The Analysis of the Microbial Populations of Two Soil Sites Contaminated with Heavy Metals

An Honors Thesis (HONRS 499)

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Abstract

The purpose of examining the microbiological flora of soils contaminated with heavy metals, particularly lead (Pb) and cadmium (Cd) is to collect information that will show the effect of heavy metal contaminants on the microbiology of the soils. The soils chosen for investigation are local sites. The eventual subsequent step would be to use the local flora of the soils to develop effective bioremediation plans. The effect of heavy metal contaminants varies with the soil being investigated. The Memorial Drive Dump site shows consistent increase in bacterial, actinomycetes, and fungi counts over a 50 day test period. The Glynwood site shows decrease in bacterial and actinomycete counts, but an increase in fungal counts over the 50 day test period. The addition of a composted sewage sludge and oat seeds were added to some of the soils of both sites. The presence of both of these additions lead to increased levels of bacteria, actinomycetes, and fungi over the soil samples without additional treatments.

Introduction

Contamination of soils is a major problem throughout the United States. This contamination occurs from the dumping of wastes created through industrialization. The United States Environmental Protection Agency (USEPA) estimates that more than one million tons of hazardous chemicals are released into the environment yearly by industrialization (Cheng, 1998). This contamination can come from a number of sources including chemical and nuclear plants, including those of the military (Francis, 1998). Some common sources of lead (Pb) include paint, gasoline additives, refining and smelting of lead, demolition of automobiles, Pb acid battery breaking, disposal of Pb acid

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batteries, and pesticide production (Pichtel, 2000). In fact car battery disposal and reprocessing sites constitute significant local hazards, not only releasing Pb but also cadmium (Cd) and other heavy metals into the soil (Pichtel, 2000).

A local example of such a problematic site is Memorial Drive Dump (MDD). Memorial Drive Dump is believed to have been a quarry prior to 1941, after which the site was filled. Though the site is privately owned, some local work by the Muncie Sanitary District revealed in 1993 battery casings on the site from improper disposal. This began an investigation of the dumpsite where blocks of black plastic and foundry sand were discovered in addition to the battery casings (USEPA, 1997). Investigation of the soil revealed elevated levels of lead, cadmium, antimony, arsenic, barium, manganese, and selenium, all exceeding exposure limits (USDHH, 1997).

When the soil of MMD was examined the Pb levels averaged 29,400mg/kg, with a maximum value of 112,500mg/kg. The global average for Pb in natural surface soils is 20mg/kg. The Cd levels at this site averaged 3.9mg/kg with a maximum value of 8.8mg/kg (Pichtel, 2000). The majority of Pb and Cd, 92.2% and 77.7%, in the soils is non-residual, meaning that it is not incorporated into the crystalline matrix, and therefore considered available to microorganisms (Pichtel, 2000). The total organic carbon (TOC) for the MDD site is 17.9%, with a pH of 6.9-7.8 (Pichtel, 2000). The MDD soil based on observation is made up primarily of clay.

The second site chosen for study is Glynwood. This soil does contain some Pb; however, the levels are much lower than those seen at the MDD site. The Pb quantity is 37.5mg/kg. Other metals in this soil include chromium, copper, nickel, and zinc. This soil is mainly made up of silt, has a pH of 6.8, and TOC 31.6g/kg.

The objective of this study is to obtain information about the microbial makeup of these two different soils. It is important to understand the microbial flora of contaminated soils in order to apply bioremediation techniques. Molecularly altered microorganisms are effective in cleaning up large contamination events, such as an oil spill, however, these engineered organisms cannot compete with indigenous microbes when contamination is more diffuse, and released over an extended period of time, as is the case in many contaminated soil sites (Cheng, 1998).

Overview

Soil is the principle environment for microorganisms. Soil is dominated by a solid phase, making a habitat that in theory should be a bad habitat for microorganisms (Stotzky, 1997). Soil provides a variety of surfaces to be colonized with various nutrient availabilities. Soil consists of sand, clay, silt, and organic matter in the form of nutrient rich humus. These materials form heterogeneous particles known as peds. Microorganisms colonize various areas on these small peds. Despite all the elements in soil, it is poor in available nutrients, especially the carbon required by microbes as a principal energy source. However, soil contains more genera and species of microbes than any other habitat, in fact at some time, soil receives all microorganisms present on this earth (Stotzky, 1997). These microorganisms include bacteria, fungi, viruses, and protozoa. Bacteria and fungi are the principle microorganisms found in soil. Bacteria are present in isolated microcolonies on the surface and in pores. Bacteria require water and immediate nutrients available, thus why they colonize surface and pore areas. Fungi grow on and between soil peds, forming bridges across the particles where moisture is available. Because of the connection of fungi between particles, they may move nutrients

and water over long distances (Prescott, 1999). Most microorganisms occur in the top few inches of soil. In the topsoil there are 10^6-10^8 bacteria per gram of soil. Fungi are the dominant microorganisms in the soil in terms of biomass (Thorn, 1997).

Microorganisms play a major role in the soil environment as well as in the interactions with other living organisms in the soil environment. One major role of microorganisms occurs in the area known as the rhizosphere, first described in 1904 by Lorenz Hiltner (Prescott, 1999). Rhizosphere organisms play a crucial role in providing nutrients, as well as organic matter synthesis and degradation (Prescott, 1999). This area consists of the region around roots of plants. As roots advance deeper into the soil the root caps secrete mucus to help the roots to move smoothly through the soil. This mucosal area is the site of microbial attachment to the roots and root hairs. This microbial attachment helps to prevent desiccation and promote absorption and transport of water and other necessary elements (Stotzky, 1997). In fact, bacteria densely colonize the living epidermal cells of plant roots and root hairs; these bacteria live in a mutualistic relationship with the plant. The bacteria are dependent on simple organic molecules from the plant, while supplying the plant with necessary nutrients from the soil that they cannot break down themselves (Stotzky, 1997). An example of a mutualistic organism that infects the plant cells is the gram negative, aerobic bacteria *Rhizobium*. *Rhizobium* is the prominent member of the rhizosphere region, though like other symbiotic organisms this bacterium is very specific to the hosts it will infect. *Rhizobium* fixes atmospheric nitrogen into ammonia and alanine, which are used by the plant cells. Not all bacteria in the rhizosphere live inside a host plant. Other associative nitrogen fixers, such as

Azobacter, *Azospirillium*, and *Acetobacter*, all utilize organic molecules released by the plant in exchange for fixing nitrogen in forms utilizable by the plant (Prescott, 1999).

Fungi also play a major role in the rhizosphere region. Mycorrhizae are fungal plant associations (Prescott, 1999). The activities of fungi in this region are key to providing or limiting nutrients to plants (Thorn, 1997). Ninety-five percent of all vascular plants are associated with a fungus that either enters the cells forming endomycorrhizae, or grows between the cells forming ectomycorrhizae. Endomycorrhizal relationships are primarily formed by fungi that fall into the zygomycete family, where as fungi that form ectomycorrhizal relationships fall primarily in the basidiomycete family (Prescott, 1999). The significant biomass of fungi in soil represents a large portion of the available nutrient pool (Thorn, 1997). In addition there are bacteria that are associated with the mycorrhizae because excess carbon provided to the fungi by the plant is released into the soil, providing an accessible nutrient. Some of these bacteria also help the fungus to establish a relationship with the plant root cells (Prescott, 1999). Through their activity as decomposers or organic matter in the soil, microorganisms lead to the maintenance of nutrient cycling and an important role in the food chain (Doelman, 1985).

Heavy metal contaminants in soils, such as lead (Pb) and cadmium (Cd) cause disturbance in these normal soil environments. There are heavy metals that exist naturally in soils, however the difference between natural and contaminant metals is their availability. Natural metals are sealed within the soil matrix, inside the peds, where bacteria and fungi do not normally colonize. Contaminant metals occur as microscopic particles throughout the soil which are more dynamic and therefore more accessible to

the inhabiting microorganisms (Doelman, 1985). Heavy metals can cause a shift of the bacterial flora to more resistant gram negative rods, as well as a higher contribution from the eukaryotes, i.e. fungi (Doelman, 1985). Microorganisms that exist in contaminated soils demonstrate one of three living characteristics. The microbes either demonstrate resistance, tolerance, or sensitivity. Resistance is the ability of microorganisms to grow under the presence of heavy metals, meaning that the metabolic processes of the microorganisms continue. Tolerance is the ability of microorganisms to survive in heavy metal contaminated soils, however, these microorganisms do not grow, and they enter a period of stasis. The third reaction of microorganisms to heavy metals in the soil is sensitivity, this means that the microbes are inhibited even at low concentrations of the metal contaminant (Doelman, 1985).

One visible characteristic of heavy metal contaminated soils is the accumulation of organic matter. This is also a sign of the inability of the soil community to resist the heavy metal contaminant. Organic matter accumulation results because the heavy metal contaminants inhibit the ability of the soil microorganisms to carry out necessary processes, including soil respiration, nitrogen mineralization and nitrification (Doelman, 1985). Organic matter accumulation due to lead may occur because lead is thought to inhibit intracellular decomposition, the ability of microbes to produce exoenzymes, as well as the function of the exoenzymes (Doelman, 1979). An example of this is the decreased activity of amylase, an exoenzyme, with an increase in heavy metals. Similar activity is seen with cellulase and urease as well, both important enzymes in decomposition and recycling of nutrients (Doelman, 1979).

