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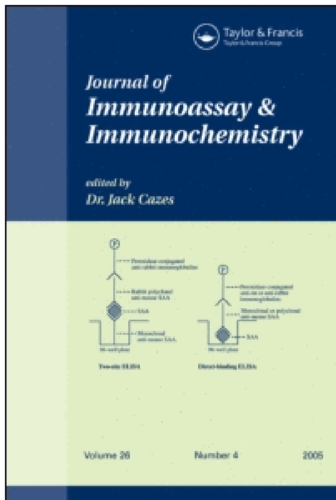

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
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To cite this Article Caruso, G. , De Pasquale, F. , Mancuso, M. , Zampino, D. and Crisafi, E.(2006) 'Fluorescent Antibody-Viability Staining and β-Glucuronidase Assay as Rapid Methods for Monitoring *Escherichia coli* Viability in Coastal Marine Waters', Journal of Immunoassay and Immunochemistry, 27: 1, 1 — 13
To link to this Article: DOI: 10.1080/15321810500403599
URL: http://dx.doi.org/10.1080/15321810500403599

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Journal of Immunoassay & Immunochemistry, 27: 1–13, 2006
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ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810500403599

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Fluorescent Antibody-Viability Staining and β-Glucuronidase Assay as Rapid Methods for Monitoring *Escherichia coli* Viability in Coastal Marine Waters

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Abstract: A faecal pollution monitoring of coastal Messina waters was performed by comparing three (microscopic, enzyme, and culture) methods. Evidence of *Escherichia coli* cells (29.99 to 96.79% of the total enteropathogenic serotypes) retaining their viability into the marine environment was shown. β-Glucuronidase activity rates suggested that living cells were also metabolically active. Heavily polluted sites were detected, where improperly treated urban wastes were discharged. Significant relationships between microscopic and enzymatic data proved both methods to be suitable alternatives to the culture method for *E. coli* detection, improving environmental quality assessment.

Keywords: Faecal pollution, Seawater monitoring, *Escherichia coli*, Viability, Fluorescent antibody, β-Glucuronidase

INTRODUCTION

Bacterial pollution represents a threat for many coastal marine areas with severe implications for water quality. Rapid and reliable analytical protocols are needed for the microbiological control of aquatic environments; improvement of the techniques available up to now constitutes the challenge

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for future developments in this field.[1] The antigen-antibody reaction, on which the fluorescent antibody (FA) method relies, and the screening for selected bacterial enzymes, e.g., β-glucuronidase, offer specific tools to detect and quantify the abundance and activity of *Escherichia coli* in seawaters.[2] The viability assessment of enteric bacteria is also of primary importance for public health preservation because, in adverse environmental conditions, they may survive in aquatic environments entering the viable but nonculturable (VBNC) state.[3,4] In *E. coli*, this phase, characterised by lost of culturability, with retention of cellular integrity and potential pathogenesis, has been widely documented.[4–10] The severe sanitary consequences that could result from the recovery of cell viability when environmental conditions become favourable[11] support the increasing interest addressed to this specific topic.

In the framework of the MIUR-Cluster 10 research programme, aimed at setting up new rapid methodologies for coastal marine monitoring, an investigation of bacterial pollution along Messina shoreline was undertaken. The aim of our research was to depict the occurrence of *E. coli* in seawater, paying attention to changes in cell viability occurring in space and in time. Improperly treated urban discharges have been recognised as the main source of the worsening quality levels of coastal waters surrounding Messina. High levels of faecal contamination occur, particularly along the Ionian coastline between Capo Peloro and Tremestieri, but the particular hydrodynamic conditions of the area leads to a strong reduction of the density of faecal coliforms already present 200 m off shore.[12] The study provided a good opportunity to compare and test, in the field, three different analytical approaches to detect the presence of *E. coli* as the main faecal pollution indicator: the standard culture method (m-FC), the β-glucuronidase activity assay (through the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide, MUG), and a specifically developed protocol that combines a viability staining (the Live/Dead staining) with an indirect fluorescent antibody (FA) procedure.

