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ORIGINAL ARTICLE

Quantification of Environmental DNA (eDNA) shedding and decay rates for three commercially harvested fish species and comparison between eDNA detection and trawl catches

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Abstract

Stock assessments are critical to inform decisions for sustainable fisheries management. Environmental DNA (eDNA) analysis is a potential tool for assessing fish biomass and populations to aid in stock assessments. To facilitate modeling of biomass based on eDNA data, shedding and decay rates are needed. We designed species-specific, probe-based qPCR assays for three economically important fish species: black sea bass (*Centropristis striata*), winter flounder (*Pseudopleuronectes americanus*), and summer flounder (*Paralichthys dentatus*). Winter flounder eDNA was measured using two qPCR assays (135 and 292 bp). We report the eDNA shedding and decay rates and the associated variability from two replicate experimental systems. The eDNA decay rates were not significantly different between all species. The eDNA shedding rates between the two replicate systems were significantly different for winter flounder (135 bp assay) and summer flounder. qPCR amplicon length did not affect the eDNA decay rates for winter flounder. The three new qPCR assays were tested in environmental waters alongside traditional trawl surveys. No eDNA from BSB, WF, or SF was detected by eDNA methods, and out of 13 bottom trawls over 6 days only 1 WF, 1 SF, and 2 BSB were caught. This research presents three new, efficient qPCR assays and shows agreement between eDNA methods and trawl surveys suggesting low abundance or absence of target fish.

KEYWORDS
black sea bass qPCR assay, eDNA decay, eDNA shedding, eDNA–trawl comparison, winter and summer flounder qPCR assays

1 | INTRODUCTION

Fisheries worldwide are threatened due to anthropogenic impacts, particularly climate change and overfishing (Dudgeon et al., 2006; Hilborn et al., 2003). Stock assessments are critical to inform management decisions for fisheries (Hilborn & Walters, 2013; Kelly et al., 2014). In marine systems, stock assessments generally require the capture of the fish through trawling, hook and line, or video and acoustic observations (Fisheries, 2019; Murphy & Jenkins, 2010). These methods can be costly, time-consuming, and destructive

for aquatic organisms and habitats (Dudgeon et al., 2006; Jenkins et al., 2014; Murphy & Jenkins, 2010). Analysis of environmental DNA (eDNA) extracted from water samples is being proposed as a novel, complementary method for fishery stock assessments (Evans & Lamberti, 2018; Hansen et al., 2018; Kelly et al., 2014; Thomsen et al., 2012, 2016).

eDNA analysis includes the capture of DNA shed by organisms such as scales, tissue, and feces into their environment (Rees et al., 2014). This eDNA can be identified using molecular tools such as quantitative PCR (qPCR) and metabarcoding (Rees et al., 2014). qPCR data and models using qPCR data can be used to identify specific species and potentially estimate biomass in space and time (Baker et al., 2018; Doi et al., 2015; Lacoursière-Roussel et al., 2016; Wang et al., 2020; Yates et al., 2020). Metabarcoding provides community-level information that could be used for biodiversity and population assessments (Kelly et al., 2014; Thomsen et al., 2016). Research on eDNA methods and applications is expanding at a rapid pace. A recent study compared eDNA metabarcoding to trawl surveys and showed that eDNA species reads correlated with species biomass (Stoeckle et al., 2020). Another recent study suggested that monitoring both mitochondrial and nuclear eDNA may provide information on population age and size as well (Jo et al., 2020). A recent review of 63 studies investigating the topic discovered that 90% of the studies reported a positive relationship between eDNA concentration and fish abundance and/or biomass (Rourke et al., 2021). Not only do eDNA methods have the potential to provide information about species presence and abundance but also they can be more sensitive and cost-effective compared with conventional methods (Davy et al., 2015; Kirtane et al., 2019; Smart et al., 2015; Tucker et al., 2016).

Models are being developed to interpret eDNA concentration measured in environmental waters and predict where and when target species were in a water body, as well as species abundance or biomass (Andruszkiewicz et al., 2019; Hansen et al., 2018; Lacoursière-Roussel & Deiner, 2019; Lacoursière-Roussel et al., 2016; Sansom & Sassoubre, 2017; Thomsen et al., 2016; Wang et al., 2020). These models require inputs including how much eDNA an organism sheds into the environment, how long that eDNA persists, and how far the eDNA is transported (Andruszkiewicz et al., 2019; Collins et al., 2018; Hansen et al., 2018; Shogren et al., 2019). Thus, empirically determining eDNA shedding and decay rates of all species of interest is a necessary step to build models that can be effective tools to augment traditional biological monitoring, for example fish stock assessments. Numerous factors inherent to the organism (e.g., biomass, life stage, density, diet) affect the eDNA shedding rate, while environmental conditions (e.g., salinity, temperature, microbial activity, sunlight exposure, nutrient availability) are known to influence the degradation rates of eDNA (Andruszkiewicz et al., 2017; Barnes et al., 2014; Eichmiller et al., 2016; Goldberg et al., 2011; Jo et al., 2017, 2019; Kelly et al., ,2014, 2018; Klymus et al., 2015; Lance et al., 2017; Maruyama et al., 2014; Minamoto et al., 2017; Sassoubre et al., 2016; Seymour et al., 2018; Strickler et al., 2015). Although eDNA is known to degrade more rapidly in marine environments than in freshwater environments, it persists in the water column for a wide time range (days) depending on the starting concentration and environmental conditions (Sassoubre et al., 2016; Sigsgaard et al., 2017; Thomsen et al., 2012). Metabolic rate and activity differ between benthic and pelagic species due to differences in behavior and physiology affecting their eDNA shedding rates, underscoring the importance of determining species-specific eDNA shedding for more species of interest (Killen et al., 2010; Thalinger et al., 2020). More research is needed on eDNA shedding and degradation rates including how the rates are modeled and replication of experiments with varying species, biomasses, and environmental conditions.

