nature microbiology

LETTERS

DOI: 10.1038/s41564-017-0030-5

Correction: Author correction

Surface properties of SAR11 bacteria facilitate grazing avoidance

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Oceanic ecosystems are dominated by minute microorganisms that play a major role in food webs and biogeochemical cycles[1]. Many microorganisms thrive in the dilute environment due to their capacity to locate, attach to, and use patches of nutrients and organic matter[2,3]. We propose that some free-living planktonic bacteria have traded their ability to stick to nutrient-rich organic particles for a non-stick cell surface that helps them evade predation by mucous filter feeders. We used a combination of in situ sampling techniques and next-generation sequencing to study the biological filtration of microorganisms at the phylotype level. Our data indicate that some marine bacteria, most notably the highly abundant *Pelagibacter ubique* and most other members of the SAR 11 clade of the Alphaproteobacteria, can evade filtration by slipping through the mucous nets of both pelagic and benthic tunicates. While 0.3 µm polystyrene beads and other similarly-sized bacteria were efficiently filtered, SAR11 members were not captured. Reversed-phase chromatography revealed that most SAR11 bacteria have a much less hydrophobic cell surface than that of other planktonic bacteria. Our data call for a reconsideration of the role of surface properties in biological filtration and predator-prey interactions in aquatic systems.

Culture-independent molecular techniques have revealed that a relatively small number of groups dominate marine microbial communities[4]. Among these, *Pelagibacter ubique* and other members of the SAR11 clade are most ubiquitous, comprising 15–60% of the total bacteria in the upper ocean[5,6].

The mechanisms underlying the extraordinary success of the SAR11 bacterial clade in open ocean ecosystems have been vigorously debated in recent years. Adaptation to resource competition has been suggested as one plausible mechanism since *P. ubique* exhibits a high surface-to-volume ratio coupled with a small and streamlined genome, containing high-affinity transporters that enable efficient metabolism in an oligotrophic environment[7]. While these traits indicate a potential for fast growth, the measured *in-vitro* growth rate of *P. ubique* is low (0.04–0.58 day^{-1})[8]. Other mechanisms, including low viral infectivity, 'cryptic escape' through reduced cell size, and elaborated K-strategy defense mechanisms have been suggested, but with the discovery of widespread 'pelagiphage' viruses infecting SAR11, these mechanisms are now considered less likely[9].

Many organisms, particularly suspension feeders, feed on bacteria, and grazing is considered a prominent mortality factor for in the ocean. Although top-down control and differential grazing pressures can potentially shape the composition of the oceanic microbial community[10,11], to date, little is known about grazing resistance of specific marine bacteria[12]. Hence, low mortality might be an additional explanation for the high abundance of SAR11[12].

To test for differential grazing efficiency for different bacteria we first focused on benthic tunicates (ascidians). These common filter feeders resemble planktonic tunicates in their use of mucous nets to filter bacteria and phytoplankton. We chose ascidians as a model organism because their strainer-like filtration apparatus is relatively simple compared to their planktonic counterparts[13] and their inhalant and exhalant siphons are sufficiently large to allow direct sampling of the water before and after filtration.

We used the InEx VacuSIP method[14] (Fig. 1a, Supplementary Information movie S1) to cleanly collect the water inhaled and exhaled by ascidians *in situ* and calculated the removal efficiencies of different microorganisms. Cell counts made with a flow cytometer indicated that the ascidian *Microcosmus exasperatus* efficiently retained photosynthetic algae, coccoid picocyanobacteria and bacteria with high nucleic acid content (52%–82%, Fig. 1b), however different taxa were retained with significantly different efficiencies (Friedman test, $P<0.001$). Notably, low nucleic acid bacteria, with which SAR11 clade is usually associated[15], were removed at significantly lower efficiency ($18\pm10\%$, CI95%, $P<0.05$, RM ANOVA, Fig. 1b). To taxonomically identify the retained and non-retained bacteria phylotypes, we extracted DNA from the microbial community in the inhaled and exhaled water and measured the relative frequencies of different bacterial phylotypes based on 16S rRNA metabarcoding analysis by SILVAngs (Fig. 1c). The ambient microbial cell count in the water inhaled by the ascidians was $8.5\times10^5\pm3.1\times10^5$ cells mL^{-1} with 20 phylotypes (clustered at 98% identity) accounting for 92% of the total 16S rRNA gene reads sequenced. SAR11 clade and picocyanobacterial phylotypes accounted for 33% and 44% of the total reads, respectively (pink and

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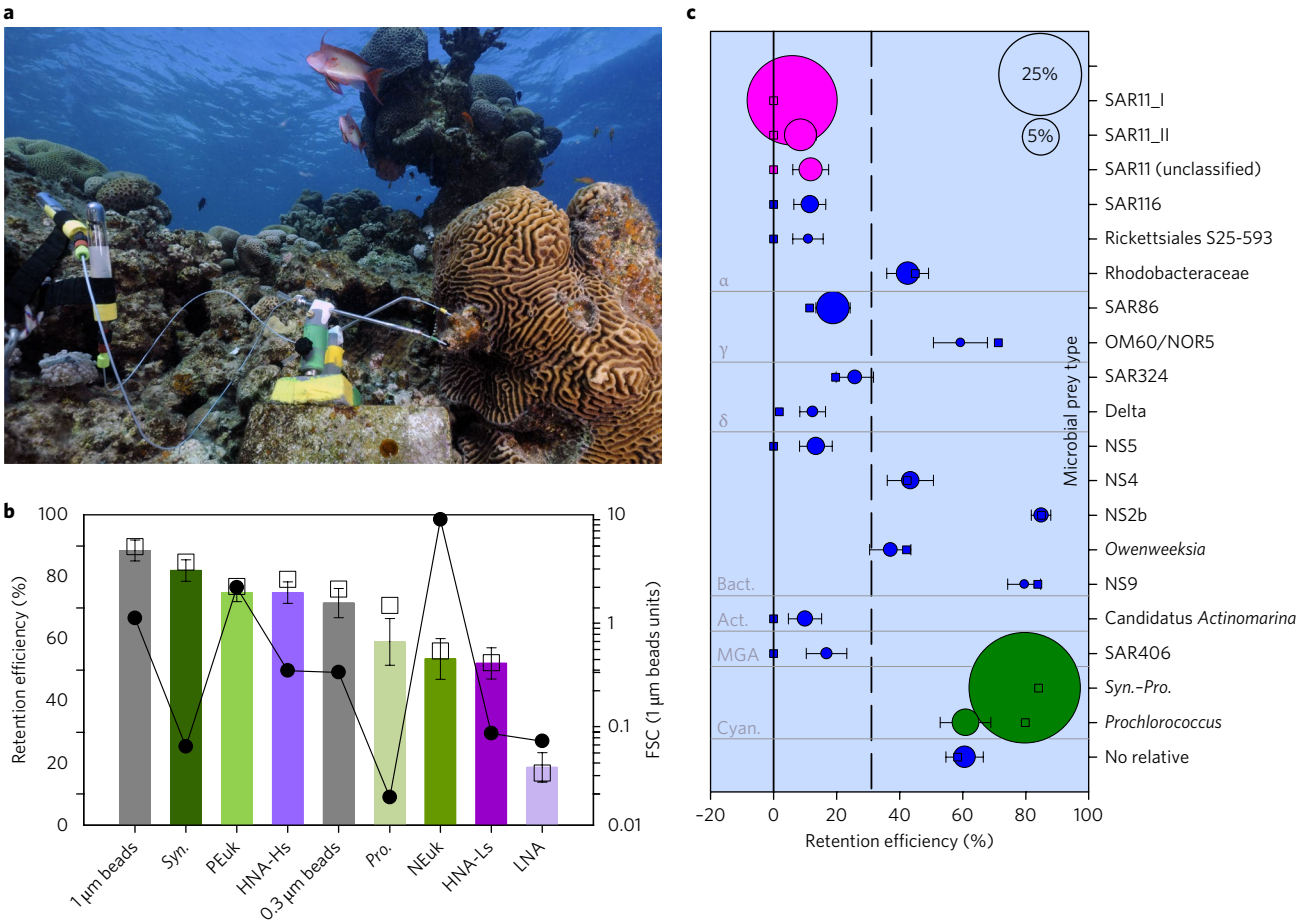

