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AQUATIC FLOW CYTOMETRY: ACHIEVEMENTS AND PROSPECTS. *M. RECKERMANN and F. COLIJN (eds.)*

Flow sorting in aquatic ecology*

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SUMMARY: Flow sorting can be a very helpful tool in revealing phytoplankton and bacterial community structure and elaborating specific physiological parameters of isolated species. Droplet sorting has been the most common technique. Despite the high optical and hydro-dynamic stress for the cells to be sorted, many species grow in culture subsequent to sorting. To date, flow sorting has been applied to post-incubation separation in natural water samples to account for group-specific physiological parameters (radiotracer-uptake rates), to the production of clonal or non-clonal cultures from mixtures, to the isolaton of cell groups from natural assemblages for molecular analyses, and for taxonomic identification of sorted cells by microscopy. The application of cell sorting from natural water samples from the Wadden Sea, including different cryptophytes, cyanobacteria and diatoms, is shown, as well as the establishment of laboratory cultures from field samples. The optional use of a red laser to account for phycocyanine-rich cells is also discussed.

Key words: flow cytometry, flow sorting, phytoplankton, bacteria, cultures.

INTRODUCTION

The essence of flow cytometry is the simultaneous measurement of different optical cell properties (light scatter and multicolour fluorescence emission), which allow the characterization and classification of individual cells in a mixture (Hofstraat *et al.*, 1991; Veldhuis and Kraay, 2000). The aquatic sciences have started to make use of flow cytometers in the late 1970s (Paau *et al.*, 1978). The autofluorescence of the naturally occurring chlorophylls and phycobilins in phytoplankton set the natural premise for the application of flow cytometry, and it was not long before flow cytometry became a widely used tool in characterizing marine phytoplankton assemblages (Yentsch and Horan, 1989). Cells having identical or similar optical properties appear as distinct clusters in a two-parameter histogram or dot plot, which are more or less separated from each other. Flow sorting takes this "virtual" separation one step further, by physically separating the cells from the mixed assemblage. Since the early days of commercially available flow cytometers, sorting has been a routine feature of many instruments.

The identification and differentiation of malignant blood cells has been the driving force for the development of flow cytometers, and especially cell sorters. However, sorting phytoplankton from a natural water sample poses slightly different requirements to the instrumentation and procedures than sorting cells from blood or tissue cultures in medicine. While in clinical applications, very high sorting speeds (up to several 10.000 cells/s) and a high yield (i.e. as many cells as pos-

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sible per unit time) are desirable and feasible due to the high concentrations of the "sorting goods", aquatic field samples are generally very dilute, and cells are sorted at rates of less than one to a few hundred per second, depending on the concentration. Large diatoms or dinoflagellates can be as scarce as a few cells per litre, making it virtually impossible to have them sorted by a standard commercially available cell sorter. At the other end of the size spectrum, autotrophic picoplankton (especially *Synechococcus* and *Prochlorococcus*) and heterotrophic bacteria can reach abundances of a few 100.000 up to several million per millilitre, and are thus much better suited for sorting, which is reflected in the current literature (see review by Davey and Kell, 1996). This paper gives a bief overview over the basic sorting methodology and describes applications of flow sorting in aquatic ecology with its associated problems.

SORTING PRICIPLES

Analytical flow cytometers (i.e. those without sorting capability) emerged in the first half of the 20th century. The first design of a fluidic flow cytometer is generally attributed to Moldavan (Moldavan, 1934). It is interesting that one of the first instruments was constructed to analyze airborne particles rather than those suspended in water (Gucker *et al.*, 1947), a work that was sponsored by the U.S. Army during World War II and was motivated by the threat of airborne pathogenic bacteria and spores in the event of biological warfare (Shapiro, 1995). Feasible methods for flow sorting in the 1960s and thereafter (Fulwyler, 1965; Kamentsky and Melamed, 1967; Friedman, 1973; Dühnen *et al.*, 1983).

To those unfamiliar with flow cytometry and sorting physics, the separation of single cells from a fluid stream at rates of several thousand per second at purities of over 99% might seem somehow magical and settled in the realm of Star Trek physics rather than in current laboratory routine. Nevertheless – the principles are easy to understand and have been applied for more than three decades. Two main sorting principles have evolved: i. the stream-in-air instruments, with an electrostatic deflection of charged fluid drops, and ii. the self-contained fluidic sorters, with a contained fluidic system. I will first briefly describe the sorting principles of the latter group before I will go into more detail with the droplet sorters.

Fluidic sorters with a closed flow cell

Fluidic sorters work according to the principle of "fluid switching" in closed flow cells. It is based on the (electro-) mechanical or -acoustic deflection of the fluid volume containing the cell to be sorted. The fluid stream is deflected by a mechanical device, such as a tiny piezoelectrically driven valve, or rendered turbulent by an acoustic transducer. Kamentsky and Melamed (1967) designed a syringe-driven sorter that concentrated the sorted cells onto a millipore filter for analysis; Friedman (1973) invented a sorter that used an acoustic transducer to continuously destroy the laminar flow shortly after the measuring point; desired cells were retained within the laminar flow and sorted by shortly switching off the transducer. A similar procedure applied to the machine of Dühnen *et al.* (1983): here, a piezo-driven valve forced a gas stream into the drain channel, thereby deflecting the cells to be sorted. Another sorting arrangement was introduced by Becton-Dickinson with its FacSort in 1991: the collection tube for the sorted cells was placed in the center of the fluid stream when a sorting event was anticipated; under no-sort conditions, the tube was placed off-center.

