Investigating the Source of PBDE Contaminant Exposure in Steelhead Trout within the Major Tributaries of the Nisqually River Basin

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Acronyms and Abbreviations

Acronyms and abbreviations used frequently in this report are listed below; those used infrequently are excluded.

BDE	Brominated diphenyl ether
CBR	Critical body residue
CCR	Continuing calibrations standards
CI	Confidence intervals
Ecology	Washington Department of Ecology
EPA	Environmental Protection Agency
ESA	Endangered Species Act
GC-HRMS	Gas chromatography - high resolution mass spectrometry
IRM	Internal reference material
JBLM	Join Base Lewis-McChord
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NWFSC	Northwest Fisheries Science Center
PBDE	Polybrominated diphenyl ether
QAPP	Quality Assurance Project Plan
QA/QC	Quality assurance/quality control
RPD	Relative percent difference
SRKW	Southern Resident killer whale
SRM	Standard reference material
TBiOS	Toxics Biological Observation System
USGS	U.S. Geological Survey
WDFW	Washington Department of Fish and Wildlife
WRIA	Water resource inventory area
WWTP	Wastewater treatment plant

Units of Measurement

cfs	cubic feet per second
L	Liter
mV	millivolt
pg/g	picogram per gram (parts per trillion)
ppt	parts per trillion (pg/g)
RM	river mile
ww	wet weight
‰	parts per thousand or per mil

Study Summary

A contaminant study conducted by Washington Department of Fish and Wildlife (WDFW) in 2014 of seaward migrating steelhead trout (*Oncorhynchus mykiss*), an ESA-listed species, documented that 33-50% of the steelhead samples collected from river, estuary and associated nearshore habitats of the Nisqually River watershed exceeded critical-body-residues (CBRs) for polybrominated diphenyl ethers (PBDE) concentrations for increased disease susceptibility whereas those collected from the Skagit and Green/Duwamish watersheds did not. Follow-up sampling in 2015 confirmed approximately 33% of steelhead trout smolts from the in-river site had levels of PBDEs known to increase disease susceptibility in salmonids. PBDEs are a group of 209 flame retardant compounds or congeners used in a large variety of products (e.g., plastics, furniture, upholstery, electrical equipment, and textiles). The main pathway for PBDEs into the environment is through household grey water that is treated and discharged via wastewater treatment plants (WWTPs).

Following the initial WDFW survey, a PBDE source assessment study was undertaken throughout the Nisqually watershed by WDFW and the Department of Ecology (Ecology) in 2017. The study concluded that PBDEs were entering the river system through the three major tributaries, the Mashel River, Muck Creek, and Ohop Creek. In particular, the Mashel River contained the highest concentrations in water and biofilm (algae, detritus and microbes) samples and congener patterns that differed from other river samples. Limited spatial sampling within the tributaries restricted our ability to definitively conclude the major PBDE sources to the Nisqually River system. As a result, a follow-up PBDE source study was conducted in 2021. The goal of the 2021 project was to identify PBDE sources within the three major tributaries of the Nisqually River, the Mashel River, Ohop Creek, and Muck Creek, and determine the impacts on the food web. Specific objectives were to 1) delineate the locations of PBDE inputs to the Mashel River, 2) investigate the presence of PBDEs in Ohop and Muck creeks, and 3) measure and describe the uptake of PBDEs in aquatic insects, an indicator of potential prey for juvenile steelhead trout. PBDE concentrations were measured in biofilms and insect larvae collected from sites within each of the three tributaries during a period of low river flow in the late summer of 2021.

The results of the 2021 study determined PBDEs are primarily entering the Nisqually River watershed in the Mashel River, with the likely source or pathway being the Eatonville WWTP outfall located at river mile (RM) 5.1. PBDE concentrations in biofilms and invertebrates from Ohop and Muck creeks were more similar to levels measured upstream of the WWTP outfall in the Mashel River and are considered background concentrations. Based on ten sampling sites in the Mashel River distributed from RM 6.5 to 0.35, we observed a 5-6-fold increase of PBDE concentrations in biofilm and invertebrates from the site just upstream of the outfall, to the site just downstream of the outfall, a half-mile stretch of the river. Indeed, the PBDE concentrations in the biofilm remained elevated for approximately half a mile downstream from the outfall before decreasing to near background levels. The relatively small geographic area where the biofilm had increased PBDEs suggest the contaminants are likely being diluted in the river from either groundwater or other sources like the Little Mashel River. Interestingly, PBDEs measured in invertebrates stayed elevated for about 3.7 miles downstream of the outfall before decreasing to almost background levels just prior to the confluence with the Nisqually River. The insect larvae are mobile and can move downstream; they also have a longer growth period than biofilms. The area of the river where PBDEs are above background could potentially be exposing predators like juvenile steelhead trout to these chemicals. Differences in PBDE congener patterns in biofilm and invertebrate samples collected below the WWTP outfall are consistent with different PBDE sources below the outfall. Furthermore, PBDE congener patterns measured in biofilm and effluent sampled directly from the Eatonville WWTP were similarly dominated by BDE-47, -99 and -100 (tetra- and penta-

BDEs), further suggesting the wastewater as the source of PBDEs to the Mashel River. In addition, enrichment in the nitrogen stable isotope, $\delta^{15}N$ below the WWTP outfall compared to those collected upstream of the outfall provided an additional line of evidence that inputs to the river from the Eatonville WWTP effluent are being incorporated into the biota of the Mashel River.

Based on the studies completed in 2017 and 2021 we were able to put together a rough idea of PBDE concentration, bioaccumulation, and biomagnification in the Mashel River system despite inconsistent periods of exposure and sample timing among all the samples. The bioconcentration of PBDEs from the water onto the biofilms collected downstream from the WWTP outfall (RM 4.9) occurred with a factor of approximately 4,500 times based on samples collected in 2017. The biomagnification factor of PBDEs from biofilm to invertebrates was approximately six, based on samples collected in 2021 from the three sites downstream of the WWTP outfall. Evidence of PBDEs bioaccumulating and biomagnifying in the Mashel River food web suggests species, such as salmonids and resident fish, that prey upon insect larvae in the system would also accumulate PBDEs, potentially at levels that may affect their health. Within Puget Sound, PBDEs have declined in recent years in species like English sole, Pacific herring, and harbor seals, likely due to PBDE-phase outs and state-wide bans. Whether PBDEs are declining in salmonids from the Mashel River is unknown. Steelhead trout migrating from the Nisqually River were sampled for PBDEs in 2014 and 2015 but not since then and were never collected directly from the Mashel River. Current levels of PBDEs are not known for Mashel River steelhead trout or other salmonids. Additional sampling of steelhead trout or other salmonids, like stream-type Chinook salmon, from the Mashel River would allow us a better understanding of the PBDEs in the food web and whether they are at levels known to impact fish health.

Introduction

Steelhead trout (*Oncorhynchus mykiss;* anadromous form) are ecologically, economically, and culturally important in the Pacific Northwest. They are an important prey item for Puget Sound's southern resident orca (*Orcinus orca*) or Southern Resident kill whale (SRKW) population and other marine mammals. Steelhead populations also support recreational and commercial fisheries, which bring economic benefits to the Puget Sound region. Furthermore, steelhead have an important cultural significance to Native Americans in Washington.

Steelhead trout like other salmonids rear in freshwater streams and require passage to marine waters. These freshwater habitats have faced pressure from urbanization, landscape development, and hydrologically altered rivers due to population growth in the Puget Sound region. The degradation of habitats from urbanization have long been known to affect salmonid species and efforts have been made to restore habitats of importance to salmonids. Habitat degradation goes beyond physical disturbance and is impacted by toxic contaminants from urban, residential, and agricultural landscapes which can degrade water quality. This toxic contamination can impact the freshwater food web steelhead rely on.

Steelhead populations across the Puget Sound region are less than 10% of their historical abundance leading to their listing as threatened under the US Endangered Species Act (ESA; Gayeski et al. 2011, Chen et al. 2018, Salish Sea Marine Survival Project 2018). Declines in Puget Sound steelhead populations are at odds with the statewide trend of population growth (Scott and Gill 2008). One cause of declining regional populations is the degradation and development of historical habitat used by steelhead, resulting in an estimated 9-30% reduction in habitat accessible to steelhead (Scott and Gill 2008). For example, the La Grande Dam may hydraulically limit the upstream travel of steelhead in the Nisqually River Basin (Figure 1), though there has been debate whether the canyon was already impassable. Furthermore, steelhead habitats are threatened by toxic contaminants from stormwater and wastewater (Chen et al. 2018). While documented studies of toxic contaminant exposure in steelhead are limited, other salmonid species are known to be exposed to environmentally relevant concentrations of toxics during their migration to marine waters (O'Neill et al. 2020b, Meador et al. 2010, O'Neill et al. 2015, Sloan et al. 2010).

A 2014 survey conducted by Washington Department of Fish and Wildlife (WDFW) of steelhead trout in the Skagit, Green/Duwamish, and Nisqually rivers identified polybrominated diphenyl ethers (PBDEs) at the highest concentration in Nisqually River steelhead (Chen et al. 2018). PBDE concentrations in Nisqually River steelhead exceeded critical body residues (CBRs) for increased disease susceptibility throughout the river system. PBDE contamination in steelhead was pervasive throughout the river system with 33-50% of fish sampled exceeding CBRs across the three sampling locations. In 2015, follow up sampling from the in-river smolt trap, identified approximately 33% of steelhead trout smolts had elevated levels of PBDEs (WDFW unpublished data). These findings were surprising due to the limited urbanized development within the Nisqually watershed. Furthermore, a previous study of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) collected in the Nisqually River estuary did not document PBDEs at concentrations above CBRs for increased disease susceptibility (O'Neill et al. 2015).
To identify potential sources of PBDEs in the Nisqually River system a 2017 study was undertaken by WDFW and Ecology, which concluded PBDEs were entering the river system through the three major tributaries, the Mashel River, Muck Creek, and Ohop Creek (O'Neill et al. 2020a). Results of the survey found the Mashel River had elevated levels of PBDEs, congener patterns that differed from other river samples, and an enriched signal of the nitrogen stable isotope, $\delta^{15}N$. The town of Eatonville's WWTP

outfall is located upstream of the 2017 study's upper Mashel River sampling site, suggesting this as the source of PBDEs entering the river (O'Neill et al. 2020a). Muck and Ohop creeks had distinct PBDE congener patterns from those found in the Mashel River, with higher concentrations of heavier PBDEs (Octa-BDEs, and Nona-BDEs). These sites also did not show an enriched signal of nitrogen stable isotope, $\delta^{15}N$, suggesting the source of the PBDEs was not wastewater (O'Neill et al. 2020a). The spatial scale of the survey limited its ability to conclusively identify the major source of PBDE inputs to the river system therefore a follow up survey of the tributaries was necessary to conclusively determine PBDE sources in the watershed.

Determining the source of PBDEs entering the Nisqually River is necessary to establish corrective management actions which may increase steelhead survival. The purpose of this study was to further investigate potential sources of PBDEs in the three major tributaries of the Nisqually River (Mashel River, Muck Creek, and Ohop Creek) and the impacts on the food web. The WDFW TBiOS team, in collaboration with the Department of Ecology, collected co-located biofilm and invertebrate samples from fourteen sites spanning the three tributaries. The specific objectives were to:

- delineate the locations of PBDE inputs to the Mashel River
- investigate the presence of PBDEs in Ohop and Muck creeks
- measure and describe the uptake of PBDEs in aquatic insects that are prey for juvenile steelhead trout.

Biofilms and invertebrate samples were used to determine potential locations of PBDE inputs to the three tributaries. Additionally, invertebrate samples provide a measure of PBDEs' impact on the food web and provides insight on how PBDEs potentially accumulate in steelhead trout. Biofilms act as a natural passive sampler, due to their carbon content, and accumulate PBDEs over their growth period on the order of several months. Invertebrates are potential prey items to steelhead trout and provide an exposure vector as they accumulate PBDEs through feeding on biofilms. To determine the location of PBDE sources into the tributaries a synoptic survey of biofilms and invertebrates was performed. PBDE concentrations and congener patterns across the tributary systems were used to identify reaches with increased PBDE concentration indicating a potential input source. Stable isotopes of carbon and nitrogen were also analyzed in biofilm and invertebrate samples. These naturally occurring stable isotopes serve as chemical tracers of trophic status and diet (Caut et al., 2009; Olson et al., 2010; Ramos et al., 2011), as well as nutrient inputs from wastewater (Cabana and Rasmussen, 1996; Loomer et al., 2015; Schlacher et al., 2005). Regionally, the enrichment of nitrogen stable isotopes beyond the background river level were used to indicate the incorporation of nitrogen derived from wastewater inputs to juvenile salmon in the Snohomish River (O'Neill et al. 2020b). Stable isotopes also provide complementary information to the analysis of PBDEs, allowing the determination of source type (i.e., wastewater, legacy dump, etc.) of PBDEs to the river system.

Methods

Species of concern

Steelhead trout are the anadromous, or sea-going, form of freshwater rainbow trout. In general, steelhead have a complex life history, are iteroparous and spawn in the spring, unlike Pacific salmon that are semelparous and mostly spawn in the fall.

After spending as many as four years at sea, adult winter steelhead enter the Nisqually River between December and May, where they wait in pools near their spawning habitat until they spawn from April

until June. Peak fry emergence occurs during mid-July. Juveniles actively rear in the rivers and creeks of the Nisqually River and its tributaries for one to three years (Nisqually Steelhead Recovery Team 2014) however, the exact rearing locations are unknown. Typically, smolts migrate to saltwater between mid-April to mid-June (Nisqually Steelhead Recovery Team 2014).

Study Area

The Nisqually River is located in west-central Washington running from its headwaters along the southern slopes of Mount Rainier to Puget Sound (Figure 1). The river is 78 miles long (Nisqually River Council 2021) and drains about 720 square miles (Nisqually Indian Tribe 2007) within Lewis, Pierce, and Thurston counties. The watershed (WRIA 11) is unique in the Puget Sound region as it has remained relatively intact and healthy, while being in close proximity to the fast-growing urban areas of Tacoma and Olympia. This is due in part to the significant portion of the watershed protected by the Nisqually Indian Reservation, Joint Base Lewis-McCord (JBLM), Mt. Rainier National Park, and Nisqually National Wildlife Refuge. There are several towns and unincorporated areas along the river's reach, including Yelm, Roy, Eatonville, Elbe, and Ashford.

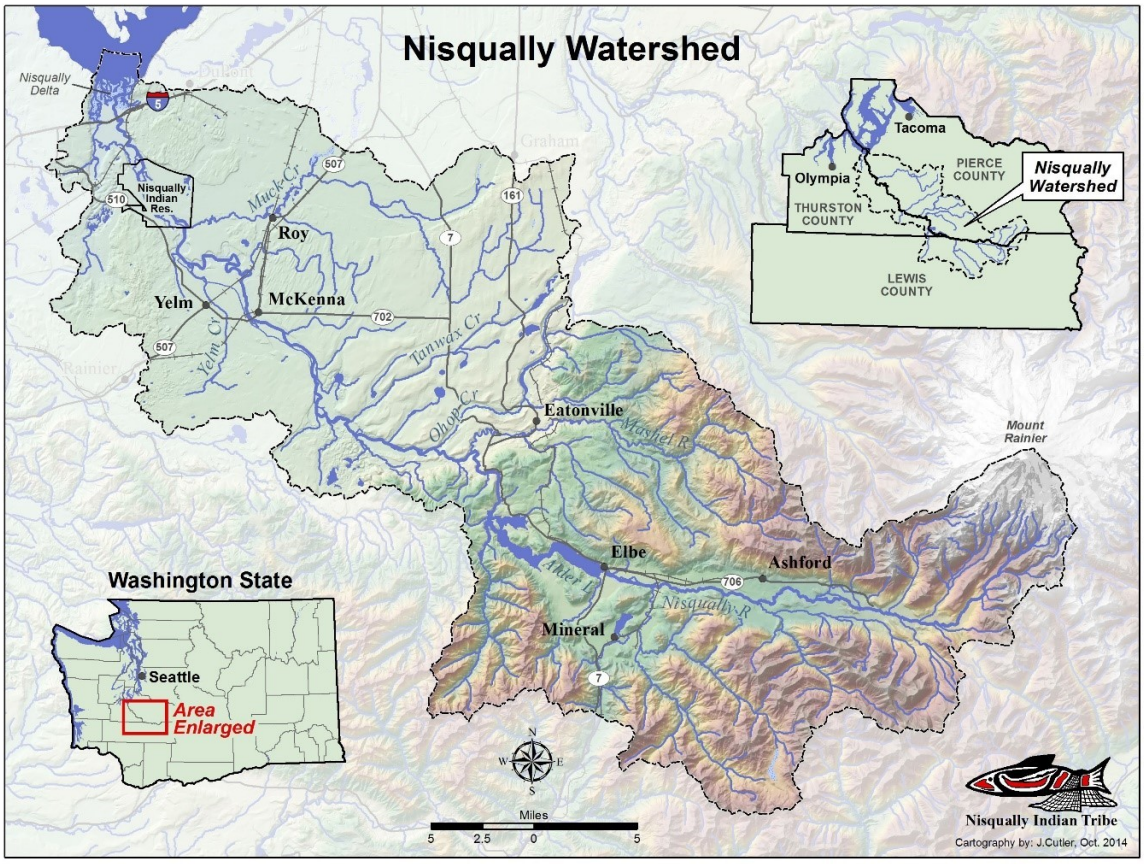

Figure 1. Map of the Nisqually River watershed . Courtesy of the Nisqually Indian Tribe.

The hydrology of the Nisqually river is both rainfall and snowmelt dominated, with peak levels associated with winter storms occurring November through April, followed by slightly lower flows in the spring associated with snow melt (Figure 2). The river travels from its headwaters at the Nisqually Glacier on Mount Rainier through montane forested regions which transition to prairie landscapes

within the Cascade Lowlands Puget Sound Ecoregion (Hobbs et al. 2019). The river is hydraulically controlled by two dams along its course, Alder Dam and La Grande Dam, which provide hydroelectricity to the region. A number of tributaries within the lowland reaches contribute to the river flows. The river enters Puget Sound approximately 8 miles northeast of Olympia, WA. Significant restoration work has been undertaken to renew the Nisqually estuary over the last three decades, restoring tidal flow to 762 acres of former farmland (Nisqually Steelhead Recovery Team 2014).

