

Proposal Cover Page

We propose to adapt and integrate a compact low-power flow cytometer with a small long-range autonomous underwater vehicle. This unique vehicle-instrument system known as *CytoAUV* will be capable of identifying and mapping microbial populations below polar ice. Steep gradients in the under-ice environment make it an ideal site to robustly test our innovative remote microbial detection approach, and the climate-sensitivity of polar regions provide a critical science target. Developing this autonomous approach is an essential prelude to under-ice astrobiology missions to Europa.

Diversity and interactions among microbial populations are key to the geochemical cycles that maintain Earth's biosphere. Current knowledge of the distribution and abundance of photosynthetic microbes in the ocean is largely due to the use of flow cytometry, in which water samples are run past a laser; the resulting scattered light and fluorescence reveal details of cell size, fluorescence emission, and cell abundance. Most commercial flow cytometers are large, power-hungry and complex, requiring continual presence of a human operator. Thus marine cytometry studies are usually limited to widely-spaced, discrete samples collected from ships. Measurement of microbial distributions in the Arctic Ocean is particularly important to understand the effects of rapid climate change on microbes and the ecosystems that depend on them, yet polar ice cover and severe weather conditions present serious limitations for ship-based sampling.

To address these challenges, we propose to integrate two new technologies developed at our institutions. *SeaFlow* is a small, automated flow cytometer that utilizes novel optics and fluidics to continuously measure microbes at rates up to 24,000 cells per second. *Tethys* is a small, low-power autonomous underwater vehicle capable of long-duration missions. *SeaFlow* and *Tethys* will be integrated to create *CytoAUV*, an instrument-vehicle system capable of mapping the three-dimensional distribution of microbial communities underneath polar ice, while simultaneously acquiring data from onboard sensors for temperature and salinity (for example) to provide physical and chemical context for the microbial data. *CytoAUV* will be designed for long-duration unattended operations by utilizing high energy-density batteries, low-power hardware components, and fault-tolerant design. Sophisticated software will be incorporated for autonomous navigation, mission planning, fault-detection and recovery, pattern recognition, and *in situ* microbial population identification. An adaptive sampling approach will utilize data from multiple sensors to autonomously adjust the sampling strategy, focusing survey efforts on regions of high microbial abundance or diversity, and gradients in physical or chemical parameters.

CytoAUV will eventually be capable of missions lasting several weeks and covering hundreds of kilometers. The adaptive sampling technologies developed here will be adjustable for detection of a wide range of Earth microbes in advance of missions to explore Europa's ocean. Following development tests along the US Pacific coast, we propose Arctic field campaigns to demonstrate the technology under ice, and to acquire microbial data as a baseline to future time-series and expanded detection capabilities. The rapidly changing Arctic environment has not been explored microbially on the proposed scale. *CytoAUV* will be launched out of Nuuk, Greenland, where our work can be integrated with other projects also hosted by the Greenland Climate Research Center with extensive support facilities. We will involve undergraduates in our proposed work and will develop an Internet telepresence capability so that students world-wide can participate in this exciting endeavor by planning simulated missions as well as viewing actual mission data from under the ice in real-time.

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1 Scientific/Technical/Management Section

1.1 Objectives and Significance

As a step towards developing suitable analysis tools and strategies for exploring life on ice-covered planetary environments, we propose a 4-year program to integrate a flow cytometer in an autonomous underwater vehicle, *CytoAUV*, to autonomously map microbial distributions in the extreme environment of the ice-covered Arctic Ocean.

1.1.1 Science

- Provide unprecedented maps of microbial populations in ice-covered Arctic waters.
- Investigate associations between microbial populations and *in situ* physical and chemical parameters in 3-D, and detect the presence of specific communities as a function of water column chemistry and depth (proximity to the ice-water interface).
- Adaptively sample fluid environments under ice cover with real-time guidance and site targeting by *CytoAUV*.

1.1.2 Technology

- Refine our current *SeaFlow* underway cytometer into a compact and low-power cytometer
- Integrate this compact cytometer into a long-range AUV (*CytoAUV*).
- Implement onboard software to analyze cytometer data and identify microbial species *in situ*, and return data to shore via low-bandwidth satellite link.
- Implement adaptive sampling to manage power usage and autonomously direct vehicle to regions of scientific interest based on output of cytometer and other contextual sensors.

1.1.3 Field Campaigns

- Deployments in Monterey Bay (USA) and Nuuk (Greenland) from ice edge to under ice.
- Demonstrate an integrated physical, chemical and biological sensor package for characterization of open water and under-ice environments.

1.1.4 Education and Outreach

- Further undergraduate training through research opportunities at MBARI and UW.
- Develop Internet telepresence capability so that any students with internet access can plan simulated missions and view mission data from under the ice in real-time.

Application of *CytoAUV* as outlined above is relevant to a number of science objectives identified in the 2008 NASA Strategic Astrobiology Roadmap, in particular:

- Goal 2 by developing reliable robotic techniques for *in situ* detection and characterization of microbes in targeted bodies of water, returning results to shore ("Earth") via a low-bandwidth long-latency communication link.
- Goal 5 by documenting microbial life that survives or thrives in ice-impacted waters along continuously changing gradients of salinity, temperature and nutrient concentrations.
- Goal 6 by documenting the ecological impact of changes in climate, habitat complexity and nutrient availability upon the microbial population structure in Arctic waters, currently attributable only broadly to rapid climate change.

1.2 Technical approach and methodology

We propose to integrate a flow cytometer in an autonomous underwater vehicle (*CytoAUV*) to autonomously detect and map microbes under ice in the Arctic Ocean in pursuit of several goals and objectives set forth in the Astrobiology Roadmap. Microbes represent both the earliest forms of life on Earth, and by biomass, the largest fraction of life in the ocean today; they are prime targets for astrobiology. Permanently ice-covered liquid water environments are among

the leading candidate sites for finding evidence of extant life elsewhere in our solar system (e.g. on Europa and possibly other Galilean satellites or Saturn's Enceladus). The high priority recently given to Europa (Clark et al., 2009) by the planetary science community in its Decadal Survey underscores the importance of developing under-ice microbial life detection systems for future missions. From an optical perspective, marine microbes are small particles with unique light scattering characteristics; photosynthetic microbes (phytoplankton) add the additional characteristics of light absorption and fluorescence. Instrumentation for detecting and characterizing small particles would provide a powerful tool for astrobiology in liquid environments on other planets. Flow cytometry is an integral tool of today's oceanography due to the sensitivity with which these instruments measure the abundance, size and fluorescent characteristic of individual cells (Sosik et al. 2010). Most existing systems have comparatively low throughput of fluid, requiring large integration times for the detection of low-abundance organisms. A key innovation here is use of *SeaFlow* system to dramatically increase throughput.

