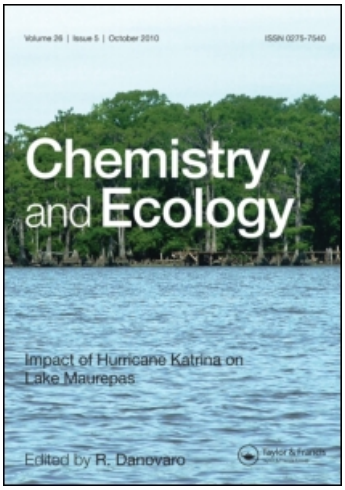

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NEW METHODOLOGICAL STRATEGIES FOR DETECTING BACTERIAL INDICATORS

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(In final form 18 February 2004)

The development of monitoring strategies for the early warning of seawater pollution, with particular reference to faecal and hydrocarbon contamination, has been the specific goal of research carried out within the Cluster 10-SAM (Advanced Systems for Coastal Marine Monitoring) Project, funded by the Italian Ministry for University and Scientific Research. Advanced analytical approaches have been designed and applied to detect bacterial species that have been selected as potential indicators of pollution in seawater samples. We report the results obtained using the fluorescent antibody and enzymatic assays for the detection of *Escherichia coli*, and a real-time PCR protocol for monitoring marine areas for hydrocarbon pollution. Immunofluorescence and enzymatic methods revealed the occurrence of different faecal pollution levels, reaching 10^5 *E. coli* cells 100 ml^{-1} in the Straits of Messina. Real-time PCR results corresponded to the different degree of oil pollution of the analysed samples. The specificity and speed make these methods promising for the detection and evaluation of marine pollution.

Keywords: Marine pollution; Bacterial indicators; Coastal monitoring

1 INTRODUCTION

Owing to the enormous density of the human population living in the coastal zone, and the increasing human activities across marine environments (transportation, drilling etc.), human disturbances to aquatic ecosystems (*i.e.*, loss of biodiversity, increasing frequency of harmful algal blooms, hypoxia, disease and declines in fisheries) have been extensively documented (Paerl, 1997); this situation is predicted to increase in the foreseeable future (Peierls *et al.*, 1991; Vitousek *et al.*, 1997). It has already been recognized that effective environmental controls result in a significant reduction in water-borne diseases (WHO, 2001). Nevertheless, the mechanisms underlying water-quality and habitat degradation remain poorly understood, so there is a need to develop and apply novel and effective ways to detect, manage and correct human-induced loss of aquatic ecosystem biodiversity, water quality, fisheries, and recreational resources.

Among aquatic biota, bacteria are generally most sensitive to environmental perturbation. They have fast growth rates, and so they are used in 'real-time' environmental monitoring.

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Moreover, bacteria respond to low levels of pollutants as well as other physical, chemical and biotic environmental changes. Therefore, bacterial indicators can play a major role in detecting and characterizing natural and human stressors, and the way those stressors drive changes in microbial diversity and function. This is especially true in aquatic environments where bacteria contribute substantially to the nutrient cycling. Bacteria-related parameters can implement a range of monitoring programs, including moored instrumentation and remote sensing on ecosystem to global scales. For bacterial detection, complementary use of analytical and molecular indicator tools shows great promise. Standard bacterial indicators usually fulfil the following basic requirements: (1) they are present in high numbers in polluted waters, and (2) they persist in the environment for a sufficient time to be easily detected through simple methods (Stevens *et al.*, 2001). Moreover, they can yield information on the specific kind of pollution that characterizes their source environments. Depending on the selected stressor, the choice of the suitable bacterial bioindicator must be optimized regarding the type and range of bacteria (or bacteria-related parameter such as the target bacterial molecules) to be used, the suitable frequency of sampling, the size of the samples to be analysed, and the reliability, low cost and speed of the analytical approaches that should be performed (IWA, 2000).

The CNR Istituto per l'Ambiente Marino Costiero of Messina has acquired long-term expertise in the monitoring of marine pollution using new, rapid techniques addressing mainly the determination of faecal indicators (Caruso *et al.*, 2000, 2002 a, b) and of marine hydrocarbon-degrading bacteria as possible indicators of oil contamination (Denaro *et al.*, accepted). These topics have been the focus of the MIUR Cluster 10-SAM Project, which aims to develop an integrated network for seawater monitoring and develop rapid methods for the use of bacterial bioindicators in marine pollution events. Issues related to the choice of more appropriate bacterial species or genes have been considered (*i.e. Escherichia coli*, rather than the total coliforms; the quali–quantitative measurement of specific genes involved in the hydrocarbon-degradation pathway, rather than the search for specific hydrocarbonoclastic bacterial taxa); the selection of suitable analytical methods bypassing the limits of culture-based approaches (low reproducibility, long analysis and response times, inability to detect 'injured' or VBNC cells) has also been addressed. Herein, we propose the immunofluorescence, the β-glucuronidase activity assay and the real-time PCR techniques as promising tools for fast and reliable screening of bacteria-related parameters potentially suitable as bioindicators of faecal and oil pollution. These methodologies represent actual challenges in the field of environmental monitoring (Caruso *et al.*, 2002 a, b; Vesper *et al.*, 2003).

2 MATERIALS AND METHODS

2.1 Activities Performed

Two different research lines have been developed to identify appropriate bacterial markers and monitoring strategies. New methodological approaches have been tested, specific for the detection of: (1) faecal contamination (*E. coli*), in the framework of the Workpackage W2-A1 of SAM Project, and (2) hydrocarbon contamination (hydrocarbonoclastic bacteria, HCB), within the Workpackage W2-A2.

2.1.1 W2-A1: Bioindicators of Faecal Pollution

For the assessment of faecal pollution, research has addressed the simultaneous detection and enumeration of *E. coli* in seawater by microscopy, enzymatic and culture-based methods. In

particular, we have prepared the protocols for the immunofluorescence (IF) technique and for the β-glucuronidase activity (MUG) assay.

2.1.1.1 Sampling and Treatment of the Samples During 2001 and 2002, monitoring cruises were performed by the *L. Sanzo* vessel in different areas (Straits of Messina, Gioia Tauro Gulf, Milazzo Gulf, Palermo Gulf) impacted by the outfall of urban sewage (Fig. 1). A total of 70 surface water samples were collected using Niskin bottles from coastal stations showing various degrees of faecal contamination and stored at +5 °C. The treatment of subsamples (100 ml or less) was different according to the method of analysis reported below.

2.1.1.2 Culture-Based Techniques Culture-based methods required the filtration of the sample through a 0.45-μm porosity membrane and further incubation of the filter on plates of a medium selective for faecal coliforms (FC) (m-FC broth (Difco) added to 1.5% agar, mFC), as suggested by APHA (1992). An estimation of the abundance of bacteria present in the sample in terms of colony-forming units (CFU 100 ml^{-1}) was obtained.

