

MECHANICS AND SELECTIVITY OF FILTRATION BY TUNICATES

by

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## DISSERTATION ABSTRACT

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Doctor of Philosophy

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Title: Mechanics and Selectivity of Filtration by Tunicates

The preferential grazing of an organism on certain particles from the environment (selective feeding) impacts particle compositions and distributions in aquatic systems.

Historically, selective feeding has been examined almost exclusively through the lens of particle size. In this dissertation, I investigated size-based selection alongside particle shape, adhesive interactions, and the mechanical operation of the filter to characterize the selective-feeding capabilities of marine mucous-mesh filter-feeders (the planktonic appendicularian *Oikopleura dioica* and the benthic ascidians *Herdmania momus* and *Styela plicata*).

I used high-speed videography to describe the feeding-filter mechanics of *O. dioica* and tested its capacity for size-based particle selection. I show for the first time how pulsatile flow coupled with elasticity of the filter facilitates prey detachment. Using synthetic beads, I show that the food-concentrating filter selectively retains smaller particles because of their increased adhesion. Appendicularian houses may therefore retain particles size-selectively, which counters the historically-held assumption that appendicularians are non-selective grazers.

I synthesized ellipsoidal microbeads to test the effect of particle length-to-width ratios on the capture efficiency of *O. dioica* and *S. plicata*. Both grazers retained ellipsoidal particles according to their minimum diameter. I identified the kinematic mechanism for retention patterns of ellipsoidal particles using high-speed videography and endoscopy of particle interactions with

the mucous filters of *O. dioica* and *H. momus*, respectively. In the filters of both animals, ellipsoids oriented parallel to fluid streamlines and the minimum dimension of the particle intercepted the filters. I provide the first mesh-scale observations of particle capture by *H. momus*, show how particle shape influences hydrosol filtration by *S. plicata*, and suggest that ascidian filtration may not be adequately described by simple sieving.

This dissertation includes published and unpublished co-authored material.

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# CHAPTER I

## GENERAL INTRODUCTION

### **The Basis for Filter-Feeding**

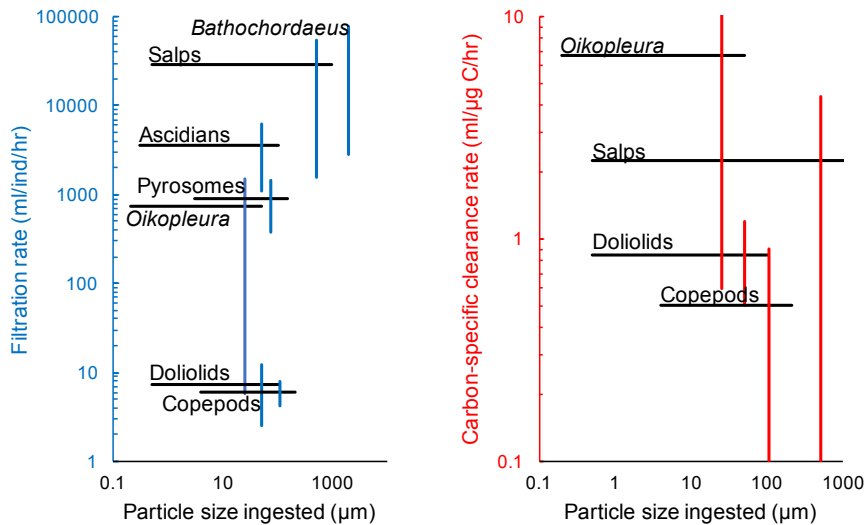
Conover (1968) described the ocean as a “nutritionally dilute environment”. Particulate food in the ocean is generally sparse, and varies both spatially and temporally; depending on the scale of the organism, nutrient variability may occur on timescales as short as seconds or minutes (Cohen and Fenchel 1994) and spatial scales as small as millimeters (Prairie et al. 2012). Suspension feeding—selecting suspended micro-organisms and detritus from the surrounding water (Hunt 1925)—is a widespread feeding strategy in both fresh and marine waters. Filter-feeding is a specific type of suspension feeding where water is filtered through “structures that retain particles mainly according to size and shape” (Jørgensen 1966). Because large volumes of water can be passed across the filter, filter-feeding is a common and effective adaptation for survival in a dilute fluid environment (Conover 1968).

### **Filtration Mechanics and Ecology of Tunicates**

Aquatic invertebrates exhibit a diverse array of filter-feeding structures, including the nets of Chaetopterid polychaete worms, the ciliary filters of bryozoans, the setal filters of amphipod crustaceans, and the cirral fans of barnacles (Riisgård and Larsen 2010). This dissertation focuses on those filter-feeders that capture suspended prey using a mucus net. Although this feeding mechanism has independently evolved in multiple animal classes (Riisgård and Larsen 2010), the area of focus for this dissertation is restricted to those mucous-mesh filter-feeders in the subphylum Tunicata. Tunicates are so named for their extracellular “tunic” that surrounds the zooid body, which is made of a material, tunicin, that resembles the

cellulose of plants. They are the closest living relatives of vertebrates, and are of great interest, both evolutionarily and ecologically (Delsuc et al. 2006, Ali and Tamilselvi 2016, Holland 2016, Henschke et al. 2016).

Tunicates occupy an important role in the marine food web, since they have high filtration rates and are one of the few metazoans that efficiently graze on picoplankton (0.2-2  $\mu\text{m}$ ) (Fig. 1). The average predator-prey size ratio in pelagic systems is 14: 1 (Sheldon, Sutcliffe Jr, and Paranjape 1977), and usually ranges from 1: 1 to ~100: 1 for planktonic predators (Hansen, Bjornsen, and Hansen 1994). The ratio for tunicates, however, can be as great as 10,000: 1 (Gorsky and Fenaux 1998) (Fig. 1). The ingestion of picoplankton by tunicates may short-circuit the microbial loop, more efficiently transferring carbon both to higher trophic levels (carnivorous zooplankton and fish) and exporting it from surface waters to depth via their fast-sinking fecal pellets (Pomeroy 1974, Yoon et al. 1996, Ramaswamy, Sarin, and Rengarajan 2005). Below I briefly summarize the feeding mechanics and ecology of each of the three tunicate classes: Ascidiacea, Larvacea, and Thaliacea. Some of the tunicate groups are less studied than others because of patchy or episodic distribution, and/or difficulties surrounding capturing, handling, and maintenance in captivity (Henschke et al. 2016). In addition, the feeding mechanisms of some taxa are easier to observe than others—for example, appendicularian houses are external and relatively large, whereas the transparent ciliary feeding mechanisms of doliolids are comparably quite difficult to observe (Deibel and Paffenhöfer 1988).

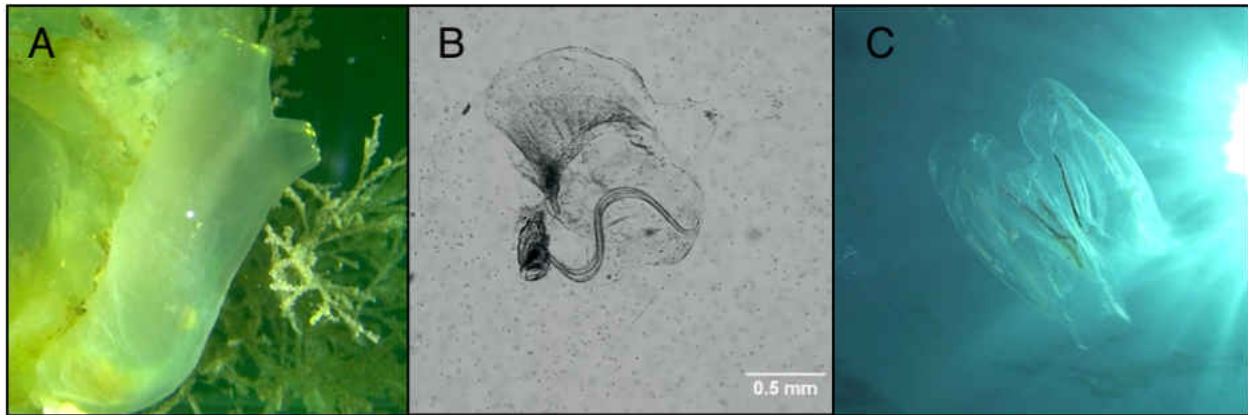


**Figure 1** Individual filtration rates and carbon-specific clearance rates of different grazers vs. their ingested particle size range (Table S1, Appendix). Point at which lines intersect represents average filtration or clearance rate.

### *Ascidiaceae*

Ascidiaceae is the only class within the Tunicata subphylum that is both benthic and sessile, and is also the most taxonomically diverse group (Tsagkogeorga et al. 2009). They are the most thoroughly studied tunicate, likely because their shallow, coastal distribution makes them easy to access (Holland 2016). They may be solitary or colonial. The ascidian mucus mesh is suspended across a basket-like structure called the branchial sac, perforated with ciliated openings called stigmata. Ascidiaceae feed by pumping water through an inhalant (or oral) siphon, into the branchial sac, and out through an exhalant (or atrial) siphon (reviewed in Millar 1971, Petersen 2007) (Fig. 2A). The three ascidian orders are classified on the basis of the structure of the branchial sac (Tsagkogeorga et al. 2009), which varies from flat to folded (Petersen 2007). In all orders, the mucus mesh is secreted by the endostyle and transported by cilia dorsally across the interior of the basket to the dorsal lamina, where it is wound into a string and conveyed to the esophagus (Petersen 2007). The speed of particle transport on the mesh varies both within and between species (Table 1). Transmission and scanning electron microscopy of the mucus mesh

revealed little structural variation between the six species studied (Flood and Fiala-Medioni 1981). Because of their fine filter, ascidians affect a broad range of particle sizes (Fig. 1). Some deep-sea ascidians do not filter-feed at all, but are thought to directly engulf particles using their mouth (Okuyama et al. 2002).



**Figure 2** The three classes of tunicates. (A) Solitary ascidian *Ciona intestinalis*; (B) Appendicularian *Oikopleura dioica*; (C) Salp *Cyclosalpa quadriluminis*. Photos A and C by K.R. Sutherland.

### *Thaliaceans*

Thaliacea, which includes salps, doliolids, and pyrosomes, were recently described as “the neglected pelagic relatives of ascidians” (Piette and Lemaire 2015). Their feeding mechanisms are less well-studied than that of ascidians or appendicularians, in part because of difficulty sampling and culturing, and also their patchy distribution (Henschke et al. 2016). In all thaliaceans, as in ascidians, the general process of feeding involves secretion of the mucous mesh by the endostyle, which moves posteriorly toward the esophagus, where it is rolled into a mucus string by cilia on the gill bar and ingested (Madin 1974).

Salps and doliolids are barrel-shaped zooids that generate a feeding current primarily by contractions of the circumferential muscles and cilia, respectively (Deibel and Lowen 2012). Locomotion and filter-feeding are coupled in salps: circular muscle bands draw water in through

the inhalant siphon, through the pharynx and mesh, and out the exhalent siphon (Fig. 2C). The feeding current of doliolids, in contrast, is achieved through beating of the cilia of the gill apertures with little movement of the zooid body (Deibel and Paffenhöfer 1988, Bone et al. 1997, Bone 1998). The mucus net of salps is suspended across the entire pharynx by peripharyngeal bands (Madin 1974), whereas in doliolids the pharyngeal wall is reduced and the filter only occupies ~a quarter of the volume of the pharynx (Bone et al. 1997). The dolioid filter movement is more complicated than that of salps: the filter is rotated in a spiral-like manner within the pharynx by the dorsal spiral volute of the peripharyngeal bands (Bone et al. 1997). This rotation causes inhaled particles to follow a curved path and be trapped between two layers of mucus rather than one. In salps the mesh appears to be secreted continuously *in situ* (Madin 1974), but laboratory observations of doliolids suggest that mucus secretion may be under neural control (Bone et al. 1997). A tangential component of particle encounter has been suggested for salp filters (Sutherland, Madin, and Stocker 2010).

Pyrosomes are permanently colonial, with zooids held side-by-side in a gelatinous tunic (Godeaux, Bone, and Braconnot 1998). The structure of individual zooids resembles that of solitary ascidians with an inhalant and exhalent siphon. The tubular colonies move slowly by the continuous jetting of fluid out of a common aperture (Bone 1998). Much less is known about the feeding behavior and mechanisms of pyrosomes compared to the other thaliaceans (Madin and Deibel 1998).

### *Appendicularians*

The class Appendicularia are solitary holoplankters that include oceanic, continental, and even coastal species (Deibel and Lowen 2012). Appendicularians are the only tunicate that retains the tadpole larval characteristics throughout their lifespan (Tsagkogeorga et al. 2009), and

are currently considered basal in the chordate phylogeny (Holland 2016). Despite their simple body architecture, the appendicularian feeding process is the most complex of any of the tunicates. They have both an external cellulose and mucus filtration apparatus (the house) and an internal mucus filter (the pharyngeal filter). Sinusoidal beating of the muscular tail drives flow into the house through the inlet filters, down the tail chamber and into the food-concentrating filter (Alldredge 1977). The food-concentrating filter is the only tunicate filter broadly recognized to function as a tangential filter, where particles travel parallel to the filtering mesh and are concentrated (Flood, Deibel, and Morris 1998). In general, the food-concentrating filter has a much finer mesh (mean=0.16 x 0.81  $\mu\text{m}$ ) (Flood, Deibel, and Morris 1998) than that of the pharyngeal filter (Table 1). After being conveyed through the food-concentrating filter, flow then moves through the buccal tube and into the mouth, where the pharyngeal filter is suspended across the pharynx. The pharyngeal filter is shared by all tunicate classes.

The aforementioned description of the appendicularians feeding process is a generalization based on the published studies of Oikopleurids. Important differences exist, however, in the house structure of the three appendicularian families (Oikopleuridae, Fritillariidae, Kowalewskidae) (Flood, Deibel, and Morris 1998). Fritillarids and Oikopleurids share a coarse pre-filter (inlet filters) that excludes large (13-170  $\mu\text{m}$ ) or spinous particles from entering the house, as well as a fine food-concentrating filter that excludes water and concentrates suspended particles (Alldredge 1977, Flood, Deibel, and Morris 1998, Gorsky and Fenaux 1998, Flood 2003). A few species, such as *Oikopleura longicauda* and the mesopelagic species *Mesochordaeus erythrocephalus*, lack inlet filters (Alldredge 1977, Hopcroft and Robison 1999). Houses from appendicularians in the Kowalewskidae family differ greatly from that of the Fritillarids and Oikopleurids, but details of their structure and function is lacking

(Flood, Deibel, and Morris 1998). The meter-sized giant larvacean houses from the mesopelagic Bathochordaeinae subfamily also differ greatly in structure from other oikopleurids (Hamner and Robison 1992, Sherlock et al. 2017).

Because of the house, appendicularians are able to retain particles without necessarily ingesting them. Any discussion of their impact on particle size spectra is thus necessarily complex. Particles can be retained on the inlet filters, food-concentrating filter, the house walls, or fecal pellets that remain stuck to the house. A variety of organisms feed upon discarded houses, and may selectively graze on different components of the house for the different particle sizes and types retained there (Alldredge 1972, Ohtsuka et al. 1993, Gorsky and Fenaux 1998).

**Table 1** Structure and hydrodynamics of the pharyngeal filter of tunicates.

Taxa	Mesh width ( $\mu\text{m}$ )	Mesh length ( $\mu\text{m}$ )	Mucus translational speed ( $\mu\text{m s}^{-1}$ )	Reynolds number	References
Ascidians	0.2-0.5 (range, multiple species)	0.5-2.2 (range, multiple species)	20-220 (range, multiple species)	$10^{-4}$	Flood and Fiala- Medioni 1981, Petersen 2007, Armsworthy, MacDonald, and Ward 2001, Flood 1982
Appendicularians	0.9-6.0 (range, <i>Oikopleura</i> <i>vanhoeffeni</i> )	1.96-14.3 (range, <i>Oikopleura</i> <i>vanhoeffeni</i> )	35-154 (range, <i>Oikopleura</i> <i>vanhoeffeni</i> )	$10^{-5}$	Acuña, Deibel, and Morris 1996, Deibel and Powell 1987
Salps	0.3-2.0 (range, multiple species)	0.9-7.5 (range, multiple species)	464-76	$10^{-3}$	Sutherland, Madin, and Stocker 2010, Bone, Carre, and Chang 2003 Sutherland, unpublished data
Doliolids	0.4? ( <i>Doliolina</i> <i>mulleri</i> )	0.45? ( <i>Doliolina</i> <i>mulleri</i> )	30-40 (range, multiple species)	$10^{-5}$	Bone et al. 1997, Deibel and Paffenhöfer 1988
Pyrosomes	0.6 ( <i>Pyrosoma</i> <i>atlanticum</i> )	0.6 ( <i>Pyrosoma</i> <i>atlanticum</i> )	Unknown	Unknown	Bone, Carre, and Ryan 2000



## **Questioning the Paradigm of Non-Selective Mucous-Mesh Filter-Feeding**

Selective feeding is defined as “an imbalance between the proportion of prey types in a predator's diet and the proportion in the environment” (Strom and Loukos 1998). Historically, mucous-mesh feeders have been assumed to filter-feed non-selectively (Bedo et al. 1993, Acuña, Deibel, and Morris 1996, Madin and Deibel 1998, Gorsky et al. 1999, González et al. 2000, Paffenhöfer and Köster 2005, Lee, Köster, and Paffenhöfer 2012), with the particle size range that the animal ingests determined solely by the dimensions of the pharyngeal filter pores (Table 1). This has increasingly been called into question, beginning with the application of aerosol filtration theory to aquatic biological filters (hereafter referred to as hydrosol filtration) by Rubenstein and Koehl (1977), followed by experimental results showing capture of particles smaller than the filter mesh pores (Loudon and Alstad 1990, Sutherland, Madin, and Stocker 2010, Acuña, Deibel, and Morris 1996, Nishikawa and Tsuda 2001, Deibel 1986). Mounting evidence shows that particle properties other than size, such as hydrophobicity or charge, also influence selection by filter-feeders (“qualitative selection”) (Labarbera 1978, Gerritsen and Porter 1982, Monger, Landry, and Brown 1999, Rosa et al. 2013, Dadon-Pilosof et al. 2017, Rosa et al. 2017). Despite this, calculations of aerosol particle capture mechanisms in models of biological filtration systems typically assume spherical particles that invariably and uniformly adhere to the filter fibers (Rubenstein and Koehl 1977, Silvester 1983, LaBarbera 1984, Loudon and Alstad 1990).

## **Dissertation Content**

This study characterizes particle-level interactions with the mucous feeding-filters of tunicates and identifies novel size- and shape-dependent selection mechanisms. I used incubation experiments, direct sampling, and image analysis to determine how particle size, shape, and filter

mechanics affect the retention efficiencies of appendicularians and ascidians. I elucidated the mechanisms for the observed retention patterns results using either high-speed videography (for appendicularians) or endoscopy (for ascidians). Chapter II provides a mechanistic basis for understanding particle selection by the cosmopolitan appendicularian *Oikopleura dioica* by showing how elastic deformation of the fibers causes size-selective retention of particles by the house. Chapter II is co-authored by Brad Gemmell, Jean-Marine Bouquet, Eric Thompson, and Kelly R. Sutherland. Chapter III, co-authored by Kelly R. Sutherland, isolates the effect of particle shape on retention by the appendicularian house and ingestion by the animal. I identified minimum particle diameter as the key variable for determining how non-spherical cells are grazed by *O. dioica*. In Chapter IV, I show that the minimum particle diameter also determines capture efficiency by benthic ascidians via hydrosol filtration mechanisms. Chapter IV is co-authored by Aviv Ben-Tal, Yuval Jacobi, Gitai Yahel, and Kelly R. Sutherland. Endoscopy observations revealed the *in vivo* hydrodynamics and mucus mesh behavior surrounding the particle capture process by ascidians. Collectively, the results reveal that the mechanics of mucous-mesh filtration are more complex than previously assumed and counter the historically-held assumption that mucous-mesh filtration is a non-selective process.

## CHAPTER II

### A SELF-CLEANING BIOLOGICAL FILTER: HOW APPENDICULARIANS MECHANICALLY CONTROL PARTICLE ADHESION AND REMOVAL

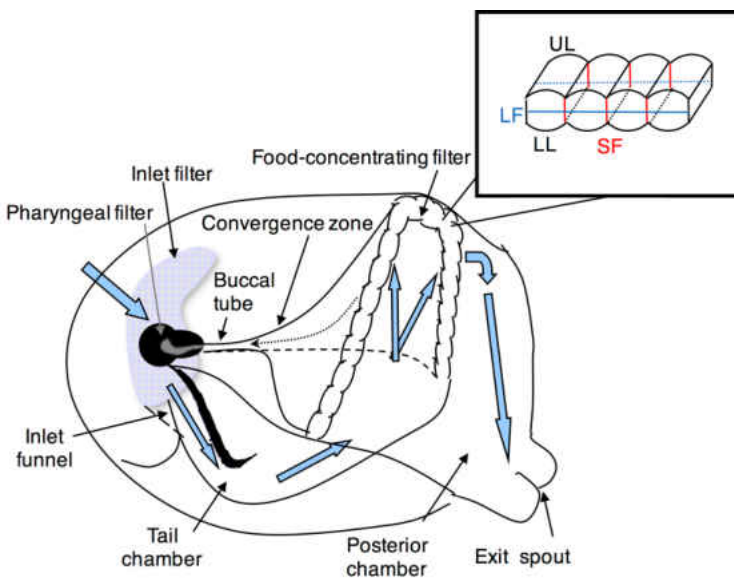
The second chapter of this dissertation has been accepted for publication in *Limnology and Oceanography* and is co-authored by Brad Gemmell, Jean-Marine Bouquet, Eric Thompson, and Kelly R. Sutherland. I helped conceive and design the experiments, performed the experiments (both high-speed videography of filter fiber elasticity and follow-up experiments to test size-selectivity of the filter fibers), analyzed and presented all of the data, contributed to funding acquisition, and wrote the initial draft of the manuscript. Co-authors contributed to funding acquisition, mentorship/supervision, helped conceive the experiments and designed methodology, helped perform a subset of the experiments (high-speed videography of filter fiber elasticity by BG, KRS, JMB, and ET), contributed expertise, and reviewed and edited the manuscript.

#### **Introduction:**

Planktonic grazers influence particle size structure, plankton diversity, and energy transfer in aquatic foodwebs (Sommer and Stibor 2002). Their influence depends upon the structure of the feeding apparatus and the hydrodynamic aspects of particle capture. Numerous grazers, such as pteropods, tunicates, lancelets, chaetopeterid polychaetes and certain gastropods, rely on a mucous filter to capture suspended prey—typically ingesting the entire mesh along with retained food particles (Riisgård and Larsen 2001). Suspended particles may either travel perpendicular to the filter (sieving) or parallel to it (tangential filtration). If suspension feeding occurs solely via sieving, the filter only retains particles larger than the mesh pores; whereas, in tangential filtration fewer particles directly contact the filter and they become concentrated as water is excluded through the filter (Brainerd 2001). The mechanisms by which a biological filter captures particles have broad implications for feeding efficiency and particle size-selection (Rubenstein and Koehl 1977).

Appendicularians (Phylum: Chordata, Subphylum: Tunicata) are a class of globally

abundant, planktonic marine grazers that can filter-feed on particles several orders of magnitude smaller than themselves, down to picoplankton (0.2-2  $\mu\text{m}$ ) (Acuña, Deibel, and Morris 1996, Gorsky and Fenaux 1998). Appendicularians have both an internal filter (the pharyngeal filter) and an extracellular mucous filtration apparatus called the house (Fig. 3). The appendicularian house is one of the most intricate structures made by an animal (Alldredge 1977). It is a spherical extracellular secretion that the animal lives inside and uses to concentrate prey particles from seawater. Appendicularian filtration shares the fundamental particle interception mechanisms employed by numerous filter-feeders, including sieving, direct interception, and diffusional deposition (Acuña, Deibel, and Morris 1996).



**Figure 3** Schematic representation of the Oikopleurid house structure and water circulation (blue arrows) (modified from Thompson et al. 2001). Inset shows magnified view of the FCF slots and the intermediate layer, composed of the lateral fibers (LF) and suspensory fibers (SF). UL: upper layer; LL: lower layer.

The oikopleurid house (Class: Appendicularia, Family: Oikopleuridae) has two distinct mucous filters: inlet filters (IF) and the food-concentrating filter (FCF) (Fig. 3). Particles that adhere to the FCF need to detach in order to be subsequently conveyed through the FCF to the

internal pharyngeal filter for ingestion (Deibel 1988, Morris and Deibel 1993). Therefore, the degree of adhesion by different particle types governs both retention in the house and ingestion by the animal. The composition of particles retained by the house has important implications for biogeochemical cycling because discarded appendicularian houses are a major source of particulate organic carbon in the ocean, possibly even exceeding phytoplankton carbon (Alldredge 1976, Katija et al. 2017). The particles that are conveyed to the pharyngeal filter for ingestion by the animal are either incorporated into animal biomass for subsequent transfer within the pelagic foodweb, or into fecal pellets that contribute to the flux of carbon to depth.

During feeding, sinusoidal beats of the animal's tail bring water into the house and control water flow through the house. There are also frequent periodic tail arrests, where the tail abruptly straightens. The house is elastic, and the tail beat and arrest cycles alternately expand and contract the house (Selander and Tiselius 2003). The FCF, the focus of the present study, has a three-layered structure similar to that of a parafoil parachute (Fig. 3) and performs tangential filtration to concentrate particles. An upper (mesh dimensions:  $0.98 \times 0.15 \mu\text{m}$ , (Flood, Deibel, and Morris 1998) and lower ( $0.24 \times 0.07 \mu\text{m}$ ; (Flood 1978, Morris and Deibel 1993) layer are held together by an intermediate screen that is connected to the filter ridges of the FCF by suspensory filaments with pores that are  $\sim 30 \mu\text{m}$  wide (Flood 1991) (Fig. 3). Two ciliated funnels called spiracles generate a current to convey particles from the FCF through the buccal tube and into the mouth (Burighel et al. 2001, Lombard, Selander, and Kiørboe 2011) (Fig. 3).