Fig. 1 | Differential retention of marine microorganisms by the ascidian *Microcosmus exasperatus* measured in situ (10 m depth) at the Eastern Mediterranean Sea ($n = 15$, Michmoret, Central Israel, September 2014). a, The VacuSIP apparatus used for direct in situ sampling of the water inhaled and exhaled by an ascidian[14]. Image courtesy of T. Shlesinger, taken in the Red Sea. **b**, Mean retention efficiency (%) of different prey types and fluorescent microspheres counted by flow cytometry (error bars show s.e.m., squares represent medians). Black circles are the median forward scatter (proxy of cell size) of each prey type normalized to the forward scatter of 1 µm reference beads (right axis, note the logarithmic scale). *Syn.*, *Synechococcus*; PEuk, pico eukaryotic algae; HNA-Hs, high nucleic acid high-scatter non-photosynthetic bacteria; *Pro.*, *Prochlorococcus*; NEuk, nano eukaryotic algae; HNA-Ls, high nucleic acid low-scatter non-photosynthetic bacteria; LNA, low nucleic acid non-photosynthetic bacteria. Removal efficiency of microspheres was measured under controlled conditions in the lab. **c**, Mean retention efficiencies of the 20 most abundant OTUs in the water. Grey lines divide OTUs into taxonomic categories; α, Alphaproteobacteria; γ, Gammaproteobacteria; δ, Deltaproteobacteria; Bact., Bacteroidetes; Act., Actinobacteria; MGA, marine group A; Cyan., Cyanobacteria. Pink indicates members of SAR11 clade, green indicates autotrophs, and blue indicates other non-photosynthetic bacteria. Dashed vertical line represents the expected retention assuming equal retention probability for all cells. Size of circles represents relative abundance in the inhaled water during sampling (circles in the upper right shows scale for 5% and 25% of total reads). Error bars show s.e.m., squares represent median retention efficiencies. It should be noted that these samples were collected during the onset of a *Synechococcus* bloom that may account for the high abundance and unusually small size of the *Synechococcus* cells. Different taxa (**b**) and phylotypes (**c**) were retained with significantly different efficiencies (Friedman test, $P < 0.001$, see supplementary Table 2 for pairwise comparison results).

green shades, respectively, in Fig. 1c). While the exhaled water had a significantly reduced frequency of picocyanobacteria reads, the frequencies of reads attributed to the SAR11 OTUs were significantly increased, suggesting low or null filtration of these phylotypes (Supplementary Information Fig. 2a). To calculate microorganism-specific retention efficiency (Fig. 1c) and selectivity coefficients (Chesson α_i[16], Supplementary Information Fig. 2b) we multiplied the relative frequency of each phylotype by the total bacterial cell counts obtained by flow cytometry. Different phylotypes were filtered with significantly different efficiencies (Friedman test, $P < 0.001$). Picocyanobacteria, Rhodobacteraceae, and Flavobacteriaceae NS2b were efficiently retained by the ascidians (medians of 81%, 44%, and 85%, respectively), while the median retention of bacteria belonging to the SAR11 clade was null (0%). Additional phylotypes that passed through *M. exasperatus* filters with very low retention included SAR86 (10%), SAR116 (0%) and Flavobacteriaceae NS5 (0%; Fig. 1c). These differences cannot be solely attributed to differences in cell size because fluorescent polystyrene beads of 0.3 µm diameter that overlapped in size with the un-filtered bacteria (0.3–1 µm) were efficiently removed by *M. exasperatus* (Fig. 1b). Moreover, *Synechococcus* cells that were extremely small during this bloom were filtered at significantly higher efficiency than both the >3 µm nano-eukaryotic algae (Fig. 1b) and other heterotrophic bacteria cells that were similar in size. Seven other ascidian species from different locations exhibited similar results of null or very low removal of SAR11 and other bacteria, both in the laboratory (Supplementary Information Fig. 3 b,c) and field experiments (Supplementary Information Fig. 4 & 5). These patterns were consistent using different primers (Supplementary Information Fig. 4a,b), different DNA extraction methods, and different sequencing protocols

