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ORIGINAL ARTICLE

Sensitive environmental DNA (eDNA) methods to detect hemlock woolly adelgid and its biological control predators *Leucotaraxis* silver flies and a *Laricobius* beetle

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Abstract

Environmental DNA (eDNA) analysis can be a powerful tool for the early detection of invasive organisms. However, research on terrestrial eDNA detection from foliage surfaces has been limited. In this study, we developed methods to capture and detect eDNA using qPCR from an invasive forest pest, hemlock woolly adelgid (*Adelges tsugae*), and three of its biological control predators *Leucotaraxis piniperda*, *Leucotaraxis argenticollis*, and *Laricobius nigrinus*. We designed four highly efficient qPCR assays with a low limit of detection (1–10 copies/reaction). The assay targeting *A. tsugae* was species-specific. The assays targeting *Le. piniperda*, and *Le. argenticollis* were biotype-specific in addition to being species-specific demonstrating applications of eDNA analysis beyond species-level detection. The *La. nigrinus* assay also detected DNA from closely related and hybridizing *Laricobius rubidus*. The eDNA methods were evaluated against traditional detection methods. We collected foliage samples from three strata (bottom, middle, and top) of eastern hemlock trees to detect the presence of *A. tsugae*. The detection of the biological control predators was evaluated using western hemlock foliage samples collected from the predators' native range in western Washington. The eDNA methods had significantly higher positive detection rates (2.8–4.5 times) than conventional methods of all target species. The strata of sampling were not significant in determining the presence of *A. tsugae* infestation. The eDNA concentration positively correlated with the observed density for all species. This study demonstrates the efficacy of eDNA analysis as a more sensitive tool for early detection of *A. tsugae* and to track the establishment of its biological control predators.

KEYWORDS
Adelges tsugae, biological control, biotype-specific qPCR, eDNA, hemlock woolly adelgid, invasive species, *Laricobius nigrinus*, *Leucopis argenticollis*, *Leucopis piniperda*

1 | INTRODUCTION

Hemlock woolly adelgid, *Adelges tsugae* Annand, is a non-native pest of eastern hemlock *Tsuga canadensis* Carrière and Carolina hemlock *Tsuga caroliniana* Engelmann, two ecologically important foundation species in eastern North America providing a unique habitat for a diversity of wildlife species (Ellison et al., 2005; Orwig et al., 2012). *A. tsugae* is native to East Asia and western North America, whereas in eastern North America, a single invasive genotype clone was introduced from Japan (Havill et al., 2016). In eastern North America, *A. tsugae* was first reported in Virginia in the early 1950s (Stoetzel et al., 2002) and has spread to 21 states in the United States and two Canadian provinces (Fidgen et al., 2014; Limbu et al., 2018). Throughout the introduced range in the east, *A. tsugae* has caused the decline and extensive mortality of its host tree species (Abella, 2018; Eschtruth et al., 2013; Gómez et al., 2015; Orwig et al., 2002). Cold temperatures in the northern introduced range can cause mortality of the overwintering *A. tsugae* sistens generation, leading to a temporary population reduction (McAvoy et al., 2017; Skinner et al., 2003; Trotter III & Shields, 2009). Adaptation of increased cold tolerance and climate change leading to milder winters are expected to accelerate the northward spread of *A. tsugae* (Elkinton et al., 2017; Ellison et al., 2018; Paradis et al., 2008), increasing the need for early detection techniques. Reliable methods of rapid and scalable early detection of *A. tsugae* infestations are necessary for effective mobilization of resources to implement management strategies and increase their rate of success (Liebhold & Kean, 2019).

Of the management strategies proposed for reducing tree mortality, silvicultural thinning (Brantley et al., 2017), chemical treatments (Doccola et al., 2007), biological control (Reardon & Onken, 2011), and host resistance (Kinahan et al., 2020; McKenzie et al., 2014) are the most researched. Classical biological control involves the introduction of specialized predators from the pest's native range to reduce pest populations to innocuous levels. Three potential specialist predators of *A. tsugae* were identified from the Pacific Northwest of the USA, where *A. tsugae* is native (Havill et al., 2016; Havill, Vieira, & Salom, 2014). These include two species of Chamaemyiidae silver flies, *Leucotaraxis argenticollis* Zetterstedt and *Leucotaraxis piniperda* Malloch, and a Derodonidae beetle *Laricobius nigrinus* Fender (Kohler et al., 2008). All three predators are most abundant as juveniles and adults during the *A. tsugae* egg-laying stages (Grubin et al., 2011; Kohler et al., 2016). Other predators sourced from Asia have been evaluated and some have been approved and released (reviewed in Havill et al. 2014), but here we focused on these three because current research indicates they hold the most promise for the management of *A. tsugae*. Evaluating the success of biological control releases requires monitoring for the establishment and dispersal of predators over time to inform management decisions such as augmenting releases and where to target future releases. This is crucial, especially for the silver flies that are the newest agents to be released (Grubin et al., 2011).

Increasing sensitivity to detect small populations, that is, early detection of *A. tsugae* and its predators, is key to improving the impact of management programs. Current methods to identify *A. tsugae* infestation largely rely on visual observation of its characteristic white wool from November through June (Costa & Onken, 2006; Fidgen et al., 2016). The only known mode of reproduction in the introduced range is through parthenogenesis (Havill et al., 2016; McClure, 1989) with new populations able to establish with as few as one individual (Tobin et al., 2013). New disjunct populations, therefore, consist of very few individuals and are difficult to detect. Adelgid predators are also likely to be present in low numbers after local establishment and are difficult to detect visually. *La. nigrinus* is usually sampled by looking for immatures under a microscope on cut adelgid-infested hemlock branches in a laboratory, using simple traps to catch adults in the field (Wiggins et al., 2016), beat-sheet sampling, or sampling larvae dropping from adelgid-infested hemlock branches in the laboratory (Mausel et al., 2010). Techniques to evaluate the establishment of *Leucotaraxis* spp. in the east have yet to be developed, but examining adelgid-infested branches under a dissecting microscope (Grubin et al., 2011) and laboratory observation of adult emergence from adelgid-infested branches have been used in the native western range (Neidermeier et al., 2020). Development of novel environmental DNA (eDNA) approaches may increase the detection sensitivity of both the pest and predators, leading to better management of the pest and resources.

