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Near real-time, autonomous detection of marine bacterioplankton on a coastal mooring in Monterey Bay, California, using rRNA-targeted DNA probes

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Summary

A sandwich hybridization assay (SHA) was developed to detect 16S rRNAs indicative of phylogenetically distinct groups of marine bacterioplankton in a 96-well plate format as well as low-density arrays printed on a membrane support. The arrays were used in a field-deployable instrument, the Environmental Sample Processor (ESP). The SHA employs a chaotropic buffer for both cell homogenization and hybridization, thus target sequences are captured directly from crude homogenates. Capture probes for seven of nine different bacterioplankton clades examined reacted specifically when challenged with target and non-target 16S rRNAs derived from *in vitro* transcribed 16S rRNA genes cloned from natural samples. Detection limits were between 0.10–1.98 and 4.43– 12.54 fmole ml^{-1} homogenate for the 96-well plate and array SHA respectively. Arrays printed with five of the bacterioplankton-specific capture probes were deployed on the ESP in Monterey Bay, CA, twice in 2006 for a total of 25 days and also utilized in a laboratory time series study. Groups detected included marine alphaproteobacteria, SAR11, marine cyanobacteria, marine group I crenarchaea, and marine group II euryarchaea. To our knowledge this represents the first report of remote *in situ* DNA probe-based detection of marine bacterioplankton.

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Introduction

Application of molecular analytical techniques has become well established in ocean science, yet the vast majority of work typically occurs in a shore-based laboratory after the return of a discrete set of samples. Although many molecular-based analyses are described as 'high-throughput', those methods generally are restricted to laboratory use and typically require a substantial effort to collect and process samples prior to batch mode analyses. Often, acquisition of data in real- or near-real time is impossible or impractical, prohibiting rapid characterization and response to dynamic, stochastic and variable biological phenomena in the environment. Limited sampling opportunities also restrict our ability to document microbial community dynamics. Samples are often obtained in time series surveys or expeditions at intervals that reflect practical and fiscal constraints of ship operations (Karl and Lukas, 1996; Fasham *et al*., 2001). While invaluable, such snapshots may not necessarily capture microbial population dynamics or patterns of gene expression on scales that reflect a continuum of environmental fluctuations.

To overcome this impediment a host of new instruments and next generation observing systems are being constructed. Most biological sensors in use today utilize optical techniques to derive presence, abundance and photosynthetic activities of organisms (Gentien *et al*., 1995; Dubelaar *et al*., 1999; Kirkpatrick *et al*., 2000; Sosik *et al*., 2003; Babin *et al*., 2005; Wang *et al*., 2005), but new molecular analytical-based sensors are also emerging (e.g. Paul *et al*., 2007). Coined 'ecogenomic sensors', these devices aim to provide measures of microbial presence and/or function at the molecular level, in many ways paralleling wet chemistry techniques used in the laboratory (NOPP, 2005). Included in the latter are the Autonomous Microbial Genosensor and Environmental Sample Processor (ESP) (Scholin *et al*., 2001; 2008; Paul *et al*., 2007). Work presented here centres on the ESP.

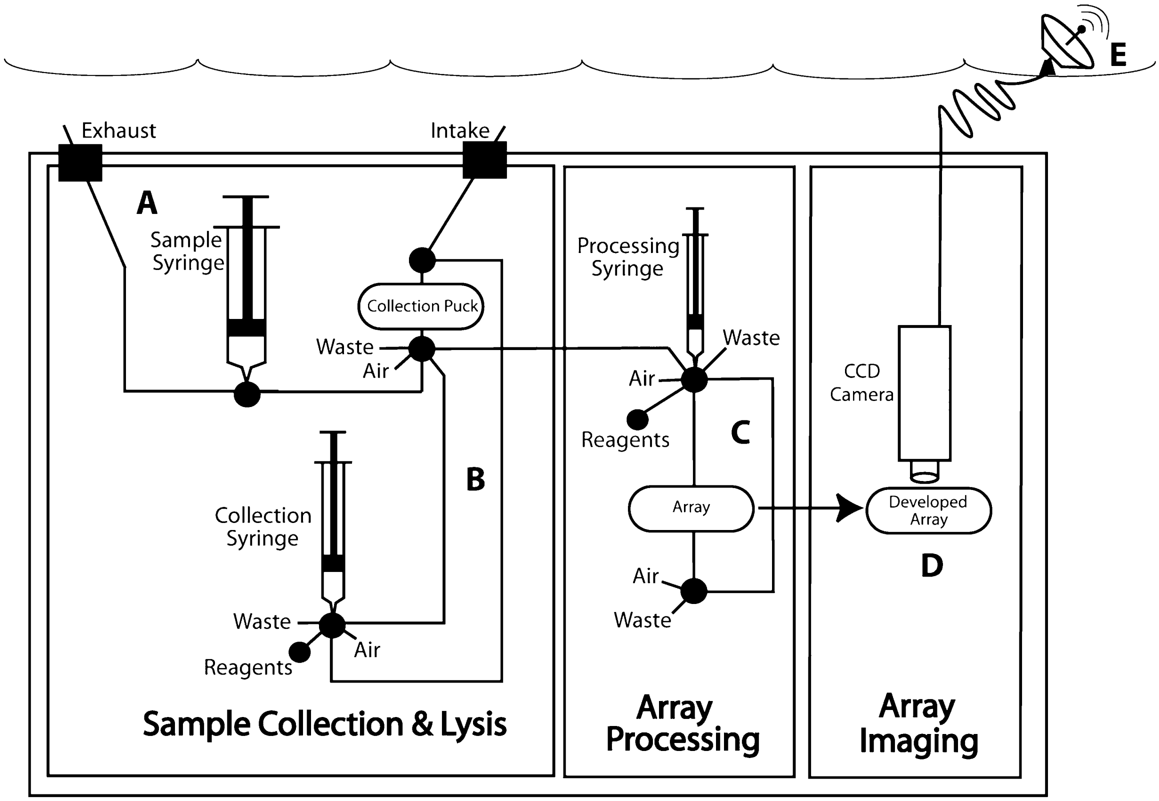

Fig. 1. Schematic diagram of the fluid path within the ESP instrument for sample collection and array processing. Seawater is brought into the instrument and filtered through a puck until the specified volume is reached or until the filter clogged.
A. The collection syringe presents various reagents to the particulates collected on the filter in the puck, resulting in cell lysis followed by dilution of the lysate.
B. The diluted lysate is passed to the processing syringe. The processing syringe delivers the lysate to a puck containing an array; then after incubation cleared to waste. The process is repeated for subsequent reagents.
C. The array is positioned under CCD camera and photographed.
D. The resulting image file is sent ashore via surface radio mooring.
E. Black circles represent valves that make connections between the syringes and puck, reagents, air or waste. See *Experimental procedures* and Greenfield *et al.* (2006) for further details.

The ESP (Fig. 1) is an electromechanical/fluidic system that collects discrete water samples from the ocean subsurface and allows for the application of DNA probe arrays to detect target rRNAs present in a crude homogenate using a sandwich hybridization assay (SHA) methodology (Roman *et al.*, 2005; Greenfield *et al.*, 2006; 2008; Haywood *et al.*, 2007; Jones *et al.*, 2008). The entire automated process of collecting a live sample to broadcast of an imaged array takes approximately 2.5 h. Previous applications of the ESP have focused on detecting harmful algae and invertebrate larvae *in situ* (Goffredi *et al.*, 2006; Greenfield *et al.*, 2006; 2008; Metfies *et al.*, 2006; Scholin *et al.*, 2008; Haywood, 2007; Jones *et al.*, 2008). Here, we demonstrate the utility of the ESP for directly detecting rRNA of marine bacterioplankton (BAC) and illustrate how this device can be used to track community population shifts remotely. A 96-well plate version of the assay was used for probe development, specificity testing and for validating results obtained using the ESP.