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EXPERIMENTAL

Collection and Treatment of Samples

From the 24th of March to the 5th of May, 2004, on a weekly basis, surface seawater samples ($n = 34$) were collected from five coastal sites (Tremestieri, Pagliarisi, Europa, Annunziata, Tennis, Fig. 1). Sub-samples (100 mL or less) were analysed using the following methods.

Plate Counts (m-FC)

Faecal coliforms (FC) were counted by membrane filtration (0.45 μm) and incubation on m-FC agar (Difco) plates.[13] Results were expressed as CFU $100\,mL^{-1}$ of water. The density of *Escherichia coli* was determined by

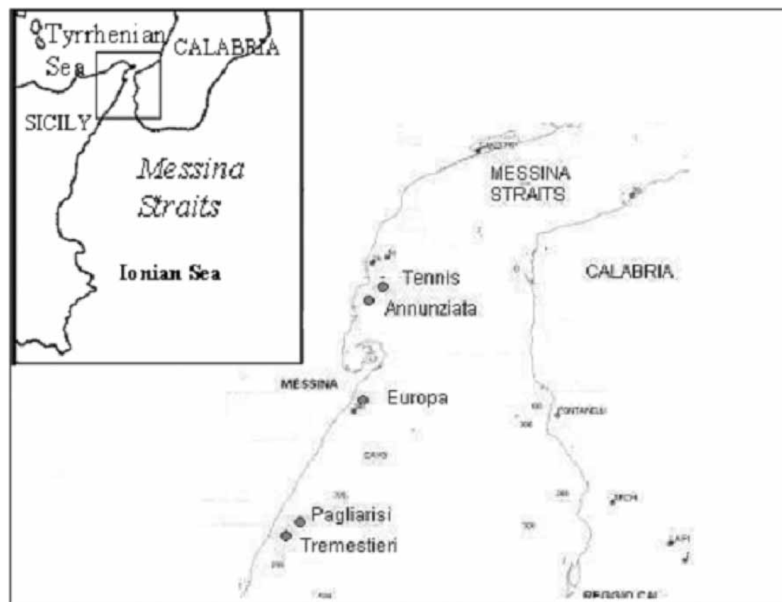

Figure 1. Sampling stations.

confirmation with James' indole reagent (Biomérieux, Marcy l'Etoile, France) of the colonies grown on m-FC agar.

MUG Assay

For the determination of β-glucuronidase activity, increasing amounts (5 to 50 μmol L^{-1}) of the fluorogenic substrate 4-methylumbelliferyl-β-D-glucuronide (MUG, Sigma, 0.5 mmol L^{-1} stock solution) were added to 10 mL aliquots of a 10-times concentrated sample.[14] The fluorescence released by enzymatic cleavage of the substrate in the product methylumbelliferone was measured at time 0, immediately after its addition, and after 3.5 h of incubation at 44°C, with a spectrofluorometer (Cary Eclipse Fluorescence spectrophotometer, Varian Inc., Palo Alto, CA, USA) at 365 and 455 nm (excitation and emission wavelengths, respectively). Calibration was performed with known concentrations (200, 400, and 800 nmol L^{-1}) of the standard MUF (Sigma, 0.5 mmol L^{-1}). Enzyme values were reported as maximum velocity of hydrolysis (Vmax, in nmol of MUF released h^{-1} 100 mL^{-1}).

Combined FA-Live/Dead (FA-L/D) Staining Procedure

This protocol combined the indirect fluorescent antibody (FA) labelling procedure[15] using isothiocyanate fluorescein (FITC)-conjugated immune sera specific for *E. coli* enteropathogenic serotypes (Behring Diagnostics

