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# *In situ* evidence for pre-capture qualitative selection in the tropical bivalve *Lithophaga simplex*

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**ABSTRACT:** Few feeding studies have been performed on tropical bivalves, and *in situ* feeding studies are lacking altogether. We investigated retention efficiencies for natural particles in the coral-boring tropical mytilid *Lithophaga simplex*. Using the *in situ* InEx technique (Yahel et al. 2005; Limnol Oceanogr Methods 3:46–58) SCUBA divers collected samples from the water inhaled and exhaled by undisturbed bivalves at the coral reef of Eilat (Gulf of Aqaba). Particle retention efficiencies were determined using flow cytometry analysis of the paired water samples. The photosynthetic bacterium *Synechococcus* ( $0.9 \pm 0.1 \mu\text{m}$ ) and larger eukaryotic algae (1 to 10  $\mu\text{m}$ ) were preferentially retained by the bivalve with removal efficiencies of up to 90% (1996 to 2000: averages of  $69 \pm 14\%$  and  $60 \pm 17\%$ , respectively,  $n = 74$  individual bivalves). The minute photosynthetic bacterium *Prochlorococcus* ( $0.4 \pm 0.1 \mu\text{m}$ ) was also moderately retained ( $41 \pm 19\%$ ). Only a small proportion of the non-photosynthetic bacteria ( $0.3 \pm 0.1 \mu\text{m}$ ) were retained ( $5 \pm 18\%$ , median of 9%), despite their numerical dominance in the plankton and considerable size overlap with *Prochlorococcus*. Size-independent preferential retention was also observed within particle types: (1) *L. simplex* more efficiently retained *Prochlorococcus* and picoeukaryotic algal cells with higher chlorophyll content and (2) the small fraction of non-photosynthetic bacteria retained did not differ in size, but had higher nucleic acid content (compared to the inhaled population) an indicator for viable and active bacteria. We conclude that particle retention is not strictly size-dependent in *L. simplex*, and probably involves other cell attributes such as cell surface properties and/or motility.

**KEY WORDS:** Suspension feeding · Nutrition · Selectivity · Coral reefs

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## INTRODUCTION

Selective feeding is an important mechanism by which animals optimize their diet, e.g. by maximizing their energy gain or avoiding harmful food (Krebs & Davies 1997). The importance of selective feeding in structuring the pelagic microbial community is well documented (Pernthaler 2005), but much less is known about selection in benthic suspension feeders (see Yahel et al. 2006).

In the past 20 yr, bivalve suspension-feeding mechanisms have been vigorously investigated using a

variety of relatively new approaches. Outcome-based techniques such as automated particle counting and flow cytometry have established the basic characteristics of suspension feeding, and provided important clues to underlying mechanisms. These have been investigated, in turn, using a variety of recently developed techniques to directly study various mechanism aspects (for reviews see Gosling 2003, Ward & Shumway 2004, Beninger 2009, this issue). While the mechanisms of particle transport, ingestion volume regulation, and pseudofaeces transport and rejection are all now reasonably well known (Gosling 2003),

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some aspects of particle processing by bivalves are still poorly understood. Among these, particle selection is, to date, a particularly intractable field, since it involves thousands of microscopic particles simultaneously passing over complex, and often occluding, processing structures, and no mechanism of recognition has yet been identified. It has long been known that bivalves are capable of particle selection (Ward & Shumway 2004). Direct observations have recently clarified the roles of the gills and the labial palps in particle selection in 3 of the 4 principal bivalve processing systems, and have established that the observed selection was effected post-capture (i.e. after the particles had been deviated from the through current onto the gill frontal surfaces: Beninger et al. 1997, 2004, 2007, Ward et al. 1998a, Cagnie et al. 2003).

Our current knowledge of bivalve feeding is derived primarily from laboratory studies carried out with either laboratory-cultured algae or natural algal assemblages. It is yet unclear how valid the predictions and models based on laboratory experiments are to actual field situations; therefore, reliable methods are needed for *in situ* study of bivalve feeding (e.g. Cranford 1999, Cranford & Hill 1999, Kotta & Møhlenberg 2002, MacDonald & Nodwell 2003). The recent development of the InEx technique has enabled *in situ* sampling of inhalant and exhalant currents from undisturbed suspension feeders (Yahel et al. 2003, 2005, 2007).

Another gap in our understanding of bivalve feeding is related to the fact that the species investigated have overwhelmingly come from temperate, eutrophic, or mesotrophic habitats (Newell & Shumway 1993). These habitats are dominated by larger phytoplankton and characterized by relatively high loads of suspended sediments and large fluctuations of temperature and food availability. In contrast, few studies have investigated feeding characteristics and mechanisms in tropical suspension-feeding bivalves. Oligotrophic tropical waters are characterized by more stable conditions and are dominated by micron-size prokaryotic phytoplankton.

In the present study, we used the InEx sampling technique and subsequent flow cytometry analysis to quantify the *in situ* retention of naturally occurring phytoplankton and bacteria by the common tropical coral-boring mytilid *Lithophaga simplex*.

## MATERIALS AND METHODS

**Study site.** The study was carried out in the fore-reef (3 to 18 m depth) of the Eilat Coral Reef Nature Reserve, northern Gulf of Aqaba, Red Sea (Israel). For a description of the study site, see Genin et al. (2009) and references therein. The concentrations of chlorophyll

(annual average:  $\sim 0.3 \mu\text{g l}^{-1}$ ; Genin et al. 2009) and particulate organic carbon ( $\sim 0.1 \text{ mg l}^{-1}$ ; Yahel et al. 2003) are low. The phytoplankton community in the Gulf of Aqaba is dominated by ultraphytoplankton ( $< 8 \mu\text{m}$ ; Yahel et al. 1998, Sommer et al. 2002). Small eukaryotic algae ( $0.6$  to  $4.0 \mu\text{m}$ ) dominate during the winter mixing, whereas the cyanobacterium *Synechococcus* ( $0.9$  to  $1.2 \mu\text{m}$ ; Sommer et al. 2002) dominates during spring blooms and is present at high concentrations ( $> 10^4 \text{ ml}^{-1}$ ) throughout the year. *Prochlorococcus* ( $0.5$  to  $0.7 \mu\text{m}$ ) is absent during the winter and dominates (numerically) the phytoplankton assemblage during the warm season, when the water is stratified and nutrient-depleted (Lindell & Post 1995). A thick (1 to 3 m) phytoplankton-depleted layer is usually found over the reef (Yahel et al. 1998), due to intense grazing by a rich guild of benthic phytoplanktivores, mainly soft corals, sponges, ascidians and boring bivalves (Yahel et al. 1998, Genin et al. 2009).

