

## CHAPTER 1

# *A Bird's Eye View of Denitrification in Relation to the Nitrogen Cycle*

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## 1.1 Introduction

Nitrate and nitrite are key players in the broad nitrogen biogeochemical cycle that is fundamental for life processes. These oxo-anions are involved in key pathways and in the turnover/recycling of nitrogen in the atmosphere, hydrosphere and geosphere. From a human perspective, nitrite and nitrate have a “Janus face”: they are important as food preservatives,<sup>1</sup> but are also implicated in oncogenic problems due to the formation of *N*-nitrosamines.<sup>2–6</sup> On the other hand, nitrite is now considered to be beneficial to health, whether formed endogenously or added therapeutically. The chapter will identify all of the components that are involved and put into context all of the diverse pathways that contribute to the nitrogen biogeochemical cycle (Figure 1.1). Emphasis will be given to the denitrification pathway—the actors, the mechanisms

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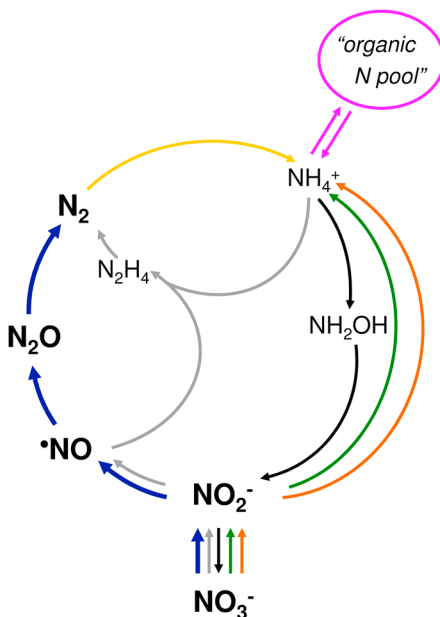
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Metalloenzymes in Denitrification: Applications and Environmental Impacts

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**Figure 1.1** Biochemical cycle of nitrogen. Denitrification, blue arrows; dinitrogen fixation, yellow arrow; assimilatory ammonification, orange arrows; “organic nitrogen pool”, pink arrows; dissimilatory nitrate reduction to ammonium, green arrows; nitrification, black arrows; anaerobic ammonium oxidation, grey arrows.

and the regulation of these “simple” chemical reactions—always keeping in mind the environmental and (bio)technological applications.

## 1.2 The Nitrogen Cycle

Hydrogen, oxygen, carbon and nitrogen are absolutely essential for life. Nitrogen is the fourth most abundant element in living organisms,<sup>7</sup> being used for the biosynthesis of *N*-containing compounds (amino acids, nucleosides and other fundamental compounds). Two nitrogen assimilatory pathways provide the reduced nitrogen that is recycled and stored in an “organic nitrogen pool”. In addition, some organisms also use nitrogen compounds as substrates for “respiration” and, for that purpose, several nitrogen dissimilatory pathways have also evolved. Nitrogen is fixed mainly from the largest pool, atmospheric dinitrogen (containing about 70% of all nitrogen) and is recycled through the dissimilatory pathways.<sup>8–12</sup>

## 1.3 Classic and New Pathways

Two nitrogen *assimilatory* pathways provide the reduced nitrogen (ammonium) that is essential for biosynthetic purposes (Figure 1.1, yellow and orange arrows). Free-living archaea, bacteria and symbiotic bacteria are

capable of dinitrogen fixation (yellow arrow) in a reaction that is catalysed by molybdenum/iron-dependent nitrogenases,<sup>13–15</sup> a reaction widely occurring in soils, oceans and the Earth's crust. Less commonly, vanadium/iron or iron-only enzymes are involved. Nitrate assimilation is dependent on the transformation of nitrate into nitrite, catalysed by molybdenum-dependent nitrate reductases,<sup>16,17</sup> and then on the reduction of nitrite to ammonium, catalysed by sirohaem-containing nitrite reductases.<sup>18–25</sup> The enzymes share similarities in prokaryotic and eukaryotic organisms.<sup>26–28</sup> In bacteria, fungi and plants, ammonium enters the “organic nitrogen pool” (Figure 1.1, pink arrows) in the form of two amino acids—glutamine and glutamate—through the concerted action of the enzymes glutamine synthase and glutamate synthase. Ammonium can also be directly incorporated into glutamate through the glutamate dehydrogenase that is present in all forms of life and is also relevant in amino acid catabolism.

