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Evidence for the role and fate of water-insoluble condensed tannins in the short-term reduction of carbon loss during litter decay

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Keywords

Poplar; Douglas-fir; proanthocyanidin; proximate chemistry; butanol-HCl; carbon sequestration

Abstract

Warmer temperatures associated with climate change have the potential to accelerate litter decay and subsequently release large amounts of carbon stored in soils. Condensed tannins are widespread secondary metabolites, which accumulate to high concentrations in many woody plants and play key roles in forest soil nutrient cycles. Future elevated atmospheric CO_2 concentrations are predicted to reduce nitrogen content and increase tannin concentrations in plant tissues, thus reducing litter quality for microbial communities and slowing decomposition rates. How the distinct condensed tannin fractions (water-soluble, acetone:MeOH-soluble and solvent-insoluble) impact soil processes, has not been investigated.

We tested the impact of condensed tannin and nitrogen concentrations on decay rates of poplar and Douglas-fir litter at sites spanning temperature and moisture gradients in coastal rainshadow forests in British Columbia, Canada. The three condensed tannin fractions were quantified using recent improvements on the butanol-HCl assay. Decay was assessed based on carbon remaining, while changes in litter chemistry were primarily observed using two methods for proximate chemical analyses.

After 0.6 and 1 year of decay, more carbon remained in poplar litter with high, compared to low, condensed tannin concentrations. By contrast, more carbon remained in Douglas fir litter than poplar litter during this period, despite lower condensed tannin concentrations. Rapid early decay was especially attributed to loss of soluble compounds, including water-soluble condensed tannins. Water-insoluble condensed tannin fractions, which were transformed to acid-unhydrolyzable residues over time, were associated with reduced carbon loss in high condensed tannin litter.

Abbreviations

acid-determined cellulose (ADC), acid-determined “lignin” (ADL), acid-hydrolyzable fraction (AHF), acid-unhydrolyzable residue (AUR), carbon (C), condensed tannins (CT), low-condensed tannin treatment (LT), low-nitrogen treatment (LN), high-condensed tannin treatment (HT), high-nitrogen treatment (HN), nitrogen (N), non-polarizable extractables (NPE), water-soluble extractables (WSE)

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Introduction

Condensed tannins (CT; proanthocyanidins) are polymers of flavan-3-ols, and the most abundant secondary metabolite produced by plants. Synthesis of CT occurs via the conserved flavonoid pathway (Barbehenn and Constabel 2011). However, broad variation in CT structure, degree of polymerisation, and level of accumulation has been documented and depends on plant species, genotype, age, and environmental conditions (Lindroth et al. 2002; Liu et al. 2005; Donaldson et al. 2006; Madritch et al. 2006; Osier and Lindroth 2006; Scioneaux et al. 2011). This leads to a broad range of CT abundances and forms in various plant tissues (Kraus et al. 2003a; Arranz et al. 2009; Norris et al. 2011; Preston and Trofymow 2015). Differences in CT polymer length, hydroxylation of B-rings, and stereochemistry has been related to a range of CT functions (Kraus et al. 2003b), which are still not yet fully characterized (Kraus et al. 2003a; Whitham et al. 2008; Barbehenn and Constabel 2011).

Condensed tannins are well known for their protein binding properties (Barbehenn and Constabel, 2011) and for broad *in-vitro* anti-microbial action (Scalbert 1991). Although commonly believed to act as anti-nutrients against insects, they are more effective as defenses against vertebrate herbivores (Constabel and Lindroth 2010; Barbehenn and Constabel 2011). Condensed tannins in soils have been experimentally observed to slow decay (Madritch et al. 2006) and inhibit nitrogen (N) mineralization and availability (Fierer et al. 2001; Madritch and Hunter 2002; Schweitzer et al. 2004). This could occur via direct or indirect effects on microbial communities. In a pan-Canadian six-year litter decomposition study, Trofymow et al. (2002) noted that winter precipitation affected decay losses only in the first year and hypothesized that the amount of tannins leached in winter might affect how much tannin remained to inhibit decay of other litter carbon (C) fractions in the summer.

Nitrogen-poor growing conditions have been associated with increased CT concentrations in foliage (Kraus et al. 2004; Harding et al. 2005; Madritch et al. 2006; Osier and Lindroth 2006). The physiological reason for this response is not clear, but it could be a mechanism for the regulation of N cycling below ground (Schimel et al. 1996; Kraus et al. 2003a; Whitham et al. 2006; Schweitzer et al. 2008; Whitham et al. 2008; Madritch and Lindroth 2015). Condensed tannins are known to bind to cell wall polysaccharides as well as proteins (Tarascou et al. 2010; Norris et al. 2011; Perez-Jimenez and Torres 2011) and form protein-polyphenol complexes progressively during senescence (Zhang et al. 2008) and decay (Lin et al. 2006; Lin et al. 2007; Zhou et al. 2012). Since CT in leaves from forest species is incorporated into litter, leaf litter contributes large CT inputs into soil systems. Increasing atmospheric CO_2 will likely increase N deficiencies in soils, since improved photosynthetic efficiency and a reduction in ribulose bisphosphate carboxylase/oxygenase (Rubisco) content of leaves will lead to reduced N in resulting litter (Hu et al. 2001; Norby et al. 2001). As a result, more CT and protein-CT complexes are expected in soils under elevated CO_2 (Liu et al. 2005; Parsons et al. 2008). The decline in litter decay rates through decreases in litter quality has been hypothesized to potentially partially offset the increase in decay rates due to climate change induced warming (Moore et al. 1999).

In *Populus tremuloides* leaves, condensed tannins can accumulate to 280 mg g^{-1} DW (Lindroth and Hwang 1996; Donaldson et al. 2006), although lower concentrations (10 - 150 mg g^{-1} DW) are typical for naturally senesced leaf litter (Lindroth et al. 2002;

Schweitzer et al. 2004; Parsons et al. 2008). *In planta*, condensed tannins exist as both solvent-soluble and insoluble (unextractable) polymers. Insoluble CT have been recently found to make up more of the CT fraction in fruit and seed than previously thought (Arranz et al. 2009; Perez-Jimenez et al. 2009) and are also found in leaves (Preston et al. 2009b). This insoluble fraction complicates analyses of tannins using solvent-based methods, although it can be measured using the butanol-HCl method directly on tissues (Preston et al. 2009b; Grabber et al. 2013). Improvements to the butanol-HCl assay now allow for quantification that is more thorough and allows for comparison of solvent-soluble and insoluble CT fractions (Mané et al. 2007; Grabber et al. 2013; Shay et al. 2017). Furthermore, quantifying CT soluble in water at room temperature (Shay et al. 2017) can help determine the fraction of CT that could readily leach out of decaying litter under natural conditions, and thus be readily available as a C source.

Here, we applied a modified butanol-HCl assay method to a leaf litter decay time series to help elucidate the fate of CT in soils. In addition to measuring changes in litter mass and C content with decay, we also applied two established proximate chemistry analyses to the same samples (Ryan et al. 1990), which provide the context of chemical changes occurring in the litter as it decayed. Proximate chemical analyses involves partitioning organic matter into mass fractions through sequential extraction in various solvents. The objectives of this study were: First, to measure water-soluble, acetone:MeOH-soluble, and insoluble CT fractions in poplar and Douglas-fir leaf litter during decay using an improved butanol-HCl protocol, and second, to jointly examine changes in litter proximate chemical fractions and CT contents in decaying leaf litter to gain insight into the fate of different CT fractions during litter decay.

Methods

Litter sampling

Naturally abscised poplar leaves (*Populus angustifolia*) and Douglas-fir needles (*Pseudotsuga menziesii subsp. menziesii*) were used as model leaf litters to track changes in condensed tannins and litter chemistry over three and a half years of decay in coastal rainshadow forests in British Columbia, Canada. The Douglas-fir litter was collected in 1991 from the Shawnigan Research forest (Vancouver Island, British Columbia) from the same material used in the Canadian Intersite Decomposition Experiment (Trofymow and CIDET Working Group 1998). Poplar litter was kindly provided by Professor Thomas G. Whitham and the Cottonwood Research Group (Department of Biological Sciences, Northern Arizona University). The litter had been collected in 2008 from a common garden established in 1991 at the Ogden Nature Center, Ogden, Utah, USA. Oven-dried, bulked Douglas-fir needles (5 g) or poplar leaves (4.6 g) were placed in fine-mesh (0.5mm) nylon bags and stapled shut using rust proof staples (Salco, Germany). Litter bags were deployed for decay in September 2011 in sites spanning temperature and moisture climatic gradients. Litter bags were placed on the forest floor in four replicate plots at zonal sites of the coastal Western hemlock and coastal Douglas-fir biogeoclimatic zones on Eastern Vancouver Island, British Columbia, along southern and northern transects (Table 1; see Online Resource 1 for map). Sites were similar in vegetation cover and all dominated by mature Douglas-fir (see Shay et al. 2015 for more site descriptions).

