# UNDERSTANDING THE MICROBES RESPONSIBLE FOR NITROGEN TRANSFORMATIONS

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## Abstract

When pondering the numerous chemical transformations of nitrogen, it may surprise some that the majority of those transformations occur exclusively inside living bacterial cells. Those wishing to better understand the details of the transformations of nitrogen must develop at least a basic understanding of taxonomy (i.e., the classification system of living organisms), the factors that bacteria face over time, and how they react to the presence of oxygen in their environment. Diatomic nitrogen gas ($N_2$) is the most abundant free element in the Earth's atmosphere and makes up about 78% of it. Nitrogen is part of various vital components of organisms, including proteins, amino acids, DNA, RNA and in the energy currency of the cell, adenine triphosphate (ATP). The chemistry of the nitrogen atom is unique and the range of nitrogen containing compounds have properties from inert to active in nature. The nitrogen cycle plays a significant role in the biogeochemical cycle by which nitrogen is converted into multiple chemical forms as it circulates among atmospheric, terrestrial, and aquatic ecosystems. In general, the nitrogen cycle has five steps: Nitrogen fixation ($N_2$ to $NH_3$/ $NH_4^+$), Nitrification ($NH_3$ to $NO_3^-$), Assimilation (Incorporation of $NH_3$ and $NO_3^-$ into biological tissues), and Ammonification (organic nitrogen and any chemical including $NH_2$ groups which are converted into ammonia ($NH_3$) or its ionic form, ammonium ($NH_4^+$) as an end product. Biological nitrogen reduction is, ironically, unfathomable until one comes to understand the organisms involved in the process and the microbial ecology of the nitrogen cycle.

## Introduction

The fields of onsite/decentralized wastewater are positioned between multiple major branches of natural science such as biology, chemistry and physics. Consequently, the theories, methods and approaches of these foundational sciences must be transferred and adopted by the onsite and decentralized wastewater disciplines. I call the fields of onsite and decentralized wastewater crossroad disciplines; a phrase I mistakenly thought I had coined (Sherman, 2008). Further research has shown a rich literature using the term already existed (Jacobs and Spillman, 2005).

Working in a crossroad discipline is both challenging and rewarding. To be sure, regardless of the new practitioner's prior experiences and background, significant new ideas and concepts will be need to also be mastered. People from a variety of backgrounds are attracted to this field because

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they recognize terms or ideas that they are familiar with in their past, but there are many more concepts that are foreign to them. Regardless of prior experience and knowledge, no one is fully prepared to perform their jobs duties without assistance. Continuous training and additional research are essential to understand the process and operation of decentralized biological wastewater treatment.

Individuals with a formal education must be prepared to broaden their areas of expertise. The flower of backgrounds (Sherman, 2015) displays all the subjects that touch on the fields of onsite and decentralized wastewater (in dark brown in the center of the flower).

Another way of describing the situation is, if you can't possibly come into the field 'knowing it all' your success will depend on a commitment to life-long learning. Here is the good news: each petal on the flower in Figure 1 has a subset of critical concepts that will be used repeatedly in the onsite and decentralized field. Understanding those concepts will give practitioners the tools they need to thrive.

For example, in my career I took one course in economics. In 30+ years, the essential information I have used in the field repeatedly from my single economics course is 1) buy low – sell high, 2) the invisible hand of Adam Smith 3) fair competition puts the price for a product at its optimum for long-term stability and 4) markets fail when monopolies dominate.

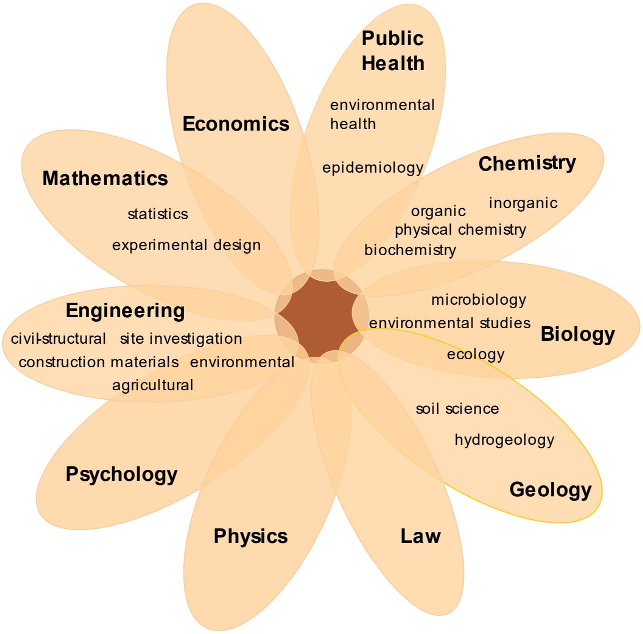


Figure 1. Flower of backgrounds after Sherman, 2015

## A primer in Biology

It will take a bit longer to describe the essential information in Biology. I should disclose that I crawled onto the flower of backgrounds from this petal, so my opinion is biased. The first subject to focus on is taxonomy. Taxonomy is the science of naming, describing and classifying living things. Living things are categorized in multiple steps starting with the most inclusive term, Domain. There are three Domains of living things, namely Archaea, Bacteria, and Eukarya. Each domain can be subdivided into groups called Kingdoms. A Kingdom can be subdivided into Phyla (singular is phylum). Phyla get further subdivided into Classes. For example, mammals are all found in the class Mammalia. Classes can be subdivided to orders and then to families. The last two designations of taxonomy are genus and species. When an organism is described, it is a two-word designation of the particular genus and species. The genus name begins with a capital letter. The species name is in lowercase throughout. The plural term for genus is genera. This word is from Latin. Multiple species in the same genus are given the designation of spp. Both genus and species are formatted in *italics*.

As an example, lets fully describe the taxonomy of a cottontail rabbit. The domain is Eukarya. The kingdom is Animal. The phylum is Chordata. The class is Mammalia. The order is Lagomorpha. The family is Leporidae (rabbits and hares). The genus is *Sylvilegus.* There are 27 species in this genus. The eastern cotton tail rabbits' scientific name is *Sylvilegus floridana*.

Let's next discuss the Biology basics for the one Domain that is the main subject of this paper, namely bacteria. Bacteria can be distinguished from Eukarya by the lack of a nucleus surrounding the genetic material in their cells. The genetic material bacteria use is almost exclusively coded using RNA (ribonucleic acid).

There are misconceptions regarding bacteria and other microbes that should be dispelled here. One is the relative size of bacteria and virus. The media often speak of bacteria and virus as a duo. The use of the term bacteria and virus can, in the minds of the man and woman on the street, allow comparisons to comedy duos like Laurel and Hardy; Abbot and Costello or Key and Peele. It gives people the inference that these two groups have common ground. They don't. The difference in their sizes is approximately 10,000 times. In an easy-to-understand analogy, if a virus were as large as an American football, a bacteria would be the size of a football field (goal line to goal line and sideline to sideline).

Bacteria also have very short lifespans compared to larger organisms. Bacteria divide their cells somewhere between once every 12 minutes to once every 24 hours. So, the average lifespan of a bacterium is around 12 hours or so, before the cell either divides asexually into two identical copies or perishes.

A defining criterion of bacteria is how they react to oxygen exposure (Table 1). Bacteria react to the presence of oxygen in their environment either positively, indifferently or negatively (sometimes fatally).

Table 1: Oxygen classifications for bacteria After: Prakash & Renade, Eds. 2021

| Group name | Definitions | Environment(s) where found | Example species |
|---|---|---|---|
| Obligate (strict) aerobe | Require abundant (22%) oxygen | Animal skin, Fast flowing streams | *Neisseria meningitides*<br>*Bacillus subtilis* |
| Microaerophile | Require oxygen concentration 1-10% | Ruminant animal gut | *Campylobacter jejuni* |
| Aerotolerant anaerobe | Indifferent to oxygen. Not used in metabolism | Drains, marshlands, swamps | *Streptococcus* spp.<br>*Lactobacillus* spp. |
| Facultative aerobe/anaerobe | Have dual metabolic pathways. Can switch between them | Fluctuating aerobic – anoxic - anaerobic | *Escherichia coli*<br>*Staphylococcus aureus* |
| Strict (obligate) anaerobe | Exposure to oxygen causes instant death | Decaying matter, petroleum seeps | *Clostridium perfringens*<br>*Bacteroides* spp.<br>*Methanobrevibacter smithii* |

## Microbial dark matter

Microbial dark matter consists of microbes that microbiologists have so far been unable to isolate in pure culture in the laboratory. Strict anaerobes are notoriously difficult to culture in the lab and require advance instrumentation such as anaerobic Glovebox, GasPac or Anoxomat. Often only mixed cultures of obligate anaerobes are produced. The slower growing strains cannot be cultured due to lack of knowledge of or inability to supply their required growth conditions in the lab.

