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GC Analysis of Chlorinated Aromatics as Metabolized by *Nitrosomonas europaea*

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Research and education focused on life below Earth's surface

Introduction

Nitrification by chemoautolithotrophic ammonia-oxidizing bacteria, such as Nitrosomonas europaea, is an important biological process in subsurface ecosystems and is a key step in the global nitrogen cycle. Nitrification, which is the production of oxidized nitrogen from cellular nitrogen and ammonia, is a biologically mediated process which is originated by the oxidation of NH_3 to NH_2OH (hydroxylamine) by the enzyme ammonia monoxygenase (AMO):

$$NH_3 + O_2 + 2e^- + 2H^+ \xrightarrow{AMO} NH_2OH + H_2O$$

Hydroxylamine is then further oxidized to nitrite by hydroxylamine oxidoreductase (HAO) by the following reaction:

$$NH_2OH + H_2O \xrightarrow{HAO} HNO_2 + 4e^- + 4H^+$$

The enzyme responsible for the initial step of nitrification, AMO, is a non-specific enzyme which oxidizes a variety of substrates. The non-specificity of AMO allows for chemical inhibition of the nitrification process by chemicals such as chlorobenzene.

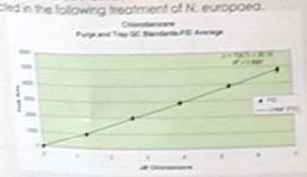
Analytical methods such as gas chromatography (GC) are necessary tools in the detection and quantification of nitrification inhibitors. Refining and developing these methods can help increase understanding of the process of cometabolism in batch cultures of N. europaea.

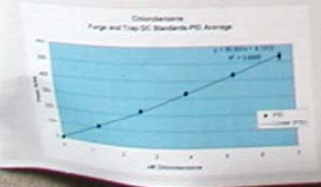
Chlorobenzene is one such compound that can inhibit nitrification in N. europaea while it is cometabolically oxidized to chlorophenol somers. Presented here are results of experiments directed at chlorobenzene inhibition of nitrification by N. europaea and analytical techniques developed to quantify chlorobenzene transformation and metabolite production.

Standard Curves

Standard curves were developed using gas chromatography (GC) for a selection of chlorinated aromatics, including chlorobenzene, 1,2-dichlorobenzene and 1,4-dichlorobenzene. Due to the low detection limits, 5mL of each standard solution was injected into a Purge and Trap prior to GC analysis. This enabled a larger sample to be collected which allowed for more accurate standard curves. Both the Photo Ionization Detector (PID) and Flame Ionization Detector (FID) took data simultaneously during each run. All standard curves were run in triplicates, from which the average linear equations and R^2 values were calculated. Small error bars denoting the 95% confidence interval confirmed the accuracy of the standard curves.

The graphs below show the average standard curves for the PID and FID of chlorobenzene, which were used to analyze data collected in the following treatment of N. europaea.





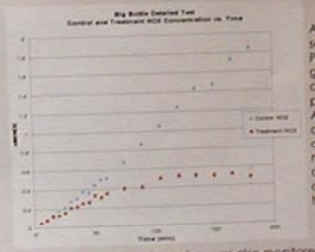
Detailed *Nitrosomonas europaea* Treatment

Experiments were performed in 2000 mL Kimax media bottles fitted with an airtight sampling port. Each bottle contained 1 L of mineral media containing 5 mM total ammonia and were inoculated to an optical density (λ = 600 nm) of approximately 0.065 with *N. europaea*. One bottle contained one of the studied concentrations of chlorobenzene, and the other bottle had no chlorobenzene added.


GC Purge and Trap

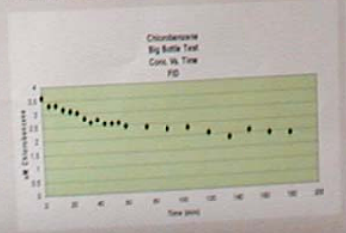
The specific goal of the treatment was to obtain data points at smaller time intervals than had been collected in previous treatments. Over the three hour treatment, 20 data points were taken for both nitrite and chlorobenzene analysis. This allowed for a detailed picture of how the above concentrations were changing over the entire span of the treatment. Samples were taken and frozen down for analysis of chlorophenol concentrations at a later date. RNA and DNA samples were also gathered.

