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Real-Time microbial concentrations by automated on-line flow cytometry for marine coastal monitoring

Casotti Raffaella[1], Balestra Cecilia[1], Van Dijk Mark[1], Passarelli Augusto[1], Sigrist Jürg[2], Hammes Frederik[2]

[1] *Stazione Zoologica Anton Dohrn, Villa Comunale, raffaella.casotti@szn.it, cecilia.balestra@szn.it, mark.vandijk@szn.it, augusto.passarelli@szn.it*
[2] *Department of Environmental Microbiology, Eawag – Swiss Federal Institute for Aquatic Science and Technology, Dübendorf, Switzerland, frederik.hammes@eawag.ch*

***Abstract* –Marine microbes are ubiquitous and play a fundamental role in shaping the oceans as we know them, through key processes such as photosynthesis and biogeochemical cycles. Their monitoring requires sampling at the correct time and space, since they show high turnover rates and patchy distributions. We have used two on-line flow cytometry systems to monitor marine microbes in the coastal area of the Gulf of Naples, as a proof-of-concept that this technology can represent a useful tool for real-time alert systems of water biological quality. Bacterial and picophytoplankton concentrations are proposed as general indicators of water quality, the first step necessary to assess the effects of changes in environmental conditions, to be followed by in-depth analyses of community composition for presence of pathogens or other indicators. During the survey we could detect hotspots of high bacterial concentrations located in areas of discharges of untreated urban wastes or subject to riverine inputs. In general, bacterial abundances were inversely correlated to salinity, suggesting that they use organic matter of terrestrial origin for their growth. The technological approach used represents a useful and fast method to monitor coastal areas also in the context of the European Directive 2008/56/EC, aimed at assessing and improving European marine waters quality.**

I. INTRODUCTION

Marine microbes are highly dynamic and react very fast to variations in ecosystem properties so as to acute pollution events [1]. In order to catch these reactions and to establish their causes, high-frequency accurate measurements are needed. Most methods used to monitor marine microbes suffer from low reproducibility, long times for responses and high demand for labor. Flow cytometry offers a solution to these constraints, allowing fast, accurate and reproducible counts of marine microbes, and in fact, it has been widely used in marine ecology studies [2]. Flow cytometry is an ataxonomic, single-cell-based method using scatter and fluorescence to discriminate and count cell types. While photosynthetic microbes can be analyzed directly due to autofluorescence of photosynthetic pigments, non-autofluorescing microbes (such as heterotrophic bacteria) need to be stained before analysis. The staining step requires sample manipulation and incubation times that are needed for a proper assessment of cell concentrations [3].

In environmental sciences, automated flow cytometry has been used for autofluorescent microbes [4, 5] and for heterotrophic bacteria after staining [6]. We have combined these two approaches by using the CytoSense (Cytobuoy bv) for the online analysis of phototrophic microbes and the onCyt automated flow cytometer (onCyt Microbiology AG) as described in [7], connected to the on-board pump of an oceanographic vessel while sailing, demonstrating that high spatial resolution monitoring of microbes distribution at surface is feasible and provides useful first-hand information that can be used as a rapid and real-time indication of water quality.

II. MATERIALS AND METHODS

Sampling was realized on June 19th, 2017 on board the RV Vettoria. The surface water was pumped by the boat pump through an online thermosalinographer (SBE45 Microthermosalinograph, SeaBird Electronics) to measure temperature and salinity and an online fluorometer for total fluorescence (Cyclops7, Turner Design interfaced with a databank). Then the water ran through a sampling device (Cytobuoy bv), splitting the flow through the CytoSense flow cytometer (Cytobuoy bv) and the onCyt on-line flow cytometer [7] in sequence. The CytoSense was set to analyse samples every 2 min, while the onCyt could analyse samples every 13 minutes (due to the staining time), corresponding to a sample

every 1 km, approximately and depending on the boat speed. Discrete samples for conventional flow cytometry were taken every 10 minutes at surface so to validate the onCyt counts using conventional flow cytometry. These samples were fixed, frozen and analyzed later in the lab after staining with SYBRGreen, as described in [8]. From the CytoSense, counts of *Synechochoccus* sp (cyanobacteria), picoeukaryotes (mixed population of size 1-3 microns), naneukaryotes (size 2- 10 microns) were obtained, while the onCyt provided concentrations of non-fluorescent, mainly heterotrophic, bacteria.

