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# Mechanisms for Soil Moisture Effects on Activity of Nitrifying Bacteria

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**Moisture may limit microbial activity in a wide range of environments including salt water, food, wood, biofilms, and soils. Low water availability can inhibit microbial activity by lowering intracellular water potential and thus reducing hydration and activity of enzymes. In solid matrices, low water content may also reduce microbial activity by restricting substrate supply. As pores within solid matrices drain and water films coating surfaces become thinner, diffusion path lengths become more tortuous, and the rate of substrate diffusion to microbial cells declines. We used two independent techniques to evaluate the relative importance of cytoplasmic dehydration versus diffusional limitations in controlling rates of nitrification in soil. Nitrification rates in shaken soil slurries, in which  $\text{NH}_4^+$  was maintained at high concentrations and osmotic potential was controlled by the addition of  $\text{K}_2\text{SO}_4$ , were compared with rates in moist soil incubations, in which substrate supply was controlled by the addition of  $\text{NH}_3$  gas. Comparison of results from these techniques demonstrated that diffusional limitation of substrate supply and adverse physiologic effects associated with cell dehydration can explain all of the decline in activity of nitrifying bacteria at low soil water content. However, the relative importance of substrate limitation and dehydration changes at different water potentials. For the soil-microbial system we worked with, substrate limitation was the major inhibiting factor when soil water potentials were greater than  $-0.6$  MPa, whereas adverse physiological effects associated with cell dehydration were more inhibiting at water potentials of less than  $-0.6$  MPa.**

As soils undergo evaporative drying, the soil solution becomes more concentrated. In order for soil microorganisms to prevent plasmolysis and maintain cell integrity, they must increase intracellular solutes to concentrations slightly greater than extracellular concentrations (3). Microorganisms create high internal solute concentrations either by producing compatible organic solutes or by taking up ions from the extracellular solution (4). High intracellular solute concentrations inhibit enzyme activity because the resulting low water potential reduces the degree of hydration of enzymes, which may change enzyme conformation (4, 10, 14). In addition, the solutes used in the cell to balance internal and external water potentials may have inhibitory effects due to interference with specific biochemical processes (2); however, these specific ion toxicities are difficult to distinguish from the direct effects of low intracellular water potential on enzyme hydration and activity.

Soil drying may also reduce substrate supply to microbial cells. As soil pores drain and water films on soil surfaces become thinner, substrate molecules must follow a more tortuous path in diffusing to cells (11, 12). This effectively increases the resistance to diffusive flow and reduces the substrate flux to the cell surface.

Although the potential importance of diffusion and dehydration effects have been recognized for a number of years (5, 6, 8), no study that we are aware of has measured the relative importance of these two factors in limiting microbial activity at different soil water potentials. One reason may be that to separate the role of the two factors, substrate supply and water potential must be uncoupled from water content and allowed

to vary independently. This is difficult to accomplish since in soil systems all three variables are normally interdependent.

Our objective was to develop techniques to separate the effects of low water potential and substrate supply and thereby determine the relative importance of cell dehydration versus diffusional limitations in controlling microbial activity. To accomplish this objective, we used nitrifying (ammonium-oxidizing) bacteria in soil as a model system and created two distinct methods of uncoupling substrate supply and water potential from soil water content. This system has three distinct advantages over other systems. First, there is only one substrate for energy generation ( $\text{NH}_4^+$ ), and thus variable diffusion rates of substrates are not a concern. Second, microbial activity can be quantified relatively easily by measuring rates of product formation ( $\text{NO}_2^-$  and  $\text{NO}_3^-$ ). Third, the substrate for nitrification can be supplied through the gas phase (as  $\text{NH}_3$ ). Since in dry soils the volume of gas is greater than the volume of liquid, and since diffusion coefficients are considerably higher in gases than in liquids (12), addition of  $\text{NH}_3$  gas should relieve the diffusional limitation of substrate supply that results from soil drying.

We also used this system because there is considerable interest in how nitrification rates are controlled by soil moisture. Nitrification can lead to increased leaching of N, resulting in N loss, acidification of soils, and pollution of groundwaters; or it can result in increased production of trace N gases (either directly or by supplying substrate for denitrification), which results in N loss and adverse effects on atmospheric ozone concentrations and radiative forcing (16). In many ecosystems, moisture is one of the most important factors controlling nitrification rates (9).

