Efficient filtration of micron and submicron particles by ascidians from oligotrophic waters

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Abstract

Suspended food particles in oligotrophic waters are scarce and the planktonic community is dominated by minute picoplanktonic (< 2 μm) cells. Consequently, suspension feeders inhabiting such environments are faced with the dual challenge of capturing extremely small particles and efficiently processing large volumes of water. We used direct in situ techniques to characterize the size-dependent capture efficiency curve of four solitary ascidian species (Chordata, Tunicata) from the ultra-oligotrophic East-Mediterranean, and two species from the oligotrophic Gulf of Aqaba (Northern Red-Sea). To control for confounding factors such as particle surface chemistry, we used a suspension of artificial polystyrene microspheres with a wide range of sizes (from 0.3 μm to 10 μm) but identical surface chemistry. This suspension was introduced into the inhalant siphon while the water inhaled and exhaled by the otherwise undisturbed ascidian was cleanly sampled. All the investigated ascidian species captured ≥ 1 μm microspheres at 95% ± 8% efficiency (mean ± 95% Confidence Interval) with no significant difference between species. Moreover, and in contrast to reports from eutrophic waters, both in situ and laboratory experiments revealed an efficient capture of submicron 0.3 μm microspheres at close to 50% efficiency. For most of the species tested, the capture efficiency of microspheres dropped below optimum between 1 μm and 0.5 μm providing an estimation of their mucus mesh pore size. We suggest that ascidians from oligotrophic waters are adapted for capturing submicron particles picoplankton and other. Such adaptation enables “oligotrophic” ascidians to access a much larger food source and facilitates the transfer of planktonic C, N, and P from the water column to the benthos.

The physical properties of aquatic environments allow living creatures and particulate matter to remain in suspension. This creates a niche for a trophic strategy unique to aquatic organisms - suspension feeding (Jørgensen 1966; Gili and Coma 1998). Suspension feeding is highly abundant in the marine environment and can be found in almost all animal phyla inhabiting the ocean (Riisgård and Larsen 2010), from small worms and copepods to large whales. By metabolizing suspended particles such as phytoplanktonic cells these creatures can potentially shape the planktonic community (e.g., Cloern 1982; Fréchette et al. 1989; Hily 1991; Bak et al. 1998; Yahel et al. 1998) and facilitate the transfer of essential nutrients and energy from the water column to the benthos (Jørgensen 1966; Koseff et al. 1993; Yamamuro and Koike 1994).

In most oceanic settings, suspension feeders reside in a highly dilute suspension of food particles (from ppm in eutrophic waters to ppb in oligotrophic waters, Miller and Wheeler 2012) with a wide range of sizes. Consequently, suspension feeders must process large volumes of water via efficient mechanisms in order to meet their energetic requirements (Jørgensen 1975). This challenge significantly intensifies for suspension feeders from oligotrophic habitats.

By definition, the planktonic biomass in oligotrophic waters is much reduced and small picoplanktonic cells (<2 micron) account for the majority of phytoplankton biomass and productivity. Most of the newly fixed organic carbon in these waters is recycled within the euphotic layer through
complex microbial food webs (the “microbial loop,” Azam et al. 1983). Thus, in oligotrophic waters, suspension feeders are faced with a dual challenge: the need to process even larger volumes of water per biomass gain while employing mechanisms able to capture the smallest cells in the ocean, many in the micron and submicron ranges.

Ascidians (phylum: Chordata, subphylum: Tunicata) are benthic suspension feeders that feed by forcing water through a mucus mesh filter (Millar 1971). The adult solitary animal has two openings: the oral, or inhalant, siphon; and the atrial, or exhalant, siphon. The inhalant siphon leads to a perforated pharynx and the site of filtration - the branchial sac. The pores of the branchial sac are lined with ciliated cells that beat synchronously to force water in, from the inhalant siphon, and through the mucus mesh. The water leaves the branchial sac and exit the body through the exhalant siphon (Monniot et al. 1991). Located along the ventral surface of the branchial sac is the endostyle, which is a glandular ciliated groove that produces the mucus mesh. Another structure, the dorsal lamina, is located on the opposite side of the branchial sac. The mucus mesh is rolled into a string along the dorsal lamina, and drawn with the trapped particles into the esophagus and stomach for digestion (Bone et al. 2003). Individual ascidians process water at a rate ranging from tens to hundreds of milliliters each minute (Fiala-Medioni 1978; Riisgård 1988; Petersen et al. 1999; Petersen and Svane 2002).