High and low CT treatments (high tannin, HT; low tannin, LT) were established for poplar-litter samples by using litter from trees with known high or low CT concentrations and known leaf chemistries (Rehill et al. 2006). All treatments included litter from the same genotypes and contained similar levels of internal N. Leaves were only selected from trees showing foliar chemistries with 5-8 mg g^{-1} N and with an inverse relationship between CT and salicortin concentrations (Pearson's correlation r = -0.866). A solution of L-Glutamine (Sigma-Aldrich, Germany) was atomized to half of the litter samples in order to create high-N treatments (HN) for each litter type. Each sample of high-N litter received two 5 ml applications of 25 g l^{-1} glutamine solution. Distilled water was used as a control in low-N treatments (LN). All litters were oven-dried (70°C overnight) prior to filling and weighing of litterbags. The composition of each litter treatment is outlined in Table 2.

Butanol-HCl assay for condensed tannins

Condensed tannins were quantified for samples decaying for 7 (0.6yr), 12 (1yr), 24 (2yr), or 43 (3.6yr) months on the forest floor, as well as for control samples not deployed into the field. For non-deployed samples, six biological replicates per poplar treatment and four biological replicates per Douglas-fir treatment were assayed. For samples collected after 7 and 12 months of decay, four replicates of each litter treatment per field site were assayed, while pooled replicates were analysed for subsequent sampling periods. Condensed tannins were assayed using a combination of improvements in the extraction protocols (Mané et al. 2007) and in the chemistry (Grabber et al. 2013) of the butanol-HCl method (Porter et al., 1986) as described by Shay et al. (2017). The improved butanol-HCl assay allowed for a more thorough and accurate quantification of total CT content, fractions soluble in water or acetone:MeOH, and fractions considered insoluble in conventional CT solvents. Briefly, quantification of total CT and CT fractions was carried out with oven-dried (70°C overnight) litter homogenized using a hammer mill (Polymix PX-MFC 90D, Kinematica, Switzerland). The water-soluble CT fraction was extracted using room-temperature dH_2O (~25 °C). The acetone:MeOH-soluble CT fraction was determined by subtracting the amount of water-soluble CT from CT soluble in an acidified (0.05% trifluoroacetic acid) mixture of 51% acetone, 34% MeOH and 15% dH_2O. Total CT content was determined by performing the butanol-HCl assay directly on tissue samples. The insoluble CT fraction was determined by performing butanol-HCl assay on residual pellet after solvent-extraction and/or by subtracting the amount of solvent-soluble CT from total CT content.

Carbon and nitrogen analyses

Carbon (C) and N concentration in ground, oven-dried litter (70 °C overnight) was analysed using an elemental combustion system with standard reactor packing for C and N along with a reduction column (Model ECS 4010 with dual-column system, Costeck Analytical Technologies Inc., USA).

Proximate chemical analyses

Proximate chemistry was determined using the forage fiber and forest product protocols (Ryan et al. 1990). Both protocols were carried out by the Chemical Services Laboratory at the Pacific Forestry Centre (Victoria, Canada).

The forest product protocol was adjusted from TAPPI standard methods (T204 cm-97, T264 cm-97, T222 om-02 and T211 om-02) to accommodate an automated soxhlet extraction apparatus (Gerhardt Soxtherm 2000 Automatic, SCP Science) with cold water condensers. Non-polarizable extractables (NPE), water-soluble extractables (WSE), acid-hydrolyzable fraction (AHF) and acid-unhydrolyzable residue (AUR) proximate fractions were determined by weight loss after stepwise series of exposure to various solvents, rinsing with deionized H_2O, vacuum filtration and overnight drying of residues at 70 °C. Ground litter samples (0.5 g) were first exposed to dichloromethane for 5 h at 150 °C (for NPE), then to deionized H_2O for 1 h at 260 °C (for WSE) and finally to 72 % sulphuric acid for 2 h at room temperature (~22°C) and to 3 % sulphuric acid for 4 h at 220 °C (for AHF and AUR) prior to ashing in a muffler furnace for 3 h at 525 °C.

The forage fiber protocol chemically separated components (all at room temperature) soluble in 0.5 M sulphuric acid and cetyltrimethylammonium bromine (soluble fraction), soluble in 72 % sulphuric acid for 3 h (acid-determined cellulose; ADC) and that are acid unhydrolyzable (so called acid-determined 'lignin'; ADL) (Ryan et al. 1990).

For both forest product and forage fiber procedures, ash content was determined using fractions remaining after acid hydrolysis (AUR and ADL). Only ash-free proximate fractions are reported. Unlike C, N and CT concentration, proximate chemistry was only determined for three undecayed leaf replicates per leaf chemistry treatment and pooled samples (four biological replicates) of decayed litter per treatment (sampling time x latitude x zone x leaf chemistry).

Data analysis

Linear (multivariate) models were used to assess the significance of latitude, zone and leaf chemistry effects on C and CT at each time period. For each sampling period, data was first modelled in response to latitude, zone and species, and then subset by species to assess the significance of leaf chemistry (using categorical data and/or initial N and CT (poplar only) concentrations), latitude and zone.

Linear multivariate and/or mixed effect models were used to test differences in proximate chemical fractions between litter chemistry (using categorical data and/or initial N and CT (poplar only) concentrations) and zonal treatments (again treating poplar and Douglas-fir litter species both jointly and separately). Differences in proximate chemistry between treatments were first assessed on litter prior to decay. Proximate chemical fractions after 0.6 and 3.6 years of decay (using dry weight, the change in dry weight, as well as the difference and fraction remaining on a C basis) were then modelled in response to litter chemistry and biogeoclimatic zone. Differences in average content and rate of change over time were also assessed. Proximate chemical fractions remaining (on a C basis) were each modelled in response to time (as a covariate or as random effect to confirm trends), litter chemistry and their interactions.

When appropriate, we transformed responses (log for % C remaining; boxcox for CT content and proximate fractions) to minimize violation of statistical assumptions. Mixed models were reduced by stepwise elimination of non-significant factors using ANOVA F-statistics and comparison of Akaike Information Criteria of reduced and non-reduced models. Significant differences between treatments were assessed by ANOVA on reduced models followed by *post-hoc* T-tests. When applicable, only significant ($p < 0.05$) Pearson's product-moment correlations are listed. All analyses were performed using R-statistics (R Core Team 2014; version 3.1.2).

Results

Relationship of condensed tannins, carbon, and nitrogen during decomposition

We used a butanol-HCl assay with improved extraction protocols that allowed us to distinguish between water- and organic solvent-extractable forms of CT (Shay et al. 2017) to better understand the dynamics of CT in decomposing leaf litter. Bulking leaf litters as outlined under Methods consistently generated low and high CT treatments (Fig.

1, Tukey HSD $p < 0.05$). However, random variability among high CT litter sampling unexpectedly led to minor but significantly different insoluble CT concentrations between N treatments (Tukey HSD $p = 0.035$; Fig. 1). These differences were small compared to the absolute concentrations and thus not expected to influence the outcome. Prior to decay, CT made up 76 to 278 mg g^{-1} of abscised poplar leaf litter dry weight. By contrast, Douglas-fir litter had 2 to 15 times less CT than low CT and high CT poplar litter, respectively (Fig. 1.a). Of the three CT fractions analysed, CT in both Douglas-fir and poplar litter was substantially insoluble, comprising 54 to 64 % of CT in poplar litter, and 59 % of CT in Douglas-fir litter on average (Fig. 1.b). Soluble CT (30 to 161 mg g^{-1} DW in poplar) were increasingly found as water-soluble forms (7 to 41 % in poplar litter) as total CT increased (Fig. 1.b). Soluble CT in Douglas-fir litter (41 % of total CT) were all classified as acetone:MeOH-soluble since no water-soluble CT were detected in this species (Fig. 1.b). After 0.6 years of decay in the litterbag experiment, across all litter treatments between 72.8 - 98.7 % of total detectable CT content was lost (Fig. 2).

