IDAHO TRANSPORTATION DEPARTMENT

RESEARCH REPORT

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RP 258

Weed-Suppressive Soil Bacteria to Reduce Cheatgrass and Improve Vegetation Diversity on ITD Rights-of-Way

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16. Abstract

Transportation departments are challenged by the invasion of downy brome (cheatgrass) and medusahead. The reduction of downy brome (cheat grass) by Weed Suppressive Bacteria (WSB) *Pseudomonas fluorescens* strain ACK55 was evaluated on roadsides of I-84, I-86 and US-95 in Idaho. Bacteria were produced and sprayed on 1-acre plots at eleven locations at seven sites in the fall of either 2014, 2015 or 2016 at 10^9 colony forming units (cfu)/m^2. The vegetation in each plot at each location was monitored in the spring and fall of each year using Sample Point. Sample Point is a digital method of monitoring vegetation across landscapes coupled with a software package that estimates cover of species or groups of plants. Percent cover of species (native and invasive), the total cover of vegetation, litter, rock, cryptogamic crust, bare soil, and GIS coordinates were recorded. The bacteria reduced downy brome (cheatgrass) by 30 to 97 percent of the control. Medusahead patches were evident in the control plots, but not found after the bacteria were applied. The volume and timing of WSB application is critical to the successful, long-term reduction and exclusion of downy brome (cheatgrass) and medusahead. Late fall applications, when air and soil temperatures were cool (below 50°F), rains were prevalent and skies were overcast, had the highest success. The greatest success with long-term reductions of downy brome (cheatgrass) and medusahead was on lands with mixed populations of native plants and moderate weed infestations. Because of its selectivity, this bacterium can be used in management of the invasive weeds downy brome (cheatgrass) and medusahead on ITD roadsides.

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METRIC (SI*) CONVERSION FACTORS

APPROXIMATE CONVERSIONS TO SI UNITS

Symbol	When You Know	Multiply By	To Find	Symbol
		LENGTH		
in	inches	25.4	millimeters	mm
ft	feet	0.3048	meters	m
yd	yards	0.914	meters	m
mi	Miles (statute)	1.61	kilometers	km
		AREA		
in^2	square inches	645.2	millimeters squared	cm^2
ft^2	square feet	0.0929	meters squared	m^2
yd^2	square yards	0.836	meters squared	m^2
mi^2	square miles	2.59	kilometers squared	km^2
ac	acres	0.4046	hectares	ha
		MASS (weight)		
oz	Ounces (avdp)	28.35	grams	g
lb	Pounds (avdp)	0.454	kilograms	kg
T	Short tons (2000 lb)	0.907	megagrams	mg
		VOLUME		
fl oz	fluid ounces (US)	29.57	milliliters	mL
gal	Gallons (liq)	3.785	liters	liters
ft^3	cubic feet	0.0283	meters cubed	m^3
yd^3	cubic yards	0.765	meters cubed	m^3
		TEMPERATURE (exact)		
^oF	Fahrenheit temperature	5/9 (^oF-32)	Celsius temperature	^oC
		ILLUMINATION		
fc	Foot-candles	10.76	lux	lx
fl	foot-lamberts	3.426	candela/m^2	cd/cm^2
		FORCE and PRESSURE or STRESS		
lbf	pound-force	4.45	newtons	N
psi	pound-force per square inch	6.89	kilopascals	kPa

Note: Volumes greater than 1000 L shall be shown in m^3

APPROXIMATE CONVERSIONS FROM SI UNITS

Symbol	When You Know	Multiply By	To Find	Symbol
		LENGTH		
mm	millimeters	0.039	inches	in
m	meters	3.28	feet	ft
m	meters	1.09	yards	yd
km	kilometers	0.621	Miles (statute)	mi
		AREA		
mm^2	millimeters squared	0.0016	square inches	in^2
m^2	meters squared	10.764	square feet	ft^2
km^2	kilometers squared	0.39	square miles	mi^2
ha	hectares (10,000 m^2)	2.471	acres	ac
		MASS (weight)		
g	grams	0.0353	Ounces (avdp)	oz
kg	kilograms	2.205	Pounds (avdp)	lb
mg	megagrams (1000 kg)	1.103	short tons	T
		VOLUME		
mL	milliliters	0.034	fluid ounces (US)	fl oz
liters	0.264	Gallons (liq)	gal	m^3
	meters cubed	35.315	cubic feet	ft^3
m^3	meters cubed	1.308	cubic yards	yd^3
		TEMPERATURE (exact)		
^oC	Celsius temperature	9/5 ^oC+32	Fahrenheit temperature	^oF
		ILLUMINATION		
lx	lux	0.0929	foot-candles	fc
cd/cm^2	candela/m^2	0.2919	foot-lamberts	fl
		FORCE and PRESSURE or STRESS		
N	newtons	0.225	pound-force	lbf
kPa	kilopascals	0.145	pound-force per square inch	psi

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Credits are provided for those that shared their photos for this report. For all photos with no credit, those were provided by Ann C. Kennedy or Chris Small, USDA-Agricultural Research Service, Pullman, WA.

Table of Contents

List of Tables

List of Figures

Acronyms and Definitions

The following acronyms and definitions are provided for further clarification for the terms used in this report.

- **Agricultural Research Service:** (ARS)
- **Bacteria:** Large domain of prokaryotic microorganisms. Typically, bacteria are a few micrometers in length and have a number of shapes, ranging from spheres to rods and spirals. Bacteria were among the first life forms to appear on Earth, and are present in most of its habitats. The organism in this study was a bacterium.
- **Bare Ground:** Soil and mineral matter smaller than one square inch in size.
- **Colony Forming Units: (cfu)** a **unit** used to estimate the number of viable bacteria or fungal cells in a sample.
- **Desired Species:** Native or exotic plant species that provide a benefit to a revegetation site.
- **Exotic Species**: Non-native species that owe their presence in a given geographic area to intentional or unintentional human mediated dispersal.
- **Geographic Information Systems:** (GIS)
- **Idaho Transportation Department: (ITD)**
- **Inoculum:** Refers to the source material used for inoculation. The word is used in three senses: In medicine, the material that is the source of the inoculation in a vaccine. In microbiology, the cells, tissue, or viruses that are used to inoculate a new culture.
- **Invasive:** Plant species on the Idaho Noxious Weed List (Appendix C), annual exotic grasses, and forbs known to be aggressive with a tendency to form monocultures and crowd out desired species.
- **Litter:** Organic matter (not decomposed) in contact with the soil surface, commonly plant matter from previous growing seasons.
- **Mile Post:** (MP)
- **Native Species:** Originated in a given geographic area without human manipulation.
- **Roadside:** Includes the sides of the road corridor beyond the paved road shoulders including impacted or maintained roadside areas within the right-of-way (ROW).
- **Rock:** Mineral matter larger than one square inch in size.
- **Sample Point:** Sample Point is software for manual image analysis. Foliar cover determinations of user-defined classes can be derived from digital images taken on the ground or aerially. Data are saved automatically to an Excel spreadsheet. Sample Point was developed by Berryman Consulting in cooperation with the USDA Agricultural Research Service and the USDI Bureau of Land Management (Booth, et al., 2006).

- **Stele:** The central core of the stem and root of a vascular plant, consisting of the vascular tissue (xylem and phloem) and associated supporting tissue.
- **Tryptic Soy Agar (TSA):** Tryptic soy agar or Trypticase soy agar is a solid growth medium for the culturing of bacteria. It is a general-purpose, nonselective media providing enough nutrients to allow for a wide variety of microorganisms to grow. It has a wide range of applications.
- **Tryptic Soy Broth (TSB):** Tryptic soy broth or Trypticase soy broth is used in microbiology laboratories as a culture broth to grow aerobic bacteria. It is a complex, general purpose medium that is routinely used to grow certain pathogenic bacteria, which tend to have high nutritional requirements.
- **United States Department of Agriculture: (USDA)**
- **Weed-suppressive Bacteria (WSB):** Bacteria that have been screened for their ability to reduce growth of weed(s) without negatively affecting desirable plants. The bacteria used in this project was *Pseudomonas fluorescens* strain ACK55.

Executive Summary

Transportation departments face the looming challenge of ever-increasing invasive, annual grasses on roadsides and rights-of-ways. Downy brome (cheatgrass, *Bromus tectorum* L.) and medusahead (*Taeniatherum caput-medusae*[L.] Nevski) negatively affect vegetation efforts on roadsides and cause ecological disaster because they alter vegetation diversity and soil quality; increase the use of herbicides and tillage; and provide fuel to wildfires. These two annual weeds are extremely competitive with perennial (desirable) grasses and other plants for available moisture and nutrients. They choke out natives in the shrub-steppe habitat of the western United States rangeland; builds fire-fuel load; and increases fire frequency and intensity. Downy brome (cheatgrass) and medusahead fuel wildfires that can destroy property and may result in loss of structures and lives.

Road construction and improvements often provide newly disturbed land that is especially vulnerable to invasion by downy brome (cheatgrass) and medusahead. Tillage, residue burning and herbicides are management practices available to land managers; however, these options individually have shown minimal results at reducing theses weed populations or sustaining desirable vegetation and healthy ecosystems.

Within the soil microbial community, plant-microbe interactions abound and can be used in restoration efforts. Naturally occurring soil bacteria can be used to reduce the competitiveness of weeds and when used in concert with present restoration efforts have the potential to change the vegetation to desired species. This study evaluatedhow weed-suppressive bacteria (WSB); *Pseudomonas fluorescens* strain ACK55 (ACK55), can be applied in a roadside setting to reduce the competitiveness of downy brome (cheatgrass) and medusahead, increase plant diversity and reduce the wildfire threat.

These weed-suppressive bacteria:

- are applied in the fall and establish in the soil microbial community as weather cools;
- inhibit root formation, root growth, and tiller initiation of these weeds;
- do not hurt native plants or crops;
- grow well in fall and spring coinciding with the early root growth of fall annual weeds; and
- grow along roots and deliver the weed-suppressive compound.

The bacterium *Pseudomonas fluorescens* strain ACK55 (ACK55; NRRL B-50848), inhibits only:

- downy brome (cheatgrass);
- medusahead; and
- jointed goatgrass *(Aegilops cylindrica* L.*).*

Pseudomonas fluorescens strain ACK55 does not inhibit economically important plants and does not injure any native plant species found in the United States. The project objective was to obtain the data needed to develop best management practices that incorporate WSB into programs that successfully reduce downy brome (cheatgrass) and medusahead, and re-establish native populations for ITD roadsides.

The reduction of downy brome (cheat grass) by the WSB was evaluated on roadsides of I-84, I-86 and US-95 in Idaho. Bacteria were produced in fall 2014, 2015, and 2016 for the field studies. The bacteria were freeze dried, vacuum packed and stored at -80^{o}C until applied to the plots. The populations of WSB were above log 9 for all sample packets. Efficacy was excellent and the minimum dose to exhibit weed suppression was log 4.7.

The downy brome (cheatgrass)-suppressive bacterium was sprayed on 1-acre plots at different locations in the fall of 2014, 2015, and 2016 at 10^9 colony forming units per m^2. Because the locations in the study had some seeded plants already established, imazapic (imidazolinone herbicide, $C_{14}H_{17}N_3O_3$; one tradename is Plateau) was not used in this study as originally planned. Bacteria were applied to eleven locations at 7 sites. Three locations were roadsides on US-95, five locations were on I-84 and two locations were on I-86.

The vegetation at each plot at each location was monitored in the spring and fall of each year and throughout the test period using Sample Point. Sample Point is a method of monitoring vegetation using digital images, a software package that calculates percent cover. Percent cover of species (native and invasive), the total cover of vegetation, litter, rock, cryptogamic crust and bare soil were recorded. For each location, the effect of the bacterial treatments on annual invasive grasses and native plant growth was determined. The relationships among these data and the variables of location, landscape, soil characteristics, and climate was also investigated. The bacteria reduced downy brome (cheatgrass) by 30 to 97% one year after bacterial application. Although medusahead patches were evident in the control plots, medusahead was not found in the bacterial plots.

The best-case scenario for optimum use and greatest success in using WSB on Idaho roadsides consists of the following six points:

1. Select a site with a healthy, dense stand of desirable plant species, such as sheep fescue, Sandberg bluegrass, crested wheatgrass, etc.
2. Select a site with moderate to low infestation of downy brome (cheatgrass) or medusahead.
3. Apply the bacteria to the site in late fall, when air and soil temperatures are below 50^{o}F and rain is forecast.
4. Apply 1 gallon of actively growing WSB to each acre.
5. Apply a broadleaf herbicide in the spring as needed.
6. The bacteria take several years to reduce downy brome (cheatgrass) or medusahead populations.

Key findings and recommendations from the study are as follows with additional details in Chapter 3.

- The timing of the WSB application is critical to the successful, long-term reduction of downy brome (cheatgrass), medusahead, and jointed goatgrass. Late fall applications, when air and soil temperatures are cool (below 50^{o}F), rains are prevalent and skies are overcast, have the highest success.
- Apply 1 gallon of actively growing WSB per acre. The carrier is water and the volume can range from 2 to 30 gallons.
- Spring applications of the WSB do not lead to consistent suppression of downy brome (cheatgrass), medusahead, or jointed goatgrass because the conditions for WSB growth, establishment and survival are sub-optimal.

- After application, the bacteria will be active in the soil for 4 to 6 years. Repeat applications may be needed depending on the site and reoccurrence of weed populations.
- The success of the WSB relies on the interaction between the weed, the bacteria, and desirable plants (native or near native). The greatest success with long-term reductions in downy brome (cheatgrass), medusahead, and jointed goatgrass has been on lands with mixed populations of native plants and moderate weed infestations.
- WSB suppress downy brome (cheatgrass), medusahead, and jointed goatgrass at the seedling (shoot emergence) stage and do not reduce the growth of established weed plants.
- As WSB reduce downy brome (cheatgrass), medusahead, and jointed goatgrass, voids are created that broadleaf weeds fill. Since WSB does not inhibit broadleaf plants, the site must be monitored carefully. Often, the use of a broadleaf herbicide is necessary in the spring of each year.
- Knowing the site history is critical to restoration success and the use of all available management tools is necessary to restore abandoned farmland.
- Use of glyphosate, imazapic, or other herbicides in the fall reduces weed growth and seed production. Tillage is needed to allow weed seed to come in contact with the soil so that it will more quickly germinate. Allowing the weed to grow only to the 2-leaf stage followed by glyphosate, imazapic, or another appropriate herbicide application is recommended.

With the reduction of the annual grass weed, other plant species are more competitive. The bacteria suppress weed roots at a time when the weed is increasing its competitive root growth. These bacteria provide a novel means to reduce invasive weeds while limiting the need for tillage and chemical use for weed control. Because of its selectivity, this bacterium can be used in management of the invasive weeds downy brome (cheatgrass) and medusahead on ITD roadsides.

Chapter 1
Introduction

Downy brome (cheatgrass, *Bromus tectorum* L.), medusahead (*Taeniatherum caput-medusae* [L.] Nevski), and jointed goatgrass (*Aegilops cylindrica* L.) are exotic, annual grass species that negatively affect cereal production in cropland; choke out native plants in the shrub-steppe of the western United States rangeland; reduce sage-grouse habitat; reduce habitat for other sagebrush-dependent wildlife; and increase fire frequency of these lands. The residue left from these three weeds increases the fire fuel load, resulting in wildfires that destroy property and cause the loss of human life. Naturally occurring bacteria from the soil and root surface have been found to inhibit these and other invasive weeds (Kennedy et al., 1991; Kennedy, 2014a, 2014b, Stubbs et al., 2014; Kennedy, 2016). [1,2,4,5]

These weed-suppressive bacteria:

- are applied in the fall and establish in the soil microbial community as weather cools;
- inhibit root formation, root growth, and tiller initiation of these weeds;
- do not hurt native plants or crops;
- grow well in fall and spring coinciding with the early root growth of fall annual weeds; and
- grow along roots and delivers the weed-suppressive compound.

The WSB used in this study, *Pseudomonas fluorescens* strain ACK55 (ACK55; NRRL B-50848), inhibits only:

- downy brome (cheatgrass),
- medusahead, and
- jointed goatgrass.

The WSB does not inhibit economically important plants and does not injure any native plant species found in the United States (Kennedy, 2014b, 2017, Kennedy et al., 2018a; Kennedy, 2018). [3,6,7,8]

In long-term field trials in the western United States, application of the bacteria resulted in almost complete suppression of these fall annual grass weeds (Kennedy, 2017, 2018; Kennedy et al., 2018b). [6, 8,9] Few of these annual grass weeds remained in the seed bank 5 to 7 years after a single application. Other plant species became more competitive with the reduction in annual grass weeds. The bacteria suppress weed roots at a time when the weed is increasing its competitive root growth and provide a novel means to reduce invasive weeds, while limiting the need for tillage and chemical weed control. Because of its selectivity, this bacterium can be used in the management of the invasive weeds downy brome (cheatgrass), medusahead, and jointed goat grass in rangeland, cropland, pasture, turf, sod production, golf courses, road sides and road cuts, construction sites, and right-of-ways (road, rail, pipeline, electrical; Kennedy et al., 2018a). [7]

Invasive Weeds

Downy brome (cheatgrass; Figure 1), and medusahead (Figure 2) are exotic, annual grass species that increase fire frequency in the western United States (Whisenant, 1990; Haubensak et al., 2009; Balch et al., 2013); reduce yields in croplands (Thill et al., 1984; Stahlman & Miller, 1990) and replace natives in rangelands (Thill et al., 1984; Duncan et al., 2004; Rice, 2005a, 2005b). [10,11,12,13,14,15,16,17] These weeds negatively affect the shrub-steppe habitat of the western United States by reducing the habitat for over one hundred sagebrush-dependent wildlife (Crawford et al., 2004), such as the sage grouse (*Centrocercus urophasianus*). [18] Downy brome (cheatgrass), medusahead and jointed goatgrass also negatively impact recreational lands (Duncan et al., 2004; Rice 2005a, 2005b) and sacred Native American lands that produce medical plants (Borins, 1995). [15,16,17,19]

More than 200 million acres of sagebrush steppe existed in North America in the 1880s. Presently, 100 million acres of this habitat in the Intermountain West remain, but more than half of these acres are infested with downy brome (cheatgrass) or medusahead. Today, downy brome (cheatgrass) can be found in all 50 states of the United States of America, as well as many provinces of Canada and several states in Mexico (Figure 3). Fire size, intensity, and frequency have increased dramatically with the expansion of downy brome (cheatgrass) and medusahead infestations (Whisenant, 1990; Brooks et al., 2004) Haubensak et al., 2009; Balch et al., 2013). [10,22,11,12] These invasive grasses quickly establish in disturbed sites leading to monocultures of downy brome (cheatgrass) or medusahead (Knapp, 1996). [23] The spread of invasive annual weeds was estimated in 1995 to be an alarming 4,600 acres a day (Asher and Harmon, 1995). [24] The dead above-ground biomass of downy brome (cheatgrass) and medusahead leaves a fine, dense mat of highly flammable fuel susceptible to ignition, which accelerates fire cycles (USGS, 2002). [25] Downy brome (cheatgrass) and medusahead infestations not only increase the frequency of wildfires, but also amplify fire intensity and size (Jackson and Sullivan, 2009). [26] Millions of acres of shrub-steppe have also been converted to annual grasslands, further exacerbating the spread of wildfires. In addition, roadsides and right of ways are invaded (or infested) with these invasive grasses that increase seed production and transport of these invasive seeds throughout the west.

Rangeland in the western United States has also been invaded by medusahead (Rice, 2005b; Davies, 2010) and will continue to spread in ways similar to other invasive grass weeds (Figure 4). [17,27] By the early 1990s, 14 million acres of public lands in the Intermountain West were infested with downy brome (cheatgrass), medusahead, or both; however, the area at risk of invasion by these two grasses is at least another 60 million acres.

Loss of sagebrush cover and increase in monoculture downy brome (cheatgrass)/medusahead stands are detrimental to nesting, foraging, and survival of sage grouse and other wildlife (Wisdom and Chambers, 2009). [28] A key step in restoration is to reduce downy brome (cheatgrass)/medusahead stands and establish native plant species. Burning, tillage, and herbicides have all been used with some success at reducing grass weeds (Epanchin-Niell et al., 2009; Davies, 2010). [29,27] Each, though, has its limitations and none appear to be successful in long-term restoration. Ecologically sound tools are

needed in restoration to reduce downy brome (cheatgrass) and medusahead stands and facilitate succession towards more native plant communities.

Restoration of annual grass weed-infested systems is a daunting task due to the competitive nature of the grasses; the slow seedling establishment of displaced native species; and the complex nature of the variety and scale of invaded sites. Alternatively, an increase in native bunchgrasses cover can reduce downy brome (cheatgrass)/medusahead abundance and competitiveness. Current methods such as herbicide application and drill seeding native grasses have had limited success at reestablishing native species and reducing annual grass weed abundance. There are several examples of larger scale restoration projects that have shown promise (Davies, 2010; Benson et al., 2011). [27,30] Thus far, few restoration efforts have reduced downy brome (cheatgrass) populations at large enough scales necessary to reduce wildfires that can lead to destruction of property, loss of life, and loss of habitat of landscape-level wildlife species.

The System

Bacteria from the soil and root surface have been found to inhibit downy brome (cheatgrass), medusahead, and jointed goatgrass and provide a valuable tool to fight these invasive weeds (Stubbs et al., 2014). [4] Building on the phenomenon of poor grass or cereal growth in the early spring, 20,000 bacteria were isolated from soil and roots just after freeze-thaw events (Kennedy, 2014a, 2014b, 2018). [2,3,8] Those isolates were screened in laboratory and greenhouse assays to obtain strains of bacteria that were selective in suppressing the growth of grass weeds but did not inhibit beneficial plants, such as cereals and native grasses. Those bacteria that were selective in their suppression were tested in field trials. Several strains of bacteria were found that inhibited downy brome (cheatgrass), medusahead, and jointed goatgrass in the field, but did not harm crops or native plants. *Pseudomonas fluorescens* strain ACK55 is one of those strains.

The WSB*, Pseudomonas fluorescens* strain ACK55, is a naturally occurring bacterium that produces a labile compound that inhibits downy brome (cheatgrass), medusahead, and jointed goatgrass, while not hurting native plants or crops. The bacterium is applied in the fall and it is active in the soil below the soil surface only during cool temperatures in late winter to early spring. The bacterium inhibits cell elongation of the weed root, reduces root growth, and reduces tiller formation, which decreases the weed competitiveness. By reducing the competitive ability of the annual grass weeds, the other plant species are able to establish and grow, allowing the bacterium and the beneficial plants to work together to reduce further weed establisment. The WSB declines with summer temperatures and will not overrun the native soil bacteria. It is not a competitive bacterium, although it can survive in the soil for up to 6 years after application. The WSB only moves in soil by traveling on the growing root or with water.

Host-range studies, investigating more than 250 select plant species, showed that only downy brome (cheatgrass), medusahead, jointed goatgrass, and their accessions were significantly inhibited by the bacteria (Kennedy, 2014a). [2] The WSB will colonize the roots of many different plants and reside on the

root or inside the plant cell wall but onouter plant cell membrane and outside the casparian strip. It does not enter the plant cell as it does not have the enzymes to break down the cell membrane, nor can it enter the plant stele, where the xylem and phloem reside. When the WSB suppresses roots, there are no visible signs of pathogenicity or lesions, just stunted roots. The inhibitory compound is made up of multiple compounds and is only active if all compounds are present. The active compound breaks down very readily and is not active in the soil solution (Kennedy, 2014b). [(3)] The weed-suppressive compound reduces plant cell elongation and is species specific. It only inhibits a few species of plants and those plant species are only grasses not broadleaf plants. The genes responsible for the weed-suppressive compound are found in many locations on the chromosome. Because of this, it would be very difficult for the inhibitory compound to change and affect other plant species, such as wheat or native blue bunch wheatgrass.

When WSB is sprayed on the soil, very few bacteria survive on the actual soil surface due to the harsh environment of UV light, low moisture, and extreme temperatures. Those bacteria that move down into the soil by rain survive and multiply. Application of WSB is best with fall rains to help the bacteria survive and move with the water to below the soil surface. (Kennedy et al,. 2018a). [(7)] The WSB, *Pseudomonas fluorescens* strain ACK55, numbers increase during cold temperatures, unlike most soil bacteria. On the other hand, they cannot compete with soil inhabitants when soil temperatures are above 10^{o}C (50^{o}F). *Pseudomonas fluorescens* strain ACK55 declines in numbers above 10^{o}C (50^{o}F) as they are inhibited by other, faster growing microbes. WSB is applied to the soil at rates higher than needed for weed inhibition because, as with any introduced organism, there is a decline in population after application. The bacteria can be applied to the soil as a spray, or coated onto native seed and then seed drilled into the soil.

This bacterium/weed interaction does not follow the normal herbicide paradigm and it takes ACK55 several years to suppress the weeds (Kennedy, 2018). [(8)] In the first few years after field application, the bacteria inhibit plant growth and populations by 20 to 50% and this inhibition increases with time. In cropland studies, the bacteria suppressed the weed and allowed the wheat to be more competitive, which then in turn reduced weed populations further. In long-term rangeland field trials in WA, application of the bacteria resulted in almost complete suppression of downy brome (cheatgrass) 5 to 7 years after a single application. With the reduction of downy brome (cheatgrass), native plant species increased over time. In these studies, there was little suppression of downy brome (cheatgrass) in the first year after fall application. The bacterium, however, also reduces the weed in the seed bank. In addition, at each site the populations of more desirable plant species increase as the downy brome (cheatgrass) becomes less competitive. The bacteria plus the native plant, turf, or wheat interact to reduce the downy brome (cheatgrass).

A study funded by the Nature Conservancy explored whether weed-suppressive bacteria could be effectively used to reduce downy brome(cheatgrass) and medusahead across a wide geography; in a variety of soil types; and in diverse climatic conditions in the shrub steppe ecosystem (Kennedy et al., 2018b). [(9)] The integration of this bioherbicide into restoration plans was also investigated. Eleven experimental sites were established in the Columbia Plateau and northern Great Basin ecoregions in

sagebrush steppe communities containing these weeds. These sites include four in Washington: Moses Coulee at Soap, WA, the Hanford Reach National Monument, and two locations in the Saddle Mountains. Seven additional sites were associated with the Ecologically-Based Invasive Plant Management project in Adin, CA; Elko, NV; Park Valley, UT; Warm Springs, ID; Burns, Jordan Valley, and Riverside, OR. The project's objective was to implement and test sagebrush steppe restoration treatments based on ecological principles for management of invasive plant species (http://ebipm.org). When *Pseudomonas fluorescens* strain D7 (D7) was applied to monoculture annual grass weed stands, the downy brome(cheatgrass) and medusahead populations were suppressed by 50% three years after application, but no further inhibition was observed in following years. Instead, weed populations recovered to pre-treatment levels by year 5. In contrast, downy brome (cheatgrass) and medusahead located in mixed stands of native or near-native plant species were inhibited at least 50% by year 3 and additional weed inhibition (60-80%) in mixed stands continued from year 3 to 5 while increases in perennial cover were observed during this period. D7 was most effective inhibiting grass weeds when a desirable plant was also present to compete with the decreasing grass weed population and fill voids created by the dead weeds. If desirable plants are not already present, D7 treatment can be coupled with drill seeding of desirable plant species.

A gallon of actively growing ACK55 (1×10^9 colony forming units mL^{-1}) is the amount needed per acre to suppress downy brome (cheatgrass) in 5 to 7 years (Kennedy et al., 2018a). [(7)] After the application of the bacterium, invasive weed root growth declines thus reducing weed competition allowing other beneficial plant species to be more competitive. Both the bacterium and the beneficial plants aid in reducing downy brome (cheatgrass) growth. This makes a good match for biocontrol, because root suppression occurs at a time when the weed's root growth increases. The benefits of weed-suppressive bacteria include dollar-valued changes in rangeland productivity for ranching, expected reduction in firefighting costs, and reduced expected losses of infrastructure due to reduced risk of wildfire (USGS, 2002). [(25)] Wildfires fueled by annual grasses destroy large portions of greater sage-grouse habitat. If unchecked, this threat could dramatically affect the long-term conservation of sage grouse and other rangeland species (Crawford et al., 2004). [(18)] In addition, downy brome (cheatgrass) invasions increase the wildfire frequency, which leads to destruction of property and loss of life. However, there are many other benefits that cannot be gauged in terms of market value. These include the value of ecological services to the general public that would be lost or reduced in quality and/or quantity if specific areas of landscape are allowed to transition to monocultures of invasive grasses. These ecological services provide benefits in the form of wildlife viewing and hunting, recreational opportunities, water quality and quantity regulation, and the bequest value of knowing that shrub-steppe ecosystems are maintained for future generations.

The Bacterium

Pseudomonas fluorescens strain ACK55 is a motile, Gram-negative rod. This bacterium was isolated from agricultural soils near Pullman, WA in early spring of 2001 during a thaw after a hard freeze (Kennedy, 2014b). [(3)] It has two polar flagella and a thick exopolysaccharide coating (Figure 5). Taxonomically, it is identified as *Pseudomonas fluorescens* biovar II or 'B' group with some similarity to biovar I or 'A' group

from MIDI analysis and it grows within pH 3.8 and 8.5. The temperature range for growth of ACK55 alone in pure liquid culture is from 0°C (32°F) to 30°C (86°F) with a growth temperature optimum of 8°C (46°F). *Pseudomonas fluorescens* strain ACK55 survival temperatures in soil differ from those in pure culture due to competition from predators. Growth in soil occurs between 0°C (32°F) and 10°C (50°F) with an optimum growth temperature of 4°C (39°F). *Pseudomonas fluorescens* strain ACK55 is an aerobe, but can function as a facultative anaerobe with nitrate as the substrate. It produces a large molecular weight compound with tertiary structure that inhibits lipopolysaccharide production and cell elongation in the roots of accessions of downy brome (cheatgrass), medusahead, and jointed goatgrass. The active fraction complex is highly labile and can only be partially purified. The compound contains chromopeptides, other peptides, and fatty acid esters in a lipopolysaccharide matrix. Separation of any of the components from the complex resulted in nearly complete loss of activity against downy brome (cheatgrass), medusahead, and jointed goatgrass. The genes responsible for the weed-suppressive compound are found on the chromosome at multiple positions, all of which are needed for activity. *Pseudomonas fluorescens* strain ACK55 has no anti-fungal activity and no anti-bacterial activity (Kennedy, 2014a; 2014b, 2018). [2,3,8] *Pseudomonas fluorescens* strain ACK55 does not produce Type 1, 2, or 3 secretions and does not produce enzymes that degrade plant cells. *Pseudomonas fluorescens* strain ACK55 reduces root growth by producing a suppressive compound that negatively affects lipid biosynthesis in the root. This inhibition is specific and inhibits cell elongation and tiller initiation. The bacteria can survive on crop residue in the soil and then move to the seed or growing roots. It survives well on roots, but is dormant at soil temperatures above 21°C (70°F). *Pseudomonas fluorescens* strain ACK55 needs to survive below the soil surface in order to suppress the weeds and requires some moisture to increase in numbers sufficient to cause weed inhibition.

The Genus *Pseudomonas*

Pseudomonas spp. are ubiquitous in the environment and can be found not only in the soil and water but on surfaces, plants, insects, and animals. The species in this genus are generally nonpathogenic and are usually involved in disease suppression or cycling of nutrients. The genus *Pseudomonas* has been divided into five groups based on metabolic characteristics and rRNA/DNA homology (Palleroni, 1984). ([31] More than 20 *Pseudomonas* species have been isolated from human clinical specimens. Chen et al. (2012) [32] list four organisms, which cause the majority of infection: *Pseudomonas aeruginosa* (homology group I) is the primary cause of ICU-related pneumonia and osteochondritis; *P. cepacia* (group II; now known as *Burkholderia cepacia*) causes foot rot in military troops and infections in children with cystic fibrosis; *P. pseudomallei* (group II; now known as *B. pseudomallei*) causes melioidosis in sheep, goats, horses, swine, cattle, dogs and cats, and is endemic in southeast Asia; *P. mallei* (group II; now known as *B. mallei*) causes glanders, a serious infectious disease of mainly horses, and also donkeys, mules, goats, dogs, and cats (not found in the U.S.; common in other parts of the world).

The majority of pseudomonad infections are caused by *Pseudomonas aeruginosa* (CDC, 2013). [33] These pseudomonads are prevalent in our environment and exist without being a pathogen. They do cause infection in the weak or ill, pneumonia in patients on breathing machines, and infections to burn victims and those with puncture wounds. In moist environments such as swimming pools they may cause skin

rashes, "swimmer's ear" and eye infections. Cystic fibrosis patients are susceptible to *Pseudomonas aeruginosa* infections in their lungs (López-Causapé et al., 2013). [34] *Pseudomonas aeruginosa* is an opportunistic organism, and in most cases only causes infection in those with compromised immune systems.

Pseudomonas fluorescens are abundant in the environment, growing mainly in soil, water, and on the surfaces of plants (Palleroni, 1984; NCBI, 2013; Hol et al., 2013), especially the rhizosphere (Rainey, 1999). [31,35,36,37] *Pseudomonas fluorescens* strains are common colonizers of potable water treatment and distribution systems (Geldreich, 1996). [38] They are colonizers of soil, residue, and roots; and are found in high numbers in every soil type (Clays-Josserand et al., 1999). [39] They also exist on and within insects and animals and are involved in the release of immobilized nutrients. *Pseudomonas fluorescens* are involved in biological control of diseases and antagonistic microbes (Howell and Stipanovic, 1979; Weller and Cook, 1983; Castrillo et al., 2000; Dekkers et al., 2000; Mazzola et al., 2007; Shalini & Srivastava, 2007). [40,41,42,43,44,45] They are rarely considered pathogens.