Our knowledge of the mucus mesh structure and dimensions is limited to electron microscopy measurements on dried and fixed specimens made by Flood and Fiala-Medioni (1981) and Turon (1990). According to those reports, the elongated rectangular mesh is composed of transverse and longitudinal filaments. The longitudinal filaments are usually thicker with a diameter of ~25 nm while the transverse filaments are ~10 nm in diameter. These form a structure with reported openings of 0.2–0.5 μm in width and 0.5–2.2 μm in length. The mesh is 90–98% porous and its dimensions reveal little variation among species (Flood and Fiala-Medioni 1981; Turon 1990).

The efficiency by which particles are captured by different suspension feeders was thoroughly investigated by numerous researchers using various methods. In most cases, parameters of filtration were recorded using electronic particle counters (such as Coulter counters) with little, if any, discrimination of the different particle types such as microorganisms vs. non-living matter and sediment grains, bacteria vs. algae, etc. (e.g., Jørgensen 1952; Jørgensen and Goldberg 1953; Randlov and Riisgård 1979; Arnowrthy et al. 2001; Sumerel and Finelli 2014). Those studies suggest that the size threshold of particles that ascidians filter is about ~2 μm. However, the capture of different types of particles might be influenced by other attributes beside size (Riisgård and Larsen 2001; Bone et al. 2003; Rosa et al. 2013, 2016). It is worth noting that the vast majority of research has been conducted on species from eutrophic areas, where microorganisms (and other food particles) tend to be relatively large, and on animals that were collected from the field but studied in the lab.

For ascidians residing in oligotrophic environments, the suggested threshold size of the ascidian filter (~2 μm) stands in sharp contradiction to the low availability of food particles above this size. The paucity of nano-planktonic (>2 μm) prey items in oligotrophic waters is exacerbated within the benthic boundary layer, where most ascidians reside. Under low flow conditions, a concentration boundary layer forms and the total cell numbers can become dramatically reduced (O’Riordan et al. 1995; Riisgård 1998; Yahel et al. 1998) making, the scarce larger food particles, even more rare. Considering the dominance of picoplankton in oligotrophic water, the postulated 2–4 μm threshold for bivalve and ascidian filtration seems counter adaptive. Moreover, evidence, collected by Yahel and coworkers (e.g., Yahel et al. 2005, 2009; Genin et al. 2009), suggests that oligotrophic suspension feeders can efficiently retain at least some marine bacteria in the sub-micron range.

To better understand the biomechanical aspects of biological filtration in the submicron range we focused this study on ascidians which have a relatively simple architecture of the filtration apparatus in comparison to that of bivalves and sponges. We measured the filtration efficiency of the ascidians using a direct method (Morganti et al. 2016) that was applied both in situ with undisturbed animals, and in the laboratory under controlled conditions. By measuring the efficiency by which ascidians capture different sizes of artificial microspheres with identical shape and chemical makeup, we circumvented the inherent bias involved in lumping together measurements of the capture of different particle types. Thus, in each experiment the only manipulated factor was particle size.

**Methods**

**Study sites**

Experiments were conducted at the School of Marine Science (Ruppin Academic Center) in Michmoret located at the Eastern Mediterranean Sea, and at the Interuniversity Institute for Marine Sciences (IUI) in Eilat (Gulf of Aqaba, the Northern Red Sea). At the Eastern Mediterranean site, experiments were conducted at ~10 m depth on a rocky ridge ~800 m west of Michmoret marina (32°24'08"N, 34°51'29"E) during September and October 2014, and August and November 2015. Water temperature during the sampling periods ranged from 23°C (November 2015) to 29°C (August 2015). At the Northern Red Sea site, experiments were conducted at ~6 m depth on the coral reef next to the IUI (33°02'02"N, 35°05'52"E) during May and August 2015 and January 2016. Water temperature during sampling ranged from 22°C (January 2016) to 27°C (August 2015).
Both sites are considered oligotrophic. Chlorophyll $a$ concentrations during the sampling periods ranged from 0.18 (August 2015) to 0.39 $\mu$g L$^{-1}$ (December 2015) at the Red Sea site (The Israel National Monitoring Program at the Gulf of Eilat, http://iui-eilat.ac.il/Research/NMP) and from 0.25 (August 2015) to 0.37 $\mu$g L$^{-1}$ (September 2014) at the Eastern Mediterranean site (Ruppin’s Estuarine and Costal Observatory, http://reco.ruppin.ac.il/).

In order to examine the effect of particle size on capture efficiency, we introduced ascidians with a homogenous mixture of artificial microspheres (Fluoresbrite® YG Carboxylate Microspheres, Polysciences) of well-defined sizes (10 $\mu$m, 3 $\mu$m, 0.5 $\mu$m, 1 $\mu$m, and 0.3 $\mu$m in diameter). We calculated the efficiency by which each microsphere size group is captured as: $CE = \frac{C_{ex} - C_{in}}{C_{in}}$, where $CE$ is the capture efficiency, and $C_{in}$ and $C_{ex}$ are the concentrations of a certain size group in the inhalant and exhalant water, respectively.

