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Denitrification

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INTRODUCTION

Denitrification refers to the dissimilatory reduction, by essentially aerobic bacteria, of one or both of the ionic nitrogen oxides (nitrate, NO_3^-, and nitrite, NO_2^-) to the gaseous oxides (nitric oxide, NO, and nitrous oxide, N_2O), which may themselves be further reduced to dinitrogen (N_2). The nitrogen oxides act as terminal electron acceptors in the absence of oxygen. The gaseous nitrogen species are major products of these reductive processes.

A dissimilatory reduction of NO_3^- and NO_2^- may occur, however, in which the major product is ammonia (NH_4^+). Such reactions occur in many of the *Enterobacteriaceae* (77), bacilli, and clostridia (57) and are reported in soils (43, 56) and marine sediments (215, 377) under very anaerobic conditions. At least some of these dissimilatory reductions to NH_4^+ can yield small amounts of N_2O as a minor product (375). Furthermore, ammonia-oxidizing nitrifiers produce small quantities of N_2O from hydroxylamine and nitrite under most conditions (24, 145, 172, 337, 438). However, these reactions are not denitrification in the strict sense and are not discussed further.

Interest in denitrification exists for several reasons. First, it is a major mechanism of loss of fertilizer nitrogen resulting in decreased efficiency of fertilizer use. Second, it is of great potential application in the removal of nitrogen from high-nitrogen waste materials such as animal residues. Third, denitrification is an important process, contributing N_2O to the atmosphere, where it is involved in stratospheric reactions which result in the depletion of ozone. Fourth, it is the mechanism by which the global nitrogen cycle is balanced. These and other aspects are discussed in this review.

Earlier reviews dealt with both assimilatory and dissimilatory reduction of nitrogen oxides (293, 301, 394), denitrification in general (94), ecological aspects (121), and the occurrence of denitrification in soil (130) and aquatic systems (36).

DENITRIFYING BACTERIA

The following summarizes the taxonomic distribution of bacteria able to use nitrogen oxides

as electron acceptors in place of oxygen, with the evolution of major gaseous products. These bacteria are biochemically and taxonomically very diverse. Most are heterotrophs and some utilize one-carbon compounds, whereas others grow autotrophically on H_2 and CO_2 or reduced sulfur compounds. One group is photosynthetic. Most possess all of the reductases necessary to reduce NO_3^- to N_2, some lack NO_3^- reductase and are termed NO_2^- dependent, and others lack N_2O reductase and thus yield N_2O as the terminal product. Still other organisms possess N_2O reductase but cannot produce N_2O from NO_3^- or NO_2^-.

Achromobacter species (most are now classified in the genus *Alcaligenes*) include denitrifying methane oxidizers (89) and NO_2^--dependent organisms able to denitrify NO_2^- but not NO_3^- (443). *Achromobacter cycloclastes* was reported to denitrify NO_2^- (184). Some denitrifying strains of *Agrobacterium radiobacter* and *Agrobacterium tumefaciens* were isolated by enrichment in an atmosphere of N_2O (310).

Alcaligenes faecalis is a numerically important denitrifier based on a survey of world soils (126). A denitrifying *Alcaligenes eutrophus* strain was isolated by autotrophic enrichment on H_2, CO_2, and NO_3^- as *Hydrogenomonas eutrophus* (299). Denitrification by a nitrifying *Alcaligenes* strain (58) and by *Alcaligenes denitrificans* and *Alcaligenes odorans* (NO_2^- dependent) (314) was also reported. Many strains of the N_2-fixing bacterium *Azospirillum brasilense* (396), previously known as *Spirillum lipoferum*, denitrify (105, 280). *Spirillum psychrophilum* also denitrifies (219).

Bacillus strains (many of which are NO_2^- tolerant, NO_2^- dependent, or thermophilic) are important numerically in rice soils (133) but are relatively less abundant on a worldwide basis (126). *Bacillus azotoformans*, a recently described species (303, 305, 307), is considered to be more important than *Bacillus licheniformis* (415), many strains of which apparently do not reduce NO_2^- to NO or N_2O to N_2 (306). Some bacilli can grow anaerobically with NO as electron acceptor (308, 311).

Chromobacterium violaceum and "*Chromobacterium lividum*" denitrify NO_3^- or NO_2^- with production of N_2O and N_2 (147). *Corynebacterium* strains were isolated in a survey of soil denitrifiers (126), and *Corynebacterium nephridii*, now of uncertain taxonomic status (161), is reported to produce N_2O as the terminal product of denitrification (161, 331). *Flavobacterium* strains may denitrify (126, 302).

Halobacterium marismortui, isolated from the Dead Sea, reduced NO_3^-, with the production of much gas. The original culture was lost (41) but a similar denitrifier was recently reisolated (425). *Hyphomicrobium* strains appear to be the only bacteria able to denitrify by using methanol (257, 285, 382). *Hyphomicrobium vulgare* grows anaerobically on NO_3^- with concomitant accumulation of NO_2^-, but direct evidence of denitrification is lacking (41).

A *Moraxella* species, isolated from soil, degrades benzoate anaerobically, reducing NO_3^- or NO_2^- to N_2 (430). Denitrifying strains of *Moraxella* group TM1 are now referred to as *Kingella denitrificans* (147). Earlier reports of N_2 production by *Neisseria* spp. (18, 19) have been confirmed. *Neisseria sicca*, *N. flavescens*, *N. subflava*, and *N. mucosa* all reduce NO_2^- to N_2O and N_2, but only *N. mucosa* denitrifies NO_3^- (147).

Paracoccus (*Micrococcus*) *denitrificans* is a facultative chemolithotroph which oxidizes H_2 and possesses electron transport systems similar to those of the mitochondrion (189). Although it has been the object of many physiological studies (e.g., 27, 304, 309), it was not isolated in a survey of denitrifiers in world soils (126) and may not be of common occurrence. *Paracoccus halodenitrificans* (41) is of uncertain importance. *Propionibacterium pentosaceum* denitrifies (136) and *Propionibacterium acidi-propionici* reduces NO_3^-, with N_2O as the terminal product (294).

The genus *Pseudomonas* includes the most commonly isolated denitrifying bacteria from both soils and aquatic sediments (126, 166) and may represent the most active denitrifiers in natural environments. Some NO_2^--dependent pseudomonads were isolated which are unable to reduce NO_3^- (407). Denitrifying pseudomonads include *P. aerogenes* and *P. aureofaciens* (126), *P. caryophylli*, and *P. chlororaphis*, some strains of which produce N_2O (148). The physiology of *Pseudomonas denitrificans* has been studied extensively. However, this organism was not observed in a survey of world soils (126), and it has been recommended that the use of the name be discontinued (41, 99).

Pseudomonas fluorescens appears to be important in soils (126), and some strains produce N_2O as terminal product (148). *Pseudomonas lemoignei* possibly (313), as well as *P. mallei* and *P. mendocina*, also contains denitrifying members. *Pseudomonas perfectomarinus* (445), although considered a nomen species (41) and rarely isolated, is a marine denitrifier on which some interesting basic studies have been done. Other denitrifying *Pseudomonas* species are *P. picketti* from rice soil (135), *P. pseudoalcaligenes* (126), *P. pseudomallei* (166), *P. solonacearum* (41), and *P. stutzeri* (1, 312).

Soybean bacteroids use NO_3^- as electron acceptor and accumulate NO_2^- (335). Some rhizobial strains reduce NO_3^- and NO_2^- to N_2

or oxides of nitrogen or to both (88, 271, 325, 444). *Rhodopseudomonas sphaeroides* is a photosynthetic nonsulfur bacterium, some strains of which apparently have the capacity for both N_2 fixation and denitrification (353–355).

Thiobacillus denitrificans couples denitrification to the oxidation of reduced sulfur compounds and can grow chemolithotrophically (7, 13, 182). *Vibrio succinogenes*, although it cannot produce gaseous products from NO_3^- or NO_2^- and therefore is not a denitrifier sensu strictu, does, however, possess N_2O reductase (440). *Xanthomonas* strains which denitrify have been reported (256) but such ability is not mentioned in *Bergey's Manual* (41).

A potential for denitrification exists in most habitats, but there is little information on which groups of organisms are responsible for the activities observed in particular systems. From their frequency of isolation, the *Pseudomonas* and *Alcaligenes* groups are perhaps of greatest significance.

PHYSIOLOGY AND BIOCHEMISTRY

When denitrifying bacteria are exposed to anaerobic conditions, in the presence or absence of NH_4^+, the dissimilatory nitrogen oxide reductases undergo derepression within a period of 40 min to 3 h (295, 296, 429). These reductases are indicated in Fig. 1 by NaR (nitrate reductase), NiR (nitrite reductase), NOR (nitric oxide reductase) and N_2OR (nitrous oxide reductase). As discussed later, the obligatory participation of the NOR in the sequence of reductions is still debatable. The model shown in Fig. 1 is based on information available for *Paracoccus denitrificans* (27, 189, 319) and is not necessarily applicable to other organisms. However, it is an example of the pathways by which electrons are transported to oxygen, or, if oxygen is absent, to the nitrogen oxides via the type *b* and *c* cytochromes.

Nitrate Reductase

Assimilatory (type B) and dissimilatory (type A) NO_3^- reductases are different proteins which are encoded by different genes (293, 301). The direct selection of dissimilatory mutants (DNaR) on the basis of resistance to chlorate (315) frequently gives rise to strains which lack both the DNaR and the assimilatory nitrate reductase activities (240, 408). However, it was shown more recently that direct selection of assimilatory NaR mutants of *Pseudomonas aeruginosa* gave strains which could still produce the DNaR (364), suggesting that different enzymes were involved. Further genetic analysis showed that different genes were involved, and it was recommended that the selection of dissimilatory DNaR mutants should be done on a background lacking assimilatory NaR rather than on a wild-type background (365).

