Analytical Methods

PAPER

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2014, **6**, 8350

Microfluidic passive samplers for *in situ* collection of live aquatic protists†

Grant M. Bouchillon,[a] Jessica Furrer Chau,[b] George B. McManus[c] and Leslie M. Shor*[ade]

This paper describes the development of microfluidic passive samplers for the collection of live protists from natural aquatic habitats. Microfluidic passive samplers provide several potential benefits over existing sampling methods. For example, they offer greater versatility, higher throughput, and do not require the disruption of specimens through the use of fixatives, stains, or by extraction. In lab testing, a marine ciliate *Cyclidium* sp. was concentrated from 600 cells per mL in a laboratory microcosm to above 2×10^8 cells per mL within individual microfluidic observation galleries. In field experiments, live protists and other microorganisms were collected from surface water and sediment in a northeastern Connecticut stream. Protists were accumulated to 1×10^7 cells per mL in individual observation galleries. Concentrating and isolating protists enables high-resolution, long-term observation of live, unstained protists. The compact arrangement of observation galleries facilitates high-throughput analysis. Sampler versions were created that differed in the degree of channel branching, the spatial density of galleries, and the size and shape of gallery entrance constrictions. Lab and field testing illustrated tradeoffs in performance among sampler variations in terms of the fraction of occupied chambers, overall on-chip biomass density, and in the types of protists and in the range of sizes of protists collected. Recommendations are provided to facilitate the adoption of microfluidic passive samplers for environmental characterization, research, and educational purposes.

Received 16th July 2014
Accepted 22nd August 2014

DOI: 10.1039/c4ay01572j

www.rsc.org/methods

Introduction

Protists are vital links in the carbon and nitrogen cycles in soil, sediment, and surface water. Protistan predation increases bacterial activity,[1] increases the recycle rate of limiting nutrients,[2,3] and enhances the breakdown of anthropogenic compounds.[4] Photosynthetic protists form the basis of much aquatic productivity.[5] Protists can also accumulate contaminants from the environment,[6] thereby making them available to higher trophic levels. Protists can serve as environmental reservoirs for human pathogens.[7,8] The composition of the protistan community structure is used as a diagnostic for wastewater treatment effectiveness.[9,10]

Currently, protist communities are reconstructed using a combination of methods. Direct microscopic examination has long been used to identify protists in environmental samples. This method is most suitable for planktonic organisms present in abundance, and when samples can be readily transported from field to lab for analysis.

Cell abundance can be increased using separation or enrichment. Separations including centrifugation and filter concentration must be employed carefully because protists can lyse under shear stress.[11] Enrichment increases concentrations gradually by cell growth in selective media. Enrichments can be done in the lab from a field inoculum, or in the field using artificial habitats (see *e.g.* ref. 12). Enrichment can complement other techniques and can be used qualitatively to broaden the set of species collected. Studies using enrichment may require laborious characterization of selectivity.

Common methods for examination of protists including density centrifugation using the Ludox-QPS[13] or Percoll-sorbitol

[a]*Department of Civil & Environmental Engineering, University of Connecticut, Storrs, CT, USA. E-mail: leslieshor@gmail.com*

[b]*Department of Physics and Engineering, Benedict College, Columbia, SC, USA*

[c]*Department of Marine Sciences, University of Connecticut, Avery Point, CT, USA*

[d]*Department of Chemical & Biomolecular Engineering, University of Connecticut, Storrs, CT, USA*

[e]*Center for Environmental Sciences & Engineering, University of Connecticut, Storrs, CT, USA*

† Electronic supplementary information (ESI) available: (1) Table S1, abundance of protists collected in field trials by deployment date and location. (2) Table S2, abundance of protists collected in field trials by sampler type. (3) Table S3, abundance of protists collected in laboratory trial with marine sediment. (4) Fig. S1, *Cyclidium* sp. collected in laboratory trial using different microcosm concentrations. (5) Fig. S2, representative micrographs from fast-acquisition scanning of protists in galleries. (6) Movie M1, highly concentrated *Cyclidium* sp. in laboratory enrichment study. (7) Movie M2, protist entering the narrow constriction of a square observation gallery. (8) Movie M3, multiple protists and a metazoan collected from the field in a heart-shaped observation gallery. (9) Movie M4, *Paramecium* sp. trapped within a heart-shaped observation gallery. (10) CAD1, computer aided design of photolithography masks of passive samplers. See DOI: 10.1039/c4ay01572j

method,[14] or fluorescent tagging[15] may involve fixing procedures that not only kill cells (thereby precluding observation of live protists), but can also compromise cell integrity and cause shrinkage.[16]

Different enrichment media can select for different species and, because growth is required before protists become observable, measured abundances will depend on both initial abundances and variations in growth rates.

A third strategy for reconstructing protist communities is to extract, amplify, and analyze genetic material directly from a field sample. Culture-independent molecular methods have revealed extensive genetic diversity in a wide range of natural environments,[17–20] but these methods have their own limitations. Protist species may be differentially recovered from environmental samples, and genetic material can be unevenly extracted and amplified.[21] The molecular tools available to characterize protist communities are generally inferior in number and degree of characterization compared with molecular tools for bacteria or macroscopic plants and animals.[22] Despite ongoing efforts to increase the functional utility of genomic information,[23] these methods cannot describe physical form, motility, grazing behavior, trophic interactions, and interspecific dependencies.