In order to describe the planktonic (pico and ultraplankton) composition of the study sites (Fig. 2) and to verify that the animals were actively filtering during the experiments, we collected inhalant and exhalant water sample pairs prior to each experiment with microspheres. Results of the natural diet of the ascidians will be published elsewhere.

**In situ sampling**

To study the filtration activity in situ, we used a modification of the In-Ex method proposed by Yahel et al. (2005, 2007) who followed (Reiswig 1971; Wright and Stephens 1978; Møhlenberg and Riisgaard 1978). This new method, termed the VacusSIPS (Morganti et al. 2016), allows simultaneous and prolonged sampling of inhaled and exhaled water of the studied organism by a slow and controlled suction of the sampled water into evacuated containers. A tube (PEEK, OD 1.6 mm, ID 0.23 mm, cat No. 1531B, IDEX) was carefully positioned a few mm into the ascidia’s inhalant siphon, the tube’s distal end was attached with a Luer connector (cat No. P-655, P-200X, and P-205, IDEX) to a hypodermic needle that was used to pierce a septum in an evacuated collecting vessel (VACUETTE®, Greiner Bio-One International, cat No. 455007). Pressure differences drove water into the tube at a slow rate, that was adjusted to $\sim$ 1 mL min$^{-1}$ by the adjusting the length of the PEEK tubing (usually 75 cm). An identical setup was simultaneously used to sample water from the exhalant siphon (Fig. 1). This setup allows the sampling of water for long periods of time with completely undisturbed animals. In order to introduce ascidians with microspheres in situ, we diluted the particles in 20 mL of filtered (0.2 $\mu$m) seawater from the sampling site to environmentally relevant final concentrations of $\sim$ 250, 1500, 10,000, 50,000, and 120,000 particles mL$^{-1}$ for the 10 $\mu$m, 3 $\mu$m, 1 $\mu$m, 0.5 $\mu$m, and 0.3 $\mu$m microspheres, respectively (Supporting Information Fig. S3). We applied this microsphere mix at a slow and steady rate using a syringe attached to a circular diffuser comprising a perforated tube that we carefully positioned around the ascidia’s inhalant siphon. A tube roller clamp from a medical infusion set (item 3 in Fig. 1c) was used to ensure a steady rate of injection. Inhalant and exhalant water samples were collected simultaneously with microsphere application (Fig. 1c, Supporting Information video 1).

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**Table 1.** Systematic affiliation, numbers, and location of studied organisms. The numbers in the columns represent the number of individuals from each species that were tested in the lab or in situ.

| Specie                  | Order          | Family     | Number of tested animals | Basin |
|-------------------------|----------------|------------|--------------------------|-------|
| Microcosmus exasperatus | Stolidobranchia| Pyuridae   | 10                       | EMT   |
| Styela plicata          | Stolidobranchia| Styelidae  | 27                       | EMT   |
| Phallusia nigra         | Phlebobranchia | Ascididae  | 5                        | EMT   |
| Herdmania momus         | Stolidobranchia| Pyuridae   | 3                        | RS    |
| Halocynthia spinosa     | Stolidobranchia| Pyuridae   | 3                        | RS    |
| Polycarpa mytiligera    | Stolidobranchia| Styelidae  | 23                       | RS    |

EMT, Eastern Mediterranean; RS, Red sea.

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Submicron particle capture by ascidians
The paired nature of the sampling design we used reduces its sensitivity to sampling biases and errors as the inhalant and exhalant water samples are taken simultaneously and thus are subjected to identical conditions, handling and analytical processes. However, the in situ sampling method is sensitive to one potential error. The natural environment contains, obviously, no microspheres. Consequently, a situation in which the exhaled water sample is “contaminated” with ambient water, either because the animal has stopped pumping or momentarily contracted and the tube has become dislodged, will result in apparent (and false) high capture efficiency measurements of microspheres. To overcome this potential bias, we carefully monitored the tubes and animals throughout the sampling, maintaining a distance of >50 cm to avoid interruption. The ultimate safeguard against this bias was accomplished by means of complimentary laboratory experiments.
Laboratory sampling

For the controlled laboratory experiments we collected three species of solitary ascidians (Styela plicata, Microcosmus exasperatus, and Herdmania momus) in the field and acclimated them for at least 3 d in the running seawater facility of The School of Marine Science at Michmoret, Israel. Each animal was placed in a separate 1-L borosilicate beaker, secured in place within a small pile of beach-rock pebbles, and supplied with \( \approx 20 \text{ L h}^{-1} \) of sand filtered seawater pumped from 3 m depth near the entrance to the Michmoret marina. Once a day the water flow was stopped for an hour and the ascidians were fed with fresh algae (Nanochloropsis sp., \( \approx 10^6 \) algal cells per beaker).