Since qPCR is often the chosen method for eDNA shedding and decay modeling studies, it is essential to understand the effect of qPCR assay design on the probability of detection. One important parameter of a qPCR assay is the amplicon length. Shorter qPCR targets are generally preferred as they provide better amplification efficiencies (Rodríguez et al., 2015; Svec et al., 2015). Thus, most eDNA studies have a target amplicon size of between 50 and 200 bp (Collins et al., 2018; Rees et al., 2014). It is hypothesized that longer fragments of eDNA might degrade faster as they provide for more potential attack points for enzymes, light, and other degrading factors (Deagle et al., 2006; He et al., 2019; Woodruff et al., 2015). One study using extracellular DNA found that a 266-bp fragment degraded 2–10 times slower than a 1017-bp fragment (He et al., 2019). However, eDNA in various states (membrane-bound/intracellular, sorbed, etc.) and variable environmental matrices may add to the complexity. Studies show contradictory results wherein some studies report longer fragments degrading faster (Collins et al., 2018; Jo et al., 2017) while others report no significant difference (Bylemans et al., 2018; Piggott, 2016).

To address the knowledge gaps discussed above, we conducted mesocosm experiments at the NOAA Northeast Fisheries Science Center James J. Howard Marine Sciences Laboratory in Sandy Hook, New Jersey, to determine the shedding and decay rates of three economically important fish species and surveys in the adjacent Sandy Hook Bay, to compare results from eDNA analysis with traditional bottom trawls. The objectives of the mesocosm studies were to: (1) develop species-specific qPCR assays for three fish species—black sea bass (BSB), winter flounder (WF), and summer flounder (SF); (2) determine the shedding and decay rates for the three species; (3) evaluate the effect of qPCR amplicon length (135 bp versus 292 bp) on eDNA decay rates for winter flounder; and (4) apply the qPCR assays for the three species to environmental water samples and compare eDNA concentrations with traditional bottom trawls.

2 | METHODS

2.1 | Environmental DNA shedding and decay rate experimental design

The eDNA degradation rate experiments were conducted at the James J. Howard Marine Sciences Laboratory in two identical flow-through tank systems (Figure 1). The water used to fill the closed

7500-L recirculating aquaculture systems was provided by the facility's intake system and originated from Sandy Hook Bay. Prior to the start of an experiment, the systems were treated mechanically using a sand filter (50 μm pore size, Arias 8000 sand filter), chemically using UV (Twin Smart UV-High-Output Sterilizer) and activated carbon (Clean & Clear Cartridge Filter), and biologically using bioreactor (Sweetwater Low-Space Bioreactor) to remove background eDNA. The two notable exceptions to this removal would be bacterial DNA for filtration processes, and eDNA from an always present background species, banded killifish (*Fundulus heteroclitus*) kept in the system. During the experiments, the UV treatment and activated carbon filter were bypassed, leaving only mechanical and biological filtration functioning for the duration of the experiment (7–14 days). For each system, water was pumped into three 1000-L holding tanks simultaneously in parallel at a flow rate of 35 L/min and then combined in a fourth tank where we sampled from (Figure 1). The three tanks separately contained the target species for these experiments and another species, banded killifish, so the sample collected from the fourth tank likely contained DNA from our target species and banded killifish. The water from the fourth tank was then pumped through the previously mentioned processes to fill two large 2000-L reservoir tanks to be redistributed to holding tanks (Figure 1). Total system volumes were calculated and adjusted for each 1 L sample removed for analysis. Water temperatures, pH, and salinity were monitored in both systems throughout the experiments (Table 1).

Black sea bass (BSB) and winter flounder (WF) eDNA shedding and decay experiments were conducted simultaneously in June 2018. Both systems 1 and 2 contained BSB and WF in different tanks, ensuring the same environmental conditions for the eDNA shedding and decay experiments. A previous study showed that the presence of two different fish species in the same tank did not significantly affect eDNA shedding and decay (Sassoubre et al., 2016) so we do not think that having multiple species in the same experimental system (separate tanks) influenced shedding and decay rates in our experiments. For the BSB and WF experiments, 10 BSB and 14 WF were added to the tanks in system 1, while 10 BSB and 13 WF were added to system 2. Fish were held in the tanks for 35 h to determine shedding rates. After 35 h, all fish were removed to determine decay rates over the next 6 days (Table 2). For the summer flounder (SF) experiments, 9 SF and 24 SF had been in systems 1 and 2, respectively, for 4 weeks prior to the eDNA experiments. In April 2019, SF eDNA was assumed to be at a steady state, water samples were collected, and then, the SF were removed to determine eDNA decay in both systems over 6 days (Table 2). Environmental variables

