ASTEP Annual Report

Project Title: An Environmental Sample Processor for Deep-Sea Seep and Hydrothermal Vent Applications

NASA Grant No: NNG06GB34G

Reporting Period: 9/23/06 – 9/25/07

P.I.: Christopher A. Scholin
Monterey Bay Aquarium Research Institute
7700 Sandholdt Rd
Moss Landing, CA 95039-0644

1.0 Introduction

The overall goals of this project center on modifying the Environmental Sample Processor (ESP; http://www.mbari.org/microbial/ESP) for autonomously sampling and detecting microbes found in Earth's deep-sea cold seep and hydrothermal vent fluids to depths of 4000m. We refer to this instrument as the deep-sea ESP, or D-ESP. The D-ESP consists of three major components: an external sampling module, the core sample processor ("core ESP") and analytical modules. The ESP can also be fitted with external environmental contextual sensors so that discrete samples acquired by the instrument can be placed in a broader context of the physical, chemical and optical properties of surrounding waters.

The project plan for this year, based largely on last year's report, is summarized in Section 2. Progress made during the past year is presented in Section 3, and an outline of plans for 2008-9 is found in Section 4.

2.0 Year-2 Project Plan

Between September 2006 and December 2007 our major objectives were to:

1) Construct an ISUS (http://www.mbari.org/chemsensor/ISUShome.htm) as a contextual sensor for detecting nitrate and bisulfide, then integrate with the core ESP.
2) Deploy the 1000m-rated D-ESP prototype in Monterey Bay (MBARI).
3) Assemble a copy of the core ESP (MBARI).
4) Construct a microfluidic interface, or microfluidic block (MFB), for the core ESP (LLNL and MBARI).
5) Complete construction of a flow-through real-time PCR module (LLNL).
6) Fit the core ESP with the MFB and attendant PCR module (LLNL and MBARI).
7) Design and initiate construction of a 4000m-rated external sampling module and core ESP pressure housing (MBARI).
8) Refine assay chemistries for use with the core ESP and MFB/PCR module (Caltech, MBARI).
9) For Education/Outreach purposes complete two animations illustrating:
    a. operation of the core ESP (in surface waters, simplest case)
    b. various applications of the D-ESP/ESP system and relationship to the Astrobiology Program.
10) As outlined in the original proposal, develop a design for an "ESP-like" device that is compatible with extraterrestrial science missions (JPL).

3. Accomplishments (9/23/06 – 9/25/07)

A project team meeting was held at MBARI this past July. In attendance were representatives of all partner institutions (MBARI, Caltech, LLNL, JPL).

Presented below is the progress made on each of the objectives in the same order as that given in Section 2. This reflects work that has taken place from the submission of last year's progress report to the present (9/23/06 to 9/25/07).

3.1 ISUS

An ISUS sensor was built in-house in collaboration with Ken Johnson's group at MBARI. It was then integrated with a core ESP and deployed two times in Monterey Bay surface waters last April-May. The ESP was programmed to carry out a variety of DNA probe and protein array assays at prescribed dates/times. The contextual sensors were programmed to sample every 20 min throughout the duration of the deployment. An example of the kind of data returned in near real-time from the ESP and attendant contextual sensors is shown in Figure 1. DNA probe arrays targeting groups of Bacteria and Archaea illustrate changes in the microbial community that correlated with changes in the chemical and physical properties of surrounding waters.

