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Field Demonstrations of Mycoremediation for Removal of Fecal Coliform Bacteria and Nutrients in the Dungeness Watershed, Washington

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Prepared for
Jamestown S'Klallam Tribe
in fulfillment of Task 2a (Mycoremediation Demonstration)
of the Dungeness River Watershed Final Workplan
for the EPA Targeted Watershed Grant Program (2004)
under a Related Services Agreement with
the U.S. Department of Energy
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Final Report
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Executive Summary

This study focused on the development and demonstration of an innovative biotechnology—mycoremediation—used in conjunction with bioretention cells, as a potential best management practice for the removal of fecal coliform bacteria and nutrients from surface waters in the Dungeness watershed of Washington State. The study is part of a larger body of work that has been conducted under a U.S. Environmental Protection Agency (EPA) Targeted Watershed Initiative in the Dungeness watershed and Bay to encourage innovative community-based solutions to protect and restore clean surface waters.

Mycoremediation is a form of bioremediation that uses conditioned native fungi and fungal mycelium applied to surface soils to remove and degrade contaminants. In this particular application, mycoremediation was used in combination with a bioretention cell (e.g., rain garden), incorporating native vegetation, a soil media mix, and natural microbial assemblages to remove and degrade fecal coliforms and nutrients. The mycoremediation treatment incorporated a layer of fungal mycelium-enhanced alder chip mulch and mycorrhizal fungi applied to plants. For this demonstration, a field site was constructed and flowing surface water was directed to a bioretention cell (control) and a mirror image mycoremediation-treated bioretention cell (treatment) for a comparative study of the bacteria and nutrient removal effectiveness at the field site.

Once the field site was constructed, three phases of the study were implemented. The first phase looked at the fecal coliform and nutrient concentrations in the source water and two outflow pipes from the control and treatment cells after the water was treated. Fecal coliform and nutrient samples from this phase were analyzed on a monthly basis after construction of the cells, plants had been established and a permanent source of water was in place. A dye study was conducted during the second phase of the study in order to better understand the retention time and attenuation rate of water moving through the site. Finally, the third phase involved an inoculation or "spike" experiment that introduced a one-time inoculation of dairy lagoon waste into the source inflow at the site. During this phase, fecal coliform and nutrient concentrations were analyzed at selected time periods from the two cells based on the results of the dye study to further assess the functionality of the system(s) to remove greater concentrations of bacteria and nutrients.

Fecal coliform bacteria were reduced to a significant degree in both the bioretention cell and the mycoremediation cell, based on the results of the monthly sampling conducted during Phase 1 and the Phase 3 spike experiment. During the Phase 1 experiment, the fecal coliform concentrations were reduced from a mean of 30 colony forming units (CFU)/100 ml in the inflow to 10 CFU/100 ml in the bioremediation (control) cell outflow and 3 CFU/100 ml in the mycoremediation (treatment) cell outflow. Although these inflow concentrations are relatively low, fecal coliform was reduced by 66% in the control cell and 90% in the treatment cell. During the Phase 3 spike experiment, a 5-ml spike of untreated dairy lagoon waste (259,000 CFU/100 ml) was introduced every minute for 15 minutes for a total addition of approximately 194,250 CFU into the site inflow. The bioretention cell outflow showed an initial spike of 376 CFU/100 ml at 1 hour, then dropped steadily over time, whereas the mycoremediation outflow never had concentrations greater than 10 CFU/100 ml and remained relatively constant throughout the duration of the experiment with a mean of 5 CFU/100 ml. An exponential decay model was used to evaluate the difference between the treatment and control fecal coliform concentration response for the 17-day duration of the experiment. There was a statistically significant difference between bioretention and mycoremediation cells between 1 and 28 hours, however after 29 hours a steady state was reached, where

the fecal coliform concentrations were reduced from a mean of 172 CFU/100 ml in the source water to a mean of 13 CFU/100 ml in the control cell and 5 CFU/100 ml in the treatment cell. Once this steady state was reached, fecal coliform was reduced by 92% in the bioretention cell and 97% in the mycoremediation cell. In both Phase 1 and Phase 3 experiments, fecal coliform bacteria were decreased significantly in the bioretention cell, and to a greater degree in the mycoremediation cell. This is likely due to the enhanced predation of bacteria through the extensive mycelial network that is associated with the fungal species in the alder chip mulch as part of the mycoremediation treatment.

The nutrient results were more difficult to evaluate, primarily because the data showed varying trends of nutrient export or removal over time. During the Phase 1 experiment, total nitrogen (TN) was exported from both the bioretention and mycoremediation cells (i.e., higher concentration in the outflow compared to the inflow) between July and October 2007; however, concentrations were reduced in the outflow of both cells compared to the inflow between October and January 2008. During the Phase 3 spike experiment, TN concentrations were reduced in both cells for the duration of the experiment (24 days), and to a greater degree in the mycoremediation cell. Alternatively, total phosphorus (TP) was consistently exported from the bioretention and mycoremediation treatment cells during both the Phase 1 and Phase 3 experiments, although the export was less in the mycoremediation cell during the Phase 3 experiment. In general, the varying trends in the nutrient data are consistent with results obtained from other field and laboratory studies of bioretention cell effectiveness. Although nutrient reduction can be achieved with bioretention and mycoremediation treatments, careful attention must be paid to the design of the field site. Other studies have shown that nitrogen reduction can be achieved if an anaerobic zone or water saturation layer is incorporated into the bioretention cell design and an organic carbon source is supplied to the site to enhance de-nitrification and removal of nitrates. In our study, the bioretention and mycoremediation cells contained zones that were submerged in water for frequent but intermittent time periods. These shifted as flow rates changed and seasonal fluctuations occurred. In the mycoremediation cell, it is likely that nutrient removal was increased by the addition of mycorrhizal fungi to the plants, which can enhance plant establishment by increasing the nutrient absorption capacity of root systems and improving soil structure.

The application of a mycoremediation treatment to a variety of field settings is relatively straight-forward and, given the appropriate landscape conditions, is appropriate for the reduction of fecal coliform bacteria. We designed a comparative field study and examined the functionality of a bioretention cell compared to a mycoremediation-treated bioretention cell as an enhanced treatment for the removal of fecal coliform bacteria and nutrients. While the bioretention cell itself performed well at reducing fecal coliform bacteria, the mycoremediation treatment provided a greater reduction of bacteria. This was particularly evident during the spike experiment where a higher concentration of bacteria and nutrients were introduced into the cells. Unfortunately, funding did not support a more thorough and quantitative evaluation of the technology (e.g., independent application of mycoremediation treatment without a bioretention cell, controlled laboratory/mesocosm settings, or rigorous replication across numerous bioretention cells). Hence, an application of a mycoremediation treatment alone would need to be evaluated on a site-specific basis taking into consideration the contaminants of interest, landscape characteristics, soil type, and hydrology. In general, bioretention cells require moderate to extensive site preparation and are viewed as somewhat permanent installations, whereas the mycoremediation treatment can be considered a temporary installation requiring no excavation and is less expensive to deploy than a bioretention cell. The application of either or both biotechnologies is highly dependent on the specific site needs being addressed.

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1.0 Introduction

1.1 Background

The Dungeness watershed is located on the Olympic Peninsula of northern Puget Sound in Washington State. The river originates in the Olympic Mountains and flows 32 miles downstream through wilderness, forested, agricultural, and residential areas to Dungeness Bay. The 200-square-mile watershed harbors more than 200 fish and wildlife species and is an important stop for migratory waterfowl. Dungeness Bay is home to the Dungeness National Wildlife Refuge, and serves as a refuge, preserve, and nursery ground for native birds, fish, and shellfish species. For over 20 years, local and regional institutions and collaborative partnerships have worked to protect and maintain ecosystem functions in the Dungeness watershed. However, the area has been slowly converted from forest to agricultural and residential land uses. The Dungeness River supports an extensive irrigation network serving the agricultural and residential community. In recent years, human-induced impacts have impaired the natural function of the river and bay. A variety of watershed health problems have ensued, including the listing of salmonid species under the Endangered Species Act and closure of Dungeness Bay to shellfish harvesting beginning in 2000 (Sargeant 2004) due to high levels of fecal coliform bacteria. Although some improvements have been made, failing septic systems, impaired in-stream flows, pollutant inputs from stormwater runoff, and floodplain development continue to persist.

In 2004, the Jamestown S'Klallam Tribe and its partners were awarded a U.S. Environmental Protection Agency (EPA) Targeted Watershed Initiative grant to focus surface water cleanup efforts in the lower Dungeness Watershed. This national grant program was established in 2003 to encourage innovative community-based approaches and management techniques to protect and restore clean water in the nation's watersheds. The Dungeness Targeted Watershed Initiative has focused its efforts from 2004 to 2008 on a number of objectives and tasks that are ultimately related to restoration activities in the watershed. The following tasks are included as part of the Initiative:

Task 1 a Microbial Source Tracking study to more precisely define pollutant sources;

Task 2 innovative best management practice (BMP) demonstrations (and market-based incentives for BMP implementation) related to water quality treatment including a mycoremediation treatment demonstration, septic system maintenance, and water conservation; and

Task 3 an Effectiveness Monitoring study, to compare the effectiveness of various BMP demonstrations within the watershed and examine the historic context within the watershed.

This document focuses on reporting the results of *Task 2(a) Mycoremediation Demonstration*, the development and demonstration of an innovative biotechnology—mycoremediation—as a BMP for the removal of fecal coliform bacteria and nutrients from surface waters in the Dungeness watershed. Mycoremediation is a form of bioremediation that uses conditioned native fungi, or mushrooms, and fungal mycelium that are usually applied to soil to remove and degrade contaminants. In this particular application, mycoremediation was used in combination with a traditional bioretention cell, or rain garden technology (Hinman 2005), thereby incorporating native vegetation, soils, and natural microbial assemblages to remove and degrade contaminants. For this demonstration, a field site with flowing surface water was used to examine the effectiveness of a bioretention cell compared to a fungal-enhanced

(mycoremediated) bioretention cell for remediating fecal coliform bacteria and nutrients in runoff water. Additional reports are available that describe *Task 1*, the microbial source tracking study (Woodruff et al. (a), in preparation) and *Task 3*, the Effectiveness Monitoring study (Woodruff et al. (b), in preparation) of the Dungeness Targeted Watershed Initiative.

1.2 Objectives

The objectives of the Dungeness Targeted Watershed Initiative fall under a larger body of ongoing activities including the short- and long-term goals of the watershed plan for Watershed Resource Inventory Area (WRIA) 18, which includes the Dungeness River (Elwha-Dungeness Planning Unit, 2005). The goals of the Dungeness Targeted Watershed Initiative that are shared with the long-term goals WRIA 18 plan include the following:

- Increase the use of BMPs associated with improving water quality.
- Improve the water quality in the Dungeness Watershed and Bay to meet shellfish harvest and freshwater standards, and to meet restoration targets.
- Mitigate the impacts of stormwater runoff.

Complementing the above goals, the Mycoremediation Demonstration task (*Task 2a*) had the following specific objectives:

- Determine the technical effectiveness of a fungal-enhanced (mycoremediation) bioretention cell at reducing fecal coliform bacteria and nutrients from surface water runoff in a field setting.
- Compare the technical effectiveness of the mycoremediation bioretention cell to that of a bioretention cell without mycoremediation.
- Provide guidance on the use and effectiveness of the technique as a BMP for reducing fecal coliform bacteria and nutrients in other watersheds.
- Improve overall functional habitat value by restoring native vegetation, while remediating contaminant levels.

1.3 Mycoremediation Overview

Mycoremediation is an innovative biotechnology that uses living fungus for *in situ* and *ex situ* cleanup and management of contaminated sites. The process typically begins with field collection of fungi from a local area and continues with steps of culturing, screening, and preconditioning of native species to remediate specific contaminants, if necessary, such as petroleum hydrocarbons, biological pathogens, organophosphates, and metals, at increased efficiency under particular environmental regimes (Thomas et al. 1999a). Once a pure culture of fungus has been obtained, it can then be transferred to sterile growth substrate to increase the culture volume and finally to an appropriate growth medium, such as wood chips, for application to field sites (Figure 1). At this stage, it can be applied in a landscaping setting and incorporated as part of planted beds, bioretention and biofiltration cells, riparian buffer zones, or bank-stabilization projects (where there is an addition of appropriate mycorrhizal fungi and partner plants).

