**Drying and rewetting effects and the effect of storage on the radiocarbon signature of heterotrophic respiration in laboratory soil incubations**

# Introduction

The laboratory soil incubation is a commonly used technique for understanding soil carbon dynamics. Soil carbon is a heterogeneous mixture of organic matter, some of which persists in the soil for months or years, while some persists for centuries or millennia. The persistence of soil carbon can be understood through the concept of different “pools” of carbon that are defined by the mechanism by which they persist in the soil and are characterized by distinct age distributions.

Natural abundance radiocarbon provides information about carbon dynamics on centennial or millennial scales, while insight into decadal scale dynamics can be gained from tracing the pulse of radiocarbon introduced into the biosphere from nuclear weapons testing (“bomb-C”) in the mid-20th century. The bomb-C pulse peaked in the atmosphere in the 1950s (Fig. 1), but due to differential rates of abiotic incorporation and biological processing, the peak is lagged in time and dampened in soils. The relative enrichment in bomb-C in different pools of soil carbon is a useful tool for inferring the relative rate at which carbon enters and leaves the pool, and for a homogenous pool it is functionally equivalent to the intrinsic decomposition rate.

Extracting and measuring the radiocarbon content of specific soil carbon pools is hampered by spatial and temporal heterogeneity of the mechanisms that lead to soil carbon persistence, such as physical occlusion in aggregates, association with minerals, or chemical recalcitrance. Defining soil carbon pools empirically with techniques such as density, size, or resistance to chemical attack can be useful, but these methods also introduce artifacts and likely result in mixtures of pools with different age distributions. In contrast, although they also introduce artifacts due to disturbance and potential alteration of the microbial community, laboratory soil incubations make use of the same fractionation agent as is found *in situ*: the microbial community. Measuring the radiocarbon signal of CO2 (Δ14C-CO2) released in laboratory incubations of bulk soils is a powerful tool for understanding the relative processing rate of carbon in soil (or transit time) as it provides an integrated measure of the weighted contribution to the release flux from pools of soil carbon with distinct processing rates.

Calculating ages and transit times of soil carbon from Δ14C requires the use of a model. However, parameterizing these models is challenging, both due to the uncertainty of the persistence mechanisms themselves as well as a lack of observational constraints. Radiocarbon observations at a single point in time are very useful, but due to the curvature of the bomb-C peak there are two points in time with the same atmospheric radiocarbon value, leading to multiple model solutions. Observations of Δ14C-CO2 at multiple points in time can greatly reduce model uncertainty by serving as additional constraints.

Air-drying soils for storage in archives is a common practice of convenience with long-recognized effects on biological, physical, and chemical properties (Bartlett and James, 1980). Soil archives have proved to be a valuable resource for looking at the change in soil carbon over time, with the most extreme example being the >150 year archives from the Rothamsted long-term experiments, used for parameterizing the well-known soil carbon model Roth-C (Jenkinson et al., 2002). However, the effect of air-drying, storage, and subsequent rewetting on 14C-CO2 observed in soil incubations has not been documented.

Following air-drying and rewetting, most soils exhibit a characteristic rapid increase in CO2 production, before returning to equilibrium respiration rates. The mechanism or mechanisms driving this pulse of CO2 have been extensively studied over the past several decades. The source of the CO2 released in the rewetting pulse has been hypothesized to come from the lysis of microbial cells subjected to osmotic shock (Williams and Xia, 2009; Warren et al 2014), disruption of soil aggregates, osmolytes released from microbes emerging from aridity induced dormancy (Fierer and Schimel, 2002/2003), desorption of mineral-associated organic matter, or a combination of these sources. [cf, Jones et al., 2019: CO2 release as a function of storage duration; only a shift in the timing of rewetting pulse].