Droplet sorting

Fulwyler's (1965) first droplet sorter was based on the principle of ink-jet printing elaborated by Sweet (1965): charged drops detach from a vibrating fluid stream and are directed to a desired position by deflection in an electrical field. The theory behind this mechanism goes back to the 19th century, when Savart and Lord Raleigh first described the tendency of fluid jets to break off into single drops after a certain distance from the ejection point. Modern droplet sorters take advantage of this. They all have the "stream-in air" design, which features a hydrodynamically focussed fluid jet leaving an orifice or nozzle at a given velocity. The fluid stream is set to very highly frequent vibrations (up to 60.000 Hz), which causes the stream to disband into separate drops at a defined distance from the laser intercept point; this distance ("drop delay") is the crucial parameter in droplet sorting. Sort decisions must be reached within fractions of milliseconds, and the charging instruction must be given at the precise point in time and space when the cell of interest is in the drop just detaching from the fluid stream (Fig. 1). As the cell of interest passes the laser beam, its

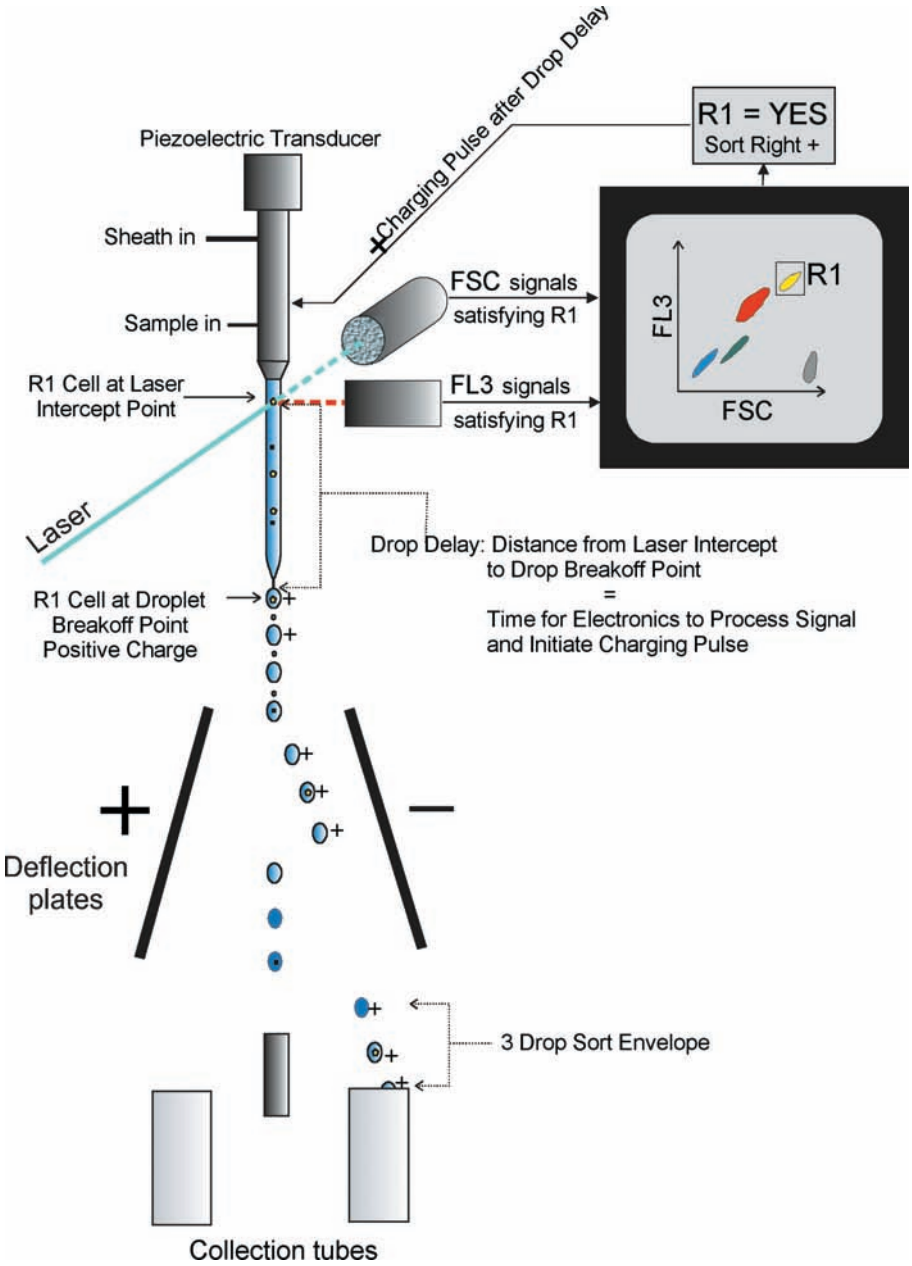


FIG. 1. – Sketch of the principal components and sequence of events in a droplet sorter. Suppose you want to sort cells from the upper right cluster in the FL3/FSC plot. You have defined a gate R1 around this cluster and decided to have R1 sorted to the right (in this example no left sort). As a cell that satisfies the R1 conditions (i.e. exhibiting the FL3 and FSC values defining that gate) passes the laser intercept point, it will be identified by the sort logic as belonging to R1. The computer knows exactly after how much time the cell will detach from the stream ("drop delay"), so a charging pulse is sent to the stream just at that moment. The drop is positively charged now, and will be deflected to the right as it passes the deflection plates. To minimize contamination, a "sort envelope" of three drops is generally set, meaning that the drop before and after the cell-containing drop are deflected as well (provided they are "clean", i.e. contain no other cells). When drops detach that do not satisfy the R1 conditions, the stream charge is suspended and the uncharged drops fall into a waste container.

charactaristic scatter and fluorescence light pulses are collected by the emission optics and passed on to their respective photomultipliers. Converted into electronic signals, the sorting electronics check whether the cell that just passed the laser beam fits the sorting criteria (defined by a sort gate). If the sort decision is "yes", then the time is calculated after which the cell reaches the drop breakoff point. At that precise moment, the entire fluid stream is electrostatically charged by an electrode placed in the sorting head. Just after the drop containing the sorted cell has detached from the stream, the charge is

suspended. The following drops will then not be charged. Generally, it is not only the very drop containing the cell that is being charged, but also the one preceeding and the one following, creating a "sort envelope" of usually three drops. This drop envelope represents a "safety margin" and can be adjusted freely. As the charged drops move through the electric field created by the deflection plates, they are deflected to the left or to the right, according to its charge (negative or positive). The whole procedure, from the cell passing the laser beam to the drop detachment point takes roughly 200 µs (Shapiro, 1995). As the drops are deflected in an electric field, the medium the cells are suspended in has to be conductive to a certain degree; this could be a problem when sorting freshwater cells.

