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A new automated flow cytometer for high frequency *in situ* characterisation of heterotrophic microorganisms and their dynamics in aquatic ecosystems

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***Abstract* – Heterotrophic microorganisms represent the main consumers and mineralisers of the organic matter in the ocean as well as in inland waters. These organisms are in strong interaction with phytoplankton and higher trophic levels and may react quickly to environmental changes. In contrast with phytoplankton, due to the lack of photosynthetic pigments they do not naturally emit fluorescence, which makes more complex their analysis by flow cytometry, and consequently, their dynamics at short time and spatial scales are poorly documented. To fill this gap and extend high frequency observation capacity of flow cytometry (FC) to heterotrophic microorganisms, an automated staining module was designed and now equips a Cytosense flow cytometer to perform *in situ* automated sampling and analysis. Here we describe the first results got with this device and demonstrate the capacity of this new FC instrument to analyse heterotrophic prokaryotes dynamics at high frequency. In a successful experiment, this prototype yielded an extensive data set generated by automated analyses run every 2 hours over 7 days in the frame of a mesocosm investigation addressing natural marine communities.**

I. INTRODUCTION

In aquatic environments, one can distinguish autotrophic phytoplankton on one side and heterotrophic microorganisms on the other side, both compartments known to play a major role in aquatic ecosystem functioning. Phytoplankton communities are the major responsible for primary production. They respond very quickly to environmental changes, even at the hour scale [1], and the automated high frequency sampling and analysis was evidenced as a crucial need to address their dynamics. The development of the first automated flow cytometers about 20 years ago, such as the Cytosense instruments (Cytobuoy, b.v..) allowed investigation of phytoplankton dynamics based on a single cell approach in almost real time while addressing their short-term variability that was previously out of reach by more conventional methods [2][3][4][5][6]. Heterotrophic microorganisms such as prokaryotes (bacteria) represent the main consumers and mineralisers of the organic matter in the ocean as well as in inland waters. Small predators such as nanoflagellates and small ciliates are also known to be very important in controlling both phytoplankton and bacteria abundances (top-down control) and transferring matter and energy to higher trophic levels. These microorganisms are in strong interaction with phytoplankton and may also quickly react to environmental changes. In contrast with phytoplankton, they do not naturally emit autofluorescence recordable by flow cytometry, which makes their analysis more complex. Their dynamics at short time and spatial scales are poorly documented. To fill this gap and extend the high frequency observation capacity of flow cytometry (FC) to heterotrophic microorganisms, an automated staining module (SM) was jointly designed by MIO and Cytobuoy company in order to be implemented on a modified version of a Cytosense flow cytometer (Cytopro) optimised for small particles. The SM features were combined with those of a Cytosense to match requirements for the sampling of fresh or sea water, staining with an accurate volume of fluorescent dye, and incubation, mandatory to observe by

flow cytometry heterotrophic microorganisms and particularly, heterotrophic prokaryotes (HP).

In this study we present the first results obtained from two experiments conducted with the SM coupled to the new Cytopro version. It demonstrates its suitability for the high frequency automated analysis of HP abundance in aquatic environments. This achievement makes a real breakthrough in aquatic microbiology since there is no other available commercial instrument than the Cytopro with the capacity to analyse heterotrophic microorganisms at high frequency and at the single cell level.

II. MATERIALS AND METHODS

A. Mesocosm experiment

A mesososm experiment with natural marine microbial communities was monitored during 7 consecutive days during the PIANO experiment (CNRS-LEFE grant). Seawater was pumped at about 5 m depth in the Bay of Marseille, and brought back to the laboratory where it was placed in a mesocosm (450 L) installed in a dedicated container, with both temperature and irradiance controled. Once in the container, the water was driven thanks to a peristaltic pump into a loop on which various instruments have been installed to monitor the biology and the environmental parameters (T,S) and peerform some discrete sampling (for nutrients analysis, microscopy counts). The seawater was thus pumped from the mesocosm container to an intermediate tank from where subsamples were collected either by a Cytosense for phytoplankton analysis or by the Cytopro for HP analysis (Fig 1). Samples for both analyses were

Fig. 1. Instalation of Cytosenses in MIO experimental container

analysed every 2 hours. The complete dataset built during this experiment, together with phytoplankton and HP counts, will be described in more detail in another article.

