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Instruments and Methods

Autonomous Microbial Sampler (AMS), a device for the uncontaminated collection of multiple microbial samples from submarine hydrothermal vents and other aquatic environments

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Abstract

An Autonomous Microbial Sampler (AMS) is described that will obtain uncontaminated and exogenous DNA-free microbial samples from most marine, freshwater and hydrothermal ecosystems. Sampling with the AMS may be conducted using manned submersibles, remotely operated vehicles (ROVs), autonomous underwater vehicles (AUVs), or when tethered to a hydrowire during hydrocast operations on research vessels. The modular device consists of a titanium nozzle for sampling in potentially hot environments ($>350\,^{\circ}C$) and fluid-handling components for the collection of six independent filtered or unfiltered samples. An onboard microcomputer permits sampling to be controlled by the investigator, by external devices (e.g., AUV computer), or by internal programming. Temperature, volume pumped and other parameters are recorded during sampling. Complete protection of samples from microbial contamination was observed in tests simulating deployment of the AMS in coastal seawater, where the sampling nozzle was exposed to seawater containing 1×10^6 cells ml^{-1} of a red pigmented tracer organism, *Serratia marinorubra*. Field testing of the AMS at a hydrothermal vent field was successfully undertaken in 2000. Results of DNA destruction studies have revealed that exposure of samples of the Eukaryote *Euglena* and the bacterium *S. marinorubra* to 0.5 N sulfuric acid at 23 °C for 1 h was sufficient to remove polymerase chain reaction (PCR) amplifiable DNA. Studies assessing the suitability of hydrogen peroxide as a sterilizing and DNA-destroying agent showed that 20% or 30% hydrogen peroxide sterilized samples of *Serratia* in 1 h and destroyed the DNA of *Serratia* in 3 h, but not 1 or 2 h. DNA AWAY™ killed *Serratia* and destroyed the DNA of both *Serratia* and the vent microbe (GB-D) of the genus *Pyrococcus* in 1 h.
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1. Introduction

The study of the ecology, diversity and function of microbes in environments such as hydrothermal vents requires the marriage of culture-independent, molecular techniques of DNA sequencing and manipulation to determine the important members of the community and culture-dependent approaches to understand their physiology and functioning. In either case, it is essential that samples be obtained that are free from cross-contamination by microbes and microbial DNA from locations other than the site of sampling. Because most devices that are used to sample remote aquatic environments must first be transported through heavily contaminating waters (e.g., air–water interface near a ship, upper water column, etc.), means must be provided for protection of collected samples from such contamination or from cross-contamination between sampling sites. Since both viable microbes and/or DNA from the habitat of interest are analyzed, protection from both types of contamination is essential.

Obtaining contamination-free microbial samples from the marine and other remote environments has long been of interest (historical account in Zobell, 1941; see also Karl and Dore, 2001), beginning with hydrowire deployed samplers (e.g., Zobell, 1941; Niskin, 1962; Lewis et al., 1963) that implemented sterile evacuated bottles, bellows-like polyethylene bags or rubber bulbs to take samples. Proximity of the sample inlets to the hydrowire and initiation of sampling by unsterile components (e.g., smashing glass inlet tube by messenger or cutting inlet tubing with unsterile knife edge) lead to significant contamination issues, however. To tackle the contamination issue, Jannasch and Maddux (1967), developed a swing arm-like sampler that oriented into the current and mechanically drew samples into sterile syringes away from the hydrowire while a sterile dialysis bag was removed from the sterile inlet prior to sampling. Field tests, where the outer surfaces were purposely contaminated with a tracer organism, revealed dramatic improvements in the collection of uncontaminated samples (Jannasch and Maddux, 1967). However, the sampler was not widely implemented.

For sampling in deep waters where hydrostatic pressure is a parameter that can affect microbial viability and growth, pressure-retaining samplers have been implemented in a variety of designs for retrieval of samples in the absence of decompression. Examples include single sample versions with (Jannasch et al., 1973; Jannasch and Wirsen, 1977) and without (Tabor et al., 1981) sample inlet protection to reduce the potential for contamination. Bianchi et al. (1999) expanded the Jannasch et al. concept to allow up to eight pressurized samples to be taken during a single deployment, though inlet protection is less rigorous (alcohol-sterilized parafilm).

Microbial studies at hydrothermal vents have catalyzed development of an array of vent samplers that can obtain samples from hot environments. Malahoff et al. (2002), for example, have developed a sampling system that is able to take vent fluid samples and maintain both in situ temperature and pressure of the sample collected. Once aboard, ship subsamples can be transferred to multiple incubators without change in either pressure or temperature. Phillips et al. (2003) designed a sampler for the capture, temperature monitoring and in situ incubation of hot smoker fluids under vent conditions. In development at the Jet Propulsion Laboratory (http://www.jpl.nasa.gov/; A.L. Lane, L.C. French) is an Underwater Volcanic Vent Mission probe, an instrumented titanium probe that can be inserted into warm and hot water hydrothermal vents for in situ measurements of temperature, imaging and spectrographic analyses within the vent crevices for evidence of microbial growth in these high-pressure liquid environments.

Recent interests in sampling the Antarctic subglacial lake, Lake Vostok, for unique microbes that have been isolated from the surface biosphere for up to tens of millions of years (e.g., Siegert et al., 2003) have catalyzed the ongoing development of sampling devices that can aseptically penetrate the ice sheet to sample the subglacial water column and sediments, while maintaining the two biospheres isolated from one another (e.g., Blake and Price, 2002). Technology is also in development (e.g., Cryobot; Subsurface Ice Probe) to search for evidence of extraterrestrial life in the Martian icecaps (e.g., Cardell et al., 2004; Carsey et al., 2005) and possible sub-ice oceans on Europa (e.g., Carsey et al., 2000; French et al., 2001).

Most microbial samplers presently in use do not have sufficient inlet protection to guarantee freedom from exogenous contamination. To address this issue, we have developed an Autonomous Microbial Sampler (AMS) that obtains six microbial samples that are free from exogenous microorganisms or DNA. A technical description of the AMS and

procedures for sterilization and DNA removal are described herein.

2. Materials and methods

2.1. Test organisms and growth conditions

Serratia "*marinorubra*", a red pigmented bacterial strain of *S. plymuthica* was isolated from coastal waters. This aerobic mesophile was grown in Marine Broth 2216 (Difco) at 23 °C. The Archaeal strain *Pyrococcus* GB-D was isolated from within the outer surface of a black smoker chimney at a Guaymas Basin 2020-m-deep hydrothermal vent site in the Gulf of California. The hyperthermophilic Archaea was grown anaerobically in sulfur-containing modified Marine Broth 2216 (Jannasch et al., 1992) at 95 °C. A culture of the protist *Euglena gracilis*, supplied by Dawn Moran, Woods Hole Oceanographic Institution, was grown in Hutner's Medium (ATCC Culture Medium 351) at 20 °C.

2.2. Measurement of heat transfer in the insulated sample chamber (ISC)

The ISC was outfitted with a complete array of sampling modules (SMs) (as shown in Fig. 1B), the interior filled with water and the assembly allowed to equilibrate to room temperature overnight. The ISC was then immersed in a refrigerated water bath maintained at a constant 4.5 °C. The bath contained a high-volume pump that rapidly circulated the water around the ISC, simulating the forced convection that would occur as the submersible, remotely operated vehicle (ROV) or hydrowire transports the AMS up through the water column. Change in the ISC internal temperature would not begin until the AMS was transported above the thermocline where temperatures exceeded ~3 °C. The temperature of the water bath and the interior of the ISC were measured using an Omega Engineering temperature unit equipped with a 1/16″ (~1.6 mm; time constant ~2 s) thermocouple probe. The thermocouple probe was inserted through one of the fittings in the ISC top to measure the internal temperature of the contained water or placed directly in the refrigerated bath to monitor the external temperature. The ISC was inverted several times every ~10 min to completely mix the interior water to simulate the semi-forced convection that would occur at the interior walls of the ISC during the intermittent lurching and rocking of the AMS during transport through the upper water column and at the sea surface.

2.3. Water sampling vessel (WSV) testing

A dye study was conducted to quantify the dynamics of mixing of sample into the WSV (Fig. 1B; see also Fig. 4). The inlet tube of a microgear pump was connected either to a vessel containing a deionized water solution of dilute sodium carbonate (pH ~9) or a vessel of the same alkaline solution containing in addition the dye phenol red (absorbance maximum, 557 nm; pH ~9 assured that the dye was completely in the 557 absorbing form). The outlet tube from the pump was in turn connected to the inlet of a 50 ml WSV. The outlet of the WSV was connected via narrow bore tubing to a spectrophotometer flow cell contained within a Shimadzu UV-1201 spectrophotometer equipped to record and display the time course of the absorbance at 557 nm of the fluid passing through the flow cell. The dead spaces in the pump and interconnecting tubing were flushed prior to experiments to assure that only the flushing dynamics of the WSV were being measured. Three minute trials were begun by simultaneously starting the pump and the spectrophotometer time course program. Absorbance at 557 nm and the cumulative volume of fluid pumped were recorded by the spectrophotometer and microgear pump software, respectively.

2.4. Test of the sampling nozzle temperature probe

The temperature response of the temperature probe was measured by alternately immersing the sampling nozzle 8–10″ (20–25 cm) into approximately 20 l freshwater temperature baths of different temperatures. The sampling nozzle was continuously agitated during measurement at a given temperature. The temperatures of the baths, measured by a mercury thermometer, did not change during the measurements.

