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NUTRIENT AVAILABILITY AS A CONTROL OF THE ABUNDANCE AND ACTIVITIES OF DEEP SUBSURFACE MICROORGANISMS

FINAL REPORT

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SUMMARY

The primary goal of our research for the expected three year grant period was to determine whether the availability of major nutrients (C, N, P) control the abundance, distribution, and activity of heterotrophic microorganisms in subsurface sediments. The specific goals of our research in the first year of the three year grant period were:

1. to develop and test a microcosm system using subsurface sediment samples from a DOE site;
2. to conduct a preliminary nutrient enrichment experiment using the microcosm system;
3. to gather and analyze data and publish the microcosm method;
4. to adapt microscopic and metabolic assays for detecting the in situ nutrient deficiencies of microorganisms in subsurface sediments.

Each of these first three specific goals was accomplished and progress was made toward the fourth goal. We designed, constructed, and tested a flow-through microcosm system that simulates conditions in natural aquifers. The system can be regulated with respect to water flow rate, temperature, oxygen concentration, and nutrient concentration. Furthermore, the system can be run aseptically, excluding exogenous bacteria from test cores.

In our preliminary nutrient enrichment experiment, we collected deep vadose sediment from the Savannah River Site and pumped sterilized well water from sampling area through microcosms that were filled with this sediment. Experimental treatments included the flow-through of this water amended with different combinations of nutrients (+N+P, +C, +N+P+C) or no additional nutrients. The experiment included sterilized control microcosms. We found that asepsis was maintained for the entire 30 day experiment. We also found that that microbial abundance, biomass, and activity increased in several microcosms in response to the flow through of the well water, which was rich in nitrate. This response, however, was variable among both microcosms containing homogenized sediments and microcosms containing intact sediment core sections. No significant treatment responses were observed, but each microcosm that had an increase in microbial growth also immobilized N from the well water. We infer that water and N availability, and water table fluctuations strongly influence microbial growth and activity in the deep vadose.

We presented these methodological developments, and the results of the nutrient enrichment experiment at the First International Conference on Ground Water Ecology, which was held in Tampa in April 1992. A manuscript was submitted and, after peer review, was accepted for publication in the Conference Proceedings. A copy of the paper (Rudnick et al. 1992) is included in this report.

We also started to examine several possible indicators of in situ nutrient deficiencies for subsurface microorganisms. Progress included the selective staining of poly-ß-hydroxyalkanoate (PHA) bodies by Nile Blue A. These storage bodies may be an indicator of organic carbon

sufficiency. We also assayed phosphatase activity in subsurface sediments; this activity may be an indicator of P deficiency.

SPECIFIC ACCOMPLISHMENTS

1. Development of flow-through experimental system for microcosms.

The flow-through system was designed and constructed (Fig. 1). All components of the system are autoclavable, including glass and silicone tubing, polypropylene connectors, and polycarbonate microcosm tubes with silicone stoppers. Because the exclusion of exogenous microorganisms is essential, air (or in the future, other gases) vents contain 0.2 μm pore size filters, which are small enough to exclude all airborne contaminants. A 0.2 μm filter and a drip trap have also been placed in-line immediately below the water reservoirs to assure that microorganisms do not move upstream from the sediments to the reservoirs. In the event of a contaminated reservoir, the in-line filter also prohibits bacteria from flowing to the cores. However, as we will describe below, we expect that some bacteria can pass through 0.2 μm filters (see below).

A set of threaded clamps assures that the silicone stoppers of each microcosm completely seal the microcosm tube ends (this is not currently shown in Fig. 1). We also carefully considered how to retain the sediment particles within the microcosm tubes. This has been achieved by pinning to each stopper a pair of glass fiber filters (Whatman GF/C and GF/D) and a stainless steel retainer (0.3 mm mesh disc) with fine stainless steel pins. These filter packets are on both the influent and effluent ends of the microcosm cores.

Sample ports were installed immediately above and below the cores in order to measure concentrations and changes in dissolved molecular oxygen. Samples can be taken aseptically by inserting a small gauge sterile needle through the port septum (after wiping the septum with disinfectant). The needle is attached to a thin autoclaved glass tube and water feeds by gravity from the flow system through this tube. After several volumes, the O_2 concentration in this water sample (<1 ml) is analyzed with a Diamond General microelectrode.