Nitrate reductase (DNaR) in denitrifiers is similar to other dissimilatory nitrate reductases in containing iron, labile sulfide, and molybdenum (394). Growth of *Escherichia coli* on tung-

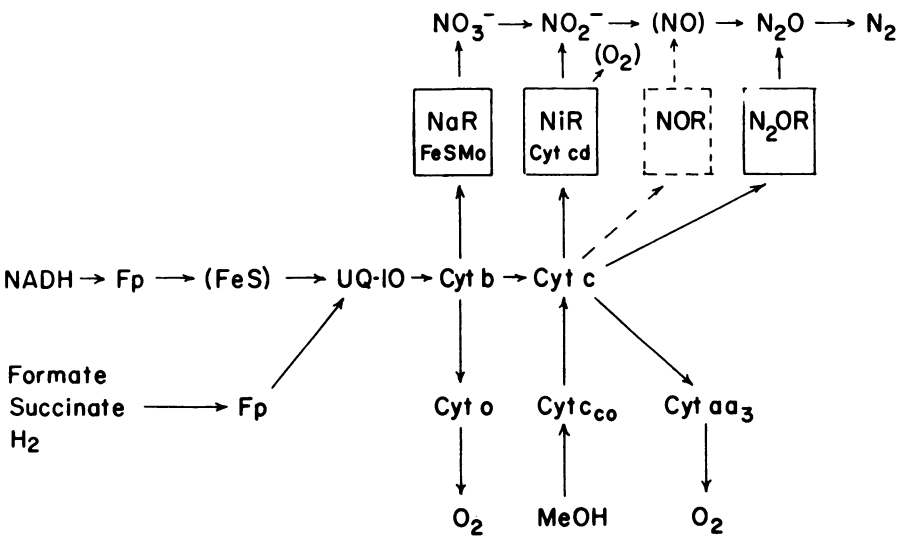

FIG. 1. Model of the probable pathways of electron transport in *Paracoccus denitrificans* (modified from references 27, 189, 319). Abbreviations: NaR, nitrate reductase; NiR, nitrite reductase; NOR, nitric oxide reductase; N_2OR, nitrous oxide reductase; Fp, flavoprotein; FeS, iron-sulfur center; UQ-10, ubiquinone-10; Cyt, cytochrome; Mo, molybdenum; MeOH, methanol.

sten gives inactive enzyme but activity can be restored by in vivo incorporation of molybdenum (358); whether this is true for denitrifiers is not known. The enzyme is associated with the membrane and is involved in energy conservation. On the basis of proton translocation studies with *Paracoccus denitrificans*, it appears to be on the inner face of the membrane (188, 222). In *B. licheniformis*, immunoferritin labeling of the NaR also occurs on the inner face of the membrane (427). In *Paracoccus denitrificans*, the NaR and the membrane cytochromes are coregulated, and the active enzyme seems to have a role in regulating its own synthesis (54, 394). Azide acts as a "gratuitous inducer," giving three to four times the NaR activity of nitrate-grown cells (54).

Immunologically cross-reacting components exist between purified NaR from *E. coli* and the purified molybdenum-containing dinitrogenase component from *Azotobacter vinelandii* and between the latter and crude extracts of *T. denitrificans* (281). These cross-reactions are consistent with the earlier theory that certain of the molybdenum-containing enzymes possess common cofactors (reviewed in reference 238).

Nitrite Reductase

NiR, or "denitrifying enzyme," catalyzes the reduction of NO_2^- to yield gaseous products and is thus a branch point which prevents the more economic recycling of nitrogen to the NH_4^+ form. The denitrifying NiR has been purified from several bacteria and appears to be of two main types. First, hemoproteins of cytochrome type *cd*, which may also have cytochrome oxidase activity (226, 356), occur in *Alcaligenes faecalis* (183, 245), *Paracoccus denitrificans* (226, 189), *Pseudomonas aeruginosa* (173, 363), *Pseudomonas perfectomarinus* (447, 448), and *T. denitrificans* (229, 356). Second, copper-containing metalloflavoproteins occur in *Achromobacter cycloclastes* (184, 185), *Pseudomonas denitrificans* (187), and denitrifying strains of *Rhodopseudomonas sphaeroides* (355). The products of NO_2^- reduction by whole cells are mainly N_2O and N_2, but with enzyme preparations of varying purity NO is commonly a major product (80, 183, 184, 265, 275, 363, 426). A membrane fraction from *Pseudomonas perfectomarinus*, however, produced N_2O and no NO (448).

Early studies indicated that NiR was a soluble enzyme (80, 183, 187). More recently, proton translocation experiments suggested an inner membrane face location in *Paracoccus denitrificans* (222), whereas in this bacterium (258) and in *Pseudomonas aeruginosa* (435) similar studies suggested a periplasmic location. Ferritin immunolabeling in *Pseudomonas aeruginosa* showed a location on the inner face of the membrane (352). A membrane location is consistent with in vivo evidence of phosphorylation associated with NO_2^- reduction in *Pseudomonas denitrificans* (213) and *B. stearothermophilus* (330). It seems reasonable to conclude that the enzyme is membrane associated but is readily solubilized so that the bulk of the protein or the activity is often found to be in the soluble fraction.

Nitric Oxide Reductase

The role of NO and the existence of NOR remain debatable. Reports mentioned earlier that purified NiR often releases NO as a major product (80, 183, 184, 265, 275) whereas whole cells do not, and the reports of semipurified NOR fractions from *Pseudomonas aeruginosa* (108), *Pseudomonas denitrificans* (264), and *Pseudomonas perfectomarinus* (296), which reduce NO to N_2O, all suggest that NO may be an intermediate in the denitrification process. The evidence for the participation of an NO-binding complex in the reduction of NO_2^- to N_2O is conflicting (229, 342, 447). Attempts to clarify the role of NO by means of isotope-trapping experiments have also given conflicting results. In *Pseudomonas aeruginosa*, denitrification of $^{15}NO_2^-$ in the presence of a pool of ^{14}NO gave negligible trapping of ^{15}NO, and the major products were $^{15}N_2$ and $^{14}N_2$ with very little $^{14}N^{15}N$ (393). It was therefore proposed (393) that NO was not an obligatory intermediate in the process, nor was it in equilibrium with another mononitrogen intermediate (Fig. 2, scheme 1). Further, similar trapping experiments with a pool of unlabeled hyponitrite showed that this compound could not be an intermediate in the denitrification process (171). On the other hand, in *Pseudomonas aureofaciens* and *Pseudomonas chlororaphis* reducing $^{13}NO_2^-$, significant label was trapped in a pool of ^{14}NO but the detailed isotopic composition of the N_2O terminal product was not determined (111). Furthermore, the data suggested that the initial reduction of NO_2^- was the rate-limiting step. In soil which is denitrifying NO_3^-, label is also trapped in an added NO pool (53). These results are consistent with either scheme 2 (Fig. 2), in which NO is in equilibrium with some unidentified intermediate, X (111), or with scheme 3 (Fig. 2), in which reduction occurs predominantly via a free NO intermediate but also via a bound intermediate (446). It has been suggested that energy conservation may occur associated with the X_{bound} pathway, and that the formation of NO_{free} may be a "means of discharging a surplus of reductant" (446). More recent ^{15}N trapping and scrambling studies of five denitrifying bacteria suggested that scheme 1 of Fig. 2

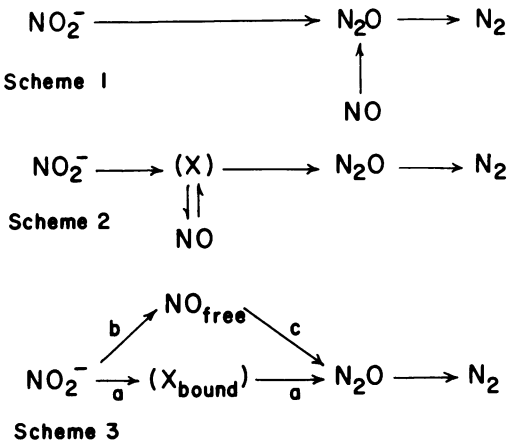

FIG. 2. Alternative models proposed for the roles of NO and N_2O in the reduction of NO_2^- to N_2.

was represented by *Paracoccus denitrificans* and *Pseudomonas aeruginosa*; scheme 2, by *Pseudomonas stutzeri* (X being a common mononitrogen intermediate); and scheme 3, by *Pseudomonas denitrificans* in which *a* was the major pathway and by *Pseudomonas aureofaciens* in which *bc* was the major pathway (127).

Nitrous Oxide Reductase

Nitrous oxide appears to be a free obligatory intermediate in the reduction of other nitrogen oxides to dinitrogen, on the basis of the following evidence. Although many bacteria, when denitrifying under conditions favorable for this process, do not release N_2O as a significant product, this gas is produced more or less transiently under certain conditions. It is the terminal product in *Corynebacterium nephridii* (161, 331) and in some rhizobia (88) and pseudomonads (111, 148), and it is the sole product of the reduction of NO by certain fractions of *Pseudomonas perfectomarinus* (296). Furthermore, the N_2O reductase is inhibited by acetylene (C_2H_2), which causes the accumulation of N_2O stoichiometrically as the terminal product of the reduction of other N oxides (14, 107, 442). The N_2OR is also inhibited by sulfide (381, 395), as is the reduction of NO (381). During reduction of $^{15}NO_3^-$ or $^{15}NO_2^-$ in the presence of a pool of unlabeled N_2O, the ^{15}N is trapped in the N_2O pool for both *Pseudomonas aeruginosa* (393) and soil (282). All of the above evidence points to N_2O as a free obligatory intermediate before final reduction to N_2. Evidence conflicting with this idea were reports (e.g., 1, 350) that azide and cyanide inhibited the reduction of N_2O to N_2 while not affecting the reduction of NO_2^- to N_2. Thus, it seemed that N_2O could not be an intermediate. However, it was recently shown (366) that NO_2^- can in some way counteract the azide inhibition of N_2OR and permit the reduction of N_2O to N_2, thus explaining the lack of accumulation of N_2O during reduction of NO_2^- in the presence of azide. The physiological basis of this effect is not known (366).

