PROJECT REPORT

To the Palo Alto Regional Water Quality Control Plant

INHIBITION OF ACTIVATED SLUDGE NITRIFICATION BY ROOT CONTROL CHEMICALS: AN INITIAL EVALUATION OF DOSAGE AND CONTACT TIME

August 14, 2007

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August 15, 2007

Mr. Phil Bobel
Palo Alto Regional Water Quality Control Plant
2501 Embarcadero Way
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Dear Phil:

Please find attached our report "Inhibition of activated sludge nitrification by root control chemicals: an initial evaluation of dosage and contact time".

Here is a summary of the major findings:

1. Razorooter and Vaporooter both inhibited ammonia oxidation; RootX had little effect at the dosages evaluated.
2. Razorooter was the most potent inhibitor of ammonia oxidation.
3. At high dosages, Razorooter and Vaporooter prevented recovery of ammonia oxidation. The higher the dosage, the longer it took for recovery to occur.
4. Both Vaporooter and Razorooter inhibited nitrite oxidation.
5. At high dosages, Vaporooter prevented recovery of nitrite oxidation. The higher the dosage, the longer it took for recovery to occur.
6. The ammonia-oxidizing bacteria differed in their response to the root control chemicls. *Nitrosomonas europaea*-like bacteria became dominant when exposed to RootX. *Nitrosopsira and Nitrosomonas europaea*-like bacteria became dominant upon exposure to Vaporooter. A *Nitrosomonas*-like bacterium found in untreated control samples remained dominant upon exposure to Razorooter.

Our report elaborates on the above findings and includes recommendations for future research. Please feel free to call if you have any concerns or questions.

Please note that because of the way in which this project developed from our earlier project (which is now closed at Stanford), we will need to resolve some administrative and budgetary issues related to this work. I will be contacting you about that shortly.

With best regards,

Craig Criddle

Craig Criddle
Professor of Environmental Engineering

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EXECUTIVE SUMMARY

Plant roots can clog sanitary sewers leading to overflows. This has led to increasing use of root control chemicals in sewers. The herbicides used for this purpose are injected into the sewers in foam formulations. These foams fill the space above the flowing sewage, coating plant roots that have penetrated the sewer, inhibiting their further growth or killing them. The objective of this study was to evaluate the effects of three different root control products - RootX, Vaporootor and Razorootor II - on the nitrifying activity of activated sludge at the Palo Alto Regional Water Quality Control Plant (PARWQCP). Each of these root control products contained different active ingredients.

This initial study focused on the effects of concentration and exposure time. A series of 80-mL batch reactors were inoculated with fresh activated sludge from PARWQCP and perturbed with eight different concentrations of each root control product. Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations were monitored over a 4-week period to determine the extent of nitrification inhibition and to assess recovery from inhibition. To evaluate the impacts of root control chemicals on the microbial community in activated sludge system, DNA analyses for the ammonia oxidizing bacteria (AOB) were carried out, using terminal restriction fragment length polymorphism (T-RFLP) of the functional gene, *amoA*.

CONCLUSIONS

7. **The root control chemicals evaluated in this study have different effects on ammonia oxidation:**

    a. **<u>Razorooter and Vaporooter inhibited ammonia oxidation; RootX had little effect for the concentration range evaluated</u>.**

    b. **<u>Razorooter is the most potent inhibitor of ammonia oxidation</u>.** The No Observable Effect Level (NOEL) for Razorooter was <12.5 mg./L (equivalent to <4.7 mg/L of diquat dibromide, the active ingredient). The NOEL for Vaporooter was 12.5 mg/L (equivalent to 0.15 mg/L of metam sodium, the active ingredient). The NOEL for RootX was 500 mg/L (equivalent to 2.75 mg/L dichlobenil).

c. **At high dosages, Razorooter and Vaporooter prevented recovery of ammonia oxidation.** Ammonia oxidation did not completely recover over a 4-week period at Razorooter concentrations > 50 mg/L (>18.7 mg/L active ingredient). No recovery occurred at Vaporooter concentrations > 1 g/L (>12 mg/L active ingredient).

d. **For exposure to Razorooter and Vaporooter, the higher the dosage, the longer it took for full recovery of ammonia oxidation.** A two-week recovery period was needed for samples incubated with 25 mg/ L Razorooter or for samples incubated with 500 mg/L of Vaporooter. For Root-X, recovery of full nitrification is rapid at all dosages examined.