There are five biotypes or biovars of *Pseudomonas fluorescens* with multiple properties used to differentiate among them (Stanier et al., 1966; Palleroni, 1984). [46, 30] Their optimal growth temperature is 25-30^{o}C (77-86^{o}F), but they may grow at temperatures as low as 4^{o}C (39^{o}F). They are rarely found in clinical settings; however, as many are considered psychrotrophic. Many products provide an environment for bacterial growth. *Pseudomonas fluorescens* are often responsible for spoilage of refrigerated foods, especially dairy, egg, fish, and meat products (Molin and Ternstrom, 1986; Sillankorva et al., 2008). [47,48] However, there are other strains of *Pseudomonas fluorescens* that thrive only at the lower temperatures.

Pseudomonas fluorescens have the ability to form biofilms under a variety of growing conditions on abiotic surfaces (O'Toole and Kolter, 1998), solid-liquid interfaces, and air-liquid interfaces (Spiers et al., 2003). [49,50] Biofilms are better able to withstand competition from other organisms, are less likely to be displaced from a surface through physical means, are better able to resist predators, and are physiologically different from free-living cells of the same bacteria; however, they have the disadvantage of being unable to escape detrimental growth conditions (Spiers et al., 2003). [50]

Pseudomonas fluorescens play a number of diverse and beneficial roles in the environment. They are valuable in agriculture due to their ability to reside in the rhizosphere of plant roots, obtaining nutrients, and protection from the plant and in turn helping to protect the plant from environmental toxins and pathogens. Several strains of *Pseudomonas fluorescens* have shown antifungal activity against a number of plant pathogens, including *Fusarium* sp., *Curvularia lunata,* and *Bipolaris* sp., as well as *Helminthosporium* in laboratory studies (Shalini & Srivastava, 2007). [45] *Pseudomonas fluorescens* strains inhibit the fungal pathogen *Pythium*, while increasing root and shoot weight of pea (*Pisum sativum*) plants[40] (Naseby et al., 2001). [51] *Pseudomonas fluorescens* SS101, biovar II, protects hyacinth bulbs from root rot caused by *Pythium intermedium* (deSouza et al., 2003). [52] *Pseudomonas fluorescens* strain WCS365 controls foot and root rot of tomato caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*[42] (Dekkers et al., 2000). [43] *Pseudomonas fluorescens* produce 2,4- diacetylphloroglucinol (DAPG), an

antibiotic that inhibits take-all disease in wheat caused by the pathogen *Gaeumannomyces graminis* var. *tritici* (Cook et al., 1995). [53] Meyer and Collar (2009) showed that DAPG inhibited many of the nematodes they tested, and that host-plant resistance to certain nematodes might be stimulated. [62] *Pseudomonas fluorescens* strain SS101 has shown the ability to control *Pythium* root rot in flower bulbs (deSouza et al., 2003), wheat and apple seedlings, and rootstock (Mazzola et al., 2007). ([52,44] *Pseudomonas fluorescens* strain 5 (Pf-5) inhibits the soil-borne plant pathogens *Rhizoctonia solani* (Howell and Stipanovic, 1979) and *Pythium ultimum* (Howell and Stipanovic, 1980). Pf-5 produces numerous antibiotics that are secondary metabolites. [40, 55] Paulsen et al. (2005) sequenced the entire genome of Pf-5. ([56] Multiple strains of *Pseudomonas fluorescens* have been isolated that inhibited the phytopathogens *Alternaria solani*, *Curvularia lunata*, *Fusarium* sp., *Bipolaris* sp. and *Helminthosporium* sp.[34] (Shalini & Srivastava, 2007). [45]

Certain strains of *P. fluorescens* produce secondary metabolites (Trippe et al., 2013) that inhibit soil-borne plant pathogens and prevent fungal diseases (Cook et al., 1995). [57, 53] *Pseudomonas fluorescens* species have been used in other applications, including pharmaceutical production, snow making, frost protection for strawberries, and disease protection for apples. Ice-nucleating *Pseudomonas fluorescens* were discovered to reduce Colorado potato beetle cold tolerance, and may have potential as an insect biological control agent (Castrillo et al., 2000). [42] Aminopeptidase produced by *P. fluorescens* [ATCC948] has been used as an additive to dairy products for debittering milk that had been contaminated by other *Pseudomonas fluorescens* species (Gobetti et al., 1995). [58] *P. fluorescens* are responsible for producing the antibiotic Mupirocin, used to treat infections caused by Methicillin-resistant *Staphylococcus aureus* (Thomas et al., 2010). [59] The naturally occurring strain *Pseudomonas fluorescens* (CL145A) is used to produce Zequanox, an aquatic biopesticide shown to be effective in controlling invasive zebra and quagga mussels. It was originally isolated from river soil in the northeastern United States (Molloy et al., 2013). [60]

Pseudomonas fluorescens strains have been alleged to cause hemorrhaging and lesions in catfish hatcheries (Meyer and Collar, 1964). [61] Many, but not all, of the growth characteristics of the causal agent were similar to those of *Pseudomonas fluorescens*. These incidences were only found in hatcheries where the populations are too high, temperatures are elevated, or the hatchlings become stressed. The disease was easily controlled by removing stressors. Further, *Pseudomonas plecoglossicida* is a fish pathogenic species that is often confused with *Pseudomonas fluorescens. Pseudomonas fluorescens* is considered to be only mildly hazardous to humans, and generally only harmful to those with compromised immune systems such as cancer patients and those with immunodeficient diseases like lupus. *Pseudomonas fluorescens* is considered to be non-virulent relative to other Pseudomonas species; although it has been linked to some infection often the causal agent is misidentified and is considered a secondary invader present after the initial infection. Cases of exogenous post-transfusion septicemia in patients may be controlled through more thorough cleaning of blood donor arms (Puckett et al., 1992). [62] Hsueh et al. (1998) reported on four hospital patients with infection caused by *Pseudomonas fluorescens* that had not received blood transfusions, and concluded that rapid identification of less common pathogens was critical to preventing an outbreak. [63] *Pseudomonas*

fluorescens infection was reported in the United Kingdom in a bone marrow transplant unit after being spread through a contaminated drinking water dispenser (Wong et al., 2011). [64] In 2005, there was an outbreak of bloodstream infections caused by *P. fluorescens*-contaminated syringes filled with intravenous catheter flush (CDC, 2006). [65] This outbreak was due in part to what was initially identified as *Pseudomonas fluorescens,* but was later identified as a specific biovar of *Pseudomonas fluorescens-Pseudomonas putida*. These few incidences are rare and *Pseudomonas fluorescens* are not considered to be pathogenic in most environments.

Pseudomonas fluorescens strains are naturally occurring, ubiquitous in soil and the environment, with only limited reports of its ability to cause disease in humans of which occurred under extremely opportunistic conditions (Gilardi, 1972). [66] *Pseudomonas fluorescens* strains are prevalent in the normal environment with only rare human disease associations of a distant relative of most *P. fluorescens* strains. Of the more than 4000 articles found in a literature search of *Pseudomonas fluorescens*, pathogen, and animal the majority refer to *Pseudomonas fluorescens* as a biocontrol organism or a plant growth promoter 28 (Howell and Stipanovic, 1979; Weller and Cook, 1983; Kennedy et al., 1991; Castrillo et al., 2000; Dekkers et al., 2000; Naseby et al., 2001; Mazzola et al., 2007; Shalini & Srivastava, 2007). [40,41,1,42,43,51,44,45] *Pseudomonas fluorescens* used in biocontrol situations are generally found in the first three biovar groups where noted. Sundh et al. (2011) found that *Pseudomonas fluorescens* were nonpathogenic and lacked the virulence factors of other plant pathogens. [67] *Pseudomonas fluorescens* that may be considered pathogens are found on the surfaces of moist environments or as a secondary invader in sick animals or humans. These *Pseudomonas fluorescens* produce antibiotics, Type II or III secretions, or enzymes that lyse cells. While few report the biovar designation of these *Pseudomonas fluorescens,* they are classified as biovar G or H and/or are closely related to *Pseudomonas putida.* ACK55 is only distantly related to these bacterial strains because it is a biovar B with no type 3 sections; has minimal protein in the cytosol; and possesses no ability to lyse cells. Identification of strains of *Pseudomonas fluorescens* in biocontrol studies is needed before release into the environment to determine biovar designation and to limit the use of a potential human or animal pathogen for biocontrol.

The *Pseudomonas fluorescens* strain under consideration, ACK55 is identified as *Pseudomonas fluorescens* biovar 'B' (type II) and is genetically distant from *Pseudomonas putida* or *P. fluorescens –P. putida complex.* The additional screening performed to select this bacterium was extensive to rule out any harmful antibiotic, cell-lytic enzymes or protein production by this strain of *Pseudomonas fluorescens*.

Field Studies

The prospect of finding a successful biocontrol agent to manage weeds has its skeptics, perhaps because researchers have prematurely spoken or published on finding a pathogen of a problematic weed before conducting extensive, non-target studies, only to find later that the microorganism inhibits more than just the target weed (Cordeau et al., 2016; Ghosheh, 2005). [68,69] It is not difficult to find bacteria or fungi that can inhibit weed growth. However, it is a completely different matter to find microorganisms that specifically suppress a weed and do not harm any other flora or fauna. A defining

characteristic of a good herbicide is its selectivity. The same criterion must be paramount in the search for biological control organisms. Deleterious rhizobacteria were identified in the 1980s, and strains that could inhibit various crops and weeds were found (Kremer et al., 2006; de Luna et al., 2005; Nehl et al., 1997; Kremer, 1986, 1987; Frederick and Elliott, 1985; Suslow and Schroth, 1982), but few bacteria were screened across many plant families or studied extensively in the field over multiple years. [70,71,72,73,74,75,76] Accurate conclusions cannot not be drawn without performing a comprehensive, methodical study of the effect of a biocontrol candidate in both small- and large-scale studies over many years in the field. Naturally occurring soil bacteria have been identified that specifically inhibit the growth of various invasive weeds by targeting the seed bank (Kennedy et al., 1991, Kennedy, 2016). [1,5]

The bacteria were applied to the soil as a spray, or coated onto crop or native seed and planted beneath the soil surface at levels of 2 x 10^{12} to 10^{13} colony forming units hectare^{-1}. (4 x 10^{12} to 10^{13} colony forming units acre^{-1}; Kennedy et al., 1991). [1] The timing of ACK55 application is critical to the success of reduction and removal of downy brome (cheatgrass). The harsh environment of the soil surface (ultraviolet light, low moisture, high temperatures) limits survival of ACK55. However, if ACK55 is mobilized down into the soil by rain or even melting snow, they survive well. *Pseudomonas fluorescens* strain ACK55 is applied in the late fall when air temperatures are below 10^{o}C (50^{o}F) and rain occurs shortly after application to ensure movement of the bacteria into the soil where it can survive and flourish. These weed-suppressive bacteria inhibit invasive grasses at the seedling stage and during early shoot emergence. The bacteria cannot significantly inhibit the growth of actively growing or mature plants. Herbicides can be used to reduce mature downy brome (cheatgrass) plant growth and limit the addition of new weed seed into the seed bank. ACK55 can be tank mixed with most herbicides; however, surfactants that are soapy or oily and adjuvants that are added to kill microbial growth may also kill the weed-suppressive bacteria. It may also be more cost effective to apply the herbicide without the bacteria earlier in the fall and/or later in the spring.

It may take ACK55 several years to suppress annual weeds (invasive grasses) as the bacteria inhibits germinating (emerging) weed seeds in the seed bank. In the first few years after field application, ACK55 inhibited downy brome (cheatgrass) growth by 20 to 50% (Kennedy, 2017). [6] The inhibition increases with time, reaching maximum weed suppression 5 to 7 years after application. In winter wheat fields, suppression of weed growth by ACK55 allowed the wheat to be more competitive, which in turn reduced weed populations further (Figure 6). In one field trial, winter wheat yields increased by 33% with the application of the bacteria (Kennedy et al., 1991). [1] This increase was equivalent yields in a weed-free control.

The bacterium must survive to reduce the downy brome and if the bacteria is applied before or during sunny weather (and in temperatures exceeding 10^{o}C (50^{o}F), little to no downy brome reduction is seen (Figure 7, Kennedy, 2017). [6] In long-term field trials in WA, spray application of the ACK55 resulted in almost complete suppression of (downy brome (cheatgrass) 5 to 7 years after a single bacterial application when a crop or perennial species were present (Figure 8, Kennedy, 2018). Monoculture downy brome (cheatgrass) plots only reached 50% inhibition with ACK55 when no native seed was present to further stress the downy brome. A desirable plant is needed to further compete with the downy brome (cheatgrass). The most significant factor in using these bacteria effectively is the timing of

application (Stubbs et al., 2014). [4] The bacteria flourish in cool soil temperatures below 50°C. They are sensitive to UV light and reproduce very poorly in warm, dry conditions. To obtain optimal weed inhibition, bacteria must be applied in the late fall or early winter to cool soil during overcast periods when rain or snow is in the immediate forecast. Without the water to move the bacteria down into the soil, many of the bacteria perish because they simply cannot survive the harsh conditions of the soil surface. The timing of application cannot be compromised.

Inhibition of target species did not occur immediately. In fact, after application, visible suppression of the weed was not usually evident until year 2. By year 4 or 5, however, weed presence was significantly reduced, the weeds were short and stunted, and very few seeds were produced per weed. Ideally, as the weeds disappear, desirable plants are able to fill the void, flourish, and prevent the return of the weed. Occasionally, the void left by the weeds is filled by undesirable broadleaf weeds and in such cases, broadleaf weed herbicide application is necessary. The growth and persistence of *Pseudomonas fluorescens strain* D7 and *Pseudomonas fluorescens strain P.f.* XJ3 in soils are very similar to ACK55 (Kennedy et al., 1991; Stubbs et al., 2014; Kennedy, 2016;). [1,4,5]

The effectiveness of weed-suppressive bacteria on lands with differing levels of weed infestation is evident (Figure 8; Kennedy, 2018). [7] In the first year after application of ACK55, little to no reduction in the downy brome (cheatgrass) population was observed. The greatest amount of reduction was in the winter wheat fields, but the reduction was less than 20% of the control population. Initially, this limited inhibition was disappointing when compared to normal herbicide application that provides immediate visual reduction in the above ground growth, but in subsequent years, a 50% reduction in weed population was observed for all three strains. By year 6, maximum inhibition was observed and the annual grass weeds in pastures, wheat fields and mixed stands were reduced to negligible levels. Treatments of monoculture weed plots resulted in a 50% reduction of weeds by year 6. Clearly, an application of weed-suppressive bacteria may not be enough to eliminate grass weed monocultures. Repeated application of the bacteria in years 3 or 6 coupled with herbicides, light tillage to stimulate germination of the weeds, followed by herbicide application and/or the introduction of native or near-native plant species by drill seeding may be required to control growth of monoculture stands of invasive weeds.

Although it may take ACK55 several years before significant weed suppression is apparent to the naked eye, in the first few years after field application, studies show the bacteria inhibit weed growth by 20 to 50% (Kennedy, 2018). [8] The inhibition increases with time, reaching maximum weed suppression three to six years after application. In winter wheat studies, the bacterial suppression of the weeds allowed the wheat to be more competitive, which in turn reduced weed populations further (Figure 8). In long-term rangeland field trials in WA, spray application of the bacteria resulted in almost complete suppression of moderate populations of downy brome (cheatgrass) (Figure 9), medusahead (Figure 10), and jointed goatgrass five to seven years after a single bacterial application when perennial species were also present. Roadside application of the bacteria also resulted in a sharp reduction of downy brome (cheatgrass), which stimulated the growth and establishment of perennial grasses that reduced soil erosion and fire potential (Figure 11, 12).

Perhaps the most effective treatment couples the one-time application of herbicides, such as imazapic (imidazolinone herbicide, $C_{14}H_{17}N_3O_3$; one trade name is Plateau) or glyphosate (N-(phosphonomethyl) glycine; one tradename is Roundup), with the bacteria. If herbicide, like imazapic, is applied when native perennials are dormant, but the weeds are still growing, the herbicide kills the growing weed plants on the soil surface and the bacteria inhibit the seeds and seedlings below. This combination leads to a rapid reduction of the target weed to non-economic levels within just a few years, especially if beneficial grasses and other plants are present to occupy the voided areas (Figure 13; Kennedy, 2018). [8] The weed-suppressive bacteria/herbicide combination aerially applied can be also extremely effective in creating fire breaks around infested lands that cannot be treated by ground application. These bacteria assist in the fight against wildfires by establishing swathes of weed-free areas that serve as fire breaks that slow down or stop the rapid progression of wildfires.

The use of biocontrol agents to inhibit the growth of weeds is not novel. The concept of searching within the entire soil microbial population to find naturally occurring bacteria that work at the seed bank level to inhibit weeds and do no harm to other organisms is still young. The approach taken here, utilized bacteria native and naturally occurring in the area by simply reapplyiing it back into the soil at higher numbers during a time when the target weed was most susceptible. The bacteria identified here are easy to propagate and very suitable for large-scale application.

Weed-suppressive bacteria can be sprayed by hand, agricultural ground spraying equipment, and aircraft. The bacteria can be coated on desirable seeds and then seed drilled into the ground. In all cases, when applied properly, excellent results have been achieved. Both the bacteria and the desirable seed, once drilled into the soil, are protected from the harsh environment of the soil surface. Both come in contact with soil and the accumulation of the limited soil water. Bacteria flourish on the desirable plant roots and spread throughout the soil. The bacteria suppress weed root growth, allow the desirable plant to take up more water and be a competitor of the weakened weed, further inhibiting weed growth.

The selection and screening process resulted in the isolation of ACK55. Further testing may identify unique, subtle traits that differentiate other strains and may single out strains that are more effective in some conditions than others. For example, one strain demonstrated a slightly higher temperature tolerance. Perhaps this strain will be more applicable in warmer temperatures. For this reason, it's necessary to carry forward multiple isolates and continue testing rather than trusting one organism to do it all.

The utilization of naturally occurring bacteria in weed control is in its infancy. These invasive weeds that infest pasture, cropland, and rangeland and illustrated the successful use of these bacteria to reduce the select weed populations in the field without harming the normal biota. This work expands on previous research on suppressive bacteria for the same three weeds (Kennedy et al., 1991; Stubbs et al., 2014) and for annual bluegrass (Kennedy, 2016). [1,4,5] Moreover, it emphasizes the weed-suppressive methodology and selection process can be directed toward many, if not all, undesirable weeds.

The addition of a synthetic herbicide to decrease weed growth further hastens the reduction of downy brome (Data not shown). At each treated site where the bacteria reduced downy brome, the populations of more desirable plant species increased as the invasive weed became less competitive. The bacteria, synthetic herbicide, plus the native plant, turf, or wheat interact to reduce the downy brome (cheatgrass). When applied to a post-wildfire site, these weed-suppressive bacteria can reduce the downy brome (cheatgrass) populations and allow desirable plant species take hold (Figure 13). As ACK55 reduces downy brome (cheatgrass), voids are created that other weeds, especially broadleaf weeds fill, and broadleaf herbicides are necessary and should be applied in the spring of each year. Restoration and ridding an area of the invasive weed, downy brome, is a long-term undertaking that involves bacteria-weed-plant-herbicide interactions, in-depth planning, and adaptive planning.

Sustainable crop production, and pasture and rangeland improvements will benefit from research directed toward the discovery, characterization, and utilization of soil bacteria that selectively suppress grass weeds. The soil contains weed-suppressive bacteria that that are extremely selective, well–matched to the weed(s) of interest, and do not harm other members of the agroecosystem. These bacteria easily and successfully fit into invasive weed management plans in roadside, cropland, and in rangeland restoration efforts. Weed-suppressive bacteria can reduce and complement synthetic herbicides, expand options in weed management, reduce state agency, farm, and ranch costs, and encourage the use of ecologically based systems. Weed-suppressive bacteria work within the seed bank and reduce weed-seed emergence. These bacteria pair well with synthetic herbicides that kill mature plant growth that the bacteria cannot suppress. In addition, synthetic herbicides can also be used on broadleaf weeds that arise in the void created by the reduction in downy brome (cheatgrass). Biological control agents such as these should reduce weeds effectively and economically, reduce weed management costs, and lead to greater sustainability.

Weed-suppressive bacteria provide novel tools for weed management. When bacteria are applied in concert with native seed and the correct herbicide is applied at the optimum rate and timing, the seed bank of the target weed is depleted and weed populations decrease to near zero. Weed-suppressive bacteria have an essential place in roadside, agriculture, and rangeland weed management by providing novel ways to reduce weed populations, reduce annual herbicide use, protect roadside soil from erosion, improve crop yields by limiting invasive weeds that can lead to sustainable crop production, improving rangelands by increasing plant diversity, and enhancing forage quality by fostering the return of native bunchgrasses and forbs. Weed management and restoration efforts will benefit from the addition of bacteria that selectively suppress weeds.

Objectives

The overall project goal was to obtain the data needed to develop best management practices that incorporate weed-suppressive bacteria into programs that successfully reduce downy brome (cheatgrass), and re-establish native populations.

The project objectives were to:

- Demonstrate the inhibition of downy brome (cheatgrass) growth by the application of downy brome (cheatgrass) -suppressive bacteria to large-scale field trials to reduce the competitiveness of downy brome (cheatgrass); and increase vegetation diversity,

- Evaluate the relative success of the bacteria on downy brome (cheatgrass) populations, on native plant establishment and the success of the bacteria at research sites and,

- Compile results of this research into a written report and incorporate this new tool into an integrated vegetation management plan for ITD or other DOT programs.

Methodology

Bacterial Grow Out

The grow out of the bacteria was conducted on solid medium in Dr. Kennedy's USDA laboratory. For the field studies, a culture of *Pseudomonas fluorescens* strain ACK55 from cryostorage was plated onto Tryptic Soy Agar (TSA) (Krieg, 1981)[(77)] in a Petri dish and grown for 2 days at 22°C (72°F). Bacterium from the Petri dish was used to inoculate Tryptic Soy Broth (TSB)[54] (Krieg, 1981). [(77)] This mixture was grown for 32 hours to mid-log stage at 22°C (72°F). The bacteria were propagated at 22°C (72°F) in Tryptic Soy Broth maintained at pH 6.5 to 7.5.

An aluminum pan (9 x 13 inches) with a sliding aluminum lid was lined with autoclavable paper (16 x 24 inches). Twelve magnets secured the paper to the bottom of cake pan to fit flat and tight against the bottom and corners while overhanging the sides. The lid was placed on pan and then the pan, paper, and lid were autoclaved on the dry cycle for 25 minutes at 16 psi and 121°C (249°F). The magnetics were removed aseptically and 500mL of sterile TSA was poured into the pans. The agar surface was made level to cover the bottom of the entire pan. After 24 hours, 7 mL of inoculum was added to the surface of the agar and spread using a sterile hockey stick. The pan was incubated at 22°C (72°F) for 7 days. The bacterial cells were then scraped off the surface of the agar and placed in plastic bags. The cells were spread into a thin shell layer in the bag, placed on a solid surface, and then frozen in a -80°C (-112°F) freezer. After the thin layer was frozen the cells were freeze dried for 16 hours at -52°C (-62°F) and -2.0 MPa pressure. The freeze-dried cells were aseptically crushed into powder. Ten grams of the cells were place into paper bags and vacuum packed. The vacuum-packed cells were stored in a -80°C (-112°F) freezer until the appropriate time for fall application.

Root Length Agar Bioassay

Before any studies could be initiated, the root length agar bioassay was used to test the effect of the bacteria on the various species and cultivars of perennials seeded on ITD roadsides and rights-of ways. Sheep fescue (*Festuca ovina* L.) was the main species being tested. The root length agar bioassay was modified from Kennedy et al. (1991). [(1)] One milliliter of mid-log bacterial growth in TSB was dispensed onto solidified, sterile water agar (0.9%) in 100 x 15-mm Petri plates for testing small seeds or 150 mL beakers with aluminum foil caps for testing larger seeds. One milliliter of sterile TSB broth was used as a control. After allowing the liquid culture to absorb into the agar for 3 to 4 hours, 10 seeds were placed on the agar surface. The seeds were from downy brome (cheatgrass), medusahead, winter wheat, blue

bunch wheatgrass, crested wheatgrass, annual bluegrass, and from plant species representing taxonomically diverse genera (Wapshere, 1974). [78] Many of the plant species tested were listed by the U.S. Environmental Protection Agency (USEPA) as the top 25+ major agricultural crops because of their economic importance, ecosystem activity, or total production values (USEPA, 2011; Table 1). [79] In addition, testing included other economically important plant species of the area, native and near native plant species, and those plants known to be involved in ecosystem maintenance to make certain that the weed-suppressive bacteria were specific. In agronomic ecosystems, the major crop species are of primary interest; however, for rangeland, the main focus of biological control was in native or near-native grass and forbs.

The bioassay plates were incubated in the dark at 15°C (59°F) for 6 days. The 15°C (59°F) incubation allowed the test bacteria and the plant species to grow, but limit the number of other microorganisms. After 6 days, the length of the longest root from each seed was measured. Two replicate plates per isolate were used and each bioassay was conducted at least twice. The desirable species used by ITD were not inhibited by the bacteria.

Field Site Selection

Sites that were moderately infested with downy brome (cheatgrass) and possibly medusahead were identified and chosen by Cathy Ford and District staff from Districts 2, 3, 4, 5 in Idaho. The various roadside locations were on I-84/I-86 and US-95 with different habitat types. ACK55 was compared with a non-inoculated control at each location. Plots were 1 acre in size in a rectangle that fit the study site the best. The study sites were all located within an area that had medium to low population of downy brome (cheatgrass) and most were previously seeded Sheep's fescue. The District staff assisted in the placement of these sites and the Maintenance Personnel were informed prior to the application.

Bacterial Application

The downy brome (cheatgrass)-suppressive bacterium was applied as a spray for a final bacterial concentration of 10^9 colony forming units (cfu) m^{-2}. Two 10 gram freeze-dried packets were used for each acre. Each packet was diluted in 1 liter of tap water and shaken overnight. The 2 liters of suspended cells were added to spray tank with 20 gallons of water per acre was used as the carrier. Cells were sprayed using two Boomless spray nozzles at 4.5 feet height with a total width of 36 inches. The bacteria were applied at 5×10^{12} colony forming units per acre. At some locations, a 10X application was applied as well.

Site Monitoring

The survival of the WSB in the soil, plant species (native and invasive) density and growth parameters along 10 m transects (within the bacterial application areas and selected control plots) were monitored each year over a period of 2 years. Monitoring of the plots, however, needs to continue for 5 to 7 years after application to determine the long-term effects of the bacterial application on vegetation diversity. Field data were collected in the spring of each year from 8 random 1.0 m^2 subplots in each plot at the eleven locations. Sample Point field data were collected in the spring and fall of each year. Precipitation, soil moisture, and soil and air temperatures were also collected over the multiyear project.

Sample Point

Sample Point is free software for point sampling of digital images for analysis. Sample Point was developed by Berryman Consulting in cooperation with the USDA Agricultural Research Service and the USDI Bureau of Land Management. A Standard Operating Procedure was developed for the use of Sample Point ™ to digitally monitor the sites where the bacteria were applied. Sample Point reduces the time, expense, and number of people needed to monitor the locations (Appendix A). The percent cover of species (native and invasive), weeds (medusahead, downy brome or cheatgrass) other annuals and perennials was assessed using Sample Point (Booth et al., 2006). [80]

Foliar and land cover measurements from nadir imagery can be quantified by using either a systematic grid form or random array of up to 225 crosshairs targeting single image pixels allowing for manual classification of those pixels. Plant species and/or land forms are labeled by the user. The image can be viewed at various magnifications at the same time to facilitate understanding the picture at various levels. Data are saved into an Excel spreadsheet automatically. Sample Point analysis reduces analysis time, cost, and environmental stress of operators. The standard operating procedure is logical and fairly easy to learn (Figure 14). The digital monitoring technique requires less time, fewer people, no prior experience of operators, minimal time in extreme temperatures, wind, rain, and insects in contrast to the traditional ground-cover measurements. The variation among Sample Point users was found to be about equal with that of users of the line-point intercept. Data verifiability and the capability to significantly increase sampling replicates are key advantages of using Sample Point with image-based monitoring. A permanent photographic record is obtained and this reduces user-related variation in the data. cover measurements across years is possible. The method also removes multi-operator variation across years.

For monitoring using Sample Point the following items are needed:

- a 1 x 1 meter PVC frame with frame to support camera and a pony leg to steady the upper frame in winds (Figure 15)
- a 3-inch angle iron secured to the underside middle of the main top PVC bar by two circular pipe clamps, (The thumb screw fits through the hole in the angle iron to the camera on the other side.)
- Extra ¼ inch common thumb screws that fit in the bottom of the camera (because they drop on the ground and are lost)
- Digital camera with more than 17 megapixels and GPS capability, remote picture taking, female screw threads in the bottom of camera to secure camera to frame
- a 1-meter stick for measuring inside the frame bottom to ensure the inner square is exactly 1 m2
- a 12-inch ruler to normalize all pictures
- a mallet to put together and take a part the frame
- a ¼ and 5/8-inch socket wrench to ensure the camera mount (the 2 pipe clamps) are tight and secure
- Several nuts and nylon spacers to make sure the thumb screws are snug against the camera and the angle iron that holds the camera in place
- Soft cloth to clean the lens

- Extra lens caps
- Clip board, plot plans, labels to mark the plot name of each picture taken
- The two pipe clamps and 3 inches "L" are placed on the top of the frame. The two pipe clamps hold one side of the "L" firmly on the PVC pipe, positioned to give you a full view of the one-meter square pipe on the ground. The sides of the picture frame (left and right) will show more of the plot. Just make sure you can see the entire outside white PVC tubing. Later you will crop to delete most of the white tubing. The sides of the picture outside the frame can be used to hold the clipboard with the site information.
- The camera needs to be set on fine or high resolution
- Screws to mount the camera onto the "L" piece

Data Evaluation

Data were compiled from the research study sites and additional established plots and bacteria populations. This work includes data obtained from the research study sites, bacteria establishment, the effect of weed-suppressive bacteria and herbicide on downy brome (cheatgrass) populations, established perennial plant populations and vegetation management activities over 2 years at select locations within ITD right-of-ways. The research service evaluated data compiled from the study sites and conduct appropriate analyses, and also complete other evaluations and summarizations of the data from additional bacteria populations, to determine the relative success of bacteria at roadside rights-of-way, the relative effects of the bacteria on native species and/or seeded species, and the ability to control a variety of weedy species including downy brome (cheatgrass) establishment. Data analyses were completed using general linear model methodology such as analysis of variance, analysis of covariance, regression, and correlation (SAS, 2015). [81] For each study, the impact of the bacterium on downy brome (cheatgrass), wheat, and native plant growth was determined and the relationships among these data and the variables of location, soil characteristics, and climate were studied.

