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Autonomous Application of Quantitative PCR in the Deep Sea: In Situ Surveys of Aerobic Methanotrophs Using the Deep-Sea Environmental Sample Processor

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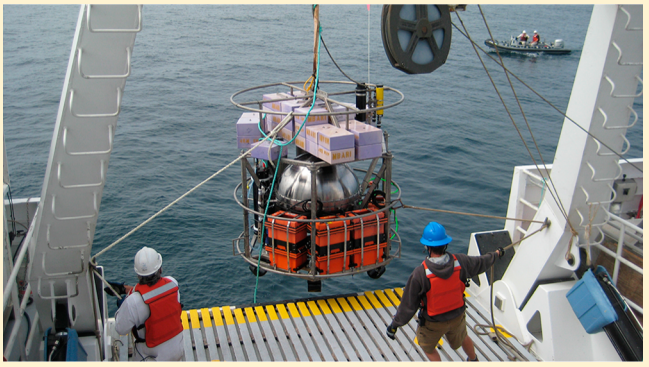
Supporting Information

ABSTRACT: Recent advances in ocean observing systems and genomic technologies have led to the development of the deep-sea environmental sample processor (D-ESP). The D-ESP filters particulates from seawater at depths up to 4000 m and applies a variety of molecular assays to the particulates, including quantitative PCR (qPCR), to identify particular organisms and genes in situ. Preserved samples enable laboratory-based validation of in situ results and expanded studies of genomic diversity and gene expression. Tests of the D-ESP at a methane-rich mound in the Santa Monica Basin centered on detection of 16S rRNA and particulate methane monooxygenase (*pmo*A) genes for two putative aerobic methanotrophs. Comparison of in situ qPCR results with laboratory-based assays of preserved samples demonstrates the D-ESP generated high-quality qPCR data while operating autonomously on the seafloor. Levels of 16S rRNA and *pmo*A cDNA detected in preserved samples are consistent with an active community of aerobic methanotrophs near the methane-rich mound. These findings are substantiated at low methane sites off Point Conception and in Monterey Bay where target genes are at or below detection limits. Successful deployment of the D-ESP is a major step toward developing autonomous systems to facilitate a wide range of marine microbiological investigations.



INTRODUCTION

The advent of integrated ocean observing systems[1] has created new opportunities for developing and deploying novel sensors. "Ecogenomic sensors" are among this emergent class. They are conceptualized as devices that will enable in situ detection of specific organisms, their genes, and gene products using molecular analytical techniques.[2] The environmental sample processor (ESP), often referred to as a "laboratory in a can", is one example of an ecogenomic sensor.[3] The ESP is designed to collect water samples and concentrate particulates by filtration and then use DNA and protein probe array technology, as well as quantitative PCR (qPCR), to detect and quantify particular biomolecules.[4,5] The instrument can also be used to preserve particulate samples for a variety of laboratory-based assays, including metatranscriptomic[6] and proteomic[7] analyses. To date, this device has been used primarily in shallow water settings.

This contribution reports on tests of a deep-sea version of the ESP (D-ESP) that were conducted along the California coast in 2010. Our objective was to carry out a series of deployments to begin developing the scientific and technical capabilities for establishing ecogenomic laboratories in the deep ocean. Toward that end, the instrument was fielded at an active methane vent located on a carbonate mound in the Santa Monica Basin and at the Monterey Accelerated Research System (MARS, http://www.mbari.org/mars) submarine cable in Monterey Bay. Both sites are ~800 m below the sea surface. Tests of the D-ESP included both free-standing and submarine cable-connected modes of operation. Assays for detecting DNA and RNA associated with aerobic methanotrophs were chosen as a focus for this investigation because methane metabolism is central to fueling and sustaining many deep-sea ecosystems.

Previous molecular investigations of the methane-rich benthic water column over and surrounding the Santa Monica mound have demonstrated the presence of a diverse assemblage

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of 16S rRNA and particulate methane monoxygenase subunit A (*pmo*A) genes that are thought to belong to specific groups of aerobic methanotrophs.[8−10] Of the six phylotypes known to contain the *pmo*A gene, OPU1 and OPU3 appear to be the most widespread and abundant groups found in the Santa Monica Basin and along the western continental margin of North America below the photic zone.[9] Because of their phylogenetic affiliation within Methylococcaceae, these are the groups most likely to be directly involved in methane oxidation and thus highly relevant to the cycling of water column methane within large expanses of the oceans. Accordingly, the D-ESP deployed at the Santa Monica site was configured to detect the 16S rRNA and *pmo*A genes indicative of the OPU1 and OPU3 phylotypes. It was also programmed to preserve particulate material for laboratory-based validation of measurements obtained in situ and to investigate the relationship between water column methane concentration and the expression of the targeted genes. Samples from low methane sites representative of open-ocean background levels (<1 nM)[11] off-shore of Point Conception, California, and at the MARS site in Monterey Bay provided additional measurements for comparative purposes.

MATERIALS AND METHODS

Deployment Locations. The D-ESP was deployed for 4 days during July 2010 on the crest of a methane-rich carbonate mound in a water depth of 800 m in the Santa Monica Basin,[12,13] off southern California, and at two off-mound sites 70 and 265 m due east of the mound (Figures 1 and 2). A list of sample locations and corresponding environmental conditions is provided in Table 1.

