**Molecular-Biological Sensing in Aquatic Environments: Recent Developments and Emerging Capabilities**

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**Abstract**

Aquatic microbial communities are central to biogeochemical processes that maintain Earth’s habitability. However, there is a significant paucity of data collected from these species in their natural environment. To address this, a suite of ocean-deployable sampling and sensing instrumentation has been developed to retrieve, archive and analyse water samples and their microbial fraction using state of the art genetic assays. Recent deployments have shed new light onto the role microbes play in essential ocean processes and highlight the risks they may pose to coastal populations. Although current designs are generally too large, complex and expensive for widespread use, a host of emerging bio-analytical technologies have the potential to revolutionise this field and open new possibilities in aquatic microbial metrology.

**Background**

Marine microorganisms are central to our relationship with the sea. In coastal regions, changes in precipitation, sewage treatment and agricultural practices fuel harmful algal blooms and the dispersal of pathogenic microorganisms, with direct impacts on food biosecurity and human health [1]. In contrast, microbes have the ability to consume anthropogenic pollutants and thereby ameliorate the global human footprint [2], as well as sustain processes that are essential to earth’s habitability. In each case there is a definite and immediate need to increase the resolution of sampling and analytics in order to accurately determine factors impacting microbial communities, their distributions and the threats they may pose. With this in mind, the Global Ocean Observing System (GOOS) has prioritised zooplankton diversity, phytoplankton, microbes and harmful algal blooms as well as fish and apex predators among their list of “Essential Ocean Variables” or EOVs, for the development of marine sensing platforms.

The advent of molecular bio-analytical methods including genetic sequence amplification and quantification has revolutionised the study of ecology by providing an accurate and sensitive means of identifying and enumerating organisms, based on their unique genetic (DNA or RNA) signatures (“molecular ecology”) [3]. Genetic assays are able to distinguish species with no phenotypic differences in complex mixed species samples [4] and are ideally suited to automation. Achieving the goal of autonomous molecular-biological sensing in aquatic habitats would represent a step-change in our capacity to measure the majority of biological EOVs by increasing spatiotemporal sampling using the most state of the art scientific methods. This review highlights the most recent developments in this field and emerging capabilities for *in situ* genetic analysis, which will influence the future development of aquatic microbial sensors.

***In situ* Microbial Sampling and Sensing Instrumentation**

The available suite of ocean-deployable instrumentation for molecular ecology is summarised in Table 1. Photographs of the apparatus are shown in Figure 1. Most of these devices are Samplers, which collect water and / or filter retentate (cells and suspended particles), with or without preservation (archival) of the material for lab-based analysis upon retrieval. Microbiological samplers such as the PhytoPlankton Sampler (PPS), Remote Access Sampler (RAS) and Water and Microplankton Sampler (WaMS) are designed to collect samples amenable to lab-based DNA measurements following deployments, quantifying changes in key phylogenetic or functional genes (indicative of key populations) over space and time. Samplers that preserve cells *in situ* allow subsequent, lab-based quantification of the activities of key functional clades via transcript and / or protein quantification. The provision of RNA and protein preservation drastically enhances the level of analytics possible post sampling. Preservation-type samplers include the Biological OsmoSampling System (BOSS), the Suspended Particulate Rosette (SPR), the Microbial Sampler-*in situ* Incubation Device (MS-SID) and the Environmental Sample Processor (ESP). While the BOSS is small and robust, low sample volumes (typically < 5 mL seawater per time point) restrict the types of analytics possible. The SPR and MS-SID are optimised to provide material for detailed lab-based analytics but are not adaptable to *in situ* sample processing and analysis (sensing) in their current forms.

In addition to samplers are “ecogenomic sensors”, which perform *in situ* genetic analysis on collected samples, obviating the time lag, economic costs and potential “bottling artifacts” associated with sample transit to the lab or research vessel. The archetype ecogenomic sensor is the ESP, a low power, submersible instrument that delivers a suite of core functions (power, communications, reagents, water sampling, sample archiving and cell lysis) to support nucleic acid analysis (quantitative DNA amplification and RNA hybridisation assays) directly in the ocean [5]. It measures zooplankton, harmful algae and bacteria [6] and major discoveries include the association of zooplankton with oceanographic fronts [7], metabolism-driven transcriptional networks [8••,9], changes in carbon-fixing cyanobacteria within daily time frames [10••,32] and triggering of a molecular “switch” that results in the production of dimethylsulfoniopropionate (DMSP) in response to microbial interactions [12].

