Testing of a Downhole Sampler Incubator (DSI) for the Uncontaminated and Exogenous DNA-Free Sampling of Crustal Fluids from Deep-Sea Bore Holes

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Low temperature hydrothermal ocean fluids (<100°C) that circulate within porous volcanic regions of the upper ocean crust result in physico-chemical conditions that form plausible habitats for a variety of microbial communities. However, this potentially important subseafloor crustal biosphere is largely unexplored because of its inaccessibility. Major questions regarding the community structure, key metabolic pathways and rates, and organic geochemistry of the crustal fluids remain uninvestigated.

The Ocean Drilling Program (ODP) has drilled a series of Borehole Observatories through the overlying sediments and into the basement rock at varying distances away from the axis of the Juan de Fuca Ridge (JFR), providing access to hydrothermal environments of differing age and vent chemistry. However, confident assessment of molecular genetic and biomarker diversity in the basement crusts is limited by uncertainties in the sampling methods used. Specifically, the fluids were collected from the top, rather than the bottom, of the steel-cased ~400 m boreholes. The extent to which the fluids were altered by their ascent up the steel-cased borehole is unknown, as is the possible contribution to the cellular biomass and molecular diversity from any biofilm or other contaminant associated with the surface of the borehole liner. To address this problem, a miniaturized sampling and tracer incubation apparatus, the Downhole Sampler / Incubator (DSI, Figure 1), is in development under NSF SGER funding for sampling and conducting experiments at the bottom of ODP boreholes where contaminating influences of the borehole liner will be minimized.

The object of this project is to test the ability of the DSI to obtain microbial samples that are free from contamination by microbes and DNA that may exist in the upper reaches of the borehole. Sampling tests are to be conducted in an apparatus that simulates the 4" ID ODP borehole using clear PVC pipe. A prototype of the borehole simulating apparatus was constructed to test proper functioning of the concept. The prototype device (Figure 2) was constructed from two 2-foot sections of 4" ID clear PVC piping joined at the center with a standard PVC union. The union was equipped with 8 inlet ports made from 18 ga. stainless steel tubing and short lengths of flexible silicone tubing to form a tracer injector (see Top View, Figure 2). A steady flow (~5 L min^{-1}, similar to the flows experienced in ODP boreholes) of filtered (≤1 μm) seawater was introduced into the base of the test device via a diffuser made from glass marbles. The diffuser resulted in an approximately laminar flow of seawater up the pipe, eventually flowing past the tracer injector where a dye and a non-virulent, red pigmented tracer organism, *Serratia marinorubra*, were introduced via a peristaltic pump. At steady state the upwardly flowing seawater resulted in a "tracer organism-free" lower zone and an upper zone that was "contaminated" by the tracer organism. The dye provided a visual indication of the behavior of the tracer introduced into the flowing seawater. As shown in the inset, the dye & tracer are transported upward as somewhat coherent tendrils until about half way up the ~2 ft length of tubing where they are gently mixed with the flowing seawater. The lower section of the pre-sterilized (5% bleach, which effectively kills the tracer organism) test apparatus possesses Sampling Ports to confirm during experiments that the lower "tracer-free" zone is indeed free of *Serratia marinorubra*. During experiments, ~50 ml samples are passed through sterile filters, which then are aseptically placed onto the surface of a nutrient medium that supports the growth of *Serratia marinorubra* and other heterotrophs. *Serratia* colonies are an intense red, compared

with the completely colorless colonies that grow from samples taken from the ≤1 μm filtered seawater.

For testing of the DSI, the apparatus shown in Figure 2 would be extended to ~20 ft in length, with the tracer injector in the middle and placed in the center well of the WHOI dock. The DSI would be lowered down through ~10 ft of water "contaminated" with the tracer organism into the lower ~10 ft "tracer organism-free" zone where samples will be taken. This experiment will directly simulate the contamination the DSI will experience in a deployment where samples collected at depth need to be free of contamination from the upper reaches of the borehole. Proper functioning of the DSI will be demonstrated when *Serratia marinorubra* will be found only in samples taken in the "contaminated zone." If the measures for protecting the DSI sample inlets are effective, samples below the tracer injector should be completely free of *Serratia*, even though the external surfaces have been contaminated with this organism by passage through the upper microbe containing waters.