In order to sample the water inhaled and exhaled by the animals, we carefully positioned a PTFE tube (75 cm long, ID 400 \( \mu \text{m} \), outer diameter 800 \( \mu \text{m} \)) a few mm into each of the ascidian’s siphons. We initialized suction through the tubes with a small syringe, and positioned the tubes’ distal end outside the water table and below water level to enable gravitational flow at an average rate of \( \approx 1 \text{ mL min}^{-1} \). Once initiated, we allowed the tubes to drip constantly. To introduce the animals with the experimental microspheres, we suspended the particles in 10 mL of filtered seawater from the lab supply system. We stopped the water supply to the beaker and gently mixed the water in the beaker with the syringe while slowly injecting the suspended microspheres into it. Before and after any addition of microspheres we collected paired water samples (2 mL each) from the inhalant and exhalant siphons into micro-centrifuge tubes (Lifegene, cat No. LMCT2.0B). We used a similar sampling setup in the experiments conducted in Eilat with the ascidian Polycarpa mytiligera that had been collected from the nearby reef. In those experiments, the ascidians were supplied with fresh and unfiltered seawater pumped from 30 m depth with no additional feeding.

Analytical methods

Flow cytometry was the standard method used to quantify the particle concentrations. We used an Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems) equipped with a syringe-based fluidic system that allows precise adjustment of the injected sample volume and hence high precision of the measurements of particle concentrations (± 5%). The optics system contained violet and blue lasers (405 nm and 488 nm, respectively) and was further adapted for the analysis of marine picophytoplankton by installing red chlorophyll fluorescence filters on the third channel of both the blue (690 ± 20 nm) and violet (685 ± 20 nm) lasers, respectively.

Samples collected before the addition of microspheres were used to quantify the abundance of nano- and pico-plankton (cells \(< 20 \) and \( 2 \mu \text{m} \), respectively). To that end, 1.8 mL of the sample were transferred into a cryovial (Corning cat No. 430659), incubated for 15 min at room temperature with Glutaraldehyde (electron microscopy grade, Sigma-Aldrich, cat No. 340855), at a final concentration of 0.1%. Samples were either kept at 4°C and analyzed within 48 h or frozen in liquid nitrogen (at least 60 min) and then stored at −80°C until analysis.

Five distinct cell groups could be discerned by the flow cytometer: non-photosynthetic microbes that were visualize with a DNA stain (hereafter referred to as Bact), and the four dominant autotrophic groups that were visualized based on their auto-fluorescence: Prochlorococcus (Pro), Synechococcus (Syn), low scatter eukaryotic algae (PicoEuk), and high scatter eukaryotic algae (NanoEuk).

Each sample was analyzed twice. First, 600 \( \mu \text{L} \) of the sample water were analyzed at a high flow rate (100 \( \mu \text{L min}^{-1} \)) for determination of the four dominant autotrophic groups (NanoEuk, PicoEuk, Syn, and Pro) with a dual threshold on the red fluorescence channels (VL3 and BL3). A second run was used to analyze cells with no auto-fluorescence, i.e., non-photosynthetic microbes (Bact). To visualize these cells, 300 \( \mu \text{L} \) of the sample water were incubated with the nucleic acid stain SYBR Green I (20–120 min dark incubation at room temperature, 1 : 10^4 of SYBR Green commercial stock) as described by Marie et al. (2001). For this run, we used a low flow rate of 25 \( \mu \text{L min}^{-1} \) and the instrument was set to high sensitivity mode. Seventy-five microliters of the sample water were analyzed with a dual threshold on the green fluorescence channels (BL1 and VL2).

Discrimination of the autotrophic groups was made based on orange fluorescence (BL2, 574 ± 13 nm) of phycoerythrin and red fluorescence (BL3, 690 ± 20 nm and VL3, 685 ± 20 nm) of chlorophyll (Marie et al. 2001) plotted against the forward-scatter of the violet laser that was used as a proxy of cell size (Robertson and Button 1989; Cunningham and Buonacorsi 1992). Due to the very weak chlorophyll fluorescence of near-surface Prochlorococcus, especially during summer, in some cases a full separation of their population from the noise signal was not possible. Reference beads (PolyScience®, cat No. 23517, Flow Check High Intensity Green Alignment 1 \( \mu \text{m} \)) were introduced as an internal standard in every run at a final concentration of \( \approx 10^3 \text{ mL}^{-1} \).

