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METHOD

Development of an environmental DNA assay and field validation for the detection of invasive pink salmon *Oncorhynchus gorbuscha*

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Abstract

1. Pink salmon *Oncorhynchus gorbuscha* was introduced from its native range in the Pacific to Northwest Russia several times since the 1950s. While this species has been regularly observed in rivers in northern Norway since then, there has been an upsurge in the numbers of odd-year *O. gorbuscha* individuals observed in rivers, particularly in northern Norway in recent years, and particularly in 2017 and 2019.
2. In the present pilot study, an assay was developed to detect *O. gorbuscha* from eDNA water samples. Positive control water samples were taken at two locations of the River Signaldalselva in northern Norway during the summer of 2019, when adults were spawning in the river. Samples showed positive detection of this species in the river, while negative control samples collected upstream migration barriers in central and southern Norway confirmed the absence of the target species.
3. These findings reveal that eDNA-based methods can be used to track the ongoing *O. gorbuscha* invasion of northern Europe and other regions where it might be or become invasive.

KEYWORDS
ddPCR, eDNA, invasion, Norway, *Oncorhynchus gorbuscha*, pink salmon, qPCR

1 | INTRODUCTION

At early stages of invasive species establishment, it is often difficult to monitor the invasion due to low densities of the invader. Early detection is particularly problematic in aquatic environments as invasive species are "out of sight" and managers often have to rely on anecdotal or opportunistic observations. Environmental DNA (eDNA) offers a rapid highly sensitive approach for early detection in aquatic environments (Klymus et al., 2015; Takahara et al., 2013). Species-specific eDNA assays are genetic survey methods that rely on the detection of taxa from extracellular and intracellular genetic material that is released into the environment. This material can be isolated from the environment (such as water, air, or soil; Taberlet et al., 2012) and interrogated using genetic markers for multi-species

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(Hänfling et al., 2016; Thomsen et al., 2012) or targeted species (Ficetola et al., 2008; Gustavson et al., 2015; Jerde et al., 2011) detection. Environmental DNA-based detection is becoming increasingly used in aquatic freshwater environments for a range of low abundance or invasive fish species (Davison et al., 2016; Klymus et al., 2015; Takahara et al., 2013)—including salmonids (Atkinson et al., 2018; Gustavson et al., 2015; Rusch et al., 2018).

Pink salmon *Oncorhynchus gorbuscha* (Walbaum 1792) are native to the Pacific Ocean, where they spawn in the rivers between the latitudes of 40° and 70° north. Adult *O. gorbuscha* migrate from the open sea and up-river for spawning, occurring between mid-July to late October depending on location, after which all spawning individuals die (Sandlund et al., 2019). Juveniles emerge the following spring, migrate down river, and out into the open sea to mature for one winter. They return as adults to freshwater in the next autumn to spawn, thus completing a strict two-year life cycle (Heard, 1991).

O. gorbuscha were introduced from their native Pacific range to northwestern Russia several times since the 1950s, when fry were stocked in several rivers that drain into the White Sea and the Barents Sea (Bakshtansky, 1980). While invasive *O. gorbuscha* have been regularly found in rivers in northern Norway since 1960 (Berg, 1961), there has been an upsurge in the observations of odd-year *O. gorbuscha* in Norwegian rivers in recent years (Mo et al., 2018), as well as in rivers in the UK and Ireland (Armstrong et al., 2018; Millane et al., 2019; Whelan, 2017). Self-reproducing populations may be established in many Norwegian rivers close to Russia in northern Norway, but likely not in southern Norway (Sandlund et al., 2019). In 2019, greater numbers of pink salmon arrived in Norwegian rivers compared to 2017, but they almost exclusively arrived in rivers in northern Norway (Mo et al., 2018; Sandlund et al., 2019).