The scientific objectives of each study determine the design requirements for each system with the primary aims of (1) fixing cells immediately (within a few minutes) in the environment due to the labile nature of mRNA or the heterogeneity of the sampling environment and (2) high resolution sampling and / or sensing under conditions that allow the interpretation of activity within the natural environment of the cells (e.g. within ocean currents or hydrothermal plumes). For sensors, the analytics should replicate state of the art bench-top accuracy and sensitivity in a compact, power-limited instrument.

**Deployment Platforms**

The vast majority of these devices sample in a Eularian framework from fixed ocean moorings or ship-based deployments. Eularian sampling is needed to collect biological datasets in a time-series and to directly connect results with fixed observatories, to capture physics driven variation in space and time as well as long-term seasonality. The design and development of custom drifters for molecular ecology meets the objective of measuring natural biological variation *in situ* by ”following the microbes” to collect a pelagic microbial community over time. While these devices are semi-Lagrangian, Acoustic Current Doppler Profiler (ADCP) measurements from drifters often show little cross-instrument current, supporting the interpretation of collected samples from a single community. Drifting instrumentation for molecular ecology include the MS-SID and ESP (Table 1), which are currently limited to short-term time-series, with maximum deployments of two weeks.

**Recent Developments in Autonomous Sampling**

 Autonomous samplers provide the essential “front end” sample collection and fixation for downstream sensing and / or lab based analytics. One of the most sophisticated of these is the SUspended Particulate Rosette (SUPR) Version 2, which has been developed for rapid seawater filtration and preservation, enabling the capture of hydrothermal venting fluids whilst minimising entrainment of background seawater in the highly dynamic hydrothermal environment [13••]. The current design incorporates a specialist pumping system featuring a dynamic flow rate, which reduces sheer-induced damage of the retentate and improves battery performance. Pilot field deployments used ROV (remotely Operated Underwater Vehicle) and CTD (conductivity, temperature and depth-measuring instrument) platforms to sample the Von Damm (2300 m depth) and Piccard (4987 m depth) hydrothermal vent sites on the Mid Cayman Rise [13••], two distinct regions with regard to geochemistry and microbial communities. While the buoyant vent plume was sampled using both the CTD and ROV, endmember fluids, characterised by higher temperatures and dissolved SiO2 concentrations, were more consistently sampled by ROV. Several deployments successfully recovered hydrothermal microbial communities, finding that compositions in the buoyant plumes at each site were similar, but with notable differences in relative abundances of members of the Archaea and Epsilon proteobacteria. Given the agility of the SUPR, which is able to collect filtered particulates (including microbes), filtrate and whole seawater, and its ability to sample at depth, it is an obvious choice for studies of the deep sea, an environment that is massively under-sampled and ill-explored. More recently the SUPR has been integrated with the REMUS 600 autonomous underwater vehicle (AUV) and used to autonomously collect seawater containing planktonic larvae for subsequent analysis by microscopy and DNA barcoding. Two recent deployments of the “SUPR-REMUS” provide a proof of concept for the use of this device in large scale studies in coastal regions and its ability to collect samples for state of the art DNA analysis indicates a capacity to revolutionise population genetics and conservation in aquatic environments [14].

***In situ* samplers for Biogeochemical Studies**

 The Microbial Sampler-Submersible Incubation Device (MS-SID) is the ideal instrument for biogeochemical studies due to its ability to measure the rates of biogeochemical processes using labeled compounds in parallel with the collection and preservation of time-series samples for molecular biology, *in situ* [15•,16]. The device is equipped to carry on-board solutions of labeled “tracer” compounds, which are mixed with a seawater sample drawn into an incubation chamber using a syringe-like pump. The sample and tracer can be incubated to infer the rate of a biogeochemical process. The incubation is terminated by filtration and the filters are collected upon instrument recovery and processed in the lab. The SID was demonstrated for the measurement of primary production, dinitrogen fixation and nitrification on a single Lagrangian drifter in the North Pacific Subtropical Gyre in 2011 (<http://hahana.soest.hawaii.edu/cmorebiolincs/biolincs.html>), indicating the adaptability of this system, with similar results measurements from shipboard incubations [17].

For molecular ecology, the MS filters up to 4 L of seawater onto each of 48 0.22 μm filters. Collected whole cells are preserved *in situ* using a preservative of choice, for example “RNAlater” (Sigma-Aldrich Ltd), which quickly stabilises the highly labile RNA pool for later analysis [16]. The MS-SID was deployed at 2222 m depth in the Eastern Mediterranean Sea in order to determine whether *in situ* preservation delivered a more accurate metatranscriptomic profile than traditional oceanographic sampling, which consists of seawater collection at depth by CTD-deployed Niskins and filtration and preservation on-board ship. As expected, the metatranscriptomic profile was dependent on the sampling method, specifically for transcripts involved in post translational modification and protein turnover. The phylogenetic groups with the largest differences between sampling methods were fungi and protists. *In situ* preservation recovered metazoan sequences more reliably than CTD Niskin sampling, with important implications for eDNA studies. Unexpectedly, more sequencing reads were obtained after *in situ* fixation, resulting in more comprehensive assemblies [15•].

