Protist News

Meeting Report: Aquatic Flow Cytometry: Achievements and Prospects, Research- and Technology Centre Westcoast (FTZ), Büsum, Germany, October 15–16, 1998

Although flow cytometry was originally targeted for use in the medical field, it has become a technique that has received wide-spread acceptance in the field of aquatic biology. Without this tool, the predominance of important and sometimes novel components of the phytoplankton, such as Synechococcus, Prochlorococcus, and the pico-eukaryotes, would not have been identified nor would their widespread abundance have been known. So, it is not surprising that a workshop on aquatic flow cytometry should be held and that it would be so well attended. With over 30 participants representing 10 countries, issues ranging from the introduction of new specialised equipment and methods to their possible application in marine biology and limnology were discussed. The meeting was divided into four main themes: Hardware, the EU/MAST III AIMS (Automated Identification and Characterisation of Microbial Populations) project, phytoplankton physiology and ecology, and microbial ecology.

The broad range of sizes, cell shapes and cell components found in phytoplankton requires a variety of specialized equipment, software and additional tools for the flow cytometric analysis of such divergent organisms. For monitoring coastal waters, a special flow cytometer, the CytoBuoy, designed by George Dubelaar (Bodegraven, The Netherlands), has been developed (EU/MAST III). It has a larger orifice than other standard flow cytometers and has been installed inside a buoy for in-situ on line monitoring. It is capable of automatically taking water samples, measuring them and sending the data by radio-transmission to shore where they can be analysed. For enhanced information, the CytoBuoy transmits not only the pre-processed data (i.e., height) of a single pulse, but also the raw data of the full pulse shape. By using the pulse shape as a kind of “fingerprint”, larger cells or cell chains common to coastal phytoplankton can be identified. Such instruments offer new possibilities to study coastal phytoplankton, which heretofore has been limited by standard flow cytometers because of their larger and more elaborate shapes.

Another technical solution for the identification of phytoplankton species was presented by Volker Kachel (Martinsried, Germany) with an integrated imaging system inside a flow cytometer. This flow cytometric imaging (FCI) or ‘image in flow’ system gives a microscopic image of a particle that is otherwise only recognised by more abstract data like fluorescence or light scatter as it passes through the flow cytometer. A light microscopic image can give valuable additional information for species identification or for verifying the identification made by ‘classical’ analytical flow cytometric (AFC) parameters. Technical aspects of this device, such as triggering the photo shot, focusing, pulsed illumination and freezing the motion of the particle in the rapid flow, were detailed. FCI systems can already be found in some flow cytometers, the MacroFlow Planktometer (EUROMAR MAROPT project), the European Optical Plankton Analyser (MAST III EurOPA project) and the FLUVO VI universal flow cytometer.

Once the particle has passed through the flow cytometer, then the more difficult task of analysing the optical data is faced. Analysis of flow cytometric data is often limited to two-dimensional scatter plots or histograms, even if the cytometer itself is capable of measuring five or more different parameters. Nevertheless, by using all of this information, it is possible to characterise and identify phytoplankton taxa even to species level. Glen Tarran (Plymouth, Great Britain) demonstrated how different multivariate analysis techniques, such as principal components analysis or artificial neural networks (ANN) can be applied to flow cytometric data to
achieve a species identification or separation of populations. The ANNs learn to distinguish species using optical data derived from unialgal cultures. Trained ANNs were later capable of identifying these organisms in mixed cultures of more than 25 nano-plankton species with more than 70% accuracy. The parameters learned by the ANN to recognise a particular taxon can later be used to set the gates on the flow cytometer so that distinct populations can be sorted for later analysis.

Richard Jonker (Amsterdam, The Netherlands) gave an overview of data analysis and management in the EU project ‘AIMS: the development of an automated system for the identification and characterisation of marine microbial communities.’ This project, with 9 European partners, plans to produce an automated identification system based on the application of artificial neural networks for flow cytometric data analysis. A number of other speakers (Dubelaar, Groben, Tarran) also presented their results in this programme. Jonker supplied additional information on digital signal procession by pulse shape analysis. Quantitative measurements by this technique have compared well with "traditional" counting of cells by light microscopy. He also stressed the need for reproducibility of measurements between flow cytometric instruments, which is often not taken into consideration. He noted that this had been done for the various flow cytometers in the AIMS project. Data was presented to show the high accuracy of counts when the same experiment was repeated with different flow cytometers following intercalibration of the flow cytometers.

