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# Effect of Copper on Hydrogenotrophic Denitrification: A Review

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**Abstract**

Nitrate contamination of water sources is a significant issue that impacts human health and the environment. This review explores the role of copper in the hydrogenotrophic denitrification (HD) process, focusing on how copper influences denitrification enzymes and microbial activity. Copper plays a critical role in activating nitrous oxide reductase, an enzyme responsible for the final step of denitrification. An imbalance in copper availability either deficiency or excess can lead to incomplete nitrate conversion and elevated nitrous oxide ($N_2O$) emissions. This study delves into the causes and effects of nitrate pollution, the mechanisms underlying hydrogenotrophic denitrification, and the impact of copper on these processes. Its findings underscore the importance of understanding the optimal copper concentrations to enhance nitrate removal and reduce $N_2O$ emissions.

***Keywords*****:** Copper effect, Hydrogenotrophic denitrification, Drinking water, Nitrate contamination

## 1. Introduction

Water is one of the essential resources for sustaining all forms of life. It plays a vital role in determining the quality of life as well as the health of an individual. United Nations' Sustainable Development (UN-SDGs) Target 6.1 demands countries "to achieve universal and equitable access to safe and affordable drinking water to all." Over 2 billion people live in water-stressed countries and it is expected to increase in the coming days due to climate change and population growth (Udmale et al., 2016). People are compelled to use groundwater which is the primary source to fulfill drinking water needs (Shinoda et al., 2021) since Kathmandu Upatyaka Khanepani Limited (KUKL), responsible for the distribution of drinking water by the government in the valley, provides approximately 25–33% of the total demand of 350 MLD (Adhikari et al., 2019). Also, there is a high disparity in water demand (472 MLD) and supply (101.24 MLD) (KUKL, 2022) indicating demand is increasing with very little supply. Groundwater appears to be a significant source of drinking water in the valley (Khatiwada et al., 2002; Udmale et al., 2016) and similar cases in many other countries (Mousavi et al., 2012).Nitrate is highly soluble and its concentration in groundwater is rising globally due to excessive use of fertilizers, improper wastewater disposal, and uncontrolled industrial discharges(Khatiwada et al., 2002). The average concentration of nitrate-nitrogen in groundwater in the Kathmandu valley is approximately 40 mg-N/l (Eamrat et al., 2017) ;which indicates treatment is necessary. Denitrification is a biological process by which a specific bacterium converts nitrates from contaminated water to nitrogen gas using nitrates or nitrites as a terminal electron acceptor. It is preferred to the physio-chemical treatment process for nitrate removal because they are economical and of a clean nature (Ghafari et al., 2009a).

Hydrogenotrophic denitrification (HD), an autotrophic technique, is a process where nitrate ($NO_3^-$) is converted to nitrogen gas by hydrogenotrophic denitrifying bacteria using hydrogen gas ($H_2$) as an electron donor bicarbonate (or carbon dioxide gas) as the carbon source. They are frequently found in anoxic conditions. This technique offers a controlled environment where conditions may be regulated for optimal performance, which makes it very important in constructed systems like water treatment plants. The existence of appropriate microbial communities, the availability of electron donors (hydrogen), the availability of essential heavy metals, and parameters like pH, temperature, carbon source and, nitrate loading concentration are some of the variables that affect the success of hydrogenotrophic denitrification (Karanasios et al., 2010; Liu et al., 2022). This method is considered highly effective for nitrate removal due to several advantages highlighted by researchers, including the absence of harmful byproducts, no need for post-treatment, target-specific action, and environmental safety (Eamrat et al., 2017; Epsztein et al., 2016, 2018; Ergas & Reuss, 2001; Ghafari et al., 2009b, 2009a; Keisar et al., 2021; Park et al., 2005; Shinoda et al., 2021; Vasiliadou et al., 2006). A well-ventilated space reduces the risk of hydrogen explosions, ensuring a safer and hazard-free treatment process.

