

**MICROBIAL REDUCTION OF NITRATE AND PERCHLORATE THROUGH  
ELECTRON STORAGE CAPACITY OF WOOD-DERIVED BIOCHAR**

by

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## ABSTRACT

Perchlorate and nitrate contamination pose significant environmental and health concerns globally. Human activities, including agriculture, livestock farming, and the production of munitions for military purposes, have significantly contributed to the elevated levels of nitrate and perchlorate pollution in the environment. Current physical and chemical remediation methods for these contaminants often have limitations and can generate additional environmental impacts. Microbial reduction has emerged as a promising approach, but traditional microbial substrates can produce toxic byproducts. In this study, we investigated the efficacy of wood-derived biochar as an exclusive electron donor for the microbial reductive transformation of nitrate and perchlorate into non-toxic products such as nitrogen gas and chloride. Our results demonstrated that biochar effectively facilitated the degradation of nitrate and perchlorate, even in small quantities. In just 4 days, 1 g of reduced biochar successfully facilitated the reductive transformation of 1.55 mM perchlorate into chloride. Similarly, within a span of 13 days, 0.5 g of biochar mediated the conversion of 3.8 mM nitrate into harmless N<sub>2</sub>. These transformation rates far exceed the established Maximum Contamination Level for perchlorate (300 times higher) and nitrate (30 times higher). Notably, these rates were reached much more rapidly compared to conventional microbiological methods, underscoring the exceptional efficacy of biochar as a remediation agent. Importantly, biochar did not generate any toxic byproducts, distinguishing it from previous methodologies. We also observed a proportionality between the mass of biochar and the rate and extent of reduction, providing valuable insights for system design. Biochar's affordability, environmental friendliness, and absence of toxic byproducts make it a promising tool for controlling

nitrate and perchlorate fate and transport. Further research may be needed to assess its feasibility and scalability in real-world scenarios. Nevertheless, this study lays a solid foundation for advancing environmental engineering practices and mitigating pollution impacts associated with these contaminants.

## **Chapter 1**

### **INTRODUCTION**

#### **1.1 Problem Statement**

The widespread utilization of chemical and natural agricultural additives, including fertilizers, has had significant repercussions on the environment and public health due to the heightened concentration of nitrate in surface water and groundwater (Panno et al., 2001; Nolan & Hitt, 2006; Ascott et al, 2017; Niu et al, 2022). Nitrate, the prevalent ionic form of nitrogen in soils and water, serves as a primary nitrogen source for plants (Gojon et al., 2011). In natural circumstances, nitrate is produced through the biogeochemical nitrogen cycle, where soil nitrifying bacteria convert atmospheric nitrogen into ammonium, nitrite, and nitrate, which plants rely on for their growth (Santamaria, 2006; Gojon et al., 2011; Kamp et al, 2015). However, human activities such as agriculture and intensive livestock production have increased nitrate levels in water bodies, leading to eutrophication and detrimental health effects, including cyanosis or blue baby syndrome (Urbansky, 1998; Santamaria, 2006; Welch et al, 2011; Wick et al., 2012; Niu et al, 2022; Singh et al, 2022).

Furthermore, perchlorate, a persistent chemical compound used as an oxidizing agent in explosives and missiles fabrication since the 1950s, has been detected in potash ores and certain fertilizers as impurities in the form of nitrate salts (Srinivasan et al, 2009; He et al, 2019). Perchlorate is not prone to sorb onto solids, and it readily dissolves in water and can contaminate aquifers and surface streams via runoff (He et al, 2019). Although perchlorate can be naturally found in arid environments (Xu et al,

2003; Srinivasan, et al 2009) and occur spontaneously through atmospheric reactions (Dasgupta et al., 2005), it poses a toxic threat to humans, particularly fetuses and infants, as it disrupts the thyroid gland and causes hormonal and developmental disorders even at low concentrations (Xu et al, 2003; Srinivasan et al, 2009; Pennino et al, 2017; El-Lateef et al, 2022).

Nitrate and perchlorate present specific challenges as they are persistent in the environment, chemically inert and resistant to degradation under anaerobic conditions, necessitating microbial enzymatic pathways for their transformation (Xu et al, 2003; Nozawa-Inoue, 2005; Bender, 2016; El-Lateef et al, 2022). Numerous studies have explored how pure cultures and microbial enrichments can utilize electron donors such as acetate, methane, and hydrogen to effectively reduce both nitrate and perchlorate. (Nozawa-Inoue et al., 2005; Srinivasan et al, 2009; Pan-Long, 2020). However, it is important to note that most of the pathways mentioned, which utilize substrates like acetate, can generate toxic reduction products such as methane, a potent greenhouse gas, and ammonium, a reduced form of nitrate known to cause plant toxicity and pose risks to public health (Singh et al, 2022).

The contamination of groundwater, primarily by nitrate, has emerged as a significant concern affecting environmental quality and public health. According to a study conducted by the US Environmental Protection Agency (EPA) in 2017, approximately 14% of the US population, about 45 million people, depends on self-supplied drinking water sources, typically unregulated domestic wells (Pennino et al, 2017), which increases the risk of consumption of contaminated water. However, the distribution of self-supplied drinking water varies across states. In states like Kansas, California, Colorado, Illinois, and Massachusetts, among others, the percentage of the

population relying on self-supplied drinking water ranges from 5% to 10%. On the other hand, in states such as Vermont, Wisconsin, New Hampshire, and Maine, the reliance on self-supplied drinking water is significantly higher, with percentages ranging from 30% to 45% (EPA, 2010).

In addition to the variation in self-supplied drinking water sources, the EPA has also estimated the percentage of state areas with groundwater nitrate concentrations exceeding 5 mg/L. It is important to note that the Maximum Contamination Level (MCL) for nitrate in drinking water is set at 10 mg/L. Table 1 provides estimates of the area and percentage of each state that has groundwater nitrate concentrations exceeding 5 mg/l. Additionally, the data includes the estimated percentage of the population in each state relying on self-supplied drinking water, with 98% of this water being sourced from groundwater wells. For instance, only 1% of Alabama's state area has groundwater nitrate concentrations surpassing the 5 mg/L threshold. While in Delaware a significant portion of the state, approximately 53%, has groundwater nitrate concentrations above the 5 mg/L limit (EPA, 2010).

| State         | Estimated area (mi <sup>2</sup> ) of state with groundwater nitrate concentrations > 5 mg/L | Estimated % of state area with groundwater nitrate concentrations > 5 mg/L | Estimated % of population with self-supplied drinking water |
|---------------|---|--|---|
| Arizona       | 12,763  | 12%  | 4%  |
| California    | 15,004  | 10%  | 7%  |
| Delaware      | 976   | 53%  | 10%   |
| Florida       | 4,975   | 9%   | 10%   |
| Louisiana     | 6,530   | 15%  | 12%   |
| Maryland      | 2,674   | 28%  | 17%   |
| Massachusetts | 951   | 12%  | 8%  |
| Nebraska      | 13,418  | 17%  | 18%   |

Table 1- Estimates of the states with higher area and percentage that has groundwater nitrate concentrations exceeding 5 mg/l, and the estimated percentage of the population in each state relying on self-supplied drinking water. Modified version of EPA, 2010.