II. RESULTS AND DISCUSSION

While for autotrophic picoplankton (*Synechococcus* spp. cyanobacteria and picoeukaryotes) a comparison with conventional flow cytometry has been shown already ([4,5]), for the heterotrophic bacteria the onCyt system has been used in freshwater systems, therefore its use in marine waters still had to be tested. For this reason, we have adapted our staining protocol (as in [8]) to accommodate the needs of the onCyt, in terms of incubation temperature and time. In order to achieve proper measurements, the samples were added a mix of paraformaldehyde and glutaraldehyde (1% and 0.05% final concentrations) before staining in presence of EDTA 50 mM. Cell counts obtained with the CytoSense and the onCyt were significantly and strongly positively correlated with counts obtained by conventional flow cytometry as shown in Fig. 1. For the bacteria, this holds true both for counts and for subpopulations (High Nucleic Acid, HNA and Low Nucleic Acid, LNA, Fig.1 c and d).

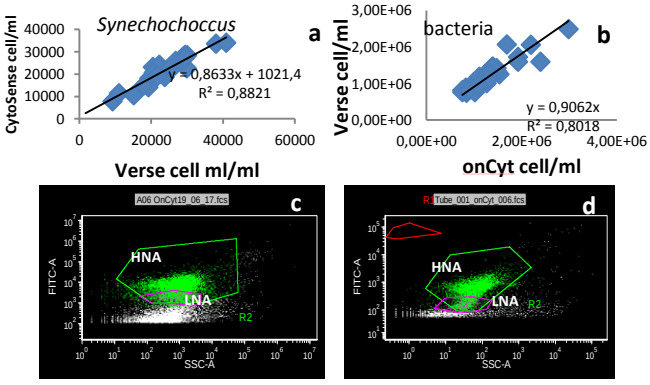

Fig. 1. Validation of data obtained by CytoSense, onCyt and conventional flow cytometry (Verse). a) correlation of cyanobacterium Synechococcus counts b) correlation of total heterotrophic bacteria counts c) cytogram of heterotrophic bacteria obtained by the onCyt d) cytogram of heterotrophic bacteria obtained by the Verse.

Once on board, the two flow cytometers were connected to the sampling line of the boat, after the thermosalinographer. The seawater was then split into two lines, one to the CytoSense, the other to the onCyt. Both instruments acquired the samples at the same time.

Fig. 2 shows the track of the boat and the values of temperature, salinity and fluorescence collected during the cruise. Two areas with low salinity were evident, one close to the harbor (Torre Annunziata) and the other in the area influenced by the Sarno river (Sarno). The first is located in correspondence of a broken collector of urban wastes, one mile from the coast. The second is in correspondence of the Sarno river, who crosses several agricultural and urban areas before discharging in the Gulf of Naples.

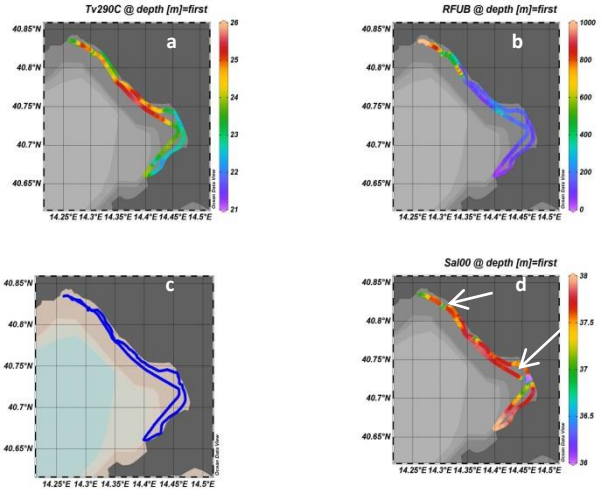

Fig.2 a) temperature, b) fluorescence from chlorophyll, c) boat track, d) salinity. The arrows indicate the site with lower salinity, Torre Annunziata (above) and Sarno (below)

Total bacterial concentrations showed peaks in correspondence of these two areas, the first (Torre Annunciata) with the highest concentration of 1.5 x 10^6 cell ml^{-1}, the second (Sarno) with 1.4 x 10^6 cell ml^{-1} (Fig. 3). While the HNA were dominant in Torre Annunziata, both HNA and LNA were present at Sarno site (not shown), indicating a shift in community composition along the sampled track. HNA and LNA in fact, are now recognized as belonging to different bacterial clusters, eventually representing the same larger clades but with different taxonomical units ([9]), and not, as previously thought as more (HNA) or less (LNA) active bacteria [9].