2.5. Contamination assessment studies

The AMS apparatus was sterilized by repeated exposure of the sampling end of the nozzle, internal conduiting, distribution valve (DV) rotor/stator and pump heads to 20% hydrogen peroxide for 3 h, followed by rinsing with sterile DNA-free deionized water (water and container bag autoclaved for 1.5 h

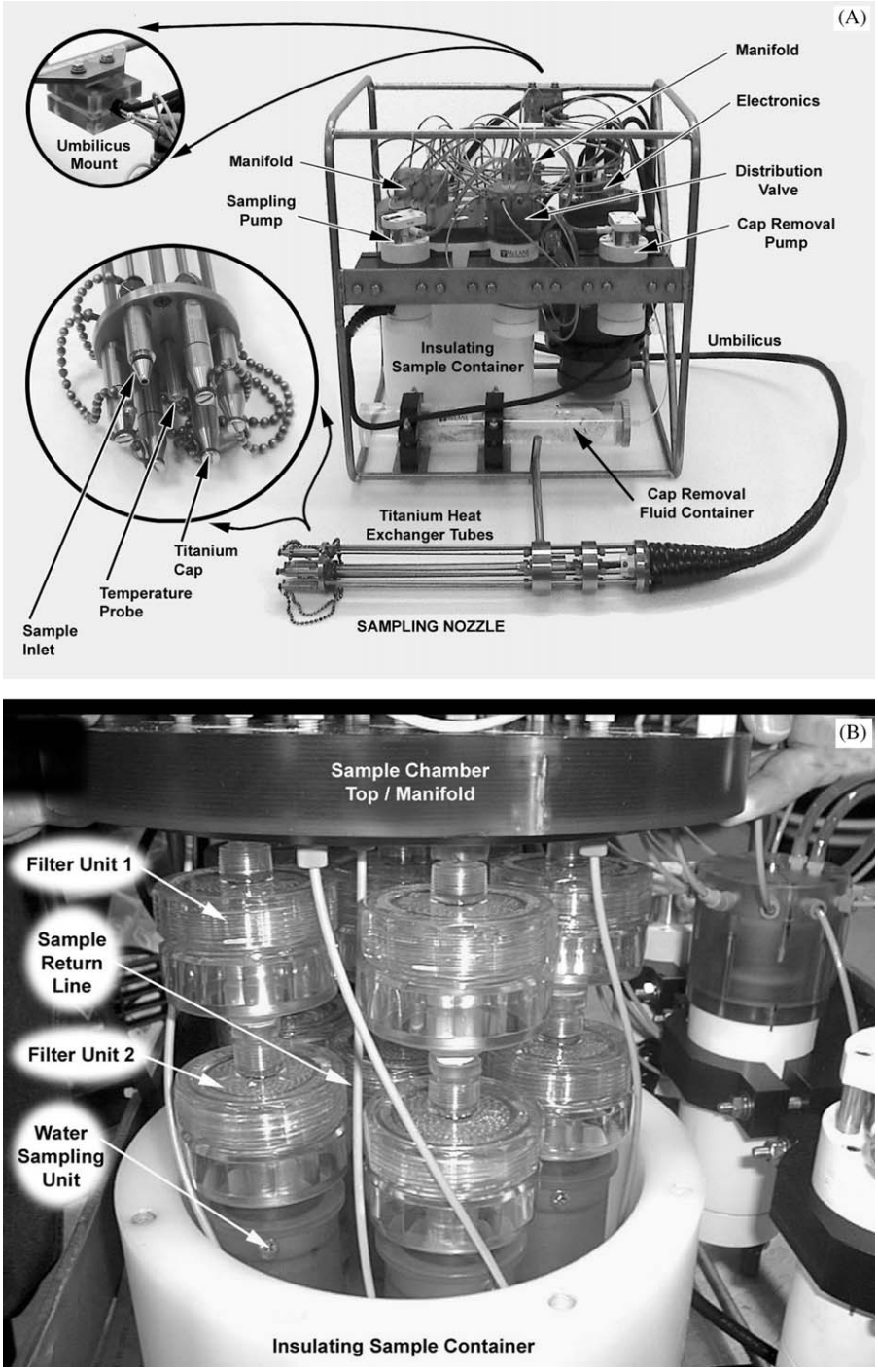

Fig. 1. Autonomous Microbial Sampler (AMS). Panel A, external hardware. Panel B, sample retaining components.

to remove DNA, see Table 3) contained within the cap removal fluid container (Fig. 1A). Sterile 50 mm, 0.45 μm Millipore™ cellulose filters housed in sterilized 50 mm in-line filter holders were filled with sterile deionized water and connected into the AMS as shown in Fig. 1B (only one filter unit per channel rather than multiple units shown). Fluids were distributed through the AMS conduiting via

the two software-controlled pumps using the software in Manual Operate mode (see Section 3.3.2).

The AMS was tested for its ability to obtain uncontaminated microbial samples using the apparatus illustrated in Fig. 2. The test apparatus was constructed from two ~60 cm sections of 4″ (~10 cm) transparent schedule 40 PVC piping that were united at the center with a standard 4″ (~10 cm) PVC union. The union was modified to form a Tracer Injector using eight 16 ga. lengths of stainless steel tubing glued into place with epoxy and affixed with silicone tubing to form a manifold with flexible injection points as shown in the *top view* inset of Fig. 2. Flowing ~20 °C seawater (nominal flow rate 4.3 l min^{-1}, filtered to nominal 1 μm using a staged large capacity filter) was introduced at the bottom of the apparatus through a flow diffuser made from an approximately ~20 cm deep bed of 1–1.5 cm marbles to guarantee laminar flow up the pipe until it reached an overflow made from another PVC union fixed with a flow outlet. Prior to use, the entire apparatus and tubing was sterilized using a 5% bleach solution, which effectively killed the red pigmented *S. marinorubra* tracer organism. The bleach solution was drained and the apparatus flushed for ~20 min with the filtered seawater. During testing, a dye solution (phenol red, in deionized water with pH adjusted to ~8 with sodium bicarbonate) and an ~1×10^9 cells ml^{-1} culture of the tracer organism

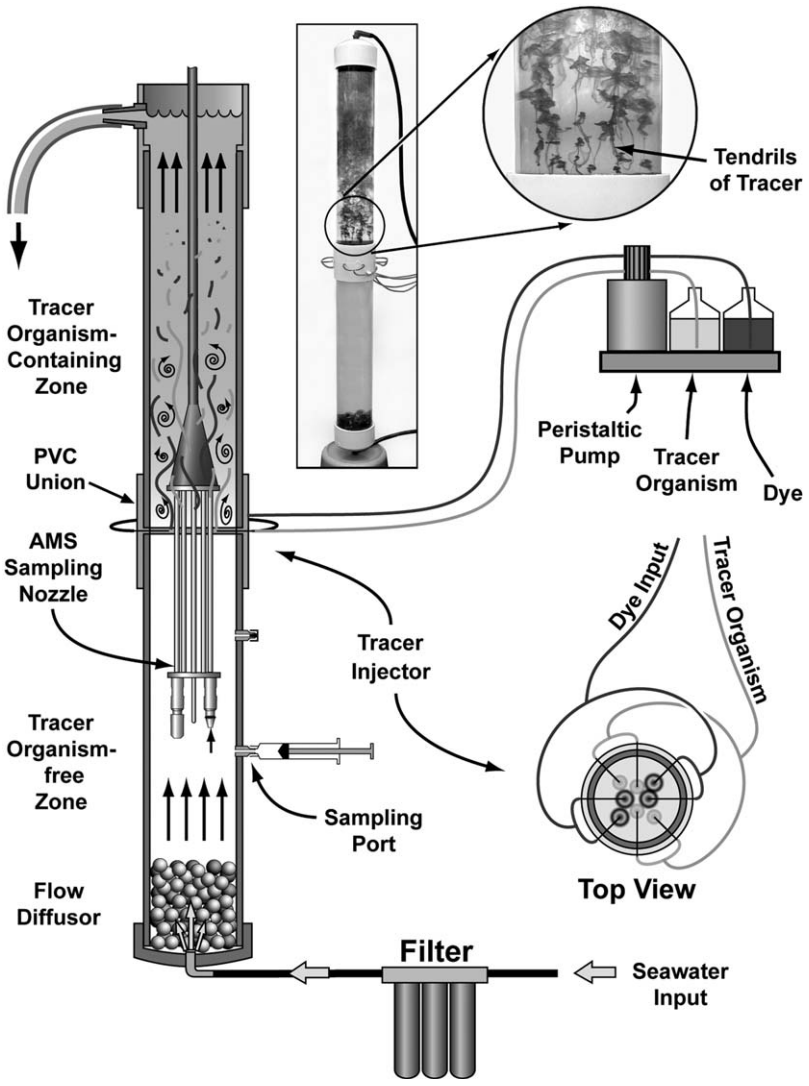

Fig. 2. Apparatus for testing the ability of the AMS to obtain uncontaminated samples.

grown in 80% seawater (to minimize any chance of the tracer settling below the Tracer Injector) containing 0.1% yeast extract and 0.5% peptone was introduced via peristaltic pump (nominal flow rate, ~4.5 ml min^{-1}) into the flowing seawater as shown in the "top view" inset of Fig. 2. The dye was implemented to provide a visual guarantee that "contaminated" water did not penetrate into the lower "uncontaminated" zone (see Fig. 2 photo inset). To guarantee that the "uncontaminated" zone was indeed free of *S. marinorubra*, samples were obtained during testing through Luer-Lok™ sample ports in the side PVC tube using sterile 60 ml syringes. Samples (50 ml) were passed through sterile 50 mm, 0.45 μm Millipore™ cellulose filters housed in sterilized 50 mm in-line filter holders for later culture.

The injected tracer/dye was allowed to come to steady state with the flowing seawater, so that a final density of ~1×10^6 cells ml^{-1} of tracer organism was attained when fully mixed in the top 1/3 of the "contaminated" zone. Because of the laminar flow of seawater in the test apparatus, the tracer suspension tended to migrate up the PVC pipe as coherent tendrils of concentrated tracer (see Fig. 2 photo insets) until approximately the halfway point in the "contaminated" zone, where gentle turbulence effectively mixed the tracer with the flowing seawater. Just prior to lowering the AMS nozzle into the test apparatus, the peristaltic pump was switched off and the tracer organism/dye front was allowed to migrate ~30 cm up the pipe (to allow mixing of the tracer organism with the flowing seawater) before the AMS nozzle was slowly (~15 s) lowered into the test apparatus. This procedure exposed the AMS nozzle to $\geqslant 1 \times 10^6$ cells ml^{-1} of tracer organism, to simulate an exposure to contaminating coastal waters prior to sampling. Replicate 75 ml samples were taken in the "tracer organism-free zone" using the AMS. During some tests, one of the AMS filters was skipped during sampling to provide a negative control. The protective caps were machined of autoclavable polypropylene plastic, which are less dense than seawater. Upon removal during sampling, the caps floated to the top of the test apparatus and were retrieved. The floating caps prevented the marble bed from being contaminated by tracer organism during the sampling process.

