Using Soil Seed Banks for Wetland Mitigation Planning: Comparison of Seed Bank Estimation Methods

Samuel M. Dutilly and Douglas A. DeBerry
Environment & Sustainability Program
College of William & Mary
Williamsburg, VA 23187

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EXECUTIVE SUMMARY

This report evaluates methods for estimating soil seed bank composition in the context of wetland mitigation planning. Soil seed banks influence plant community resilience and play a crucial role in the success of wetland creation and restoration projects. Accurately assessing seed bank composition before site disturbance can help promote desirable native species while mitigating the spread of invasive species, thereby decreasing long-term management costs. This is particularly relevant for wetland mitigation projects that are designed to take advantage of existing organic content in the surface soils by either leaving the topsoil intact, or removing it, stockpiling during construction, and re-spreading to establish final grades.

Three seed bank estimation methods were tested: 1) **in-house seed extraction**, 2) **greenhouse emergence**, and 3) **offsite laboratory seed extraction**. All methods used representative soil samples from a potential wetland mitigation site. The first method involved washing soil through a series of sieves to separate seeds from the non-seed soil fraction. The second method involved spreading soil samples evenly over a seed-free growing medium in the greenhouse to encourage seedling emergence, with seedlings being removed once mature enough to be identified to species. The third approach involved submitting samples to an offsite laboratory that specializes in seed identification from soil samples. The latter two methods (greenhouse and offsite lab) were then tested against artificially created soil seed bank controls that were pre-mixed from sterile soil and a known quantity and species composition of seeds.

The results revealed significant trade-offs in the effectiveness and efficacy of the methods used. The in-house seed extraction method identified the greatest number of seeds but was found to be time consuming, inaccurate, and difficult to execute. Therefore, after an initial pilot study using this approach, this technique was removed from further experimentation. Greenhouse emergence was found to underestimate seed diversity but provided more reliable species identification. The offsite laboratory extraction method yielded a higher number of seeds but was prone to identification inaccuracies. Individual samples in both approaches (greenhouse and offsite lab) were very different when compared to the true composition of the artificial seed mix

based on Jaccard's dissimilarity index, but the overall seed bank composition across all samples was estimated reasonably well by greenhouse trials. The offsite lab identified more seeds in the overall sample, but the lab's report contained several errors and omissions and displayed among the highest dissimilarity values in our study.

We conclude that performed individually, emergence and extraction lack the precision required for detailed vegetation forecasting in wetland mitigation planning. However, targeted seed identification for large-seeded invasive species may be a feasible application for extraction studies, and combining sample units into larger composite samples by site area or community could streamline greenhouse emergence studies. A more prudent approach would be to combine both techniques with a complete floristic inventory of the proposed mitigation area, with an emphasis on marginal habitats (e.g., ditches, pond fringes, etc.) that could serve as sources of seeds and for the types of species that could colonize a wetland mitigation project once constructed.

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Achenes from a species in the sedge family (Cyperaceae) viewed at 10x magnification during the in-house seed extraction pilot study. Given the cross-reticulations on the achene surfaces, this could be a species in either Rhynchospora *or* Eleocharis, *but positive identification could not be verified for this sample based on available seed ID protocols.*

INTRODUCTION

Soil seed banks serve as reservoirs of plant diversity, influencing vegetation composition over time. They contribute to plant community resilience by allowing species to regenerate following disturbances such as flooding, fire, or habitat alteration (Leck, 1989; Parker et al., 1989). However, the composition of a seed bank is not always reflective of the aboveground vegetation due to differences in seed persistence, dormancy, and dispersal mechanisms (Naumann and Young, 2007; Bossuyt and Honnay, 2008). Therefore, it can be beneficial to analyze the seed bank of a site before causing disturbance or soil alterations, which would help to identify potential future volunteers and understand risk of undesirable plant species.

Seed Banks and Wetland Mitigation

In the field of wetland mitigation, soil seed banks can play a major role in determining the success of a project (DeBerry and Perry, 2000b). Wetland mitigation – the process of compensating for wetland losses by replacing wetlands on the landscape – is typically implemented by either creating a new wetland in an area where it previously did not exist (wetland creation) or restoring a wetland that was previously disturbed or drained (wetland restoration) (Brooks and Gebo, 2013). **Wetland creation** projects frequently depend on the reuse of topsoil to restore native vegetation. This often involves removing the topsoil, stockpiling onsite, and re-spreading the topsoil to increase organic matter in the surface soils once the site has been constructed (DeBerry et al. 2004). This practice may also increase the opportunity for any seeds that are in the topsoil to germinate once the material has been reincorporated onsite. However, this could also increase the potential for invasive or undesirable plants to become established, the management efforts for which can take five to seven years to remove (van der Valk and Pederson, 1989). These species often emerge in the years following initial planting, further complicating restoration efforts.

Invasion risk may be even higher for **wetland restoration** projects, which focus on re-establishing wetland hydrology on sites that have been previously drained (e.g., low-lying farm fields previously established on drained wetlands) (Brooks and Gebo, 2013). A common practice is to simply dam or plug drainage ditches, an approach that could be used to restore wetland hydrology without changing existing elevations within the site (Biebighauser, 2007). In such circumstances, the topsoil could be left in place, and the existing seed bank could be used to help re-establish desirable wetland species; however, the number of wetland species that return has been shown to decrease with time since the original wetland was drained or disturbed (van der valk and Pederson, 1989). Accurately estimating the soil seed bank composition before project implementation could significantly enhance restoration outcomes by allowing practitioners to decide whether to retain or replace topsoil based on level of risk.

Seed Bank Estimation Methods

Among available seed bank estimation methods, the **emergence method** is the most widely used in seed bank assays. This approach relies on germinating seeds directly from a soil sample in a greenhouse (or similar controlled setting) and identifying the plants to species level from the seedlings that emerge (DeBerry and Perry, 2000a). Specific techniques vary, but most emergence trials involve subjecting a soil seed bank sample to a cold stratification treatment, then spreading the sample over a seedless growing medium (e.g., greenhouse grade potting soil) and allowing the seeds to germinate (Gross, 1990; Mahé et al., 2021). This process can take up to three years, and the controlled setting of a greenhouse may not be the ideal germination conditions for many of the plants in the seed bank (Price et al., 2010; Mahé et al., 2021). Despite these drawbacks, the emergence method has been implemented in planning wetland restoration projects in the U.S. (Minnesota Board of Water and Soil Resources, 2008).