Presently, the spawning time of *O. gorbuscha* in Norway does not appear to overlap with native salmonids (Sandlund et al., 2019, for example, Atlantic salmon *Salmo salar* and brown trout *S. trutta*). However, *O. gorbuscha* and *S. salar* have similar preferences for spawning habitats and so there is a risk of competition for optimal spawning sites (Sandlund et al., 2019). While *O. gorbuscha* juveniles emerge ready to migrate to sea, observations in Norwegian rivers suggest that they spend some time feeding in freshwater (from weeks to months) and during this time there may be interactions between juveniles of native salmonids (Sandlund et al., 2019). However, it is also possible that the eggs and fry of *O. gorbuscha* can provide a source of food for other native salmonid species (Rasputina et al., 2016). In order to fully assess the impacts of the presence of *O. gorbuscha* in Norwegian (and other European) rivers, it is first necessary to determine the spatial, as well as the temporal distribution of this species.

The primary objective of this study was to develop a species-specific probe-based assay for detection of *O. gorbuscha*. This assay will allow for researchers to also use mitochondrial (mt)DNA *COI* region as a qPCR target in addition to the *CytB* region (Duda et al., 2021). We also aimed through a pilot study to validate our assay by employing the non-intrusive method of eDNA collection and analysis of water samples from Norway, where *O. gorbuscha* has been reported.

2 | MATERIALS AND METHODS

2.1 | Water sampling and eDNA extraction

During August 2019, coinciding with the spawning season of *O. gorbuscha*, four water samples were collected from two localities in River Signaldalselva (where *O. gorbuscha* has previously been detected, Berntsen et al., 2020) in northern Norway (2.4 and 11.6 km upstream from the river mouth; Table 1 and Figure 1). Furthermore, six samples (at three localities) were collected above an impassable barrier in the anadromous River Driva, central Norway, where

TABLE 1 Details of water sampling for *Oncorhynchus gorbuscha* environmental DNA in the three rivers Signaldalselva, Driva and Straumen

Sample ID	River	Locality	County	Latitude	Longitude	Date	Water volume
1	Signaldalselva	Upstream	Storfjord	69.220955	19.978007	14.09.2019	8
2	Signaldalselva	Upstream	Storfjord	69.220955	19.978007	14.09.2019	8
3	Signaldalselva	Downstream	Storfjord	69.260257	19.897654	14.09.2019	5
4	Signaldalselva	Downstream	Storfjord	69.260257	19.897654	14.09.2019	5
5	Driva	Risfossen	Oppdal	62.520994	9.595627	27.08.2019	10
6	Driva	Risfossen	Oppdal	62.520994	9.595627	27.08.2019	10
7	Driva	Hengebrua	Oppdal	62.537810	9.613437	27.08.2019	10
8	Driva	Hengebrua	Oppdal	62.537810	9.613437	27.08.2019	10
9	Driva	Bjørbekk	Sunndal	62.570157	9.054907	28.08.2019	10
10	Driva	Bjørbekk	Sunndal	62.570157	9.054907	28.08.2019	10
11	Straumen	Røyemål	Nome	59.320695	9.010238	14.09.2019	5
12	Straumen	Hegna	Nome	59.323014	9.001926	15.09.2019	5
13	Straumen	Hogga	Nome	59.305295	9.043420	15.09.2019	5

