'In situ' Characterization of Microorganisms Indigenous to Water-Table Aquifers

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Prepared for

Robert S. Kerr Environmental Research Lab.
Ada, OK

Sep 84

EPA-600/D-84-232
September 1984

IN SITU CHARACTERIZATION OF MICROORGANISMS INDIGENOUS TO WATER-TABLE AQUIFERS

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Cooperative Agreement
CR-806931

EPA Project Officer
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U.S. ENVIRONMENTAL PROTECTION AGENCY
ADA, OK 74820

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completing)

1. REPORT NO.	2.	3. RECIPIENT'S ACCESSION NO.
EPA-600/D-84-232		PB85 101731

4. TITLE AND SUBTITLE	5. REPORT DATE
In Situ Characterization of Microorganisms Indigenous to Water-Table Aquifers	September 1984
	6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
D.L. Balkwill and W.C. Ghiorse	

9. PERFORMING ORGANIZATION NAME AND ADDRESS		10. PROGRAM ELEMENT NO.
Dept. of Biological Sci. Florida State University Tallahassee, FL 32306	Dept. of Microbiology Cornell University Ithaca, NY 14853	CBPC1A 11. CONTRACT/GRANT NO. CR-806931

12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
U.S. EPA, R.S. Kerr Environ. Research Laboratory P.O. Box 1198 Ada, OK 74820	14. SPONSORING AGENCY CODE EPA 600/15

15. SUPPLEMENTARY NOTES

16. ABSTRACT

Core material from the deeper subsurface was examined for the presence and activity of microbes. Methods included acridine orange direct counts of the total number of cells, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride reduction assays of the number of respiring cells, plate counts of the number of viable cells, and examination of the ultrastructural characteristics of any microbes by transmission electron microscopy. The results demonstrated conclusively that appreciable numbers (1-10 million per gram) of bacteria reside in shallow, water-table aquifers. This observation is important because (1) it contradicts the traditional belief that such environments are almost devoid of life and (2) the numbers are large enough to potentially affect ground water quality.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Ground water quality Ground water microbiology Soil microbiology	AODC INT Reduction Tetrazolium dye reduction	6F 6M

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	Unclassified	16
	20. SECURITY CLASS (This page)	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

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INTRODUCTION

Water-table aquifers are environmentally significant because they contain ground water reserves that represent most (95% in the United States; Josephson, 1980) of the freshwater available for irrigation or consumption. With the occurrence of organic pollutants in ground waters becoming an increasingly widespread problem (Council on Environmental Quality, 1981), it is important to define and develop an understanding of factors that affect ground water quality.

Microorganisms could play a major role in maintaining ground water quality, considering that they can profoundly affect biological and chemical activities in surface soils and other environments (Alexander, 1977). However, the microorganisms in aquifers have been studied only rarely (Dunlap and McNabb, 1972), perhaps because early reports of soil microbiologists (e.g. Waksman, 1916) indicated that the number of microorganisms in soil drops sharply with increasing depth. More recent studies have shown that microorganisms can be present at considerable depths in the subsurface (Dockins et al., 1980; Dunlap et al., 1972; Whitelaw and Edwards, 1980: Whitelaw and Rees, 1980), but problems of contamination by surface soil have hampered the interpretation of these results. Thus, detailed data on the occurrence and numbers of microorganisms in aquifers remains scant (Dunlap and McNabb, 1973). Even less is known about the *in situ* metabolic activities of such organisms or about how these activities may affect organic contaminants of ground water.

In 1979, we initiated efforts to obtain more detailed information on the aquifer microflora by direct observation of *in situ* microorganisms in subsurface samples. Traditional cultural methods were deemed mostly unsuitable for these studies because they were not likely to select for many of the significant organisms in subsurface samples (Ghiorse and Balkwill, 1981). Instead,

light and electron microscopical methods for direct observation of microbial cells in surface soils were modified for application to aquifer and other subsurface materials. The present report reviews the information regarding the characterization of microorganisms in water-table aquifers and other subsurface materials that we have obtained with this approach (Ghiorse and Balkwill, 1981, 1983; Wilson et al., 1983).

MATERIALS AND METHODS

Description of Samples

Samples were collected from a total of four sites in Louisiana, Oklahoma, and Texas, from above and below the water table at each site. Subsurface regions situated above aquifers were sampled because they are likely to affect water that travels from the surface to aquifers below. The samples and their origins are listed in Table 1; for more detailed information, see the original references cited in the table. Aquifer and subsurface samples were collected aseptically by using a modification (Wilson et al., 1983) of the procedures developed by Dunlap et al. (1977).