2. Preparation of sterile groundwater for system reservoirs.

We tested methods of sterilization to minimize alteration of the chemistry of the water that we will feed to the microcosms. The feed water is groundwater collected at the same time as the sediment cores, from the same formation and depth; the use of this water will enable our experimental system to optimally mimic the *in situ* environment.

To avoid introduction of exogenous bacteria into the test cores, the water that we feed to the cores must be sterile. We wish to avoid autoclaving the water because the extreme heat and pressure may alter the composition and concentrations of dissolved organic matter and trace elements in the water. Membrane filtration is the method of choice; however, we have found that the commonly used filter "sterilization" procedure using 0.2 μm filters is unsatisfactory. In experiments using nutrient-enriched lake water, we found that these filters could effectively sterilize small volume water samples (<200 ml) but not those consisting of 2 or more liters of water; bacteria consistently grew in the filtrate of the latter. It seems likely that extremely small bacteria pass through 0.2 μm filters. Such "miniaturized" bacteria are known to be more common in oligotrophic systems than eutrophic systems (Morita, 1985) and thus are likely to be found in groundwater.

We improved our protocol for filter sterilization by using 0.1 μm polycarbonate filters (Poretics Corp.), and water prefiltered through glass fiber filters and 0.2 μm filters. In order to avoid airborne contaminant microorganisms, the filtration is done in a closed system. All lines, glass frits, filters, and reservoirs are autoclaved before filtration. In line filters are supported on glass frits instead of threaded filter holders which were found not to be reliable in several experiments. 0.1 μm filters supported by glass frits provided reliable, bacteria free water in three experiments.

From our tests of filtration methods, we are very cautious about reliance on filtration as a reliable sterilization method. While we succeeded in sterilizing water with 0.1 μm filters, it is likely that any given filter may have flaws that allow some bacteria to pass. Apparently, such flaws can be disturbingly common (Stockner *et al.*, 1990). Therefore, we have decided to further treat our 0.1 μm filtered groundwater by a pasteurization process in which the water will be heated to 80°C for 15 minutes and then cooled. This temperature cycle can be repeated two or three times on successive days to insure sterility of the feed water.

3. Preliminary tests of the maintenance of asepsis in a sterilized system.

To test the robustness of the system with regard to asepsis, we set up a sterile system that included microcosm tubes filled with lake sediment. In this test, the feed water was enriched lake water which had been filtered and then autoclaved in the reservoir. Sediments from a lake bottom, which were rich in organic matter also were autoclaved, first in a beaker (for 1 h on 3 consecutive days) and finally in the microcosms, (20 min). Water was pumped through the sediments for 4 days. Subsequently, water from the reservoir and collection bottles was filtered, and the filters were incubated in PTYG medium for one week. No bacteria grew in the medium. Sediment samples were dispersed in pyrophosphate and PTYG medium was added to encourage bacterial growth. Bacteria were found by microscopic observations of the pyrophosphate suspension, but no colonies developed from the suspension on PTYG plates. Some bacteria in the sediment remained intact during autoclaving, yet none were viable.

4. Preliminary tests for back-contamination of water reservoirs from microcosm sediments.

A system was assembled with lake bottom sediments, which have high densities of bacteria. Reservoirs contained autoclaved water with nutrients added. After pumping water through the sediments for 4 days, water in the reservoirs, collection bottles, and sediments were tested for the presence of bacteria. The reservoir remained sterile, while bacteria were very abundant in the sediments. Bacteria also fouled the outflow lines, and were detected in the collection vessels. However, no fouling was observed in the lines upstream of the microcosms and the reservoir water remained sterile. The drip trap and the in-line filter protected the reservoir from back contamination.