Microfluidic devices are becoming increasingly common in microbiology. Specific applications include characterization of bacterial chemotaxis,[24] analysis of contaminant mixing enhancement due to bacterial motility,[25] and high-throughput methods for bacterial detection and enumeration.[26] Literature reports on the use of microfluidic devices to study protists are somewhat more limited. Prior work has employed microfluidic devices to study chemosensory behavior of *Tetrahymena*,[27] methods for detection of *Cryptosporidium* in drinking water,[28] vortex generation by feeding ciliates in small chambers,[29] dynamics of amoeba lysis with biocide exposure,[30] and restrictions on protist motility by microchannels and microchannel constrictions.[31,32] There have also been several reports where protists were used to illustrate new methods for on-chip cell handling or imaging. For example, Kumano *et al.*[33] used protists to demonstrate hydrodynamic trapping and Zhu *et al.*[15] used protists to demonstrate cell alignment for greater sensitivity in an immunofluorescent assay. Lee *et al.*[34] used the inherent motion of *Euglena* sp. to illustrate a new approach for higher-resolution imaging.

There are few examples of microfluidic devices being used for field-based applications involving protists. Mudanyali *et al.*[35] created a field-portable imaging system for fluorescently-labeled cells. Similarly, Zhu *et al.*[36] developed a cell phone-based system for in-field imaging of waterborne pathogens. In both cases, microfluidics were not used for sampling, rather cells were independently collected, stained and mounted prior to use. Jonsson *et al.*[37] described an aquatic sampling device using acoustic radiation for trapping cells from water samples,

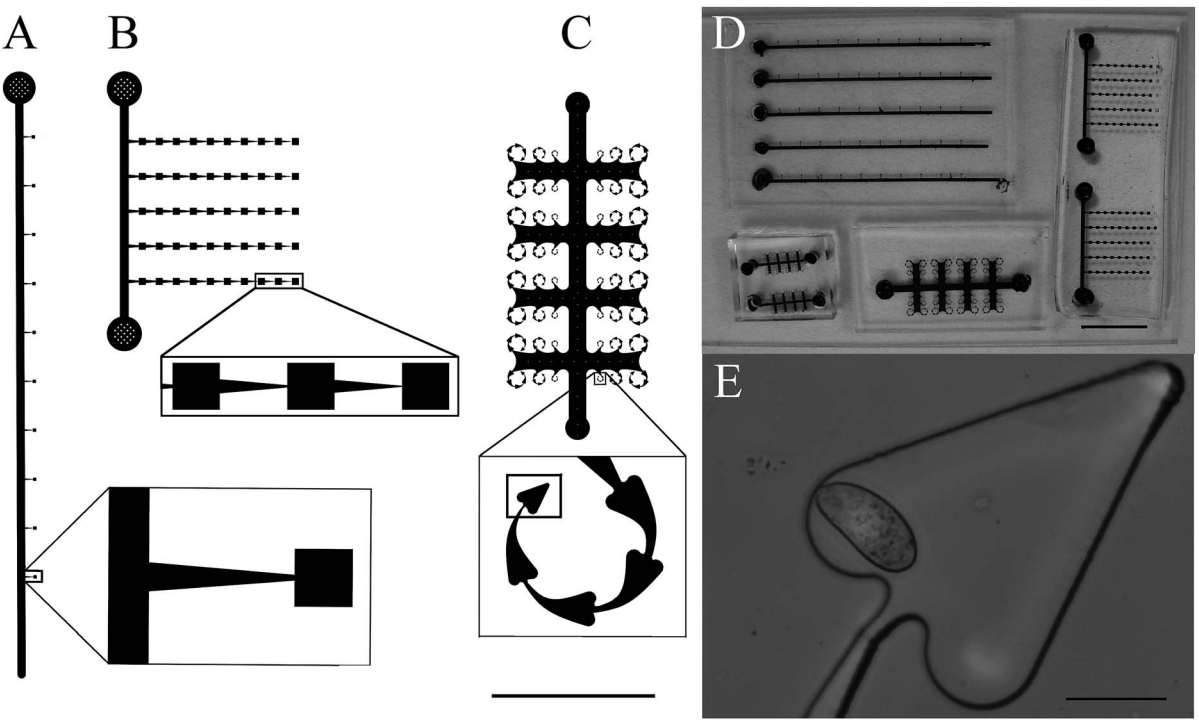

Fig. 1 Microfluidic passive samplers were created in three variations. (A) The simplest variation, "secondary in parallel" has 10 square galleries opening from funnel-shaped side-channels arranged in parallel. (B) Another variation, "secondary in series" has five identical side channels each with ten square galleries. (C). The most complex design, "tertiary" has an additional degree of branching and 48 curved channels in three sizes, each with five heart-shaped galleries. Scale of A, B, C is 1 cm. AutoCAD designs are available in supplemental information. (D) The composite sampler used for field testing was comprised of 5 secondary in parallel, two secondary in series, and two small and one large tertiary samplers arranged as shown. Microfluidic structures have been filled with black dye. Scale 1 cm. (E) Example micrograph of a trapped protist in a terminal gallery in the tertiary sampler (400×, phase contrast, scale 50 μm).

including at great depth. Their device was intricate, with 10 layers in the design, and was tested exclusively in a lab setting using beads and GFP-labeled yeast cells. To our knowledge, a microfluidic approach has never been demonstrated in the field as a sampling tool to characterize protist communities from natural environments.

Here we describe the development and validation of microfluidic passive samplers designed to concentrate and isolate live planktonic or benthic protists from aquatic habitats. Each passive sampler is comprised of a network of branching microchannels opening onto nanoliter-scale observation galleries (Fig. 1). The size and shape of gallery entrances tends to concentrate motile protists, just as lobster pots and fish weirs concentrate larger aquatic organisms. When contained within an observation gallery, protists move freely. Most of our galleries fit fully within the field of view of a 10× objective. Protist containment permits extended-time, high-magnification characterization of protist morphology and other behaviors without the need for mobility inhibitors, fixatives, or stains. The technology lends itself to both high-content and high-throughput imaging of entrapped protists. Three variations were successfully developed and tested, illustrating the robustness and versatility of the approach.