There is debate about how the FCF works, including the mechanisms controlling particle adhesion and detachment. The prevailing assumption has been that the fine lower layer of the FCF concentrates particles (Flood 1978), while the intermediate screen simply attaches the two layers (Deibel 1986). Since the pores of the intermediate screen are up to twice as wide as those

of the IF, a possible straining function has been proposed (Alldredge 1977) and disputed (Flood 1991). Previously, pulsatile flow has been suggested to play a role in the particle adhesion detachment process by the FCF of the cold-water appendicularian *O. vanhoeffeni*, but this mechanism was not investigated in detail (Deibel 1988, Deibel and Paffenhöfer 1988, Morris and Deibel 1993). Acquiring a better understanding of the mechanisms of filtration by the FCF is important because of the filter's remarkable capacity to concentrate particles up to 1000x the concentration of ambient seawater (Morris and Deibel 1993). Furthermore, understanding the mechanisms of biological self-cleaning systems has practical applications for biomimetics (Liu and Jiang 2012).

In this study, we tested three hypotheses: 1) that pulsed flow is the mechanism for particle detachment; 2) that the intermediate layer of the FCF plays a purely structural role, and 3) that no size-selection by the house occurs through particle interactions with the FCF.

## **Methods:**

### *Hydrodynamics of the appendicularian house*

All visualizations were conducted at the Sars Centre for Marine Molecular Biology, Bergen, Norway, using cultured *Oikopleura dioica* (Bouquet et al. 2009). We used high-resolution, high-speed microvideography to visualize the hydrodynamics of filtration within the house. The setup for visualizations followed that previously described (Gemmell, Jiang, and Buskey 2014). Briefly, an individual appendicularian (day 5 or 6) was added to a 50 mL glass cuvette. Flow was traced using live, unicellular microalgae *Rhinomonas reticulata* for observations of the whole house at low magnifications (4x) or *Isochrysis galbana* at high magnifications of particular areas (40x). Images were recorded using an Edgertronic high-speed camera (1280×1024 pixel resolution, 500 frames s<sup>-1</sup>) with brightfield illumination from a fiber

optic light source. The filming vessel was positioned on a manually adjustable stage between the light source and the camera. A long working-distance microscope objective (4x or 40x) was mounted to an adjustable-height optics clamp positioned between the filming vessel and the camera.

Videos were converted to an image stack in QuickTime Pro. ImageJ software was used for subsequent velocity measurements. The speed of the detaching particles was assessed by particle-tracking velocimetry, performed either manually, by tracking individual particles between frames using the plugin MTrackJ (Meijering, Dzyubachyk, and Smal 2012), or automatically using the ParticleTracker plugin for videos with high particle densities. The instantaneous velocity of the filter fibers was measured as the change in arc length between the extended and relaxed fiber divided by the change in time. All results are reported as the mean  $\pm$  95% confidence interval from day 5 animals unless stated otherwise.  $N$  is used throughout to refer to the number of individual animals and  $n$  for number of observations. In all cases,  $N$  was used to calculate the confidence interval.

#### *Morphology and function of the intermediate screen*

The setup for visualizing the filtration apparatus structure was the same as that used for visualizing the hydrodynamics, except that animals were placed in a dilute milk (Tinemelk® low-fat milk, 1.2% fat) bath (~1:10000 milk: seawater) to filter for ~1 hr and then rinsed in clean seawater prior to videography. Deposition of milk fat particles facilitated visualizations of the filter fibers of the FCF intermediate screen. ImageJ was used for subsequent velocity and morphometric measurements.

#### *Particle adhesion to the food-concentrating filter*

To experimentally determine how particle size affects adhesion to the appendicularian

FCF, we used latex microspheres that allowed us to maintain constant surface properties while varying only particle size. The animals were fed a mixture of different sized (3, 6, 10, and 20  $\mu\text{m}$  diameter) fluorescent polystyrene microspheres (Polysciences, Inc.) ( $10^{-1}$  to  $10^{-2}$  beads  $\text{mm}^2$  of FCF). To visualize bead interactions with the FCF, we used a Sony 4K FDR-AX100 HD camcorder (1280 x 720 pixel resolution, 120 frames  $\text{s}^{-1}$ ) mounted to a Nikon Eclipse E400 microscope with a 10x objective using a Martin Microscope M99 Camcorder Adapter. Immature day 5 ( $N=5$ ) or day 6 ( $N=2$ ) animals were first placed in 0.2  $\mu\text{m}$  FSW to inflate a new, clean house. Animals were then filmed individually in a glass embryo dish with a suspension of the microspheres. Because the visualizations were kept very short, the number of tail beating cycles prior to the measurements was small and approximately equal across treatments.

Videos were converted to image stacks and analyzed using ImageJ as described above. Percentage particle adhesion was calculated by counting the number of microspheres adhering to the FCF immediately prior to a single tail arrest and then tracking the number of particles that detached or remained adhered after the FCF re-inflated upon recommencement of tail beating. Only new adhesion events from incoming particles were analyzed (beads that were already stuck to the filter prior to commencement of videography were excluded from the analysis).

### *Statistical analysis*

Analysis of size-dependent adhesion was conducted using R Studio (version 1.0.143 © 2009-2016) using particle size (3, 6, or 10  $\mu\text{m}$ ) as a predictor variable of percent adhesion to the FCF. Twenty-micron particles were excluded from statistical analysis because of the comparably low sample size for adhesion measurements ( $n=11$ ,  $N=3$ ). The distribution of percent adhesion did not adhere to the ANOVA assumption of normality (evaluated using a normal probability plot) and arcsin-transformed data still differed significantly from a normal distribution (Shapiro-

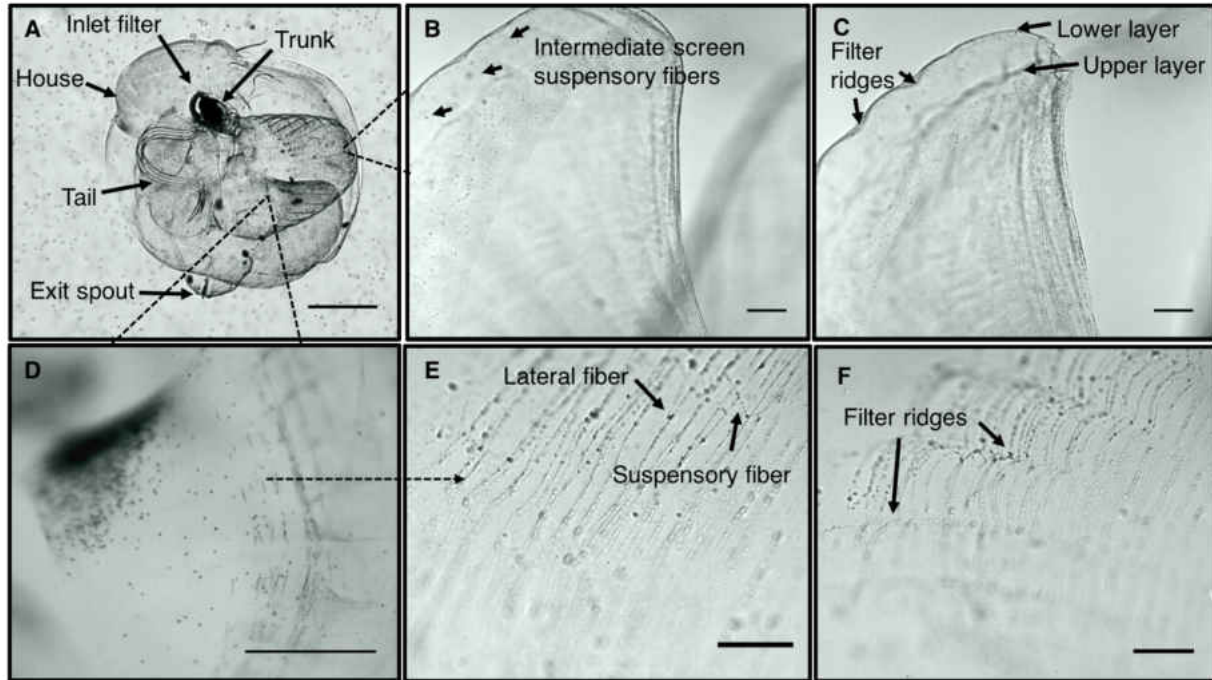


Wilk normality test,  $W = 0.749$ ,  $P < 0.001$ ). Size-dependent adhesion was therefore tested using a nonparametric Kruskal-Wallis one-way ANOVA on ranks, followed by a Nemenyi test for pairwise multiple comparisons.

## **Results:**

### *Hydrodynamics of the appendicularian house*

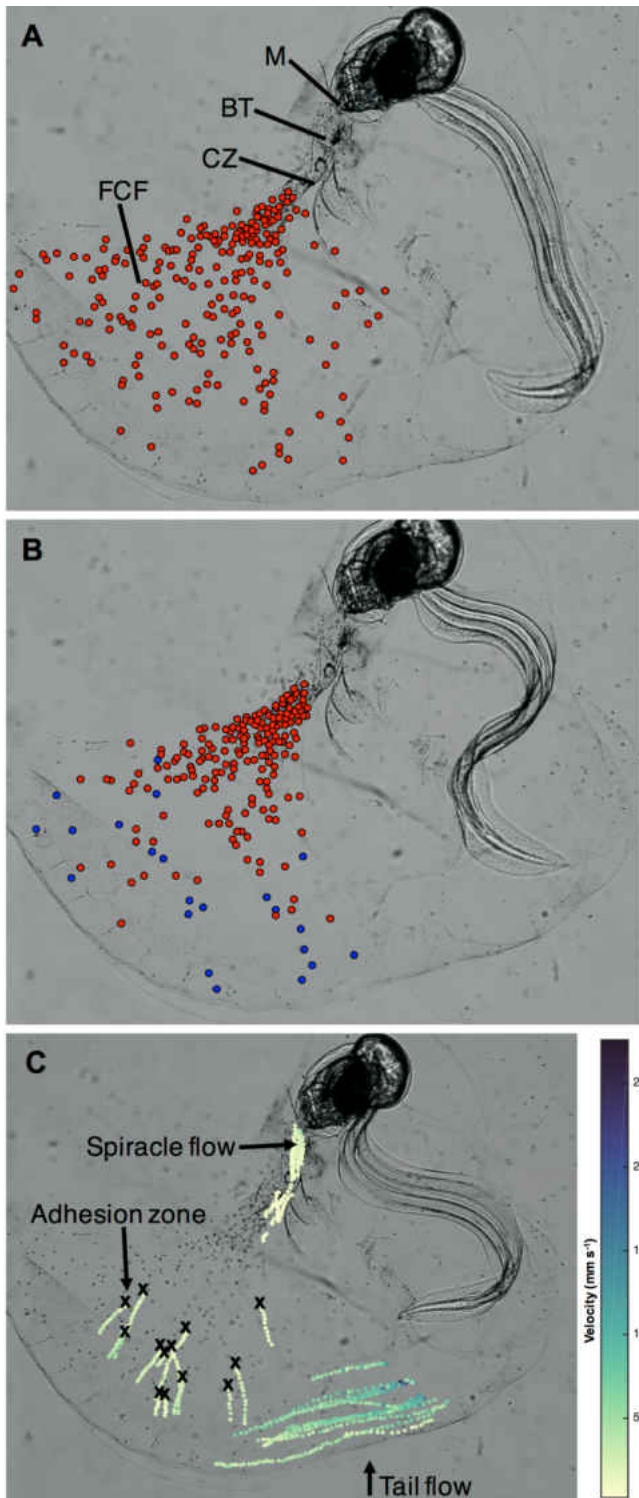
Appendicularians forced water into the house through the sinusoidal beating of the tail, which inflated the house like a balloon. The tail beat cyclically, alternating between continual beating and periodic tail arrests. The tail arrest partially deflated the house, whereby water exited out of the two inlet filters and cleared accumulated particles from the inlet and food-concentrating filters as previously described (Deibel 1988, Flood 1991, Selander and Tiselius 2003). Alternation between tail beating and tail arrest not only deflated and re-inflated the FCF (Fig. 4B, C), it also changed the shape of the individual mesh fibers of the intermediate layer (Fig. 4E, F). The mesh fibers exhibited an elastic, accordion-like conformational change: when the house partially deflated during a tail arrest due to a presumed decrease in fluid pressure, the fibers bent and shortened. We were not able to resolve the individual fibers of the lower or upper layers of the FCF, but the elasticity of the intermediate fibers was mirrored by the entire FCF. When the house re-inflated as the tail resumed beating, the bent fibers recoiled, which, in combination with the pulsatile flow induced by the tail, caused a pulse of previously-adhered particles to detach and travel down the filter toward the buccal tube (Fig. 5A and B).



**Figure 4** Brightfield images extracted from microvideography of free-swimming *Oikopleura dioica* showing the effect of the tail beat and arrest cycle on the structure of the food-concentrating filter. (A) *O. dioica* with *Rhinomonas* as tracer particles (4x objective). (B) Inflated wing of food-concentrating filter during tail beat (40x objective). (C) Deflated wing of food-concentrating filter during tail arrest. (D) Magnified view of the food-concentrating filter showing fibers of intermediate screen (11.5x objective). (E) The extended fibers of the intermediate screen during tail beating (40x objective). (F) The compressed fibers of the intermediate screen during period of tail arrest (40x objective). Scale bars: A, D: 0.5 mm; B, E, F: 0.1 mm.

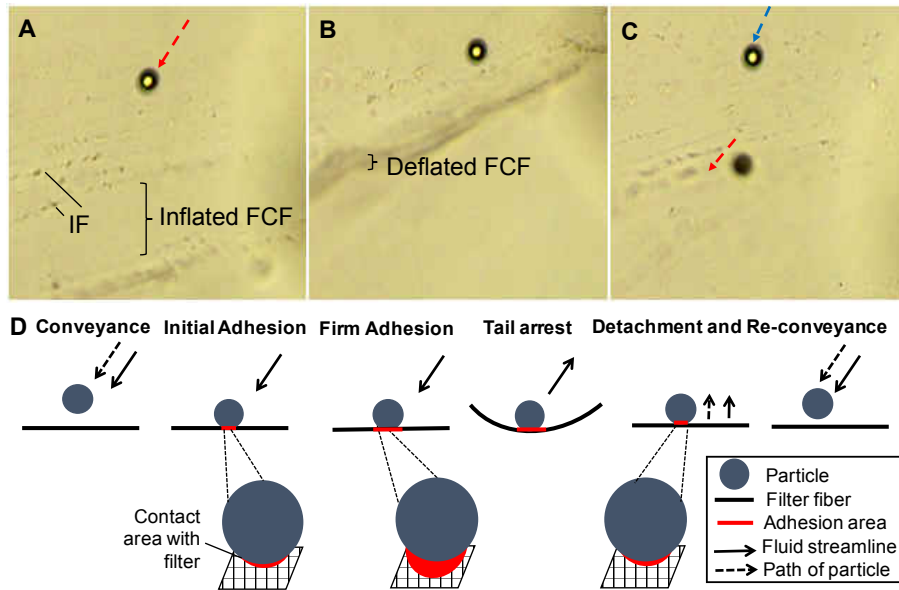
Particles that entered the FCF first adhered in an “adhesion zone” on the outer margin of the FCF, characterized by both low flow from the tail and low flow from the spiracles (Fig. 5C). An average of  $90\% \pm 25\%$  of *R. reticulata* that entered the FCF first adhered to the FCF prior to being conveyed to the pharyngeal filter ( $n=30$ ,  $N=3$ ). Particles also detached most frequently from this outer part of the FCF, opposite the trunk (Fig. 5A and B). After detachment, particles were laterally conveyed toward the buccal tube at  $1.35 \pm 0.94 \text{ mm s}^{-1}$  ( $n=3366$ ,  $N=8$ ). The percentage change (%) in length of the fibers between their maximally contracted and extended state was  $10 \pm 16$  ( $n=10$ ;  $N=3$ ). The average velocity of the fibers was  $0.19 \pm 0.58 \text{ mm s}^{-1}$  ( $n=21$ ,

$N=3$ ), which gives a Reynolds number of  $10^{-4}$  for individual filter fibers, calculated using diameter of the lateral fibers of the intermediate screen ( $\sim 2 \mu\text{m}$ ) as the characteristic length scale. The maximum measured instantaneous velocity of the fibers was  $0.97 \text{ mm s}^{-1}$ . The phases of particle adhesion and detachment to the FCF, and the hypothesized role of filter elasticity, are summarized in Fig. 6.



**Figure 5** Impact of the tail on the distribution of *Rhinomonas* particles in the food-concentrating filter of *Oikopleura dioica*. (A) Attached particles during the tail arrest cycle (red) and (B) pulse of detached particles collecting near the entrance of the buccal tube from the recommencement of the tail beating. Particles in blue were not present in (A) but entered the filter from the tail chamber as the filters re-inflated. (C) Map of adhesion of *Rhinomonas* particles to the FCF during

continuous beating of the animal's tail. Black Xs show the location of particle adhesion. Heatmap shows particle velocities in the tail chamber, the FCF, and the buccal tube. FCF: food-concentrating filter; CZ: convergence zone; BT: buccal tube; M: mouth.



**Figure 6** The process of particle adhesion to and detachment from the food-concentrating filter of *Oikopleura dioica*. (A) A 20 μm latex bead is conveyed laterally along the intermediate screen of the FCF (red arrow) toward the buccal tube due to the hydrostatic pressure generated by the animal's tail beating and by suction from the mouth. (B) Bead adheres to the FCF. A tail arrest deflates the FCF, which results in a temporary reversal of flow away from the buccal tube in a “backflush,” and causes the individual mesh fibers of the intermediate screen to bend. (C) The tail resumes beating, the FCF re-inflates and the mesh fibers return to their extended conformation, causing the bead to detach and move toward the buccal tube. Blue arrow shows a new bead entering the field of view as a pulse of particles progresses down the FCF. (D) Schematic representation of the steps of the adhesion and detachment process that occur in A-C, with the inferred changes in adhesive contact between the particle and the filter fiber. Firm adhesion occurs as time-dependent chemical adhesive interactions take place between the particle and the filter fiber. The elasticity of the fiber resets this process.

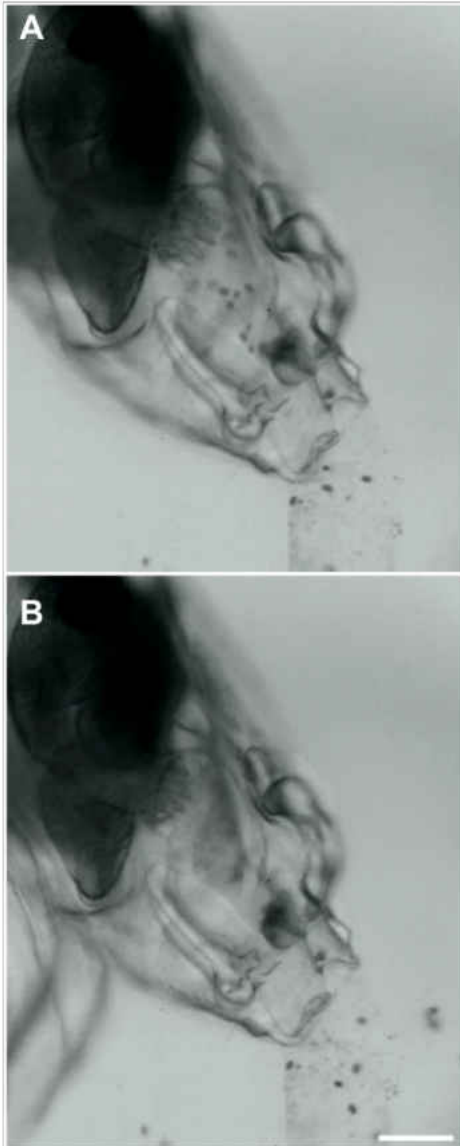
Although Flood (1991) reported that the tail arrests serve to aggregate particles, we observed that beads remained mostly disaggregated (i.e., did not stick together) during conveyance through the food-concentrating filter and buccal tube, and were usually captured as singlets by the pharyngeal filter. The deflation of the house during tail arrests reduced the inter-particle distance, but beads rarely adhered to each other when the FCF re-inflated.

### *Observations of feeding behavior*

We observed that appendicularians are able to decouple filtration driven by tail beating from suction driven by the spiracles. Animals could draw particles into the house, but prevent the entry of particles from the food-concentrating filter into the buccal tube through a previously undescribed mechanism: flow could be halted at the convergence zone of the two fans of the food-concentrating filter such that no particles entered the buccal tube while the buccal tube remained attached to the mouth. Alternatively, flow could enter the buccal tube and particle selection could be controlled through a valve in the buccal tube. In the absence of suction from the mouth, the valve was forced open during the tail arrest, then resealed when tail beating resumed. When suction from the mouth caused flow into the buccal tube, the valve closed, sealed by the influx of water, even during tail arrests. Inverted flux from water forcefully exiting the mouth caused the valve to open and particles in the buccal tube were drawn into the exit chamber.

At low frame rates, the ciliary spiracles appear to rotate due to a stroboscopic effect, but at higher frame rates the rotation is evidently a metachronal wave. The mechanism that accomplishes flow reversals to expel particles from the pharyngeal cavity has been vague, described as a “ciliary reversal” that may involve a change in the orientation of cilia, a change in the direction of ciliary beating, or both (Galt and Mackie 1971, Fenaux 1986, Lombard, Selander, and Kiørboe 2011). Although at lower frame rates (120 fps) this reversal appears to occur in the clockwise direction, this is due to stroboscopic effect. At high-speed (500 fps) we observed the reversal occurred only in the dorsal-ventral direction. Reversal of the ciliary beat direction changed the overall shape of the spiracles and appeared to reduce the size of the canal

between the cilia (i.e., formed a constriction). Each episode of flow reversal was associated with such a constriction event (Fig. 7).

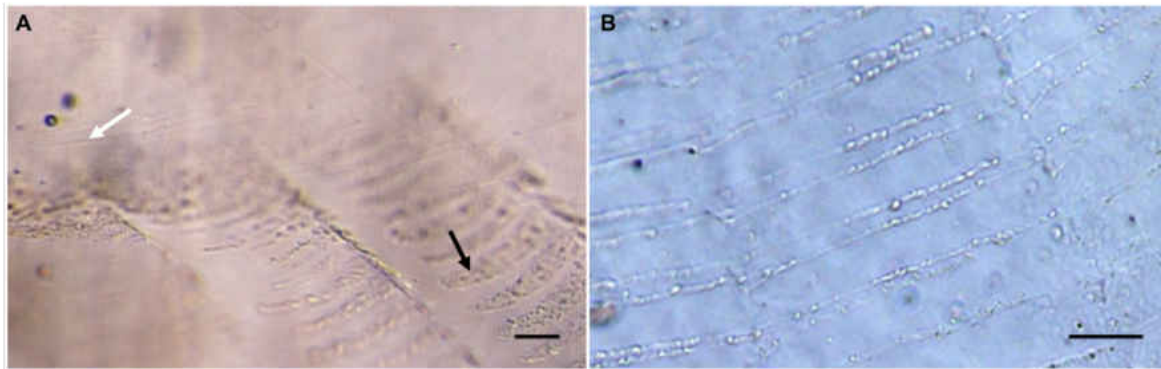


**Figure 7** Changes in the conformation of the ring of spiracle cilia of *Oikopleura dioica*. (A) Structure of the spiracle cilia when water is drawn into the mouth. (B) Structure of spiracle cilia during reversal of flow and particles out the mouth. Scale bar: 0.1 mm.

#### *Morphology and function of the intermediate screen*

The milk bath allowed visualization of the coarse fibers of the intermediate layer of the FCF (Fig. 8). The fibers were arranged in a rectangular mesh with a mean width and length of  $W=22 \mu\text{m} \pm 7 \mu\text{m}$  and  $L=160 \mu\text{m} \pm 22 \mu\text{m}$  ( $n=25$ ,  $N=5$ ) (Fig. 8B). The length of the intermediate

mesh was determined by the diameter of the filter ridges of the FCF. The margins of the ridges had vertical suspensory fibers connected to the lower and upper layers and crossed with the lateral fibers of the intermediate layers (Fig. 4B, E). The lateral fibers of the intermediate screen with deposited milk fat particles had a mean width of  $2 \pm 0.8 \mu\text{m}$  ( $n=25$ ,  $N=5$ ). Milk particles ( $<1 \mu\text{m}$ ) primarily collected on the lower layer (Fig. 8A), not the upper layer. Through milk-stained visualizations of the intermediate layer of the FCF, we were able to observe particles being conveyed directly along the intermediate screen (Fig. 8A).

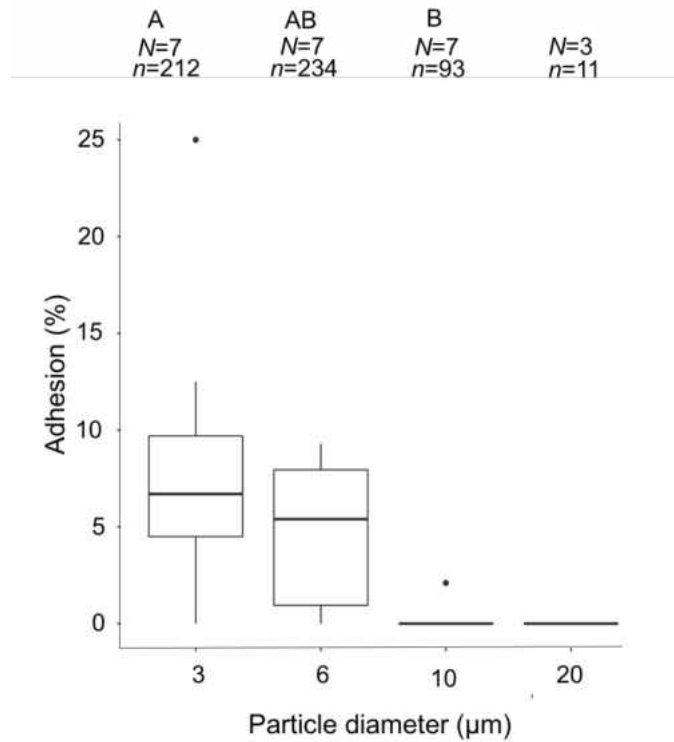


**Figure 8** Milk fat particles adhered to the food-concentrating filter of *Oikopleura dioica*. (A) Black arrow shows colloidal milk particles caked on the lower layer of the food-concentrating filter; white arrow shows the fibers of the intermediate screen suspended above the lower layer. A  $10 \mu\text{m}$  microsphere is shown being conveyed along the intermediate screen. (B) The rectangular mesh of the intermediate screen of the food-concentrating filter with adherent milk particles. Scale bars:  $0.05 \text{ mm}$ .

