

3-1-2015

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## Publication Information

Laramie, Matthew B.; Pilliod, David S.; and Goldberg, Caren S. (2015). "Characterizing the Distribution of an Endangered Salmonid Using Environmental DNA Analysis". *Biological Conservation*, 183, 29-37. <http://dx.doi.org/10.1016/j.biocon.2014.11.025>



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Special Issue Article: Environmental DNA

## Characterizing the distribution of an endangered salmonid using environmental DNA analysis

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## ARTICLE INFO

## Article history:

Received 28 February 2014

Received in revised form 3 November 2014

Accepted 14 November 2014

Available online 11 December 2014

## Keywords:

eDNA

Methow

Okanogan

*Oncorhynchus tshawytscha*

Spring Chinook

## ABSTRACT

Determining species distributions accurately is crucial to developing conservation and management strategies for imperiled species, but a challenging task for small populations. We evaluated the efficacy of environmental DNA (eDNA) analysis for improving detection and thus potentially refining the known distribution of Chinook salmon (*Oncorhynchus tshawytscha*) in the Methow and Okanogan Subbasins of the Upper Columbia River, which span the border between Washington, USA and British Columbia, Canada. We developed an assay to target a 90 base pair sequence of Chinook DNA and used quantitative polymerase chain reaction (qPCR) to quantify the amount of Chinook eDNA in triplicate 1-L water samples collected at 48 stream locations in June and again in August 2012. The overall probability of detecting Chinook with our eDNA method in areas within the known distribution was 0.77 ( $\pm 0.05$  SE). Detection probability was lower in June (0.62,  $\pm 0.08$  SE) during high flows and at the beginning of spring Chinook migration than during base flows in August (0.93,  $\pm 0.04$  SE). In the Methow subbasin, mean eDNA concentration was higher in August compared to June, especially in smaller tributaries, probably resulting from the arrival of spring Chinook adults, reduced discharge, or both. Chinook eDNA concentrations did not appear to change in the Okanogan subbasin from June to August. Contrary to our expectations about downstream eDNA accumulation, Chinook eDNA did not decrease in concentration in upstream reaches (0–120 km). Further examination of factors influencing spatial distribution of eDNA in lotic systems may allow for greater inference of local population densities along stream networks or watersheds. These results demonstrate the potential effectiveness of eDNA detection methods for determining landscape-level distribution of anadromous salmonids in large river systems.

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### 1. Introduction

Salmon populations once abundant throughout the Pacific Northwest have declined dramatically, due largely to hydropower development, habitat degradation and overharvest (Mullan, 1987; Nehlsen et al., 1991; FR 76:42658, 2011). The Columbia River drainage once supported some of the largest known runs of Chinook salmon (*Oncorhynchus tshawytscha*) (Chapman, 1986; Utter et al., 1989). Spring Chinook of the Upper Columbia River Evolutionarily Significant Unit (ESU) are now among the most imperiled North American salmon and are currently listed

as Endangered under the Endangered Species Act (ESA) (FR 64:41839, 1999). Costly conservation efforts such as hatchery supplementation, habitat restoration and harvest management have been implemented to conserve remaining populations (LCFRB, 2010; GAO RCED-93-41, 1993). The ability to accurately monitor changes in distribution and to rapidly track responses to management strategies is important for assessing the status and effectiveness of conservation efforts and informs effective decision making (Hernandez et al., 2006; Stem et al., 2005). One major challenge of determining or confirming the distribution of an aquatic species such as Chinook across large landscapes is the low detection rate with conventional methods, especially when the species is present at low densities.

An emerging method that improves detection of many aquatic species is environmental DNA (eDNA) analysis. This method determines presence of a species based on the collection, extraction, and amplification of DNA from the environment (Ficetola et al., 2008;

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Goldberg et al., 2011; Jerde et al., 2011). Recent studies have demonstrated that eDNA detection can be a reliable method for determining the distribution of various species of fish in freshwater ecosystems (Jerde et al., 2011; Dejean et al., 2011; Minamoto et al., 2012; Takahara et al., 2012; Thomsen et al., 2012a; Takahara et al., 2013; Wilcox et al., 2013) as well as in oceans (Thomsen et al., 2012b). eDNA detection methods have been shown to be more sensitive than traditional sampling methods, such as electrofishing or visual surveys, particularly when determining presence of rare or low-density species. Studies have also shown positive correlation between eDNA concentration and relative abundance of the target organism (Thomsen et al., 2012b; Takahara et al., 2012; Goldberg et al., 2013; Pilliod et al., 2013). Inference to the upstream location of stream organisms detected using eDNA is uncertain (Pilliod et al., 2014), but a recent study showed that invertebrate DNA can be transported and detected downstream from known populations as far as 12 km (Deiner and Altermatt, 2014).

Despite the demonstrated effectiveness of detecting fish with eDNA detection methods, few fisheries management programs are currently taking advantage of this state-of-the-art tool for determining the presence of sensitive, native species. This study was designed to test the effectiveness of eDNA detection methods for determining the distribution of threatened and endangered Chinook salmon populations in the Methow and Okanogan Subbasins of the Columbia River by comparing a distribution resulting from eDNA detection to the current, known distribution of the species. We also examined several factors that may influence Chinook eDNA concentrations, such as time of sampling, water temperature as it relates to Chinook habitat preference and tolerance, and sample location along a stream.

## 2. Methods

### 2.1. Study species

Interior Columbia River Chinook are comprised of two lineages, described as ocean- and stream-type, each with a different life history strategy (Healey, 1991; Waples et al., 2004). Ocean-type Chinook adults migrate to freshwater throughout summer and fall and spawn primarily in mainstem rivers. Stream-type Chinook migrate upstream during peak spring flows, which allow them to access preferred spawning habitat in higher headwater tributaries. Spawning takes place in the late summer and fall for both strains, but in different habitats resulting in near complete reproductive isolation (Waples et al., 2004; Beacham et al., 2006; Narum et al., 2007). Upon emergence, juveniles of ocean-type Chinook migrate to the ocean their first spring, as sub-yearlings, while stream-type juveniles remain in freshwater until their second spring before migrating to the ocean as yearlings (Healey, 1991). Therefore, stream-type Chinook are likely present in freshwater systems throughout the year, while ocean-type Chinook are likely only present a portion of the year. Hereafter, we will refer to stream- and ocean-type Chinook by their more commonly used names: *spring* and *summer* Chinook, respectively.

