The coupling of biotechnology with electrochemistry:
Electrodenitrification of water using the enzyme nitrate reductase

Cyril Geyer
University of Northern Iowa

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THE COUPLING OF BIOTECHNOLOGY WITH ELECTROCHEMISTRY:
ELECTRODENITRIFICATION OF WATER USING
THE ENZYME NITRATE REDUCTASE

An Abstract of a Thesis
Submitted
In Partial Fulfillment
Of the Requirements for the Degree
Master of Science

Cyril Geyer
University of Northern Iowa
August 2001
Nitrate is a worldwide contaminant that causes environmental (eutrophication) and human health (methemoglobinemia, cancers) concerns. Very few techniques are available to eliminate it from natural environment and they suffer from major drawbacks, such as production of unwanted byproducts (toxins or brine). The electrochemical reduction of nitrate on any electrode is relatively slow and catalysts or electron mediators are needed. The goal of this project was to develop a new process that could overcome these disadvantages. The project demonstrates the feasibility of using an enzyme, nitrate reductase, and a mediator, methyl viologen to transfer the electrons from the electrode to nitrate, either on glassy carbon or on copper. This enzyme system was shown to accelerate the rate of reduction. An electrode composed of a membrane filled with copper shot and nitrate reductase was constructed. This electrode succeeded in reducing nitrate from a solution containing the electron mediator. Without further optimization, the electrochemical efficiency was approximately 20%. The main advantage of this system is the production of a gaseous product, which is probably nitrogen, an environmentally safe gas. The results are promising since no special conditions are required to run the system. Indeed, the reduction takes place in an air atmosphere, at room temperature and the two electrodes do not need to be separated.
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This study by: Cyril Geyer

Entitled: THE COUPLING OF BIOTECHNOLOGY WITH ELECTROCHEMISTRY:
ELECTRODENITRIFICATION OF WATER USING THE ENZYME NITRATE
REDUCTASE

has been approved as meeting the thesis requirement for the

Degree of Master of Science in Environmental Science.

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Dr. John W. Somervill, Dean, Graduate College
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INTRODUCTION

The Nitrate Problem and its Current Solutions

The Nitrate Problem

Since the beginning of industrialization, the concentration of nitrate in water has increased all over the world. Spalding and Exner (1993) reviewed the occurrence of nitrate in groundwater. In order to compare the different forms of nitrogen in the environment, the concentration of nitrate is often given as the concentration of nitrogen in nitrate present in the sample as NO₃-N. They reported that, in 1991, NO₃-N concentrations range from 4 to 11 mg/L in groundwater from agricultural areas in Belgium. They also predicted that by 1995, 20% of French people, 8% of the former Federal Republic of Germany population and 5% of Danes would be supplied by water that exceeds the European Community limit of 11.3 mg/L NO₃-N (50 mg/L NO₃). In 1991, several agricultural areas of the Southern Ontario, Canada, were found to exceed 10 mg/L NO₃-N, which is the World Health Organization guideline and the U.S. Environmental Protection Agency (EPA) Maximum Contaminant Limit (MCL). Nitrate contamination was also shown in Ivory Coast, in areas that were deforested, in Israel, beneath sewage-irrigated land, and in Australia, in the Gambier Plain. The United States is in a similar situation. Nolan and coworkers (1996, 1997) established a map about the risk of contamination of ground waters in the US based on the land-use practices and the geology (Figure 1). The Mid-West (including Iowa) and Far-West are particularly at risk. They reported that about 25% of the wells in high-risk areas were exceeding the MCL.
Figure 1: Risk of Nitrate in Groundwater from Nolan and Ruddy, 1996

This high occurrence of nitrate in water is alarming because it is a potential threat for human health and the environment. First, high levels of nitrate can cause a serious disease called methemoglobinemia or "blue-baby syndrome" (Cornblath and Hartmann, 1948; Kross et al., 1992). Nitrate is reduced to nitrite in the less acidic and lower oxidizing environment of the stomach in young infants by fecal microorganisms. Nitrite reacts with hemoglobin and oxidizes Fe (II) to Fe (III) to produce methemoglobin. Unlike adults, young infants are not able to reduce methemoglobin back to hemoglobin whereas adults are. Since methemoglobin is not able to carry oxygen, young children may die by suffocation or may have severe mental problems due to oxygen deprivation of the brain. Treatment of methemoglobinemia consists of the administration of methylene
blue, which reduces back Fe (III) to Fe (II) (i.e., reduction of methemoglobin to hemoglobin).

Nitrate is also suspected to have long-term exposure effects. Odashima (1980) reported that nitrite can react with secondary amines, amides and carbamates in the stomach, producing N-nitroso compounds that are known carcinogens for animals. Spalding and Exner (1993) cited several studies of the relationship between nitrate in water and hypertension, infant mortality, central nervous system birth defects, and non-Hodgkin's lymphoma. But none are conclusive and more research about these effects needs to be carried out.

However, nitrate primarily threatens the environment, especially the aquatic ecosystems. Indeed, nitrate is one of the major nutrients for plants and micro-organisms and, with sufficient phosphate, an increase in its concentration in water causes an abnormal growth of algae and macrophytes known as eutrophication (Organisation for Economic Cooperation and Development, 1982). Ryding and Rast (1989) and Henderson-Sellers and Markland (1987) have shown that phosphates (or phosphorus sources in general) are the limiting nutrient in most aquatic systems and a decrease in its concentration is enough to decrease or stop the effect of eutrophication. However, in coastal sea areas, nitrate is limiting and it is mandatory to diminish its concentration. Eutrophication is characterized by growth of aquatic plants. These plants block the sunlight for other algae, and their death leads to a depletion of oxygen, since oxygen is consumed during degradation of the algae. This can significantly affect an ecosystem by killing fishes and the less resistant algal species.
Current Solutions to the Nitrate Problem

Several techniques and practices have been developed to face the nitrate problem. Among them, the most obvious is to decrease the nitrate input in nature. A policy, called the best management practices policy, can be used (Addiscott et al., 1991; Strebel et al., 1989). It includes the following measures:

- No application of nitrogen fertilizer in fall, because no crop can take it up.
- Less bare soil in winter, i.e., winter crop will take up nitrate throughout winter.
- Early sowing of winter crop in fall, because it will take up more nitrogen, the weather being warmer.
- Use of animal manures (when possible), because they are a source of nitrogen that will enter the environment.
- Use of nitrogen fertilizer in accordance with professional advice, as needed by the type of soil.

These practices will improve the quality of natural waters, however remedial means for actual contaminated waters are needed. Even though the major problem is in the environment, very few techniques are available to clean a waterbody, either overland or underground, and soils. Most of these remedial techniques are based on electrochemistry (e.g., Chew and Zhang, 1998; Lin et al., 2000), on redox chemistry (e.g., Ottley et al., 1997; Huang et al., 1998) or on the enhancement of the natural biodegradation by applying a source of carbon (e.g., EPA, 1996; Smith et al, 2001). The electrochemical technique developed by Chew and Zhang (1998) combines electromigration of nitrate ions towards an anode surrounded by iron powder. Two electrodes are placed in soils and
a current is applied between them. Nitrate ions, thus attracted to the positive anode, pass through a mixture of soil and powder iron. Nitrate is reduced by metallic iron to ammonium ions and nitrogen gas. This reduction consumes protons and usually occurs at low pH. However, the water electrolysis occurs at the anode produces protons, which sustains the reduction of nitrate by iron.

Lin et al. (2000) obtained a patent for nitrate reduction based on Pourbaix diagrams. These diagrams show the stable form of an element according to the redox potential of the medium. Two electrodes are inserted into the medium to be treated (e.g., water, soil) and a potential is applied which corresponds to the potential of nitrogen gas stability. However, it is not clear if such an approach will really be effective for the reduction of nitrate. Indeed, the Pourbaix diagrams are based on the thermodynamics of the reactions, i.e., they only take into account the theoretical feasibility of the reactions. Although it is well known that the reduction of nitrate is thermodynamically feasible, the kinetics of the reaction is slow.

Ottley et al. (1997) suggested that nitrate could be reduced by iron (II) in presence of copper (II). However, extremely low oxygen concentrations are required to allow the reduction because iron (II) is oxidized quickly by oxygen. A major drawback is the production of ammonium ions, which are also pollutant. Huang et al. (1998) used almost the same approach. Nitrate was reduced by iron (0). The two major drawbacks are the production of ammonium ions and iron (II).

Lastly, Smith et al. (2001) stimulated the natural denitrification of an aquifer by applying formate to the water. Groundwater was withdrawn, amended with formate and
pumped back. Microorganisms used formate as a source of electron donors to grow and reproduce. Meanwhile, they used nitrate as electron acceptors, the water being anoxic.

The U.S. EPA report (1996) used the same approach to remediate soils and aquifer polluted by organic compounds. They applied nutrients (e.g., nitrate, phosphate, potassium) on the ground to enhance the biological activity. They observed a depletion in nitrate. Nevertheless, this technique is applicable only in bodies of water in anoxic state and it presents the risk of replacing one pollutant with another one (e.g., nitrate by formate).

Techniques have also been developed to improve the quality of drinking water. The first type is biological denitrification. The water to be treated flows through a reactor containing bacteria. These bacteria use nitrate instead of oxygen to grow and reproduce. Therefore, the medium needs to be in anoxic condition. This treatment is efficient but an organic source of carbon needs to be added to the water. This carbon source is usually methanol, which is toxic and thus an excess should be avoided. In addition, the bacteria can produce toxins and a post-treatment (e.g., chlorination or ozonation) needs to be carried out. The second type is a physical process such as reverse osmosis or an ion-exchange treatment. But both physical processes are energy consuming in order to significantly decrease the nitrate concentration. Moreover, these processes produce brines that are heavily enriched in ions, because these techniques withdraw other ions in addition to nitrate. The brines have to be either disposed or treated.

Fonseca et al. (2000) developed a technology that combines these two major types of techniques. They called it an ion-exchange membrane bioreactor. The technology is
composed of two separated parts, one containing the solution to be treated and the other one the medium of biological denitrification. The two cells are separated by an anion-exchange membrane, which equilibrates the concentration of ions on both sides. Nitrates pass through the membrane to the other cell where they are consumed. Consequently, their concentration decreases in the treated solution. In contrast, the other ions are not consumed, so that they can go back and forth through the membrane and no change in their concentration is observed. Moreover, the organic carbon source cannot go through the membrane. Therefore, no contamination of organic carbon can occur. However, the residence time is high (4.4 hr) and this technique cannot yet be industrially applied.

Nitrate Chemistry and Biochemistry

Chemistry of Nitrate

Solutions for nitrate reduction that are currently available suffer due to several disadvantages and the goal of this research is to develop a technique based on electrochemical and enzymological processes, that would enhance the denitrification rate. However, before going further, some additional discussion of the chemistry and biochemistry of nitrate are necessary.