Fungus also can be used in combination with their mycorrhizal partner plants for remediation and restoration. In a mycorrhizal association, a symbiotic relationship forms between a fungal species and the roots of a host plant. For example, if a nutrient, such as nitrogen, is in short supply, mycorrhizal fungi will transfer it to the host plant in exchange for carbohydrates the plant has derived from photosynthesis. Benefits to both species can include bidirectional movement of nutrients and minerals, enhanced uptake of water, and protection against pathogens. Plants with a mycorrhizal symbiont are often better able to tolerate environmental stress as well.

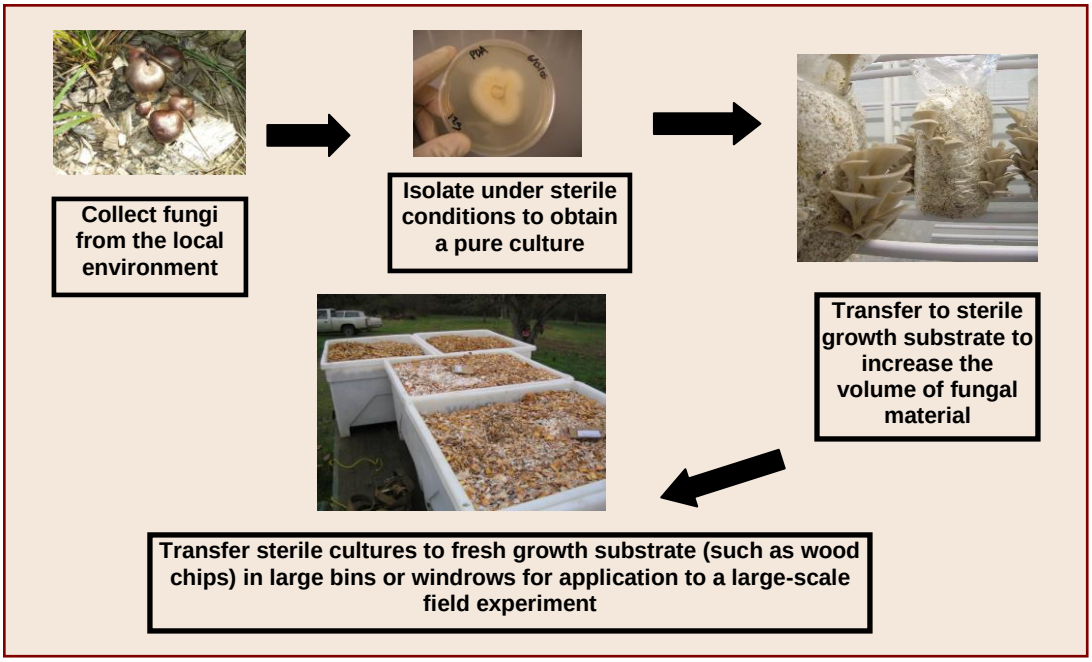

Figure 1. Mycoremediation Preparation Process for Application to Field Settings

Filamentous fungi spread underground by sending threadlike mycelium throughout the soil. Mycelium is the perennial body of the fungus: a loosely organized mass of cells that permeates the substrate at a density of ~1 mile of mycelium/in^3. Fruiting bodies, which are commonly known as mushrooms, are the visible parts usually seen above the soil; they are formed from the mycelia at certain times of the year to carry out the sexual reproduction of the organism. It is in the underground (mycelial) portion of the fungal system where the nutrient uptake and exchange and bacterial predation take place.

1.3.1 Mycoremediation of Bacteria

It has long been known that certain fungi produce antibiotics that kill bacteria (Barron 1992). Various species of fungi, particularly the wood-degrading Basidiomycetes, are predators of bacteria and nematodes, whereas other species use spores as their specialized food source. For example, *Pleurotus ostreatus*, the oyster mushroom, typically preys on fecal coliform bacteria (e.g., *Escherichia coli*) as a source of nitrogen (Barron 1988, 1992). *Agaricus bisporus*, the edible table mushroom and other fungi, have degraded both Gram-positive and Gram-negative bacteria in experimental tests by their natural release of a suite of enzymes (Fermor and Wood 1981). These fungi have demonstrated the capability to chemically sense the presence of bacteria colonies, initiate specialized growth to reach them, and secrete

compounds to digest the bacterial colonies (Barron 1988; Barron and Thorn 1987; Fermor and Wood 1981). Another experiment documented that motile bacteria were attracted to and concentrated around crystal exudates produced by proprietary fungal strains, in large numbers, and were subsequently immobilized and digested (Word et al. 1998; Thomas et al. 1999b, 2000).

In a pilot-scale field application of mycoremediation in Mason County, Washington, using fungal mycelium grown in straw/wood chips, demonstrated that the fecal coliform concentration of waste produced from horse pasture runoff was reduced by 50% before the effluent was transported by natural waters to commercial shellfish-growing beaches. In this case, the runoff was retained for a sufficient time period as it passed through the natural drainage areas containing the fungal-enhanced wood chips to allow predation of the bacteria by the fungal system. Based on the pilot-scale study results, mycoremediation was recommended as a BMP in Mason County to control bacterial pollution from agricultural runoff (Hayward and Stamets 1998).

1.3.2 Mycoremediation of Nutrients

Fungi require nitrogen for synthesis of proteins, nucleic acids, coenzymes, and chitin, and can obtain nitrogen from various sources. Most fungi can use ammonium as the sole source of nitrogen, and many (such as ecto/endomycorrhizal species) have the necessary enzymes to make use of nitrate and nitrite as nitrogen sources as well. The metabolic process within the fungal body yields ammonia as a product that is either transformed and used by the fungus or transferred to a partner plant for its benefit. In addition, all fungi can use organic nitrogen-containing compounds to supply their needs (Jennings 1995).

Phosphorus also is required by fungus for its growth. In many artificial growth media, phosphorus is supplied in the form of orthophosphate, which is naturally found in soils, and which can be absorbed and used by plants. In nature, fungi readily break down organic phosphorus compounds in the remains of dead plants and other organisms, and can release phosphorus from organic phosphates in soil through its phosphatase enzymes, bringing into solution many otherwise insoluble phosphorus compounds.

A 2003 bench-scale experiment used a consortium of fungal species to reduce a dairy lagoon waste cap and to test its nutrient and coliform reduction ability (Thomas et al. 2003). Nitrogen and phosphorous reduction targets were met or exceeded: organic nitrogen (target 25% reduction) was reduced by up to 31%, and phosphorus (target 20% reduction) by up to 46%. In addition, the cap volume was reduced by 25% to 40%.

1.4 Bioretention Cells for Water Treatment

Bioretention cells are used increasingly as an alternative approach to conventional stormwater runoff management practices in urban and agricultural areas to reduce sediment loads, manage water release, and treat selected contaminants through the use of natural processes (e.g., urban and general applications – Baker and Revel 1999; Hammer 1992; Reed et al. 1995; Sands et al. 1999; Kadlec 1999; Scholes et al. 1999; Kim et al. 2003; Hunt 2003; Hseih et al. 2007; and agricultural applications – Geary and Moore 1999; Khatiwada and Prolprasert 1999). Bioretention cells are generally used for managing stormwater runoff from developed areas that include layers of engineered soil/sand/organic media or shredded hardwood mulch supporting a mixed vegetative layer. Bioretention cells are considered a stormwater BMP that is integral to the low-impact development philosophy (Davis et al. 2006). In most cases,

stormwater is directed to a bioretention area where it pools (typically 15 to 30 cm) and infiltrates. Between precipitation events, the bioretention cell is designed to remain relatively dry. Biologically mediated pathways that involve both microorganisms and plants are recognized as important components of effective conversion and reduction of nutrients and bacteria (Davis et al. 2006). Bioretention cells can be designed with an under-drain, although this feature is normally not installed except where the underlying soil has poor infiltration characteristics, where sampling of effluent is desired, or where infiltration is not allowed due to infrastructure or groundwater considerations.

The fate of nutrient compounds in bioretention cells is highly dependent on the timeframes for infiltration through these cells. Usually limited contact times during a rapid runoff infiltration event will allow physical process reactions and some very rapid chemical reactions to take place, including adsorption and ion exchange. However, contact times are too short for slower biogeochemical transformations (Hsieh et al. 2007). For example, ammonium, which is cationic in aqueous solution, is typically immobilized by negatively charged clays and organic matter in soils through sorption and ion exchange processes (Hook 1983). Alternatively, nitrate may be minimally held by typical bioretention media and, as an anion, is very mobile in soils and will not adsorb to soil media to any significant extent, usually accounting for poor removal of nitrate from bioretention cells. A second timeframe comes into play between infiltration events, where bioretention cells may drain and dry significantly. The second timeframe allows sufficient time for more complex chemical and biological transformations to occur in the bioretention media, such as aerobic nitrification or biological denitrification in anoxic zones. Bioretention cells can be designed and constructed with an anaerobic zone for targeted removal of nitrogen. This is typically done by providing a storage reservoir for treatment water.

Although very few bioretention field sites have been extensively tested, results vary widely in terms of the reduction of nutrient levels. The performance of bioretention cells is dependent on a number of factors, including the composition and concentration of input nutrients, infiltration frequency and rate, seasonal variations regarding flow, the fill media composition, and the inclusion of anaerobic zones of saturation and fill media amendments (e.g., straw, leaf compost) in the overall design (Davis et al. 2001; Davis et al. 2006; Hsieh et al. 2007; Hunt et al. 2003). Limited data regarding stormwater infiltration show bioretention cells to be moderately effective in nutrient removal overall. The Center for Watershed Protection (CWP) lists median removal efficiencies for total phosphorus (TP) as 70%, and total nitrogen (TN) as 51% (CWP 2004). Alternatively, a number of studies have shown bioretention cells to export both nitrogen and phosphorus under certain conditions (Davis et al. 2001; Davis et al. 2006; Hsieh et al. 2007). In addition, a compilation of data using wetlands to treat stormwater runoff shows nitrate removals ranging from -193% (nitrate export) to 99% reduction. The TP data ranged from -55% (TP export) to 89% (Carlton et al. 2001). However, none of these studies specifically address the enhancement of bioretention cells with the addition of conditioned fungal-enhanced mulch (mycoremediation) for contaminant removal as we have done here, and we are not aware of any other studies or demonstrations that have conducted this type of enhancement.

1.5 Approach

In this field demonstration, we constructed two identical bioretention cells, including native plants planted in an identical pattern for both cells; however, one cell received an enhanced fungal surface mulch layer with the addition of a mycorrhizal fungi mix that was applied to each plant (treatment cell) and the second cell contained a traditional surface mulch layer with no addition of mycorrhizal fungi to

plants (control cell) for comparison. We used mycorrhizal fungi to address nutrient reduction and lignin (wood) degraders that are predatory to bacteria for fecal coliform reduction. The cells received a continuous source of water, rather than intermittent sources, as is traditionally done in stormwater runoff bioretention cells designed to handle first-flush runoff from a rain event (Davis et al. 2001). Because this was a field study, and due to the nature of the site location and design, our goal was to understand, in general terms, what the removal efficiency (or export) of bacteria and nutrients was based on effluent collected from the underdrain pipe outflow. We studied the system as part of our regular monthly monitoring for the Dungeness Targeted Watershed Initiative and also during a time when a spike of dairy lagoon waste was added to the influent.

2.0 Methods

2.1 Demonstration Site Construction

Methods used to construct the demonstration site, monitor fecal coliform and nutrients, conduct a fluorescein dye study, conduct a dairy lagoon waste-loading experiment, and analyze results are described in the following sections.

2.1.1 Site Description

The study site was located in an agricultural setting in the lower Dungeness Watershed. At one time, the site had been used as an irrigation overflow ponding area, but it had been inactive and dry in recent years. The site was located on residential property, adjacent to pasture land and tidal wetlands that are connected to the Strait of Juan de Fuca (Figure 2). A continuous source of water was supplied to the site from a nearby irrigation ditch.

2.1.2 Construction Methods

Overview. Construction of the bioretention cells and selection and placement of native plants followed modification of methods referenced by Hinman (2005). The mycoremediation approach and application of fungal-enhanced mulch were methods derived from Thomas et al. (1999a, 2003). The demonstration project involved a multi-phased process that included site construction, routine monitoring of fecal coliform and nutrients conducted as part of *Task 3* – the Effectiveness Monitoring study, and an enhanced loading field experiment using dairy lagoon waste.