The other most widely used method is **seed extraction** from soil samples (DeBerry and Perry, 2000a; Price et al., 2010). This technique uses some filtering mechanism to remove the seeds from a sample (sieve, cloth bag, flotation, etc.) and then relies on identifying seeds to species using the morphological features of the seeds themselves (Gonzalez and Ghermandi, 2012). Seed extraction has been shown to increase the number of different species found within a sample, but seed identification can be difficult, time consuming, and inaccurate (DeBerry and Perry, 2000a; Price et al., 2010; Gonzalez and Ghermandi, 2012). Additionally, the extraction method can be bias toward larger seeds with well documented morphology (Mahé et al., 2021). Notwithstanding these issues, there are professional laboratories that offer fee-based seed identification services using seed bank extraction as the primary seed identification protocol.

Study Purpose

Despite the value of seed bank assessments, there is currently no rapid, cost-effective method for estimating soil seed banks in the context of wetland mitigation. This study aims to evaluate existing methods and determine their efficiency and accuracy in predicting soil seed bank composition. We did this by replicating two approaches that could be used by a wetland mitigation practitioner (e.g., consultant, mitigation banker, agency scientist, etc.), and one involving outsourcing the approach to a seed identification laboratory. The three methods are: 1) **in-house extraction**, 2) **greenhouse emergence**, and 3) **offsite laboratory extraction**.

METHODS

Field Sampling

To evaluate the potential for a seed bank assay to be used in wetland mitigation feasibility, we evaluated a low-lying farm field adjacent to an existing wetland mitigation bank (Cedar Run Mitigation Bank) in Fauquier County, Virginia (Figure 1). At the time of our study (mid-May

2022), the field was being used as a cattle pasture but because of its close proximity to the existing wetland mitigation bank, it served as a representative potential area of expansion for the bank and thus suited our evaluation criteria for mitigation feasibility (i.e., an area in which wetlands could be created or restored). The field was approximately 2 hectares in size.

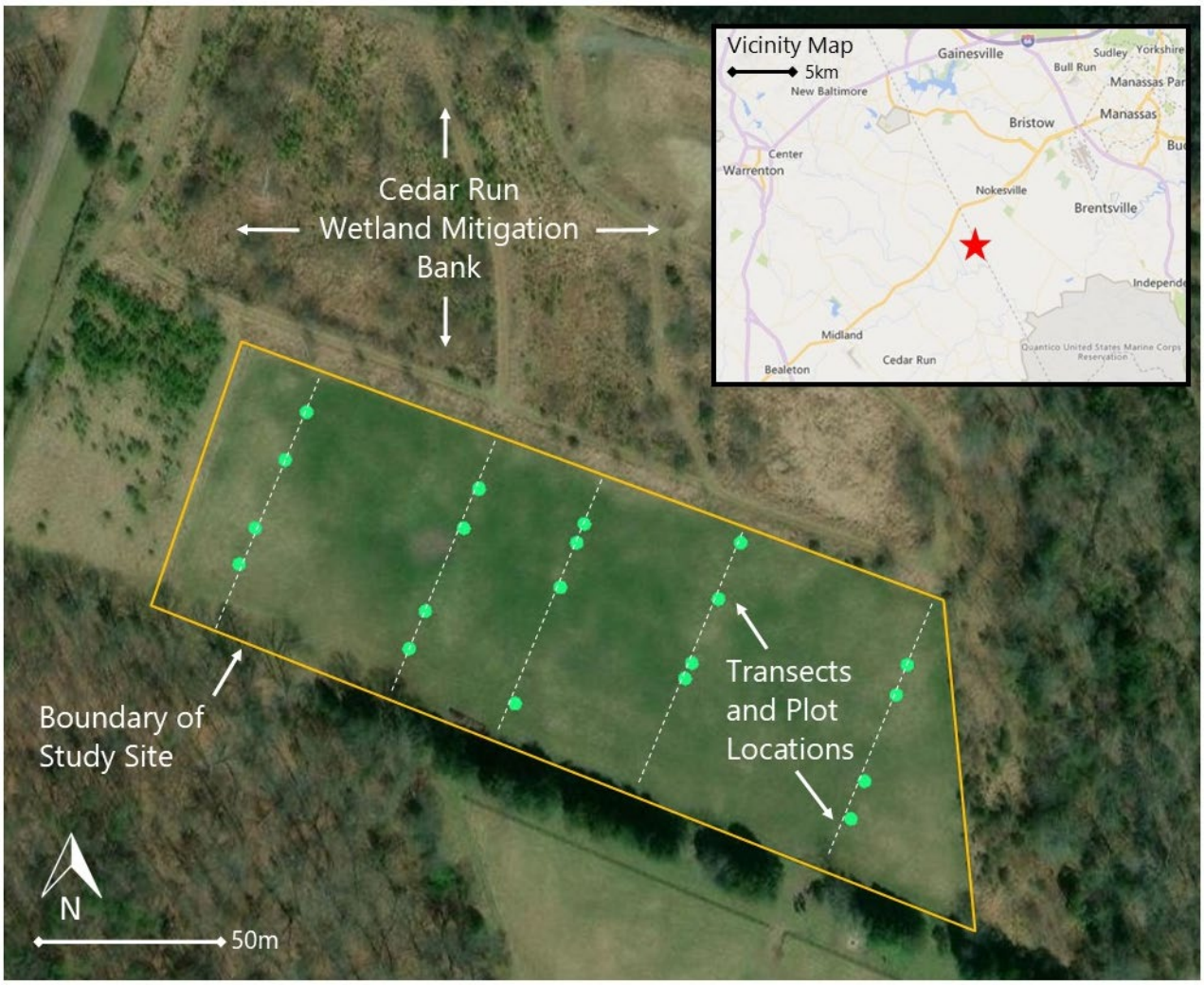

Figure 1. Field site location and study design.

Study Design: Sampling was completed using the stratified-random configuration described in DeBerry (2020). For this site, we established a baseline along one side of the field and divided it into five 40m segments, then drew a random number between 1 and 40 from a random numbers generator to determine baseline position for a perpendicular transect in each segment (Figure 2).

We then divided each perpendicular transect into four equal segments and selected another random number to position one plot in each segment, taking segment length as the domain for the random numbers draw. This resulted in four plots per transect over five transects, for a total of 20 plots. Plot dimensions were $1m^2$ in accordance with DeBerry (2020), and the center of each plot was mapped using a sub-meter GPS receiver (Figure 1).

