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Water Potential and Starvation Stress in Deep Subsurface Microorganisms

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Abstract Nine intact core samples, collected aseptically from depths of 10-436 m near the Savannah River Plant in South Carolina, were tested for water potential, microbial numbers, and microbial activity. Although all samples were collected from below the water table, two samples (a Pee Dee clay from 238 m and a Middendorf clay from 324 m) showed unsaturated conditions (-2.7 and -2.1 MPa, respectively). Both of these samples had very low numbers of culturable cells, low microbial biomass (ATP assay), and low microbial activities (measured as respiration), suggesting that low matric water potentials in these strata are limiting factors to microorganisms. An Acinetobacter sp. isolated from the 324 m depth was found to maintain viability under starvation conditions in sterilized aquifer material, even when subjected to severe desiccation (-22 MPa). A Pseudomonas sp., with the ability to oxidize thiosulfate to sulfate, was isolated from the 378 m Middendorf clay sample. This organism survived nutrient deprivation reasonably well; however, the presence of thiosulfate appeared to interfere with its normal ability to maintain viability by endogenous metabolism. Cells cultured in the presence of thiosulfate did not undergo dwarfing and cell viability declines. These are two examples of indigenous subsurface microorganisms, each with different adaptations for long-term survival under conditions of desiccation and/or starvation.

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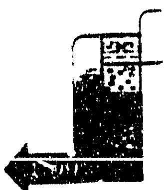


Introduction

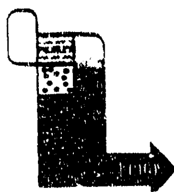
Concerns regarding the safety of groundwater supplies have fostered an intense interest in the microbiology of subsurface terrestrial environments. Early studies of relatively shallow groundwater sediments (10-30 m) clearly demonstrated that microbial life extends well below surface soil horizons.[4,5,7,9,14,16,17,30] These studies have been followed more recently by far deeper probes of subsurface environments, and these have also unearthed a diverse microflora.[2,3,11,13,15,24] The existence of indigenous communities of microorganisms in these groundwater sediments suggests a potential for *in situ* microbiological degradation of organic pollutants in contaminated groundwater supplies. With the development of bioremediation as an eventual goal, a number of basic studies have been undertaken to determine the presence and abundance of microorganisms in deep subsurface environments and to assess their potential for survival and metabolic activity under the limiting environmental conditions of the subsurface.

As in most natural environments, the numbers of microorganisms which can be directly observed by microscopy exceed by one or more orders of magnitude those which can be quantified by culture methods (e.g., standard plate counts).[7,9,14,17,24] Examples of non-culturable microorganisms include: (1) cells which are dead or dying, (2) cells which are fastidious (i.e., requiring growth factors that are not provided by standard plate count media), and (3) cells which are starving or injured. The latter two groups comprise viable nonculturable cells, which require non-culture methods for assessing their potential for metabolic activity. Given the low concentrations of organic carbon typically found in these subsurface sediments, it is to be expected that a high proportion of microorganisms are in starvation-survival mode. In addition to nutrient stress, reduced water availability may be a factor which is limiting to microbial activity, particularly in the vadose zone, but possibly in regions below the water table as well. In the present study, subsurface samples from near the Savannah River Plant (SRP) in South Carolina were characterized in terms of water potential and microbiological parameters. An effort was made to use nonculture methods which have been developed for soil microbiology, along with standard plate counts, to determine biomass, rates of *in situ* metabolic activity, and the physiological status of deep subsurface microorganisms. Desiccation and starvation responses of individual isolates from the Middendorf formation were also examined.

Materials and Methods

Sample collection. Subsurface sediment samples were collected aseptically from borehole C10 as part of the United States Department of Energy's Deep Subsurface Microbiology Program. The C10 site is located approximately 40 km east of the Savannah River Plant. Samples were collected from August through October 1988, from depths of 10-436 m. The aseptic sampling technique consisted of a subcoring procedure described by Phelps et al.[23] Portions of the intact subcores were sent by overnight express to New Mexico Tech for immediate microbiological testing.