These statistics highlight that a considerable portion of the population in the United States potentially faces exposure to nitrate concentrations that are near or even exceeding the established MCL. This situation warrants attention and emphasizes the need for effective measures to monitor and address the issue of elevated nitrate levels in groundwater, particularly in states with higher percentages of self-supplied drinking water sources like Delaware, Maryland, Rhode Island, and Louisiana, among others.

## **1.2 Remediation approaches and Research gaps**

Various physical and chemical methods have been proposed and employed in the past for the remediation of water bodies contaminated with anions such as nitrate and perchlorate. Among the commonly utilized techniques are Reverse Osmosis, chemical denitrification, ionic exchange, electrodialysis, and adsorption (He et al, 2019; El-Lateef et al., 2022). Most of these methods necessitate costly equipment, involve multiple steps, require a wide range of reagents and metallic catalysts/electrodes, and operate at pH levels significantly different from those found in natural water, potentially leading to environmental repercussions.

For instance, the process of catalytic reduction of nitrate using metallic electrodes can produce harmful byproducts, including ammonia, hydroxylamines, and hydrazines (Singh et al., 2022). Furthermore, the effectiveness of certain ion exchange resins in removing nitrate diminishes considerably over time due to the presence of chloride and sulfate ions, which are commonly found in natural environments (Sigh et

al, 2022). Additionally, the effectiveness of this method is compromised by the need for specific disposal of concentrated residual waste brines and the requirement to regenerate the resin after limited removal cycles (He et al, 2019).

Most physical and chemical methods commonly exhibit relatively modest removal efficiencies, ranging from as low as 10-25% to a maximum of 42%-97% (El-Lateef et al., 2022; Singh et al., 2022). Achieving higher reduction percentages often necessitates specific conditions such as pH levels, redox potential, and ionic strength, among other factors. This specificity makes their application in natural systems challenging. In contrast, microbial reduction has emerged as a widely employed approach in water treatment for the degradation of nitrate and perchlorate. Microbial nitrate/perchlorate reduction treatment offers several advantages, including low operating costs, high efficiency, and suitability for in-situ remediation (Chaudhuri et al, 2002; He et al, 2019). As a result, microbial reduction treatment appears to be a favorable course of action (He et al, 2019; Xia et al., 2020; Bi et al., 2022).

However, it is important to note that some of the studies mentioned, which employed pure and mixed microbial cultures with traditional carbon sources or electron donors for nitrate and perchlorate reduction, have resulted in the generation of toxic byproducts such as methane or CO<sub>2</sub> (common greenhouse gases) through acetate oxidation, or ammonium (harmful to plants, fish, and human health) as a byproduct of incomplete nitrate mineralization. Therefore, there is a pressing need to explore alternative methodologies and microbial electron donors to develop a clean, sustainable, and environmentally friendly system for degrading these compounds and transforming them into harmless substances.

### 1.3 Electron Storage Capacity of Pyrogenic Black Carbon

Biochar is produced by a thermochemical conversion of dry biomass through pyrolysis at 400-800°C under oxygen-limited conditions. This thermally altered wood-derived biomass (char) is more and more extensive in natural under global warming and anthropogenic activities, however, is recognized as an important geo-constituent involved in various environmental implications including carbon sequestration, soil improvement, and environmental remediation (Yao et al, 2012; Yavari et al, 2015; Xin et al, 2018, 2022). It has been demonstrated in numerous studies that biochar can serve as a sorbent for immobilizing various organic and inorganic pollutants due to its large surface area (Chen & Yuan, 2011; Yao et al., 2012; Yavari et al., 2015; Xin et al., 2022).

Recent research has also highlighted biochar's capability to store and exchange electrons with abiotic and biotic agents due to their electroactive quinoid functional moieties formed during pyrolysis, enabling it to accept, store, and donate electrons (Klüpfel et al., 2014; Xin et al., 2018, 2021). This attribute, termed Electron Storage Capacity (ESC) (Xin et al, 2018, 2021, 2022) or Electron Exchange Capacity (EEC) (Yuan et al, 2021), can range from several millimoles of electrons per gram of biochar, depending on the biomass and pyrolysis temperature (Klüpfel et al., 2014; Xin et al., 2018). ESC enables biochar to mediate abiotic and biotic electron transfer, where chemical reductants/oxidants or microorganisms capable of utilizing biochar's redox functional groups, such as quinones, can serve as electron sources or terminal electron acceptors (Saquing et al., 2016; Xin et al., 2018).

Figure 1, adapted from Xin et al. (2022), provides a compelling illustration of the remediation capabilities of wood-derived biochar. The plot displays the abiotic reductive degradation of 2,4-dinitroanisole (DNAN), a major component in the

insensitive formulation IMX-101, facilitated by the sorption and electron storage capacity of biochar. Two forms of the same Rogue biochar, oxidized and reduced, were employed to assess their effectiveness in removing and reducing DNAN through sorption and reduction processes.

In Panel a (left), the results demonstrate that oxidized biochar (red circles) successfully removed 56% of DNAN from the solution, primarily through adsorption mechanisms. Intriguingly, reduced biochar (Panel a, blue circles) exhibited an additional 13% removal of DNAN, accompanied by the formation of 2-Amino-4-nitroanisole and 4-amino-2-nitroanisole, which are reduction intermediates of DNAN (Panel b). This unequivocally indicated that biochar not only removed the pollutant from the aqueous phase via sorption but also played a significant role in the reductive transformation of DNAN, leveraging its electron storage capacity.

The reversible nature of ESC allows for oxidation-reduction cycles without significantly affecting the overall electron transfer, rendering biochar a promising tool for environmental remediation purposes (Xin et al, 2018). Recent studies have further demonstrated that microbial species such as *Geobacter metallireducens* (Saquing et al, 2016) and *Shewanella oneidensis* (Kappler et al, 2014) can utilize oxidized biochar as terminal electron acceptor for the oxidation of organic substrates that are naturally available, like acetate, and reduced biochar as an electron donor for the reduction of nitrate into ammonium (Saquing et al, 2016; Kappler et al, 2014). Biochar has also been found to enhance the rate and extent of microbial reduction of nitrate and perchlorate when alternative electron donors or bacterial substrates are present (Sathishkumar et al., 2020; Tian et al., 2019). Zhu et al. (2019) conducted a study demonstrating that a microbial mixed culture from a digested sludge was able to use

humic acids and anthraquinone-2,6-Disulfonate (AQDS), common quinone-based redox mediators (QRM) and presumably biochar analogs, to enhance the rate of nitrate and perchlorate reduction with acetate as the electron donor. This is significant because through the microbial access of stored electrons in biochar, contaminants that do not strongly bind to biochar can still be degraded.