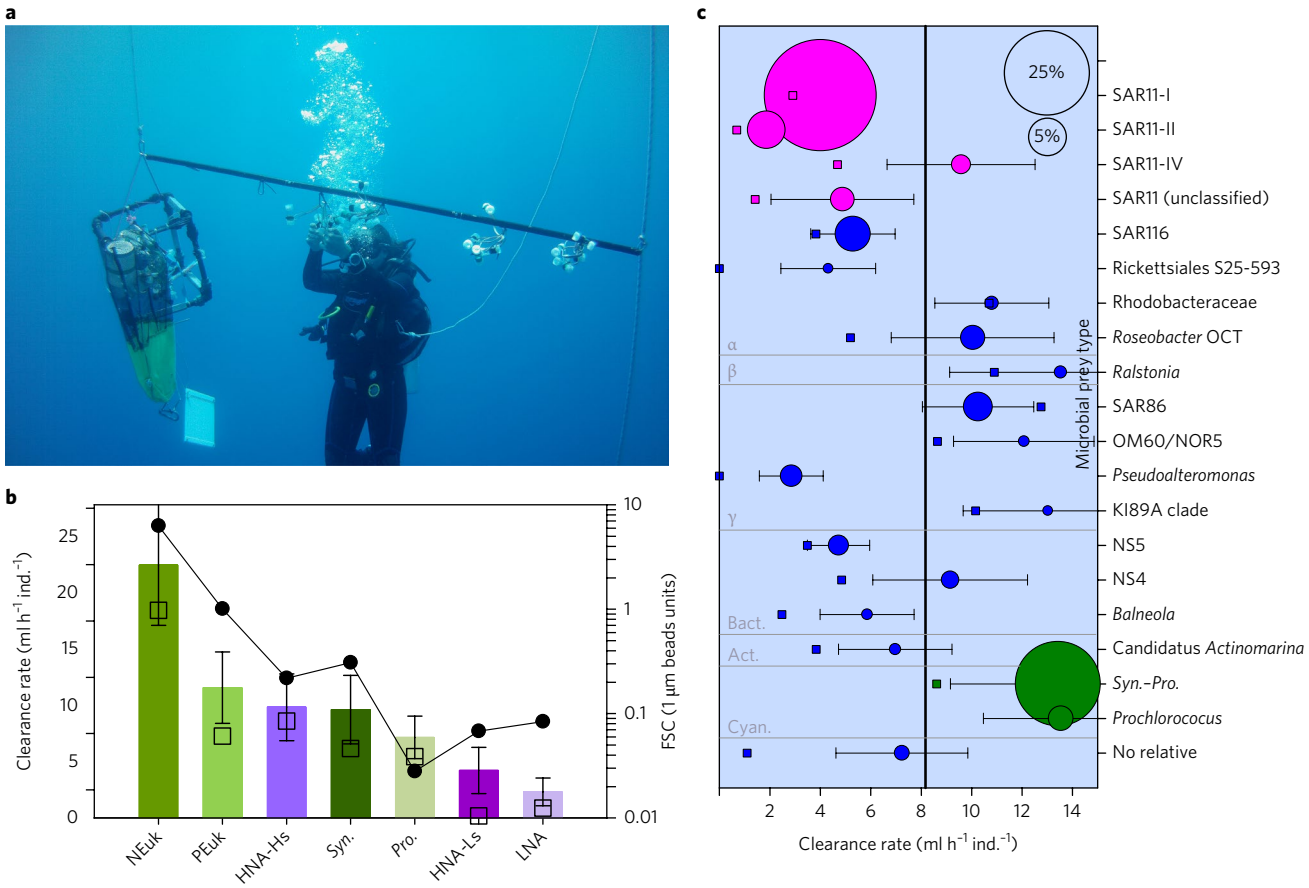

Fig. 2 | Differential clearance rate of marine microorganisms by the appendicularian *Oikopleura albicans* measured by in situ incubations in the NW Mediterranean Sea during April 2014, $n = 15$. a, In situ incubations at 6–8 m depth using blue-water diving techniques. **b**, Average clearance rates of different prey types counted by flow cytometry (error bars show s.e.m., squares represent medians). Black circles are the median forward scatter (proxy of cell size) of each prey type normalized to the forward scatter of 1 µm reference beads (right axis, note the logarithmic scale). Image courtesy of A.D.-P. **c**, Clearance rates of the 20 most abundant OTUs in the water, grey lines divide OTUs into taxonomic categories. Pink indicates members of the SAR11 clade, green indicates autotrophs, and blue indicates other non-photosynthetic bacteria. Vertical line represents the expected clearance rate assuming equal clearance rate probability for all cells. Size of circles represents relative abundance in the inhaled water during sampling (circles in the upper right shows scale for 5% and 25% of total reads). Error bars show s.e.m., squares represent median clearance rates. Different taxa (**b**) and phylotypes (**c**) were retained with significantly different efficiencies (Friedman test, $P < 0.001$, see supplementary Table 3 for pairwise comparison results).

(Supplementary Information Fig 5), and were also confirmed by CARD-FISH (Supplementary Information Fig. 4c,d,e). Evidence for differential and size-independent filtration of particles has been previously reported for bivalves[17] and sponges[18], but the mechanism has so far remained elusive.

Since differential benthic bacterivory is unlikely to have a significant effect on the abundance and distribution of pelagic bacteria, we sought to measure differential predation by appendicularians, the pelagic relatives of ascidians. While appendicularians (Tunicata) may only dominate open ocean bacterivory during temporary population blooms, they are important pico-planktivores in most of the world's oceans[19]. In large numbers, appendicularians can remove more than half of the marine microbial populations in a matter of days[20,21] thus playing a central role in pelagic food webs[22]. They filter small particles using a complex mucus 'house' built of fine-meshed filter elements[23]. Discarded appendicularian houses and fecal pellets sink to the ocean's interior and therefore remove grazed particles from surface waters[24].

To measure the impact of appendicularian grazing, individual *Oikopleura albicans* (trunk length 1–3 mm) were located underwater using blue-water SCUBA diving. A transparent open-ended 30 mL cylinder was slowly positioned over the animal and both sides were gently closed. The animal was allowed to feed undisturbed in this incubation chamber for 40–120 min (Fig. 2a). To quantify the grazing induced by the appendicularian on different microbial phylotypes, the microbial community in the chamber was compared with paired controls (identical chambers that were simultaneously closed with no animal inside). The experiment was replicated with 15 individual appendicularians in the Ligurian Sea (Northwest Mediterranean Sea).

Cell counts obtained with a flow cytometer indicated that *O. albicans* filtered photosynthetic algae with high efficiency, consistently exhibiting high clearance rates (7–18 mL h^{-1}, Fig. 2b), comparable to previously published values. However, different taxa were cleared with different efficiencies (Friedman test, $P < 0.001$). Small picocyanobacteria as well as bacteria with high nucleic acid content were also removed but at a reduced rate (clearance rate of 5–10 mL h^{-1}, Fig. 2b). No significant removal was observed for low nucleic acid bacteria, usually associated with the SAR11 clade[15] (Fig. 2b).

The microbial cell count in the water filtered by the appendicularians was $5.6 \times 10^5 \pm 1.5 \times 10^5$ cells mL^{-1} with 20 phylotypes accounting for 94% of the total 16 S rRNA gene reads sequenced. SAR11 and picocyanobacteria phylotypes accounted for 45% and 27% of the total reads, respectively (Fig. 2c). Calculation of frequencies of reads, clearance rates (Fig. 2c) and selectivity coefficients (Supplementary Information Fig 6a) indicated that, with the