All the filters were placed onto the surface of Marine Agar 2216 (Difco) (medium containing sea salts, 0.1% yeast extract, 0.5% peptone and 1.5% agar) for growth of colonies. The nominal 1 μm filtered flowing seawater did contain viable microorganisms that would grow on the above medium (~33 [±6 SD] cells ml^{-1}) but none contained a red pigment that could be confused with *S. marinorubra*, which formed bright red colonies when allowed to grow on the filters for ⩾2 days at room temperature.

2.6. Chemical procedures for sterilization and DNA removal in samples

2.6.1. Acid/base hydrolysis

Aliquots (50 μl) of rinsed suspensions containing 3×10^4 cells of *Euglena* or 2×10^8 cells of *Serratia* were distributed among a number of sterile 1.5 ml microfuge tubes (6 × 35 mm glass tubes for the 200 °C treatment). The microfuge tubes were subjected to alkaline treatment (0.5 N NaOH ± 1.5 N NaCl) or acid treatment (0.5 N H_2SO_4) at two temperatures (~25 and ~50 °C). Additional treatments involved autoclaving for periods up to 1 h and exposure to 200 °C dry heat for select periods ranging from 0 to 24 h. At the end of a specified treatment, the aliquots subjected to acid/base hydrolysis were neutralized prior to extraction of DNA.

2.6.2. Treatment with hydrogen peroxide or DNA AWAY™

Aliquots (50 μl) of rinsed suspensions containing 3×10^7 cells of *Serratia* or *Pyrococcus* were added to microfuge tubes, centrifuged for 7 min at 12,200 × *g* (10,000 rpm) to pellet the cells and the supernatant discarded. Hydrogen peroxide (50 μl of 10%, 20% or 30% solutions) or DNA AWAY™ (Molecular BioProducts) were added as treatments. Following specified times of 1, 2, or 3 h, the contents were evaporated to dryness so that the treatment solutions did not interfere with the DNA extraction procedure. After drying, 150 μl sterile DNA-free TE buffer (10 mM Tris–HCl, 1 mM EDTA), pH 8, was added to each sample (made from deionized water that was autoclaved for 1.5 h). A control that was not evaporated was run to rule out DNA destruction by evaporation.

2.7. Viability studies

Aliquots (50 μl) of *Serratia* containing 4×10^7 cells added to sterile microfuge tubes. In Treatment

I, 50 μl of 10%, 20% or 30% hydrogen peroxide was added to yield a final concentration of 5%, 10% or 15%. Treatment II removed any effect of the liquid medium. In this treatment, cells were first pelleted by centrifugation, the supernatant discarded and 50 μl 10%, 20% or 30% hydrogen peroxide or DNA AWAY™ added to the tubes. A control tube was resuspended in sterile 3.5% NaCl. Treatment III checked for effects of residues from the treatments on viability of newly added cells. Tubes from Treatment I containing 15% hydrogen peroxide or DNA AWAY™ were evaporated to dryness. Fresh cells in medium were pelleted in separate tubes by centrifugation, resuspended in an equal volume of sterile 3.5% NaCl and 50 μl added to each tube. An untreated control tube received 50 μl of the sterile 3.5% NaCl resuspended cells. At designated time intervals of 1, 18 and 41 h, tube contents were resuspended using a Vortex mixer and then 2 μl was spotted on the surface of Marine Agar 2216 plates. Plates were examined over 7 days for growth of red colonies of *Serratia*.

2.8. Extraction of remaining DNA for PCR assays

DNA was extracted into phenol according to the procedure of Maniatis et al. (1982). Any intact cells were lysed by the addition of 417 μl TE buffer, pH 8, 30 μl 10% sodium dodecyl sulfate (SDS) and 3 μl Proteinase K (20 mg ml^{-1}) followed by incubation at 37 °C for 1 h. Next, 100 μl of 5N NaCl and 80 μl CTAB/NaCl were added and the mixed suspension incubated at 65 °C for 10 min. An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added and thoroughly mixed before centrifuging in a microfuge at 15,900 × *g* (13,000 rpm) for 5 min. The supernatant was removed and treated again in the same manner. The supernatant was transferred to another sterile microfuge tube. After adding 0.6 volume of ice-cold isopropanol to precipitate any DNA, the preparation was stored at −20 °C overnight. The DNA was pelleted in a microfuge at 15,900 × *g* (13,000 rpm) for 20 min and the supernatant discarded. Following the addition of 200 μl ice-cold 70% (vol/vol) ethanol, the preparation was centrifuged in a microfuge as above and the supernatant discarded. After drying under a stream of filtered nitrogen, the precipitated DNA was dissolved in 30 μl 3 × autoclaved DNA-free deionized water.

2.9. Polymerase chain reaction (PCR) amplification of 16s rDNA

DNA extracted as above was used as template in the PCR procedure. The Bacterial primer pairs 8F(5′-AGRGTTTGATCCTGGCTCAG-3′) and 1492R(5′-CGGCTACCTTGTTACGACTT-3′) as well as Archaeal primer pairs 8F(5′-TCCGGTTGATCCTGCC-3′) and 1492R(5′-GGCTACCTTGTTACGACTT-3′) used for PCR were produced by Operon Technologies, Inc., Alameda, CA. For *Euglena*, modified forward primer A (ATCTGGTTGATYCTGCCAG) and reverse primer B (CACTTGGACGRCMWCCTAGT) were supplied by Michael S. Atkins, Woods Hole Oceanographic Institution (Atkins et al., 2000). The GeneAmp® PCR System 9700, PE Applied Biosystems, Norwalk, CT, was used for all samples. PCR reaction mixtures totaling 100 μl contained 10 μl 10 × PCR buffer (Teske et al., 2002), 8 μl deoxyribonucleoside triphosphate (dNTP), 1 μl of each primer, 76.5–78.5 μl PCR water, 0.5 μl Taq polymerase and 1–3 μl DNA template.

The PCR-amplified samples were loaded on 1% (wt/vol) agarose gels. Following electrophoresis, the gels were stained with ethidium bromide and were examined for DNA bands with the ChemiImager™ 4000 Low Light Imaging System, Alpha Innotech Corp., San Leandro, CA.

2.10. Materials

All chemicals were of reagent or analytical grade. The following crucial items were obtained from the companies indicated: temperature probe, Omega Engineering, Inc.; polysulfone filter units, Nalge Nunc International Corporation (Nalgene®); fittings, Upchurch, Inc.; Tedlar™ bags containing sterile, DNA-free deionized water for cap removal, McLane Research Labs, East Falmouth, MA.

3. Results and discussion

3.1. Description of the AMS

There are two major technical directions one can take in the development of devices for obtaining microbial samples from the deep ocean, that of maintaining both the hydrostatic pressure and temperature of the site of sample collection, or allowing the samples to decompress while focusing on temperature control to protect psychrophiles.

Studies of Yayanos and colleagues (Yayanos and Dietz, 1983; Yayanos and DeLong, 1987) suggest that the detrimental effects of decompression on viability of psychrophilic barophiles are relatively slow and secondary to the effects of elevated temperature. While abyssal barophiles (from depths between ~3000 and 6000 m) that are in culture grow poorly when decompressed, their viability is likely to remain intact during typical periods required for sample recovery and recompression to in situ pressures (~4 h; Yayanos and DeLong, 1987), so long as low temperatures are maintained. Hadal "obligate" barophiles (from depths >6000 m) are significantly more sensitive to decompression. Cultures of the hadal strain MT-41, for example, lose approximately 60% of their viability when decompressed for ~4 h (Yayanos and Dietz, 1983). Because of the technical difficulties associated with the routine use of high-pressure equipment and the reasonable likelihood of obtaining viable microbes to at least abyssal depths, we chose to focus on a design that does not retain hydrostatic pressure during sample retrieval.

The AMS is shown in Fig. 1. The dimensions of the cage are 49.5 cm (19.5″) wide × 46 cm (18″) deep × 56 cm (22″) high, and the instrument weighs 38.6 kg (85 lbs) in air when the ISC contains no water and 46.7 kg (103 lbs) in air with the ISC full of water, which would be the typical weight when handled on deck. The instrument weighs 16 kg (35 lbs) in freshwater or 15.4 kg (34 lbs) in seawater (ISC full of water). The major components of the AMS include the following: (1) A sterilizable sampling assembly (Fig. 1B) composed of the ISC lid into which are mounted six SMs that each consist of an assembled series of interchangeable filter units and/or containers for the collection of particulate or water samples according to user needs. The sampling assembly and SMs are removable for the processing of samples and for sterilization. (2) Fluid-controlling components (Fig. 1A) comprised of a DV and two positive displacement microgear pumps (cap removal pump, sampling pump). (3) A sterilizable sampling nozzle and associated umbilicus that will permit uncontaminated sampling of the environment by a submersible or autonomous underwater vehicle (AUV) manipulator arm or by direct mounting on the vehicle. A temperature probe has been incorporated into the nozzle to permit continuous measurement and recording of temperature at the site of sampling. (4) A Tattletale 8™ (TT8™)-based electronic controller/data recorder for controlling sampling events and interfacing with the user.

A functional schematic of one channel of the AMS is shown in Fig. 3. Between sampling events, the unit is configured as illustrated in panel A, where the DV rotor is positioned between ports, sealing the valve. The first sampling event is initiated by advancement of the valve rotor to cap removal port 1 as shown in panel B, followed by activation of the cap removal pump (P1). Sterilized, DNA-free deionized water (autoclaved 1.5 h) is briefly pumped, as shown by the arrows (fluid path unshaded), through the valve to a sampling line "T" manifold, to the umbilicus and sampling nozzle. Pressure accumulates within an "energizer coil" that suddenly hydraulically expels the titanium (or polypropylene for sterility testing described below) cap to expose a sterile titanium sample inlet tube. P1 is turned off and the DV advanced to sampling port 1 (panel C). Sample collection begins by activating the sampling pump (P2) that draws sample (arrows) from the sampling nozzle through SM 1 of the sampling unit, the DV, pump P2 and ultimately back into the environment (fluid path unshaded). Upon completion of sampling, pump P2 is turned off and the DV is advanced to the next sealed position as shown in panel D. The process is repeated for each of the five remaining samples. Sample contained within the SM is protected from contamination by the long diffusion path in the sampling umbilicus and by the sealed valve.