The extent of the effect of the heavy metal contaminants on the soil is related to the buffering capacity of the soil. The buffering capacity is in essence the extent to which the makeup of the soil prevents adverse effects on the microbial flora when exposed to a heavy metal contaminant. The buffering capacity is dependent on the type of soil. Soil can be characterized as sand, clay, or peat. The inhibition of the decomposition of organic matter in soils contaminated with lead is more pronounced in sandy soils, less pronounced in clay, and not noticeable in peat soils (Doelman, 1979). Upon closer examination of effect of lead on soil respiration it is noted that soil respiration is seriously inhibited at intermediate as well as high concentrations of lead in the soil. However, respiration in clay is not retarded until the higher concentrations of lead are added to the soil. And again peat shows no effect on soil respiration despite lead concentration. The effect on soil respiration follows the same trend that buffering capacity has on inhibition of decomposition (Doelman, 1979).

Bioremediation seeks to eliminate contaminants from soils using natural processes of soil organisms, including plants and microbes. Cheng by definition describes a contaminant as a natural or synthetic element in excessive amounts. The attraction of bioremediation is to maintain the natural functioning of the soil, which is often lost through harsh abiotic remediation techniques such as incineration. New focus of concern not only for human life, but also the sustainability of the ecosystem (Cheng, 1998). Another goal of bioremediation is to decrease the cost of remediating contaminated sites, which as of now remains high (Francis, 1998).

There are several characteristics that must be considered during a bioremediation task of soil, and it is difficult to develop a global standard because soil varies

substantially, even within a single region (Cheng, 1998). Chemical properties of the soil that effect the bioremediation of a site are, pH, cation and anion exchange, organic matter content and surfaces, mineral content and surfaces, nutrients, salts, and heavy metals. The composition of the soil is also important, clay particles are more reactive because of the greater surface area they offer as opposed to sand and silt particles in soil. The retention characteristics of the soil also important, because the greater retention capacity of the soil the less the contaminant is available for breakdown or transformation (Cheng, 1998).

Not all bioremediation projects result in the elimination of a contaminant. In fact metal contaminants are very difficult to eliminate because they cannot be destroyed, they can be removed or transformed (Francis, 1998). Transformation is the conversion of a toxic metal contaminant to a nontoxic form (Cheng, 1998). Transformation is primarily done by the microorganisms in the soil that seek to use the metal as an electron acceptor during respiration. Some mechanisms that the microorganisms use to transform toxic metals include: hydrolysis, hydroxylation, dehalogenation, demethylation, methylation, nitrogen reduction, deamination, ether cleavage, conversion of a nitrile to an amide, and conjugation (Cheng, 1998). Most often the breakdown of a contaminant occurs due to a consortia of microorganisms functions in various steps of the process (Cheng, 1998).

There are many factors to consider in a bioremediation program, and the result will be influenced by the contaminant, the microbes involved, and the environment. All three elements of the situation must be considered to develop an effective bioremediation program for the individual site (Sadowsky, 1998).

Materials and Methods

Soil was sampled from the two above described sites. This soil was potted into small black planting pots. Each treatment contained three pots. The treatments included: MDD no addition, MDD plus a composted sewage sludge (CSS) in a ~75%/25% ratio (the CSS contains TOC of 262g/kg), MDD plus oat seeds, three sets of Glynwood soil with no additions, two sets of Glynwood soil plus CSS, and one set of Glynwood soil plus oat seeds. These pots were kept moist throughout the duration of the experiment.

The pots were left for approximately three weeks before the first samples were taken, denoted as day 0. The second sample was taken 50 days after the first sample, denoted day 50. These samples were frozen for storage until microbiological testing was done.

For each pot replicate the sample was plated for day 0 and day 50. The agar used for growth of microorganisms included, Plate Count Agar (Difco), as a nutrient agar for bacterial growth, Actinomycete Isolation Agar (Difco), as a selective agar for actinomycete isolation, and Sabouraud Dextrose Agar (Difco), as a selective agar for fungal growth. Serial dilutions of soil solution were prepared to be plated on the different agar types. The dilutions were prepared by adding 1g of soil to 99ml of sterilized water, giving a 10^{-2} dilution. One ml was taken from this dilution and placed in another 99ml of sterilized water to give a 10^{-4} dilution, and so on up to 10^{-8}. All water used in making the dilutions was sterilized in an autoclave at 121°C for 15 minutes.

The dilutions were then plated on all agar types from 10^{-3} to 10^{-8}. The odd numbered dilutions were plated by taking 100µl of the higher even dilution and adding to the plate. For example, to make the 10^{-3} dilution 100µl was taken from the 10^{-2} dilution

and plated by aseptic spread plate technique. The even numbered dilutions were plated by taking 1ml of solution and plating it by the aseptic spread plate technique.

All plates were incubated at room temperature. After 48 hours of growth the Plate Count Agar plates and the Actinomycete Isolation Agar plates were checked and the colonies counted, giving a total of colony forming units (CFU) for each plate. The 10^{-3} plates for the Plate Count Agar and Actinomycete Isolation Agar were not counted because the large amount of colonies did not allow for accurate counts to be made. The fungi on the Sabouraud Dextrose Agar plates were counted after 1 week of growth, and the CFU determined.

The CFU of the three replicates for each soil treatment were averaged together to give a result for that treatment that was then graphed and the standard deviations determined.

From the Plate Count Agar plates some colonies were removed and attempted to be identified using BioLog plates. First a pure culture was obtained on either Plate Count Agar or the BioLog BUG Agar, and a gram stain done. If the gram stain was negative an oxidase test was performed using small prefilled ampules of oxidase reagent. If the bacteria turned blue when inoculated onto a piece of filter paper with a drop of oxidase reagent, then the oxidase test was positive. This helped to further narrow the computer selection criteria in the BioLog computer program. When the initial gram stain and oxidase test done, and the bacterium characterized as a gram positive or negative rod or coccus, then the colonies were inoculated from the pure culture into the BioLog GP/GN inoculating fluid. The turbidity range of the inoculated GP/GN fluid was determined using the BioLog Turbidiometer, which gives a percent transmittance value. The GP/GN

inoculating fluid was within +/- 3% transmittance of the BioLog standard for the type of organism. After the proper transmittance was obtained, 150µl of inoculating fluid was added to each of the 96 wells of the BioLog plate. The BioLog plates were incubated at room temperature as well for 48 to 72 hours, and read by looking for a purple positive color in each well and those positives marked in the BioLog computer database, and an identification obtained based on the pattern of positive wells. The A1 well of the BioLog plate is the control well, all other wells were compared to this one to determine positive purple color change.

Results

Day 0 addition of CSS to soils. There are two sets of treatments of Glynwood soil with CSS. One of these sets of treatments shows greater CFUs than the Glynwood soils without additional treatment on plate count agar. This difference is statistically significant in comparison to the two lower Glynwood soil treatments at a dilution of 10^{-5}, however, this statistical difference is not maintained throughout the graphs (Figure 1). One set of Glynwood soil alone shows a high CFU average at 10^{-4} and 10^{-6}. These points can be viewed as outliers, because the other two sets of Glynwood soil alone do not show such large CFU averages at either of these dilutions. Also the graph containing the outlier is more variable than the repetitions of the same Glynwood soils without additional treatment (Figure 1). The second Glynwood+CSS treatment does not show as large of a difference between it and the Glynwood soil alone curves; however, it too does remain visibly higher than the Glynwood soil alone curves (Figure 1).

At Day 50 the Glynwood+CSS treatments remain greater than the Glynwood soil alone, except for a few points of variability at 10^{-4} and 10^{-6}. The Glywood+oats is also

lower than both of the Glynwood+CSS treatments at 10-4, and remains below one of the treatments until 10^{-7} (Figure 4). However, there is a decrease in the average numbers of CFU between day 0 and day 50. Both day 0 Glynwood+CSS curves show higher CFU than the same treatments at day 50, though the comparable curves do show the same patterns (Figure 10).

The MDD treatments were not repeated as more than one set as with the Glynwood soils. The MDD+CSS treatment did show visible increase in CFU over the MDD soil lacking this treatment at day 0 (Figure 1). The MDD+CSS treatment does shows visibly greater CFU than the MDD soil alone at day 50 as well. This difference is statistically significant at 10^{-5}, however, this statistical difference is not maintained throughout the curves (Figure 4). However, unlike the Glynwood curves, the day 50 curves of MDD+CSS show greater quantities of CFU (Figure 7).

Overall, the addition of CSS to soil seems to increase the bacterial CFU on plate count agar over soils without this additional treatment. The effect of the 50 day incubation period on the soil with CSS varies based on the site. The Glynwood site does not show increased CFU on plate count agar over the 50 days, where as the MDD site does show increased CFU on plate count agar over the 50 days.

The treatment of Glynwood soil and MDD soil with show greater average CFU on actinomycete isolation agar when compared to soils without treatment at day 0. There again is variability among the Glynwood+CSS treatments. The first set of Glynwood+CSS treatments shows visibly greater CFU than all other Glynwood treatments, however, this is not the same for the second Glynwood+CSS treatment, which fall below the Glynwood+oats, and one Glynwood soil without treatment (Figure 2).

However, at day 50 the average CFU of Glynwood+CSS drop below those seen in untreated Glynwood soil (Figure 5). However, when comparing the day 0 and day 50 treatments there is some variability seen. For one set of Glynwood+CSS treatments the day 50 shows lower average CFU than the comparable treatment at day 0, with the two time points being statistically different at 10^{-4}. The second set of Glynwood+CSS treatments do not match the first. The Glynwood+CSS day 50 time point has higher CFU than day 0, however, this is reversed after 10^{-6} dilution (Figure 11). The standard deviations for both sets of time points are large at 10^{-4}, which displays the variability of the data, and the need for more repetitions.