8. **The root control chemicals evaluated in this study also have different effects on nitrite oxidation:**

   a. **Both Vaporooter and Razorooter inhibit nitrite oxidation, as indicated by the persistence of nitrite and absence of nitrate in test samples.** Based on the active ingredient concentration, nitrite oxidation is most sensitive to Vaporooter. The NOEL for Vaporooter was 25 mg/L (equivalent to 0.3 mg/L of the active ingredient metam sodium). The NOEL for Razorooter was also 25 mg/L (equivalent to 9.3 mg/L of the active ingredient diquat dibromide). Minimal nitrite accumulation occurred for RootX at the highest level tested (1 g/L, equivalent to 5.5 mg/L dichlobenil).

   b. **At high dosages, Vaporooter prevented recovery of nitrite oxidation.** No recovery occurred at Vaporooter concentrations > 1 g/L (>12 mg/L active ingredient). The methods used in this study dud not enable a determination of the recovery time required for nitrite oxidation due to inhibition by Razorooter.

   c. **For exposure to Vaporooter, the higher the dosage, the longer it takes for recovery of nitrite oxidation.** A two-week recovery period is required for samples incubated with 500 mg/ L of Vaporooter.

9. **The dominant lineages of ammonia-oxidizing bacteria differ in their susceptibility to inhibition by different root control chemicals.**

   a. *Nitrosomonas europaea*-like bacteria became dominant upon exposure to RootX.

   b. *Nitrosopsira and Nitrosomonas europaea*-like bacteria became dominant upon exposure to Vaporooter.

   c. A *Nitrosomonas*-like bacterium found in untreated control samples remained dominant upon exposure to Razorooter. This organism appears to be the same AOB group dominant in the activated sludge of aeration basin at the PARWQCP for May 2005 to June 2005 and September 2005 to January 2006 in a year-long study.

RECOMMENDATIONS

The results of this study establish that root control chemicals do inhibit nitrification and that concentration and exposure time are important variables. However, the following questions remain unanswered, and additional work is recommended to address them:

1. **How do application practices influence the delivery of root control chemicals to the wastewater treatment facility?** Information is needed on current application practices, and how these processes influence the concentrations of root control chemicals that enter the wastewater treatment facility.

2. **How are root control chemicals entering the plant diluted and degraded as they pass through the treatment facility?** Information is needed on how root control chemicals that enter the treatment facility are diluted and degraded as they pass through the treatment train. A tracer study is thus recommended to understand system hydraulics, and batch degradation studies are recommended to assess the kinetics of biodegradation of root control chemicals.

3. **How does the structure of the microbial community influence its ability to adapt to different root control chemicals?** Further studies are needed to assess the effects of root control chemicals on community structure, how nitrifying activated sludge communities adapt to exposure to different root control chemical, and to determine whether specific microbial lineages impact stability and adaptation. Future studies should also examine nitrifying Archaea to determine whether these organisms contribute to the stability of nitrification upon exposure to inhibitors.

4. **What policies will best protect the treatment facility from the adverse effects of root control chemical applications?** A synthesis of data from the studies recommended above is needed to establish a set of protocols or guidelines that will ensure stable nitrification and prevent adverse effects on system performance.

INTRODUCTION

Nitrification is an important process in biological wastewater treatment because it removes ammonia from wastewater, protecting water bodies from N-stimulated eutrophication, nitrogenous oxygen demand, and the toxicity of ammonia. It is a two-step process carried out by two distinct functional groups of chemolithoautotrophic bacteria: (1) ammonia-oxidizing bacteria (AOB) that oxidize ammonia (NH_3) to nitrite (NO_2^-), and (2) nitrite-oxidizing bacteria (NOB) that oxidize nitrite to nitrate (NO_3^-). Recent studies by our group have revealed that ammonia-oxidizing archaea (AOA) are also present at the PARWQCP. We have observed seasonal changes in the lineages of AOB, and these shifts correlated to changes in temperature. It is unknown how different lineages respond to different chemical inhibitors. Given this complex ecology and the increasing stringency of effluent standards, it is important to establish operating procedures and policies that ensure consistent plant performance.

