RECENT ADVANCES IN ASSESSING THE DYNAMICS OF PHYTOPLANKTON ASSEMBLAGES BY HIGH FREQUENCY ANALYSIS AT THE SINGLE CELL LEVEL

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Glossary : CNRS : Centre National de la Recherche Scientifique ; INSU : Institut National des Sciences de l'Univers ; IRD : Institut de Recherche pour le Développement ; MIO : Mediterranean Institute of Oceanography ; UMR : unité mixte de recherche ; CEL2SAT: acronyme of the project entitled "*Diversité structurelle et fonctionnelle du phytoplancton à l'échelle régionale de la Manche Occidentale : de la cellule à l'image satellite » ;* PRECYM : Plate-forme Régionale de Cytométrie pour la Microbiologie ; GIPREB : Groupement d'Intérêt Public pour la Réhabilitation de l'Etang de Berre.

Abstract
Phytoplankton plays a major role in oceanic biogeochemical processes by converting inorganic material into organic matter, the feeding source of the entire marine food chain. Its large diversity and short cell cycle make it very sensitive to environmental changes. To overcome difficulties linked to the very large size range and species number, and correctly account for phytoplankton dynamics in natural environments, it is critical to observe it at the single cell level, at an hour time scale, as well as at a spatial submesoscale. The availability of commercialised, dedicated automated flow cytometers (Cytosense), eventually submersible (Cytosub), enables to address the dynamics of phytoplankton temporal and spatial distributions.
The reported recent developments in that field stem from 9 years of experience and leadership. They concern investigations with a Cytosense conducted on a lagoon near Marseille, on a research vessel in the English Channel (coupled to a FerryBox), on a EOL (Environnement Observable Littoral, CNRS-Mobilis) buoy in the Bay of Villefranche sur Mer (France), in the Western Mediterranean Sea (2 cruises in 2013) and on the ferry Armorique connecting Roscoff (France) to Plymouth (UK) (coupled to a FerryBox). Except that in the English Channel, the Cytosense was remotely controlled. New developments under progress target heterotrophic microorganisms.

1 INTRODUCTION

Phytoplankton is the major producer of organic matter on earth, contributing up to 45% of annual global photosynthesis, though it only represents about 2% of the overall photosynthetic biomass (Field et al., 1998; Falkowski et al., 2003). Such a capacity stems from its existence essentially in the form of single cell organisms that can grow and divide rapidly (up to 2 divisions day^{-1} (Alpine and Cloern, 1988; Furnas, 1991) and spread over 70% of the earth surface. This short cell cycle makes phytoplankton able to react promptly (hour time scale) to any change in its environment. The current phytoplankton is the outcome of about 3 billion year evolution, from a time where life was only present in the form of single cell organisms. These organisms had to adapt to an infinite number of environmental changes, generating over years an extreme diversity that still remains to be fully unveiled. Because it is at the basis of the so called "food chain" in the ocean, being the major source of matter and energy for the higher trophic levels, in both pelagic and benthic environments (Li et al., 2006), phytoplankton was and still is the subject of intensive investigations. Many techniques were used. Optical microscopy is the only one giving access to cell identification, at least the larger (>20 µm) ones and is commonly used in phytoplankton monitoring. However, analysis by optical microscopy is time consuming, requires highly specialised staff and therefore cannot handle large numbers of samples. Consequently, it is unsuitable to investigate short-term variability or spatial distribution of phytoplankton. Pigment analysis by high-performance liquid chromatography (HPLC) opened the way to a better and faster quantification of

dominant groups (Jeffrey and Vest, 1997). The lack of pigment specificity makes interpretations difficult and limits the benefit of this approach even when coupled to optical microscopy (Havskum et al., 2004), particularly regarding the dynamics of phytoplankton. Fluorometry is also a bulk approach, and by dealing with optical signals, it is suitable for high frequency recording of the fluorescence that is emitted by chlorophyll *a* upon appropriate excitation. This is why fluorometry is easily and widely used to estimate the chlorophyll *a* content in water masses and to address its variability at short temporal and spatial scales (Rantarjavi et al., 1998). Serious limitations are linked to the conversion of chlorophyll *a* content to carbon biomass and also to the limited seawater volume generating the collected fluorescence signal. It means that large cells that are weakly concentrated may easily escape to observation.

