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EFFECTS OF EXOGENOUS ORGANIC SUBSTRATES ON NICKEL-DEPENDENT ANAEROBIC CARBON MONOXIDE OXIDATION BY WETLAND SEDIMENTS

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Contents

ABSTRACT

Anaerobic carbon monoxide (CO) oxidation has been known since the 1920's. In the 1970s its mechanisms and enzymology were studied to determine its microbiology, genetics, and biochemistry. Although CO is found throughout the environment, there is a lack of research on the role of CO oxidation in microbial communities under anaerobic conditions. Through the use of sediment from the Bluebonnet swamp in Baton Rouge, the effect of simple organic substrates including glucose, pyruvate, acetate, casamino acids, and pyruvate on CO oxidation in these microbial communities was tested. These substrates are often used as sources for carbon, fermentation, and energy for growth. Each substrate had five replicates and was tested at both ambient temperatures and 60°C. A gas chromatograph was used to measure CO concentrations in the samples to which 20% CO had been added (final concentration). After analyzing the lag times and CO uptake rates of the samples, it was determined that at ambient temperatures, the addition of glucose increased the lag time before CO oxidation. The addition of an amino acid mixture, casamino acids, decreased the lag time before CO oxidation at ambient temperatures; while the other substrates had no effect. At 60°C, the addition of acetate decreased the lag time before CO oxidation took place, while other substrates had no effect. Casamino acids at ambient temperatures also increased the rate of CO uptake. At 60°C, the addition of glucose and syringate inhibited CO uptake rate. These findings support the hypothesis that the rate of Ni-dependent CO oxidation is dependent upon on specific substrates available to the microbes found within the sediment. They also suggest that while substrates such as glucose and pyruvate are more commonly used by these microbial communities, less commonly used substrates such as amino acids might increase CO uptake rates.

INTRODUCTION

Anaerobic CO oxidation (an-coox) by soil has been known since 1926, and by sewage sludge and a methanogenic culture since the 1930s (Schlegel, 1974). Beginning in the 1970s, research focused primarily on the microbiology, biochemistry, and genetics of an-coox. Numerous studies have established the enzymology and mechanisms involved (Schlegel, 1974). When a nickel-dependent CO dehydrogenase (Ni-dependent CODH) proved essential for an-coox, it was distinguished from a respiratory oxidation based on a molybdenum-dependent CO dehydrogenase (Mo-dependent CODH). Other studies documented the diversity and activity of a relatively small group of bacteria capable of growing with CO as a carbon and energy source (Kochetkova, 2019). These thermophilic bacteria used CO and water as an energy source and converted them to hydrogen and CO2. Many of these isolates grew preferentially as thermophiles (optimum growth > 60 ^{o}C) with heterotrophic substrates, e.g., simple organic acids, sugars, and amino acids, but could switch their metabolism if CO was abundant.

Although CO is ubiquitous in the environment, very little is known about its use by natural microbial communities under anaerobic conditions. The lack of oxygen occurs commonly in freshwater and marine sediments, hot springs and even some soils (Brazelton, Nelson, and Schrenk, 2012). These systems are known to harbor anaerobic bacteria that can potentially use CO **(**Momper et al., 2017). Nonetheless, variables that affect communities of Ni-dependent CO oxidizers and their potential activities are largely unexplored. Research on the impacts of temperature, pH, organic matter, and other variables has only recently been initiated.

The existing Ni-dependent CO oxidizers typically use simple organic substrates in a various fermentation or sometimes respiration reactions. Substrates such as glucose, pyruvate, amino acids, simple aromatic acids, and acetate occur naturally, and serve as sources of carbon

and energy for growth by a wide range of anaerobic bacteria. Glucose and pyruvate are used by the largest number of anaerobes, while amino acids and aromatic acids are less commonly used. The use of acetate in the absence of oxygen is restricted to very few anaerobes; methane-producing bacteria are among the most important acetate degraders in freshwater sediments and anaerobic sludges (Ginkel, 2005).

Since populations and activities of bacteria in natural systems are typically limited by substrate availability, it is possible that the potential for Ni-dependent CO oxidation depends on substrates in addition to CO. To test this hypothesis, sediments from a freshwater swamp were incubated anaerobically under various conditions that included temperatures of 25^{o}C or 60^{o}C, with additions of selected organic substrates (glucose, pyruvate, an amino acid mixture, syringate, and acetate) or deionized water. Lag times and rates for oxidation of CO at 20% headspace concentrations were compared to values of unamended control sediments also incubated at 25^{o}C or 60^{o}C. The results supported the hypothesis that the addition of some substrates to sediment does have an effect on Ni-dependent CO oxidation.

Data showed the addition of glucose at ambient temperatures significantly increased the lag time before CO uptake, while the addition of casamino acids at ambient temperatures significantly decreased the lag time. When acetate was added to samples stored at 60^{o}C, the lag time was significantly decreased. The addition of casamino acids at ambient temperatures resulted in a significantly higher rate of CO uptake while the addition of glucose and syringate at 60^{o}C resulted in significantly decreased CO uptake rates. These results support the idea that, although glucose is more commonly used by these CO oxidizing bacteria, amino acids such as a mix of casamino acids as a source of carbon could allow for faster growth of the anaerobic bacteria. The increase in CO uptake caused by the addition of casamino acids supports the

hypothesis that the ability to use CO under anaerobic conditions is limited by the availability of anaerobic substrates.

MATERIALS AND METHODS

Study design. To assess the impacts of exogenous organic substrates on CO uptake rates, sediments from a local wetland were incubated with the following general treatments: (1) unamended sediment (referred to as controls) provided a baseline of activity with limited disturbance; (2) sediments with deionized water added (referred to as "DI") provided an indication of responses to the effects of dilution; (3) sediments with organic substrates in a deionized water solution provided an indication of responses to specific substrates (referred to by the substrates used).

Each of these treatments included 5 replicates (15 samples total for 3 treatments) for each of the following exogenous substrates: acetate, casamino acids, glucose, pyruvate, and syringate (a total of 75 samples for all 5 substrates). One set of five substrates with each of the three treatments was incubated at ambient laboratory temperature (25$_o$C), and a second set was incubated at 60$_o$C.

After adding CO to a final concentration of about 20% in anaerobic sample headspaces, two variables were observed. Lag times represented the amount of time that elapsed from the addition of CO to the first indication of a decline in concentration. Uptake rates were estimates of the greatest rate of decline in CO concentrations. The results were used to test hypotheses that substrate additions would decrease lag times and increase uptake rates at both ambient temperature and at 60$_o$C.