Materials and procedures

Device fabrication

Microfluidic passive samplers were fabricated from polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) using standard methods of photolithography and soft lithography as described elsewhere.[38–40] Briefly, master templates of each variation were fabricated by selectively cross-linking SU-8 2025 negative photoresist (Microchem, Newton, MA) coated to a desired thickness onto a 3 inch-diameter silicon wafer (Nova Electronic Materials, test grade, Flower Mound, TX). Coated wafers were selectively exposed to ultraviolet light at 50 mW cm^{-2} using a chrome-on-glass photomask (Advanced Reproductions, Andover, MA). The time of UV exposure increased with photoresist height and ranged from 3.0 s to 6.1 s. Where exposed to UV light, photoresist was chemically cross-linked during a subsequent post-exposure bake (95 °C, 9 min). Non-cross-linked photoresist was removed using SU-8 developer (Microchem, Newton, MA, 15 min). Masters were washed with isopropyl alcohol (70% v/v, Fisher Scientific, Waltham, MA) and acetone (HPLC grade, Fisher Scientific, Waltham, MA) then solvent was evaporated in N_2 (99.0%, Airgas Inc., Salem, NH). Photoresist heights were verified to ±1 μm by profilometry (Dektak 150, Veeco, NY).

Replicate samplers were created by casting masters in PDMS. PDMS was mixed 10 : 1 with cross-linker and de-gassed for 20 minutes in a vacuum desiccator. Uncured PDMS was poured over each master to a depth of 2–3 mm then cured (60 °C for at least 3 h). Castings were peeled from the master, trimmed, and entrance wells punched with a 2 mm diameter biopsy punch (Miltex Inc., York, PA). PDMS was cleaned with isopropyl alcohol, dried, and then exposed to oxygen plasma to activate surface groups (Model PDC-32G, 18W RF Coil, Harrick Plasma, Ithaca, NY). Finally, PDMS was bonded feature-side down to a methanol-cleaned, plasma-treated 2 × 3-inch glass microscope slide in the arrangement shown in Fig. 1. Prior to deployment in the field, devices were filled with sterile 0.2 μm-filtered natural waters. With the aid of capillary action, trapped air in dead-end channels permeates out of the PDMS allowing channels to fill with water within 10 minutes.

Laboratory microcosms

Laboratory testing of passive microfluidic samplers was done using pure cultures of marine ciliates in a seawater and sand microcosm with the "tertiary series" variation. First, 550 g clean sand was combined with 700 mL 2/3 ocean strength Artificial Seawater for Protozoa (ASWP)[31,32] in a sterile 170 × 90 Kimax glass dish with glass lid, autoclaved for 60 min, then held at room temperature for 72 h to allow any cysts to excyst. The supernatant was decanted and fresh ASWP was added, then the microcosm was autoclaved again. Finally, the decanting, replacing ASWP, and autoclaving steps were repeated once more.

Next, stationary-phase cultures of live marine ciliates *Cyclidium* sp. and *Euplotes vannus* were washed to remove excess bacteria then added to the microcosm. The total protist concentrations in the microcosm was approximately 4.5×10^5 *Cyclidium* and 3×10^4 *E. vannus* cells in 700 mL. Finally, the "tertiary series" sampler was suspended in the water column. Protists in galleries were enumerated after 36 h.

In some cases, bacteria in galleries were enumerated at higher magnification. When gallery size was greater than the field of view, bacteria in three 50 μm × 50 μm frames were enumerated and the average count extrapolated to the total gallery area.

Field testing

Composite samplers configured as shown in Fig. 1D were field tested in the Willimantic River in northeastern Connecticut. Samplers were placed in sediment and surface water locations 1 m, 10 m, and 60 m downstream of the University of Connecticut wastewater treatment plant outfall (41.784N, 72.281W, near USGS Station 1119500). Composite samplers were attached to custom-built stainless steel holders and placed either 3 cm below the sediment surface or 0.3 m below the water surface. Stream depth was 1 m at the time of sampling.

Field sampling was conducted in two consecutive trials. Samplers were placed in the field from 08-Nov-2011 to 13-Nov-2011, recovered, and new samplers placed from 13-Nov-2011 to 16-Nov-2011. Approximate surface water conditions during sampling were 8 °C, pH 7.4, and dissolved oxygen concentration 6.8 mg L^{-1}.

At recovery, composite samplers were rinsed with stream water to remove adhering sediment from the flat surfaces, but any debris in entrance wells was retained. Entrance wells were covered with sterile cured PDMS, and samplers were placed into individual Petri dishes. Dishes were wrapped in parafilm and contained a small amount of sterile deionized water to prevent water loss. Samplers were returned to the laboratory and stored

in the dark at 4 °C up to 24 h prior to imaging, although most of the samplers were observed within 6–8 hours of retrieval. As the storage time is short compared to the 72 hours of sampling time, we are confident that most of the protists were captured during the sampling time.

Imaging of field-captured protists

Passive samplers were imaged directly on a fully-automated inverted microscope (Zeiss AxioObserver.Z1 AX10) using a programmable scanning stage and a 10× objective (Zeiss Plan-Apochromat 10×/.45 M27) equipped with differential interference contrast (DIC). Video images were captured at 15 frames per second (fps) using an MRm high-speed CCD camera at a resolution of 1388 × 1040 pixels. In some cases, higher-magnification, higher-resolution images and videos were collected following low-magnification scans.