#### *Particle adhesion to the food-concentrating filter*

Particle adhesion to the FCF following a tail arrest and re-inflation cycle was consistently low (grand mean =  $4.5 \pm 5.6\%$ ,  $n=21$ ,  $N=7$ ) (Fig. 9). One outlying individual had 25% adhesion of  $3 \mu\text{m}$  beads; however, this mean was calculated from comparatively few particles ( $n=1$  adhering particle out of 4 particles).





**Figure 9** Tukey box-and-whiskers plot showing the percentage adhesion of different sized latex microspheres to the food-concentrating filter of *Oikopleura dioica*. Adhesion was calculated from the mean percent adhesion, summed across tail arrests for each animal. *N* indicates the number of animals from which mean percentage adhesion was measured, *n* indicates total number of particles analyzed from all animals. The letters A and B indicate significant differences with Nemenyi posthoc test.

Particle size significantly affected adhesion (Kruskal-Wallis  $\chi^2=8.344$ , d.f.=2,  $P=0.0154$ )

(Fig. 9). Although no difference in adhesion was found between 3 and 6 µm beads ( $P=0.77$ ) or between 6 and 10 µm beads ( $P=0.117$ ), 3 µm beads adhered significantly more to the FCF than 10 µm beads ( $P=0.021$ ). Very few 20 µm beads of the mixed particle suspension were observed in the FCF, presumably because the width of the IF mesh, which is a function of the animal's body size, is of similar size (Lombard et al. 2010) and excluded these beads from entering the house.

### Discussion:

We observed that the filtration process of *O. dioica* involved particles regularly attaching to and detaching from the FCF. The mechanism of the FCF is therefore not entirely analogous to

an industrial tangential flow filtration system, where adhesion to the filter is generally low and usually irreversible (Altmann and Ripperger 1997). Rather, we describe the appendicularian FCF as a “self-cleaning filter” and provide a thorough mechanistic explanation for how the FCF must detach particles in order for them to reach the pharyngeal filter for ingestion. Detachment only occurred after a tail arrest during re-inflation of the filter, and most particle adhesion was reversed through this process (Fig. 9). We propose that detachment is caused by increased viscous drag on the particle as water flow increases through the FCF during re-inflation, combined with a reduction in contact area of the particle to the elastic mesh (Fig. 6). We provide experimental evidence showing size-selective retention by the filter (Fig. 9), which is consistent with the size-dependent detachment patterns predicted by Stokes drag force (Fig. 10A). Collectively, these results provide a mechanistic basis for understanding particle selection by these ecologically important grazers.

#### *Theoretical framework of adhesion and detachment forces*

Pulsatile flow generated by the tail arrest has previously been acknowledged to play an important role in clearing the IF of accumulated particles (Flood 2003, Selander and Tiselius 2003, Tiselius et al. 2003), but its role in cleaning the FCF has heretofore been less widely recognized (Flood 1978). To test if pulsatile flow alone can explain the detachment process, we can simplify the system of the FCF to two forces: the drag force for detachment ( $F_d$ ) and the adhesion force ( $F_a$ ). Because particle collection by appendicularians occurs in laminar flow at low Reynolds numbers (Morris and Deibel 1993, Acuña, Deibel, and Morris 1996), we can use a modified Stokes drag, which increases linearly with particle size as:

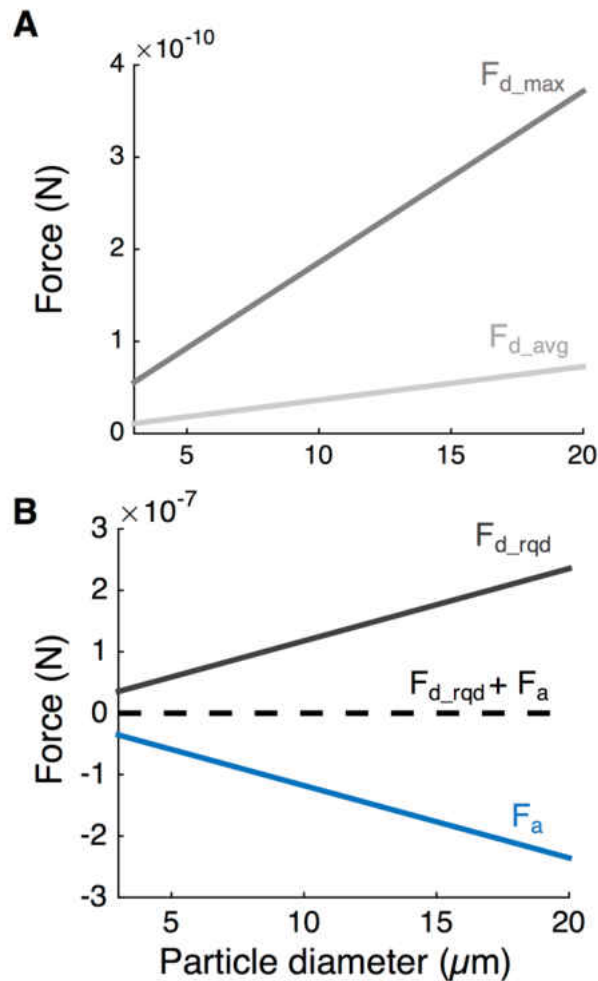
$$F_d = 1.7009 \cdot 6\pi\mu r v$$

where  $\mu$  is the dynamic viscosity of seawater ( $1.07 \cdot 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$ ),  $r$  is the radius of the sphere

( $1.5 \cdot 10^{-6}$  -  $1 \cdot 10^{-5}$   $\mu\text{m}$ ),  $v$  is the velocity ( $\text{m s}^{-1}$ ), and 1.7009 is a constant to account for the effect of a surface near the fluid stream (O'Neill 1968, Burdick, Berman, and Beaudoin 2001).  $F_d$  is calculated from the average ( $1.9 \cdot 10^{-4}$   $\text{m s}^{-1}$ ) and maximum ( $9.7 \cdot 10^{-4}$   $\text{m s}^{-1}$ ) instantaneous velocities of the intermediate filter fibers during a re-inflation of the house to estimate the respective changes in the drag force caused by pulsatile flow (Fig. 10A). Although the adhesive interactions between particles and the mucus filter are complex and beyond the scope of the present study, we estimate the adhesive force using the Johnson, Kendall and Roberts (JKR) model of adhesive elastic contacts, where the pull-off force required to detach an elastic sphere from a hard, flat substrate scales linearly with the particle radius as:

$$F_a = -\frac{3}{2}\pi\omega R$$

where  $\omega$  is the surface energy ( $\text{J m}^{-2}$ ) due to the van der Waals interactions between the particle and the substrate (Johnson, Kendall, and Roberts 1971, Persson 2003) and  $R$  is the radius of curvature, here assumed equivalent to  $\frac{1}{2}r$  (Attard and Parker 1992). Although the surface energy is unknown,  $0.01 \text{ J m}^{-2}$  represents a conservatively low, biologically relevant estimate (Gay 2002). Pulsatile flow caused by the tail arrest and re-inflation cycle increased the Stokes drag force by an order of magnitude when calculated from the average and maximum fiber velocities (Fig. 10A). However, these empirically calculated values of  $F_d$  (Fig. 10A) were both orders of magnitude lower than the estimates of  $F_a$  (Fig. 10B). Our results therefore support our hypothesis that pulsed flow facilitates particle detachment, but pulsed flow may not be the sole mechanism for detachment since our calculations of increased viscous drag alone cannot account for our observations that particle detachment consistently occurs after a re-inflation of the house.



**Figure 10** Adhesion and detachment forces in the food-concentrating filter. (A) Empirically calculated average and maximum Stokes drag force ( $F_d$ ) on individual filter fibers of the food-concentrating filter during filter re-inflation after a tail arrest. (B) Theoretical Stokes drag force required ( $F_{d\_req}$ ) to equal the estimated attractive adhesion pull-off force ( $F_a$ ). Dashed black line shows the sum of these forces. When  $F_a - F_d > 0$ , particles should remain adhered  $F_a - F_d < 0$ , particles should detach.

Numerous factors may account for the inadequate drag force compared to the estimated adhesive force. One possible explanation for the discrepancy is that the conformational changes of the fibers from relaxed to stiff during re-inflation of the FCF occur at instantaneous velocities rapid enough to impose sufficient drag. We used the change in arc length to measure the overall conformational change of the fibers from relaxed to straight, which occurs over multiple tail

beats during re-inflation of the FCF (~0.5 s, 250 frames on our camera). However, the fibers stiffen abruptly (often in less than 2 ms, or 1 frame) over very short distances (a few microns), and these spatial and temporal resolution limitations affect the maximum velocity that we can measure (Adrian 1991) during the period of fiber stiffening. Thus, our measured maximum instantaneous velocity of the fibers is likely an underestimate. In order to mathematically satisfy the velocity that would be required to overcome the adhesion force (i.e., for  $v > \frac{F_a}{1.7009 \cdot 6\pi\mu r}$ ), an instantaneous fiber velocity of  $\sim 0.6 \text{ m s}^{-1}$ , or a drag force of  $10^{-7}$  to  $10^{-8}$  N, would be required (Fig. 10B) and an intermediate Re number of 1. Although this speed may seem prohibitively fast, there are numerous biological examples of small, cellulose-based structures that passively achieve speeds up to an order of magnitude greater than this (Edwards et al. 2005, Nimmo et al. 2014, Forterre et al. 2016), and therefore could provide a mechanistic explanation for particle detachment by the appendicularian FCF. However, further investigation of the adhesive forces involved and the precise detachment mechanism is required.

#### *Experimental observations of adhesion and detachment*

The elasticity of the filter may also facilitate particle detachment in other ways. Deformable materials can undergo time-dependent adhesion, whereby the contact area between the particle and the substrate increases the longer the two are in contact (Krishnan et al. 1994) (Fig. 6D). Thus, as appendicularians filter-feed and accumulate adhered particles on the FCF, it should seemingly become more difficult to detach particles the longer the particles remain adhered. We propose that the pulsatile flow caused by periodic tail arrests and the associated elastic behavior of the filter fibers act in concert to counteract this problem: the elastic recoil of the filter following a tail arrest and filter re-inflation cycle reduces the contact area between the particle and the filter fiber, reducing the adhesive force and facilitating detachment (Fig. 6D).

The importance of fiber elasticity as a driving mechanism for detachment is supported by our observation that particle detachment occurred most frequently on the outer margins of the FCF (Fig. 5A and B). Because the distance between the lateral fibers and suspensory fibers shortens towards the buccal tube (Fig. 4D), the lateral fibers at the outer margins of the FCF are able to deform more during deflation than lateral fibers closer to the buccal tube. This increased deformation produces greater adhesive area change that favors detachment at the filter margins. The elasticity of the filter may also indirectly aid detachment by increasing the achievable peak flow rate and increasing shear stress at the walls (San and Staples 2012).

Historically, appendicularians have been assumed to feed non-selectively (Bedo et al. 1993, Acuña, Deibel, and Morris 1996, Gorsky et al. 1999). Our results, however, show that size-dependent adhesion may cause selective particle retention by the appendicularian house, with smaller particles being more likely to remain adhered to the FCF (Fig. 9). We therefore reject our hypothesis that no size-selection by the house occurs through particle interactions with the FCF. This study is the first experiment to isolate particle retention by the FCF, and therefore it is difficult to make direct comparisons with prior results on size-retention by the whole house, since particles may exhibit different adhesion patterns onto different house components (house walls, IF, or FCF). For example, the combined animal (*O. dioica*) and house system retained smaller beads with lower efficiency (Fernández et al. 2004), but, since these measurements were influenced by the pharyngeal filter, this finding does not conflict with our results. Fernández et al. (2004) also showed reduced retention of the largest beads (6  $\mu\text{m}$ ) by the IF of small animals. Similarly, Conley & Sutherland (2017) showed 10  $\mu\text{m}$  beads were positively selected in the houses compared to 3  $\mu\text{m}$  ones; however, it is likely that 10  $\mu\text{m}$  beads mostly adhered to the IF rather than the FCF. They found smaller particles (0.3  $\mu\text{m}$ ) were consistently higher in the house

than larger ones (1.75  $\mu\text{m}$ ), which is consistent with our observations of size-selection by the FCF. Previous flow cytometric analysis of *O. dioica* houses showed 0.2  $\mu\text{m}$  beads accumulated in the house at a higher rate than 0.75  $\mu\text{m}$  ones (Bedo et al. 1993). Although Bedo et al. (1993) proposed that the 0.2  $\mu\text{m}$  beads adhered to the internal house walls, it is probable that adhesion to the FCF also contributed. Smaller particles are also known to accumulate more on the membranes of industrial crossflow filtration systems (Stamatakis and Tien 1993). Collectively, these observations suggest that appendicularians houses may exhibit a bimodal distribution of accumulated particles: large particles adhered to the IF and small particles selectively adhered to the FCF.

In general, however, we found that adhesion across particle sizes remained consistently low—attesting to the efficacy of the self-cleaning ability of the FCF. Bochdansky and Deibel (1999) offered wide bounds for the loss of particles in the appendicularian house (~15-300%), but only one prior measurement has been made for the adhesion to the house: an estimated ~30% of filtered phytoplankton (<30  $\mu\text{m}$ ) removed by *O. dioica* remained adhered over the lifetime of the house (Gorsky, Fisher, and Fowler 1984). Since this estimate included particle adhesion to the entire house, it provides an upper bound for the overall percentage adhesion of particles to the FCF over multiple tail arrests. Undoubtedly, particle sizes, types, and concentrations also influence overall adhesion to the house, and as the house ages and the IF become clogged, pulsatile flow may become less effective. Nevertheless, our results show that, in our experimental conditions, the FCF is quite effective at detaching adhered particles through a single tail arrest and re-inflation cycle. The frequent replacement of the entire house by newly synthesized and inflated structures (~ every 4 h for *O. dioica* at 15°C, Troedsson et al. 2009) provides a regular filter system reset to overcome accumulated adhesive clogging.

### *Function of the intermediate screen*

The functional role of the intermediate layer of the appendicularian FCF has remained uncertain, with speculation that it serves a purely structural role (Deibel 1986), or possibly a straining function (Alldredge 1977). Although the intermediate layer certainly serves a structural role in connecting the upper and lower layers of the FCF as previously suggested (Deibel 1986), it is evident that various sizes of particles, from 20  $\mu\text{m}$  beads (Fig. 6A-C) to milk fat and charcoal particles (Fig. 8, Deibel 1986), interact directly with the intermediate screen and sometimes adhere to its fibers. The intermediate fibers, like the rest of the FCF, exhibited a marked deformability (Fig. 4E, F). Therefore, we reject our hypothesis that the intermediate layer serves a solely structural role, since our results show it is involved in particle collection and that the elasticity of its fibers may facilitate particle detachment.

### *Observations of feeding behavior*

Our observations of the valve in the buccal tube and the role of the spiracles in flow reversals help further explain appendicularian feeding behavior. Fenaux (1986) described two valve-like openings on either side of buccal tube through which filtered water exits the buccal tube; however, we observed only one valve in the center of the tube. We believe the center valve is the same structure previously described as two side valves, since the stream of ejected particles makes it appear that the valve is located on the side. Lombard et al. (2011) described a “pipe-smoking” behavior in which reversal of the ciliary beat of the spiracles causes particle rejection into the exit chamber. Our findings suggest that the valve in the buccal tube provides an additional pathway for pipe-smoking behavior.

Furthermore, our observations lessen some ambiguity regarding the mechanism of the previously described “ciliary reversals” (Galt and Mackie 1971). Our video observations show



that, during a reversal, the ciliary beat does not reverse in the clockwise direction, but does reorient in the dorsal-ventral direction. This reorientation appears to reduce the effective diameter of the spiracle funnel, forming a constriction that is associated with flow reversal (Fig. 7).

### *Implications*

While particle-filter adhesion is a micro-scale process, understanding the effect of adhesion on selective particle retention has predictive value for large-scale processes such as carbon transformation and transport. The implications of particle adhesion to the appendicularian FCF is of particular importance because appendicularians represent a shortcut in the aquatic trophic web, linking ultraplankton directly to many marine fish (Purcell et al. 2005). Particles that adhere to the appendicularian house are more nutritive than those packaged in the refractory fecal pellets (Bochdansky and Deibel 1999). Because of this, discarded houses are preyed upon by numerous taxa, including calanoid and harpacticoid copepods, euphausiid larvae, and flat, forage, and reef fishes, and contribute to remineralization of particulate organic carbon in the ocean (Alldredge 1976, Steinberg et al. 1994, Purcell et al. 2005).

### *Bridge to Chapter III*

In Chapter II, I established how the mechanics of the appendicularian food-concentrating filter (namely, pulsatile flow and elasticity) contribute to size-dependent adhesion patterns. In Chapter III, I elaborate on size-dependent adhesion patterns by examining both particle retention by the external filtering house and by the internal pharyngeal filter. I examine a broader array of particle sizes, down to the submicron range, and also investigate the effect of particle shape.

CHAPTER III

PARTICLE SHAPE IMPACTS EXPORT AND FATE IN THE OCEAN THROUGH  
INTERACTIONS WITH THE GLOBALLY ABUNDANT APPENDICULARIAN  
*OIKOPLEURA DIOICA*

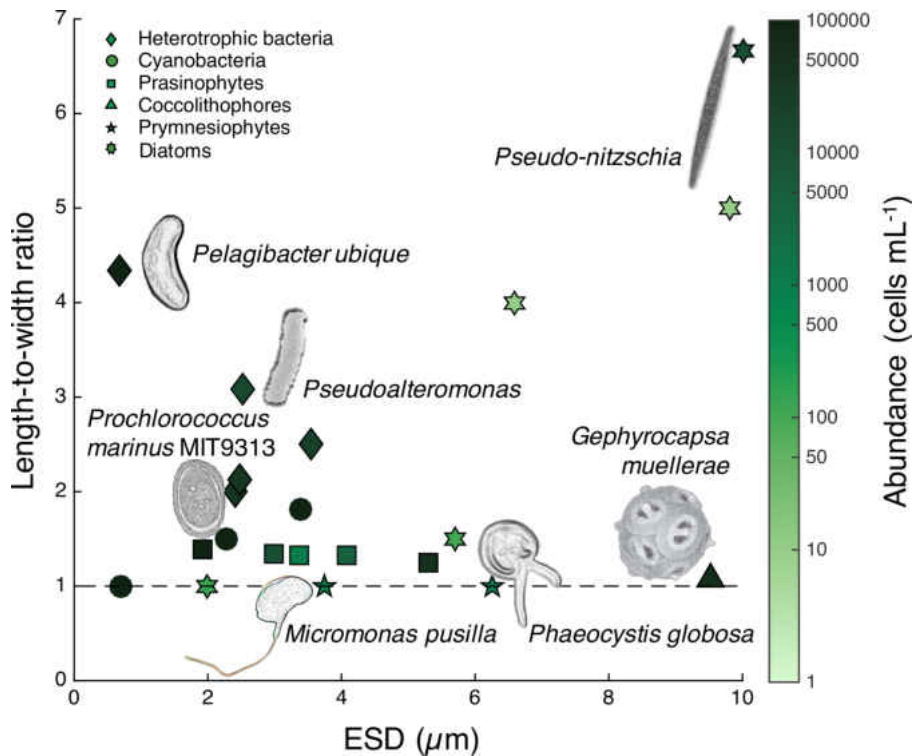
The third chapter of this dissertation has been accepted for publication by *PLoS ONE* and is co-authored by Kelly Sutherland. I conceived and designed the experiments, performed the experiments (both incubation experiments and videography of ellipsoidal bead behavior), contributed to funding acquisition, analyzed and presented the data, and wrote the initial draft of the manuscript. K.R. Sutherland contributed to funding acquisition, mentorship/supervision, helped conceive the experiments and design of methodology, helped perform the incubation experiments, and reviewed and edited the manuscript.

**Introduction:**

The ocean is dominated by microorganisms that account for more than half of oceanic primary production and strongly mediate biogeochemical cycling (Azam 1998). Many of these marine microbes are non-spherical (Fig. 11) (Jonasz 1987, Gibson, Atkinson, and Gordon 2007, Guasto, Rusconi, and Stocker 2012, Leblanc et al. 2012). For example, the cosmopolitan SAR11 bacterial clade accounts for a third of the total microbial community in the upper ocean, and the cells have a curved rod morphology (Morris, Rappé, Connon, and Vergin 2002).

*Prochlorococcus*, a genus of cyanobacteria that contributes up to half of the photosynthetic biomass in certain oligotrophic regions, has differently shaped strains, ranging from spherical to oval (Fig. 11) (Johnson et al. 2006, Ting et al. 2007). Among prokaryotes, rod shape is more common than spherical (Perry and Staley 1997): a meta-analysis of the shapes of free-swimming bacterial genera found only 21% were spherical—the majority instead having rod-like shapes (Dusenbery 1998). The pico- and nano-eukaryotes encompass a highly diverse group of organisms whose morphologies are also wide-ranging and often non-spherical (Lewis 1976, De

Vargas et al. 2015). Their shapes may be further complicated by structural features such as spines, plates, or scales, and the formation of long filaments or chains.



**Figure 11** Morphological diversity of marine microbes. Length-to-width ratio versus surface-equivalent sphere diameter (ESD) (Pabst and Gregorova 2007) of some abundant free-living microbes in the upper ocean (Table S2, Appendix). Surface area ( $S$ ) of ESD ( $\mathbf{ESD} = \frac{6}{\pi} S^{1/2}$ ) was calculated based on the assumption of a prolate spheroid (Pabst and Gregorova 2007). Color of points shows average abundances. Dashed black line represents the length-to-width ratio of a perfectly spherical cell.

Cell shape is central from an evolutionary perspective for both prokaryotes and eukaryotes. Rod- or filamentous-shaped bacterial cells are ancestral, while coccoid morphology is derived (Young 2006). Cell morphology can exhibit rapid, phenotypic plasticity and may also be subject to selective pressures over longer evolutionary time scales. The selective forces influencing cell shape are varied (Young 2006). Elongated shapes, for example, are advantageous for nutrient acquisition by increasing total surface area relative to volume for diffusion. Rod-like shape enables the establishment of poles and the sequestration of

biochemicals such as attachment complexes, protein domains, and cell wall differentiation, and may facilitate adhesion in high-shear environments (Young 2006). Eukaryotic phytoplankton are commonly rod-shaped since rods sink more slowly than spheres of equivalent volume, thereby promoting retention in the photic zone (Lewis 1976, Padisák, Soróczki-Pintér, and Reznér 2003). Predation is certainly another important selective force on cell shape; however, our understanding of this interaction is much less complete (Young 2006).

Despite the morphological diversity of aquatic particles, the effect of particle size on predation has been more thoroughly studied than that of shape. Size-dependent predation has been extensively investigated using synthetic microspheres (Børsheim 1984, Pace and Bailiff 1987, Deibel and Lee 1992, Bedo et al. 1993, Christaki et al. 1998, Fernández et al. 2004, Sutherland, Madin, and Stocker 2010), which isolate the effect of size without the other confounding variables of live prey (e.g. surface properties, shape, density, motility). Presently, non-spherical polymer micro-particles are not commercially available (Champion, Katare, and Mitragotri 2007), and thus there is no comparable experimental method with which to test the potential for shape-based selection. Instead, the relationship between particle shape and predation has been primarily examined through the lens of predator-induced phenotypic plasticity (Corno and Jürgens 2006, Bergkvist et al. 2012). Predation often imposes a selection pressure for filamentous cells, small cells, and asymmetrical grazing-resistant morphologies (Jürgens et al. 1999), but shape remains a difficult variable to isolate and test experimentally.

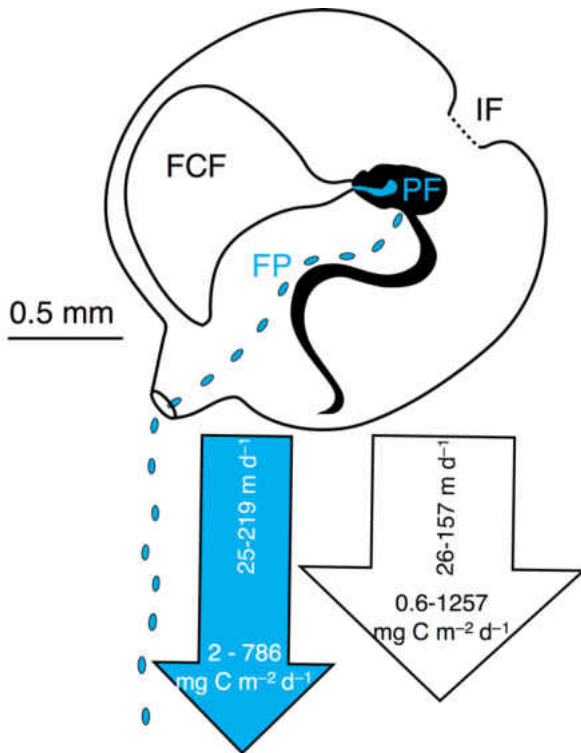
Particle-grazer interactions in fluid are governed by hydrodynamics (Guasto, Rusconi, and Stocker 2012). At microbial-length scales, the hydrodynamics are low Reynolds number and dominated by viscous forces rather than inertial ones (Purcell 1977). Rod-shaped particle behavior in a viscous fluid is known to be more complicated than that of spheres, characterized

by tumbling behavior with varying orientations (Bretherton 1962, Kim and Klapperich 2010). Previous studies have also shown that particle shape has important ramifications for drag and the detachment of particles adhered to a surface (Zimon 2012). The majority of models of marine particle behavior nonetheless assume spherical cell shape (Gibson, Atkinson, and Gordon 2007). We hypothesized that the short axis of ellipsoidal particles should determine how they are captured by filter-feeders because theory predicts ellipsoidal particles in laminar flow tend to orient with the long axis parallel with fluid streamlines (Jeffery 1922, Bretherton 1962) an orientation that minimizes drag (Loth 2008).