First of all, nitrogen has 9 different oxidation states (Table 1) ranging from +5 (nitrate) to -3 (ammonia) and the nitrate reduction leads to several intermediates or products (Table 2).
Table 1: Examples of the Different Oxidation States of Nitrogen

<table>
<thead>
<tr>
<th>Oxidation state</th>
<th>Acid solution</th>
<th>Example of molecule</th>
<th>Alkaline Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>NH₄⁺</td>
<td>NH₃</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td>N₂H₅⁺</td>
<td>N₂H₄</td>
<td></td>
</tr>
<tr>
<td>-1</td>
<td>NH₃OH⁺</td>
<td>NH₂OH</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>N₂</td>
<td>N₂</td>
<td></td>
</tr>
<tr>
<td>+1</td>
<td>N₂O</td>
<td>N₂O</td>
<td></td>
</tr>
<tr>
<td>+2</td>
<td>NO</td>
<td>NO</td>
<td></td>
</tr>
<tr>
<td>+3</td>
<td>HNO₂</td>
<td>NO₂⁻</td>
<td></td>
</tr>
<tr>
<td>+4</td>
<td>N₂O₄</td>
<td>N₂O₄</td>
<td></td>
</tr>
<tr>
<td>+5</td>
<td>HNO₃</td>
<td>NO₃⁻</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Reduction of Nitrate and Thermodynamic Potentials in Acidic Conditions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Charge</th>
<th>Thermodynamic Potential</th>
</tr>
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<tbody>
<tr>
<td>NO₃⁻ + 2 H⁺ + e⁻ → NO₂⁻ + H₂O</td>
<td>+0.775 V</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻ + 2 H⁺ + 2 e⁻ → NO₂⁻ + H₂O</td>
<td>+0.835 V</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻ + 4 H⁺ + 3 e⁻ → NO + 2 H₂O</td>
<td>+0.957 V</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻ + 5 H⁺ + 4 e⁻ → ½ N₂O + 5/2 H₂O</td>
<td>+1.116 V</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻ + 6 H⁺ + 5 e⁻ → ½ N₂ + 3 H₂O</td>
<td>+1.246 V</td>
<td></td>
</tr>
<tr>
<td>NO₃⁻ + 10 H⁺ + 8 e⁻ → NH₄⁺ + 3 H₂O</td>
<td>+0.875 V</td>
<td></td>
</tr>
</tbody>
</table>

Nitrite ion (NO₂⁻) is toxic and thus it is undesirable as a product of denitrification.

Nitrogen dioxide (NO₂), nitric oxide (NO) and nitrous oxide (N₂O) are known greenhouse-effect gases and NO₂ and NO are precursors to urban smog. In addition, N₂O reacts with ozone in the upper atmosphere participating to the stratospheric ozone depletion. Ammonium ion (NH₄⁺) is also a pollutant. Some plants use it as a source of nitrogen instead of nitrate. In other words, nitrogen gas (N₂) is the only environmental friendly end product as it composes about 80% of the atmosphere.
The chemical and electrochemical reduction of nitrate has been studied for the purpose of developing quantitative analytical techniques for the determination of nitrate in aqueous solutions. However, many of these techniques use toxic chemicals, such as cadmium and copper salts (Bodiny and Sawyer, 1977) and suffer from poor selectivity. In addition, nitrate has been found to be reduced by metals, such as iron (Van Cleemput and Baert, 1983; Cheng et al., 1997; Huang et al., 1998) or zinc (Carlson, 1986), and by metallic ions, e.g., ferrous ions (Ottley et al., 1997; Petersen, 1979; Van Hecke et al., 1990; Buresh and Moraghan, 1976; Moraghan and Buresh, 1977; Van Cleemput and Baert, 1983). It should be noted that the rate of reduction and the products depend on the conditions. For example, Van Hecke et al. (1990) reported a fast rate of denitrification at pH 8.1 whereas it was slow at pH 7.5. Moreover, these conditions require a copper catalyst. The product was ammonium ion and a temporary accumulation of nitrite and hydroxylamine was observed. Buresh and Moraghan (1976) noted a small production of nitrous oxide and nitrogen gas in the same conditions. Huang et al. (1998) observed a reduction of nitrate by metallic iron to ammonium only at pH<4. Cheng et al. (1997) reported a reduction even at pH 7 but in buffered solution. In all of these treatments, chemicals, such as metallic iron and iron (II), were added to the solution to be treated.

Biochemistry of Nitrate

In contrast to chemical reduction of nitrate, several organisms (plants, fungi, bacteriae) have developed several systems to use nitrate either as an acceptor of electrons (denitrification) or as a nitrogen source to produce the amino acids they need (assimilation).
(Takashi et al., 1963). In denitrification, the products are N₂ and N₂O with NO₂⁻ as an intermediate. The organisms use this pathway to produce energy when oxygen is missing (anoxic condition). For that purpose, the organisms have developed two enzymes, nitrate reductase (NaR) and nitrite reductase (NiR). NaR catalyses the reaction of reduction of NO₃⁻ to NO₂⁻ whereas NiR catalyses the reduction of NO₂⁻ to N₂, N₂O or NH₄⁺ (depending on the organism). These reactions occur at physiological pH, i.e., between 6.5 and 8.5 and the rates of reduction are relatively high.

Campbell (1999) has recently reviewed the properties of NaR, which catalyses the following reaction:

\[
\text{NO}_3^- + \text{NADH} \rightarrow \text{NO}_2^- + \text{NAD}^+ + \text{OH}^- 
\]

Nitrate reductase models are described in Figure 2. Cofactors, which are electron mediators for nitrate reductase, other than NADH can be used (e.g., methyl viologen (MV) or bromphenol blue (BPB)) by NaR to reduce nitrate. In addition, ferricyanide (Fe(CN)₆³⁻) or mammalian cytochrome c (Cyt c) can be reduced by NaR, the oxidation of NADH driving the reaction. Moreover, NO₂⁻ is an inhibitor of the NaR enzyme, in order to regulate its activity (feedback inhibition). In other words, the product is usually an inhibitor of the enzyme to limit the concentration of a product in the cell, especially when it is toxic.

Nitrate reductase has been isolated from various organisms, including bacteria (*Escherischia coli* (MacGregor, 1978), *Veillonella alcalescens*, *Pseudomonas perfectomarinus*, *Enterobacter aerogenes* and *Paracoccus denitrificans* (Payne, 1978)) or plants (*Corn* (Campbell and Campbell, 1996)). Different types of nitrate reductase have
different structural features, however they appear to effectively catalyze the reduction of nitrate in a similar general way. The differences can be seen in their molecular weights or in the cofactors (NADH or NADPH) that are involved.

![Diagram of Nitrate Reductase Models](image)

**Figure 2: Nitrate Reductase Models.** (a) Functional Model of the Enzyme; MV, Methyl Viologen; BPB, Bromphenol Blue. (b) Sequence Model of the Enzyme; DI, Dimer Interface; From Campbell, 1999

**Electrochemistry of Nitrate**

Natural denitrification appears not to be sufficient to remedy the anthropogenic input of nitrate in nature. It would appear that the electrochemical reduction of nitrate could be an attractive alternative. Electrochemical reduction has been considered for the treatment of radioactive waste, which contains significant amount of nitrate in acidic and basic solutions (Bockris and Kim, 1996, 1997; Kim, 1996; Epstein et al., 1964; Li et al.,
The overall electrochemical reduction of nitrate ion on a metal electrode is usually kinetically controlled. In other words, overpotentials (potentials much greater than thermodynamics would predict) have to be applied in order for the reaction to occur at a significant rate. As a result, homogeneous catalysts (i.e., electrocatalysts) have been introduced to improve the kinetics of the overall electrode reactions. Plieth (1978) reviewed the catalyzed electroreduction of nitrate. The reaction that takes place at the electrode is the direct reduction of the catalyst or electron mediator, which in its reduced form transfers electrons to nitrate. Mo(VI) or U(V) are examples of such catalysts:

\[
\begin{align*}
\text{U(VI)} + 2 \, e^- & \rightarrow \text{U(IV)} \text{ on the electrode} \\
\text{U(IV)} + \text{NO}_3^- & \rightarrow \text{U(V)} + \text{products in solution} \\
\text{Or Mo(VI)} + 2 \, e^- & \rightarrow \text{Mo(IV)} \text{ on the electrode} \\
\text{Mo(IV)} + \text{NO}_3^- & \rightarrow \text{Mo(VI)} + \text{products in the solution}
\end{align*}
\]

Ogawa and co-workers (1991 and 1992) studied other metals for the same purpose, such as alkaline earth metal ions (Mg(II), Ca(II), Sr(II) and Ba(II)). These ions act as intermediary for the transfer of charge between the electrode and nitrate. However, it is not clear how they transfer the charge. A complex nitrate-metal is probably formed, and nitrate ion in this complex could be easier to reduce. In addition, the hydrolysis of the metal ion in the vicinity of the electrode supplies protons for the nitrate reduction. For instance:
Ca^{2+} + H_2O \rightarrow Ca(OH)^+ + H^+

Boese and Archer (1982) reported the reduction of nitrate in presence of electrogenerated Yb(II). Two mechanisms were found. The first one is the reduction of nitrate by Yb(II), which produces Yb(III) and then the regeneration of Yb(II) from Yb(III) on the electrode. The second process is the reduction of nitrate directly on a layer of Yb(III) hydroxide.

Organometallic compounds (cationic \( \eta^5 \)-Cyclopentadienyl)(\( \eta^6 \)-arene)iron(II), ferrocene like compounds) have also been used as homogeneous catalysts (Buet et al., 1979). Ferrocene is a well-known reversible redox couple, which exchanges easily one electron with an electrode. Therefore, they act in the same way as inorganic ions.

Heterogeneous catalysis can be effected by the modification of the electrode surface. Metals, including platinum (Horányi and Rizmayer, 1985) or cadmium (Xing et al., 1990), can be deposited on a substrate to improve the rate of nitrate reduction. Organic compounds, such as polyaniline (Mengoli and Musiani, 1989), organometallic polymers, such as poly-(n-Bu_4N)₂[Mo_2Fe_6S_8(SPh)_9] (Kuwabata et al., 1986) or inorganic chemicals, such as doped diamond (Tenne et al., 1993) can also be used to modify an electrode surface. Lastly, new alloys have been developed, which result in surface with better properties for nitrate reduction (Ureta-Zañartu and Yáñez, 1997).

Modifications of an electrode surface change its adsorption properties, and the enhancement of the reduction rate can be explained by the improvement of the adsorption properties. Indeed, Petrii and Safonova (1992) and Horányi and Rizmayer (1982, 1985) showed that the rate depended on the competition between adsorption of nitrate and other
compounds, especially hydrogen. For instance, the rhodium-nitrate bond is stronger than
the platinum-nitrate bond with the result that rhodium appears to be a better surface to
electrochemically reduce nitrate.

Sunohara et al. (1993) showed that metal oxide layers (Mn, Fe, Co, Ni and Cu)
deposited on graphite substrate catalyzed the reduction of nitrate. However, an increasing
amount of oxide deactivates the electrode. The electrode becomes more resistant, when
the oxide layer is thicker, and the electrode is deactivated. Mengoli and Musiani (1989)
reported that a polyaniline layer, a conductive polymer, prevents the electrode from
undergoing passivation. In other words, the oxide layer, which would normally appear on
the surface at rest potential, does not develop thanks to the polyaniline coating, and thus
the electrode is not deactivated.

