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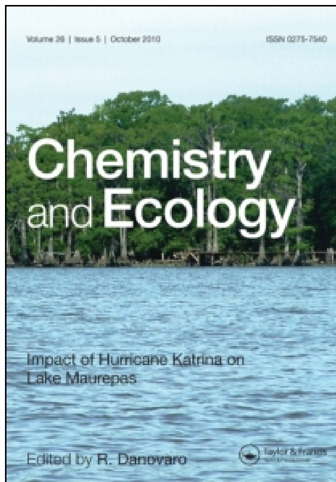

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Luigi Vezzulli[a]; Elisabetta Pezzati[a]; Mariapaola Moreno[b]; Monica Stauder[a]; Mauro Fabiano[b]; Carla Pruzzo[a]
[a] Department of Biology (DIBIO), University of Genoa, Genoa, Italy [b] Department for the Study of Territory and its Resources (DIP.TE.RIS), University of Genoa, Genoa, Italy

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Molecular ecology of marine sediments: determination of Real-Time PCR efficiency for quantifying microbial cells

Luigi Vezzulli[a]*, Elisabetta Pezzati[a], Mariapaola Moreno[b], Monica Stauder[a], Mauro Fabiano[b] and Carla Pruzzo[a]

[a]*Department of Biology (DIBIO), University of Genoa, Viale Benedetto XV 5, 16132 Genoa, Italy;* [b]*Department for the Study of Territory and its Resources (DIP.TE.RIS), University of Genoa, Corso Europa 26, 16132 Genoa, Italy*

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Polymerase chain reaction (PCR) efficiency is the rate at which a PCR amplicon is generated. More efficient amplification of the DNA fragment (amplicon) will generate more products in fewer cycles, thus improving accuracy and sensitivity of quantitative PCR. The efficiency of Real-Time PCR to detect and quantify microbial cells was compared in marine sediment samples of different origin and chemical composition, and in standard samples. Real-Time PCR efficiencies of marine sediment samples ranged from 1.48 ± 0.1 to 1.83 ± 0.1 and were significantly different from those of standard samples, most probably due to different concentrations of PCR inhibitors. The Real-Time PCR efficiency was higher using species-specific primers (>1.7), and lower using universal primers (<1.7). Generally, when the PCR efficiency was higher, its detection limit was lower. In addition, the sensitivity of the Real-Time capillary assay over the traditional assay was generally greater. We suggest that for quantifying microbial cells in marine sediment samples using Real-Time PCR, standard curves should be constructed for both the standard and sediment samples, and a correction factor should be applied. For qualitative PCR a real-time capillary assay should be used for the detection of small quantities of DNA.

Keywords: PCR; Real-Time PCR; marine sediment; bacteria

1. Introduction

Molecular techniques provide an exciting opportunity to overcome the requirement for culturing, and have therefore greatly increased our understanding of microbial diversity and functioning of the marine environment [1]. In particular, PCR-based methods have wide application for the detection, enumeration, functional characterisation and biodiversity assessment of marine bacteria and marine microbial communities [2]. Optimal PCR performance using marine sediment is dependent upon the extraction and purification of nucleic acids [3,16]. In addition, PCR amplification can be severely hampered by the presence of inhibitory substances which are co-extracted with nucleic acids, such as humic acids, organic matter and clay particles [4]. While a number of studies have been conducted to assess and improve the methods used to extract and purify

*Corresponding author. E-mail: luigi.vezzulli@unige.it

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DNA from marine sediments [3,5], little information is currently available on the efficiency of PCR reactions of the DNA from those samples. The efficiency of PCR is measured in terms of its specificity, yield and fidelity. More efficient amplification will generate more products using fewer cycles, thus improving sensitivity and accuracy of quantitative PCR. In this study, PCR efficiency was determined by the Real-Time PCR method using marine sediment samples of different origin and chemical composition, and in standard samples spiked with known concentrations of target bacterial cells. In the analysis, a diagnostic species-specific primer set targeting the *toxR* gene of *Vibrio parahaemolyticus* and a universal primer set targeting bacterial 16S rRNA were tested. The comparison between reaction efficiency and sensitivity in the described experimental settings was carried out and discussed to improve the detection and quantification of microbial cells in marine sediments using Real-Time PCR.

