Effect of Water Potential on Growth and Iron Oxidation by *Thiobacillus ferrooxidans*

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Received for publication 29 November 1974

The effect of water potential on the growth of two strains of *Thiobacillus ferrooxidans* was determined by adding defined amounts of sodium chloride or glycerol to the culture medium. The two strains differed slightly, and the most tolerant strain had a minimum water potential for growth of $-15$ to $-32$ bars when sodium chloride was used and $-6$ bars when glycerol was used. In another approach, the limiting water potential was determined by equilibrating small amounts of culture medium with atmospheres of relative humidities equivalent to specific water potentials, and the ability of the organism to grow and oxidize ferrous iron was determined. Under these conditions, which are analogous to those which might control water potential in a coal refuse pile or copper leaching dump, the lower limit at which iron oxidation occurred was $-23$ bars. The water potential of some coal refuse materials in which *T. ferrooxidans* was present were determined, and it was found that the water potentials at which the organism was active in these habitats were similar to those at which it was able to grow in culture. However, marked variation in water potential of coal refuse materials was found, presumably due to differences in clays and organic materials, and some coal refuse materials would probably never have water potentials at which the organism could grow. Some literature on the water potentials in copper leach dumps is reviewed, and it is concluded that control of water potential is essential to maximize the success of leaching operations. Because adequate drainage is necessary in a leach dump to ensure sufficient aeration, in many cases water availability in leach dumps may restrict the development of the bacterium necessary for the process.

The importance of water potential in controlling the activity of microorganisms in nature is well known. Especially in matric systems such as soil and foods, water potential is often low or variable and may severely limit the growth or activity of microorganisms (7, 10, 15, 17, 19, 20). An important outcome of recent work in this area is the understanding that water content of a substrate does not itself control microbial activity, but activity is controlled by water availability, which is determined not only by water content but by osmotic and absorptive properties of the substrate. Water availability is most precisely expressed in terms of water potential (a pressure term) or by water activity (usually abbreviated $a_w$). In the soils literature, water potential rather than $a_w$ is most frequently used, and this expression will be used in the present paper.

Water potential as it applies to soil microorganisms has been reviewed most clearly and extensively by Griffin (8-10) and has also been discussed in some of the papers in the volume edited by Brown and Van Haveren (6). Briefly, water potential is an energy term and can be defined as the difference in free energy between the system under study and a pool of pure water at the same temperature. Water potential can be expressed in a number of different units, but the most widely used unit is the bar, which is equivalent to $10^4$ dyne/cm$^2$ or 0.986 atm. Water potentials of natural systems are always less than that of pure water and are expressed as negative bars. Water potential can be reduced either by adsorption of water to surfaces, in which case the water potential reduction is referred to as matric, or it can be reduced by the presence of solutes in the water, in which case the reduction is referred to as osmotic. In many cases, microorganisms show an equivalent response to matric and osmotic reduction in water potential. Osmotic reduction is more convenient to use in practice, as it merely involves the addition to the culture medium of a defined...
amount of a solute such as NaCl or glycerol; thus, osmotic water potentials are more often studied than matric potentials. To control water potentials matrically, it is necessary to equilibrate the material against an atmosphere of known relative humidity or to add or subtract water from the system experimentally. It should be emphasized that the relationship between water potential and water content varies from one system to another, depending on how tightly the components of the system bind water. Thus, a measurement of the water content of a soil will not provide a direct measurement of water potential or water availability. Water potential can only be equated with water content if a calibration curve has been prepared for each soil type.

Although considerable work has been done on the physiology and metabolism of *Thiobacillus ferrooxidans*, little ecological work has been done, and we have little understanding of the factors in nature which influence its development. This is in spite of the fact that *T. ferrooxidans* is of considerable practical importance, being primarily responsible for the development of acid-mine drainage through its ability to oxidize pyritic materials associated with coals and ores. Further, *T. ferrooxidans* is of crucial importance for the success of leaching operations used in the recovery of copper from low-grade ores, and water availability in leaching tanks may play an important role in controlling the growth and activity of the organisms and thus in the effectiveness of the leaching operation.