The MDD+CSS shows visibly larger CFU averages than the MDD soil alone, however, the standard deviations do not show that these treatments are statistically different. Also the difference in the treatments is lost at 10-5 because of the low colony formation in the higher dilution for both treatments (Figure 2). The MDD+CSS day 50 treatment is only marginally higher than the MDD soil alone at day 50 between dilutions 10^{-5} and 10^{-8} (Figure 5). At 10^{-4} the MDD soil alone shows a higher CFU average than the MDD+CSS, however, because two of the three trials show high CFU, this data point cannot be regarded as an outlier, therefore more repetitions would determine a more accurate average. Unlike the Glynwood soil time point data, the MDD+CSS curve for day 50 shows more average CFU than the curve for day 0. Though this difference is distinguishable, it is not significant, nor a large visible difference (Figure 8).

Treatment with CSS shows increased CFU of fungi on sabouraud dextrose agar at day 0. Both the Glynwood+CSS treatments show visibly higher CFU curves, however, large standard deviations hides any statistical differences between Glynwood+CSS

treatments and the other Glynwood treatments (Figure 3). The higher CFU averages of the Glynwood+CSS treatment over the Glynwood soil alone is maintained at day 50, however, some variability is seen at 10^{-6}, with one of the Glynwood soil sets, caused by a high CFU count of one of the replicates. The difference between the Glynwood+CSS treatments and the Glynwood soil alone is statistically significant at 10^{-4} (Figure 6). The Glynwood+CSS day 50 samples showed higher average CFU than the comparable curves for the day 0 time point (Figure 12).

The MDD+CSS also shows visibly higher CFU curves when compared to both the MDD soil alone and MDD+oats. These values show statistical difference at 10^{-3}; however, this difference is lost in higher dilutions due to the large standard deviation of the MDD+CSS curve (Figure 3). The day 50 treatments of MDD+CSS show increased average CFU over the day 0 treatments (Figure 9).

Addition of oats. The effect of the CFU with the addition of oats was looked at for both the Glynwood and MDD soils. At day 0 on plate count agar more bacterial CFU were present with the addition of oats when compared to the Glynwood soil with no additional treatment (again ignoring the outlier at 10^{-4} for the untreated Glynwood soil) (Figure 1). However, at day 50 the CFU averages of the Glynwood+oats treatment is highly variable showing both lower and higher average CFU than the untreated Glynwood soil (Figure 4). When comparing the two time points the day 0 curve shows higher average CFU than day 50 for the lower half of the dilutions, and the opposite is true for the higher dilutions (Figure 10).

At day 0 on the actinomycete isolation agar, a similar trend is seen as that for the day 0 plate count agar Glynwood soil with oats. The Glynwood+oats treatment at day 0

shows higher average CFU than the untreated Glynwood soils, however, because of the large standard deviations a statistical difference cannot be determined (Figure 2). At day 50 the Glynwood+oats treatment maintains larger overall average CFU, and this increased CFU show significance at 10^{-5}, 10^{-6}, and 10^{-7} (Figure 5). The curve of day 50 Glynwood+oats treatment is more continuously decreasing through the dilutions as compared to the day 0 time point (as well as the other treatments) that have a drastic decreasing slope between the first two dilutions (Figure 11). This may suggest that the presence of oats help to stabilize the actinomycete population in the Glynwood soil.

In the MDD soil at day 0 the MDD with oats shows higher CFU than the MDD soil with no treatment on plate count agar (Figure 1). However, the curve at day 50 is more variable, beginning below the non treated soil, and rising above in the later dilutions (Figure 4). An increase in CFU is also seen with the addition of oats on actinomycete isolation agar. This greater CFU average is also seen when comparing the MDD+CSS treatment (Figure 2). However, the curve at day 50 resembles that for the plate count agar, there is a low CFU average at 10^{-4}, this average varies little with the next two dilutions giving a plateau appearance to the curve. Therefore, the first dilution does not show a greater average CFU than the untreated MDD soil, however, the greater CFU is seen in the next two dilutions, replicating that seen on day 0 (Figure 5). Like the Glynwood soil with oats, the MDD soil with oats shows a more stable graph, further supporting the possible correlation between the presence of oats and the actinomycete population.

The Glynwood and MDD soils with oats were also plated on sabouraud dextrose agar. At day 0 the Glynwood soils with oats showed more average CFU at 10^{-3} than the

Glynwood soils alone, however, they were not higher than the Glynwood soils with CSS. At 10^{-4} the Glynwood+oats curve is nearly equal to two of the three untreated Glynwood soil curves (Figure 3). However, by day 50 the distance between the Glynwood+oats and Glynwood soil alone curves increases, with Glynwood+oats showing more average CFU, however, the error bars do not reveal a statistical difference (Figure 6). There is an increase in the average CFU of the day 50 curve compared to the day 0, this could be due to the interaction of fungi within the rhizosphere (Figure 12).

The MDD soil shows more CFU than the non-treated soil, however, this varies at the 10-4 dilution because of a high count for the non-treated soil. This variation is due to the large standard deviation of the non-treated soil, because the MDD+oats maintains a small standard deviation throughout the curve (Figure 3). At day 50 the MDD untreated soil falls below the MDD+oats treatment, however, there is not a great difference in CFU (Figure 6). When comparing the day 0 and day 50 curves the MDD+oats shows greater average CFU at day 50 than day 0, however, the day 50 curve is also more variable, so the significance of this distance cannot be determined (Figure 9).

Diversity. The actinomycete isolation agar showed little diversity at both 0 and 50 days. Other colonies of organisms not characteristic of actinomycetes grew up as well on this isolation agar, which would imply a problem with the media in isolating actinomycetes, or very viable non-actinomycete species that will grow on any nutrient source. In general on the plate count agar the samples taken from day 0 had more diversity than those taken from day 50. The predominant organism in the day 0 plates was identified as *Brevibacterium otiditis*, a gram positive rod, growing as a large cream granular colony. At day 50 plates the predominant colony morphology was a white granular colony, also a

gram positive rod. Though an identification of the day 50 sample could not be obtained using the BioLog plate, it is most likely that this is the same species, just more resistant due to growth in the presence of the heavy metals. The colonies on the day 50 plates were typically smaller, and because of the decreased diversity the plate was more monochrome in color. When looking at the diversity of organisms seen on the plates it must be remembered that the majority of microorganisms that live is soil are non-culturable, so the diversity seen on the plate only displays one small portion of the diversity with in the soil (Bakken, 1997).

The fungi were not identified, however, when observing the plates at day 0 and day 50 there was often a shift to growth of different fungal colonies, based on visible observation of the colors and mycelial growth. The day 50 plates had a more colorful array of organisms. Based on observation many of the plates were overrun with what appeared to be *Aspergillus niger*.

Identification. The bacteria identified by the BioLog system are listed in Table 1. Also listed are the probability given by the BioLog computer system and the BioLog system calculated similarity and distance of the test organism to the identification organism. The identification of *Pseudomonas fluorescens* is a logical identification. *Pseudomonas sp* are part of normal soil microflora. *Pseudomonas fluorescens* is also looked at as an organism that can be genetically altered for increased agricultural production. Its name is derived from its ability to fluoress under UV light. The *Burkholderia sp* are in the same group as the *Pseudomonas sp* also present in soils, some of the species are important plant pathogens. *Cellumonas cellasea* is a coryneform bacteria, which are a common bacteria type found in soils. As its name indicates the *Cellumonas sp* is important in

degrading cellulose. *Francisella sp* are often found in animals and arthropods, given this it is very likely that this species would show up in the soil.

Discussion

When comparing the different average CFU curves, it is clear that more replicates of the different types of treatments are needed to make conclusive results, and bring out any statistical significance which is alluded to by the presence of some statistical difference between treatments at particular dilutions. The addition of CSS did increase bacterial and fungal growth at both sites over the soil without the CSS additive. This is most likely due to the increased level of organic matter provided by the CSS, which could be used as a nutrient source. However, it is not conclusive whether the addition of CSS increased the growth of actinomycetes. The Glynwood soil showed variable results as to CFU of actinomycetes with the addition of CSS; however, the MDD soil did show an increased average CFU with the addition of CSS.

The addition of oats showed increased bacterial, actinomycete, and fungal growth in both soils. This occurrence is most likely due to the important rhizosphere interactions that occur between bacteria and fungi. This symbiotic relationship allows for increased growth of microorganisms over soils lacking this interaction.

The effect of the 50 day span was different based on the site from which the soil was taken. The MDD soil consistently showed increased average CFU at day 50 than when the comparable treatments were examined at day 0. The Glynwood soils showed decreased levels of bacteria over the 50 day time period, however the fungi levels increased over the 50 day time period. This same occurrence is seen in another

experiment using Glynwood soil containing a petroleum contaminant in combination of differing levels of two types of chromium (Pichtel, 1992).

It is obvious that there is a difference in the two soil sites, and the subsequent effect on the microorganisms. This difference could be due to the nature of the contamination of the soil. The high amounts of heavy metals, particularly Pb and Cd could allow for quicker selection of resistant microorganisms, and then proliferation of those microorganisms. The lower Pb levels of the Glynwood soil may inhibit growth of microorganisms, particularly the bacteria, because the nonresistant bacteria may metabolically function longer providing more competition and not allowing for the increased proliferation of resistant microorganisms. In addition small successive doses of contaminant metals have a stronger inhibitory effect than one large dose (Doelman, 1985). Because it is unclear as to if the contamination of each site occurred slowly or in large increments it is impossible to say definitely if this could cause the difference in the reaction of the soil microorganisms of the different sites. However, it does remain as a possibility that if the Glynwood soil is being continuously contaminated by small doses of Pb, that the greater inhibition of growth over time could be due to the means by which the soil is being contaminated.