The sorting goals of purity (i.e. the complete separation of the desired cell group from all other cells) and yield (i.e. sorting speed) are in conflict with each other. Therefore, sorting instruments generally offer several sorting modes, depending on the experimental objective. If highest purity is the prime objective, then drops containing the desired cell will be deflected only if they have no other cells in a predetermined number of neighboring drops. If highest sorting speed is desired (e.g. for enrichments), then this condition is relaxed, so that every drop containing the target cell is deflected, regardless if the neighboring drops are "contaminated" by other cell types. If a predetermined number of cells are to be sorted (e.g. one cell per microtiter well to establish clonal strains), then the sorter can be set to stop sorting after that predetermined number.

An alternative to the positive selection and physical separation of a desired cell group is the elimination of unwanted cells without isolating them from the sample. This procedure ("zapping") requires a specified instrument, equipped with a laser that can generate intense UV laser pulses which kill the undesired cells by photodamaging the DNA (Martin and Jett, 1981; Keij *et al.*, 1995). Whatever the principle, a prerequisite is the precise cooperation of the fluidic, optical and electronical components of the instrument. An extensive overview over the different sorting methodologies, which in principle have not changed during the last 10 years, is given by Lindmo *et al.* (1990) and Shapiro (1995). An early but comprehensive presentation of the droplet sorting technique was given by Herzenberg and coworkers (Herzenberg *et al.*, 1976).

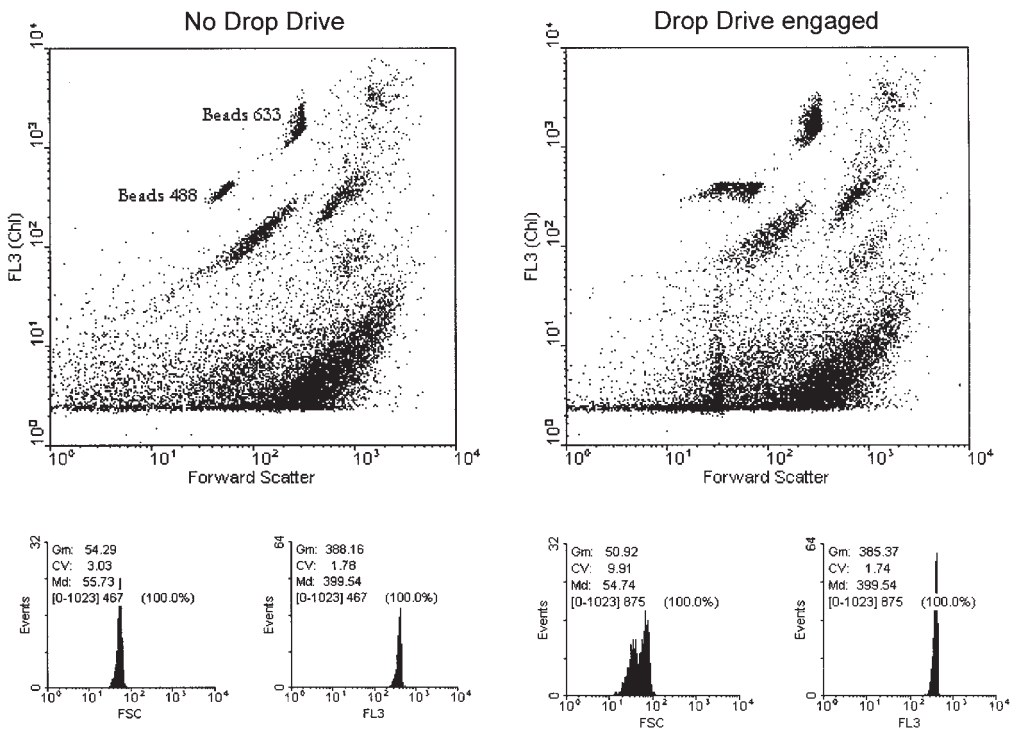


FIG. 2. – Flow cytometric dot plot of a natural water sample from the Wadden Sea, containing 4 phytoplankton clusters and 2 bead poulations (Beads 488 and Beads 633), with and without the drop drive engaged. Histograms represent the gated cluster "Beads 488". Note the difference in FSC-CV´s. Nozzle size 100µm, sheath pressure 9.0 PSI, sample differential 0.8 PSI, threshold parameter FL3 (70V threshold) at 450V. Sort mode: Enrich; Drop Delay: 12.0 drops; Drop Drive Frequency: 17,376 kHz; Drop Drive Amplitude: 29; 3 drop sort envelope.

Problems associated with droplet sorting

Dean (1985) and Shapiro (1995) draw attention to a widely underestimated effect that may occur when a high number of the same cell type is to be sorted: drops of the same charge entering the same collection vessel cause the charge to accumulate in that vessel, thereby decreasing the yield with progressing sorting duration by repelling incoming drops. A (sterile) grounding wire could help here, but the grounding of every single well in a disposable microtiter plate seems not very feasible.

During sorting, the fluid stream is forced to vibrate at a given frequency and amplitude, so the scatter and fluorescence signals also vibrate to some degree. This of course has some effect on the measuring precision: CV´s may increase (Fig. 2). While this poses only a minor problem to well defined and separated clusters, slightly overlapping clusters and clusters of very small cells may be daubed in a way that makes it difficult to define proper sort gates.

The large size and high energy demand (most instruments use large water-cooled lasers) of droplet sorters have made them unsuitable for employment on a ship. Although some have been used successfully at sea (e.g. Olson *et al.*, 1985; Veldhuis and Kraay, 1990; Tarran and Burkill, 1993), these instruments are rather inconvenient to take on board a reseach ship. Small bench-top instruments (featuring fluidic sorters, if any) are much better suited for this purpose. Fluidic sorters with a closed flow cell are safer than droplet sorters when biohazardous material is to be sorted (Shapiro, 1995; Ferbas *et al.*, 1995). Moreover, fluidic sorters should in theory be more precisely controllable than droplet sorters (Shapiro, 1995), but nevertheless, the latter have proven superior in terms of sort speed and purity.