Lastly, the products are dependent on the reaction conditions, including both the
electrode materials and the solution conditions. For instance, ammonia is the principal
product in acidic solutions on copper (Pletcher and Poorabedi, 1979; Cattarin, 1992;
Desrochers and Bélanger, 1998; Kaczur et al., 1994), whereas hydroxylamine is the main
product in acidic conditions on platinum (Van der Plas and Barendrecht, 1980). Nitrogen
and nitrogen oxides are produced by electrochemical reduction at under-potential-
deposited cadmium on gold or silver when the pH is 1 while nitrite is produced at pH of 3
(Xing et al., 1990). Moreover, Kaczur et al. (1994) reported that on copper the current
efficiency increases with decreasing pH.
Electrochemistry and Biotechnology

Enzymes have been recently utilized in the development of sensors (reviewed by Bartlett and Cooper (1993) and Cosnier (1997)). Glucose sensors based on glucose oxidase entrapped in a polymer (such as polypyrrole, polyaniline, or poly(o-phenylenediamine) have been developed by several groups (Gros et al., 1996; Sung and Bae, 2000; Dumont and Fortier, 1996; Sangodkar et al., 1996; Shin et al., 1996; Almeida et al., 1993; Appleton et al., 1997). Moreover, Kuhn (1998) reported a glucose sensor where glucose oxidase is in solution and a mediator of electron (ferricyanide) is used to shuttle the electrons from the enzyme to the electrode. Other biosensors have been developed: dopamine and glutamate (Cosnier et al., 1997b), nitrite (Wu et al., 1997), parathion (Sacks et al., 2000) or hydrogen peroxide (Jönsson and Gorton, 1989; Gorton et al., 1992). In addition, an electrode capable of reducing hydrogen peroxide at relatively high rates has been reported by Bartlett and co-workers (1996, 1997).

Nitrate reductase (NaR) has also been used in several biosensors. Aylott et al. (1997) entrapped NaR in sol-gel. The reaction of nitrate with NaR changes the UV/visible spectrum of the enzyme allowing a determination of the nitrate. Willner et al. (1993) used the photoreduction of a semi-conductor (TiO₂) to reduce the bipyridinium units of the polymer. This reduction regenerates the oxidized form of TiO₂. NaR entrapped in the polymer oxidizes this bipyridinium units back and is thus reduced. This allows the reaction between NaR and nitrate. Another photoinduced determination, which also uses bipyridinium compounds, has been developed by Willner et al (1989).
Nitrate reductase has also been used in electrochemical sensors, in which a mediator is needed to carry the electrons from the electrode to the enzyme. Cosnier and coworkers (1994, 1997a) and Willner et al. (1992) used bipyridinium moieties attached to a conductive polymer (polypyrrole, polythiophene respectively). Nitrate reductase in that case is encapsulated in the polymer. Patolsky et al. (1998) and Narvaez et al. (1997) developed a nitrate sensor using microperoxidase-11 (an electro-active heme-peptide) as a mediator for electrons. Mellor et al. (1992) showed that different dyes could mediate the reduction of NaR on an electrode (Azure A, Safranin T, Neutral red, Bromphenol Blue, Cibacron blue see Figure 3). These dyes have the advantages of not being as toxic as methyl viologen. However, these mediators are not as effective electron transfer agents as methyl viologen.

Glazier et al. (1998) have described a nitrate sensor which incorporates methyl viologen (MV, see Figure 3) in a Nafion layer (insulator polymer with high cation exchange capacity). The layer acted as a reservoir for the electron mediator, since MV was immobilized at some percentage of saturation in the presence of other inorganic cations. Nitrate reductase was retained behind a dialysis membrane to prevent any leaching. Therefore, a smaller amount of NaR could be used and the activity decreased slowly. For the same purpose, Ugo et al. (1998) and Moretto et al. (1998) developed a nitrate sensor based upon an anion-permselective composite membrane that allows only nitrate to go through. Nitrate reductase and methyl viologen were retained with a small volume inside the probe.
Figure 3: Chemical Structure of some Electron Mediators Used with Nitrate Reductase
Ferreyra et al. (2000) studied the impact of nitrite on a nitrate biosensor that uses methyl viologen. They found that MV reacts with nitrite to reduce it further and thus it could influence the response of such biosensors by increasing the current.

Lastly, Campbell and Campbell (1996) have reported on a process of denitrification based on three reductases, nitrate, nitrite and nitrous oxide reductase. The process is based upon two cells: the first containing nitrate reductase and the second nitrite reductase and nitrous oxide reductase, all being entrapped in gel (granular diatomaceous earth activated with polyimine) with the dye Azure A. No decline of activity was observed after four days of activity at 22 °C nor after one month at 4 °C. However, the rate seems to be relatively slow since about 30 hrs are needed to treat 250 mL of water containing 10 ppm (10 mg/L) of nitrate.

Goal of this Research Project

The purpose of this research is to develop a process of denitrification of water, which would be fast, simple, and economically able to sustain an industrial process. The entrapment of the enzymes is rather complicated. Therefore, this project will initially investigate an in-solution process, using membranes to retain both the enzymes and the mediator in a treatment compartment. Moreover, testing on electrode materials will be carried out to determine an industrially applicable one. In addition, the rate of nitrite reduction by an electron mediator without the biological enzyme will be studied in order to avoid the use of nitrite reductase, if possible. All these experiments will be carried out
at the laboratory level and if they appear to be successful, a pre-industrial flow cell will be developed.
CHAPTER 1: ELECTROCHEMISTRY OF METHYL VIOLOGEN IN PRESENCE OF NITRATE AND NITRATE REDUCTASE

Materials and Methods

All solutions were prepared with ultra-pure water from a MilliQ system (Millipore S.A.). The background electrolyte solution was a phosphate buffer (0.1 M, pH 7.5) prepared with Na₂HPO₄ (Fisher Scientific Company, S-373) and NaH₂PO₄ (Fisher Scientific Company, S-369). 1,1'-Dimethyl-4,4'-bipyridinium dichloride (methyl viologen, MV²⁺) (ICN Pharmaceuticals, Inc., 195323), NaNO₃ (Fisher Scientific Company, S-343) and nitrate reductase EC 1.6.6.2 (NaR) (Sigma Chemical Company, N7265, from Aspergillus Species, cofactor: NAD[P]H, pH 7.5) were added to the buffer in the desired concentration, from stock solutions of 10 mM, 10 mM and 200 U/mL respectively. All chemicals were reagent grade and used as received. Solutions were deoxygenated with nitrogen for about ten minutes prior to the experiments and a nitrogen atmosphere was maintained throughout the measurements.

The reference electrode was Ag | AgCl | (3M) Cl⁻Na⁺ (RE-5B MF 2052 BAS, Bioanalytical Systems, Inc.), and all potentials in this part are referred to this electrode. The working electrode was glassy carbon (MF 2012 BAS), of 4.8 × 10⁻² cm² geometric area determined electrochemically. It was polished with wet alumina powder of particle size 1 µm (Moyco Precision Coated Abrasives, LPA-010), and then rinsed with pure water. The counter electrode was a platinum wire (MW 4130 BAS).
Cyclic voltammetry measurements were performed with a BAS 100B Electrochemical Analyzer (BAS) using the equipment software package BAS 100W (BAS). Each set of experiments was carried out at room temperature in a 10 mL electrochemical cell (VG-2 BAS). The potential sweep rate was 50 mV/s. The cell is described in Figure 4.

Figure 4: 10 mL Cell used to Study the Electrochemistry of MV$^{2+}$

Results and Discussion

Electrochemistry of Methyl Viologen

Figure 5 shows cyclic voltammograms for different concentrations of methyl viologen (0, 0.2, 0.5, 1 and 1.5 mM). In the range of potentials analyzed, no faradic current are evident for the buffer alone. As expected, the cathodic peak current is proportional to the concentration of MV$^{2+}$ (Figure 6). MV$^{2+}$/MV$^+$ is a well-known reversible couple
(reaction 1). In Figure 5, the peak currents ratio is close to one (1.056) and the peak potential separation $\Delta E_p$ approaches 59 mV (65 mV). These features are typical of reversible systems. In addition, the reduction potential $E_0$ is equal to -670 mV. These data are consistent with the work of Ferreyra et al. (2000). The system is working correctly, and the catalytic effect of NaR and NO$_3^-$ can be studied.

$$MV^{2+} + e^- \rightleftharpoons MV^+. \quad (1)$$

Figure 5: Cyclic Voltammograms of MV$^{2+}$ in Phosphate Buffer at 50 mV/s. Nitrogen Atmosphere on Glassy Carbon ($4.8 \times 10^{-2}$ cm$^2$). Concentration: 0, 0.2, 0.5, 1 and 1.5 mM at Room Temperature.
Figure 6: Plot of Cathodic Current versus the Concentration of MV$^{2+}$ in Phosphate Buffer at 50 mV/s. Nitrogen atmosphere on glassy carbon ($4.8 \times 10^{-2}$ cm$^2$) at Room Temperature

Catalysis of Methyl Viologen by Nitrate Reductase and Nitrate

Figure 7 shows cyclic voltammograms of MV$^{2+}$ (0.2 mM) in presence of increasing NaR concentration (0, 0.5, 1.5, 2, 3, 4 and 5 U/mL) and with NO$_3^-$ at a constant concentration (1 mM). When MV$^{2+}$ and NO$_3^-$ are both in solution, the current for the reduction of MV$^{2+}$ equals the current for the reduction of MV$^{2+}$ alone (Figure 7 B and C). Thus, NO$_3^-$ does not seem to react neither with MV$^{2+}$ nor directly at the electrode or at least the reactions are not fast enough to be seen at this sweep rate. Therefore, no catalytic effect with MV$^{2+}$ as an electron mediator can be attributed to the reduction of NO$_3^-$. The cathodic peak current increases with NaR concentration, whereas the anodic peak current decreases. In fact, NaR oxidizes MV$^+$ to MV$^{2+}$ in the vicinity of the
electrode and then the reduced form of NaR reduces NO$_3^-$ to NO$_2^-$ (reactions 1, 2 and 3). Thus, MV$^{2+}$ is regenerated at the electrode with concomitant decrease in MV$^+$. The result is that the reduction current for MV$^{2+}$ increases, while the oxidization current for MV$^+$ decreases.

\[
MV^{2+} + e^- \rightarrow MV^+ \quad (1)
\]

\[
MV^+ + NaR_{ox} \rightarrow MV^{2+} + NaR_{red} \quad (2)
\]

\[
NaR_{red} + NO_3^- + 2H^+ \rightarrow NaR_{ox} + NO_2^- + H_2O \quad (3)
\]

Figure 7: Cyclic Voltammograms of MV$^{2+}$ (0.2 mM) in Phosphate Buffer (0.1 M, pH 7.5) in presence of NO$_3^-$ (1 mM) at 50 mV/s. Nitrogen Atmosphere on Glassy Carbon (4.8 x $10^{-2}$ cm$^2$) at Room Temperature. Concentrations of NaR: 0 (A, B and C), 0.5 (D), 1.5 (E), 2 (F), 3 (G), 4 (H) and 5 U/mL (I) (A is Buffer alone. B is MV$^{2+}$ alone in Buffer. C is MV$^{2+}$ and NO$_3^-$)
Figure 8 represents the relation between NaR concentration and the cathodic peak current. It appears linear up to 3 U/mL and, thereafter, the current becomes independent of the enzyme concentration. At concentration lower than 3 U/mL, the peak current depends upon the concentration of NaR. Hence, the rate of reaction 2 is proportional to the NaRox concentration. Since NaRox is formed in reaction 3, the latter is the rate limiting step. In other words, reaction 3 is controlling the rate of the overall reaction. After 3 U/mL, the rate of the reaction does not depend upon the NaRox concentration. Therefore, reaction 2 becomes the rate controlling step. This means that a enzyme concentration greater than 3 U/mL for 0.2 mM of MV²⁺, the denitrification process reaches a maximum rate, and the concentration of enzyme does not need to be increased further.

