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InEx—a direct in situ method to measure filtration rates, nutrition, and metabolism of active suspension feeders

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Abstract

Sponges, bivalves, and tunicates play an important role in the trophic dynamics of many benthic communities. However, direct in situ measurements of their diet composition, filtration, and excretion rates are lacking for most species, and knowledge of these rates is based mostly on indirect, in vitro measurements. This paper presents and evaluates an in situ, nonintrusive technique of direct measurement of the rate and efficiency by which an active suspension feeder removes (or discharges) substances from (to) the water it filters. The technique, termed "InEx," is based on the simultaneous, pair-wise collection of the water inhaled and exhaled by the animal. It was specifically adapted to allow reliable sampling of common, small suspension feeders with excurrent aperture as small as 2 mm. The difference in concentrations of a certain substance between a pair of samples provides a measure of the retention (or production) of the substance by the animal. Calculations of feeding (or production) rates are obtained through multiplying the concentration difference by the pumping rate. The latter is concurrently measured by recording the movement of a dye front in a transparent tube positioned within the excurrent jet. An important quality of the InEx technique is that it does not manipulate the studied organisms and thus allows realistic estimates of the organism's performance under natural conditions. Preliminary results showing the diet composition, feeding rates, and removal efficiencies of some coral reef sponges, bivalves, and tunicates are presented and discussed.

The paramount role of active suspension feeders in benthic food webs (Gili and Coma 1998), as well as their commercial value (Bayne 1998), has inspired a host of studies concerning their diet composition, food acquisition mechanisms, feeding rates, and water transport (reviewed by Jørgensen 1966; Bayne et al. 1976; Bayne and Newell 1983; Levinton et al. 1996; Bayne 1998; Gili and Coma 1998; Iglesias et al. 1998; Riisgård and Larsen 2000; Riisgård 2001*a*). Our current understanding of the nutritional ecology and physiology of active suspension feeders is based, by and large, on studies conducted in closed vessels (Ribes et al. 2000; Riisgård 2001*a*; Petersen et al. 2004), using robust taxa adapted to the highly dynamic, turbid, and productive environments of temperate and boreal coastal waters (Hawkins et al. 1998). As such, these taxa are "pre-adapted" to laboratory conditions.

The vast majority of these closed vessel studies (reviewed by Jørgensen 1966; Wildish and Kristmanson 1997; Riisgård 2001*a*), have relied on indirect laboratory measurements (*see* Jørgensen 1966; Petersen et al. 2004), where removal/production rates are commonly deduced from concentration shifts of markers such as algal cells, colored beads, radioactive label, or excretion products in the vessels containing the experimental organisms. Since indirect methods cannot differentiate pumping rates and retention efficiency, both parameters are indistinguishably combined and reported as "clearance rate," that is, the volume of water cleared (100% removal) of suspended particles per unit of time (Jørgensen 1966; Riisgård 2001*a*). Similar indirect incubation methods have also been applied in the field (e.g., Frost and Elias 1985) for the measurements of sus-

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pension feeders' feeding rates. Hopkinson et al. (1991) used in situ bell jars to study the metabolism and nutrient fluxes of benthic organisms. More recently, Ribes et al. (1998, 1999, 2000) introduced a recirculating bell jar system. This indirect technique uses natural seawater under natural temperature and illumination and considerably minimizes disturbance to the studied animals. Bio-deposition methods (reviewed by Iglesias et al. 1998; Petersen et al. 2004) estimate intake rates by comparing the inorganic (and thus indigestible) content of seston with the inorganic content of bivalve bio-deposits.

A direct laboratory method for the study of the intake and production of active suspension feeders that involves minimal interference was suggested by Wright and Stephens (1978). Under this method, the exhaled water is sampled directly after a single passage across the animal's filtration apparatus using a capillary tube. Unfortunately, this method has not gained much attention since its initial use (but *see* Yukihira et al. 1999).

While indirect techniques lump together the pumping rates and filtration efficiency, a considerable effort has also been devoted to the study of water transport rates of various active suspension feeding taxa (as reviewed by Jørgensen 1966; Famme and Riisgård 1986; Jones et al. 1992; Wildish and Kristmanson 1997; Riisgård 2001*a*). The methodology used in these laboratory studies ranges from the straightforward quantification of dyed water movement within a tube inserted into a sponge osculum (Parker 1914; Gerrodette and Flechsig 1979), an algal clearance rate method (assuming 100% removal efficiency, e.g., Møhlenberg and Riisgård 1979; Petersen and Riisgård 1992; Riisgård et al. 1993; Riisgård 2001*a*), a constant-level tank method (in which the exhaled water is channeled into a measuring flask, Jørgensen 1966 and references therein), to complex mechano-optic devices (e.g., Jones et al. 1992; Famme and Riisgård 1986; Riisgård 2001*a*). Tank head pressure (e.g., Jørgensen et al. 1990; Riisgård et al. 1993), temperature differences between the animal interior and its environment (Defossez et al. 1997), microscopic observations (e.g., Turon et al. 1997), and video endoscopy (Ward and MacDonald 1996) have also been used in laboratory studies.

There have been far fewer attempts to measure active suspension feeder pumping rates in situ. These attempts have relied mostly on the use of hot wire or hot film thermistors (Reiswig 1974; Vogel 1974, 1977; Fiala-Medioni 1978*a*; Savarese et al. 1997). These instruments allow a continuous record of excurrent velocity but estimates of water transport rates require additional information on aperture area and ambient flow (Fiala-Medioni 1978*b*). When such information is available, pumping rates can be recorded for prolonged periods. Reiswig (1974) estimated instantaneous pumping rates with a hand-held flow meter over the oscula of the giant Caribbean sponges he investigated, whereas Savarese et al. (1997) injected dye into inhalant canals and subsequently measured the dye jet trajectories within the ambient flow field. Most recently, an Acoustic Doppler Velocimeter was used to measure the excurrent velocity of a coral reef sponge (Yahel et al. 2003), and time-lapse videography of exhalant siphon area was suggested as a proxy for bivalve feeding activity in the field (MacDonald and Nodwell 2003).

Despite intense research efforts, many basic aspects of the nutritional ecology and physiology of active suspension feeder are not yet fully resolved, and the optimal methodology for measuring these parameters is still hotly debated (e.g., Jørgensen 1996; Bayne 1998; Ward et al. 2000; Cranford 2001; Riisgård 2001*a*, 2001*b*; Petersen et al. 2004). As pointed out by Riisgård (2001*a*) and Petersen et al. (2004), closed vessel assay results are sensitive to the experimental conditions, including vessel geometry, flow characteristics, and the type of suspended particle diet offered. Comparisons between coral reef suspension feeders transferred to the laboratory and undisturbed in situ animals indicated that pumping behavior was considerably altered in the laboratory even when animals were positioned in very large tanks with ample supply of fresh reef water (Yahel unpubl. data unref.). Some coral reef sponges and ascidians alter their feeding rate, modify their food preference, and most often stop feeding when placed in closed vessels (Yahel and Hadas unpubl. data unref.). In several experiments, clearance rates were independent of food availability in the vessels and, instead, varied as a function of the supply of fresh seawater into the vessel. For example, the clearance rate of the *Synechococcus* cyanobacteria by the reef sponge *Negombata magnifica* was reduced by 4-fold when water residence time in the experimental tank was increased from 5 to 20 min (Hadas unpubl. data unref.). Moreover, numerous active suspension feeders in coral reefs are either boring or encrusting (Yahel et al. 1998) and cannot be separated from their substrate and brought to the laboratory without severely damaging their body.

