THE INFLUENCE OF OXYGEN ON NITRATE AND NITRITE REDUCTION

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Since the classical experiments of Gayon and Dupetit (1886), it has been known that oxygen inhibits the reduction of nitrate and the formation of nitrogen by denitrifying bacteria. Several investigators have studied the effect of oxygen on denitrification by more or less qualitative methods, but there has been very little work of a quantitative nature showing the magnitude of the inhibition in relation to the partial pressure of oxygen during growth of the bacteria or during the denitrification process. The present investigation was undertaken to obtain quantitative data on the influence of oxygen on nitrate and nitrite reduction.

LITERATURE

Weissenberg (1897) tested the ability of three denitrifying bacteria to reduce nitrate and nitrite in shallow layers of medium exposed to air and in the complete absence of oxygen. Oxygen was eliminated from the anaerobic cultures by the use of a pyrogallol seal, a hydrogen atmosphere, or glass-stoppered bottles completely filled with the nutrient medium. Weissenberg found that complete denitrification occurred in the anaerobic cultures, whereas aerobically nitrate was reduced only as far as nitrite. Thus he demonstrated the greater sensitivity of nitrite reduction to inhibition by oxygen. However, Seiser and Walz (1925) observed a considerable nitrogen loss from nitrate-containing cultures of *Pseudomonas putida* exposed to air, though this was less than under anaerobic conditions.

Lloyd and Cranston (1930) measured the gas exchange that occurred when denitrifying cultures were grown in air or in a nitrogen atmosphere in a closed system. They observed a large nitrogen evolution under anaerobic conditions and an almost equally large oxygen uptake in air. They concluded that nitrate was only slightly attacked aerobically, although some nitrogen was lost from the medium even under their most aerobic conditions.

The first quantitative approach to the problem of nitrate reduction was made by Stickland (1931), who determined the influence of oxygen at various partial pressures on the reduction of nitrate to nitrite by cell suspensions of *Escherichia coli*. Under conditions of aeration that should have maintained an equilibrium of oxygen distribution between the liquid and gas phases, he found as little as 0.36 per cent oxygen caused a 21 per cent inhibition of nitrate reduction, 1 per cent oxygen caused approximately 50 per cent inhibition, and 3.76 per cent oxygen caused 93 per cent inhibition. A tenfold increase in nitrate concentration did not modify these results, thus demonstrating that the inhibition was noncompetitive. He found further that carbon monoxide partially relieved oxygen

inhibition of nitrate reduction and concluded that different enzymes are involved in the activation of nitrate and oxygen, since they show different affinities for their substrates and carbon monoxide.

Meiklejohn (1940) investigated the effect of oxygen on denitrification, maintaining that the notion that oxygen interferes with this process is a "neat teleological explanation" never adequately verified. Her experiments were similar to those of Seiser and Walz; using an unidentified strain of *Pseudomonas* she observed that denitrification occurred to almost the same extent in "aerated" and anaerobic cultures. The interpretation of these results is complicated by the fact that the method of aeration was certainly not adequate to keep the culture medium saturated with oxygen at atmospheric pressure. The partial pressure of oxygen in some parts of the "aerated" medium may have been very low.

Van Olden (1940) was the first investigator to apply modern manometric techniques to the study of denitrification. Using *Micrococcus denitrificans* he made the important observation that the ability of washed bacteria to produce nitrogen from nitrate is dependent upon their previous history. Bacteria that had grown anaerobically with nitrate were capable of causing rapid denitrification of nitrate under anaerobic conditions, whereas bacteria grown aerobically either with or without nitrate denitrified very slowly or not at all. Van Olden concluded that "nitrate reductase" is an adaptive enzyme in the sense of Karstrom (1937). It must be pointed out, however, that from his results it is impossible to decide which enzyme or enzymes failed to develop under conditions unsuitable for denitrification. Since nitrite was formed by some of the bacteria grown aerobically, it is quite possible that their inability to denitrify was at least partially due to the absence of a nitrite reductase or some other enzyme mediating a reaction between nitrite and nitrogen.