2.1.1.3 IF Technique This technique relies on the treatment of the sample with fluorescently labeled immune sera specific for the target bacteria (*E. coli*) and further observation of the antigen–antibody complex under an epifluorescence microscope. Briefly, the IF protocol involved filtering the sample through a Nuclepore black membrane (0.22 μm), then incubating the filter with hydrolysed gelatine (final concentration 2%) for 20 min; after washing with phosphate-buffered saline (PBS), the filter was incubated with a 1:80 dilution of Murex *E. coli* immune sera (pool A + B + C) for 30 min and then incubated with Goat anti-rabbit IgG-FITC (1:160 in PBS) for 30 min. Under an epifluorescence microscope (Axioplan Zeiss, filter set BP 490, LP 510, FT 520), IF-labelled *E. coli* cells were easily detected as rod-shaped cells with green fluorescing outlines (Caruso *et al.*, 2000; 2002a). The number of cells present in the sample (cells 100 ml^{-1}) was determined.

For further development of the IF technique, two protocols of IF-viability assays were set up, combining the labelling with immune sera with propidium iodide (PI) or 5-cyano-2,3 ditolyl tetrazolium chloride (CTC), as markers of dead and viable respiring cells, respectively. Using this method, described in detail by Caruso *et al.* (2003), it has been possible to obtain information on the different physiological states that may coexist within the heterogeneous *E. coli* population.

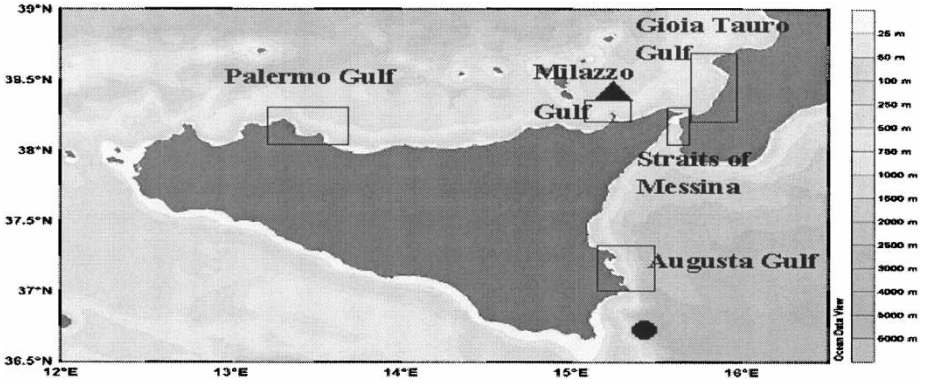

FIGURE 1 Monitoring areas (kindly provided by Dr. F. Raffa, CNR IAMC Messina). ▲: MH station; ●: CP station.

2.1.1.4 MUG Assay The MUG assay is an indirect method to estimate faecal pollution by determination of the β-glucuronidase activity; this enzyme is specific to *E. coli* and some strains of *Shigella* (Gauthier *et al.*, 1991; Farnleitner *et al.*, 2001). The experimental procedure involved incubating the sample with increasing amounts (10–50 nM) of the fluorogenic substrate MUG (4-methylumbelliferyl-β-D-glucuronide). This is subsequently cleaved into its measurable fluorescent product, methylumbelliferone (MUF), by the enzyme β-glucuronidase (Caruso *et al.*, 2002b). The increase in fluorescence intensity, measured using a spectrofluorometer, provided an estimation of the amount of substrate hydrolysed, which was dependent on the amount of enzyme present in the sample. Values obtained were expressed in terms of potential enzymatic activity (maximum velocity, V_{max}, of substrate hydrolysis, in nanomoles of MUF released per hour).

2.1.1.5 Statistical Analysis An analysis of variance (ANOVA) test was performed to evaluate whether the mean values of data obtained by two methods were statistically different. Prior to statistical analysis, data were log-transformed to stabilize the variance and attain the normal distribution. The same ANOVA analysis was used to test values obtained by each method for their temporal and spatial differences. After discarding data that were negative, the statistical analysis by linear regression of the $\log_{10}$-transformed m-FC data vs. the $\log_{10}$-transformed IF and MUG data was used to determine the strength of the reciprocal correlation between the values obtained.

2.1.2 W2-A2: Bioindicators of Oil Pollution

With respect to point (2), research activities have been aimed at testing the possibility of using HCB-related parameters as indicators of oil contamination.

HCB are marine obligate bacteria, characterized by a highly specialized metabolism, high affinity for hydrocarbons, formation of blooms in oil-spill-contaminated waters and potential production of biosurfactants. Among the HCB, *T. oleivorans* has been isolated from the sediments of Milazzo harbour (Yakimov *et al.*, 2004). This seems to be highly specialized in the use of aliphatic hydrocarbons with C7 to C20 carbons. A real-time polymerase chain reaction (PCR) approach was applied to test the use of functional genes of HCB (such as alkane hydroxylase, *alk*B, involved in alkane catabolism) or 16S rDNA, as markers of the presence of HCB and, indirectly, of oil pollution. Primers for the amplification of 16S rDNA and *alk*B genes of *T. oleivorans* were designed for this assay.

2.1.2.1 Samples and Preliminary Sample Treatment The sampling was performed in the Sicilian area during two different cruises onboard the *L. Sanzo* vessel, in 2001, and the R/V *Urania*, in 2002. Two surface water samples were collected by means of Niskin bottles: one from a polluted station located in front of Milazzo harbour (38° 13.47′ N–15° 14.80′ E) (MH sample), and one from a pelagic unpolluted station located in front of Capo Passero (36° 30′ N-15° 50′ E) (CP sample), respectively (Fig. 1). Samples were filtered immediately after collection and stored at −20 °C. The CP sample was partially used to inoculate an oil enrichment culture (CP_{oil} sample), as detailed below.

2.1.2.2 Oil-Enrichment Culture A subvolume (1 l) of the CP sample at the time of sampling (t_0) was filtered through 47 mm Nuclepore filters (0.2 μm pore size), and the filter was then placed in an Erlenmeyer flask containing 200 ml of ONR7a medium (Dyksterhouse *et al.*, 1995) supplemented with crude oil (Arabian light, 0.1% v/v) and nutrients ($(NH_4)H_2PO_4$; 0.2% w/v). Incubation was carried out in the dark at 22 °C, by shaking,

until the increase in turbidity, due to bacterial growth, ceased (7 days). A volume of this enrichment (5 ml) was then filtered for DNA extraction, as detailed below.