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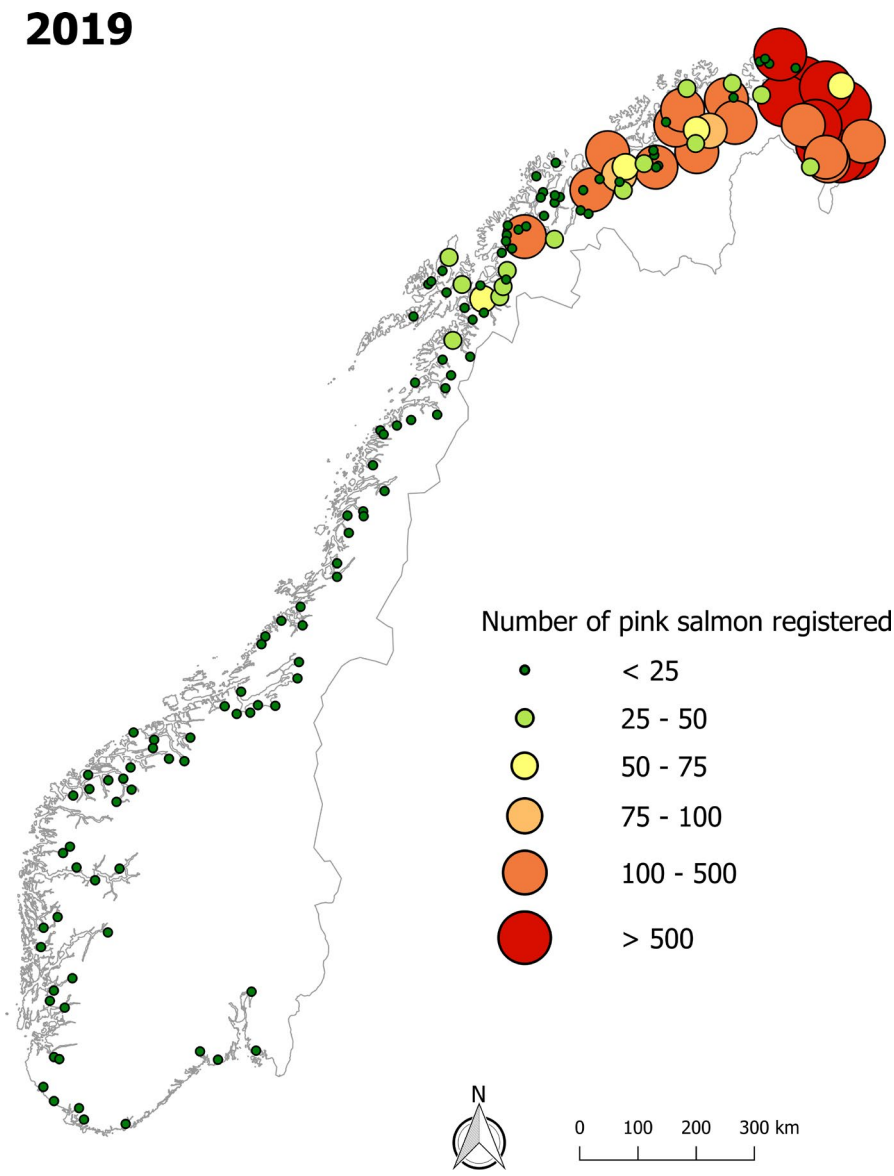

FIGURE 1 Water sampling locations (blue triangles) and distribution of *Oncorhynchus gorbuscha* detections throughout Norway in 2019 (colored circles). Map originally published in Berntsen et al. (2020) and used with permission

TABLE 2 Details of the assay that was designed, tested, and deployed for detection of a 98bp region of the mitochondrial *COI* of pink salmon *Oncorhynchus gorbuscha* in this study.

Primer Type	Name	Sequence 5′–3′	Length (bp)
Forward primer	*PinkF*	CACCGCCMTAAGCCTACTAA	20
Reverse primer	*PinkR*	AGGCATGGGCTGTAACGATT	20
Probe[†]	*PinkPr*	CGCTCTTCTAGGGAATGACCA	21

[†]5′ VIC labelled reporter dye and 3′ non-fluorescent quencher.

O. gorbuscha are not able to reach, and three samples from the non-anadromous River Straumen, southern Norway, were included as negative field controls (Figure 1). As these samples were not expected to amplify *O. gorbuscha*, the presence of amplifiable DNA was confirmed in these samples by ddPCR for the presence of *S. trutta*, as this species is present at all sampling locations, by deploying the specific assay (Gustavson et al., 2015).

Each sample was filtered on 2.0 μm glass fiber filters (Merck Millipore) using a peristaltic pump (Vampire sampler; Bürkle, Bad Bellingen). Filters were placed in 5 ml tubes with 4050 μl ATL buffer (Qiagen). All samples were stored at room temperature until further processing in the genetics laboratory. All field equipment (e.g., filtering tubes and collection bottles) were sterilized between collections of each sample using 10% bleach solution for approximately 60 min.

In the laboratory, 450 μl Proteinase-K (Qiagen) was added to the sampling tubes and incubated overnight at 56°C. DNA was isolated using NucleoSpin Plant II Midi kit (Macherey-Nagel), following the manufacturers protocol except that Qiagen buffers were used instead of those supplied with the kit. Extracted DNA was eluted in 200 μl AE buffer. Samples were re-eluted for maximizing the output of DNA.