Differences in C loss were mainly related to species and N treatments, yet also related to CT up to 1 year after the onset of decay. Carbon concentrations in non-decayed litter were 12 % greater in Douglas-fir litter, and were significantly correlated only with CT concentrations in poplar litter ($r = 0.936$; Table 2). After 0.6 year of decay, % C remaining was 25 % greater in Douglas-fir than poplar litter ($p < 0.001$; Fig. 3), but was not affected by latitude or zone ($p = 0.586$ and 0.304, respectively). At this time, more C remained in litter treatments with lower initial N concentrations (regardless of species; $p < 0.014$), and more initial CT concentrations (poplar only; $p < 0.001$; Fig. 3). The similar magnitudes of N and CT effects resulted in no significant differences in % C remaining between LTLN and HTHN litters after 0.6 year of decay ($p = 0.871$). Significant differences in % C remaining between poplar and Douglas-fir litter ($p < 0.001$), as well as the influence of N ($p = 0.001$) and CT ($p = 0.008$) on C content in poplar litter, remained detectable through 1 year of decay (Fig. 3). At this time, significantly more % C remained in the coastal Western Hemlock than the coastal Douglas-fir zone in poplar litter (5.4 % on average; $p = 0.014$), and in Douglas-fir litter samples placed in northern vs. southern field sites (8.7 %; $p = 0.008$; data not shown). The effects of latitude on % C remaining in Douglas-fir litter remained detectable through to 3.6 years of decay ($p < 0.039$), and no other significant differences in % C remaining between treatments were shown in samples decaying for 2 years ($p > 0.05$). After 3.6 years of decay, % C remaining in Douglas-fir was significantly less than in poplar litter (19 %; $p < 0.001$; Fig. 3) and when decaying in coastal Western Hemlock sites (12 %; $p = 0.018$; data not shown). While latitude, zone and CT content did not significantly affect the % C remaining in poplar litter after 3.6 years ($p = 0.354$ and 0.574, respectively), high initial N concentrations significantly reduced % C remaining at this sampling time (13 %; $p = 0.005$; Fig. 3).

Early reductions in detectable CT could not all be attributed to C loss. Total CT made up 2.53 % (Douglas-fir), 10.27 % (LT) and 35.97 % (HT) of the C in non-decayed poplar litter, assuming an average tannin C mass fraction of 0.62 based on the molecular structures of procyanidin and prodelphinidin monomers (Barbehenn and Constabel 2011). On average, the loss of detectable tannins after 0.6 year (differences between Fig. 1.a and 2) was equivalent to 24 %, 95 % and 7 % of the C loss during this period for LT, HT and Douglas-fir litters, respectively. In some cases (such as HTLN decaying at the southern coastal Douglas-fir site), the loss of detectable CT in C equivalents was even greater than the actual C loss. This suggests that portions of the CT carbon was likely still present, possibly transformed, in the litter after 0.6 year of decay despite the majority of CT no longer being detectable by the butanol-HCl assay.

Changes in forage fiber proximate chemistry within the first 0.6 year of decay

Early loss of soluble proximate fraction were proportional to losses of water-soluble CT among treatments. The soluble proximate fraction of litter prior to decay was 23 % greater in poplar than Douglas-fir litter ($p < 0.001$) and was positively correlated with CT concentrations in poplar litter ($p = 0.005$). After 0.6 year of decay, poplar litter had lost ~2 times more of its initial soluble proximate chemical fraction compared to Douglas-fir litter ($p <0.001$; Fig. 4.a). More of the soluble proximate chemical fraction was lost in poplar litter samples with high initial CT concentrations than those with low CT concentrations (6.3 %; $p = 0.003$; Fig. 4.a). This difference was similar in magnitude (6.5 %) to the difference in water-soluble CT lost between these two litter types. The soluble proximate fraction, and its loss during decay, was not associated with initial N concentrations ($p > 0.05$), suggesting no preferential leaching of N in high N treatments. Thus, loss of the soluble proximate chemical fraction corresponded to differences in litter composition between species and in water-soluble CT among poplar litter.

Early changes in ADL content correlated with loss of detectable insoluble CT in poplar litter. Acid Determined “Lignin” (ADL) concentrations prior to decay was 22 % greater in poplar than Douglas-fir litter treatments ($p = 0.001$), while Acid Determined Cellulose (ADC) was 1.7 times greater in Douglas-fir than poplar litter ($p < 0.001$). Prior to decay, ADL and ADC proximate fractions did not correlate with differences in N and

CT ($p > 0.05$). In contrast, ADC fraction of poplar litter remained relatively unchanged after the first 0.6 year of decay ($p = 0.443$; Fig. 4.b). On average, change in ADL content after 0.6 year of decay did not differ between species ($p = 0.958$), however it was affected by initial CT concentrations in poplar litter ($p = 0.002$; Fig. 4.c). Low and high initial CT concentrations led to 6.76 % loss versus 4.16 % gain in ADL content after 0.6 year of decay, respectively (Fig. 4.c). The net difference in ADL content between high and low CT litter is similar in magnitude to the 10 % difference in insoluble CT content prior to decay.

Changes in proximate chemistry, as determined by the forage fiber technique, indicated that net C loss after 0.6 year of decay was mostly attributable to loss of soluble compounds across species, while loss of ADC-like compounds further accounted for net C loss in Douglas-fir litter. High CT concentrations in poplar litter increased the ADL content in leaf litter after 0.6 year of decay, thereby offsetting the greater concurrent C losses associated with greater soluble compounds (i.e. water-soluble CT) and leading to 6.6 % greater C remaining in high CT compared to low CT poplar litter.

Changes in forage fiber and forest product proximate chemical fractions after 3.6 years of decay

Loss of extractable (soluble) proximate chemical content corresponded to initial CT and N concentrations among litter. Prior to decay, both NPE and WSE content were greater in poplar than Douglas-fir litter (57 % and 15 % greater, respectively). In poplar litter, NPE increased with greater N ($p < 0.019$) and greater CT ($p < 0.001$) concentrations, while WSE showed no significant associations ($p > 0.05$). Trends in the loss of the soluble proximate chemical fraction using the forage fiber method were best correlated with the summed changes in NPE and WSE determined by the forest product method ($r = 0.91$; $p < 0.001$), but more closely matched changes in NPE ($r = 0.90$; $p < 0.001$) than WSE ($r = 0.61$; $p = 0.034$). Only the loss of NPE content was associated with total initial CT concentrations in poplar litter ($p = 0.004$).

Summed loss of acid-extractable (ADC or AHF) and unextractable (ADL or AUR) proximate chemical content was associated with species, N and CT treatments, yet quantification of these proximate chemical fractions prior to and after decay differed depending on the method used. Unlike ADC and ADL, both AHF and AUR were inversely related to CT concentration prior to decay ($p < 0.001$). Irrespective of the method used, all proximate chemical fractions, except for ash, generally decreased with decreasing %C remaining over 3.6 years of decay ($p < 0.001$; Fig. 4 and 5).The summed loss of AHF and AUR was highly correlated with that of ADC and ADL ($r = 0.98$; $p < 0.001$), which was similar to correlations between the summed content of these proximate chemical fractions prior to and after decay ($r = 0.91$ and 0.99, respectively; $p < 0.001$). However, AHF and ADC content correlated better prior to, rather than after 3.6 years of decay ($r = 0.85$ versus 0.53), while AUR and ADL content showed the opposite trend ($r = 0.30$ versus 0.85). Modelling of AHF + AUR or ADL + ADC both showed the same greater loss in Douglas-fir than poplar litter ($p < 0.001$), while loss in poplar litter was directly related to initial N concentrations ($p < 0.040$) and inversely related to initial CT concentrations ($p < 0.021$). Nevertheless, ADL + ADC and AHF + AUR content after 3.6 years of decay only differed between species ($p < 0.001$) and initial N concentrations ($p = 0.020$).

Discussion

Future atmospheric CO_2 concentrations are predicted to increase the amount of condensed tannins in foliar litter (Liu et al. 2005; Parsons et al. 2008), which may favour future C sequestration. However, the impact of higher CT is still unclear, in particular since the biological effects of the distinct CT forms is still unresolved. Our previous refinements of the butanol-HCl assay allowed us to better categorize CT into water-soluble, acetone:MeOH-soluble, and insoluble fractions (Shay et al. 2017). This is particularly relevant for studies of decaying litter, where CT fractions with different chemical properties (e.g. solubility) can serve different ecological functions, and thus impact C cycling. In conjunction with proximate chemical analyses, our data show that in poplar litter, the water-soluble CT component corresponds to differences in soluble proximate fractions, and that other CT forms, mainly insoluble CT, are converted to acid-unhydrolyzable products during litter decay. The absence of water-soluble CT in Douglas-fir litter contributed to smaller early loss of soluble C compared to poplar litter; however, factors other than CT content were responsible for the overall slower early decay.