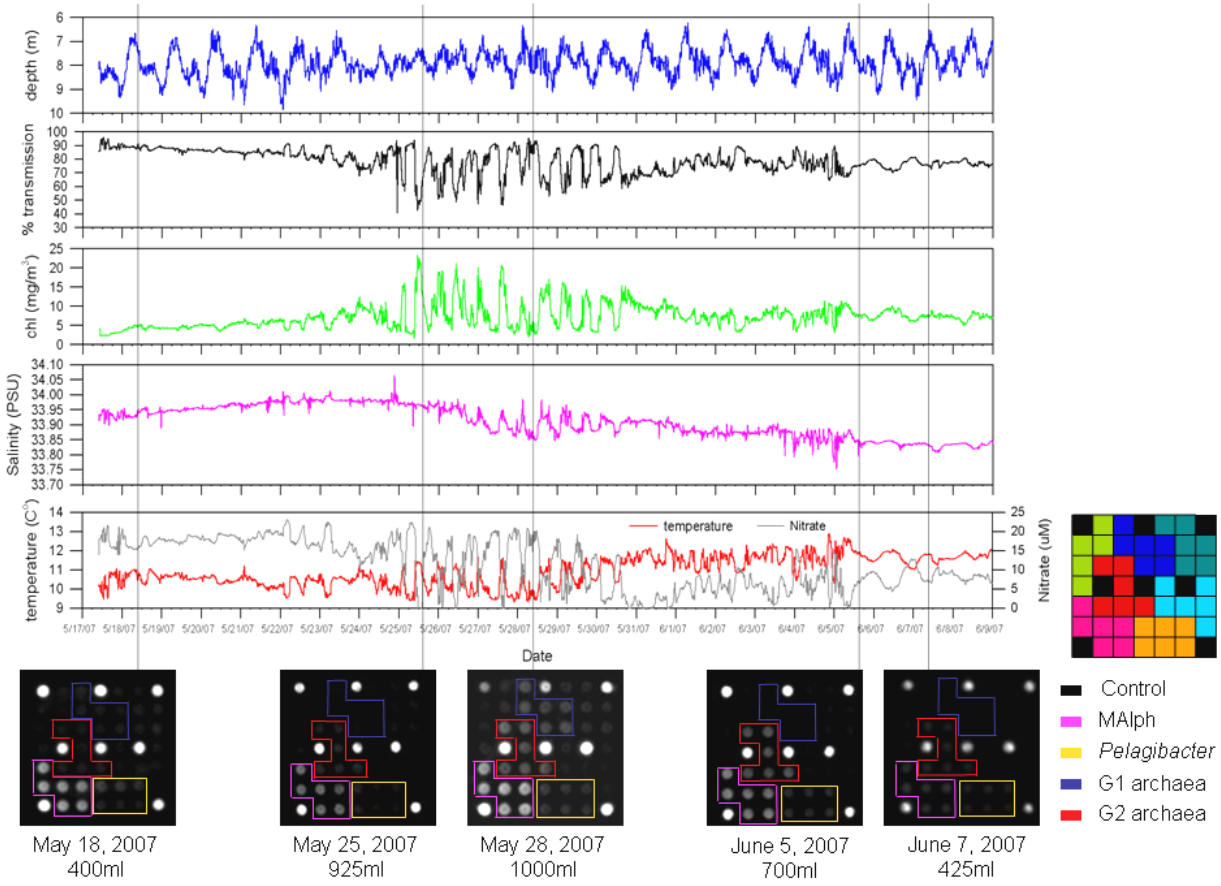

Figure 1. *In situ* detection of marine bacterioplankton using ESP DNA probe arrays during a spring field deployment. The ESP was moored in Monterey Bay, May 17-June 11, 2007. Top four graphs show physical and chemical data collected by contextual sensors on the ESP mooring during the deployment. The bottom array images show the developed BAC arrays colored boxes indicate the different bacterioplankton groups detected. Sample volume is shown underneath the array. The arrays shown are ~15mm x 15mm

3.2 D-ESP prototype deployments

The 1000m-rated D-ESP, or "1km D-ESP," was designed and manufactured in large part with a grant from the Keck Foundation prior to the start of this project. This ASTEP grant provided funds to bring that system to operational status and fit to the ROV Ventana. The 1km D-ESP consists of a deep-water sampling module (DWSM) mated to a core ESP contained in a separate housing. The DWSM is capable of collecting ~1.75L of water from depth, isolating that volume from the external environment, and depressurizing to ~1ATM prior to delivery to the core ESP.

The performance of the D-ESP prototype was evaluated over the course of three different deployments in Monterey Bay during 2007 using the R/V Pt. Lobos and ROV Ventana (http://www.mbari.org/dmo/vessels_vehicles/ventana/ventana.html). First, the DWSM subsystem was deployed in February to verify functionality prior to connecting to the core ESP. It was then combined with the core ESP and deployed in March. We succeeded in operating the D-ESP from near surface to 1000m, using DNA probe array assays for groups of common marine microbes (as in Fig. 1). The March deployment proved that the concept of capturing a volume of raw seawater and processing it with the ESP was viable, but in deep waters it appeared that we did not accumulate sufficient biomass to detect microbial rRNA using the direct detection methodology proven to work in surface waters.

The D-ESP was deployed once more in August. It was fitted with a sample wand that allowed a mechanical arm attached to the ROV to precisely position the sample intake at ~2-3m from the D-ESP itself (Fig 2). A pump was used to move fluid continuously through the wand, past the DWSM intake, and then through a CTD/transmissometer and ISUS. In this fashion we were able to we were able to capture contextual data at the same time the DWSM collected a sample. The ESP was loaded with reagents and probe arrays for marine microbes (as before) as well as for invertebrates. Samples were collected from the water column as before, then cold seeps and whale falls.