In summary, drawing conclusions from results of closed-vessel experiments regarding the actual performance of the animals in the field is controversial, even under optimal experimental conditions (e.g., Cranford and Hill 1999; Cranford 2001, and references therein).

The in situ indirect method developed by Ribes et al. (1998, 2000) bypasses some of the problems inherent in the laboratory system. Nevertheless, it suffers from the same limitation of the laboratory-based clearance method discussed above (*see* Riisgård 2001*a*), and it is quite demanding in terms of the amount of equipment and work required per specimen sampled (Ribes et al. 2000).

Reiswig, in his comprehensive fieldwork on the ecology of Caribbean sponges (Reiswig 1971*a*, 1971*b*, 1974), pioneered the use of direct in situ techniques for the study of active suspension feeder diet composition, metabolic performance, and water transport rates. His direct methods were based on comparing the content of the water inhaled and exhaled by sponges and, therefore, were free of most of the methodological pitfalls associated with both indirect and in vitro experiments. Combined with pumping rate estimates obtained by hot wire thermistors or hand-held flow-meters, Reiswig's data

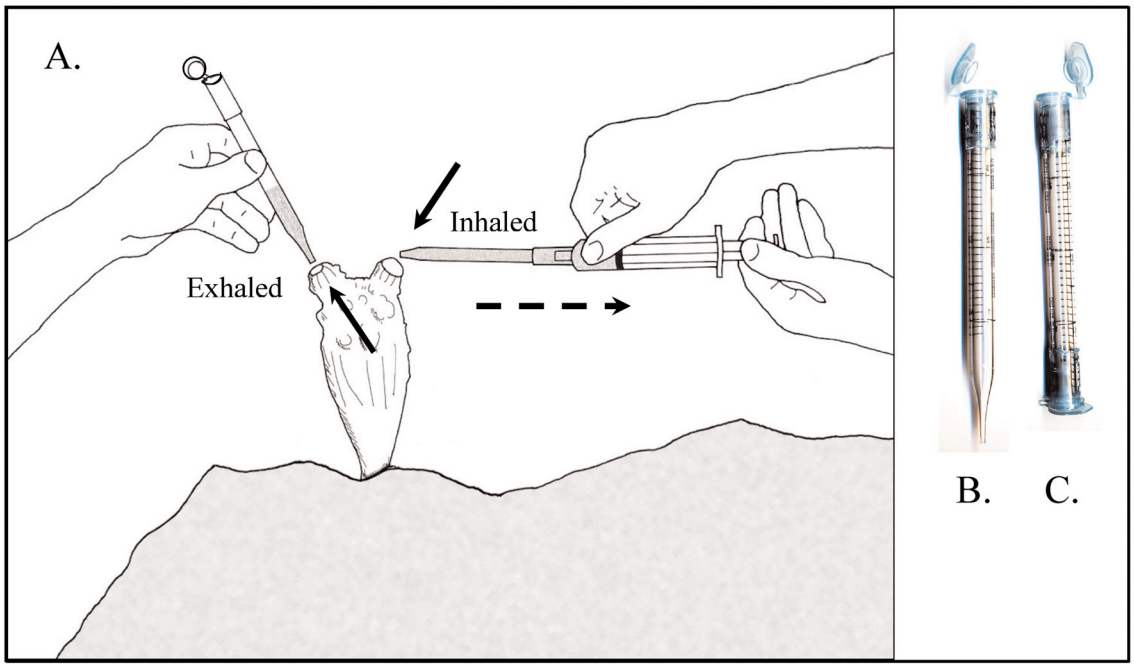

Fig. 1. (A) Schematic representation of the InEx technique as applied to a solitary ascidian. Exhaled water is collected passively using the positive pressure at the exhaled water jet to flush and replace the water within an open-ended tube carefully positioned within the excurrent jet. Inhaled water is sampled into an identical sampler attached to a syringe. In this example, the samplers are Pt10 (Table 1) designed for the collection of relatively large water quantities (7 mL) from organisms with minute excurrent aperture (~3 to 5 mm). Arrows indicate the direction of water flows. (B) Photograph of a Pipette-shaped (Pt) InEx sampler. (C) Photograph of a tube-shaped (Tu) InEx samplers (for sampler make-up, *see* Materials and procedures).

allowed calculation of the sponge's actual grazing rates. Unfortunately, the methodology used by Reiswig for his giant Caribbean sponges (e.g., trapping the exhaled water in large plastic bags) cannot be easily applied to smaller sponges (Wilkinson 1978) and even less so for more sensitive organisms such as bivalves and ascidians (Yahel unpubl. data unref.). Use of samples obtained by active suction of the exhaled water (e.g., using a syringe, Reiswig 1974, 1985; Wilkinson 1978; Pile et al. 1996; Pile 1997) requires proper control over the risk of "contaminating" the exhaled water sample by sucking in ambient water, thus the suction rate should be negligible in comparison to the excurrent flow rate (e.g., Wright and Stephens 1978). Alternatively, suction samples can be obtained from the excurrent atrial cavity, provided that the sample volume is negligible with respect to the cavity volume. Unfortunately, many common benthic suspension feeders do not possess large excurrent cavity or high pumping rate. Therefore, hand-held syringe sampling is inappropriate for most active suspension feeder species. If suction sampling is to be used with small suspension feeders, water samples should be withdrawn using a device that allows slow and well-controlled suction (e.g., Wright and Stephens 1978).

In the present article, we present a combination of earlier in vitro methods used by Parker (1914), Wright and Stephens (1978), and Møhlenberg and Riisgård (1979), and the pioneering in situ approach of Reiswig (1971*a*, 1971*b*). Our aim is to develop a reliable, rapid, and nonintrusive sampling strategy that enables both individual and community-scale studies of the diet composition, pumping rates, grazing rates, and metabolism of a wide range of active suspension feeders in situ.

Materials and procedures

Water sampling—The InEx method is based on a simultaneous collection of paired water samples from the water inhaled and exhaled by active suspension feeders. Two scuba divers carry the sampling underwater. Samples of inhaled water are drawn slowly by one diver with the use of a small, open-ended tube attached to a syringe as the diver holds its inlet next to the animal's inhaling aperture (Fig. 1, *see* Video clip 1 <http://www.aslo.org/lomethods/free/2005/XXXXa1.html>). The exhaled water is sampled by a second diver with an identical tube held within the exhalent jet and aligned with it, as close

Video clip 1. Highlights from an InEx sampling at Race Rocks, British Columbia, Canada. The sampled organism is the sponge *Isodictya rigida*. Video courtesy of G. Fletcher, Pearson College, www.racerocks.com. Video can be seen at http://www.aslo.org/lomethods/free/2005/0046a1.pdf.

