Modification of the Environmental Sample Processor (ESP) to Enable Remote Detection of Marine Microbes and Genes They Express

A proposal submitted to the Moore Foundation in response to a request from Dr. Lita Proctor

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Introduction

The study of aquatic organisms at the molecular level is a mainstay of modern biological oceanography. Numerous diagnostic procedures exist for identifying marine organisms and their genes and gene products, and for elucidating the roles they play in biogeochemical cycles. While such techniques form the cornerstone of many research initiatives around the world, their application typically requires the return of discrete samples to a modern laboratory for analysis. Logistical difficulties associated with collecting those samples at multiple locations routinely and synoptically, and processing them rapidly in the field, limits the application of molecular analytical technologies in an environmental research and monitoring context. In stark contrast, many physical, chemical and gross biological properties of the water column are routinely assessed remotely at high frequency using a variety of air borne and in-water sensor arrays. The disparity between our capacity to measure physical and chemical oceanographic parameters versus our ability to understand the distribution, abundance and activities of specific microorganisms within that milieu, in real-time, impedes our ability to characterize and respond to a wide range of biological phenomena. The overriding goal of my research program is to develop methods and instrumentation that make it possible to overcome this limitation, to conduct molecular biological analyses in real-time, in the field, autonomously if desired. This proposal focuses on developing that capability further, specifically for marine microbes in collaboration with Dr. Jon Zehr and others at the Microbial Genomics Experimentation and Remote Sensing Laboratory, the University of California at Santa Cruz.

The Environmental Sample Processor (ESP)

Many of the diagnostic procedures for detecting molecular signatures share an overlapping set of requirements, such as collecting, concentrating, preserving and disrupting cells, applying a series of reagents in a timed sequence, removing particulates, applying solid phase extraction chemistries, and so on. Operationally, these methods can be divided into those that depend on intermolecular reactions, like and antibody/antigen binding, nucleic acid hybridization and enzyme mediated processes, and those that rely on separation and identification of target molecules based on their inherent physical properties. Optical and electrochemical transducers are used commonly to detect and quantify numerous molecular signatures. By developing a relatively low-power, compact instrument that meets this core set of functional requirements, it is possible that one could conduct many commonly applied molecular biological analyses remotely, in situ.

In an effort to realize that goal, a group of scientists and engineers at the Monterey Bay Aquarium research Institute (MBARI) undertook development of the Environmental Sample Processor (ESP; Scholin et al. 1998, 2001, 2005). The ESP is an electromechanical/fluidic instrument system designed to collect discrete water samples from the ocean subsurface, concentrate microorganisms (particulates), and automate application of molecular probes to identify microorganisms based on ribosomal RNA (rRNA) signature sequences. In addition, the

ESP archives discrete samples for a variety of nucleic acid analyses, microscopy and other types of analytical procedures after the instrument is recovered. To date, we have focused our analytical capacities on a suite of marine planktonic organisms ranging from heterotrophic and photosynthetic bacteria, archaea and eucarya to small invertebrates found in the upper ocean. "First generation" (1G) prototypes of the ESP have been deployed in Monterey Bay and the Gulf of Maine (e.g., Goffredi et al. in revision, Scholin et al. 2005).

To demonstrate the feasibility of detecting of target organisms remotely in the ESP we devised custom DNA probe arrays that target rRNA sequences indicative of specific species or groups of species (e.g., Figs 1, 2). To develop a probe array the ESP first collects a sample and removes seawater, then homogenizes material retained using a chaotrope and heat. The resulting crude homogenate is applied to the array, followed by a sequence of reagents that reveal target molecules retained at specific locations on the array grid using sandwich hybridization and chemiluminescent signaling (Scholin et al. 1997, 1999, 2005). An image of the resulting array is captured by a CCD camera and transmitted to a remote location for interpretation. The entire automated process, from collection of a live sample to broadcast of an imaged array, takes ~2 hours and occurs subsurface. The reagents employed in these assays are stable for extended periods (none used in the ESP require refrigeration), and the chemical reactions themselves are amenable to microfluidic scaling. Different arrays can be tailored to specific groups of organisms such as 'planktonic microbes', 'harmful algae,' or 'invertebrate larvae,' etc. In this fashion we have demonstrated that the ESP can support detection of many different rRNA target sequences using a common methodology, suite of reagents and core sample processing instrumentation.