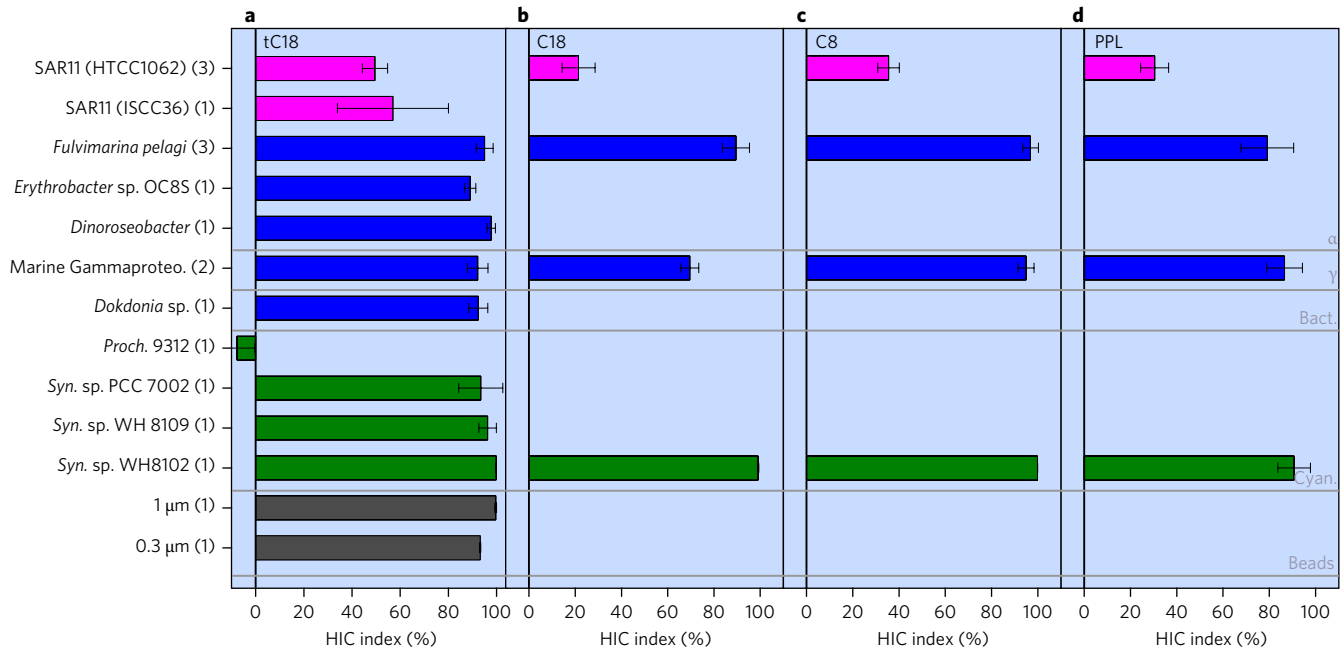

Fig. 3 | Hydrophobicity of cultures bacteria measured by HIC for four autotrophs, two heterotrophic copiotrophs and five heterotrophic oligotrophic bacterial cultures. a–d, The HIC of some bacteria was measured by several type of affinity columns: tC18 (**a**), C18 (**b**) C8 (**c**) PPL (**d**). Grey lines divide OTUs into taxonomic categories. Beads, fluorescent microspheres. Pink indicates members of the SAR11 clade, green indicates autotrophs, blue indicates other heterotrophic bacteria, and grey indicates fluorescent microspheres. Error bars show HIC range (max–min), numbers in parentheses indicate number of biological replicate (different batches of culture run at different times). Each biological replicate had three technical replicates (the same batch tested with three different column pairs).

exception of subclade SAR11-IV, members of the SAR11 clade and several other phylotypes (SAR116, *Pseudoalteromonas*, *Rickettsiales* S25–593 and *Flavobacteriaceae* NS5) were removed at a significantly lower rate (Tukey Pairwise Multiple Comparison Test, $P < 0.05$) when compared to other abundant bacteria such as *Prochlorococcus*, *Synechococcus*, SAR86, *Roseobacter* OCT, Rhodobacteraceae and *Ralstonia* (Fig. 2c).

Our grazing experiments showed that both benthic ascidians and planktonic appenicularians efficiently remove some bacteria but not others, suggesting that size and shape are not the sole determinants of the capturability of sub-micron size cells. Alternatively, attachment of the removed microorganisms to particles can be invoked to explain the observed patterns. That is, bacteria associated with particles and aggregates will be efficiently captured whereas free-living bacteria may pass through the filter. However, this is clearly not the case for the coccoid picocyanobacteria, since they are free-living and were efficiently removed. Moreover, in an experiment designed to test this hypothesis we compared the bacterial community in Eastern Mediterranean surface water filtered on 5 µm, 1 µm and 0.2 µm membranes. Several abundant phylotypes that were efficiently removed by the tunicates (SAR86, Rhodobacteraceae, and the OM60) passed freely through the 1 µm membrane, the size threshold for "free-living". Furthermore, these groups are consistently found worldwide in pre-filtered bacterioplankton samples and are not known to be especially particle-associated. We therefore examined the potential role of cell surface properties in determining the likelihood of bacteria being trapped by the grazers' mucous nets.

Hydrosol filtration theory predicts that the encounter rate between cells and their predators should be a function of the cell concentration, size, and motility[25], whereas post-contact particle capture is predicted to be a function of the particle stickiness index[26]. Surface properties have been suggested to mediate the adhesion of particles and marine microorganisms to solid surfaces[26,27] and the capturability of microbial prey[28,29]. Variation in the attractive solvation force associated with cell-surface hydrophobicity can significantly affect the stickiness index of picoplanktonic cells and hence their grazing resistance[28].

To test for possible relationships between microbial grazing, surface properties, and grazing resistance, we compared the retention of different marine bacteria (Fig. 3) from cultures and natural microbial assemblages (Fig. 4) by several types of affinity columns and calculated a Hydrophobic Interaction Chromatography index (HIC[28]) for each bacterium. The cell-surface hydrophobicity of SAR11 phylotypes, including two culture representatives (HTCC1062 and ISCC36 isolated by L. Steindler), was significantly lower than that of all other cultured (Fig. 3) and environmental (Fig. 4) bacteria. The only exceptions were SAR11-IV bacteria that were efficiently retained by the hydrophobic columns (Fig. 4) and were also the only SAR11 member efficiently retained by the tunicates (Fig. 2c, Supplementary Information Fig. 3c), and *Prochlorococcus* 9312 that was not retained at all by the hydrophobic column (Fig. 3). These results suggest that hydrophobicity may determine the likelihood of cell being retained by a predator's mucus net.

Our data show variable grazing resistance of different microbial phylotypes filtered with different efficiencies. For SAR11, low removal efficiency is correlated with low HIC index however, other phylotypes that were removed at low efficiency had high HIC index (e.g., NS5, SAR116, Figs. 1, 2 and 4) suggesting the likely involvement of other cell surface properties (for example, ref. [30]).

Most predation in the open ocean is thought to occur via contact and adhesion on either a filter apparatus, ciliated gills, ciliated part of tentacles, or direct adhesion to protoplasm. Our findings suggest that *P. ubique* and other marine bacteria can slip through the mucous nets of common filter-feeders. We hypothesize that a guild of free-living planktonic bacteria, most notably the SAR11 clade, have a non-sticky cell surface, which may reduce cell adhesion to nutrient-rich organic particles, but also confer resistance to grazing by mucous-net suspension feeders. This trade-off may help further