Flow cytometry is a technique analysing at high rate optical properties of suspended individual particles flowing in a stream through an optical beam. When intercepted by the optical beam (usually a laser beam) each particle generates scatter and fluorescence (naturally or after staining) signals. Data analysis groups particles sharing similar optical properties and some instruments are able to sort out cell groups for further investigations. This technique was initially developed for the needs of the biomedical field. Phytoplankton being essentially represented by single cells in suspension in seawater and containing fluorescent photosynthetic pigments appeared suitable flow cytometry analysis. The potentialities of flow cytometry analysis and cell sorting were thus investigated in the early 1980s (Yentsch at al., 1983) and shortly after led to the breakthrough that represented the discovery of *Prochlorococcus* (Chisholm et al., 1988) that makes the largest group in the oligotrophic ocean (Partensky et al., 1999).

This finding fostered the development of new flow cytometers specifically designed for the analysis of phytoplankton in natural samples such as the optical plankton analyser (OPA ; Dubelaar et al., 1989 ; Jonker et al., 1995). This effort went on with the design of the Cytobuoy flow cytometer, conceived to be operated *in situ* in an automated way (Dubelaar et al., 1999). A specific feature of this instrument was the recording of the pulse shape of the signals generated by phytoplankton cells when they are intercepted by the laser beam, enabling the analysis of chains which is not possible with conventional flow cytometers. The number of cells composing a given chain is defined by the number of repeated shape-patterns representing the flow cytometric signature of each cell. Another automated flow cytometer was developed at Woods Hole Ocean Institute to investigate pico and phytoplankton (Olson et al., 2003) but did not include pulse shape recording.

The first commercialised submersible flow cytometer (Cytosub) developed by the Cytobuoy company (Cytobuoy.com) was bought by our group in 2004. This new approach was first validated by Thyssen et al. (2008a) then applied to investigate the dynamics of the phytoplankton assemblage in the Bay of Marseille (Thyssen et al., 2008b) and its spatial distribution in the north eastern Atlantic (Thyssen et al., 2009). The Cytosub was further involved in a mesocosm experiment in the frame of a Canadian programme (Thyssen et al., 2011).

Meanwhile, the Cytobuoy company kept upgrading its instruments (Cytosub, Cytosense) and included an image in flow device, particularly efficient for large (>20 µm) cells. The Cytosense, a lab-top flow cytometer, can be run in an automated way on pumped water which provides a large flexibility in coastal monitoring stations and in studies conducted on research vessels or ships of opportunity.

2 RECENT APPLICATIONS

2.1 Phytoplankton dynamics remotely monitored from a buoy

Our Cytosub was adapted by the constructor so that it could be run inside the EOL (Environnement Observable Littoral, CNRS-Mobilis) buoy deployed in the Bay of Villefranche sur Mer ((43.682°N, 7.319°E; north western Mediterranean Sea) by the Universe Science Observatory (OSU) of Villefranche sur Mer (Fig. 1).

Figure 1 View of the EOL (Environnement Observable Littoral, CNRS-Mobilis) buoy deployed in the Bay of Villefranche sur Mer (France). The water column depth at this location is about 100 m.

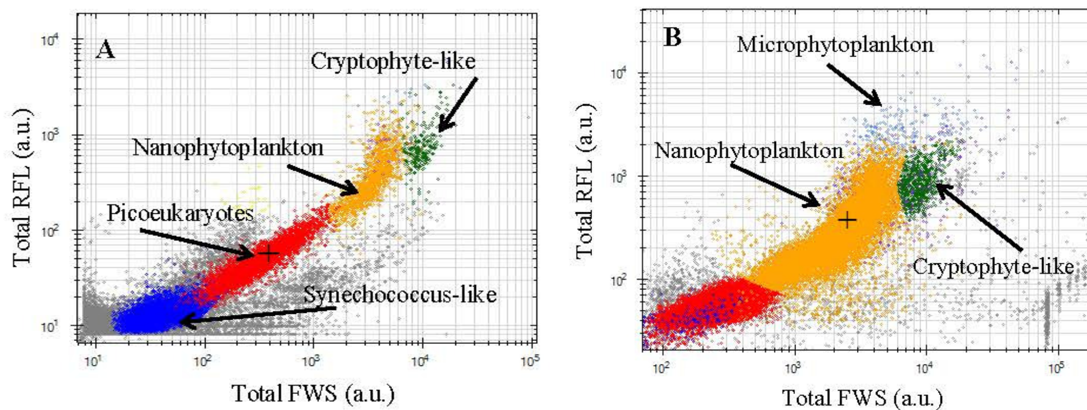

Figure 2 Typical cytogrammes illustrating the cluster resolution when using a low threshold (a) to focus on the smallest cells, and a higher threshold (b) to analyse a larger volume and account for bigger cells while skipping the smallest ones. Total FLR : integrated red fluorescence signal with respect to time. Total FWS: integrated forward scatter signal with respect to time. a.u. : arbitrary unit.