Figure 2. Location of Mycoremediation Site Adjacent to Tidal Wetlands

Bioretention cell construction. The construction design of the bioretention cells used methods described by Hinman (2005) with the following modifications. Because the primary purpose of the study was to examine the effectiveness of an enhanced fungal application to the site (e.g., mycoremediation), we designed twin biofiltration cells that were adjacent to each other and separated by an impermeable barrier. Each cell received water from the same source, with separate effluent pipes for measuring contaminant concentrations. A schematic of the twin cells is shown in Figure 3. When the bioretention cells were fully functional, source irrigation water was fed into a flow-splitter distribution vault (38 cm by 56 cm by 30 cm). The influent water was then gravity fed from the distribution vault to the cells, located at a slightly lower elevation. Water was discharged into the two cells at the surface over a layer of river rock used for energy dissipation. Water flowed across and down through the cells. Water was removed from the cells by either 1) a gravity-fed underdrain outflow pipe, accessible for capturing effluent from the treatment and control cells, 2) exfiltration, or 3) evapotranspiration. A cross-sectional view of the bioretention cell schematic is shown in Figure 4.

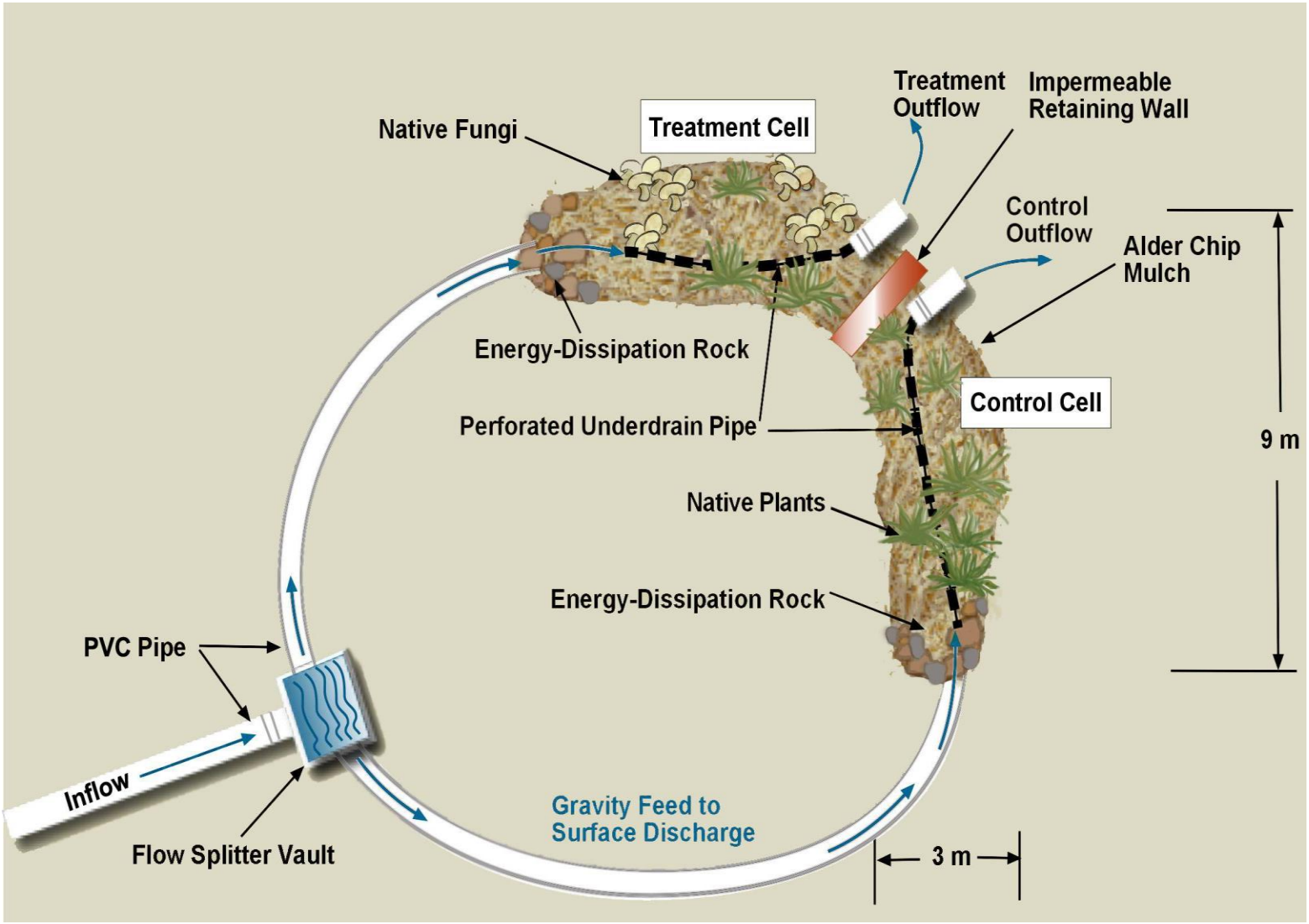

Figure 3. Schematic of Twin Biofiltration Cells, with Native Plants and Fungi in the Treatment Cell and Native Plants only in the Control. Inflow water is split in the distribution vault with equal volumes gravity fed to the two cells (not to scale).

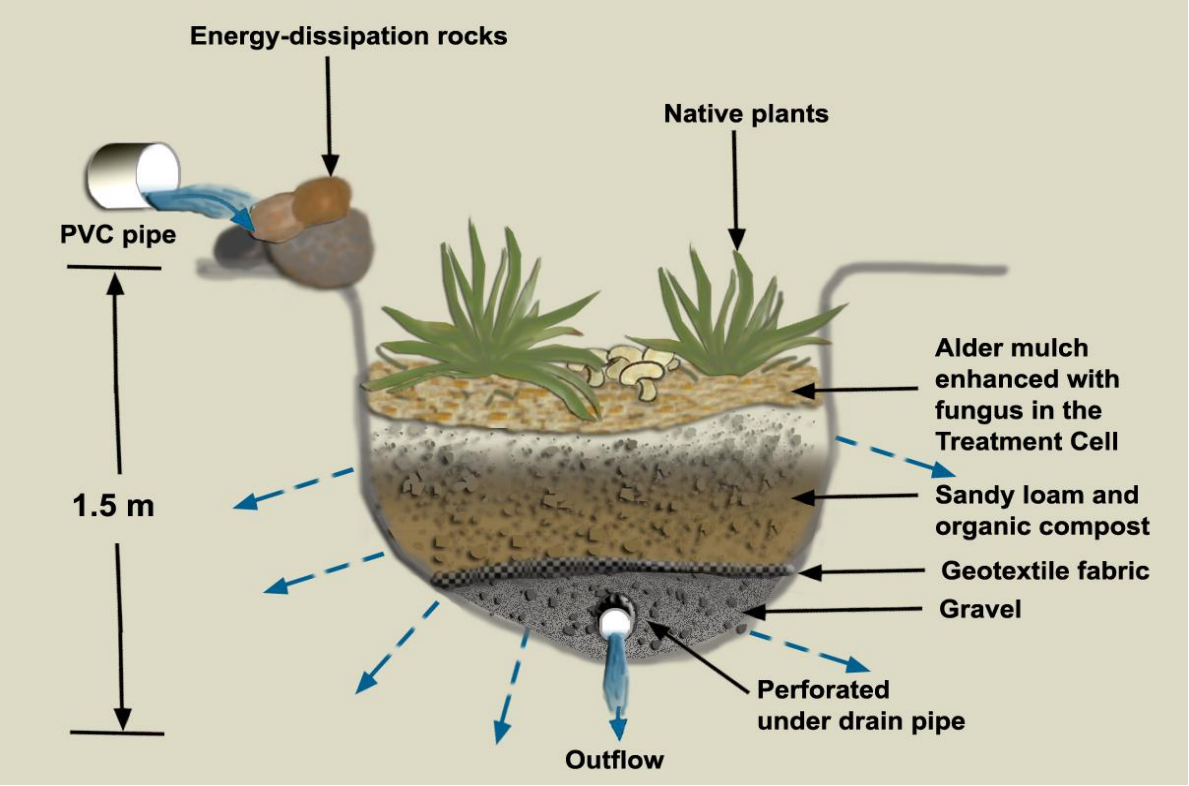

Figure 4. Cross-Sectional Schematic of the Enhanced, Excavated, Biofiltration Treatment Cell: Sand/Organic Material Fill over Perforated Drainage Pipe, Native Plants with Fungal Inoculation of Mulch Layer (not to scale)

Initial site construction and excavation occurred in September of 2005. Figure 5 shows the construction process. Each cell was excavated to the approximate dimensions of 3 m wide by 9 m long by 1.5 m deep (Figure 5A). A gravel layer and 15-cm diameter perforated underdrain pipe were added to each cell (Figure 5B) and connected to an outflow (effluent) pipe. The under-drain was installed primarily for sampling purposes. In addition, the under-drain was elevated approximately 1 ft above the base of the bioretention cell to provide an anaerobic zone below the outflow point to encourage nitrogen removal by denitrification. A layer of permeable geo-textile fabric was added to allow water movement but prevent passage of fine sediments that could clog the underdrain (Hinman 2005), and an impermeable barrier was placed between the two cells (retaining wall timber with plastic liner) (Figure 5C). Each cell was backfilled with approximately 75 m^3 of a sandy loam soil that included organic compost, sand, and local soil mixed to specifications provided by Hinman (2005) (Figure 5D).

Figure 5. Construction of Twin Biofiltration Cells: (A) Excavation of Twin Cells; (B) Placement of Perforated Underdrain Pipe in Gravel Envelope, (C) Placement of Permeable Landscape Fabric and Impermeable Barrier Between Cells (red arrow), and (D) Energy-Dissipation Rock at Surface Outlet with Layer of Sand and Organic Material

Introduction of native plants and fungal mulch. Native plants were selected based on recommendations from local nursery staff and Hinman (2005). Plant species that were used are listed in Table 1. The planting plan was based on the general scheme shown in Figure 6, with plants selected for particular zones related to wetness:

- *Zone 1* – Area of periodic or frequent standing or flowing water
- *Zone 2* – Periodically moist or saturated during larger storms
- *Zone 3* – Dry soils, infrequently subject to inundation or saturation.

Small revisions were made (e.g., siting of the *Malus fusca* – Oregon crab apple trees) to suit the landowners' preferences. For each cell, 12 shrubs were planted in Zones 1 and 2, on 1.2-m centers, and approximately 600 herbaceous plants were installed on 30-cm centers. For Zone 3, 16 shrubs were planted on 1.5-m centers. All plants and mulch were added to the site in November 2005 and a deer fence was placed around the site for protection of the plants.