Acridine Orange Direct Counts (AODC)

Epifluorescence light microscopy (LM) of acridine orange (AO)-stained samples was used to determine the morphological characteristics and the total numbers of cells by direct counts (AODC). A modification of Trolldenier's (1973) method was used to determine the AODC as described by Ghiorse and Balkwill (1983).

Respiring Bacteria

The proportion of AODC bacteria capable of reducing 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), i.e., the proportion of respiring bacteria (Zimmermann et al. 1978), was determined by mixing 2.5 g of

subsurface material with 20 ml of filter-sterilized 0.1 % sodium pyrophosphate (SPP) in a 125-ml Erlenmeyer flask. The mixture was shaken at room temperature for 15 min at 160 rpm, 2.5 ml of 0.2 % aqueous INT was added, and shaking was resumed for an additional 15 min. Excess INT was removed by decanting and centrifuging the entire contents of the flask at 10,000 rpm for 10 min. The supernatant fluid was decanted and the pellet was washed by centrifugation in 10 ml of 0.1 % SPP. The final pellet was resuspended in 22.5 ml of 0.1 % SPP and the AODC procedure described by Ghiorse and Balkwill (1983) was followed.

To count bacteria containing INT-formazan deposits, green fluorescent cells were first identified under epi-illumination. These were then inspected for the presence of INT-formazan employing a 100 X bright field objective lens. Care was taken to use bright field illumination conditions that optimized recognition of the red formazan deposits in the cells. This included adjusting the substage iris diaphragm and the illuminator rheostat to the same setting each time, as well as the use of neutral density filters to reduce brightness of the field.

INT-containing bacteria of two types were counted. One type was characterized by diffuse but distinctly reddish cells with no apparent granules. The second type was characterized by the presence of distinct red granules inside the cell.

Plate Counts

Standard plate counts in triplicate were used to estimate the number of viable microorganisms in subsurface environments. Both nutritionally rich (PYG and/or 1-5% PYG agar; Ghiorse and Balkwill, 1983) and low-nutrient (SEA; Wilson et al., 1983) media were used in all cases. All plates were incubated aerobically at 25 C, and colonies were counted after 1-2 weeks.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to determine the ultrastructural characteristics of subsurface microorganisms. Microbial cells were released and concentrated from subsurface materials prior to TEM examination with the centrifugal washing method described by Ghiorse and Balkwill (1983).

RESULTS

Morphological Characteristics of Aquifer Microorganisms

Epifluorescence light microscopy (LM) of AO-stained preparations readily detected microbial cells in all of the aquifer and other types of subsurface samples examined (see Table 1). Objects that fluoresced bright green, thereby indicating that they contained double-stranded DNA (Daley and Hobbie, 1975), and that possessed appropriate morphological characteristics were considered to be microbial cells. These cells stood out clearly against a dull orange background of fluorescing abiotic material.

Epifluorescence LM was useful for assessing the range of morphological diversity in each sample and for detecting the occurrence of microcolonies (for illustrations of these results, see Fig. 1 in Ghiorse and Balkwill, 1983 and Fig. 2 in Wilson et al., 1983). Microcolonies (groups of cells with similar morphological characteristics) were present in all samples, but the range of morphological diversity varied considerably in samples from one location to another. Texas and Oklahoma (both Lula and Pickett) samples contained mostly small, coccoid bacterial cells that were similar in shape to those found in surface soils (Bae et al., 1972; Balkwill and Casida, 1973; Balkwill et al., 1975, 1977). Few, if any, eukaryotic forms were detected. In contrast, the samples from Louisiana contained a greater variety of bacterial forms, including: small coccoid cells, rod-shaped cells of varying dimensions, and actinomycetes or other filamentous types. Some of these

samples contained small numbers of microeukaryotic forms. Very recently, a cyst-forming amoeba and a fungus have been detected in Oklahoma samples by special cultural methods (J. Sinclair, personal communication). These microeukaryotes were present in low numbers in comparison to bacteria and, therefore, they do not appear to account for a significant portion of the biomass in the sample.