We used the appendicularian *O. dioica* (Phylum: Chordata, Subphylum: Tunicata) as a model grazer because of the importance of appendicularians in ocean biogeochemical cycling. Appendicularians are planktonic herbivores whose abundance and individual grazing rates can equal or even exceed that of copepods (Alldredge 1981, Tiselius et al. 2003). Appendicularians filter-feed by passing large volumes of water across an external mucous filter to consume microbe-sized particles down to four orders of magnitude smaller than themselves—encompassing the bacterial size range (Acuña, Deibel, and Morris 1996, Gorsky and Fenaux 1998). The mucous filter, called the “house,” is periodically discarded and re-secreted at a rate of 2-40 houses day<sup>-1</sup> (Sato, Tanaka, and Ishimaru 2003). Discarded appendicularian houses, containing concentrated, non-ingested prey, therefore constitute a major source of marine snow, contributing ~28-39% of total particulate organic carbon export to the ocean’s interior (Alldredge et al. 2005).

We used incubation experiments coupled with high-speed videography of ellipsoidal and spherical microbead trajectories through the appendicularian house to determine how particle shape affects grazing and fate. Appendicularians can differentially affect the fate of microbial

prey through their complex feeding mechanisms, which result in particles either being retained in the external mucous house, or being ingested by the animal (Fig. 12). Appendicularian grazing occurs through a series of distinct filtration steps. Prior to entering the house, particles are first screened by two coarse-meshed bilateral inlet filters that exclude large or spinous particles. Particles are then conveyed across the food-concentrating filter, which acts as a tangential filter to exclude water and concentrate particles (Morris and Deibel 1993). The final filtration step occurs at the pharyngeal filter, which is suspended across the animal's pharynx and collects particles for ingestion. Particles that adhere to the inlet or food-concentrating filters remain associated with the house (which is ultimately discarded), whereas particles captured by the pharyngeal filter are ingested and, depending on their digestibility, either incorporated into animal biomass or into fecal pellets (Fig. 12). Dense fecal pellets from *O. dioica* tend to be expelled from the house and thus represent a separate pathway for vertical flux (Fenaux 1986) sinking up to  $200 \text{ m day}^{-1}$  (Urban, Deibel, and Schwinghamer 1993) (Fig. 12). In some areas, *O. dioica* has been observed to be the second-largest contributor to total fecal carbon flux of any individual species (González, Kurbjeweit, and Bathmann 1994, Vargas et al. 2002). Appendicularian grazing can therefore profoundly alter planktonic particle diversity and fate, both within and below the upper mixed layer.



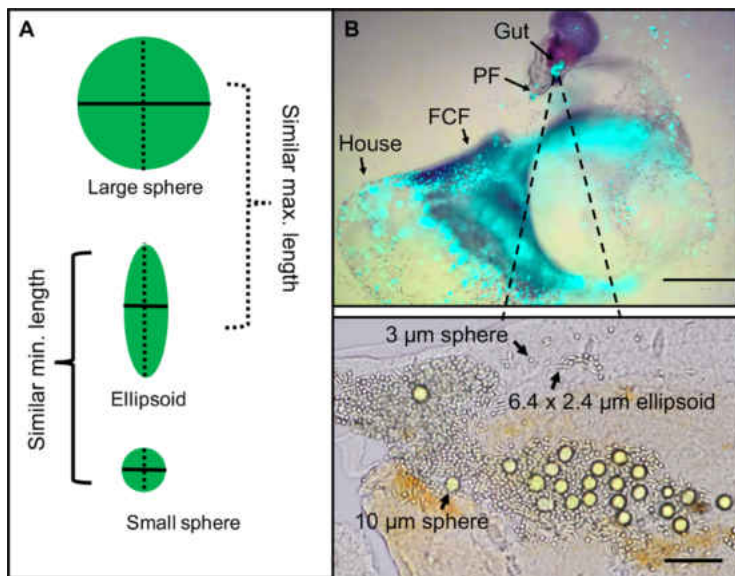
**Figure 12** Grazing by *Oikopleura dioica* influences particle fate. Different fates of particles grazed by the appendicularian *Oikopleura dioica*: particles associated with the discarded house (white arrow) via retention on either the inlet filters (IF), food-concentrating filter (FCF), or house walls; particles captured on the pharyngeal filter (PF), ingested, and incorporated into fecal pellets (FP) (blue arrow). Arrow widths represent the average flux of houses ( $703 \text{ mg C m}^{-2} \text{ d}^{-1}$ ) and fecal pellets ( $446 \text{ mg C m}^{-2} \text{ d}^{-1}$ ) (Dagg and Brown 2004). Arrow lengths represent the average sinking rates of houses ( $50 \text{ m day}^{-1}$ ) (Alldredge et al. 2005 and references therein) and fecal pellets ( $60 \text{ m day}^{-1}$ ) (Dagg and Brown 2004). Values show the range of flux for houses (Alldredge et al. 2005 and references therein) and fecal pellets (Vargas et al. 2002, Dagg and Brown 2004) and the sinking rates of houses (Dagg and Brown 2004) and fecal pellets (Dagg and Brown 2004, Dagg et al. 1996). Schematic of *O. dioica* by Jenna Valley.

Because of the ubiquity of rod-shaped microbes in the ocean (Fig. 11), the scarcity of data on how shape affects the fate of particles in the ocean represents a notable gap. We show that particle interactions with the appendicularian feeding-filters depends on particle shape and also particle size, and that, irrespective of size, ellipsoidal particles behave like spheres of equivalent width. We propose that ellipsoidal cells experience the benefits of an increased surface-to-volume ratio without incurring an increased cost of predation.

## Materials and methods:

### *Incubation experiments*

We tested a mix of three polystyrene bead types (small spheres, ellipsoids, and large spheres) using three separate grazing incubation experiments (Table 2). Ellipsoidal particles were synthesized using a toluene-stretching technique (Champion, Katare, and Mitragotri 2007). To account for the possible effect of the stretching procedure on bead surface properties, we prepared control spherical microbeads using the same polyvinyl film embedment procedure but without mechanically stretching the film. The ellipsoids were had one axis similar to the diameter of the small spheres, and one axis similar to the diameter of the larger spheres (Fig 13A). The large spheres were always slightly larger than the maximum dimension of the ellipsoids due to constraints of the commercial availability of different sizes of microspheres (Fig 13A). The large spheres were always slightly larger than the maximum dimension of the ellipsoids due to constraints of the commercial availability of different sizes of microspheres (Fig 13A, Table 2). In each incubation experiment, all three bead types were offered simultaneously. Particle concentrations were selected based on the concentration of similarly-sized cells in the upper ocean (Sutherland, Madin, and Stocker 2010) (Table 2).



**Figure 13** Experimental bead mixture for incubations. (A) Schematic of experimental bead mixture used in each of three incubation experiments with *Oikopleura dioica*. (B) Top: *O. dioica*



in house filtering a mixture of *Rhinomonas reticulata* (red, ~17  $\mu\text{m}$  diameter) and fluorescent 10  $\mu\text{m}$  microspheres (green). PF: pharyngeal filter; FCF: food-concentrating filter. Scale bar 0.5 mm. Bottom: experimental bead mixture (3-10  $\mu\text{m}$ ) in the gut post-incubation. Scale bar 50  $\mu\text{m}$ .

1 **Table 2** Experimental conditions for three incubation experiments.

2 Incubation experiment	Bead mixture	Diameter ( $\mu\text{m}$ )	Volume ( $\mu\text{m}^3$ )	Emission (nm)	$T_0$ conc. (mean beads $\text{mL}^{-1} \pm \text{S.D.}$ )	$T_f$ conc. (mean beads $\text{mL}^{-1} \pm \text{S.D.}$ )	Control $T_0$ conc. (mean beads $\text{mL}^{-1} \pm \text{S.D.}$ )	Control $T_f$ conc. (mean beads $\text{mL}^{-1} \pm \text{S.D.}$ )
1	Small sphere	0.32	0.14	660	$8.1 \times 10^5 \pm 5.9 \times 10^5$	$1.0 \times 10^6 \pm 6.2 \times 10^5$	$5.0 \times 10^5 \pm 2.8 \times 10^5$	$6.0 \times 10^5 \pm 1.4 \times 10^5$
	Ellipsoid	0.3 x 0.7	0.14	441	$1.1 \times 10^5 \pm 7.1 \times 10^4$	$1.3 \times 10^5 \pm 1.0 \times 10^5$	$1.8 \times 10^5 \pm 3.5 \times 10^4$	$1.0 \times 10^5 \pm 7.0 \times 10^4$
	Large sphere	1	4.2	441	$1.8 \times 10^5 \pm 1.8 \times 10^5$	$7.8 \times 10^4 \pm 6.2 \times 10^4$	$1.8 \times 10^5 \pm 3.5 \times 10^4$	$1.3 \times 10^5 \pm 1.8 \times 10^5$
2	Small sphere	0.5	0.065	763	$2.6 \times 10^6 \pm 6.8 \times 10^5$	$2.6 \times 10^6 \pm 8.4 \times 10^5$	$2.9 \times 10^6 \pm 5.0 \times 10^5$	$2.5 \times 10^6 \pm 6.0 \times 10^5$
	Ellipsoid	0.5 x 1.4	0.065	441	$1.7 \times 10^6 \pm 5.5 \times 10^5$	$1.6 \times 10^6 \pm 4.5 \times 10^5$	$1.5 \times 10^6 \pm 3.2 \times 10^5$	$1.9 \times 10^6 \pm 2.8 \times 10^5$
	Large sphere	1.75	22.5	441	$1.4 \times 10^6 \pm 6.4 \times 10^5$	$1.2 \times 10^6 \pm 4.6 \times 10^5$	$1.0 \times 10^6 \pm 5.8 \times 10^4$	$1.5 \times 10^6 \pm 3.3 \times 10^5$
3	Small sphere	3	14	529	$2.1 \times 10^5 \pm 6.2 \times 10^4$	$2.1 \times 10^5 \pm 5.8 \times 10^4$	$2.6 \times 10^5 \pm 6.1 \times 10^4$	$2.1 \times 10^5 \pm 2.0 \times 10^4$
	Ellipsoid	2.4 x 6.4	14	441	$2.6 \times 10^5 \pm 8.9 \times 10^4$	$2.4 \times 10^5 \pm 6.6 \times 10^4$	$3.0 \times 10^5 \pm 4.3 \times 10^4$	$3.1 \times 10^5 \pm 5.9 \times 10^4$
	Large sphere	10	524	441	$6.4 \times 10^4 \pm 2.3 \times 10^4$	$5.7 \times 10^4 \pm 2.1 \times 10^4$	$7.8 \times 10^4 \pm 3.4 \times 10^4$	$5.9 \times 10^4 \pm 6.4 \times 10^3$

All experimental animals were obtained from the appendicularian culture facility at the Sars Centre for Marine Molecular Biology in Bergen, Norway. For each incubation, either late day-5 or early day-6 animals were used to ensure consistent animal size (Table 3). Animals were transferred from 1- $\mu\text{m}$  filtered seawater (FSW) to a 0.2- $\mu\text{m}$  FSW bath and probed to abandon their houses using a wide-bore pipette. We then transferred animals into a second 0.2- $\mu\text{m}$  FSW bath in order to allow them to build a new, clean house prior to use in the experiment. Incubation chambers (44 mL) were pre-rinsed with 0.2- $\mu\text{m}$  FSW and filled with 0.2- $\mu\text{m}$  FSW. Experimental bead mixtures (small spheres, ellipsoids, and large spheres) were diluted with seawater and then pipetted into the incubation chamber. The chambers were gently inverted several times to homogenize the beads prior to the addition of an animal. One animal was randomly assigned to each incubation container, and two or three chambers with no animal (i.e., only experimental particles) served as controls. All incubations were performed on a lab bench at 20°C. Since we sought to compare the relative proportion of different bead types in the house and guts, all incubations were 10 min in duration, which is the approximate gut clearance time for *O. dioica* (López-Urrutia and Acuña 1999). *O. dioica* has a maximum *in situ* filtration rate of 12.5 ml animal<sup>-1</sup> hr<sup>-1</sup> (Alldredge 1981), so the volume of the incubation container allowed for a relatively constant ambient particle field (~5% of the chamber volume filtered). Each chamber was sampled for 1 mL of the initial water prior to the addition of an animal ( $T_0$ ) and the water at the end of the incubation ( $T_{\text{final}}$ ). At the conclusion of the incubation, we recorded whether each animal was still in the house. We then carefully pipetted the animal and house using a wide-bore pipette and transferred them to a glass embryo dish with 0.2- $\mu\text{m}$  FSW. If animals were still filtering,

they were probed to abandon their house, photographed for size measurements, and then the whole animal was collected using a Pasteur pipette for subsequent gut content analysis. Houses were also collected with a micropipette set to a fixed volume (300  $\mu$ L). All samples were collected into 1.8 mL cryotubes, promptly fixed with 0.1% (by volume) of 25% gluteraldehyde, and refrigerated until analysis.

**Table 3** Observational results from incubation experiments with *Oikopleura dioica*.  $T_{\text{final}}$  is the water sampled at the end of the 10-minute incubations.

Experimental observations	3-10 $\mu$ m	0.5-1.75 $\mu$ m	0.3-1.0 $\mu$ m
<i>O. dioica</i> trunk length, mm (mean $\pm$ SD)	0.79 $\pm$ 0.12 (n=12)	0.79 $\pm$ 0.10 (n=12)	1.0 $\pm$ 0.18 (n=10)
Proportion of animals filtering at $T_{\text{final}}$	33.3%	100.0%	66.7%
Proportion of animals with beads in gut	50.0%	58.3%	55.6%
Proportion of animals with >10 beads in gut	33.3%	58.3%	44.4%
Total number of beads in gut (mean $\pm$ SD)	195 $\pm$ 262	266 $\pm$ 160	141 $\pm$ 111

Beads were quantified using a Nikon Eclipse Ei compound microscope. Beads in the 3-10  $\mu$ m size range were analyzed using epifluorescence microscopy with a 10x objective. Concentrations of  $T_0$  and  $T_{\text{final}}$  samples for 3-10  $\mu$ m beads were determined using a Reichert Bright-Line hemacytometer (0.1 mm deep, CAT # 1492). Concentration measurements for beads <3  $\mu$ m were made using a Petroff-Hausser Bacteria Counter (0.2 mm deep, Fisher Scientific CAT # 02-671-13) with a 20x or 40x objective using dark-field light microscopy (Abdel-Fattah, El-Genk, and Reimus 2002). All samples were vortexed prior to counting and a sufficient number of grids were counted to achieve an average total bead count of  $\sim$ 100 (20  $\mu$ L per sample).

Gut samples were prepared by directly mounting animals onto a glass slide and treating them with 10  $\mu$ l recombinant PCR Grade Proteinase K solution from *Pichia*

*pastoris* (Sigma-Aldrich, Cat No. 3115887001) to degrade the tissue for better differentiation of beads (Fig. 13B). Gut samples were analyzed using a 40x objective and photographed using a Canon EOS 5D. We performed manual particle counting using the Multi-point Tool in Image J (Schneider, Rasband, and Eliceiri 2012). Houses from the 3-10  $\mu\text{m}$  were intact enough to mount on a microscope slide and perform particle counts identically to the guts. Houses from the other incubations had dissolved and thus were counted identically to the water samples using a Petroff-Hausser chamber. Data analysis for gut samples was restricted to those animals with  $>10$  beads. Because of the inherent fragility of house and gut samples, all parameters could not be measured for all samples.

### *Videography*

Immature day-6 *O. dioica* were filmed individually using a Sony 4K FDR-AX100 (HD resolution, 120 fps) mounted to a Nikon Eclipse E400 with a 10x objective using a Martin Microscope M99 Camcorder Adapter. Animals were filmed in a glass embryo dish filtering a suspension of 10  $\mu\text{m}$ -diameter spheres and ellipsoids of similar width ( $L=22 \pm 2.4 \mu\text{m}$  and  $W=7.8 \pm 1.1 \mu\text{m}$ ;  $N=5$ ; mean  $\pm$  SD). Videos were converted to image stacks for velocity and trajectory analysis in ImageJ (Schneider, Rasband, and Eliceiri 2012). Particle tracking velocimetry was performed by manually tracking individual particles between frames using the plugin MTrackJ (Meijering, Dzyubachyk, and Smal 2012). Particle coordinates were used to calculate velocities and net-to-gross displacement ratios (NGDR) for spherical and spheroidal particles. All trajectories were obtained from one day-6 individual. Trajectories for net-to-gross displacement were restricted to distances  $>100 \mu\text{m}$ .

For measurements of ellipsoidal particle orientation, the image stack was registered using the StackReg plugin with an Affine transform to account for the inflation and deflation of the feeding mesh. Images were then inverted and frames for trajectory analysis were color-coded in one of six channels using the Stack-to-Hyperstack tool. Stacks were Z-projected and the composite image was used to show trajectories. Measurements of ellipsoidal particle orientation were made using the straight-line angle tool in ImageJ (Schneider, Rasband, and Eliceiri 2012) and converted to be relative to the fluid flow.

### *Statistics*

Selection for the different shaped beads was calculated using the Chesson's selectivity index (Chesson 1983):

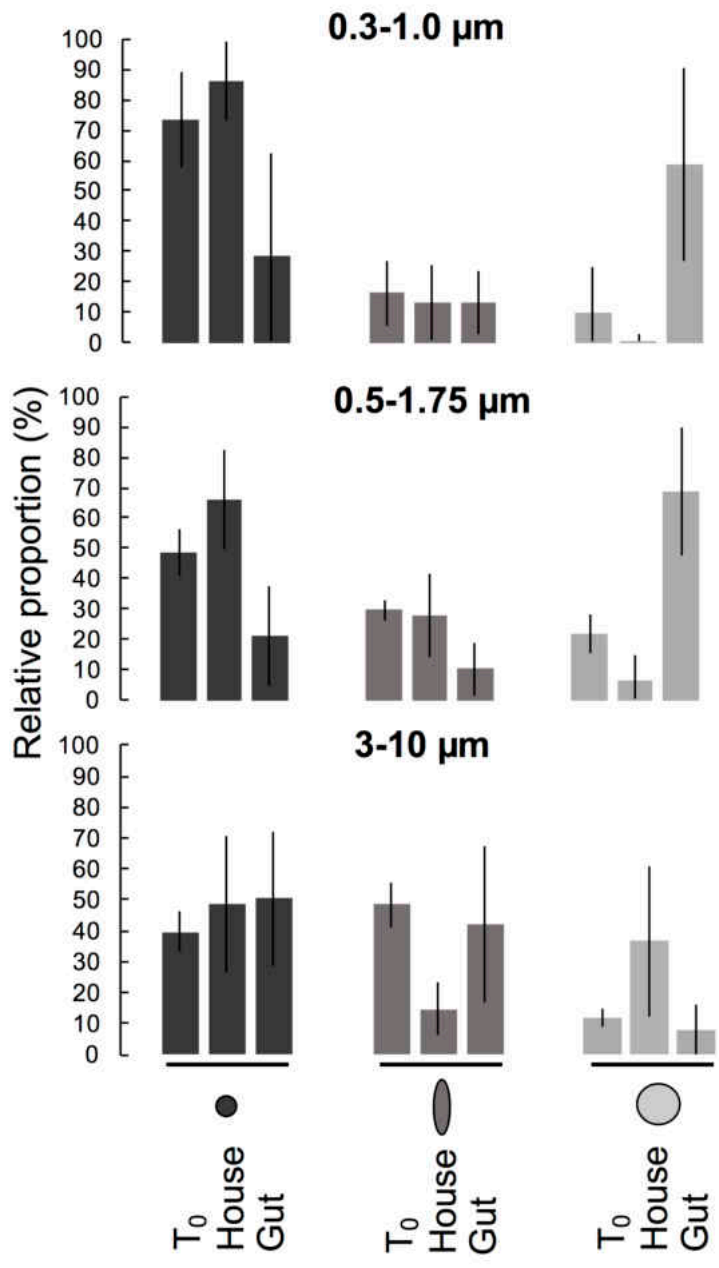
$$\alpha_i = \frac{d_i/e_i}{\sum_{j=1}^k \left( \frac{d_j}{e_j} \right)}$$

where  $i= 1, 2, \dots, k$

$k$  is the number of prey categories,  $d$  is the proportion of prey type  $i$  in the diet,  $e$  is the proportion of prey type  $i$  in the environment. The selectivity coefficient  $\alpha_i$ , which is independent of the relative abundance of different prey types, ranges from 0 to 1 (Chesson 1983). Random feeding is defined by  $\alpha_i = \frac{1}{k}$ , with values  $<\alpha_i$  and  $>\alpha_i$  indicating negative and positive selection, respectively. In this study,  $\frac{1}{k} = 0.333$  since each incubation tested three bead types. The null hypothesis of no selection ( $\alpha_i = \frac{1}{k}$ ) was tested using t-tests. Data were arcsine-square root transformed to meet the assumption of normality and a Bonferroni correction of alpha level (0.05/number of t-tests) was used to provide an overall error rate of 0.05 (Scheiner 2001).

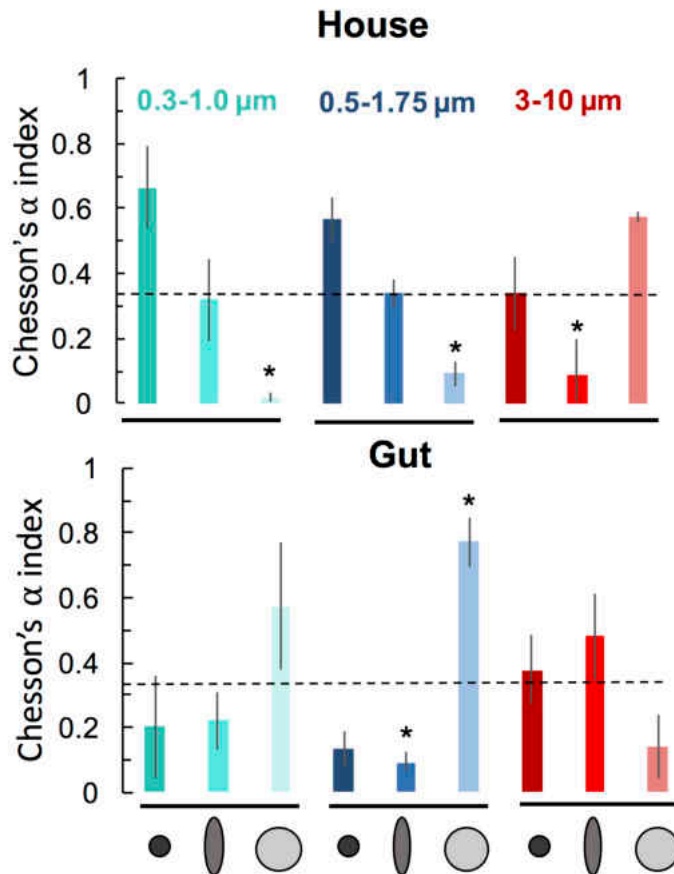
## *Results*

Three separate incubation experiments with *O. dioica* grazing on environmentally relevant particle sizes (0.3-10  $\mu\text{m}$ ) showed that shape differentially affected particle retention patterns in the house and gut (Fig. 14). Retention patterns in the house were more variable than that in the gut and interrelated with particle size. Regardless of size, ellipsoids were always ingested according to their minimum diameter (Figs. 14 and 15). The mean Chesson's  $\alpha$ -index for ellipsoids was always more similar to that of spheres of similar minimum diameter than to spheres of similar maximum diameter (Fig. 15), indicating that the minimum dimension of the ellipsoid appears to determine ingestion rates.



**Figure 14** Fate of different shaped beads from three incubation experiments. Relative proportions (mean  $\pm$  SD) of various bead mixes in the ambient water at the start of the incubation (T<sub>0</sub>), gut, and house of the appendicularian *Oikopleura dioica*.





**Figure 15** Particle shape affects selection by the appendicularian house and gut. Selectivity coefficients (mean Chesson's  $\alpha$ -index  $\pm$  SE) for different bead types in the houses and guts from each of three incubation experiments with *Oikopleura dioica*. \* indicates selectivity values that were significantly different from non-selectivity ( $\alpha = 0.33$ , dashed line) tested using t-tests with a Bonferroni correction of alpha level ( $p \leq 0.0028$ ) (Table 4).

