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Salmon carcasses influence genetic linkages between forests and streams

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Abstract

Biodiversity at many scales (functional group, species, genetic) can result in emergent ecological patterns. Here we explore the influence of tree genotypic variation and diversity on in-stream ecosystem processes and aquatic communities. We test whether genetically diverse inputs of leaf litter interact with a keystone organism, anadromous salmon, to influence in-stream ecosystem function. We used reach-level manipulation of salmon carcasses and leaf litter bags to examine how nutrient inputs interact with genetic variation in leaf litter decomposition. Genotypic variation in black cottonwood (*Populus balsamifera* ssp. *trichocarpa*) significantly influenced leaf litter chemistry, litter mass loss, and fungal biomass, but these variables were only weakly influenced by salmon carcass presence or a genotype*salmon (G x E) interaction. Mixtures of genotypes tended to demonstrate antagonistic effects (slower than expected decomposition) in the absence of salmon, but synergistic effects (faster than expected decomposition) when salmon were present. Our findings suggest that the influence of plant genotypic variation in linking aquatic and terrestrial ecosystems may be altered, and in some cases intensified in the presence of a keystone vertebrate species.

Keywords: biodiversity-ecosystem function, genes-to-ecosystems, aquatic-terrestrial interaction, litter mixtures

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Introduction

Over the last decade, a body of research has shown that genetic variation in several foundation species can influence associated communities and ecosystem function, and these influences can be as important as the influences of species-level diversity (Whitham *et al.* 2006; Hughes *et al.* 2008; Bailey *et al.* 2009). A subset of this research shows significant intraspecific (within species) variation in litter chemistry across a suite of plant species (including *Populus* [cottonwood, aspen], *Quercus* [oak], and *Betula* [birch]) and subsequent litter decomposition and nutrient cycling in both terrestrial and aquatic systems (Schweitzer *et al.* 2004; Madritch *et al.* 2006; LeRoy *et al.* 2007; Silfver *et al.* 2007). The majority of previous genes-to-ecosystems research has been conducted in common garden environments, but several recent studies have taken a broader scope to examine genetic variation across environmental gradients. It is crucial to move genes-to-ecosystems research outside the realm of common gardens because this will help to place genetic variation within the context of broader environmental variation.

Understanding genetic by environment (G x E) interactions is important because they may elucidate situations in which the influence of genetic variation is either attenuated or amplified by environmental factors. For example, in previous studies, G x E interactions were rare for sea grass communities (Tomas *et al.* 2011), but relatively more common for terrestrial insect communities (Johnson and Agrawal 2005; Tack *et al.* 2010; Rowntree *et al.* 2010; Genung *et al.* 2012; Tétard-Jones *et al.* 2013). In the case of influences on ecosystem functions, clear G x E interactions have been shown in both terrestrial (Madritch *et al.* 2006; He *et al.* 2012) and aquatic (LeRoy *et al.* 2012) leaf litter decomposition studies and a soil nitrogen transformation study (Pregitzer *et al.* 2013). In most cases, the environmental factor examined is abiotic (such as nutrient enrichment), but occasionally the factor is biotic, such as herbivory (Schweitzer *et al.* 2005b) or the presence of another genotype (Genung *et al.* 2012).

Emergent biodiversity patterns have been shown for both species mixtures and genotype mixtures of litter. Diversity in detritus research represents a subset of the broader

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biodiversity-ecosystem function literature (as reviewed by Gessner *et al.* 2010; Swan and Kominoski 2012) and shows that litter mixtures have three possible effects on the decomposition process: 1) additive effects where litter mixtures decompose at rates expected based on each litter type in isolation 2) non-additive, synergistic effects where mixtures decompose faster than expected, and 3) non-additive, antagonistic effects where mixtures decompose slower than expected (Lecerf *et al.* 2011). Several recent papers have also shown that mixtures of genotypes within species can influence decomposition and carbon cycling. As with species diversity studies, these few results show that litter genotype mixtures can exhibit significantly faster litter decomposition than expected (Schweitzer *et al.* 2005a), significantly slower decomposition than expected (Madritch *et al.* 2006), or no difference between observed and expected mass loss (Madritch *et al.* 2006; LeRoy *et al.* 2007).

Variation in environmental contexts may help explain the unpredictable additive and non-additive results observed in past diversity studies; however, very few studies have manipulated litter diversity and environmental variables together to test the consistency of emergent effects. Rosemond *et al.* (2010) manipulated litter species diversity in the presence and absence of elevated in-stream nutrients and showed that litter mixtures decomposed even faster in mixture when also exposed to high in-stream nutrient concentrations. In a similar study, Bretherton *et al.* (2011) examined litter species mixtures in the presence and absence of salmon carcasses (a natural source of in-stream nutrients and organic matter) and also showed more synergistic responses to litter mixing when salmon carcasses were present. This study further explores these relationships, but goes to a finer level to ask how genotypic diversity effects may be altered by nutrient enrichment via salmon carcass inputs.

Because anadromous salmon represent a major ecosystem-level influence on streams and forests throughout the northern hemisphere (Gende *et al.* 2002), salmon presence is an ideal biotic environmental factor to examine in this context. The presence of salmon carcasses tends to increase in-stream algal and microbial productivity (Fisher-Wold and Hershey 1999)

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and alters the structure of macroinvertebrate communities (Wipfli *et al.* 1998). Although much is known about inputs of both leaf litter and salmon carcasses to streams separately, only a few studies have examined their interactions and the results have been mixed. For example, salmon presence accelerated litter decomposition for relatively labile species like *Acer* (maple; Yanai and Kochi 2005), *Salix* (willow; Kohler *et al.* 2008), mixed *Alnus* (alder) + *Acer* litters (Claeson *et al.* 2006), and mixed *Alnus* + *Populus* (cottonwood) and *Alnus* + *Acer* litters (Bretherton *et al.* 2011), but slowed decomposition for both *Alnus* and *Acer* litters in isolation (Zhang *et al.* 2003), and mixtures of *Populus* + *Acer* and *Populus* + *Alnus* litters (Bretherton *et al.* 2011).