Table 1. List of Agronomically Important Plants Tested in the Root Length Bioassay to Determine if the Weed-suppressive Bacteria (*Pseudomonas fluorescens* strain ACK55) Negatively Impacted Growth

Common	Latin Name
Alfalfa	*Medicago sativa L.*
Apple	*Malus* spp. Mill.
Barley	*Hordeum vulgare L.*
Beans	*Phaseolus* spp. L.
Brocolli	*Brassica oleracea*
Camelina	*Camelina sativa* L.
Canola	*Brassica napus* L.
Celery	*Apium* spp. L.
Chick peas	*Cicer arietinum*
Clover	*Trifolium* spp. L.
Corn	*Zea mays* L.
Cotton	*Gossypium* spp. L.
Cucumber	*Cucumis sativus* L.
Faba bean	*Vicia faba*
Flax	*Linum narbonense* L
Lentil	*Lens culinaris*
Lettuce	*Lactuca sativa* L.
Magnolia	*Magnolia* spp. L.
Mint	*Mentha* spp. L.
Oat	*Avena* spp.
Onion	*Allium* spp. L.
Pea	*Pisum sativum* L.
Peanuts	*Arachis* L.
Pepper	*Capsicum* L.
Ponderosa	*Pinus ponderosa*
Potato	*Solanum spp.*
Rice	*Oryza sativa L.*
Rose	*Rosa* spp. L.
Soybeans	*Glycine max* L. Mer.
Squash	*Cucurbita* spp.
Sugar beets	*Beta vulgaris*
Sunflower	*Helianthus* spp. L.
Tomato	*Solanum* spp.
Vetch	*Vicia* spp. L.
Wheat	*Triticum aestivum L.*

Figure 1. Downy Brome or Cheatgrass (*Bromus tectorum* L)

Figure 2. Medusahead (*Taeniatherum caput-medusae*[L.] Nevski)

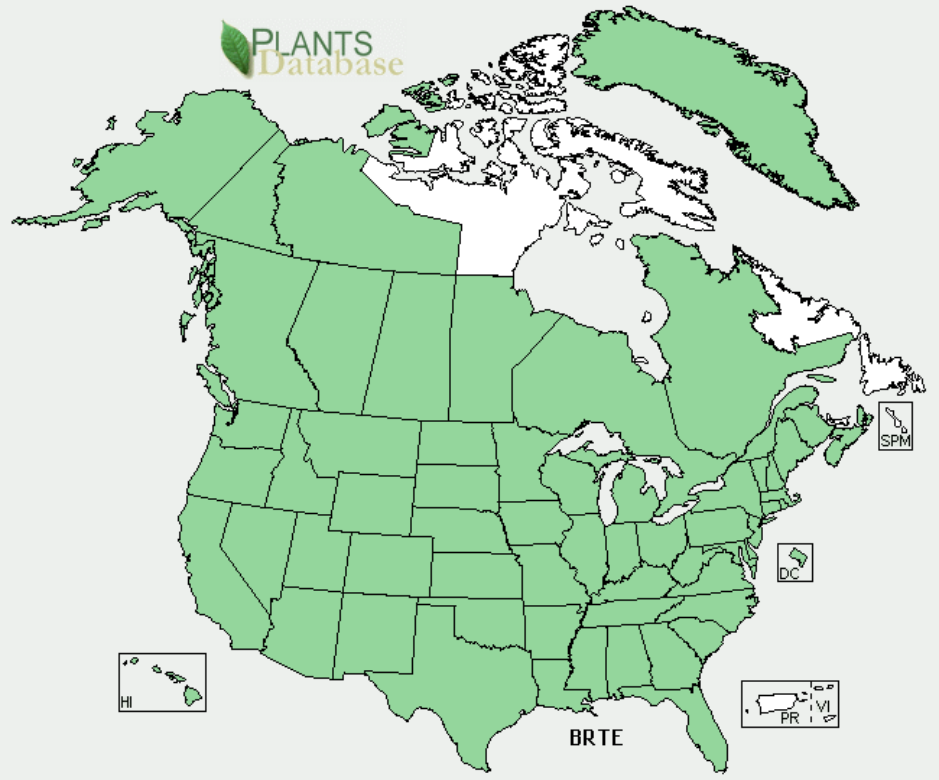


Figure 3. The Distribution of Downy Brome (Cheatgrass) in North America (NRCS, 2014a)

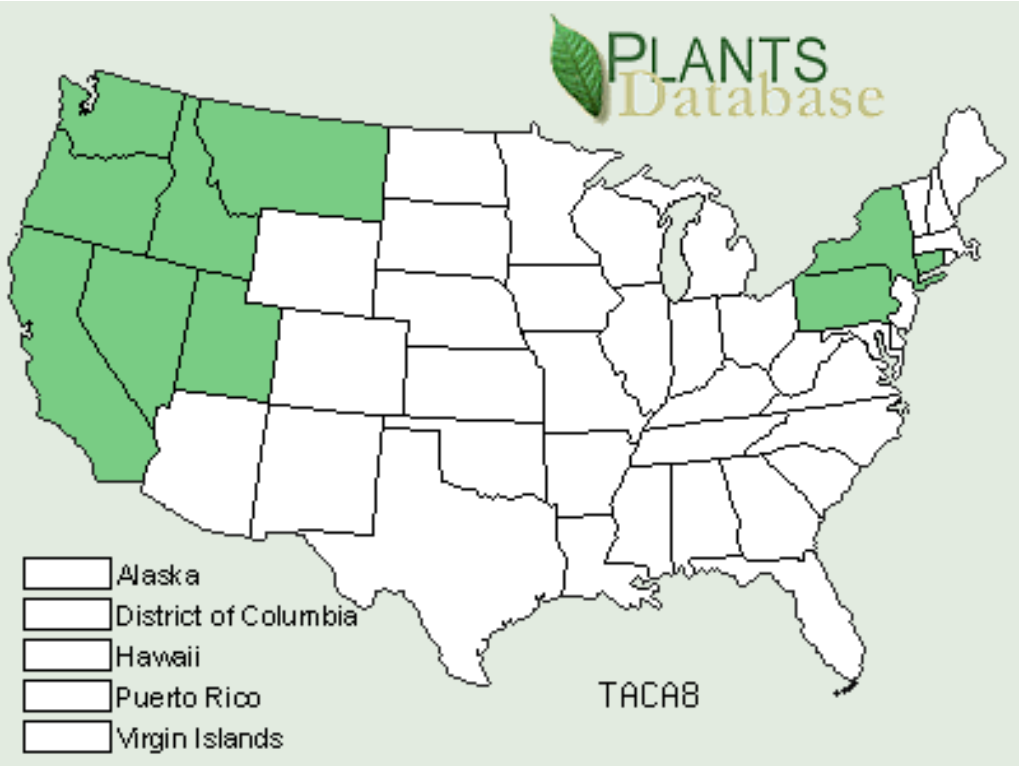
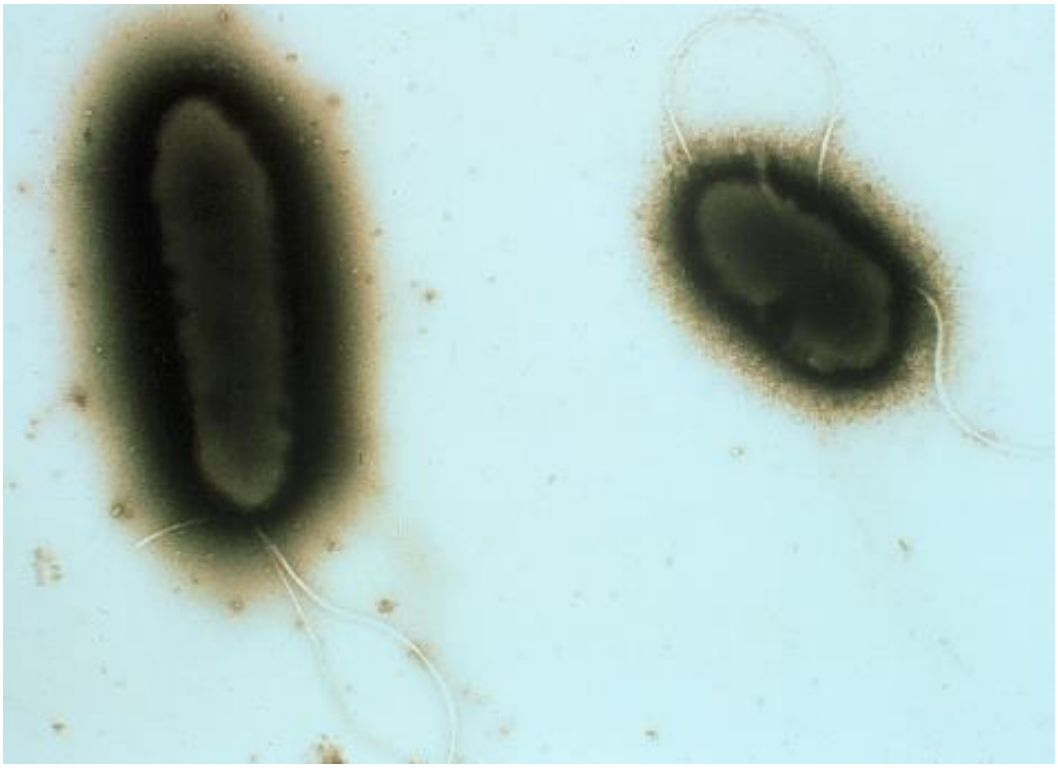


Figure 4. Distribution of Medusahead in North America (NRCS, 2014b)

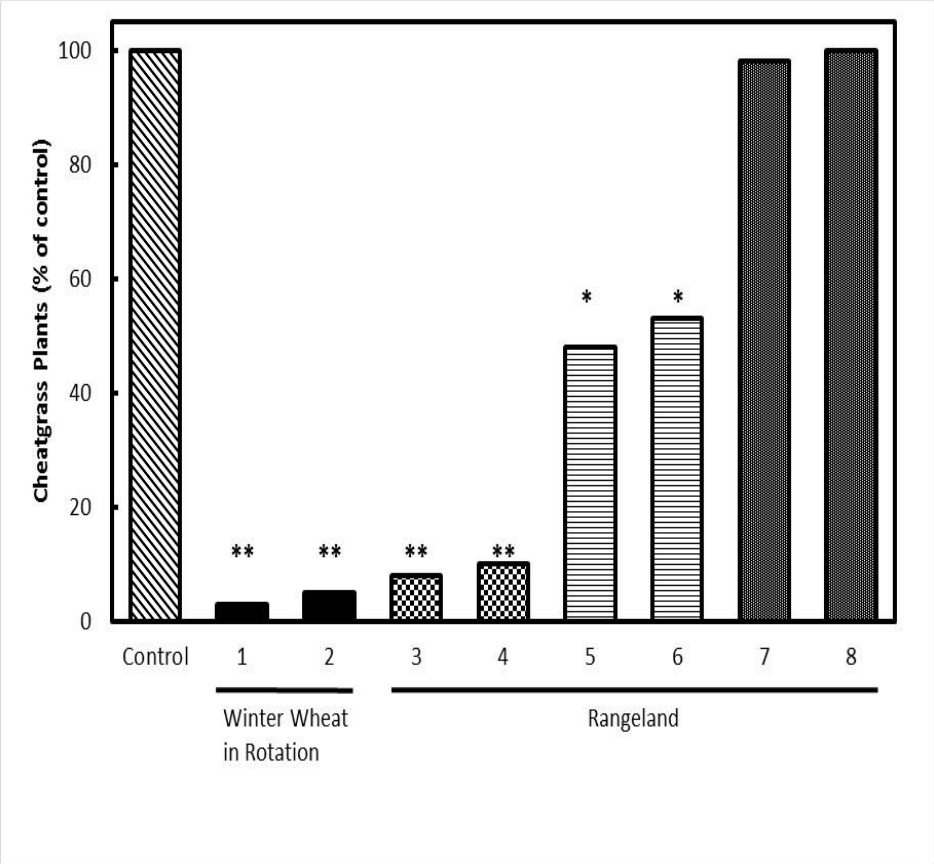


Note: Magnification is 100,000X.

Figure 5. Transmission Electron Micrograph of *Pseudomonas fluorescens*

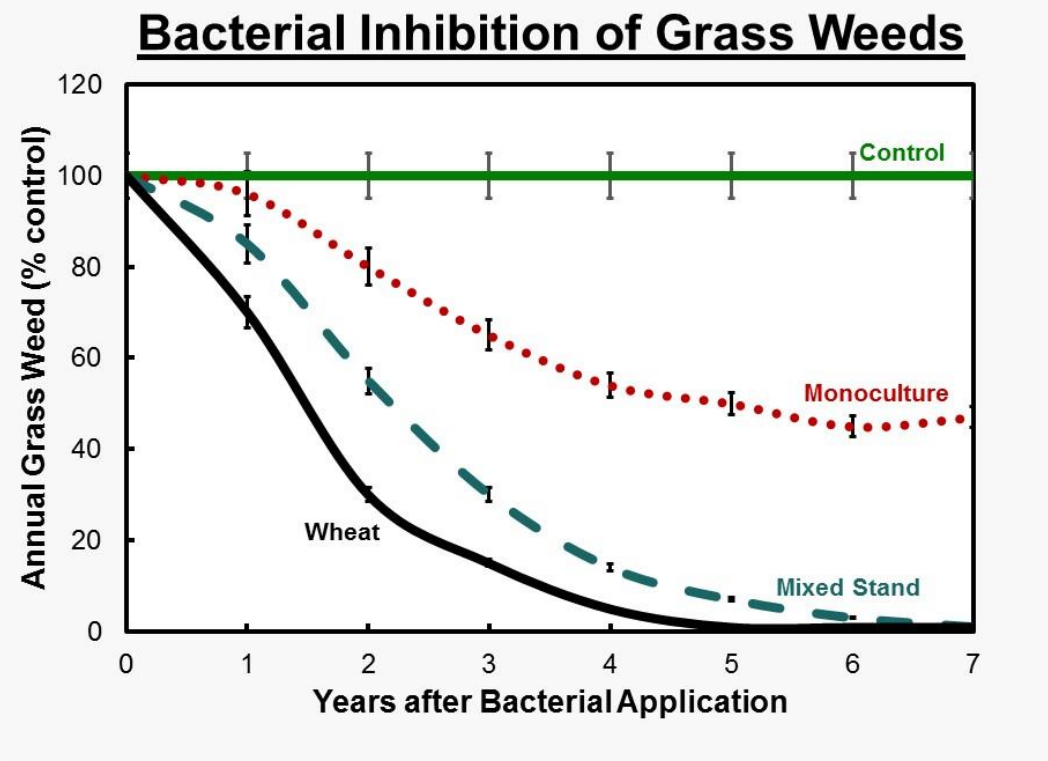
Note: The untreated control plot (left) and plots treated with the weed-suppressive bacteria (right) 5 years after application of the bacteria. The bacteria reduced downy brome (cheatgrass) populations by 64 percent. Winter wheat frames the sides of each picture with downy brome (cheatgrass) in the inter row. Weed-suppressive bacteria reduce the number of downy brome (cheatgrass) plants by 64% compared to the control.

Figure 6. Winter Wheat Fields Containing Downy Brome (Cheatgrass) at Benge, WA



Note: WSB was applied to the soil as a spray at 4 x 1011 cells hectare-1 (8 x 1011 cells A-1) in the fall of the year. Each bar represents four sites with five replications of 0.4 hectare (1 acre) plots. Control for each site was set at 100%. 1) Winter wheat/spring wheat rotation in Washington, ACK55 applied in rain; 2) Winter wheat/spring wheat rotation in Idaho, ACK55 applied in rain; 3) Mixed natives/cheatgrass rangeland in Washington, ACK55 applied in rain; 4) Mixed natives/cheatgrass rangeland in Idaho, ACK55 applied in rain; 5) Monoculture Cheatgrass rangeland in in Washington, ACK55 applied in rain; 6) Monoculture cheatgrass rangeland in Washington, ACK55 applied in rain; 7) Mixed natives/cheatgrass rangeland in Washington, ACK55 applied in dry conditions, Sunny skies and no rain for at least two days after application; 8) Mixed natives/cheatgrass rangeland in Idaho, ACK55 applied in dry conditions, Sunny Skies and no rain for at least two days after application. Bars were significantly different from control at P ≤ 0.10 = * and P ≤ 0.05 = **.

Figure 7. Downy Brome (Cheatgrass) Plants (Percent of Control) 5 Years after Different Application Treatments

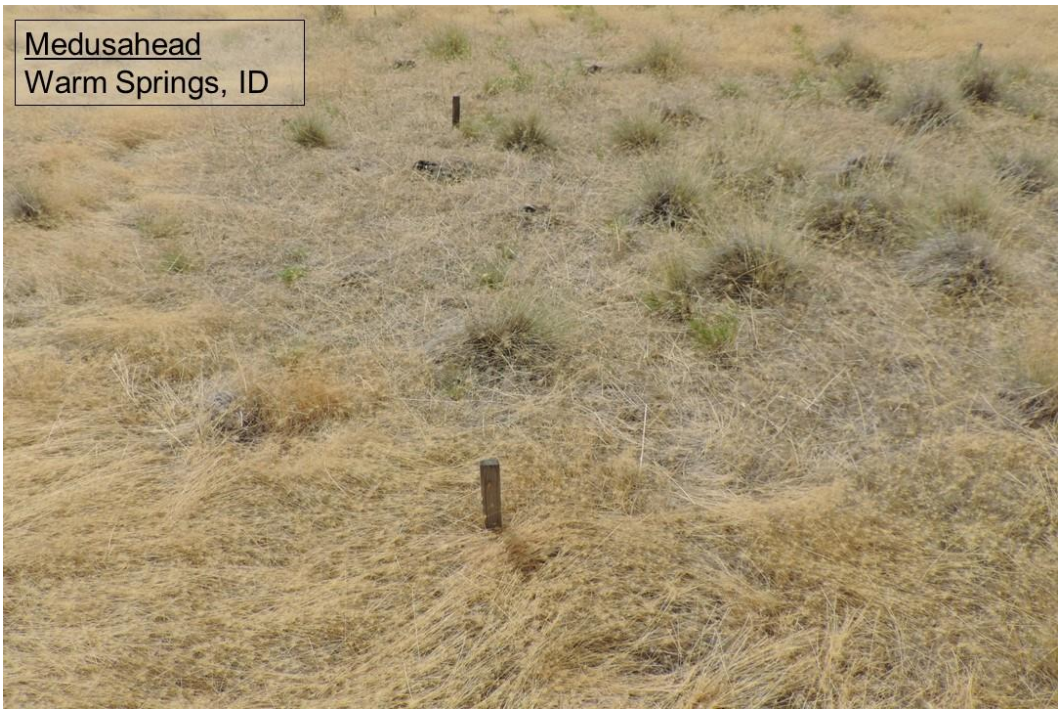


Note: 1) pasture and agricultural cropland with winter wheat as the rotational crop; 2) mixed stand rangeland containing weeds and native plants, and; 3) rangeland covered by a weed monoculture with no natives visible. Each line represents the mean of five sites in Central and Eastern Washington State.

Figure 8. *Pseudomonas fluorescens* Strain ACK55 Was Tested for Suppression of Downy Brome (Cheatgrass) Over Time in Three Plant Ecosystems

Note: The bacteria reduced downy brome (cheatgrass) populations by 56 percent.

Figure 9. Rangeland Field Plots at Park Valley, UT of Untreated Control (Left) and Plots Treated with Weed-suppressive Bacteria (Right) Three Years After Application of the Bacteria

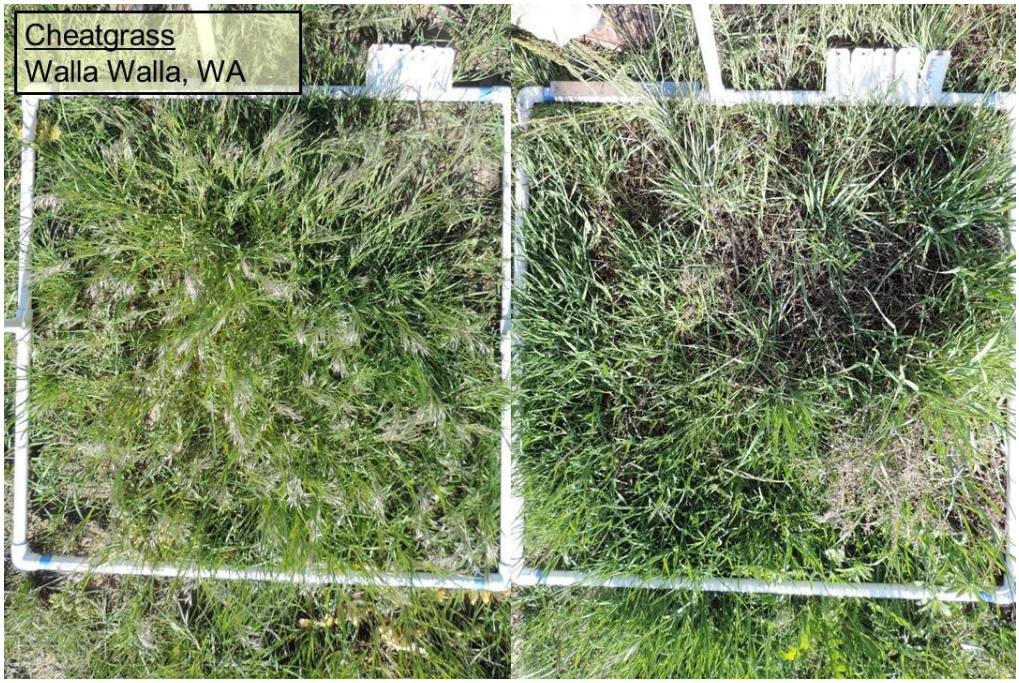


Note: The bacteria reduced medusahead populations by 89percent. Note drift of bacteria to left of stakes.

Figure 10. Rangeland Field Plots at Warm Springs, ID of Untreated Control (Left) and Plots Treated with Weed-suppressive Bacteria (Right) Five Years After Application of Bacteria

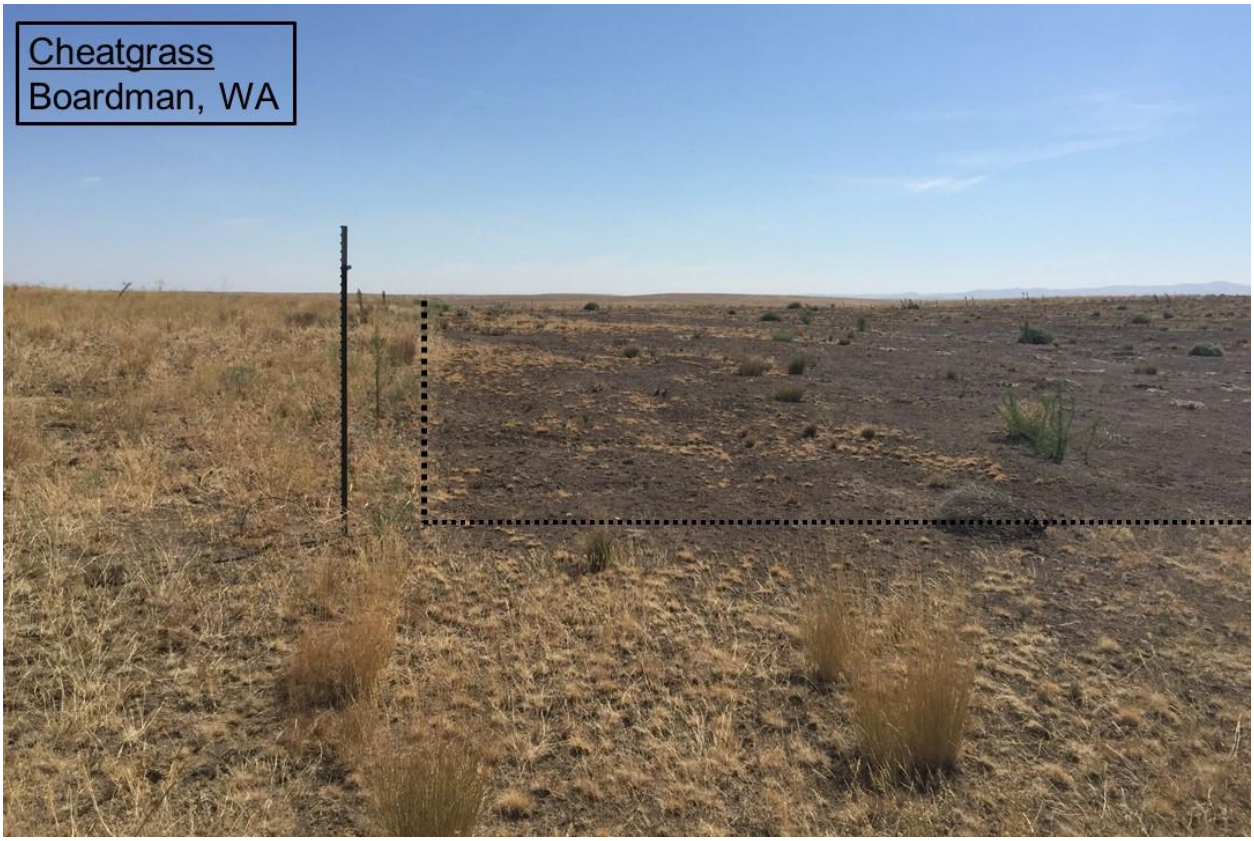
Note: The bacteria reduced downy brome (cheatgrass) populations by 98 percent.

Figure 11. Untreated Control (left) and Plots Treated with Weed-suppressive Bacteria (right) at Pullman, WA 10 Years after Application of the Bacteria



Note: Pseudomonas fluorescens strain ACK55 was coated on intermediate wheat grass seed and drilled into the roadside three years prior to this picture.

Figure 12. Untreated Control (left) and Plots Treated with Weed-suppressive Bacteria (right) on Washington State DOT Right-of-Way Near Walla Walla, WA



Note: The control plot is to the left of the fencing. To the right of the fencing, ACK55 applied as a spray at 4 x 1011 cells hectare-1 (8 x 1011 cells A-1). The whole site was burned by wildfire in spring 2015; Weed- suppressive bacteria was sprayed Dec 2015; Picture was taken Sept 2016. The bacteria were sprayed along the fence row and to the right of the fence. Photo from Jerry Benson.

Figure 13. Burned Land Treated with ACK55

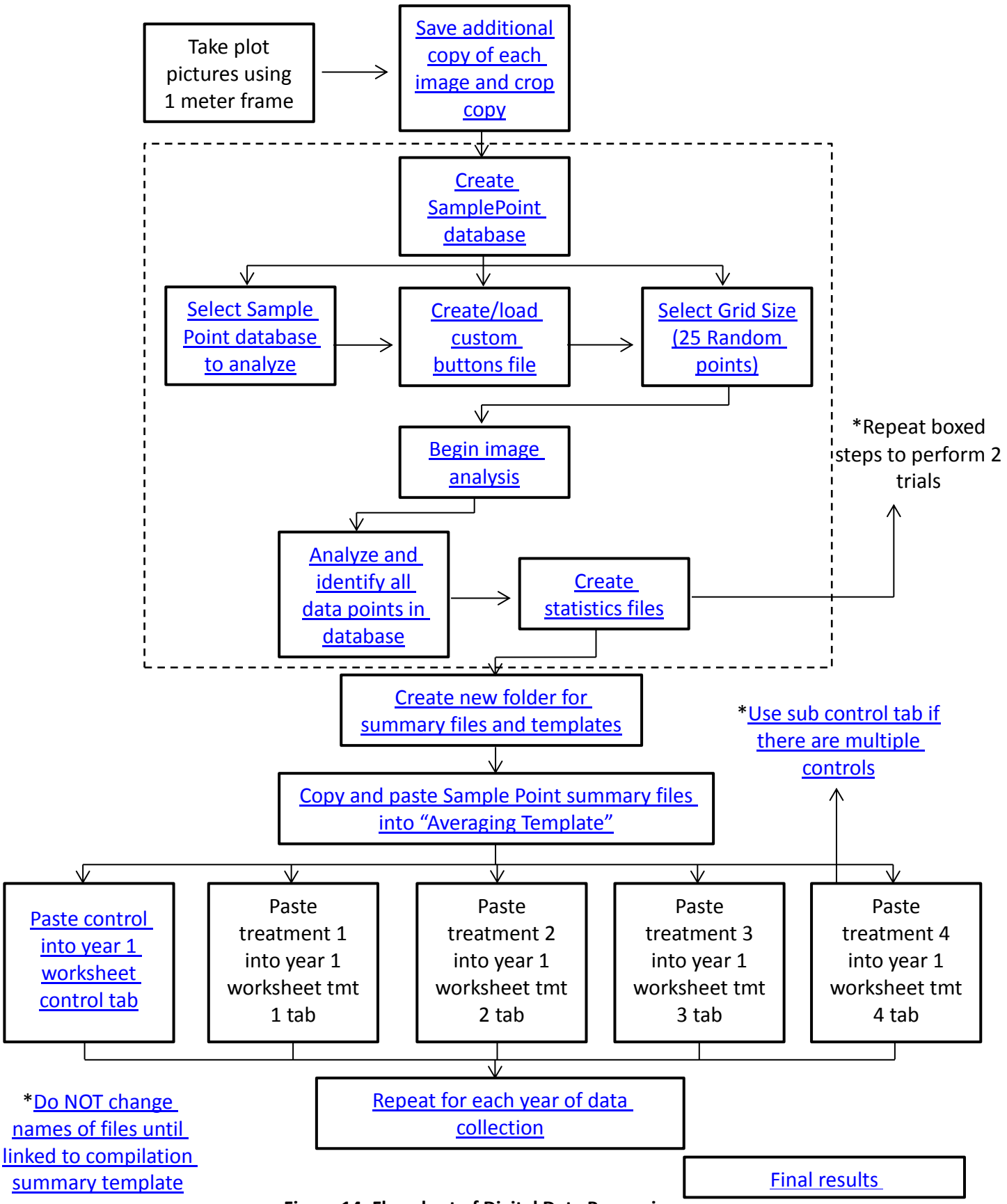
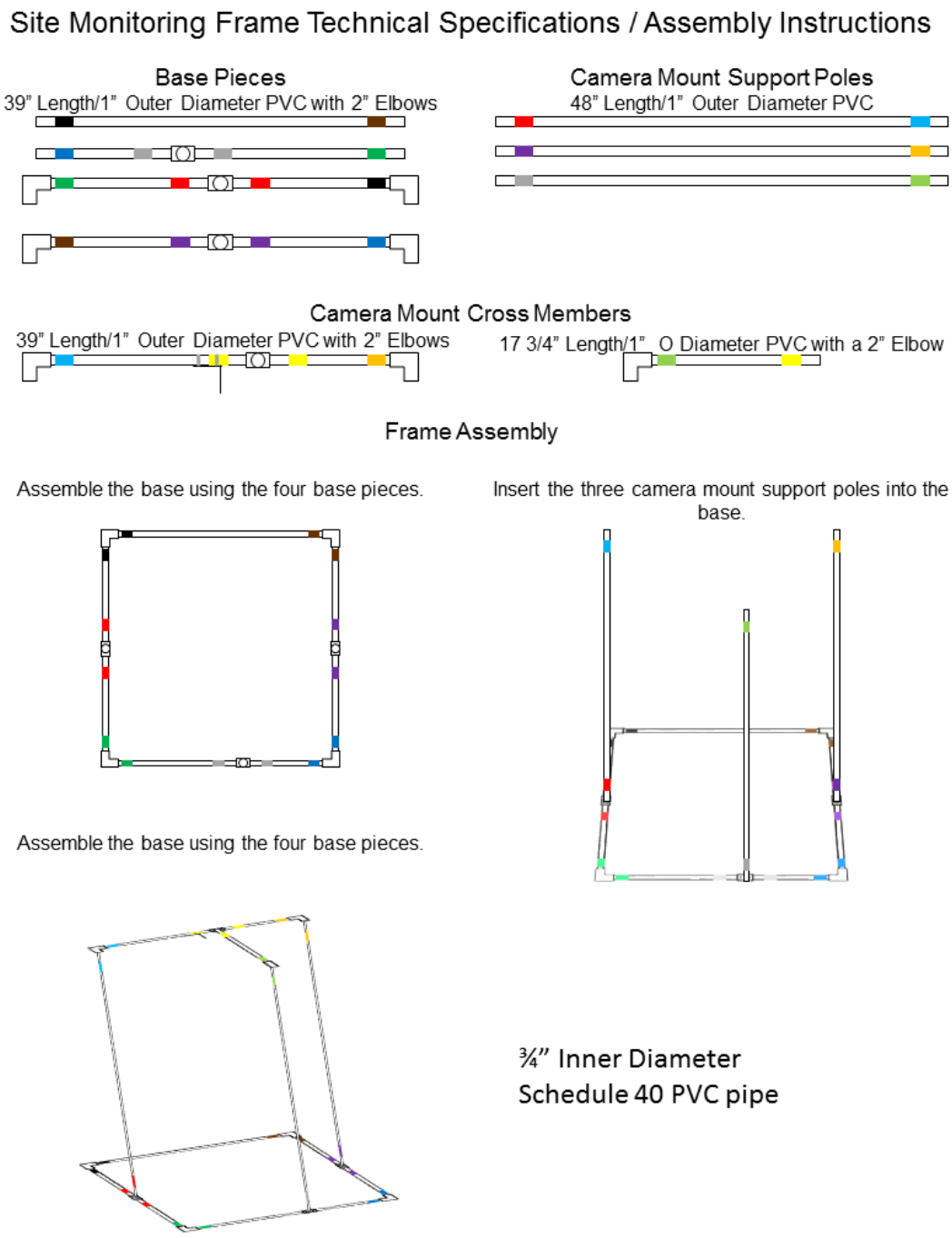


Figure 14: Flowchart of Digital Data Processing



Note: This picture appears distorted, all angles are at 90 degrees. The frame is to be square all around

Figure 15: SamplePoint Monitoring Frame Parts and Assembly

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Chapter 2
Idaho Roadside Bacterial Sites

The sites used in this study were located in ITD Districts 2, 3, 4, and 5. Eight sites were located on I-84 & I-86 from the east end of Boise and extended east towards Pocatello. There were six locations on I-84 and two locations on I-86 (Table 2, Figure 16). Three sites were located along US-95 as far north as Genesee, ID and extended south to the Idaho/Oregon border.

I-84, MP59, Eisenman Exit, #1

There were three sites at I-84 MP59 because of the varied previous land management, vegetation management and landscape of the cloverleaf. The bacteria were applied in the fall of 2014 (Figures 17, 18).

The Eisenman Exit, #1 plots are located on the northeast side of the interstate clover leaf and consist of a large flat area with a heavy population of downy brome (cheatgrass) and a diverse healthy mix of beneficial vegetation throughout the site (Figure 19). There was little evidence of seeded plants establishing and surviving in high numbers at this first site of three. Tumble mustard, Sandberg bluegrass, and Western Yarrow were present at this site. A buried utility line transects the area east to west and consists of a soil depression and a line of monoculture downy brome (cheatgrass). The utility line area did not received treatments and was not monitored or included in this study. In fall 2014, bacteria were spray-applied to three sections within this first site (Table 2).

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 85% as compared to the control when measured in the spring of 2015 after the 2014 fall application and 95% inhibited when monitored in the fall of 2016 a full year after application of the bacteria. In these two monitoring dates, the downy brome (cheatgrass) populations in control plots were quite low and the high reduction in downy brome (cheatgrass) could be due to the low populations of downy brome (cheatgrass) during those years. The spring of 2017 was wetter than other years since bacteria were applied and downy brome (cheatgrass) flourished in these plots (Figure 19, Figure 20). Populations of downy brome (cheatgrass) were high in both the control and the bacteria plots (Figure 21). In the spring 2017, the percent reduction of downy brome (cheatgrass) was only 54% which was lower than the previous years. The fluctuation in percent reduction of the weed by the bacteria varies widely from year to year in some studies. Usually, the control plots compensated for the moisture differences. In this case, however, that did not seem to be the result (Table 3). The bacteria generally do not reduce downy brome (cheatgrass) populations greatly in the first few years as seen in past studies. The major effect of the bacteria will be evident in 5 to 7 years after application of the bacteria. However, the outline of the bacteria plots showed a change in the plant diversity with the application of the bacteria (Figure 19).

I-84, MP59, Eisenman Exit, #2

The Eisenman Exit, 2 plots are located on the southeast side of the interstate and on both sides of the on and off-ramps (Figure 22). This second site of three actually consists of two areas both with a west facing aspect. The areas consist of a toe slope, side slope and ridge top (Figure 23). The south half of the area was burned in 2014 from the road shoulder up the hill beyond the fence line. The burn pattern allowed for monitoring burned and unburned areas across the toe slope and side slope. The dominant vegetation included perennial sheep fescue, annual downy brome (cheatgrass), annual medusahead and various forms and some grasses. The sheep fescue was sparsely distributed in some parts of the burned area. Site cover consisted of 20 percent bare ground, which was lower, but not statistically significant from the non-burned area (Figures 24, 25).