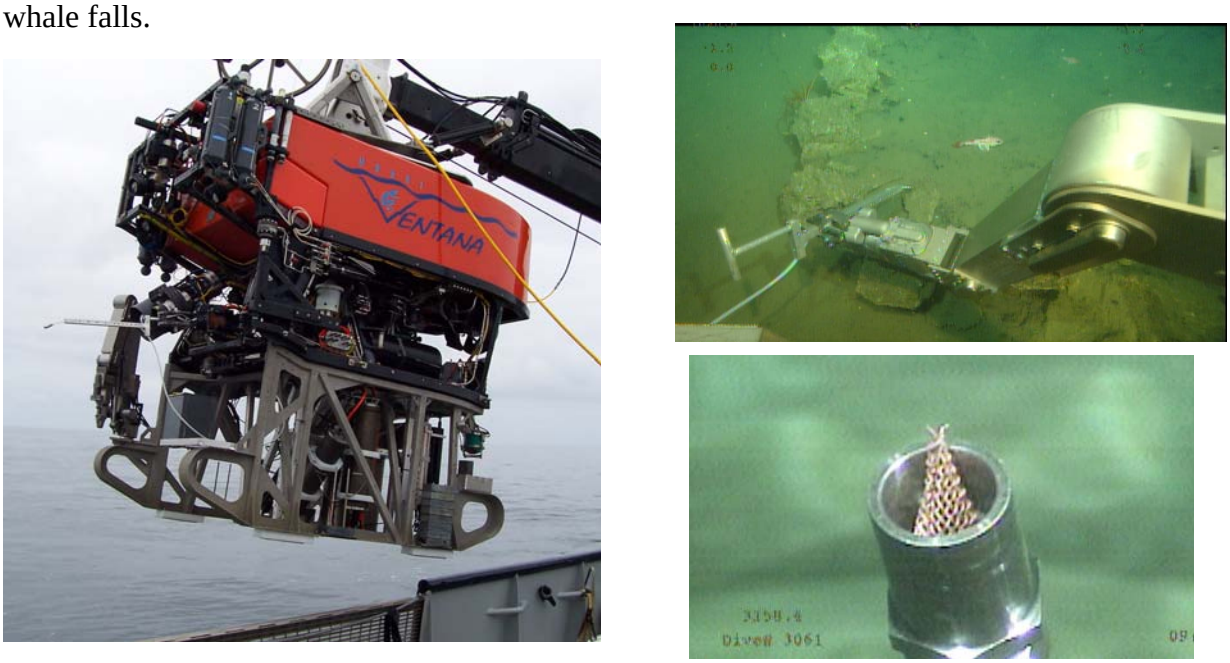

Figure 2. The 1km D-ESP fitted with sample wand being deployed with the ROV Ventanna (left), sample wand extended sampling the bottom at 1000m (top right), and close up of the wand sample intake (bottom right). The D-ESP and CTD/ISUS sensors are fitted to the sled under the ROV.

Bacterioplankton arrays were developed at 30m and 1000m. We observed the presence of certain groups in shallow waters, as would be expected. However no positive reactions were observed at 1000m despite successfully processing the sample and developing the array. Samples collected from depth using Niskin bottles mounted on the ROV were returned to the laboratory. We confirmed that the ESP must handle larger sample volumes (accumulate more biomass) to trigger a positive reaction using our current direct detection chemistry, presumably because the rRNA content of targeted cells is less than those at the surface. Since that time we have developed a "high volume" sampling scheme that should overcome this limitation (see 3.8).

In contrast, unambiguous positive arrays came from sampling a relatively fresh whale carcass (if there is such a thing!), "Pebbles", at 633m depth (Fig. 3). We demonstrated that the D-ESP is capable of detecting a variety of rRNA signatures in the deep-sea, consistent with eukaryotes, polycheates, *Osedax* and crustaceans associated with the carcass. A much older carcass that is largely degraded and devoid of *Osedax* and other organisms ("Francisco" at 1000m depth) did not return a positive reaction, consistent with the minimal biomass compared to Pebbles. The whale carcasses are an excellent example of a "biological hotspot" in deep water.

