Methods - Aboveground Harvest - M.E. Kubiske

2009 Field Season – Final Harvest

Our analytical tree biomass harvest will provide the following information for each ring section (our main experimental unit): total above- and belowground biomass, biomass of individual plant organs, and total leaf area. In addition, we will be able to determine above- and belowground biomass components by species and aspen genotype.

Beginning with leaf-out in 2009, we will fumigate the experiment with the treatment gasses for *ca*. 4 weeks (*e.g.*, mid May to mid June). After which, two field crews will begin working simultaneously to harvest two rings within one block. We will harvest our experiment on a block-by-block basis. The first two rings to be harvested from each block will be chosen randomly. Julian date at the beginning and end of harvesting activities in each ring will be recorded and used as a potential covariate to account for variation due to change in phenological state throughout the harvest.

Within the treatment core area of each ring, we will randomly delineate areas that encompass 4m x 4m in the aspen-only community and 4m x 2m in the mixed-species communities (Figure 1). These delineated areas will define the trees that will be harvested both above- and below-ground. *We will simultaneously harvest the above- and belowground tree portions in the delineated areas of each ring section, working in a block-by-block fashion until the entire experiment is harvested.*

<u>Coordination with the belowground harvest</u>*:* Harvesting and measurement of belowground biomass will be the exclusive responsibility of Drs. D.R. Zak (University of Michigan) and K.S. Pregitzer (University of Nevada – Reno; letter of collaboration attached). Their procedures were thoroughly described in a proposal submitted to the US DOE Office of Science by Karnosky *et al*. in 2007. Briefly, they will excavate a volumetric 4m x 4m x 1m-deep pit within the core area of each aspen-only ring section, and a 4m x 2m x 1m-deep pit in each mixed-species ring section, to determine coarse and fine root biomasses and their depth distributions. The belowground harvest protocol has been carefully planned to coincide with, and complement, the above-ground harvest described here.

The below-ground harvest will consist of two field crews, operating in tandem. They will use a backhoe (provided by the FS) to excavate 4x4 and 4x2 m plots from which all the trees were cut and removed the previous day. They will utilize a FS field laboratory, located at the Aspen FACE site, to extract all the roots from the excavated soil. A dry-run of the concurrent above- and below-ground harvest procedures is scheduled for June 2&3, 2008.

Aboveground Biomass

Each 4x4 m plot in the aspen monoculture, and 4x2 m plot in the mixed species sections, will, hypothetically, contain 16 and 8 trees, respectively (Figure 1). In reality, due to mortality since the trees were planted, a number of trees in each sampling plot will be either missing or dead. Our assumption is that the density and composition (genotype & species) of the delineated plots will be representative of that for the core area of each ring section. *For consistency with the belowground harvest in terms of tree density, only trees within the delineated 4x4 m or 4x2 m plots will be used for calculating above- versus below-ground biomass partitioning for hypothesis 2.* However, our target sampling intensity to fully characterize variation in above-ground allometry for hypothesis 1 is 3 trees of each aspen genotype, and 3 of each species from the two mixed-species ring sections. If the numbers of trees located within each delineated sampling plot do not achieve our sampling target, additional trees of the appropriate genotype or species will be randomly selected from the remaining core area of each ring section.

