

Effects of Adding Sewage Sludge and Urea-Phosphate Fertilizers to the Great Sippewissett Salt Marsh, Falmouth, MA on Heavy Metals and Microbial N-Cycling

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ABSTRACT

Nitrification and denitrification are essential components of the nitrogen cycle by making nitrogen available for plant uptake and returning nitrogen to the atmosphere as a gas. Microbial nitrogen cycling processes were studied in Great Sippewissett Marsh, MA and Eel Pond, MA. The nitrogen cycle is important in regulating the exchange of nitrogen through the air, soil and organic matter. In this study, the effects of added metals and fertilizer on nitrification, denitrification and bacterial productivity were examined. Fertilizer in the form of urea phosphate was found to increase nitrification as was fertilizer in sludge form. Metals were not found to inhibit nitrification nor denitrification. Neither fertilizer nor metals were found to have any effect on bacterial productivity.

Key words: Great Sippewissett Marsh, microbes, nitrogen cycle, sewage sludge, urea phosphate fertilizer, heavy metals

INTRODUCTION

Pollution of toxic heavy metals has become an environmental global issue. In recognition of the toxicity of metals, the Clean Water Act and Clean Air Act were passed to help combat the increase of metal contamination to the environment. Metals have been found to affect organisms as diverse as marsh grasses (Giblin 1980), crabs (Giblin 1980) and nitrifying bacteria. This study examines the effects of metals and fertilization on nitrification and denitrification. Changes in the rates of nitrification and denitrification are important because they alter the relative amounts of nitrogen available for organisms to use and the amount of nitrogen gas in the atmosphere.

Salt marshes have been used as disposal sites because they act as filters by adsorbing toxic substances and excess nutrients from the sludge fertilizer (Hamlett 1986). It is not well understood what the impact of the sludge has on the microbial nitrogen cycle. It is important to see how metals affect microbes in the sediment. To determine the effect of metals on microbes, I looked at bacterial productivity, nitrification and denitrification. Bacterial productivity is a measure of the growth of heterotrophic bacteria in the sediment. Nitrification and denitrification are both components of the nitrogen cycle. The nitrogen cycle generates nitrogen in forms usable to plants.

Nitrification is a two step process driven by two genera of aerobic autotrophic bacteria: *Nitrosomonas* sp. and *Nitrobacter* sp. *Nitrosomonas* sp. are responsible for the oxidation of ammonia to nitrite while *Nitrobacter* sp. converts nitrite to nitrate. Overall, nitrification is the process of oxidizing ammonia to nitrate. Nitrifying bacteria have a slow growth rate and are especially sensitive to heavy metals (Blum and Speece 1991).

Denitrification is the conversion of nitrate to nitrogen gas by anaerobic heterotrophic bacteria. Denitrifying bacteria aren't as sensitive to metals as nitrifying bacteria, however because denitrification uses the product of nitrification, inhibiting nitrification also affects denitrification.

I measured the concentration of four metals: lead, zinc, copper and chromium. The Clean Water Act limited the discharge of toxic pollutant metals, like lead, copper and zinc (Wernick & Themelis 1998). Lead is highly toxic and its use was limited in the 1970's by the government. Until then however, leaded gasoline was used in cars and boats, generating a large amount of lead pollution. Today, the largest use of lead in the United States is in car and industrial lead-acid batteries (Wernick & Themelis 1998).

Zinc is the least toxic metal I measured however it is known to inhibit nitrification even at low concentrations (Bott 2005). The majority of zinc today is used as a corrosion-inhibiting coating on steel products (Wernick & Themelis 1998). Copper is very toxic in high concentrations however is required for the normal functioning of nearly all organisms. Copper addition experiments have been shown to suppress nitrification (Lee *et al.* 1997). The final metal I measured was chromium. Chromium is inert in most forms and generally not available for uptake by organisms.

METHODS

Sampling Sites

For my sampling sites, I used Great Sippewissett Marsh in Falmouth, MA and Eel Pond in Woods Hole, MA. I used experimental plots that were established in April 1974 (Valiela *et al.* 1975). For my study I used two control plots, two Urea-Phosphate plots and two High-Fertilized plots at Great Sippewissett Marsh. Each plot has a 10 meter radius and are flooded twice daily with seawater from Buzzards Bay (Hamlett 1986). The Urea-Phosphate plots are fertilized with urea and phosphate. The High-Fertilized plots receive sewage sludge fertilizer containing metals. The metal content in the sludge has varied since 1974. Between 1980 and 2000 Zinc was 1,100 to 547 ppm in the sludge. Chromium was 6,160 to 148 ppm, Copper was 470 to 201 ppm and lead was 390 to 63 ppm. The concentration of each metal has decreased over the years. From each of the six plots I sampled in Great Sippewissett Marsh I took two samples. Each sample was approximately 2 cm thick from the surface sediment. Marsh sediments are normally anoxic below 1 cm (Teal & Kanwisher 1961). I took samples along the creek that ran through each plot and all samples were taken in *S. alterniflora* dominated sites.