Because the DSI won't be completed until this summer we used the test apparatus in Figure 2 to test the sampling nozzle of the Autonomous Microbial Sampler (AMS) for the ability to obtain contamination-free samples. The AMS (Figure 3) is the prototype microbial sampler, upon which the miniaturized DSI is based. This device is designed for use by DSV Alvin or ROVs where a sampling nozzle attached to an umbilicus is manipulated to the sampling site via manipulator arm. Figure 2 shows the AMS sampling nozzle positioned in the "tracer organism-free" zone during typical sampling. Results of an early experiment are shown in Figure 4 and are very encouraging. In this experiment a *Serratia marinorubra* culture at a population density of ~10^9 cells ml^{-1} was pumped into the flowing stream of seawater in the test apparatus, which mixed down to ~10^5 cells ml^{-1} near the top. This level of contamination is about the levels one sees in typical coastal seawater and ~10x the levels seen in a typical ODP borehole. Because, as mentioned above, the tracer tends to remain in concentrated "tendrils" near the tracer injector, the nozzle was actually exposed to contamination levels ~10,000-fold higher than it would experience in a typical coastal environment. Even then, only a single *Serratia* colony, in three 50-ml samples taken, was able to penetrate the nozzle's protective barrier to contamination. Subsequent experiments (data not shown) where the nozzle was exposed to only ~4 x 10^5 cells ml^{-1} contamination levels, more in line with environmental levels of contamination, revealed that no *Serratia* tracer organisms got by the protective cap system used to prevent sample contamination.

Results thus far have demonstrated that the test apparatus to be employed in future testing of the DSI works as intended. It provides for controlled exposures to contaminating tracer organisms, while successfully maintaining a completely "tracer organism-free" zone that will effectively test the ability of the DSI to obtain contamination-free samples.