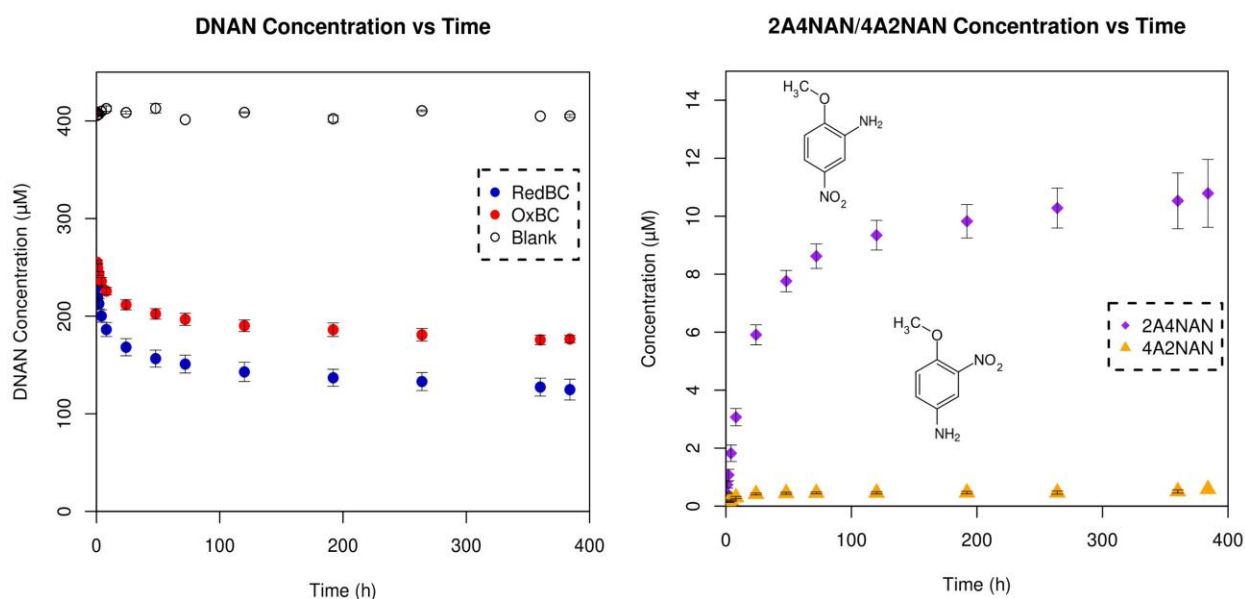


Figure 1 – DNAN removal and reductive transformation by wood-derived Rogue biochar. Modified from Xin et al, 2022.

#### 1.4 Hypothesis and objectives

As discussed, previous studies have investigated the biological reduction of perchlorate and nitrate by a series of inorganic and organic electron donors (e.g.,

methane, acetate, hydrogen), and the ability of microorganisms to access the ESC of biochar and other QRM as electron shuttles to reduce different compounds like iron, nitrate and perchlorate (Urbansky, 2002; Chaudhuri et al., 2002; Tipton et al, 2003; Xu et al., 2003; Nozawa-Inoue et al., 2005; Van Trump and Coates, 2008; Srinivasan et al., 2009; Kappler et al, 2014; Saquing et al, 2016; Lv et al., 2019; Zhu et al, 2019). Biochar has also been found to enhance the rate and extent of microbial reduction of nitrate and perchlorate when alternative electron donors or bacterial substrates are present (Sathishkumar et al., 2020; Tian et al., 2019). However, it has not yet been demonstrated if biochar can sustain the microbial reduction of both nitrate and perchlorate as the sole electron donor. In contrast with a previous study conducted in 2016 by Saquing et al, where a pure strain was utilized to reduce nitrate into ammonia, a toxic reduction product, using reduced biochar as the electron donor, we hypothesize that a mixed soil culture should be able to reduce Nitrate directly to N<sub>2</sub> without ammonia being produced.

Given the potential of biochar as a cost-effective remediation tool, this study aims to explore the efficiency of wood-derived biochar to serve as the exclusive electron donor to support the microbial reductive transformation of nitrate and perchlorate into non-toxic reduction products like nitrogen gas and chloride, respectively. The results of this study could imply important advances in the environmental engineering field, especially as a potential solution for pollution impacts generated by industries such as agriculture, livestock, and military activities.

## Chapter 2

### MATERIALS AND METHODS

#### 2.1 Chemicals

$\text{KNO}_3$  ( $\geq 99\%$ ) was purchased from Thermo-Fisher Scientific (Waltham, MA).  $\text{K}^{15}\text{NO}_3$  (98% atom  $^{15}\text{N}$ ) was obtained from Sigma-Aldrich (St. Louis, MO).  $\text{NaClO}_4$  ( $\geq 99\%$ ),  $\text{Na}_2\text{CO}_3$  ( $\geq 99.5\%$ ),  $\text{NaHCO}_3$  (100.3%),  $\text{Na}_2\text{S}_2\text{O}_4$  (~85%),  $\text{H}_2\text{SO}_4$  (95-98%), all acquired from Fisher Chemical (Hampton, NH).  $\text{H}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  ( $\geq 99\%$ ) acquired from Fluka Biochemika (Charlotte, NC).  $\text{NH}_4\text{Br}$  (99%) obtained from Alfa Aesar (Haverhill, MA).  $\text{KH}_2\text{PO}_4$  (99%) acquired from ICN Biomedicals INC (Costa Mesa, CA). Trace mineral solution and Vitamin supplement, both obtained from ATCC (Manassas, VA). All chemicals were used as received.

#### 2.2 Oxidation and reduction of Rogue Biochar

The oxidation process applied in this study was a modified version of the method described by Xin et al. (2018). Initially, raw rogue biochar obtained from Oregon Biochar Solutions was subjected to a drying process at  $65^\circ\text{C}$  and then ground to achieve a particle size below  $100\ \mu\text{m}$ . The ground biochar was suspended in phosphate buffer at a pH of  $7.0 \pm 0.2$  and purged with air using an air diffuser. The suspension was then shaken at a speed of 100 RPM for a duration of four weeks. This extended period allowed for the removal of any residual electrons that may have been generated during the pyrolysis process and stored by redox functional groups, such as quinones, present in the biochar. Following oxidation, the wet biochar was filtered using a Buchner funnel vacuum filtration setup and Whatman glass microfiber filters

with a pore size of 0.4  $\mu\text{m}$ . The filtered biochar was subsequently dried at 65°C and stored in a desiccator for future use.

For the reduction process, a modified version of the procedure described by Saquing et al. (2016) was employed. A subsample of the previously oxidized biochar was placed inside a Coy anaerobic glove box under an atmosphere of  $\text{N}_2/\text{H}_2$  (98:2, v/v). In a basal salt medium with a pH of  $7.0 \pm 0.2$ , 500 mL of 25 mM dithionite, a commonly used and well-studied reductant (Xin et al., 2018), was added to initiate the reduction. The solution and biochar were then agitated at 100 RPM for a duration of 24 hours. This process was repeated two additional times in 24-hour intervals to enhance the contact between the biochar and the reductant, ensuring a more extensive reduction. Between each cycle, the biochar was separated from the solution using a Buchner funnel vacuum filtration setup and Whatman glass microfiber filters with a pore size of 0.4  $\mu\text{m}$ . The separated biochar was subsequently resuspended in fresh dithionite solution. Finally, the reduced biochar was thoroughly rinsed using deoxygenated deionized water, vacuum dried within the glovebox, and stored under anaerobic conditions in a desiccator.