I also sampled from Eel Pond, an anthropogenically contaminated site in Woods Hole. I chose to sample from Eel Pond because it likely was highly metal contaminated but would not be receiving an influx of nutrients. Eel Pond is used as a boat harbor and therefore receives all the pollutants associated with boats. It is also surrounded by roads, residential and commercial buildings. I took two samples from *S. alterniflora* areas in the marsh section of Eel Pond.

After collecting all the samples, each sample was homogenized and stored in a refrigerator.

Measurement of nitrification

To measure potential nitrification in the sediment I measured one to two grams of fresh soil into a 50-mL centrifuge tube. From the fourteen plots, each sample had its own tube: T1, T2 and T3. The exact weight was recorded to 0.01 grams. To this tube I added 30 mL of a solution

containing: 200 μM NH_4Cl and 60 μM KH_2PO_4 in sea water. The capped tubes were shaken on a shaker table lying on their sides at room temperature in the dark. After the tubes were shaken, I took the T1 samples off the shaker table, centrifuged them and syringe filtered the supernatant into a scint vial. The scint vial was immediately frozen. 24 hours after that the same procedure was followed for the T2 tubes. Finally, 48 hours after the procedure had been started, the final group, T3 tubes were centrifuged, syringe filtered (GF/F swinex filters) and frozen. The amount of nitrate in the scint vials was measured using a Lachat machine. The amount of nitrate was compared and plotted between the T1, T2 and T3 vials for each plot.

Measurement of denitrification

Potential denitrification was measured by calculating the slope of the concentration of nitrate at three time points. For T0, T1 and T2, 10 grams wet weight of soil sample was measured into separate Erlenmeyer flasks. I put 30 mL into each flask of a solution in artificial seawater that contained 150 μM nitrate, 100 μM glucose and 100 μM ammonium. The ammonium was added to provide a nitrogen source for biosynthesis so that the nitrate wouldn't be used for assimilative nitrate reduction. The T0 flasks were shaken immediately for five minutes and filtered through Whatman 1 Filter Paper and frozen for future nitrate analysis. The T1 and T2 flasks were sealed with suba seals, with each seal punctured by two needles that allowed the option of air flow. Each flask was evacuated of air and filled with nitrogen gas three times. The flasks were then sealed from outside air so as to remain completely anaerobic. After twenty four hours, T1 flasks were shaken, filtered and frozen for nitrate analysis. After twenty four more hours, the same procedure was repeated for the T2 flasks. The nitrate analysis was run on a Lachat machine. I calculated the rate of denitrification by determining the slope from the T0 and T1 points. I discounted the T2 points because there appeared to be a nitrate limiting factor.

Bacterial Production

From each of my samples, I weighed out 0.5 g into each of four small capped centrifuge vials. One tube was marked T0, the other three were T1. Each sample had four vials (and each vial had 0.5 g of sample). The T0 vial acted as a control, while the three T1 vial were duplicates. To the T0 vial I added 20 μL 100% TCA and shook to distribute the TCA. The T0 samples are killed right away to account for abiotic adsorption of leucine onto particulate matter. To each of the T1 vials I added 0.5 mL of 50 μM 3H Leucine working solution and shook gently. I placed the vials in a tub of water and incubated them for one hour. After one hour, I removed the vials from the incubator and spun each sample down in a minicentrifuge to force all the substances to the bottom of the vial. I then added 20 μL 100% TCA to the three T1 vials and shook. The samples were stored in a refrigerator for one week until I was ready to process them. After a week, I sonicated the samples for one minute and centrifuged at 14,000 g for 10 minutes. I filtered the supernatant through 0.2 μm polycarbonate filters using a clockface manifold with 25 mm GF/F backing filters. The filters and the pellets (in the vials) were both rinsed twice with 5% TCA, once with 40 mM leucine, once with 80% ethanol and once with nanopure water. After the rinsing I combined the filter and pellet in 2 mL centrifuge tubes and filled them with an alkaline solution containing 0.5 N NaOH, 25 mM EDTA and 0.1% SDS. I heated the vials for one hour at 90°C. Then I centrifuged the vials at 14,000 g for 10 minutes. I put 100 μL of the supernatant in 20 mL scintillation vials and added 10 mL Hionic-Fluor scintillation cocktail (Perkin-Elmer #6013319). Finally, I read the bacterial productivity counts on a scintillation counter. The

scintillation counts were converted in pmol leucine per mL per hour using a formula derived by Jen Bowen.

