THE INFLUENCE OF COPPER, LEAD AND IRON
ON STREAM SEDIMENT NITRIFICATION

A THESIS

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BY
NICHOLAS C. REISING

DR. MELODY BERNOT, ADVISOR
DR. KLAUS NEUMANN, ADVISOR
BALL STATE UNIVERSITY
MUNCIE, INDIANA

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Introduction

Bacteria are single celled prokaryotic organisms ubiquitous across ecosystems (Madigan and Martinko 2006). Although some bacteria are pathogens, the majority are beneficial and fundamental to both human and ecosystem function. For example, bacteria are key in the cycling of nutrients, such as carbon and nitrogen, thereby providing resources to higher organisms. Without microorganisms, nutrients would not be cycled with the efficiency needed to sustain life. Bacteria are also essential to degrading toxins and recalcitrant compounds in the environment; providing more suitable habitat for higher organisms. Many genera of bacteria have adapted to specific niches, each using the metabolic waste of another organism as a primary energy source. The cyclic role microbes play in Earth’s biosphere is often referred to as the microbial loop (Cole 1994). The microbial loop describes microbial activity in ecosystems connected by the metabolic relationships of different bacterial genera. Nitrification is one example of how the metabolic waste of a prokaryotic or eukaryotic organism is an energy source for a bacterium.

Specialized bacteria are the primary organisms involved in nitrification; though some plants and fungi can participate (Sprent 1987). Nitrification requires at least two bacterial genera, one being ammonia-oxidizing and the other nitrite-oxidizing, predominantly from the genera *Nitrosomonas* and *Nitrobacter*, respectively (Madigan and Martinko 2006, White 2007). Nitrification is the two-step microbial-mediated process of oxidizing ammonia to nitrite ($\text{NH}_4^+ \rightarrow \text{NO}_2^-$) and subsequently to nitrate ($\text{NO}_2^- \rightarrow \text{NO}_3^-$) (Figure 1; Hovanec and Delong 1996; You et al. 2009). Bacteria that oxidize
ammonia and nitrite use the compounds as an electron source, a process that provides the energy for inorganic carbon to be incorporated into biomass via the Calvin cycle (Sprent 1987; Hagopian and Riley 1998).

The first organisms involved in nitrification are referred to as ammonia-oxidizing (nitrosifying) bacteria that include the genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus and Nitrosovibrio* (Hagopian and Riley 1998; Madigan and Martinko 2006). The second group of organisms involved is the nitrite-oxidizing (nitrifying) bacteria that include the genera *Nitrobacter, Nitrococcus, Nitrospira and Nitrospina* (Hagopian and Riley 1998; Madigan and Martinko 2006). These microbial genera are Gram negative, aerobic chemoautotrophs with a mean generation time of 20 - 60 h, relatively slow compared to other genera (Sprent 1987; Hagopian and Riley 1998). *Nitrosomonas* has an ideal pH range of 7.9 - 8.5 and optimal growth temperature of 35 - 40°C (Table 1; Jones and Hood 1980; Villaverde, et al. 1996; Grunditz and Dalhammar 2000). *Nitrobacter* has an ideal pH range of 7.2 - 7.9 and optimal growth temperature of 38°C (Table 1; Boon and Laudelout 1962; Keen and Prosser 1987; Villaverde et al. 1996; Grunditz and Dalhammar 2000). Both ammonia- and nitrite-oxidizing bacteria are photo-inhibited and must be bound to a surface for optimal growth (Hagopian and Riley 1998).

*Environmental Factors Influencing Nitrification*

The rate of nitrification in the environment is dependent on multiple factors including the biological community, dissolved organic carbon (DOC), temperature,
dissolved oxygen (DO), and pH (Table 1; Kemp and Dodds 2001; Strauss et al. 2002; Earl et al. 2006). Primarily, nitrification is limited by the concentration of ammonia and nitrite in an environment which can be influenced by stream organisms (Table 1; Jones and Hood 1980; Villaverde et al. 1996; Lee et al. 1997; Ciudada et al. 2007) as well as surrounding land use (Galloway 1998). Both ammonia and nitrite are toxic to some aquatic organisms at concentrations exceeding 0.2 mg/L and 1.8 mg/L, respectively (Hagopian and Riley 1998). However, the end product of nitrification, nitrate, is toxic to organisms at concentrations greater than that of ammonia and nitrite (> 20 mg/L; Hagopian and Riley 1998). Further, nitrite and nitrate can be toxic to humans. When nitrite or nitrate is mixed with hemoglobin, a reduction in oxygen carrying capacity of the molecule occurs, leading to methemoglobinemia (blue baby’s syndrome) in humans or brown blood disease in fish and other animals (Frances et al. 1998). Because of the potential toxicity of these nitrogen compounds, successful conversion via nitrification is important to prevent high ammonia and nitrite concentrations that could threaten organisms.

Ammonium, ammonia, and nitrite can also be toxic to nitrifying bacteria. *Nitrosomonas* shows inhibition via nitrite at 5 mg/L and ammonium at 0.5 mg/L (Jones and Hood 1980; Lee et al. 1997). *Nitrobacter* is inhibited by ammonium at 0.1 - 1 mg/L (Villaverde et al. 1996; Ciudada et al. 2007). The overall toxicity of ammonium, ammonia, and nitrite to nitrifiers depends on the organisms present.
Effect of Metals on Nitrification

The effect of metals on nitrification rate is related to the type and concentration of metal and the species of nitrifying microbes present (Table 2; Mertoglu et al. 2008). Metals are found naturally in the environment but can also enter a system via human activity. Metals can enter an environment as atmospheric pollution following fossil fuel combustion and through leaching from improperly disposed items like batteries and other metal products (Guinee et al. 1999; Rimmer et al. 2006). Even as recycling efforts increase, the mining of many metals continues to grow, leading to an increase of metals entering the environment; although this is not an issue in the study area. (Guinee et al. 1999). Landscape remediation and construction can cause metals trapped within soil to more readily enter aquatic systems, such as lakes and streams, causing the sediment to act as a metal sink (Blake et al. 2007). Depending on water flow, sediment-bound metal may diffuse into the water. The rate of diffusion is a function of water chemistry as well as discharge yielding variable metal concentrations over time. It is imperative freshwater metal concentrations and their potential effects on the ecosystem are more comprehensively assessed.

