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A Novel eDNA-Based Approach for the Monitoring and Management of the Endangered Beluga (*Huso huso*, Linnaeus, 1758) and Adriatic (*Acipenser naccarii*, Bonaparte, 1836) Sturgeon

Caterina Maria Antognazza [1,*], Fausto Ramazzotti [2,3], Antonia Bruno [2], Andrea Galimberti [2,3], Monica Di Francesco [4] and Serena Zaccara [1]

[1] Department of Theoretical and Applied Sciences, University of Insubria, Via H. Dunant 3, 21100 Varese, Italy; serena.zaccara@uninsubria.it
[2] Department of Biotechnology and Biosciences, University of Milano-Bicocca, P.za Della Scienza 2, 20126 Milan, Italy; fausto.ramazzotti@unimib.it (F.R.); antonia.bruno@unimib.it (A.B.); andrea.galimberti@unimib.it (A.G.)
[3] National Biodiversity Future Centre, 90133 Palermo, Italy
[4] Settore Fauna—Parco Lombardo della Valle del Ticino, Via Isonzo 1, 20013 Pontevecchio di Magenta, Italy; monica.difrancesco@parcoticino.it
* Correspondence: caterina.antognazza@uninsubria.it

Abstract: Beluga sturgeon (*Huso huso* Linnaeus, 1758, acipenseridae) and Adriatic sturgeon (*Acipenser naccarii*, Bonaparte, 1836, acipenseridae) within the Po River basin have been recently assessed for the IUCN Red List of Threatened Species and were found to be Extinct in the Wild and Critically Endangered, respectively. Significant declines in both species' abundance have spurred major research efforts and management actions in recent decades. Recently, specific actions have been conducted to recover habitat connectivity through projects of river defragmentation and reintroduction plans have been implemented for both sturgeon species. To manage effective conservation efforts, knowledge of a species' distribution and abundance is critical, especially for adult sturgeon that are able to move hundreds of kilometers away from release sites. Here, two new quantitative PCR (qPCR) assays to detect beluga sturgeon and Adriatic sturgeon environmental DNA (eDNA) in water samples have been developed with the goal of providing an alternative method to monitor their presence. Two Taqman-based assays targeting the mitochondrial cytochrome *b* region were developed and showed no amplification of other related and co-occurring fishes. A mesocosm within the Ticino Park on the Ticino River (a main tributary of the Po River), where both species are bred, was used to develop and validate the assays. The LOQ for *H. huso* assay corresponded to $C_t = 41$ (7.33×10^7 DNA counts/µL of reaction) and for *A. naccarrii* it was Ct = 37 (2.23×10^{16} DNA counts/µL of reaction). Additionally, water samples were taken from the discard drainage, which flows directly into the Ticino River, testing positive detection of eDNA within a distance of up to 2 km. Overall, the results suggested that the two assays developed in this study could represent a promising new tool for monitoring both beluga and Adriatic sturgeon.

Keywords: conservation; sturgeon; eDNA; qPCR; freshwater ecosystem

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1. Introduction

Sturgeon (order Acipenseriformes, Berg 1940) represent one of the most important aquatic natural resources, both scientifically and commercially. With over 85% of species listed as endangered or threatened, sturgeon are considered the world's most imperiled vertebrate group (IUCN 2022). All species are migratory, either anadromous or potamodromous. Typically, they are slow-growing animals, and they reach sexual maturity very late in life (aged up to 20 years), and some species do not spawn yearly [1]. Over the past century, sturgeon' populations have been reduced by human-mediated alterations to riverine habitats, such as channelization, dam construction, and degradation of the water

quality [2]. Specifically, to the North of Italy, Po River drainages into the Adriatic Sea have affected the beluga sturgeon (*Huso huso* Linnaeus, 1758) and the Adriatic sturgeon (*Acipenser naccarii*, Bonaparte, 1836), which have been recently assessed for the IUCN Red List of Threatened Species and were found to be Extinct in the Wild and Critically Endangered, respectively (IUCN 2022). Due to their long life cycle and anadromous behavior, they are very susceptible to overfishing, pollution, and habitat fragmentation [3]. A significant drop in catches began in the early 1920s, primarily as a result of overfishing and habitat fragmentation [4]. Recently, species reintroductions (i.e., the translocation of individuals to areas where a species has been extirpated with the aim of re-establishing a self-sustaining population) have become a widespread practice in sturgeon' conservation biology [5], and this also contributes to the safeguarding of both beluga and Adriatic sturgeon [6].