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Inc., Westwood, MA, USA) together with propidium iodide (PI) stain, a component of the Live/Dead (L/D) BacLight bacterial viability kit (Molecular Probes, Eugene, Oregon, USA). The use of PI, which penetrates cells with damaged membranes only, allowed the distinction of dead cells, fluorescing red, within the total enteropathogenic *E. coli* population. Cells were enumerated by an epifluorescence microscope (Axioplan 2, Carl Zeiss S.p.A., Arese, Milano, Italia) equipped with an image analysis system and viewed separately with fluorescein (blue light, BP 450–490, FT 510, LP 520) and rhodamine (green light, BP 510–560, FT 580, LP 590) filter sets. When excited under blue light, total (living + non-living) *E. coli* cells showed green fluorescing outlines due to FITC (emission peak: 520 nm); by switching to the green light, the dead (PI-positive) *E. coli* cells fluoresced red, due to PI labelling (emission peak: 617–623 nm). The fraction of living cells was determined as the difference between the total FITC-labelled *E. coli* cells and PI+ dead cells. Prior to its application to the analysis of natural samples, a suspension of killed *E. coli* (by boiling) was used as a negative control, in order to verify that dead cells were efficiently stained by our FA-L/D protocol. Counts were performed on a minimum of 20 randomly selected microscopic fields. Cell abundance was expressed as the mean value of *E. coli* cells per 100 mL of sample.

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Statistical Analysis

Values obtained by each method were log-converted to stabilize the variance and attain normality; the differences between mean counts were tested for statistical significance using an analysis of variance (ANOVA). Paired data were analysed using Pearson correlation coefficients to determine if values were statistically correlated.

RESULTS

The main steps of our FA-L/D labelling protocol are presented schematically in Table 1. The concentration of 30 μM L^{-1} PI, chosen from a variety of concentrations ranging from 5 to 50 μM L^{-1}, proved to be effective in labelling dead cells without any interference with the antigen-antibody reaction. The use of PI from a L/D BacLight bacterial viability kit was conceived to answer to the need of standardizing the viability staining protocol. After this treatment, microscopic fields observed, using fluorescein or rhodamine filters, were also overlapped to observe the simultaneous labelling of both fluorochromes and to discriminate, within the total enteropathogenic *E. coli* cells, the ones that were dead.

Figure 2 shows the distribution, during each sampling, of FC values, as obtained by plate count, and of total enteropathogenic *E. coli*, as determined

Table 1. Main steps of the combined FA-L/D protocol

Filtration through a 0.22 μm Nuclepore black membrane
Incubation with hydrolysed gelatine (2% final concentration), 20 min
Washing 3 times with 5 mL PBS
Incubation with a 1 : 40 dilution in PBS of Murex *E. coli* antisera (pool A + B + C), 30 min
Washing 3 times with 5 mL PBS
Labelling with a 1 : 80 dilution of goat-anti rabbit–IgG-FITC, 30 min
Incubation with PI stain from L/D kit 15 min in the dark
Mounting with L/D BacLight mounting oil
Observation under epifluorescence microscope

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by FA-L/D; β-glucuronidase activity rates, measured by MUG assay, are also reported. Total enteropathogenic *E. coli* values ranged from 1.31×10^3 to 4.49×10^5 cells $100\,mL^{-1}$, with peaks at Pagliarisi and Europa stations. Except for one sampling (April, 30) only, their lowest concentrations were found at Annunziata station, which differed significantly from those recorded at the stations Pagliarisi ($F = 11.90$, $P < 0.01$, $n = 14$) and Europa ($F = 19.13$, $P < 0.01$, $n = 14$).

FC showed values 1–2 orders of magnitude lower than *E. coli*, ranging from 9.0×10^0 to 4×10^4 CFU $100\,mL^{-1}$, suggesting the existence of a fraction of VNCB cells. However, similar trends were reported for FC and *E. coli* values, highlighting no significant differences between culture and microscopic methods. This finding was also confirmed by results of ANOVA ($F = 2.82$, $P < 0.05$, $n = 34$). As far as the enzyme measurements are concerned, the potential β-glucuronidase activity rates oscillated between 0.002 and 726.88 nmol h^{-1} $100\,mL^{-1}$, with the lowest values recorded at Annunziata station. Their spatial distribution was similar to that observed for *E. coli* and FC counts, as further demonstrated by ANOVA values ($F = 3.35$ and 2.57, $P < 0.05$, $n = 34$, respectively).

