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Effectiveness Monitoring in the Columbia Wetlands: e-DNA Metabarcoding Pilot Study

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Columbia Wetlands Stewardship Partners

Photo from Columbia Wetland Stewardship Partners

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Abstract

The “Climate change mitigation in the Columbia Wetlands project” aims to restore ecosystem functions of the wetlands to aid in the conservation of Species at Risk (SARA) that are dependent on these wetlands. Restoration techniques included the use of beaver dam analogues to improve habitat for Species at Risk.

This report focuses on the use of environmental DNA (eDNA) metabarcoding to investigate climate change vulnerability of wetlands and restoration effectiveness. The eDNA method allows estimation of species richness from collected benthic samples without the need for traditional microscopic taxonomic identification. We are using eDNA metabarcoding method to quantify the genetic composition and the biodiversity of vulnerable wetlands and assess the effects of restoration activities in the context of an adaptive-management framework.

eDNA monitoring includes analysis of samples for macroinvertebrates, fish, and diatom genomic sequences to evaluate use of these taxa as a component of wetland effectiveness monitoring. In addition, to the genomics work we are monitoring other key ecosystem attributes including hydrology, SARA-listed species, migratory birds and vegetation and mapping.

We have conducted spring and summer sampling sessions. In the 2023 spring sampling session, we completed an initial assessment of dominant trends in species richness. We found that a least four replicates per wetland were required were needed to improve precision, reduce variance to improve the power to detect differences between wetlands. Graphical inspection of percent richness of Orders Odonata, Ephemeroptera and Trichoptera (OET) suggests that decreasing connectivity to the Columbia River may favor these group of species.

We received eDNA data from the summer session on March 10, 2024, which prevented detailed analyses in the current report. Analyses of the 2023 summer session data will provide more in-depth analyses of the biodiversity of the wetlands. This report details results from the spring session.

Contents

Introduction

The overarching goal of the "Climate change mitigation in the Columbia Wetlands project" is to conserve species at risk and the abiotic and biotic components of vulnerable wetland ecosystems that support these species through restoration activities including the use of Beaver Dam Analogues (BDAs) (Figure 1). The Columbia Wetlands are an important globally significant wetland complex, but these wetlands are vulnerable and drying because of alterations in the runoff regimes and loss of open water and aquatic habitats due to climate change (Rodrigues et al. 2023, Goodbrand and MacDonald, 2022, Hopkinson 2020).

The Columbia Wetland Stewardship Partners (CWSP) aims to retore open water habitats in the Columbia Wetlands for Species At Risk (SARA), migratory birds, fish and ecosystem functions using BDAs. BDAs are artificial beaver dams which can be used to restore abandoned natural beaver dams and the timing of wetland hydrology by slowing the post-freshet recession and increasing water storage. Beaver dam analogues essentially "plug leaks" in the wetlands where natural beaver dams have failed mimicking natural ecological processes and restoring open water areas for wildlife.

The wetland effectiveness monitoring plan for the project is a collaborative effort to assess the effectiveness of beaver dam analogues as a nature-based restoration tool in the Columbia Wetlands and describe how BDAs restore the hydrology, and ecology and of vulnerable wetlands. eDNA metabarcoding and other methods are being used to track restoration goals in a wetland effectiveness monitoring plan.

The project led by Dr. Suzanne Bayley and the Columbia Wetlands partnership includes multiple indicators of key ecosystem attributes (Gann et al. 2019) as restoration targets. Specifically, the CWSP wetland effectiveness monitoring plan includes monitoring of the following ecosystem attributes: eDNA metabarcoding (macroinvertebrates, vegetation, diatoms described here), Species At Risk, migratory birds, fish, water quality, sediment parameters, wetland hydrology and mapping at restored and reference sites.

Component leads include Darcie Quamme, Integrated Ecological Research (eDNA, this report), and Catriona Leven (MSc thesis with Dr. R. Rooney University of Waterloo on beaver dam metrics/hydrology, and CWSP monitoring of migratory birds and vegetation) and Jessica Holden (CWSP/Living Lakes Canada, LLC, mapping/drone, bathymetry), Dr. Ryan MacDonald (hydrology), CWSP/LLC (outreach, social, cultural, fiscal) and the Kootenay Conservation Partnership's Kootenay Connect initiative (ecological connectivity).

As a result of this diverse evaluation team, the CWSP wetland effectiveness monitoring plan exceeds the Society of Ecological Restoration (SER) "Standards of practice to guide ecosystem restoration" overall and meets the criteria for baseline monitoring in 2024 (Nelson et al 2023).

> *"Measure multiple indicators of key ecosystem attributes (abiotic condition, species composition, ecosystem structure and function, external exchange and threats) and cultural and socioeconomic attributes. If feasible include indicators of genetic composition and ecological connectivity. Selected indicators should match those specified in the monitoring and evaluation plan as well as those that will be collected from reference sites"*
>
> *From the Society of Ecological Restoration (SER) "Standards of practice to guide ecosystem restoration" (Gann et al 2019).*

Volunteers helping with BDA construction in 2022.

BDA complete. Photo from May 2023.

Site 38: BDA pictured at left blocking gap connecting wetland to river shown at right. Oct. 2023.

Site 38: 54 ha of restored wetland, October 2023.

Figure 1: **Beaver dam analogue restoration at Thresher's wetland (Site 38) completed in 2022 by CWSP**. The completed BDA pictured here restored 54 ha of open water habitat for migratory birds increasing the duration of water storage and restoring habitat for migratory birds and SARA listed species. Photos from CWSP and D. Quamme (L to R) and drone photos from Jessica Holden.

The current report focuses on the use of environmental DNA (eDNA) metabarcoding as an indicator of biodiversity, genetic composition, and a measure of restoration effectiveness.

eDNA metabarcoding is a rapid genetic method that allows the identification of taxa at a higher taxonomic resolution resulting in identifications of taxa compared to identification using microscopy (Taberlet 2012a and 2012b, Figure 2). eDNA metabarcoding is effective at detecting a greater number of species compared to morphological taxonomic analysis because the method reveals life stages and species that are not easily identified or unknown based on morphology (Robinson and Hajibabaei 2021). The method is particularly effective where an understanding of taxa richness and biodiversity, food webs and trophic dynamics are important or where species information is limited (Taberlet 2012a and 2012b).

