

CytoBuoy: a step forward towards using flow cytometry in operational oceanography*

G.B.J. DUBELAAR and P.L. GERRITZEN

Dubelaar Research Instruments Engineering, Zeelt 2, 2411 DE, Bodegraven, The Netherlands.
Fax +31 (0)172 615219. E-mail: dubelaar@cytobuoy.com
Datawell b.v. Laboratory for Instrumentation, Zomerluststraat 4, 2012 LM Haarlem, The Netherlands.

SUMMARY: While the performance of biological sensors in real time monitoring networks is limited to bulk values like chlorophyll fluorescence, in practice the implementation of automated phytoplankton taxonomy remains a remote option. Aiming to reduce this gap we developed a flow cytometer called CytoBuoy for autonomous in situ operation, for instance in a moored buoy with wireless data transfer. Although not comparable to microscopy, flow cytometers detect and count particles allowing a limited level of particle characterization based on the light scatter and fluorescence properties of the individual particles. CytoBuoy analyses a large size range of particles, typical for marine coastal zones and fresh waters. The 'field' design implies a tradeoff between the accuracy and versatility of laboratory flow cytometers and the qualities needed for trouble free autonomous operation *in situ*. The optics and electronics however were designed for maximal reflection of the particle morphology in the measured signals. Whereas standard cytometers reduce these to single peak or area 'listmode' numbers, the signal courses are preserved fully by CytoBuoy and transferred to the computer as raw data, which allows more extended morphological analysis. Extended field tests will have to show how the system holds in various environments and weather conditions.

Key words: CytoBuoy, in situ flow cytometry, phytoplankton analysis, marine monitoring.

INTRODUCTION

There is a growing concern with the marine environment. We fear depletion of fish stocks, pollution and possible impacts of climate change, such as sea level rise and its consequences for coastal areas. These are strong reasons for a modern system for information on the marine environment. We need both continuous descriptions of the present state of the sea and continuous forecasts of the future condition of the sea. Together this is known as "Operational Oceanography" (Tent, 1997). All this infor-

mation should be rapidly available in a usable format, based on real time data input from integrated networks of fixed and moving monitoring systems operating in complementary slots in time, space and resolution. Real time information depends on a reliable sensor signal. Although sensor systems have improved, there is still far to go before we can monitor many analytical parameters on a long term basis without a great deal of manual intervention (Meredith, 1996). Whereas chemical and physical sensors are more and more available, there is a lack of sensors and instruments for measuring biological parameters in real time monitoring networks. This holds particularly for the monitoring of phytoplankton.

*Received June 14, 1999. Accepted March 13, 2000.

Clearly things get complicated when one tries to automate the detection, counting and basic analysis of particulate matter in surface waters, with the huge variety in concentrations of biological and non-biological particles, and their diversity in size, shape and physiological properties. This diversity causes ambiguity in the relation between the measured entity and particle size, or exceeds physical limits of sensitivity, analysis resolution or measurement range. An even more remote option would be the automated online determination of the species composition of the phytoplankton community. Today the best available on-line technology yields a single fluorescence number per water sample, while manual light microscopy is the standard off-line technology. Because even relatively simple phytoplankton community qualifications such as size class characterization still require microscopical determination, there is no reason to work with anything less than species composition. However, the huge costs involved limit the spatial and temporal sampling frequencies applied as well as the numbers of individuals counted by microscopy to questionable confidence levels in terms of accuracy and coverage of the dynamic biological processes in the environment. In addition, a description in terms of species composition gives no quantitative answer to questions on the physiological state of species nor the variance between individuals, important for the analysis of population and community structure. By the absence of routinely applicable and validated alternatives this situation dominates the existing infrastructure of monitoring programs and data interpretation frameworks. Without further entering the discussion of what parameters should be monitored we expect that the discrepancy in the current monitoring situation may be reduced by an online sensor probing on the level of individual particles, generating quantitative data with an information depth somewhere in between the bulk value and the species composition.

While flow cytometric analysis is inherently fast owing to the flow through principle, several correlated optical measurements are made simultaneously from each individual particle. This means that a higher level of particle differentiation can be reached than size analysis alone. Another basic advantage of flow cytometry (FCM) over other optical particle analysis techniques also relates to the 'flow system': the use of a clean, particle free sheath fluid surrounding the sample stream and restricting it to the center of the flow cuvette. Flow cytometers

are therefore relatively insensitive to fouling, which is a major issue for many in situ applications, particularly when the sample contains solids of varying size and consistency (Meredith, 1996). Flow cytometry is a quantitative and semi-automated laboratory technique, universally accepted as method for scientific analysis of phytoplankton. The simple physical measuring principle and the moderate data generation allow integration in realtime monitoring systems in principle. With the CytoBuoy Project (Dubelaar *et al.* 1998), we aimed to transfer flow cytometer technology to an in situ platform with wireless data transfer. The objective was to build a phytoplankton flow cytometer into a small moored buoy. We used the widely known Datawell Waverider buoy having a 90 cm spherical hull and a 380 x 480 mm cylindrical sensor compartment, in which the instrument should fit. The cytometer was tested for realistic rolls on the Datawell wave simulating swing. Another important design feature was limited power consumption to allow for battery powered operation, not a trivial aspect in the light of the history of flow cytometers as major electrical power consumers. All these criteria influenced the choice of components and the way the instrument works - with less performance in terms of accuracy and versatility common to laboratory instruments but with the basic requirements for hands-off operation with acceptable mean time between failure even when subjected to incidental shock impact and severe environmental conditions. As a new functionality of the CytoBuoy flow cytometer we designed the optics and electronics for maximal reflection of the particle morphology in the measured signals. Whereas standard cytometers reduce these to single peak or area 'listmode' numbers, the signal courses are preserved fully by CytoBuoy and transferred to the computer as raw data, which allows more extended morphological analysis. We sent a questionnaire to various potential users to find out preferred sampling regimes, target particle types and concentrations. We chose the preliminary specifications to accommodate the widest practical application area common to most of the responses, as discussed below. The photos in Fig. 1 show the simple layout of the instrument and its dimensions in combination with the buoy. While extended field tests are scheduled for reliability tests, this paper describes the technical structure and operational mode of the CytoBuoy system including a discussion of problems and possibilities with regard to generated data, instrument use and integration in

