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Forest age influences freshwater biodiversity in temperate watersheds

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ABSTRACT

Forests can affect stream structure and function, but evaluations linking freshwater biodiversity to watershed-scale forest-stand conditions are limited. We evaluated competing hypotheses about spatial patterns of freshwater biodiversity by combining species inventories of vertebrates and invertebrates from traditional and environmental DNA (eDNA) methods across 24 temperate watersheds of young to old forests (37 to 124 y old). Freshwater taxonomic and functional richness of invertebrates were, on average, 1.2-fold and 1.5-fold higher, respectively, in older forests (>75 y) than in younger forests, consistent with the late-seral hypothesis. Vertebrate taxonomic and functional richness were 1.6-fold and 2.4-fold higher, respectively, in older forests, marginally supporting the late-seral hypothesis. Collectively, these findings suggest that heterogeneous habitat conditions of older forests support more rare, specialized, or intolerant freshwater species with diverse community roles. Evenness generally did not vary with mean watershed stand age, thus the diversity of habitats within a watershed may be shaped by other factors, such as disturbance history, watershed characteristics, and landscape heterogeneity. However, as measured by traditional sampling, invertebrate taxonomic evenness declined with increasing mean watershed stand age, slightly supporting the early-seral hypothesis, as younger forests may promote more evenly distributed assemblages. Ultimately, although greater freshwater richness is supported by older forests, evenness may depend more heavily on other factors in forested watersheds. Our findings provide empirical support for long-held ideas about the tight relationship between forests and freshwater biodiversity, emphasizing the interconnectedness between terrestrial and aquatic ecosystems and the importance of considering forests in watershed-scale conservation.

1. Introduction

Forests are crucial for shaping freshwater ecosystems by regulating water availability, water quality, nutrient cycling, and inputs of organic matter and large wood into streams (Warren et al., 2016 and references therein). However, compared to terrestrial forest ecosystems, the biodiversity of freshwater ecosystems in forests remains less well understood. Many taxa lack comprehensive inventories, making it critical to evaluate their current status and assess the influence of watershed-scale characteristics, including the frequency and intensity of prior forest disturbances. Freshwater biodiversity is declining globally, with one-quarter of freshwater fauna threatened with extinction (IPBES, 2019; Sayer et al., 2025). Native forests support over 85 % of amphibians worldwide (Urbina-Cardona et al., 2024). In the United States, 21 % of freshwater fish species are associated with forests (Bury et al., 2021), but among all vertebrates, freshwater fish have the highest global extinction rate, declining by 83 % since 1970 (WWF, 2018; Reid et al., 2019). Freshwater invertebrates in forests have not been systematically inventoried, though there are greater associations of macroinvertebrates with riparian cover (Tanaka et al., 2016) and global assessments show an overall decline in status of invertebrates (Cumberlidge et al., 2009; Richman et al., 2015; De Grave et al., 2015). Understanding patterns of freshwater biodiversity is critical to inform conservation and to understand the ecological significance of forests in supporting overall biodiversity (Lo et al., 2020).

Forest structural complexity has been positively linked to biodiversity (Pan et al., 2018), and elements of complexity change with forest successional age and stages. These changes can significantly influence

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stream structure and function by mediating light availability, large wood input, resource subsidies, and trophic interactions (Warren et al., 2016 and references therein). In addition to floods, droughts, and wildfire, forested watersheds experience a range of disturbances and stressors including insect and pathogen outbreaks and forest harvest, leading to a range of watershed stand ages and structural conditions. In some cases, contemporary forest-management practices can incorporate additional protections, including smaller harvest units and wider riparian buffers (Stednick, 2008; Cristan et al., 2016), further contributing to mixed standages within a watershed (Steel et al., 2017). Forest-cover information at the watershed scale may be most relevant to assess freshwater biodiversity, considering that many freshwater species distributions change at broad scales, such as watersheds (Frissell et al., 1986; Schlosser, 1991). Mean area-weighted watershed stand age is a metric for capturing time since stand-replacing disturbances as the proportion of a watershed affected by recent versus historical disturbances. Empirical data spanning a broad gradient of watershed stand ages across diverse landscapes offer an opportunity to understand the relationship between watershed stand age and freshwater biodiversity.

Several hypotheses have been developed to interpret global gradients of biodiversity. To better understand how mean forest stand age at the watershed scale affects freshwater biodiversity, we explore these relationships through four hypotheses (Fig. 1a). The *early-seral* hypothesis posits that watersheds with young forest stands with stand-initiation structures, on average, are positively related to the highest biodiversity as they support many pioneer species (e.g., Swanson et al., 2011). Conversely, the *late-seral* hypothesis suggests that older forest stands with gap-dynamic structures (Spies and Franklin, 1996) support higher biodiversity (e.g., bats (Avila-Cabadilla et al., 2009); lichen (Kuusinen and Siitonen, 1998); fungi (Redecker et al., 2001; vascular plants (Halpern and Spies, 1995)). Older forests consist of stands that support diversified microclimates, ecological niches, and complex habitat structures that can maintain rare (Zeni et al., 2019; Lo et al., 2020), specialized, intolerant, and endemic species (Jankowski et al., 2021). The *mid-seral* hypothesis proposes that mid-aged forest stands, on average, maximize species richness, as the conditions created may support both pioneering and specialized species from both young and old forests. In this context, watersheds with patches of forests of different ages will have stands with a range of forest-seral conditions that support freshwater biodiversity. The *watershed mosaic* hypothesis suggests that biodiversity responds to factors unrelated to forest age, as observed with detritivore richness in Swedish forests (Frainer and McKie, 2021). In streams, freshwater biodiversity has been linked to current and historical streamflow paths (Dias et al., 2014), latitude (Rypel and David, 2017), stream disturbances (Lepori and Hjerdt, 2006), and the surrounding land use (Allan, 2004; Dudgeon et al., 2006; Feld et al., 2016), with declines driven by human activities, including logging, mining, urban settlements, and land conversion (Cantera et al., 2022).

Here, we evaluate how freshwater biodiversity in temperate forested watersheds, measured as species richness and evenness, varies as a function of mean watershed forest stand age across a gradient of young to old stands (37 to 124 y) (Fig. 1b). Contributions of both richness and evenness contribute to a more comprehensive understanding of freshwater biodiversity, although many prior analyses have focused strictly on richness for its simplicity (Soininen et al., 2012). Evenness is likely independent of richness in freshwater ecosystems (Soininen et al., 2012) and is often considered important for maintaining ecosystem function (Hillebrand et al., 2008; Wittebolle et al., 2009). Using both traditional survey methods and environmental DNA (eDNA) metabarcoding, we aim to understand watershed-scale factors driving freshwater biodiversity patterns in 24 temperate watersheds by quantifying taxonomic and functional richness and evenness. Understanding spatial variation and patterns of freshwater biodiversity across a gradient of mean watershed stand age highlights watershed-scale forest conditions that support greater richness or evenness and may reveal additional complexities of

