Refinement and validation of eDNA surveys for detecting shortjaw kōkopu (*Galaxias postvectis*) in New Zealand streams

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Shortjaw kokōpu observed during a spotlighting survey in Flowery Creek.
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1. Introduction

1.1 Background

This report presents the results of field trials for the detection of shortjaw kōkopu (*Galaxias postvectis*) populations using environmental DNA (eDNA). Shortjaw kōkopu are a widespread yet geographically sparse fish species with a conservation status of 'threatened' in the New Zealand Threat Classification System (Dunn et al. 2018). As such, they are the most threatened of five migratory galaxiids contributing New Zealand's whitebait fishery, which targets the migrating juvenile fish as they return to freshwater from a larval development phase at sea (McDowall 1984). Adult shortjaw kōkopu are often found in small to medium-size streams with cover provided by in-stream substrate, large wood, or riparian vegetation, and are more common on the west coasts of both the North and South islands (Bowie & Henderson 2002; Goodman 2002; McDowall et al. 1996).

As with other migratory fish species, shortjaw kōkopu face a multitude of challenges associated with the availability of critical habitat at the necessary times (McDowall 1992). These habitats are dispersed across generally large scales that encompass mountains-to-sea geographies and connectivity influences that interact with the various life stages. These diverse aspects and localities can be difficult to accommodate within conservation planning systems that operate on site-based approaches which may overlook the role of connectivity between habitats and consequential effects. Improving these aspects of aquatic species conservation is being addressed by the Migratory Fish Recovery Programme initiated by the Department of Conservation (DOC). The programme has a current focus on improving the knowledge and security of four species: īnanga (*Galaxias maculatus*), shortjaw kōkopu (*G. postvectis*), long-finned eel (*Anguilla dieffenbachii*) and kanakana / piharau / lamprey (*Geotria australis*) (Richarson 2022). To support this programme there is a general need to upscale existing field survey efforts and/ or develop new methods for distribution mapping and monitoring at broad scales. However, this is typically challenging for commonly-used freshwater fish survey approaches such as spotlight surveys and electric fishing (Allibone 2013; Joy et al. 2013). In this context, the use of aqueous eDNA sampling offers potential advantages associated with transport of source DNA material to distant sampling sites which can facilitate the detection of target species (Ficetola et al. 2008; Goldberg et al. 2011; O'Donnell et al. 2017; Rees et al. 2014). Furthermore, it has been successfully applied to survey species that are difficult to monitor using other techniques (e.g., Biggs et al. 2015; Nester et al. 2020).

As with other survey methodologies, it is essential to establish the level of uncertainty associated with the survey approach, which for eDNA involves both field and laboratory procedures. Establishing the sensitivity of eDNA tests and associated margin of errors is particularly important for their interpretation (Ficetola et al. 2008; Ficetola et al. 2015; Rees et al. 2014).

For aqueous eDNA applications such as the detection of rare species, some of the important topics for consideration include:

- the sensitivity of the test to detect presence when presence is there (absence of false Type II errors or 'false negatives')
- prevalence of Type I errors (false positives)
- the dispersal distances of eDNA from distant source populations
- the duration of eDNA in the environment
- the effect of other environmental parameters on the above

In this report the first of these is referred to as detection sensitivity. Its evaluation requires assessment against an independent dataset which is comparable for the variables of interest. These data will often require a purposeful study design, such as those based on parallel monitoring and subsequent comparison of the two datasets for the same response variable (e.g., presence/absence). Where one of the methods is an established or standard procedure, this approach can also be used to support optimisation of the newer procedure through investigating the effect of various sampling design, laboratory or equipment alternatives on attributes of interest (e.g., sensitivity). This was a specific focus of the present study in which active and passive eDNA sampling equipment and associated field procedures were compared in a parallel monitoring experiment.

1.2 Objectives

This project had two key objectives: 1) evaluating the sensitivity of eDNA survey methodologies for their ability to detect shortjaw kōkopu, and 2) detecting new shortjaw kōkopu populations in catchments where they had not previously been recorded.

For the first objective the general approach involved parallel monitoring whereby a selection of eDNA sampling protocols were trialled and compared to near-concurrent spotlighting surveys in the same waterways over two consecutive summers. The second objective was primarily addressed in the first year of the study through selecting catchments in which there were no previous records of shortjaw kōkopu in the New Zealand Freshwater Fish Database (NZFFD). There was less emphasis on this objective in the second year of the study due to a focus on gathering additional sensitivity data.

Report layout

This report combines the results from the two field monitoring campaigns and two independent eDNA analyses. These used the original Wilderlab 'basic freshwater' assay panel, and a modified version that was developed in response to results obtained in the first iteration of laboratory testing. To provide a complete picture of the relevant findings and their application in the two iterations, results are included from both the original and newly-developed eDNA assay panels in section 3. The improved sensitivity of the new assay panel and updated assessment of field survey methods are briefly discussed in section 4 and key conclusions from each component of the study summarised in Section 5.

2. Methods

2.1 Site selection criteria

Two survey campaigns were conducted over successive years on the West Coast of the South Island. The study design involved parallel monitoring of the same waterway using fixed reach spotlight surveys and different eDNA sampling protocols. Initial criteria for the selection of study sites were based on an evaluation of species distribution models for shortjaw kōkopu (Crow et al. 2014; Leathwick et al. 2008). Areas of agreement and discrepancy for predicted shortjaw kōkopu presence were mapped between models to identify priority locations for future surveys and threat assessments (Orchard 2020). Four classes of waterways were identified which differ in the strength of evidence for shortjaw presence (Table 1). The classification can be performed for individual reaches within the River Environment Classification (REC) framework (Snelder 2010; Snelder & Biggs 2002) or intersected with management units such as catchments and subcatchments as illustrated in Figure 1.

The general approach to study site selection involved identifying candidate sites based the above classes and accessibility for survey logistics. Travel time logistics were particularly important due to the intensive sampling schedule for the planned trials which required repeat sampling at two different study sites on the same day for the majority of the field campaign (see section 2.3 for further details).

In 2021, the surveys were located in the Hokitika area and had a focus on class 2 and 3 waterways where it was hoped that some new shortjaw kōkopu populations would be found. In 2022, the focus changed to prioritising the further evaluation of eDNA survey methods following the finding of lower-than-expected sensitivities in waterways with confirmed shortjaw kōkopu presence in the 2021 trials. A greater proportion of class 1 waterways were included in the 2022 surveys to facilitate further sensitivity assessments (see section 2.2 for details).

Table 1 Four classes of shortjaw kōkopu habitat and predicted habitat that account for different lines of the evidence for fish presence at a nationwide scale. These classes are assigned using the best available information which spans a considerable date range includes different survey types. In additional to the limitations inherent in each of the information sources there are uncertainties associated with landscape changes since the date of the measurements that contribute to each. These include environment changes affecting the sites of historical fish observations and environmental parameters used in the SDMs (Orchard 2020). SDM = species distribution model. The models referred to are described in Leathwick et al. (2008) and Crow et al. (2014).

Class	Status	Conservation priority
1	Shortjaw kōkopu presence confirmed by the results of field surveys	very high
2	Presence predicted by both SDMs	high
3	Presence predicted by one of the SDMs but not the other	medium
4	No evidence for presence as judged by SDM probabilities of capture below the modelled threshold for presence, and the absence of confirmed sightings	low

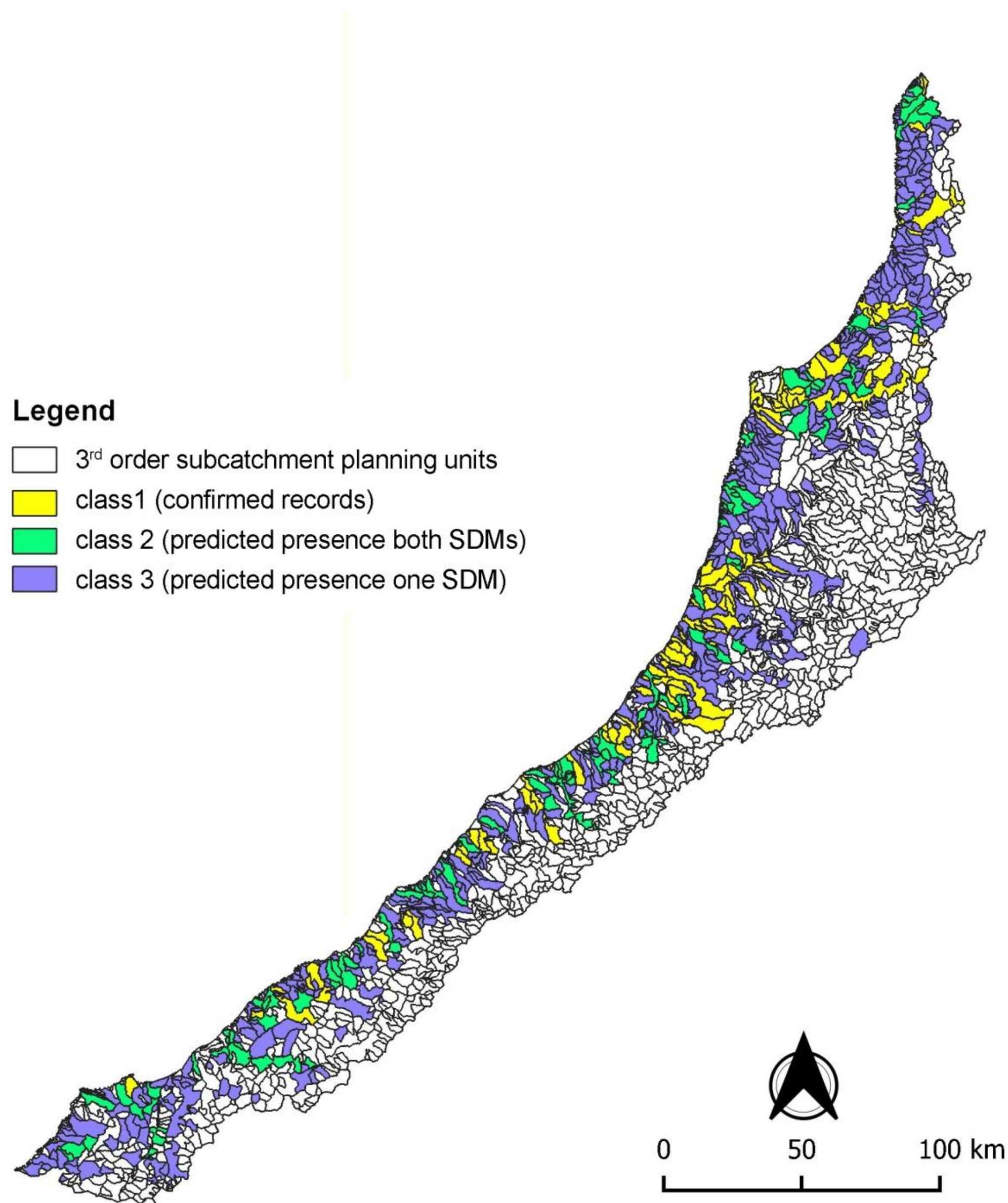


Figure 1 Example of a region-wide classification of 3rd order subcatchment planning units (PLUs) for shortjaw kōkopu. The yellow polygons represent confirmed records and the green and blue polygons indicate survey priorities for location new populations based on the probability of capture thresholds reported for species distribution models (SDM) developed in 2008 (Leathwick et al. 2008) and 2014 (Crow et al. 2014).