Sample characterization. Moisture content was determined gravimetrically after 24 hours of desiccation at 105°C. Water potentials were measured by thermocouple psychrometry (Decagon Devices, model SC-10, Pullman, WA).



Standard plate counts. Initial ten-fold dilutions were made in 0.1% $Na_4P_2O_7 \cdot 10H_2O$ (pH 7.0). Further serial 10-fold dilutions were made in phosphate-buffered saline.[14] Diluted sediment suspensions were spread plated in triplicate onto PTYG and 1%PTYG agar.[3] Colonies were counted after eight days of incubation at 22°C.

Basal respiration. One-gram (wet weight) sediment samples were placed in 8-ml serum vials, sealed, and incubated at 22°C. Carbon dioxide was measured by gas chromatography immediately after sealing and after 24 hours. Immediately prior to gas sampling, the vials were vortexed for five seconds to mix headspace and sediment gases. Three replicates of each sediment sample were tested. Controls poisoned with 0.5% NaN_3 were used to quantify abiotic CO_2 production and the resulting values were subtracted from those of the live treatments.

Substrate-induced respiration. Biomass carbon was estimated by the SIR assay as modified by West and Sparling.[1,29] One-gram (wet weight) sediment samples, along with 30 mg glucose, were placed in 8-ml serum vials, sealed, and incubated at 22°C. Carbon dioxide in the headspace was measured by gas chromatography at 0.5 and 2.5 hours after the vials were sealed. Immediately prior to gas sampling, the vials were vortexed for five seconds to mix headspace and sediment gases. Biomass was calculated using the following relationship[1]: $y = 40.04x + 0.37$, where y = biomass C (μg 100 /gdw) and x = respiration rate (ml CO_2 100 /g of sediment (h^{-1})). Five replicates of each sample were tested.

ATP and adenylate energy charge. Adenylates were extracted by a modification of the method of Vaden et al.[28] A 10-ml solution of extractant containing 2 N H_3PO_4, 0.02 M ethylenediamine tetraacetic acid (EDTA), 2 M urea, and 0.24 M dimethylsulfoxide (DMSO) was added to one-gram (wet weight) sediment samples. The samples were sonicated on ice for five minutes at a constant output of 75 watts, centrifuged at 12,100 x g for 10 minutes at 5°C, and decanted. The resulting extract was stored at -80°C prior to analysis. The efficiency of extraction was determined by amending the extractant to contain 0.5 μM each of ATP, ADP, and AMP (Sigma Chemical Co., St Louis, MO). The amended extractant (10 ml) was added to a one-gram sample of the same sediment and sonicated and centrifuged as described above. Adenosine triphosphate standards were made up in the extractant, and the plain extractant served as the blank. Prior to analysis, the pH of standards, blanks, and sediment extracts were adjusted to 7.70 - 7.90 with 0.1 M tricine buffer (pH 10.0) and 5 M ethanolamine. Adenylates were measured as ATP by a luciferin-luciferase assay. Adenosine triphosphate was measured directly. Adenosine diphosphate and AMP were measured as ATP following enzymatic conversion as described by Martens.[21] Adenylate energy charge (AEC) was calculated according to the formula: $AEC = ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP])$.

Isolation and characterization of a starvation-tolerant microorganism. Although initial plate counts yielded no viable microorganisms from the 324 m depth sample in the Middendorf formation, plate counts performed using 1%PTYG agar after several weeks storage of the core material at 5°C showed the presence of 1.9×10^2 Colony Forming Units (CFU)/g of a single colony type. One colony was selected and