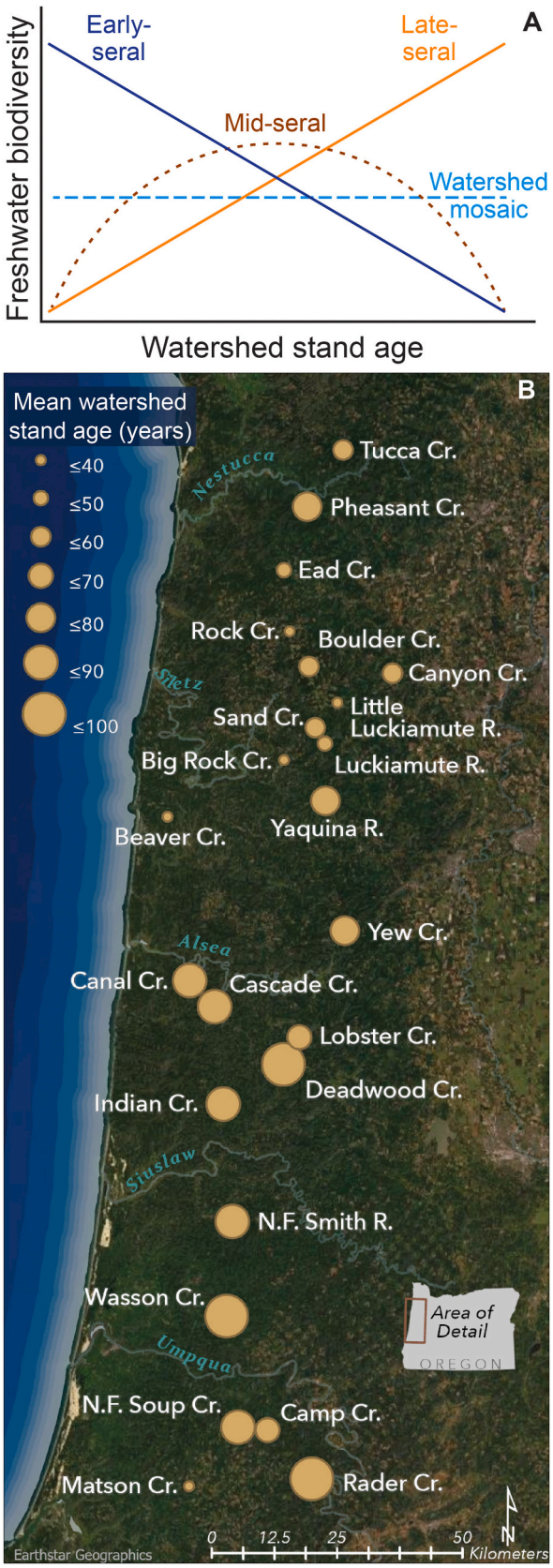

Fig. 1. Study area map and hypothesis diagram. (A) Hypothesized patterns of freshwater biodiversity in (B) 24 temperate watersheds across a gradient of watershed stand age in coastal Oregon, United States. The size of study site dots corresponds to the watershed stand age, with bigger dots representing older forest stands and smaller dots for younger forest stands.

forested watersheds (e.g., Frainer and McKie, 2021).

2. Results

2.1. Freshwater biodiversity (richness and evenness) across watershed stand age

Both taxonomic and functional richness of invertebrates and vertebrates increased with watershed stand age, on average, but patterns differed slightly among metrics and taxa (Fig. 2; Table S1). Average taxonomic and functional richness of freshwater invertebrates were 1.2-fold (20 %; 11 taxa) and 1.5-fold (49 %; 4 functions) higher respectively, in older forests than in younger forests, consistent with the late-seral hypothesis. Vertebrate richness showed even larger proportional differences, with taxonomic richness 1.6-fold (61 %; 7 taxa) higher and functional richness 2.4-fold (144 %; 27 functions) higher in older forests. Although these patterns are consistent with the late-seral hypothesis, statistical support was stronger for invertebrates than for vertebrates. Watersheds with older mean stand ages supported cold-water vertebrates, especially salmonids, *Cottus* spp. (sculpins), and *Rana* spp. (frogs). Invertebrates that were more prevalent in older forests include sensitive taxa, such as *Rhithrogena* (mayflies), *Zapada* (stoneflies), *Zaitzevia* (riffle beetles), *Micrasema* and Lepidostoma (caddisflies), and *Calineuria* (stoneflies). This contrasts with watersheds having younger mean watershed stand ages, which exhibited a greater presence of pioneering invertebrates, such as larval Ephemeroptera or Diptera that comprised about 10 % of all species detected in young forests.

Patterns of taxonomic and functional evenness generally did not vary with watershed stand age, except for invertebrate taxonomic evenness as measured by traditional surveys (Fig. 3; Table S2). Taxonomic evenness for invertebrates as measured by eDNA, taxonomic evenness for vertebrates using both methods, and functional evenness for invertebrates and vertebrates using both methods did not show associations with mean watershed stand age. However, there was a marginal decline in taxonomic evenness for invertebrates by traditional surveys ($F_{1-22} = 3.4$, $R^2 = 0.13$, $p = 0.079$), which decreased slightly with increasing watershed stand age. Although no vertebrate evenness metrics showed meaningful trends ($p \geq 0.58$), invertebrate taxonomic evenness as measured by traditional sampling declined by 1.1-fold (from 0.80 to 0.70 species) with increasing mean watershed stand age, supporting the early-seral hypothesis. Interestingly, for invertebrates, functional richness increased by a similar magnitude (+1.4-fold) compared to the decline in functional evenness (−1.1-fold), demonstrating an almost inverse relationship for these two common biodiversity metrics. We found no support for the mid-seral hypothesis, as incorporating quadratic terms did not improve model fits for any of the biodiversity relationships examined.

As expected, in addition to mean watershed stand age, taxonomic richness of both vertebrates and invertebrates combined was positively associated with stream temperature (mean 7-day maximum) and Beck's biotic index where an increase in intolerant species (less-tolerant species) is associated with greater species richness (Fig. 4; Table S3). Taxa richness was also negatively associated with road density, canopy cover, and elevation. Taxonomic evenness for both vertebrates and invertebrates as measured by traditional surveys had a negative relationship with stream temperature (mean 7-day maximum), whereas, as measured by eDNA, vertebrates had a marginally positive relationship and invertebrates had no relationship (Fig. 5; Table S4). Taxonomic evenness had a positive correlation with distance to historic splash dam locations for vertebrates as measured by eDNA, but no relationship was found for vertebrates by traditional surveys or for invertebrates by either method.