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 86% as compared to the control when measured in the spring 2015 after the 2014 fall application and 92% inhibited when monitored in the fall of 2015, a full year after application of the bacteria. In the spring of 2016, the bacteria suppressed downy brome (cheatgrass) by 90%. In the spring of 2017, downy brome (cheatgrass) inhibition was only 50% (Figure 25). The burn areas did not differ from the non-burned area in the downy brome populations. Medusahead was present in patches at this site, but no Medusahead patches were observed in the bacteria plots (Figure 23).

I-84, MP59, Eisenman Exit, #3

The Eisenman Exit 3 plots are located on the southwest side of the interstate and on and off-ramp (Figure 26, 27). This site contains a roadside ditch with a slight slope increasing from west to east. The area burned in 2014 and extends along Eisenman Rd south. The vegetation consisted of predominantly sheep fescue with established plants sparsely distributed in some areas. The dominant annual grass was downy brome (cheatgrass). In addition, there were forbs present and bare ground areas (Figure 27).

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 87% as compared to the control when measured in the spring 2015 after the 2014 fall application and 89% inhibited when monitored in the fall 2015 a full year after application of the bacteria (Figure 26, 28). Downy brome (cheatgrass) growth was extensive and widespread in this part of the area (Figure25). In the spring of 2016, downy brome (cheatgrass) was inhibited by 89%. In the spring 2017, the above average spring precipitation caused a flush of early downy brome (cheatgrass) growth; however, the bacterial inhibition and reduction of downy brome (cheatgrass) population was 72%. There was a visual demarcation between the bacteria and the control plots as indicated (Figure 26).

I-84, MP64, Blacks Creek Exit

The Blacks Creek Exit plot is located on the northwest side of the interstate clover leaf and starts at the fence line that parallels the ramp to the west (Figure 29). The site consisted of a south facing slope. The plot is located at the top of the slope facing towards Black Creek Road at the beginning of the west ramp. (Figure 29). The vegetation consisted of predominant evenly distributed stands of sheep fescue

and a medium to light infestation of downy brome (cheatgrass). Downy brome (cheatgrass) populations were greatest at the midslope. Western yarrow, rubber rabbitbrush and small sagebrush plants were found at this location. The bacteria plot extended half way down the bowl and less than half way across the south side of the bowl. Spring forbs were present and downy brome (cheatgrass) was interspersed in the spaces between the sheep fescue in the control plot and less so in the bacterial plot. The southeast corner of the bacterial plot ended just before a definite rise in the slope. Old-wood sagebrush plants were occasionally distributed in heavy patches throughout the area and were not included in the plots.

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 75% as compared to the control when measured in the spring 2016 after the 2015 fall application and 89% inhibited when monitored in the fall of 2016; a full year after application of the bacteria (Figures 30, 31). In the spring of 2017, downy brome (cheatgrass) was inhibited only 43% and there was no differences seen or counted between the control and bacteria plots (Figure 28). The sheep fescue was dominant in 2015 and 2016, but was not a major plant component in 2017.

I-84 MP173 / US-93 Exit Twin Falls 1, South & Close to I-84

The US-93 Twin Falls 1 & 2 plots are located on the southeast section of the cloverleaf at the intersection of I-84 and US-93 (Figures 32, 33). Two plots were established in a relatively flat area with a slight slope from west to east (Figure 33). The east border of both plots consisted of 2-4 ft high rock ledge. Each plot was set in a slightly shallow depression. Untreated control plots were located between the treated plots and to the south side of the second plot. A rock face bordered the west end of plot #1 near I-84. The soils were gravelly and mixed with little organic matter. Near the entrance to this site entering from US-93, there is a large gravel area which was used as a parking lot and waste site. The site contained oil, gasoline stains, nuts, bolts, and pieces of metal. Large equipment had been stored here. A rough gravel road bordered the northern most side of plot 1. Plots were established so that the rock out cropping demarked the plot borders and separated them from the control plot. The dominant perennial vegetation consisted of established crested wheatgrass and several forb patches. Downy brome (cheatgrass) was moderate in abundance and the area was dominated by many broadleaf weeds, such as prickly lettuce and tumble mustard. The broadleaf weeds became even more abundant in the bacteria plots when the bacteria reduced the downy brome (cheatgrass) levels (Figure 34).

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 76% as compared to the control when measured in the spring 2016 following the 2015 fall application and 85% inhibited when monitored in the fall of 2016; a full year after application of the bacteria. In the spring 2017, no differences could be seen or counted between the control and bacteria plots; bacteria was inhibited only 30% (Figure 35). There were no competing plants at these sites as seen previously. The downy brome (cheatgrass) exhibited two growth forms: 1) green, tall and vigorously growing plants and 2) short in stature, tan and senescent plants with few seed. In the spring 2017, downy brome (cheatgrass) populations throughout this site, both in the control and bacteria plots were high. There were no seeded perennial grasses observed in

either the control or bacteria plots in 2017; however seeded perennial grasses were evident in 2015 and 2016. Few broadleaf plants were visible in either plot and they were evident in 2016. Perennial grasses are needed to compete with the weekend downy brome (cheatgrass) due to the bacterial application and fill the void as the bacteria reduces the competitiveness of the downy brome (cheatgrass).

I-84 MP173 / US-93 Exit Twin Falls 2, South & Away from I-84

The US-93 Twin Falls 1 & 2 plots are located on the southeast section of the cloverleaf at the intersection of I-84 and US-93. Two plots were established in a relatively flat area with a slight slope from west to east. The east border of both plots consisted of 2-4 ft high rock ledge (Figure 32, 33). Each plot was set in a slightly shallow depression. Untreated control plots were located between the treated plots and to the south side of the second plot. A rock face bordered the west end of plot #1 near I-84. The soils were gravelly and mixed with little organic matter. Near the entrance to this site entering from US-93, there is a large gravel area which was used as a truck parking lot and waste site. The site contained oil, gasoline stains, nuts, bolts, and pieces of metal. Large equipment had been stored here. A rough gravel road bordered the northern most side of plot 1. Plots were established so that the rock out cropping demarked the plot borders and separated them from the control plot. The dominant perennial vegetation consisted of established crested wheatgrass and several forb patches. Downy brome (cheatgrass) was moderate in abundance and the area was dominated by many broadleaf weeds, such as prickly lettuce and tumble mustard. The broadleaf weeds became even more abundant in the bacteria plots when the bacteria reduced the downy brome (cheatgrass) levels.

Site Summary and Recommendations US-93 MP 0-1

Downy brome (cheatgrass) was inhibited by 75% as compared to the control when measured in the spring 2016 following the 2015 fall application and 84% inhibited when monitored in the fall of 2016; a full year after application of the bacteria (Figure 34, 35). As stated above, in the spring of 2017 the area was inundated with downy brome (cheatgrass) from two growth types: 1-tall and vigorously growing and still green and the other short, with few seed heads and beginning to senesce.

I-86 MP32, West of Neely Exit

The I-86 Exit, MP32 plot is located in the Interstate median between two grates (Figure 32). Two plots were established in the median; one on each side of the Emergency Vehicle Access & Turnarounds. Controls were adjacent to both bacteria plots. There were very few plants observed and plant growth was sparse, but downy brome (cheatgrass) was present (Figure 36, 37). No perennial grass was evident when the bacteria were applied in fall of 2015. In 2016 and 2017, wheatgrasses, possibly western or streambank, were dominant and other plant species, such as pepper weed, tumble mustard and/or clover or alfalfa had developed.

Site Summary and Recommendations

The site was mowed prior to monitoring in the fall of 2016 and no information could be recorded. In the spring of 2017, the plant species were more diverse and downy brome (cheatgrass) had increased in numbers (Figure 38).

I-86 MP58, Farm Tank Road Exit

The I-86 Exit, MP58 plots are located on the northeast side of the Interstate and overpass. A small rectangle plot was established in a relatively flat area which consisted of a newly seeded patch of lush non-bunch grass plants that resembled wheat, perhaps intermediate wheatgrass or triticale (Figure 39). Downy brome (cheatgrass) was moderate in abundance in between the seeded rows (Figure 39). The wheat-type grass growth and vigor indicated recent use of some type of fertilizer or growth stimulator. The seeded perennial grass was intermixed with downy brome (cheatgrass). The downy brome (cheatgrass) probably increased in percent cover with the unusual spring rains (Figure 40). Alfalfa or clover was intermixed with the grass stand.

Site Summary and Recommendations

In the fall 2016, downy brome (cheatgrass) was inhibited 86% by the bacteria as compared to the control; a full year after application of the bacteria. In the spring of 2017, the total area had been sprayed with herbicides which damaged the grasses. The area was dry and brown. Some downy brome (cheatgrass) was observed in the control plots; however less downy brome (cheatgrass) was observed in the bacteria plots. There was a 45% reduction of downy brome (cheatgrass) observed at this location. The reason for the herbicide treatment kill is not known. A demarcation line was evident at the plot edges (Figure 39, 41).

US-95 MP 0-1 Idaho/Oregon Border

The US-95 MP0-1 plot is located in a flat area west of US-95, east of the fence line, and south of the gravel road (Figure 38). The plot is located parallel to the highway and in between two telephone poles (Figures 42, 43). The area was burned in the 2015 Soda Fire. The vegetation consisted of old-grown sage brush that was burned in the Soda Fire, crowns of burned sheep fescue and Sandberg bluegrass were evident along with new spring forbs. Blue bunch wheatgrass was present but not in high numbers. Fiddleneck tarweed, Western yarrow and tumble mustard along with prickly lettuce was evident at this site. Some rock and bare soil were obvious and in several patches throughout the area (Figure 42).

Site Summary and Recommendations

Downy brome (cheatgrass) was inhibited by 70% as compared to the control when measured in the spring of 2016; after the 2015 fall application and 82% inhibited when monitored in the fall of 2016; a full year after application of the bacteria (Figures 44, 45). In the spring 2017, the percent reduction of downy brome (cheatgrass) was 75% slightly lower than the previous year.

US-95 MP327 Uniontown Rd, Genesee

The area is agricultural with the wheat and canola being grown on the surrounding land. This plot was in the median strip consisting sheep fescue, Canada bluegrass, Ventanata grass, reed canary grass, and downy brome (cheatgrass) in moderate populations with no forbs evident (Figure 41). The plots were sprayed with the bacteria in November of 2016. The bacteria were applied to the median north of the and the controls were to the north and south (Figures 46, 47).

Site Summary and Recommendations

In spring of 2017, the plots were monitored and Ventanata was the dominate grass in both plots. Downy brome (cheatgrass) was observed with higher concentrations in the control plot as compared to the bacteria plots. Downy brome (cheatgrass) populations were reduced by 40% in the bacteria plot as compared to the control (Figures 46, 48; Table 3). Ventanata and reed canary grass were observed to increase in cover, while the seeded sheep fescue was declining. Ventanata is a serious invader species and action is needed to reduce its spread in these median strips. Reed canary grass also needs to be managed in these locations. The reduction in downy brome (cheatgrass) by the bacteria will allow these two weeds to spread and the seeded desirable plant species will decline (Figure 42). Additional seedings of desirable perennial grasses is needed at this location (Figure 43).

US-95 MP330 Kluss Rd, Genesee

The area is agricultural with the wheat and canola being grown on the land past the roadside (Figure 49, 50). This plot was in the median strip and the vegetation consisted of Canada bluegrass, Ventanata, reed canary grass and downy brome (cheatgrass) in moderate populations and sheep fescue with no forbs evident. The plots were sprayed in November of 2016 (Figure 49). The bacteria were applied to the median north of the and the controls were to the north and south.

Site Summary and Recommendations

In spring of 2017, the plots were monitored and Ventanata was the dominate grass in both plots. Downy brome (cheatgrass) was observed with higher concentrations in the control plot as compared to the bacteria plots. Downy brome (cheatgrass) populations were reduced by 40% in the bacteria plot as compared to the control. Ventanata and reed canary grass were observed to increase in cover, while the seeded sheep fescue was declining. Prickly lettuce was increasing (Table 3; Figure 51). This site will require additional seeding and establishment of sheep fescue or other desirable plant species to fill the voids as the downy brome (cheatgrass) declines (Figure 51). If no desirable plant is provided the Ventanata and reed canary grass will invade the entire area as downy brome (cheatgrass) declines. If the Ventanata, in particular, is not managed for decline, this weed will spread into the agricultural lands and cause crop yield reductions. Ventanata is a more serious weed in these areas because the plant is smaller, with less dry matter production than downy brome (cheatgrass). Ventanata also contains silica that can degrade teeth of cattle and ruin agricultural equipment.

Discussion

The survival of the bacteria in the soil, plant species (native and invasive) density and growth parameters within the bacterial application areas, and selected control plots) were monitored each year over a period of 2 years. Monitoring of the plots, however, needs to continue for 5 to 7 years after application to determine the long-term effects of the bacterial application on vegetation diversity.

All of the eleven ITD locations where the weed-suppressive bacteria were applied had some sort of reduction in downy brome (cheatgrass) and medusahead cover in the first year after application of the bacteria. The annual grass weeds were of medium to low infestations initially and sheep fescue was a good competitor. At most locations, there were few broadleaf weeds observed dominating the

landscapes. The Twin Falls plots were the only sites that had broadleaf weeds occupying the site after downy brome (cheatgrass) cover was reduced in year one. Additional broadleaf herbicides are needed these sites in both plot locations.

The WSB resulted in reductions in downy brome (cheatgrass) cover greater that 70% compared to the control plots. The spring weather in 2017 was marked by cool temperatures and above average precipitation. A record amount of downy brome (cheatgrass) germinated in the spring compared to other years. At several of the sites, the downy brome (cheatgrass) populations increased dramatically, as compared to previous years. In the fall of 2017, at some locations, the downy brome (cheatgrass) seed in the seed bank may be too high for the bacteria to inhibit. The bacteria do inhibit downy brome (cheatgrass) populations, but desirable plants are also needed to compete with the downy brome (cheatgrass) for water and space. If there are no desirable plants to compete with downy brome (cheatgrass), then the space will again be occupied with either downy brome (cheatgrass) or broadleaf weeds. Several of the locations had been seeded with perennial grass species, however at some locations the perennial grasses did not survive or establish well. Additional seeding is needed to allow desirable plants for the bacteria to work effectively and to rid the area of downy brome (cheatgrass).

Conclusions

- Bacteria were able to inhibit downy brome (cheatgrass) and medusahead in the ITD roadside plots without a fall application of Imazapic.
- The bacteria effectively reduce stands of downy brome (cheatgrass) that are medium-sized and smaller populations, as well as sheep fescue or Sandberg bluegrass, Canada bluegrass, or blue bunch wheatgrass or other wheatgrasses can re-establish and fill in the voided areas.
- Sheep fescue is an excellent competitor plant and when present effectively fills in the voids created by the weakened downy brome (cheatgrass). Other desirable plants that are successful with the bacteria include crested wheatgrass, Idaho fescue, Sandberg bluegrass and other bluegrass species and quickly fill in and establish in the voids created by reduced downy brome (cheatgrass) populations.
- Medusahead was found in patches on the ITD roadsides. The bacteria were able to stop the growth and spread of medusahead and in effect reduce the populations to near zero.
- The abundant precipitation in spring 2017 caused a greater amount of downy brome (cheatgrass) to germinate and in some cases the downy brome (cheatgrass) dominated the landscape. This was especially true when no perennial plants were able to establish.
- The bacteria reduce weed populations slowly over time. Since it has only been two years after bacterial application for most of the sites, we would not expect consistent low weed populations at this time. We expect greater suppression of the weed by the bacteria in the coming years.

Table 2. Locations, Mile Post Markers and GIS Coordinates for Sites Used in this Study

Road	Milepost (MP)/Exit Name	North	West	Location Description	Bacteria Applied	Town
I-84	**MP59, Eisenman Exit #1**	43.50747	116.14627	South of I-84, East of Overpass, by fence 1/2 burned	Nov. 2014	Boise, ID
I-84	**MP59, Eisenman Exit #2**	43.50720	116.14174	Northeast of I-84, On either side of designated pipeline area	Nov. 2014	Boise, ID
I-84	**MP59, Eisenman Exit #3**	43.50728	116.14174	South of I-84, East of Overpass, in circle	Nov. 2014	Boise, ID
I-84	**MP64, Blacks Creek Exit**	43.46608	116.09176	North of I-84 by Kuna Rd and ON-ramp heading west	Nov. 2015	Boise, ID
I-84	**MP173, US-93 South Exit, #1**	42.63898	114.44341	Southeast of I-84, left off US-93 North, Entrance was a break in fence	Nov. 2015	Twin Falls, ID
I-84	**MP173, US-93 South Exit, #2**	42.63866	114.44391	Southeast of I-84, left off US-93 North, Entrance was a break in fence	Nov. 2015	Twin Falls, ID
I-86	**MP32, West of Neely Exit**	42.71782	112.91567	Median, in between two grates	Nov. 2015	Pocatello, ID
I-86	**MP58, Farm Tank Rd Exit**	42.91397	112.52913	Northeast of I-86, East of parking lot	Nov. 2015	Pocatello, ID
US-95	**MP0-1**	43.25935	117.01366	West side of US-93, South of gravel road, in burned area	Nov. 2015	Marsing, ID
US-95	**MP327, Uniontown Rd**	46.55291	116.94934	Median	Nov. 2016	Genesee, ID
US-95	**MP330, Kluss Rd**	46.59535	116.94354	Median	Nov. 2016	Genesee, ID

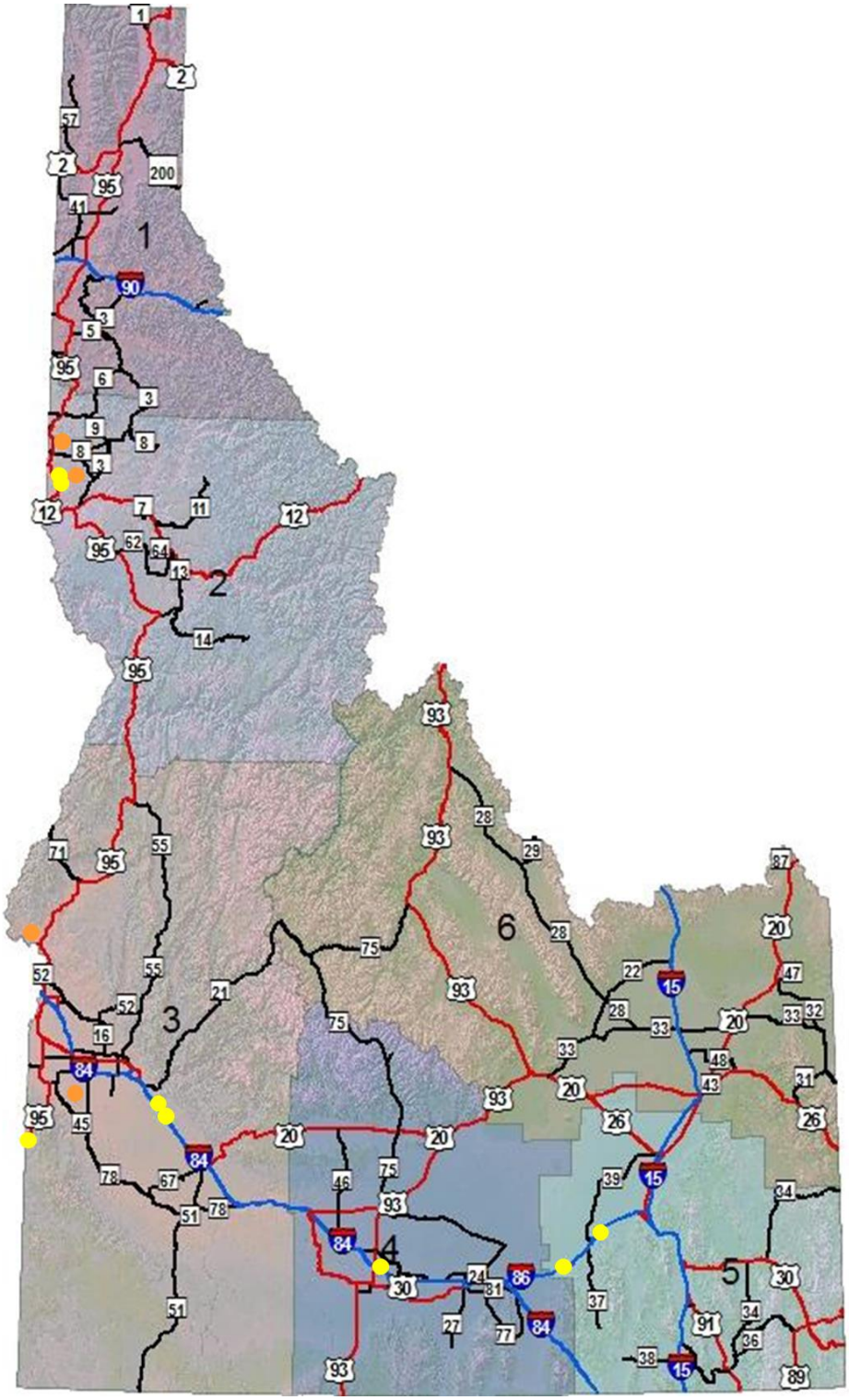


Figure 16. Location of Sites for ITD Study (Yellow Dots) and Additional Study Sites (Orange Dots)

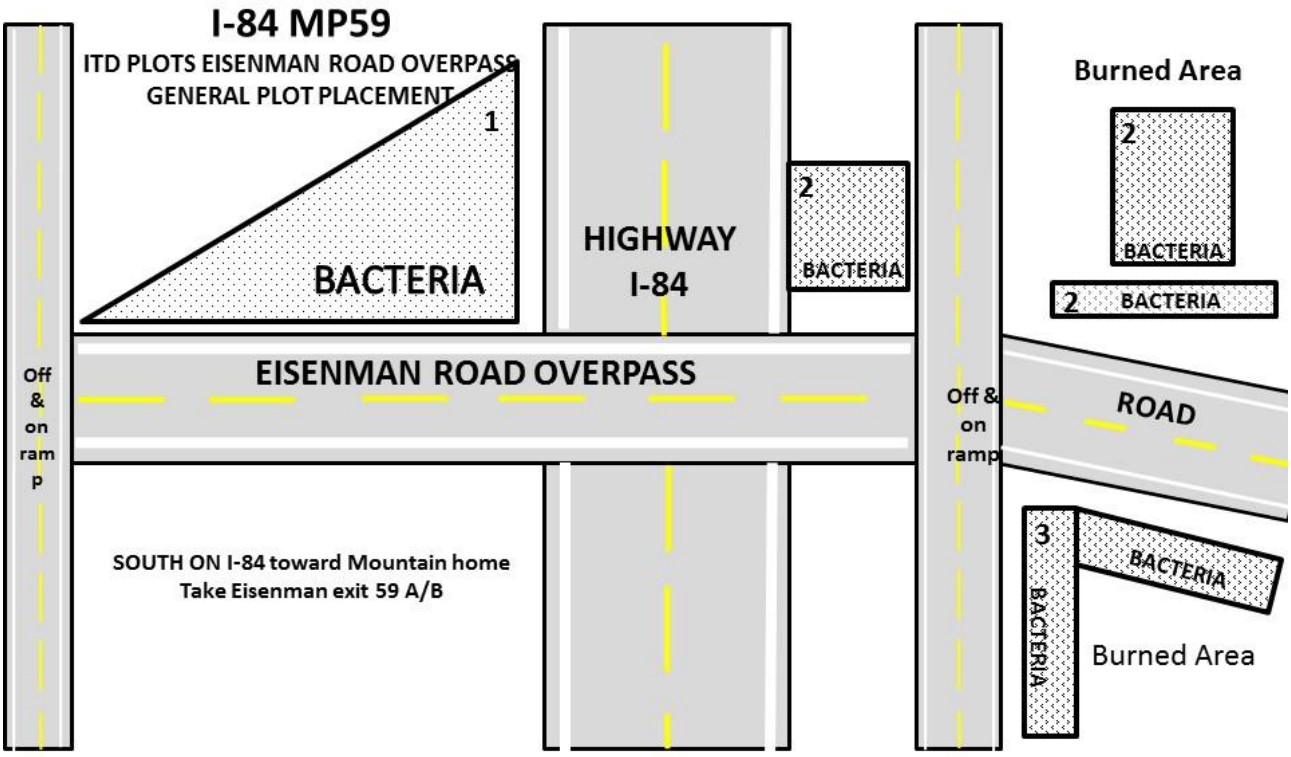


Figure 17. Plot Plans of ITD Bacterial Plots at I-84 Eisenman Exit - All Three Locations

Note: Bacteria were applied within the dotted lines. Control plots are outside the lines.

Figure 18. Pictures of ITD Bacterial Plots at I-84 MP59 Eisenman Exit - Location #1, Spring 2017

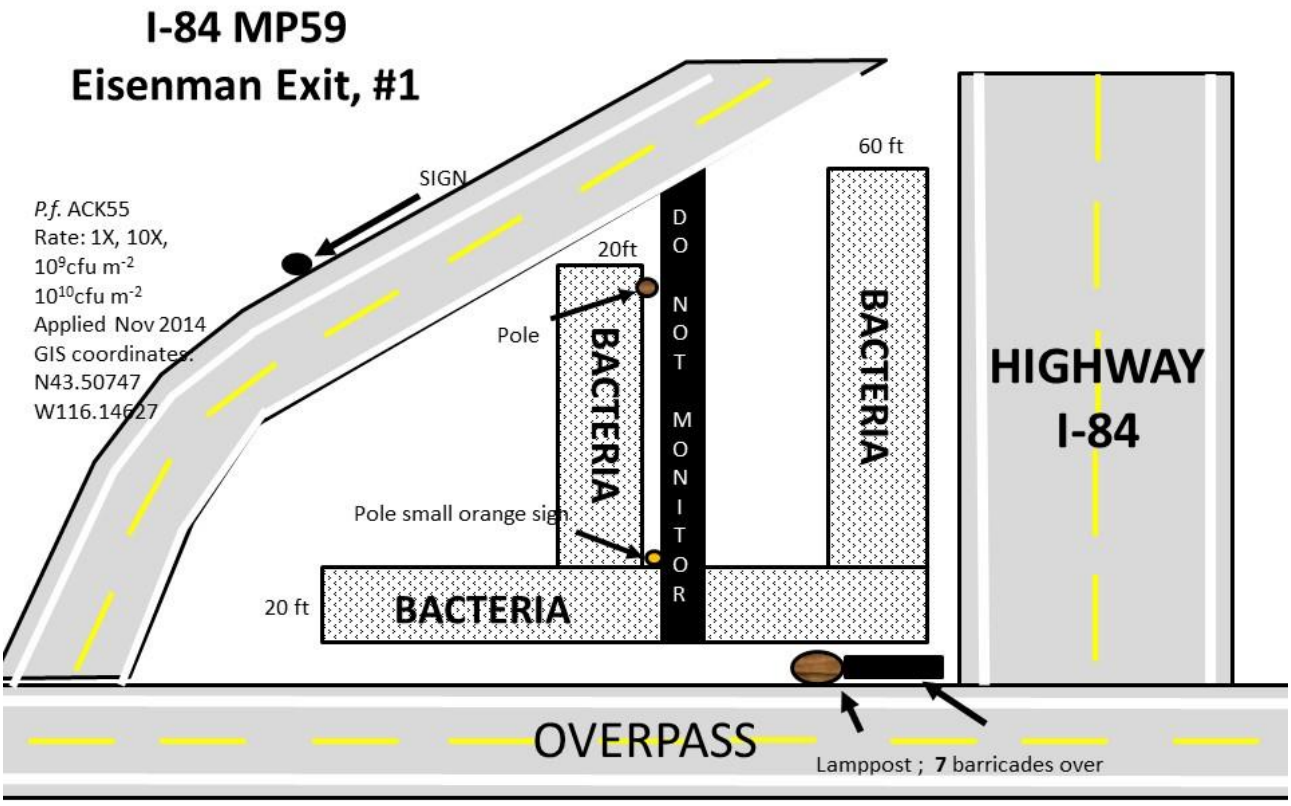


Figure 19. Plot Plans of ITD Bacterial Plots at I-84 MP59 Eisenman Exit - Location #1

Note: Less downy brome (cheatgrass) was found in the bacteria plot.

Figure 20. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-84 MP59 Eisenman Exit - Location #1, Spring 2016

Figure 21. Pictures of ITD Bacterial Plots, Control (Left) and bacteria (Right), at I-84 MP59 Eisenman Exit - Location #1, Spring 2017

Table 3. Locations and Downy Brome (Cheatgrass) Inhibition by the Weed-suppressive Bacteria for Spring 2015, Spring and Fall 2016, and Spring 2017 at Sites Used in this Study.

	Spring 2015	Spring 2016	Fall 2016	Spring 2017
Site	**Downy brome (cheatgrass) Inhibition**			
ND	**Percent**			
I-84, MP59 Eisenman Exit, #1 Northeast	35	85	95	54
I-84, MP59, Eisenman Exit, #2 Southeast, Burned	30	86	92	63
I-84, MP59, Eisenman Exit, #2 Southeast, Unburned	32	84	90	62
I-84, MP59 Eisenman Exit, #3 Southwest	36	87	89	72
I-84, MP64, Blacks Creek Exit	ND	75	89	43
I-84 MP173 US-93 exit Twin Falls				
North, Close to I-84	ND	76	85	30
South, Away from I-84	ND	75	84	30
I-86, MP33, West of Neely Exit	ND	ND	Mowed	45
I-86, MP58, Farm Truck Road Exit	ND	ND	86	45
US-95, MP 0-1, ID, OR Border	ND	70	82	75
US-95, MP274, Uniontown Road	ND	ND	ND	40
US-95, MP330, Kluss Road	ND	ND	ND	40

Note: Values are average of 8 digital images and observations on Sample Point by 3 individuals.

ND, Not Determined

Note: Bacteria plot to the right of the dotted line. Control to the left. This area was burned.

Figure 22. Pictures of ITD bacterial Plots at I-84 MP59 Eisenman Exit - Location #2.

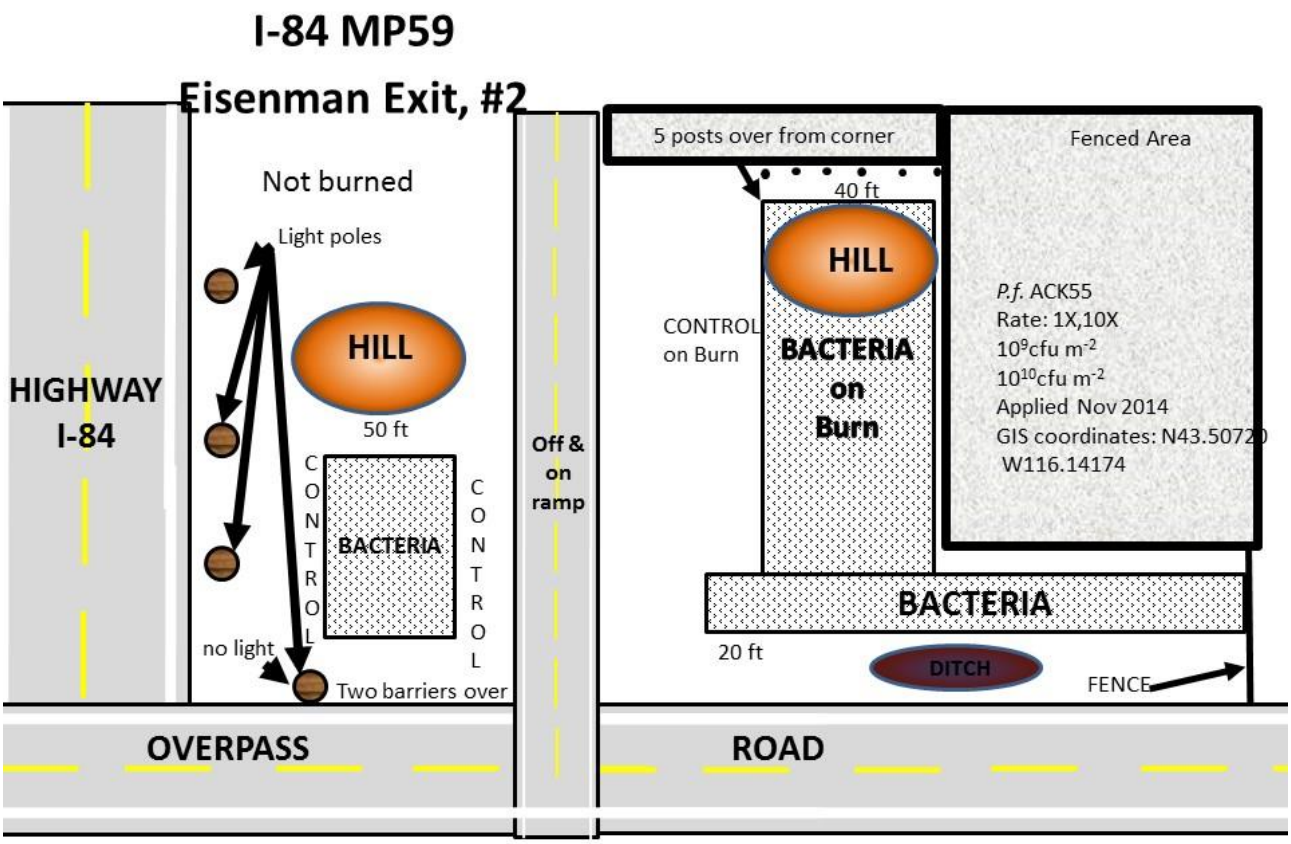


Figure 23. Plot Plans of ITD Bacterial Plots at I-84 MP59 Eisenman Exit - Location #2.