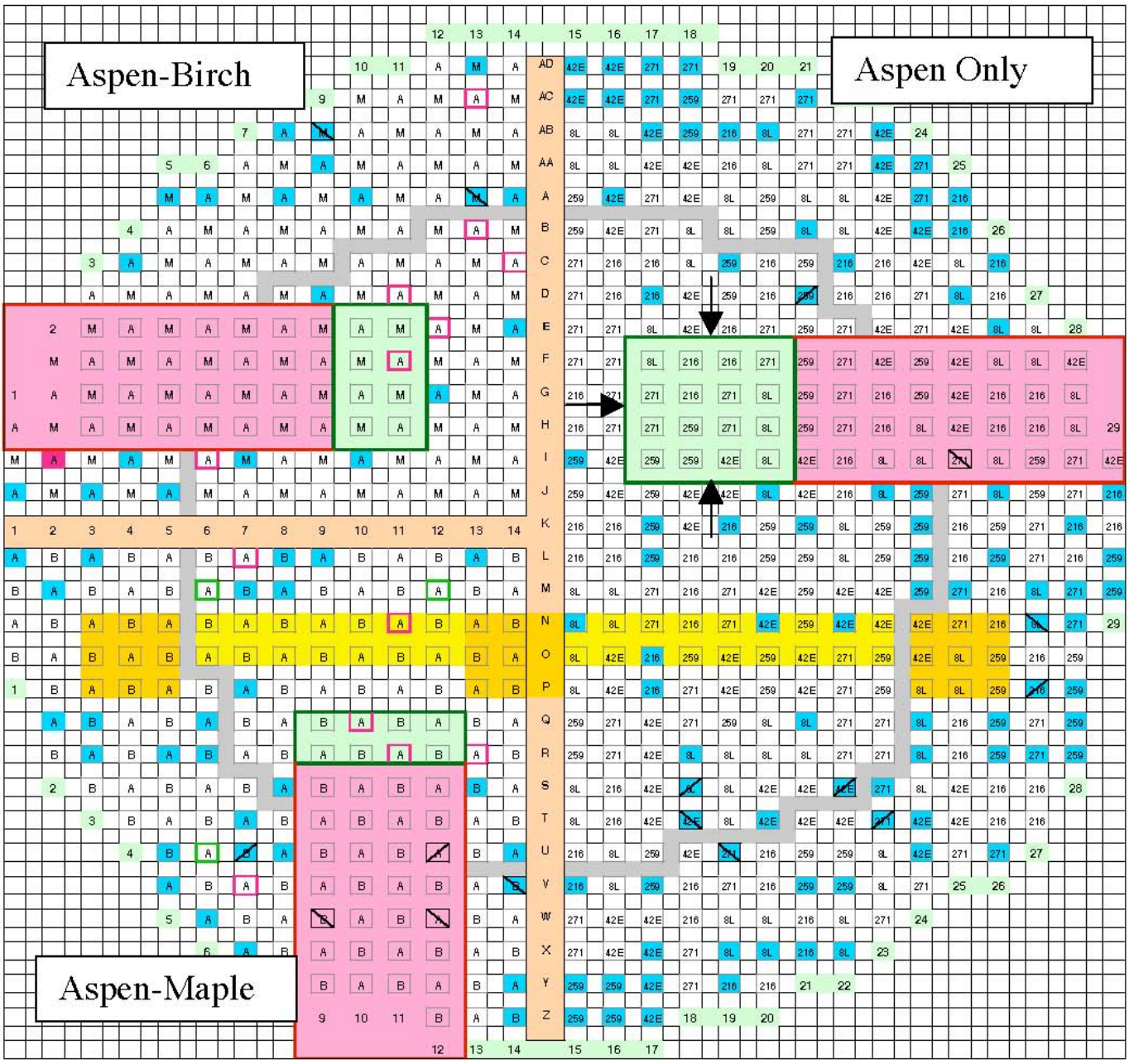

Figure 1. Schematic of a treatment ring at Aspen FACE showing exact locations of tree species (left half, A = aspen clone 216, M = maple, B = birch) and aspen clones (right half). Each tree is identified by its location in a grid of letters (rows) and numbers (columns) and by a unique ID number. Also shown are 3 ring sections divided by wooden walk-ways (tan), location of canopy access scaffolds (yellow & orange), delineation of the treatment "core" area where treatment gas concentrations are most stable (gray), and missing or dead trees (blue). Example locations of cleared access paths are shown as pink rectangles and delineated 4x4 m and 4x2 m harvest plots as green rectangles for the final harvest in 2009. The arrows show the locations of horizontal cores for sampling fine roots to be extracted from the exposed face of the 4x4 pit. Cross-hatched trees were removed in 2000 & 2002.