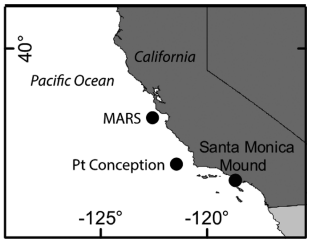

Figure 1. Sampling locations are shown for the D-ESP during the July 2010 expedition to the Santa Monica Basin in southern California and off-shore of Point Conception and the December 2010 deployment on the Monterey Accelerated Research System (MARS) submarine cable in Monterey Bay.

Sulfur-oxidizing bacterial mats mantle the mound, and streams of methane bubbles occasionally rise out of cracks in the hard authigenic carbonate carapace. Analysis of water samples collected in Niksin bottles from water column casts using a CTD-rosette or mounted on an ROV have documented previously a persistent methane-enriched water column in the immediate vicinity of the mound.[8,11] The Santa Monica mound and surrounding seafloor are within the most oxygen depleted portion (dissolved oxygen ≤0.1 mL/L) of the extensive oxygen minimum zone distributed along the western North American continental margin.[14] Bottom water temperature and oxygen concentration were stable at approximately 5.2 °C and 0.09 mL/L, respectively, during the deployment (Table 2).

Immediately following the Santa Monica Basin deployment, water was collected using Niskin bottles on a CTD rosette launched from MBARI's R/V *Western Flyer* at a site

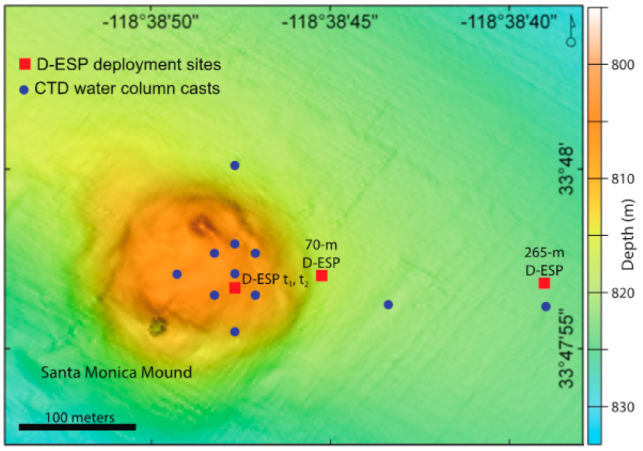

Figure 2. D-ESP deployment locations and CTD water column casts are plotted on this high-resolution AUV-acquired multi-beam image of the Santa Monica mound and the surrounding seafloor. Methane concentration measurements for the CTD water column casts are illustrated in Figure S1-S11 of the Supporting Information.

approximately 230 km WNW of Point Conception, CA (Table 1). That sample was processed immediately by the D-ESP upon its arrival on deck, being subjected to the same suite of analyses and preservation methods used during the Santa Monica mound deployment. Samples that were collected from the Monterey Bay MARS site using the D-ESP in December 2010 were also included in this investigation (Table 1) to provide background measurements from a low methane benthic reference site far removed from any seafloor source of methane.[11] The seafloor at the MARS site comprises relatively smooth, but firm, tan hemiplegic sediment with a biologically diverse and relatively abundant benthic fauna (Figure S1-S2, Supporting Information).

Overview of the D-ESP. The ESP instrument used in this investigation is the same as that used in previous shallow water deployments,[5] but it is contained in a different housing and uses an external sampling module (see below) to collect and depressurize seawater samples prior to particle filtration. The ESP instrument housing is a 1 m diameter titanium sphere, rated for use in water depths up to 4000 m. The sphere, external sampling module, contextual sensors, and associated equipment are mounted on an aluminum frame that serves as a benthic lander (Figure 3) designed to be deployed from the deck of a ship.[15]

Control of free-fall descent and ascent of the 1850 kg (mass in air) lander is provided by syntactic foam flotation mounted on the upper portion of the benthic lander and a 120 kg in air drop weight that can be released by an ROV or acoustically activated burnwire. The D-ESP is capable of operating autonomously for short periods of time (days) on battery power. Longer-term deployments on submarine cables are constrained by availability of reagents and sample processing capacity.

Communication with the D-ESP while on the seafloor was accomplished using either a hull-mounted acoustic transducer on the R/V *Western Flyer* or an ROV-manipulated wet-mate connection while on the seafloor.[16] The D-ESP used the same type of wet-mate connection while attached to the MARS node.

The D-ESP can be deployed with a variety of chemical and physical sensors that provide a contextual framework for evaluating factors controlling microbial populations. This suite

Table 1. D-ESP Deployment Locations and Water Sample Characteristics

						water sample characteristics				
location	latitude	longitude	water depth (mbsl)	ROV dive/ CTD cast[a]	date/time (PDT) of water sampling[b]	sampling depth (mbsl)	temp °C	O_2 mL/L	percent transmission	CH_4 (nM)	environment
Santa Monica mound crest	33.799979°	−118.646841°	800	DR-169	July 23, 2010/1027	798	5.21	0.09	89.4	5900 ± 140; n = 2	high methane water column
off mound, 70 m east	33.799603°	−118.645780°	815	DR-170	July 24, 2010/1539	813	5.21	0.08	89.3	8.4 ± 0.4; n = 2	low methane water column
off mound, 265 m east	33.799703°	−118.644197°	825	DR-171	July 25, 2010/1108	824	5.21	0.08	89.3	2.1 ± 0.1; n = 2	low methane water column
Point Conception	34.600167°	−122.150000°	805	DESP2010c12	July 27, 2010/0940	620	5.91	0.56	90.8	0.4 ± 0.1; n = 3	background methane water column sample using CTD rosette
MARS site	36.712552°	−122.186920°	881	V-3596 and V-3601	December 1, 2010 to December 22, 2010	879	4.16	0.28	90.7	0.7 ± 0.1; n = 4	background methane water column