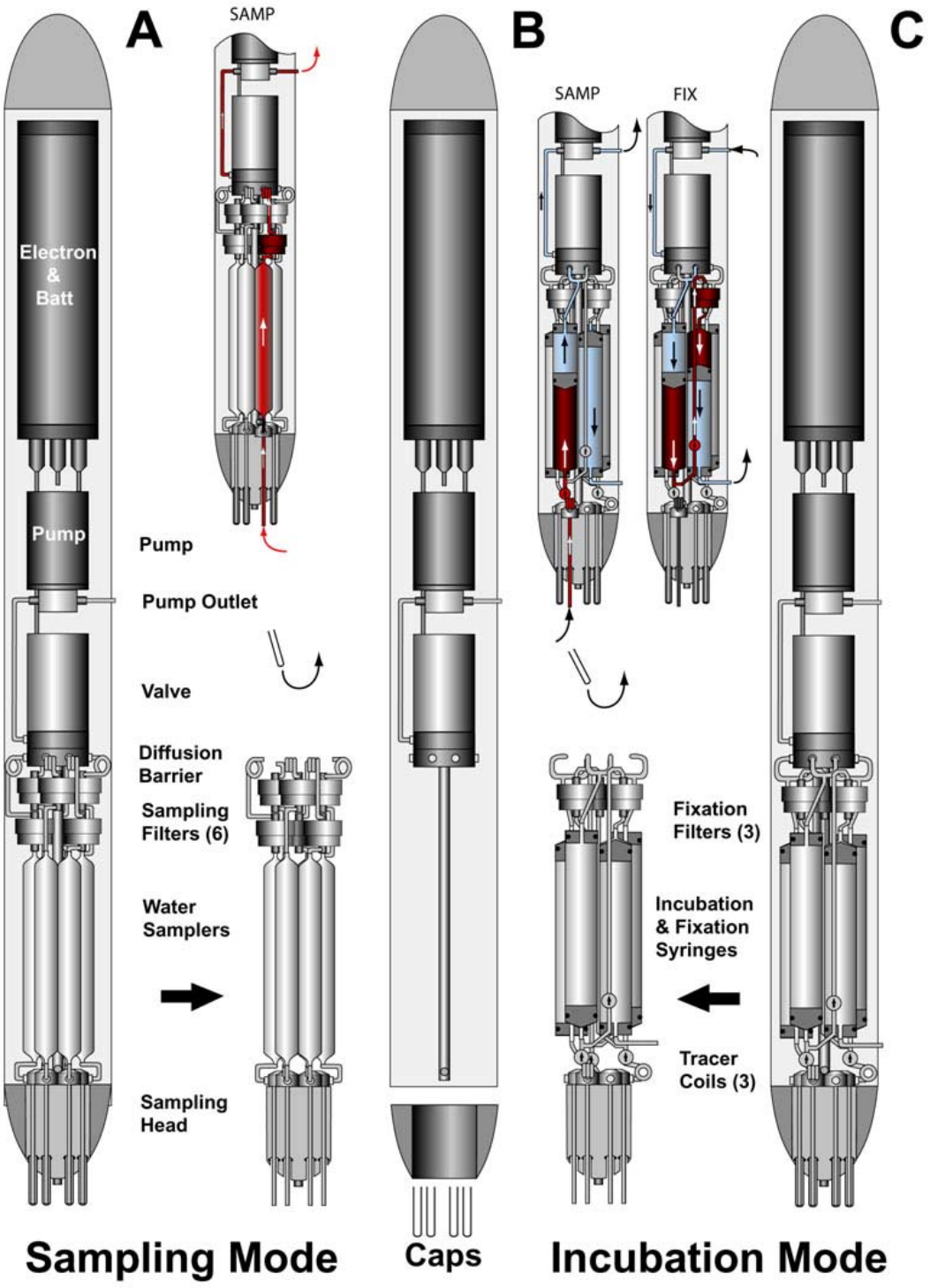

Figure 1. ***Conceptual schematic of the downhole Sampler Incubator (DSI).*** The DSI is to penetrate into 4" ID boreholes to sample 400 m into the seafloor subsurface in off-axis hydrothermal vent remote environments. The device (3" diameter x ~7 ft length) is in development. The device will collect uncontaminated microbial samples and conduct in situ tracer incubation experiments. **Panel A**, microbial sampling configuration. Sample flow is indicated in red. **Panel B**, device when sampling hardware removed for sterilization and DNA removal. **Panel C**, tracer incubation configuration. Incubated sample indicated in red.