APPLICATIONS

Taxonomic identification

In order to identify unknown flow cytometric clusters, their cells must be sorted onto a microscopic slide, and can then be identified subsequently by microscopy. Absolute purity is not necessary in this case; a large number of sorted cells is more important, hence a fast sort mode (e.g. for enrichments) can be chosen. After a few seconds of sorting, a tiny drop appears on the slide. Drop sizes depend on the chosen orifice diameters and vary between 50 to 400 µm. A quick examination of the sorted drop is crucial, as it evaporates very fast. Cells larger than ~5µm can be morphologically identified by light microscopy in most cases, provided they remain intact after the brutal impact on a glass surface at bicycle speeds of 30 - 50 km/h (high speed sorters can generate jet stream velocities of up to 180 km/h (!), (Lindmo *et al.*, 1990). Large fragile flagellates, such as *Fibrocapsa japonica*, disintegrate soon after sorting (Fig. 3), but remain mostly identifiable (provided the investigator has some anticipation what to expect). Cells with a stronger cell wall, such as diatoms, bear the sorting visibly undamaged. Smaller cells like ultra- and picoplankton require an additional concentration method and subsequent examination by epifluorescence microscopy. For this purpose, the cells of interest can be sorted into a vial that had been preloaded with filtered and fixed medium (e.g. 1%

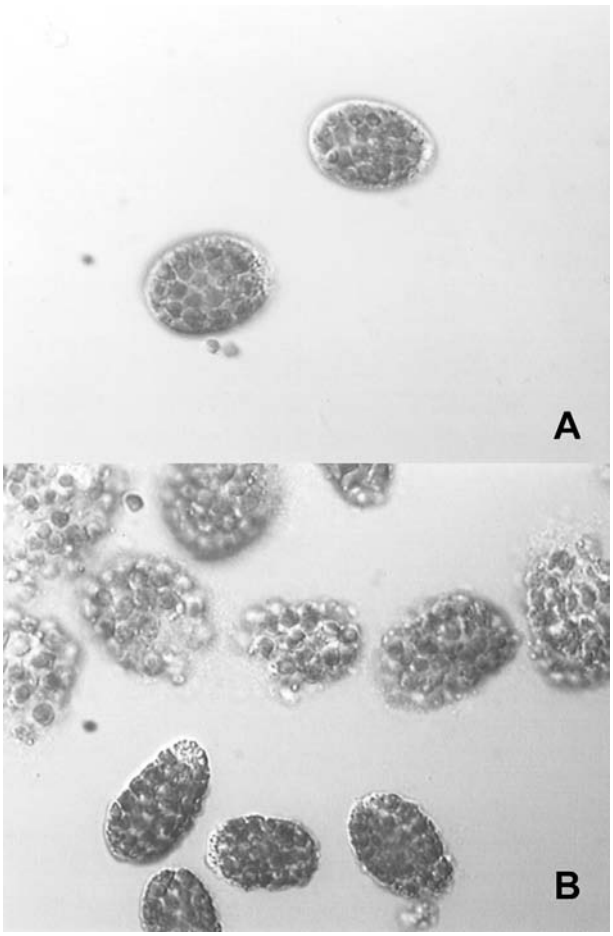


FIG. 3. – A: *Fibrocapsa japonica* cells, live, motile and intact; B: cells from the same culture, shortly after sorting onto a microscopic slide. The post-sort cells (B) showed no sign of viability, a rougher surface structure than the pre-sort cells (A), and disintegrated within a few minutes.

formaldehyde). For a reasonable number of cells to inspect, it is necessary to sort a much higher number than with the slide method, so the sorting may have to last much longer (several minutes). After sorting, the contents of the vial can be treated like a natural water sample to be analysed by epifluorescence (e.g. Knap *et al.*, 1996): concentration onto a black polycarbonate filter and staining with a preferred nucleic acid stain, if applicable (e.g. DAPI, 5µg/mL final conc.). The advantage of this method is that the slides can be deep-frozen (-20 °C) and stored for several months. It is very difficult to specify the small phytoflagellates <5µm and picophytoplankton <2µm on morphological grounds; however, the presence of a flagellum or the fluorescence colour can give some decisive clues. Sorting cells directly onto a wet glassfibre filter may be a direct method to acquire enough cells for an HPLC pigment analysis (c.f. Mantoura and Llewellyn, 1983) in a single or multiple sort run. This, however, depends on the concentration of the wanted cells in the water sample, and may only be feasible during bloom conditions, due to the small water volume used for sorting. For a bulk HPLC pigment analysis, water volumes of 100 mL to several Litres are filtered, while a filled sample tube on a flow sorter contains only 3 mL. Some authors have circumvented the problem that cell concentrations were too low for their molecular analyses of sorted cells by concentrating the dilute samples by tangential flow filtration (Porter *et al.*, 1993), centrifugation (Wallner *et al.*, 1997), or on filters (Urbach and Chisholm, 1998) prior to sorting.