Another factor influencing the effect of the heavy metal contaminants is the makeup of the soil. Glynwood soil has a high concentration of silt. Soils showing the greatest effect of low concentrations of metals are sandy soils (Doelman, 1985). Though silt is not sand, it most likely acts more like sand in the effects of concentrations of heavy metals, and less like clay, which can sustain higher concentrations of metal contaminants before an effect is seen (Doelman, 1985). The fact that the Glynwood soil reacted

similarly in two different experiments, with two different types of contaminants, one being solely heavy metal contaminant, the other also containing a petroleum contaminant, shows how important the soil make up is in contributing to the effect of the contaminants. The development of a bioremediation plan must incorporate the soil type, however, it is not safe to say that universal bioremediation techniques can be developed based on soil type. The contaminant present still requires consideration. In an experiment with Glynwood soil contaminated with fly ash of a powerplant showed decreases in the numbers of bacteria, actinomycetes, and fungi (Pichtel, 1990).

There is also Cd present in the MDD soil. Cd has a more drastic effect on the soil community than Pb (Doelman, 1985). The consistent increase of CFU over the 50 day period could be influenced by the Cd found in the MDD soil, which is not present in the Glynwood soil. Cadmium may select resistant microorganisms more rapidly, and without competition the increased proliferation is seen. More experimentation would have to be done with just Cd and Pb being the contaminants of the soil to make a conclusive observation.

Doelman stated, that heavy metal contaminants often cause a shift to more gram negative rods, and eukaryotes. However, it is inconclusive in this experiment if this is true because many of the original isolates were gram negative rods (data not shown). It is possible that the gram negative species grew more readily on the plate count agar, and therefore masked the presence of more gram positive species. For this observation to be made confidently an experiment must be designed to differentially select for gram negative and gram positive species based on additives that may be added to the medium. Also to truly display this shift other testing of the soil bacteria must be done, such as PCR

identification, because the majority of soil bacteria are nonculturable (Bakken, 1997). These bacteria may be contributing to the change in the microbial population due to the contaminant, as well as any elimination of the contaminant that may ocuur.

However, it is clear that the fungi did fair better in both soil types over the 50 day time period. The presence of, what is thought to be *Aspirgillus niger*, supports the finding of Babich that *Aspergillus sp.* were able to tolerate high levels of Cd (Babich, 1977). The *Aspergillus* was present in both soil types, suggesting that this species can tolerate high levels of Pb as well as other toxic metals, which are present in smaller quantities in both the soils. The results of the fungal analysis would suggest that there is an increased ability of fungi to grow in contaminated soils, and therefore the increased use of fungi in bioremediation projects.

This experiment led to a number of possible conclusions, however, for these conclusions to be effectively supported experiments must be performed to delineate the validity of these conclusions. Increased repetitions would also help to remove the variability seen in growth. A ground work is set for a number of possibilities to be explored further by looking at the effects the different soils as well as the different treatments have on the soil microbial populations.