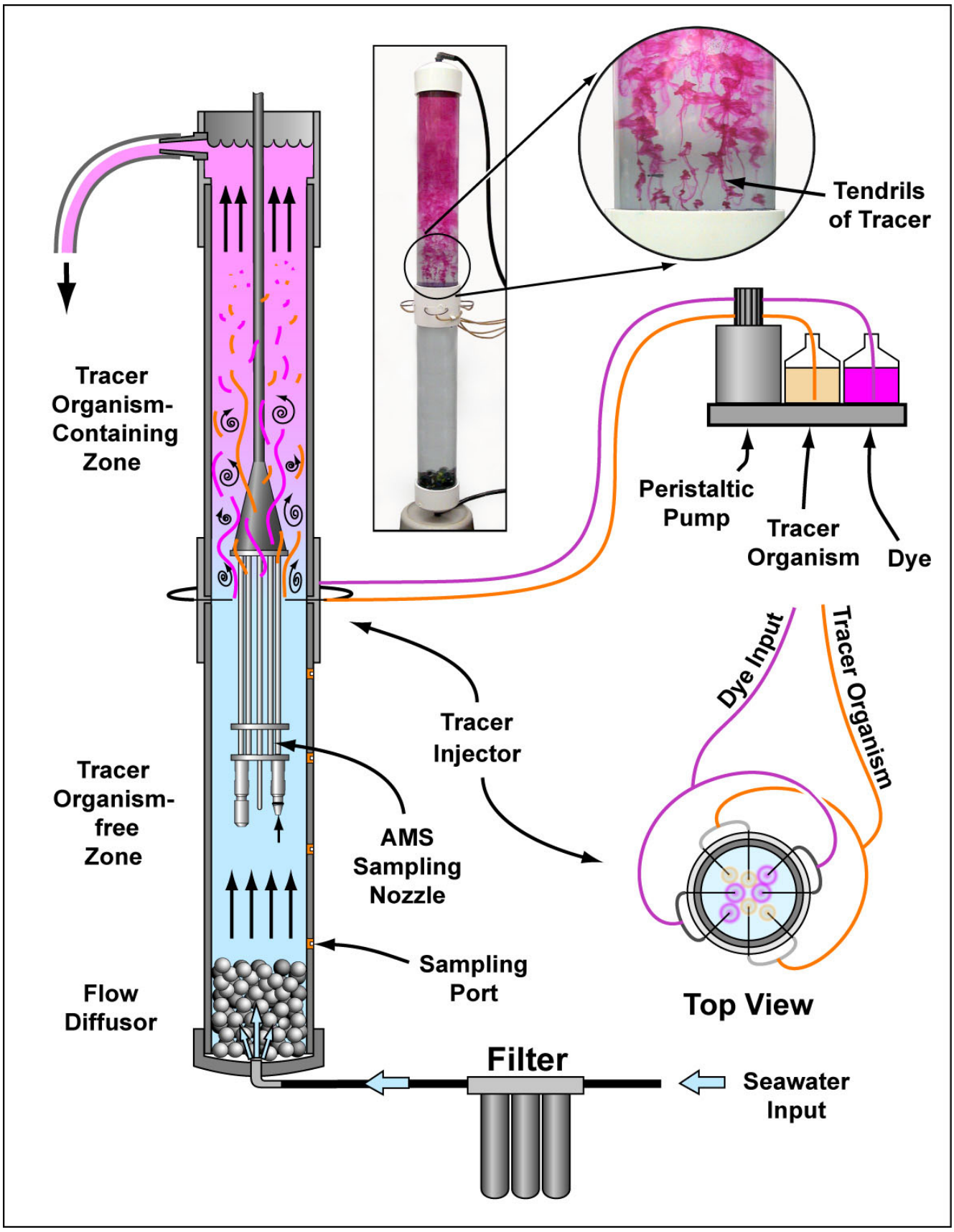

Figure 2. ***Schematic of apparatus used to test the ability of the AMS to obtain contamination-free samples in the face of heavy contamination of the sampling nozzle.***