Sediment Collection. The sediment used in this study was collected from the Bluebonnet Swamp located in Baton Rouge, Louisiana. Bluebonnet Swamp is comprised of 103 acres that are used for education, tourism, and conservation (BREC, 2020). Sediment samples were collected from the upper 5-10 cm of unconsolidated sediment in a cypress-tupelo bottomland swamp (BREC, 2020). Triplicate samples were collected with a hand trowel then transferred to ziplock storage bags. The sealed bags were transported to a laboratory at Louisiana State University and stored at room temperature.

Figure 1. View of sampling site on Bluebonnet Swamp boardwalk.

Sediment treatments and assays. Sediment in each of the ziploc bags was homogenized by manually mixing the contents of the sealed bags. Five-gram (fresh weight) sub-samples were obtained from the ziplock bags with a 25-ml pipette from which the tip had been removed; sub-samples were transferred to 60-ml serum bottles. Sediment mass was weighed to the nearest 0.01 g using a portable balance. For each substrate assay, 5 bottles were designated as controls, 5 as DI, and 5 were labeled with the added substrates. For the ambient temperature treatments, 0.25 mL of substrate stock solutions (see below) plus 0.75 mL of deionized water were added to the samples. One ml volumes of substrate stock solutions were added to bottles incubated at 60$_{o}$C;

stock solutions for these treatments were 4-fold more dilute than those for the ambient temperature incubation.

All bottles were vortexed for about 5 seconds. An anaerobic headspace was created by flushing 15 bottles for a given set of treatments with deoxygenated nitrogen gas. Headspaces were flushed for about 120 minutes. Afterwards, 18 cc (mL) of 100% CO was added to the bottle headspaces to yield a starting concentration of approximately 20%.

Initial and all subsequent headspace CO concentrations were measured by removing 0.4 cm^3 volumes for analysis with a SRI Instruments Model 8610 gas chromatograph equipped with a thermal conductivity detector. Sample responses were standardized using 20% CO. Samples were obtained at intervals until concentrations were depleted to the instrument detection limit (less than 1000 ppm).

Statistical Analysis. Following the collection of data for all substrates at both temperatures, the data was compiled into Excel and Kaleidagraph software packages for analysis. The rates and lag times were calculated and averaged. Lag times were defined as the point in time at which CO began to decline clearly. Rates were based on the maximum change in CO during the incubation based on a linear regression analysis of the time points selected. Standard deviations for both were calculated as well. Analysis of Variance (ANOVA) were performed using XLSTAT to determine which substrates were significantly different from both the control samples as well as each other.

RESULTS

Glucose:

When glucose was added to sediment samples stored at an ambient temperature, the lag time ranged from 1.61 days to 2.95 days between the five replicates. The average lag time for these samples were 2.682 ± 0.599 days (Table 1). The average CO uptake rate for the glucose samples stored at an ambient temperature was 2.500 ± 0.502 percent d^{-1} (Table 2). For the glucose sediment samples stored at 60°C, the lag time ranged between 0.151 days and 0.574 days. The average lag time for these samples was 0.358 ± 0.201 days (Table 1). The average CO uptake rate for the glucose samples stored at 60°C was 4.538 ± 2.173 percent d^{-1} (Table 2).

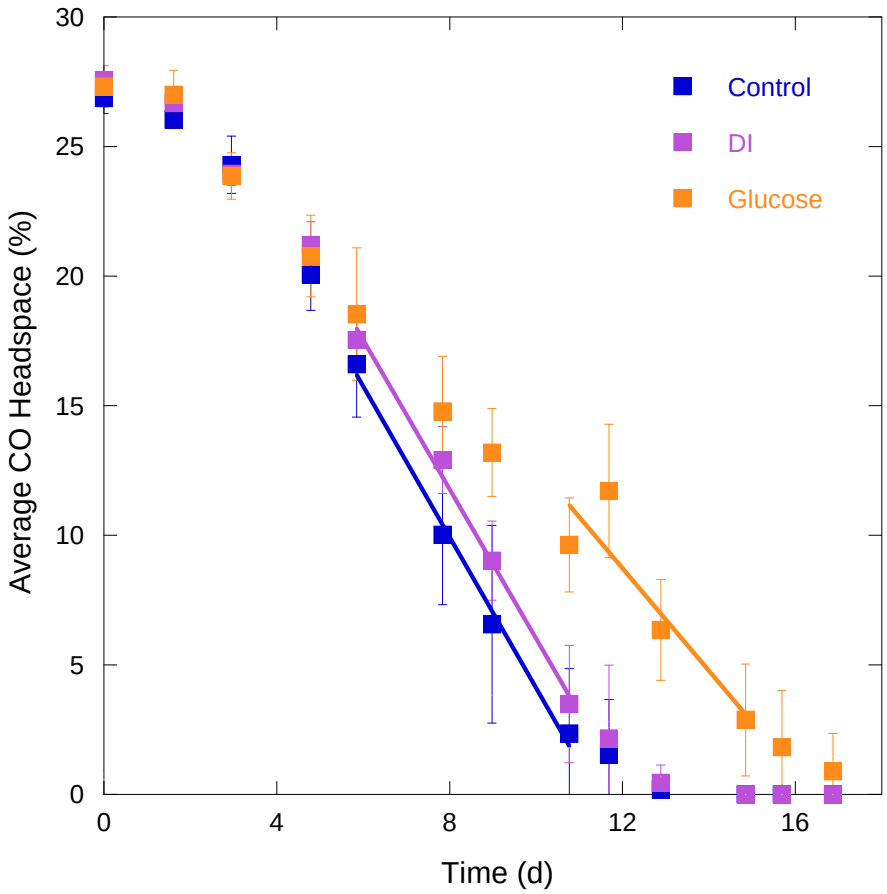

Figure 2. Average CO headspace of glucose samples stored at ambient temperatures over time.