Environmental DNA methods have aided in increasing the detection sensitivity of rare and invasive species in both aquatic and terrestrial systems (Beng & Corlett, 2020; Rees et al., 2014; Ruppert et al., 2019). eDNA is the DNA released by organisms through dead cells, feces, mucus, etc. into the environment. This can be extracellular DNA, or intracellular in individual cells or tissue. This eDNA can be collected and identified using molecular methods such as quantitative polymerase chain reaction (qPCR) for sensitive and species-specific detection. While eDNA-based detection methods have led to increased sensitivity in detecting rare or invasive species in aquatic systems, their development and use in terrestrial systems are limited (Beng & Corlett, 2020; Rees et al., 2014; Ruppert et al., 2019). This is primarily because eDNA released in aquatic environments disperses readily allowing the eDNA to travel away from the release site and still be detected (Andruszkiewicz et al., 2019; Sansom & Sassoubre, 2017). Unlike aquatic environments, eDNA released on terrestrial substrates is not likely to homogeneously disperse in the environment. Early studies investigating eDNA on terrestrial substrates relied on collecting soil samples for analysis (Buxton et al., 2018; Kirtane et al., 2019; Leempoel et al., 2020). However, eDNA adsorbed to soil particles may remain preserved from degradation and not represent the current occupancy of a species (Cai et al., 2006; Demanèche et al., 2001; Kirtane et al., 2020). Recently, species from Diptera and Coleoptera orders were identified using eDNA metabarcoding via non-invasive baited traps (Camila et al., 2021). However, few studies have focused on eDNA analysis directly from plant surfaces. eDNA methods have been employed to identify foraging mammals using DNA left behind on browsed woody vegetation (Nichols et al., 2012). Valentin et al. (2020) used crop washing stations to aggregate eDNA from an invasive crop pest, the brown marmorated stink bug (*Halyomorpha halys*, Stål). This

study also concluded that their eDNA method was more sensitive in determining the presence of the pest, compared to conventional blacklight or pheromone traps. However, it might be more challenging to aggregate eDNA from forest pests than crop pests and thus require the development of additional capture methodologies. Spray aggregation and tree rolling methods to capture eDNA from another invasive forest pest in Northeastern USA, the spotted lanternfly (*Lycorma delicatula*, White), were able to detect their presence ahead of visual surveys (Valentin et al., 2020).

While eDNA analysis is proven to be a useful tool for determining presence/absence, an increasing body of research is geared towards estimating the abundance of the target species (Yates et al., 2019). Most of the studies to date determining the abundance of target organisms using eDNA signals have focused on aquatic organisms (Rees et al., 2014; Ruppert et al., 2019). eDNA concentration is determined by numerous factors influencing eDNA shedding, decay, and transport (e.g., life stage, density, temperature, sunlight exposure, pH, and microbial activity), apart from the number or biomass of the organism (Andruszkiewicz et al., 2017; Barnes et al., 2014; Collins et al., 2018; Eichmiller et al., 2016; Goldberg et al., 2018; Jo et al., 2017; Lance et al., 2017; Seymour et al., 2018). Thus, the first step in developing models to determine abundance using eDNA concentration is to determine an empirical relationship between the two.

In this study, we develop and evaluate novel qPCR assays for the detection of *A. tsugae* (HWA), *Le. argenticollis* (LA), *Le. piniperda* (LP), and *La. nigrinus* (LARI). The acronyms, HWA, LA, LP, and LARI are used to refer to the qPCR assays, while the species themselves are referred to using their Latin name in this manuscript. LA and LP qPCR assays were designed to be specific to the western biotype in addition to being species-specific. Using paired field samples of eastern hemlock foliage from *A. tsugae* northern invasion front, we compare the sensitivity of the eDNA (using HWA assay) approach with the currently established visual detection methods. Using western hemlock foliage infested with the western lineage of *A. tsugae* and potentially with its native predators, we evaluated the sensitivity of detection of the predator species via eDNA and traditional sampling methods. We also determined the relationship between eDNA concentration and the density of the target species.

2 | METHODS

2.1 | Study species

A. tsugae is a member of a small family of sap-sucking insects, which use conifers as host plants and have complex life cycles (Havill & Foottit, 2007). The largest of the *A. tsugae* adults average 1.4 mm long and are covered with white wax extruded from pores to form a wool-like ovisac and are sedentary except for a brief first-instar dispersal stage (for photographs, see Limbu et al., 2018). During the late summer months, the smaller settled first-instar nymphs enter aestivation and lack conspicuous wool, thus making detection extremely difficult (McClure, 1989). In late winter through spring, adult *A. tsugae* lay eggs in their woolly ovisacs in the native western and introduced eastern range. During the time of this study, any eastern *A. tsugae* present on branches would be expected to range from first to third-instar nymphs (Gray & Salom, 1996; Joseph et al., 2011). *Pineus strobi* Hartig, *Pineus pini* Macquart, *Pineus pinifoliae* Fitch, *Adelges abietis* Linnaeus, *Adelges cooleyi* Gillette, *Adelges laricis* Vallot, and *Adelges piceae* Ratzeburg are closely related species to *A. tsugae* and included in this study to test the specificity of the eDNA methods.

La. nigrinus belongs to a genus of beetles that have specialized on adelgids and is one of the target biological control predators in this study (Montgomery et al., 2011). Closely related non-target species include that *Laricobius rubidus* LeConte is native to eastern North America and hybridizes with the target *La. nigrinus*, *Laricobius laticollis* Fall is native to western North America and co-occurs with the target *La. nigrinus*, and *Laricobius osakensis* Montgomery is native to Japan and released in the eastern United States for biological control of *A. tsugae* (Montgomery et al., 2011; Toland et al., 2018). These related and potentially co-occurring species were included in this study to test the specificity of eDNA methods. Photographs of all life stages of *La. nigrius* are available in Limbu et al. (2018).

The silver flies (Diptera: Chamaemyiidae) are a large group with at least 340 described species whose larvae are predatory on sternorrhynchus Hemiptera (Gaimari & Havill, 2021). Both *Leucotaraxis* species of interest, *Le. argenticollis* and *Le. piniperda*, have two biotypes with a different host and prey preferences (Havill et al., 2018; Kohler et al., 2008; Motley et al., 2017). The non-target eastern lineages (defined as North America east of the Great Plains) feed primarily on pine bark adelgid (*Pineus strobi*), while the target western lineages (defined as North America west of the Great Plains) feed on *A. tsugae* (Havill et al., 2018; Kohler et al., 2008; Motley et al., 2017). Since the eastern and western lineages cannot be distinguished morphologically, post-release identification of *Leucotaraxis* spp. is one of the major challenges in monitoring the establishment of the silver flies released as biological controls. Photographs of these silver flies are available in Limbu et al. (2018) and Gaimari and Havill (2021). Closely related non-target species *Neoleucopis pinicola* Malloch, and *Neoleucopis atratula* Ratzeburg were also included to test the specificity of the eDNA methods.

2.2 | Field sample collections

We collected foliage of two hemlock species, eastern hemlock from Dome Island at Lake George, New York, USA, and western hemlock from Washington, USA. Eastern hemlock foliage was collected to test methods for detecting the invasive *A. tsugae* clone, while the western hemlock foliage was collected to detect the biological control predators.