2. Material and methods

2.1. *Preparation of standard and sediment samples with known concentration of target bacterial cells*

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Sediment samples of different origin were collected at three sampling locations in the Mediterranean Sea (Table 1). The measure of organic matter content was obtained by loss of weight on ignition at 350 °C (Table 1). More details on the characteristics of the sediment are reported elsewhere [6,7].

Standard bacterial suspensions in sterile saline water and sterile sediment samples were prepared as follows.

Mixed bacterial cultures were obtained by overnight enrichment of the *in situ* sediment community in marine broth at 20 °C, followed by centrifugation at 10,000 *g* for 10 min, and collection and resuspension of bacterial cells in sterile saline water (0.9% NaCl) to final concentrations of 10^8 CFU/ml.

Pure bacterial culture of *V. parahaemolyticus* were obtained by overnight enrichment of *V. parahaemolyticus* (ATCC 17802) cells prepared in alkaline peptone water (APW) at 37 °C followed by centrifugation at 10,000 *g* for 10 min and collection and resuspension of bacterial cells in sterile saline water (0.9% NaCl) to final concentrations of 10^8 CFU/ml.

The sterile sediment samples were prepared for Real-Time PCR efficiency determination using aliquots of mixed and pure culture bacterial suspension to spike calcinated sediment (450 °C for 2 h) to final concentrations of 10^8 CFU/ml.

To prepare marine sediment samples for Real-Time PCR efficiency determination using species-specific primers, the collected samples were first checked for the presence of the *toxR* gene of *V.parahaemolyticus* using the LightCycler, as described in next paragraph. If PCR was negative for *toxR* DNA, aliquots of *V. parahaemolyticus* (ATCC 17802) suspension were then spiked into sediment samples to a final concentration of 10^8 CFU/ml. Concentration of *V. parahaemolyticus*

Table 1. Main features of sediment samples used in the study.

Sediment origin	Location	Depth (m)	Total organic matter content (%)	Total prokaryotic count (cell g^{-1})	References
Coastal	Portofino Marine Protected Area, Ligurian Sea	10	2 ± 0.2	$1 \times 10^8 \pm 1 \times 10^7$	This work
Fish-farm	Vibo Marina (Tyrrhenian Sea)	40	4 ± 0.5	$4 \times 10^8 \pm 2 \times 10^7$	[6]
Harbour	Sanremo-Portosole Harbour, Ligurian Sea	9	6 ± 0.7	$3 \times 10^9 \pm 8 \times 10^8$	[7]

in pure culture suspension and spiked sediment samples were checked using the CFU plate count method in thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Difco) [8].

To prepare marine sediment samples for Real-Time PCR efficiency determination using universal primers, the collected samples were left untreated and the total prokaryotic count of the *in situ* microbial community was carried out by the acridine orange direct count (AODC) procedure described by Hobbie et al. (1977) [9] (Table 1).

All the preparations described were performed in triplicate.

2.2. *Nucleic acid extraction and construction of dilution series*

DNA was extracted from the sediment samples of pure *V. parahaemolyticus* (10^8 CFU/ml) and the mixed bacterial suspensions (10^8 CFU/ml) in sterile saline water by using the Ultraclean Soil DNA Kit (MoBio Laboratories, Solana Beach, CA, USA). Purified DNA was quantified by using a PicoGreen dsDNA Quantification Kit (Molecular Probes).

For each sample, a series of 10-fold dilutions of extracted DNA was prepared in a sterile TE Buffer.