2.2 | Molecular assay development and specificity testing

An assay was designed to amplify a 98 bp region of the mtDNA *COI* of *O. gorbuscha* (Table 2). This assay consisted of primers and a 5′ VIC labelled TaqMan® minor groove binding (MGB) probe. The primers and probes were designed using Primer3 software (Rozen & Skaletsky, 2000). Specificity of the assay was checked *in silico*, by aligning the primers and probe with the consensus sequence generated from publicly available *O. gorbuscha* sequences as well as those from other salmonids commonly occurring in the study area (e.g., *S. salar* and *S. trutta*). The primer and probe sequences were also checked against the NCBI database (using BLAST) to ensure specificity to the target organism. Furthermore, specificity of the assay was checked using qPCR, with tissue-extracted DNA from *O. gorbuscha*, as well as from *S. salar* and *S. trutta*. Tissue-extracted DNA was also acquired and tested by qPCR for rainbow trout *O. mykiss*, which, while not a native salmonid, has been widely introduced throughout Europe (Stanković et al., 2015). In addition, DNA extracted from the closely related chum salmon *O. keta* was tested to check the specificity of the assay. The latter is not currently found in Europe, but this species overlaps with *O. gorbuscha* in its native range.

All assay development qPCR reactions took place in a 20 µl reaction volume, containing 10 µl of TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher), 2 µl of each primer (2 µM), 2 µl of probe (2 µM; Applied Biosystems), and 2 µl of template DNA (where extracts were normalized to 34 ng/µl). The PCR program consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. All qPCR reactions were carried out using QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems).

To further test the potential cross-amplification of other salmonids occurring in Norway (*O. mykiss*, *S. salar*, *S. trutta*, Arctic char *Salvelinus alpinus*, brook trout *Salvelinus fontinalis*, lake trout *Salvelinus namaycush*, and grayling *Thymallus thymallus*) the O. gorbuscha assay was also tested using droplet-digital-(dd)PCR on these species. Detection and concentration of target-DNA were assessed using droplet-digital-PCR (QX200 Droplet Digital PCR system with AutoDG; Bio-Rad Laboratories). A tissue-extracted DNA sample of *O. gorbuscha* was included in the analysis, as a positive control, and a no-template control was included as a negative control. The ddPCR reactions took place in a final reaction volume of 22 µl and consisted of 0.9 µM forward and reverse primers, 0.25 µM of the probe, ddPCR™ Supermix for Probes (No dUTP) (Bio-Rad Laboratories), dH_2O, and 5 µl template. To generate droplets, an AutoDG Instrument (Bio-Rad Laboratories) was used, with subsequent PCR amplification in a Veriti96-Well Thermal Cycler (Applied Biosystems). The following thermal cycling conditions were used: an initial denaturation step at 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 sec, annealing and extension at 60°C for 1 min, a final step of denaturation at 98°C for 10 min, and a final hold at 4°C. PCR plates were transferred to a QX200 Droplet Reader (Bio-Rad Laboratories) to automatically detect the fluorescent signal in the droplets. QuantaSoft software v.1.7.4 (Bio-Rad Laboratories) was used to separate positive from negative droplets according to manufacturer's instructions. A threshold of minimum 3 positive droplets was used as a criterion for positive detection (Wacker et al., 2019).

Quantitative PCR of eDNA field samples from River Signaldalselva (where *O. gorbuscha* was detected in 2017, Berntsen et al., 2020), River Driva, and River Straumen was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Briefly, a standard curve was generated using a quantified (NanoDrop®-1000, Thermo Scientific, Wilmington, DE) DNA extract of *O. gorbuscha* tissue (DNA Concentration of 19.57 µg/l) using seven 10:1 serial dilutions as template for qPCR. The standard curve for *O. gorbuscha* ($y = -3.345 + 23.927$, $R^2 = 0.999$, efficiency = 99.03%) was generated using a 3 µl DNA template in a total reaction volume of 30 µl and run in triplicates. All field samples were quantified in triplicate (three technical replicates), to ensure consistency, with two laboratory negative controls. The average C_T across technical replicates (n = 3) was used for quantification.