2.2. eDNA and traditional sampling

We evaluated eDNA from 192 stream-water samples across 24

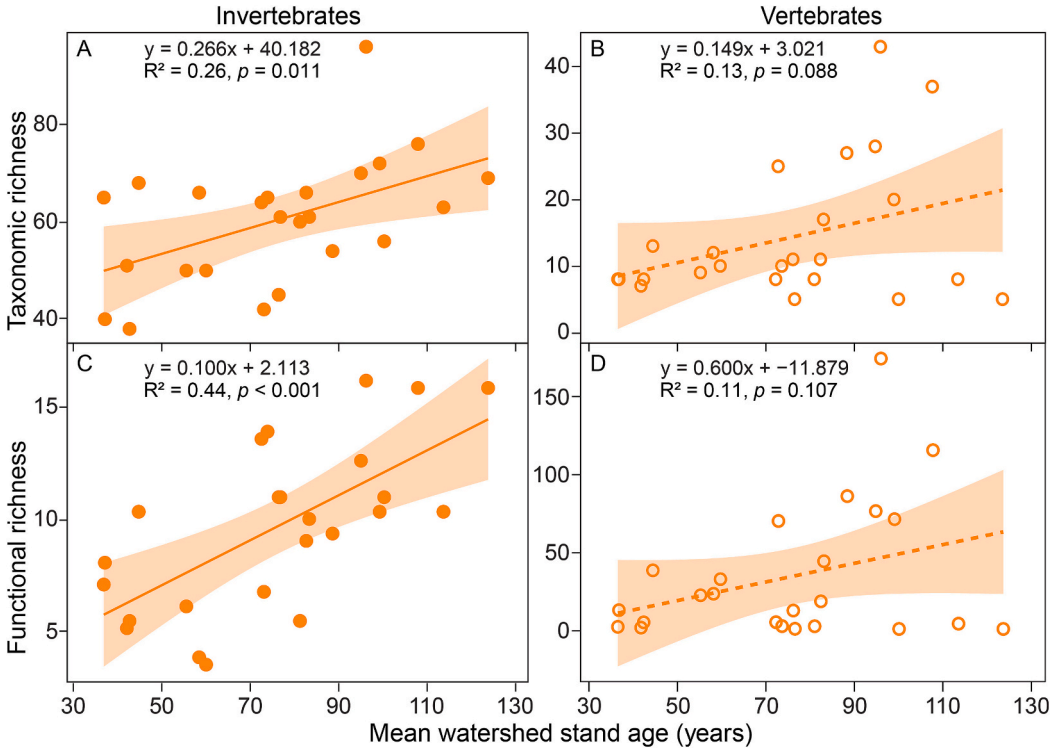

Fig. 2. Watershed stand age and freshwater richness. (A) Freshwater invertebrate (solid line and circles) and (B) vertebrate (dashed line, open circles) taxonomic richness for species and unique genera and (C &D) their function(s) as a proportion of multidimensional trait space across 24 watersheds in coastal Oregon, United States. Richness numbers are sums of species and unique genera combined across traditional sampling (electrofishing or Hess sampling) and eDNA. Color corresponds to the supported hypothesis of late-seral in Fig. 1 for significant relationships.

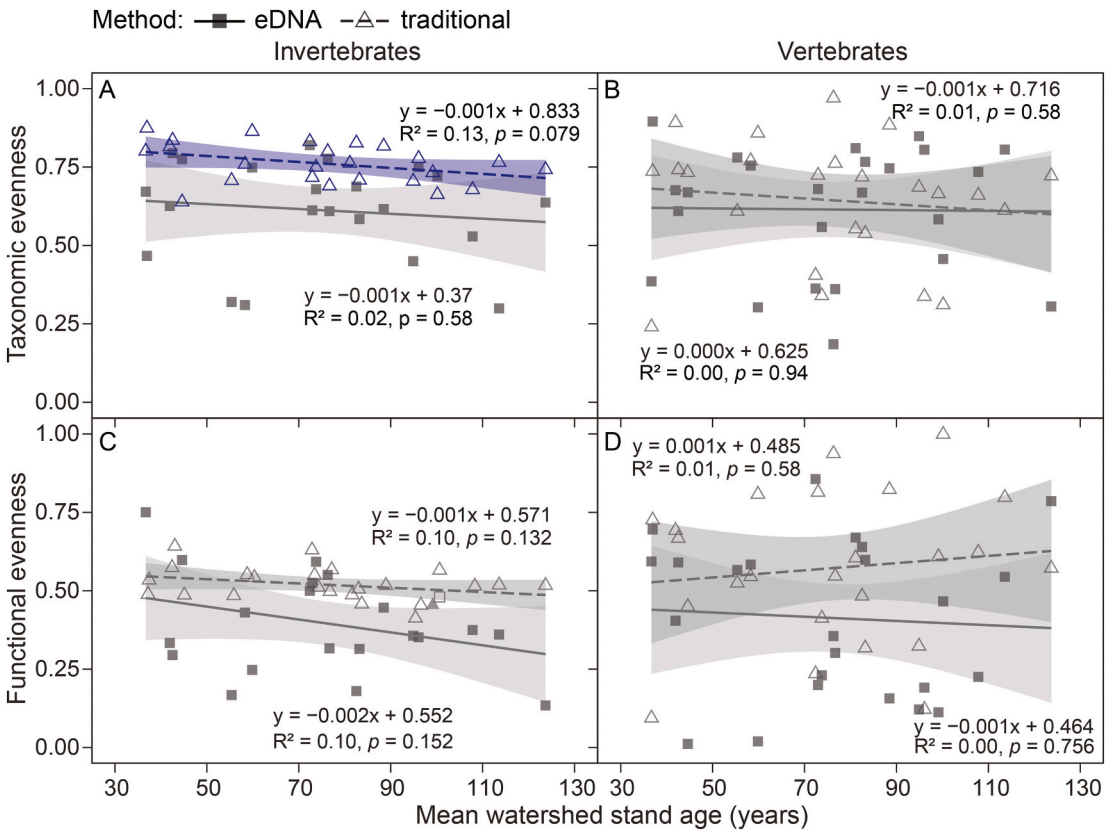

Fig. 3. Watershed stand age and freshwater evenness by method. (A) Freshwater invertebrate and (B) vertebrate taxonomic evenness (relative abundance of a species or unique genera) and (C & D) their function(s) as a proportion of relative abundance across multidimensional trait space across 24 watersheds in coastal Oregon, United States. Evenness was derived separately for eDNA (solid, squares) and traditional (dashed, triangles) survey methods. Color corresponds to the supported hypothesis of early-seral in Fig. 1 for significant relationships.

watersheds (Supplementary Table S5). These samples yielded 57.58 million quality-filtered DNA sequences from 48 different primer pairs that targeted fishes, amphibians, invertebrates, mammals, and pathogens. For fishes, amphibians, and invertebrates, we detected 73 families and 237 unique taxa, including 51 freshwater vertebrates and 186 freshwater invertebrates identified to species or unique genus level (Table S5). All eDNA taxa were confirmed to be native to the Pacific Northwest, except for the *Cottus* spp. (sculpins), which also genetically corresponded to related species elsewhere, likely representing unique lineages that have yet to be assigned bioinformatically for this region. Individual mitochondrial eDNA markers do not fully resolve *Cottus* species identities (Young et al., 2022), so eDNA matches are indicative of species complexes. In some complexes, the best matching eDNA sequence is for a highly similar species from elsewhere in the world. For example, 11.4 % of DNA sequences classified as *Cottus* show highest sequence similarities with sculpin taxa native to Lake Baikal region in Russia.