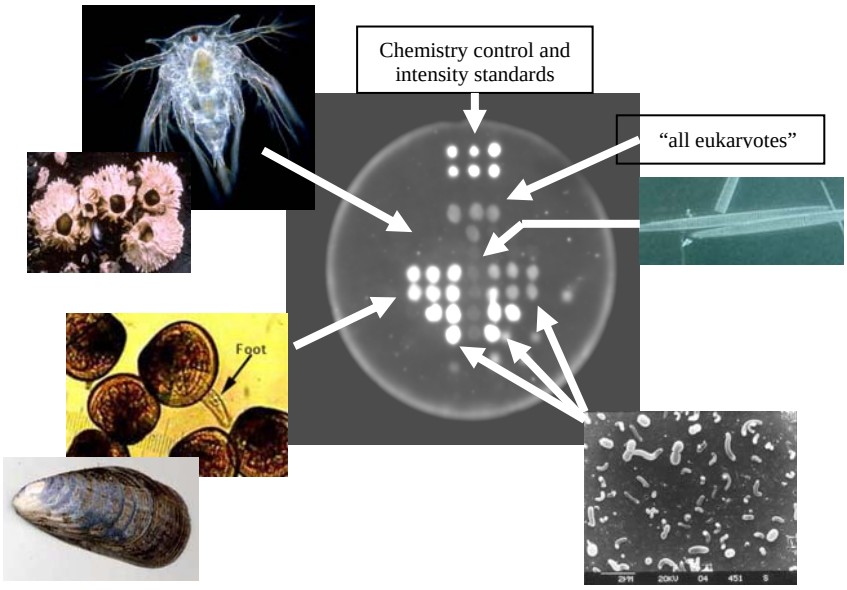

Figure 1. Example of a custom 25mm DNA probe array developed automatically in the ESP using a natural seawater sample. Printed for demonstration purposes are (top center) probes for chemistry controls and array intensity standards, and (clockwise, right to left) small-subunit rRNA-targeted, probes for "universal eukaryote," "pennate diatoms," specific groups of marine bacteria (*Roseobacter*and *Cytophaga*), mussel larvae and barnacle larvae. This array illustrates the simultaneous detection of mussel larvae, *Roseobacter* (weak) and *Cytophaga* (strong) and pennate diatoms (weak); barnacle larvae were not present in the sample.