Microbial dark matter is unrelated to the dark matter of physics and cosmology, but is so-called for the difficulty in effectively studying microbes as a result of their inability to be cultured by current methods. It is difficult to estimate the relative magnitude of microbial dark matter, but the accepted gross estimate is that roughly one percent of microbial species in a given ecological niche are culturable. Consequently, we are unaware of 99 percent of the microbial species in nature. In recent years, progress has been made in culturing bacteria, primarily because of the valuable industrial chemicals produced by obligate anaerobic species.

## A primer in Chemistry

The focus of this paper is the atom Nitrogen and its chemistry. The atomic number (number of protons) of Nitrogen is 7. The atomic weight of nitrogen (sum of protons and neutrons) is 14.01 (Figure 3). Electrons are deemed to have zero weight. The inner shell of electrons (called S) contains two of nitrogen's 7 electrons. The second shell of electrons (called D) contains 5 of 8 possible spots filled by the remaining electrons (Figure 1). There are five valence electrons in the Nitrogen atom.

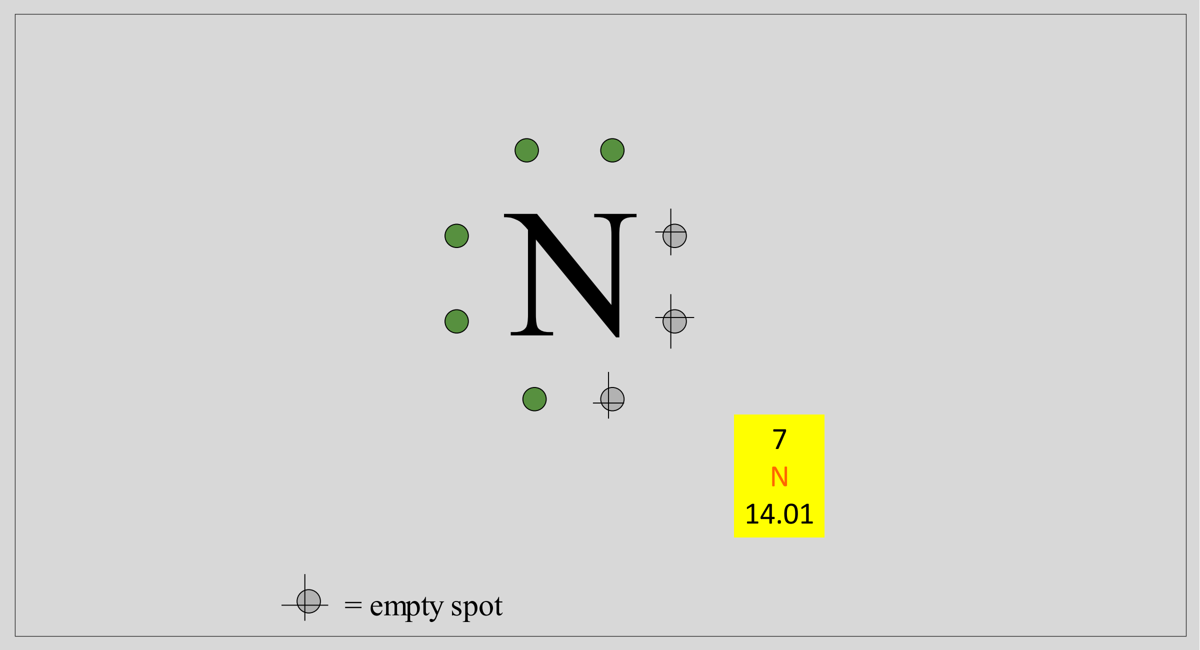
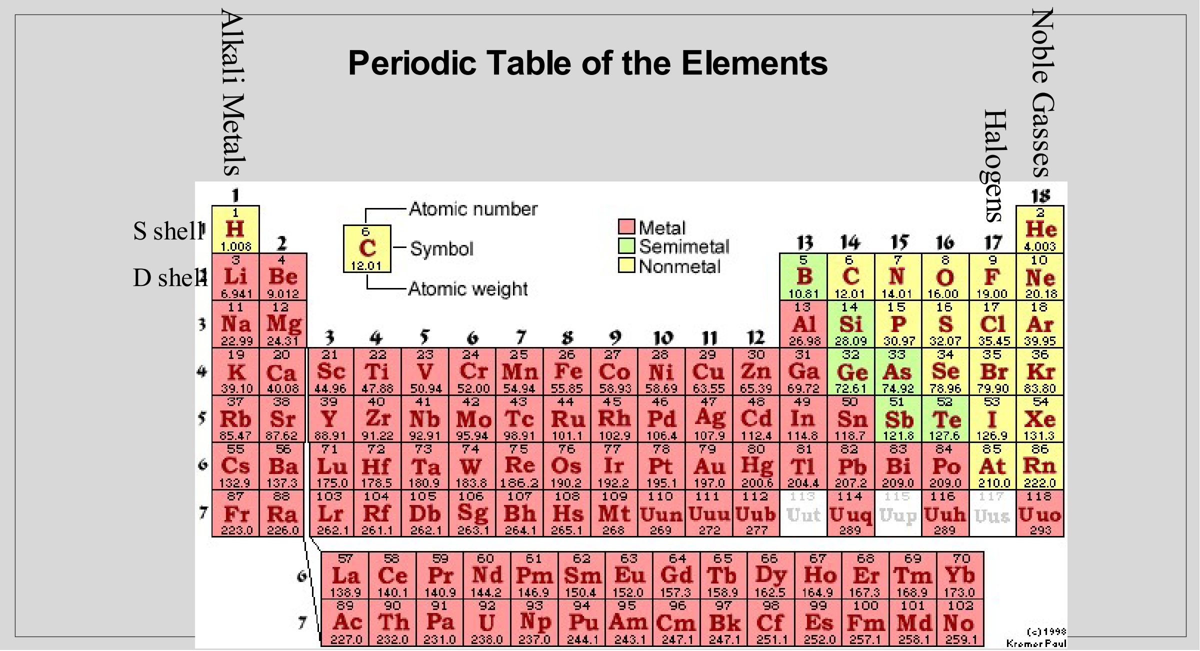


Figure 2. Lewis electron dot diagram for an atom of nitrogen



Periodic Table of the Elements

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S shell 1 | 1 H 1.008 | | | | | | | | | | | | | | | | | 2 He 4.003 |
| D shell 2 | 3 Li 6.941 | 4 Be 9.012 | | | | | | | | | | | 5 B 10.81 | 6 C 12.01 | 7 N 14.01 | 8 O 16.00 | 9 F 19.00 | 10 Ne 20.18 |
| 3 | 11 Na 22.99 | 12 Mg 24.31 | | | | | | | | | | | 13 Al 26.98 | 14 Si 28.09 | 15 P 30.97 | 16 S 32.07 | 17 Cl 35.45 | 18 Ar 39.95 |
| 4 | 19 K 39.10 | 20 Ca 40.08 | 21 Sc 44.96 | 22 Ti 47.88 | 23 V 50.94 | 24 Cr 52.00 | 25 Mn 54.94 | 26 Fe 55.85 | 27 Co 58.93 | 28 Ni 58.69 | 29 Cu 63.55 | 30 Zn 65.39 | 31 Ga 69.72 | 32 Ge 72.61 | 33 As 74.92 | 34 Se 78.96 | 35 Br 79.90 | 36 Kr 83.80 |
| 5 | 37 Rb 85.47 | 38 Sr 87.62 | 39 Y 88.91 | 40 Zr 91.22 | 41 Nb 92.91 | 42 Mo 95.94 | 43 Tc 98.91 | 44 Ru 101.1 | 45 Rh 102.9 | 46 Pd 106.4 | 47 Ag 107.9 | 48 Cd 112.4 | 49 In 114.8 | 50 Sn 118.7 | 51 Sb 121.8 | 52 Te 127.6 | 53 I 126.9 | 54 Xe 131.3 |
| 6 | 55 Cs 132.9 | 56 Ba 137.3 | 71 Lu 175.0 | 72 Hf 178.5 | 73 Ta 180.9 | 74 W 183.8 | 75 Re 186.2 | 76 Os 190.2 | 77 Ir 192.2 | 78 Pt 195.1 | 79 Au 197.0 | 80 Hg 200.6 | 81 Tl 204.4 | 82 Pb 207.2 | 83 Bi 209.0 | 84 Po 209.0 | 85 At 210.0 | 86 Rn 222.0 |
| 7 | 87 Fr 223.0 | 88 Ra 226.0 | 103 Lr 262.1 | 104 Rf 261.1 | 105 Db 262.1 | 106 Sg 263.1 | 107 Bh 264.1 | 108 Hs 265.1 | 109 Mt 268 | 110 Uun 269 | 111 Uuu 272 | 112 Uub 277 | 113 Uut | 114 Uuq 289 | 115 Uup | 116 Uuh 289 | 117 Uus | 118 Uuo 293 |