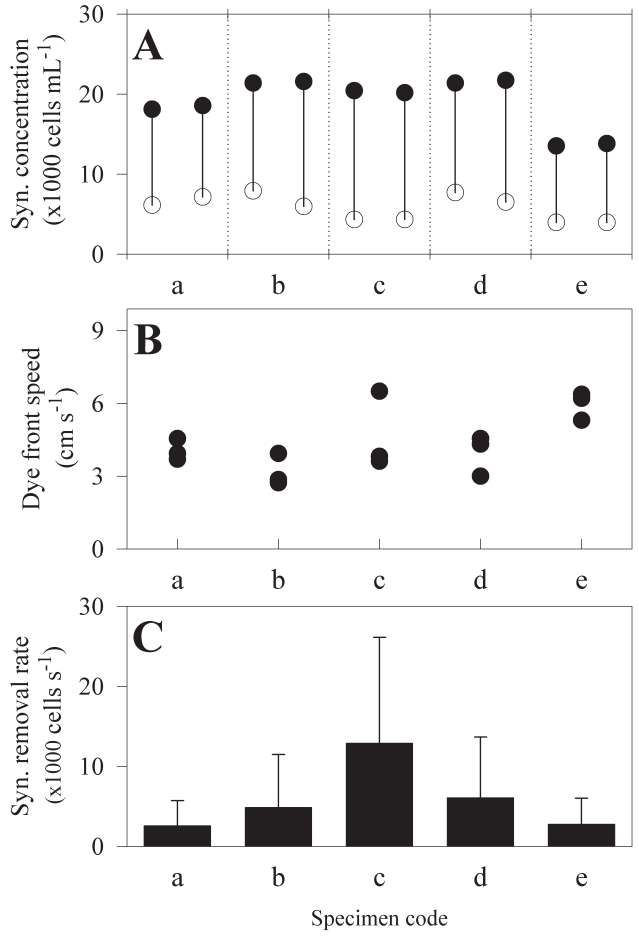

Fig. 2. Examples of typical parameters measured with the InEx method. (A) Concentrations of *Synechococcus* (Syn) in the water inhaled (●) and exhaled (○) by five specimens of the solitary ascidian *H. gangelion* (two consecutive replications collected from each animal). Lengths of connecting lines indicate number of cells removed. (B) Three consecutive excurrent jet velocity measurements (dye-front speed) of the same specimens as in (A) obtained immediately after water sampling. (C) Instantaneous grazing rate estimates for the same specimens calculated as the product of the data presented in A and B and the Tu10 sampler cross-section (Table 1). Error bars (SD) represent the total variation contributed by individual animals (variation of pumping rate and retention efficiency), plankton patchiness in the ambient water, and sampling error: $SD_{composite} = (SD^2_{cell\ removal} \times \text{pumping rate}^2 + SD^2_{pumping\ rate} \times \text{cell removal}^2)^{0.5}$.

as possible (<2 mm) to the animal's exhaling aperture, but with no physical contact (Fig. 1, Video clip 1). When sampling is accomplished, the inhalant sampling tube is plugged first, starting at its proximal end, then the exhalant sampling tube is carefully plugged, starting at its distal end, the proximal end is then plugged while still positioned within the excurrent jet. Filling time is determined individually for each pair as 1.5 × the time it took the exhalent jet to flush clear the sampling tube prefilled with dyed seawater (~5 mg L^{-1} fluorescein). If the dye is suspected to interfere with water analysis, flushing time should be measured with an identical tube a few minutes prior to sampling. Fluorometric measurements of dye concentrations in these tubes indicated that this filling time ensured a replacement of greater than 99% of the water contained in the tube prior to the start of sampling (*see* Sampler flushing time). The difference between the inhaled and the exhaled water samples (InEx pair) indicates the net retention (or production) of the substance of interest.

Multi-oscular organisms—To ensure genuine pairing of InEx samples for multi-oscular organisms (e.g., sponges), entry and exit points of the water path through the animal are visualized with vital dye (fluorescein, 10 mg L^{-1}); their locations are marked for subsequent sample collection. Sampling is delayed to minimize dye effect on water properties or animal behavior. The duration of water passage through the filtration apparatus may also be determined by the same method. However, with the exception of glass sponges, all organisms surveyed so far exhibited a passage time of less than 2 s, which is an order of magnitude smaller than our typical sampling duration.

Excurrent velocity measurement—Excurrent jet speed and water transport rates can be measured concurrently with each InEx sample. A small amount of dyed seawater (fluorescein, 100 mg L^{-1}) is placed just inside one end of a transparent, cylindrical tube while the sampler's finger seals the other side. The tube is positioned within the excurrent jet and aligned with it, as close as possible to the exhaling aperture (but avoiding physical contact) and the finger is released to allow the exhalent jet to flow through the tube. The movement of the dye within the tube is videotaped, and the dye-front speed is determined with frame-by-frame analysis using marks on the tube as a scale. This rapid measurement (a few seconds) is typically replicated 3 to 5 times.

Rate calculations and statistical analysis—The method yields two independent measurements: the difference between InEx (Inhaled-Exhaled) samples (Fig. 2A) and instantaneous water flux per excurrent aperture. The latter is calculated as the product of the mean dye-front speed (Fig. 2B) and the tube or excurrent aperture cross-section area (*see* Pumping rate measurements). These parameters can be combined to produce instantaneous filtration rate estimates (Fig. 2C). Alternatively, traditional "clearance rate" estimates can be calculated as the product of filtration efficiency [(In – Ex)/In)] and the water transport rate. Due to the inherent patchy distribution of plankton and other water constituents, care should be taken to collect the InEx samples in a way that will ensure genuine pairing of the samples and facilitate true pairwise analysis. In cases in which several aliquots are being analyzed from each water sample, the appropriate statistical test is a repeated measures analysis of variance (ANOVA) or its aparametric equivalents.

Materials for InEx samplers—A variety of sizes and shapes of sampling tubes were examined in preliminary trials. The final design samplers were easily fabricated from standard disposable polystyrene pipettes (e.g., Bibby Sterilin Ltd, Staffordshire, U.K.), and they consist of a shortened pipette with the upper half of a chopped Micro-Tube (e.g., Eppendorf) fitted

Table 1. Types of InEx samplers used

Sampler name	LPt 1	LPt 2	Pt 5	Pt 10	Tu 5*	Tu 10*	DFS 1	DFS 2	G 32
Length (mm)	282	282	65	160	145	111	128	130	151
Volume (mL)	1.6	2.7	2.5	6.9	3.9	6	0.7	5.5	32.0
Inlet									
Inner diameter (mm)	2.0	2.0	2.0	3.0	6.3	9.4	2.8	3.6	10.0
Outer diameter (mm)	3.0	3.0	3.0	4.0	14.5	13.7	4.5	8.3	12.3
Body									
Inner diameter (mm)	2.8	3.6	5.8	8.0	5.8	8.0	2.8	3.6	24.6
Outer diameter (mm)	4.6	5.0	7.1	10.0	7.1	10.0	4.6	5.0	22.5
Outlet									
Inner diameter (mm)	4.9	4.6	6.3	9.4	6.3	9.4	2.8	3.6	12.0
Outer diameter (mm)	8.3	8.3	14.5	13.7	14.5	13.7	4.5	5.0	16.3
Cross-section area (cm^2)					0.26	0.50	0.06	0.10	
Number of stoppers	1	1	2	1	2	2	0	0	2

Samplers were fabricated from polystyrene Sterilin pipettes fitted with chopped micro-tube as stoppers, except for the G 32, which was custom-made from glass. LPt samplers were intact pipettes fitted with a stopper on the distal end. Pt samplers had the same design but the pipette was shortened (*see* Fig. 1B). Tube-shaped samplers were simple cylinders with stoppers fitted on both sides (*see* Fig. 1C). DFS samplers were simple transparent tubes with no stoppers, used solely for pumping rate measurements.
*Samplers that were used for both water collection and dye-front speed measurements.

directly or with an external Tygon tube as a stopper to one or both ends. Pipette-shaped samplers with only one stopper (LPt and Pt, *see* Fig. 1B) were kept sealed by inserting their narrow tip (2 to 3 mm) into a compatible 15 mm deep hole drilled in a 30 mm silicon pad. Custom-made precombusted glass samplers were used for special applications (e.g., dissolved organic carbon [DOC] sampling, Yahel et al. 2003). Some relevant examples of InEx samplers are described in Table 1.