### **2.3 Culture Preparation and Maintenance**

Topsoil (25-30 cm) collected from a garden outside Harker ISE Building at the University of Delaware in Newark, DE, in March 2022 (39.6788666, -75.7489206) was used as the seed to set the microbial mixed culture. A soil suspension was prepared inside of the previously mentioned anaerobic glovebox using 3 g of soil and 1.125 L of basal salt medium prepared with deoxygenated deionized water. The salt media formula is a modified version of the one proposed by Zhu et al (2019)

containing 0.42g/L NaHCO<sub>3</sub>, 0.78g/L NaH<sub>2</sub>PO<sub>4</sub>\*2H<sub>2</sub>O, 0.012g/L NH<sub>4</sub>Br, 0.1g/L KH<sub>2</sub>PO<sub>4</sub>, 10 mL trace mineral solution and 10 mL vitamin supplement. The microbial culture bottle was filled completely with no headspace to prevent H<sub>2</sub> intrusion and minimize hydrogenotrophic growth. The bottle was also wrapped with aluminum foil to prevent photosynthesis. Perchlorate/Nitrate and reduced biochar were added at different times as the sole electron acceptor and donor, respectively. No other potential donor or acceptor was added. Samples of perchlorate/nitrate and chloride were taken over time and their concentrations were monitored periodically using Ion Chromatography.

#### **2.4 Perchlorate Reduction experiment**

Four sets of batch reactors were prepared using 160-mL serum bottles in triplicates inside an anaerobic glovebox: a) 1g Reduced Biochar (1g RedBc): 100 mL of liquid culture with 1 g of freshly reduced Rogue biochar, b) 2g Reduced Biochar (2g RedBc): 100 mL of liquid culture with 2 g of freshly reduced Rogue biochar, c) 1g Oxidized Biochar (OxBc) Control: 100 mL of liquid culture with 1 g of fully oxidized Rogue biochar, d) Abiotic control: 100 mL of fresh sterile basal salt medium with 1 g of reduced Rogue biochar. Each reactor was sealed with a rubber stopper and aluminum crimp to ensure anaerobic condition and was wrapped with aluminum foil to avoid photosynthesis. The initial perchlorate concentration for all the reactors was 3.3mM. The reactors were removed from the glovebox and purged with grade-five N<sub>2</sub> for 20 min to completely remove H<sub>2</sub>. Liquid samples were taken at pre-determined times using a sterile syringe with needle and passed through a 0.22-µm PTFE syringe filter (Thermo-Fisher Scientific, MA) for IC analysis.

## 2.5 Nitrate Reduction Experiment

Nitrate microbial reduction mediated by reduced biochar was assessed by using labeled  $\text{K}^{15}\text{NO}_3$  (98% atom). Nitrogen isotope 15 has a low natural abundance in comparison to  $^{14}\text{N}$  (<0.4%), is stable, and it was used to differentiate from the  $^{14}\text{N}$  present in the headspace of the reactors. To set the experiment four different types of reactors and controls were prepared: a) RedBc+Microbes: 120 mL solution, 3.5mM  $\text{K}^{15}\text{NO}_3$  inoculated with 1% (v/v) nitrate-reducing bacteria and 0.5g of chemically reduced Rogue biochar; b) OxBc control: 120 mL solution, 3.5mM  $\text{K}^{15}\text{NO}_3$  inoculated with 1% (v/v) nitrate-reducing bacteria and 0.5g of oxidized Rogue biochar; c) Biotic control: 120 mL solution, 3.5mM  $\text{K}^{15}\text{NO}_3$  inoculated with 1% (v/v) nitrate-reducing bacteria in absence of biochar; d) Abiotic control: 120 mL sterile solution, 3.5mM  $\text{K}^{15}\text{NO}_3$  and 0.5g of reduced biochar in absence of bacteria.

The experiment was prepared in duplicates inside of an anaerobic glovebox (98%  $\text{N}_2$  and 2%  $\text{H}_2$ ), and for all the setups 160 mL sterile glass serum bottles were used and sealed with rubber stoppers and aluminum crimps. Next, the reactors were taken out for purging with high purity  $^{14}\text{N}_2$  to remove any  $\text{H}_2$  in the headspace and avoid overestimation of the nitrate reduction due to hydrogenotrophic reactions. Reactors were covered with aluminum foil to prevent any autotrophic or photolytic reactions and shaken at 100 RPM. Samples were taken over time until the system reached equilibrium. The  $^{14}\text{N}$  mass that was transferred from the mixed culture to the reactors was <1% (~6  $\mu\text{mol}$ ), meaning that most N initial mass and reduction products (99%) were strictly coming from the labeled  $^{15}\text{N}$ .

To investigate the impact of nitrate on perchlorate reduction, a pair of duplicate reactors were set up following the same methodology as described for nitrate reduction, with the exception that approximately 4.5 mM of perchlorate and 0.3 g of reduced biochar were introduced. Biotic control without the presence of reduced biochar was also included. Consistent with the previous experiment, no additional electron donors were supplied to the system, thereby demonstrating the ability of biochar to serve as the sole electron donor. All reactors were handled in the same way as described above, same thing for sampling and analytes quantification.

## **2.6 Analytical methods**

Liquid samples collected using a sterile syringe needle, filtered using 0.2 $\mu$ m PTFE syringe filter (Thermo-Fisher Scientific, MA) and stored at 4°C in polypropylene centrifuge tubes (Globe Scientific, NJ) for further analysis. Perchlorate, nitrate, and chloride concentrations were measured using a Metrohm Eco Ion Chromatogram (IC) equipped with a Metrosep a Supp 5-100/4.0 anion column. The eluent solution used was HCO<sub>3</sub><sup>-</sup>/CO<sub>3</sub><sup>2-</sup> (1.0 mM/3.2 mM) and the regenerant solution was 0.1 mM H<sub>2</sub>SO<sub>4</sub>. Elution times for Chloride, Nitrate and Perchlorate were 3.67 min, 6.8 min and 34 min, respectively. Considering the late elution time for perchlorate, and to prevent measurement imprecisions due to the saturation of the suppressor, the running method was designed as follows: a flowrate of 0.7 mL/min and two injections of 20 min each, with the first one being the actual sample containing the three analytes, and the second one containing deionized water. During the first injection Chloride and Nitrate peaks were observed at the mentioned elution times, while perchlorate was eluted after 14 min during the second injection. The

analyte's concentration was calculated by dividing the area under the peak by the response factor obtained from the slope of the calibration curve after plotting concentration in mM and area in [ $\mu\text{S}/\text{cm}^*(\text{min})$ ]. Corrections were made for the solution volume withdrawn during sampling.

Ammonium samples were measured with a Vernier LabQuest 2 UV-vis spectrophotometer (Vernier, OR), using the Salicylate method (Hach Method 10031). 0.1 mL of sample were added to the solution ampule to a ratio of 1:50 (v/v) with the Ammonia salicylate and Ammonia cyanurate reagent powder pillows. After shaking and a 20-minute reaction time, 3 mL of sample were collected into a quartz cell to measure using UV-vis.  $\text{NH}_4^+$  peak was read in the spectrum at 655 nm. Ammonium concentrations were obtained by dividing the absorbance read at 655 nm by the extinction coefficient obtained from the slope of the calibration curve after plotting concentration vs absorbance.