## MATERIALS AND METHODS

The soil used in all experiments was a silt loam collected from the 0 to 9-cm layer of a California oak woodland-annual grassland ecosystem. This soil had a

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pH (1:1, soil/water) of 6.1 and total carbon and nitrogen concentrations of 4.9 and 0.34%, respectively. Soil was collected during summer in an air-dry state, sieved (<2-mm grain size), and stored at 5°C until experiments could be performed (approximately 14 days). Water potentials of all solutions, slurries, and soil samples were measured by using the dew point mode of a Wescor HR-33T Dew Point microvoltmeter (Wescor Inc., Logan, Utah). Soil gravimetric water content was determined by oven drying samples at 110°C for 48 h.

The first method was designed to evaluate how much nitrification rates decline from changes in water potential alone. Soil slurries with different water potentials were prepared by placing approximately 10 g of soil in 250-ml Erlenmeyer flasks with 100 ml of solution. The solutions contained 1 mM potassium phosphate and sufficient  $K_2SO_4$  to create 18 different osmotic potentials ranging from  $-0.01$  to  $-3.2$  MPa (approximately 0 to 0.67 M  $K_2SO_4$ ). We chose  $K_2SO_4$  as the osmolyte because  $K^+$  and  $SO_4^{2-}$  are common ions in many dry soils, and these ions have relatively low specific ion toxicities (13). Sufficient  $(NH_4)_2SO_4$  was also added to the solutions to create slurry concentrations of approximately 0.5 mM  $NH_4^+$  (70 mg of N kg of soil $^{-1}$ ). Preliminary experiments showed that in slurries with high  $K_2SO_4$  concentrations adsorbed  $NH_4^+$  was released into solution, and less  $(NH_4)_2SO_4$  was required to create 0.5 mM  $NH_4^+$ . The actual amount of added  $(NH_4)_2SO_4$  ranged from 1 ml of 50 mM  $(NH_4)_2SO_4$  for slurries with no  $K_2SO_4$  to 0.1 ml for slurries nearly saturated with respect to  $K_2SO_4$ . The slurries were shaken for 1 h at 180 rpm on an orbital shaker, and then each slurry was adjusted to pH 6.3 with 5% KOH.

Subsamples (10 ml) of the slurries were removed after 6, 8, 24, and 31 h of shaking. Each subsample was centrifuged, and the supernatant was collected and stored frozen until it could be analyzed. Nitrate and nitrite concentrations were determined colorimetrically by a Lachat flow injection autoanalyzer (Lachat Chemicals, Inc., Mequon, Wis.). Nitrification rates were estimated by measuring the linear increase in  $NO_2^-$  plus  $NO_3^-$  during the incubation period.

In these slurried samples, substrate was supplied in excess and substrate supply was independent of water potential. Therefore, changes in nitrification rates should be a direct result of changes in water potential.

A second method was designed to evaluate how much of the decline in rates at low water potential could be attributed to substrate limitation. Soil samples were adjusted to 12 different water contents ranging from 0.4 to 0.08 kg kg $^{-1}$  ( $-0.01$  to  $-3.8$  MPa) by spraying the soil with a fine mist of deionized water and shaking the sample in a plastic bag. The moist soils were allowed to equilibrate overnight, and then soil water potential was measured as described previously. A subsample of soil was extracted in 2 M KCl (approximately 10:1 solution/soil weight), and initial  $NH_4^+$ ,  $NO_3^-$ , and  $NO_2^-$  concentrations were determined colorimetrically. The soils were spread into 5-mm-thick layers in polyethylene containers (18 by 10 by 5 cm) with snap-fit lids (Rubbermaid Inc., Wooster, Ohio). The containers were sealed and incubated at 23°C, and after 24 h a second subsample was extracted in KCl.

For a subset of samples, we increased substrate supply by injecting  $NH_3$  gas into the headspace of the containers. A preliminary experiment showed that recovery of  $NH_3$  gas in soil (as KCl-extractable  $NH_4^+$ ) was linearly related to the amount of  $NH_3$  added, up to the highest addition rate of 640 mg of  $NH_3$ -N kg of soil $^{-1}$ . Recovery was 78% for soils at  $-0.3$  and  $-0.9$  MPa and 69% for soil at  $-3.0$  MPa. On the basis of these results, either 0, 4, 8, 12, 16, or 20 ml of  $NH_3$  was injected through rubber septa in the sides of plastic containers of soil adjusted to the same 12 different water potentials described previously. These amounts of  $NH_3$  were selected to increase soil  $NH_4^+$  concentrations by approximately 0, 20, 40, 60, 80, or 100 mg of N kg of soil $^{-1}$ , respectively.