To assist site selection, landowner information, the location of potential access points and an impression of streambed and riparian vegetation conditions were compiled from desktop data sources. Preliminary field visits were also conducted to check the feasibility of access, nature of hazards, and other site-level planning considerations such as water depths and velocity for spotlight surveys. The combined results of these steps informed the final selection of study sites. Figure 2 illustrates some aspects of this process for the Hokitika area together with the final set of study sites selected for 2021. A similar process was followed in 2022 to select study sites in the Greymouth area.

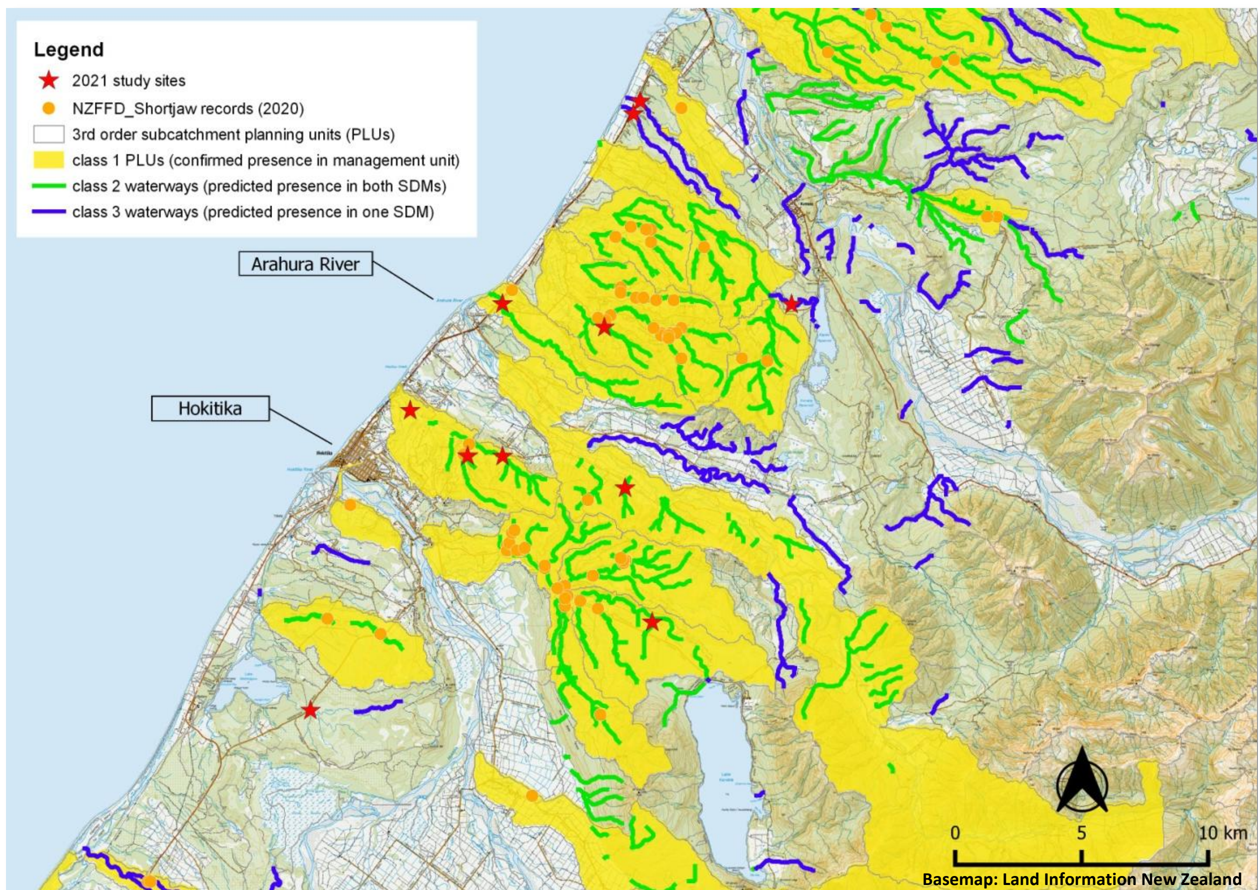


Figure 2 An example classification of waterways and planning units using to support study site selection in the Hokitika area using the classes described in Table 1. The location of shortjaw kōkopu observations recorded in the New Zealand Freshwater Fish database (NZFFD) is also shown along with the final selection of 2021 study sites.

2.2 Study site locations

2021 surveys

The 2021 surveys were located in the Hokitika area. Ten of the 11 study sites were located in catchments or subcatchments with no previous records of shortjaw kōkopu either within the study reach or further upstream (Table 2a). The remaining sampling site (Houhou Creek) was located downstream of previous shortjaw kōkopu records and also downstream of two other study sites (Foley and McIntyres creeks) (Figure 3).

2022 surveys

The 2022 surveys were located in the Mawhera / Grey area and included a greater number of reaches (6 of the 10) for which shortjaw kōkopu presence had been confirmed in previous field surveys (Table 2b, Figure 4). This decision was taken with DOC staff to increase the potential number of sampling sites at which shortjaw kōkopu presence might be confirmed in the parallel monitoring campaign. Additionally, advances in the design of eDNA passive samplers had resulted in a new equipment option being available. The new 'pod' passive samplers were therefore included in the 2022 trials in place of the older 'drogue' samplers which were used in 2021 trails (see details in section 2.5).

Further details of each study site are provided in Appendix 1.

Table 2 Study site details.

(a) 2021 surveys

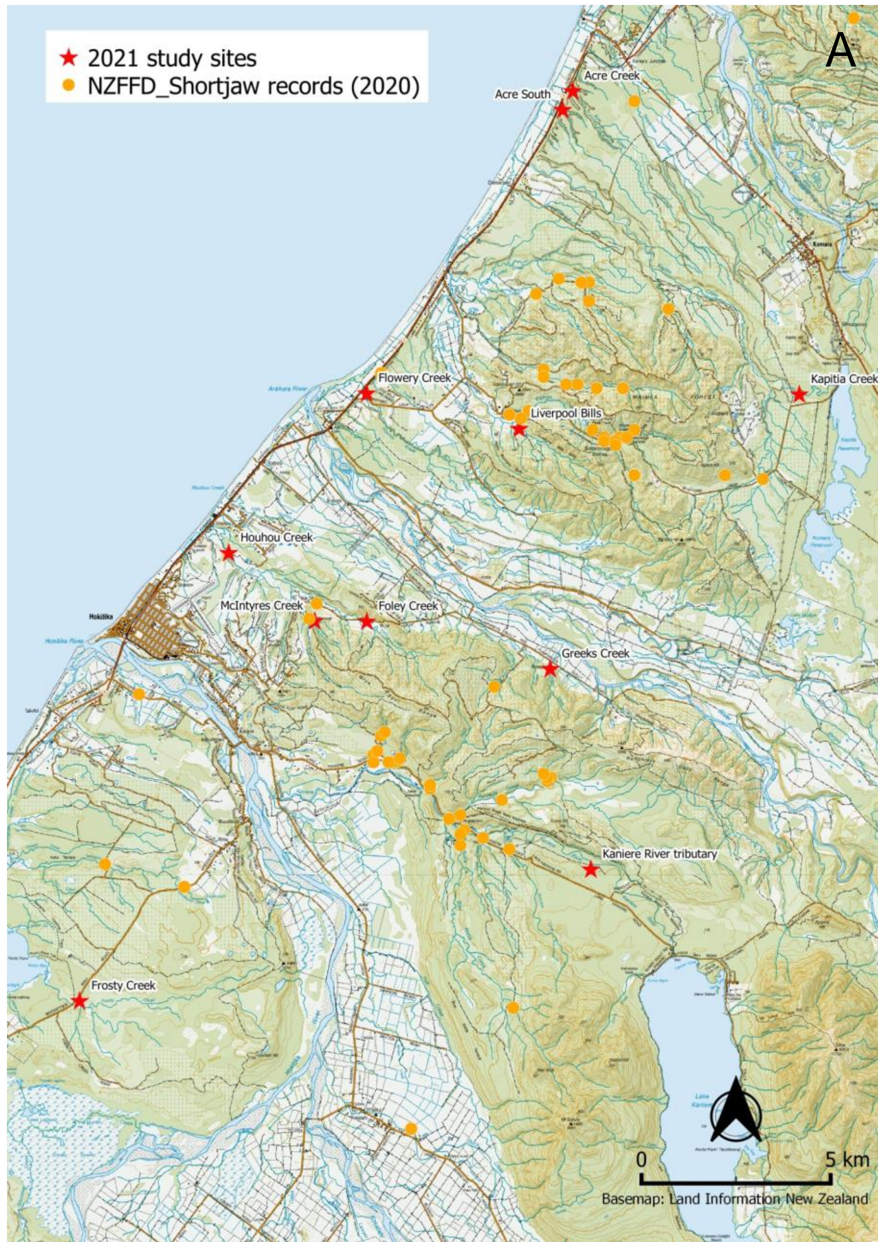
Waterway	Access point	Survey reach class prior to this study*	Spotlighting survey date	Coordinates (WGS84)†	
				X	Y
McIntyres Creek	Blur Spur Rd	2	6/4	171.023403	-42.716694
Kapitia Creek	Greenstone Rd	3	7/4	171.181230	-42.666494
Liverpool Bills	Stafford Loop Rd	2	7/4	171.091787	-42.671254
Acre Creek	SH6	3	8/4	171.110253	-42.591913
Acre South Creek	SH6	3	8/4	171.108022	-42.595375
Foley Creek	Blue Spur Rd	2	30/4	171.039731	-42.716892
Greeks Creek	Humphreys Gully Rd	2	1/5	171.101431	-42.728332
Kaniere River tributary	Ward Rd	2	1/5	171.112574	-42.776458
Flowery Creek	SH 6	2	6/5	171.042830	-42.661876
Frosty Creek	Woodstock Rimu Rd	4	7/5	170.944341	-42.807114
Houhou Creek	Keoghans Rd	1	7/5	170.997330	-42.699891

(b) 2022 surveys

Waterway	Access point	Survey reach class prior to this study*	Spotlighting survey date	Coordinates (WGS84)†	
				X	Y
Rough n Tumble Creek	Notown Rd	4	23/3	171.440860	-42.449489
Woolley Creek	Taylorville Rd	1	24/3	171.370171	-42.421847
Rocky Creek	Taylorville Rd	1	25/3	171.383204	-42.416222
Dowan Creek	Notown Rd	1	26/3	171.401022	-42.435050
Granite Creek	SH6	2	28/3	171.330484	-42.243449
Canoe Creek	SH6	3	29/3	171.340519	-42.217149
Mill Creek	Forestry track	1	27/3	171.309774	-42.456206
Card Creek	Rutherglen Rd	3	30/3	171.221788	-42.556749
Cariboo Creek	Ogilvies Rd	1	31/3	171.227668	-42.562169
Stony Creek	Taylorville Rd	1	1/4	171.344541	-42.431984

* see Table 1 for class descriptions.