Nitrifying communities in activated sludge bioreactors are constantly subject to various levels of inhibitory chemicals discharged from households and industries. Chemicals are also added to sewers to alleviate problems related to the distribution systems. An important issue that municipalities are currently facing is the intrusion of roots into sewage collection pipes and storm drains. Motivated by mandates from the U.S. Environmental Protection Agency (EPA) to reduce sanitary sewer overflows (SSOs), municipalities have implemented root control programs to clear out root-infested pipes and to restore pipe flow capacity. While mechanical removal is possible, chemicals are usually applied to treat the sewer lines. The active ingredients in many of these formulations are herbicides. They are usually applied with foam so that the herbicides are brought into direct contact with the roots for the active ingredients to inhibit further root growth or kill the plants. A problem with this strategy is that episodes of nitrification instability have occurred in the downstream wastewater treatment plant within hours of the chemical application (Ake, 1995). Complete recovery usually requires several days. Such long recovery periods (i.e., low resilience of the microbial community) can lead to periods in which the effluent ammonia concentrations exceed regulatory limits. It is also possible that undesirable nitrite discharges could occur during these upsets.

The purpose of this study was to provide improved understanding of the impacts of root control products on nitrification. The results should be of value to municipalities as they develop recommendations for contractors or clients regarding the application of root control products to their distribution systems. The results will also facilitate more comprehensive investigation of factors affecting the stability of nitrification in activated sludge systems.

OBJECTIVES

1. To evaluate how the dose of major root control chemicals and exposure time affect nitrification.

2. To compare the relative effects of different root control products on the nitrifying bacteria within activated sludge systems.

MATERIALS AND METHODS

Root Control Chemicals

Staff of the PARWQCP supplied three root control products for testing. Each product contained different percentages of active ingredients and foaming agents as listed in Table 1. We attempted to adjust the root control product concentration to achieve equivalent active ingredients concentrations for each product in the batch assays. However, for Root-X, excessive foaming and a low percentage of the active ingredient (dichlobenil at 0.55%) made it impossible to achieve the target level required for comparison with the other products. Accordingly, we decided not to adjust the active ingredient concentration but rather to apply all three products at the same product mass concentration level. All root control products were prepared following instructions provided by the suppliers.

Table 1. Compositions of Root Control Products

Root Control Products	Active Ingredients (% Wt)	Concentration Range in Test
RootX	2,6-dichlorobenzonitrile (dichlobenil) (0.55%)	0.07 – 5.5 mg/L dichlobenil
Vaporooter	Sodium methyldithiocarbonate (metam-sodium) (30% of powder); 2,6-dichlorobenzonitrile (dichlobenil) (50% of liquid)	0.01 – 0.90 mg/L dichlobenil 0.15 – 12 mg/L metam sodium
Razorooter II	6,7-Dihydrodipyrido(1,2-a:2',1'-c)pyrazinediium dibromide (diquat dibromide) (37.3%)	4.7-373 mg/L diquat dibromide

Batch Assays

Batch assays were performed to assess the effects of root control chemicals on nitrification. These studies were based on Arvin's Minntox assay (1994). The standard assay uses a fixed exposure period of 2 hours, but for this work, the period of exposure was increased to 4 weeks to evaluate the effects of exposure time. A sample of mixed liquor suspended solids (MLSS) was collected from the aeration basin at PARWQCP on September 20, 2006, and centrifuged. The pellet was rinsed with phosphate buffer and dilute media to remove inhibitors, then resuspended in 80-mL of sterile media containing 50 mg NH_3-N/L. Seven concentrations of each root control product (1,000, 500, 250, 100, 50, 25, 12.5 mg/L) were added to each batch at time 0, and triplicate controls prepared. All batches were covered with aluminum foil and shaken at 200 rpm at room temperature. Solution pH was monitored for each incubation with 1N NaOH or 1N HCl to maintain it to the pH range 7.0-7.5. Mixed liquor samples were also collected periodically for chemical analysis and the cell pellets were stored at -20 ºC for community analysis.

Chemical Analysis

Ammonia concentrations were measured at the start of the batch assay using a colorimetric method (Hach Test N' Tube -salicylate method (0.4-50.0 mg N/L)). Nitrite (NO_2^-) and nitrate (NO_3^-) concentrations of the supernatant were measured by ion chromatography (Dionex ICS-250 with column AS11-HC).