The energy was supplied by solar panels and stored on batteries located inside the EOL cabin, above sea level. The experiment lasted from January 24 to April 6 2012. The Cytosub analysed seawater pumped at 1 m depth. Thanks to a wifi connexion between the EOL buoy and the OSU, the Cytosub could be remotely controlled from our laboratory and data downloaded every day. Seawater samples were analysed every two hours by using in sequence two protocols, one focusing on the smallest cells, the other skipping the smallest cells to account for the larger ones by analysing more seawater as illustrated by Figure 2 displaying for each case cytogrammes of red fluorescence (emitted by chlorophyll a) versus forward scatter (linked to cell size). The key point was the trigger level set for the detection of the red fluorescence signal. A total of six phytoplanktonic cell groups were resolved throughout the validated 532 flow cytometry analyses.
The study, conducted in collaboration with scientists of the Laboratory of Oceanology of Villefranche sur Mer (LOV), succeeded in describing the onset of the spring bloom in the north western Mediterranean Sea and to document the influence of environmental pulse events on the *in situ* hourly-scale dynamics of the phytoplankton structure (Thyssen et al., 2014). By using a loess procedure, a controlled smoothing highlighted the global trend of the signals and of the abundance of each cluster. Three abundance pulses were observed and could be correlated to pulses of environmental variables (wind speed, rain fall, nutrient concentration). By working on

the periodic signals it was possible to characterise the cell cycle of the resolved clusters and differentiate their responses to the observed environmental changes (see Thyssen et al., 2014).

2.2 High frequency phytoplankton monitoring in surface waters

The first attempt to address the heterogeneity of phytoplankton surface distribution in the ocean with a Cytosub, clearly evidenced the dependence of the community structure on crossed water masses (Thyssen et al., 2009). In addition, it was possible to discriminate the periodic components from the longer-term trends in abundance so that cell cycles could be estimated. The large diversity of phytoplankton prevents to expect identifying the role of each species in biogeochemical processes. As a possible answer to this difficulty, the initial work on phytoplankton dynamics investigated at the single cell level revealed that cell clusters behaved as a single entity (Thyssen et al., 2008), and were recognised as functional response groups. This viewpoint was in line with the suggestion by LeQuéré (2005) to consider phytoplankton functionalities with respect to biogeochemical processes, each functionality being possibly shared by several species.
This approach was applied in particular to the dynamics of phytoplankton assemblages in the English Channel in the frame of the CEL2SAT (CNRS-INSU) project, by combining automated instruments, the Cytosense of the PRECYM regional flow cytometry platform of the MIO (http://precym.mio.univ-amu.fr) and a FerryBox instrument already installed on the ferry Armorique connecting Roscoff (France) to Plymouth (United Kingdom) twice a day. The coupling of the Cytosense with the FerryBox lasted from 16 May to 17 September 2013 and yielded technological and software developments that will be ready for use when implementing these instruments onboard ships of opportunity. The CEL2SAT project yielded an unprecedented amount of data on the phytoplankton distribution across the English Channel between Roscoff and Plymouth and the exploitation of the whole dataset is still under progress.

2.3 Response of the phytoplankton assemblage to a gust of wind.

The Berre lagoon, close to Marseille (France) is one of the largest brackish lagoons in the Mediterranean area with its 155 km^2 surface and makes a shallow water body (maximum depth: 9m). The construction of a hydroelectric power plant on its edge completely disturbed its ecosystem by frequent and large freshwater discharges that added to natural freshwater outflows. The lagoon is also under the influence of surrounding oil refineries and chemical factories. The seawater inflow goes through the Caronte channel and prevailing winds (northerly and easternly) make additional strong natural forcings. To alleviate the impact of the hydroelectric plant, its freshwater discharge was constrained with respect to time and volume. An agency (GIPREB), a public consortium for the restoration of the Berre lagoon, was set up to implement a detailed survey and monitor the lagoon eutrophication since 1995. However the short-term variation of the phytoplankton compartment was not taken into account in that survey. To fill this gap, we addressed the response of the phytoplankton to the large and sudden changes in its environment, particularly gusts of wind. To achieve this goal, we installed an automated flow cytometer (Cytosense instrument from the PRECYM regional flow cytometry platform of the MIO; http://precym.mio.univ-amu.fr) equipped with an image in flow device, inside the GIPREB building on the lagoon shore and ran hourly analyses of the lagoon water pumped 250 m off the shore, at 1 m above bottom. The experimental setup enabled parallel collection of hydrological variables on the same water. Subsamples from the same analysed water were taken at lower frequency to determine nutrient concentrations. Two flow cytometry protocols were run in sequence, one focusing on small cells, the other one on larger cells by setting an appropriate threshold on the red fluorescence signal (triggering the data acquisition) to skip the small cells and analyse a larger volume, bigger cells being less abundant. Cells sharing similar scatter and fluorescence properties were regrouped and thus we could define 12 distinct clusters labelled C1 to C12, C1 corresponding to the largest observed cells (56.4 ± 12.2 µm) and C12 to the smallest (0.9 ± 0.1 µm). Cells from C1 to C4 belonged to the microphytoplankton size class, those from C5 to C10 to the nanophytoplankton size class and cells of C11 and C12 were picoplankton. Most of the microphytoplankton cells were identified from their images. Figure 3 displays a typical cytogramme of red fluorescence (FLR) versus sideward scatter (SWS) in which clusters C4 and C5 are singled out with corresponding images of microphytoplankton cells. During the October 2011 experiment, a strong north wind (Mistral) event occurred with mean speed >20 ms^{-1} and the lagoon water temperature dropped by 5.2°C between 5 and 11 October. The meteorological data (wind speed and direction) were provided by the meteorological station of the Mediterranean Institute of Oceanography (MIO) located on the Frioul archipelago, in the Bay of Marseille. The high frequency observation of