Figure 8: Plot of Cathodic Current Change versus NaR Concentration for the Reduction of 0.2 mM MV²⁺ in presence of 1 mM NO₃⁻ in Phosphate Buffer at 50 mV/s. Nitrogen Atmosphere on Glassy Carbon (4.8 × 10⁻² cm²) at Room Temperature
Figure 7 (H and I) shows a shoulder before the cathodic peak. Ugo et al. (1998) observed the same phenomenon. They also noted a small decrease of the cathodic peak in presence of NaR and in absence of NO$_3^-$ . They believed that the shoulder and the decrease are due to the adsorption of NaR at the surface. The adsorption would modify the properties of the surface. Another explanation for the shoulder would be a direct reduction of NaR at the surface. The direct reduction would be slow, and a high concentration of NaR would be required in order to observe the reaction.

Figure 9 shows cyclic voltammograms of MV$^{2+}$ (0.2 mM) in presence of NaR (1 U/mL) and with increasing concentration of NO$_3^-$ (0.5, 1, 1.5, 2, 3, 4, 6, 8 and 10 mM). When only NaR is present, the current is close to the background current (buffer only). Nevertheless, the small difference might be due to the direct reduction of NaR. In the presence of NaR$^{\text{red}}$, the peak current due to MV$^{2+}$ reduction increases with NO$_3^-$ concentration. Indeed, MV$^+$ reduces NaR$_{\text{ox}}$ so that the concentration of MV$^{2+}$ available at the electrode is increased and thus the cathodic current is increased. However, if NO$_3^-$ is not present, no enhancement of the current is observed in the presence of NaR$_{\text{red}}$. In contrast, if NO$_3^-$ is present, it reacts with NaR$_{\text{red}}$ to oxidize it. Therefore, the concentration of MV$^{2+}$ and the cathodic current increase.
Figure 9: Cyclic Voltammograms of MV$^{2+}$ (0.2 mM) in Phosphate Buffer (0.1 M, pH 7.5) in presence of NaR (1 U/mL) at 50 mV/s. N$_2$ Atmosphere on Glassy Carbon (4.8 $\times$ 10$^{-2}$ cm$^2$) at Room Temperature. Concentrations of NO$_3^-$: 0 (A, B and C), 0.5 (D), 1 (E), 1.5 (F), 2 (G), 3 (H), 4 (I), 6 (J), 8 (K) and 10 mM (L) (A is Buffer alone. B is NaR alone in Buffer. C is NaR and MV$^{2+}$)

Figure 10 presents the cathodic current as a function of the nitrate concentration. The relation is linear up to 4 mM and, thereafter, the current becomes independent of nitrate concentration. The cathodic current for the reduction of MV$^{2+}$ is a measure of the rate of NO$_3^-$ reduction. At concentration lower than 4 mM, the peak current depends upon the concentration of NO$_3^-$. Since NO$_3^-$ is present only in reaction 3, the latter is the rate limiting step. In other words, reaction 3 is controlling the rate of the overall reaction. After 4 mM, the rate of the reaction does not depend upon the NO$_3^-$ concentration.
Therefore, reaction 2 becomes the rate controlling step. This means that a nitrate concentration greater than 4 mM per 1 U/mL of enzyme, the denitrification process reaches a maximum rate.

Figure 10: Plot of Cathodic Current Change versus NO$_3^-$ Concentration for the Reduction of 0.2 mM MV$^{2+}$ in presence of 1 U/mL NaR in Phosphate Buffer at 50 mV/s. Nitrogen Atmosphere on Glassy Carbon ($4.8 \times 10^{-2}$ cm$^2$) at Room Temperature

These three sets of experiments (Figures 5, 7 and 9) show that a system with MV$^{2+}$ and NaR may be able to reduce NO$_3^-$. Even though a direct reaction of NaR at the electrode might be possible, MV$^{2+}$ is needed to accelerate the process. Therefore, the next step will be to run an electroreduction in a small volume to demonstrate its feasibility.
CHAPTER 2: BULK ELECTROLYTIC REDUCTION OF NITRATE

Materials and Methods

All solutions were prepared with ultra-pure water from a MilliQ system (Millipore S.A.). Three different background electrolyte solutions were used. A carbonate buffer (0.1 M, pH 7.5) was prepared with NaHCO₃ (Fisher Scientific Company, S-233-3) and H₂SO₄ (Fisher Scientific Company, A-300-500). A Tris-HCl buffer (0.1 M, pH 7.5) was prepared with Tris(hydroxymethyl)aminomethane (Aldrich Chemical Company, 25,285-9) and HCl (Fisher Scientific Company, A-144-500). A Tris-Sulfate buffer (0.1 M, pH 7.5) was prepared with Tris(hydroxymethyl)aminomethane (Aldrich Chemical Company, 25,285-9) and H₂SO₄ (Fisher Scientific Company, A-300-500). 1,1'-Dimethyl-4,4'-bipyridinium dichloride (methyl viologen, MV²⁺) (ICN Pharmaceuticals, Inc., 195323), NaNO₃ (Fisher Scientific Company, S-343), NaNO₂ (Baker Chemical Co., 3780), and nitrate reductase EC 1.6.6.2 (NaR) (Sigma Chemical Company, N7265, from Aspergillus Species, cofactor: NAD[P]H, pH 7.5) were added to the proper buffer in the desired concentration. All chemicals were reagent grade and used as received. Solutions were deoxygenated with nitrogen or argon for about ten minutes prior to the experiments and a nitrogen or argon atmosphere was maintained throughout the measurements.

The quasi-reference electrode was a silver wire (E.H. Sargent and Co., S-85235-B), which had a potential of +40 mV vs Ag/AgCl (3 M NaCl). All potentials in this section are referred to this electrode. The working electrode was rotating glassy carbon (AFE2M050GC, Pine Instrument Company), of 0.508 cm² geometric area determined
electrochemically. It was polished with wet alumina powder of particle size 1 µm (Moyco Precision Coated Abrasives, LPA-010), and then rinsed with pure water. Another working electrode was built using 450 copper shot (Fisher Scientific Company, C-430) of 26.32 g total weight (mean diameter: 2.32 mm; mean specific surface area: 76 cm²). The copper shot was placed in a cellulose Spectro/Por2 molecular porous membrane tubing (cut-off 12,000-14,000 Da, 16 mm diameter, 2 mL/cm) (Spectrum Laboratories, Inc., 132678; distributed by Fisher Scientific Company, 08-670-3BB). The tubing was filled with buffer containing the required amount of NaR (2.8 mL). The counter electrode was a platinum coil of about 15 cm length.

Bulk electrolysis were performed with a BAS 100B Electrochemical Analyzer (BAS) using the equipment software package BAS 100W (BAS). Each set of experiments was carried out at room temperature in two electrochemical cells, which are described in Figures 11 and 12.

Nitrate and nitrite ions were analyzed by ion chromatography (DX-100 ion chromatograph, Dionex Company) using an IonPac AS4A-SC 4mm column (Dionex Company). The eluent was a carbonate buffer, 1.8 mM sodium carbonate (Thom Smith) / 1.7 mM sodium bicarbonate (Fisher Scientific Company, S-233-3). Ammonium ion was analyzed using a testing kit (Pulse Instruments, 942-043). Nitric oxide was analyzed using a specific probe (Harvard Apparatus, AmiNO-700 Nitric Oxide Sensor), in which the response was recorded using a MacLab interface on a Macintosh computer. Nitric oxide (Aldrich Chemical Company, 29,556-6) and NH₂OH, HCL (Eastman Organic Chemicals, P340) were used to demonstrate the efficiency of the tests.
Platinum counter electrode  Argon or Nitrogen  Working electrode: Either rotating glassy carbon; or cellulose bag filled with copper

Separation between the two electrodes: fritted

Silver quasi-reference electrode

Figure 11: Cell used to Study the Electroreduction of NO₃⁻ on Small Volume (about 10mL)

Working electrode: cellulose bag filled with copper shots and 3 U of NaR

Platinum counter electrode separated or not from the solution

Argon or nitrogen

Silver wire as quasi-reference electrode

Figure 12: Cell used to Study the Electroreduction of NO₃⁻ (30 mL)
Results and Discussion

Reduction of Nitrate on Glassy Carbon

A solution containing approximately 1 mM of $\text{NO}_3^-$ was kept at constant potential (-900mV) for 9 hours in presence of 1 mM of $\text{MV}^{2+}$ and 3 U/mL of nitrate reductase. The solution was prepared with 7 mL carbonate buffer, because the phosphate buffer, used for the cyclic voltammograms, interferes during the analysis of $\text{NO}_3^-$ and $\text{NO}_2^-$ by ion chromatography. The solution was deoxygenated by nitrogen. The experiment was carried out in the cell described Figure 11, in which the working electrode and the counter electrode were separated by a fritted glass. The rotating glassy carbon electrode served as the working electrode and it was rotated at 2000 rpm in order to favor the mass transfer of methyl viologen from the bulk solution onto the electrode.

The beginning concentration of nitrate was set to 1 mM. This corresponds to 14 mg/L $\text{NO}_3^-$, which is greater than the maximum concentration limit (10 mg/L) for drinking water. In the previous chapter, nitrate reductase concentration has been shown to limit the reduction of nitrate at concentrations lower than 0.25 U/mL per 1 mM nitrate. Hence, 3 U/mL nitrate reductase will not limit the reduction. In addition, a methyl viologen concentration of 1 mM was shown to limit the nitrate reduction rate only at a concentration of nitrate reductase greater than 15 U/mL.

Figure 13 shows the concentration of $\text{NO}_3^-$ and $\text{NO}_2^-$ according to the elapsed time. The concentration of $\text{NO}_3^-$ is substantially diminished after 9 hrs. However, the reduction rate seems faster at the beginning and slows down after 4 hrs. This decrease in the reduction rate is due to the lesser concentration (and thus availability) of $\text{NO}_3^-$ in the
solution since it is consumed. Therefore, the contact between NaR and NO$_3^-$ is less likely to happen, the rate of reduction decreases. This variation in rate could also be due to the change in pH of the solution in the course of the reduction. Indeed, the reduction of NO$_3^-$ and NO$_2^-$ requires protons (reactions 4 and 5).

\[
\begin{align*}
\text{NO}_3^- + 2 \text{H}^+ + 2 \text{e}^- & \rightarrow \text{NO}_2^- + \text{H}_2\text{O} \quad (4) \\
2 \text{NO}_2^- + 8 \text{H}^+ + 6 \text{e}^- & \rightarrow \text{N}_2 + 4 \text{H}_2\text{O} \quad (5)
\end{align*}
\]

More interesting, NO$_2^-$ appears as a product of reduction of NO$_3^-$ but then disappears. It does not accumulate in stoichiometric amount. The data suggest that nitrite can be reduced by MV$^+$ (reactions 6 and/or 7). The same electrochemical cell was then used,
replacing NO$_3^-$ by NO$_2^-$, and in which NaR was not present. The results are displayed in Figure 14. The NO$_2^-$ decreases regularly over 12 hrs. However, no test without MV$^{2+}$ has been conducted. Therefore, it is not possible to know if MV$^-\cdot$ reduces NO$_2^-$ or if the reduction takes place directly at the cathode (reaction 5).

$$6 \text{MV}^+ + 2 \text{NO}_2^- + 8 \text{H}^+ \rightarrow 6 \text{MV}^{2+} + \text{N}_2 + 4 \text{H}_2\text{O} \quad (6)$$

$$4 \text{MV}^+ + 2 \text{NO}_2^- + 6 \text{H}^+ \rightarrow 4 \text{MV}^{2+} + \text{N}_2\text{O} + 3 \text{H}_2\text{O} \quad (7)$$

Figure 14: Plot of NO$_2^-$ Concentration versus Elapsed Time. Electrolysis at $-900 \text{ mV}$ in presence of 1 mM MV$^{2+}$ on Glassy Carbon (0.508 cm$^2$). 7 mL Carbonate Buffer under a Nitrogen Atmosphere at Room Temperature. Rotation Rate 2000 rpm

Table 3 shows the efficiency of the overall denitrification to nitrogen gas. The calculation of the efficiency is shown in Appendix I. It takes into account the reduction of NO$_3^-$ to NO$_2^-$ and the reduction of NO$_2^-$ to N$_2$. The efficiency of the electrochemical
process is fairly constant and relatively high (40-45%). Therefore, the reduction process can take place until a high degree of reduction is reached since it is as efficient throughout the experiment. The efficiency suggests that approximately one half of the current is used to reduce NO$_3^-$.

