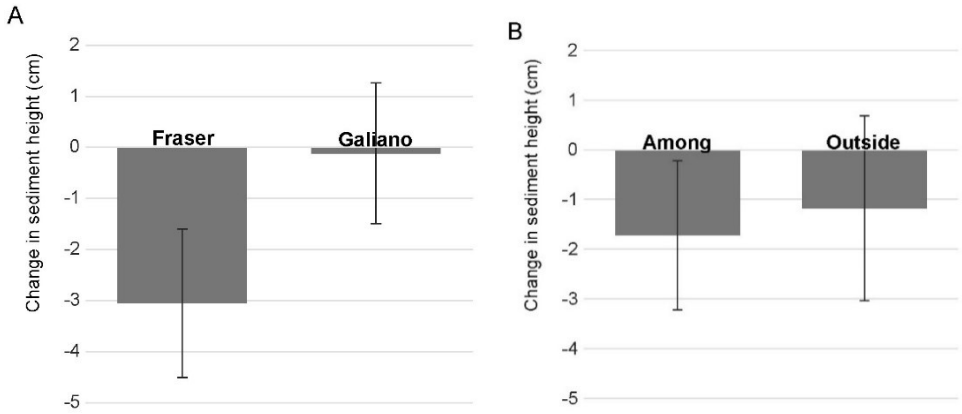

Figure S2.2. Mean (± SE) changes in sediment height were measured using sediment poles surveyed between 2005 and 2013. Not all poles could be visited during each research cruise. Changes in sediment height were compared between poles deployed at Fraser or Galiano reefs (A), and between poles deployed among or outside of patches of reef (B).

Supplemental Material S2.3. High-definition videos of structure-from-motion 3D models of BACI experiments are available on ERA. Each video file is named depending on site [impacted site either before (BI) or after (AI) damage, and two control sites, C1 and C2] and year surveyed (2011, 2013, and 2014).

Appendix 3

These figures accompany Chapter 4. Cell replacement in sponges sheds light on stem cell origins in Metazoa.

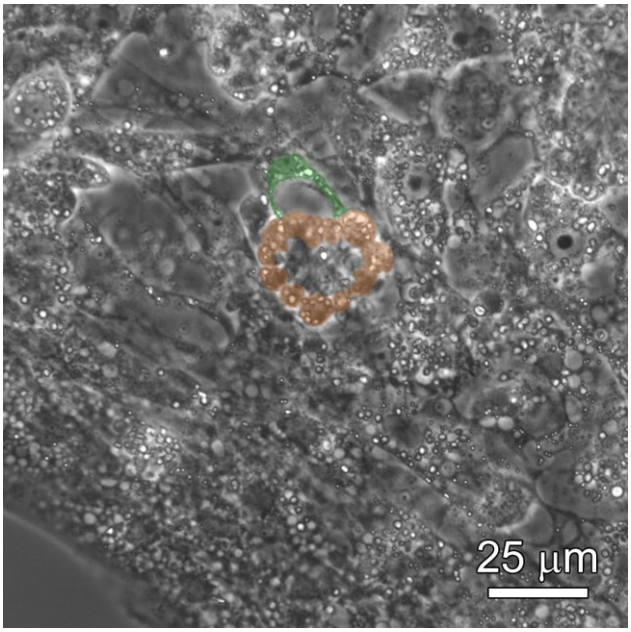

Figure S3.1. Choanocytes move as a unit and in this instance a choanocyte chamber (pseudocolored orange) was being pulled by a cell from the mesohyl that attached to it with two pseudopodia (pseudocolored green). Scale bar: 25 µm.