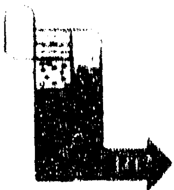
identified by morphological and biochemical tests. This isolate was tested for its ability to survive in the core material under conditions of nutrient limitation and desiccation. Core material (G-14) was crushed, mixed, placed in 1.0 g aliquots in 8-ml serum vials, and sterilized by Tyndallization. The success of the Tyndallization was tested by plating onto 1% PTYG agar. The isolate was cultured in 1%PTYG broth at room temperature on a shaker (200 rpm). When the culture reached a late logarithmic stage of growth, cells were centrifuged (5,000 x g, 10 minutes) and resuspended in a sterile starvation medium (inorganic constituents only from 1%PTYG). This cell suspension, containing 9.6×10^6 cells/ml, was then added in 300 μl aliquots to each serum vial. Sediments were mixed in the vials on a vortex mixer, then subjected to varying degrees of desiccation by exposing them for varying lengths of time to the flow of air in front of a HEPA filter in a sterile transfer hood (Environmental Air Control, model TT 4830, Hagerstown, MD). After varying periods of desiccation, the vials were sealed with sterile septa and incubated at 22°C. Numbers of surviving cells were quantified immediately, and after 13, 26, and 53 days by standard plate counts on 1%PTYG agar as described above. Final water potentials and water contents of the desiccated samples were measured as described above after 53 days of incubation.

Isolation and characterization of a heterotrophic thiosulfate oxidizer. A bacterial strain isolated from the Middendorf formation (378 m) on ATCC Medium 290, containing thiosulfate and solidified with 15 g/l agar,[12] was characterized by standard morphological and biochemical tests and by the use of a Biolog GN plate (Biolog, Inc., Hayward, CA).

This organism was tested for thiosulfate oxidation in medium 290 and amended with 0.10 g of glucose, 0.1 g of yeast extract, 0.05 g of peptone, and 0.05 g of tryptone per liter. The medium was inoculated with a starter culture which had been incubated for four days in the same medium on an orbital shaker (200 rpm) at 26°C. Cultures were incubated at 26°C and shaken at 200 rpm. Concentrations of sulfate were measured by barium sulfate precipitation; thiosulfate, tetrathionate, and trithionate concentrations were measured by a cyanolysis method.[20] Cell numbers and cell sizes were monitored by phase contrast microscopy.

Results

Physical and biological characterization of core samples. The majority of the core samples showed saturated conditions; however, two samples (G-10 and G-14) had low water potentials (−2.70 and −2.07, respectively) indicative of unsaturated conditions (Table 1). Standard plate counts yielded no viable cells from four of the nine samples; the others had values ranging from 5.0×10^2 to 4.0×10^4 CFU/g. Respiration rates ranged from below detection (actually appearing to be negative due to slightly higher CO_2 production in the poisoned control) to 1.08 μg CO_2/gw/h. Microbial biomass was detected by the SIR method in eight of the nine samples, whereas ATP was detected in only five samples. Five of the eight samples for which the AEC could be calculated had values below 0.5. Respiration rates were



correlated with ATP values ($r = 0.77$) and adenylate energy charge values ($r = 0.86$).

Starvation/desiccation survival. The strain isolated from sample G-14 was identified as an Acinetobacter based on its being a Gram-negative, strictly aerobic, oxidase negative, catalase positive, nonmotile rod. It proved to be remarkably tolerant of nutrient deprivation, even under conditions of severe desiccation (-21.9 MPa) (Figure 1). Only those exposed to the most severe desiccations (-7.7 and -21.9 MPa) declined in numbers and even these declined by a factor of less than two during 53 days incubation.