1.2.1 Flow Cytometry Technology: *SeaFlow*

Flow cytometers typically inject samples into the center of a particle-free sheath fluid such that cells flow single-file past a laser light focused on the center of the stream. Photomultiplier tubes measure scattered light (roughly proportional to cell size) and specified fluorescence emission of individual cells at rates of several thousand per second. These parameters are used to identify phytoplankton populations with similar size and fluorescence characteristics (Olson et al. 1989, Li 1990). Flow cytometry at sea remains relatively limited because of the large size and complexity of these instruments, the frequent need for a dedicated instrument operator, and the requirement for the ultra-clean sheath fluid used as a carrier for the sample fluid.

We created *SeaFlow* (Fig. 1) in response to the need to autonomously and continuously

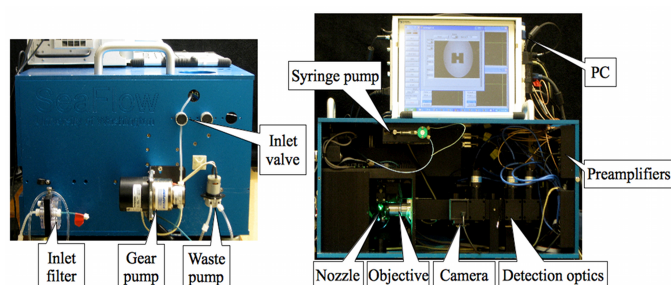


Fig. 1. *SeaFlow* left side and front view

(Ribalet et al., 2010, Ribalet et al., 2011). Currently, *SeaFlow* has collected the equivalent of 70,000 measurements across 35,000 km of the North Pacific Ocean. The technical breakthrough that enables *SeaFlow* measurements is an optical system we developed known as Virtual Core cytometry (Swalwell et al. 2009) that eliminates the need for sheath fluid and instead performs the measurement directly and continuously on a 200 μm -diameter stream of seawater. The simplicity and robustness of this system has opened the way for continuous measurements on flowing seawater either from a ship's seawater intake (*SeaFlow*) or directly from the environment as proposed here. UW has the facilities necessary to design, fabricate and test new flow cytometer instruments, including *SeaFlow*.

By definition, only a fraction of cells are in the center of the *SeaFlow* stream and thus in focus. In-focus cells are defined as the optimally positioned particles (OPP) and are identified

measure phytoplankton cells (0.5-20 μm in size) in surface waters while underway aboard a ship. The first long-term (2 weeks) use at sea occurred in 2008. *SeaFlow* engineering development has been led by Swalwell (Swalwell et al. 2009, Swalwell et al. in press) and data analysis by Ribalet

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using position sensitive detectors to determine the side-to-side position of a particle; the axial position (relative to focal plane) of a particle is based on scaling these detectors with the forward light scatter signal. Application of the OPP software filter creates flow cytometry bivariate dot plots indistinguishable from those obtained with conventional flow cytometers (Fig. 2). To

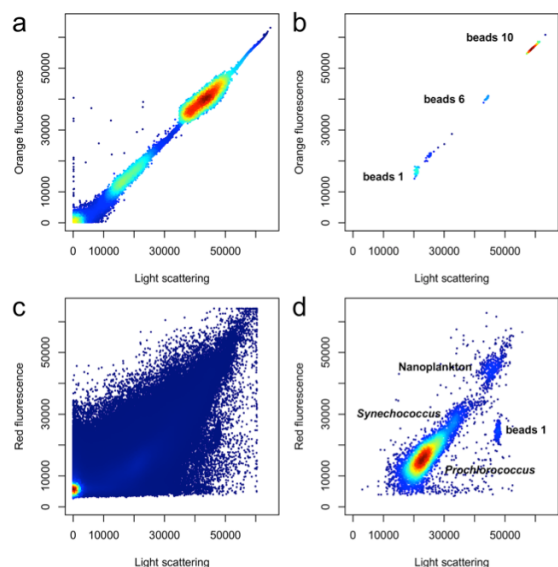


Fig. 2. Flow cytometric measurements of light scattering and fluorescence of calibration beads (a,b) and a Pacific Ocean seawater sample (c,d). Total detectable particles (a,c) and OPP (b,d) are shown. Warm colors represent higher particle abundance. Note the abundant population of the tiny cyanobacterium *Prochlorococcus* well above the baseline.

samples obtained with *SeaFlow* agree with those obtained with the BD Influx, a gold-standard laboratory cytometer for analysis of discrete samples.

A critical driver of *SeaFlow* capabilities is the fact that oceanographic fieldwork requires a robust instrument that can perform in harsh conditions on an unstable platform. High detection sensitivity is necessary to perform measurements on small marine microbes (e.g. *Prochlorococcus*, a cyanobacterium less than 0.8 μm in size) whose diameter can be close to the wavelength of the light source (Fig. 2). Our design work has entailed a total ‘re-think’ of the mechanical and optical components that compose a flow cytometer. *SeaFlow* has a rigid optical axis and enclosure to isolate it from the effect of a ship’s motion in rough seas (Fig. 1). Specifically optimized optical filters and photodetectors, combined with our low noise electronics and real-time processing of the digitized pulses, allow detection of *Prochlorococcus*, a performance benchmark achieved by few high-end commercial instruments. *SeaFlow* was installed on the *R/V Thompson* in March 2010 and has operated semi-autonomously via remote

determine cell concentrations of the different populations, the volume of the OPP region (the Virtual Core) is first calculated by multiplying the volume of the detectable region by the ratio of OPP particles to total detectable particles. The detectable region is defined as the cross sectional area within the stream where particles are detected; it has a fixed volume set by the field of view of the optical system. The detectable region is calibrated by analyzing known concentrations of 1, 6 and 10 μm calibration beads (Cs) and comparing the *SeaFlow* bead count (Pd) to the known concentration. The effective volume of the detectable region is calculated by multiplying the total analyzed volume of the stream by the ratio Cs/Pd. This calibration step need only be done once (Swalwell et al., in press). Total cell concentration is determined by dividing the cell count by the volume of the detectable region. The number of cells within a given population divided by the OPP volume is the concentration (cells/ml). Cell concentrations of cultures and natural

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desktop control since May 2011. Since then, we have collected ~130 days of data or the equivalent of 60,000 flow cytometric samples.