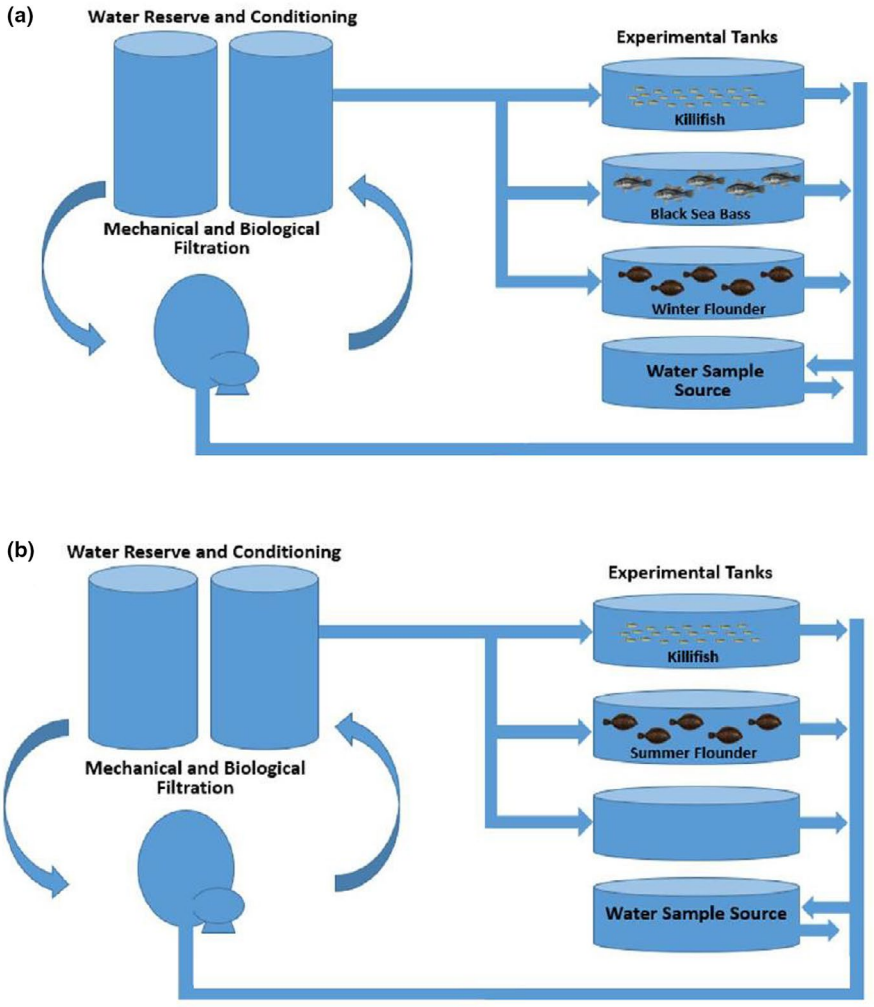

FIGURE 1 Design of recirculating aquaculture system (RAS) for measuring eDNA shedding and decay rates. (a) Black sea bass and winter flounder experimental setup. (b) Summer flounder experimental setup. Each set of experiments was run simultaneously in two replicate RAS referred to as systems 1 and 2

TABLE 1 Environmental conditions in systems 1 and 2 measured over the study period. Error is represented by the standard deviation

Experiment number	Species	pH		Temperature (°C)		Salinity (ppt)		Water volume (L)	
		System 1	System 2	System 1	System 2	System 1	System 2	System 1	System 2
1	Black sea bass, winter flounder	7.88 ± 0.07	7.93 ± 0.2	19.86 ± 1.45	19.06 ± 0.4	27.92 ± 2.19	27.99 ± 2.18	8112	7945
2	Summer flounder	8.04 ± 0.08	7.88 ± 0.04	16.21 ± 0.12	15.87 ± 0.15	33.85 ± 0.05	33.86 ± 0.08	8028	7676

including temperature, salinity, and pH were measured in each system every day of the experiments (Table 1).

For BSB and WF experiments, water samples were collected at 24 h, 10 h, and 3 h before the fish were added to check for a background BSB or WF eDNA signal in the tank water. After BSB and WF were added to the tanks, samples were collected at 0.5, 3.5, 8, 12, 15.5, 20, 24, 27, 32, and 34 h to determine the steady-state concentration of their eDNA and calculate shedding rates. The BSB and WF were removed after 35 h, and water samples were collected at 1, 3, 6.5, 9, 12, 15, 18, 21, 24, 27, 30, 33.5, 37, 41, 46, 51, 60, 84, and 120 h to determine the eDNA decay rates. For SF, duplicate samples were collected 2 h prior to fish removal to establish steady-state concentration and then 2, 9.5, 12.5, 15.5, 12.5, 24.5, 27.5, 22.5, 36.5, 38.5, 46, 50, 62, 71.5, 85, 97, 109, 120 and 132 h after SF were removed.

At each sampling, duplicate 1 L water samples were collected from the fourth tank in the system in order to homogenize water from the other tanks as completely as possible (Figure 1). Water samples were vacuum-filtered through 0.4-μm polycarbonate filters (Whatman, UK) using a vacuum pump (Millipore, Model #WP6111560). Blanks made from 1 L of DI water were filtered each day of the experiments (n = 17). At the beginning of each experiment, 1 L of source seawater from the facility was filtered to check for BSB, WF, or SF eDNA signal from the source waters. The filters were stored at −20°C before being shipped on dry ice to the laboratory at the University at Buffalo (UB). At UB, eDNA was extracted from the filters and analyzed by qPCR, and shedding and decay rates were calculated.

2.2 | Trawl sampling and eDNA collection

A total of 26 water samples were collected and filtered alongside thirteen trawls done over 6 days during May–November 2019 in Sandy Hook Bay, NJ. An 18′ otter trawl was towed for 10 min with 200 ft of cable at an average speed of 3 kts. At the end of the trawl, species of interest (BSB, WF, and SF) were counted and released. Water samples were collected at the beginning and end of each trawl, 1 m above the bottom, which was between 2.5 m and 19 m from the water surface. Water was collected using a Kemmerer bottle (WildCo, Yulee, FL). The water sample was transferred into a sterile 1-L bottle and vacuum-filtered onboard using sterile, single-use disposable filtration funnels (Thermo Fisher Scientific, Waltham, MA) and 0.4-μm polycarbonate filters (Whatman, UK). Different volumes (400–700 ml) were filtered based on the turbidity of the sample and clogging of the filters (Table 3). The filters were transferred to 5-ml transport tubes, stored, and shipped to the laboratory on dry ice where it was stored at −20°C until DNA extraction. Blanks made from 100 ml DI water were filtered onboard at the beginning and end of each day of sampling to check for contamination. Given the time of year of sampling and the expected fish, water samples for eDNA were tested for BSB and WF in May, and BSB and SF in September–November.