Certain metals are known to influence biological processes, including microbial activity. Microbial nitrification processes are typically not affected by low concentrations of metals; however, as concentrations increase they can inhibit activity (Table 2; Hu et al. 2004). Toxicity is related to free metal ion concentration, rather than total metals, so compounds containing metals that dissociate in water are generally more toxic (Semerci and Cecen 2007). Interestingly, nitrifying microbes exposed in vitro to low
concentrations of a metal can develop a tolerance to the metal and even resist future exposures of high, normally inhibitory concentrations (Mertoglu et al. 2008). For example, in a stream exposed to mining runoff, long term exposure to copper and lead has been shown to cause changes in metal tolerance levels of the stream microbes and alters the dominant genera (microbial succession) of nitrifying bacteria present (Satchanska et al. 2005), indicating microbial adaptation following metal exposure (Mertoglu et al. 2008).

**Influence of copper.**

Copper is an essential trace element in nitrifying bacteria but becomes toxic as concentrations increase to levels that disrupt normal cellular function (Sato et al. 1988). The concentration at which the metal becomes toxic depends on bacterial physiology. Copper concentrations between 1.27 – 12.7 mg/L are important for optimal ammonia monooxygenase (AMO) enzyme function (Table 2; Ensign et al. 1993). In both *Nitrosomonas* and *Nitrobacter*, higher copper ion concentrations can decrease nitrification rates (Braam and Klapwijk 1980; Lee et al. 1997; Hu et al. 2004). At higher copper concentrations (>30 mg/L), ammonia oxidizing bacteria, such as the genus *Nitrosomonas*, show signs of growth delay greater than that of nitrite oxidizers, such as *Nitrobacter* (Lee et al. 1997). Concentrations of only 0.5 mg/L copper can cause significant reduction (50%) in nitrification rates for the genus *Nitrosomonas* (Sato et al. 1988). Copper concentrations in sewer sludge have been reported at 0.10 mg/L (Sato et al. 1988). Copper concentrations recorded in the EPA STORET database document
streams of the Upper White River Watershed of central Indiana ranging from 0.63-19.8 µg Cu/L stream water.

**Influence of lead.**

Lead ions are highly toxic to nitrifying microbes and cause greater inhibition relative to copper (Mittal *et al.* 2004). Lead binds to soil particles and also to living and dead microbial cells (Stucznksi *et al.* 2002; You *et al.* 2009). Since lead adheres to cells, it is theorized that lead may not have a direct influence on bacterial enzymes (Stucznski *et al.* 2002), like copper and other metals, as it may not enter the bacterial cell (Sato *et al.* 1988). *In vitro* studies have found that lead had no significant influence on either step of the nitrification process when compared to cadmium and nickel (You *et al.* 2009). Lead concentrations in sewer sludge have been reported at 0.12 mg/L (Sato *et al.* 1988). The EPA STORET database documents streams of the Upper White River Watershed ranging from 0.01 -120 µg Pb/L stream water.

**Influence of iron.**

Similar to copper, iron is an essential element for nitrifying bacteria. The optimal concentration for nitrification is 6 mg/L for *Nitrosomonas* and *Nitrobacter* (Meiklejohn 1957). The lowest concentration of iron needed for nitrification is 0.1 mg/L for *Nitrosomonas* and 0.3 mg/L for *Nitrobacter* (Meiklejohn 1957). Both *Nitrosomonas* and *Nitrobacter* can tolerate iron concentrations of 112 mg/L, though activity is reduced (Meiklejohn 1957). Iron concentrations in sewer sludge have been reported at 3.0 mg/L.
(Sato et al. 1988). In central Indiana, iron concentrations range from <100 µg/L to 27,000 µg/L in the White River basin (Holdeman et al. 1999).

To assess the influence of metals on freshwater integrity as sediment microbial nitrification, metal concentrations in central Indiana freshwaters were measured. Further, the influence of metals on sediment nitrification rates was experimentally quantified. The primary goal of this research was to quantify the influence of copper, lead, and iron on sediment nitrification rates in the streams of the Upper White River Watershed of central Indiana. It was hypothesized that microbial responses to metals are a function of the history of metal exposure and stream physiochemical characteristics. It was further hypothesized that sediment metal concentrations would be more influential than dissolved metal concentrations on nitrification rates.
Methods

Site Selection

Seven sites were selected in the Upper White River Watershed of central Indiana to represent a range of agricultural and urban land use in the surrounding sub-watersheds (Figure 2). All sites selected were 3rd order streams based on the criteria described by Cole (1994). Topographic maps and aerial photographs obtained from the Indiana University GIS spatial data portal (topographic maps from USGS 1984; and aerial photography from Google Earth 2010) were used to determine stream order. The White River Watershed covers an area of 174,830 acres and is located in the Tipton Till plain of east-central Indiana. It contains mostly sand and gravel from glacial deposits. The predominant soil type has been classified as silt loam and highly erodible (White River Watershed Project 2001).

