DENITRIFICATION

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Preface

Appreciation for the significance of universally and unceasingly active microbial phenomena such as photosynthesis, fixation of nitrogen, synthesis of antibiotics, and production of foodstuffs by fermentation has not been difficult to attain. Each of those processes is productive and beneficial for human life; and, not surprisingly, each has been the subject of an intensive, long-term series of investigations. In contrast, enthusiasm for the importance of at least one other broad-scope microbial activity has been much more difficult to generate. That phenomenon is denitrification, which is responsible for the loss of many tons of nitrogen fertilizer annually. Yet despite its impact in nature, the number of investigators studying the process remains small and funds for research support are minimal.

Evidence consistent with those assertions comes readily to hand. As recently as 1953, a scientist of the stature of Albert Jan Kluyver (200) opened a symposium on nitrate reduction in a distinctly defensive, if not apologetic, tone by noting the following:

> It seems a somewhat risky enterprise to make bacterial nitrate reduction the subject of a contribution to a modern symposium on bacterial metabolism. Most bacteriologists will consider the subject distinctly demoded. . . . Does not the answer to the question "nitrates reduced, or not?" yield a diagnostic character in quite common use, and is there not a very convenient routine procedure for the establishment of this characteristic? . . . Usually it is not realized that formation of nitrite out of nitrate is only the first step in nitrate reduction, and that there are numerous examples in the literature which testify to the ability of certain microorganisms to reduce nitrate to further reduction stages, like nitrous oxide and nitrogen. . . . The foregoing may only serve as an illustration that the way in which living cells react on the presence of nitrate in their medium may differ considerably, and this suggests at once the desirability of some further analysis of the situation.

To his great credit, before leaving the subject Kluyver (200) laid tentativeness aside and forcefully indicated the direction we should take:

> The full splendor of bacterial nitrate reduction, however, reveals itself in the case in which the nitrate.... decides over life and death of the organism, and it is this *true dissimilatory nitrate reduction* (Kluyver's emphasis) which seems to offer the best chances for closer investigation....

The current volume is devoted to just that: "closer investigation" of the history and development of attitudes toward denitrification, and of the microbiology, physiology, biochemistry, genetics (what little we know of it), ecology, and applied aspects of the process. The chapters are fairly well self-contained so that the reader intent on selection of only one or a few topics may profitably do so. But reading from front to back rather than selectively seems to offer greater promise for better understanding in even the narrowest area of interest. Considerable attention is given to development of improved analytical capacity, for part of the blame for the relative neglect of this phenomenon in the past may be laid to the lack, over several decades, of simple and inexpensive yet precise methods of assay. Each technical advance has been followed by a spate of discovery in domains that investigators could not previously penetrate. The recent availability (143), even in the most restricted sense, of radioactive but rapidly decaying ^{13}N-labeled nitrate is a case in point.

Additional specific attention is devoted to the pivotal reaction, denitrifying nitrite reduction, because of the uniquely deleterious effect it exerts, on a global scale, on the standing stocks of nitrogenous plant nutrients. Throughout the book, an attempt is made to emphasize the particularly promising areas for ever "closer investigation" that have come to light since Kluyver's call to action. Fortunately, such areas are numerous and easy to identify—if not yet accessible, in every case, to analysis.

Several people aided me greatly in the production of this book. I am grateful to M. A. Grant and Larry Evans for the preparation of illustrations and to Lucy Campbell for her guidance and assistance as a reference librarian. The editorial aid given by Sue Mealor is worthy of specific note and is particularly well appreciated. This book is dedicated to my wife and my daughter, with love and affection—but also to the memory of R. A. Smith, U. Gayon, and G. Dupetit, with deepest respect.

