TRACKING ORGANIC SUBSTRATE ALTERATIONS IN PASSIVE REACTIVE ZONES FOR PLANNING AND MONITORING[1]

Daphne L. Place [2], Elise Claveau [3], Linda Figueroa [2]

Abstract. Microorganisms within passive reactive zones (e.g. wetlands, SR bioreactors, PRB's, etc.) utilize organic substrates, such as wood, in the process of reducing sulfate and immobilizing the metals from mine drainage. The rate and extent of substrate utilization controls the performance and longevity of the passive treatment system. This paper evaluates alterations in substrate composition for four reactive mixtures, over time. The alterations are determined using a method adapted from a sequential extraction technique used to determine carbohydrate and lignin composition in agricultural products. Tracking substrate alterations in this way can be used as a tool for substrate selection as well as evaluation of substrate performance over time. The data collected by tracking organic substrate alterations from pre-operational to post-operational conditions for the four bioreactors can be tied with performance data to better understand the function of the bioreactors. Analysis of the data shows that using total carbon to predict the longevity of passive treatment systems is not enough because the carbon must be in a form that is bioavailable to the microbial community. Analysis also shows that using cellulose to lignin ratios may be useful in the substrate selection process. Tracking substrate alterations over time also allow for the estimation and prediction of substrate utilization that can be correlated with sulfate reduction rates. Applicability of tracking substrate alterations over time is not limited to lab scale bioreactors. It can be used to analyze both spatial and temporal samples for within any passive treatment system to provide valuable insight for the planning and monitoring of passive reactive treatment zones. It can show if the proposed reactive mixture contains carbon in the forms that can be used by the microorganisms. It can also show why a system may be reducing sulfate at a lower than expected rate. Finally, it can help manage the sustainability of the passive treatment system by showing when and if the reactive mixture needs to be refreshed.

Additional Key Words: reactive mixture, sulfate reducing bacteria, substrate utilization

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Introduction

Microorganisms within passive reactive zones (wetlands, sulfate reducing bioreactors, permeable reactive barriers) utilize organic substrates, such as wood, in the process of reducing sulfate and metals produced from acid mine drainage (AMD). The rate and extent of substrate utilization controls the performance and longevity of the passive treatment. Sulfate reducing bacteria (SRB) are the target organism for the removal of sulfate from AMD. However, SRB are one group of organisms in a microbial community. Like a food chain, each group of organisms in the community provides food for another group of organisms. The first group of organisms hydrolyzes the organic substrate, providing food for the fermenting bacteria. These bacteria then conduct their metabolic processes and provide food for the SRB. A breakdown in the utilization of the substrate, such as not making enough food for the next microbial group, can cause the chain to collapse and thus the passive treatment system to fail.

An effective substrate is one that is biodegradable in a way that is sufficient to support the metabolic needs of the microbial community, and sustainable over the expected life of the passive treatment system. Kalin (2004) acknowledges that this has been difficult to do. "The engineering challenge...[has been to] design systems capable of operation for years to decades with little or no maintenance" (Kalin, 2004 page 229). Opinions vary as to what length of time constitutes the expected life of passive treatment systems. Long term performance of passive bioreactors (Gusek et al., 1998) and passive reactive barriers (Blowes et al., 2000), treating inorganic contaminants suggest longevity between 12 and 20 years, respectively. Unfortunately, the majority of passive treatment systems are not meeting this expected life. Tracking alterations in the organic substrate of these passive treatment systems may improve overall performance and treatment system sustainability by helping with organic substrate selection and providing an additional means of monitoring the system over time.

Other research that considered substrate alterations, such as Waybrant et al. (1998), Cocos et al. (2002), use total carbon, total nitrogen and/or total phosphorus to characterize alterations in the solid phase organic substrate in the passive treatment system. Looking solely at total carbon is not enough. The carbon must be in a form that is available to the microbial community. Longevity expectations based on total carbon will likely overestimate the sustainability of the system. The technique used in this research is capable of tracking the usable forms of carbon throughout the life of the treatment system and may provide better planning of system longevity.

This paper evaluates the changes in substrate composition, in two points in time, of four reactive mixtures using a sequential extraction method. The data demonstrate how the method can be used to evaluate substrate sustainability over time, and to select the most appropriate substrate. Pre-operational data, the initial point in time, for the four reactive mixtures is presented herein (Figueroa et al., 2004) and compared with post-operational data, the final point in time, collected as part of the research effort. The solid phase substrates evaluated include: oak wood, dried alfalfa, pine wood, and corn stover.