Streams were sampled in May and August 2010 to encompass stream flow at generally higher (May) and lower levels (August) corresponding to spring runoff and base flow. The May and August sampling times also facilitated incorporation of various seasonal changes in stream physiochemical properties such as changes in water temperature, stream biology, riparian characteristics, and land use.

Sediment and Water Collection

At each site and sampling event, sediment and water were collected for laboratory nitrification assays. Specifically, a composite sediment sample was collected from the top 5-10 cm of the benthos at several points along the width of the stream channel. Sediment was placed into an acid-washed bucket with a lid. Additionally, ~2.5 L of
stream water (unfiltered) was collected from a well-mixed portion of the stream into acid-
washed 1 L Nalgene bottles. An additional 250 mL of stream water was collected and
immediately filtered using a syringe fitted with glass fiber filters (Whatman GF/F, 0.7 μm
pore size) into a 250 mL Nalgene acid-washed bottle for subsequent analysis of dissolved
nutrient and metal concentrations. At each site, stream physiochemical parameters were
also measured in the stream thalweg including pH, dissolved oxygen concentration,
turbidity, total dissolved solids (TDS), and temperature using a Hydrolab minisonde
equipped with an LDO oxygen sensor. After collection, samples were immediately
placed on ice for transport to the laboratory. Filtered water samples were frozen within 12
h of collection for subsequent analyses of nutrient and metal concentrations. Sediment
and unfiltered water were placed in a refrigerator at 4°C (< 24 h) until assay start. Filtered
water samples were analyzed for anion and cation concentrations including nitrate (NO₃⁻
-N), phosphate (PO₄-P), chloride (Cl⁻), sulfate (SO₄²⁻), bromide (Br⁻), ammonium (NH₄⁺-
N), lithium (Li⁺), potassium (K⁺), magnesium (Mg²⁺), and calcium (Ca²⁺) using ion
chromatography (DIONEX, ICS-3000 and 2000).

Laboratory Mesocosms

Laboratory mesocosms were prepared by sieving collected sediment, separately
for each site, using a nylon (2.54 mm x 1.25 mm) screen to homogenize the sediment and
remove debris. After sediment homogenization, sediment from each site was separately
sub-divided for preparation of laboratory mesocosms by filling a graduated cylinder with
40 cm³ of sediment and placing into 250 mL glass containers. After sediment addition, 56
mL of site-appropriate stream water was added to each mesocosm. Five paired replicate
mesocosms (N = 10) were prepared for each site and treatment. Prepared laboratory mesocosms from each site were randomly assigned one of 4 treatments including a control (no metal addition), copper addition (127 mg/L), lead addition (127 mg/L), and iron addition (127 mg/L). Metal additions were made using 14 mL of prepared TraceCERT standards for ICP (1000 mg/L Cu, Pb, Fe dissolved in a 2% nitric acid solution) metal stock (Sigma Aldrich) which is 7.62 mg metal per 60 mL total flask content volume. The control mesocosms received 14 mL of deionized water. Each treatment had 5 paired replicates prepared for each stream sampled (N = 280 total mesocosms).

Nitrification Activity

Nitrification activity was measured using nitrapyrin-inhibition assays on paired replicate mesocosms. Specifically, five replicate mesocosms were treated with nitrapyrin dissolved in dimethy sulfoxide (DMSO) to reach a mesocosm concentration of 10 mg/L nitrapyrin (Kemp and Dodds 2001). The remaining 5 paired replicates were treated with an equal volume of DMSO only. After nitrapyrin and DMSO were added to mesocosms, the mesocosms were gently bubbled with air for ~10 s and covered with a tarp to block light. Mesocosms were incubated for 5 d with all mesocosms briefly uncovered, bubbled with air for ~10 s, and immediately re-covered every 24 h to ensure mesocosms remained oxic.

After incubation, ammonium was extracted from sediment by adding 10 mL of 1 N potassium chloride (KCl), mixing the flasks, then incubating for 10 min, followed by a
30 min sediment settling period. Overlying water was then filtered with glass fiber filters (Whatman GF/F, 0.7 µm pore size) into an acid washed 15 mL Falcon tube and immediately refrigerated (< 24 h) for subsequent analyses of ammonium concentrations using the phenol-hypochlorite technique (Weatherburn 1967). Remaining water was decanted and mesocosms were placed in a 75°C drying oven overnight, followed by measurement of sediment dry mass in each individual mesocosm. Nitrification rates were calculated for each paired replicate mesocosm (N = 5 for each treatment) by subtracting the measured ammonium concentration in the nitrapyrin-treated paired replicate from the DMSO-only paired replicate, and then dividing by mesocosm sediment dry mass and total incubation time for expression of the nitrification rate as µg NH₄-N/gdm/d.

**Bioavailable Sediment Metal Concentrations**

Biologically available metal concentrations in sediments were quantified according to McKeague (1978). Using the collected homogenized and dried stream sediment from each site, 0.5 g of sediment was placed into a 15 mL acid-washed Falcon tube. For each stream site, two replicates were prepared for a total of two tubes per site, in addition to three water and acid oxalate blanks. Acid oxalate (10 mL) was added to each tube and the tubes were capped. All tubes were then shaken horizontally in the dark for 4 h. After shaking, tubes were centrifuged at 2000 X g for 13 min, and the supernatant decanted and saved for metal analysis on the ICP-OES, Perkin Elmer Optima 2100 DV. Remaining sediment was discarded. Due to high iron concentrations in the sediment, a 1/10 dilution was used to keep the samples within the standard range.
**Total Sediment Metal Concentrations**

To determine the total metal concentration in stream sediment, a multi-acid digestion was used, modified from Briggs *et al.* (2002). To perform the assay, 0.2 g of dry homogenized stream sediment was placed into a Teflon vessel with subsequent addition of 3 mL (30% w/v) H₂O₂. After 24 h, 2 mL of concentrated nitric acid (65% w/v) was added to each vessel, followed by 1 mL of concentrated (40% w/v) hydrofluoric acid. The vessels were then capped and heated (~100 °C) overnight, then uncapped and heated until dry. The nitric and hydrofluoric acid steps were repeated 3 times due to undissolved materials. Three mL of H₂O₂ was then added to each vessel and heated until dry to remove remaining organics. The H₂O₂ addition was also repeated 3 times. Due to high iron concentrations in the sediment, iron samples were run with an additional 1/10 dilution.