In addition to particle shape, size also had a pronounced effect on particle fate. The relative proportions of different bead types were consistently inverse in the house and in the guts (Fig. 14). In the 3-10  $\mu\text{m}$  incubation, the 10  $\mu\text{m}$  spheres had a positive selectivity index in the house, while the 2.4 x 6.4  $\mu\text{m}$  ellipsoids had a negative selectivity index, and 3  $\mu\text{m}$  spheres had neutral selectivity (Fig. 16; Table 4). The reverse pattern occurred for both the 0.5-1.75  $\mu\text{m}$  incubation and the 0.3-1.0  $\mu\text{m}$  incubation: large spheres were disproportionately ingested while small spheres and ellipsoids

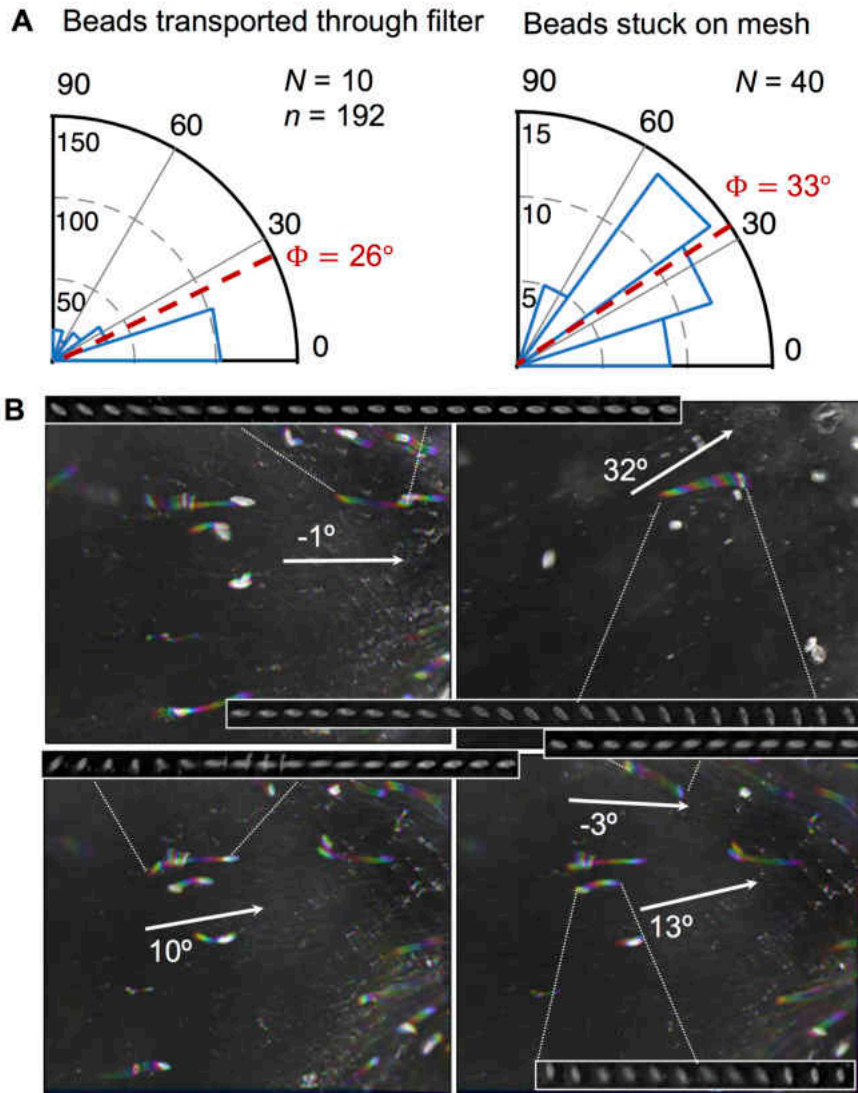
predominated in the house (Fig. 15). In the houses from the two incubations with smaller-sized beads (0.3-1.75  $\mu\text{m}$ ), ellipsoidal beads had a mean Chesson's  $\alpha$ -index in between that of the small and large spheres, indicating intermediate, but non-selective, retention. Absolute counts of beads in guts, along with the change in relative proportions of beads from  $T_0$  suggest a sharp reduction in ingestion of particles below  $\sim 1.0 \mu\text{m}$  (Fig. 14). The proportion of animals filtering at  $T_{\text{final}}$  was lowest for the incubation with the largest size particle assemblage (3-10  $\mu\text{m}$ ), and the animals in this experiment also had the lowest proportion of animals with  $>10$  beads in the gut (Table 3), implying reduced efficacy of feeding with this particle regime. We therefore infer that while *O. dioica* houses may affect a broader range of particle sizes, the animals themselves are restricted to ingesting a size range of 1.0  $\mu\text{m}$  to  $<10 \mu\text{m}$ . Both particle shape and size therefore affect predation by appendicularians and influence the ultimate fate of the particles.

**Table 4** Statistical results from incubation experiments with *Oikopleura dioica*. Statistical comparisons are from T-Tests on the selectivity coefficient  $\alpha_i$  values versus the null hypothesis of no selection ( $\alpha_i = \frac{1}{k}$ ). \* $p \leq 0.1$ , \*\* $p \leq 0.05$ , and \*\*\* $p \leq 0.0028$  (Bonferroni correction).

Bead size	House			Gut		
	<i>t</i> -statistic	df	p-value	<i>t</i> -statistic	df	p-value
0.3	2.4725	6	0.0483**	-1.1696	3	0.327
0.3 x 0.7	-0.72759	6	0.494	-1.3777	3	0.262
1	-11.179	6	$<0.001$ ***	1.1402	3	0.337
0.5	3.4275	9	0.00754**	-3.6531	6	0.0107**
0.5 x 1.4	-0.047972	9	0.963	-5.385	6	0.002***
1.75	-5.1398	9	$<0.001$ ***	5.2286	6	0.002***
3	-0.19261	8	0.852	0.001788	5	0.999
2.4 x 6.4	-7.8573	8	$<0.001$ ***	-1.0277	5	0.351
10	2.9004	8	0.0199**	-2.4019	5	0.0615

High-speed micro-videography of the food-concentrating filter revealed the mechanism behind the similar retention of ellipsoids and spheres of equivalent width: no significant difference was observed between the net-to-gross displacement ratio (NGDR) of 8 x 22  $\mu\text{m}$  ellipsoids and 10  $\mu\text{m}$  spheres (ANOVA,  $F_{1,54} = 0.457$ ,  $P = 0.502$ ,  $n = 26$  and  $n = 29$  for spheres and ellipsoids, respectively), nor was there a significant difference in their speeds through the filter (two-factor ANOVA,  $F_{1,34} = 2.133$ ,  $P = 0.153$ ,  $n = 31$  for both spheres and ellipsoids). The kinematics of ellipsoidal particles were thus similar to spheres of equivalent width, consistent with results from the incubation experiments.

Ellipsoids tended to passively orient short-end forward when they moved through the low Reynolds number regime of the food-concentrating filter and were more variably oriented when adhered to the filter mesh (Fig. 16). Beads suspended in the fluid had a lower mean orientation angle ( $\Phi = 26^\circ$ ), indicating greater alignment with the fluid flow than beads stuck to the mesh ( $\Phi = 33^\circ$ ) (Fig. 16). Ellipsoidal particles exhibited a tumbling behavior, changing orientation as they were conveyed through the filter (Fig. 16B).



**Figure 16** Trajectories and orientation of ellipsoidal microbeads through the feeding-filter of *Oikopleura dioica*. (A) Frequency histograms show the angles for ellipsoidal microbeads ( $7.8 \times 22 \mu\text{m}$ ) suspended in the fluid of the food-concentrating filter and adhered to the filter mesh of *Oikopleura dioica*. Sectors correspond to number of beads observed,  $\Phi$  is the grand mean direction for all beads,  $N$  is the number of independently measured beads,  $n$  is the instantaneous angles pooled for all beads. Angle measurements for beads adhered to the mesh represent independent measurements for  $N=40$  beads, whereas measurements for beads suspended in the fluid represent instantaneous angles pooled for all individuals,  $n=192$ , from the trajectories of  $N=10$  beads. All angles are relative to the fluid flow. (B) Five sample trajectories of beads transported through the food-concentrating filter. Frames were color-coded (red, yellow, green, cyan, blue, magenta) so that the color order shows direction and white indicates a particle has not moved. Arrows show directions of fluid flow. Montages show the bead orientations for the respective trajectory.

## **Discussion:**

The upper ocean is dominated by non-spherical particles (Fig. 11), which are central in biogeochemical cycling. Our findings demonstrate that the shape of microbe-sized particles affects their fate through their interactions with the filtration apparatus of a globally abundant marine grazer, *O. dioica*. The results from our incubation experiments show that, regardless of the particle size, the minimum particle diameter is the key variable affecting how particles are grazed (Figs. 14 and 15). This study represents the first experimental evidence of differential grazing based solely on particle shape and implies grazing by appendicularians can cause non-uniform export of different shaped particles.

Although feeding structures may vary widely (Jørgensen et al. 1984), encounter between a prey particle and a food-collecting element is a somewhat general process because of the finite number of hydrodynamic particle encounter mechanisms (Rubenstein and Koehl 1977, Humphries 2009). The appendicularian pharyngeal filter can capture particles smaller than the mesh pores through direct interception and diffusional deposition of particles onto the sticky filter fibers (Acuña, Deibel, and Morris 1996). These small-scale interactions between a particle and filter fiber are applicable to other planktonic grazers. Phagotrophic protists, dominant bacterivores in aquatic systems, exhibit a wide variety of feeding strategies, but food acquisition depends initially on prey contact and capture by a food-collecting element prior to ingestion (Montagnes et al. 2008). Copepods capture prey using bristled appendages at Reynolds numbers  $\sim 10^{-2}$  to  $10^{-1}$  (Koehl and Strickler 1981). Previous work has shown that elongated diatoms in the siphon flow of a copepod's feeding appendages tend to orient with their long axes

parallel to streamlines (Visser and Jonsson 2000), corroborating our high-speed videography observations that prolate spheroids in the laminar flow regime of the appendicularian food-concentrating filter tend to passively orient short-end forward (Fig. 16). Mathematical predictions and empirical evidence also support that spheroidal particles at low Reynolds numbers preferentially align with fluid streamlines, periodically flipping orientation depending on the hydrodynamic shear (Jeffery 1922, Bretherton 1962, Seymour et al. 2011). The kinematics and differential retention of spheroidal particles by appendicularians may therefore have broader applicability to other pelagic and benthic bacterivores that rely on different feeding appendages to capture prey.

The few studies that have examined the ramifications of microbe shape for predation (Turley, Newell, and Robins 1986, Pedley and Kessler 1987, Jürgens et al. 1999, Visser and Jonsson 2000, Troedsson et al. 2007) are subject to the methodological limitations of live prey—namely, the possible confounding effects of surface properties, motility, and morphological variability (e.g. flagella, pili, fibrils, gel matrices, coccoliths or protist scales). Ours is the first study to isolate the effect of particle shape on differential grazing using synthetic particles of uniform dimensions, densities, and surface properties. Our findings demonstrate particle length-to-width ratios influence particle fate—specifically, whether particles were ingested or remained adhered to the appendicularian house (Figs. 14, 15). Particle shape can therefore affect the composition of marine snow aggregates produced by mucous filter-feeders.

Non-uniform selection has implications for particle removal from the upper ocean and for carbon export to the ocean's interior. Appendicularians are a major contributor to marine snow in the euphotic and mesopelagic zones through the production of discarded

houses and fecal pellets (Alldredge and Silver 1988, Robison, Reisenbichler, and Sherlock 2005). Although houses and fecal pellets are aggregations of small particles, particles in these forms are subject to different fates (Urban, Deibel, and Schwinghamer 1993, Vargas et al. 2002) (Fig. 14). The carbon content of discarded houses varies by species and is also affected by retained particles, ranging from 2.6 to 56 ug C house<sup>-1</sup> with flux rates spanning 0.6 to 1257 mg C m<sup>-2</sup> d<sup>-1</sup> (Alldredge et al. 2005). An individual *Oikopleura* can produce over 300 fecal pellets per day (Taguchi 1982), with carbon content ranging from 3.55 x 10<sup>-14</sup> to 1.18 x 10<sup>-11</sup> µg C fecal pellet<sup>-1</sup> (Sato et al. 2008) and flux rates of 40 to 1310 mg C m<sup>-2</sup> d<sup>-1</sup> (Dagg and Brown 2004). Houses and fecal pellets are also subject to different sinking rates (Fig. 14) and microbial mineralization may occur during their descent. Our results suggest that large (≥10 µm diameter) and submicron particles are more likely to remain associated with the appendicularian house, which generally contain more labile carbon than fecal pellets (Bochdansky and Deibel 1999) and contribute to greater carbon flux rates (Fig. 14), whereas micron-sized particles (<10 µm diameter) are more likely either to be assimilated into biomass or compacted into fecal pellets that sink at faster rates than the houses (Fig. 14). These findings therefore have ramifications for the microbial loop (Pomeroy 1974), indicating that cell size and shape may influence which cells are recycled into the food chain via the microbial loop versus exported from the surface ocean.

Consistent with previous studies (Bedo et al. 1993, Acuña, Deibel, and Morris 1996, Fernández et al. 2004), we found that particle size also strongly influences selection and particle fate. Appendicularians efficiently ingested particles in the intermediate size range (1-3 µm), leaving a higher-than-ambient proportion of large

particles (10  $\mu\text{m}$ ) and submicron particles ( $<0.5 \mu\text{m}$ ) in the house. Previous authors have similarly shown reduced ingestion of particles below  $\sim 1 \mu\text{m}$  (Bedo et al. 1993, Fernández et al. 2004). Since size and shape are interrelated metrics, our results extend the current understanding of size-based selection by identifying how length-to-width ratios affect retention efficiencies. It is well-established that a cell's length-to-width ratio is of fundamental importance for a variety of physical and physiological processes, including photosynthesis, diffusion, active motility and passive dispersal (Young 2006). We show for the first time that micron and submicron ellipsoids are ingested at rates comparable to a sphere of equivalent width. Our results suggest elongated cells may gain the various physiological advantages of an increased surface-to-volume ratio without incurring any increased cost of predation. This identifies an additional, potential explanation for the prevalence of rod-shaped cells in the ocean.

#### *Bridge to Chapter IV*

For the present study (Chapter III), I used a toluene-stretching technique to synthesize rod-shaped particles from the latex spherical microbeads historically fed in size-selection experiments. I used the beads in incubation experiments with the appendicularian *Oikopleura dioica* and found that the minimum particle diameter was the key variable for determining how non-spherical cells were grazed. The kinematic mechanism invoked to explain these retention patterns was that rod-shaped beads oriented parallel to fluid streamlines, and thus that the minimum diameter of the rod presumably intercepted the filter fiber. Appendicularians have a complex external filtration apparatus called the house, which is unique to the class, and particles must pass through a series of three different filters (the inlet, food-concentrating, and pharyngeal



filter) prior to ingestion. In Chapter III, I tested the broader applicability of these results to by conducting comparative experiments on the effect of particle shape on retention by a benthic ascidian (*Styela plicata*), which filter-feeds using a different mechanism.

## CHAPTER IV

# NOT-SO-SIMPLE SIEVING BY ASCIDIANS: MESH-SCALE OBSERVATIONS OF NON-SPHERICAL PARTICLE CAPTURE AND RETENTION BY HYDROSOL FILTRATION

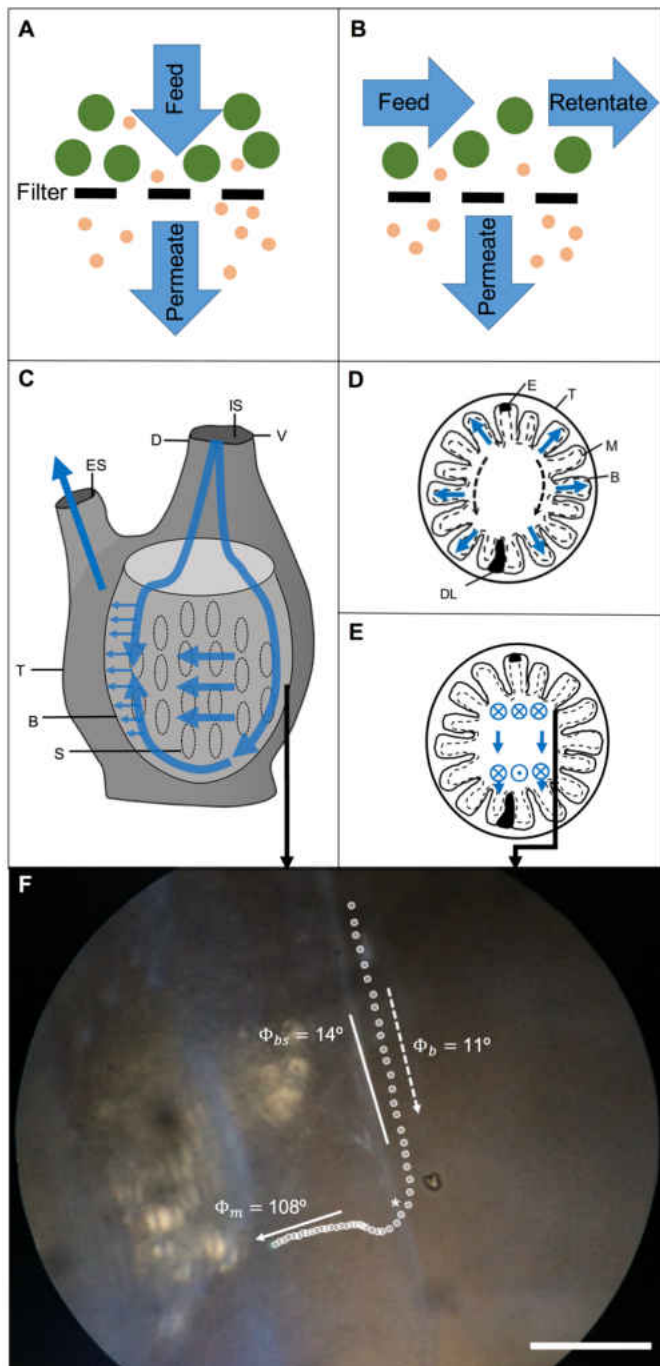
The fourth chapter of this dissertation is in preparation for submission to *Marine Biology* and is co-authored by Aviv Ben-Tal, Yuval Jacobi, Gitai Yahel, and Kelly R. Sutherland. I conceived and designed the experiments, synthesized ellipsoidal particles, performed a subset of the endoscopy experiments, analyzed and presented the data, contributed to funding acquisition, and wrote the initial draft of the manuscript. Co-authors contributed to funding acquisition, mentorship/supervision, helped conceive the experiments and designed methodology, helped perform a subset of the experiments (endoscopy and direct sampling by A.B.T.), and reviewed and edited the manuscript.

### **Introduction:**

Filter-feeding particulate food from the surrounding water is a common feeding strategy that occurs across organisms spanning a range of sizes including protists, zooplankton, fish, and whales. Different mechanisms, however, are responsible for bringing particles in contact with the filter-feeding structure (Kjørboe 2011). The mechanism of particle collection depends on the particle size, density, surface properties, and also on the hydrodynamics of the filtration system. Some common types of filtration include: 1) dead-end sieving (direct flow filtration), 2) crossflow filtration, and 3) hydrosol filtration.

The term “sieving” can be used broadly to describe 100% capture of particles larger than the pore size of the filter or appendage (Shimeta and Jumars 1991). However, the term often implicates a set of hydrodynamic criteria, wherein there are only two fluid streams: the feed (raw water that has not yet passed through the filter), which is perpendicular to the filter, and the permeate (water that has passed the filter) (Fig. 17A).

A pressure gradient moves all of the feed through the filter, and particles are only captured if they are larger than the filter pores (Li and Li 2015).



**Figure 17** Schematic of filtration mechanisms and ascidian feeding. (A) Dead-end sieving (B) Crossflow filtration (C) Side view showing water flow through *Herdmania momus*. IS: inhalant siphon; ES: exhalant siphon; S: stigmata; T: tunic; B: branchial sac. V: ventral side; D: dorsal side. (D) Cross-section of an ascidian showing classical

depiction of water flow through the branchial sac. Branchial sac is shown folded. E: endostyle, where mucus mesh is initially secreted; M: mucus mesh; DL: dorsal lamina, where mucus mesh is wound for ingestion. (E) Cross-section showing proposed depiction of water flow supported by this study.  $\otimes$  indicates flow into the page. (F) Endoscope micrograph showing trajectory of particle pre- and post-capture by *H. momus*. Each point represents one frame.  $\Phi_{bs}$ : angle of branchial sac;  $\Phi_b$ : angle of 6  $\mu\text{m}$  bead prior to capture on the mesh;  $\Phi_m$ : angle of mesh movement. \* indicates position of particle upon contact with the mesh. Black arrows in panels C and E show approximate position of endoscope in the branchial sac. Scale bar: 0.05 mm.

In industrial crossflow (tangential) filtration, there are three fluid streams: the feed, the permeate, and the retentate (concentrated particles) (Bowen and Jenner 1995, Bhave 1996, Mota, Teixeira, and Yelshin 2002). As in sieving, the permeate is perpendicular to the filter, but the feed and retentate both move parallel to the filter (Fig. 17B) (Bhave 1996). A pressure gradient causes the permeate to diverge orthogonally from the parallel crossflow (Song and Elimelech 1995). As such, crossflow filtration is often used to fractionate large particles from small ones, usually by recirculation of the retentate (Schwartz and Seeley 2002). Industrial crossflow filtration considerably reduces filter clogging because tangential shearing forces, which arise from the permeate turning to exit the filter pore, lessen the deposition of large particles on the filter surface and delaying cake buildup (Song and Elimelech 1995, Sanderson et al. 2001). In contrast, large particles in dead-end sieving often accumulate on the filter, reducing its effective pore size and increasing the pressure drop (Schwartz and Seeley 2002). There are a few examples of biological filters that resemble industrial crossflow filters. Video endoscopy revealed that several species of suspension-feeding fishes (*Dorosoma cepedianum*, *Carassius auratus*, *Oreochromis esculentus*) use a crossflow-style filtration: a high velocity crossflow prevented ~95% of particles from contacting the filtering gill rakers (Sanderson et al. 2001). The crossflow filtration process of these fishes was therefore

characterized by lower amounts of particle deposition than is typical of industrial crossflow filters (Sanderson et al. 2001).

Hydrosol filtration predicts intermediate capture efficiency of particles smaller than the mesh pores via different mechanisms—direct interception, inertial impaction, gravitational deposition, and diffusional deposition—that all cause particles to encounter a filter fiber (Rubenstein and Koehl 1977). The relative influence of the different hydrosol capture mechanisms depends on the Reynolds number of the filter. Direct interception and diffusional deposition are the primary mechanisms at low Reynolds numbers (Shimeta 1993). Hydrosol filtration can occur independently or may accompany another type of filtration. The particle capture mechanisms of a biological filter determine the particle size spectrum that filter-feeding animals affect and therefore have broad ecological implications (Sanderson et al. 2001).

One such group of globally abundant filter-feeders are ascidians, a class of tunicates considered by some to be a keystone component of the benthic megafauna community (Ali and Tamilselvi 2016). Solitary ascidians are cylindrically shaped with an inhalant siphon arranged opposite to an exhalent (atrial) siphon (Fig. 17C). Throughout the manuscript, we will refer to the orientation of the ascidian using the terminology described by (Herdman 1899), where the anterior end refers to the side of the animal where the siphons are located, the posterior end is the region attached to the substrate, and the dorsal and ventral sides are toward and opposite the exhalent siphon, respectively (Fig. 17C). Ascidians feed by using cilia to pump water from the inhalant siphon, into the pharynx (branchial sac), and ultimately out the exhalent siphon. The branchial sac is perforated with stigmata, and the beating of the lateral cilia on the stigmata generates the

water current (Fig. 17C) (reviewed in Petersen 2007). The arrangement of the siphons and *in situ* orientation of the animals also facilitates passive flow by dynamic pressure through the inhalant siphon (Young and Braithwaite 1980, Knott et al. 2004). Turbulence occurs at the inhalant siphon, but flow at the filter is viscous and laminar (Kustin et al. 1974, LaBarbera 1984). The viscous pump driven by the lateral cilia of the stigmata is an energetically undemanding system because of the low pressure drop of the filter (0.1-0.4 mm H<sub>2</sub>O) (Jørgensen 1983, Jørgensen et al. 1984, Riisgård 1988).

Particle capture occurs by adhesion onto a fine mucous mesh. The dimensions of the ascidian mesh are generally accepted to be ~0.5 µm wide x 2 µm long with little variation between species (Flood and Fiala-Medioni 1981, Barrera 1990, Petersen 2007), although one study found a finer mesh whose longest dimension did not exceed 0.5 µm (Pennachetti 1984). The mesh is secreted by the endostyle and conveyed dorsally in two separate sheets along the interior surface of the branchial walls by cilia on the secondary structures of the branchial sac (i.e., ciliary mucus transport is separate from the ciliary water current) (Orton 1913, Holley 1986, Mackie et al. 1974, Goodbody 1975). At the dorsal lamina, the mesh is wound into a mucous cord and conveyed to the esophagus for subsequent ingestion (Petersen 2007). Textbook descriptions and the primary literature all depict flow in the branchial sac to be exclusively perpendicular to the mucus mesh in all directions (Fig. 17D) (Werner 1954, Alexander 1981, Jørgensen et al. 1984, Pennachetti 1984, Petersen 2007), except for the endostyle and dorsal lamina, which are non-filtering regions (MacGinitie 1939, Werner 1954). Particles are prevented from being deposited along the endostyle by an additional set of cilia, which effectively redirect

particles to the left and right sides of the endostyle (i.e., onto the mucus wall of the pharynx) (Orton 1913, Holley 1986).

Ascidians are considered a characteristic “simple sieve” (Riisgård and Larsen 2001, Petersen 2007). They are assumed to feed continuously and non-selectively, with the dimensions of the mucus mesh serving as the primary determinant of the particle size captured (Jørgensen 1966). However, since the ascidian filter uses mucus as an adhesive for captured particles, previous authors have acknowledged that it likely does not function solely by dead-end sieving but also presumably captures particles via hydrosol filtration (Rubenstein and Koehl 1977, Wotton 1994). The extent to which hydrosol filtration adds to capture of submicron particles was recently examined for multiple species of oligotrophic ascidians, which showed relatively high removal efficiency ranging from 31-97% for 0.3  $\mu\text{m}$  microspheres (Jacobi 2016).

Despite such meticulous prior investigations, the hydrodynamics of particle capture in ascidians remain vague. Most papers provide a similar general description of water flowing in to the incurrent siphon and out through the exhalent siphon, but mention of flow in the branchial sac is often entirely omitted. Descriptions of particle capture in the branchial sac are primarily from the older literature (Orton 1913, Jørgensen 1949, Werner 1954, Millar 1971), or often vague or nonspecific about where and how it occurs. In this study, we sought to investigate the hydrodynamics of ascidian filtration and the process of particle capture by testing three hypotheses: 1) Ascidians filter using aspects of cross-flow filtration; 2) Particle shape affects interactions with the fluid and mesh; 3) As a consequence of 2), particle shape (and also size) is a major determinant of capture efficiency.

In most biological applications of hydrosol filtration theory, particles are assumed to be spherical (Rubenstein and Koehl 1977), which is at odds with the morphological diversity of aquatic particles (Conley and Sutherland 2017). To test the first hypothesis, we used video endoscopy to determine the incident angle at which particles approached the filter of *Herdmania momus*. To test the second hypothesis, we targeted the particle size range expected to be captured with imperfect efficiency via hydrosol filtration mechanisms (0.3–1.0  $\mu\text{m}$ ) (Jacobi 2016). We directly sampled polystyrene beads in the inhaled and exhaled water to determine how particle shape (spherical vs. ellipsoidal) affects the capture efficiency by hydrosol mechanisms.