[a]DR = *Doc Ricketts* ROV; V = *Ventana* ROV. [b]Acquisition of a sample was completed 16 min after start time.

of instruments includes an in situ mass spectrometer (ISMS),[17] in situ underwater spectrometer (ISUS),[18] a CTD, and optical sensor packages.

Sample Acquisition and Particle Filtration Using the Deep Water Sampling Module. The ESP was originally designed to operate within 50 m of the sea surface. Use of the instrument below that depth required the development of a deep-water sampling module (DWSM) that acquires and then decompresses a water sample before passing it to the core ESP instrument for filtration and processing (Figures 3 and 4).

The DWSM can collect up to 10 L of water; once depressurized, the ESP can process multiple aliquots of that primary sample. In the core ESP instrument, variable volumes of decompressed seawater are filtered through "pucks".[19] Filtered particulate material is then either homogenized and processed immediately using downstream molecular assays (Figure 4) or preserved with RNA*later* (Life Technologies, Carlsbad, CA) for laboratory-based molecular investigations.[6,7,20] Upon recovery of the instrument, unprocessed preserved filters are removed from pucks and stored at −80 °C until analyzed.

Autonomous qPCR 5′-Nuclease Assays of Particulate Samples Using the D-ESP qPCR Module. Preston et al.[5] provide details of sample collection and analysis using an ESP instrument fitted with a qPCR module. A brief summary of this process follows and is shown schematically in Figure 4. Sample particulates were concentrated on a 25 mm diameter, 0.22 μm pore size Durapore filter (#GVWP02500, Millipore Corporation, Bellerica, MA). Particulate material retained on the filter was homogenized using 3 M guanidine thiocyanate-based chaotrope solution (pH 8.9; Spyglass Biosecurity, San Francisco, CA) and heating to 85 °C for 10 min. The lysate was filtered through a second filter puck (0.22 μm Durapore), and the nucleic acids were purified using a silica-based solid-phase extraction (SPE) system (see Preston et al.[5] and Supporting Information for details). Purified nucleic acids were eluted from the SPE column in 60 μL molecular biology grade water (Sigma, St. Louis, MO).

Quantitative PCR analysis of each target gene was obtained by mixing 6 μL of the purified nucleic acid sample, 6 μL of a selected primers and probe mixture for the 5′-nuclease assay and, 18 μL of a DNA polymerase enzyme mixture. The resulting 30 μL mixture was precisely positioned within the qPCR module, thermal-cycled, and fluorescence intensity was measured using photodiodes.

Thermal cycling conditions for the D-ESP qPCR module comprised an initial hold at 93 °C for 75 s, followed by 42 amplification cycles of annealing and extension at 59 °C for 30 s, and denaturation at 93 °C for 15 s. A temperature gradient (1 °C intervals between 55 and 62 °C) was used to establish optimal annealing temperature for each qPCR assay. Fluorescence measurements for both linearized plasmid standards and environmental samples were background corrected using a mean value method.[21] Fractional C_t was computed at the intersection of a 4-point linear regression fit to C_t vs log background-corrected fluorescence and a fluorescence threshold of 200 units.

Pre- and post-deployment standard curves spanning 4 orders of magnitude concentrations were obtained using linearized plasmid templates[8] containing the targeted genes. Standard curves were compiled and evaluated using statistical criteria outlined by Burns et al.,[22] and outliers were identified using the Grubbs test.[23] The standard curves used to estimate gene

Table 2. Methane Concentration and Water Sample Characteristics of ROV and CTD Rosette-Collected Water Samples

ROV dive/Niskin bottle	date/time (PDT)	location	depth (mbsl)	temp °C	O_2 (mL/L)	percent transmission	CH_4 (nM)
DR-168/5+6	July 22, 2010/1730	mound	804	5.23	0.09	82.4	170, 120[a]
DR-168/7	July 22, 2010/1742	mound	804	5.22	0.09	83.1	55000
DR-168/8	July 22, 2010/1833	mound	805	5.23	0.09	83.2	7.7
DR-169/5+6	July 23, 2010/1027	mound	798	5.21	0.09	89.4	5800, 6000[a]
DR-169/7	July 23, 2010/0825	mound	804	5.21	0.09	89.4	65
DR-169/8	July 23, 2010/1712	mound	800	5.21	0.08	89.4	2800
DR-170/5+6	July 24, 2010/1539	70 m site	813	5.21	0.08	89.3	8.1, 8.6[a]
DR-170/7+8	July 24, 2010/1634	mound	798	5.21	0.08	88.5	2200, 5400[a]
DR-171/5+6	July 25, 2010/1108	265 m site	824	5.21	0.08	89.3	2.2, 2.0[a]
DR-171/7+8	July 25, 2010/1211	mound	800	5.21	0.09	89.3	2.9, 2.9[a]
DR-172/5+6	July 26, 2010/1149	160 m site	818	5.21	0.09	89.3	2.2, 2.1[a]
DR-172/7+8	July 26, 2010/1157	265 m site	821	5.21	0.09	89.3	2.2, 2.2[a]

[a]Replicate from the adjacent Niskin bottle.