Harvesting and processing of aboveground biomass will closely follow methods used in a partial harvest in 2007 (unpublished), which were modified from prior partial harvests in 2000 and 2002 (King *et al.* 2005). From these methods, described here in detail, we will have detailed information of bark, wood, and total stem mass; bark wood, and total branch mass; and leaf mass and area by annual height growth increment for each aspen clone and for paper birch and sugar maple. These variables will be used to create allometric relationships using total tree height and diameter at breast height (1.3 m) measured on all core trees, which will then be used to estimate biomass and leaf area by ring section, species, and genotype. Thus, prior to harvesting each ring, we will measure height and diameter of all core trees in each respective ring, which will provide the basis for scaling biomass and allometry to the stand level.

In addition, we will collect samples for wood quality analysis (Kaakinen *et al.*, 2004, Kostianinen *et al.*, 2007), photographs of tree crowns, and fine root samples for root DNA fingerprinting.

Field Work: We will harvest all living trees in each delineated sample area. On each tree to be harvested, we will label the 1.3 m line (DBH) and draw an upward-pointing arrow on the north aspect for photographs (Figure 6). All reachable dead branches will be removed and placed in a plastic bag. The trees will be cut at ground level and carried out of the stand by hand.

Outside of the rings, we will lay the cut trees on a plastic tarp and measure the total length and the length of the live crown. We will label the annual height-growth increments (HGI) on the main stem, starting at the top and ending with the earliest full HGI containing live crown; the main stem will be severed at that point. HGIs will be visually identified by examining bud scars and branching patterns. Next, we will collect 20 fresh, fully expanded leaves (about 8-10 g) from each HGI, immediately immerse them in liquid N, and store them on dry ice for transport to the lab. These leaves will

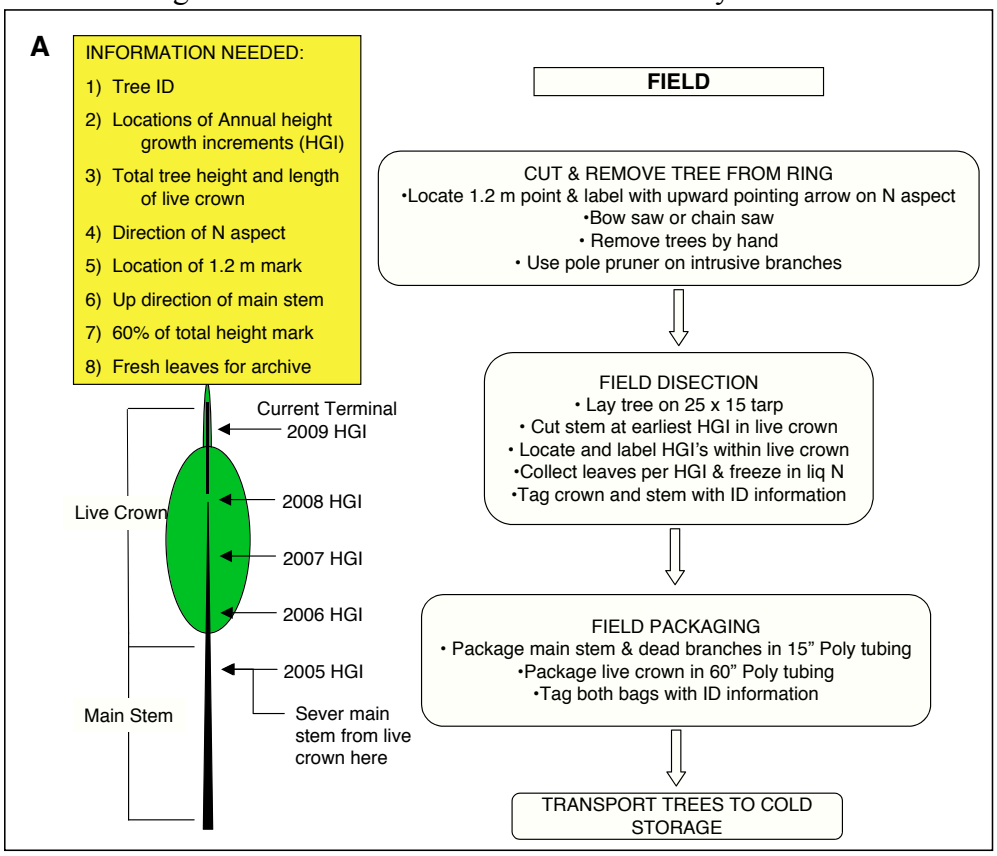

Figure 6. A) Flow chart showing field processing of 2009 harvest trees at Aspen FACE. B) Packaging harvested trees during the 2007 analytical harvest.