Air-drying has been shown to result in the formation of new or stronger mineral-organic associations, increased aggregate stability, decreased microbial biomass, and a higher quantity of water-extractable organic matter. Air-drying and rewetting effects appear to be soil-specific, with desorption of minC upon rewetting observed for smectite-rich or highly charged soils, and differences in the quantity and rate of CO2 release following rewetting varying with soil texture and degree of aggregation (Kaiser et al., 2014).

During short-term incubations, the majority of CO2 can be assumed to derive from the substrates consumed by the microbial community *in situ*. In longer duration incubations, the lack of new inputs to the system is assumed to lead to shifts in substrate utilization, from easily accessible, shorter-cycling pools to less accessible pools, i.e. protected from decomposition in some manner (Schädel et al. 2020). If the relative contribution to respiration from soil organic matter pools with different intrinsic cycling rates changes in a short-term incubation following air-drying and rewetting, this should be detectable in the 14C-CO2.

For example, disruption of soil aggregates following drying and rewetting would likely lead to greater availability of soil organic matter formerly protected from decomposition via physical occlusion. The effect on 14C-CO2 would be to increase the contribution to respiration from this relatively slower soil organic matter pool. However, if the rewetting pulse derives mainly from lysed microbial cells or the release of microbial osmolytes little change in 14C-CO2 would be expected.

The promise of improving soil carbon models by obtaining 14C-CO2 measurements from archived soils is tantalizing, but first the possible effects of air-drying and rewetting, as well as the effect of the duration of storage, must be quantified. The direction and magnitude of any change in 14C-CO2 induced by these disturbances should be indicative of the change in substrate, i.e. increased contribution of either faster or more slowly cycling carbon pools.

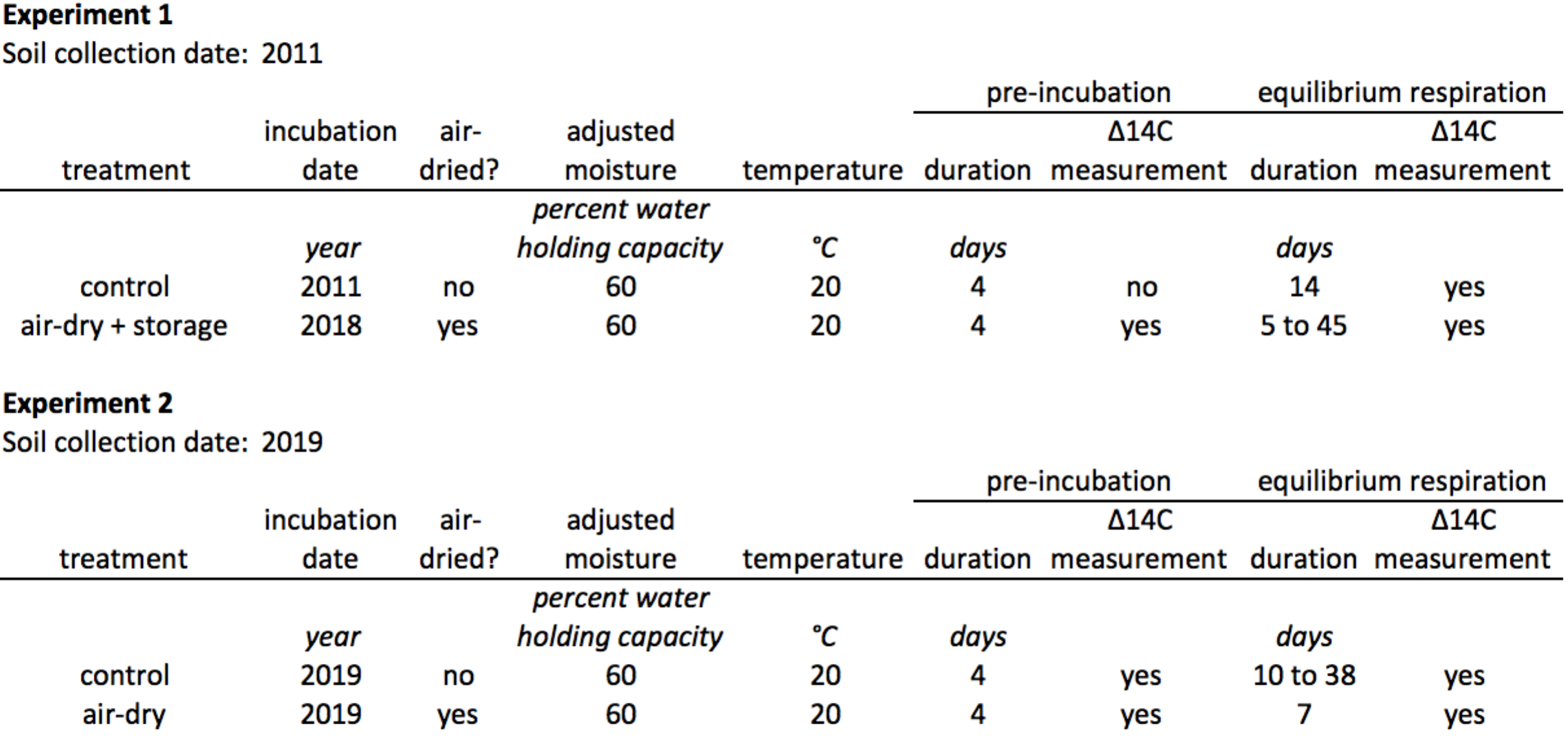
We developed the following hypotheses regarding the potential effects of air-drying and rewetting, and storage duration, on 14C-CO2 observed in laboratory soil incubations:

1. Air-drying and rewetting will lead to transient mobilization of a small pool of slower cycling carbon, shifting the 14C-CO2 of the CO2 pulse released immediately following rewetting;
2. 14C-CO2 released during the equilibrium respiration period will not be significantly different from that of control samples rewet from field-moist conditions;
3. Storage duration will not affect 14C-CO2 observed in equilibrium period respiration.

# Methods

We devised three experiments to assess the feasibility of measuring 14C-CO2 in incubations of archived soils. Experimental conditions for the first two experiments, looking at air-drying and rewetting in combination with storage, and at the effect of air-drying and rewetting alone (i.e. without the storage effect), are described in Table 1 (below). We conducted a third experiment to assess the impact of storage duration on observed 14C-CO2, but as the control sample incubations had been conducted by different investigators as part of different experiments, incubation conditions were more variable.

**Table 1.** Experimental design



## Sample selection and field sampling

Control incubations conducted for Experiment 1 (in 2011) were part of a larger study from the Biodiversity Exploratories project (Solly et al. 2014). We choose a subset of the samples for the present study to cover two ecosystem types (forest and grassland) and to span a range of soil textural classes, from the relatively sandy soils of the Schorfheide-Chorin geographic region to the more clay-rich soils from the Hainich-Dunn (Table 2 [sites, depths, texture, c/n, 13C, moisture]). We omitted samples that showed the presence of inorganic C during the control incubations using the δ13C signature: any samples with δ13C > -25‰ were considered as potentially affected by the release of inorganic C. We then selected three grassland and three forest samples from the interquartile range of 14C-CO2 observed in 2011 for each geographic region (n total = 12 sites).

For Experiment 2, we returned in July 2019 to the Hainich-Dunn region to collect new samples from the same sites that were originally sampled in 2011. As we did not observe significant treatment differences between the two geographic regions in the results of Experiment 1, we restricted the resampling to just one region to save on cost and time. As in 2011, for each plot three 10cm cores (0-10 cm depth) were collected and homogenized to yield one composite sample per plot. Any aboveground vegetation was clipped, and organic horizons were scraped away prior to coring at the forest sites.

Samples for Experiment 3 were obtained from the archives of S. Trumbore. Soils were originally collected from various locations around the United States and had been in storage for 4 to 14 years following the control sample incubations. All samples came from forest ecosystems, as no grassland samples were available. Owing to a lack of samples from deeper soil horizons, the samples included in this study were restricted to the A horizon only.