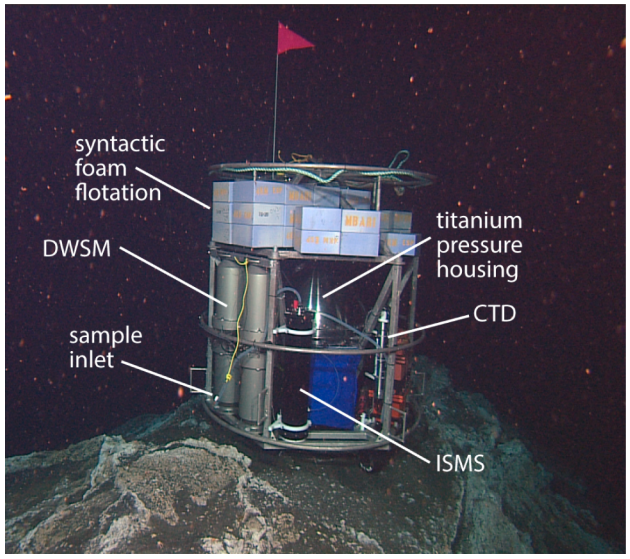

Figure 3. D-ESP is shown deployed on the sharply defined crest of the Santa Monica mound at a water depth of 800 m below sea level. A copper filter screen attached to the sample inlet for the deep water sampling module (DWSM) restricts the sample to particle sizes less than 0.5 mm. A separate parallel sampling tube provides water to the in situ mass spectrometer (ISMS) and a conductivity–temperature–depth (CTD) instrument mounted on the benthic lander. Blocks of syntactic foam on the upper portion of the 2 m tall D-ESP frame provide flotation.

abundances in the environmental samples had amplification efficiencies of approximately 95% for all assays (Table S2-S8, Supporting Information), which is comparable to those obtained in the laboratory for the same assays. When a C_t value for an environmental sample exceeded the C_t for the lowest concentration standard, the target gene was deemed detectable but not quantifiable.

Quantitative PCR assays that targeted the *pmo*A[8] and corresponding 16S rRNA[10] genes of OPU1 and OPU3 (see Supporting Information for details) were utilized for all laboratory investigations and for in situ analyses performed by the D-ESP at the Santa Monica Basin site. Only the *pmo*A assays were utilized in situ during the MARS D-ESP deployment. Table S1-S5 of the Supporting Information lists the four environmental gene target primer and probe sequences and final concentrations employed. The qPCR assay protocol at each D-ESP deployment site was to run an internal positive control followed by a series of environmental targets. It took approximately 17 h to collect a sample, extract and purify the nucleic acids, run a full suite of qPCR assays, collect and preserve one or more aliquots of the same sample, and complete cleaning steps to prepare the instrument for the next sampling event.

Post-Deployment qPCR Gene and Gene Expression Assays of Preserved Particulate Material. DNA and RNA fractions were extracted from the same D-ESP preserved particle filter (25 mm diameter, 0.22 μm Durapore) using bead beating and a Qiagen AllPrep DNA/RNA Mini kit (Qiagen, Valencia, CA) following a modified method of Varaljay[20] as described in the Supporting Information. Prior to cDNA synthesis, the RNA fraction was DNase-treated (Life Technologies, Carlsbad, CA) according to the manufacturer's directions, except the incubation step was 1 h in duration. cDNA was then synthesized from the DNase-treated RNA fraction using random hexamers and the Superscript III enzyme system (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcriptase (RT) and no reverse transcriptase (NRT) reactions were run for each sample. The extracted DNA fraction and cDNA (RT and NRT reactions) were analyzed by qPCR on an Applied Biosystems StepOnePlus qPCR instrument with the aforementioned assays (Table S1-S5, Supporting Information). Parameters for standard curves obtained using the same plasmid stock standard solutions as used for the in situ qPCR assays are listed in Table S2-S8 of the Supporting Information.

Contextual Measurements. A Seabird SBE 16*plus* CTD (Seabird Electronics, Bellevue, WA) equipped with a high-precision pressure sensor provided a hydrographic context for the Santa Monica Basin and MARS deployments. An ISMS provided high frequency in situ measurements of methane concentration on the crest of the Santa Monica mound where streams of methane gas bubbles are periodically released. ISMS operation was limited to the first 13 h of the deployment because of exhaustion of an internal battery pack. Details of operation and calibration of the ISMS are described in the Supporting Information.

Contextual water samples were collected using Niskin bottles mounted on a CTD rosette or ROV to document the spatial variation of methane concentration over and surrounding the Santa Monica mound and at the open-ocean background sampling sites. Methods of sample collection and analysis are provided in the Supporting Information.