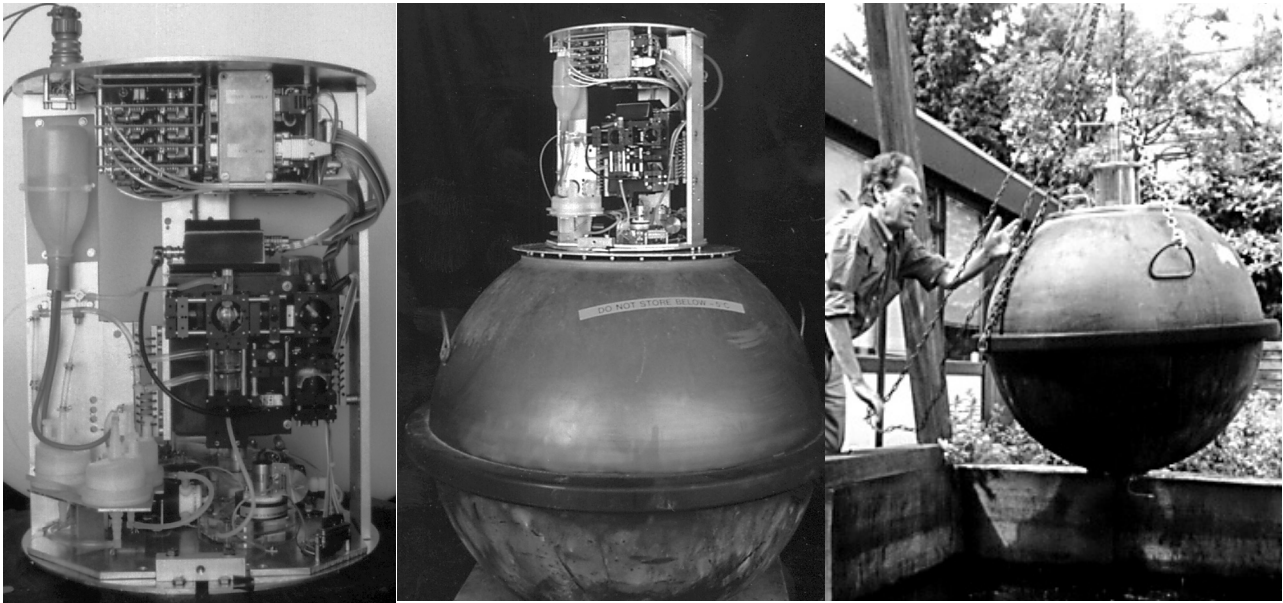


FIG. 1. – Left panel: Prototype of the CytoBuoy flow cytometer module without cover (top section = electronics, middle section (black) = optics, lower section = fluidics). Middle panel: Flow cytometer module placed on top of the 90 cm buoy for comparison. When mounted the module sits in the center on the bottom, surrounded by up to 70 kg of batteries, and data transmission electronics above the module. The lid has a flashing beacon on top with an antenna. Right panel: Buoy with cytometer inside being hoisted into the Datawell outside water basin. The sample inlet tube is visible right underneath the buoy.

monitoring systems as well as some recent developments. Technical design details were reported elsewhere (Dubelaar *et al.*, 1999).

OPERATIONAL POSSIBILITIES AND LIMITATIONS

Particle size and measurement principle

The oceans are dominated by small single phytoplankton cells like *Synechococcus* sp. and the Prochlorophytes (Veldhuis and Kraay, 2000). Phytoplankton in coastal waters however shows a greater diversity in cell sizes and shapes, particularly with colony forming species, extending the particle size spectrum to hundreds of micrometers and up to over a millimeter occasionally. Because mid-ocean application of an autonomous cytometer requires fully matured technology with high costs for satellite data transfer, we aimed at the coastal application first and consequently for a large size range of particles. Because many larger phytoplankton cells or colonies are asymmetrical, we make a distinction between the length of these particles and their largest diameter perpendicular to this length axis. Asymmetrical particles are stretched out in the fluid system in the direction of flow before passing the laser beam. There are two general types of size

restrictions: first the smallest orifice in the flow system and secondly the linearity of the signal transducing system. The inflow of particles is nonselective to one third of the tubing diameter (R. Tuk and M. Donze, Techn. Univ. Delft, unpublished results). With the 800 μm tubing internal diameter of the CytoBuoy this means that the sampling of particles up to about 265 μm is nonselective, whereas bigger particles are gradually undersampled with increasing size, particularly the rigid type of particles such as large diatom cells like *Coscinodiscus* sp. How efficiently the particles are actually being measured depends on the optics and electronics which set space limits to precisely where a (part of a) particle intersects the laser beam and time limits to the duration of its traversal through the laser beam respectively. These settings are too narrow for many larger or longer phytoplankton particles, which then causes nonlinearity and sometimes artefacts in signal processing. Upper size values may vary from about 20 - 50 μm depending on the instrument and the settings. The CytoBuoy has an optical field of view of about 400 μm , and there is no electronic time limit to the pulse duration. The latter relates to the use of an optical scanning method as earlier developed for the experimental Optical Plankton Analyzer (Balffoort *et al.*, 1992; Dubelaar *et al.*, 1989; Hofstraat *et al.*, 1991, 1994; Peeters *et al.*, 1989) and its successor, the European Optical Plank-

ton Analyzer (Jonker *et al.*, 1995; Dubelaar *et al.*, 1995). It allows the analysis of field samples, even covering the size spectrum of natural *Microcystis aeruginosa* (Dubelaar and van der Reijden, 1995). In these systems the linearity is not affected by the length of the particles, and therefore there is no length limit to the measured particles. This is particularly useful if big diatoms or filamentous or chain forming colonies are present, while the wide field of view means that only particles with a very large diameter suffer from nonlinear signal generation. A trade off with this large size range design is a somewhat lower sensitivity for the smallest particles as compared to standard cytometers, with a current detection limit of 1-2 μm particles depending on their index of refraction.