In some previous work from this laboratory (4), the ecology of *T. ferrooxidans* was studied in one of its natural habitats, pyrite-rich coal refuse material, using a direct radioisotope technique. In that work, it was shown that this bacterium was active in a variety of coal refuse materials, but that activity varied widely probably as a result of availability of its main natural substrate for energy generation, iron pyrite. In the previous work, moisture content of coal refuse materials was measured, but water potential was not considered. It is the purpose of the present work to examine the effect of water potential on the growth of *T. ferrooxidans* and to compare the results with the water potentials actually found in pyritic substrates. Since *T. ferrooxidans* is an obligate acidophile and requires pH values of 2 to 3, it had been considered that it might be more sensitive to reduced water potentials than common soil bacteria. However, it will be shown that *T. ferrooxidans* resembles many other bacteria in its sensitivity to reduced water potential, and that many times in its natural habitat the development of the organisms will be limited by water availability. The practical significance of these observations in promoting the growth *T. ferrooxidans* in leaching operations and in inhibiting its growth in acidogenic coal-associated materials will be discussed.

**MATERIALS AND METHODS**

**Culture methods.** The basal mineral salts medium of Allen (1), to which 10 g of filter-sterilized FeSO₄·7H₂O per liter was added, was used. The final pH was 2.5 unless otherwise noted. Incubations were at room temperature in unshaken stainless-steel-capped culture tubes or flasks. The cultures used were isolated from weathering pyritic materials in mine dumps from former lead-zinc mining operations southwest of Shullsburg, Wisconsin.

Both cultures came from moist sites deep within the piles of rock. The pH of a 1:1 slurry of material used to isolate culture 57-5 was 2.5; that for 58-1 was 3.9. Initial enrichments were set up by adding small amounts of the weathering crumbs to culture tubes. After positive growth was noted, as evidenced by ferrous iron oxidation and the formation of a ferric hydroxide precipitate, transfers were made, and pure cultures were isolated by a most-probable-number method. Although not characterized in detail, the organisms seemed to be typical of *T. ferrooxidans*, consisting of small, actively motile rods. After initial isolation, cultures were maintained by weekly transfers.

**Water potential control.** The principles of osmotic and matric control of water potential have been discussed by Griffin (10). In the present work, osmotic control of water potential was effected by adding sodium chloride or glycerol to the standard culture medium. The actual water potentials were calculated using freezing-point depression data, following the formula given by Lang (13): water potential (joules/kilogram) = \((1,332) \times (\text{freezing-point depression})\). Freezing-point depression data were obtained from tables in the *Handbook of Chemistry and Physics* (21). Following standard practice (6), water potentials are expressed in this paper as bars (1 bar = 100 J/kg).

For matric control of water potential, the agar plate method of Harris et al. (11) was used. Solutions of sodium chloride or glycerol corresponding to desired water potentials were prepared and agar was added; after autoclaving petri plates were poured. Materials (soil for the data in Fig. 1 or the culture medium for the data in Table 3) whose water potentials were to be adjusted were placed in the lids of inverted plates and allowed to equilibrate in a foam-capped chamber at room temperature for 4 days. In earlier work (19), preliminary experiments had shown that this time period was sufficient for equilibration of small amounts of soil or other materials.

**Growth and iron oxidation on cover slips.** With an Eppendorf pipette, 100-μl amounts of Allen's medium at pH 2 containing 10 g of FeSO₄·7H₂O per liter were placed on cover slips. The initial ferrous iron amount per cover slip was around 16 to 20 μg, well
within the sensitivity of the phenanthroline assay. The cover slips were then equilibrated with atmospheres of known relative humidities which were equivalent to specific water potentials using the petri plate method of Harris et al. (11). The plates containing the cover slips were equilibrated for 24 h in an insulated chest, and then each medium droplet was inoculated with approximately 1 μl of culture, using a platinum loop. The plates were quickly sealed and returned to the foam chest. After 5 and 16 days, some of the cover slips were removed and assayed for ferrous iron. Each cover slip was placed in a beaker with 7 ml of water, and ferrous assays were performed on 0.1-, 0.9-, and 6-ml volumes.