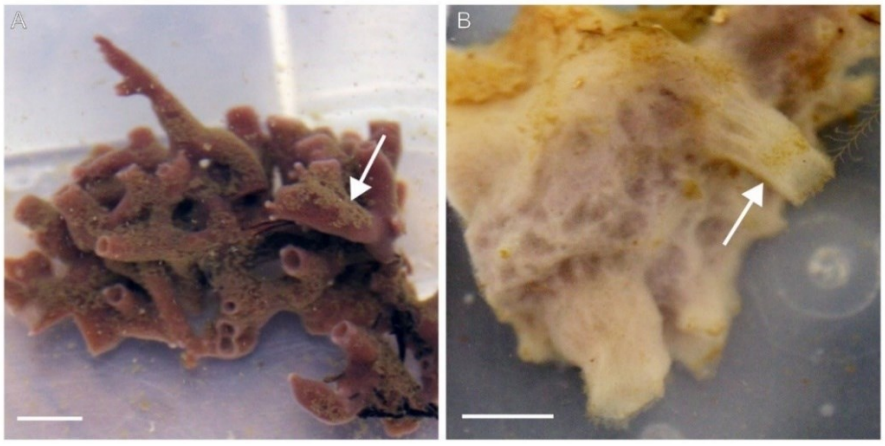

Figure S3.2. Sponges incubated in bacteria-enriched seawater produced brown detritus in their tanks whereas those in filtered seawater produced less detritus. A. *Haliclona permollis*, an intertidal demosponge. B. *Haliclona mollis*, a subtidal demosponge. Arrows point to the brown detritus, which was often found near the oscula of the sponges. Scale bars: 1 cm.

Table S3.1. Cell cycle lengths harvested from the literature for a variety of cell types from mature and larval/embryonic animals, plants, unicellular eukaryotes, bacteria, and syncytial tissues. Relevant notes are listed in the notes section. References match those listed in Figure 4.6.