Therefore, half of the current is used for other reactions, such as reduction of protons or water (reactions 8 and 9).

\[
\begin{align*}
2 \text{H}_2\text{O} + 2 \text{e}^- & \rightarrow 2 \text{OH}^- + \text{H}_2^+ \quad (8) \\
2 \text{H}^+ + 2 \text{e}^- & \rightarrow \text{H}_2 \quad (9)
\end{align*}
\]

Table 3: Time, Charge, NO$_3^-$ and NO$_2^-$ Concentrations and Efficiency (from NO$_3^-$ to N$_2$ Accounting for NO$_2^-$ that has not Reacted). Electrolysis at $-900$ mV in presence of 1 mM MV$^{2+}$ and 3 U NaR on Glassy Carbon (0.508 cm$^2$). 7 mL Carbonate Buffer under a Nitrogen Atmosphere at Room Temperature. Rotation Rate 2000 rpm

<table>
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<tr>
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<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>NO$_2^-$ (mM)</td>
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<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>-</td>
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<td>45</td>
<td>42</td>
</tr>
</tbody>
</table>

Table 4 shows the efficiency of the reduction of NO$_2^-$ (see Appendix I for calculation details) in the absence of an enzyme, but in the presence of an electron mediator, MV$^{2+}$.

The efficiency (37% at the beginning, which decreased to 26% after 9 hrs) is lower than the efficiency of the overall denitrification. Thus, the transfer of the electrons from the electrode to NO$_2^-$ (either directly or mediated by MV$^{2+}$) is probably less effective than the transfer to NO$_3^-$. However, in the case of nitrate reduction, an enzyme, NaR, is present to
favor the transfer of electrons to NO$_3^\text{-}$. Thus, this result is not unexpected. Moreover, the efficiency drops over time. It is likely that the conditions of the solution (e.g., pH) have changed during the course of the reaction. Indeed, the pH rises from 7.5 at the beginning to 8 after 12 hrs. This change would favor other reactions to occur at the electrode.

Table 4: Time, Charge, NO$_2^\text{-}$ Concentration and Efficiency (from NO$_2^\text{-}$ to N$_2$). Electrolysis at $-900$ mV in presence of 1 mM MV$^{2+}$ and on Glassy Carbon (0.508 cm$^2$). 7 mL Carbonate Buffer under a Nitrogen Atmosphere at Room Temperature. Rotation Rate 2000 rpm

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<tbody>
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<td>5.4</td>
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</tr>
<tr>
<td>NO$_2^\text{-}$ (mM)</td>
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<td>1.1</td>
<td>0.9</td>
<td>0.9</td>
<td>0.8</td>
<td>0.7</td>
<td>0.5</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>-</td>
<td>37</td>
<td>34</td>
<td>25</td>
<td>23</td>
<td>23</td>
<td>26</td>
<td>18</td>
<td>19</td>
</tr>
</tbody>
</table>

After the 12 hrs of electrolysis at $-900$ mV, the solution was tested for NH$_4^+$ and NO. Neither species were found in the electrolysis solution. The absence of ammonia could be due to a degassing by nitrogen or more likely it is not a product of the reduction. Moreover, the kit for NH$_4^+$ was used on a solution of NH$_2$OH, which gave an excellent response as was the case for NH$_4^+$. Thus, the kit also detects NH$_2$OH (it is not specific to NH$_4^+$ but to amines) and NH$_2$OH is not a product of the reaction.

In collaboration with Dr J. Bumpus, a solution of MV$^{2+}$ was reduced electrochemically to MV$^+$ under argon (the solution becomes blue because of MV$^+$). The NO probe was then placed in the solution and an aliquot of NO saturated water was injected. The blue-colored MV$^+$ solution immediately turned colorless, indicating that
MV$^+$ was oxidized to MV$^{2+}$. Furthermore, no peak of NO was observed. A second aliquot was injected in the now colorless solution, i.e., which contained MV$^{2+}$ and no more MV$^+$; a peak of NO was observed. In consequence, NO is thought to be readily reduced by MV$^+$ (reactions 10 and/or 11). Thus, if it is an intermediary of the reaction, it does not accumulate. All these experiments suggest that the product(s) is (are) gaseous, i.e., N$_2$ or/and N$_2$O.

\[
\begin{align*}
4 \text{MV}^+ & + 2 \text{NO} + 4 \text{H}^+ \rightarrow 4 \text{MV}^{2+} + \text{N}_2 + 2 \text{H}_2\text{O} \quad (10) \\
2 \text{MV}^+ & + 2 \text{NO} + 2 \text{H}^+ \rightarrow 2 \text{MV}^{2+} + \text{N}_2\text{O} + \text{H}_2\text{O} \quad (11)
\end{align*}
\]

**Reduction of Nitrate on Copper**

**Reactions on copper.** Since nitrate reductase and glassy carbon are relatively expensive, copper was tested as a material for the working electrode in order to reduce the cost of the cell. Cyclic voltammetric data on copper indicate that the reduction of MV$^{2+}$ occurs at almost the same potential as on glassy carbon. As in any electrochemical reaction, the overall current or rate of reduction depends on the surface area of the electrode. Therefore, copper shots were used to increase the surface area and to keep the volume of the electrode small. In contrast to copper powder, the porosity of copper shots is high enough to allow effective mass transfer.

In addition, no nitrate reductase is needed in the bulk solution but a high concentration is required close to the electrode, where the highest concentration of MV$^+$ is present. For that purpose, copper shots were confined in a cellulose membrane with 3 U of NaR (2.8 mL). Unfortunately, MV$^{2+}$ and MV$^+$ can cross the membrane and MV$^{2+}$ (1
mM) was put in the solution (10 mL of carbonate buffer) with NO$_3^-$ (1 mM). The solution was continuously flushed with argon and stirred. The bulk electrolysis was carried out at -900 mV vs silver quasi-reference electrode.

Figure 15 illustrates the change in the concentration of NO$_3^-$ and NO$_2^-$ as a function of the elapsed time. After 5.5 hrs, the concentration of NO$_3^-$ is substantially diminished. The rate of reduction is much greater than on glassy carbon, which is consistent with the electroactive area being much larger. Thus, more MV$^+$ is available to reduce NaR. In addition, the same amount of NaR is present but in a smaller volume. Therefore, NaR and MV$^+$ are highly concentrated, and the rate increases. Nitrite seems to accumulate in the solution throughout the experiment, but not in a stoichiometric amount. The data suggest that the reactions on copper are similar to those on glassy carbon.

![Figure 15: Plot of NO$_3^-$ (+) and NO$_2^-$ (x) Concentrations versus Elapsed Time. Electrolysis at -900 mV in presence of 1 mM MV$^{2+}$. Working Electrode: 6 mL Copper Shots and 3 U NaR (2.8 mL) in a Cellulose Membrane. 10 mL Carbonate Buffer under an Argon Atmosphere at Room Temperature](image)
The same system was used with NO$_2^-$ instead of NO$_3^-$ in the absence of NaR (12 mL of solution). Figure 16 shows the results. As expected, the NO$_2^-$ concentration decreases over time, because it is reduced in solution. Figure 17 represents the results carried out on copper foil (9.6 cm$^2$ geometric area) with and without MV$^{2+}$ (11 mL of Tris-sulfate buffer, flushed by argon, -800 mV). The rates of reduction at the beginning are equal (0.54 mM/hr). Therefore, the reduction of NO$_2^-$ takes place directly at the electrode and, at least on copper, the presence of an electron mediator MV$^+$ is unnecessary.

Figure 16: Plot of NO$_2^-$ Concentration versus Elapsed Time. Electrolysis at -900 mV in presence of 1 mM MV$^{2+}$. Working Electrode: 6 mL Copper Shots in a Cellulose Membrane. 12 mL Carbonate Buffer under a Nitrogen Atmosphere at Room Temperature
Table 5 shows the efficiency of the overall reduction of NO$_3^-$ to N$_2$ on copper shots (see Appendix I for calculation details). As can be seen, the efficiency is lower than on glassy carbon, i.e., initially at 34% decreasing to 19%. Thus, it appears that there can be significant side reactions that can occur at a copper electrode. However, the system has not been optimized and the efficiency is fairly high. With optimization, the efficiency will probably be improved up to 50% or more. In addition, the efficiency drops over time as would be expected due to a decrease in concentration of nitrate. However, this is probably not a problem because an industrial process would keep the concentration of nitrate fairly constant.
Table 5: Time, Charge, NO$_3^-$ and NO$_2^-$ Concentrations and Efficiency (from NO$_3^-$ to N$_2$ Accounting for NO$_2^-$ that has not Reacted). Electrolysis at -900 mV in presence of 1 mM MV$_{2+}$. Working Electrode: 6 mL Copper Shots and 3 U NaR (2.8 mL) in a Cellulose Membrane. 10 mL Carbonate Buffer under an Argon Atmosphere at Room Temperature

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Charge (C)</td>
<td>0</td>
<td>4.4</td>
<td>6.5</td>
<td>9.6</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>NO$_3^-$ (mM)</td>
<td>1</td>
<td>0.7</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>NO$_2^-$ (mM)</td>
<td>0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>-</td>
<td>34</td>
<td>26</td>
<td>25</td>
<td>23</td>
<td>19</td>
</tr>
</tbody>
</table>

Effect of pH on the reactions. Experiments were designed to decrease the applied potential and, thus, to consume less energy, and, meanwhile, to avoid some unwanted reactions. Figure 18 represents the electrolysis at -700 mV of 12 mL of solution (1 mM NO$_3^-$ and 1 mM MV$_{2+}$, flushed by argon). The reaction occurs, even though it is slower. Several experiments were performed and, each time, the reaction seems to slow down after a few hours. Meanwhile, the pH rises faster than would be expected according to the reactions of reduction (4 and 5). When acid was added (Figure 18) after 9 and 11 hrs, the reaction started again. It was suspected that CO$_2$ was degassed from the solution, which changed the pH of the solution. Since NaR works at pH 7.5, after few hours, the reaction ended or, at least, slowed down. Therefore, Tris-HCl buffer was used later on. Unfortunately, the peak of Cl$^-$ overlaps the ion chromatographic peak of NO$_2^-$ and only the concentration of NO$_3^-$ can be analyzed.
Electrolysis at -700 mV with 1 mM MV$^{2+}$. Working Electrode: 6 mL Copper Shots and 3 U NaR (2.8 mL) in a Cellulose Membrane. 12 mL Carbonate Buffer under an Argon Atmosphere at Room Temperature. Acid Added after 9 and 11 hrs.