Here we expand on previous research demonstrating genes-to-ecosystems linkages across aquatic-terrestrial boundaries by examining genotypic variation in litter chemistry for *Populus balsamifera* L. ssp. *trichocarpa* Torr. and A. Gray ex Hook (black cottonwood, hereafter *Populus trichocarpa*; this study includes Nisqually-1, the first tree to be genomically sequenced; Tuskan *et al.* 2006). We first examine how genetic variation influences leaf litter chemistry, then we examine the influence of genetic variation and genotype mixing effects on leaf litter decomposition as it interacts with an important biotic environmental factor, the presence of anadromous salmon carcasses. We hypothesised that: 1) genetic variation in *P. trichocarpa* would lead to differences in litter chemistry, 2) this genetic variation, as well as the presence of salmon carcasses, and their interaction (genotype * salmon) would influence leaf litter mass loss at all harvest dates, fungal biomass accumulation, and aquatic macroinvertebrate community metrics; 3) leaf litter mass loss would be related to the suite of litter chemicals measured; 4) genotypic mixing would accelerate mass loss and fungal biomass accumulation; and 5) salmon carcasses, by providing nutrients and organic matter, would interact with genotype mixtures to increase synergisms in mass loss, increase synergistic responses of fungal biomass accrual, and alter macroinvertebrate communities.

Materials and Methods

Study site

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This study was conducted between 15 January and 4 April 2009 in McKenna Creek (46.93498 N 122.56394 W, elevation 107 m), a tributary of the Nisqually River, WA, USA. The stream reaches were relatively low gradient channels with sandy substrate and ranged in width from 1 to 3 m. Although salmon spawning was not occurring in this location during our study period (peak spawning is from September to November), salmon carcasses are seasonally present at this location as detrital inputs earlier in the fall and the carcasses left by late-November spawners are not likely to be fully degraded until mid-February. Access to this site was granted by the Nisqually Land Trust and no further permits were required for this study location. Throughout the study period, average stream temperatures ranged from 5.3 - 5.7°C, average pH ranged from 6.3 - 7.2, and average dissolved oxygen ranged from 8.9 - 10.2 mg L⁻¹. The riparian zone was dominated by *P. trichocarpa*, *Alnus rubra* Bong (red alder), *Acer macrophyllum* Pursh (bigleaf maple), *Thuja plicata* Donn ex D. Don (western redcedar), and *Pseudotsuga menziesii* (Mirb.) Franco (Douglas-fir). Average annual precipitation at the site was approximately 129 cm and average max and min temperatures were 25 and 0° C, respectively.

Litter collection

Leaf litter was collected at the Puyallup Research and Extension Center of Washington State University's (WSU) R.L. Goss Research Farm in a 38-year old common garden of *P. trichocarpa*. To establish the common garden, branches were collected randomly from naturally existing stands across OR, WA and BC in 1976 and planted at this one location to isolate genetic differences among genotypes (**Fig. 1**). Branches of full-grown, individual genotypes were wrapped in mesh to collect genotype-specific litter. Naturally abscised litter was collected weekly from 20 October to 21 November 2008 for two replicate clones of each of six *P. trichocarpa* genotypes (**Fig. 1**; Chilliwack 61-154 [C], Arlington 88-596 [A], Snoqualmie 5-52 [S], Nisqually-1 [N], Longview 9-91 [L], and Hoh 95-876 [H]). Genotypes dropped their leaves throughout this period, but their leaf fall timing differed. We needed to wait until we had collected

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154 enough leaf litter from all genotypes before we could implement the study. Access to this site
155 was granted by WSU and no further permits were required for litter collection.

156 *Study Design*

157 Air-dried leaf litter was weighed into 2 g ($\pm$ 0.05 g) quantities and experimentally placed into 1
158 cm-mesh litterbags (23 x 28 cm) for each of the 6 genotypes in isolation. Sixteen replicate
159 litterbags were created per genotype and for each of 3 harvest dates, yielding a total of N = 288
160 litterbags. Additionally, a suite of litter mixtures was created to address both the influence of
161 genotype richness and mixture compositional influences on litter mass loss. Five different
162 genotype mixtures included: one equal-weight mixture of all six genotypes (6-genotype), and
163 four equal-weight mixtures of 2 genotypes each (C+L, N+L, N+C, N+S). Genotype pairs were
164 chosen to represent a gradient in geographic distances among genotypes (see **Table S1**).
165 Sixteen replicate litterbags were created per mixture treatment for each of 3 harvest dates,
166 yielding an additional N = 240 litterbags.

167 The sixteen replicate litterbags for each single genotype and mixture treatment were
168 placed at 16 different locations in McKenna Creek using another common garden design. The
169 study was designed so that one replicate litterbag of each treatment and each intended harvest
170 date were placed along 2.5 m pieces of rebar similar to a blocked design with all 33 litterbags
171 randomly placed along each rebar. In an ideal situation, 8 blocks would have been randomly
172 treated with salmon carcasses and 8 would have been control blocks, but the inherent lack of
173 independence between up- and downstream locations in a riverine environment made a fully
174 randomized block design impossible. Instead, eight replicate “blocks” were placed perpendicular
175 to streamflow in downstream salmon manipulation reaches and eight more were placed directly
176 upstream in no-salmon control reaches. Blocks were separated by roughly 10 - 20 m to maintain
177 independence among experimental units and to place rebar lengths in similar environmental
178 conditions. We placed rebar lengths in depositional pools at similar depths (average depths
179 were 31 and 33 cm for salmon and no salmon rebars, respectively). On each rebar length,

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litterbags were randomly attached with colored cable ties to facilitate removal from the stream on the appropriate harvest date (14, 28 or 78 d). We based collection dates on past studies that showed only 25% mass remaining after 78 d (Bretherton *et al.* 2011).

