Title: Methods for Environmental Monitoring of DOE Waste Disposal and Storage Sites

Period: 11-1-87 to 3-31-88

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Proposal for Optimizing a Biological Treatment System for Denitrification of Y-12 Waste Streams.

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Co-Investigator - Nathaniel Revis

Introduction

Problem - The denitrification process at Y-12 involves the use of sludge to denitrify aqueous plating waste containing relatively high levels of NO3. The process from time to time does not denitrify. The factors associated with the failure of the process remains to be resolved. We propose to resolve those factors by taking the following research approaches.

1) Isolation and identification of microorganisms originating from sewage sludge which are associated with denitrification.

2) Define physiological factors required for denitrification in this process system.

3) Define toxic factors associated with the aqueous waste that may affect the process of denitrification.

Background - Microorganisms play an important role in controlling the nitrogen balance in nature. A release of excess nitrogen into the environment has the potential for creating a nature imbalance and thereby alter life forms currently existing. Therefore, it becomes imperative that methods be developed to impede this imbalance and ecosystems are allowed to develop to their natural potential.
Nitrogen in decomposing organic matter found in soils is largely converted to ammonia (NH₃), a volatile nitrogen form. Nitrogen is stabilized by oxidation of NH₃ to nitrate (NO₃). This is a two stage biochemical process carried out by bacteria called nitrifying. Two major genera of bacteria are responsible for the oxidation, although Bergey's list a total of five. The two are Nitrosomonas (NH₃ — NO₂) and Nitrobacter (NO₂ — NO₃), both strict aerobes. The total process whereby NH₃ is converted to NO₃ is called nitrification.

The reduction of NO₃ — N₂ is through the use of oxygen in nitrate as a hydrogen acceptor. Free oxygen tends to inhibit the reduction reaction. A variety of bacteria can carry out the first stage of the reduction reaction (NO₃ — NO₂), especially the coliforms, facultative microorganisms. However, only bacteria known as denitrifiers can take nitrite (NO₂ — N₂) to elemental nitrogen. Some denitrifiers while reducing NO₂ to N₂ may form an intermediate, nitrous oxide (N₂O). This intermediate, however, is not obligatory for N₂ to be formed. Some bacteria form it and some do not.

Another reduction-type reaction of nitrate is NO₃ — NH₄ (assimilatory nitrate reduction). This reaction is carried out by bacteria such as Cl. Welchii, Desulfovibrio, and others.

Thus, several microorganisms are involved in maintaining the balance of nitrogen in soils. Some are more important than others. Arriving at complete denitrification of Y-12 waste stream treatment tanks may take on various physiological characteristics and involve several bacterial strains.
Milestones - The proposed time-frame for accomplishing the above research objectives are as follows. The project will officially begin Sept. 1, 1986 and end April 1, 1987. Three total reports will be written during this time. Reports will provide updated information as to project accomplishments. From Sept. 1, 1986 - Nov. 15, 1986 research on item number one will be done and final report written. Between Nov. 16, 1986 - Feb. 1, 1987 research on item two, and between Feb. 1, 1987 - April 1, 1987, item three, with report and final reports written.

The budget for pursuing this project is provided below.

**Budget**

<table>
<thead>
<tr>
<th>A) Personnel</th>
<th>Cost</th>
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<tbody>
<tr>
<td>Microbiologist - Grenetta Hicks (800 hours)</td>
<td>$16,240.00</td>
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<td>Technician - Cynthia Tolman (800 hours)</td>
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</tr>
</tbody>
</table>

| B) Materials and Supplies | |
| **Total Direct** | **$36,343.00** |

| C) Indirect Cost @ 30% | |
| **Total Direct & Indirect** | **$47,246.00** |

| D) Fee @ 10% | |
| **Total** | **$51,971.00** |
Detailed Contract Objectives

A) To define the denitrification process of Y-12 waste stream treatment tanks, in terms of:

1) Isolation, purification, and characterization of microorganisms most important for the denitrification process.
2) Provide these pure cultures as microorganisms to be used for seeding Waste Stream tanks.
3) Provide methodology for seeding tanks.
4) Determine physiological parameters necessary to achieve repeated denitrification of Waste Streams.
5) Determine possible inhibitors from Waste Stream to the process of denitrification.

If time permits,

B) To amplify the denitrification process in tanks. To be achieved mainly by genetic manipulation of those microorganisms important to the denitrification process. Genetic experimentation to take place will include:

1) Mutagenesis of whole bacterial cells
2) Screen pertinent bacteria for plasmid DNA associated with denitrification process.
3) If #2 is applicable, isolate the plasmid DNA and either
   a) mutate DNA and transform back into host cells or
   b) reconstruct plasmid efficiency through rDNA technology.
Discussion

Isolation, Purification, and Characterization of Microorganisms

It is not surprising that individual denitrification waste stream tanks vary as to their success from process to process. Two major factors probably contribute to the variation. One is the undefined microbial population entering the system through denitrification sludge inoculum and two, variation in constituents entering the system as a part of the waste streams. Both factors influence the type of microbial growth developing in the tanks with time.