$^{15}\text{N}_2$  mass was quantified with a 6890N Network GC system, coupled with a 5973 Network Mass Selective detector, both from Agilent Technologies (Santa Clara, CA). For each sample, 50 $\mu\text{L}$  of gas were taken from the headspace of the sealed vials and injected immediately into the GC-MS inlet using a 250 $\mu\text{L}$  VICI Sideport gas needle. The GC-MS was equipped with an Agilent 19091P-Q03 column, capillary of 15 m x 320  $\mu\text{m}$  x 20  $\mu\text{m}$ , the carrier gas was Grade 5 Helium with a constant flow rate of 1.6 mL/min. The temperature for the injector and the oven were set as 250°C and 35°C, respectively. The running time was 1 minute. The selected method uses Selected Ion Monitoring mode (SIM), scanning for m/z ratios of 28, 29 and 30, to quantify and differentiate between the abundance of bimolecular  $^{14}\text{N}_2$ , bimolecular  $^{15}\text{N}_2$ , and a combination of both. Peak retention time for  $\text{N}_2$  was 0.48-0.49 min. Even if

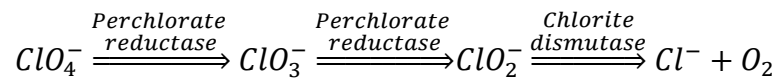
a general calibration curve from 0% to 20% (v/v)  $^{15}\text{N}_2$  in pure  $^{14}\text{N}_2$  was performed at the beginning of the experiment, calibration standards of 0%, 2.5%, 5% and 10%  $^{15}\text{N}$  were injected and quantified before each sampling batch to ensure the quantification accuracy.

## Chapter 3

### RESULTS AND DISCUSSION

#### 3.1 Microbial Perchlorate Reduction

Several microbial perchlorate reduction studies have postulated the following pathway as the potential mechanism for reduction (Rikken et al, 1995; Xu et al, 2003; Nozawa-Inoue et al, 2005; Bender, 2016; He et al, 2019):



During our experiments chlorate was transitionally observed at extremely low concentrations while chlorite was never detected. Figure 2 shows the biological reduction of perchlorate mediated by a) 1 g and b) 2 g of reduced biochar. RedBc and OxBc stand for those reactors containing Reduced Biochar and Oxidized biochar, respectively. Solid lines with circles represent changes in perchlorate concentration over time. Dashed lines with diamonds represent changes in chloride concentration over time. The black solid line with squares represents the mass balance ( $\text{ClO}_4^- + \text{Cl}^-$ ). Chloride was scaled to an initial concentration of 0 mM for plotting purposes. Error bars represent the standard deviation from the different triplicate sets.

During the first 24 hours, rapid consumption of perchlorate was observed in reactors containing reduced biochar, indicated by red circles in Figure 2. This consumption was accompanied by a concomitant formation of chloride. The reduction rates for perchlorate were measured as 0.049 mM/h and 0.082 mM/h for reactors with 1 g and 2 g of reduced biochar, respectively. These rates closely matched the chloride formation rates of 0.054 mM/h and 0.086 mM/h for the corresponding biochar

quantities. Interestingly, the rates of reduction and production of  $\text{ClO}_4^-$  and  $\text{Cl}^-$  were nearly double (1.7x) when using 2 g of biochar compared to 1 g, suggesting a proportional relationship between the mass of biochar or the available electrons, and the reaction rate. After 24 hours, the perchlorate reduction and chloride formation rates decreased significantly for the 1 g RedBc reactors. Conversely, for the 2 g RedBc reactors, the initial rate continued almost linearly until approximately 32 hours, after which the reaction gradually slowed down. In both cases, the reaction reached a plateau between 72 to 96 hours, indicating no further observable transformation. This can be attributed to the depletion of readily available electrons in the biochar that are accessible to microbes.

The total transformation of perchlorate observed was 1.55 mM and 2.8 mM for 1 g RedBc and 2 g RedBc, respectively. Similarly, chloride production amounted to 1.52 mM in reactors with 1 g of reduced biochar and 3.0 mM in reactors with 2 g of reduced biochar. The total electron transfer remained consistent at 1.2 mmol of electrons per gram of biochar for both cases (1 g and 2 g of reduced biochar). Two significant findings can be highlighted from these observations: first, there appears to be a proportionality between the mass of available biochar and the rate and extent of perchlorate reduction and chloride formation; and second, the stoichiometric balance between perchlorate and chloride suggests that most electrons derived from biochar, as the sole electron source, were strictly utilized for microbial respiration.

To ensure that perchlorate reduction was not due to abiotic processes such as biochar-mediated abiotic reduction or adsorption, two controls were included: a) 1 g of reduced biochar with perchlorate in sterile basal salt media, and b) 1 g of Oxidized biochar (OxBc) with perchlorate reducing microbes. As shown in Figure 2, neither

control showed significant changes in perchlorate and chloride concentrations (indicated by blue circles and blue diamonds in panels a and b of Figure 2). The slight decrease in  $\text{ClO}_4^-$  concentration after 40 hours could be attributed to minor variations in the response factor of the IC, likely caused by suppressor saturation after running multiple samples, rather than representing genuine reduction.

This assumption could be supported by the similar behavior observed in perchlorate concentration for both the abiotic control and Oxidized biochar control after 40 hours. Another potential explanation is related to the minimal chloride formation (0.18 mM) observed in the OxBc control (blue diamonds in Figure 2a) during the initial 12 hours. To the best of our knowledge, this phenomenon could be explained by the utilization of residual electrons stored within microbial cells for perchlorate reduction during periods of starvation, microbial autophagy processes, or the consumption of necromass as a response to the absence of an external energy source. These hypotheses will be further explored and discussed in the forthcoming section to provide a comprehensive understanding of the underlying mechanisms.

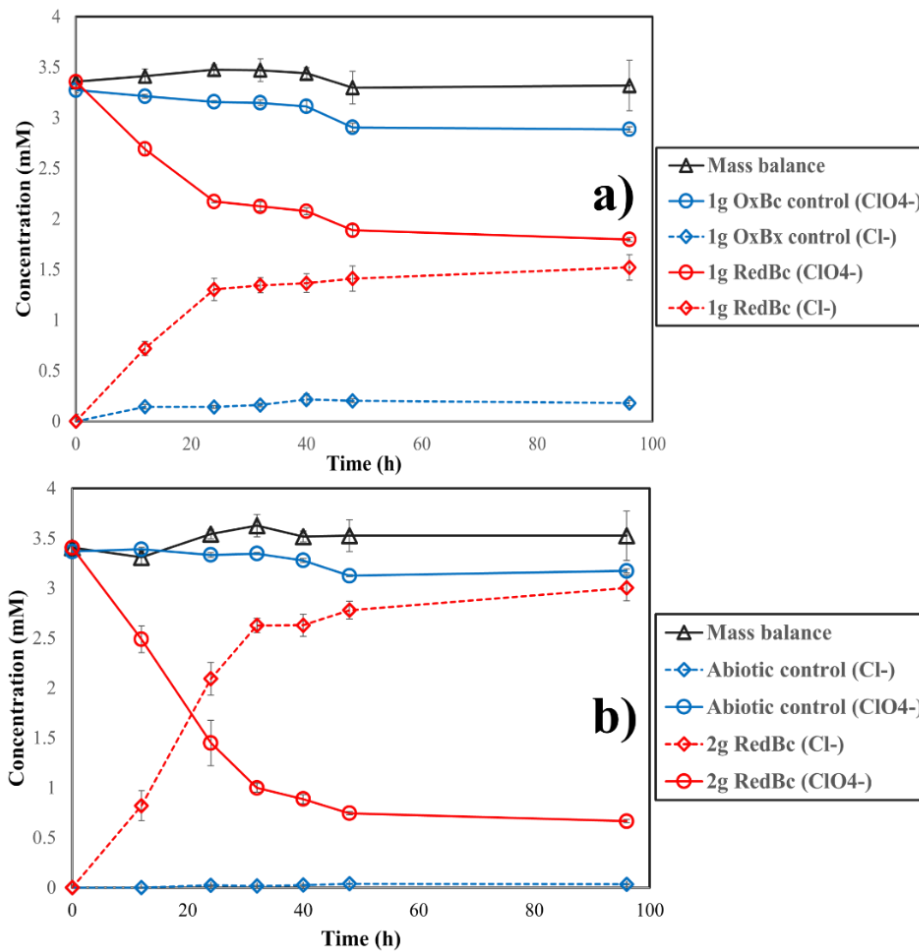


Figure 2 - Perchlorate biological reduction mediated by a) 1 g and b) 2 g of reduced biochar.

