ABUNDANCE AND DISTRIBUTION OF ACTIVELY RESPIRING BACTERIA IN A COASTAL-OFFSHORE TRANSECT OF THE TYRRHENIAN SEA(APRIL 2007)

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Abstract
The abundance and distribution of actively respiring bacteria within the total prokaryotic community were estimated along the water column in a coastal-offshore transect crossing the Tyrrhenian Sea during spring 2007. Specific fluorochromes (SyBr Green and 3,5-cyano-dytolyl-tetrazolium chloride, CTC) were used to detect by flow cytometry the total prokaryotic community and the fraction of actively respiring bacteria, respectively. Bacterial numbers were compared to those determined by epifluorescence microscopy. Respiratory activity rates, leucine aminopeptidase and beta-glucosidase activity rates were also analysed, in order to relate the abundance of living cells to their functional role in the ecosystem functioning.
Keywords: Bacteria, Tyrrhenian Se, Analytical Methods

Introduction
The determination of cell viability is a critical issue in assessing the role of bacterial biomass in aquatic environments [1, 2]. Increased attention has recently been paid in determining the fraction of bacteria that are metabolically active and can be actively involved in biogeochemical cycles. Flow cytometry (FCM) with specific fluorochromes used allow us to discriminate bacterial cells according to their living attributes [2]. SyBr Green is a nucleic acid probe to stain the total prokaryotic community (TP), which fluoresces in the green range (maximum emission: 521 nm) of the light spectrum, when excited by a 488-nm wavelength light. The 5-cyano-2,3 ditolyl tetrazolium chloride (CTC) is a redox stain conventionally used as a marker of actively respiring cells, since in the presence of oxygen it is converted into formazan, which accumulates as red fluorescing granules within CTC+ cells [3]. The search aimed at evaluating, along a coastal-offshore transect crossing the Tyrrhenian Sea, the distribution of actively respiring bacteria within the prokaryotic community by FCM, in comparison with epifluorescence microscopy (EPI).

Materials and Methods
During April 2007, 31 seawater samples were collected by R/V "Universitatis", at depths from 5 to 3250 meters, in an area of the central Tyrrhenian Sea (Lat. 39°30'00''N-Long. 13°30'00''E to Lat. 40°36'30''N-Long. 14°08'30''E). The study was carried out within the VECTOR (VulnErability of the Italian coastal areas and marine ecosystems to Climate changes and Their rOle in the Mediterranean caRbon cycle) Project. Three stations (VTM, 2 and 5) were sampled; VTM was the most off-shore station (maximum depth: 3450 m), about 80 nautical miles SW of Naples, while station 5 was the most coastal one (maximum depth: 700 m). Before FCM and EPI analysis, a 5-ml water sample was pre-filtered on a 100 µm-mesh size net to prevent clogging of the flow cytometer. A 2 ml volume of the filtered sample was stained with SyBr Green II (Molecular Probes, final concentration: a 10^{-4} dilution of a 5 mg ml^{-1} stock solution) for 10 minutes in the dark at room temperature. A 1-ml aliquot of the SyBr Green-stained sample was added with a 5 mmol l^{-1} solution of CTC (final concentration; Polyscience), and analysed using an Apogee 40 flow cytometer (Apogee Flow Systems). Green fluorescence was collected in the FL1 channel(515-545 nm, specific for SyBr Green); red fluorescence was collected in the FL3 channel (650-690 nm, specific for CTC). The remaining 1 ml of the SyBr Green- and CTC-stained sample was stored at + 5°C until EPI analysis, performed within 2 hours of sampling.For EPI method, the water sample was filtered on a Nuclepore black (0.22 µm pore size) polycarbonate filter; the filter was observed with a Zeiss Axioplan 2 epifluorescence microscope, equipped with specific filter sets (blue light: BP 450-490, FT 510 and LP 520, for SyBr Green+ cells; green light: BP 510-560, FT 580 and LP 590, for CTC+ cells). On the same samples treated for FCM and EPI,temperature, salinity, oxygen, fluorescence, extracellular enzymatic activities (leucine aminopeptidase, LAP, β-glucosidase, β-Glu, alkaline phosphatase, AP) and microbial community respiration by the Electron Transport System (ETS assay)[4], were measured.

Results
Temperature ranged from 13.39 to 17.79°C; salinity from 37.56 to 38.73. TP counts obtained by FCM varied between 3.03 x 10^5 cells ml^{-1} (station VTM, 500 m) and 6.92 x 10^6 cells ml^{-1} (station 5, 100 m). CTC+ bacteria ranged from 1.94 x 10^4 to 1.19x10^6 cells ml^{-1}, recorded at station VTM (500 m) and at station 2 (1000 m); on average, they accounted for 8.8 (station VTM) and 16.28% (station 2) of TP. At station VTM, a decreasing trend for both TP and CTC+ cells was observed; an opposite distribution was found at stations 2 and 5. CTC+ cells were particularly abundant at station 2, between 500 and 1500 m, where values of 32.8-38% of TP were reached. TP counts obtained by EPI varied between 8.25 x 10^3 (station 5, 50 m) and 1.79 x 10^5 cells ml^{-1} (station VTM, 25 m). CTC+ bacteria ranged from 9.45 x 10^2 (station VTM, 500 m) to 2.39 x 10^4 cells ml^{-1} (station 2, 1000 m); on average, they represented 40.88% of TP. Spatial distribution of TP and CTC+ cells found by EPI followed the same vertical patterns as FCM. CTC+ cells predominated at station 2, accounting for over 70% of TP between 500 m and 1500 m. Significant relationships were always found between TP and CTC counts by FCM (Pearson r: 0.975, 0.845, 0.881, $P<0.01$, stations VTM, 2, and 5, respectively), since CTC+ cells belong to TP. Analysis of variance performed on samples grouped according to the main water masses (surface Tyrrhenian waters: 0-200 m, intermediate mixed waters: 200-1000 m, and deep waters: > 1000 m), showed that CTC+ cells were significantly higher in intermediate mixed layers than at surface ($F= 4.751$, $P<0.05$ for FCM). On average, FCM counts were two orders of magnitude higher than EPI counts, although no statistical differences were found between both the methods. At station 2, TP and CTC+ counts by FCM correlated positively with temperature (r= 0.818 and 0.836, $P<0.01$, respectively) and negatively with salinity (r= -0.72 and -0.637, $P<0.05$, respectively); CTC+ cells correlated negatively with oxygen (r= -0.851, $P<0.01$), suggesting its consumption during respiration. No relationships between CTC+ cells and ETS activity rates was generally detected, while CTC+ cells were related to LAP, β-Glu and AP (station VTM, r=0.781, 0.702, 0.798, $P<0.01$).

Discussion
FCM method is a suitable approach to study the physiological state (i.e. active respiration) of specific microbial populations which are active in the organic matter biogeochemistry. Actively respiring cells in the examined Tyrrhenian transect ranged in the same order of abundance as other marine environments [3, 5]; they prevailed in the intermediate layers, suggesting their distribution could be affected by water masses, like the Levantine Intermediate Water. The lack of relationships between CTC+ cell numbers and ETS activity rates confirms that cell abundance and activity may sometimes be uncoupled, because CTC method detects only the most highly active cells [5]. FCM and fluorochromes could represent a powerful tool also for monitoring bacterial cells for their living properties (enzyme activity, metabolism, respiration) in clinical samples, helping to better assess their potential pathogenic role.

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