TABLE 2 Fish counts and biomass for each species in each experimental system

	Number of fish		Average mass per fish (g) (± standard deviation)		Total fish mass (g)	
Species	**System 1**	**System 2**	**System 1**	**System 2**	**System 1**	**System 2**
Black sea bass	10	10	580.6 ± 141.3	521.5 ± 135.6	5806.4	5215.1
Winter flounder	14	13	99 ± 32.8	96.5 ± 43.4	1386.2	1255
Summer flounder	9	24	42.6 ± 3.8	42.4± 4.4	340.9	636.4

2.3 | eDNA extraction

Genomic DNA from tissue of four species used in this study (BSB, WF, SF, and banded killifish) and Atlantic silverside (*Menidia menidia*), which was studied in the same laboratory, was extracted for primer development to ensure no cross-amplification. Genomic DNA was extracted using the Qiagen Blood and Tissue Kit (Qiagen, Valencia, CA) protocol with minor modifications as described in a previous study (Sansom & Sassoubre, 2017). All genomic DNA was diluted to 1 ng/μl after extraction. eDNA from filters was extracted using the same protocol. Extraction blanks (no tissue or filter) were run with each batch of DNA extractions. Eluted DNA was quantified using the dsDNA High Sensitivity Kit for the QUBIT 3.0 fluorometer (Applied Biosystems, Foster City, CA).

2.4 | Primer design and qPCR optimization

Species-specific primers and TaqMan probes were designed to target the mitochondrial cytochrome oxidase subunit 1 (COI) gene of BSB, WF, and SF. Primers were initially designed using Primer-BLAST (Ye et al., 2012) with at least 3 mismatches on each primer to ensure no amplification with closely related and co-occurring species. The target sequences were then aligned in MEGA7 (Kumar et al., 2016), and TaqMan probes were specifically designed to maximize mismatches with the non-target species, especially those co-occurring in our experimental systems. The oligonucleotides were then analyzed using OligoAnalyzer (IDT, Coralville, CA) to ensure optimal melt temperature and ensure a minimal possibility of dimerization during qPCR amplification. For winter flounder, two species-specific overlapping assays, one short (135 bp) and one long (292 bp), were designed to test the hypothesis that a longer DNA strand would experience more rapid decay (Figure S1). The specificity of the designed primers was tested against genomic DNA extracted from non-target species to determine cross-reactivity, including BSB, WF, SF, Atlantic silversides, and banded killifish. All qPCRs were run on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA). Primer concentrations and cycling parameters were optimized for each assay to achieve the highest efficiency. Cycling parameters for BSB, SF, and WF-short assays were initial incubation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 10 s followed by annealing and extension at 60°C for 45 s. qPCR efficiency generally decreases with an increased amplicon size (Bylemans et al., 2018). To address this, an additional extension step at 70°C for 30 s was added after annealing at 60°C for 45 s for the WF-Long assay. All 20 μl qPCRs were run in triplicate on 96-well plates using TaqMan Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA) with 2 μl of template DNA and optimized primer and TaqMan probe concentrations listed in Table 4. Triplicate no-template controls (NTCs) were run on each plate. To determine assay efficiency and sensitivity, a 6-point standard dilution curve made using synthetic DNA gBlock® Gene Fragment (IDT, Coralville, CA) was run in triplicate on each plate. Standard curves on each plate were used to calculate the eDNA concentrations of samples analyzed on the given plate. Primer sequences, concentrations, and efficiency are shown in Table 4 in compliance with MIQE guidelines (Bustin et al., 2009).

2.5 | qPCR data analysis

Limit of detection (LOD) was defined as the lowest standard with 95% amplification across all replicates, and the limit of quantification (LOQ) was defined as the lowest standard with a coefficient of variation (CV) below 35% (Klymus et al., 2019). All qPCR results were separated into three categories: quantifiable eDNA concentration, below limit of quantification (BLOD), and non-detects (ND). All samples with triplicate qPCRs at Cqs greater than the average Cq of the lowest standard were considered to have quantifiable eDNA concentrations. Only these samples were used in modeling the eDNA shedding and decay rates. Samples with one or two positive qPCR replicates and/or with Cqs below the average Cq of the lowest standard were categorized as BLOD. All samples with no qPCR amplification were categorized as ND. The lowest standard run for each assay was 10 copies per reaction (Table 4). All samples categorized as BLOD in the tank experiments were checked for inhibition by diluting the samples 1:10 before adding them to the qPCR.

2.6 | Environmental DNA decay rate modeling

To determine the shedding and decay rates for each fish species, the experimental tanks were modeled as completely mixed batch reactors following previous studies (Sansom & Sassoubre, 2017; Sassoubre et al., 2016) (Equation 1).

$$V\left(\frac{dC}{dt}\right) = S - kCV \quad (1)$$

TABLE 3 Sampling date, trawl number, trawl count, volume filtered, and eDNA assay tested for the samples collected alongside bottom trawls

Date	Sample type	Fish species captured during trawl (count)	Volume filtered (ml)	eDNA assays tested
5/2/2019	NTC before		100	BSB, WF
	Trawl 1 before		400	BSB, WF
	Trawl 1 after		400	BSB, WF
	Trawl 2 before		400	BSB, WF
	Trawl 2 after		400	BSB, WF
	Trawl 3 before	Winter flounder (1)	400	BSB, WF
	Trawl 3 after		400	BSB, WF
	Trawl 4 before		400	BSB, WF
	Trawl 4 after		400	BSB, WF
	Trawl 5 before		400	BSB, WF
	Trawl 5 after		400	BSB, WF
	Trawl 6 before		400	BSB, WF
	Trawl 6 after		400	BSB, WF
	NTC after		100	BSB, WF
9/26/2019	NTC before		100	BSB, SF
	Trawl 7 before		400	BSB, SF
	Trawl 7 after		500	BSB, SF
	Trawl 8 before	Black sea bass (1)	400	BSB, SF
	Trawl 8 after		700	BSB, SF
	NTC after		100	BSB, SF
10/3/2019	NTC before		100	BSB, SF
	Trawl 9 before	Black sea bass (1)	400	BSB, SF
	Trawl 9 after		400	BSB, SF
	NTC after		100	BSB, SF
10/23/2019	NTC before		100	BSB, SF
	Trawl 10 before		400	BSB, SF
	Trawl 10 after		400	BSB, SF
	NTC after		100	BSB, SF
10/31/2019	NTC before		100	BSB, SF
	Trawl 11 before		500	BSB, SF
	Trawl 11 after		500	BSB, SF
	NTC after		100	BSB, SF
11/14/2019	NTC before		100	BSB, SF
	Trawl 12 before	Summer flounder (1)	500	BSB, SF
	Trawl 12 after		500	BSB, SF
	Trawl 13 before		400	BSB, SF
	Trawl 13 after		400	BSB, SF
	NTC after		100	BSB, SF