Statistical analysis—Statistical analysis was carried out using STATISTICA, version 6 (data analysis software system, StatSoft Inc. 2002). Parametric tests were used whenever possible, and data were transformed when necessary to meet the requirements of normality and homogeneity of variance. The equivalent nonparametric tests were used whenever data did not meet the ANOVA assumptions. For repeated measure ANOVA, we also tested the compound symmetry and sphericity assumptions (i.e., cases in which differences between levels were correlated across subjects) and compared the results of the univariate test with Wilks' λ (a multivariate criterion).

Assessment

Comparison of inhaled and exhaled water—The InEx technique was used for the measurement of a variety of metabolic parameters, including respiratory gases, plant nutrients, plant pigments, total organic carbon (TOC), DOC, and alkalinity (Yahel et al. 2003; Yahel et al. unpubl. data unref.). Ultra-plankton (<10 μm, Murphy and Haugen 1985) was used to examine the technique's efficiency. Ultra-plankton cell counts are ideal for this purpose as they are reliably made with a flow cytometer and require a small sample volume (1 mL). Moreover, ultra-plankton consists of several cell populations that are selectively removed by different active suspension feeder taxa (Pile et al. 1996; Ribes et al. 1998, 1999) thus providing "internal control" for the method.

Three locations were used to evaluate the method. Our primary location was the coral reef in front of the Steinitz Marine Laboratory of Eilat, Gulf of Aqaba, Red Sea (29°30′N, 34°56′E). Further evaluation was carried out at the reefs of Alphonse Island, Seychelles (06°59′S, 52°43′E), and at Race Rocks (123°32′W 48°18′N), British Columbia, Canada. The latter site is characterized by high currents (>2 m s^{-1}) and cold water (6°C to 10°C). All field samples were collected by two divers using standard SCUBA techniques. A flow cytometer (FACSort, Becton Dickinson) was used to estimate the concentrations of 4 groups of ultra-plankton: nonphotosynthetic bacteria (Bact, median size ~0.4 μm) and the three major autotrophic groups, *Synechococcus* (*Synechococcus*, ~1 μm), *Prochlorococcus* (Pro, ~0.6 μm), and pico-eukaryotes (Eukaryotic algae, ~2 to 3 μm), as described in Yahel et al. (1998).

InEx measurements of removal efficiencies exhibited high repeatability. For example, the average deviation between duplicate InEx samples obtained from 5 specimens of the solitary ascidian *Halocynthia gangelion* was 4% (Fig. 2A). In this case, the mean removal efficiency of the *Synechococcus* was 70% and the standard deviation was 6% (coefficient of variance [CV] = 9%). In a similar experiment, 11 oscula from 3 specimens of the boring sponge *Cliona mussae* were sampled. The average deviation between replications was 1%, and the mean removal efficiency was 94% ± 4% (CV = 4%). The fact that very few cells were found in the samples of the exhaled water indicated that the exhaled water was sampled cleanly, with negligible "contamination" by ambient, nonfiltered water.

The presence of different ultra-planktonic groups in the ambient water provided internal control for the method. To

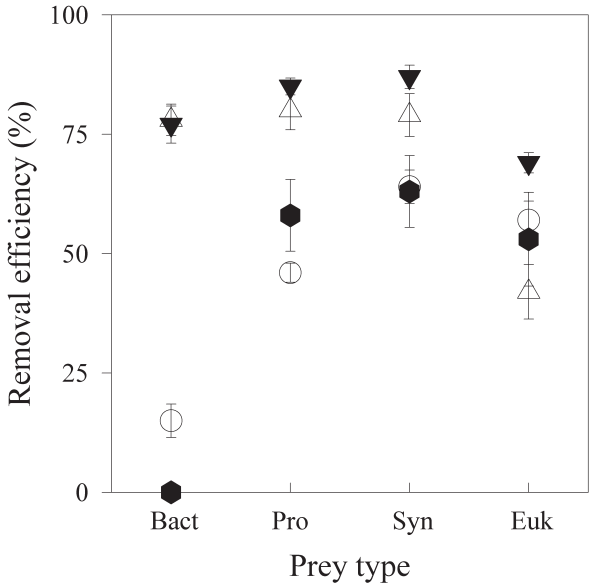

Fig. 3. Sampler type effect: Comparison of ultra-plankton retention efficiency of various active suspension-feeder taxa using two types of InEx samplers. Two organisms possessing small (<3 mm) excurrent apertures, the bivalve *Lithophaga simplex* (○, *n* = 4), and the sponge *Cliona mussae* (△, *n* = 6) were sampled by a pipette-shaped LPt 1 sampler (open symbols). A cylindrical Tu 10 sampler (filled symbols) was used for the sponge *S. clavatus* (▼, *n* = 8) and the ascidian *H. gangelion* (⬢, *n* = 3), both possessing much larger excurrent apertures (>9 mm). All samples were collected at the same site during a single sampling dive (23 September 1997). Pro, *Prochlorococcus*; Syn, *Synechococcus*; Euk, eukaryotic algae; Bact, nonphotosynthetic bacteria. Error bars = SE.

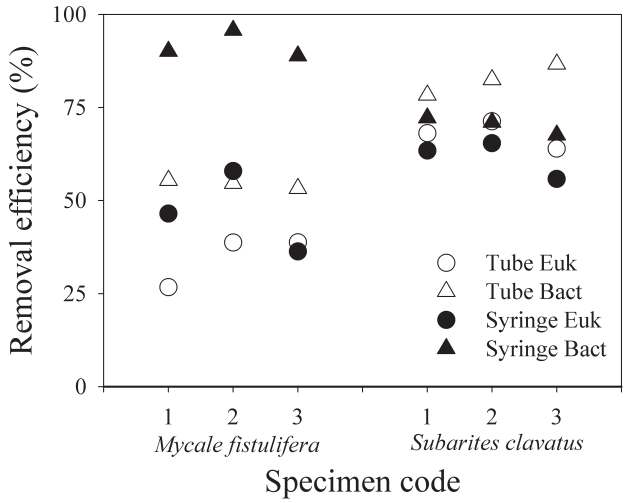

Fig. 4. A comparison of two methods of sampling the water inhaled and exhaled by two large reef sponges. Removal efficiency of nonphotosynthetic bacteria and eukaryotic algae was measured concurrently over the same sponge oscula. Exhaled water was captured using both the InEx technique (empty symbols) and slow active suction by a syringe (filled symbols). For the purpose of this comparison, sponges possessing relatively large volume oscula (>5 mL) with strong excurrent gets (>120 mL min^{-1}, Yahel unpubl. data unref.) were selected. For each osculum, a syringe sample was slowly drawn immediately after an InEx sample. The apparent high removal efficiency of bacteria by *M. fistulifera* may be a consequence of accidental suction from within the loose tissues of this sponge (*see* text).

test the significance of these differences, a within-between repeated measure ANOVA design was used, with the removal efficiencies [(In – Ex)/In] for each prey taxon (Pro, Syn, Euk, Bact) as the within-subject, repeated measure factor, and the grazer taxa as the between-subject, fixed factor. The analysis showed that removal efficiencies of the different ultra-planktonic prey types varied significantly both within and between active suspension feeder species (Fig. 3, $F_{3,54} = 131.2$, $P < 0.0001$; and $F_{3,18} = 25.5$, $P < 0.0001$, respectively). For example, the overall retention efficiency measured for the sponge *Suberites clavatus* (80%) was almost twice that of the ascidian *H. gangelion* (44%), both sampled by an identical sampler. A consistent removal pattern was exhibited by each of the active suspension feeder species regardless of the sampler type used (Fig. 3). Hence, no significant removal ($P > 0.2$) of nonphotosynthetic bacteria was measured for the ascidian (Tu 10 sampler) and bivalve (Pt 10 sampler) samplers. Using the same samplers at the same time on nearby sponges revealed high filtration efficiencies (>77%) of nonphotosynthetic bacteria by these organisms (*S. clavatus*, Tu 10 sampler; and *C. mussae*, Pt 10 sampler).