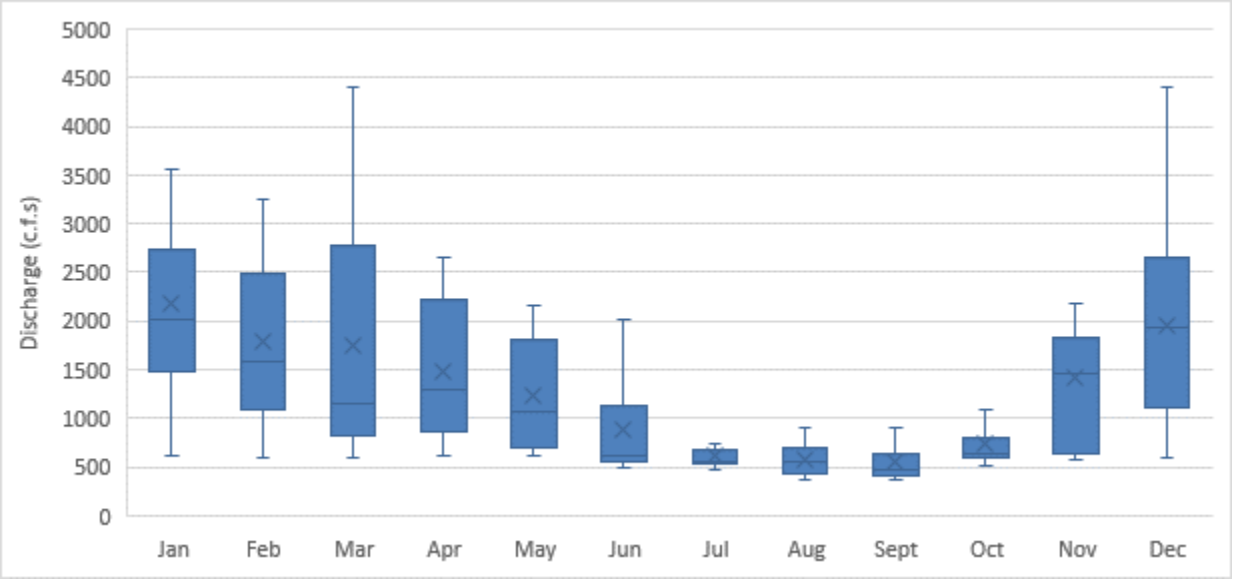

Figure 2. Nisqually River discharge (cubic feet per second; cfs) from 2000-2019 recorded at the USGS flow station (gage #12089500) in the Nisqually River near McKenna, WA (USGS 2022). X = mean monthly discharge

The Mashel River is the second largest Nisqually tributary by watershed area, draining an area of over 80 square miles, and entering the Nisqually River approximately 3 miles downriver of the La Grande Dam (Pierce County 2014; Nisqually Steelhead Recovery Team 2014). The river primarily flows through forested land (Nisqually Steelhead Recovery Team 2014). The city of Eatonville is located along the river, and discharges treated wastewater from the Eatonville WWTP into the river.

Ohop Creek is the third largest Nisqually River tributary with a total drainage area of 40 square miles (Pierce County 2014; Nisqually Steelhead Recovery Team 2014). Running through the Ohop valley the drainage's historical land use was agricultural but recently has moved to more residential use (Nisqually Steelhead Recovery Team 2014).

Muck Creek is the largest Nisqually tributary by watershed area, draining approximately 93 square miles (Pierce County 2005). Primarily, the creek flows across undeveloped land such as natural prairies and second growth forests, in addition to agricultural and low-density residential areas while the lower portion of the creek runs through the southeastern portion of JBLM lands (Pierce County 2005, Nisqually Steelhead Recovery Team 2014).

The flow of the three main tributaries into the Nisqually are predominantly rain driven but also impacted by snow melt in the spring. Consistent with the long-term mainstem flow pattern (Figure 2), stream discharges for the Mashel River and Muck Creek from January to December 2021 recorded peak flow levels from winter storms during the period of November through April (Figure 2). Flow levels then declined during the spring melt off period before leveling off during the summer low flow period.

Potential PBDE Sources

WDFW and Nisqually tribal staff have identified several potential PBDE sources in the Nisqually watershed. Specific PBDE sources of potential impact to the three major tributaries of the Nisqually river are outlined in Table 1. These sources include a WWTP outfall, a major municipal stormwater outfall, and surface stormwater runoff from a former (i.e., legacy) dump used by Weyerhaeuser, the Fort Lewis facility/lands, the University of Washington's (UW) Charles L. Pack Experimental Forest research facility/lands (UW Pack Forest) and a biosolids application along the Little Mashel River (Hodgson pers. comm.). Additionally, diffuse sources of PBDEs could enter the river system through atmospheric deposition and runoff from the landscape. The results from the 2017 PBDE source assessment (O'Neill et al. 2020a) suggested the Eatonville WWTP was a major source of PBDEs, however, the spatial coverage within the Mashel River was too limited to conclude whether it was the primary source within the Nisqually watershed.

Table 1. Table of potential PBDE sources in the three Nisqually River tributaries, the Mashel River, Ohop Creek and Muck Creek.

River or Tributary Receiving Water	Potential PBDE source	Location of Potential Inputs
Muck Creek	Fort Lewis facilities/lands	associated watershed flows to Muck Creek
Ohop Creek	Eatonville stormwater outfall	discharges to a tributary of Ohop Creek
Mashel River	City of Eatonville WWTP outfall	discharges to Mashel River, just below Eatonville
Mashel River	Biosolids application	Little Mashel River
Mashel River	Legacy dump (used by Weyerhaeuser)	associated watershed flows to Mashel River
Mashel River	UW Pack Forest - occasional application of sewage sludge as an experimental fertilizer	associated watershed flows to Mashel River
All Tributaries	Atmospheric deposition to watershed	flows to all tributaries

Wastewater Treatment Discharge

Daily discharge rates of treated wastewater from the Eatonville Wastewater Plant (a potential PBDE source) vary throughout the year from 0.15 to 0.96 MGD (Washington Department of Ecology PARIS database 2022). Peak discharges occur concurrent to peak river flows in the Mashel River, its receiving body (Figure 4). This is potentially due to stormwater influence on the wastewater conveyance system. Wastewater discharges during the summer low flow period were on average 0.2 MGD. These discharges were within permitted limits and met the criteria for acute and chronic dilutions within the mixing zone at RM 5.1 of the Mashel River. WWTP discharges did not exceed permitted limits during the sampling period. PBDEs are not a permitted or regulated group of compounds for Washington State facilities.

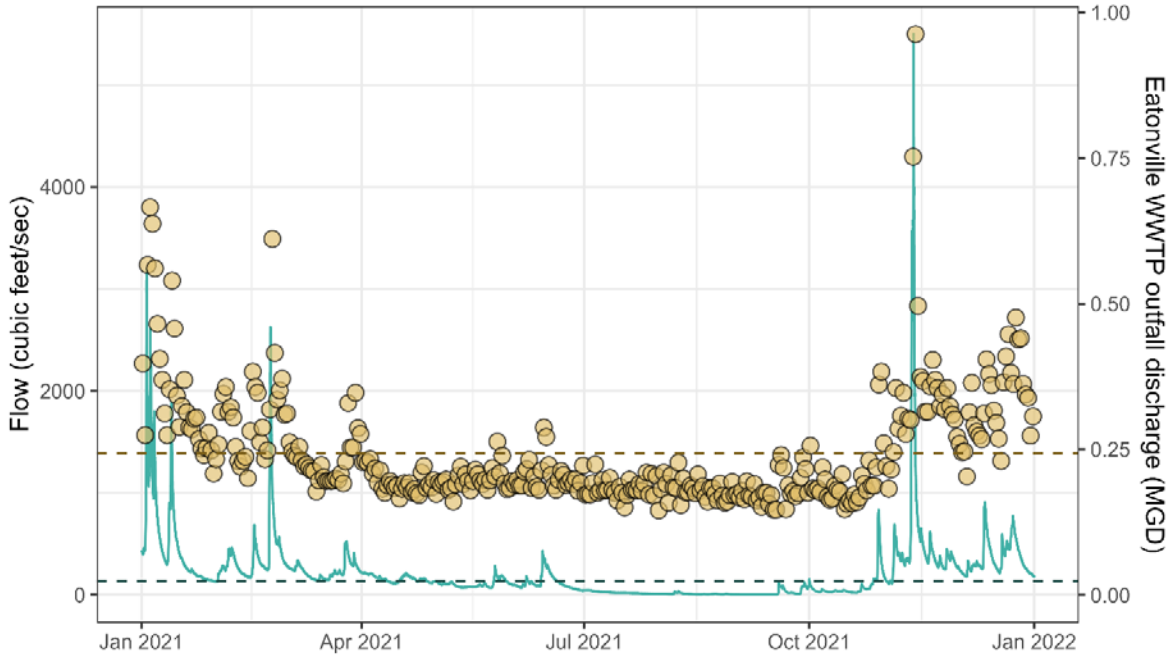

Figure 3. Mashel River flow compared to the Eatonville WWTP discharge. The 2021 stream flow (cubic feet per second) data collected from an USGS stream gage (U.S. Geological Survey 2021) in the Mashel River (gage # 12087000) is displayed on the left y-axis (teal line). While the 2021 daily discharge rates (millions of gallons per day; MGD) from the Eatonville wastewater treatment plant (WWTP) outfall located at approximately river mile 5.1 in the Mashel River is displayed on the right y-axis (tan circles; Washington Department of Ecology PARIS database 2022). The dashed horizontal line represents the mean 2021 river flow (teal) and the mean discharge rate from the WWTP outfall (tan) for 2021.

The summer low flow period (July to August) was selected for sampling with the objective to measure PBDEs at their potential peak concentration in the tributaries. The combination of low river flow and steady discharge volumes from potential PBDE sources (i.e., wastewater treatment plants) may cause the highest in-river PBDE concentration. Additionally, sampling during late summer allows for the collection of biofilm mats which accumulate PBDEs over a longer growth period, providing the integration of PBDEs which spans the low flow period. The 2021 sampling period (August 30th to September 1st; Figure 4) captures similar river flow conditions to those sampled in the 2017 Nisqually River PBDE study (O'Neill et al. 2020a).

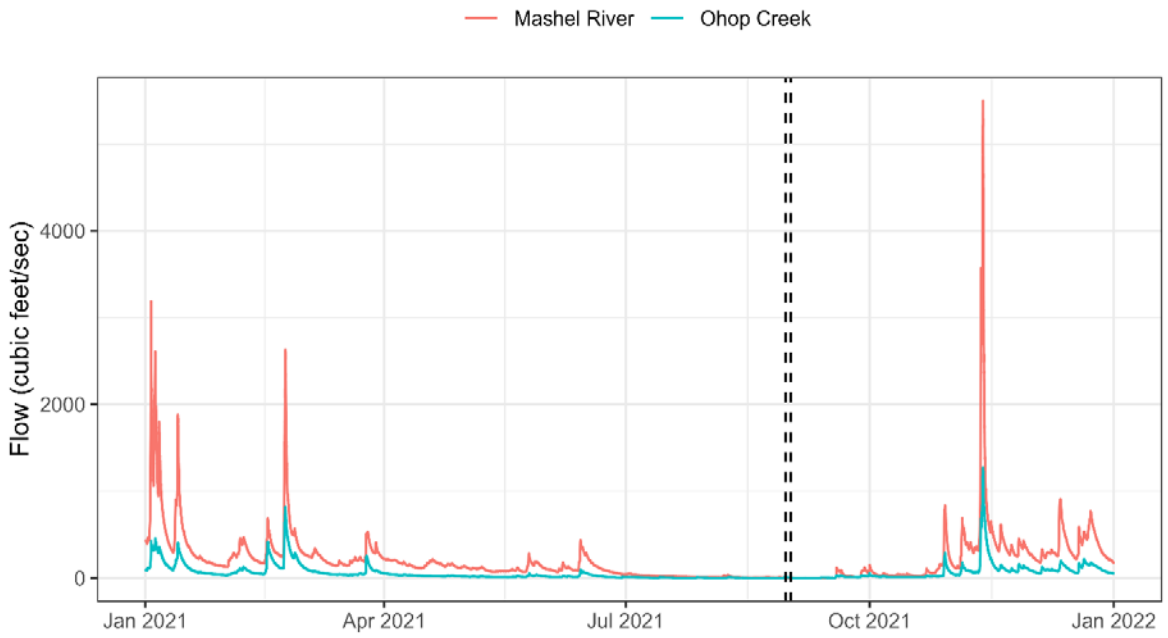

Figure 4. Stream flow (cubic feet per second) data collected from two USGS stream gages (U.S. Geological Survey 2021) in Ohop Creek (gage #12088000) and the Mashel River (gage # 12087000). The area between the two dotted lines signifies the sample collection period in both tributaries during the driest period of the year. Flow data from U.S. Geological Survey 2022.

Sample Collection

As detailed below, the concentrations of PBDEs and stable isotopes were assessed in biofilms and invertebrates from 14 sites along the Mashel River, Muck Creek and Ohop Creek. Table 2 provides site information including location, type of samples collected, and analyses performed. Site locations within the Mashel River, Muck Creek and Ohop Creek are shown in Figure 5 and Figure 6. Biofilm and invertebrate samples were co-collected at thirteen sites. Samples were collected during the period of August 30th to September 1st (Figure 4).

Table 2. Location and number (n) of biofilm and invertebrate samples collected from each tributary (trib.) for PBDE and stable isotope (SI) analyses and invertebrate taxonomy (Tax.). Site numbers correspond to collection locations in Figure 5 and Figure 6. Samples collected for PBDE analyses were analyzed at SGS Axys Laboratories in Sidney, British Columbia, Canada. Samples collected for SI analysis were analyzed by the NOAA Northwest Fisheries Science Center and all invertebrate taxonomy samples were assessed by an entomologist at the Department of Ecology. RM = river mile, NC = not collected

Trib.	RM	Site Num	Site Name	Latitude	Longitude	Collection Date	Biofilm Samples		Invertebrate Samples		
							n PBDEs	n SIs	n PBDEs	n SIs	n Tax.
Mashel River	6.5	MR01	Boxcar Canyon	46.867523	-122.244504	8/30/2021	NC	NC	1	1	1
	6	MR02	Bridge on Alder cutoff Rd	46.863639	-122.252449	8/30/2021	1	1	1	1	1
	5.4	MR03	Smallwood Park	46.860097	-122.263388	8/30/2021	1	1	1	1	1
	4.9	MR04[a]	Downstream Outfall	46.857911	-122.270903	8/30/2021	2[b]	2[b]	2[b]	2[b]	1
	4.6	MR05	439th St	46.856447	-122.275411	8/30/2021	1	1	1	1	1
	4.4	MR06	Above Little Mashel	46.857616	-122.280285	8/31/2021	1	1	1	1	1
	4.2	MR07	Below Little Mashel	46.857064	-122.282630	8/31/2021	1	1	1	1	1
	3.2	MR08	Hwy 7	46.856221	-122.303111	8/31/2021	1	1	1	1	1
	1.4	MR09	Downstream Dump	46.855044	-122.325158	9/1/2021	1	1	1	1	1
	0.35	MR10[a]	Lower Mashel	46.847322	-122.330669	9/1/2021	1	1	1	1	1
Ohop Creek	6	OC01	Below lake	46.880886	-122.279125	8/31/2021	1	1	1	1	1
	2.1	OC02	Peterson Rd	46.867840	-122.343253	8/31/2021	1	1	1	1	1
	0.1	OC03[a]	Lower Ohop	46.846017	-122.368850	8/30/2021	1	1	NC	NC	NC
Muck	0.25	MC01[a]	Lower Muck	46.996288	-122.627072	9/1/2021	1	1	1	1	1
						Total	14	14	14	14	13

[a]Sites sampled in 2017 study (O'Neill et al. 2020)

[b] Duplicate samples collected

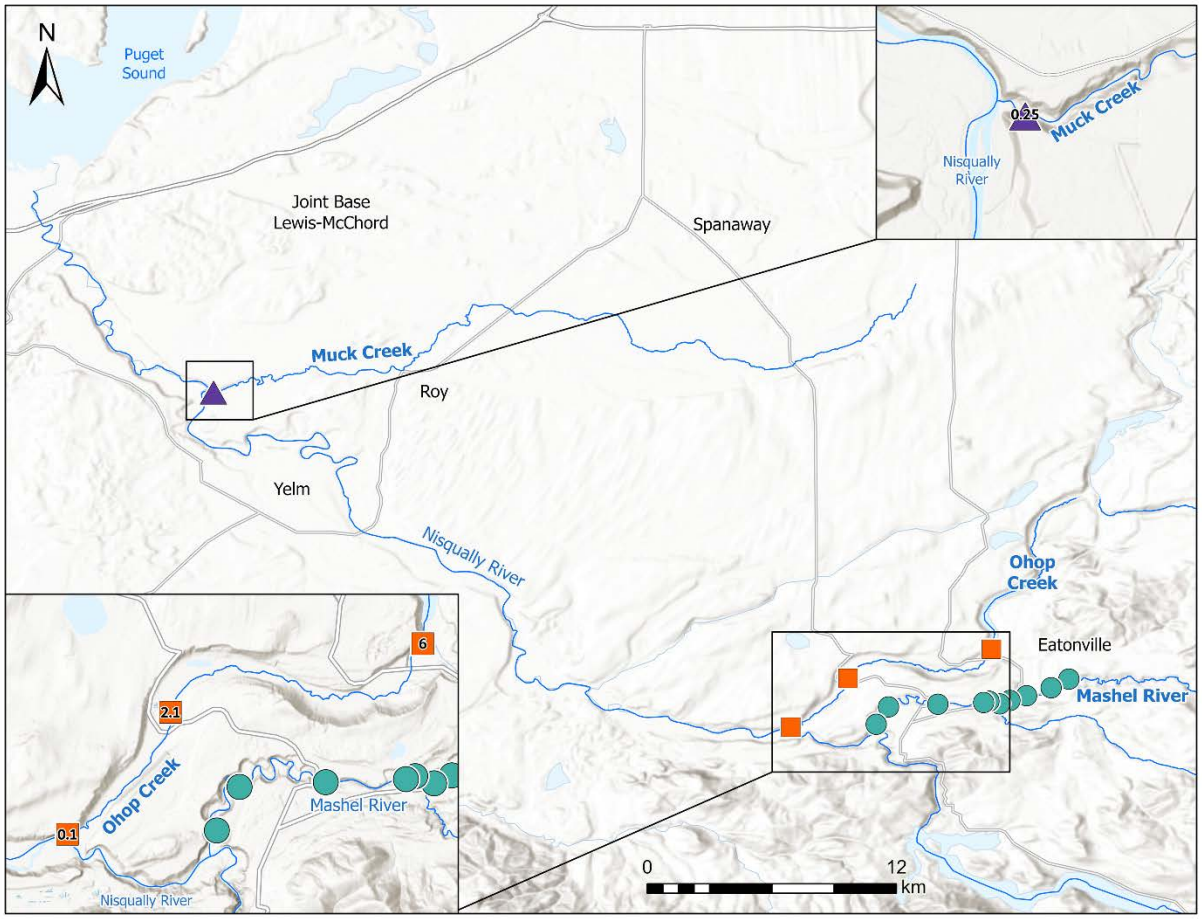

Figure 5. Map of the sampling sites in the Nisqually River watershed. Insets show collection site locations in Muck Creek (purple triangle) and Ohop Creek (orange squares). Collection sites for the Mashel River (blue circles) are shown in an expanded view in Figure 6. Collection sites are labeled with the approximate river mile. Site names, coordinates and sample collection information are detailed in Table 2.