### 2.2. Study area – Methow Subbasin

The Methow Subbasin in western Okanogan County, Washington USA drains 2900 km<sup>2</sup> via the Methow, Chewuch and Twisp Rivers before emptying into the Columbia River near Pateros, Washington (Fig. 1). The Methow contains both spring and summer Chinook (UCSRB, 2007). In 2012, 52,846 Chinook were counted as they migrated from the ocean upstream past Wells Dam, on their way to the Methow and Okanogan Subbasins (DeHart, 2013).

We used existing Chinook distribution maps (UCSRB, 2007) to select sites ( $n = 32$ ) categorized a priori as (1) Chinook likely present (i.e. within the known distribution of Chinook,  $n = 21$ ), or (2) Chinook likely absent (i.e. outside of the known distribution of Chinook,  $n = 11$ ) (Fig. 1, Appendix A). These site-types will be referred to hereafter as *Chinook likely present* and *Chinook likely absent*. Three sample sites of the latter category were physically inaccessible to Chinook (above barriers to anadromy) and served as stream negative controls. All sites in the Methow Subbasin were sampled twice, once during high, spring flows from 22 to 27 June 2012, and again during reduced late-summer flows from 9 to 13 August. We also collected three water samples from a juvenile spring Chinook rearing tank at US Fish and Wildlife Service (USFWS) Winthrop National Fish Hatchery (WNFH, Winthrop, WA USA) on 26 June 2012. These samples served as laboratory positive controls, and were omitted from the distribution analysis. In general, stream flows were approximately 10 times higher during spring runoff in June than later in August, as flows approached base-flow. During June sampling, flows ranged from 242 m<sup>3</sup>/s in the mainstem Methow River (USGS stream gage 12449950) to approximately <1 m<sup>3</sup>/s in small tributaries (visual estimate).

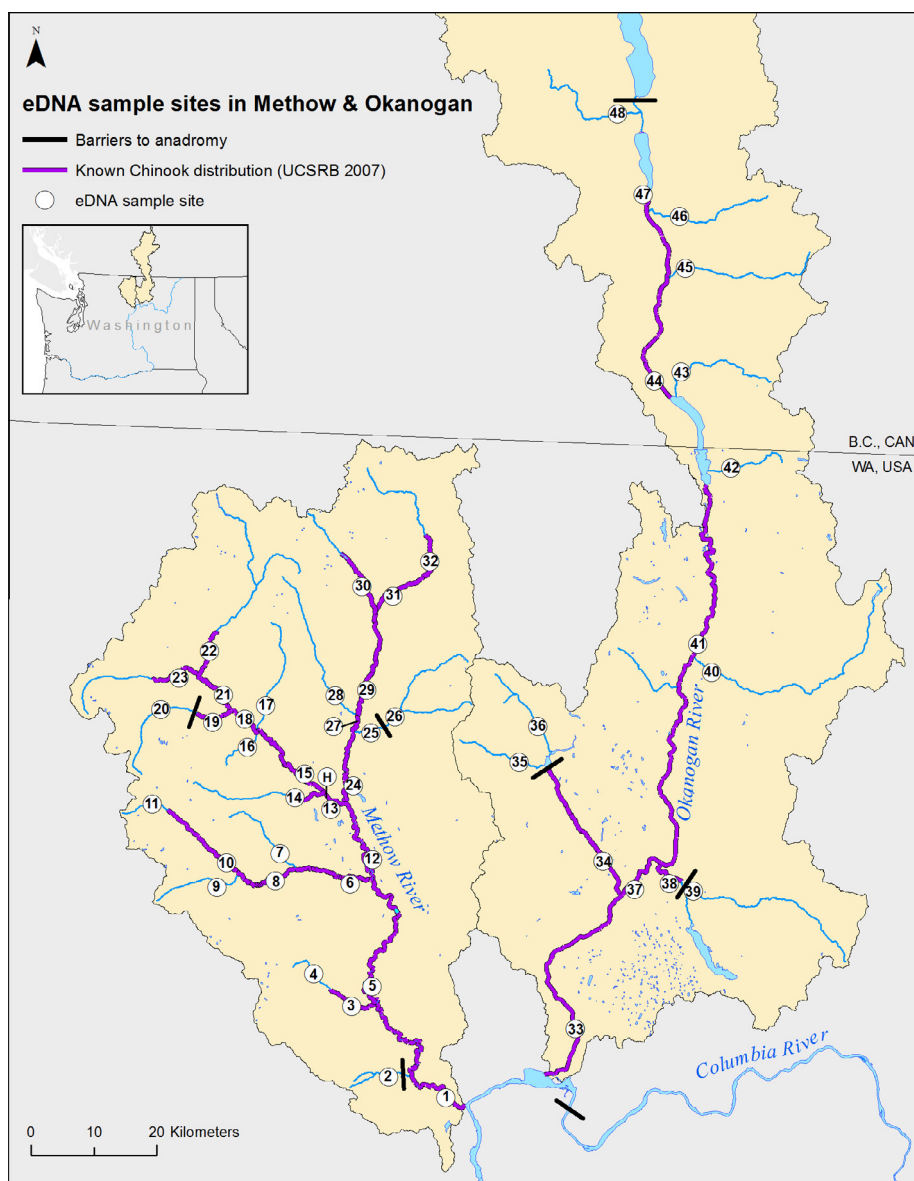
### 2.3. Study area – Okanogan Subbasin

The Okanogan Subbasin is adjacent to and east of the Methow and spans the border between Washington, United States and British Columbia, Canada (Fig. 1). The Okanogan Subbasin is more than four times the size of the Methow, draining approximately 13,000 km<sup>2</sup>. The Okanogan contains summer Chinook; spring Chinook were extirpated from this subbasin by the 1930s (UCSRB, 2007). Migrating spring Chinook adults from nearby subbasins may occasionally stray into the Okanogan, suggesting potential for presence of a very low-density population (J. Arterburn, CCT F&W biologist, personal communication). The Colville Confederated Tribes plan to reestablish spring Chinook throughout much of their historic range in the Okanogan as an experimental population under section 10(j) of the ESA (FR 76:42658, 2011). The source stock for the Okanogan reintroduction would initially come from the adjacent Methow Subbasin. We sampled 16 sites in the Okanogan Subbasin (Fig. 1, Appendix A), consisting of both Chinook likely present sites ( $n = 7$ ) and Chinook likely absent sites ( $n = 9$ ). All sites were sampled twice, once during high spring flows from 18 to 21 June 2012, and again during reduced late-summer flows from 14 to 17 August. These surveys will serve as the baseline distribution (prior to the reintroduction of spring Chinook to the Okanogan Subbasin) and can be used as part of a monitoring program to track changes in Chinook distribution following their reintroduction.