A critical aspect for developing and managing effective conservation efforts is the knowledge of a species' distribution and abundance through monitoring [7]. Assessing the occurrence and distribution of freshwater species using eDNA-based approaches is becoming established as a powerful conservation management tool [8,9], especially in the case of fish taxa [10–13] eDNA-based approaches have been proven to be reliable and non-invasive strategies suitable for monitoring rare, elusive, anadromous and imperiled fishes, including sturgeon [14–18]. Requiring only the collection of water samples, eDNA can exhibit considerable time and cost benefits over traditional sampling [19], thus allowing for greater spatial distribution of effort, which is critical for species like adult sturgeon that can move over hundreds of kilometers in the Po River basin. Unfortunately, no eDNA-based tools specific for the target detection of *H. huso* and *A. naccarii* have ever been developed, limiting the efficacy of monitoring efforts. Therefore, this study aimed (i) to develop and test two probes-based qPCR assays for detecting *H. huso* and *A. naccarii* eDNA in water samples from a mesocosm (Figure 1), targeting the variable mitochondrial region Cytochrome *b* (Cyt *b*), and (ii) to test the detection distance of the two assays by collecting the water from the drainage of the mesocosm, which directly flows into the Ticino River.

Figure 1. Individual (**a**) *Huso huso* and (**b**) *Acipenser naccarii*, swimming in the mesocosm where water was collected for testing the two probes-based qPCR assay or detecting *H. huso* and *A. naccarii* eDNA.

2. Materials and Methods

2.1. Mesocosm Sampling Site

The mesocosm with sturgeon is located in Ticino Park along the Ticino River, a main Alpine tributary of Po River (Figure 2; [20]). The mesocosm consisted of two semi-natural communicating tanks. Each tank is bordered by grids, with natural bottoms and banks, each about 350 m long and 15 m wide. The tanks are mainly fed by a large natural spring channel and partly by small springs along the banks and on the bottom, and directly flowing into the Ticino River (around 2 km) (Figure 1). The depth of the tanks up to the river confluence does not exceed 1.6 m. In the upstream tank (tank 1), *Huso huso* is bred with a few juvenile individuals of *Acipenser naccarii*. In total, at the moment of sampling, there were around 30 *H. huso* individuals for an approximate total biomass of 700 kg. The *A. naccarii* juveniles were around 1000 individuals with an approximate biomass of 300 kg. Downstream (tank 2), divided by a grid, which impedes fish movements, about 400 adult individuals of *A. naccarii* (60–120 cm) are bred, with an approximate biomass of 1900 kg. In the system, a few trout and barbels were also present.