3 | RESULTS AND DISCUSSION

Studies have shown that eDNA methods are powerful to monitor ongoing aquatic invasions (e.gKlymus et al., 2015; Takahara et al., 2013), and the qPCR assay presented here was developed in 2017 in response to the emergent invasion of *O. gorbusha* throughout northern Europe. The assay is currently being deployed for monitoring the ongoing invasion of *O. gorbusha* in Europe (e.g., https://1000rivers.net/). The rapid implementation of the assay by monitoring bodies (such as The Norwegian Institute for Nature Research (NINA), Fossøy et al., 2018) demonstrates the capability to quickly develop and adopt eDNA-based methods for tracking invasive aquatic species. The years 2017 and 2019 were unprecedented in terms of the number of *O. gorbuscha* that were observed in Norwegian rivers (Mo et al., 2018), as well as in Ireland (Millane et al., 2019; Whelan, 2017) and Scotland (Armstrong et al., 2018). These events highlighted the need for an effective method to monitor the presence of *O. gorbuscha* throughout its invasive (and potentially invasive) range. The assay developed in our study and deployed on samples from 2019 was successful at detecting *O. gorbusha* both *in silico* and *in vitro*. The lowest detectable concentration of DNA was 0.0001 µg/l based on the standard curve (average over three technical replicates $C_T = 37.16$, SD = 0.04).

Testing of the *O. gorbuscha* assay with non-target salmonids using tissue-extracted DNA and qPCR revealed that the assay did not cross-amplify *S. salar*, *S. trutta*, or *O. mykiss*. Furthermore, ddPCR testing of non-target salmonid species (*O. mykiss*, *S. salar*, *S. trutta*, *S. alpinus*, *S. fontinalis*, *S. namaycush*, and *T. thymallus*) did not produce any detectable amplification. However, amplification was observed for *O. keta* DNA analyzed by qPCR (data not shown). This species is closely related to *O. gorbuscha*, and there are very few nucleotide differences between these species for the *COI* region targeted by our assay (Figure 2). While there are sporadic anecdotal reports of

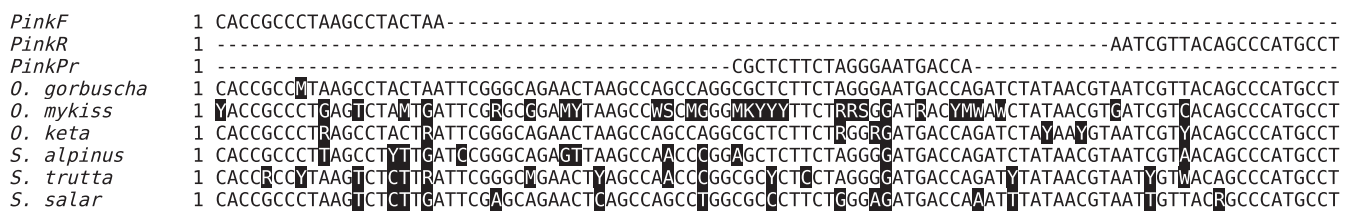

FIGURE 2 Primers (PinkF and PinkR) and probe (PinkPr) designed for targeted detection of *Oncorhynchus gorbuscha* are shown aligned to the target consensus sequence, as well as consensus sequences for other salmonid species. Mismatches are highlighted by shading. Underlined species names indicate that the assay confirmed to amplify tissue from the target

TABLE 3 Results of qPCR-analyses of *Oncorhynchus gorbuscha* in the three rivers Signaldalselva, Driva, and Straumen

Sample ID	River	Locality	PCR replicates	Cт mean	Cт SD	Quantity mean	Quantity SD
1	Signaldalselva	Upstream	3/3	35.83	0.57	0.000293	0.000124
2	Signaldalselva	Upstream	3/3	37.63	0.72	0.000088	0.000048
3	Signaldalselva	Downstream	3/3	31.10	0.33	0.007282	0.001724
4	Signaldalselva	Downstream	3/3	31.09	0.16	0.007270	0.000841
5	Driva	Risfossen	0/3				
6	Driva	Risfossen	0/3				
7	Driva	Hengebrua	0/3				
8	Driva	Hengebrua	0/3				
9	Driva	Bjørbekk	0/3				
10	Driva	Bjørbekk	0/3				
11	Straumen	Røyemål	0/3				
12	Straumen	Hegna	0/3				
13	Straumen	Hogga	0/3				

Note: All samples were run in triplicates and PCR replicates show the number of positive reactions. C_T is reported as mean and SD for across the triplicates (note that Sample ID 2 from Signaldalselva is outside the dynamic range as per standard curve).