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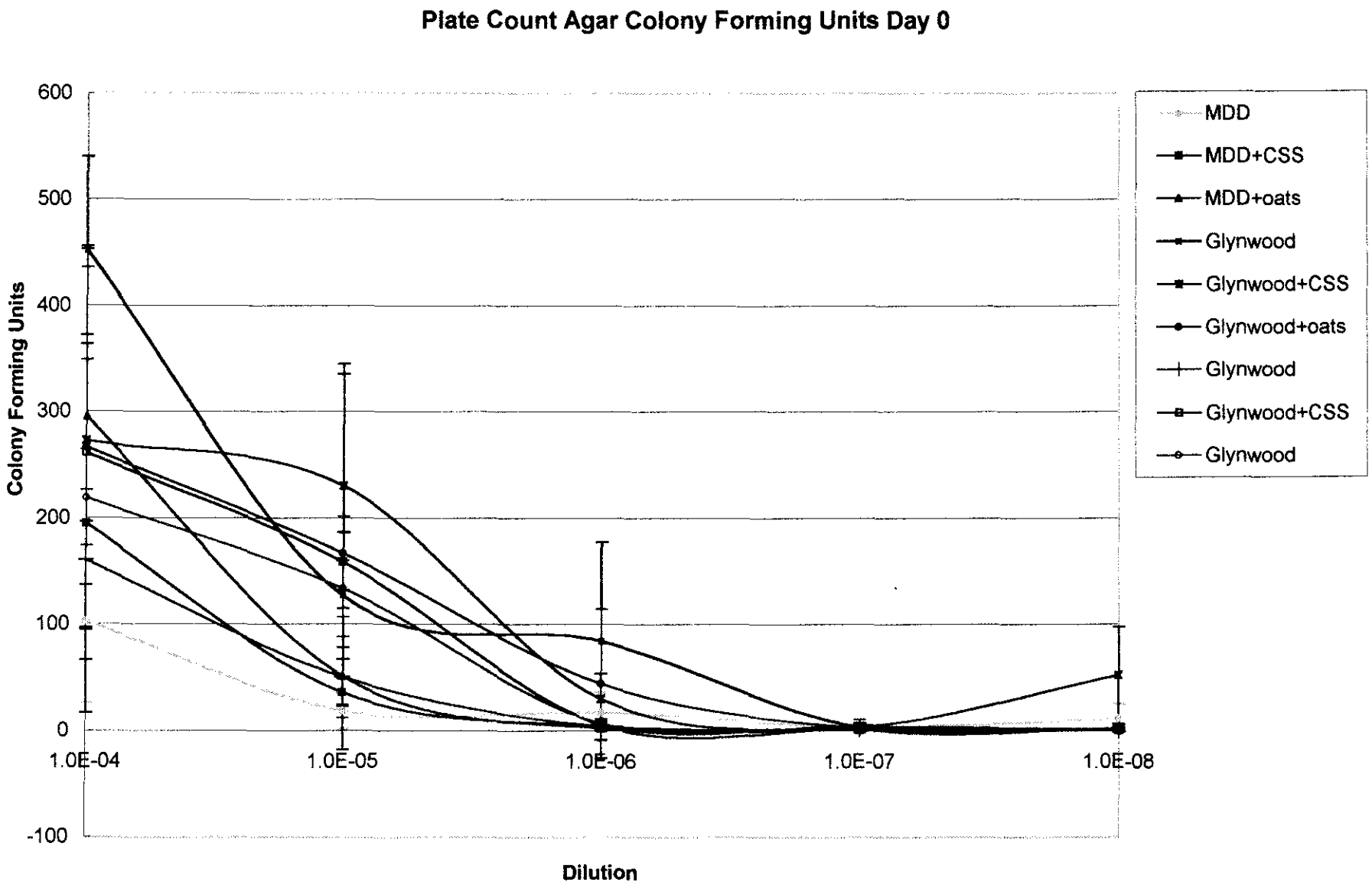
Figure 1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300
200
100
0
-100
Colony Forming Units
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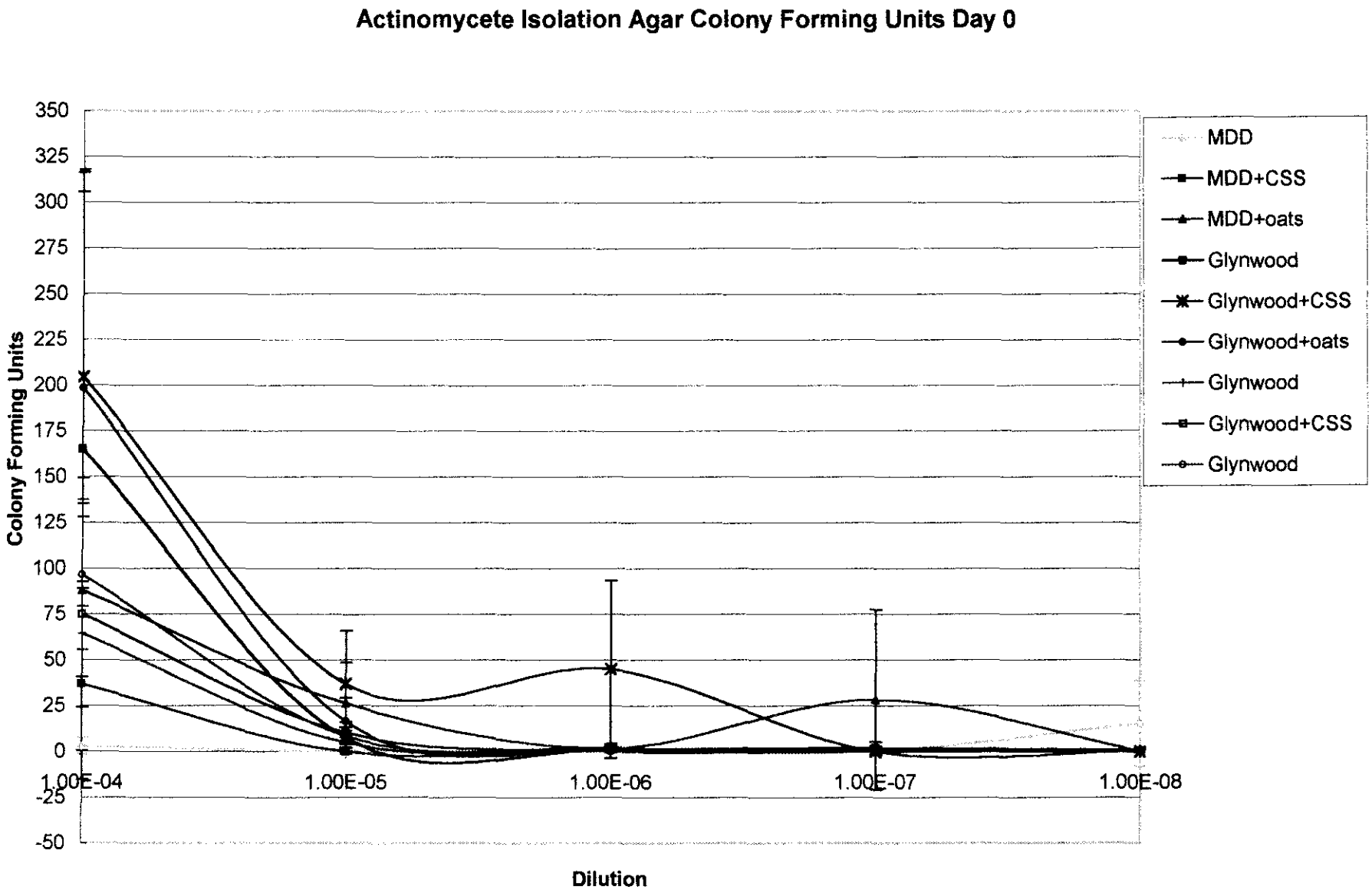
1.0E-05
1.0E-06
1.0E-07
1.0E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 2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175
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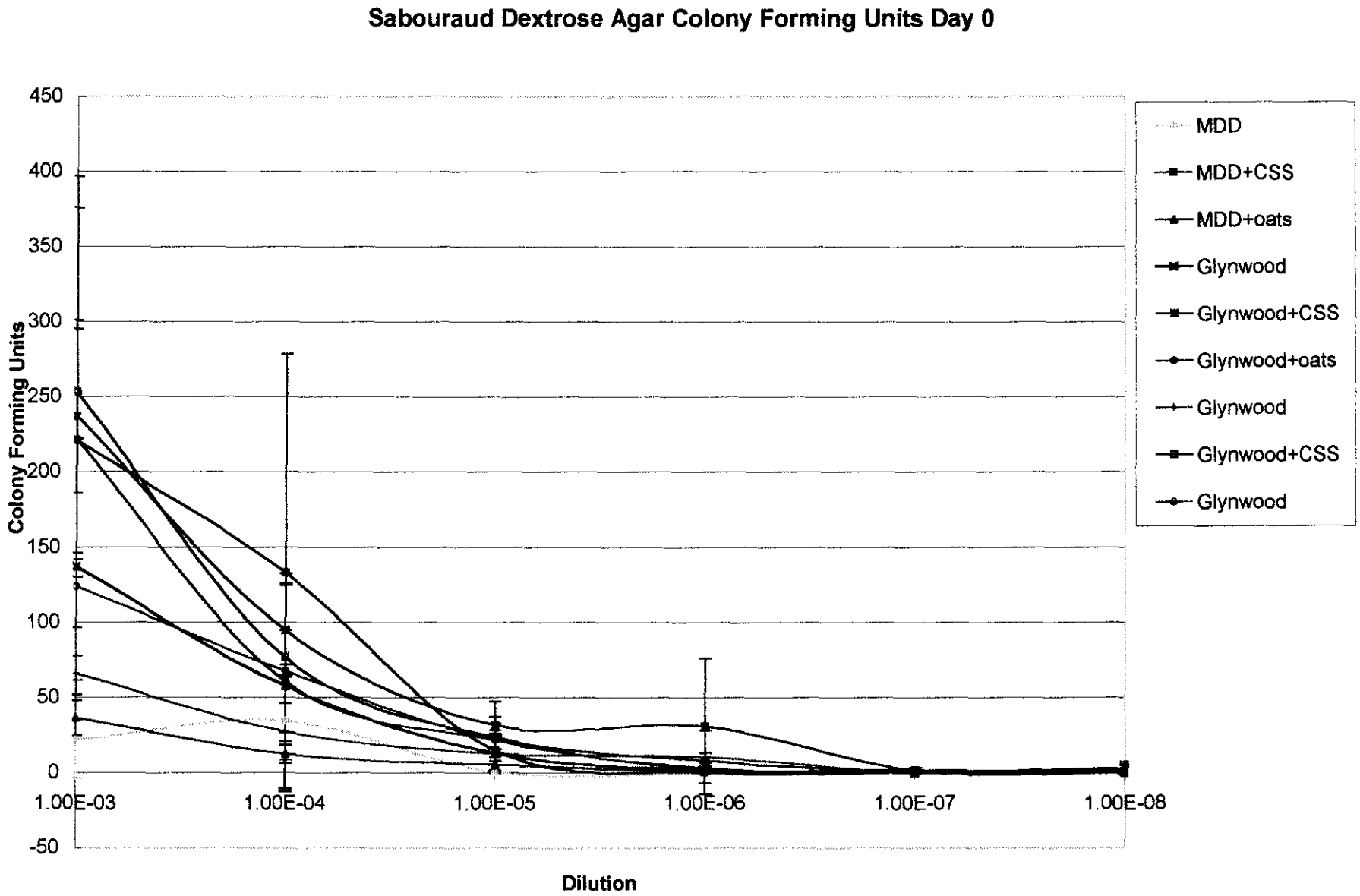
125
100
75
50
25
0
-25
-50
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 3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Colony Forming Units
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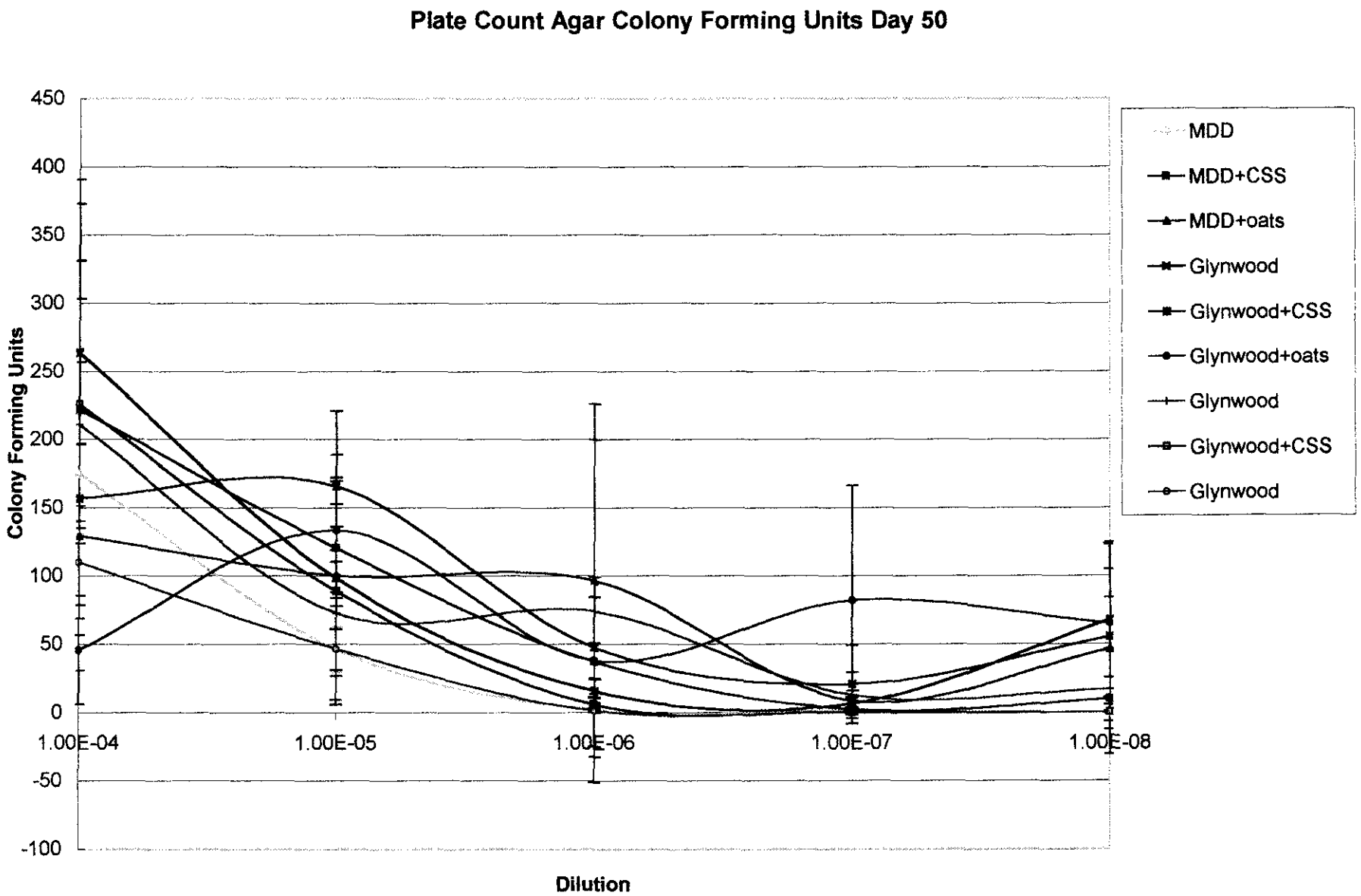
300
250
200
150
100
50
0
-50
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 4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Colony Forming Units