Although we observed diatoms and other microalgae from both surface water and benthic habitats, this report focuses on non-pigmented forms. Due to the limited resolution of high-speed image capture, only protists with a major axis >10 μm were cataloged. For organisms of sufficient size, major and minor axes were measured at 2.5 μm resolution from a representative frame of the scan video. Protist heights were not measured. For calculation of biovolume, heights were set to the minimum of the gallery height or the measured minor axis.

Sampler designs

To illustrate the effects of sampler geometry on field sampling performance, three variations of microfluidic passive samplers were developed. Variations differed in the degree of branching and in the arrangement of galleries along channels (Fig. 2). In each case, primary channels are defined as those that open from entry ports, and observation galleries are arranged along either secondary or tertiary channels.

The simplest sampler variation featured ten observation galleries arranged in parallel (Fig. 2A, "secondary in parallel"). Each gallery was located at the end of a short entrance channel, and the ten entrance channels are arranged in parallel along the primary channel. Reminiscent of miniature lobster traps, each gallery measuring 200 μm × 200 μm opens from a funnel-shaped entrance channel (Fig. 1A, detail). The funnel-and-gallery design is based on our previous work, where we found crawling marine ciliates successfully traversed channels that narrowed gradually, but were unable to re-enter narrow channels from a large chamber.[32] Versions of the secondary in parallel variation were created with five different entrance constrictions: 20, 15, 10, 5, or 2 μm wide. One copy of each version was included in the composite sampler (Fig. 1D). The measured depth of microfluidic features was 20 μm.

In the second variation, ten 200 μm × 200 μm galleries are arranged in series along each of five identical secondary channels (Fig. 2B, "secondary in series"). Gallery constrictions are sequentially smaller, measuring 100, 90, 80, 70, 60, 50, 40, 30, 20, and 10 μm wide. The measured depth of microfluidic features was 37 μm.

The third variation featured a fern-like arrangement of eight tertiary channels each with 5 sequentially-smaller heart-shaped galleries arranged in series (Fig. 2C, "tertiary series"). Here, reentrant gallery entrances are flanked by lobes. The design is reminiscent of fish weirs, which have been used for centuries to concentrate fish in surface waters, and similar to "ratchet" features in a microfluidic bacteria sorter.[41] The tertiary channels are curved to increase gallery area density. Two versions of the tertiary variation were created: "large tertiary" has gallery entrance constrictions ranging from 5 to 75 μm wide and was fabricated 25 μm high. "Small tertiary" is an exact 2/5 scale replica, with gallery entrance constrictions ranging from 2 to 30 μm wide and it was fabricated 10 μm high.

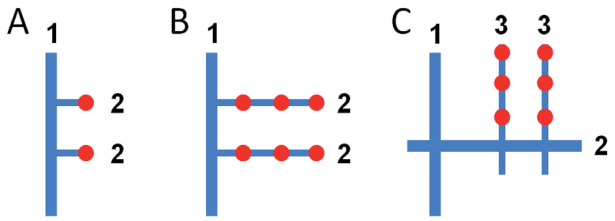

Fig. 2 Schematic showing degree of branching of microchannels (blue lines) and arrangement of galleries (red circles) in the three sampler variations. (A) In the simplest variation, observation galleries terminate secondary channels that are arranged in series. (B) In the next variation, observation galleries are arranged in parallel along secondary channels arranged in series. (C) In the third variation, observation galleries are arranged in parallel along tertiary channels arranged in series.

Results and discussion

Microfluidic passive samplers act as an artificial protist habitat, enriching protists *in situ*, and isolating protists into individual observation galleries. Samplers are passive. There is no fluid flow during sampling, and no mechanical or electrical components are required. Protists move through samplers by their own motility.

Unlike other recent reports, the samplers described here require no complex flow control[20,22,41] or integrated imaging systems.[15,34,42] The drawback of this approach is the particular geometry of the sampler creates an inherent bias, selectively enriching certain forms based on size, shape, and locomotion characteristics.

Protist concentration from lab microcosms

Microfluidic passive samplers were effective in concentrating protists from lab microcosms. Pure cultures of the ciliated protist *Cyclidium* sp. were increased from a concentration of 600 cells per mL in a microcosm to a maximum gallery concentration of 2.3×10^8 cells per mL in the tertiary sampler (Fig. 3). In some cases, galleries were entirely filled with protists (see movie M1 in ESI,† and "Max Abundance" line in Fig. 3B).

Protist concentrations increased steadily in successive galleries arranged in series (Fig. 3B). Consecutive galleries are numbered as shown in Fig. 3: "Gallery 1" is closest to the entrance and "Gallery 5" is the channel terminus. The average protist concentration Gallery 1 was 3.9×10^6 cells per mL. The