 Metatranscriptomic results from the MS-SID validated conclusions of previous studies using the *In situ* Filtration and Fixation Sampler (IFFS; [18]) and Automatic Flow Injection Sampler (AFIS; [19]). While RNA quality and quantity are high in samples collected using the MS-SID and IFFS, target functional genes in each study (*actR*, coding for Actinobacteria actinorhodopsin at 1 m depth [18], Archaeal *amoA,* coding for ammonia monooxygenase and Sulfurimonas spp. GD-1 *recA*, coding for DNA recombinase from 70 – 120 m depth [19]) were found to be differently transcribed when fixed *in situ* than when collected by Niskin. The results of these studies argue for the expansion of autonomous *in situ* fixation methods in molecular ecology.

**Recent developments in autonomous sensing**

 The Autonomous Microbial Genosensor (AMG; [20••]) is the first microfluidic system capable of *in situ* RNA amplification and detection using NASBA [21], an RNA-based amplification technique that is useful for the analysis of the viable cell fraction. The Environmental Sample Processor, or ESP, is a molecular biology “lab in a can” capable of *in situ* rRNA sandwich hybridisation [7,22-28], protein ELISA [29,30], and quantitative PCR [10••, 11,25•,28,31,32]. The latter is implemented using the Microfluidic Block (MFB) analytical module which employs the principles of sequential injection and zone microfluidics to perform solid phase extraction and quantitative PCR on ESP-processed water samples. The core ESP instrument can also archive filtered whole cells and frustules [33] for later lab analyses using targeted probes [11,12,34], metatranscriptomics [8••,9,35,36••] or proteomics [37]. The ESP has been deployed for six months on a mooring or elevator and eleven days on a drifter and is rated to 4000 m depth.

 The major advantage of the ESP is its scientific versatility. Multiple hypotheses can be addressed targeting bacteria, harmful algae, invertebrates and toxins within a single deployment [26,30]. An ESP drifter deployment in the North Pacific Subtropical Gyre targeted nitrogen fixing bacteria using qPCR, prokaryotic species using rRNA hybridisation and archived samples which were later returned to the lab for metatranscriptomic analyses. This deployment demonstrated the patchiness of nitrogen fixing populations in the environment relative to more ubiquitous clades, spanning three orders of magnitude in abundance over 30 hours in a single water mass [10••]. Metatranscriptomic sequencing of high-resolution archived samples showed that heterotrophic bacterial clades have diel gene transcription, seemingly in response to the release of fixed carbon by the dominant phytoplankton genus *Prochlorococcus* [9]. Targeted metatranscriptomic analysis of archived samples using the MicroTOOLs microarray [38] showed that *in situ* transcriptional patterns reflected frequent diapycnal mixing events in the proximity of an anti-cyclonic eddy, supplying the photic zone with nutrients and fueling a phytoplankton bloom in this oligotrophic region (J. C. Robidart, unpublished). These three ESP-based studies from a single deployment have contributed new insights to our understanding of the microbial ecology underpinning biogeochemistry in the open ocean.

 While the ESP drifter is an ideal platform to study microbial dynamics within a single water mass, the instrument is large and is dependent on intensive ship time and personnel for deployment and recovery. The third generation of the instrument is smaller and deployable on a long-range AUV, capable of Lagrangian drift (<http://www.mbari.org/successful-launch-new-genomic-sensor/>). The ease of deployment of this vehicle will undoubtedly expand the user base and frequency of deployment for this versatile molecular-biological sensor.

**Emerging Analytics**

Current deployable sensor instrumentation including the AMG [20••] and ESP [5] can perform state of the art genetic assays *in situ*, but are limited by their large complex fluidics and high operational costs. New innovations, specifically in the field of microfluidic Lab on a Chip-based molecular analytics have the potential to alleviate these constraints and open new possibilities for *in situ* microbial metrology. Novel technologies under development include low-power, portable thermal cycling systems for PCR, new microfluidic Lab on a Chip devices supporting isothermal DNA and RNA amplification modalities, and novel methods of genetic sequence detection and quantification. The custom-built qPCR module integrated with the ESP-MFB is a reusable, low power (10 – 14 V) design featuring up to six replicate reactions and optical configurations for a range of fluorescent probe-based assays [11]. The Rotary Zone Thermal Cycler (RZTC) is a prototype, miniaturised and low powered thermal cycler design that has demonstrated better thermal performance than a high-powered Peltier-based bench-top system [39]. In its current form the RZTC can perform up to seven replicate reactions, but can be easily adapted to increase the number of parallel runs without significant increase in size or weight, making it a good choice for integration onto a deployed sensor. Digital Droplet PCR (ddPCR) is an emerging technology which requires minimal reaction volumes, preserving precious sample, and is intrinsically quantitative without the need for standards, making it an attractive candidate for a simplified *in situ* analytical platform. Recent developments include successful integration of ddPCR into conveyor belt [40], Lab on a Chip [41] and Lab on a Disk [42] miniaturised formats.

