THE ENVIRONMENTAL SAMPLE PROCESSOR - A NOVEL INSTRUMENT FOR THE AUTONOMOUS COLLECTION AND REAL-TIME DETECTION OF MICRO-ORGANISMS

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INTRODUCTION

Understanding the presence, abundance distribution and population dynamics of micro-organisms requires frequent collection of discrete water samples at many locations and depths. However, collecting appropriate water samples over relatively large spatial and temporal scales (e.g., several square kilometers: months to years) is limited by the frequency one can visit a particular location and the amount of time one can occupy that station. Similarly, providing quantitative measures of the abundance of a wide variety of organisms that may inhabit a suite of sampling sites is severely restricted by the time and labor necessary for sample processing, In stark contrast, many physical, chemical and gross biological properties of the water column may be determined in real-time using a variety of air borne, shipboard, moored and/or drifting sensor arrays (e.g.,htt;://www.mbari.org/oasis/index.html).

Once samples are collected, molecular probe assays (DNA, antibody, etc.) offer one means to speed and ease the detection and quantification of an enormous variety of organisms as well as the particular genes they harbor and express. However, such applications are presently hindered by the need for highly repetitive operations that demand trained personnel and specialized laboratory facilities. These requirements restrict the utilization of molecular probes for real-time ecological studies because the rate of sample processing is often limited and application of the technology outside of a laboratory can be difficult, impractical or impossible.

In an effort to overcome these problems, a novel instrument, the environmental sample processor (ESP), is being designed. The ESP will collect discrete water samples autonomously, concentrate particles contained within those samples onto filter disks and automate application of species-specific DNA probes to identify and quantify the organisms captured. In addition to archiving discrete samples, the instrument is intended to transmit results of the probe assays in real-time to a remote location for data processing and interpretation. By integrating a network of these sensor with those aimed at characterizing physical and chemical properties of the water column, we aim to generate synoptic views of the distribution and abundance of target species in an environmentally relevant context, remotely and in real-time. Although the current focus of the project is the detection of harmful algae using species-specific DNA probes, the ESP could be modified to detect a wide range of water borne microbes by changing the probes applied by the instrument.

ESP FUNCTIONALITY

The methods used to process phytoplankton samples for species determination using either standard microscopy or molecular probe methods require particles (organisms) from a water sample to be concentrated on a suitable media, followed by sequential additions to and/or exchanges of reagents across the collected cell material. Once this is accomplished, cells may be 1) archived for microscopy, toxin analysis, nucleic acid extraction, etc. 2) intact cells may be labeled with lectin, antibody or DNA probes, 3) cells may be homogenized with subsequent applications of molecular probes to reveal specific molecules (toxins, proteins, nucleic acid sequences) indicative of certain species or groups of species (e.g., Scholin et. al. 1997, Miller and Scholin 1998, Scholin and Anderson 1999, Scholin et. al. 1999, 2000). The ESP has been designed to automate and repeat these processes as specified by a program designed by the user. In addition, the ESP is intended as a stand alone instrument for submerged operation on moorings and drifters. In order to support these type of remote operations, the instrument is designed to allow complete remote control and data upload capabilities via radio modem.