The earlier research of Figueroa et al. (2004) consisted of four bench-scale up-flow anaerobic passive bioreactor columns that were operated for more than one year to compare sulfate removal of various organic substrates. Each column was initially packed in duplicate with one of four different organic solid phase substrates: oak wood, dried alfalfa, pine wood or corn stover. Corn stover is the ground up stalk left behind in the field after harvest. The bioreactors were glass columns, 30 cm long and 5 cm in diameter, with four 1 cm threaded side ports distributed

at 6 cm increments up from the base of the column. Each column contained 150 grams of reactive mixture, on a dry weight basis having a packed volume of 380 cm^3 ± 90 cm^3. This mixture was composed of 45% #8 mesh silica (67.5g), 40% specific organic substrate, 4 mm size (60g), 10% fresh dairy manure (15g), and 5% # 10 mesh limestone (7.5g). All columns received an influent of synthetic mine water containing 1000 mg/L sulfate, and 50 mg/L zinc and manganese. The influent was delivered by an isometric peristaltic pump, set to 30mL/day. The pH of the influent was 6. As part of the previous study, effluent samples were collected and analyzed for sulfate, zinc and manganese concentrations. The early data (first 3 months) indicated that the alfalfa and corn stover based columns allowed for a higher reduction in sulfate, with effluent concentrations between 75 and 125 mg/L. Comparatively, effluent concentrations for oak and pine based columns was between 200 and 300 mg/L. From this data, it was concluded that alfalfa and corn stover were better substrates for the reduction of sulfate in the treatment system as designed.

As time passed, the oak, pine and alfalfa columns either stopped flowing or stopped reducing sulfate and zinc concentrations. After more than 18 months of operation one of the duplicate alfalfa based columns continues to flow but is not removing a detectable amount of sulfate or zinc and one of the duplicate corn stover based columns continues to flow removing about 500 mg/L of sulfate and 50 mg/L of zinc. During the last 10 months of operation, effluent quality data was collected periodically, however flow data was not collected. For this reason, only relative performance comparisons can be made and mass balances cannot be done with any meaning.

Methods

When the columns were decommissioned all the reactive mixture for each column was homogenized by mixing in a beaker. The reactive mixture was put into 50 mL falcon tubes, labeled and placed in the freezer for later analysis. Because the entire column was homogenized before being placed in the sample tubes, it is assumed that each tube contains a representative sample of the entire column. Prior to analysis, these sample tubes were placed in the refrigerator to thaw. The technique for tracking substrate alterations is adapted from a method, by Hall, for determining the carbohydrate composition in agricultural products (Hall, 2000) and a method, by Templeton and Ehrman, for determining acid insoluble lignin in biomass (Templeton and Ehrman, 1995). According to these techniques organic material can be subdivided into four operationally defined main categories: (1) organic acids, mono- and oligosaccharides; (2) polysaccharides, starch, fructans, pectin substances and β-glucans; (3) cellulose and hemicellulose; and (4) lignin (Hall, 2000; Templeton and Ehrman, 1995). The proportion of each category present in organic material is determined through a successive extraction technique that is shown in the diagram below. As can be seen, the insoluble portion of an extraction is the initial sample for the next extraction. To follow is a detailed explanation of each step in the process. The substrate sample is subjected to the ethanol extraction, separating the ethanol soluble from ethanol insoluble fraction. A sample of the ethanol insoluble fraction is then subjected to the neutral detergent extraction, separating the neutral detergent soluble from the neutral detergent insoluble fraction. A sample of the neutral detergent insoluble fraction is then subjected to the acid hydrolysis extraction, separating the acid soluble from acid insoluble fractions. The acid insoluble fraction will contain both organic and inorganic components (Templeton and Ehrman, 1995). Therefore, the lignin is the organic component of the acid insoluble fraction (Templeton and Ehrman, 1995).