Results

Tests of the SHA capture and signal probes

Individual capture probes in combination with the two signal probes (Table 1) were tested against samples containing target and non-targets using the 96-well plate format. Negative reactions returned an average A_{450} of < 0.08, while those greater than 0.110 were scored as positive (Table 2). Probes for marine alphaproteobacteria, SAR11, SAR86 subgroups, and marine cyanobacteria hybridized only to their intended targets. Both the marine group 1 (G1) crenarchaea and marine group 2 (G2) euryarchaeal probes showed specificity for archaea. However, both probes cross-reacted with rRNA transcript from marine group III (G3) euryarchaeota (pcr clone 1#6F, V. Orphan, unpublished). Although not specific for their intended archeal clades, the G3 euryarchaeota are not routinely recovered in libraries constructed from Monterey Bay BAC (Beja *et al.*, 2000; Suzuki *et al.*, 2004), and more

Table 1. Sandwich hybridization capture and signal probes used for the ESP array and 96-well plate formats.

Probe name	Probe type	Target clade	Sequence (5′–3′)[a]	Comments	Reference
CF319a	Capture	*Cytophaga-Flavobacteria*	Biotin-(C9)3-TGGTCCGTGTCTCAGTAC	Cross reacts with many groups; not used in study	Manz *et al.* (1996)
Malph-1_488	Capture	Marine alphaproteobacteria	Biotin-(C9)3-GCCGGGGTTTCTTTACCA	Gel purified	Gonzalez *et al.* (1996) Gonzalez and Moran (1997)
Picophyto496	Capture	Marine cyanobacteria	Biotin-(C9)3-GGCACGGAATTAGCCGWGGCTTA	Weakly targets *Synnechococcus* and *Prochlorococcus*	Modified from Suzuki *et al.* (2000)
SAR11_441	Capture	SAR11	Biotin-(C9)3-TACAGTCATTWTCTTCCCCKACGAAGAG		Modified from Morris *et al.* (2002)
SAR86i–ii_470	Capture	SAR86 subgroups I and II	Biotin-(C9)3-GCAGGTAACATCASGGWTATAGG		This study
SAR86iii_470	Capture	SAR86 subgroup III	Biotin-(C9)3-GATGGTAATGTCACGGTTATTGG		This study
MarineCren_554	Capture	Marine G1 crenarchaea	Biotin-(C9)3-GATGCTTTAGGCCCAATAATCAMCCT	Cross reacts with marine G3 euryarchaea	Modified from Massana *et al.* (1997)
MarineEuryII_547	Capture	Marine G2 crenarchaea	Biotin-(C9)3-TTAGGCCCAATAAAAKTGACKACCACT	Cross reacts with marine G3 euryarchaea	Modified from Suzuki *et al.* (2000)
MarineEuryIII_682b	Capture	Marine G3 crenarchaea	Biotin-(C9)3-GGATTACAACATTTCACCGCTCCCC	Cross reacts with Raphidophytes, not used in study	This study
Eub338	Signal	Bacteria	Dig-C9-GCWGCCWCCCGTAGGWGT-C9-Dig	Includes *Planctomycetales* and *Verrucomicrobia*	Daims *et al.* (2006)
Univ519ab	Signal	Universal	Dig-C9-TTACCGCGGCKGCTGGCAC-C9-Dig	Universal but biased towards Archaea and Bacteria	Modified from Suzuki *et al.* (2000)
AlexComp	Capture		Biotin-(C9)3-GGGAAATATGAAAAGGACTTTGAA	Positive control on arrays	Greenfield *et al.* (2006)
Alex-alt-S	Signal		Dig-C9-GTCCTTTTCATATTTCCCTCATGG-C9-Dig	Complimentary to AlexComp	Greenfield *et al.* (2006)

a. Underlined base of signal probe indicate the position of the internal dioxygenin (Dig); (C9)3 = 3 C9 spacers.

specifically have not been recovered from the ESP deployment site (C. Preston, unpublished data). Thus, both were advanced to field trials. The probe for G3 euryarchaea failed to react with its intended target but did react with chloroplast rRNA (not shown). The capture probe for *Cytophaga-Flavobacteria* cross-reacted with seven of the eight non-target transcripts tested. Consequently, probes targeting G3 euryarchaea and *Cytophaga-Flavobacteria* were not considered further.

To assess probe specificity using the ESP, homogenates as used in the 96-well plate assay were provided directly to the instrument, which then developed and imaged an array. Background signal from non-spotted regions on the arrays averaged 1814 ± 128 counts. All non-targets and negative controls gave reaction intensities below array background except for marine cyanobacteria where counts where consistently 1000 counts above background (Table 2). Control probes reacted successfully on every array. Targets positive in the 96-well plate assay also reacted successfully in the array format. Probe spots exceeding three standard deviations above overall array background were considered positive for marine alphaproteobacteria, SAR11, SAR86 subgroup i–ii, SAR86 subgroup iii, G1 crenarchaea and G2 euryarchaea. A positive reaction for the marine cyanobacteria probe exceeded that definition by an additional 1000 counts.

No cross reactivity was observed when eight non-target bacterial transcripts (each at 50 ng ml^{-1} lysate) and lysed *Escherichia coli* cells (1×10^{8} cells ml^{-1} lysate) were combined. When all target and non-target transcripts were mixed, signals from the marine alphaproteobacteria, SAR11, SAR86i–ii and SAR86iii capture probes were within 10% of the pure transcript. In contrast, a lower signal (82–83% of the pure signal) was observed in the complex mixture for both archaeal probes (Table 2).

Standard curves were determined by diluting the six target transcripts in a constant background of eight non-target bacterial transcripts each at 50 ng ml^{-1} lysate (Fig. 2). For every probe tested, the 96-well plate format was more sensitive (Fig. 2, Table 2). An A_{450} value of at least 0.25 was required to obtain a positive signal on the array. The lower limit of detection for each capture probe was in the low fmol ml^{-1} range for both assay formats (Table 2). The reproducibility of the arrays was assessed using samples that contained six target transcripts (each at 12.5 ng ml^{-1} lysate) and eight negative transcripts (each at 50 ng ml^{-1} lysate). Three replicate arrays had a coefficient of variation below 20%. Similar results and signal intensities were obtained after combining the transcripts in a background of 10^{8} cells ml^{-1} *E. coli*. Although the signal was reproducible within a single batch of arrays, the absolute signal varied when the same samples

Table 2. The specificity of the various capture probe in the 96 well and array format SHA using target and non-target rRNAs.