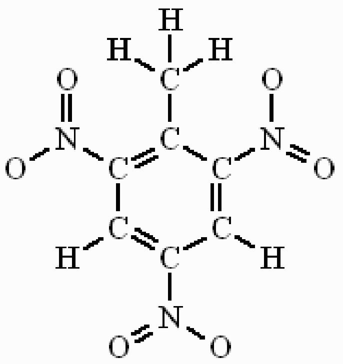
| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 6 | 57 La 138.9 | 58 Ce 140.1 | 59 Pr 140.9 | 60 Nd 144.2 | 61 Pm 146.9 | 62 Sm 150.4 | 63 Eu 152.0 | 64 Gd 157.3 | 65 Tb 158.9 | 66 Dy 162.5 | 67 Ho 164.9 | 68 Er 167.3 | 69 Tm 168.9 | 70 Yb 173.0 |
| 7 | 89 Ac 227.0 | 90 Th 232.0 | 91 Pa 231.0 | 92 U 238.0 | 93 Np 237.0 | 94 Pu 244.1 | 95 Am 243.1 | 96 Cm 247.1 | 97 Bk 247.1 | 98 Cf 251.1 | 99 Es 252.0 | 100 Fm 257.1 | 101 Md 258.1 | 102 No 259.1 |

Figure 3. Periodic Table of the Elements (Nitrogen is found on top of column 15)

Valence electrons predict the chemical reactions Nitrogen will make with other atoms. The goal for every element it to reach a full electron shell such as those of Noble Gasses on the far-right column of the periodic table. If nitrogen were able to lose 5 valence electrons, it would have the full valence electrons of Helium (2S). If nitrogen were able to gain three electrons, the D shell electrons would be eight as in the Noble Gas Neon (8D).

Nitrogen is found in 7 oxidation states. Oxidation state is a hypothetical charge of an atom in a compound. It shows the total number of electrons which have been removed or added to an element to get to its present state. It indicates the degree of oxidation (loss of electrons) or reduction (gain of electrons) of an atom.

Depending on the other atoms it is combined with, nitrogen compounds could be nonreactive (also called inert). However, other compounds can be explosive. The structural formula of TNT is shown to the left (Figure 4). TNT is an acronym for Tri Nitro



Toluene. The compound is composed of 7 Carbons, 5 Hydrogens, 3 Nitrogens and 6 Oxygens. The chemical formula is $C_7H_5N_3O_6$. In a compound, the subscript after the element is the number of them e.g.: $H_2O$, or water is called H two O (2 hydrogens, 1 oxygen).

When a nitrogen atom is found in an environment where hydrogen is abundant and oxygen is absent, it can take the electrons from three hydrogen atoms and fill Nitrogen's D shell, making the compound ammonia (Figure 5). Ammonia has a pH of 11 in a one molar solution. In an aqueous environment (in water), ammonia will protonate and take another hydrogen atom, making ammonium ($NH_4^+$).

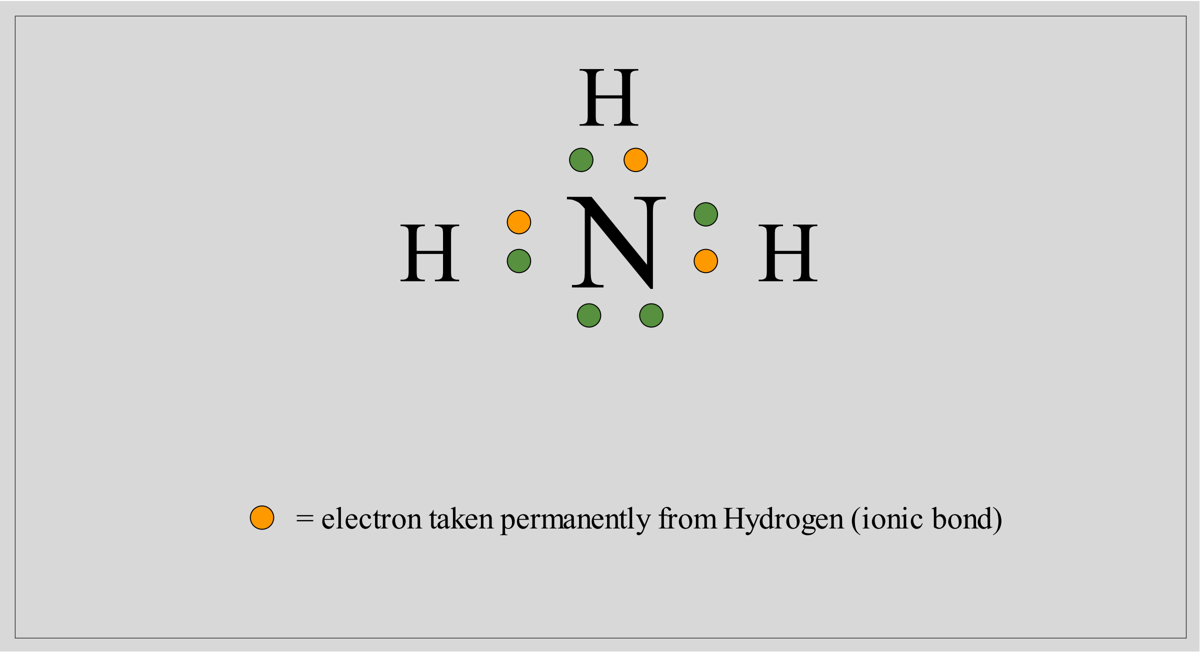


Figure 5. Lewis electron dot diagram for the nitrogen compound $NH_3$ (ammonia)

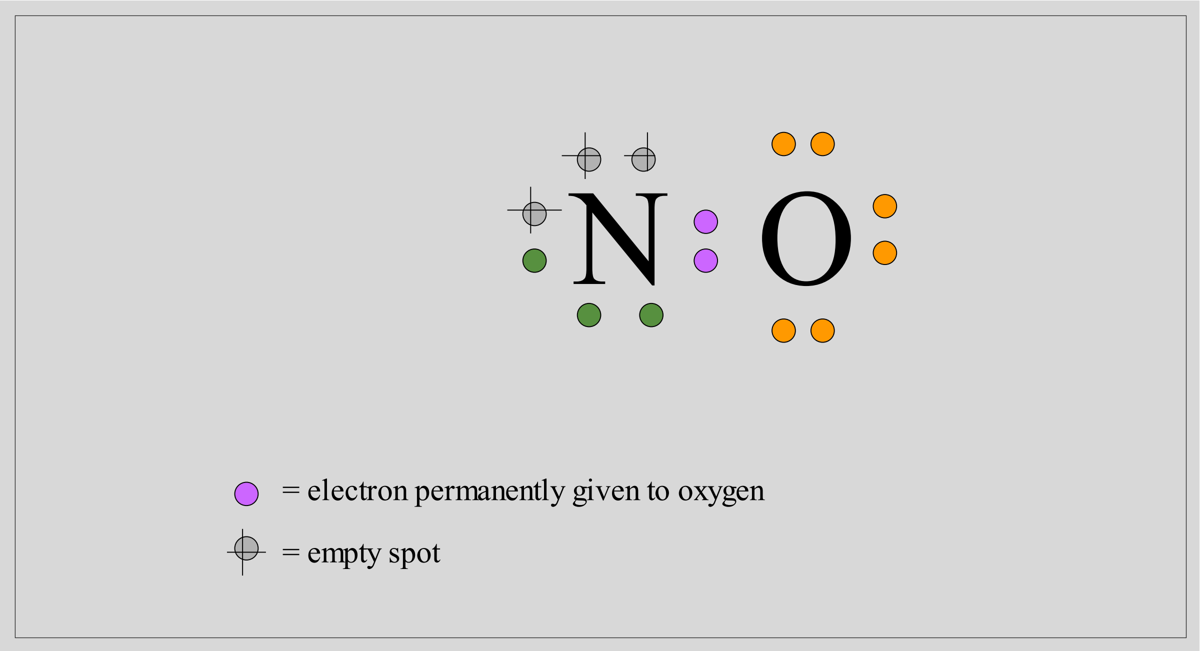


Figure 6. Lewis electron dot diagram for the nitrogen compound NO (Nitric Oxide)