In these moist soil incubations, nitrification rates were calculated from increases in  $NO_3^-$  and  $NO_2^-$  concentrations during the 24-h incubation. The occurrence of nitrate consumption in the soil samples was checked by isotopically labelling the  $NO_3^-$  pool with  $^{15}N$ -nitric oxide (NO) gas and then monitoring disappearance of the  $^{15}N$  from the  $NO_3^-$  pool during the 24-h incubation. Use of  $^{15}NO$  gas allows labelling of the  $NO_3^-$  pool without addition of water (15). The  $^{15}N$  data showed that  $NO_3^-$  consumption was not significant in any of the samples, and thus  $NO_3^-$ -plus- $NO_2^-$  accumulation was a suitable measure of nitrification rates.

Supplying substrate to nitrifying bacteria through the gas phase allowed us to bypass diffusional bottlenecks created by low soil water contents and to effectively uncouple substrate supply from water content and water potential. Increased activity of nitrifiers following exposure of the moist soils to  $NH_3$  would indicate that the nitrifiers were substrate limited. Since the slurry method allows direct measurement of adverse physiological effects associated with cell dehydration, and the moist soil incubations allow direct measurement of substrate limitation, the relative importance of each factor can be determined. In addition, because each factor is directly measured rather than estimated by difference, we were able to determine if these factors explain all of the decline in activity that accompanies soil drying or if other factors must be identified.

## RESULTS

Lowering water potentials in slurries by adding  $K_2SO_4$  resulted in an exponential decline in nitrification rates (Fig. 1). The relationship fit the following equation:  $k = 15.4 e^{0.58\psi}$ , where  $k$  is the nitrification rate in mg of N kg $^{-1}$  day $^{-1}$  and  $\psi$

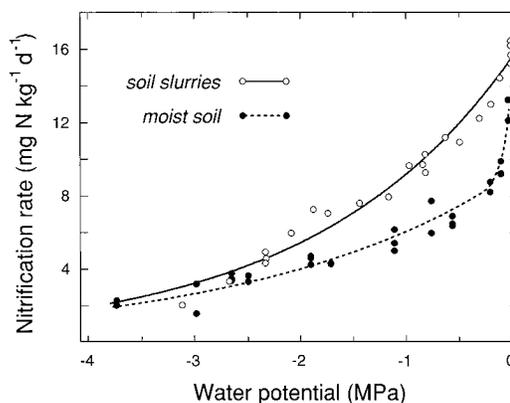


FIG. 1. Effect of water potential on nitrification rates in shaken soil slurries and in moist soil samples. In soil slurries,  $NH_4^+$  was supplied in excess and water potential was controlled by varying the  $K_2SO_4$  concentration.

is the water potential in MPa ( $r^2 = 0.958$ ). In the moist soil incubations, however, nitrification rate had a very different relationship to water potential (Fig. 1). At water potentials of greater than  $-0.1$  MPa, nitrification rates were relatively high, but as the water potential was lowered to  $-0.2$  MPa, rates declined more rapidly than an exponential curve would predict. At water potentials below  $-0.2$  MPa, however, rates in the moist soils followed an exponential decline. At very low water potentials ( $< -2.5$  MPa), there was little difference between nitrification rates in the moist soil samples and in the slurries, while at moderate to high water potentials ( $> -2.0$  MPa), rates in the moist soils were substantially lower than rates in the slurries.

Addition of  $NH_3$  increased nitrification rates in all moist soil incubations (Fig. 2), and in general, the highest  $NH_3$  concentrations resulted in the highest rates. Mean soil  $NH_4^+$  concentrations during the 24-h incubation ranged from 6 mg of N kg $^{-1}$  in unamended samples to 93 mg of N kg $^{-1}$  in samples receiving the highest  $NH_3$  additions. Addition of  $NH_3$  stimulated rates such that the highest rates in the moist soil incubations were not significantly different from rates in the slurries ( $P > 0.05$ ).

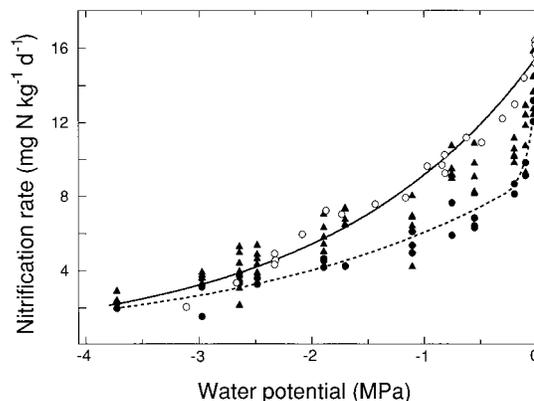


FIG. 2. Effect of water potential on nitrification rates in soil slurries and in moist soil after supplying  $NH_3$  through the gas phase. Symbols:  $\circ$ , rates in soil slurries;  $\bullet$ , rates in moist soil;  $\blacktriangle$ , rates in moist soil after exposure to various amounts of  $NH_3$  gas. For a given water potential, the highest  $NH_3$  concentrations generally correspond to the highest nitrification rates.