As in the Methow Subbasin, stream flows in the Okanogan were approximately 10 times higher during spring runoff in June than in August, as flows approached base-flow. During June sampling, flows ranged from 390 m<sup>3</sup>/s in the mainstem Okanogan River to 0.03 m<sup>3</sup>/s in small tributaries (USGS stream gages 12447200 and 12438900, respectively).

### 2.4. Field methods

At each sample site, we filtered three 1-L stream water samples, treated as replicates, followed by one 1-L negative control composed of distilled water. Water was filtered through a Whatman Disposable Filter Funnel with 47 mm diameter, 0.45 μm pore size cellulose nitrate type WCN sterile filter membrane (Whatman International Ltd., England). The filter funnel was connected to a Masterflex L/S Econodrive peristaltic pump. We held the filter funnel just below the surface of the stream, facing upstream, into the current. The pump was engaged until 1-L of stream water was collected. We collected water samples at approximately an arm's



**Fig. 1.** Numbered eDNA sample sites throughout Methow and Okanogan Subbasins. Inset shows study area location in north central Washington State, USA and southern British Columbia, Canada. 'H' indicates location of Chinook hatchery (WNFH).

length from the stream bank, thus reducing potential contamination resulting from entering the water at each site.

After filtering, we removed the filter from the disposable funnel and placed it into a sterile 2-ml vial with 200-proof molecular grade ethanol for preservation. Sample vials were stored at 4 °C until DNA could be extracted. Water temperature was collected at each site, approximately 5 cm below the water surface, at the time of sampling.

### 2.5. Molecular assay design and verification

We developed a species-specific qPCR assay for Chinook targeting a 90 base-pair sequence of the cytochrome c oxidase subunit I (COI) region within the mitochondrial genome, a region that has been targeted and sequenced for a wide range of organisms for DNA barcoding (Hebert et al., 2003). The probe was labeled with 6-FAM at the 5'-end and a minor groove binding non-fluorescent quencher (MGB-NFQ; Thermo Fisher Scientific Inc., Waltham, MA) at the 3' end. We used Primer Express 3.0 software

(Thermo Fisher Scientific Inc.) to evaluate and select the target amplicon with F-primer: 5'-CTG GCA CMG GGT GAA CAG TCT ACC-3', R-primer: 5'-AAT GAA GGG AGA AGA TCG TYA GAT CA-3' (Integrated DNA Technologies, Inc., Coralville, IA), and probe: 6FAM-CTC CTG CGT GGG CTA G-MBG-NFQ). A BLAST search was conducted to check specificity of the assay, ensuring that a 100% match existed for Chinook, and not for sequences from non-target species likely present in the study area. The selected assay contains a minimum of 3 dissimilar bases between Chinook and closest relative Coho salmon (*O. kisutch*) (Healey, 1991); our primer and probe design placed these dissimilar bases towards the 3' ends of each primer to reduce amplification of non-target DNA (Wright et al., 2013).

Target species (Chinook) fin clips from the Columbia River region ( $n = 20$ ), were obtained from various agencies as were fin clips from non-target, co-occurring related species (*Oncorhynchus mykiss*, *O. clarki*, *O. kisutch*, *O. nerka*, *Cyprinus carpio*, *Lepomis macrochirus*, *Micropterus salmoides*, *M. dolomieu*, *Pomoxis nigromaculatus*, *Cottus bairdii*, *Ictalurus punctatus*, *Catostomus columbianus*, *Ptychocheilus oregonensis*, *Prosopium williamsoni*, *Salmo trutta*, *Perca*

*flavescens*, *Ameiurus* spp., *Richardsonius balteatus*) to directly test assay specificity.

To verify our assay would detect only Chinook DNA, we extracted DNA from the fin clips from our target and co-occurring non-target species in the study area using a Qiagen's DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's protocol. All Chinook tissue samples produced positive detections using the Chinook assay, while none of the non-target tissue samples produced a positive detection with the exception of Coho salmon (*O. kisutch*). However, 0.01× dilutions of Coho DNA extracted from fin clips failed to amplify using the Chinook assay. This 0.01× Coho DNA dilution was higher concentration than any of the Chinook eDNA extracts from environmental samples collected in this study, and thus we believe to be higher than is likely to be encountered in natural systems. To further ensure that this cross-amplification would not be a problem in environmental samples, we tested three 1-L water samples collected from Peterson Creek (Southeast, AK USA), a stream without Chinook, but with a high concentration of Coho (Johnson and Daigneault, 2013); none of the samples tested positive using the Chinook assay.

## 2.6. DNA extraction of filtered eDNA

We extracted eDNA from filter samples using a QiaShredder and Qiagen's DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) following the protocol of Goldberg et al. (2011). One half of the filter was used for extraction, the remaining half was archived. Ethanol was allowed to evaporate from the extraction filter half for 24 h prior to extraction. Final elutes were stored in 100 µl AE buffer.