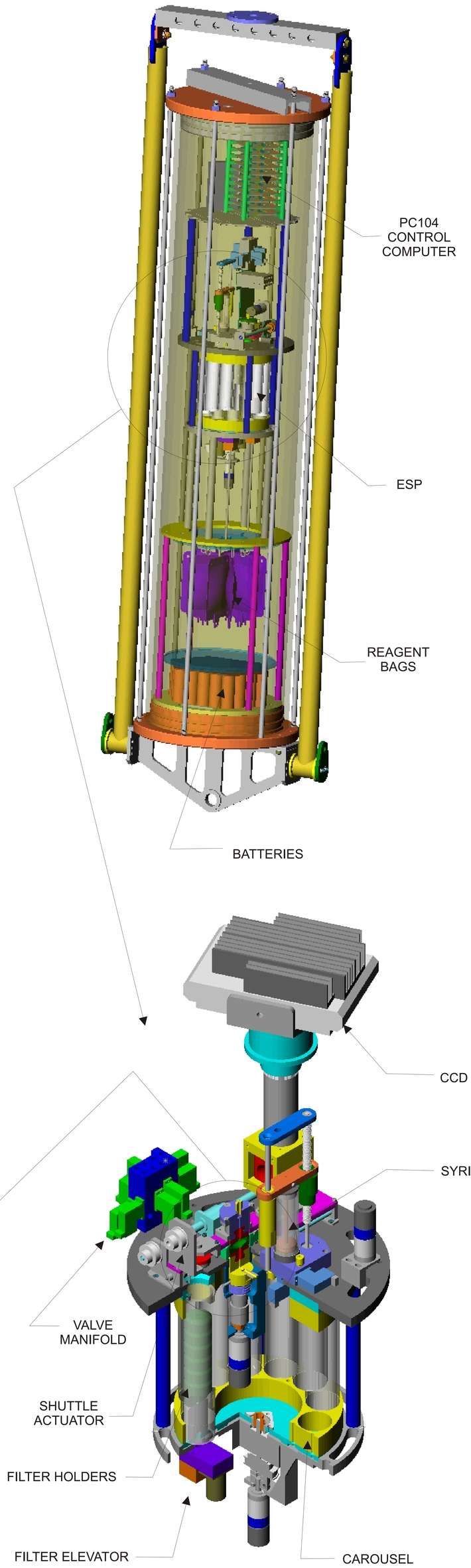
The general functionality of the instrument is as follows. Up to 100 filter holders can be stored in the rotating carousel. Several types of filters are used in these filter holders. Currently we use a glass fiber filter for toxin analysis, a polycarbonate filter for whole cell analysis, a hydrophillic Durapore filter for creating cell homogenate and a custom oligonucleotide array for sandwich hybridization. A carousel tube with the appropriate filter is positioned over the filter elevator which lifts the filter into the shuttle. The shuttle slides the filter holder into the filter seal clamp. The clamp seals to the top and bottom of the filter holder providing a connection to the upper and lower valve manifolds. Additional valves allow the syringe pump to pull reagents top to bottom across the filter from the upper manifold or push reagents from the lower manifold bottom to top across the filter. Waste may be discharged overboard or captured on board in a dedicated reservoir. The filter manifolds are organized as blocks of 8 and can be cascaded for additional capability. In the current format, we have 16 valves (2 manifold blocks) connected to the top of the seal clamp and 8 valves (1 manifold block) connected to the bottom.

Once the filter holder is clamped in the filter seal clamp, the syringe pump pulls sea water through the filter holder concentrating particles (organisms) on the filter media. Now the syringe pump is used to sequentially add and exchange reagents across the collected material. The seals used in the filter seal clamp have been designed with embedded heater pads. This allows the filter holder to be heated above 100°C at any point in the sequence. At this point, the product may be pulled into the syringe pump and used in a protocol required a different filter media (e.g., sandwich hybridization). Alternatively, the product may be an image captured by the scientific grade CCD camera integrated in the ESP. The last step stores the processed filter holder in a empty tube. At his point the ESP can continue processing using another filter holder or got to sleep, waiting for the next wake up call to start processing the next sample.

CONTROLLING THE ESP - THE ESP MACRO LANGUAGE

A primary design goal of the ESP is to develop an instrument capable of automating a wide variety of chemical protocols. To this end, we have designed in a number of features, modular valving to support a relatively large number of reagents, filters holders which can support a wide variety of media, a highly accurate and repeatable syringe pump capable of accurately drawing, mixing and dispensing fluid volumes from 10s of µl to 20 ml and the ability to heat the sample at any time during the protocol to temperatures above 100°C. Perhaps the most important feature of our modular approach is the ESP macro language, a very simple ASCII test based language used to define the exact sequence of steps which are automatically performed by the ESP. Dozens of protocols have been developed to automate the processes described in this poster. A typical protocol consists of predefined macro calls. At the lowest level, a macro call performs a basic operation on the ESP such as loading a filter, setting the valve configuration, heating to a user specified temperature for a given time, acquiring an image, drawing a user specified volume of sea water across the filter, dispensing a user specified volume of reagent into the filter holder, etc. Sequences of these low level macros can be written as a subroutine to perform a specific protocol e.g., whole cell archive, toxin archive or sandwich hybridization. Any number of these subroutine macros can be called from a higher level macro that wakes up the instrument, performs a sequence of subroutines, goes to sleep and waits for the next wake up time. A segment of a typical used written subroutine macro looks like the following (comments are preceded by the # character).

```
# Program test file for the Genosensor DELTA unit
# This macro represents the steps taken to create lysate as part 1 of the 2 step (filter) Sandwhich Hybridization process.
# CREATED 11/29/99 BY C.Scholin, R. Marin; modified3/6/00
# This program uses pre-mix lysis/sample buffer in lower valve 3 the 5xSET 0.01% IGEPAL rinse is included through the sample &
#Setrinse ports, air and toggle manifold used to clear lower loop, 1.5 ml lysate, heating time 14 min, no backlash comp required
# lysis push @.2 lots to reduce foaming
PROGRAM: SANDHYBE1test35
SYRINGE PULL 7.5 Sample
DELAYSEC 30
SYRINGE PULL 5.0 Sample          # this is FSW to clear line
DELAYSEC 10
SYRINGE PUSH ALL Waste
SYRINGE PULL 5.0 Setrinse  # 5x SET with 0.01% IGEPAL
DELAYSEC 10
 SYRINGE PULL 5.0
DELAYSEC 10
 SYRINGE PULL 5.0
```