Samples collected during the microsphere experiments were preserved with 0.1% Glutaraldehyde and kept at 4°C until analysis (within a few weeks). This preservation method had been validated in a preliminary experiment that had shown no significant loss of microspheres up to 3 months post collection. The different size groups of microspheres were measured with a dual threshold on the green and blue fluorescence channels (VL1 and VL2). To ensure reliable quantification of all size groups, the flow rate was set to either 25 \( \mu \text{L min}^{-1} \) or 100 \( \mu \text{L min}^{-1} \) so that the event rate would be kept below 1000 events s\(^{-1}\).

The sizes of microspheres were validated with a fluorescence microscope (BX 53, Olympus). Small (\(< 1 \mu \text{m} \)) microspheres were visualized and measured using an eSEM.
microscope (Quanta 200 FEG, FEI) at the Center for Nano-science and Nanotechnology, Tel-Aviv University.

Capture efficiency model

Following previous work (Loudon 1990; Sutherland et al. 2010), predictions of encounter efficiency were calculated by adapting the model presented by Silvester (1983). This model takes into account the dimensions of the mesh, the size of particles, water velocity through the filter, and the mechanisms that control the probability of encounter between particles that are smaller than the mesh size, and the mesh elements.

The encounter of polystyrene microspheres with the ascidian filter is likely to be limited to the non-inertial mechanisms: direct interception and diffusional deposition. This is because in the marine environment, electrostatic attraction is considered to be unimportant due to the high ionic strength of seawater (Riisgård and Larsen 2010). The density of polystyrene microspheres (1050 kg m$^{-3}$) is close enough to that of seawater to render gravitational deposition negligible as well. The significance of inertial impaction in the encounter of particles depends on the value of the Stokes number (Silvester 1983), $N_l = d_p^2 \rho_p \mu/18 \rho_{sw} v d_f$, where $d_p$ is the particle diameter, $\rho_p$ the particle density, $\mu$ flow through the filter, $\rho_{sw}$ is seawater density, $v$ is seawater kinematic viscosity, and $d_f$ the diameter of filter fibers. For the size range of particles used in this work, $N_l$ is considerably lower than one (for parameter notation and values see Supporting Information Table S1).

As the exact dimensions of the mucus mesh as well as the velocity through the filter were not measured, calculations of model predictions were done using the reported range of values for these parameters (Supporting Information Table S1). For additional details see Supporting Information.

Data analysis

Flow cytometry data files were processed with “FCS express 4” software (De-Novo TM). The forward scatter of the instrument was calibrated to allow size estimates using a standard and a sub-micron flow cytometry particle size reference kits of non-fluorescent polystyrene beads (Molecular probes, cat No. F13838, F13839). The forward scatter data of these calibration particles was fitted with a second-order function, as previously suggested (Julià et al. 2000), and this was used to estimate the size of planktonic cells (hereafter reported as “FSC-size”). Since the refraction index of the polystyrene calibration beads we used is higher than that of planktonic cells (Foladori et al. 2008), it is possible that we overestimated the size of planktonic cells (Foladori et al. 2008). It should be noted that size estimates for particles smaller than the wavelength of the laser we used (405 nm) should be regarded with caution although smaller particles did have a lower forward scatter (Supporting Information Fig S1). While the relative size estimates of all particles below 0.6 $\mu$m are probably a good representation of reality, the absolute size estimates of minute particles are suspect but suggest very small cells. We used the cell sizes to estimate their carbon content using conversion factors from Houlbrèque et al. (2006) and references therein except for the non-
photosynthetic bacteria (Bact) for which we used a fixed value of 14 fg C cell$^{-1}$.

We performed the statistical analysis in “RStudio” (version 0.99.879 – © 2009–2016 RStudio) with the “ez” (version 4.2-1) package. For power analysis, we used the G*Power software (version 3.1.9.2, written by Franz Faul, University of Kiel, Germany). For ANOVA, the assumptions of Homogeneity of variances, normality and (for repeated measures) sphericity were checked and where violated, data were transformed (percentage data were usually arcsine transformed) or the alternative a-parametric test was used.

**Results**

Picoplanktonic cells (all cells < 2 μm) numerically dominated the planktonic community, accounting for >97% of the total cell numbers. Minute non-photosynthetic microbes (< 1 μm), labelled as “Bact” in Fig. 2, Table 2, accounted for most of the planktonic biomass (> 50%, Fig. 2). As expected for oligotrophic environments, the total phytoplanktonic (microalgae and cyanobacteria) biomass in both study sites was very low (< 7 μg carbon L$^{-1}$). However, the rare nanoeukaryotic algae accounted for a major part of the phytoplanktonic biomass (> 20%). Overall submicron cells comprised more than 60% of the total planktonic biomass.