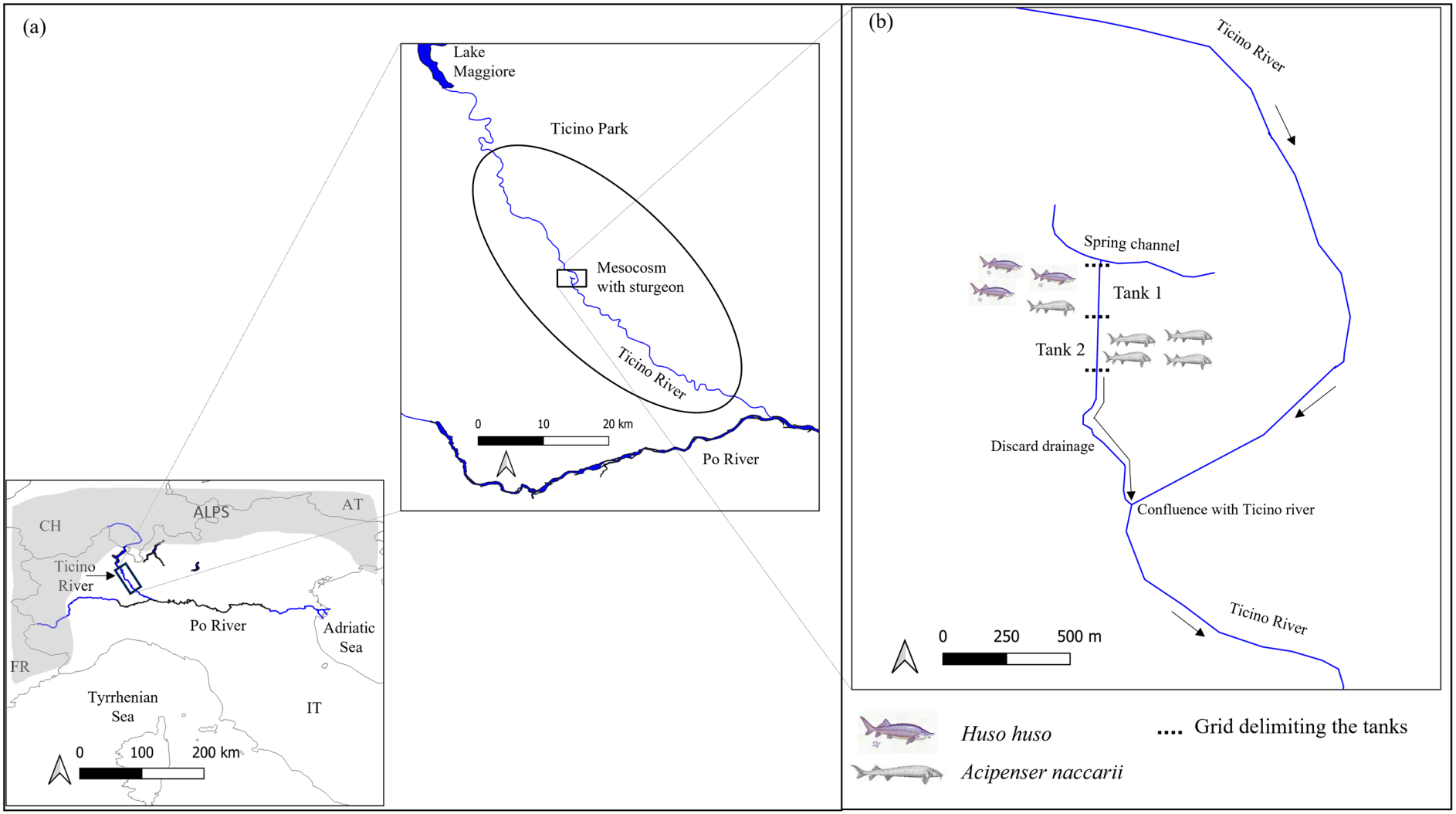

Figure 2. (**a**) Map of mesocosm with sturgeon (45.386836° N, 8.838726° E) located in Ticino Park along Ticino River, Alpine tributary of Po River (North Italy); (**b**) zoom on the mesocosm structure (tanks with stocked sturgeon) and discard drainage. Two water samples were taken from the spring channel (negative controls) and twenty-eight water samples were taken up to the confluence with the Ticino River.

2.2. Water Sampling

Sampling took place in June 2023 and in September 2023; water samples were collected using a portable eDNA sampler (Smith-Root) [21] through an 0.45 um eDNA Sampler self-preserving filter (Smith-Root) [22] standing from the edge of the tanks and discarded drainage. At each sampling point, 1 L of benthic water and 1 L of water column were filtered, separately, and a total of 2 L of water was filtered from each tank. Starting from the outflow of tank 2 up to the confluence with the Ticino River (around 2 km), 12 sites were sampled at regular distances between sites. At each site, 2 L of water (1 L benthic and 1 L column water) was filtered to investigate the distance detection of eDNA in natural conditions (Figure 1). Upstream of tank 1, 2 L of water was filtered as a negative control. Filters were kept cool and dark during the transport to the laboratory and stored at −80 °C until the eDNA extraction process (within a week after collection). Total eDNA was extracted from the filters using DNeasy® PowerSoil® Kit (Qiagen, Milano, Italy) according to the manufacturer's protocol. The eDNA was eluted in 75 µL of warmed elution buffer (40 °C) to increase the final eDNA concentration.

All laboratory procedures of the pre-amplification steps were carried out in separate rooms from the post-amplification steps, with dedicated personal protective equipment.

2.3. Assay Design

In order to identify possible genomic region sequences suitable for designing specific primers and probes for *A. naccarii* and *H. huso*, a multi-species sequence alignment was created from publicly available sequence data from the National Center for Biotechnology Information's (NCBI) Genbank repository (http://www.ncbi.nih.gov/genbank, accessed on 26 May 2023). The alignment was created by considering Cyt *b* nucleotide sequences, since all the species of sturgeon present in the Po River basin, and the species involved in hatchery activity for caviar production, were largely abundant for this marker. The dataset included 123 Acipenseridae sequences belonging to *Acipenser baerii* (Brandt, 1869), *A. gueldenstaedtii* (Brandt & Ratzeburg, 1833), *A. naccarii* (Bonaparte, 1836), *A. ruthenus* (Linnaeus, 1758), *A. stellatus* (Pallas, 1771), *A. sturio* (Linnaeus, 1758) (extinct in Italy), *A. trasmontanus* (Richardson, 1836) and *Huso huso* (Linnaeus, 1758) (Supplemental Table S1). Primers and probes were designed using "Primer Quest™Toll" (https://www.idtdna.com/Primerquest/Home/Index, accessed on 27 May 2023) [23], while the presence of dimers and hairpin formations was checked with OligoCalc v. 3.27 (http://www.operon.com/tools/oligo-analysis-tool.aspx, accessed on 27 May 2023). A further validation step was performed to assess the specificity of the designed assays and the possible mismatches with other non-Acipenser species by using NCBI Primer-BLAST tool [24].