Frozen whole carcasses (1 - 2 carcasses, approx 1 - 2 kg) of Chinook salmon (*Oncorhynchus tshawytscha*; average length: 70 cm; average width: 17 cm) were wrapped in wire mesh and attached along the top edge of the rebar lengths in downstream salmon reaches to retain the carcasses directly upstream of litterbags, *sensu* Bretherton *et al.* (2011). Whole carcasses were used instead of exact masses of salmon tissue to better simulate natural spawning influences, but this may have resulted in unmeasured variation in salmon nutrient loadings among blocks. Control blocks were identical and included the litterbags attached to the rebar and the mesh envelope, except these envelopes did not enclose salmon carcasses. A previous study used sand bags as no-salmon controls, but found significant microbial colonization of the sand and bag (Bretherton *et al.* 2011) and so in this study, nothing was used in place of the carcasses, which may have resulted in slightly altered flow environments in control blocks. By 78 d the salmon carcasses were reduced to bones and small pieces of amorphous tissue, and leaf litter bags contained between 0.0 and 0.97 g of AFDM (ash-free dry mass) leaf material (representing 100% and 50% mass loss, respectively).

Litter chemistry

Subsamples of air-dried litter were prepared for litter chemical analysis by grinding to pass a 0.42 mm-mesh screen using a Wiley mill (Thomas Scientific, Swedesboro, NJ). Initial litter % N and % C were determined using elemental analysis (Carlo Erba NC2100 Elemental Analyzer).

A modified version of EPA Method 365.3 was used for litter % P analysis. Approximately 500 mg of dry leaf litter powder was combusted in a muffle furnace at 550°C for 3 h, to which a 10 mL solution of dilute *aqua regia* was added. Diluted extracts were analysed using the spectrophotometric ascorbic acid – phosphomolybdate method using a diode-array spectrophotometer (Hewlett-Packard 8453, Germany). Proximate cellulose and lignin

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percentages were determined using a gravimetric method modified by Gessner (2005). Values for acid detergent cellulose (ADC) and acid detergent lignin (ADL) were converted to percentages based on the initial dry weight of each sample. We analysed condensed tannins using a modified butanol-HCl method (Porter *et al.* 1986; LeRoy *et al.* 2007). Tannin standard for *P. trichocarpa* was prepared by exhaustive extraction using the methods of Hagerman and Butler (1989). All standards and samples were analysed for absorbance at a wavelength of 550 nm using a diode-array spectrophotometer.

Leaf litter mass loss

Leaf litter bags collected from the stream were placed in polyethylene zipper bags and transported to the lab for processing. Leaves were gently rinsed of sediment and macroinvertebrates and 10 leaf punches (11 mm dia) were taken from leaf laminae in each litter bag for ergosterol analysis (see below). The remaining leaf material was dried at 70°C for 72 h, weighed, ground using a Wiley mill to pass a 0.42-mm mesh screen and subsampled (0.250 g) for combustion in a muffle furnace at 550° C for 3 h to determine ash-free dry mass (AFDM) remaining fraction.

Fungal biomass

Ergosterol concentrations were used to estimate fungal biomass from leaf discs via gas chromatography-mass spectroscopy (GC-MS). Leaf discs were extracted in 5 mL of methanol then spiked with 50 µL of 7-Dehydrocholesterol and 10 mL of 15% KOH/methanol. Vials were incubated in a drying oven at 80° C for 90 min, cooled and then ergosterol was partitioned into a pentane solution (1:3, DI water:pentane). After the extracts were evaporated to dryness under nitrogen gas and slight warming, a derivatization reagent (15 µL of neat pyridine and 50 µL of (N)- Bis(trimethylsilyl)trifluoroacetamide) was added to each sample vial. Vials were vortexed for 5 s, dried in an oven at 60°C for 30 min, then cooled and 500 µL of toluene was added prior to analysis by GC-MS (Agilent 7890A/5975C). Ergosterol concentrations were converted to fungal

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biomass (mg g^{-1} leaf) assuming an ergosterol concentration of $5.5 \mu\text{g mg}^{-1}$ of mycelial dry mass (Gessner and Chauvet 1993).

Macroinvertebrate analyses

Aquatic macroinvertebrates were separated from leaf matter and preserved in 70% ethanol for identification. All macroinvertebrates from the 28 d harvest were identified to the lowest taxonomic level possible using Merritt *et al.* (2008) and dissecting microscopy. Aquatic macroinvertebrates were collected under a Washington State Department of Fish and Wildlife Scientific Collection Permit granted to the Evergreen State College. This study complied with all relevant regulations, and no protected species were sampled.

Statistical Analyses

In order to examine genotypic differences in initial leaf litter chemistry (%), we transformed percent data using the arcsine-square root transformation (this transformation normalizes the distributions of percent data) and used analysis of variance (ANOVA) to compare differences among the six *P. trichocarpa* genotypes. Significant ANOVAs were followed by Tukey's Honest Significant Difference (HSD) posthoc tests. All data analyzed using parametric ANOVA met the assumptions of normality and homogeneity of variances and tests were run in JMP Pro (11.0, SAS Institute Inc., Cary, NC, 1989-2015) with an $\alpha = 0.05$. All figures show back-transformed means ± 1 standard error (SE).