Our approach uses eDNA metabarcoding of macroinvertebrates as indicators of biodiversity and genetic composition funded through the "Sequencing: The Rivers for Environmental Assessment and Monitoring" project (STREAM) https://stream-dna.com carried out at the University of Guelph. These methods have been implemented in other restoration activities as a method of measuring effectiveness (Quamme et al. 2022, 2021a, 2021b 2018, Blair 2022). More recently, the University of Guelph has developed methods for sampling diatoms (Maitland et al. 2020) and fish (pers. com Micheal Wright) using the CABIN sampling methods (ECCC 2018). These taxa were also included in our pilot study to evaluate whether these taxa are useful in our wetland effectiveness monitoring.

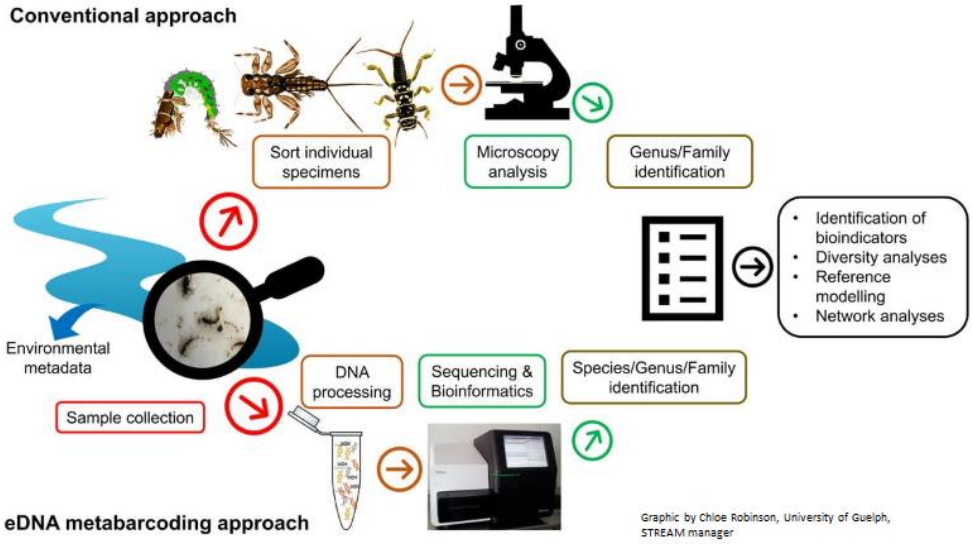

Figure 2: eDNA metabarcoding methods for taxonomic identification and indicators of biodiversity versus morphological identification of taxa from Robinson and Hajibabaei (2021). In the current report, we use eDNA metabarcoding methods to identify macroinvertebrates, diatoms and fish species.

We are using a "Before, After, Reference, Test" sampling design to compare restoration actions between reference sites and test sites (restored sites) with sampling to incorporate seasonality and spatial variability within a wetland over the 5-year project time frame (Figure 3). Seasonal sampling will be blocked by spring and fall sampling periods with rates of sampling appropriate to each parameter. Spatial designs and sample numbers are to be determined based on a statistically valid sampling design. A

sampling schedule, and site number will be developed for each parameter monitored that will be repeatable on an annual basis.

This project will:

1. Help evaluate decision making and plans to move forward with wetland restoration.
2. Provide inference to wildlife populations and Species At Risk which may be difficult to assess directly because of appropriate scale and population movements while macroinvertebrates integrate localized condition.
3. Track ecological lift by examining the rate at which wetland macroinvertebrate taxa and richness increase over time following reestablishment of natural hydrological patterns.
4. To identify actions that encourage the development of a diverse macroinvertebrate community providing a base for higher trophic levels in wetland ecosystems.

In the May of 2023, we collected pilot data to determine the required samples sizes required for eDNA sampling focussed on the lower Columbia wetlands using the Canadian Biomonitoring Network (CABIN) methods for wetlands (ECCC 2018).

We sampled more broadly in August 2024 including wetlands prioritized for wetland restoration based on our findings from the early season. We also sampled sites in reference condition with varying connectivity to flooding conditions. Baseline sampling will be used as benchmarks for assessing restoration outcomes (Gann et al. 2019).

The present report focuses on genomic data and eDNA monitoring outcomes from May of 2023. Data from August 2023 was delivered on March 10, 2024 from the University of Guelph STREAM project and has not been analyzed fully due to the recent arrival of the data set.