## 2.7. qPCR of field collected samples

DNA extracted from filters was analyzed using Quantitect multiplex PCR mix (Qiagen GmbH) and checked for inhibition in one of two ways: (1) the Chinook assay was run first, followed by analysis with Taq-Man Exogenous Internal Positive Control Reagents (Exo-IPC) (Thermo Fisher Scientific Inc.) for samples testing negative (i.e. not duplexed), or (2) the Chinook assay was run in duplex with IC (Qiagen GmbH). Non-duplexed samples contained 1X Quantitect MasterMix, 0.2 µM forward primer, 0.2 µM reverse primer, 0.2 µM probe and 3 µl DNA extract in 15 µl total reaction volume. Reactions testing for inhibition using Exo-IPC consisted of 1× Quantitect MasterMix, 0.4× Exo-IPC assay, 1X Exo-IPC DNA and 3 µl DNA extract in a 15 µl total reaction volume. Reactions duplexed with IC consisted of: 1× Quantitect MasterMix, 0.2 µM forward primer, 0.2 µM reverse primer, 0.2 µM probe, 0.5× IC assay, 0.5× IC template and 3 µl DNA extract in a 15 µl total reaction volume. Cycling conditions for all reactions consisted of 15 min PCR initial heat activation at 95 °C, followed by 50 cycles of 60 s denaturing at 94 °C, with 60 s annealing/extension at 60 °C for Exo-IPC reactions, and 70 °C for all others. A standard curve was calculated using DNA extracted from Chinook tissue, quantified using a NanoDrop (Thermo Fisher Scientific Inc.), and then included in serial dilution ( $10^{-2}$  thru  $10^{-6}$ ) with each plate. All plates also contained a PCR non-template control (molecular grade water). Samples were run on 96-well clear optical plates on an ABI 7300 or ABI 7500FAST Real-time PCR System (Thermo Fisher Scientific Inc.). All field samples were run in triplicate to ensure detection of degraded or low-quantity DNA (Waits and Paetkau, 2005). When DNA was detected in all three qPCR triplicates, we averaged the DNA concentration from the three to create a single value for each 1-L field sample. qPCR triplicates that produced ambiguous results (i.e. not all positive or all negative) were re-run to ensure that the mixed results were not due to contamination within the plate. If the results remained ambiguous,

all 6 replicates were averaged. If all three qPCR triplicates came back negative on the second run, then the sample was considered negative for eDNA. Test results were considered negative if no exponential phase occurred during the 50 cycles of PCR. Any samples that showed signs of inhibition (delayed or non-amplification of internal controls) were diluted to 0.1× to reduce inhibition and re-run in triplicate (Pilliod et al., 2013). Analysis of qPCR data was conducted using AB Sequence Detection Software (Version 1.4.0.25, Thermo Fisher Scientific Inc.). The qPCR product from a subset of field samples ( $n = 11$ ) were sent to GeneWiz Inc. (South Plainfield, NJ), where they were purified using ExoSAP-IT (Affymetrix Inc., Santa Clara, CA) and sequenced using Sanger sequencing to verify that the PCR product obtained from field samples was comprised only of our intended target sequence (Appendix B).

## 2.8. Data analysis

To determine compatibility in detection of Chinook distribution between the eDNA method and conventional methods we used a Pearson's Chi-squared test with Yates' continuity correction to compare detection at Chinook likely present sites and Chinook likely absent sites.

We calculated detection probability during June and August sampling events as the average of individual site detection rates during each sampling event  $\pm$  SE. We calculated individual site detection rates as the number of 1-L replicates that tested positive for Chinook eDNA ( $n = 0-3$ ) at a site divided by the number of replicates collected at that site ( $n = 3$ ). This was calculated for the June ( $\rho_{\text{June}}$ ) and August ( $\rho_{\text{August}}$ ) sampling events separately, as well as combined ( $\rho_{\text{Combined}}$ ), for Chinook likely present sites ( $n = 28$ ).

For analyses of eDNA concentration, we calculated and used the average eDNA concentration across the three 1-L field samples (i.e., field replicates) at each site during each sampling event. This reduced the variability sometimes found in eDNA concentration estimates (Pilliod et al., 2013) and allowed us to calculate variance around each estimate.

We expected eDNA concentrations to decrease as distance upstream increased, due to fewer Chinook inhabiting the higher headwater tributaries, and also due to a downstream accumulation of genetic material. We examined this by plotting eDNA concentrations by distance upstream, for sites in the three primary watersheds within the Methow Subbasin (Twisp, Methow and Chewuch watersheds) and fitting linear regressions to each. Total distance ranged from 94 km in the Twisp watershed (sites 1, 5, 6, 8, 10, 11), 110 km in the Methow watershed (sites 1, 5, 12, 13, 15, 18, 21, 23) and 117 km in the Chewuch watershed (sites 1, 5, 12, 24, 27, 29, 31, 32) (Fig. 1). We measured the distance upstream from the Columbia River confluence using ArcGIS v10.1 software (ESRI, Inc.).

To test for a difference in Chinook eDNA concentrations present in June and August, we used separate Wilcoxon signed rank tests (with continuity correction) to compare mean eDNA concentrations collected in June and August for the Methow mainstem ( $n = 3$ ), Okanogan mainstem ( $n = 4$ ), Methow tributaries ( $n = 21$ ) and Okanogan tributaries ( $n = 5$ ). Analysis included only sites that had Chinook eDNA detected during at least one sampling event (i.e., given presence).

To examine the rate of false-negatives (and the number of necessary 1-L replicates that might be collected at each site in future eDNA studies), we considered only sites with confirmed presence of Chinook, indicated by at least one positive 1-L replicate at a site, during a sampling event. Our rate of false-negatives was then the number of 1-L replicates in which no Chinook eDNA was detected divided by the total number of replicates collected at sites where Chinook were confirmed present.