To determine metal concentrations in the water column, 15 mL acid washed Falcon tubes were used. To each tube, 10 mL of acidified stream water (5 ml of 65% w/v nitric acid per 100 mL of stream water) was added. Three replicates were made for each site for a total of 21 tubes. The samples were then refrigerated until analyzed on the ICP-OES for metal concentration. All transference of liquid was performed using acid washed bottles and pipette tips.

**Calculations and Statistical Analyses**

Differences in control nitrification rates and metal concentrations among streams were compared using one way analysis of variance (ANOVA). Two Sample t-tests were
used to compare differences for nitrification rates, sediment and water metal concentrations between the sampling events (May, August). Bonferroni corrected Pearson correlations were used to identify relationships between stream physicochemical parameters, nitrification rates, and metal concentrations. ANOVA and t-tests were performed using MiniTab 16 Software, and correlation statistics were performed using SAS Statistical software using $p$-values $\leq 0.05$ to determine significance.
Results

Bioavailable Metal in Sediment

Overall, bioavailable copper, lead, and iron concentrations in stream sediment varied among sites ($N = 7$). Sediment concentrations ranged from 654 – 1,985 mg Fe/kg sediment and from 1.00 - 2.91 mg Cu/kg sediment. Lead concentrations were below detection limits at all sites except in May at one site (Pleasant Run Creek, 0.472 mg Pb/kg sediment) and in August at one site (Mud Creek, 0.38 mg Pb/kg sediment). Across sites, significant differences in bioavailable metal concentrations were identified between sampling events (May vs. August; $p < 0.05$). Specifically, bioavailable iron in Buck Creek was ~25% higher in May (876 mg Fe/kg) relative to August (654 mg Fe/kg; $p = 0.030$; Figure 3). Similarly, bioavailable iron in Killbuck Creek was ~41% higher in May (1,985 mg Fe/kg) relative to August (1161 mg Fe/kg; $p = 0.01$; Figure 3). Bioavailable copper concentrations in Killbuck Creek were ~51% higher in May (2.63 mg Cu/kg) relative to August (1.29 mg Cu/kg; $p = 0.004$; Figure 3).

Bioavailable Metal in Water Column

Copper and iron concentrations in stream water varied among sites and ranged from below detection to 0.10 mg Fe/L, and from 0.01 – 0.02 mg Cu/L. Lead concentrations were below detection limits at all sites and sampling events. Overall, bioavailable iron concentrations (mean = 0.026 mg/L) in stream water were greater than copper concentrations (mean = 0.013 mg/L; $p = 0.01$). Across sites, significant
differences in bioavailable iron concentrations in the water column were identified between sampling events (May, August; \( p < 0.05 \)) in all streams, except White Lick (\( p = 0.904 \)), with higher dissolved iron concentrations in August relative to May. Killbuck Creek was the only site with significant difference in dissolved copper concentrations between May and August (0.02 mg/L vs. 0.01 mg/L; \( p = 0.037 \); Figure 4).

**Control Nitrification Rates**

Overall, control nitrification rates were ~76% greater in May (mean = 4.31 µg NH\(_4\)-N/gdm/d) than August (mean = 1.05 µg NH\(_4\)-N/gdm/d; \( p = 0.001 \)). Across sites, significant differences in control nitrification rates were identified between sampling events (May, August; \( p < 0.05 \)) only in Killbuck Creek (\( p = 0.001 \)). No other stream showed significant differences in nitrification rates with sampling time (Figure 5).

**Nitrification Response to Metals**

Overall, there was not a consistent nitrification response to metal enrichment across sites (Figure 6). Nitrification response to metal enrichment did not differ between May and August (\( p > 0.05 \); *data not shown*). Iron enrichments did reduce nitrification rates compared to the control during August (\( p < 0.05 \)): Buck Creek (0.00 vs. 1.72 µg NH\(_4\)-N/gdm/d), Mud Creek (0.00 vs. 8.89 µg NH\(_4\)-N/gdm/d), Pleasant Run Creek (0.02 vs. 1.29 µg NH\(_4\)-N/gdm/d) \( p = 0.017 \), and White Lick Creek (0.03 vs. 0.715 µg NH\(_4\)-N/gdm/d) \( p = 0.038 \) (Figure 6). Copper enrichments significantly decreased nitrification
rates in May compared to the control in Killbuck Creek (1.34 vs. 7.58 µg NH₄-N/gdm/d; \( p = 0.020 \)) and Pleasant Run Creek (0.00 vs. 3.86 µg NH₄-N/gdm/d; \( p = 0.001 \); Figure 6). Copper enrichments also increased nitrification rates compared to the control during August in Killbuck Creek (1.01 vs. 0.279 µg NH₄-N/gdm/d; \( p = 0.024 \)). Significant decreases in August nitrification rates for copper enrichment compared to the control were found in Buck Creek (0.17 vs. 1.72 µg NH₄-N/gdm/d; \( p = 0.032 \)), Pleasant Run Creek (0.09 vs. 1.29 µg NH₄-N/gdm/d; \( p = 0.021 \)), and White Lick Creek (0.05 vs. 0.715 µg NH₄-N/gdm/d; \( p = 0.045 \)) (Figure 6). Lead enrichments decreased nitrification rates in May compared to the control in Killbuck Creek (0.99 vs. 7.58 µg NH₄-N/gdm/d; \( p = 0.001 \)) (Figure 6). Lead enrichments also significantly decreased August nitrification rates compared to the control in Buck Creek (0.13 vs. 1.72 µg NH₄-N/gdm/d; \( p = 0.029 \)), Cold Creek (0.00 vs. 1.14 µg NH₄-N/gdm/d; \( p = 0.001 \)), Pleasant Run Creek (0.00 vs. 1.29 µg NH₄-N/gdm/d; \( p = 0.001 \)), Stony Creek (0.01 vs. 1.13 µg NH₄-N/gdm/d; \( p = 0.037 \)), and White Lick Creek (0.00 vs. 0.715 µg NH₄-N/gdm/d; \( p = 0.001 \)) (Figure 6).