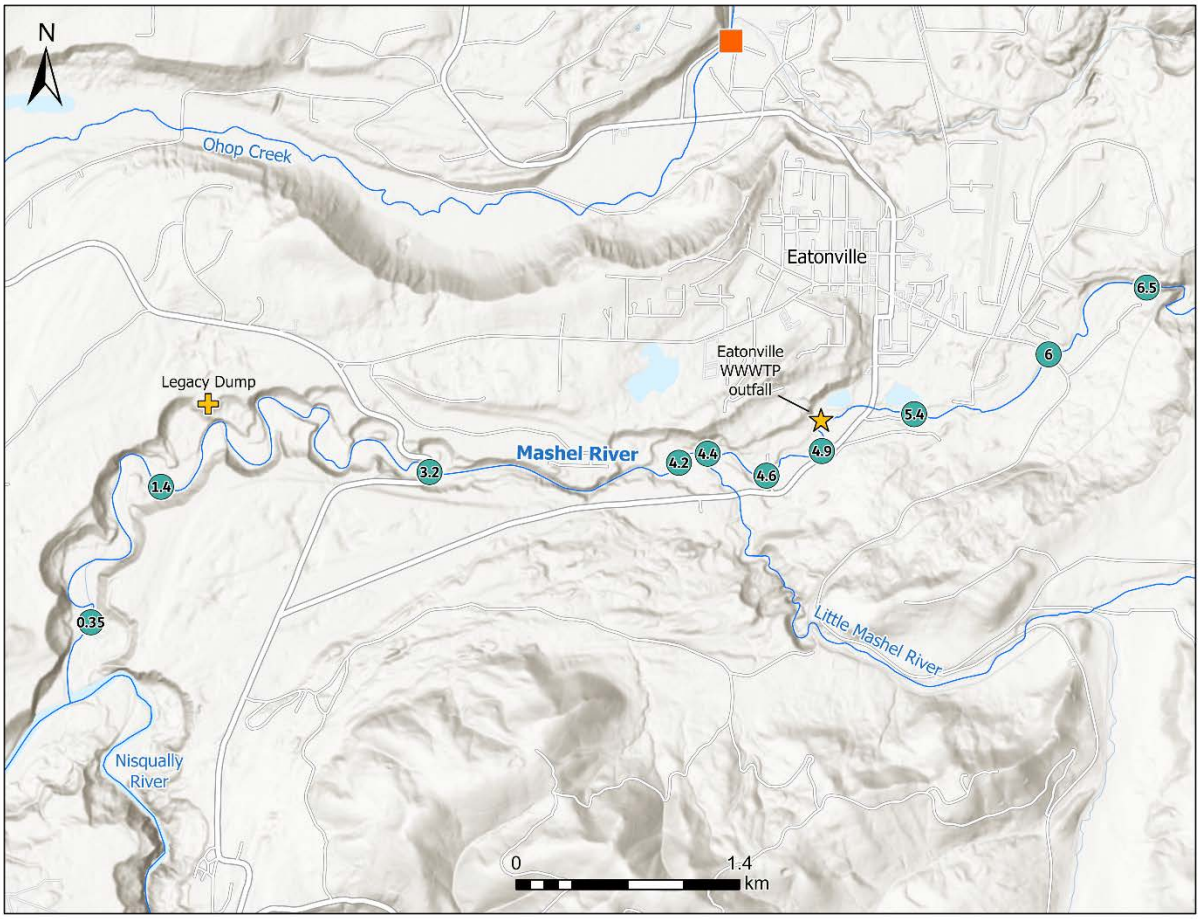

Figure 6. Map of collection locations for biofilm and invertebrate samples in the Mashel River (blue circles) as well as three possible PBDE sources to the river , the Eatonville wastewater treatment plant (WWTP) outfall (yellow star), the legacy dump (yellow cross) and the Little Mashel River. Collection sites are labeled with the approximate river mile. Site names, coordinates, and sample collection information are detailed in Table 2.

Biofilm Collection

Biofilms refer to a mixture of periphyton, microbial biomass, and fine sediments found on underwater rocks and debris. Periphyton is algae attached to the river bottom, rocks, or debris in the river. Fourteen biofilm samples were collected from thirteen sites, with a replicate sample collected at one site, MR04. Due to a lack of biofilms in the sampling area, a sample was not collected at MR01. Standard protocols for sampling biofilms were followed as outlined in Stevenson and Bahls (1999) and Larson and Collyard (2019). Briefly, biofilms were scraped from rocks using stainless steel blades (Figure 7) and collected in a stainless bowl for weighing in the field to confirm that sufficient biomass was retrieved (>10 g ww). Excess water was decanted from the sample before transferring to a labeled and certified cleaned glass jar. Biofilm samples were stored on ice immediately following collection and during transfer to the laboratory. Prior to stable isotope and PBDEs analysis, samples were stored at -20°C and -4°C, respectfully.

Figure 7. An example of (left) biofilm being scraped from a rock and (right) caddisfly larvae casings after removing larvae.

Invertebrate Collection

In order to measure PBDE concentrations and potential bioaccumulation of PBDEs in juvenile steelhead prey, aquatic invertebrates were collected at almost all sample locations. Fourteen invertebrate samples were collected from thirteen sites with a replicate sample collected at one site, MR04. Due to the absence of invertebrates at site OC03, no sample was collected. The same invertebrate casing was targeted at each sample location (Figure 7). Invertebrates were picked from the river substrate, removed from their casings, and combined until a minimum of 20 grams of tissue mass was collected. Invertebrates were transferred to a certified clean glass jar, transported on ice, and stored at -20°C before being processed for analysis. Before submission for analyses, each sample was homogenized using a Bamix hand blender to create a tissue composite of all individuals collected at each site. Reference samples for taxonomic identification were collected at each site and stored in ethanol. Taxonomic identification was performed by Stephanie Estrella, freshwater benthic taxonomist at the Washington State Department of Ecology.

We sampled larval aquatic insect from the benthic habitat in the Mashel River and Ohop and Muck creeks as a proxy for prey consumed by steelhead in streams. Salmonids and trout diet varies seasonally, depending on the availability of prey but is often selective towards larger sized prey. Larval stages of insects that drift downstream (i.e., drifting aquatic insect larvae) are the primary food of stream salmonids, although adult aquatic insects and terrestrial insects are taken at the surface when they are abundant, and other invertebrates are eaten as well (Quinn 2018). These larval aquatic insects are typically benthic, and crawl along the bottom feeding on algae scaped off rocks, leaves and other organic material which they shred, or prey they catch (Quinn 2018) but can passively or actively drift

downstream (Naman et al. 2016). Chironomid (Diptera), mayflies (Ephemeroptera), caddis flies (Trichoptera, especially Hydropsychidae) are primary aquatic insect consumed by juvenile steelhead or rainbow trout (Angradi and Griffith 1990, Bisson 1978; and Merz 2002) but their importance varies seasonally. For example, Li et al. (2016) noted that the diet of trout (cutthroat and steelhead) in coastal Oregon streams switched from 45% bottom or stream water column prey (primarily Ephemeroptera and Trichoptera) and 36% terrestrial (primarily Coleoptera and Diptera) in the spring, to 85% terrestrial insects in the summer (primarily Hymenoptera), returning to more mixed winter diets in the fall.

Wastewater Treatment Plant Influent/Effluent

On November 2, 2021, the influent and effluent of the Eatonville WWTP was collected for PBDE analysis. A three-part composite sample was collected over an 8-hour period. Sample aliquots were collected using a stainless-steel transfer jug and poured directly into 1L amber glass jars. Duplicate samples were collected of the effluent from within the treatment plant after the final UV disinfection stage of treatment, just prior to discharge to the Mashel River. The influent sample was collected from the main influent pipe prior to preliminary treatment and solids removal. A transfer blank was collected midway through the sample period to assess the possible introduction of PBDEs during the sampling process; ultra-clean laboratory-grade reagent water was poured into the stainless-steel transfer jug and then into a sample jar for analysis.

PBDE Analysis

Invertebrate, biofilm and water samples were analyzed for PBDEs at SGS AXYs Analytical laboratory in Sidney, BC, Canada. Samples were analyzed by high-resolution mass spectrometry using EPA Method 1614, AXYS method MLA-033, providing concentrations for 46 congeners at sub pg/g levels. Briefly, a sample aliquot is homogenized and spiked with twelve isotopically labeled analogs before being dried with anhydrous sodium sulfate in preparation for Soxhlet extraction with methylene chloride for 18 to 24 hours. Extracts are evaporated to dryness and the lipid content is determined. Extracts are then cleaned up before being analyzed by gas chromatography high resolution mass spectrometry (GC-HRMS). PBDE congeners are identified by comparing retention time and ion- abundance to authentic standards. Quantitation is performed through a multi-point calibration where concentration is determined by isotope dilution. Isotopic dilution relies on a series of isotopically labeled analogs which are spiked into the sample before extraction. Each of the twelve labelled analogs is used in the quantitation of one or more native PBDEs. Quantitation is performed during analysis based on the ratio of native and labeled PBDEs in a calibration solution and in the sample. This method of quantitation provides a measure of correction for the loss of target compounds' mass during extraction and analysis.

Stable Isotope Analysis

To assist with detecting changes in nutrient and wastewater inputs over the study area, biofilm and invertebrate samples were analyzed for bulk stable isotopes of nitrogen (N) and carbon (C) at NOAA's Northwest Fisheries Science Center, following methods detailed in Gates et al. (2020). Briefly, frozen biofilm and invertebrate samples were freeze-dried and ground to a fine powder using a micro ball-mill and then weighed into tin capsules. The capsules were combusted in a Thermo Fisher Scientific Flash 2000 Elemental Analyzer coupled with the Conflo IV interface and analyzed using a Delta V Advantage Isotope Ratio Mass Spectrometer. Values were calibrated against internal laboratory standards (aspartic acid and 15N-enriched histidine), which were analyzed after every 10 field samples. Quality assurance measures for stable isotope ratios included the analysis of both continuing calibration standards and a fish tissue, SRM 1946 (National Institute of Standards and Technology, Gaithersburg, MD, USA), with each batch of samples of 10 field samples (Sloan et al., 2019).

Stable isotopes of carbon and nitrogen were expressed in standard delta notation ($\delta^{13}C$, and $\delta^{15}N$),

$$\delta\ (‰) = 10^3\ [(R_{sample}/R_{standard}) - 1],$$

where R is the ratio of heavy and light isotopes in a sample (13C:12C and 15N:14N). We expressed stable isotope ratios in units of permil (‰ –parts per thousand) and are relative to international standards: Vienna PeeDee Belemnite (VPDB) for $\delta^{13}C$, and atmospheric nitrogen for $\delta^{15}N$.

Data Analysis

PBDEs

PBDE concentration data were provided by SGS SXYS in the form of an electronic data deliverable (EDD). In addition to PBDE results, the EDD contained detection limits, reporting limits, result qualifiers, and labelled analog recoveries for each environmental sample. The EDD was screened for completeness before data analysis took place. Laboratory qualifiers were than consolidated to "U" "UJ" or "J" or non-qualified based on the definition of each provided in the section 11.2 of the project QAPP. Result concentrations less than the method detection limit (qualified "U") were censored and reported as non-detected. Results greater than the method detection limit but less than the reporting limit (qualified "UJ") were excluded from the analysis and reported as non-detected. Results which were positively identified but the associated numerical value represented an approximate concentration (qualified "J") were included in the analysis. Qualified and non-qualified results included in the analysis were censored against laboratory method blanks specific to the sample batch and media. Samples were censored and considered not detected at concentrations less than five times the laboratory blank concentration for each congener. Individual results for 46 congeners analyzed in the method were reported in this study. Total PBDE concentrations were calculated for each sample based on the congeners' summed concentration.

For the assessment of congener patterns, the detected congeners for each sample were summed by PBDE homolog group (i.e., di-BDE, tri-BDE, tetra-BDE, penta-BDE, hexa-BDE, hepta-BDE, octa-BDE, nona-BDE, and deca-BDE) and the proportion of the nine summed homolog groups were calculated using the total PBDE concentration. Stacked bar plots of the homolog proportions for each sample were created in R and then visually inspected.

Stable Isotopes

Stable isotopes of nitrogen and carbon were visually inspected to determine if the concentrations varied among tributaries and among sites within tributaries. The concentrations of PBDEs in biofilm and inverts were compared to isotopes of nitrogen and carbon in the same samples to see if they co-varied. Linear regression was used to test for significant relationships between $\delta^{15}N$ and $\sum_{46}$PBDEs, two independent metrics that can both be affected by sources such as wastewater.

Quality Assurance/Quality Control (QA/QC)

Data QA/QC for PBDE analyses for this project included measures of precision, bias, and sensitivity. Duplicate field and lab samples provided a measure of analysis precision. An assessment of laboratory PBDE recoveries provided a measure of bias. Instrument detection limits and laboratory blanks for PBDEs were assessed to provide a measure of the analyses' sensitivity.

Data QA/QC for stable isotopes also included measures of precision, bias, and sensitivity. Laboratory calibration standards were normalized against known primary standards (IAEA CH-7, USGS 40, and USGS 41a) before use. Replicate analyses of an internal reference material (IRM; NIST SRM 1946 which has been defatted, freeze dried, and homogenized) with each set provided a measure of analytical precision

and bias for stable isotopes. Data were rejected if the precision and bias fell outside of laboratory criteria and the sample set was rerun after addressing any problems. Calibration standards were analyzed throughout the instrument run at specified intervals. Data were rejected if calibration standard precision fell outside of laboratory criteria, assuring stability of measurements over the entire set. Data were rejected for samples where peak amplitudes fell outside of a given range, assuring that the carbon and nitrogen signals fell within the linear range of the detector. Several method blanks were run at the beginning of each set and peak amplitudes and were required to fall below a maximum value to ensure lack of system contamination.

PBDEs Analyses

Total PBDE concentrations for invertebrate field duplicates from MR04 were within 20% relative percent difference (RPD), well within the QC target for precision (± 50% RPD). The biofilm field duplicate from the same station had a RPD of 128% for total PBDEs. This difference in duplicate biofilm sample concentrations can be explained by a slightly different composition of algae species between the field replicates that were collected independently at MR04. Individual PBDE congener RPDs ranged from 3% to 149% for the invertebrate duplicates and 63% to 145% for the biofilm duplicates. The large range of RPDs is in part due to low precision at concentrations near the detection limit. A field duplicate of the effluent sample from the Eatonville WWTP met all the project QC limits with an overall RPD of 8% and congener RPDs ranging from 3% to 50% with a median of 9%.

Laboratory recoveries of labelled analog spikes for invertebrate samples were all within the methods QC limits, as detailed in the QAPP (20 -200% for 209L and 25–150% for other labelled analogs), except for BDE-209L, which had low recoveries (<20%) for six samples (Table 3). It should be noted that the recoveries met the QC limits of the analytical laboratory (10 – 400%). The low recoveries for BDE-209L may bias concentrations higher for nona- and deca-BDE congeners (BDE- 203, -206, -207, 208, 209) due to the isotope dilution calculations as BDE-209L is used to calibrate all of these congeners. These congeners account for 0.1% to 16% of total PBDE concentrations in invertebrates with all but one sample accounting for less than 5%. Due to the low fraction of nona- and deca-BDEs in invertebrate samples, BDE-209L recoveries likely introduce limited bias on total PBDE concentrations. Labelled analog spike recoveries for biofilms were within QC limits for all samples. Surrogate recoveries of lab blanks and matrix spikes were within the QC acceptance criteria for all but BDE-209L in the invertebrate analysis. Labelled analog spikes were generally well within the QC limits for the whole water samples collected from the WWTP. The sole exception was the labelled BDE-209 compound in the influent sample, which had a low recovery of 17%.

Native PBDE laboratory recoveries for matrix spike samples ranged from 92% to 105 % and 92% to 104% for biofilm and invertebrate analyses, respectfully. Recoveries for the whole water WWTP samples ranged from 97% to 104%. These recoveries were within method QC limits and indicate accurate measurements of PBDEs in both biofilms and invertebrate matrixes.

Detection limits for PBDE analysis in all media met the sensitivity necessary for the project as outlined in the QAPP. Laboratory blanks were assessed to provide a measure of background contamination inherent to the sample extraction and analysis process. Samples were censored based on these results as described in the data analysis section of this report. The percentage of congeners censored by the laboratory blanks ranged from 2% to 9% for invertebrate sample, 0% to 4% for biofilms and 0%-5% for the whole water WWTP samples.