The repeatability of the inhaled water collection method was tested by comparing three sets of triplicate samples collected in ~3-minute intervals next to three different incurrent siphons (an ascidian and two bivalves). In all cases, cell counts of each of the pico-planktonic groups were almost identical (CVs ranged from 0% to 6%). Comparison of more spatially dispersed samples taken next to different individuals located 5-30 m from each other over a similar time scale indicated much higher CVs (ranging from 13% to 22%). This finding highlights the strengths of the paired-sample design, in which there is an inhaled sample for every exhaled sample.

To compare the passive InEx sampling technique to active syringe suction of exhaled water (e.g., Reiswig 1985; Pile 1997), immediately after the collection of an InEx pair, water was carefully and slowly drawn with either 10 or 30 mL syringes next to the ostia (inhalant aperture) of some large sponges as well as from within their oscula (exhalant aperture). The samples were analyzed with a flow cytometer and an epi-fluorescence microscope (a method described by Lindell and Post 1995). Flow cytometry analysis yielded indistinguishable estimates of pico-plankton removal efficiency for samples collected from the relatively large sponges *Mycale fistulifera* (*n* = 3), *S. clavatus* (*n* = 3), and *Theonella swinhoei* (*n* = 2) (repeated measure ANOVA of pooled, arcsine transformed data, $F_{1,5} = 4.46$, $P = 0.883$). These results (Fig. 4) indicate that sampling using controlled suction could be an efficient approach for sufficiently large taxa with strong excurrent jets and/or large excurrent atrial cavities, provided that the sampling rate is negligible in comparison to the excurrent flow

Table 2. Average sampler flushing time (SFT) required for the excurrent jet to displace and flush the ambient water contained in the sampler and the average ratio of sampler volume (SV) to the volume of water pumped via the sampler during that time (WV)*

Sampler type and species	Taxonomic grouping	*n*†	SFT (s)	SV/WV ratio
Tu 10				
Didemnum candidum	Colonial tunicate	13	73 (15)	14.1
Halocynthia gangelion	Solitary tunicate	28	53 (16)	16.7
Mycale fistulifera	Sponge	49	42 (15)	14.7
Suberites clavatus	Sponge	57	27 (10)	16.4
Pt 10				
Cliona mussae	Sponge	20	95 (20)	3.4
Lithophaga simplex	Bivalve	20	152 (32)	3.4
LPt 2				
Cliona mussae	Sponge	14	132 (32)	28.3
LPt 1				
Lithophaga simplex	Bivalve	28	113 (44)	12.0

*Pumping rate was determined by the dye-front speed method (*see* text).
†*n* is the number of excurrent jets sampled.

rate. However, efforts to apply the syringe method for taxa possessing smaller excurrent apertures (e.g., *Lithophaga* bivalves or boring sponges) were unsuccessful. In such small taxa, there was no simple way to control the sampling rate, and the results yielded no interpretable signal.

Microscopic analysis of syringe samples collected from within the sponge's oscula revealed a dense population of large eukaryotic algae (>10 μm) in samples taken from *M. fistulifera*. These cells, too large to be detected by the flow cytometer, are presumably similar to the eukaryotic algae reported by Pile (1997, and references therein) to be "produced" and expelled by several sponge species. Such algae were absent from the corresponding exhaled (and inhaled) water samples obtained by the InEx method, as well as from the ambient water samples obtained by syringes. It is suggested that the apparent production of eukaryotic algae reported by Pile (1997) was an artifact of using the syringe method due to forceful suction of either periphyton or endo-symbionts (Wilkinson 1978) from the oscula walls. Suction from within the oscula wall tissues may also explain the apparent high removal efficiency of bacterial cells measured with the syringe technique for *M. fistulifera* (Fig. 4).

Sampler flushing time—Field experiments. The time required for the excurrent jet to displace and flush the ambient water contained in the sampling tube was designated "sampler flushing time." Flushing efficiency was measured in the field by holding the tube (prefilled with dyed seawater) within the excurrent jet, 1 to 2 mm from the excurrent aperture, until no dye was visible within the tube. The tube was then sealed and the residual dye concentration was determined fluorometrically (Turner AU-10, Turner Designs). An identical tube was simultaneously held in a similar position over an inert surface as control. By the time the samplers were determined "clear" by underwater visual inspection, they contained, on average, 0.4% ± 0.7% of the initial fluorescein concentration (10 to 100 mg L^{-1}). Despite the somewhat subjective manner of this method, its repeatability was fairly high with 8% ± 7% deviation from the mean (n = 110 duplicate sampler flushing time measurements). This convenient method was thus adopted as the standard mean for sampler flushing time determination in subsequent experiments. The ratio of sampler volume to the volume of water required to flush it clear was nearly identical for the same sampler type and independent of the sampled taxa (Table 2). Sampler volume (Table 2), sampler geometry, and ambient hydrodynamics affected sampler-flushing time. When samplers were left open, a gradual exchange of the sampler and ambient water occurred also in the absence of an excurrent jet as a consequence of wave action, ambient currents, and sampler movements. However, even under extreme conditions (e.g., the excurrent jet oriented into a strong ambient current), whenever a sampler was located over an excurrent jet, the bulk of the dyed water was flushed out by the excurrent within seconds (*see* Video clip 1), while identical samplers simultaneously held over inert surfaces retained >20% of the initial dye concentrations for several minutes.

Flow tank experiments. To test the method under controlled conditions, an artificial excurrent jet was fitted into a flume flow chamber with transparent walls (Fig. 5; Kiflawi and Genin 1997). The excurrent model was built with a 20-mm internal diameter (ID) hose capped by a flat (1 mm) replaceable lid into which an orifice was drilled. This arrangement assured an excurrent profile as close as possible to "plug flow" (Charriaud 1982; Savarese et al. 1997). Ambient current velocity, jet flow rate, jet orientation, and aperture diameter were adjustable. A flow meter (Gravity ball, Gilmon STD nr 13) installed between the head tank and the excurrent model was used to measure excurrent jet flows. Flow visualization was carried out by dyeing either the tank (ambient) water or jet water, or by injecting small amounts of dyed water into or around the samplers. A video camera (Sony Handycam, High-8