*Factors Correlated with Metal Concentrations and Nitrification Rates*

Stream pH, temperature, total dissolved solids (TDS), and dissolved oxygen concentrations (DO) were not significantly correlated with sediment iron concentrations \( (p > 0.05; \text{Figure 7}) \). Dissolved iron concentrations were negatively correlated with stream DO \( (r = -0.75, p = 0.003) \). Stream pH, temperature and TDS were not significantly correlated with dissolved iron concentrations \( (p > 0.05; \text{Figure 8}) \). Sediment copper concentrations were negatively correlated with stream temperature \( (r = -0.66, p = 0.010) \).
Stream pH, TDS, and DO were not significantly correlated with sediment copper concentrations ($p > 0.05$; Figure 9). Stream pH, temperature, TDS, and DO were not significantly correlated with dissolved copper concentrations ($p > 0.05$; Figure 10).

Control nitrification rates were positively correlated with sediment copper concentrations ($r = 0.78$, $p = 0.001$; Figure 11). There was no significant correlation between control nitrification rates and sediment iron concentrations, water iron concentrations, water copper concentrations, stream pH, or TDS ($p > 0.05$; Figure 11). Nitrification response to metal enrichment was positively correlated to total iron concentrations ($r = 0.61$, $p = 0.02$; Figure 12) and total copper concentrations ($r = 0.742$, $p = 0.002$; Figure 12).
Discussion

Metal Concentrations in Central Indiana Streams

These data suggest iron is the most abundant metal in the selected study sites relative to copper, and lead. Copper was the second most abundant metal with lead being undetectable in all but two samples (N = 14 total). These findings are consistent with previous reports from the Indiana Department of Environmental Management (IDEM) (Holdeman et al. 1999; Table 3). The higher presence of iron compared to copper and lead may be attributed to less federal and state monitoring of these contaminants as iron is not regulated due to minimal adverse effects at environmentally-relevant concentrations (Holdeman et al. 1999). In contrast, copper is both toxic to humans and can function as a biocide to aquatic organisms at environmentally-relevant concentrations (Nirel and Pasquini 2010; Moore and Ramamoorthy 1984). Similarly, lead is also highly toxic to both humans and aquatic organisms at environmentally-relevant concentrations (Moore and Ramamoorthy 1984). Higher concentrations of iron relative to copper and lead in central Indiana streams may also be due to greater natural occurrences of these elements.

Factors Controlling Metal Concentrations in Central Indiana Streams

Geochemical processes and sediment dynamics (i.e., sorption) may influence dissolved metal concentrations in streams, with evidence supporting metal concentrations may follow diel cycles (Nimick et al. 2003). Urban et al. (1990) found soluble Fe
concentrations in lakes to be correlated to dissolved organic carbon (DOC) concentrations and decreasing stream pH. Dissolved organic carbon enhances Fe mineral phase solubility above pH 5 and buffers dissolved Fe content below pH 5, by binding to dissolved metal and starting a flocculation process (Urban et al. 1990). Wen et al. (1998) found as pH increases, the adsorption rate of Cu increases. Aquatic plants have also been shown to absorb metal from aquatic environments, reducing overall metal content (Miretzky et al. 2004). The rate at which aquatic plants can remove metal depends on plant species and water conditions such as DO content (Miretzky et al. 2004). The relationship between DO and plant metal uptake may be the cause of decreasing dissolved iron concentrations as steam DO increases (Figure 8). Observed relationships between metal concentrations and physiochemical parameters (Figures 7-10) may be due to biogeochemical processes and factors not measured in this study, such as DOC concentration and macrophyte abundance.

Factors Controlling Stream Nitrification Rates

Stream sediment nitrification rates measured in this study (1-7 μg NH₄-N/gdm/d) were comparable to rates previously measured in lake sediments (0.4 - 2.3 μg NH₄-N/gdm/d; Strauss and Dodds 1997). Differences in stream physiochemical factors may have influenced control nitrification rates measured across sampling events and explain variation between the May and August sampling events. Overall, control nitrification rates were greater in the May relative to August (Figure 5). In general, pH was less than 9 in May and greater than 8.5 in all August across streams. May samples showed an
increase in nitrification rate as pH increased from around 7.0 to just above 8.5, while August samples are closely clustered between pH 8.5 and 9 (Figure 11). Previous studies (Strauss et al. 2002) show nitrification rate tends to increase as stream pH increases from acidic to basic, with pH 7.5 being optimal. Their findings were similar to the observations made in this study for May samples, but August samples had higher pH (above the optimum of pH 7.5) and lower nitrification rates potentially due to confounding factors. This also supports the conclusion noted by Strauss et al. (2002) that additional factors other than pH may influence nitrification responses including levels of organic carbon and available ammonium as well as available light. Light can inhibit the growth of nitrifying bacteria (Hagopian and Riley 1998) and even under ideal growth conditions, nitrifying bacteria have a relatively slow mean generation time, up to 60 h or 2.5 d (Sprent 1987; Hagopian and Riley 1998). Ward et al. (1982) suggested that light levels may strongly influence the location and depth at which nitrifying bacteria are found, with higher nitrification activity occurring below the photo zone of coastal waters. The greater depth of streams in May relative to August (data not shown) may have shielded the stream sediment from light, thus allowing for increased bacterial numbers in May relative to August.