Ultrastructural Characteristics of Aquifer Microorganisms

Transmission electron microscopy (TEM) of aquifer and other subsurface materials confirmed the presence of microorganisms in all samples by revealing objects that possessed ultrastructural features (such as cell walls, membranes, and intracytoplasmic inclusions) unequivocally characteristic of microbial cells (for illustrations, see Figs. 2 and 3 in Ghiorse and Balkwill, 1983 and Fig. 3 in Wilson et al., 1983). The dimensions and shapes of these cells corresponded to those of the green-fluorescing objects considered to be cells in AO-stained preparations for LM (above).

TEM of thin-sectioned microbial cells that were released and concentrated from aquifer or other subsurface samples by blending and centrifugal washing (see Materials and Methods) provided important information on these organisms that could not be obtained readily with other approaches. For example, it was possible to determine the relative proportions of Gram-positive and Gram-negative bacteria in aquifer environments because thin sectioning revealed the architectural details of their cell walls. Both Gram-positive and Gram-negative forms were present in all samples, but the former were always clearly predominant (two-thirds or more of the bacteria observed were Gram-positive).

The cytoplasm of many subsurface bacterial cells was partially depleted of the intracellular constituents (ribosomes and nuclear material) commonly found in laboratory-cultured cells. Control experiments involving addition of

laboratory-cultured cells to subsurface samples established that these cytoplasmic constituents were not lost during preparation of the samples for TEM. Some of the in situ bacterial cells with a depleted cytoplasm contained mesosome-like internal membranes and intracellular storage bodies such as polyphosphate granules (Jensen, 1968), or more frequently, poly- -hydroxybutyrate (PHB) granules (Dunlop and Robards, 1973). In contrast, other subsurface bacteria lacked such inclusions and contained the "normal" or "healthy-looking" cytoplasm that is characteristic of laboratory-cultured cells. A few of these bacteria possessed cross-walls or division septa, implying that they were in the process of dividing when the samples were fixed. This was observed in both coccoid and filamentous forms. A small number of bacterial cells in the samples from Louisiana also contained internal membrane systems that were reminiscent of those found in nitrifying or methane-oxidizing bacteria. Ruthenium red staining indicated that many subsurface bacteria were surrounded by polysaccharide-based capsules and glycocalyx layers. The polysaccharide strands of these structures often extended from the cell surface to surrounding pieces of abiotic materials.

As was true of morphological diversity (above), the internal ultrastructural diversity of the bacteria in samples from Louisiana was greater than that of the bacteria in Oklahoma samples. TEM also confirmed that the overwhelming majority of subsurface microorganisms were prokaryotic.

Numbers of Microorganisms in Aquifer Environments

Epifluorescence LM of AO-stained samples was an effective way to obtain direct counts (AODC) of total microbial cells in aquifers and other subsurface environments. The resulting counts (Table 2) were lower, sometimes by two or three orders of magnitude, than those that have been reported for typical surface soils (see Alexander, 1977), and they were remarkably consistent from

one sampling site to another. Samples from Oklahoma, Louisiana, and Texas typically contained between 1-10 million AODC-cells per gram (dry weight) of subsurface solids. Somewhat surprisingly, the numbers of cells did not decrease appreciably with increasing depth at any site.

Plate counts on media with differing nutrient concentrations were used to estimate the numbers of viable cells present in subsurface samples (Table 2). Although traditional cultural methods of this type were of limited value for obtaining meaningful information on aquifer microorganisms (see Discussion), we included plate counts in our studies to provide a basis for comparison with other environmental investigations. Plate counts on nutritionally rich media like PYG agar were generally lower (sometimes much lower) than on low-nutrient media like SEA (Table 2). The highest plate counts, which were usually obtained on SEA, were always lower than the AODCs of the same sample. The magnitude of this discrepancy varied from one sampling site to another. Plate counts for samples from Oklahoma were sometimes as high as 50% of the AODC, but those for samples from Louisiana and Texas were generally much lower (0.01% of the AODC or less).

Metabolic Activities of Aquifer Microorganisms

Plate counts demonstrated that some of the microorganisms residing in aquifer and other subsurface samples were capable of growth, but this information provided little or no indication of their activities in situ. Similarly, direct observation of microbial cells with LM and TEM provides only limited and indirect information on the metabolic activities of these organisms. Therefore, it was of interest to apply methods designed to reveal in situ metabolic activity more directly. One such method involves the use of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as a measure of respiratory activity of microbial cells (Zimmermann et al., 1978).

Preliminary results with the INT method suggest that 1-10 % of the AODC bacterial cells in samples from Texas were capable of respiration-linked INT reduction. In most cases, however, INT-formazan-containing bacteria contained the diffuse type of deposit. Very few cells contained distinct granules. These results suggest a low level of respiratory activity in the subsurface bacterial population.