Lemoigne *et al.* (1946) found that when *Bacillus megatherium* was grown in a medium containing nitrate as the sole nitrogen source, a pure oxygen atmosphere greatly increased the lag period. This did not occur if there was a source of organic nitrogen in the medium or if the atmosphere contained less than 64 per cent oxygen. They concluded that oxygen arrests the mechanism involved in the assimilation of nitrate, a conclusion that seems to harmonize with the findings of Weissenberg (1897) that the reduction of nitrite is especially susceptible to the inhibitory action of oxygen.

Korochkina (1936) reported that high rH values (24 to 25) did not prevent denitrification and concluded that the process probably could not be eliminated by aeration. She observed, however, that in a medium of rH 35 the rate of reduction of nitrate to nitrite was reduced.

Korsakova (1941) recently reported that, when an organic carbon source was supplied in an amount 5 to 10 times in excess of that required to reduce the available nitrate, the reduction of the latter was as complete under "aerobic" as under anaerobic conditions. This result is difficult to interpret in view of the absence of information on the relative rates of nitrate reduction and on the precise conditions of aeration.

With the exception of the work of Seiser and Walz and of Meiklejohn, the evidence summarized above strongly indicates that oxygen has a deleterious effect on the reduction of nitrate and nitrite and that one or more of the enzymes involved in denitrification is adaptive in the sense that it is only formed in bacteria grown anaerobically in the presence of nitrate. The apparent inconsistencies in the literature may be due to differences in the behavior of different bacteria. However, a more likely explanation is variation in methods of maintaining "aerobic" conditions. Unless special precautions are taken to maintain equilibrium between the atmosphere and the culture medium, it cannot be assumed that all parts of the medium are adequately supplied with oxygen.

MATERIALS AND METHODS

The organism used in these studies was isolated from soil after enrichment in a succinate-nitrate medium. It was identified as *Pseudomonas denitrificans* (Breed *et al.*, 1948). The original culture was kept at 3 to 5 C and remained viable for over 3 years. At intervals of 3 to 6 months, the original culture was transferred to fresh slants of peptone-acetate-nitrate (medium A) agar having the following composition in grams per liter: $Na_2C_2H_3O_2 \cdot 3H_2O$, 8.5; KNO_3, 10; Difco peptone, 4; and agar, 20; pH 7.2. After growth at 28 C these slants were maintained at 3 to 5 C and were used as inocula for all cultures needed for the experimental work. Two or three rapid transfers were made in medium A broth before inoculating large liquid cultures used for growing cells for manometric experiments.

Anaerobically grown cells were obtained by inoculating 0.2 to 0.3 ml of a 48-hour culture in medium A broth into 1 liter of the same medium in a deep vessel (volumetric or Erlenmeyer flask). Cells were harvested after vigorous gas evolution was apparent (usually 35 to 40 hours) and were washed in M/60 phosphate buffer, pH 7.0. Prior to centrifugation, dissolved gases in the culture medium were removed by transferring the entire culture to a suction flask and applying a vacuum. If this step is omitted, gas bubbles form on sedimented organisms and carry them back to the surface. After being washed, the cells were resuspended in 5 to 8 ml of M/40 phosphate buffer, pH 7.0, and centrifuged lightly to remove clumps. The latter operation is important in order to obtain a relatively homogeneous suspension in which all the cells are subjected to a uniform environment.

Cells grown in air were obtained by inoculating the bacteria into 1-liter wide-mouthed Erlenmeyer flasks containing 300 ml of medium A broth. These flasks were incubated in a shaking machine at 28 C. Cells grown at reduced oxygen tensions were cultivated in 2-liter flasks provided with sintered glass aerators, the desired O_2–N_2 mixture being bubbled through the medium at a rate of approximately 2 liters per minute. Since at this rate the quantity of oxygen passing through the medium was greatly in excess of the requirements of the bacteria and since the gas was very finely dispersed and the liquid vigorously stirred, it is probable that the partial pressure of oxygen in the medium was very close to that in the gas at all times. A few drops of "neo-fat 17" were added to the

medium to minimize foaming during aeration. Cells grown in the presence of oxygen were always harvested near the end of the logarithmic growth phase, as determined by photoelectric turbidity measurements.