Our leaf litter was naturally senesced when it was collected, but for consistency it was oven-dried it prior to deployment. Oven-drying fresh plant material has been shown to cause species-dependent decreases in soluble CT content and increases in insoluble forms compared to freeze-drying (Wolfe et al., 2008), but it is unlikely to have a strong effect on already dry senesced material. We measured the proportions of tannin forms in the same litter batches used for our experiments, and correlated these with effects observed in the field; nevertheless we cannot exclude the possibility that proportions of tannin forms are slightly different from naturally senesced leaves as found in nature.

Rapid loss of water-soluble condensed tannins

Detectable CT in all forms was rapidly lost in all our litter samples within 0.6 year of decay (Fig. 2). A similar rapid loss or transformation of CT was previously observed in other systems (Schofield et al. 1998; Kraus et al. 2003a; Parsons et al. 2008; Preston et al. 2009b; Norris et al. 2011; Zhou et al. 2012). The improved butanol-HCl assay allowed for more complete extraction of bound CT (Shay et al. 2017) and suggest that in decaying litter, CT are not simply more tightly bound to substrates, but are subjected to other processes. Leaching and/or mineralization are likely not adequate to explain the total loss of detectable CT, since greater C loss was observed in poplar with low CT compared to high CT concentrations, and since the loss of detectable CT in some cases exceeded the loss of litter when converting values to C equivalents. The loss of water-soluble CT between high and low tannin poplar litter closely matched that of soluble proximate chemical fractions, suggesting the leaching or rapid microbial utilization of water-soluble CT. Preferential leaching of low molecular weight CT during early leaf litter decay has previously been reported (Schofield et al. 1998). The initial loss of soluble compounds in decaying litter was not influenced by our N treatments, indicating that the glutamine applied to half of our litter samples did not preferentially leach out of litterbags during the seasonally rainy winters at the onset of decay. We suspect this N source to be rapidly taken up by saprophytic microbes (Schimel and Bennett 2004) allowing for the more rapid decay of our N-rich litter, although the possibility remains that some was bound to compounds such as CT in the litter matrix (Nierop et al. 2006; Norris et al. 2011; Zhou et al. 2012).

Few studies on the breakdown of CT have been published (Preston et al. 2009b), but degradation of CT by microbes (i.e. mineralization), including the depolymerization of complex proanthocyanidin structures, would need to proceed to the molecular state of terminal-CT units and phloroglucinol carboxylic acid in order for the proanthocyanidins not be detected by the butanol-HCl assay (Porter et al. 1986, Bhat et al. 1998). Rapid degradation of CT to these forms may be possible, given rapid decay in the first 0.6 year but this is not likely since C loss by microbial respiration could not account for the inverse relationship between loss of detectable CT and loss of C. Microbial communities are therefore unlikely to be utilizing the CT in our litter as primary C sources, with exception of those in water-soluble form. Leaching of inhibitory tannins in response to winter precipitation (Trofymow et al. 2002) does not apply to this study as all sites had high winter precipitation and only water-soluble tannins were likely leached by 7 months. Therefore, the effects of CT in slowing decay of litter C fractions during the first year were related to the amounts of acetone:MeOH-soluble and insoluble tannins and their transformation.

In our study, total initial CT concentrations in poplar litter and loss over time were linked to soluble proximate chemical fractions (forage fiber method) and NPE (forest product method), not WSE as observed by Preston et al. (2009b). However, the *Populus angustifolia* leaf litter used in our study contained less WSE (136 mg g^{-1} DW) than the other species used by Preston et al. (370 - 400 mg g^{-1} DW; 2009b). For comparison, differences in NPE and WSE using same bulked Douglas-fir litter were less than 27 and

10 mg g^{-1} DW, respectively, which indicate comparable results for proximate chemistry between these two studies. Therefore, our results demonstrate that the WSE chemical fraction cannot be assumed to represent total CT, at least when studying senescent and decaying leaf litter, since various CT forms will elute in WSE, NPE, AHF and AUR fractions depending on their structure and binding affinity.

Transformation of water-insoluble condensed tannins was associated with a reduction in carbon loss during early decay

If leaching and degradation by microbes cannot explain the rapid loss of detectable acetone:MeOH-soluble and insoluble CT, these are rapidly transformed, either chemically or via biological action, into products undetectable using butanol-HCl. For example, direct cleavage of one of the three main flavan-3-ols C-rings while still polymerized would lead to a reduction in detectable CT with concurrent low C loss. In our study, change in ADL after 0.6 year correlated with initial CT concentration, suggesting that CT was being transformed to ADL (or AUR) products. This is consistent with findings from the Canadian Intersite Decomposition Experiment, which show the AUR fraction of decaying litter as being partially composed of CT derived structures that resist decay (Preston et al. 2009a, b; Preston and Trofymow 2015). The increase in acid-unhydrolyzable residue associated with CT transformation was detectable until 2 years of decay when using the forage fiber methodology, while evidence of such transformation were also significant after 3.6 years of decay using the forest product method. These two protocols differ mostly in terms of heating during successive extractions steps, which would suggest that products of CT are still being modified after 2 - 3.6 years of decay. However, high correlations between CT forms in litter prior to decay and rapid loss of detection of the majority of CT prevented us from clearly differentiating the fates of acetone:MeOH-soluble and insoluble CT. Nevertheless, our findings demonstrate the ecological significance of water-insoluble CT forms below-ground, in contrast to previous suggestions that these tannins were less relevant than water-soluble forms (Yu and Dahlgren 2000). Determining the short term fate of CT beyond the leachable fraction would require isotopic labelling of CT and better tracking of components via ^{13}C NMR (Preston et al. 1997).

The effects of CT on C loss were limited to 1 year and no longer evident after 2 years of decay. This is similar to findings by Liu et al. (2009) as well as by Madritch and Lindroth (2015). Comparable to most litters in a pan-Canadian study (Preston et al. 2009b; note AUR values were not ash corrected), all proximate chemical fractions except for ash content decayed to some extent over 3.6 years of decay at our sites. The greater losses of acid-hydrolyzable and unhydrolyzable content in Douglas-fir than poplar litter could be due to either general chemical differences between the litter types (e.g. CT structure, cutin content) or to the endemic nature of the Douglas-fir litter to our field sites, to which local microbial communities are adapted (Ayres et al. 2009). Contrary to commonly held assumptions, the ADC chemical fraction decayed more slowly than ADL in our litter treatments, especially since the decay of CT and cutins were contributing to the accumulations of AUR-like (i.e. ADL) products over time (Preston et al. 2009a). However, the slow apparent decay of ADC chemical fractions, especially in poplar litter, could also be the result of increasing contributions to such chemical fraction by turnover of microbial biomass.

The stronger correlation between AUR+AHF and ADL+ADC rather than between AUR and ADL or AHF and ADC prior to and after 3.6 years of decay suggests that heat (the main difference in quantification of acid (un)hydrolyzable fractions between the forage fiber and forest product methods) was leading to the different proportions of proximate chemistries deemed acid hydrolyzable (ADC or AHF) or not (ADL or AUR). Furthermore, the correlation between AUR and ADL was stronger after decay, while the reverse was true of AHF and ADC; this provides further support to the idea that water-insoluble CT that are solubilised by acid hydrolysis in the presence of heat (e.g. AHF or butanol-HCl) are being transformed to non-CT, true-acid-unhydrolyzable residues during decay. The generally better correlations of CT and N concentrations with proximate chemical fractions obtained via the forest product method, compared to the forage fiber method, would reinforce the greater suitability of the former method in characterizing litter during decomposition studies. However as shown above, using multiple approaches to characterize litter composition can help elucidate (partial) trajectories during decay.

Conclusion

Our data suggest that the rapidly lost water-soluble CT may be less relevant than other CT forms in slowing C loss during litter decay, in contrast to the existing paradigm. Thus, rapid initial transformation of water-insoluble CT to ADL-like products was associated with the slower early decay in the high CT litter. These ADL-like products of CT transformation appear to be further transformed to AUR-like products between 2 and 3.6 years of decay, although with minor impact on concurrent C losses during this period. Due to reduced rates of early decay, and greater C transformation to decay-resistant products, yearly inputs of high-CT foliar litter can therefore lead to greater C sequestration in soils. Increases in litter CT content, as predicted under elevated future atmospheric CO_2 concentrations, could thus have long-term consequences for C sequestration in forest soils.