***Lithophaga simplex*.** The Lithophaginae (Bivalvia: Mytilidae) are important borers of a wide variety of calcareous substrata, including dead and live coral skeletons. In the Red Sea, the mussel *L. simplex* Iredale, 1939 (Fig. 1) inhabits the massive coral *Astropora myriophthalma* (Lamarck, 1816) (Fig. 1) and, less frequently *Goniastrea pectinata* (Mokady et al. 1992), but is specific to other corals in other localities (Morton 1983). The bivalve reaches a few centimetres in length and is completely enclosed by the coral skeleton (Fig. 1A), with only the distal end of the siphons (1 to 3 mm in diameter) extending into the surrounding seawater (Fig. 1B). As a mytilid, it probably possesses a homorhabdic filibranch gill structure (i.e. all filaments are of 1 simple tubular type, and the gill is not capable of post-capture qualitative selection), but we are not aware of any histological studies of the gill structure for this species. *A. myriophthalma* forms large colonies (up to several metres), often carrying dense populations of *L. simplex*. In the Gulf of Aqaba, several hundred *L. simplex* specimens may colonize a single coral head, with a mean density of  $22 \pm 11$  mussels  $100 \text{ cm}^{-2}$  of coral surface (Mokady et al. 1998). The overall density of *L. simplex* at the study site was  $2.5 \text{ ind. m}^{-2}$ , about one-quarter of the overall *Lithophaga* spp. individuals we surveyed (Genin et al. 2009). The known benefits for the bivalves from this association include protection against predators and possibly the nutritional use of coral mucus (Shafir & Loya 1983). While boring bivalves are commonly regarded as parasites, *L. simplex* was shown to provide ammonium from its nitrogenous waste to the photosynthetic symbiotic algae of its coral host (Mokady et al. 1998).

**Water sampling.** An *in situ*, unintrusive technique, based on the simultaneous pairwise collection of the water inhaled and exhaled by the animal (InEx), was

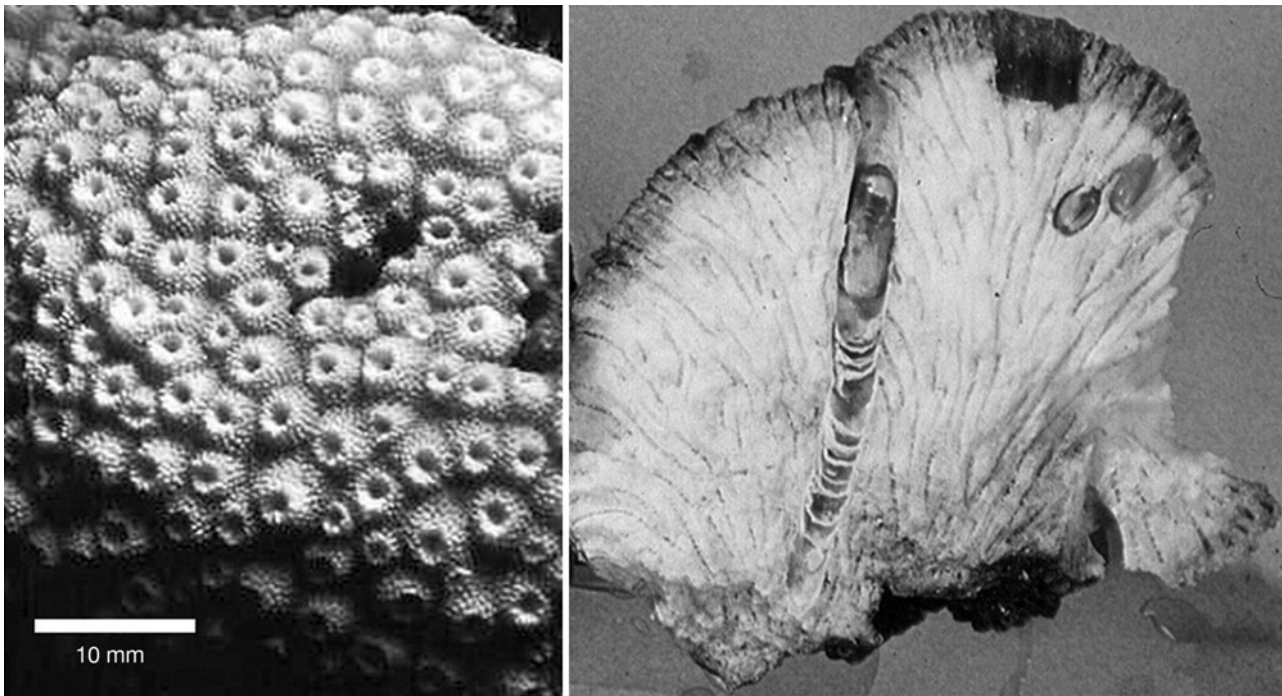


Fig. 1. (A) *Lithophaga simplex* inside the massive hermatypic coral *Astreopora myriophthalma*, as viewed from the outside (siphon diameter: ca. 2 to 3 mm). (B) The boring bivalve *L. simplex* inside its host *A. myriophthalma*. Photographs courtesy of I. Brikner and Y. Loya

used to directly measure the rate and efficiency of particle removal from the water filtered by the studied animals as detailed in Yahel et al. (2005). Briefly, a SCUBA diver sampled the inhaled water by slowly ( $\sim 0.5 \text{ ml s}^{-1}$ ) withdrawing water into a modified plastic pipette, attached at its proximal end to a syringe, while holding its open (intake) end (3 to 5 mm) next to the bivalve inhalant siphon. A sample of exhaled water was taken simultaneously using an identical tube held within the exhalant jet, with the tube's intake end positioned  $< 2 \text{ mm}$  above the bivalve exhalant siphon, using the excurrent jet to flush and then fill the tube. We used 2, 3 and 7 ml samplers (LPt1, Pt5 and Pt10, respectively; in Yahel et al. 2005). Care was taken to avoid physical contact with the bivalve, and, when such contact occurred accidentally, the sample was aborted. Filling time (1 to 6 min, depending on the sampler volume) was determined individually for each pair so that it would last 150% of the time it took the exhalant jet to flush clear an identical tube pre-filled with fluorescein dye (measured a few minutes prior to each sampling). Sampling duration was  $> 50$  times longer than the few seconds it took the water to pass through the bivalve. Thus, each 'InEx' pair represented a several-minute integration of bivalve activity. The difference in particle concentration in the 2 samples provides a measure of the retention efficien-

cies of different particle sizes and types (Yahel et al. 2005).

The present study focused on the boring bivalve *Lithophaga simplex* living in *Astreopora myriophthalma* corals. Overall 74 pairs of InEx samples were collected from *L. simplex* in 16 dives during 10 sampling sessions spanning October 1996 to September 2000 and covering all 4 seasons.