Heavy metals can significantly influence denitrification processes, depending on their type, valence state, and concentration. They may either stimulate or inhibit the microbial activity crucial for denitrification. The relationship between heavy metals and denitrification is complex, involving various interactions at the enzymatic, microbial, and process levels. Therefore, optimizing denitrification in water treatment systems especially in environments contaminated with heavy metals requires a thorough understanding of these interactions.(Liu et al., 2022).

Copper is an essential metal that functions as a co-factor for several enzymes, including those involved in the denitrification process. Nitrous oxide reductase (Nos) requires copper to catalyze the last stage of reducing $N_2O$ to $N_2$. Copper is necessary for the proper functioning of enzymes which must be controlled and used in optimal concentration as a higher concentration than the threshold will inhibit the denitrification process (Liu et al., 2022).

This review aims to explore the various roles of copper in hydrogenotrophic denitrification, with a focus on its effects on microbial activity and denitrification efficiency. Understanding these processes, we can optimize nitrate removal and lower $N_2O$ emissions by managing copper concentrations. In addition to highlighting existing research gaps, this review provides recommendations for future studies to better elucidate the complex interactions between copper and denitrification processes. This information can help reduce the adverse health effects of nitrate pollution and guide the development of more efficient water treatment techniques.

## 2. Methods

This review was conducted by analyzing existing scholarly work related the impact of copper on hydrogenotrophic denitrification. Research articles were gathered from platforms including Scopus, Web of Science, and Google Scholar. Keywords such as "copper and denitrification," "hydrogenotrophic denitrification," "nitrate in groundwater," and "heavy metals and microbes" were used to filter relevant content. Studies considered were published between 1990 and 2024 and were chosen for their direct relevance to nitrate removal processes, with an emphasis on microbial and enzymatic interactions with copper. Factors such as pH, temperature, hydrogen levels, residence time, carbon sources, and heavy metal presence were assessed in relation to HD efficiency.

## 3. Nitrate in Water Sources

Nitrate contamination in groundwater is a major global concern (Keisar et al., 2021) and continues to increase over time (Benyoucef et al., 2013). Nitrate pollution in terms of concentration and affected areas has resulted due to rapid and uncontrolled urbanization in recent decades (Eamrat et al., 2020). According to WHO (2022), the guideline value of Nitrate is 50 mg/l as nitrate ion (or 11 mg/l as nitrate-nitrogen), and nitrite is 3 mg/l as nitrite ion (or 0.9 mg/l as nitrite-nitrogen).

### *3.1. Sources of nitrate*

Human activity and natural processes are the leading causes of nitrate contamination in water. Since nitrogen-based fertilizers are widely used in agriculture, runoff and leaching into surface and groundwater are the primary anthropogenic sources. Excess nitrates end up in water systems because these fertilizers, which are meant to boost crop development, frequently absorb more than plants can handle. Manure, which can release nitrates into the environment if not adequately handled, is another way that animal husbandry contributes to nitrate contamination (Glass & Silverstein, 1998; Rezvani et al., 2017).

One of the main contributors is sewage leaks and septic tank discharge. Another significant cause of nitrate contamination is industrial operations. Industries that process food, make chemicals, or manufacture pharmaceuticals can have wastewater effluents with high nitrate concentrations (Bouchard et al., 1992; Eamrat et al., 2017)

Managing nitrate levels in water systems is complicated, as seen by these several causes of nitrate contamination. A comprehensive understanding of the anthropogenic causes of nitrate pollution is necessary for developing effective mitigation methods. The focus should be on controlling anthropogenic activities, as the contribution from natural atmospheric deposition is minimal and can be considered negligible (Karanasios et al., 2010).

### *3.2. Harmful effects of nitrate*

Water with high nitrate levels causes serious health and environmental hazards. Methemoglobinemia, or "blue baby syndrome," is a significant health risk that arises when infants drink water contaminated with nitrates (Khanitchaidecha et al., 2012). The body transforms nitrate into nitrite, which attaches to hemoglobin and lowers its ability to carry oxygen, and if treatment is delayed, it may become life-threatening.