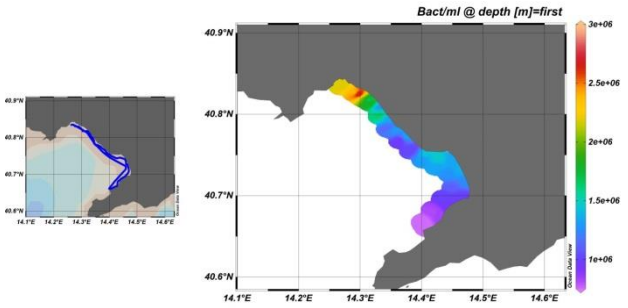

Fig.3 Sample track (on the left) and total heterotrophic bacteria concentrations (right) in cells x ml^{-1}

Samples taken at the Sarno site have shown presence of *E. coli*, indicator of recent faecal contamination, but not at the Torre Annunziata site (Milva Pepi, pers. Comm.), indicating the importance of further bacteriological analyses before properly assessing a risk for human health only based on total bacterial concentrations. The same is true for toxic algae, where taxonomical identification alone does not always correspond to presence of toxins that has to be proven using other appropriate detection methods.

The autotrophic microbial components also showed a clear separation between the two areas, with the cyanobacterium *Synechococcus* dominating the southern, and picoeukaryotes and cryptophytes the northern areas (Fig. 4). This also confirms previous observations showing that the prokaryotes (cyanobacteria) respond to different environmental cues than the larger eukaryotes (picoeukaryotes and cryptophytes) and that the latter are generally responsible for the peaks in total chlorophyll (as from the fluorescence patterns, Fig. 1b).

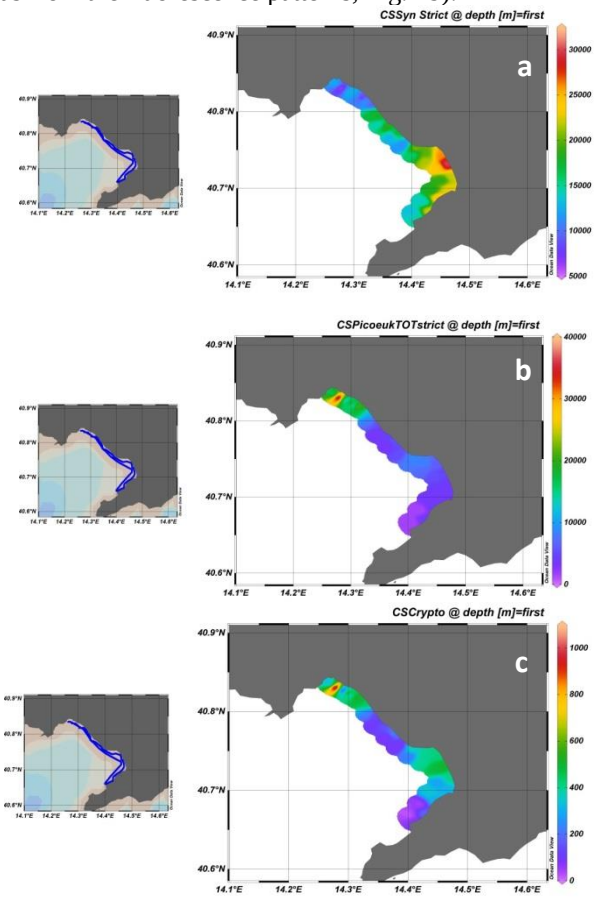

Fig.4 Sample track (on the left) and a) Synechococcus b) picoeukaryotes c) Cryptophytes concentrations in cells x ml^{-1}

III. CONCLUSIONS

Automated high frequency flow cytometry proves to be a cost-effective, fast method for the detection of marine microbes, allowing real-time measurements of cell concentrations to be correlated with environmental factors, in order to be interpreted and potentially used as markers of water quality and pave the way to further intervention. Microbial communities, in turn, and heterotrophic bacteria in particular, appear to be useful markers of changed environmental conditions, that need to be considered and included in water quality assessment such as those sought by the European Directive 2008/56/EC, which aims at reaching a Good Environmental Status (GES) for all European waters within 2020.

Caution must be adopted, though, since total bacterial cell numbers may not further reflect a risk for human health, in terms of pathogenicity or toxicity. However, flow cytometry may represent a valuable method for a first screening, highlighting potential critical sites in real time, to be further monitored and controlled by other methods.

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