**Flow cytometry.** A FACSort flow cytometer (Becton Dickinson, 15 mW, 488 nm, air-cooled Argon-ion laser) was used to measure the concentration and cell characteristics of non-photosynthetic microbes (hereafter referred to as non-photosynthetic bacteria [Bact]) and the 3 dominant autotrophic groups in the reef waters (*Prochlorococcus* [Pro], *Synechococcus* [Syn] and pico-eukaryotes [Euk]). Taxonomic discrimination was made based on the orange fluorescence (Fl2) of phycoerythrin and the red fluorescence (Fl3) of chlorophyll (Marie et al. 1999), and on side scatter (SSC, a proxy of cell volume; Simon et al. 1994) and forward scatter (FSC, a proxy of cell size; Cunningham & Buonacorsi 1992, Robertson et al. 1998). Each sample was analyzed twice. First, 150 to 300  $\mu\text{l}$  of the sample water ( $> 2.5 \times 10^4$  cells) was analyzed during 2 to 3 min, for determination of ultra-phytoplankton with the discriminator set to Fl3. A second run was used to analyze cells with no autofluorescence, i.e. non-photosynthetic



microbes. To visualize these cells, a 250 µl volume of the sample water was incubated with the nucleic acid stain SYBR Green I (20 min dark incubation at room temperature, 1:10<sup>4</sup> of SYBR Green commercial stock) (Marie et al. 1999). About 50 µl of sample water (>4 × 10<sup>4</sup> cells) was analyzed during a 2 min run with a low flow rate, and the discriminator was set to green fluorescence (Fl1). *Prochlorococcus* has very weak chlorophyll fluorescence near the surface, especially in summer. Thus, in some cases, when full separation from the noise was not possible, it was necessary to apply a Gaussian fit to a density distribution plot of the SSC or Fl3; this extrapolation allows better estimates of *Prochlorococcus* cell concentration. Only samples where the non-photosynthetic bacteria and *Prochlorococcus* populations could be fully resolved, from the noise and from each other, were used for subsequent analysis of cell population attributes.

Yellow-green beads (Polysciences; diameter: 1 µm) were used as an internal standard in each sample, and (unless stated otherwise in the text) all cellular attributes were normalized to the beads using the equation:

$$\overline{\text{Norm}}_{i,j} = \frac{\overline{\text{Population}}_{i,j}}{\overline{\text{Beads}}_{i,j}}$$

where  $\overline{\text{Norm}}_{i,j}$  is the normalized mean cell population  $i$  (e.g. *Synechococcus*) for the mean cell attribute  $j$  (e.g. SSC) (Marie et al. 1999). This normalization allows proper comparison of results obtained using different instrument settings.

The comparisons of cell properties were especially robust due to the paired sampling design applied in the present study (the same populations were compared in the same water prior to and after passage through the bivalve). The normalization to the calibration beads provided additional protection against instrumental drifts and shifts in sheath fluid properties.

Raw (list mode) data were recorded using 4 decades (10<sup>0</sup> to 10<sup>4</sup>) log scale and 256 bins (channels) and analyzed using Cytowin (Version 4.1 developed by D. Vaultot, [www.sb-roscoff.fr/Phyto/cyto.html#cytowin](http://www.sb-roscoff.fr/Phyto/cyto.html#cytowin)) or WinMDI (Version 2.8 developed by J. Trotter, <http://facs.scripps.edu/software.html>).

FSC is correlated to the size and the equivalent sphere diameter (ESD) of particles. Because these relationships are roughly linear for up to ~5 µm particles (Cunningham & Buonaccorsi 1992, Robertson et al. 1998, Cavender-Bares et al. 2001), FSC is widely used as a relative or absolute measure of size (Grob et al. 2007). For simplicity we report the ratio of FSC of particles to the FSC of 1 µm beads as cell size in micrometre units. Since we neither measured the cells microscopically, nor calibrated our flow cytometer measurements (Cavender-Bares et al. 2001, Shalapyonok et al. 2001, Grob et al. 2007), the sizes given here should be regarded as

approximations, especially for larger particles (>8 µm) at the limits of the dynamic range used. Due to this limitation the flow cytometer counts were not transformed to cell biomass.

Log to linear transformation was done using the equation  $x_{\text{lin}} = 10^{4 \times x_{\text{log}} \times 256^{-1}}$ . No normalization to beads was applied to the density distribution examples presented below; thus, care was taken to average and compare only a few (>12) samples from adjacent water samples, which were run consecutively using exactly the same instrument settings.

Discrimination of the 4 particle types was not on the same taxonomic level. Moreover, the accuracy of discrimination was also variable; it was close to 100% for *Synechococcus* and the eukaryotic algae, but much lower for *Prochlorococcus* and non-photosynthetic bacteria that, on occasion, merged with the noise. To be conservative, we have omitted from the cell property analysis any samples in which the non-photosynthetic bacteria, *Prochlorococcus*, and noise could not be fully resolved. As a result, we have most likely overestimated the actual differences between the non-photosynthetic bacteria and *Prochlorococcus* populations. Population size has an important effect on the accuracy of statistical description of cell properties. Thus, larger confidence intervals were usually associated with smaller populations such as the eukaryotic algae (and in some seasons also *Prochlorococcus*), as well as with the reduced populations in the exhaled water.

Eventual differences in retention of live versus dead bacteria were investigated using the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining technique (Sieracki et al. 1999) for the InEx experiments undertaken in September 2000. An aliquot taken from each water sample was incubated in the dark, at room temperature, with 2 mmol l<sup>-1</sup> CTC (Polysciences). Incubation was terminated after 15 min by the addition of 0.1% glutaraldehyde. The sample was then analyzed in a third run. In a positive control, established by enriching sample water with 1% LB medium, the CTC positive cell concentration doubled within 20 min.

**Statistical analysis.** The sampling design (InEx) was specifically developed as a 'pairwise comparison' (Yahel et al. 2005). Therefore, a 'within-subject' design (Rao 1997, i.e. paired *t*-test, repeated-measure ANOVA, and their nonparametric alternatives) was used throughout the analysis to test the null hypothesis of unselective retention. Data are reported as averages (±1 SD) unless stated otherwise. Statistical analyses were done using STATISTICA for Windows (Ver. 6.0, StatSoft).

Comparisons of cell size-frequency distributions were done within runs using samples stained with the nucleic acid stain SYBR Green I. *Prochlorococcus* was distinguished from the non-photosynthetic bacteria on the SSC versus red fluorescence cytogram based on their

red chlorophyll fluorescence. Bin averages were calculated for cell frequencies within each of the 256 logarithmically spaced bins (channels) and normalized as the percentage of the total number of cells in the respective population.