Other methods. The phenanthroline method of the American Public Health Association (2) was used to assay for ferrous iron. Moisture contents of soils and coal refuse material were determined by drying at 105°C overnight. All water contents are expressed as percent of water of oven-dry soil.

**RESULTS**

Osmotic water potential. Experiments were performed to determine the effect of osmotic water potential on growth of *T. ferrooxidans*. Table 1 shows results obtained in initial experiments when sodium chloride was used to control the water potential. The water potentials given are only approximate, since they do not take into consideration the water potential contributed by the medium ingredients. The two main medium ingredients affecting water potential are g/liter, used to adjust pH, and FeSO₄·7H₂O. The concentration of the former is less than 0.01 N and will contribute less than 0.5 bars. The concentration of the latter was generally 10 g/liter, which will contribute around 3 bars (calculated from freezing-point depression data for FeCl₃, given in the Handbook of Chemistry and Physics [21]), but when 44 g/liter was used (second experiment in Table 1) the contribution was about 12 bars. This added water potential contribution from the higher concentration of ferrous sulfate probably explains the lower tolerance to NaCl seen in the second experiment in Table 1 for strain 59-1.

As seen, the two strains showed minor differences in sensitivity, strain 57-5 tolerating somewhat lower water potentials. In the second experiment, where closer intervals were used, the lowest water potential for strain 57-5 was −32 bars (including the ferrous contribution), no growth occurring at −36 bars. This value is similar to that of a number of other common soil and water bacteria (17). It should be noted that the salinity of seawater corresponds approximately to the water potential at which *T. ferrooxidans* is unable to grow.

Because of the possible toxicity of sodium chloride, these experiments were repeated using glycerol, another common water potential-controlling agent. Sensitivity to glycerol was even greater than to sodium chloride, the limiting water potential at which growth occurred being about −6 bars for strain 57-5 (Table 2). Again, strain 59-1 was somewhat more sensitive.

**Controlling water potential without adding anything to the medium.** Because of the concern that salt or glycerol might be affecting the organism in a way other than by altering the water potential, experiments were set up using the cover slip procedure, in which the water potential was varied without adding anything to the medium. The equilibration resulted in a concentration of the culture medium to amounts equivalent to the water potential of the controlling humidity. In effect, this method results in an osmotic control of water potential using the dissolved ingredients of the medium as regulatory agents. This method is analogous to a way in which water potential might be controlled in a coal refuse pile or copper leach dump if the water percolating through the pile.

| NaCl (g/liter) | Water potential (bars) | Strain 57-5 | Strain 59-1 |
|---------------|------------------------|-------------|-------------|
|               | Due to NaCl            | Total       | Total       |
| Expt 1        |                        |             |             |
| 100           | −82                    | −85         |             |
| 80            | −66                    | −69         |             |
| 50            | −40                    | −43         |             |
| 20            | −15                    | −18         | +           |
| 10            | −7                     | −10         | +           |
| 5             | −4                     | −7          | +           |
| 2             | −2                     | −5          | +           |
| 0             | 0                      | −3          | +           |
| Expt 2        |                        |             |             |
| 80            | −66                    | −78         |             |
| 50            | −40                    | −52         |             |
| 45            | −36                    | −48         |             |
| 40            | −32                    | −44         |             |
| 35            | −28                    | −40         |             |
| 30            | −24                    | −36         |             |
| 25            | −20                    | −32         | +           |
| 10            | −8                     | −20         | +           |
| 5             | −4                     | −16         | +           |
| 0             | 0                      | −12         | +           |