2.1.2.3 DNA Extraction For DNA extraction, optimal volumes of CP, CP_{oil} and MH samples (5 l, 5 ml and 200 ml, respectively) were filtered onto 47 mm Nuclepore membranes (0.2 μm pore size). DNA was extracted from the Nuclepore filters according to the method of Zhou *et al.* (1996), with minor modifications. Briefly, each filter was treated in Petri dishes with 0.5 ml of extraction buffer: 100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate buffer (pH 8.0), 70 μl of lysozyme (50 mg ml^{-1}), and 30 μl of 10% SDS with 8 μl of RNA_{ase} and 8 μl of proteinase K (10 mg ml^{-1}) were added. After shaking at 50 °C for 1 h, the sample was added to 100 μl of NaCl 5 M and 80 μl of 10% CTAB (hexadecylmethylammonium bromide)/0.7 M NaCl and incubated at 65 °C for a further 10 min. A two-step extraction using (1:1 v/v) phenol:chloroform:isoamyl alcohol (24:24:1) and chloroform:isoamylic alcohol (24:1) was carried out, each step followed by centrifugation (13,000 rpm for 2 min). The aqueous phase was transferred to a sterile tube, and isopropanol (0.7 vol) and 10 M sodium acetate (0.2 vol) were added. After mixing by inversion, centrifugation (13,000 rpm for 20 min) was again carried out. The pellet was then washed with 500 μl of 70% ethanol and, after centrifugation (13,000 rpm for 2 min), air-dried and resuspended in 50 μl of MilliQ water.

2.1.2.4 Real-Time PCR: Primer Optimization and Quantitative Measurements With the aim to determine the minimum primer concentration giving the maximum yield and the minimum non-specific amplification, the first experiment was performed on a pure culture of *T. oleivorans.* Following the manufacturer's instructions, several master mixes were prepared with different amounts of primers (both 16S and *alk*B specific primers). Amplifications were carried out using a GeneAmp 5700® (PE Applied Biosystems, Foster City, CA) on three replicates of each combination of the forward and reverse primers (50:300, 300:300, 300:900 nM). PCR was performed using an initial denaturation step at 95 °C for 3 min, followed by 40 cycles (denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min, final extension at 72 °C for 10 min). The reaction volume was 50 μl, consisting of: 1X SyBR Green PCR Master Mix (containing AmpliTaq Gold), 1.5 mM $MgCl_2$, 100 μM dNTP, 10 ng DNA. The primer concentration giving the lower threshold cycle (C_T) value was used for further experiments.

Standard curves for both 16S rDNA and *alk*B genes were generated by a stock solution (4 μg ml^{-1}) of total extracted DNA from a pure culture of *T. oleivorans.* Serial dilutions were tested and run both like standard point and unknown samples. The tests for the sensitivity, detection limit, specificity and fishing capability of the primers were also carried out in a comparative analysis of optimal volumes of the CP, CP_{oil} and MH samples. The *alk*B genes were then quantified in the CP, CP_{oil} and MH samples.

3 RESULTS

3.1 W2-A1: Bioindicators of Faecal Pollution

The results of mFC and IF counts (Fig. 2) have demonstrated the presence of low faecal contamination levels in the Milazzo Gulf, in Palermo and Gioia Tauro Gulfs. The mean values of FC and *E. coli* (IF) were 7.5×10^1 CFU 100 ml^{-1} and 2.59×10^4 cell 100 ml^{-1} (respectively, Palermo) and 2.38×10^2 CFU 100 ml^{-1} and 8.19×10^3 cell 100 ml^{-1} (respectively, Gioia Tauro). Because of the consistent inputs from urban wastes, the Straits of Messina

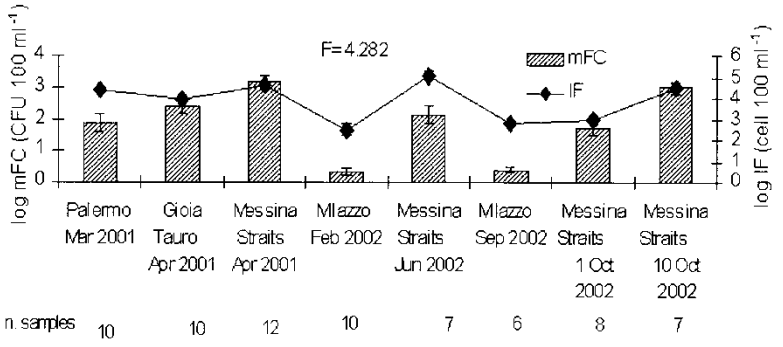

FIGURE 2 Comparison between plate (mFC) and immunofluorescence (IF) counts. The *F*-value, obtained by the ANOVA test, is shown.

suffered higher levels of pollution, with the highest microbial densities recorded by IF in June 2002, when a maximum of 1.12×10^5 cell 100 ml^{-1} was reached. No statistical differences were detected from the ANOVA test performed between the IF and the FC data ($F = 4.282$, $n = 70$). The significance of temporal and spatial variations was also evaluated by the ANOVA test (Tabs. I and II).

Despite the discrepancy between direct (IF) and indirect (FC) counts, owing to the ability of the IF method also to estimate VNBC cells not otherwise evaluated, analysis by linear regression ($R^2 = 0.4432$, $n = 45$, Fig. 3) suggested that there was some agreement in the data obtained with the two methods.

The IF-viability assays have indicated that in seawater samples collected from the Straits of Messina (Fig. 4), culturable cells varied from 1.4×10^1 to 2.96×10^2 CFU 100 ml^{-1}, representing only a low percentage (less than 9%) of total IF counts, while non-culturable

TABLE I Results of the ANOVA test between sites carried out on plate (FC) and immunofluorescence (IF) counts.

	FC	*IF*
Palermo vs. Gioia T.	0.458	1.056
Palermo vs. M E Straits	4.023	0.338
Palermo vs. Milazzo	2.276	2.59
Gioia T. vs. M E Straits	3.573	1.931
Gioia T. vs. Milazzo	1.001	1.391
M E Straits vs. Milazzo	4.458	3.129

$^*P < 0.05$.

TABLE II Temporal variability of data: results of ANOVA test carried out on plate (mFC) counts, immunofluorescence (IF) counts and enzyme (MUG) values.

	mFC	*IF*	*MUG*
Messina Straits Apr 01 vs. Jun 02	2.757	2.429	21.25**
Messina Straits Apr 01 vs. 1 Oct 02	3.119	4.696*	16.83**
Messina Straits Apr 01 vs. 10 Oct 02	1.704	0.48	42.31**
Messina Straits Jun vs. 1 Oct 02	0.052	64.82**	1.728
Messina Straits Jun vs. 10 Oct 02	3.675	0.246	2.409
Messina Straits 1 vs. 10 Oct 02	4.219	4.037	0.029
Milazzo Feb vs. Sep 02	2.524	0.091	25.59**

$^*P < 0.05$; $^{**}P < 0.01$.