Eastern hemlock foliage from Dome Island was collected between October 21 and 23, 2020. The island was divided into 16 sectors (of ~1 acre each), and one eastern hemlock tree was chosen from each of the 16 sectors based on height, branch distribution

(high live crown ratio), and structural integrity for safe climbing using single rope technique (Figure 1). We collected three samples from each tree at different heights, resulting in a total of 48 samples from the island. We collected the first sample from the lowest possible point on the tree, the second from the highest point that could be climbed, and the third approximately halfway between them. The height of each sample collection, as well as the height of the tree, was determined using a hypsometer. We collected 15–60 g samples of foliage (needles and stems) and sealed them in a 1 gal zip-seal bag at each sampling height. Gloves were changed, and clippers were rinsed with 4.5% hydrogen peroxide (Peroxigard™, Oakville, ON) before each climb to reduce eDNA contamination. Preliminary observations had indicated denser infestation at the southern end of the island; thus, we sampled from North to South to reduce eDNA contamination through clothes, ropes, and climbing gear that could not be sterilized. Foliage samples were stored and transported on ice to the laboratory, where they were stored at −20°C for up to 3 weeks before visual inspection and eDNA analysis.

We collected western hemlock foliage samples infested with *A. tsugae* from three sites at Point Defiance Park (Marina: 47.31, −122.52; Aquarium: 47.30, −122.52; Fort: 47.30, −122.53) and one location at Shannon Point (48.51, −122.69) in western Washington, USA, between April and October 2020 (Table S2). Nine tree-level samples were collected from Point Defiance Park on April 15, May 13, June 10, and October 10, and two tree-level samples from Shannon Point on May 1 and 27 (Table S2). All western hemlock foliage samples were collected from the lower and mid-canopy between 1 and 7 meters using a 5-meter pole pruner. The pruner was sterilized and gloves change between each sample collection. Collaborators placed 15–60 g of foliage in 1 gal zip lock bags and shipped overnight to the quarantine laboratory, Sarkaria Arthropod Research Laboratory (SARL), in Ithaca, NY (USDA APHIS permit P526P-18-00945). Samples were stored at −20°C for up to three weeks before visual inspection and eDNA analysis.

2.3 | Visual inspection

We conducted a visual inspection under a dissecting microscope (10x-40x magnification) to count *A. tsugae* nymphs on eastern hemlock foliage. A similar process was used to find *Leucotaraxis* flies and *La. nigrinus* larvae on the western hemlock foliage. During the time of western collections for this study, we could expect to find three larval instars or puparia (enclosed or intact) of *Leucotaraxis* flies, and larvae of *La. nigrinus* on western hemlock foliage (Dietschler et al., 2021; Grubin et al., 2011; Kohler et al., 2016; Rose et al., 2020; Zilahi-Balogh et al., 2003). For the enumeration of *A. tsugae* on eastern hemlock, approximately half of the sampled

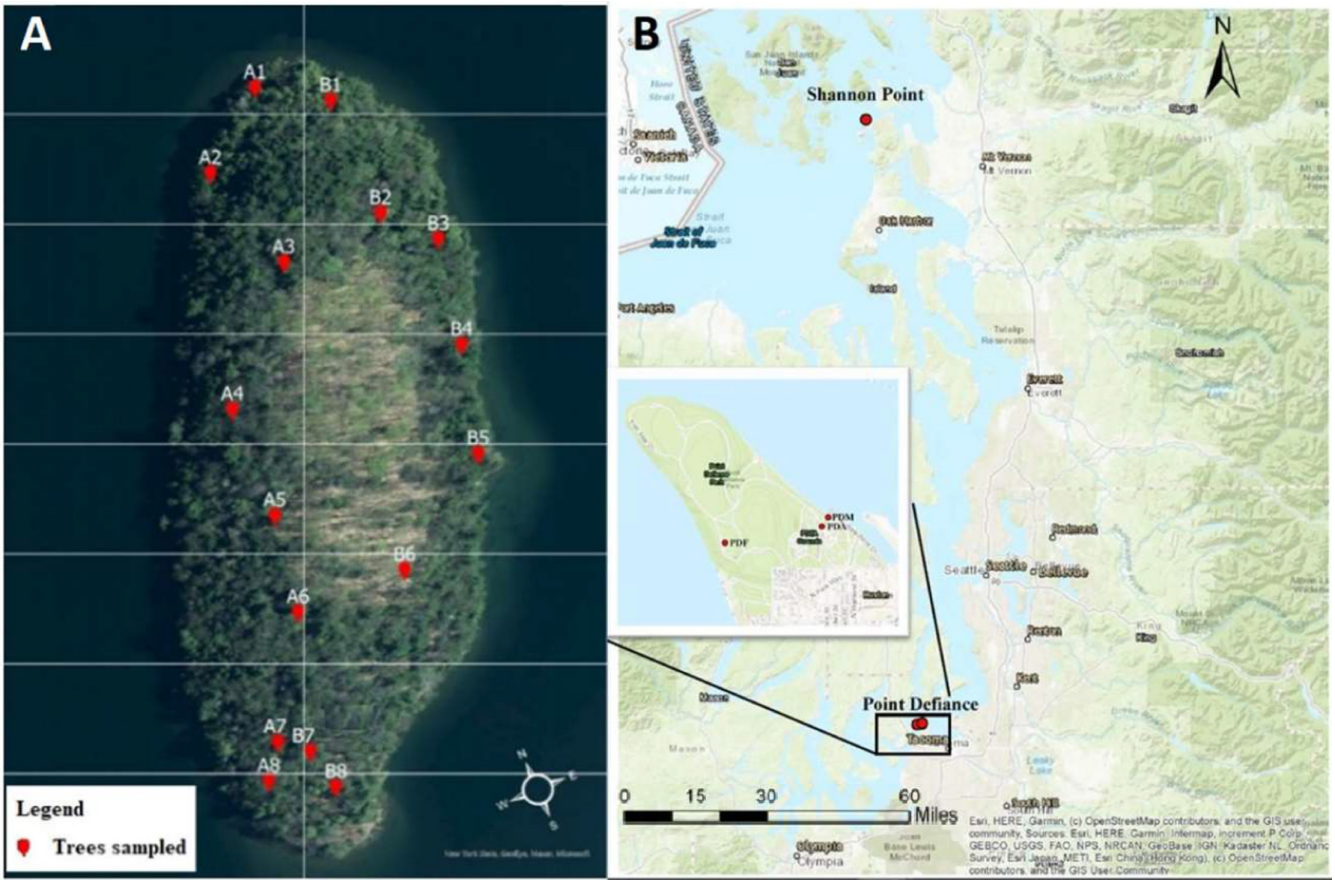

FIGURE 1 (a) Map of Dome Island in Lake George, NY, indicating trees sampled in each of the 16 quadrants for detection of *A. tsugae*. (b) Map showing sampling sites at Point Defiance and Shannon Point

foliage was separated (total length ranging from 64 to 270.5 cm) and the remainder was stored at −20°C for subsequent eDNA analysis. Foliage length refers to the cumulative length of hemlock branchlets in the sample measured during the visual inspection process. Measuring the foliage while collecting and dividing can increase the probability of eDNA contamination and was thus avoided. All results were normalized by the foliage length (e.g., individuals per cm) before analysis. We counted all visible nymphs on the subsample under a dissecting microscope. Then, the cumulative length of the stems on the foliage sample was measured to the nearest 0.1 cm and discarded. This sub-sampling was done to reduce the probability of eDNA contamination between samples during the enumeration.