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Mature animal tissue						
Spongilla lacustris	Animalia	Porifera	choanocyte	159		this study
Sycon coactum	Animalia	Porifera	choanocyte	30.2		this study
Haliclona mollis	Animalia	Porifera	choanocyte	176		this study
Aphrocallistes vastus	Animalia	Porifera	choanoblast	170		this study
Spongilla lacustris (gemmule)	Animalia	Porifera	choanocyte	34.3		this study
Hymeniacidon sinapium	Animalia	Porifera	choanocyte	20-40		(Shore 1971)
Halisarca caerulea	Animalia	Porifera	choanocyte	5.4		(De Goeij et al. 2009)
Hydra attenuata	Animalia	Cnidaria	epithelial cell	96-168	Longer cell cycle during food-poor or starved conditions.	(Bosch and David 1984)
Hydra oligactis	Animalia	Cnidaria	interstitial stem cells	43.2-96		(Holstein and David 1990)
Mytilus galloprovincialis	Animalia	Mollusca, Bivalvia	gill cell	24-30		(Martínez-Expósito et al. 1994)
Mytilus galloprovincialis	Animalia	Mollusca, Bivalvia	epithelia (stomach & digestive gland)	12	Tides drive rhythm of cell cycle.	(Zaldibar et al. 2004)
Riftia pachyptila	Animalia	Annelida	epidermis	6	Hydrothermal vent worm	(Pflugfelder et al. 2009)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Riftia pachyptila	Animalia	Annelida	peripheral trophosome	6	Hydrothermal vent worm	(Pflugfelder et al. 2009)
Riftia pachyptila	Animalia	Annelida	median trophosome	3	Hydrothermal vent worm	(Pflugfelder et al. 2009)
Riftia pachyptila	Animalia	Annelida	central trophosome	1	Hydrothermal vent worm	(Pflugfelder et al. 2009)
Lamellibrachia luymesi	Animalia	Annelida	epidermis	3	Cold seep worm	(Pflugfelder et al. 2009)
Lamellibrachia luymesi	Animalia	Annelida	peripheral trophosome	3	Cold seep worm	(Pflugfelder et al. 2009)
Lamellibrachia luymesi	Animalia	Annelida	median trophosome	3	Cold seep worm	(Pflugfelder et al. 2009)
Lamellibrachia luymesi	Animalia	Annelida	central trophosome	3	Cold seep worm	(Pflugfelder et al. 2009)
Styela clava	Animalia	Chordata, Tunicata	epithelial cells of stomach & esophagus	420	Funnel epithelium of dorsal tubercle, lip epithelium of dorsal tubercle, mucus cells of esophagus, crest population of stomach, groove population of stomach	(Ermak 1975)
Styela clava	Animalia	Chordata, Tunicata	intestinal & rectal epithelia	840	Intestinal epithelium, rectal epithelium	(Ermak 1975)
Styela clava	Animalia	Chordata, Tunicata	Cells of endostyle & esophagus	several months	Zone 1 of endostyle, band cells of esophagus	(Ermak 1975)
Mus musculus	Animalia	Chordata, mouse	duodenal crypt	10.4-13.5		Reviewed by (Guiguet et al. 1984)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Mus musculus	Animalia	Chordata, mouse	colon	20.9-21.8		Reviewed by (Guiguet et al. 1984)
Mus musculus	Animalia	Chordata, mouse	jejunal mucosa	18.75		Reviewed by (Van'T Hof 1965)
Mus musculus	Animalia	Chordata, mouse	colon mucosa	16		Reviewed by (Van'T Hof 1965)
Mus musculus	Animalia	Chordata, mouse	periodontal fibroblasts	33.9-42.4		Reviewed by (Guiguet et al. 1984)
Mus musculus	Animalia	Chordata, mouse	thymus	9.4-9.6		Reviewed by (Guiguet et al. 1984)
Mus musculus	Animalia	Chordata, mouse	uterine cervix	22.8		Reviewed by (Guiguet et al. 1984)
Mus musculus	Animalia	Chordata, mouse	erythroblasts	5.3-7.3		Reviewed by (Guiguet et al. 1984)
Mus musculus	Animalia	Chordata, mouse	hair follicle	13		Reviewed by (Van'T Hof 1965)
Mus musculus	Animalia	Chordata, mouse	esophagus	181		Reviewed by (Van'T Hof 1965)
Rattus norvegicus	Animalia	Chordata, rat	jejunal crypt	10.0-15.5		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	bone	38.1		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	cartilage cells	23		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	tibia cartilage	54.2		Reviewed by (Guiguet et al. 1984)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Rattus norvegicus	Animalia	Chordata, rat	leukocytes	10.4-34.6		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	erythroblasts	7.4-11.1		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	thyroid follicle	120.9		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	brain	15.3-23.3		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	dentate gyrus	16.4		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	dentate gyrus	18.7		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	dentate gyrus	15.8		Reviewed by (Guiguet et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	tibial metaphysis	36		Reviewed by (Van'T Hof 1965)
Rattus norvegicus	Animalia	Chordata, rat	tibial endosteum	57		Reviewed by (Van'T Hof 1965)
Rattus norvegicus	Animalia	Chordata, rat	tibial periosteum	114		Reviewed by (Van'T Hof 1965)
Rattus norvegicus	Animalia	Chordata, rat	condylar cartilage prechondroblast	78-114	Slows with time of year/age.	Reviewed by (Petrovic et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	sphenooccipital synchondrosis chondroblast	55.6		Reviewed by (Petrovic et al. 1984)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Rattus norvegicus	Animalia	Chordata, rat	cultured skeletoblasts	72-187		Reviewed by (Petrovic et al. 1984)
Rattus norvegicus	Animalia	Chordata, rat	cultured prechondroblasts	19-55	Slowed with age.	Reviewed by (Petrovic et al. 1984)
Embryonic/larval animal tissue						
Baikalospongia bacillifera	Animalia	Porifera	choanocyte in larva	13-15		(Efremova and Efremov 1977)