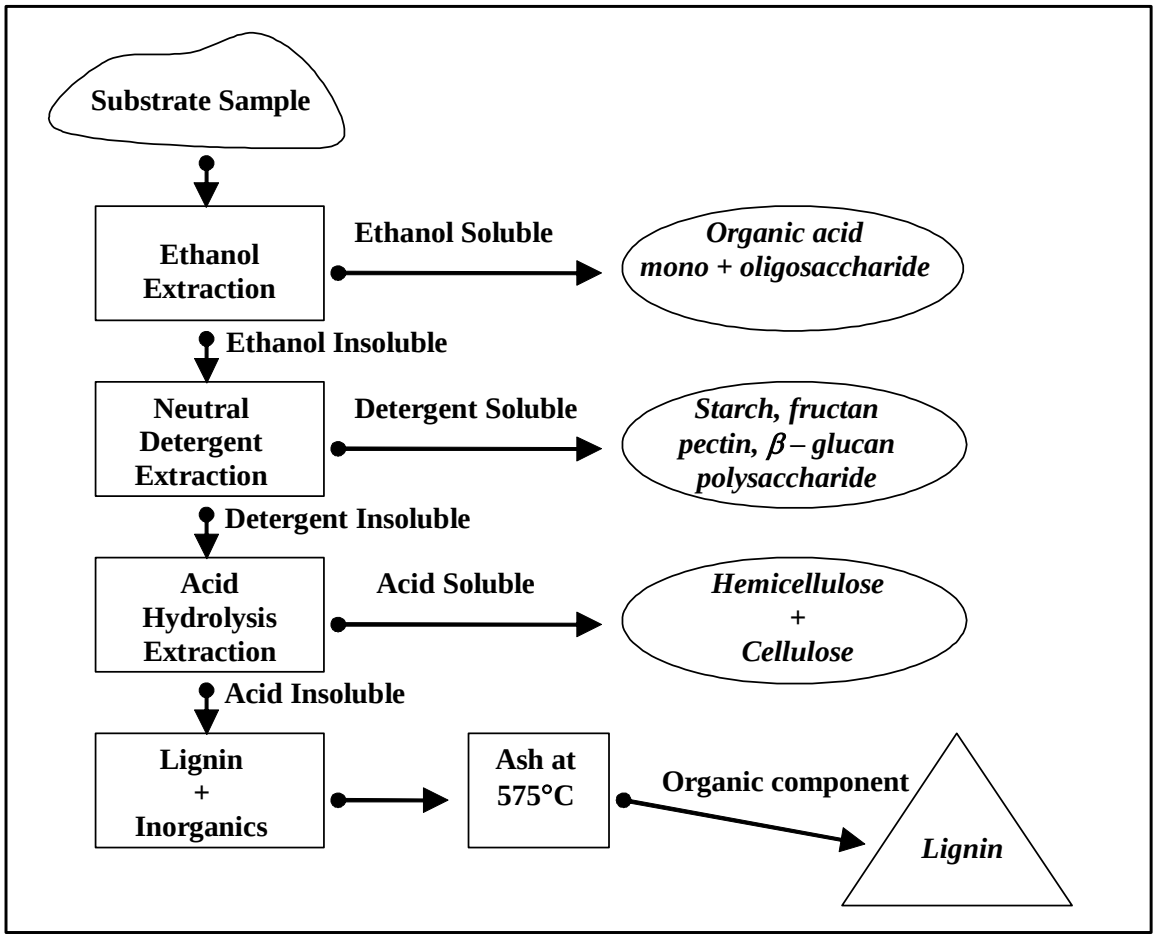

Figure 1. Diagram of sequential extraction process with fractions and categories (adapted from Hall (2000) and Templeton and Ehrman (1995)).

For each of the four reactive mixtures, every extraction was conducted in replicates of two to six, depending on the amount of insoluble fraction produced by each previous step. The post-operational data is an average of the analysis of each replicate. Specifically, the number of replicates in the average of data for the oak based reactive mixture is greater than the number of replicates in the average of data for the alfalfa based reactive mixture. As mentioned earlier, the pre-operational data is taken from the previous research described in Figueroa et al. (2004).

Ethanol Extraction

The purpose of the first phase of the sequential extraction is to separate lower molecular weight mono- and oligosaccharides from polysaccharides present in the solid phase substrate. The procedure requires a sample to ethanol ratio of 1.0 ± 0.04 g : 200 mL. An eighty percent ethanol solution, by volume, was used for all extractions, as called for in the procedure by Hall (2000). Once the ethanol and substrate were combined, the extraction mixture was agitated, by shaker table or stir plate, overnight or about 18 hours. After being agitated the mixture was filtered under a vacuum using Whatman 541 filter paper, having a nominal pore size of 20-25 μm. The portion of the mixture that passes through the filter is the ethanol soluble fraction,

which consists of mono- and oligosaccharides. The portion of the mixture that is retained on the filter is the ethanol insoluble fraction, which is used in the neutral detergent residual method. The filters are dried and pre-weighed. The ethanol insoluble fraction, with filter, was dried and weighed (Hall, 2000).

Neutral Detergent Residue Method

The purpose of this next phase of the sequential extraction is to separate polysaccharides, starch, fructans, pectin substances, and β-glucans from cellulose and hemicellulose. This separation is accomplished by using the neutral detergent residual method. The procedure requires that one gram of sample be added to 100 mL of neutral detergent solution and 0.2 mL of heat-stable α-amylase. The one gram sample is from the ethanol insoluble fraction produced above. The neutral detergent is a mixture of 1.8 L distilled water, 54 g sodium lauryl sulfate, 26.3 g EDTA (ethylenediaminetetra acetic acid), 7.2 g sodium hydroxide, 12.26 g sodium borate, 8.21 g disodium hydrogen phosphate, and 18 mL triethylene glycol, prepared using the method presented in Hall (2000). The heat stable α-amylase is added to ensure the dissolution of the starch present in the sample. This mixture is heated to a rolling boil for 1 hour, swirling every 15 minutes. Once boiled, the mixture was filtered under a vacuum using a Whatman 541 filter paper, like that used for the ethanol extraction. The portion of the mixture that passes through the filter is the neutral detergent soluble fraction, which consists of polysaccharides, starch, fructans, pectin substances, and β-glucans. The portion of the mixture that is retained on the filter is the neutral detergent insoluble fraction, which is used in the acid-insoluble lignin determination. The filters are dried and pre-weighed. The neutral detergent insoluble fraction, with filter, was dried and weighed (Hall, 2000).