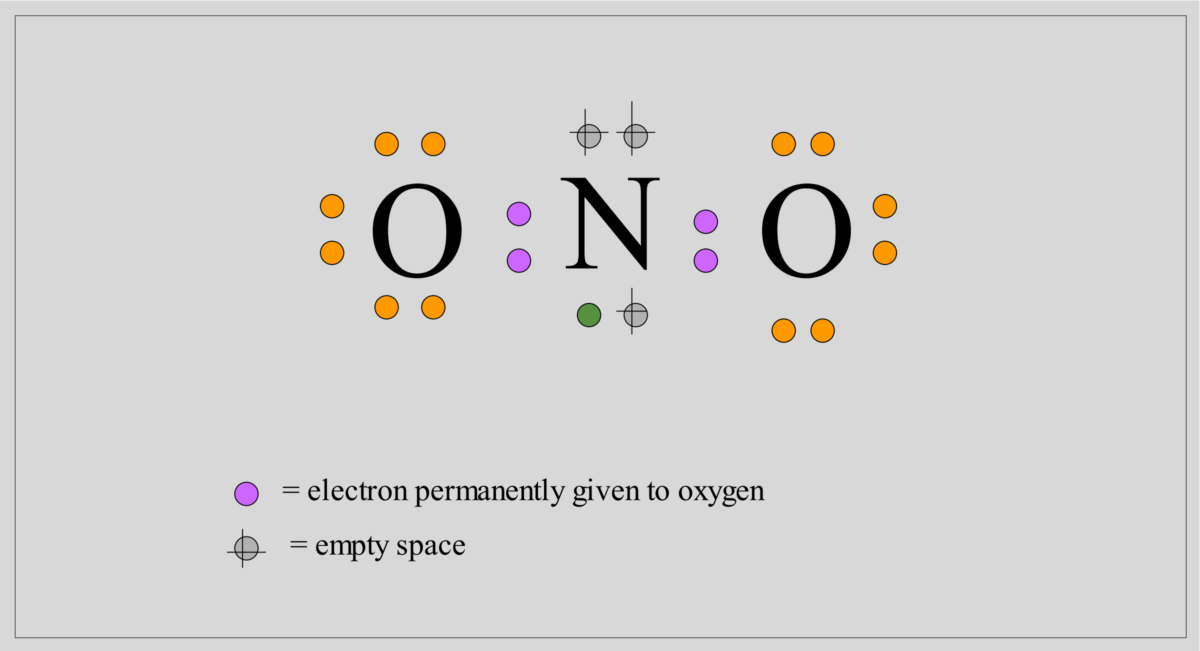


Figure 7. Lewis electron dot diagram for the nitrogen compound $NO_2$ (Nitrite)

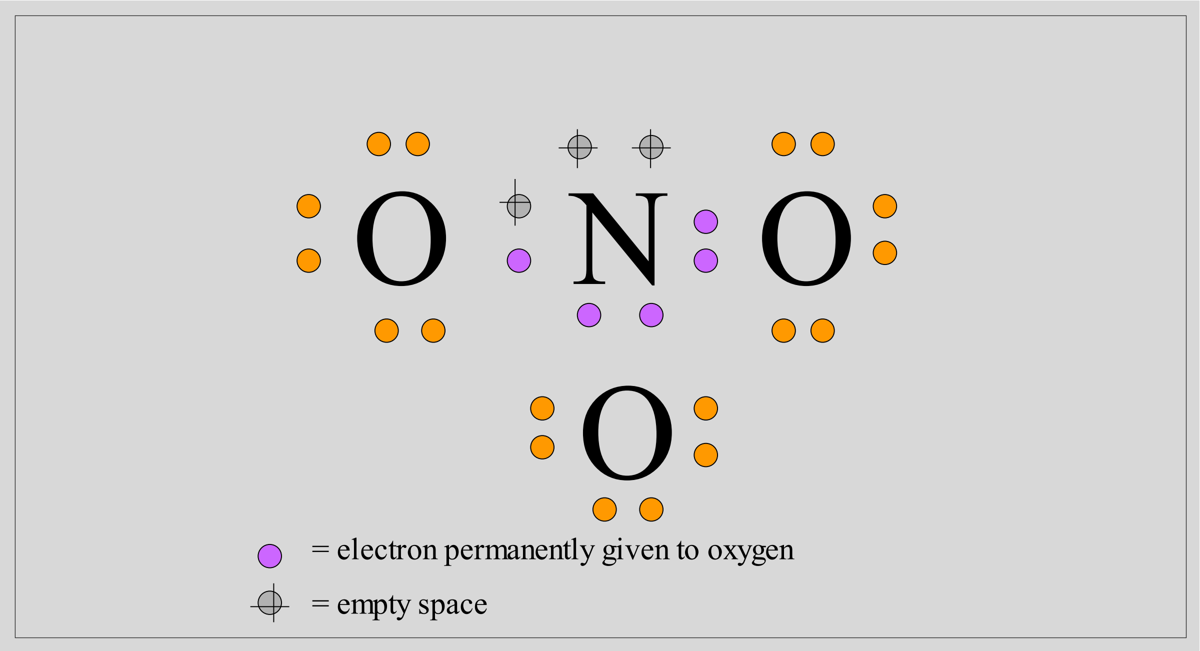


Figure 8. Lewis electron dot diagram for the nitrogen compound $NO_3$ (Nitrate)

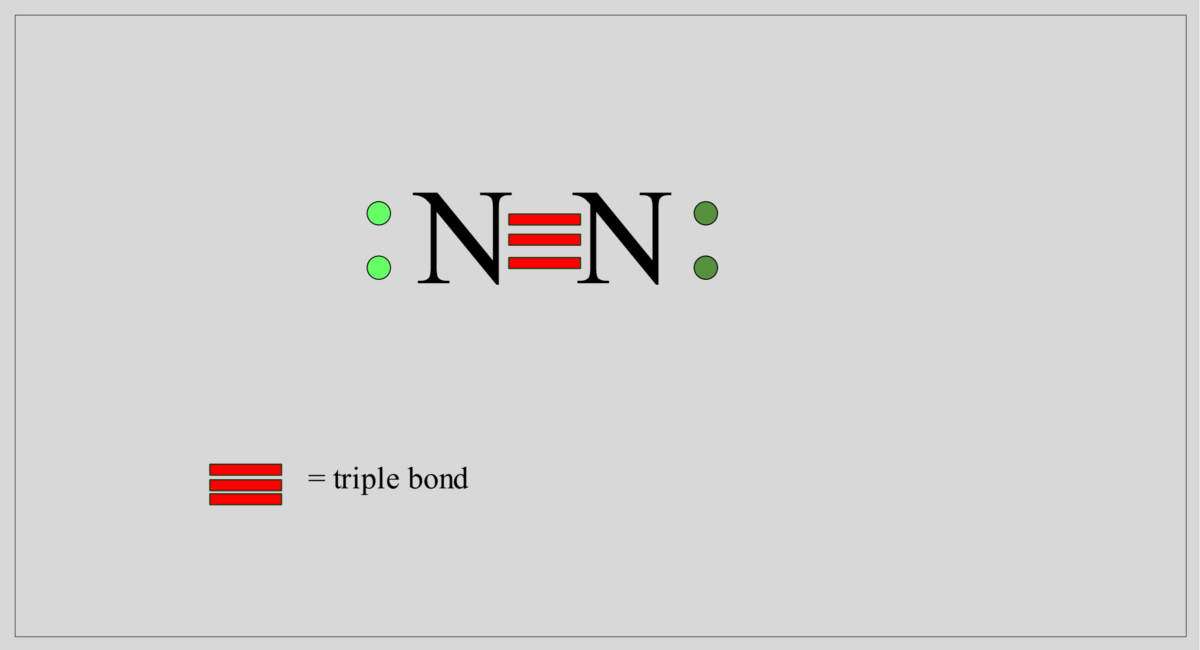
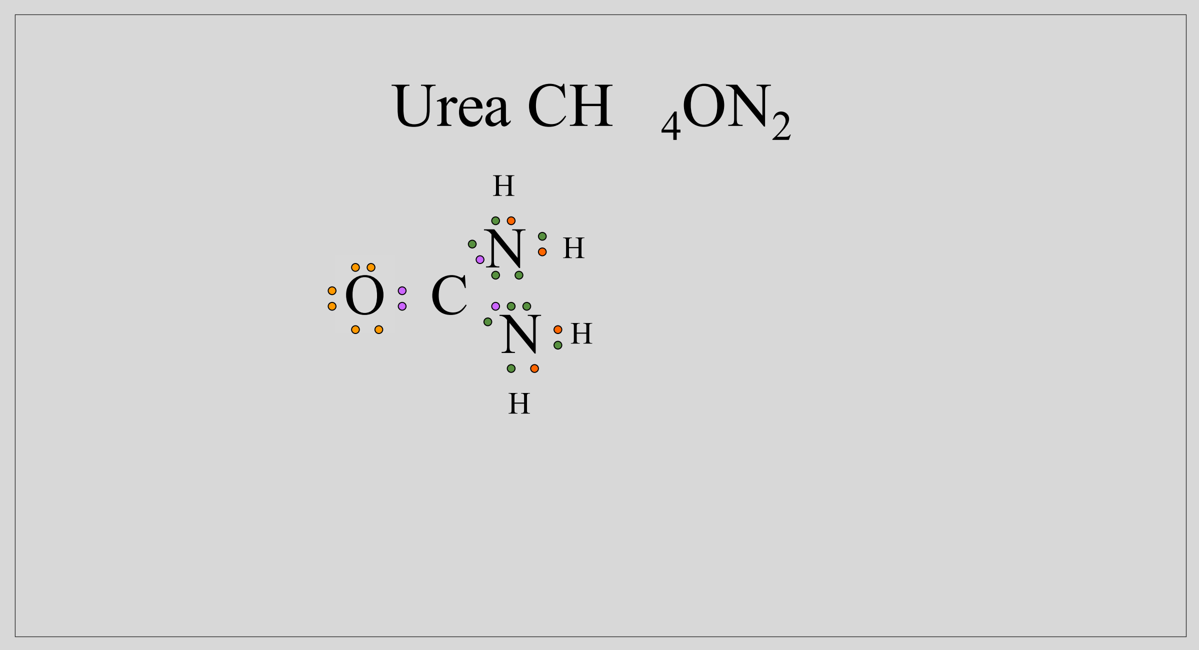