then be archived at –80C. Both the lower main stem and the intact live crown will be enclosed in a sheath of 8 mil polypropylene, which will be closed at each end with cable ties. Trees will be transported on a flatbed trailer and placed into cold storage prior to processing at the lab, which will occur within 24 hrs. In 2007, the longest a harvested tree remained in cold storage was 21:55. Most trees were processed within 1-5 hours of being cut. We will operate two harvesting crews in tandem, one in each of two treatment rings.

Laboratory Work One at a time, trees will be removed from cold storage and will enter a "production line" system of measuring, dissection, and sampling (Figure 7). We will operate two production lines in tandem. Harvested trees will *not* be assigned to one production line or the other.

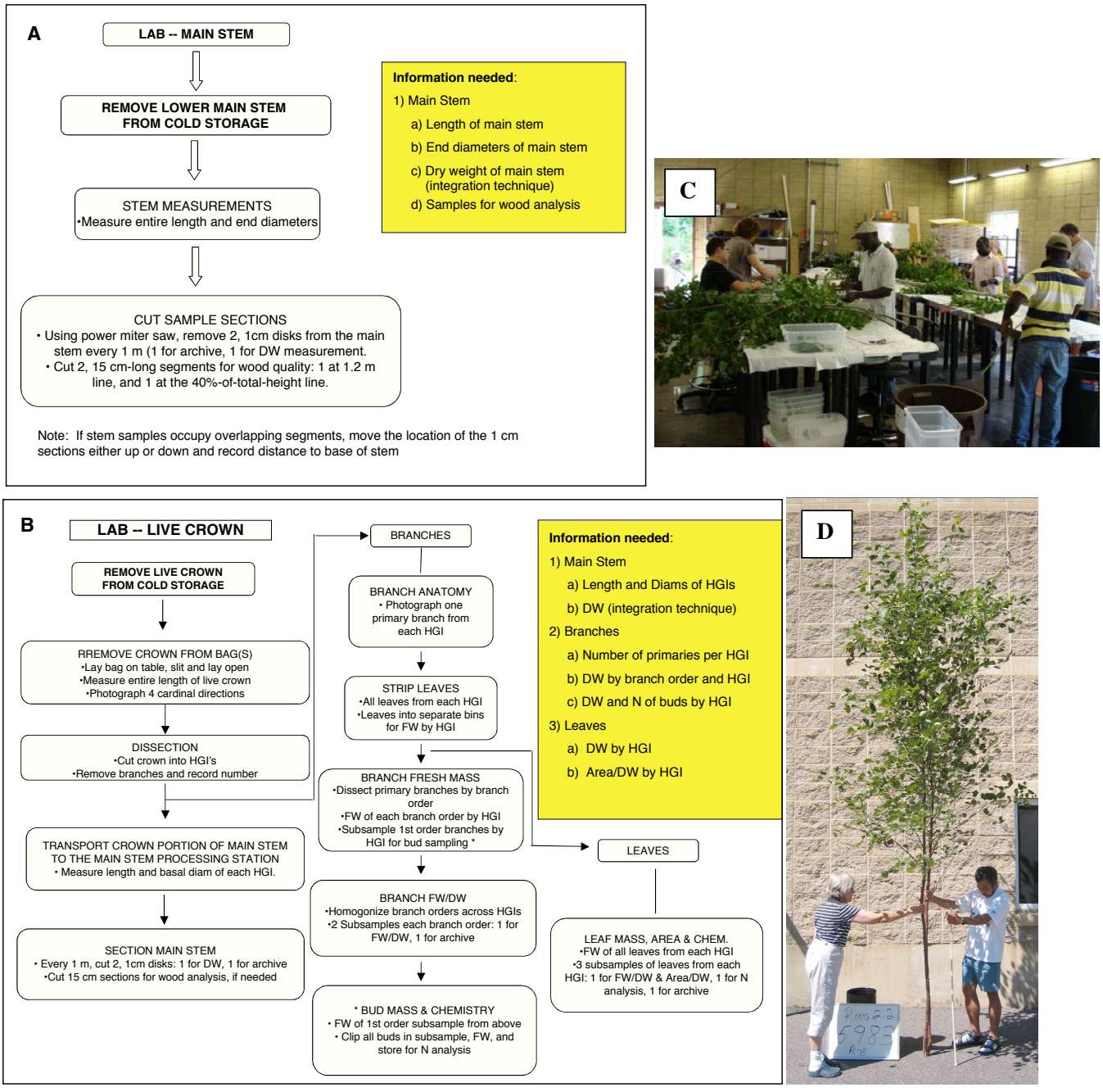

Figure 7. A) Flow chart showing processing of the lower (non-live crown) portion of the main stem. B) Flow chart showing processing of the live crown. C) Dissecting the live

From the lower (non-live crown) main stem, we will cut wood samples using a power miter saw: two, 15 cm-long sections located at the 1.2 m line and at 40% total height for wood properties analysis (Figure 7A). These sections will be frozen. This sampling protocol was used previously for wood properties analysis at Aspen FACE by E. Vapaavuori *et al.