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Fig. captions

Fig. 1 Average condensed tannin concentrations (± SE) in naturally abscised poplar (n = 6) and Douglas-fir (Fd; n = 4) leaf tissue with low and high nitrogen (LN and HN) and condensed tannin content (LT and HT; poplar only), prior to decay. Butanol-HCl assays with 50 % acetone were performed (a) directly on samples (total CT) or (b) on soluble extracts (Water-Sol. and Ace:MeOH-Sol.) and residual pellet after soluble extraction (Insoluble). For each type of CT, letters indicate significant pair-wise differences using TukeyHSD ($p < 0.001$ for each six-way ANOVA). In some cases error bar are too small to see at the plotted scales. Water-soluble condensed tannins were not detected in Douglas-fir litter (Shay et al. 2017)

Fig. 2 Average condensed tannin concentrations in naturally abscised poplar and Douglas-fir (Fd) leaf tissue with low and high nitrogen (LN and HN) and condensed tannin content (LT and HT; poplar only). Data shown for 0.6 or 1 year of decay, in coastal Douglas-fir (DF) and coastal Western Hemlock (WH) zones. Letters indicate significant pair-wise differences using TukeyHSD. n = 8, consisting of four samples decayed at northern transects and four samples decayed at southern transects (± SE)

Fig. 3 Boxplots of % carbon remaining in naturally abscised poplar and Douglas-fir (Fd) leaf tissue with low and high nitrogen (LN and HN) and condensed tannin (LT and HT; poplar only) content, after 0.6, 1, 2 and 3.6 years of decay. Averages (n = 16) represent litter decaying throughout four sites located along the northern and southern transects spanning in the coastal Western Hemlock and coastal Douglas-fir zones (see Shay et al. 2015 for further site details)

Fig. 4 Proximate chemical analysis fractions expressed as % carbon remaining in naturally abscised poplar and Douglas-fir (Fd) leaf litter with low and high nitrogen (LN and HN) and condensed tannin (LT and HT; poplar only) content, over 3.6 years of decay (± SE). Analysis determined using the forage fiber protocol: soluble fraction (a; solubles), acid-determined cellulose (b; ADC), acid-determined ”lignin” (c; ADL) and ash (d)

Fig. 5 Differences in the proximate chemical fractions between 0 and 3.6 years of decay. Samples include naturally abscised poplar and Douglas-fir (Fd) leaf litter with low and high nitrogen (LN and HN) and condensed tannin content (LT and HT; poplar only). Positive values represent gains while negative values represent losses in the % dry weight (a, b) or % C remaining (c, d), ± SE. Proximate chemical fractions for the forest product method (a, c) include: non-polar extractables (NPE), water soluble extracts (WSE), acid-hydrolysable fraction (AHF), acid-unhydrolysable residue (AUR) and ash. Those for the forage fiber method (b, d) include: soluble fraction (Solubles), cellulose (ADC), acid-determined “lignin” (ADL) and ash

Table 1: Location and climate of field sites located along northern and southern transects spanning the coastal Western Hemlock (WH) and coastal Douglas-fir (DF) bioecoclimatic zones. Climate parameters were measured at each site for the duration of the study (from May 1st, 2011 to April 30th, 2015).

Transect	Zone	Latitude	Longitude	Soil moisture (%)[a]	Soil temperature (°C)[a]	Air temperature (°C)[b]	PET[c]	DD[d]
North	DF	N 49° 25’ 30.0”	W 124° 40’ 00.3”	13.93 (2.27, 51.30) [3.85, 40.97]	9.55 (2.56, 16.03) [3.73, 15.37]	8.99 (-7.51, 26.23) [-4.86, 23.90]	88.2	3296
	WH	N 49° 28’ 22.4”	W 124° 48’ 39.2”	9.05 (0.94, 28.13) [3.03, 20.62]	8.97 (1.73, 15.17) [2.96, 14.71]	8.97 (-7.80, 27.48) [-5.90, 26.32]	90.5	3296
South	DF	N 48° 28’ 30.4”	W 123° 28’ 58.8”	11.93 (2.93, 26.53) [4.03, 24.72]	9.94 (2.58, 16.47) [3.91, 16.18]	9.59 (-7.74, 32.50) [-5.18, 30.00]	89.3	3513
	WH	N 48° 34’ 05.2”	W 123° 39’ 46.3”	11.25 (3.03, 19.77) [6.00, 18.68]	8.40 (1.50, 15.76) [2.31, 13.55]	8.61 (-8.33, 29.39) [-5.97, 28.50]	87.8	3168

[a] Mean annual (extreme minimum, maximum over 4year span) [mean annual minimum, maximum] measured 30cm below forest floor

[b] Mean annual (extreme minimum, maximum over 4year span) [mean annual minimum, maximum] measured 30cm above forest floor

[c] Mean potential evapotranspiration from May to September, using the Thornthwaite method

[d] Mean annual degree days above 0°C

Table 2: Initial chemical variables measured on leaf and needle litter with low and high nitrogen (LN and HN) and low and high condensed tannins (LT and HT; poplar-leaf litter only). Means and standard errors (±) were calculated using six poplar samples or four Douglas-fir (Fd) samples per treatment, except for proximate chemical fractions for which three samples were used per treatment. Values represent mg g^{-1}. Condensed tannin (CT) values were previously reported in Shay et al. 2017.

Species	**Treatment**	**Carbon**	**Nitrogen**	**C / N**	**Solubles**[a]	**ADC**[a]	**ADL**[a]	**Ash**[a]
Poplar	LTLN	457.0 ±1.1	8.1 ±0.2	56.39 ±1.45	435.0 ±12.5	188.0 ±17.2	374.0 ±11.6	4.3 ±1.5
	HTLN	481.8 ±4.3	6.9 ±0.2	70.36 ±1.75	451.0 ±35.2	168.7 ±11.2	379.0 ±30.2	2.7 ±0.7
	LTHN	461.4 ±5.5	15.7 ±0.3	29.41 ±3.56	435.1 ±2.9	203.0 ±38.7	359.0 ±39.6	6.3 ±0.9
	HTHN	477.5 ±5.6	14.3 ±0.3	33.31 ±3.41	491.0 ±16.5	177.9 ±11.0	326.4 ±6.2	4.7 ±0.9
Douglas-fir	FdLN	512.7 ±1.5	6.1 ±0.1	83.52 ±1.37	348.3 ±20.7	317.7 ±2.3	305.3 ±14.9	28.3 ±0.9
	FdHN	511.1 ±1.5	11.3 ±1.3	45.46 ±9.83	387.7 ±11.1	295.0 ±8.7	290.6 ±0.8	26.7 ±1.7

Species	**Treatment**	**Water-Soluble CT**	**Ace:MeOH-Sol. CT**[b]	**Insol. CT**	**Total CT**[c]
Poplar	LTLN	1.6 ±1.3	20.7 ±11.7	44.0 ±12.4	66.4 ±8.1
	HTLN	71.9 ±17.6	92.4 ±13.9	139.5 ±15.2	264.9 ±7.9
	LTHN	3.1 ±1.4	33.8 ±14.9	53.9 ±9.2	85.8 ±6.4
	HTHN	60.1 ±10.1	97.5 ±7.7	158.6 ±8.7	291.5 ±5.3
Douglas-fir	FdLN	0	8.3 ±0.8	11.9 ±0.6	20.8 ±0.4
	FdHN	0	8.3 ±0.6	12.0 ±0.4	20.9 ±0.3