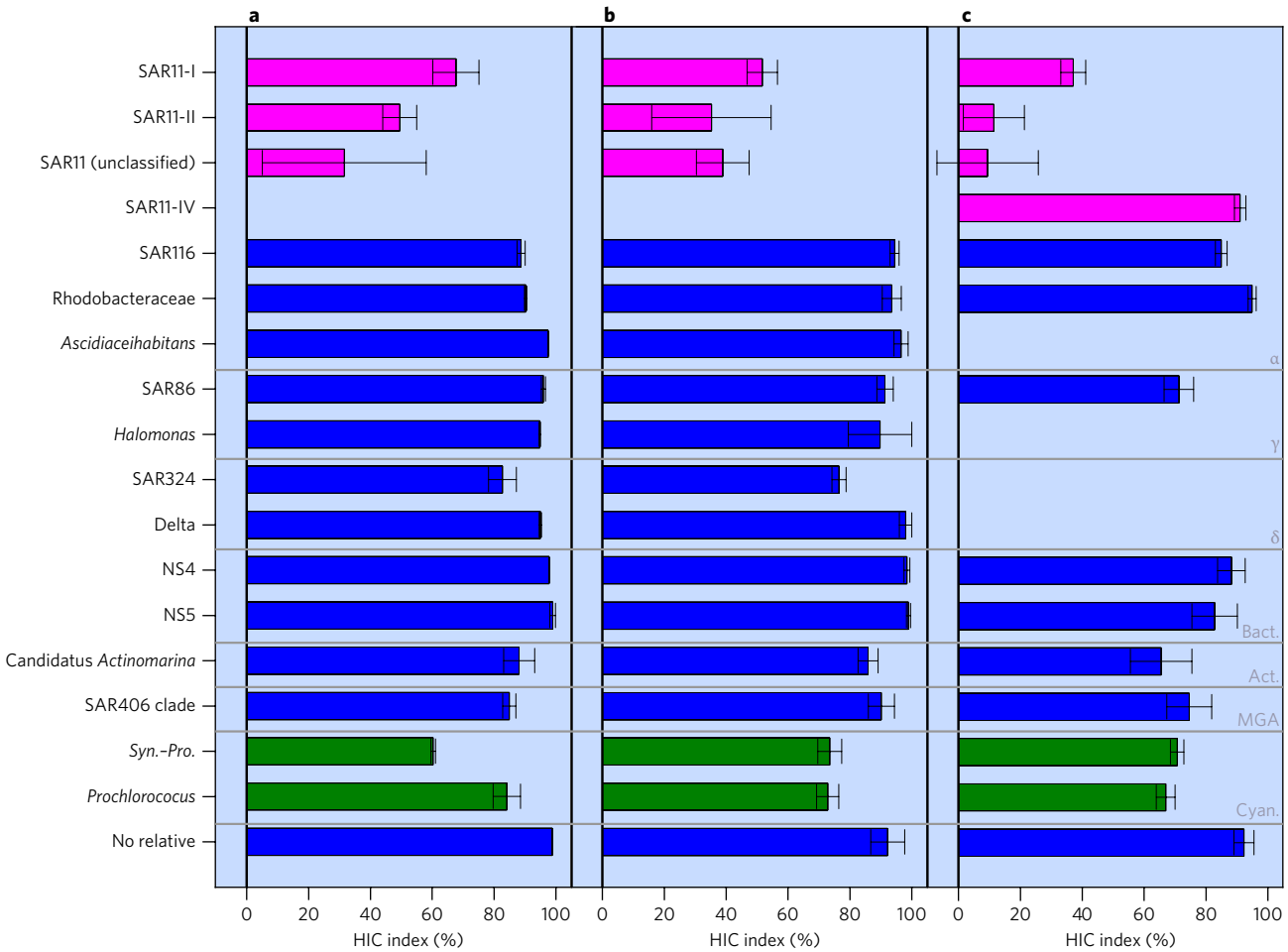

Fig. 4 | Hydrophobicity of naturally occurring marine bacteria measured by HIC in surface seawater samples collected at ~10 m depth. a, Eastern Mediterranean Sea, April 2015 $n=2$. **b**, Eastern Mediterranean Sea, July 2015 $n=4$. **c**, Gulf of Aqaba, May 2015, $n=11$. Grey lines divide OTUs into taxonomic categories. Pink indicates members of SAR11 clade, green indicates autotrophs, and blue indicates other heterotrophic bacteria. Error bars are the range in panel **a** ($n=2$) and s.e.m. in panels **b** and **c**. Each biological replicate had three technical replicates.

explain their success in the ocean, especially if this type of grazing avoidance will be confirmed for micro-grazers such as nanoflagellates. A non-stick surface can clearly benefit oligotrophic bacteria in other ways and its biochemical basis, physiological and ecological roles requires further study.

Since bacterial recognition and mucociliary mechanisms are effective defense mechanisms against pathogen invasion in humans and most other animals, a better understanding of the interaction of bacterial cell wall composition with mucous filters could have far-reaching implications beyond marine biology (for example, ref. [31] and references therein).

Methods

Study sites. In situ sampling of ascidian filtration was conducted in the oligotrophic Eastern Mediterranean Sea in front of Michmoret (Central Israel) at 10 m depth and in the Gulf of Aqaba (Northern tip of the Red Sea) in front of the Interuniversity Institute for Marine Science in Eilat (IUI) at 6 m depth. In situ sampling of appendicularian feeding was done in the more productive waters of the Ligurian Sea (Northwest Mediterranean Sea near Nice, France). Sampling period, location, the taxa sampled, number of replicates, and the sampling method of all experiments presented in this paper are summarized in Supplementary Information Table 1.

Sample collection. Direct in situ sampling of ascidians (InEx). Sampling of the water inhaled and exhaled by ascidians was conducted by SCUBA diving at 6–20 m. Representative specimens from each species were selected during preparatory dives based on ease of access. The pumping activity of each specimen was visualized before and after each sample collection. Seawater collected next to the studied specimen was dyed with sodium fluorescein and was gently released through a 0.2 µm filter near the inhalant siphon. The speed and magnitude of the exhalant jet provided a clear indication of the animal's pumping activity.

To cleanly collect inhaled and exhaled water, we used the VacuSIP[13] technique (movie S1) that allows simultaneous, clean, and controlled collection of the water inhaled and exhaled by the suspension feeders without any contact or interference with the studied animal. Water samples were collected by carefully positioning minute tubes (PEEK, external diameter 1.6 mm, internal diameter 0.27 mm, IDEX 1531) inside the exhalant siphon and next to the inhalant siphon of the sampled ascidian. Piercing the septum of an evacuated 10.5 mL tube (VACUETTE® Urine Tube, Round Bottom 10.5 ml, Greiner Bio-One, cat No. 455007) with a hypodermic syringe needle attached to the distal end of each tube allows the external pressure to slowly force the sampled water into the vessel through the sampling tube. The slow and controlled sampling rate (0.5–1 mL min^{-1}) enables integration of the animal's feeding activity and the inherent patchiness of plankton in the water while ensuring contamination-free sampling. Sampling duration was 10–15 minutes and sample volume was ~10 mL. After collection, samples were kept on ice until processing (within 2 hours) for cell counts with a flow cytometer and for DNA extraction (See below). A detailed description of the experimental procedure and a link to a step-by-step video can be found in[13]. The retention efficiency (RE) of planktonic cells was calculated from the difference in concentration between the inhaled (In) and exhaled (Ex) water using the formula: RE (%) = 100*(In-Ex)/In.

Laboratory sampling of ascidians (InEx). For controlled laboratory experiments, ascidians were carefully collected by scuba divers from 3–20 m depth in front of Michmoret, Israel (East Mediterranean). Animals were transferred underwater with the substrate they were attached to and kept in a running seawater facility at Michmoret. Each specimen was placed in a 1 L borosilicate beaker on a pile of small beach-rock pebbles and was supplied with ~0.33 L min^{-1} of sand-filtered

seawater pumped from 3 m depth. Animals were fed with cultured *Nanochloropsis* (~10^6 algal cells) once a day. All beakers were positioned inside a water table with ample supply of pumped seawater that maintained stable ambient temperature. After a few days of acclimation, samples of the water inhaled and exhaled by the animals were directly collected using a similar InEx method as for in situ sampling. In most cases the VacuSIPs were replaced with slow suction induced by gravitational siphoning of the water outside the water table. Similar experiments were also conducted in the running seawater facility of Villefranche-sur-Mer with a random assembly of ascidians collected locally and from the Thau lagoon (Northwest Mediterranean). There, all water samples were collected using the VacuSIPs.