1.2.2 Long-Range AUV

A new class of propeller-driven AUV, called *Tethys*, has been created to carry ‘intermediate power’ sensors for long ranges and/or for long duration expeditions (Bellingham et al., 2010). The vehicle is small, about 120 kg dry weight, and easily handled (Fig. 3). As currently configured with a variety of sensors, the vehicle is routinely operated for week-long deployments at a speed of 1 m/s (2 kts). Endurance is currently ten days on secondary batteries, and two to three times this on primary batteries. The vehicle is typically shore-launched and recovered, using a Boston Whaler to tow the vehicle to and from a boat launch ramp. Operators interact with the vehicle via an Iridium satellite link, recovering data snippets in near real-time and sending new mission commands to the vehicle as desired. Real-time data reports from 2011 missions can be viewed at: <http://aosn.mbari.org/TethysLogs/2011/>.

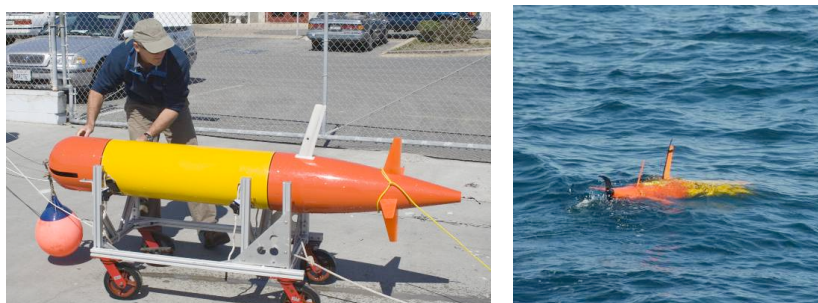


Fig. 3. *Tethys* before and during operation

Tethys operates a version of layered control called ‘state-configured layered-control’ (Bellingham and Consi, 1991; Godin et al., 2010) which is capable of autonomous adaptive operations for long periods. The vehicle software detects and responds to faults in critical components and in some cases can continue the mission even though components have failed. *Tethys*’ variable buoyancy system is ideally suited to high latitude operations, as surface waters in the Arctic Ocean can be quite fresh during the melting season. Variable buoyancy enables more efficient operations at low speed, but also permits the vehicle to surface and sink at zero speed, which would allow the vehicle to surface in open water between ice floes, for example, for satellite communications and a navigation fix. Incorporation of a USBL system allows homing on a transponder, which would allow recovery of the vehicle through an ice hole, as was accomplished for the *Odyssey* AUV in 1994.

Tethys development started at MBARI in 2006, with the first sea deployment in December 2009. The leaders in *Tethys* development, Bellingham (co-I) and Hobson, have extensive sea experience with AUV operations, including in the Arctic (Bellingham et al, 2008), and have developed a number of AUVs before, including *ALTEX* (Bellingham, et.al., 2000) the MBARI *Dorado* AUV (Sibenac, et.al., 2002), the *Odyssey II* AUVs (Bellingham et.al., 1994), the *Odyssey* (Bellingham et.al., 1992), the *CETUS* hovering AUV (Curcio et.al., 1998), and the *Sea Squirt* (Bellingham et.al., 1989). MBARI has extensive facilities to support AUV development, including engineering labs, machine shop, and 10-m deep saltwater test tank.

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1.2.3 Science strategy

Our ultimate goal is to adaptively sample fluid environments under ice cover with real-time guidance and site targeting by *CytoAUV*, providing an unprecedented image of microbial communities under ice. Our first step towards achieving this goal begins with our focus on the phytoplankton because of their natural fluorescence signal and their critical role within marine systems. Microbial populations in the Arctic Ocean are undergoing change (Li et al., 2009), in concert with climate-driven alterations to the ice cover (reductions in thickness and areal extent; Nghiem et al. 2007, Stroeve et al. 2007, Boe et al. 2009). Potential shifts in community structure of the phytoplankton (PI Armbrust and Co-I Worden expertise) and bacteria (Co-I Deming expertise) will have major repercussions on carbon sequestration at depth in the ocean (Forest et al. 2011, Kellogg et al. 2011). Apart from large-scale correlations with changes induced by climate warming (regional freshening of Arctic waters and higher temperatures), the drivers behind these changes are not well understood.

1.2.4 Under-Ice Field Program

Under-ice operations of *CytoAUV* will occur near Nuuk, Greenland, in the ice-covered Kobbefjord (Fig. 4), which has been characterized through an annual cycle (Mikkelsen et al. 2007). Work will be staged from the Greenland Climate Research Center (GCRC), led by Søren Rysgaard (see support letter), in concert with their ongoing programs on the ice-water system (Jensen and Rasch 2009). While Kobbefjord is covered with ice during winter, the surrounding

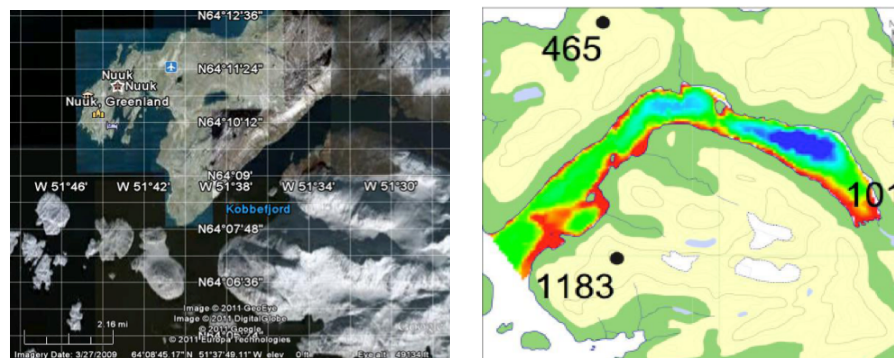


Figure 4. Satellite image of Greenland (left) highlighting the Kobbefjord study region and bathymetry of the region based on multi-beam analysis (red:0m, yellow: 25m, green: 50m and dark blue: 150m depth).

waters are navigable by boat in spring. Indeed, Nuuk is considered an open-water port and is also accessible by from Iceland or Canada. Both small and large boats and a range of specialized equipment are available from the GCRC (see Rysgaard letter). We will deploy our system in late spring/early summer to observe the microbial community changes associated with ice breakup and freshwater runoff from shore. The characteristics of Kobbefjord enable a comparatively simple operation in which the AUV is launched from a small boat in open water, or possibly even launched from Nuuk, which is less than 10 km from Kobbefjord. The standard method to operate *Tethys* is to interact with the vehicle sporadically from shore via an Iridium satellite link. After launch, the vehicle would run into Kobbefjord under ice, make its measurements, then return to open water to surface to rapidly communicate results to operators on shore. Depending

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on the operational cycle of the vehicle and the power consumption of the cytometer, we anticipate deployments of several days to a week.