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2.3. *Primers*

The primers used in this study were: (a) universal primer BR1/F AGAGTTTGATCCTGGCT and BR1/R GGACTACCAGGGTATCTAAT, targeting the highly conserved regions of bacterial 16S rRNA genes that produced PCR products ca. 798 bp in size [10,17]; (b) species-specific primer ToxR/F GTCTTCTGACGCAATCGTTG and ToxR/R ATACGAGTGGTTGCTGTCATG, targeting the *toxR* gene of *V. parahaemolyticus* that produced PCR products ca. 369 bp in size [11]. The primers were synthesised by TIB Molbiol (Berlin, Germany).

2.4. *Amplification with the LightCycler and determination of Real-Time PCR efficiency*

Real-time amplification was performed on the dilution series using a LightCycler instrument 1.5 (Roche Diagnostics, Mannheim, Germany). The LightCycler-FastStart DNA Master SYBR Green I Kit, optimised for use with glass capillaries and containing a hot start polymerase, was used as the master mix base for all reactions. Each reaction mixture contained 5.0 mmol of $MgCl_2$ and 500 nmol of each primer in a final volume of 20 µl. Specific reaction conditions and cycling regimes for each of the two assay types were as follows:

Primers toxR: initial denaturation at 95 °C for 10 min, subsequently 40 cycles of denaturation at 95 °C, 5 s, annealing at 60 °C, 5 s and elongation at 72 °C, 12 s, followed by final elongation at 72 °C for 10 min.

Primers BR1: initial denaturation at 95 °C for 10 min, subsequently 40 cycles of denaturation at 95 °C, 5 s, annealing at 52 °C, 5 s and elongation at 72 °C, 29 s, followed by final elongation at 72 °C for 10 min.

PCR runs were analysed directly in the LightCycler using melting analyses and the analysis software provided with the instrument. The correct size of the products was confirmed in some cases by traditional agarose gel electrophoresis.

Then 5 µl of DNA extract (20 ng/µl) was added to the reaction mixture. Each template was tested at least three times to confirm the reproducibility of the assays. For each run, a positive (standard pure culture) and negative control (sterile milliQ water) were loaded. The efficiency and linear dynamic range of the Real-Time PCR reaction was evaluated by constructing a standard curve using the second derivative maximum method included in the LightCycler data analysis software, Version 3.5.3. Using this method, the log of the CFU concentration of a dilution series

of the sample was plotted versus the cycle number at which the fluorescent signal increased above the background or threshold (Ct value). This approach was used because, for detection and quantification of bacterial cells in environmental samples, it is easier to understand results in actual CFU numbers than in DNA concentrations or copy numbers. Each point of the standard curve was obtained from the mean value of three replicates of the same sample and was considered acceptable only when all the replicates scored positive with a coefficient of variation $<5\%$.

Efficiency is derived from the idealised function for the amount of PCR product formed: $N = N_0 \times E^n$, where N is number of amplified molecules, N_0 is the initial number of molecules, n is the number of amplification cycles and E is the efficiency, which is ideally 2. The standard curves were derived from the function described above: $n = -(1/\log E) * \log N_0 + (\log N/\log E)$. Therefore, the slope of the line equals $-(1/\log E)$ and the efficiency can be calculated from the slope: efficiency $= 10^{-(1/\mathrm{slope})}$ [12].

The actual concentration of the template is not needed when determining the efficiency, as it depends only upon the slope of the line.