A. tsugae predators were sampled from their native range on western hemlock foliage. Since *A. tsugae* predators were expected to be less abundant than *A. tsugae* on the foliage, we passed the entire (1.8–13.3 m) samples of western hemlock foliage through visual inspection before eDNA analysis. All *Leucotaraxis* larvae and puparia found on the foliage were removed from the foliage and stored individually in 2-ml microcentrifuge tubes for subsequent DNA extraction and identification to the species level using multiplex qPCR. *Laricobius* larvae were removed and discarded as they can be accurately identified morphologically on western hemlock sampled from selected sites. This was done to ensure no intact target organisms were accidentally included in the eDNA analysis. After visual inspection, the foliage was placed back in the bags and frozen at −20°C for eDNA analysis.

2.4 | eDNA collection from hemlock foliage samples

We processed both eastern and western hemlock foliage samples similarly to capture the eDNA of target species. We removed the foliage from the bags and placed it in bleach-sterilized 500-ml Nalgene bottles. Gloves were changed before handling each sample. We added 450 ml of DI water to each Nalgene, then capped and agitated bottles by hand for 10 s to dislodge eDNA from the foliage and into the water. We pre-filtered the water through 0.3-mm nylon mesh to remove whole organisms and any large debris that may clog the filters. The water was then directly poured into 500-ml vacuum filtration funnels with a 0.8-µm track-etched polycarbonate membrane (Whatman, Pittsburg, PA). The filtration was powered by a built-in laboratory vacuum port in the fume hood. After the filtration, we removed the filters using sterilized forceps and stored them in 2-ml microcentrifuge tubes at −20°C until DNA extraction. Preliminary experiments showed that samples with over 60 g of foliage either clogged the filters during filtration or the silica spin column during DNA extraction. Thus, we divided samples with over 50 g of foliage into two portions. We filtered and extracted the two portions separately, but their eluates were combined at the end of DNA extraction. This process was performed for 5 eastern hemlock samples, and 24 western hemlock samples. All materials including Nalgene bottles, nylon mesh, filtration funnels, and tweezers were cleaned with freshly prepared 10% bleach after use. After every ten foliage samples, one full-process negative control (total of 8 throughout the experiment) of 500 ml of DI water was filtered and processed alongside the samples, including filtration, DNA extraction, and qPCR to monitor for the presence of eDNA contamination during the laboratory procedure.

2.5 | DNA extraction

We conducted all DNA extractions using the Qiagen DNeasy Blood and Tissue kit. For eDNA extraction from filters, whole filters were used in place of animal tissues in the manufacturer's protocol and incubated with 180 µl buffer ATL and 20 µl proteinase K for 3 h at 56°C with constant shaking at 300 rpm. The resulting lysate (no filter) was transferred to another microcentrifuge tube, and the remaining procedure was carried out according to the manufacturer's protocol. DNA extracted from filters was eluted in 50 µl of buffer AE, while the DNA from *Leucotaraxis* larvae and reference samples was eluted in 100 µl of buffer AE. At least one full-process negative control was included in each batch of DNA extractions to track contamination at each stage of the process.

2.6 | qPCR assay development and testing

We designed novel qPCR primers to amplify the mitochondrial cytochrome oxidase subunit 1 (COI) gene of *A. tsugae* (HWA), *Le. argenticollis* (LA), *Le. piniperda* (LP), and *La. nigrinus* (LARI) using NCBI Primer-BLAST (NCBI, Bethesda MD) and verified them for optimal annealing temperature using Oligo Analyzer (IDT, Coralville, CA). For LA and LP assays, sequences of only the western biotypes were used as input to generate primers in Primer-BLAST. The accession numbers for all sequences used for qPCR assay design are provided in Table S4. For designing the HWA and LARI assays, we aligned the sequences of the PCR targets generated by Primer-BLAST in MEGA7 (Pennsylvania State University, PA) along with the sequences of closely related and co-occurring species. The TaqMan® probes were then designed "by-eye" probes that maximize species specificity and verified for specificity using the BLAST sequence analysis tool (Madden, 2013). For designing the biotype-specific assays for silver flies, the species-specific primers generated in Primer-BLAST with most mismatches with the eastern biotypes were selected. *Le. argenticollis* and *Le. piniperda* target sequences were aligned in MEGA7 with their corresponding sequences from the eastern biotypes of the silver flies. The TaqMan® probes were designed "by-eye" to maximize mismatches with the eastern biotypes (Figure S1). The LA and LP assays targeting the western biotype had 6 and 8 mismatches with the corresponding sequences of the eastern biotypes *Le. argenticollis* and *Le. piniperda*. Hairpin, self-dimer, and hetero-dimer analyses were also conducted on the primer and probe sequences using Oligo Analyzer to ensure efficient qPCR. For simplex qPCR, we labeled all TaqMan probes with FAM fluorophore and double

quenched with ZEN™ quencher (IDT, Coralville, CA). For multiplex qPCR, we designed an additional probe for *Le. argenticollis* with the same sequence but with TAMRA fluorophore and quencher (IDT, Coralville, CA).

While all qPCR assays were tested for specificity in silico using Primer-BLAST during the design process, we also tested the specificity of the assays experimentally using qPCR with the template DNA from all possibly co-occurring and closely related species to our targets. In the case of *A. tsugae* (HWA assay), this included testing the specificity using DNA extracted from *P. strobi*, *P. pini*, *P. pinifoliae*, *A. abietis*, *A. cooleyi*, *A. laricis*, and *A. piceae*. Both LA and LP assays (targeting *Le. argenticollis* and *Le. piniperda*, respectively) were tested against DNA extracted from closely related and co-occurring *N. pinicola* and *N. atratula*. The LA qPCR assay targeting *Le. argenticollis* was tested against *Le. piniperda* DNA and vice versa to check for non-specific amplification of the two closely related species often released at the same sites for biological control. Apart from being species-specific, the LA and LP assays were also designed to be specific to western biotypes of *Le. argenticollis* and *Le. piniperda*. The biotype level specificity was tested with triplicate qPCR using a 7-point standard curve (1–10^6 copies/reaction) made from Gene Fragments (Twist Bioscience, San Francisco, CA) designed using corresponding sequences from eastern biotypes of *Le. argenticollis* and *Le. piniperda* (Figure S1). The LARI assay (targeting *La. nigrinus*) was tested for specificity using the DNA extracted from *La. rubidus*, *La. laticollis*, and *La. osakensis*.