2.4. Primer and Probes Validation on Positive Tissue Samples

Genomic DNA was extracted from a fin clip sample collected from *A. naccarii* and *H. huso*, using the DNeasy Blood&Tissue® Kit (Qiagen, Milano, Italy) following manufacturer's instructions. The concentration and quality of the genomic DNA were checked with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The two assays were tested on DNA from both sturgeon species. To select the optimal annealing temperature of each primer pairs, gradient PCR trials were conducted in 20 µL total volume reactions containing 4 µL of 10× Buffer solution (5× WonderTaq reaction Buffer EuroClone®, Pero (MI), Italy), 1 µL of each primer (10 µM), 0.25 µL Taq polymerase (WonderTaq EuroClone®), and 2 µL of DNA template, under the following conditions: an initial step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 60 s, with annealing temperature ranging from 50 to 60 °C for 45 s, elongation at 72 °C for 60 s, and a final extension at 72 °C for 7 min. Gel electrophoresis runs of the resulting PCR products were performed on a 2% agarose gel and visualized under UV light. A subset of amplicons of the expected length were excised and sequenced bidirectionally at Eurofins GenomicsDNA. After primer trimming, the presence of an open reading frame was verified for the obtained consensus sequences by using the online tool EMBOSS Transeq

(http://www.ebi.ac.uk/Tools/st/emboss_transeq/, accessed on 27 May 2023). Species identification was validated by querying the obtained nucleotide sequences with BLASTn tool [25].

2.5. qPCR Assay Set Up

Quantitative Real Time PCR (qPCR) on a Real-Time PCR StepOne® (Applied Biosystems®, Waltham, MA, USA) instrument to test the primer pairs assays. First, each primer pair was used on tissue samples to determine the amplification efficiency (E), limit of detection (LOD), and limit of quantification (LOQ), according to [26,27]. To generate the standard curve, tenfold serial dilutions of quantified positive tissue controls were used at a starting concentration of 340 ng/µL in both cases (Supplemental Table S3). The samples were run in duplicate, together with negative controls (NTC: no template control samples). The qPCR analysis was performed in 10 µL reaction mix containing 5 µL of buffer, 0.25 µL of each primer (10 µM), 0.15 µL of dual-labeled (5′-FAM, 3′-MGBEQ) Taqman Probe (10 µM) and 2 µL of eDNA template with the same thermal conditions used for eDNA samples analysis. Amplification efficiency (E) for both primer sets was calculated according to the following formula:

$$E = 10^{\left(-\frac{1}{-slope}\right)}$$

2.6. eDNA-Based Detection in Environmental Samples

Each eDNA sample was analyzed in six technical replicates (Supplemental Table S2) together with positive (PTC) and negative controls (NTC), respectively. PTC consisted of 2 µL of gDNA extracted from tissue samples, while NTC included ultrapure water. The qPCR was performed following standard thermal conditions, with the annealing step varying depending on the target: *A. naccarii*: 60 °C annealing temperature for 45 s, 45 cycles; *H. huso*: 58 °C annealing temperature for 60 s and 45 cycles. eDNA samples were considered positive when a sigmoidal signal was observed in at least two qPCR technical replicates for each run and above the LOD, whereas their status was uncertain when it was observed only in a single technical replicate, even if it was above the LOD. When the qPCR copy number output was below the LOQ but above the theoretical qPCR limit (three copies per reaction according to [26], they were considered as 'detectable but not quantifiable' (DBNQ). The C_t (Cycle threshold) values were converted into DNA counts (DNA copies) as follows:

$$DNA\ counts = E\hat{}\left(C_T max - C^T\right)$$

$$\frac{[DNA]ng}{\mu l} = 10\hat{}(C_{T-y})/-slope$$

Randomly, some amplicons from eDNA samples were sent for sequencing (Eurofins, Hamburg, Germany) to confirm the detection. Moreover, to further confirm the specificity of our assays, both were tested on eDNA extracts from three water bodies where the absence of Acipenseridae is certain, all of which were located within the Po River basin (i.e., Parco Nord Milano, 45.5314° N, 9.2123° E; San Genuario, 45.1982° N, 8.1889° E). These sites are representative of the fish community inhabiting the Po River basin [28,29]).

3. Results

3.1. Initial qPCR Assays Testing

Preliminary tests of the Cyt *b* qPCR assays on both *A. naccarii* and *H. huso* on DNA from tissue samples demonstrated that the method was specific to the selected mitochondrial region of the target species. The species-specificity test was checked through sequence match analysis from tissue-based amplicons and did not return any high similarity value with the teleost fish or other taxa present in Italian rivers (Table 1).

Table 1. Primer, probe, and qPCR information for the Cyt *b* assays developed.