The ascidian species we studied exhibited efficient capture of micron and sub-micron particles (10 μm, 3 μm, 1 μm, 0.5 μm, and 0.3 μm in diameter, Fig. 3). Microspheres as small as 0.3 μm were captured with at least 41% efficiency (H. momus, lab experiments). In situ experiments showed that >1 μm microspheres were captured with 95% ± 8% (mean ± 95% CI) efficiency with no significant difference between the ascidian species investigated (RM-ANOVA of arcsine transformed capture efficiency, p > 0.08). H. spinosa showed the highest efficiency in capturing microsphere particles with 91% ± 6% efficiency on 0.3 μm microspheres. The capture efficiency of this ascidian did not seem to drop in the size range we studied. The least efficient ascidian was H. momus which, during lab experiments, demonstrated reduced efficiency for both small and large microspheres (41% ± 19% and 61% ± 26% for 0.3 μm and 3 μm, respectively), suggesting that its filtration may have been impaired.

P. mytiligera and M. exasperatus were studied both in lab and in situ experiments. P. mytiligera exhibited a higher capture efficiency of small particles (0.3 μm) when kept in the running seawater laboratory (90% ± 3%) in comparison to the field (64% ± 10%, pairwise t test of arcsine transformed capture efficiency, Bonferroni corrected p < 0.05). The capture efficiency of M. exasperatus on 0.3 μm microspheres suggests a similar trend, with capture efficiency of 68% ± 13% in the lab vs. 43 ± 26 in the field, but this difference was not significant (Bonferroni corrected p = 0.06, Power > 0.8, Fig. 3).

The number of captured microspheres was linearly related to their concentration in the inhalant water (Fig. 4) in the range of 10²–10⁵ particles mL$^{-1}$ indicating that capture efficiency was concentration independent under the experimental concentration range. This was found to be true for all the studied ascidians species excluding H. momus. For this species, the relationship between inhalant concentration and the number of captured microsphere per mL differs from linearity for most microsphere size groups (Supporting Information Fig. S2). Since H. momus also presents relatively low capture efficiency, we advise the readers to consider the data of this species with caution.

**Discussion**

The use of artificial particles allowed us to examine the effect of size on capture efficiency as an isolated explanatory variable. Previous studies (e.g., Randløv and Riisgård 1979; Jørgensen et al. 1984; Armsworthy et al. 2001; Sumerel and Finelli 2014) have examined the size limits of the ascidian filter by measuring the removal of many types of particles with no discrimination between the different particle types. This approach assumes that size is the sole attribute of the particle that will affect its “catchability.” However, theory predicts that other characteristics, most notably an ambiguous “stickiness” property, may also have a profound influence on “catchability” (Shimeta and Jumars 1991; Bone et al. 2003; Riisgård and Larsen 2010). Moreover, a number

| Group   | FSC-diameter (mean ± SD, μm) | Concentration (mean ± SD, 10⁶ cells L⁻¹) |
|---------|-----------------------------|------------------------------------------|
|         | EMT | RS | EMT | RS |
| NanoEuk | 6.26 ± 3.54 | 4.36 ± 2.08 | 2.3 ± 1.6 | 2.69 ± 2.76 |
| PicoEuk | 1.8 ± 1.34 | 0.85 ± 0.54 | 1.61 ± 2.4 | 0.38 ± 0.51 |
| Syn     | 0.62 ± 0.19 | 0.68 ± 0.19 | 99.9 ± 62.4 | 15.82 ± 6.16 |
| Pro     | 0.64 ± 0.17 | 0.61 ± 0.18 | 9.6 ± 11.15 | 20.49 ± 30.15 |
| Bact    | 0.41 ± 0.06 | 0.42 ± 0.06 | 679.4 ± 345.85 | 361.23 ± 270.81 |

NanoEuk, high scatter eukaryotic microalgae; PicoEuk, low scatter eukaryotic microalgae; Pro, Prochlorococcus; Syn, Synechococcus.
Fig. 3. Capture efficiency of different ascidians, in situ (left column) and in the laboratory (right column). Ascidians were introduced with chemically identical microspheres of different sizes (10 μm, 3 μm, 1 μm, 0.5 μm, and 0.3 μm in diameter) while water was sampled from the inhalant and exhalant openings. (a) - M. exasperatus in situ n = 5, (b) - lab n = 10; (c) - P. mytiligera in situ n = 23, (d) - lab n = 3; (e) - H. spinosa in situ, n = 4; (f) - H. momus lab, n = 7; (g) - P. nigra in situ, n = 7; (h) - S. plicata lab, n = 27. Error bars are 95% confidence interval.