To test for the efficiency of the qPCR assays, gBlocks™ Gene Fragments were obtained from IDT (Coralville, IA) (Table S3). We placed *Le. piniperda* and *La. nigrinus* target sequences individually on separate gBlock fragments, while *Le. argenticollis* and *A. tsugae* target sequences were placed adjacent to each other on the same gBlock fragment (Table S3). We quantified the copy number of the gBlocks using Qubit 4.0 (Life Technologies, Carlsbad, CA). We ran 7-point gBlock standard curves ranging from 1 to 10^6 copies per reaction in triplicate on each qPCR plate of a given assay to ensure consistently efficient qPCRs. Limit of quantification (LOQ) was defined as the lowest standard concentration at which all qPCR replicates amplified above the threshold (Ambruster & Pry, 2008; Kirtane et al., 2019; Xia et al., 2018). All samples above the LOQ were converted from cycle threshold (Ct) values to copies/reaction using the standard curve equation of the given assay (Table 1). For analysis, copies/reaction was then converted to copies/g foliage for eastern hemlock and copies/cm foliage for western hemlock foliage. The standard curve equation was determined by pooling all the standard curves obtained throughout the experiment. All samples with Ct values above the LOQ or in which only one or two replicates were amplified were retested to check for contamination at the qPCR level. If the results remained unchanged after the re-test, indicating no qPCR contamination, the samples were considered positive, but below the limit of quantification (BLOQ).

2.7 | qPCR protocol

All qPCRs were run in 96-well plates in a ViiA 7 Thermocycler (Applied Biosystems, Foster City, CA) at the Biotechnology Resource Center at Cornell University. All environmental samples were analyzed in triplicate. Each qPCR plate had a triplicate standard curve and three reaction wells designated for qPCR negative controls. Each reaction well contained 10 µl 2x TaqMan Environmental Mastermix, 0.6 µM final concentration of each primer, 0.3 µM concentration of TaqMan probe, 2 µl template DNA, and molecular grade water to bring volume

TABLE 1 qPCR parameters for assays developed in this study

Target species	Oligonucleotide sequence (5′→3′)	Slope	Intercept	Efficiency	LOQ (copies / reaction)[a]	Amplicon size (bp)
Adelges tsugae	HWA forward—ACAGGATGAACAATTTACCCAC HWA reverse—AGCACCTGCTAGAACAGGTAAGG HWA probe—CCATTATTCCCATGATCAATTTTAATTACTGC	−3.43	41.62	0.96	1	246
Leucotaraxis argenticollis	LA forward—ACCCGGGAGCATTAATTGGA LA reverse—ACCAGCTCCATTTTCCACTCT LA probe—TGTAATTGTAACAGCCCACGCATTTGTAATAAT	−3.34	39.33	0.99	1	242
Leucotaraxis piniperda	LP forward—AGGAGCCCCTGATATGGCTT LP reverse—ACAGAAGCACCTCTATGGGC LP probe—AGGTTGAACAGTTTACCCCCCTTTATCATCTAA	−3.43	42.38	0.96	1	168
Laricobius nigrinus	LARI forward—GGCGCCTGAGCAGGAATAGT LARI reverse—AGCATGGGCTGTTACAATAACG LARI probe—ACTTCTCTTAGACTTTTAATTCGGGCAGA	−3.47	39.85	0.94	10	120

[a]LOQ refers to the limit of quantification defined as the lowest gBlock standard concentration at which all qPCR replicates amplify above the threshold.

up to 20 µl. Cycling parameters started with incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, and an annealing/extension step at 60°C for 45 s. The qPCR threshold was set at 0.03 ΔRn throughout the experiment for consistency in comparing results throughout the study (Baccari et al., 2020; Cao et al., 2013; Kirtane et al., 2019).

Simplex qPCRs were run in triplicate for all environmental samples, and the average of the three values was used in statistical analyses. Simplex qPCR was also used for all the standard curves and assay specificity tests. We conducted duplex qPCR to identify *Leucotaraxis* larvae and puparia from foliage samples to the species level. For the duplex PCR, we followed the same protocol as simplex but added both assays, LP (FAM-labeled) and LA (TAMRA-labeled), in the qPCR mix.

2.8 | Statistical analysis

We performed McNemar's test to determine whether there was a significant increase in positive detection rates using eDNA methods when compared with conventional visual inspection methods for each of the four target species. We used single-factor ANOVA to determine differences in *A. tsugae* infestation between the three strata (Bottom, Mid, and Top) using both nymph density (visual inspection) and *A. tsugae* eDNA concentration. We analyzed the relationship between density as measured by visual inspection, and eDNA concentration using linear regression for each of the four target species. We log-transformed eDNA concentration since the data deviated from a normal distribution. The target eDNA concentration in samples categorized as BLOQ is likely to be very low. Thus, for the regression analyses, we assigned them a concentration value of one order of magnitude lower than the lowest quantifiable concentration for the given target. The BLOQ samples with *A. tsugae*, *Le. piniperda*, *Le. argenticollis*, and *La. nigrinus* targets were assigned eDNA concentration values of 0.3 eDNA copies/g, 53.4 eDNA copies/cm, 0.5 eDNA copies/cm, and 3.4 eDNA copies/cm, respectively. Alpha value was set to 0.05 for all statistical analyses. All statistical analyses we conducted using R (R Core Team, 2021).

3 | RESULTS

3.1 | Performance of qPCR assays

All qPCR assays were tested for specificity and efficiency before using them for sample analysis. No cross-amplification was detected using the HWA assay against DNA from any of the non-target adelgid species. For the LARI assay, no cross-amplification was detected for *La. laticollis* and *La. osakensis*, but DNA from *La. rubidus* did amplify. Despite multiple attempts to re-design and improve the specificity of the LARI assay, non-specific amplification on *La. rubidus* DNA was still detected in all five individuals of *La. rubidus* tested. Neither the LA nor the LP assays amplified DNA from *N. pinicola* or *N. atratula*. The LA assay and LP assay did not cross-amplify DNA from *Le. piniperda* and *Le. argenticollis*, respectively, allowing multiplex qPCR for the identification of larvae and pupae dissected from foliage samples. The LA and LP assays were also determined to be biotype-specific in addition to being species-specific. The LA assay did not detect gene fragments from the eastern biotype of *Le. argenticollis* on a gBlock standard curve of 10^6 to 1 copy/reaction. The LP assay only amplified one of the three replicates of *Le. piniperda* eastern biotype gBlock at the highest concentration tested, 10^6 copies/reaction, with a high Ct value (37.4) and showed no amplification at lower concentrations. This reduces the probability of accidentally detecting eDNA from native eastern biotypes of the two species (Table 1). All assays had high amplification efficiencies (94–99%). The LA, LP, and HWA assays had a low limit of detection (LOD) of 1 copy/reaction and 10 copies/reaction for the LARI assay (Table 1). None of the full process negative controls or the qPCR negative controls showed amplification for any of the target species indicating a low chance of contamination during the sample process and qPCR analysis.