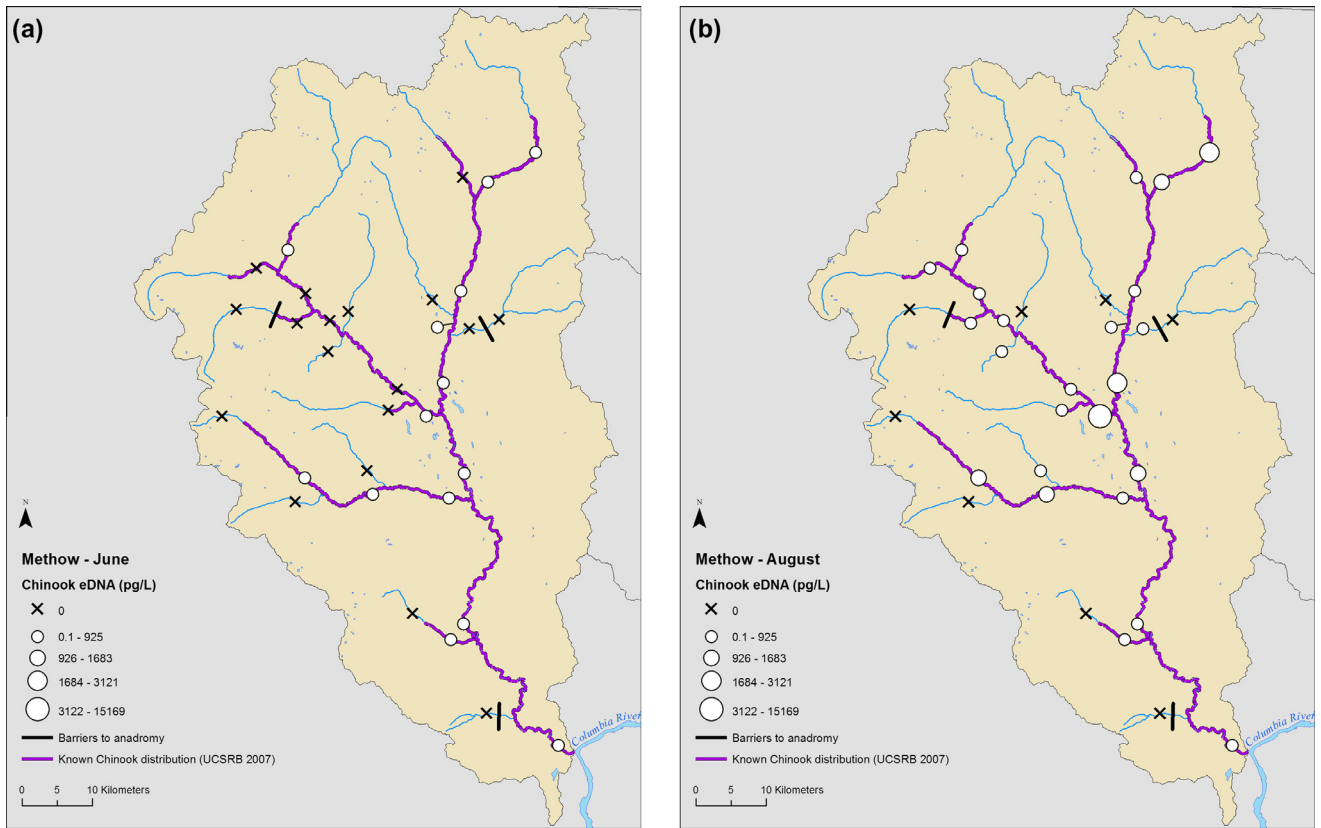


Fig. 2. Chinook eDNA concentrations in the Methow Subbasin. (a) June 2012 and (b) August 2012.

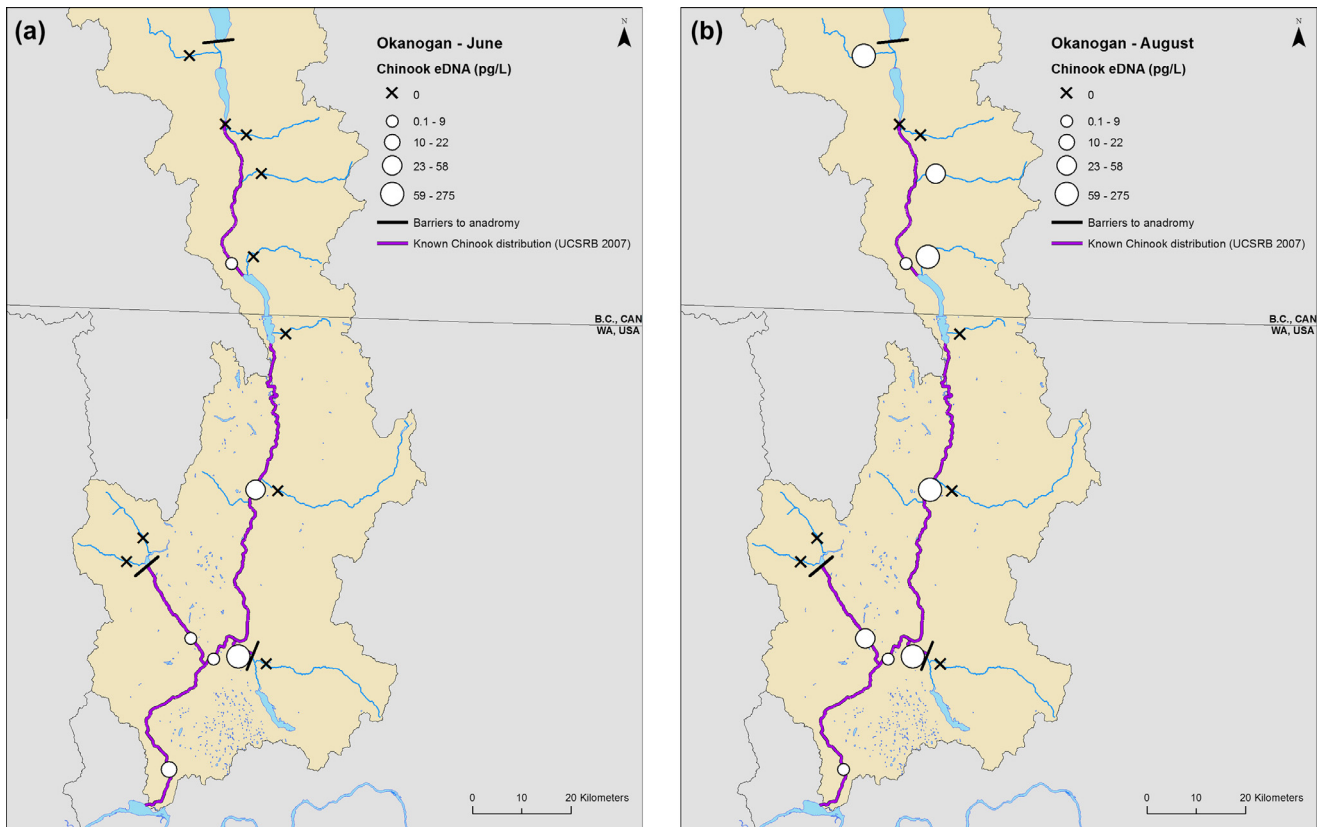


Fig. 3. Chinook eDNA concentrations in the Okanogan Subbasin. (a) June 2012 and (b) August 2012.