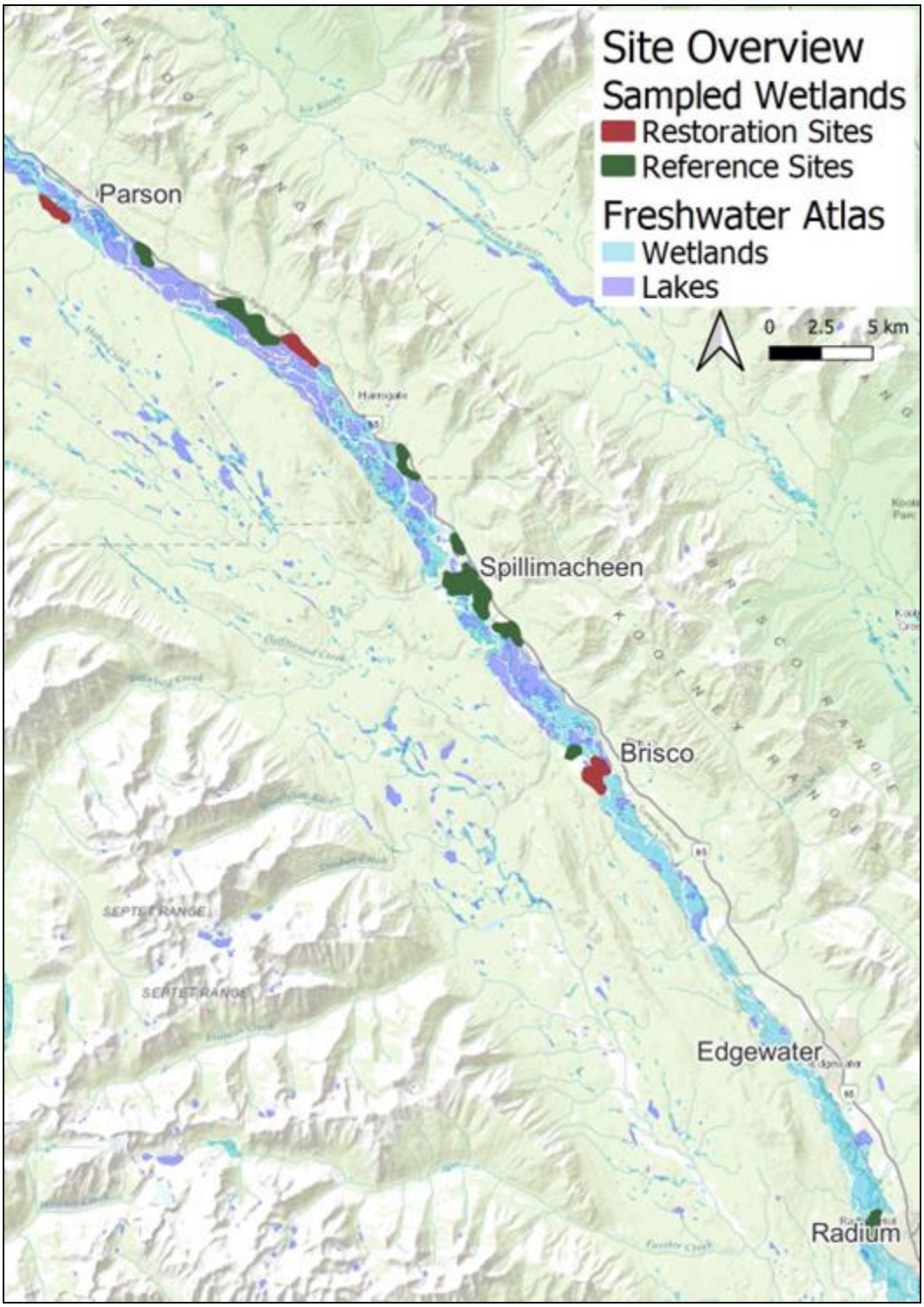

Figure 3: Study location including restoration and reference wetlands within the Columbia wetland complex.

Spring 2023 pilot study: Methods

We conducted a pilot study to assess optimal sampling strategies and provide an initial assessment of the eDNA approach. The main objectives of fieldwork and subsequent analyses were as follows:

- Graphical inspection of percent composition by hydrological category for Class Insecta and Orders of dragonflies, mayflies and caddisflies to aid in determining the length of gradient needed to assess an effect.
- Obtain preliminary estimates of total species richness for sampled wetland areas using two types of resampling methods including bootstrapping and mark-recapture.
- Determine the number of within-wetland samples needed to obtain precise estimates of richness.
- Relate measures of biodiversity and genetic composition to the hydrological classifications of the Columbia Wetlands (continually connected, partially connected and poorly connected to the Columbia River).

Field methods

In our pilot study, we sampled six wetlands in May 2023 of with varying hydrological regimes and restoration potential or reference condition. Hydrological classifications were based on MacDonald (2021) and Goodbrand and MacDonald (2022) (Table 1). Hydrological classifications, gap and beaver dam measurements are currently being updated as part of Catriona Leven's MSc. thesis (pers com., Leven 2024).

Table 1: Hydrological classifications of the Columbia Wetlands

Hydrologic connectivity to Columbia River	**Hydrologic flux and residence time of water in wetland**	**Topography**	**Wetland classification**
Continually connected	Fluctuates with river stage.	River gap present.	**Group A**-wetland stage is dependent on river stage, wetland has a similar hydrograph to river.
Partially connected	Wetland stage increases with flooding over levee and beaver dams, drains easily, slower recession than continually connected.	Some gaps to river, creeks or other wetlands, outlets blocked by beaver dams.	**Group B-**early June peak stage, faster maximum annual peak recession rate than Group C. **Group C**-early June peak stage and maximum annual peak is subdued.
Poorly or not connected	Wetland stage increases with flooding overbanks and beaver dam, slow drainage, greater water storage in fall season.	Minimal gaps with no outlets due to blockage by levee and/or beaver dams.	**Group D-** no early June peak due to dams, slow maximum annual peak recession. **Group E**- early June peak event but with a slower maximum annual peak recession rate.

Classifications from Goodbrand and MacDonald (2022).

We sampled reference sites and targeted restoration sites over a range of hydrological classifications including wetlands that were continually connected, partially connected and poorly connected. This is because BDA restoration should change the hydrology of targeted wetlands from one that fluctuates with

the stage of the Columbia River (continually connected) to a wetland with greater water storage, a longer residence time and more open water for wildlife (partially connected, Table 2, Figure 4).

Table 2: Pilot study, initial testing on a subset of six wetlands sampled May 2023

Site#	Letter code	Hydrological classification	Restoration or reference site	Size (ha)	Number of 2- min kick samples
21	RES	**C: Partially connected**	Reference	49.47	6
24	NAB	**A: Continually connected**	Restoration potential	45.77	2
31	GAL	**B: Partially connected**	Reference	40.22	6
38	THR	• **Originally continually connected (Group A)** • **Restored to partially connected**	Restoration completed	53.77	8
69	TUR	**E: Poorly connected**	Reference	11.56	6
71	ELK	**A: Continually connected**	Restoration potential	22.28	6

May 2023, during pilot sampling, reference site, partially connected to the Columbia River

May 2023 October 2023

Figure 4: **Reference site that is partially connected to the Columbia River with ~20 ha open water by the end of the season** (Galbraith's wetland #31 GAL). Natural beaver dams and levees at this site slow the recession of water to the Columbia River following freshet. Top photo and bottom left photo in May 2023. Bottom right, October 2023. Top photo by D. Quamme. Bottom photos from J. Holden.

Two-minute CABIN kick net samples were collected for submission to the STREAM program (Sequencing The Rivers for Environmental Assessment and Monitoring. Thirty-six samples were submitted to the University of Guelph in May 2023 (Figure 5).

CABIN for wetland protocols (ECCC 2018) were used to characterize the macroinvertebrate community that inhabit the emergent and submergent zones of the wetlands where the macroinvertebrate diversity is greatest (De Szalay and Resh 2000). Sampling was timed for mid-May in the pilot study to coincide with low water levels prior to spring freshet when there is the largest difference between wetland in water storage. The sampling procedure in wetlands involved a gentle disturbance of bottom sediments and two-minute sweeps of the water column by foot or by kayak using a CABIN net (ECCC 2018, Figure 5).

Macroinvertebrates were sampled throughout the shallow wetlands (mean depth <1.4m, 2020-22) using a CABIN kick-net of length 45.7 cm, width 25.4 cm, and depth 25.4 cm with a 400 µm mesh net and a handle length of 1.78 m (ECCC 2007, Tall et al 2008) (Figure 5).