3.2 | Detection of *A. tsugae* on eastern hemlock using visual inspection and eDNA analysis

Sub-samples collected from each of the eastern hemlock samples for visual inspection at Dome Island ranged from 64 to 270.5 cm of cumulative branch length per sample (Table S1). The presence of *A. tsugae* was detected visually on 11of 48 samples. The densities of *A. tsugae* nymphs ranged from 0.005 to 6.921 nymphs/cm. These 11 positive foliage samples were collected from 6 out of the 16 trees sampled (Table 2 and Table S1).

TABLE 2 Cross-tabulation of the number of detects and non-detects of target species using visual and eDNA based methods

	eDNA detects	eDNA non-detects	Total
Target species: *Adelges Tsugae*			
Visual detects	11	0	11
Visual non-detects	31	6	37
Total	42	6	48
Target species: *Leucotaraxis piniperda*			
Visual detects	12	0	12
Visual non-detects	22	4	26
Total	34	4	38
Target species: *Leucotaraxis argenticollis*			
Visual detects	6	1	7
Visual non-detects	14	17	31
Total	20	18	38
Target species: *Laricobius nigrinus*			
Visual detects	8	1	9
Visual non-detects	23	6	29
Total	31	7	38

The remainder of eastern hemlock foliage (i.e., the sub-sample not used in visual inspection) collected from Dome Island was used for eDNA analysis. The mass of the foliage samples ranged from 16.1 to 58.7 g (Table S1). *A. tsugae* eDNA was detected on each of the 16 trees sampled. qPCR amplification using the HWA assay was observed in 43 out of 48 foliage samples, in at least one of the three qPCR replicates (Table 2 and Table S1). Out of the 43 foliage samples that were positive for *A. tsugae* eDNA, 40 amplified in all three qPCR replicates, while 3 samples amplified in one or two replicates (Table S1). We repeated triplicate qPCR on these three samples and obtained the same result (i.e., amplification below the limit of quantification or amplification of fewer than three replicates). This confirmed the absence of contamination at the qPCR level in these samples. Thus, these samples were considered positive, but below the limit of quantification (BLOQ). The concentrations of quantifiable samples ranged from 3.12 to 203521.31 eDNA copies / g of eastern hemlock foliage (Table S2). McNemar's test reported a significant increase in positive detection rates using eDNA analysis to indicate the presence of *A. tsugae* ($p < 0.001$). There was no significant difference in *A. tsugae* infestation between the three strata of eastern hemlock trees sampled on Dome Island when measured by both ovisac density (ANOVA, $p = 0.58$) and *A. tsugae* eDNA concentration (ANOVA, $p = 0.39$) (Figure 2). There was a weak positive relationship between *A. tsugae* density as measured by visual inspection and its eDNA concentration ($n = 42$, $R^2 = 0.22$, $p = 1.0e\text{-}03$) (Figure S2).

3.3 | Detection of biological control species using visual inspection and eDNA analysis

A total of 234.93 m of foliage over 38 western hemlock samples was carefully examined under a dissecting microscope in search of *A. tsugae* predators, with an average length per sample of 6.18 m ranging from 1.63 m to 13.33 m (Table S2). In total, 92 silver fly larvae and puparia were removed, and their DNA extracted and identified using duplex qPCR. *Le. argenticollis* larvae and puparia ($n = 28$) were identified from 7 samples with densities ranging from 0.09–1.54 individuals/m (Table S2). *Le. piniperda* larvae and puparia ($n = 63$) were identified from 12 foliage samples with densities ranging from 0.11 to 4.17 individuals/m (Table S2). One larva did not amplify using either LA or LP qPCR assays, so could have been *N. atratula*, which is occasionally found feeding on *A. tsugae* in western North America (Neidermeier et al., 2020). Only 2 samples had individuals from both *Leucotaraxis* species, while neither species was observed in 21 of 38 samples (Table S2). A total of 67 *La. nigrinus* larvae were identified from seven foliage samples with densities ranging from 0.09 to 7.14 individuals / m (Table S2). Either *Le. argenticollis* or *Le. piniperda* were found alongside *La. nigrinus* on 5 of those samples, while 17 samples had none of the three predators (Table S2). At Point Defiance, the April 15 sample had *Le. piniperda* and *La. nigrinus*, May 13 had all three predators, June 10 had *Le. argenticollis* and *La. nigrinus*, and October 10 had *Le. piniperda* and *Le. nigrinus*. Shannon Point had *La. nigrinus* present in both samples, and *Le. argenticollis* was present in only the May 27 sample. While nine tree-level samples were collected at each Point Defiance sampling period, the presence of both species was only found in one tree level sample during the May 13 and October 10 sample periods, respectively. Using the conventional visual inspection methods, none of the samples had all three predator species.

After visual inspection, removal, and identification of target predators, the same 38 foliage samples were assayed using eDNA analysis. *Le. argenticollis* eDNA was detected on 21 samples out of which 9 were categorized as BLOQ and 12 were quantified with concentrations ranging from 5.4 to 1431.9 copies / m (Table 2 and Table S2). *Le. piniperda* eDNA was detected in 34 samples out of which 9 were categorized as BLOQ and 25 were quantified with concentrations