Metal analysis

I measured between 200 to 300 mg of sediment of each sample into its own centrifuge tube. To each tube I added 5 mL of full strength HNO₃ acid. I also added the acid to two empty centrifuge tubes for controls. I heated the tubes for one hour at 70°C, swirling once halfway through. The tubes cooled for an hour. After cooled, I added 5 mL HCl to the tubes and heated them for one hour at 70°C, swirling halfway through. The tubes cooled overnight. The next day I filtered the liquid into volumetric beakers using Whatman 42 filter paper. I diluted the acidic liquid to 100 mL with deionized water. I then transferred the solution to 200 mL bottles for storage. I used an Atomic Absorption Spectrophotometer to measure concentrations of lead, copper, zinc and chromium in each sample.

Carbon and nitrogen analysis

A small amount of wet sediment from each sample was weighed and then put in a drying oven. Once dry, the dry weight was taken for use as a wet/dry weight conversion factor. The dried samples were ground into powder using a mortar and pestle. The samples were then analyzed for carbon and nitrogen content using a CHN Analyzer.

Sulfur analysis

I used approximately 200 mg of dried sample from each plot and used a sulfur combustion machine to measure the levels of sulfur in each sample.

RESULTS

Potential Nitrification

The rate of potential nitrification was highest in the HF plots, followed closely by the UP plots. Both the control and Eel Pond samples had significantly lower rates than the HF and UP (Fig. 1). One sample from the HF plots was a very large outlier and the data obtained from this sample was not used in calculating the average potential nitrification for the HF plots.

Potential Denitrification

The control plot had the highest rate of denitrification of all the plots (Fig. 2). It was not significantly different from any of the other sites. The rates of denitrification were very similar between the UP, HF and Eel Pond samples (Fig. 2).

Bacterial Productivity

There was no statistically significant difference in the bacterial productivity between any of the plots (Fig. 3).

Metals

Eel Pond has a very high concentration of lead and is significantly different from all the other sites (t test, $p \leq 0.05$) with more than six times the lead of all others (Fig. 4). Eel Pond also has a high concentration of copper, nearly twice that of the HF plots (Fig. 5). The HF plot has a

copper concentration of almost four times the UP plots. The HF plot has a much higher concentration of zinc and chromium than all the other plots (Fig. 6 & 7). The control, UP and Eel Pond have similar concentrations of zinc and chromium but are all significantly different from the HF plots (t-test, $p \leq 0.05$).

C:N

On analysis of the sediments for carbon and nitrogen, all the plots had similar values for both elements. Eel Pond and the HF plot had slightly greater amounts of nitrogen (Fig. 8), but similar carbon values so therefore had lower C:N ratios (Fig. 9). The difference in nitrogen between Eel Pond and HF plots is statistically significant ($p \leq 0.05$), the HF plots have a greater amount of nitrogen present in the surface sediment. The UP and control plots had lesser, but nearly equal percent nitrogen values (Fig. 8).

My data collection showed there to be no correlation between C:N and bacterial productivity and no correlation between %C and bacterial productivity.

Sulfur

The HF plots had almost twice the amount of sulfur as the control and UP plots, and more than three times the amount of Eel Pond (Fig. 10). These differences are all statistically significant ($p \leq 0.05$). The control and UP plots had equal amounts of sulfur, with Eel Pond having a significantly smaller amount of sulfur present.

DISCUSSION

Nitrification

Nitrification is the process of converting ammonium to nitrate by nitrifying bacteria. My data suggests that metals are not inhibiting nitrification. This is shown by the high rates of nitrification in both the HF and UP plots. Both plots are fertilized but the HF plots receive fertilizer that contains metals. The HF plot had slightly higher nitrification rates than the UP, suggesting that the metals are not inhibiting nitrification. The CT and EP sites have low nitrification rates because they don't receive additional fertilizer. The difference between the low levels of nitrification in the control and EP plots versus the high rates of nitrification in the UP and EP is due to the fertilizer added to these sites. Fertilizer fuels productivity which increases the rates of nitrification. The increased rates of nitrification in the fertilized plots are because there is more organic matter present that undergoes decomposition.

In 1995, Komulainen and Mikola performed a similar experiment on soil processes using fertilizer containing copper and nickel. They found that there was a higher concentration of ammonium in fertilized sites. They attributed this increase in part to decreased nitrification. They also found that the release of nitrate was reduced by the presence of heavy metals. My results indicate otherwise however, with increased nitrification rates (Fig. 1) in the heavy metal fertilized plots.