* (Kaakinen *et al.*, 2004, Kostianinen *et al.*, 2007), who will analyze wood properties on these samples, also (letter of collaboration attached). We will also cut 2, 1-cm-long sections at 1-m increments along the lower main stem and at the midpoints of each HGI in the live crown. In the event sections to be cut will otherwise overlap, the 15 cm-long wood analysis sections will have location priority and the 1-cm-long dry mass and archive samples will be cut as close as possible to their intended positions, making note of their actual distance from the cut base of the stem.

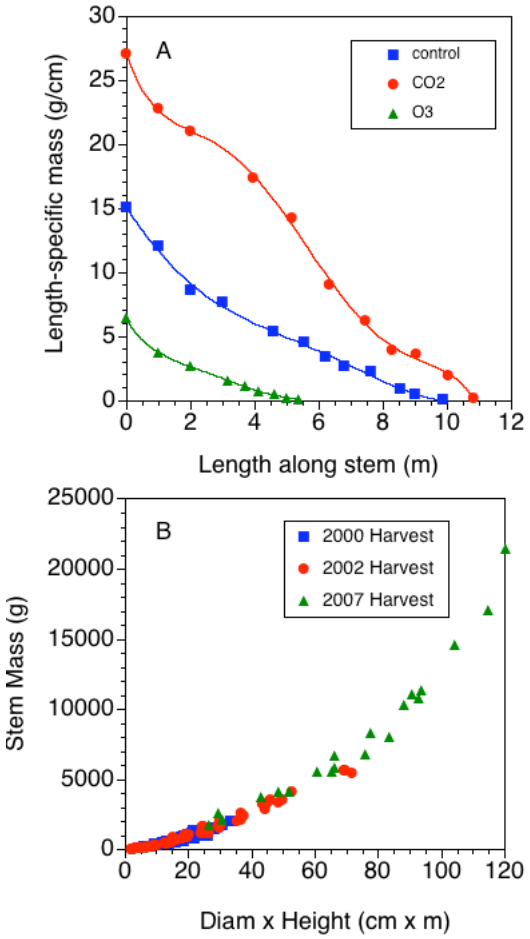

Figure 8. Stem mass data from partial harvest at Aspen FACE in 2007. A) Examples of length-specific stem mass functions from three trees. Total stem mass (g) of each stem is the area below the curves. To obtain the area, a 5th order polynomial is fit and then integrated. B) Stem mass versus independent Diameter x Height measurements, from three harvests. Stem Masses in 2000 and 2002 were determined by oven drying and weighing entire stems. Mass in 2007 was determined by curve integration as in panel A..