Heterotrophic sulfur oxidizer. The bacterium isolated from sample G-15 on a thiosulfate-containing medium was identified as a Pseudomonas sp. based on its being a Gram negative, strictly respiratory, oxidase positive, catalase positive, motile rod. It was identified as P. testosteroni by the Biolog test. This identification was supported by the finding that the isolate could degrade testosterone as a sole carbon source in minimal medium. In thiosulfate-containing growth medium amended with organic substrates, the isolate was shown to oxidize thiosulfate slowly to sulfate, with the production of tetrathionate and trithionate as intermediates (Figure 2a). Growth and thiosulfate oxidation in medium containing only thiosulfate was very poor. Oxidation of thiosulfate did not appear to enhance the growth rate of P. terstosteroni in organic media; generation times were approximately 0.5 hours in both media (Figure 3). However, the presence of thiosulfate in the growth medium had a profound effect on cell size (Figure 2b) and viability. Cells grown in a heterotrophic medium underwent dwarfing beginning in the stationary phase of growth, whereas cells cultured with thiosulfate grew to an average length of 3.3 μm and width of 1.5 μm after 25 hours and diminished in size only slightly after prolonged incubation. Long-term survival of cells cultured with thiosulfate was much lower than those cultured in plain organic medium. Per cent viability, calculated as (viable counts/direct counts) x 100, declined steadily to 0.6% during 31 days incubation with thiosulfate, while 77.5% of cells cultured without thiosulfate remained viable after the same incubation period.

Discussion

The application of assays borrowed from soil microbiology has the advantage that the methods are intended for microbial populations which are frequently in a relatively inactive state. Also, as in soil systems, many subsurface microbial populations are undoubtedly forced to subsist on diets of recalcitrant polymeric compounds such as humic and fulvic acids. The respiration- and adenylate-based assays used in this study are appropriate for the viable nonculturable cells which comprise large proportions of the microbial communities in soils and in subsurface sediments. The disadvantage of these methods for subsurface applications lies in their sensitivity. Biomass estimates and rates of activity of microorganisms in subsurface environments are typically several orders of magnitude lower than those in surface soils, and so they are often below detection. Nonetheless, the values obtained here provide some insights into life in the deep subsurface.

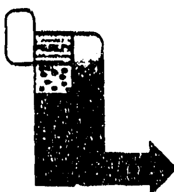
Adenosine triphosphate concentrations have been shown to be good predictors

of contaminant biodegradation rates in shallow aquifer material.[33] In the SRP samples, AEC was even more strongly correlated than ATP with overall microbial respiration rates, suggesting that AEC may be a more accurate predictor than ATP of potential for contaminant biodegradation.

Ratios of microbial biomass carbon to total organic carbon (TOC) have been used in soil studies as indicators of the degree of development of microbial communities, with high values corresponding to relatively "new" or recently disturbed communities, and low values occurring in later successional stages.[18] These ratios also reflect the forms of available carbon, in that "older" microbial communities are coincident with higher proportions of stabilized, recalcitrant compounds, such as humic and fulvic acids. Total organic carbon measurements were available for four of the nine sediment samples received, and of these four, three had biomass levels which were detectable by the SIR method. The TOC's for samples G-5, G-6, and G-20 were 0.52, 2.2, and 11.0 mg of carbon/gdw, respectively. Combining TOC's with the SIR biomass values as ratios yields values of 11.0, 5.18, and 3.68 mg biomass-C/ mg organic carbon for the three samples. These ratios are considerably lower than those typically found in surface soils and, therefore, are in agreement with what we already know or can surmise about these communities(i.e., that they are extremely "old," stable, and surviving amid predominantly stable, recalcitrant organic carbon).

The biological characterization of these deep subsurface samples indicates relatively inactive, severely nutrient-stressed microbial communities, a conclusion which is consistent with the data of other subsurface investigations.[2,14] Most samples showed some biological activity, as evidenced by the respiration-based biomass and activity assays; however, not all of these samples showed viable cells in the standard plate count assays. This suggests the presence of viable non-culturable cells which may be starved, injured, or nutritionally fastidious. As an example, cells could not be cultured from sample G-14 in the upper Middendorf formation until after a prolonged period of storage at 5°C, a step which may resuscitate starving or injured cells. Indications of highly stressed cells in this upper Middendorf region have also been found in the phospholipid analyses.[31] The AEC values for most of the strata suggest inactive and physiologically stressed cells. Adenylate energy charge values in the range of 0.50-0.75 are considered to represent dormant or inactive cells; values below 0.4 are thought to indicate dead or dying cells.[19] In at least two of the samples from the Pee Dee and Middendorf formations, desiccation may have contributed additional stress to microorganisms. Desiccation in porous media inhibits microbial metabolism directly by loss of water and indirectly by the reduction of solute diffusion.