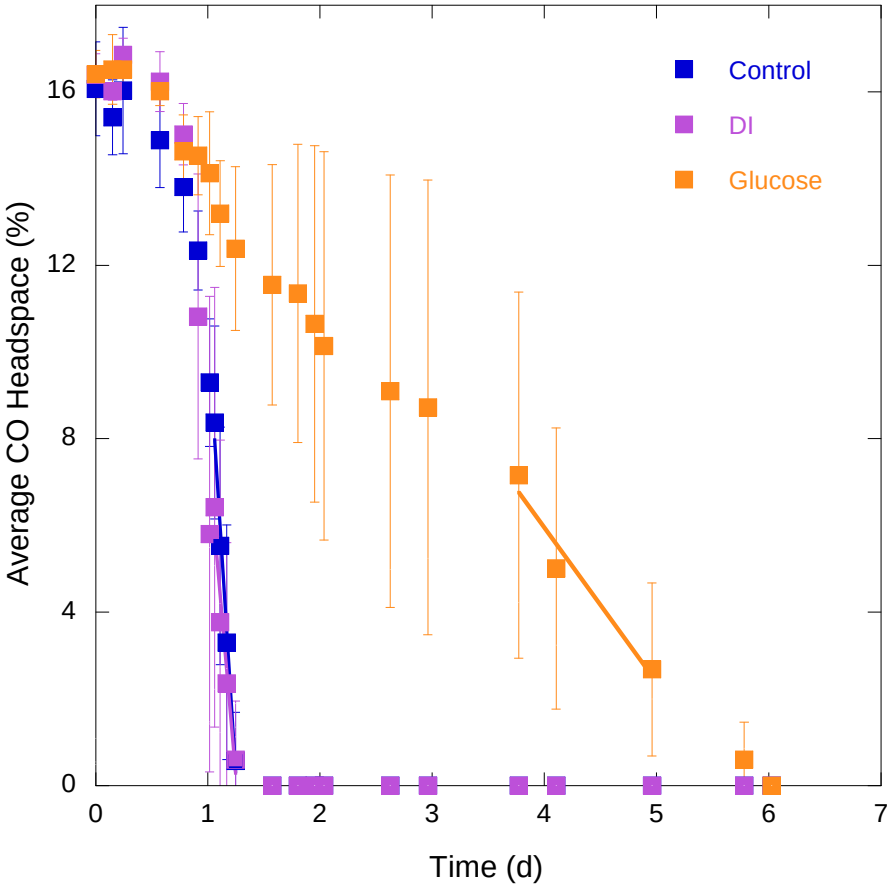

Figure 3. Average CO headspace of glucose samples stored at 60°C over time.

Pyruvate:

When pyruvate was added to sediment samples stored at an ambient temperature, the lag time was 2.13 days with no variation between the replicates (Table 1). The average CO uptake rate for the pyruvate samples stored at an ambient temperature was 2.546 ± 0.459 percent d^{-1} (Table 2). For the pyruvate sediment samples stored at 60°C, the lag time ranged between 0.16 days and 0.59 days. The average lag time for these samples was 0.246 ± 0.192 days (Table 1). The average CO uptake rate for the pyruvate samples stored at 60°C was 48.875 ± 6.274 percent d^{-1} (Table 2).

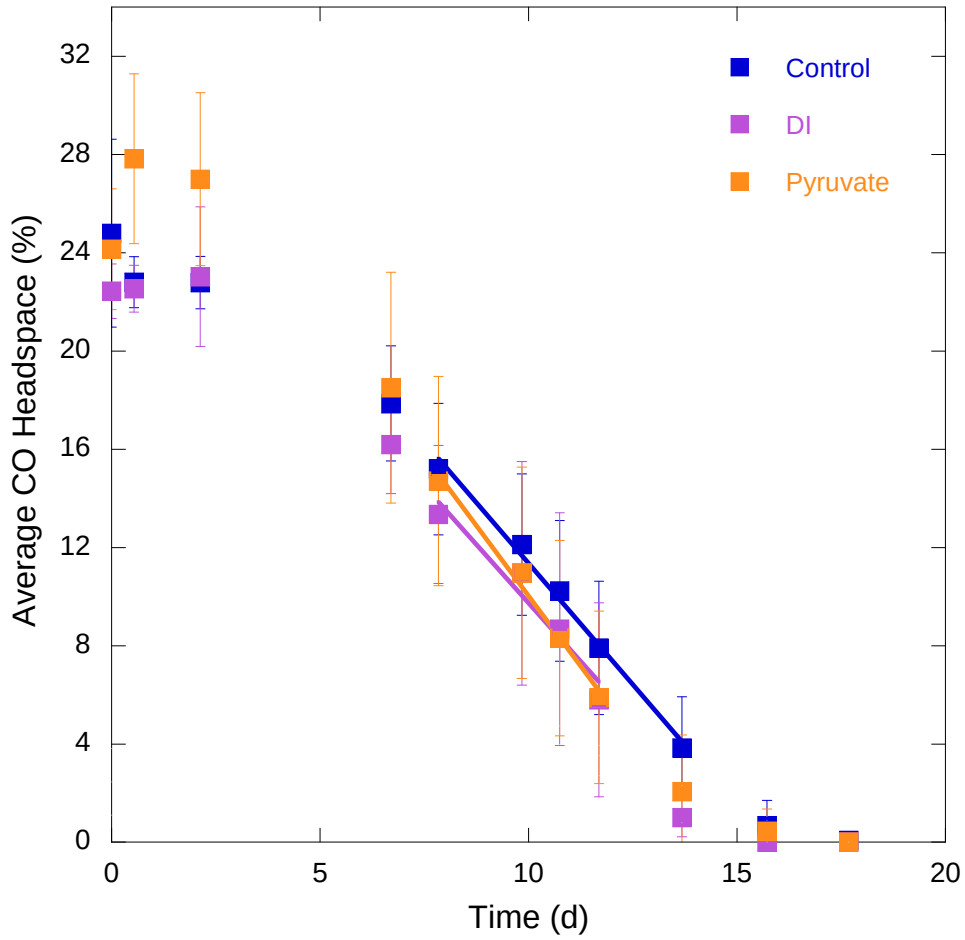

Figure 4. Average CO headspace of pyruvate samples stored at ambient temperatures over time.