Bacterial densities followed a variable temporal distribution, with the highest culture and microscopic counts during the first samplings, particularly at Europa station. During the following samplings, bacterial peaks moved towards Pagliarisi station, more southern than Europa station. Bacterial counts dropped to minimum values (on average 2.48×10^2 CFU $100\,mL^{-1}$ and 2.91×10^3 cells $100\,mL^{-1}$ for FC and *E. coli*) during the last sampling, when they were significantly different from those of the first one ($F = 5.637$, $P < 0.05$, and 17.25, $P < 0.01$, $n = 14$, by m-FC and FA-L/D, respectively). In a similar way, the levels of β-glucuronidase activity reached their minimum values during the May sampling.

For each sampling, significant relationships between m-FC and FA-L/D ($0.742 \le r \le 0.995$, $P < 0.01$) and m-FC and MUG ($0.746 \le r \le 0.999$, $P < 0.01$) results were detected by Pearson correlation coefficients calculated comparing the culture *vs* FA-L/D combined staining counts and the

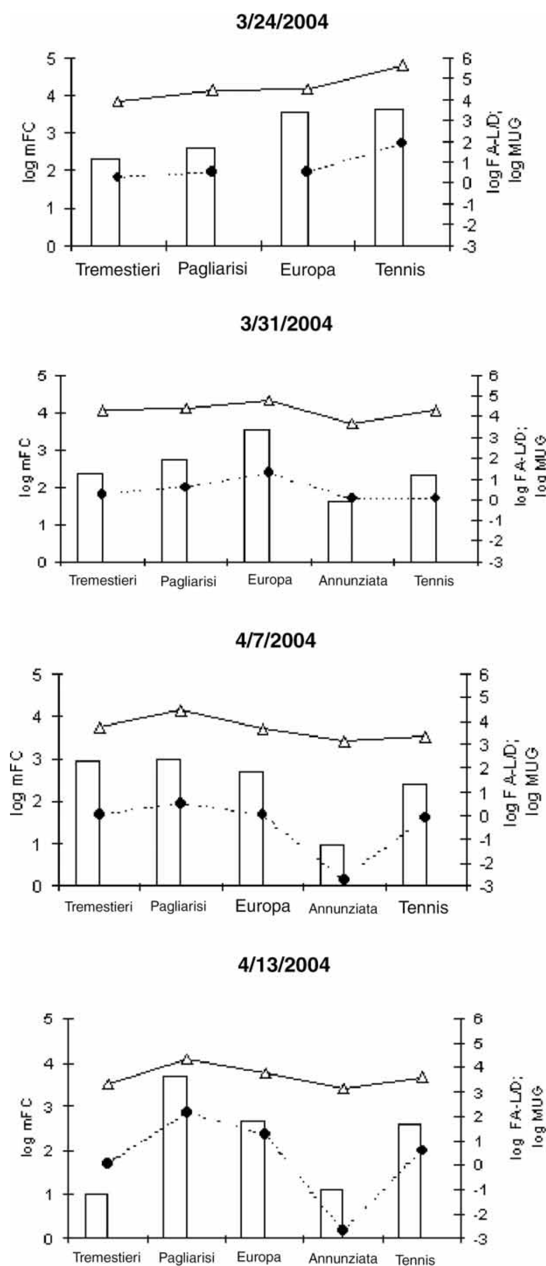

Figure 2. Total counts by culture (m-FC) and microscopic (FA-L/D) methods and β-glucuronidase activity rates (MUG) detected in all the samplings. Data are expressed as CFU $100\,mL^{-1}$ (white bars, m-FC), cells $100\,mL^{-1}$ (white triangle, FA-L/D) and nmol h^{-1} $100\,mL^{-1}$ (black circles, MUG).