Primer Name	Sequence (5′-3′)	Amplicon	qPCR Conditions
NacFw	GTCACACAAATCCTAACAGGACT		
NacProbe	[FAM]TTCAACAGCCTTCTCCTCTGTTGC[MGBEQ]	156 bp	60 °C 45 s. 45 cycles
NacRev	TATATACTATGGTTCATACCTCC		
HusFw	AGTAACATTCCACCCATAC		
HusProbe	[FAM]ATTCATCCTAATGTTAGTTGGGC[MGBEQ]	120 bp	58 °C 60 s. 45 cycles
HusRev	CCAGACAACTTCACACC		

The amplification efficiency of the *A. naccarii* primers was 84.5%. For the 10-fold serial dilution of *A. naccarii* DNA, the LOD was 340 × 10^{-6} ng/µL, with a mean cycle threshold value (C_t) of 43 (SD ± 0.17). The LOQ corresponded to a C_t = 37 (2.23 × 10^{16} DNA counts/µL of reaction) (Figure 3). The *H. huso* amplification efficiency was 91.5%. The measured LOD corresponded to 340 × 10^{-6} ng/µL, with a mean cycle threshold value (C_t) of 39 (SD ± 0.13) (Figure 3). The LOQ corresponded to C_t = 41 (7.33 × 10^7 DNA counts/µL of reaction) (Figure 3). R^2 values were ≥99.8% for *A. naccarii* and ≥99.9% for *H. huso* (Supplemental Table S4).

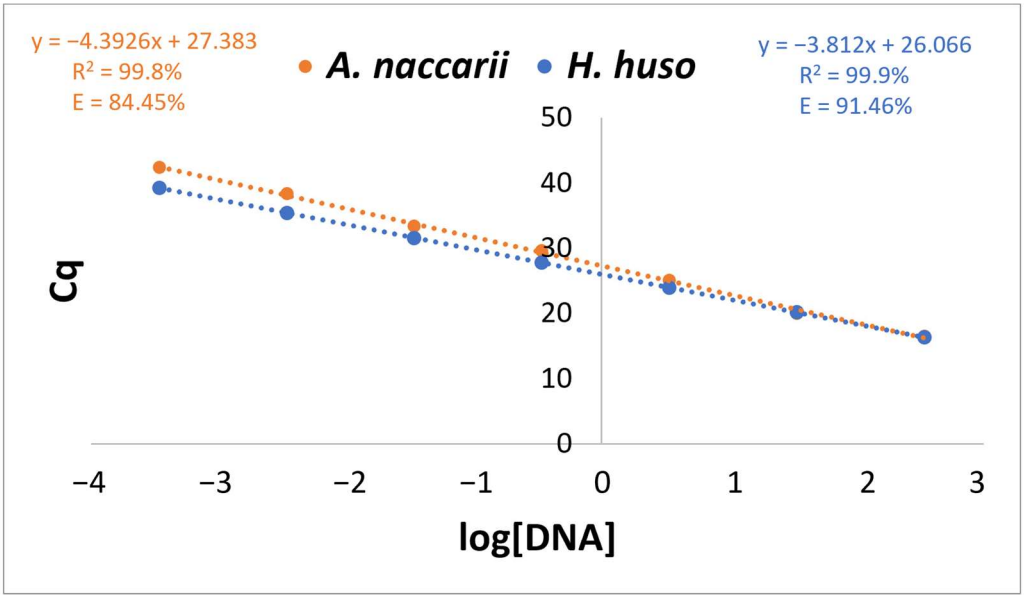

Figure 3. Standard regression line of the qPCR positive control samples. Y-axis gives the quantitative cycles (Cq), and x-axis the log of the starting gDNA concentration of each dilution (ranges 340–0.00034 ng/µL). gDNA from *A. naccarii* is reported in orange circles, and gDNA from *H. huso* in blue ones (*cf* Table S3).

3.2. eDNA Detection in Environmental Samples

The Cyt *b* qPCR assays were then tested on the environmental DNA, showing species-specific detection. No amplification was detected in the field negative controls (including the two eDNA extracts from water bodies where no Acipenseridae species occur) for both assays. In tank 1, *H. huso*'s Cyt *b* qPCR assay amplified both benthic and column water samples (sample S1) at 30 cycles, whilst *A. naccarii*'s Cyt *b* qPCR was amplified during later cycles (31–32 cycles) according to the lower abundance of this species (Figure 1; Table 2). In tank 2, *A. naccarii*'s Cyt *b* qPCR was amplified at lower cycles (sample S2, C_t = 30–31), according to the higher biomass in tank 2 (Figure 2; Table 2).

Table 2. Samples' details and results based on the two Cyt *b* assays. Sample ID, type of sample (column water, benthic water), distance from the outflow of tank 2 (distnacc), distance from the outflow of tank 1 (disthuso), average C_t values based on six replicates (successful replicates indicated), average eDNA concentration in (ng/µL) ([eDNA]), and DNA counts/L (DNA copies) are indicated.