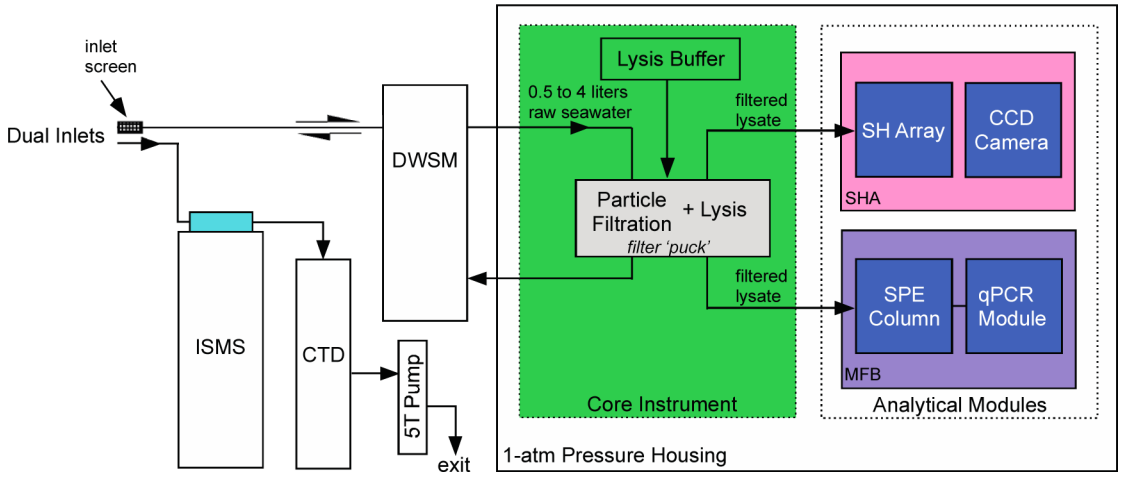

Figure 4. High-level process flow diagram illustrates the major components and fluid pathways for the D-ESP. The Deep Water Sampling Module (DWSM) acquires up to a 10 L sample and decompresses it before pushing user-selected aliquots of raw seawater through a filter "puck" inside the core instrument. Filtrate is returned to the DWSM, repressurized to ambient pressure, and expelled through the inlet after sample acquisition has been completed. An independent fluid pathway inside the pressure housing controlled by rotary switching valves provides lysis buffer to the filter puck and distributes processed lysate downstream to the sandwich hybridization array (SHA) and the microfluidics block (MFB). In the MFB, nucleic acids are purified in the heated solid-phase extraction (SPE) column prior to use in the qPCR module. A Seabird 5T pump pulls unfiltered water from an inlet co-located with the DWSM sampling inlet through the in situ mass spectrometer (ISMS) equilibration chamber (cyan shading) and a Seabird SBE 16*plus* CTD.

RESULTS AND DISCUSSION

The sampling scheme at the Santa Monica mound site was based on the hypothesis that the abundance of OPU1 and OPU3 16S rRNA and *pmo*A genes would co-vary with dissolved methane concentration, decreasing with distance from the mound. The qPCR results obtained in situ would be validated by laboratory-based qPCR assays of preserved particulate material collected by the D-ESP. Preserved particulate material would also allow for investigation of the correlation between methane concentration and expression of the OPU1 and OPU3 16S rRNA and *pmo*A genes.

Water column methane concentrations determined in this study (Figure 2, Figure S2-S11, Supporting Information, and Table 2) along with previous measurements document a widespread occurrence and persistence of methane-enriched benthic water in the Santa Monica Basin relative to waters found at comparable depths in the open ocean, off Point Conception, and at the MARS site in Monterey Bay (Table 1). On-mound methane concentrations show a high degree of meter-scale spatial and temporal heterogeneity (range 2.9–55,000 nM) over the four day deployment of the D-ESP (Table 2). This scale of heterogeneity is supported by ROV-based visual and sequential scanning sonar imaging that revealed periodic release and rapid dissipation of methane bubble streams emerging from the mound (data not shown). Methane concentration measurements obtained by the ISMS approximately 1 m above the mound surface show that rapid temporal fluctuations occur near the mound crest, consistent with the visual and sonar observations (Figure 5).

However, assuming that a tidally induced decrease in hydrostatic pressure at the seafloor increases methane bubble release rates,[24] the broader-scale change in methane concentration over the observation period is out of phase with water depth. Thus, it is unlikely that the variations in methane concentration are related directly to seafloor pressure modulation of methane bubble release rates at the mound crest. In contrast with the on-mound measurements, methane

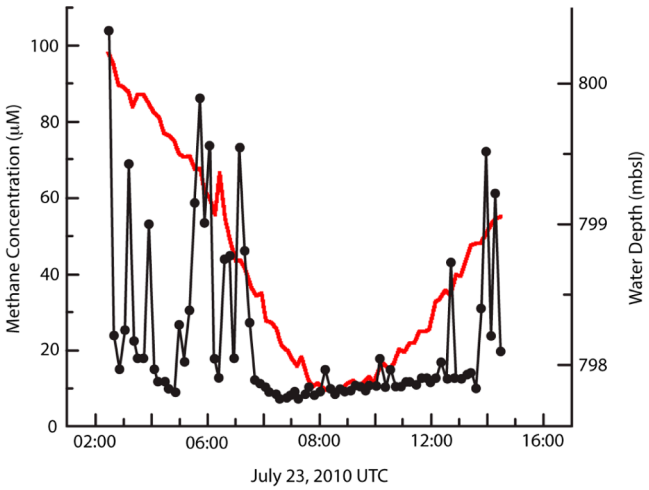

Figure 5. Fluctuations in methane concentration (black symbols) observed at the Santa Monica mound crest during 13 h of continuous monitoring by the in situ mass spectrometer on July 23, 2010 and corresponding measurements of water depth (red line) obtained by the CTD are illustrated. Six hours of relatively quiescent conditions were flanked by periods of highly variable methane concentrations and, at times, visible bubble streams emanating from the mound crest. Detection limit is 5 μM.