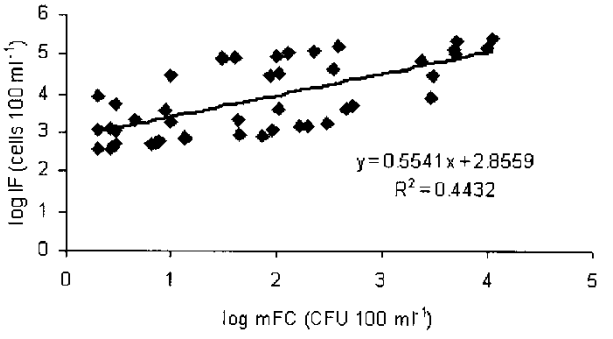

FIGURE 3 Linear regression between culture-based methods (mFC) and microscopy (IF) methods.

but actively respiring cells ranged from 4.8×10^1 to 8.90×10^2 cells 100 ml^{-1} (47.45% of total IF counts) and can be detected only by microscopy techniques.

The MUG assay carried out on the samples collected from the Straits of Messina showed that β-glucuronidase activity values showed interannual variations, being one order of magnitude higher in 2002 than in 2001; enzyme activity was measured at enhanced levels in the Straits of Messina in June 2002 (79.11 nM MUF h^{-1}, mean value). β-Glucuronidase measurements were not statistically different from plate counts, as shown by the ANOVA test values ($F = 1.644$, $n = 42$, in 2001, and 1.161, $n = 28$, in 2002) (Fig. 5).

The ANOVA test confirmed that no significant spatial differences were detected by the MUG assay (Tab. I); in contrast, temporal variations were significant and mainly ascribable to the peak value of β-glucuronidase detected in April 2001 in the Messina Straits (Tab. II).

As observed for IF, analysis through linear regression (Fig. 6) showed a significant correlation between the values obtained by MUG and m-FC methods ($R^2 = 0.2277$, $n = 45$). Comparison by linear regression between MUG data vs. IF values and MUG data vs. m-FC values revealed that MUG values correlated with IF counts ($R^2 = 0.3981$, $n = 52$)

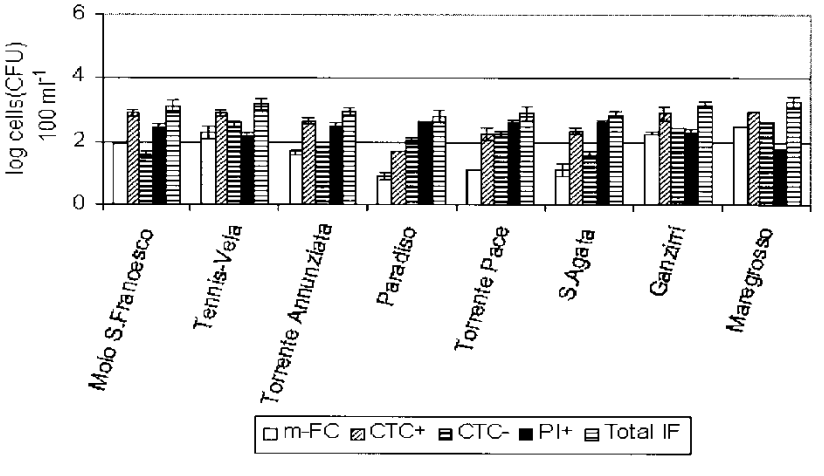

FIGURE 4 Results of combined immunofluorescence (IF)-viability assays. Abbreviations: m-FC, plate counts; CTC+, viable respiring cells, positive by 5-cyano-2,3 ditolyl tetrazolium chloride (CTC)-IF procedure; CTC−, cells negative by CTC-IF procedure, non-respiring but still viable; PI+, dead cells, positive by propidium iodide (PI)-IF procedure; total IF, cells labelled by IF. Error bars indicate the standard deviation from the mean value ($n = 4$ samples collected for each site).

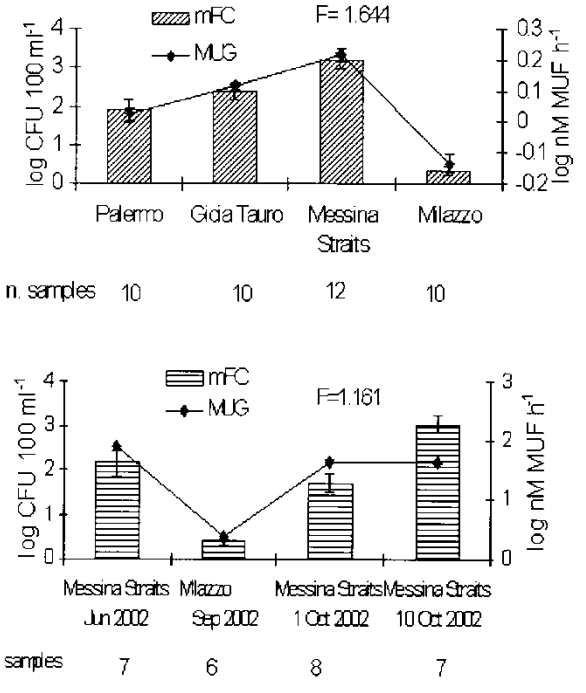

FIGURE 5 Comparison between enzymatic (MUG) and plate (mFC) counts. For each data set, the *F*-value, obtained by the ANOVA test, is reported.

more significantly than with m-FC counts ($R^2 = 0.3889$, $n = 26$), suggesting the higher selectivity of the MUG assay for *E. coli* rather than for the FC group on the whole (Fig. 7a and b).

In conclusion, the main advantages related to the use of IF and MUG methods consist in their specificity, reduced analysis and response times, low reagent costs, and in the possibility of detecting VNBC cells. Disadvantages include, for IF, the narrow range of reactivity of the sera and the subjectivity of readings, and, for MUG, the possibility of false positives (*Shigella* spp.) and negatives (β-glucuronidase-negative *E. coli* strains). For both methods, the threshold value is 10^2 cells 100 ml^{-1}.