The finding of a starvation-tolerant Acinetobacter in the deep subsurface is perhaps not surprising since members of this genus are nearly ubiquitous in other natural environments, including soil and aquatic environments.[6] Acinetobacters are found in high concentrations in groundwaters[8]; they are common isolates from the SRP boreholes[3]; and they have been found in subterranean volcanic tuff at the



Nevada Test Site (Penny Amy, personal communication). The extraordinary starvation tolerance as well as desiccation tolerance of this bacterium is reminiscent of the frequently studied soil bacteria of the genus Arthrobacter. In fact, Acinetobacter might be considered a Gram-negative subsurface counterpart of soil arthrobacters. The actual physiological basis of this starvation tolerance has yet to be studied, but one may assume that it includes a limiting of the rates of respiration and endogenous metabolism as has been found in Arthrobacter crystallopoietes and numerous other starvation-tolerant bacteria.[10]

Conclusion

Evidence of heterotrophic sulfur oxidation has been found by other deep subsurface investigators (J. K. Fredrickson, personal communication). Thiosulfate oxidation by strictly heterotrophic bacteria has been found in bacteria from soil and marine habitats and has been the subject of sporadic study for decades.[22,25-27] The selective advantage, if it exists for this phenomenon, has remained a mystery. Most investigators have failed to demonstrate an increased growth rate when thiosulfate is present. One might hypothesize an advantage in long-term survival during organic nutrient deprivation. However, the data of the present study fail to support this hypothesis and, in fact, indicate a distinct disadvantage of thiosulfate oxidation in this particular strain of P. testosteroni. Thiosulfate appears to interfere with the normal dwarfing response in this isolate. This is perplexing in that thiosulfate is actually present in the porewater of the Middendorf formation; and so presumably these microorganisms are similarly disadvantaged in their natural environments. We have also tested the starvation/desiccation responses of this P. testosteroni isolate by the system described above for Acinetobacter; in this system, P. testosteroni remains viable only under moist conditions (data not shown).

Starvation survival appears to be a requisite trait for existence in subsurface systems. Desiccation tolerance may be advantageous in selected groundwater microhabitats, and it may also indicate that at least some groundwater microorganisms are derived from more water-stressed environments such as surface soils and unsaturated subsoils.

Acknowledgements

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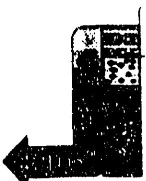

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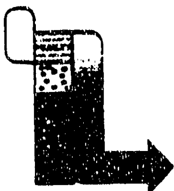

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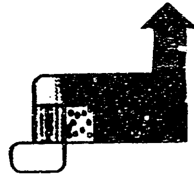
Table 1. Characteristics of subsurface samples from borehole C10 near the Savannah River Plant in South Carolina.

Sample Number	Depth (m)	Geological Formation	Percent Moisture	Water Potential (MPa)	Viable counts PTGY Log(CFU/gdw)	Viable counts 1% PTGY Log(CFU/gdw)	Respiration Rate (μCO_2/gdw/h)	Biomass (SIR Method) (µg BiomassC/gdw)	ATP (nmol/gdw)	Adenylate Energy Charge
0	10	Tobacco Road	23.7	-0.16	n.g.[a]	n.g.	0.056	29.6	0.0087	0.39
5	70	McBean	29.3	-0.05	n.g.	n.g.	0.103	5.72	0.0377	0.29
6	119	McBean	20.3	-0.14	4.6	4.6	1.00	11.40	0.0342	0.76
8	194	Ellenton	34.7	-0.15	2.6	2.7	-0.0436	n.d[b]	n.d.	0.24
9	212	Ellenton	27.4	-0.04	3.4	3.6	0.260	21.8	n.d.	0.23
10	238	Pee Dee	15.1	-2.70	n.g.	n.g.	0.152	18.3	n.d.	i[c]
14	324	Middendorf	32.0	-2.07	n.g.	n.g.	0.186	12.8	n.d.	0.45
15	378	Middendorf	12.7	-0.18	2.4	3.0	0.300	30.6	0.005	0.51
20	436	Cape Fear	17.8	-0.66	3.3	3.2	1.08	40.5	0.127	0.63