Tectura scutum	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	0.87-0.55	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Calliostoma ligatum	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	1.57-1.03	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Littorina scutulata	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	3-1.68	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Littorina sitkana	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	4.13-2.8	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Lacuna vincta or variegata	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	3.85-2.6	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Haminaea vesicula	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	3.77-1.98	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Haminaea callidegenita	Animalia	Mollusca, Gastropoda	2-4 cell stage embryo	10.17-4.8	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Helobdella triserialis	Animalia	Annelida, Hirudinea	embryonic cells	4-9	From Table 1, page 112	(Bissen and Weisblat 1989)
Asplanchna brightwelli	Animalia	Rotifera	Embryo (first 10 cleavages)	0.25-0.50	Lifespan ~4 d	(Birky et al. 1967)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Asplanchna brightwelli	Animalia	Rotifera	Adult/post-mitotic	0	Lifespan ~4 d	(Birky et al. 1967)
Asplanchna brightwelli	Animalia	Rotifera	Syncytial vitellarium	0	Lifespan ~4 d	(Birky et al. 1967)
Terebratalia transversa	Animalia	Brachiopoda, Articulata	2-4 cell stage embryo	1.03-0.63	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Terebratulina unguicula	Animalia	Brachiopoda, Articulata	2-4 cell stage embryo	1.43-0.8	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Phoronis pallida	Animalia	Phoronida	2-4 cell stage embryo	1.25-0.68	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Phoronis vancouverensis	Animalia	Phoronida	2-4 cell stage embryo	2.88-2.02	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Luidia foliolata	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.93	Reared in 10°C	(Strathmann et al. 2002)
Evasterias troschelii	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.5	Reared in 10°C	(Strathmann et al. 2002)
Orthasterias koehleri	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	2	Reared in 10°C	(Strathmann et al. 2002)
Pisaster ochraceus	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.57	Reared in 10°C	(Strathmann et al. 2002)
Leptasterias hexactis	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	6.55	Reared in 10°C	(Strathmann et al. 2002)
Crossaster papposus	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	2.03	Reared in 10°C	(Strathmann et al. 2002)
Pteraster tesselatus	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.68	Reared in 10°C	(Strathmann et al. 2002)
Henricia leviuscula	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.98	Reared in 10°C	(Strathmann et al. 2002)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Henricia sp. (gray armpit)	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	1.73	Reared in 10°C	(Strathmann et al. 2002)
Henricia sp. (brooder)	Animalia	Echinoderm, asteroid	2-4 cell stage embryo	2.93	Reared in 10°C	(Strathmann et al. 2002)
Oikopleura dioica	Animalia	Chordata, Tunicata	2-4 cell stage embryo	0.33-0.23	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Corella inflata	Animalia	Chordata, Tunicata	2-4 cell stage embryo	0.87-0.58	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Ascidia paratropa	Animalia	Chordata, Tunicata	2-4 cell stage embryo	1.08-0.7	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Boltenia villosa	Animalia	Chordata, Tunicata	2-4 cell stage embryo	0.92-0.59	Reared in 10°C and 14°C	(Strathmann et al. 2002)
Mus musculus	Animalia	Chordata, mouse	neural progenitor cell	8-18		(Al-Kofahi et al. 2006)
Mus musculus	Animalia	Chordata, mouse	fetal neocortex (cerebral wall)	15.1		(Takahashi et al. 1993)
Rattus norvegicus	Animalia	Chordata, rat	retina during development	14-55		(Alexiades and Cepko 1996)
Homo sapiens	Animalia	Chordata, human	embryonic stem cell	15-16		(Becker et al. 2006)
Homo sapiens	Animalia	Chordata, human	normablast	15-18		Reviewed by (Van'T Hof 1965)
Unicellular Eukaryotes						
Amphidinium carteri	Protista	dinoflagellate	Whole cell	27		(Olson and Chisholm 1986)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Amphidinium carteri	Protista	dinoflagellate	Light-limited	82	Cell volume also decreased with light limitation.	(Olson and Chisholm 1986)
Amphidinium carteri	Protista	dinoflagellate	N-limited	139		(Olson and Chisholm 1986)
Alexandrium fundyense	Protista	dinoflagellate	Whole (strain GtCA29)	60-150	Varies depending on temperature & phosphorus.	(Taroncher-Oldenburg et al. 1999)
Chlamydomonas eugametos	Plantae	unicellular green algae	Whole cell	20-48	High temperatures = longer cell cycle & larger cells	(Zachleder and Van Den Ende 1992)
Saccharomyces cerevisiae	Fungiae	yeast	Whole cell	1.65-2.37		(Brewer et al. 1984)
Saccharomyces cerevisiae	Fungiae	yeast	Whole cell	2.1	Good N source (ammonia)	(Rivin and Fangman 1980)
Saccharomyces cerevisiae	Fungiae	yeast	Whole cell	1.8	Good N source (glutamine)	(Rivin and Fangman 1980)
Saccharomyces cerevisiae	Fungiae	yeast	Whole cell	6.7	Poor N source (proline)	(Rivin and Fangman 1980)
Aspergillus nidulans	Fungiae	ascomycete (filamentous fungus)	Whole cell	1.5-2		(Bergen and Morris 1983)
Euglena gracilis	Protista	euglena	Whole cell	24-30		Reviewed by (Adams et al. 1984)
Monosiga brevicollis	Protista	Choanoflagellata	Whole cell	6		(King et al. 2003)
Physarum polycephalum	Fungiae	Myxomycete (slime mold)	syncytial plasmodium	8-12		Reviewed by (Tyson and Sachsenmaier 1984)