3.2. Electronic controller

The electronics stack is composed of four 2″ × 3″ printed circuit boards. The first of these is a TT8™ single board microcontroller manufactured by Onset Computer Corporation. The system's control program is run on the TT8's Motorola 68332 microprocessor and operates the system through the peripheral boards, designed and built by McLane Research Laboratories, East Falmouth, MA.

The first of these boards, the AUX/Stepper, is composed of two portions. The AUX portion accepts power from the main battery or an external power source, provides the regulated voltages necessary to run the rest of the system and a port for connection of a 9-V battery for memory backup, and the precision circuit to which the sampling nozzle temperature probe is connected. Power to the valve and pumps is switched under software control.

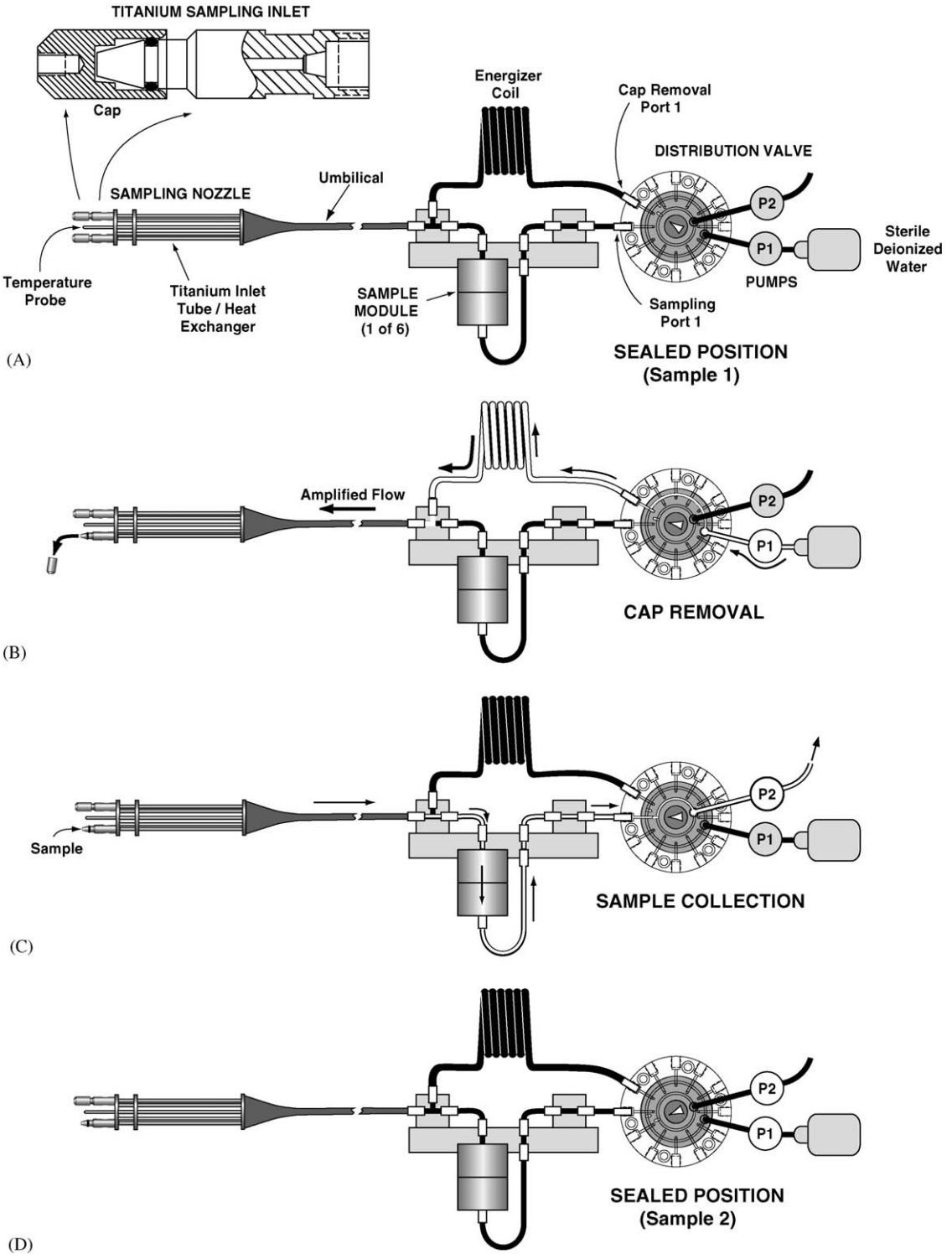

Fig. 3. Functional schematic of one channel of the AMS fluid-handling components.

The wiring harness is configured to accept main battery and external power sources simultaneously. Current is automatically drawn from the higher voltage source. The temperature sensor itself, a thin film or wire-wound platinum element, is mounted at the end of the sampling umbilicus (Fig. 1A, large circled inset). The Stepper portion of this board contains the hardware for translating TT8™ control signals into the commutation signals that drive the stepper motor of the multiport valve. The binary signal from a slotted disk and a proximity switch is used in conjunction with software algorithms to locate the "home port" and to perform precise port alignment and identification during operation.

The remaining two boards in the stack are both 3-phase motor control boards. Each is associated with one of the two system pumps for operation as described above. The pump circuit is based on a high-performance Motorola chip set which is controlled by the TT8™. An analog signal is generated and combined in a supervisory chip with the position of the motor shaft to construct 3-phase commutation signals. A triad of Hall sensors mounted in the motor senses the position of the shaft and the commutation signals are applied to the motor through power FETs in the drive chip. A final chip returns shaft speed information derived from the Hall sensor signals to the TT8™ to close the flow rate control loop in software (see below).

3.3. Software

3.3.1. Operator interface

The operator controls the microbial sampler through a layered menu structure. From the Main Menu, the operator has access to the real-time clock, system diagnostic and test routines, deployment programming and control, and the instrument data file. The user is presented with the Main Menu when the sampler is first powered up and can return to the Main Menu at anytime.

The communications interface is a standard, 3-wire, RS-232 connection passing ASCII characters. The "operator" works through a laptop PC running a terminal emulator (Cross Cut™) that is provided by Onset Computer Corporation. The interface can also be controlled by a supervisory microprocessor such as might be used to coordinate the actions of an AUV.

3.3.2. Main Menu options

Set time: Permits the operator to set the real-time clock of the TT8 microcontroller which is used to schedule sampling in some modes of operation during a deployment and provides time stamps for sample information stored in the instrument data file.

Diagnostics: Displays the output of the analog to digital channels monitoring main battery voltage and the microbial sampler temperature sensor. The display is repeated at 1-s intervals until terminated by the operator. This option is useful during bench testing and when calibrating the temperature sensor.

Manual operation: Allows direct control of the pumps and valve by the operator through selections from the Manual Operation Menu. This option is intended primarily for bench use during system testing and for instrument preparation prior to deployment.

Sleep: Places the instrument in a low-power mode to extend battery life. The instrument enters this mode automatically if left without operator input or a task list for more than 20 min or during deployment when a task list is complete.

Deploy system: Several different modes of operation are available to the operator. In addition to the various sampling modes, the operator has full control over the pumping parameters for each event. As noted above, the pump is dynamically controlled to track a desired flow rate and to protect the sample. Data acquisition is automatic throughout a deployment.

Offload data: During each deployment, a data file containing the volumes pumped, estimated sample vessel exchange percentage (discussed below), initial and final probe temperatures, start and stop times, ports used, initial and final battery voltages, and diagnostic messages is created. This information is stored both in battery-backed-up RAM and in non-volatile flash memory for use in sample analysis. The file is recovered from the instrument in ASCII text format using the "capture to file" capability of the Cross Cut™ terminal emulator running on the user's PC. A time history of each pumping event, including flow rates, cumulative volumes pumped, battery voltages and probe temperatures, is created. Because of space constraints, this information is stored only in RAM. The pumping histories are also recovered as text files using the terminal emulator. The transfer of the data file is controlled through the Offload Menu.

3.4. Laboratory testing

3.4.1. Water sampling vessel

The WSV unit (Fig. 4A) is designed to collect filtered or unfiltered water samples. Before a deployment, the WSV is pre-filled with sterile, DNA-free deionized water (autoclaved 1.5 h) to prevent contamination while compensating for the pressure at depth. Collection entails pumping sample water into the chamber where it mixes with the precharge water before exiting. The vessel inlet consists of a low-sheer, ducted nozzle (Fig. 4B, inset) that directs the fluid into the sample vessel in a spiral motion to effect complete mixing within the vessel as quickly as possible but without damage to cells (Fig. 4B–F).

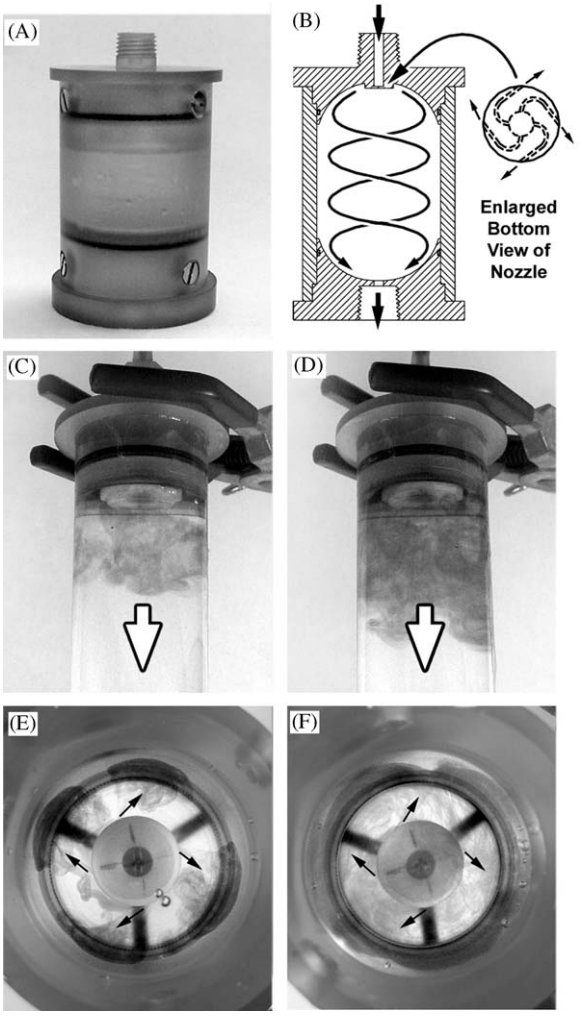

Fig. 4. AMS water sampling vessel (WSV) and demonstration of effectiveness of mixing using a dye.