† coordinates are for the downstream end (start point) of each 400 m fixed reach which was also the eDNA sampling point.



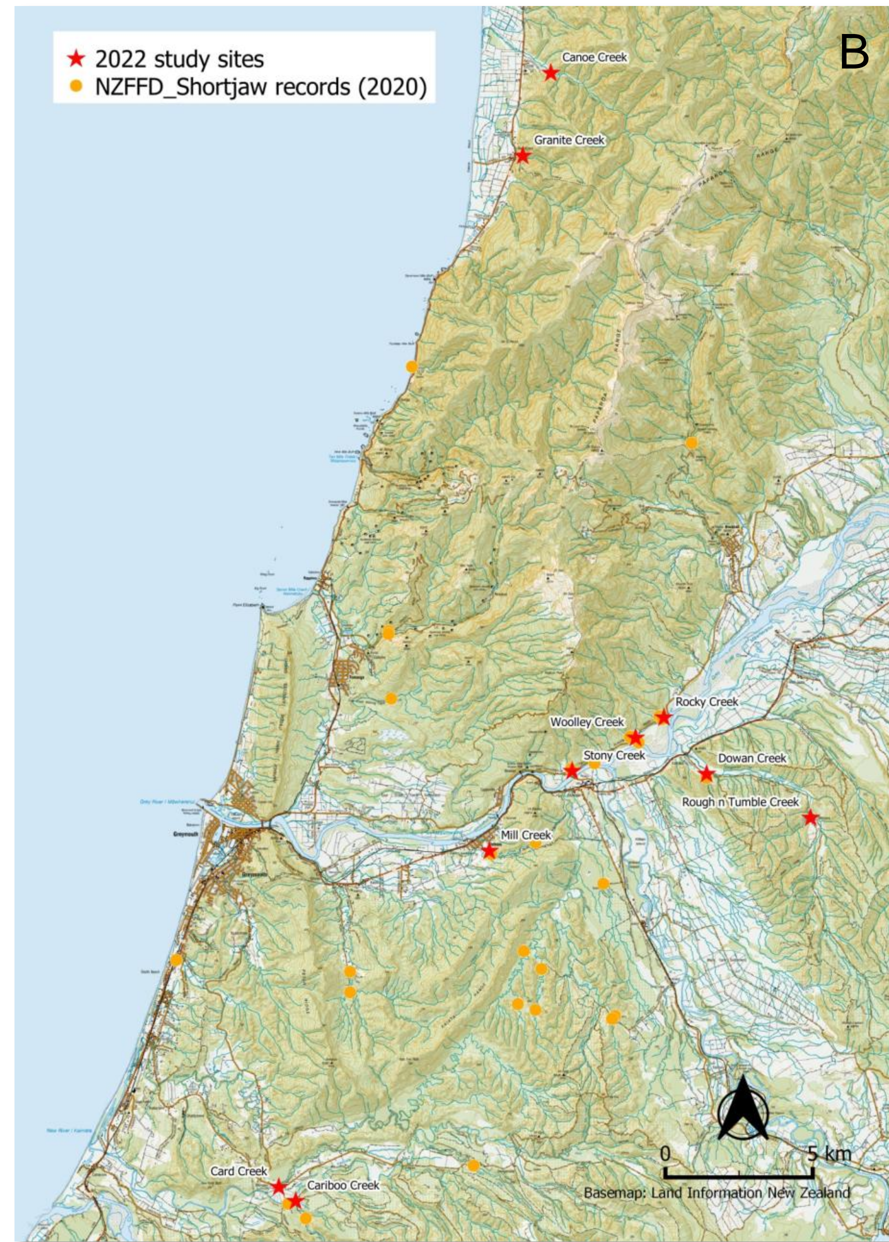


Figure 3 Study site names and locations. The coordinates shown are located at the downstream end of a 400 m fixed reach which was also the eDNA sampling site.
A. 2021 surveys in the Hokitika area. B. 2022 surveys in the Mawhera / Grey area.

2.3 Spotlighting surveys

Spotlighting for shortjaw kōkopu has been found to be one of the best methods for monitoring this species and is particularly useful for obtaining relative abundance data (Allibone 2013; Jack 2020; Jack et al. 2001; Joy et al. 2013). To support the objective of discovering new populations the methodology for this project was based on spotlight surveys within 400 m fixed reaches in a relatively fast yet robust procedure generally following Jack (2020) and Orchard (2021b). This involves a targeted survey for shortjaw kōkopu which aims to capture any suspected individuals to confirm identification and obtain length measurements. All other fish species are recorded and their length estimated but no effort made to capture.

The daytime habitat surveys were done prior to spotlighting to mark out the fixed reaches and complete habitat assessments. Each 400 m reach was established by measurement from a pre-determined origin point along the channel centreline using a 50 m tape. Width and depth measurements were made every 50m. Other habitat assessments were completed within four 100 m sub-reaches that were marked using brightly coloured flags for visual identification at night. Environmental parameters measured within each sub-reach included maximum depth, water colour, temperature, conductivity, habitat type, substrate composition, instream cover and riparian vegetation cover and type. Habitat conditions were also assessed at the 400 m reach scale using the Pfankuch Stability Assessment (Pfankuch 1975), and National Rapid Habitat Assessment Protocol (NRHAP) (Clapcott 2015). Additional notes on threats and pressures affected the streambed or riparian zones were noted when observed, and in some cases included observations made downstream of the study reach (e.g., presence of fish passage barriers where previously known or seen).

Spotlight surveys began a minimum of 1 hour after dark at the downstream end of the survey reach which was also the eDNA sampling point. Either one or two reaches were surveyed on a given night depending on factors such as ease of access, length of time to complete the first reach, weather conditions and energy levels of survey team. The spotlighting process generally followed the descriptions in Joy et al. (2013) for a single-pass survey with a team or two or three people. The primary spotlight was a Narva Colt (1000 lumen) lamp operated by one team member and all team members were equipped with powerful head torches. The team works systematically upstream surveying all of the wetted area including shallow areas on the fringes and backwaters on the riparian margins including those that may have been temporarily disconnected from surface water flow. A second 1000 lumen lamp was also used in some of the larger waterways to reduce survey time.

All fish observed were recorded individually along with their measured or estimated length using total length in all cases. All fish species seen were identified to the closest confident taxonomic level and their total length estimated to the nearest 5 mm or closest 1 mm if caught. An attempt was made to catch any suspected shortjaw kōkopu to confirm identification and calibrate visual size estimates. Galaxiids that could not be positively identified to species level were recorded as 'unidentified galaxiid'. No attempt was made to catch eel species and they were mostly recorded as 'unidentified eel' unless a positive identification was possible. Following the spotlighting of each sub-reach the percentage fishable area was estimated to record the proportion of the wetted area in which fish could be reliably seen.

2.4 eDNA surveys

2.4.1 Sampling equipment and treatments

The 2021 eDNA sampling protocols use two types of equipment: active syringe sampling and passive sampling using a drogue, both provided by Wilderlab NZ Limited (Figure 4). Syringe sampling involves the filtration of water on-site using a 60 ml plastic syringe fitted with a 1.2 micron filter capsule (Figure 5a). The drogue devices are a passive sampler that directs stream flow through a filter positioned within the water column and their performance is thus somewhat influenced by stream flow. In comparison the syringes, the drogues use a coarser Dacron (synthetic wool) filter that that is less prone to clogging but can lose a significant amount of DNA through the greater effective pore size. Each drogue was deployed positioned at the top of the water column using a nylon tether attached to existing instream wood or rocks (Figure 5b). Syringe sampling was done using two different protocols: day and night. The day time samples were taken in the early afternoon and night time samples a minimum of 1 hour after dark. Both day and night syringe sampling protocols were completed at all 11 sites. We aimed for a total sample volume of 1000 ml that was sampled as 20 x 50 ml sub-samples, each taken from 5 cm below the water surface. At some sites the full 1000 ml sample could not be obtained due to clogging of the filter and these instances the final sample volume was recorded to enable normalisation of eDNA count data. Drogue sampling was completing at 7 of the 11 sites with the drogues being deployed overnight (approximately 12 hours) at the same sites used for syringe sampling.

The 2022 eDNA sampling protocols also used two types of equipment but the passive sampling was done using the newly designed 'pod' samplers. In comparison to the drogue samplers the pod samplers are fitted with a pre-filter that excludes larger particles such as detritus and whole organisms, and a coarse internal Dacron filter as used in the drogues. They were deployed following the recommended combination of manifold housings and individual pod samplers mounted on short aluminium stakes. The manifolds secured on warratah stakes are well suited for deeper sites, while arrays of individual pods can be useful in shallow water situations. To investigate the potential effect of a longer sampling period, two pod sampler treatments were trialled (16 and 26 hours). Day and night syringe samples were taken at all sampling sites with three replicates each for all treatments.

A summary of the sampling treatments and replicates used in each year are shown in Figure 4.

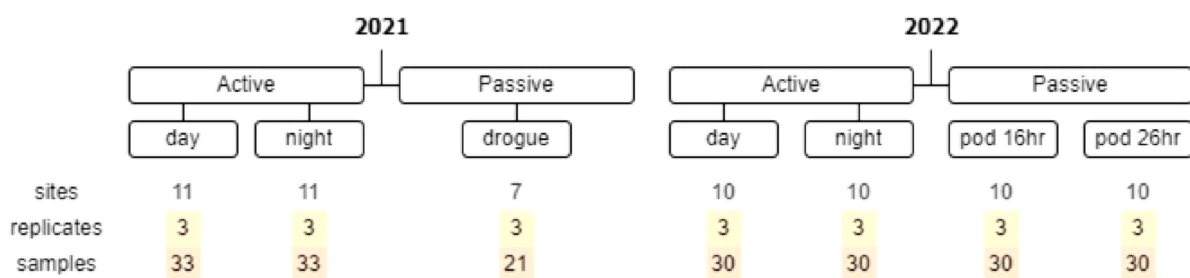


Figure 4 eDNA sampling design used in the 2021 and 2022 parallel monitoring trials.

