Retention efficiencies of the coral reef sponges *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* determined by Coulter counter and plate culture analysis

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Abstract
Sponges are the dominant organisms on many coral reefs and through feeding they may greatly reduce the concentration of suspended food particles. Retention efficiencies of the tubular sponges *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* were examined on a coral reef located in the Florida Keys. Replicate ambient and exhalant water samples were collected in situ from individuals of each species and analysed using two methods. Retention efficiencies of suspended particles (0.75–18 μm) examined using Coulter counter analysis were similar among the three sponge species, averaging 86%. For all sponges, particle retention decreased as particle size increased from 0.7 to 18 μm. Water samples plated on to Marine Agar produced 54 microbial types. Retention efficiencies of culturable microbes were similar among the three species, averaging 82%. This study suggests that the coral reef sponges *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* play an important role in the transfer of energy between the pelagic and benthic environments.

Key words: *Feeding, microbes, retention, sponges*

Introduction

Sponges are common macro-invertebrates on many coral reefs and may dominate the benthic community in the Caribbean and Florida Keys (Schmahl 1990; Wilkinson & Cheshire 1990). Most coral reef sponges in the western Atlantic are heterotrophic feeders (Wilkinson & Cheshire 1990), filtering suspended particulate matter such as bacteria and microalgae. Sponges are capable of filtering large volumes of water and thus may remove a significant amount of suspended food (Reiswig 1971; Pile et al. 1996; Ribes et al. 1999). Sponges capture food particles by pumping seawater into their internal canal system. Water entering through incurrent pores or ostia passes through diverging incurrent canals into flagellated choanocyte chambers, which drive the water current. Water exits the sponge through excurrent pores, or oscula. Food particles, depending on their size, are either captured by choanocytes or engulfed through phagocytosis by pinacocytes lining the incurrent canals (Reiswig 1971; Weissenfels 1992).

Sponge feeding studies have often compared particle type and concentration found in inhalant (ambient) water with that present in exhalant water, with particle retention determined using a variety of techniques. For example, direct microscopic analysis and chemical analysis were used by Reiswig (1971) to investigate particle feeding of sponges common on Jamaican coral reefs. Microscopic analysis allowed particles to be categorized to size and morphology. The effect of particle size on sponge feeding can also be examined using Coulter counter analysis (e.g. Stuart & Klumpp 1984; Duckworth et al. 2003; Duckworth & Pomponi 2005). Flow cytometric analysis separates individual populations (e.g. heterotrophic bacteria) from the microbial community and can thus help determine the influence of particle type on sponge feeding (e.g. Pile et al. 1996; Ribes et al. 1999). The seston community can be further broken down into individual species using plate

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culture analysis, whereby inhalant and exhalant water samples are plated on to a culture medium such as Marine Agar. The culturable microbes can then be sequenced and identified.

Sponge retention patterns were investigated in this study using the Demospongiae *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis*. All three species are common on coral reefs in the Caribbean, Bahamas and Florida Keys (Humann 1992) and have a tubular morphology, with most individuals consisting of two to five tubes about 30 cm long. Because of their abundance and size, all three sponge species may play an important role in the transfer of energy from the pelagic to benthic communities. For each sponge species, particle retention was examined using Coulter counter and plate culture analysis. The aims of this study were to examine particle retention patterns among sponges and to compare the two analytical methods for investigating sponge feeding.