Acid-Insoluble Lignin Determination

The final phase of the sequential extraction is to separate cellulose and hemicellulose from lignin. This is accomplished using the acid-insoluble lignin determination (Templeton and Ehrman, 1995). The procedure for this phase requires that 0.30 ± 0.01 grams of solid phase substrate be mixed with 3 mL of 72% (by weight) sulfuric acid and placed in a 30°C water bath to hydrolyze for 2 hours. The sulfuric acid hydrolyzes the cellulose and hemicellulose, but does not affect the lignin, which is mostly insoluble in mineral acids (Templeton and Ehrman, 1995). After hydrolysis is complete, the mixture is diluted to a 4% sulfuric acid solution by bringing the volume to 84 mL, using deionzed water, and then placed in an autoclave at 121°C for 1 hour. The mixture is then filtered under a vacuum through a pre-weighed fritted crucible. The portion of the mixture passing through the crucible is the acid soluble fraction, which consists of cellulose and hemicellulose. The portion of the mixture retained in the crucible is the acid insoluble fraction, which consists of lignin, inorganic materials, and proteinaceous materials (lignin-like substances). The crucible and contents are dried at 105°C until constant weight is reached. The crucible is then placed in a 575°C muffle oven for at least 3 hours. The crucible is cooled and weighed. The material left in the crucible is the acid insoluble ash. The acid insoluble fraction less the acid insoluble ash is the lignin content (Templeton and Ehrman, 1995).

Calculations

To follow is an explanation of the calculations for determining the various extraction fractions:

EIF (wt.) = mass retained on filter

ESF (wt.) = (mass of sample) – EIF

NDIF (wt.) = mass retained on filter

NDSF (wt.) = (mass of EIF) – NDIF

AIF (wt.) = mass retained in crucible

AIA (wt.) = inorganic fraction of AIF

ASF (wt.) = (mass of NDIF) – AIF

AIL (wt.) = AIF – AIA

EIF ≡ ethanol insoluble fraction

ESF ≡ ethanol soluble fraction

NDIF ≡ neutral detergent insoluble fraction

NDSF ≡ neutral detergent soluble fraction

AIF ≡ acid insoluble fraction

AIA = acid insoluble ash

AIL ≡ acid insoluble lignin

ASF ≡ acid soluble fraction

Results

Below is a table showing the carbohydrate fractions of the organic portion in each of the four reactive mixtures in both pre-operation and post-operation conditions. Just looking at changes in the percent fractions is not enough; you must look at how these changes affect the total mass. To establish a mass basis for comparison, the percent composition data was converted to mass data by assuming that lignin was not significantly degraded. This assumption is supported by research conducted in anaerobic composting where lignin was found to have a degradability of 0% (Haug, 1980). This is further supported by the use of lignin content as a part of an empirically based calculation for determining the biodegradable fraction of volatile solids in organic waste components, where high lignin content correlates with low biodegradability (Tchobanoglous et al., 1993).

Table 1. Percent carbohydrate fraction for four reactive mixtures in pre-operation (Figueroa et al., 2004) and post-operation conditions.

Column Condition and Organic Substrate	**ESF %**	**NDSF %**	**ASF %**	**AIL %**
Pre-Operation Oak	4.9	9.3	53.3	17.6
Post-Operation Oak	4.0	13.4	61.1	20.8
Pre-Operation Alfalfa	11.9	26.7	28.3	7.6
Post-Operation Alfalfa	7.1	9.4	37.8	36.0
Pre-Operation Pine	2.5	7.3	57.1	19.3
Post-Operation Pine	2.6	6.9	62.5	27.4
Pre-Operation Corn Stover	8.7	12.4	50.7	10.2
Post Operation Corn Stover	1.1	47.8	29.0	12.6

Another assumption necessary to establishing total organic mass of the post-operation mixtures is that the net accumulation of mass was negligible when compared to the net loss of mass. Estimates of bacterial mass by Pruden et al. (2005) on similar bioreactors suggest cell-mass accounts for less than 10^{-5} g per gram of reactive mixture. Total zinc accumulations, based on complete removal of zinc over a one year period, is only 0.5 g, compared to the initial reactive substrate mass of 75 grams. Thus, the assumptions of lignin conservation and negligible mass accumulation within the column are reasonable. Post-operation total organic mass was estimated by equating the post-operation lignin percent to the initial mass of lignin. Once the mass of lignin is established, the total organic mass and the mass of the other fractions are calculated. Table 2 shows the organic mass of the reactive mixture in both the pre-operation and post-operation condition, using the aforementioned assumptions.