The organic nitrogen, in the form of amino and amide groups, can be transferred, transformed and incorporated into other amino acids and several other nitrogen-containing biomolecules (Figure 1.1, pink arrows). The organic nitrogen is then recycled between all living organisms through food chains, waste products and organic decay. Mineralisation also takes part in the process of returning ammonium back to the environment.

The biogeochemical cycle of nitrogen continues with four well-understood nitrogen *dissimilatory* pathways, in which nitrogen compounds are used as electron donors/acceptors to derive energy *via* respiration.

### 1.3.1 Dissimilatory Nitrate Reduction to Ammonium and Nitrification

Dissimilatory reduction of nitrate to ammonium (DNRA; Figure 1.1, green arrows) is used for anaerobic growth. In this pathway, nitrite is directly reduced to ammonium using a multi-*c*-type haem-containing nitrite reductase.

Nitrifiers use the “opposite” aerobic oxidation of ammonium to nitrite (and to nitrate) for the chemoautotrophic fixation of inorganic carbon (nitrification; Figure 1.1, black arrows). Hence, DNRA and nitrification can be regarded as alternative short-circuits within the vast dinitrogen reservoir.<sup>29,30</sup> Aerobic ammonium-oxidising bacteria use ammonium almost exclusively as the electron donor in “respiration” and oxidise it to hydroxylamine (catalysed by ammonium monooxygenase) and then to nitrite (catalysed by hydroxylamine oxidoreductase; Figure 1.1, black arrows). In addition, archaea (from marine and soil environments) seem able to oxidise ammonium for chemolithoautotrophic growth.<sup>31–34</sup>

Nitrite-oxidising bacteria are responsible for the final oxidation of nitrite to nitrate through a reaction that is catalysed by a molybdenum-containing nitrite oxidoreductase (Figure 1.1, black arrow). These bacteria are chemolithoautotrophs that derive energy from the oxidation of nitrite to nitrate in a strictly aerobic process. Here, all of the carbon that is needed is satisfied by carbon dioxide assimilation (*via* the Calvin cycle).<sup>35–41</sup>

Nitrite oxidation is a relevant step of the nitrification pathway and probably the main source of nitrate under aerobic conditions. However, as described below, nitrite is also oxidised through other pathways.

### 1.3.2 Anaerobic Oxidation of Ammonium to Dinitrogen

Ammonium can also be oxidised anaerobically *via* the anaerobic ammonium oxidation (AnAmmOx) pathway (Figure 1.1, grey arrows) and is used for anaerobic chemoautotrophic growth of planctomycetes.<sup>42–48</sup> In this pathway, ammonium is first oxidised by nitric oxide (NO) to yield hydrazine in a reaction that is catalysed by the di-haem-*c*-containing enzyme hydrazine synthase. Hydrazine is then oxidised to dinitrogen by the octa-haem-*c*-containing enzyme hydrazine oxidoreductase.<sup>44,49–51</sup> The necessary NO is formed *via* nitrite reduction by a *cd*<sub>1</sub> haem-containing nitrite reductase, which is a well-described enzyme of the denitrification pathway. In this way, AnAmmOx is coupled to nitrite reduction. Recently, a bacterial strain KSU-1 that follows this pathway was hypothesised to contain a copper-containing nitrite reductase enzyme (which is also a well-known enzyme of the denitrification pathway), suggesting that the enzymatic machinery might be more flexible than initially thought.<sup>52,53</sup>

This AnAmmOx pathway is presently one of the challenging and unknown features of the nitrogen biogeochemical cycle, operating in marine, freshwater and terrestrial ecosystems.