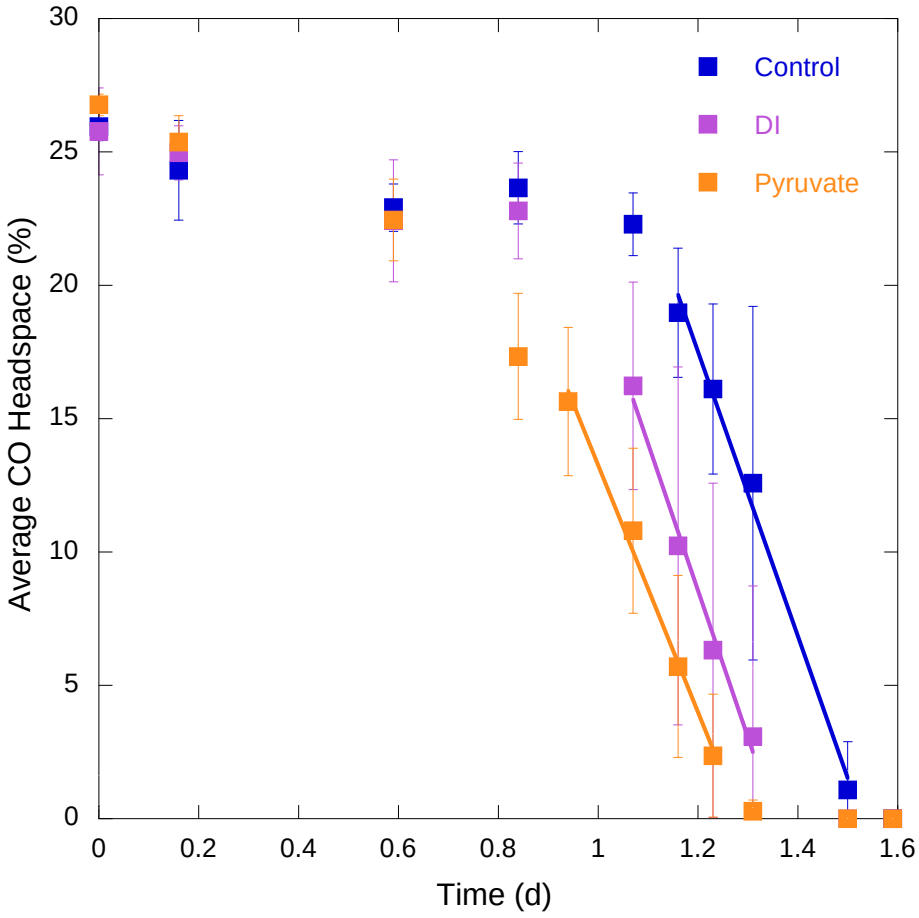

Figure 5. Average CO headspace of pyruvate samples stored at 60°C over time.

Acetate:

When the acetate was added to sediment samples stored at an ambient temperature, the lag time ranged from 0.72 days to 7.7 days between the five replicates. The average lag time for these samples were 3.948 ± 3.454 days (Table 1). The average CO uptake rate for the acetate samples stored at an ambient temperature was 3.210 ± 1.086 percent d^{-1} (Table 2). For the acetate sediment samples stored at 60°C, the lag time was 0.17 days, with no variation between replicates (Table 1). The average CO uptake rate for the acetate samples stored at 60°C was 42.712 ± 14.688 percent d^{-1} (Table 2).

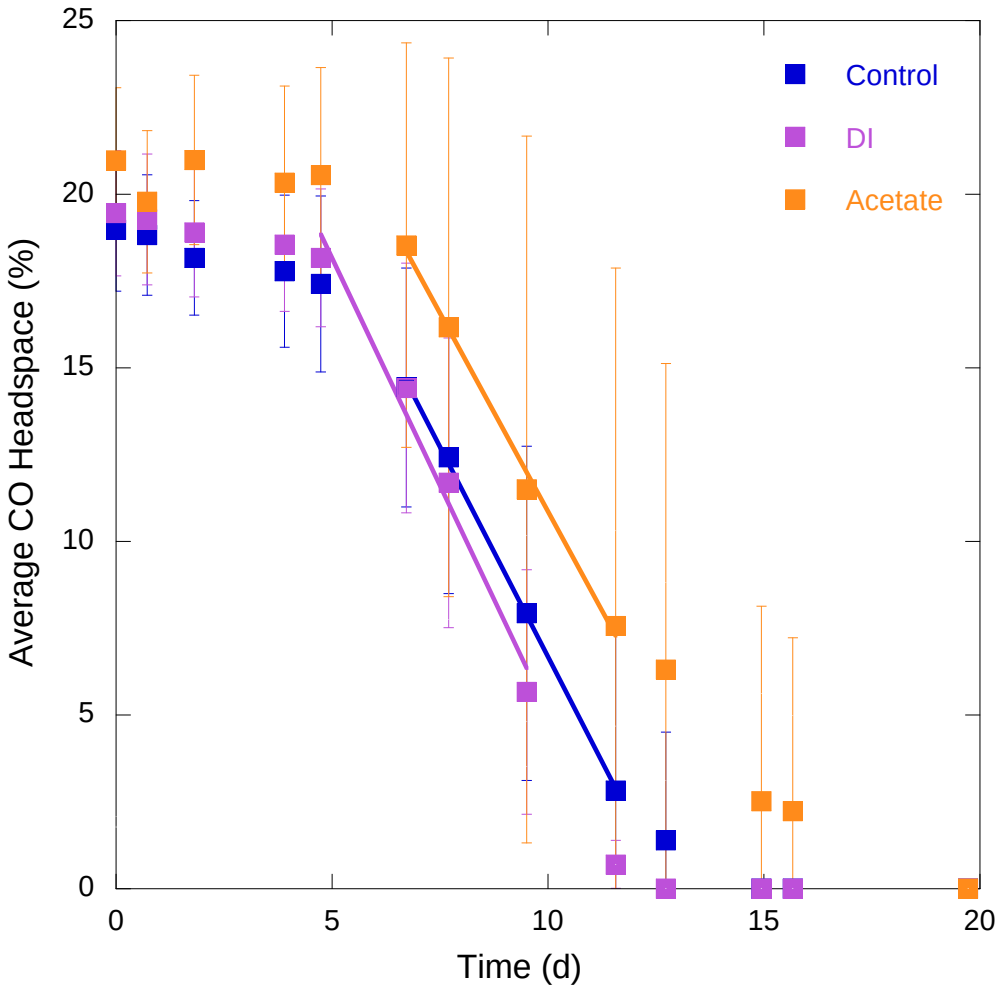

Figure 6. Average CO headspace of acetate samples stored at ambient temperatures over time.