1.3 Relevance to NASA's programs

Our project will develop technology relevant to unpiloted missions to icy moons. These missions are subject to long communication latencies and low communication bandwidth. The cytometer's onboard data summarization capability will enable return of invaluable science data over limited communication bandwidth. *CytoAUV*'s adaptive sampling capability will enable autonomous onboard mission planning, science targeting, and resource management; the vehicle's fault-tolerant design enables untended long-duration missions.

In proposing autonomous scientific exploration via continuous under ice sampling instrumentation, our project embraces Decadal Survey decisions and the high prioritization of missions to Europa. The NASA/ESA Joint Summary Report on the Europa Jupiter System Mission (Clark et al., 2009) is built on the hypothesis that ice-covered Europa with its potentially vast under-ice ocean was, and may remain, habitable (Pappalardo et al. 1999, Hand and Chyba 2007). Although we recognize that available evidence suggests an absence of oxygen in this ocean and likely no opportunity for photosynthesis, our plans begin with under-ice Arctic oxygen-generating phytoplankton as our test communities for two reasons. These populations are critical to the future of ecosystems in Earth's ice-covered ocean during rapid climate change and their natural autofluorescence eases initial development and science return of *CytoAUV*. Success with this program can lead immediately to adaptive sampling of other types of microbes (non-fluorescent, anaerobic, prokaryotic) as analogs for life in an ice-covered ocean beyond Earth.

1.4 Work Plan, Specific Objectives and Milestones

1.4.1 General Work plan

1.4.1.1 Integration of *SeaFlow* flow cytometer in AUV

We will build upon the optics and fluidics developed for *SeaFlow* to design and construct a new compact cytometer system for integration on *Tethys*. Together, engineers from UW and MBARI will define power, mechanical and software interfaces between instrument and vehicle, and set upper limits on instrument size, mass and power consumption. The cytometer package will utilize the same Virtual Core technology developed for *SeaFlow*, but will be implemented in a closed loop fluidic system. The design will incorporate *SeaFlow* technologies with redesigned low-power electronics and data acquisition system. Specific design modifications include:

- A compact and low power (50-100 mW) 488 nm laser will be used for light scatter and fluorescence excitation with five detectors: two for position sensing, one for side scatter, one each for fluorescence emission of phycoerythrin (characteristic of the smallest under-ice phytoplankton, *Synechococcus*) and chlorophyll (feature of all phytoplankton). The photomultiplier tubes (PMT) will be a compact low-power voltage output style.
- The light collection path will be reduced with a shorter focal and parfocal length objective and a decrease in the filter and lens size from a 25 mm standard to 9 mm.
- The flow rate will be measured by calibrating a differential pressure sensor with pressure ports located at the seawater entrance port and after the sample pump.
- *Seaflow* digitizes then low-pass filters the light pulses in software prior to peak height analysis. The new cytometer will use analog electronics to low-pass filter light pulses and store the peak heights on peak detectors.

- A data acquisition and processing system will be developed that is capable of operating at 20,000 events/second to allow accurate measurement of dense bloom concentrations of 10^8 cells/L. This system will perform the tasks of data storage, communication with the AUV control processor, and processing of the position sensitive detection.
- The processed data will be used in simple cytometric analysis (see below: diversity index, simple gating) and passed to the AUV processor for guidance decision-making and telemetry back to shore.

1.4.1.2 Modification of *Tethys* and optimization of polar operations

While *CytoAUV*'s core vehicle and software will be identical to existing *Tethys* vehicles, certain characteristics will be modified to accommodate the cytometer and to support under-ice operations. The cytometer will be housed in the payload portion of the vehicle, which is the most forward section. This portion will be completely customized for *CytoAUV*. Externally the vehicle will look like a slightly longer version of the existing *Tethys*. In addition to the cytometer, sensors will also be integrated to provide physical (temperature, salinity, current), optical (scattering, fluorescence) and chemical (nitrate and dissolved oxygen) context for the flow cytometer measurements. These sensors are currently installed on the *Tethys*, and are well understood. Other modifications include possible power system upgrades to support higher peak loads and increasing the variable buoyancy system's maximum displacement.

Polar operations impose special requirements for AUV performance. Risks associated with these operations are outlined below, along with strategies for mitigating these risks.

- Magnetic compasses are a preferred heading reference because of their low power and cost, however they perform poorly in proximity to the North magnetic pole. The Earth magnetic inclination around Nuuk is ~ 78 degrees, so minimizing vehicle-induced magnetic influences on the compass and the use of calibration methods should ensure adequate compass performance.
- Arctic surface waters can be quite fresh, requiring a vehicle to manage a wider buoyancy range. *Tethys* is uniquely suited to deal with this range as it has a variable buoyancy system. Depending on the displacement of the cytometer package, the existing buoyancy system can be doubled in size to ensure adequate reserve buoyancy.
- Low temperatures can alter the property of certain materials (e.g., fluids too viscous, plastics below glassy transition, battery performance degraded). Temperatures in Nuuk in May-June are typically around 0°C , and seldom fall below -10°C , mitigating this concern.
- A range of task-level control capabilities specific to under-ice operations will be required. For example, sampling near the ice may require reducing vehicle speed to zero, and using the buoyancy system to bring the vehicle up to the ice canopy. Also, vehicle behaviors for fault-detection and recovery will have to be less conservative. Usually vehicles will 'bail' to the surface at the first hint of failure, where they will either communicate home by satellite or be recovered. Under-ice bailing is not acceptable, consequently the vehicle must be capable of operating even in the event of failures.
- In shallow Arctic water, the ice canopy can ground on the bottom, creating a fully three-dimensional environment in which the vehicle must navigate. We will restrict AUV operations to regions where the water depth is much greater than the ice thickness. An upward-looking altimeter will provide the vehicle the ability to sense the overhead ice and navigate to avoid it in the same manner as AUVs navigate near the seafloor.