2.5. *Amplification with conventional PCR*

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In addition to Real-Time PCR, amplification of each DNA extract was carried out on a Mastercycler Personal (Eppendorf, Germany) using the Master Taq Kit (Eppendorf, Germany). For amplification, 10 µl of DNA extract (10 ng/µl) was used in the presence of 0.2 mmol/l dNTPs. The concentrations, in 50 µl PCR buffer were: 1X Taq Buffer with Mg+, primers, 0.5 µmol/l; 1X Taq Master PCR enhancer and 2.5 U of *Taq* polymerase (Eppendorf). The temperature profile for the PCR was as follows: an initial step of 10 min at 94 °C, followed by denaturation for 30 sec at 94 °C, annealing for 30 sec at 55 °C (primers toxR) or 46 °C (primers BR1), and primer extension for 1 min at 72 °C. After the 35th cycle, the extension step was prolonged for 7 min to complete synthesis of all strands, and then the samples were kept at 4 °C until analysis. Negative and positive controls were included in every experiment. PCR products were detected by gel electrophoresis. Samples (10 µl) of final PCR products were loaded onto 1.5% agarose gels and subjected to electrophoresis in 1 × TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) for 5–10 min at 120 V. The gels were stained with ethidium bromide and gel images were visualised under UV light and were captured by using a gel documentation system (GelDoc, Bio-Rad, Hercules, CA, USA). A 100-bp DNA Ladder (TIB Molbiol, Berlin, Germany) was included in each gel as a molecular size standard.

2.6. *Statistical analysis*

For each sample type (sterile seawater suspension, sterile sediment and marine sediments) the construction of a standard curve was performed in three independent replicates following the procedure described in Section 2.4. A one-way analysis of variance (ANOVA) was performed to compare the efficiency of the Real-Time PCR assay for the different sediment samples. Differences between individual samples were calculated by the Tukey's studentised range (HSD) test. These analyses were performed using the StatistiXL statistical software (Version 1.8). Significance was determined with a value of $\alpha \leq 0.05$.

3. Results and discussion

Real-Time PCR quantification relies on the assumption that amplification efficiencies are the same for unknown samples and in the standard used for calibration. In order to check this assumption for

application of Real-Time PCR for quantifying microbial cells in marine sediments, we determined the efficiency of Real-Time PCR in marine sediment samples of different origin and chemical composition, as well as in standard samples. PCR efficiency in the analysed samples was estimated via the calibration dilution curve and slope calculation (Figure 1), and is reported in Table 2.

Generally, it was found that the Real-Time PCR efficiency in marine sediment samples was significantly lower than those in sterile saline bacterial suspension. It is possible that the presence of inhibitors in the DNA purified from the marine sediment samples resulted in a higher cycle number at which the DNA level is determined (CT value) and flatter amplification curves (Table 2).

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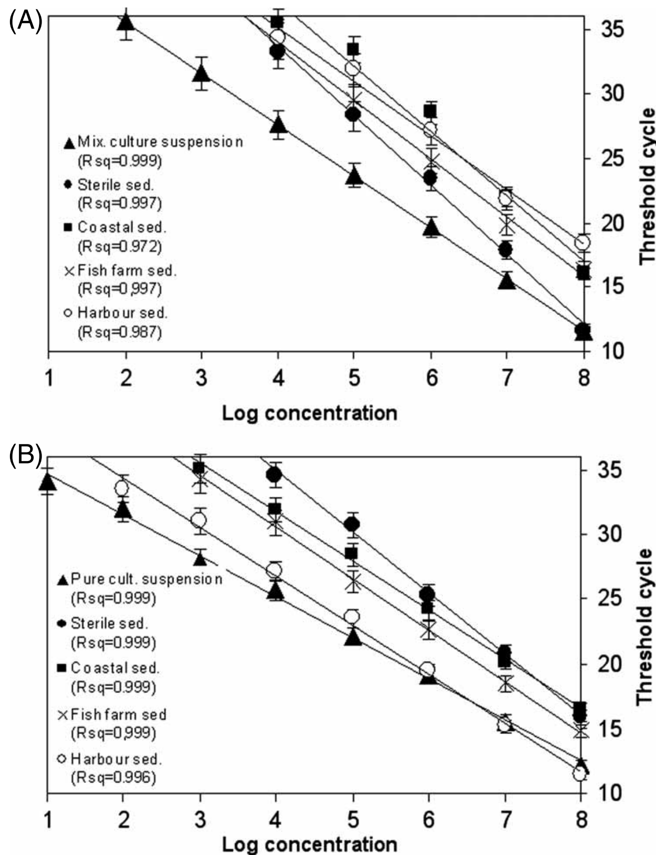