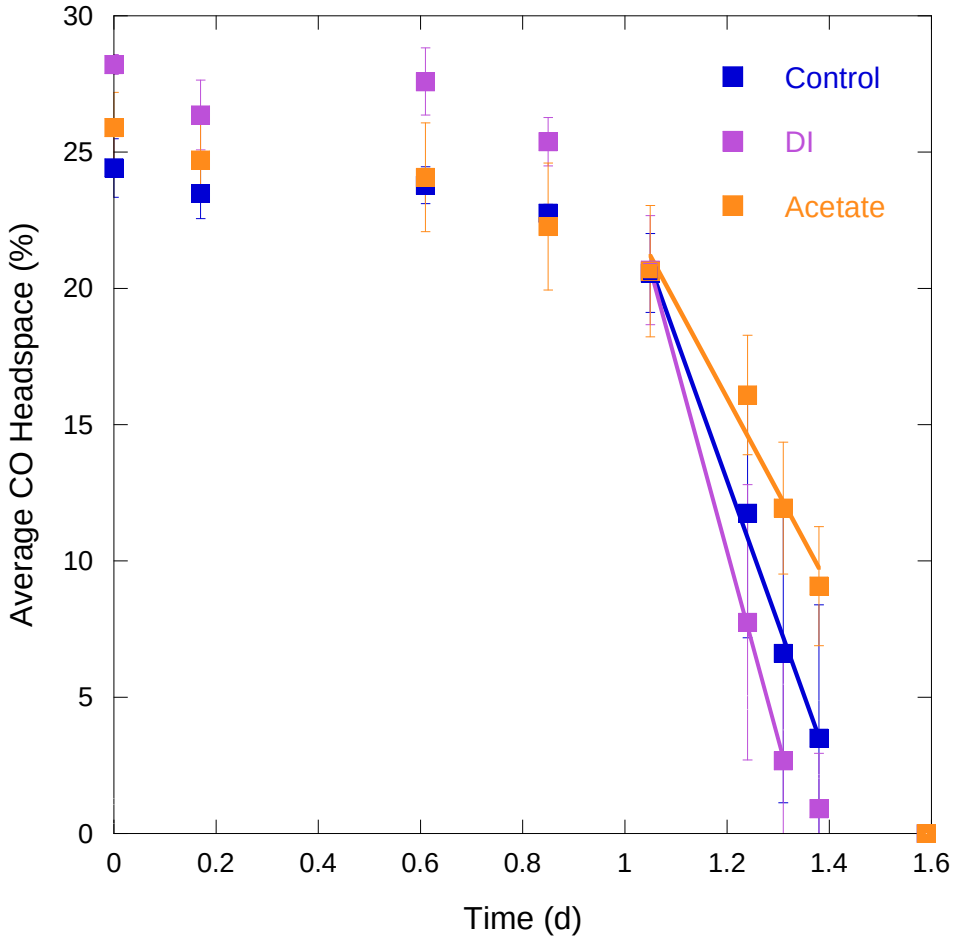

Figure 7. Average CO headspace of acetate samples stored at 60°C over time.

Casamino Acids:

When the casamino acids were added to sediment samples stored at an ambient temperature, the lag time was 0.71 days, with no variation between the replicates (Table 1). The average CO uptake rate for the casamino acids samples stored at an ambient temperature was 4.714 ± 0.415 percent d^{-1} (Table 2). For the casamino acids sediment samples stored at 60°C, the lag time ranged between 0.35 days and 1.04 days. The average lag time for these samples was 0.602 ±

0.258 days (Table 1). The average CO uptake rate for the casamino acids samples stored at 60°C was 32.013 ± 9.926 percent d^{-1} (Table 2).

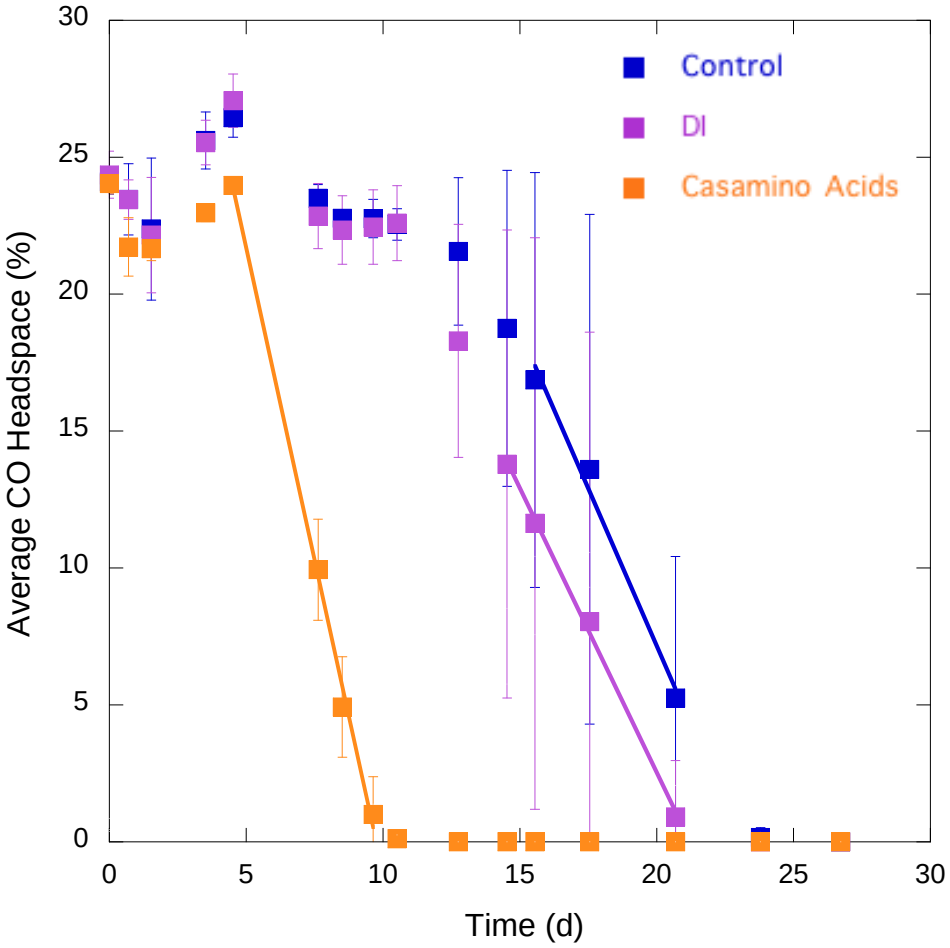

Figure 8. Average CO headspace of casamino acids samples stored at ambient temperatures over time.