T-RFLP Analysis of AOB Populations

AOB community structures after exposure to root control chemicals were analyzed using the amoA-based T-RFLP method developed by Park and Noguera (Park, 2004). This method used a

HEX-labeled forward primer (amoA-1F) and a FAM-labeled reverse primer (amoA-2R) to amplify 491-bp amoA gene. Duplicate PCR products were pooled and purified using the Montage PCR filter units (Millipore, Billerica, MA). The purified products were digested with *TaqI* restriction endonuclease (Fermentas, Glen Burnie, MD). After re-purification, the digested PCR products were sent to the Research Technology Support Facility of Michigan State University for analysis in a capillary electrophoresis machine (ABI 310 genetic analyzer, Foster City, CA). The length of T-RFs and peak heights were analyzed using the Genemarker Software version 1.4 (Soft Genetics, State College, PA)

RESULTS AND DISCUSSION

A. Effects of different root control chemicals on ammonia and nitrite oxidation

Both nitrite and nitrate concentrations were monitored to determine the extent of inhibition. A decrease in the production of nitrate plus nitrite indicated inhibition of ammonia oxidation. An accumulation of nitrite indicated inhibition of nitrite oxidation.

In controls that received no inhibitor, nitrite accumulated to 6 mg N/L (12% of the added NH3-N) in the first 24 hours but completely oxidized to nitrate thereafter (Figure 1). This pattern was reproducible, and consistent with normal batch nitrification kinetics. In test samples that received inhibitors, the persistence of nitrite for longer time periods indicated inhibition of nitrite oxidation.

The detailed patterns of nitrification inhibition for each root control product are discussed below.

RootX – 0.55% dichlobenil (concentration range for the test: 0.07 – 5.5 mg dichlobenil/L)

RootX addition inhibited ammonia oxidation (i.e., the rate of production of nitrate plus nitrite was lower for the test samples than for the control) only at the highest concentration tested (1 g/L). The lower nitrite and nitrate production during the first 24 hours in this test sample (Figure 1A & 2A), suggests that higher level (5.5 mg/L) of dichlobenil inhibited ammonia oxidation during the early exposure period. After 48 hours, ammonia oxidation was fully recovered (Figure 2A). The AOB populations might have adapted to the presence of dichlobenil and thus resumed their ammonia oxidation capability.

The initial rate of nitrate production was similar for all concentrations evaluated. After 48 hours, however, the rate decreased due to complete consumption of ammonia (Figure 2A). Nitrite was also detected in all incubations (Figure 1A), but the level was lower than that of the controls. Nitrite concentrations in all batches peaked within 24 hours then decreased to below the detection limit after 48 hours, similar to the pattern of the control samples. Therefore, it was concluded that inhibition of nitrite oxidation was minimal in the test range of RootX concentrations

RootX contained a low percentage of active ingredients. The low solubility of the inactive ingredients and foaming made testing of higher concentrations of the active ingredient impractical. Therefore, a more comprehensive study on the extent of foaming effects on

nitrification inhibition would be useful for establishing regulatory standards for root control chemicals with low percentage of active ingredient.

Vaporooter – 30% metam sodium in 5 gallon liquid, 50% dichlobenil in 30 ounces powder, mixed to 125 gallons (concentration range for the test 0.15-12 mg/L metam sodium)

The pattern of nitrite accumulation following exposure to different concentrations of Vaporooter differed substantially from the other two root control chemicals (Figure 1B). It was an important indicator of both ammonia and nitrite oxidations for this particular root control chemical, because nitrate concentrations remained low or increased slowly until or after nitrite concentrations peaked (Figure 2B). As determined from the delay in increase in nitrate concentration, the NOEL of ammonia oxidation for Vaporooter was 12.5 mg/L. Following exposure to medium to high concentrations of Vaporooter (25, 50, 100, 250, 500 mg/L, equivalent to 0.3, 0.6, 1.2, 3 & 6 mg/L of metam sodium), nitrite concentrations peaked at sequentially later points in time and at higher levels while nitrate concentrations also increased subsequently. This demonstrated that that the recovery time for ammonia oxidation was responding to Vaporooter concentrations (i.e. the higher the concentration, the later the appearance of nitrite peak and the increase of nitrate levels, indicating longer recovery time for ammonia oxidation). At lower Vaporooter concentrations (25, 50 & 100 mg/L), ammonia oxidation recovered within 3 days. However, 2 weeks were required for full recovery of ammonia oxidation in samples exposed to higher Vaporooter concentrations (250 & 500 mg/L).