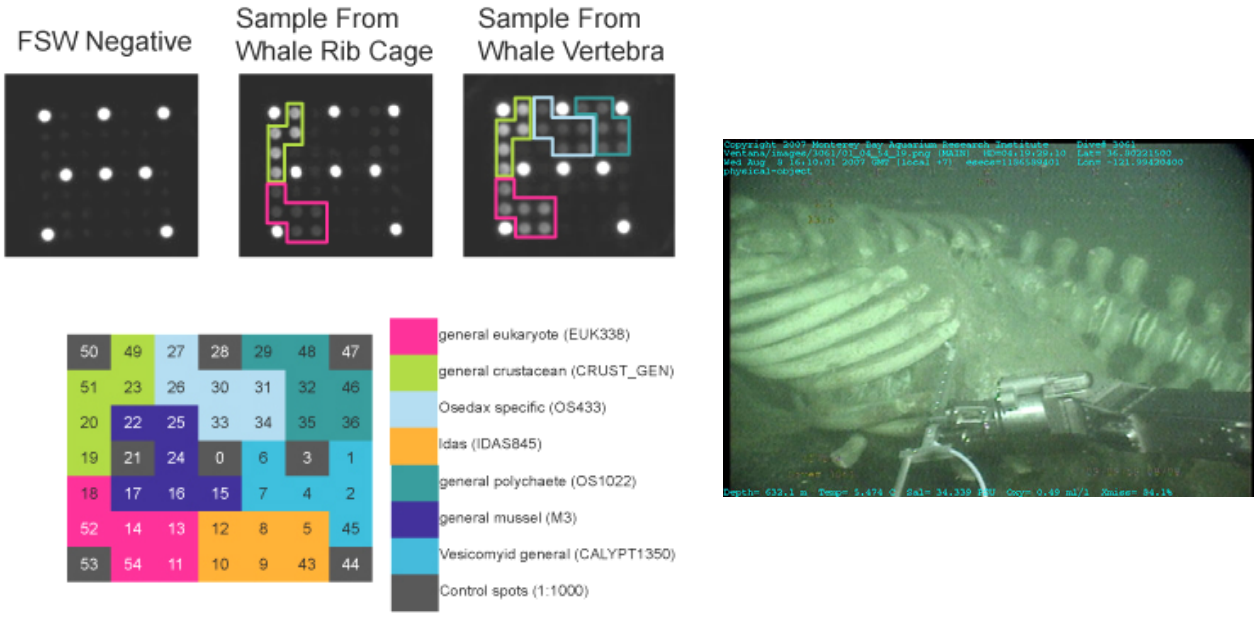

Figure 3. Detection of invertebrates associated with "Pebbles" whale fall (633m) August 8, 2007. At left are arrays from pre-deployment negative control (filtered seawater) and two arrays collected from different portions of the carcass. Below the arrays is a map showing locations of probe spots. Colored boxes surrounding probe spots on arrays correspond to invertebrate species detected. At right is a frame grab showing the sample wand extended from the ROV during sampling of the rib cage.

Cold seeps with clam communities were sampled as well. No positive reactions were retuned using the invertebrate arrays (as per Fig 3). However, we did detect bisulfide with the ISUS when the sample wand was driven into the sediment.

3.3 Production of a copy of the core ESP

A copy of the core ESP used was completed, run in the laboratory and cold tested to 4-10^{o}C. The instrument was named NEO (NASA ESP Observatory). The core ESP used for surface and deep water deployments reported above is referred to as GG, for 2nd Generation (2G) ESP.

The new instrument (NEO) was successfully operated to 10°C, but the syringe pumps (http://www.kloehn.com/online_catalog/page_ten.htm) leaked at 4°C. Syringe pumps of the same make and model do perform adequately at that temperature in the original core ESP (GG). We believe this problem can be overcome through OEM manufacturing, carefully matching syringe barrel ID with plunger OD.

NEO was deployed in surface waters of Monterey Bay (just off Santa Cruz) August 30. It is fitted with a CTD/fluormeter/tranmissometer and ISUS. It is programmed to carry out 5 different probe array assays: bacteria/archea, harmful algae, invertebrate larvae, pufM mRNA (gene involved with synthesis of bacterial chlorophyll) and domoic acid (algal toxin). The first 4 use DNA probes, the last uses antibodies. A radio modem allows for two-way communications with the instrument. The deployment is accompanied by AUV surveys using a variety of chemical, physical and optical sensors. Near NEO's mooring is also a moored vertical profiler (MVP) and upward looking acoustic Doppler current profiler (ADCP). Recovery of NEO is set for September 28.

3.4 MFB

The ESP must collect large volumes of natural samples and then render material collected for analysis on a microfluidic scale. To meet that need we have designed a separate fluid handling station that can be added to the core ESP. This work was carried out with in collaboration with workers at LLNL. We refer to this device as the "microfludic block", or MFB (Fig 4). The MFB is take-off point for distributing samples and reagents to one or more detection modules. The current emphasis is to support a reusable solid phase extraction column for purifying nucleic acids and a 4-channel real-time PCR module. The MFB is designed to support reagents for a variety of PCR master mixes, primer/probe combinations and control templates. Ultimately we aim to use the MFB to gang multiple functions in an analytical cascade (e.g., employ probe arrays down stream of PCR).

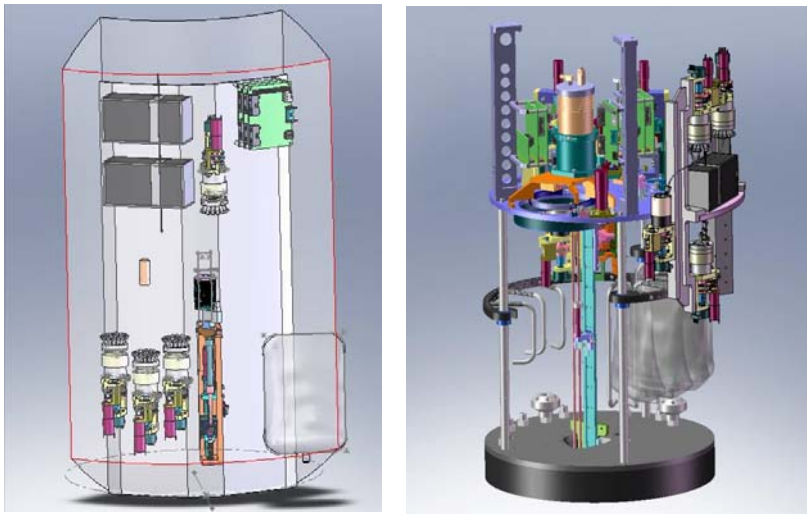

Figure 4. Solid model showing the microfluidic block. Left panel: standalone unit fitted with two LLNL PCR modules. Right panel: an earlier version of the same block and single PCR module attached to a core ESP (on far right). Parts of the ESP have been removed to improve clarity. For scale, the ESP is approximately 16" in diameter, 30" tall.