Methods

Sampling regime

Four sponge replicates each of *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* were sampled on a coral reef located off Long Key (24°45′N 80°47′W), Florida Keys, in June 2003. The sample area was approximately 10×10 m in size, situated at a depth of 20 m, so that all sponges experienced similar environmental conditions. In addition, all sponges were sampled during one dive, thus avoiding any possible temporal variation among water samples. All sponges used in this study were larger than 30 cm in height, free of macro-epibionts and were actively pumping water, determined by observing the disruption of suspended sediments as they passed over the oscula. To increase the accuracy of particle counts, three ambient and three exhalent water samples were collected from each sponge. Each water sample was approximately 5 ml in volume, collected using a syringe. The ambient samples were taken approximately 20 cm away from and up-current of each individual, approximately midway up its height. The exhalant samples were taken approximately 5 cm into the sponge osculum, and were carefully drawn at a slow rate of 1 ml s^{-1}. Considering that water samples were drawn at a rate lower than water is expelled from coral reef sponges (Reiswig 1974) and that all three sponge species in this study have large atria, it is unlikely that exhalant water samples were contaminated with ambient water (Yahel et al. 2005). All three sponge species have chimney-like tubes with the oscula diameter of sufficient width to allow the penetration of a syringe without it touching the osculum wall, thus particle "production" is also unlikely (Yahel et al. 2005). For each sponge, all water samples were collected within 2 min, with the syringe immediately capped after use. Using the ambient and exhalant water samples, sponge retention was examined using two distinct methods.

Sampling analysis

For Coulter counter analysis, a 1.5 ml subsample was placed into a 2 ml vial and fixed with 0.2 ml of 5% formalin. The samples were analysed on a Multisizer 3 Coulter Counter (Beckman) using a 30 μm aperture tube, with particles broken down into three size classes: 0.75–2, 2–5 and 5–18 μm. Particles <0.75 or >18 μm could not be accurately counted. The Coulter counter does not differentiate between organic and inorganic particles.

For plate culture analysis, 0.1 ml of each water sample was spread onto the surface of Difco™ Marine Agar 2216 (Becton Dickinson, 212185) plates within 6 h after collection and cultured at 25°C in the dark. After 6 days, the colony number per plate was counted. Each distinct colony type was then isolated and plated to obtain pure cultures. Pure cultures with colonies with like appearance (pigmentation, morphology, opticity, margin and surface structure) were counted as the same organism. Cell size and motility were determined by growing a pure culture in Marine Broth 2216 (Difco, 079-01-2) and microscopic analysis. The Gram reaction of each microbe was determined using a standard method: air drying of the test sample, staining with crystal violet (1 min), followed by iodine (1 min), destaining with acetone and counter-staining with safranin (1 min). In addition, each culture was identified using sequencing with DNA extracted using a standard phenol:chloroform protocol. Polymerase chain reaction using universal bacterial primers Ecoli9 (16S Eco9.for: 5′-GAG TTT GAT CCT GGC TCA G-3′) and Loop 27 (16S Lop.rev: 5′-GAC TAC CAG GGT ATC TAA TC-3′) amplified ~750 bp of the 16S small subunit rRNA gene region as described by Sfanos et al. (2005). Positive polymerase chain reactions were shipped overnight to Northwoods DNA (Solway, Minnesota, USA) for sequencing before being proofread (Chromas DNA v 1.45, Griffith University, Southport, Queensland, Australia) and queried with NCBI BLAST (Basic Local Alignment Search Tool) v 2.2 (www.ncbi.nlm.nih.gov/blast/). The plate culture analysis used in this study only detected heterotrophic bacteria.

Data analysis

All results from each method were standardized to 1 ml for interpretational and statistical comparisons. For each method, replicate water samples were averaged per sponge to obtain an average ambient and exhalant concentration. The percentage retention for each group, such as size class or colony number, was determined by the formula:

$$\% \text{ retention} = [(\text{Conc}_{amb} - \text{Conc}_{exh})\ \text{Conc}_{amb}^{-1}] \times 100$$

where Conc_{amb} is the mean ambient concentration and Conc_{exh} is the mean exhalant concentration (Pile et al. 1996). For the Coulter counter data, repeated measures ANOVA was used to statistically analyse differences in percentage retention among the sponge species (between factor) and the three size classes (within factor). For the plate culture data, a one-way ANOVA examined differences in percentage retention among the three sponge species. For both methods, the Modified Levene Equal Variance test determined that the data had similar variances ($P > 0.05$), thus no transformation was required for the analyses. To examine retention differences for each microbe characteristic (e.g. motility), chi-squared tests were used to compare exhalant ("actual data") with ambient ("expected data") proportions. The exhalant data for each species was pooled to increase the sample number for each characteristic. Analyses were performed using the Number Cruncher Statistical System (NCSS).