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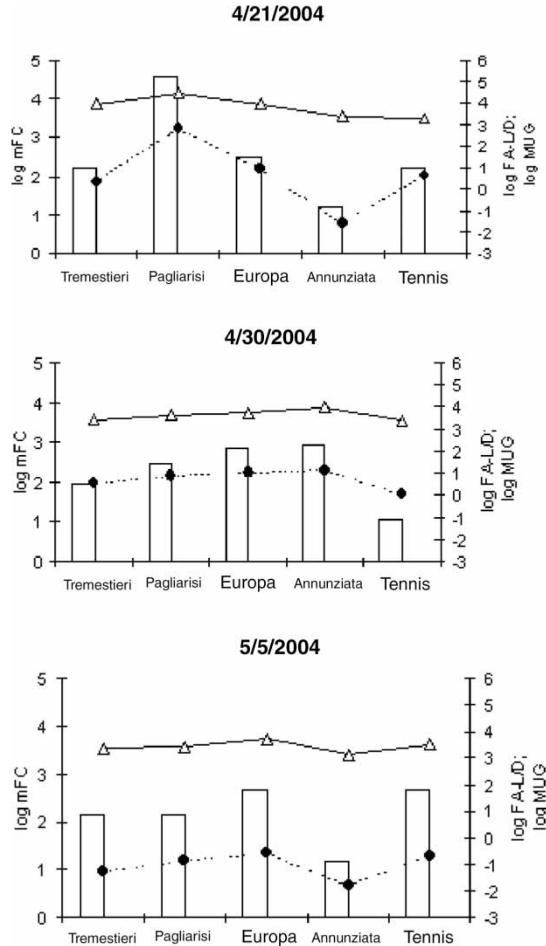

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culture counts *vs* MUG rates (data not shown in the figure). Overall, MUG and FA-L/D values were also significantly related ($r = 0.673$, $P < 0.01$).

The contribution of live and dead fractions to the total enteropathogenic *E. coli* population at each station is reported in Fig. 3. No clear temporal trend was observed in their distribution. Within the total enteropathogenic *E. coli* population, identified by FITC labelling, living cells globally represented a percentage ranging from 29.99 to 96.79%, with the highest mean values recorded at Tremestieri and Tennis stations (98.21 and 91.92%, respectively). Dead cells accounted for a similar fraction (15% of the total) at Pagliarisi and Europa stations. As revealed by ANOVA ($F = 3.16$, $P < 0.05$, $n = 34$), the values concerning the living fraction were not significantly different from MUG values; this result suggested that both fluorescent-antibody/viability

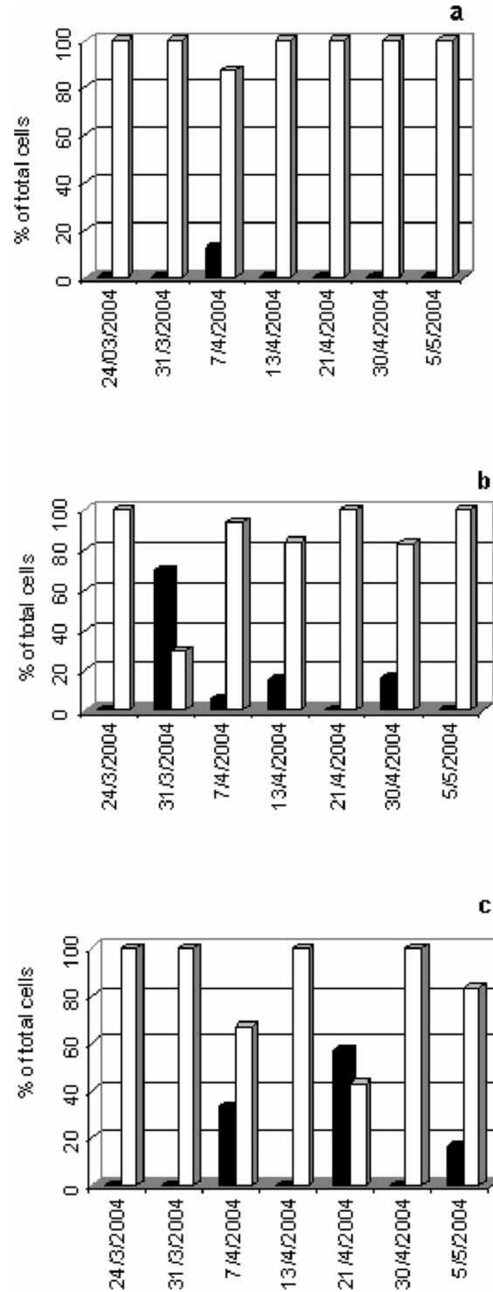

Figure 3. Percentages of live (white bars) and dead (black bars) *E. coli* cells obtained by the combined FA-L/D protocol at the different stations: a, Tremestieri; b, Pagliarisi; c, Europa; d, Annunziata; e, Tennis.