We compared all species detected using eDNA to species detected using traditional approaches of backpack electrofishing (targeting fish, amphibians, and crayfish) and Hess sampling (targeting benthic macroinvertebrates, which are invertebrates that can be seen with naked eye). Using electrofishing, we identified 12 vertebrate species (with sculpins grouped as a single *Cottus* spp.) versus 49 lineages identified by eDNA. Native coastal cutthroat trout (*Oncorhynchus clarkii clarkii*) was the most common taxon detected across watersheds by both eDNA and traditional sampling. With Hess sampling, we identified 164 macroinvertebrate taxa compared to 43 invertebrate taxa detected by eDNA (Table S5). We did not use traditional sampling to evaluate freshwater mussels, but all mussel species identified by eDNA are native to our study area.

Rare mussels were detected exclusively by eDNA. We detected western pearlshell (*Margaritifera falcata*; IUCN: near threatened, NatureServe G3 vulnerable) in Lobster (mean watershed stand age, 63.7), Yew (72.9 y), and Roder Creeks (37.7 y), and the Yaquina River (76.6 y). We detected western ridged mussel (*Gonidea angulata*; IUCN: vulnerable, NatureServe G3 vulnerable) in Deadwood Creek (98.5 y) and the Yaquina River. We detected Oregon floater (*Anodonta oregonensis*; IUCN: least concern, NatureServe G5 secure) in the Yaquina River. Lastly, we detected the nonnative rusty crayfish (*Faxonius rusticus)* in Deadwood Creek and the Yaquina River.

3. Discussion

Freshwater richness increased in watersheds with older forests, whereas evenness was unchanged or decreased with increasing mean watershed stand age, suggesting that different metrics of freshwater biodiversity support different forest-age hypotheses. For example, averaged over watersheds, watersheds draining older forests yielded greater taxonomic and functional richness for invertebrates than younger forests, which is a pattern also marginally observed for vertebrates, supporting the late-seral hypothesis. Evenness generally did not vary with watershed stand age, except for invertebrate taxonomic evenness as measured by traditional sampling, which slightly declined in watersheds draining younger forests, supporting the early-seral hypothesis. The mid-seral hypothesis was not supported, suggesting that middle-aged forests do not support the highest levels of freshwater biodiversity, though older forests pass through this stage in the course of development. The watershed mosaic hypothesis was also not supported, evidence that watershed stand age is important to freshwater biodiversity. By examining the complexities of different biodiversity metrics, we

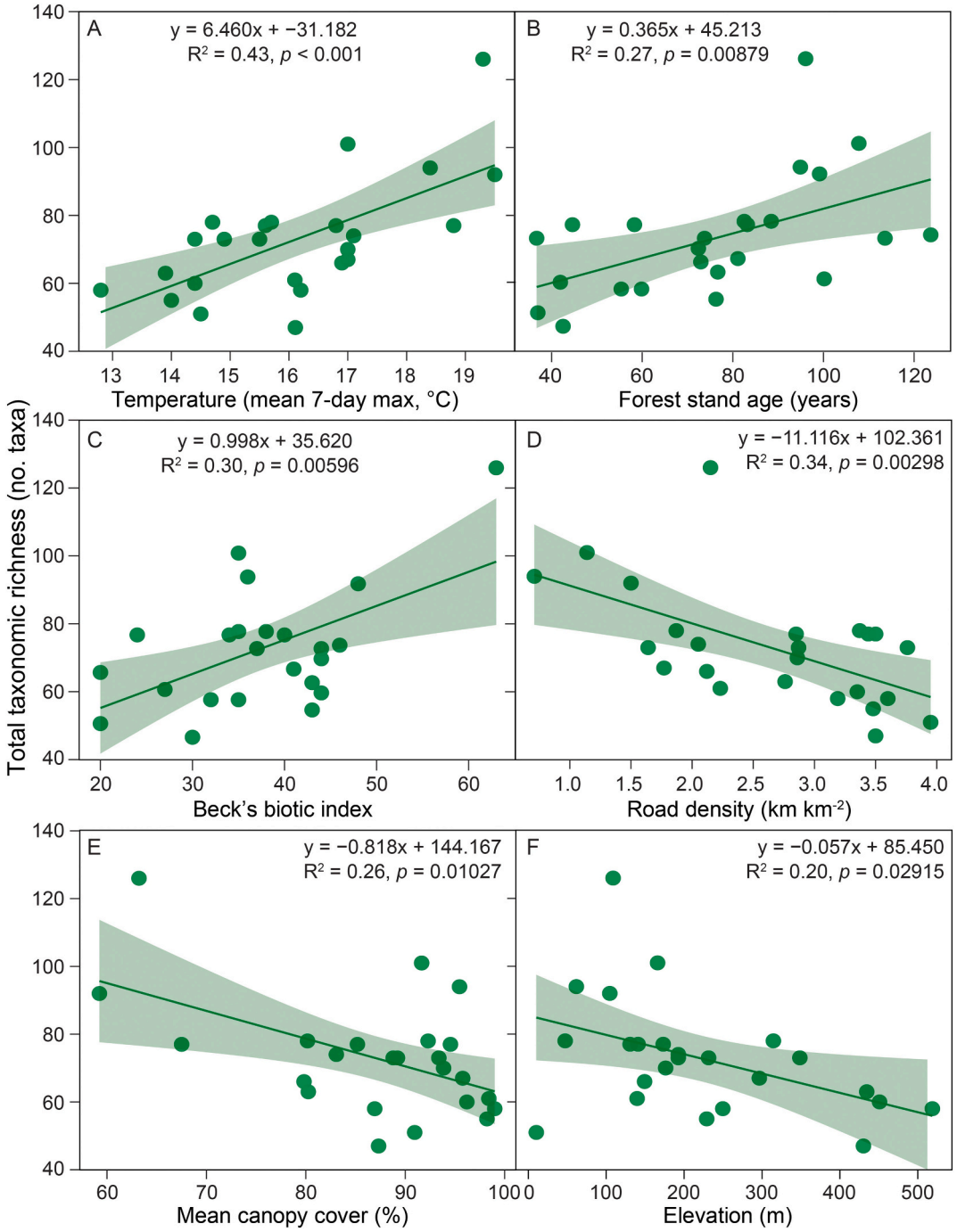

Fig. 4. Factors influencing taxonomic richness of freshwater taxa. Relationship of taxonomic richness of invertebrates and vertebrates to (A) stream temperature (7-day max, °C); (B) mean watershed stand age of forest (yr); (C) Beck's biotic index; (D) road density (km km^{-2}); (E) canopy cover (mean %); and (F) elevation (m). Richness numbers are sums of species and unique genera combined across traditional sampling (electrofishing or Hess sampling) and eDNA for invertebrates and vertebrates; only significant relationships will be in color.

provide empirical support for long-held ideas about the tight relationship between forests and freshwater biodiversity (e.g., Reeves et al., 1995; Reeves et al., 2004; Reeves and Bisson, 2009) and the importance of considering forests in watershed-scale conservation (Williams et al., 1997).