Particle concentration

A flow cytometer analyses the particles on a one by one basis. Before passing the laser beam, the sample stream is injected into a particle free sheath fluid that stretches it out into a very thin laminar thread of fluid with well-separated particles. However, if the concentration of particles in the sample is too high or if the sample flow is too large, the particles in this sample fluid thread will be closer to each other and some of them may coincide while passing the laser beam. This causes a breakdown of the individual particle analysis character. In laboratory devices, the sample rate is adjustable to optimize the speed for various concentrations of the samples, and the number of particles to be analyzed is adjustable also. Autonomous operation implies the absence of an operator observing the results on a sample by sample basis. CytoBuoy therefore has a fixed sampling flow rate (0.3 ml per minute), small enough to prevent particle coincidence in bloom situations or high sediment load, and large enough to prevent very long running times with clear waters and accompanying problems such as large energy use and particles settling out in the tubing. Regarding the maximum concentration, the unwanted particle coincidence starts to become significant (10%) at analysis rates of about 30,000 particles per second or a particle concentration in the sample of about 6 million particles per milliliter. We found it most convenient to let CytoBuoy acquire a fixed amount of data per sampling. The 64 kbyte data buffer used for each detector typically contains between 4500 and 1500 particle pulses, corresponding to samples with small particles and samples containing also large

particles. At the high concentration mentioned, giving a counting rate of thirty thousand particles per second, the buffers are filled in about 0.15 s after analysis of 0.75 μl of sample. Normally, concentrations are much lower and analysis takes tens of seconds to some minutes. The maximum running time is arbitrarily limited to 10 minutes per sample for practical reasons, giving a maximum sample volume of 3 ml for a single analysis run. This sets the lower concentration to about 100 particles per ml to allow some statistical analysis.

Optical configurations

CytoBuoy measures forward light scatter, side scatter, orange fluorescence and red fluorescence. Forward light scatter is measured with a small photodiode (BPX61, Siemens, München, Germany). Instead of beamsplitters, dichroic mirrors and a photomultiplier for each of the side scatter and fluorescence parameters, CytoBuoy uses a spectrograph (CP140, I.S.A., Longjumeau, F) and a multipixel solid state detector (proximity focussed PP0380D, D.E.P., Roden, Netherlands). Currently only 3 pixels are used to probe side scatter and the two fluorescence bands, but this configuration allows easy upgrading to detectors with more pixels to probe more fluorescence emission bands. Flow cytometers may have one or more lasers to excite the natural and/or artificially introduced fluorescent pigments of the particles. CytoBuoy accommodates one laser at this moment. Many laboratory instruments use Argon ion gas lasers with their strong blue-green (488 nm) or green (515 nm) lines. These lasers can also be set to produce UV (350-360 nm) emission. The most commonly used artificially fluorescent probes are designed for these wavelengths. Instruments used to analyze phytoplankton are often equipped with a 633 nm 'red' Helium-Neon gas laser as a second laser. Combination with the main laser set to 488 or 528 nm yields good possibilities to excite the different natural pigments of phytoplankton in fresh and marine waters respectively (Hofstraat, 1994) that emit predominantly in the red band of the spectrum (Chlorophyll: 680-690 nm) and in the yellow/orange band (Phycocerythrin: 580-630 and Phycocyanin: 640-670 nm). Gas lasers have the best beam quality, but they cannot be used in the CytoBuoy because they are too big and eat too much power. Bare solid state lasers on the other hand are as small as a thimble and the corresponding temperature and light output stabilized packages are not

bigger than a pack of cigarettes. Such laser modules having sufficient output power (>20 mW continuous) are available now in the 635 - 680 nm region. Particularly interesting are the 635 nm types probing the Phycocyanin absorption maximum, and there are 675 nm lasers that emit right on top of the chlorophyll red absorption maximum (in which case some fluorescence may be found above the 700 nm wavelength). Shorter wavelength beams of sufficient quality from small sized packages are restricted at this moment to the green 532 nm line of the diode pumped frequency doubled NdYag lasers. This wavelength is not too far from the Phycoerythrin absorption band (540-570 nm) but rather far away from the blue Chlorophyll a and b absorption maxima (420-450 and 470-490 nm respectively). This laser type is used in the CytoBuoy prototype (Compass 315-20, Coherent, San Francisco, Ca, USA), allowing the measurement of orange and red fluorescence. At the moment there are no small 'blue' lasers available with sufficient quality in terms of noise, beam pointing stability and power. This may change very soon however. A 473 nm version 'diode pumped' laser (Bremas, Bremen, Germany) is available already for testing, whereas the Japanese Nichia Chemical company started the commercial sales of the first blue laser diodes. These already have 5 mW output power at 390-420 nm. In this case, the blue excitation of natural Chlorophyll with its fluorescence emission at 680 nm may be combined (in the laboratory) with artificial pigments with smaller Stokes shifts.