Table 1. Native Plants Used in Biofiltration Cells

Plant type	Genus/Species	Common Name
Tree	*Malus fusca*	Pacific crab apple
	Salix lucida	shining willow
	Crataegus douglasii	black hawthorn
Shrub	*Cornus sericea*	red-osier dogwood
	Lonicera involucrata	twinberry honeysuckle
	Myrica gale	sweetgale
	Physocarpus capitatus	Pacific ninebark
	Oemleria cerasiformis	Indian plum
	Symphoricarpos albus	common snowberry
	Ribes lacustre	black swamp gooseberry
	R. sanguineum	red-flowering currant
	Crataegus douglasii	black hawthorn
	Spiraea densiflora	rosy spiraea
	S. betulifolia	white spiraea
Emergent	*Carex lyngbyei*	Lyngbye's sedge
	C. mertensii	Mertens' sedge
	C. obnupta	slough sedge
	C. pachystachya	chamisso sedge
	C. pansa	sanddune sedge
	C. sitchensis	Sitka sedge
	C. spectabilis	showy sedge
	Eleocharis palustris	common spikerush
	Juncus effusus	common rush
	J. tenuis	poverty rush
	Scirpus microcarpus	panicled bulrush
Herbaceous	*Aster chilensis*	Pacific aster
	Iris tenax	toughleaf iris
	Sisyrinchium angustifolium	narrowleaf blue-eyed grass
	Fragaria chiloensis	beach strawberry
	Potentilla fruticosa	shrubby cinquefoil

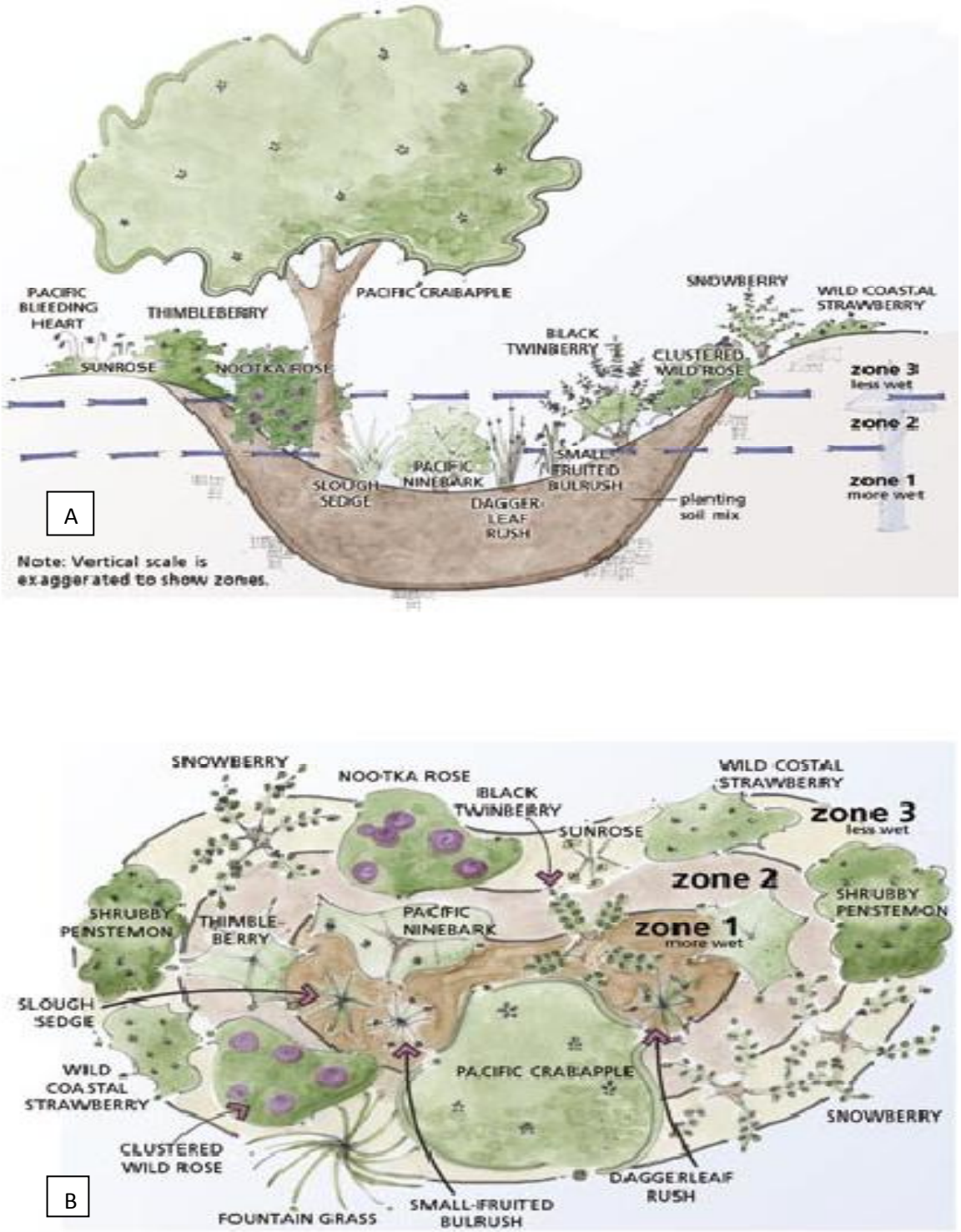

Figure 6. Generalized Native Planting Scheme Showing (A) Planting Zones and (B) General Layout of Native Plants to be Introduced to Each Cell (from Hinman 2005)

The native vegetation was planted in both cells (Figure 7 A-C). An equal number of each species was planted in each of the two cells in a mirror-image layout. In the fungal-treatment cell, all plants were inoculated with a commercial blend of mycorrhizal fungi (Down to Earth®) that contained a soluble powdered blend of endomycorrhizae, ectomycorrhizae, and humic acid. This blend was watered into each plant and surrounding soil during planting. The plants in the control cell were not inoculated with mycorrhizal fungi. All plant "starts" were covered with potting containers for protection prior to addition of a surface mulch layer (Figure 7D). A total of 14 m^3 of alder chip mulch was added to each cell. The control cell mulch did not contain fungus; however, the treatment cell mulch contained a fungal-enhancement. To create the treatment mulch, three species of preconditioned lignin degrading fungi (*Pleurotus ostreatus, Pleurotus ulmarius*, and *Stropharia rugoso-annulata*) were used to inoculate 3 m^3 of alder mulch (Figure 8 A-B) offsite several weeks prior to addition at the site in the process outlined in Figure 1. The fungal-enhanced mulch was then mixed onsite with the remaining 11 m^3 (Figure 9). Mulch was distributed to each cell by a conveyor system (Figure 9A) and spread by hand. The potting containers covering the plant starts were then removed (Figure 9 B-D). The control cell was handled first in all cases to prevent cross-contamination.

Figure 7. Plants Were Added to the Site in Mirror-Image Distribution in the Two Cells (A-C). After planting, the plant "starts" were covered to protect them from being crushed during mulch application (D).

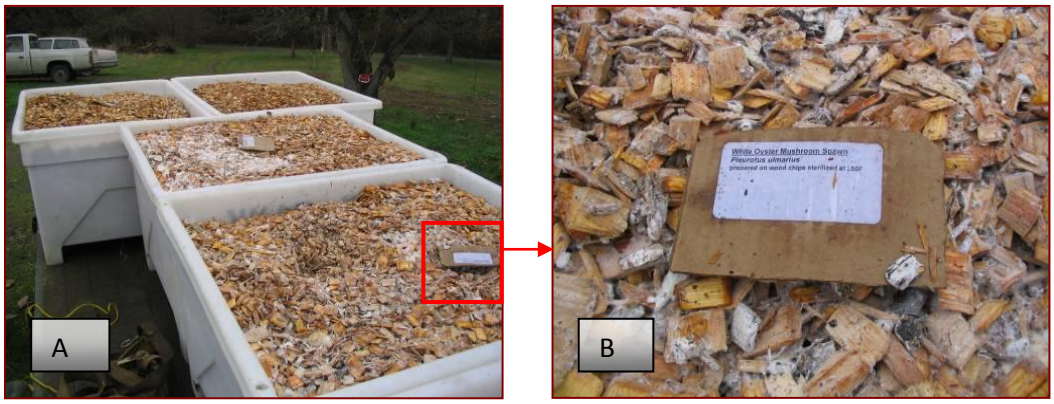

Figure 8. Fungal-Inoculum-Enhanced Alder Chip Mulch Before Application to the Treatment Cell (A) and Closeup Detail of Mulch (indicated by red box) (B)

Figure 9. Fungal-Enhanced Mulch was Added by Conveyor System (A), Spread Around the Plants (B), Protective Potting Containers Removed (C), and Energy-Dissipation Rocks Left Uncovered (red arrow) (D). Note deer fence surrounding cells (green arrow).

Initial maintenance of the site consisted of watering the newly planted vegetation using a sprinkler system, as needed. In December 2005, a flow-splitter distribution vault was added to the site. A temporary and intermittent source of water was provided to the site until August 2006 when a permanent

source was installed. A short time later, a blockage was discovered in the nonperforated pipe flowing to the treatment cell, at which point all piping from the flow-splitter distribution vault to each cell was replaced with more durable, polyvinyl chloride (PVC) pipe. At the same time, flow levelers were added to the distribution vault to refine the regulation of flows to the two biofiltration cells. Maintenance of the site continued throughout the study and consisted primarily of weed removal, regulating the flow at the site, and attending to exclusion of site intruders, such as moles and dogs. A photographic record documented all stages of site construction and development of the site over time. Water quality was analyzed from the inflow distribution vault and the control and treatment outflows on a routine basis.

2.2 Fecal Coliform and Nutrient Monitoring

Fecal coliform and nutrient samples from water were collected on a monthly basis as part of *Task 3* – the Effectiveness Monitoring study, for the duration of the study. For the purposes of the mycoremediation study, analysis focused on samples that were collected during the latter part of the study when a permanent water source was flowing to the site and plants were well established, between July 2007 and January 2008. Field samples were collected from the distribution vault (inflow) and from the treatment outflow and control outflow following standard methods described in the Quality Assurance Project Plan (Streeter 2005). Flow rates were measured on a routine basis.

2.3 Fluorescein Dye Study

A fluorescein dye study was conducted in November of 2007 to better understand the attenuation rate of water moving through the site and water retention capability. This information also was used in the planning and design of a dairy lagoon waste loading experiment (Section 2.4). Concentrated fluorescein dye (7.5 L; mean concentration 91,700,000 ppb) was added to the flow-splitter distribution vault as one volume; samples were taken and concentrations were measured over time. Samples were taken from the control and treatment outflows after introduction of the dye (Time = 0 hour) at regular intervals for several days and periodically for weeks following the experiment. Samples collected at 1, 2, 6, 22, 46, 74, 120 hours, and 36 and 62 days were shipped to Ozark Underground Laboratory, Inc. for analysis. All samples were stored at 4 ºC immediately upon collection and shipped cold.

2.4 Dairy Lagoon Waste Loading Experiment

An experimental loading of a local liquid dairy lagoon waste was conducted in May 2008. At 1-minute intervals for 15 minutes, 5 ml of undiluted liquid waste was added to 1 L of inflow water, mixed briefly, then added to the flow-splitter distribution vault. The total volume of liquid waste added was 75 ml (5 ml × 15 min). Samples of inflow and treatment and control outflow were taken over a time span of 17 days for fecal coliform analysis and 24 days for nutrient samples (TP and TN). The sampling schedule for fecal coliform bacteria was as follows: Day 0 (0 – pre-spike, 1, 2, 3, 4, 5, 6, and 12 hours), Day 1 (24, 26, 28, 30, and 36 hours), Day 2 (48, 51, 54, and 60 hours), Day 3 (72, 75, and 78 hours), then once each on Day 8, 9, 10, 14, 15, 16, and 17. Samples (50 ml) were collected in triplicate at the flow-splitter distribution vault, and the treatment and control outflows and analyzed by the Clallam County Health Department. Flow rates were measured at each sampling interval from the flow-splitter distribution vault and the two outflow pipes.

Nutrient samples were collected less frequently over a time span of 24 days. Samples from the inflow and the treatment and control outflow were taken on Day 0 (0-pre-spike, 2, 4, 6 hours), Day 1 (24 hours), Day 2 (48 hours), Day 3 (72 hours), and once each on Day 5, 9, 10, 15, 17, and 25. Samples were collected in duplicate and held at 4 ºC until analysis occurred. Only one set was analyzed, with the second set held as backup and archived.

2.5 Analytical Methodology

Fecal coliform samples were analyzed using Standard Method SM18 Membrane Filtration 9222D at the Clallam County Environmental Health Laboratory. TN and TP were analyzed using the methods of Valderrama (1981) at the University of Washington. Fluorescein dye samples were measured at an emission wavelength of 503 nm on a Shimadzu spectrofluorophotometer (Model RF-5000U) at Ozark Underground Laboratory, Inc.

Statistical analysis for this project used Minitab, Version 13.32 (Minitab, Inc.) and Prism 4, Version 4.03 (GraphPad Software, Inc.). The mean, standard deviation, and the coefficient of variation of fecal coliform concentrations were calculated and compared between sampling locations (the flow-splitter distribution vault, treatment cell outflow, and control cell outflow) as appropriate. A one-sided, one-sample t-test was used to test whether the difference in mean fecal coliform concentration was statistically significant (inflow vs. treatment outflow, inflow vs. control outflow, and control vs. treatment outflows). A one-sided, one-sample t-test also was used to test whether the difference in mean flow rates was statistically significant (inflow vs. treatment, inflow vs. control, treatment vs. control).