NTCs were made from filtering 100 ml of DI water.

where V is the volume of the reactor, C is the concentration of eDNA (copies/L), t is time in hours, S is the shedding rate, and k is the decay rate constant.

A decay rate constant was, k, was calculated for the data following first-order decay by fitting a line to $\ln(C/C_0)$ versus time (h), where C_0 is the steady-state concentration of DNA (copies/L), and C represents the concentration of DNA (copies/L) at time, t. For experiments with BSB, WF, and SF, data fit first-order decay between 9 and 17 h after the fish were removed. The decay rate constant was used to calculate the shedding rates (in copies per hour per fish and copies per hour per kg of fish biomass) using Equation (1) and assuming steady state, $dC/dt = 0$. Propagated error for shedding rates was

TABLE 4 Oligonucleotides designed and used in this study. LOD refers to the limit of detection, and LOQ refers to the limit of quantification as defined in Klymus et al., (2019)

Assay Name	Target species	Sequence (5′- 3′)	Reaction concentration (nmol)	Amplicon size (bp)	Average Efficiency (%)	LOD (LOQ) Copies/reaction
BSB-F	Black sea bass	GAGCTGGCATGGTAGGTACG	300	116	94	10 (100)
BSB-R		TTGCCGAAGCCCCCAATTAT	300			
BSB-P		FAM-AGCCTTCTTATCCGAGCTGAGCTAAG-BHQ	150			
WF-Short-F	Winter flounder	CCTTCTAGCCTCTTCAGGCG	900	135	95	10 (1000)
WF-Short-R		TGAAATTCCGGCAAGGTGGA	900			
WF-Short-P		FAM-CAGGGACAGGATGAACCGTGTATCCC-BHQ	150			
WF-Long-F	Winter flounder	AGCAGAACTAAGCCAACCCG	600	292	94	10 (1000)
WF-Long-R		ATTTCCAGCTAGTGGGGGATAC	600			
WF-Long-P		FAM- CGTCGAAGCTGGGGCAGGGACAGGA-BHQ	150			
SF-F	Summer flounder	GTGGGAACAGCCCTGAGTTT	300	103	92	10 (10)
SF-R		AGGCGTGTGCAGTAACGATT	300			
SF-P		FAM-CTTAGCCAACCCGGCGCCCTGC-BHQ	150			

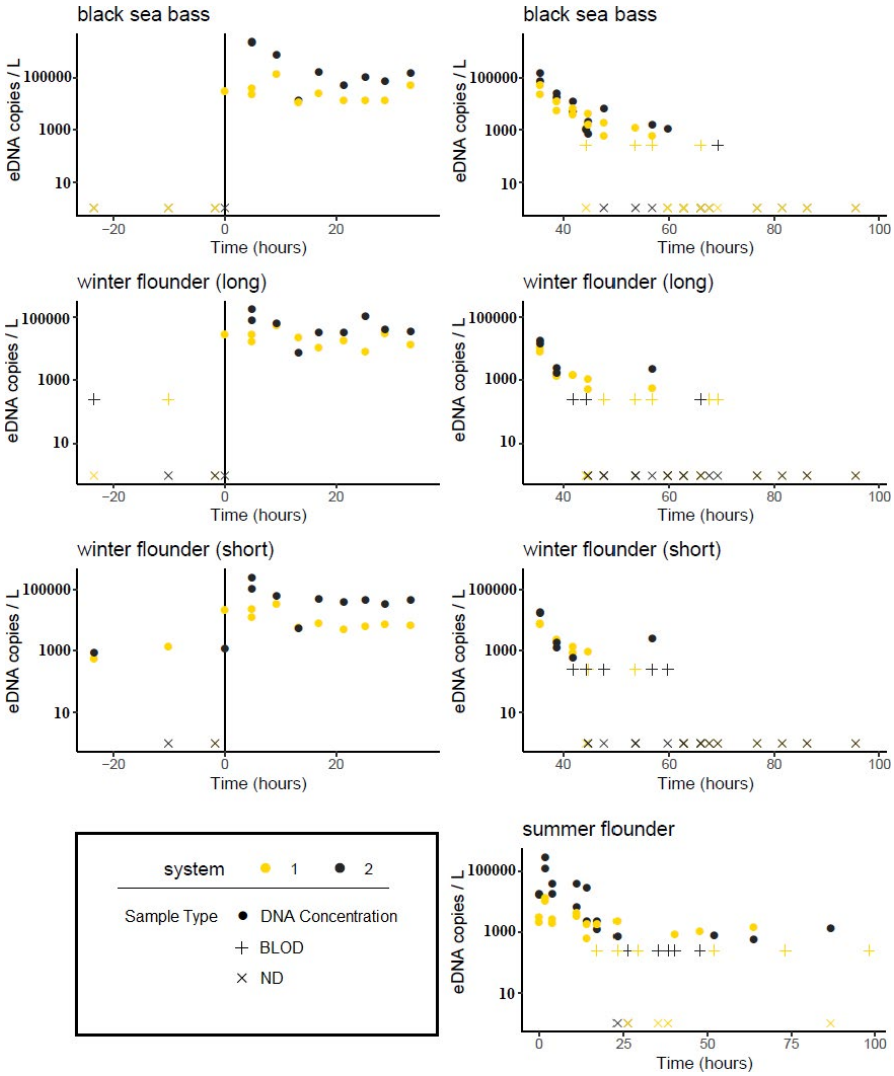

FIGURE 2 eDNA concentrations before fish were added to the tanks and while the fish were in the tanks (shown in the left column) and eDNA concentrations after the fish were removed (shown in the right column). The solid vertical black line marks the time when fish were added to the systems. Colors represent the two experimental systems run simultaneously. There is only one graph for summer flounder showing eDNA concentrations after the fish were removed because experiments were not performed while the fish were in the tanks. For this reason, the *x*-axis for the summer flounder graph starts at 0 h when collection of experimental data began