B. A new staining module

The staining module is a new device developed to enable automated staining of a given sample volume confined in a loop during the appropriate incubation time before its transfer to the Cytosense flow chamber for the usual FC analysis. It also includes a system to flush and clean the tubing in order to avoid intercontamination between samples and remove the free dye in suspension. Due to the required incubation time (in general about 20 to 30 min), the automated analysis of heterotrophic microorganisms may be scheduled up to every half hour or more.

C. Cytopro flow cytometer

The Cytopro coupled to the SM was set to collect and stain water samples every 2 hours. Upon its collection from the intermediate tank, the sample was sent to the staining loop of the SM were SYBR Green I (Invitrogen™) was added drop by drop from a small dye container (up to final concentration 1:10,000 volume/volume). The sample-dye mixture was homogeneised by a continuous looping in the tubing of the loop. It was subsequently incubated for a time determined by the operator (15 min in this case) and afterwards automatically pumped into the Cytopro flow-chamber. In order to avoid the contamination of the sheath liquid by the fluorescent dye, a new chemical filter has been designed and implemented on the sheath pathway in order to catch the free fluorescent molecules remaining in suspension after the samples have been mixed with the sheath liquid. This step is mandatory as on this automated flow cytometer the sheath liquid is recycled and obviously crosscontamination between samples must be avoided. All the operations were under the control of the CytoUSB software implemented with new features to pilot the SM. After each measurement, a cleaning cycle was initiated by CytoUSB in order to flush the SM with the sample water to avoid inter-contamination. Steps including a cleaning solution (mixture of bleach and ethanol) were also implemented in order to remove the dye in the tubing. The raw FCM data files generated by the Cytopro were analysed with the custom software CytoClus that enables batch processing of large data sets. Trigger for data acquisition was performed on the the yellow green fluorescence. Gating of the heterotrophs was done on a cytogram total Sideward scatter (SWS) vs total Yellow Green fluorescence signals (Fig 2). Sample flow rate was set up at 0.55 mm^3s^{-1}. Acquisition was set up to 2 min.

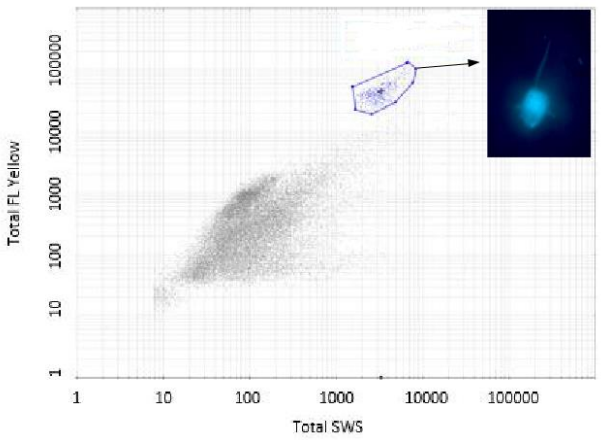

Fig.2. Cytogram of Total SWS vs total Yellow Green fluorescnece (gated cluster indicate heterotrophic nanoflagellates)

D. Epifluorescence microscopy

Samples for enumeration of heterotrophic nanoflagellates (HNF) were fixed with formaldehide (final concentration 2%), after which they were filtered on 0.6 µm polycarbonate filters and stained with 4,6-diamidino- 2-phenylindol (DAPI) (0.5-mg-ml^{-1} final concentration) for 10 min [9]. HNF cell counts were performed with the Olympus (BX61) epifluorescence microscope of the PRECYM platform of the M.I.O, equipped with a 100X objective and a cooled CCD digital camera (RETIGA SRV, QImaging, USA). Images were recorded by using ImageProþ software (MediaCybernetics,USA).