*Table 3. Labelled analog recoveries and congener blank censoring for PBDE analysis. The EPA Method 1614 recovery range for all labelled analogs excluding BDE-209L is 25-150%. BDE-209L recovery range is 20-200%. Percent of congeners blank censored for each sample is based on 5x method blank concentration. * Range excludes 209L*

		Invertebrates			Biofilms		
		Labelled Analog Recovery		Blank	Labelled Analog Recovery		Blank
Site Name	River Mile	Range*	209L	Censored	Range*	209L	Censored
MR01	6.5	35% - 94%	15%	7%	NA	NA	NA
MR02	6	34% - 97%	13%	7%	30% - 69%	29%	2%
MR03	5.4	35% - 103%	13%	4%	39% - 87%	38%	0%
MR04	4.9	39% - 109%	14%	7%	33% - 78%	50%	2%
MR04	4.9	37% - 104%	13%	9%	36% - 74%	33%	0%
MR05	4.6	34% - 104%	13%	7%	26% - 60%	27%	2%
MR06	4.4	43% - 109%	34%	2%	26% - 67%	25%	0%
MR07	4.2	43% - 113%	37%	7%	31% - 79%	30%	0%
MR08	3.2	36% - 97%	40%	7%	37% - 89%	35%	2%
MR09	1.4	40% - 100%	39%	7%	39% - 92%	39%	0%
MR10	0.35	40% - 101%	36%	7%	30% - 69%	25%	4%
OC01	6	39% - 102%	37%	7%	35% - 78%	51%	2%
OC02	2.1	38% - 102%	35%	7%	28% - 65%	27%	4%
OC03	0.1	NA	NA	NA	29% - 74%	35%	0%
MC1	0.25	35% - 84%	34%	7%	27% - 68%	27%	2%
Lab Blank		40% - 96%	14%		35% - 66%	27%	
Matrix Spike		33% - 69%	15%		45% - 66%	47%	

Stable Isotopes

Isotopes of nitrogen and carbon measured for invertebrate field duplicates from MR04 were 2.9% and 6.9% RPD, respectively, well within QC target for precision (± 20% RPD) outlined in the QAPP for this project. Likewise, isotopes of nitrogen and carbon measured for biofilm field duplicates from MR04 were 0.11% and 14.7% RPD, respectively. The more enriched nitrogen signal in the biofilm duplicate at MRO4 is consistent with the observation that the algae species differed among duplicates. During laboratory analytical runs, three samples were run in triplicate, which provided precisions (1 sigma) of ± 0.05‰ for carbon and ± 0.1‰ nitrogen.

The QC results indicated the stable isotopes values were not biased as the control limits were met for IRM values (i.e., the upper and lower control limits of the reference values are within ± 0.3 per mil (‰) and ± 0.2‰ of the reference value for $\delta^{15}N$ and $\delta^{13}C$, respectively). Currently, there is not tissue Standard Reference Material (SRM) for rations of stable isotopes of carbon and nitrogen. Instead, the NWFSC Laboratory uses the National Institute of Standards and Technology (NIST) SRM 1946 (fish muscle tissue) as IRM, for which the assigned reference values for $\delta^{15}N$, $\delta^{13}C$ for SRM 1946 are the mean of repeated in-house analyses of this IRM for stable isotopes of carbon and nitrogen, a minimum of three samples of per batch. Additionally, the standard deviation of isotopic values in the replicate analyses of each standard continuing calibrations standards (CCR) met the QC laboratory requirement of ≤0.25 (‰) for $\delta^{15}N$ and ≤0.35‰ for $\delta^{13}C$. The $\delta^{13}C$ and $\delta^{15}N$ values can be affected if the mass

spectrometer responses for the CO2 and N2 peaks are too small or too large. For the biofilm and invertebrate samples, all samples met the required peak amplitudes for N2 and CO2 (between 500 and 12,000 millivolt (mV)). For the biofilm sample from MR03, the peak amplitudes are near their limits for N2, so the accuracy of the results should be interpreted with caution, however the results are similar to other background samples in the Mashel, suggesting there is minimal bias.

Stable isotopes analysis in all media met the sensitivity necessary for the project. Three laboratory blanks (i.e., tin cups with no added sample analyzed in in the same manner as the field samples) were assessed at the beginning of every batch to provide a measure of background contamination inherent to the sample analysis process. For all method blanks, the N2 mass 28 and CO2 mass 44 peak amplitudes for all the method blanks were <50 mV, the QC requirement for this method (Sloan et al. 2019), indicating there was no blank contamination that would affect the sensitivity of the analyses.

Results/Discussion

PBDEs Concentrations in Biofilm and Invertebrates

Concentrations of PBDEs measured in biofilm and invertebrate samples collected in 2021 from three major tributaries of the Nisqually River varied widely among tributaries and sampling locations. Higher concentrations were only observed in the Mashel River, suggesting a PBDE source within that tributary compared to Ohop and Muck creeks (Figure 8, Table 4). For example, the highest TPBDEs concentration measured in biofilm collected from RM 4.9 in the Mashel River (1,850 pg/g ww; Table 4) was over five times higher than the average TPBDEs in biofilm from the background sites in the Mashel River, upstream of the WWTP outfall (277 pg/g) and all sites in Ohop and Muck creeks (350 pg/g ww). Most notably, invertebrates collected from the Mashel River (RM 4.9-RM 3.2) had a mean TPBDE concentration (6,080 pg/g) that was ~7 times higher than invertebrates from the background locations (mean 907 pg/g) and ~13 times higher than tissue concentrations from Ohop and Muck creeks (mean 461 pg/g). These results indicate sources in the Mashel River are a major contributor of PBDEs to the Nisqually River watershed and are consistent with findings from the 2017 PBDE source assessment (O'Neill et al. 2020a).

The invertebrate tissue samples analyzed for PBDEs were of similar composition across all the sample sites and were composed of larval and pupal stages of the caddisfly, *Disosmoecus gilvipes*. *D gilvipes* is a common invertebrate in montane streams of western North America (Resh et al., 2011; Li et al., 1989; Wiggins and Richardson, 1982). All individuals sampled in this study appeared to be in the last instar stage (fifth) which is generally when they are transitioning from a larval to pupal stage; the casing is constructed of coarse sand and gravel. At this stage of life history *D. gilvipes* is likely close to a year old and therefore has a much longer period of exposure to PBDEs than the sampled biofilms do. The functional feeding guild of *D. gilvipes* is scraper-grazer, meaning the diet of the specimens we sampled was like our biofilm samples.

Spatially, TPBDEs in biofilm and invertebrates varied widely in the Mashel River, indicating a source of PBDEs in the river downstream of RM 5.4 (Figure 8). The three upstream sites at RM 6.5, RM 6, and RM

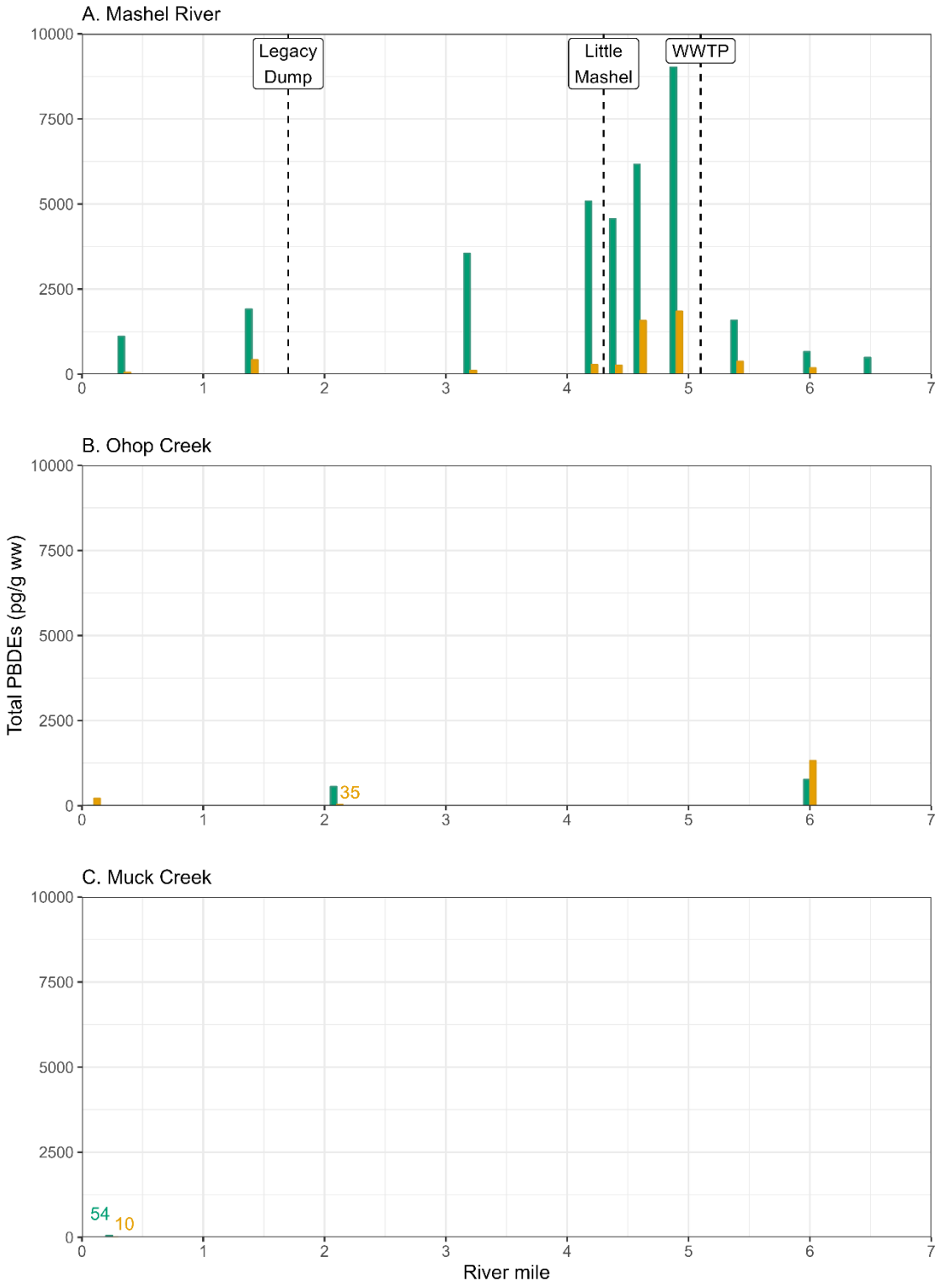

Figure 8. Total PBDEs (pg/g wet weight) measured in both invertebrates (green bars) and biofilm (orange bars) collected from three tributaries of the Nisqually River, A) Mashel River, B) Ohop Creek and C) Muck Creek. The approximate river mile of the collection site is on the x-axis with points of possible PBDE sources in the Mashel River (A) marked with a dashed line and labeled. The low TPBDE concentrations measured in biofilm and invertebrates from Ohop and Muck creeks are labeled on the figure in their respective colors. Note that the duplicate field samples were excluded.

Table 4. Concentrations of total PBDEs (pg/g wet weight), lipids (%), $\delta^{15}N$ (‰), and $\delta^{13}C$ (‰) measured in biofilm and invertebrates collected from the Mashel River, Ohop Creek and Muck Creek in August/September 2021. See Table 1 for site details.

Nisqually Tributary	River Mile	Site Number	Site Name	Lipids (%)		Total PBDEs (pg/g ww)		$\delta^{15}N$ (‰)		$\delta^{13}C$ (‰)	
				Biofilm	Inverts	Biofilm	Inverts	Biofilm	Inverts	Biofilm	Inverts
Mashel River	6.5	MR01	Boxcar	NC	1.2	NC	485	NC	1.19	NC	-17.89
	6	MR02	Bridge on Alder cutoff Rd	0.19	4.2	180	657	0.003	1.47	-22.97	-17.96
	5.4	MR03	Smallwood	0.081	3.6	374	1580	0.04	1.48	-22.21	-17.71
	4.9	MR04	Downstream Outfall	0.16	6.5	1850	9020	8.96	10.49	-14.85	-14.75
	4.9	MR04b	Downstream Outfall Rep	0.14	6.0	402[a]	8130	8.95	9.86	-17.19	-15.19
	4.6	MR05	439th St	0.21	5.0	1570	6160	8.5	8.97	-20.69	-19.75
	4.4	MR06	Above Little Mashel	0.15	5.6	259	4560	11.17	10.36	-13.98	-16.03
	4.2	MR07	Below Little Mashel	0.17	6.0	278	5080	10.75	9.46	-15.03	-15.68
	3.2	MR08	Hwy 7	0.16	5.2	108	3550	9.67	8.29	-13.21	-14.53
	1.4	MR09	Downstream Dump	0.16	4.4	418	1910	6.33	6.69	-17.07	-15.04
	0.35	MR10	Lower Mashel	0.096	2.9	49	1110	5.22	6.33	-18.71	-16.39
Ohop Creek	6	OC01	Below lake	0.16	6.2	1320	768	2.22	3.79	-23.9	-19.16
	2.1	OC02	Peterson Rd	0.13	5.4	35	561	5.56	7.05	-22.47	-23.36
	0.1	OC03	Lower Ohop	0.079	NC	214	NC	6.09	NC	-32.83	NC
Muck Crk	0.25	MC01	Lower Muck	0.19	2.6	10	54	3.96	6.24	-26.74	-27.81

[a] This biofilm field replicate sample was a slightly different species of algae than the other samples and so was excluded from any data analysis

5.4 represent the background concentrations for both biofilm and invertebrates as they are upstream from any known sources, and they had relatively low concentrations of TPBDEs (Table 4). Also, the concentrations measured in those five samples (no biofilm was collected at RM 6.5) are comparable to concentrations measured in the samples from Ohop and Muck creeks, further suggesting that samples in Ohop and Muck creeks also represent background concentrations. Most striking however, was the increase in TPBDE concentrations within a half mile stretch of the river from RM 5.4 to RM 4.9 where TPBDEs in biofilm increased five-fold from 374 pg/g ww to 1,850 pg/g ww and TPBDEs in invertebrates increased six-fold from 1,580 pg/g ww to 9,020 pg/g. This drastic increase in TPBDEs in both biofilm and invertebrates suggests there was a source of PBDEs to the river between RM 5.4 and RM 4.9. Indeed, the outfall for the Eatonville WWTP is located at approximately RM 5.1, which insinuates effluent discharged from the outfall as the source or pathway of PBDEs to the Mashel River.

TPBDE levels in biofilm were elevated over upstream background concentrations for approximately 0.5 miles downstream of the outfall, with biofilm samples located at both RM 4.9 and 4.6 outside the 95% CI of our upstream biofilm background concentrations (277 $\pm$ 66 pg/g ww). Slightly downstream from the outfall at RM 4.9 and 4.6 the biofilm TPBDE levels were 1,850 and 1,570 pg/g ww, respectively. From there, the TPBDEs in biofilm decreased and remained low, ranging from a high of 418 pg/g ww at RM 1.4 to a low of 49 pg/g ww at the most downstream site at RM 0.35. This decrease of TPBDEs in the biofilm suggests the PBDEs are likely being diluted in the river, possibly from groundwater inputs and the Little Mashel River, and are not as readily available as they are in the immediate vicinity downstream of the WWTP outfall. Alternatively, it is possible that PBDEs are only elevated in substrate in the immediate vicinity (i.e., 0.5 miles) of the WWTP outfall. In contrast to biofilm samples, the PBDE concentrations in invertebrate were elevated relative to background upstream stations for at least 3.7 miles, with all invertebrate samples located between RM 4.9 and 1.4 outside the 95% CI of our upstream background samples (908 $\pm$ 230 pg/g ww). Near the mouth of the river, at RM 0.35, the PBDE concentration had declined to 1,110 pg/g ww, just within the 95% CI of the upstream background samples.

The zone for PBDE enrichment in the Mashel River (i.e., the measured distance over which PBDEs concentrations were elevated relative to background) was far greater for invertebrates than biofilm samples, possibly because the invertebrates are more mobile and the growth period is longer. As noted above, PBDE concentrations in biofilm were only elevated 0.5 miles downstream of the WWTP, whereas PBDEs in invertebrates were elevated for at least 3.7 miles – almost eight times the distance, with all invertebrate samples located between RM 4.9 and 1.4 outside of the 95% CI of the upstream samples. These data suggest a broader area of influence from PBDE inputs extending through much of the lower Mashel River and potentially exposing juvenile steelhead (and other species) inhabiting this reach of the river.

In addition to accumulating PBDEs, the invertebrates also showed evidence of biomagnification, with mean TPBDE concentrations (2,897 pg/g ww, ± 3,014 SD) over six times higher than mean TPBDEs in biofilm (476 pg/g ww, ± 620 SD) collected from the same sites in the three tributaries. However, the degree of biomagnification was exceptionally variable among sampling sites. TPBDEs measured in the invertebrates at each site was four to 33 times higher than TPBDEs measured in the biofilm from the same site, with one exception. A biofilm sample from RM 6 (OC01) in Ohop Creek had a much higher level of PBDEs than the invertebrates from the same location (Figure 8). Moreover, the sample had an unusually high proportion (83%) of the congener BDE-209 compared to the total summed PBDEs. Similarly, in the 2017 study, high PBDE concentrations measured in biofilm from Muck and Ohop creeks were mostly driven by the heavier PBDE-congeners, like BDE-209, or deca-BDE, which caused us to exclude them from analyses. There is some uncertainty with how the BDE-209 results should be

interpreted as the congener can be difficult to measure in the lab due to equipment contamination. Moreover, BDE-209 readily adheres to sediment and fine particles which could indicate that the biofilm samples from some sites contained some amount of sediment (Wang et al. 2017). Presence of sediment in the samples, could explain the variable and inconsistent levels of BDE-209 in biofilm from some of the sites and between samples from the two studies. For example, the proportion of heavier congeners in the biofilm samples from Muck Creek changed from 95% in 2017 to 28% in 2021. Similarly, the proportion of heavier congeners changed in the samples from RM 0.1 in Ohop Creek between the two studies. As little has changed in the immediate area surrounding these collection sites between 2017 and 2021, it might be possible that sediment contaminated with BDE-209 contributed to some of our biofilm samples and not others during collection. Other sites with high proportions (i.e., > 75%) of BDE-209 measured in biofilm included the most downstream site in Ohop Creek at RM 0.1 (OC03), and two sites in the Mashel River at RM 6 (MR02) and 5.4 (MR03).

Given the difficulty of measuring BDE-209, in the following sections we included data evaluations with and without BDE-209. When removed from the total summed PBDEs, the levels of TPBDEs in the biofilm from RM 6 in Ohop Creek decreased and were much lower than what was measured in the invertebrates (Figure 9). As a result, invertebrates from this site showed that PBDEs are biomagnifying, as expected, with levels in invertebrates three times higher than PBDE levels measured in biofilm, albeit at much lower concentrations than were measured in samples from the Mashel River.