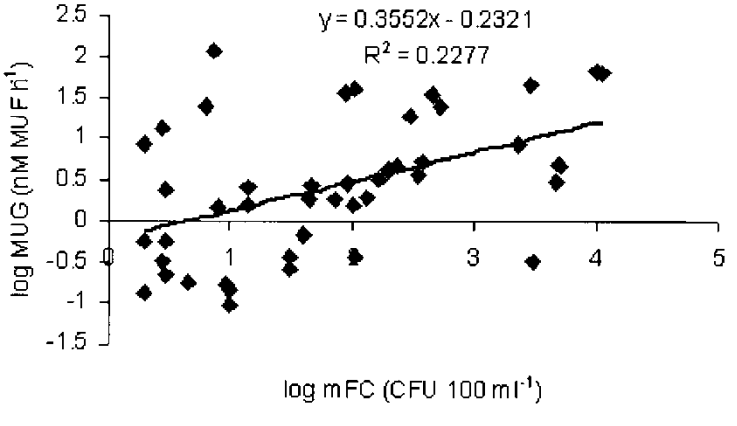

FIGURE 6 Linear regression between enzyme (MUG) and culture-based (mFC) methods.

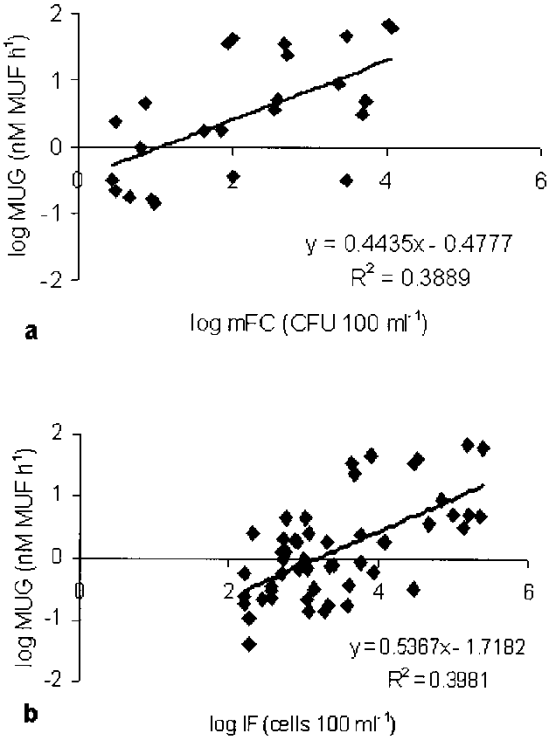

FIGURE 7 Comparison of enzyme values (MUG) vs. (a) plate (mFC) and (b) immunofluorescence (IF) counts.

3.2 W2-A2: Bioindicators of Oil Pollution

Primers designed for *Alcanivorax borkumensis alk*B produced aspecific products, and the dissociation curves were detected at different melting temperatures. On the contrary, primers related to *T. oleivorans* both for 16S rDNA and *alk*B genes yielded only one pattern of dissociation curves at melting temperature (84 °C). Therefore, subsequent experiments were carried out using *T. oleivorans* as a target microorganism. The sequences of the primers used are shown in Table III. The detection limit of the instrument was 10^{-9} ng (Fig. 8).

According to the test on the *T. oleivorans* DNA, the optimum concentration of both *alk*B and 16S rDNA specific primers for PCR was the combination of 50 nM forward/50 nM

TABLE III Sequences of the used primers for quantification of *Thalassolituus oleivorans.*

Primer	*Sequence*	*Position*
ThlsF	cctaaggggaaagcggg	145
ThlsR	cagctatgtgctattaacacac	434
ThlalkF	gacgtcgccacacctgcc	125
ThlalkR	aacgaacgagacataccggg	342

Note: Thls F and R (forward and reverse) were designed on 16S rRNA; Thl *alk* F and R were constructed on the *alk*B sequence of *Thalassolituus oleivorans.*

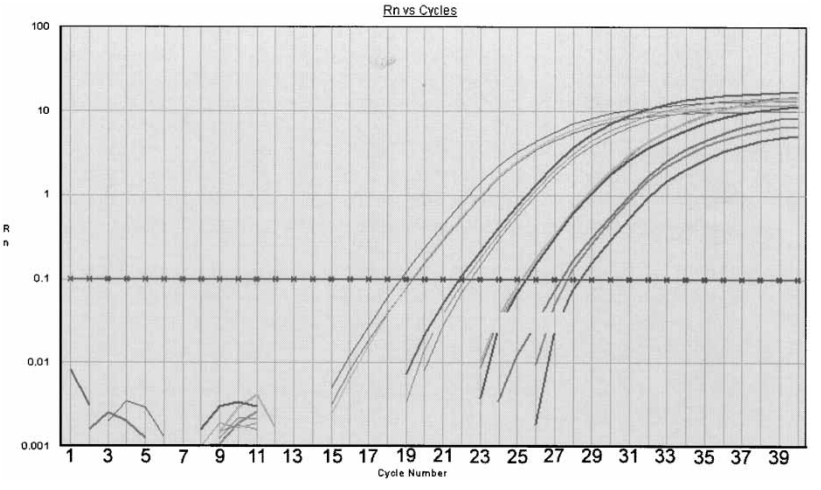

FIGURE 8 Amplification curves of serial dilution of DNA of *Thalassolituus oleivorans* to evaluate the detection limit of the instrument.

reverse that generated an amplification curve at a C_T value of 22 and a specific dissociation curve with a melting temperature of 84 °C. Moreover, the theoretical and detected melting temperatures were the same, and the dissociation curves did not show any aspecific amplification. The standard curve is shown in Figure 9.

Analysis carried out on different subvolumes of the *T. oleivorans* pure culture revealed a stable quantitative ratio of 1:3 between the 16S rDNA and *alk*B genes. Repetitive ratios were also observed between the concentration of the extracted DNA (DNA_{TOT}), the amount of *alk*B genes and the total number of cells measured by epifluorescence microscopy on DAPI stained filtered samples (Porter and Feig, 1980) ($0.11 \pm 0.001_{DNATOT}$: $10^{-17} \pm 0.003_{alkB}$:1; pg/pg/cell). This value was used as a reference for calculating cell numbers related to the real-time measurements on natural samples (Tab. IV).

When applied to the natural samples, the *T. oleivorans* 16S rDNA-targeted primers were found to be non-selective, since they led to aspecific amplification products. On the contrary, the *alk*B gene concentrations were reliable, since they were yielding overlapping dissociation

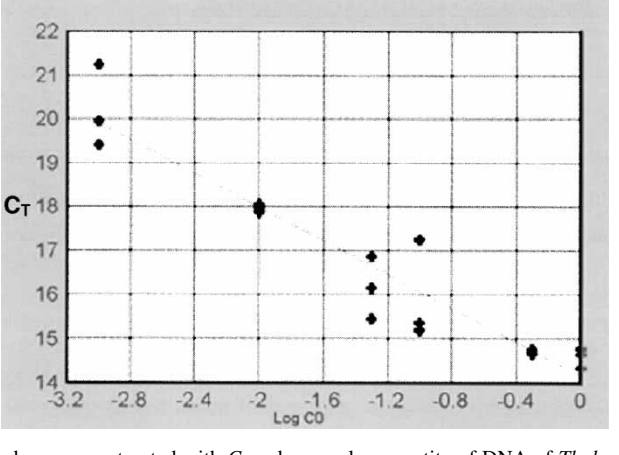

FIGURE 9 Standard curve constructed with C_T values vs. log quantity of DNA of *Thalassolituus oleivorans.*

TABLE IV Number of cells of *Thalassolituus oleivorans* detected on the analysed samples.