For the fluorescein dye experiment, the amount of dye passing through the treatment and control outflow pipes over time was estimated by summing of the concentrations of dye from successive sampling events. The proportion of the cumulative dye recovered for up to 120 hours was calculated for each sampling period and modeled as a sigmoidal (nonlinear) logistic model. The two-parameter logistic model was expressed as

$$y = \frac{1}{(1+10^{(\text{LogEC50} - x)\bullet \text{Hillslope}})} \quad (1)$$

where y = was the proportion of the total dye recovered
LogEC_{50} = the $\log_{10}$ of the time associated with 50% recovered
x = the log10 hour
Hillslope = the slope of the sigmoid curve.

For the dairy lagoon waste spike experiment, an exponential decay model was used to evaluate the decay in the difference between the treatment and control outflow median fecal coliform concentrations over 17 days. The three-parameter decay model was expressed as

$$y = S\, e^{-Kx} + P \quad (2)$$

where y was the difference between the control and treatment outflow median fecal coliform concentrations, which starts at $S + P$ and decays to P with a rate of constant K.

Where appropriate, the percent of removal, or export, was calculated for fecal coliform and nutrients as a concentration-based removal of the outflows from the inflow. We were unable to express results on a mass-balance basis due to the open nature of the bioretention cell in the field setting and the inability to account for all of the source water in the system.

3.0 Results

The results for monitoring fecal coliform and nutrients, the fluorescein dye study, and the dairy lagoon waste spike experiment are described below.

3.1 Fecal Coliform Monitoring

Routine monitoring for fecal coliform was conducted between August 2007 and January 2008 during six sampling events on a monthly basis (Figure 10). Samples were collected in triplicate from the inflow, treatment outflow, and control outflow. The mean fecal coliform concentration in the flow-splitter distribution vault was 30 colony forming units (CFU)/100 ml (±4). The average concentration of fecal coliform from the treatment outflow (mycoremediation) was 3 CFU/100 ml (±2) and 10 CFU/100 ml (±4) from the control outflow (bioremediation only). These differences were statistically significant between locations when the fecal coliform concentrations were above the detection limit (2 CFU/100 ml), based on a one-sided, one-sample t-test (i.e., inflow-treatment, inflow-control, and treatment-control).

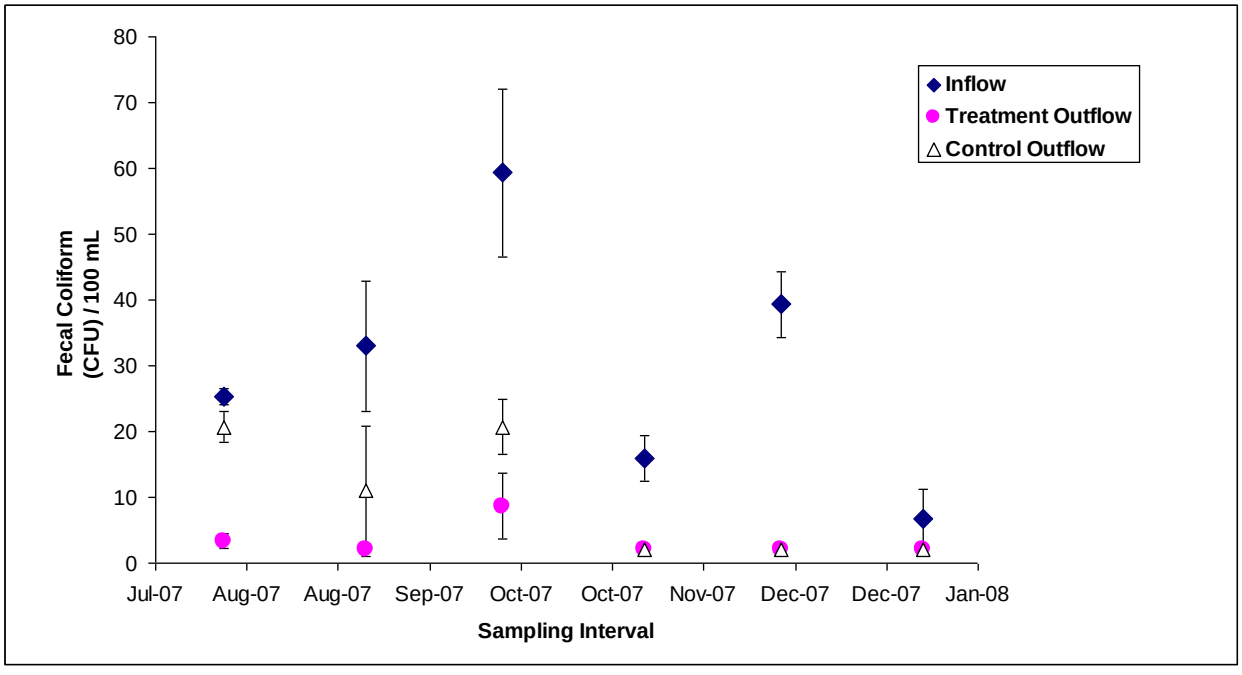

Figure 10. Fecal Coliform Concentration (CFU/100 ml) over a 6-Month Time Period (mean ± standard deviation).

During this 6-month time period, flow rates were measured on an intermittent basis and flows were subject to change from the source supply. When the flow was measured, it ranged between 19.5 and

23.5 L/min at the flow-splitter distribution vault with a mean of 22 L/min (±1.75). This was split between the two cells, so that each cell was receiving an average of 11 L/min. The mean treatment outflow rate was 3.71 L/min (±0.42) while the control outflow rate was 3.55 L/min (±0.70). The overall reduction of fecal coliform (concentration-based removal) in the control cell (bioremediation only) was 66% (concentration-based removal). Fecal coliform was reduced by 90% in the treatment cell.

3.2 Nutrient Monitoring

Monthly monitoring of the inflow and two outflows for nutrients occurred between July 2007 and January 2008 at the same time as fecal coliform monitoring. The TN input to the system was highly variable and ranged between 733 µg/L and 4101 µg/L. The mean input was 1977 µg/L ± 1220 (Table 2) (Figure 11). Outflows were also variable with a steady decrease occurring between July and November 2007. An overall export of TN (i.e., greater outflow concentration than inflow) occurred between July and September, while a reduction was observed between November and December (Table 2 and Fig 11).

The inflow concentration of TP remained relatively constant throughout the sampling period at 86 µg/L (±44) (Table 2 and Figure 12). The outflow concentrations were generally much higher than the inflow, and the treatment concentrations (558 ± 344 µg/L) were greater than the controls (421 ± 283), although there was a steady decline in the outflow concentrations between September and January. The export of phosphorus ranged from 1826% in July in the control outflow to a 24% reduction in January. While both TN and TP show a general decrease over time in concentration from the outflows, the inflow concentration of TN varied over the duration of the routine monitoring compared to the relatively constant concentration of TP during the same time.

Table 2. Summary Table of Nutrient Concentrations and Percent of Removal Efficiency by Sampling Interval

	TN(µg/L)			TP (µg/L)		
Sampling Interval	Inflow	Treatment Outflow	Control Outflow	Inflow	Treatment Outflow	Control Outflow
July 2007	773	3104	4043	42	785	802
(% removal)		*(-301)*	*(-423)*		*(-1786)*	*(-1826)*
August 2007	814	2828	2903	35	799	513
(% removal)		*(-248)*	*(-257)*		*(-2179)*	*(-1365)*
September 2007	1472	2785	2946	60	1046	780
(% removal)		*(-89)*	*(-100)*		*(-1641)*	*(-1198)*
October 2007	2827	2363	2913	110	592	327
(% removal)		*(16)*	*(-3)*		*(-437)*	*(-197)*
November 2007	2491	1505	1239	121	381	241
(% removal)		*(40)*	*(50)*		*(-216)*	*(-100)*
December 2007	4101	2063	1034	85	153	167
(% removal)		*(50)*	*(75)*		*(-81)*	*(-98)*
January 2008	1362	1362	1874	153	153	117
(% removal)		*(0)*	*(-38)*		*(0)*	*(24)*

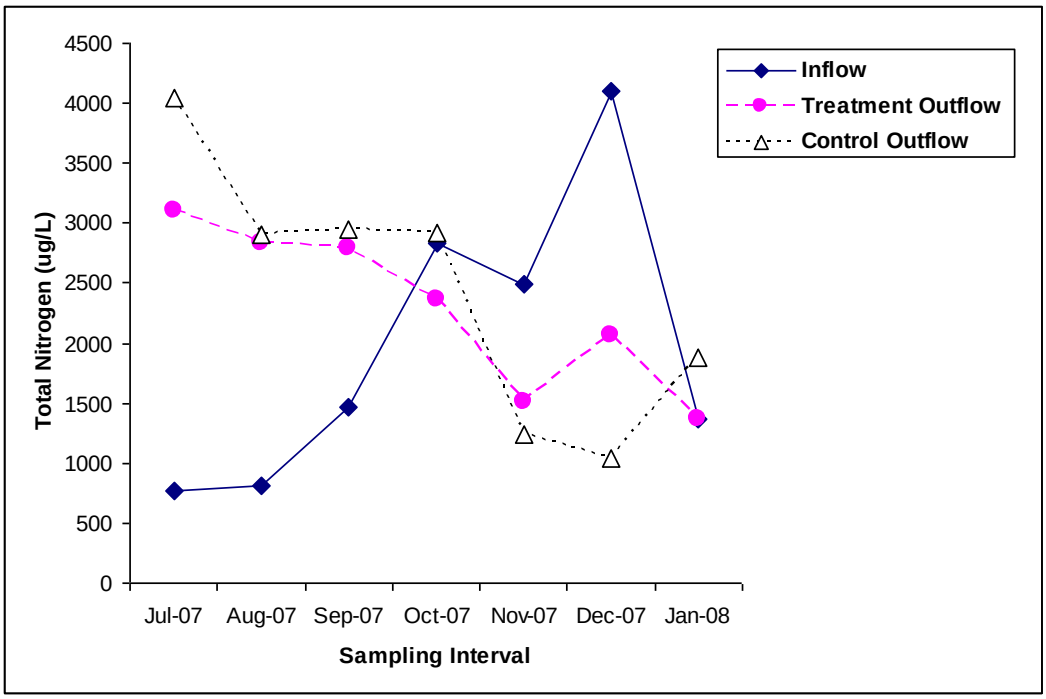

Figure 11. Total Nitrogen Concentration (µg/L) Between July 2007 and January 2008 for Inflow, Treatment Outflow, and Control Outflow

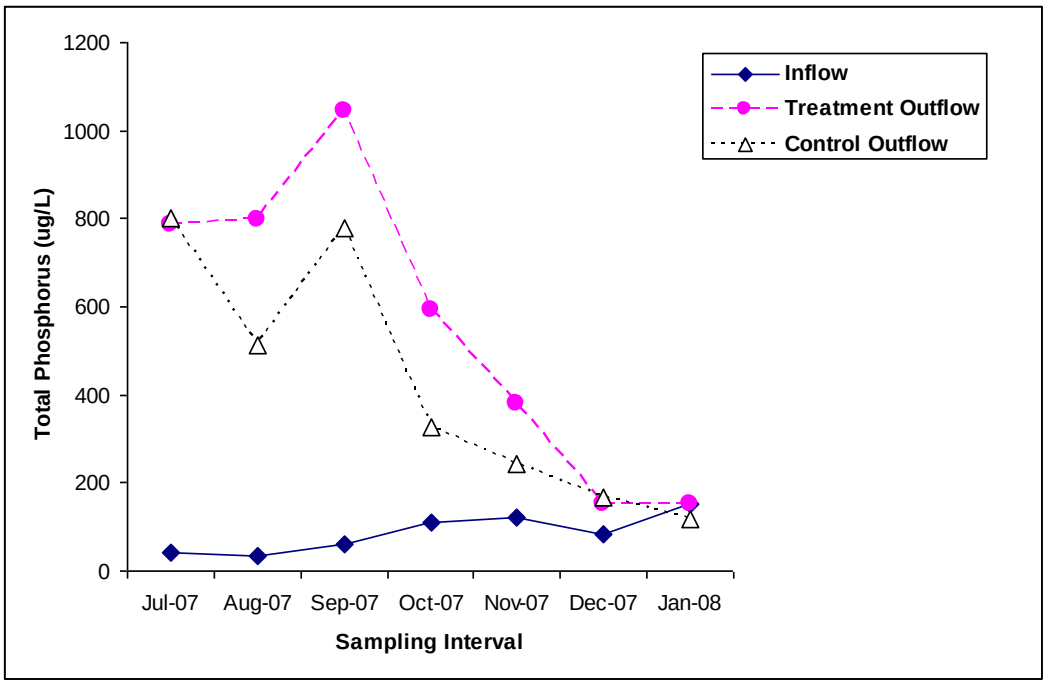

Figure 12. Total Phosphorus Concentration (µg/L) Between July 2007 and January 2008 for Inflow, Treatment Outflow, and Control Outflow

3.3 Fluorescein Dye Study

The fluorescein dye study was conducted in November 2007 to better understand the timing of water parcel movement through the cells. Fluorescein was selected for its mobility, because it could best represent the movement of soluble compounds through the cells. The dye was introduced at one point in time and concentrations were measured at various time periods thereafter until Day 63. Flow rates were measured at each sampling interval. Figure 13 shows relatively steady flow rates for both treatment outflows for the duration of the experiment with a drop during the last sampling period. The inflow varied more as the length of time increased between sampling periods. For the duration of the dye study, the mean inflow was 11.2 L/min (±2.0), while the treatment outflow was 1.4 L/min (±0.6) and control outflow was 1.0 L/min (±0.3). The flow rates between the control and treatment outflows were not statistically different.