TABLE 5 eDNA steady-state concentrations, decay rate constants, half-lives, and shedding rates for each species and each experimental system

Species	System number	Steady-state concentration (copies L^{-1} g^{-1})[a]	Decay rate constant (h^{-1})[b]	eDNA half-life ($T_{1/2}$) (h)	Shedding rate per fish (copies h^{-1} $individual^{-1}$)[c]	Shedding rate per gram (copies h^{-1} g^{-1})[c]
Black sea bass	1	3.77 ± 3.07	0.19 ± 0.030	3.71	$3.32 * 10^6 \pm 2.75 * 10^6$	$5.72 * 10^4 \pm 4.74 * 10^4$
	2	20.33 ± 9.24	0.17 ± 0.050	4.00	$1.46 * 10^7 \pm 7.85 * 10^6$	$2.79 * 10^5 \pm 1.50 * 10^5$
Winter flounder (short target)	1	4.69 ± 0.88	0.25 ± 0.038	2.77	$9.27 * 10^5 \pm 2.56 * 10^5$	$1.31 * 10^5 \pm 3.62 * 10^4$
	2	33.23 ± 4.99	0.57 ± 0.091	1.22	$5.58 * 10^6 \pm 3.07 * 10^6$	$7.52 * 10^5 \pm 4.14 * 10^5$
Winter flounder (long target)	1	11.54 ± 6.32	0.25 ± 0.050	2.82	$2.31 * 10^6 \pm 1.32 * 10^6$	$3.27 * 10^5 \pm 1.86 * 10^5$
	2	38.25 ± 24.30	0.22 ± 0.12	3.21	$1.66 * 10^7 \pm 1.37 * 10^7$	$2.24 * 10^6 \pm 1.47 * 10^6$
Summer flounder	1	7.91 ± 2.04	0.070 ± 0.035	9.9	$1.68 * 10^5 \pm 9.43 * 10^4$	$3.54 * 10^4 \pm 1.99 * 10^4$
	2	28.46 ± 1.34	0.24 ± 0.044	2.89	$1.39 * 10^6 \pm 2.63 * 10^5$	$7.86 * 10^5 \pm 1.49 * 10^5$

[a]Error represents standard deviation.
[b]Error represents standard error.
[c]Error represents propagated error.

calculated as described by Sansom & Sassoubre, 2017. We assumed steady-state concentration, *C*, was achieved for BSB and WF approximately 38.75 h after the fish were added based on previous studies (Sansom & Sassoubre, 2017; Sassoubre et al., 2016). *C* for BSB and WF was determined by averaging the eDNA copy number of five observations before t = 38.75 h after the fish were added based on the plateauing of eDNA concentration observed (Figure 2). Since SF were in the system for well beyond the time it takes to reach steady state (4 weeks) before the experiment, steady state was assumed and eDNA concentration before the removal of SF was considered as its steady-state concentration. All BLOD, ND, and quantifiable samples after 24 h of fish removal were excluded from decay rate calculations because they did not follow first-order decay, which was assumed for the decay rate constant, *k*, calculation. However, the positive samples after 24 h do suggest biphasic decay or resuspension as observed in previous studies (Andruszkiewicz Allan et al., 2020; Eichmiller et al., 2016; Harrison et al., 2019; Jo & Minamoto, 2020). Since the eDNA persistence has been described using decay rate constants (*k*) and half-life ($T_{1/2}$), both were calculated in this study (Collins et al., 2018; Hansen et al., 2018).

eDNA half-life ($T_{1/2}$) was calculated using Equation (2), where *k* is the calculated decay rate constant using Equation (1).

$$T_{1/2} = \frac{0.693}{k} \quad (2)$$

2.7 | Statistical analysis

ANCOVA statistical test was used to compare the slopes representing the eDNA decay overtime in R (RCore, 2016) with respect to the species of fish, WF assay lengths, and replicate systems. The paired *t* tests, conducted in MS Excel, were used to evaluate the null hypothesis that the shedding rates are not significantly different between the two systems when paired by species assays. Statistical significance was set at an alpha level of 0.05 for all tests.

3 | RESULTS

3.1 | Species-specific assay performance

All four species-specific assays had an amplification efficiency of over 90% for all qPCR plates with a limit of detection (LOD) of 10 copies per reaction (Table 4). The limit of quantification (LOQ) as defined by Klymus et al., (2019) was 10 copies per reaction for SF, 100 copies per reaction for BSB, and 1000 copies per reaction for WF short and WF long (Table 4). No cross-amplification between species was observed when tested with genomic DNA (1 ng/L) extracted from BSB, WF, SF, and killifish tissue. A small concentration of WF eDNA was detected in the tanks prior to the addition of the fish in both systems but was two orders of magnitude lower than the steady-state concentration (Figure 2). Since all filtration blanks, extraction blanks, qPCR blanks (NTCs), and samples from treated source water showed no amplification for winter flounder eDNA prior to addition of fish, we concluded