III. RESULTS

A. Staining module validation

The initial validation of the staining module was conducted by MIO through comparisons of staining and counting by using the conventional approach, i.e. (i) manual staining, incubation on a bench in the dark at room temperature, and analysis with a FACS Calibur (Becton dickinson) flow cytometer, and (ii) staining the sample within the loop of the SM, then analysing it after 15 min incubation with the Cytopro. Once validated, the process was automated by Cytobuoy by including the SM control within the CYTOUSB software

B. Enumeration of heterotrophic microorganisms with the Cytopro

The Cytopro analyses of the mesocosm microbial community done during 7 consecutive days showed stable staining and countings of heterotrophic prokaryotes. The measurments of HP abundances revealed shift in their counts between day 1 and day 3 (Fig 3), while at the same time we observed the appearance and increase in abundance of another population, potentially identified as their predators due to their higher sideward scatter and yellow green fluorescence intensities. Sideward scatter is related to the size, shape and structure of the particles. Yellow green fluorescence is related to the amount of fluorescent dye, correlated to the nucleic acid content.

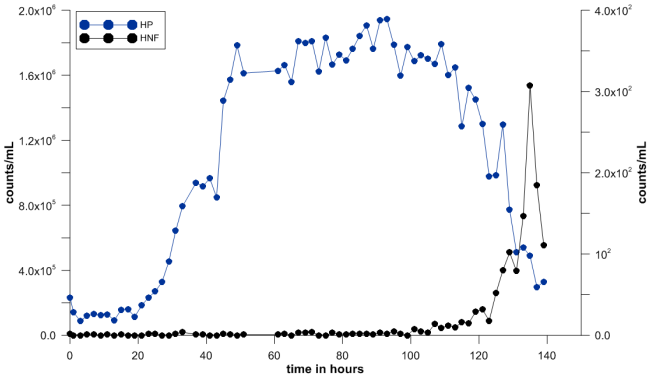

Fig.3. HP and HNF dynamic (abundances per ml during days)

Microscopy observations identified this new population as heterotrophic nanoflagellates (HNF) (Fig 2). HNF microscopy counts gave good comparison with the flow cytometer (data not shown), which was confirmed with Pearson test ($R=0.89$, $p<0.0001$).

IV. DISCUSSION AND CONCLUSIONS

The reported Cytopro experiment addressed for the first time the dynamics of a natural microbial community including HP and HNF conducted during 7 consecutive days. The automated HP abundance analysis revealed a shift in their abundance between day 1 and day 3, while at the same time the increase of another heterotrophic group was evidenced, identified as small predators (HNF) by microscopy. The innovation brought by this study resides in the successful use of a new generation of commercial flow cytometer model coupled to a staining module prototype to perform the automated *in situ* HP analysis at high frequency and remotely controlled during extended time periods (days-to-weeks). A commercial version of the staining module is ongoing and should be released very soon.

The capability to investigate heterotrophic prokaryotes and heterotrophic nanoflagellate increase the range of microorganisms that can be investigated directly in situ, automaticaly and at high frequency. This capacity should offer new insights on the structure, dynamics and thus

functioning of the microbial network.
Subsequently to the PIANO experiment, the Cytopro and its SM were placed on a scientific research vessel for the PEACETIME (ProcEss studies at the Air-sEa Interface after dust deposition in the MEditerranean sea) cruise in the Mediterranean Sea. The seawater was continuously pumped from subsurface along the ship track. Both Cytopro and SM were remotely controlled and operated from land by a MIO scientist thanks to satellite connection to perform both phytoplankton and bacteria analyses at high resolution in the Mediterranean Sea. The reported preliminary results demonstrate that Cytopro is suitable for investigating dynamics of both phytoplankton and heterotrophs in aquatic ecosystems at high temporal resolution over several weeks. Although in the PIANO case study we used two Cytosense flow cytometers to address abundances of both heterotrophs and autotrophs, the Cytopro is able to analyse both, staining or not the samples.
There is no doubt that with the technical improvements that a prototype requires, the Cytopro will offer a powerful instrument to explore the dynamics of the aquatic microbial compartment. For instance, the implementation of two dye containers is already on going to perform viability tests using the nucleic acid double staining (NADS) protocol [8].
The staining module attached to the Cytopro represents a real breakthrough in aquatic microbiology. Indeed, on top of providing in real time a complete picture of the microbial compartment dynamics, it also enables the use of viability or physiological dyes [7][8] and thus opens the way to a more complete picture of the microbial compartment structure, dynamics and thus functioning.

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