## **Methods:**

### *Animal collection and husbandry*

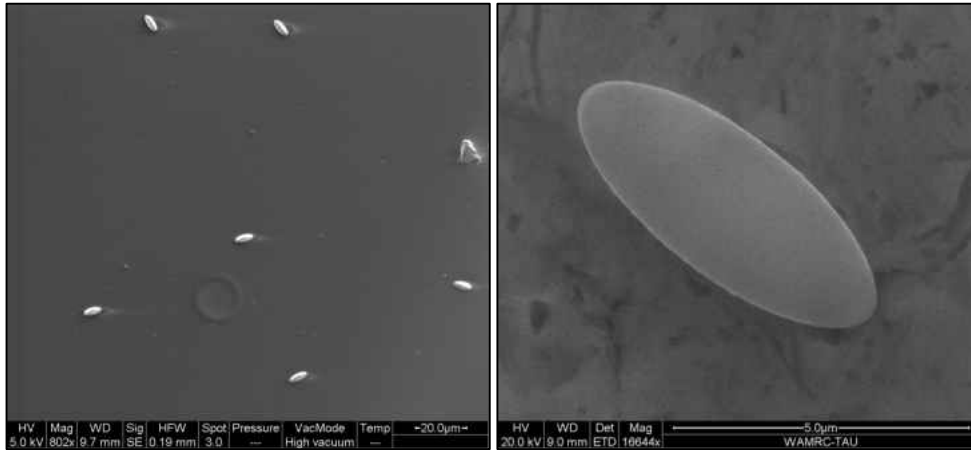
Direct sampling of particle capture was conducted using *Styela plicata* ( $N=12$ ) that were collected by SCUBA divers from ropes of the Sea Bream Farm off the coast of Mikhmoret, Israel, at ~5-18 m depth (N 32°24.700', E 034°50.250'). Animals were quickly transported to The School of Marine Sciences, Ruppin Academic Center, Mikhmoret campus, secured in a cup of gravel, and kept in a sea table with running seawater at ambient temperature of 28°C and salinity of 39-40 PSU. Animals were fed daily using an automated drip system with a constant concentration of *Nannochloropsis* sp. (~5000 cells  $\text{ml}^{-1}$ ). Endoscopy was conducted on field-collected *Herdmania momus* obtained from the same site. All ascidians were fed a pure culture of *Nannochloropsis* sp. a few hours before direct sampling or endoscopic examination to stimulate secretion of the mucus net. Endoscopy was conducted within three days to one month of animal collection.



### *Experimental particles*

Ellipsoidal beads were synthesized using a toluene-stretching method (Champion et al. 2007, Ho et al. 1993, Conley and Sutherland 2017). Briefly, latex microbeads were embedded in a polyvinyl film and mounted on a custom-built stretching device. The stretcher was immersed in a toluene bath for 3 h to partially dissolve the latex beads, and then the film was stretched 2-fold to elongate the beads. The film was then left overnight for beads to re-solidify, and then the polyvinyl was dissolved in water to re-suspend the ellipsoidal microbeads. Control spherical beads were treated with the same toluene bath procedure but the polyvinyl film was not stretched. Submicron ellipsoid beads used for direct sampling of capture efficiency had uniform dimensions with a mean length and width of  $L=0.7 \pm 0.4 \mu\text{m}$  and  $W=0.3 \pm 0.3 \mu\text{m}$  ( $N=21$ ; mean  $\pm$  SD).

The effect of stretching on the surface characteristics of microbeads was confirmed by visualizing ellipsoidal beads using an environmental scanning electron microscope (ESEM). Samples were prepared by placing a 5  $\mu\text{l}$  drop of the bead solution on a freshly-cleaved piece of silicon and fixed onto an aluminum stub using double-sided carbon tape. ESEM images were obtained using an FEI Quanta 200F ESEM scanning electron microscope at 5.0 kV in low vacuum mode. Beads had smooth, featureless surfaces and showed no signs of alterations from the stretching procedure other than shape (Fig. 18).



**Figure 18** Environmental scanning electron micrographs of ellipsoidal microbeads, showing that the stretching procedure does not alter the surface characteristics of the beads.

*Endoscopy: hydrodynamics and mesh movement*

Endoscopic examination was conducted using a rigid borescope with HOPKINS rod lens optical system (Karl Storz, viewing angle: 30°; field angle: 40°; outer diameter: 1.9 mm; working length: 100 mm). Three different cameras were used at different times throughout the experiment: 1) a Telecam camera head connected to a Telecam DX II Camera Control Unit with a Techno LED Nova 150 light source (720 x 480 pixel resolution); 2) an iPhone 5S (1080 x 1920 pixel resolution) and 3) an iPhone 5 SE (2160 x 3840 pixel resolution), the latter two with a SmartScope adapter mounted directly to the endoscope (Karl Storz). For all setups, the endoscope was mounted on an MM-33 micromanipulator and positioned above the ascidian on a lab stand. The optical insertion tube (OIT) was lowered gently into the inhalant siphon of the experimental ascidian and video recording initiated when both siphons were open and the animal appeared relaxed. No “densensitization” of animals to the OIT was performed (Armsworthy et al. 2001). Recording speed for all videos was 30 frames s<sup>-1</sup>. Fluorescent microspheres (20 μm) were used to calibrate the spatial scale.

A high concentration of fluorescent 0.5  $\mu\text{m}$  beads (486 nm emission) was used to visualize mucous mesh behavior ( $\sim 10^8$  beads  $\text{mL}^{-1}$  added to inhalant siphon). Videos for measurements of mesh speed were taken from the dorsal side of the animal at the lower part of the branchial sac, an area that provided views of the mesh moving in a single plane.

*Endoscopy: ellipsoidal particle behavior*

Three sizes of fluorescent ellipsoidal beads (4.5 x 13, 8 x 22, 15 x 44  $\mu\text{m}$ , 486 nm emission) were each observed pre- and post-capture on the mesh of *H. momus*. Beads were gently injected directly above the inhalant siphon using a pipette. Each size of bead was applied and observed separately. Observations of the process of ellipsoidal particle capture were collected from the endostyle side because particle capture was observed there more frequently. Angular data were collected on a 0°-90° scale, with 0° representing a spheroidal bead oriented parallel to the direction of flow.

*Direct sampling of particle retention efficiency*

To test the effect of particle shape on particle capture via hydrosol filtration, we compared the capture of 0.3  $\mu\text{m}$  ( $\pm 0.01$   $\mu\text{m}$  SD) spherical microbeads, 0.3 x 0.7  $\mu\text{m}$  ellipsoidal microbeads, and 1.0  $\mu\text{m}$  ( $\pm 0.01$   $\mu\text{m}$  SD) spherical beads using a direct sampling method (Wright and Stephens 1978). Ascidians in gravel cups were placed individually in 1 L Pyrex beakers with water flow from the sea table turned off. Small tubing (PTFE tube, 60-90 cm long, ID 400  $\mu\text{m}$ , outer diameter 800  $\mu\text{m}$ ) was inserted carefully a few mm into the ascidians siphons and used to simultaneously sample the water inhaled and exhaled by the studied ascidian by slow gravity siphoning. Samples were collected into 2 mL Eppendorf tubes. Each sample was fixed with 3.6  $\mu\text{L}$  of 50%

glutaraldehyde for fixation and kept at 4°C until analysis with flow cytometry. The difference in concentrations of beads between a pair of samples provides a direct measure of the capture, and capture efficiency was calculated as  $E = \frac{C_{in} - C_{ex}}{C_{in}}$ , where  $CE$  is the capture efficiency, and  $C_{in}$ , and  $C_{ex}$  are the concentrations of a certain particle group (e.g. size group) in the inhalant and exhalant water, respectively.

Prior to the addition of fluorescent beads, *Nannochloropsis* was added and a control sample was collected to confirm normal feeding behavior. Fluorometry was used to determine that a similar concentration of *Nannochloropsis* ( $10^2$ - $10^4$  cells  $ml^{-1}$ ) was applied to each treatment. A subsample of the *Nannochloropsis* were filtered onto a 0.2  $\mu m$  polycarbonate membrane for size measurements using epifluorescence microscope (BX53, Olympus) and followed by analysis using ImageJ. Then the beaker was flushed with fresh seawater, and a combination of *Nannochloropsis* and the fluorescent beads of each treatment type were slowly syringe-injected into the beaker and sampling was initiated by gravity flow. Beads were always vortexed prior to sampling.

Since the capture efficiency of a single particle type was found to vary temporally for a single animal, repeated measurements were made for each animal. Results were restricted to those in which *Nannochloropsis* were captured above 70%, based on the assumption that animals with <70% capture of *Nannochloropsis* were not feeding constantly throughout the sampling period because previous work with *S. plicata* showed >70% capture efficiency of 1.0  $\mu m$  beads in lab. Capture efficiency data that adhered to this quality criterion were then analyzed at the average level for each animal.

### *Flow cytometry*

Inhalant and exhalant samples were analyzed fresh using an Attune Acoustic-Focusing Flow Cytometer. Populations were analyzed based on forward scatter versus green fluorescence using violet laser excitation (405 nm). Three distinct groups could be discerned by the flow cytometer: 0.3  $\mu\text{m}$  beads, 1.0  $\mu\text{m}$  beads, and *Nannochloropsis*. Because of this, the 0.3  $\mu\text{m}$  control beads were always applied separately from the ellipsoidal beads for the direct sampling because the forward scatter signal of the two groups could not be reliably differentiated. See Jacobi et al. (submitted) for a detailed description. Only beads that appeared as singlets (based on the forward scatter signal) were included in the grouping on the flow cytometer. No evidence of bead aggregation was observed using forward scatter as a proxy for particle size. To ensure reliable quantification of all size groups, the flow rate was set to either 25 or 100  $\mu\text{L min}^{-1}$  to ensure that the event rate was kept below 600 events  $\text{s}^{-1}$ .

### *Particle tracking*

Recorded videos from the endoscope were converted to an image stack in QuickTime Pro for subsequent velocity, particle orientation, and morphometric analysis in ImageJ (NIH, USA). Particle tracking velocimetry for mucus mesh speeds and free-stream velocities was done manually by tracking individual beads between frames using the plugin MTrackJ (Meijering et al. 2012). To test the null hypothesis that ascidians filter by dead-end sieving, we analyzed the incident angle at which particles approached the filter prior to capture. Incident angles and ellipsoidal bead orientation were both measured using the straight line angle tool in ImageJ (Schneider, Rasband, and Eliceiri 2012). Ellipsoidal bead orientation was converted relative to the fluid flow, while the

incident angle at which particles approached the filter was converted relative to the branchial sac. Since many particle trajectories curved directly prior to capture (1-5 frames), orientations were measured both during the straight period of the trajectory (i.e., the first point of particle appearance in the field of view and the last point of the trajectory prior to any curvature) and during the period of curvature (hereafter referred to as the angle pre-curvature and the angle of curvature, respectively).

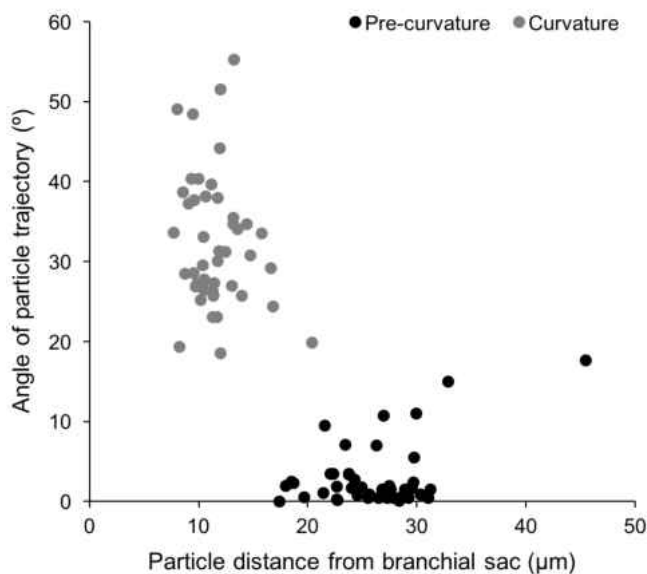
### *Statistical analyses*

Analysis of capture efficiency data was conducted using R (R Core Team, 2013). Since multiple measurements of capture efficiency (2-7) were obtained from each individual, average capture efficiency for each individual was analyzed statistically as paired samples with a balanced design. Three negative values of capture efficiency (-2% to -15%) were converted to zeroes prior to statistical analysis on the basis that negative capture efficiencies reflect the limited precision of the flow cytometer rather than a real biological signal. The capture efficiency of particles by *S. plicata* was left-skewed and violated the assumptions for a repeated-measures ANOVA when evaluated using a normal probability plot and a Bartlett's test for homogeneity of variance. Transformations did not homogenize the variance, so the capture efficiency of different particle types was tested using a nonparametric Friedman's test, the nonparametric equivalent of a repeated measures ANOVA, followed by a Nemenyi test for pairwise multiple comparisons. Throughout the manuscript, values are reported as mean  $\pm$  95% CI unless stated otherwise.

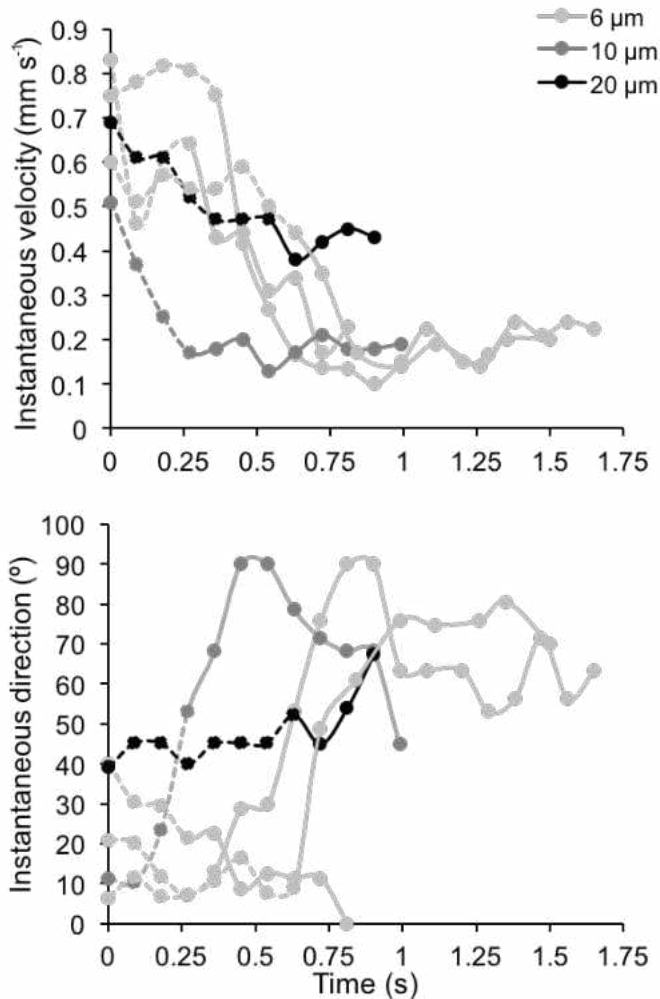
## Results:

### *Endoscopy: hydrodynamics and mesh movement*

Visualization of flow in the branchial sac of *H. momus* using fluorescent beads (6  $\mu\text{m}$ ) as a tracer showed that bulk flow at tens of microns away from the filter was parallel with the filter (Figs. 17F, 19). Particle capture was identified based on the abrupt change in the trajectory and speed of the particle upon adhesion to the mesh (Fig. 20). Particles decelerated prior to encountering the mesh, presumably due to the boundary layer at the mesh, and then, once adhered to the mesh, traveled laterally along the interior wall of the branchial sac (Fig. 20). In the period immediately prior to capture (0.07-0.2 s), particles curved toward the filter (Figs. 19, 20). The mean particle speed in the 0.1 s prior to particle capture was  $0.36 \pm 0.39 \text{ mm s}^{-1}$  ( $n=14$ ,  $N=3$ ). The mean angle of the particle arrival trajectory prior to curvature was  $3 \pm 1^\circ$  ( $n=48$ ,  $N=1$ ), while the mean angle of curvature was  $32 \pm 3^\circ$  ( $n=45$ ,  $N=1$ ) (Fig. 19).



**Figure 19** Angles of the pre-capture trajectories of 6  $\mu\text{m}$  beads (relative to branchial sac,  $0^\circ$ ) near the endostyle pre-curvature ( $>10\text{s}$  of  $\mu\text{m}$  from the mesh) and during curvature ( $\leq 20 \mu\text{m}$  from the mesh).



**Figure 20** Speed and direction of five ellipsoidal microbeads pre-capture (dashed segment) and post-capture (solid segment of line) on the mesh of *Herdmania momus*. The precision of speed and direction measurements is  $\pm 3$  frames or 9.9%.

Beads near the endostyle traveled on a downward trajectory from the inhalant siphon toward the bottom of the branchial sac. In one individual, we observed that 20  $\mu\text{m}$  beads came into close proximity to the mesh but 72% (18 out of 25) of the particles in the field of view did not appear to be captured on the mesh but rather accelerated ( $1.4 \pm 0.69 \text{ mm s}^{-2}$ ,  $n=10$ ) on a downward trajectory toward the bottom of the branchial sac, suggesting that this parallel crossflow component is sufficient to keep at least some particles in suspension as they travel toward the bottom of the branchial sac.



We were able to indirectly visualize the behavior of the mucus mesh by observing the intercepted particles. Particles caught on the mucus mesh moved uniformly along the branchial wall at a much slower speed than particles in the fluid (Table 5, Fig. 19) and did not flow out the stigmata. Although the mean mesh speed was consistently between 0.1-0.2 mm s<sup>-1</sup> across individuals (Table 5), an individual's mesh speed varied by a factor of 2 to 10. During the same filming session for ascidian II, the mesh speed increased from 0.04 to 0.41 mm s<sup>-1</sup> after the addition of 10 µm beads. Mucus mesh conveyance along the branchial sac could also stop temporarily and then resume, which is inconsistent with previous statements that mesh secretion is continuous (Petersen, Mayer, and Knudsen 1999, Jørgensen 1966). Although we did not attempt to directly test the potential for an active behavioral response to particle loads, it is evident that the ciliary transport speed of the mucus can be quite variable, at least for short periods of time.

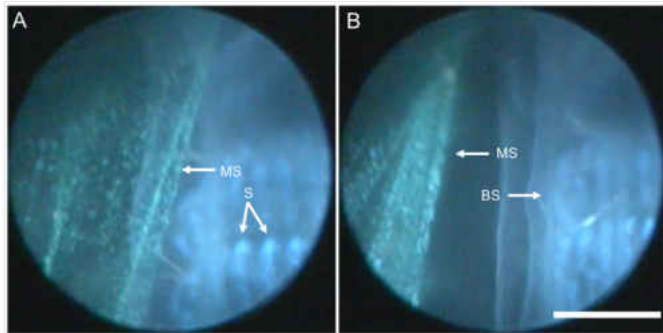
**Table 5** Measurements of mesh and fluid speeds determined from endoscopy on *Herdmania momus*. *N* is the number of independently measured beads, *n* is the total number of instantaneous velocities measured. For consistent comparisons, all measurements were obtained from the dorsal lamina area at the lower part of the branchial sac using 10 or 20 µm beads as tracers.

Individual	Mesh speed, mm s <sup>-1</sup> mean ± 95% CI (range)	<i>N, n</i>	Fluid speed, mm s <sup>-1</sup> mean ± 95% CI	<i>N, n</i>
1	0.20 ± 0.05 (0.05-0.33)	14, 126	1.5 ± 0.40	10, 63
2	0.17 ± 0.05 (0.05-0.41)	14, 147	2.4 ± 0.74	5, 37
3	0.12 ± 0.04 (0.04-0.17)	6, 110	0.62 ± 0.14	7, 174
4	0.10 ± 0.04 (0.03-0.18)	8, 79	1.7 ± 1.0	6, 79

The mean free-stream fluid velocity in the branchial sac of *H. momus* was 1.5 mm s<sup>-1</sup> (Table 5), which is comparable to measurements at the inhalant siphon of *Ciona intestinalis* using particle image velocimetry (0.4–3.8 mm s<sup>-1</sup>) (Du Clos 2016). Based on the characteristic length scale of the diameter of the inhalant siphon of *H. momus* (1.1 · 10<sup>-2</sup> ± 1.3 · 10<sup>-2</sup> m, *N*=5), the average free-stream fluid velocity (1.5 · 10<sup>-3</sup> m s<sup>-1</sup>), the

dynamic viscosity of seawater ( $1.07 \cdot 10^{-3} \text{ kg m}^{-1} \text{ s}^{-1}$ ), and the density of seawater ( $1.02 \cdot 10^3 \text{ kg m}^{-3}$ ), the Reynolds number for the fluid of the branchial sac is  $\sim 15$ . Based on the characteristic length scale of a single filter fiber (diameter  $\sim 10^{-8} \text{ m}$ , Flood and Medioni 1981), and the average pre-capture particle approach speed ( $3.6 \cdot 10^{-4} \text{ m s}^{-1}$ ), we calculated the Reynolds number for ascidian filtration to be  $3 \times 10^{-6}$ .

At high enough particle concentrations, the mucus sheet became visible (Fig. 21A), allowing us to observe the mesh behavior in addition to the particle trajectories. Contraction of the animal, often in response to disturbance from the endoscope or high particle concentrations, caused the movement of the mesh along the branchial sac to cease or detached the mesh from the branchial sac (Fig. 21B). This behavior was not observed in actively filtering animals at low particle concentrations.

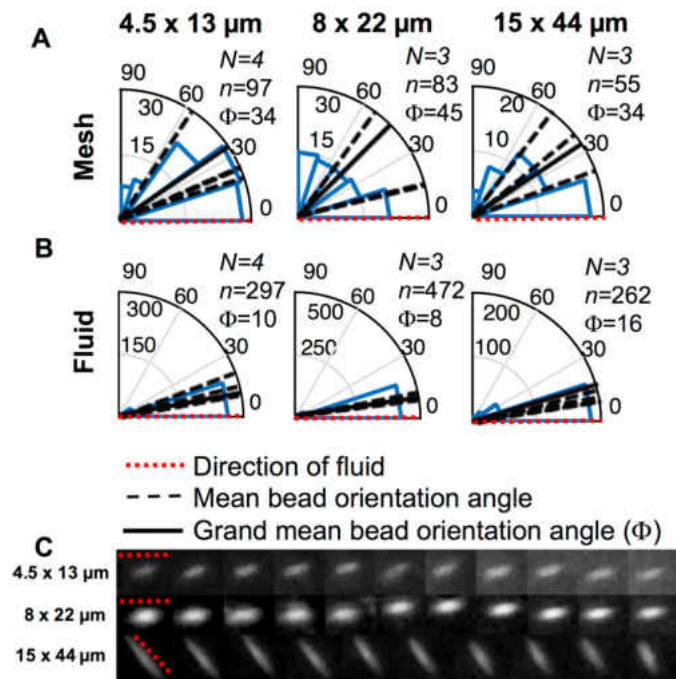


**Figure 21** Endoscope micrographs of the mucus sheet on the branchial sac of *Herdmania momus*. (A) Mucus sheet (MS) visualized using  $0.5 \mu\text{m}$  fluorescent green microspheres. Mesh shown traversing branchial sac (BS) prior to a contraction. S: stigmata. (B) Mucus sheet detached from a fold of the branchial sac during contraction. Scale bar:  $0.1 \text{ mm}$ .

#### *Endoscopy: ellipsoidal particle behavior*

Using endoscopy, we were able to observe the capture process of ellipsoidal particles. All sizes of ellipsoidal beads in the fluid of the branchial sac oriented with their long axis parallel with fluid streamlines (Fig. 22). Ellipsoids approached the mesh with their short axis toward the mesh, and upon contact with it, they reoriented in such a way

as to lie with their long axes against the mesh. Post-capture, 6 and 20  $\mu\text{m}$  ellipsoids were directionally oriented ( $\sim 35^\circ$  relative to the fluid streamlines) with the long axis roughly oriented with the direction of mesh movement (Fig. 22). The mean orientation angle was similar among all three sizes of beads (Fig. 22).

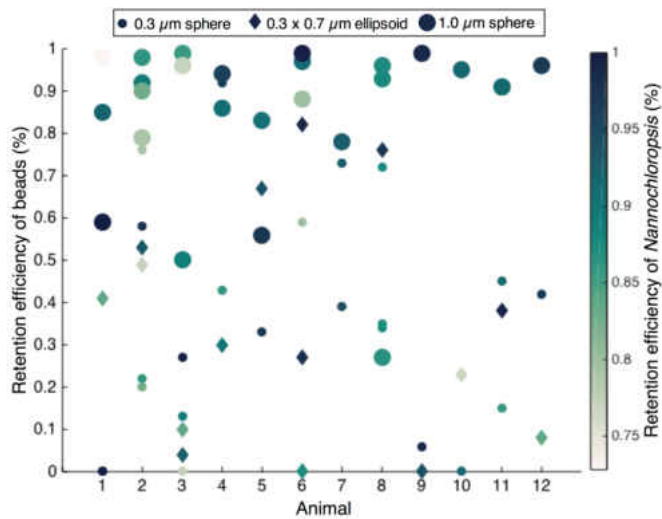


**Figure 22** Orientation of three sizes of ellipsoidal microbeads (4.5 x 13, 8 x 22, 15 x 44  $\mu\text{m}$ ) in the fluid of the branchial sac and on the mesh of *Herdmania momus*. (A) Rose diagrams showing the orientation angles of in the fluid of the branchial sac. (B) Rose diagrams showing the orientation angles of microbeads on the mucus mesh. All angles are relative to the fluid flow (i.e., direction of fluid flow=0°). The area of each sector is proportional to the frequency in the corresponding angle group. Dashed black lines show the mean for each individual animal, solid black line shows the overall mean ( $\Phi$ ).  $N$  signifies number of individuals;  $n$  signifies the total number of observations. (C) Image sequences show sample bead trajectories in the fluid of the branchial sac. Time between frames is 0.03 s. Red dotted line indicates the direction of the fluid.