Decomposition rates were compared using a general linear model (PROC GLM in SAS 8.01, SAS Institute, Inc. 1999-2000) with fixed effects and Type I Sums of Squares to determine significant treatment effects, *sensu* Kominoski *et al.* (2007). Time was treated as a continuous variable in the model, and each term (time, litter genotype presence or absence, salmon carcass presence or absence, and diversity terms – genotype richness and composition) was added sequentially to the model. The two diversity terms were composed of genotype richness (one-, two-, or six-genotype litter mixtures) and genotype composition (C+L, N+C, N+L, N+S, 6-geno) and allowed for separation of these two diversity effects.

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Linear relationships between decomposition rates and litter chemicals were determined using multiple linear regression in JMP. Decomposition rate constants (k) were determined for each leaf litter treatment with and without salmon by regressing the natural log of AFDM against day (Jenny *et al.* 1949).

Two-way ANOVAs with tree genotype, salmon treatment, and genotype*salmon (G x E) interaction as main factors were used to explore patterns in mass loss at individual harvest dates, fungal biomass and simple invertebrate metrics (taxa richness, evenness, diversity, and total abundance). Tukey's HSD post hoc tests were used to determine differences among all pairwise treatments. In addition, mass loss and fungal biomass at individual harvest dates were compared across genotype richness levels (1, 2 and 6 genotypes) using ANOVA.

To test for non-additivity of leaf litter mass loss in these same mixtures, Chi-square tests were used to compare observed mass loss values in mixtures to expected values based on the mass loss of each genotype in isolation at each harvest date. Additive responses were those that did not vary from expectation while synergistic responses showed significantly higher mass loss than expected and antagonistic responses showed significantly lower mass loss than expected. Multiple comparisons required a Bonferroni-adjusted alpha to be set at $\alpha = 0.0016$.

Macroinvertebrate data from litter bags were analysed using multivariate community analysis methods. Non-metric multidimensional scaling (NMS) ordination and multi-response permutation procedures (MRPP) were performed in PC-ORD (4.34, MJM software 1995-2005). Relativization to taxa maximum was used to reduce the effects of hyper-abundant taxa. Two-dimensional ordinations were chosen as the least-stressful representation of this complex multidimensional dataset based on scree-plot evaluations. MRPP was used to determine significant differences in macroinvertebrate assemblages among different leaf litter and salmon treatments. Indicator Species Analysis was used to determine species-specific associations with litter or salmon treatments (Dufrene and Legendre 1997).

Results

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Litter chemistry

Leaf litter chemistry differed among *P. trichocarpa* genotypes for all litter chemicals measured: % N, % P, C:N, cellulose, lignin and condensed tannins (**Fig. 2**). Genotype accounted for large percentages of the variation in litter chemistry variables, ranging from explaining 30% of the variation in cellulose to 96.7% of the variation in N (see R^2 values in **Fig. 2**).

Leaf litter decomposition

Because leaf litter decomposition can be analysed with respect to both the overall rate of decomposition through time, as well as the actual mass loss at individual harvest dates, we present data on both the rate of decomposition ($k \text{ day}^{-1}$) and mass loss (%). Multiple factors significantly explained leaf litter decomposition rates overall, including mixed-genotype litter richness ($p = 0.0318$) and individual litter genotype presence/absence for the genotypes Hoh ($p = 0.0038$) and Nisqually ($p = 0.0200$, **Table 1**; Overall model: $F_{(65,327)} = 19.6$, $p < 0.0001$). We originally hypothesised that salmon carcasses, by providing a pulse of nutrients, would increase litter decomposition for all genotypes; however, this was not the case (**Table 1**; $p = 0.6947$). Consistently for both salmon and control treatments through time, the genotype from the Hoh River (H; Olympic Peninsula, WA) showed slower decomposition, and Nisqually (N; Nisqually River, WA) showed accelerated decomposition. Both of these genotypes showed a significant presence/absence effect on mixed litter decomposition rates (**Table 1**). Contrary to our predictions, however, salmon carcasses did not significantly affect overall decomposition rates, and instead interacted with genotypes and genotype mixtures differentially (see below).

Multiple linear regression models revealed that when salmon were absent, % P and % cellulose significantly negatively influenced decomposition rates ($F_{(2,36)} = 10.31$, $p = 0.0003$), but in the presence of salmon carcasses, % condensed tannins and % lignin negatively influenced decomposition ($F_{(2,18)} = 17.36$, $p < 0.0001$). Decomposition rates for single genotypes of *P. trichocarpa* litter ranged from 0.0084 (± 0.00124) for Longview litter in the presence of salmon to 0.0120 (± 0.00075) for Arlington litter in the absence of salmon. Decomposition rates for

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genotype mixtures were slightly more variable and ranged from 0.0076 ($\pm$ 0.0009) for the Nisqually x Longview mixture in the absence of salmon to 0.0125 ($\pm$ 0.0004) for the Nisqually x Snoqualmie mixture in the presence of salmon (**Table S2**). All exponential regressions used to determine decomposition rates were significant at $p < 0.05$.

Leaf litter mass loss

Two-way ANOVAs for mass loss at individual harvest dates showed a significant genotype effect, but no salmon effect, or genotype*salmon (G x E) interaction (**Table 2**). *Post hoc* tests revealed that when salmon carcasses were present, genotypic effects on decomposition were weaker (**Fig. 3a vs 3b**). Litter from different genotypes lost mass at significantly different rates in the absence of salmon on both days 14 and 28 (**Fig. 3a and 3e**, respectively); however, in the presence of salmon carcasses, no significant differences in mass loss among genotypes were detected until day 28 (**Fig. 3f**), and all genotype effects were weaker in the presence of salmon. By day 78, genotype effects disappeared in both treatments as remaining litter and salmon biomass were both low (**Fig. 3i and 3j**).