Long-term exposure to high nitrate levels in adults has been linked to an increased risk of gastric cancer in particular, as it causes the stomach to produce carcinogenic N-nitroso compounds (Dahab et al., 1994). Some scientific data points to the possibility that nitrites and nitrates may cause mutagenicity, teratogenicity, and birth abnormalities as well as increase the risk of non-lymphoma, Hodgkin's bladder cancer, and ovarian cancer when consumed(Camargo & Alonso, 2006).

## 4. Copper and Hydrogenotrophic Denitrification (HD)

### *4.1. Hydrogenotrophic denitrification (HD)*

HD reduces nitrate to nitrogen gas by using hydrogen ($H_2$) as an electron donor with the aid of specialized bacteria. It involves a sequence of biochemical reactions. The steps involved are (Albina et al., 2019; Du et al., 2016);

**Nitrate ($NO_3^-$) Reduction to Nitrite ($NO_2^-$):** The first step is the nitrate reductase (NaR) enzyme that reduces nitrate to nitrite. This is an important reaction because it starts the denitrification process.

**Nitrite Reduction to Nitric Oxide (NO):** Nitrite reductase (NiR) enzymes further reduce nitrite to nitric oxide. Environmental factors like pH and temperature can have an impact on the activity of the enzymes and their overall efficiency.

**Nitric Oxide Reduction to Nitrous Oxide ($N_2O$):** The third stage is the conversion of nitric oxide into the potent greenhouse gas nitrous oxide. Nitric oxide reductase (NoR) helps in this reaction.

**Nitrous Oxide Reduction to Nitrogen Gas ($N_2$):** Nitrous oxide reductase (Nos) catalyzes the last step, which is the reduction of nitrous oxide to nitrogen gas. This step is vital because it stops $N_2O$ from escaping into the atmosphere.

The denitrification rate was affected by the variation of nitrate concentration, pH, temperature, alkalinity, carbon and microbial community involved.

- pH: The optimal pH range for HD is between 7.0 and 8.0 (Ghafari et al., 2010) and 7.6 to 8.6 (Karanasios et al., 2010). This process is pH-dependent. Outside of this range, a pH change may impact the activity of the denitrifying bacteria and decrease the efficiency.
- Temperature: The rate of HD is temperature-dependent, and the optimum temperature range is generally between 25°C and 35°C. Higher values up to 42°C were also reported (Karanasios et al., 2010). Denitrification can be enhanced by elevated temperatures; however, excessively high temperatures may inhibit the activity of denitrifying bacteria.
- Residence time: The amount of time the wastewater spends in contact with the biomass in the bioreactor. A longer residence time allows for a more thorough conversion of nitrate to nitrogen gas, but it also raises the bioreactor's costs. A shorter residency period lowers the capital and operating expenditures but may result in incomplete conversion of nitrate to nitrogen gas.

- Hydrogen concentration: HD depends on the availability of hydrogen. If the concentration of hydrogen is too low, the process will be limited by the availability of the electron donor. On the other hand, if the hydrogen concentration is too high, denitrification may be inhibited.
- Nitrate concentration: HD is also influenced by nitrate content. Different $NO_3$ -N concentrations have distinct effects. Denitrification rates have been shown to rise at nitrate concentrations as high as 492 mg $NO_3$-N/l. Some studies, however, discovered that denitrification was hindered at nitrate concentrations greater than 30 mg$NO_3$ -N/l. (Karanasios et al., 2010).
- Carbon sources: HD requires a carbon source for bacterial growth and metabolism. The denitrification rate is affected by the quantity and quality of carbon sources.

HD is influenced by the availability of hydrogen as an electron donor, appropriate microbial communities, and environmental parameters like pH, temperature, and the presence of additional nutrients or inhibitors. Those factors must be optimized to make HD more effective. Significantly, the presence of heavy metals also highly impacts the denitrification efficiency.