#### *Direct sampling of particle retention efficiency*

Cultured *Nannochloropsis* cells were approximately spherical,  $\sim 2 \mu\text{m}$  (length=2.1  $\mu\text{m} \pm 0.24$ , width= 1.9  $\mu\text{m} \pm 0.19$ ,  $N=6$ ), and captured at an average of  $90\% \pm 2\%$  ( $N=12$ ). Using the capture of *Nannochloropsis* as a quality control indicator of normal

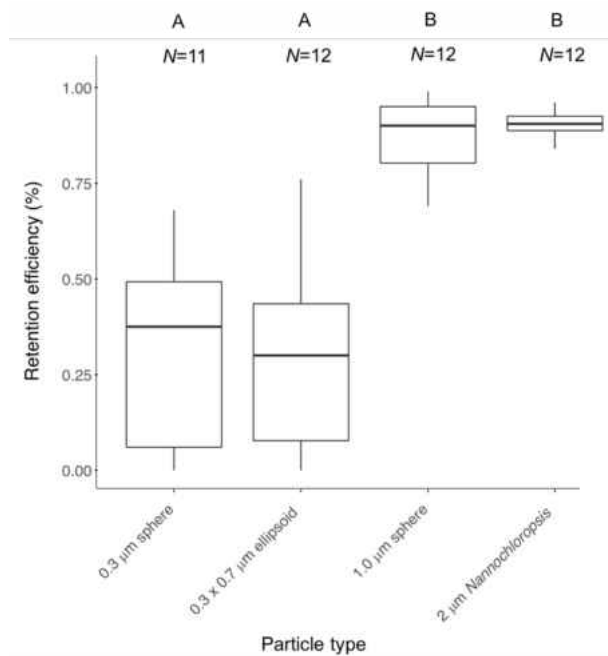
feeding behavior, we were able to detect that the capture patterns for the same individual could vary with sampling (Fig. 23). For example, in individual 6, the capture efficiency of ellipsoidal particles varied from 27% to 82%, while the capture efficiency of *Nannochloropsis* was 98% and 99% for the respective samplings.



**Figure 23** Repeated measures of the retention efficiency of *Styela plicata* fed microbeads and *Nannochloropsis* showing variability of capture patterns within and between individuals. Measurements were obtained through direct sampling of the water inhaled and exhaled by ascidians in the lab. Retention of *Nannochloropsis* was used as an indicator that animals were feeding normally.

Particle type (size and shape) significantly affected the average capture efficiency of *S. plicata* (Friedman  $\chi^2=26.9$ , d.f.=3,  $P<0.001$ ). *Nannochloropsis* and 1.0  $\mu\text{m}$  spherical control beads were both captured with high efficiency ( $86\% \pm 10\%$  for 1.0  $\mu\text{m}$  beads), with no significant difference between the two ( $P=0.96$ ) (Fig. 24). Ellipsoidal beads were captured with significantly lower efficiency than *Nannochloropsis* and 1.0  $\mu\text{m}$  beads ( $P=0.0016$  and  $P=0.0085$ , respectively), as were spherical 0.3  $\mu\text{m}$  beads ( $P=0.0023$  and  $P=0.0016$ ). The capture of ellipsoidal beads ( $32\% \pm 26\%$ ) was not statistically different from that of the 0.3  $\mu\text{m}$  spherical control beads ( $31\% \pm 27\%$ ,  $P=0.96$ ); however, the ellipsoidal beads had a higher maximum average capture efficiency (76%) than the

0.3  $\mu\text{m}$  spherical control beads (68%). The capture efficiency of both 0.3  $\mu\text{m}$  spherical and ellipsoidal beads was more variable than capture of 1.0  $\mu\text{m}$  beads (Fig. 24).



**Figure 24** Average retention efficiency of different sized and shaped prey by the ascidian *Styela plicata*. Measurements were obtained through direct sampling of the water inhaled and exhaled by ascidians in the lab. In the boxplots, the box top and box bottom represent the first and third quartile, respectively; the horizontal line in the box shows the median; whiskers show the range. Negative retention values indicate more beads were measured at the exhalent siphon than the inhalent siphon. Any groups sharing the same letter are not significantly different based on Nemenyi post-hoc test ( $\alpha=0.05$ ).

### Discussion:

Collectively, our results suggest that the depiction of ascidians as continuous, non-selective direct sieves does not reflect the behavioral and hydrodynamic complexity of ascidian feeding. Previous schematic representations of flow in the branchial sac of ascidians (Fig. 17D) oversimplify the different spatial scales of flow patterns (Werner 1954, Alexander 1981, Jørgensen et al. 1984, Pennachetti 1984, Petersen 2007). The capture efficiency of small particles ( $\leq 1 \mu\text{m}$ ) can vary widely for an individual animal even when larger particles are captured at high efficiency ( $>70\%$ ) (Fig. 23). Mesh

secretion is not always continuous, and an individual's mesh speed can also change (Table 5). Therefore, the size of the mesh pores is not the sole determinant of capture patterns. Local flow conditions and particle dynamics are an important part of the filter-feeding process, since local flow can be nonuniform even at low Reynolds numbers (Shimeta and Jumars 1991). This is the first attempt to address how fine-scale flow and particle kinematics influence ascidian feeding.

*Endoscopy: hydrodynamics and mesh movement*

Ascidians adhere to the sieving model in that pressure differences move all of the feed through the filter, filtered particles adhere to the filter (Fig. 21), and *Nannochloropsis*, which is larger than the mesh pores, was captured with an average efficiency of 98% (Fig. 24). However, ascidian filtration also shares some hydrodynamic similarities to crossflow filtration (Fig. 17B, E). Structurally, ascidians resemble a tubular membrane crossflow filtration system where the filter is on the inside wall of the tube. There is a “crossflow” component in the branchial sac, where the fluid being filtered is pumped along the surface of the filter rather than exclusively perpendicular to it (Fig. 17E, F) (Sanderson et al. 2001), but the crossflow velocity is evidently insufficient to keep all particles suspended. Collectively, these results support our first hypothesis that ascidians filter using aspects of cross-flow filtration, and we suggest that ascidian filtration acts as a hybrid-flow filtration system rather than a classical direct sieve. Although the tubular morphology of ascidians seems to necessitate some parallel flow, we could find no prior studies that have previously described flow in the branchial sac to be tangential to the branchial sac.

Similar endoscopic observations with bivalves suggested particles encountered the gill filaments with a low angle of approach  $\sim 30^\circ$  (Ward et al. 1998); however, subsequent work showed that streamlines are essentially normal to the gill surface ( $70\text{-}90^\circ$ ) within a distance of  $\geq 0.10\text{-}0.15$  mm, while a more parallel flow fluid stream ( $30^\circ$ ) may occur in the far-field flow (Riisgård and Larsen 2000). Both the pre-and post-curvature flow stream near the mesh of ascidians is therefore more parallel than that of bivalves (Fig. 19), although it is important to acknowledge that such measurements are inherently sensitive to the angle of observation, as well as the temporal and spatial scale being considered (Riisgård and Larsen 2000). Our measurements of capture trajectories were viewed from the side of the branchial sac (Fig. 17F). From this angle, the particle approach angle was consistently parallel to the filter even tens of microns from the mesh, and the curvature of the trajectories was  $\sim 30^\circ$  at  $\sim 10$   $\mu\text{m}$  from the mesh (Fig. 19). Because of the pressure drop across the filter, the near-field flow may become more orthogonal to the mesh at spatial and/or temporal scales beyond the resolution of our video endoscopy system, but if so, this transition occurs on smaller spatial scales than that in bivalves.

Some authors have previously acknowledged that many animals use filters whose fibers are not oriented normal to the direction of flow (Rubenstein and Koehl 1977, Braimah 1987), and that this affects both the efficiency of particle capture via hydrosol mechanisms and the pressure drop of the filter (Spielman and Goren 1968). Two design criteria influence the optimum function of a biological feeding-filter: 1) high contact with edible particles and 2) low resistance to flow (Vogel 1994). These criteria are usually opposing because fine-meshed filters, such as those used by ascidians, have a high

resistance to flow (Vogel 1994). While a strictly sieving filter is subject to excessively high pressure drop and high clogging rates (Chen 1955), bulk flow parallel to the mesh offers several advantages in terms of resistance. Cylindrical filter fibers in parallel flow experience less drag (Vogel 1994) and a lower pressure drop (Chen 1955) than those subjected to normal flow.

One prior study used endoscopy to observe ascidian feeding (Armsworthy et al. 2001). These experiments described the feeding process of *Halocynthia pyriformis* during exposure to low and high particle concentrations. Two distinct feeding modes were observed: one, at low particle concentrations, in which the mucus mesh traversed the entire surface of the branchial basket (crest and folds) in the classically described way, and a second, at high particle concentrations or sediment loads, when squirting turned the mucus sheets into strands that traversed solely the crests of the basket. These two modes occurred at significantly different speeds—a fast ( $0.074 \text{ mm s}^{-1}$ ) and slow speed ( $0.015 \text{ mm s}^{-1}$ ). Another study used a dissected window in the tunic to observe mucus mesh behavior of *Ascidia paratropa* and found that the mesh could detach from the surface of the branchial sac, moving as a flat sheet from the endostyle to the dorsal lamina through the “pulling” of the dorsal lamina rather than by the cilia of the stigmata (Pennachetti 1984). This form of mucus transport was reportedly often associated with high concentrations of particulate material and prior to the animal squirting (Pennachetti 1984). Our observations of the detachment of the mucus mesh from the branchial sac (Fig. 21B) at high particle concentrations appear quite consistent with this description provided by Pennachetti (1984). However, because we only observed this behavior when animals were fed a very high concentration of  $0.5 \text{ }\mu\text{m}$  latex beads ( $\sim 10^8 \text{ beads mL}^{-1}$ ), we



do not consider it a component of normal (i.e., undisturbed) feeding behavior.

Nonetheless, it may play a role during exposure to high sediment loads from episodic events such as storms and stormwater runoff (Ali and Tamilselvi 2016), as suggested by Armsworthy et al. (2001).

The transport speed of the mucus filter influences its exposure time and hence the amount of water filtered through each area of the filter. The speed of ascidian mesh transport may vary with animal size (Flood 1982), particle concentration (Armsworthy et al. 2001), and likely with temperature (Flood 1982). Previous studies have reported ascidian mesh speeds ranging from 0.015 to 0.22 mm s<sup>-1</sup> (Flood 1982, Armsworthy et al. 2001). Our measurements of the mean mesh speed fall within this range, but reveal a faster upper limit (0.83 mm s<sup>-1</sup>) for mucus mesh transport speed (Table 5). Different species or sizes of animals may operate at different mesh speeds (Flood 1982). Temperature might also play an important role, since it affects the beating speed of cilia (Flood 1982). Variation in mesh speed may also be caused by an increased rate of secretion from the endostyle, or by stretching the mesh through pulling by the dorsal lamina. In either case, variation in mesh speed could indirectly contribute to the different capture patterns observed within individuals (Fig. 23)—faster mucus transport speeds would lower exposure time of the mesh and lessen the likelihood of clogging, or rapid pulling of the mesh could effectively stretch the mesh pores.

#### *Endoscopy: ellipsoidal particle behavior*

Ellipsoidal particles oriented non-randomly in both the fluid of the branchial sac and post-capture on the mesh (Fig. 22), which supports our second hypothesis that particle shape affects interactions with the fluid and mesh. Endoscopic observations of

ellipsoidal particle capture by *H. momus* revealed that the minimum axis of the ellipsoid intercepts the filter fiber, thereby providing the mechanism for the capture patterns found through direct sampling of *S. plicata*. These results are consistent with those from appendicularians (Conley and Sutherland 2017), copepods (Visser and Jonsson 2000), and carp larvae (Drost, Osse, and Muller 1987). The behavior of ellipsoidal prey thus appears quite consistent across predator feeding structures, and our work extends the applicability of these mechanisms from planktonic grazers to a benthic suspension-feeder.

The measured speed of ellipsoidal particles in the 0.1 s prior to particle capture ( $0.36 \pm 0.39 \text{ mm s}^{-1}$ ) is consistent with previous calculations of flow velocities through the filter based on the pumping rate at zero back pressure ( $0.2\text{--}0.37 \text{ mm s}^{-1}$ ) (Jørgensen 1983, Jørgensen et al. 1984, Riisgård 1988, Petersen 2007).

#### *Direct sampling of particle capture efficiency*

Our direct sampling results show near 100% capture of particles  $>1.0 \mu\text{m}$  and intermediate capture efficiency of  $0.3 \mu\text{m}$  beads (Fig. 24). While dead-end sieving predicts 100% capture of particles larger than the mesh pores and 0% capture of particles smaller than the mesh pores, hydrosol filtration predicts intermediate capture efficiency of particles smaller than the mesh pores (Rubenstein and Koehl 1977). Our results suggest hydrosol capture mechanisms add to particle capture by ascidians, consistent with Jacobi (2016). Hydrosol mechanisms have been shown to contribute to capture of small particles by salps and appendicularians (Deibel and Lee 1992, Acuña et al. 1996, Fernández et al. 2004, Sutherland et al. 2010, Kiørboe 2011), but such mechanisms have not been as well documented for ascidians (Jacobi 2016). Previous studies have

suggested that ascidians can only capture particles down to 1-2  $\mu\text{m}$  with 100% efficiency, but the lower size limit of efficient capture is not universally agreed upon (Jørgensen et al. 1984). A review of ascidian suspension feeding asserted that there are no studies demonstrating capture of submicron particles, and that if submicron particles are captured, they cannot constitute a quantitatively important food source (Petersen 2007). Endoscopic observations showed that 0.5  $\mu\text{m}$  beads were effectively captured on the mesh of *H. momus* (Fig. 21), and we did not observe any 0.5  $\mu\text{m}$  beads flowing through the mesh, even at lower particle concentrations. Although 0.3  $\mu\text{m}$  beads could not successfully be visualized by the endoscope, results from direct sampling with *S. plicata* (Fig. 24) indicate that capture of particles  $<1.0 \mu\text{m}$  occurs through hydrosol mechanisms, as previously suggested (Jacobi 2016). These observations support the notion that ascidians have a very fine mucous mesh that is able to efficiently capture prey the size of solitary bacteria.

The application of aerosol filtration theory to biological filters revolutionized our understanding of the mechanisms for size-selective feeding (Riisgård and Larsen 2001). The theory's assumption of spherical prey particles (Rubenstein and Koehl 1977) is nonetheless at odds with the wide-ranging morphologies of aquatic particles: bacteria range from coccoid to bacilloid to vibrioid; flagellates are often asymmetrical, with spines, scales, or collars; diatoms encompass filamentous, pennate, centric, and discoid forms (Jonasz 1987, Dusenbery 1998, Clavano et al. 2007, Guasto et al. 2012, Conley and Sutherland 2017). Our results show how the length-to-width ratio of particles affects particle-encounter efficiency through hydrosol capture mechanisms: the minimum diameter of ellipsoidal particles determines the capture efficiency (Fig. 24). These results

support our third hypothesis that particle shape and size determine the capture efficiency, although our results indicate that ellipsoidal particles may be appropriately modeled as spheres of equivalent minimum diameter.

#### *Future directions*

Further work examining the flow fields of the entire branchial sac would enhance our understanding of ascidian filtration. For example, the possibility of recirculation in the branchial sac has not been explored. Flow in the pharyngeal filter of salps has been observed to be circular, and such flows may serve to concentrate particles via recirculation (Sutherland 2009, Sutherland et al. 2010), similar to the role of the food-concentrating filter of appendicularians (Morris and Deibel 1993). In describing ascidian feeding, Jørgensen (1949) described “a certain amount of aggregated particles was always observed when the [filtered] suspensions were examined under the microscope,” suggestive of a possible role of recirculation and concentration in the branchial sac. Video observations and dye visualizations of the fine filtering combs of the cladoceran *Daphnia*, previously believed to function as a sieve, showed most fluid moved tangential to the filtering combs rather than through them. The authors similarly suggested that tangential filtration may operate in concert with hydrosol filtration and sieving (Gerritsen et al. 1988). Given the great morphological diversity of aquatic filter- and suspension-feeders, the flows of many biological filters may be more hybrid-type in nature and the process of particle capture may be more complex than simply sieving.

CHAPTER V  
SYNOPTIC DISCUSSION

**Selective Feeding by Tunicates**

Selective feeding influences the composition and stability of communities (Murdoch 1969, Strom and Loukos 1998). The mechanisms for selective feeding in aquatic systems are less easily observed than in terrestrial systems (Porter 1977), yet their impacts remain just as far-reaching: feeding selectivity by aquatic grazers has been shown to induce morphological shifts in prey (Hahn and Höfle 2001), direct seasonal succession (Sterner 1989), and affect inter-species competition (Kirk 1991) and trophic structure (Fuchs and Franks 2010).

Selective feeding is often viewed in opposition to filter-feeding (Britannica 2016, Jørgensen 1966). However, selective filter-feeding mechanisms have been identified for bivalves, such as the inclusion of particles in mucus to either prevent food loss or to facilitate rejection (Shumway et al. 1985, Ward et al. 1993). The particle selection process of bivalves involves the rejection of particles as pseudofeces (Kiørboe and Mohlenberg 1981), a unique mechanism that is not widely applicable to other filter-feeding taxa. This dissertation identified novel selective-feeding mechanisms by tunicates, a group of filter-feeders historically assumed to feed non-selectively (Jørgensen 1966, Bedo et al. 1993, Acuña, Deibel, and Morris 1996, Gorsky et al. 1999). I review the current knowledge of tunicate selective-feeding mechanisms below, highlighting and contextualizing the contributions from this dissertation. The review is broadly organized into two different sections covering physical selection mechanisms that depend on the properties of the particles (size, shape, and surface properties) and mechanisms that

depend on predator behavior separately. Although this organizational framework suggests these mechanisms are discrete, in many cases the selection process depends on the interaction between particle properties and a behavioral response, and there is therefore some inherent overlap between sections.

### *Physical Selection Mechanisms*

#### *I. Size-dependent Selection*

All tunicates exhibit mechanical, size-dependent selection with imperfect (<100%) retention efficiency of submicron particles by the pharyngeal filter (Kremer and Madin 1992, Sutherland, Madin, and Stocker 2010, Jacobi 2016). Ascidians have a left-skewed retention efficiency curve, with reduced retention between 1-2  $\mu\text{m}$  (Jørgensen et al. 1984, Petersen 2007, Petersen 2016). Submicron particles can be retained through hydrosol mechanisms (Chapter IV, Jacobi 2016), but the retention efficiency varies considerably between species (Flood and Fiala-Medioni 1981, Stuart and Klumpp 1984, Bak et al. 1996, Lesser and Slattery 2015, Stabili et al. 2015, Jacobi 2016, Stabili et al. 2016, Dadon-Pilosof et al. 2017). *Halocynthia spinosa* retains submicron particles with the highest reported efficiency:  $91 \pm 6\%$  (mean  $\pm$  95% CI) for 0.3  $\mu\text{m}$  microspheres (Jacobi 2016). In comparison, in Chapter IV, I showed that the ascidian *Styela plicata* retains particles  $\geq 1 \mu\text{m}$  with  $\sim 100\%$  efficiency, but retains 0.3  $\mu\text{m}$  beads with only  $31 \pm 27\%$  efficiency (mean  $\pm$  95% CI). Its congener, *S. clava*, also feeds size-selectively, with a bell-shaped retention efficiency curve that is maximal at intermediate size particles (5-9.5  $\mu\text{m}$ ) and declines  $\sim 3.5$  and 14  $\mu\text{m}$ , depending on particle concentrations (Jiang et al. 2008). In Chapter IV, I also showed that the size-dependent patterns of retention efficiency are not always constant, and that size-dependent selection may also depend on

the animal's behavior, the hydrodynamics of filtration, or possibly the mesh secretion rate. Therefore, a single measurement of an individual's retention efficiency may not reflect the true variability.

In addition to lower retention of particles smaller than the mesh pores, some ascidian species exhibit reduced retention of particles larger than the mesh pores. *Ciona intestinalis* had significantly decreased retention efficiency for particles  $>4.5 \mu\text{m}$ , possibly because larger particles were often silt (Pascoe, Parry, and Hawkins 2007, Dadon-Pilosof et al. 2017). Dadon-Pilosof et al. (2017) found that multiple species of oligotrophic ascidians removed nano-eukaryotic algae (2-20  $\mu\text{m}$ ) at a lower efficiency than 1  $\mu\text{m}$  beads, pico-eukaryotic algae, and *Synechococcus*. Similar results were also shown with ascidians by (Yahel, Marie, and Genin 2005). The Antarctic ascidian *Cnemidocarpa verrucosa* had a higher filtration efficiency for heterotrophic bacteria (mostly *Archaea*) than for picoeukaryotes (Lesser and Slattery 2015). The mechanism for reduced retention of particles larger than the mesh pores, but smaller than the inhalant siphon, remains somewhat unclear, although contracting the inhalant siphon to reduce the opening diameter has been suggested as a behavioral mechanism to prevent the entry of large silt particles (Armsworthy, MacDonald, and Ward 2001, Pascoe, Parry, and Hawkins 2007).

The appendicularian pharyngeal filter also has a left-skewed retention efficiency curve with declining efficiency of submicron particles. Like the ascidian filter, the pharyngeal filter can capture particles smaller than the mesh pores through hydrosol mechanisms (Acuña, Deibel, and Morris 1996). Models and experimental evidence show the pharyngeal filter of *O. vanhoeffeni* ( $\sim 3 \mu\text{m}$  mesh pores) can retain particles  $\sim 0.1 \mu\text{m}$

with ~10-25% efficiency (Flood, Deibel, and Morris 1992, Acuña, Deibel, and Morris 1996). A similarly low retention (3-27%) of submicron particles (0.2  $\mu\text{m}$ ) was also shown for *O. dioica* and *Fritillaria borealis* (Fernández et al. 2004). The retention efficiency of the pharyngeal filter of *O. dioica* and *O. vanhoeffeni* increases to 14-80% for larger submicron particles (0.5-0.75  $\mu\text{m}$ ) (Deibel and Lee 1992, Bedo et al. 1993, Fernández et al. 2004), implying efficient bacterivory and less efficient filtration of viruses. Large virus-like particles were found in the guts of appendicularians from the Sargasso Sea and the coastal North Pacific (Gowing 1993). Intriguingly, *O. dioica* was recently confirmed to filter the *Emiliana huxleyi* virus at rates comparable to that of larger particles ( $\sim 2\text{-}50 \text{ mL}^{-1} \text{ ind}^{-1} \text{ day}^{-1}$ ) (Lawrence et al. In Review).

Size-dependent retention patterns of the thaliaceans are less thoroughly characterized than that of the other two tunicate classes, but in general the pharyngeal filter of thaliaceans appears less efficient at retaining small particles than ascidians or appendicularians. Experimental evidence shows that many species of salps retain particles  $< 2\text{-}4 \mu\text{m}$  with  $< 100\%$  efficiency (Harbison and Gilmer 1976, Harbison and McAlister 1979), although mathematical models predict higher retention of smaller particles through hydrosol mechanisms (Sutherland, Madin, and Stocker 2010). Some authors have suggested that smaller salps may have a mesh with smaller pores that would more efficiently retain small particles (Harbison and Gilmer 1976, Harbison and McAlister 1979, Bone, Carre, and Chang 2003), but even small salps (*Salpa aspera*) retained 1.0  $\mu\text{m}$  particles with only  $\sim 15\%$  efficiency (Kremer and Madin 1992). Experimental results show *Pegea confederata* can ingest particles down to 0.5  $\mu\text{m}$  and mathematical modeling suggests it may capture particles as small as 0.05  $\mu\text{m}$  through



hydrosol mechanisms, but only at <2% efficiency (Sutherland, Madin, and Stocker 2010). Evidence from chemostats (Katechakis et al. 2002) mesocosms (Katechakis et al. 2004), incubations (Crocker, Alldredge, and Steinberg 1991), and fecal pellet analysis (Köster et al. 2011) indicates doliolids capture submicron free-living bacteria, but with unknown efficiency. Retention patterns of pyrosomes also remain unclear (Bone, Carre, and Chang 2003). Measurements of the mesh (Bone, Carre, and Ryan 2000) suggest submicron particle capture is likely (Table 5), but the only study to date on size-selectivity of pyrosomes showed favorable selection of particles >10 µm (Perissinotto et al. 2007). The smallest cells identified in pyrosome fecal pellets were 3-5 µm phytoplankton (Drits, Arashkevich, and Semenova 1992). A recent study hypothesized that a swarm of *Pyrosoma spinosum* was sustained by high densities of *Synechococcus* and flagellates ~1 to 3 µm (Gauns et al. 2015). Submicron filtration is therefore now established for all tunicates except pyrosomes, but apparently with low overall efficiency.

In addition to the size-dependent selection by the pharyngeal filter, appendicularians have the unique potential to also influence particle size spectra through particle interactions with the house. The house necessarily causes size-dependent selection because it prevents some particles from being ingested (Vaugeois, Diaz, and Carlotti 2013, Conley and Sutherland 2017). In most appendicularian species, size-dependent selection first occurs at the inlet filter, which excludes large or spinous particles from entering the house (Alldredge 1977, Sherlock, Walz, and Robison 2016). The restricted size of particle varies between species from ~15-54 µm (Alldredge 1977). Spinous particles, such as *Trichodesmium* or foraminifera, as well as diatoms, large dinoflagellates, detritus, and metazoans are among the particle types often excluded from

entering the house (Alldredge 1976, Alldredge 1977, Sherlock, Walz, and Robison 2016). These particles may or may not remain associated with the house when it is discarded, depending on how strongly the particles adhere to the filter and whether or not they are able to be removed during back-flushing of the filters during tail arrests (Fenaux 1986, Flood 2003). Some appendicularians lack filters on the incurrent openings and thus the dimensions of the incurrent openings are the only limitation on the size of particles that may enter the house (Alldredge 1977). Flood (2003) observed that *Fritillaria borealis* has a second mechanism, in addition to an inlet filter, to exclude large ( $>30\ \mu\text{m}$ ) particles: particles can be arrested against the anterior wall of the tail chamber and washed out of the house prior to entering the food-concentrating filter.