In manometric experiments involving oxygen at less than the atmospheric level, a suitable mixture of oxygen and nitrogen was prepared by the use of flow meters, and the gas was flushed for several minutes through the vessels. The oxygen content of a sample of the gas was usually determined by standard methods of gas analysis. For very low oxygen tensions, however, the pyrogallol methods of Elliot and Henry (1946) were used to estimate the actual oxygen concentration in the experimental vessels. Oxygen-free nitrogen was prepared by passing tank nitrogen over hot copper.

In manometric experiments, the bacteria were suspended in 0.025 M phosphate buffer, pH 7.0. Alkali was present in the center well of each Warburg vessel. Usually 0.2 ml of 0.1 M sodium acetate were used as the oxidizable substrate, and 0.2 ml of 0.04 M sodium nitrate or nitrite were added from the side arm after equilibration. All manometric experiments were done at 37 C.

Nitrite was estimated by the method of Rider and Mellon (1946).

Medium B. Medium B contained the following compounds in grams per 100 ml of distilled water: KNO_3, 5.00; sodium acetate, 4.21; $K_2HPO_4 \cdot 3H_2O$, 3.97; KH_2PO_4, 0.24; $MgSO_4$, 0.057; $CaCl_2$, 0.0087; and sodium glutamate, 0.096. Four volumes per cent of yeast autolyzate were also included.

RESULTS

Preliminary manometric experiments showed that suspensions of bacteria grown under anaerobic conditions cause a rapid denitrification with either nitrate or nitrite. Figure 1 shows that nitrogen was formed from nitrite at an almost constant rate until the nitrite was used up. The slight hump in the curves for both nitrite and nitrate during the first 20 minutes is due to a lag in the absorption of carbon dioxide. With nitrate the rate of nitrogen evolution was at first slower than with nitrite, but it increased gradually until the two rates were nearly equal. In some experiments the difference in rates with the two substrates was not so great, but qualitatively the same effect was always observed. This effect can undoubtedly be ascribed to a competition between nitrate and nitrite as hydrogen acceptors. Since nitrate is always reduced somewhat more rapidly than nitrite, the formation of nitrogen from nitrite is retarded until most or all of the nitrate has been used up.

Inhibition of nitrite reduction by oxygen. The manometric method cannot be used to follow denitrification in the presence of oxygen, because no simple method is available for simultaneously determining nitrogen evolution and oxygen uptake. It was necessary, therefore, to follow denitrification of nitrite by means of nitrite analyses. Since the conversion of nitrite to nitrogen involves several steps, nitrite analyses will only give a true measure of nitrogen evolution when there is no accumulation of intermediate products such as have been detected under special conditions by Korsakova (1927) and Elema *et al.* (1934).

The accumulation of possible intermediates between nitrite and nitrogen was

investigated by an experiment in which the disappearance of nitrite was followed chemically and the formation of nitrogen was determined manometrically. The results, presented in table 1, show that the nitrogen evolved was equivalent

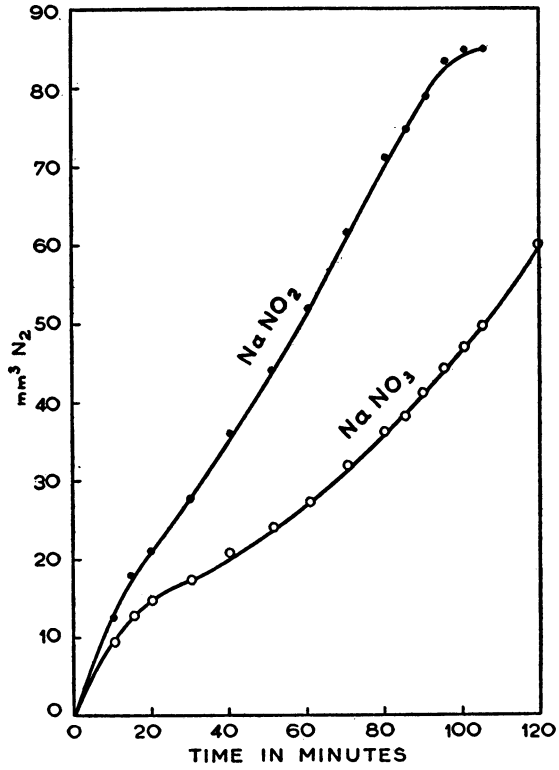

Figure 1. Nitrogen formation from nitrite and nitrate.