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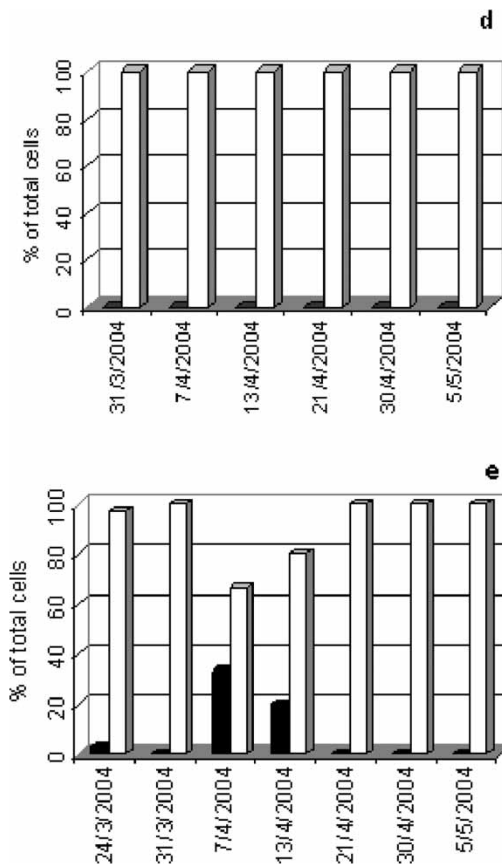

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and enzyme methods identified the same bacterial sub-population or, in other words, that cells scored as living by epifluorescence microscope were also still metabolically active.

Furthermore, the linear regression analysis of log-transformed m-FC *vs* FA-L/D and m-FC *vs* MUG data (Fig. 4) showed that all the three methods yielded statistically similar results; in particular, enzyme rates were correlated with FC counts more significantly ($R^2 = 0.6161$) than FA-L/D ($R^2 = 0.4873$). Moreover, FA-L/D data displayed a poor dispersion with respect to the theoretical regression line.

DISCUSSION

The viability of faecal bacteria in seawaters is a critical issue for public health preservation; in our study, living properties of *E. coli* cells have been detected

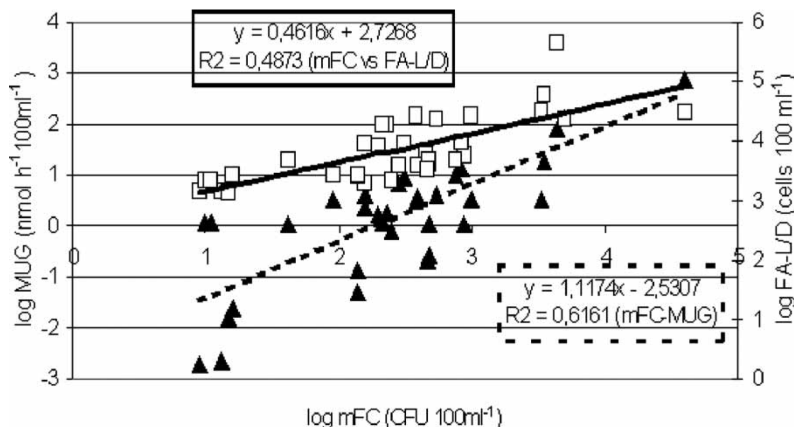

Figure 4. Regression analysis of m-FC values *vs* FA-L/D (white squares, solid line) and MUG values (black triangles, dotted line).