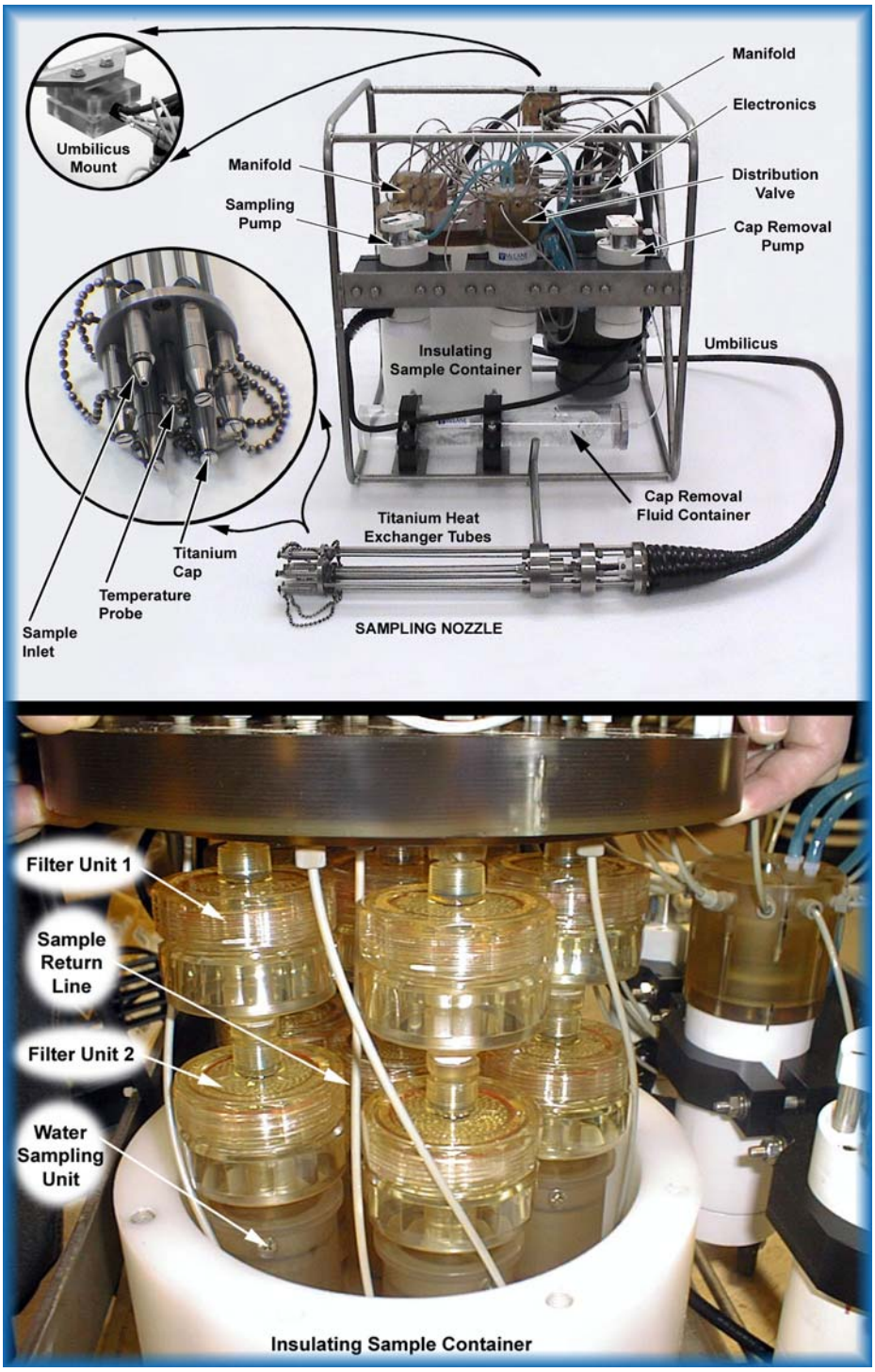

Figure 3. ***Autonomous Microbial Sampler***. The major components of the AMS include the following: 1) A sterilizable Sampling Unit accommodating 6 Sampling Modules that each consist of an assembled series of interchangeable filter units and/or containers for collection of particulate or water samples according to user needs (lower panel). 2) Fluid controlling components comprised of a Distribution Valve and two positive displacement microgear pumps . 3) A sterilizable Sampling Nozzle and associated umbilicus that will permit uncontaminated sampling of the environment by manipulator arm or by direct mounting on the vehicle. A temperature probe has been incorporated into the nozzle to permit continuous measurement of temperature at the site of sampling. 4) A Tattletale 8-based electronic controller/data recorder for controlling sampling events and interfacing with the user. The inlets of the Sampling Nozzle are protected by removable caps that are hydraulically removed just prior to sampling.