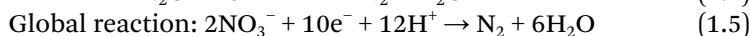
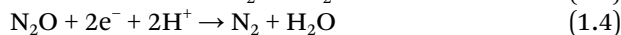
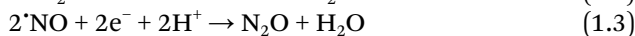
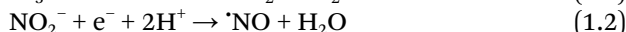
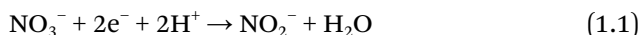
### 1.3.3 Anaerobic Reduction of Nitrate to Dinitrogen—Denitrification

This book is devoted to the denitrification pathway, an anaerobic process that is used by a wide range of microorganisms for energy generation in which nitrate, present in the soil or water, is reduced to gaseous dinitrogen. Nitrate accumulation and the release of nitrous oxide into the atmosphere due to the excessive use of fertilisers in agriculture are examples of two environmental problems in which denitrification plays a central role.

The denitrification<sup>54,55</sup> metabolic pathway is found in a wide range of organisms, from archaea to eubacteria, and even in some eukaryotes (*e.g.*, fungi, protozoa and benthic *Foraminifera* and *Gromiida*).<sup>55–57</sup> It occurs in both autotrophic and heterotrophic organisms, coupled to the anaerobic oxidation of organic carbon.

The reduction of nitrate to dinitrogen gas (eqn (1.5)) is accomplished by five different types of metalloenzymes in four surprisingly simple steps (eqn (1.1)–(1.4)): nitrate is reduced to nitrite, then to NO, then to nitrous oxide and finally to dinitrogen. A battery of enzymes is utilised, namely the reductases of nitrate, nitrite, NO and nitrous oxide. There are also multiple electron donors that interact with these enzymes to transfer the electrons that

are required in these stepwise reactions.<sup>54,58–60</sup> The importance of the pathway has inspired efforts to understand the structures of the participating enzymes and their enzymatic mechanisms.



The four sequential steps involve three one-oxygen atom abstractions (eqn (1.1), (1.2) and (1.4)) and a one-oxygen atom transfer coupled with N–N bond formation (eqn (1.3)). Each step is catalysed by specific metalloenzymes whose 3D structures have all been determined.<sup>54,59</sup>

The metal diversity detected in this pathway is acknowledged, being a delight in bioinorganic chemistry. A dependence upon molybdenum, iron and copper is observed. Two different types of nitrite reductases (eqn (1.2)) can be found: an iron-dependent enzyme, featuring *c*-type and *d*<sub>1</sub>-type haem centres (*cd*<sub>1</sub> haem-containing nitrite reductase) and a copper-dependent enzyme (copper-containing nitrite reductase).<sup>54,57,58,61,62</sup> The initial step of nitrate transformation to nitrite (eqn (1.1)) is carried out by nitrate reductase, which is part of the bis-pyranopterin guanosine dinucleotide-containing family of mononuclear molybdo-enzymes.<sup>63</sup> The last two steps of denitrification use exquisite machineries. NO reductase (eqn (1.3)) contains both haem iron and non-haem iron, building up an active site that resembles cytochrome *c* oxidase (*i.e.*, is copper/iron dependent).<sup>64–66</sup> Nitrous oxide reductase (eqn (1.4)), the last enzyme of this pathway, contains a novel tetranuclear copper centre (Cu<sub>4</sub>), which is a unique and challenging structure in biology.<sup>67,68</sup>

All of the diverse pathways interact and close the nitrogen biogeochemical cycle (Figure 1.1). The fixed nitrogen is eventually lost to the atmosphere, soils, crust and oceans.

### 1.3.4 New Avenues/New Challenges

In addition to the well-recognised four nitrogen dissimilatory pathways, several new processes have been identified, including anaerobic phototrophic nitrite oxidation,<sup>69,70</sup> microaerobic nitrification/AnAmmOx coupling,<sup>69–75</sup> hyper-thermophilic dinitrogen fixation/methane production<sup>76</sup> and “denitrification/intra-aerobic methane oxidation”.<sup>77,78</sup>

Over recent years, the discovery and addition of new organisms participating in the nitrogen cycle and the scrutiny of new ways to transform the nitrogen oxo-anions have added more complexity to this biogeochemical cycle, revealing an intricate network of pathways. The regulation and control of these complex pathways is still a challenging problem, and future research will certainly clarify some of the presently established dogmas.