		Acipenser naccarii				*Huso huso*			
ID	**Type**	**Distnacc (m)**	**C_t**	**[eDNA]**	**DNA Copies**	**Disthuso (m)**	**C_t**	**[eDNA]**	**DNA Copies**
S1	Column	-	32 (3/6)	0.1027	3.79×10^{31}	-	30 (3/6)	0.0809	9.28×10^{34}
	Benthic		31 (6/6)	0.1787	4.12×10^{33}		30 (6/6)	0.0885	1.82×10^{35}
S2	Column	-	31 (4/6)	0.1267	2.25×10^{32}	0	32 (4/6)	0.0214	4.43×10^{30}
	Benthic		30 (6/6)	0.2623	1.06×10^{35}		31 (6/6)	0.0384	5.80×10^{32}
15c	Column	0	31 (6/6)	0.1298	2.76×10^{32}	450	31 (6/6)	0.0427	7.81×10^{32}
15b	Benthic		31 (6/6)	0.1740	3.29×10^{33}		32 (6/6)	0.0267	2.32×10^{31}
16c	Column	50	44 (0/6)	-	-	500	33 (6/6)	0.0182	1.33×10^{30}
16b	Benthic		44 (0/6)	-	-		40 (0/6)	-	-
17c	Column	100	35 (6/6)	0.0192	2.55×10^{25}	550	33 (6/6)	0.0193	2.08×10^{30}
17b	Benthic		36 (6/6)	0.0114	3.19×10^{23}		34 (6/6)	0.0064	5.27×10^{26}
1c	Column	150	35 (6/6)	0.0211	5.74×10^{25}	600	39 (5/6)	0.0004	2.29×10^{17}
1b	Benthic		35 (6/6)	0.0158	4.99×10^{24}		40 (0/6)	-	-
2c	Column	250	36 (4/6)	0.0107	1.84×10^{23}	700	35 (6/6)	0.0044	3.30×10^{25}
2b	Benthic		36 (6/6)	0.0091	4.67×10^{22}		35 (6/6)	0.0037	8.25×10^{24}
3c	Column	400	36 (6/6)	0.0112	2.77×10^{23}	850	38 (3/6)	0.0007	2.08×10^{19}
3b	Benthic		36 (3/6)	0.0099	9.87×10^{22}		40 (0/6)	-	-
4c	Column	600	34 (6/6)	0.0260	3.44×10^{26}	1050	36 (5/6)	0.0025	4.90×10^{23}
4b	Benthic		34 (6/6)	0.0318	1.85×10^{27}		35 (6/6)	0.0038	1.11×10^{25}
5c	Column	800	37 (6/6)	0.0083	2.23×10^{22}	1250	36 (6/6)	0.0028	1.03×10^{24}
5b	Benthic		34 (6/6)	0.0314	1.67×10^{27}		35 (6/6)	0.0047	5.61×10^{25}
6c	Column	1000	35 (6/6)	0.0212	6.06×10^{25}	1450	35 (5/6)	0.0044	3.05×10^{25}
6b	Benthic		34 (6/6)	0.0365	5.94×10^{27}		35 (6/6)	0.0047	5.62×10^{23}
7c	Column	1250	36 (6/6)	0.0115	3.50×10^{23}	1650	36 (5/6)	0.0026	6.41×10^{23}
7b	Benthic		34 (6/6)	0.0266	4.06×10^{26}		39 (4/6)	0.0005	4.48×10^{18}
8c	Column	1500	32 (6/6)	0.1061	5.00×10^{31}	1950	34 (1/6)	0.0067	7.78×10^{26}
8b	Benthic		36 (6/6)	0.0134	1.27×10^{24}		36 (2/6)	0.0019	5.41×10^{22}
9c	Column	2000	33 (6/6)	0.0453	3.73×10^{28}	2450	40 (0/6)	-	-
9b	Benthic		34 (6/6)	0.0347	3.87×10^{27}		40 (0/6)	-	-

The detection of eDNA for both assays covered the 2 km range investigated in the mesocosm for both column and benthic water (sampling point 9 for *A. naccarii*'s Cyt *b* qPCR assay and sampling point 8 for *H. huso*'s Cyt *b* qPCR assay) (Table 2; Figure 4). Out of 24 samples, only 2 samples (16c and 16b), for the detection of *A. naccarii* eDNA provided positive signals in all replicates at higher C_t values than the dilutions, and thus their detection is considered unreliable, whilst 5 samples were unreliable for the detection of *H. huso* eDNA (Table 2). Specifically, for *H. huso* eDNA detection, sampling point 9 provided a positive but unreliable signal due to the detection at a high C_t value, probably due to the outreach of the distance range detection of the assay (approximately 2.5 km) (Table 2). Detection of *A. naccarii*'s eDNA was successful in all six qPCR replicates in all samples except samples 2c and 3b, where successful replicates were four and three, respectively. In comparison, the detection of *H. huso*'s eDNA provided less successful replicates for a higher number of samples (eight in total) (Table 2). However, only at sampling point 8 was the number of successful qPCR replicates low (less than 50%), probably approaching the range of the detection limit (Table 2). For both assays, the eDNA detection did not show a linear trend from the outflow of the tanks to the confluence of the Ticino River (Figure 4). A higher eDNA concentration was retrieved in benthic water in five out of eleven sampling points for *A. naccarii*'s Cyt *b* qPCR assay; specifically, four benthic samples collected between 600 m and 1250 m from the outflow of tank 2 (Figure 4; Table 2). For

H. huso's Cyt *b* qPCR assay, a higher eDNA concentration was retrieved in benthic water in only three samples (17b, 5b and 6b) (Figure 4; Table 2).