For appendicularians, which are too small and fragile for direct sampling as described above, we modified the indirect clearance rate method techniques described in ref. [32]. Organisms were collected in drift dives using blue-water SCUBA into an open-ended cylinder. A control sample was immediately collected with an identical cylinder. The closed cylinders were incubated at the collection site (depth, 6–8 m) for ~0.5–1.5 hr. At the end of the incubation, the incubators were pulled onto the boat and water samples were collected from each incubator and preserved for further analysis as described below.

Sample analysis. *Flow cytometry.* Flow cytometry was the standard method used to quantify total concentrations of the microbial community in the seawater, cell characteristics of non-photosynthetic microorganisms (hereafter referred to as HB, non-photosynthetic bacteria) and the three dominant autotrophic groups [*Prochlorococcus* (Pro), *Synechococcus* (Syn), and eukaryotic algae (Euk)]. 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 cell concentrations (±5%). The optics system contained violet and blue lasers (405 and 488 nm, respectively) and was further adapted for the analysis of marine ultra-plankton samples as described below.

Aliquots of 1.8 mL were collected from each water sample and transferred into 2 mL cryovials (Corning cat No. 430659). Samples were first incubated for 15 min at room temperature with Glutaraldehyde 50% (electron microscopy grade, Sigma-Aldrich, cat No. 340855), at 0.1% (final concentration) for the oligotrophic East Mediterranean and Red-Sea water and 0.2% (final concentration) for the more productive NW Mediterranean water. 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 (within a few weeks).

Each sample was analyzed twice. First, 600 µl of the sample water was analyzed at a high flow rate (100 µL min^{-1}) for the determination of ultra-phytoplankton with a dual threshold (trigger) on the red fluorescence channels of the violet and blue lasers. A second run was used to analyze cells with no autofluorescence, i.e., non-photosynthetic microorganisms. To visualize these cells, a 300 µL aliquot of the sample water was 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 previously described[32]. For this run we used a low flow rate of 25 µL min^{-1} and the instrument was set to high sensitivity mode. Seventy-five µl of the sample water were analyzed with a dual threshold (trigger) on green fluorescence channels of the violet and blue lasers. Taxonomic discrimination 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[29]; side-scatter (SSC), a proxy of cell surface complexity and cell volume[33], and forward-scatter (FSC) proxy of cell size[34,35]. Given the very weak chlorophyll fluorescence of near-surface *Prochlorococcus*, especially in summer, in some cases, full separation of their population from the noise signal was not possible.

Reference beads (Polysciences™, cat# 23517, Flow Check High Intensity Green Alignment 1.0 µm) were used as an internal standard in each sample.

DNA extraction. Only small water volumes can be reliably collected in standard in situ feeding experiments. We therefore tested different extraction methods to obtain the highest microbial DNA yield from seawater samples as small as 1 mL. Comparisons of the community composition of marine bacteria obtained from extractions of 1 versus 30 mL seawater (using 454 pyrosequencing) and 5 versus 300 mL (using Illumina sequencing) indicated that the relative abundance of the most common phylotypes (those that accounted for >0.1% of total reads) could be reliably quantified from small volume extractions ($R^2 > 0.95$, $P < 0.001$, Supplementary Information Fig. 1).

Water samples (5–10 mL) from all in situ experiments were filtered on a ø25mm, 0.2 µm polycarbonate membrane (GE Healthcare Biosciences, cat No. 110606) under low vacuum immediately after collection and frozen in 1.5 mL micro-tubes at −20 °C until analysis. DNA from each filter was extracted using the DNeasy 'Blood & tissue kit' (QIAGEN, cat. No. 69504) with the following modifications to the manufacturer's protocol. ATL buffer (180 µL) and 20 µL of proteinase K were added and samples were incubated at 56 °C for 1 hr. Then 200 µL of AL Buffer and 200 µL of 95–100% ethanol were added to the sample and the mixture was pipetted into spin columns and placed in a 2 mL collection tube. Tubes were centrifuged at 6000 RCF for 1 min. The flow-through was discarded and 500 µL of AW1 buffer was added to the column, centrifuged at 6000 RCF for 1 min, and the flow-through discarded. This step was repeated for the third time, with 500 µL Buffer AW2 and a spin of 18,000 RCF for 1 min to dry the membrane before elution. For the elution step the spin column was placed on a new collection tube. Two hundred µL of buffer AE preheated to 56 °C were pipetted at three steps (50 µL, 50 µL and 100 µL) into the column, each step followed by 6000 RCF centrifugation for 1 min. The sample was then incubated at room temperature for at least a minute and stored at −20 °C.

Next-generation sequencing. Samples were amplified for sequencing using a forward and reverse fusion primer (28F-519R, 16 S V1-V3 region). The forward primer was constructed with (5′-3′), the Illumina i5 adapter (AATGATACGGCGACCACCGAGATCTACAC), an 8–10 bp barcode, a primer pad, and the 5′– GAGTTTGATCNTGGCTCAG –3′ primer. The reverse fusion primer was constructed with (5′-3′), the Illumina i7 adapter (CAAGCAGAAGACGGCATACGAGAT), an 8–10 bp barcode, a primer pad, and the 5′–GTNTTACNGCGGCKGCTG –3′ primer. Primer pads were designed to ensure the primer pad/primer combination had a melting temperature of 63 °C–66 °C according to methods developed by the lab of Patrick Schloss (http://www.mothur.org/w/images/0/0c/Wet-lab_MiSeq_SOP.pdf). Amplifications were performed in 25 µL reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1 µL of each 5 µM primer, and 1 µL of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95 °C for 5 min, then 35 cycles of 94 °C for 30 sec, 54 °C for 40 sec, 72 °C for 1 min, followed by one cycle of 72 °C for 10 min and 4 °C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was size selected in two rounds using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana) in a 0.7 ratio for both rounds. Size selected pools were then quantified using the Qubit 2.0 fluorometer (Life Technologies) and loaded on an Illumina MiSeq (Illumina, Inc. San Diego, California) 2 × 300 flow cell at 10pM.

Bioinformatics. All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3; ref. [36]). Each read was aligned using the SILVA Incremental Aligner (SINA SINA v1.2.10 for ARB SVN (revision 21008))[36] against the SILVA SSU rRNA SEED and quality controlled[37]. Reads shorter than 50 aligned nucleotides and reads with more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artifacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded from downstream analysis. After these initial steps of quality control, identical reads were identified (dereplication), the unique reads were clustered (OTUs), on a per sample basis, and the reference read of each OTU was classified. Dereplication and clustering were done using cd-hit-est (version 3.1.2; http://www.bioinformatics.org/cd-hit)[38] running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.98, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 123; http://www.arb-silva.de) using blastn (version 2.2.30+; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with standard settings[39].

The classification of each OTU reference read was mapped onto all reads that were assigned to the respective OTU. This yields quantitative information (number of individual reads per taxonomic path), within the limitations of PCR and sequencing technique biases, as well as multiple rRNA operons. Reads without any BLAST hits or reads with weak BLAST hits, where the function "% sequence identity + % alignment coverage)/2" did not exceed the value of 93, remained unclassified. These reads were assigned to the meta group "No Relative" in the SILVAngs fingerprint and Krona charts[40].