concentrations in ROV-collected water samples from the two off-mound D-ESP deployment sites (Figure 2 and Figure S2-S11, Supporting Information) are more uniform and substantially lower than on-mound samples (range 2.0 to 8.6 nM) but distinctly higher than open-ocean methane concentrations measured at Point Conception and the MARS site.

The eastward trend of the three-site deployment transect (Figure 2) was roughly perpendicular to the <20 cm/sec northwestward–southeastward contour parallel flow of benthic bottom water.[13] This orientation minimized local effects of methane venting at the mound and provided regionally

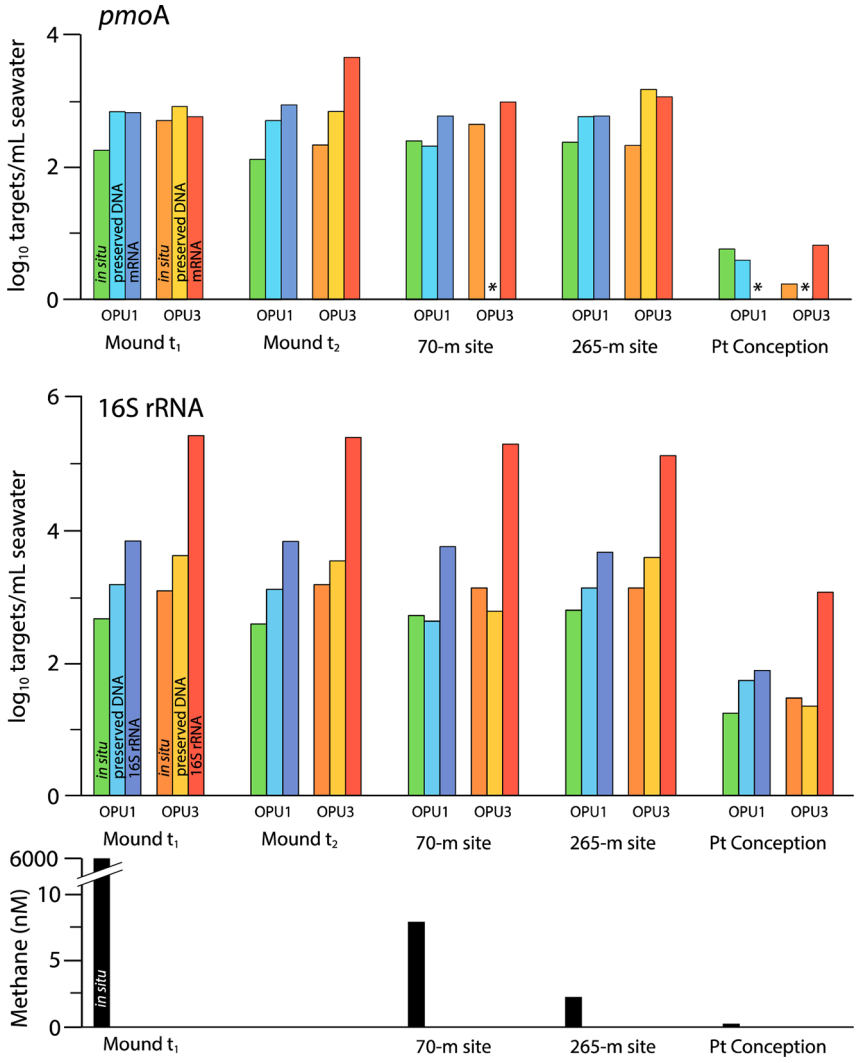

Figure 6. Whether performing in situ qPCR assays by the D-ESP (in situ) or processing preserved particulate material (preserved DNA), gene abundances were similar for the OPU and OPU3 affiliated *pmo*A and 16S rRNA genes. Additionally, although methane concentration drops with distance from the mound, there is no correlative change in gene abundance (in situ or preserved DNA) or gene expression (16S rRNA or mRNA). RNA transcript levels are minimum values because some mRNA decay is likely to have occurred during sample acquisition prior to RNA*later* stabilization. An asterisk indicates the molecular target was not detected.

integrated samples of the aerobic methanotroph community near the seafloor.

Results of in situ qPCR assays obtained from the transect show, compared to the open-ocean background sites, an overall high and relatively uniform abundance of 16S rRNA and *pmo*A genes from both the OPU1 and OPU3 phylotypes (120−1600 copies/mL), but there is no correlation with methane concentration (Figure 6). The same genes found at Point Conception (0.4 ± 0.1 nM methane) are at or below the level of quantification (~25 copies/mL seawater in a 500 mL sample). At the MARS site (0.7 ± 0.1 nM methane), OPU3 *pmo*A was detected but not quantifiable (below detection limit of 7 copies/mL seawater in a 2000 mL samples); OPU1 *pmo*A was not detected in any sample.