To examine the relationship between eDNA concentration at sample sites and water temperature, we plotted June and August values and fit an exponential regression model to eDNA concentrations for samples with temperatures >18 °C, the EPA recommended maximum habitat temperature to safely protect against lethal conditions for both juvenile and adult salmonids (EPA, 2003). If eDNA concentrations reflect the density of individuals at sampling sites, we would expect a decrease in eDNA with increasing temperatures above this threshold.

Site 40 was omitted from statistical analysis due to PCR inhibition that was not resolved using dilution. Statistical analyses were performed using R-Statistical Software (Version 2.15.3, 2013-03-01, © 2013 The R Foundation for Statistical Computing).

### 3. Results

We detected Chinook at 27 of 28 sites where they were expected to be present (Table 1) and an additional 6 sites where they were not expected given the reference maps (sites 7, 16, 25, 43, 45 and 48; Fig. 1). These sites were all accessible to Chinook (i.e., above no known barriers). Sequenced qPCR product from these sites confirmed that the fragment consisted only of the intended target sequence (Appendix B). The effectiveness of our eDNA methods in determining the distribution of Chinook in these large watersheds was not likely due to chance ( $\chi^2 = 19.7654$ ,  $df = 1$ ,  $p$ -value <0.0001).

We did not detect Chinook DNA in any of our distilled water field negative controls ( $n = 97$ ), laboratory DNA extraction negative controls ( $n = 23$ ) or qPCR negative controls ( $n = 20$ ). With triplicate sampling (three 1-L replicates collected per site, per sampling event) there were 4 possible non-ordered site detection outcomes (Table 2). We found 13 cases of false negatives within our replicates from sites that produced at least one positive detection for Chinook eDNA during a sampling event (i.e., Chinook were confirmed present at the site). This represented an 8.2% (13 of 159) rate of false negatives where replicates failed to detect Chinook eDNA when the species was present.

In both the Methow and the Okanogan Subbasins, we found an increase in the percentage of sites that tested positive for presence of Chinook DNA between the June and August sampling events (Table 3). Detections increased by 71.4% from June to August in the Methow and by 50.0% from June to August in the Okanogan. These increases tended to be further upstream in the mainstem rivers and in smaller tributaries (Figs. 2a and b; 3a and b). At Chinook likely present sites (i.e. assuming presence), the  $\rho_{\text{Combined}}$  was  $0.77 \pm 0.05$  SE (June and August combined), while  $\rho_{\text{June}}$  was  $0.62 \pm 0.08$  SE, and  $\rho_{\text{August}}$  was  $0.93 \pm 0.04$ .

Among sites that tested positive for Chinook eDNA during at least one sampling event: eDNA concentrations increased from June to August in tributaries to the Methow River (11.75 and 1391.87 pg/L, respectively,  $p$ -value <0.0001) but not the mainstem Methow River (measured at 50.72 and 743.26 pg/L, respectively) (Fig. 4). June and August concentrations did not differ in the

**Table 1**  
eDNA detection versus distribution determined using traditional survey methods.

Known distribution <sup>a</sup>	eDNA methods		
	Detected	Not-detected	Number of sites
Chinook likely present sites	27	1	28
Chinook likely absent sites	6	13	19

<sup>a</sup> 'Known distribution' adapted from Upper Columbia River Salmon Recovery Board Spring Chinook and Steelhead Recovery Plan 2007 (UCSRB, 2007) and personal communication with regional fisheries biologists.

**Table 2**

Percentage of 1-L water samples that tested negative and positive for presence of Chinook DNA out of 282 replicates (47 sites \* 2 sampling events \* 3 1-L replicates), based on triplicate sampling at each sample site.

Possible detection outcomes at a site (with three, 1-L replicates) <sup>a</sup>	% of sites
000	43.6
100, 010, 001	5.3
110, 101, 011	3.2
111	47.9
Total	100

<sup>a</sup> Note: 0 = No Chinook eDNA detected, 1 = Chinook eDNA detected.

**Table 3**

Increase in sites with positive detection of Chinook eDNA from June to August 2012 sampling events (a site was considered positive (+) when at least one 1-L replicate tested positive).

	Sites	Sites (+) June	Sites (+) August	Increase (%)
Methow	32	14	24	71.4
Okanogan	15	6	9	50.0
Combined	47	20	33	65.0

Okanogan mainstem (22.32 and 15.57 pg/L, respectively) or tributaries (57.05 and 93.21 pg/L, respectively). The 10 highest concentrations of Chinook eDNA were collected at sites with water temperatures ranging from 12.1 °C to 18.2 °C (Fig. 5). Concentrations decreased exponentially at sites with water temperatures >18 °C ( $R^2 = 0.87$ ).

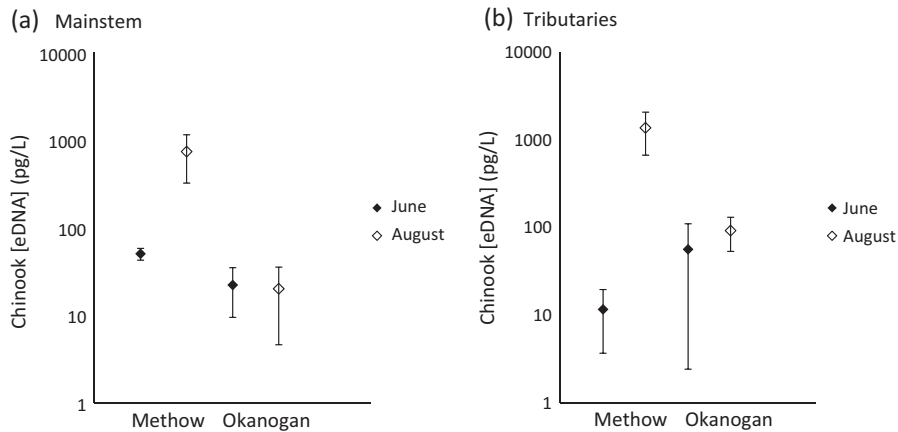
Samples collected at a spring Chinook rearing tank at WNFH produced the highest eDNA concentrations throughout the study (Appendix C) and served only as positive controls. Of true field samples, the highest Chinook eDNA concentrations were found at site 13, which was the site located immediately downstream from the hatchery.