For the remainder of the report, we will be focusing on the Mashel River as it is the tributary with overwhelmingly high values of PBDEs in biofilm and invertebrates compared to samples collected from Ohop and Muck creeks. Appendix A and B have additional graphical displays of PBDE composition and stable isotopes signals, respectively, for biofilm and invertebrate samples collected from Ohop and Muck creeks.

PBDE Composition in Biofilm and Invertebrates

The concentrations and proportions of PBDE congeners in biofilm and invertebrates from the Mashel River varied, with the heavier homolog groups, especially deca-BDE, detected more frequently and at higher proportions in the biofilm (Figure 10A, Figure 11, Table 5). Moreover, the proportion of deca-BDEs in the samples was greatest when there were low overall PBDE concentrations in the samples (Table 5). Given the uncertainty of analyzing deca-BDE in biota, we opted to exclude it from analysis.

When deca-BDE is excluded from the biofilm samples (Figure 10B), there is still a higher proportion of nona- and octa-BDEs in the biofilm compared to invertebrate samples, especially upstream of the Eatonville WWTP outfall at RM 5.1 and RM 6. Immediately downstream of the Eatonville WWTP outfall at RM 5.1, where PBDEs were elevated, the biofilm samples were dominated by the tri- (RM 4.4 only; 52%), tetra- (20-36%) and penta-BDE (19-36%) homolog groups (Figure 10B). The PBDE pattern was similar in biofilm from RM 4.4 to 0.35, with the tetra- and penta-BDE homolog groups dominating the biofilm samples and accounting for 36-48% and 35-37% of the congeners in the samples, respectfully (Figure 10B).

In contrast to the biofilm samples, BDE-209 was only detected in the invertebrate sample at RM 5.3 (MR03), and whether it was included or not did not greatly affect the PBDE composition among sites

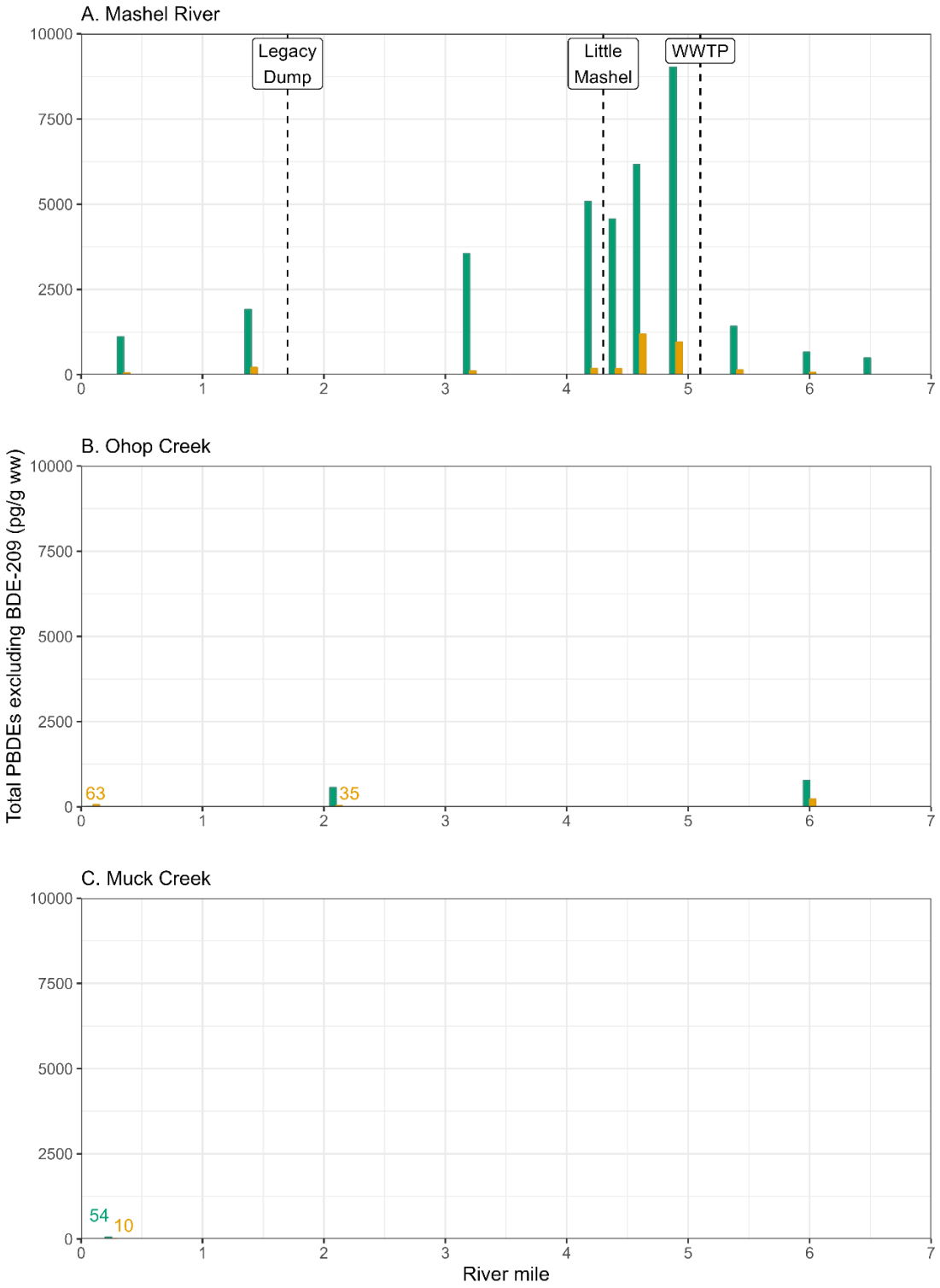

Figure 9. Total PBDEs excluding BDE-209 (pg/g wet weight) measured in both invertebrates (green bars) and biofilm (orange bars) collected from three tributaries of the Nisqually River, A) Mashel River, B) Ohop Creek and C) Muck Creek. The approximate river mile of the collection site is on the x-axis with points of possible PBDE sources in the Mashel River (A) marked with a dashed line and labeled. The low TPBDE concentrations measured in biofilm and invertebrates from Ohop and Muck creeks are labeled on the figure in their respective colors. Note that duplicate field samples are excluded from these graphs.

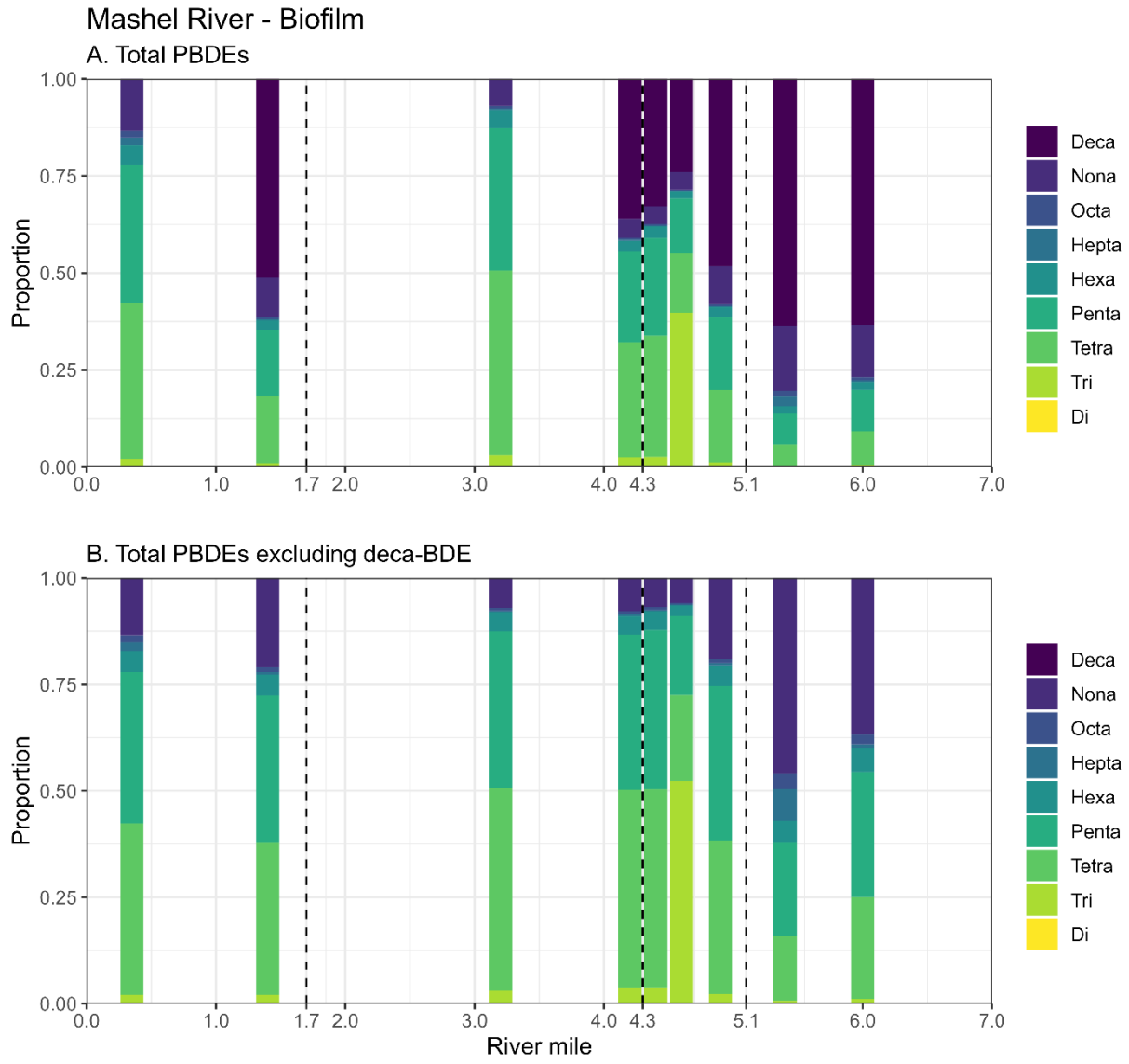

Figure 10. Proportion of detected PBDE congeners in biofilm from the Mashel River summed as homolog groups for A) all measured PBDE congeners (total PBDEs) and B) total PBDEs excluding BDE-209, or deca-BDE. The approximate river mile of the collection site is on the x-axis. The potential PBDE sources (dotted lines) are labeled with their river mile (RM); RM 1.7 = legacy dump, RM 4.3 = Little Mashel River, and RM 5.1 = Eatonville WWTP outfall. Note the duplicate samples are excluded from this graph.

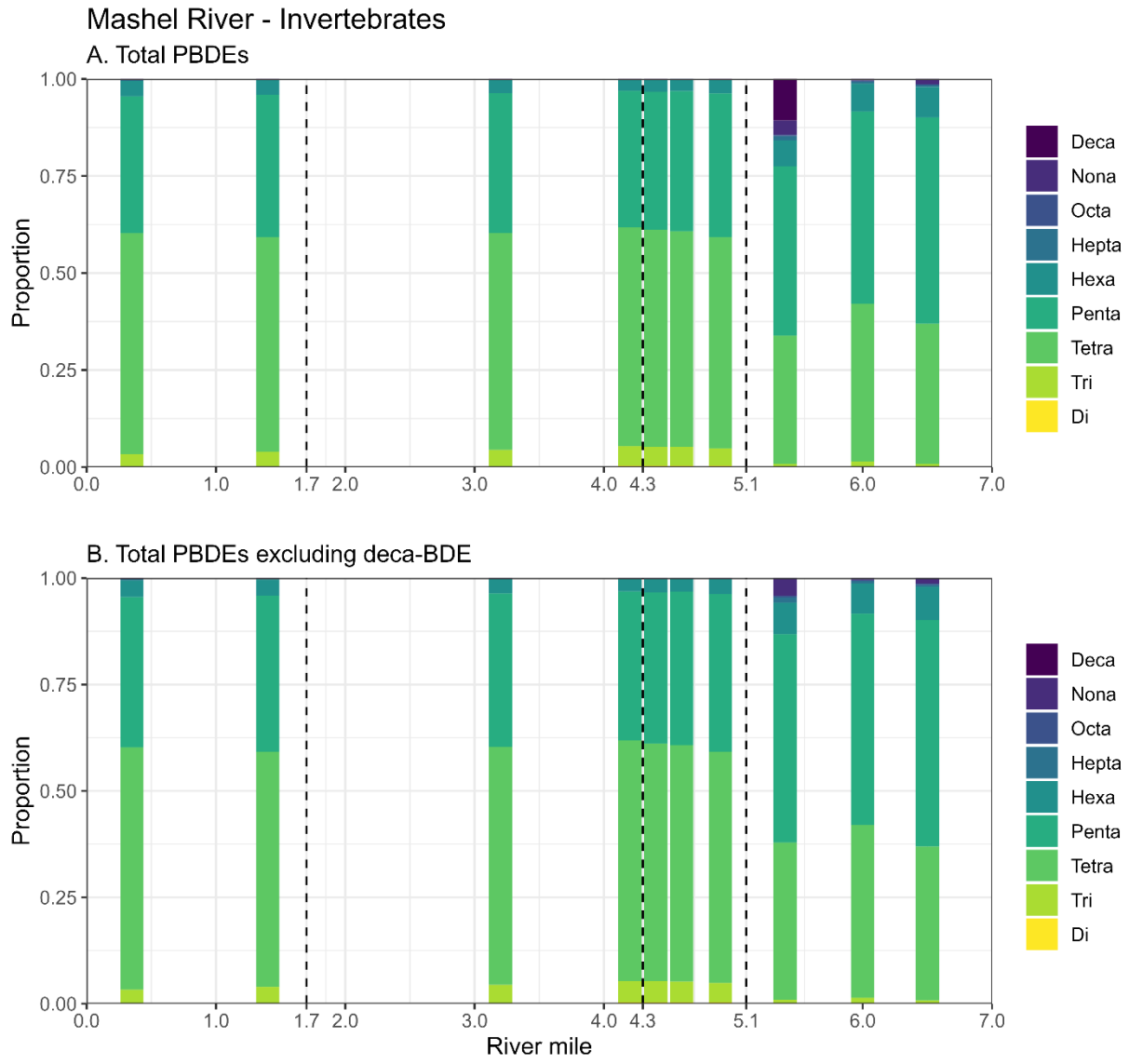

Figure 11. Proportion of detected PBDE congeners in invertebrates from the Mashel River summed as homolog groups for A) all measured PBDE congeners (total PBDEs) and B) total PBDEs excluding BDE-209, or deca-BDE. The approximate river mile of the collection site is on the x-axis. The potential PBDE sources (dotted lines) are labeled with their river mile (RM); RM 1.7 = legacy dump, RM 4.3 = Little Mashel River, and RM 5.1 = Eatonville WWTP outfall.

Table 5. Summed detected values of nine PBDE homolog groups for all biofilm and invertebrate samples. When two or more samples had non-detected concentrations of PBDEs in a homolog group, the average limit of quantitation was calculated. For octa- and deca-BDEs where only congener was in the homolog group, the limit of quantitation was used as the homolog concentration. The number of PBDE congeners included in the sum are in parentheses below the homolog name. NC = not collected, X = sample excluded, bio = biofilm, inv = invertebrates

Site	River	Di-BDE (n = 5)		Tri-BDE (n = 6)		Tetra-BDE (n = 8)		Penta-BDE (n = 7)		Hexa-BDE (n = 6)		Hepta-BDE (n = 3)		Octa-BDE (n = 1)		Nona-BDE (n = 3)		Deca-BDE (n = 1)	
Num	Mile	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv	Bio	Inv
OC01	6	0.17	1.72	1.28	17.6	22.5	345	25.4	350	5.54	50.1	0.868	2.03	4.17	0.774	161	1.33	1100	<21.7
OC02	2.1	0.089	2.43	0.735	16.9	13.3	293	10.7	202	2.88	37.2	2.24	7.07	0.604	0.577	4.41	1.98	<24.2	<20.1
OC03	0.1	0.10	NC	0.477	NC	14.6	NC	12.5	NC	3.02	NC	1.57	NC	1.79	NC	29.3	NC	151	NC
MC01	0.25	<0.151	0.325	0.294	1.91	3.75	27	2.98	20.5	0.366	2.41	<0.266	0.246	<0.171	0.289	2.82	1.59	<18.9	<24.7
MR01	6.5	NC	<0.263	NC	3.96	NC	175	NC	258	NC	37.9	NC	2.42	NC	1.21	NC	6.42	NC	<84.8
MR02	6	<0.147	0.435	0.771	8.86	15.7	267	19.3	326	3.57	47.1	0.712	3.65	1.51	1.60	24.1	2.72	114	<23.3
MR03	5.4	<0.295	0.416	0.913	13.2	20.7	522	30.0	692	7.08	105	10.1	18.1	5.08	4.18	62.5	59.2	238	169
MR04	4.9	1.40	31.2	20.5	408	344	4900	347	3340	46.6	315	5.36	12.1	8.72	4.26	182	5.74	890	<31.4
MR04b	4.9	X	27.9	X	397	X	4510	X	2900	X	276	X	11.4	X	4.00	X	<5.12	X	<40.8
MR05	4.6	0.927	19.1	623	303	240	3420	222	2220	29.4	185	1.84	5.03	4.31	2.1	70.2	<5.527	377	<56.1
MR06	4.4	0.228	14.5	6.52	227	80.8	2550	65.1	1620	7.76	142	0.5	4.16	1.1	2.1	11.9	4.25	84.9	<25.8
MR07	4.2	0.292	17.4	6.60	254	82.4	2870	64.9	1780	7.74	147	0.768	4.89	1.27	1.8	13.9	3.79	100	<55
MR08	3.2	0.19	8.08	3.09	150	51.3	1980	49.7	1280	4.94	120	0.340	5.32	0.785	2.3	7.49	1.6	36.1	<30.1
MR09	1.4	0.174	3.22	4.08	71.6	72.9	1050	70.8	701	10.1	70.4	1.14	3.57	2.52	1.52	42.6	2.25	214	<23.6
MR10	0.35	<0.169	1.22	1.04	35.6	19.8	630	17.5	391	2.44	42.8	0.988	2.85	0.843	1.44	6.61	1.94	<32.4	<14.6

(Figure 11A and Figure 11B). In the invertebrates collected upstream of the WWTP outfall from RM 6.5 to RM 5.4, the tetra-BDEs and penta-BDEs dominated and the summed concentrations of the tetra- plus penta-homologs accounted for the majority (86-90%) of the BDE congeners in the invertebrate samples (Figure 11B). Additionally, those same samples contained a greater portion of penta-BDEs than tetra-BDEs. While the PBDEs increased in the invertebrates at RM 4.9 and stayed fairly elevated to RM 0.35, the proportion of tetra-BDEs plus penta-BDEs in the samples was similar to the samples collected above the outfall, accounting for 91-92% of the congeners (Figure 11B). Unlike the samples collected upstream of the outfall, the invertebrate samples from RM 4.9 to RM 0.35 were predominantly tetra-BDEs, consistently ranging from 54 to 57% while penta-BDEs accounted for 35-37% of the congeners at the same site (Figure 11B).