Figure 2: Perpendicular transect layout and representative view of study site.

Soil/Seed Bank and Vegetation Sampling: From the center of each $1m^2$ plot, we extracted a soil sample using a 5cm diameter soil corer to a depth of 10cm to use in the seed bank estimation procedures (Figure 3). In addition, we collected data on species composition and abundance of the standing vegetation so that we could get an understanding of the existing species in the field for comparison with the seed bank results. Within each plot, we identified all plants to species level based on Weakley et al. (2020) and assigned a cover class to each species using modified Daubenmire cover classes (Mueller-Dombois and Ellenberg, 1974). The

Figure 3: Soil/seed bank field sample.

cover classes were as follows (midpoints rounded to nearest integer in parentheses): 0-1% (1%), 1-5% (3%), 5-25% (15%), 25-50% (38%), 50-75% (63%), 75-95% (85%), 95-100% (98%).

Pilot Study – Seed Bank Estimation Methods

This project tested three methods for estimating soil seed banks: in-house seed extraction, greenhouse emergence, and offsite laboratory seed extraction. Methods were initially tested in **pilot studies** using a subset of five 100g soil samples composed of one random sample from each of the five transects. The purpose of the pilot study phase was to get an initial understanding of level-of-effort, time, and accuracy based on comparisons among methods and with the standing vegetation in the field.

Figure 4: Soil sieves used for in-house extraction method.

Pilot In-house Extraction Method: To replicate an in-house seed extraction protocol, we washed the five individual 100g soil samples through a series of standard soil texturing sieves to remove as much non-seed matter as possible (Figure 3; Gonzalez and Ghermandi, 2012; Oklahoma State University, 2025). The residual material was examined and further separated under a dissecting microscope until all seeds were removed. Seeds were then identified using seed ID references (Seed Identification Guide, 2018; Abbott et al., 2025; McDonald et al., 2025), counted for each sample, and verified by a senior botanist.

Pilot Greenhouse Emergence Method: For the greenhouse emergence method, we cold stratified the five 100g samples at around four degrees Celsius for three weeks prior to initiating the trials – a technique that is used to encourage cessation of dormancy for many species (Gross, 1990; DeBerry and Perry, 2000a). Emergence trials were carried out in the College of William & Mary Greenhouse beginning on June 6, 2022 and ending on May 23, 2023. Soil samples were spread in grow trays over sterile Promix flexible purpose potting mix as a seedless


Figure 5: Pilot study greenhouse emergence germination trays.

germination base (Figure 5). Trays were watered regularly and monitored for new species germination. Plants were left in the trays until large enough to identify to species, then removed.

Pilot Offsite Laboratory Extraction Method: We researched laboratories that provide seed bank estimation services using the extraction method and selected a reputable lab based on web-available information and reviews. To develop an initial understanding of the output from an offsite lab analysis, we sent one set of five soil samples from the field study to the seed identification lab in mid-May 2022, with testing complete and results provided in less than two weeks at a cost of $251 per sample. The laboratory used an extraction method similar to the one described above (i.e., samples were washed and sieved then screened to a desired particle size before being examined under magnification).

Full-scale Trials – Seed Bank Estimation Methods

From the initial results of the pilot study, we narrowed the options for seed bank estimation to the greenhouse emergence and offsite laboratory extraction methods to take to full-scale trials (see Results below for the rationale behind removing the in-house extraction). To test the accuracy of both approaches, we created an artificial seed bank in a set of controlled soil samples containing a known seed quantity and species composition. This approach has been successfully used by others to test seed bank estimation methods (Leon and Owen, 2004).

For the full-scale trial, our artificial seed bank was formulated to mimic field conditions. This seed mix featured a greater proportion of small seeds and fewer large seeds, which would be representative of a natural seed bank (Shipley and Dion 1992; Turnbull et al., 1999; Jakobsson and Eriksson, 2000; Moles et al., 2004). We also used a known quantity of native seeds provided by a seed supplier (Ernst Conservation Seeds), as well as the seeds of three invasive species hand-harvested by the authors (*Arthraxon hispidus*, *Lespedeza cuneata*, and *Microstegium vimineum*). The intent here was to create an artificial "invasion risk" with our controlled seed bank and determine how effective these methods would be at identifying the risk. All seeds had been cold stratified either by the seed provider (native seeds) or the authors (invasive seeds) prior to inclusion in

Figure 6: Harvesting seed-free soil from natural subsoil for artificial seed banks used in full-scale trials. The blade of the tile spade in the image is approximately 40cm long.

the seed mix. The full artificial seed mix was comprised of 23 species and a total of 53 seeds per sample, the composition of which is provided in Table 5.

The soil medium for the artificial seed bank was collected from a natural silty clay loam subsoil below a seasonally saturated pine forest in James City County, Virginia. The surface soil layers were removed to a depth of 40cm with a tile spade, then the subsoil material was collected with a 7cm diameter dutch auger to an additional 10cm depth (i.e., the soil used to create the artificial seed bank was collected between 40cm and 50cm in the profile) (Figure 6). This process ensured that no pre-existing seed bank from the forest would be included in the samples. Ten identical seed bank replicates were created for each full-scale trial.

Full Greenhouse Emergence Trials: Using the 10 replicates of the artificial seed bank, we repeated the greenhouse emergence trials in 10 separate germination trays following the methods described above (Figure 7). The full greenhouse trials were initiated on August 20, 2022 and terminated on May 23, 2023.

Figure 7: Full-scale greenhouse emergence study shown at trial initiation (left), and with seedlings after one month (right).

Full Offsite Laboratory Extraction Trials: Finally, we sent a set of 10 artificial seed bank samples to the same seed identification laboratory used in the pilot study, requesting the same species identification protocol.

Data Analysis

To test for accuracy of seed estimation methods, we calculated Jaccard's Dissimilarity Index between the species composition of the artificial seed bank samples and the results acquired from both the full trial greenhouse emergence and offsite lab studies. We also used the index to compare composition of the standing vegetation to the results of all three pilot studies to see if the methods could be used to detect seed bank species that were not in the extant flora. Jaccard's Dissimilarity is an ecological distance measure of the form:

$$J(i,j) = sim(i,j) = 1 - \frac{a}{a+b+c}$$

where i and j represent separate species groups, a = species in common, b = species unique to i, and c = species unique to j. This index gives a number between 0 and 1, with values closer to 0 representing small differences between species groups and values closer to 1 representing larger differences.