### 3.2 Microbial Nitrate Reduction

Figure 3 shows Nitrate biological reduction mediated by reduced biochar. Panel A shows the changes in nitrate mass over time, panel B depicts labeled  $^{15}\text{N}_2$  produced by  $^{15}\text{NO}_3^-$  biological reduction, and panel C represents Total N mass balance in form of 1) nitrate (green circles), 2) Labeled 15 nitrogen gas (blue squares), and 3) ammonium (red triangles). The black vertical dashed arrow in panels A and B

represents the extent of nitrate reduction and nitrogen gas production, respectively, that was caused specifically by reduced biochar (0.38 mmol N), subtracting the portion that was caused by the unknown alternative electron source observed in the controls (0.057 mmol N). The horizontal dashed line in panel C represents the total labeled  $^{15}\text{N}$  mass added at the beginning of the experiment (0.43 mmol). Error bars represent the standard deviation from the duplicates. Dashed curves represent the trend between samples but do not follow any fitting/model.

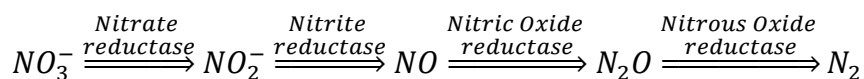
As expected, the abiotic control exhibited no nitrate reduction, nor labeled  $^{15}\text{N}_2$  gas production as shown in Figure 3a and 3b (black squares and black triangles, respectively). This confirms that just as perchlorate, nitrate is chemically inert to the electron storage capacity of biochar and cannot be chemically reduced. Parallely, the biotic and oxidized biochar controls exhibited a nearly identical and limited extent of nitrate reduction (0.057 mmol N) and simultaneous labeled nitrogen gas production (0.056 mmol N) over the initial 9 days. However, no further reaction was observed thereafter, suggesting the depletion of a limited alternative electron source. This behavior like what was observed in perchlorate OxBc control suggests that even in the absence of reduced biochar, microbes were able to utilize a minimal alternative electron source potentially coming from autophagy or necromass synthesis.

Numerous studies have demonstrated that soil microorganisms, such as *Chlamydomonas reinhardtii*, can accumulate disaccharides such as sucrose, or polysaccharides like starch, during periods of starvation or desiccation, limited nitrogen, or  $\text{CO}_2$  conditions, and in the presence or absence of light (Potts, 1994; Ball et al., 1989). These compounds can serve as alternative carbon/electron sources for microbial metabolism. Another survival mechanism utilized by soil microorganisms

like *Saccharomyces cerevisiae* during encystment processes is autophagy, where cellular components such as ribosomes, membranes, mitochondria, cortical elements, and even parts of the nuclear apparatus are degraded as a self-preservation strategy to synthesize new macromolecules (Gutierrez et al., 2001; Kraft et al., 2008).

Additionally, soil microbes have been known to recycle dead cell biomass or necromass as an alternative substrate source to support growth, which plays a crucial role in soil carbon storage (Buckeridge et al., 2020). These metabolic mechanisms provide possible explanations for the alleged perchlorate/nitrate reduction and chloride/nitrogen gas formation even in the absence of an external electron source like reduced biochar.

In reactors containing reduced biochar, a noticeable decrease in nitrate concentration is observed after an initial lag of 0-24 hours, indicating microbial adaptation at the initial stages of the reaction. Subsequently, reduction rates accelerate until approximately 200 hours (9 days), after which the reaction begins to slow down. Finally, complete reduction of nitrate is achieved between 12 and 13 days. This indicates that microbes are utilizing reduced biochar as their primary electron donor for nitrate respiration. The complete conversion of a total mass of 0.43 mmol N to nitrogen gas (Presumably ~10% due to the discussed potential alternative and yet unknown electron source and 90% due to reduced biochar) corresponds to a net electron transfer of 3.8 mmol e<sup>-</sup>/g of biochar. Notably, no NH<sub>4</sub><sup>+</sup> production was observed throughout the entire reaction, suggesting that the soil mixed culture directly reduced nitrate to nitrogen gas without yielding ammonia as an intermediate product, potentially via denitrification pathway:



This finding differs from the observations made by Saquing et al. (2016), who reported that *G. metallireducens* converted nitrate to ammonium through the oxidation of biochar that had undergone prior biological reduction by acetate consumption. This implies that mixed cultures have the potential to directly reduce nitrate to harmless nitrogen gas, thus preventing the buildup of a potentially more toxic reduction product like ammonium.

As previously mentioned, a notable difference was observed in the net electron transfer between perchlorate (1.2 mmol e-/g) and nitrate (3.8 mmol e-/g) reduction. One potential explanation for this disparity lies in the thermodynamic and kinetic differences in the reduction process, influenced by the structural factors of both molecules. Perchlorate reduction, due to its tetrahedral structure, requires a higher energy input and has a higher activation energy requirement compared to nitrate reduction (Urbansky, 1998, 2002; Srinivasan and Sorial, 2009; He et al., 2019). The Gibbs free energy associated with nitrate and perchlorate reduction further supports the energetically favorable nature of nitrate microbial reductive respiration, and potentially a higher extent of reduction (Tipton et al, 2003). The values of -1120 kJ/mol for nitrate reduction (Latham et al., 2015) and -1066 kJ/mol for perchlorate reduction (Baidas et al., 2023) confirm that nitrate reduction is more favorable from an energetic standpoint and could suggest a potential explanation for the higher electron transfer (Tipton et al, 2003).