A prototype of the MFB is undergoing testing at the time of this writing. The majority of the costs required for this development are borne by a grant from the Gordon and Betty Moore Foundation (to C.S.).

3.5 PCR module

The LLNL module is a 4-color, real-time flow-through PCR device (Fig. 5). A CDR was held at LLNL last May, and a final design for the production modules was completed. The first production unit has been fabricated, assembled, and checked out electronically. The next step is to test it thermally and optically, including running PCR reactions. The LLNL team has parts in hand for the four production modules for MBARI, but they are waiting on assembly until testing of the first module is completed. The same group has submitted a record of invention to the LLNL intellectual property office for the electronics design, mechanical design, and software of the PCR module.

The next major steps are optical and thermal tests of the production version of the PCR module. The thermal tests will be done to verify that the module can maintain consistent reaction temperatures in a wide range of ambient temperatures.

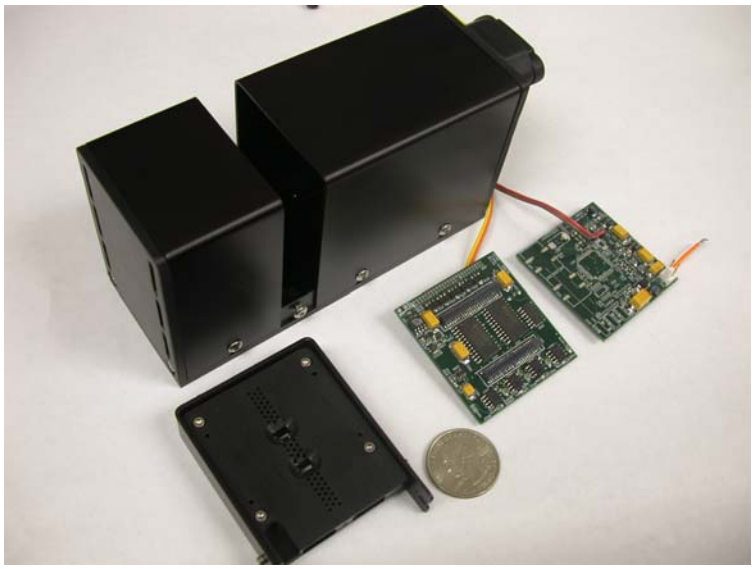

Figure 5. Some of the major components of the prototype ESP flow-though PCR module developed at LLNL (as shown Feb 07). Bottom left to right: heater module, U.S. quarter for scale, analog board, digital board. Top: enclosure. .

3.6 Integration of MFB, PCR module and NEO

Pending. The current plan is to mate the MFB and PCR modules later this year and then integrate with NEO by Q2 2008.

3.7 4km D-ESP

Preliminary designs for the 4000m-rated sample collection module and housing for the core ESP have been completed. The sampler design employs the same concept of capturing a volume of water up to 10L and depressurizing prior to delivery to the core ESP. Development of the sample module will go forward under the auspices of this grant. Development of the housing for the ESP, a 40” diameter titanium sphere, will be paid for by a combination of funds from this grant and a separate award from the National Science Foundation (to C.S.). A comparison of the 1km D-ESP attached to the ROV sled and the preliminary design for the 4km D-ESP is shown in Fig. 6.

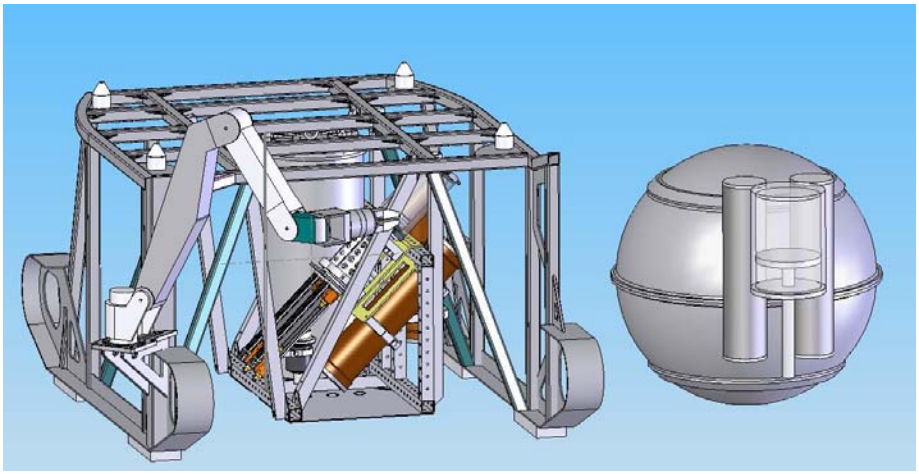

Figure 6. Solid model showing at left the 1km D-ESP mounted on the ROV sled as per Fig. 2. At right is the outer envelope of key elements of the 4km D-ESP. The core ESP fitted with the MFB/PCR modules (etc) will be housed in a 40" ID titanium sphere. Parts of the sampling module are shown at the front of the sphere.