An alternate approach to investigating potential metabolic activities of aquifer microorganisms was developed and used by J. T. Wilson (Wilson et al., 1983). This approach involved the use of microcosms constructed from subsurface materials to determine whether the organisms indigenous to those materials could degrade selected organic pollutants. Toluene was degraded rapidly in subsurface samples from above and below the water table at Lula, Oklahoma. Comparison of autoclaved and non-autoclaved samples indicated that the degradation was a biological process. Chlorobenzene was also degraded in these samples, but (i) its degradation rate was considerably slower than that of toluene and (ii) degradation took place only in samples from above the water table. Bromodichloromethane was also degraded slowly, but it was not clear whether this was a direct or indirect result of microbial metabolism. In contrast, there was no detectable degradation of 1,2-dichloroethane, 1,1,2-trichloroethane, trichloroethylene, or tetrachloroethylene in any of the Lula, Oklahoma samples.

DISCUSSION

The results reviewed here demonstrate conclusively that appreciable numbers (1-10 million per gram) of microbial cells reside in aquifer material. This observation is important for two reasons: (i) it contradicts the traditional belief that such environments are almost devoid of microbial life and (ii) the numbers of cells detected were great enough to potentially affect ground water quality, provided that these cells were metabolically active.

Morphological and ultrastructural data indicated that, even though the total number of microorganisms was quite consistent from one sampling site to another, the identity of those organisms may have varied considerably. This has important implications with respect to the potential effects of microorganisms on ground water quality, since different microbial types carry out different metabolic reactions and will respond differently to specific pollutants. Equally important is the fact that the range of microbial types varied widely from one site to another. A pollutant compound might destroy all microbial life in an aquifer that contained only a few types of bacteria, whereas a more diverse microbial community would be more likely to include a species that could survive or even degrade the pollutant compound. In defining the various factors that control ground water quality, then, it probably will be necessary not only to consider microorganisms in general, but also to consider the specific microbial population of each aquifer system.

Some of the microorganisms in aquifer and other subsurface environments must be viable because the studies reviewed here showed that they were capable of growth on plates. However, most of the AODC cells in typical samples did not grow on plates. This could mean that these organisms were not viable, but it is more likely that the growth media used for plating simply failed to meet their possibly complex growth requirements (see also Ghiorse and Balkwill, 1983.) There is a need, then, to characterize the growth requirements of subsurface microorganisms so that more realistic procedures for enumerating viable cells can be developed. Alternatively, modifications of direct LM approaches like the INT method might also serve to solve this problem.

The fact that plate counts of aquifer and other subsurface samples usually were higher on nutritionally rich media than on relatively dilute media implies that the in situ microorganisms in these samples may prefer low levels of nutrients for growth. Morphological and ultrastructural data in the

studies reviewed here also point to adaption by subsurface microorganisms to low-nutrient and/or starvation conditions. The overwhelming predominance of prokaryotes, for example, probably occurred because oligotrophic prokaryotes are much better adapted than eukaryotes to live in environments with very low levels of organic matter (Poindexter, 1981). The PHB granules seen in subsurface bacteria also indicated an adaptation to low-nutrient conditions, since synthesis of these and other storage materials is a common bacterial strategy for surviving periods of nutrient shortage (Poindexter, 1981; Shively, 1974). The depleted cytoplasm of many subsurface bacteria suggests that these cells actually were either nutrient-limited or starving at the time of sampling and, therefore, probably were relatively inactive members of the microbial community. On the other hand, the bacteria with a "healthy" cytoplasm or with division septa must have learned both to survive and to grow actively under low-nutrient conditions.

Although data obtained by direct observation of subsurface microbial cells with LM and TEM allowed us to draw reasonable conclusions about the likely physiological characteristics of subsurface microorganisms (above), we still know very little about the specific in situ or potential in situ metabolic activities of these organisms. Specialized techniques like the INT procedure may prove helpful in this regard, but there is a need to develop more powerful and sophisticated LM and TEM methods for determining in situ metabolic activities in subsurface environments. Such information will be critical in order to understand the biology of subsurface microorganisms. It will also be critical to understand how subsurface microorganisms may affect ground water quality and how these microorganisms themselves may be affected by pollutants.

ACKNOWLEDGEMENTS

This work was supported by Subcontract No. 6931-5 under U.S.E.P.A. Cooperative Agreement No. CR806931-02.

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