Unpolluted	0.86 cell l^{-1}
Polluted area	1.5×10^4 cell l^{-1}
Unpolluted enrichment	2×10^7 cell l^{-1}

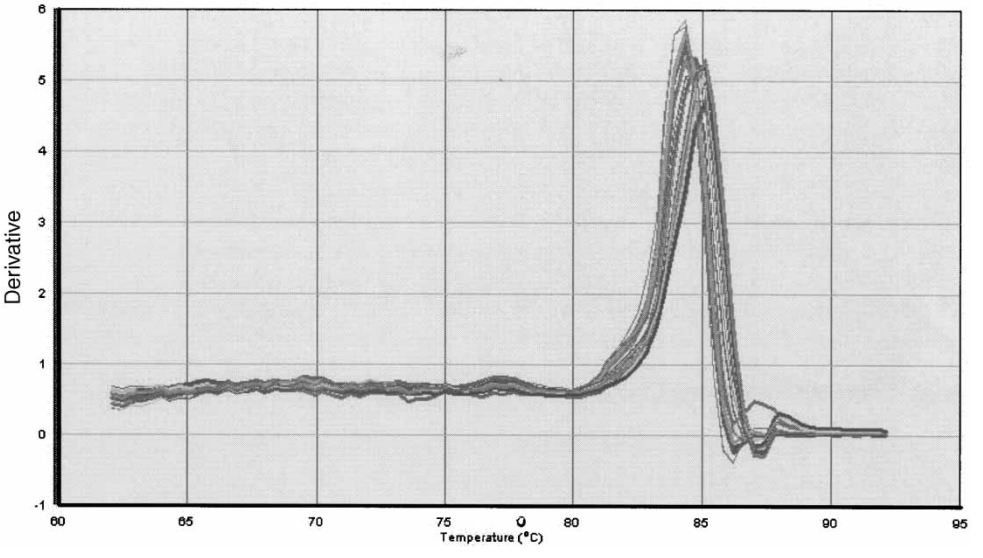

FIGURE 10 Real-time PCR *alk*B *Thalassolituus oleivorans* dissociation plot.

curves for each of the analysed mixed samples (CP, CP_{oil}, MH) (Fig. 10). The *alk*B gene measurements obtained showed a maximum value corresponding to the CP_{oil} sample. The minimum value was measured in the CP sample and was several orders of magnitude lower than the CP_{oil} and MH samples (Tab. IV).

4 DISCUSSION

4.1 W2-A1: Bioindicators of Faecal Pollution

Water-borne diseases have major public-health and socio-economic impacts, making rapid and reliable routine monitoring of the microbiological water quality of fundamental importance for mitigation (Sobsey *et al.*, 1993; Ashbolt *et al.*, 2001). Because of the wide variety of pathogens that may be present in waters, and because many of the detection techniques are very difficult, laborious and expensive, their detection is unsuitable in routine analysis. Therefore, current guidelines for the assessment of water quality impose the quantification of microorganisms that are indicators of the health status of aquatic environments and provide early warnings about potential, but not actual, health hazards (WHO, 2001). The concept of bacteria as indicators of water quality has received increasing interest with the development of more sensitive microbiological techniques (Stevens *et al.*, 2001).

For faecal or sewage pollution, the routine water quality controls rely on the search for FC (primarily *E. coli* and *Klebsiella* spp.), as the group more closely related to the occurrence of

enteric pathogens than total coliforms. Recent opinions of the scientific community seem to agree with the choice of *E. coli* as a specific indicator for faecal pollution. The use of this bacterium is appropriate because of the large numbers of *E. coli* present in the human intestine, and the fact that they are not generally present in other environments (Edberg *et al.*, 2000). Besides FC, the importance of other faecal indicators (such as enterococci) in water quality monitoring has also been suggested, due to the relationship found between their presence in contaminated waters and the outbreak of gastrointestinal diseases (Cabelli *et al.*, 1979).

However, the failure of current analytical methods to recover stressed or injured bacteria, which may survive in waters but are undetectable by conventional plate assay, has been highlighted by a number of studies (Roszack and Colwell, 1987; Pommepuy *et al.*, 1996; Rozen and Belkin, 2001). Culture-based assays also suffer from long response times, low accuracy and a lack of sensitivity in detecting environmental pathogens (Pyle *et al.*, 1999). In light of limitations in approaches to the monitoring of the pollution of recreational waters, a consistent revision of the efficacy of these procedures has been suggested. In addition, alternative rapid methods for microbiological analysis have been proposed and are often welcomed in environmental monitoring studies, since they provide rapid responses on the degree of anthropogenic disturbance and are compatible with basic laboratory facilities (Sartory and Watkins, 1999). Real-time analysis would be ideal for the management of microbial water quality and the protection of public health (IWA, 2000); this consideration supports the need for rapid analysis. To be practical, new rapid methods should meet minimum requirements such as specificity and sensitivity, the availability of data within the same working day or in a shorter time than in standard methods, repeatability and reproducibility of results. Both MUG and IF techniques satisfy these characteristics. From data obtained in our study, MUG values correlated with IF counts more significantly than with mFC counts. This finding could result from the possible underestimation of FC numbers by sample filtration on a membrane with a high porosity (0.45 instead of 0.22 μm) and by the lack of a preliminary incubation step on non-selective medium aimed at recovering stressed cells.

4.2 W2-A2: Bioindicators of Oil Pollution

Up to now, the most sensitive method available for DNA sequence detection has been PCR. This technique may be able to amplify even one copy of a DNA template by several million. Methods based on the PCR and DNA hybridization probes have received particular attention (Sandu *et al.*, 1995; Martin *et al.*, 2000; Chang *et al.*, 2001). The more recent advent of fluorescent probe-based PCR technology (Heid *et al.*, 1996) has led to the development of homogeneous methods for detecting organisms that require relatively short periods of time. Quantitative PCR (QPCR) has been found to be useful for quantitative analysis of microorganisms in environmental samples (Suzuki *et al.*, 2000; Takai and Horikoshi, 2000; Stulz *et al.*, 2001), but to our knowledge, this approach has not been used in the analysis of HCB-related parameters in marine environments.