		Capture probe[b]							
Transcript/RNA source[a]	Clade	CF319a	Malph-1_488	SAR11 441	SAR86i–ii 470	SAR86iii 470	MarCren554	MarEury547	Picophyto496
Lysis buffer	NA	0.072 ± 0.002 NVS	0.057 ± 0.001 NVS	0.065 ± 0.007 NVS	0.061 ± 0.003 NVS	0.061 ± 0.002 NVS	0.065 ± 0.007 NVS	0.076 ± 0.009 NVS	0.079 ± 0.000 2458 ± 105
M0_P4A7	*Cytophaga*	**3.5** **10005 ± 457**	0.050 ± 0.002 NVS	0.051 ± 0.003 NEG	0.059 ± 0.002 NVS	0.058 ± 0.008 NVS	0.075 ± 0.010 NEG	0.070 ± 0.009 NEG	0.069 ± 0.015 2666 ± 202
M0_P1G4	Marine alphaproteobacteria	**0.102 ± 0.004**	**2.812 ± 0.052** **8914 ± 688**	0.064 ± 0.007 NVS	0.069 ± 0.003 NVS	0.074 ± 0.010 NVS	0.052 ± 0.007 NVS	0.083 ± 0.009 NVS	0.071 ± 0.006 2212 ± 67
M0_P1D2	SAR11	**0.351 ± 0.008**	0.050 ± 0.001	**1.738 ± 0.066** **10781 ± 748**	0.051 ± 0.001 NEG	0.061 ± 0.006 NEG	0.063 ± 0.003 2233 ± 134	0.059 ± 0.003 NEG	0.084 ± 0.003 2293 ± 99
M0_P1H12	SAR86 subgroup II	**0.170 ± 0.008**	0.050 ± 0.001 NVS	0.068 ± 0.003 NVS	**0.729 ± 0.010** **5028 ± 246**	0.063 ± 0.007 NVS	0.057 ± 0.009 NVS	0.057 ± 0.002 NVS	0.075 ± 0.004 2476 ± 109
M0_P2C4	SAR86 subgroup III	**0.170 ± 0.009**	0.055 ± 0.002 NVS	0.067 ± 0.010 2183 ± 154	0.060 ± 0.003 NVS	**3.5** **21828 ± 797**	0.053 ± 0.001 NVS	0.055 ± 0.001 NVS	0.067 ± 0.000 2593 ± 160
M0_050405a8	Marine G1 crenarchaea	**0.458 ± 0.017**	0.054 ± 0.001 NVS	0.093 ± 0.001 NVS	0.066 ± 0.013 NVS	0.070 ± 0.001 NVS	**3.5** **19903 ± 379**	0.065 ± 0.001 2400 ± 127	0.060 ± 0.003 2442 ± 78
M0_050405A5	Marine G2 euryarchaea	0.056 ± 0.053	0.056 ± 0.001 NEG	0.056 ± 0.000 NVS	0.066 ± 0.01 NVS	0.064 ± 0.006 NVS	0.059 ± 0.005 NVS	**3.5** **15816 ± 1122**	0.067 ± 0.001 2501 ± 437
Synecococcus sp. CCMP 1334[c]	Marine cyanobacteria	**0.546 ± 0.008**	0.073 ± 0.007 NVS	0.067 ± 0.004 NVS	0.056 ± 0.002 NVS	0.070 ± 0.012 NVS	0.058 ± 0.003 NVS	0.057 ± 0.002 NVS	**0.208 ± 0.018** **3966 ± 292**
1#6F	Marine G3 euryarchaea	ND	0.056 ± 0.001	0.057 ± 0.001	0.056 ± 0.002	0.055 ± 0.002	**0.523 ± 0.019**	**0.190 ± 0.016**	0.060 ± 0.002
M0_P1B1	ARCTICBD96-19	ND	0.053 ± 0.004 NVS	0.052 ± 0.002 NVS	0.062 ± 0.002 NVS	0.057 ± 0.001 NVS	0.057 ± 0.013 NVS	0.073 ± 0.01 NVS	0.062 ± 0.008 2098 ± 27
M0_P3C11	*Verrucomicrobiales*	ND	0.095 ± 0.017 NVS	0.092 ± 0.014 NVS	0.057 ± 0.002 NVS	0.058 ± 0.001 NVS	0.067 ± 0.007 NVS	0.062 ± 0.004 NVS	0.075 ± 0.005 2929 ± 85
M0_P3A6	HTCC230	ND	0.055 ± 0.001 NVS	0.054 ± 0.001 NVS	0.056 ± 0.001 NVS	0.063 ± 0.004 NVS	0.060 ± 0.007 NVS	0.058 ± 0.002 NVS	0.058 ± 0.003 2172 ± 38
M0_P3B5	NAC11-7	ND	0.057 ± 0.008 NVS	0.051 ± 0.001 NVS	0.060 ± 0.005 NVS	0.062 ± 0.006 NVS	0.061 ± 0.003 NVS	0.060 ± 0.004 NVS	0.055 ± 0.005 × NVS
M0_P3D12	0M60	ND	0.060 ± 0.005 NVS	0.061 ± 0.004 NVS	0.057 ± 0.001 NVS	0.061 ± 0.003 NVS	0.069 ± 0.013 NVS	0.058 ± 0.003 NVS	0.068 ± 0.002 2462 ± 71
M0_P3C12	EB036A07	ND	0.057 ± 0.006 NVS	0.049 ± 0.002 NVS	0.048 ± 0.001 NVS	0.058 ± 0.006 NVS	0.051 ± 0.005 NVS	0.049 ± 0 NVS	0.049 ± 0.002 2489 ± 63
M0_P3A5	KTC1119	ND	0.048 ± 0.001 NVS	0.054 ± 0.007 NVS	0.052 ± 0.004 NVS	0.056 ± 0.008 NVS	0.052 ± 0.002 NVS	0.048 ± 0 NVS	0.053 ± 0.001 2394 ± 46
M0_P1A8	SAR156	ND	0.053 ± 0.007 NVS	0.047 ± 0.001 NVS	0.063 ± 0.003 NVS	0.062 ± 0.001 NVS	0.061 ± 0.007 NVS	0.062 ± 0.007 NVS	0.052 ± 0.004 NVS
E. coli[c]	Enterobacteriaceae	ND	0.056 ± 0.001 NVS	0.054 ± 0 NVS	0.054 ± 0.002 NVS	0.060 ± 0.001 NVS	0.067 ± 0 NVS	0.055 ± 0.004 NVS	0.054 ± 0 2288 ± 74
Non-target transcripts + *E. coli*[d]		ND	0.053 ± 0.003	0.058 ± 0.001	0.053 ± 0.004	0.052 ± 0.001	0.050 ± 0.002	0.058 ± 0.008	0.058 ± 0.013
Target & non-target transcripts[e]		ND	**2.979 ± 0.030**	**1.642 ± 0.044**	**0.662 ± 0.013**	**1.803 ± 0.043**	**1.030 ± 0.033**	**1.332 ± 0.029**	ND
Target transcript[e]		ND	**2.812 ± 0.052**	**1.738 ± 0.066**	**0.729 ± 0.010**	**1.714 ± 0.098**	**1.238 ± 0.057**	**1.628 ± 0.092**	ND
Limit of detection[f] (fmoles ml^{-1} lysate)		NA	0.22 7.31	1.98 4.59	0.10 12.54	0.11 3.66	0.18 5.61	0.37 4.43	NA

a. Transcripts and native RNA at 50 ng ml^{-1} lysate (approximately $2.1–9.7 \times 10^{10}$ copies ml^{-1}) for specificity tests except for the array using *Synecchococcus* RNA (150 ng ml^{-1} lysate).
b. Top value is the A_{450} (standard deviation) from 96 well plate assay; bottom comment from ESP developed array as follows: NVS, no visible spot; NEG, less than three standard deviations above background; raw CCD pixel intensity (standard deviation); ND, not determined. Bolded values indicate positive hybridization signals; grey-shaded boxes indicate probe-target matches.
c. RNA isolated from culture.
d. Eight non-target bacterial transcripts (each at 50 ng ml^{-1}) with lysed *E. coli* cells (1×10^8 cells ml^{-1} lysate).
e. Eight non-target bacterial transcripts at 50 ng ml^{-1} and target transcript concentration as follows 50 ng ml^{-1} lysate for M0_P1G4, M0_P1H12, M0_P1D2, 25 ng ml^{-1} lysate for M0_P2C4, M0_050405A5 and 12.5 ml^{-1} lysate for M0_050405a8.
f. Limit of detection determined from Fig. 2. Positive signal > A_{450} = 0.11 or 2500 CCD counts; NA, not applicable.