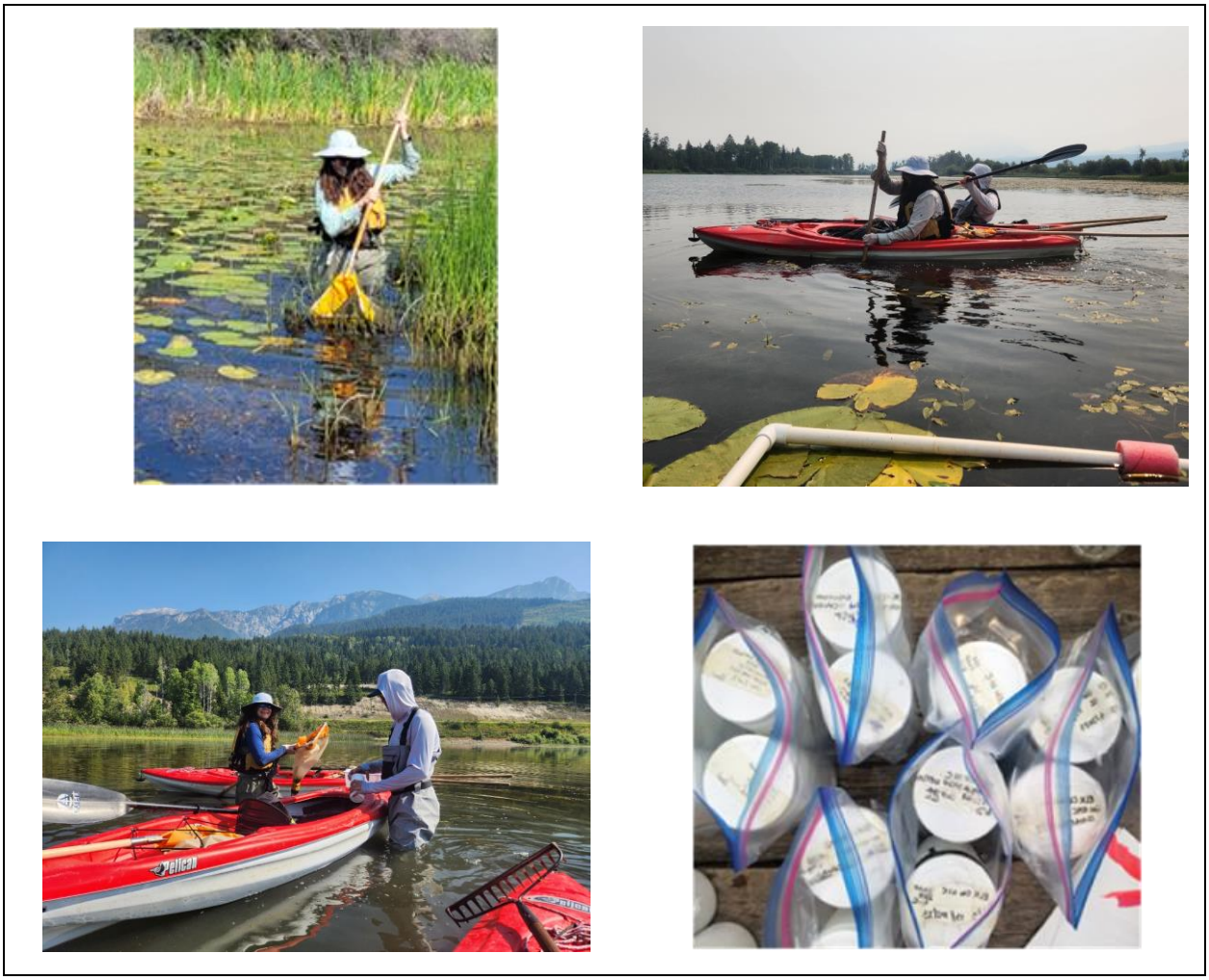

Figure 5: **eDNA field collection.** Sampling took place on the Columbia Wetlands near Brisco, B.C. using a 2-minute sweep by foot (top right) or by kayak (bottom left) using the Canadian Aquatic Biomonitoring Protocols. Darcie Quamme and Braeden Toikka in photo. Samples were preserved with glycol in 1-liter jars (bottom right). Photos by B. Toikka and C. Leven.

The sweep sample collected macroinvertebrates from the water column, bottom sediments and from aquatic plants at each site (ECCC 2018). Sampling was carried out by foot if there was a hard bottom and from a kayak (Keith et al. 2022) where soft benthic zones made wading too dangerous. The orientation of the CABIN net was not adjusted for kayak sampling as in Keith et al. (2022).

Meta-data collection included vegetation (SAV) plots, water quality (methods in Leven et al. 2022, Rooney et al. 2013) and associated habitat variables (ECCC 2018), (Figures 6 and 7).

Figure 6: Meta-data collection included vegetation (SAV) plots (n=10 per wetland), water quality sampled (n=1 site per wetland) and habitat variables targeted for restoration and reference sites. Catriona Leven upper right, Paige Thurston (upper left), Jessica Holden (bottom left). Photos by D. Quamme.