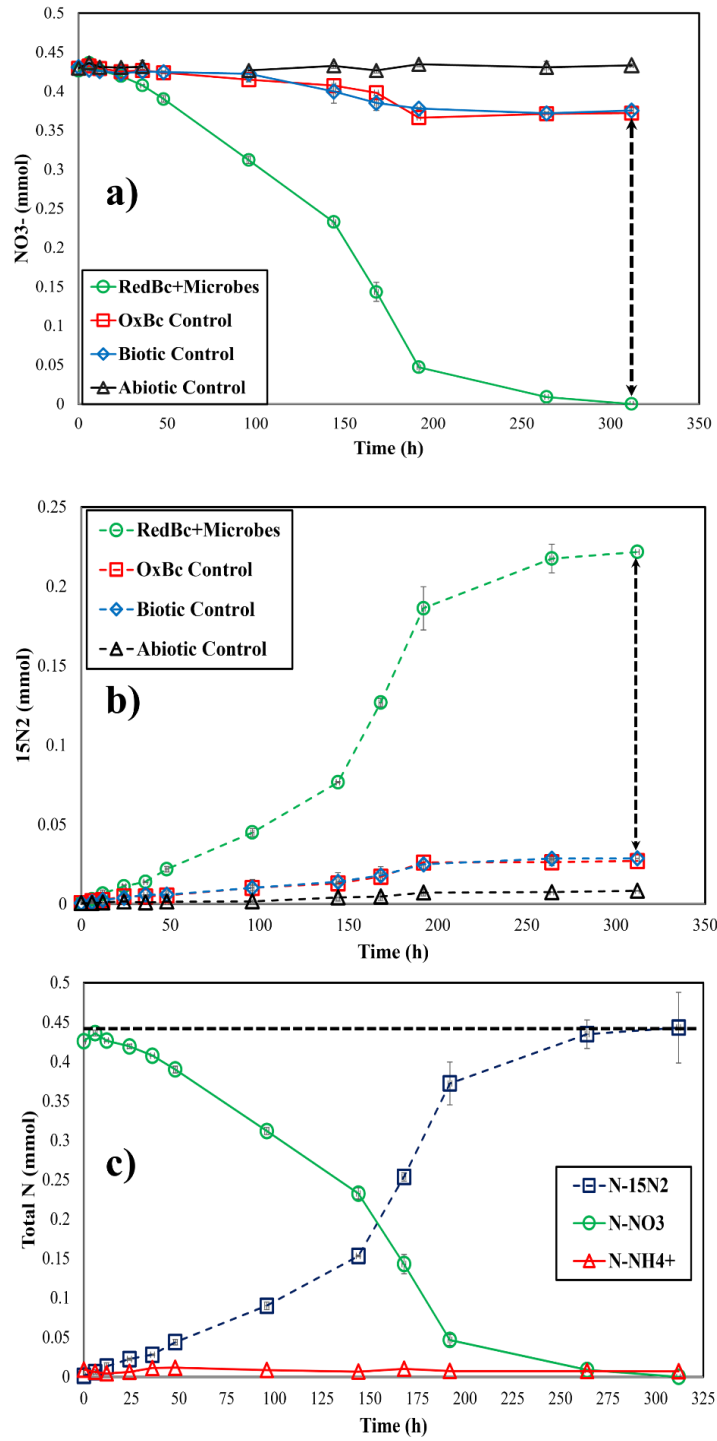


Figure 3 – Nitrate biological reduction (a) and  $^{15}\text{N}_2$  production (b) mediated by reduced biochar. Total Nitrogen in form of  $\text{NO}_3^-$ ,  $^{15}\text{N}_2$  and  $\text{NH}_4^+$  (c)

Multiple studies have demonstrated that in the presence of both nitrate and perchlorate, pure and mixed microbial cultures tend to preferentially select nitrate as the primary electron acceptor. Perchlorate reduction rates decrease, and in some cases, cease entirely until nitrate is completely depleted (Urbansky, 2002; Chaudhuri et al., 2002; Tipton et al, 2003; Xu et al., 2003; Nozawa-Inoue et al., 2005; Van Trump and Coates, 2008; Srinivasan et al., 2009; Lv et al., 2019).

According to Chaudhuri et al. (2002), with the exception of *D. agitata*, all Perchlorate Reducing Bacteria (PRB) have the capability to use nitrate as an alternative terminal electron acceptor. In the presence of both anions, and using acetate as the electron donor, nitrate is consistently consumed first, and no perchlorate reduction is observed. Van Trump and Coates (2009) conducted a study investigating the impact of nitrate on perchlorate reduction using a soil culture derived from soil sediment. In their experiments, they utilized anthraquinone-2,6-Disulfonate (AH<sub>2</sub>QDS), a widely used quinone redox mediator (QRM) and biochar analog, as the electron donor. Similarly to the previous study, perchlorate reduction and chloride formation were inhibited until all nitrate was microbially reduced.

Another example of this was shown in the study performed by Xu et al. (2003), where they examined the reduction of perchlorate and nitrate in bacteria acclimated to only one of the two electron acceptors. Their findings revealed that the enzymes responsible for perchlorate and nitrate reduction are expressed separately. Interestingly, the bacteria that were cultivated in the presence of both nitrate and perchlorate exhibited enhanced perchlorate reduction compared to bacteria grown solely on perchlorate. Conversely, bacteria cultivated exclusively on nitrate did not demonstrate any perchlorate reduction.

These observations are consistent with an experiment conducted during our study (Figure 4), where our soil mixed culture that by that time had been exclusively fed with nitrate for approximately three months was transferred to a fresh medium containing similar concentrations of nitrate and perchlorate. A known reduced biochar mass was added as the sole electron donor. Following a lag period of approximately 24 hours, and similarly to what was observed during the individual nitrate reduction,  $^{15}\text{NO}_3^-$  rapidly decreased, and  $^{15}\text{N}_2$  was produced concurrently. However, the perchlorate concentration remained unchanged throughout the entire reaction period of 13 days, and no chloride formation was observed.

Figure 4 depicts the results obtained during the mixed experiment including both nitrate and perchlorate. Black solid and dashed lines with “X” symbol represent the change in Perchlorate and Chloride mass expressed in mmol, respectively. Green solid and dashed lines with circles and squares represent  $^{15}\text{NO}_3$  and  $^{15}\text{N}_2$  mass (Reactors containing both anions) expressed in mmol, respectively. Red triangles represent ammonium in the system.

These findings suggest that reduced wood-derived biochar has the potential to serve as a viable electron donor for nitrate and perchlorate reduction, based on its similar behavior to previous experiments using different commonly employed electron donors like  $\text{H}_2$ ,  $\text{CH}_4$ , and Acetate. In contrast to the previously mentioned methods, the approach described in this study does not produce environmentally harmful reduction byproducts such as  $\text{CH}_4$  and  $\text{CO}_2$  (common greenhouse gases) or  $\text{NH}_4$  (highly toxic for fish and plants). Our study demonstrated that a native soil mixed culture exhibited the ability to efficiently reduce both perchlorate and nitrate using reduced biochar as the electron donor. Interestingly, when both perchlorate and nitrate were present, the

microbial community displayed a clear preference for nitrate. This preference can be attributed to the lower activation energy and thermodynamically favorable reaction associated with nitrate reduction, which is consistent with previous findings in the literature. To optimize the adaptation of the mixed culture for practical applications, we recommend implementing growth cycles that alternate between perchlorate and nitrate as the sole electron acceptor or even supplying both contaminants simultaneously. This approach would enhance the culture's ability to effectively degrade both contaminants and improve its overall performance in remediation efforts.