Fig. 4. Ascidian response to variations in inhalant particle concentrations. The number of captured microspheres per mL pumped (calculated as the difference between the inhalant and exhalant concentration) is plotted as a function of the concentration of microsphere in the inhalant water for each size group. (a) - 10 μm, (b) - 3 μm, (c) - 1 μm, (d) - 0.5 μm, and (e) - 0.3 μm microspheres. Different ascidian species are indicated by different colors. Both axes are log scaled, the black line represents the linear regression (regression R² values: 0.98, 0.94, 0.97, 0.98, and 0.86 for 10 μm, 3 μm, 1 μm, 0.5 μm, and 0.3 μm, respectively).
of studies have shown evidence of size-independent differences in the capture rates of different cell types by some suspension feeders (Gerritsen and Porter 1982; Monger et al. 1999; Yahel et al. 2006, 2009; Rosa et al. 2013, 2015, 2016). The size limits for particle capture in suspension feeding should, therefore, be studied using a uniform particle type so that size is the only manipulated variable, as in the present study.

Although the use of artificial microspheres circumvents the inherent bias of lumping together capture measurements of different types of particles, our sampling scheme is sensitive to some potential biases. Dislocation of the exhalant sampling tube will lead to overestimation of capture efficiency measurements in situ experiments and underestimation of capture efficiency in laboratory experiments (where microspheres were mixed into the aquaria water rather than directly injected into the inhalant siphon). As a partial remedy, we used fluorescein dye to visualize the exhalant water jet prior to any sampling and verified that the sampling tube was located amidst the jet. Furthermore, after sampling and analysis, we screened our data by observing the removal of naturally occurring prey. Because the population of *Synechococcus* is usually easy to define and count in the flow cytometer we monitored its removal by the animals and used it as an indication of active feeding and proper location of the sampling tube.

The submicron threshold for optimal feeding we report here for the six “oligotrophic” ascidians we studied, is in agreement with microscopic observations of ascidians’ mucus mesh filter dimensions reported form eutrophic waters (Flood and Fiala-Medioni 1981; Turon 1990). It is therefore unclear whether the >2 µm threshold reported in previous studies (Randløv and Riisgård 1979; Armsworthy et al. 2001; Sumerel and Finelli 2014) was a consequence of a methodological bias (e.g., limited sensitivity of the Coulter counter) or adaption to the larger plankton that dominate these waters.

Since particles smaller than the suggested pore size (~0.5 in width and ~2.2 in length, Flood and Fiala-Medioni 1981) are efficiently captured (>50%, Fig. 3), we conclude that filtration in ascidians cannot be described by simple sieving. Rather, it is better described by the mechanisms detailed in the Hydrosol Filtration theory (Rubenstein and Koehl 1977) that takes into account the several different mechanisms that may lead to the encounter of a particle with the filtering element. In ascidians, these are probably limited to direct interception and diffusional deposition (see the description of the capture model in the “Methods” section).

A previous study of salps, the pelagic relatives of ascidians, adapted the model suggested by Silvester (1983) to describe the encounter of particles with the salp filter (Sutherland et al. 2010). Following Sutherland et al. we calculated the expected encounter efficiency of particles with the ascidian filter. For most species, the calculated range of predicted encounter efficiencies is higher than the measured capture efficiency (Fig. 5). This discrepancy suggest that our current understanding of the ascidian filtration apparatus may be incomplete. For particles that are smaller than the pores of the rectangular mucus mesh, adhesion to the filter fibers will greatly influence capture efficiency. The model we adapted (Silvester 1983; Loudon and Alstad 1990; Sutherland et al. 2010) predicts encounter rather than capture efficiency. If the encounter of a particle does not necessarily imply its capture it may also explain the difference between encounter predictions and the observed capture data. For example, encountered particles may dislodge and slip through the mesh-pore or tumble over the mesh and slip over its rim. Our observations emphasize the long-recognized need for a better understanding of particle retention mechanisms, and their dependency on additional particle traits besides size, such as adhesiveness.

Given that submicron cells account for a large portion of the suspended planktonic biomass in oligotrophic waters (Fig. 2), the capture of small, submicron particles might be a necessity for suspension feeders adapted to living in these environments. Thus, the efficient capture of submicron particles, displayed by the ascidians we studied is to be expected.
in oligotrophic suspension feeders and may explain the general similarity in the size spectra that apparently can be captured by the animals we tested. *H. spinosa* stood out among the tested species in possessing exceptional efficiency in capturing submicron particles (91% ± 6% efficiency on 0.3 μm microspheres, Fig. 3). This species usually resides in hidden crevasses (Shmuel and Shenkar 2017) where it is likely to experience lower flows and therefore lower particle fluxes. Thus, its high efficiency in particle capture is perhaps an adaptation to the niche it occupies.