Effects of the cellulose membrane on the reactions. The copper shots were washed with concentrated H$_2$SO$_4$ and a new electrode was constructed with a cellulose membrane and NaR (3 U). It was used on three consecutive days to denitrify three different solutions containing about 1 mM of NO$_3^-$ in 30 mL of Tris-HCl buffer containing 1 mM of MV$^{2+}$. Each experiment was carried out at -900 mV. The first day, the two electrodes were separated by a fritted and the solution was deoxygenated with argon. The second day, the solution was still deoxygenated with argon but the electrodes were not any longer separated. Lastly, the third day, the electrodes were not separated and the solution was not deoxygenated.
Figure 19 shows the results. It also presents the effect of MV$^{2+}$ and NaR on the reduction, (electrode not separated and solution deoxygenated). The reaction is faster when the two electrodes are in the same solution. It is likely that the resistance of the system is diminished when the fritted glass is not present, thus the current of reduction and the rate of reaction are greater. In addition, MV$^+$ is concentrated behind the membrane and what leaves the inner solution can react at the anode since it is not useful for the reaction. In contrast, if NaR is placed in the solution with MV$^{2+}$ and the anode, MV$^+$ is oxidized at the anode as soon as it is produced and, thus, it is no longer available for NaR. No reduction can be observed in that case. Therefore, the cellulose membrane plays the role of the fritted glass, which is no longer needed. In addition, the membrane hinders the transfer of oxygen from the solution to the cathode. Thus, MV$^+$ is consumed by the few oxygen molecules at the beginning at the electrode, then, MV$^+$ is available for NaR. The small amount of oxygen that might cross the membrane does not significantly affect the rate of reaction. This will simplify any industrial usage of this system since the solution to be treated would not need to be deoxygenated and the two electrodes could be placed directly in the effluent. The end solution in presence of oxygen was tested for NH$_4^+$. None was found. A gaseous product is still expected.
Figure 19: Plot of NO$_3^-$ Concentration versus Elapsed Time. Electrolysis at -900 mV with 1 mM MV$^{2+}$ (except ×). Working Electrode: 6 mL Copper Shots and 3 U NaR (2.8 mL) (except -) in a Cellulose Membrane. 30 mL Tris-HCl Buffer under an Argon Atmosphere (except o) at Room Temperature. Electrodes not Separated by a Fritted (except Δ). (+) follows all the conditions

Table 6 shows the efficiency of the different treatments illustrated in Figure 18 (see Appendix I for calculation details). Although the separation of the two electrodes slows down the reduction, it gives a higher efficiency (63% to 34% after 10 hrs compared to about 20%). In the presence of oxygen, the efficiency is lower than in its absence, but this difference does not appear as a problem in an industrial process. Indeed, the deoxygenation of the solution would probably be more expensive than the additional current. Moreover, the difference can also reflect experimental error. Even if less efficient, the non-separation of the electrodes requires less time to achieve the degree of
denitrification. In addition, the efficiency can probably be improved by optimization of the different conditions.

Table 6: Time, Charge, NO$_3^-$ Concentration and Efficiency (from NO$_3^-$ to N$_2$).
Electrolysis at $-900$ mV with 1 mM MV$_2^+$ (except $\times$). Working Electrode: 6 mL Copper Shots and 3 U NaR (2.8 mL) (except -) in a Cellulose Membrane. 30 mL Tris-HCl Buffer under an Argon Atmosphere (except o) at Room Temperature. Electrodes not Separated by a Fritted (except $\Delta$). (+) follows all the conditions

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>0</th>
<th>1.5</th>
<th>2</th>
<th>2.5</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta$</td>
<td>NO$_3^-$ (mM)</td>
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<td>0.7</td>
<td>0.6</td>
<td>0.4</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ NO$_3^-$ (mM)</td>
<td>1</td>
<td>0.5</td>
<td>0.4</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efficiency (%)</td>
<td>-</td>
<td>24</td>
<td>25</td>
<td>24</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>o NO$_3^-$ (mM)</td>
<td>1</td>
<td>0.7</td>
<td>0.5</td>
<td>0.3</td>
<td>0.2</td>
<td></td>
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<tr>
<td>Efficiency (%)</td>
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<td>20</td>
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<tr>
<td>- NO$_3^-$ (mM)</td>
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<td>Efficiency (%)</td>
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<td>15</td>
<td>20</td>
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<tr>
<td>$\times$ NO$_3^-$ (mM)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effects of NaR and MV$^{2+}$ on the reactions. Figure 19 shows that there is almost no denitrification in absence of NaR. Thus, NO$_3^-$ reacts neither with MV$^{2+}$ nor at the electrode. In addition, in the absence of MV$^{2+}$, but in presence of NaR, the rate of reduction is slow but denitrification occurs. This confirms that NaR is reduced slowly directly at the electrode, as it has been suggested by cyclic voltammetry. However, the rate is too slow to get rid of a mediator. Therefore, the system where MV$^{2+}$ and the enzyme are present is required to denitrify the solution. Denitrification using the enzyme
alone is fairly efficient (Table 6, about 15%). However, it is much too slow to be considered as an industrial process.
CHAPTER 3: GALVANIC REDUCTION OF NITRATE

Materials and Methods

All solutions were prepared with ultra-pure water from a MilliQ system (Millipore S.A.). The background electrolyte solution was a Tris-Sulfate buffer (0.1 M, pH 7.5) prepared with Tris(hydroxymethyl)aminomethane (Aldrich Chemical Company, 25,285-9) and H₂SO₄ (Fisher Scientific Company, A-300-500). 1,1'-Dimethyl-4,4'-bipyridinium dichloride (methyl viologen, MV²⁺) (ICN Pharmaceuticals, Inc., 195323), NaNO₃ (Fisher Scientific Company, S-343), and nitrate reductase EC 1.6.6.2 (NaR) (Sigma Chemical Company, N7265, from Aspergillus Species, cofactor: NAD[P]H, pH 7.5) were added to the buffer in the desired concentration. All chemicals were reagent grade and used as received.

The galvanic cell was composed of a copper cathode, of approximately 10 cm² geometric area and 3 bolts coated by zinc (DRI Industries, 8-32x1”). In this galvanic system, the potential of the copper electrode is approximately -800 mV vs Ag/AgCl (3 M NaCl). The copper electrode and the zinc-coated bolts were clamped and placed in a 50 mL beaker, containing 40 mL buffer, 0.99 mM nitrate, 1 mM methyl viologen and 2 U nitrate reductase (Figure 20). The solution was stirred and degassed with argon.
Nitrate and nitrite ions were analyzed by ion chromatography (DX-100 ion chromatograph, Dionex Company) using an IonPac AS4A-SC 4mm column (Dionex Company). The eluent was a carbonate buffer, 1.8 mM sodium carbonate (Thom Smith) / 1.7 mM sodium bicarbonate (Fisher Scientific Company, S-233-3).

**Results and Discussion**

During the development of a cell made of a copper electrode, a plexiglass frame and a membrane, a contact was created between copper and galvanized bolts. When placed in an aqueous medium, the methyl viologen entrapped between the membrane and the copper turned blue, which is the color of methyl viologen partially reduced. It was suspected that the contact between the bolt and the copper constituted a galvanic cell. It
should be noted that this cell was abandoned, because a seal between the copper electrode and the membrane could not be achieved.

To test this hypothesis of a zinc/copper galvanic cell, an experiment was designed. Forty mL of buffer, containing 0.99 mM of nitrate, 1 mM of methyl viologen and 2 U of nitrate reductase, were placed in a 50 mL beaker. The system was purged and maintained under an argon atmosphere because oxygen can react with the reduced form of methyl viologen, MV\(^+\), which is necessary to reduce nitrate reductase. During the course of the experiment the solution turned blue. In addition, the blue color could initially be observed at the surface of the copper electrode. After 3 days (time to consume the zinc coating of the three bolts, which were added individually), the nitrate concentration decreased to 0.39 mM and the nitrite concentration was equal to 0.25 mM. It should be noted that nitrite does not accumulate in a stoichiometric amount and thus nitrite must be further reduced. Therefore, the galvanic cell, using methyl viologen and nitrate reductase, has the same ability to reduce nitrate and nitrite as the electrolytic cell previously described (chapter 2). In other words, the galvanic cell, using the same electron-transfer system, reduces nitrate to nitrite. Copper exhibits the same catalytic properties towards the nitrite reduction.

The reactions that occur in this system are the following:

At the copper cathode:

\[
\begin{align*}
MV^{2+} + e^- & \rightleftharpoons MV^+. \\
2 \text{NO}_2^- + 8 \text{H}^+ + 6 e^- & \rightarrow \text{N}_2 + 4 \text{H}_2\text{O}
\end{align*}
\]
In solution:

\[ \text{MV}^{+} + \text{NaR}_{\text{ox}} \rightarrow \text{MV}^{2+} + \text{NaR}_{\text{red}} \quad (2) \]

\[ \text{NaR}_{\text{red}} + \text{NO}_3^- + 2 \text{H}^+ \rightarrow \text{NaR}_{\text{ox}} + \text{NO}_2^- + \text{H}_2\text{O} \quad (3) \]

At the zinc anode:

\[ \text{Zn} \rightarrow \text{Zn}^{2+} + 2 \text{e}^- \quad (12) \]

General equation:

\[ 5 \text{Zn} + 2 \text{NO}_3^- + 12 \text{H}^+ \rightarrow 5 \text{Zn}^{2+} + \text{N}_2 + 6 \text{H}_2\text{O} \quad (13) \]

This system could be used in place where electricity is not available. Zinc metal would only have to be periodically added. The major disadvantage is that zinc ion will be introduced into the environment.
CHAPTER 4: NITRATE REDUCTION IN A FLOW CELL

Materials and Methods

All solutions were prepared with ultra-pure water from a MilliQ system (Millipore S.A.). Two different background electrolyte solutions were used. A carbonate buffer of constant concentration (0.1 M, pH 7.5) was prepared with NaHCO₃ (Fisher Scientific Company, S-233-3) and H₂SO₄ (Fisher Scientific Company, A-300-500). A Tris-Sulfate buffer (0.1 M, pH 7.5) was prepared with Tris(hydroxymethyl)aminomethane (Aldrich Chemical Company, 25,285-9) and H₂SO₄ (Fisher Scientific Company, A-300-500). 1,1'-Dimethyl-4,4'-bipyridinium dichloride (methyl viologen, MV²⁺) (ICN Pharmaceuticals, Inc., 195323), NaNO₃ (Fisher Scientific Company, S-343), NaNO₂ (Baker Chemical Co., 3780), and nitrate reductase EC 1.6.6.2 (NaR) (Sigma Chemical Company, N7265, from Aspergillus Species, cofactor: NAD[P]H, pH 7.5) were added to the proper buffer in the desired concentration. All chemicals were reagent grade and used as received.

The working electrode was copper (4-40109-03, Electrosynthesis Company), of approximately 10 cm² geometric area. The counter electrode was graphite (4-440107, Electrosynthesis Company).