**The Effect of Metals on Sediment Nitrification Rates**

Metal concentrations in the stream sediment may have more of an influence on nitrifying microbes than the metal dissolved in the water column (Figure 11). Since nitrifying microbes grow best when bound to a surface and shielded from light (Hagopian
and Riley 1998), the stream sediment is the ideal habitat for nitrifying bacteria. Thus, sediment metal would be more influential than water column metal. The correlation between control nitrification rates and sediment iron and copper concentration (Figure 11, 12) suggest that nitrification may be facilitated with increasing concentrations under some conditions. Observations by Dollhopf et al. (2005) indicated an increase in nitrification rate as sediment Fe (III) content increased in salt marshes. This increase was attributed to possible protection of AMO by iron (III) from sulfide. While sulfide was not measured in this study, iron could be performing a similar type of protection which would explain increased nitrification rates as iron concentrations increase. Copper has also been shown to protect nitrifying bacteria against some nitrification inhibition compounds (Campbell and Aleem 1965) which also supports the relationship between increased copper concentrations and increased nitrification rates (Figure 12).

Copper enrichment of the Killbuck Creek mesocosm in May decreased nitrification rates but increased rates in August when sediment concentrations were lower (Figure 3, 6) suggesting microbial adaptation and optimization to the higher naturally occurring May copper concentrations. Thus an increase in nitrification rate was observed when additional copper was added to the lower in situ copper concentration of the Killbuck Creek August mesocosms, supporting observations of microbial adaption to metal increases by Mertoglu et al (2008).

The lack of a significant increase or decrease in nitrification rate with iron enrichments in May (Figure 6) suggest iron additions of 127 mg/L may not influence microbial nitrification during the May conditions. Sediment iron concentrations were
similar in May compared to August with only Killbuck Creek and Buck Creek having significant decreased in August (Figure 3). Killbuck Creek had the highest dissolved iron concentration and the only evidence of significant reduction in nitrification rates with enrichment in August (Figure 4, 6). However, there were significantly lower sediment iron concentrations in August. Based on the hypothesis that sediment metal is more influential than dissolved metal concentrations on nitrification rate; reduced rates may be attributed to lower sediment iron concentrations which may reduce microbial protection from certain compounds (Dollhopf et al. 2005).

The overall lack of a significant difference between control nitrification rates and iron enriched mesocosms (Figure 6) may be due to the higher concentrations of iron naturally found in the sampled streams compared to copper (Figure 3, 4). Significant differences were more prevalent for copper treatments including an increase in Killbuck Creek (Figure 6). Nitrifying microbes sampled were from an environment with higher iron concentrations compared to copper. Thus 127 mg/L iron addition did not consistently influence stream nitrification rates. Rather, the total metal concentrations (sum of in situ concentration and experimental addition) will dictate nitrification response (Figure 12).

Since a majority of the stream samples had lead concentrations that were below detection in both sediment and water column (Figure 3, 4) and inconsistent response to metals (Figure 6), it is difficult to determine with certainty, the influence of lead on microbial nitrification. Observations by You et al. (2009) found that 40 mg/L of lead did not affect nitrification rates in sludge. Though enrichment concentrations were greater in
this study, the addition of 127 mg/L of lead did not have a direct influence on nitrification in stream sediment.

Conclusions

Previous research has indicated that certain metals can influence the physiology of nitrifying microbes (Table 2) though the influence of metals at environmentally-relevant concentrations on stream sediment nitrification rates is not well understood. These data suggest that sediment metal concentrations in the streams may have more of an influence on nitrifying microbes than dissolved metal concentrations. Nitrification rates in central Indiana streams were comparable to previous nitrification estimates in aquatic ecosystems. Similarly, sediment and dissolved metal concentrations were within previously reported ranges. The nitrification response to metal enrichment in stream ecosystems is likely a function of both physiochemical characteristics of the stream ecosystem and the history of exposure. Nitrification rates were much lower during August compared to May sample times. This difference in nitrification rate was observed in both treated and control mesocosms groups.

The May sampling event was characterized by lower water temperature and pH, but greater dissolved oxygen concentration compared to the August sampling event. Relationships between these physiochemical characteristics and stream sediment nitrification rates suggest that stream physicochemical properties are more influential on microbial nitrification than an increase of 127 mg/L of the metals copper, lead, and iron.
Though a 127 mg/L metal increase may reduce stream sediment nitrification rates; stream physiochemical characteristics such as DO, pH and light exposure likely dictate the majority of observed microbial responses by possibly influencing bacterial communities.
Acknowledgements

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Literature Cited


of Coho salmon and the acute toxicity of ammonia in swimming and resting rainbow trout. Aquatic Toxicology 59: 55–69.


Figure Legends

**Figure 1:** Diagram of the nitrification process and the bacterial genera involved as well as energy produced in each step. Modified from Sprent (1987) and Hagopian and Riley (1998).