The SAR11 OTUs were named using the widely recognized clades initially described as clades I and II[41] later expanded to clades II and IV[42] and used subsequently by recognized authors in the SAR11 field[43,44,45]. The OTUs were reassigned by inserting representative sequences from each SILVA SAR11 clade into a SAR11 phylogenetic tree constructed in the ARB program using full length sequences that defined clades I-IV[45]. The OTUs defined by SILVA as SAR11 S1 and S1* were grouped together in the same surface clade I. To avoid a mixture of general and specific labels, the OTU classed as "SAR11" which did not fall into a defined cluster was classed as SAR11_Unclassified.

CARD-FISH. Fluorescence In Situ Hybridization (FISH) and CAtalyzed Reporter Deposition FISH (CARD-FISH) were used to quantify the concentration of specific bacterial phylotypes such as *Pelagibacter ubique*. Briefly, 15–20 mL samples were fixed with freshly-made, filtered paraformaldehyde 32% to a final concentration of 2% and incubated for 8 h at 4 °C. The sample was then filtered on a ø25mm, 0.2 µm polycarbonate membrane filter and stored frozen (−20 °C). The CARD-FISH protocol was modified after[46,47]. Cells on filter sections were hybridized with the EUB338 general eubacteria probe[48], a SAR11 specific probe[5] and a negative control probe NON338[49,50]. Filters were examined and imaged under an Olympus BX61 epifluorescence microscope using 100x magnification. Cell counts were carried out using ImageJ scientific image analysis (NIH).

Cell surface hydrophobicity. We compared cell surface hydrophobicity for cultured bacteria of prominent environmental clades using a method suggested by M. Landry and previously described[28]. Briefly, triplicates of ambient seawater or bacterial cultures were passed through hydrophobic and hydrophilic solid phase columns (each triplicates used separate pair of columns). Cell concentrations were measured by flow cytometry before and after the sample passage through each column. The relative frequency of the prominent bacterial taxa in the environmental (seawater) samples was also quantified by DNA extraction and 16S rRNA gene sequencing as described above.

To calculate the HIC (Hydrophobic Interaction Chromatography) index for each population we used the formula: $HIC = 100*(C_{hydrophilic} - C_{hydrophobic})/C_{hydrophilic}$

Where $C_{hydrophilic}$ is the cell concentration after passage through the hydrophilic column and $C_{hydrophobic}$ is the cell concentration after passage through the hydrophobic column. Therefore, a HIC index value close to 100 indicates that the hydrophobic column retained most of the cells at question whereas the same cells have passed through the hydrophilic column. In contrast, low HIC values (close to zero) indicate a relatively hydrophilic cell surface, so that most cells passed through both columns at a similar rate.

To insure that the measure HIC index is a consequence of cell surface hydrophobicity and not of the experimental columns, we have replicated the HIC index experiments with four different column types: HPLC sample preparatory columns, (a) tC18 (Sep-pak C18, Waters, cat No. WAT036810) (b) C18 (Sep-pak, Waters, cat No. WAT020515) (c) C8 (Sep-pak, Waters, cat No. WAT036775) (d) PPL (Agilent Life Sciences, cat No. 12105006). Waters hydrophobic columns (a-c, above) (tC18, C18, and C8) were run against a hydrophilic Diol column (Waters, WAT020530) and the Agilent hydrophobic column (PPL) was run against a 2OH column (Agilent Life Sciences, cat No. 12102036).

Bacteria cultivation for column assays. Bacteria were grown to logarithmic phase in 40 mL medium in 125 mL polycarbonate, acid-washed and autoclaved flasks at 17 °C under 12:12 hr light:dark cycles unless otherwise indicated. Cultures were transferred at least once after revival from glycerol stocks before being used in the assay. Growth was monitored on an easyCyte HT Guava flow cytometer (Merck Millipore) after staining for 60 min with SYBR Green I (Invitrogen by Thermo Fisher Scientific, Eugene, OR, USA) as described previously[51]. Sterilized seawater for media was collected in acid-washed polycarbonate carboys, 0.2 µm filtered, autoclaved and sparged (6 h with 0.1 µm filtered CO_2 followed by 20 h with 0.1 µm filtered air). Cultures were diluted to ~10^5 cells per mL with their appropriate medium or seawater before the assay.

HTCC1062 (SAR11) was grown in the dark using the basal salt and trace metal mix of the artificial seawater medium ASM1[52] supplemented with (final concentrations): 1 mM NH_4Cl, 100 µM K_2HPO_4, 1 µM $FeCl_3$, 80 µM sodium pyruvate, 40 µM oxaloacetate, 1 µM betaine, 50 µM glycine, 40 µM taurine, 50 µM methionine and a vitamin solution consisting of 593 nM vitamin B1, 227 pM B3, 81 nM B5, 59 nM B6, 74 pM B12, 555 nM myo-inositol and 409 pM 4-aminobenzoic acid. HTCC2506 (*Fulvimarina pelagi*) was grown in sterilized seawater supplemented with 100 µM NH_4Cl, 100 µM K_2HPO_4, 1 µM $FeCl_3$ and 0.1 µM methionine. For culturing HTCC2143 (marine gammaproteobacterium) the same medium was further supplemented with 0.9 µM (1 µM total) methionine, 25 µM sodium pyruvate and D-glucose, 1 µM glycine. Both media were supplemented with the same vitamin solution as used for HTCC1062. Seawater for HTCC2143 and HTCC2506 medium was collected from the Eastern Mediterranean Sea (32°15′N; 034°9.6′ E) at 10 m depth on December 2014. *Synechococcus* sp. WH8102 was grown in PRO99 medium and *Synechococcus* sp. PCC7002 in L1 medium[53]. Both strains were grown under continuous light. Strains WH8102 and PCC7002 were grown with shaking (50 rpm) at 21 °C and at room temperature (20–25 °C), respectively. Seawater for these two media was collected at 10 m depth from the Eastern Mediterranean Sea (32°27′N; 034°23E) on April 2015. *Dokdonia* sp. MED134 and *Dinoroseobacter shibae* DLF12 were grown in Marine Broth 2216.

Statistical analysis. The sampling design (InEx and before/after incubations) was specifically developed as a "pairwise comparison". Therefore, a "within subject" design (i.e., paired t-test, repeated measure ANOVA, and their nonparametric alternative: Wilcoxon signed-rank test and Friedman Repeated Measures Analysis of Variance on Ranks, with Tukey post-hoc Pairwise Multiple Comparison, respectively) was used throughout the analysis to test the null hypothesis of unselective retention. Our comparisons of cell identity and properties were especially robust due to the paired sampling design applied throughout sample collection and analysis (the same populations were compared in the same water prior to and after the passage via the tunicate filtration apparatus or affinity column using identical analytical methods). The normalization to calibration beads provided additional protection against instrumental drifts.