Data format and analysis

Although CytoBuoy measures the standard flow cytometry parameters forward light scatter, side scatter and two fluorescence bands, the data format is different. When passing the laser beam, each particle emits a short flux of photons by scattering or fluorescing. The time course of this short flux we call a 'pulse' or 'signal'. Photodetectors convert these photon pulses into electrical pulses. The duration of these pulses, typically in the order of microseconds, roughly equals the combined height of the laser beam as traversed by the particles and the particle length, divided by the particle flow speed at the intersection point. The shape of the pulses is a convolution of the intensity profile of the laser beam as traversed by the particles and the shape (or distribution of optical properties) of the particles themselves along their long axis. Normal-

ly, this convolution is dominated by the bigger Gaussian laser beam profile. The corresponding photodetector output pulses are quantified by digitizing their peak or area value that are then sent to a computer in so called list mode data files. However, CytoBuoy's laser is focussed to a very thin sheet of light (5 μm as measured between e^{-2} points) laser beam height, and the pulses are more and more dominated by the shape of the particle for particles larger/longer than 5 μm . With forward scatter, the gross shape or 'particle shadow' governs the signal, whereas with side scatter the distribution of small cellular structures determines the signal. The fluorescence pulses correspond to the intracellular location of the fluorescent pigments over the length of the particle. The morphological information of these pulses is valuable. With only one laser, a limited amount of pigment composition information can be obtained. This means that morphological data is even more important to discriminate species. Digital signal processor boards (DSP's) may acquire and analyze this type of pulses in real time (Zilmer 1995), and such a system was developed recently for the phytoplankton signals of the (Eur)OPA flow cytometer. Real time (within milliseconds) analysis could be used to perform particle sorting or imaging-in-flow based on specific pulse characteristics, but this is not relevant to autonomous flow cytometry. Because DSP's are not particularly small or suited for low power applications, CytoBuoy has specially designed data grabber cards to fully capture and store the pulses in data buffers, one grabber card for each parameter. When the buffers are full (64 kbyte for each parameter), the series of pulses are transferred to the computer via serial or parallel line or by radio as raw data. Digitizing means probing a continuously varying signal, and assigning a numeric value

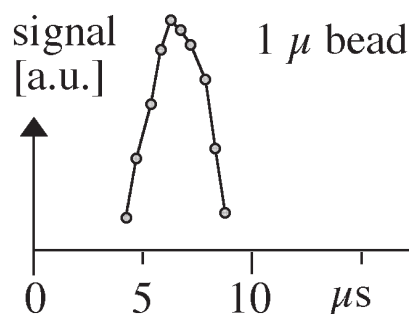


FIG. 2. – The forward light scatter signal of a 1 μm plastic sphere.