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- Nuuk has a large tidal excursion requiring vehicle operations to be timed carefully to coincide with either low or favorable currents. The acoustic Doppler system will be useful in allowing the vehicle to measure local currents and time activity.
- We will use four levels of navigation for the AUV. First, a GPS system built into the *Tethys* antenna will routinely obtain GPS fixes when the vehicle surfaces. Second, the vehicle will dead-reckon using a Doppler sonar to measure velocity relative to the bottom, and a compass for heading. Third, an ultrashort baseline (USBL) acoustic system will measure range and direction to a transponder and allow acoustically marking interesting sites for revisit by the vehicle. Finally, we will use geophysical navigation methods based on bathymetric maps of Kobbefjord as a backup navigation scheme.

1.4.1.3 Instrument data processing

Onboard processing: Two key project objectives are return of science data to shore in near real-time, and autonomous adaptive sampling, both of which will be enabled by onboard data processing. Onboard software will calculate a cytometric diversity index of richness in physiological and genetic variations of phytoplankton cells, as defined by Li et al. (1997). The index will be calculated based on light scatter and chlorophyll fluorescence, making it possible to estimate the structure of the phytoplankton communities in real-time and track blooms (high cell concentration, low cytometric diversity index; black circle, Fig. 5) or biological fronts (high cell concentrations, high diversity index; red circle, Fig. 5). The raw data acquisition rate of *CytoAUV* (several gigabytes per day) exceeds the vehicle's 7800 baud Iridium satellite modem

capacity to transfer data to shore. The diversity index is composed of several floating-point numbers computed every minute and can easily be returned over the low-bandwidth satellite link. Thus the science team will have an unprecedented real-time view of diversity while the mission is underway. Moreover, the diversity index will be available to the onboard adaptive sampling software (see below).

Post-mission processing: The raw cytometer data will be stored onboard for post-mission retrieval. Flow cytometry data typically consists of multiparametric descriptions of millions of individual cells. Because of the high volume of data

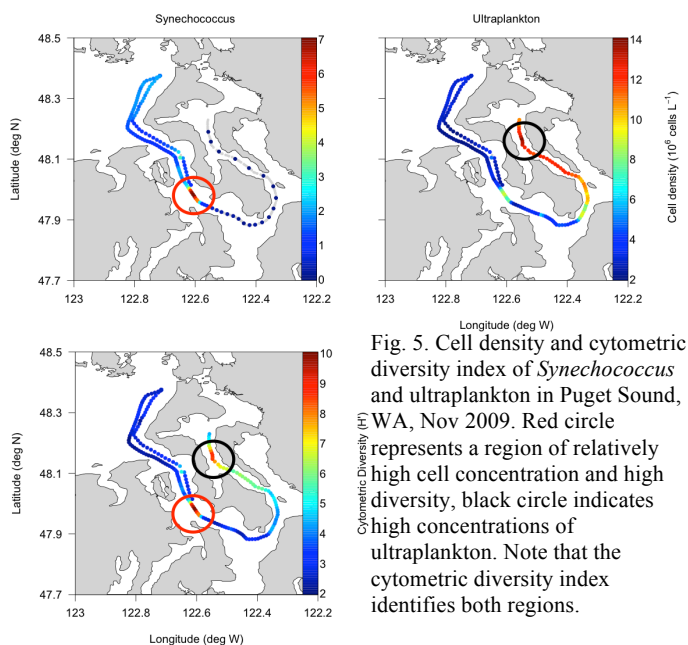


Fig. 5. Cell density and cytometric diversity index of *Synechococcus* and ultraplankton in Puget Sound, WA, Nov 2009. Red circle represents a region of relatively high cell concentration and high diversity, black circle indicates high concentrations of ultraplankton. Note that the cytometric diversity index identifies both regions.

created with continuous measurements, we developed a first-generation software package

(www.bioconductor.org/packages/2.8/bioc/html/flowPhyto.html) that pipelines a collection of functions to turn raw data files into data tables and plots of concentration, size and fluorescence characteristics of the various phytoplankton populations (Ribalet et al. 2011). The software uses a flexible statistical model-based clustering approach for identifying and clustering cell populations. Most of the analysis consists of setting up the statistical priors for the cell population-clustering algorithm; the overall pipeline requires minimal human intervention.

1.4.1.4 Development of CytoAUV adaptive sampling algorithm

CytoAUV's onboard sampling software addresses risks imposed by operating under ice, which limit interactions with the vehicle while underway. This sampling software 1) determines the optimal AUV survey strategy (e.g. speed, horizontal and vertical trajectory) to focus on areas of scientific value, and 2) powers on the cytometer and collects data within these regions of interest. Data from the cytometer and other onboard sensors will be used to autonomously adjust the sampling strategy, focusing survey efforts on regions of high cell abundance, high cytometric diversity, and/or steep gradients in nutrient concentrations or salinity. Development of

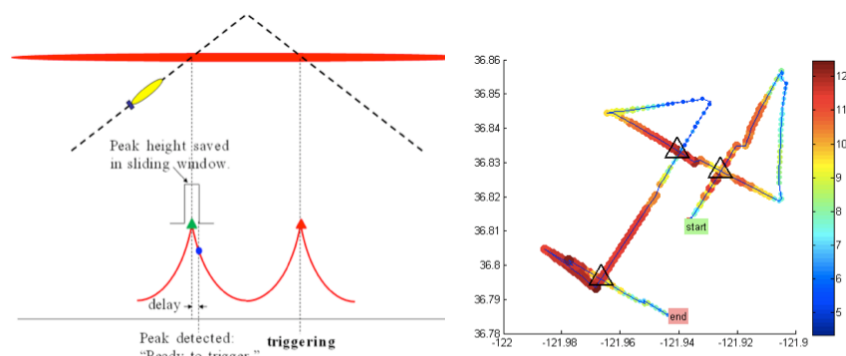


Fig. 6. Left panel: As the AUV's sawtooth trajectory carries it through a phytoplankton patch, the vehicle detects the fluorescence peak on the first crossing, with a delay. It captures the peak on the second crossing with no delay (from Zhang et al. 2010). Right panel: On 24 April 2011, *Tethys* ran the algorithm to find and track the center of a moving phytoplankton patch. Triangles correspond to successively updated patch center estimates (from Godin et al. 2011).