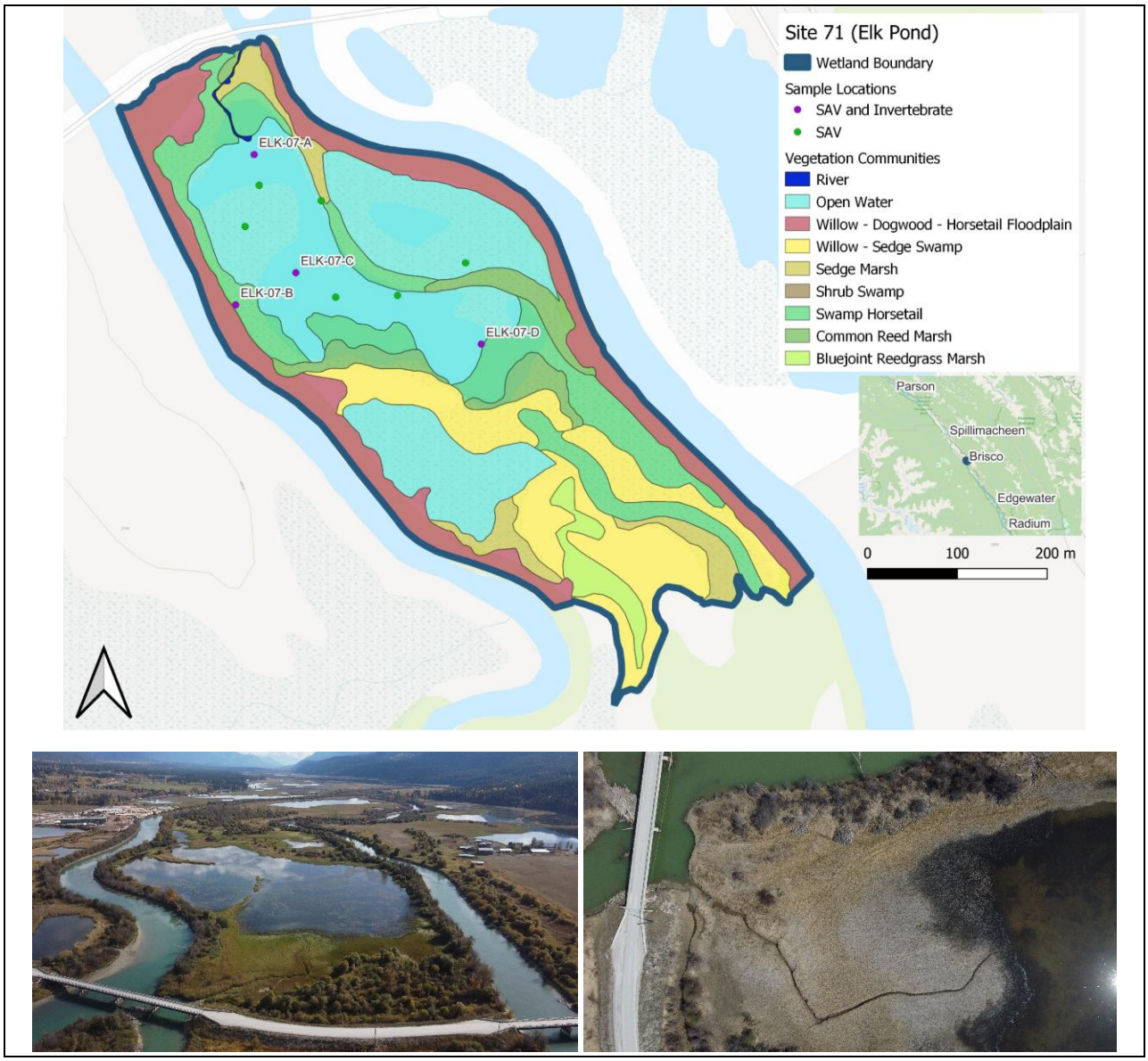

Figure 7: **Pre-restoration sampling of eDNA and vegetation (SAV) plots** at Site 71 (Elk Pond) a "continually connected" targeted for future BDA restoration at the north-east gap. eDNA plus SAV plots show in purple with a CABIN labelling. SAV only plots shown in green. Bottom left photo take of wetland facing south. Bottom right is an overhead photo of the gap and potential location for BDA restoration. Map and drone photos by Jessica Holden.

eDNA meta-barcoding methods

Sample collection for DNA was carried out as recommended by the Hajibabaei Lab, University of Guelph, Center for Genomic Biodiversity STREAM protocols for DNA collection (Baird, D. J. and Hajibabaei, M. 2012). Gloves were used so as not to contaminate the sample and no attempt to reduce the sample was made to handle the sample as little as possible. The sample was filled to under 50% of the jar to facilitate sample preservation. No reduction of samples was carried out post-sampling to minimize handling of the sample.

Samples were shipped to the University of Guelph and stored in freezers at -20°C in the lab until they could be processed. Samples consisting of mud, vegetation, bulk tissue DNA for diatoms and macroinvertebrates and fragments of DNA from fish. Bulk tissue DNA (actual organism) was collected for macroinvertebrates and diatoms while fragments of DNA resulting from mucus, feces or sloughed cells of fish but not the whole organism.

Samples were coarsely homogenized in a sterile blender and DNA was extracted using a DNeasy® PowerSoil® kit (Qiagen, CA) (Figure 8). A small amount of extracted DNA was then processed following the standard Hajibabaei Lab protocol for Next-Generation Sequencing (NGS), using Illumina that allows sequencing billions of DNA strands in parallel.

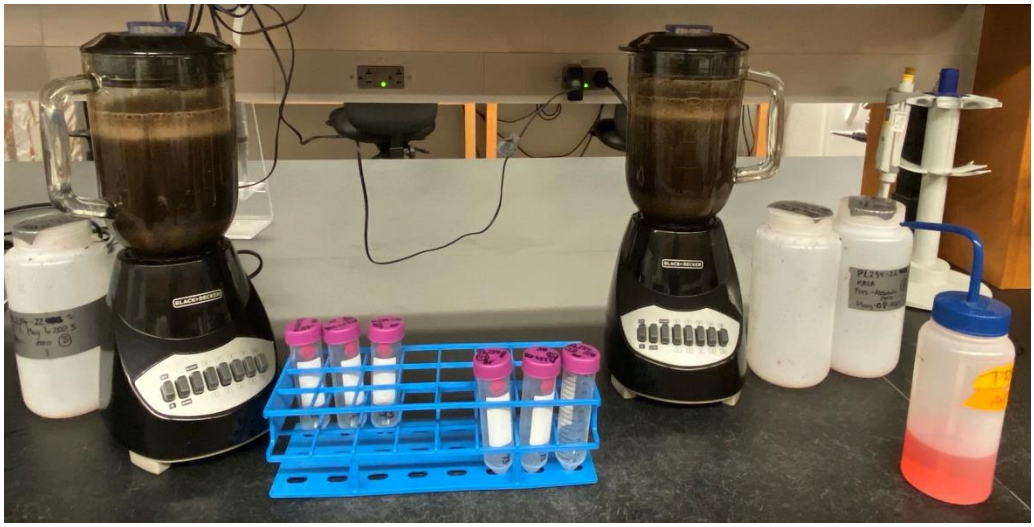

Figure 8: Sample homogenization of plant material, biota and sediment at the Hajibabaei laboratory in preparation for Next Generation Sequencing (NGS). Photo from STREAM (2023).