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in coastal samples by comparing different methods: culturability by the plate method; metabolic (β-glucuronidase enzyme) activity by fluorogenic measurement, and membrane integrity by an indirect FA assay combined with PI. It is commonly recognised that standard culture methods for the specific detection and quantification of *E. coli* involve some restrictions in terms of both accuracy and sensitivity (i.e., failure to detect injured bacteria), which make their application to seawater monitoring inadequate.[1,16] Consequently, the search for rapid, highly sensitive methods is encouraged as a reliable and effective tool for early warning of sewage pollution. Our study aimed at proposing new analytical approaches, such as microscopic and enzymatic methods, that are more specific, require less time and are less cost-consuming than the standard culture method, involving the use of basic laboratory facilities. Being growth independent, both FA-L/D and MUG were able to identify viable but nonculturable cells; they differed in their basic principles, the first relying on membrane integrity and the second considering the expression of enzyme activity. On the other hand, it is recognised that the parameter "cell activity" could not be used as a surrogate estimation of "cell culturability" and, therefore, activity rates are not directly comparable with abundance data. Furthermore, the assessment of β-glucuronidase activity only could not be considered as an adequate predictor of the complete metabolic activities, and this could result in a possible underestimation of the amount of active cells. In spite of these limitations, our basic opinion is that data concerning both bacterial metabolism and density, if yielding reciprocally consistent results, could contribute to a more actual and precise estimation of the viability of faecal bacteria in natural marine samples. As previously stated,[17] in fact, the assay of one property of living cells cannot provide sufficient proof of viability.

In the samples examined, *E. coli* represented the main component within the total FC population (data not shown); this result was confirmed by the significant relationships between *E. coli* and FC values. Compared with FA-L/D, the m-FC method, however, yielded an underestimate of bacterial concentrations; this quantitative discrepancy could be explained by the large percentage of nonculturable cells present in our natural samples that were still alive and also expressed β-glucuronidase activity *in situ*, as shown by the significant relationship between FA-L/D and MUG. Analysis of total data by linear regression suggested the reciprocal agreement of results obtained by the three methods; enzyme rates were correlated with FC values at a significance level higher than FA counts. This finding, previously observed, both in freshwater[9] and highly-contaminated marine environments,[18] led us to suppose that the high level of organic matter available at these sites could support a great percentage of metabolically active cells. As a fact, 76.47% (on average) of the samples examined exceeded the threshold values allowed by Italian laws for bathing waters (FC $<$ 100 CFU $100\,mL^{-1}$), showing the occurrence of improperly treated urban sewage along the shoreline.

In our study the FA-viability staining procedure previously applied for the detection of *E. coli* in natural seawaters[19] was also modified: the propidium iodide reagent came from a standardised L/D viability kit, making it easier to set up the appropriate concentration of the stain and also resulting in advantages for the operator safety. Our protocol has proved to be effective in discriminating, in seawaters, the living and the dead fraction within a specific (*E. coli*) bacterial population. Compared with β-glucuronidase activity measurements, sometimes biased by the occurrence of false-positive and false-negative results (i.e., coliforms other than *E. coli* or β-glucuronidase negative *E. coli*, respectively), the microscopic method offers the advantages of simplicity of execution, specificity, and sensitivity of the immunological techniques, rapid and quantitative responses, providing rapid information about the physiological state in which pathogenic bacteria may persist in natural environments.[6,9] The high incidence (on average 91.64%) of viable cells detected by the combined FA-L/D protocol and the measured values of β-glucuronidase activity higher than the maximum value (79.11 nmol MUF h^{-1} $100\,mL^{-1}$) previously reported in the same Sicilian area,[2] suggested that anthropogenic inputs were recent and that the large nutrient and organic matter inputs supported bacterial survival and activity. If we compare the present data with counts obtained in 2002, when dead cells accounted for 22% of the total bacterial density in Messina Straits,[19] this result proved evidence of a worsening of the microbiological quality of our coastal waters. It also confirmed the importance, for human health, of rapid procedures able to detect, *in situ*, the fraction of bacteria retaining their viable and/or active properties that could be scored as negative and, therefore, underestimated by conventional plate counts. The assessment of the viability of specific bacteria using a "battery" of

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different methods may yield a more sensitive estimate of the persistence of human faecal indicators in coastal waters, offering new, interesting perspectives in the field of aquatic environment monitoring.

ACKNOWLEDGMENTS

The work has been funded by Italian MIUR (Ministero Università e Ricerca) within the Cluster 10-SAM "Realizzazione ed attivazione di una rete integrata di piattaforme e mezzo mobile attrezzati per il monitoraggio dell'ambiente marino costiero" Project.

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Received June 3, 2005
Accepted July 2, 2005
Manuscript 3180

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