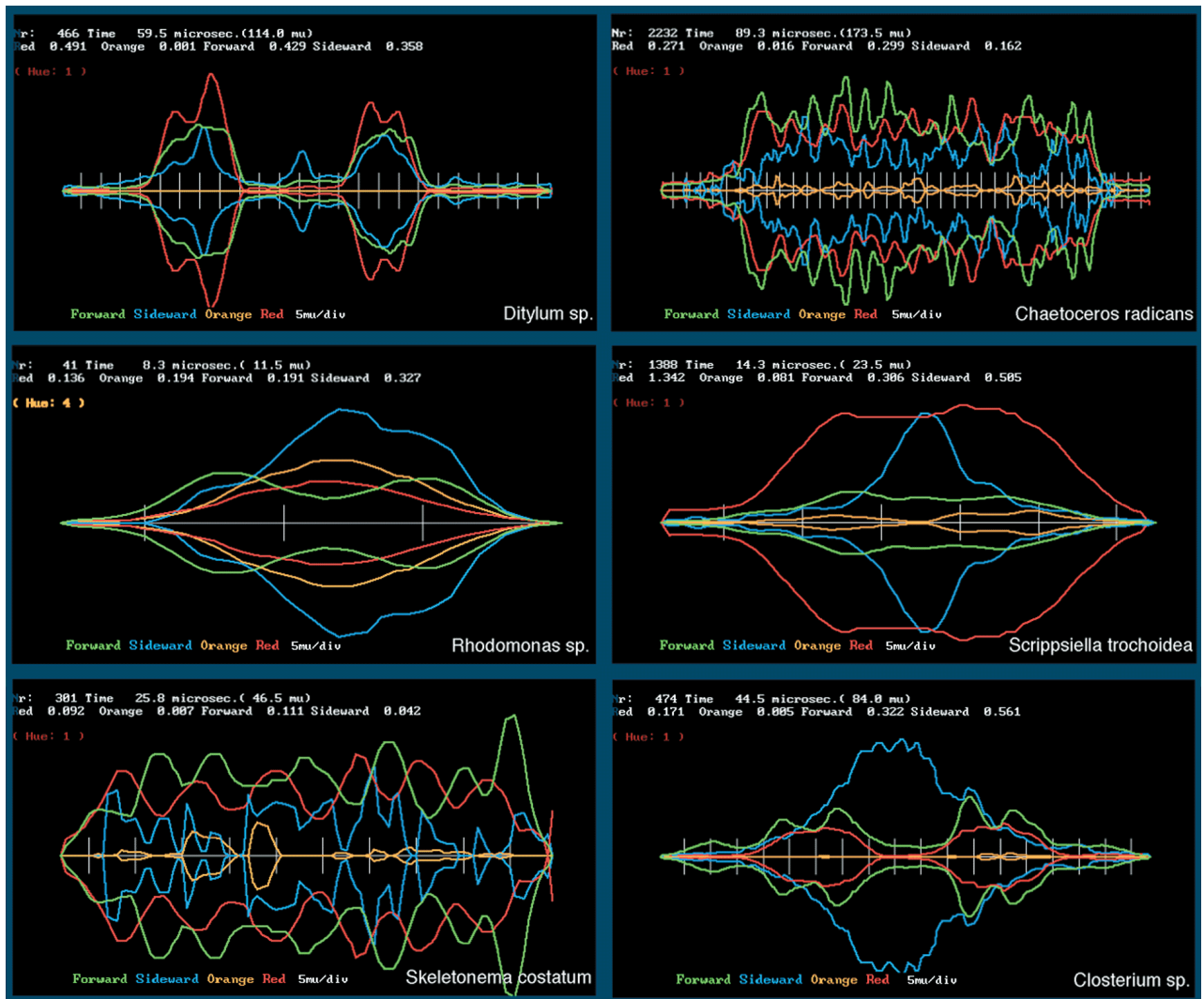


FIG. 3. – Light scatter and fluorescence profiles of a typical individual cell or colony from several species as measured by CytoBuoy. The pulses are vertically mirrored for better visual interpretation. The particle length [$5 \mu\text{m}/\text{division}$] is calculated from the duration of the pulse [μs]. Forward and Sideward refer to measured light scattering directions; Orange and Red refer to the measured fluorescence bands. The vertical scales are normalized, the mean values are indicated in the headings of the graphs.

repeatedly in fixed time intervals. With large time intervals, much of the fine detail of the signal is lost, but very small intervals lead to many more numbers to process for the same signal. The smallest signal is characterized by at least 10 points in CytoBuoy, as is shown in Figure 2 for a very small particle. The pulse duration and shape of such a small particle is still dominated by the $5 \mu\text{m}$ height of the laser focus, whereas the longer signals clearly show the morphology of the particle. Figure 3 shows examples of the measured and stored pulse shapes of some particles from various species.

These pulses need to be processed in order to compute particle characteristics, and to allow subsequent classification of the particles. From each pulse, various list mode parameters can be derived,

like pulse length, integral, maximum, number of peaks etc. The pulse length can be used in order to compute particle length and the size of the chlorophyll containing part of the cell. The number of peaks per pulse can be used to calculate the number of cells per colony. Also, list mode parameters can be computed from multiple pulse parameters, like ratio, difference etc. The data analysis software for the CytoBuoy, called CytoWave, combines general purpose flow cytometry data analysis features with specific features for the translation of the pulse data from the CytoBuoy into list mode data. General features include dotplots, histograms, selection of data using nonrectangular gates, multiple selections, reading and writing of the internationally accepted FCS (flow cytometry standard) data format and sta-

tistical analysis of subpopulations. The original pulse data of cells selected in a dotplot or histogram is displayed to get insight into the morphological structure of the cells. Phytoplankton can be discriminated from sediment particles, debris and noise. Within the phytoplankton, groups and sometimes species can be discriminated.

Sampling

There is no sample treatment in CytoBuoy. As mentioned earlier, instead of sample prefiltration, CytoBuoy uses just a simple sample inlet tube hanging freely underneath the buoy. Everything that fits in the tubing will be analyzed. Other kinds of sample preparation to be considered are the preservation of the sample and the introduction of fluorescent probes. Whereas preservation of the sample is not needed with in situ analysis, current protocols to label the cells for species recognition or cell cycle analysis are too complicated to automate in a small device. Some work is being done to design protocols based on filtration of cells instead of centrifuging, which would be better from the perspective of possible automation of these sample handling protocols. We expect that early warning applications may justify development of automated sample preprocessing if fluorescent probes become available with sufficiently simple protocols, for the detection of specific plankton groups or species of special interest, e.g. toxic species.

The sample is taken just below the water surface (about 60-70 cm) underneath the buoy, and the particles are analyzed within about one minute after entering the sample tubing. Sampling at other depths is not possible currently although processes like vertical migration of algae, stratification of water layers, vertical currents etc. would give reasons for measuring at different depths. An auxiliary intake system should be used, but this needs development, or a submerged flow cytometer. Sampling frequencies may be programmed by the user. The maximum sampling frequency depends on the situation. Before the next sample can be analyzed, the contents of the data buffers should be transferred to a storage medium. This takes about a minute if the computer or data logger is connected to the instrument, but it may take about 25 minutes with radio transmission of the data. In the case of more analyses of the same sample, analysis can continue directly after data transfer, but if a new sample has to be analyzed, flushing of the sample line takes one minute. If the sample frequency is low as

with buoy operation (radio transmission), the laser is started and shut down again at each sampling. The laser starting procedure takes 3-4 minutes. So in buoy operation a practical frequency would be one sampling per hour, but in other cases frequencies of 10 samples per hour can be reached if the concentrations are high enough.

Operational cycle

Lithium batteries have a much higher power density than conventional batteries, but they are also more expensive. Lithium is better than other types of batteries with respect to short peaks of power such as required at laser start-up. The maximum battery load for the buoy is about 70 kg. We have now 40 kg of specially shaped lithium batteries that fit nicely in the buoy and deliver 22 kWh, sufficient for the flow cytometer to take 3000 - 4000 samples including data transmission by radio. A main problem with batteries is shipping however, especially the lithium types, which are subject to stringent safety restrictions. This situation is not so stringent for alkaline batteries, which could give 15 kWh with a full buoy load. Battery life is more or less inversely proportional to the sampling frequency, and depends also on the particle concentration, the type of radio transmission and 'overhead' such as the flashlight and standby use. The particle concentration plays a role because with rather low concentrations the analysis takes longer which costs more power.