The raw output from NGS produced sequences (barcodes) for macroinvertebrate, diatom and vertebrate using the gene cytochrome oxidase c subunit 1 (CO1) part of mitochondrial DNA (Figure 9). These sequences were then reduced to sequences that were of high enough quality to match to reference sequences. All sequences were matched to morphologically identified specimens reference sequences from the Barcode Of Life Datasystem (BOLD). Only species taxonomically assigned with high confidence (bootstrap support >= 0.70) were included to indicate species present.

Methods and results from eDNA metabarcoding will also be provided by the STREAM (2024) companion report from the University of Guelph produced by project manager, Micheal Wright.

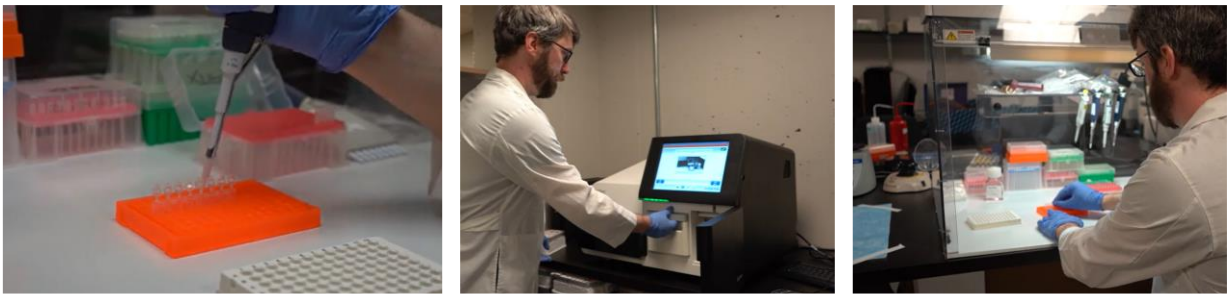

Figure 9: Samples were sent to the Centre for Biodiversity Genomics, University of Guelph for analysis under STREAM funding. Pictured here is Michael Wright, Program manager, Photo credit: Hannah James.

Results

The spring macroinvertebrate data was received on July 25, 2023. The goal of this preliminary analyses was to inform and finalize sampling design for July 31, 2023 and beyond as well as assess dominant trends in richness and other indicators of biodiversity and genetic composition from the preliminary data.

Analysis of macroinvertebrate response to wetland hydrology

Total species richness as a response variable can mask responses to a gradient because of species turnover. As a result, we initially graphed two important macroinvertebrate groups including the Class Insecta and the aquatic insect Orders Odonata, Ephemeroptera and Trichoptera (OET), dragonflies, mayflies and caddisflies. Percent composition from the May 2023 sampling were inspected for patterns. Site 69, a wetland with no connectivity to the river had 1.3-2.7 times greater richness for the Class Insecta than other wetlands sampled in the A and C classifications (continually connected – partially connected to the Columbia River) (Figure 10).

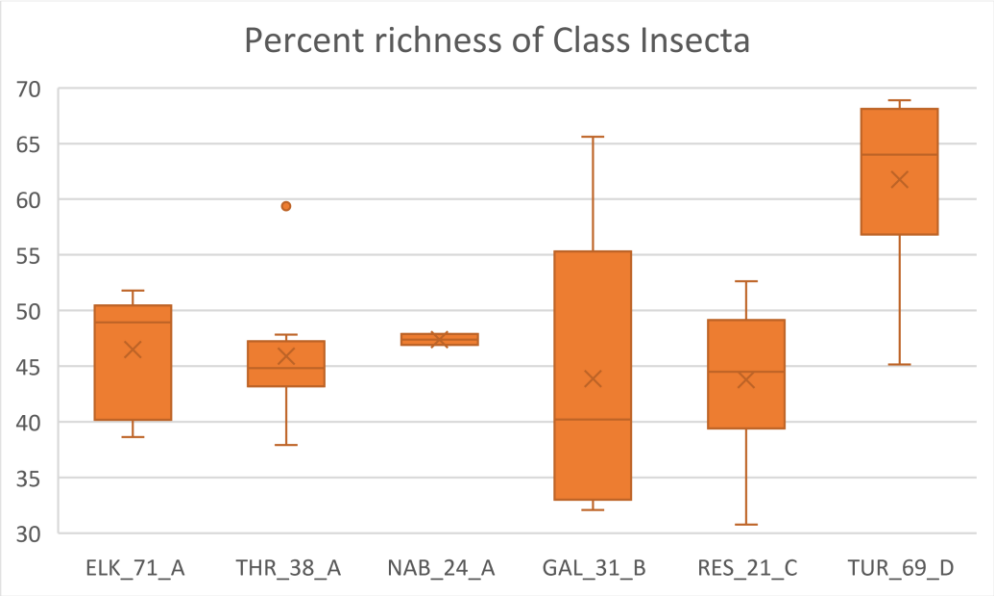

Figure 10: Percent richness of Class Insecta by wetland sampled. Letters indicate CABIN site letters, numbers indicate CWSP site numbers, letters A-D indicate hydrological category of wetland. n= 6 except for THR_38_A (n= 8) and NAB_24_A (n = 2).

Graphical inspection of percent richness of OET suggests that decreasing connectivity to the Columbia River may favor these group of species (Figure 11). Further sampling is needed to establish this trend. Overall, these findings suggest that further sampling should include the longest wetland hydrological gradient possible within the Columbia wetlands incorporating A (continually connected), B/C (partially connected and D/E (poorly or not connected) wetland classifications to allow for the greatest effect size. Further replicates of wetland categories will aid data analyses. With more time for analysis, numerous metrics can be examined, and more elaborate analyses can be carried out.