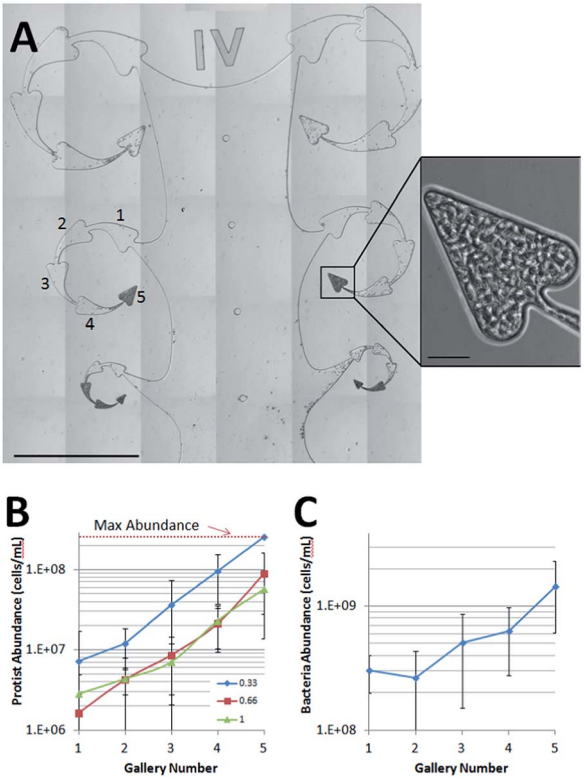

Fig. 3 (A) Tiled micrograph images of a portion of a "tertiary series" sampler used for lab testing. Concentrations of *Cyclidium* sp. become greatly enriched towards the 5th gallery in the series, in some cases, completely filling the terminal gallery with protists (inset). Scale 1 mm and 50 μm for inset. (B) Protist abundance in same-sized tertiary channels by gallery number. The data series for the largest-sized channels is labeled "1" and smaller channels are labeled to reflect relative size: average and standard deviation of 18 channels in a tertiary sampler. (C) Average bacteria abundance within six channels, two of each size: average and standard deviation. Note log scale.

average concentrations in the next four galleries were 6.9×10^6, 1.7×10^7, 4.6×10^7, and 1.3×10^8 cells per mL, respectively. Concentrations of *E. vannus* also increased steadily in successive galleries (data not shown).

Bacteria were also enumerated in successive galleries (Fig. 3D), and bacteria concentrations were also greatest in the smallest terminal galleries. Overall, bacteria concentrations were about 2 orders of magnitude higher than *Cyclidium* concentrations, and the highest bacteria concentrations were about 5×10^9 cells per mL.

Passive microfluidic enrichment of motile microorganisms has been described previously for *Escherichia coli* by Hulme *et al.*[41] These authors reported the mechanism for "ratcheting" was the shape of microchannels, where outward-moving bacteria are re-directed more often than inward-moving bacteria. In our work, enrichment may likewise result from channel shape. Additional mechanisms resulting in concentration along galleries may include chemotaxis toward higher prey concentrations, or some form of coordinated motility among protists, as suggested previously by Lawrence & Snyder[43] and Ogata *et al.*[44]

Although keeping the microcosm in line with the total protist biomass, the single-species biomass available for concentration in the microcosm is much higher than would be found in natural environments: this microcosm contained 1.3×10^4 cells per mL of *Cyclidium* sp., compared with 1×10^4 cells per mL for all species of protists combined in a typical surface water.[14] The high concentration was used to demonstrate the ratcheting effect of the samplers. Real populations would have a diversity of sizes, shapes, and modes of motility. Thus, the extreme efficiency of concentration achieved in individual observation galleries in the microcosm test would be less likely to occur during field sampling.

When seeking to identify the efficiency of the devices, an experiment, like that of the lab microcosm mentioned previously, was undertaken with the main variable being the concentration of the microbes in starting solution.

The samplers of the small tertiary variation were put in 9 different cultures of *Cyclidium* sp. in ASWP. The concentration of the cultures was modified by varying the amounts of carbon available. A range of *Cyclidium* sp. concentrations in the cultures varied from 2.1×10^3 cells per mL to 2.7×10^4 cells per mL. The amount of captured organisms in the sampler after 24 hours (Fig. S1†) had no correlation with the concentration of the cultures it was placed in. The average amount of organisms captured per sampler was 223 with a standard deviation of 125. A hypothesis for this is that the samplers may have "capture capacity" or a maximum flux of organisms based on the entrance size. This leads to the conclusion that over a certain period of time the sampler, although able to trap and concentrate under short-term conditions, may possibly come to equilibrium with the surrounding environment. This would allow for long-term experimentation and continuous monitoring of natural environments as the ecosystem develops around the sampler.

An experiment was conducted wherein 20 samplers, secondary in series variation, were placed in natural marine sediment. The samplers were observed, counted, and repositioned each day for 4 days (Table S1†). Baiting the samplers with algae was also employed. The protists within the sampler were compared with protists captured using conventional methods such as filtration. It was revealed that the relative abundance of species differed. It was seen that *Holosticha* sp. and *Prostome* sp. were much more likely to be represented than that of *Strombidium sulcatum* when compared to the actual abundance within the sediment sample, although no more than a 20% difference in any case. The mechanism for this bias is unknown and would be an avenue for further study. Thus this method would be better suited when studying an ecosystem's species richness rather than relative abundance.

Field-sampling and imaging of live protists

Field testing was performed using composite samplers comprised of all sampler variations (Fig. 1D). High-content imaging is facilitated by protist isolation. Composite samplers were comprised of hundreds of individual galleries. The excess number of galleries means trapped protists tend to be

distributed individually in galleries. In our field test, 82% of protists cataloged were observed alone, 13% were observed in a group of two, and 5% were observed in groups of three or more. The maximum group size was seven protists. This shows the high degree of sample isolation that may occur within the device, for easy reference, cataloging, or observation.

Protists contained in galleries can be observed over extended periods. In both lab and field trials, we have routinely observed live protists for 48–72 hours, similar to durations achieved by other trapping mechanisms.[33] In some cases entrapped protists from laboratory cultures remained viable for up to 2 weeks if protected against desiccation and if the buffer was periodically refreshed.

In our work, approximately 9960 galleries were imaged within 24 hours of retrieval on each of two sample retrieval dates. Due to the short storage time, protist reproduction was possible but limited. It was only observed once during the entire sampling procedure. High-speed scanning is facilitated by the compact, fixed arrangement of galleries along a single focal depth. Rapid sample processing avoids sample deterioration. At 15 fps, there was ample opportunity to image each individual protist in multiple orientations, thereby capturing motility characteristics of live protists. Such information is seldom available with other sampling methods but can greatly aid identification. Representative frames (Fig. S2†) illustrate the quality of images collected during fast acquisition and used for cataloging individual protists.