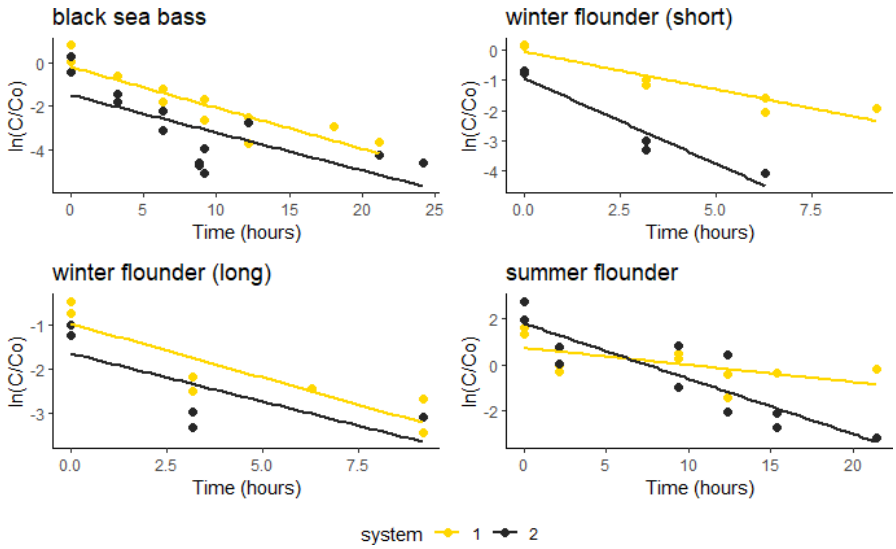

FIGURE 3 eDNA decay of the three species of fish in the two systems modeled assuming first-order decay. Yellow represents system 1, and black represents system 2. Experiments were performed in both systems simultaneously for each species and for BSB and WF at the same time

that the contamination was not from source water or laboratory procedures. The source of this small signal in the qPCR data was likely residual eDNA from previous experiments in the system, and it is unlikely that it influenced the experiments or eDNA shedding and decay rate calculations. Samples were diluted 1:10 to test for potential inhibition (in BLOD and environmental samples). eDNA was not detected in the diluted samples suggesting qPCR inhibition could not account for the lack of eDNA detected.

3.2 | eDNA shedding rates for black sea bass, winter flounder, and summer flounder in experimental systems 1 and 2

eDNA was detected in the samples as soon as the fish were added to the tanks as shown by the detection at $t = 0$. For BSB and WF, a steady state was achieved before the fish were removed at 35 h (Figure 2, left column). SF were in the tanks for 4 weeks before they were removed; thus, a steady-state eDNA concentration was assumed. The average steady-state concentrations ranged from 3.8 to 38.3 copies per L of water per gram of fish biomass (Table 5). The calculated shedding rate was reported as eDNA copies per hour per fish (copies h^{-1} $fish^{-1}$) to account for differences in the number of fish in each system and copies per hour per biomass (copies h^{-1} g^{-1}) to account for differences in total biomass between the systems. The average shedding rates ranged from $1.68 * 10^5$ to $1.66 * 10^7$ copies h^{-1} $fish^{-1}$, and $3.54 * 10^4$ to $2.24 * 10^6$ copies h^{-1} g^{-1} (Table 5). The shedding rates in systems were not significantly different when paired by species-specific assays (paired t test, $p = 0.095$).

3.3 | eDNA shedding rates for black sea bass, winter flounder, and summer flounder in each experimental system and by amplicon length

Once the fish were removed, an initial increase in eDNA concentration was observed for BSB in systems 1 and 2, WF short in system 1, and SF in systems 1 and 2 (Figure 2), possibly due to additional eDNA released due to stress as the fish were moved, which has been observed previously (Maruyama et al., 2014; Sansom & Sassoubre, 2017; Sassoubre et al., 2016; Thalinger et al., 2020). After fish removal, the eDNA concentration decreased rapidly and was not reliably detected after ~12 h for BSB, ~9 h for WF, and ~17 h for SF (Figure 3). Since the time until the eDNA is undetectable depends on the steady-state concentration, decay rate constants (k) were used to compare the persistence of eDNA between species and systems. The most rapid eDNA decay was observed in WF-short system 2 ($k = 0.567 \pm 0.091$ h^{-1}), while the slowest was observed in SF system 1 ($k = 0.07 \pm 0.035$ h^{-1}) (Table 5). The decay rate constant was not significantly different between the species for each experimental system (ANCOVA, $p = 0.758$). However, the decay rate constants were significantly different between the two systems for WF short (ANCOVA, $p = 6.6 * 10^{-3}$) and SF (ANCOVA, $p = 9.3 * 10^{-3}$). qPCR amplicon length did not have a significant effect on the decay rate constants for WF when tested with WF-short and WF-long assays (ANCOVA, $p = 0.685$).

3.4 | Comparison of eDNA analysis to catch from bottom trawls

No quantifiable eDNA of any of the target species was detected in environmental samples collected alongside bottom trawls. Across all 13 trawls over 6 days, only 1 WF, 1 SF and 2 BSB were caught. The lack of detection by either method (eDNA or trawling methods) suggests there were few, if any, WF, SF, and BSB .

4 | DISCUSSION

Sensitive and species-specific qPCR assays for three commercially harvested and economically important fish species (black sea bass, winter flounder, and summer flounder) were developed. These assays were then applied to water samples from mesocosm experiments

conducted to determine eDNA shedding and decay rates for each fish species. Calculating eDNA shedding and decay rates is a necessary first step to relate eDNA concentrations to biomass and species abundance. eDNA shedding and decay rates are important inputs for models that parameterize when and where fish were in a water body based on eDNA concentrations from water samples. To test the qPCR assays developed in the marine environment, water samples were collected alongside traditional bottom trawl surveys and analyzed for all three species. All three species were not detected by eDNA methods, and only 1 WF, 1 SF, and 2 BSB were caught across 13 trawls over 6 days, suggesting few, if any, fish were in the waters sampled. While previous studies have shown agreement between trawl catches and eDNA methods when the species of interest were present (Rourke et al., 2021), this study shows agreement between these methods when the species of interest was likely not present or in very low abundance. Determining when a species is not present using eDNA methods is an important and understudied area of research, especially given the potential applications of eDNA methods to identify rare, threatened, endangered, or invasive species, which can be challenging to identify using conventional methods (Rees et al., 2014). The research presented in this paper provides evidence for the use of eDNA methods to confirm low abundance or the likely absence of target species.