## Experimental conditions

### Experiments 1 and 2 (air-drying and rewetting, with and without storage)

In Experiment 1 we looked at the effect on 14C-CO2 from air-drying and rewetting in combination with storage (treatment: air-dry + storage). The control samples for this experiment were collected and incubated in 2011. 14C-CO2 from control sample incubations were then compared to a second set of incubations performed seven years later (in 2018) on splits of the original samples. Following sample splitting, treatment sample splits were air-dried and stored in sealed plastic bags.

A second experiment was designed to assess the effect of air-drying and rewetting directly, without the potentially confounding effect of storage. For this experiment additional soils were collected in 2019 from a subset of the sites sampled in 2011. After collection, soils were homogenized and split into two subsamples, one of which was air-dried (treatment: air-dry) prior to incubation, the other of which was incubated without air-drying (control).

Incubation conditions were the same for both experiment 1 and experiment 2. Soils were sieved to <2mm at field-moisture, and water holding capacity was determined on a subsample. Soils were weighed out as duplicates into 200 ml beakers and placed into 1L mason jars with airtight lids fitted with two sampling ports. Prior to sealing the jars moisture content was adjusted to 60% of soil water holding capacity (either from field-moist conditions for control samples or from an air-dried state for treatment samples). Following moisture adjustment, jars were flushed with CO2-free air and left to incubate for a four-day pre-incubation period. Following pre-incubation jars were flushed again, and CO2 was then allowed to accumulate for a second period under equilibrium respiration conditions.

Note that respiration rates had not yet reached equilibrium levels for the majority of samples by the end of the pre-incubation period, but as a four-day pre-incubation was used in the initial 2011 incubations for the control samples in experiment 1, we maintained the same duration for the treatment incubations in 2018 and for the air-drying and rewetting experiment conducted in 2019 (Experiment 2).

### Experiment 3 (storage duration)

We obtained soil samples from studies conducted in various laboratories over the past two decades (n total sites = 39). Since the control incubations were conducted in different laboratories by different investigators, incubation conditions such as temperature, quantity of soil incubated, and pre-incubation period duration varied. However, the moisture content for the treatment incubations, i.e. those conducted after air-drying and storage, were adjusted to the same level as in the control incubations. When possible treatment incubations were conducted in duplicate or triplicate, but owing to limited quantities of soil, single sample incubations were performed for some sites. Further details on incubation conditions, headspace gas sampling, and sample provenance for Experiment 3 are given in Supplementary Table 1.

## Headspace gas sampling

### Experiment 1 (air-dry + storage)

For control incubations, headspace CO2 concentrations were measured at the end of the pre-incubation period and then on days 1, 3, 7 and 14 during the equilibrium respiration period. For the air-dry + storage treatment incubations, headspace CO2 concentrations were measured daily during the pre-incubation, and on days 3, 5, and 7, then additionally on days 10, 38, and 45 for those samples that had not yet reached target CO2 concentrations.

CO2 concentration targets for the air-dry + storage treatment incubations were set to the amount of CO2 respired by the corresponding control sample during the equilibrium respiration period.

Headspace samples were collected and analyzed for 14C and 13C content at the end of the equilibrium respiration period for control incubations, but were collected after both the pre-incubation and equilibrium respiration periods for the air-dry + storage treatment incubations. However, only nine of the twelve samples respired adequate CO2 to measure 14C following the pre-incubation period for the air-dry + storage treatment incubations.

*Experiment 2 (air-dry only)*

Headspace CO2 concentrations were measured daily during the pre-incubation period for both control incubations and the air-dry treatment incubations. During the equilibrium respiration period for the control incubations, CO2 concentrations were measured on days 3, 5, and 7, and then weekly until adequate CO2 had been respired for measuring 14C. Similar to Experiment 1, CO2 concentration targets for the treatment incubations were determined by the amount of CO2 respired by the corresponding control samples during the equilibrium respiration period. Due to higher respiration rates, all air-dry treatment samples reached the CO2 targets after seven days of incubation, with CO2 concentrations measured on days 3, 5, and 7.