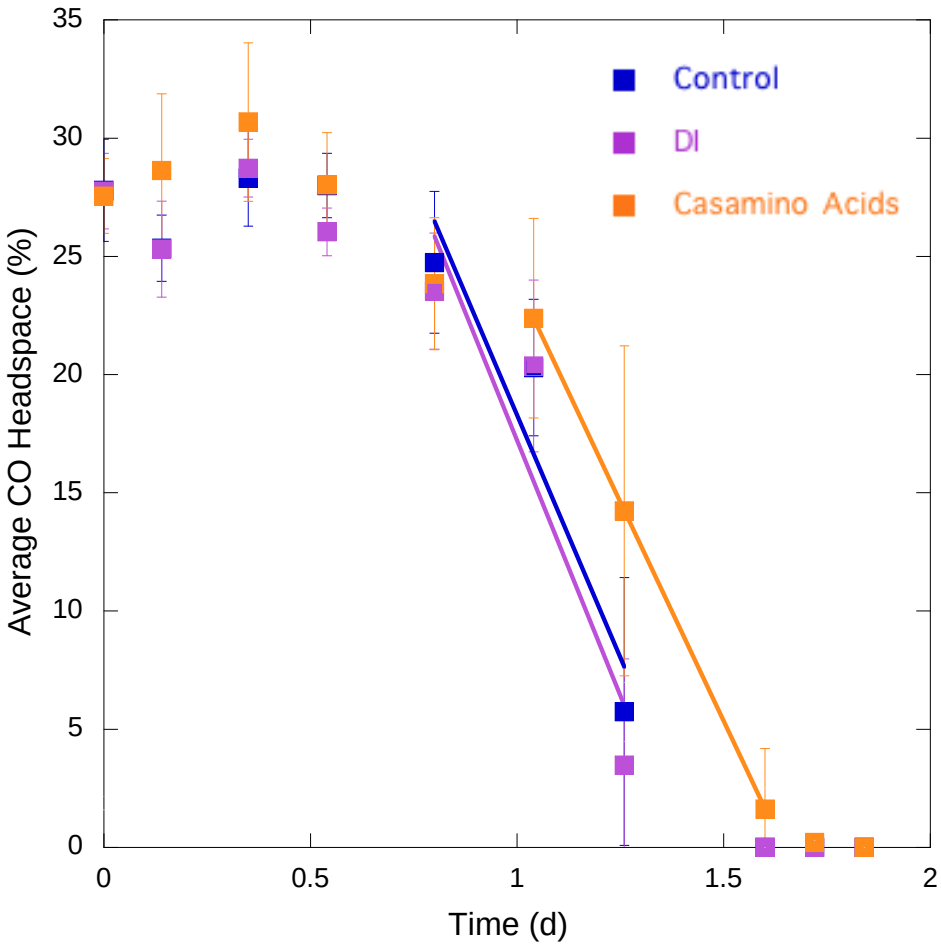

Figure 9. Average CO headspace of casamino acids samples stored at 60°C over time.

Syringate:

When the syringate was added to sediment samples stored at an ambient temperature, the lag time ranged from 0.69 days to 3.45 days between the five replicates. The average lag time for these samples were 1.242 days ± 1.234 (Table 1). The average CO uptake rate for the syringate samples stored at an ambient temperature was 2.995 ± 0.758 percent d^{-1} (Table 2). For the syringate sediment samples stored at 60°C, the lag time ranged between 1.04 days and 1.36 days. The average lag time for these samples was 1.226 ± 0.170 days (Table 1). The average CO uptake rate for the syringate samples stored at 60°C was 34.435 ± 8.531 percent d^{-1} (Table 2).

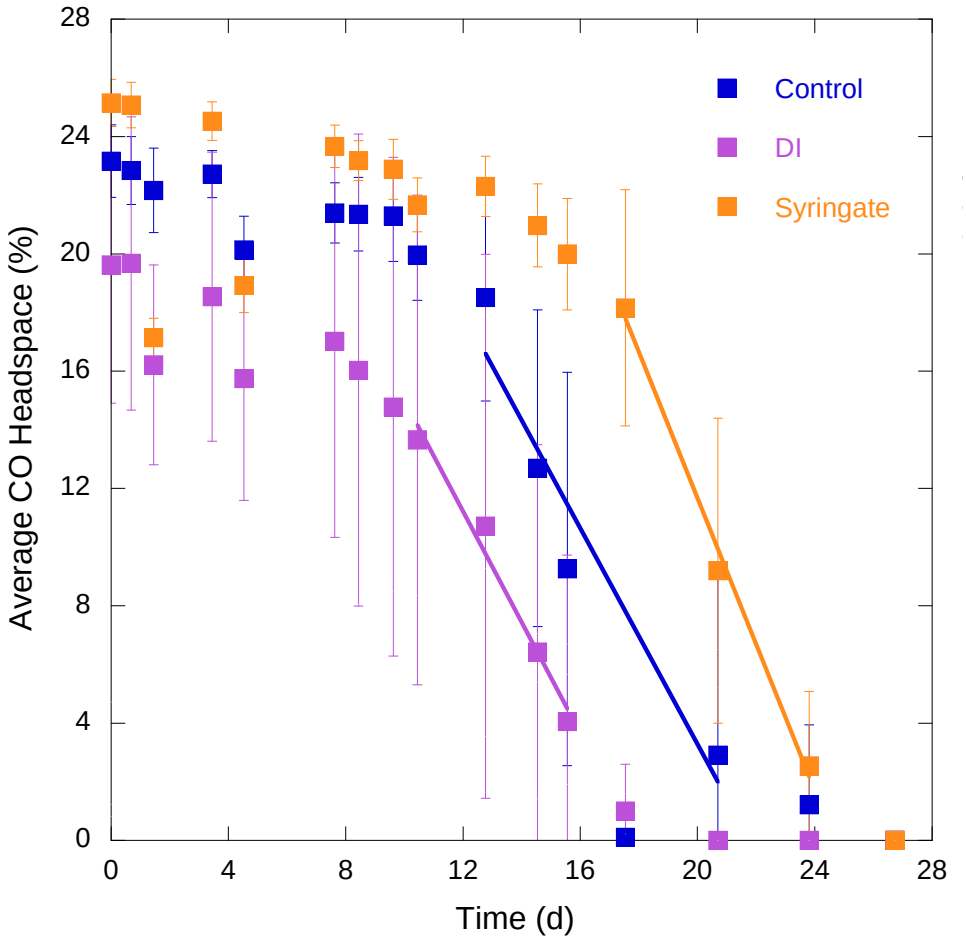

Figure 10. Average CO headspace of syringate samples stored at ambient temperatures over time.

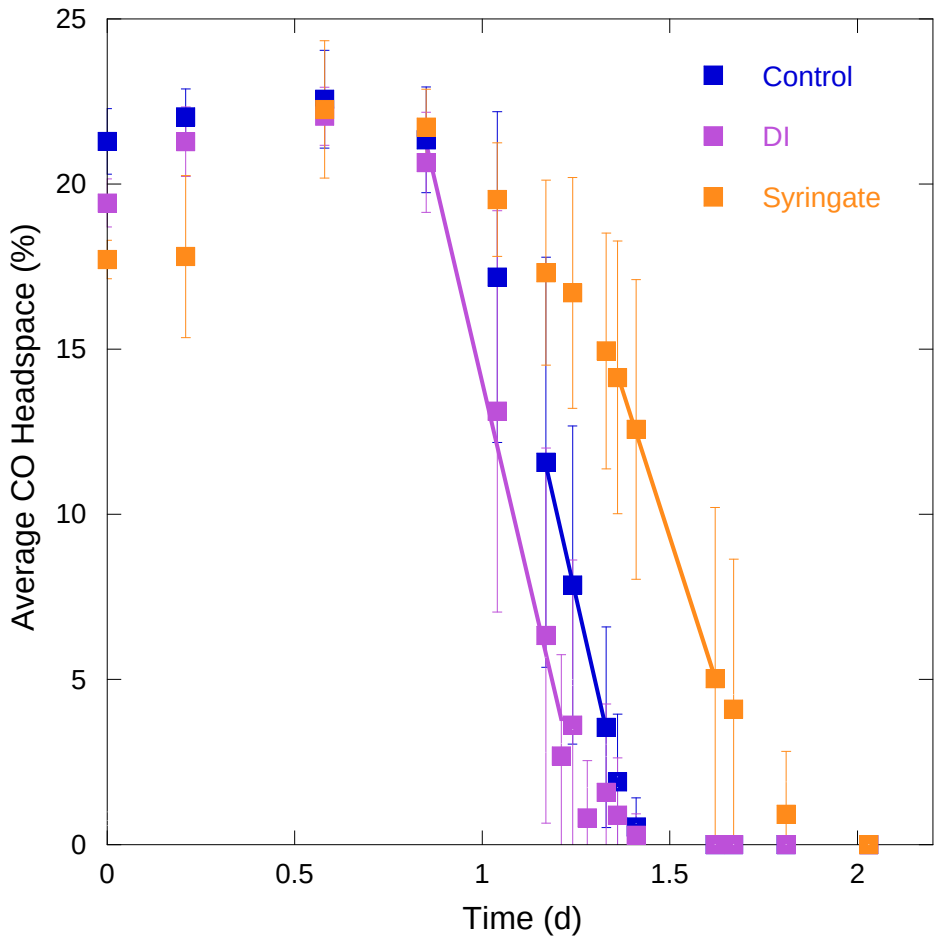

Figure 11. Average CO headspace of syringate samples stored at 60°C over time.