3.8 Assay Chemistries

Work at MBARI has concentrated on developing the means for acquiring high concentrations of biomass using modified sample collection pucks, establishing an appropriate PCR positive control for use in multiplexed reactions that may be deployed in a variety of oceanic regimes, and testing of the MFB.

For sample collection, we are experimenting with combining a 25mm diameter wafer of sintered titanium material (~0.2 or 10µm pore size, ~2mm thick) stacked on top of a standard COTS 25mm filter (0.2µm Durapore). When fitted into an ESP sample puck, larger organisms/particles are captured in the sintered material, thus reducing the load applied to the 0.2µm conventional filter. With this arrangement we could filter three times more surface water compared to our traditional single filter format. When water collected from 1000m was filtered through the stacked filters, we were able to increase our sample volume 10-fold. By concentrating more biomass from water collected at 1000m we were able to detect microbial groups using our direct rRNA capture method (as per Figs. 1, 3). This should allow us to overcome the limitation imposed by sampling with the standard, 0.2µm pore size conventional filter, and may circumvent filter clogging under bloom conditions.

We have identified and evaluated several internal positive control (IPC) candidates for the PCR modules. These are being tested against DNA samples extracted from a wide range of oceanic regimes, including vent fluids and seawater from various geographic locations. Two primer/probe/template combinations appear to be promising (i.e., are not reactive towards environmental sequences). Next we will evaluate the IPC in multiplexed reactions containing Taqman primer/probe combinations for environmentally relevant genes.

The research group at Caltech has completed the characterization of particulate methane monooxygenase gene (pmoA) diversity affiliated with planktonic aerobic methanotrophs above active methane venting from methane seeps off the coast of Northern California (Eel River Basin) and Southern California (Santa Monica Basin). Application of newly designed PCR primers specific for the water column sequences increased both the amplification efficiency and overall recovered diversity from the planktonic methanotrophic assemblages. Most notable is the detection of a novel clade of sequences from both Eel River and Santa Monica Basin, likely representing an as yet unknown marine methanotrophic lineage.

Additional comparisons with sediment-hosted bacterial methanotrophs indicate distinct, non-overlapping populations reside in the sediment and water column, and support the notion that the pmoA gene sequences detected in the water column are indigeneous and not a product of sediment resuspension. To our knowledge, this is the first study that has compared the differences in methanotroph diversity between water column and sediment. Furthermore, the high similarity in the diversity of planktonic pmoA clades recovered between the California sites as well as from other geographically distant locations (Western Pacific) indicate the potential for the design of universally applicable quantitative PCR assays for planktonic methanotrophs distributed globally.

The second phase of the Caltech study has been focused on developing and testing quantitative PCR assays based on the sequence data amassed in years 1 and 2. Goals for the coming months will be to further define the detection limits of the primer probe combination for samples collected at a greater distance from the methane seep source as well as directly test and integrate these newly developed assays with the D-ESP/quantitative PCR module.

3.9 Education and Outreach: animations

Working with external contractors, we have developed two animations to illustrate concepts behind current ESP operations and a variety of applications targeted in the future. The first has no audio and illustrates near surface water, moored deployment and basic operations involved in collecting water samples, preparing extracts, developing probe arrays, and telemetry of data to shore. The second has audio, and is geared more towards a general audience that might visit a technology museum, for example. The latter illustrates use of the ESP in several scenes: hydrothermal vent, “red tide”, fish aquaculture, harbor/sewage, and finally a connection with the Astrobiology program to look for signs of life beyond Earth. Both animations will be made available on the ESP web page soon, and we are also distributing them ad hoc (via MBARI’s ftp server) on request.

3.10 JPL

The JPL team initiated work February 2006. Since that time they have:

- Completed conceptual design for flight-compatible ESP.
- Purchased environmental chamber for component evaluation in vacuum, temperature (-85C to +100C), or Mars atmosphere.
- Evaluated a variety of candidate “detection (or analytical) modules” including microfluidic CE and HPLC-chip that can be coupled to the core ESP sample processing system.
- Purchased glass ampoule sealer for storing liquid reagents (method used by Viking Landers).
- Began design for microfluidic CE injection system.

Conceptual design of flight-like ESP was originally proposed as a Year-3 deliverable (Fig. 7). The flight-ESP design concept is based on the following considerations, which are intended to make this instrument a strong candidate for future planetary missions.

- **Flight heritage and compatibility**.
- **Thermal cycling and lifetime considerations.**

- **Established experimental procedure**.
- **Design simplicity and ease of refurbishability.**
- **Miniature, low power, and rugged components.**
- **Planetary protection (PP) and contamination control (CC) compatibility.**

The flight- ESP conceptual design is based on the use of glass and materials such as Teflon, PEEK, and Durapel that are compatible with flight-like thermal cycling temperatures and a wide range of extraction solvents and reagents. The flight-ESP design concept utilizes a combination of well-established fluid manipulation techniques which are compatible with planetary surface applications in order to move liquids from the macroscale to the microscale: **gravity feed** (suitable for macro-scale extraction), **solenoid pumps and valves** (suitable for meso-scale derivatization, filtering, and other fluidic manipulations), and **electrophoretic transport** (used to inject and separate during micro-CE analysis).