As can be seen, the oak and corn stover based columns changed the least with respect to total mass, followed by the pine based column and the alfalfa based column, respectively. Fig. 2 shows a mass comparison of the pre-operation and post-operation organic reactive mixture. The four categories shown are related to the operationally defined fractions as presented in Fig. 1.

The total height of each bar in the graph represents the weight of organic material within the reactive mixture. As previously mentioned, the reactive mixture consists of 15 grams of manure and 60 grams of oak wood, dried alfalfa, pine wood or corn stover. The pre-operation weight of the organic material within the mixture is less than 75 grams because the manure contained approximately 60% inorganic material (Figueroa et al., 2004). The pre-operation and post-operation mass of lignin is equal, as set forth by the assumptions stated above. Within the oak based column the organic acid fraction was reduced by 32%, the starch fraction was increased by 21%, and the hemicellulose fraction was reduced by 3%. For the alfalfa based column the organic acid fraction was reduced by 88%, the starch fraction was reduced by 92%, and the hemicellulose fraction was reduced by 72%. Within the pine column, the organic acid fraction was reduced by 26%, the starch fraction was reduced by 32%, and the hemicellulose fraction was

reduced by 23%. Finally, for the corn stover based column the organic acid fraction was reduced by 89%, the starch fraction increased by 213%, and the hemicellulose fraction decreased by 53%. The gain of mass in the starch fraction may be due, in part, to inorganic interferences with the sequential extraction.

Table 2. Organic mass of four reactive mixtures in pre-operation and post operation condition.

Column Condition and Organic Substrate	**Organic Mass (grams)**
Pre-Operation Oak	63.8
Post-Operation Oak	63.5
Pre-Operation Alfalfa	55.9
Post-Operation Alfalfa	15.8
Pre-Operation Pine	65.0
Post-Operation Pine	52.7
Pre-Operation Corn Stover	61.5
Post Operation Corn Stover	60.9

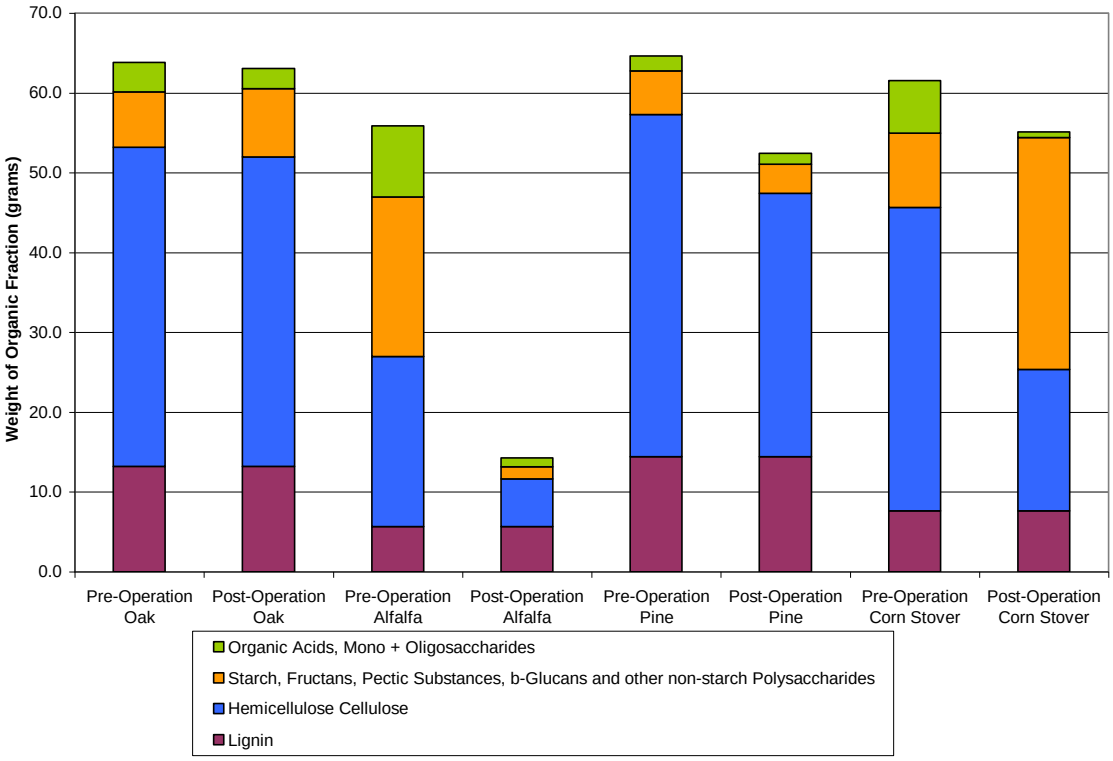

Figure 2. Pre-operation (Figueroa et al., 2004) and post-operation mass of the carbohydrate fractions and lignin for the four reactive mixtures tested.