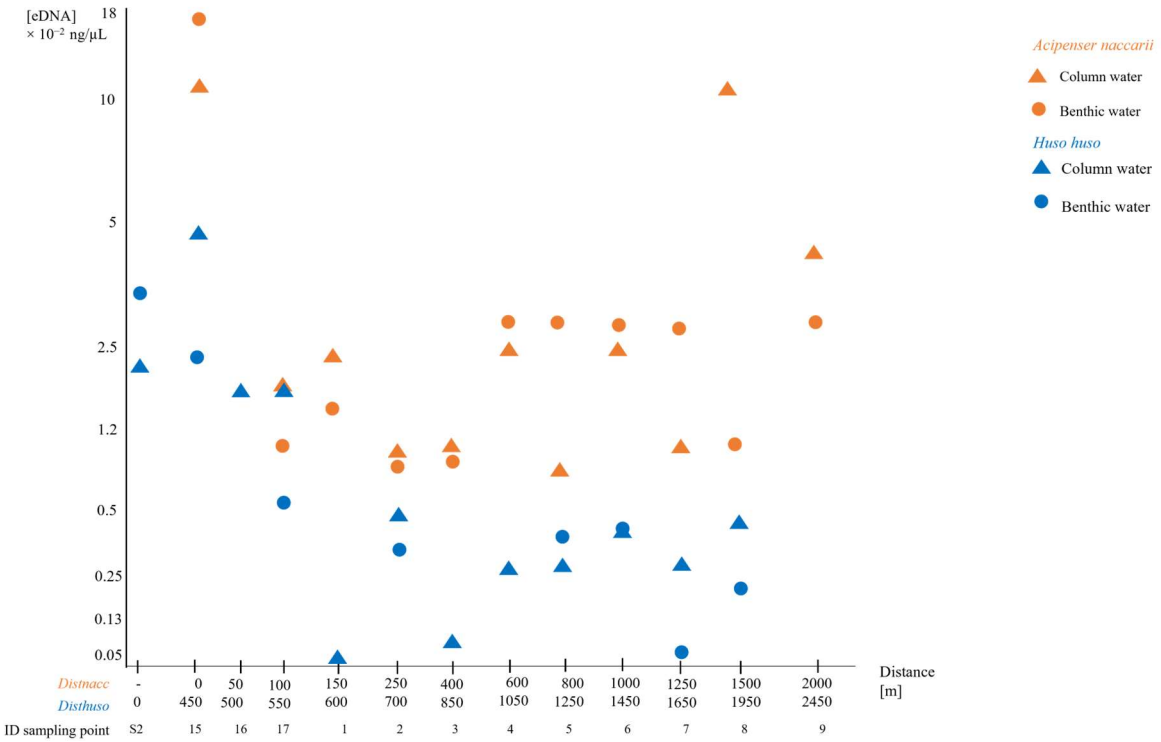

Figure 4. qPCR amplifications result for *Acipenser naccarii* eDNA assay (orange) and *Huso huso* (blue) across the range of DNA samples (*cf* Figure 2; Table 2). The average of the eDNA concentrations (six replicates each sample) for benthic water (circle) and column water (triangle) is plotted. On the x-axis, the distance (m) from the outflow of tank 2 (Dissnacc) and tank 1 (Disshuso) is indicated, alongside the ID of the sampling point (*cf* Table 2).

4. Discussion

This study was the first one devoted to the tracking of eDNA from the rare and endangered sturgeon *H. huso* and *A. naccarii*, in freshwater ecosystems, based on qPCR Cyt *b* assays. The two qPCR assays for detecting *H. huso* and *A. naccarii* eDNA in field sampling were robust, highly efficient, and species-specific. The trials conditions from the mesocosm allowed us to simulate the sympatry conditions found in the Po River basin of the two sturgeon species, where the abundance of the rare *H. huso* in the tanks was 1:3 compared to the presence of *A. naccarii*.