Figure 1. Linear regression line of the standard curve calculated using (A) universal (BR1) primers, and (B) species-specific (*ToxR*) primers in sediment samples using the LightCycler. The threshold cycle (CT) (average of three replicates, coefficient of variation <5) is the cycle number at which the fluorescent signal is first detectable above the background. Cell concentration is set as log numbers with ten-fold difference.

Table 2. PCR efficiency values of sediment samples calculated using the standard curve method with the LightCycler.

Sample	Universal primers	Species-specific primers
Saline water suspension	1.83 ± 0.2	1.98 ± 0.1
Sterile sediment	1.53 ± 0.1	1.60 ± 0.2
Coastal sediment	1.48 ± 0.1	1.83 ± 0.1
Fish-farm sediment	1.66 ± 0.1	1.77 ± 0.1
Harbour sediment	1.65 ± 0.2	1.79 ± 0.1

In contrast, Real-Time PCR efficiency in marine samples was usually significantly higher than that in sterile sediments, as calcinations may enhance the concentration of PCR inhibitors (Table 2). As the efficiency in the sediment samples is lower compared with the one of pure culture suspension, the concentration of the unknown samples will be underestimated. The underestimation factor can be calculated for each cycle number according to the following equation: $Estd^n/Eunk^n$, where n is the number of cycles, Estd is the efficiency of the standard samples and Eunk is the efficiency of the unknown sample. For example, for *V. parahaemolyticus* quantification in coastal sediment samples, at cycle number 30 and with the described difference in efficiency from the pure culture suspension, the concentration would be underestimated 11-fold. This value can be used as a correction factor for an improved quantification of *V. parahaemolyticus* cells in these sediments. In contrast, high amplification efficiency in the sediment samples (as against sterile sediment as the calibrator), led to overestimation of the microbial cell number. For example, there was 56-fold overestimation of *V. parahaemolyticus* cells count at cycle number 30 for coastal sediment samples. Differences in PCR efficiency are caused by different primers, enzymes, reaction settings and by inhibitors in the sample. In particular, the use of different primers and the presence of PCR inhibitors can be considered the primary factors influencing PCR efficiency in our experiment, since other additional factors were kept constant.

Our results indicate that Real-Time PCR efficiency did not differ significantly between marine sediment samples when using species-specific primers, suggesting that PCR inhibitors are active at similar levels in these samples (Tables 3 and 4). The Real-Time PCR efficiency was higher for *toxR* than BR1 and, assuming the effect of inhibitors is the same for both conditions, it is probable that the use of universal primers significantly contributed to this loss of efficiency. In turn, this loss of efficiency may depend on primer specificity and the microbial community composition of the samples. The occurrence of different microbial communities might help to explain the significant changes in PCR efficiency found for the different marine sediment samples when using primers BR1 (Tables 3 and 4).

High PCR efficiency, together with efficiency of DNA extraction (data not shown) would also put constraints on detection limits on a gene-copy or cell-number basis, as shown in Table 5. Generally, when the PCR efficiency was higher, its detection limit was lower (Table 5). The sensitivity of Real-Time PCR to detect microbial cells in marine sediment was generally greater (up to 10 times more sensitive) when compared with the results obtained with conventional PCR (Table 5). This improved sensitivity may be due to the small amount of a sample placed in a thin, narrow glass tube which, in turn, allows for an improved rate of heat conduction where air is used as the medium for temperature change.

Target reaction temperatures can be rapidly achieved, and reaction times can be shortened, probably improving efficiency and thus sensitivity of the Real-Time PCR reaction [13–15].