Discussion

As stated earlier an effective substrate is one that is biodegradable in a way that is sufficient to support the microbial community, and sustainable over the expected life of the passive treatment system. Projections vary widely as to what length of time constitutes the expected life of passive treatment systems, ranging from 12 to 20 years (Blowes et al., 2000; Gusek et al., 1998). Tracking alterations in the organic substrate shows promise for improving overall performance and system sustainability by helping with organic substrate selection and providing an additional means of monitoring the system over time. To follow is a discussion of how tracking alterations in organic substrate can be used for substrate selection, can clarify understanding substrate utilization, promotes treatment system sustainability, and is applicable to field scale systems.

Selection

Cellulose, a significant molecule used in the structure of plants, contains lignin and forms a complex structure (Haug, 1980). Lignin is a three-dimensional molecule that retards the decomposition of cellulose because it creates a physical barrier between the cellulose fibrils (Haug, 1980). This physical barrier reduces the exposed surface area of cellulose there by making it inaccessible to microorganisms (Haug, 1980). Referring to Table 2, alfalfa, or similar herbaceous materials, are biodegradable, but the extent is too great and the supported community stops reducing sulfate because it is out of food. The results suggest that woods and corn stover tend to retain their relative proportions of carbohydrate fractions to a greater extent than herbaceous substrates. This is due to the cellular structure of the substrate and its biodegradability. As mentioned earlier, empirical calculations of biodegradability assume lignin is not readily degraded (Tchobanoglous et al., 1993). Knowing that lignin interferes with the ability of the microbes to access the cellulose, it is valuable to look at the cellulose to lignin ratio of substrates as part of the selection process.

Fig. 3 shows the mass of cellulose and lignin in each of the four bioreactors, in both pre-operation and post-operation condition. It also shows the ratio of cellulose to lignin (C:L) for these columns. Looking only at the pre-operation cellulose values shown above, one might conclude that pine would be a superior substrate because pine contains the most cellulose, followed by oak, corn stover and alfalfa. Likewise, looking only at the pre-operation lignin values shown above, one might conclude that the alfalfa would be a superior substrate because it contains the least amount of lignin, followed by corn stover, oak and pine. Now, looking at the C:L ratio for the pre-operation data, keeping in mind that a higher C:L ratio means that there is more surface area of cellulose available to the microorganisms, one might conclude that corn stover is the superior substrate having the highest C:L ratio of 4.94, followed by alfalfa, oak and pine. Finally, looking at the changes in C:L ratio for the two points in time, the oak based column does not change much over the operating time, from 3.03 to 2.94. The ratios for the pine based column change slightly from 2.96 to 2.28. However, the ratios for the alfalfa based column and the corn stover based column change substantially between the two points in time, from 3.74 to 1.05 and 4.94 to 2.3, respectively.

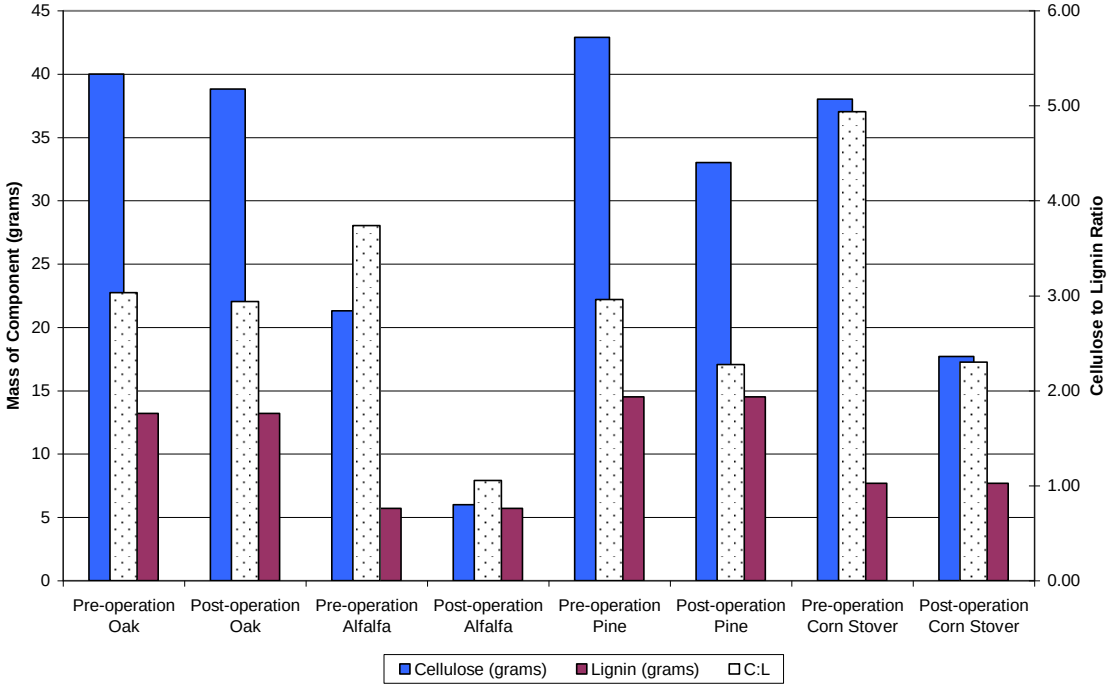

Figure 3. Graph of cellulose mass, lignin mass and the ratio of cellulose to lignin for four bioreactors at two points in time (pre-operation data by Figueroa et al., (2004)).