## 1.4 Book Outlook

This book, devoted to metalloenzymes in denitrification, aims to give a concise update of the multiple and complex aspects of denitrification. Two initial overarching chapters (Chapter 1—A Bird's Eye View of Denitrification in Relation to the Nitrogen Cycle, by Isabel Moura, Luisa Maia, Sofia R. Pauleta and José J. G. Moura; and Chapter 2—Nitric Oxide Production, Damage and Management During Anaerobic Nitrate Reduction to Ammonia, by Jeffrey A. Cole) will provide a general overview of the subject, placing the denitrification pathway in the nitrogen cycle and connecting some of its substrates and products, such as nitrate and nitrite, with the other pathways of the nitrogen cycle. The role of NO as a by-product of these enzymes, and its role as a signalling molecule, as well as the new functions of proteins such as Hcp and YtfE, is reviewed.

The following chapters will cover the key enzymes and bioinorganic aspects of denitrification, including nitrate reductase (Chapter 3—Structure, Function and Mechanisms of Respiratory Nitrate Reductases, by Pablo J. Gonzalez, Maria G. Rivas and José J. G. Moura), iron-dependent nitrite reductase (Chapter 4—Nitrite Reductase—Cytochrome *cd*<sub>1</sub>, by Serena Rinaldo, Giorgio Giardina and Francesca Cutruzzolà), copper-dependent nitrite reductase (Chapter 5—Structure and Function of Copper Nitrite Reductase, by Masaki Nojiri), NO reductase (Chapter 6—Structure and Function of Nitric Oxide Reductases, by Takehiko Tosha and Yoshitsugu Shiro) and nitrous oxide reductase (Chapter 7—Insights into Nitrous Oxide Reductase, by Sofia R. Pauleta, Cíntia Carreira and Isabel Moura). The book will also cover the efforts that have been made to model the active sites and the reactions involved (Chapter 8—Model Compounds for Molybdenum Nitrate Reductases, by Amit Majumdar and Sabyasachi Sarkar; Chapter 9—Model Compounds for Nitric Oxide Reductase, by Abhishek Dey and Somdatta Ghosh Dey; and Chapter 10—Model Compounds of Copper-Containing Enzymes Involved in Bacterial Denitrification, by Brittany J. Johnson and Neal P. Mankad). Electron transfer and molecular recognition in denitrification and nitrate dissimilatory pathways will be addressed in Chapter 11 by Rui M. Almeida, Simone Dell'Acqua, Sofia R. Pauleta, Isabel Moura and José J. G. Moura. Aspects on nitrate and nitrite transport will also be covered (Chapter 12—Channels and Transporters for Nitrogen Cycle Intermediates, by Susana L. A. Andrade and Oliver Einsle), as well as the regulation of denitrification (Chapter 13—Regulation of Denitrification, by Stephen Spiro). The denitrification pathway in fungi and Gram-positive bacteria will also be addressed (Chapter 14—Denitrification in Fungi, by Hirofumi Shoun and Shinya Fushinobu; and Chapter 15—Denitrification and Non-Denitrifier Nitrous Oxide Emission in Gram-Positive Bacteria, by Yi-hua Sun, Paul De Vos and Kim Heylen). Emphasis will also be given to the environmental impact, water treatment (biotechnology) and modelling/mitigating of its negative impacts (Chapter 16—Denitrification Processes for Wastewater Treatment, by Bing-Jie Ni, Yuting Pan, Jianhua Guo, Bernardino Virdis, Shihu Hu, Xueming Chen and Zhiguo Yuan). Finally, the novel human

nitrate/nitrite/NO signalling pathway will be discussed within the nitrogen cycle scenario (Chapter 17—Lessons from Denitrification for the Human Metabolism of Signalling Nitric Oxide, by Luísa B. Maia and José J. G. Moura).

The book will update the state of the art and new aspects on the structure and function of the enzymes involved in denitrification, the regulation of this pathway, mechanistic revelations/implications and the efforts on the chemical modelling of the reaction centres. Applied aspects are an added value in respect to environment, regulation, measurement and mitigation.

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