Electron transport to N_2OR generates a membrane potential only slightly smaller than that associated with O_2 utilization (249). It appears to involve cytochromes of types *b* and *c* (27, 244) (Fig. 1). Since these cytochromes are membrane associated in *Pseudomonas denitrificans* (244) and since continuous-culture studies of energy yields in this organism growing on various nitrogen oxides suggests that energy conservation is associated with the use of N_2O as electron acceptor (213), it is usually assumed that the N_2OR is located at the membrane (293). Indeed, a membrane-bound N_2OR was reported in *Pseudomonas perfectomarinus* (296). It has been claimed more recently, for a thermophilic bacillus, that the N_2OR sedimented at greater than $4{,}000 \times g$, but actual data were not presented (134). Cell-free N_2OR activity has been extremely elusive in most laboratories. However, reproducibly active preparations of N_2OR were recently prepared by gentle osmotic lysis of spheroplasts of *Paracoccus denitrificans* (220). The enzyme was present in the soluble fraction and, although extremely labile, with a half-life of about 1 h at room temperature, it was shown that acetylene, CO, azide, and cyanide all inhibited the N_2OR non-competitively with respect to N_2O (220). Further studies have achieved a 60-fold purification with respect to the crude lysate. The enzyme is sensitive to O_2 and to common salts; it has an apparent molecular weight of about 85,000; and although it does not contain Mo or Fe, it possibly contains Cu (221, 246). This is supported by recent cultural evidence that Cu is required for anaerobic denitrifying growth on N_2O and for the biosynthesis of N_2OR in *Alcaligenes* (186).

FACTORS CONTROLLING DENITRIFICATION

Oxygen

It is generally assumed that the nitrogen oxide reductases are repressed by O_2 and that, when this gas is removed, even in the absence of nitrogen oxides, the reductase enzymes are derepressed within a period of 40 min to 3 h (293, 296). It is noteworthy that gradual depletion of O_2 (296) or provision of "semianaerobic" conditions (54) appears to allow normal synthesis of at least the DNaR, whereas a rapid shift to anaerobiosis by sparging with helium apparently does not (296, 448). The picture is not quite clear,

however, since although 0.35% O_2 repressed NaR in continuous cultures of *Azospirillum brasilense* (278), derepression of *Pseudomonas denitrificans* NaR was observed in batch cultures at 5% O_2 (349) and of *T. denitrificans* NaR in continuous cultures at 15 μM (1.3%) O_2 (195). In the latter case, and also in a denitrifying *Hyphomicrobium* strain (257), NO_3^- was required as an inducer even in anaerobic cultures. In *Pseudomonas aeruginosa*, NaR was repressed when the O_2 supply rate was equal to or greater than the O_2 consumption rate (0.4 mmol of O_2 liter^{-1} min^{-1}) but was fully derepressed when O_2 was supplied at 1/20 of this rate (364). A bacterium isolated from activated sludge was reported to have NaR one-fifth derepressed even in the presence of 15 mg of O_2 liter^{-1} (>21% O_2) (224). Continuous cultures of a denitrifying *Hyphomicrobium* strain were considerably less sensitive to O_2 repression of NaR when growing at low growth rates than when growing at higher rates of 0.1 to 0.15 h^{-1} (257). Stouthamer (394) suggested that the repression or derepression of the DNaR may be controlled by the redox state of components of the electron transport chain. Although there is little direct evidence supporting this idea, it is consistent with the action of azide as a gratuitous inducer, causing the development of four times the NaR activity shown by an anaerobic culture exposed to NO_3^- (54).

Nitrite reductase requires somewhat longer for derepression than does the NaR (408, 429). Furthermore, it is more strongly repressed by O_2 than is the NaR (224, 257, 349). However, it is reported to be partially derepressed in *T. denitrificans* at 91 μM (7.8%) O_2 (195). Nitrous oxide reductase is derepressed in the absence of O_2 (243, 293, 296), but there is no information on the concentration of O_2 which controls the repression-derepression threshold of this enzyme.

Preformed reductases in whole cells are generally immediately or gradually inactivated by O_2, possibly due to competition for electrons rather than to an inactivation of the enzyme itself by O_2 (293, 394). Membrane vesicles of *Paracoccus denitrificans* were reported to use both O_2 and NO_3^- simultaneously but the reason for this was not apparent (188). Semipurified N_2O reductase from *Paracoccus denitrificans* was 50% inactivated by air, but 80% of the original activity could be regained by incubation with reduced benzyl viologen for several hours (221).

The later reductases in the denitrification sequence are somewhat more O_2 sensitive than are the earlier reductases (20, 224, 293, 349), and this is supported by studies of complex systems such as soil, in which low O_2 concentrations decrease the overall rate of denitrification, at the same time causing a larger mole fraction of N_2O in the products (116).

In soils and sediments the O_2 concentration depends on (i) the O_2 consumption rate, (ii) the O_2 diffusion rate, (iii) the geometry of the diffusion path, and probably other factors. Thus, in soils there is frequently an interaggregate air-filled porosity surrounding intraaggregate water-filled pores (82) which become more or less anaerobic, permitting denitrification to occur (370). These anaerobic zones can be of the order of 200 μm in diameter (149). When an aerobic soil becomes anaerobic, there are changes in the absolute and relative activities of the denitrifying reductases (374). For up to 3 h, activities apparently reflect the endogenous preexisting conditions, and N_2 is a dominant product. However, the NO_3^- and NO_2^- reductases then undergo derepression before the N_2O reductase does, with the result that N_2O becomes the dominant product. After about 1 to 2 days, N_2O reductase increases and N_2 again becomes the major product (113, 374). Time-dependent changes in N_2O metabolism are evidently not due to changes in redox potential, since they occur even when redox potential is constant (233). It is clear that such complex spatial and temporal variations in conditions (115) make analysis of the denitrification process in soil very difficult.

In flooded soils and sediments, there is no air-filled pore space and an O_2 gradient is established in the upper several millimeters of the surface (104, 409, 422). The thickness of the oxidized layer affects the rate of nitrification and the provision of NO_3^- for the underlying zone of denitrification (288).

Organic Carbon

The availability of electrons in organic carbon compounds is one of the most important factors controlling the activity of the heterotrophs, which comprise the bulk of denitrifiers. Although this factor has not been much studied in cultures, it is clear that denitrifying activity is related to organic carbon contents in both sediments (411) and soils. In soils, activity is highly correlated with water-extractable organic carbon (34, 46) and extractable reducing sugars (385) and is frequently stimulated by the addition of exogenous carbon (28, 128, 282, 428). Interestingly, different organic compounds which support equal rates of denitrification may nevertheless give different mole fractions of N_2O in the products (31), suggesting that they may exert differential effects on the reductases involved. Under some conditions, there is no effect of organic carbon addition (399), indicating that this factor is not rate limiting. Furthermore, with abundant carbon and complete anaerobiosis re-

duction proceeds significantly towards NH_4^+ rather than the gaseous products (57, 377, 384).

Nitrogen Oxides

In some organisms, such as *Pseudomonas denitrificans* (243) and *Pseudomonas perfectomarinus* (296), the nitrogen oxide reductases are derepressed under anaerobiosis even in the absence of the oxides. However, in *Pseudomonas aeruginosa* (364), *Paracoccus denitrificans* (54, 394), and *B. stearothermophilus* (100), the presence of NO_3^- is required for or very much stimulates derepression of DNaR, and it is reported that NO_2^-, chlorate, and azide also induce or stimulate the production of DNaR (54, 394). The role of the oxides in the derepression of the respective reductases is not clear.

The apparent K_m values for the dissimilatory reduction of nitrogen oxides by in vivo and cell-free systems are in the range of 5 to 290 μM (20, 109, 122, 220, 246, 355), although a value as high as 1.3 mM has been reported (340). However, in soils, water-logged soils, and sediments the diffusion of NO_3^- to the denitrifying sites can become an important factor (48, 300, 329), and the reactions are frequently reported to be first order up to at least 50 or 100 μg of N g^{-1} in soils (282, 348, 368, 428) or 10 mM sediment (409), although lower values are also reported (4, 59, 239).

The gaseous oxides are not reported to affect the reduction of the ionic oxides. The latter, on the other hand, are usually preferentially reduced before the gaseous oxides, causing the often observed transient accumulation of denitrification intermediates. This phenomenon may be due to competition for electrons or to intrinsic differences in the specific activities of the reductases (20). In *Pseudomonas denitrificans*, NO_2^- does not affect the rate of reduction of NO_3^- but causes partial uncoupling of energy metabolism, apparently by disrupting the transmembrane proton gradient (259). In soil, NO_2^- may cause a lag in the reduction of $^{15}NO_3^-$ (282) and partially inhibit the reduction of N_2O (112).