We found strong support for the late-seral hypothesis for both taxonomic and functional richness for invertebrates and marginal support for vertebrates independently, likely because heterogeneous structure of older forests supports more rare, specialized, or intolerant species with diverse community roles. Our findings reveal that watershed stand age is an important driver of both the number of species streams can support and also the breadth of ecological strategies represented, evidence that freshwater habitat specialization is promoted by watershed-scale forest age. For example, abundant salmonid populations have historically been associated with intact streams (no land use conversion) coupled with forest structural complexity (Sedell and Luchessa, 1982). Invertebrates have been linked to late-seral and intact ecosystems globally (Wallace and Webster, 1996; Feio et al., 2023). The larger array of invertebrate taxa in older forests in our watersheds are typically associated with cold, stable, and well-oxygenated streams, in

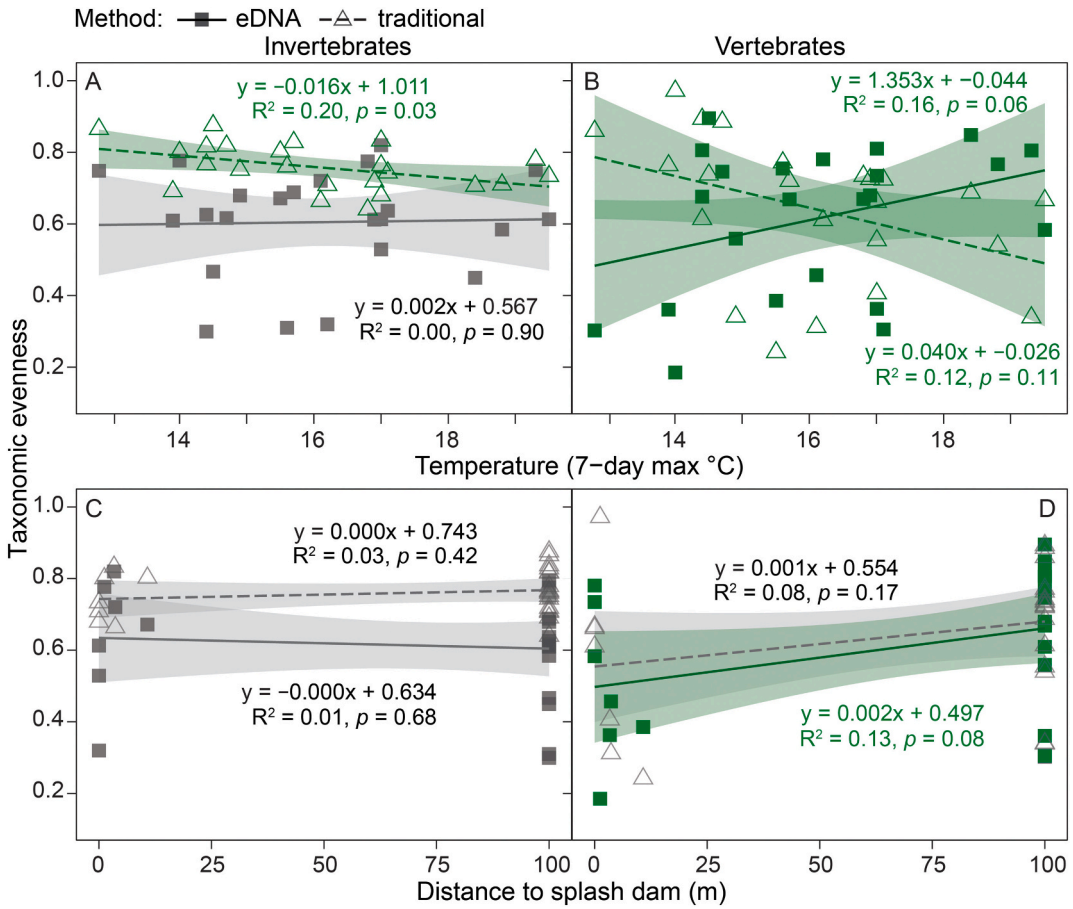

Fig. 5. Factors influencing taxonomic evenness of freshwater taxa by method. Relationship of taxonomic evenness of vertebrates and invertebrates to (A & B) stream temperature (mean 7-day max, °C) and (C & D) distance to historic splash dam locations (m). Evenness was derived separately for eDNA (solid, squares) and traditional (dashed, triangles) surveys; only significant relationships will be in color.

contrast with Diptera and Ephemeroptera found in younger forests, suggesting a shift toward more specialized and disturbance-intolerant invertebrate communities as forests age and mature. For instance, western ridged mussels and Oregon floaters were found in watersheds with forests averaging over 76 years old, suggesting that those watersheds offer stable conditions for long-lived species, likely owing to their age, but also owing to the geospatial position in the stream network of those watersheds being lower in elevation (Bonacina et al., 2023). Generally, when functional richness is high, there is a high degree of ecological niche differentiation and less resource competition (Mason et al., 2005), likely owing to greater habitat heterogeneity that supports the many community roles of unique species. More generally, it is thought that functional richness benefits ecosystem functions and is linked to an ecosystem's capacity to respond to environmental change (Hooper et al., 2005).

As expected, freshwater richness of vertebrates and invertebrates combined was related to various characteristics of the watershed, including stand age. Aquatic ecosystems include multiple, more complex drivers of biodiversity than terrestrial ecosystems (Moi et al., 2020), highlighting the importance of identifying key relationships. For example, total freshwater richness was negatively correlated with road density, elevation, and canopy cover. Freshwater richness declines with elevation, which also aligns with plant and terrestrial vertebrate and invertebrate communities (Peters et al., 2016). Increased road densities and increased canopy cover in younger forests following forest management suggest that legacies of anthropogenic stressors can reduce freshwater richness. In contrast, total freshwater richness was positively related to stream temperature and watershed stand age, with greater richness where forests were older and cold water temperatures were elevated and likely closer to optimal for taxa. In support, at a continental scale, fish richness in rivers was related to temperature across North America likely owing to the latitudinal gradient and less so, but also related in Europe (Griffiths et al., 2014). Macroinvertebrate richness has been shown to increase with temperature (Bonacina et al., 2023). Beck's biotic index showed more tolerant invertebrates being found when richness was lower, with more tolerant and intolerant invertebrates when richness was greater. However, species richness of invertebrate detritivores did not vary with forest age in Sweden (Frainer and McKie, 2021), potentially because those systems naturally have lower taxonomic and functional complexity as they lie at higher latitude than our study streams.

Taxonomic and functional evenness responses had little response to watershed stand age, except for invertebrate taxonomic evenness, which weakly supported the early-seral hypothesis. Taxonomic evenness for invertebrates using traditional sampling was lower in older forests than in younger forests, as expected because species composition in older forests have increased specialization and more uneven abundances across taxa. Communities with greater evenness may be more resistant when faced with change, whereas when they are more uneven, their resistance may be less (Wittebolle et al., 2009).The lack of a relationship for the other evenness responses is likely owing to aspects of the watershed other than watershed stand age, such as disturbance history (Lepori and Hjerdt, 2006; Cantera et al., 2022), watershed characteristics (Rypel and David, 2017), and/or landscape heterogeneity (Dudgeon et al., 2006; Feld et al., 2016).