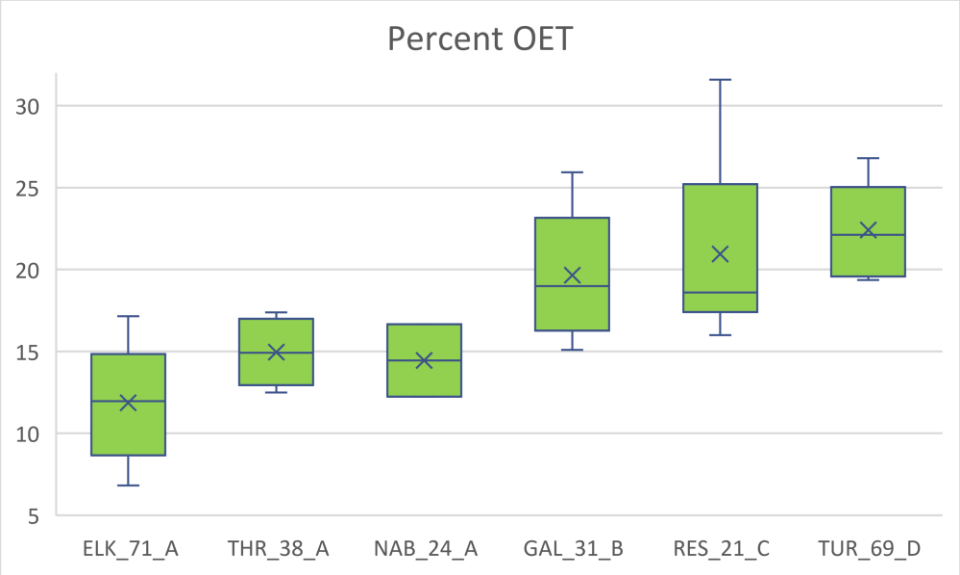

Figure 11: Percent richness of the Orders (Odonata, Ephemeroptera and Trichoptera) by wetland sampled. Letters indicate CABIN site letters, numbers indicate CWSP site numbers, letters A-D indicate hydrological category of wetland. n= 6 except for THR_38_A (n= 8) and NAB_24_A (n = 2).

Total macroinvertebrate species richness detected by genomic methods.

Species richness was initially summarized by the mean number of species counted per sample. Bootstrap methods were used to provide confidence limits on mean estimates (Table 3).

Table 3: Mean estimates of macroinvertebrate diversity

Wetland		Mean	Bootstrap				
	samples	observed	estimated	SE	CI		CV
ELK (A)	6	55.0	54.3	5.0	44.6	64.0	9.1%
THR (A)	8	43.0	42.6	3.7	35.4	49.9	8.7%
GAL (B)	6	37.5	37.9	5.2	27.7	48.2	13.8%
RES (C)	6	27.2	27.4	2.3	22.9	31.9	8.4%
TUR (D)	6	49.3	50.4	6.1	38.5	62.3	12.1%
NAB(A)	2	48.5	48.5	0.3	47.8	49.2	0.7%

Estimated total species richness using resampling techniques.

Total species richness was then estimated using bootstrap and mark-recapture estimators based on all the samples (Figure 12 a and b). Both approaches attempt to estimate the number of species that were

not detected in samples. The bootstrap approach used resampling to estimate proportion missed based on proportion of species present in resamples of the data set. The mark-recapture method estimates detection probabilities of species based on detection frequencies across all samples conducted. The mark-recapture approach is more robust to uneven detection probabilities of species (Boulinier et al., 1998). The estimator of Chao (1984) was used for mark-recapture estimates. *Rich* (Rossi, 2011) and *SPECIES* (Wang, 2011) *r* packages were used for calculations with data being plotted using the *ggplot* package (Wickham, 2009). Below are estimates of total species richness. Estimates using both approaches were similar both with reasonable precision. The NAB wetland should be treated cautiously given that it was based on only two samples.

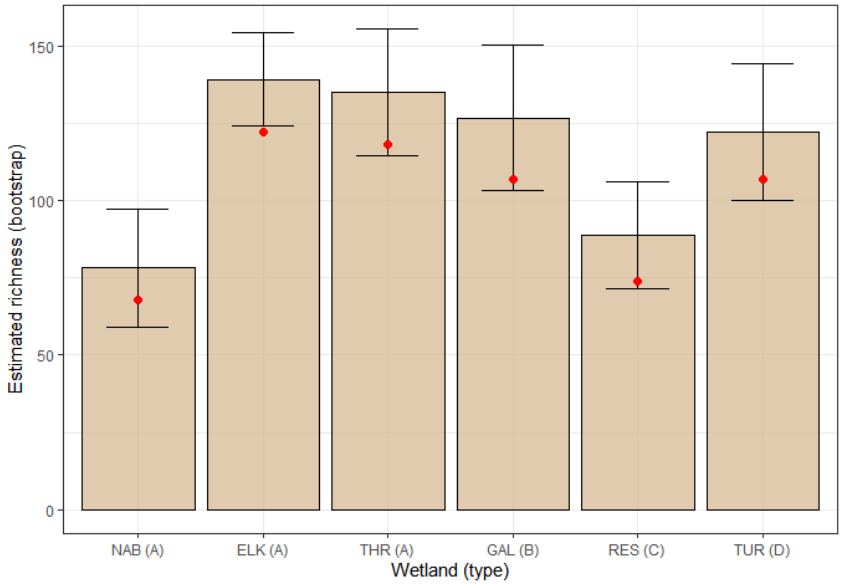

Figure 12a

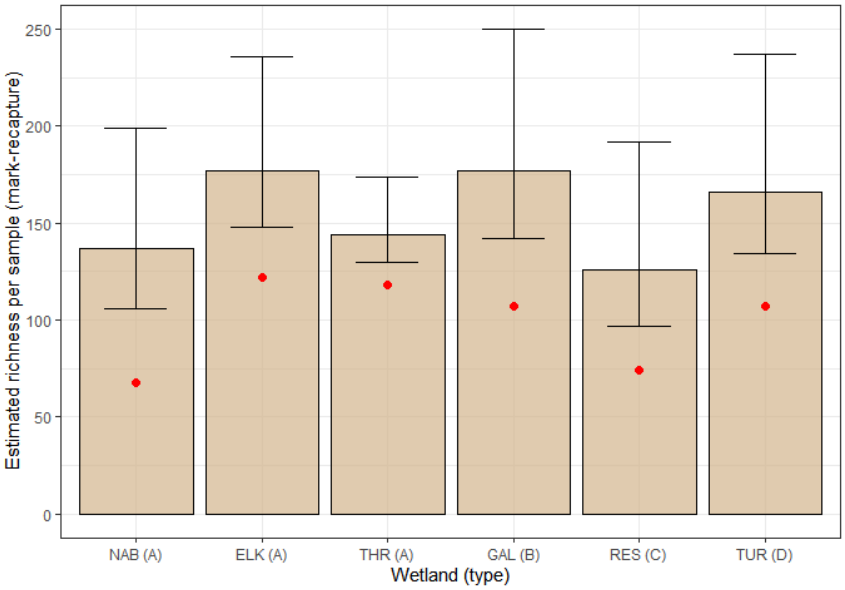

Figure 12b

Figures 12 a and b. Estimated total richness using resampling techniques including species not detected by eDNA using bootstrapping and mark-recapture. The red dot is the cumulative species of species at each wetland detected by eDNA.