Figure 5 Study sites. (a) Active sampling using syringes assisted by a caulking gun at Liverpool Bills Gully. (b) Drogue samplers in position in McIntryres Creek.

2.4.2 Controls on cross-contamination

The strategy for eDNA sampling was closely interwoven with the fixed-reach spotlighting surveys to avoid potential for cross contamination. All eDNA sampling was done prior to the spotlighting survey to eliminate the potential for equipment used elsewhere (e.g., waders) to have been in contact with the water column upstream of an eDNA sampling point. The eDNA sampling points were established at the

downstream end of the 400 m fixed reach for each waterway (Table 2). Three individual sampling points were identified across the channel (left, centre, right), each positioned in a section of relatively even flow that avoided eddies and other recirculating features. To address contamination risks associated with repeat sampling of the same sites each successive sample was taken a small distance upstream of the previous sampling position. New sterilised equipment was used for each individual eDNA sample with the exception of those using manifolds which were re-used between sites after treatment by bleaching and heat sterilisation. Disposable gloves were used and changed after collection of each sample. Once fixed, eDNA sampled were labelled and stored in cool dark conditions before processing the laboratory, and are effectively sealed from any form of cross-contaminant through the design of the filtration and packaging system.

2.5 Parallel monitoring schedule

A relatively complex set of sampling logistics were required to implement the parallel monitoring approach. Each study site required up to five separate visits to complete the set of measurements. These were done following a schedule which directed the order of different surveys to eliminate potential eDNA contamination above the sampling point (Figure 6). The logistics for concurrent sampling with a downstream eDNA sampling point are more difficult than when it can be located at the upstream end of the spotlighting reach, in which case a more compressed format could be used. However, the best access to nearly all of the study sites was from the downstream end. In most instances this was the only feasible access for eDNA sampling within the required timeframes due to rough terrain of the streambeds and difficulties of sampling at night. These logistics also needed to align with periods of flow river low which were comparable over the survey period and also between years. In 2021 the surveys were completed in three separate field campaigns targeting low flow periods within a 33 day period overall (Table 2a). In 2022 the survey campaigns were fortunate to coincide with an extended period of low flow that allowed all of the surveys to be completed on consecutive days (Table 2b).

Day	Time	Site 1		Site 2		Site 3
1	day					
1	night	Passive samplers deployed late afternoon				
2	day	16hr passive sample retrieved Syringe-day sample taken				
2	night	26hr passive sample retrieved Syringe-night sample taken	↔	Passive samplers deployed late afternoon		
3	day	Habitat survey of fixed reach	↔	16hr passive sample retrieved Syringe-day sample taken		
3	night	Spotlight survey of fixed reach	↔	26hr passive sample retrieved Syringe-night sample taken	↔	Passive samplers deployed late afternoon
4	day			Habitat survey of fixed reach	↔	16hr passive sample retrieved Syringe-day sample taken
4	night			Spotlight survey of fixed reach	↔	26hr passive sample retrieved Syringe-night sample taken
5	day					Habitat survey of fixed reach
5	night					Spotlight survey of fixed reach

Figure 6 Parallel monitoring schedule for four eDNA sampling treatments and near-concurrent spotlighting surveys as used in 2022. The 2021 survey schedule was similar but with only one (overnight) passive sample being taken at each site that was deployed in the evening on the previous day.

2.6 Laboratory procedures

Sample extraction, library preparation, sequencing and bioinformatics broadly followed the method outlined in David et al. (2021), except that DNAs were extracted using the Genolution GD141 cartridge on the Nextractor NX-48S system (Genolution), and pooled libraries were run on the Illumina iSeq 100 platform at Wilderlab NZ Limited in Wellington.

In the original round of eDNA analyses the Wilderlab 'basic freshwater' assay panel was used for sequencing (herein referred to as the 'original assay panel'). The contributing primer sets are the LV, CI, RV, DG and WV assays shown in Table 3.

2.7 Development of new 'YG' primer set

Upon review of the initial results from the parallel monitoring campaigns using the original assay panel (see details below), the identification of low detection rates (particularly from the original LV (12S) and DG (16S) assays) prompted the development of a new assay/primer-set targeting a short but hypervariable region of the mitochondrial cytochrome b gene (assay code 'YG'). This assay produces a short (101 bp) amplicon that is highly specific for *Galaxias* spp. and can distinguish between all New Zealand Galaxiid species with the exception of *G. anomalus* and *G. pullus*, which share an identical barcode at this locus. All 2021 and 2022 samples were subsequently extracted from -20C storage and re-analysed using the new assay in June 2022, in order to ascertain whether detection rates for shortjaw kōkopu could be improved with the new *Galaxias*-specific assay.

Following development of the new 'YG' primer set, archived eDNA from all samples was re-analysed using the 'new assay panel' in which the DG primer was dropped and replaced with YG (Table 3).

Table 3 Components of the metabarcoding assay panels used in this study.

Assay	Gene	Forward Primer	Reverse Primer	Reference
LV	mt12S	TCGTGCCAGCCRCCGC	CATAGTGGGGTATCTAATCCCAGTTTG	[1]
CI†	COI	DACWGGWTGAACWGTWTAYCCHCC	GTTGTAATAAAATTAAYDGCYCCTARAATDGA	[2]
RV†	mt12S	TTAGATACCCCACTATGC	TAGAACAGGCTCCTCTAG	[3]
DG	mt16S	TCTTCGGTTGGGGCGAC	GGATTGCGCTGTTATCCCT	[4]
WV†	mt16S	GACGAGAAGACCCTWTGGAGC	CCRYGGTCGCCCCAAC	[5]
YG*	Cyt-b	CBGAYATCTCYACCGCYTTYTC	AAAGAAAGATGCGCCRTTRGCATG	[6]

[1] Forward primer developed by Wilderlab; 'MiFish' reverse primer from Miya et al. (2015).
[2] Primers developed by Wilderlab.
[3] Forward primer from Riaz et al. (2011); reverse primer from Kelly et al. (2014).
[4] Forward primer developed at Wilderlab; reverse primer from Deagle et al. (2009).
[5] Forward and reverse primers adapted from Nester et al. (2020).
[6] Forward and reverse primers developed by Wilderlab (this study).
* Included after the original analysis of 2021 and 2022 samples, by reanalysing samples from -20C storage.
† Unable to definitively resolve *Galaxias postvectis* to species level due to non-diagnostic DNA barcode sequence.

2.8 eDNA sensitivity evaluation

The focus of data analysis for this project involved evaluating the sensitivity of eDNA sampling methods for the detection of shortjaw kōkopu. This was done by calculating the detection rate for the waterways in which shortjaw kōkopu presence had been confirmed by at least one method in the study. This treatment of uncertainty essentially ignores the potential for Type I (false positive) errors which were not specifically tested for. The absence of Type I errors is an assumption that was instead addressed through the proactive measures to eliminate them as described in section 2.4.2. Subject to this assumption, the survey design provides a direct test of the Type II errors resulting from imperfect detection, or in other words, the failure to detect presence when presence was there (Underwood & Chapman 2003).

Confirmed presence was established from the results of both the spotlighting surveys and eDNA surveys, and waterways not meeting this condition were discarded from the sensitivity analysis. Therefore, some waterways which returned negative spotlighting results but positive eDNA results were included, with the latter being identified on a per-sample basis. In this situation the test considered whether detections were made across all eDNA samples from the same and other eDNA sampling methods. Waterways were only dropped from the analysis where there was no evidence of shortjaw kōkopu presence across all the available data sources.

The primary comparisons of interest were:

- sampling methods (syringe-day, syringe-night, drogue-12hr, pod-16 hr, pod-26 hr).
- equipment types (syringe, drogue, pod)

Binomial Generalized Linear Mixed Models (GLMMs) were used to evaluate detection rate differences between sampling equipment and the sampling methods. GLMMs were fitted by maximum likelihood (Laplace Approximation) using the *lme4* package (Bates et al. 2015), and assumed that the detection rate varied randomly by site (Site), for fixed effects of sampling method (Method), and sampling equipment (Equipment).

DetectionRate ~ Method + (1 | Site)
DetectionRate ~ Equipment + (1 | Site)

These tests were applied to detection rates calculated on both a per-sample and per-site basis for each survey campaign. All analyses were completed in R version 4.1 (R Core Team 2021).

3. Results

3.1 Spotlighting surveys

The 2021 spotlighting surveys identified a total of 15 shortjaw kōkopu (Table 4a). The greatest abundance was found in Acre Creek (n=6 fish), followed by Flowery Creek (n=5). The Foley Creek study site (n=4 fish) is a tributary of Houhou Creek, where previous shortjaw kōkopu observations have been made in another tributary (Brennans Creek), and the Houhou Creek study site lies further downstream. In this case, the spotlighting survey was abandoned in Houhou Creek due to water clarity concerns, but we regarded Houhou Creek as having 'confirmed presence' in the eDNA evaluations on the basis of the observations made upstream. The catchments where shortjaw kōkopu were found have markedly different land-uses patterns. Acre Creek represents a largely unmodified catchment and Flowery Creek has a relatively high proportion of intensive land uses including farmland and forestry (Appendix 1).

In 2022, the spotlighting surveys recorded a total of 82 shortjaw kōkopu (Table 4b). There were particularly high numbers in two waterways (Stony Creek and Cariboo Creek), and positive identifications in eight of the 10 waterways. This partly reflects the sampling strategy which targeted a greater proportion of sites where shortjaw kōkopu had previously been recorded in comparison to the waterways selected in 2021 (Table 2). This facilitated the parallel monitoring approach for eDNA evaluation, as was desired, using the balanced sampling design to compare syringe sampling and pods at all sites. Details of other fish species recorded in these surveys are available in a separate report for the 2021 surveys (Orchard 2021b), and in NZFFD records for both years.

Figure 8 A large gravid shortjaw kōkopu caught in Dowan Creek.

Table 4 Summary of shortjaw kōkopu abundance and fishing effort metrics recorded in 2021 and 2022 spotlighting surveys.