The effects of NO_3^- on various systems are probably the best studied. It seems clear that at low concentrations NO_3^- controls the rate of the denitrification reaction with first-order kinetics, as discussed earlier, and, in a system which has not previously been exposed to NO_3^-, it may promote derepression of the reductases and stimulate N_2O reduction (23). However, it appears that at higher concentrations it may (i) increase the rate of diffusion to anaerobic microsites (48) and thus affect the apparent kinetics of NO_3^- reduction in unstirred water-saturated systems (300, 329), (ii) cause more prolonged poising of the Eh at about +200 mV which might otherwise drop to about −300 mV (9, 233), (iii) inhibit the enzymatic reduction of NO and cause accumulation of NO_2^- (295), and (iv) inhibit the reduction of N_2O and thus cause a greater mole fraction of N_2O in the products (22, 79, 234, 282, 397). However, up to 100 mM NO_3^- did not inhibit N_2O reduction in an *Alcaligenes odorans* strain incapable of NO_3^- reduction (20). This suggests that the effects of NO_3^- in other systems may not be direct.

pH

In pure culture, as well as in natural systems, the denitrification rate is positively related to pH, with an optimum in the range of 7.0 to 8.0 (94, 270, 282, 405, 428). Denitrification may occur in wastes up to about pH 11 (321). At low pH values, the nitrogen oxide reductases, especially that which reduces N_2O, are progressively inhibited such that the overall rate of denitrification decreases but the mole fraction of N_2O produced increases, and at pH 4.0 N_2O may be the major product (282, 428). In acid peat, the low pH of 3.5 was reported to be the only factor which prevented the occurrence of denitrification (207). Thus, it appears that both decreasing pH and increasing O_2 concentration tend to decrease the overall denitrification rate, at the same time increasing the proportion of N_2O in the products evolved (116).

Interpretation of denitrification processes at low pH values is complicated because of the simultaneous occurrence of abiological reactions of NO_2^- which yield one or more of the gaseous products NO, N_2O, N_2, and CH_3NO_2. These reactions have been best studied in soil systems (reviewed in reference 210).

Temperature

Most of the studies of the effect of temperature on denitrification have been carried out with soil in which there appears to be a marked temperature dependence. This is in contrast to aquatic sediments in which surprisingly little variation of rate with temperature is reported (4, 59, 190).

Exposure of soil or activated sludge to temperatures in the range of 10 to 35°C gives Q_{10} values in the range of 1.5 to 3.0 (11, 90, 282, 383). Rates increase up to a maximum in the region of 60 to 75°C and then decline rapidly above this temperature (34, 203). At high temperatures the mole fraction of N_2O in the products tends to be very high (203, 211). It is usually assumed that enrichment with thermophilic bacilli (415) or other bacteria is responsible for the observed activity at high temperature, but it is speculated that abiological reactions may also be important (203).

In the low-temperature range, soil denitrification decreases markedly, but is nevertheless

measurable even at 0 to 5°C (11, 34, 368), where relatively larger mole fractions of N_2O (282) and NO (8) are reported.

Inhibitors

The reductases involved in denitrification are susceptible to inhibition by a variety of compounds (Table 1). The mechanism of action is not clear for any of the inhibitors, many of which exert metabolic effects other than those on specific reductases involved in denitrification. Sulfide appears to inhibit specifically the reduction of NO and N_2O and is probably responsible for the accumulation of these gases reported in certain sulfidic marine sediments (379). The role of sulfide in relieving the C_2H_2 inhibition of N_2O reductase is discussed below in connection with the use of C_2H_2 as a tool for the measurement of denitrification rate.

METHODS

The precise quantitation of long-term losses of nitrogen through denitrification in nitrogen balance studies is extremely difficult. Negative balances of 10 to 50% of applied fertilizer nitrogen were ascribed largely to denitrification in early long-term studies in agricultural systems (2, 39). These have been confirmed by more recent experiments in which significant nonrecoveries of added $^{15}NH_4^+$ or $^{15}NO_3^-$ fertilizer compounds were observed in laboratory (119) or field (65) experiments. In all of these studies a major factor limiting the precision of the denitrification estimate is the determination of total nitrogen in the system (164). Nitrogen balance methods cannot generally be applied to natural habitats.

The disappearance of endogenous or added nitrogen oxides is frequently used as a measure of denitrification rate in both terrestrial and aquatic systems. The commonly made assumption that added NO_3^- or NO_2^- is reduced only to gaseous products was supported by some ^{15}N studies with soils (282, 428) and lake water (142). However, as pointed out in the Introduction, a dissimilatory reduction of NO_3^- to NH_4^+ occurs in certain enterobacteria (77), bacilli (181, 415), and clostridia (57, 157). Such organisms appear to be responsible for the formation of significant amounts of NH_4^+ (up to 55%) from NO_3^- by carbon-rich and highly anaerobic soils (56, 66, 384) and sediments (215, 377), and it is therefore dangerous to make the untested assumption that NO_3^- or NO_2^- is reduced exclusively to N_2O or N_2 (164).

Calculations of NO_3^- deficits associated with so-called "nitrate anomalies" in the ocean have been extrapolated to yield denitrification estimates. As discussed below for aquatic systems, oxygen-minimum regions are associated with low NO_3^- concentrations which are not correlated with high concentrations of NO_2^- or other nitrogen fractions. This deficit, or anomaly, is assumed to be equal to the denitrification loss of gaseous N_2O and N_2 which occurred since the water mass became separated from the main oceanic water mass (72, 74, 162).

TABLE 1. Some inhibitors of denitrification

Inhibitor (concn)	Reaction inhibited	Reference(s)
Acetylene (10^{-3} atm)	$N_2O \rightarrow N_2$	14, 107, 442
Azide, cyanide, DNP[a] (ca. 10^{-4} M)	$NO_3^- \rightarrow N_2$, $N_2O \rightarrow N_2$	1, 246, 304, 350
Nitrapyrin (nitrification inhibitor)		
14 ppm in soil	$NO_3^- \rightarrow N_2$	263
50 ppm in soil	No effect	167
50 ppm in culture	$NO_3^- \rightarrow N_2$	167
N-serve formulation (20 ppm in enrichment culture)	$NO_3^- \rightarrow N_2O$ and N_2	250
Pesticide		
Vapam (20 ppm in soil)	$NO_3^- \rightarrow N_2$	263
Dalapon (10 ppm in soil)	$NO_3^- \rightarrow N_2$	423
Toluidine derivatives	NO_2^-, $N_2O \rightarrow N_2$	26
Sulfur compound		
SO_4^{2-} (100–500 μg of S g^{-1})	NO_3^- disappearance in soil	218
S^{2-}		
40 mmol g^{-1}	$NO_3^- \rightarrow$ gaseous products	272
0.3 mM	$NO \rightarrow N_2O$	381
0.3 mM, 8 μmol g^{-1}	$N_2O \rightarrow N_2$	381, 395

[a] DNP, 2,4-Dinitrophenol.

The disappearance of added N_2O was used as a measure of denitrification potential in slurries of rice soils (129, 131) and salt marsh soil (362). This method is basically nondisturbing in that only gas-phase manipulations are required. However, it may overestimate a rate if the introduced concentration of N_2O saturates reducing sites which were not saturated in situ, or it may underestimate if endogenous NO_3^- or NO_2^- competes significantly with the N_2O for the available electron supply.

The ideal estimate of denitrification activity by the precise measurement of the amount of N_2O and N_2 produced (15) is difficult to achieve in practice because of the N_2 present in the system as a naturally high air background or as a frequent contaminant from occlusion or leakage. Most direct assays of denitrification have involved the addition of $^{15}NO_3^-$ followed by mass spectrometric detection and measurement of the $^{15}N_2O$ and $^{15}N_2$ released. Such methods were used successfully in closed incubations of aquatic (64, 142–144, 217) and terrestrial (52, 53, 67, 120) samples. Precautions necessary in the mass spectrometric analysis of mixtures of N_2, N_2O, and CO_2 include the differential freezing out of gaseous components (152, 283, 284) and the measurement of each of the 28, 29, and 30 mass/charge peaks for subsequent calculations (164, 165).

Much greater sensitivity and shorter (0.1 to 2 h) experiments are possible when the short-lived isotope ^{13}N ($t_{1/2}$ ca. 10 min) is used. An accelerator is required to bombard distilled water with a proton beam, yielding mainly $^{13}NO_3^-$ of very high activity, which can then immediately be used experimentally (139, 399, 400). The evolved N_2O is trapped in a liquid N_2-cooled U-tube, the N_2 is trapped in a liquid N_2-cooled molecular sieve, and activities are monitored continuously with gamma ray detectors (400).

In situ field measurements of $^{15}N_2$ production were achieved in water-sediment enclosures supplemented with small amounts of $^{15}NO_3^-$ (60, 401, 402) but gas leakage, pressure fluctuation, and other problems make such measurements in terrestrial systems difficult (117, 237, 254). Fluxes of N_2O alone from soils can be measured either with in situ enclosures (78, 95–97, 117, 176, 247, 255) or by concentration gradient measurements (50, 338, 339, 345). In the latter, there are many problems in the determination of soil-air exchange rates (231). Not least among the difficulties is the accurate definition of the N_2O concentration gradient in the 0- to 5-cm depth which is so important in the control of the diffusive flux under varying moisture conditions (338).

Nitrogen/argon ratio measurements can provide estimates of denitrification in aquatic systems. The degree of under- or oversaturation with N_2 of water samples is obtained by comparing the ratio of observed N_2 and Ar concentrations (N_2/Ar) with the ratio of their respective solubilities (N_2^1/Ar^1) in water equilibrated with air under the observed temperature and chlorinity conditions (16, 163). Such studies may be more qualitative than quantitative in view of the possible effects of N_2 fixation (418) and CH_4 stripping (241) on the N_2/Ar ratio.

Acetylene (C_2H_2) was reported to inhibit the reduction of N_2O during denitrification (107) and this was confirmed in pure cultures of several denitrifying bacteria (14, 442). The inhibition of N_2O reduction in soil was complete at C_2H_2 concentrations down to 10^{-3} atm, and NO_3^- was stoichiometrically converted to N_2O (441). The phenomenon thus provided the basis for a relatively simple and sensitive assay for denitrification (207, 441) in which a simultaneous estimate of N_2 fixation as C_2H_2-reducing activity can also be obtained (441). The validity of this procedure was confirmed for soils by ^{15}N and ^{13}N studies (292, 346, 372), but it was reported that at very low NO_3^- concentrations more than 0.1 atm of C_2H_2 may be required to give complete inhibition of the reduction of N_2O (201, 372). There is as yet no explanation for this phenomenon.