O. keta finds in Europe, there is currently no evidence of *O. keta* being establish in northern Europe, and as such the cross-amplification of the *O. gorbuscha* assay with *O. keta* should not present a major concern for researchers wishing to apply this assay for *O. gorbuscha* detection in other European locations. Note also that the cross-amplification occurred when using tissue extracts with high DNA concentrations and that cross-amplification might be less probable using environmental samples (c.f. Duda et al., 2021). Nevertheless, if *O. keta* were to become established in Europe and co-exist with *O. gorbuscha*, the assay could detect their presence. Although the methods could not differentiate the two species, eDNA could be used in concert with traditional methods and would be well suited for an early detection system. A detection of *O. keta* or *O. gorbuscha* would indicate the potential presence of an invasive species and such reports should be followed up with traditional methods capable of discriminating between the two species. Perhaps more importantly, the assay does not amplify *O. mykiss*, a species that have been spread over large areas of northern Europe and in some cases has established self-supporting populations (c.f. Stanković et al., 2015). In addition, should any eradication measures be employed in the future, eDNA methods could be used to determine the efficacy of such efforts (Banks et al., 2016). Before we can recommend the deployment of the assay in the Pacific region that supports a range of native *Oncorhynchus* species, it would be important to assess the species specificity in comparison with the other *Oncorhynchus* species (c.f. Duda et al., 2021).

The *O. gorbuscha* assay detected target eDNA in water samples from both locations in River Signaldalselva (Table 3), while no samples from River Driva nor River Straumen (negative field control sites) contained detectable eDNA from the target species. While there was no detection of *O. gorbuscha*, all samples contained amplifiable *S. trutta* DNA. The literature is currently not clear about how far downstream eDNA transport occurs in running water (c.f. Berntsen et al., 2020). However, the concentrations of *O. gorbuscha* eDNA were almost 40x lower in the upper reaches of Signaldalsevla as compared to the lower reaches (Table 3). While this could reflect the additive nature of eDNA in running water over shorter distances with increasing concentrations further downstream, it might also be indicative of higher concentrations of *O. gorbuscha* in the lower reaches.

We detected a mismatch in the forward primer sequence (A/C, M c.f. Table 3), based on the *O. gorbuscha* consensus sequence generated from publicly available *COI* records on GenBank. The source of this mismatch is a sequence from an odd-year individual

(Accession No. MG951587.1) from the White Sea that was submitted to the NCBI database after the assay was developed and tested. It is, therefore, possible that this haplotype is found in Norwegian rivers. This mismatch is found on the 5′ end of the forward primer, and therefore, it is unlikely that our assay efficiency was severely compromised in the present study. Furthermore, the variant should not affect the species specificity as there are several mismatches in the forward primer to other salmonid species. However, to ensure optimal efficiency in the assay (and particularly to ensure sensitive detection of the low copy numbers frequently found in eDNA samples), we recommend that the forward primer incorporate a degenerate base at this position should the assay be deployed in other studies.

In conclusion, the results of our pilot study show that the assay presented here can be used to detect the presence of *O. gorbuscha* in running water. Subsequently, we suggest that eDNA is a suitable, rapid, and highly sensitive method for tracking the ongoing invasive spread of *O. gorbuscha* throughout Europe. Furthermore, the assay can be used to monitor *O. gorbuscha* presence following any future eradication efforts that may be implemented. While the utility of the assay developed in this study is limited to those areas where *O. gorbuscha* does not overlap with *O. keta*, this should not be of concern for researchers using this assay to detect *O. gorbuscha* within its European invasive range as *O. keta* has not been reported to be invasive in Europe. Finally, the eDNA approaches presented here can be used for tracking many invasive aquatic species.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

All authors conceived and planned the study. Laura M. Gargan assisted in study design, drafted the ms and carried out laboratory work together with Jeanette E. L. Carlsson and Bernard Ball. Tot A. Mo and Frode Fossøy carried out the field work. Jens Carlsson conceived, designed, and had the oversight of the project with assistance from Frode Fossøy. All the authors contributed to the interpretation of the results, provided critical feedback, and helped shape the research, analyses, and manuscript.