(a) 2021 surveys

Study site	Reach length (m)	Wetted area (m^2)	Fished area (m^2)	Shortjaw kōkopu abundance
Acre Creek	400	1910	835	6
Acre South Creek	400	970	532	0
Flowery Creek	400	3110	2343	5
Foley Creek	400	1620	1180	4
Frosty Creek	400	3900	1597	0
Greeks Creek	400	990	679	0
Houhou Creek	*	*	*	*
Kaniere River trib.	400	740	598	0
Kapitia Creek	400	1670	879	0
Liverpool Bills	400	1450	1050	0
McIntyres Creek	400	485	184	0
TOTALS	**4000**	**16845**	**9877**	**15**

* a spotlighting survey was not completed in Houhou Creek due to weather conditions

(a) 2022 surveys

Study site	Reach length (m)	Wetted area (m^2)	Fished area (m^2)	Shortjaw kōkopu abundance
Canoe Creek	400	2787	731	1
Card Creek	400	3293	2270	0
Cariboo Creek	400	2983	2020	27
Dowan Creek	400	727	520	1
Granite Creek	400	1350	607	7
Mill Creek	400	1123	945	2
Rocky Creek	400	1480	928	3
Rough n Tumble Creek	400	1640	941	0
Stony Creek	400	2310	1579	36
Woolley Creek	400	1780	1176	5
TOTALS	**4000**	**19473**	**11717**	**82**

3.2 eDNA analysis with original assay panel

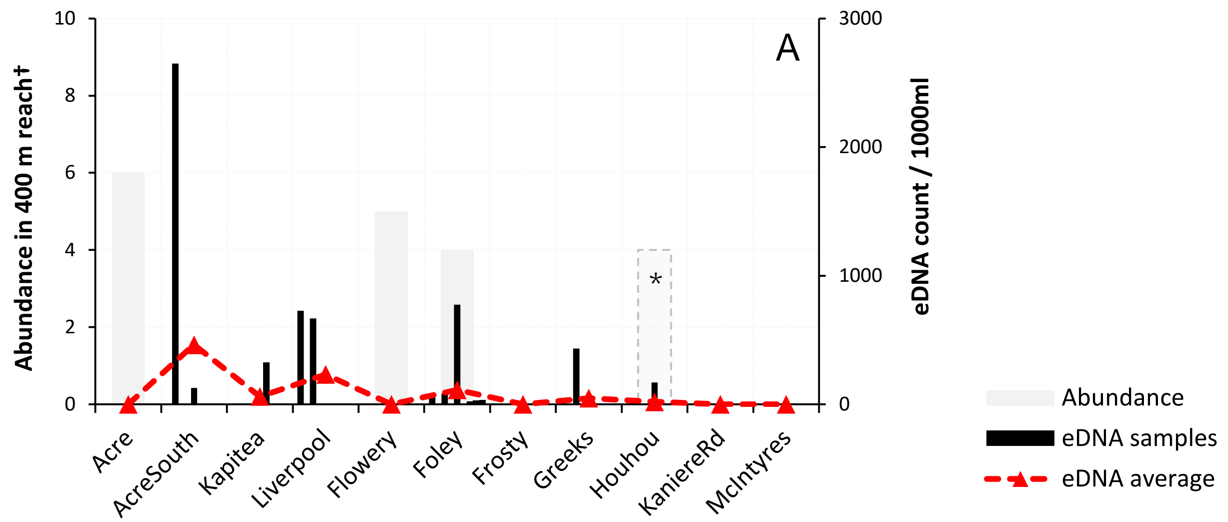
3.2.1 Overview

The eDNA results featured relatively high non-detection rates at sites with confirmed shortjaw kōkopu presence, as detailed further below. However, they also included examples of positive detections at sites where the spotlighting surveys had not identified shortjaw kōkopu in the upstream reach. Another general observation that can be made is that the combined results of spotlighting and eDNA surveys generated more detections that would have been possible from either method alone.

3.2.2 eDNA sequence counts

eDNA sequence counts from individual samples varied widely both within and between years (Figure 9, Appendix 2). In 2021, the highest counts were recorded in Acre South and Liverpool Bills streams (2774 and 1395, respectively). In both of these waterways, the spotlighting surveys failed to detect shortjaw kōkopu suggesting that the eDNA source might lie further upstream. In 2022, the average eDNA counts were generally much lower, and the total count across all sites (170), was much lower than the 2021 total (6152), despite there being a greater number of samples taken in 2022 (n=120).

2021



2022

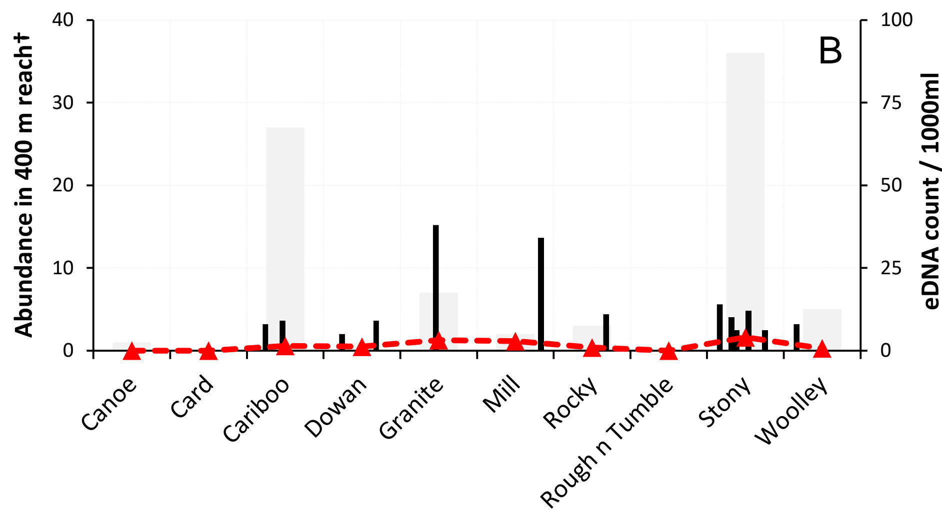


Figure 9 Results from eDNA analyses with the original assay panel. Results from spotlighting surveys are also shown with abundance (Y axis) being the number of fish observed in the reach upstream of the eDNA sampling point. Per-sample (black bars) and average (red triangle) eDNA counts are shown for each site. Note marked differences in the Y-axes for both abundance and eDNA count between years. * a spotlighting survey was not completed for Houhou Creek due to weather conditions but another sampling site (Foley Creek) with confirmed shortjaw kōkopu presence is located upstream.

3.2.3 Sensitivity of sampling methods

For completeness, this section reports on results from the sensitivity evaluation using the original assay panel. However, due to the low eDNA counts in 2022, and their implications (see discussion in section 4.1), these analyses were regarded as inconclusive and instead became the focus of further investigations. Detection rates at study sites with confirmed shortjaw kōkopu presence showed low per-sample detection rates (maximum of 0.25) in both survey campaigns (Tables 5 and 6).

2021 trials – syringe and drogues

In 2021, the syringe-night and drogue-12hr sampling methods both outperformed the syringe-day detection rate on a per-sample basis (Table 5a). However, differences between the sampling methods were not statistically significantly in the GLMMs and similarly for the comparison between syringe and drogue sampling equipment ($z = -0.32$, $p = 0.75$).

On a per-site basis (n=3 replicates), the best results were achieved by the syringe-night and drogue-12hr methods which each resulted in a detection rate of 0.5 (Table 5b). Based on pooled data for equipment type, the same detection rate (0.5) was achieved by the syringes and drogues. An analysis of pooled data from all eDNA sample-method combinations (n=9 at each site) shows that raising the number of effective replicates could achieve a detection rate of 0.75, meaning that the target species was still missed by all eDNA samples at a quarter of the study sites.

Table 5 eDNA sensitivity results from the 2021 field trials using the original assay panel. The per-site calculation represents the presence / absence sensitivity obtained from n=3 replicate eDNA samples. This analysis is based on study sites with confirmed shortjaw kōkopu presence, at which all eDNA sampling methods were deployed (n=4).

(a) Sampling method comparison

Sampling method	Sampling strategy	n	Sensitivity (detection rate)	
			per-sample (±SE)	per-site
syringe-day	active	12	0.17 ±0.11	0.25
syringe-night	active	12	0.25 ±0.13	0.50
drogue-12hr	passive	12	0.25 ±0.13	0.50
Average (all samples)	combined	36	0.23 ±0.07	0.75

(b) Sampling equipment comparison

Sampling equipment	Sampling strategy	n	Sensitivity (detection rate)	
			per-sample (±SE)	per-site
syringe	active	24	0.21 ±0.08	0.50
drogue	passive	12	0.25 ±0.13	0.50

2022 trials - syringe and pods

The 2022 trials returned generally lower per-sample detection rates than those recorded in 2021 (Table 6), which is consistent with the markedly lower eDNA counts that year. Both syringe methods performed better than the pods on a per-sample basis (Table 6a) but the average detection rate of the syringes (0.17) was nonetheless lower than that achieved in 2021 (Table 6b). These trends were also reflected in the per-site detection rates in which the syringe sampling methods generally outperformed the pods (Table 6b). However,

the differences between sampling methods and sampling equipment were not statistically significant in the GLMMs.

Using pooled data from all eDNA sample-method combinations from each site (n=12) returned a per-site sensitivity of 0.88 (Table 6a). This represents the lack of a positive detection at one of the eight study sites with confirmed presence (Canoe Creek). At that site, only a single shortjaw kōkopu was identified in the spotlighting surveys and it was located approximately 300 m upstream of the eDNA sampling point. This is also a fast-flowing waterway which suggests that dilution effects may have influenced this result, providing an insight into the detection limits of the eDNA sampling procedures despite a high number of individual samples being taken from each waterway.

Table 6 eDNA sensitivity results from the 2022 field trials using the original assay panel. The per-site calculation represents the presence / absence sensitivity obtained from n=3 replicate eDNA samples. This analysis is based on study sites with confirmed shortjaw kōkopu presence, at which all eDNA sampling methods were deployed (n=8).

(a) Sampling method comparison

Sampling method	Sampling strategy	n	Sensitivity (detection rate)	
			per-sample (±SE)	per-site
syringe-day	active	24	0.17 ±0.08	0.50
syringe-night	active	24	0.17 ±0.08	0.38
pod-16hr	passive	24	0.13 ±0.07	0.38
pod-26hr	passive	24	0.08 ±0.06	0.25
Average (all samples)	combined	96	0.14 ±0.04	0.88

(b) Sampling equipment comparison

Sampling equipment	Sampling strategy	n	Sensitivity (detection rate)	
			per-sample (±SE)	per-site
syringe	active	48	0.17 ±0.05	0.63
pod	passive	48	0.10 ±0.04	0.50

3.3. eDNA analysis with new assay panel

3.3.1 eDNA sequence counts

The new assay panel featuring the new 'YG' primer set had a dramatic effect on eDNA sequence counts, particularly in the 2022 samples which had returned low counts with the original primer set (Table 7). This improvement also resulted in new detections in samples for which shortjaw kōkopu had not been detected using the original assay panel. The number of detections per waterway ('presence/ absence hits' in Table 7) was slightly more than double the original result in both years. This reflected at least one eDNA sample having made a detection where previously that sample had not.