TABLE 1

A comparison of nitrite utilization with nitrogen formation

TIME	NO_2-N USED*	N_2 FORMED
minutes	*μM N*	*μM N*
5	0.4	1.0
40	3.2	2.8
80	5.9	5.1
150	7.4	8.5
215	12.6	12.1
265	15.3	14.7

* The initial quantity of nitrite N was 20.0 μM.

to the quantity of nitrite reduced within the limit of error of the determinations. This means that under the conditions of our experiments there was no significant accumulation of intermediate reduction products.

Preliminary experiments showed that air inhibits denitrification of nitrite by cell suspensions. Before the main experiments to determine the relation between the rate of denitrification and oxygen partial pressure were done, it was important to find out whether the inhibition by oxygen is reversible. If the action of oxygen were irreversible over short periods of time, extreme precautions would have to be taken in handling the bacteria prior to and during the experiment.

To investigate the reversibility of oxygen inhibition, the rates of nitrogen evolution from nitrite by anaerobically grown bacteria were compared manometrically after different periods of exposure to air. In one vessel that served as an anaerobic control, the cells were maintained under a nitrogen atmosphere throughout the experiment. In a second vessel the cells were shaken in air and the rate of denitrification was followed by periodic nitrite analyses. In two other vessels, the cells were shaken in air for different lengths of time; then the air was flushed out with nitrogen and the subsequent anaerobic rate of nitrogen evolution was followed manometrically.

TABLE 2

Reversibility of oxygen inhibition of denitrification

CONDITION	TIME OF RATE MEASUREMENT	RATE OF DENITRIFICATION
	minutes	*mm³ N_2/hr*
Anaerobic	0–160	30.0
Anaerobic following 15-min preaeration	30–160	28.0
Anaerobic following 62-min preaeration	80–160	23.2
Aerobic	0– 80	10.1
	80–135	3.8

The results presented in table 2 show that oxygen inhibition of denitrification is almost completely reversible after a 15-minute exposure to oxygen, but is only partially reversible after 1 hour. It is probable that a much more prolonged exposure to oxygen might cause a permanent inhibition. It should be mentioned that the rate of nitrite disappearance in the vessel continuously shaken with air did not decline continuously, but stayed almost constant for about 60 minutes and then dropped rather suddenly to the lower rate shown in the table. The most important conclusions in relation to further experimental work are that the effect of oxygen is largely reversible over short periods of time and the rate of denitrification responds very rapidly to a change in experimental conditions.

The main experiments were done in the Warburg apparatus, although only with the anaerobic vessels were the manometric data useful for the final calculations. Six vessels were filled with the same cell suspension and reagents, and each vessel was then flushed with a different mixture of oxygen and nitrogen. The vessels were shaken at a rapid rate (150 oscillations per minute) to ensure equilibrium between the gas and liquid phases. The period of incubation was sufficiently short so that the partial pressure of oxygen would not change ap-

preciably during the experiment. The nitrite content of the suspension was determined at zero time and again after a 25- to 50-minute period of incubation. From these results the average rate of nitrite reduction was calculated for each vessel. The results are plotted in the lower curve of figure 2 as a function of the oxygen content of the gas phase. The curve shows that nitrite reduction is inhibited about 45 per cent by 2.5 per cent oxygen. At higher oxygen levels there is a further gradual decrease in rate until in air (20.6 per cent oxygen) the inhibition is 73 per cent. In other experiments the percentage inhibition by air varied from 65 to 75 per cent. Oxygen levels above atmospheric were not used.