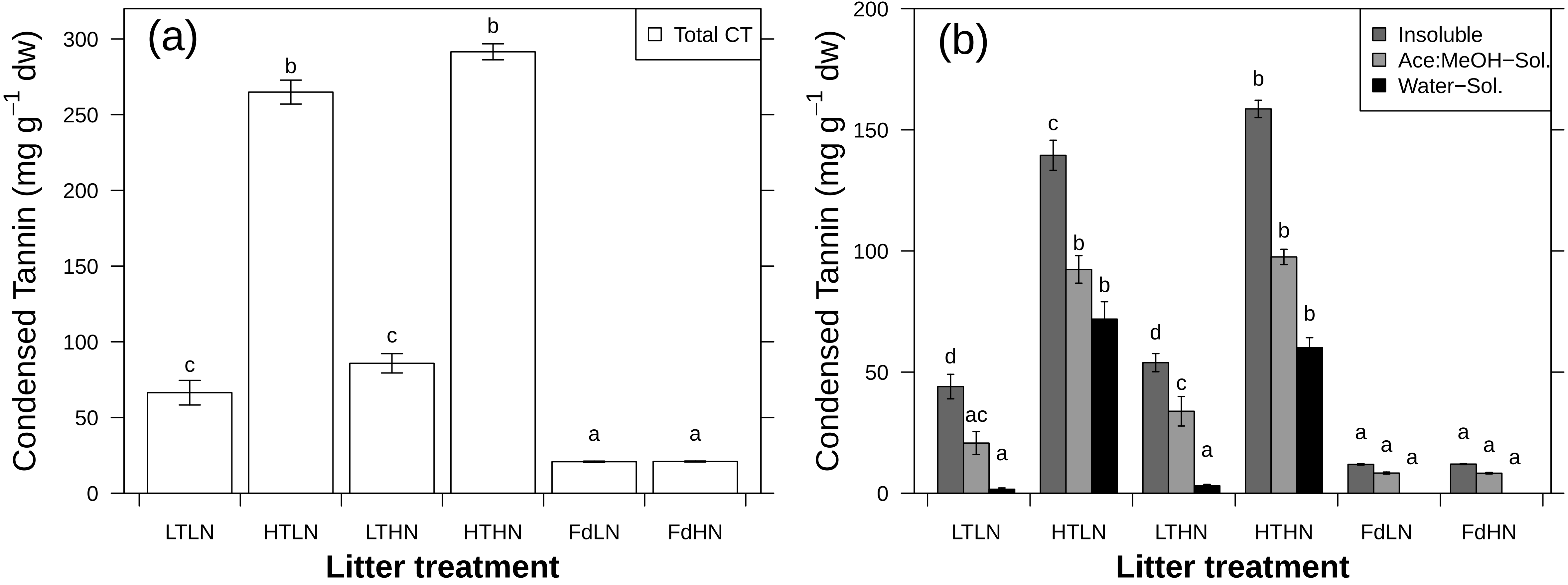
Species	**Treatment**	**NPE**[d]	**WSE**[d]	**AHF**[d]	**AUR**[d]	**Ash**[d]
Poplar	LTLN	137.6 ±15.2	130.7 ±0.1	304.2 ±5.0	414.8 ±6.6	12.7 ±0.6
	HTLN	177.7 ±15.2	154.0 ±11.1	283.8 ±10.9	373.1 ±5.4	11.4 ±0.8
	LTHN	152.4 ±5.6	128.4 ±7.5	307.8 ±4.4	398.1 ±2.7	13.3 ±1.6
	HTHN	230.2 ±6.5	132.3 ±12.4	274.3 ±3.0	353.0 ±5.5	10.1 ±0.5
Douglas-fir	FdLN	99.5 ±3.5	119.5 ±1.0	362.6 ±0.6	394.0 ±3.4	24.4 ±0.6
	FdHN	121.6 ±3.2	117.2 ±1.5	355.1 ±1.2	382.6 ±0.5	23.4 ±0.4

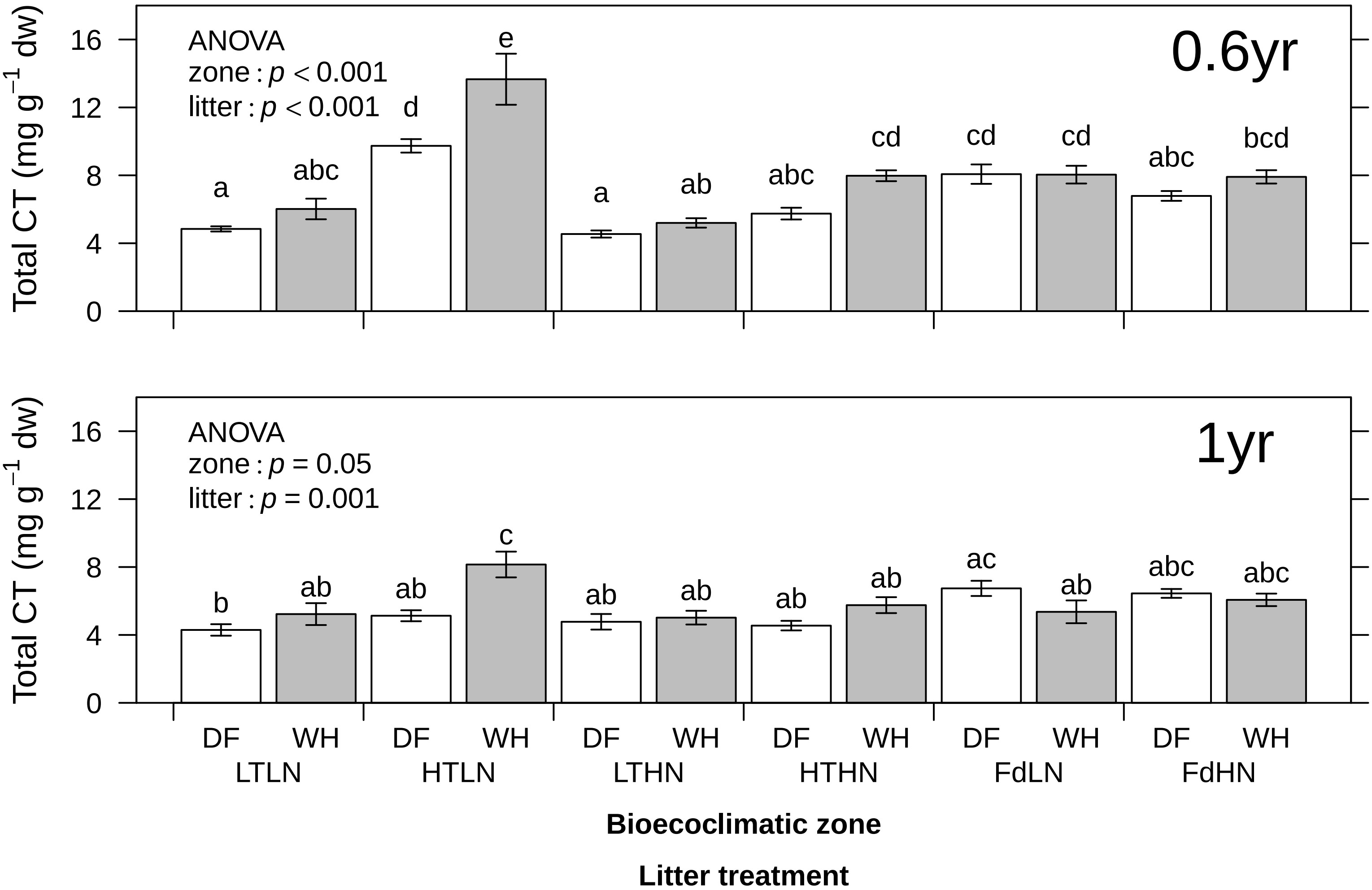
[a] Proximate chemical fractions determined using forage fiber method: soluble fraction (Solubles), acid-determined cellulose (ADC), acid-determined lignin (ADL) and ash