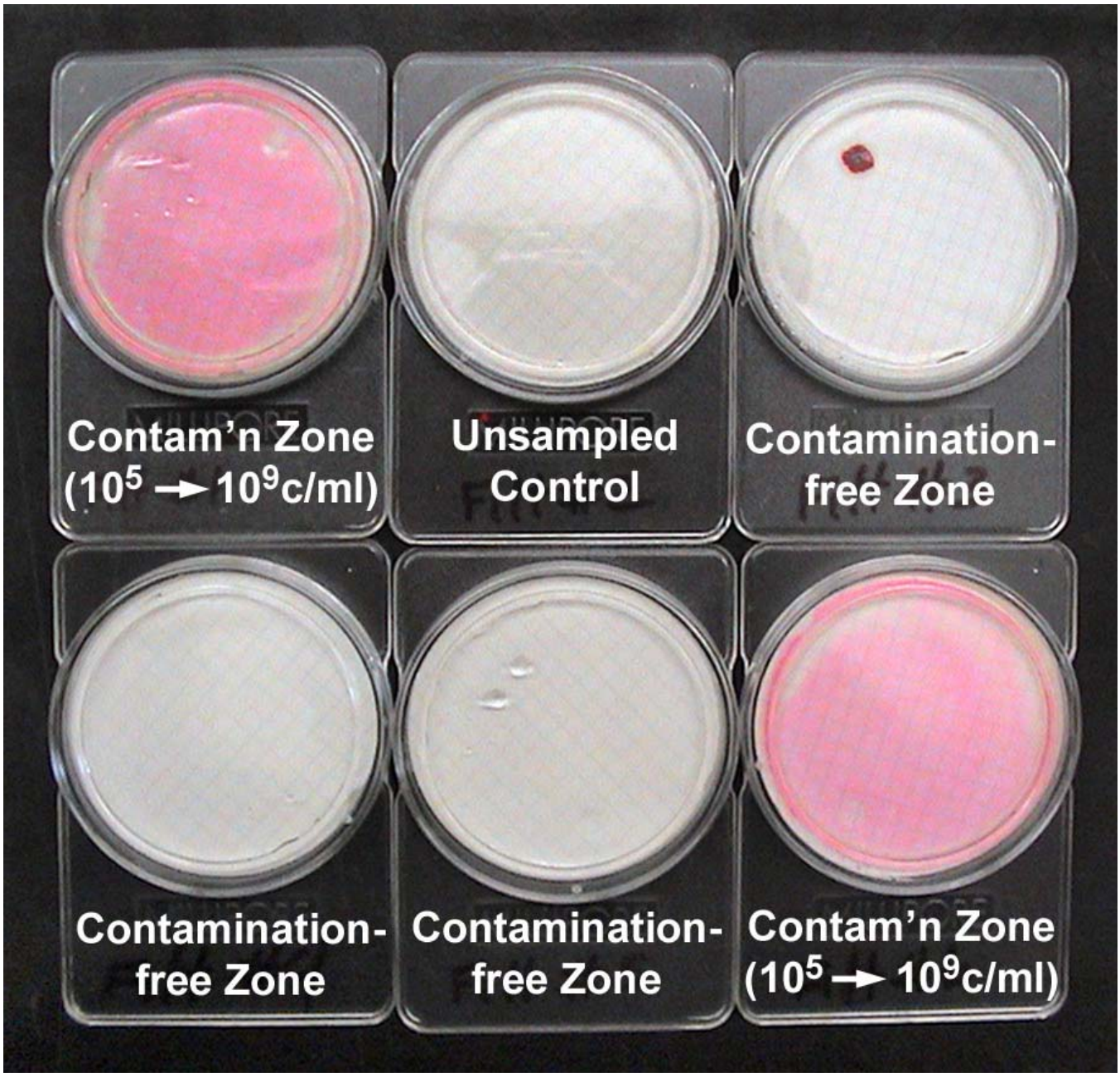

Figure 4. ***Results of AMS sampling experiment.*** Two samples (50 ml) were taken in the contaminated zone (the pink coloration is the result of the numerous bacteria in these samples growing into a confluent layer rather than individual colonies), 3 in the uncontaminated zone, one negative control. Appearance of one red colony means that only a single tracer organism got into the sample, against a contamination background of ~10^9 cells per ml. This is a good result, given that the level of contamination was ~10,000 x above that which would be experienced in typical oceanic sampling. Additional experiments closer to true environmental contamination levels were undertaken and no contaminating colonies were observed.