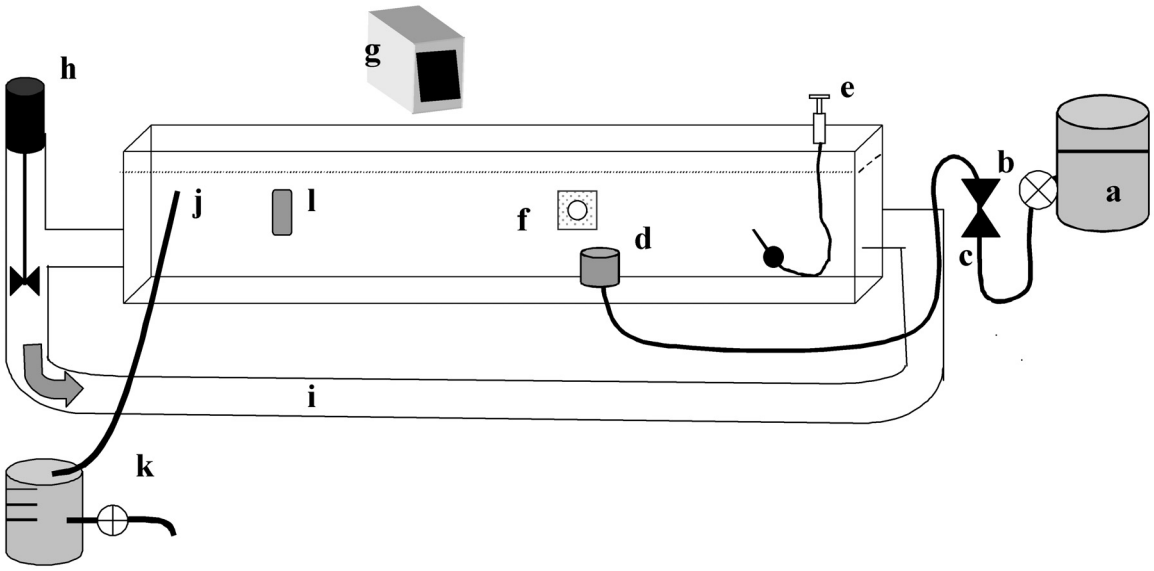

Fig. 5. Schematic representation of the setup used for testing and calibrating the InEx technique in the flume. a. Head tank; b. Flow regulator; c. Flow meter; d. Excurrent model with interchangeable orifice; e. Dye injector; f. Video camera; g. Video monitor and recorder; h. Controlled propeller unit; i. Return circuit; j. Water outlet; k. Additional measuring flask; l. Thermostat (drawing is not to scale, *see* Kiflawi and Genin [1997] for tank specifications).

CCD-V700E) mounted in front of the excurrent model was used to record and analyze the fluid motion.

Sampler flushing time measurements were carried out in the flume by filling a set of identical samplers with tank-water containing a known dye concentration; samplers were then positioned above the excurrent jet for different flushing durations (3 to 100 s). At the end of each run, the sampler was sealed, and the concentration of the dye remaining in the tube was determined fluorometrically. Ratio of initial-to-final concentration was used as a measure for flushing efficiency. As depicted in Fig. 6, sampler flushing-rate approximated exponential decay after an initial phase characterized by either a short delay (pipette-shaped samplers) or an abrupt concentration drop (tube-shaped samplers). This exponential mode of sampler flushing, a consequence of boundary layer formation along the sampler walls (J.R. Kossef pers. comm. unref.), enforced prolonged sampling whenever clean exhaled samples were sought. For ~6 mL samplers, sampler flushing time ranged from 0.5 min for high-pressure sponges (jet flow rate ~250 mL min^{-1}) to 2.5 min for small bivalves (~10 mL min^{-1}).

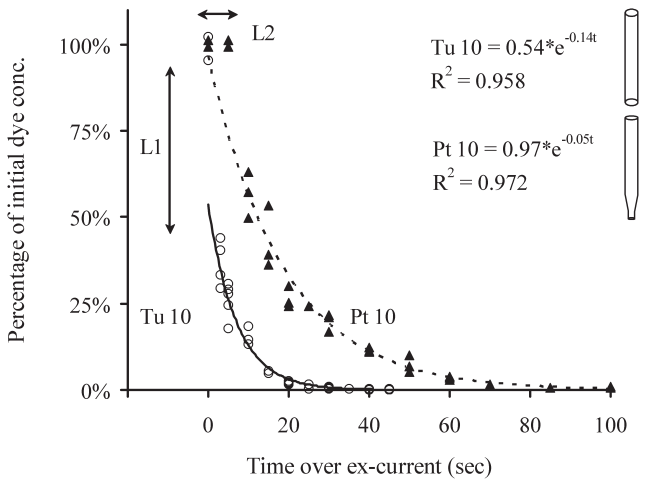

Fig. 6. The time course of sampler flushing (SFT) of two samplers (in percentage of initial dye concentration) as a function of the time the sampler was held open over the artificial excurrent jet. Double-sided arrows mark the gap between the observed flushing time-courses and the prediction of an exponential decay model. The Tu 10 sampler was held over a 10-mm excurrent model (flow rate = 6.7 mL s^{-1}). Half of the sampler content was replaced within the first 5 s (L1) because of piston effect of the excurrent jet, followed by an exponential dilution. The Pt 10 sampler was held over a 2-mm excurrent model (flow rate = 0.88 mL s^{-1}). The exponential dilution started after a typical lag required for the flow field to develop within the sampler (L2). See Table 1 for sampler descriptions.

Contamination by ambient water—Intrusion of ambient water into the exhaled water sampler during sample collection (hereafter "contamination") is of major concern. We used visualization of ambient water invasion by dying either "exhaled" or "ambient" tank water and testing various combinations of ambient conditions, excurrent models, and sampler types. In cases in which the sampler's inlet internal diameter (ID) was larger than the excurrent diameter, dyed tank water was entrained by the Venturi effect (Vogel 1978) into the sampler. This effect led to significant underestimation of the retention efficiency, as demonstrated in Fig. 7A. Nevertheless, dye visualization and fluorometric measurements indicated that as long as the sampler ID was similar to or smaller than the diameter of the animal's excurrent aperture, only exhaled water entered the Ex sampler. Upward dye movement and flushing of the samplers may be evident, even in cases in