Yong-Woo Lee et al. set up a laboratory experiment in 1997 where they could test the effects of copper and nickel on the nitrifying bacteria: *Nitrosomonas* sp. and *Nitrobacter* sp. Lee et al. added 5 mg/L of copper over sixty hours to cultivated nitrifying bacteria and measured the change in ammonium concentration. They added 50 mg/L of nickel over sixty hours to another set of nitrifying bacteria. They found that after sixty hours nitrification was partially suppressed

in the bacteria contaminated with copper. The bacteria contaminated with nickel were also inhibited and became severely inhibited after the concentration of nickel passed a certain threshold. In contrast, the bacteria with copper were gradually inhibited by the increasing concentration of the metal. From this it was determined that nitrifying bacteria are more sensitive to copper than nickel. It was also found that *Nitrosomonas* sp. are equally or more sensitive than *Nitrobacter* sp. bacteria to copper. Therefore, the first step of nitrification is the rate limiting step under conditions with copper as a stressor. The 5 mg/L of copper that was added in this experiment is much lower than the amount of copper that is retained in the HF and EP plots (Fig. 6). Since metals did not appear to be inhibiting nitrification in my study, it is possible that a significant amount of copper on the plots is simply unavailable to the microbes.

Lee et al.'s conclusion that *Nitrosomonas* sp. are more sensitive than *Nitrobacter* sp. is corroborated in a study by Bott et al. (2005) in which zinc was used as the metal stressor. A short term nitrate rate experiment found that 0.5 mg/L zinc did not inhibit nitrification, while 50 mg/L Zn moderately inhibited the bacteria. They found that ammonia oxidizing bacteria are more sensitive to metal addition than nitrite oxidizing bacteria.

Denitrification

Denitrification is lowest in the UP and HF plots because the increased fertilization promotes the growth of the tall form of *S. alterniflora* to grow (Rogers *et al.*, 1998). The tall form of *S. alterniflora* takes up more water from the sediment, creating oxygen air pockets in the sediments. The plants also return oxygen to the sediment to the roots through the aerenchyma which further oxidizes the soil around them. Retranslocation is a method plants use to cope with anoxic sediment conditions (Giblin 1983). Regardless of the shift of dominance to the tall form of *S. alterniflora*, an increase in primary production fueled by fertilizer would increase evapotranspiration and retranslocation. This drives the surface sediment more oxic in the UP and HF plots than the control. Therefore, denitrification (an anaerobic process) must take place deeper in the sediment. Since I only sampled the top 2 cm of the sediment, I was not able to capture all the denitrification that was occurring in the HF and UP plots. In addition, the final time set of nitrate values (which I did not use in calculating the rate of denitrification) was likely low because the denitrifying bacteria were limited by nitrate. The final nitrate values plateaued instead of decreasing as expected.

Bacterial Productivity

Although there were no significant differences between any of the plots, the UP and HF plots had higher bacterial production than the control, suggesting that increased plant substrate in the fertilized plots may contribute to increased bacterial production.

Jennifer Bowen used the same procedure to obtain bacterial productivity at the Great Sippewissett Marsh however she sampled the marsh during the summer and also sampled more plots. In addition to the CT, UP and HF plots, Bowen sampled a Low-Fertilized (LF) and Extra High Fertilized (XF) plot for a wider spectrum of data. She found that the bacterial productivity peaked in the LF plots followed (in decreasing order of bacterial production) by the CT, HF, UP and XF plots. Despite the slight differences between plots however, there didn't appear to be a significant effect from metals.

Metals

The primary reason behind the differences in concentration of each metal in each plot is due to a difference in inputs. In the 1970's lead was present in high quantities both in the sludge added to the HF plot and also in Eel Pond because of leaded gasoline (introduced to the pond via runoff and boat engines). Presently, due to environmental protection laws, lead has become less prevalent and therefore less lead is present in the HF sludge and Eel Pond. Copper and chromium are both present in high concentrations in Eel Pond because of boat paints and pressure treated lumber. The sludge fertilizer added to the HF plots is high in copper, and even higher in chromium. In the HF plot, where both fertilizer and metals are added, the fertilizer fuels plant production. This increases evapotranspiration and retranslocation which causes there to be a deeper oxic zone. Lead is retained in sediment as PbS which forms anaerobically. Due to the deepening of the oxic zone, lead is not retained well in the surface sediment. Outside researchers have found that the concentration of lead is highest 10 cm deep in Great Sippewissett Marsh, where anoxic conditions are likely to exist (Harrold 2005).

Zinc is highest in the HF plots along with the highest concentration of sulfur. Zinc is not well retained in the sediment because zinc sulfides are easily oxidized. Zinc also combines with soluble chloride complexes which are retained in the sediment. In general, zinc is easily remobilized and the majority of zinc input is lost from the marsh and enters deeper water (Giblin 1983) Giblin suggests that the zinc (and other metals) are not lost from the plots as particulates, instead they are being chemically remobilized.

Copper is one of the more toxic metals I measured and is present in very high amounts in Eel Pond. Copper has the highest concentration in Eel Pond because of contamination from boats and pressure treated lumber. The HF plots also have a high concentration of copper, which they receive directly from the sludge fertilizer. When conditions in the sediments are anoxic, copper combines with sulfide to form copper sulfide. When the sediments are oxic, copper adsorbs to hydrous oxides so copper is retained in the sediment regardless of oxygen conditions.