Species	Kingdom	group	cell type	T_c (h)	Notes	Reference
Dictyostelium discoideum	Fungiae	slime mold	cell	9		(McDonald and Durston 1984)
Euplotes eurystomus	Protista	ciliated protozoan	syncytial macronuclei	14	Divides amitotically.	(Prescott et al. 1962)
Euplotes eurystomus	Protista	ciliated protozoan	syncytial micronuclei	14	Divides while macronucleus is also dividing.	(Prescott et al. 1962)
Bacteria						
Salmonella	Bacteria	bacterium	Whole cell	0.42		Reviewed by (Van'T Hof 1965)
Escherichia coli	Bacteria	bacterium	Whole cell	0.67-1.5		Reviewed by (Poole 1984)
Alcaligenes eutrophus	Bacteria	bacterium	Whole cell	1.267		Reviewed by (Poole 1984)
Bacillus subtilis	Bacteria	bacterium	Whole cell	1.33		Reviewed by (Poole 1984)

Appendix 4

These supplemental tables accompany Chapter 5. Feeding and excretion by the glass sponge *Aphrocallistes vastus*.

Table S4.1. Results of post-hoc Bonferroni tests comparing $\delta^{13}C$ and $\delta^{15}N$ values from sponge tissue collected from (A) Fraser and (B) Galiano reefs in 2007, 2009, 2011, and 2014. (C) Bonferroni post-hoc tests were also used to compare $\delta^{13}C$ and $\delta^{15}N$ values between different sampling locations (Fraser Reef; Howe Reef, Galiano Reef, and outside of the SoG (Non-SoG). Statistically significant p-values (α=0.05) are in bold.

A.

Fraser Reef: overall p=0.000

	2007	2009	2011	2014
2007	-	1.00	**0.00**	
		0	**1**	0.091
2009	**0.00**	-	**0.00**	**0.03**
	0		**0**	**5**
2011	**0.00**	0.59	-	0.60
	0	8		4
2014	**0.00**	1.00		-
	0	0	0.713	

15N

13C

B.

Galiano Reef: overall p=0.000

	2007	2009	2011	2014
2007	-	**0.000**	**0.000**	**0.001**
2009	**0.025**	-	**0.015**	**0.002**
2011	1.000	**0.000**	-	0.870
2014	**0.000**	0.073	**0.000**	-

15N

13C

C.

By reef (sponge tissue): overall p=0.000

	Fraser	Galiano	Howe	Non-SoG
Fraser	-	**0.000**	1.000	**0.002**
Galiano	**0.000**	-	**0.000**	1.000
Howe	0.569	**0.000**	-	**0.020**
Non-SoG	**0.000**	1.000	**0.002**	-

15N

13C

Appendix 5

This figure accompanies Chapter 6. Spicule and flagellated chamber formation in a growth zone of *Aphrocallistes vastus*.

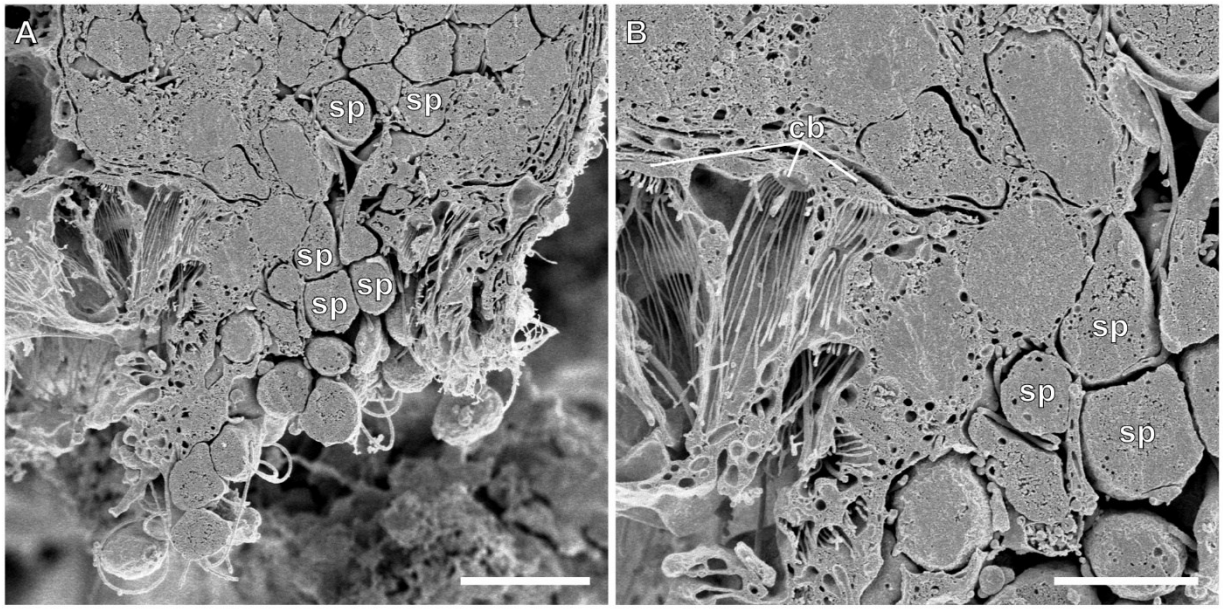

Figure S5.1. Sperm found near a flagellated chamber. A. Sperm (sp) were visible extruding out of a spermatocysts near a chamber. B. Closer view shows that collar bodies (cb) were surrounding the area that sperm were extruded into. Scale bars: A. 10 μm; B. 5 μm.