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1 Historical Developments

Recognition of the Phenomenon

We are not privileged to know or to have any way of estimating when denitrification began, but it is surely an age-old process. Margulis and Lovelock (229) postulate that bacteria generated nearly all of the nitrogen of the earth's atmosphere and now maintain the standing stock by continual denitrification. Down through geologic time, the volume of nitrogen repeatedly removed from the soils and waters of the biological world through denitrification must have been enormous and the tonnage far beyond the reckoning. The action is now widespread and unrelenting and probably has long been so, but no one knew of, even suspected, its existence until the latter part of the nineteenth century. For all the hints of such a phenomenon we can now recognize in hindsight, or from reading modern interpretations into the words of ancient reports (412), no one in earlier days recorded a flash of true perceptiveness. Our scientific predecessors remained oblivious of denitrification right through the centuries.

We can excuse such ignorance, of course. It is easy now to see that discernment of any process as arcane as denitrification had to await the maturation of both concept and technique in chemistry and microbiology. Consequently progress was uneven. Until the 1860s, the mind of man had neither the power to conceive of, nor the methodology to visualize, so gratuitously damaging a process in nature.

Identification of nitrogen and oxygen as elements, demonstration of their existence as the main components of air, and determination of their occurrence together in acidic and gaseous compounds all took place as recently as the last three decades of the eighteenth century. Considering the need then for time for development of insights, it is not hard to see that analytical procedures in chemistry could not have been advanced enough to reveal denitrification until the mid-nineteenth century. At that time microbiology was still an infant science.

Still in all, the discipline flourished. Both the methods needed for the routine isolation, culture, and description of microorganisms and an appreciation of the scope and variety of their chemical activities reached maturity in the workers' intellects during the latter half of the nineteenth century. It is a tribute to human ingenuity that the first fragmentary (and puzzling) reports of events we can now ascribe to denitrification began to appear just as soon as appropriate techology was available.

Realization of the significance of the phenomenon grew out of pragmatism, which is nothing strange in science. Interest in a number of microbiological functions boasts of practical beginnings. The systematic study of denitrification is no exception. It started with workers carrying out a series of elemental analyses on soils, water and sewage, fermenting juices, and manures and decaying vegetable matter. Their primitive assays first revealed the losses of nitrogen that led others, in time, to true recognition of the microbial etiology of denitrification (242, 243, 312, 332).

We are indebted to Smith (359) for the initial description of a systematic study of denitrification, which he made public in 1867. Noting that the nitrogen of nitrate disappeared from standing, organic-rich waters, he indicated that "nitrogen, therefore, may be removed from water either as ammonia, or organic matter, or nitric acid, *every trace of it disappearing*"; and further, with impressive discernment, "the oxygen seems to be removed as oxygen of the air is, probably leaving *nitrogen to pass off as gas*" (emphasis added in both quotations).

Nitrate was well recognized even then as a plant fertilizer, and processes that resulted in its loss were given immediate attention by European soil scientists. In 1868, the production "*du gaz nitreux*" during the fermentation of the sugars in beet root juice led Reiset (312) to ascribe the release to oxidation of ammonia. Before that year ended, however, Pasteur was complimented for confirmation of Schloesing's (and, we know now, the correct) idea that reduction of nitrate was responsible for the liberation of nitrogenous gas from urine and tobacco juice (332). Doing field work, he demonstrated an interdependence among anoxia, the combined consumption of organic matter and nitrate, and the loss of nitrogen from soil. A curious reaction known to a few sanitary engineers and fermentation scientists suddenly seemed threatening to the entire agricultural community.

The prospect was dismaying. No one wanted to encourage the destruction of nitrate. But, there it was, a defect that discomfited everyone. Among the first to feel disquieted were those who depended on the manufacturing process then widely used for the natural, aerobic production of nitrate in soils (i.e., nitrification) (98, 152, 333). At times they lost all the nitrate they produced.

Despite the realization that bacteria produced nitrate, workers of the day failed for some time to connect microorganisms with the losses of nitrogen observed in water, soil or their manufacturing plots. However, within a few years, Meusel (242, 243) suggested that bacteria were responsible for destruction of nitrate in soils or water and conjectured that the nitrate served as an oxidant that aided in the destruction of cellulose. And with acceptance of that notion, order began to emerge.