* All experiments were done with replicate tubes, which showed the same results. The incubation period was 2 weeks. In the first experiment, 10 g of FeSO₄·7 H₂O per liter was used; in the second experiment, 44 g/liter was used. The latter concentration is equivalent to that used in the 9 K medium of Silverman and Lundgren (18). +, Visible iron oxidation and microscopically visible growth; −, no iron oxidation or microscopically visible growth.
TABLE 2. Effect of osmotic water potential (glycerol) on growth of T. ferrooxidans*

| Glycerol (g/liter) | Total water potential (bars) | Strain 57-5 | Strain 59-1 |
|-------------------|----------------------------|-------------|-------------|
| 184               | -61                        | -           | -           |
| 147               | -49                        | -           | -           |
| 92                | -32                        | -           | -           |
| 74                | -26                        | -           | -           |
| 55                | -20                        | -           | -           |
| 37                | -15                        | -           | -           |
| 18.4              | -8.8                       | +           | -           |
| 9.2               | -6                         | +           | +           |
| 3.7               | -4.2                       | +           | +           |
| 0                 | -3                         | +           | +           |

*All experiments were done with replicate tubes which showed the same results. The incubation period was 3 weeks. Iron concentration in the medium, 10 g of FeSO₄·7H₂O per liter. +, Visible iron oxidation and microscopically visible growth; -, no iron oxidation or microscopically visible growth.

evaporates until the liquid is in equilibrium with the atmosphere of the pile.

The lowest water potential causing complete inhibition of ferrous iron oxidation was -32 bars, although partial inhibition occurred at -23 bars, as evidenced by the fact that no iron oxidation occurred at this water potential after 5 days of incubation, but oxidation was found after 16 days of incubation (Table 3). These results are similar to those found above when sodium chloride was used to control water potential, but the organism seems able to tolerate a somewhat lower water potential when controlled by the ingredients of the culture medium rather than with sodium chloride or glycerol.

**Water potential of coal refuse materials.**

Belly and Brock (4) had used a radioisotope method to measure the activity of T. ferrooxidans directly in pyritic materials associated with coal. During that work, moisture contents of all of the samples studied were determined. However, there is no simple relationship between moisture content and water potential, since variations in clay minerals and other substances with strong binding properties for water can greatly influence water potential. Two soils may have the same water content but may differ markedly in water potential. To determine water potential from moisture content, a calibration curve for each soil is necessary. Consequently, in the present work calibration curves were determined for several of the coal refuse soils which had been studied by Belly and Brock (4). These soils had been stored in sealed plastic bags for about 1 year and presumably had not changed significantly in moisture-binding capacity.

Some typical calibration curves of coal refuse materials are shown in Fig. 1. Quite wide variations are seen, presumably due to differences in clay and other moisture-binding materials present. These data emphasize the importance of knowing water potential rather than moisture content, because in two of these soils (165-1 and 192-1) quite low water potentials occur even at relatively high water contents. Thus, at 40% water, both of these soils have water potentials lower than -20 bars, whereas at the same water content two other soils have water potentials higher than -5 bars. On three of these soils, activity of T. ferrooxidans had been measured by the radioisotope method, and viable counts had been made by a most-probable-number method. The results (Table 4) show that in those soils in which high numbers and significant ¹⁴CO₂ incorporation had occurred the native water potentials were in the range found suitable for the organism in the present work. In the one soil with a low water potential, activity of the organism was low and most-probable-number counts were low.