Higher nitrite peak levels indicated a higher nitrite oxidation inhibition levels in samples exposed to higher Vaporooter concentrations. While nitrite peak levels were below 10 mg N/L for most samples, it reached as high as 32 mg N/L after incubation with 500 mg/L of Vaporooter (6 mg/L of metam sodium) for 1 week. It took another week for the full recovery of nitrite oxidation in this assay, as shown by the rapid increase in nitrate concentration (Figure 2B). Over the 4-week experimental period, nitrite and nitrate concentrations were below the detection limits for the batch that received the 1 g/L of Vaporooter (12 mg/L of metam sodium). Therefore, both ammonia and nitrite oxidations did not recover. Whether this level represented the toxic level for AOB and NOB in the system will require further study. Since dichlobenil concentrations remained low in all assays, this indicates that metam sodium in Vaporooter played the major role in inhibiting ammonia and nitrite oxidations.

Razorooter – 37.3% diquat dibromide (concentration range for the test 4.7-373 mg/L diquat dibromide/L)

The concentration range of active ingredient in Razorooter tests was higher compared to the other two root control products because Razorooter contains a significantly higher percentage of the active ingredient - diquat dibromide. Even samples applied with lowest concentrations of Razorooter (12.5 and 25 mg/L) showed delayed increase in nitrate concentrations (Figure 2C) without any nitrite accumulation (Figure 1C). This suggests the inhibition of ammonia oxidation only. Thus the NOEL for Razorooter was estimated to be below 12.5 mg/L. Ammonia oxidation in these two batches started to recover after 24 hours, but the rate was slower compared with the controls (see slope on Figure 2C). The full recovery of ammonia oxidation did not occur until after one week of exposure to 12.5 mg/L or two weeks of exposure to 25 mg/L of Razorooter

At a higher Razorooter concentration (50 mg/L), nitrite started to accumulate after 2 weeks (Figure 1C), but did not reach the peak level even after four weeks of exposure. Ammonia oxidation in this sample had not achieved full recovery by the end of the experiment. On the other hand, nitrate concentration in this assay remained at low levels throughout the 4-week period, indicating the continuous inhibition of nitrite oxidation. The NOEL of nitrite oxidation for this root control chemical was therefore 25 mg/L. For all other samples exposed to higher Razorooter concentrations (100, 250, 500 mg/L and 1 g/L), both nitrite and nitrate concentrations remained at minimal levels after 4 weeks of exposure. Recovery of ammonia and nitrite oxidation had not been observed. Longer exposure period would be required for understanding the recovery pattern from exposure to Razorooter.

B. AOB Community Structures

Betaproteobacterial *amoA* genes were successfully amplified from some of the DNA extracts at the end of the exposure period. Figure 3 presents a typical T-RFLP electropherograms for the AOB lineages. With dual labeling method, the electropherograms contain peaks corresponding to the forward terminal restriction fragments (T-RFs) and peaks corresponding to the reverse T-RFs. This enabled higher resolution for a better analysis of AOB communities. For example, based on an in silico T-RFLP analysis and a previous study (Park & Noguera, 2004), the 283-bp forward T-RF was associated with the *Nitrosospira* lineage, while the 270-bp reverse T-RF was mostly observed in the *Nitrosomonas europaea* lineage. By combining the forward and reverse T-RFs, we would be able to estimate the dominant, and thus more resistant AOB group under inhibitory conditions to various root control active ingredients.

The *amoA* T-RFLP histograms in Figure 4 demonstrated variations in the relative abundance of different AOB groups under exposure to various concentrations and types of root control chemicals. The *Nitrosomonas europaea*-like clusters, represented by 219/270-bp T-RF pairs, became the dominant AOB group under exposure to of 1 g/L of RootX (5.5 mg/L dichlobenil). However, upon exposure to 1 g/L of Vaporooter (12 mg/L of metam sodium), both *Nitrosopsira and Nitrosomonas europaea*-like lineages, as indicated by the signature 283/206-bp and 219/270-bp T-RF pairs respectively, became more dominant. The 219/206-bp T-RF pairs, which represent the dominant *Nitrosomonas*-like AOB cluster in activated sludge of aeration basin in PARWQCP for May to June, 2005 and September 2005 to January 2006 during previous year-long study and in the control batches for this study, remained the major AOB group in batches exposed to 25 mg/L of Razorooter (9.3 mg/L of diquat dibromide).