Bulk electrolysis were performed with a potentiostat/galvanostat (model 173, EG&G Princeton Applied Research) equipped with a digital coulometer (model 179, EG&G Princeton Applied Research). Each set of experiments was carried out at room temperature in an electrochemical cell (micro-01, Electrosynthesis Company), which is described in Figure 21.
Gathering Bolts
Frame
Solution Flow
Frame gasket
Copper electrode (cathode)
Electrode gasket (1)
Membrane (nitrate permeable) (2)
Electrode gasket (3)
Circulation frame (4)
Membrane gasket (5)
Membrane (Nafion) (6)
Membrane gasket (7)
Circulation frame (8)
Electrode gasket (9)
Graphite electrode (anode)
Frame gasket
Frame
Gathering Nuts

Figure 21: Flow Cell Schematic
Two pumps (model BC-2CP-MD, March MFG., Inc.) were used to circulate the solutions in each compartment. In the cathode compartment, nitrate or nitrite (1 mM) was circulated in 200 mL of the desired buffer. When needed, one liter of Tris-Sulfate with 0.1 mol of Na₂SO₄ was used in the anode compartment. Three types of membranes were used to separate the compartments. An anion-exchange membrane (Soda Neosepta AMX, Tokuyama Corp.) and a cation-exchange membrane (Nafion 450, DuPont de Nemours) were purchased from the Electrosynthesis company. A cellulose Spectro/Por2 molecularporous membrane tubing (cut-off 12,000-14,000 Da, 16 mm diameter, 2 mL/cm) (Spectrum Laboratories, Inc., 132678) was purchased from Fisher Scientific Company.

Nitrate and nitrite ions were analyzed by ion chromatography (DX-100 ion chromatograph, Dionex Company) using an IonPac AS4A-SC 4mm column (Dionex Company). The eluent was a carbonate buffer, 1.8 mM sodium carbonate (Thorn Smith) / 1.7 mM sodium bicarbonate (Fisher Scientific Company, S-233-3).

Results and Discussion

The efficiency of a nitrate reductase-assisted reduction of nitrate has been proven in a batch cell. However, to be practical as a field device, the reduction should be able to take place in a continuous process, such as a flow cell. To demonstrate the functioning of such a cell, a first set of experiments was carried out using nitrite without methyl viologen or nitrate reductase. The cell is illustrated in Figure 20 and was constructed as indicated with the exception of the nitrate permeable membrane and the second electrode.
gasket (2 and 3). The nitrate permeable membrane was not needed because nitrate reductase was not used. The Nafion membrane was necessary to separate the anode and cathode compartments. When run without this Nafion membrane, nitrite was apparently oxidized back to nitrate on the anode and therefore no net reaction occurred.

The results of the electrolysis at different current densities are shown on Figure 22. The electrolysis at 0.75 mA cm\(^{-2}\) reduces more nitrite than any other. It is expected that the reduction occurs faster when the current density increases. Indeed, as the current density is increased, more charge is transferred and more nitrite is reduced. If the current density is increased beyond 1 mA cm\(^{-2}\), gaseous evolution, probably hydrogen, is observed. Horányi and Rizmayer (1985) showed that the rate of nitrate and nitrite reduction on platinum depended on the adsorption competition between hydrogen and nitrate or nitrite. Increasing the hydrogen gas concentration in the solution would therefore increase its competitive capacity and decrease the rate of nitrite reduction. Even though hydrogen adsorption is less effective on copper than on platinum, it is possible that this mechanism accounts for the decrease in the amount of nitrite reduction.

Figure 23 presents the efficiency of the nitrite reduction at the different current densities. With the exception of 0.5 mA cm\(^{-2}\), the efficiency decreases with increasing current density. This result is expected since the cathodic potential becomes more negative when the current density increases. Therefore, more side reduction reactions can occur and the efficiency drops. The choice of the current density used in a process would be based on the respective costs of electricity and time.
Figure 22: Plot of NO$_2^-$ Concentration versus Elapsed Time. Bulk Electrolysis in Flow Cell without 2 and 3. 200 mL of Tris-Sulfate Buffer. Anode Compartment: 0.1 M Na$_2$SO$_4$ in Tris-Sulfate. (◊) 0.3 mA cm$^{-2}$; (Δ) 0.5 mA cm$^{-2}$; (×) 0.75 mA cm$^{-2}$; (○) 1 mA cm$^{-2}$; (*) 1.5 mA cm$^{-2}$.

Figure 23: Efficiency of Bulk Electrolysis of Approximately 1 mM NO$_2^-$ in Tris-Sulfate. Flow Cell without 2 and 3. Anode Compartment: 0.1 M Na$_2$SO$_4$ in Tris-Sulfate. (◊) 0.3 mA cm$^{-2}$; (Δ) 0.5 mA cm$^{-2}$; (×) 0.75 mA cm$^{-2}$; (○) 1 mA cm$^{-2}$; (*) 1.5 mA cm$^{-2}$.
Since the flow cell design works on a nitrite system, it was adapted to treat a solution containing approximately 1 mM of nitrate. Each experiment is described in Table 7. A small volume of an inner solution was created close to the cathode by adding a nitrate permeable membrane, either Neosepta AMX or cellulose membrane. The area of the electrode was about 10 cm$^2$ and the electrode gasket was about 1 mm thick. Therefore, the volume of the inner solution was less than 1 mL since the gasket was compressed to seal the cell. Inside this inner volume, nitrate reductase and methyl viologen were incorporated in buffer (the added volume before assembly and the amount of methyl viologen and nitrate reductase are described in Table 7). The inner solution between the cathode and the nitrate permeable membrane was quiet, whereas the solution to be treated was circulated. The anode and cathode were not separated by a Nafion membrane.

None of these designs were effective for the reduction of nitrate. Several parameters can pose problems. The first one is the current density. Indeed, the current density determines the cathode potential. If it is too low, the potential will not reach $-700 \text{ mV}$, which is mandatory to reduce methyl viologen $\text{MV}^{2+}$ to $\text{MV}^+$. In contrast, if the current density is too high, the potential will be negative enough to reduce methyl viologen, $\text{MV}^{2+}$, to its non-ionic form, MV, which will precipitate out of the solution. This precipitated form is no longer available to reduce nitrate reductase. Therefore, the problem is to find the range of current densities that sets the cathode potential in the $\text{MV}^+$ production range.
Table 7: Flow Cell Designs for the Treatment of Nitrate Solution. Anode and Cathode Compartments not Separated, i.e., Elements 6 to 9 not Included.

<table>
<thead>
<tr>
<th>Run number</th>
<th>Cell assembly</th>
<th>Nitrate-permeable membrane</th>
<th>Added (mmol)</th>
<th>MV$^{2+}$ (U)</th>
<th>NaR (U)</th>
<th>Current density (mA/cm)</th>
<th>Buffer</th>
<th>Time of the experiment (hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MV2+ and NaR before assembly</td>
<td>Neosepta AMX</td>
<td>2</td>
<td>1</td>
<td>5</td>
<td>0.5</td>
<td>Tris-Sulfate</td>
<td>4.4</td>
</tr>
<tr>
<td>2</td>
<td>MV2+ and NaR before assembly</td>
<td>Neosepta AMX</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0.05</td>
<td>Tris-Sulfate</td>
<td>29.5</td>
</tr>
<tr>
<td>3</td>
<td>Assembly Neosepta AMX then MV2+ and NaR injected</td>
<td>0.7</td>
<td>0.5</td>
<td>3</td>
<td>0.1</td>
<td>Tris-Sulfate</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MV2+ and NaR before assembly</td>
<td>Neosepta AMX</td>
<td>1.2</td>
<td>1</td>
<td>3</td>
<td>0.05</td>
<td>Tris-Sulfate</td>
<td>46</td>
</tr>
<tr>
<td>5</td>
<td>MV2+ and NaR before assembly</td>
<td>Neosepta AMX</td>
<td>0.2</td>
<td>100</td>
<td>3</td>
<td>0.05</td>
<td>Tris-Sulfate (1 M)</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>MV2+ and NaR before assembly</td>
<td>Neosepta AMX</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>0.05</td>
<td>Carbonate</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>MV2+ and NaR before assembly</td>
<td>Cellulose</td>
<td>1</td>
<td>0 inside; 1 mM outside</td>
<td>3</td>
<td>0.05</td>
<td>Tris-Sulfate</td>
<td>22</td>
</tr>
</tbody>
</table>
The second parameter is the membrane type used to permit nitrate exchange but retain nitrate reductase and methyl viologen. Most experiments were carried out with an anion-exchange membrane, which should retain methyl viologen and nitrate reductase close to the electrode. However, the transport rate of nitrate through this membrane is not known. It may be very slow and therefore mass transfer will limit the nitrate reduction. Other membranes need to be tested, especially in a batch system to have an understanding of their transfer capabilities. The Neosepta AMX membrane cannot be used in a batch design because it is too rigid to be folded in a tube form. In contrast, the cellulose membrane was successfully used in the batch cell. However used in the flow cell, it did not exhibit better results than the Neosepta AMX membrane. In addition, other parameters could have been the cause of the non-efficiency. The residence time of the solution in the cell could have been too short to allow the transfer of nitrate through the membrane, especially with the Neosepta AMX membrane, which is fairly thick. Therefore, slower flow rates, i.e., longer residence time, need to be tested.

The third parameter is the buffer. Indeed, the proton in the Tris-Sulfate is carried by an amine, i.e., as a cation. Therefore, protons cannot be transferred through the anion-exchange Neosepta AMX membrane. Since protons are necessary for the reduction of nitrate, the reaction will be limited by the availability of protons in the inner buffer. The carbonate buffer used was not more effective than the Tris-Sulfate but additional experiments need to be carried out.

The fourth parameter is the isolation of the cathode and anode compartments of the cell. As the solution flows by the anode, nitrite, produced by the reduction of nitrate at
the cathode, can be oxidized back to nitrate. This did not happen in a batch cell, but the anode materials were different. Indeed, in the batch cell, the anode was platinum whereas it is modified carbon in the flow cell. The separation of the two compartments needs to be tested. However, initial testing did not succeed in reducing nitrate when the anode and the cathode compartments were separated.

Lastly, nitrate reductase and methyl viologen could be lost during the assembly of the cell or the concentrations could be too low. As a result, the amount of methyl viologen and nitrate reductase entrapped behind the membrane would be low and the reaction would be very slow. However, most experiments were carried out for at least 22 hrs. This was believed to be sufficient to observe a decrease in the concentration of nitrate due to its reduction.

To test these parameters, initial testing was carried out with nitrite. The current density was set at 0.5 mA cm\(^{-2}\), the anion-exchange Neosepta AMX membrane was placed in the system, the anode and cathode compartments were separated and the solution contained 0.9 mM of nitrate (200 mL). After 3 days, the nitrite concentration was 0.56 mM. This result shows that either the transport of nitrite through the membrane is very slow or that the proton availability is limiting the reaction. It should be noted that nitrate is a little bigger than nitrite. Thus, the transfer of nitrate should be slower than the transfer of nitrite. In addition, the current density was 10 times higher in the experiment than in most nitrate experiments. The reduction rate would therefore be slower in the case of nitrate reduction.
CONCLUSION AND FUTURE WORK

Nitrate is a worldwide contaminant that causes environmental (eutrophication) and human health (methemoglobinemia, cancers) problems. Very few techniques are available to remove it from the environment and techniques that do exist suffer from major drawbacks, such as production of unwanted byproducts (toxins or brine). The electrochemical reduction of nitrate on any electrode is relatively slow and catalysts or electron mediators are needed. The goal of this project was to develop a new process that could overcome these disadvantages. The project demonstrate the feasibility of using an enzyme, nitrate reductase, and a mediator, methyl viologen to transfer the electrons from the electrode to nitrate, either on glassy carbon or on copper. This enzyme system was shown to accelerate the rate of reduction. An electrode composed of a membrane filled with copper shot and the enzyme, nitrate reductase, was constructed. This electrode succeeded in reducing nitrate from a solution containing the electron mediator. Without further optimization, the electrochemical efficiency was approximately 20%. The main advantage of this system is the production of a gaseous product, which is probably nitrogen gas, an environmentally safe gas. In addition, a zinc/copper galvanic cell can provide the electrons needed for the nitrate reduction. Zinc is oxidized at the anode while methyl viologen is reduced at the copper cathode. Such a system could be used in situations where electricity is not available. However, the zinc anode will need to be periodically added.
This project has not yet resulted in the development of a practical flow cell for nitrate reduction. Several parameters need to be explored and the project will be continued by others. First, the current density range of methyl viologen, $\text{MV}^{2+}$, reduction to methyl viologen partly reduced, $\text{MV}^+$, needs to be determined. Meanwhile, the effect of the mixing patterns and, thus, the suitability of a divided cell must be determined. In addition, other anion-exchange membranes will be tested in order to obtain a better nitrate transfer through the membrane to the electrode area. Lastly, these experiments should be conducted with a buffer that can carry the protons inside the electrode area. This means that the proton carrier needs to be anionic, like phosphate or carbonate.