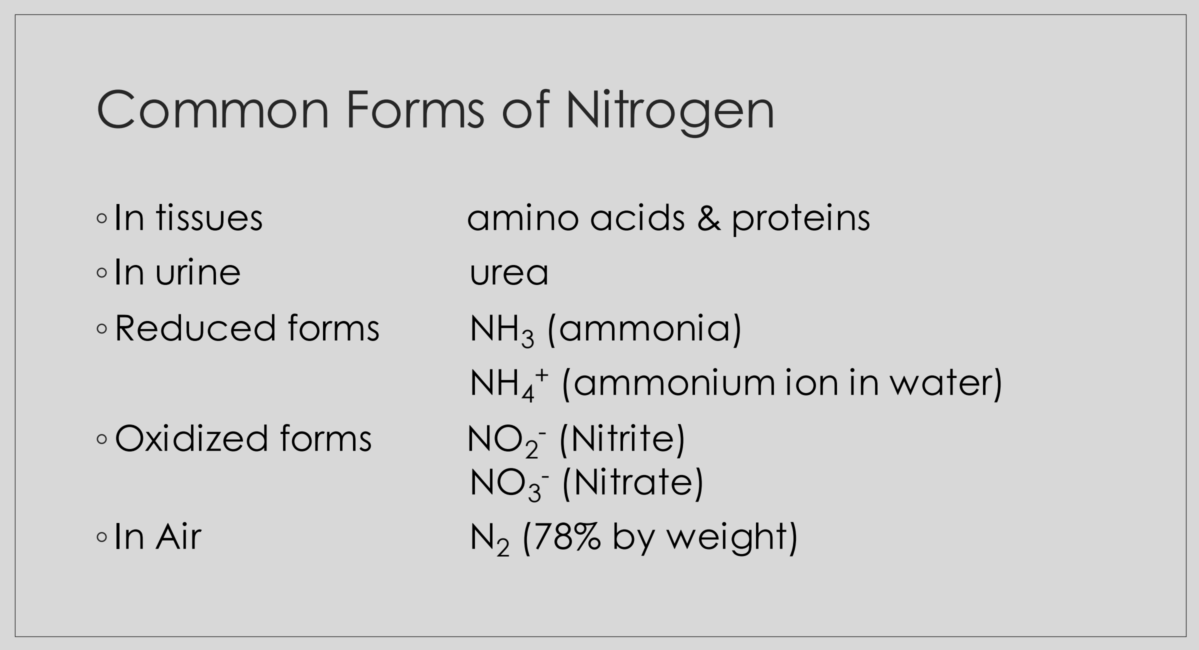
Figure 9. Lewis electron dot diagram for the nitrogen compound $N_2$ (diatomic Nitrogen gas)



Urea $CH_4ON_2$

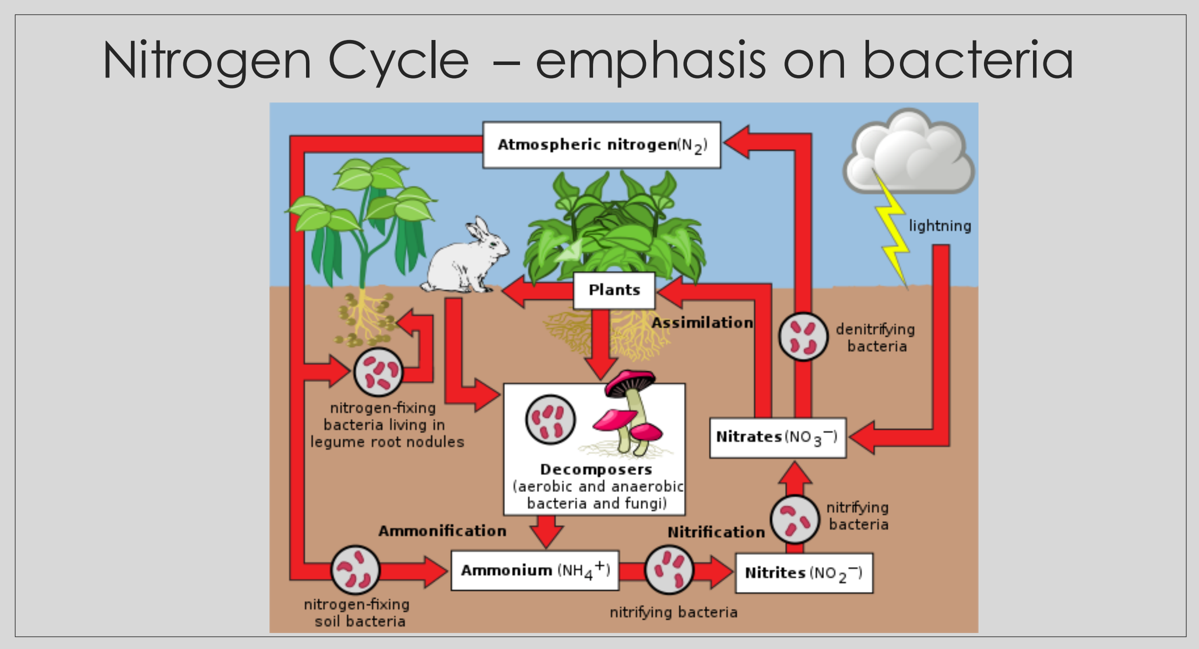
Figure 10. Lewis electron dot diagram for the nitrogen, carbon. oxygen and hydrogen compound $CH_4ON_2$ (urea)

Table 2: Common forms of Nitrogen



## Common Forms of Nitrogen

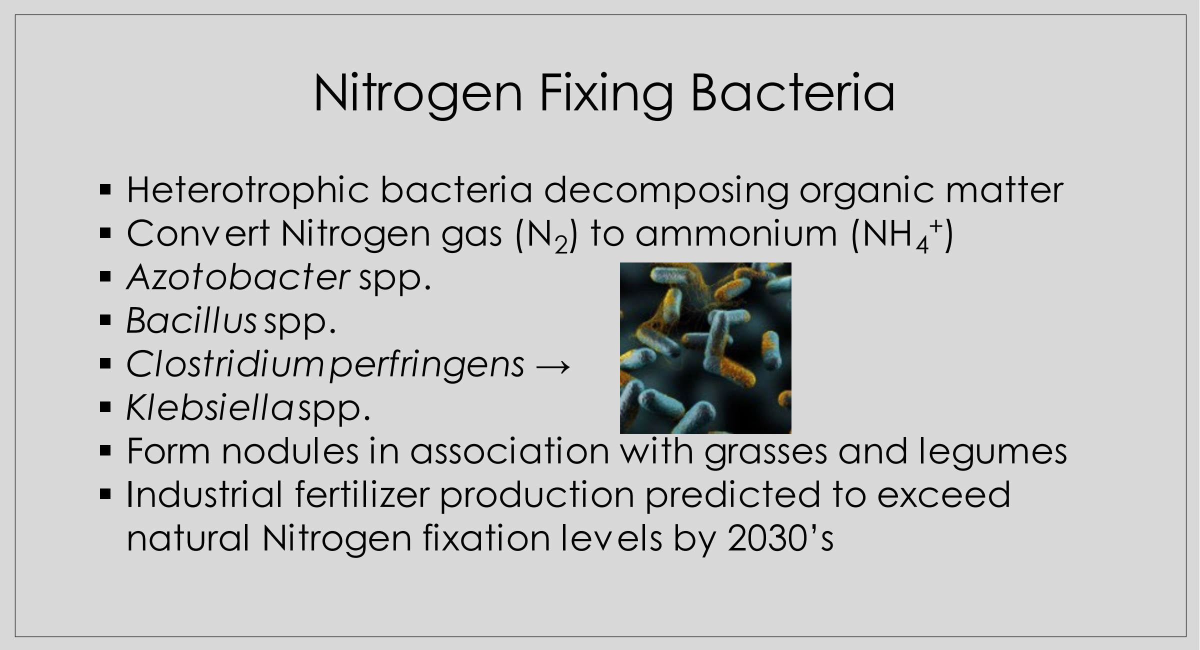
| | |
|---|---|
| ◦ In tissues | amino acids & proteins |
| ◦ In urine | urea |
| ◦ Reduced forms | $NH_3$ (ammonia)<br>$NH_4^+$ (ammonium ion in water) |
| ◦ Oxidized forms | $NO_2^-$ (Nitrite)<br>$NO_3^-$ (Nitrate) |
| ◦ In Air | $N_2$ (78% by weight) |