The stem disks will be frozen. One set of the disks will be remain frozen in archive, and the other used for determining main stem dry mass and volume. We will use procedures similar to those used in the 2007 partial harvest. The disks will be thawed, and diameters measured with a diameter tape. We will measure their volumes by displacement of water. Because the disks will not be precisely 1 cm thick, disk volume and diameter will be used to calculate the mean thickness, or length, as: l = volume/(πr^2). This is needed to standardize disk mass and volume to 1 cm of stem length for the calculations below. We will remove bark, cambium, and phloem from the wood and oven dry them at 65 °C for 48 h. We will then plot length-specific stem mass (M; g cm^{-1} stem length) of the wood and bark portions of each disk versus height above ground (h), and integrate M with respect to h from 0 to total tree height (ht) to obtain total stem wood and bark dry mass (Figure 8):

$$Total\ stem\ mass = \int_0^{ht} M \frac{dm}{dh}$$

This method for estimating total stem mass of each harvested tree is accurate and cost effective. It is superior to either oven drying all the tree stems, which is impractical with trees this large, or using published allometric equations, which would obscure the unique characteristics of each stem (Figure 8A).

A similar procedure will be used for determining stem volume, using volumes of the wood disks determined from displacement of water:

$$Total\ stem\ volume = \int_0^{ht} V \frac{dv}{dh}$$

Where V is length-specific stem volume (cm^3 cm^{-1} stem length).

The live crown will be photographed from the 4 cardinal directions using a high-resolution digital camera. The crown will be held vertically against the

outdoor side of the building providing a solid background (Figure 7D). A dry erase board with ID information and 1 m scale will be included with each photograph. The photographs will preserve the visual image of each tree. Following photographs, the live crown will be taken to the processing room (Figures 7B&C). We will remove all primary branches (we define a primary branch as one that connects directly to the main stem) from the main stem and count them by HGI, keeping them separated by HGI. The upper (live-crown) main stem will go to the main stem processing station described above (Figure 7A).

For each HGI, we will remove all leaves, leaving branches intact. We will remove three subsamples of leaves of each HGI: one for leaf area and specific leaf area determination (subsample 1; about 20 leaves), one for N analysis (about 20 leaves), and one for archival purposes (about 150 g). We will measure fresh weight (FW) on the bulk HGI sample and the three subsamples simultaneously, so that moisture content will be consistent among the four pools of leaves (bulk HGI pool and three sub samples). From subsample 1, we will determine DW/FW and Leaf Area/FW, which will enable us to calculate total leaf DW and leaf area for each HGI.

We will individually photograph all intact, leafless primary branches with a high-resolution digital camera. After photographing, we will dissect the primary branches into annual growth segments numbering them as 1^{st}, 2^{nd}, 3^{rd}, etc., order branches, counting from distal to proximal ends, so that the current year, leaf-bearing shoots will be 1^{st} order branches, the previous year's leaf-bearing shoots will be 2^{nd} order branches, etc. We will measure the FW of each branch order for each HGI; any 1^{st} order branches exhibiting indeterminate growth will be weighed separately.

After weighing each branch order by HGI, we will combine HGI's for each order, homogenize, and remove a subsample of at least 10% of the branches from each branch order pool. We will then measure the subsamples for FW/DW which will allow us to determine the DW of each branch order by HGI. Paper birch branch morphology is somewhat different from the other two species, wherein leaves occur on both short shoots and long shoots. Long shoots are the current year terminals of each branch, and short shoots occur as spurs along previous years' growth. The spurs will be treated the same as first order branches. Any flowers or flower buds will be collected from the branches and frozen by HGI for later analysis. If any seed-bearing catkins remain on the birch trees, they will be collected for later analysis which could include germination rate, mass, and chemistry (Hussain *et al*. 2001, Darbah *et al*. 2007).