Taxonomic evenness for vertebrates as measured by eDNA increased

with increasing distance from historic splash dam locations, suggesting that legacies of anthropogenic stressors may contribute to the imbalance of stream community structure (Miller, 2010). Conversely, taxonomic evenness as measured by traditional surveys declined with rising stream temperature for invertebrates and vertebrates, highlighting warmer temperatures as a potential stressor that negatively affected community evenness in forested watersheds, though warmer cold water conditions benefit taxonomic and functional richness. There were mixed temperature responses across watershed stand ages for taxonomic evenness as measured by eDNA, showing no relationship for invertebrates and marginal significance for vertebrates of warmer temperature with increasing watershed stand age. Notably, we documented an inverse relationship between richness and evenness for invertebrate taxonomic diversity, proposing that a larger variety of functional niches may be available to invertebrates in watersheds with younger forests.

There is little information in the literature on the contributions of early-seral forests to freshwater biodiversity (e.g., Brooks et al., 2012), though a wide range of terrestrial species are associated with early-successional forest conditions (Swanson et al., 2011; Betts et al., 2013). Despite a lack of information on freshwater biodiversity, fish density and biomass have been found to be greater immediately after forest harvest, including for brook trout (*Salvelinus fontinalis*) in New England, USA (Nislow and Lowe, 2003, 2006) and for coastal cutthroat trout in the US Pacific Northwest (Bateman et al., 2018, 2021) owing to either an increase in basal resources (e.g., Kiffney et al., 2014; Warren et al., 2016) or an increase in available habitat that supports young-of-year rearing along stream fringes resulting from the increase in flow following harvest (Penaluna et al., 2015).

It is possible that different relationships for freshwater biodiversity may be observed across a greater range of mean watershed stand-age conditions. The gradient of mean watershed stand ages in our study was representative of our study region at the watershed scale (Steel et al., 2017). However, this gradient does not include endpoints that when averaged across the watershed incorporate stand initiation (0 y) or old-growth (>200 y) conditions. For example, streams in our study are well shaded (canopy cover >60 %) and do not exhibit open stand-initiation conditions of early-seral forests that are rarely available on the landscape to be evaluated for freshwater ecosystems (but see LeRoy et al., 2023). In addition, understanding the relationship of watershed stand age to freshwater biodiversity where stand age is affected only by natural processes is lacking.

The relative role of riparian versus watershed stand age could be further evaluated. Many forested watersheds retain riparian buffers of unharvested trees on both sides of the stream, especially in Canada and the United States (Lee et al., 2004), across landownerships. Riparian areas interface between freshwaters and forests (Gregory et al., 1991), mediating effects from adjacent and upland disturbances to streams. As ecotones, riparian areas surrounding streams incorporate landforms and environments connecting land to streams, often across a gradient of environmental factors. The general intention of riparian buffers is to both insulate streams from stressors, such as forest-management activities, and to support various ecosystem services to the stream, including serving as sources of sediment, nutrients, and metal retention, offering erosion prevention, moderating light for stream temperature, and contributing large wood and organic matter (Lee et al., 2004; Richardson et al., 2012; Swartz et al., 2024). However, some riparian buffers may not be adequately fulfilling these roles, for instance, if their area is too small or if there is a certain structural stage or age of maturity necessary for them to provide certain services such as large wood recruitment (Steel et al., 2017; Martens et al., 2019). Over time, if a riparian buffer in a younger forested watershed reaches its intended average condition of mature and late-seral condition, the question remains as to whether these buffers could contribute to conserving freshwater richness by providing "life-boating" and supersede the watershed-scale response seen here.

Although multigene eDNA metabarcoding, electrofishing, and Surber sampling together reveal freshwater biodiversity, further research could identify sources of error for each method. For example, in the metabarcoding process it can be difficult to distinguish erroneous detections from real detections (e.g., Rees et al., 2014; Deiner et al., 2017). Surber sampling targets macroinvertebrates but can miss invertebrates that are not detectable with the naked eye or at lower taxonomic resolutions. However, eDNA metabarcoding detects fewer invertebrates than morphological methods such as Surber sampling, as shown in a recent review of the worldwide literature (Poyntz-Wright et al., 2024), and accordingly further work could improve freshwater invertebrate detections with eDNA metabarcoding to capture an entire stream community. Electrofishing is optimal under ideal conditions for trout, but it is less understood how well the method characterizes fish communities across and within taxa for stream assemblages of multiple taxa. Despite these challenges, we demonstrate the utility of combining eDNA with traditional methods in answering applied ecological questions at the watershed scale.

Overall, we found that watershed stand age from young to old (37 to 124 y old) influences freshwater biodiversity, especially richness, and that forests and watersheds are tightly linked. By maintaining a range of forest ages and optimizing riparian buffer widths, freshwater richness and evenness could perhaps be optimized and predicted. Conservation strategies could prioritize conserving watersheds with older forest stands while fostering structural complexity in younger forest stands. Ultimately, integrating freshwater biodiversity into management plans alongside currently considered water-quality metrics could play a key role in reversing the decline of freshwater biodiversity and reveal new priority areas and strategies for enhanced conservation efforts.

4. Methods

4.1. Study area and design

We assessed freshwater biodiversity in 24 forested watersheds in a temperate zone with seasonally dry, productive forests of the Pacific Northwest of the United States (Fig. 1a) focusing on taxonomic and functional richness and evenness. We used a stratified random sampling design to select 24 sites from 3rd- to 5th-order streams in watersheds, stratified by mean watershed stand age across a gradient of young to old forests. Temperate forests with wide ranges of biogeographical and climate variation, and with sufficient complexity in forest structure and variety of tree species, are ideal for exploring relationships related to forested ecosystems (Pan et al., 2018). Although tree-age data are not readily available everywhere, individual trees can grow up to 800 years old in temperate watersheds of the Pacific Northwest (Pan et al., 2011). Most old forests historically would have undergone stand-replacing disturbances, such as those caused by fire, insect outbreaks, or wind events, but since the late 19th century, forest harvest and associated management are mostly responsible for initiating succession of current forest stands (Sedell and Luchessa, 1982; Birdsey et al., 2006). Variable management objectives and silvicultural regimes across landownership types have led to a range of distributions of stand ages at broader spatial scales. In coastal Oregon, federally owned forests currently comprise approximately 30 % of forests >120 years old and <5 % of these older forests are currently found on private lands (Steel et al., 2017). Mean area-weighted watershed stand age was calculated using a 2016 forest structure raster derived from a 30-m Landsat imagery Forest Inventory and Analysis data (USFS) (publicly available at: https://lemma.forestry.oregonstate.edu/data/structure-maps).