In situ soil measurements which exploit the C_2H_2 inhibition of N_2O reduction may use flow-through enclosures, with C_2H_2 injected into the soil through perforated tubes and trapping of evolved N_2O on a molecular sieve trap (347). Alternatively, a more or less gas-tight enclosure may be used, with C_2H_2 injected into and samples removed from the gas phase (237, 290). In water-sediment systems, the loss of gases by diffusion around the walls of an enclosure appears to be less of a problem (61), but appropriate concentrations of dissolved C_2H_2 are more difficult to achieve, and the extraction and determination of dissolved gases by the multiple phase equilibration technique (248) is more time-consuming.

Less than complete inhibition of N_2O reduction has been observed in some soil (437), freshwater sediment (209), salt marsh (412), and marine sediment (208) systems. The reason for incomplete inhibition could include a utilization of the C_2H_2 by *Nocardia* (197), by aerobic soils (137, 138), and by anaerobic soils and sediments (87, 421). Concentrations of C_2H_2 may also be depleted through nitrogenase activity (441) but this is generally insufficient to relieve the inhibitory effect of C_2H_2 on the N_2O reductase. The presence of sulfide was reported to promote the reduction of N_2O in soil even in the presence of normally inhibitory concentrations of C_2H_2 (395), and it was suggested that this phenome-

non may be the cause of incomplete C_2H_2 inhibition of N_2O reduction in marine or other sulfidic systems.

In view of the increasing use of C_2H_2 for the assay of N_2 fixation since 1966 and of denitrification since 1976, it is important to remember that C_2H_2 shows biological effects other than those of interest in the assays mentioned. It is clear that some of the inhibitory effects listed in Table 2 have important implications for the assay of nitrogenase activity in natural systems. Some effects could also clearly interfere with the measurement of denitrification as discussed above. Of particular significance here is the strong C_2H_2 inhibition of NH_4^+ oxidation by nitrifying bacteria, a phenomenon which precludes the use of C_2H_2 for the measurement of naturally coupled nitrification-denitrification processes.

TERRESTRIAL SYSTEMS

Soil

Soil is a three-dimensional matrix of solid, liquid, and gaseous phases which receives pulsed inputs of water (and therefore O_2) in rainfall, nitrogen compounds in fertilizers, and organic carbon in plant residues. The conditions within the soil aggregates, therefore, vary markedly in space and time. Concentration gradients exist on a scale of micrometers, and the adequate quantitation of biochemical activities in such microenvironments is virtually impossible given the methods currently available. The relations between O_2 concentrations, anaerobic microsites, and denitrification must therefore be examined on a statistical basis (114, 115) or with the help of theoretical models, two of which were recently reported (228, 370). A rainfall of 20 to 30 mm increases the total anaerobic soil volume and promotes denitrification (81), but theoretical considerations suggest that this volume is more dependent on the magnitude and distribution of the respiratory activity and on the gaseous diffusion coefficient in the aggregates (228).

Spatial and temporal variations in conditions, and thus in denitrifying activity, are also caused by cycles of wetting and drying, which promote nitrification followed by denitrification (289, 328) and increase the availability of organic carbon (125, 291). Freezing and thawing of soil also increases its denitrifying potential (253), presumably by releasing organic carbon. The addition of nitrogenous fertilizers or of plant or animal residues creates localized zones having relatively high concentrations of organic or inorganic nitrogen or both as well as organic carbon. Mineralization of nitrogen in animal residues is followed by its nitrification and subsequent denitrification, and all of these processes vary greatly both spatially and temporally (45, 49, 151). All of the variability associated with denitrification in soils contributes to the difficulty of its adequate quantitation in the field, an aspect which will be considered later. However, a further factor which causes microenvironment heterogeneity is the presence of plant roots.

TABLE 2. Biological effects of acetylene

Effect	Reference(s)
Inhibition of	
N_2O reduction by denitrifiers	14, 442
N_2 fixation (can impose N limitation)	51
N_2ase-catalyzed H_2 evolution	177
Proliferation of clostridia	40
Hydrogenases	
Conventional	371
Uptake	62, 371
NH_4^+ oxidation	
By *Nitrosomonas*	178
By soil	179, 420
Methanogenesis	323
Methane oxidation	92
Ethylene cooxidation	91
Utilization of C_2H_2 by	
Nocardia rhodochrous (aerobic)	197
Aerobic soils	137, 138
Anaerobic soil and sediment	87, 421

The Rhizosphere

The plant root affects its immediate soil environment, the rhizosphere (12), by releasing soluble root exudates, sloughing off root surface and root cap cells, and producing mucigel polysaccharide. It acts as an oxygen sink in light-textured soils, thus depleting oxygen in the rhizosphere. In anaerobic water-saturated soils and sediments the root may release oxygen, which is transported to the root via a system of gas spaces or lacunae (286). Clearly, the root causes the establishment of concentration gradients of most of the regulating factors discussed earlier.

Large populations of denitrifiers frequently exist in the rhizosphere region (386, 434) where they may be 10 to 100 times more numerous, for oak seedlings, than in the root-free soil (279) or up to 514 times more numerous, for rice, than in the corresponding root-free soil (128). Denitrifiers are also enriched in the rhizosphere of the salt marsh grass *Spartina alterniflora* (362). Most of the roughly 10^7 denitrifiers per g of root or rhizosphere soil observed for rice were *Pseudomonas* or *Alcaligenes*, but bacilli represented a large proportion of the rhizoplane (root surface) population (341).

Studies using ^{15}N-labeled fertilizer compounds in closed systems showed that the pres-

ence of plants increased denitrification of the added fertilizer (236, 389–391) but caused a decrease in the mole fraction of N_2O produced (387, 388). Direct measurements showed that denitrification occurred in the rhizosphere soil rather than in the root tissue itself (433) and that N_2O-reducing activity of the rice rhizosphere was up to 14 times that of the root-free soil (128, 129, 132, 324). Denitrification was stimulated by decapitated plant roots (8), by low availability of K^+ (403), and by periods of irrigation (417). It seems clear that plants have the potential to stimulate denitrification in their rhizosphere by virtue of their creation of O_2 and organic carbon gradients. However, this is true only if there are significant sources of nitrogen oxides. If the oxides are in relatively short supply or the plant roots act as very strong assimilatory sinks for inorganic nitrogen or both, the plant may exert a negative effect on denitrification in its rhizosphere (42, 290, 297, 373).

Field Measurements of Gaseous Fluxes

Because of the changing conditions and pulsed inputs associated with both natural and agricultural soils, denitrifying bacteria undergo flushes of activity of short or long duration. The precise measurement of denitrification over long periods of time is, therefore, difficult and has depended in the past on ^{15}N balance and recovery studies. However, direct measurements of gaseous fluxes were recently reported. After the addition of 300 kg of NO_3^--^{15}N ha^{-1}, the fluxes of $^{15}N_2O$ and $^{15}N_2$, measured in temporary enclosures over 1- to 2-h periods, showed marked time-dependent variations, with peak fluxes of N_2O and N_2 up to 7.5 and 60 kg of N ha^{-1} day^{-1}, respectively (339). The N_2O released represented 5 to 26% of the total denitrification, which in turn accounted for 1 to 73% of the fertilizer added, depending on whether the plots were uncropped or manure treated (339). Field measurements with the C_2H_2 inhibition method also showed sharp peaks of denitrification up to 3.4 kg of N ha^{-1} day^{-1}. The N_2O flux represented 13 to 30% of the total denitrification, which in turn accounted for 14 to 52% of the fertilizer added (344).

The advent of the sensitive electron capture method for the detection of N_2O encouraged field studies of N_2O fluxes, using short-term enclosure or concentration gradient measurements. At least 14 such studies have appeared since 1978. All reported great variations in flux rate as a function of time, with maxima (Table 3) occurring 2 to 7 days after fertilizer addition or very shortly after periods of irrigation or rainfall. The higher flux rates, which are assumed to be a result of denitrification, were associated with the larger applications of fertilizer nitrogen in conditions of high moisture, organic carbon, and temperature. In many cases a diurnal variation in N_2O emission occurred, with a maximum in the early afternoon coinciding with the temperature maximum (96, 97, 247).

Some of the lower flux rates shown in Table 3 were obtained in soils with relatively low moisture contents which might be considered to be too low to permit denitrification (30, 73, 78, 97). Such losses are frequently greater from the nitrifiable compounds, NH_4^+, anhydrous NH_3, and urea, than from NO_3^- (30, 69, 78). It appears that this N_2O production in dry soils is due to nitrification (32) since NH_4^+-oxidizing *Nitrosomonas*, *Nitrosolobus*, *Nitrosospira*, and *Nitrosococcus* can all produce small quantities of N_2O at high as well as at low concentrations of O_2 (24, 145). This source of N_2O may be important for atmospheric chemistry but it apparently represents a <0.1% loss of applied fertilizer nitrogen (30, 359).

It seems clear that soils generally act as sources of N_2O (268, 359) through either denitrification or nitrification reactions. They can, however, act as N_2O sinks under conditions of complete anaerobiosis, ready availability of organic carbon, and absence of NO_3^- (21). The dissolved N_2O in equilibrium with the atmosphere is about 9 nM at 20°C, very much lower than K_m values reported for N_2O reduction, which range from 500 nM to 60 μM (20, 220, 246). However, a field grassland has been reported to act as an N_2O sink on many occasions (343). It is noteworthy that a biological removal of N_2O under highly aerobic conditions was reported not to produce N_2 and not to be inhibited by acetylene (413). This process was catalyzed by a consortium of five corynebacteria, not one of which alone could reduce N_2O in air (414).