Figure 24. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-84 MP59 Eisenman Exit- Location #2, Spring 2016

Figure 25. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-84 MP59 Eisenman Exit- Location #2, Spring 2017

Figure 26. Picture of ITD Bacterial Plots at I-84 MP59 Eisenman Exit- Location #3

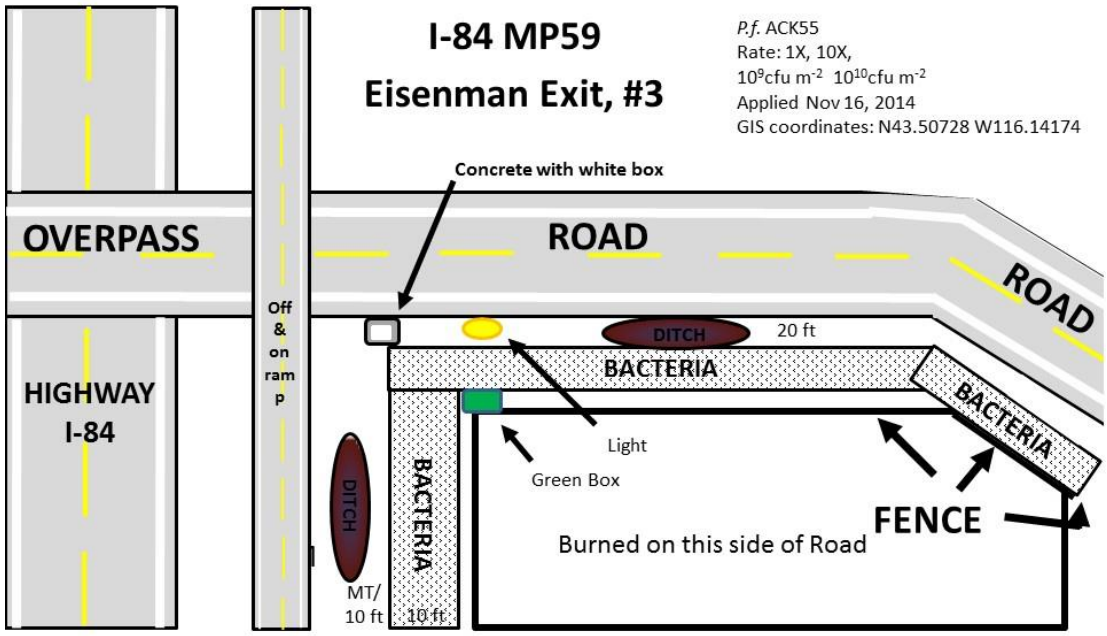
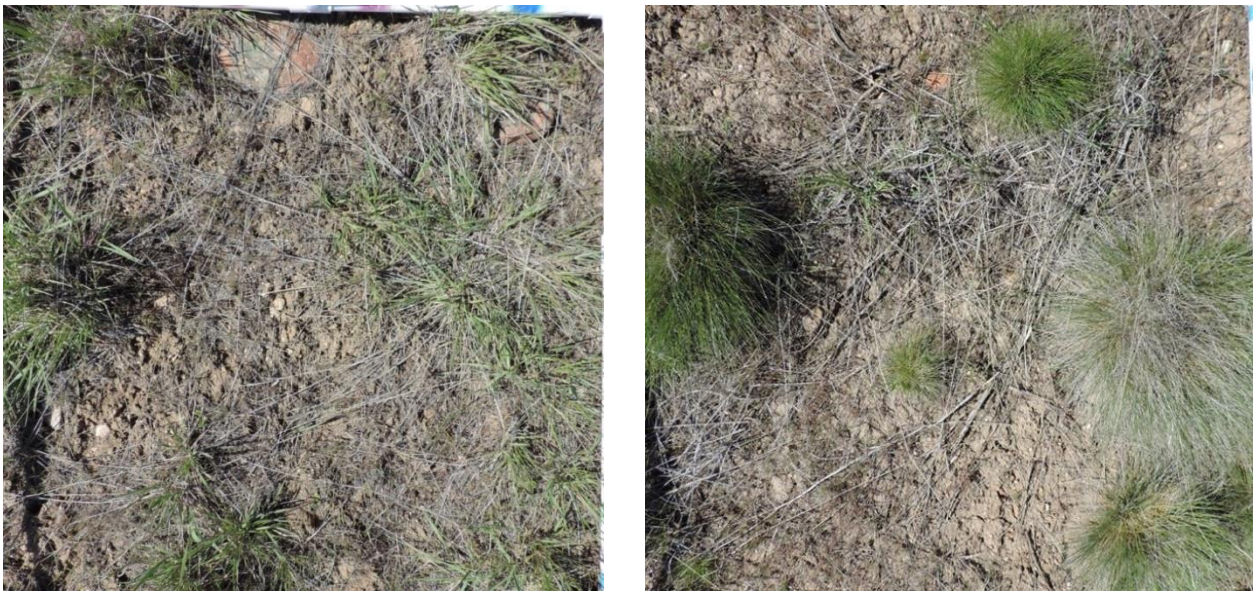


Figure 27. Plot Plans of ITD Bacterial Plots at I-84 MP59 Eisenman Exit- Location #3



Note: Higher populations of downy brome (cheatgrass) in control than bacterial plots. Sheep fescue increased in number and size in bacteria plots.

Figure 28. Pictures of ITD Bacterial Plots, Control (Left) and bacteria (Right), at I-84 MP59 Eisenman Exit- Location #3, Spring 2016.

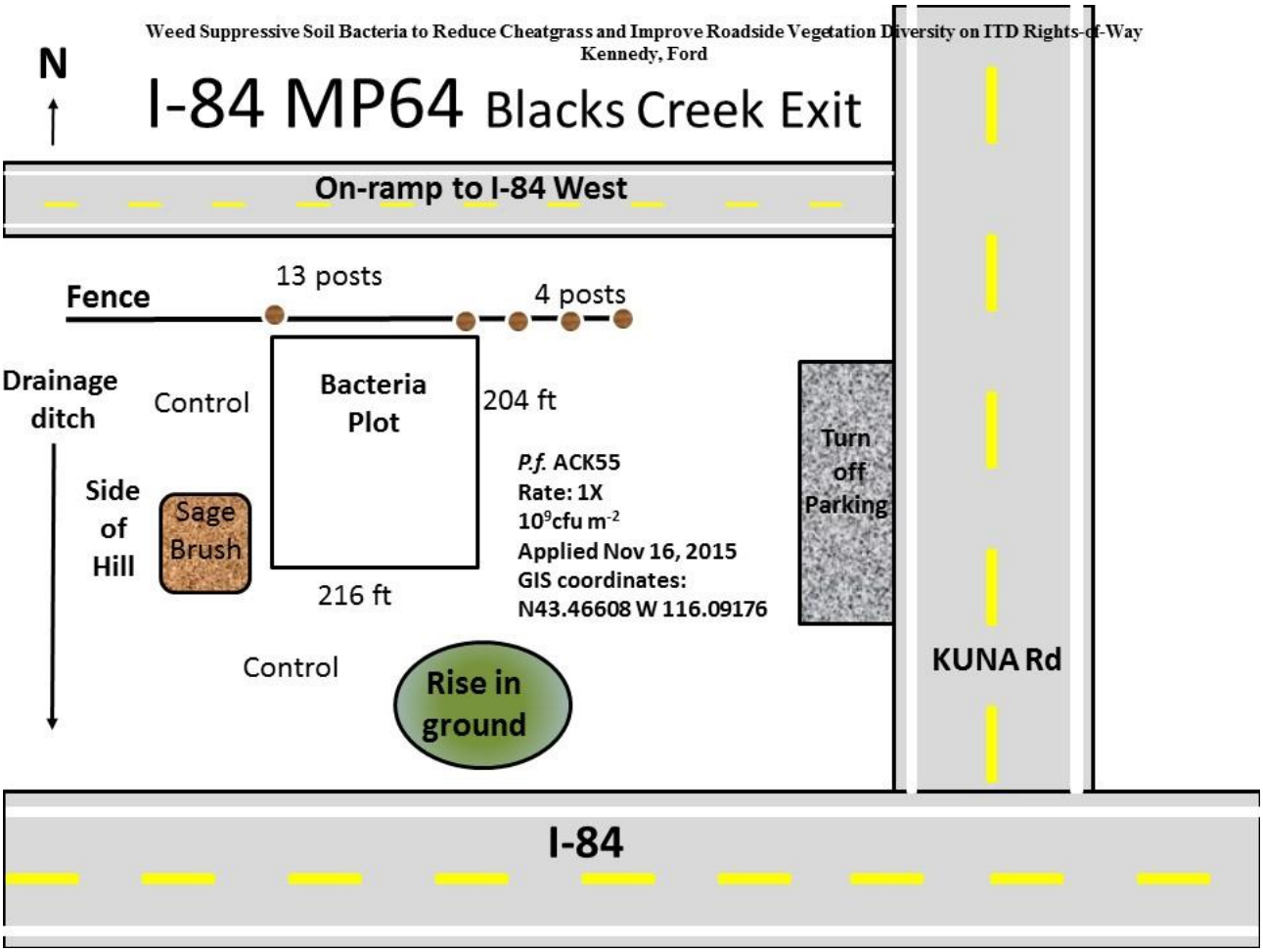


Figure 29. Plot plans of ITD bacterial plots at I-84 MP64 Blacks Creek Exit.

Figure 30. Pictures of ITD Bacterial Plots Control (Left) and Bacteria (Right) at I-84 MP64 Blacks Creek Exit, Spring 2016

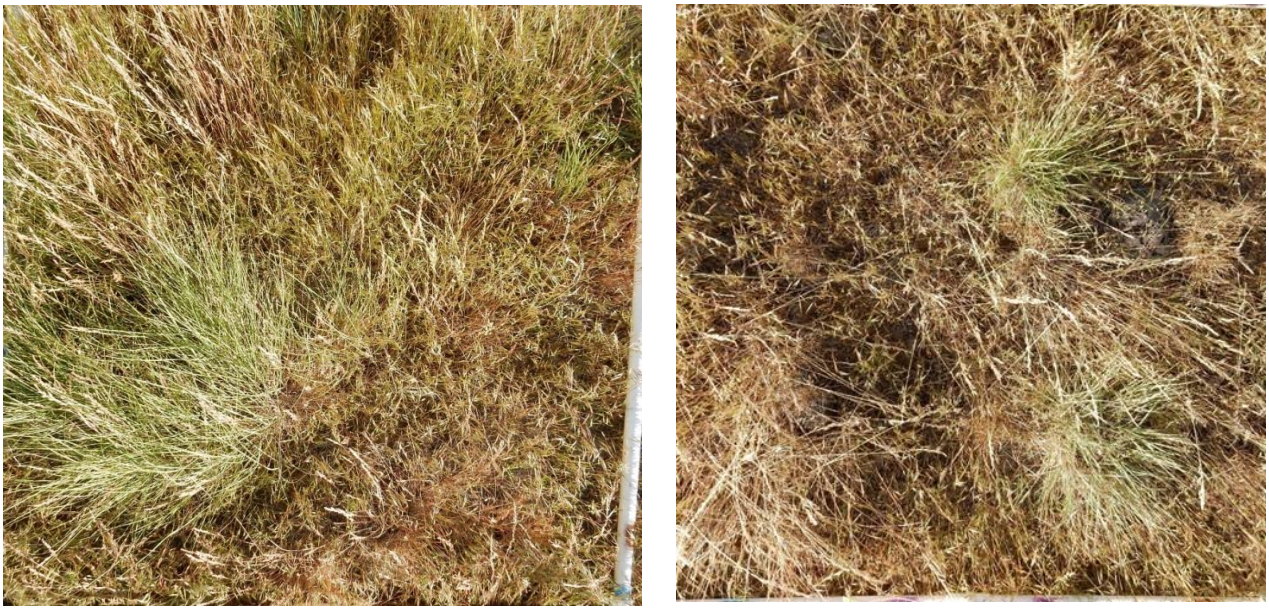


Figure 31. Pictures of ITD Bacterial Plots Control (Left) and Bacteria (Right) at I-84 MP64 Blacks Creek Exit, Spring 2017

Note: Downy brome (cheatgrass) was the dominate species throughout both the control and bacteria plots, few seeded plants were evident. No demarcation between bacteria and control plots were evident in spring 2017.

Figure 32. Picture of ITD Bacterial Plots at I-84 MP173 and US-93 Twin Falls North and South Plots, Spring 2017

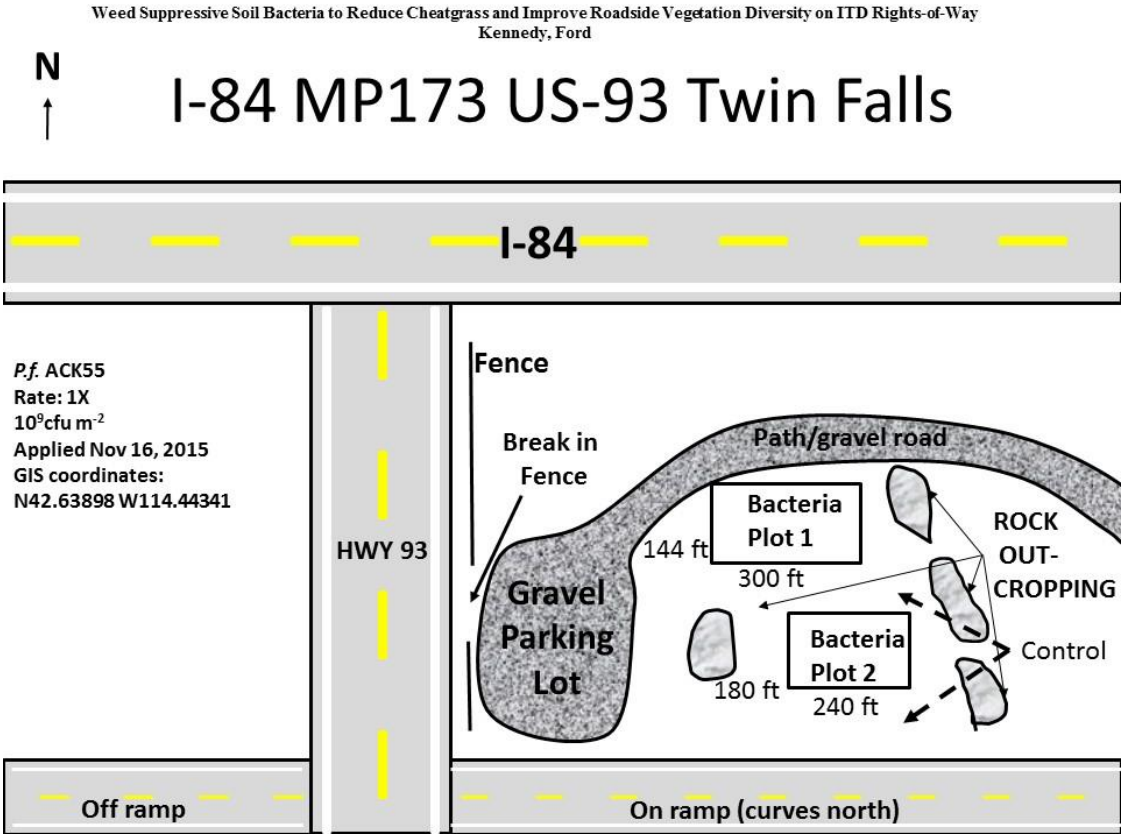


Figure 33. Plot Plans of ITD Bacterial Plots at I-84 MP173 and US-93 Twin Falls North and South Plots

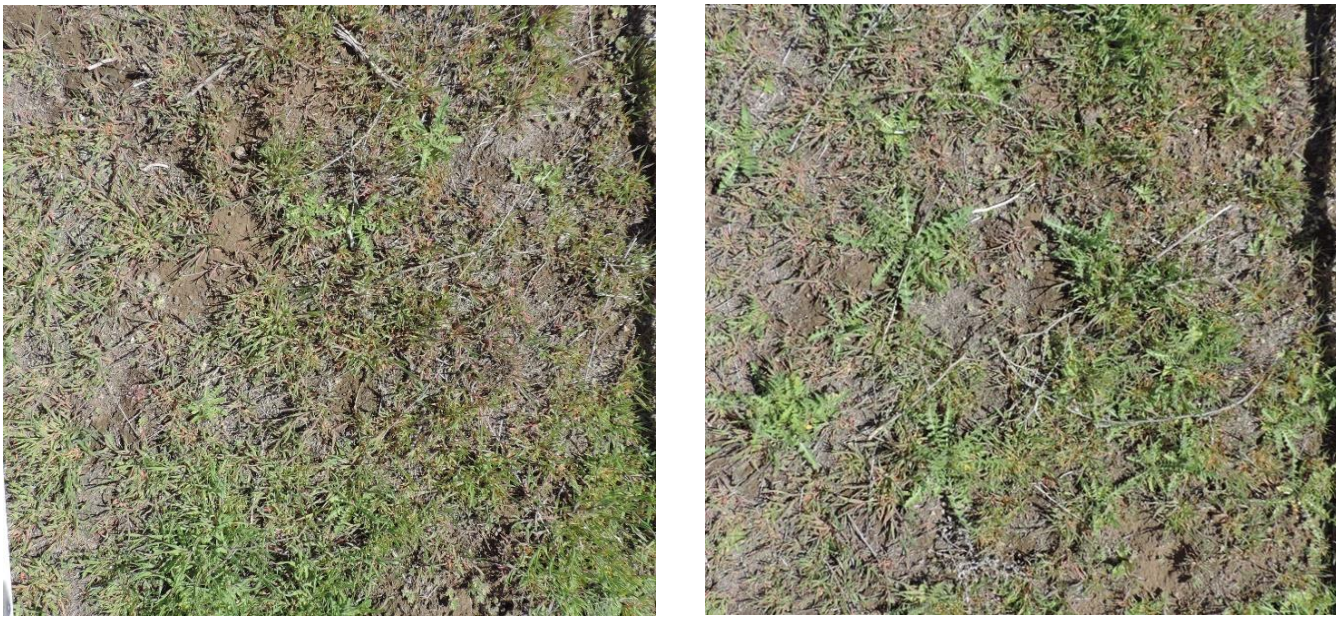


Figure 34. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-84 MP173 and US-93 Twin Falls North and South Plots, Spring 2016

Note: Downy brome (cheatgrass) was only slightly less in the bacteria plots than in the control plots.

Figure 35. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-84 MP173 and US-93 Twin Falls North and South Plots

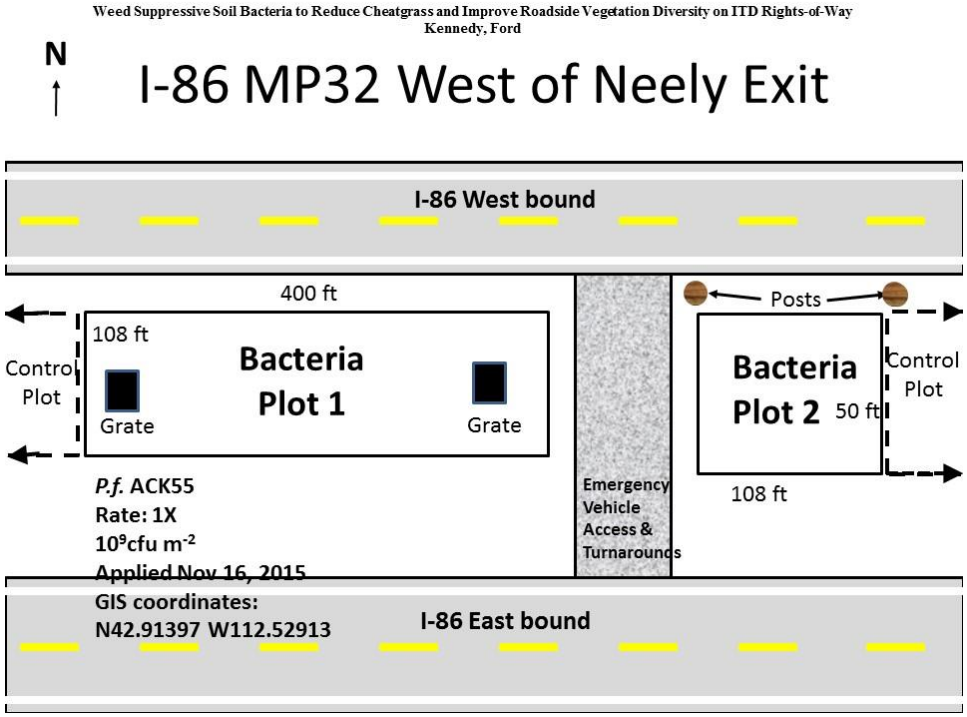


Figure 36. Plot Plans of ITD Bacterial Plots at I-86 MP32

Figure 37. Picture of ITD Bacterial Plots at I-86 MP32, Spring 2017

Figure 38. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-86 MP32

Note: Bacteria plot to left of line, one of the control plots to right in up per photo.

Figure 39. Pictures of ITD Bacterial Plots at I-86 MP58, Spring 2017

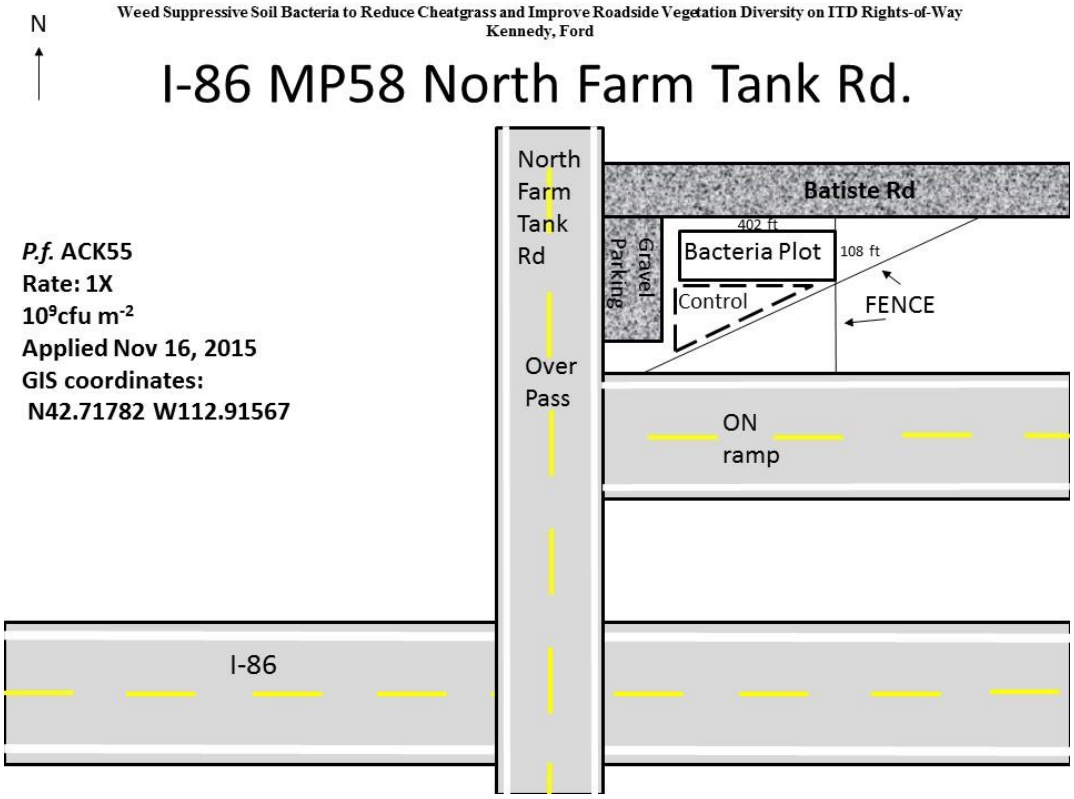


Figure 40. Plot plans of ITD bacterial plots at I-86 MP58

Figure 41. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right), at I-86 MP58, Spring 2017

Note: Bacteria plots to right of dotted line. Control plots to left of dotted line.

Figure 42. Picture of ITD Bacterial Plots on US-95 MP 0-1, Spring 2017

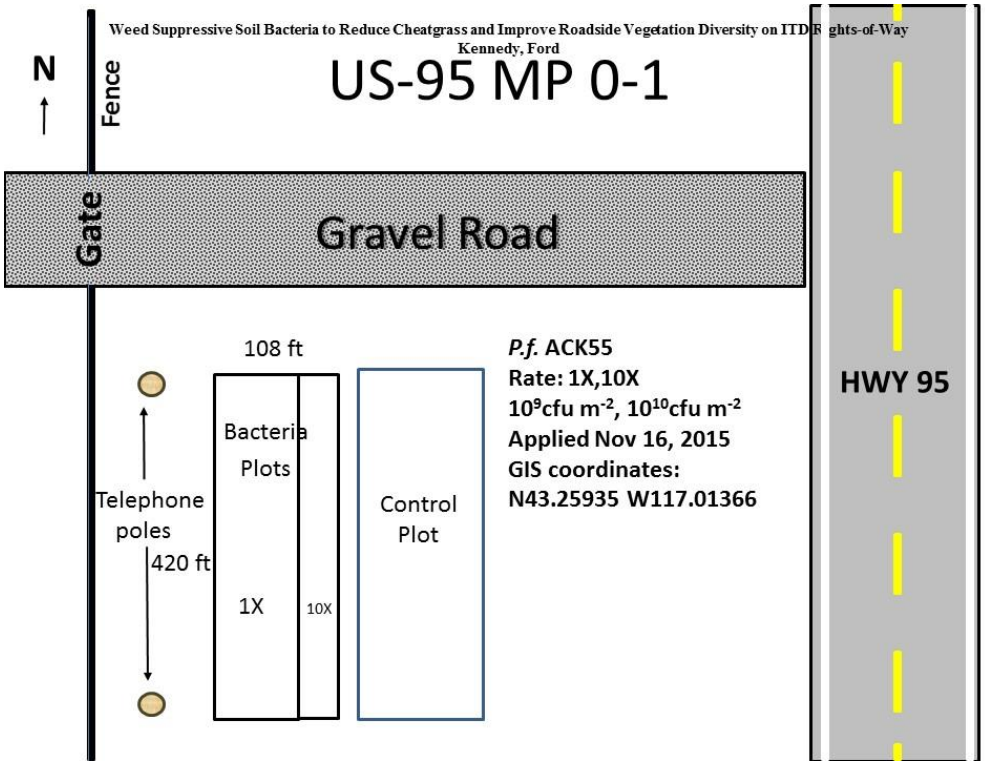


Figure 43. Plot Plans of ITD Bacterial Plots on US-95 MP 0-1

Figure 44. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right) on US-95 MP 0-1, Spring 2016

Note: Medusahead was present in control plots, but not present in bacteria plots.

Figure 45. Pictures of ITD Bacterial Plots; Control (Left) and Bacteria (Right) on US-95 MP 0-1, Spring 2017

Figure 46. Pictures of Area around ITD bacterial plots at US-95 MP327

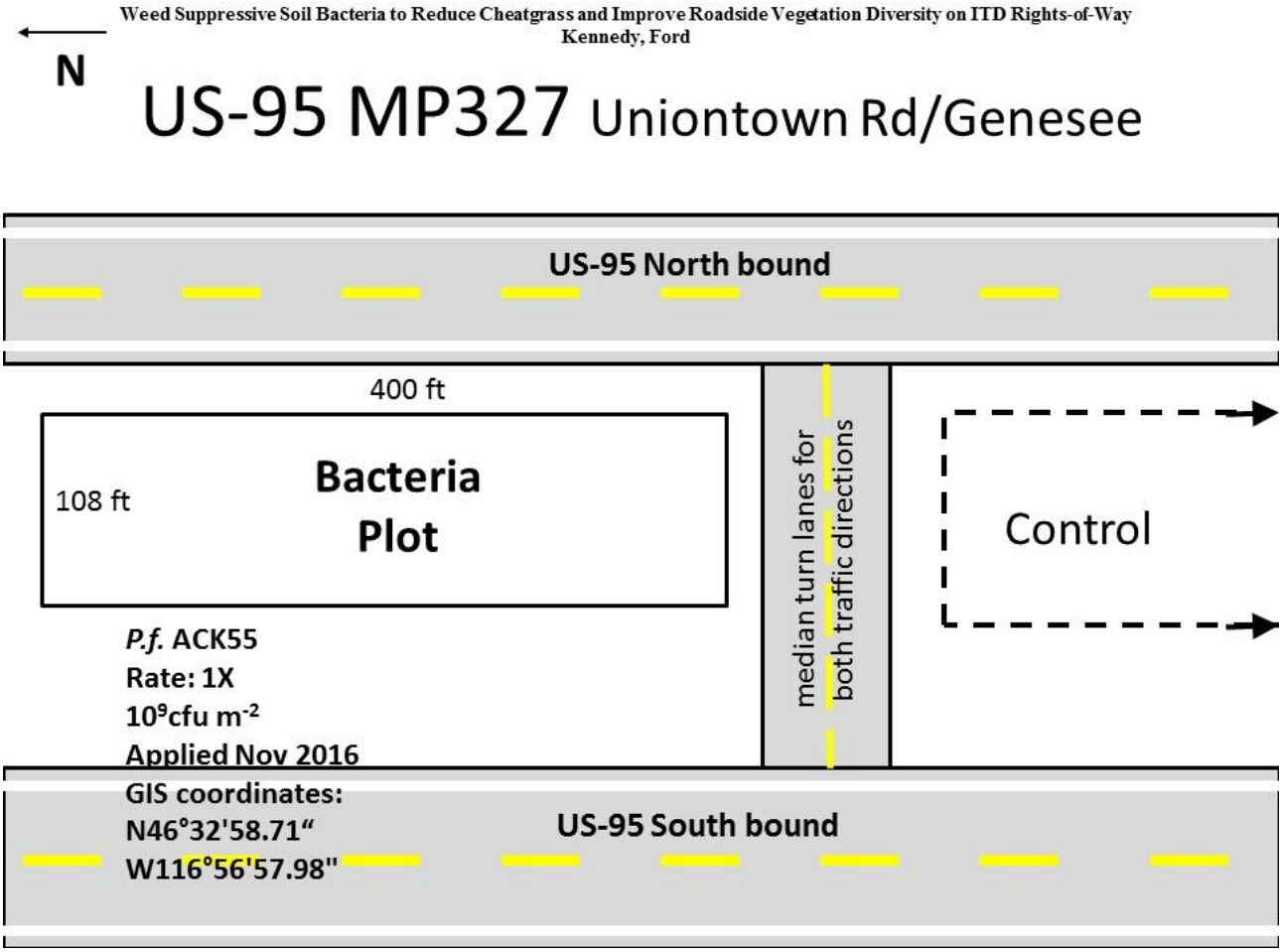


Figure 47. Plot Plans of ITD Bacterial Plots on US-95 MP327 near Genesee, ID, Spring 2017

Figure 48. Pictures of ITD Bacterial Plots, Control (Left) and Bacteria (Right) at US-95 MP327

Figure 49. Pictures of ITD Bacterial Plots on US-95 MP 330, Spring 2017

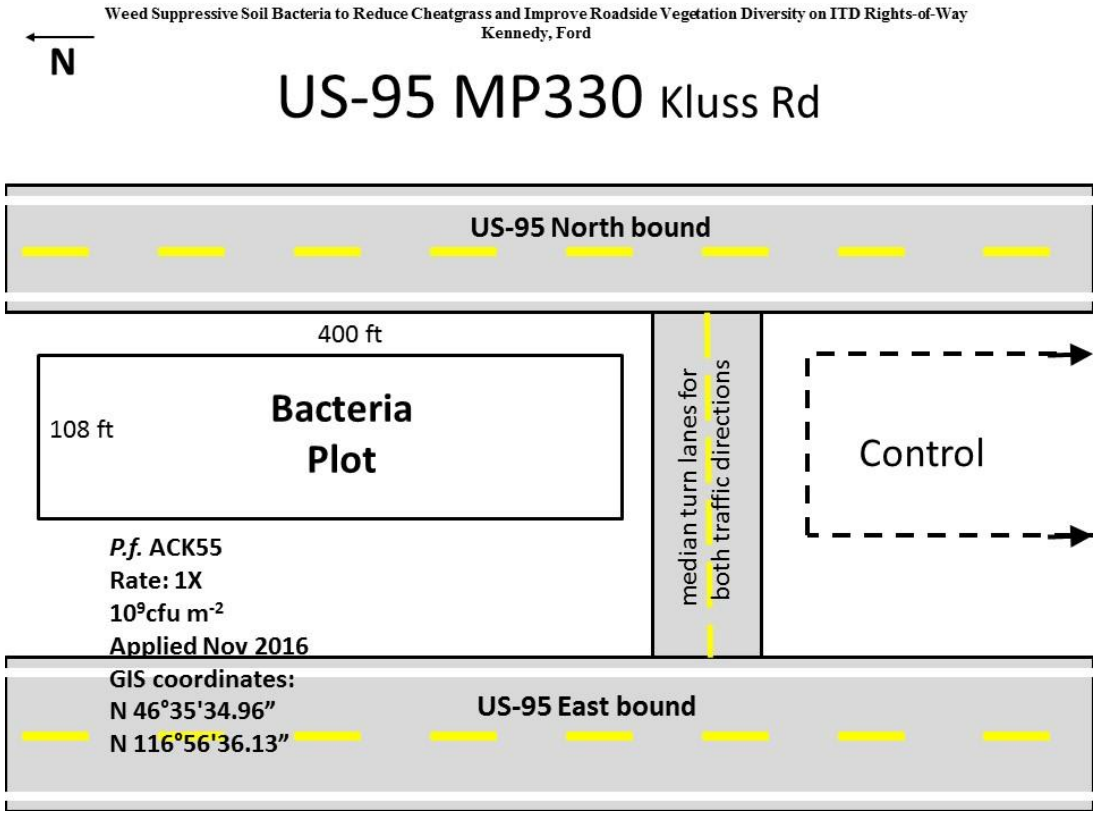


Figure 50. Plot Plans of ITD Bacterial Plots on US-95 MP 330

Note: Downy brome (cheatgrass) was dominant in the control plots and a minor plant in the bacteria plots.