Headspace samples were collected and analyzed for 14C and 13C content after both the pre-incubation and equilibrium respiration periods for both control and air-dry treatment incubations.

### Experiment 3 (storage duration)

For the control sample incubations, CO2 concentrations were only measured during the equilibrium respiration period (with the exception of two samples, see Supplementary Table 1). Owing to the lack of pre-incubation respiration data, treatment incubation CO2 measurements were only made for a single period of the treatment sample incubations. Incubation vessels were sealed immediately following rewetting and samples were allowed to respire until an equivalent amount of CO2 had been released (per g of soil C) as during the control sample equilibrium respiration period. Headspace CO2 concentrations were measured every three days for the first two weeks of incubation, and weekly as needed thereafter.

## Additional measurements

We measured both organic and inorganic carbon content of all soils, as well as total nitrogen content and particle size distribution. Radiocarbon analyses were conducted at the Max Planck Institute for Biogeochemistry accelerator mass spectrometer facility (Experiments 1 and 2) or the University of California Irvine Keck Facility for Accelerator Mass Spectrometry (samples from USA, Experiment 3). All radiocarbon measurements are reported with respect to the international standards (Stuiver and Pollack 1977; Steinhoff...?).

## Statistics

We determined the statistical significance of treatment effects using paired t-tests (alpha = 0.05). In order to identify potential influences on the observed treatment effects we performed a linear regression analysis using the difference between treatment and control 14C-CO2 as the response variable, and the difference in CO2 respired (control – treatment), soil carbon and nitrogen content, change in moisture content upon rewetting, and particle size as explanatory variables.

# Results

## Respiration rates

### Experiment 1 (air-dry + storage treatment)

Respiration rates increased dramatically following rewetting for the air-dry + storage treatment in comparison to control samples, similar to what has been observed in other air-dry/rewetting studies [cite]. However, the magnitude and timing of the peak in respiration rates was significantly different between grassland and forest sites [statistics; other forest/grassland studies for comparison?] **(Fig. 3)**.

Among the air-dry + storage samples, respiration rates were more than twice as high in grassland soils than in forest soils, reaching a maximum of 3.8 mg CO2 g soil C-1 d-1 after 92 h, followed by a sharp decline. Mean respiration rates in forest sites peaked at 1.5 mg CO2 g soil C-1 d-1after 166 h, followed by a much more gradual decline than in grassland sites. Control samples responded more weakly and more gradually to rewetting, although as in the treatment samples respiration was greater in grassland soils than in forest soils. Peak respiration rates for control incubations were 1.9 and 0.6 mg CO2 g soil C-1 d-1 after 115 h for grassland and forest soils, respectively.

### Experiment 2 (air-dry treatment, 2019 samples)

Respiration rates for the air-dry only treatment samples showed a similarly dramatic increase in comparison to the controls as was observed for the air-dry + storage treatment samples in Experiment 1. However, unlike the air-dry + storage treatment, peak respiration rates were not significantly different (p > 0.05) between forest and grassland soils in Experiment 2, peaking at 3.0 and 3.3 mg CO2 g soil C-1 d-1 after 95 h for grassland and forest soils, respectively **(Fig. 3)**.

## Radiocarbon results

### Pre-incubation versus equilibrium respiration 14C-CO2

Despite the significant differences in respiration, and in contrast to hypothesis 1, we did not observe significant differences between 14C-CO2respired during the pre-incubation period and 14C-CO2 respired during the equilibrium respiration period: neither for the air-dry + storage treatment nor for the air-dry treatment alone **(Fig. 5)**. The interactions with land use was not significant nor was the interaction with experiment, so all data were pooled for statistical analysis.

in Experiment 1 since pre-incubation 14C-CO2 was not measured for these samples in 2011.