The number of study sites at which shortjaw kōkopu were detected increased dramatically in the 2021 samples with the new assay panel making a positive detection at all 11 study sites. In contrast, it resulted in only one new detection in the 2022 samples (Card Creek) and two study sites remained without a single eDNA hit (Canoe and Rough n Tumble creeks) (Table 7).

Table 7 Comparison of results from the original assay panel and new assay panel featuring the new 'YG' cytochrome b primer set.

(a) 2021 surveys

	Acre	Acre South	Flowery	Foley	Frosty	Greeks	Houhou	Kaniere tributary	Kapitea	Liverpool	McIntyres	Total
eDNA count												
Original assay panel	0	2774	0	781	0	433	17	0	367	1189	0	**5561**
New assay panel	213	3356	1584	1078	560	965	17	829	1125	1846	275	**11848**
Difference	213	582	1584	297	560	532	0	829	758	657	275	**6287**
Presence/absence hits												
Original assay panel	0	2	0	6	0	1	1	0	2	2	0	**14**
New assay panel	3	2	1	8	2	2	1	3	4	3	1	**30**
Difference	3	0	1	2	2	1	0	3	2	1	1	**16**

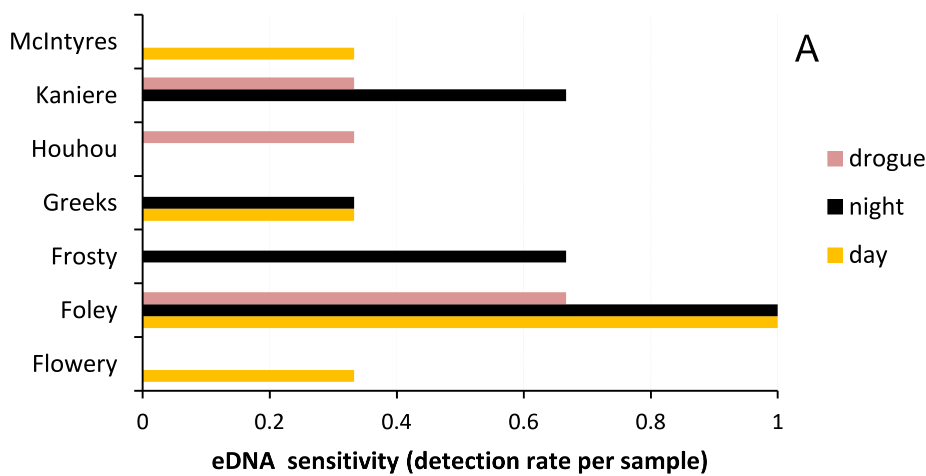
(b) 2022 surveys

	Canoe	Card	Cariboo	Dowan	Granite	Mill	Rocky	Rough n tumble	Stony	Woolley	Total
eDNA count											
Original assay panel	0	0	17	14	38	34	11	0	48	8	**170**
New assay panel	0	41	1009	1172	1234	34	11	0	5113	579	**9193**
Difference	0	41	992	1158	1196	0	0	0	5065	571	**9023**
Presence/absence hits											
Original assay panel	0	0	2	2	1	1	1	0	5	1	**13**
New assay panel	0	1	4	4	5	1	1	0	10	3	**29**
Difference	0	1	2	2	4	0	0	0	5	2	**16**

3.3.2 Sensitivity of sampling methods

Detection rates for the various eDNA sampling methods ranged from 0 – 100% at study sites with confirmed shortjaw kōkopu presence (Figure 10).

Detections were made by all three replicates for both day and night syringe sampling at Foley Creek in 2021, and similarly for both pod sampling methods deployed in Stony Creek in 2022. For all other methods and site combinations, one or more detections were made by the various sampling methods. Interestingly, all of the methods made detections at least one site that were not picked up by the other methods deployed at that site, with the exception of syringe-night method in 2022 (Figure 10).



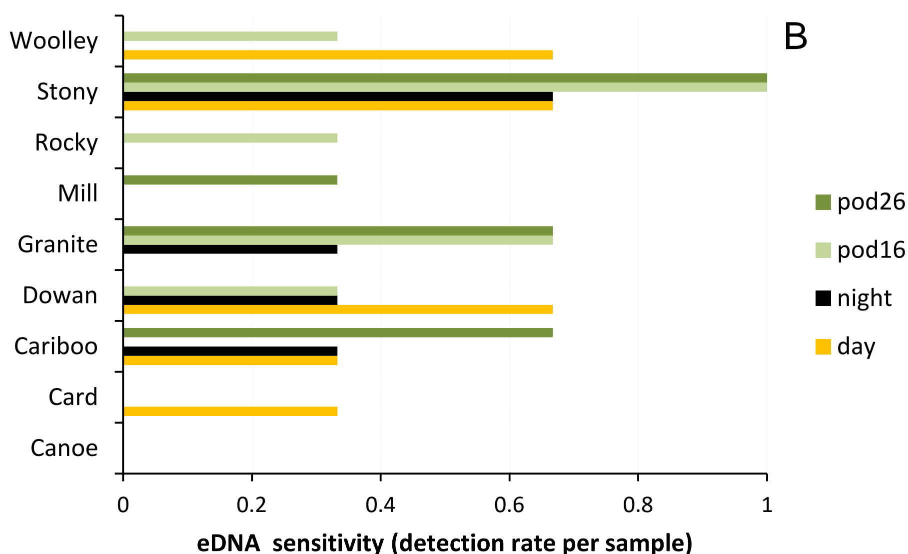


Figure 10 Per-sample eDNA detection rates at sites with confirmed shortjaw kōkopu presence using the new assay panel. For each year only the sites sampled with all methods are included in the analysis. All methods were sampled with n=3 replicates in both years.

The sensitivity comparison between methods was applied to seven of the 11 sites sampled in 2021, and nine of the 10 sites sampled in 2022. These represent the sites with confirmed shortjaw kōkopu presence at which all of the sampling methods were deployed in each year (i.e., the same rationale underpinning the original analysis).

2021 trials – syringe and drogues

Comparisons between the methods used in 2021 showed that syringe-night sampling had the highest detection rate per sample (0.38), but the syringe-day sampling achieved a better detection rate across all three replicates deployed at a given site (0.57) (Table 8). The lack of a clear trend is consistent with the somewhat haphazard pattern of syringe hits from the various samples and site combinations as shown in Figure 10. In comparison, the drogues returned a lower per-sample detection rate (0.19) but achieved the same per-site detection as rate as syringe-night sampling (0.43). The syringe versus drogue equipment comparison using pooled data showed markedly better performance from syringes on both a per-sample and per-site basis (Table 8b), but it should be noted that this is derived from an unbalanced design. The GLMM results also showed that the difference was not-statistically significant in this case ($z = 1.33$, $p = 0.18$).

Overall, it can be concluded that the passive drogue sampling method offers no advantage over the active syringe sampling methods. Across all eDNA sampling methods we tested, there was also a considerable likelihood of non-detection with n=3 replicates. However, by pooling the syringe sampling results (from day and night time samples), sensitivity improved to 0.86. This reflects a successful detection at six of the seven sites from n=6 effective replicates (Table 8b). Taken together, these results suggest that raising the number of replicates (to a minimum of 6) may be warranted to improve the detection sensitivity of the sampling methods that we trialled.

Table 8 Results from 2021 evaluations of eDNA sensitivity for the detection of shortjaw kōkopu from parallel monitoring studies at sites where all sampling methods were deployed (n=7). The per-site calculation represents pooled data from n=3 replicates. eDNA analyses used the new assay panel featuring the 'YG' primer set.

(a) Sampling method comparison

Sampling method	**Sampling strategy**	**n**	**Sensitivity (detection rate)** per-sample (±SE)	per-site
syringe-day	active	21	0.29 ± 0.10	0.57
syringe-night	active	21	0.38 ± 0.11	0.43
drogue-12hr	passive	21	0.19 ± 0.09	0.43
Average (all samples)	combined	63	0.29 ± 0.06	0.48

(b) Sampling equipment comparison

Sampling equipment	**Sampling strategy**	**n**	**Sensitivity (detection rate)** per-sample (±SE)	per-site
syringe	active	42	0.44 ± 0.07	0.86
drogue	passive	21	0.27 ± 0.14	0.43

2022 trials - syringe and pods

The per-sample detection rates were the same for three of the four methods tested in 2022 (0.30), but markedly lower for the syringe night sampling method (0.19) (Table 9). As with the 2021 results, the detection sensitivity per-site was in the 0.4 – 0.6 range for all methods when n=3 replicates were considered (Table 9a). When the samples were pooled by equipment type, the pods performed better than syringes on both a per-sample (0.30) and per-site basis (0.78), with this test representing n=6 effective replicates across the nine study sites. This provides some evidence for a passive sampler advantage attributable to the new pod design, but the difference was not statistically significant in the GLMMs.

Key findings from the 2022 analyses included further evidence that a minimum of n=6 eDNA samples is needed to reliably detect shortjaw kōkopu using these sampling methods. Further trials of the new pod samplers may be warranted given their favourable results in this study, and could include a focus on the sampling contexts or conditions in which they may outperform other methods. Indications from this study suggest that such conditions might be presented by clear fast-flowing waterways. It is also notable that the deployment times we trialled (16 and 26 hours) produced very similar results. It would be useful to consider the effect of a greater range of deployment times and these might include longer periods to ascertain any potential for improved detection and/ or identify inflection points at which sensitivity may drop-off.

Table 9 Results from 2022 evaluations of eDNA sensitivity for the detection of shortjaw kōkopu from parallel monitoring studies at sites where all sampling methods were deployed (n=9). The per-site calculation represents pooled data from n=3 replicates. eDNA analyses used the new assay panel featuring the 'YG' primer set.