Nitrogen Cycle – emphasis on bacteria

Figure 11. Nitrogen cycle (Mid slide and bottom on left nitrogen fixing bacteria, right bottom nitrifying bacteria upper right denitrifying bacteria)

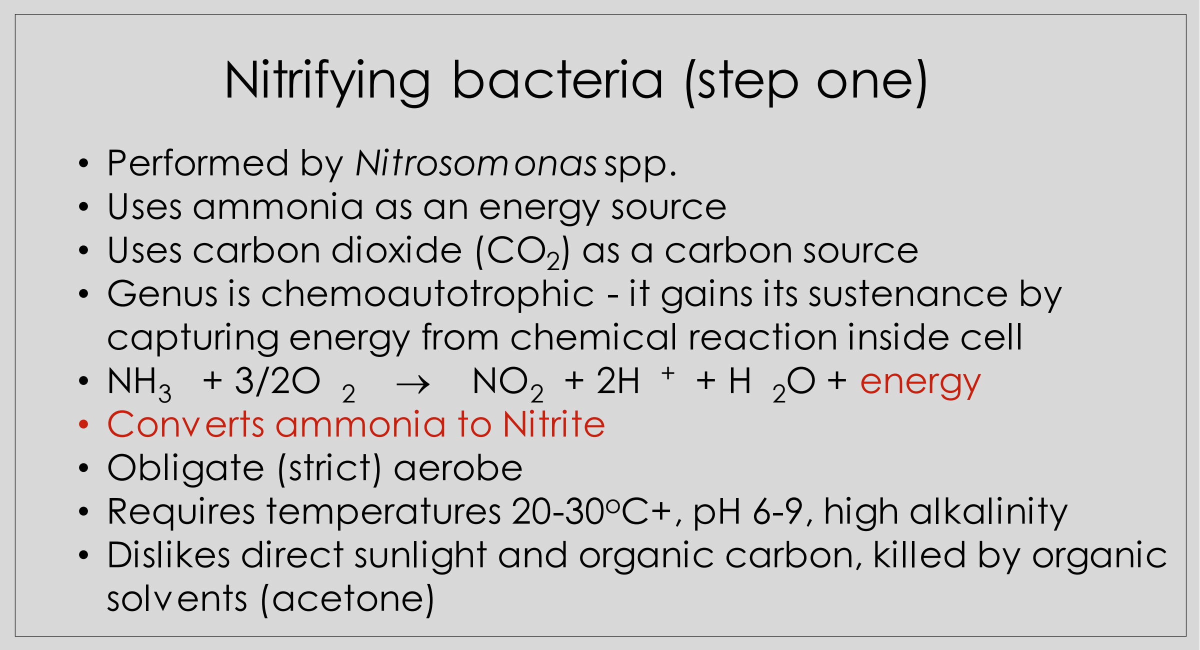
Most nitrogen cycle figures don’t emphasize that all transformations except lightning occurs inside a bacterial or fungal cell.  Many different bacterial types are involved in transforming nitrogen.



## Nitrogen Fixing Bacteria

- Heterotrophic bacteria decomposing organic matter
- Convert Nitrogen gas ($N_2$) to ammonium ($NH_4^+$)
- *Azotobacter* spp.
- *Bacillus* spp.
- *Clostridium perfringens* →
- *Klebsiella* spp.
- Form nodules in association with grasses and legumes
- Industrial fertilizer production predicted to exceed natural Nitrogen fixation levels by 2030's

Figure 12. Nitrogen Fixing Bacteria summary



## Nitrifying bacteria (step one)

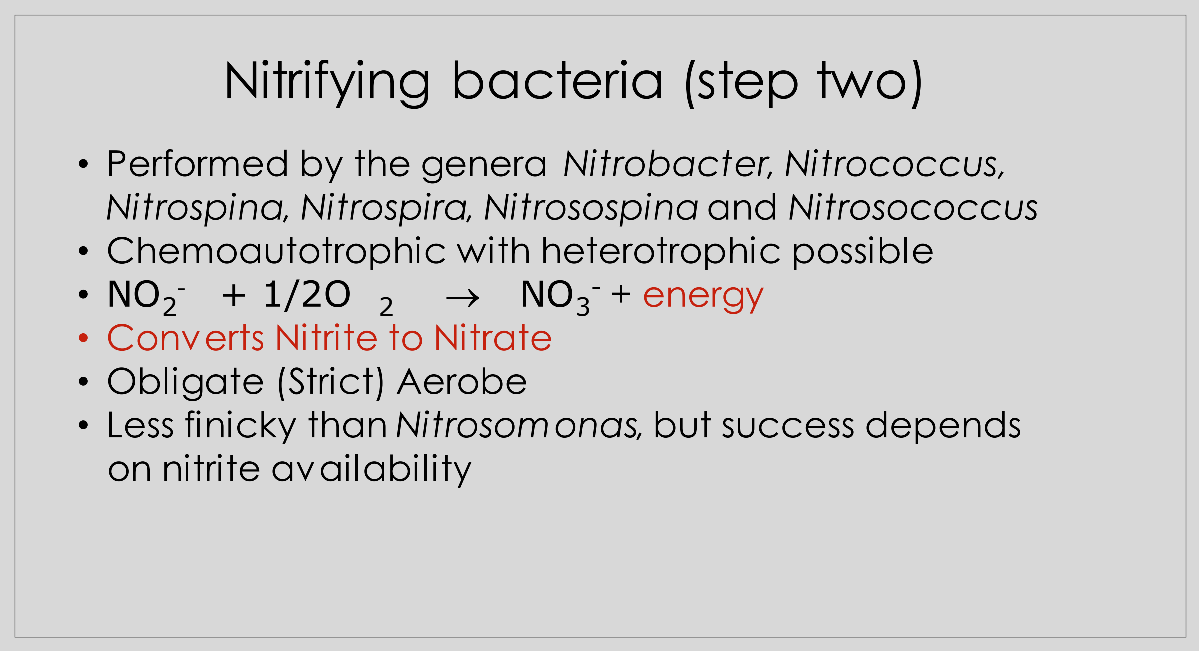
- Performed by *Nitrosomonas* spp.
- Uses ammonia as an energy source
- Uses carbon dioxide ($CO_2$) as a carbon source
- Genus is chemoautotrophic - it gains its sustenance by capturing energy from chemical reaction inside cell
- $NH_3 + 3/2O_2 \rightarrow NO_2 + 2H^+ + H_2O +$ energy
- Converts ammonia to Nitrite
- Obligate (strict) aerobe
- Requires temperatures 20-30°C+, pH 6-9, high alkalinity
- Dislikes direct sunlight and organic carbon, killed by organic solvents (acetone)

Figure 13. Nitrifying bacteria (step one)

The genus *Nitrosomonas* consists of 10 known species. Typical environments are soils, lakes, streams, estuaries, oceans and salt lakes. The type species of the genus (first to be described and accepted) was *Nitrosomonas europaea*. It was discovered on a limestone grave marker shielded from sunlight by a lichen by Winogradsky in 1892. This genus is photophobic. They usually generate a biofilm matrix, or form clumps with other microbes, to avoid direct sunlight.