Results

Coulter counter analysis

The ambient concentration of suspended (organic and inorganic) particles 0.75–18 μm in size averaged 220,450 particles ml^{-1}. The retention of particles 0.75–18 μm in size was similar between the three species ($F_{df(2,27)} = 1.19$, $P = 0.319$), averaging 86% (Figure 1). The power of this analysis was low (0.24), however, indicating the possible occurrence of a type II error. This probably resulted from the low number of sponge replicates per species. The percentage retention varied significantly among the three size classes ($F_{df(2,27)} = 28.50$, $P < 0.001$) (Figure 1). Overall, the percentage retention of 0.75–2, 2–5 and 5–18 μm particles was 87, 64 and 41%, respectively. There was no significant interaction effect ($F_{df(4,27)} = 1.02$, $P = 0.414$).

Plate counting analysis

After 6 days, the mean ambient number of culturable microbes was 1350 colonies ml^{-1}. Retention efficiencies of culturable microbes were similar among the three species ($F_{df(2,9)} = 0.15$, $P = 0.864$), averaging 82% (Figure 2). The power of this analysis was also low (0.17). Fifty-four microbial types were isolated from the Marine Agar culture, with 46 positively matched to Genbank (Table I). Several strains could only be sequenced and identified to a high taxonomic level such as family or order (e.g. Bacteroidetes). The majority of culturable microbes were < 2 μm in size and most were Gram negative and motile (Table I). Of the 54 culturable microbes, 42 types were detected in ambient water samples, whereas the remaining 12 types were only isolated from sponge exhalant water. Only microbe types found in ambient water were used to examine the influence of microbial characteristics on retention

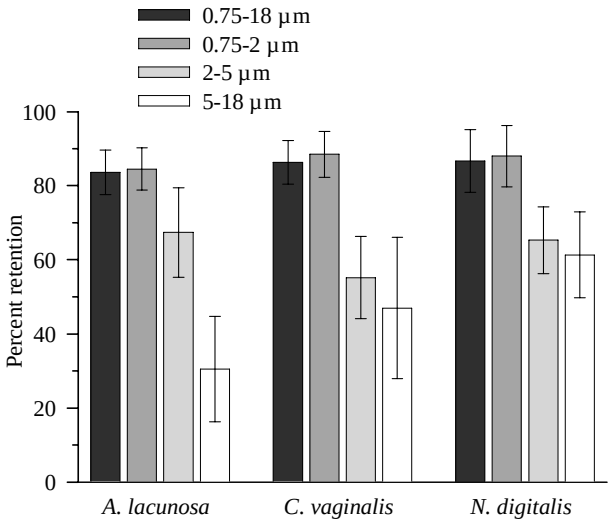

Figure 1. Percentage retention of suspended particles by *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* using Coulter counter analysis and broken down into size classes. Error bars represent 95% confidence limits between sponge replicates.

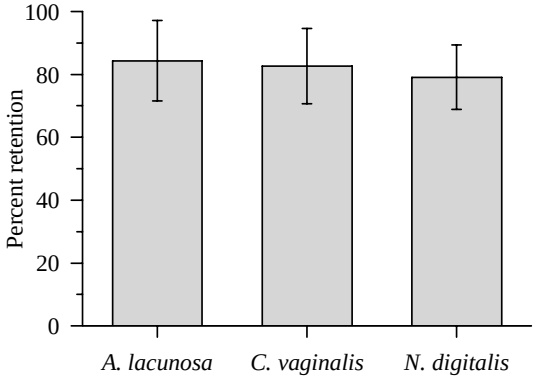

Figure 2. Percentage retention of suspended particles by *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* using plate culture analysis. Error bars represent 95% confidence limits between sponge replicates.

Table I. List of the culturable microbes and their characteristics present (×) in the ambient water and exhalant waters of each sponge species. Microbe size is grouped into three classes, similar to the Coulter counter analysis: * <2 μm; ** 2–5 μm; *** 5–18 μm.