Within wetland variation in total macroinvertebrate species richness

Of importance to the study is to establish how many plots are required for each wetland to obtain reasonable estimates of richness. To explore the data estimates were using the mark recapture estimator based on increasing numbers of plots. The coefficient of variation (CV) of estimates is plotted below (Figure 13). A spline trend line was then fitted to look at overall trend in precision across all sites. Estimates of reasonable precision (CV<15%) were obtained with four or more samples for most sites. This is an initial assessment; an approach that randomly resamples plots would provide a more robust comparison (to be conducted in future analyses).

This preliminary analysis suggests four or more plots are needed to improve precision. If less plots are sampled, then estimate precision will be low which will increase variance between sites (due to sampling) and therefore lower power to detect differences between wetlands.

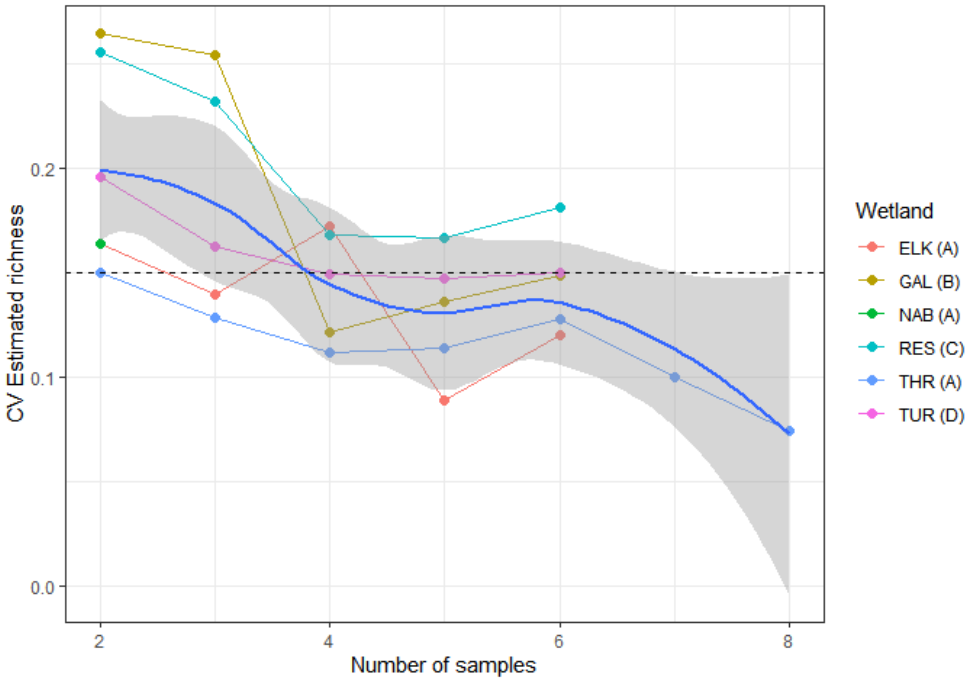

Figure 13. Coefficient of variation (CV) of the estimated richness with increasing sample (plot) number at each wetland. Note there is only one point for NAB because only two plots were sampled at this wetland.

Summary

In May of 2023, we collected pilot data to determine the required samples sizes required for eDNA metabarcoding on the lower Columbia wetlands using the Canadian Biomonitoring Network (CABIN) methods for wetlands (ECCC 2018). We also sampled sites in reference condition with varying connectivity to flooding conditions. We found that a least four replicates per wetland were required were needed to improve precision, reduce variance to improve the power to detect differences between wetlands.

Graphical inspection of percent richness of Orders Odonata, Ephemeroptera and Trichoptera (OET) suggests that decreasing connectivity to the Columbia River may favor these group of species.

Spring pilot sampling in May 2023 also suggested that:

- Further sampling should include a long hydrological gradient incorporating A (continually connected), B/C (partially connected and D (poorly or not connected) wetland classifications to allow for the greatest effect size.
- At least four or more plots were needed to obtain precise within wetland estimates and thus the power to detect differences between wetlands based on mark-recapture estimates of total richness.
- Between wetland variance should be evaluated after wetland sample size is expanded.
- Approaches that utilize trends in species richness and related demography (fidelity of species to a wetland and turnover rate of new species in a wetland) (Cam et al 2000) and/or multivariate analyses should be considered.

We used information from the spring pilot to inform and plan a more intensive sampling program for August 2024 including sampling 16 wetlands with three wetlands prioritized for restoration, one completed restoration project and 14 reference sites (Appendix 1, Table 3). We collected a total of 96 samples in the Lower Columbia Wetlands in spring and summer sampling in 2024. Baseline sampling will be used as benchmarks for assessing future restoration outcomes (Gann et al. 2019). Detailed analyses are planned and will inform future sampling.

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Appendix 1: August sampling design for the Lower Columbia wetlands

Table 2: August sampling of Lower Columbia Wetlands

Site#	Letter code	Hydrological classification	Restoration or reference site	Size (km^2)	Number of 2-min kick samples
21	RES	**C: Partially connected**	Reference	0.49	**4**
24	NAB	**A: Continually connected**	Restoration potential	0.46	**4**
30	JUB	**A: Continually connected**	Reference	1.11	**4**
49	RAD	**C: Partially connected**	Reference	0.61	**4**
31	GAL	**B: Partially connected**	Reference	0.4	**4**
38	THR	• **Originally continually connected (Group A)** • **Restored to partially connected**	Restoration completed	0.54	**4**
62	SSP	**D: Poorly connected**	Reference	0.55	**4**
69	TUR	**E: Poorly connected**	Reference	0.12	**4**
71	ELK	**A: Continually connected**	Restoration potential	0.22	**4**
127	PAL	**B: Partially connected**	Reference	0.13	**4**
131	BCK	**B: Partially connected**	Reference	0.29	**4**
132	CAS	**A: Continually connected**	Restoration potential	0.75	**4**
140	GRA	**C: Partially connected**	Reference	0.17	**4**
141	BHL	**B: Partially connected**	Reference	0.24	**4**
144	ZGS	**A: Continually connected or B: Partially connected**	Restoration potential	0.19	**2**
145	DAV	**A: Continually connected**	Restoration potential	0.13	**2**
Total					**60**