To identify sites, we initially stratified watersheds at the hydrologic unit code 12 (HUC-12) scale. For all HUC-12 options within Oregon's Coastal Ecoregion: mean stand age ranged from 3 to 143 y (mean 53 y, minimum = 0, maximum = 849). Our range of final watersheds selected reflects similar mean watershed stand ages 37 y to 124 y (Table S6). We limited the pool of candidate HUC-12 options to the central section of the Coastal Ecoregion in Oregon to facilitate travel among sites. We

excluded any watersheds with small private landowners or towns to ensure selected watersheds were predominantly forestland. We initially categorized HUC-12 s by forest ownership, creating two separate pools: 1) HUC-12 basins with >60 % federal ownership [US Forest Service (USFS), Bureau of Land Management (BLM)]; and 2) all other HUC-12 basins. Within each group, we randomly selected 15 watersheds and evaluated watershed size for consistency across groups, while ensuring mean watershed stand age differed across groups. Within each HUC-12, we identified potential sampling locations within 4th-order streams and, in some cases, adjusted our reach to 3rd- or 5th-order segments owing to safe access or permit limitations. Typically, only one 4th-order stream occurred within each HUC-12 watershed, but when more than one occurred, we randomly selected a single stream. From this initial pool of 30 streams, we randomly selected 24 streams representative of each stratification level to include in this study.

We sampled during seasonal low flow in July and August of 2020 across a range of landownerships, including USDA Forest Service, Bureau of Land Management, Oregon Department of Forestry, Hampton Resources, Inc., Manulife Investment Management, Roseburg Forest Products, Stimson Lumber Company, and Weyerhaeuser Company.

4.2. Traditional field methods

4.2.1. Macroinvertebrate sampling

We collected macroinvertebrate samples using a Hess sampler following standard methods (243 μm mesh net; 33 cm diam x 40 cm deep) in three locations within each stream. Approaching each sample location from downstream, we placed a Hess sampler on the streambed and pushed it in to create a seal with the funnel collection bag downstream. We disturbed the stream substrate to a depth of 10 cm for two to three minutes of effort. We composited samples into a single sample per stream in high-density polyethylene (HDPE) bottles preserved with 95 % ethanol. All macroinvertebrate samples were sorted, enumerated, and identified to the lowest practical taxon level (typically genus) by Benthic Aquatic Services (Lawrence, KS, USA). We also calculated invertebrate density (individuals m^{-2}).

4.2.2. Vertebrate sampling

We quantified fish and stream-associated amphibians using triple-pass depletion backpack electrofishing (model LR-20B Smith-Root Vancouver, WA, United States) after we collected eDNA samples (see eDNA methods below). We electrofished three 20-m sections of a contiguous 60-m stream reach, which were temporarily blocked to prevent movement of individuals in and out of reaches during sampling. We anesthetized individuals using buffered tricaine methanesulfonate and identified individuals to species when possible. We placed animals in an aerated bucket for recovery prior to being released at the same location where they were collected.

4.2.3. Environmental and geospatial habitat variables

We extracted elevation from 30-m National Elevation Dataset included in the National Hydrography Dataset Plus Version 2, which was also used to identify stream order in our initial site-selection process. To calculate road density, we clipped a statewide road layer to watershed boundaries, then divided the sum length of roads within a watershed by the watershed area. We acquired road data from the Oregon Transportation dataset (available from: https://geohub.oregon.gov/datasets/oregon-geo::all-public-roads/about (accessed 16 October 2018).

We measured canopy cover at each of six transects spaced at 20-m intervals for each 100-m reach for each stream. While standing in the stream's thalweg, we used a concave densiometer to measure canopy cover in all four directions (upstream, downstream, right bank, left bank). We determined mean canopy cover across each transect within a watershed. At the beginning of the summer, we deployed temperature data loggers (miniDOT, PME, San Diego USA, 0.1 °C accuracy) at the downstream end of each stream reach. We housed each logger in a PVC pipe and secured it to the bottom of the stream. The logging interval to 10-min intervals. To calculate the 7-day moving average maximum temperature, we used only the period of simultaneous logging across all sites (6–20 August 2020), which was limited owing to remoteness of sites.

4.3. eDNA field methods and lab work

We collected 3-L water samples (2-L thalweg +1-L adjacent slack water) at each stream including 4 replicates. Using a vacuum pump, we pumped sample water through 0.45-μm single-use cellulose nitrate filters (Sterlitech, Kent, WA, USA). To prevent cross-site contamination, we entered sites downstream from where samples were taken, and we decontaminated equipment (bottles, tweezers, waders) with a 50 % bleach solution followed by a triple rinse of deionized water. Filters were loosely rolled, stored cold in 5-mL vials on wet ice during collection and transport, and then frozen at −20 °C within 6 h of collection until DNA extraction. Using DNeasy PowerWater® kit (Qiagen, Hilden, Germany), we extracted DNA from each filter per manufacturer's instructions. Post-extraction samples were cleaned and concentrated using the Zymoclean™ Large Fragment DNA Recovery Kit (Zymo Research, Irvine, CA, USA). DNA was quantified using fluorometry (Qubit™ High Sensitivity kit; ThermoFisher, Waltham, United States),

4.4. eDNA amplification and sequence analysis

Multigene eDNA metabarcoding enables taxon detections using multiple genetic targets, thereby improving detection probability for rare species and taxonomically diverse communities. Our target strategy includes gene regions that discriminate both higher taxonomic levels of orders and families (e.g., mitochondrial 12S and 16S Ribosomal DNA) and lower taxonomic levels of genera and species (e.g., cytochrome oxidase I [COI] and cytochrome B [cytB]. Our strategy for primer design and amplification methods are detailed briefly below and in detail in Hauck et al. (2019). The full list of primers and gene targets used in this study are in Table S7.

For the amplification of eDNA targets, we used a Fluidigm 48.48 Access Array with one-step Fluidigm cycling parameters and a modified annealing temperature of 58 °C at the Roy J. Carver Biotechnology Center at University of Illinois Urbana-Champaign. The Fluidigm Access Array performs microfluidic PCR reactions in nanoliter-scale volumes in an array of 48 samples × 48 target primer pairs (2304 reactions). Traditional biases in a multiplex PCR, such as uneven amplification due to primer competition, do not occur in the Access Array because each primer × sample combination is amplified separately. We indexed the samples using an 8 bp barcode, and reactions were pooled in equal volumes prior to sequencing. For reactions, we used >15 ng of sample eDNA, and included bovine serum albumin (BSA) at 0.2 μg·μL − 1 final volume to mitigate PCR inhibition. Each array included positive and negative controls for quality control. Our positive control contained a mixture of genomic DNA derived from targeted species plus λ bacteriophage DNA (New England Biolabs, Ipswich, MA, USA), that we mixed at equal mass and diluted to a final concentration of 10 ng/μl. Our negative controls consisted of λ DNA and we submitted at 10 ng/μl. Positive controls ensured that the primers were amplifying as expected, and negative controls allowed us to detect DNA contamination on the array.

Amplified eDNA targets were sequenced on an Illumina NovaSeq 6000 with 2 × 250 bp paired-end reads, sequences were demultiplexed by index and by primer, and ϕX174 DNA control sequences were removed at the Roy J. Carver Biotechnology Center. Demultiplexing steps allowed up to one mismatch per index and two mismatches per primer. Raw sequence reads are deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession SUB15108232. Sorted sequence reads were as described in Hauck et al. (2023), with the exception that reads 1 and 2 were joined

into contigs prior to trimming the primer portions of sequence read with Trimmomatic v. 0.33. To avoid the inclusion of primer dimers, we set a minimum length filter at 150 bp and adjusted where necessary.