RESULTS

Standing Vegetation Data

Eighteen (18) species were documented in sample plots in the field, with dominants including tall fescue (*Schedonorus arundinaceus*, 37.2% relative cover), white clover (*Trifolium repens*, 22.8%), and annual bluegrass (*Poa annua*, 20.9%). Other common species included bulbous buttercup (*Ranunculus bulbosus*, 8.9%), English plantain (*Plantago lanceolata*, 3.0%), and sweet vernal grass (*Anthoxanthum odoratum*, 1.2%). Three non-dominant forbs were unable to be identified to species due to immaturity and lack of flowering or fruiting at the time of the study. Vegetation data are presented in Table A1 (Appendix).

Pilot Study – Seed Bank Estimation Results

In-house Extraction Pilot Results: The results of the pilot in-house extraction method are summarized in Table 1, and the detailed results are presented in Table A2 (Appendix). As Table 1 shows, the in-house extraction method successfully isolated 338 seeds from the five 100g soil samples. Of these, 153 (45.3%) were able to be identified to species level, and 243 (71.9%) were able to be identified to genus (including the species group). Positive species identifications varied between samples, ranging from 84.8% (sample A) to 8.6% (sample E), although there was significantly less variation in positive genus identifications (91.3% to 37.1%, samples A and E, respectively). There were 16 species and 31 genera positively identified across all five samples, and 57 unknown taxa that were able to be differentiated as separate seeds based on morphology but could not be identified to species or genus due to database and resource limitations. However, as noted in Table A2 (see Appendix), nearly all of the unknowns were represented by only one seed in a given sample.

Table 1. Summary of pilot in-house extraction results.

	Seed Bank Samples					
	A	B	C	D	E	Totals
Total Seeds Counted	46	22	175	60	35	338
# Seeds Identified to Species	39	8	75	28	3	153
% Seeds Identified to Species	84.8%	36.4%	42.9%	46.7%	8.6%	45.3%
# Seeds Identified to Genus	42	10	149	29	13	243
% Seeds Identified to Genus	91.3%	45.5%	85.1%	48.3%	37.1%	71.9%
Species Positively Identified	7	4	10	5	2	16
Genus Positively Identified	8	5	16	6	4	31
Unknown	3	4	19	22	19	57

Although progressive sieving was able to remove most of the mineral soil fraction, there was a significant amount of non-seed organic material in the samples that could not be separated out using sieves. Thus, the sieved samples had to be parsed under a dissecting microscope to physically remove smaller seeds from the non-seed material. Each sample took approximately 6 hours for full seed extraction (sieves plus hand removal), and another 3 hours to identify to species or genus using the dissecting microscope and seed identification resources.

Jaccard's dissimilarity between the standing vegetation (Table A1, Appendix) and the in-house extraction results (Table A2, Appendix) was 0.83, suggesting very little overlap between the extant vegetation on our field site and the seeds that were able to be identified from the seed bank (see Table 4).

Greenhouse Emergence Pilot Results: Twelve (12) taxa were identified in the greenhouse emergence pilot study, 10 of which were positively identified to species and two only to genus (Table 2). Of the 31 total seedlings counted, the most commonly encountered species in germination trays were smooth crabgrass (*Digitaria ischaemum*) and spotted spurge (*Euphorbia maculata*), neither of which was documented in the standing vegetation (see Table A1, Appendix). Jaccard's index for the pilot emergence results versus the extant vegetation was 0.88 (see Table 4). We also used the Jaccard's distance to compare the greenhouse emergence pilot data to the in-house extraction data, and the result was 0.74. From trial initiation to final seedling removal, the pilot greenhouse emergence study took nearly one year to complete (June 6, 2022 to May 23, 2023).

Table 2. Greenhouse emergence pilot results (seedlings per sample).

	Seed Bank Samples					
	A	B	C	D	E	Total
Agrostis sp.		1	1			2
Acalypha rhomboidea					1	1
Digitaria ischaemum	1	1	2	7		11
Erigeron canadensis				1		1
Euphorbia maculata	1		2	1	1	5
Oxalis dillenii				2		2
Plantago lanceolata	1					1
Panicum sp.					1	1
Setaria parviflora		2				2
Solanum carolinense		3				3
Trifolium campestre				1		1
Trifolium repens					1	1
Total:	3	6	4	12	4	

Offsite Laboratory Extraction Pilot Results: The offsite seed lab found 112 seeds and identified 17 taxa from the five samples submitted in the pilot study (Table 3). Among these, 12 were identified to species, four were identified only to genus, and one was simply identified as a member of the grass family (Poaceae). Common species included Virginia three-seeded mercury (*Acalypha virginica*), paspalum (*Paspalum* sp.), yellow bristlegrass (*Setaria pumila*), and white clover. Jaccard's index for the offsite pilot results compared to the standing vegetation was 0.85 (Table 4). We also compared the offsite pilot data to the in-house extraction and greenhouse emergence results, and the Jaccard's values for those were 0.71 and 0.72, respectively.

Table 3. Offsite extraction pilot results (seeds per sample).

	Seed Bank Samples					
	A	B	C	D	E	Total
Acalypha virginica					21	21
Amaranthus spp.				6		6
Ambrosia artemisiifolia			1			1
Carex spp.					9	9
Elusine indica		2				2
Mollugo verticillata		2				2
Oxalis stricta		1				1
Panicum capillare					3	3
Paspalum sp.	6				15	21
Plantago lanceolata	4					4
Plantago major				2		2
Poaceae (grass)		1				1
Setaria pumila		3	1	11		15
Taraxacum officinale	1					1
Trifolium pratense				1		1
Trifolium repens	1	14	1	4		20
Verbena sp.		2				2
Total:	12	25	3	24	48	

Table 4. Jaccard's dissimilarity matrix for all pilot studies.

	Standing Veg	In-house Extraction	Greenhouse Emergence
In-house Extraction	**0.83**		
Greenhouse Emergence	**0.88**	**0.74**	
Offsite Laboratory	**0.85**	**0.71**	**0.72**

Full-scale Trials – Seed Bank Estimation Results

As noted above, following the pilot studies we narrowed the options to **greenhouse emergence** and **offsite laboratory extraction** to carry forward to a full-scale trial. Although the in-house extraction methods produced useable results, it was decided that the output was not worth the labor, time, training, and potential expense required to competently execute a self-guided extraction seed bank assay (see Discussion below). Further, the in-house extraction protocol would not have been an unbiased trial under the selected full-scale protocol because our research team would have already been aware of the species composition and seed density of our artificial seed bank samples and, more importantly, would have already known how to identify the seeds from our hands-on work with the species when we created the samples.