The particulate material preserved using the D-ESP allowed for laboratory confirmation of qPCR assay data obtained in situ and provided an opportunity to assess levels of gene expression relative to the standing stock of methane. Quantitative PCR assays of preserved particulate material reveal a 16S rRNA and *pmo*A gene abundance pattern for the OPU1 and OPU3 phylotypes that is similar to the qPCR results obtained in situ in real-time by the D-ESP (Figure 6).

However, the gene abundance measurements obtained in situ are generally lower by less than a factor of 3 compared to those obtained from the same water sample preserved using the D-ESP and then processed later in a laboratory. The small differences in gene abundance estimates between in situ and preserved samples likely reflect the different cell lysis methods used in this investigation (heating in the D-ESP for real-time in situ assays vs more efficient mechanical shear for the preserved material).

RNA transcripts of the OPU1 and OPU3 16S rRNA and *pmo*A genes are abundant in the preserved samples from the Santa Monica site (16S rRNA OPU1−6.3 × 10^3 cDNA copies/mL and OPU3−2.5 × 10^5 cDNA copies/mL; *pmo*A ~1000 cDNA copies/mL). However, they also do not vary along the deployment transect and show no correlation with the 3-order of magnitude difference in methane concentration between the on-mound and the two off-mound sites (Figure 6). The expression of these genes reveals an active aerobic methano-

troph community within the general vicinity of the Santa Monica methane mound, one that is not being influenced solely by methane from the mound itself. This finding is consistent with the Santa Monica Basin being generally enriched with methane from multiple sources. The community of aerobic methanotrophs is responding to methane-enriched waters on a regional scale, rather than to a local point source of methane. The water column samples collected from sites with lower methane concentrations (Point Conception and MARS) substantiate this finding.

The deployments of the D-ESP described here are a significant step forward in proving that autonomous molecular analytical laboratories can be used in the deep ocean. To our knowledge, these tests are the first successful deployments of an ecogenomic sensor that unequivocally detected the abundance of microbial genes, in real-time, at water depths greater than ~800 m. The use of RNA*later* to stabilize particulate material in situ for expanded molecular assays in the laboratory was also demonstrated. The general agreement between results obtained in real time and those gathered post-deployment proves that particle concentration, lysis, DNA purification, and qPCR methods can be automated and applied in extreme environmental settings. These operations demonstrate also that the liquid reagents used in these procedures can be stabilized for extended periods. The analysis of particulate material preserved in situ highlights the opportunity for making complementary measurements that may not be possible to carry out in real time.

The next step in D-ESP technology development is to increase the duration of deployments by utilizing submarine cable technology. The capability to conduct substrate utilization experiments to assess microbial community response in ways that cannot be accomplished by laboratory-, ship-, or ROV-based technologies will also be sought. This work is being done with the expectation that coastal and global ocean observatories (http://www.ioos.gov/) will be expanded, and so opportunities for fielding ecogenomic sensors on a variety of fixed and mobile ocean-observing platforms will increase in the near future. For the first time, ocean observing systems that allow investigators to carry out interactive experiments and test hypotheses remotely in situ from a molecular biological perspective are within reach.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of qPCR assay development, nucleic acid extraction, ISMS operation and calibration, and dissolved methane concentration analysis. Figure S1-S2 shows characteristic features of the seafloor at the MARS deployment site. Figure S2-S11 illustrates water column methane concentration profiles from above the Santa Monica mound. Table S1-S5 lists primer and probe sequences used for the qPCR 5′-nuclease assays. Table S2-S8 summarizes qPCR standard curve parameters for in situ and postdeployment gene expression assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ocean Observatories Initiative (OOI) Scientific Objectives and Network Design: A Closer Look. http://www.oceanleadership.org/files/Science_Prospectus_2007-10-10_lowres_0.pdf (accessed December 17, 2012).

(2) Scholin, C. S. What are "ecogenomic sensors?" A review and thoughts for the future. *Ocean Sci.* **2010**, *6*, 51−60, http://www.ocean-sci.net/6/51/2010.

(3) Scholin, C. S.; Doucette, G.; Jensen, S.; et al. Remote detection of marine microbes, small invertebrates, harmful algae, and biotoxins using the environmental sample processor (ESP). *Oceanography* **2009**, *22*, 158−161.

(4) Greenfield, D. I.; Marin, R., III; Doucette, G. J.; Mikulski, C.; Jones, K.; Jensen, S.; Roman, B.; Alvarado, N.; Feldman, J.; Scholin, C. Field application of the second generation environmental sample processor (ESP) for remote detection of harmful algae: 2006−2007. *Limnol. Oceanogr.: Methods* **2008**, *6*, 667−679.

(5) Preston, C. M.; Harris, A.; Ryan, J.; Roman, B.; Marin, R., III; Jensen, S.; Everlove, C.; Birch, J.; Dzenitis, J. M.; Pargett, D.; Adachi, M.; Turk, K.; Zehr, J. P.; Scholin, C. A. Underwater application of quantitative PCR on an ocean mooring. *PLoS One* **2011**, *6* (8), e22522. DOI: 10.1371/journal.pone.0022522.