Some examples from the Wadden Sea

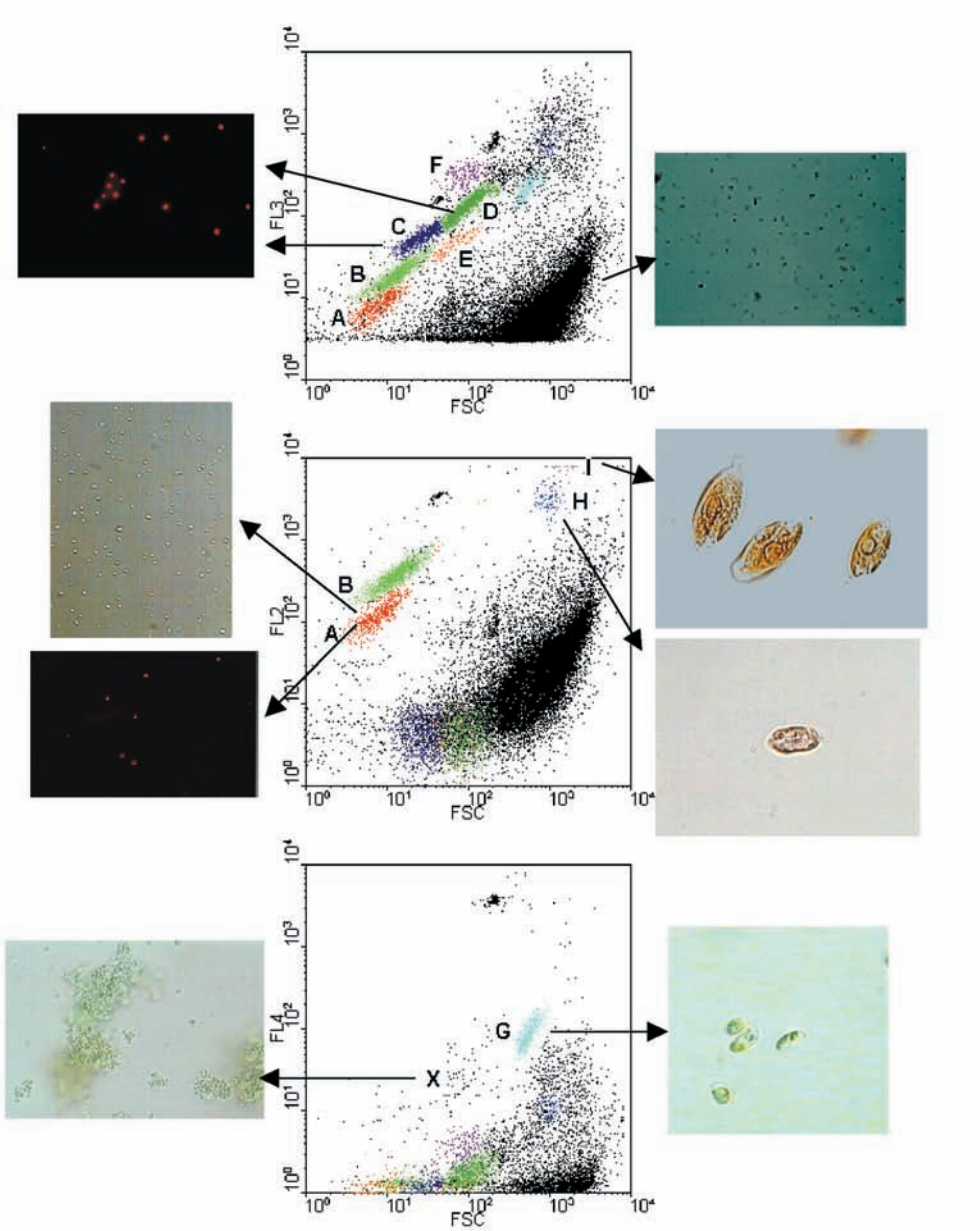
Figure 4 shows dot plots of a water sample from the German Wadden Sea. By sorting cells of different clusters onto a microscopic slide, it was possible to identify most of them. We were able to differentiate two clusters of *Synechococcus* cells rich with phycoerythrine (PE, Type I and II, Clusters A and B in Fig.4A, upper panel) and two PE-containing cryptophytes (*Teleaulax amphioxeia* (H) and *Rhodomonas marina c.f.* (I), middle panel in Fig. 4A). Additional information could be gained by employing a red laser line (633 nm) to specifically excite phycocyanine (PC, emission at 660/20). This made it possible to visually separate two other cyanobacteria and cryptophyte species that otherwise would have overlapped with other phytoplankton: the PC-rich cyanobacteria (*Microcystis viridis* (X)) and the small PC-containing cryptophytes *Hemiselmis virescens* (G) (lower panel in Fig. 4A). The cryptophyte genera *Hemiselmis* is interesting in that it is the only cryptophyte genus with some species possessing phycoerythrine and others phycocyanine (Hill and Rowan, 1989). In addition to these clusters, which are characterized by their specific phycobilin fluorescence, other eukaryotic phytoplankton were differentiated by their light scatter and red fluorescence properties. Three clusters of picoautotrophic cells could be differentiated (C, D and E), and also the small filamentous diatom *Leptocylindrus minimus* (F). Figure 4B shows an example from a bloom of the potentially ichtyotoxic *Fibrocapsa japonica*. The FL3 and FL2 voltages were reduced to have the large *Fibrocapsa* cells on scale. In addition to *Fibrocapsa* (M) and other eukaryotic phytoplankton presented in Fig 4A (clusters D, H, and I), two more diatom species could be identified by sorting: *Nitzschia closterium* (K) and *Thalassiosira minima* (L).

Molecular analysis

As discussed above, the smaller the cells get, the more uniform they look, both autotrophic and heterotrophic. Thus, methods other than microscopy must be applied here for taxonomic classification. HPLC pigment analysis was mentioned before, but it can only give a rough allocation to a taxonomic entity. Recent developments in biotechnology, and most prominently the "polymerase chain reaction" (PCR), have given rise to a new research field in aquatic ecology: the molecular analysis of genetic material and the concurrent development of taxonomic affinities. The design and construction of flu-

FIG. 4. – Flow cytometric dot plots of summer samples from the German Wadden Sea (station Büsum-Pier). FL3 represents the red fluorescence (excited by a 488nm laser line, emission at 675/20), indicative for Chlorophyll, FL2 stands for the orange fluorescence of phycoerythrine (PE, excited by 488nm, emission at 575/20), FL4 is excited by a second laser line (red, 633nm) and represents phycocyanine fluorescence (PC, emission at 660/20). Forward Scatter is indicative for cell size. **A**.: optimized for the small phytoplankton;. **B**: during a bloom of *Fibrocapsa japonica* with reduced FL3 and FL2 voltage to resolve larger species. Clusters identified as follows: A=*Synechococcus* type I, B=*Synechococcus* type II (both PE-rich coccoid cyanobacteria), C=Pico-Eukaryotes type I, D=Pico-Eukaryotes type II, E= Pico-Eukaryotes type III, F=*Leptocylindrus minimus* (diatoms), G=*Hemiselmis virescens* (PC-containing cryptophytes), H=*Teleaulax amphioxeia c.f.* (PE-containing cryptophytes), I=*Rhodomonas marina c.f.* (PE-containing cryptophytes), K=*Nitzschia closterium* (diatoms), L=*Thalassiosira minima* (diatoms), M=*Fibrocapsa japonica* (raphidophytes), X=*Microcystis viridis* (cyanobacteria); small dense clusters represent beads (0.961 µm beads for 488nm excitation, Duke Scientific; 2.5 µm beads for 633 nm excitation, Molecular Probes).