If mixing is rapid and complete, sample dilution (with the precharge water) should follow a predictable function of the cumulative pumped volume. To test this, quantitative flushing experiments were conducted at 4 flow rates spanning the range used during typical AMS sampling (Fig. 5, 25–100 ml min^{-1}). Dye-containing sample entering the WSV followed the same mixing dynamics independent of the flow rate (Fig. 5A) and agreed quite well with the algorithm:

$$S_V \approx S_E \times [1 - \exp(-k \times (V - D))], \quad (1)$$

where S_V is the absorbance of dye exiting the WSV, representing the concentration of sample after volume V has been pumped through the vessel, S_E is the absorbance of the undiluted dye, in this case representing the concentration of the sample in the environment, k is the dilution constant (ml^{-1}) and D corrects for the fact that ~4.2 ml (~8% of the WSV volume) of fluid was pumped before there was any appearance of dye. This is because the dye does not instantaneously mix with the precharge water as seen in Fig. 4C and D. Best fit values for S_E, k and D were 1.23 ($\pm$0.04 SD) absorbance units, 0.0232 ($\pm$0.0024 SD) ml^{-1} and 4.2 ($\pm$0.9 SD) ml, respectively. Dynamics of dye removal from the WSV (i.e., charge water removal) was also measured by pumping in alkaline deionized water (to prevent the phenol red from changing color) (Fig. 5B) and fit to the equation

$$S_V \approx S_E \times \exp(-k(V - D)). \quad (2)$$

The fit produced essentially the same estimates for k and S_E (concentrated dye originally present in the WSV, representing the charge water). Convergence onto an estimate for D was not possible and was assigned the value 4.2 ml to allow best fit estimates of the other variables. While the above equations did not exactly model the mixing dynamics of the WSV, the best fit estimates obtained were adequate for incorporation into the AMS software to provide a conservative estimate of Sample Vessel Exchange Percentage, $(S_V/S_E) \times 100$, so the operator will know when the contents of the WSV are representative of the environment sampled.

3.4.2. Thermal transfer of heat into the ISC

A high-density polyethylene (HDPE) ISC has been incorporated into the design of the AMS to protect cold collected samples from large temperature excursions during retrieval of the apparatus through

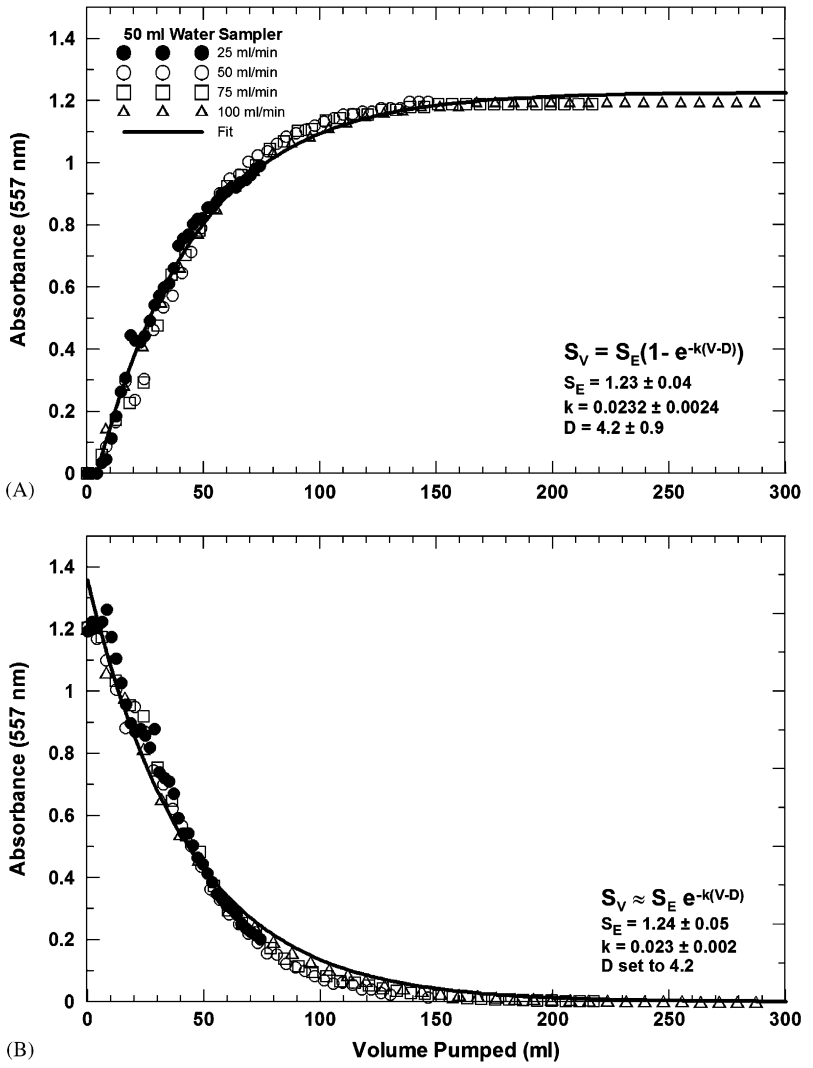

Fig. 5. Mixing dynamics in 50 ml AMS water sampling vessel. Panel A, fraction of sample contained in the WSV vs. volume of sample pumped through system. Panel B, fraction of precharge water remaining in the WSV vs. volume of sample pumped through system.

warmer waters above the thermocline. Recovery of manned submersibles, ROVs or AUVs can take 30–60 min in waters approaching 30 °C in the tropics. Deep-sea microbes residing at environmental temperatures of 3 °C typically possess growth temperature maxima of 8–10 °C and can tolerate temperatures 6 (±3 SD) °C above growth temperature optima (e.g., Hamamoto, 1999; Madigan et al., 1997). Above this upper limit, however, viability is dramatically reduced. Moderate to tropical waters at 25–30 °C are therefore potentially lethal and protection of collected samples from thermal shock is essential.

Given the complex geometry of the internal array of SMs, accurate quantification of the heat flow properties of the enclosure required that the dynamics of heat flow be determined experimentally under conditions simulating a typical AMS recovery. The temperature of the water contained within the ISC (i.e., sample temperature) follows a second-order linear-lumped-parameter response equation (Kreith and Bohn, 1993, pp. 124–126):

$$T(t) = \left[m_2 \times \frac{(T_0 - T_i)}{(m_2 - m_1)} \times \exp(m_1 \times t)\right] - \left[m_1 \times \frac{(T_0 - T_i)}{(m_2 - m_1)} \times \exp(m_2 \times t)\right] + T_i, \quad (3)$$

where $T(t)$ is the ISC internal temperature at time t, T_0 the initial internal temperature (at $t = 0$) and T_i

the temperature of the infinite heat source or sink (temperature bath or upper ocean). A least-squares fit of the data with $T_0 = 18.5\,^{\circ}\mathrm{C}$ and $T_i = 5\,^{\circ}\mathrm{C}$ permitted the determination of rate constants m_1 and m_2 ($-5.00\,\mathrm{h}^{-1}$ and $47\,\mathrm{h}^{-1}$, respectively) that were dependent on the thermal properties of the ISC and assembly of SMs only. It took ~0.13 h before any change in temperature was detected, which reflects the time required for heat to be conducted through the HDPE wall of the ISC (Fig. 6A, closed circles). Once m_1 and m_2 were determined for the ISC under the forced convection conditions at the exterior surface of the container and semi-forced convection at the interior surfaces of the container, it was possible to estimate temperature change for any temperature regime. Given samples collected from the deep ocean ($T_0 = 3\,^{\circ}\mathrm{C}$) and exposure of the AMS to a typically warm upper ocean ($T_i = 25\,^{\circ}\mathrm{C}$) for 0.5 and 1 h, the internal temperature [$T(t)$] would increase by ~3 and ~7 °C, respectively. An acceptable temperature increase for cold adapted samples from the deep ocean occurs within this window. If additional thermal protection is necessary, one could place an open cell foam jacket over the ISC for an incremental improvement in insulating capacity (e.g., a 1/4″-thick and 1/2″-thick jacket would slow heat transfer by ~10% and ~20%, respectively).

3.4.3. Temperature sensor

To determine the response of the temperature sensor, the sampling nozzle was alternately placed into ice water, hot and warm water that was vigorously stirred. Results of the study are shown in Fig. 6B. The data (symbols) were least-squares fit (solid lines) to Eq. (3), where $T(t)$ is the indicated probe temperature at time t, T_0 is the initial temperature of the probe at $t = 0$, T_i the temperature of the bath into which the probe is immersed and m_1 and m_2 the temperature response constants (min^{-1}). The data were reasonably fit by the theoretical equations, though the ceramic electrical insulator within the probe, the titanium mounting plate and sample inlets had some effect on the dynamics of the temperature probe and resulted in small deviations from theoretical two-lump thermal responses. The range of temperature response constants from the experiments shown in Fig. 6B were m_1, -56 (± 13 SD) min^{-1}; m_2, -7.0 (± 0.5SD) min^{-1}. The time required, on average, for the temperature sensor to come within 99% of equilibration was 0.72 min (43 s). Accurate temperature readings of the environment can therefore be made in just under a minute. Adjustments in probe calibration may be made in software.