Clogging of the fluid system is a major source of annoyance with lab cytometers but is absolutely disastrous for an autonomous instrument. The problem of sample preparation - normally by prefiltration - down to a size that is acceptable to the analyzer without affecting the true value of the "whole" sample is difficult to overcome (Meredith, 1996). Instead of some automatic 'filtration' device inside the CytoBuoy, the only physical limitation of the particle size is the orifice of the sample inlet tubing (hanging right underneath the buoy) which has an internal diameter of 800 μm currently. This is sufficiently wide for virtually all phytoplankton species, and other types of suspended particles. An almost non pulsating tubing pump allows the particles to flow from the outside of the buoy into the measuring cuvette in one single piece of very smooth tubing, without valves or sharp bends that might block the passage of large particles. The cuvette has a 1000 μm square cross section. We have experienced no clogging with the current system so far.

Fouling may also shorten operational life. Whereas fouling in the optical cuvette increases the level of scattered light and impairs the signal to noise ratio of light scatter measurements, fouling and growth in the fluid tubing may cause unwanted particles to appear and in the worst case cause complete system clogging. The first problem is relatively unimportant because as all flow cytometers, the CytoBuoy has a sheath fluid system providing a particle free fluid surrounding the sampled sea water, as it runs through the optical measurement cuvette at high speed (2 m/s). The system is therefore inherently clean, whereas the data does not consist of images but of light intensity measurements that can easily be calibrated with microscopic standard beads. The fact that CytoBuoy has a closed circulating sheath fluid system allows the addition of preservative to prevent growth in the tubing and filters. The most important filter is placed directly downstream of the measuring cuvette to catch all particulate material present in the mixture of sheath and sample fluid leaving the cuvette. The currently used small capsule filters are in service for months, and a big type should last a year. In addition we try to prevent fouling by using smooth wall types of tubing materials, and by back flushing the teflon sample tube with the particle free sheath fluid after every analysis. This also may reduce the probability of an organism of about the size of the tubing orifice to enter, attach and block the inlet tubing between samplings. At sampling startup the sample line is flushed inwards with sea water during the laser warmup time therewith removing any remains of the preservative fully before the actual measurement starts. Because the system is a 'closed system' the amount of backflushing after each sampling is equal to the sea water volume taken in previously which is more than the actually analyzed volume. Field tests will show how this solution holds in practice.

Radio transmission

The currently installed UHF (>300 MHz) transmission type does not penetrate much over the optical horizon. The range is therefore limited to about 10 km unless the receiving antenna is very high. Short wave (25-40 MHz) ground wave propagation over sea water is usable up to 50 km distance, but present equipment has insufficient data rate. Development of new equipment is ongoing. Satellite (Inmarsat etc.) communications to geostationary satellites is getting significantly cheaper but it still

needs too much power. A new possibility is probably ORBCOM. This is a network of many satellites in low orbits that gives continuous coverage. Not usable for the transfer of megabytes, but limited list mode data could be possible.

DISCUSSION AND OUTLOOK

Data quality and reliability

The importance of accuracy is sometimes overestimated in considering autonomous monitoring equipment. Stability and reliability should be the prime considerations (Meredith, 1996). In many cases, reliable trend measurements are required and high accuracy analyses may be started when consent limits have been breached. For the autonomous flow cytometer, we pursued reliability and stability by using fixed settings, fixed mountings, and solid state technology for both laser and detector, a wide field of view, and a wide bore fluid system without failure or blocking prone syringes or valves. These aspects, including the large dynamic range design, imply a trade off with aspects such as accuracy and sensitivity, for instance in comparison with the high beam quality of large gas lasers, the sensitivity of photomultiplier tubes, the better accuracy and sensitivity related with a small optical field of view and conventional detection and pulse processing. The standard deviation is indeed 2-3 times larger as compared to standard flow cytometers, about 10% CV measured on the forward light scatter signal of 4 μm calibration beads. This may still improve after fine tuning of the amplifiers, and removing much of the stray light still present in the prototype optics and increasing the light collection efficiency. Over months of more or less regular use in the lab no adjustments were necessary. The instrument was transported to various sites and no adjustments or alignment were required even after an unintended hard collision with a door post while carrying the instrument on board. Data quality may be impaired by severe roll motion of the buoy in bad weather conditions. This is related to sample core movements in the flow cuvette that were reduced by a special sample injection design but cannot be excluded for 100% when rolls are heavy. In these cases, meteorological data should be used to decide to discard data sets or not. Other sources of failure may be component failure. During the total prototype phase we experienced only three major failures:

the initially applied valveless piston sample pump blocked, an electrical connection short circuited owing to air transport vibrations and a fluid switch valve clogged with large *Coscinodiscus* cells. The pump was replaced by a specially designed tubing pump; the antivibration design was improved, and the valve was removed completely. So far no new failures were experienced. In buoy operation, the radio link should also be reliable. Actual buoy based operation with wireless data transfer was demonstrated during a short feasibility test reported elsewhere including instrumental design details (Dubelaar *et al.*, 1999). Thereafter, the cytometer unit was used on several occasions and trials on ships and labs for periods up to a month, and a second generation prototype is being built now that will be used for extended field and buoy tests to show how the system holds in various environments and weather conditions.