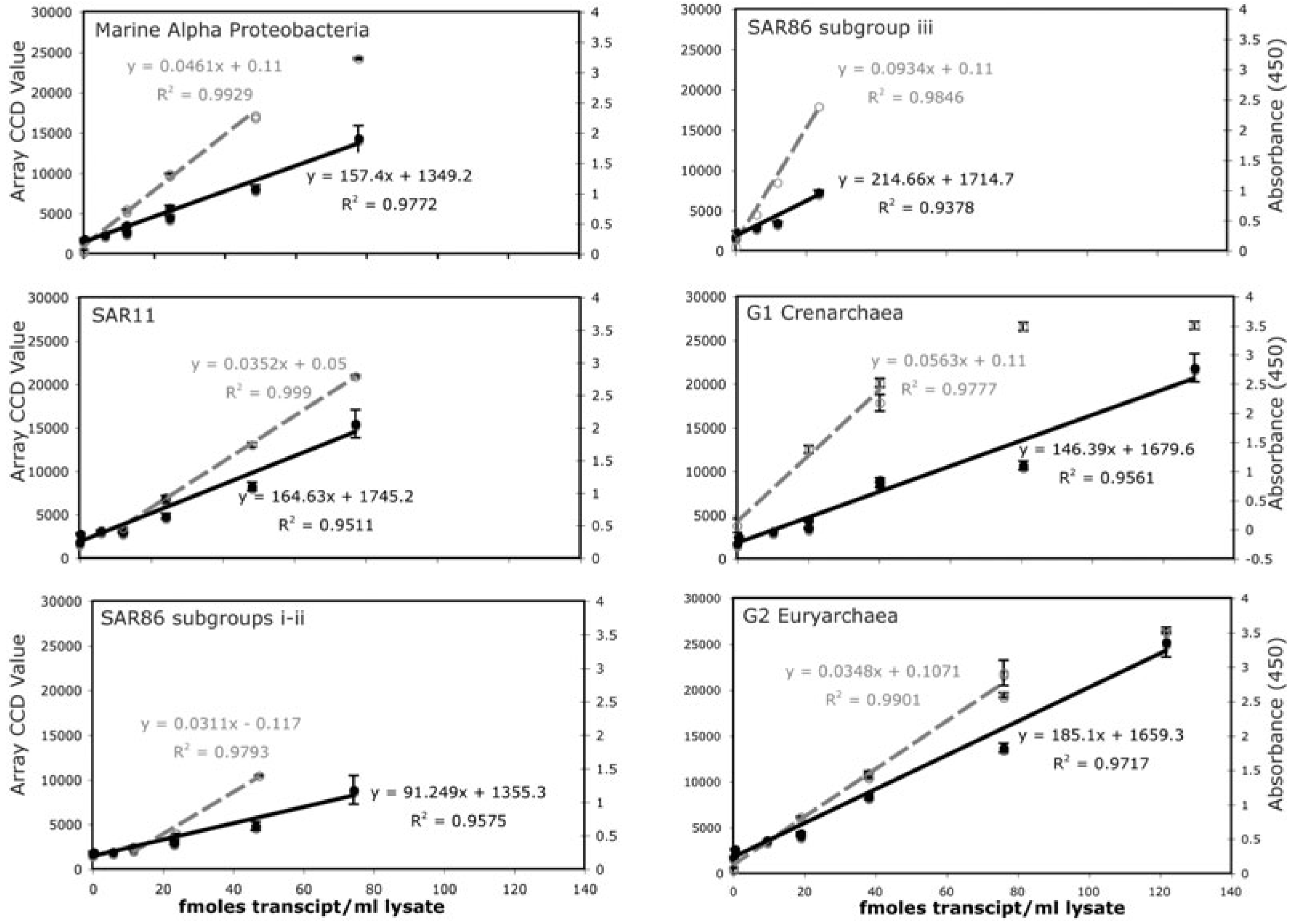

Fig. 2. Standard curves showing the response of the SHA for different targets using the 96-well plate (open circles, grey dashed line) and ESP array formats (black circles, solid black line) for the targeted bacterioplankton groups. The concentration of target transcripts was varied in a constant background of non-target transcripts (see *Experimental procedures*). Probes are listed in Table 1.

were run using different batches of arrays (data not shown).

Samples prepared by the ESP were similar to if not more reactive than those prepared manually. For example, native seawater samples collected, lysed and diluted for hybridization by the ESP ($n = 1$) were compared with the same sample collected, lysed and diluted manually ($n = 3$). Samples processed by the ESP for G2 euryarchaea yielded an A_{450} of 3.128 ± 0.099, whereas those processed manually had A_{450} values of 2.911 ± 0.171, 3.033 ± 0.060 and 2.575 ± 0.028. Experiments targeting marine alphaproteobacteria yielded similar results: 0.309 ± 0.020 from the ESP-processed sample versus 0.217 ± 0.007, 0.230 ± 0.005 and 0.214 ± 0.099 for samples processed manually. In addition, positive SHA values indicated the presence of rRNA rather than rRNA genes as both untreated and DNase-treated samples resulted in positive reactions (0.437 ± 0.004 and 0.454 ± 0.010 respectively), and samples treated with RNase produced no signal (0.084 ± 0.008).

Environmental Sample Processor field deployments

Arrays fielded on the ESP were printed with capture probes for marine alphaproteobacteria, SAR11, marine cyanobacteria, G1 crenarchaea and G2 euryarchaea (Fig. 3A). Prior to deployment, the ESP was provided filtered sterilized seawater and native surface seawater for negative and positive controls respectively (e.g. Fig. 3B and C). For the positive test, the ESP detected the presence of marine alphaproteobacteria and G2 euryarchaea (Fig. 3C, Table 3). The 96-well plate SHA using the same sample and sample volume confirmed the presence of marine alphaproteobacteria and G2 euryarchaea. SAR11 and marine cyanobacteria were also present but below the detection limit of the arrays. G1 crenarchaea were not detected in either SHA format.