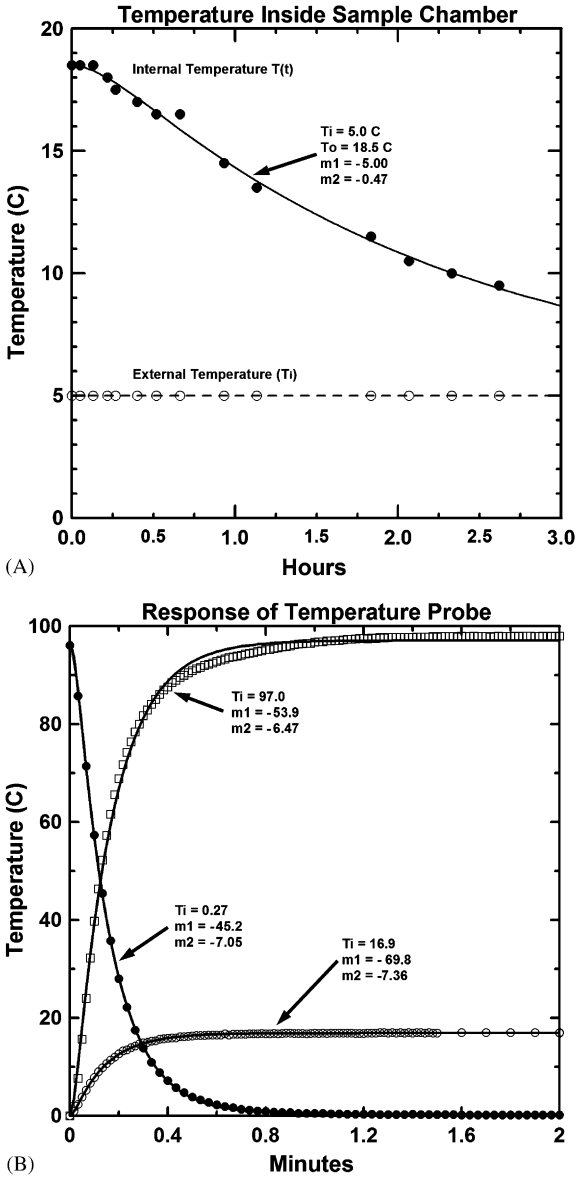

Fig. 6. Time course measurements of the thermal properties of AMS components. Panel A, thermal dynamics of the insulated sample chamber. Panel B, response of the sampling nozzle temperature sensor to abrupt temperature changes (symbols, measured data; solid lines, least-square fits to Eq. (3)). Best fit parameter values are shown in the insets.

3.4.4. Thermal transfer of heat in the sampling nozzle

Because the titanium sampling nozzle is connected to an umbilicus that is composed of

PEEK™ tubing with an upper temperature limit of approximately 200 °C, there is potential for damage if the nozzle is inadvertently or intentionally thrust into very hot vent water (~400 °C). To avoid this, we incorporated a heat exchanger into the design of the nozzle that would be able to lower the temperature of incoming sample fluid from 400 to 200 °C. The heat exchanger was constructed of 0.3175″ OD × 0.23144″ ID Grade 2 titanium tube with a length that was calculated to mediate the desired change in temperature. The fluid heat loss (Q_f, Watts) required to lower the temperature of the sample fluid from T_{in} to T_{out} (°K) can be found from steam tables (Meyer et al., 1968) and the following equation:

$$Q_f = m(H_{in} - H_{out}), \quad (4)$$

where m is the maximum mass flow of sample fluid through the heat exchange tubing (~60 ml min^{-1} = 1.04 g s^{-1}, flow rate at the sampling pump), and (H_{in}–H_{out}) (J g^{-1}) is the enthalpy change in the incoming and exiting fluid at T_{in} and T_{out}, respectively. A linearized estimate for the needed length of the titanium heat exchanger tube was made by equating the heat loss required (Q_f) to the heat transferred through the tubing at the mean fluid temperature (Q_{ex}):

$$Q_f = m(H_{in} - H_{out}) = Q_{ex} = \frac{((T_{in} + T_{out}/2) - T_\infty)L}{(1/\hbar_{ci}2\pi r_i) + (\ln(r_o/r_i)/2\pi K_{Ti}) + (1/\hbar_{co}2\pi r_o)}. \quad (5)$$

The titanium heat exchange tubing possessed an inside radius (r_i) and outside radius (r_o) of 0.116 and 0.159 cm, respectively. The thermal conductivity of Grade 2 titanium, K_{Ti}, was determined from tables to be 0.2 W cm^{-2} °K^{-1} at the temperature range of interest (Kreith and Bohn, 1993). The average heat transfer coefficient between the internal wall of the heat exchange tubing with the internal fluid is $\hbar_{ci}$ (W cm^{-2} °K^{-1}), and the average heat transfer coefficient of the external wall of the heat exchanger tubing that is in free convection with seawater is $\hbar_{co}$ (W cm^{-2} °K^{-1}). Their values are calculated from the equations for forced convection inside the tubing and for a single horizontal cylinder in free convection outside the tubing (Kreith and Bohn, 1993), respectively. These equations were evaluated using the tables of thermodynamic properties of water (Meyer et al., 1968) at the average bulk temperature of the fluid ($(T_{in} + T_{out})/2 - T_\infty$), where ($T_{in} + T_{out}$)/2 is the average fluid temperature and T_∞ is the temperature of the surrounding seawater (3 °C). The term $1/\hbar_{ci}2\pi r_i$ is the thermal resistance to convection heat transfer per unit length from the sample fluid to the inside wall of the exchange tubing (forced convection, cm °K W^{-1}), $\ln(r_o/r_i)/2\pi K_{Ti}$ is the thermal resistance to conduction of heat per unit length through the titanium wall of the heat exchanger tubing (cm °K W^{-1}) and $1/\hbar_{co}2\pi r_o$ is the thermal resistance to heat transfer from the outer wall of the heat exchange tubing to the surrounding seawater (free convection, cm °K W^{-1}). If a value for Q_f is equated to Q_{ex} and substituted into Eq. (5), the required length of heat exchange can be found by solving for L (cm).

Because the properties of the sample fluid passing through the heat exchanger dramatically change as it is cooled from a super critical fluid at 400 °C to water at 200 °C (e.g., velocity decreases ~3.7×, density increases ~3.7×, thermal conductivity increases ~3.4×, specific isobaric heat capacity decreases ~5.7×, viscosity increases ~3.8×), the calculation in Eq. (5) was conducted over smaller increments of the tubing. Rather than conduct a calculation for L over the whole 400–200 °C temperature range ($L_{400-200\,°C}$), where there are large non-linear changes in the parameters of Eqs. (4) and (5), we chose to calculate and sum the length of heat exchange tubing required to cool the fluid over this range in 50 °C steps (i.e., $L_{400-200\,°C} = L_{400-350\,°C} + L_{350-300\,°C} \cdots + L_{250-200\,°C}$). The parameters in Eqs. (4) and (5), averaged over the narrower temperature intervals, are more easily determined and the summed result, $L_{400-200\,°C}$, more accurate. At a constant pressure of 275 bar (depth ~2710 m), the value for $L_{400-200\,°C}$ was found to be 29 cm (calculation results summarized in Table 1). Because of the errors inherent in determining $\hbar_{ci}$ and $\hbar_{co}$, we added ~20% to the calculated value of $L_{400-200\,°C}$ to provide a margin of safety. The length of the heat exchange tubing in the sampling nozzle was therefore increased to 36 cm.

3.5. Field testing

3.5.1. Assessment of potential for microbial contamination

The ability of the AMS sampling nozzle (Fig. 1A, inset) to protect collected samples from external contamination was tested using the apparatus illustrated in Fig. 2 and procedures described in Methods. The tracer organism, *S. marinorubra* (cell

Table 1
Average heat transfer coefficients[a] (h_{ci}, h_{co}, and K_{Ti}), average heat loss (Q_f) and computed length (L) of titanium heat exchange tubing required to reduce the temperature of water by the indicated 50 °C increments (ΔT_f)

ΔT_f (°C)	Q_f (Watt)	h_{ci} (W cm^{-2} °K^{-1})	h_{co} (W cm^{-2} °K^{-1})	K_{Ti} (W cm^{-1} °K^{-1})	$\bar{T}-T_\infty$ (°C)	L (cm)
400–350	799	0.690	0.350	0.2	372	11.0
350–300	296	0.620	0.319	0.2	322	5.2
300–250	252	0.590	0.281	0.2	272	5.7
250–200	233	0.552	0.242	0.2	222	7.2
400–200					Sum	**29.1**

[a]Pressure of system, 275 bar. See Eqs. (4) and (5) and the associated text for definition and units of indicated parameters. ΔT_f = the 50 °C temperature interval over which the average heat transfer properties were determined. T_∞ = temperature of the infinite sink, $\bar{T} = (T_{in} + T_{out})/2$ is the average fluid bulk temperature over the indicated 50 °C temperature interval. See Eq. (5).

dimensions 0.8–1.0 × 1.0–2.0 μm), was introduced into filtered seawater flowing in the apparatus at 4.3 l min^{-1} at a steady state concentration of $1.01(\pm 0.21\ \text{SD}) \times 10^6$ viable cells per ml (determined from samples collected from the "tracer organism-containing zone"). *S. marinorubra* was used as a surrogate for all bacteria when it comes to potential for contamination and has the desired properties that (1) it can be easily seen when grown into colonies (red pigment) and (2) it remains completely viable when introduced and sampled in the test apparatus. Viability of the culture was ~100% when the above viable counts were compared to direct cell counts, [$1.0\ (\pm 0.2\ \text{SD}) \times 10^6$ total cells per ml]. To simulate exposure of the sampling nozzle to contaminating waters prior to sample collection, it was slowly lowered through the upper "tracer organism-containing zone" of the test apparatus into the "tracer organism-free zone" as shown in Fig. 2 and outlined in Methods. Results of the sampling test are shown in Fig. 7 where six 75 ml samples were taken in the "tracer organism-free zone" (i.e., Clean Zone in Fig. 7) after the nozzle was purposely contaminated as described above. The filtered source water contained no organisms that grew into red pigmented colonies, but did contain a host of organisms developing colorless to yellowish colonies (~33 [±6 SD] culturable cells ml^{-1}) and occasional brown colonies (magnified insets). Also, no red colonies were found in control samples collected from the "tracer organism-free zone" just prior to AMS sampling, indicating that the test apparatus was successful in segregating and maintaining a "clean zone" separate from the "contaminated zone." Confluent red growth was observed in samples withdrawn from the "contaminated zone" as expected. An example of what a "contaminated" AMS sample would look like is

Fig. 7. Protection of samples from contamination after exposure of the AMS nozzle to a 1×10^6 viable cells ml^{-1} seawater suspension of *S. marinorubra*.

shown in the plate labeled "contam. example", which occurred when the contaminated AMS nozzle was accidentally plunged into the marble bed during one test, effectively contaminating the "clean zone" with *S. marinorubra*. In a separate experiment (data not shown), the AMS nozzle was lowered past the Tracer Injector (Fig. 2) while the peristaltic pump was left running, effectively exposing the nozzle to tendrils (Fig. 2, inset; due to the laminar flow regime) of essentially undiluted culture containing ~10^9 cells ml^{-1} of the tracer organism (vs. the above experiment where the pump was turned off to avoid

such high level, less controlled levels of contamination, see Methods). In this experiment, three AMS samples were obtained in the "clean zone" (one sample was skipped as a negative control, which contained no colonies, and two samples were obtained in the "contaminated zone" as a positive controls, which resulted in confluent red growth). Two of the three AMS samples were free of the tracer organism; one, however, contained a single *S. marinorubra* colony. Only at extreme levels of contamination, approaching 1000× the levels one would typically expect to experience in coastal seawater (e.g., DeLong et al., 1999), and ~2000–20,000× the contamination levels one may experience in the open ocean (Whitman et al., 1998; claim $\sim 5 \times 10^5$ prokaryotes ml^{-1} for the open ocean above 200 m and $\sim 5 \times 10^4$ ml^{-1} below 200 m), is the AMS sampling nozzle less than 100% successful at protecting collected samples from contamination.