Greenhouse Emergence Full Trial Results: Of the original 23 species included in the artificial seed bank samples, 12 emerged as seedlings during the full-scale greenhouse emergence study across all 10 trials (Table 5). Species richness in individual seed bank samples ranged from 5 to 10, with an average of 6.9 per sample. There were 101 positively identified seedlings in total with an average of 10.1 per sample, and only four seedlings across all samples that could not be identified due to early mortality. The most prevalent species were mistflower (*Conoclinium coelestinum*), switch grass (*Panicum virgatum*), spreading panic grass (*Panicum dichotomiflorum*), deer tongue (*Dichanthelium clandestinum*), and joint-head grass (*Arthraxon hispidus*).

Jaccard's dissimilarity per sample averaged 0.70 (i.e., comparing original artificial seed bank with germination results), but the index across all trials was 0.48. The entire study took 39 weeks (August 22, 2022 to May 23, 2023) to complete before seedlings were no longer emerging from germination trays. The germination trial was successfully able to identify two of the three invasive species that were included in the seed bank samples – joint-head grass and sericea lespedeza (*Lespedeza cuneata*) – but failed to identify Japanese stiltgrass (*Microstegium vimineum*).

Offsite Extraction Full Trial Results: The offsite seed identification lab provided 24 separate taxa from the 234 (out of 530) seeds they found in the 10 artificial seed bank samples we submitted (see Table A3, Appendix). Of these, 13 were listed to species level, nine were listed to genus level, and two were listed just to family level. Jaccard's dissimilarity averaged 0.93 per sample (i.e., lab list compared to true list from artificial seed bank samples). Considering only the taxa that the lab reported to species level, Jaccard's dissimilarity across all samples was 0.83. Making some allowances for congeners (e.g., if the lab listed only genus, counting that as "correct" in the index, as in "*Carex* spp."), Jaccard's index was 0.70.

Of the 13 taxa that the lab provided to species level, seven were not in the original artificial seed bank samples and were therefore reported by the lab in error. Likewise, of the lab's 24 total taxa listed (species, genus, family), 10 were not actually in the original samples, and one species listed by the lab (*Panicum bergii*) is not found north of Georgia in the Atlantic states. Finally, although

the lab was consistently able to identify the invasive sericea lespedeza in samples, it failed to locate any of the invasive joint-head grass or Japanese stiltgrass seeds.

Table 5. Full-scale greenhouse emergence results (seedlings per sample). Left two columns include full species list from the artificial seed bank samples and initial seed density per sample (n=10). Rows with no values in sample columns indicate that the species did not emerge.

	Initial Seed Density per	Artificial Seed Bank Samples										
Full Artificial Seed Bank List	Sample	**G-1**	**G-2**	**G-3**	**G-4**	**G-5**	**G-6**	**G-7**	**G-8**	**G-9**	**G-10**	Total
Arthraxon hispidus	**1**	1	1	1	1	1	1	1	1	1		9
Bidens aristosa	**1**											
Carex frankii	**1**				1							1
Carex lurida	**1**				1	2					1	4
Carex vulpinoidea	**3**		1		1	1		1		1	1	6
Chamaecrista fasciculata	**1**	1		1	1	1	1	1				6
Chasmanthium laxum	**2**											
Cinna arundinacea	**1**											
Elymus virginicus	**1**											
Conoclinium coelestinum	**3**	2	2	3	3	2	2	1	3	3	2	23
Euthamia graminifolia	**5**											
Juncus effusus	**5**											
Juncus tenuis	**5**											
Leersia oryzoides	**1**											
Lespedeza cuneata	**1**			1							1	2
Ludwigia alternifolia	**5**		1	1							1	3
Microstegium vimeneum	**1**											
Dichanthelium clandestinum	**2**		1	1	1	1	2	2	2	1	1	12
Panicum dichotomiflorum	**3**	3		1	2	2	1	3	2		1	15
Panicum virgatum	**2**	2		2	3	2	1	2	3	1		16
Polygonum sagittatum	**2**											
Solidago rugosa	**3**		1	1	1					1		4
Verbena hastata	**3**											
Total:	**53**	9	7	12	15	12	8	11	11	8	8	101

DISCUSSION

This study evaluated the efficacy of using soil seed bank estimation methods to predict future colonizers in a wetland mitigation scenario. Our research goals were motivated in part by the tacit belief that the seed bank is critical to vegetation community outcomes in wetland creation and restoration – a mantra that has been around even since the "early years" when wetland mitigation emerged as a separate discipline in the U.S. (e.g., 1980s and early 1990s; Kusler and Kentula, 1989; Hammer 1992). Since that time, studies investigating this topic have produced variable results with varying degrees of reliability (van der Valk et al., 1992; ter Heerdt and Drost, 1994; Brown, 1998; DeBerry and Perry 2000b, Middleton, 2006; Mahé et al., 2021); thus, we were

interested in determining if there had been any advancements made in seed bank estimation techniques and then testing available approaches in the applied scenario of a wetland mitigation feasibility study.

The questions we were pursuing could be restated as follows: 1) What techniques are currently available for soil seed bank estimation? 2) How should the seed bank be sampled? 3) Which technique is most effective and efficient? 4) Is a seed bank study worth it? We will discuss each question individually below.

What techniques are currently available for soil seed bank estimation?

One commonality among nearly all the studies cited in this report is that the methods available for seed bank estimation have not changed over the years. Seed bank assays generally involve sampling soils and either 1) germinating the seeds in emergence trials, or 2) extracting the seeds directly from the soil and identifying the seeds themselves. Therefore, these are the methods that were evaluated in this study.

There are also available methods for determining seed viability in a sample [e.g., elutriation, a process that recognizes fine root production from seeds in the soil (Gross 1990)]; however, since the focus of a seed bank assay in wetland mitigation is primarily to determine species composition, isolating seed viability is perhaps an unnecessary complication and of course would already be addressed in a germination trial. One advancement that was apparent during this study is that seed identification resources are much more available via web databases and online identification tools than in the previous decades of seed bank research, and the likelihood that these resources will only improve with time should make extraction a more manageable technique in future years.

How should the seed bank be sampled?