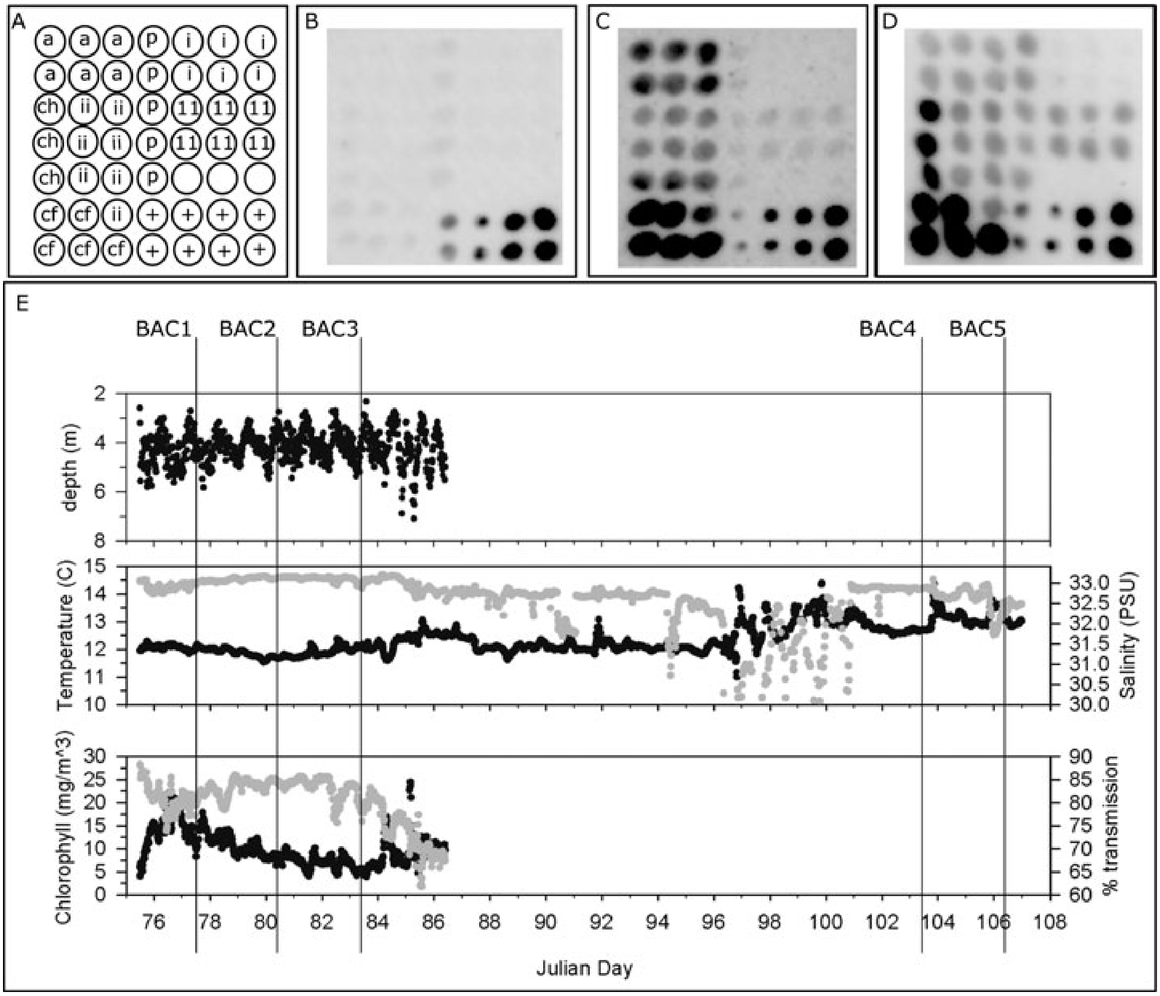

Fig. 3. Custom 25 mm diameter probe arrays printed for use with the ESP (A–D) and environmental conditions during the March and April ESP field deployments (E). The location of capture probes on DNA arrays for deployments (A) are as follows: *Cytophaga-Flavobacteria* (cf), marine alphaproteobacteria (a), SAR11 (11), G1 crenarchaea (i), G2 euryarchaea (ii), G3 euryarchaea (ch), and '+' (Positive control, AlexComp) (Table 1). Empty white circles indicate non-printed areas. Arrays represent a filtered seawater negative control (B), a pre-deployment positive control using 225 ml surface seawater (C), and the BAC5 array (JD106) from the deployed ESP (D). Results from the *Cytophaga-Flavobacteria* and marine G3 euryarchaea probes are not considered due to their cross reactivity (see Table 2). Depth, temperature and salinity (black and grey symbols respectively), and chlorophyll and percent transmission (black and grey symbols respectively) recorded during the field deployments. Contextual data up to JD86 was obtained from the CTD mounted on the ESP, and from JD86–108 from the surface water CTD on the CIMT/M0 mooring. Horizontal lines indicate timing of the BAC arrays 1–5. No contextual data was available for BAC6 and BAC7 arrays. Results from BAC1–7 arrays are presented in Table 3.

All BAC arrays ($n = 7$) were processed and imaged successfully during the deployments and were similar in appearance to those processed in the lab (e.g. Fig. 3C and D). Non-probe background values for the arrays during the March deployment were slightly lower (average 2153 ± 345 counts) than in April (average 2497 ± 342 counts). These values are similar to the native sample positive controls run prior to the deployment and 200–600 CCD counts higher than backgrounds observed during the transcripts tests. Volumes sampled by the ESP during the deployments ranged from 225 to 600 ml (Table 3). The depth of the sample intake valve during the first deployment averaged 4.2 m (range 2.3–7.1 m) and the chlorophyll concentration ranged from 3.8 to 24.36 mg m^{-3}. There was a general warming trend during the two deployments (Fig. 3E).

Positive reactions were observed for all capture probes on at least one array developed *in situ* (Table 3). Arrays indicated the presence and persistence of the marine alphaproteobacteria, SAR11 and G2 euryarchaea during the two deployments. The marine cyanobacteria probe was positive from Julian day 83 (JD83) through the end of the deployment, while that for G1 crenarchaea weakly positive on JD83 and JD103. The sample collected on

Table 3. Summary of control and 2006 deployed ESP arrays for the detection of various bacterioplankton groups.[a]

Sample	Sample date	Sample volume (ml)	Malph	SAR11	Marine cyanobacteria	G1 crenarchaea	G2 euryarchaea	Background
Negative	NA	50	NVS	NVS	NEG	NVS	NVS	1653 ± 67
Monterey Wharf	NA	225	6106 ± 420	NEG	NEG	NVS	4284 ± 218	2346 ± 95
BAC1	JD77.6	400	5388 ± 347	4502 ± 176	NEG	NEG	6276 ± 194	2550 ± 343
BAC2	JD80.4	275	6297 ± 501	4119 ± 121	NEG	NEG	4786 ± 146	1933 ± 130
BAC3	JD83.4	275	7733 ± 764	4299 ± 164	4407 ± 339	2870 ± 101	5805 ± 315	1974 ± 184
BAC4	JD103.4	575	4481 ± 254	5063 ± 152	5640 ± 405	2749 ± 104	4887 ± 246	2105 ± 158
BAC5	JD106.4	600	5600 ± 469	6146 ± 396	6158 ± 382	NEG	7348 ± 221	2470 ± 290
BAC6	JD109.4	225	4984 ± 131	4596 ± 308	4846 ± 550	NEG	4634 ± 332	2470 ± 290
BAC7	JD112.4	575	6658 ± 549	6270 ± 341	5949 ± 204	NEG	6519 ± 483	2940 ± 304

a. For example of array image see Fig. 2. Julian day (JD) in 2006. See Fig. 3 for environmental conditions for BAC1–5. NA, not applicable.

JD83 showed the presence of all five targeted BAC groups.

In addition to samples collected by the ESP, five samples were also collected manually from a boat near the ESP mooring and were analysed using the 96-well plate assay (Fig. 4, open symbols). Of the five samples obtained, two corresponded with ESP BAC array runs (JD83 and 103). Those two samples confirmed the presence of groups detected on the arrays except for the weak G1 crenarchaea signal on JD103. In that case, the concentration of the sample used in the ESP exceeded that of the 96-well plate assay (287 ml seawater ml^{-1} lysate versus 100 ml seawater ml^{-1} lysate, respectively).