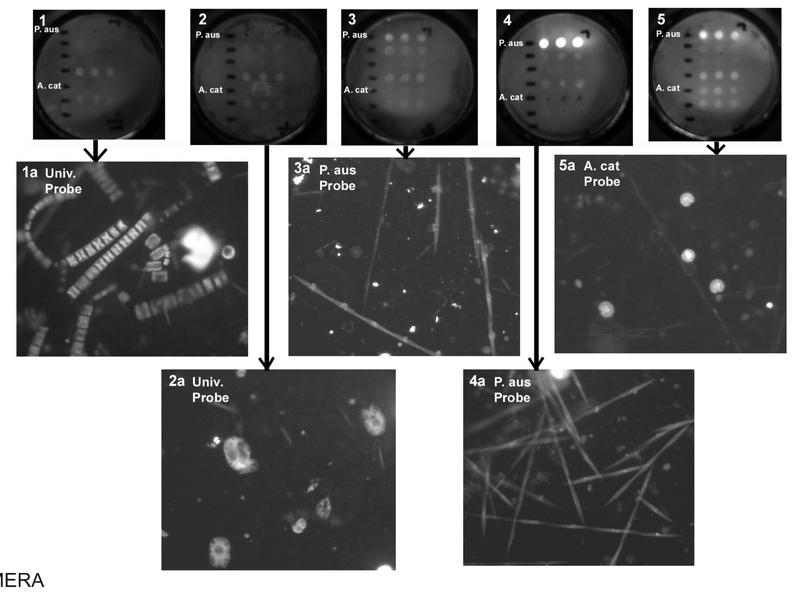
ESP FIELD RESULTS FROM THE MUSE EXPEDITION

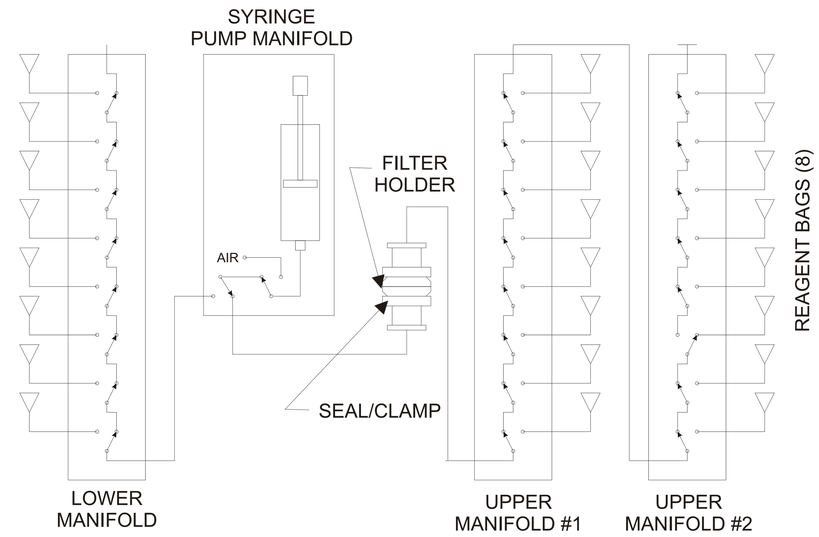
Two prototypes of the Environmental Sample Processor, or ESP, were deployed in Monterey Bay this past August during the Monterey Bay Upper Water Column Experiment (MUSE). One instrument is built for lab/shipboard use, while the other is battery-powered and designed for in situ, subsurface, autonomous operation. The shipboard unit was used to generate cell homogenates (crude cell lysates) and develop prototype oligonucleotide probe arrays to reveal the presence of several different harmful algal species in near real-time. The same instrument allowed us to archive discrete samples for subsequent whole cell DNA probe application and toxin analysis. The in situ ESP was successfully moored in 100m of water, with the sample processor module immobilized at 5m and tethered to a surface float. This system underwent an electro-mechanical test that exercised pre-programmed macros for wake-up/run/sleep computer control functions and a sequence of loading/unloading of filter holders as well as pumping of reagents internally. The log files generated during the operation were successfully telemetered to shore via radio modem.