Microbial classification	Size	Gram	Motile	Ambient	*Aplysina lacunosa*	*Callyspongia vaginalis*	*Niphates digitalis*
Alteromonas spp.	*	−	Yes	×			
Bacillus cereus	*	+	Yes		×		
Bacillus macroides	*	+	No				×
Bacillus marisflavi	*	+	Yes		×		
Bacillus pumilus	*	+	Yes	×			
Bacteroidetes	*	−	No	×	×	×	×
Beijerinckiaceae	*	−	Yes	×			×
Cytophaga spp.	**	−	Yes	×	×		
Dietzia spp.	**	+	No	×			
Ertythrobacter citreus	*	−	Yes	×			
Flavobacteriaceae	*	+	No	×			
Flexibacter aggregans	**	−	Yes	×			
Flexibacter echinicida	**	−	Yes	×			
Flexibacter spp.	**	−	Yes	×		×	
Flexibacteriaceae	**	−	Yes	×		×	
Gammaproteobacterium	*	−	Yes	×			
Gordona terrae	*	+	No	×			
Klebsiella spp.	*	−	No			×	
Lysobacter spp.	*	−	Yes	×	×		
Marinomonas protea	*	−	Yes	×			
Methylarcula spp.	*	+	Yes		×		×
Microbacteium spp.	*	+	No				×
Microscilla arenaria	***	−	Yes	×	×		
Microscilla spp.	***	−	Yes	×			×
Photobacterium damselae	*	−	Yes	×			
Photobacterium leiognathi	*	−	Yes	×			
Photobacterium spp.	*	−	Yes	×		×	
Phyllobacteriaceae	*	−	Yes		×		×
Porphyrobacter spp.	*	−	Yes		×		
Pseudoalteromonas spp.	*	−	Yes	×			×
Pseudomonas spp.	**	−	Yes		×		
Psychrobacter submarinus	*	−	No				×
Rhizobiales	*	−	Yes	×			
Rhodobacter spp.	*	−	Yes	×		×	
Roseobacter spp.	*	−	Yes	×	×		
Sphingobacteria	*	+	Yes	×	×	×	
Sphingomonas spp.	*	+	Yes	×		×	
Staphylococcus warneri	*	+	No	×			×
Stappia spp.	*	−	Yes			×	
Sulfitobacter dubium	*	−	No	×	×	×	
Vibrio agarivorans	*	−	Yes	×			×
Vibrio campbelli	*	−	Yes	×			
Vibrio carchiariae	*	−	Yes	×			
Vibrio nigripulchritudo	*	−	Yes	×		×	×
Vibrio spp.	*	−	No	×		×	×
Vibrio tubiashi	*	−	No	×	×		×
Unidentified 1	*	+	No	×			×
Unidentified 2	*	+	Yes	×	×		
Unidentified 3	*	+	No	×			
Unidentified 4	*	+	No	×			
Unidentified 5	*	+	No			×	
Unidentified 6	*	+	No	×	×		
Unidentified 7	*	+	No	×	×		
Unidentified 8	*	+	No	×			

rates. Chi-squared tests determined that microbe retention was unaffected by microbe size ($\chi^2_{df(2)} = 0.909$, $P = 0.635$), Gram stain ($\chi^2_{df(1)} = 0.187$, $P = 0.665$) or motility ($\chi^2_{df(1)} = 0.00$, $P = 1.00$).

Discussion

The coral reef sponges, *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis*, have a high retention for small suspended particles, generally

>80%. This finding supports other studies that have determined that sponges have retention efficiencies ranging from 70 to 99% for small suspended particles (Reiswig 1971; Pile et al. 1996, 1997; Ribes et al. 1999) and thus indicates that sponge feeding represents an important coupling between the benthic and pelagic communities. The retention efficiency of *Callyspongia vaginalis* has been examined previously. Using flow cytometry, Pile (1999) found that *Callyspongia vaginalis* retained *Synechococcus*-type cyanobacteria with 90% efficiency, whereas heterotrophic bacteria, *Prochlorococcus*, and picoeucaryotes were poorly retained. The low overall retention rates of *Callyspongia vaginalis* may have resulted from the relatively low ambient concentration (Pile 1999). In comparison, the retention of prokaryotic microbes such as heterotrophic bacteria by all three sponge species in this study was high ($\geq$ 80%). In addition, similar particle retention between the two methods indicates that all three species retained culturable microbes at similar rates to all suspended particles 0.75–18 μm in size. These findings suggest that *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* were generally unselective feeders for a given particle size. *Prochlorococcus* cells are $\leq$0.7 μm (Partensky et al. 1999) and too small to be detected using the Coulter counter.