Figure 51. Pictures of ITD Bacterial plots; Control (Left) and bacteria (Right) on US-95 MP 330 Spring 2017

Chapter 3
Best Management Practices

Abstract

Weed-suppressive bacteria (WSB) offer a fresh, innovative way to implement weed management techniques for the Idaho Transportation Vegetation Specialist. When the suppressive bacteria are applied in combination with the correct herbicide applied at the optimum rate and timing, the seed bank of the target weed can be effectively reduced to low levels. The WSB used in this study, (ACK55), is most effective when combined with the optimal seeding of a desirable plant or species mix with the best genetics and best placement to fill in the void created by the absence or reduction of the target weed. After proper application, ongoing management is necessary to maintain a healthy, desirable plant community that can compete against the ever-challenging weeds. A healthy, flourishing plant community is the best weed control. Initially, light tillage may be prescribed to uncover buried weed seed and foster germination of weeds that become more susceptible to herbicides. Surface tillage may also foster good seed establishment of the desired plants; however, care must be taken to avoid loss of soil or organic matter to wind and water erosion. Additional management practices need to be considered as situations arise. The WSB represents one of the newest weed management practices and ultimately may end up being one of the most powerful, but it is not a stand-alone approach. There are multiple field scenarios and addresses how to optimally apply WSB to achieve effective, long-term management of invasive weeds.

Background

Downy brome (cheatgrass, *Bromus tectorum* L.), medusahead (*Taeniatherum caput-medusae* [L.] Nevski*),* and jointed goatgrass *(Aegilops cylindrica* L.*)* are annual grass weeds, increasingly prevalent in the western United States (Rice 2005a,b).[16,17] They impair cereal crop production (Stahlman and Miller 2009); choke out native plants (Knapp 1996; Brooks et al. 2004); degrade rangelands (Asher and Harmon 1995; Epanchin-Niell et al. 2009); and reduce sage-grouse and other sagebrush-dependent wildlife (Crawford et al. 2004; Duncan et al. 2004; Rice 2005a, b; Wisdom and Chambers, 2009).[14, 23,22,24,29,18,15,16,17,28] In addition, the dry residue left from grass weeds provides abundant, readily available fuel for wildfires (USGS 2002; Brooks et al. 2004; Epanchin-Niell et al. 2009; Balch et al. 2013) that can destroy property and result in the loss of human life (US Fire Administration 2012; Yan & Hanna 2013).[25,22,29,12,82,83] Naturally-occurring bacteria isolated from native soil and root surfaces have been found to inhibit downy brome (cheatgrass), medusahead, and jointed goatgrass (Kennedy et al. 1991; Kennedy, 2014b, 2016, 2018).[1,2,5,8] One such bacterial strain is *Pseudomonas fluorescens* strain ACK55 (ACK55); however, there are several other bacterial isolates that have been found to inhibit other grass weeds and screening in the Kennedy Lab identified other unique isolates that selectively suppress other invasive weeds and plants (Kennedy, 2016).[5] WSB inhibits root formation, root growth, and tiller initiation. It **does not** inhibit economically important plants and **do not** injure any native plant species found in the United States. The full list of traits is presented in Table 4.

One of the greatest natural resource challenges that transportation departments face is the invasion of exotic, annual grasses. Downy brome (cheatgrass) and medusahead negatively affect vegetation efforts on roadsides and cause ecological disaster because they alter vegetation diversity, soil quality, and increase the use of herbicides and tillage. Downy brome (cheatgrass) is extremely competitive with perennial (desirable) grasses and other plants for the available moisture and nutrients. Downy brome (cheatgrass) chokes out the natives in the shrub-steppe habitat of the western United States rangeland; builds fire-fuel load; and increases fire frequency and intensity.

Road construction and improvements often result in newly disturbed land that is especially vulnerable to invasion by downy brome (cheatgrass). Tillage, residue burning and herbicides are management practices available to land managers; however, these options have been ineffective at reducing downy brome (cheatgrass) populations or sustaining desirable vegetation and healthy ecosystems. Tillage practices can lead to greater erosion at a site.

The WSB, *Pseudomonas fluorescens* strain ACK55, offers another option to the control of downy brome (cheatgrass), but it **must be** applied under the proper conditions to achieve favorable results. The bacteria thrive in the subsurface soil during cold temperatures, therefore, it is critical to apply WSB in the fall to soils below 50^{o}F with rain or snow in the forecast. If simply applied to the surface of warm, dry soil, bacteria survival is poor due to the harsh environment of UV light, low moisture, and extreme temperatures. However, if applied at the proper time, those bacteria that are mobilized down into the soil by fall rains survive and flourish. When soil temperatures rise above 50^{o}F, ACK55 numbers drop and the WSB is unable to compete with other, faster-growing soil bacteria. To compensate for poor growth and survival at higher temperatures, it is applied to the soil at rates higher than actually needed for weed inhibition. The bacteria can be applied to the soil as a spray, or applied onto native seed and planted beneath the soil surface with a seed drill.

Although it may take the WSB several years before significant weed suppression is apparent to the naked eye, in the first few years after field application, studies show the bacteria inhibit weed growth by 20 to 50 percent (Kennedy, 2018). [(8)] The inhibition increases with time, reaching maximum weed suppression three to six years after application. In winter wheat studies, the bacterial suppression of the weeds allowed the wheat to be more competitive, which in turn reduced weed populations further (Figure 8). In long-term rangeland field trials in WA, spray application of the bacteria resulted in almost complete suppression of moderate populations of downy brome (cheatgrass) (Figure 9), medusahead (Figure 10), and jointed goatgrass five to seven years after a single bacterial application when perennial species were also present. Roadside application of the bacteria also resulted in a sharp reduction of downy brome (cheatgrass), which stimulated the growth and establishment of perennial grasses that reduced soil erosion and fire potential (Figure 11, 12).

After application of WSB, weed growth decreased and weeds became less competitive, allowing the populations and growth of native plant species to increase and establish. The bacteria plus the native plant, turf, or wheat interact in concert to reduce the downy brome (cheatgrass), medusahead, and/or jointed goatgrass. One gallon of actively growing WSB (1 x 10^{12} cfu or cells per acre) is the prescribed

amount to suppress these three weeds in three to five years. The benefits of the WSB include dollar-valued changes in rangeland productivity for ranching, reduction in firefighting costs, and reduced losses of infrastructure due to reduced risk of wildfire (USGS, 2002).[25] It represents a new and powerful tool in weed management and restoration efforts. However, the integration of this new method into existing practices of weed management requires careful consideration, timing and foresight.

The Best Management Practice

The best-case scenario for optimum use and greatest success in using weed-suppressive bacteria on Idaho roadsides consists of the following six points:

1. Select a site with a healthy, dense stand of desirable plant species, such as sheep fescue, Sandberg bluegrass, crested wheatgrass, etc.

2. Select a site with moderate to low infestation of downy brome (cheatgrass) or medusahead.

3. Apply the bacteria to the site in late fall, when air and soil temperatures are below 50^{o}F and rain is forecast.

4. Apply 1 gallon of actively growing WSB to each acre.

5. Apply a broadleaf herbicide in the spring as needed.

6. The bacteria may take several years before one can visually see a reduction of downy brome (cheatgrass) or medusahead populations.

Additional information is provided below for other situations.

Bacterial Application

- The WSB inhibits downy brome (cheatgrass), medusahead, and jointed goatgrass at the seedling stage and does not reduce the growth of established weed plants.

- **The timing of WSB application is critical** to the successful, long-term reduction and exclusion of downy brome (cheatgrass), medusahead, and jointed goatgrass. **Late fall applications, when air and soil temperatures are cool (below 50^{o}F) and rains are prevalent, have the highest success.**

- The bacteria can be applied to frozen soils or snow, especially if more snow is in the forecast. The limiting factors in late-season bacterial application are cold temperatures that freeze spray nozzles and hoses.

- Soil texture and soil nutrient content do not affect the efficacy of the bacteria. In extensive studies across the west, few differences were observed in bacterial survival or ability to suppress downy brome (cheatgrass).

- Application of the bacteria before fall rains transports the bacteria from the plant surface into the soil. There is no need to remove residue to facilitate the movement of the bacteria into the soil. In studies of residue removal in the field, a negative effect was found. The residue removal uncovered additional downy brome (cheatgrass) that then germinated. The high numbers of weed seed germinating reduced the ability of the bacteria to reduce high numbers of the germinating seed resulting in less inhibition of the downy brome (cheatgrass).

- Spring applications of the WSB do not lead to consistent suppression of downy brome (cheatgrass), medusahead, or jointed goatgrass because the conditions for bacteria growth, establishment and survival are sub-optimal.

- The proper and current State **herbicide applicator license is needed to use the WSB5**. Personal protective equipment for applying the bacteria include waterproof gloves, long-sleeved shirt and long pants, and shoes plus socks. After application, triple rinse the spray tank and flush hoses and nozzles well. The nozzle size is not critical; however, the larger the orifice the better. Do not allow spray pressure to exceed 30 psi.

- Apply 1 gallon of actively growing WSB, *Pseudomonas fluorescens* strain ACK55, mixed in a tank of water for each acre. The actively growing bacteria is most effective and results are seen more quickly. The carrier is water and the volume can range from 2 to 30 gallons.

- The bacteria, in concentrated form, can also be sprayed onto seed while the seed is tumbling in a seed coater or concrete mixer. The number of bacteria needed for seed coating is the same as for spray application per unit area. The bacteria should be applied to the seed to deliver 5×10^{12} colony forming units per acre. The liquid slurry containing the bacteria should be low to avoid early germination of the seed. Six to ten ounces of liquid to coat sixty pounds of seed should be sufficient to mix the bacteria onto the seed without causing seed germination. The seed can be drilled into the soil. Applying the bacteria to the soil as a spray or coating desirable seed with the bacteria have been equally effective at delivering the bacteria to the soil so that weed suppression can take place.

- Do not apply the liquid to dry soil. Apply the bacteria when it is raining or rain is in the immediate forecast. Rain is needed to mobilize the bacteria and help the bacteria migrate into the soil. Bacteria survival is poor if air temperatures are too high or the bacteria are exposed to sunlight on the soil surface for too long.

- After application, the bacteria will be active in the soil for 4 to 6 years. **Repeat applications may be needed in 4 to 6 years depending on the site and reoccurrence of weed populations.**

- The success of the WSBrelies on the interaction between the weed, the bacteria, and desirable plants (native or beneficial). The greatest success with long-term reductions in downy brome (cheatgrass), medusahead, and jointed goatgrass has been on lands with mixed populations of native plants and moderate to low weed infestations.

- The bacteria will not inhibit the growth of standing weed plants. Fall weed growth from late summer or early fall rains will require herbicide treatment to reduce already standing/existing plants and speed up the reduction of grass weeds.

- As the WSB reduces downy brome (cheatgrass), medusahead, and jointed goatgrass, voids are created that broadleaf weeds fill. Since it does not inhibit broadleaf plants, the site must be monitored carefully. In many cases, the use of a broadleaf herbicide is necessary in the spring of each year to control the advancement of broadleaf weeds due to the reduction of downy brome (cheatgrass) infestations.

- It is important to monitor the land regularly so that a recurrence of downy brome (cheatgrass), medusahead, and jointed goatgrass (through wind or water movement) or the appearance of other weeds can be addressed and suppressed quickly and completely.

- Knowing the site history is critical to restoration success and the use of all available management tools is necessary to restore disturbed areas.

- Use of glyphosate, imazapic, or other herbicide in the fall reduces annual grasses and weed growth. Tillage may be useful to allow weed seed: soil contact and facilitate weed seed germination prior to herbicide application. Allowing the weed to grow only to the 2-leaf stage followed by glyphosate, imazapic, or other appropriate herbicide application is recommended.

- Depending on the level of invasion, it may be necessary to apply herbicides multiple times to reduce weed growth especially in the first year.

- WSB, *Pseudomonas fluorescens* strain ACK55, is not as effective without native plant populations or desirable plants to actively compete with the grass weed. The bacteria work by decreasing the weed seed bank and suppressing seedling vigor.

- If native or desirable plants are not present, apply herbicides to reduce weed populations prior to applying the soil bacteria. This may take a couple of seasons. Once weed populations are reduced, apply a mix of native/desirable plant seed using optimal equipment such as a drill seeder with a flexible packer arm, at the correct time in the fall, at the correct depth in between 1/4 and 3/8 inches deep. The bacteria can be coated onto desirable seed and drilled into the soil.

Rangeland

Optimal suppression and long-term reduction of downy brome (cheatgrass), medusahead, and jointed goatgrass by WSB is achieved in rangelands with mixed populations of native plants, a moderate infestation of the weeds, and a herbicide application (3 oz. imazapic, or a low rate of glyphosate in the fall) at the correct time to reduce the existing weed plants while not injuring perennial grasses.

- Native or desired plant species must be present for optimal inhibition of the weed. Application

of bacteria alone without some desirable plant present will result in a short-lived reduction of target weeds.

- Drill seeding of desirable plants may be necessary, if no native plants are present in the seed bank or at the site. If the area is a monoculture of these three grass weeds and lacks native plants, herbicides may be needed to rid the area of the first year's growth of the grass weeds. The bacteria can be coated onto the desirable seed to deliver 1 x 10^{12} cfu per acre.

- Ideally, reseeding desired plants should follow application of the bacteria either in the same year or in the fall of the next year or perhaps reseeding could wait until two falls after bacterial application.

- Grazing should be delayed in areas that have been reseeded until the seeded plants have a chance to establish a sufficient root system.

- It is important to monitor the restored land regularly so re-infestation can be addressed early if the native plants populations are not increasing.

- Each site is different and unique restoration plans must be developed for each site to achieve the desired outcome.

Post-Fire Restoration

Post-fire restoration can be successful when WSB are included in the restoration plan. The removal of the thick residue that can build up from these weeds exposes a large quantity of weed seed ready to germinate. When coupled with herbicides, perhaps surface tillage, and drill seeding of natives, WSB can be an integral part of the restoration of these lands.

The actions needed to be taken in post-fire restoration on ITD land are as follows.

- In the early fall, the land can be harrowed lightly 1 or 2 times and left to over winter to mix the weed seed with soil and hasten germination. An herbicide application can be used to rid the area of any germinating weeds or wait until spring.

- The next spring (April to May), spray with glyphosate and AMS (no R-11 needed) or imazapic when the annual weeds are actively growing, but haven't yet produced seed. Continue to monitor for additional weed emergence, spraying with glyphosate or imazapic as needed.

- In early summer, harrow 1 to 2 times, being careful to not disturb the soil too much. Avoid wind erosion and soil loss by tilling lightly or not at all.

- In June and July, monitor weeds; spray with 2,4-D, glyphosate, and/or imazapic, or other appropriate herbicide as needed.

- In October, monitor weeds; spray with 2,4-D, glyphosate and/or imazapic , or other appropriate

herbicide as needed.

- Spray the bacteria in the fall as indicated above or coat the desirable seed with the bacteria to be mechanically seeded or hydroseeded.

- Drill seed grasses in October to November making sure the seeding depth is between 1/4 and 3/8 inches and that soil: seed contact is made. The bacteria can be coated onto the desirable seed to deliver 1 x 10^{12} cfu per acre.

- Early the next spring, monitor weeds and spray with a broadleaf herbicide like Bronate and Dicamba. If there is no basin wildrye or mustard present, consider applying a low rate of a Sulfuron Urea herbicide, like Express. Use other appropriate herbicides as needed.

- Monitor for weeds, development of planted species, and spray herbicides as needed in March through May.

- Two years of double fallow may be necessary before planting, if the weed populations are extremely high.

Disturbed or Eroded Land

Soil that has been tilled excavated or moved from its original site contains few native seeds and often, past disturbance has resulted in reduced organic matter and loosened or eroded soil. These conditions pose the greatest challenge in using the WSB to restore native habitats and rangeland. Restoration is less effective on soil that has had long term monoculture or on heavily eroded sites. More time, tillage, herbicide spray passes, drill seeding, energy, and funds are needed when working with heavily disturbed lands with few native species.

- Knowing the site history is critical to restoration success and the use of all available management tools is necessary to restore abandoned farmland.

- Use of glyphosate, imazapic, or another herbicide in the fall reduces weed growth. Tillage is needed to allow weed seed to come in contact with the soil contact and germinate. Allowing the weed to grow only to the 2-leaf stage followed by glyphosate, imazapic, or other appropriate herbicide application is recommended.

- Depending on the level of invasion, it may be necessary to apply herbicides multiple times to reduce weed growth especially in the first year.

- Weed-suppressive bacteria are not as effective without native plant populations or desirable plants to actively compete with the grass weed. Weed-suppressive bacteria work by decreasing the weed seed bank and suppressing seedling vigor.

- If native plants are not present, weed plants are first reduced with herbicides. Then, the correct mix of native seeds should be seeded with the correct drill having a flexible packer arm, at the

correct time, at the correct depth in between 1/4 and 3/8 inches deep. The bacteria can be coated onto the desirable seed to deliver 1 x 10^{12} cfu per acre.

Table 4. Traits of Weed-suppressive Bacteria *Pseudomonas fluorescens* strain ACK55

- In the field, the WSB **suppresses** multiple accessions or biotypes of the annual grass weeds; downy brome (cheatgrass), medusahead, and jointed goatgrass.
- **The WSB is very selective and does not suppress** other weed species.
- **Does not injure desirable plant species** such as crops or native and near-native plant species in the field (over 250 grass species tested).
- Increases in cell numbers in the soil only in the late fall, winter and early spring (< 50^{o}F). Bacteria populations are low during the warmer temperatures of the plant growth season.
- Does **not enter the plant cell.**
- Produces a weed-suppressive compound in the root intercellular spaces. The compound breaks down very easily, has **no residual and** is not active in the soil.
- Is not competitive and survival in field soil is less than six years. These **bacteria do not naturally persist in high numbers in most soils**.
- **Does not grow in natural waters including ditches, rivers, lakes, and oceans**.
- Has **no anti-microbial activity**.
- Has no means of producing **enzymes that lyse cell membranes and enter plant cells**.
- Has **no protein secretions** (Type I, II, III) that could harm non-target plants.
- Has low to **no allergenic protein secretions**.
- Does not elicit hypersensitivity reactions to humans or domestic animals.
- Has the weed-suppression **gene located at multiple positions on the chromosome**. This reduces risk of gene transfer to 1:10^{30}.
- Has **no plasmids that could be transferred among organisms**.
- Does not alter **soil microbial communities after application**.
- Does not harm **Daphnia** growth or populations.
- Does not harm **wireworm** larvae growth or populations.
- Does not harm **Lemna** growth or populations.
- Does not harm **lady bug** growth or populations.
- Does not harm **Honey bee** growth or populations.
- Has no negative effect on **avian** growth.
- Has no negative effect on **rabbit** eyes or skin.
- Has no negative effect on **rat** toxicology or pathology.

Chapter 4
Conclusions and Recommendations

One of the greatest natural resource challenges that transportation departments face is the invasion of exotic, annual grasses. Downy brome (cheatgrass) and medusahead negatively affect vegetation efforts on roadsides and cause ecological disaster because they alter vegetation diversity, soil quality, and increase the use of herbicides and tillage. Downy brome (cheatgrass) is extremely competitive with perennial (desirable) grasses and other plants for the available moisture and nutrients. Downy brome (cheatgrass) chokes out the natives in the shrub-steppe habitat of the western United States rangeland; builds fire-fuel load; and increases fire frequency and intensity.

The downy brome (cheatgrass)-suppressive bacterium was sprayed on 1-acre plots at different locations in the fall of 2014, 2015, and 2016 at 10^9 colony forming units (cfu)/m^2. Because all locations in the study had some seeded plants already established, imazapic was not used in this study as originally planned. Bacteria were applied to eleven locations at 7 sites. Three locations were roadsides on US-95, six locations were on I-84 and two locations were on I-86.

The bacteria reduced downy brome (cheatgrass) by 25 to 97percent in the short time period (1 to 3 yr) of this study, but this reduction varied with year. In the spring of 2017, above average spring precipitation resulted in a flush of downy brome (cheatgrass) growth, which reduced the percent inhibition of downy brome (cheatgrass) in the bacteria plots compared to the control. Although medusahead patches were evident in the control plots, medusahead was not found in the bacterial plots after bacterial application. As these plots are monitored in the next several years, more inhibition is expected in those locations with desirable plant populations. A two- or three-year monitoring period is not sufficient to illustrate the bacteria's ability to reduce downy brome (cheatgrass) when normal time period for bacterial suppression is from 5 to 7 years after application.

The Best Management Practice for achieving greatest success in using weed-suppressive bacteria on ITD roadsides consists of the following six points:

1. Select a site with a healthy, dense stand of desirable plant species.
2. Select a site with moderate to low infestation of downy brome (cheatgrass) or medusahead.
3. Apply the bacteria to the site in late fall, when air and soil temperatures are below 50^oF and rain is forecast.
4. Apply 1 gallon of actively growing WSB to each acre.
5. Apply a broadleaf herbicide in the spring as needed.
6. The bacteria take several years to reduce downy brome (cheatgrass) or medusahead populations.

Key findings and recommendations from the study are as follows:

- The timing of WSB application is critical to the successful, long-term reduction and exclusion of downy brome (cheatgrass), medusahead, and jointed goatgrass. Late fall applications, when air and soil temperatures are cool (below 50^{o}F), rains are prevalent and skies are overcast, have the highest success.

- The 1 gallon of actively growing WSB per acre or the equivalent of 5×10^{12} colony forming units per acre are the best application rate to date. The bacteria were able to survive and flourish and effectively inhibit the downy brome (cheatgrass) or medusahead as quickly as the next fall. That inhibition continued into the next year. The bacteria can be sprayed on the soil surface or applied to the seed to deliver the same amount of bacterial populations to the soil.

- The success of the WSB relies on the interaction between the weed, the bacteria, and desirable plants (native or beneficial species). The greatest success with long-term reductions in downy brome (cheatgrass), medusahead, and jointed goatgrass has been on lands with mixed populations of native plants and moderate weed infestations.

- As WSB reduce downy brome (cheatgrass), medusahead, and jointed goatgrass, voids are created that broadleaf weeds fill. Since WSB does not inhibit broadleaf plants, the site must be monitored carefully. In many cases, the use of a broadleaf herbicide is necessary in the spring of each year to control the advancement of broadleaf weeds due to the reduction of downy brome (cheatgrass) infestations.

These bacteria provide a novel means to reduce invasive weeds while limiting the need for tillage and chemical use for weed control. Because of its selectivity, this bacterium can be used in management of the invasive weeds downy brome (cheatgrass) and medusahead on ITD roadsides. Long-term management of ITD lands will benefit from the inclusion of the bacteria into management programs along with drill seeding and establishment of desirable plants.

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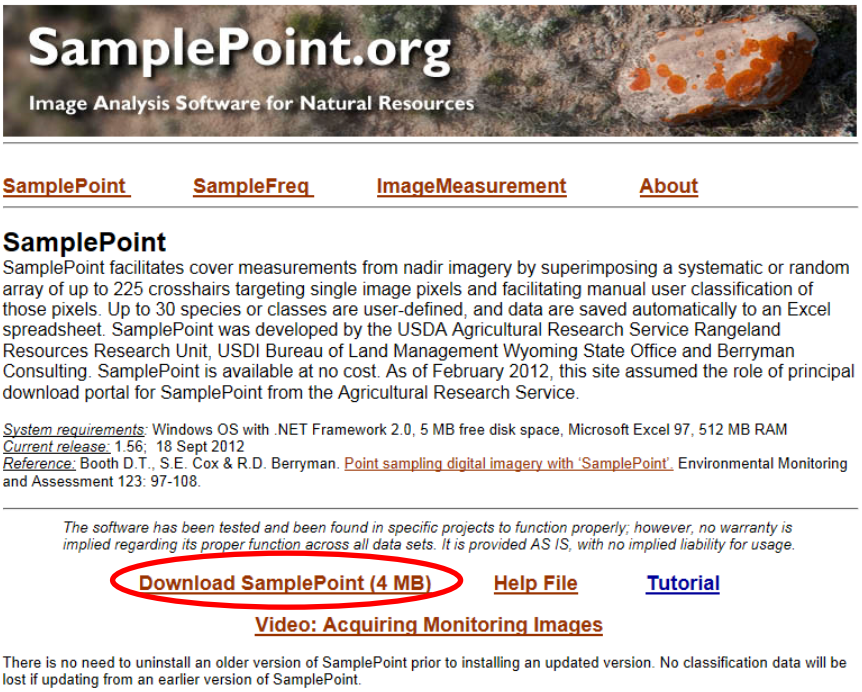
Appendix A
SamplePoint Standard Operating Procedures

Compiled by Jacob Mutch, Eugene Winkler and Dr. Ann Kennedy

SamplePoint facilitates point-sampling of digital images. SamplePoint identifies objects within the parameter of a picture and calculates data that is representative of a population. It can be used for various forms of data collection, including counting cheatgrass and other plant species in a research plot with multiple bacteria treatments. The data produced through SamplePoint can determine the effectiveness of Battalion Pro bacteria at a specific research site.

1. To download SamplePoint onto a computer, go to www.samplepoint.org. This will take you to the SamplePoint homepage (shown below). On the homepage, select Download SamplePoint (4 MB).



2. Once the above link has been selected, a box will appear at the bottom of the screen (shown below). In this box, click the arrow next to the "save" icon and scroll down to "save and run." Selecting this option will install the program to the computer. Once downloaded, SamplePoint should appear in the Start menu and can be opened from there.

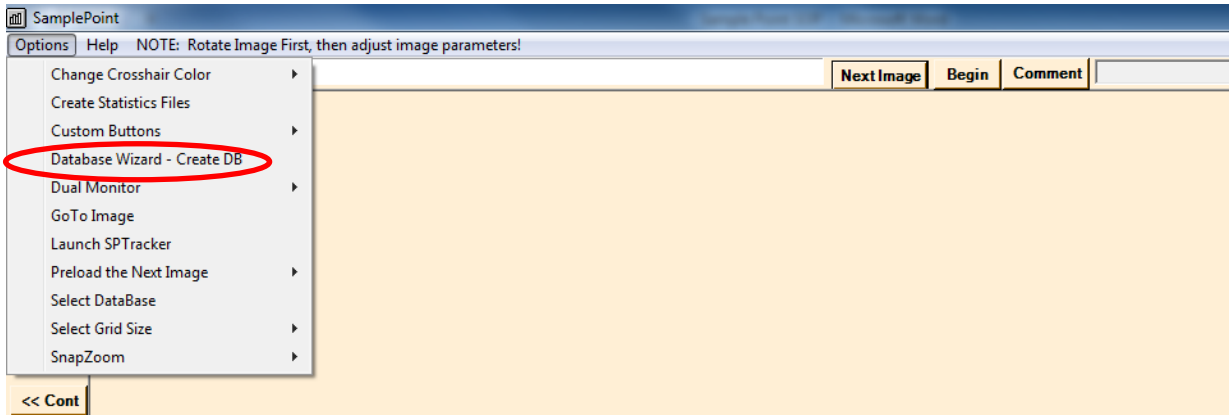
3. Prior to using SamplePoint to identify data points, all pictures must be saved with a specific name that can identify that image without viewing it.

4. Before uploading images to a database within SamplePoint, images should be cropped so that only the desired sample area is shown. For accurate data collection, the entire sample area must be shown. Leaving a small portion of the perimeter will not impact data collection as SamplePoint does not sample the outside edges of images. An example is given below. This can be done using Microsoft Paint or any photo editing program.

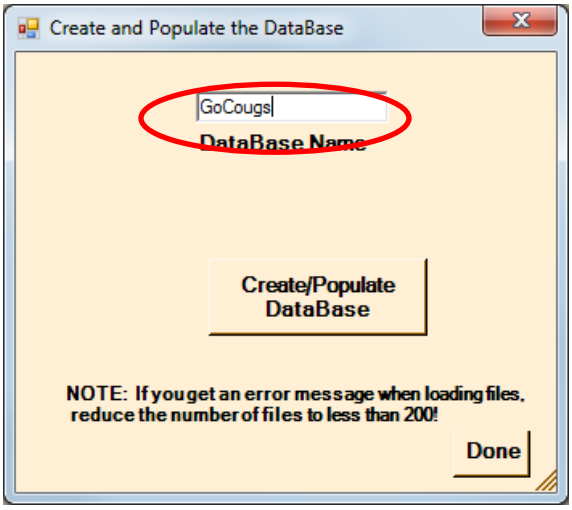
5. To improve image quality, files may be saved in BMP or TIFF format. The software recommends using either the BMP or TIFF format and these may be selected from the "Save as" menu after cropping in Microsoft paint. JPEG images seem to work as well if the image has a high enough resolution.

6. Once all images have been cropped and saved with an appropriate identifying name, a database may be created within SamplePoint to begin data collection. To create a database, open SamplePoint by selecting the SamplePoint icon in the Start menu. If using repeatedly, the icon can be dragged from the start menu to the taskbar for convenience.

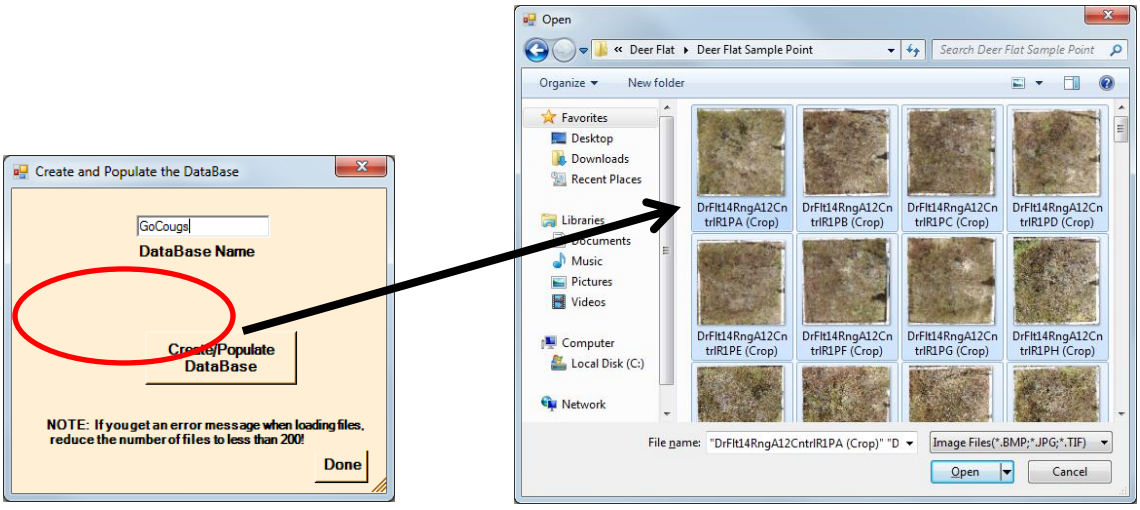
   Once SamplePoint has been opened, select the "Options" icon in the top left corner. From the options menu, select "Database Wizard – Create DB" (shown below).



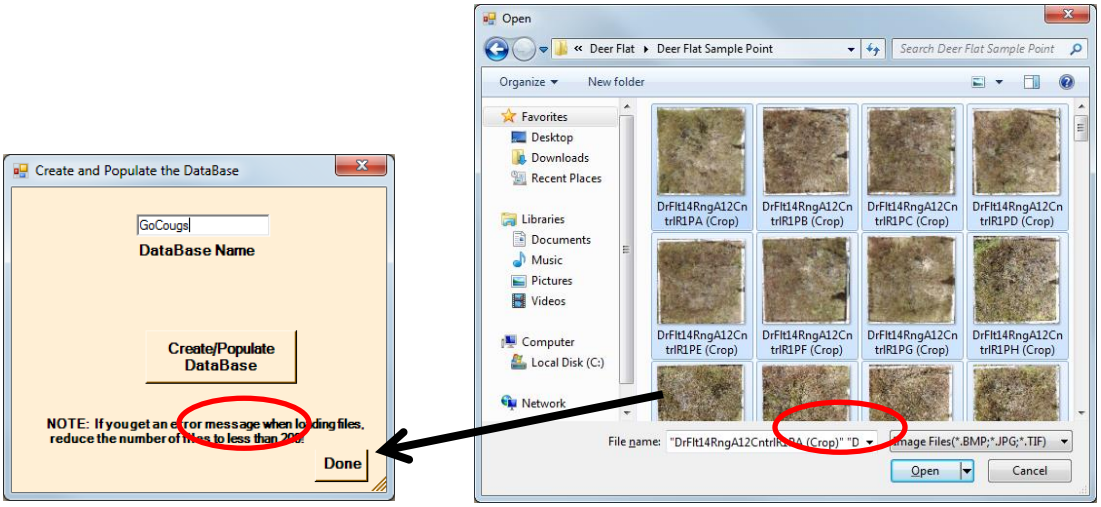
7. Selecting Database Wizard will open an additional window in the center of the SamplePoint screen titled "Create and Populate the Database." This window allows the database to be given an appropriate title that will identify it from other databases and to be populated with pictures. Before uploading pictures to the database, enter a title for the database in the box titled DataBase Name.



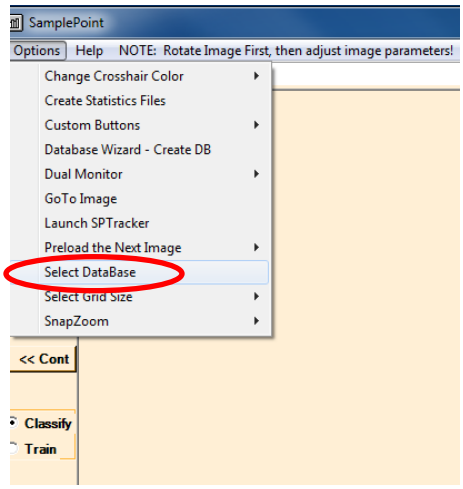
8. Once the database has been titled, it may be populated with images. To add images, select the "Create/Populate DataBase" icon. After selecting this icon, another window will appear from which images may be selected and added to the database. Up to 200 images may be loaded to one database. If the desired data set is more than 200 pictures, multiple databases may be created to analyze the data set.