Chromium is highest in the HF plots, a reflection of the inputs rather than the retention rate. Giblin (1983) found that less than 20% of the chromium in the XF plots she tested was labile. Chromium is considered a carcinogen to humans, however most forms of chromium in the environment are essentially inert. Chromium is poorly absorbed and is almost completely immobile. It is generally not bioavailable and therefore doesn't make its way up the food chain to humans. Hexavalent chromium is toxic however, and accumulates in plant roots (ICDA Guidelines).

Carbon:Nitrogen

All the plots had similar C:N ratios, however both the HF and EP plots had significantly more nitrogen than the UP and control. The difference between HF and EP was also significant (t test, $p \leq 0.05$) but small. From this we would expect to see high nitrification rates in both the HF and EP plots however the rates are high only in the HF plots. The rate of nitrification in Eel Pond is negligible. This suggests that the ammonium added to the HF plots and the urea in the UP plots are fueling nitrification, rather than particulate nitrogen as measured by the C:N analysis. Also, the high concentrations of lead and copper (and perhaps other metals that were not measured) may be inhibiting nitrifying bacteria in Eel Pond.

Sulfur

The presence of high concentrations of sulfur in the HF plots may help retain metals in the sediment. Sulfur is a product of anaerobic respiration so its occurrence in the oxic surface sediments in the HF plots is interesting to note.

CONCLUSION

Metals do not appear to inhibit nor promote nitrification and denitrification. Fertilizers in the form of urea and phosphate increased nitrification. Sludge fertilizer containing metals also increased nitrification, although not significantly more than the urea phosphate fertilizer. The highest denitrification rates were found in control plots, likely due to the more oxic surface sediment suppressing denitrification in the UP and HF plots. Finally, neither metals nor fertilizer appear to have affected bacterial productivity.

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APPENDIX

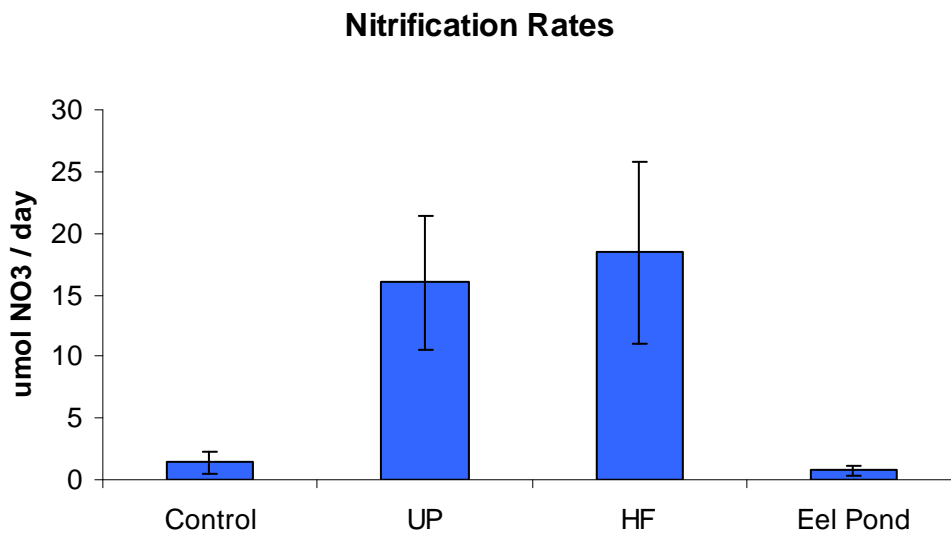


Fig. 1: The UP and HF plots have high but similar rates of nitrification. The control plot and Eel Pond have very low rates of nitrification. Nitrification rates determined by calculating the slope of nitrate concentration over three time periods. Nitrate concentration was measured on a Lachat.