Table 3: Ten known species of the genus Nitrosomonas with details (see Koops et. al, 1991)

| **Species name** | **Preferences or environment** | **Motile or sedentary** | **Has genome been sequenced?** |
|---|---|---|---|
| N. aestuarii | Requires salt, uses urea | | |
| N. communis | Soils | | |
| N. europaea | Soils and fresh water | | yes |
| N. eutropha | High ammonia tolerant | Motile through flagellum | |
| N. halophila | Requires salt | Motile through flagellum | |
| N. marina | Requires salt, uses urea | | |
| N. nitrosa | Requires low ammonia, uses urea | | |
| N. oligotropha | Requires low ammonia, uses urea | | |
| N. stercoris | Composted cattle manure | | |
| N. ureae | Uses urea | | yes |



## Nitrifying bacteria (step two)

- Performed by the genera *Nitrobacter, Nitrococcus, Nitrospina, Nitrospira, Nitrosospina* and *Nitrosococcus*
- Chemoautotrophic with heterotrophic possible
- $NO_2^- + 1/2O_2 \rightarrow NO_3^-$ + energy
- Converts Nitrite to Nitrate
- Obligate (Strict) Aerobe
- Less finicky than *Nitrosomonas*, but success depends on nitrite availability

Figure 14 Nitrifying bacteria (step two)

## Discussion

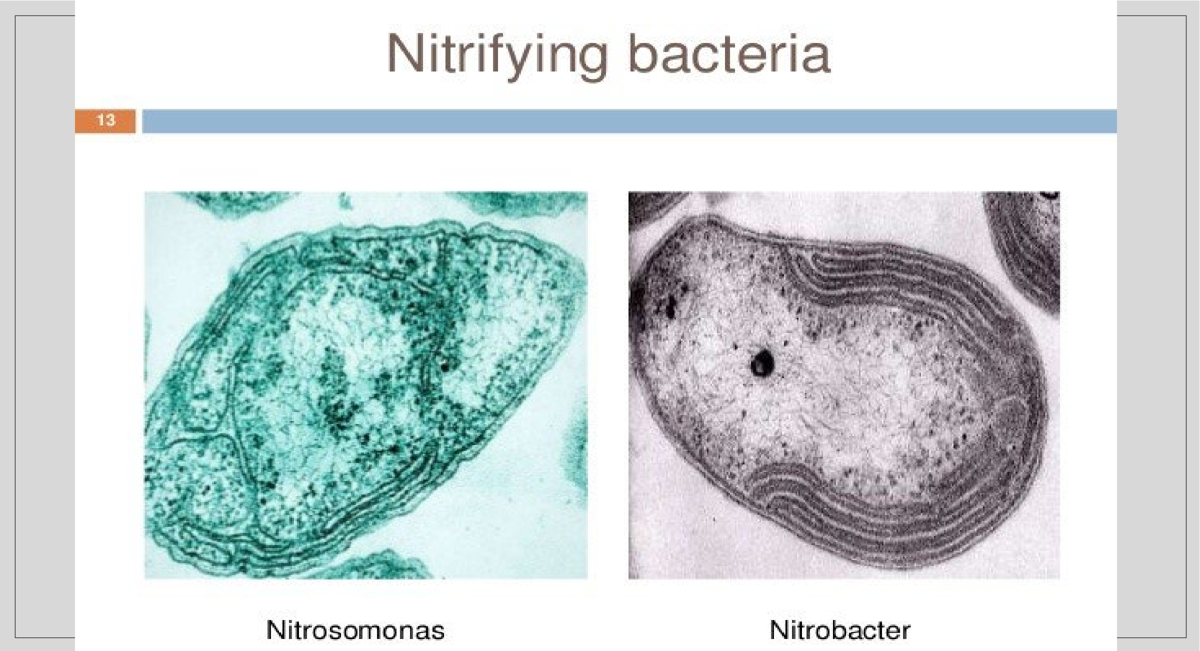
To obtain a complete understanding of how nitrifying bacteria function, we must use information that comes from the field of Physical Chemistry. Physical chemistry is the branch of chemistry that deals with the study of macroscopic and microscopic phenomena in chemical systems in terms of the principles, practices, and concepts of physics such as motion, energy, force, time, thermodynamics, quantum chemistry, statistical mechanics, analytical dynamics and chemical equilibria.

Chemicals have a measurable quantity of energy associated with them. Chemical reactions transforming one compound to another will either require energy expenditure or will release energy. In the case of nitrifying bacteria (part 1 and part 2) both bacterially mediated reactions yield energy. The amount of energy released can be tracked by using the Gibbs Free Energy for the chemicals in question. A negative Gibbs Free Energy means the reaction will yield energy.

Table 4: Gibbs Free Energies for Ammonia, Nitrite and Nitrate and the net energy gained per step

| Chemical name | Gibbs Free Energy | Net energy gained (max.) |
|---|---|---|
| Ammonia | -16.4 kJ/mole | |
| Nitrite | -32.2 kJ/mole | -15.8 kJ/mole |
| Nitrate | -111.3 kJ/mole | -79.1 kJ/mole |

The analogy I use to describe what nitrifying bacteria are doing is think of the helical spring toy slinky perched at the top step of basement stair. When the slinky is pointed down the stairs it will convert potential energy (elevation) into kinetic energy (motion) by dropping lower down the stairs. In the case of nitrifying bacteria, only a fraction of the total energy released by the transformation will be captured inside the cell. The payoff for the nitrite to nitrate conversion (step two) is five times greater than the first step.



## Nitrifying bacteria

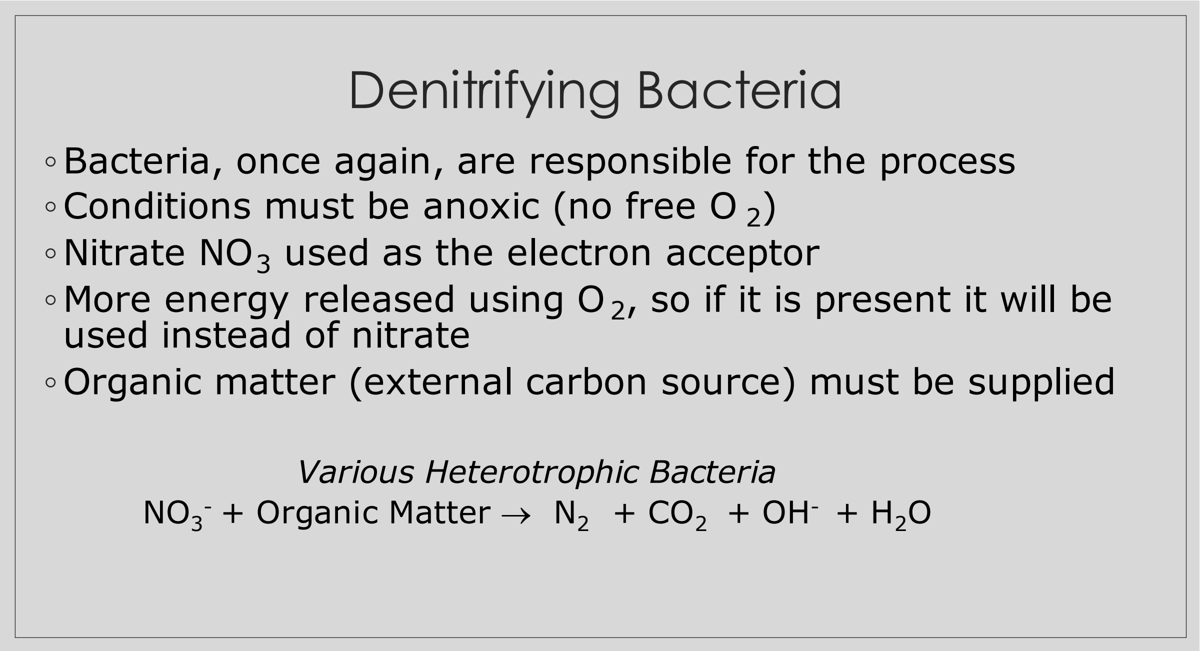
13

Nitrosomonas

Nitrobacter

Figure 15 Photos of Nitrifying bacteria through a light microscope

Images were taken through a light microscope. The subjects are about 1-2 μm in diameter. These organisms are metabolically complex, but no mouth nor anus is present. Well-developed organelles inside cells to carry out chemical reactions. These autotrophs would <u>never</u> drool.



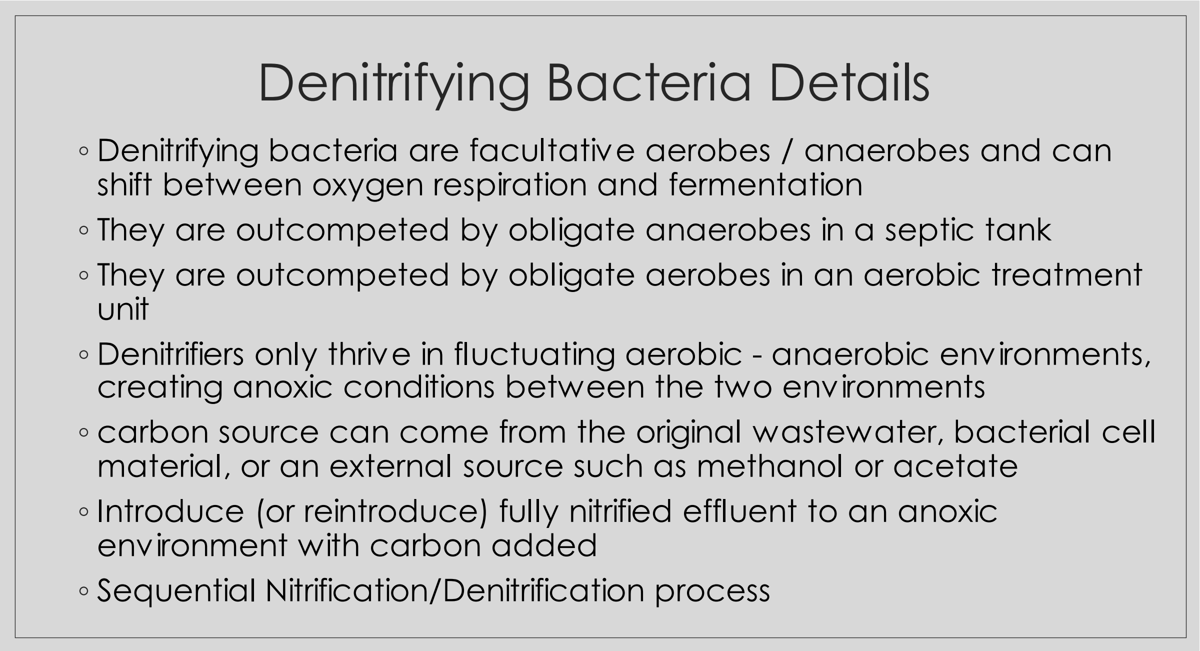
## Denitrifying Bacteria

- Bacteria, once again, are responsible for the process
- Conditions must be anoxic (no free $O_2$)
- Nitrate $NO_3$ used as the electron acceptor
- More energy released using $O_2$, so if it is present it will be used instead of nitrate
- Organic matter (external carbon source) must be supplied