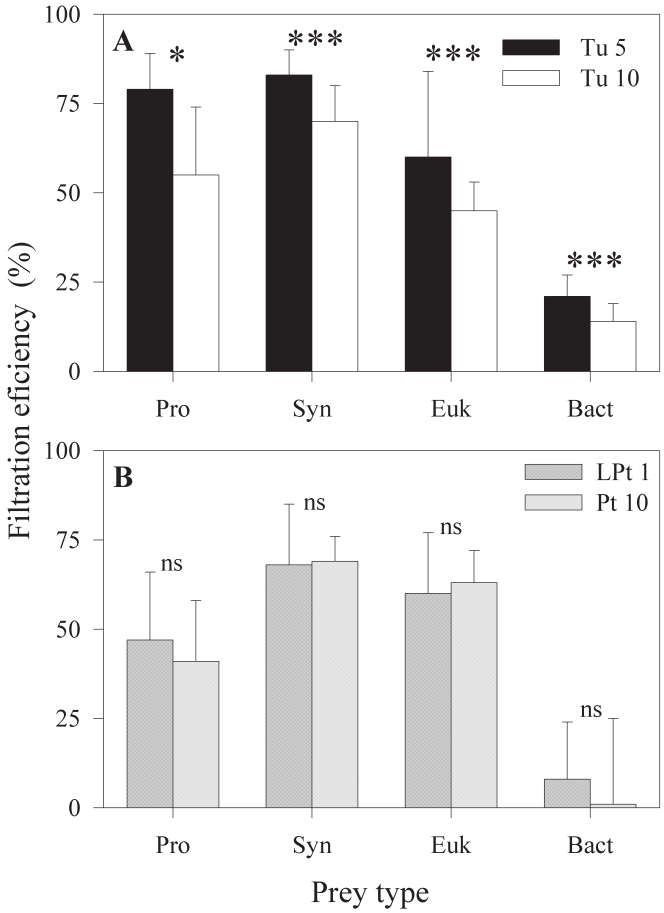

Fig. 7. An example of proper and improper fitting of sampler ID to excurrent aperture and their effect on ultra-plankton retention efficiency estimates (error bar, SD). (A) Two samplers of the same type but with different ID were used to measure the filtration efficiency of the colonial tunicate *Didemnum candidum*. Tu 10 ID > excurrent orifice; Tu 5 ID = excurrent orifice. Mann-Whitney U test indicated that efficiency estimates obtained with the Tu 10 sampler (ID > excurrent orifice) were significantly lower (*$P < 0.05$; ***$P < 0.001$; $n = 13$ and 12 for Tu 10 and Tu 5, respectively). (B) Two different sampler types, both with inlet ID ≤ excurrent orifice were used to estimate filtration efficiency of the boring bivalve *Lithophaga simplex*. No significant difference was found between the samplers (Mann-Whitney U test, $P > 0.15$ for all comparisons; $n = 23$ and 36 for LPt 1 and Pt 10, respectively). Note that the Pt 10 sampler has a conical, pipette-shaped inlet end and its volume is ~4 times larger than the cylindrical LPt 1.

which active pumping by the studied organism is absent. This artifact is generated by higher ambient water velocities away from the boundary at the outlet of the sampler (Bernoulli effect, Vogel 1974). Various capping devices and configurations aimed to offset this effect were tested in the flow tank with no success. However, this problem was significant only with long samplers (>25 cm, e.g., LPt type, *see* Table 1) oriented perpendicular to the ambient flow or in the presence of strong currents. This shortcoming can be resolved by selecting sampling positions where the sampler is oriented with a slight angle (<5°) into the ambient flow. In general, pipette-shaped samplers and samplers with a smaller diameter-to-length ratio were less susceptible to contamination by ambient water intrusion.

To assess the potential of ambient water contamination, a comparison of direct filtration efficiencies measurements was carried out in the lab using the Wright and Stephens (1978) method, with InEx measurements for the same individuals. Five specimens of *N. magnifica* previously attached to polyvinyl chloride plates and grown suspended over the reef were transferred to a 4-L aquarium with a fresh reef water supply (residence time < 3 min). Using a micromanipulator, a micro-capillary tube was mounted inside one of the sponge's oscula without touching the sponge. A similar tube was located within 2 mm of the sponge's ostia. The slow suction through the capillary tubes ensured clean collection of exhaled and inhaled water (Wright and Stephens 1978). Water droplets from the distal end of the tube were collected and analyzed by a flow cytometer. The sponges were then returned to the field and sampled by the InEx method 3 d later, using the minute InEx sampler (Pt 5, volume 2.5 mL, length-to-outlet ID ratio = 10, Table 1), which is predicted to be most susceptible to ambient water contamination. Filtration efficiencies measured at the laboratory were on average 6% ± 4% higher than those measured in the field (Hadas unpubl. data unref.). Therefore, InEx-based estimates are expected to underestimate the "true" filtration efficiencies by < 10%.

Pumping rate measurements—Calibration. Calibration of dye-front speed measurements was carried out in the flume (Fig. 5) under low ambient flow conditions (0 to 3.5 cm s^{-1}), with the excurrent model jet in vertical position. The water flux from the model excurrent jet ranged from 6 to 160 mL min^{-1} (jet speed 3 to 83 cm s^{-1}) for the 2 mm orifice, and from 10 to 2100 mL min^{-1} (jet speed 0.2 to 45 cm s^{-1}) for the 10 mm orifice. Dye-front speed measurements were replicated 10 times for each flow rate.

The dye-front was first tracked without a tube (as suggested by Savarese et al. 1997). However, for realistic flow rates (e.g., 2.5 cm s^{-1} for a 6-mm orifice, Savarese et al. 1997), significant deflection of the excurrent jet was evident even in very low ambient current velocities (<2 cm s^{-1}). Due to the extreme sensitivity of this measurement to ambient currents and the necessity of an external scale to be held within the plane of the dyed jet, this technique was not further employed.

Dye-front speed measurements through conducting tubes were short (<5 s) and exhibited good repeatability (average CV 18% ± 10%, $n = 59$). Calibration of dye-front speed to water velocity via the excurrent model exhibited good linear correlation ($R^2 > 0.96$, Fig. 8) up to flow rates of 100 mL min^{-1}, for a Tu 10 sampler (ID 9.4 mm over a 10-mm orifice) and 65 mL min^{-1}, for a DFS 1 sampler (ID 2.8 mm over a 2-mm orifice).

Sampler size effect. Since a perfect match between the tube diameter and the animal's excurrent aperture is not practically feasible, the same protocol was used to test the performance of

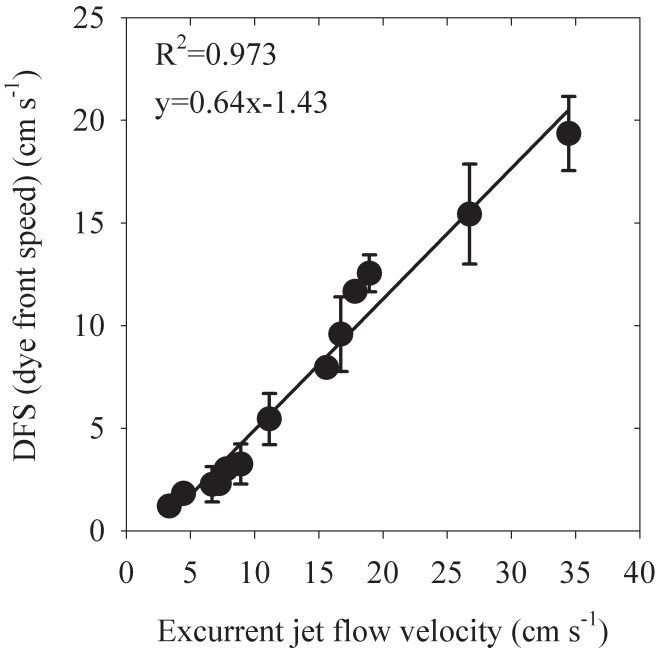

Fig. 8. An example of a pumping rate (dye-front speed) calibration experiment. Dye front speed was recorded by video analysis within a transparent tube located just above an artificial excurrent orifice. DFS 1 sampler (ID 2.4 mm) was used to sample a 2-mm orifice. The experiments were performed in the flume. Error bars = SD.

sampling tubes that differed from the excurrent model orifice. This comparison was undertaken using all possible combinations of samplers with 2.8-, 6.3-, and 9.4-mm ID (DFS 1, Tu 5, and Tu 10, respectively) over 2-, 6-, 10-, and 12-mm excurrent orifices. Results indicated that for IDs smaller than the model's orifice, the dye-front speed within the tube overestimated the predicted (average) excurrent jet speed by no more than 15%. For sampler IDs larger than the excurrent orifice, dye-front speed was lower than the actual excurrent speed. Nevertheless, for IDs up to 40% larger than excurrent orifice, dye-front speeds were in good agreement with the predicted speeds within the tube, calculated as actual excurrent flow rate/tube cross-section area. Even when the samplers' cross-section area was twice that of the excurrent orifice (e.g., Tu 10 sampler over a 6-mm orifice), dye-front speed deviated from the predicted speed by less than 20% (average 12%, SD 17%, n = 12). Thus, pumping rates should be calculated using measurements of dye-front speed with tube IDs similar to or slightly larger than the excurrent orifice. Water flux could then be calculated as the product of sampler cross-section area and dye-front speed within the tube. Alternatively, tubes with ID smaller than the excurrent orifice can be used. In this case, the excurrent flux should be calculated as the product of the dye-front speed and the aperture cross-section area (which should be measured separately). If a plug flow is assumed (e.g., Savarese et al. 1997), this method may overestimate the actual flow rate. The use of a dye-front speed sampler with a much smaller ID is not recommended as, in such case, knowledge of the velocity distribution across the excurrent jet is also required (Charriaud 1982; Savarese et al. 1997; Riisgård 2001*a*).