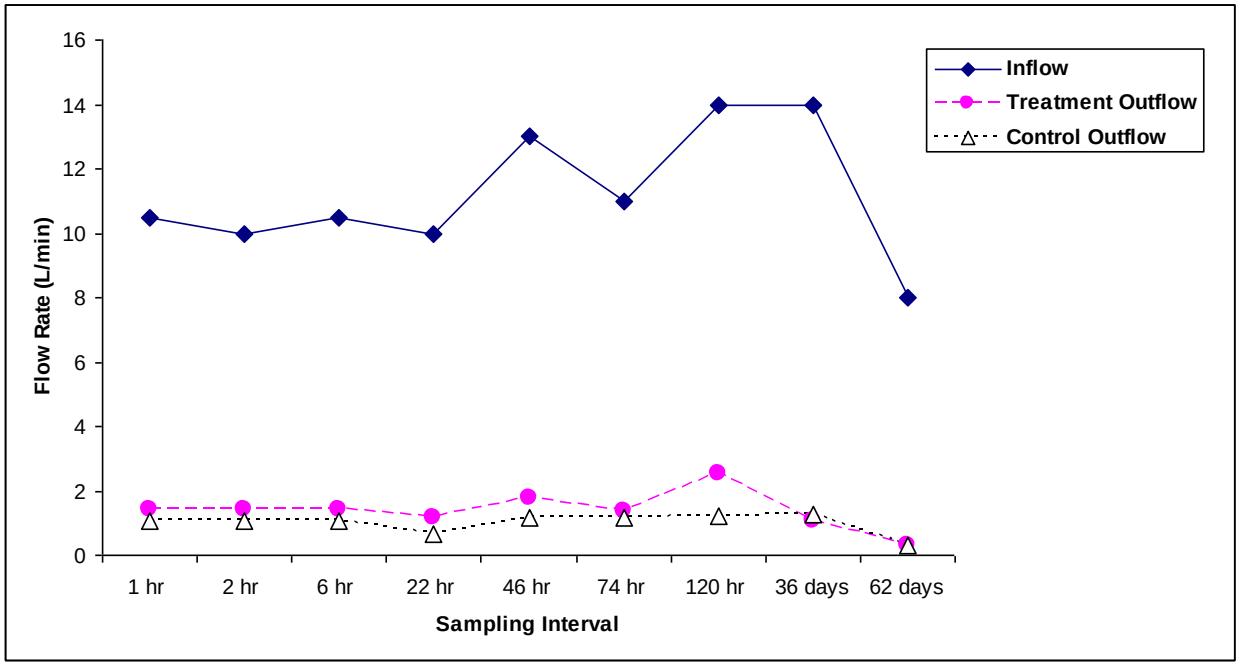

Figure 13. The Flow Rate over Time During the Dye Study at the Inflow, Treatment Outflow, and Control Outflow. Note X-axis scale is not proportional.

A stock volume of 7.5 L of concentrated liquid fluorescein dye (mean concentration 91,700,000 ppb) was added to the flow-splitter distribution vault at Time = 0. At the 1- hour sampling interval, there was no dye detected in the distribution vault, indicating it had moved into the two cells. The concentrations of dye were measured beginning at 1 hour in the outflows. Table 3 shows an initial concentration of dye measured in the control cell outflow of 214 ppb at 1 hour. Dye was not observed until the 2 hour sampling from the treatment outflow (4.2 ppb).

Figure 14 shows the concentration of dye through time. The highest observed concentration was measured at 2 hours from the control outflow (13,800 ppb), while the highest observed concentration from the treatment outflow was approximately half (6,920 ppb) at 46 hours.

Table 3. Fluorescein Dye Measured in the Control and Treatment Outflows

Sampling Interval	Control Dye (ppb)	Treatment Dye (ppb)
1 hr	214	0
2 hr	13800	4.2
6 hr	7060	144
22 hr	7970	5980
46 hr	3790	6920
74 hr	2660	3410
120 hr	802	1190
Day 37	214	141
Day 63	23.6	74.3

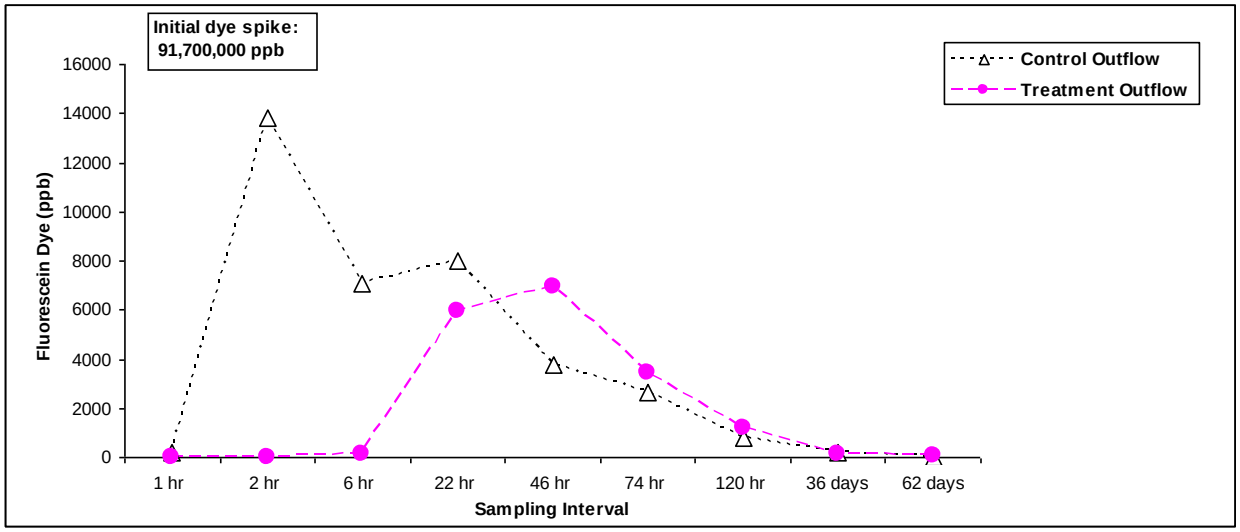

Figure 14. Concentration of Fluoroscein Dye in Control Versus Treatment Outflow Over Time

Because the average flow rates between the control and treatment outflow were not statistically different, the cumulative dye recovered from the treatment and control outflows over time was estimated by summing the concentrations of dye from successive samplings through 120 hours. Figure 15 shows the cumulative dye recovered from the treatment and control outflows. Approximately 2 times more dye was discharged from the control outflow than from the treatment outflow during the 120 hours of measurement.

Using the calculated cumulative dye released over 120 hours, the proportion recovered was calculated for each sampling period and modeled as a sigmoidal (nonlinear) logistic model (Figure 16). Based on the model fit of the cumulative dye released, the length of time associated with 50% of the maximum dye discharged was estimated to be from 27 to 31 hours for the treatment outflow—approximately 5 times longer than the 3 to 8 hours estimated for the control outflow.

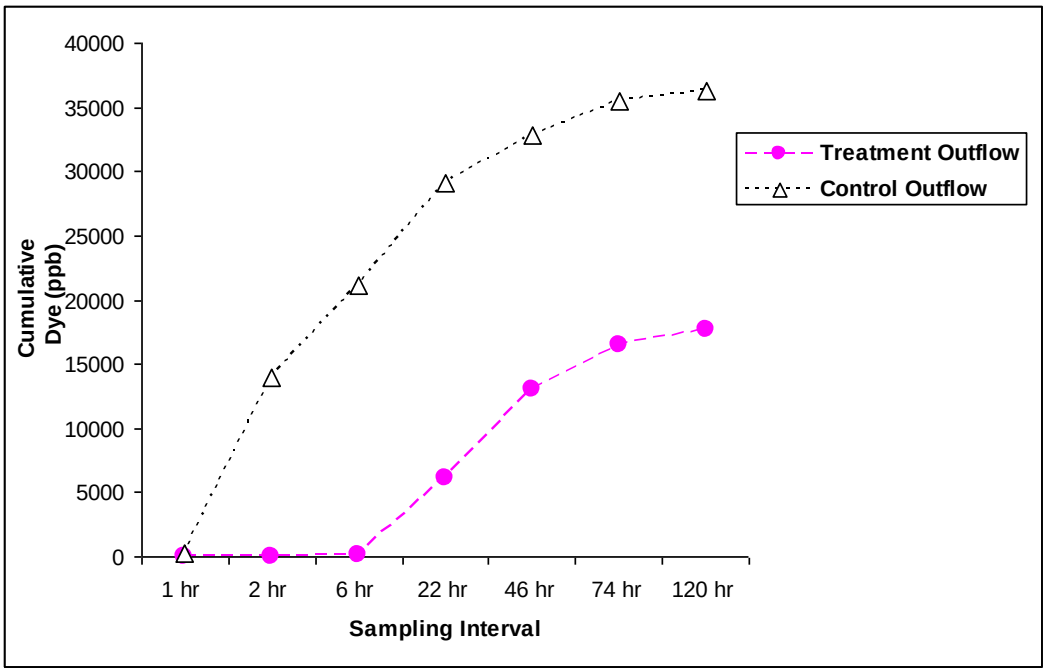

Figure 15. Concentration of Cumulative Dye Recovered Up to 120 Hours from the Treatment and Control Outflows. Note X-axis is not proportional.

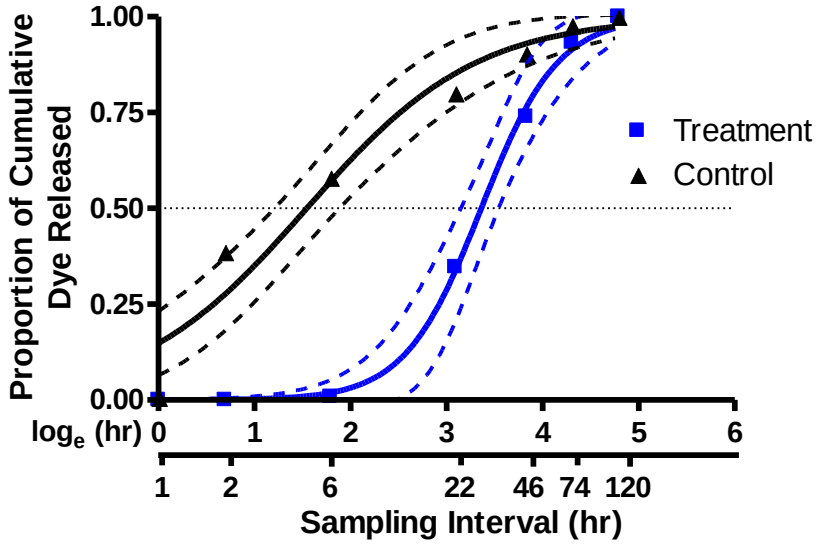

Figure 16. Modeled Fit of the Proportion of Cumulative Dye Released from the Treatment and Control Outflows

3.4 Dairy Lagoon Waste Spike

The results for fecal coliform and nutrients are described in the following sections.