a n.g., no growth 10^{-2} dilution plates

b n.d., not detected.

c i, indeterminant; i.e., no adenylates were detected.



Figures

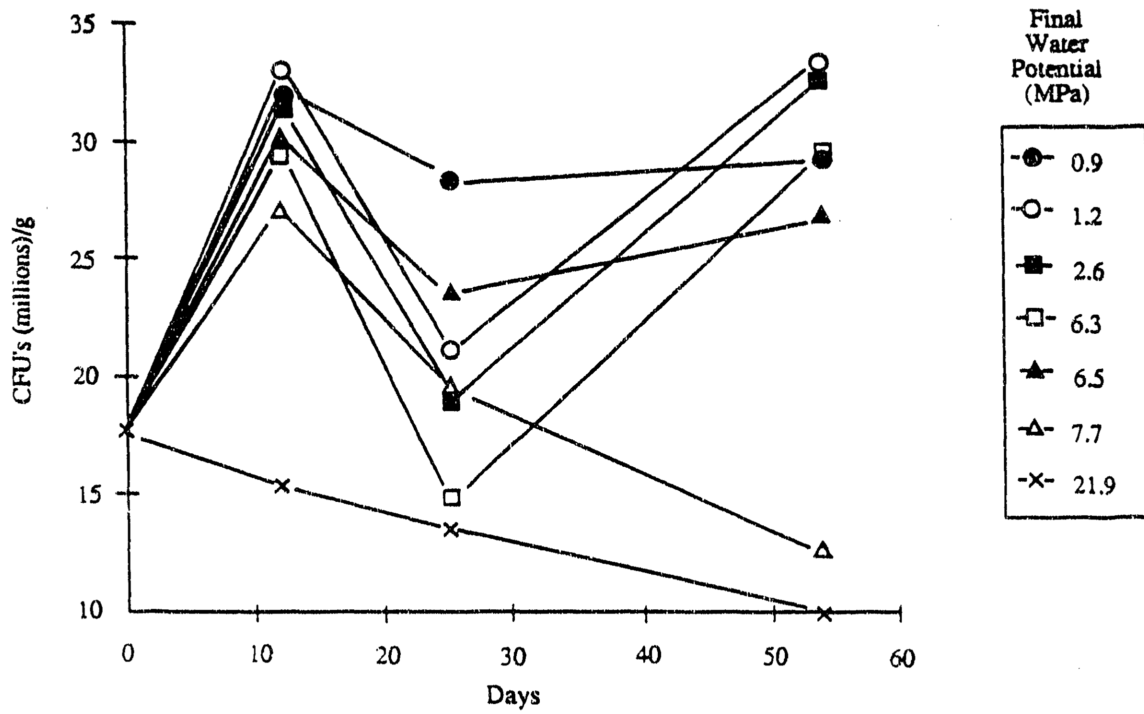


Figure 1. Starvation survival of an Acinetobacter sp. inoculated into sterilized Middendorf clay from SRP and incubated under varied degrees of desiccation stress.