CytoAUV's adaptive sampling algorithm will draw upon a suite of adaptive sampling algorithms we developed, exemplified by two algorithms: AUV peak-capture and AUV patch-tracking.

AUV peak-capture algorithm: MBARI's *Dorado* AUV is equipped with ten fast water samplers (called "gulpers"), each of which takes in a 1.8-L water sample in 1~2 sec. The problem for the Gulper is similar to that of the cytometer: how to obtain representative samples of organisms, especially when organisms often aggregated in very thin layers? For Gulper, the limited resource is number of samples, for the cytometer, the resource is energy. We have developed an adaptive triggering algorithm for the AUV's gulpers to autonomously trigger the gulpers at fluorescence peaks characteristic of high phytoplankton abundance (Zhang et al. 2010a). The principle of the method takes advantage of the AUV's sawtooth (yo-yo) trajectory. In one yo-yo cycle, a descent leg followed by an ascent leg, the vehicle crosses the phytoplankton bloom

twice. On the vehicle's first crossing (descent leg), the peak detection (by tracking the fluorescence signal's slope) comes with a delay, but the vehicle remembers the peak value. On the second crossing (ascent leg), as soon as the fluorescence signal reaches the saved peak value, a sampling is triggered. This way, the sampling is right on the peak. This algorithm has been successfully used by the *Dorado* AUV in numerous field programs for studying phytoplankton thin layers and intermediate nepheloid layers (Zhang et al. 2010b), and for capturing water samples in a deep hydrocarbon plume in the Gulf of Mexico (Zhang et al. 2011). Cell-rich layers and convective brine plumes can be expected in the under-ice and ice-edge regions accessible from Nuuk, facilitating first-time tests of this approach in ice-covered waters.

AUV patch-tracking algorithm: Biological processes such as phytoplankton blooms appear in patches that evolve from initiation to decline. A challenge is to detect and track these features in real time. We have developed an algorithm for an AUV to autonomously localize and track the center of a phytoplankton bloom patch based on *in vivo* chlorophyll *a* fluorescence. The algorithm takes advantage of the responsiveness of the AUV platform to minimize the chance of losing track of the patch and maximize the rate at which the patch center is revisited. The field performance of patch tracking by the MBARI *Tethys* AUV is shown in Fig. 6. Importantly, *Tethys* currently responds to bulk fluorescence properties that result from mixed populations; it cannot discriminate between different populations of phytoplankton despite their different ecosystem roles. *CytoAUV* will distinguish between different types of cells based on differences in cell size and pigment content.

1.4.2 Timeline

1.4.2.1 Major project milestones

Activities and milestones: start date April 1, 2012	Date complete; Year
Vehicle-instrument interface review	Aug, 2012; Yr1
Instrument PDR / Vehicle CDR	Nov, 2012; Yr1
Instrument CDR	April 2013; Yr 2
<i>CytoAUV</i> vehicle complete, instrument complete	Sept, 2013; Yr 2
Integration and testing complete	Dec, 2013; Yr 2
Monterey Bay sea trials complete	March 2014; Yr 2
First Arctic campaign (timing dependent on ice conditions)	May-June, 2014; Yr 3
Data analyses, system refinements	April 2015; Yr 4
Second Arctic campaign (timing dependent on ice conditions)	May-June, 2015; Yr 4
Post-mission data processing software	Dec, 2015; Yr 4
Finalize analyses, publications	Jan-Apr; Yr 4

1.4.2.2 Year 1

1.4.2.2.1 Vehicle-instrument interface review

The UW and MBARI engineering teams will determine realistic limits to cytometer dimensions, mass, and power consumption, as well as electrical, mechanical and software interfaces between it and the vehicle. These results will be captured in an interface control document (ICD). To fit within the vehicle payload fairing, the cytometer diameter must not exceed 11.5 inches, and the length should not exceed 12 inches, a more flexible constraint dictated by hydrodynamic control considerations. Ideally, the cytometer should draw 50 watts or less to be compatible with the present *Tethys* power system. Higher power draw would be

accommodated with modifications to the vehicle power system. During this phase, the team will utilize modeling tools, prototypes, and other analytical methods to determine reasonable limits. Within a few months of project start, the team will conduct a formal vehicle-instrument interface review and will finalize the ICD. The vehicle and instrument teams will then begin construction of their subsystems.

1.4.2.2.2 Instrument preliminary design review (PDR)

Modifications necessary to miniaturize the *SeaFlow* cytometer for packaging in the *Tethys* vehicle will be investigated leading up to the preliminary design review. In this period, a new compact microscope objective, small photo-detectors, two candidate flow cells, and modified *SeaFlow* front-end electronics will be assembled on a *SeaFlow* flow cytometer and evaluated with cultured marine phytoplankton and Puget Sound samples. A variable power laser will be used as the excitation source to determine the minimum necessary power to detect the small marine cyanobacterium *Synechococcus* (~2 μm diameter) in natural samples. This level of performance is commensurate with well-regarded commercial bench top cytometers that utilize similar components.

In parallel, MBARI will select a suitable microprocessor for instrument control. Requirements driving the microprocessor selection include small size, low power, and ability to acquire and log data at high speeds (~100KB/sec while the instrument is “on”).

1.4.2.2.3 Vehicle critical design review (CDR)

A CDR for the vehicle with a particular focus on payload integration will occur at the end of Year 1. At this stage the ICD will have been written (see Vehicle-instrument interfaces, above) and the characteristics of the cytometer payload will be defined in sufficient detail to make final necessary modifications (see Modification of *Tethys* and optimization of polar operations).

1.4.2.3 Year 2

1.4.2.3.1 Instrument CDR

The optical components tested in Year 1 will be incorporated into a new miniature light collection system based on the *SeaFlow* collection optics but with a change from 25-mm lenses and filters to 9 mm. We anticipate these modifications will yield an optical system roughly 40% shorter than the *SeaFlow* design. This new system may be folded as necessary to fit within the AUV payload section.