Ambient flow effect. The effect of sampler orientation with respect to the ambient flow was examined using the same protocol as above for three different orientations: 90° (sampler perpendicular to the flow), 45° (sampler outlet facing into the flow), and 135° (sampler outlet facing downstream). In each orientation, three different samplers were tested: DFS 1, Tu5, and Tu 10 over 2-, 6-, and 10-mm orifices, respectively. Current velocity was fixed to 4 cm s^{-1}. Under such moderate flows, the effect of the sampler's orientation relative to the flow was negligible. For instance, a Tu 10 sampler directed 45° into or along with the flow had a mean deviation of –4% and +5% from the predicted dye-front speed, respectively. This deviation was not significantly different from zero (t test, $P > 0.22$).

The effect of flow velocity over dye-front speed flux estimates was examined for DFS 1 and Tu 10 sampler in an upright 90° orientation. Four ambient velocities were examined: 0, 4, 6, and 11 cm s^{-1}, with the sampler oriented perpendicular to the flow. No influence of flow on dye-front speed was found (one-way ANOVA, $F_{3.35} < 2.2$, $P > 0.1$). Nevertheless, the power of the performed tests was small (>0.3) and thus these negative findings should be interpreted cautiously. In the setup used here, stable excurrent velocity could not be maintained with higher ambient flows and therefore, no quantitative data are available for the effect of higher ambient flows. Nevertheless, qualitative observation under higher ambient flow velocities with various sampler orientations indicated that under such conditions excurrent flow rates could be grossly underestimated.

Comments and recommendations

The direct in situ nature of the InEx technique circumvents many pitfalls inherent to traditional methods used thus far in the study of the physiology and ecology of active suspension feeders (Riisgård 2001*a*). The organisms are studied in their natural habitat with no manipulation or disturbance. When properly applied, the InEx technique provides independent estimates of both water transport rates and removal/production efficiencies. Sampling is rapid and requires simple equipment. The InEx technique can be used with a wide range of common suspension feeders, including fairly small animals with excurrent jet aperture as small as 2 mm in diameter. It can be applied to a wide range of measurements including feeding and respiration (e.g., Yahel et al. 2003), exchange of heavy metals and toxic compounds, and nutrient uptake. InEx-based retention efficiencies, pumping rates, and clearance rates (a product of water flux and retention efficiency) are comparable to published values (*see* Table 3 in Yahel et al. 2003) and are less susceptible to contamination and/or sampling bias. Measurements of dye-front speed were in excellent agreement with continuous in situ excurrent measurements made with

Acoustical Doppler Velocitometer (MicroADV, 16 MHz) over several individual sponges in Eilat (Yahel et al. 2003).

Since the InEx technique requires divers to hold the sampler in a stable position, it is less applicable in rough environments, such as the surge zone. Sampling in extremely turbid water may be problematic since adequate visibility is also required. Nevertheless, underneath the surge zone, sampling is limited mostly by diving safety considerations, and the method was effectively applied in low temperatures (6°C, using dry suits) and high tidal currents (<1 m s^{-1}). Note, however, that while reliable water sampling collection can be carried under such conditions, pumping rate estimates may be affected to an unknown degree by high ambient current. In addition, the InEx technique is not suitable for active suspension feeders that do not have a defined excurrent aperture.

The "pureness" of exhaled water samples depends to a large extent on sampling time and sampler type (*see* below). However, as some contamination of exhaled water with ambient water is unavoidable, the resulting estimates of removal/production efficiencies can be considered to be lower bound. A minimum of four sampler volumes should be pumped through a sampler to flush it clear (Table 2). Samples from organisms with low pumping capacity are thus restricted to few milliliters. In order to use a larger sample volume, which is required for some analytical procedures (e.g., particulate organic carbon [POC], attempts to adapt the plastic bag method of Reiswig (1971*a*) to minute active suspension feeders were made, but with no success. In such cases, the optimal solution seems to be to use a controlled suction device (Yahel unpubl. data unref.).

The selection of sampler size and type for water collection has significant bearing on sampling duration and quality. A smaller sampler reduces sampling time and improves the sample's quality. However, it is recommended that the ratio between the sampler's length and its outlet ID (upper-end) will be kept between 10 and 25. Whenever possible, samplers with narrow tips (e.g., Pt 10, Table 1) are preferable.

Several studies used the product of jet speed and aperture area to estimate water-transport rate (e.g., Fiala-Medioni 1978*a*, 1978*b*, Jones et al. 1992; Savarese et al. 1997). Jet speed–based estimates assumed a known distribution of water velocities across the excurrent aperture (Charriaud 1982). This latter assumption may not be valid for many sponge species (e.g., *Mycale* spp., *Suberites* spp., and *Theonella* spp.) in which large exhalent canals are merged from different directions just below the osculum opening. Our observations with dyed water revealed that excurrent jets in many suspension feeders may be highly turbulent and are frequently composed of several independent jets. In such cases, reliable estimates of the actual water flux may best be achieved through measuring the pumping rate with a short tube that captures the excurrent jet (either with the DFS method as described above or with a flow meter that measures the flow velocity within the tube). This method may be better than measuring the unconfined flow and multiplying it by the excurrent aperture area.

A perfect match between the dye-front speed sampler's inlet and the animal excurrent aperture is hard to achieve under field conditions. Nevertheless, fairly good estimates can be obtained using a set of commercially available glass tubes with ID increments of 1 or 2 mm. If only excurrent jet velocities are of interest, we recommend the use of a dye-front speed sampler with ID smaller than excurrent orifice. However, if pumping rates are under study, the sampler ID should be as close as possible but slightly larger than the diameter of the excurrent orifice. The water flux in this case will be calculated as the product of sampler ID and dye-front speed. This procedure circumvents the need for an additional measurement of excurrent orifice cross-section area (Charriaud 1982; Savarese et al. 1997) and the complications associated with the variable velocity distribution across the excurrent jet.

Rates of uptake (or release) of commodities by active suspension feeders are traditionally reported per animal mass or volume units (Wildish and Kristmanson 1997). The ability to attribute such values to field populations is hampered in many cases when quantification of biomass or living volume of active suspension feeders in the field is impractical, inaccurate, or excessively destructive. Furthermore, colonial taxa often accumulate and incorporate large quantities of exogenous material over or into their tissues (e.g., Reiswig 1985), while the quantification of the biomass or living volume of boring taxa is practically impossible. An important advantage of the InEx technique is that all fluxes are estimated per excurrent jet (Fig. 2), in situ, with no manipulation of the studied organism. This technique thus allows realistic estimates of the organism's performance under natural conditions. Hence, InEx results are suitable for extrapolations to community fluxes, simply by surveying the density of relevant excurrent apertures in the system.

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