### Equilibrium respiration (Experiments 1 and 2)

Note the one outlier (forest, control) for which the pre-incubation CO2 was substantially depleted relative to equilibrium period respiration. However, even when this outlier was included in the statistical analysis, the difference between pre-incubation 14C-CO2 and equilibrium 14C-CO2 was not significant. Due to lower respiration rates during pre-incubation only three of the six forest samples in Experiment 1 generated enough CO2 to measure radiocarbon, and additionally, it was not possible to compare pre-incubation and equilibrium respiration 14C-CO2 for the control samples in Experiment 1 as pre-incubation 14C-CO2 was not measured for these samples in 2011.

### Treatment effects on observed equilibrium period 14C-CO2

Relative to the controls the air-dry + storage treatment (Experiment 1, open squares in **Fig. 5**) led to enrichment in grassland samples, but depletion in forest samples. In contrast, the air-dry only treatment (Experiment 2, open circles, **Fig. 5**) led to enrichment for both forest and grassland samples (2019 points). Treatment effects on 14C-CO2 were signifcant for both forests and grassland soils in Experiment 1 (2011 points, **Fig 5**), and significant for grassland samples but not forest samples in Experiment 2 (2019 points, **Fig. 5**). The absolute mean difference in 14C-CO2 between control and treatment samples was greater in grassland samples (21.4‰) than in forest samples (12.1‰) for both experiments.

ΔΔ14C of respired CO2 was enriched relative to the atmosphere for all samples in both experiments. Looking across experiments, the decline in 14C-CO2 between 2011 and 2019 paralleled that of atmospheric 14C for forest control samples and both control and treatment grassland samples, but was much smaller for the forest treatment samples.

### Effect of cumulative respired carbon on 14C-CO2

[maybe expand with stats for other explanatory factors? e.g. texture, N content, change in moisture upon rewetting, etc…]

We looked at the possible effect of the difference in the amount of carbon respired (mg CO2-C g soil C-1) on the differences between control and treatment 14C-CO2 using a linear regression model, but it was not significant overall. When data from Experiment 1 and Experiment 2 were considered separately, we observed a slight positive trend between the difference in respired carbon and the difference in 14C-CO2 within Experiment 2, but it was only marginally significant (p = 0.063).

## Treatment effect on 14C-CO2 for all samples (Experiments 1, 2, and 3)

Difference between control and treatment samples from all experiments show that treatment effects, i.e. air-drying followed by rewetting or air-drying followed by storage and subsequent rewetting, typically result in changes in 14C-CO2 between ±20‰ to ±40‰, with the majority within ±20‰. These difference are equivalent to the decline in atmospheric radiocarbon over 5 and 10 years, respectively, during the period of 2000 to 2020. The samples from Tennessee (magenta points) are an exception. However, these points do not show only bomb-C enrichment, but rather the results of exposure to a localized plume of 14C enriched CO2 from a nearby incinerator four years prior to sample collection (Trumbore et al., 2002). Treatment 14C-CO2 for these highly enriched samples were more depleted relative to the controls than were the samples only labeled with bomb-C.

Grassland samples tend to be above 1:1 line, while forest samples are generally below, regardless of origin. A notable exception to this trend are the three German forest samples that are above 1:1 line, which were analyzed in 2019 (air-dry only treatment).

# Discussion

The increase in respiration rates seen in this study following air-drying and rewetting align with what many others have seen (cite). However, the significant difference in the 14C of respired CO2 between the control and treatment samples in this study show that this increased respiration appears to be fueled by a different substrate source than would otherwise be available to the microbial community without air-drying and rewetting.

In contrast to our initial hypothesis, the 14C of respired CO2 respired immediately after rewetting (during the pre-incubation period) was not significantly different than what was observed later during the equilibrium respiration period. This suggests that the change in substrate availability initiated by air-drying and rewetting persists throughout the incubation. Previous researchers have found evidence for microbial osmolytes or lysed cells providing the fuel for the pulse of CO2 observed following rewetting of dried soils, but the results from this study suggest that either this pool of substrate is large enough to sustain respiration for....[quantify with percent of soil C pool] or that the newly available substrate incorporates organic material from other soil pools as well.