These patterns of penta- and tetra-BDEs in both biofilm and invertebrate samples are not surprising as biota tend to accumulate higher proportions of BDE-47 (tetra-BDE), BDE-99 (penta-BDE) and BDE-100 (penta-BDE) compared to other congeners (Ecology 2006). In fact, BDE-47 and BDE-99 are the most abundant congeners in the commercial mixture of Penta-BDE, primarily used in the USA, phased out of use in 2004, and most often detected in wildlife and humans around the world (Birnbaum and Staskal 2004; Noyes and Stapleton 2014; Arkoosh et al. 2015). Indeed, a study of freshwater fish in Washington showed that tetra- and penta-BDEs were the major congeners detected in the samples from various locations across the state (Ecology 2006).

The change in BDE-congeners from upstream of the outfall to downstream suggests a change in the PBDE source in the river, likely the effluent from the WWTP outfall. The higher proportion of tetra-BDE relative to penta-BDE homologs downstream of the WWTP outfall suggests the WWTP effluent was dominated by BDE-47 or other BDE congeners that are bio-transformed to BDE-47. The amount of BDE-47 and BDE-99 present in the samples, specifically in the invertebrates, shows these contaminants are in the Mashel River food web in large proportions which can be passed on to higher trophic species that consume these invertebrates. Biomagnification of the PBDEs could lead to other species accumulating PBDEs at harmful levels that can affect their health, as was measured in steelhead trout from the Nisqually River in 2014 (Chen et al. 2018) and 2015 (WDFW unpublished data).

PBDE Sources in the Mashel River

Samples collected from the influent pipe to the Eatonville WWTP had total PBDE concentrations of 93,594 pg/L; samples collected of the effluent had an average concentration of 1,855 pg/L. Background contributions of PBDEs from the field sampling equipment were negligible (9 pg/L). It is clear the treatment process of the plant is removing a considerable amount of the incoming PBDEs from the surrounding residential area. Because of the unknown time of travel of wastewater through the plant it is not possible to estimate a percent reduction of PBDEs due to the treatment process.

The measured PBDE concentration in the influent sample entering the Eatonville WWTP was within the range of a few samples which were collected from the City of Everett treatment plant in 2020 and 2021 (unpublished data) (Table 6). The effluent PBDE concentrations from the Eatonville plant are lower than those measured in other Puget Sound WWTPs (Table 6). It should be noted that samples in this study represent a one-day manual composite and a more comprehensive assessment of possible PBDE inputs to the WWTP may be warranted. A recent study by Wong (2022) found a considerable range in the PBDE concentrations from a variety of industrial sources discharging to WWTPs (Table 6).

Table 6. Summary statistics of PBDE concentrations from various sources throughout Puget Sound. The majority of samples are single day grab or composite samples. All concentrations are in pg/L.

Media or source	n	Min	Median	Mean	SE	Max	Reference
Combined Sewer Overflow	10	2,567.1	23,622.5	35,868.5	15,216.9	166,384.0	King County, 2013
WWTP influent	3	70,345.4	86,000.4	108,656.6	30,816.8	169,623.9	City of Everett, unpublished data
WWTP effluent	32	5,039.0	20,794.5	34,779.4	6,808.2	134,736.5	WDOE and Herrera, 2010; Meador et al., 2022; City of Everett, unpublished data
Pre-treatment industrial	9	29.0	9,200.0	420,918.0	384,139.3	3,490,000.0	Wong, 2022

Although, the Eatonville WWTP samples do show a reduction in PBDE mass during treatment, measurable amounts of BDEs are being discharged to the Mashel River (Figure 12). The effluent sample contained mainly BDE-47, -99 and -100, making up approximately 67% of the total PBDE mass. BDE-209 was measured in the effluent sample, but due to blank contamination the result was qualified as non-detect, meaning we cannot treat it as a positive identification of the compound. The PBDE composition is generally compatible with the composition of the biofilm samples collected in 2017 and 2021 near the effluent discharge. The exception being several lighter congeners in the effluent (BDE-8/11, -15, -17/25, -and 28/33) that generally do not appear to concentrate much in the biofilm. It is worth noting that the biofilm results from sample MR05 (RM 4.6) contained elevated BDE-17/25 relative to all other samples, including the

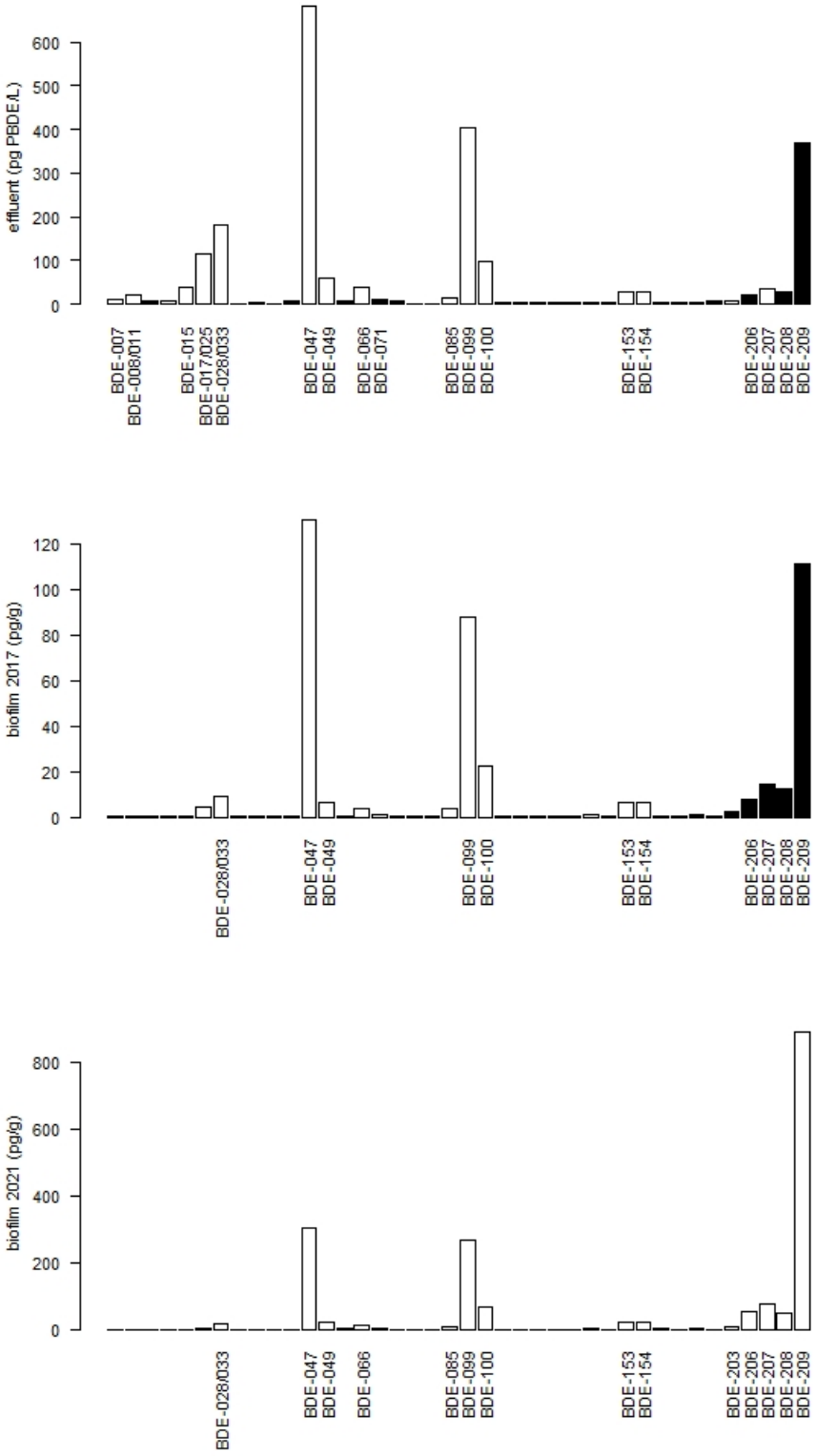

Figure 12. Concentrations of PBDEs congeners detected in wastewater effluent in 2021 (upper panel) that discharges into the Mashel River at river mile 5.1 and in biofilm samples collected below the outfall at river 4.9 in 2017 (middle panel) and 2021 (lower panel). Black bars represent results qualified as non-detect due to blank contamination. White bars are detected results.

samples collected closer to the WWTP outfall. It's unclear why this is the case, but BDE-17/25 does not appear to bioaccumulate in the invertebrates.

Stable Isotopes in Biofilm and Invertebrates

Across the three tributaries and 14 collection sites, the stables isotopes of $\delta^{15}N$ and $\delta^{13}C$ in samples ranged broadly, from 0.003 to 11.17‰ and -32.8 to -13.2‰ respectfully for biofilm samples, and from 1.19 to 10.49‰ and -19.75 to -14.53‰ respectively for invertebrate samples (Table 4). However, the samples with higher enriched values of $\delta^{15}N$ and $\delta^{13}C$ were only observed in the Mashel River.

Within the Mashel River, the isotopic values $\delta^{15}N$ and to a lesser extent $\delta^{13}C$ in biofilm and invertebrate samples were starkly enriched downstream of the Eatonville WWTP at RM 5.1. At sampling locations downstream of the outfall (from river miles 4.9 to 0.35), average (± Std dev) ratios of $\delta^{15}N$ in biofilm and invertebrates were 8.69‰ (±2.04‰) and 8.81‰ (±1.60‰), well above the average upstream $\delta^{15}N$ in biofilm (0.02‰) and invertebrates (1.38 ‰ ± 0.16‰) (Figure 13, Table 4). Peak $\delta^{15}N$ enrichment occurred at RM 4.4 for biofilm (11.17‰) and RM 4.9 for invertebrates (10.49‰), then gradually became less enriched moving towards the mouth of the tributary at RM 0.35 where measured $\delta^{15}N$ was 5.22‰ in biofilm and 7.05‰ in invertebrates. (Figure 13, upper panel). The $\delta^{15}N$ measured in biofilm and invertebrates at the mouth of the Mashel River were much more comparable to the $\delta^{15}N$ measured in biofilm and invertebrates at the mouths of Ohop and Muck creeks (Table 4). In contrast, in samples collected upstream of the WWTP outfall (RM 5.4 to 6.5) the maximum $\delta^{15}N$ in background biofilm and invertebrate samples were 0.04‰ and 1.48‰ (Table 4). The depleted $\delta^{15}N$ signal in sites upstream of the outfall are possibly influenced by atmospheric deposition of nitrogen released from the melting glacier at the head of the river. The accepted standard for $\delta^{15}N$ is atmospheric dinitrogen gas (i.e., N_2) with a $\delta^{15}N$ of 0‰, close to the observed $\delta^{15}N$ values observed in the upstream biofilm and invertebrate samples. Assuming the stable isotope signals in the upstream sites in the Mashel River are influenced by input of deposition of atmospheric nitrogen released from the melting glacier at the head of the river, we would expect a gradual enrichment in $\delta^{15}N$ as you moved downstream, but not the dramatic shift we observed between RM 5.4 and 4.9.

The isotopic values of $\delta^{13}C$ in biofilm and invertebrate samples collected in the Mashel River were also generally enriched downstream of the WWTP outfall, except for the biofilm and invertebrate samples collected at RM 4.6 (MR05), but the magnitude of the enrichment was much smaller (Figure 13, lower panel) than observed for $\delta^{15}N$. At sampling locations downstream of the outfall average (± Std dev) of $\delta^{13}C$ in biofilm and invertebrates were -16.34‰ (±2.54‰) and -15.92‰ (±1.67‰), which is more enriched than biofilm collected upstream of the WWTP outfall (-22.6‰) and only marginally so for invertebrate samples(-17.9 ‰ ± 0.13‰) respectively (Figure 13, Table 4).

The isotopic ratio of $\delta^{15}N$ is predicted to increase (become more enriched) by approximately 3.4‰ with each trophic level in a food web, which is known as trophic enrichment (Post, 2002). Trophic enrichment was observed in the invertebrate samples compared with co-located biofilm sample for sites collected upstream of the WWTP outfall (RM 5.4 to 6.6) and to a lesser extent downstream of the legacy dump (RM 1.4 to 0.35) but not at those between the WWTP outfall and the legacy dump. The lack of apparent trophic enrichment downstream in the invertebrate samples collected in the Mashel River between the WWTP outfall and the legacy dump was surprising because the diet of *D. gilvipes,* the caddis fly larvae species composing the invertebrate tissue samples, was similar to our biofilm samples. We hypothesize that the discharge from the WWTP contributed to the lack of apparent trophic enrichment, discussed in more detail below.

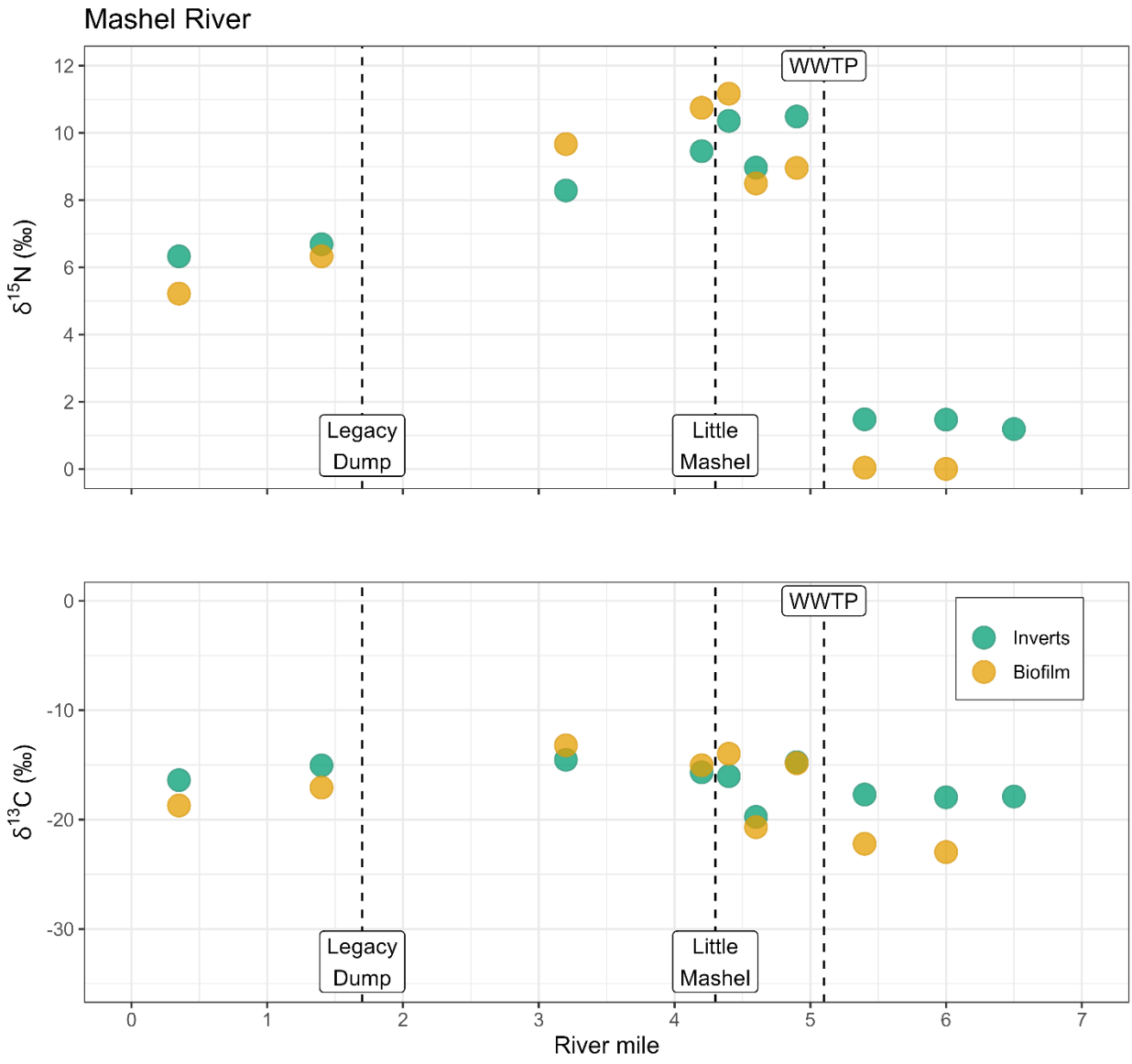

Figure 13. Stable isotopes of $\delta^{15}N$ (top) and $\delta^{13}C$ (bottom) measured in both invertebrates (green circles) and biofilm (orange circles) collected from the Mashel River. The approximate river mile of the collection site is on the x-axis with points of possible PBDE sources in the Mashel River marked with a dashed line and labeled.

The enriched isotopic values of $\delta^{15}N$ and $\delta^{13}C$ downstream of the Eatonville WWTP outfall are consistent with an incorporation of a different nitrogen and carbon source (Figure 13), likely the Eatonville WWTP effluent. Biota exposed to secondary and tertiary sewage treatment that removes excess nitrogen with nitrifying and denitrifying bacteria typically have an enriched $\delta^{15}N$ signal compared to background values (Heaton, 1986; Savage 2005; Valiela et al., 2000). In addition to the ambient nitrogen load in the environment, nitrogen in wastewater is incorporated into aquatic food webs through 1) the uptake of dissolved nutrients by primary producers and/or 2) consumption of particulate-organic matter by primary consumers (Tucker et al., 1999). In our study, the dissolved N from the wastewater could be taken up by the microbes and algae in the biofilm (i.e., the primary producer) and then passed to the invertebrates (*D. gilvipes, a primary consumer*) when they graze on the biofilm. The other possible mechanism is enriched particulate N and C is being deposited on the substrate and shows up as part of

the mixture in the biofilm, and similarly being grazed by the invertebrates. Because our biofilm samples are scraped from rocks within the stream, they likely contained some detritus (including inorganic material) and certainly contained microbial biomass which can affect the bulk isotopic ratios. Overall, the lack of apparent trophic enrichment in the caddis fly larvae compared to biofilm in some samples likely reflects variability in the nutrient uptake pathways described previously, possible inorganic deposition on biofilms, and differences in periods of growth.