Laboratory time series

Seven native samples from 5 m at station M0 in Monterey Bay were collected between JD73 and JD270, 2006, and were analysed using the 96-well plate and ESP SHA, and RT-qPCR. Samples obtained represented microbial communities associated with varying environmental conditions (Table 4). Arrays included the same groups used during field deployments and both SAR86-targeted capture probes. The same batch of arrays and reagents was used for processing all samples. Using the 96-well plate assay, positive signals were observed on each day for marine alphaproteobacteria, SAR11 and G2 euryarchaea. Assays for G1 crenarchaea, marine cyanobacteria, SAR86i–ii, and SAR86iii were below the limit of detection of the assay in at least one sample and never exceeded an A_{450} of 0.5 (Fig. 4). SAR11 and marine cyanobacteria had a biomodal distribution with peaks in abundance in the spring and late summer. In contrast, marine alphaproteobacteria and the SAR86 subgroup i–ii peaked in late summer. G1 crenarchaea was detected only once during an upwelling event (low temperature, low chlorophyll seawater; Table 4).

Sandwich hybridization assay capture probes for SAR11, G1 crenarchaea and G2 euryarchaea target a similar phylogenetic clade and are located near the primers and Taqman probe used in RT-qPCR analyses, so are well matched for comparison. For those groups the SHA and PCR analyses revealed similar trends (Fig. 4B–D). In contrast, the phylogenetic affinities of SHA capture probes for marine alphaproteobacteria, SAR86 and marine cyanobacteria differ significantly from the RT-qPCR assays. Consequently direct comparison of results of those two assays is problematic, but the RT-qPCR assays did confirm the presence of those targets as detected by SHA (data not shown). With two exceptions, the ESP arrays revealed the presence of the same BAC groups as were detected using the 96-well

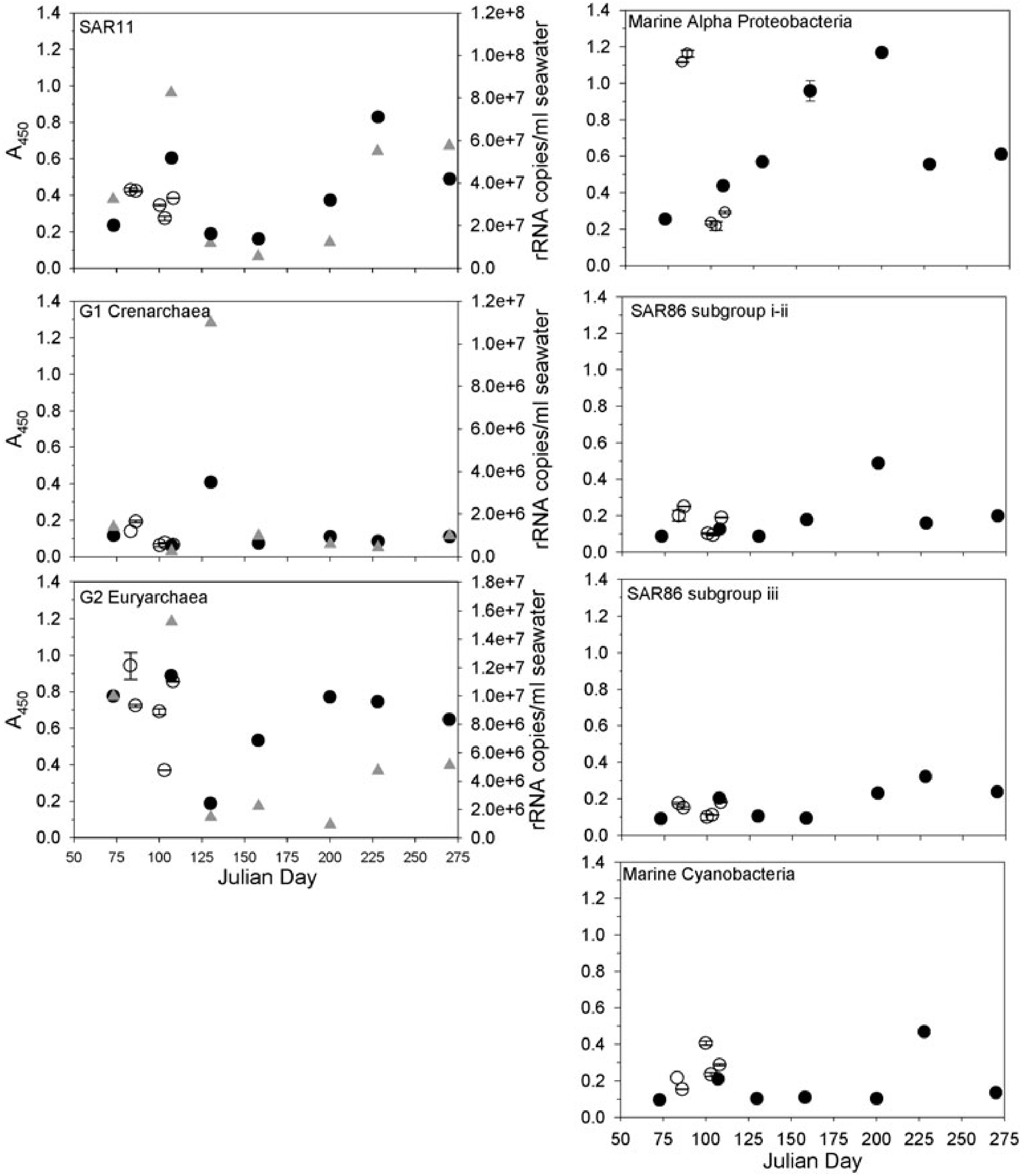

Fig. 4. Results from the 96-well plate SHA of field samples (100 ml seawater ml^{-1} lysate) collected from Station M0 during monthly sampling (black circles) or during the March and April ESP deployments (open circles). In addition for SAR11, G1 crenarchaea and G2 euryarchaea, the rRNA copy number ml^{-1} seawater (grey triangles) was also estimated using RT-qPCR from extracts made from the same lysate used in SHA. Environmental conditions for the monthly samples are presented in Table 4 and for the ESP deployments in Fig. 2.

assay, so long as the A_{450} was above 0.25 (Fig. 5). Relative to the plate assay, the sample from JD200 returned a false positive for marine GI crenarchaea and a false negative for G2 euryarchaea.

Discussion

A major advantage of the SHA methodology is that it allows for simultaneous detection of a variety of target

Table 4. Environmental conditions of monthly M0 5 m seawater samples.

Julian day (2006)	Temperature (°C)	Salinity (PSU)	Transmission (%)	Chlorophyll (mg m^{-3})
73	11.9	33.12	84.6	0.8
107	12.9	32.47	76.2	2.0
130	10.5	33.59	83.4	2.5
158	11.4	33.79	50.7	26.3
200	13.2	33.80	68.7	9.2
229	14.3	33.55	75.7	5.0
270	14.0	33.50	82.0	2.4

sequences in near real-time without invoking nucleic acid purification or amplification. In this investigation, we tested whether this technique was extensible to nine common groups of marine bacterioplankton. Of those, five assays targeting different groups of bacteria were shown to be specific and two assays for archaea were deemed useful (with the caveat of potentially cross reacting with G3 euryarchaea) based on *in vitro* transcribed 16S rRNA genes and extracted RNA. Using those probes we proved that direct detection of rRNA indicative of marine alphaproteobacteria, SAR11, SAR86 subgroups i–ii and subgroup iii, marine cyanobacteria, G1 crenarchaea and G2 euryarchaea, collected from natural seawater, is feasible. Further, we demonstrated for the first time that such molecular signatures can be assessed remotely, *in situ*, using probe array technology and the ESP. Deployment of the instrument coincided with a relatively stable period, so the observed shifts in microbial populations were minimal. However, the time series from samples collected during monthly CTD casts (Fig. 4) clearly shows that the SHA technique reveals major shifts in microbial rRNA community structure that are in keeping with changing environmental conditions. Thus, the ESP thus offers a novel means for accessing the ocean and microbes that inhabit it.