Although genotypic variation influenced leaf litter mass loss, genotype richness in mixtures was a weak predictor of mass loss. In most cases, leaf litter bags with 2 or 6 genotypes did not lose mass faster or slower on average than single genotype litter bags (**Fig. 3c,d,g,k,l**), regardless of the presence of salmon. In only one case, mass loss on day 28 for leaf litter mixtures in the presence of salmon carcasses, was there a significant increase in mass loss for the 6-genotype mixture compared to single genotypes (**Fig. 3h**); however, looking more closely at different mixtures of genotypes reveals further non-additive patterns. The direction (both synergistic and antagonistic) and magnitude of mass loss effects depended on compositional effects (which genotypes were present), as well as the presence of salmon (**Fig. 4, Table S3**). In the absence of salmon, antagonistic responses (significantly less mass lost than expected; symbols fall below the 1:1 line) were more common and were found for the 6-genotype mixture on day 14, and most mixtures on day 78 (**Fig. 4a**). In contrast, significant

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synergistic responses (significantly more mass lost than expected; symbols fall above the 1:1 line) occurred for the C+L mixture on day 28 and the N+C mixture on day 78 (**Fig. 4a**). In the presence of salmon, synergistic responses were more common and were seen for the N+C mixture on day 14, the 6-genotype mixture on day 28 and most mixtures on day 78 (**Fig. 4b**). In contrast, only one antagonistic response was shown in the presence of salmon for the N+C mixture on day 28 (**Fig. 4b**). The mean deviation in mass loss from expectation (observed – expected) in the absence of salmon shows a significant antagonistic response, while the mean deviation for mixtures in the presence of salmon shows a significant synergistic response (**Fig. 4c**).

Fungal Biomass

Aquatic fungal biomass at day 28 was most strongly influenced by litter genotype (**Table 2**). Qualitatively, the difference appears stronger in the absence of salmon carcasses (**Fig. 5**), but there was no significant salmon effect or G x E effect. Litter genotype mixing led to an overwhelmingly synergistic response in fungal biomass with significantly more fungal biomass on leaf litter mixtures than expected based on single genotype litterbags. This result was observed for all treatments except for the 6-genotype mixture in the absence of salmon and N+C mixture in the presence of salmon (**Fig. 5c**). Additionally, fungal biomass in the salmon-treated reach showed even stronger synergistic responses to litter mixing than control reaches, though both mixture effects were significantly synergistic (**Fig. 5d**). Finally, fungal biomass was significantly influenced by genotype richness in both the absence and the presence of salmon carcasses ($F_{(2,38)} = 3.4230$, $p = 0.0430$; $F_{(2,37)} = 4.5609$, $p = 0.0170$, respectively). Tukey's HSD post hoc tests reveal that fungal biomass on single genotypes is significantly lower than that found on 2-genotype mixtures in both experimental treatments, but the 6-genotype mixture did not differ from either single or 2-genotype mixtures.

Macroinvertebrates

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Macroinvertebrate communities were not significantly different among litter genotypes when compared using multi-response permutation procedure (MRPP) analysis; however, communities differed between the salmon and no-salmon treatments (MRPP A = 0.035, $p < 0.0001$). Salmon carcass presence significantly reduced macroinvertebrate taxa diversity as shown using Shannon's and Simpson's Diversity Index values (**Table 2**). Overall macroinvertebrate taxa abundance, richness and evenness did not significantly differ between salmon carcass and no-salmon reaches. Invertebrate taxa abundance was significantly correlated with fungal biomass in the no-salmon control reach ($R^2 = 0.1216$, $F_{(1,39)} = 5.4$, $p = 0.0254$) but not in the salmon reach ($R^2 = 0.0056$, $F_{(1,37)} = 0.21$, $p = 0.6510$).

Several macroinvertebrate taxa were significant Indicator Species for either the no-salmon control or the salmon carcass treatments. Members of the Trichopteran genus *Lepidostoma* ($p = 0.0010$), and the Dipteran families Tipulidae ($p = 0.0050$) and Simuliidae ($p = 0.0100$) indicated for the no-salmon control treatments, while members of the Plecopteran genus *Malenka* ($p = 0.0480$), the Trichopteran genus *Onocosmoecus* ($p = 0.0010$), the Dipteran family Psychodidae ($p = 0.0130$) and the Mollusc family Physidae ($p = 0.0020$) indicated for the salmon carcass treatment.

Discussion

As predicted, genetically distinct clones of *P. trichocarpa* differed in leaf litter chemistry, mass loss at mid-stages of decay, and fungal biomass accrual. Contrary to our predictions, salmon carcasses did not stimulate decomposition overall, which was somewhat unexpected because water chemistry downstream of salmon carcasses can be elevated in ammonium (Claeson *et al.* 2006), and nutrient enrichment has been shown to stimulate leaf litter decomposition by 50% in a large, recent meta-analysis (Ferreira *et al.* 2014). Instead, in this study, salmon carcass presence altered the non-additive responses of genotype mixtures, stimulated fungal biomass, and altered macroinvertebrate community structure. Although salmon influenced several in-stream variables, we cannot clearly show any genotype by environment interactions with

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salmon carcasses using this experimental approach. Previous research in an aspen (*Populus tremuloides*) system found evidence for G x E interactions between leaf litter and nutrient additions (LeRoy *et al.* 2012), but there are some key methodological differences between these two studies. First, the nutrient addition in LeRoy *et al.* (2012) took place while the aspen were growing, and leaf litter decomposition for nutrient-enriched trees was compared to decomposition for control trees. It is possible that the form of nutrient addition or the location of the addition (in the forest versus in the stream) may alter G x E responses. Second, because of key experimental design issues, this previous study also involved better randomization of nutrient-enriched and control litterbags. The results we provide here suffer the same issues as in-stream nutrient addition studies in terms of the complications of upstream versus downstream treatments which may have confounded treatment effects with environmental effects.