The pairs of primers (forward, reverse) for real-time amplification were especially designed for selective amplification of 150 bp length fragments, that is the optimal compromise with the required length for real-time PCR-based measurements (optimal length: 50–70 bp, max detectable: 150 bp) and the need to obtain specific amplifications. Owing to the non-specificity of the *Alcanivorax* targeted primers, further analyses are in progress to design new, substitutive primers for this targeted species. The stable quantitative ratio between *T. oleivorans* 16S rDNA and *alk*B genes confirmed previous results (Yakimov *et al.*, 2004).

The *T. oleivorans* 16S rDNA targeted primers were not specific when applied to the mixed samples. In any case, a further development of new, more specific, taxonomic primers does

not seem to be of particular interest. In fact, according to previous monitoring activities, the dominant HCB groups show a competitive geographical distribution and characterize different areas depending on as-yet undefined controlling factors, probably including nutrient availability (Giuliano *et al.*, in preparation). For the foreseeable future, the development of taxonomic targeted primers covering a wider range of HCB species may not be possible because of significant 16S rDNA differences between the different taxa. Therefore, the functional genes involved in the hydrocarbon degradation pathways (such as the *alk*B genes) appear to be more suitable as potential biomarkers for oil pollution. Results showed a clear difference in the relative content of the *Thalassolituus alk*B genes of the CP, CP_{oil}, and MH samples. The relative concentrations were logically distributed according to the level of pollution of the samples analysed. The detection limit of the instrument was low enough for us to expect reliable measurements also for non-polluted samples, characterized by a possible low number of HCB cells. According to preliminary results, the possible suitability of the *alk*B genes as markers for marine oil-polluted areas is stressed. The *alk*B selected primers that have been used in the present work were designed to be specific for the *T. oleivorans* species, but work is in progress for the optimization of *alk*B targeted primers that can amplify genes simultaneously from a wide range of HCB taxa.

5 CONCLUSIONS

Besides the technical development, the microbiological safety of waters must be guaranteed by a series of actions, including implementation of active surveillance, and by assessing multiple parameters, public education and technology transfer (Ford and Colwell, 1995). Integrated programmes of environmental monitoring and health surveillance represent challenges for future developments in the field of seawater quality assurance.

In this work, we provide advances in the choice and optimization of new, highly promising technologies for the detection of faecal and oil-pollution events. In particular, an improved approach has been proposed for monitoring faecal pollution events, which better reflects health risks and provides results for effective management of seawater. According to Torrance (1998) and Farnleitner *et al.* (2001), the detection of specific bacteria by the analysis of their antigenic cell determinants (IF method) or their metabolic pathway signatures (MUG assay) has proved to be highly sensitive and suitable for the purpose. Both methods have been successfully optimized for detecting *E. coli* cells in natural marine waters. The detection of the viability status of bacteria by the combination of IF with a marker of membrane integrity or respiratory activity is a new and interesting development of the immunological approach that is regarded as very promising for assessing the physiological heterogeneity of *E. coli* population in seawater (Caruso *et al.*, 2003).

The real-time PCR provided rapid amplification and quali-quantitative analysis of HCB related target molecules without the electrophoresis step. From the preliminary obtained results, the real-time analysis of the *T. oleivorans alk*B genes seems to be highly promising for monitoring oil-pollution events in marine environments. To our knowledge, our study is the first application of the real-time PCR methodology for the detection and quali-quantitative analysis of HCB in seawater. Further analyses are planned on a different kind of bacterial functional genes as well as on the quantification of *E. coli*-specific sequences.

New analytical (microscopy, enzymatic and molecular) methods have been set up as alternative approaches to standard culture-based methods. The use of an appropriate combination of these technologies with the measurement of additional bacterial indicators (*i.e.* HCB) is

suggested. This is in agreement with what was already established by Ashbolt *et al.* (2001), since the detection of a single indicator cannot fulfil all the needs of water-quality surveillance.

The great potential of the new methods, especially molecular biology methods, for the control of contamination by chemical and microbiological pollutants, lies in their high sensitivity and speed, which make it possible to develop an immediate response for the safety of marine environment.

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References

APHA (American Public Health Association) (1992). *Standard methods for the examination of water and waste water*. APHA, Washington, DC, 18th edition.

Ashbolt, N., Grabow, W. O. K. and Snozzi, M. (2001). Indicators of microbial water quality, in Fewtrell, L. and Bartram, J. (eds.), *Water quality: guidelines, standards and health, World Health Organization (WHO)*. IWA, London, Chap. 13, pp. 289–316. [www http://www.who.int/water_sanitation_health/Documents/IWA/iwachap13.pdf].

Cabelli, V. J., Dufour, A. P., Levin, M. A., McCabe, L. J. and Haberman, P. W. (1979). Relationship of microbial indicators to health effects at marine bathing beaches. *American Journal of Public Health*, **69**, 690–696.

Caruso, G., Crisafi, E. and Mancuso, M. (2002a). Immunofluorescence detection of *Escherichia coli* in seawaters: a comparison of different commercial antisera. *Journal of Immunoassay and Immunochemistry*, **23**, 479–496.

Caruso, G., Crisafi, E. and Mancuso, M. (2002b). Development of an enzyme assay for rapid assessment of *Escherichia coli* in seawaters. *Journal of Applied Microbiology*, **93**, 548–556.

Caruso, G., Mancuso, M., Crisafi, E. (2003). Combined fluorescent antibody assay and viability staining for the assessment of the physiological states of *Escherichia coli* in seawaters. *Journal of Applied Microbiology*, **95**, 225–233.

Caruso, G., Zaccone, R. and Crisafi, E. (2000). Use of the indirect immunofluorescence technique for detection and enumeration of *Escherichia coli* in seawater samples. *Letters in Applied Microbiology*, **31**, 274–278.

Chang, H. C., Leaw, S. N., Huang, A. H., Wu, T. L. and Chang, T. C. (2001). Rapid identification of yeasts in positive blood cultures by a multiplex PCR method. *Journal of Clinical Microbiology*, **39**, 3466–3471.

Denaro, R., D'Auria, G., Di Marco, G., Genovese, M., Troussellier, M., Yakimov, M. M. and Giuliano, L. (accepted). Assessing T-RFLP suitability for the description of bacterial community structure and dynamics in oil-polluted marine environments. *Environmental Microbiology*.

Dyksterhouse, S. E., Gray, J. P., Herwig, R. P., Lara, J. C. and Staley, J. T. (1995). *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. *International Journal of Systematic Bacteriology*, **45**, 116–123.