Root DNA fingerprinting - Because the aspen stands at the Aspen FACE site are a mixture of five clones, we will use highly discriminating microsatellite markers to generate distinct DNA fingerprints for each aspen clone (Brunner *et al*. 2004; Saari *et al*. 2005). This will enable us to identify fine roots of individual clones, to estimate belowground biomass of each clone, and, coupled with our aboveground allometry, will facilitate our analysis of clonal competitive interactions.

The genetic basis for each of the five aspen clones will be determined by generating unique reference DNA fingerprints from aspen bud and leaf tissue collected during the 2009 growing season. Using the polymerase chain reaction, genomic simple-sequence-repeat variation will be surveyed with ten-microsatellite markers isolated from trembling aspen. We will use 4 loci identified by Dayanandan *et al*. (1998) to distinguish among 32 aspen genotypes, and will augment our tests with up to six additional microsatellite loci developed by Rahman *et al*. (2000) to uniquely identify our 5 genotypes. Genotyping will start with the most variable of the 10 loci and proceed until we obtain discriminating fingerprints for each clone. Individual root fragments isolated from root cores will be genotyped and compared to the reference genotypes. The fine

root biomass for individual clones will then be estimated using the relative abundance of each genotype compared to all aspen fine root fragments identified (Brunner et al. 2004).

Three, 10 cm x 10 cm horizontal cores will be extracted from three exposed faces of each volumetric 4x4 m pit in the aspen-only sections (Figure 1). The cores will be located in the same position in each treatment ring, with randomization being provided by the unique placement of clones in each ring, and the random distribution of fine roots. The three cores will be homogenized, and a subsample of fine roots collected. We will make no distinction between suberized and non-suberized fine roots. The root material will be placed in plastic bags, moistened with water and stored at 4 ºC until the fresh tissues can be transported to the lab. Each sample will be sorted carefully using forceps and frequent washing with water to separate the individual root fragments. We will determine the total fresh mass of roots in each sample, and the fresh mass of individual root fragments. Individual root fragments will then be subjected to DNA extraction and genotyping protocols adapted from Marquardt et al. (2007), and Marquardt and Epperson (2004). We will then be able to determine, as a proportion of total sample mass, the fine root mass of individual aspen clones. Results from this part of our proposal will be provided to Zak & Pregitzer for use with their root biomass data.

Methods - Belowground Harvest - D.R. Zak and K.S. Pregitzer

Once the aboveground biomass is removed in a ring section, we will excavate a volumetric soil pit to a depth of 1 m within the harvested area to determine coarse and fine root biomass, as well as their depth distribution. Pits will be 4 m x 4 m in the aspen-only section and 4 m x 2 m in the mixed species sections. The protocol will follow that developed by King et al. (2007), and the pit will be excavated to a depth of 1.0 m. The root crown of any tree that lies within the surface dimension of a pit will be excavated by hand; its fresh weight will be determined, and a 100 g subsample will be removed for dry weight determination. The remaining belowground plant material will be removed using a small tracked back hoe, and all soil removed from the pit will be transported to an area just outside the FACE ring and then passed through a mechanically-driven soil screen specifically built to sieve large volumes of excavated soil. The openings of the soil-screen mesh are rhomboid and measure 1.5 cm x 4 cm. This large mesh allows for rapid soil sieving without significant loss of coarse root material (King et al. 2007). Coarse root systems will not be divided into size classes, and we will recover roots > 1 mm in diameter using this mechanical device. Pregitzer et al. (2000) has shown that virtually all the roots of *Populus tremuloides* > 1.0 mm is diameter are woody and have undergone secondary thickening. The roots of *Betula papyrifera* are similar to *Populus tremuloides* in terms of branching morphology and diameter distribution, and the roots of *Acer saccharum* are more finely divided. Greater than 95% of the belowground biomass will occur in roots > 1 mm in diameter (Pregitzer et al. 1990, 2000); and the mechanical sieving approach will effectively recover this plant material from soil.