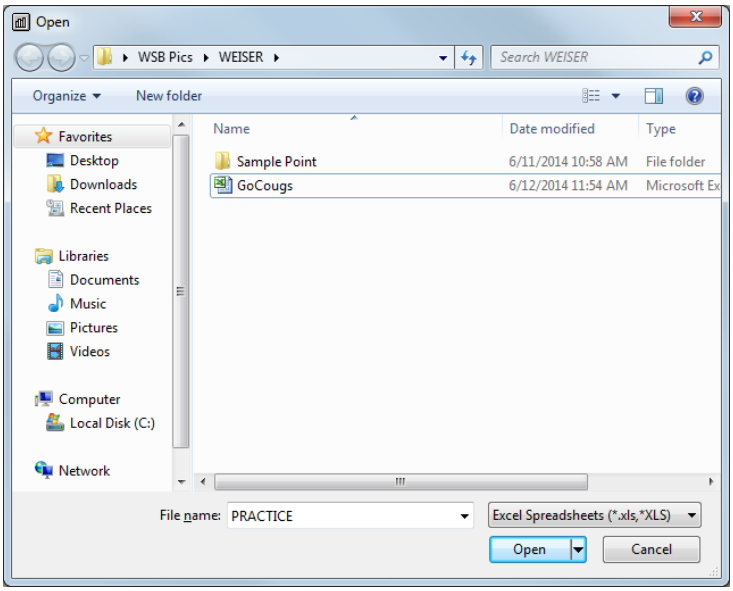


9. Once desired images have been selected, click “Open” to add images to the database. After all images have been added, clicking the “Done” icon will finalize the database and add selected images.

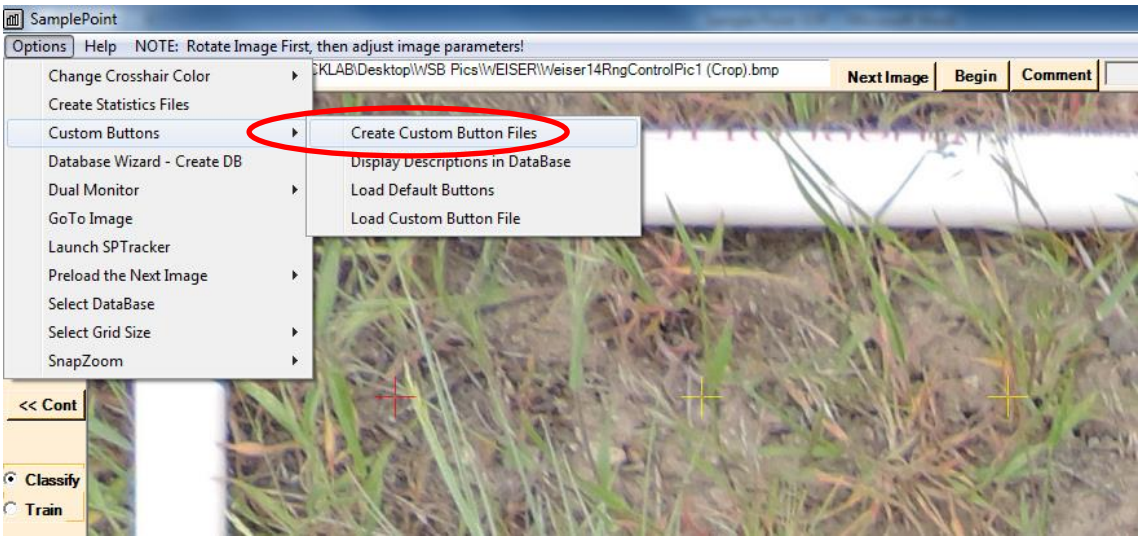


10. To begin analyzing images in the database, select the options menu in the top left corner. Scroll down to the “Select DataBase” icon to open the database and begin data collection. Clicking “Select DataBase” will open a window from which the database may be selected. The database will appear as an Excel file with the name given above. Select the appropriate file and then click “Open” to begin data analysis.

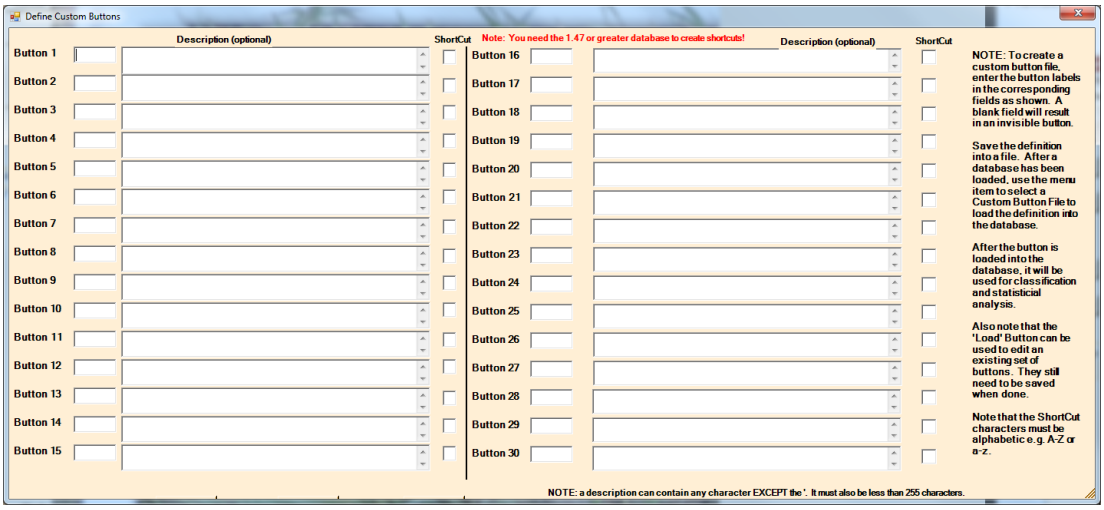




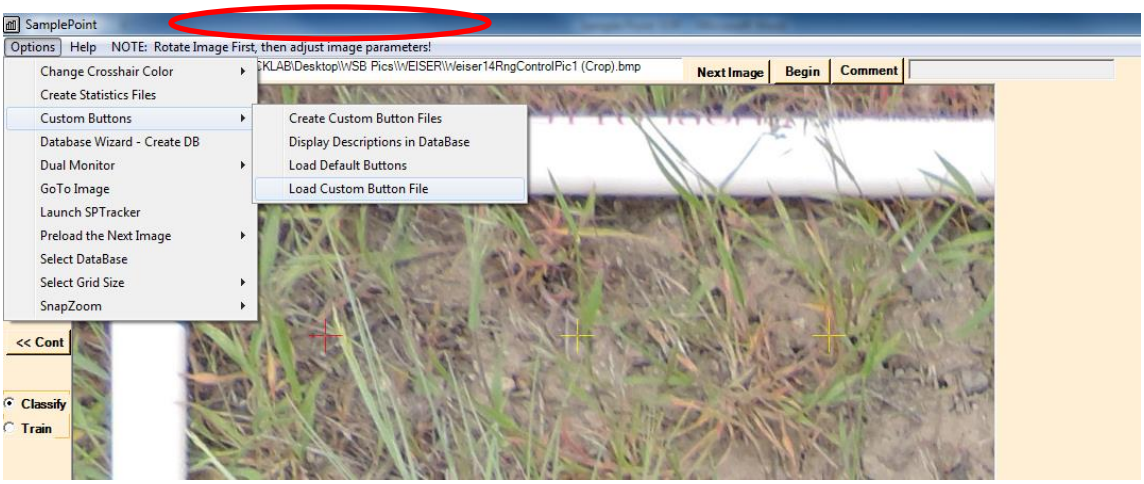
11. To identify each data point, use the buttons at the bottom of the screen. The default buttons are shown, but can be changed to fit each data set. You can load existing custom button files that coincide with your data. If no file fits, you can create your own. To change the button options, select “Custom Buttons” and “Create Custom Button Files” from the options menu.



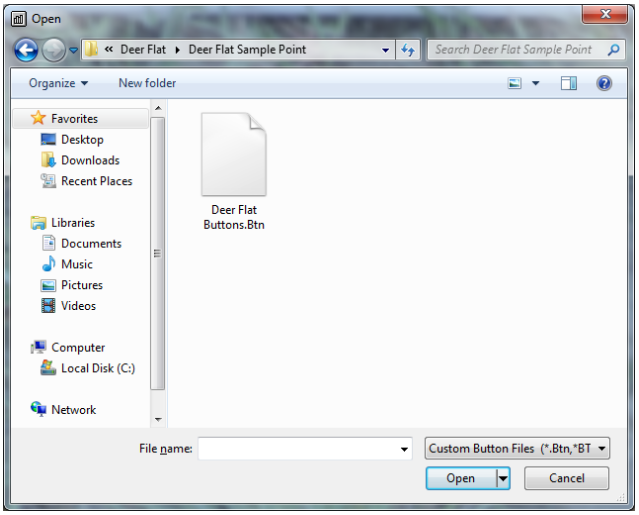
This will open an additional window in which up to 30 buttons may be created to fit the current data. After creating the desired buttons, click the “save” icon to save the buttons to a file within the computer. Keyboard shortcuts and descriptions of each button may also be added to the buttons file if desired.



12. The custom buttons file may be uploaded and used to analyze data after it has been saved. To load the buttons file, again scroll to custom buttons under the start menu, this time selecting "Load Custom Button File."



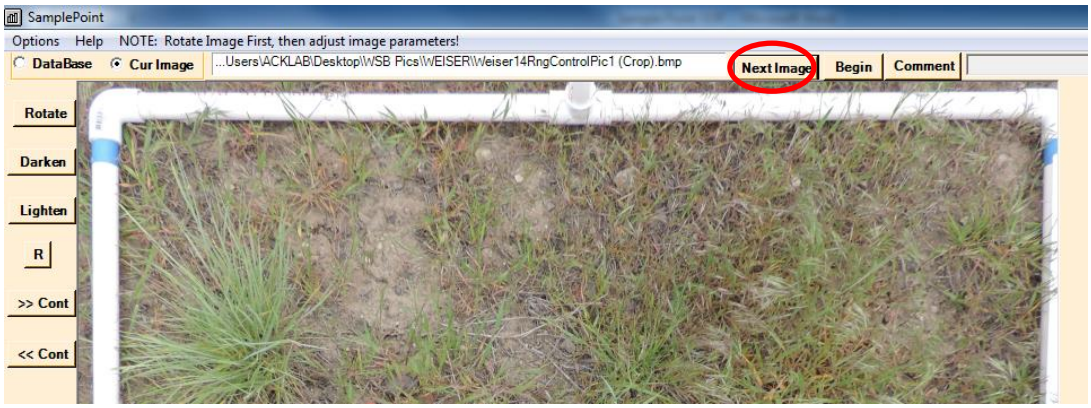
This will open a window from which the custom buttons file may be accessed and uploaded for use. Select the desired buttons file (new or existing) and click "Open" to begin using the custom buttons.



13. The default setting is 100 data points per image. This may be adjusted by choosing “Select Grid Size” under the options menu.

For our procedure choose “Random Points” and then input 25.

14. Once the database has been selected, the first image of that file will open. To begin analyzing the data, click “begin.”

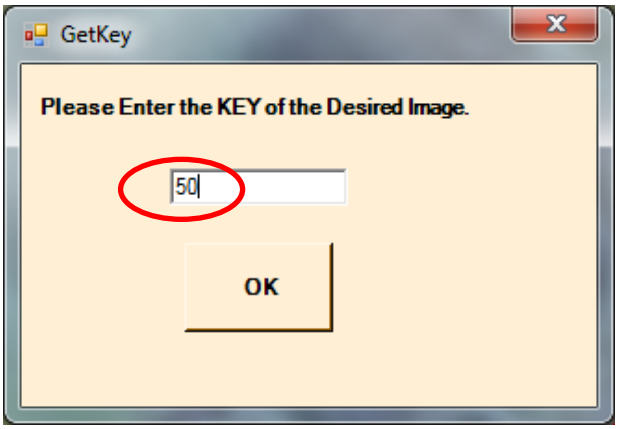


This will open the image with multiple crosshairs. The red crosshair is the current data point to be analyzed. The yellow crosshairs will be analyzed later. To adjust the zoom, use the up and down arrows or scroll up and down with the mouse.

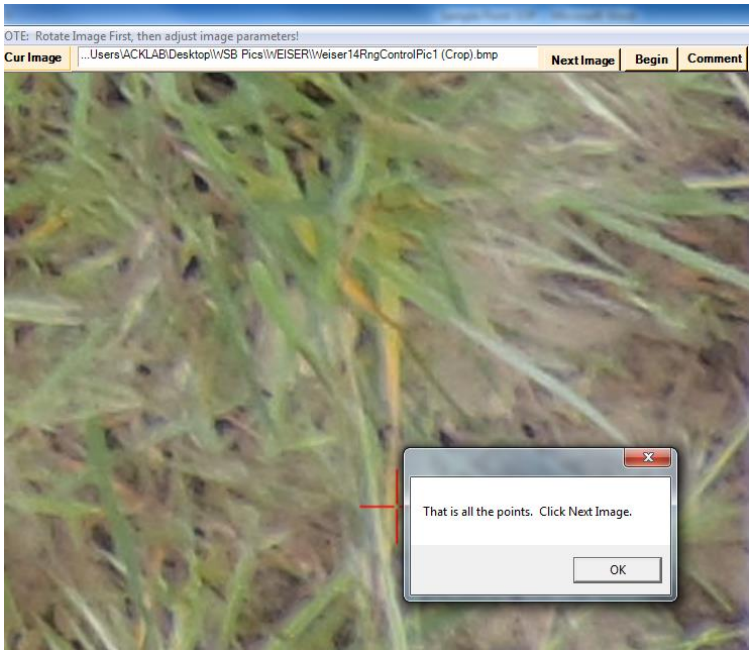
15. After creating and loading appropriate buttons for the data set, data analysis may begin. To properly identify a point in the picture, zoom in so objects can be easily identified. Ideally, the object or plant species in the very middle of the cross hair will be identified.

Click on the appropriate button to identify the data point. This will save the data in the Excel worksheet which may be opened by viewing the original folder from which the images were uploaded, after you finish analyzing images.

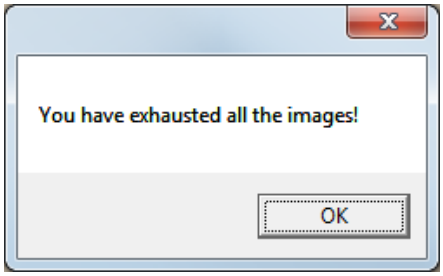
16. SamplePoint saves all data to an Excel database and there is no need to save completed work. SamplePoint may be closed at any time and reopened without losing any data. To resume at the latest data point, select your database and choose “Go to Image” under the options menu and type in the next image number that has not yet been completed.



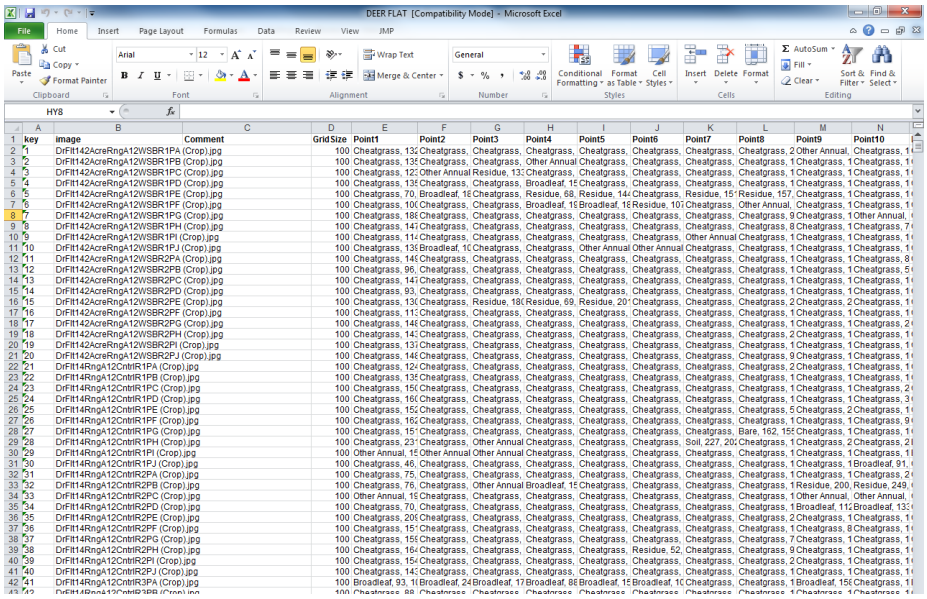
17. Continue analyzing specific data points until all images have been completed. After completing all points for one image, a notification will appear. At this point, click OK on the notification and select “Next Image” on the top right of the screen.



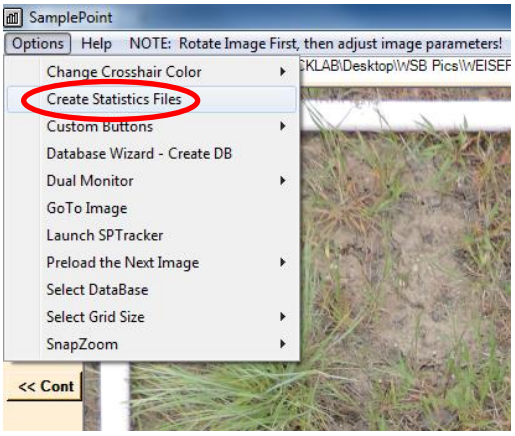
18. Continue this process until all points have been analyzed. After the last image in the data set has been analyzed, a notification will appear.



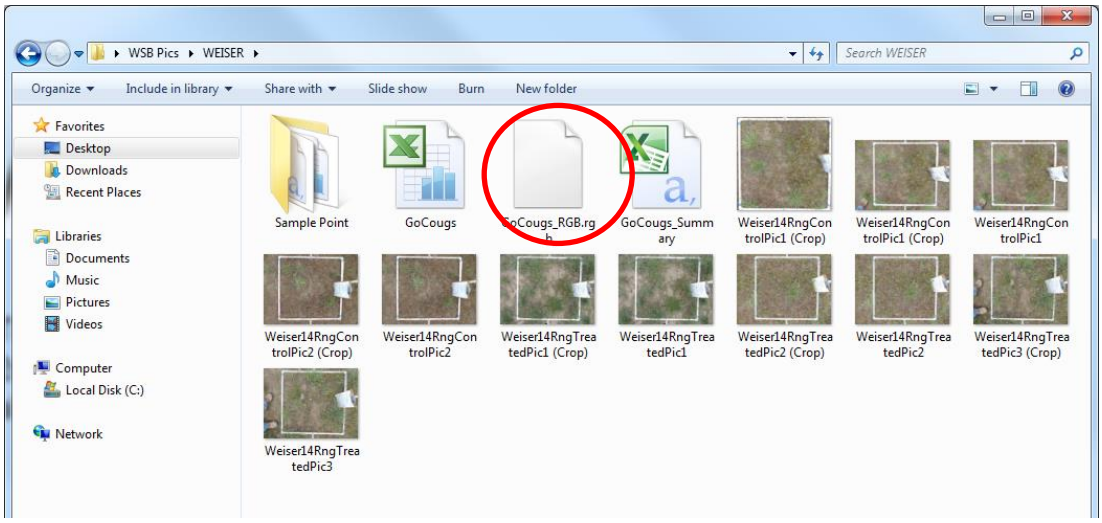
19. A complete data set may be viewed by opening the Excel worksheet in the original folder in which the images were located. This data set will include the identifications for each data point and every image within the database.



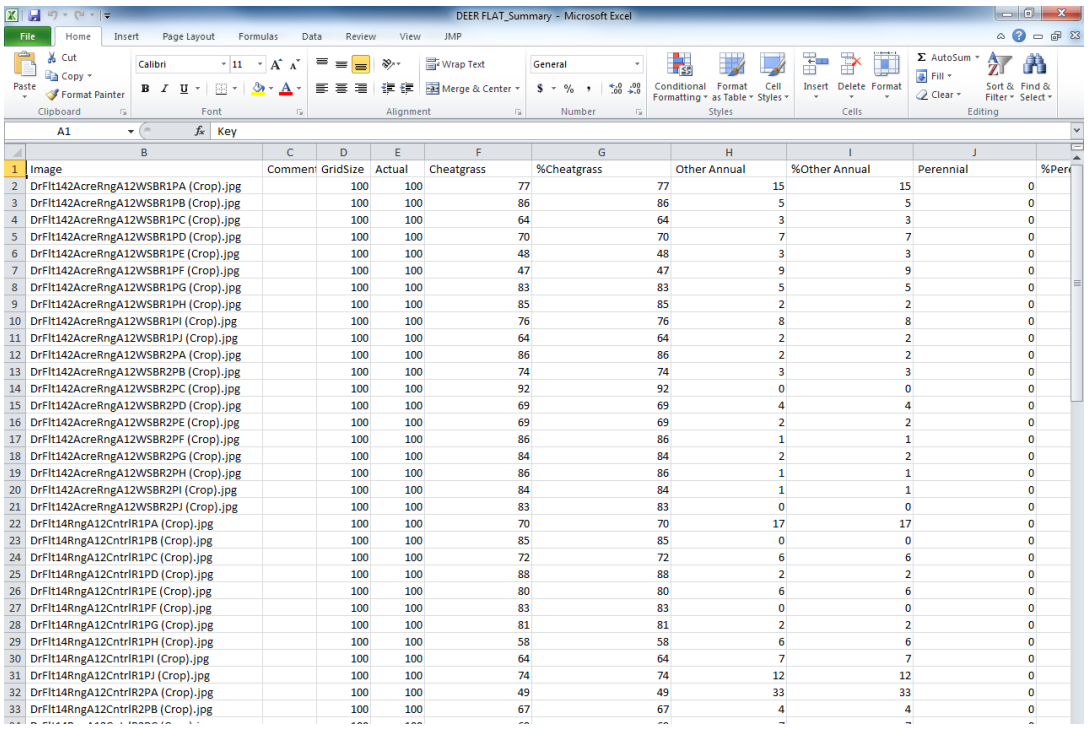
20. The database may be converted to an Excel file that includes statistics and percentages for each image, calculated from the identifications made at each data point. To generate this file, select "Create Statistics Files" from the options menu. If you have closed SamplePoint, the database must be reloaded prior to creating this file. It may take a couple of minutes to generate a statistics file, depending on the size of the database.



21. Once the statistics file has completed, it may be accessed in the same folder as the original images and the previous excel document with all identified data points. The statistics file will have the same title as the database, followed by "_summary."



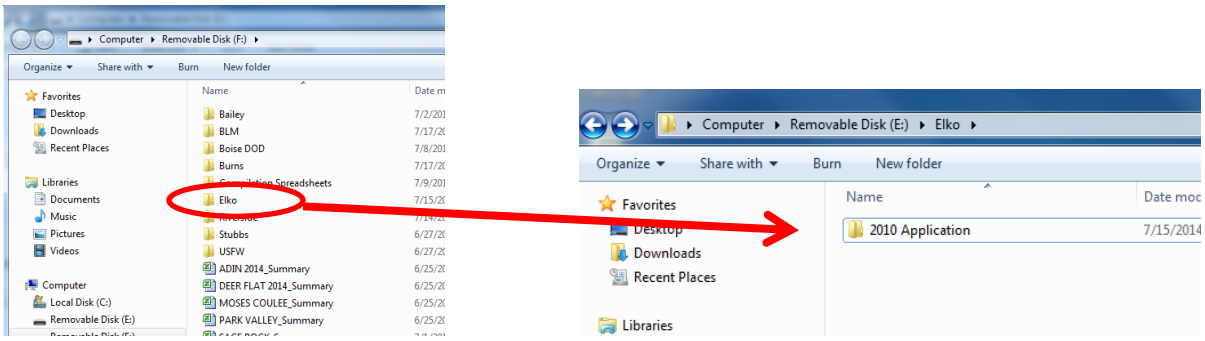
This document will contain the number of each type of data point and percentages for all images, as shown below.



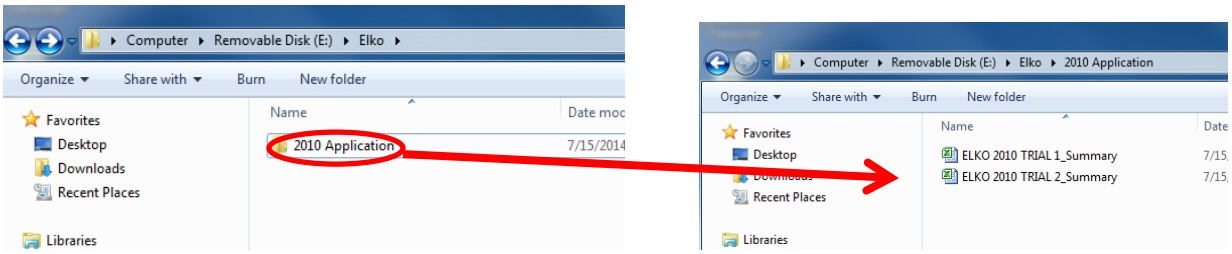
	B	C	D	E	F	G	H	I	J	
1	Image	Comment	GridSize	Actual	Cheatgrass	%Cheatgrass	Other Annual	%Other Annual	Perennial	%Per
2	DrFlt142AcreRngA12WSBR1PA (Crop).jpg		100	100	77	77	15	15	0	
3	DrFlt142AcreRngA12WSBR1PB (Crop).jpg		100	100	86	86	5	5	0	
4	DrFlt142AcreRngA12WSBR1PC (Crop).jpg		100	100	64	64	3	3	0	
5	DrFlt142AcreRngA12WSBR1PD (Crop).jpg		100	100	70	70	7	7	0	
6	DrFlt142AcreRngA12WSBR1PE (Crop).jpg		100	100	48	48	3	3	0	
7	DrFlt142AcreRngA12WSBR1PF (Crop).jpg		100	100	47	47	9	9	0	
8	DrFlt142AcreRngA12WSBR1PG (Crop).jpg		100	100	83	83	5	5	0	
9	DrFlt142AcreRngA12WSBR1PH (Crop).jpg		100	100	85	85	2	2	0	
10	DrFlt142AcreRngA12WSBR1PI (Crop).jpg		100	100	76	76	8	8	0	
11	DrFlt142AcreRngA12WSBR1PJ (Crop).jpg		100	100	64	64	2	2	0	
12	DrFlt142AcreRngA12WSBR2PA (Crop).jpg		100	100	86	86	2	2	0	
13	DrFlt142AcreRngA12WSBR2PB (Crop).jpg		100	100	74	74	3	3	0	
14	DrFlt142AcreRngA12WSBR2PC (Crop).jpg		100	100	92	92	0	0	0	
15	DrFlt142AcreRngA12WSBR2PD (Crop).jpg		100	100	69	69	4	4	0	
16	DrFlt142AcreRngA12WSBR2PE (Crop).jpg		100	100	69	69	2	2	0	
17	DrFlt142AcreRngA12WSBR2PF (Crop).jpg		100	100	86	86	1	1	0	
18	DrFlt142AcreRngA12WSBR2PG (Crop).jpg		100	100	84	84	2	2	0	
19	DrFlt142AcreRngA12WSBR2PH (Crop).jpg		100	100	86	86	1	1	0	
20	DrFlt142AcreRngA12WSBR2PI (Crop).jpg		100	100	84	84	1	1	0	
21	DrFlt142AcreRngA12WSBR2PJ (Crop).jpg		100	100	83	83	0	0	0	
22	DrFlt14RngA12CntrlR1PA (Crop).jpg		100	100	70	70	17	17	0	
23	DrFlt14RngA12CntrlR1PB (Crop).jpg		100	100	85	85	0	0	0	
24	DrFlt14RngA12CntrlR1PC (Crop).jpg		100	100	72	72	6	6	0	
25	DrFlt14RngA12CntrlR1PD (Crop).jpg		100	100	88	88	2	2	0	
26	DrFlt14RngA12CntrlR1PE (Crop).jpg		100	100	80	80	6	6	0	
27	DrFlt14RngA12CntrlR1PF (Crop).jpg		100	100	83	83	0	0	0	
28	DrFlt14RngA12CntrlR1PG (Crop).jpg		100	100	81	81	2	2	0	
29	DrFlt14RngA12CntrlR1PH (Crop).jpg		100	100	58	58	6	6	0	
30	DrFlt14RngA12CntrlR1PI (Crop).jpg		100	100	64	64	7	7	0	
31	DrFlt14RngA12CntrlR1PJ (Crop).jpg		100	100	74	74	12	12	0	
32	DrFlt14RngA12CntrlR2PA (Crop).jpg		100	100	49	49	33	33	0	
33	DrFlt14RngA12CntrlR2PB (Crop).jpg		100	100	67	67	4	4	0	

When you have finished you should have two statistics files for each location. Each one represents one trial of 25 random points.

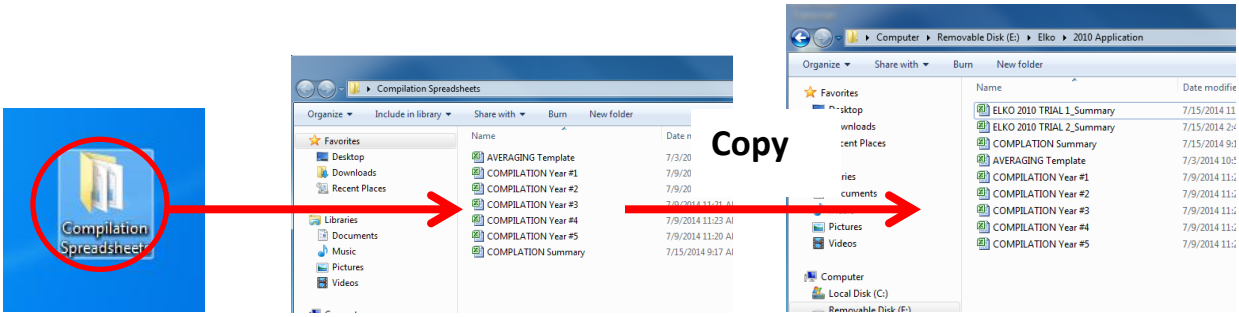
1. Inside the location folder create two folders for the different application (of the treatment) years. If you only have data for one, create one now and another when you have data for that application.



2. Copy the two summary files into this new folder.

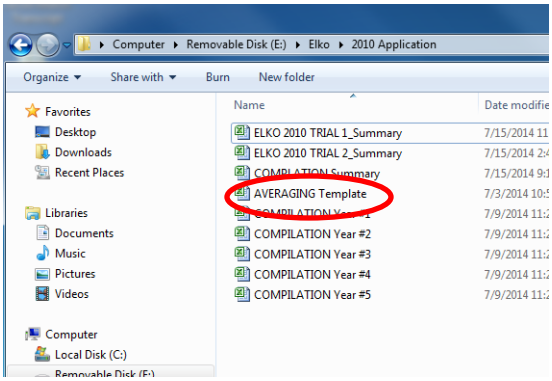


3. Copy the Files within the folder “Compilation Spreadsheets” to your new folder also.

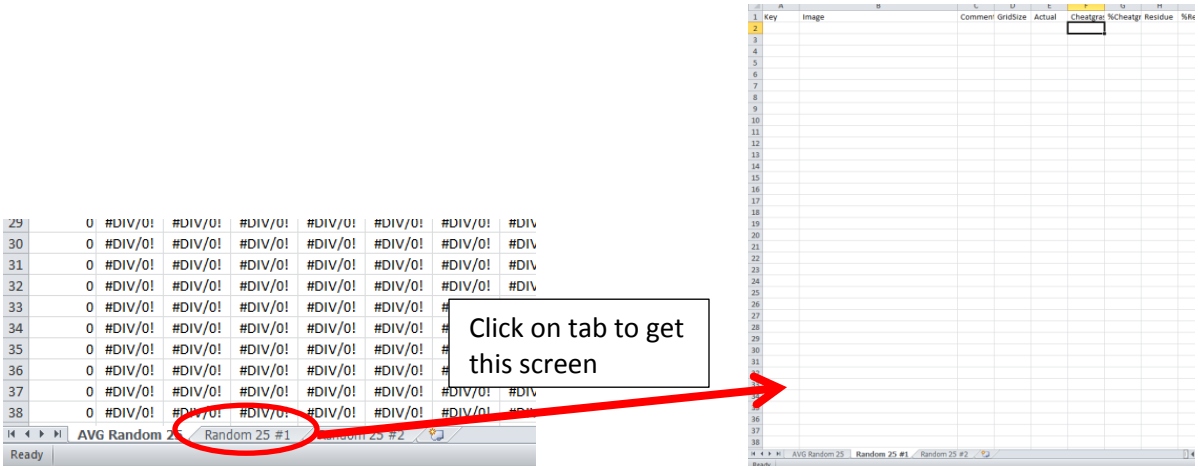


4. Do NOT change the names of the files from the template.

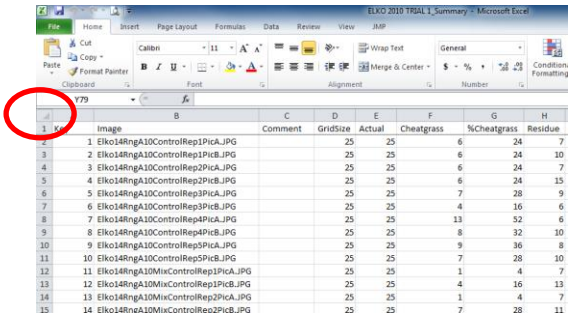
5. Open the "AVERAGING Template" file within new folder.