Few of the incoming microorganisms will survive the total denitrification process. Those that survive will be the ones mainly responsible for controlling the system and the success of the process. A sampling of end process material will be a good source for isolating pertinent bacteria involved in the process. Samples have been provided by Y-12 engineers of contents from rapid and slow denitrification process tanks.

Once bacteria have been purified and characterized they will be taken through a laboratory scale process of denitrification, mimicking that occurring in large Y-12 tanks. During the lab scale process, samples will be taken periodically to test for microbial number and type, nitrogen content, along with a host of physiological parameters that are discussed below. It is during this testing that seeding conditions and methodology will be developed for insuring maximum success and reproducibility of denitrification on a large scale in Y-12 waste stream treatment tanks. Since the overall denitrification process from beginning to end in the large tanks is very complex, we may find that more than one type of
microorganism is important to the process and that a multiphase seeding procedure will be necessary to insure success.

Physiological Parameters

Throughout the denitrification process, the physiological environment of the tank changes with time due mainly to microbial activity. Microbial metabolism important to the denitrification process is controlled by these parameters. It is imperative that changes in the tank environment be monitored at various time intervals so that a better understanding is gained of the process. Each physiological parameter to be monitored is discussed below.

1) pH—Although the waste stream material is neutralized prior to entering the tank, growth of microorganisms during the process alter the pH due to excretion of acid or alkaline waste products into the tank environment. Enzymes involved in oxidation–reduction reactions of nitrogen compounds have optimum pH ranges. The detection and maintenance of the appropriate pH is necessary for successful denitrification in the tanks.

2) Dissolved oxygen (D.O.)—The D.O. level in the tank is highly influenced by the microbial population. Microorganisms scavenge available oxygen from the system, using it for oxidation reactions involved in growth. Since oxygen is not mechanically replaced in the tank once it is removed, microorganisms will vary based on their ability to survive in high, low, or no O₂ present in solution. It is perceived at this time that both aerobic and anaerobic bacteria are important to the overall tank process. The aerobic bacteria remove O₂ in the tank that enters with sewage.
and waste stream content. This causes the conditions in the tank to become anaerobic. A high D.O. level drops and is monitored as a low D.O. reading. Denitrification begins to occur under anaerobic conditions. Most denitrifying bacteria are facultative, but carry out denitrification only under anaerobic conditions. The more versatile an organism, in terms of its O₂ tolerance, the more likely it will survive from beginning to process end, owing to no drastic shifts in pH or tank temperature.

3) Temperature - Enzymes involved in nitrification and denitrification have temperature optimums for maximum efficiency. Negative temperature shifts in the treatment tanks could affect the denitrification process.

4) Nitrogen content - At each sampling during the overall process various nitrogen forms will rise and fall in quantity. Tests will be conducted to determine N content (NH₃, NO₂, NO₃, N₂O, N₂) of samples. By doing this one can assess whether the denitrification is taking place and what tank parameters are important for the process to move in a favorable direction.

5) Phosphate content - Since P0₄ is important to the success of the denitrification process it might be important to monitor its disappearance with time and assess whether feeding of P0₄ into the tank at various intervals might be necessary to increase the efficiency of the process.

6) Acetate Carbon Content - Since acetate is the carbon source used in the Y-12 treatment system its importance to the denitrification process will be assessed.
Amplification of Denitrification Process

This phase of the project is difficult to define at the present time. Reason being that overall denitrification occurring in Y-12 waste stream treatment is not yet understood. There may be one or a combination of microorganisms needed to direct the treatment process to success. The bacterial metabolism controlling denitrification may be of plasmid or chromosomal origin. Until this is known, general rather than specific methodologies will be discussed.

Amplification of any microbial system by genetic means is good since genetic changes are usually stable. Increasing the rate of a metabolic process carried out by bacteria is an example of amplification (i.e., reduce the time denitrification takes place in the tanks from 7 days to 4 days). Metabolic rates are controlled by enzyme synthesis. Enzymes are derived from proteins which come from amino acids that were originally coded for by DNA in the form of genes. Therefore, changing genes by altering base composition can affect metabolic rates.

Genetic alterations can be accomplished by different means. For example, chemical or physical mutagenesis of DNA or DNA reconstruction through cloning and rDNA technology. Since no specific microorganism(s) have been targeted to characterize and use as seed candidates, the exact genetic approach to be taken can be revealed at a later date. The main goal directive however, for amplification will be to increase the rate of denitrification by genetic means.
In summary, it can be said that the overall denitrification system in Y-12 waste stream treatment tanks is a complex microbial operation, involving several species of microorganisms, their significance occurring at various stages of the process. It is believed, however, that only a few of the microorganisms entering the system play an important part in insuring the success of denitrification. There may be one or several microbes involved. Seeding the tanks at various points during the overall process with these important microbes may be useful in "driving" the system in a positive direction towards denitrification. Important to the system in addition to the microorganisms is the correct physiological conditions necessary for denitrification to take place. Providing both sets of parameters for the waste stream treatment tanks may be the route to go to insure repeated success of the denitrification process.