We focused our sampling in the topsoil layer because it contains the majority of seeds in the seed bank (Thompson, 1992). Further, we assumed that our analysis would be appropriate for either wetland creation or wetland restoration, because in the former the topsoil is likely to be stripped, stockpiled, and respread on the site during the construction phase, and in the latter the topsoil is likely to be left in place to encourage regeneration from the historic wetland seed bank once hydrology is restored (DeBerry et al., 2004; Middleton, 2006). We also assumed that in either scenario seeds would be non-uniformly distributed across a given study site, and that seed bank estimation should therefore be based on replicate soil samples from the entire study area to adequately represent the distribution and abundance of species in the seed bank (van der Valk et al., 1992). To accomplish this, we used a sampling procedure that was designed to spread the sample across the study area while also ensuring that samples were independent and random for purposes of statistical analysis (DeBerry, 2020). However, because the ultimate goal of a seed bank assay in wetland mitigation feasibility is to determine presence and composition

of species in the seed bank, one could accomplish the same with systematic sampling (e.g., sampling on a grid or regularly spaced intervals on transects), an approach that has shown to yield results comparable to random sampling (Benoit et al., 1989). For wetland practitioners who are not accustomed to ecological sampling theory, this might simplify the field sampling phase of a seed bank study and would not diminish the reliability of the results obtained.

For the purposes of this research project, we used DeBerry (2020) as a guideline for determining the number of aboveground vegetation plots (i.e., sample units), and plots were then used to determine the locations of our soil samples. Therefore, the seed bank sampling effort was aligned with the aboveground sampling effort, which is an intuitive approach that accords with other reviews (Warr et al., 1993). It should be noted that our study site was relatively homogeneous with respect to aboveground community composition; however, if the study area had crossed community boundaries, it would have been appropriate to stratify the sample effort by community type (Krebs, 1999).

Which technique is most effective and efficient?

Effectiveness: The real value of a seed bank assay in wetland mitigation feasibility is that it gives wetland practitioners the opportunity to determine the identity of the plants in the species pool (*sensu* Taylor et al., 1990) that cannot be observed by studying the standing vegetation alone. Given the high Jaccard's dissimilarity between the aboveground vegetation and the techniques tested (see Table 4), all three of the strategies used in this study (in-house extraction, greenhouse emergence, offsite laboratory extraction) succeeded in finding species in the seed bank that were not sampled in the aboveground plots. Thus, all show some measure of effectiveness along these lines. However, dissimilarity was also high *among* the techniques (Table 4), suggesting very little overlap in the species found by each approach and casting doubt on the prospect determining a superior technique.

An effective seed bank estimation procedure also needs to be ***accurate***, and this is where we discovered some departures among the methods tested. Because we did not carry the in-house extraction procedure forward to the full-scale study, accuracy for this procedure could not be assessed in the same manner as the other two approaches. However, the initial in-house extraction data from the pilot study point to some preliminary conclusions. Although there were several dozen taxa in the samples that could not be identified (see Table A2, Appendix), nearly half (45.3%) of the total *seeds* were able to be positively identified to species, and over two-thirds (71.9%) were identified to at least the genus level (Table 1). Given that the identifications were verified by a senior botanist, this result suggests that a self-directed extraction study could produce meaningful composition data for use in mitigation planning. Furthermore, since most invasive taxa can be differentiated at the genus level (e.g., *Arthraxon*, *Microstegium*), an in-house extraction study would increase the predictive power of an invasive species risk assessment based on our results. Practitioners could presumably maximize the utility of such a study by focusing on the seed morphology of invaders that pose the highest risk.

Accuracy was assessed more directly in the full-scale trials for the greenhouse emergence and offsite laboratory extraction protocols because we used an artificially created seed bank and therefore had full knowledge of the complete seed composition and abundance in each sample. The results of these trials showed striking differences between the two approaches. Notably, over half (12 out of 23) of the original seed bank species were positively identified in the greenhouse emergence trials, and this resulted in the lowest Jaccard's dissimilarity recorded in the entire study (0.48) (i.e., emergence-derived species composition was closest to the true seed bank). By contrast, over half (seven out of 13) of the species reported by the offsite lab were incorrect, resulting in one of the highest dissimilarity values in comparison with the original seed bank (0.83). Further, the greenhouse trials successfully identified two of the three invasive species in the seed bank, whereas the offsite lab only found one. These results clearly indicate that, at least for a scenario like the one used in this study, a self-directed greenhouse emergence assay would produce more reliable species composition results than outsourcing to a seed identification lab.

Finally, effectiveness can also be evaluated by looking at the ***total number of seeds*** identified in the soil, and on this score seed extraction is the clear front-runner. In the three pilot studies, the in-house extraction protocol identified 338 seeds, which is an order of magnitude larger than the total number found in the greenhouse emergence pilot trials (31). This result was similar for the offsite lab pilot study (112 seeds). Likewise, in the full-scale trials, the offsite lab extraction found 234 seeds, which is over twice the number confirmed in the greenhouse emergence trials (101). The underestimation of the seed bank via the emergence method is consistent with previous experimental results in the literature (Price et al., 2010; Mahé et al., 2021) and highlights one of the most significant drawbacks to the approach. However, as noted above, the higher seed density produced from extraction results did not confer an advantage in accuracy, so there are certainly tradeoffs between the two approaches.

Efficiency: Notwithstanding the value provided by the in-house extraction method in terms of seed density and discovery (i.e., finding species in the seed bank not in the standing vegetation), the **overall level of effort** required to execute a reliable self-directed extraction procedure is probably not scalable to the level of a typical wetland mitigation feasibility study. At over 9 hours per sample, the amount of labor required just to separate the seeds from the soil is almost certainly more than the average wetland professional can commit to such a task, especially given the that the results were only somewhat reliable. This was ultimately our rationale for excluding the in-house extraction trials from further review after the pilot study.

From an effort standpoint, a greenhouse emergence study is much less labor intensive, requiring only periodic watering, as well as plant identification and removal once seedlings are old enough to be verified. The obvious downside to a greenhouse emergence assay is the total amount of time required to complete the study (Price et al., 2010; Mahé et al., 2021), which for our trials took up to a year. Of course, another important consideration is that such a study requires unlimited access to a greenhouse, and this is likely to be the more important limiting

factor for most professionals. However, if a greenhouse is available, the length of time needed to complete an emergence study may not be an issue for practitioners who have the benefit of several months lead time to plan and execute the study. In terms of labor efficiency, the offsite laboratory extraction was by far the superior approach. In both the pilot and the full-scale trials, the lab results were available within a few weeks of sample submission. As noted above, this approach also produced the least reliable data, but there were some taxa correctly identified, so there may be some cost-benefit decisions to weigh when considering use of this approach.