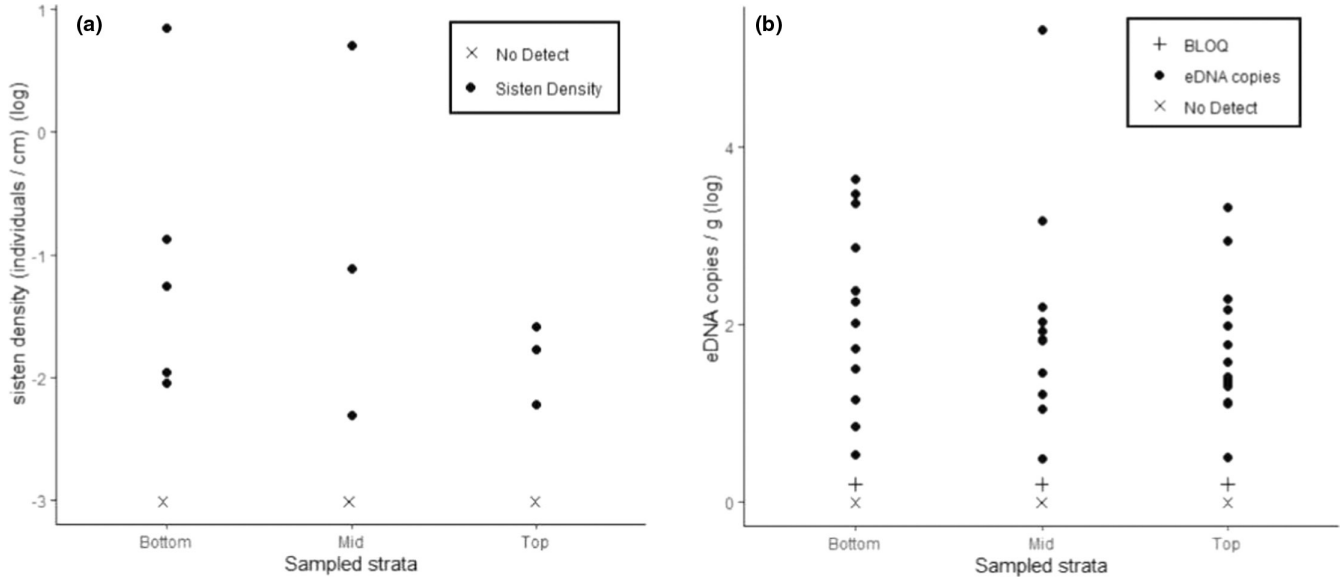

FIGURE 2 Detection of *A. tsugae* on three strata of eastern hemlock trees on Dome Island, NY, using (a) visual and (b) eDNA identification

ranging from 533.7 to 5,261,438.5 copies / m (Table 2 and Table S2). eDNA, from both *Le. piniperda* and *Le. argenticollis*, was detected in 18 samples with only one sample with no eDNA from either of the silver flies (Table 2 and Table S2). *La. nigrinus* eDNA was detected in 31 samples out of which 3 were categorized as BLOQ and 28 were quantified with concentrations ranging from 34.6 to 226,951.5 copies / m (Table 2 and Table S2). eDNA belonging to all the three species was detected in 16 samples, and there were no samples from which eDNA was not detected from any of the three predators (Table S2). McNemar's test showed an increase in positive detection rates for all three biological control predator species (*Le. piniperda*, $p = 9.44E\text{-}07$; *Le. argenticollis*, $p = 1.15E\text{-}03$; *La. nigrinus*, $p = 1.81E\text{-}05$). There was a weak positive relationship between eDNA concentration and the density of *Le. piniperda* ($n = 34$, $R^2 = 0.22$, $p = 5.0E\text{-}03$) and *La. nigrinus* ($n = 31$, $R^2 = 0.18$, $p = 1.0E\text{-}02$), but the relationship was not significant for *Le. argenticollis* ($n = 21$, $R^2 = 0.12$, $p = 0.14$) (Figure S2).

4 | DISCUSSION

Adelges tsugae infestation is a critical threat to eastern hemlocks, a foundation species impacting the dependent ecosystems and wildlife (Orwig et al., 2002). Rapid and early detection of infestations is critical for making effective management decisions and their success (Liebhold & Kean, 2019). The northward movement of the infestations and the recent first discovery in Adirondack Forest Preserve in New York are especially concerning due to the abundance of hemlock in the region (Albright et al., 2020; DEC, 2020). The eDNA methods described in this study may be a step in widespread simultaneous monitoring of very early-stage *A. tsugae* infestations and detecting the establishment of three of its most promising specialist biological control predators. The positive detection rate of the pest and its biological control predators was 2.8–4.5 times higher using eDNA methods than conventional methods (Tables 2 and 3). A similar increase in detection sensitivity has been reported in a previous study detecting invasive spotted lanternflies in vineyards (Allen et al., 2021). Furthermore, the visual inspections in this study were considerably time-consuming with a total of 58.2 m of eastern hemlock and 234.9 m of western hemlock foliage being visually inspected. Due to additional benefits of the eDNA methods such as lower cost and higher efficiency especially when scaled up (Davy et al., 2015; Kirtane et al., 2019; Smart et al., 2016), we strongly recommend the incorporation of this technique for regular monitoring of *A. tsugae* infestations and evaluating successes of biological control releases.

4.1 | qPCR assay performance

All the qPCR assays designed and tested in this study had a high amplification efficiency and low limit of detection making them ideal for use in identifying and quantifying eDNA. All qPCR assays except LARI, targeting *La. nigrinus*, were species-specific. The LA and LP assays targeting *Le. argenticollis* and *Le. piniperda*, respectively, were not only species-specific but also biotype-specific. This broadens the scope of eDNA applications to monitor biological diversity beyond species-level to population-level detection. Studies using metabarcoding approaches have also been able to acquire population-level genetic information from eDNA samples collected from seawater (Parsons et al., 2018; Sigsgaard et al., 2016). Identification below the species level is especially important for tracking introduced biological control agents because there can be an intraspecies variation that is correlated with the differences in biological control specificity or efficiency (Andersen & Wagner, 2016).

The LARI assay amplified non-target *La. rubidus*, a co-occurring species that hybridizes with *La. nigrinus* in eastern release sites (Fischer et al., 2015; Havill et al., 2012). While the LARI assay may not be ideal for monitoring the establishment and dispersal of *La. nigrinus* in the east, this non-specific amplification did not impede *La. nigrinus* detection on western hemlock samples as *La. rubidus* is not distributed in western North America (Brown, 1944; Lawrence & Vaurie, 1983). It may be possible to design additional qPCR assays targeting other mitochondrial or nuclear genes specific to *La. nigrinus*. This would require efforts to build up reference libraries for new target genes for both species. However, currently, we cannot eliminate the chance of detecting hybridized *La. nigrinus* x *La. rubidus* individuals or pure *La. rubidus* individuals co-occurring in the same forest stand without further tests like sequencing qPCR amplicons, or PCR restriction fragment length polymorphism (Davis et al., 2011).

4.2 | Interpreting positive detection rates of eDNA and traditional sampling methods

As eDNA methods gain more widespread use in pest and natural enemy detection (Valentin et al., 2020), it is important to clarify

TABLE 3 Positive detection rate of target species using conventional methods and novel eDNA based methods developed in this study

Species	Number of samples	Positive detection rate (visual inspection)	Positive detection rate (eDNA analysis)
Adelges tsugae	48	22.9%	89.6%
Leucotaraxis argenticollis	38	18.4%	55.3%
Leucotaraxis piniperda	38	31.6%	89.6%
Laricobius nigrinus	38	18.4%	81.6%

how the data can be interpreted and what conclusions can be drawn. McNemar's test indicated the results provided by the eDNA test were significantly more sensitive than visual inspection for all target species in this study. In the case of *A. tsugae* detection, the eDNA was detected on all 16 trees, while visual inspection detected them on only 6 trees. A similar increase in sensitivity using eDNA detection of another terrestrial insect pest, the spotted lanternfly, has been reported (Allen et al., 2021). Visual inspection methods can be limited spatially and temporally, while eDNA can persist over time and be transported easily through space. While studies exploring eDNA degradation rates on foliage are limited, agromyzid leafminer (*Liriomyza sativae* Blanchard) eDNA was found 28 days after the leafmines became empty (Pirtle et al., 2021) and mammal eDNA has been reported to persist for up to 24 weeks on foliage after browsing (Nichols et al., 2012), but eDNA associated with *A. tsugae* wool may be protected for longer periods through shading or adsorption (Kessler et al., 2020; Kirtane et al., 2020; Valentin et al., 2021).