The modified *SeaFlow* electronics prototyped in Year 1 will be laid out on new printed circuit boards with a move to thin small outline package (TSOP) scale integrated circuit packages. At this point, MBARI will provide a first revision of the CPU and data acquisition system so that all instrument components may be bench tested as a unit. The sensitivity of the new instrument will be determined by using beads of different diameters (1, 6 and 10 μm) and phytoplankton cultures relevant to the anticipated particle sizes and shapes of Arctic communities. We will evaluate the performance of the instrument in underway use in Puget Sound, WA, on board the UW's *R/V Thomas G. Thompson*. The cytometer prototype will be run in parallel with *SeaFlow* using the same water intake.

The instrument microprocessor will summarize the cytometry data into a cytometric diversity index of microbial communities. This index as defined by Li (1997) expresses richness in terms of physiological and genetic variations of phytoplankton cells. A novel algorithm to calculate this index based on light scatter and chlorophyll fluorescence will be developed and implemented to estimate the diversity of phytoplankton communities in real-time. *SeaFlow* has

collected more than 70,000 samples across a range of environmental conditions; this dataset will be used to develop and optimize the diversity index. We will test various combinations of cytometric categories and numbers of cells necessary to optimize the sensitivity to changes in community structure.

1.4.2.3.2 *Vehicle build complete*

Core vehicle components will be completed by September 2013, making it ready for payload integration. Because the payload is an integral element of the vehicle (in effect the entire nose section), in-ocean tests of the vehicle will not be possible prior to cytometer integration. However, tank tests of an appropriately weighted vehicle will be used to verify subsystem functionality in a salt-water environment.

1.4.2.3.3 *Instrument complete*

The bench cytometer system evaluated for the critical design review will be packaged into a system that fits within the vehicle payload section and is integrated with the vehicle power and communication systems. To assist integration of the cytometer – the primary source of schedule and cost risk – we will use a ‘breadboard vehicle’ for software integration prior to electrical and mechanical integration. This approach, combined with use of the vehicle simulator, greatly streamlines integration of sensor systems by decoupling software development from hardware. The completed system will undergo testing prior to integration on the AUV, including testing for pressure effects on cytometry measurements, assessed by injecting a bead solution of known concentration into the fluidic system while at different pressures while in a pressure chamber.

1.4.2.3.4 *Integration and test*

Following benchtop testing, the cytometer will be integrated onto the AUV, and will undergo a series of integration tests. *CytoAUV* will be tested by immersion in MBARI's 10-m deep salt-water test-tank to verify housing integrity, vehicle trim and buoyancy, and basic cytometer functionality.

1.4.2.3.5 *Monterey Bay sea trials*

Field trials will be carried out in Monterey Bay. Initial deployments will be attended by a small boat; longer deployments will be controlled from shore via Iridium. This will provide ample opportunity for exploring cytometer performance dependence on vehicle flight characteristics, and to experiment with different operational profiles and adaptive sample strategies. Subsequently, *CytoAUV* performance will be validated along the California Cooperative of Fisheries Investigations (CalCOFI) Line 67, an environmental gradient that traverses Monterey Bay and provides a rich 60-year dataset of physical-chemical and biological properties. By following a well-characterized transect, we will validate performance and gain insights into the communities that drive marine productivity in this region. Paired *CytoAUV* and shipboard sampling will allow performance of all sensors to be validated, as MBARI's *R/V Western Flyer* has the same equipment as *CytoAUV* for detection of environmental parameters. A shipboard *SeaFlow* will validate *CytoAUV* cytometry measurements and discrete samples will be collected using traditional shipboard sampling for analysis with our Influx flow cytometers. Thus the initial benchmark measurements from the prototype cytometer performed at UW with cultures and natural population from Puget Sound will be seamless with those performed during integration with the AUV and in Monterey Bay sea trials.

Tom O'Reilly 9/2/11 5:55 AM

Comment [3]: Provided by Jim

CytoAUV adaptive sampling algorithms will be optimized during these trials. The patch-tracking algorithm (Godin et al. 2011) will track the centroid of a biological patch allowing the temporal evolution of the cytometric diversity index to be tracked in real time and at very high spatial and temporal resolution. The variation of the cytometric diversity index with depth will be used to concentrate measurements in the depth range where signals are the strongest. CytoAUV can maintain a pre-programmed sawtooth trajectory (in the vertical dimension) and increase the cytometer's duty-cycle (i.e., more intensive sampling) in the depth range with the peak signals. The depth-dependent duty-cycle adaptation algorithm will be developed based on the AUV peak-capture algorithm (Zhang et al. 2010a; see Section 1.4.1.4).

1.4.2.4 Year 3

1.4.2.4.1 *First Arctic campaign*

The first Nuuk field program will have a heavy technical and operational emphasis. Testing will include: launch and recovery procedures, verifying basic vehicle functionality and cytometer performance, and testing performance of AUV dead-reckoning, the USBL acoustic system, and geophysical navigation. Once navigation performance is understood, a sequence of progressively more ambitious science missions will be carried out. These will start with measurements at the entrance of Kobbefjord over a 24hr period, then graduate to under-ice missions. Excursions under ice will test a progression of capabilities such as using the vehicle buoyancy engine to drift up to the ice canopy, operating further up the fjord and near the shore, and testing adaptive sampling strategies. These maneuvers will develop a comfort level of vehicle capabilities under-ice, as well as microbial maps of different regions of Kobbefjord. By the end of the field program, we would carry out multi-day deployments in which the vehicle repeatedly maps a section from open water to a location well under the ice. Vehicle operations will be controlled via Iridium, with results will be made available on the web immediately. Although Iridium reported data would necessarily be small summary messages, the entire vehicle data set will be downloaded between deployments and also made available via the web. This strategy will allow participants at UW and MBARI to analyze vehicle and cytometer data, greatly accelerating our ability to tune CytoAUV's performance. Finally, we will collect discrete samples for identification of populations encountered by CytoAUV at the ice-edge and in open-water (Demir-Hilton et al. ISME 2011, Cuvelier et al, PNAS 2010).

1.4.2.4.2 *System refinements*

Instrument and vehicle subsystems may be modified based on lessons learned from the first Arctic field campaign. In particular, we will likely refine the adaptive sampling algorithm based on cytometer and contextual sensor data acquired during the campaign.