In addition, this current study may underestimate the influence of both litter genetic variation and salmon carcasses on the detrital food web based on the timing of the study. The need to wait to collect litter from all genotypes and then prepare hundreds of litter bags meant the study was placed in the stream 1-2 months later than peak litter fall and fewer shredding and salmon-adapted invertebrates may have been present at this later time. Future research should work to prepare and deploy both litter bags and salmon carcasses earlier to better match the experimental inputs with natural allochthonous inputs.

Genotype mixture effects on mass loss were often non-additive, but highly idiosyncratic and dependent on the composition of the genotype mixture and the environmental context in which the litter decomposed (in the presence or absence of salmon). Similar patterns have been shown in previous studies exploring mixtures of litter species (Lecerf *et al.* 2011), and so this was not unexpected. In litter mixtures, the presence of salmon did not completely overwhelm diversity effects, which was seen in a recent example using a long-term nutrient enrichment experiment (Rosemond *et al.* 2010), but had the interesting effect of altering non-additive outcomes. Several mixtures switched from additive mass loss in the absence of salmon to

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synergistic (faster than expected) mass loss in the presence of salmon. Additionally, several litter mixtures that showed antagonistic (slower than expected) mass loss in the absence of salmon switched to additive or synergistic mass loss in the presence of salmon carcasses. These switches are not isolated to this study, but two other recent papers show a similar switch when litter mixtures were exposed to a nutrient source in the stream environment (Rosemond *et al.* 2010; Bretherton *et al.* 2011; **Fig. 6**). The pattern of slightly to very antagonistic mixture effects for control treatments compared to synergistic effects for elevated nutrient treatments (through fertilizer or salmon additions) is largely consistent across habitats, leaf species, and nutrient environments, and should be explored in future studies. Further research could provide evidence for a more broadly applicable Nutrient-Diversity Synergism Hypothesis (**Fig. 6**), but this pattern would need to be further tested under other conditions and in other systems, especially in light of a very recent study which showed synergistic litter effects in streams with low nutrients compared to eutrophic systems (Lima-Fernandes *et al.* 2015). In this case, since an environmental gradient in eutrophication was used instead of a nutrient manipulation, it is possible that the eutrophic streams were otherwise degraded or had different shredder of microbial communities that may have influenced mass loss in the opposite direction.

It is possible that idiosyncratic litter mixing responses may be due to environmental variation across our study reaches. Since microhabitats were chosen to be as similar as possible among both salmon and no-salmon control blocks, environmental differences were not explicitly measured as covariates. In this study, any environmental variation would have resulted in error in the leaf litter mass loss data, making it more difficult to see differences among genotypes or mixtures. The fact that there are evident differences in mass loss among intraspecific leaf litter treatments at mid-stages of decay shows that environmental variation was not large enough to swamp these patterns. This does not suggest that environmental variation was not present or important, just that genotypic variation was more important at mid-stages of decay. At early and late stages of decay, it is possible that unmeasured environmental variables

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were relatively more important because neither genotype nor salmon carcass presence explain much variation in mass loss at these time periods.

In contrast to the idiosyncratic mass loss effects in mixtures discussed above, genotype mixture effects on fungal biomass accrual were consistently synergistic across treatments, and more so in the presence of salmon carcasses. These results are relatively novel. One previous study examined aquatic fungal biomass across many different genotypes of two *Populus* species and their hybrids and found significant differences in fungal biomass accumulation on genotypes within species (LeRoy *et al.* 2007), but work with mixtures of genotypes and environmental interactions is new in this study, and should be further explored.

While litter genotype was a driver of patterns in litter mass loss and aquatic fungal biomass, stream invertebrates were more sensitive to salmon carcass presence. Specifically, the presence of salmon carcasses had a negative influence on the richness, evenness, and diversity of the macroinvertebrates that colonized leaf surfaces. It is likely that the carcasses provided more appealing substrate for a variety of macroinvertebrates and had the influence of drawing certain components of the community away from litter bags, similar to the findings of Zhang *et al.* (2003). Although there were no differences in overall macroinvertebrate abundances on litter bags in no-salmon control and salmon carcass treatments, there was an overall shift in the community structure found in litter bags from these two treatments. We found no evidence of macroinvertebrate discrimination among *P. trichocarpa* genotypes or litter mixtures, which is supported by previous studies comparing genotypes within species of *Populus fremontii* and *Populus angustifolia* (LeRoy *et al.* 2007) and species mixtures in the presence and absence of salmon (Bretherton *et al.* 2011). The timing of leaf fall in the Pacific Northwest is generally October through November and the timing of fall salmon runs in these rivers is generally August through November. The need to collect and prepare leaf litter and salmon carcasses prior to the study required us to wait until just after these major natural inputs and may have influenced in-stream invertebrate responses to both of these detrital inputs.

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We demonstrate that genotypically distinct leaf litters and salmon carcasses interact in streams to influence leaf litter mass loss and fungal biomass growing on leaf surfaces, but only carcass presence influenced aquatic macroinvertebrates. Thus while ecosystem function may be sensitive to the interaction of G x E effects, stream invertebrates (often considered bioindicators of stream health) are more sensitive to major biotic environmental factors like salmon carcass presence than to the complex interactions between salmon, tree genetics, and carbon-cycling through decomposition.