In some cases the standard data provided by flow cytometry, i.e., autofluorescence, forward and side scatter, are insufficient for a clear identification of phytoplankton species. This problem may be overcome with the development and application of species-specific probes for different phytoplankton taxa. René Groben (Bremerhaven, Germany) presented his progress with the development of probes using rRNA sequences for the AIMS project. Specificity tests of these probes include comparison with sequences from computer databases, their hybridisation to membrane-bound algal DNA with chemiluminescent detection and in situ hybridisation of fluorochrome-labelled probes to whole cells. These cells can either be analysed directly with a flow cytometer or viewed with a fluorescence microscope, i.e., to identify an algal species or group of taxa after flow cytometric sorting to confirm the identification made by an ANN. A broad range of probes from higher group level (i.e., specific for chlorophytes) to species level (i.e., for Phaeocystis globosa) will be available in the near future.

The application of rRNA probes in combination with flow cytometry to field samples was shown by Jo Brenner (Stuttgart, Germany). During two cruises with the research vessel ‘Heincke’ in 1997 and 1998 at the Orkney Islands (Scotland), blooms of the toxic dinoflagellate Alexandrium tamarense were detected by using species-specific rRNA probes as part of the German BMBF project TEPS (The Development, Occurrence and Distribution of Toxicity in Eukaryotic/Prokaryotic Assemblages). The population measurements using the probes correlated well with cell counts using microscopic methods and with toxin concentrations measured by HPLC onboard ship. Such good correlations show excellent promise for species identification and quantification by a combination of flow cytometry and specific molecular probes. The use of the HPLC onboard ship offers another opportunity to have real-time estimates of the development of toxic algal blooms.

On the other hand, using flow cytometric methods coupled with molecular probes for the analysis of marine bacteria have failed so far because of methodological difficulties in probe hybridisation after filtering the bacterial samples and because of problems with low RNA-content of the field samples as compared to culture populations. With such low signal to noise ratios, bacterial identifications cannot be resolved from the background, even if specific identification of the marine bacteria can be shown by fluorescence microscopy. Certain dyes like Cy5 or combinations of dyes, such as Cy3 and SYTOX for counter-staining the cells, were not suitable for flow cytometric analyses of bacterial populations (Gunnar Gerds, Helgoland, Germany). Clearly this area of research requires more technological development before it can be widely applied to field populations.

The flow cytometers, FACSCalibur and FACSVersion SE, were introduced by Rico Bongaarts (Fa. Becton Dickinson). Both machines offer possibilities for a wider range of measurements and have also cell sorters included, which makes them useful for different applications in aquatic sciences. Marcus Reckermann (Büsum, Germany) showed specific examples of how he has applied flow sorting to reveal phytoplankton community structure, to obtain mono-specific algal cultures, and to define group specific physiological parameters of sorted species (i.e., those cells associated with 14C or 15N uptake).

The application of flow cytometry for answering questions in aquatic ecology was the topic for more than half of the talks at the workshop. Analytical flow cytometry can be used for growth rate studies of phytoplankton as it was shown for Skeletonema costatum (Frank Jochem, Hamburg, Germany),
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The abundance of the dominant species of cyanobacteria in different water depths in the Mediterranean can be applied for the study of vertical distributions through the flow cytometer. This method has even provided a very elaborate demonstration of the saturable fluorescence of the cell cycle. In situ growth rates of these cultures could be calculated by combining these data with division rates of the species. The use of specific fluorescence-labelled antibodies in addition to nuclear staining results in even more information. It was possible to identify *Gymnodinium mikimotoi* with AFC in pure cultures, in enriched and in natural seawater samples by incubating them with a species-specific antibody. Together with the PI stain, the growth rate of this species could be measured even in mixed field samples (Peperzak). Physiological studies were done by Jochem with a fluorochrome-labelled antibody against the nitrate reductase protein, an enzyme which is necessary for nitrate metabolism. Even under high nitrate conditions, the enzyme is present only in small amounts in the cell, which makes it sometimes difficult to analyse. With the high sensitivity of AFC, it was possible to detect the labelled protein even with low enzyme concentrations following growth under low nitrate conditions. Also the accumulation of nitrate reductase protein shortly after transferring the cultures from low to high nitrate conditions could be measured, giving a more detailed picture of the response of *S. costatum* to changing amounts of nitrate. Veldhuis showed the abundance of *Prochlorococcus* in different water depths and analysed also the cell cycle of this species by staining the DNA prior to AFC and measuring its content in the cells. He also provided information on vital stains that can be used to track physiological states.