phytoplankton cells enabled to characterise the dynamics of each resolved cluster before, during and after the wind event. One major result of this experiment was the confirmation of clusters as functional response groups as initially demonstrated by Thyssen et al. (2008b).

In this experiment, a particular attention was given to the impact of gusts of winds on specific growth rates. Because the cell optical properties are linked to cell size, high frequency single cell analysis provides a good alternative to estimate growth rates. Indeed, the periodic increase and decrease of the cell scatter-signal intensity expresses the cell growth and division occurring during the cell cycle (Durand, 1995 ; Binder et al., 1996). Sosik et al. (2003) developed a model to calculate growth rates of natural phytoplanktonic groups from the diel variations of the phytoplankton cell dimensions derived from automated high frequency flow cytometry surveys. This approach can be further improved by estimating cell dimensions derived from image in flow attachments (Sieracki et al., 1998). The Cytosense that we used in the Berre Lagoon experiment being equipped with an image in flow device, we took advantage of this capacity to estimate biovolumes. We adapted the size-structured matrix population model of Sosik et al. (2003) to process the single cell analysis of two dinoflagellate clusters and estimate their division rates before, during and after a gust of wind. Both clusters were composed of cells large enough to make images suitable for determining cell dimensions and abundant enough to yield robust results. Indeed, clusters with larger cells were made of cells in low abundance. Cells in one selected cluster were identified with *Gymnodinium sp*. from the collected pictures. Their mean cell biovolume increased one hour after dawn before it progressively decreased upon mitotic divisions. The amplitude of both processes varied before, during and after the wind event so that growth rates ranged between 0.22 d^{-1} (<1 division per day) and 0.85 d^{-1} (>1 division per day). In contrast, pictures could not help in identifying cells in the other cluster. Nevertheless, results showed that the ratio of max/min mean biovolume varied from 1.26 to 1.55 during the gust of wind, and growth rates progressively increased with regard to the variation of mean biovolume amplitude to reach 0.46 d^{-1} on 11 October. The striking result of this investigation was to demonstrate, thanks to the automated high frequency observation of phytoplankton, that cell physiological changes can prevail over the response of abundance. The full study is reported in Dugenne et al. (2014). As a major consequence, variations in phytoplankton growth rates would generate a mismatch between the related photosynthetic carbon fixation and net primary production derived from ocean color data. Indeed, usual low frequency (monthly or bimonthly) surveys cannot take into account pulse events and the subsequent phytoplankton biomass pulses that significantly contribute to biogeochemical fluxes and budgets at the annual scale (Lomas et al., 2009).

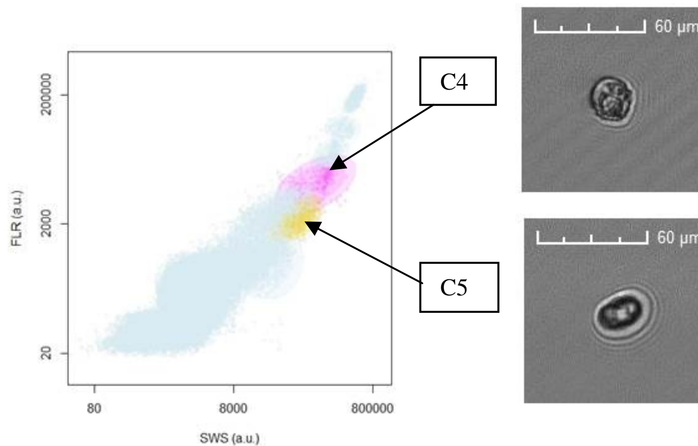

Figure 3 Typical cytogramme of the phytoplankton analysis in the Berre lagoon, displaying red fluorescence (chlorophyll) with respect to side scatter linked to cell structure. Pictures of cells composing clusters C4 and C5 are also shown. a.u. : arbitrary unit.