AQUATIC SYSTEMS

Water Column

Oxygen-depleted regions of the oceans are frequently associated with particular distributions of the nitrogen oxides (29) characterized by two separate NO_2^- maxima and a NO_3^- minimum. For example, in the eastern tropical North Pacific there is a "primary NO_2^- maximum" at 80 m underlain by an N_2O maximum at about 105 m, both in the thermocline region in which O_2 is partially depleted (76, 317). At greater depths of 180 to 500 m, where O_2 is depleted to below about 0.2 mg $liter^{-1}$, there is a "secondary NO_2^- maximum" associated with depletion of both NO_3^- and N_2O (72, 76, 317). The NO_2^- accounts for only a small part of the NO_3^- depletion (this is referred to as the NO_3^- anomaly), and it is assumed that the nitrogen oxide deficit is due to denitrification to dinitrogen (29,

TABLE 3. Maximum reported rates of N_2O flux from soils

System	Addition(s) (kg of N ha^{-1})	N_2O flux (g of N ha^{-1} day^{-1})	Reference(s)
Manure treated	300 ($^{15}NO_3^-$)	7,500	339
Rye grass	300 ($^{15}NO_3^-$)	900	338, 339
Uncropped	300 ($^{15}NO_3^-$)	400	339
Direct drilled	Not stated	700	47
Corn	200 (NH_4^+)	550	267
Sugar beet	200 (NH_4^+)	8	267
Vegetables, irrigated	120 (NH_4^+)	400	345
Not stated	Not stated	518	95
Corn, irrigated	200 (NH_4^+)	270	175
Grassland	250 (NH_4NO_3)	212	343
Flooded field	None	302	96
Grass sward	None	43	97
Vegetables	(NH_4^+, urea)	200	344
Fallow	250 (NH_4^+)	60	30
Annual pasture	112 (NO_3^-)	50	48
Soybean	None	44	33
Tobacco	224 (NH_4NO_3)	30	255
Fallow	220 (NH_3)	15	73
Short-grass prairie	450 (urea)	35	268
Short-grass prairie	None	10	268
Uncultivated	100 (NH_4NO_3)	10	78
Uncultivated	100 (NH_4^+)	5	78
Uncultivated parkland	None	3	359

71, 72, 74, 110, 398, 436). Nitrate-reducing bacteria were isolated from such a secondary NO_2^- maximum (55), and bottle assays of over 200-h duration showed, in one study, reduction of NO_3^- beyond NO_2^- with insignificant production of NH_4^+ and N_2O (141) or, in another, reduction of added $^{15}NO_3^-$ to $^{15}NO_2^-$ and $^{15}N_2$ (140). Some estimates of rate of denitrification obtained by direct assay, or by calculation based on the NO_3^- anomaly, are shown in Table 4.

The primary NO_2^- (and N_2O) maximum observed in the eastern tropical Pacific is ascribed to nitrification (29, 76, 155) or to leakage of NO_2^- during the assimilation of NO_3^- by phytoplankton (204) or to both processes. There does not appear to be any direct evidence that either of these processes, or denitrification, is specifically responsible for the primary NO_2^- maximum.

In both marine and freshwater systems, the depletion of O_2 below about 0.2 mg $liter^{-1}$ favors the occurrence of denitrification, which proceeds until the nitrogen oxide concentrations are very low, for example, NO_3^- below 10 μg of N $liter^{-1}$ (60). A high potential for denitrification may exist even though the countable denitrifying bacteria are not more than 100 per ml (217). Water masses which can become entirely depleted in the ionic as well as the gaseous nitrogen oxides include anaerobic hypolimnia (35, 37, 60, 198, 212, 416), anoxic lakes under ice (142), and anoxic upwellings (101) and inlets (85).

During summer stratification in dimictic lakes (e.g., Fig. 3) the anoxic hypolimnion may be entirely depleted of all nitrogen oxides, as described earlier. The aerobic mixed epilimnion may be depleted of the ionic nitrogen oxides by assimilation and of gaseous N_2O by diffusion to the atmosphere. Between these two regions, in the metalimnion, there are sometimes persistent maxima of NO_2^- and NO_3^- (35, 37, 159, 212, 416) as well as of N_2O (212, 416). The maintenance of such maxima (Fig. 3) may be due to the simultaneous occurrence of nitrification and denitrification in adjacent microsites, or they may be the product of nitrification activity alone (416). However, it has recently been suggested that the NO_2^- maximum may be due to the cooxidation of NH_4^+ by methylotrophic bacteria, which show a peak of activity restricted to the same depth (159). Since these bacteria also release N_2O during oxidation of NH_4^+ (T. Yoshinari, personal communication; E. Topp and R. Knowles, unpublished data), they may be responsible also for the persistence of the N_2O maximum between the dissimilatory sink in the hypolimnion and diffusion loss to the epilimnion.

It is clear that N_2O concentrations in aquatic systems range from undersaturations in anoxic regions to supersaturations in lake metalimnia, as noted above. Nitrous oxide supersaturations also are reported from rivers (198, 251) and from parts of the oceans where O_2 remains at least 50% saturated (76, 103, 317, 439, and other work reviewed in reference 155), but most of these reports suggest that nitrification is probably the

TABLE 4. Estimates of denitrification rates in the water column of aquatic systems

System	Method	Rate (μg of N liter^{-1} day^{-1})	Reference
Marine[a]			
Galapagos Bay, ETSP	$^{15}NO_3^-$, bottle	12–18	143
Galapagos Bay, ETSP	In situ concn	2.3	334
ETNP, O_2 minimum	$^{15}NO_3^-$, bottle	7.2–254	140
ETNP, O_2 minimum	NO_3^-, bottle	67	141
ETNP	Isotope fractionation	0.13	71
ETSP	NO_3^- anomaly	0.41	74
Arabian Sea	NO_3^- anomaly	0.003–0.027	98
Freshwater			
Subarctic lake	$^{15}NO_3^-$, bottle	90	142
Brackish lake	$^{15}NO_3^-$, bottle	0–476	217
Lake Mendota hypolimnion	$^{15}NO_3^-$ disappearance	8–26	37
Lake 227, summer stratification	$^{15}NO_3^-$, bottle	0–30	60
Antarctic lake	C_2H_2, bottle	0–0.25	416

[a] ETSP, Eastern tropical South Pacific; ETNP, eastern tropical North Pacific.

major N_2O-producing mechanism in these locations. Similar supersaturations in lakes (212, 232) could also be due to nitrification.

Sediments

In freshwater sediments, denitrifying bacteria occur usually in numbers ranging from 10^5 (190) to 10^{10} g (dry weight) of sediment^{-1} (193), indicating considerable potential for denitrification. The identity of these organisms is not well characterized, although they appear to be frequently fluorescent pseudomonads (190). Bacteria reducing NO_3^- to NO_2^- also occur abundantly and are represented by the genera *Aeromonas*, *Pseudomonas*, and *Acinetobacter* as well as by members of the enterobacteria

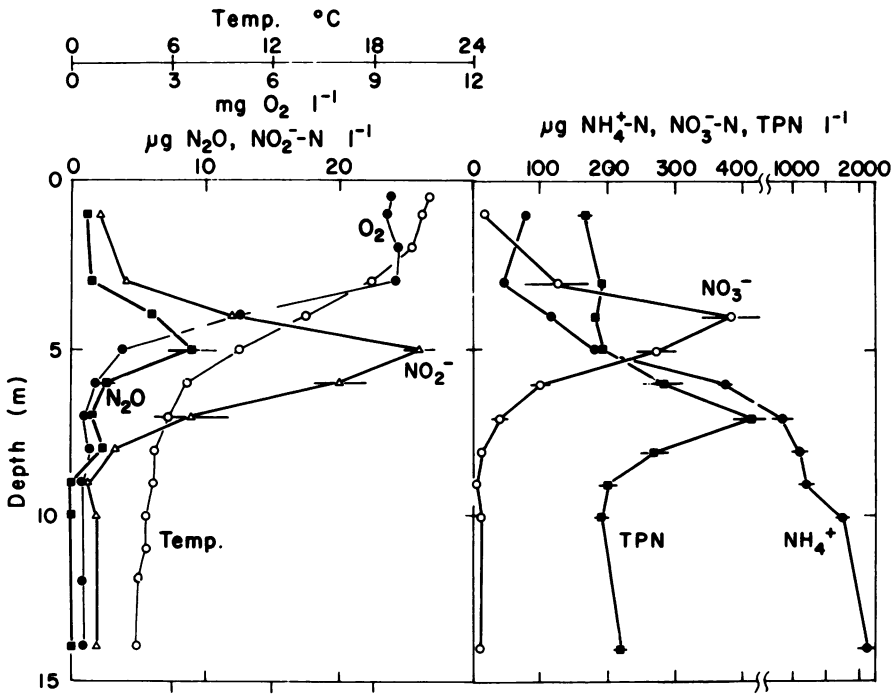

FIG. 3. Peaks of nitrogen oxides occurring in the metalimnion of Lake St. George, Ontario. Data are the means (± standard errors) of values observed between 21 June and 11 September (from reference 212). TPN, Total particulate nitrogen.

(102, 174). Denitrification activity is related to the organic matter content of the sediment (411) but is not nearly as well related to the trophic state of the lake as is the water column behavior (193).

For sediment denitrification to occur, NO_3^- must be present in the water column or must be produced at the sediment surface. There is ample evidence that nitrification frequently occurs when O_2 is present at the surface of marine (170) and freshwater epilimnetic and other sediments (6, 146). Under these conditions, denitrification can occur when the O_2 consumption rate of the sediment is sufficient to create anaerobiosis. The presence of up to 2 mg of O_2 liter^{-1} in the water may somewhat reduce denitrifying activity in the sediment (409), but activity is not significantly inhibited by even 6 to 8 mg of O_2 liter^{-1} (209, 402, 409); indeed, epilimnetic sediments may show greater denitrifying activity than hypolimnetic sediment (60). The simultaneous occurrence of nitrification and denitrification is supported by successful model simulations for marine sediment systems (168, 406).