DATA AVAILABILITY STATEMENT

Data from the ms will be made available on request.

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REFERENCES

Armstrong, J. D., Bean, C. W., & Wells, A. (2018). The Scottish invasion of pink salmon in 2017. *Journal of Fish Biology*, *93*, 8–11. https://doi.org/10.1111/jfb.13680

Atkinson, S., Carlsson, J. E. L., Ball, B., Egan, D., Kelly-Quinn, M., Whelan, K., & Carlsson, J. (2018). A quantitative PCR-based environmental DNA assay for detecting Atlantic salmon (*Salmo salar* L.). *Aquatic Conservation: Marine and Freshwater Ecosystems*, *28*, 1238–1243. https://doi.org/10.1002/aqc.2931

Bakshtansky, E. L. (1980). 'Pink salmon in the Kola Peninsula', In: J. E. Thorpe (ed) *Salmon ranching*. Academic Press, , pp:245–259.

Banks, J. C., Demetras, N. J., Hogg, I. D., Knox, M. A., & West, D. W. (2016). Monitoring brown trout (*Salmo trutta*) eradication in a wildlife sanctuary using environmental DNA. *New Zealand Natural Sciences*, *41*, 1–13.

Berg, M. (1961). Pink salmon (*Oncorhynchus gorbuscha*) in northern Norway in the year 1960. *Acta Borealia*, *A Scientia*, *Tromsø*, *17*, 1–24.

Berntsen, H. H., Sandlund, O. T., Thorstad, E., & Fiske, P. (2020). *Pukkellaks i Norge*, *2019*. NINA Rapport 1821. Norsk institutt for naturforskning. https://brage.nina.no/nina-xmlui/handle/11250/2651741

Davison, P. I., Creach, V., Liang, W.-J., Andreou, D., Britton, J. R., & Copp, G. H. (2016). Laboratory and field validation of a simple method for detecting four species ofnon-native freshwater fish using eDNA. *Journal of Fish Biology*, *89*, 1782–1793. https://doi.org/10.1111/jfb.13086

Duda, J. J., Hoy, M. S., Chase, D. M., Pess, G. R., Brenkman, S. J., McHenry, M. M., & Ostberg, C. O. (2021). Environmental DNA is an effective tool to track recolonizing migratory fish following large-scale dam removal. *Environmental DNA*, *3*(1), 121–141. https://doi.org/10.1002/edn3.134

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, *4*, 423–425. https://doi.org/10.1098/rsbl.2008.0118

Fossøy, F., Thaulow, J., Anglès d'Auriac, M., Brandsegg, H., Sivertsgård, R., Mo, T. A., Sandlund, O. T., & Hesthagen, T. (2018). *Bruk av miljø-DNA som supplerende verktøy for overvåkning og kartlegging av fremmed ferskvannsfisk*. NINA Rapport 1586. Norsk institutt for naturforskning. ISSN: 1504-3312, ISBN: 978-82-426-3325-5.

Gustavson, M. S., Collins, P. C., Finarelli, J. A., Egan, D., Conchúir, R., Wightman, G. D., King, J., Gauthier, D., Whelan, K., Carlsson, J., & Carlsson, J. (2015). An eDNA assay for Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. *Journal of Fish Biology*, *87*, 1254–1262. https://doi.org/10.1111/jfb.12781

Hänfling, B., Lawson Handley, L., Read, D. S., Hahn, C., Li, J., Nichols, P., Blackman, R. C., Oliver, A., & Winfield, I. J. (2016). Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. *Molecular Ecology*, *25*, 3101–3119. https://doi.org/10.1111/mec.13660

Heard, W. R. (1991). Life history of pink salmon (*Oncorhynchus gorbuscha*). In C. Groot, & L. Margolis (Eds.), *Pacific salmon life histories* (pp:121–123). UBC Press.