Finally, efficiency should consider **overall cost**, and this is easiest to judge for the offsite laboratory analysis since each sample was analyzed at a lump sum cost (for our study, ca. $250/sample). For larger sites that might require dozens of samples or more to adequately estimate the seed bank, this expense could prove cost prohibitive, especially if the results are only marginally reliable. Assuming that most wetland professionals have access to a dissecting microscope and a set of soil sieves (or could acquire them inexpensively), the cost for an in-house extraction would simply be the labor required to execute the study. However, as noted above, this process was extremely labor-intensive and, in our estimation, would be cost-prohibitive for most professionals just based on the labor required. Finally, the cost for the materials needed to conduct a greenhouse emergence trial is minimal and amounts to some germination trays, potting soil, and the time required to attend to the trays. Assuming that greenhouse access is available, at face value an emergence study would be the least expensive; however, practitioners should consider the overall time required to complete the study (up to a year based on our results), which could cause labor expenses to compound especially if the greenhouse is offsite and requires travel for periodic maintenance and seedling identification.

Is a seed bank study worth it?

Given the discussion above, there is no clear "winner" among the methods currently available for seed bank estimation in wetland mitigation feasibility studies. Tradeoffs to consider include which method produces the most accurate list of species (emergence), which finds the greatest number of seeds (in-house extraction), which one can identify species not found in the standing vegetation (presumably all), and which is most efficient in terms of time commitment (offsite extraction) and overall cost (emergence). For this reason, studies focused on a comprehensive understanding of the seed bank have recommended using a combination of both emergence and extraction protocols (e.g., Gonzalez and Ghermandi, 2012), although some suggest that time is better spent conducting a thorough floristic inventory of the study area and focusing on marginal habitats that could serve as refugia for future wetland colonizers (e.g., ditches, pond fringes; Brown 1998).

From our results, we feel reasonably confident that a full in-house extraction study would not easily scale up to the level of a typical wetland mitigation project and therefore is probably not worth the time investment. However, we do believe that the process could be streamlined if the goal was to target specific invasive species in the seed bank. In this case, invasives with larger or

morphologically unique seeds would be easy to target in the extraction process (Mahé et al., 2021). This may be a viable way to estimate the future impact of invasive species at a site and, perhaps more importantly, determine whether or not the *in situ* topsoil could be used in the wetland project without invasion risk. For many, this could be the most important piece of information to gain from a seed bank study.

The inaccuracy and large blind spots yielded from the offsite laboratory data suggest to us that outsourcing seed identification to a lab is not worth the expense. However, following the same logic as noted above, if the goal is to target invasive species it may be plausible to identify which invasives are most likely to be present in a given region and then communicate species information to the lab ahead of processing. With that knowledge, the laboratory could run targeted extraction trials, which would almost certainly improve accuracy and might also reduce the cost per sample.

Finally, assuming that a mitigation professional has access to a greenhouse and can easily work sample maintenance and surveillance into a schedule that can be maintained for several months, an emergence study could be worth the effort under many wetland mitigation feasibility scenarios. The results will underestimate seed density, but based on our study, species composition data will be reasonably reliable and can provide valuable information on future colonizers. To improve efficiency and also conserve space in the greenhouse, van der Valk et al. (1992) recommend conducting a stratified-random sample for each study area similar to the approach that we used, but instead of keeping the soil samples in separate trays, they suggest combining all samples into one tray per study area or stratum. This would streamline the sampling process and also reduce the amount of time and effort required in the greenhouse with fewer containers to oversee.

Ideally, a scenario that combines a simplified greenhouse emergence approach with some targeted extraction trials (e.g., invasive-focused trials) would have the potential to produce valuable information and also minimize the inaccuracies and inefficiencies inherent in these techniques. We believe that such an approach could be profitably combined with a detailed floristic inventory of the proposed mitigation area, with an emphasis on marginal habitats (e.g., ditches, pond fringes, etc.) that could serve as sources of seeds for the types of species that could colonize a wetland mitigation project after construction. Once mitigation professionals have had time to standardize this type of three-pronged approach to the point where it could be executed efficiently and effectively, it would be hard to imagine a more comprehensive set of analyses for seed bank estimation in wetland mitigation.

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APPENDIX

Tables A1, A2, and A3

Table A1. Cedar Run Mitigation Bank - Adjacent Field Existing Vegetation

May 10, 2022

Fauquier County, VA

Investigators: Sam Dutilly and Doug DeBerry

Scientific Name	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3	C4	D1	D2	D3	D4	E1	E2	E3	E4
Allium vineale														1						
Ambrosia artemisiifolia						3										3				
Anthoxanthum odoratum	15	15	3																	
Carex sp.																		3	3	
Cerastium glomeratum														1			3			
Juncus tenuis			1	3								1				3			3	15
Plantago lanceolata	3	3	3	15			3	3		3	3	15		15	3	15				
Plantago rugelii									1				1							
Poa annua	3	3	3	15	3	63	63	38	3	15	63	63	38	15	38	38	38	38	15	38
Ranunculus bulbosus	3	38	15	3	15	15	3	3	38	3	38	15	15	3	3	3	38	3		
Schedonorus arundinaceus	63	85	15	63	15	38	15	38	38	85	15	63	38	85	63	63	63	63	63	85
Solanum carolinense														1						
Taraxacum officinale	1	1				1		3			3	3		3						
Trifolium repens	15	1	63		63	15	38	38	63	15	63	38	63	15	38	15	15	38	38	15
Unidentified forb 1				3					3	3	1	15			38	38				
Unidentified forb 2	1							1				1	1			1		1		
Unidentified forb 3							1							1						
Veronica arvensis						1								1						

Table A2. In-house Extraction Pilot Study Results.