Selective data acquisition

Before analysis, a particle should be detected. A flow cytometer operator normally chooses one of the available measurement entities to serve as the detection criterion. If the output of the corresponding detector is forced above the noise level by the presence of a particle in the laser beam, the electronics systems are 'triggered' to read out all detectors and process the signals. Whereas light scattering is common to all particles, the choice of fluorescence as the trigger parameter allows the operator to limit the analysis and counting to fluorescing particles. This is a selective manner of data reduction. The autonomous character of CytoBuoy requires a fixed trigger setting however. It is most convenient to use forward light scattering to detect all particles; discrimination of phytoplankton and further analysis can be done afterwards with the data analysis software. In the case of application of CytoBuoy in sediment rich water, the data may be dominated by small non-fluorescent particles leaving only a small number of the larger phytoplankton cells and colonies in the data file. These data files contain less particles (1500-4000 particles depending on the size distribution) as compared to normal flow cytometer files (10000 or more, typically) because CytoBuoy generates about 20 to 60 times more numbers to preserve the individual particle scans. The huge data acquisition rate of up to 20 million numbers per second in combination with the small and low power electronics still poses limitations to the data buffer

size. Therefore we are implementing an enrich feature for large particles, typically big phytoplankton cells and colonies. The method works without changes in trigger parameters or levels to keep full data compatibility and prevent selectivity for certain particle types. After a normal measurement run i.e. the acquisition of a full data buffer, a subsequent series of acquisition runs is executed in which only large particles are stored in the buffer on the basis of their pulse length.

Data reduction and automated analysis of the pulse shapes

CytoBuoy's high data yield is no problem at all with the current generation of data storage devices, but for the buoy operated situation it is the radio transmission which still consumes much time and therewith battery power. Direct data processing of the pulse data into list mode data inside the buoy CPU is possible; this however would mean the loss of possibilities to extract more features from the data in off line analysis. A mixed solution would be to do this only for the smallest particles, because their pulses are dominated by the Gaussian laser beam shape and contain little morphological information. Another general approach is to convert the pulse data into mathematical functions such as a Fourier transform or discrete sine or cosine functions or wavelet transform. Preliminary results show that data reductions of 50-80 % can be achieved for short to long particles, whereas the original pulses can be restored from these data with high resemblance. These general approaches would require rather heavy calculating jobs inside the buoy, but this may still be advantageous in terms of power use. After receiving the raw data, the CytoWave software extracts predetermined types of list mode data from the pulse profiles for regular user interactive data analysis, supported by selective display of the full pulse shapes. We expect that automated classification of the 'raw' pulse data with artificial neural networks (ANN) would speed up analysis combined with more discrimination potential, which is currently investigated. This would allow a more efficient use of the large data generation capacity of this type of cytometric analysis. However, specific repeated interactive analysis of the flow cytometer data may be standardized to a high extent by the user in day to day practice already. Promising general developments are ongoing in the AIMS project (Jonker *et al.*, 2000) with ANN processing of list-

mode flow cytometer data of phytoplankton and coupling to a data base of complementary data.

Pursuit of a higher analytical resolution

A way of upgrading the analytic resolution is to add independent optical parameters, or to add resolution to existing parameters. As mentioned before, the CytoBuoy detection system allows easy upgrading to more fluorescence emission bands. Upgrading to two laser excitation wavelengths would certainly improve the capability for pigment group discrimination but this would require more complicated electronics or a reduction of the particle count rate. The CytoBuoy pulse data format added one-dimensional morphological resolution to the standard flow cytometry parameters. Further upgrading would mean to acquire images instead of the pulse shapes. Autonomous operation implies nonselective data acquisition first, meaning one image for each particle. This does not seem realistic for the near future, at least until we have more insight into the possibilities of low power fast acquisition, storage and subsequently highly efficient data reduction of images. Imaging generates massive amounts of data (Kachel and Wietzorrek, 2000). A moderate resolution of 256x256 pixels uses 64 kbyte for a single particle, which is the present full grabber capacity. Even with a huge data reduction, the radio link probably will remain a bottleneck.

Other platforms and integration with other devices

The Datawell wave rider buoy is a perfect waterproof housing for the sensor. The CytoBuoy flow cytometer module is small enough to be easily installed in various other places also. A wind and rain proof version is straightforward and allows installation on a fixed land or shore position or on a ship. The latter application requires a 'soft' mounting to eliminate engine/screw vibrations, and is particularly promising with respect to the 'ship of opportunity' principle in which instrument packages are installed on for instance ferry ships to generate high frequently monitoring time series over the ships route. Real underwater deployment requires a pressure proof housing and a sample loop system to separate the cytometer fluidics from the high water pressure at depths to 200 m. An aluminium "diver version" is being developed in the framework of a scientific demonstration program for the Autosub

autonomous underwater vehicle (Griffiths *et al.*, 1998). This submersible version will also be applicable for lowering on a cable from a research vessel or some fixed mounting on a submerged construction (offshore platform), but prolonged underwater operation requires the use of better corrosion resistant housing material. The CytoBuoy can also be triggered from another monitoring device, or vice versa. This is a matter of creating a suitable electronic control link. Triggering another device on the basis of CytoBuoy's measurements is difficult because this needs off line analysis first. In the case of monitoring, e.g. in the presence of a specific group of particles with distinct optical properties, this could be preprogrammed and used to trigger for instance an automatic sampling device (D. Mills, CEFAS, personal communication). There is a substantial interest in small (<1 μm) particles. An autonomous CytoBuoy type of instrument for these smallest particles requires a different design of optics, fluidics and electronics. The cuvette should be narrower as well as the sample stream for instance to allow a much narrower (intenser) focus, and more efficient light collection (higher numerical aperture objective with a small depth of focus). In addition there would be no pulse shape information, and photomultipliers for higher gain. Because of the narrower cuvette and tubing, a prefiltration system would have to prevent large cells and sediment particles from entering and clogging the system. To solve this latter problem for a selective and reproducible autonomous operation may be the biggest challenge.

CONCLUSION

The direct online estimation of phytoplankton biomass and the ability to discriminate between different phytoplankton groups allows the analysis of undisturbed natural samples with the advantage of having continuous quantitative *in situ* observations in time or space (tracks), yielding more insight into the seasonal or spatial dynamics and patchiness of ecosystems. The long term operational aim is the large scale implementation and integration of rugged 'individual particle analysis' devices in monitoring systems, as a complementary tool to narrow the gap between continuous bulk value measurements and low frequency microscopical observations. The CytoBuoy development represents a significant step in this process.