The above results indicate that different AOB lineages differ in their susceptibility to the nitrification inhibitors that are present in root control chemicals. A more quantitative study is needed to elucidate the combined effects of concentration and exposure times on AOB and NOB in the activated sludge.

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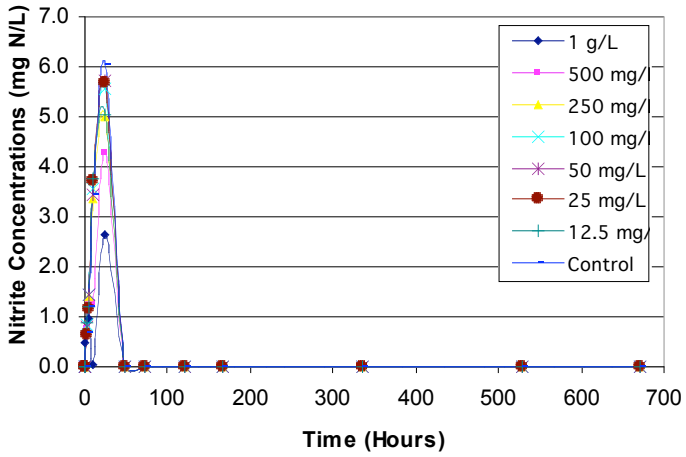

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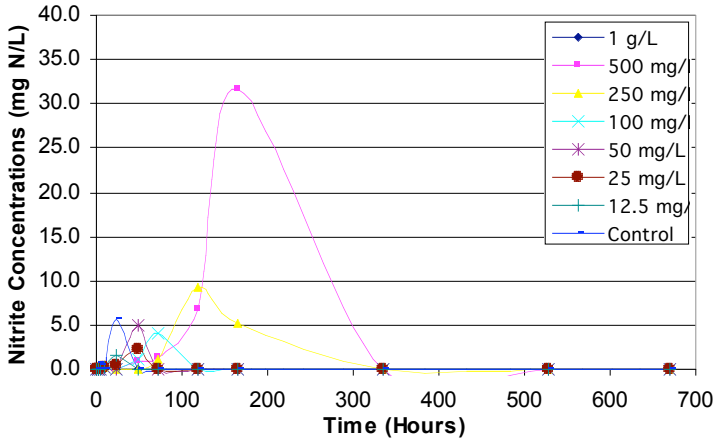

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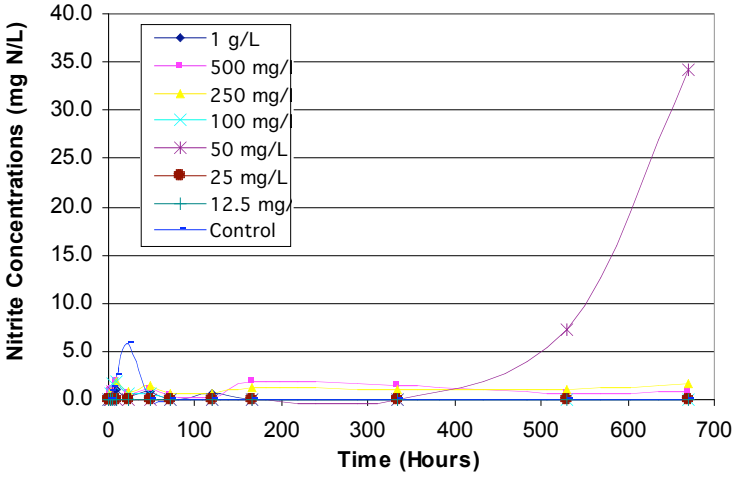
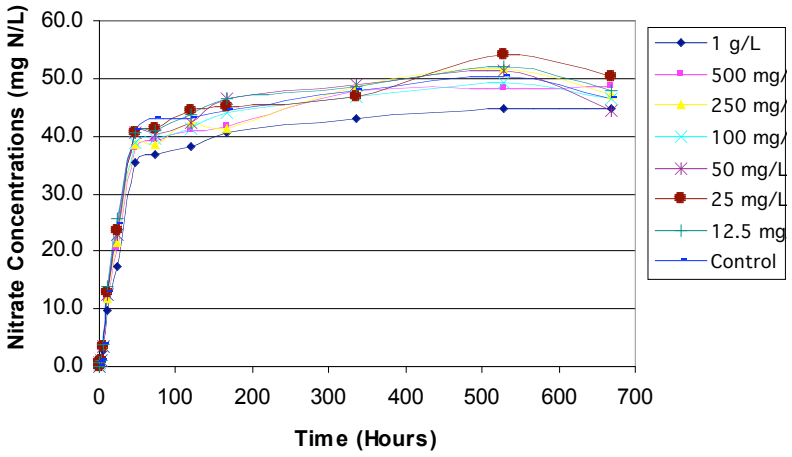