Alexander Chekalyuk (Wallops Island, USA) provided a very elaborate demonstration of the saturating-flash fluorescence techniques (pump-and-probe) as applied to flow cytometry to estimate the photosynthetic capacity of single cells as they pass through the flow cytometer. This method has even been tried on-board ship and has revealed a fourfold difference in the photosynthetic capacity of different species and offers new possibilities to obtain population-specific photosynthetic characteristics. Michael Denis (Marseille, France) showed how flow cytometry can be applied for the study of vertical distribution of phytoplankton. Time-series samples from different water depths in the Mediterranean Sea were analysed by flow cytometry, the species composition identified and their numbers counted. The abundance of the dominant species of cyanobacteria in a water layer was greatly affected not only by grazing or epodic events like strong winds, but also by diel vertical migration (DVM). This periodic event and the resulting presence or absence of large numbers of organisms in the upper water region can have a great impact on the carbon uptake and should therefore always be taken into consideration when calculating carbon fluxes.

Feeding experiments of *Peridiniopsis* on *Rhodomonas* and *Cryptomonas* were introduced by Thomas Weisse (Mondsee, Austria). Grazing of the algae by the dinoflagellate was measured by flow cytometry and with a Coulter counter as well as estimated directly by light microscopy. Although the Coulter counter was quite rapid in counting cells, which is a requirement for any grazing study, it has its limitations because the Coulter counter can only analyse numbers and sizes of cells. For analysing cells of similar sizes, the additional parameters afforded by the flow cytometer, such as fluorescence, were necessary. In the second study, algal concentrations decreased as the cells were grazed, this was followed by a concomitant increase in autofluorescence of the predators from ingested algal chloroplasts. Grazing rates calculated with the flow cytometer and the Coulter counter also correlated well with those obtained by light microscopy. Feeding is a highly variable process, depending on the physiological status both of the predator and its prey. Starving predators were less selective in their prey and the feeding process was very fast, before it slows down or even stops after some time. In both studies, only about 50% of the predator population could be shown to be feeding. With flow cytometry, the intricate details of grazing can be better studied, time series can be made, and digestion rates calculated.

Applications for the use of flow cytometry to determine the population structure of the bacterial community was reviewed by Josep Gasol (Barcelona, Spain). He showed how the use of nuclear stains has facilitated the identification of high and low DNA assemblages in the bacterial communities. There is some indication that predators prefer the bigger bacteria, those with the higher DNA content. The fluorescence of the bacteria stained with nuclear dyes correlated with their size. Also good correlations were presented between flow cytometric counts of bacteria and those based on fluorescence microscopy.

All in all, this workshop provided an excellent overview of the 'state of art' of flow cytometric analyses of the aquatic environment. The participants showed the enormous potential that this technique has for aquatic sciences by the introduction of new equipment and methods and how to apply them to...
different fields. Also the results obtained by AFC correlate well so far with those obtained by other "classical", but often more time consuming or laborious methods, such as light microscopy. Flow cytometry can speed up and simplify research in some fields of aquatic science. Therefore it can be expected that flow cytometry will become a major tool in answering ecological and physiological questions in oceanography and limnology in the near future. Readers who want more information about the workshop should visit the homepage of the FTZ Westküste at: http://www.uni-kiel.de:8080/ftzwest/ ag1/projekte/Workshop.html or become part of the discussion list about aquatic flow cytometry at http://www.flowcytometry.org.
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