The study of salmon and leaf litter interactions is not simply a pairing of major energy players in stream ecosystems. Salmon-derived nitrogen provides a limiting nutrient for trees and vascular plants growing in riparian zones in the northern hemisphere and significantly fertilizes riparian forests and increases above-ground net primary productivity (Helfield and Naiman 2001; Reimchen *et al.* 2003). The interaction between salmon carcasses and riparian trees could result in a feedback to litter quality and potentially further influence litter dynamics (Madritch *et al.* 2009; LeRoy *et al.* 2012), especially in systems with healthy salmon runs. For example, Morris and Stanford (2011) found that salmon carcasses significantly enriched riparian plants and lowered C:N ratios across the entire 2.5 km floodplain of the Kol River on the Kamchatka Peninsula, Russian Far East. Further research into the plasticity of litter phenotypes when exposed to salmon carcass additions could provide more insight into these interactions.

Our results highlight the importance of asking questions across the boundaries of emerging fields of research, such as genes-to-ecosystems (Whitham *et al.* 2008), biodiversity-ecosystem function (Lecerf and Richardson 2010; Cook-Patton *et al.* 2011; Lecerf *et al.* 2011), and terrestrial-aquatic interactions (Richardson *et al.* 2010). More specifically, effects of genotypic variation on ecosystem function can be dependent on biotic and abiotic environmental contexts (Madritch *et al.* 2009; LeRoy *et al.* 2012; Pregitzer *et al.* 2013). Using leaf litter and salmon carcass interactions highlights two major allochthonous energy inputs to streams and riparian forests of the northern hemisphere and widens our understanding of terrestrial-aquatic

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interactions. The presence of salmon carcasses resulted in more frequent synergistic responses in mixtures both for litter mass loss and fungal biomass. Understanding the circumstances under which genetic variation may influence ecosystem function is a crucial area of research since a variety of factors influence genetic diversity in both foundation and keystone species. For example, range shifts under future climate conditions are likely to influence genetic variation in foundation tree species (Excoffier *et al.* 2009). Both genetic variation in keystone species and the overall influence of these organisms on ecosystem functions are likely reduced in situations where the species (like anadromous salmon) have been extirpated or greatly reduced in most natural riparian systems (only 6 to 7% of historic Pacific Northwest populations persist; Gresh *et al.* 2000).

In this paper, salmon carcass presence altered the way in which leaves from genetically diverse trees interacted with one another in mixtures, both through litter mass loss and fungal decomposers, as well as altered the litter-dwelling aquatic macroinvertebrate community. It is in these ways, and not in clear overall or interactive ways, that a major detrital input of nutrients and organic matter influences the brown food web in this stream system. Thus, a genetic perspective on ecosystem function becomes more important as riparian systems witness the loss of key ecological players like anadromous salmon. The highly complex nature of these systems warrants further investigation into the extended community and ecosystem effects of plant genotypic variation across gradients of both abiotic and biotic environmental variation and predictions for these systems as they experience large scale changes (Kominoski *et al.* 2013).

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Table 1. ANOVA results for mass loss analysis. ANOVA results for a general linear model describing the effects of incubation time (Time), leaf genotype presence (Snoqualmie, Longview, Chilliwack, Arlington, Hoh, and Nisqually genotypes), salmon carcass presence (Salmon), genotype richness (Richness), and genotype composition (Composition) within leaf litter bags as well as interactions among these factors on leaf litter decomposition. This model uses fixed effects and Type I sums of squares. Significant effects are denoted in bold and with asterisks (*).

Source	DF	Type I SS	Mean Square	F Value	p-value
Time	2	64.8993	32.4496	600.68	<0.0001*
Snoqualmie	1	0.0184	0.0184	0.34	0.5600
Longview	1	0.0180	0.0180	0.33	0.5646
Chilliwack	1	0.0697	0.0697	1.29	0.2569
Arlington	1	0.0004	0.0004	0.01	0.9295
Hoh	1	0.4578	0.4578	8.47	0.0038*
Nisqually	1	0.2951	0.2951	5.46	0.0200*
Salmon	1	0.0083	0.0083	0.15	0.6947
Richness	1	0.2511	0.2511	4.65	0.0318*
Composition	3	0.0792	0.0264	0.49	0.6903
Snoqualmie*Salmon	1	0.0090	0.0090	0.17	0.6828
Longview*Salmon	1	0.0169	0.0169	0.31	0.5767
Chilliwack*Salmon	1	0.0327	0.0327	0.61	0.4371
Arlington*Salmon	1	0.1240	0.124	2.30	0.1307
Hoh*Salmon	1	0.0421	0.0421	0.78	0.3781
Nisqually*Salmon	1	0.0900	0.0900	1.67	0.1977
Salmon*Richness	1	0.0059	0.0059	0.11	0.7418
Salmon*Composition	3	0.0647	0.0216	0.40	0.7537
Time*Snoqualmie	2	0.0072	0.0036	0.07	0.9354
Time*Longview	2	0.1133	0.0567	1.05	0.3515
Time*Chilliwack	2	0.3880	0.1940	3.59	0.0287*
Time*Arlington	2	0.0861	0.0431	0.80	0.4514
Time*Hoh	2	0.0397	0.0199	0.37	0.6926
Time*Nisqually	2	0.2800	0.1400	2.59	0.0764
Time*Salmon	2	0.1362	0.0681	1.26	0.2848
Time*Richness	2	0.0432	0.0216	0.40	0.6705
Time*Composition	6	0.4610	0.0768	1.42	0.2053
Time*Snoqualmie*Salmon	2	0.1252	0.0626	1.16	0.3152
Time*Longview*Salmon	2	0.0372	0.0186	0.34	0.7090
Time*Chilliwack*Salmon	2	0.0861	0.0431	0.80	0.4514
Time*Arlington*Salmon	2	0.0081	0.0040	0.07	0.9279
Time*Hoh*Salmon	2	0.1970	0.0985	1.82	0.1632
Time*Nisqually*Salmon	2	0.0579	0.0289	0.54	0.5858

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Table 2: Genotype, environment and G x E interactions. Two-way ANOVA results showing effects (p-values) of genotype, environment and G x E interactions on leaf litter decay and consumers. Specific responses are shown for mass loss at days 14, 28 and 78, fungal biomass at day 28, and the abundance, richness, evenness, Shannon’s diversity index and Simpson’s diversity index values of aquatic macroinvertebrates at day 28. Significant effects are denoted in bold and with asterisks (*).