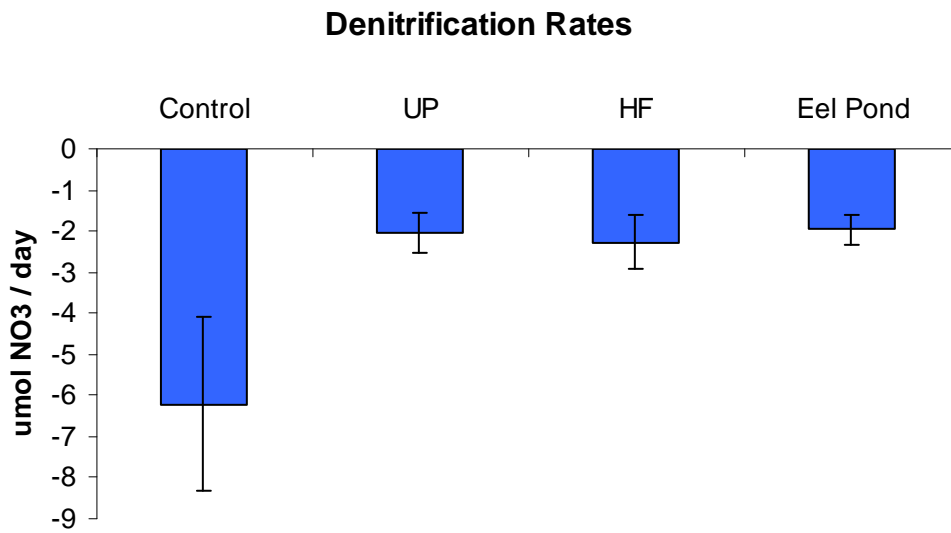


Fig. 2: The control plots had the highest rates of denitrification. The UP, HF and Eel Pond samples had similar rates of denitrification. Denitrification rates determined by calculating the slope of nitrate concentration over three time periods. Nitrate concentration was measured on a Lachat.

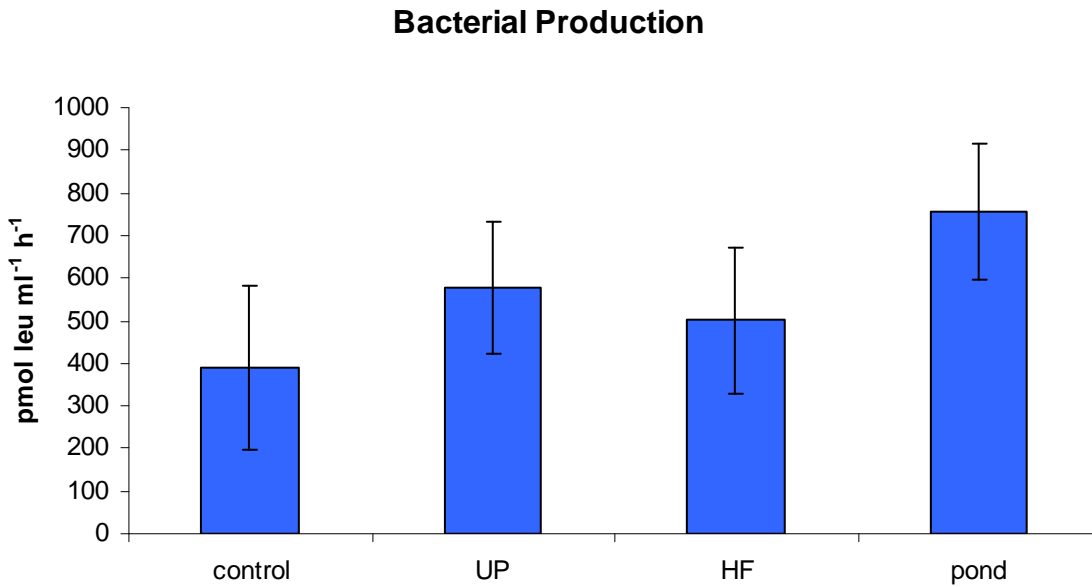


Fig. 3: Bacterial productivity was not significantly different between any of the plots.

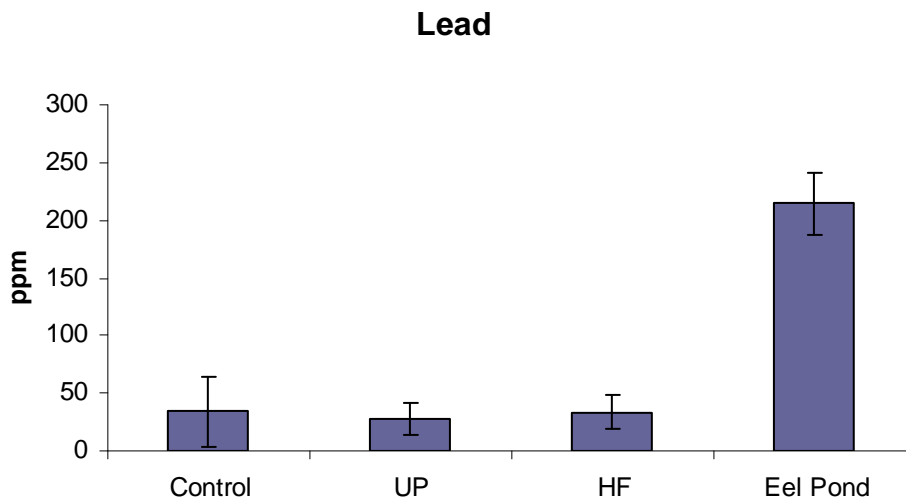


Fig. 4: The concentration of lead was highest in Eel Pond. Lead concentration was low among the other plots. Lead concentration was measured on an Atomic Absorption Spectrophotometer.