**Figure 2:** Location of study sites in the Upper White River Watershed in central Indiana.

**Figure 3:** Mean sediment copper and iron concentrations in sampled streams during May and August ± SE. Different letters denote significant difference in concentrations between May and August for a given stream.

**Figure 4:** Mean water column copper and iron concentrations in sampled streams during May and August ± SE. Different letters denote significant difference in concentrations between May and August for a given stream.

**Figure 5:** Mean control sediment nitrification rate in sampled streams during May and August ± SE. Different letters denote significant difference in nitrification rates between May and August for a given stream.

**Figure 6:** Mean nitrification rates in metal enriched mesocosms in May and August ± SE. “a” indicates value is significantly greater than the control. “b” indicates significantly less than the control and “c” denotes the control value used to determine “a” and “b” per stream, per sample time. P-values to the right are for the comparisons of control rates compared to metal treatments (a,b,c) (*) indicates no detected nitrification.

**Figure 7:** Correlations between sediment iron concentrations and stream physiochemical parameters: DO, temperature and pH. N=14.

**Figure 8:** Correlations between water column iron concentrations and stream physiochemical parameters: DO, temperature and pH. N=14.

**Figure 9:** Correlations between sediment copper concentrations and stream physiochemical parameters: DO, temperature and pH. N=14.

**Figure 10:** Correlations between water column copper concentrations and stream physiochemical parameters: DO, temperature and pH. N=14.

**Figure 11:** Correlations between control nitrification rates and stream sediment and water column iron and copper concentrations as well as stream physicochemical parameters: DO, temperature and pH. N=14.

**Figure 12:** Correlations between nitrification response and total metal concentration (sum of sediment, dissolved and enriched addition concentration) for iron and copper. N=14.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Optimal <em>Nitrosomonas</em> Activity</th>
<th>Optimal <em>Nitrobacter</em> Activity</th>
<th>Citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity</td>
<td>0.3-0.5</td>
<td>0.3-0.5</td>
<td>Jones, et al. 1980</td>
</tr>
<tr>
<td>NH$_4$-N</td>
<td>≤0.5mgN/L</td>
<td>≤0.1 mgN/L</td>
<td>Jones, et al. 1980; Villaverde, et al. 1996; Lee, et al. 1997; Ciudada et al. 2007</td>
</tr>
<tr>
<td>Metal</td>
<td>Genera</td>
<td>Concentration</td>
<td>Effect</td>
</tr>
<tr>
<td>-------</td>
<td>----------------</td>
<td>---------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Copper</td>
<td><em>Nitrosomonas</em></td>
<td>30mg/L</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td><em>Nitrosomonas</em></td>
<td>1.27–12.7 mg/L</td>
<td>Ideal enzyme (AMO) function</td>
</tr>
<tr>
<td>Iron</td>
<td><em>Nitrosomonas</em></td>
<td>0.1mg/L</td>
<td>Minimal concentration required</td>
</tr>
<tr>
<td></td>
<td><em>Nitrobacter</em></td>
<td>0.3mg/L</td>
<td>Minimal concentration required</td>
</tr>
<tr>
<td></td>
<td>Combined*</td>
<td>6mg/L</td>
<td>Ideal concentration for nitrification</td>
</tr>
<tr>
<td></td>
<td>Combined*</td>
<td>112mg/L</td>
<td>Inhibitory</td>
</tr>
<tr>
<td>Lead</td>
<td>Unspecified</td>
<td>10mg/L</td>
<td>Inhibitory</td>
</tr>
<tr>
<td></td>
<td>Unspecified</td>
<td>2–40mg/L</td>
<td>Inhibitory</td>
</tr>
</tbody>
</table>

*Combined refers to effects on both *Nitrosomonas* and *Nitrobacter*
Table 3. Concentrations of metals quantified in freshwater ecosystems and wastewater sludge

<table>
<thead>
<tr>
<th>Metal</th>
<th>Water Source</th>
<th>Concentrations</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>Sludge</td>
<td>0.10 mg/L</td>
<td>Sato, et al. 1988</td>
</tr>
<tr>
<td></td>
<td>Stream Sediment</td>
<td>1.1-4990 mg/Kg</td>
<td>Newhouse, et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Stream Water</td>
<td>&lt;1.0-27.0 μg/L</td>
<td>Holdeman, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Stream Sediment</td>
<td>0.996–2.91 mg/Kg</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td>Stream water</td>
<td>0.01 – 0.02 mg/L</td>
<td>This Study</td>
</tr>
<tr>
<td>Iron</td>
<td>Sludge</td>
<td>3.0 mg/L</td>
<td>Sato, et al. 1988</td>
</tr>
<tr>
<td></td>
<td>Stream Water</td>
<td>&lt;100 – 27600 μg/L</td>
<td>Holdeman, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Stream Sediment</td>
<td>654– 1985 mg/Kg</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td>Stream Water</td>
<td>0.00 -0.10 mg/L</td>
<td>This Study</td>
</tr>
<tr>
<td>Lead</td>
<td>Sludge</td>
<td>0.12 mg/L</td>
<td>Sato, et al. 1988</td>
</tr>
<tr>
<td></td>
<td>Stream Sediment</td>
<td>&lt;1.0 – 27.5 μg/L</td>
<td>Holdeman, et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Stream Water</td>
<td>5 – 11500 mg/Kg</td>
<td>Newhouse, et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Stream Sediment</td>
<td>0.381 – 0.472 mg/Kg</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td>Stream Water</td>
<td>Below Detection</td>
<td>This Study</td>
</tr>
</tbody>
</table>
Figure 1

Ammonia (NH₃)

\[ \text{NH}_3 + \frac{1}{2} \text{O}_2 \rightarrow \text{NH}_2\text{OH} = 0.7 \text{kcal} \]