In classical grazing experiments, suspension-feeder activity affects food concentrations in the experimental vessel (Chesson 1983, Riisgård 2001a). Measuring direct filtration efficiency, as was done here, allows estimation of the Chesson selectivity index ( $\alpha_i$ ) as the maximum-likelihood estimator:  $\bar{\alpha}_i = F_i \left( \sum_{i=1}^m F_i \right)^{-1}$  (Case 1 in Chesson 1983), where  $m$  is the number of particle types and  $F_i$  is the filtration efficiency for the  $i$ th particle type, calculated as:  $F_i = (In_i - Ex_i)/In_i$  (where  $In_i$  and  $Ex_i$  are the concentrations of the  $i$ th particle type in the water inhaled and exhaled by the studied animal, respectively). A separate  $\alpha_i$  was calculated for each paired water sample. For better visualization  $\alpha_i$  were rescaled to  $\varepsilon_i$  using the equation  $\varepsilon_i = (m\alpha_i - 1)/[(m - 2)\alpha_i + 1]$ , where  $m$  is the number of particle types available (Chesson 1983). Values of  $\varepsilon_i$  range from  $-1$  when none of the  $i$ th type particles are taken, to  $1$  when the  $i$ th type particles are the only ones retained. However, since the statistical properties of the  $\varepsilon_i$  are not fully resolved, statistical inference was done solely with  $\alpha_i$  values (Chesson 1983). To meet ANOVA requirements of homogeneity of variance and normality (verified by Cochran and Lilliefors tests, respectively), filtration efficiency was square-root and arcsine transformed, and Chesson  $\alpha_i$  values were square-root transformed. Pairwise, post hoc comparisons of  $\alpha_i$  values were made using the Tukey honestly significant differences (HSD) test for unequal  $n$ .

## RESULTS

### Ambient conditions

The water temperature at the study site ranged from 20 to 26°C, with rare occurrences of warmer (up to 28°C) days. The seasonal succession of phytoplankton was similar to that reported by Lindell & Post (1995), characterized by concentration shifts of several orders of magnitude for *Prochlorococcus* (null to  $2.5 \times 10^5$  cells  $ml^{-1}$ ) and eukaryotic algae ( $10^2$  to  $1.5 \times 10^4$  cells  $ml^{-1}$ ). *Synechococcus* and the non-photosynthetic bacteria demonstrated much less variability, with changes between  $10^4$  to  $3 \times 10^4$  cells  $ml^{-1}$  for *Synechococcus* (except for a few days during the spring blooms) and  $0.5 \times 10^6$  to  $1 \times 10^6$  cells  $ml^{-1}$  for non-photosynthetic bacteria. The ambient concentration of CTC positive (active) bacteria in September 2000 was  $6 \times 10^4 \pm 3 \times 10^4$   $ml^{-1}$  ( $n = 32$ ).

### Particle size and type

Over the entire study period, the mean size of non-photosynthetic bacteria populations in the ambient water averaged 0.3  $\mu m$  ( $n = 140$  flow cytometer runs) and those of the *Prochlorococcus* and *Synechococcus* were 0.4 and 0.9  $\mu m$ , respectively. Eukaryotic algae were much larger with a mean FSC to 1  $\mu m$  beads ratio of  $7.4 \pm 3.3$ . Within each water sample, the populations showed a wide size distribution, reflected by the large CVs of the population means (average: 262, 173 and 206 % for the non-photosynthetic bacteria, *Prochlorococcus* and *Synechococcus*, respectively). As a result, the size distributions of the *Prochlorococcus* population within each water sample had typically >25 % overlap with the non-photosynthetic bacteria on the one hand (Fig. 2) and with the *Synechococcus* population on the other (Fig. 3). Similarly, the size of *Synechococcus* cells overlapped with both the *Prochlorococcus* from below and the eukaryotic algae from above, albeit to a lesser degree.

### Particle type retention

Particle retention by *Lithophaga simplex* varied according to particle type (repeated-measure ANOVA,  $F_{3,117} = 254$ ,  $p < 0.0001$ ; Fig. 3). *Synechococcus* cells

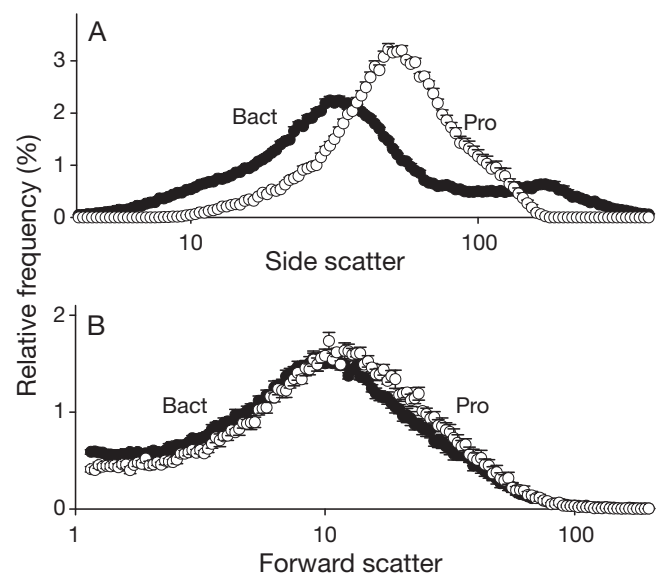


Fig. 2. Frequency distributions of the size and cellular properties of *Prochlorococcus* (Pro,  $\circ$ ) and non-photosynthetic bacteria (Bact,  $\bullet$ ) in 10 representative samples collected in September 1998 from the ambient water next to the studied specimens. (A) Side scatter is related primarily to cell texture and volume of the cell. (B) Forward scatter is related to cell size. Horizontal axes are plotted using arbitrary, log-scale units. Error bars: 95% confidence intervals of the means. Note the large size overlap between the 2 bacterial populations