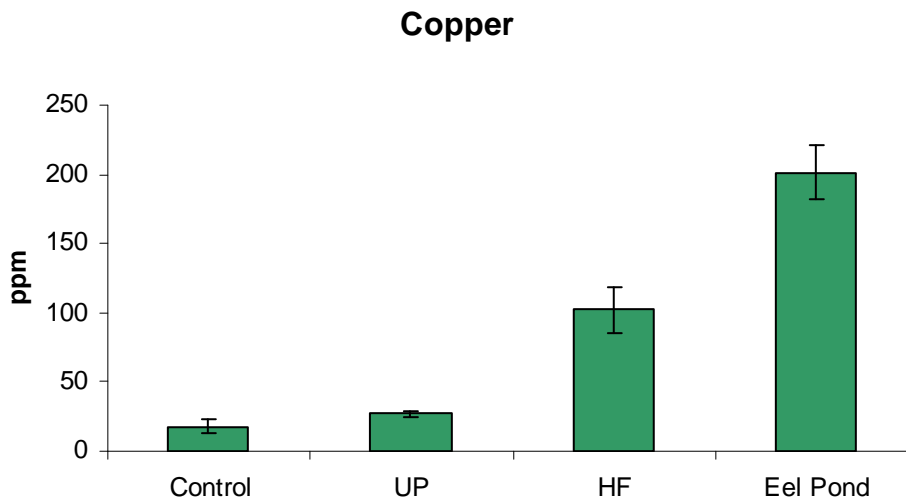


Fig. 5: Copper concentration was highest in Eel Pond, followed by the HF plots. The control and UP plots were low in copper. Copper concentration was measured on an Atomic Absorption Spectrophotometer.

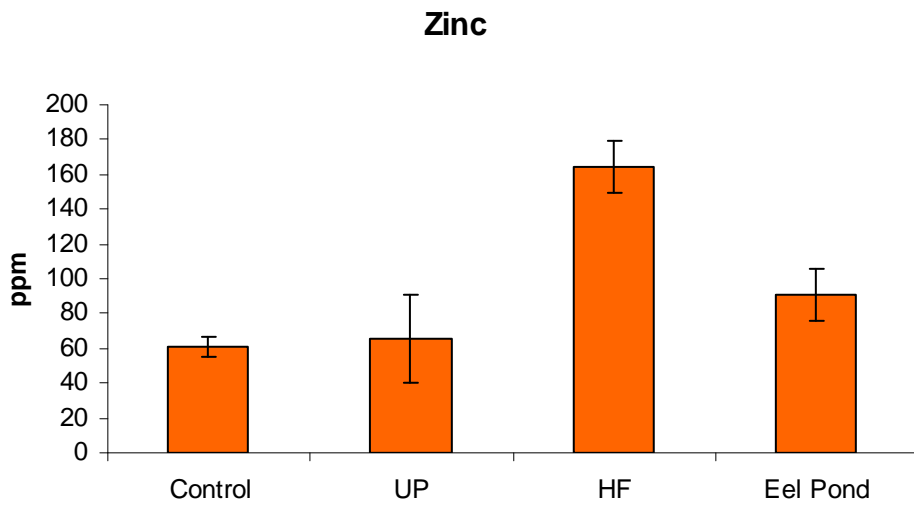


Fig. 6: Zinc concentration was highest in the HF plots, followed by control, UP and Eel Pond. Zinc concentration was measured on an Atomic Absorption Spectrophotometer.

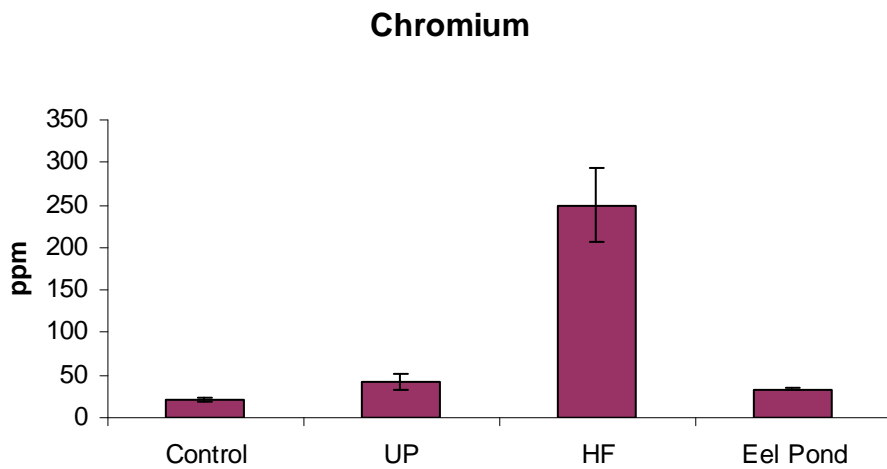


Fig. 7: Chromium concentration was highest in the HF plots, followed by the Control, UP and Eel Pond. Chromium concentration was measured on an Atomic Absorption Spectrophotometer.