Table 1. Average Lag Times for Each Substrate. Lag times are given in days for 5 replicates.

	Ambient Average Lag Time	Ambient Standard Deviation	60°C Average Lag Time	60°C Standard Deviation
Control Glucose	1.61	0	0.358	0.201
1mL DI Glucose	2.414	0.734	0.550	0.193
Glucose Added	2.682	0.599	0.358	0.201
Control Pyruvate	1.812	0.711	0.246	0.192
1mL DI Pyruvate	2.408	2.528	0.332	0.236
Pyruvate Added	2.13	0	0.246	0.192
Control Acetate	1.960	1.647	0.61	0
1mL DI Acetate	1.692	1.298	0.394	0.318
Acetate Added	3.948	3.454	0.17	0
Control Casamino Acids	6.348	2.691	0.644	0.142
1mL DI Casamino Acids	4.53	0	0.44	0.201
Casamino Acids Added	0.71	0	0.602	0.258
Control Syringate	4.17	3.316	1.002	0.085
1mL DI Syringate	0.842	0.340	0.834	0.164
Syringate Added	1.242	1.234	1.226	0.170

Table 2. Average Rates of CO Uptake for each Substrate. Rates are in percent of CO oxidized per day (mean of 5 replicates).

	Ambient Average Rate	Ambient Standard Deviation	60°C Average Rate	60°C Standard Deviation
Control Glucose	3.167	0.712	46.309	6.998
1mL DI Glucose	2.879	0.304	42.926	14.072
Glucose Added	2.500	0.502	4.538	2.173
Control Pyruvate	2.559	1.128	72.531	36.977
1mL DI Pyruvate	2.135	0.240	55.522	21.761
Pyruvate Added	2.546	0.459	48.875	6.274
Control Acetate	2.756	0.784	61.286	7.384
1mL DI Acetate	3.224	0.659	65.521	10.346
Acetate Added	3.210	1.086	42.712	14.688
Control Casamino Acids	3.299	0.236	34.071	3.440
1mL DI Casamino Acids	3.567	0.912	53.423	26.227
Casamino Acids Added	4.714	0.415	32.013	9.926
Control Syringate	3.090	0.955	50.913	5.667
1mL DI Syringate	4.204	0.316	48.892	6.365
Syringate Added	2.995	0.758	34.435	8.531

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	29	88139.74	3039.30	26.38	**< 0.0001**
Error	118	13594.22	115.21		
Corrected Total	147	101733.96			

Table 3. ANOVA for analysis of variance of the CO uptake rate among the various samples. A p value of <0.0001 indicates a significance among between the samples.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Temperature	1	69605.62	69605.62	604.19	**< 0.0001**
Treatment	28	18534.12	661.93	5.75	**< 0.0001**
Temperature*Treatment	0	0.00			

Table 4. Type I Sum of Squares analysis to determine the significance of temperatures (ambient versus 60$_o$C) and treatments (the various substrates) on uptake rates. A p value of <0.0001 signifies there is there were both significant temperature and treatment effects.

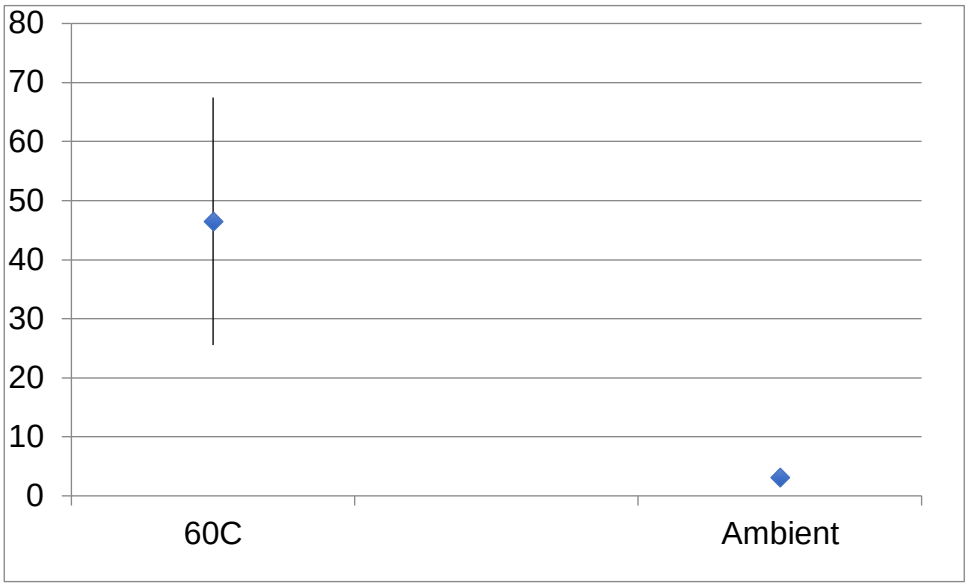

Figure 12. Plot of CO uptake rate means and standard deviation across all substrates as a function of incubation temperature, 60ₒC or ambient (25ₒC). Note that rates (percent per day) are substantially higher at 60ₒC. The CO uptake rates between the two temperatures are significantly different from one another across all substrates.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Model	15	322.13	21.48	15.38	**< 0.0001**
Error	132	184.33	1.40		
Corrected Total	147	506.46			

Table 5. ANOVA for analysis of variance of the CO uptake lag between control, DI, and substrate samples. A p value of <0.0001 indicates a significant difference among the samples.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Temperature	1	155	155	111	< **0.0001**
Treatment	0	0			
Temperature*Treatment	14	167	12	9	< **0.0001**

Table 6. Type I Sum of Squares analysis. A p value of <0.0001 signifies there is a significant difference between the lag times at the two different temperatures, but there was not a significant effect of treatment on lag time.