Figure 1. Nitrite concentrations versus exposure time for assays with different root control product concentrations as indicated in the legend. The active ingredients are listed with the root control product **(A)** RootX – dichlobenil; **(B)** Vaporooter- major: metam sodium; minor dichlobenil; **(C)** RazoRooter – diquant bromide.

A.



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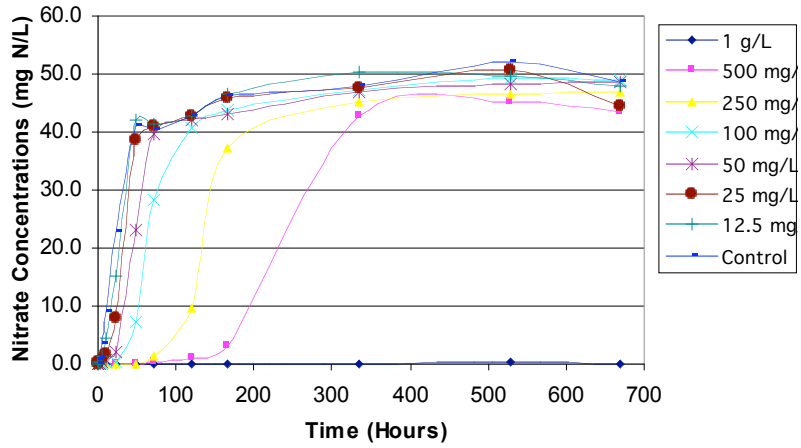

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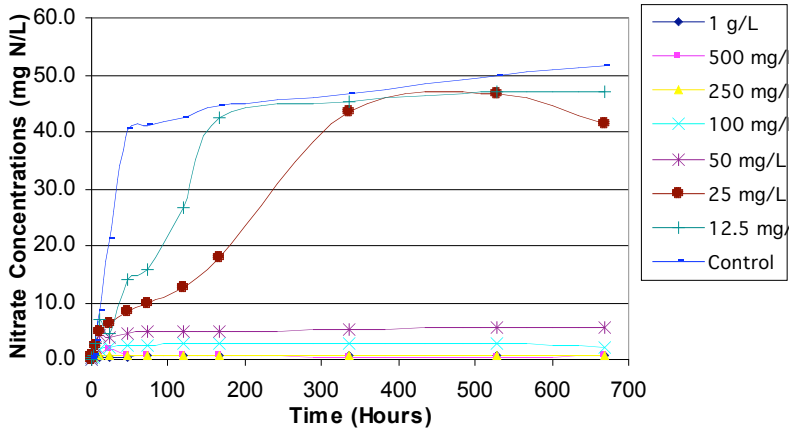


Figure 2. Nitrate concentration versus exposure time for assays with different root control product concentrations as indicated in the legend. The active ingredients are listed with the root control products **(A)** RootX – dichlobenil; **(B)** VapoRooter- major: metam sodium; minor dichlobenil; **(C)** RazoRooter – diquant bromide.