Glass can readily withstand the extremes of temperature encountered during flight qualification testing, and has already been demonstrated as a suitable material for transporting mL quantities of liquids to the surface of Mars on the two Viking Landers, which stored the "nutrient broth" for the Labeled Release experiments in sealed glass ampoules (Levin and Straat, 1977). Solid reagents will be stored in on-chip glass reaction chambers that can be sealed by spot-welding metal foils onto the top surface. This technique is currently being developed for Sample Analysis at Mars (SAM) GC/MS instrument on the MSL'09 mission.

The flight-ESP can potentially contribute to the science goals of future NASA missions to the Moon (to analyze for exogenous organics at the poles), Mars (to search for past or present life and to better understand exogenous delivery of organics and the impact of hydrothermal production), Europa (to search for evidence of biotic or prebiotic chemistry through hydrothermal production), or Asteroids/Comets/KBOs (to better understand the early chemistry of the solar system). In particular, we expect that this instrument will be a strong candidate for inclusion on a *Mars Astrobiology Field Laboratory*, a *Europa Surface Lander*, and a *Titan Aerobot*.

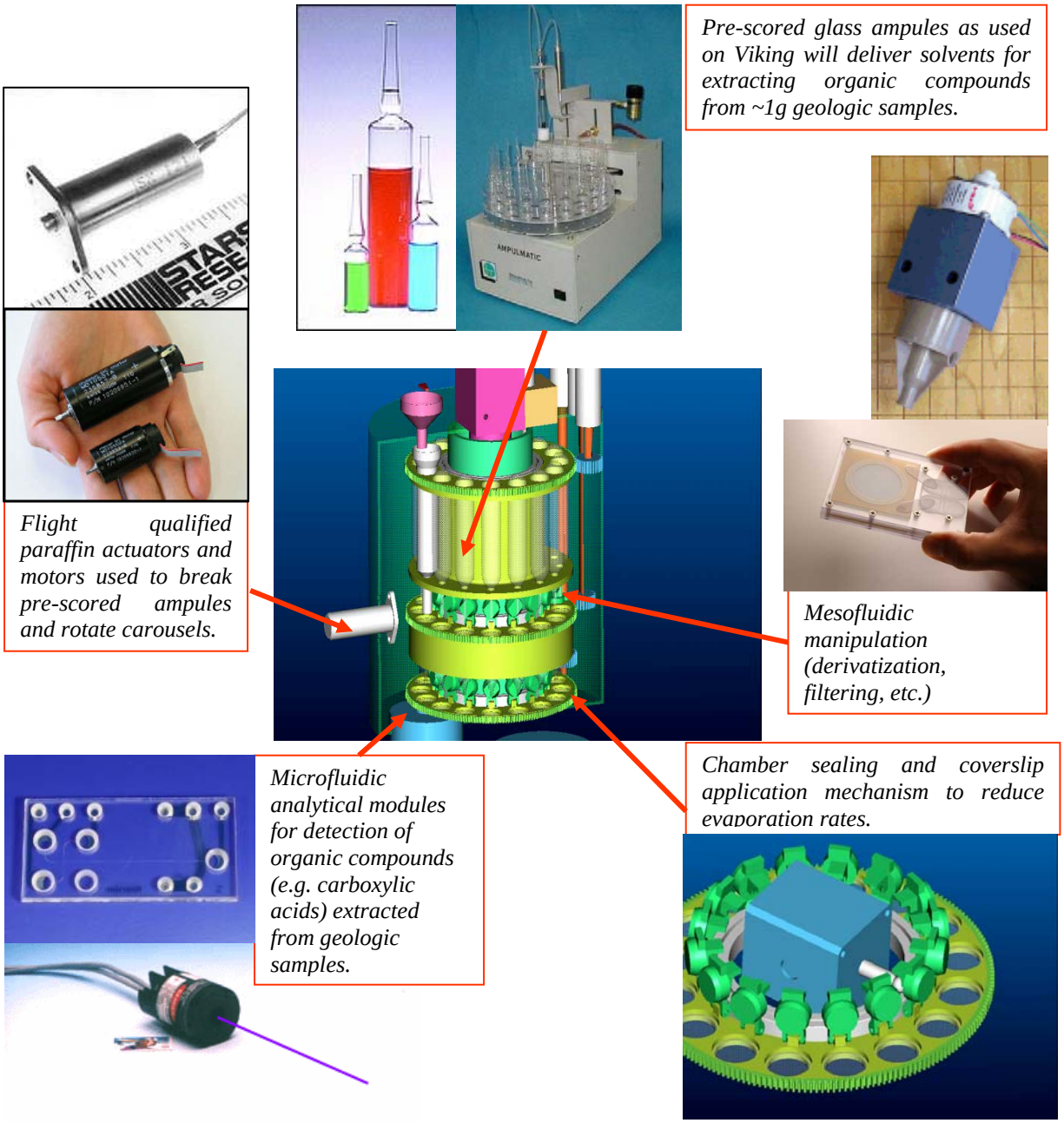

Figure 7. Conceptual design for a Flight-ESP.