Composite samplers can be scanned in as little as 5 minutes based on currently-available high-speed imaging capabilities. An automated system with referenced gallery positions could further reduce the time needed for scanning. Additionally, cataloging and image recognition software[42] could be employed to reduce operator bias and reduce image acquisition time.

Sampler performance

Composite samplers were deployed in consecutive field trials to each of three locations in a Connecticut stream in both water column and sediment habitats. In the first trial, a total of 78 protists were collected from surface water, and 83 protists were collected from sediment. In the second trial, 139 protists were collected from surface water and 76 protists were collected from sediment (Table S2†). Protist counts in consecutive sampling trials differed by a factor of 2 for surface water and by 10% for sediment. This sampling technology is an enrichment method, and therefore should not be considered quantitative without extensive characterization of enrichment efficiency. However, repeat sampler performance at the same site provides an indication of the reproducibility of the sampling method.

The accumulation of protists was also consistent between the three sites along the river and the two habitat types (Table S1†). Overall, 58% of protists were captured from surface water and 42% from sediment. Among the three locations, 33% were found at Site 1, 37% at Site 2, and 30% at Site 3.

Sampling performance in terms of the number and size of captured protists differed widely with sampler variation. The majority of protists were found in tertiary samplers. Overall, 40% of protists were found in large tertiary samplers, 30% were found in small tertiary samplers, 25% were found in secondary in series samplers and the remaining 5% were found in secondary in parallel samplers (Table S3†).

The secondary in parallel variation collected the fewest total protists, but had the highest chamber occupancy rate. For an example of protists trapped in this gallery form, see movie M2 in the ESI.† Of approximately 600 total galleries from secondary in parallel samplers, 16% contained a protist. The next highest occupancy rate was 5.2% for the large tertiary device. The secondary in series and small tertiary devices each had a gallery occupancy rate below 2%. The fraction of occupied galleries has implications for the scan time required to search for protists among empty galleries while lower abundance may prolong survival time of protists in galleries.

Each sampler variation and version featured a different range of gallery constriction sizes. Biovolumes are plotted against the cross-sectional area of the gallery constriction for each sampler variation (Fig. 4). All sampler varieties captured at least one protist across the entire range of entrance constrictions created. For example, the secondary in parallel variation captured protists in at least one of the observation galleries connected to each constriction size across the entire 40–400 μm^2 range of entrance constriction sizes.

The secondary in series variation contained the broadest range of protist biovolumes. Protists with biovolumes ranging from 130 to 740 000 μm^3 were captured in different galleries in this sampler version.

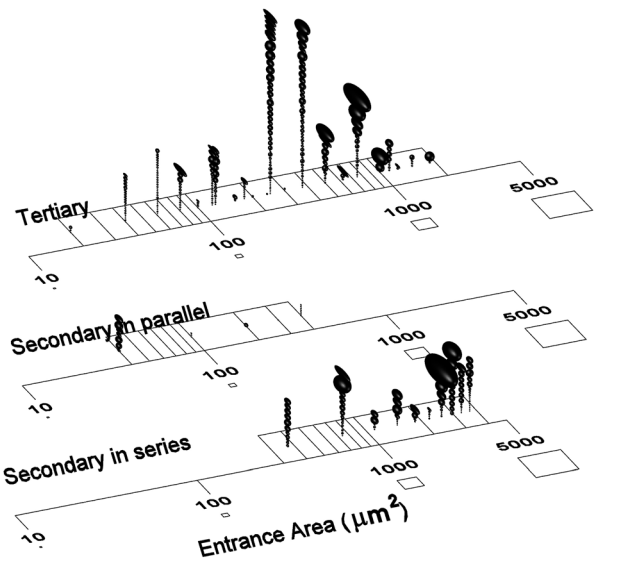

Fig. 4 Sizes of trapped protists *versus* sampler constriction dimensions. Cross-sectional areas of individual protists plotted against the area of the entrance constriction of the gallery where they were found. Protists are stacked on each position to show abundance of protists and range of sizes found. Relative constriction sizes are shown by the open boxes along the axis and are on the same scale as the approximate 3D reconstructions of protists. Some protists are apparently larger than the constrictions they have passed, suggesting exceptional ability to squeeze through narrow openings.

The small tertiary samplers with gallery entrance constrictions ranging from 20 to 300 μm^2 were effective in capturing the smaller protists. These protists had cross-sectional areas ranging from 26 to 923 μm^2. Large tertiary samplers with gallery entrance constrictions ranging from 125 to 1875 μm^2 captured protists with cross-sectional areas ranging from 26 to 4334 μm^2. Large tertiary devices contained about 50% more protists than secondary in series devices in about 50% less total gallery volume.

The variability in protist sizes found within the even individual galleries was due to both the shape and size of constrictions and size, shape, plasticity and motility mode of protists. For an illustration of a variable range of protist forms found in a single gallery, see movie M3 in the ESI.† Nearly one-quarter of protists had cross-sectional areas >150% of the cross-sectional area of the gallery constriction. Nearly 3% of protists were observed in galleries with entrance constrictions 5 times smaller than their estimated ellipsoid cross-sectional area. Protists with cross-sectional areas significantly larger than gallery entrance were observed and imaged for extended periods (for an example of a protist trapped within a gallery due to a small constriction, see movie M4 in ESI†).

Limitations, implementation, and extensions

The samplers described here can be placed in a range of different habitats, including seawater or freshwater, and in water column or sediment. Devices can also be deployed for short times or extended periods. Unlike other reports,[15,34,36,37] our samplers require no post-processing, staining or fixing of specimens. No sampler maintenance or monitoring was required. Upon retrieval, live protists are already contained within an observation cell that can be placed directly on a microscope.