Although retention efficiencies were high overall for *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis*, approximately 15% of the available seston was not retained. Particle size is clearly an important factor, as the results of the Coulter counter analysis determined that particle retention decreased as particle size increased. Similar retention rates among particle sizes from the plate culture analysis probably resulted from analysing presence/absence data (Table I) and not actual counts. The highest retention efficiency for small food particles is typical for many sponge species (Stuart & Klumpp 1984; Ribes et al. 1999; Duckworth et al. 2003) and it probably results from the different mechanisms of particle capture. Particles <5 μm are generally captured by choanocytes, whereas larger particles are primarily engulfed through phagocytosis by pinacocytes lining the incurrent canals (Reiswig 1971; Weissenfels 1992). Particle type and digestibility may also be important factors influencing retention rates for some sponges. Reiswig (1971), for example, found that three Jamaican coral reef sponges had higher retention efficiencies for bacteria and unarmoured cells than for armoured cells (e.g. diatoms), possibly because the latter cell type is more difficult to digest. Most armoured cells are also relatively large (>5 μm), which would further reduce retention rates. The results of the plate culture analysis in this study determined that neither bacterial motility nor Gram reaction affected their retention by the three sponge species. In general, particle type did not appear to influence the retention efficiencies of *Aplysina lacunosa*, *Callyspongia vaginalis* and *Niphates digitalis* in this study.

Overall retention rates may also be lowered by the production of faecal products (Stuart & Klumpp 1984) or cells expelled by the sponge. In separate studies, Pile (1997) and Pile et al. (1997) found that the exhalant waters of some sponges contained significantly more picoeukarytes than present in ambient water, possibly because they were expelled after living in the mucous layer lining the sponge's aquiferous system. The results of the plate culture analysis in this study identified 12 microbes present in exhalant water that were not detected in the ambient water. Because sponges often contain large symbiotic microbial populations (Reiswig 1981) it is possible that some of these microbes are expelled periodically by sponges. Many of the microbial species detected in ambient water were also found in the exhalant water (Table I), indicating that microbes can pass through the sponges without being captured. It is therefore possible that the 12 microbial species were uncommon in ambient water and simply not detected.

Clones and/or isolates belonging to the α, β, γ and δ subdivisions of the *Proteobacteria* are common from marine pelagic microbial communities (Ravenschlag et al. 2001; Ansede et al. 2002). Similar results were found in this study, with a strong bias towards the α and γ subdivisions of the *Proteobacteria* containing commonly isolated "marine" bacterial species. Molecular techniques have made it increasingly clear that a large proportion of bacterial diversity in natural habitats is non-culturable and therefore unexplored. Thus, a large number of molecular techniques have been employed to assess the bacterial diversity independent of cultural methods (Amann et al. 1995). These studies revealed that 99% or more of the bacterial diversity remains uncultured and unexplored. However, the use of molecular techniques is limited, as it does not give all the phenotypic and functional information and therefore was not employed in this study.

The retention efficiencies of the three sponges were similar between the two analytical methods. Each method, however, examined the water samples differently, thus each provided a different perspective on sponge feeding. The Coulter counter method allowed the seston to be broken down into various size classes, so the influence of particle size on retention efficiency could be determined. However, a limitation of this method is that is does not discriminate between organic and non-organic

particles. The plate culture method provided identification and information of the culturable microbes present in sponge inhalant and exhalant waters. Because plating is a simple technique, it may be suitable for researchers in developing countries and remote locations.

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References

Amann RL, Ludwig W, Schleifei K. 1995. Phylogenetic index and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59:143–69.