ACKNOWLEDGMENTS

The 'CytoBuoy project' was supported by the European Commission in the Marine Science and Technology program, contract MAS3-CT95-0030 as a joint effort of Datawell (Haarlem), Oceanor (Trondheim), DRIE (Bodegraven), Kantakun (Delft) and Pyxilla (Delft). We thank the reviewers for their valuable contribution.

REFERENCES

- Balfoort, H.W., Th. Berman, S.Y. Maestrini, A. Wenzel and T. Zohary. – 1992. Flow cytometry: instrumentation and application in phytoplankton research. *Hydrobiologia*, 238: 89-97.
- Dubelaar, G.B.J., A.C. Groenewegen, W. Stokdijk, G.J. van den Engh and J.W.M. Visser. – 1989. The optical plankton analyser (OPA): a flow cytometer for plankton analysis, II: Specifications. *Cytometry*, 10: 529-539.
- Dubelaar, G.B.J. and C.S. van der Reijden. – 1995. Size distributions of *Microcystis aeruginosa* colonies: a flow cytometric approach. *Water Science and Technology*, 32 (4): 171-176.
- Dubelaar, G.B.J., A. Cunningham, A.C. Groenewegen, J. Klijnstra, L. Boddy, M.F. Wilkins, R.R. Jonker and J. Ringelberg. – 1995. A European optical plankton analysis system: flow cytometer based technology for automated phytoplankton identification and quantification. In: Weydert M, Lipiatou E, Goñi R, Fragakis C, Bohle-Carbonell M, Barthel KG (eds.), *Marine Science and Technology, Second MAST days and EUOMAR Market. Project reports*. Vol. 2: 946-956. Commission of the European Community, Luxembourg.
- Dubelaar, G.B.J., K. Tangen, P.L. Gerritzen, A.E.R. Beeker and R.R. Jonker. – 1998. CytoBuoy: In situ optical scanning of individual particles with a buoy mounted flow cytometer. In: K.-G. Barthel *et al.* (eds.), *Marine Science and Technology, Project Synopses, Volume IV: Advanced Systems*. pp. 1500-1511. Commission of the European Community, Luxembourg.
- Dubelaar, G.B.J., P.L. Gerritzen, A.E.R. Beeker, R.R. Jonker and K. Tangen. – 1999. Design and first results of the CYTO-BUOY: an autonomous flow cytometer with wireless data-transfer for in situ analysis of marine and fresh waters. *Cytometry* 37: 247-254.
- Griffiths, G., N. W. Millard, S. D. McPhail, P. Stevenson, J. R. Perrett, M. Pebody, A. T. Webb, D. T. Meldrum and D. Russell. – 1998. Towards Environmental Monitoring with the Autosub Autonomous Underwater Vehicle, *Proceedings Underwater Technology '98*, Tokyo, April 1998.
- Hofstraat, J.W., M.E.J. de Vreeze, W.J.M. van Zeijl, L. Peperzak, J.C.H. Peeters and H.W. Balfoort. – 1991. Flow cytometric discrimination of phytoplankton classes by fluorescence emission and excitation properties. *J. Fluorescence* 1(4): 249-265, 1991.
- Hofstraat, J.W., W.J.M. van Zeijl, M.E.J. de Vreeze, J.C.H. Peeters, L. Peperzak, F. Colijn and T.W.M. Rademaker. – 1994. Phytoplankton monitoring by flow cytometry. *J. Phytoplankton Res.* 19(9): 1197-1224.
- Jonker, R.R., J.T.M. Meulemans, G.B.J. Dubelaar, M.F. Wilkins and J. Ringelberg. – 1995. Flow cytometry: a powerful tool in analysis of biomass distributions in phytoplankton. *Water Science and Technology* 32(4): 177-182.
- Jonker, R.R., J.T.M. Meulemans, G.B.J. Dubelaar, M.F. Wilkins and J. Ringelberg. – 1995. Flow cytometry: a powerful tool in analysis of biomass distributions in phytoplankton. *Water Science and Technology* 32(4): 177-182.
- Jonker, R., R. Groben, G. Tarran, L. Medlin, M. Wilkins, L. Garcia, L. Zabala and L. Boddy. – 2000. Automated identification and characterization of microbial populations using flow cytometry: The AIMS Project. *Sci. Mar.*, 64(2): 225-234.
- Kachel, V. and J. Wietzorrek – 2000. Flow Cytometry and Integrated Imaging. *Sci. Mar.*, 64(2): 247-254.
- Meredith, W.D. – 1996. On-line monitoring of water quality. *New World Water* 1996: 103-108.
- Peeters, J.C.H., G.B.J. Dubelaar, J. Ringelberg and J.W.M. Visser. – 1989. The optical plankton analyser (OPA): a flow cytometer for plankton analysis, I: Design considerations. *Cytometry* 10: 522-528.
- Tent, H. – 1997. European dimensions of ocean and climate forecasting. In: J.H. Stel, H.W.A. Behrens, J.C. Borst, L.J. Droppert and J. v.d. Meulen (eds.), *Operational Oceanography. The Challenge for European Co-operation*. Elsevier Oceanography Series 62: 7-10.
- Veldhuis, M.J.W. and G.W. Kraay. – 2000. Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Sci. Mar.*, 64(2): 121-134.
- Zilmer N.A., M. Godavarti, J.J. Rodriguez, T.N. Yopp, G.M. Lambert and D.W. Galbraith. – 1995. Flow cytometric analysis using digital signal processing. *Cytometry* 20: 102-117.