In Oikopleurids, size-dependent selection then occurs at the food-concentrating filters, on which smaller particles are more likely to remain stuck and thus remain associated with the house (Conley et al. 2017). The greater adhesion of smaller particles is assumed to be a product of the lower viscous drag force, which scales linearly with the particle radius and as such would make smaller particles harder to detach (Conley et al. 2017). Tiselius et al. (2003) found that discarded houses had higher pigment concentrations from small algae ( $4\text{-}11\ \mu\text{m}$ ) and that larger algae ( $30\text{-}90\ \mu\text{m}$ ) were not collected on houses. Flow cytometric analysis of *O. dioica* houses showed  $0.2\ \mu\text{m}$  beads accumulated in the house at a higher rate than  $0.75\ \mu\text{m}$  ones (Bedo et al. 1993), and video observations show colloidal particles, including milk and charcoal, irreversibly adhere to the fibers of the intermediate screen (Deibel 1986, Conley et al. 2017). These results suggest that smaller particles are more likely to remain adhered to the appendicularian food-concentrating filter and contribute to size-dependent selection by the animal.

## II. Shape-dependent Selection

Despite Jørgensen's (1966) often-cited definition of filter-feeding as "feeding by passing the surrounding water through structures that retain particles mainly according to size and shape," very little work has addressed the effect of particle shape on retention by filter-feeders. Only one study prior to this work has explicitly examined its effect on selectivity by appendicularians. Troedsson et al. (2007) investigated the fate of three, different shaped cultured algae (*Isochrysis* sp., 6- $\mu\text{m}$  diameter; *Chaetoceros calcitrans*, 4- x 3- $\mu\text{m}$  length x width; *Rhinomonas* sp., 17- $\mu\text{m}$  diameter) captured by *Oikopleura dioica*. The effect of shape was not tested in isolation and the retention patterns depended both on the algal concentration and whether the animals were fed a monoculture or a mixed algal suspension. The main shape-dependent effect identified was that *C. calcitrans*, a smaller alga with projecting spines, tended to be retained on the inlet filters and blocked the entry of the larger *Rhinomonas* particles into the house. A few others have suggested that tunicates may exhibit reduced ingestion of spinous prey (Alldredge 1976, Tiselius et al. 2003, Scheinberg, Landry, and Calbet 2005), but the effects of particle shape aside from spines remains largely overlooked.

Mackie et al. (2006) observed that particle shape can influence squirting and crossed reflex response of ascidians (described in detail below in "Behavioral Selection Mechanisms"). *Corella inflata* did not exhibit a rejection response when fed thin, flat *Ulva* fragments ( $\leq 800 \mu\text{m}$ ), but did so when fed the smaller but chunkier fragments of *Chondracanthus*. *Ulva* fragments oriented longitudinally and did not intercept the oral tentacles at the inhalant siphon, whereas *Chondracanthus* was detected by the hair cells of the tentacles.

This work (Chapters III and IV) provides the only experimental evidence to isolate the effect of particle shape without any other conflating variables (e.g. size, surface properties, density), and is also the first to address the potential impacts of microbial cell shape for fate and carbon flux. My results indicate that the minimum particle diameter is the key variable for determining how rod-shaped cells are retained by the pharyngeal filter, and that this pattern is consistent across particle sizes and different tunicate classes.

### *III. Surface Property-dependent Selection*

Surface properties, including charge (Labarbera 1978, Gerritsen and Porter 1982, Sanders 1988, Rosa et al. 2017,), biochemical coatings (Matz et al. 2002), hydrophobicity (Monger, Landry, and Brown 1999), and wettability (Gerritsen and Porter 1982, Rosa et al. 2013, Rosa et al. 2017), have been shown to affect particle retention by ciliates, bivalves, brittle stars, flagellates, and crustaceans. Aside from one study that showed a higher proportion of negatively charged carboxyl- and sulfate-functionalized microbeads in the guts of *O. dioica* (Fig. S1, Appendix), and a higher proportion of positively-charged amino-functionalized beads in the houses (Fig. S1, Appendix), all studies of surface property effects in tunicate feeding to date have focused on ascidians.

Growing evidence supports chemical-based selection by ascidians. (Young 1988) first observed that ascidians reject conspecific sperm, which appeared “based at least partly on chemical characteristics of the food, not just size.” Two recent studies support this: Dadon-Pilosof et al. (2017) found that the ascidian *Microcosmus exasperatus* efficiently retained picocyanobacteria, Rhodobacteraceae, and Flavobacteriaceae NS2b, with a significantly higher efficiency (64%, 42% and 84%, respectively) than

*Pelagibacter ubiquus*, SAR 116, and Flavobacteriaceae NS5, and SAR 86 (8%, 11%, 13%, and 18%, respectively). Seven other ascidian species and the appendicularians *Oikopleura albicans*, *O. fusiformis* and *O. longicauda* also had similarly null or low retention of the SAR 11 clade. The differential retention of these bacterial phylotypes could not be explained by size alone because fluorescent latex microbeads (0.3  $\mu\text{m}$ ) at the lower size range of the un-filtered bacteria (0.3-1  $\mu\text{m}$ ) were more efficiently removed by *M. exasperatus* (~70%). Cell surface hydrophobicity was invoked as a probable mechanism for the observed retention patterns because the SAR 11 clade had a lower hydrophobic interaction chromatography index (i.e., more hydrophilic cell surface) than other bacterial phylotypes measured (Dadon-Pilosof et al. 2017). A second study found biochemical coatings influenced the retention efficiency of hydrosol filtration by ascidians (Jacobi 2016). Submicron latex spheres coated with the surfactant Poloxamer 188, a polymer with hydrophobic and hydrophilic moieties, were retained at significantly lower efficiencies by five different ascidian species (Jacobi 2016).

#### *Behavioral Selection Mechanisms*

Tunicates exhibit a limited capacity for particle selection via behavioral mechanisms. The main behavioral mechanism for particle rejection is the prevention or exclusion of large particles from contacting the mucus filter. Ascidians may actively reject large particles and appendicularians have several mechanisms to control what particles are ingested. To date, little evidence supports behavioral selection by thaliaceans.

Ascidians can reject particles either by mechanical exclusion through capture on the oral tentacles at the inhalant siphon aperture to prevent large particles from entering

the pharynx (much like the inlet filters of appendicularians), or by hydrodynamic exclusion through ejection in a fluid jet (Petersen 2007). A mechanoreceptor coronal organ consisting of zig-zagging hair cells borders the oral tentacle surface and is posited to perceive incoming particles and trigger a rejection reaction (Burighel et al. 2003, Burighel, Caicci, and Manni 2011). Contraction of the sphincter muscle reduces the gaps between the oral tentacles by ~25%, which can cause incoming particles to become lodged in the tentacle mesh (Mackie et al. 2006). Klumpp (1984) observed that the buccal tentacles of the ascidian *Pyura stolonifera* closed more frequently when fed an increasing load of Sephadex® beads (20-100 µm), whereas Mackie et al. (2006) observed consistent rejection responses only for much larger particles (355-600 µm). Young (1998) implicated the buccal tentacles as a mechanism for rejection of conspecific larvae by solitary adults. Particles lodged on the mesh may either eventually slip through the mesh or may be dislodged by hydrodynamics (Mackie et al. 2006). One study observed that the buccal tentacles of *Clavelina lepadiformis* and *Ciona intestinalis* could secrete mucus, which hung from the tentacles and collected fine particles (Werner 1954).

Hydrodynamic exclusion can occur either through squirting or the crossed reflect response—closing the exhalant siphon while the inhalant siphon remains open and contracting the body wall to expel particles (Hecht 1918)—or through squirting—contraction of the body wall to forcefully eject water and particles from both the inhalant and exhalant siphons (Burighel, Caicci, and Manni 2011). The crossed response is elicited by light stimulation, including single large particles (355–425 µm), whereas squirting occurs in response to stronger stimuli, such as aggregates of large particles (Mackie et al. 2006). Squirting is accompanied by arrest of the lateral cilia of the stigmata

(MacGinitie 1939, Mackie et al. 2006) and has been suggested to serve a similar function as the production of pseudofeces by bivalves (Carlisle 1966, Klumpp 1984, Armsworthy, MacDonald, and Ward 2001,). It may aid in the rejection of large (Klumpp 1984, Mackie et al. 2006) or undesirable (MacGinitie 1939, Young 1988, Bingham and Walters 1989) particles. Spontaneous squirting increases with increasing seston load (Robbins 1981).

All thaliaceans have ciliated lips that are presumed to serve a chemosensory function, and all can perform a behavior analogous to the crossed reflex response of ascidians (Bone and Mackie 1982, Caicci et al. 2013). The crossed reflex of salps has been hypothesized to help prevent large objects from entering the pharynx (Mackie and Bone 1977). At particle concentrations  $> 5.0$  ppm, the mesh of *Pegea confederata* can become clogged with a bolus that cannot be ingested, and they may swim backward in an apparent attempt to dislodge the bolus (Harbison, McAlister, and Gilmer 1986). However, because swimming and feeding are coupled in salps, the crossed reflex response interrupts their locomotion (Mackie and Bone 1977). Although salps do not have a coronal organ comparable to that of ascidians, doliolids and pyrosomes do (Caicci et al. 2013). In the laboratory, doliolids have been observed to perform an escape response similar to the crossed reflex response, although it is unclear if this is solely used as a predator avoidance mechanism or if it also could be used to reject or otherwise manipulate prey (Deibel and Paffenhöfer 1988). Similarly, *Pyrosoma* can respond to large particles by contracting the oral siphon and arresting the cilia of the gill bars (Caicci et al. 2013) and *Doliolum nationalis* also arrests the cilia of the gill bars when large or noxious particles contact the mouth (Deibel and Paffenhöfer 1988). *Dolioletta*

*gegenbauri* gonozooids have been observed to rotate their entire body to help re-position large cells into the mouth (Gibson 2000).

Appendicularians exhibit the greatest array of behavioral mechanisms for selection of particles. They have sensory cells in the mouth considered homologous to the ascidian coronal organ (Rigon et al. 2013), and the ciliary spiracles can serve a similar function to the ascidian squirting response by preventing large particles from entering the mouth. *Oikopleura dioica*, *O. albicans*, and *Bathochordaeus* have inner sensory cells just below the lower lip that are hypothesized to play a role in particle rejection, since the addition of particulate material to the incoming water or tactile stimulation of the lips caused a reversal of the feeding current (Lohmann 1933, Galt and Mackie 1971, Bassham and Postlethwait 2005, Rigon et al. 2013, Sherlock, Walz, and Robison 2016). Reversal of the rotation of the ciliary spiracles has been long invoked as the mechanism that accomplishes these flow reversals to expel particles from the pharyngeal cavity (Galt and Mackie 1971, Fenaux 1986, Lombard, Selander, and Kiørboe 2011). However, high-speed videography of the spiracle cilia of *O. dioica* shows that the rotation of the cilia does not, in fact, reverse as previously assumed; rather, a constriction of the spiracles is associated with each episode of flow reversal (Chapter II). The ciliary spiracles of *Fritillaria* apparently also do not reverse, but instead undergo periodic arrests mediated by stimulation of the receptors of the lower lips (Bone, Gorski, and Pulsford 1979).

At least four ways are known for particles to be rejected by *O. dioica* after conveyance through the food-concentrating filter: 1) the pharyngeal filter may be damaged or cease to be secreted, causing particles to exit via the spiracles (Flood 1991); 2) individual particles may be rejected after entering the mouth via “pipe-smoking



behavior,” whereby a flow reversal caused by the spiracles ejects particles out of the mouth when the buccal tube is detached from the upper lip (Fenaux 1986, Lombard, Selander, and Kiørboe 2011); 3) a flow reversal caused by the spiracles can eject particles out of a large valve-like opening in the buccal tube while the tube remains attached to the mouth (Chapter II, Fenaux 1986); 4) at high particle concentrations, the animal may close its mouth by flipping up the lower lip, causing bulk particles to be rejected non-selectively, exiting via the posterior chamber and out of the house through the exit spout (Flood 1991, Acuña and Kiefer 2000, Selander and Tiselius 2003, Lombard, Selander, and Kiørboe 2011). The latter mechanism has been implicated as a satiation response at high particle concentrations ( $>20,000$  cells  $\text{ml}^{-1}$ ) (Selander and Tiselius 2003). Ascidians may also exhibit a “satiation response” to prevent overloading of the gut, spontaneously squirting and arresting the lateral cilia of the stigma after a particle-laden mesh reaches the dorsal lamina and starts to be ingested (Van Weel 1940, Robbins 1981, Mackie et al. 2006).

An additional mechanism for behavioral selection by appendicularians and some thaliaceans is through active swimming behavior. Anecdotal observations of cultured *Oikopleura dioica* suggested that animals are able to move through different particle environments and select favorable patches to remain in by regulating the speed of the tail beat (Fenaux 1986). This was later supported by quantitative analysis of the swimming speed and tail behavior in response to changing food concentration (Selander and Tiselius 2003). They found *O. dioica* can separate swimming from feeding, and allocate more time to swimming at low particle concentrations. At high particle concentrations, total tail beat frequency declined and animals made more tail arrests, leading the authors to

conclude that “*O. dioica* clearly possess behavioural mechanisms to reduce the negative effects of high food concentrations” (Selander and Tiselius 2003). No follow-up studies have tested for a behavioral swimming response to different particle types, although some have suggested that house abandonment may occur in response to an undesirable particle field (Bedo et al. 1993).

The potential for a plastic response in filtering structure in response to environmental forcing factors remains ripe area for future investigation. The house renewal rate of *O. dioica* is unaffected by the food regime, but animals raised in a food-limited regime have been shown to produce smaller houses than those in a standard food regime (Troedsson et al. 2009). One study showed that older *O. dioica* animals (day 5) exhibited translational regulation of oikosins, with a higher oikosin 7-to-oikosin 3 ratio under a standard food regime than a limited food regime (Troedsson et al. 2009). Since oikosin 7 is associated with the house walls and oikosin 3 is located in the food-concentrating filter, this was proposed as a possible mechanism to conserve energy by creating a smaller house without compromising particle capture from low food concentrations (since reducing the house size should not affect the ingestion rate of small particles).

Other possible behavioral selection mechanisms are anecdotal and largely speculative. For example, if disturbed, ascidians can cease production of the mucus mesh (MacGinitie 1939, Chapter IV), although this has not been implicated as a response to undesirable particles. The mesh translational speed can also vary (Chapter IV), which changes the exposure time of the mesh and may alter size-dependent retention patterns. MacGinitie (1939) also raised the possibility that ascidians may be able to drop particles

from the mucus using cilia bordering the dorsal groove, but this has not yet been confirmed. Lee et al. (2012) found *Dolioletta gegenbauri* did not ingest oil droplets in the absence of phytoplankton, although no mechanism was proposed to explain how ingestion of particles was prevented.

### **Concluding remarks**

The tunicate feeding process has more potential for particle selection than historically assumed. Filtration is often described using the analogy of a colander, coffee or tea strainer (Sanderson et al. 2001). It is important to acknowledge that this analogy, although familiar, does not fully represent the behavioral and mechanical complexity of how these animals feed. In fact, whether tunicates can even be described as true “filter-feeders” (Jørgensen et al. 1984) remains ambiguous. Although particles can be retained by passing water through the mucous filter (Jørgensen 1966), particles can also be retained by contacting the mucus surface as water is carried along the filter rather than exclusively through it, which is more consistent with the definition of suspension-feeding (Jørgensen 1966, p. 131).

As particles become deposited on a filter, the pressure drop of the filter increases. This implies a trade-off between the broadness of a filter-feeder’s capture efficiency curve and the energetic cost associated with clogging of the filter. As described in the above review, selective filter-feeding can arise through multiple different mechanisms. Depending on the mechanism, selective feeding may represent an advantageous, or even adaptive, strategy (Selander and Tiselius 2003, Troedsson et al. 2009), or can also passively result from the constraints of the filter’s form, function, and hydrodynamics (Chapters II, III).

The studies comprising this dissertation inform our understanding of the mechanics and selectivity of tunicate filtration. The results revealed how the reversible process of particle adhesion to the food-concentrating filter of *O. dioica* differs from that of an industrial tangential filtration system, and how this filtration process causes size-dependent selection by the animal. I showed how the hydrodynamics of the pharyngeal filter of *H. momus* differ from an industrial dead-end sieve, and that the size-dependent selection of ascidian filtration is inconstant. I also identified how particle shape influences retention by hydrosol filtration, tangential filtration, and sieving.

Many follow-up questions arise from this work and related, recent research: How do other microbial cell morphologies, such as the curved rod shape of *Pelagibacter ubique*, affect its kinematics and interactions with biological filters? How variable are the adhesive forces between live prey and mucus filter fibers, and to what extent does this influence measured retention rates? More broadly, to what extent does predation function as a selective agent for bacterial survival and evolution (Matz and Kjelleberg 2005)? What is the true prevalence of sieving vs. crossflow vs. hybrid-type filtration among aquatic invertebrates? Answers to these and other questions will further our understanding of the grazing impact of mucous-mesh filter-feeders.

## APPENDIX

**Table S1** Data and references for Fig. 1.

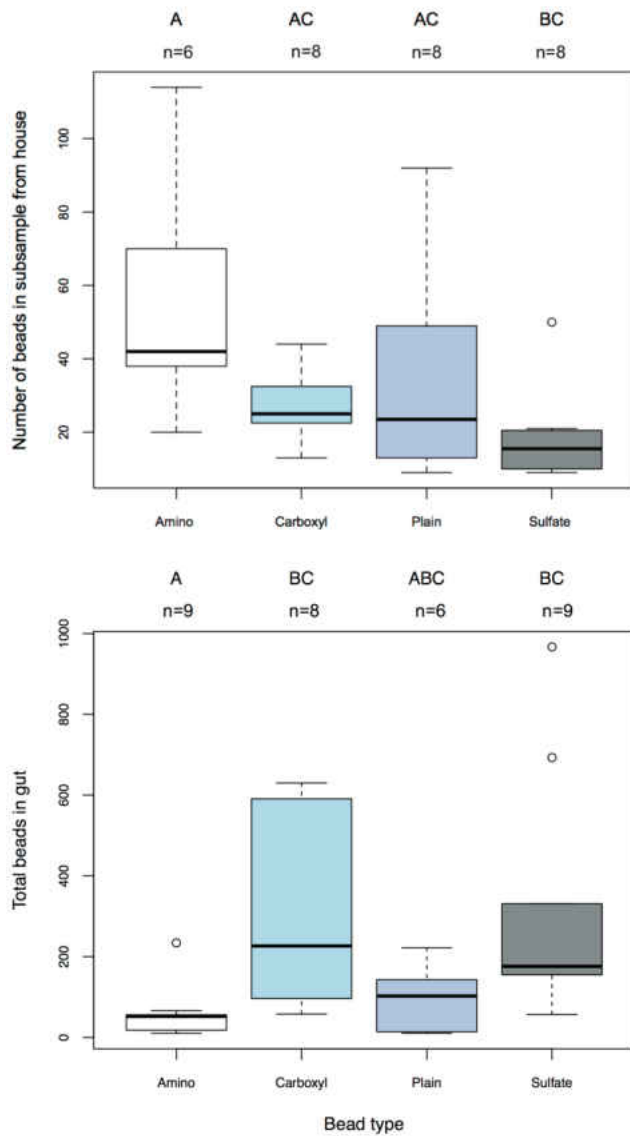
<b>Taxa</b>	<b>Size range ingested</b> ( $\mu\text{m}$ )	<b>Filtration rate</b> ( $\text{ml ind}^{-1}$ $\text{hr}^{-1}$ )	<b>Carbon-specific clearance rate</b> ( $\text{ml } \mu\text{g C}^{-1}$ $\text{hr}^{-1}$ )
Ascidians	0.3-100 (Jacobi 2016, Klumpp 1984)	1084-6195 (Fiala-Medioni 1978)	Unknown
<i>Oikopleura</i>	0.2-50 (Fernández et al. 2004, Gorsky et al. 1999, Flood 2003)	5.8-1477 (Alldredge and Madin 1982, Deibel 1998)	0.6-12.9 (Sato, Tanaka, and Ishimaru 2004)
<i>Bathochordaeus</i>	2000 <sup>†</sup> (Sherlock et al. 2017)	2800-76200 (Katija et al. 2017)	Unknown
Salps	0.5-1000 (Sutherland, Madin, and Stocker 2010, Fuchs and Franks 2010)	1584-55188 (Sutherland and Madin 2010)	0.1-4.4 (Sato, Tanaka, and Ishimaru 2004)
Doliolids	0.5-100 (Katechakis et al. 2002, 2004)	2.5-12.3 (Madin and Deibel 1998, Alldredge and Madin 1982)	0.5-1.2 (Sato, Tanaka, and Ishimaru 2004)
Pyrosomes	3-150 (Drits, Arashkevich, and Semenova 1992)	381-1442 (Perissinotto et al. 2007) <sup>‡</sup>	Unknown
Calanoid copepods	4-210 (Katechakis et al. 2004, Hansen, Bjornsen, and Hansen 1994) for <i>Acartia</i>	4.3-7.9 (Alldredge and Madin 1982) for <i>Calanus</i> , <i>Psuedocalanus</i> , and <i>Temora</i>	0.1-0.9 (Sato, Tanaka, and Ishimaru 2004) for <i>Calanus</i> and <i>Eucalanus</i>

<sup>‡</sup>Values are for colony, not individual zooids.

<sup>†</sup>Minimum size unknown; value represents maximum upper bound based on the maximum diameter of the adult mouth.

**Table S2** Data and references for Fig. 10.

Category	Genus, species	Mean width (μm)	Mean length (μm)	Source for size measurements	Abundance (cells mL <sup>-1</sup> )	Source for abundance
Heterotrophic bacteria	<i>Pelagibacter ubique</i>	0.15	0.65	Munn 2011	3.45 x 10 <sup>5</sup>	Morris, Rappé, Connon, Vergin, et al. 2002
Heterotrophic bacteria	<i>Roseobacter denitrificans</i>	0.75	1.5	Shiba 1991	3.5 x 10 <sup>4</sup>	Wietz et al. 2010
Heterotrophic bacteria	<i>Roseobacter litoralis</i>	0.75	1.6	Shiba 1991	3.5 x 10 <sup>4</sup>	Wietz et al. 2010
Heterotrophic bacteria	<i>Vibrio</i> spp.	0.65	2	Vos et al. 2011	1.3 x 10 <sup>4</sup>	Wietz et al. 2010
Heterotrophic bacteria	<i>Pseudoalteromonas</i>	1	2.5	Vos et al. 2011	2.1 x 10 <sup>4</sup>	Wietz et al. 2010
Cyanobacteria	<i>Prochlorococcus marinus</i> MIT9313	0.8	1.2	Ting et al. 2007	1 x 10 <sup>5</sup>	Johnson et al. 2006
Cyanobacteria	<i>Prochlorococcus marinus</i> MED4	0.7	0.7	Ting et al. 2007	1 x 10 <sup>5</sup>	Johnson et al. 2006
Cyanobacteria	<i>Synechococcus</i>	1.1	2	Waterbury et al. 1979	1.9 x 10 <sup>5</sup>	Waterbury et al. 1979
Prasinophytes	<i>Micromonas pusilla</i>	1.1	1.5	Booth, Lewin, and Norris 1982	1 x 10 <sup>4</sup>	Vaulot et al. 2008
Prasinophytes	<i>Ostreococcus tauri</i>	0.7	1	Chrétiennot-Dinet et al. 1995	1.0 x 10 <sup>5</sup>	Vaulot et al. 2008
Prasinophytes	<i>Bathycoccus prasinos</i>	1.5	2	Eikrem and Thronsen 1990	3.8 x 10 <sup>3</sup>	Collado-Fabbri, Vaulot, and Ulloa 2011
Prasinophytes	<i>Pycnococcus provasolii</i>	3.4	3.4	Guillard et al. 1991	1 x 10 <sup>3</sup>	Zingone et al. 2011
Prasinophytes	<i>Prasinoderma coloniale</i>	2	2.5	Hasegawa et al. 1996	1 x 10 <sup>3</sup>	Vaulot et al. 2008
Coccolithophores	<i>Gephyrocapsa muelleriae</i>	3.8	4.1	Winter and Siesser 2006	1 x 10 <sup>4</sup>	Winter and Siesser 2006
Prymnesiophytes	<i>Phaeocystis pouchetii</i>	6.25	6.25	Medlin and Zingone 2007	1 x 10 <sup>3</sup>	Vogt et al. 2012
Prymnesiophytes	<i>Phaeocystis globosa</i>	3.75	3.75	Medlin and Zingone 2007	1 x 10 <sup>3</sup>	Vogt et al. 2012
Diatoms	<i>Pseudo-nitzschia brasiliiana</i> small	1.8	12	Villac et al. 2005	1 x 10 <sup>4</sup>	Villac et al. 2005
Diatoms	<i>Skeletonema costatum</i>	2	3	Leblanc et al. 2012	1 x 10 <sup>2</sup>	Leblanc et al. 2012
Diatoms	<i>Thalassionema nitzschioides</i> small	2	10	Leblanc et al. 2012	1 x 10 <sup>1</sup>	Leblanc et al. 2012
Diatoms	<i>Nitzschia</i> sp. small	1.5	6	Leblanc et al. 2012	1 x 10 <sup>1</sup>	Leblanc et al. 2012
Diatoms	<i>Chaetoceros</i> spp. <i>pequeñas</i>	2	2	Leblanc et al. 2012	1.2 x 10 <sup>2</sup>	Leblanc et al. 2012



**Figure S1** Tukey box-and-whiskers plot showing counts of 0.5  $\mu\text{m}$  latex microspheres with different surface functional groups in the houses and guts of *Oikopleura dioica* following incubation. The letters A, B, and C indicate significantly different groups with Tukey post-hoc tests after one-way ANOVA. Any groups sharing the same letter are not significantly different. Animals were incubated in 44-mL chambers for 10-min using the protocol described in Chapter III, except that animals were filtered a suspension of a single particle type. Beads were enumerated using a Petroff-Hausser Bacteria Counter (0.2 mm deep, Fisher Scientific CAT # 02-671-13) with dark-field light microscopy (Abdel-Fattah, El-Genk, and Reimus 2002). Because of the high particle concentrations, beads in the houses were subsampled, whereas all beads in the guts were counted.

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