Sediments which are overlain by anoxic waters tend to release NH_4^+ into the water column (6, 146) since nitrification cannot occur. Under these conditions denitrification is dependent on a supply of NO_3^- from the water column, and the depth of the layer which actively denitrifies depends on the rate of NO_3^- diffusion into the sediment. The diffusion of NO_3^- is an important factor, whether the overlying water is oxic or anoxic, and it is responsible for the observations that only the top 1 to 5 cm of sediment participate in the denitrifying activity (216, 287, 351, 409) and that the kinetics appears to be first order up to concentrations of 10 (4, 239, 287) or even 100 (351) μg of NO_3^--N ml^{-1}. Relatively high concentrations (up to 200 μg of N ml^{-1}) of NO_3^- can poise the Eh of a sediment at about +100 mV (192), inhibit methanogenesis, and cause an intensification of the oxidized surface brown zone (411). Indeed, it has been suggested that NO_3^- addition could be used to bring about the oxidation of surface sediment, reducing the solubility of phosphorus compounds and thus decreasing the exchange of phosphate with the water column (298, 336).

Because of the different redox potentials involved, denitrification in coastal marine sediment occurs in a zone above that at which SO_4^{2-} reduction is occurring (380). The accumulation of NO and N_2O which may occur between the two activity zones (379) is probably due to the inhibition of NO and N_2O reductases by sulfide (381, 395), which would prevent the complete denitrification of all nitrogen oxides to dinitrogen. Other products of the reduction of NO_3^- in sediments, NH_4^+ and organic nitrogen, may amount to only about 3 to 5% of the NO_3^- disappearing under some conditions (402, 410) but elsewhere they may be a very significant proportion, of the order of 20 to 70% in marine sediments (214, 215, 360, 377) as well as in lake sediments (202).

The temperature dependence of denitrification in aquatic systems has not been rigorously studied. However, in view of the generally cool conditions of many aquatic systems it is interesting that denitrification activity in lake sediments is reported not to vary with temperature in the range of 5 to 23°C (3, 4, 190). It is suggested that this may be due to denitrifying activity being limited by NO_3^- concentration and diffusion rather than by biochemical reactions (4).

The availability in sediments of O_2 for nitrification and of NO_3^- and NO_2^- for denitrification is affected by the activity of benthic infauna, including chironomids, tubificid and polychaete worms, and crustacea. The burrows and water-pumping activity of such animals increase the rate of exchange of O_2 and NO_3^- and stimulate both nitrification and denitrification (5, 63, 150, 169, 377). Benthic worms supported in glass beads also show denitrification activity, suggesting a role for the gut microflora in this process (63).

Reported estimates of denitrification rates in marine and lake sediments obtained by a great variety of methods (Table 5) range from low values of 0.042 and 0.14 mg of N m^{-2} day^{-1} for generally aerobic deep-sea sediments of the eastern Atlantic Ocean to values of the order of 100 mg of N m^{-2} day^{-1} for sediments of relatively rich eutrophic coastal and freshwater systems. Low rates associated with very extensive areas of the deep ocean and continental shelf can clearly assume significance in the global nitrogen cycle.

WASTE TREATMENT

Considerable interest has developed in the removal of nitrogen from organic and high-nitrate wastewaters to reduce the contamination and eutrophication of receiving waters. Nitrogen removal utilizes denitrification frequently coupled with nitrification, and such processes have been recently reviewed (118, 225, 260, 320, 361).

The direct addition of animal residues to soil, perhaps the most primitive disposal method, leads to the development of zones of mineralization, nitrification, and denitrification within the upper layers of soil and the accumulation of ethylene (44), methane, nitrogen, and nitrous oxide in the soil atmosphere (45, 49). The total nitrogen losses from ammonia volatilization and denitrification in a feedlot, for example, can amount to 700 to 2,830 kg of N ha^{-1} over a 2-year period (419). A study of sewage water

TABLE 5. Estimates of denitrification rates in aquatic sediments

System	Method	Rate, mg of N m^{-2} day^{-1} (μg of N g^{-1} day^{-1})	Reference(s)
Marine			
Deep east Atlantic	NO_3^- profiles	0.042	17
Deep northeast Atlantic	N_2 profiles	0.14	431
Bering Sea shelf	$^{15}NO_3^-$, flask	3.0	216
Alaska shelf	C_2H_2 inhibition	0.034–8.6	156
U.S. salt marsh	N_2 production	(7.2–36)	199
U.S. salt marsh	N_2/Ar, in situ enclosure	0–120	200
U.S. coastal	N_2 production, core	33.6	360
Japanese coastal	$^{15}NO_3^-$, flask	(0.05–3.7)	214, 215
Danish coastal	$^{15}NO_3^-$, bottle	2.0	287
Danish coastal	C_2H_2 inhibition, core	(0–3.8 ml^{-1})	379
Danish coastal	$^{15}NO_3^-$, bottle	(1–12 ml^{-1})	377
Danish coastal	C_2H_2 inhibition, core	14	378
Freshwater lakes			
Danish	N balance	0–129	3
Danish	NO_3^- disappearance	100–500	4
Swedish, unpolluted	$^{15}NO_3^-$, in situ enclosure	5–25	401
Swedish, eutrophic	$^{15}NO_3^-$, in situ enclosure	54–96	401, 402
Japanese river, sessile bacteria	$^{15}NO_3^-$, N_2/Ar ratio	8–16	276
Canadian, eutrophic	$^{15}NO_3^-$, in situ enclosure	15	60
Canadian, mesotrophic	C_2H_2 inhibition	2.4–7.2	61
Canadian stream	$^{15}NO_3^-$ balance, columns ± worms	50–90	63

applied to soil columns showed that there was a negative relationship between log(infiltration rate, in centimeters per day) and log(percentage of N removal). Over 80% removal of nitrogen was achieved with a cycle of 9 days flooded and 5 days dry, and an infiltration rate of 15 cm day^{-1} (226). Significant nitrogen removal rates, averaging 85%, can also be achieved by lagooning fish and meat-packing plant wastes with a 10.6-day retention time and intermittent aeration (261, 262), but the maintenance of appropriate oxygen concentrations is very difficult.

For the large-scale treatment of municipal or other wastes, more sophisticated processes are used, involving either suspended cultures or attached biofilm cultures (68), or a combination of both methods. Agitated stirred-sludge reactors are examples of suspended cultures. Conditions favoring nitrification and denitrification may be provided either in different regions of the same reactor or at different times (by intermittent aeration). Such systems utilize the endogenous carbon sources of the waste to support denitrification and, therefore, there is no primary sedimentation. High biomass is maintained in the reactor by a variable recycling of active sludge (68). The activity is controlled by such factors as the temperature, the carbon/nitrogen ratio of the influent waste, its content of organic and inorganic nitrogen, and the ratio of aerobic retention time/anaerobic retention time. The latter factor is important in matching the nitrification and denitrification activities and maintaining the respective biomass populations. Such an operation (having a capacity of about 6×10^5 m^3 day^{-1}) was described for the Vienna Blumental plant (242). Aeration times varied from 2 to 5 h day^{-1}, and the total nitrogen removal ranged between 60 and 80%. Higher contents of mixed-liquor suspended solids (6 to 7 kg m^{-3}) appeared to be advantageous to maintain denitrification rates. It was also necessary to control the oxygen supply according to the oxygen uptake rate in order to maintain nitrification and BOD_5 removal (242).

Since proper control of single-stage or single-sludge systems is difficult, the oxidation stage of nitrification and BOD removal is frequently separated from the anaerobic denitrification stage. In such systems the carbon and energy requirement for denitrification is provided by introducing some of the untreated waste (205) to give >2.3 mg of BOD mg NO_3^--N^{-1} (277) or by adding other sources of organic carbon. Methanol is the preferred source (206, 285), but it is expensive, and a recent survey of industrial carbonaceous wastes showed that distillery fusel oil supported three times more rapid a denitrification rate (0.33 mg of N denitrified mg mixed liquor volatile suspended $solids^{-1}$ day^{-1}) than did methanol (266). The design of the denitrification stage depends somewhat on the particulate

content of the NO_3^--containing influent. For high particulate wastes a stirred anaerobic fermentor may be appropriate, but if the particulate content is reasonably low, various types of packed-bed reactors may be used with upward or downward flow of the waste to be treated. The packing material used in such a reactor may be sand or gravel, and if the void volume is less than about 50%, it is referred to as an anaerobic submerged filter. Lighter packing materials, such as carbon, and greater upward flow rates give rise to a "fluidized bed" in which the packing is kept in suspension (68). In the absence of packing, 1- to 5-mm-diameter pellets of biomass may develop and remain suspended in an upward flow of 8.5 m h^{-1} (205). In all packed-bed reactors the packing becomes coated with a layer of biomass, known as a biofilm, of the order of 300 μm in thickness (333), which effectively increases the biomass retention time and permits liquid phase retention times as short as 3 h (223). However, too high a flow rate can result in washout of biomass (206). Rates of denitrification in these systems may be 10 times those attainable in suspended culture systems (124, 392). Nitrate usually penetrates the biofilm only partially, with the result that the kinetics are not zero order but more likely about 0.5 order (269, 392) because of the limitation of nitrate diffusion rate through the film. Figure 4 shows diagrammatically the zones which develop within a biofilm in a system which is nitrate limited, with organic carbon in excess, and the presence of some oxygen in the suspending fluid. Denitrification occurs only in that part of the anaerobic layer through which nitrate penetrates.