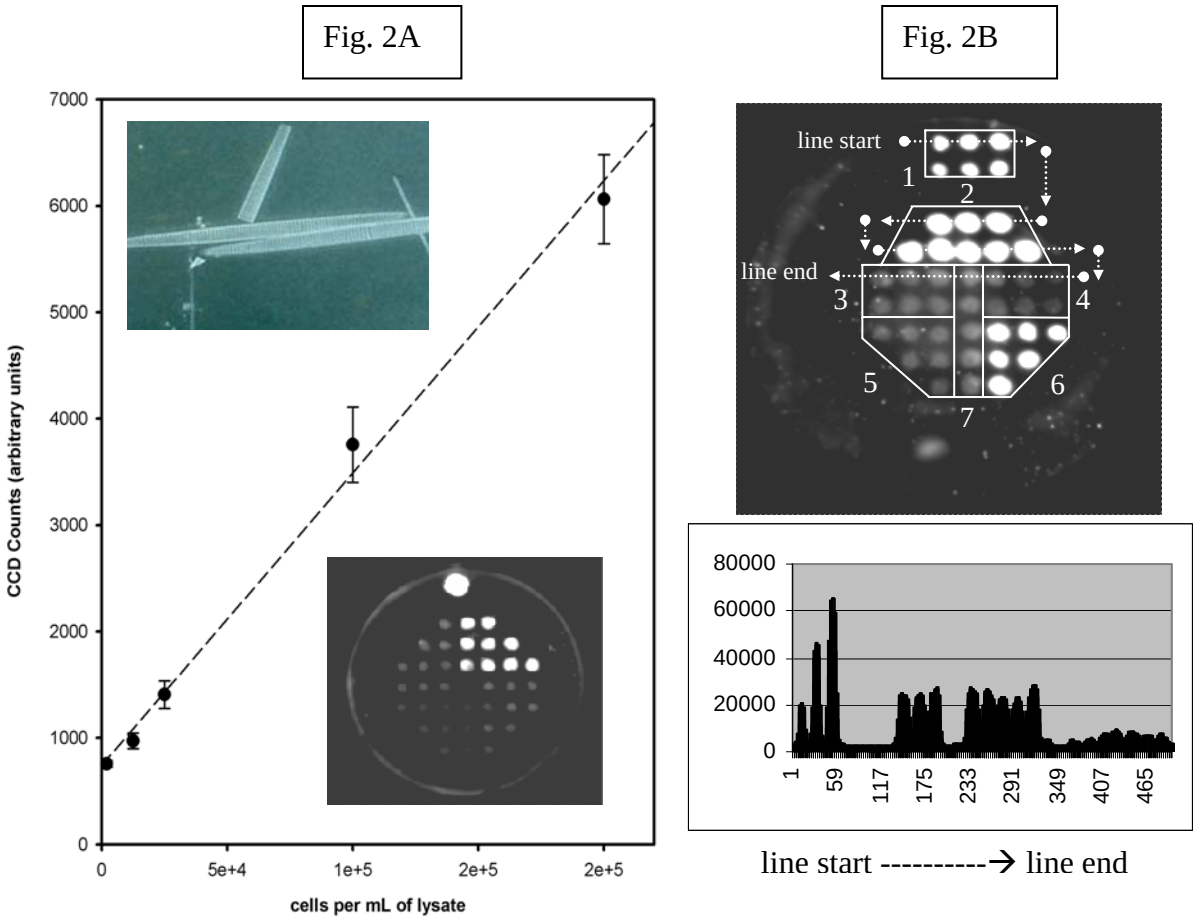

Figure 2. Examples of 25mm diameter DNA probe arrays for the ESP. A) Standard curve showing response of species-specific rRNA probes to *Pseduo-nitzschia australis* (inset above), a toxic marine alga. The array shown (inset bottom) carries probes for a variety of toxic phytoplankton present in Monterey Bay, CA. The low end of the curve represents the equivalent of 1e4 cells L^{-1} and the upper end 1e6 cells L^{-1} when ~400ml raw water is concentrated and homogenized in 2ml lysis buffer. The arrays used to generate this data were processed manually in a lab test fixture. B) Top: Marine bacteria array developed in the ESP printed with integral chemistry controls and intensity standards (1) and probes for a variety of marine microbes (2- *Cytophoga*; 3-Sar11; 4-Arch Grp II; 5-Sar 86; 6-*Roseobacter*; 7; picophytoplankton). The sample consisted of 50ml raw seawater concentrated through a 0.2µm pores size filter. Each group of probes is printed as replicates. B) Bottom: An example of data extracted from the images using a line tool to define pixel value (y axis; CCD counts) as a function of total line length (x axis; pixel counts).