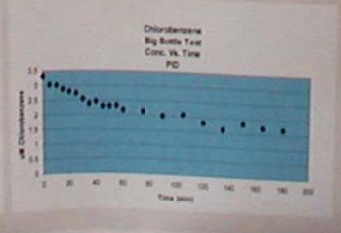
Experimental Results



Ammonia oxidation by *N. europaea* was found to be highly sensitive to the presence of small amounts of chlorobenzene. Previous treatments had shown that increasing inhibition with greater exposure time to the higher chlorobenzene concentrations implied a toxic effect on *N. europaea* from the presence of chlorobenzene. Chlorobenzene was oxidized by AMO during the tests and it was uncertain whether it was chlorobenzene or its transformation products that caused the apparent toxic effect. From the present treatment the exact nitrite inhibition is modeled with detailed time points. At the left is a plot of nitrite production versus time for the cells exposed to chlorobenzene compared to the cells left untreated. In the treatment, nitrite production ceased at about 100 minutes.

Chlorobenzene transformation was also monitored using GC purge and trap techniques. Previous treatments had indicated that chlorobenzene concentration dropped off in the first hour, but insufficient data had been taken to model that behavior accurately. Taking data points every five minutes for the first hour allowed detailed graphs and a better understanding of the initial behavior of the system. Concentrations were calculated using the standard curves produced earlier, producing slightly different concentration profiles. Future detailed treatments will be performed to confirm the data curves shown below.

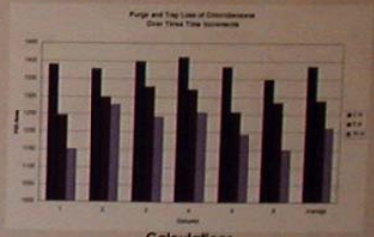




GC Purge and Trap Loss Over Time

While the Purge and Trap is an excellent device to help detect the concentrations of chlorobenzene in our samples, it is not a speedy process. The Purge and Trap consists of 16 columns, which take 30 minutes each to purge the sample through the GC. In our experimental set-up, 20 samples were acquired over the three hour treatment to be analyzed through the Purge and Trap. Accordingly, some of the samples were sitting in the Purge and Trap columns for over 8 hours before they were analyzed.

If loss of chlorobenzene were occuring in the Purge and Trap columns over time, it would be important to know the percentages lost so these factors could be taken into consideration for future analyses. A time test was performed on the most commonly used columns (1- 6). A standard solution of ~2µM chlorobenzene was loaded and analyzed with the Purge and Trap over three different time periods. The first was loaded and run immediately. The second was loaded, then analyzed after an 8 hour delay, and the third was loaded, then analyzed after a 16 hour delay. From the average of all six columns, the percent chlorobenzene lost during the 8 hour wait and the 16 hour wait was found. For this study the FID detector was used alone. The bar graph below also demonstrates how different columns produce slightly different measurements of the same standard solution, and how the discrepancies become greater over longer time periods.



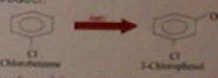
Calculations

Standard Deviation at 0 hrs = 21.3
Standard Deviation at 8 hrs = 33.8
Standard Deviation at 16 hours = 55.5

Average percentage lost over 8 hrs : 6.9 %
Average Percentage lost over 16 hrs : 12.8 %

Future Analytical Developments

Understanding the oxidation of chlorobenzene in the treatment of N. europaea is the next step in the analytical process. Chlorobenzene is an inhibitor that can be oxidized by the bacteria into what is believed to be a chlorophenol by-product.



Unfortunately, detection of the chlorophenol can not be performed similar to chlorobenzene using the GC/Purge and Trap due to its non-volatile properties. The chlorophenol must de derivatized and then extracted out of water prior to analysis on the GC. Current methods involve using acetic anhydride as a derivatization reagent, followed by extraction into hexane. After ample mixing, the hexane layer can be removed and analyzed on GC using an Electron Capture Detector (ECD) for quantification.

The next step is to refine the method on the GC to produce sufficient peak separation and sensitivity with the ECD. Ideally, an auto sampler will be used to inject a volume of 1µL onto the column to ensure repeated accuracy. With future successes, we hope to identify the concentrations of the chlorophenol isomers as they are formed over the treatment period and determine their toxicity to *N. europaea*.