(6) Ottesen, E. A.; Marin, R., III; Preston, C. M.; Young, C. R.; Ryan, J. P.; Scholin, C. A.; DeLong, E. F. Metatranscriptomic analysis of autonomously collected and preserved marine bacterioplankton. *ISME J.* **2011**, *5*, 1881−1895.

(7) Saito, M. A.; Bulygin, V. V.; Moran, D. M.; Taylor, C.; Scholin, C. Examination of microbial proteome preservation techniques applicable to autonomous environmental sample collection. *Front. Aquat. Microbiol.* **2011**, *2*, article 215; DOI: 10.3389/fmicb.2011.00215.

(8) Tavormina, P. L.; Ussler, W., III; Orphan, V. J. Planktonic and sediment-associated aerobic methanotrophs in two seep systems along the North American margin. *Appl. Environ. Microbiol.* **2008**, *74*, 3985−3995.

(9) Tavormina, P. L.; Ussler, W., III; Joye, S. B.; Harrison, B. K.; Orphan, V. J. Distributions of putative aerobic methanotrophs in diverse pelagic marine environments. *Int. Soc. Microbial. Ecol. J* **2010**, *4*, 700−710.

(10) Tavormina, P. L.; Ussler, W., III; Steele, J. A.; Connon, S. A.; Klotz, M. G.; Orphan, V. J. Abundance and distribution of diverse membrane-bound monooxygenase (Cu-MMO) genes within the

Costa Rica oxygen minimum zone. *Environ. Microbiol.* **2013**, *5*, 414−423.
(11) Ussler, W., III; Paull, C. K. Detection of methane sources along the California continental margin using water column anomalies. Presented at the 6th International Conference on Gas Hydrates [Online], Vancouver, Canada, July 2008. http://circle.ubc.ca/handle/2429/1089 (accessed November 13, 2012).
(12) Ussler, W., III; Paull, C. K.; Normark, W. Methane gas emanation from an active carbonate mound in Santa Monica Basin, offshore Southern California. *Geophys. Res. Abstr.* **2008**, *8*, 05223.
(13) Paull, C. K.; Normark, W. R.; Ussler, W., III; Caress, D. W.; Keaten, R. Association among active seafloor deformation, mound formation, and gas hydrate growth and accumulation within the seafloor of the Santa Monica Basin, offshore California. *Mar. Geol.* **2008**, *250*, 258−275.
(14) Levin, L. A. Oxygen Minimum Zone Benthos: Adaptation and Community Response to Hypoxia. In *Oceanography and Marine Biology: An Annual Review*; Gibson, R. N., Atkinson, R. J. A., Eds.; Taylor and Francis: New York, 2003; Vol. *41*, pp 1−45.
(15) Conway, M.; McGill, P. Transporting equipment and samples using benthic elevators. *Sea Technol.* **2008**, *49*, 10−14.
(16) Barlow, S.; Flynn, J.; Terada, S.; Mudge, W. Power and Communication Architectures for Cabled Subsea Observatories. Presented at the Symposium on Underwater Technology and Workshop on Scientific Use of Submarine Cables and Related Technologies, Tokyo, Japan, April 17−20, 2007, DOI: 10.1109/UT.2007.370956.
(17) Wankel, S. D.; Germanovich, L. N.; Lilley, M. D.; Genc, G.; DiPerna, C. J.; Bredley, A. S.; Olson, E. J.; Girguis, P. R. Influence of subsurface biosphere on geochemical fluxes from diffuse hydrothermal fluids. *Nat. Geosci.* **2011**, *4*, 461−468.
(18) Johnson, K. S.; Coletti, L. J. *In situ* ultraviolet spectrophotometry for high resolution and long-term monitoring of nitrate, bromide, and bisulfide in the ocean. *Deep-Sea Res. I* **2002**, *49*, 1291−1305.
(19) Greenfield, D. I.; Marin, R., III; Jensen, S.; Massion, E.; Roman, B.; Feldman, J.; Scholin, C. A. Application of environmental sample processor (ESP) methodology for quantifying *Pseudo-nitzschia australis* using ribosomal RNA-targeted probes in sandwich and fluorescent *in situ* hybridization formats. *Limnol. Oceanogr.: Methods* **2006**, *4*, 426−435.
(20) Varaljay, V. A. Quantitative Analysis of Bacterial DMSP-Degrading Gene Diversity, Abundance, and Expression in Marine Surface Water Environments. Ph.D. Thesis, University of Georgia, Athens, GA, 2012.
(21) Wittwer, C. T.; Kusukawa, N. Real-Time PCR. In *Molecular Microbiology: Diagnostic Principles and Practice*; Persing, D. H., et al., Eds.; ASM Press: Washington, DC, 2004; pp 71−84.
(22) Burns, M. J.; Nixon, G. J.; Foy, C. A.; Harris, N. Standardisation of data from real-time quantitative PCR methods: Evaluation of outliers and comparison of calibration curves. *BMC Biotechnol.*. **2005**, *5*, (31), DOI: 10.1186/1472-6750-5-31.
(23) Grubbs, F. E. Procedures for detecting outlying observations in samples. *Technometrics* **1969**, *11*, 1−21.
(24) Chanton, J. P.; Martens, C. S.; Kelley, C. A. Gas transport from methane-saturated, tidal freshwater and wetlands sediments. *Limnol. Oceanogr.* **1989**, *34*, 807−819.