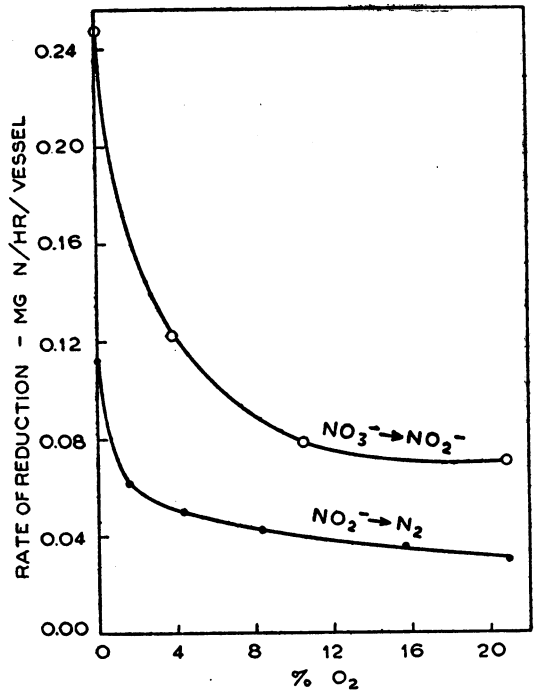

Figure 2. Inhibition of nitrate and nitrite reduction by oxygen at various partial pressures.

Inhibition of the reduction of nitrate to nitrite by oxygen. In a preliminary experiment the effect of air on nitrate reduction to nitrite was determined by measuring the nitrite concentration at intervals in two identical suspensions, one shaken in air, the other in a nitrogen atmosphere. During the sampling of the anaerobic suspension, nitrogen was flushed through the vessel to prevent contamination with oxygen. Parallel analyses were done on suspensions in which nitrate was replaced by nitrite. The results presented graphically in figure 3 show that under anaerobic conditions the reduction of nitrate by cell suspensions of *P. denitrificans* is much faster than the reduction of nitrite, thus causing a temporary large accumulation of nitrite. The rate of nitrite disappearance after the maximum has been passed is almost identical with the rate of nitrogen for-

mation in the anaerobic suspensions initially supplied with nitrite. In the presence of air, nitrate reduction to nitrite was considerably slower than in nitrogen. Thus air inhibits the reduction of nitrate as well as nitrite.

To determine the influence of various oxygen tensions on nitrate reduction an experiment similar to the main experiment described for nitrite reduction was performed. The data gave the rate of nitrite accumulation at various oxygen

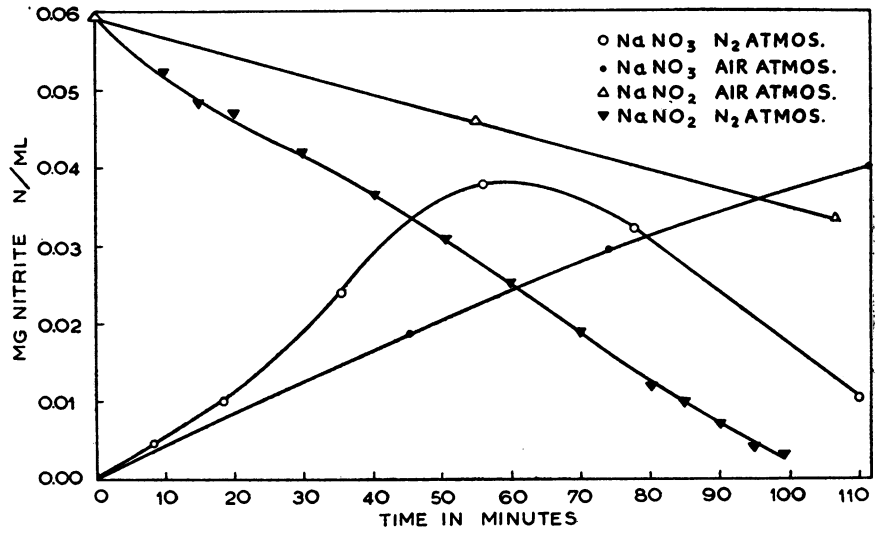

Figure 3. Influence of air on nitrate and nitrite reduction.