**DISCUSSION**

The influence of water potential on the

**TABLE 3. Effect of osmotic water potential on ferrous iron oxidation by T. ferrooxidans using the culture medium itself to control water potential**

| Water potential (bars) | 57-5 | 59-1 |
|------------------------|------|------|
|                       | 5 days | 16 days | 5 days | 16 days |
| -82                    | -     | -     | -     | -     |
| -67                    | -     | -     | -     | -     |
| -40                    | -     | -     | -     | -     |
| -32                    | -     | -     | -     | -     |
| -23                    | +     | -     | +     | -     |
| -15                    | -     | +     | +     | +     |
| -7                     | +     | +     | +     | +     |
| -4                     | +     | +     | +     | +     |
| 0                      | +     | +     | +     | +     |

*Growth took place on cover slips containing medium which had been equilibrated with relative humidities equivalent to various water potentials. +, Ferrous iron oxidation occurred, resulting in a decrease from 16 to 20 µg of ferrous iron per cover slip to 1 µg per cover slip or less; -, no ferrous iron oxidation. Uninoculated controls never showed ferrous iron oxidation. Duplicate cover slips showed similar results. See text for procedure for equilibrating cover slips containing culture medium to specific water potentials.
growth and function of soil bacteria has been reviewed by Scott (17), Griffin (10), and Dommergues and Mangenot (7). In general, it can be stated that soil bacteria are more sensitive to reduction in water potential than are fungi, although there is considerable variation from species to species. Among the bacteria, the actinomycetes are able to function best at low water potentials, although the gram-positive cocci also have some tolerance to low water potentials. Although it is generally considered that -15 bars is the lowest limit at which many soil bacteria can develop, certain organisms can develop, although slowly, at lower water potentials. However, only among the fungi do organisms exist which have optima below -15 bars (e.g., the fungus Xeromyces bisporus; 17). According to Dommergues and Mangenot (7), nitrogen fixation by Azotobacter ceases at about -10 bars, and nitrification of ammonia generally ceases at -15 bars, although it was found to persist, but slowly, in certain arid West African soils at -50 bars. Very little work has been done on sulfur oxidation in soil, but Moser and Olson (16) found sulfur oxidation in a Kansas soil to be optimal at -0.03 to -0.06 bar matric and to cease completely at -15 bars. Since our data show that T. ferrooxidans is able to grow and oxidize ferrous iron at water potentials of -15 to -20 bars, this organism is not especially unusual and could be considered to respond to water availability in a manner similar to that of many other soil bacteria. Thus, the fact that T.

**Fig. 1. Relationship between moisture content and water potential for four coal refuse materials from southern Indiana coal fields. Approximately 1-g amounts of soil were placed in the lids of petri plates containing water-potential-controlling agar (see reference 11). After 2 days, the agar was changed, and equilibration was allowed to proceed for another 2 days. The moisture contents of the equilibrated soil samples were then determined. Values are percent of water of oven-dry soil. Coal refuse samples were collected by Belly (see ref. 4) and had been stored in sealed plastic bags for about 1 year before the study was performed.**

| Sample | pH | Native moisture content (%) | Calculated water potential (bars) | CO$_2$ uptake (counts/min per g) | Viable count (most probable no./g of soil) |
|--------|----|----------------------------|----------------------------------|---------------------------------|------------------------------------------|
| 163-2  | 2.10 | 15.3                     | -13                              | 10,607                          | 1.5 x 10$^4$                           |
| 162-2  | 2.20 | 27.0                     | -8                               | -                                | -                                         |
| 165-1  | 1.40 | 5.4                      | >-90                             | 2,405                           | 9.3 x 10$^4$                           |
| 192-1  | 2.15 | 35.7                     | -29                              | 19,069                          | 9.3 x 10$^4$                           |

*Data of R. T. Belly (unpublished) and Belly and Brock (4). Calculated water potentials are from native moisture contents using the calibration curves in Fig. 1. Radioisotope uptake data were determined using the method of Belly and Brock (4); incubation times, 18 to 24 h.

*No data were obtained on this sample, but other samples with similar characteristics showed high values, similar to that of 192-1.

ferrooxidans is an obligate acidophile does not seem in itself to affect its ability to grow and function at reduced water potential.