Hydroxylamine (NH₂OH)

\[ \text{NH}_2\text{OH} + \text{G}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + \text{H}^+ = 83.3 \text{kcal} \]

Nitrite (NO₂⁻)

\[ \text{NO}_2^- + \frac{1}{2} \text{O}_2 \rightarrow \text{NO}_3^- = 17.8 \text{kcal} \]

Nitrate (NO₃⁻)

Ammonia-Oxidizing Bacteria (Nitrosomonas)

Nitrite-Oxidizing Bacteria (Nitrobacter)
Figure 2
Figure 3
Figure 4

Water Cu (mg/L) vs. Water Fe (mg/L) for different sites and months:

- **Buck**: May vs. Aug,
  - p = 0.106
  - No significant difference

- **Cold**: May vs. Aug,
  - p = 0.102
  - No significant difference

- **Killbuck**: May vs. Aug,
  - p = 0.037
  - Significant difference, marked with a and b

- **Mud**: May vs. Aug,
  - p = 0.106
  - No significant difference

- **Pleasant Run**: May vs. Aug,
  - p = 0.293
  - No significant difference

- **Stony**: May vs. Aug,
  - p = 0.465
  - No significant difference

- **White Lick**: May vs. Aug,
  - p = 0.512
  - No significant difference

Note: For some sites, significant differences are indicated by a and b labels.
Figure 5

Control Nitrification Rate (µg NH₄-N/gdm²/d)

- Buck: p = 0.560
- Cold: p = 0.247
- Killbuck: p = 0.001
- Mud: p = 0.135
- Pleasant Run: p = 0.093
- Stony: p = 0.336
- White Lick: p = 0.229

May  Aug
Figure 6

May Nitrification Rate (μg NH₄-N/g dm/d)

Buck
- Control
- Response

Cold

Killbuck

Mud

Pleasant Run

Stony

White Lick

August Nitrification Rate (μg NH₄-N/g dm/d)

Buck

Cold

Killbuck

Mud

Pleasant Run

Stony

White Lick

Cntrl Cu Fe Pb

May
- Cu: p=0.864
- Fe: p=0.712
- Pb: p=0.736

Aug
- Cu: p=0.032
- Fe: p=0.188
- Pb: p=0.029

May
- Cu: p=0.102
- Fe: p=0.389
- Pb: p=0.154

Aug
- Cu: p=0.091
- Fe: p=0.124
- Pb: p=0.900

May
- Cu: p=0.020
- Fe: p=0.994
- Pb: p=0.001

Aug
- Cu: p=0.024
- Fe: p=0.124
- Pb: p=0.900

May
- Cu: p=0.367
- Fe: p=0.446
- Pb: p=0.753

Aug
- Cu: p=0.109
- Fe: p=0.719
- Pb: p=0.719

May
- Cu: *
- Fe: p=0.930
- Pb: p=0.670

Aug
- Cu: p=0.021
- Fe: p=0.017
- Pb: *

May
- Cu: p=0.303
- Fe: p=0.342
- Pb: p=0.523

Aug
- Cu: p=0.530
- Fe: p=0.688
- Pb: p=0.037

May
- Cu: p=0.736
- Fe: p=0.935
- Pb: p=0.884

Aug
- Cu: p=0.045
- Fe: p=0.038
- Pb: *
Figure 7

- **Sediment Iron (mg/Kg)**
  - pH
    - $r = -0.195$, $p = 0.524$
  - Temperature (°C)
    - $r = -0.360$, $p = 0.206$

- **TDS**
  - May
  - August
    - $r = -0.285$, $p = 0.323$

- **Dissolved oxygen (mg O₂/L)**
  - $r = -0.207$, $p = 0.498$
Figure 8

Water Column Iron (mg/L) vs pH, TDS, Temperature (°C), and Dissolved oxygen (mg O₂/L)
Figure 9

The plots show the relationship between sediment copper concentration (mg/Kg) and various environmental factors:

- **pH**: The data points for May and August show a negative correlation, with a correlation coefficient of $r = -0.277$ and a p-value of $p = 0.456$.
- **Temperature (°C)**: The relationship is negative with $r = -0.659$ and $p = 0.010$.
- **TDS** (Total Dissolved Solids): The correlation is negative with $r = -0.466$ and $p = 0.093$.
- **Dissolved oxygen (mg O₂/L)**: There is no significant correlation with $r = 0.049$ and $p = 0.874$.
Figure 10

Water Column Copper (mg/L)

- **pH**
  - May: $r = -0.218$, $p = 0.474$
  - August: $r = 0.312$, $p = 0.278$

- **Temperature (°C)**
  - May: $r = 0.267$, $p = 0.356$
  - August: $r = 0.410$, $p = 0.164$

- **TDS**

- **Dissolved oxygen (mg O₂/L)**
Figure 11

Control Nitrification Rate (μg NH₄-N/gdm/d)

- Sediment Fe (mg/kg)
  - May: $r = 0.518$, $p = 0.058$
  - August: $r = 0.777$, $p = 0.001$

- Sediment Cu (mg/kg)
  - $r = -0.151$, $p = 0.606$
  - $r = 0.004$, $p = 0.990$

- Water Fe (mg/L)
  - $r = -0.500$, $p = 0.082$
  - $r = -0.370$, $p = 0.193$

- Water Cu (mg/L)
  - $r = 0.004$, $p = 0.990$
  - $r = 0.004$, $p = 0.990$

- Stream pH
  - $r = -0.500$, $p = 0.082$
  - $r = -0.370$, $p = 0.193$

- Stream TDS
  - $r = -0.500$, $p = 0.082$
  - $r = -0.370$, $p = 0.193$