Jerde, C. L., Mahon, A. R., Chadderton, W. L., & Lodge, D. M. (2011). "Sight-unseen" detection of rare aquatic species using environmental DNA. *Conservation Letters*, *4*(2), 150–157. https://doi.org/10.1111/j.1755-263X.2010.00158.x

Klymus, K. E., Richter, C. A., Chapman, D. C., & Paukert, C. (2015). Quantification of eDNA shedding rates from invasive bighead carp *Hypophthalmichthys nobilis* and silver carp *Hypophthalmichthys molitrix*. *Biological Conservation*, *183*, 77–84. https://doi.org/10.1016/j.biocon.2014.11.020

Millane, M., Walsh, L., Roche, W. K., & Gargan, P. G. (2019). Unprecedented widespread occurrence of Pink Salmon *Oncorhynchus gorbuscha* in Ireland in 2017. *Journal of Fish Biology*, *95*, 651–654. https://doi.org/10.1111/jfb.13994

Mo, T. A., Thorstad, E. B., Sandlund, O. T., Berntsen, H. H., Fiske, P., & Uglem, I. (2018). The pink salmon invasion: A Norwegian perspective. *Journal of Fish Biology*, *93*, 5–7. https://doi.org/10.1111/jfb.13682

Rasputina, E. N., Shustov, Y. A., & Tyrkin, I. A. (2016). Eggs of pink salmon *Oncorhynchus gorbuscha* as additional nontraditional food of juvenile Atlantic salmon *Salmo salar* in rivers of the Kola Peninsula. *Russian Journal of Biological Invasions*, *7*, 294–296.

Rozen, S., & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics methods and protocols* (pp. 365–386). Humana Press. Totowa, NJ.

Rusch, J. C., Hansen, H., Strand, D. A., Markussen, T., Hytterød, S., & Vrålstad, T. (2018). Catching the fish with the worm: A case study on eDNA detection of the monogenean parasite *Gyrodactylus salaris* and two of its hosts, Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Parasites and Vectors*, *11*, https://doi.org/10.1186/s13071-018-2916-3

Sandlund, T., Berntsen, H. H., Fiske, P., Kuusela, J., Muladal, R., Niemelä, E., Uglem, I., Forseth, T., Mo, T. A., Thorstad, E., Veselov, A. E., Vollset, K. W., & Zubchenko, A. V. (2019). Pink salmon in Norway: The reluctant invader. *Biological Invasions*, *21*, 1033–1054. https://doi.org/10.1007/s10530-018-1904-z

Stanković, D., Crivelli, A. J., & Snoj, A. (2015). Rainbow trout in Europe: Introduction, naturalization, and impacts. *Reviews in Fisheries Science & Aquaculture*, *23*, 39–71. https://doi.org/10.1080/23308249.2015.1024825

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. (2012). Environmental DNA. *Molecular Ecology*, *21*, 1789–1793. https://doi.org/10.1111/j.1365-294X.2012.05542.x

Takahara, T., Minamoto, T., & Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. *PLoS One*, *8*, e56584. https://doi.org/10.1371/journal.pone.0056584

Thomsen, P. F., Kielgast, J., Iversen, L. L., Wiuf, C., Rasmussen, M., Gilbert, M. T. P., Orlando, L., & Willerslev, E. (2012). Monitoring endangered freshwater biodiversity using environmental DNA. *Molecular Ecology*, *21*, 2565–2573. https://doi.org/10.1111/j.1365-294X.2011.05418.x

Wacker, S., Fossøy, F., Larsen, B. M., Brandsegg, H., Sivertsgård, R., & Karlsson, S. (2019). Downstream transport and seasonal variation in freshwater pearl mussel (*Margaritifera margaritifera*) eDNA concentration. *Environmental DNA*, *1*, 64–73. https://doi.org/10.1002/edn3.10

Whelan, K. (2017). *'Pink invaders'*, *Off the Scale*, *issue 18*, *Sept– Oct 2017*, pp. 14–21. [pdf] (2019 March 22). http://www.offthescaleangling.ie/issue18/offthescale_issue18.pdf

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