TABLE 3

Influence of oxygen on the rate of nitrate reduction to nitrite

1. OXYGEN IN GAS PHASE	2. FRACTION OF ANAEROBIC NITRITE REDUCTION OCCURRING AT THIS O_2 TENSION*	3. RATE OF NITRITE REDUCTION	4. RATE OF NITRITE ACCUMULATION	5. RATE OF NITRATE REDUCTION (3 + 4)
%		*mg N/hr*	*mg N/hr*	*mg N/hr*
0	1.00	0.206	0.043	0.249
3.65	0.46	0.095	0.026	0.121
10.5	0.36	0.062	0.017	0.079
20.8	0.27	0.055	0.016	0.071

* From lower curve of figure 2.

tensions. Now the rate of nitrate reduction is equal to the rate of nitrite accumulation plus the rate of nitrite reduction. The latter quantity was calculated from the anaerobic rate of nitrite reduction in this experiment and the percentage inhibition of nitrite reduction at each oxygen tension obtainable from the lower curve in figure 2. The rate of nitrate reduction estimated in this way is plotted against oxygen concentration in the upper curve of figure 2, and the primary data are given in table 3. The data results show that nitrate reduction is in-

hibited by oxygen in much the same manner as nitrite reduction. Low oxygen tensions cause a large percentage decrease, but even in a solution fully saturated with air the rate of nitrate reduction is 29 per cent of the anaerobic rate.

Influence of oxygen tension during growth on the formation of nitrate- and nitrite-reducing enzyme systems. Van Olden (1940) observed that denitrifying bacteria cultivated under aerobic conditions were almost or entirely unable to reduce nitrate to nitrogen and concluded that the formation of one or more enzymes essential to denitrification was prevented by air. In view of this observation it was of interest to investigate the ability of bacteria grown under lower oxygen tensions to reduce nitrate and nitrite.

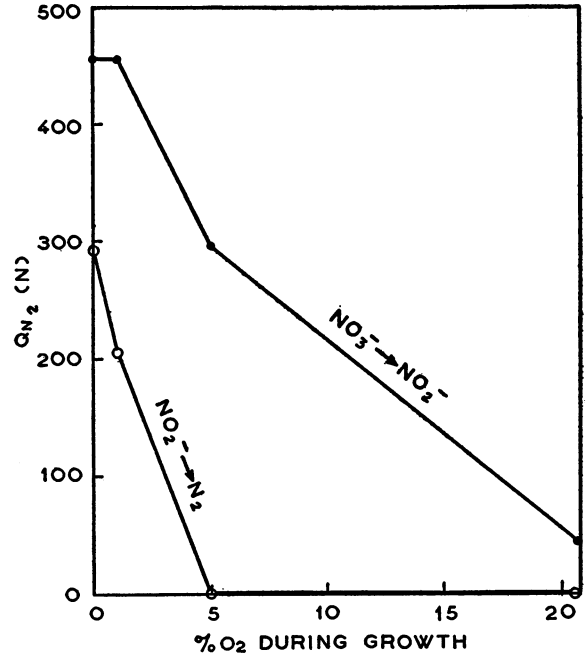

Figure 4. Influence of oxygen concentration during growth on the formation of nitrate- and nitrite-reducing enzymes. The activities of the enzymes were measured under anaerobic conditions.

The bacteria were grown under four conditions of aeration, namely, anaerobically, and aerobically while being vigorously aerated with 1.0, 5.0, or 20.6 per cent oxygen in nitrogen. The cells, harvested at about the same phase of growth, were tested for their ability to reduce nitrate to nitrite and to reduce nitrite to nitrogen under anaerobic conditions. The results presented graphically in figure 4 show that the oxygen tension during growth has a tremendous effect upon the rates of both processes. The formation of nitrite-reducing enzymes is decreased 29 per cent by 1 per cent oxygen and is completely prevented by oxygen at a level of 5 per cent or higher. The lowest oxygen level capable of preventing the formation of nitrite-reducing enzymes may be considerably below 5 per cent; no data are available in the range between 1 and 5 per cent oxygen. The for-

mation of nitrate-reducing enzymes is much less sensitive to oxygen during growth; 1 per cent oxygen causes no detectable inhibition and even saturation of the culture medium with air does not completely prevent the formation of such enzymes.