(a) Sampling method comparison

Sampling method	**Sampling strategy**	**n**	**Sensitivity (detection rate)**	
			per-sample (±SE)	per-site
syringe-day	active	27	0.30 ± 0.08	0.56
syringe-night	active	27	0.19 ± 0.07	0.44
pod-16hr	passive	27	0.30 ± 0.08	0.56
pod-26hr	passive	27	0.30 ± 0.08	0.44
Average (all samples)	combined	27	0.27 ± 0.04	0.50

(b) Sampling equipment comparison

Sampling equipment	**Sampling strategy**	**n**	**Sensitivity (detection rate)**	
			per-sample (±SE)	per-site
syringe	active	54	0.24 ± 0.06	0.67
pod	passive	54	0.30 ± 0.06	0.78

4. Discussion

4.1 Importance of validation studies

The series of studies reported here shows the value of evaluating eDNA survey results against independent data to inform the refinement of methodologies and underpin the interpretation of results. This validation approach produced several unexpected findings. The most pronounced among these was the marked difference in eDNA sequence counts between years when using the original assay panel. These differed by two orders of magnitude despite being comprised of samples from multiple sites. The results were not obviously explained by dilution effects and instead suggested the influence of another unknown source of variance that was greater than the effect of target species abundance within the study reach.

Discussions with Wilderlab and DOC staff, and between members of an informal eDNA monitoring group have identified two broad categories of variance that may have influenced these results.
They are:

- variance in the concentration of eDNA fragments in the sampling environment due to factors such as the shedding rate of source material, which in turn could be influenced by seasonal, demographic or behavioural aspects of the target species, or the effect of environmental conditions such as temperature, chemical or microbial differences in the sampling environment on the transport or persistence of eDNA.
- variance in the binding properties of primers with their target sequences in laboratory protocols.

The decision to embark on a primer development project provided an opportunity to explore these issues further. It should be noted that comparisons between results from the original and new assay panels were enabled by the ability to re-assess the same samples from archived eDNA. Archiving is therefore recommended as a standard practice and may be particularly valuable where the assessment methodologies are not well established for the target species and/ or study environment.

In this case the re-assessment process showed significantly improved eDNA sequence counts and target species detection rates (Table 7). The validation process based on a parallel monitoring design supported this sequence of methodological developments and resulted in an improved basis for revisiting the original research questions around the comparison of field sampling methods and typical error rates.

4.2 Insights for eDNA detection of shortjaw kōkopu

There was no apparent relationship between eDNA sequence counts and the number of fish observed upstream of the eDNA sampling points. This cautions against the interpretation of eDNA counts as an indicator of fish abundance, at least at this fixed-reach assessment scale. Results from individual streams provide additional insights on the relationship between observed fish abundance, spatial distribution, and eDNA detection rates. For example, at Canoe Creek where a single shortjaw kōkopu was located approximately 300 m upstream from the eDNA sampling point, none of the 12 eDNA samples were able to detected presence. The intervening section of stream is a fast flowing waterway which may increase effective dilution of eDNA fragments in the sampling environment in this case. In Dowan Creek, which was the only other study site where a single fish was recorded in the 2022 spotlighting surveys, four of the 12 eDNA samples returned a successful detection. In this case the fish involved was a large gravid female (Figure 8) located in a slow flowing pool only 50 m from the eDNA sampling point. This site also returned the 3rd highest eDNA count which was higher than several other sites with greater shortjaw kōkopu abundance. The poor correlation between eDNA counts and abundance appears to have been influenced by the presence of a single large individual close to the eDNA sampling point in Dowan Creek in relation to other study sites. The spatial distribution and also size of individuals may therefore influence the relationship between species abundance and eDNA sequence counts in samples taken from downstream sampling points.

As was expected, the eDNA sampling also resulted in new detections (i.e., not picked up in the spotlighting surveys). These may reflect the presence of fish that were not seen during spotlighting due to factors such as cryptic behaviour or being located in effectively unfished parts of the reach (e.g., swift water), or may reflect the presence of fish located upstream of the survey reach that were detectable via the dispersal of their eDNA. There is currently very little information on the dispersion and detection distances of eDNA from upstream resident populations of target species such as shortjaw kōkopu. This is likely to have species-specific aspects and would assist with the interpretation of both positive and negative eDNA results. In this case, it would be particularly useful to inform the upscaling of eDNA sampling campaigns for shortjaw kōkopu and other rare or invasive species which are difficult to detect over large scales using other techniques.

5. Conclusions

With still by far the majority of New Zealand's waterways with potential shortjaw kōkopu habitat yet to receive an initial survey, the development of more time and cost-effective survey approaches can make a significant contribution to the understanding and management of this culturally-important fish species. Urgent needs for shortjaw kōkopu conservation include the identification of population strongholds to establish catchment-specific priorities for the protection of critical habitat and hydrological connectivity, and consideration of whitebait fishing effects on juvenile shortjaw kōkopu (Goodman 2018; Orchard 2020). Improved distribution data can also assist with the ground-truthing and refinement of species distribution models which can complement empirical data but require validation.

The development of targeted conservation measures for this species is important for biodiversity conservation and sustainable use in the context of whitebait fisheries. An improved knowledge of important catchments can assist these needs by informing the evaluation of existing strategies, such as fisheries closures, and design of new approaches for shortjaw kōkopu and other whitebait species conservation. As the most endangered of the whitebait species, there is a particular need to evaluate and pinpoint measures that are effective for shortjaw kōkopu.

Results from the study provide an initial evaluation of the sensitivity and uncertainties associated with eDNA sampling protocols in common use in New Zealand. The results may assist with decisions on sampling method selection and replication levels, as summarised in the following section. Applications include species-specific monitoring, metabarcoding studies and index development projects using eDNA data. The latter area of application is most relevant where it is desirable that shortjaw kōkopu are represented in metabarcoding outputs. The evaluation of commonly-used sampling and laboratory procedures contributes to these needs by helping to identify detection biases, or conversely, provide evidence for their absence. Similar evaluation approaches are recommended for other rare or cryptic species for which detection is desirable in eDNA survey methodologies.

As with other techniques for surveying rare and cryptic species, eDNA survey approaches require optimisation and validation to assess to potential for both false-positive and false-negative error sources with non-uniform effects across taxa (Goldberg et al. 2016; Zinger et al. 2019). The study demonstrates the importance of conducting such evaluations, and shows how the results obtained can inform methodological improvements.

5.1 Summary of key findings

1. The new assay panel developed in response to this study produced significantly higher eDNA counts and a more than 2-fold improvement in eDNA detection hits across 207 independent samples, in comparison to the original primers used in Wilderlab's 'basic freshwater' assay panel. The new 'YG' primer set is now included in place of the original 'DG' primer set, as the default primers for shortjaw and giant kōkopu detection, although clients may opt to include an analysis with the original primers where needed to provide backwards-compatibility (e.g., in long-term monitoring programmes).

2. Replication considerations:

- Relatively poor performance from n=3 replicates across all sampling methods that were trialled
- Markedly improved performance from n=6 replicates across all sampling methods; but this was still insufficient to deliver 100% sensitivity. Therefore a minimum of 6 (and potentially more) replicates should be included in any standardised method for eDNA surveys where the detection of shortjaw kōkopu is the focus, or important component of, the survey objectives.

3. Comparisons between sampling methods:

- no advantage of passive drogue samplers over active syringe sampling
- some evidence for improved performance from passive pod samplers over active syringe sampling but with no difference between the two pod deployment times that were tested in this trial. However, these modest gains in sensitivity need to be considered in the context of the extra costs associated with having to make an extra site visit for retrieval of passive samplers such as pods, over active samplers such as syringes.

4. Comparisons between eDNA and spotlighting

- eDNA sampling made more detections than fixed-reach spotlighting and included the first records of shortjaw kōkopu in some catchments and a large number of catchment overall. This indicates some potential advantages over fixed-reach methods for detecting upstream or cryptic populations, particularly if the above sensitivity aspects are accounted for in survey design. Further research on detection distances would assist with the interpretation of both positive and negative eDNA results.

5. Future research recommendations include:

- further trials to investigate the potential benefits of passive pod samplers (or absence thereof), and with particular attention to the performance of longer deployment times and interactions with environmental co-variables in the sampling environment.
- further investigation of the dispersal and downstream detection characteristics of shortjaw kōkopu eDNA from populations upstream of the eDNA sampling point across a representative range of catchment morphologies and associated environmental conditions.
- additional validation of any standardised approach that emerges or is proposed with regards to the selection of sampling method(s) and replication, ideally across a wide range of study sites.
- Markedly improved performance from n=6 replicates across all sampling methods; but this was still insufficient to deliver 100% sensitivity. Therefore a minimum of 6 (and potentially more) replicates should be included in any standardised method for eDNA surveys where the detection of shortjaw kōkopu is the focus, or important component of, the survey objectives.

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Appendix 1. Study site details

Table S1. Description of study sites

(a) 2021 surveys

Study site	Description	Survey date	Shortjaw kōkopu presence†
McIntryes Creek	McIntryes Creek is a tributary of Brennans Creek in the Houhou Creek catchment. The study reach is a 1st order stream with an upstream catchment area of 0.9 km^2. As noted above, shortjaw kokōpu have been previously recorded in Brennans Creek and Houhou Creek, but they were not detected in the McIntyres surveyes despite the study reach being located only a short distance upstream from the confluence with Brennans Creek. The stream is relatively small with a predominantly cobble bottom and established forest cover in most places with exception of a small open area near the gun club. A notable aspect of the spotlighting results was the large number of banded kokōpu recorded (n=95) despite the relatively small size of the wetted area in the reach.	6/4	N
Kapitia Creek	This study site is in upper section of Kapitia Creek beginning approximately 2 km below the Kapitia Reservoir. The study reach is a 3rd order stream with an upstream catchment area of 16 km^2 when the waterways associated with the reservoir are included. There is an abundance of moss and algal cover on the instream substrates throughout the study reach indicative of relatively stable conditions that are influenced by the regulating effect of the reservoir. The riparian zone is generally well-vegetated with scrub or tussock cover and adjacent land cover includes regenerating forest and patches of cleared land.	7/4	Y
Liverpool Bills	Liverpool Bills Gully is a tributary of Waimea Creek (Figure 1). The study reach is a 2nd order stream with an upstream catchment area of 3.4 km^2. The landscape features low hill country and terraces with extensive areas of plantation forest and associated roading, especially to the south. The riparian zone features remnants of plantation forestry, patches of tussock and scrub, and regenerating native forest. There is a thriving fish population that included the highest number of redfin bullies recorded in the wider study and several large giant kokōpu as detailed in Orchard (2021a).	7/4	Y
Acre Creek	Acre Creek is a tributary of the Kapitia, joining the lower Kapitia lagoon from the north. The study site is a 2nd order stream with an upstream catchment area of 4 km^2. The riparian margins are largely unmodified and the river corridor downstream is also in good condition featuring established native forest cover and fenced buffer zone from the adjacent pastural land with the exception of a single stock crossing downstream of SH6.	8/4	Y
Acre South	Acre South is an un-named creek located a short distance south of Acre Creek. The study site is a 1st order stream with an upstream catchment area of 2.4 km^2. Upstream of SH6 the riparian margins and catchment in general are unmodified with established native forest cover. Downstream of SH6 the riparian margins are in relatively poor condition due to the close proximity of pastural land. Another important feature is an in-stream barrier associated with the culvert beneath SH6 which has a 0.5 m vertical concrete wall at the pipe end (Orchard 2021a).	8/4	Y
Foley Creek	Foley Creek is a tributary of the Houhou Creek situated north of Hokitika. The study reach upstream of Blue Spur Road is a 2nd order stream with an upstream catchment area of 4.2 km^2. Although the riparian zone is well vegetated within the survey reach there are areas of cleared land and earthworks nearby.	30/4	Y
Greeks Creek	Greeks Creek is a 2nd order tributary of the Arahura with an upstream catchment area of 1.2 km^2. The upper catchment has mainly regenerating native forest cover. Some high and partly unstable scarps are present in the riparian corridor towards the upstream end of the study reach.	1/5	Y
Kaniere River tributary	This study site is an un-named tributary of the Kaniere River. It is a 1st order stream with an upstream catchment area of 1.4 km^2 accessed via the West Coast Wilderness trail from Ward Road. The reach has an established podocarp canopy with the occasional small light gap formed by fallen trees. It was one of the smallest streams surveyed but featured a range of habitat types including pools, and relatively large giant kōkopu were present.	1/5	N
Flowery Creek	Flowery Creek is a tributary of the Arahura River that joins the northern rivermouth lagoon system downstream of SH6. The study site is a 3rd order stream with an upstream catchment area of 10 km^2. The riparian margins are located in private land with a mixture of vegetation types including some regenerating forest, interspersed with grassy banks, tussocks, sedges and scrub. The upper catchment	6/5	Y