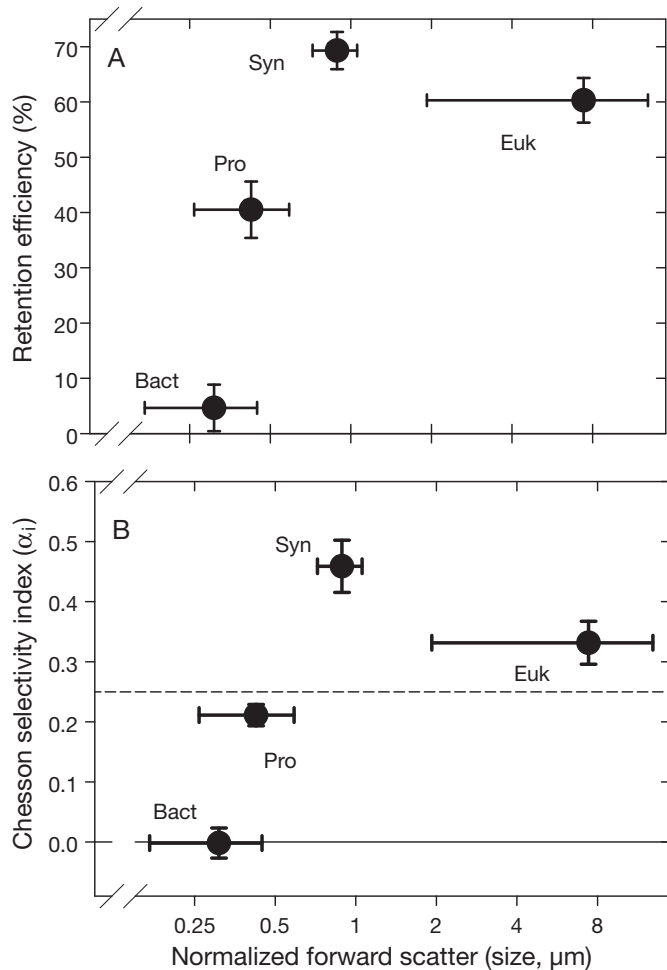


Fig. 3. *Lithophaga simplex*. (A) Average retention efficiency for each of the 4 particle types (Bact: non-photosynthetic bacteria; Euk: eukaryotic algae; Pro: *Prochlorococcus*; Syn: *Synechococcus*), plotted as a function of particle size. Vertical bars: 95% confidence intervals for mean retention efficiencies; horizontal bars: 90% prediction intervals for mean particle sizes. (B) Average selectivity index  $\alpha_i$  (Chesson 1978) calculated for all InEx pairs when data for all 4 particle types were available. The expected value for non-selective feeding was 0.25 (dashed line). Vertical bars: 95% confidence intervals; horizontal bars: as in Panel A. Note the logarithmic scale of the x-axis

( $0.9 \pm 0.1 \mu\text{m}$ ) were removed at efficiencies of up to 90% (average:  $69 \pm 14\%$ ; Fig. 3A). Larger eukaryotic algae (FSC:  $7.4 \pm 3.3$ ) were removed with significantly reduced efficiency ( $60 \pm 17\%$ , paired *t*-test,  $p < 0.001$ ), but comparison of selectivity indices indicated they were not significantly less preferred (Tukey HSD,  $p = 0.066$ ). The minute photosynthetic bacterium *Prochlorococcus* ( $0.4 \pm 0.1 \mu\text{m}$ ) was also readily captured ( $41 \pm 19\%$ ), but its removal efficiency was significantly lower (Tukey HSD,  $p < 0.001$ ) than that of the other 2 photosynthetic groups. Surprisingly, only a small proportion of the non-photosynthetic bacteria ( $0.3 \pm 0.1 \mu\text{m}$ ) were removed ( $5 \pm 19\%$ ) despite the large size

overlap between those bacteria and *Prochlorococcus* (Fig. 2B). When present,  $>20\%$  of the *Prochlorococcus* cells were removed in 88% of the InEx pairs (Fig. 4). In contrast, a removal of  $>20\%$  of non-photosynthetic bacteria was observed in only 7 of 74 cases, with zero removal in 25% of the cases (Fig. 4).

Despite the marked seasonal changes in particle abundance, the removal of photosynthetic particles remained nearly constant, resulting in a quasi-linear functional response (Fig. 5A–C), and the particle retention ranking did not change in the different sampling seasons (Fig. 5E). Seasonal variations in retention efficiencies were only evident for the eukaryotic algae (Kruskal-Wallis ANOVA,  $p < 0.05$ ), but without a clear pattern. Despite the low retention efficiency of the non-photosynthetic bacteria, their removal was also correlated with ambient (inhaled) concentrations ( $r_p = 0.58$ ,  $p < 0.001$ ), indicating that removal occurred mostly in higher than average bacterial concentrations ( $>5 \times 10^5 \text{ ml}^{-1}$ ; Fig. 5D).

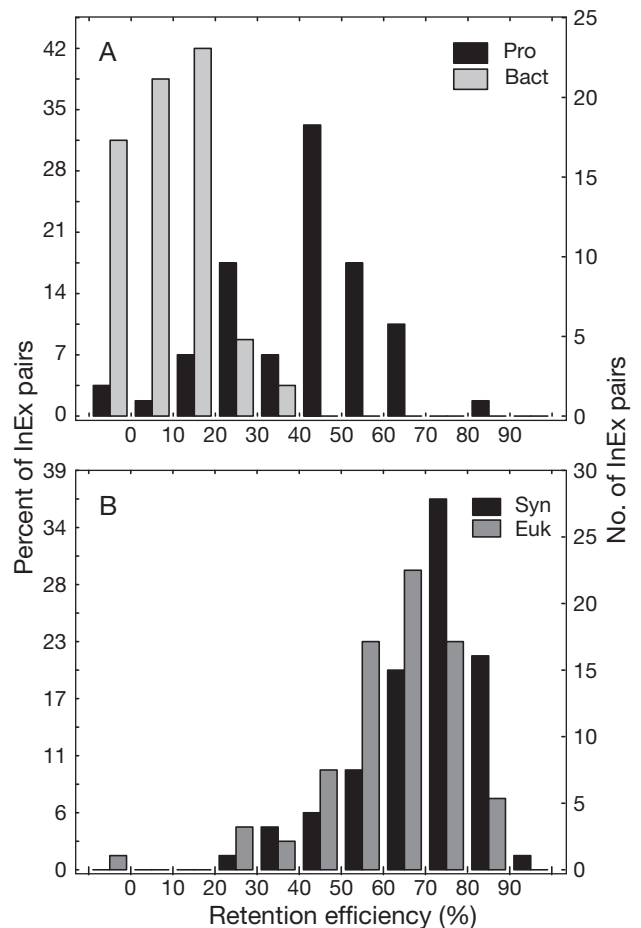


Fig. 4. Frequency of measured cell retention efficiencies for all 74 InEx pairs: (A) *Prochlorococcus* (Pro) and the non-photosynthetic bacteria (Bact) and (B) *Synechococcus* (Syn) and eukaryotic algae (Euk)

### Selective retention within particle types

Selective retention was also observed within particle types. For example, within the *Prochlorococcus* and eukaryotic algae, cells with higher pigment content and higher SSC were always preferentially retained. Similarly, within the more homogeneous *Synechococcus* population such selectivity was observed at certain times of the year, notably the spring bloom of 1997. Here *Lithophaga simplex* exhibited a clear preference