Once the pit has been excavated, we will use a square core (10 cm x 10cm), which will extend 10 cm into the pit face; it will be used to determine the mass and depth distribution of fine roots. We will extract square cores in 10-cm increments from the surface of the mineral soil to the bottom of the pit (1.0 m); a set of cores will be collected randomly on each of the four faces of each pit. Roots will be sorted by hand into two size classes, < 1.0 mm and > 1.0 mm. A subsample of the remaining soil will be elutriated to recover the remaining fine roots (*sensu* Pregitzer et al. 2000); fine root subsamples will be oven dried at 65 ^{o}C. We will archive 10-g subsamples of each root class in frozen and dry states. Subsamples of root-free mineral soil (see

Loya et al. 2003) from each 10-cm depth increment will also be analyzed for C, N ^{13}C and ^{15}N (4 pit faces x 10 depth increments x 3 community types per ring x 12 rings =1,440 soil samples). Adjacent to the place where we sample roots and mineral soil along each pit face, we will also use a soil core (10 cm x 10 cm) to determine mineral soil bulk density. Samples of root-free mineral soil from each depth increment will be air-dried and archived.

In the aspen-birch and aspen-maple ring sections, we will be able to identify coarse roots by species (*Populus*, *Betula*, *Acer*, and understory) using their outward morphology (bark, color, woody vs. non-woody, etc.). However, it is not possible to distinguish among the coarse roots of aspen genotypes, nor will it be practically possible to distinguish among the fine roots of the different species, although it is physically possible because their morphology is quite distinct. Inasmuch, we will be able to estimate the mass of fine roots by each community type, and we will also be able to roughly estimate the mass of the coarse roots of each species in the mixed-species communities. Estimates of belowground fine root biomass of each aspen clone will be obtained will be made post-harvest using the root DNA fingerprinting described previously.

Sample Archive

We will archive air-dried and frozen subsamples of leaves, branches, stemwood, coarse roots and fine roots harvested from each ring section. Frozen leaves and fine roots will be stored at -80^{o}C, and the other plant parts will be stored at -20^{o}C. The plant samples archive will be maintained at the USFS lab in Rhinelander. We anticipate archiving 100 g of air-dried material of each plant component, except fine roots. We will archive all of the fine root samples that are not used for the analyses described above and we anticipate the mass will be substantially less than 100 g of oven-dried tissue. In addition, we will freeze 5 separate 10 g samples of each plant component collected in each ring section, again, except for fine roots. This will enable us to provide 5 potential users with freshly frozen tissue from our experiment; we have selected this number due to anticipated demand and the constraints of freezer space. We will also archive air-dried (100 g) and frozen (5, 10-g subsamples; -80 ^{o}C) forest floor samples for future chemical analyses as well as molecular analyses of microbial communities. Five 10-g subsamples of surface soil (0 to 20 cm) collected in 2008 will be stored at - 80 ^{0}C for future molecular analysis of microbial communities. It is our experience that the integrity of microbial DNA is maintained longer when forest floor and mineral soil are stored at - 80 ^{0}C; this will enable future users of this material to extract DNA directly. Additionally, we have archived DNA extracted from forest floor and surface mineral soil on three sampling dates during the 2007 field season. This material is currently stored at the University of Michigan, and D.R. Zak will make it available after it has been analyzed for microbial community composition and function. Forest floor and soil archives will be maintained at D.R. Zak's lab at the University of Michigan. A database of archived samples will be posted on the Aspen FACE website which will be maintained for 5 years following decommissioning of the site.

Data Archive

All data and metadata from our harvest will be archived at the CDIAC FACE data archive http://public.ornl.gov/face/index.shtml and the Aspen FACE website http://aspenFACE.mtu.edu and will be available for use by other researchers per our usual on-line data request protocol.

2010 Field Season

We will remove the aboveground biomass of all trees in the experiment during the 2010 field season as we prepare to decommission the Rhinelander FACE site. We will hire professional chain saw operators to sever each remaining tree, which will be carried out of the ring by hand. We will measure total height and length of the live crown. The live crown will be severed from the lower stem, and photographed at the four cardinal directions as described above. The entire tree will then be discarded.