A



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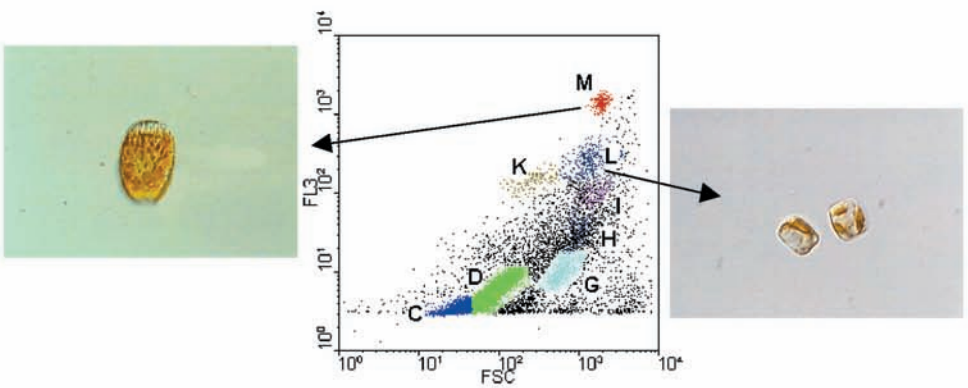

orescently labelled ribosomal 16S RNA (rRNA) oligonucleotide probes has become a widely used technique to make specific cell types visible in a mixed assemblage (DeLong *et al.*, 1989; Jonker *et al.*, 2000). To achieve rRNA probes that are specific to a desired level (family, order, genus, species), it is necessary to have that specific genetic material beforehand, which is generally provided by cultures. Unfortunately, it is generally approved that "good laboratory rats" are not necessarily species, that are in any way important under field conditions. For aquatic heterotrophic bacteria, it is estimated that less than 1% of the total cell counts are cultivable (Amann *et al.*, 1995). For phytoplankton, this number will probably be higher, but especially the picoeukaryotic (<2 µm) phytoplankton remains for a large part unspecified (for an extensive flow cytometric inventory of available strains see Simon *et al.*, 1994). This dilemma may be resolved, at least partly, by flow sorting. Provided the desired cell cluster is well defined (i.e. no major overlap with other clusters), and in some way related to a specific taxonomic group (which may or may not be the case), and there are enough cells of that cluster in the sample, it is possible to obtain genetic material for PCR amplification and subsequent probe design and synthesis. Owing to the PCR technique, the number of cells needed for molecular analysis and probe construction (several 1000 cells) is achievable within a short sorting duration (a few minutes), and formaldehyde fixation does not severely interfere with amplification, at least for some bacterial strains (Wallner *et al.*, 1997). For the small phytoplankton, Urbach and Chisholm (1998) have demonstrated a high genetic variability within local field populations of oceanic *Prochlorococcus* by sequencing cloned PCR products amplified from flow sorted cells. This tiny photoautotrophic prokaryote is ubiquous in tropic and subtropical oceans and has often been observed to show at least two sub-populations in flow cytometric analyses, which have recently been shown to feature different growth and grazing characteristics (Reckermann and Veldhuis, 1997, Moore *et al.*, 1998).

Establishing clonal or monospecific cultures

Creating monospecific, or even clonal cultures from natural populations has been a difficult but unavoidable task in aquatic ecology. In order to obtain reliable information on ecophysiological features of plankton species, it is necessary to physically isolate them from their natural environment and culture them. Large cells can be isolated individually by pipetting with a drawn out glass (pasteur) pipette (Hoshaw and Rosowski, 1973). This procedure works relatively well with non-motile species like centric diatoms, as well as with some ciliates and large flagellates, but normally fails with small motile flagellates. The serial dilution technique (Throndsen, 1995), in turn, is most suitable for fast-growing small flagellates. Principle of the method is the step-wise dilution of a natural water sample until only one specimen is left, which is then allowed to start a clonal strain. A third method is best suited for heterotrophic bacteria, but may also be applicable to some small phytoplankton: sterilized, nutrient-rich agar is plated out in a petri dish, and some µl of the natural water sample is spread across the agar by an inoculating loop (Koch, 1881; Reichardt, 1978). After a few days, some colonies will have formed which stem from a single cell. From these, clonal cultures can be grown. The latter two techniques work well with very small plankton, but have the disadvantage, that the cells of interest cannot be chosen beforehand.

Although flow sorting is far from being a low-cost routine technique, it has proved to be an attractive alternative to the above mentioned isolation methods. Not long after the advent of flow cytometry in phytoplankton research, Yentsch *et al.* (1983) reported on the successful sorting and growing of small cyanobacterial cells (presumably *Synechococcus*) in culture. In an attempt to create axenic strains from bacteria-contaminated phytoflagellate cultures, Sensen *et al.*, (1993) used flow sorting of single cells into Erlenmeyer flasks. They reported that 20-30% of the sorted cultures grew successfully; of those, at least 20% proved to be axenic. Visible growth in the flasks required 6-12 weeks after inoculation.

To obtain viable cells after sorting, some basic requirements have to be met. First of all, the sheath fluid must be appropriate for the sorted cells to grow in; all tubing must be free of any toxic agents that might diffuse into the medium; and the laser power should be turned down as far as possible to avoid or minimize photodamage of the sorted cell. Haugen *et al.* (1987) examined the viability of four flagellate species *(Croomonas salina, Micromonas* sp., *Tetraslmis* sp. and *Gyrodinium* sp.*)* after flow cytometric analysis (tested for fluidic and laser illumination stress); although some physiological damage was found (evidenced by a temporary depression in

growth rates), cells in all treatments recovered after 2 d to full previousely measured growth rates.