A-2	#	B-3	#	C-4	#	D-1	#	E-2	#
Carex bushii	7	Brassica rapa	2	Ambrosia artemisiifolia	2	Carex bushii	14	Egrostis sp.	1
Datura stramonium	1	Brassica rapa	2	Carex bushii	2	Orobanche uniflora	1	Juncus sp.	9
Digitaria ischaemum	4	Digitaria ischaemum	1	Carex sp.	2	Setaria pumila	7	Paspalum dilatum	1
Mollugo verticillata	10	Mollugo verticillata	1	Digitaria ischaemum	12	Solanum carolinense	1	Trifolium repens	2
Paspalum dilatatum	6	Oxalis sp.	2	Digitaria sp.	52	Trifolium repens	5	Unknown 11	1
Paspalum laeve	10	Trifolium repens	2	Juncus sp.	13	Verbena sp.	1	Unknown 20	3
Taraxacum officinale	1	Unknown 11	2	Mollugo verticillata	1	Unknown 13	2	Unknown 49	1
Trifolium sp.	2	Unknown 6	1	Oxalis sp.	5	Unknown 20	1	Unknown 5	1
Unknown 1	1	Unknown 7	1	Panicum sp.	2	Unknown 30	1	Unknown 50	1
unknown 2	2	Unknown 9	8	Paspalum dilatatum	2	Unknown 31	1	Unknown 51	1
unknown 4	2			Paspalum laeve	22	Unknown 33	1	Unknown 52	1
				Paspalum sp.	1	Unknown 34	1	Unknown 53	1
				Persicaria maculosa	1	Unknown 35	1	Unknown 54	1
				Plantago lanceolata	1	Unknown 36	1	Unknown 55	1
				Setaria pumila	1	Unknown 37	1	Unknown 57	1
				Trifolium pratense	31	Unknown 38	1	Unknown 58	1
				unknown 12	1	Unknown 39	1	Unknown 59	1
				unknown 13	1	Unknown 40	1	Unknown 60	1
				unknown 14	1	Unknown 41	2	Unknown 61	1
				Unknown 15	1	Unknown 42	2	Unknown 62	1
				Unknown 16	1	Unknown 43	1	Unknown 63	1
				Unknown 17	1	Unknown 44	1	Unknown 8	1
				unknown 18	1	Unknown 45	1	Unknown 9	2
				Unknown 19	1	Unknown 47	1		
				Unknown 20	1	Unknown 48	1		
				Unknown 21	1	Unknown 64	1		
				Unknown 22	4	Unknown 8	6		
				Unknown 23	1	Unknown 9	2		
				Unknown 24	1				
				Unknown 25	1				
				Unknown 28	1				
				Unknown 29	1				
				Unknown 6	1				
				Unknown 8	1				
				Unknown 8	2				
				Unknown 9	2				

Table A3. Full Trial Offsite Extraction Results

F1	#	F2	#	F3	#	F4	#	F5	#
Bidens spp.	1	Adropogon spp.	1	Bidens spp.	1	Bidens spp.	1	Bidens spp.	1
Carex spp.	2	Asteraceae	3	Carex spp.	3	Carex sp.	2	Carex sp.	3
Lespedeza cuneata	1	Bidens spp.	1	Chamaecrista fasciculata	1	Chamaecrista fasciculata	1	Chaemcrista fasciculata	1
Oryza sativa	1	Carex spp.	3	Cyperus spp.	2	Cyperus spp.	2	Cyperus spp.	2
Panicum capillare	2	Chamaecrista fasculata	1	Elymus spp.	1	Eragrostis sp.	1	Elymus spp.	1
Panicum spp.	4	Cyperus spp.	2	Eragrostis sp.	1	Lespedeza cuneata	1	Lespedeza cuneata	1
Panicum virgatum	2	Lespedeza cuneata	1	Lespedeza cuneata	1	Ludwigia alternifolia	3	Oryza sativa	2
Polygonum punctatum	2	Oryza sativa	1	Orzya sativa	1	Panicum capillare	3	Panicum capillare	2
Sporobolus spp.	2	Panicum capillare	3	Panicum capillare	3	Panicum spp.	1	Panicum spp.	2
Verbena sp.	3	Panicum spp.	2	Panicum spp.	1	Panicum viragatum	2	Panicum virgatum	1
		Polygonum punctatum	2	Polygonum punctatum	2	Poaceae	3	Polygonum punctatum	2
		Schizachyrium scoparium	1	Schizachyrium scoparium	1	Polygonum punctatum	2	Schizachyrium scoparium	1
		Sporobolus spp.	2	Unknown	1	Rudbeckia hirta	1	Sporobolus spp.	2
		Verbena sp.	3	Verbena sp.	2	Schizachyrium scoparium	1	Verbena sp.	3
						Sporobolus spp.	2		
						Verbena sp.	2		

F6	#	F7	#	F8	#	F9	#	F10	#
Andropogon spp.	1	Andropogon spp.	1	Andropogon spp.	1	Andropogon spp.	1	Andropogon spp.	1
Bidens spp.	1	Bidens spp.	1	Bidens spp.	1	Bidens spp.	1	Bidens spp.	1
Carex sp.	3	Carex sp.	3	Carex spp.	3	Carex spp.	3	Carex spp.	3
Chaemaecrista fasciculata	1	Chamaecrista fasciculata	1	Chamaecrista fasciculata	1	Chamaecrista fasciculata	1	Chamaecrista fasciculata	1
Cyperus spp.	2	Cyperus spp.	2	Cyperus spp.	1	Cyperus spp.	2	Cyperus spp.	1
Lespedeza cuneata	1	Elymus virginicus	1	Eragrostis sp.	1	Ludwigia alternifolia	3	Lespedeza cuneata	1
Oryza sativa	2	Leersia oryzoides	1	Lespedeza cuneata	1	Oryza sativa	1	Panicum capillare	2
Panicum capillare	1	Lespedeza cuneata	1	Oryza sativa	1	Panicum capillare	2	Panicum spp.	1
Panicum spp.	2	Ludwigia alternifolia	2	Panicum capillare	1	Panicum spp.	2	Polygonum punctatum	2
Panicum virgatum	1	Panicum bergii	2	Panicum virgatum	1	Panicum virgatum	2	Schizachyrium scoparium	1
Polygonum punctatum	2	Panicum capillare	2	Polygonum punctatum	1	Polygonum punctatum	2	Sporobolus spp.	2
Schizachyrium scoparium	1	Panicum virgatum	1	Rudbeckia hirta	1	Rudbeckia hirta	3	Verbena sp.	1
Sporobolus spp.	2	Polygonum punctatum	2	Sporobolus spp.	2	Sporobolus spp.	2		
Verbena sp.	3	Rudbeckia hirta	2	Verbena sp.	2	Verbena sp.	3		
		Senecio vulgaris	2						
		Sporobolus spp.	2						
		Verbena sp.	3						