Ansede JH, Friedman R, Yoch DC. 2001. Phylogenetic analysis of culturable dimethyl sulfide-producing bacteria from a *Spartina*-dominated salt marsh and estuarine water. Applied Environmental Microbiology 67:1210–7.

Duckworth AR, Pomponi SA. 2005. Relative importance of bacteria, microalgae and yeast for growth of the sponge *Halichondria melanadocia* (De Laubenfels, 1936): a laboratory study. Journal of Experimental Marine Biology and Ecology 323:151–9.

Duckworth AR, Samples GA, Wright AE, Pomponi SA. 2003. In vitro culture of the tropical sponge *Axinella corrugata* (Demospongiae): effect of food cell concentration on growth, clearance rate and biosynthesis of stevensine. Marine Biotechnology 5:519–27.

Humann P. 1992. Reef Creature Identification. Jacksonville: New World Publications.

Partensky F, Hess WR, Vaulot D. 1999. *Prochlorococcus*, a marine photosynthetic prokaryote of global significance. Microbiology and Molecular Biology Reviews 63:106–27.

Pile AJ. 1997. Finding Reiswig's missing carbon: quantification of sponge feeding using dual-beam flow cytometry. Proceedings of the 8th International Coral Reef Symposium 2:1403–10.

Pile AJ. 1999. Resource partitioning by Caribbean coral reef sponges: is there enough food for everyone? Memoirs of the Queensland Museum 44:457–61.

Pile AJ, Patterson MR, Savarese M, Chernykh VI, Fialkov VA. 1997. Trophic effects of sponge feeding within Lake Baikal's littoral zone. 2. Sponge abundance, diet, feeding efficiency, and carbon flux. Limnology and Oceanography 42:178–84.

Pile AJ, Patterson MR, Witman JD. 1996. In situ grazing on plankton <10 μm by the boreal sponge *Mycale lingua*. Marine Ecology Progress Series 141:95–102.

Ravenschlag K, Sahm K, Amann R. 2001. Quantitative molecular analysis of the microbial community in marine arctic sediments (Svalbard). Applied Environmental Microbiology 67:387–95.

Reiswig HM. 1971. Particle feeding in natural populations of three marine Demosponges. Biological Bulletin 141:568–91.

Reiswig HM. 1974. Water transport, respiration and energetics of three tropical marine sponges. Journal of Experimental Marine Biology and Ecology 14:231–49.

Reiswig HM. 1981. Partial carbon and energy budgets of the bacteriosponge *Verongia fistularis* (Porifera: Demospongiae) in Barbados. Marine Ecology 2:273–93.

Ribes M, Coma R, Gili JM. 1999. Natural diet and grazing rate of the temperate sponge *Dysidea avara* (Demospongiae, Dendroceratida) throughout an annual cycle. Marine Ecology Progress Series 176:179–90.

Schmahl GP. 1990. Community structure and ecology of sponges associated with four southern Florida coral reefs. In: Rützler K, editor. New Perspectives in Sponge Biology. Washington: Smithsonian Institution Press. p 376–83.

Sfanos K, Harmody D, Dang P, Ledger A, Pomponi S, McCarthy P, Lopez J. 2005. A molecular systematic survey of cultured microbial associates of deep-water marine invertebrates. Systematic and Applied Microbiology 28:242–64.

Stuart V, Klumpp DW. 1984. Evidence for food-resource partitioning by kelp-bed filter feeders. Marine Ecology Progress Series 16:27–37.

Weissenfels N. 1992. The filtration apparatus for food collection in freshwater sponges (Porifera, Spongillidae). Zoomorphology 112:51–5.

Wilkinson CR, Cheshire AC. 1990. Comparisons of sponge populations across the Barrier Reefs of Australia and Belize: evidence for higher productivity in the Caribbean. Marine Ecology Progress Series 67:285–94.

Yahel G, Marie D, Genin A. 2005. InEx — a direct in situ method to measure filtration rates, nutrition, and metabolism of active suspension feeders. Limnology and Oceanography Methods 3:46–58.