If a clonal strain is desired, purity is of highest priority. The use of multi-well plates and a one-cell-per-well sort allows the establishment of clonal strains. This can be achieved by sorting only one cell (after which sorting is suspended), and the application of a specialized unit offered by the vendor, or just by moving the plate by hand. Provided the instrument offers the space to place the wells of the micotiter plate directly into the trajectory of the deflected drops, the manual method is easy. After the instrument signalled a successful single sort into a well, the plate is moved one well further, the next sort is started, and so on. Using this technique, we sucessfully sorted and grew phytoplankton species from the Wadden Sea, including the cyanobacterial species *Synechococcus* ssp. and *Microcystis viridis*, the cryptophytes *Hemiselmis virescens, Teleaulax amhioxeia*, and *Rhodomonas marina c.f.* , the diatom *Thalassiosira minima*, and some unidentified prymnesoid flagellates and two types of pico-eukaryotes. However, the culturing success was quite different between these species. While almost every sorting of the cryptophytes (Fig. 4A, B) and the cyanobacterium *Microcystis viridis* yielded a sucessful culture after 4-12 weeks after the inoculation sort, *Synechococcus* and especially the pico-eukaryotic cells were quite difficult: only after several attempts, we managed to grow these isolates in culture, and some stains died after an initial growth phase. Nevertheless, the pico-eukaryotes and (clusters C and D in Fig. 4, region R2 in Fig. 5) could eventually be brought into culture and were classified as chlorophytes by HPLC pigment analysis (presence of Chl.b and Lutein). A very small cyanobacterium containing phycocyanine was also sorted and grew into culture (Fig. 5). Diatoms are easy to sort and grow, but rarely form a distinct cluster that is well separated from the others. Still, it is possible to place the sort window into a cluster of mixed taxonomic composition and try to get clonal strains from any of the species represented by that cluster.

The improvement of biotechnologically interesting algae, bacteria or yeast has also been promoted by flow sorting. The desired cell type must form a distinct cluster, and its important property must be in some way analysed after sorting. Following the identification of the relevant strain, it can be selected for by flow sorting and brought into culture. In this way, strains of the ß-carotene producing chlorophyte *Dunaliella salina* that produced twice the amount of the carotenoid than the wild type, was successfully sorted using their specific fluorescence and scatter characteristics, and grown (Benamotz, 1991). Betz *et al.* (1984) sorted and cultured subpopulations of the bacterium *Rhizopus arrhizus* that were different in their light scatter characteristics; some of these subpopulations showed an increased lipase production, and could subsequently be selected for further lipase production. An elegant way of selecting for a desired property of a specific cell type is the coupling of that property with the inherent fluorescent characteristics of the cells, which can be readily measured by the flow cytometer. An *et al.* (1991) were able to isolate and grow astaxanthin-hyperproducing mutants of the yeast *Phaffia rhodozyma*, in which the astaxanthin content of the cells was related with their autofluorescence intensity. Nir *et al.* (1990 a, b) developed an interesting technique for signal amplification of small bacteria. They produced "gel microbeads" of reproductible size, in which bacterial clones grew and formed colonies. These microbeads could be flow sorted conveniently according to their ß-Galactosidase activity, which was quantified by a fluorecent substrate. Porter *et al.* (1993) and Porter *et al.* (1995) used immunofluorescence to sort and grow specific target cells of wild lake and sewage bacteria, and physiologically different strains of the cultured bacterium *Micrococcus luteus* following a starvation period were isolated and recultivated by Kaprelyants *et al.* (1996).

Post-experimental separation of cell groups

Uptake experiments in natural communities usually suffer from the fact that uptake rates of individual groups cannot be distinguished from the bulk measurements. Size fractionation has been applied extensively to account for e.g. ^{14}C uptake rates of different size groups (e.g. Larsson and Hagström, 1982). However, the size of a plankton organism and its function in the ecosystem are two different categories. For example, in a sample containing a diverse diatom and flagellate community, it is impossible to separate their respective uptake rates, as the two groups largely overlap in size. Although flow cytometric analysis does not necessarily yield a separate cluster for each single species or even functional group in a natural water sample, it is still superior to size fractionation when it comes to separation of different plankton groups, be they func-