Development of a second generation (2G) ESP is being carried out with support from the National Science Foundation (NSF, OCE-0314222) and by MBARI. Surface ocean (<50m) deployments of the first 2G ESP are scheduled for summer 2005. The design goal underlying the 2G ESP is to develop a modular core instrument system that can be re-configured/modified to suit a wide variety of deployment and analysis scenarios. It will build on the same functional concepts and chemistries validated in the existing unit, with emphasis placed upon making a more, robust, compact, lower power, and user-friendly device. Three major components comprise this new instrument:

Core Sample Acquisition & Analysis System (2G ESP)

- Suitable for collecting small to moderate sized samples (ml's to 2L)
- Automates sample archival (for whole-cell and cell-free assays), as well as development of DNA probe arrays and competitive ELISAs in siti, in near real-time.
- Utilizes modular subsystems to enable re-configuration for unique applications.
- Sample collection and processing stages are separated, providing dedicated subsystems for inherently "messy" operations of collecting/preserving/extracting samples and for meeting "clean" requirements associated with sample analysis on a mircofluidic scale.

External Sample Acquisition Modules

- Used for meeting specialized sampling requirements (e.g., for large sample volume concentration, constraints posed by deep-water environments, etc.).
- Can be added to or removed from a core ESP system as needed.

Analytical modules

- Custom analytical devices that require upstream sample collection and processing.
- Integral to the core ESP, accessed as a separate modular subsystem.
- Core ESP provides housing, power, fluids (pre-processed samples, reagents if needed, etc.), and data/control communications.
- Can be added to or removed from a core ESP system as needed.

Under the auspices of a grant from the Keck Foundation, MBARI is building a prototype pressure housing and sample collection module suitable for deploying the 2G ESP at depths to 4000m. This version of the instrument is known as the deep-sea ESP, or D-ESP. Our goal is to deploy the D-ESP in areas of intense geological and microbiological interest (e.g., hot vents, cold seeps) and link the relationships between tectonic activity, fluid flow and microbial flux from below the seafloor to overlaying waters in time series fashion using the same detection chemistries as those developed for surface water applications. To realize that goal, we will first mount the prototypical D-ESP on an MBARI ROV so that it can be tested at variable depths and locations. Once that is done successfully, we hope to transition the D-ESP in 2006 to benthic observatories, including stand-alone moorings and cabled systems.

Research objectives with support from the Moore Foundation

The long-term goal of my work is to enable scientists and resource managers to utilize a distributed set of ESPs in an interactive manner, as a configurable set of remote laboratories that can be commanded either manually or in pre-programmed fashion to carry out a variety of molecular biological analyses. One of the most exciting prospects for developing and implementing that vision rests with investigators studying marine microbes and the role they play in mediating oceanic biogeochemical cycles (e.g., Béjá et al. 2000, Suzuki et al. 2001, Zehr, J. P. and P. J. Turner 2001, Hallam et al. 2004, Jenkins et al. 2004, Venter et al. 2004). In particular, there are numerous opportunities for developing in situ instrumentation that involve detecting functional genes and assessing their expression in response to varying chemical and physical regimes. Using the ESP to carry out such investigations requires a quantum leap in development of its analytical capability. For example, beyond collecting samples and creating

crude homogenates to develop rRNA-based arrays, current methods used in the laboratory for microbial genomics work dictate nucleic acid purification and amplification, application of microarrays and interrogating amplification reactions in real-time, and detecting specific gene products. Developing those capabilities within the envelope of the ESP is within the realm of possibility, but doing so requires a dedicated team of scientists and engineers that can focus on prototyping custom hardware, developing new protocols, experimenting with alternative methods of acquiring data (e.g., eliminating the need for PCR), and doing all of that on just a few watts (!), etc. Raising support for that effort is exceedingly difficult to do via conventional grants given the perceived risk associated with the project and unattractive high salary cost. This is not "safe science." With support from the Moore Foundation, however, MBARI can assemble a team to begin to address this challenge, a group already experienced in developing and deploying the ESP. The collaboration with Dr. Jon Zehr et al. offers further opportunity as that input can help refine and guide instrumentation development and marine operations activities that target specific applications of microbial genomics information. An apt acronym for this work might be the "Microbial Environmental Genomics Applications (MEGA) Project."