*Various Heterotrophic Bacteria*

$$NO_3^- + \text{Organic Matter} \rightarrow N_2 + CO_2 + OH^- + H_2O$$

Figure 16. Denitrifying bacteria summary

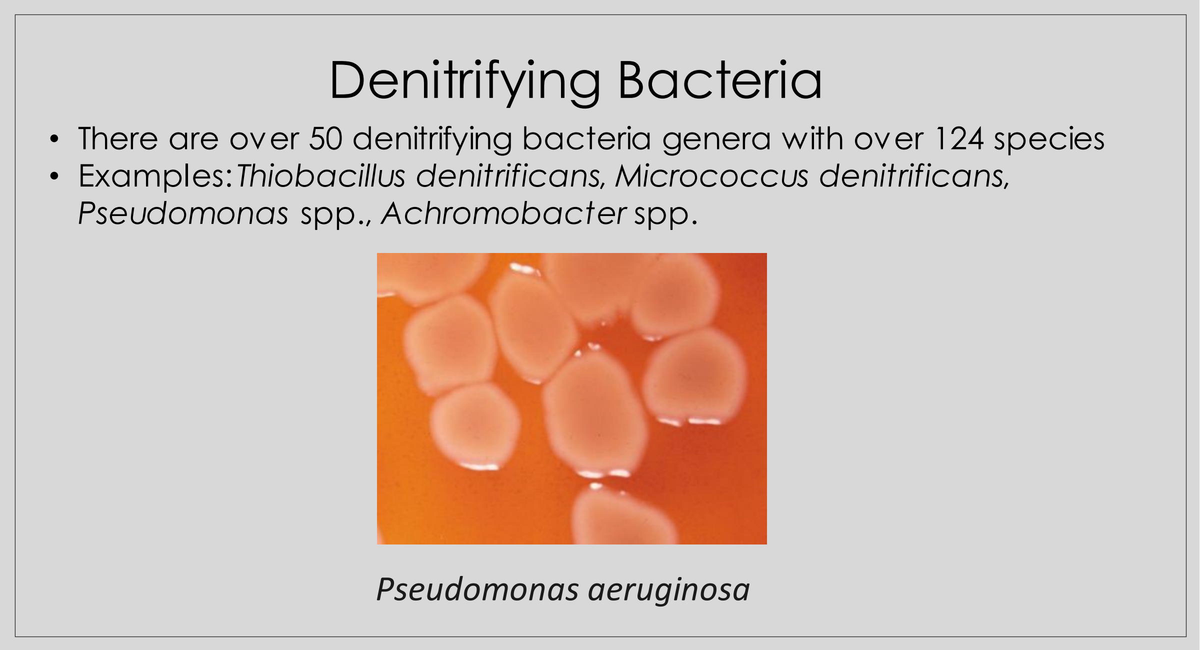


Denitrifying Bacteria Details

- Denitrifying bacteria are facultative aerobes / anaerobes and can shift between oxygen respiration and fermentation
- They are outcompeted by obligate anaerobes in a septic tank
- They are outcompeted by obligate aerobes in an aerobic treatment unit
- Denitrifiers only thrive in fluctuating aerobic - anaerobic environments, creating anoxic conditions between the two environments
- carbon source can come from the original wastewater, bacterial cell material, or an external source such as methanol or acetate
- Introduce (or reintroduce) fully nitrified effluent to an anoxic environment with carbon added
- Sequential Nitrification/Denitrification process

Figure 17. Denitrifying bacteria details

The analogy I use is denitrifying bacteria are the switch-hitters of the bacterial world. Two metabolic pathways cost the organism having them so in a consistently aerobic or anaerobic environment, they will be at a competitive disadvantage. The only time they have the upper hand is when conditions flip-flop between aerobic and anaerobic so lots of anoxic conditions are found in between. Anoxic is to oxygen content a dusk and dawn is to level of sunlight. In anoxic conditions there is no free oxygen but oxygen containing compounds (carbon dioxide or nitrate) are available and are combined with oxygen. Denitrifying bacteria breathe the oxygen off of nitrate and release nitrogen gas.



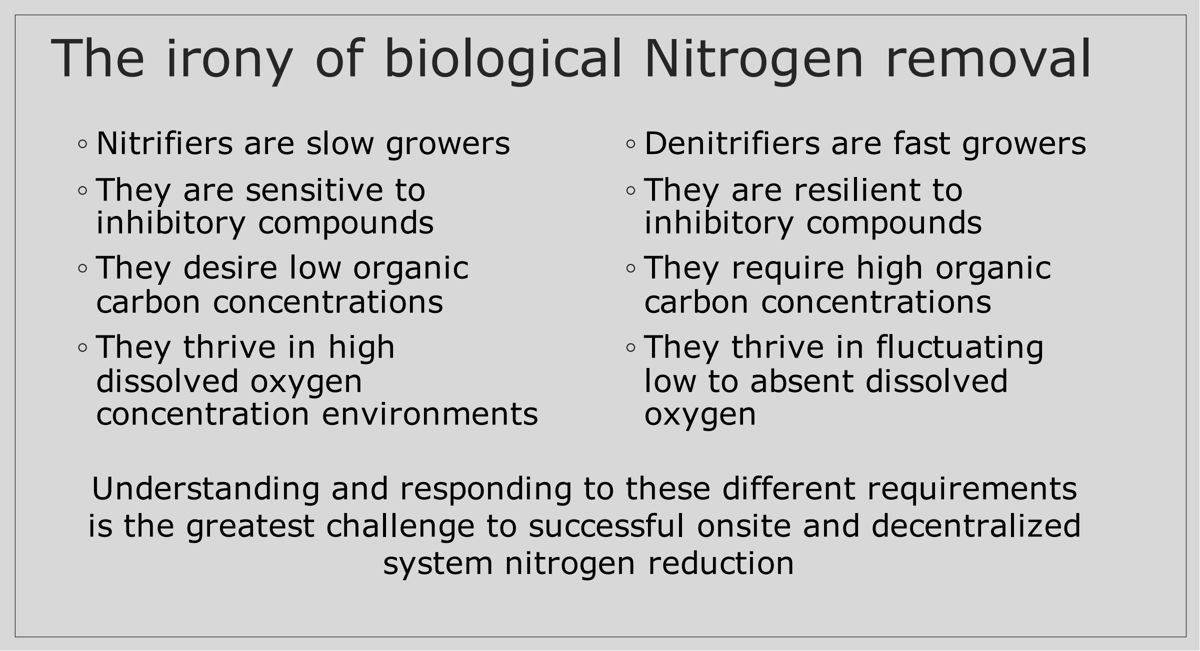
## Denitrifying Bacteria

- There are over 50 denitrifying bacteria genera with over 124 species
- Examples: *Thiobacillus denitrificans, Micrococcus denitrificans, Pseudomonas* spp., *Achromobacter* spp.

*Pseudomonas aeruginosa*

Figure 18. Denitrifying bacteria genera and species examples

**Conclusion**



## The irony of biological Nitrogen removal

- Nitrifiers are slow growers
- They are sensitive to inhibitory compounds
- They desire low organic carbon concentrations
- They thrive in high dissolved oxygen concentration environments

- Denitrifiers are fast growers
- They are resilient to inhibitory compounds
- They require high organic carbon concentrations
- They thrive in fluctuating low to absent dissolved oxygen

Understanding and responding to these different requirements is the greatest challenge to successful onsite and decentralized system nitrogen reduction

Figure 19.  A comparison of preferences and dislikes of nitrifying and denitrifying bacteria

## Acknowledgements

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