Protists may be differentially accumulated, or may differentially survive, within sampler galleries. From our testing, we suspect the devices described in this report may be best suited to surface-associated crawling ciliates, which are found in both benthic and pelagic habitats, including the surfaces of suspended flocs and other solids. Protist sampling biases of current variations are unknown without further testing. Development of adapted sampler geometries that favor the truly pelagic protists, such as the ciliates responsible for much of the planktonic herbivory in oceans and lakes, is ongoing.

Initial experimentation on protists also suggests a maximum "capture capacity" for individual samplers may exist. As a result, sampler variations described here may be better suited for characterizing relative community composition and species richness *versus* quantitatively determining abundance.

Future work can implement microfluidic passive samplers in a range of different environments. These environments may include wastewater treatment plants, the deep ocean, or surface waters during a harmful algal bloom. In selecting a sampler variation for a particular application, the sampler features should be matched to the application's requirements. For example, the simplest sampler geometry, the secondary in parallel design, is well suited to characterize the protist community in wastewater treatment plants. More than 175 species of ciliates have been reported from wastewater, including large species including *Amphileptus*, *Euplotes*, and *Carchesium* spp.[45] The secondary in parallel variation offers high occupancy rates and entrance sizes that can be tailored to the protist forms that indicate good process performance of aerobic digestion in wastewater treatment.

In deep sea environments, a variety of novel ciliate forms have been observed, including lineages not previously described in surface waters. Where little is known about a protist community, the secondary in series variation offers features suited to initial characterization. This sampler variation offers the advantages of a large volumetric capacity suitable for extended sampling, and a broad range of entrance sizes for collecting a wide distribution of protist sizes.

Monitoring of potential bloom species of dinoflagellates (*e.g. Dinophysis* spp.) currently involves frequent sampling and microscopy or expensive *in situ* flow cytometer instruments (*e.g.* Campbell *et al.* 2010 ref. 46). Our devices, deployed *in situ*, may serve to concentrate bloom species before they attain high abundance in the water column. The large concentrating effect and compact gallery composition of the tertiary variation could make this sampler the ideal choice for this application.

Integration of passive sampling with imaging *in situ* will further enhance performance. For example, automated imaging would eliminate the need for skilled operators to periodically observe protists *in situ* for characterization of wastewater performance.[47] Integrated sampler/imaging systems could be deployed to extreme environments using deep ocean "gliders" to build sea plankton and protist inventories. Systems affixed to buoys could remain in place over long periods and provide early warning of periodic events such as harmful algal blooms. Finally, advanced imaging techniques such as axially distributed sensing[48] could be incorporated to allow live organisms to be visualized with high resolution and in 3D, possibly in the field.

Use of these samplers in conjunction with molecular genomics techniques such as sequencing, fluorescence *in situ* hybridization, and analysis of gene expression patterns could further delineate linkages between form and function for individual protists in diverse ecosystems.

Microfluidic devices may also be cost-effective as a tool for K-12 education. PMDS microfluidic devices have a total materials cost of around $1 each. While production of masters using photolithography requires specialized equipment and skills, masters may be ordered from fabrication vendors, or fabricated more easily using 3D printing for large designs.

The samplers described here offer many advantages over existing methods, but they also have some limitations. Like all environmental sampling methods, the inherent selectivity and bias of microfluidic passive samplers must be carefully characterized for each application. This research offers a starting point in the development of a family of microfluidic passive samplers tailored for different applications through further testing and development.

Acknowledgements

This study was supported by grant 1027125 from the National Science Foundation Division of Biological Infrastructure. We thank Jinzi Deng for designing an early version of the Tertiary device, Steven J. Tuorto and Gary L. Taghon for testing early prototypes, and Guleid Awale, Megan Nolan, and Erika P. Orner for assistance with method development and image analysis.