Similar to understanding its persistence, it is important to account for the influence of eDNA transport while interpreting positive results. eDNA may be transported over short distances through wind or from higher branches through rainfall (Valentin et al., 2021). Just like the spread of *A. tsugae* themselves, their eDNA can be transported over long distances via birds and other insect vectors (Russo et al., 2016; Russo et al., 2019). For these reasons, it is important to acknowledge that eDNA methods may result in false positives and may not indicate localized infestations. However, these false positives can be used to predict new invasions ahead of time, especially in the case of *A. tsugae*, as the dispersal agents of eDNA likely also transport the pest. Furthermore, the scalability and cost-effectiveness of eDNA-based methods could be used for regular surveying of vast expanses of habitat and can help narrow down specific areas for subsequent visual surveys or management (Biggs et al., 2015; Davy et al., 2015; Evans et al., 2017; Huver et al., 2015; Kirtane et al., 2019). For example, sites with positive signals for *A. tsugae* eDNA can be prioritized for visual inspection. Sites with visual confirmation of the pest can then be treated to eradicate or contain the spread as early detection increases the success rate of these measures (Liebhold & Kean, 2019).

Understanding the relationship between eDNA concentration and observed density can help estimate the level of infestation of the pest and the success of the establishment of the predators. There were positive relationships among visually observed density and eDNA detection for *A. tsugae*, *Le. piniperda*, *La. nigrinus*, and *Le. argenticollis* but the observed density only accounted for 22%, 22%, 18%, and 12% of the variation, respectively (Figure S2). The relationship was not considered statistically significant ($p > 0.05$) in the case of *Le. argenticollis*. A recent study investigating a similar correlation with spotted lanternflies in grape vineyards found that 71% of the variation in visual observations could be explained by their eDNA signal (Allen et al., 2021). Determining a more precise level of *A. tsugae* infestations could greatly enhance the utility of eDNA data generated from surveys and in turn aid in better management decisions. This study only collected samples from the invasion front which typically has smaller populations, thus the concentration of data points near zero (Figure S2). Future studies could utilize contrived samples from varying levels of infestation to potentially determine a relationship between *A. tsugae* eDNA concentration and infestation level for *A. tsugae* over a broader range. Understanding environmental factors that contribute to the temporal persistence of eDNA on foliage surfaces can further bolster the model predictions.

4.3 | Ecological insights from detection of biological control predators on western hemlock

Data from eDNA surveys in conjunction with traditional sampling techniques can also be used to advance the ecological knowledge of target species. Previous studies have found a temporal overlap of these two *Leucotaraxis* species during the larval stage when aggregating observations from multiple locations (Dietschler et al., 2021; Rose et al., 2020), but that adult emergence is temporally asynchronous at the site level (Dietschler et al., 2021; Neidermeier et al., 2020). Our study only found both species in the same tree sample in two out of the 21 samples where *Leucotaraxis* were detected, with overlap occurring in only the May 13 and October 10, 2020, sampling periods. Only *Le. piniperda* were detected in Point Defiance samples collected in April, with species overlap in May, transitioning to only *Le. argenticollis* in June, suggesting site-level sequential trends at the tree level. By contrast, 18 samples had positive eDNA detection for both *Le. argenticollis* and *Le. piniperda*, likely due to persistence of eDNA on foliage. While further exploration of site-level community dynamics and niche separation of the predators in this system is needed, our data suggest that larval presence could follow similar trends as adults when evaluated at site and/or tree level. The potentially limited physical overlap of the two species of *Leucotaraxis* highlights the importance of being able to detect the presence of preserved eDNA in the absence of the organism. Further studies exploring the persistence of eDNA associated with foliage surfaces and *A. tsugae* wool will enhance the interpretation of positive eDNA detections, and aid in developing strategies to use visual inspection complimentary with eDNA surveillance.

4.4 | Next steps and new frontiers

This study provides progress towards developing comprehensive rapid detection techniques for monitoring forest pests. Improvements in sampling protocol and eDNA processing that reduce costs and control contamination, as well as the development of on-site methods, will enhance the utility of this tool. Our data from Dome Island showed no significant difference between eDNA concentrations detected on three strata of eastern hemlock sampled, demonstrating that collections from ground level (low strata) may be sufficient for detecting infestations thus reducing survey time,

costs, and potential contamination from the use of climbing gear. Sampling water from streams or rivers for *A. tsugae* and its predators may also be a viable method. Small amounts of rain can significantly reduce eDNA on aboveground surfaces (Valentin et al., 2021), causing it to flow into streams or rivers. Since eastern hemlocks dominate riparian habitats (Ellison et al., 2005), rivers could act as "conveyor belts" collecting and transporting eDNA used to detect presence in the entire watershed (Deiner et al., 2016; Deiner & Altermatt, 2014). If *A. tsugae* eDNA can be detected in rivers and streams, it could result in broader resolution sampling that could be followed up by targeted sampling to zero in on infestation. Recent advances in the development of air eDNA capture methodology may present an additional route to monitor the terrestrial insect community, including invasive species (Clare et al., 2021; Roger et al., 2022). With novel capture methods, advancements in on-site eDNA processing will allow surveyors to process eDNA samples within 30 min, as demonstrated in the case of invasive silver carp (*Hypophthalmichthys molitrix* Valenciennes) (Doi et al., 2020). Furthermore, development in species-specific Loop-Mediated Isothermal Amplification (LAMP) could be developed to replace qPCR (Alzaylaee et al., 2020; Williams et al., 2017). LAMP analysis can be conducted with minimal equipment and report the presence/absence of target DNA based on a color change observable through the naked eye (Alzaylaee et al., 2020; Williams et al., 2017) can further improve rapid on-site detection of eDNA.

AUTHOR CONTRIBUTIONS

AK, ND, TDB, and MW conceptualized the experiments. All authors contributed to sample collection and/or processing. All authors contributed to drafting and revising the manuscript.

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CONFLICT OF INTEREST

The authors of this manuscript report no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data generated in this study is publicly available in a Dryad repository DOI https://doi.org/10.5061/dryad.rn8pk0pcz

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