3 DISCUSSION

If the concept of automated flow cytometry analysis first emerged in the biomedical field (see Abu-Absi et al., 2003 and references herein) like flow cytometry itself, it was developed in parallel in the marine field to address the *in situ* dynamics of phytoplankton (Dubelaar et al., 1999; Olson et al., 2003), and further on in fresh water microbial ecology (Hammes et al., 2012; Besmer et al., 2014). Because we were the first to purchase the only commercialised automated and submersible flow cytometer (Cytosub, Cytobuoy bv) most of all the investigations reported above and conducted with the Cytosub and the new generation of Cytosense with image in flow made breakthroughs in the field of the phytoplankton dynamics. It started by giving ground to the concept of functional response groups for clusters resolved by flow cytometry. It went on by demonstrating the efficiency of this approach to conduct time series at high frequency (hour scale), to investigate spatial heterogeneity of phytoplankton at sub-meso-scale, to operate these instruments on pumped water, whether from a sailing boat, a ferry, a buoy, a research vessel. For the most recent experiments, they were run being remotely controlled. The great flexibility of this approach is now demonstrated and consequently open the way to new fields of investigation that were largely out of reach up to now. However, if the instrumentation gap can be considered as resolved now, one coming challenge is the automation of the data treatment. Indeed, the high frequency analysis is generating a huge amount of data and there is an urgent need for automated treatments. Some solutions were already explored (Malkassian et al., 2011) but more investigations are needed. The image in flow device re-establishes to some extent the identification capacity of optical microscopy, at least for large enough cells. This property was exploited to anticipate the development of harmful algae (Campbell et al., 2010). Opening the access to phytoplankton dynamics at the single cell level gives access to the functioning of this microbial community and its reaction to environmental changes. These automated flow cytometers are the only instruments able to generate biological information in an automated way and at a rate compatible with the dynamics of the observed microorganisms. It is expected that they will be soon implemented in the ocean observing systems to supply biogeochemical models with more robust biological information.

4 NEXT CHALLENGES

As presented in this report, automation of flow cytometry in the aquatic environment exclusively concerned phytoplankton because it is essentially composed of single cells in suspension in water and is able to generate fluorescence signals upon excitation of its photosynthetic pigments. In contrast, heterotrophic microorganisms in the aquatic environment require to be stained with fluorescent dies in order to be analysed by flow cytometry. This is currently achieved in the laboratory where this process implies more handling and more time with staining, incubation and analysis. To extend the automated high frequency flow cytometry analysis to aquatic heterotrophic microorganisms, it is necessary to automate the whole staining procedure. To solve this problem, in collaboration with the Cytobuoy company, we designed a new instrument (Cytopro) with an automated staining module, dedicated to heterotrophic prokaryotes and microzooplankton. The Cytopro is currently under evaluation and validation before being applied to the aquatic environment, both marine and fresh water. The coupling of Cytosense and Cytopro instruments will open the way to the investigation of the joint dynamics and interactions of autotrophic and heterotrophic microorganisms, another new field of research made accessible. The next big challenge ahead will be to make this instrument submersible to address the dynamics of heterotrophic microorganisms in the entire water column which is still out of reach but necessary to decipher this still largely unknown world (Aristegui et al., 2009). Meanwhile, other groups involved in microbial ecology in fresh water developed different solutions for the automation of the staining procedure and the flow cytometry analysis with conventional instruments, to address microbial dynamics in aquatic ecosystems (Besmer et al., 2014; Fontana et al., 2014). Finally, considering the needs generated by global ocean observing systems, a major challenge already mentioned in this report is the automation of the data treatment and the handling of the related huge data bases.

A really appealing and thriving new research domain is now made available to a number of scientists.

Note: this report is derived from a conference given by Michel Denis at the CISB (Centro Interdipartimentale per l'analisi dei modelli e dell'informazione nei sistemi biomedici) meeting "The CISB scientific activity: recent and seminal achievements". Rome, May 29-30, 2014, Palazzo Baleani. Melilotus Thyssen headed the CEL2SAT project.

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