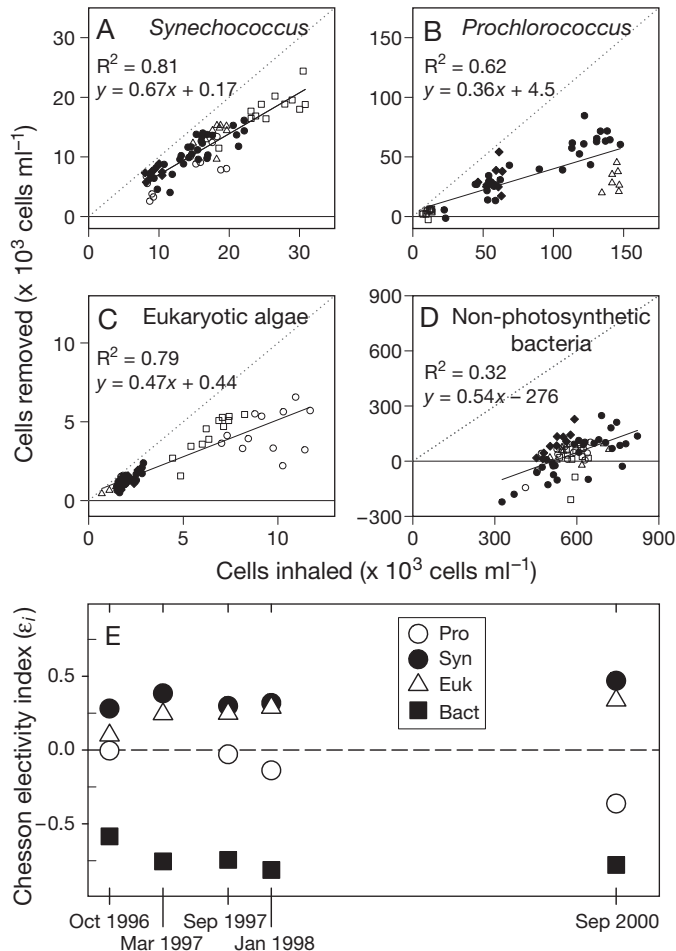


Fig. 5. Removal of each of the 4 ultra-planktonic particle types: (A) *Synechococcus* (Syn), (B) *Prochlorococcus* (Pro), (C) eukaryotic algae (Euk) and (D) non-photosynthetic bacteria (Bact), plotted against its ambient (inhaled) concentration. Dotted lines ( $x = y$ ) represent 100% removal. Different symbols denote different sampling periods.  $\blacklozenge$ : October 1996;  $\circ$ : March 1997;  $\bullet$ : September 1997;  $\square$ : January 1998;  $\triangle$ : September 2000. Linear regression statistics are for all seasons pooled. (E) Particle preference in the different sampling periods. Chesson  $\alpha_i$  values were rescaled to  $\epsilon_i$  so that they would be independent of the number of particle types available.  $\epsilon_i$  values range from  $-1$  to  $1$ , where  $-1$  indicates none of the  $i$ th type particles are retained, and an  $\epsilon_i$  of  $1$  indicates cases when the  $i$ th type particles are the only ones selected. Zero is the expected value for  $\epsilon$  if there is no selection. Error bars were omitted for clarity of presentation (Chesson 1983)

for *Synechococcus* cells with higher fluorescence (proxy of pigments and nitrogen content) and stronger SSC (proxy of cell texture and pigment content). Fig. 6 shows an example of a shift to the left in the optical properties distribution of the cells that were not re-

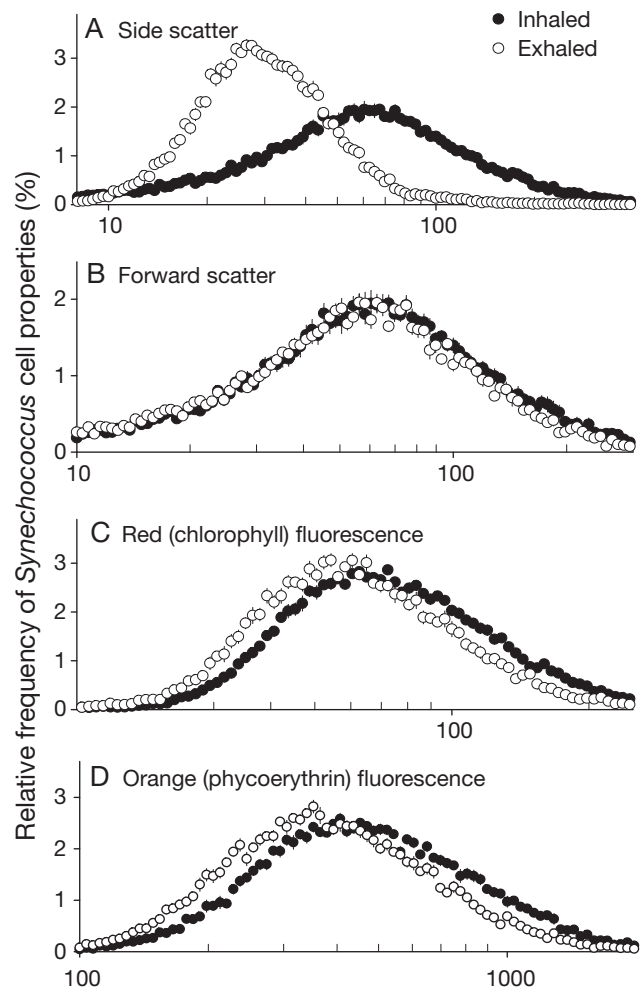


Fig. 6. An example of selective retention among a population of *Synechococcus* cells. The normalized frequency distributions of the optical cell properties (A to D) in the water inhaled ( $\bullet$ ) and exhaled ( $\circ$ ) by 12 *Lithophaga simplex* were plotted. InEx samples were collected during 2 d in the 1997 eukaryote spring bloom (18 and 25 March 1997). As each sample was internally normalized, similar relative frequencies (y position) do not indicate similar concentrations (exhaled concentrations were considerably lower). List mode data were transformed from log to linear, and the skewness (Sk) and kurtosis (Ku) were calculated separately for each sample before normalization. The difference in the location of the distribution (population mean) was highly significant in all cases (paired  $t$ -test,  $p < 0.001$ ). The shapes of the distributions were also significantly different for all but the phycoerythrin fluorescence distribution (Wilcoxon matched pairs test,  $p < 0.05$  for the comparison of both the Sk and Ku within each pair). Orange fluorescence is related to *Synechococcus* phycoerythrin content (a light-harvesting and nitrogen-storage pigment). Horizontal axes are plotted using arbitrary, log-scaled units. Error bars: 95% confidence intervals of the means