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200
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50
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-100
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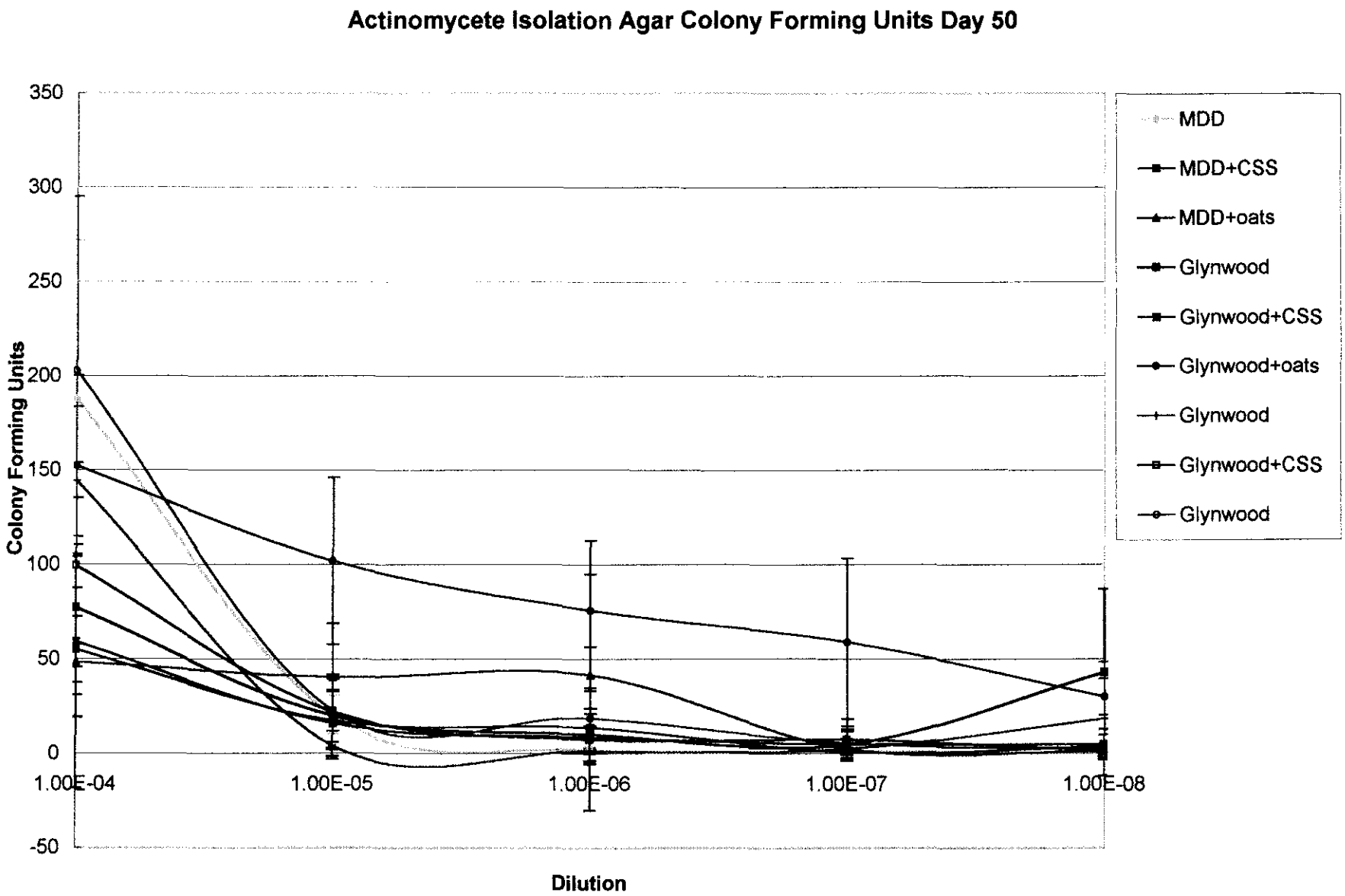
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 5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Colony Forming Units
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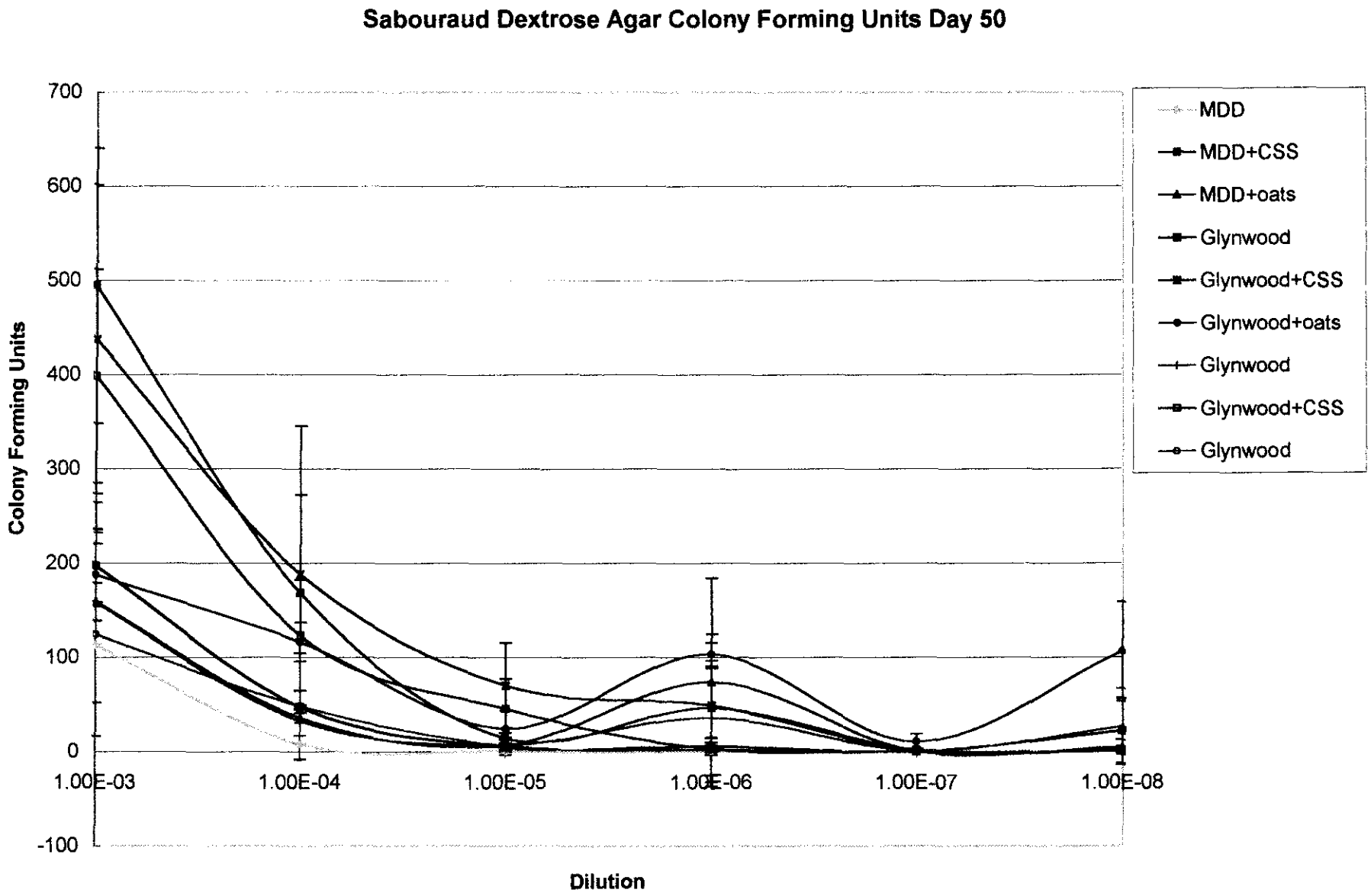
250
200
150
100
50
0
-50
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 6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Colony Forming Units
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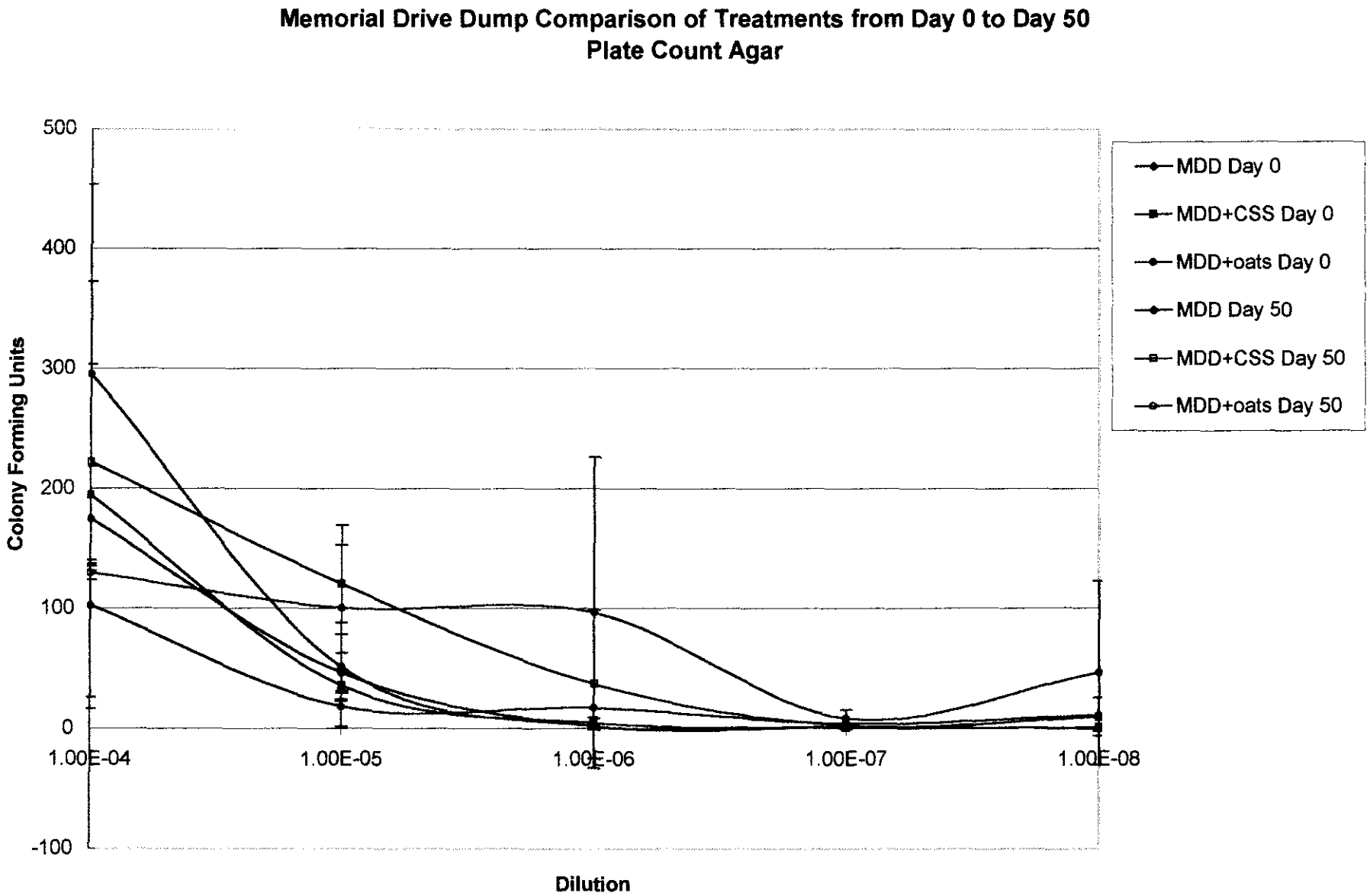
400
300
200
100
0
-100
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD
MDD+CSS
MDD+oats
Glynwood
Glynwood+CSS
Glynwood+oats
Glynwood
Glynwood+CSS
Glynwood