1.4.2.5 Year 4

1.4.2.5.1 *Second Arctic campaign*

The second Nuuk field program will focus on developing a full time-series of cytometer observations in Kobbefjord over a two-week period. The details of this expedition will be driven by results of the first deployment, but will likely involve a vehicle mission sequence like:

- Run a section up Kobbefjord, return to entrance, surface, telemetry back result summary
- Assume low power configuration at depth, and loiter for some fraction of a day
- Surface to communicate with shore for new mission directives
- Inform small boat operations and ice operations for sample collection at hotspots

AZW 9/2/11 7:08 AM

Comment [4]: ginger we could site e.g. Demir-Hilton et al. ISME 2011, Cuvelier et al, PNAS 2010 - they aren't critical but would give an idea of what we are talking about and would involve some references to work by me

identified by *CytoAUV* (adaptive sampling validation in extreme environment)

- Either carry out new mission, or if no new mission, repeat the section.

The most likely scenario will be to run the vehicle sensor suite almost continuously and recover and redeploy the system often, given the convenience of Kobbefjord to Nuuk.

1.4.2.5.2 Post-mission data processing

Data archiving, reprocessing of the cytometry diversity index, and fine-tuned clustering analysis of microbial populations will occur after vehicle recovery and raw flow cytometric data retrieval. Raw data will be quality checked prior to data archiving. Optimally positioned particles will be identified using our fine-tuned filtration algorithm (Ribalet et al. 2011) and the resulting derived data set will be saved in the standard flow cytometric data format (.fcs). The cytometry diversity index will be re-calculated and size and fluorescence characteristics of individual cells will be normalized by the bead signals (used as an internal standard) to correct for instrument drift and allow comparison between deployments. Fine-tune clustering of microbial populations, especially the eukaryotic phytoplankton, requires sophisticated clustering algorithms. We will further develop our first-generation software and pipeline that automates population clustering to minimize the required human guidance. We will focus on reducing clustering ambiguities by implementing new statistical priors and testing newly developed cytometric clustering algorithms (e.g. Aghaeipour et al. 2011). We will also check our results in general against complementary datasets generated by other research teams at the GCRC in Nuuk (see Rysgaard letter).

1.5 Project Management

1.5.1.1.1 Responsibility of team members

Ginger Armbrust will serve as PI and will oversee scientific integration, coordination and direction of research projects as needed. She has overseen development of *SeaFlow* and is an expert on phytoplankton. James Bellingham has extensive experience with development of AUVs and AUV operations including under ice. He will oversee engineering and development of the AUV and will be responsible for executing AUV field programs. Alexandra Worden is a phytoplankton expert and has years of experience working with flow cytometry at sea. Worden will lead the science program in Monterey Bay. Jody Deming is a recognized expert on microbial communities in and around sea ice in the Arctic and close colleague of Søren Rysgaard (e.g., Bowman et al., 2011) who leads the GCRC in Nuuk, Greenland, and will host our operations there (see support letter). Deming will be responsible for developing the scientific program for operations at Nuuk, coordinating analysis of results. Worden and the MBARI postdoc will lead the design and analysis of the Pacific validation cruises. Deming, Armbrust and the UW postdoc will lead design and analysis of Nuuk datasets. Postdocs from both institutions will be involved in field work, data analysis, synthesis and manuscript preparation for results from both regions and will develop complementary analysis strategies for validation of identified populations, providing these trainees with exposure to different types of mission planning and dramatically different ecosystems. Jarred Swalwell and Francois Ribalet have worked closely together to innovate at the engineering and computer science interface and develop *SeaFlow* and interpret the resulting data. Thomas O'Reilly will function as system engineer for the overall effort, to ensure that elements developed at UW and MBARI integrate successfully. He and the software engineers Mike Risi and Yanwu Zhang will develop software for the instrument and vehicle, including data acquisition and logging, diversity index calculation, and adaptive sampling algorithms. Mechanical engineers Brett Hobson and Jon Erickson and electrical

engineer Ed Mellinger will design vehicle-instrument interfaces and build an Arctic-capable vehicle. Technician Thomas Hoover will help build and operate the vehicle. Shelly Carpenter and Rhonda Morales will provide technical assistance during field campaigns and laboratory experiments. Rita Peterson will provide assistance for logistical coordination, including organized interactions between UW and MBARI teams. Two undergraduates per year, supervised by Armbrust and Deming, will conduct summer research at UW.

1.5.1.1.2 Research integration and communication plan

Project members will communicate by conference call to review project goals and research progress and to make adjustments as necessary. This degree of exchange will lead to the professional growth of individual scientists, development of new projects and ideas and likely result in new collaborative ventures. The program will require frequent interactions between members of the different labs and students, with post-docs and staff likely spending time in more than one laboratory. An annual workshop will be held at UW in Years 1 and 3 and at Monterey in Years 2 and 4. All team members will work together during planned field campaigns in year 2 in Monterey Bay and in Nuuk, Greenland in Years 3 and 4. Contingency work will be performed in Monterey Bay given easy boat access and crew familiarity with *Tethys* operations.

1.5.1.1.3 Data and information management plan

Research results will be disseminated via publications, meeting presentations, and the workshops. The breadth of our team ensures broad dissemination across many fields, including Astrobiology (e.g., Deming and Eicken 2007). We will develop an authorship policy to ensure appropriate credit for each team member such as recommended by the International Committee of Medical Journal Editors (www.icmje.org). We are dedicated to timely release of data.

1.6 Education and Outreach

Educational opportunities that involve the intersection of engineering, math and biology are all too rare. This project provides an exciting opportunity for undergraduate students to be exposed to interdisciplinary science and engineering in two exciting natural environments, with experiences that are highly relevant to space missions. The leadership team includes one male and three female investigators (including PI) at different career stages and with different backgrounds and expertise. Each year, 2 UW undergrads will be recruited for the summer to work on cytometry development and analysis. In the past, we have recruited students with background in engineering, computer science, oceanography, and biology and we will continue to recruit in these areas. In Years 2 and 4 we will host a hands-on 'Integrated Science and Engineering Careers' workshop for University of California Santa Cruz students where Co-I Worden has a joint appointment. The workshop will be held on dates adjacent to the annual project science meeting when UW participants will be at MBARI. Students will come to MBARI and rotate between the offerings of the different core disciplines involved in the project, including machine shops, flow cytometry, biological interpretation and integration with instrumentation, AUV testing (in the MBARI test-tank), mission planning for extreme environments and leading integrative research efforts. During the summer the project will recruit interns from the MBARI internship program to participate in development of an internet telepresence for the project. Interns will work directly with software engineer O'Reilly, and Co-Is Worden and Bellingham. Arctic Mission data will be web accessible to the broader community in near real-time (as near real-time as Satellite links allow).

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