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Table 3. Output of the one-way ANOVA test used to compare the efficiency of Real-Time PCR for sediment samples using (A) universal (BR1), and (B) species-specific (*ToxR*) primer pairs.

Source	DF	Mean sq.	F	*p*
(A)				
Model	4	0.050	57.30	$<$**0.0001**
Error	10	0.001		
Total	14			
(B)				
Model	4	0.057	64.65	$<$**0.0001**
Error	10	0.001		
Total	14			

Table 4. Output of the Tukey's studentised range (HSD) test used to compare the efficiency of Real-Time PCR for the individual sediment samples using (A) universal (BR1), and (B) species-specific (*ToxR*) primer pairs.

(A)

Contrast	Mean diff.	q	*p*
Mixed cult suspension vs Sterile sed	0.300	17.516	**0.000**
Mixed cult suspension vs Coastal sed	0.323	18.879	**0.000**
Mixed cult suspension vs Fish-farm sed	0.163	9.537	**0.000**
Mixed cult suspension vs Harbour sed	0.167	9.731	**0.000**
Sterile sed vs Coastal sed	0.023	1.362	0.865
Sterile sed vs Fish-farm sed	−0.137	7.980	**0.002**
Sterile sed vs Harbour sed	−0.133	7.785	**0.002**
Coastal sed vs Fish-farm sed	−0.160	9.342	**0.000**
Coastal sed vs Harbour sed	−0.157	9.147	**0.001**
Fish-farm sed vs Harbour sed	0.003	0.195	1.000

(B)

Contrast	Difference	q	*p*
Pure cult suspension vs Sterile sed	0.387	22.576	**0.000**
Pure cult suspension vs Coastal sed	0.173	10.120	**0.000**
Pure cult suspension vs Fish-farm sed	0.220	12.845	**0.000**
Pure cult suspension vs Harbour sed	0.193	11.288	**0.000**
Sterile sed vs Coastal sed	−0.213	12.456	**0.000**
Sterile sed vs Fish-farm sed	−0.167	9.731	**0.000**
Sterile sed vs Harbour sed	−0.193	11.288	**0.000**
Coastal sed vs Fish-farm sed	0.047	2.725	0.364
Coastal sed vs Harbour sed	0.020	1.168	0.917
Fish-farm sed vs Harbour sed	−0.027	1.557	0.802

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Table 5. Detection limits (expressed as log concentration of *V. parahaemolyticus*) of real-time PCR and conventional PCR in standard and marine sediment samples using species-specific (ToxR) primer pairs.

Samples	Real-Time PCR (log concentration)	Conventional PCR (log concentration)
Saline water suspension	1	1
Sterile sediment	4	4
Coastal sediment	3	4
Fish-farm sediment	3	4
Harbour sediment	2	3

4. Conclusion

It is concluded that either the use of pure culture suspension or sterile sediment as standard for Real-Time PCR are not suitable for quantifying microbial cells in marine sediments due to the difference in PCR efficiency.

Removal of PCR inhibitors and improved primer design are factors that may greatly improve the efficiency of Real-Time PCR reaction and its sensitivity for detection and quantification of microbial cells in marine sediment samples. Since in practice it would not be feasible to obtain optimal PCR performance, the reaction efficiency should always be checked when setting a new PCR assay for a specific microbial target and sediment sample. In particular, for quantitative PCR, a standard curve should be constructed and evaluated, taking into account efficiency measurements of both the standard and sediment samples to estimate the effect of difference in efficiency and

calculate correction factors. The evaluation of PCR detection limit, in turn, should be interpreted with care and always in the context of the particular experimental settings.