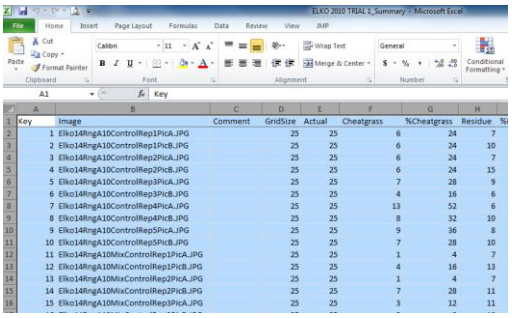


6. You will notice that there are three tabs for different worksheets at the bottom. Click on the tab titled "Random 25 #1".



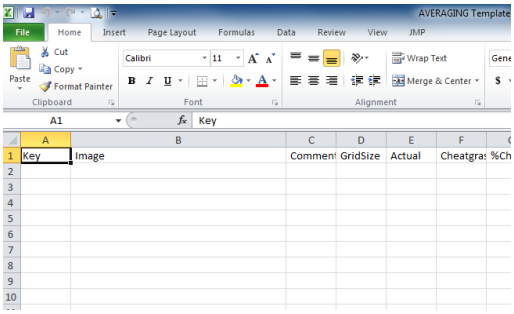
7. Open one of the Summary file from Sample Point.
8. Click on the arrow at the upper left hand corner to select the entire sheet.

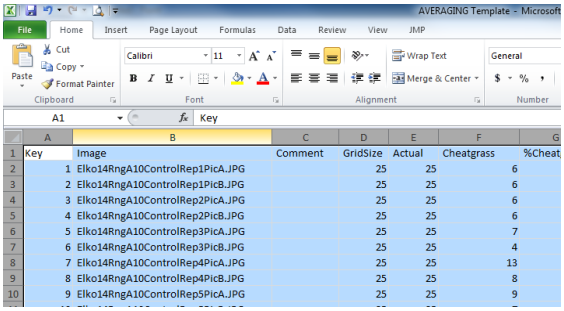




9. Click on copy
10. Go back to the "AVERAGING Template" and click on cell A1 at the top left of the sheet and paste.

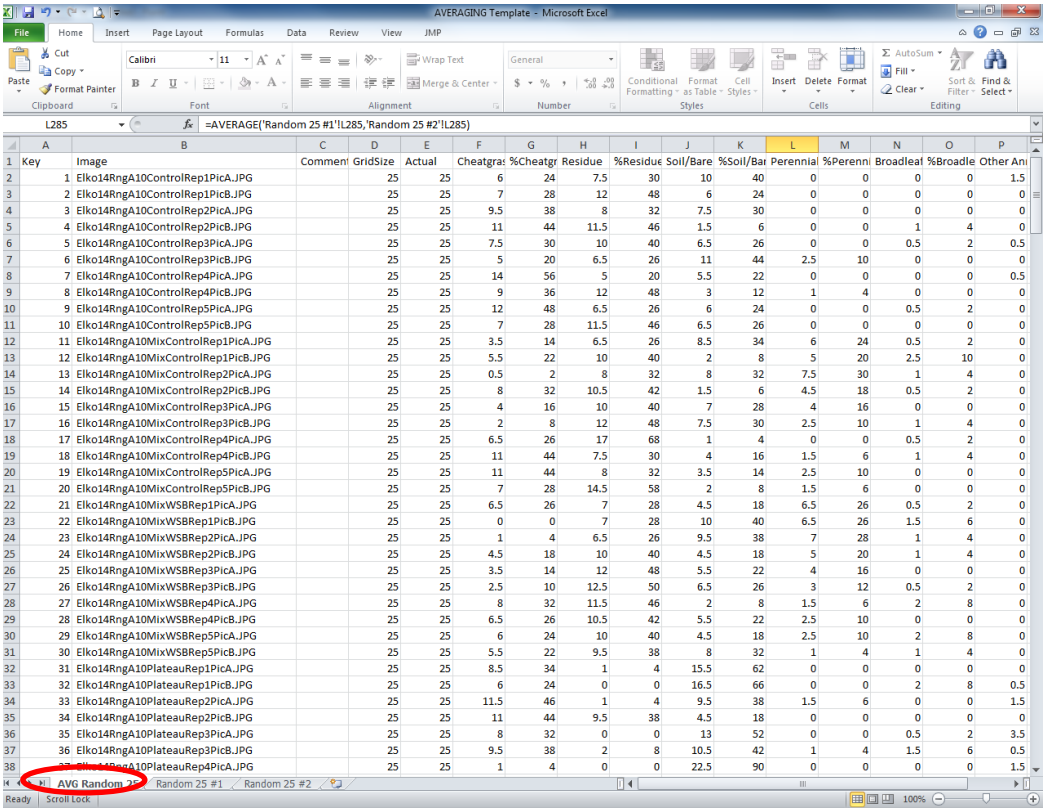
11. All the data from the first trial should be visible now in the AVERAGING Template.





12. Repeat the process with the second Sample Point Summary file except you will paste it into the "Random 25 #2" tab at the bottom of the "AVERAGING Template" file.

13. Now click on the "AVG Random 25" tab at the bottom to open this sheet.



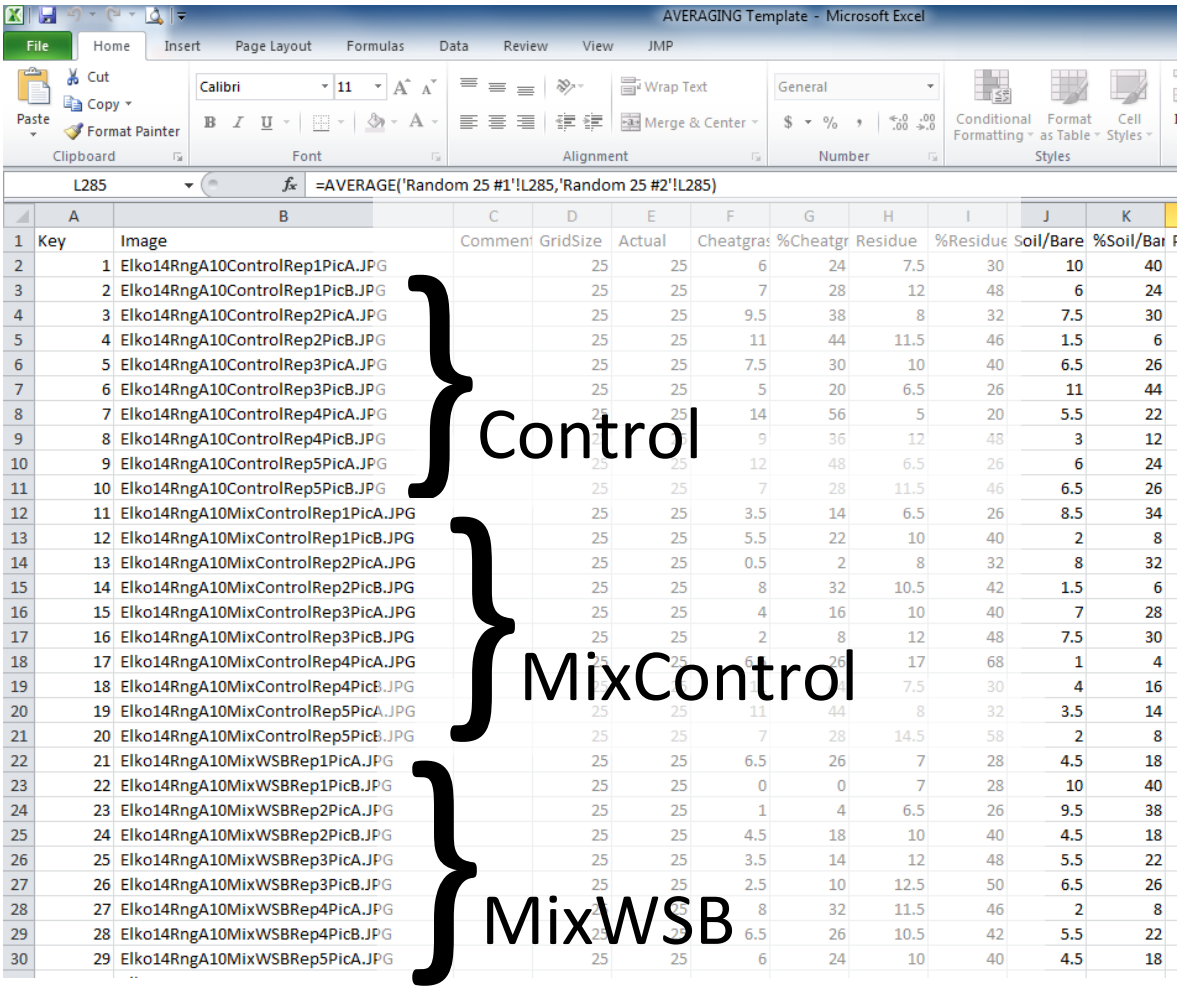
You should see a screen like this. As a check you will now see a combination of whole numbers and decimals (#.5) indicating the average has been taken.

14. You should have all of your data averaged from the two trials on this worksheet of the file.

15. Leave the AVERAGING Template file open on the "AVG Random 25" sheet. Be careful you are not on one of the other tabs for individual trials

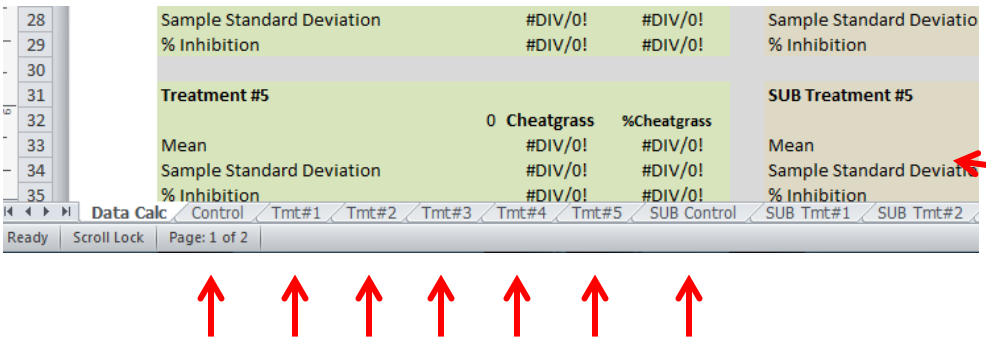
16. Save this worksheet but leave this file open for the next step

1. Within the AVERAGING Template file you will need to identify the different types of plots by the names of the pictures. Some will be control plots, burn plots, wsbspray, etc.



	A	B	C	D	E	F	G	H	I	J	K
1	Key	Image	Comment	GridSize	Actual	Cheatgras	%Cheatgr	Residue	%Residue	Soil/Bare	%Soil/Bar
2	1	Elko14RngA10ControlRep1PicA.JPG		25	25	6	24	7.5	30	10	40
3	2	Elko14RngA10ControlRep1PicB.JPG		25	25	7	28	12	48	6	24
4	3	Elko14RngA10ControlRep2PicA.JPG		25	25	9.5	38	8	32	7.5	30
5	4	Elko14RngA10ControlRep2PicB.JPG		25	25	11	44	11.5	46	1.5	6
6	5	Elko14RngA10ControlRep3PicA.JPG		25	25	7.5	30	10	40	6.5	26
7	6	Elko14RngA10ControlRep3PicB.JPG		25	25	5	20	6.5	26	11	44
8	7	Elko14RngA10ControlRep4PicA.JPG		25	25	14	56	5	20	5.5	22
9	8	Elko14RngA10ControlRep4PicB.JPG		25	25	9	36	12	48	3	12
10	9	Elko14RngA10ControlRep5PicA.JPG		25	25	12	48	6.5	26	6	24
11	10	Elko14RngA10ControlRep5PicB.JPG		25	25	7	28	11.5	46	6.5	26
12	11	Elko14RngA10MixControlRep1PicA.JPG		25	25	3.5	14	6.5	26	8.5	34
13	12	Elko14RngA10MixControlRep1PicB.JPG		25	25	5.5	22	10	40	2	8
14	13	Elko14RngA10MixControlRep2PicA.JPG		25	25	0.5	2	8	32	8	32
15	14	Elko14RngA10MixControlRep2PicB.JPG		25	25	8	32	10.5	42	1.5	6
16	15	Elko14RngA10MixControlRep3PicA.JPG		25	25	4	16	10	40	7	28
17	16	Elko14RngA10MixControlRep3PicB.JPG		25	25	2	8	12	48	7.5	30
18	17	Elko14RngA10MixControlRep4PicA.JPG		25	25	6	26	17	68	1	4
19	18	Elko14RngA10MixControlRep4PicB.JPG		25	25	10	44	7.5	30	4	16
20	19	Elko14RngA10MixControlRep5PicA.JPG		25	25	11	44	8	32	3.5	14
21	20	Elko14RngA10MixControlRep5PicB.JPG		25	25	7	28	14.5	58	2	8
22	21	Elko14RngA10MixWSBRep1PicA.JPG		25	25	6.5	26	7	28	4.5	18
23	22	Elko14RngA10MixWSBRep1PicB.JPG		25	25	0	0	7	28	10	40
24	23	Elko14RngA10MixWSBRep2PicA.JPG		25	25	1	4	6.5	26	9.5	38
25	24	Elko14RngA10MixWSBRep2PicB.JPG		25	25	4.5	18	10	40	4.5	18
26	25	Elko14RngA10MixWSBRep3PicA.JPG		25	25	3.5	14	12	48	5.5	22
27	26	Elko14RngA10MixWSBRep3PicB.JPG		25	25	2.5	10	12.5	50	6.5	26
28	27	Elko14RngA10MixWSBRep4PicA.JPG		25	25	8	32	11.5	46	2	8
29	28	Elko14RngA10MixWSBRep4PicB.JPG		25	25	6.5	26	10.5	42	5.5	22
30	29	Elko14RngA10MixWSBRep5PicA.JPG		25	25	6	24	10	40	4.5	18

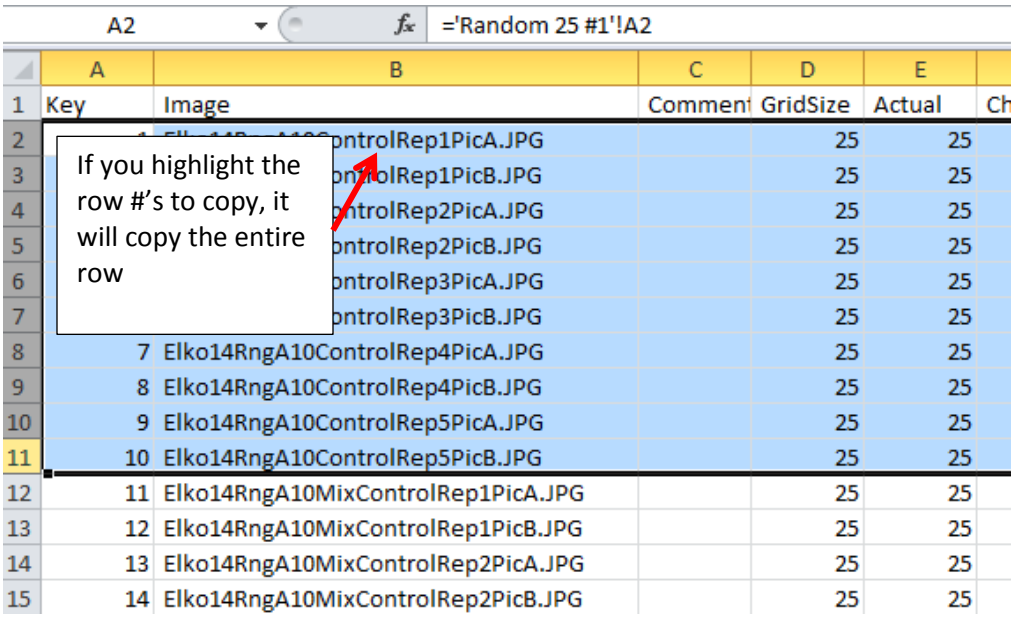
2. Open the file "COMPILATION Year #1" and notice the tabs at the bottom. The first tab is a summary page that will automatically run calculations. The second tab is for the Control plot, and the others are for various other treatments used on that plot.



This is what the "**Data Calc"** Tab looks like. Do NOTHING to the Data Calc tab, it will calculate on its own

There is a tab for each treatment.

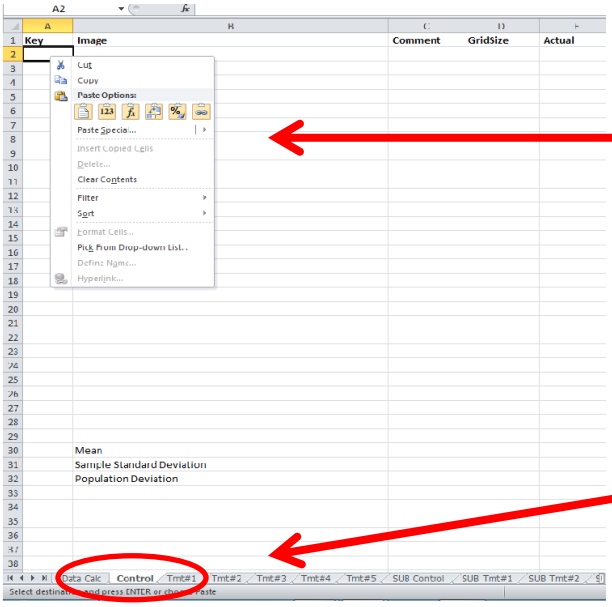
3. Inside the AVERAGING Template file, place the cursor over the "2" that labels the row number. Click and drag down to the end of the first plot type (eg. Control). You have now highlighted entire rows for that plot type.



	A	B	C	D	E
1	Key	Image	Comment	GridSize	Actual
2		ntrolRep1PicA.JPG		25	25
3		ntrolRep1PicB.JPG		25	25
4		ntrolRep2PicA.JPG		25	25
5		ntrolRep2PicB.JPG		25	25
6		ntrolRep3PicA.JPG		25	25
7		ntrolRep3PicB.JPG		25	25
8	7	Elko14RngA10ControlRep4PicA.JPG		25	25
9	8	Elko14RngA10ControlRep4PicB.JPG		25	25
10	9	Elko14RngA10ControlRep5PicA.JPG		25	25
11	10	Elko14RngA10ControlRep5PicB.JPG		25	25
12	11	Elko14RngA10MixControlRep1PicA.JPG		25	25
13	12	Elko14RngA10MixControlRep1PicB.JPG		25	25
14	13	Elko14RngA10MixControlRep2PicA.JPG		25	25
15	14	Elko14RngA10MixControlRep2PicB.JPG		25	25

4. Go to the "COMPILATION Year #1" file and click on the appropriate tab along the bottom. Likely the first set will be a control so then click on the control tab.

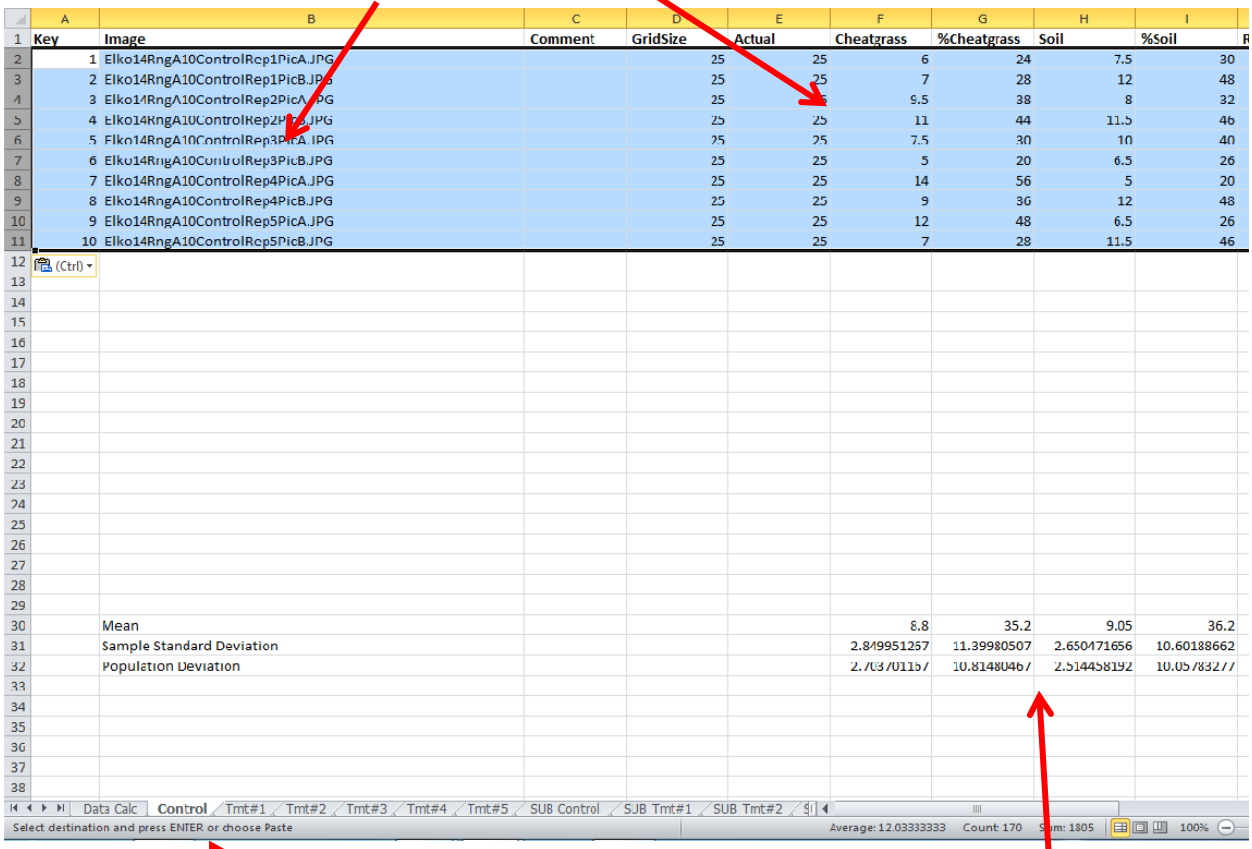
5. Click on cell A2 which is the first open cell on the sheet. RIGHT-click the mouse and choose "Paste Special", and then choose "Values". The information should appear in this sheet.



Make sure you are in cell A2, and choose Paste – Special - Values

Paste into "**Control**" Tab

This is a good place to check: Make sure the correct name and data is here. If you forget to "Paste Special", Excel may grab different data than you want as its default



Key	Image	Comment	GridSize	Actual	Cheatgrass	%Cheatgrass	Soil	%Soil
1	Elko14RngA10ControlRep1PicA.JPG		25	25	6	24	7.5	30
2	Elko14RngA10ControlRep1PicB.JPG		25	25	7	28	12	48
3	Elko14RngA10ControlRep2PicA.JPG		25	25	9.5	38	8	32
4	Elko14RngA10ControlRep2PicB.JPG		25	25	11	44	11.5	46
5	Elko14RngA10ControlRep3PicA.JPG		25	25	7.5	30	10	40
6	Elko14RngA10ControlRep3PicB.JPG		25	25	5	20	6.5	26
7	Elko14RngA10ControlRep4PicA.JPG		25	25	14	56	5	20
8	Elko14RngA10ControlRep4PicB.JPG		25	25	9	36	12	48
9	Elko14RngA10ControlRep5PicA.JPG		25	25	12	48	6.5	26
10	Elko14RngA10ControlRep5PicB.JPG		25	25	7	28	11.5	46
	Mean				8.8	35.2	9.05	36.2
	Sample Standard Deviation				2.849951257	11.39980507	2.650171656	10.60188662
	Population Deviation				2.703701157	10.8148046	2.514458192	10.05783277

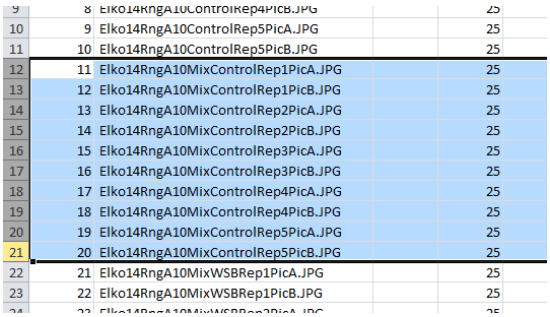
Make sure you paste into correct treatment

You can see automatic calculations for this treatment

At the bottom of the sheet you should see the spreadsheet displaying the averages and standard deviations for this treatment.

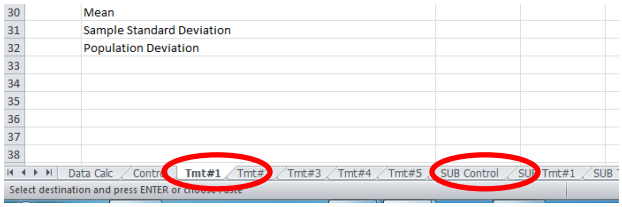
6. Repeat this process for the other treatments. Each separate treatment will go into its own sheet labeled as a tab at the bottom.

7. If you have a second "control" (eg. Some plots will have a burn control and a burn treated) you can paste it twice. Once into a treatment tab #1-5, and also into the "SUB Control" tab. Any treatments to compare against this will ALSO go into a treatment tab #6-10 to compare to the "SUB Control".



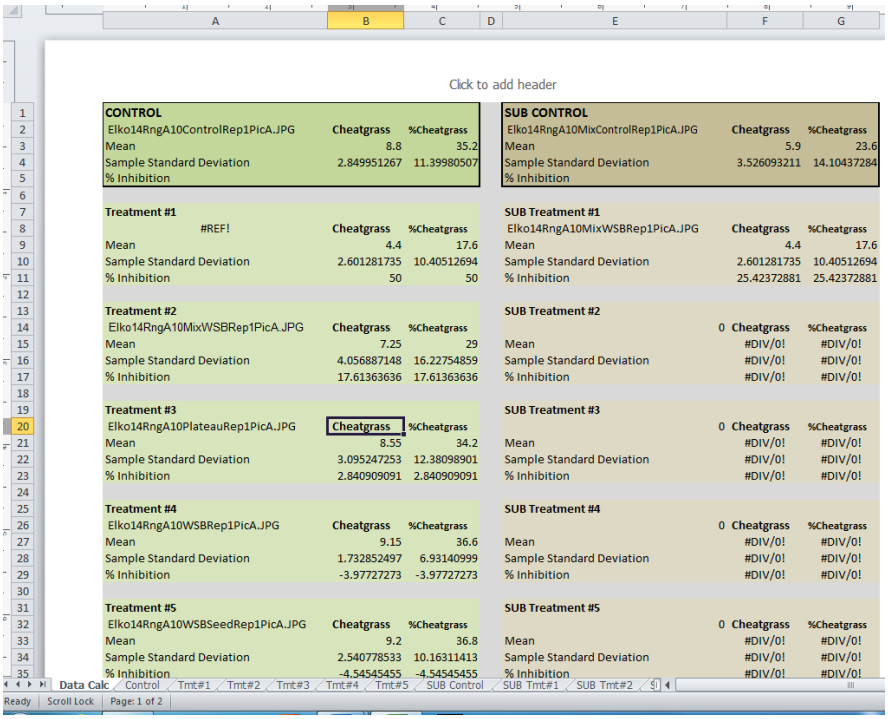
9	8	Elko14RngA10ControlRep4PicB.JPG 25
10	9	Elko14RngA10ControlRep5PicA.JPG 25
11	10	Elko14RngA10ControlRep5PicB.JPG 25
12	11	Elko14RngA10MixControlRep1PicA.JPG 25
13	12	Elko14RngA10MixControlRep1PicB.JPG 25
14	13	Elko14RngA10MixControlRep2PicA.JPG 25
15	14	Elko14RngA10MixControlRep2PicB.JPG 25
16	15	Elko14RngA10MixControlRep3PicA.JPG 25
17	16	Elko14RngA10MixControlRep3PicB.JPG 25
18	17	Elko14RngA10MixControlRep4PicA.JPG 25
19	18	Elko14RngA10MixControlRep4PicB.JPG 25
20	19	Elko14RngA10MixControlRep5PicA.JPG 25
21	20	Elko14RngA10MixControlRep5PicB.JPG 25
22	21	Elko14RngA10MixWSBRep1PicA.JPG 25
23	22	Elko14RngA10MixWSBRep1PicB.JPG 25

Example: The "MixControl" data wil copy into…



Both the "Tmt#1" tab AND the "SUB Control" tab

8. After you have pasted all treatments, click on the "DATA Calc" Tab to see the results for this year. There should be mean, standard deviation, and % inhibition calculations for each treatment and sub-treatments as compared to the controls.



Click to add header

CONTROL				**SUB CONTROL**		
Elko14RngA10ControlRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**		Elko14RngA10MixControlRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**
Mean	8.8	35.2		Mean	5.9	23.6
Sample Standard Deviation	2.849951267	11.39980507		Sample Standard Deviation	3.526093211	14.10437284
% Inhibition				% Inhibition		
Treatment #1				**SUB Treatment #1**		
#REF!	**Cheatgrass**	**%Cheatgrass**		Elko14RngA10MixWSBRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**
Mean	4.4	17.6		Mean	4.4	17.6
Sample Standard Deviation	2.601281735	10.40512694		Sample Standard Deviation	2.601281735	10.40512694
% Inhibition	50	50		% Inhibition	25.42372881	25.42372881
Treatment #2				**SUB Treatment #2**		
Elko14RngA10MixWSBRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**			0 **Cheatgrass**	**%Cheatgrass**
Mean	7.25	29		Mean	#DIV/0!	#DIV/0!
Sample Standard Deviation	4.056887148	16.22754859		Sample Standard Deviation	#DIV/0!	#DIV/0!
% Inhibition	17.61363636	17.61363636		% Inhibition	#DIV/0!	#DIV/0!
Treatment #3				**SUB Treatment #3**		
Elko14RngA10PlateauRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**			0 **Cheatgrass**	**%Cheatgrass**
Mean	8.55	34.2		Mean	#DIV/0!	#DIV/0!
Sample Standard Deviation	3.095247253	12.38098901		Sample Standard Deviation	#DIV/0!	#DIV/0!
% Inhibition	2.840909091	2.840909091		% Inhibition	#DIV/0!	#DIV/0!
Treatment #4				**SUB Treatment #4**		
Elko14RngA10WSBRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**			0 **Cheatgrass**	**%Cheatgrass**
Mean	9.15	36.6		Mean	#DIV/0!	#DIV/0!
Sample Standard Deviation	1.732852497	6.93140999		Sample Standard Deviation	#DIV/0!	#DIV/0!
% Inhibition	-3.97727273	-3.97727273		% Inhibition	#DIV/0!	#DIV/0!
Treatment #5				**SUB Treatment #5**		
Elko14RngA10WSBSeedRep1PicA.JPG	**Cheatgrass**	**%Cheatgrass**			0 **Cheatgrass**	**%Cheatgrass**
Mean	9.2	36.8		Mean	#DIV/0!	#DIV/0!
Sample Standard Deviation	2.540778533	10.16311413		Sample Standard Deviation	#DIV/0!	#DIV/0!
% Inhibition	-4.54545455	-4.54545455		% Inhibition	#DIV/0!	#DIV/0!

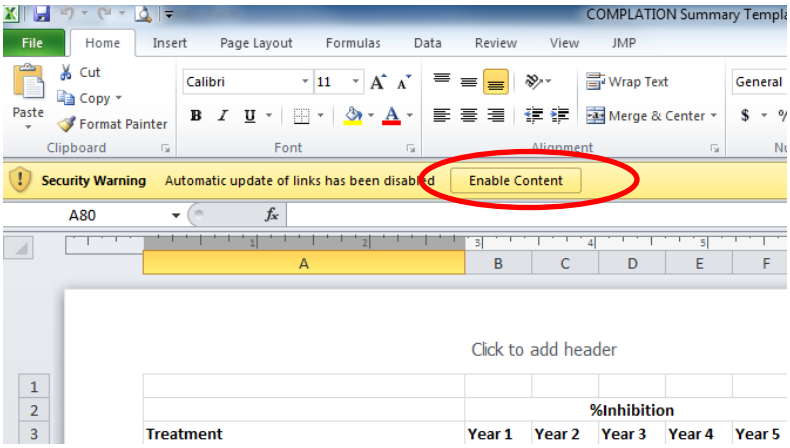
a. There will be a separate file for each year of data collection at each site.

b. Repeat the same process for each year except you will paste items into the Year #2 file.

c. Continue with as many years of data you need to enter.

d. DO NOT change the name of the "COMPILATION Year#1" type files. They are linked to the "COMPILATION Summary" file and will not link if you change the name before the link is made.

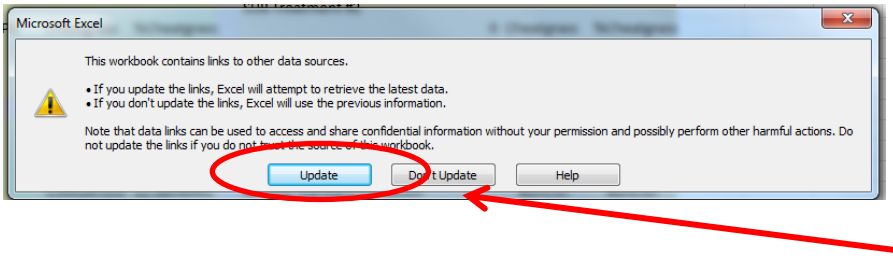
9. After you have finished transferring data to the COMPILATION "Year#1" file, save and close it. Then open the "COMPILATION Summary" file and enable the link. The calculated data should appear in the Year 1 tab. Save and close.



The first time the screen will look like this. Click on the "Enable Content"

10. After these data has been linked and saved you can change the name of "COMPILATION Year#1" to something that identifies it with the plot location.

11. When you add subsequent year's data into the "COMPILATION Year#__" file you should open the "COMPILATION Summary" to update it. Since it has already been linked once, the screen might look different.



After it has been linked once the screen will probably look like this. In this case click on the update button to complete the link.