Our hypothesis that Chinook eDNA concentrations would decrease as distance upstream increased (i.e. that eDNA would accumulate downstream) was not supported. Concentrations varied in a non-linear fashion in the Methow, Twisp and Chewuch watersheds (all  $p$ -values >0.05, linear regression  $R^2 = 0.0012$ , 0.0625 and 0.2275, respectively; Fig. 6).

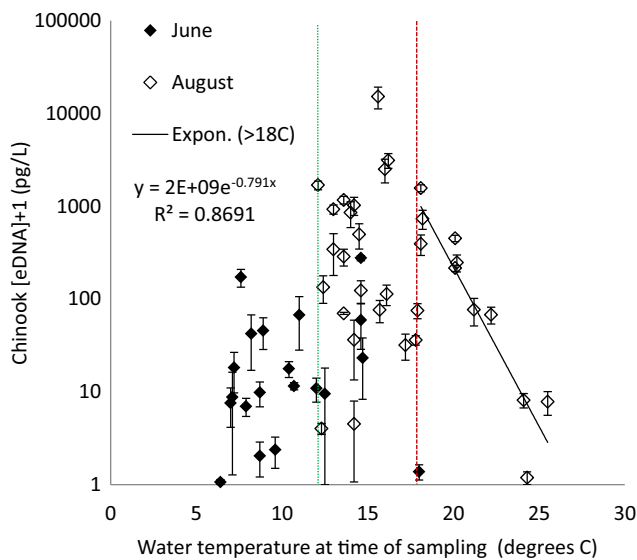
## 4. Discussion

### 4.1. High detection rate of eDNA

Our overall detection probability (0.77) and the detection of Chinook eDNA at 6 additional sites outside of their known range indicates that eDNA detection is likely to be an effective method for determining the distribution of Chinook throughout large watersheds. These additional detections also indicate that the distribution of Chinook in these subbasins may be larger than previously described. To reduce false-negatives, studies using eDNA detection have tended to collect water in triplicate at a sample site (Ficetola et al., 2008; Goldberg et al., 2011; Pilliod et al., 2013). We found that with confirmed presence of Chinook eDNA at a site, 8.2% of our 1-L replicates produced false negatives (Table 2). This suggests that, at least in our application, fewer samples could be collected at a site to reduce the cost of eDNA sampling by up to two-thirds (1 sample versus 3), while still maintaining a low rate of false negatives (<10%).



**Fig. 4.** (a) Mean Chinook eDNA concentrations from mainstem Methow and Okanogan River sites, Methow June, August:  $n = 3$ , Okanogan June, August:  $n = 4$ . (b) Mean Chinook eDNA concentrations at sites in tributaries of the Methow and Okanogan Rivers. Data only include sites where Chinook were detected during at least 1 sampling event (June or August), Methow June, August:  $n = 21$ , Okanogan June, August:  $n = 5$ . Diamonds indicate sample means, bars represent standard error.

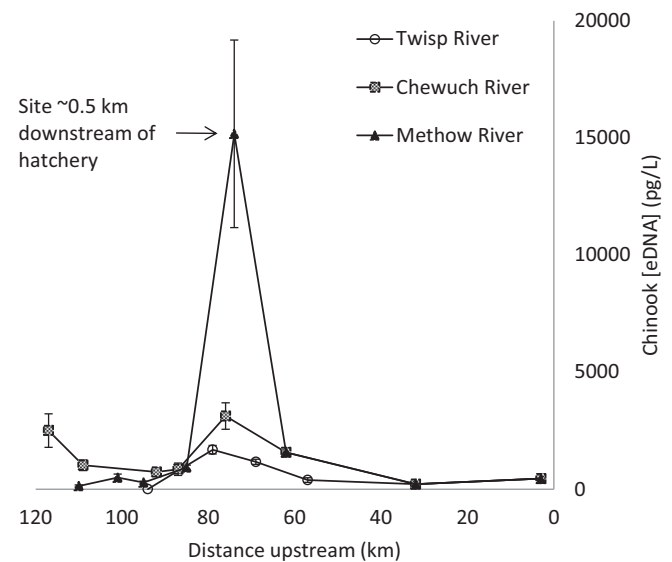


**Fig. 5.** Concentration of Chinook eDNA at sites throughout study area by water temperature at time of sampling. Green line represents optimum Chinook rearing temperature, 12.5 °C (Beschta et al., 1987). Red line represents EPA recommended maximum habitat temperature for salmonids, 18 °C (EPA 2003). Points indicate eDNA concentration at a sample site, bars represent standard error. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Temporal variation in eDNA detection and concentration

The high detection probability (0.93 in August) suggests a high sensitivity of the eDNA method to species presence, as has been determined by other studies (Ficetola et al., 2008; Jerde et al., 2011; Goldberg et al., 2011; Takahara et al., 2012; Spear et al., 2015). Reduced detection rates during the June sampling event (0.62) suggests that either higher flows dilute available eDNA (reducing probability of collecting DNA in our 1-L samples) or that Chinook were not present in some stream reaches during June. It is likely that additional Chinook migrated into the system between sampling events. Therefore, differences in our detection rates between June and August may reflect true differences in occurrence, rather than solely an increase in detection probability due to reduced flow.