Utilization

The data collected from the four bioreactors was from two points in time. If the substrate alterations are tracked over time, it may be possible to calculate a microbial utilization rate. This historical utilization rate can then be used to project the life of the passive treatment system.

Figure 4 is a graph of the substrate changes over time for a hypothetical reactive mixture in a passive treatment system. Each time (t) represents a sample point in the operational life of this treatment system. From the changes in substrate composition, calculated using the aforementioned methods, one could begin to see trends in substrate utilization. Some trends may include rate changes as the reactive mixture ages and seasonal fluctuations in substrate utilization. If the data for the passive treatment system is compared with similar data from other passive treatment systems, optimum applications may become apparent. That is to say that some mixtures may have faster start up times, more sustainability or be better suited for maintaining rates of utilization during seasonal fluctuations.

Sustainability

According to Kalin (2004) current techniques of chemical monitoring "can provide little predictive measure of the 'health' or 'sustainability' of the microbial community doing all the work" (Kalin, 2004 page 230-231). One of the major challenges to the successful application of passive treatment systems is constructing and implementing a system that is sustainable over the expected life of operation. As stated earlier, passive treatment systems are failing to meet the expected life. Tracking substrate alterations over time allows us an opportunity to learn from the microbial community to better understand what they would prefer to eat. For example, oak, or similar hard woods, are somewhat biodegradable, but the extent is low, compared to other

substrates, like herbaceous material. Research in the author's lab has shown that when hard wood is used in a passive treatment system, the supported community stops reducing sulfate because it cannot access the cellulose. When herbaceous material is used in a passive treatment system, the supported community stops reducing sulfate because is has utilized all the available carbohydrates. This phenomenon with oak based columns failing to continue reducing sulfate over time has been noted in other research efforts conducted by the author's research group. Specifically, six columns with oak based reactive mixture, like the reactive mixture analyzed for this research, were operated over a 1 year period. After a few months of operation, these columns continued to flow but failed to reduce sulfate. In contrast, corn stover is sufficiently biodegradable to support the microbial community. The duplicate corn stover based column continues to flow and sulfate reduction has stabilized at 500 mg/L, 50% of the influent concentration. Of the solid phase organic substances studied by Figueroa et al., (2004) corn stover has proven to be the most promising at satisfying the expectation of substrate to support the microbial community and remain sustainable over the operation time of the system. Lab based experiences like these can help with field applications, but analyzing field data in this way will provide more valuable information. This may or may not lead to the development of a "super" substrate but it will go a long way to help develop more sustainable passive treatment systems in the field.