Due to the small sample size, data are reported as medians unless stated otherwise. In classical grazing experiments, grazing by the suspension feeder affects food concentrations in the experimental vessel[16,32]. Measuring direct filtration efficiency as was done here allows estimation of Chesson selectivity index (α_i) as the maximum likelihood estimator $\tilde{\alpha}_i = F_i\left(\sum_{i=1}^{m} F_i\right)^{-1}$ (case 1 in[16]) where m is the number of prey types and F_i is the filtration efficiency for the ith prey type, calculated as $F_i = (In_i\text{-}Ex_i)/In_i$ (where In_i and Ex_i are the concentrations of the ith prey type in the water inhaled and exhaled by the studied animal, respectively). A separate α_i was calculated for each paired water sample. To meet ANOVA requirements of homogeneity of variance and normality, filtration efficiency was square root and arcsine transformed and Chesson αis were square root transformed. For Repeated Measure ANOVA (RM ANOVA) we also tested the compound symmetry and sphericity assumptions (i.e., cases in which differences between levels were correlated across subjects) using Mauchly's Test of Sphericity and compared the results of the univariate test with Wilks' λ (a multivariate criterion). Statistical analyses were done using STATISTICA for Windows (Ver 10.2, StatSoft, Inc. 2011).

Potential biases and pitfalls. The Illumina sequencing grossly overestimated the relative abundance of picocyanobacterial cells in comparison to the flow cytometer counts (up to 10-fold), pointing to potential biases inherent in any analytical method. Furthermore, the application of different primer sets to the same DNA samples also generated different community composition results. To mitigate against such analytical biases, we applied a paired design methodology throughout our experiments, in which each and every analysis was carried out on paired samples that were compared before and after exposure to a mucus net, grazer, or an affinity column, therefore controlling for any internal analytical bias.

The different analytical methods resulted in similar patterns. Bacteria in the SAR11 clade were the least retained microorganisms, whereas picocyanobacteria as well as Flavobacteria NS2 were efficiently filtered by all suspension feeders examined, regardless of the analytical method used. For example, in our data set, primers 28F-519R provided considerably higher estimates of SAR11 abundance compared to primers 515F-Y-926R[54]. Nevertheless, both primers provided very similar grazing trends (Spearman correlation $r = 0.80$, $P < 0.001$, and compare Supplementary Information Figs. 3a and 3b). Very similar trends were also found using different DNA extraction methods and 454 Pyrosequencing (Supplementary Information Fig 5) and using qPCR (tested for both appendicularians and ascidians). CARD-FISH also provided no indication of SAR11 removal by the ascidian *Polycarpa mytiligera*.

Data availability. Sequence data was deposited in the European Nucleotide Archive[55] (ENA) using the data brokerage service of the German Federation for Biological Data (GFBio, ref. [56]), in full compliance with the Minimal Information about any (X) Sequence (MIxS) standard[57]. The data is accessible under the INSDC accession number PRJEB21921. Any other data that support the findings of this study are available from the corresponding author upon request.

Received: 22 November 2016; Accepted: 18 August 2017;
Published online: 02 October 2017

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Acknowledgements

We thank S. Giovannoni for providing strains HTCC1062, HTCC2506 and HTCC2143, and L. Gomez-Consarnau for providing strains MED134 and DLF12 and D. Roth-Rosenberg and D. Sher lab for providing strain PRO9312. M. Landry for useful discussion and help with HIC application. M. Gilboa and staff of the School of Marine Science, Ruppin for technical assistance. BSF grant 2012089 to K.S, Y.T. and G.Y. ISF grant 1280/13 to G.Y. ECOGELY ANR-10-PDOC-005-01 to F.L. and ANR RHOMEO 11-BSV7-0021 to M.T.S. We thank the Bio2Mar, and Cytometry-Imaging platforms of the OOB and EMBRC-zooplankton of OOV for access to instrumentation. Support provided to A.D.P by the Mediterranean Sea Research Center of Israel and IUI PhD award.

Author contributions

A.D.-P. was involved in study design, performed the in situ and lab experiments, fine-tuned protocols, compiled and analyzed FCM, FISH, HIC and sequencing data and prepared the manuscript. G.Y. designed the study, and participated in field experiments, data analysis and manuscript preparation. A.G. was involved in planning the study. Y.T. and L.S. were involved in study design. L.S. and M.H. cultured oligotrophic and copiotrophic bacteria utilized for column experiments. K.R.C., K.S. and F.L. participated in planning some of the experiments, field and lab sampling. N.J.W. and M.T.S. were involved in the FISH experiments and data analysis. M.R. designed and performed bioinformatics analyses F.O.G. designed bioinformatics analyses Y.J. performed beads experiments. All authors discussed the results and commented on the manuscript during its preparation.

Competing interests

The authors declare no competing financial interests.

Additional information

Supplementary information is available for this paper at doi:10.1038/s41564-017-0030-5.

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Experimental design

1. Sample size

Describe how sample size was determined.

To minimize experimental bias we have made every possible effort to collect most of the samples underwater from animals that were minimally disturbed. This is especially important when working in oligotrophic water where the microbial community is notoriously sensitive to handling. Therefore, the actual sample size was mostly limited by diving logistics (e.g., limited time underwater, sea conditions, and cost) and animals availability. For example, salps (planktonnic tunicates) were a major target for us, but during the three years of study we have repeatedly failed in our effort to collect reilable InEx samples from salps. it should be noted however, that the paired nature of our sampling design allows a "within subject" comparison that minimize the the required N.

2. Data exclusions

Describe any data exclusions.

Our goal was to search for, and quantify microbe specific filtration. In some cases, cell counts made with a flow cytometer showed null or very small differences between the cell concentrations in inhaled and exhaled water, indicating poor filtration or poor sampling, these (few) InEx pairs were excluded for downstream analysis.
After sequencing, Reads shorter than 50 aligned nucleotides and reads with 365 more than 2% of ambiguities, or 2% of homopolymers, respectively, were excluded from further processing. Putative contaminations and artifacts, reads with a low alignment quality (50 alignment identity, 40 alignment score reported by SINA), were identified and excluded 368 from downstream analysis

3. Replication

Describe whether the experimental findings were reliably reproduced.

Our findings were reliably reproduced: After the initial findings with one ascidian species in the Eastern Mediterranean Sea , we have tested 7 more asicidians species form two additional basins. To rule out methodological biases, we have also replicated the analysis with different sets of primers and sequencing methods (see text for details).

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Organisms were selected arbitrarily in each dive. For example, during blue-water dives for appendicularia, we have sampled the animals (tens out of many millions) that were drifted by the currents and spotted within reach. For benthic ascidians, we have selected animals that were actively pumping and that a VacuSip sampler could be positioned nearby.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

NA

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or the Methods section if additional space is needed).

n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☐	☒ A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly.
☐	☒ A statement indicating how many times each experiment was replicated
☐	☒ The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒ A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒ The test results (e.g. *p* values) given as exact values whenever possible and with confidence intervals noted
☐	☒ A summary of the descriptive statistics, including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒ Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

STATISTICA for Windows (Ver 471 10.2, StatSoft, Inc. 2011

For all studies, we encourage code deposition in a community repository (e.g. GitHub). Authors must make computer code available to editors and reviewers upon request. The *Nature Methods* guidance for providing algorithms and software for publication may be useful for any submission.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All material are readily available

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

NA

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

NA

b. Describe the method of cell line authentication used.

No Eukaryotic call lines were used

c. Report whether the cell lines were tested for mycoplasma contamination.

NA

d. If any of the cell lines used in the paper are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

NA

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Our text meets the ARRIVE guidelines.
Benthic tunicates remained intact during the experiments . Few planktonic tunicates were collected for identification under the microscope

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	NA