Examples of some of the results obtained with the shipboard ESP unit are shown in Fig X. Detection of two toxic species, the pennate diatom Pseudo-nitzschia australis and the dinoflagellate Alexandrium catenella, is emphasized. The top series of panels (samples 1-5) show images of the oligonucleotide probe arrays captured using the shipboard ESP CCD camera. Locations of probes, spotted in triplicate, for Pseudo-nitzschia australis and Alexandrium catenella are denoted "P. aus" and A. cat," respectively. To develop an oligonucleotide probe array, the ESP 1) collects 400 ml of surface sea water, concentrates particles > ~5µm onto a 25 mm Durapore filter leaving less than 0.1 ml of residual sea water behind, 2) injects 1.5 ml of lysis reagent, 3) heats the sample to ~85°C and 4) the resulting sample homogenate is drawn into the syringe. Using the shuttle and elevators, the Durapore sample collection filter is replaced with an oligonucleotide probe array. The sample homogenate is passed over the array, followed by a sequence of reagents that reveal rRNA molecules retained at specific locations on the array grid. In the examples shown here neither Pseudo-nitzschia australis nor Alexandrium catenella was detected in panels 1 and 2. In contrast, Pseudo-nitzschia australis was detected in panels 3-5, albeit at different concentrations as indicated by the variable intensity associated with the Pseudo-nitzschia australis-specific probes. Alexandrium catenella was detected in sample 5 only.

The ESP was also programmed to archive samples for whole cell and toxin analyses, enabling post-deployment verification of the data collected in real-time by the ESP on the oligonucleotide probe array. An example of the whole cell analysis is shown in panels 1a-5a. During the MUSE deployment and immediately after development of an oligonucleotide array, the ESP was programmed to concentrate and preserve an aliquot of the same sample used to generate the array. For this application, 40 ml of water was passed through a 25mm, 3 µm pore size polycarbonate filter. The sea water was then displaced by addition of a saline ethanol solution ("modfied EtOH/SET," Miller and Scholin 2000) and allowed to stand for 1 hr. The ethanol solution was withdrawn and the preserved sample stored dry onboard the ESP. Following the deployment, the ESP was plumbed with a fresh suite of reagents and programmed to process the archived samples using in situ hybridization (see Miller and Scholin 2000). Once the in situ hybridization process was completed, the sample filter was recovered, mounted on a slide, and viewed using epifluorescence microscopy. In the examples shown, several different rRNA probes were used: "Univ." (labels rRNA from all organisms), "P. aus" (labels rRNA specific to Pseudo-nitzschia australis), and "A. cat" (labels rRNA specific to Alexandrium catenella). Panels 1a and 2a reveal very different phytoplankton communities, neither of which harbor significant numbers of Pseudo-nitzschia australis nor Alexandrium catenella. Both were processed using the universal probe, labeling all species. The sample seen in 1a was collected near a site of upwelling where centric diatoms and heterotrophic dinoflagellates were abundant. The sample seen in 2a came from an area where nutrients were depleted, the abundance of phytoplankton much less than that in 1a, and the phytoplankton community was dominated by several species of dinoflagellates as well as very small pennate diatoms. Contrary to the first two samples, the probe array seen in panel 3 indicated the presence of Pseudo-nitzschia australis. To confirm this prediction, the corresponding, archived whole cell sample was processed using a probe that labels that Pseudo-nitzschia australis specifically. As predicted, the archived sample did indeed contain that species (panel 3a). Similarly, the probe array seen in panel 4 indicated Pseudo-nitzschia australis was very abundant; in situ hybridization of the corresponding sample revealed very high numbers of the expected species (panel 4a). The probe array seen in panel 5 predicts the presence of a number of species represented on the grid, in particular Pseudo-nitzschia australis and Alexandrium catenella. In this case, the archived whole cell sample was processed using a probe specific for Alexandrium catenella and that species was in fact found embedded within the phytoplankton community (panel 5a). In that same sample, one can also see the faint outline of large pennate diatoms (not labeled by the "A. cat" probe), the likely source of the Pseudo-nitzschia australis signal seen on the corresponding array.

Pseudo-nitzschia australis and Alexandrium catenella both produce potent neurotoxins, although different in structure and mode of action (Hallegraeff 1989). Thus, the oligonucleotide arrays make it possible to not only predict what samples (i.e., specific locations, times) contain what species, but also predict what samples contain what suite of toxins. To confirm those predictions, the ESP was programmed to collect a 750 ml aliquot through a 25mm GFF filters of the same sample used to generate the arrays and used for whole cell archives. The resulting "toxin archive filter" was stored dry onboard the ESP, recovered post-deployment and shipped to the NOAA Marine Biotoxins analytical facility in Charleston, SC. Analysis of these samples is on-going.





ESP PLUMBING SCHEMATIC