We classified sequence reads to taxon using the programs KMA v. 1.2.23 (Clausen et al., 2018) and CC-Metagen v. 1.2.2 (Marcelino et al., 2020). The classification database used was the Marcelino indexed database (11 June 2019) that includes all sequences from the NCBI nucleotide collection, except environmental or artificial sequences. Our data include multiple gene targets of varying conservation, length, and taxonomic origin, so we modified CC-Metagen thresholds to optimize species richness. Using positive controls to guide parameters, we selected the following taxon threshold settings, which are percentages, for this specific data set: class = 80.91, order = 81.21, family = 88.4, genus = 96.25 and species = 98.0. These parameters provided the most accurate identities possible in the positive control sequences across all genes and species targeted. For each site, we summed the number of reads for each sample × primer combination. We deduplicated data and summed where haplotypes resulted in multiple reports of the same species at a single site by primer combination. Finally, we removed samples with detections of a subset of species found in the negative controls from the study. Index hopping (barcode swaps) are known to occur in patterned flow cells (Costello et al., 2018) and we see them as a long tail of low frequency randomly distributed detections in our metabarcoding data (Flitcroft et al., 2025). To mitigate barcode swaps, we removed the lowest 1 % of reads tallied by primer and sample, which for this dataset is equivalent to <396 reads for each sample x barcode combination.

4.5. Statistical analysis

We integrated eDNA and traditional survey data in our biodiversity assessments as suggested by others (Deiner et al., 2017; McElroy et al., 2020). Diversity metrics were derived separately for invertebrates and vertebrates and for eDNA and traditional methods before analyses. For each watershed, we calculated taxonomic richness at the finest resolution possible (species or unique genus) by summing taxa lists for each method together for each watershed. We avoided double counting taxa between methods in each watershed. We calculated taxonomic evenness using Pielou's J index, which ranges from 0 (uneven species abundance distributions) to 1 (complete evenness) for each method to account for different abundances between methods. For eDNA data, we included the summed number of reads per taxa for all primers for targeted taxa and traditional sampling included counts. We likewise used Pielou's J index to assess functional richness (the range of unique functional traits within the community) and functional evenness (the uniformity of trait abundances in functional space) for each watershed. Functional richness was estimated using the convex-hull volume in trait space (FRic) with the dbFD function from the *FD* package. We compiled occurrence matrices from eDNA and traditional surveys that were used for evenness calculations. For evenness, we calculated Shannon indices from abundance matrices and standardized them by log-transformed richness to obtain taxonomic evenness, whereas functional evenness (FEve) was estimated with dbFD using trait and abundance data. Trait matrices for invertebrates and vertebrates were harmonized across eDNA and traditional datasets, retaining only informative traits (variance >0.15–0.20) to avoid collinearity.

To calculate traits for the functional metrics, we assessed a range of ecological and life-history traits for detected fishes, amphibians, and invertebrate freshwater taxa. Traits included habitat preference (e.g., aquatic, terrestrial, benthic), diet (e.g., algae/phytoplankton, detritus, invertebrates, fish/crustaceans, blood), reproductive characteristics (e.g., fecundity, egg adhesion, spawning season, parental care), developmental traits (e.g., metamorphosis, larval dispersal), physiological adaptations (e.g., hypoxia tolerance, cold-water adaptation, cold- vs. warm-blooded), and behavioral or morphological features (e.g., benthic lifestyle, nest building, longevity, maximum total length, and age at maturity). Trait values were compiled from peer-reviewed literature, species trait databases, and published field guides (Supplementary Methods).

We evaluated whether taxonomic and functional diversity across watersheds varied as a function of mean watershed stand age by targeting invertebrates and vertebrates independently. We tested the null hypothesis that diversity does not vary with mean watershed stand age, termed the *watershed mosaic* hypothesis (Fig. 1b). If a statistically significant increase or decrease was detected in the relationship for richness or evenness versus mean watershed stand age, we assigned the *late-seral* or *early-seral* hypotheses, respectively. To determine if/when the *mid-seral* hypothesis was supported, we compared negative quadratic model fits with linear regression model fits and retained a quadratic fit if it improved overall model fit (R-squared value and *p*-value).

We evaluated relationships of taxonomic richness with other important variables that may aid in explaining correlated variables, including stream temperature (7-day max, °C), road density (km km^{-2}), mean canopy cover (mean %), elevation (m), distance to historic splash dam location (m), and Beck's biotic index (Figs. 4 and 5). We fitted linear regression models relating each richness or evenness response to these environmental predictors. Model fits were evaluated with scatterplots and regression lines including regression equations, R^2, and associated *p*-values extracted directly from model summaries. Beck's biotic index was calculated for invertebrates collected with traditional field methods, including species relative abundance and tolerance values (Beck, 1955). Lower values are associated with greater stressors and indicate more tolerant species whereas higher values are associated with lower stressors and more intolerant species. We used the historic splash dam database compiled for the Oregon Coastal Province by Miller (2010). Then, we calculated stream distance to the splash dam from our study site if it was downstream and in the same watershed. We examined taxonomic evenness with the same habitat variables considered for richness (Fig. 5). All analyses were conducted in R v4.3.1 (R Core Team, 2023) using the *FD* package for functional diversity metrics (Laliberté and Legendre, 2010), the *vegan* package for diversity and evenness indices (Oksanen et al., 2022), and *ggplot2* for data visualization (Wickham, 2016).

We systematically processed eDNA data to compile detected taxa and cross-referenced them against biodiversity records in Global Biodiversity Information Facility (GBIF), iNaturalist, Biocollections (iDigBio), and VertNet using the *spocc* R package. This step ensured that all taxa had documented occurrences across the study area to minimize false positives. To evaluate congruence between eDNA and traditional survey results for vertebrates (fishes and amphibians) and invertebrates, we conducted comparative analyses. These analyses leveraged eDNA's sensitivity to detect cryptic or rare species and validated findings against benchmarks from traditional surveys. We used the *FD* and *vegan* R packages to link taxonomic information with trait data curated from multiple sources (Supplementary Methods) enabling the calculation of functional diversity indices. All R scripts and workflows used in the analyses are available in the Supplementary Information.

CRediT authorship contribution statement

Brooke E. Penaluna: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Ashley A. Coble:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Arif Jan:** Writing – review & editing, Visualization, Validation, Investigation, Formal analysis. **Richard Cronn:** Writing – review & editing, Supervision, Methodology, Investigation. **Laura L. Hauck:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation. **Ivan Arismendi:** Writing – review & editing, Visualization, Methodology, Formal analysis. **Jessica Homyack:** Writing –

review & editing, Resources, Investigation, Funding acquisition.

Declaration of competing interest

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Relationships

There are no additional relationships to disclose.

Other activities

There are no additional activities

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2025.111530.

Data availability

Data is in supplemenary material and code is being uploaded to GitHub. Raw eDNA data is in NCBI's Sequence Read Archive SUB15108232

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