	also passes through similar production landscapes with a relatively narrow riparian corridor being typical throughout. It was one of the more modified catchments that was surveyed.		
Frosty Creek	Frosty Creek is a tributary of the Lake Mahinapua catchment located south of Hokitika. The lake is located approximately 1.5 km downstream of the Woodstock Rimu Road. The study reach is a 3rd order stream with an upstream catchment area of 15.6 km^2. This reach is characterised by a relatively wide and confined channel bordered by steep forested banks in most places. The stream features dark tannin-stained waters and deep pools and runs broken by riffle sections. The riparian margins are largely a mixture of established and regenerating native forest. To the south there is an area of plantation forestry which comes close to the stream in several places. Despite the presence of this nearby production land the stream appears relatively stable and is largely unmodified in the riparian corridor.	7/5	N
Houhou Creek	The study reach is a 3rd order stream with an upstream catchment area of 15.9 km^2 containing a mix of production land and pockets of regenerating native forest and scrub. Shortjaw kokōpu have been previously recorded in this reach (Table 1) and also in the upstream catchment in Brennans Creek.	7/5	Y

(b) 2022 surveys

Study site	**Description**	**Survey date**	**Shortjaw kōkopu presence†**
Rough n Tumble Creek	Rough n Tumble Creek is a 2nd order stream with an upstream catchment area of 2.3 km^2. The catchment has a mix of plantation forestry and native forest cover. Water diversion tunnels associated with historical mining activities are present in the lower section of the catchment. The streambed is relatively mobile with evidence of significant bed-load yet to stabilise.	23/3	N
Woolley Creek	Woolley Creek is a 2nd order stream with an upstream catchment area of 2.6 km^2. Most of the catchment has native forest cover. The streambed is relatively stable but some recent slips were noted in river corridor.	24/3	Y
Rocky Creek	Rocky Creek is a 2nd order stream with an upstream catchment area of 3.2 km^2. Most of the catchment has native forest cover.	25/3	Y
Dowan Creek	Stony Creek is a 1st order stream with an upstream catchment area of 1.1 km^2. Most of the catchment has plantation forestry cover with some patches of regenerating native forest cover. Some recent slips were noted in the river corridor.	26/3	Y
Stony Creek	Stony Creek is a 2nd order stream with an upstream catchment area of 1.9 km^2. Most of the catchment has native forest cover with the exception of a few open paddocks adjacent to the study reach. An instream barrier was noted at the road culvert which has a vertical headwall at the outlet.	27/3	Y
Granite Creek	Granite Creek is a 2nd order stream with an upstream catchment area of 2.7 km^2. The stream has a dry streambed for 1 km upstream from SH6 and the study reach was located above this section. The stream has a relatively high gradient with predominantly native forest cover in the catchment upstream.	28/3	Y
Canoe Creek	Canoe Creek is a 3rd order stream with an upstream catchment area of 21.0 km^2 above study reach which was located approximately 2 km upstream from SH6. The lower section of the catchment features an open weedy riverbed that is used for 4wd access for several km upstream from SH6.The upper catchment has predominantly native forest cover.	29/3	Y
Mill Creek	Mill Creek is a 2nd order stream with an upstream catchment area of 1.8 km^2. The land cover is primarily plantation forestry and with patches of established natives in the riparian corridor. Despite the small volume of the stream there are several deep pools in the study reach. .	30/3	Y
Card Creek	Card Creek is a 4th order stream with an upstream catchment area of 24.9 km^2 featuring a mixture of land uses and cover types. Within the study reach and further upstream the stream is protected by high scarps that form a narrow canyon in places. The study reach featured several deep pools and lad a relatively low gradient.	31/3	N
Cariboo Creek	Cariboo Creek is a 3rd order stream with an upstream catchment area of 14.8 km^2. Land cover types include plantation forestry and native forest. Within the study reach the streambed has an open partly braided character.	1/4	Y

† based on detection at the study site using eDNA sampling or visual observation.

Appendix 2 Results from parallel monitoring trials

Table S2. Results from eDNA analysis using the original primer set. Abundance figures represent shortjaw kōkopu observations from spotlighting surveys at the same sites.

(a) 2021 surveys

Study site	Acre	AcreSouth	Kapitea	Liverpool	Flowery	Foley	Frosty	Greeks	Houhou*	KaniereRd	McIntyres
Abundance	6	0	0	0	5	4	0	0	*	0	0
eDNA samples											
syringe-day1	0	0	0	534	0	0	0	0	0	0	0
syringe-day2	0	0	0	655	0	73	0	0	0	0	0
syringe-day3	0	127	41	0	0	23	0	0	0	0	0
syringe-night1	0	0	0	0	0	0	0	0	0	0	0
syringe-night2	0	2647	0	0	0	82	0	433	0	0	0
syringe-night3	0	0	326	0	0	33	0	0	0	0	0
drogue-16hr1	no sample	no sample	no sample	no sample	0	542	0	0	17	0	0
drogue-16hr2	no sample	no sample	no sample	no sample	0	0	0	0	0	0	0
drogue-16hr3	no sample	no sample	no sample	no sample	0	28	0	0	0	0	0
eDNA total count	**0**	**2774**	**367**	**1395**	**0**	**1013**	**0**	**433**	**170**	**0**	**0**

(b) 2022 surveys

Study site	Canoe	Card	Cariboo	Dowan	Granite	Mill	Rocky	Rough n Tumble	Stony	Woolley
Abundance	1	0	27	1	7	2	3	0	36	5
eDNA samples										
syringe-day1	0	0	0	0	0	0	0	0	0	0
syringe-day2	0	0	0	0	0	0	0	0	14	8
syringe-day3	0	0	8	5	0	0	0	0	0	0
syringe-night1	0	0	0	0	0	0	0	0	10	0
syringe-night2	0	0	0	0	0	0	0	0	6	0
syringe-night3	0	0	9	0	38	0	0	0	0	0
pod-12hr1	0	0	0	0	0	0	0	0	12	0
pod-12hr2	0	0	0	0	0	0	0	0	0	0
pod-12hr3	0	0	0	9	0	0	11	0	0	0
pod-26hr1	0	0	0	0	0	0	0	0	6	0
pod-26hr2	0	0	0	0	0	34	0	0	0	0
pod-26hr3	0	0	0	0	0	0	0	0	0	0
eDNA total count	**0**	**0**	**17**	**16**	**38**	**34**	**11**	**0**	**48**	**8**

* a spotlighting survey was not completed for Houhou Creek due to weather conditions but another sampling site (Foley Creek) is located in the catchment upstream.

Table S3. Results from eDNA analysis using the new YG primer set. Abundance figures represent shortjaw kōkopu observations from spotlighting surveys at the same sites.

(a) 2021 surveys

Study site	Acre	AcreSouth	Kapitea	Liverpool	Flowery	Foley	Frosty	Greeks	Houhou*	KaniereRd	McIntyres
Abundance	6	0	0	0	5	4	0	0	*	0	0
eDNA samples											
syringe-day1	108	0	0	534	1584	32	0	0	0	0	0
syringe-day2	0	0	456	1032	0	73	0	532	0	0	275
syringe-day3	15	273	120	0	0	23	0	0	0	0	0
syringe-night1	0	0	0	0	0	265	489	0	0	524	0
syringe-night2	0	3083	155	280	0	82	0	433	0	0	0
syringe-night3	90	0	394	0	0	33	71	0	0	66	0
drogue-16hr1	no sample	no sample	no sample	no sample	0	542	0	0	17	0	0
drogue-16hr2	no sample	no sample	no sample	no sample	0	0	0	0	0	0	0
drogue-16hr3	no sample	no sample	no sample	no sample	0	28	0	0	0	239	0
eDNA total count	**213**	**3356**	**1125**	**1846**	**1584**	**1078**	**560**	**965**	**17**	**829**	**275**

(b) 2022 surveys

Study site	Canoe	Card	Cariboo	Dowan	Granite	Mill	Rocky	Rough n Tumble	Stony	Woolley
Abundance	1	0	27	1	7	2	3	0	36	5
eDNA samples										
syringe-day1	0	41	0	355	0	0	0	0	0	268
syringe-day2	0	0	0	0	0	0	0	0	886	199
syringe-day3	0	0	8	5	0	0	0	0	257	0
syringe-night1	0	0	0	0	0	0	0	0	10	0
syringe-night2	0	0	0	803	0	0	0	0	923	0
syringe-night3	0	0	9	0	38	0	0	0	0	0
pod-12hr1	0	0	0	0	0	0	0	0	525	0
pod-12hr2	0	0	0	0	183	0	0	0	560	0
pod-12hr3	0	0	0	9	166	0	11	0	366	112
pod-26hr1	0	0	0	0	408	0	0	0	519	0
pod-26hr2	0	0	626	0	439	34	0	0	288	0
pod-26hr3	0	0	366	0	0	0	0	0	779	0
eDNA total count	**0**	**41**	**1009**	**1172**	**1234**	**34**	**11**	**0**	**5113**	**579**

* a spotlighting survey was not completed for Houhou Creek due to weather conditions but another sampling site (Foley Creek) is located in the catchment upstream.