Figure 7


Memorial Drive Dump Comparison of Treatments from Day 0 to Day 50
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Colony Forming Units
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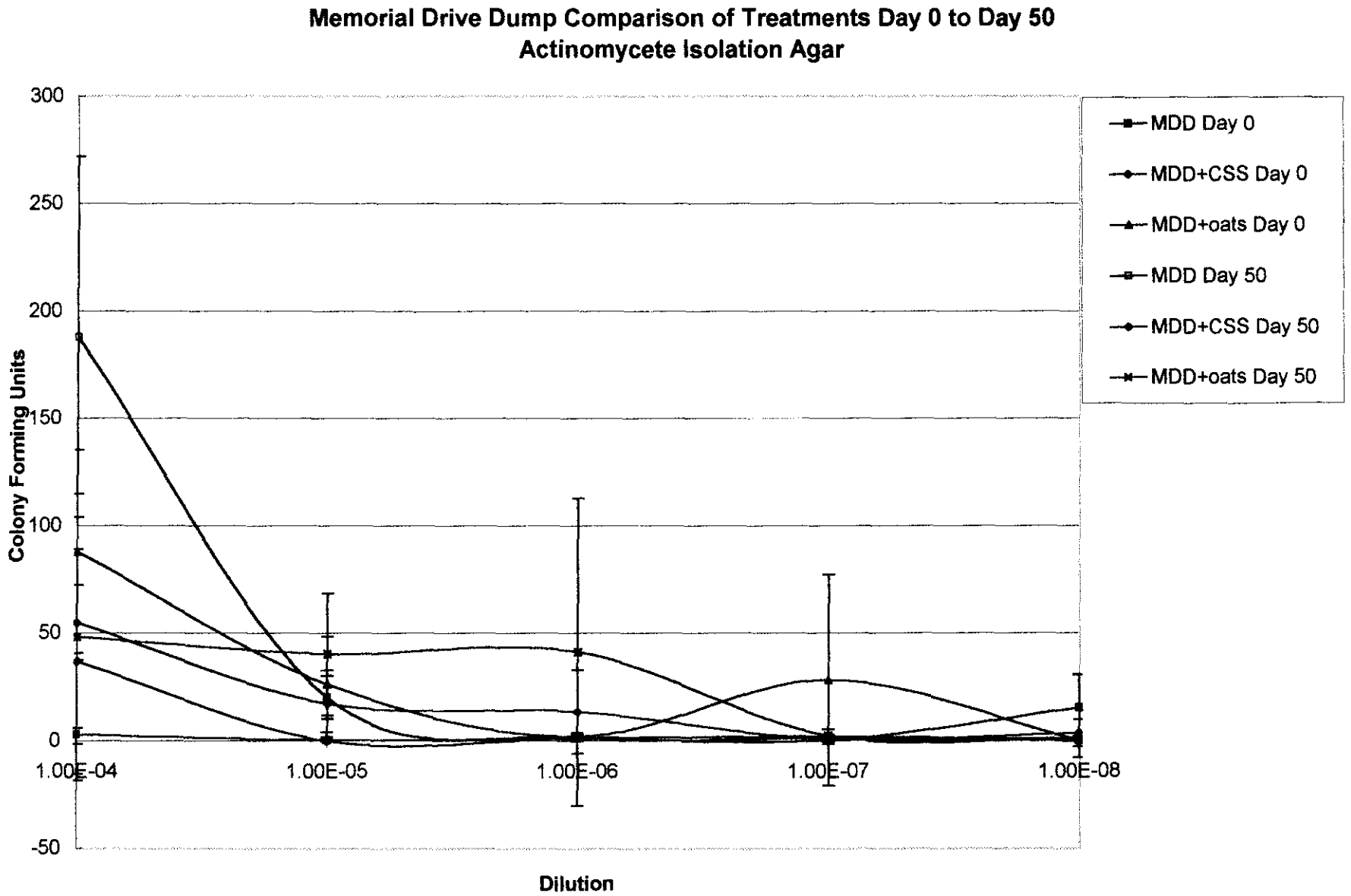
400
300
200
100
0
-100
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD Day 0
MDD+CSS Day 0
MDD+oats Day 0
MDD Day 50
MDD+CSS Day 50
MDD+oats Day 50

Figure 8


Memorial Drive Dump Comparison of Treatments Day 0 to Day 50
Actinomycete Isolation Agar
Colony Forming Units
300
250
200
150
100
50
0
-50
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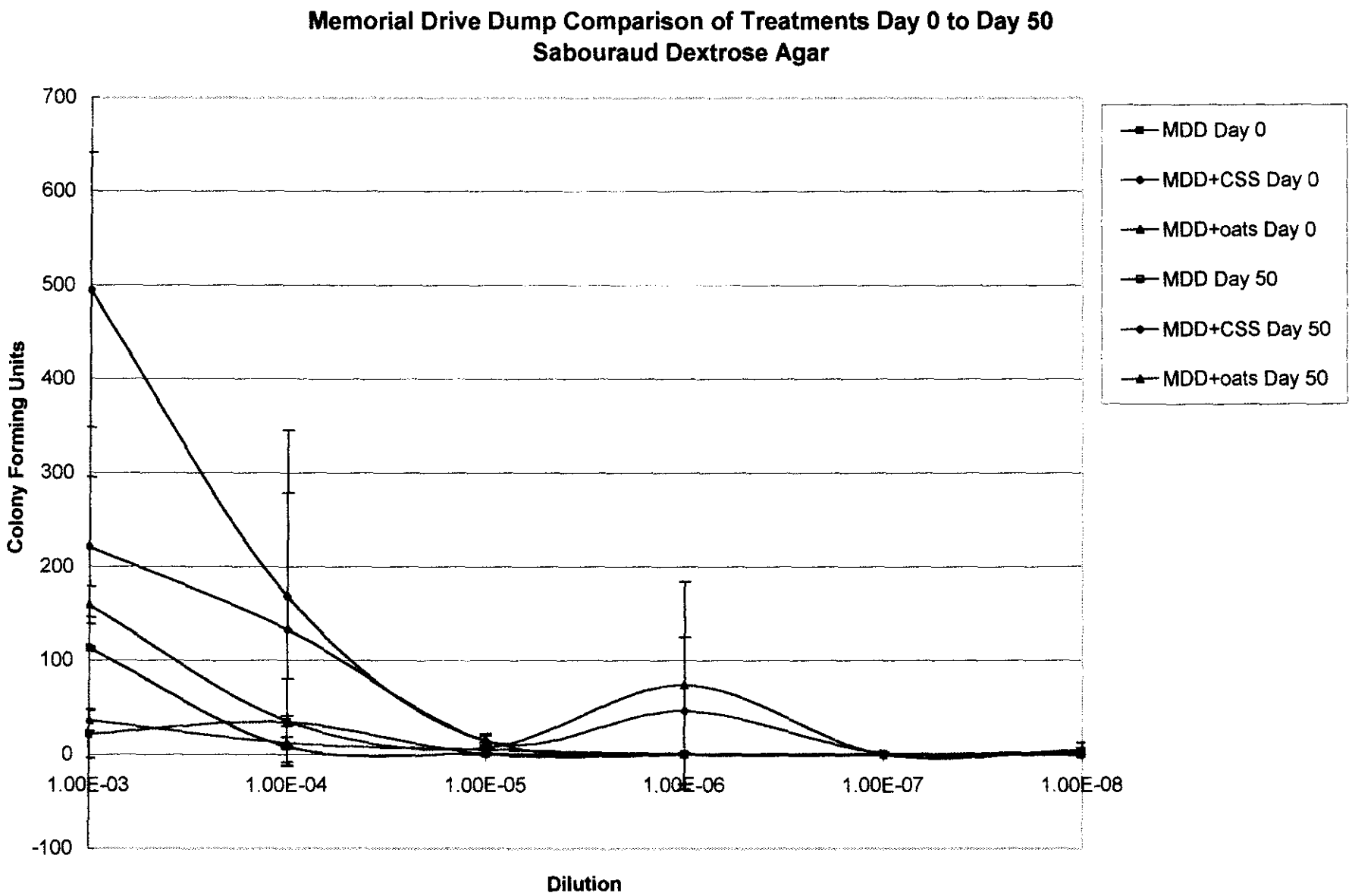
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD Day 0
MDD+CSS Day 0
MDD+oats Day 0
MDD Day 50
MDD+CSS Day 50
MDD+oats Day 50