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References

[1] N.L. Thakur, R. Jain, F. Natalio, B Hamer, A.N. Thakur, and W.E.G. Muller, *Marine molecular biology: An emerging field of biological sciences*, Biotechnol. Adv. 26 (2008), pp. 233–245.
[2] V.L. Wilson, B.C. Tatford, X. Yin, S.C. Rajki, M.M. Walsh, and P. LaRock, *Species-specific detection of hydrocarbon-utilizing bacteria*, J. Microbiol. Methods 39 (1999), pp. 59–78.
[3] G.M. Luna, A. Dell'Anno, and R. Danovaro, *DNA extraction procedure: A critical issue for bacterial diversity assessment in marine sediments*, Environ. Microbiol. 8 (2006), pp. 308–320.
[4] J.L. Kirk, L.A. Beaudette, M. Hart, P. Moutoglis, J.N. Klironomos, H. Lee, and J.T. Trevors, *Methods of studying soil microbial diversity*, J. Microbiol. Methods 58 (2004), pp. 169–188.
[5] J. Zhou, M.A. Bruns, and J.M. Tiedje, *DNA recovery from soils of diverse composition*, Appl. Environ. Microbiol. 62 (1996), pp. 316–322.
[6] L. Vezzulli, M. Moreno, V. Marin, E. Pezzati, M. Bartoli, and M. Fabiano, *Organic waste impact of capture-based Atlantic bluefin tuna aquaculture at an exposed site in the Mediterranean Sea*, Estuar. Coast. Shelf 78 (2008), pp. 369–384.
[7] M. Moreno, L. Vezzulli, V. Marin, P. Laconi, G. Albertelli, and M. Fabiano, *The use of meiofauna diversity as an indication of pollution in harbours*, ICES J. Mar. Sci. 65 (2008), pp. 1428–1435.
[8] L. Clesceri, A. Greenberg, and A. Eaton, *Standard Methods for the Examination of Water and Wastewater*. 20th ed. American Public Health Association, United Book Press, Washington, DC, 1998.
[9] J.E. Hobbie, R.J. Daley, and S. Jasper, *Use of Nuclepore filters for counting bacteria by fluorescence microscopy*, Appl. Environ. Microbiol. 33 (1977), pp. 1225–1228.
[10] S. Nikkari, F.A. Lopez, P.W. Lepp, P.R. Cieslak, S. Ladd-Wilson, D. Passaro, R. Danila, and D.A. Relman, *Broad-range bacterial detection and the analysis of unexplained death and critical illness*, Emerg. Infect. Dis. 8 (2002), pp. 188–194.
[11] Y. Bu Kim, J Okuda, C. Matsumoto, N. Takahashi, S. Hashimoto, and M. Nishibuchi, *Identification of* Vibrio parahaemolyticus *strains at the species level by PCR Targeted to the toxR Gene*, J. Clin. Microbiol. 37 (1999), pp. 1173–1177.
[12] R. Rasmussen, Q*uantification on the LightCycler*, in *Rapid Cycle Real-time PCR*, S. Meuer, C. Wittwer, and K.I. Nakagawara, eds., Springer, New York, 2001, pp. 21–34.
[13] C.T. Wittwer and S.J. Garling, *Rapid cycle DNA amplification: Time and temperature optimization*, BioTechniques 10 (1991), pp. 149–168.
[14] L. Ducharme and J.H. Weis, *Modulation of integrin expression during mast cell differentiation*, Eur. J. Immunol. 22 (1992), pp. 2603–2607.
[15] J.H. Weis, S.S. Tann, and C.T. Wittwer, *Detection of rare mRNA species via quantitative RT-PCR*, Trends Genet. 8 (19992), pp. 263–264.
[16] K.A. Kormas, D.C. Smith, V. Edgcomb, and A. Teske. *Molecular analysis of deep subsurface microbial communities in Nankai Trough sediments (ODP leg 190, Site 1176)*, FEMS Microbiol Ecol. 45 (2003), pp. 115–125.
[17] G. Zheng, C. Wang, H.N. Williams, and S.A. Pineiro, *Development and evaluation of a quantitative real-time PCR assay for the detection of saltwater Bacteriovorax*, Environ. Microbiol. 10 (2008), pp. 2515–2526.

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