Direct detection of marine BAC using SHA

Previous studies using SHA to detect RNA have primarily utilized NaCl-based buffers, with or without formamide, with capture probes attached to either glass slide arrays (Small *et al*., 2001; Chandler *et al*., 2003; Chandler and Jarrell, 2004) or magnetic beads (Spiro *et al*., 2000; Rowan *et al*., 2005). Direct detection of rRNA genes has also been accomplished using suspension arrays and a Luminex flow cytometer (Ellison and Burton, 2005). The majority of these assays required target purification and/or long incubations at high hybridization temperatures. Here, we used a GuSCN-based reagent that is effective at disrupting cells, inactivates nucleases and permits direct, specific hybridization at much lower temperatures compared with NaCl-based buffers (Van Ness and Chen, 1991). Detection limits using SHA in either format are similar to other microarray-based detection techniques (Small *et al*., 2001; El Fantroussi *et al*., 2003; Peplies *et al*., 2004).

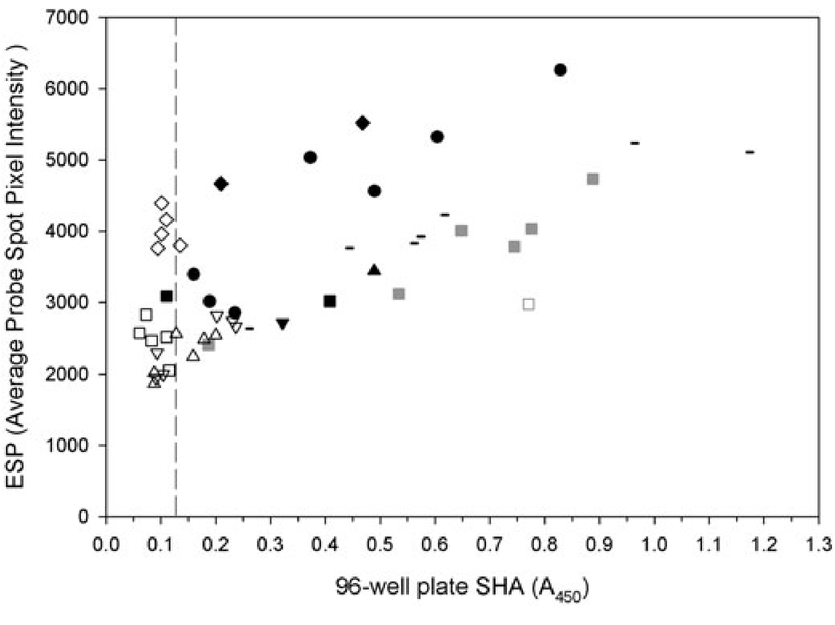

Fig. 5. Comparison of results obtained using the ESP array and 96-well plate SHAs on matched field samples. Array signals above background + 3SD (solid symbols) and below (open symbol) are shown for marine alphaproteobacteria (dash), SAR11 (circle), SAR86i–ii (up triangle), SAR86iii (down triangle), marine cyanobacteria (diamond), G1 crenarchaea (black squares) and G2 euryarchaea (dark grey squares). Dashed line indicates the limit of detection ($A_{450} < 0.110$) for the 96-well plate assay. Linear regressions for capture probes with least two field samples with an $A_{450} > 0.25$ were as follows: marine alphaproteobacteria, $y = 2674x + 2364$ ($r^2 = 0.891$); S11, $y = 4901x + 2332$ ($r^2 = 0.867$); and G2 euryarchaea, $y = 2681x + 1893$ ($r^2 = 0.630$).

As theoretical predictions of probe specificity and Tm based on sequence homology often differ from laboratory tests (Pozhitkov *et al*., 2006; Haywood *et al*., 2007), probes were tested experimentally. Capture-target-signal combinations that worked well in the 96-well plate format, also worked well using the array format. Reaction conditions reported here were acceptable (i.e. specific), but were not necessarily optimal for any given probe. Each probe had its own characteristic reactivity towards the target (Fig. 2, Table 2). Thus, similar signal intensities obtained using different capture probes do not indicate a similar abundance of the target rRNA. Moreover, recorded signal variations could reflect changes in the abundance of organisms or cellular rRNA content (DeLong *et al*., 1989; Smith *et al*., 1992; Kemp *et al*., 1993), volume of seawater collected, or some combination thereof. Non-specific hybridizations cannot be ruled out conclusively when considering results of tests that employed native samples. Nevertheless, signals reported here are reasonable, as none of the field samples gave results that exceed values tested in controlled laboratory tests using dominant non-target clades. In addition, the distribution and population dynamics of the target organisms were similar to those observed previously (C. Preston, M. Suzuki and E. DeLong, unpublished data). Thus, the assays appear to reflect natural variations in the native rRNA pool.

Environmental Sample Processor performance and validation

We interpreted positive signals on the arrays as indicating the presence or absence of targeted clades, and proof that the concepts underlying the detection methodology are promising and worthy of further investigation. No attempt was made to normalize arrays to account for variations that arise outside of the actual abundance of the targeted sequence or to estimate relative changes in rRNA abundance of the various clades. Presently, abundance estimates for the BAC groups would have to rely on standard curves generated by *in vitro* transcribed rRNA (Fig. 2) rather than native rRNA. In the future, the performance of the SHA will be characterized using cultures when available as well as natural samples. Given current protocols, we found that the ESP arrays were susceptible to variable and sometimes elevated background with field samples collected at different times, whereas the 96-well plate SHA was very stable and appears insensitive to sample matrix. The reasons underlying this difference are under investigation.

The best means of validating the performance of the ESP was to apply different analytical techniques to a large volume of lysate created from replicate samples. Attempts to confirm SHA results using RT-qPCR were successful for those assays whose probes targeted near identical clades. Clearly, matching specificity of probes for a variety of assays including SHA, RT-qPCR and qPCR assays will improve opportunities for comparing performance of different detection methods. However, discrepancies even with matched target clades are possible as the two approaches have their own biases and limitations. Regardless of the approach, the use and development of the sample archival function of the ESP will enhance options for assessing quality of data obtained in real-time using the SHA arrays.

Conclusions

The 96-well and ESP array SHA formats accurately reflected the presence of various bacterial and archaeal clades in both laboratory and field settings. To our knowledge this represents the first report of remote *in situ* DNA probe-based detection of marine BAC. The requirements associated with obtaining and processing a sample make data rates from the ESP slow compared with other sensors that yield almost nearly continuous chemical and physical measurements (e.g. CTD or optical sensors). Such high-frequency measurements can be incorporated into an event detection capability to trigger sampling events.

As with any methodology there are both advantages and limitations. The SHA method is simple, employs reagents that are stable for extended periods at temperatures 4–25°C, and is highly amenable to automation. However, when there is a need to detect low copy number targets, then more demanding methodologies such as those that use nucleic acid purification and amplification may be required (Suzuki *et al*., 2001; Casper *et al*., 2004; Short and Zehr, 2005). The choice of methodology will depend on the specific target analytes, detecting requirements, and questions being addressed.