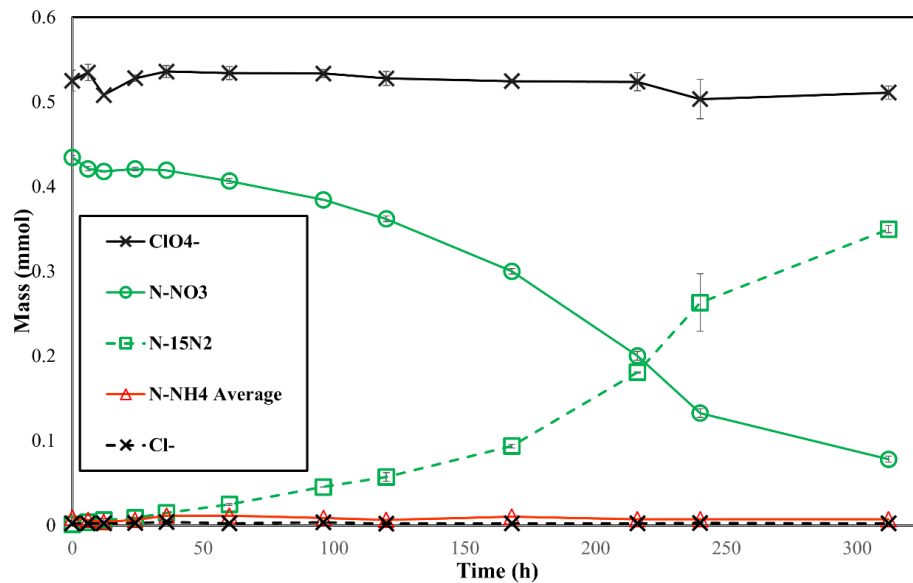


Figure 4- Nitrate and perchlorate mixed microbial reduction.

## Chapter 4

### CONCLUSIONS AND ENVIRONMENTAL IMPLICATIONS AND APPLICATIONS

The presence of perchlorate and, more significantly, nitrate contamination is a global concern with far-reaching consequences. Not only does it impact ecosystems and the environment at large, but it also poses a significant threat to human health. This issue is not isolated or temporary; it is an ongoing environmental problem that has persisted for decades and will continue to generate negative impacts. The continuous production of nitrogenated compounds for agricultural and livestock purposes, coupled with the substantial input of nitrate and perchlorate resulting from explosive production, exacerbates the issue.

As previously described, various physical and chemical methods have been proposed and utilized for the remediation of water bodies contaminated with nitrate and perchlorate anions. Nonetheless, these methods often require costly equipment, involve multiple steps, and utilize various reagents and metallic catalysts/electrodes that can generate further impacts. In contrast with these methods that can achieve modest removal efficiencies, microbial reduction has emerged as a widely employed approach in water treatment for the degradation of nitrate and perchlorate. Microbial reduction treatment offers advantages such as low operating costs, higher efficiency, and suitability for in-situ remediation. However, it carries a risk of generating toxic byproducts with the utilization of traditional microbial substrates.

Given these circumstances, environmental engineers and scientists face a formidable challenge and an urgent need to explore and develop alternative methodologies with microbial electron donors generating clean, sustainable, and

environmentally friendly system capable of degrading these compounds and transforming them into harmless substances.

This pressing need aligns perfectly with the main objective of our study. We investigated the efficacy of wood-derived biochar as an exclusive electron donor for the microbial reductive transformation of these contaminants into non-toxic reduction products such as nitrogen gas and chloride. Biochar, a ubiquitous and naturally abundant material, offers advantages of being cheap, easily obtainable, and environmentally friendly.

Our results demonstrated the effectiveness of biochar as a microbial electron donor, comparable to traditional bacterial substrates used in previous methodologies. Even lesser amounts of reduced biochar, such as 0.5 g and 1 g, were able to mediate the degradation of significant quantities of nitrate (0.38 mmol) and perchlorate (0.26 mmol), respectively. These concentrations were more than 30 times and 300 times the Maximum Contaminant Level (MCL) for nitrate and perchlorate, respectively. This indicates that under environmental conditions, a remediation system utilizing biochar should provide an ample supply of electrons to facilitate the microbial degradation of these targeted compounds.

One of the most significant findings of our study was the absence of any toxic byproducts generated by biochar. This distinguishes biochar from previous physical, chemical, and even microbiological methodologies and highlights its potential as a clean and environmentally friendly approach to controlling the fate and transport of perchlorate and nitrate contaminants. By utilizing biochar as an electron donor, we offer a sustainable and effective solution that mitigates the production of harmful byproducts commonly associated with other methods. Additionally, our findings

suggested a significant proportionality between the mass of available biochar and the rate and extent of perchlorate and nitrate reduction. This observation holds important implications for the design and implementation of remediation systems in field applications. By understanding this relationship, calculations can be made to extrapolate the required amount of biochar for effective remediation and to design systems that optimize the reduction rates and extent of contaminant degradation. This proportionality offers valuable insights for developing practical and efficient strategies to address nitrate and perchlorate contamination in real-world scenarios.

In addition to these findings, it is important to highlight several other benefits of biochar that make it an excellent option for field application as a tool to control the fate and transport of similar contaminants:

- Natural production during wildfires: Wildfires are a common occurrence, and they naturally produce significant amounts of black carbon, which can potentially be utilized for remediation and agricultural purposes. On December 31st of 2021 the National Interagency Fire Center's (NIFC) reported an average of 61.5 thousand wildfires in the United States that had burned approximately 7.5 million acres of land mainly on Western States like Arizona, California, and Colorado among others (US Center for Disaster philanthropy, 2022). This represents tons of available black carbon naturally produced that could be potentially utilized for environmental remediation and agricultural amendment purposes. This abundant resource can help save economic resources that would otherwise be allocated to support research on different environmental issues.

- Clean and environmentally sustainable production through pyrolysis: Biochar can be produced from renewable feedstocks such as agricultural and forestry waste, as well as waste plastic and tires. The pyrolysis process for biochar production reduces volatile emissions and decreases the amount of waste sent to landfills, making it a clean and sustainable approach (USDA, 2023).
- Strong scientific support as a remediation agent: as referenced in this study, numerous studies have demonstrated the effectiveness of biochar as an agricultural amendment, as well as a potential remediation agent for contaminated soils and water. The sorptive and electron storage capacities of biochar have been extensively researched and proven to be effective in decontamination systems.
- Non-invasive and environmentally friendly: Since most biochar used for agricultural or remediation purposes is derived from biomass feedstocks, its application in the field is not expected to cause significant harmful environmental impacts. In fact, it often improves soil structure quality, reduces surface runoff, and enhances microbial activity for the degradation of common water contaminants like perchlorate and nitrate.
- Rechargeable and enduring sorbent: Biochar stands out from other electron donors due to its ability to be recharged (reduced) through the biodegradation of organic substrates. This characteristic makes biochar a long-lasting sorbent and electron repository in soil. Its high porosity and surface area not only enable the absorption of contaminants but

also create anaerobic and reducing microenvironments within its pores, facilitating the microbiological reductive degradation of contaminants.

In conclusion, our study demonstrates that wood-derived biochar holds great promise as an exclusive electron donor for microbial degradation of nitrate and perchlorate. Its effectiveness, coupled with its low-cost and environmentally friendly nature and absence of toxic byproducts, positions biochar as a valuable tool for remediating and controlling the fate and transport of these contaminants. Further research and application of this technique could contribute to mitigating pollution impacts and advancing environmental engineering practices. It is essential to note that the current study focused on laboratory-scale experiments, and further investigation is required to assess the feasibility and scalability of implementing wood-derived biochar as an electron donor in real-world remediation scenarios. However, the results presented in this study lay a solid foundation for future research and practical applications in the field of environmental engineering.

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