Response Variable	Genotype	Salmon	G x E	R ²	F	df	P
<i>Leaf litter decay</i>							
Mass loss (14 d)	0.2466	0.1764	0.2065	0.2132	1.55	11, 74	0.1357
Mass loss (28 d)	<0.0001*	0.3540	0.9264	0.3427	3.32	11, 81	0.0010*
Mass loss (78 d)	0.3248	0.7391	0.9725	0.0982	0.61	11, 73	0.8103
<i>Consumers</i>							
Fungal biomass	0.0095*	0.1926	0.2114	0.4115	2.35	11, 48	0.0256*
Invert Abundance	0.2907	0.1108	0.2577	0.3159	1.55	11, 48	0.1543
Invert Richness	0.5466	0.6298	0.9640	0.1261	0.49	11, 48	0.9003
Invert Evenness	0.6897	0.1003	0.4398	0.1368	1.01	11, 48	0.4477
Shannon’s Index	0.9486	0.0002*	0.4490	0.2385	1.99	11, 48	0.0420*
Simpson’s Index	0.8129	0.0012*	0.4894	0.2098	1.69	11, 48	0.0936

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Figure Legends

Figure 1: Map of study locations. Map showing the collection locations for each *Populus trichocarpa* genotype (●), the location of the common garden site at the Puyallup Research and Extension Center (WSU, Puyallup, WA) used for litter collection (▲) and the location of the stream (McKenna Creek; ▲) used for decomposition experiments. Map created in Arc-GIS 10.0 by Dylan G. Fischer.

Figure 2. Intra-specific variation in black cottonwood litter chemistry. Initial litter chemistry differed significantly among genotypes of *Populus trichocarpa* leaf litter: a) % nitrogen, b) % phosphorus, c) C:N ratio, d) % acid-detergent cellulose, e) % acid-detergent lignin, and f) % condensed tannins. Bars represent means $\pm$ 1 SE, and lower case letters denote significant differences among genotypes.

Figure 3. Mass loss through time for individual genotypes and genotype mixtures.

Percent mass loss for 6 genotypes of *Populus trichocarpa* leaf litter in isolation and compared to mixtures of 2 and 6 genotypes after 14 d (a, b, c, d) 28 d (e, f, g, h), and 78 d (i, j, k, l) in the stream. Gray bars and gray open circles represent control litter bags and black bars and black open circles represent litter bags exposed to salmon carcasses. Bars represent means $\pm$ 1 SE, and lower case letters denote significant differences among genotypes. Open circles represent mass loss observations for genotype mixtures at different levels of genotype richness, and horizontal dashed lines represent treatment means.

Figure 4. Observed versus expected mass loss for genotype mixtures in the presence

and absence of salmon carcasses. Observed % mass loss for all genotype mixture treatments plotted as a function of the expected % mass loss based on average values for each genotype in isolation: a) no-salmon controls, b) salmon carcass treatments, and c) overall non-additive treatment effects ($\pm$ 1 SE). Leaf litter treatments included the following mixtures: 6-genotype mixture (6-geno), Chilliwack + Longview (C+L), Nisqually + Chilliwack (N+C), Nisqually + Longview (N+L), and Nisqually + Snoqualmie (N+S). Diagonal lines represent 1:1

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equilibrium, and ellipses contain all means from each harvest date. Asterisks denote significant non-additive responses in mixture (at Bonferroni-corrected $\alpha = 0.0016$); asterisks above the 1:1 line show synergistic mass loss, asterisks below the line show antagonistic mass loss.

Figure 5. Aquatic fungal biomass on individual genotypes and genotype mixtures

comparing mixtures in the presence and absence of salmon carcasses. Mean aquatic fungal biomass (mg g^{-1} leaf) on leaves of each *Populus trichocarpa* genotype in: a) no-salmon control litter bags, and b) salmon carcass treatment litter bags. Gray bars represent means for control litter bags and black bars represent means for litter bags exposed to salmon carcasses (± 1 SE); c) Observed fungal biomass for each genotype mixture (y-axis) compared to expected values of fungal biomass based on average values for each genotype in isolation (x-axis). Leaf litter treatments included the following mixtures: 6-genotype mixture (6-geno), Chilliwack + Longview (C+L), Nisqually + Chilliwack (N+C), Nisqually + Longview (N+L), and Nisqually + Snoqualmie (N+S). Gray symbols represent control litter bags and black symbols represent litter bags exposed to salmon carcasses. Non-significant additive responses denoted with “ns,” all other treatments were significantly non-additive (synergistic) at Bonferroni-corrected $\alpha = 0.005$. Diagonal line represents 1:1 equilibrium; and d) overall non-additive treatment effects (± 1 SE) on fungal biomass.

Figure 6. Patterns in synergistic and antagonistic mass loss across studies. Additive mass loss, synergistic mass loss (more mass lost than expected) and antagonistic mass loss (less mass lost than expected) patterns from three studies comparing mixed leaf litter mass loss in control and nutrient-enriched environments. Rosemond *et al.* (2010) used dripped fertilizer as the nutrient enrichment and examined effects on species mixtures and two studies used salmon carcasses as nutrient enrichment, Bretherton *et al.* (2011) examined effects on species mixtures and the this study examines effects on genotype mixtures. Values represent mean non-additive responses ± 1 SE, and may represent patterns that support a Nutrient-Diversity Synergism Hypothesis.

Observed fungal biomass (FB)