When we compare the limiting water potential of T. ferrooxidans with the water potentials in its natural environment, it can be seen that under some conditions natural water potentials in otherwise suitable environments are lower than those at which this bacterium can grow. This is shown specifically for soil 156-1 (Table 4), but is almost certainly the case in other soils associated with pyritic materials. As
seen from the soil moisture-water potential curves in Fig. 1, there is marked variation in these pyritic soils in water content at equivalent soil moistures. This is likely because of the wide variation in organic materials and clays which occur in these soils. Although there are many clay minerals in the initial coal refuse or pyritic material at the mine face, further clay mineral synthesis probably occurs by the breakdown of feldspars and other rock-forming minerals as a result of acid attack, the acid arising as a result of the action of T. ferrooxidans on the pyritic materials of the coal. The importance of acid conditions in accelerating the conversion of silicates to clay minerals has been reviewed by Loughnan (14). Thus, by oxidizing pyritic materials to sulfuric acid, T. ferrooxidans in effect promotes the development of conditions (a clay-rich environment) in which the moisture necessary for its own development will be less available. Conceivably, the low activity of T. ferrooxidans reported by Belly and Brock (4) in some old, extensively weathered coal refuse materials might have been due to the low water potential of these materials as a result of conversion of all of the silicate minerals into clays, although other factors such as pH, availability of nutrients, or energy sources could also be responsible. However, it seems almost certain that some coal refuse materials would probably never have water potentials at which the organism could grow, especially in regions where rainfall was limited.

The present study is also of significance in developing means for optimizing the activity of T. ferrooxidans in copper leach dumps. In such dumps, acid is allowed to percolate through the pile of low-grade ore, and the bacteria develop and oxidize the sulfide minerals, liberating soluble copper sulfate (3). Because of the aerobic nature of the process, it is essential that considerable pore space be maintained within the pile, which means that the leach dump cannot be flooded with leach solution. However, if the leach rate is to be maintained so that air space remains, water availability will likely be restricted, thus inhibiting the growth of T. ferrooxidans. There are two studies on the copper-leaching operation at the Chino mine, Santa Rita, New Mexico, which relate to this situation. Kennedy and Stahl (12) determined with a pressure plate method the water potential of Chino leach dump material as a function of water content. Although they did not extend their analyses to water potentials much below 15 to 20 lb/in² (equivalent to about −1 to −2 bars), the shapes of the curves they did obtain suggest that the leach dump material holds water very tightly, so that rather low water potentials will obtain even at rather high water contents (50% or higher). Further, these workers showed that there was considerable variability in the shape of the water content-water potential curves, depending upon the size of the particles. The fine material held water much tighter than did the coarse material, as would be expected. In another study at the same mine, Bhappu et al. (5) determined the number and activity of T. ferrooxidans at different levels within the leach dump and related this to the water content of the material. Except for samples taken just at the surface of the dump, all of the samples had water contents of 10% or less, and bacterial activity and numbers were very low. From the data of Kennedy and Stahl (12) it can be concluded that the water potentials where T. ferrooxidans was active were all above −15 bars, whereas those where bacterial activity was low or undetectable were considerably lower, probably in the range of −50 to −100 bars. These results agree with the data provided in the present paper and suggest that an important parameter in controlling the success of a leaching operation is the water potential of the environment in which the bacteria are growing.

In conclusion, T. ferrooxidans resembles many other common soil bacteria in requiring a fairly high water potential for its activity. This finding is of considerable practical importance in controlling the activity of this organism in habitats generating acid-mine drainage and in promoting its activity in copper leaching dumps. Additionally, because the oxidation of sulfide minerals by this organism and its close relative, T. thiooxidans, is an important biogeochemical process in nature, further studies on the effect of water availability on potential or actual habitats of these bacteria would be desirable to be able to better predict their distribution and activity in nature. Because increased water availability restricts the entry of air which is so necessary for the development of this obligate aerobe, and because conditions promoting best aeration will lead to restricted water availability, many habitats which might otherwise be suitable for the development of these bacteria might be essentially uncolonized.

ACKNOWLEDGMENTS

This work was supported by a research grant from the National Science Foundation (GB-35046).

The technical assistance of Charlene Knaack is gratefully acknowledged.

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