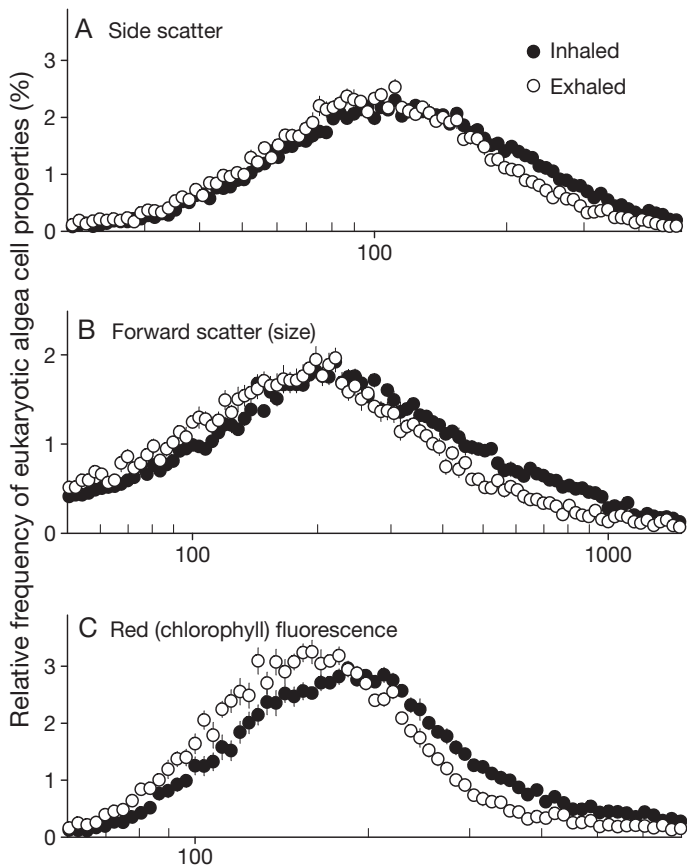


Fig. 7. As for Fig. 5A–C, but for eukaryotic algal (2 to 10 μm) cells. Since these cells normally do not contain phycoerythrin, the orange fluorescence signal was not plotted. The difference in the location of the distribution (population mean) was highly significant in all cases (paired *t*-test, *p* < 0.001). The shapes of the distributions were also significantly different for all but the side scatter distributions (Wilcoxon matched pairs test, *p* < 0.05)

tained by 12 *L. simplex* specimens. Selection for larger *Synechococcus* cells (shift to the left in the cells' FSC, a proxy of cell size) is much more muted in this example, but was evident in other seasons. Cell distributions were always peaked (*Ku* > 0), with a long right tail (*Sk* > 0) in both the inhaled and the exhaled waters, but the exhaled populations were significantly less skewed and less peaked (except for the red chlorophyll fluorescence). These results show that over and above the general preferential retention for *Synechococcus*, *L. simplex* preferentially retained higher quality *Synechococcus*. The same trend was also evident for the more diverse group of eukaryotic algae (Fig. 7).

The within-particle type preferences are summarized in Fig. 8, based on each of the cellular attributes recorded by the flow cytometer. Most notable is the preferential retention of cells with higher chlorophyll content within the populations of *Prochlorococcus* and eukaryotic algae (Wilcoxon matched pairs test, *p* < 0.001). *Lithophaga simplex* also preferentially retained larger *Synechococcus* and eukaryotic algae, whereas no such size preference was observed for the *Prochlorococcus* population. While *L. simplex* showed clear preference for *Synechococcus* cells with higher pigment content (e.g. Figs. 6 & 7) during some seasons (spring and summer 1997, autumn 2000), this was not the case during the autumn of 1996 and winter of 1998.

Interestingly, the small fraction (5%) of non-photosynthetic bacteria retained by *Lithophaga simplex* had significantly higher green fluorescence (nucleic acid content) in comparison to the nucleic acid content of the bacteria in the exhaled waters. A comparison between CTC positive and negative cells (Gasol et al. 1995) in 8 InEx pairs (September 2000) showed no significant difference (paired *t*-test, *p* > 0.05).

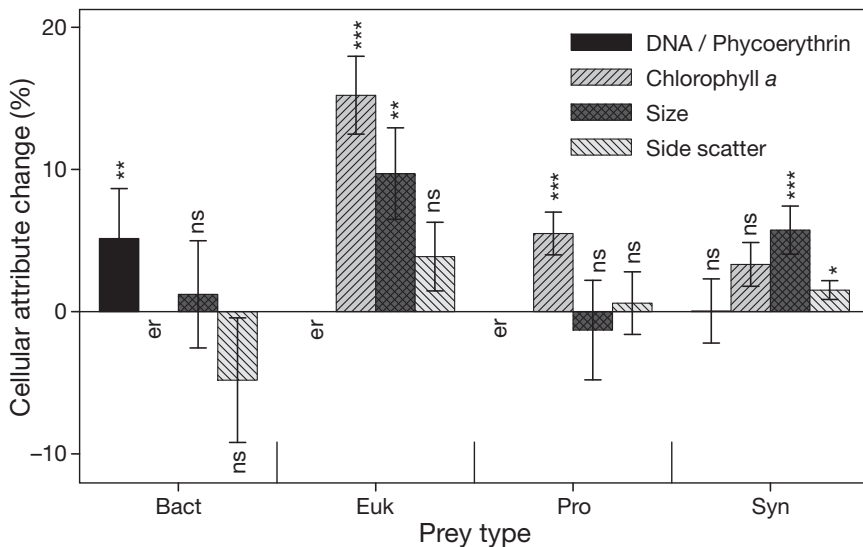


Fig. 8. Differences in cells' optical characteristics between inhaled and exhaled populations. Positive bars indicate positive selection for particles with higher cell attributes (fluorescence or scatter); negative bars indicate negative selection for the measured optical attribute of the cells. Each bar represents the average change calculated separately for each optical attribute in InEx pairs as:  $100 \times (A_{in} - A_{ex})A_{in}^{-1}$ , where *A* is the respective attribute. The significance of the difference between inhaled and exhaled population attributes was tested using Wilcoxon matched pairs test (ns: no significant difference; \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001). Attributes that were not relevant for the respective particle population are indicated as 'er'. Error bars: SE

## DISCUSSION

The present study demonstrates *in situ* the selective, size-independent capture of planktonic microorganisms by the tropical boring mytilid *Lithophaga simplex*. Use of the InEx technique ensured that only the first step in the feeding process was targeted, namely, particle capture and retention from the highly diluted medium in which these tropical bivalves reside. Such pre-capture qualitative selection is biologically interesting from several standpoints. (1) It has not yet been documented for any bivalve (but see Yahel et al. 2006 regarding size-independent selectivity in sponges). (2) It obviously raises the question of possible mechanisms. Mucus trapping of particles deflected onto the gill filament frontal surface has been assumed to be the norm for homorhabdic-gill bivalves (Silverman et al. 1999, Beninger et al. 2003, Beninger & Decottignies 2008), but this is obviously incongruent with the results observed for *L. simplex*. (3) It is at variance with theoretical predictions of little or no selection under conditions of low particle concentration (Sierszen & Frost 1992). Concerning the latter point, it may be argued that suspension feeders in oligotrophic habitats are adapted to a different scale of relative particle concentrations.

The pioneering studies by Møhlenberg & Riisgård (1978), Palmer & Williams (1980), Jørgensen et al. (1984) and Riisgård (1988) established that retention efficiencies by a variety of bivalves representing the main processing systems were size-dependant, with an asymptotic increase in retention efficiency with increasing size of the food particles. Such size-dependant asymptotic curves may be typical for many particle types, but there is persistent evidence of partially size-independent retention for certain particle types (Kjørboe & Møhlenberg 1981; for review see also Ward & Shumway 2004). Reports of size-independent retention are particularly intriguing (Newell et al. 1989, Bougrier et al. 1997), since capture and retention are thought to be purely mechanical or hydro-mechanical processes (Jørgensen 1981, Beninger et al. 1992, 2003, Nielsen et al. 1993, Silverman et al. 1996b, 1999, Ward et al. 1998b, Beninger & Decottignies 2008, present paper).