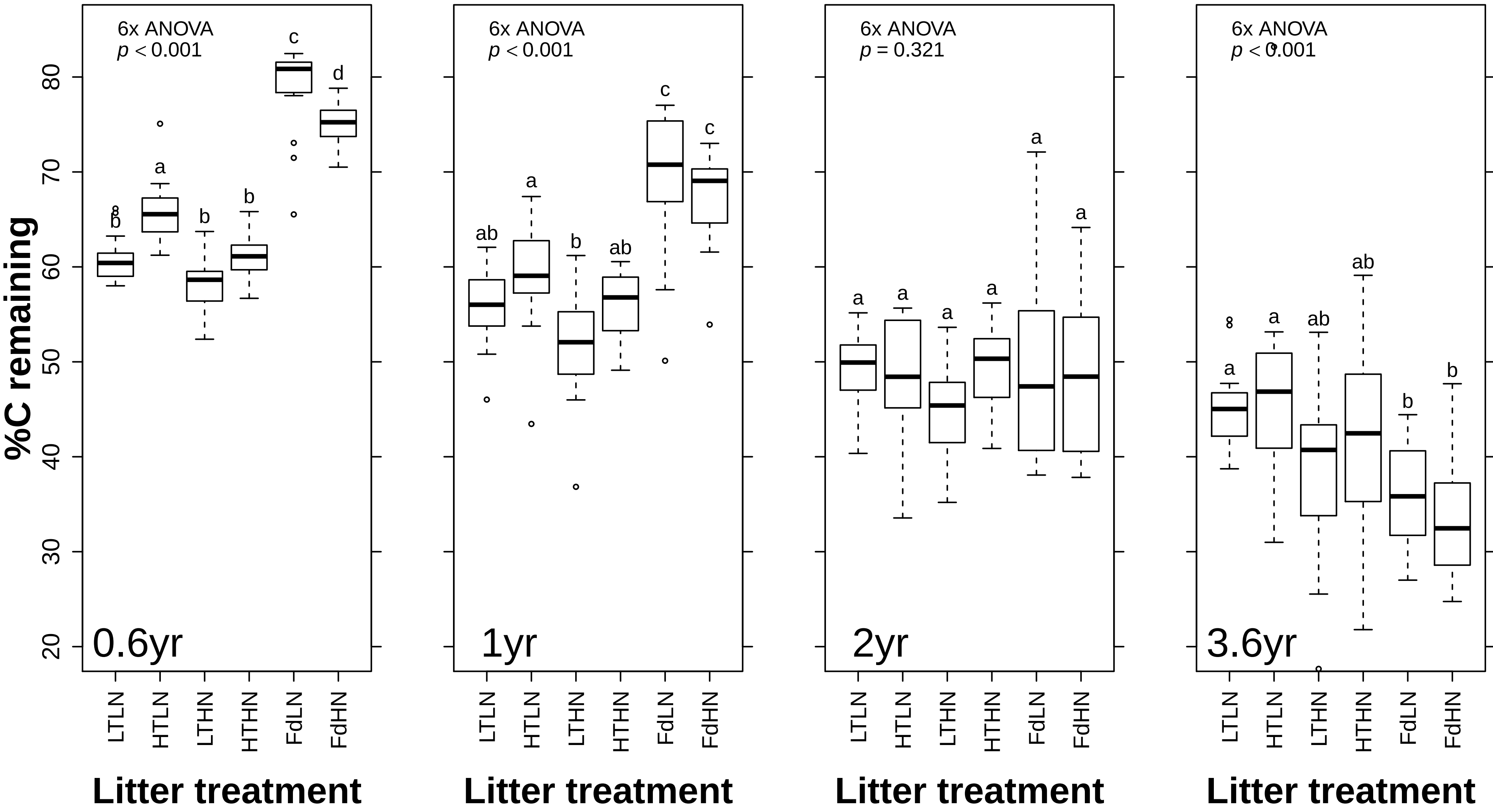
[b] Soluble in acetone:MeOH:water minus water-soluble content

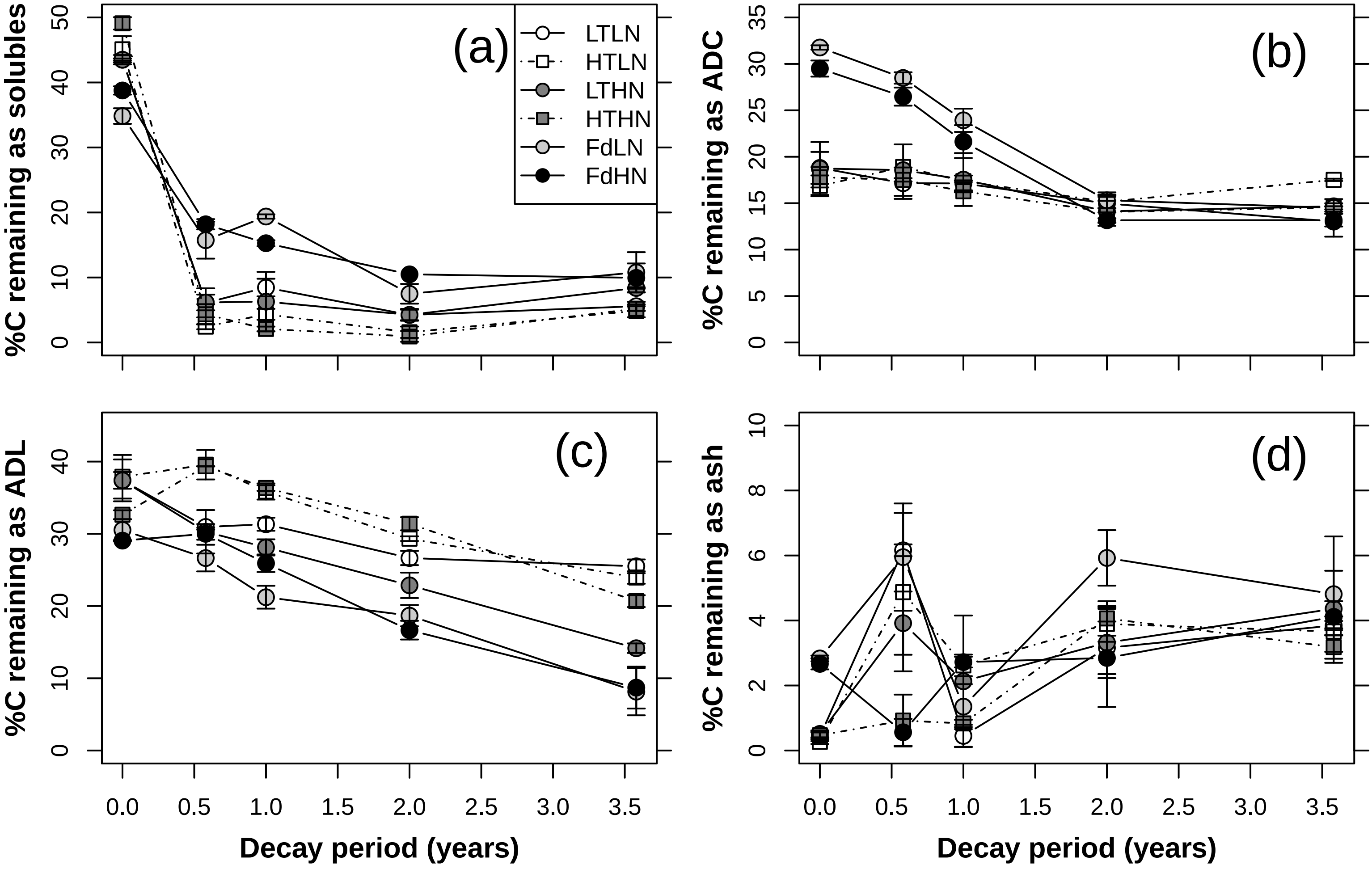
[c] Using butanol-HCl assay directly on litter sample

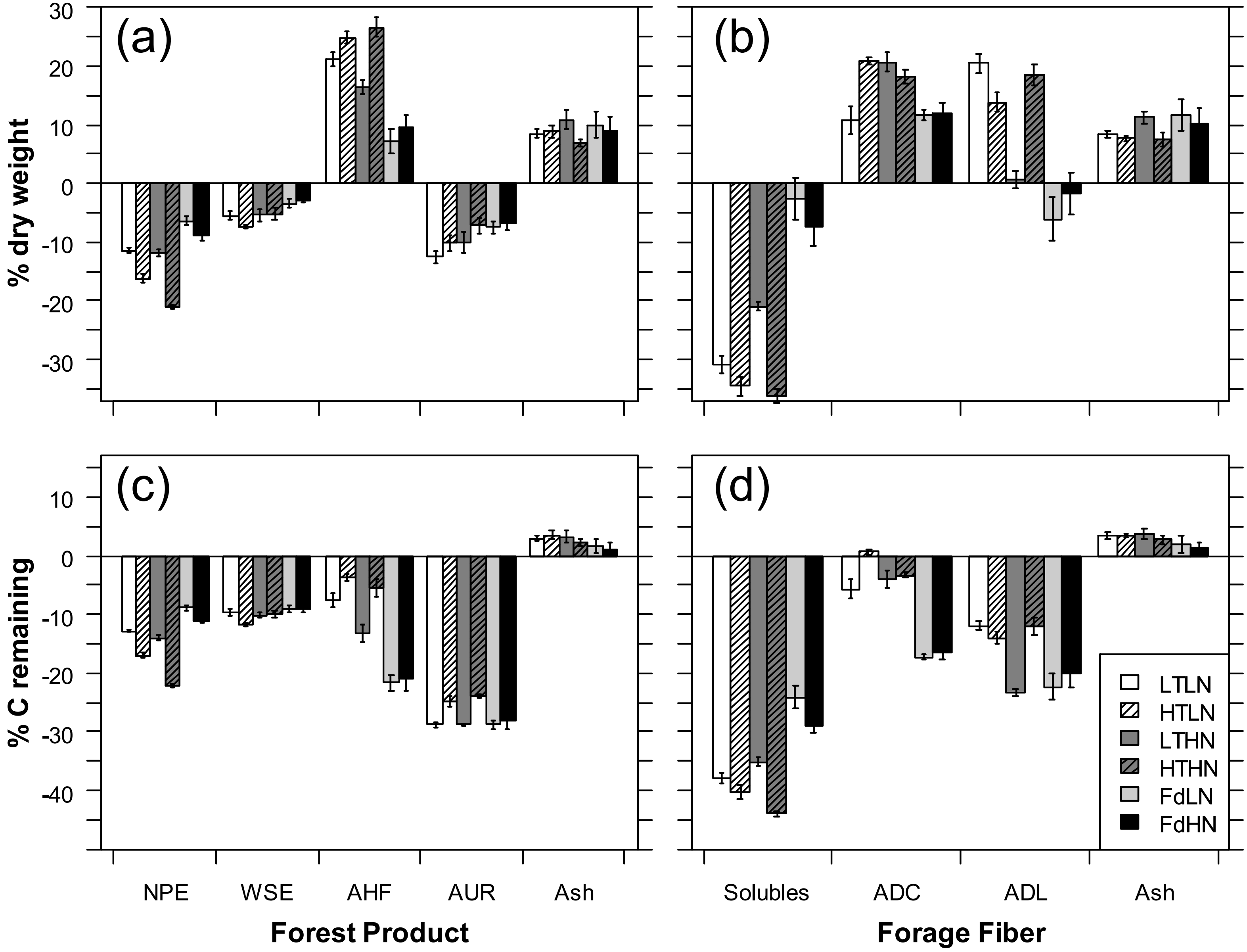
[d] Proximate chemical fractions determined using forest product method: non-polar extractables (NPE), water-soluble extracts (WSE), acid-hydrolysable fraction (AHF), acid-unhydrolysable residue (AUR) and ash