Experimental procedures

In vitro *T7 Transcription of 16S rRNAs*

Selected 16S bacterial or archaeal rRNA genes were cloned from DNA extracted from seawater samples collected in 2004 from station M0 (36.8342 N, 121.898 W), and transcribed *in vitro* to produce synthetic rRNA for probe specificity studies. Cloned 16S rRNA genes were amplified using M13 forward and reverse primers using the 1× reaction buffer, 0.2 μM dNTP, 3 mM $MgCl_2$, 0.5 μM each primer, 0.025 U μl^{-1} Platinum Taq (Invitrogen) and 20 ng plasmid. Reactions were carried out on a ABI9700 (Applied Biosystems) using a temperature profile of 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and lastly 72°C for 7 min. Amplifications producing the expected size fragment were used in transcription reactions (T7 or T3 Ampliscribe Kit, Epicentre) according to the manufacturer's instructions. Reactions were treated with DNase for 15 min after transcription. Transcripts were further purified using Turbo DNA-free (Applied Biosystems).

Sample collection and preparation for SHA

Cultures of *E. coli* were grown overnight in Difco Luria–Bertani broth (Benton, Dickinson and Co.). Bacterial cell concentrations were estimated by direct count on 0.2 μm GTBP polycarbonate filters (Millipore, Bedford, MA) using an epifluoresence microscope after DAPI staining (Porter and Feig, 1980). *Synnechococcus* CCMP 1334 cells were provided by J. Zehr.

Bacterial cultures or 200 ml native seawater samples were collected using gentle vacuum (< 10 mmHg) onto 25 mm 0.2 μm Durapore hydrophilic membranes (Millipore). Filters were transferred to a 2 ml screw top polypropylene vial and used immediately or frozen in liquid N_2 until use. Frozen samples were thawed to near room temperature before proceeding with lysis.

To homogenize samples, 1 ml of lysis buffer [3 M GuSCN, 50 mM Tris, 15 mM EDTA, 2% Sarkosyl and 0.2% SDS (v/v), at pH 8.9; modified from 48, Saigene Corp.] was added to each filter, vortexed, and incubated at 85°C for 10 min, with a brief vortexing midway through the heating. Thereafter, an equal volume of diluent [50 mM Tris, 15 mM EDTA, 2% Sarkosyl and 0.2% SDS (v/v), at pH 8.9] was added, and then filtered through a 0.2 μm hydrophilic Durapore syringe filter (Millipore). To generate volumes > 1.75 ml, replicate samples were homogenized, diluted, combined and filtered. The resulting lysate was used in the 96-well plate and/or ESP array SHA formats. For some samples, RNA was purified from an aliquot of the undiluted lysate (see below). *In vitro* rRNA transcripts or purified RNA from cultures were added directly to lysis buffer and treated as above.

To determine that molecules captured were rRNA or rDNA, a 600 ml seawater sample was collected as above. Total nucleic acids were then extracted (Massana *et al.*, 1997). The sample was split into three aliquots, each containing approximately 15.4 μg total nucleic acid. Two aliquots were incubated for 1 h at 37°C with either 2 U DNase (Epicentre) or 8 μg RNase (Promega). The DNase-treated aliquot was then incubated at 70°C for 10 min to inactivate the enzyme. The last aliquot was left untreated. Volumes were adjusted to 35 μl with RNase-free water after treatment. To each, 0.5 ml lysis buffer was added, heated to 85°C, diluted for hybridization, and applied to the 96-well plate SHA.

Sandwich hybridization assay

The SHA employs capture and signal probes (Scholin *et al.*, 1996). In this study, capture probes were group-specific while signal probes targeted universally conserved regions of 16S rRNA (Table 1). Capture and signal probes (Oligo's Etc., Eugene, OR) were handled and designed as described elsewhere (Greenfield *et al.*, 2006; Haywood, 2007; Jones *et al.*, 2008). Capture and Alex-comp (positive control) probes (Table 1) were printed on Predator® membrane (Pall Corp., East Hills, NY; after Greenfield *et al.*, 2006). Signal probes Eub338 and Univ519ab were combined (each at 100 ng ml^{-1}) in 500 mM GuSCN, 50 mM Tris pH 8.55–8.65, 10 mM EDTA. For a positive control on arrays, 3.125 ng ml^{-1} Alex-alt-S was added (Greenfield *et al.*, 2006). Sandwich hybridization assays in the 96-well plate format were carried out using a robotic processor (Saigene Corporation, Seattle, WA) (Scholin *et al.*, 1998; Tyrrell *et al.*, 2001; Goffredi *et al.*, 2006; Greenfield *et al.*, 2006). At least three replicate wells were performed for each sample and capture probe combination; the averaged A_{450} values are reported. Replicate samples for each capture-signal probe combination showed less than a 10% coefficient of variation.

Environmental Sample Processor deployment, array processing, and sample archiving

The ESP was deployed in Monterey Bay, CA, at Station M0 (36.83 N, 121.90 W) March 16–27 (JD75–86) and again April 10–23, 2006 (JD100–113), on a mooring that positions the instrument subsurface (Scholin *et al.*, 2008). For the first deployment, the ESP was fitted with a Seabird SBE 16+ CTD (Bellevue, WA) with fluorometer (Turner Cyclops-7) and transmissometer (WetLABS Cstar) that provided environmental measurements every 20 min. During the second deployment the ESP CTD failed and temperature and salinity data were obtained from the CIMT/M0 mooring (Ryan *et al.*, 2005) that was located within 0.5 km of the ESP mooring.

Environmental Sample Processor deployments included assays for harmful algal bloom species and phycotoxins, invertebrates and BAC (Greenfield *et al.*, 2006; Jones *et al.*, 2008). Only the results from the BAC arrays are presented here. A 'BAC phase' consisted of a series of operations that included sample collection and lysis, dilution and filtration of lysate for hybridization, SHA probe array development, and sample archival. The ESP initiated sampling daily at 9AM local time. It was programmed to collect a 0.4 l sample onto a 0.2 μm duropore filter during the March deployment and 1 l sample in April During sample filtration a ~10 psi differential was maintained using pressure transducers mounted above and below the filter puck until the volume specified was reached. If the instrument could not filter 25 ml within 2.5 min, filtering was terminated and the sampled volume was recorded. The material retained on the filter was homogenized with 1.1 ml 3 M GuSCN lysis buffer at 85°C for 10 min. The lysate was recovered, diluted 1:1 as above, passed through the collection puck once more and recovered. This lysate was filtered through a second 0.2 μm Duropore before passage to the array. The remainder of the operations were as previously described (Greenfield *et al.*, 2008). Array images (when available), data from the CTD and a log of instrument operations were transmitted to shore hourly using a radio modem. The intensity of probe spots was determined using ImageJ v.1.36b (W. Rasband, NIH, Bethesda Maryland) by defining a constant circular area from which pixel intensity was derived. Probe intensities reported here were averaged from five to seven replicate probe spots (Fig. 3A).

RNA extraction and RT-qPCR analysis

RNA was purified from field samples collected and lysed as above. To 1 ml of undiluted lysate, NaOAc (pH 5.2) and ethanol were added to a final concentration of 0.4 M and 40% (v/v) respectively. The entire sample was then applied by centrifugation in multiple aliquots to an RNeasy column (Qiagen, Valencia, CA). Subsequent washes and elution were as per the manufacturer's recommendation. DNA contamination was eliminated using Turbo DNA-free. cDNA was synthesized from 2 μl RNA using 2.5 ng μl^{-1} random primers and Superscript III (Invitrogen) as per manufacturer's instruction. RT reactions (2.5 μl) were used in group-specific qPCR assays as previously described (Suzuki *et al*., 2000; 2001; Shi, 2005).

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