Additionally, we observed a strong positive relationship between $\delta^{15}N$ and total PBDE concentration for invertebrate samples collected downstream of the Eatonville WWTP outfall; the higher the PBDE burden in invertebrate tissues, the more enriched they were in $\delta^{15}N$ ($y = 2.03x - 8,03$; slope $p = 0.0013$ $r^2 = 0.81$), suggesting a similar source for nitrogen and contaminants (Figure 14). In contrast, there was no positive relationship between total PBDE concentration and $\delta^{15}N$ in invertebrate samples collected upstream of the WWTP outfall (RM 5.4, 6, and 6.5). Collectively, these results support our hypothesis that the source of PBDEs and nitrogen to the Mashel River is the effluent from the Eatonville WWTP.

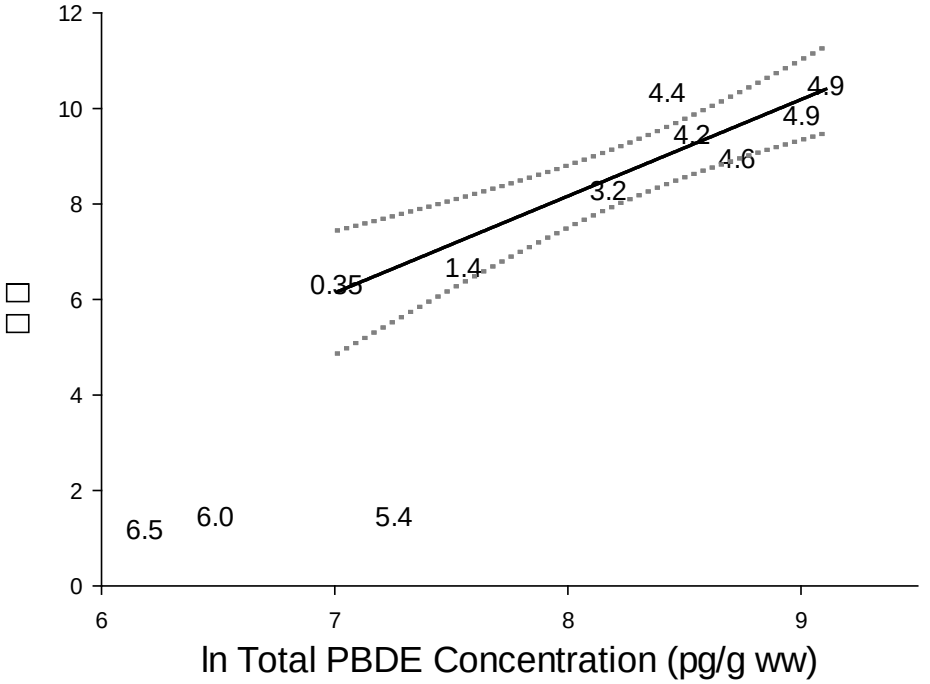

Figure 14. Relationship between log transformed total PBDEs (pg/g wet weight) and $\delta^{15}N$ (‰) measured in invertebrates samples collected at each river mile (RM) in the Mashel River (shown as symbols) showing that PBDE concentrations are positively correlated downstream of the Eatonville WWTP (RM 4.9 to 0.35; $y = 2.02 x - 8.03$; $p= 0.0013$, $r^2 = 0.81$), but not at the upstream sites (RM 5.4 to 6.5). BDE-209 was excluded from the total PBDE concentration.

Ecological relevance

Bioaccumulation and Biomagnification of PBDEs

Despite inconsistent periods of exposure and sample timing among all the samples we have collected in the Mashel River during our investigations in 2017 and 2021, we are able to put together a rough idea of PBDE concentration, bioaccumulation and biomagnification in this system (Figure 15). The bioconcentration of PBDEs from the water onto the biofilms at RM 4.9 occurred with a factor of approximately 4,500 times based on samples collected in 2017 (Figure 15, left panel). There has not been much research on bioconcentration of PBDEs from water into biofilms or primary producers

(algae), but it appears that factors measured in the Mashel River are similar to limited examples from the marine environment (Magnusson et al., 2007). The biomagnification factor of PBDEs from biofilm to invertebrate was approximately six based on samples collected in 2021 from the three sites downstream of the WWTP outfall, MR04, MR05 and MR06 (Figure 15, right panel).

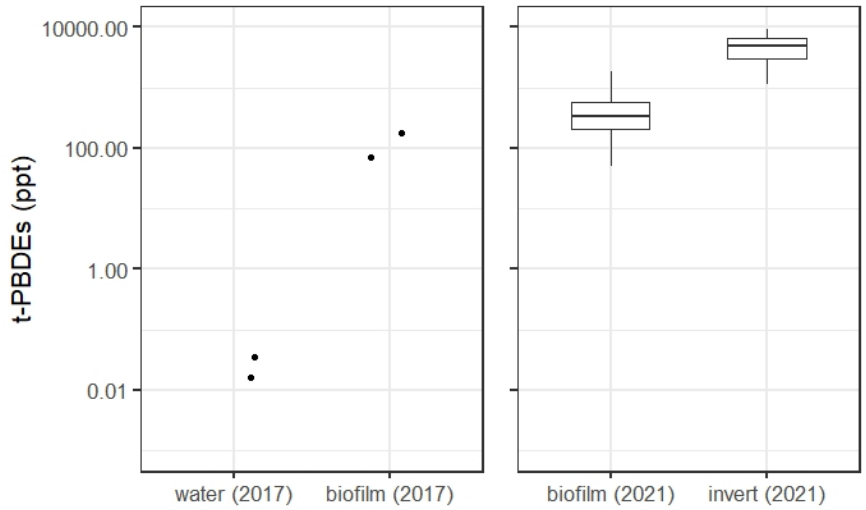

Figure 15. Concentratioon of (total) t-PBDEs (ppt- part per trillion) in water and biofilm collected in 2017 (left panel) and in biofim and inverts collected in 2021(right panel) from river miles 4.9 to 4.4, demonstrating bioaccumualtion from water to invertebrates.

While biomagnification of PBDEs is well established in the literature, the compositional and possible metabolism of certain PBDE congeners is less constrained. Comparing the biofilm and invertebrate tissue samples demonstrates how many of the heavier (deca-) PBDE compounds are not dominant in the invertebrate tissues. Whether these compounds pass through the invertebrates bound to sediment or whether the invertebrate metabolizes or de-brominates some of the compounds is not well-understood (Figure 16). Either way, the composition of PBDEs in the invertebrates is similar to that measured in the steelhead trout from the Nisqually basin (Chen et al., 2018).

Juvenile steelhead are known to consume the fifth instar stage of *D. gilvipes* (Tippets and Moyle 1978). However, their primary prey includes many other aquatic insects including Chironomid (Diptera), mayflies (Ephemeroptera), other caddis flies (Trichoptera), especially Hydropsychidae, (Angradi and Griffith 1990, Bisson 1978; and Merz 2002). The energetic density of steelhead prey varies greatly (McCarthy et al. 2009) and scraper/grazers like *D. gilvipes* are likely not the most energetically important prey but they can contribute to the overall diet. Concentrations of PBDEs measured in steelhead collected from the Nisqually River and estuary in 2014 was 9.9 (±10) ppb (Chen et al. 2018) or 9,900

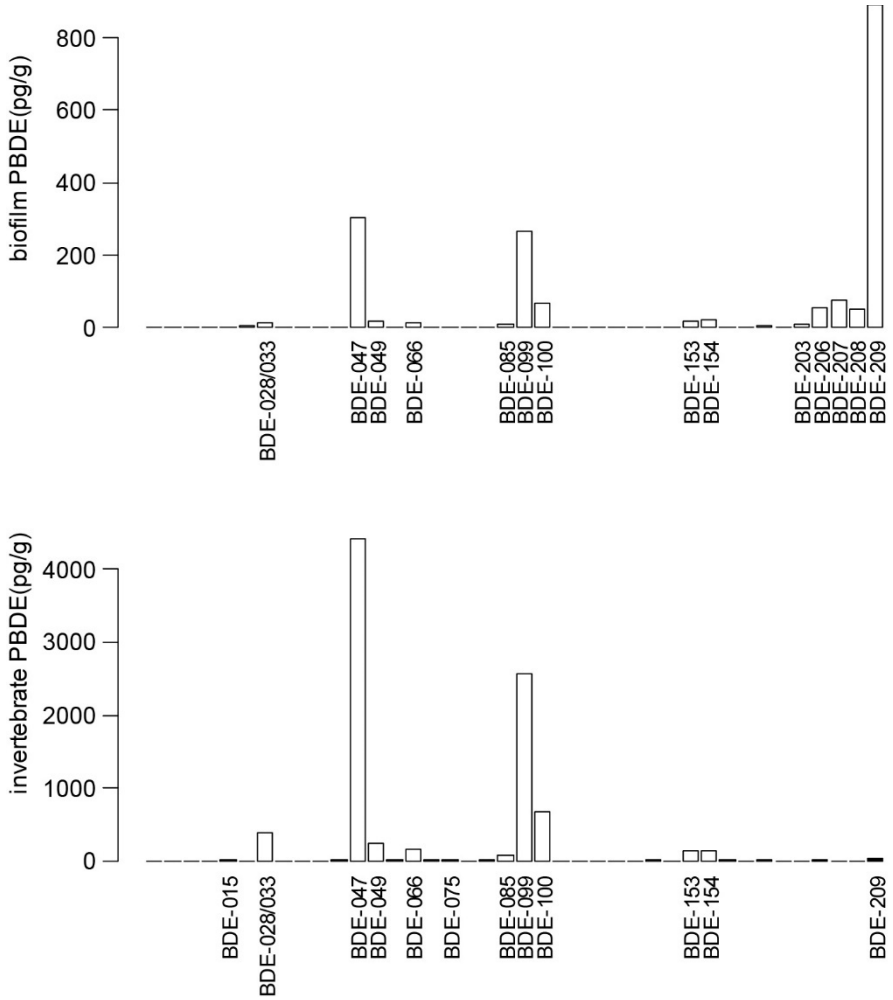

Figure 16. Measured concentrations of PBDE congeners in biofim and invertebrate samples collected in the Mashel River in 2021.

(±10,000) ppt, approximately twice as high as those measured in invertebrates in 2021, suggesting there is some biomagnification through the food web from water to steelhead. However, these data are not the most appropriate for evaluating PBDE bioaccumulation in the food web of the Mashel River given the five-year gap between the collection time of the invertebrates and the steelhead samples. Furthermore, the steelhead samples collected in 2014 were collected from a smolt trap on the lower mainstem of the Nisqually River, so there is uncertainty as to where these fish resided in the Nisqually River system. Despite these issues the results from both the 2017 and 2021 PBDE source tracing study support our hypothesis that the lower reach of the Mashel River, downstream of the Eatonville WWTP, is likely the main source of PBDEs to juvenile Steelhead rearing in the Nisqually River drainage and likely contributed to the elevated PBDE concentrations we observed in 2014 (Chen et al. 2018) and 2015 (WDFW, unpublished data).

Within Puget Sound currently, there are no regulatory criteria for environmental concentrations of PBDEs in Washington State. Previous laboratory studies exposing juvenile Chinook salmon to PBDEs (congeners BDE-47 and BDE-99) and subsequent disease challenges found concentrations of PBDEs (BDE 47 + BDE-99) from ≥ 470 to ≤ 2,500 ng/g lipid (9.8 to 40 ng/g ww) increased the Chinook salmons' susceptibility to disease and altered thyroid hormones (Arkoosh et al. 2010, 2013, 2017 and 2018, see

Supplementary Material in Chen et al. 2018). Indeed, these PBDE levels are used to assess the health of juvenile Chinook salmon and Pacific herring as part of the Puget Sound Partnership's Toxics in Aquatic Life Vital Sign, one of many indicators used to measure the health of the Puget Sound ecosystem. Similarly, the PBDE concentrations measured in steelhead from the Nisqually River system were compared to the Arkoosh et al. (2010, 2013, 2017 and 2018) studies to screen for fish with levels that may increase their susceptibility to disease (Chen et al. 2018).

Regional PBDE Trends

The current concentrations of PBDEs in juvenile steelhead are unknown, and they may have declined since our last sampling in 2015. Based on data collected in the early 2000s, Osterberg and Pelletier (2015) identified publicly-owned treatment works (POTWs) as the primary pathway through which PBDEs move from their terrestrial sources (households and commercial/industrial dischargers) to Puget Sound waters. However, statutory and voluntary PBDE controls on production and use of these chemicals have occurred in Washington State over the past 20 years. In 2008, Washington state banned the sale of select Penta- and Octa-BDE mixtures, however they can still be found in materials produced before the ban. Currently, the manufacture (including import), processing, and distribution of Deca-BDE, or Deca-BDE-containing products is banned by the US Environmental Protection Agency, effective February 2021, though there are exceptions.

PBDEs have declined in the marine benthic and pelagic food web of Puget Sound over the past 25 years (West et al. 2017; Ross et al. 2013) and currently concentrations are generally below effect levels in Pacific herring, English sole, and adult Chinook salmon (PSP 2023), likely in response to the regulatory controls imposed in Washington State. Overall, PBDE concentrations in seaward migrating juvenile Chinook salmon from four Puget Sound rivers (Snohomish, Duwamish, Puyallup/Hylebos and Nisqually) combined are showing a declining trend since 2006, but data at individual rivers is limited for testing statistically significant trends (O'Neill et al. in prep). Additional years of data will be needed to confirm whether PBDE levels in juvenile salmon are truly declining or whether biological covariates (e.g., fish size, lipid content, natural- or hatchery-origins) are influencing the declining trends. Moreover, although PBDEs have declined broadly in Puget Sound's marine food web over the past two decades and appear to be declining in juvenile salmon collected from Puget Sound rivers PBDE concentrations may still be at high enough concentration to impair fish health in localized areas. For example, PBDE contamination at levels of concern has been reported in seaward-migrating juvenile Chinook salmon of the Snohomish River and Puyallup rivers (Puget Sound Partnership 2023). O'Neill et al. (2020) identified POTW discharges in the Snohomish River as the likely source of PBDEs to juvenile Chinook in that system. These results suggest that timing and proximity of discharges with salmon migration in a restricted water body (river) may be key factors in the exposure of fish to chemicals discharged by POTWs.

Potential impacts to other species

Although this study was focused on identifying sources of PBDEs to steelhead trout to the Nisqually River, other salmonid species may also be affected by PBDE exposure. Like steelhead, stream-type Chinook salmon, coho salmon, and cutthroat trout generally reside in freshwater longer than ocean-type juvenile Chinook salmon (Quinn 2018) and are generally distributed in the upstream portions of the freshwater habitat. Indeed, most Chinook salmon in the Nisqually River watershed are ocean-type and migrate to marine waters as subyearlings, but there is data indicating a subset of Chinook salmon are stream-type and overwinter in the system (Nisqually Chinook Recovery Team 2001; Klungle et al. 2018). Thus, if the health of juvenile steelhead trout from the Nisqually River watershed are impaired via exposure to PBDEs while rearing in the Mashel River, then other juvenile salmonids, including stream-type Chinook salmon, coho salmon, and cutthroat, may also be impaired.

Because of the importance of the Mashel River to various salmonid species, 3.9 miles of shoreline along the Mashel River are protected from development (Nisqually Land Trust 2017) and over 60 log jams have been constructed in the river specifically to improve habitat for salmonids (Regional Fisheries Coalition 2016). While the protected shoreline and additional habitat created from the log jams are beneficial to the salmonids in the system, the high levels of PBDEs are potentially detrimental to those species residing and rearing in the Mashel River. O'Neill et al. (2020b) acknowledged that contaminants from wastewater may be undermining restoration efforts in areas of the Pacific Northwest as substantial amounts of money and time are spent to restore habitat for salmonids, but water quality is not considered in the process. Not only is it important to understand the habitat needs of the species of concern during restoration efforts, but the contaminant quality of that habitat needs to also be assessed and understood. Given the Eatonville WWTP's outfall potential to expose salmonids to harmful levels of PBDEs, additional consideration must be taken to protect salmonids when planning restoration work in the Mashel River.

Conclusions

The results of the 2021 PBDE source assessment in three Nisqually River tributaries show that while PBDEs were measured at all sites in each tributary at varying levels, the Mashel River represents the most likely location where juvenile steelhead trout, an ESA-listed species, are taking up PBDEs. The source of PBDEs to the Mashel River is likely the effluent from the Eatonville WWTP based on the location, magnitude, and pattern of PBDEs and stable isotopes in biofilm and invertebrates. PBDEs measured in samples from upstream of the WWTP outfall in the Mashel River and samples from Ohop and Muck creeks were low and are considered background concentrations. Biofilm and invertebrate insect larvae exhibited elevated PBDE levels and an altered PBDE congener pattern downstream of the Eatonville WWTP outfall, located at RM 5.1. Indeed, within a half mile stretch of the river between RM 5.4 and 4.9, PBDEs increased five- and six-fold in invertebrates and biofilm, respectively. Similarly, an enrichment of the stable isotopes $\delta^{15}N$ and $\delta^{13}C$ in biofilm and invertebrates downstream of the outfall confirm the influence of effluent on the river biota. A single composite sample from the WWTP influent and effluent confirmed that PBDEs are entering the WWTP from the surrounding residential inputs, and the current treatment process is removing a considerable amount of PBDEs. However, measurable concentrations are being discharged in the effluent with a composition that is compatible with that measured in river biota.

Relative to background PBDE levels measured in samples upstream of the outfall, PBDEs remained elevated in biofilm a half mile downstream of the WWTP outfall, while PBDEs were elevated in invertebrates for 3.7 miles downstream. The zone for PBDE enrichment in the Mashel River was far greater for invertebrates than biofilm samples, possibly because the invertebrates are more mobile and can travel downstream and have a longer period of growth in the river. Our data suggests a broader area of influence from PBDE inputs extending through much of the lower Mashel River and potentially exposing juvenile steelhead (and other species) inhabiting this reach of the river.