Isothermal sequence amplification methods are increasingly implemented with miniaturised and portable analytical devices due to an uncomplicated thermal regime (reduced complexity) and lower overall reaction temperatures compared to PCR (potentially reduced power consumption). Nucleic Acid Sequence Based Amplification (NASBA) is a process that mimics retroviral replication enabling the direct amplification of either RNA or DNA targets. Recently, NASBA integrated microarray analysis (NAIMA) has been demonstrated for the multiplexed detection of microorganisms with DNA and RNA-based genomes on a single LOC platform [43•,44]. The use of microarray detection and quantification has also been demonstrated in tandem with the Recombinase Polymerase Amplification (RPA) method, an isothermal DNA amplification technique, which, unlike NASBA, doesn’t require an initial, high temperature template denaturation and primer annealing incubation step, leading to further reductions in complexity versus PCR [45].

Most genetic amplification instruments require high performance optics, which quantify the amount of amplified product in real time using fluorescent reporters, but increase the cost, size, complexity and power requirements of the analytical module. The “Nuclemeter” is a new concept in LOC genetic assay design that can measure a Loop-Mediated Amplification (LAMP) reaction using a simple LED excitation source and a CCD camera, powered by four AA batteries [46]. The sample is amplified in a reaction chamber adjoining a narrow conduit containing a fluorescent reporter dye. The reaction product diffuses into the conduit liberating a fluorescent signal, and the distance travelled is proportional to the starting template concentration. Other LOC genetic assays have omitted the use of optical apparatus altogether. Label-free, real-time PCR and LAMP have been developed utilising miniaturised pH sensors based on Ion Sensitive Field Emission Transducers (ISFETs) [47,48••]. Polymerisation of new RNA or DNA strands liberates H+ which, in an unbuffered solution, leads to a reduction in pH, proportional to the rate of amplification. This principle has been demonstrated using an SD Card-based genetic assay featuring an integrated circuit and reaction chamber with thermal controls and ISFET pH sensors, demonstrating the potential scale of miniaturisation that can, currently be achieved [48••]. The diversity of miniaturised analytical modules currently available at the prototype level has the capacity to revolutionise ocean molecular-biological sensing pending further development and integration with existing sampling and sensing instrumentation.

**Conclusions**

The deployment of molecular biological sensing and sampling platforms has demonstrated the utility of *in situ* microbiology, enabling researchers to study microbial communities in their natural environment. Although most of the current instrumentation can only sample the ocean, the development of integrated sampling and analysis platforms (ecogenomic sensors) has also been demonstrated, most notably using the Environmental Sample Processor. This revolutionary sensor technology has the potential for widespread use, not only for fundamental ocean research but for the implementation of, for example, coastal and offshore monitoring networks for harmful algal blooms, and the detection and enumeration of water quality indicators. This will be realised from the new innovations in technology and biochemistry that will continue to reduce the size, complexity and cost of the sampling, processing and analytics without sacrificing analytical power.

**Table 1.** Sensor and sampler technologies and their specifications. WHOI: Woods Hole Oceanographic Institute; MBARI: Monterey Bay Aquarium Research Institute; instruments manufactured at McLane Research Laboratories are commercially available. Filter pore sizes ≥ 0.22 μm include prokaryotes. Max volume depends on cell concentration sampled. Instrument sizes are (S)mall (< 5L), (M)id-sized (5 – 100 L) and (L)arge (>100L). M: Mooring, P: Pier, FB: Ferry Box, ROV: Remotely Operated Vehicle, Df: Drifter, AUV: Autonomous Underwater Vehicle. \* The 3rd Generation ESP (in development) is small and deployable on AUV platforms. \*\* WaMS typical sample volume is 150ml and it is being developed to preserve cells with Lugols Solution after sample collection.

**Figure 1. Figure 1.** Instrumentation discussed in this review: A) SID drifter and B) ESP drifter from BioLINCS cruise, September 2011, images courtesy of Kendra Turk-Kubo; C) WaMS with sampling bags, image courtesy of SAHFOS D) AMG on dock pictured next to housing, Figure 6 from Paul et al., 2007; E) SUPR on HROV *Nereus* during the 2013 Cayman cruise led by C.R. German, image courtesy of J. Breier.

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