Air-drying and subsequent rewetting has a significant effect on the 14C of respired CO2, but our results show that the direction and magnitude of the trend is dependent on when the sample was collected and upon the type of ecosystem. Relative to un-dried control samples, respiration from forest soils analyzed in this study tend to show depletion in 14C-CO2 following air-drying and rewetting, while grassland soils show enrichment. The forest soils incubated in Experiment 2, which were collected in 2019, stand out as a counter example in that the air-dry and rewetting treatment lead to enrichment in 14C-CO2 relative to the controls. Yet the soils collected in 2011 from these same sites showed the same trend of depletion in response to treatment as observed elsewhere.

The switch from depletion to enrichment in 14C-CO2 following treatment, as observed in the forest soils from Central Germany between 2011 and 2019, could be explained by a corresponding shift in the relative enrichment of the more slowly cycling soil carbon pool in comparison to the fast cycling pool. This scenario is illustrated in Fig. 1 as a crossing of the slow and fast pool 14C curves between the (hypothetical) observation of the system in 1992 and 2019.

If we assume that the respiration flux is dominated by the fast cycling soil carbon pool, then control sample 14C-CO2 should decline at nearly the same rate or just slightly slower than atmospheric 14C, as is observed for the forest and grassland soils sampled in both 2011 and 2019 for Experiments 1 and 2. The relative increase in ∆∆14C-CO2 seen in the grassland soils suggests that there is a more slowly cycling carbon pool that is more enriched than the fastest cycling pool, and it is carbon from this more slowly cycling pool that is contributing more to respiration in treatment samples than in control samples. Similarly in forest soils, as can be seen from larger dataset shown in Fig. 7, the trends suggest that treatment leads to a greater contribution to respiration from a more slowly cycling pool of soil carbon. However, in forest soils collected prior to 2019, the depletion in 14C-CO2 observed in comparison to control sample incubations suggests that older carbon is being respired in forest soils in response to treatment than in grassland soils. This explanation is also consistent with what is seen in the highly enriched samples from TN: increased depletion in 14C-CO2 relative to the controls due to a much greater difference in 14C between the most recently fixed carbon and the older carbon in the soil.

Soils in this study spanned a relatively small range of storage duration, from 0 to 14 years, but within this range the duration of storage did not have a significant effect on the difference observed in 14C-CO2. To test this effect fully, it would be ideal to measure splits of the same sample at multiple points in time, but this was not possible within the confines of this study. The slight increase in the difference between control and treatment sample 14C-CO2 seen with increased duration of storage in the highly enriched samples from Oak Ridge, TN, analyzed in Experiment 3, suggests that some of the most recently fixed carbon may be preferentially lost over time. These samples were included precisely because the highly enriched label was concentrated in the most recently fixed carbon, and therefore should be a sensitive indicator of whether or not storage leads to losses. However, as the incubations conducted after 4 years of storage were done in a different laboratory under different conditions than the incubations after 14 years of storage, we caution that this may not represent a real trend.

Overall, the slight increase in the apparent age of respired CO2 seems to be consistent across the soils studied, but stronger in grassland soils than in forest soils. In the context of modeling applications, the differences in 14C-CO2 caused by air-drying and subsequent rewetting observed in this study would lead to shifts in apparent transit time of soil carbon by 5 to 10 years relative to estimates from incubations of soils that have not undergone air-drying. Depending on the needed resolution, this difference may be negligible, but future studies should consider the possible consequences of this shift. In conclusion, we believe the radiocarbon incubation technique for archived soils is promising approach for improving soil carbon models, and that the benefit of having observations of the system at multiple time points outweighs the slight shift in 14C-CO2 caused by the processes of air-drying, storage, and rewetting.