A decrease in the mesh “pore” size as an adaptation for the capture of smaller particles would imply a large increase in the energetic demand of the filtration process. For example, decreasing the pore size from 2 μm to 0.5 μm implies an approximate 16-fold increase in the mesh resistance to flow (Silvester 1983; Riisgård 1988). However, since the energetic cost of filtration in ascidians is considered to be extremely low (< 0.2% of total metabolism, Riisgård 1988), the overall impact on the metabolic energy budget of the animal may be negligible.

A recent study has presented evidence that some bacteria, most notably members of the SAR11 clade, the most abundant bacteria in the world, are able to completely avoid being captured by ascidians and other suspension feeders (Dadon-Pilosof et al. 2017). That study posited a hypothesis that the minute SAR11 cells have developed “non-stick” surface properties that enable them to evade predation by suspension feeders, facilitating the clade’s exceptional success. Behind this argument is the idea that SAR11 cells are able to evade being captured due to a combination of “non-stick” surface properties and their small size. The data presented here, providing evidence of the efficient capture of particles in the size range of SAR11 cells, supports the idea that size alone cannot account for the findings presented by Dadon-Pilosof et al. of near zero capture efficiency of SAR11 cells by ascidians.

An intriguing observation is that 100% removal efficiency is rarely observed, even for particles that are clearly larger than the mesh “pore” size (Fig. 3). The use of microspheres at low concentrations, as we did with the larger size groups (10 μm and 3 μm, ~ 10^5–10^3 particles mL^-1), makes these measurements more sensitive to background noise that might lower the calculated capture efficiency. To control for such potential bias, we have routinely measured the background noise in each run of the flow cytometer using both DDW and 0.2 μm filtered seawater. Moreover, the fact that the linear relationship between the number of captured particles and the inhalant concentration, excluding the case of *H. momus*, was maintained at lower inhalant concentrations (Fig. 4, Supporting Information Fig. S2), indicates that the capture efficiency is concentration independent. It is therefore suggested that < 100% filtration efficiency of large particles (> 2 μm) is a true phenomenon that may be an outcome of a “damaged” mucus mesh or possibly incomplete mesh production.

Notably, we found different capture efficiencies of submicron microspheres between the ascidians tested in the lab and those tested in situ (Fig. 3). The facilities we used for the lab experiments are equipped with a constant supply of fresh seawater. Nonetheless, the planktonic community in these waters were highly depleted due to the filtering system they passed through and to the inevitable settling of suspension feeders in and along the supply system (Alexander et al. 2015). *P. mytiligera* and *M. exasperatus* acclimated to lab conditions, in which the water supply was depleted from plankton (< 25% of ambient concentration, Yahel, unpubl. data) presented a more efficient removal of sub-micron microspheres in comparison to animals studied in the field (Fig. 3). The conventional wisdom is that the dimensions of the mucus mesh filter in ascidian are constant (Bone et al. 2003), meaning that the animal cannot alter them as a response to external changes. Nonetheless, the increase in capture efficiency observed in the lab experiments suggest some plasticity in the dimensions of the filter in response to an altered composition of prey.

**Conclusions**

Ascidians possess a relatively simple filtration apparatus that allows a straight forward inference regarding its operation. Furthermore, solitary ascidians have only two openings, one for inhaling and the other for exhaling water. This, and the relatively large dimensions of these openings, simplified sampling and allowed us to conduct many measurements in situ. These features allow the collection of unbiased and relevant ecological and physiological data and render ascidians an ideal model for the study of biological filtration.

Using inert and chemically identical particles revealed that the lower size limit of the ascidian filter is submicron. This may very well be an adaptation to the fact that, in oligotrophic waters, submicron cells account for a major part of the planktonic biomass. It was previously suggested that submicron particles cannot constitute a quantitatively important food source for ascidians (Petersen 2007). However, all the ascidians we studied were able to efficiently capture submicron particles as small as marine bacteria, suggesting that ascidians likely exploit this large nutrient and energy pool, which in oligotrophic habitats is very significant (Fig. 2). By capturing and metabolizing pelagic pico-plankton, ascidians can provide their neighboring benthic primary producers with otherwise unavailable nutrients (e.g., Ribes et al. 2007), thus playing an important role in coupling pelagic primary and bacterial production with the benthic food web.

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Conflict of Interest

None declared.