3.4.1 Fecal Coliform

In May 2008, an experiment was conducted to introduce a spike of dairy lagoon waste into the flow-splitter distribution vault to better understand how the treatment and control cells would respond to higher concentrations of nutrients and bacteria. As in the dye study, the flow rates were measured at each sampling interval through the 17 days of the experiment (Figure 17). The mean flow rate from the flow-splitter distribution vault (inflow) was 19 L/min (±3.6); therefore, the flow rate to each cell was approximately 9.5 L/min. The outflow from the treatment cell was 3.87 L/min (±1.03) and the outflow from the control cell was 3.65 L/min (±0.84). Statistically, the flow rates between the inflow and treatment outflow, and the inflow and control outflow are significantly different, whereas the flow rates between the treatment and control outflows are not significantly different.

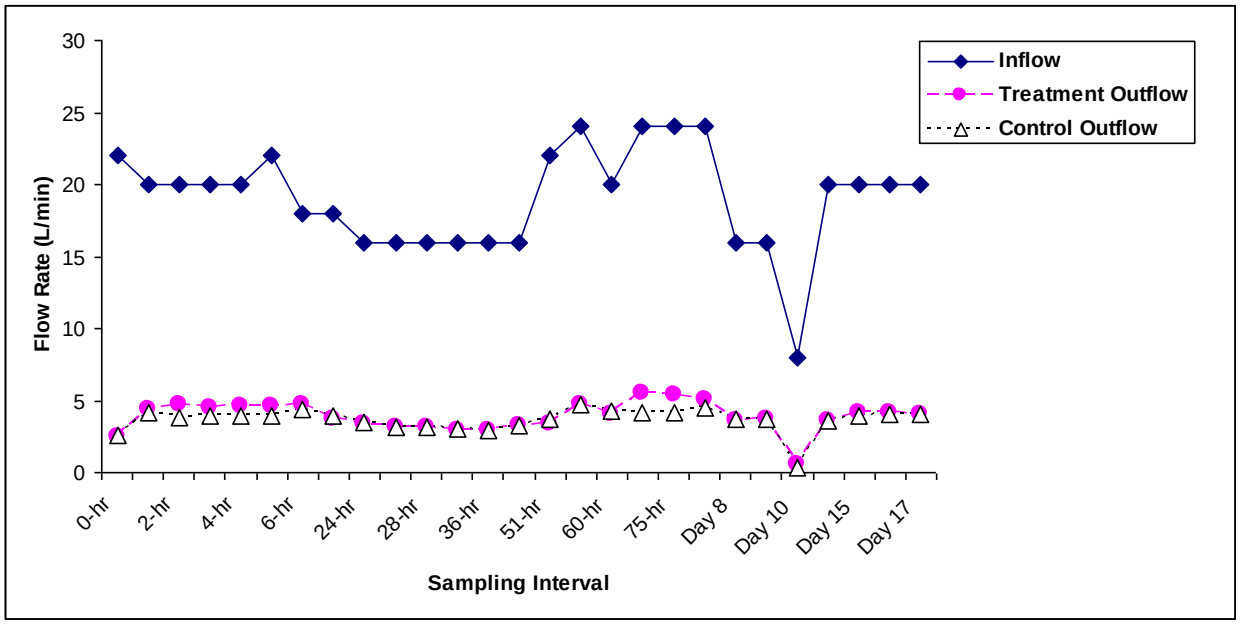

Figure 17. Flow Rate Over Time During the Dairy Lagoon Waste Spike Experiment at the Inflow, and at the Treatment and Control Outflow. Note X-axis is not proportional.

The mean fecal coliform concentration of the spike stock was 259,000 CFU/100 ml. Every minute for 15 minutes, 5 ml, or ~12,950 CFU, were added for a total addition of ~194,250 CFU. Figure 18 shows a fecal coliform concentration of 200 CFU/100 ml in the inflow just prior to the start of the experiment (Time = 0 hr). Inflow concentrations increased slightly, up to 365 CFU/100 ml at 3 hours, but generally showed a decrease by the 4-hour sampling time, indicating that the initial spike had been flushed from the flow-splitter distribution vault. The control outflow also showed an initial spike at 1 hour up to 376 CFU/100 ml, then dropped steadily over time. The control outflow concentrations are significantly higher than the treatment outflow concentrations up until the 28-hour sampling. The treatment outflow remained relatively constant throughout the duration of the experiment with a mean of 5 CFU/100 ml (±4).

An exponential decay model was used to evaluate the difference between the treatment and control fecal coliform concentration response over the 17-day duration of the experiment. Figure 19 shows the modeled results of the decrease in difference over time. After 29 hours, there was no significant difference between the median concentration of the treatment and control outflow. However, the

concentration of fecal coliform in the control outflow was significantly greater than in the treatment outflow between 1 and 28 hours, with a constant linear decrease in the log difference until 29 hours when no difference was observed.

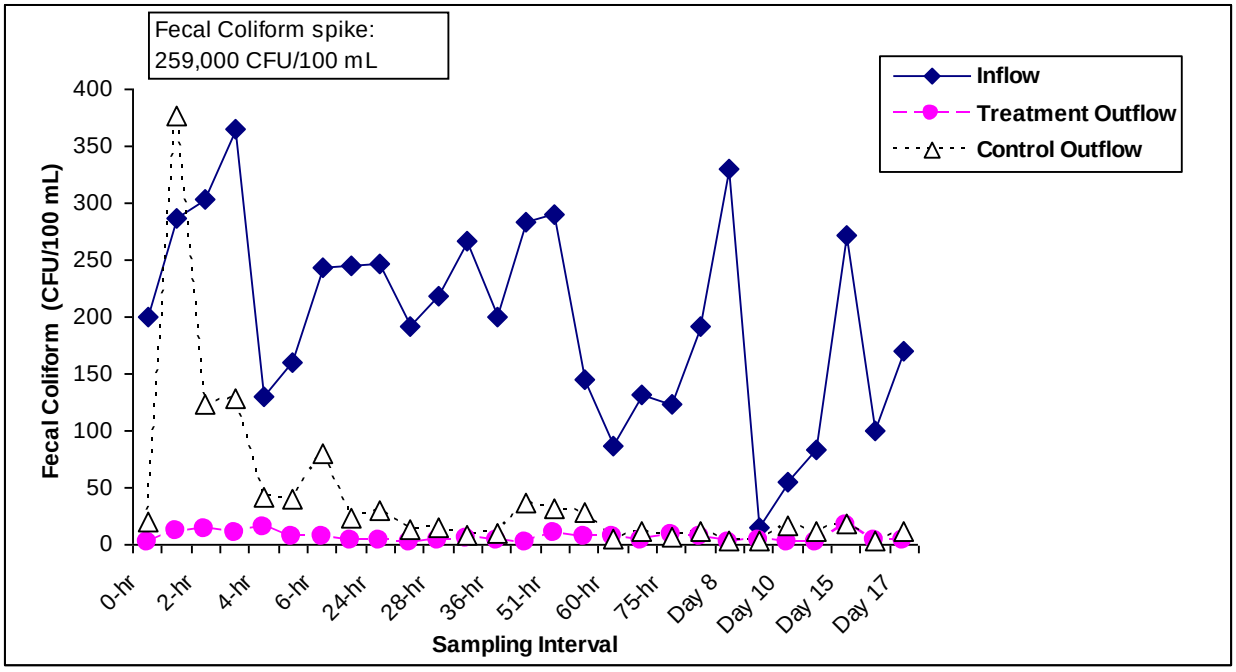

Figure 18. Fecal Coliform Concentration in the Inflow, and Treatment and Control Outflow from the 17-Day Spike Experiment. Note X-axis is not proportional.

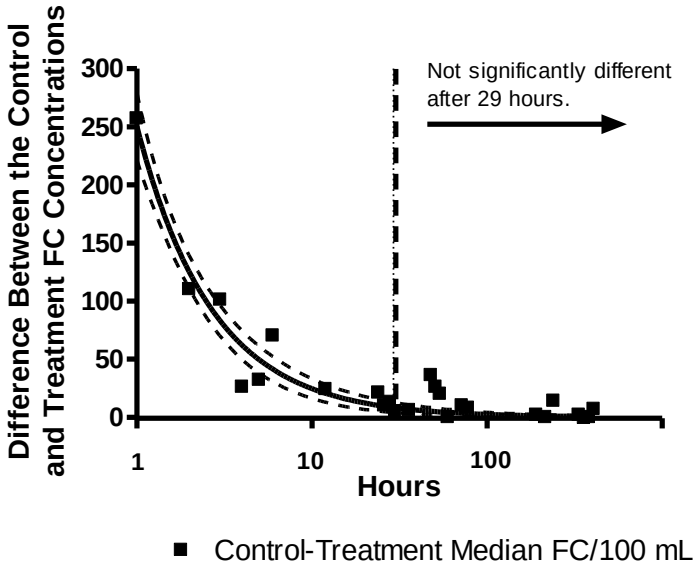

Figure 19. Modeled Fit of the Difference Between the Control and Treatment Outflow Fecal Coliform Concentrations over Time

Once the biofiltration cells had reached a steady state (i.e., after 29 hours), the average concentration in the inflow was 172 CFU/100 ml, the treatment outflow was 5 CFU/100 ml, and the control outflow was 13 CFU/100 ml. The overall reduction of fecal coliform after equilibrium concentration was reached in the inflow was 97% in the treatment cell and 92% in the control cell.

3.4.2 Nutrients

Concentrations of TN and TP were monitored for a total of 24 days. The mean concentration of the TN spike from the dairy lagoon waste added to the flow-splitter distribution vault was 12,813 µg/L. The pre-spike concentration of TN was 2370 µg/L (Time = 0 hr). Figure 20 shows that the 2-hour inflow sampling interval had a slight decrease in TN (2332 µg/L), indicating that the flow-splitter distribution vault had been flushed shortly after introduction of the spike. This confirms the same observation that the fecal coliform data show. The inflow concentration was relatively stable for the first 72 hours of sampling (2197 µg/L ±181). The concentration decreased, then increased again between 8 and 15 days. The mean control outflow concentration was 1157 µg/L (±150) and was lower than the inflow concentration at all sampling intervals. The mean treatment outflow concentration was 680 µg/L (±256), which was lower than the control outflow during all sample intervals. During the first 72 hours, when the least variability in the data occurred, the percent reduction of TN from the control cell outflow was 44% and the reduction in the treatment cell outflow was 72%.

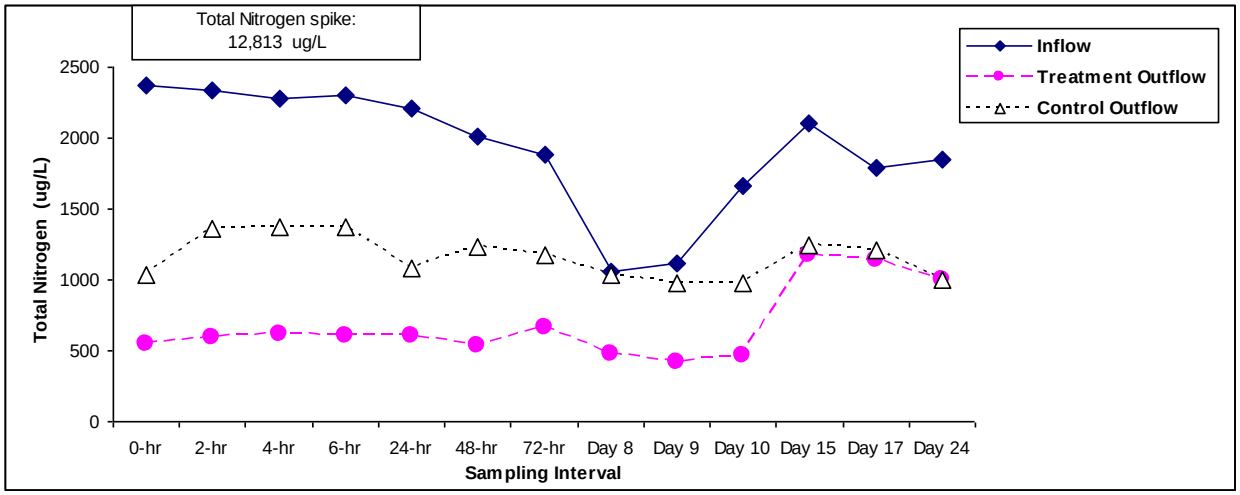

Figure 20. Concentration of TN from the Inflow and from the Treatment and Control Outflow During the Spike Experiment. Note X-axis is not proportional.