5. Construction of a device to extrude sandy sediments aseptically into microcosm tubes.

An apparatus was designed and built specifically to load the central portion of Shelby tube cores into our microcosms with minimal disturbance and minimal microbial contamination. In the current extruder design, the Shelby core is held vertically in place by braces attached to a rigid frame. The height of the braces and the core is adjustable. Below the Shelby core, an hydraulic jack pushes the sediment up through the fixed tube. The top of the jack piston is fitted with a metal disc, which pushes against a rubber stopper that fits tightly against the tube's inner wall, beneath the sediment. We have found that extruding sandy sediment in a vertical position and in a smooth and relatively undisturbed manner is necessary for properly loading the microcosms. This requires that the jack and core must be well aligned vertically and the jack must be operated slowly. The design of the frame assures that the core is vertical and the piston is kept in the same vertical line by a set of annular rings that fit snugly against the inner wall of the core and around the jack piston. In the absence of these design features, we found that saturated sands frequently could not be extruded. Presumably off-line rapid extrusion caused a shift in grain geometry (K. Sargent and T. Phelps, personal communication) that caused the core to swell. Not even a four-ton jack could move the core without damaging the extruder. Furthermore, we found that with the strict maintenance of vertical extrusion, water from the sand is not squeezed out the bottom of the core.

At the top of the frame, the microcosm tube is held in an adjustable block. Contaminant bacteria are kept out of tube by a plastic cap and a bottom covering of aluminum foil. Each tube is autoclaved with the cap and foil in place. The cap is translucent so that we can see the sediment entering the microcosm tube and know how far to extrude the sediment into the tube. The bottom of the microcosm tube is bevelled to a sharp edge to cause minimal disturbance of the sediment. After the microcosm top is inserted into the retaining block, the sediment is slowly extruded to the bottom of the microcosm. A thin layer of the sediment that is exposed to the air is pared off with a flamed spatula, the bottom foil cap is removed and the sediment is smoothly extruded into the microcosm. All

peripheral sediments, which are contaminated with bacteria from shallower depths as the core tube penetrates the ground, are excluded from the microcosms.

6. Selection of cytological indicators of bacterial nutrient status.

We have found previously (N. Magill 1989. Ph.D. Thesis, Cornell University; Sinclair and Ghiorse, 1989; Karunaratne *et al.*, 1990) that many subsurface bacteria isolates can store two or three of the common storage products found in prokaryotes [poly-ß-hydroxyalkanoate (PHA), polyglucose, and polyphosphate]. Approximately seventy-five percent of all isolates from the C10 borehole were able to store PHA. Bacteria normally store one or more of the three products above in response to unbalanced nutrition. For example, bacteria may store large amount of PHA when carbon is available in excess while N or P are limiting (Neidhardt *et al.*, 1990). Thus, the presence of PHA storage bodies is an indicator of a nutrient limitation other than carbon. As described in the original proposal, there are numerous procedures for detecting storage inclusions. We prefer to use a fluorescent microscopic procedure, if possible because of the high reliability and ease of detection afforded by these procedures. We have successfully used the Nile Blue A staining procedure (Ostle and Holt, 1982) for staining of PHA in bacteria in subsurface sediments. We also are testing other fluorescent dyes that react specifically with polyphosphate and polyglucose. Excitation by blue (488 nm), green (514 nm) or red (630 nm) is a key criterion for dye selection because we hope to use these dyes in the CLSM. Because fluorescent dye selection and testing is not yet completed for polyphosphate and polyglucose, we plan to use standard procedures in the first experiments, acid-toluidine blue for polyphosphate and Lugol's iodine or PAS for polyglucose.

7. A 30 day experiment using the flow through apparatus.

The first nutrient enrichment experiment was set up and run in December 1991 and January, 1992. Its objectives were to: 1) examine the practicality and effectiveness of our procedures for subcoring and microcosm set-up under real experimental conditions; 2) test experimental asepsis over a one month period; 3) compare the abundance and growth rates of microorganisms in mixed sediments versus intact cores; and 4) obtain some preliminary information on nutrient limitations in the sandy subsurface sediments of DOE's Savannah River Site. A full description of the experiment, including results and discussion, are included in the appended manuscript (Rudnick et al. 1992), which was presented for the First International Conference on Ground Water Ecology and accepted for publication in the Conference Proceedings after peer review.

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