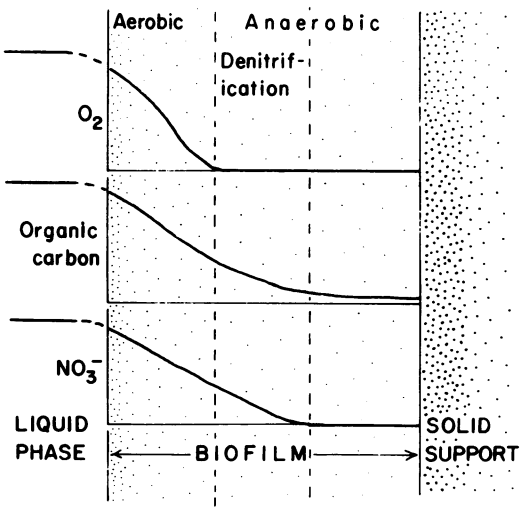

FIG. 4. Hypothetical distribution of O_2, organic carbon (in excess), and NO_3^- (limiting) within the biofilm of an attached growth reactor in which O_2 is present in the liquid phase. The penetration of the NO_3^- and the size of the zone of denitrification depend on the concentration of NO_3^- and its diffusion rate (after reference 160).

High nitrate concentrations (500 to 5,000 mg of NO_3^--N $liter^{-1}$) found, for example, in uranium oxide plant wastes can be treated effectively by using attached biofilm (123, 180) and packed-bed (25) and fluidized-bed reactors (124). Concentrated animal wastes, such as poultry manure, may have high concentrations of NH_4^+ which inhibit the oxidation of NO_2^- such that it is this oxide which is subsequently denitrified. This process is termed nitritification-denitritification, and it apparently proceeds at a higher rate than conventional nitrification-denitrification via NO_3^- and is therefore preferable (321). However, it does not appear to have been frequently applied.

The bacteria mainly responsible for denitrification in waste treatment systems are *Alcaligenes* (196), *Pseudomonas aeruginosa* (106), *Achromobacter* and *Bacillus* (369), and bacteria similar but not identical to *Moraxella* and *Acinetobacter* (106). The addition of methanol causes the enrichment largely of denitrifying *Hyphomicrobium* spp. (285) but consortia may also be involved. For example, a methanol-utilizing *Vibrio extorquens* produces citrate and isocitrate, which support the growth of denitrifying *Pseudomonas stutzeri* (332). A similar association can occur (but here separated in space or time or both) between aerobic methane-utilizing bacteria and *Pseudomonas stutzeri* (332). Facultatively methylotrophic denitrifiers which required growth factors were isolated from a low-temperature (5°C) process, further suggesting the importance of consortia in waste treatment systems (158). *T. denitrificans* enrichment cultures can be supported by elemental sulfur as energy source, and such a system is effective in removing NO_3^- from nitrified septic tank effluent. The effluent is passed through a 1:1 mixture of limestone (1-cm fragments) and lump sulfur (2-cm fragments) inoculated with *T. denitrificans*, and the following reaction occurs (367): $5S + 6KNO_3 + 2CaCO_3 \rightarrow 3K_2SO_4 + 2CaSO_4 + 2CO_2 + 3N_2$. However, it is pointed out that SO_4^{2-} contamination of ground water may limit the practical application of this system (367). A capacity for denitrification can be maintained for up to 2.5 years in anaerobic sludge and methanogenic enrichment cultures, which suggests that these systems contain denitrifying bacteria having alternative anaerobic modes of energy metabolism (201).

The contribution of waste treatment systems to atmospheric N_2O is of some concern, but there is little information available. It is note-

worthy, however, that fermentation wastewater acclimated to or supplemented with NO_3^- released small quantities of N_2O during denitrification whereas that adapted to or supplied with NO_2^- produced none (273, 274).

GLOBAL ASPECTS

The Nitrogen Cycle

Microbial denitrification is a major factor in the global nitrogen balance. However, because of the great variation in rates of denitrification in terrestrial and aquatic systems, and the inadequacy of methods for its measurement in nature, there are great uncertainties in estimates of global denitrification. Many estimates were arrived at by difference; that is, a value was assigned equal to the discrepancy between other estimates of inputs and outputs. Four selected recent compilations of global gaseous flux rates provide maximum and minimum values (Fig. 5). It is clear that the uncertainty in the denitrification estimates is greater than that associated with the other fluxes except for the biological N_2 fixation in aquatic systems. The high maximum estimate for aquatic denitrification is a result of an attempt to accommodate some high estimates of N_2O fluxes out of the oceans (153–155, 194) based on dissolved N_2O concentrations in surface waters, while assuming that (i) this N_2O is a result of denitrification and (ii) the mole fraction of N_2O in the products of denitrification is <0.2 or 0.3.

Denitrification and Atmospheric Chemistry

The stratospheric ozone layer above about 16 km altitude is important because it absorbs the potentially harmful UV component (280 to 320 nm) of solar radiation. It is subject to at least four hazards: emissions of water vapor and nitrogen oxides from supersonic jet planes or wide-bodied jets in the stratosphere (404), nuclear reactions in the stratosphere, upward diffusion of chlorofluoromethanes used in aerosol propellants and as refrigerants (432), and upward diffusion of tropospheric N_2O into the stratosphere. It is beyond the scope of this review to discuss these aspects in detail. However, it is clear that denitrification in many systems acts as a source of N_2O to the troposphere. In the stratosphere it undergoes the photochemical reaction $N_2O + O(^1D) \rightarrow 2NO$, and the NO produced then catalyzes the destruction of ozone as follows (83, 191, 152): $NO + O_3 \rightarrow NO_2 + O_2$; $NO_2 + O \rightarrow NO + O_2$. These reactions caused great concern that increases in the global usage of inorganic fertilizers, or indeed of any forms of nitrogen fixation, could lead to increased denitrification and production of N_2O. Early predictions suggested that

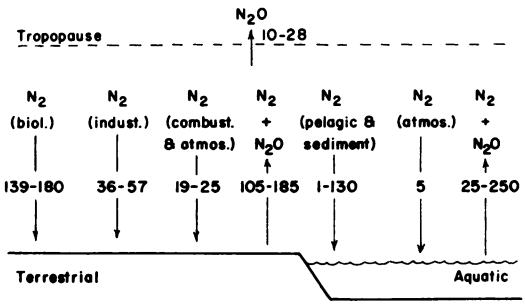

FIG. 5. Global gaseous fluxes in the nitrogen cycle. The minimum and maximum estimates shown are based on four compilations (84, 155, 322, 376). The units are teragrams per year.

>8% depletion of the O_3 concentration might occur (84, 252). However, evaluation of the potential threat is difficult for two reasons. First, new photochemical reactions of nitrogen oxides which lead to O_3 formation are being discovered (86):

$$\begin{aligned} NO + HO_2 &\rightarrow NO_2 + HO \\ NO_2 + h\nu &\rightarrow NO + O \\ O + O_2 + M &\rightarrow O_3 + M \\ \hline \text{net } HO_2 + O_2 &\rightarrow HO + O_3 \end{aligned}$$

Second, the quantitation of all sources and sinks of N_2O is far from complete (70, 93, 154, 235). In addition to the probably large but indeterminate source from denitrification, other sources include nitrification in terrestrial (22) and aquatic (439) systems, enteric bacteria (375), lightning (84), coal-utilizing and other power stations (316), automobile exhausts (70), nitric acid production by catalytic oxidation of ammonia (230), and the burning of biomass (85). Certain eutrophic aquatic systems can act as sinks for N_2O as mentioned earlier, but the major known natural sink is the photolysis of N_2O in the stratosphere (154; Fig. 5). These processes apparently result in a remarkably constant concentration of N_2O in the troposphere of 300 (357, 424) or 330 ppb by volume (70, 317, 318, 326, 327). Short-term diel fluctuations with an amplitude of about 30 ppb (by volume) were reported to indicate an important N_2O sink at the ground surface (38) but other diel studies could not confirm this (70). The results of a comprehensive series of troposphere measurements in the northern and southern hemispheres between 1976 and 1980 showed that the concentration of N_2O is increasing at about 0.2% per year (424). This increase is largely accounted for by combustion, suggesting that agricultural sources are less than has been estimated earlier (424).

CONCLUDING REMARKS

It is clear from the foregoing discussion that many aspects of the denitrification process and its significance are inadequately understood. The mechanisms by which the synthesis and activity of the reductases are regulated by such factors as O_2, the redox state of the electron carriers, or the nitrogen oxides themselves are not entirely clear. Furthermore, it remains debatable whether the reductases are subject to sequential or to coordinate derepression. There appears to be some diversity in the nature of the nitrogen oxide reductases, and of the electron transport components and pathways, between different genera and species. The very existence of a specific NO-reducing enzyme and the role of NO as an obligatory intermediate are questioned. Nitrous oxide, on the other hand, appears to be established as a free obligatory intermediate, but studies of the enzymology of N_2O metabolism are only now beginning. The concerted application of classical techniques, together with the newer methods of molecular genetics, should permit great advances in our understanding of the comparative biochemistry of denitrification.

Methodological inadequacies continue to dog those who study the occurrence of denitrification outside the laboratory. Despite some improvements in methods, the quantitation of denitrification in natural and manipulated terrestrial and aquatic systems remains a goal which is not yet achieved. The bacteria largely responsible for denitrification in aquatic systems are still virtually unknown.

A potential for the practical management of denitrification clearly exists. This is being exploited in various waste treatment processes for nitrogen removal in which denitrification is promoted in specially designed reactors. However, the universal application of denitrification in the tertiary treatment of municipal wastes is still a considerable way off. Agricultural management of denitrification to reduce nitrogen losses is at present dependent on the manipulation of soil conditions in such a way as to inhibit denitrification. However, with greater understanding of the basic mechanisms involved, it may yet become possible to promote the dissimilatory reduction of the ionic nitrogen oxides to ammonia rather than to the gaseous products which are otherwise lost to the atmosphere.

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