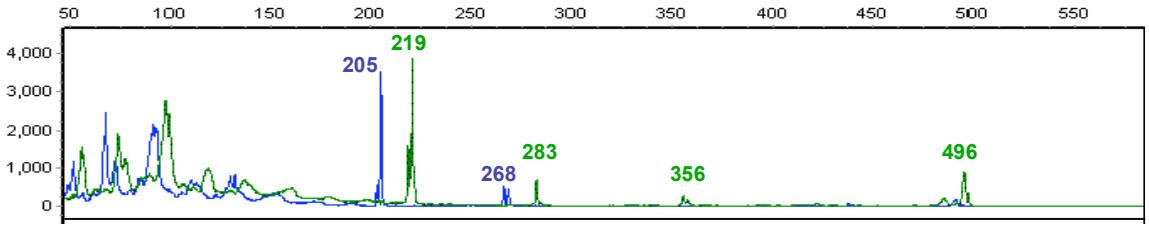
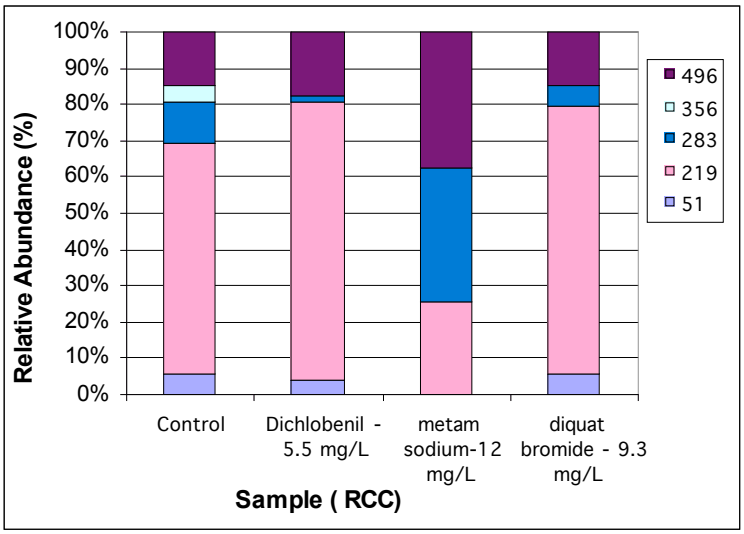


Figure 3. Typical *amoA* T-RFLP electropherogram from the batch assays. The forward T-RFs are represented with the green panel and the reverse T-RFs are represented with the blue panel. The lengths of T-RFs are indicated on peaks determined by in-silico and experimental T-RFLP analysis.

A.



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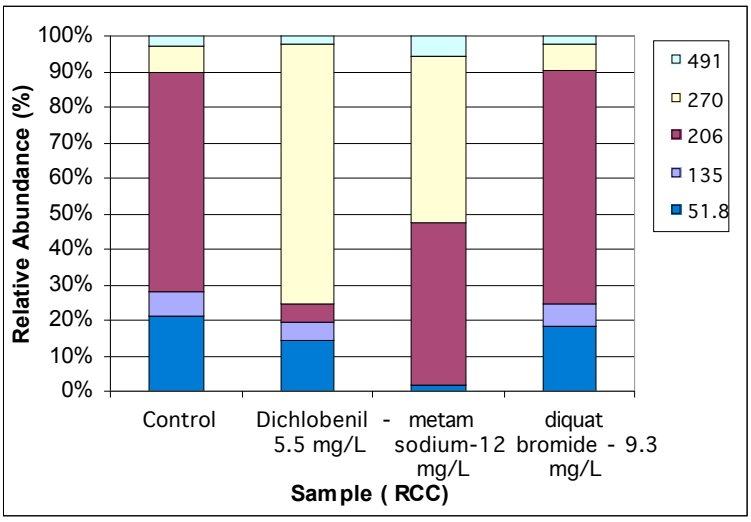

Figure 4. Histogram representations of *amoA* T-RFLP electropherograms with *TaqI* from the batch assays (fragment sizes are indicated in the legends). (**A)** Forward T-RFs; (**B)** Reverse T-RFs.

REFERENCES

1. **Ake, T. N.** 1995. The Evaluation of Metam Sodium and Dichlobenil Impacts on Activated Sludge Nitrification. Thesis for Masters of Science in Environmental Engineering, Virginia Polytechnic Institute and State University.
2. **Arvin, E., S. Dyreborg, C. Menck, and J. Olsen**. 1994. Mini-nitrification test for toxicity screening, MINNTOX. *Water Research* **28**:2029.
3. **Park, H. D., and D. R. Noguera.** 2004. Evaluating the effect of dissolved oxygen on ammonia-oxidizing bacterial communities in activated sludge. *Water Research* **38**:3275.