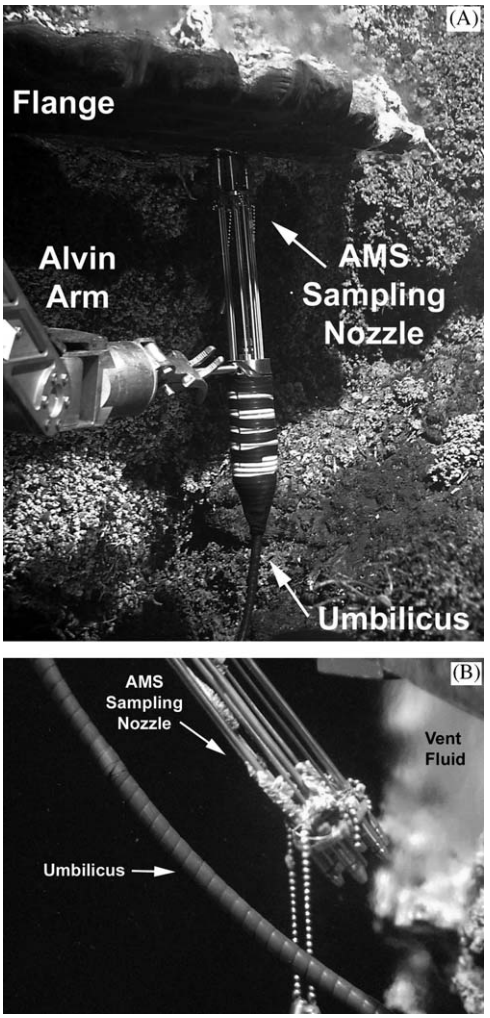

Fig. 8. AMS sampling at hydrothermal vents using DSV Alvin. Panel A, sampling at a flange vent (variable temperature between 140 and 200 °C) ambient and at the Lobo site on Endeavor Ridge, Juan de Fuca Ridge vent field (~47°53′N, ~129°9′W; R.V. *Atlantis cruise* AT03-54). Panel B, sampling of high-temperature vent fluid (~280 °C) emanating from the Chowder Hill vent site at Juan de Fuca Ridge, (48°27.6′N, ~128°42.6′W).

3.5.2. *Sampling of hydrothermal vents by the AMS*

Figs. 8A and B illustrate AMS sampling of various flange and chimney hydrothermal vents. The AMS sampling nozzle proved to be very robust during sampling. Occasional drifting of Alvin during sampling sometimes resulted in "plowing" of the sample nozzle into the rocky surfaces of vent chimneys and inadvertent immersion of the sampling tip into 400 °C water without damage. The Perfluro-elastomer O-rings used to seal the protective caps are in fact rated for continuous exposures to temperatures of 260 °C (intermittent exposure to ~350 °C) without damage and we have proved this to be the case. The O-rings did not carbonize or "weld" the protective caps to the sample inlet, preventing their removal. They remained pliant and were completely undamaged by exposure to

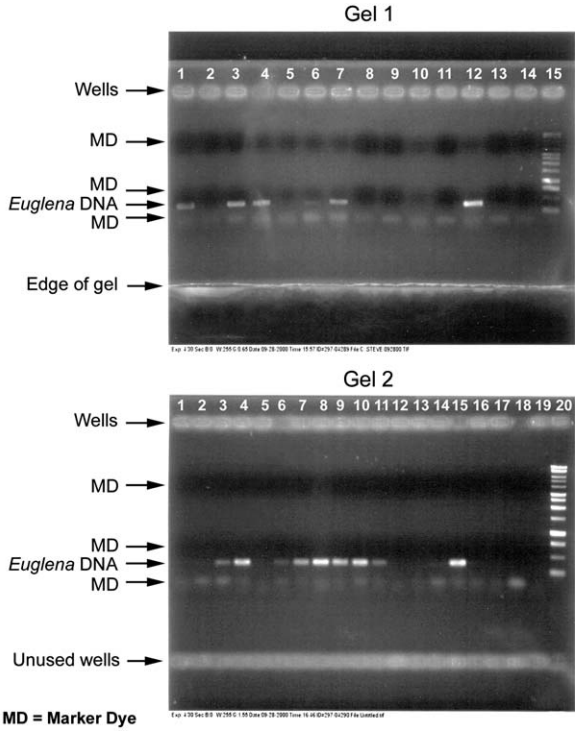

Fig. 9. Examples of agarose gels of PCR-amplified *Euglena gracilis* DNA after indicated treatments.

~400 °C water (the titanium metal in fact was slightly discolored by the high temperatures). The ~12″ titanium tube heat exchangers also worked well for cooling very hot samples to temperatures that did not damage the PEEK™ tubing of the umbilicus. During one dive, the sampling nozzle was totally immersed in ~270 °C water (figure not shown; not the intended mode of sampling). These temperatures were high enough to melt the boot material used to interface the umbilicus with the nozzle and the plastic material used to consolidate the PEEK™ tubes of the umbilicus. Nevertheless, the PEEK™ tubing in the umbilicus remained intact and a good sample was obtained. We believe that with proper sampling procedures, the AMS can be used for sampling high-temperature water and survive exposure to temperatures >350 °C.

3.6. Testing of procedures for sterilization and DNA removal

Various treatments were tested for effective destruction of DNA. Destruction of DNA was verified by conducting PCR DNA amplification

Table 2
Score of presence (+) or absence (−) of *Euglena gracilis* DNA in the gels of Fig. 8 after treatments

Well no.	Incubation temperature (°C)	Hours incubated	Treatment	Score
Gel 1				
1	50	0.25	NaOH	+
2	Negative control	0	NaOH + NaCl	−
3	50	0.25	NaOH + NaCl	+
4	25	0.25	H_2SO_4	+
5	50	0.25	H_2SO_4	−
6	200	0.25	Dry	+
7	25	18	NaOH	+
8	50	24	NaOH	−
9	25	18	NaOH + NaCl	−
10	50	24	NaOH + NaCl	−
11	Negative control	0	Reagent	−
12	Positive control	0	Untreated cells	+
13	—	—	NA	−
14	—	—	NA	−
15	—	—	DNA marker	+
Gel 2				
1	50	2	H_2SO_4	−
2	200	2	Dry	−
3	121	0.5	Autoclave	+
4	25	2	NaOH	+
5	50	5	NaOH	−
6	25	2	NaOH + NaCl	+
7	50	5	NaOH + NaCl	+
8	25	0.5	NaOH	+
9	50	1	NaOH	+
10	25	0.5	NaOH + NaCl	+
11	50	1	NaOH + NaCl	+
12	25	1	H_2SO_4	−
13	50	1	H_2SO_4	−
14	200	1	Dry	−
15	121	0.25	Autoclave	+
16	25	24	H_2SO_4	−
17	50	24	H_2SO_4	−
18	200	24	Dry	−
19	Negative control	0	NaOH	−
20	—	—	DNA marker	+

NA = lane not applicable to present experiment.
(+) indicates visible DNA bands on the gels, (−) indicates no visible bands.
(—) = treatment not conducted or relevant.

studies of treated samples of three test organisms, *E. gracilis,* a representative of eucaryotic organisms, *S. marinorubra*, a representative of procaryotic organisms (bacteria) and *Pyrococcus* GB-D, a hyperthermophilic sulfur respiring vent archaeal heterotroph. Our intent was to establish a minimum procedure that assures complete destruction of the DNA of these test organisms.

3.6.1. Acid/base treatment procedures

Aliquots of rinsed suspensions of the test organism(s) were distributed among a number of identical microfuge tubes and subjected to autoclaving for up to an hour, alkaline treatment (0.5 N NaOH; 0.5 N NaOH + 1.5 N NaCl) or acid treatment (0.5 N H_2SO_4) at two temperatures (~25 °C and ~50 °C or 200 °C dry heat for select periods ranging from 0 to 24 h). At the end of a specified treatment, the acid- or base-treated samples were neutralized, subjected to a standard SDS lysis/protease digestion/phenol extraction procedure, followed by alcohol precipitation of DNA for preservation until analysis. Small aliquots of the DNA were subjected to PCR using a universal primer pair for amplification of 23s RNA gene sequences unique to the Eukaryote *Euglena* and 16 s RNA gene sequences unique to Bacteria. Analysis of the PCR product by gel electrophoresis was undertaken and ideally resulted in a single band with an intensity (after staining) that is proportional to the initial concentration of intact sequence between the primer pairs. As the DNA-destroying treatment proceeds in time, the probability of a PCR-disrupting lesion appearing in the sequence between the primer pairs increases, resulting in a decrease in PCR product. With increasing treatment time, the intensity of the PCR product band should decrease exponentially. An appropriate treatment will result in no detectable PCR product.

Results of these studies are shown in Fig. 9 and Table 2. Fig. 9 shows photographs of the agarose gels used for scoring the presence or absence of a discernable DNA band. If there was the slightest indication of a DNA band, as illustrated by lane 6 of both gels 1 and 2, the result was scored positive. Lanes 2 and 5 (among others) in gel 1; lanes 1 and 2 (among others) in gel 2 are examples of samples that contained no amplifiable DNA and were scored negative (see also Table 2). Positive and negative controls behaved as expected, indicating that the DNA extraction/PCR amplification procedures were working normally and that reagents did not contain exogenous DNA.