A

Sulfur (mEq/l)

$S_2O_3^{2-}$

$S_4O_6^{2-}$

$S_3O_6^{2-}$

SO_4^{2-}

B

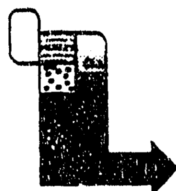
cell size (μm^3/cell)

With Thiosulfate

Without Thiosulfate

Times (hours)

Figure 2. Growth of <u>Pseudomonas testosteroni</u> isolated from a Middendorf clay. (a) Concentrations of thiosulfate and its oxidation products in the polythionate pathway in an organic medium amended with thiosulfate. (b) Average cell sizes when cultured in organic broth with and without thiosulfate amendment.



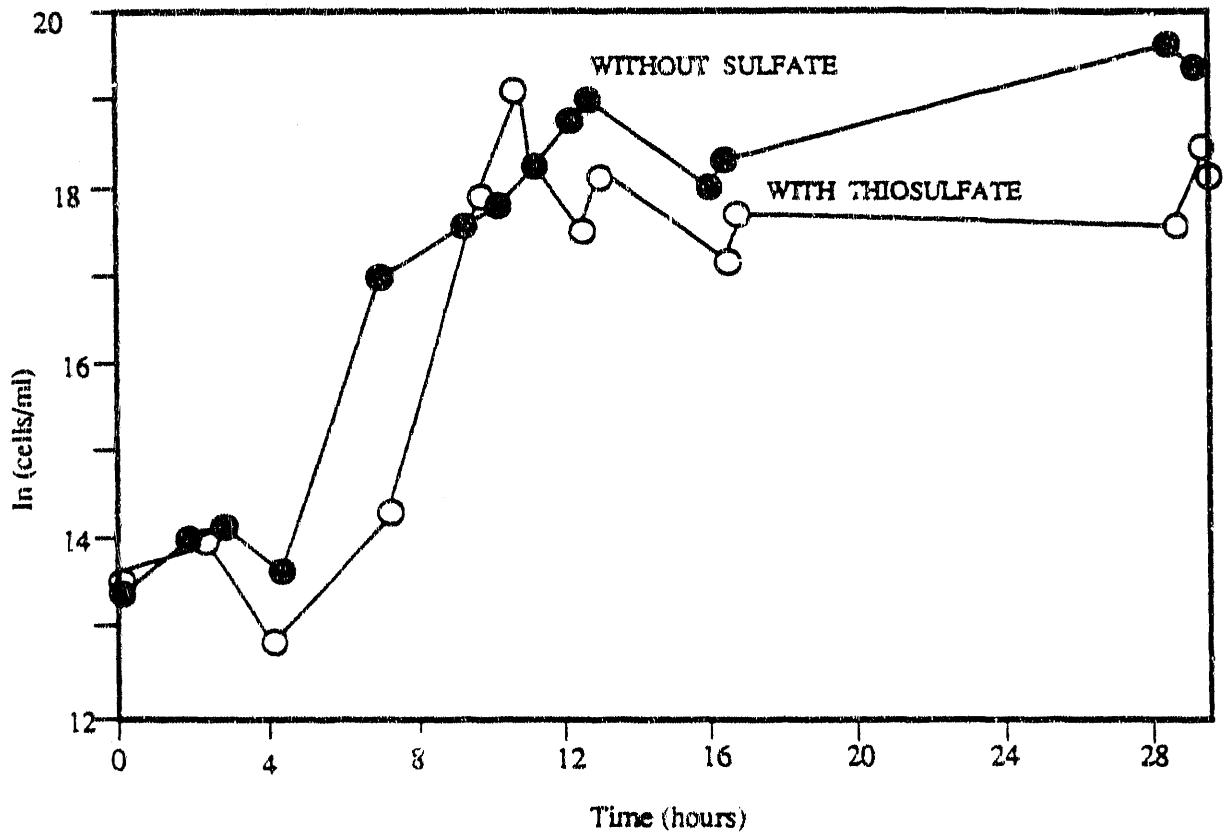
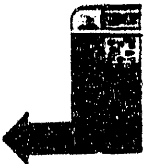


Figure 3. Growth of *Pseudomonas testosteroni* (direct microscopic counts) cultured in organic broth with and without thiosulfate amendment.



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