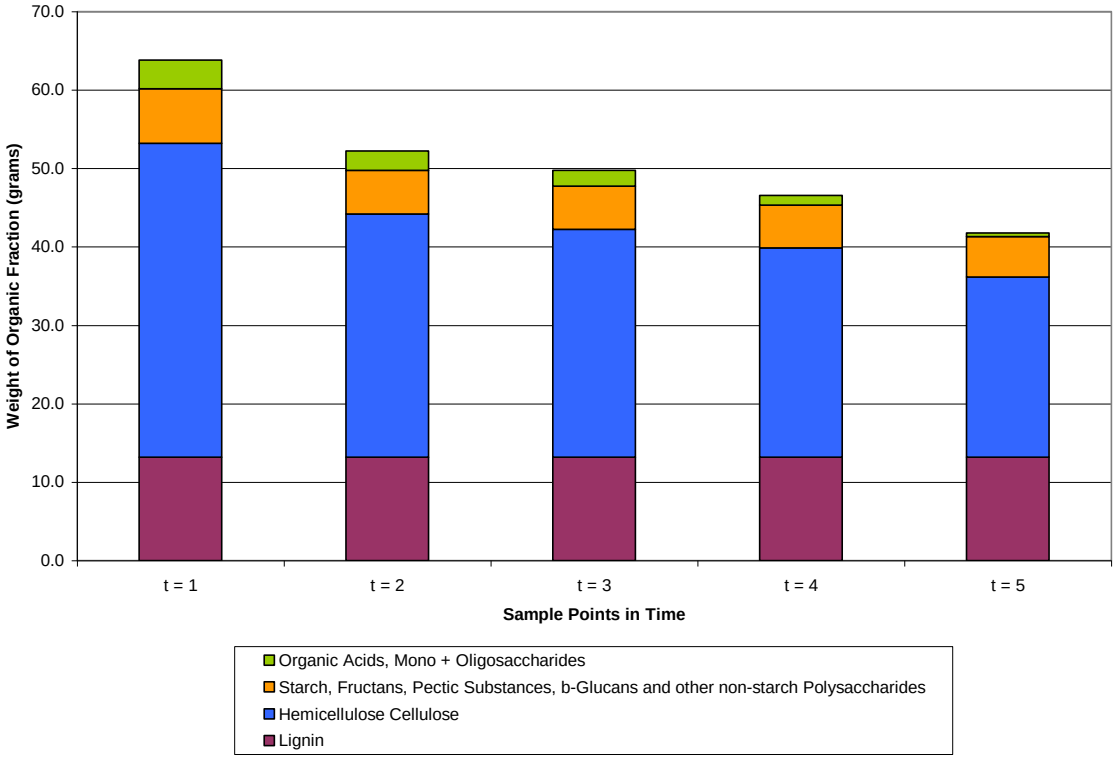

Figure 4. Changes in mass of the carbohydrate fractions and lignin for a hypothetical reactive mixture over time of operation.

Applicability

The applicability of tracking substrate alterations is not limited to bench scale bioreactors, as it can be used on field scale passive treatment systems as well. The tracking method discussed earlier can be used to monitor alterations in the reactive mixture both spatially and temporally.

Samples of the reactive mixture can be taken at various locations, both vertically and horizontally. Analysis can show spatial variations in substrate utilization that may be used along with liquid phase sampling to monitor sulfate reduction rates, metal accumulation and even short circuiting of flow. Samples of the reactive mixture taken over time can be used in conjunction with liquid phase sampling, to evaluate microbial utilization of the substrate. Coupled with sulfate removal data, viability of the reactive mixture can be assessed. For example, if sulfate removal is lower than expected while the substrate composition data shows adequate cellulose, as was the case for the oak based bioreactor, one could conclude that the organic substrate is not sufficiently biodegradable to support the microbial community, and thus the goal of sulfate and metals removal. On the other end, if sulfate removal is lower than expected and substrate composition data shows inadequate cellulose, as was the case for the alfalfa based bioreactor, one could conclude that either the organic substrate was biodegraded too quickly or that is in time to refresh the reactive mixture.

Interferences

During analysis of the post-operation reactive mixture, some potential interferences were noted. First, the accumulated biomass is able to pass through a 20 μm filter. It was thought that the biomass could cause an artificially high assessment of soluble fractions. However, based on research conducted by Pruden et al. (2005), the mass of bacteria present in the sample material may be on the order of 10^{-5} grams per gram of sample. The extraction process requires 2-3 grams of sample, thus the possible mass contribution for the microbial biomass is very small, below the accuracy of the scales being used.

Next, accumulated zinc sulfide could be influencing the mass of the column. Further research into this issue will be addressed using a total metals digestion of both soluble and insoluble fractions, notably that from the neutral detergent extraction. The reason for this is the likelihood that the EDTA present in the neutral detergent solution may have, under the conditions of the extraction, complexed with the metal, allowing the sulfide to volatilize. The stability constant for zinc sulfide is pK = 24.5 (CRC, 2003) while the stability constant for zinc-EDTA is pK = 16.5 (Stark et al., 1982). Under standard conditions the metal would remain associated with the sulfide; however, the extraction technique requires the solution be heated to 100°C for 1 hour, which changes the kinetics of the reactions. In addition, the ratio of mass to solution is 1g : 100 mL compared to the ratio for common metal extractions in soil using EDTA, 5g : 25 mL (Carter, 1993). Both solutions contain 0.05M EDTA.

Finally, it is possible that during the acid-insoluble extraction, the sulfide could leave the system as hydrogen sulfide gas. This would result in a loss of mass which would influence the determination of acid soluble and insoluble fractions.

Conclusions

In conclusion, tracking substrate alterations over time shows great promise as a tool for selecting solid phase organic substrate and monitoring the sustainability of the substrate and microbial community over the operating time of the passive treatment system. Other research that considered substrate alterations used total carbon, total nitrogen and/or total phosphorus. As stated previously, looking solely at total carbon is not enough. Total carbon will not show the loss of specific carbohydrate groups utilized by the microbial community that supports the SRB. A key to the biodegradability of a substrate is a function of both the amount of lignin relative to

the amount of cellulose and the arrangement of the lignin within the cellulose. Noting the loss of these specific carbohydrate groups can be critical in the monitoring and maintenance of a successful passive treatment system. Comparing performance data with the substrate utilization data will help explain what may be causing these systems to fail. Even more importantly it gives another tool that will help understand and anticipate declines in function, so appropriate actions can be taken to maintain the usefulness and longevity of passive treatment systems. The interferences of the technique can be understood and overcome. Therefore, this extraction technique is a relatively simple procedure that provides valuable insight for the planning and monitoring of passive reactive treatment zones.

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