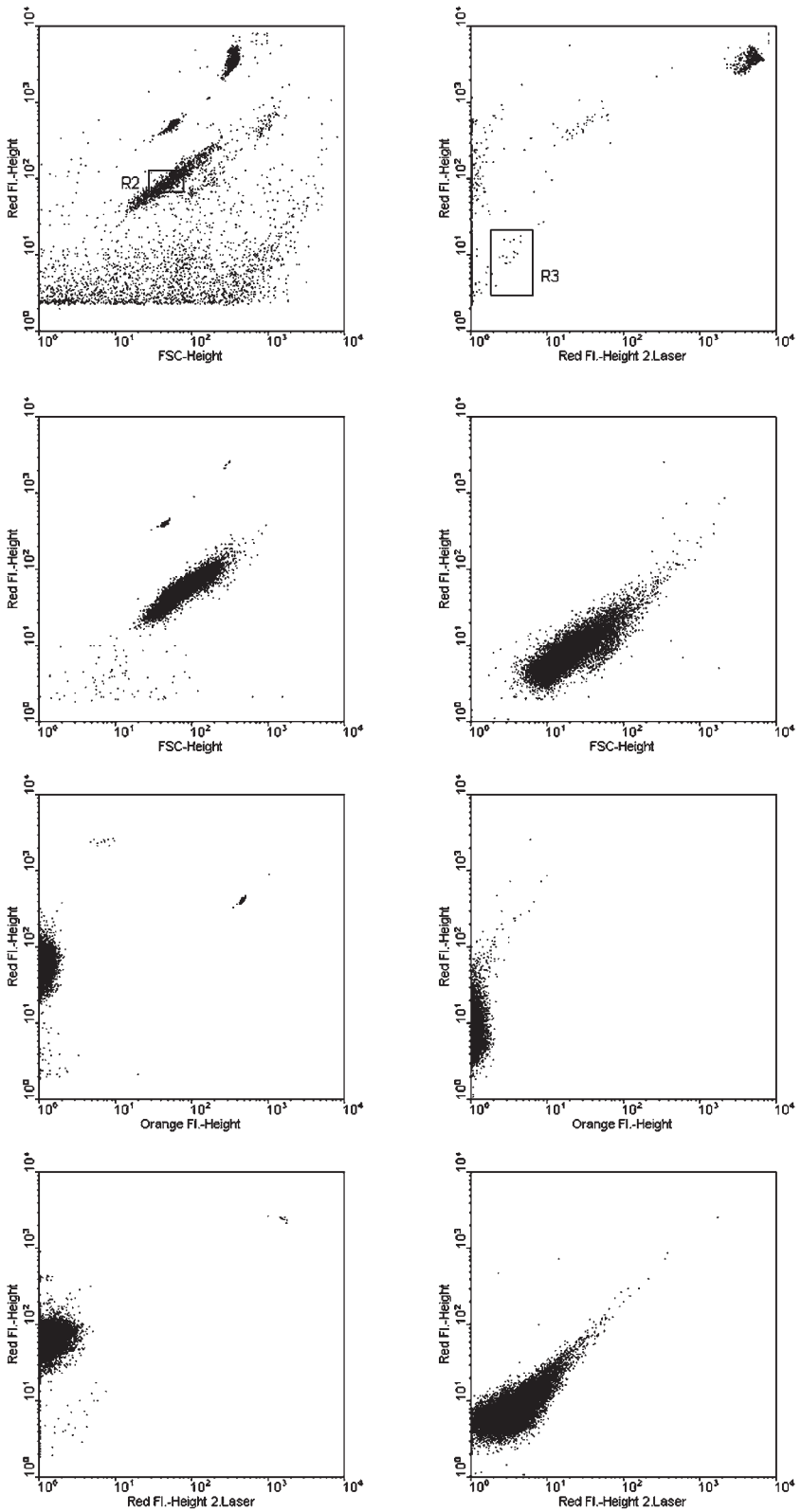

FIG. 5. – Flow cytometric dot plots of an autumn sample from the German Wadden Sea (station Büsum-Pier) with sort regions (R2 and R3), and of the respective cultures. R2 and R3 were sorted into multi-well plates and grew to dense monospecific cultures after approximately 8 weeks in a light cabinet at 100µE and 16 °C. R2 (left column) is characterized by the absence of both phyoerythrine (Orange-Fl.) and phycocyanine fluorescence (Red Fl. 2. Laser). HPLC pigment analysis revelaed the presence of both lutein and chl.b, which is a strong indication that this group belongs to the chlorophyceae or prasinophyceae, possibly *Micromonas pusilla.* R3 (right column) is presumably a very small cyanobacterium with a strong phycocyanine signal (Red Fl. 2. Laser), but no phyoerythrine fluorescence (Orange-Fl.). HPLC confirmed the presence of zeaxanthin.

tionally coherent, or not. Rivkin *et al.* (1986) tested carbon uptake rates of various diatom and flagellate cultures and in a natural sample, with pre- and post-sort incubation with ^{14}C. They clearly showed that sorting does affect the photosynthetic activity of the cells, be it by physical stress, or photo-oxidation by the laser; so they concluded that it is not advisable to put the cells through the sorting procedure if physiological parameters are to be measured that shall have some relevance for natural field conditions. They also found, however, that post-incubation sorting does not significantly affect the intracellular label, i.e. the radioactivity remains within the cell and thus allows the separation of specific cell groups and the measurement of cell specific uptake rates, and therefore the contribution of a given group to total carbon uptake. In oceanic water, Li (1994) was able to sort three different ultraphytoplankton groups following ^{14}C incubations. He found that the eukaryotic cells generally contributed substantially more to bulk uptake than the smaller prokaryotic groups *Synechococcus* and *Prochlorococcus*, due to their much larger cell size and hence cell-specific uptake rates. Only exceptionally, the prokaryotes were more important. Similarily, Lipschultz (1995) used post-incubation sorting to account for ^{15}N uptake rates of two phytoplankon size classes and non-chlorophyll containing particles (heterotrophic bacteria, protozoa, detritus). He was able to show that ammonium is the preferred N source over nitrate for small phytoplankton (in this case <10 µm), whereas the opposite holds for the large phytoplankton (>10 µm). The restriction to only two phytoplankton sort gates (chlorophyll containing cells <10 µm and >10 µm, not taking full advantage of flow cytometric discriminatory possibilities), obviously resulted from the high biomass required for a mass spectrometric ^{15}N analysis. Servais *et al.* (1998) demonstrated differential growth rates of different size classes of heterotrophic bacteria. The bacterial samples were first incubated with leucine, then stained with the DNA-specific stain SYTOX 13, after which the differently stained cluster were sorted and independedly analysed for leucine incorporation (see also Gasol and del Giorgio, 2000).

PROSPECTS

Due to its sophistication and high costs, flow sorting (and flow cytometry in general) will probably not become a routine technology for aquatic research in the nearer future. Especially droplet sorting requires a minimum of expertise and experience. So it will be mainly specialized labs that will use flow sorting in the future, as has been mostly the case so far. Especially the advent of new fluorescent probes and molecular techniques to the analysis of bacteria and phytoplankton (Porter *et al.*, 1995; Wallner *et al.*, 1997; Urbach and Chisholm, 1998), and the isolation and screening of biotechnologically interesting microorganisms (reviewed in Davey and Kell, 1996) open new prospects for the use of flow sorting in aquatic ecology. As aquatic ecology moves more and more from basic towards applied research, the screening of aquatic microorganisms from different ecosystems, including exotic environments, becomes an increasingly important field of research. Flow sorting can be a valuable tool to achieve this.

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