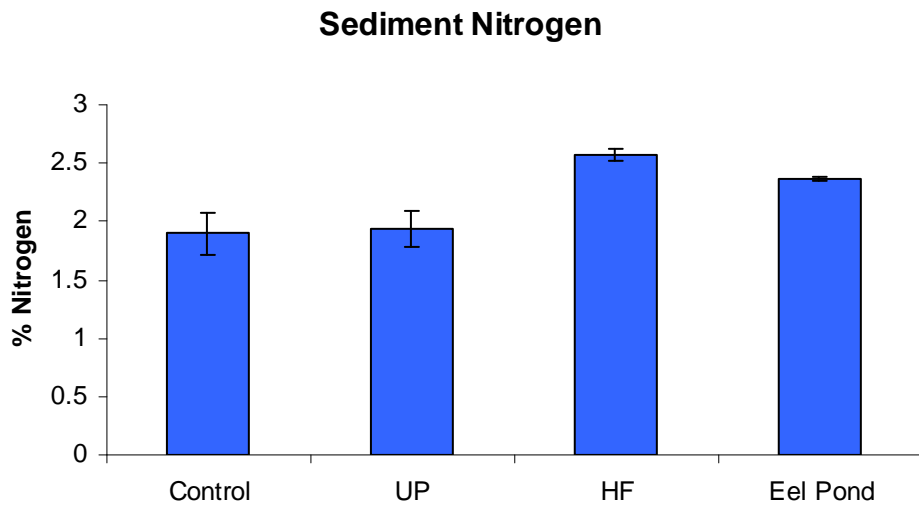


Fig. 8: The HF plots and Eel Pond have the highest percentage of nitrogen. The Control and UP plots have slightly lower percentages of nitrogen.

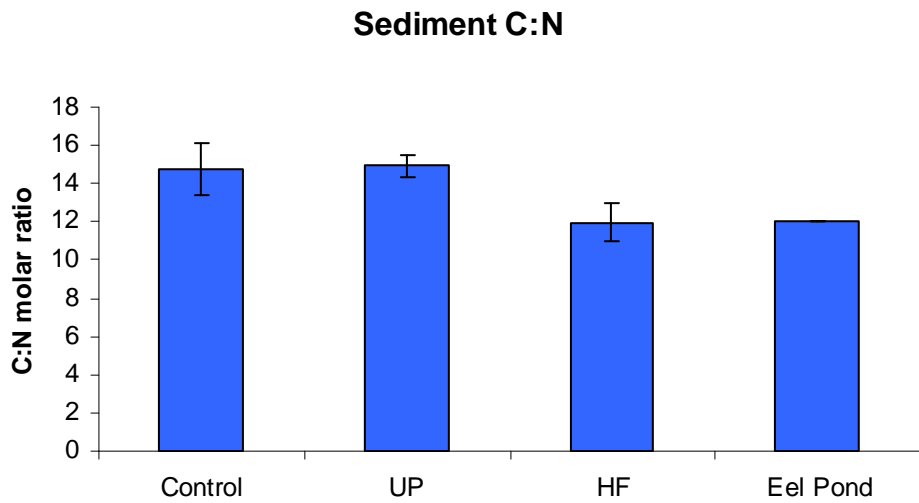


Fig. 9: The C:N ratio is highest in the Control and UP plots. The HF plots and Eel Pond have slightly lower C:N ratios.

Average % Sulfur in Sediments

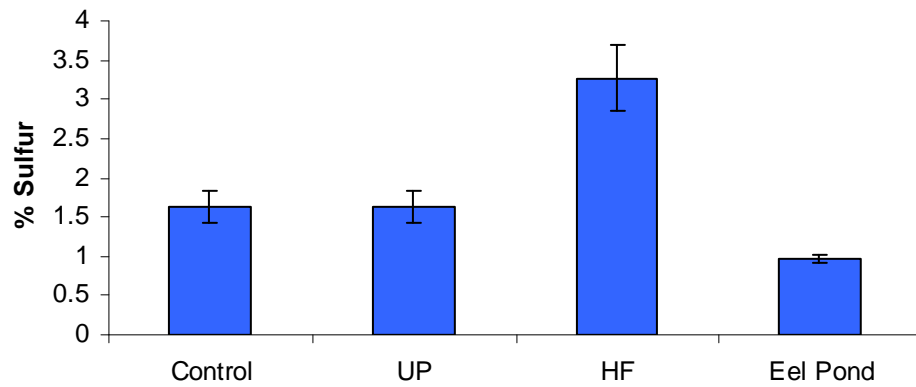


Fig. 10: The HF plot has significantly higher percent sulfur than other plots.