### *4.2. Copper and hydrogenotrophic denitrification (HD) relationship*

The relationship between copper and HD is complex and influenced by several factors, including copper concentration, microbial community composition, and environmental conditions.

Copper is a necessary trace metal for many biological systems. It functions as a co-factor for several enzymes, including those connected to the denitrification process. Nitrous oxide reductase (Nos) requires copper to reduce $N_2O$ to $N_2$ (Granger & Ward, 2003) and it is a very significant step as $N_2O$ is a potent greenhouse gas that has a far higher potential to cause global warming than $CO_2$ (Felgate et al., 2012; Woolfenden et al., 2013).

Previous studies have demonstrated that trace levels of copper (10–100 µg/L) enhance total nitrogen removal and lower $N_2O$ emissions. Higher concentrations (such as 0.95 mg/L) can stop 50% of the anaerobic methanogenic granular sludge's denitrifying bacteria's activity(Liu et al., 2022; Ochoa-Herrera et al., 2011).

In a study done by Magalhães et al. (2011), they found that the diversity and abundance of genes involved in nitrite and nitrous oxide reduction are decreased, and denitrification is inhibited by increasing copper concentrations. Similarly, study performed in *Arthrobacter nicotianae* D51 strain, the maximum growth rate and denitrification capacity happened when 0.05 mg/l of $Cu^{2+}$ was present, but $Cu^{2+}$ severely inhibited both growth and aerobic denitrification capacity at ≥0.1 mg/l (Cai et al., 2019).

The microbial communities engaged in denitrification might vary in composition and activity depending on the availability of copper. For example, studies have shown that copper availability affects the expression of genes, protein levels, and enzyme activities associated with the denitrification pathway in *Bradyrhizobium diazoefficiens* (Pacheco et al., 2022). Copper-dependent enzymes perform optimally in the presence of adequate copper, thereby enhancing the overall efficiency of the denitrification process.

In addition to copper, other trace metals involved in denitrification include iron, manganese, and molybdenum. However, copper distinguishes out because of its special function in promoting nitrous oxide reductase activity. Understanding the interactions between copper and other trace metals is essential for gaining a comprehensive insight into the factors influencing denitrification efficiency.

In the context of Kathmandu Valley, where groundwater is a primary drinking water source due to limited surface supply source, nitrate contamination remains a growing concern. Studies have reported nitrate concentrations exceeding WHO standards, driven by uncontrolled urbanization, overuse of fertilizers, and poor wastewater management. Implementing HD systems in this region could provide an efficient and eco-friendly solution. However, copper concentrations in local water sources and treatment systems must be carefully managed to ensure that microbial communities' function effectively for sustained nitrate removal. Proper copper management is essential to balance enzymatic activity and microbial viability. Optimizing this balance can significantly improve nitrate removal efficiency and reduce greenhouse gas emissions, particularly in nitrate-affected regions such as Kathmandu Valley.

## 5. Conclusions and Recommendations

The complex interaction between copper and HD has been studied in this review, with particular attention paid to copper's dual function as a crucial co-factor and possible inhibitor of the denitrification process. Hydrogenotrophic denitrification is a microbial route that converts nitrate to nitrogen gas, which is important for removing nitrate contamination in water sources. The activity of nitrous oxide reductase (Nos), an enzyme required for the conversion of nitrous oxide ($N_2O$) to nitrogen gas ($N_2$), depends critically on copper, a vital trace metal. This last stage of the denitrification process is crucial because it stops the atmosphere from filling with the powerful greenhouse gas $N_2O$.

Based on the review's findings, the following recommendations can be made to maximize hydrogenotrophic denitrification and decrease the harmful effects of nitrate pollution and $N_2O$ emissions;

- Optimal copper management is essential.
- Copper and microbial interactions study is needed to understand their complex interactions.
- Policy and guidelines should be developed to regulate copper concentrations in agricultural and industrial effluents.
- Raising public awareness and educating stakeholders—farmers, business owners, and legislators—about the significance of copper management in denitrification.

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