The results showed promise for applying the assays for monitoring *H. huso* and *A. naccarii* in their natural freshwater habitat. The maximum detection distance of *H. huso* in this study was at around 2 km, with a signal that dropped at around 2.5 km, whilst the maximum detection distance for *A. naccarii* could be greater than 2 km, but a further sampling site was not accessible during this study. A greater detection distance of *A. naccarii* might be related to the higher abundance in the tanks; indeed, quantitative comparison of eDNA detection versus acoustic detections revealed significant relationships between eDNA and Atlantic sturgeon in two river systems [30]. However, when Atlantic sturgeon were in lower abundances, in deeper areas, and were not actively migrating, eDNA detection in surface water failed [31]. Whilst also other studies have found the quantity of eDNA to be correlated with species density (i.e., [31–33], there is still limited knowledge of how conditions such as DNA shedding rates (fish behavior), water chemistry, flow, and temperature affect eDNA concentrations. River flow could influence the detectability by moving eDNA downstream up to 100 km from where the fish are located [34–36]; however, there is some evidence that eDNA does not necessarily accumulate in downstream sampling

sites [7,31] and the transport of eDNA is highly variable and complicated to trace [37]. Further work in natural habitats should include measuring the river flow at each sampling site, and deeper investigations are needed to examine the factors influencing variability in eDNA detection rates. Indeed, to design a satisfactory survey method, an understanding of the sensitivity of eDNA detection rates downstream in running water is critical [38]. In addition to abiotic factors, the distribution and movement of fish can also be affected by the flow [39,40]. Further complicating the interpretation of eDNA results is the historical eDNA transport between river sediments and water column [41]. Sediment eDNA may be present at a higher concentration than that in the water column [41], but it could also not be correlated with the actual aquatic eDNA [42]. However, given that sturgeon tend to live at the bottom of the river over sand and mud, and feed on bottom-living invertebrates and small fishes, eDNA-based detection is not likely to detect traces that have been retained in the environment in the long term. Although, in this study, the depth of the tanks where sturgeon were bred did not allow testing difference between surface/column water and benthic water, a previous study on sturgeon suggested that there may be a higher eDNA concentration in benthic water [16], which must be taken in account during further investigations. Moreover, increased eDNA detection was observed in Alabama sturgeon during the spawning seasons, when fish were likely migrating and producing gametes [16], which is likely to be the same for both *H. huso* and *A. naccarii* during their migration up to the Po River basin.

Overall, these results indicate that qPCR eDNA-based detection of *H. huso* and *A. naccarii* has broad potential applications. eDNA is a viable approach for monitoring this important endangered species and can inform DNA-based trophic dynamics studies [43]. Given the relatively low eDNA abundances in river samples, increasing the water volume sampled, the sampling frequency, and varying the depths sampled may improve the detection probability, especially for the rarest *H. huso*.

Many efforts have been put in place to promote the ecological requirements of the Po River catchment, restoring its connectivity and opening migratory routes for anadromous species like sturgeon, with evidence that the Po River remains suitable for the reproduction of the Adriatic sturgeon [44]. The removal of the main impoundment in the lower Po River, Isola Serafini Dam (EU-LIFE project ConFluPo, 2012–2017), is critical for the current reintroduction program for both sturgeon species [6], allowing them to reach the upstream-located suitable spawning sites. Furthermore, the engineered fish pass, built in Isola Serafini, is equipped with a station for observing fishes' movements in both directions [45], which can effectively support eDNA monitoring. Indeed, eDNA sampling seems to be especially suited to monitoring the reproduction and spawning of migratory anadromous fish in particular when it is paired with other methods to confirm that reproduction is taking place, such as upstream spawning migration [13,46,47]. For migratory species, repeated sampling can determine the timing and spatial extent of spawning [48,49] recovery dynamics following dam removal [50]. Moreover, high-frequency temporal sampling of water could effectively provide a more accurate representation of spawning abundances [13,51,52] that is a critical issue for optimizing conservation and management actions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/environments11080160/s1, Table S1: List of GenBank from Cyt *b* sequences used to design the two assays specific to *Huso huso* and *Acipenser naccarii*. Each sequence was trimmed to match only the region targeted by the assay. Species, GenBank accession number (GenBank) and reference are indicated. The sequences were downloaded from NCBI-GenBank (Nucleotide Archive) on 26 May 2023. Table S2: Detailed results obtained for each environmental sample regarding the detection of *Acipenser naccarii* and *Huso huso*. Sample name (ID), sample type (column–benthic water), cycle thresholds (C_t) for each replicate (Rep) and the average cycle thresholds with the standard deviation ($C_t \pm$ s.d.) values are also reported. Table S3: Detailed results obtained for tissue sample of *Acipenser naccarii* and *Huso huso* used to generate the standard curve. Eight dilutions were tested, concentration of DNA ([DNA] ng/µL), cycle thresholds (C_t) for each

replicate (Rep), average cycle thresholds (C_t) value are reported. Table S4: qPCR outcome summary of *Acipenser naccarii* and *Huso huso* environmental samples analysis. Sample name (ID), sample type (column–benthic water), average cycle thresholds (C_t), concentration of DNA (ng/µL), DNA counts/µL and DNA counts/L are indicated.

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