To date, all studies reporting size-independent retention have used either cultured algae in laboratory experiments, or natural algal assemblages in laboratory experiments. Overwhelmingly, the majority of bivalve and algal species investigated have come from temperate, eutrophic, or mesotrophic habitats. Size-independent retention of phytoplankton has been reported for some bivalves feeding on  $>2 \mu\text{m}$  particles in temperate zones and conjectured to be due to differences in shape, flexibility, or motility (reviewed by Ward & Shumway 2004). Ward & MacDonald (1996) studied 2 sub-tropical bivalves in Bermuda (*Arca zebra*,

*Arciidae* and *Pinctada imbricata*, Pteriidae). In the laboratory, both species showed typical size-dependant, pre-ingestive feeding responses, with an asymptotic increase in retention efficiency with increasing size of the food particles. *A. zebra*, but not *P. imbricata*, demonstrated particle selection, rejecting (in the pseudofeces) material with significantly higher carbon and lower nitrogen concentrations, thereby increasing the quality of material ingested by approximately 31%. Qualitative selection in the tropical pearl oyster *P. margaritifera* has also been inferred from gut content analysis (Loret et al. 2000); however, the techniques of both studies do not allow the distinction between pre- and post-capture selection.

The selective pattern observed for *Lithophaga simplex*, whereby pico-planktonic cells of similar size are retained in different efficiencies, argues for qualitative rather than mechanistic selection, presumably an adaptation for enhancement of the quality of the retained particles. For example, the photosynthetic bacterium *Prochlorococcus* was efficiently removed (up to 88%), whereas non-photosynthetic bacteria that were essentially of the same size and shape were retained in null to very low efficiencies. The majority (90 to 95%) of the non-photosynthetic bacteria in the oligotrophic waters at our study site were CTC negative, indicating dead, inactive, starved, or growth-arrested cells (see Davidson et al. 2004 for a review and contrasting data). Such bacteria are less preferred by protozoan grazers (del-Giorgio et al. 1996, Jürgens & Matz 2002).

Retention efficiencies and Chesson selectivity indices were significantly different among all 4 prey taxa examined (non-photosynthetic bacteria, *Prochlorococcus*, *Synechococcus* and eukaryotic algae) despite the large overlap of the cell size distribution (typically  $>25\%$ ; Fig. 2, and compare Figs. 6B & 7B).

Within each of the photosynthetic cell populations, there was a general pattern of preferential retention of cells with higher chlorophyll content. Similarly, the small fraction of non-photosynthetic bacteria that was retained by the bivalve had a significantly higher nucleic acid content, compared to the rejected bacteria. These selectivity patterns prevailed regardless of large seasonal shifts in the planktonic community composition and abundance. The most notable exception was the reduced retention efficiency of *Prochlorococcus* in September 2000 (Fig. 5B,E).

It should be noted that, while only a small portion of the non-photosynthetic bacteria was retained by *Lithophaga simplex*, other homorhabdic bivalves such as *Geukensia demissa* and *Dreissena polymorpha* efficiently retained such small cells (Kreeger & Newell 1996, 2001, Silverman et al. 1996a). Nevertheless, assimilation of bacteria seems to be less efficient in comparison to phytoplankton (Kreeger & Newell 1996,

Nichols & Garling 2000). Microscopic observation using nucleic acid staining (DAPI) reveals that, while most of the picoplankton was freely suspended (unattached), some bacteria were attached to aggregates, mucus, or marine snow (G. Yahel unpubl. data). It is therefore plausible that the small proportion of non-photosynthetic bacteria removed by *L. simplex* may have been ingested with such aggregates (Kach & Ward 2008). Indeed, most of the bacteria removal occurred during spring blooms, when organic aggregates were prevalent in reef waters (G. Yahel pers. obs.).

Preference for cyanobacteria over diatoms (all >3 µm) has been reported for several freshwater unionids (Baker & Levinton 2000, 2003). However, in these cases, clear size differences existed between the different particle types, and selection was post-retention. The preferential retention of *Synechococcus* observed in the present study has also been observed in other coral reef suspension feeders examined using the InEx technique (Yahel et al. 2005, G. Yahel unpubl. data). The concentration of *Synechococcus* pigments was significantly reduced in comparison with other algal pigments in the water flowing over the reefs in Curacao, indicating its selective grazing (van Duyl et al. 2002). This contrasts with the situation observed for the pelagic habitat in the Gulf of Aqaba, where *Synechococcus* was shown to be the least-preferred cell type (Sommer et al. 2002). As the phytoplankton supply to the studied reef is mostly affected by pelagic processes (Genin et al. 2009), the seston available to reef benthic suspension feeders such as *L. simplex* may be impoverished of cells other than *Synechococcus*; thus, these benthic suspension feeders may have evolved to accept, or even select, these cyanobacteria.

Capture and retention of particles are generally considered to be purely mechanical or hydro-mechanical processes (Jørgensen 1981, Beninger et al. 1992, 2003, Nielsen et al. 1993, Silverman et al. 1996a,b, 1999, Ward et al. 1998b, Beninger & Decottignies 2008). This view is at variance with the observations of size-independent retention reported here. There is at present no theoretical or practical framework for understanding differential retention of particles of similar sizes, shapes, flexibilities and densities. The most prominent shift between the inhaled and exhaled populations (Fig. 6) is observed in the SSC, which is generally associated with internal complexity and external cell surface traits. It is thus tempting to suggest that selection is made according to external cell characteristics that are revealed by differences in SSC. However, preference for higher pigment content (see also Fig. 8) suggests that other mechanisms such as chemosensory detection may also be at work (Beninger et al. 2008a). Shimeta (1993) and Shimeta & Koehl (1997) suggested that the clearance of sub-micrometer par-

ticles should be greatly enhanced by cell motility. Indeed, swimming behaviour has been reported in several cultured *Synechococcus* strains (Brahamsha 1999), and these are the particles that were most efficiently retained in the present study. Motility has not been documented for *Prochlorococcus* cells, which were much less efficiently retained in the present study. Differences in cell motility may thus be a factor in size-independent particle retention.

Another feature that might help account for size-independent particle retention is some form of particle recognition prior to capture. Such recognition has been reported post-capture in 2 marine bivalve species for the perfrustular envelope of diatoms (Beninger et al. 2004, 2005, 2008a,b). This would be an interesting subject for future research on the mechanisms of pre-capture, size-independent particle retention.

If the preferential retention of *Synechococcus* is related to quality (e.g. Ward et al. 1997), it is of interest to determine what quality features may be discriminating. High-energy, low N and P coral mucus, rather than particles, was suggested as an important source of carbon for *Lithophaga simplex* (Shafir & Loya 1983). If this is so, then we suggest that the pre-capture qualitative selectivity observed in *L. simplex* may not maximize carbon or energy gain, but rather other nutrients, as has been observed in some freshwater bivalves (Nichols & Garling 2000) and oysters (Ward et al. 1997).

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