Stability of denitrifying capacity under various conditions. There is some evidence in the literature (Giltay and Aberson, 1892) that denitrifying bacteria gradually lose their ability to reduce nitrite as a result of being grown under laboratory conditions. It seemed desirable therefore to find out whether our culture would undergo such degeneration and whether the change, if it occurred, was influenced by the availability of oxygen.

To investigate these questions, bacteria that had been grown in medium B for a considerable time under (a) aerobic and (b) anaerobic conditions were tested from time to time for their ability to denitrify. In the aerobic series 10 successive daily transfers were made in small Erlenmeyer flasks incubated on a shaker to ensure adequate aeration. In the anaerobic series 16 successive daily transfers were made using test tubes provided with pyrogallol, potassium carbonate seals.

The ability of the bacteria in each culture of the aerobic series to denitrify was measured by inoculating them into an anaerobically incubated medium and determining the growth turbidimetrically after 48 hours. Since, under anaerobic conditions, denitrification coupled with the oxidation of organic compounds was the only energy-yielding process available to the bacteria, it could be assumed that a decreased ability to denitrify could be reflected by a decreased rate of growth. Although there was some variation in the turbidity of different cultures, there was no consistent trend in the results. The last two cultures in the series grew just as rapidly as the first two. These results indicate that no marked decline in denitrifying ability occurred as a consequence of repeated subculturing under strongly aerobic conditions.

This conclusion was further substantiated by comparing the pH changes that occurred when the bacteria from the final cultures of the aerobic and anaerobic series were grown for 48 hours in medium B under anaerobic conditions. The pH change may be taken as a direct measure of the amount of denitrification. The observed increase, from pH 6.8 to 8.3, was the same in both cultures. Also nitrite was absent from both cultures. These results confirm the conclusion that the denitrifying capacity of the organism is independent of its previous history of exposure to oxygen.

DISCUSSION

The experimental results clearly show that oxygen affects nitrate reduction and denitrification in two ways, by suppressing the formation of the enzyme systems that catalyze these reactions and by directly interfering with the action of the enzyme systems when they are present in the bacteria. The first effect is the more striking in the organism we have studied, but both effects are of great importance in determining the actual rate of these processes.

Exposure of the bacteria to oxygen during growth suppresses the formation

of enzyme systems responsible for nitrite reduction much more than those responsible for the reduction of nitrate to nitrite. As a result, at oxygen tensions of about 5 per cent, nitrate can be reduced only as far as nitrite, which accumulates in the medium. At lower oxygen tensions there is also an abnormally large accumulation of nitrite, but this is accompanied by denitrification. In this range of oxygen tensions, both the accumulation of nitrite and the rate of denitrification are greatly affected by relatively small changes in the oxygen level. In view of these relations it is easy to understand why apparently contradictory results have been obtained in the past by different investigators who used different methods of aerating their cultures.

Under natural conditions such as exist in the soil or in composts the occurrence of denitrification is generally dependent upon the formation of nitrite or nitrate by nitrifying bacteria. Since nitrification requires oxygen whereas denitrification is generally thought of as an anaerobic process, there has been some question as to whether both can occur simultaneously under the same conditions. Our results, taken in conjunction with the data of Meyerhof (1917) on the relation between oxygen partial pressure and the rate of nitrification, indicate that at oxygen tensions below 5 per cent this is possible. In a region of higher oxygen tension, denitrification will not occur together with nitrification unless the bacteria have been able to move from an adjacent region of lower oxygen tension within a short period of time. In this connection it should be emphasized that in a heterogeneous system such as soil the oxygen tension may change greatly over very short distances. Under such circumstances the diffusion of nitrite and nitrate as well as the movement of bacteria will be a factor in determining the rate of denitrification.

SUMMARY

Oxygen has a twofold action on denitrification: it suppresses the formation of nitrate- and nitrite-reducing enzyme systems, and when these systems are present it decreases the rate of the reduction processes. Quantitative data are given illustrating both of these effects of oxygen. It has been shown also that the inhibition of denitrification by air is largely reversible over short periods of time. Ability to denitrify did not decline appreciably as a result of continuous growth under aerobic conditions.

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