Euglena cell suspensions were exposed to the following treatments: (1) 0.5 N NaOH, (2) 0.5 N NaOH + 1.5 N NaCl and (3) 0.5 N H_2SO_4 at 25 and 50 °C for incubation periods ranging from 15 min to 24 h. It is clear from the results in Table 3 that exposure to acid was far superior to any of the alkaline treatments. Exposure to 0.5 N H_2SO_4 for 1 h at 25 °C was sufficient to destroy all traces of DNA and <15 min was required at 50 °C. DNA could be destroyed by exposure to NaOH as well, but much longer incubations were required. At 25 °C, a full 24 h were required to destroy DNA in 0.5 N NaOH. The addition of NaCl reduced the time required by half (12 h) and incubation at 50 °C resulted in a modest improvement with DNA destruction occurring in 5–8 h. With respect to recycling the AMS for the next deployment, it is abundantly clear that acid treatment is the only procedure that works quickly enough at room temperature to be practical. A 1–2 h treatment with 0.5 N H_2SO_4 falls well within a practical time window for recycling the AMS. Based on the effective time intervals observed when *Euglena* was treated with acid, another similarly prepared aliquot of *Serratia* was treated with 0.5 N H_2SO_4 for periods of 1, 2 and 3 h. The DNA extraction and PCR procedures were repeated. The treatment also proved effective in destroying DNA from *Serratia* for all three time periods tested.

It is of interest to note that exposure of samples to dry heat (200 °C) for 1 h and autoclaving for 1 h also destroys DNA. For materials that tolerate these conditions and are of a size to fit into an autoclave or oven, this will be useful in preparation of solutions and hardware for use with the AMS.

3.6.2. Hydrogen peroxide and DNA AWAY^{TM} studies

We have explored the possibility of using chemical treatments with strong solutions of hydrogen peroxide for both sterilizing the internal conduits of the AMS and for oxidative destruction of exogenous DNA via iron-catalyzed radical reactions resulting in the cleavage of the sugar backbone, derivatization of the purine and pyrimidine bases and other oxidative reactions (e.g., Henle and Linn, 1997). Studies in the literature have shown that 30% hydrogen peroxide was effective for sterilizing glass surfaces and biological indicator disks inoculated with viable cells and spores of

Table 3
Destruction of *Euglena gracilis, S. marinorubra* and *Pyrococcus* GB-D DNA following intervals of chemical and high-temperature treatments

Euglena gracilis

Treatment (h)	Pos. ctrl	Neg. ctrl	Treatment 25 °C			50 °C			200 °C	121 °C
			0.5 N NaOH	1.5 N NaCl + 0.5 N NaOH	0.5 N H_2SO_4	0.5 N NaOH	1.5 N NaCl + 0.5 N NaOH	0.5 N H_2SO_4	Dry heat	15 psi Autoclave
0[a]	+	—	—	—	—	—	—	—	—	—
0 (cell free)[b]	—	–	–	–	—	—	—	—	—	—
0.25	—	—	+	+	+	+	+	–	+	+
0.5	—	—	+	+	+	+	+	–	+	+
1	—	—	+	+	–	+	+	–	–	–
2	—	—	+	+	–	+	+	–	–	—
5	—	—	+	+	–	–	+	–	–	—
8	—	—	+	+	–	–	–	–	–	—
12	—	—	+	–	–	–	–	–	–	—
18	—	—	+	–	–	–	–	–	–	—
24	—	—	–	–	–	–	–	–	–	—

Serratia marinorubra

Treatment (h)	Pos. ctrl[a]	25 °C				
		0.5 N H_2SO_4	30% H_2O_2	20% H_2O_2	10% H_2O_2	DNA AWAY™
0[a]	+	—	—	—	—	—
1.3	—	–	—	—	—	—

2.3	—	–	—	—	—	—
3.3	—	–	—	—	—	—
0 (evap)[c]	+ (+)[d]	—	—	—	—	—
1.3	—	—	+	+	+	–
2.3	—	—	+	+	+	–
3.3	—	—	–	–	+	–

Pyrococcus GB-D

Treatment (h)	Pos. ctrl[a]	25 °C				
		0.5 N H_2SO_4	30% H_2O_2	20% H_2O_2	10% H_2O_2	DNA AWAY™
0 (evap)[c]	+ (+)[d]	—	—	—	—	—
1.3	—	—	—	—	—	–
2.3	—	—	—	—	—	–
3.3	—	—	—	—	—	–

Aliquots (50 μl) containing 3×10^4 cells of *Euglena gracilis* were treated as indicated above. Aliquots (50 μl) containing 2×10^8 cells of *S. marinorubra* (H_2SO_4 treatments); 3×10^7 cells of *S. marinorubra* or *Pyrococcus sp.* (H_2O_2 & DNA AWAY™ treatments) were treated as indicated above. The samples were extracted and taken through PCR amplification as described in Methods. PCR products were examined for the presence of DNA by agarose gel electrophoresis, followed by ethidium bromide staining. Visible DNA bands on the gels are indicated by a (+) and no bands by a (−). If not applicable or an experiment was not conducted, (—) was entered.

[a]No treatment, positive control.

[b]Reagent negative control.

[c]Cell aliquot evaporated as in the H_2O_2 and DNA AWAY™ treatments prior to DNA extraction.

[d]Additional positive controls (+) in which cell aliquots were added to microfuge tubes in which H_2O_2 and DNA AWAY™ solutions that were evaporated indicated that remaining residues do not destroy DNA.

Bacillus subtilis and *B. stearothermophilus* after <30 min exposure at room temperature (Wilkins et al., 1994). If hydrogen peroxide is effective, the AMS could be sterilized and freed of exogenous DNA without disassembly of the unit. This would greatly simplify the logistics of recycling the AMS between deployments. Effectiveness of hydrogen peroxide and the commercial product DNA AWAY™ for destroying *Serratia* DNA is also shown in Table 3. Hydrogen peroxide at 20% or 30% will destroy DNA after a 3 h exposure, but not at the shorter exposure times of 1 or 2 h. DNA AWAY™ was effective at destroying DNA in the shortest incubation period tested, 1 h. Tests of the effectiveness of DNA AWAY™ were made on the additional test organism, *Pyrococcus* GB-D. As shown in Table 3, it was identically effective at destroying the DNA of this organism as it was for *Serratia*.

We have conducted a viability study with our bacterial test organism in which cell suspensions and pelleted cells were exposed to final concentrations of ~5%, 10%, 15%, 20% and 30% hydrogen peroxide for 1–24 h (Table 4). When $\sim 1.6 \times 10^6$ cells were then plated on a nutrient medium, no growth was found in any of the cases, suggesting that hydrogen peroxide can be quite effective at killing vegetative bacterial cells. Residues left over after air drying hydrogen-peroxide-treated cultures did not appear to affect the viability of freshly added cells.

The commercial product, DNA AWAY™, made by Molecular BioProducts is used for treatment of

Table 4
Viability of *S. marinorubra* following exposure to hydrogen peroxide (H_2O_2) and DNA AWAY™

Treatment type	Exposure time (h)	Control resuspended in 3.5% NaCl	Treatment agent			
			H_2O_2 (5%)	H_2O_2 (10%)	H_2O_2 (15%)	DNA AWAY™
I. Cells in medium	1	—	–	–	–	—
	2	—	–	–	–	—
	3	—	–	–	–	—
	4	—	–	–	–	—
	5	—	–	–	–	—
	6	—	–	–	–	—
	7	—	–	–	–	—
	19	—	—	—	–	—
			H_2O_2 (10%)	H_2O_2 (20%)	H_2O_2 (30%)	
II. Pelleted cells	0.5	+	—	—	–	–
	1	+	–	–	–	–
	2	+	–	–	–	–
	3	+	–	–	–	–
	4	+	–	–	–	–
	5	+	–	–	–	–
	6	+	–	–	–	–
	7	+	–	–	–	–
	19	+	—	—	–	–
	22	+	—	—	–	–
	24	+	–	–	–	–
III. Cells added to residue	1	+	—	—	+	+
	18	+	—	—	+	+
	41	+	—	—	+	+

Aliquots (50 µl) containing 4×10^7 cells of *S. marinorubra* were introduced into sterile microfuge tubes and treated as indicated above (see also Methods). After treatment, 2 µl aliquots (1.6×10^6 cells) were spread onto the surface of Marine Agar 2216 plates for growth. Appearance of one or more colonies after 7 days incubation at room temperature scored as (+), no colonies scored as (−). If not applicable or an experiment was not conducted, (—) was entered.

Treatment I: Cells in Marine Broth 2216 cultures exposed to H_2O_2 at the indicated final concentration.

Treatment II: Cells from above broth cultures centrifuged to remove medium and exposed to H_2O_2 at the indicated final concentrations. Control cultures were resuspended in 3.5% NaCl.

Treatment III: Cells in Marine Broth 2216 cultures added to sterile microfuge tubes in which H_2O_2 and DNA AWAY™ were evaporated to determine if toxic residues were left behind after evaporation.

glass, plastic ware and stainless steel to destroy DNA and may also have application with the AMS. Experiments shown in Table 4 suggest DNA AWAY™ does kill our test bacterium, *S. marinorubra*, with the same effectiveness as hydrogen peroxide, though this result is not likely to hold for all organisms. If DNA AWAY™ is used for treating AMS conduits, care should be taken to assure that it is completely removed to avoid killing sample organisms or destroying desired sample DNA. Residues left from treatment after air drying, however, do not affect viability of *Serratia* or destroy DNA of either test organism.

The experiments conducted indicate that chemical means can be implemented for the combined requirement of sterilization and destruction of DNA. Exposure of the AMS conduits to high strength (20%) hydrogen peroxide for at least 3 h will be effective for this dual purpose. It is of interest to note that titanium is chemically attacked by 30% hydrogen peroxide, as evidenced by the appearance of a yellowish color in the hydrogen peroxide solution and probably should be avoided. This was not observed when titanium was exposed to 20% hydrogen peroxide. If desired, additional exposure of the conduits to the commercial agent DNA AWAY™ will provide an increased margin of safety with respect to the removal of exogenous DNA.

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