4. Plans for 2008

Over the next year our objectives are to:

1) Integrate the MFB and PCR module with NEO (Q4 2007-Q2 2008).
2) Qualify the system (1) for use at 2-25^{o}C (Q2 2008)
3) Fabricate, integrate and test the 4000m-rated pressure sampling module and core ESP housing (Q1-3 2008).
4) Deploy NEO with MFB/PCR in Monterey Bay surface waters using the existing surface mooring (Q2-3).

5) Deploy the 4000m-rated D-ESP (4km D-ESP) in Monterey Bay and contiguous waters using the R/V Western Flyer and ROV Tiburon II (Q3-4 2008). We will target use of PCR module for detecting microbes and specific genes in the water column and benthic communities (>3200m, cold seeps, whale falls). At this time we plan two, 4-day cruises (http://www.mbari.org/dmo/vessels_vehicles/Western_Flyer/flyer.html).
6) Install the 4km D-ESP on the MARS (http://www.mbari.org/mars/) cabled observatory for extended trials/qualification for subsequent deployments (Q4 2008).
7) Demonstrate application of the MFB/PCR unit in surface and deep-water deployments.
8) Plan for deployment of the 4km D-ESP at Axial Seamount in 2009 (Fig. 8) in collaboration with David Clague and others.
9) Flight-ESP: Future tasks will focus on refining the design, evaluating detection strategies and candidate "detection modules," and demonstrating key sub-components including fluidic valves and reagent storage techniques in order to increase their TRL level. Another focus will be to develop a strategy for sample injection into microfluidic devices.
10) Submit papers for publication: several describing the ESP system, its application in surface and deep-waters, probe chemistries, diversity and stratification of aerobic methanotrophs.
11) Update the ESP web site.
12) Seek out Education/Outreach opportunities through EARTH (http://www.mbari.org/earth/default.htm), CMORE (http://cmore.soest.hawaii.edu/; http://cmore.soest.hawaii.edu/cmore_theme3.htm), and the Monterey Bay Aquarium (http://www.montereybayaquarium.org/efc/efc_mbari/mbari_home.asp).

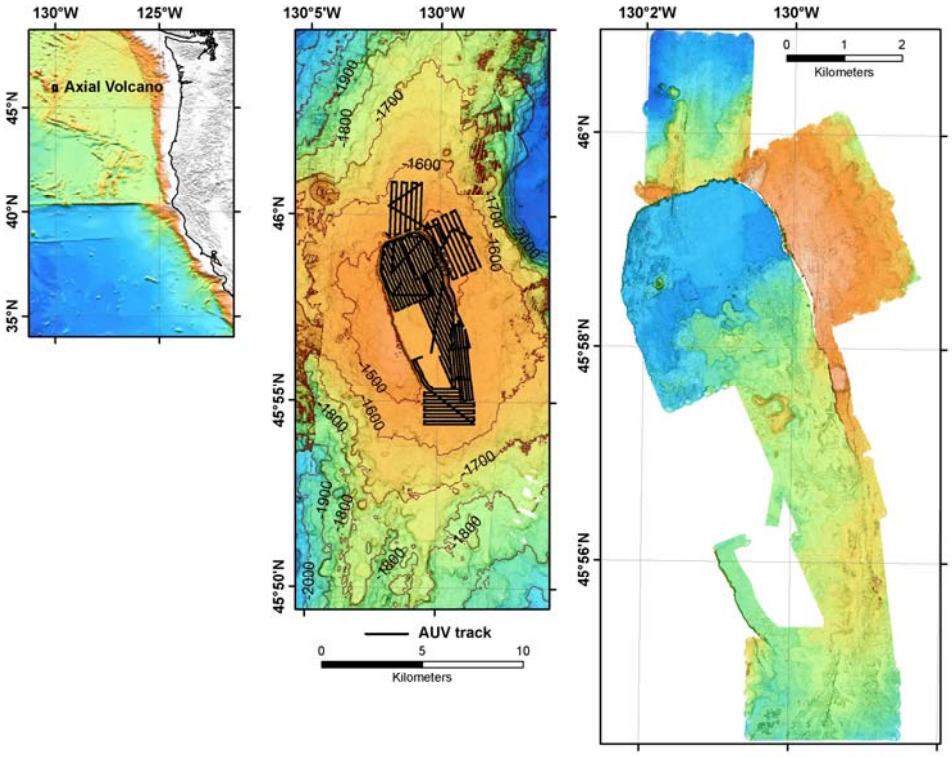

Figure 8. From left to right, site map, AUV mapping track, and detailed bathymetry from the mapping AUV (http://www.mbari.org/auv/MappingAUV/Default.htm). Work by David Clague's group at MBARI is providing a detailed view of Axial Seamount, including location of hydrothermal vents that were previously unknown. We plan to deploy the 4km D-ESP at this site and elsewhere along the Juan de Fuca plate in 2009.