Edberg, S. C., Rice, E. W., Karlin, R. J. and Allen, M. J. (2000). *Escherichia coli*: the best biological drinking water indicator for public health protection. *Journal of Applied Microbiology*, **88**, 106S–116S.

Farnleitner, A. H., Hocke, L., Beiwl, C., Kavka, G. G., Zechmeister, T., Kirschner, A. K. T. and Mach, R. L. (2001). Rapid enzymatic detection of *Escherichia coli* contamination in polluted river water. *Letters in Applied Microbiology*, **33**, 246–250.

Ford, T. E. and Colwell, R. R. (1995). A global decline in microbiological safety of water: a call for action, in *American Academy of Microbiology, Report*, pp. 1–40. [www http://www.asmusa.org/acasrc/Colloquia/waterquality1995.pdf].

Gauthier, M. J., Torregrossa, V. M., Babelona, M. C., Cornax, R. and Borrego, J. J. (1991). An intercalibration study of the use of 4-methylumbelliferyl-β-D-glucuronide for the specific enumeration of *Escherichia coli* in sea water and marine sediments. *Systematic and Applied Microbiology*, **14**, 183–189.

Giuliano, L., Denaro, R., Genovese, M., Cappello, S., D'Auria, G., Chernikova, T. N., Timmis, K. N., Golyshin, P. N. and Yakimov, M. M. (in preparation). Natural microbial diversity occurring in superficial sediments of Milazzo harbour (Sicily, Italy) and communities succession during microcosm enrichments on various hydrocarbons as revealed by 16S rDNA-based molecular phylogenetic analyses.

Heid, C., Stevens, J., Livak, K. and Williams, P. (1996). Real time quantitative PCR. *Genome Research*, **6**, 986–994.
IWA (International Water Association) (2000). Rapid microbiological monitoring methods: the status quo, in *IWA, The Blue Pages, July 2000*, pp. 1–13. [www http://www.iwahq.org.uk/pdf/bp0002.pdf].
Martin, C., Roberts, D., van der Weide, M., Rossau, R., Jannes, G., Smith, T. and Maher, M. (2000). Development of a PCR-based line probe assay for identification of fungal pathogens. *Journal of Clinical Microbiology*, **38**, 3735–3742.
Paerl, H. W. (1997). Coastal eutrophication and harmful algal blooms: importance of atmospheric deposition and groundwater as nitrogen and other nutrient sources. *Limnology and Oceanography*, **42**, 1154–1165.
Peierls, B. L., Caraco, N. F., Pace, M. L. and Cole, J. J. (1991). Human influence on river nitrogen. *Nature*, **350**, 386–387.
Pommepuy, M., Butin, M., Derrien, A., Gourmelon, M., Colwell, R. R. and Cormier, M. (1996). Retention of enteropathogenicity by viable but nonculturable *Escherichia coli* exposed to seawater and sunlight. *Applied and Environmental Microbiology*, **62**, 4621–4626.
Porter, K. G. and Feig, Y. S. (1980). The use of DAPI for identifying and counting aquatic microflora. *Limnology and Oceanography*, **25**, 943–948.
Pyle, B. H., Broadaway, S. C. and McFeters, G. A. (1999). Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. *Applied and Environmental Microbiology*, **65**, 1966–1972.
Roszack, D. B. and Colwell, R. R. (1987). Metabolic activity of bacterial cells enumerated by direct viable count. *Applied and Environmental Microbiology*, **53**, 2889–2893.
Rozen, Y. and Belkin, S. (2001). Survival of enteric bacteria in seawater. *FEMS Microbiology Reviews*, **725**, 1–17.
Sandu, G. S., Kline, B. C., Stockman, L. and Roberts, G. D. (1995). Molecular probes for diagnosis of fungal infections. *Journal of Clinical Microbiology*, **33**, 2913–2919.
Sartory, D. P. and Watkins, J. (1999). Conventional culture for water quality assessment: is there a future? *Journal of Applied Microbiology Symposium Supplement*, **85**, 225S–233S.
Sobsey, M. D., Dufour, A. P., Gerba, C. P., LeChevallier, M. W. and Payment, P. (1993). Using a conceptual framework for assessing risks to health from microbes in drinking water. *Journal of American Water Works Association*, **85**, 44–48.
Stevens, M., Ashbolt, N. and Cunliffe, D. (2001). Microbial indicators of water quality. An NHMRC discussion paper, in *Public Consultation Draft*, September 2001. [www http://www.health.gov.au/nhmrc/advice/microb.pdf].
Stultz, J. R., Snoeyenbos-West, O., Methe, B., Lovley, D. R. and Chandler, D. P. (2001). Application of the 5′-fluorogenic exonuclease assay (TaqMan) for quantitative ribosomal DNA and rRNA analysis in sediments. *Applied and Environmental Microbiology*, **67**, 2781–2789.
Suzuki, M. T., Taylor, L. T. and DeLong, E. F. (2000). Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′-nuclease assays. *Applied and Environmental Microbiology*, **66**, 4605–4614.
Takai, K. and Horikoshi, K. (2000). Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. *Applied and Environmental Microbiology*, **66**, 5066–5072.
Torrance, L. (1998). Immunological detection and quantification methods, in *OECD Workshop Molecular Methods for Safe Drinking Water, Interlaken 1998, Proceedings*, pp. 1–21. [www http://www.eawag.ch/publications_e/proceedings/oecd/proceedings/Torrance.pdf].
Vesper, S. J., Brinkman, N. E., Haugland, R. A., Wymer, L. J., Byappanahalli, M. and Whitman, R. L. (2003). Evaluation of a rapid, quantitative real-time PCR method for enumeration of pathogenic Candida cells in water. *Applied and Environmental Microbiology*, **69**, 1775–1782.
Vitousek, P. M., Mooney, H. A., Lubchenco, J. and Melillo, J. M. (1997). Human domination of the earth's ecosystems. *Science*, **277**, 494–499.
WHO (World Health Organization) (2001). Bathing water quality and human health: fecal pollution, in *Outcome of an Expert Consultation*. Farnham, UK, April 2001, pp. 1–58. [www.http://www.who.in/water_sanitation_health/Recreational_water/wsh01-2.pdf].
Yakimov, M. M., Giuliano, L., Denaro, R., Crisafi, E., Chernikova, T., Abraham, W.-R., Luensdorf, H., Timmis, K. N. and Golyshin, P. N. (2004). *Thalassolituus oleivorans* gen. nov., sp. nov., and new marine bacterium confined to the utilization of hydrocarbons. *International Journal of Systematic and Evolutionary Microbiology*, **54**, 141–148.
Zhou, J., Bruns, M. A. and Tiedje, J. M. (1996). DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*, **62**, 316–322.