Between the two studies in 2017 and 2021, we have been able to show that PBDEs are present in the water of the Mashel River, accumulate in biofilm and then in invertebrates with evidence of biomagnification. But while PBDEs were measured at elevated levels in biofilm and invertebrates from the Mashel River in 2021, we remain unsure of PBDE levels in steelhead trout from the Mashel River. Our last sampling of steelhead from the Nisqually River smolt trap took place in 2015, and a third of the samples had PBDE levels at concentrations known to impact salmonid health. Given the magnitude of PBDEs measured in the biofilm and invertebrates during this study, we hypothesize that those past steelhead with elevated PBDE concentrations likely accumulated them while residing in the Mashel

River. However, sampling juvenile steelhead trout directly from the Mashel River would be necessary to confirm whether the PBDEs are elevated enough to cause harm. Moreover, PBDE concentrations may have declined since the 2014 and 2015 steelhead sampling in the Nisqually River watershed as has been recently documented in marine species of Puget Sound.

Recommendations

We recommend a limited collection of steelhead trout, stream-type (yearling) Chinook or other salmonids to update the PBDE data for this river system. Current levels of PBDEs in steelhead and other salmonids from the Mashel River are unknown as the last sampling of steelhead in the Nisqually River system took place in 2015 and the collection site was located roughly 27 miles downstream from the Mashel River confluence. Sampling steelhead and/or other salmonids, such as stream-type (yearling) Chinook, coho or cutthroat trout, directly from the Mashel River would allow better understanding of whether salmonids rearing in this reach are accumulating detrimental levels of PBDEs. Additionally, concurrent sampling of invertebrates at salmonid collection sites would allow for better assessment of PBDE biomagnification in the Mashel River food web.

Other possible future studies include:

- analyses of PBDEs in resident fish (sculpins, whitefish), and
- monitoring of PBDEs in Eatonville WWTP influent and effluent to assess trends in potential PBDE exposure for steelhead and other species.

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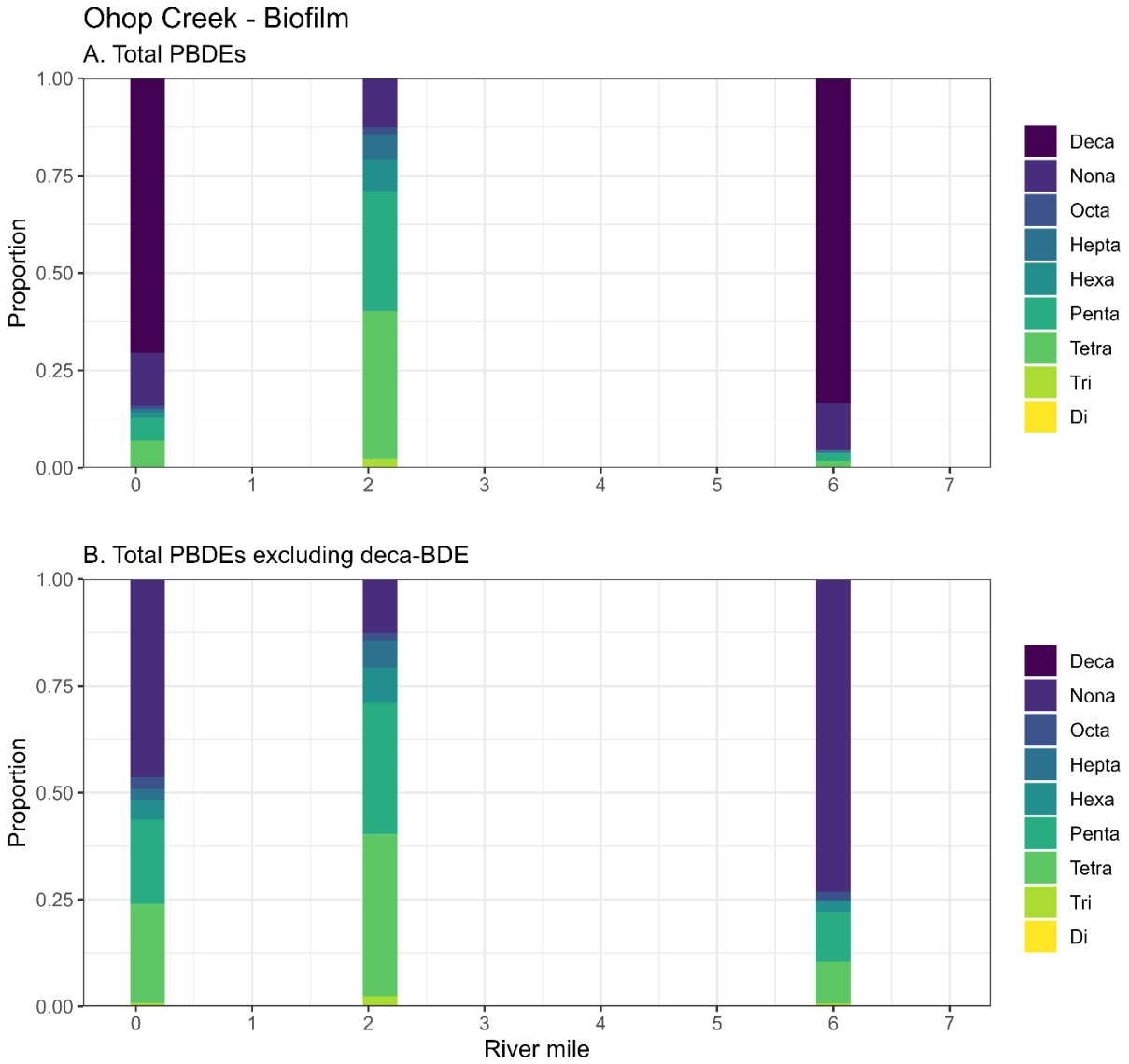
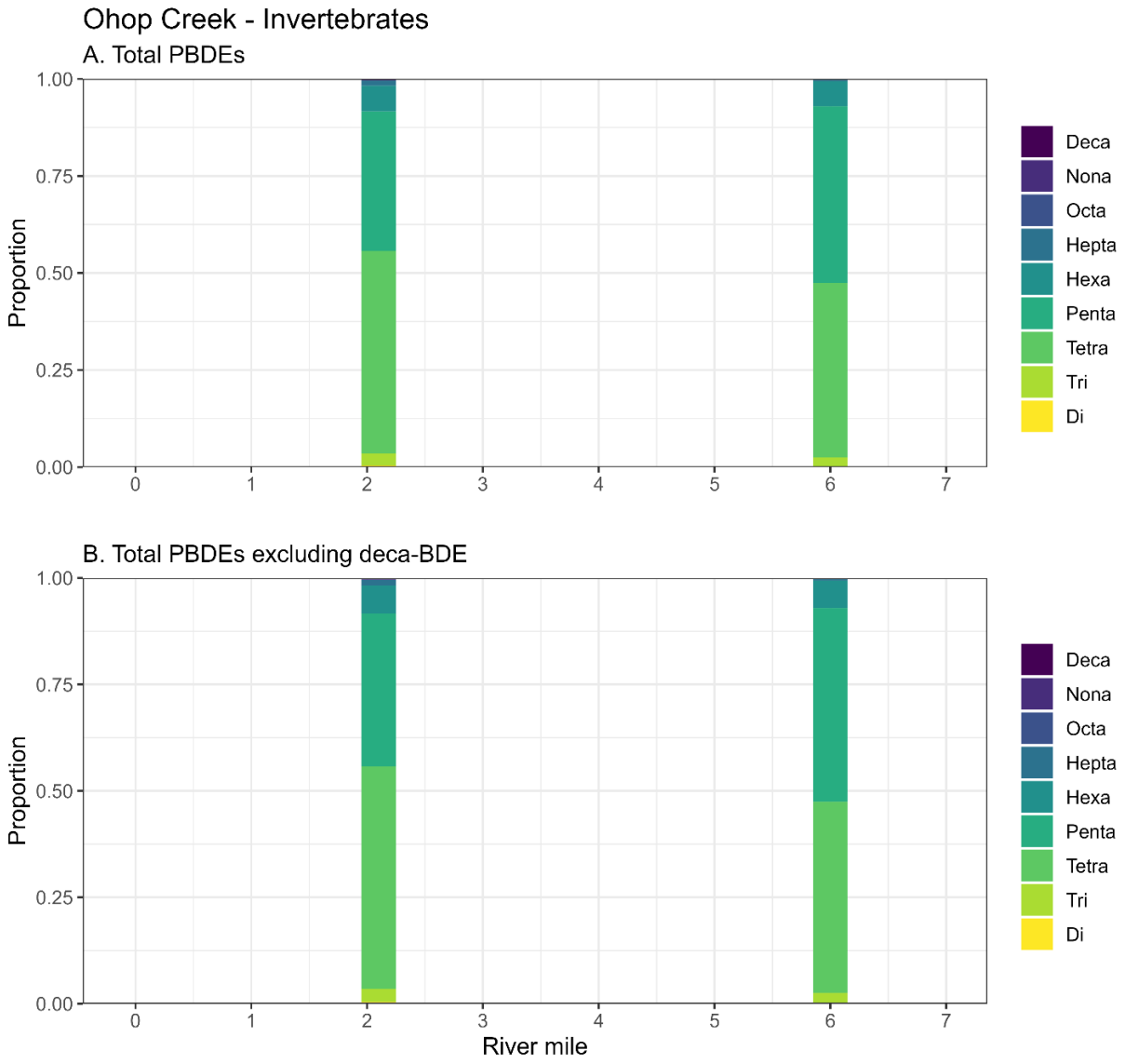
Appendix A

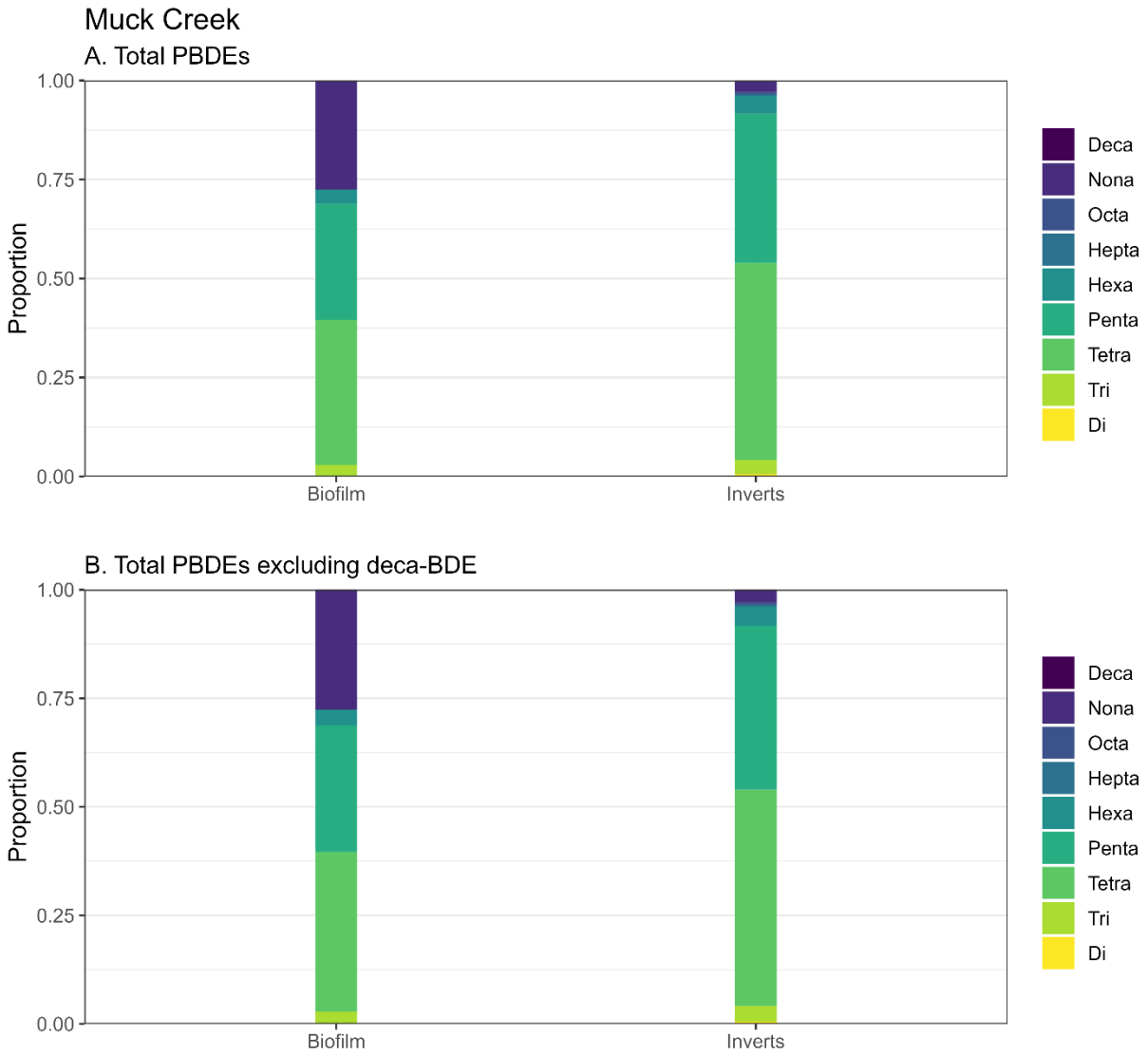
PBDE homolog patterns of biofilm and invertebrates from Ohop and Muck creeks



Appendix A1. Proportion of detected PBDE congeners in biofilm from Ohop Creek summed as homolog groups for A) all measured PBDE congeners (total PBDEs) and B) total PBDEs excluding BDE-209, or deca-BDE. The approximate river mile of the collection site is on the x-axis.



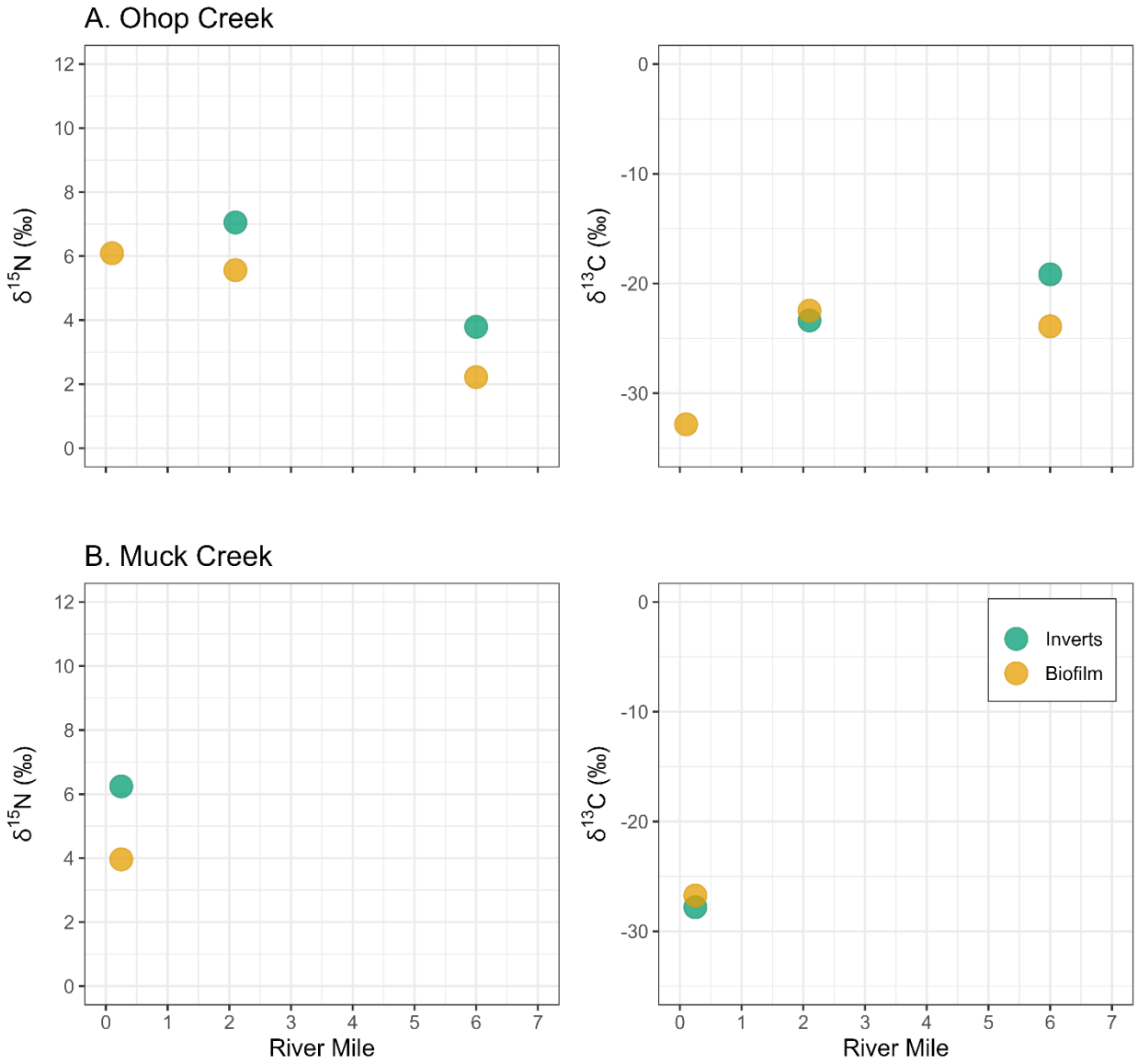
Appendix A2. Proportion of detected PBDE congeners in invertebrates from Ohop Creek summed as homolog groups for A) all measured PBDE congeners (total PBDEs) and B) total PBDEs excluding BDE-209, or deca-BDE. The approximate river mile of the collection site is on the x-axis.



Appendix A3. Proportion of detected PBDE congeners in biofilm and invertebrates from Muck Creek summed as homolog groups for A) all measured PBDE congeners (total PBDEs) and B) total PBDEs excluding BDE-209, or deca-BDE. Both biofilm and invertebrate samples were collected at approximately river mile 0.25.

Appendix B

Ohop and Muck creeks biofilm and invertebrate stable isotope results



Appendix B1. Stable isotopes of $\delta^{15}N$ (left) and $\delta^{13}C$ (right) measured in both invertebrates (green circle) and biofilm (orange circles) collected from Ohop Creek (A) and Muck Creek (B). The approximate river mile of the collection site is on the x-axis.