Another area of research will be to entrap nitrate reductase in a polymer. This will facilitate the transfer of nitrate to nitrate reductase, since the polymer will be thinner than a membrane. This polymer will also avoid the leaching of the enzyme. The polymers used in the literature are polypyrrole (Cosnier and coworkers, 1994, 1997a), and polythiophene (Willner et al., 1992). These polymers have viologen moieties attached on them because they are cationic and will, therefore, hinder the diffusion of methyl viologen into them. An undergraduate student will study the feasibility of a denitrification process using the polypyrrole coating.

Lastly, two other areas should be explored. The first is the study the material of the electrode. Current results indicate that copper exhibits catalytic properties toward nitrite reduction. Therefore, other materials will also likely allow the direct reduction of nitrite on the electrode. The second area would be to develop new geometry to favor the mass transfer. For example, a cylindrical geometry would allow a homogeneous current
density, a high surface area and a good mass transfer (Figure 24). The space between the two electrodes would be limited (in the centimeter range). In addition, the flow rate would be slow to permit a long residence time.

![Cylindrical Cell for Nitrate Reduction](image)

**Figure 24: Cylindrical Cell for Nitrate Reduction**

Solution to be treated

Anode

Copper tube cathode

Solution containing nitrate reductase and methyl viologen

Nitrate permeable membrane
REFERENCES


Van Hecke, K.; Van Cleemput, O.; Baert, L. Environ. Pollut. 1990, 63, 261-274.


APPENDIX I: CALCULATION OF THE ELECTROREDUCTION EFFICIENCY

Tables 3 and 5:

\[
E = \frac{([NO_3^-]_0 - [NO_3^-]_i) \cdot 2 \cdot V \cdot F + ([NO_3^-]_0 - [NO_3^-]_i - [NO_2^-]_i) \cdot 3 \cdot V \cdot F}{Q_t}
\]

Table 4:

\[
E = \frac{([NO_2^-]_0 - [NO_2^-]_i) \cdot 3 \cdot V \cdot F}{Q_t}
\]

Table 6:

\[
E = \frac{([NO_3^-]_0 - [NO_3^-]_i) \cdot 5 \cdot V \cdot F}{Q_t}
\]

\(E\): Efficiency in %

\([NO_3^-]_0\): Initial concentration of NO3–

\([NO_3^-]_i\): Concentration of NO3– at time t

\([NO_2^-]_0\): Initial concentration of NO2–

\([NO_2^-]_i\): Concentration of NO2– at time t

2, 3 or 5: Number of electrons transferred during the reduction of NO3– to NO2–, NO2– to N2 or NO3– to N2

\(V\): Volume of the solution

\(F\): Faraday constant (96500 C/mol)

\(Q_t\): Charge passed at time t
APPENDIX II: ENTRAPMENT OF PEROXIDASE IN POLYANILINE

Materials and Methods

All solutions were prepared with ultra-pure water from a MilliQ system (Millipore S.A.). A citrate phosphate buffer (Mcllvaine's standard buffer, pH 5) was prepared with Na₂HPO₄ (Fisher Scientific Company, S-373) and citric acid (Fisher Scientific Company, A-940-500). The background electrolyte solution was a solution of 0.5 M Na₂SO₄ (Fisher Scientific Company, S-419-500) in the buffer. Horseradish peroxidase EC 1.11.1.7 (HRP) (Sigma Chemical Company, P6782) and 1,2-diaminobenzene (Aldrich Chemical Company, P2393-8) were added to the buffer in the desired concentration. H₂O₂ (Fisher Scientific Company, H-325-500) was diluted to 0.3% weight every day and increasing amounts were added to the electrolyte solution during chronoamperometric measurements. Nafion (Solution Technology, Inc., 1100W) was diluted to 0.5% by C₂H₅OH (Aldrich Chemical Company, 45,984-4) and one drop was deposited onto the electrode and allowed to dry. All chemicals were reagent grade and used as received.

The reference electrode was Ag | AgCl | (3M) Cl⁻Na⁺ (RE-5B MF 2052 BAS, Bioanalytical Systems, Inc.), and all potentials in this section are referred to this electrode. The working electrode was rotating glassy carbon (AFE2M050GC, Pine Instrument Company), of 0.508 cm² geometric area determined electrochemically. It was polished with wet alumina powder of particle size 1 µm (Moyco Precision Coated Abrasives, LPA-010), and then rinsed with pure water. The counter electrode was a platinum wire (MW 4130 BAS).
Cyclic voltammetry and chronoamperometry measurements were performed with a BAS 100B Electrochemical Analyzer (BAS) using the equipment software package BAS 100W (BAS). Each set of experiments was carried out at room temperature in a 10 mL electrochemical cell (VG-2 BAS). The cell is described in Figure 11.

![Figure 11: Cell used to Study the Electroreduction of NO₃⁻ (30 mL)](image)

The surface of the electrode was modified using a protocol adapted from Bartlett and coworkers (1996 and 1997). A polyaniline film was electrodeposited at the surface of the electrode from a solution of aniline (0.44 M) (Sargent-Welch Scientific Company, SC-10697) in 1.9 M H₂SO₄ (Fisher Scientific Company, A-300-500), 0.5 M Na₂SO₄ (Fisher Scientific Company, S-419-500) and 5 mM 1,4-diaminobenzene (Aldrich Chemical Company, P2396-2) at 940 mV. Each set of experiments had a different layer thickness (see the results and discussion section). Then, HRP was adsorbed from a buffer solution
of the desired concentration and crosslinked by glutaraldehyde (Acros, 11998-0250). A layer of 1,2-diaminobenzene (5 mM in buffer) was deposited at 340 mV for 4 minutes. The horseradish peroxidase catalyses the reduction of hydrogen peroxide to water. Therefore, the effectiveness of each electrode was tested with $\text{H}_2\text{O}_2$ (equation 14) by chronoamperometry at 90 mV and rotated at 500 rpm (see the results and discussion section for more details).

$$\text{H}_2\text{O}_2 + 2 \text{H}^+ + 2 \text{e}^- \rightleftharpoons 2 \text{H}_2\text{O} \quad (14)$$

**Results and Discussion**

For the first set of experiments, the electrode was dipped in a 0.44 M aniline solution in 1.9 M sulfuric acid for 120s and rotated at 500 rpm. The electrode was then immobilized and aniline was polymerized until a charge of 1 C was passed (1.97 C/cm$^2$ or about 25 µm thick). The electrode was then thoroughly washed using the phosphate citrate buffer. Fifty µL of HRP solution (300 U/mL) were deposited on the polymer and allowed to dry, followed by 50 µL of glutaraldehyde, which were also allowed to dry. The drying times were in the order of an hour and a half. Polyaniline was chosen because polyaniline layers are conductive and the coating of 1,2-diaminobenzene is expected to prevent HRP from leaching. In addition, glutaraldehyde is known to cross-linked enzymes, immobilizing them in polymers.

Responses of such electrodes are shown on Figure 25. It can be seen that the responses of the different preparations are not similar, although the same protocol was used. Thus, this preparation lacks reproducibility, probably because of the drying.
Indeed, HRP is exposed to O₂, when it is allowed to dry, and it is sensitive to oxidation. Therefore, some differences in the drying time can explain a part of the lack of reproducibility. Some preparations could have not been dried completely and therefore less oxidation could have happened. In addition, the cleaning of the electrode (its polishing) is a step, which is sometimes difficult to reproduce.

**Figure 25:** Plot of current versus H₂O₂ concentration. Chronoamperometry at 90 mV for 60s on Glassy Carbon/polyaniline/HRP electrodes (0.508 cm²). 20 mL buffer at Room Temperature. Rotation Rate 500 rpm. Each curve represents a different electrode prepared with polyaniline (1 C), 50 µL of HRP solution (300 U/mL), 50 µL of glutaraldehyde and 1,2-diaminobenzene (4 min)

Figure 26 shows the effect of storage on the behavior of the polyaniline/HRP electrode. Such a preparation of the electrode can not be stored in a freezer. Indeed, the polyaniline layer is very fragile and some of the polyaniline coating was lost during the thawing of the electrode, resulting in the loss of the enzyme adsorbed on it. Therefore, a
Nafion coating was deposited onto the polyaniline in order to stabilize the polyaniline layer. Although Nafion is an ionophore, it is not electrically conductive. However, when Nafion was used, the polyaniline/HRP electrode did not respond to the presence of $\text{H}_2\text{O}_2$. The Nafion layer might be too thick to allow hydrogen peroxide to penetrate to the enzyme. Lastly, it is not possible to store the electrode at room temperature because HRP is not stable (Figure 26). Other means of stabilization need to be tested.

![Figure 26: Plot of current versus $\text{H}_2\text{O}_2$ concentration. Chronoamperometry at 90 mV for 60s on Glassy Carbon/polyaniline/HRP electrodes (0.508 cm$^2$). 20 mL buffer at Room Temperature. Rotation Rate 500 rpm. (×) first preparation of the electrode; (+) same stored one night in freezer; (-) same plus Nafion coating; (Δ) second preparation; (o) same stored at room temperature](image)

A new protocol of preparation of the electrode was tested. Polyaniline was deposited from the same solution until 0.5C was passed (0.98 C/cm$^2$ or about 12 µm). The
electrode was then thoroughly washed using the phosphate citrate buffer. The electrode was dipped in 8 mL of buffer containing of 300 U/mL HRP, 5 mM 1,2-diaminobenzene and 0.5 M Na$_2$SO$_4$. HRP was adsorbed for 10 min at 100 rpm. The electrode was then rotated for 10 min at 100 rpm in glutaraldehyde. After, the polymerization of 1,2-diaminobenzene was done at 90 mV under quiet conditions for 4 minutes in the HRP solution. The electrode was then tested with H$_2$O$_2$ and the results are shown in Figure 27. Most responses are similar. Therefore, this protocol appears more reproducible than the previous one. However, once again, O$_2$ seems to denature the enzyme (see Figure 27 o) and the differences between electrodes might come from a difference in the O$_2$ exposure.

Figure 27: Plot of current versus H$_2$O$_2$ concentration. Chronoamperometry at 90 mV for 60s on Glassy Carbon/polyaniline/HRP electrodes (0.508 cm$^2$). 20 mL buffer at Room Temperature. Rotation Rate 500 rpm. Each curve represents a new preparation of the electrode (10 min at 100 rpm in HRP solution then 10 min at 100 rpm in glutaraldehyde). (o) was left 10 min in air before testing.
The study of HRP was not carried out any longer because it was a model for NaR. Unfortunately, it was not possible to obtain the reversible couple of methyl viologen on polyaniline by cyclic voltammetry. Therefore, no electrode using this model has been developed. However, other polymers (with viologen moieties) or other mediators should be tested.