Sites that tested positive for Chinook DNA during the June sampling event ( $n = 20$ ), especially those in the smaller tributaries,



**Fig. 6.** Variation in eDNA concentration along the entirety of three watershed in the Methow Subbasin; Twisp River (sites, 1, 5, 6, 8, 10, 11), Chewuch River (sites 1, 5, 12, 24, 27, 29, 31, 32) and Methow River (sites 1, 5, 12, 13, 15, 18, 21, 23). Asterisk (\*) indicates distance upstream of Winthrop National Fish Hatchery along the Methow River (artificial input of Chinook genetic material).

likely indicate the presence of spring Chinook (rather than summer Chinook). These are either adults arriving on their early upstream migration or sub-yearling juveniles that had yet to migrate to the ocean. Although the Okanogan Subbasin spring Chinook population is currently listed as extirpated (UCSRB, 2007), there is evidence of PIT tagged spring Chinook straying from nearby subbasins into the Okanogan (J. Arterburn & B. Miller, CCT F&W biologists, personal communication). This could account for the early detections. However, summer Chinook juveniles that fail to migrate to the ocean, potentially up to about 40% of males in a hatchery population (Larsen et al., 2004; Larsen et al., 2013), could also contribute to detectable Chinook eDNA during the June sampling event, particularly at sites lower in the basin.

4.3. Spatial variation of eDNA along streams

The positive control samples collected from the rearing tank at WNFH (site H) held high densities of juvenile spring Chinook at the time of sampling. The tanks were designed to allow a mix of



groundwater and Methow River water to flow into and through the tanks, and then through an outflow just below the hatchery, feeding back into the Methow River. This 'artificial input' of Chinook genetic material resulted in a spike in eDNA at the site immediately downstream (site 13, Fig. 6). Concentrations were greatly reduced at sites successively further downstream of this hatchery, suggesting that eDNA concentrations are somewhat localized and do not appear to simply accumulate downstream, at least at the scale of this study. This is supported by the findings of Denier and Altermott (2014) that showed a 12 km transport and detection distance for aquatic invertebrate eDNA, and Spear et al. (2015) that found amphibian eDNA varied in concentration, but without a consistent pattern, along several stream reaches. A confounding factor, not accounted for in this study, was the discharge input of tributaries between sites, which may have either added to the Chinook eDNA concentration, or diluted the eDNA concentration. Further research is necessary to better understand eDNA transport and the limitations of upstream inference.

Our baseline survey of Chinook presence in the Okanogan Subbasin shows distribution primarily along the mainstem Okanogan, typical of summer Chinook distributions, with occasional occurrence in some of the larger tributaries as mentioned. These baseline data will serve as an initial monitoring survey, prior to the release of an experimental population of spring Chinook in the Okanogan Subbasin by the Colville Confederated Tribes. With future monitoring, and by comparing back to these survey data, they should be able to track changes in Chinook distribution following the reintroduction, helping them to assess its progress.

#### 4.4. Future directions for eDNA detection of Chinook

Water temperature has been shown to affect both the likelihood of Chinook presence, in terms of habitat suitability (Brett, 1952; Coutant, 1977) as well as the degradation rate of DNA (Zhu, 2006; Strickler et al., 2015). Our results show highest maximum eDNA concentrations at sites with water temperatures slightly above the ideal temperature range of the species. An exponential decrease in Chinook eDNA concentrations occurred from 18 °C to 25.5 °C (our highest water temperature), which is similar to results from studies using traditional detection methods that found decreased rearing densities of several salmonids around the same temperature range (Frissell, 1992). While our results are somewhat confounded by the increased rate of DNA degradation that occurs at higher temperatures, this may suggest a potential use of eDNA detection methods to help determine habitat preference or confirm habitat suitability across large habitats.

The molecular assay used in this study was designed to detect the presence of Chinook salmon at the species level. To better understand distributions of spring versus summer Chinook within a subbasin, without relying on spatial and temporal assumptions, an assay capable of accurately differentiating between the two life-history types, perhaps targeting single-nucleotide polymorphisms in additional regions of the Chinook genome, would be a necessary and valuable tool.

## 5. Conclusions

Salmonid distributions can be determined using the molecular methods described in this study. While we admittedly did not control for stream discharge, concentrations of genetic material do appear to be localized, or limited, in terms of their ability to be transferred and accumulated downstream by flowing water. These localized concentrations of Chinook eDNA may be caused by spatial variation in fish densities. Further research should focus on identifying limiting factors of eDNA transport and persistence

and modeling local fish densities across large landscapes using eDNA analysis.

This study can help inform the development of monitoring programs using eDNA to determine the distribution of salmonids in large watersheds. For the purpose of population monitoring, this method is not necessarily intended to replace traditional survey methods such as electrofishing or snorkel count surveys (especially where count data or actual, rather than relative, density is required) but could be a valuable, complementary tool to rapidly determine distributions and assess and prioritize stream reaches to better assign limited resources.

## Acknowledgements

Funding for this study was provided by Colville Confederated Tribes Fish & Wildlife Department (CCT F&W), U.S. Geological Survey, and the USFWS Great Northern Landscape Conservation Cooperative. C.S. Goldberg was funded by the Department of Defense Environmental Security Certification Program under ESTCP Project RC-201204. We thank Katherine Strickler, Jim Smith, Peter Koetsier and others for their manuscript reviews. Laboratory space, equipment and training were provided by Lisette Waits & Jennifer Adams at University of Idaho Department of Fish and Wildlife Sciences and Jim Smith & Kevin Feris at Boise State University Department of Biological Sciences. We are grateful for fish tissue sample contributions from Jeff Stephenson at Hagerman Fish Culture Experiment Station (CRITFC); Matthew Campbell (Idaho Department of Fish and Game); Brian Miller, Darin Hathaway & others at CCT F&W; Matt Cooper and Christian Smith at USFWS Abernathy Fish Technology Center. Joe Peone, John Arterburn, Keith Wolf, Brian Miller, Jennifer Panther (CCT F&W) provided data support, tissue samples and regional information. Charles Frady (Washington Department of Fish and Wildlife) provided regional information regarding site selection. The USFWS allowed us to collect water samples from WNFH. Jeffrey Williams (Alaska Department of Fish and Game) provided water samples from Peterson Creek and Herbert River. Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

## Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocon.2014.1.025>.

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