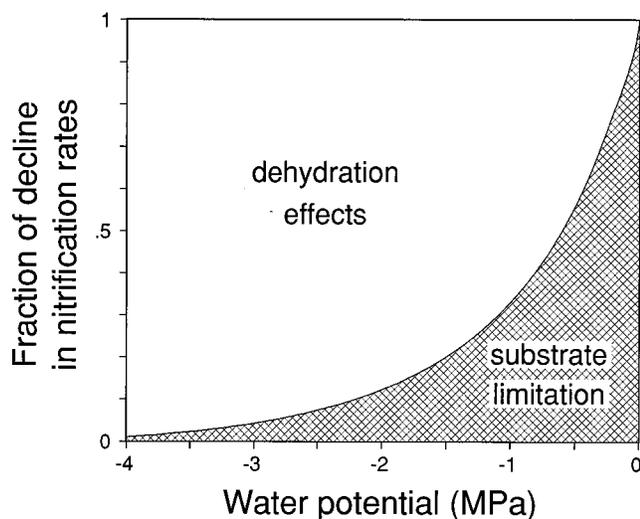


FIG. 3. Proportion of decline in nitrification rates in moist soil due to either substrate limitation or adverse physiologic effects of cell dehydration. At each water potential, the decline attributable to dehydration effects was calculated from the difference between the maximum rate (the mean of rate measurements in slurries at  $-0.01$  MPa) and the rate in slurries at the lower water potential (indicated by the solid line in Fig. 1). The decline due to substrate limitation was calculated from the difference between rates in the slurries and rates in the moist soils (i.e., the difference between the solid line and the dashed line in Fig. 1). These values were expressed as a fraction of the total decline in moist soil (the maximum rate minus rates indicated by the dashed line in Fig. 1).

### DISCUSSION

Results from the slurry incubations indicate that adverse physiologic effects associated with cell dehydration cause a 75% decline in rates at  $-2.7$  MPa and a 25% decline in rates at  $-0.5$  MPa (relative to rates at  $-0.1$  MPa). The declines in moist soil at these water potentials, however, were 79 and 49%. Therefore, dehydration effects account for almost all of the decline in rates at  $-2.7$  MPa but for only a portion of the decline at  $-0.5$  MPa. The  $\text{NH}_3$  addition experiment showed that this additional decline in rates seen in the moist soils could be eliminated by increasing substrate supply. Therefore, essentially all of the decline in nitrification rates can be accounted for by either dehydration effects or substrate limitation.

The relative importance of the two factors changes at different water potentials. This can be seen by calculating for various water potentials the decline due to either dehydration effects (i.e., the difference between the maximum rate, at  $-0.1$  MPa, and rates in the slurries) or substrate limitation (i.e., the difference between the rates in the slurries and the rates in the moist soil) and expressing this as a fraction of the total decline in the moist soil (i.e., the difference between the maximum rate and rates in the moist soil). These values are plotted as a function of water potential in Fig. 3. At water potentials of greater than  $-0.6$  MPa, substrate supply was the most important factor controlling nitrification rates, whereas at water potentials of less than  $-0.6$  MPa, dehydration effects were most important.

The relationship shown in Fig. 3 will undoubtedly change for different soil types and microbial communities, depending on soil water potential-water content relationships, concentration and diffusion characteristics of important substrates, and the relative tolerance of the microorganisms to dehydration stress. For example, at a given water potential, coarse-textured soils have lower water contents than fine-textured soils, and thus diffusional limitations should be more severe in coarse-textured soils. For microbial populations subsisting on high-mo-

lecular-weight substrates, or other compounds with low diffusion coefficients, substrate diffusion should be more limiting at low water potentials. Likewise, for xerotolerant microbial populations such as many fungi, the adverse physiological effects of cytoplasmic dehydration should be less severe, and substrate diffusion may represent the primary limiting factor at lower water potentials. The filamentous nature of fungi and actinomycetes, however, may provide a benefit in dealing with diffusional limitations by allowing them to exploit microsites isolated by thin or discontinuous water films (1, 18).