Figure 9


Memorial Drive Dump Comparison of Treatments Day 0 to Day 50
Sabouraud Dextrose Agar
Colony Forming Units
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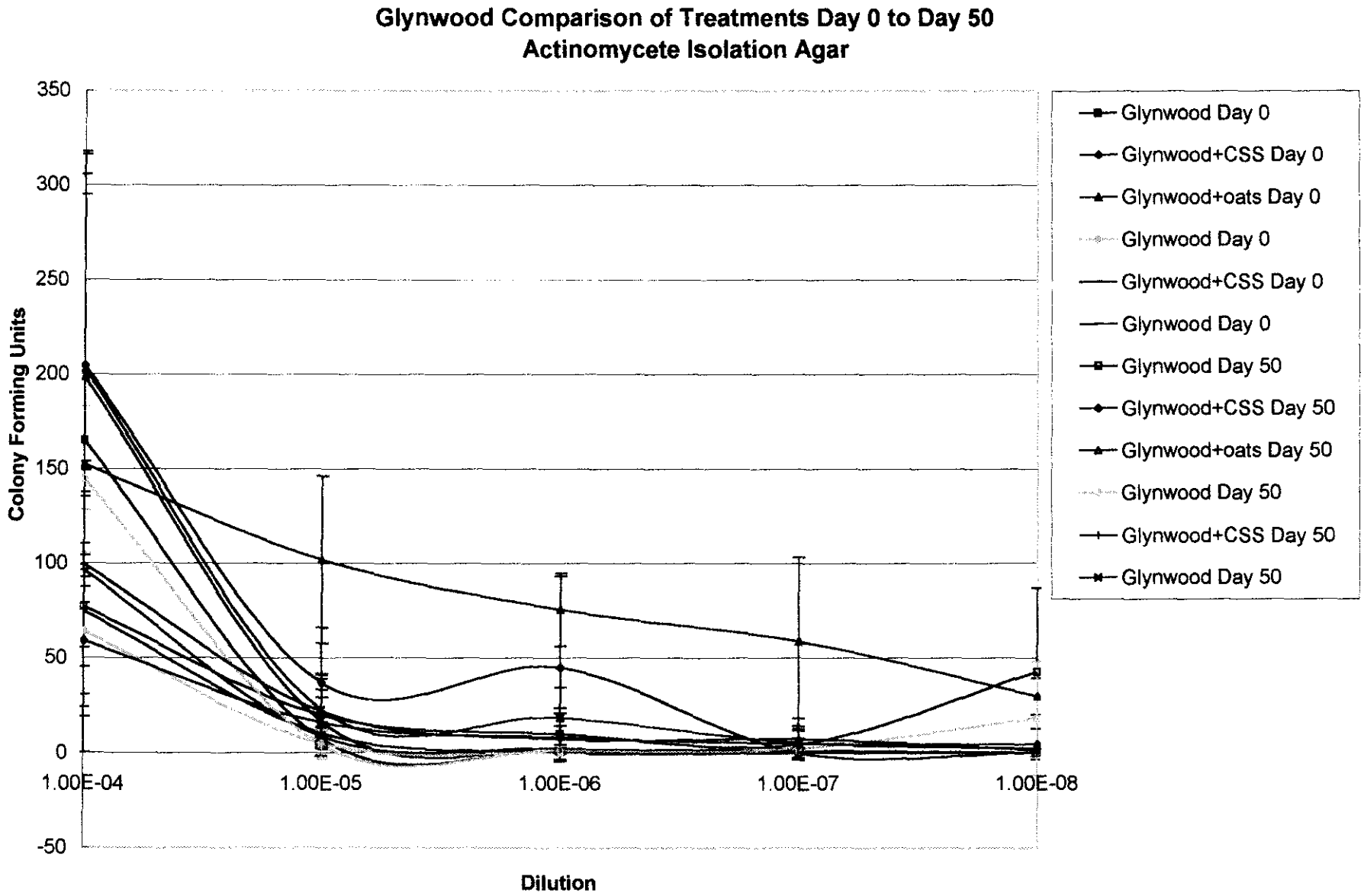
600
500
400
300
200
100
0
-100
1.00E-03
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
MDD Day 0
MDD+CSS Day 0
MDD+oats Day 0
MDD Day 50
MDD+CSS Day 50
MDD+oats Day 50

Figure 11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Colony Forming Units
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200
150
100
50
0
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1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
Glynwood Day 0
Glynwood+CSS Day 0
Glynwood+oats Day 0
Glynwood Day 0
Glynwood+CSS Day 0
Glynwood Day 0
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Glynwood+CSS Day 50
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Glynwood Day 50
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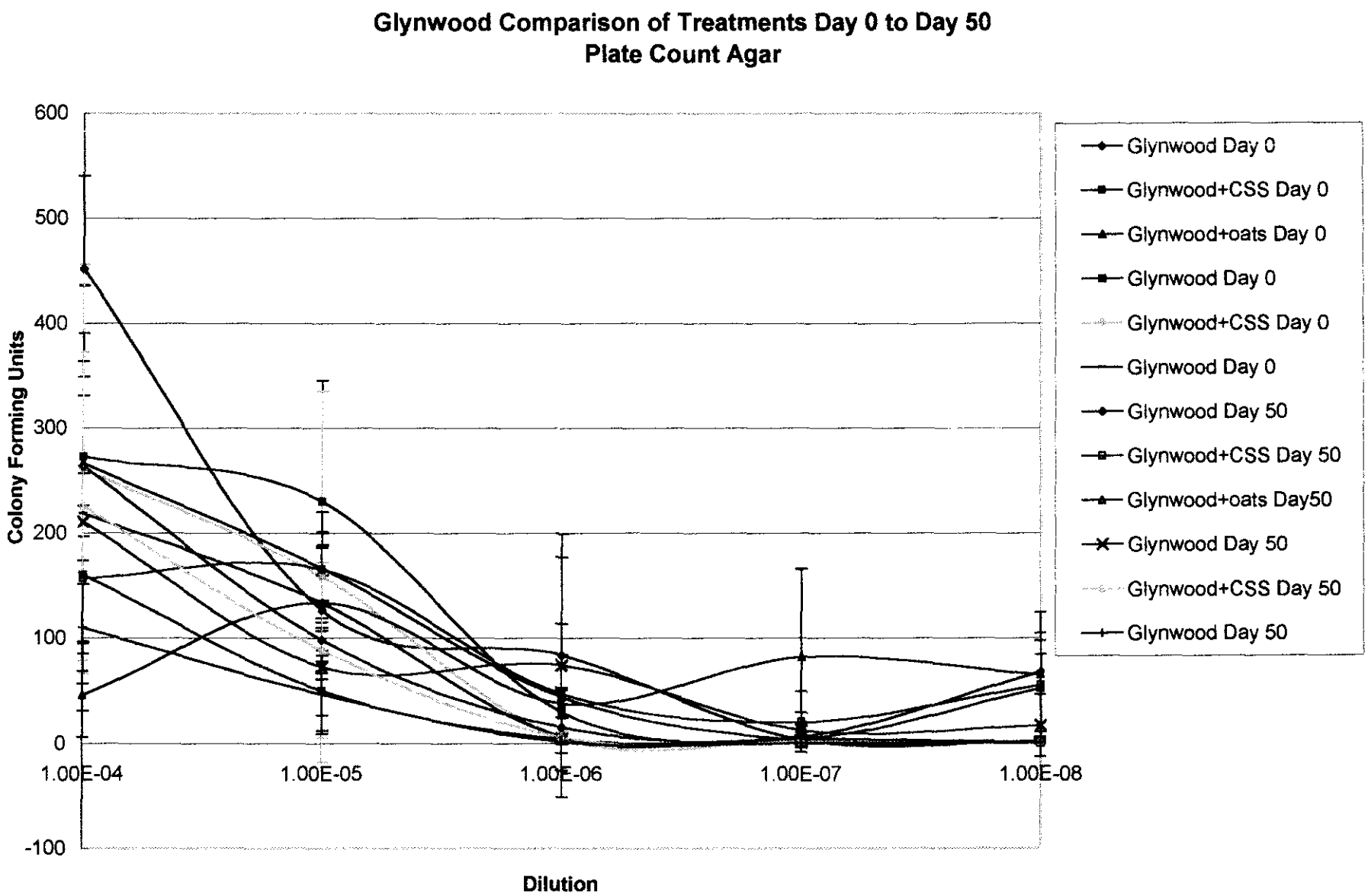
Glynwood Day 50

Figure 10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Colony Forming Units
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300
200
100
0
-100
1.00E-04
1.00E-05
1.00E-06
1.00E-07
1.00E-08
Dilution
Glynwood Day 0
Glynwood+CSS Day 0
Glynwood+oats Day 0
Glynwood Day 0
Glynwood+CSS Day 0
Glynwood Day 0
Glynwood Day 50
Glynwood+CSS Day 50
Glynwood+oats Day50
Glynwood Day 50
Glynwood+CSS Day 50
Glynwood Day 50