The mean concentration of the TP spike from the dairy lagoon waste added to the flow-splitter distribution vault was 1977 µg/L. The pre-spike concentration in the inflow was 53 µg/L (Time = 0) with very little change noted at 2 hours, indicating a complete flushing of the distribution vault (Figure 21). Concentrations were relatively stable for the inflow, and for the treatment and control outflow through Day 9 of the experiment, with an increase noted on Day 10 for all samples, then a slight decrease or leveling. The mean concentration for the inflow through Day 9 was 50 µg/L (±4), while the mean concentration for the control outflow was 149 µg/L (±23) and 114 µg/L (±10) for the treatment outflow. An export of TP was observed during almost all sampling intervals with export higher from the control cell compared to the treatment cell. For the first 9 days of the experiment, the percent of export was 198% for the control cell and 128% for the treatment cell.

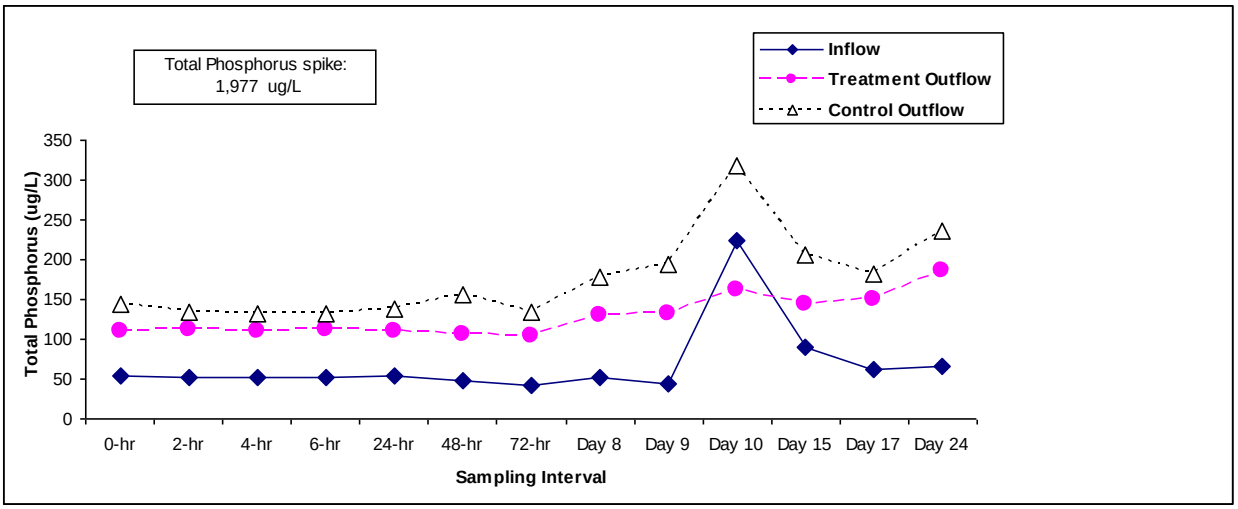

Figure 21. Concentration of TP from the Inflow and from the Treatment and Control Outflow During the Spike Experiment. Note X-axis is not proportional.

4.0 Discussion and Conclusions

The purpose of this study was to demonstrate the use of an innovative best management practice to reduce fecal coliforms and nutrients from surface runoff. Mycoremediation was applied in combination with bioretention cells to an area that received runoff on a continual basis from an irrigation ditch. The incorporation of a mycoremediation treatment into the design of one of the two bioretention cells and the addition of a spiked experiment allowed us to explore the characteristics of both on a comparative basis.

The study incorporated three phases of the mycoremediation demonstration. The first phase was initiated once the cells had been constructed, a mycoremediation treatment applied, and a permanent water source established. It included routine monitoring of fecal coliform and TN and TP concentrations at the site on a monthly basis. A dye study was conducted during the second phase to better understand the retention time and attenuation rate of water moving through the site, and the third phase involved a one-time addition of a dairy lagoon waste spike to evaluate the function of the cells with a higher loading of bacteria and nutrients.

Our BMP study was located at a field site, hence our results are based on what might be expected in a natural setting; however, it did not afford the stringent controls that would have provided additional information, such as mass balance evaluations of inputs and outputs. Rather, the site received a constant source of input water, although water flows were variable, and underdrain pipes captured a portion of the outflow water from both the treatment and control cells. It should be noted that over half of the water piped to the cells went beyond the boundary of the constructed area. Thus, the water not moving through the outflow pipes was subject to increased soil contact and the potential for further degradation of the pollutants of interest. The underdrain pipes were installed only for sampling purposes in this case. The relatively high infiltration rate of the surrounding soils indicates that bioretention could be very effective for removal of selected pollutants at this site.

When average flow rates at the site were calculated for the inflow and combined outflows, the routine monitoring phase showed 67% removal of water through the cell into surrounding soil (i.e., 33% exited via the outflow pipes). During the dye study, 78% of the water from the dye study was removed through the cell, and 60% was removed through the cell during the spike experiment. Under a natural bioretention cell setting, the water would be subjected to increased soil contact and longer retention/reaction time, presumably leading to further reduction of fecal coliform and nutrient exchange and uptake by plants.

The reduction of fecal coliform bacteria at the demonstration site is clearly the easiest to evaluate, because all trends indicated a significant reduction. During the Phase 1 experiments, a period of relatively low fecal coliform concentration in the inflow (30 CFU/100 ml), statistically significant reductions were noted for the bioretention cell (66%) and for the mycoremediation treatment (90%). In this case, the mycoremediation treatment was 24% more effective than the bioretention cell alone. During the Phase 3 spike experiment, after the inflow concentrations reached a steady state of 172 CFU/100 ml at 29 hours, the treatment outflow was on average 5 CFU/100 ml and the control outflow was 13 CFU/100 ml. In this case, the percent reduction in fecal coliform was 97% for the mycoremediation treatment and 92% for the control. In addition, the concentrations of fecal coliform measured in the control outflow were significantly higher than those measured from the treatment outflow for the first 28 hours (control outflow maximum was 376 CFU/100 ml versus a treatment outflow maximum concentration of 20 CFU/100 ml (Figures 18 and 19). The data suggest that bioretention cells can reduce fecal coliform bacteria under a range of concentrations (e.g., lower concentrations during Phase 1 and higher concentrations during Phase 3) and that mycoremediation treatment enhances or increases that reduction. However, based on these experiments, we do not know what the upper threshold of fecal coliform concentration is, beyond which the system would not function effectively to reduce fecal coliform.

Nutrients were more difficult to evaluate, primarily because the data showed varying trends of export or removal through time. For example, TN was exported from the site (i.e., higher concentration in the outflow relative to the inflow) between July and October 2007, but showed a reduction between October 2007 and January 2008. However, during the course of the 7 months of monitoring, the TN inflow concentration increased over five-fold while the outflow concentrations decreased by over half. The TN removal efficiencies ranged from -423% (TN export) to 75% removal (Table 2). During the relatively short spike experiment (24 days), TN also showed a reduction or removal in the bioretention cells, where the observed treatment outflow concentrations were less than the control outflow concentrations. The TN data from both of these studies would suggest that once the soil was saturated with water below the under-drain, an anaerobic zone became an effective part of the TN removal and treatment. On the other hand, TP was consistently exported from the bioretention and mycoremediation treatment cells during the routine monitoring and the spike experiment. Export of TP during the routine monitoring was extremely high (-2179% removal efficiency), and during the first 9 days of the spike experiment removal efficiency averaged -128% from that of the treatment cell and -198% from that of the control cell.

A number of studies have examined the functional role of bioretention cells in both field settings as well as controlled laboratory and mesocosm conditions for the reduction of nutrients (Davis et al. 2001; Hunt et al. 2006; Davis et al. 2006; Hsieh et al., 2007). Again, results have varied depending on the bioretention cell design, the retention time of water in the cells, the soil media layer configuration, and the extent of aerobic and anaerobic zones in the cells. For nitrogen removal, the importance of an aerobic zone coupled to an anaerobic zone is acknowledged (Hsieh et al. 2007). Through aerobic nitrification processes involving *Nitrosomonas* and *Nitrobacter* bacterial species, captured ammonium ions in soils

may be oxidized, ultimately becoming nitrate. At this point, nitrate may be exported or leached from the bioretention cell. However, with poor draining soils and increased retention times, anoxic zones can be created with redox potentials suitable for denitrification (Meyer et al. 2002). When nitrate is trapped in these anoxic zones, it can undergo biological denitrification, resulting in transformation of nitrate to gaseous nitrogen species. An organic carbon source is required to sustain this process. Recent designs of bioretention cells and media have focused on allowing a portion of the cell to remain continuously submerged, promoting these denitrification processes. In our study, ponding occurred in each bioretention cell and portions of the cell were submerged for frequent but intermittent time periods. The extent and frequency of ponding changed as the flow rates shifted and seasonal fluctuations occurred. In unpublished data from this study, we did see a reduction of nitrate in almost all cases and a periodic reduction in TN.

Different pathways may dominate when comparing the long-term fates of phosphorus and nitrogen compounds (Davis et al. 2006). Although phosphorus is biologically active, there is no significant ecological transport pathway in which it can be converted to a gaseous form, as with nitrogen. Hence, phosphorus may accumulate in the retention cell, be exported with outflow, or be taken up by vegetation. In this study, we saw a consistent export of phosphorus from both cells. In field studies, Hunt (2003) found that the phosphorus index (P-index) of the fill soil was an important factor in the ability of soils to adsorb additional phosphorus. The higher the P-index, the less likely the soil was able to adsorb additional phosphorus, and the more likely the site was to export TP. In this study, we did not measure the P-index of the fill soil, hence if the P-index was high this may have contributed to the high export rate. Alternatively, the slightly reduced phosphorus export that was seen in the mycoremediation treatment compared to the control cell could have been caused by enhanced mycorrhizal fungal activity (resulting from the initial inoculation at plant installation) allowing vegetative uptake of phosphorus (Sylvia 2003).

Nutrient pathways are complex, involving microbial conversion of both nitrogen and phosphorus compounds to various forms that are transported through soil, water, and plants. It is important when designing bioretention cells to select design criteria that target the pollutant of interest (Hunt et al. 2006). For example, a bioretention area designed to remove phosphorus should include a soil media mix with a low P-index to enhance phosphorus uptake in the soils, rather than export. If nitrogen species are targeted, then design features should include a soil media mix with a high organic content and features that allow for submerged or anaerobic zones to allow denitrification processes to take place. Mycorrhizal fungi and native plant additions are also an important component of nutrient pathways, and could be included as design features to enhance plant uptake of nutrients and in targeted cases certain contaminants.

One of the objectives of the mycoremediation demonstration site was to develop functional habitat that included native plants and incorporated appropriate mycorrhizal fungi. The addition of fungi in the treatment cell serves two primary purposes: 1) to enhance the establishment and growth of the plants and 2) to ultimately enhance the nutrient removal. Although most plants are eventually colonized by their appropriate mycorrhizal symbiont, inoculating during installation speeds up the process and provides the plant with the benefits of the symbiotic relationship from the start. Mycorrhizal fungi provide an abundance of benefits to partner plants which include (but are not limited to) mobilization, release, and transfer of nutrients and trace metals; increased drought resistance by way of transfer of water from pore spaces too small for plant fine roots to access; increased seedling establishment; increased and influenced diversity of soil microfauna; protection of the plant host from pathogens; improvement of soil structure and aeration; resistance to erosion; suppression of weeds; and connecting the plant community via a

complicated web of underground networks of mycelia. Figure 22 shows the complement of native plants after 30 months of growth.

The benefits of the mycoremediation treatment application to a bioretention cell or other type of site (e.g., stream bank, riparian buffer) include the following:

- a technology based on natural systems
- only native fungal species used; can locally source all materials (plants and fungi)
- minimal handling and low maintenance
- visible improvement to a site
- non-toxic byproducts; no secondary waste streams produced
- protects local water quality
- mobile and flexible; no structures, no minimum batch size
- economical
- effective at reducing fecal coliform and nutrients when properly designed
- applicable to a variety of other contaminants (e.g. PAHs, PCBs, metals)

Figure 22. Biofiltration Cells at Study Site After 3 Years with a Complement of Native Plants. Treatment (mycoremediation) cell is on the left, control (bioretention only) cell on the right

5.0 References

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