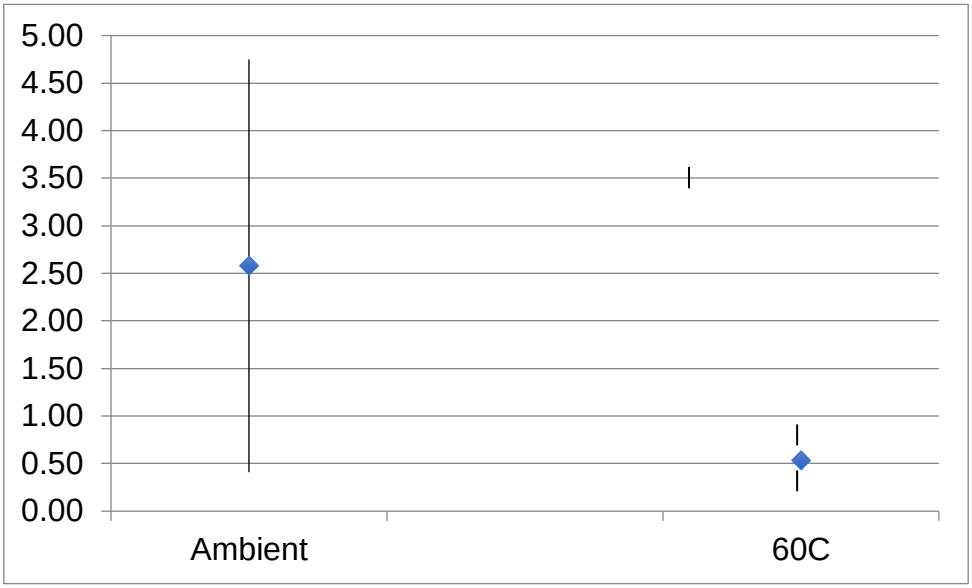

Figure 13. Plot of CO uptake lag times and standard deviation across all substrates as a function of incubation temperature, 60$_o$C or ambient (25$_o$C). Note that average lag time is substantially shorter at 60$_o$C. There was no significant difference between the lag times of the two temperatures.

DISCUSSION

Results from this study suggest that Ni-dependent CO-oxidizing microbes within swamp sediment are affected by specific substrates whether they are incubated at ambient temperatures or 60 ^{o}C. Both CO uptake rates and lag times varied among the five substrates, (Tables 1-6). In general, CO uptake rates were significantly higher at 60 ^{o}C than at ambient temperatures (Figure 12). Anaerobic microbes that oxidize CO have been reported to grow best at temperatures of 60 ^{o}C or higher (Baker *et al*., 2016). The incubation at 60 ^{o}C created an optimal environment for thermophiles to use CO as a carbon source for growth, which could cause the difference seen between the samples incubated at different temperatures. However, we also observed notable activity at ambient temperatures, which has been rarely reported before. In addition, in spite of considerable variability in lag times for ambient temperature incubations, the average lag times were lower at 60 ^{o}C (Figure 13), which is consistent with a process that is mostly thermophilic.

The addition of glucose at ambient temperature significantly increased the lag time of CO uptake in comparison to the control samples**.** It did not, however, significantly change the lag time of CO uptake in the 60 ^{o}C samples. There was no significant difference between the control and glucose sample CO uptake rates at ambient temperatures. This finding was in line with a study that tested anaerobic microbial growth when exposed to CO and glucose. This study found glucose did not have a significant effect on the growth rate when glucose was added to the control samples (Kochetkova *et al.,* 2019). Glucose is commonly used as a source of carbon source for microbes undergoing CO oxidation. The sediment's tendency to use glucose as a source could explain the absence of a significant change in CO uptake rate because the control sediment could have previously been using glucose as a source for growth in situ. When glucose was added to the samples at 60 ^{o}C, it significantly decreased the CO uptake rate in comparison to

the control samples. A decrease in CO uptake is indicative of changes in the microbial populations that are present possibly with an increase in abundance of those that don't use CO.

Pyruvate showed no significant effect on the sediment samples. Although it is likely pyruvate affected the populations of anaerobes found within the sediment, the addition of pyruvate may not have had an effect on the amount of CO oxidizers. If the abundance of anaerobes that undergo CO oxidation within the sediment did not change, then CO uptake would not change.

While the addition of acetate did not have a significant effect on the CO lag time at ambient temperatures or the uptake rates for both temperatures, it did significantly decrease the lag time before CO uptake in samples at 60 ^{o}C. The use of acetate in the absence of oxygen not common among many anaerobes. This decrease in lag time could have been caused by a preference for CO over acetate by these anaerobes.

Syringate significantly decreased the CO uptake rate at 60 ^{o}C, but not at ambient temperatures. A decrease in CO uptake was likely due to a change in the microbial population caused by the addition of syringate. This addition either increased the amount of anaerobes that do not use CO within the population or decreased the amount of microbes that use CO in the absence of oxygen. It also had no effect on the lag times of either the ambient or 60 ^{o}C samples. Much like pyruvate, it is likely that the abundance of CO oxidizers present within the soil did not change due to syringate addition, having no effect on the lag times or rate of CO uptake at ambient temperatures for the syringate samples.

The addition of casamino acids significantly decreased the lag time before CO oxidation at ambient temperatures. This decrease in lag time was most likely due to a preference for CO over the use of casamino acids. It did not have a significant effect on the lag time at 60 ^{o}C.

Although casamino acids did not have a significant effect on the CO uptake rate at 60 ^{o}C, it did significantly increase the rate of CO uptake at ambient temperatures. It is likely that the addition of casamino acids increased the presence of CO oxidizing anaerobes within the soil, which consequently increased the CO uptake rate.

One limitation of this study was the time of sampling. Because this study was conducted over the span of about eight months, seasonal changes have affected the composition of the sediment. The assays were conducted one time for each substrate at each temperature throughout the eight months. Some sediment was collected and run during summer temperatures, while others were conducted during the winter. Another limitation was the inability to run the assays for each substrate at each incubation temperature more than once. Due to the limited time frame of the experiment and the length of the ambient assays, the samples were only run one time for each substrate at each temperature.

CONCLUSION

The addition of substrates glucose, syringate, casamino acids, and acetate had a significant effect on the CO uptake rates and lag times at both ambient temperatures and 60$_{o}$C. Significant variance between the substrate samples was also observed. The ability to use CO under anaerobic conditions is limited by the availability of casamino acids. For all other substrates, the preexisting capacity exists as a whole, without the addition of the substrates. While decreases in CO uptake rates could be observed in other sediment samples, casamino acids was the only substrate in which the CO uptake rate significantly increased. This increase indicates that the sediment is limited by the presence of casamino acids. In future experiments, the assays could be conducted for each substrate within each season to ensure environmental factors did not affect the results. If time allowed, future assays should also be run for each substrate multiple times, to ensure the results could be replicated.

ACKNOWLEDGEMENTS

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