We owe much of the credit for the change for the better to two pioneering investigators. In 1882, Gayon and Dupetit (141) introduced the era of carefully planned, microbiologically sound experiments when they reported the first of a strikingly perceptive series of observations. It was they who introduced the term *denitrification* to describe the gas-producing, anaerobic destruction of nitrate carried out in their laboratory by bacteria from sewage. It was also they who noted that only a small part of the nitrate nitrogen supplied in an experimental system was assimilated into the growing microbial cells. Gayon and Dupetit were the first to abandon complex animal and plant products as culture media and to compile a list of simpler carbon compounds that served as electron donors for denitrification. Olive and sweet almond oils, glycerine, glycol, sucrose, alcohols of the fatty acid series, tartrate, ethanol, and propanol were found most effective.

That same year, 1882, Deherain and Maquenne (84) confirmed the observations of others by reporting that denitrification occurred in organic-rich, but not organic-poor, soils. They further indicated that nitrogen oxides were released sporadically from soil during the process. Employing the same criteria (86) used in their earlier attempts to gain acceptance for the notion that nitrification is a microbial process, they showed the following:

1 Soil lost the capacity for denitrification when heated for several hours at 110–120°C.
2 After cooling down, such heat-inactivated soil regained denitrifying capability only when supplemented with a quantity of normal soil.
3 Exposure of ordinary soil to chloroform vapors destroyed the capacity for denitrification.

A short time later, an American, Alfred Springer, reinforced the idea that denitrification is creditable to microbial action when he demonstrated that the "ferments" (i.e., microorganisms) clinging to tobacco roots released nitric oxide from nitrate (367). Investigators immediately asked, "Is it possible that the microorganisms which both form and destroy nitrate can live side by side in the soil?" An affirmative answer to this naive question seems obvious now; but at the time that Duclaux (98) showed that both nitrifying and denitrifying bacteria could, "*vivre côte à côte*," prospects for solving such a puzzle were anything but encouraging. How a soil could either yield or destroy nitrate had long perplexed the agronomists of the era. They were delighted when the causative agents were unmasked.

[As an interesting aside, it might be noted that the capacity of nitrate reducers to reduce chlorate (and bromate and iodate as well) was first recognized at this early stage of development (250). The observation was not to be exploited until many years later. With the development of microbial genetics, it was noted that the loss in a bacterial strain of the lethal capacity for the reduction of chlorate provided a useful selective marker for recovering nitrate reductase-negative mutants from mutagenized populations (307, 381, 382); see also Chapter 9).]

Focusing on the Etiological Agents

Continuing their ingenious investigations, Gayon and Dupetit stamped 1886 as a particularly memorable year in the history of nitrogen metabolism by their isolation of pure (axenic) cultures of two denitrifying strains from sewage (142). The isolates were designated *Bacterium denitrificans* α and β. With a remarkable insight shared by several early workers, the French investigators noted that denitrification is not a fermentation but a kind of "combustion" by nitrate of organic material such as citrate or asparagine. Like others, they presumed in error that the oxygen atoms were separated from the nitrate and used to accomplish the combustion by the ordinary (of course, then unknown) series of biochemical reactions of which the bacteria were capable. Early in the course of their monumental studies, Gayon and Dupetit recognized the release of nitrogen oxides, as well as dinitrogen, by one of their bacterial isolates.

Within two years of the pure culturing of denitrifiers, investigators incorporated into the routine descriptive procedures used for taxonomic characterization a test for the ability of all newly isolated bacterial strains to display denitrification (128, 331, 432).

When combined with Schloesing's observations of an earlier time (332), acquisition of the ability to isolate denitrifiers greatly sobered the thinking of agronomists by calling an ancient agricultural practice into question. For centuries, animal manures had been routinely mixed with soil in hopes of increasing yields from crop plants. But, several studies conducted by soil scientists during the nineteenth century showed that use of cattle and horse