References

1 T. Posch, K. Simek, J. Vrba, J. Pernthaler, J. Nedoma, B. Sattler, B. Sonntag and R. Psenner, *Aquat. Microb. Ecol.*, 1999, **18**, 235–246.
2 P. J. Kuikman, A. G. Jansen, J. A. Veen and A. J. B. Zehnder, *Biol. Fertil. Soils*, 1990, **10**, 22–28.
3 D. A. Wright, K. Killham, L. A. Glover and J. I. Prosser, *Appl. Environ. Microbiol.*, 1995, **61**, 3537–3543.
4 S. F. Tso and G. L. Taghon, *Microb. Ecol.*, 2006, **51**, 460–469.
5 P. Falkowski, *Photosynth. Res.*, 1994, **39**, 235–258.
6 C. Adam and J. Garnier-Laplace, *Limnol. Oceanogr.*, 2003, 2303–2313.
7 M. R. W. Brown and J. Barker, *Trends Microbiol.*, 1999, **7**, 46–50.
8 G. Greub and D. Raoult, *Clin. Microbiol. Rev.*, 2004, **17**, 413–433.
9 J. Liu, M. Yang, R. Qi, W. An and J. Zhou, *Water Res.*, 2008, **42**, 1907–1918.
10 C. A. Papadimitriou, A. Papatheodouiou, V. Takavakoglou, A. Zdragas, P. Samaras, G. P. Sakellaropoulos, M. Lazaridou and G. Zalidis, *Desalination*, 2010, **250**, 378–382.
11 D. J. Gifford, *Mar. Ecol.: Prog. Ser.*, 1985, **23**, 257–267.
12 J. Cairns Jr, D. Kuhn and J. Plafkin, *Methods and Measurements of Periphyton Communities: A Review*, STP, 1979, vol. 690, pp. 34–57.
13 K. Xu, Y. Du, Y. Lei and R. Dai, *European Journal of Protistology*, 2010, **46**, 263–270.
14 D. M. Alongi, *Estuarine, Coastal Shelf Sci.*, 1986, **23**, 443–450.
15 L. Zhu, Q. Zhang, H. Feng, S. Ang, F. S. Chau and W.-T. Liu, *Lab Chip*, 2004, **4**, 337–341.
16 C. A. Jerome, D. J. Montagnes and F. Taylor, *J. Eukaryotic Microbiol.*, 1993, **40**, 254–259.
17 D. Bass and T. Cavalier-Smith, *Int. J. Syst. Evol. Microbiol.*, 2004, **54**, 2393–2404.
18 M. Doherty, B. A. Costas, G. B. McManus and L. A. Katz, *Aquat. Microb. Ecol.*, 2007, **48**, 141.
19 A. Dopheide, G. Lear, R. Stott and G. Lewis, *Appl. Environ. Microbiol.*, 2008, **74**, 1740–1747.
20 L. A. Katz, G. B. McManus, O. L. Snoeyenbos-West, A. Griffin, K. Pirog, B. Costas and W. Foissner, *Aquat. Microb. Ecol.*, 2005, **41**, 55–65.
21 F. Zhao and K. Xu, *Acta Ecol. Sin.*, 2012, **32**, 209–214.
22 D. A. Caron, *BioScience*, 2009, **59**, 287–299.
23 C. Bowler, D. M. Karl and R. R. Colwell, *Nature*, 2009, **459**, 180–184.
24 J. R. Seymour, Marcos and R. Stocker, *Am. Nat.*, 2009, **173**, E15–E29.
25 R. Singh and M. S. Olson, *Environ. Sci. Technol.*, 2011, **45**, 8780–8787.
26 C. Sakamoto, N. Yamaguchi and M. Nasu, *Appl. Environ. Microbiol.*, 2005, **71**, 1117–1121.
27 S.-W. Nam, D. V. Noort, Y. Yang and S. Park, *Lab Chip*, 2007, **7**, 638–640.
28 H. Bridle, M. Kersaudy-Kerhoas, B. Miller, D. Gavriilidou, F. Katzer, E. A. Innes and M. P. Y. Desmulliez, *Water Res.*, 2012, **46**, 1641–1661.
29 D. van Noort, *μSwimming pools for ciliates*, Groningen, The Netherlands, 2010.
30 M. F. Santillo, M. L. Heien and A. G. Ewing, *Lab Chip*, 2009, **9**, 2796–2802.
31 W. Wang, L. M. Shor, E. J. LeBoeuF, J. P. Wikswo, G. L. Taghon and D. S. Kosson, *Appl. Environ. Microbiol.*, 2008, **74**, 1945–1949.
32 W. Wang, L. M. Shor, E. J. LeBoeuf, J. P. Wikswo and D. S. Kosson, *Appl. Environ. Microbiol.*, 2005, **71**(8), 4628–4637.
33 I. Kumano, K. Hosoda, H. Suzuki, K. Hirata and T. Yomo, *Lab Chip*, 2012, **12**, 3451–3457.
34 S. A. Lee, G. Zheng, N. Mukherjee and C. Yang, *Lab Chip*, 2012, **12**, 2385–2390.
35 O. Mudanyali, C. Oztoprak, D. Tseng, A. Erlinger and A. Ozcan, *Lab Chip*, 2010, **10**, 2419–2423.
36 H. Zhu, O. Yaglidere, T.-W. Su, D. Tseng and A. Ozcan, *Lab Chip*, 2011, **11**, 315–322.
37 J. Jonsson, S. Ogden, L. Johansson, K. Hjort and G. Thornell, *Lab Chip*, 2012, **12**, 1619–1628.
38 D. A. Markov, P. C. Samson, D. K. Schaffer, A. Dhummakupt, J. P. Wikswo and L. M. Shor, *J. Visualized Exp.*, 2010, **39**, e1741.
39 D. A. Markov, S. Manuel, L. M. Shor, S. R. Opalenik, J. P. Wikswo and P. C. Samson, *Biomed. Microdevices*, 2010, **12**, 135–144.
40 J. Deng, A. Dhummakupt, P. C. Samson, J. P. Wikswo and L. M. Shor, *Anal. Chem.*, 2013, **85**, 5411–5419.
41 S. E. Hulme, W. R. DiLuzio, S. S. Shevkoplyas, L. Turner, M. Mayer, H. C. Berg and G. M. Whitesides, *Lab Chip*, 2008, **8**, 1888–1895.
42 A. Schaap, T. Rohrlack and Y. Bellouard, *Lab Chip*, 2012, **12**, 1527–1532.
43 J. Lawrence and R. Snyder, *Can. J. Microbiol.*, 1998, **98**, 238–244.
44 M. Ogata, T. Hondou, Y. Hayakawa, Y. Hayashi and K. Sugawara, *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.*, 2008, **77**, 011917.
45 C. R. Curds, *An illustrated key to the British freshwater ciliated protozoa commonly found in activated sludge*, HMSO, 1969.
46 L. Campbell, R. J. Olson, H. M. Sosik, A. Abraham, D. W. Henrichs, C. J. Hyatt and E. J. Buskey, *J. Phycol.*, 2010, **46**, 66–75.
47 A. Nicolau, N. Dias, M. Mota and N. Lima, *Res. Microbiol.*, 2001, **152**, 621–630.
48 R. Schulein, M. DaneshPanah and B. Javidi, *Opt. Lett.*, 2009, **34**, 2012–2014.