This proposal is conceived around the idea of the "analytical module" concept. That is, to utilize the existing core ESP, as well as emerging sampling module and target molecule fractionation/purification capabilities, to fulfill the generic steps of sample acquisition and processing that are needed to "feed" new analytical systems essential for applying microbial environmental genomics data in an autonomous sensory context. Three major objectives fall roughly over 1-year periods as follows:

Year 1: Definition of functional requirements: What methods does the ESP need to support, what are the requirements associated with them, and how can we rapidly assess feasibility of meeting those needs? During the first phase of this project, the MBARI team will work with Zehr et al. to define in engineering terms requirements of translating essential analyses used in the laboratory to ones that are suitable and most desirable for deployment in situ. Detecting organisms and genes involved in nitrogen cycling will serve as an initial focus for this work. Defining functional requirements involves a broad suite of activities, ranging from identifying candidate assays along with their sample acquisition and handling requirements, to assessing the reagents required (their stability, needs for storage, possibility of using substitutes such as dehydrated reaction cocktails, etc.), then narrowing the field to those that appear most promising for autonomous deployment. We will then evaluate existing "analytical modules" that could be used to meet identified needs (e.g., small, portable nucleic acid amplification devices already prototyped). This will involve travel to a number of different labs that we have budding collaborations with, including Lawrence Livermore National Laboratory (Mariella et al.; e.g., Belgragder et al. 2003), Univeristy of Miami (RMAS, Goodwin et al.; e.g., LeGier et al. in press), and Univerity of Tokyo (Fuji et al.; e.g., Fukuba et al. 2004). Based on identified functional requirements, opportunities for developing formal collaborations, and/or our own custom design solutions, we will then undertake development of a dedicated laboratory test fixture that incorporates components of the ESP system alongside appropriate electrical/fluidic connections for incorporating new analytical modules. Using "gold standard" laboratory techniques and equipment (e.g., provided by Zehr et al.) for comparison, we will then determine if basic upstream sample collection

and processing requirements are met with existing hardware and protocols, and if not then undertake research and development activities to ensure those needs are met. The same methods will be applied to prototype downstream analytical technologies, to assess their utility for carrying out necessary reactions with appropriately prepared samples. This test fixture will allow us to swap out components readily, and thus modify designs and applications at a faster rate than would be possible by working with an existing ESP that is compacted for deployment in a pressure housing. A key goal associated with this phase of the project is to ensure that tools and techniques being developed are generic, and are clearly applicable to a host of environmental research efforts that extend well beyond oceanic N cycling. Collaborations and consultations with researchers outside of UCSC (e.g., E. DeLong, D. Karl, et al.) will help guide us in this regard.

Year 2: Instrumentation prototyping and modification of the existing ESP. Based on activities carried out in Year 1, we will select assays that we believe can be supported by the ESP and create detailed designs for integrating them into a dedicated instrument. The various electrical and mechanical components required will be fabricated and once more tested in the laboratory, comparing results against "standard" procedures and methods used in the Microbial Genomics Experimentation and Remote Sensing Laboratory. This will involve building custom hardware, modifying designs or manufacturing procedures as necessary, etc., until satisfactory results are achieved. We will strive to have complete assays – from live sample collection, to processing, to reporting of results – running on the dedicated test fixture side-by-side standard lab equipment by the end of Year 2.

Year 3: Field studies to assess the feasibility of conducting microbial genomics investigations remotely, in situ. By year 3 we will seek to build and deploy a customized ESP that meets analytical requirements defined, refined and evaluated during Years 1 and 2. The specific location(s), duration and a detailed list of objectives of these deployments will be determined near the end of Year 2. Our overall goal is to demonstrate the feasibility of sensing microbes, specific genes and expression of those genes remotely, in situ, using the ESP system, and to link variations associated with those parameters to changes in the chemical and physical environment.

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