In this study, the adverse physiologic effects associated with low water potential were measured by lowering the osmotic potential in soil slurries. Although low water potential in soil is usually due to low matric potential rather than low osmotic potential, in theory either type of potential will have the same effect on intracellular water activity (7), and thus the physiologic effects should be equivalent. There are some additional differences between conditions in slurry and moist soil incubations, however, that should be acknowledged. Although the water potential in dry soil is dominated by matric forces, there is also an osmotic component, since the soil solution contains a wide variety of dissolved compounds. As the soil solution becomes more concentrated because of soil drying, many of the compounds become insoluble and precipitate, leaving only highly soluble ions such as  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{NH}_4^+$ ,  $\text{Cl}^-$ ,  $\text{NO}_3^-$ , and  $\text{SO}_4^{2-}$  and low-molecular-weight organic compounds. In contrast, the composition of the slurry solution used in this study was dominated by  $\text{K}^+$  and  $\text{SO}_4^{2-}$ , with relatively small amounts of the other species. To the extent that ions more toxic than  $\text{K}^+$  dominate in soil solutions, the slurry will underestimate the dehydration effects that actually occur. To the extent that less toxic ions dominate (such as some organic compounds), the slurry will overestimate dehydration effects. These differences may explain slight, but nonsignificant, differences between rates in the slurries and rates in  $\text{NH}_3$ -augmented soils at very low water potentials (Fig. 2).

Another difference between conditions present in the slurry and in moist soil is that  $\text{PO}_4^{3-}$  is supplied in excess in the slurry. If the  $\text{PO}_4^{3-}$  supply limited activity of nitrifiers in soil, then slurry rates would be higher than those obtainable in moist soil. In fact, the high water content of the slurry should increase diffusional supply of all nutrients, not just  $\text{NH}_4^+$ . If other nutrients limited nitrification rates in this study, however, addition of  $\text{NH}_3$  gas would not have increased rates in moist soil to slurry values. Therefore, nutrients other than  $\text{NH}_4^+$  do not appear to limit nitrification rates in this soil.

In addition to increasing  $\text{NH}_4^+$  concentrations in the soil, one of the effects of  $\text{NH}_3$  addition is to raise the soil pH slightly. The highest rates of  $\text{NH}_3$  addition resulted in pH increases of 0.2 to 0.4 U. The pH of the soil slurries (6.3) was higher than that of the unamended moist soil (6.1), however, and increasing the pH of the moist soil by  $\text{NH}_3$  addition made the pH, at most, 0.1 to 0.2 U higher than in the slurries. In addition, one of the primary effects of increasing pH on nitrification is to increase the amount of  $\text{NH}_3(\text{aq})$  relative to  $\text{NH}_4^+$  (17). Since  $\text{NH}_3$  is considered to be the actual substrate utilized by nitrifying bacteria, the addition of  $\text{NH}_3$  gas probably increased substrate supply both by increasing the total concentration of  $\text{NH}_4^+$  plus  $\text{NH}_3$  and by changing the ratio of the two species.

Although in this discussion we have considered the effects of substrate limitation and cell dehydration to be distinct, there may be interactions between the two factors. For example, the ability of a microorganism to produce compatible solutes may be dependent on the supply of some external resource (e.g., energy-supplying substrates). If this is the case, then reduced

soil water contents would restrict supply of the resource by lowering diffusion rates and prevent the microorganism from synthesizing compatible solutes. The microorganism might then suffer from increased specific ion toxicities because of diffusional limitations. A decline in microbial activity caused by this interaction could be categorized either as dehydration effects or as substrate limitation effects. In this study, however, if nitrifying bacteria were unable to produce compatible solutes because of insufficient energy, addition of  $\text{NH}_3$  would allow compatible solute production and thus reduce specific ion toxicities. Therefore, this interaction would be considered one of the effects of substrate limitation.

The general paradigm discussed here should be applicable to a wide variety of solid matrices in addition to soils. Since diffusion rates are a function of water content whereas dehydration effects are a function of water potential, the water content-water potential relationship of each matrix will effect the relative importance of diffusion and dehydration effects. Although water content-water potential relationships differ for each matrix, many show curvilinear relationships in which water content declines rapidly at high water potentials and slowly at low water potentials. For these matrices, diffusion of substrates will limit microbial activity most at high water potentials, whereas the adverse physiologic effects associated with cell dehydration will be the most limiting factor at low water potentials. In addition, because the two factors interact, high substrate concentrations may at least partially offset the adverse effects of low water potential.

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