(a)
(b)
Total CT
Insoluble
Ace:MeOH−Sol.
Water−Sol.
Condensed Tannin (mg g^{-1} dw)
Litter treatment
LTLN
HTLN
LTHN
HTHN
FdLN
FdHN
0
50
100
150
200
250
300
c
b
c
b
a
a
d
ac
a
c
b
b
d
c
a
b
b
b
a
a
a
a
a
a


Total CT (mg g^{-1} dw)
0
4
8
12
16
ANOVA
zone : $p < 0.001$
litter : $p < 0.001$
0.6yr
a
abc
d
e
a
ab
abc
cd
cd
cd
abc
bcd
ANOVA
zone : $p = 0.05$
litter : $p = 0.001$
1yr
b
ab
ab
c
ab
ab
ab
ab
ac
ab
abc
abc
DF
WH
DF
WH
DF
WH
DF
WH
DF
WH
DF
WH
LTLN
HTLN
LTHN
HTHN
FdLN
FdHN
Bioecoclimatic zone
Litter treatment


%C remaining
80
70
60
50
40
30
20
6x ANOVA
$p < 0.001$
0.6yr
b
a
b
b
c
d
6x ANOVA
$p < 0.001$
1yr
ab
a
b
ab
c
c
6x ANOVA
$p = 0.321$
2yr
a
a
a
a
a
a
6x ANOVA
$p < 0.001$
3.6yr
a
a
ab
ab
b
b
LTLN
HTLN
LTHN
HTHN
FdLN
FdHN
Litter treatment
Litter treatment
Litter treatment
Litter treatment


(a)
(b)
(c)
(d)
LTLN
HTLN
LTHN
HTHN
FdLN
FdHN
%C remaining as solubles
%C remaining as ADC
%C remaining as ADL
%C remaining as ash
Decay period (years)
Decay period (years)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
0
10
20
30
40
50
0
5
10
15
20
25
30
35
0
2
4
6
8
10


(a)
(b)
(c)
(d)
% dry weight
% C remaining
30
20
10
0
-10
-20
-30
-40
NPE
WSE
AHF
AUR
Ash
Solubles
ADC
ADL
Ash
Forest Product
Forage Fiber
LTLN
HTLN
LTHN
HTHN
FdLN
FdHN

Supplementary Material

Evidence for the role and fate of water-insoluble condensed tannins in the short-term reduction of carbon loss during litter decay

Biogeochemistry

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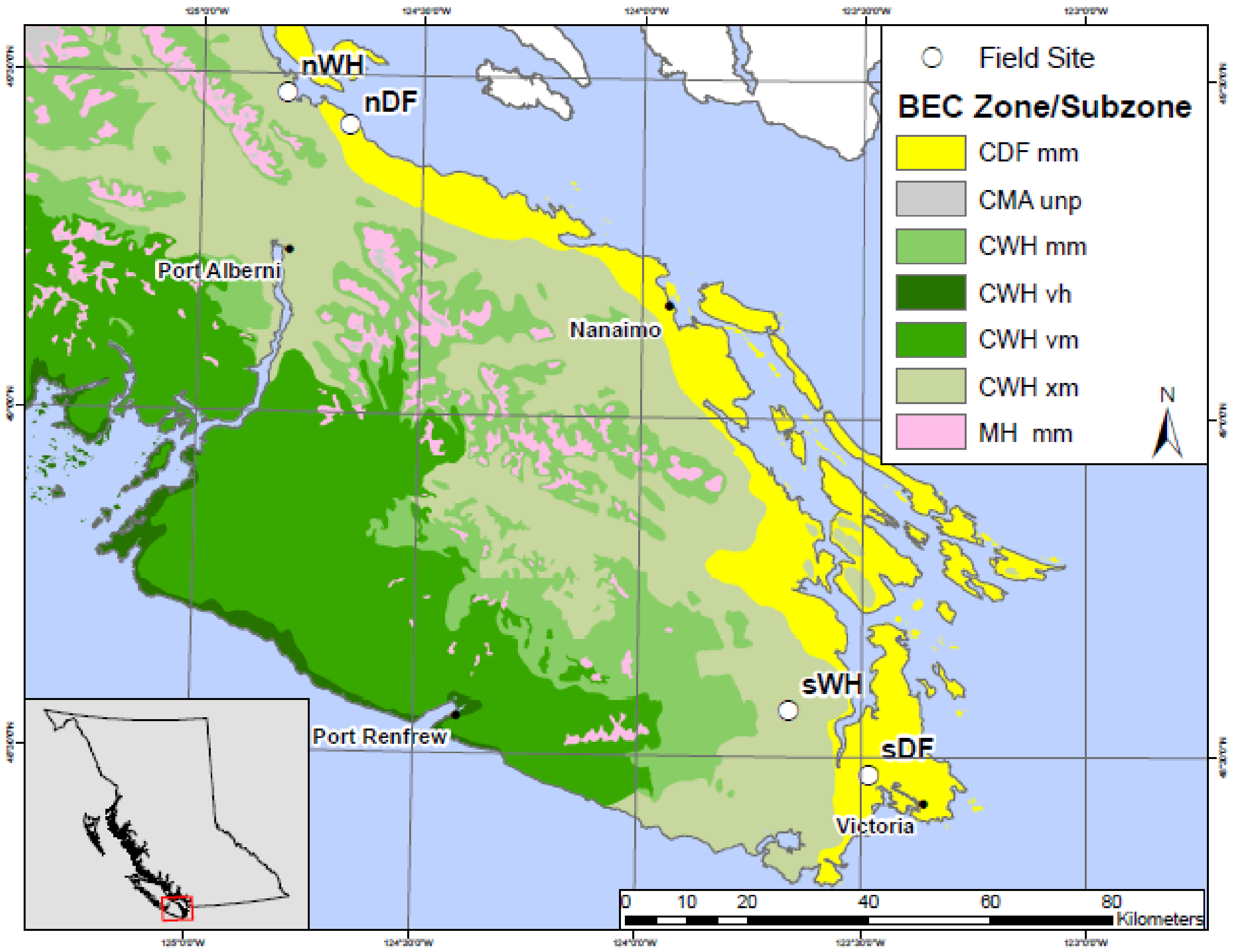

Supplementary Figure S1: Map of southern Vancouver Island, British Columbia, Canada, showing location of southern (s) and northern (n) transects, each with zonal sites in coastal Western Hemlock (WH) and coastal Douglas-fir (DF) biogeoclimatic zones. Colors represent coastal Douglas-fir (CDF), coastal Western Hemlock (CWH), coastal Mountain-Heather Alpine undifferentiated and parkland (CMA unp) and Mountain Hemlock (MH) Biogeoclimatic (BEC) zones as well as moist maritime (mm), very wet hypermaritime (vh), very wet maritime (vm) and very dry maritime (xm) subzones.