4.1 | eDNA shedding rates across species and between experimental systems

The calculated eDNA shedding rates were not significantly different between the two systems when paired by species-specific assay. It is worth noting that the calculated propagated error of the shedding rates was very large, mostly attributed to the error associated with the decay rate constants and steady-state concentrations. This analysis and propagated error calculations from previous studies highlight the variability involved in eDNA shedding and decay, even in relatively controlled experimental systems (Andruszkiewicz Allan et al., 2020; Sansom & Sassoubre, 2017). Not only is variability in environmental waters likely higher than in controlled mesocosm experiments, but also the behavior of the fish, especially benthic fish such as WF and SF, may be different in a natural environment than in the mesocosm experiments, which would influence eDNA shedding rates (Thalinger et al., 2020).

4.2 | Effect of species and environmental conditions on decay rate constants

The decay rate constants (k) were not significantly different between species leading to the conclusion that the mechanisms responsible for eDNA decay are not heavily dependent on the species but rather on the conditions of the water the eDNA is in. This is especially useful in monitoring fish stocks as the same eDNA decay rate constant could be used to determine biomass for different species that are in the same water body. The decay rate constants were also similar to previous studies with marine fish: 0.024–0.029 h^{-1} for shanny (Collins et al., 2018); 0.057–0.068, 0.055–0.07, and 0.101 h^{-1} for Pacific sardine, Pacific chub mackerel, and Northern anchovy, respectively (Sassoubre et al., 2016); 0.013 and 0.026 h^{-1} for European flounder and three-spined stickleback, respectively (Thomsen et al., 2012); 0.037–0.467 h^{-1} for Japanese jack mackerel (Jo et al., 2019); and 0.059–0.092 h^{-1} for mummichog (Andruszkiewicz Allan et al., 2020). We did observe differences in decay rate constants between systems for WF and SF. Additionally, the steady-state concentration of system 2 was consistently higher than that of system 1 for all species. Since the two systems were designed to be identical and the abiotic water quality variables measured were not very different (Table 1), there must be another variable affecting eDNA decay that we did not measure. One possible variable contributing to different eDNA decay rates is the microbial community and nutrients in the systems (Barnes et al., 2014; Salter, 2018). These results suggest that variability between experimental systems is important and should be considered in future studies estimating eDNA decay rates. It is also likely that eDNA decay rates for the same species might be different in different natural environments. Ideally, eDNA decay rates would be determined specifically for the water matrix or environment that the decay rate would be used to determine fish biomass in.

4.3 | Effect of amplicon length on eDNA decay rate

There was no significant difference between the decay rate constants for the WF-long (292 bp) and WF-short (135 bp) assays (Table 5). A previous study concluded that crab DNA degraded 1.2 times faster than fish eDNA, with a likely explanation that the crab assay was 21 bp longer (Collins et al., 2018). Another study reported a significantly faster eDNA degradation of 719-bp assay than a 127-bp assay targeting Japanese jack mackerel, although the assay efficiencies were not reported, which could have influenced the comparison (Jo et al., 2017). The results of this study indicate the contrary. The two overlapping qPCR assays, 135 bp WF short and 292 bp WF long, with similarly high amplification efficiencies, did not affect the calculated eDNA decay rate constants (Figure S1). For amplicon length to have a significant impact on decay rate kinetics, the difference between the lengths may need to be greater (He et al., 2019). Additionally, the hypothesis that longer fragments of eDNA degrade more rapidly due to more potential attack points may only be valid for free extracellular eDNA and not membrane-bound or particle-bound DNA (He et al., 2019). In marine eDNA samples, most aqueous eDNA captured on filters is in the range of between 1 and 10 μm, suggesting its presence inside a cell, mitochondria, or adsorbed to larger particles (Sassoubre et al., 2016; Turner et al., 2014; Wilcox et al., 2015). Long-range PCR techniques revealed that entire mitochondrial genomes were intact in eDNA samples, again suggesting the presence of intracellular DNA in eDNA samples (Deiner et al., 2017). A recent meta-analysis further emphasized the need to understand the state of the eDNA to determine which environmental or sampling variables were

important to consider (Jo & Minamoto, 2020). This study used 0.4-μm pore size filters; thus, a majority of the eDNA captured is likely to be intracellular. A species whose eDNA is more extracellular (due to a majority being shed through feces or through an invertebrate siphon) would likely have a different decay rate constant than a species whose eDNA is more intracellular (majority shed through scales or tissue) when targeting a longer amplicon. However, more research in this area is needed.

4.4 | Using eDNA to predict biomass in coastal marine waters

No eDNA from target species was detected in any of the environmental samples. This is likely due to a small population of the target species or no individuals in the water, which is consistent with results from the bottom trawls that showed only 0–1 individuals captured per trawl (Table 3). While no direct comparison could be made between eDNA concentrations and organism counts from bottom trawls, the eDNA and trawl data agree. Studies have argued that eDNA as a survey tool can be more sensitive than conventional methods in many cases (Fernández et al., 2018; Kirtane et al., 2019; Smart et al., 2015). However, the data presented in this manuscript suggest that when only a few individuals are in a large water body, trawling may result in species capture when eDNA methods do not detect any species-specific eDNA, likely due to the volume of water the trawl passes through versus the limited volume that can be filtered for eDNA analysis.

Applications of eDNA methods range from detecting the presence of a species to estimating the abundance to assessing biodiversity. While it is extremely useful to demonstrate that a high concentration of eDNA in a sample correlates with a high abundance of target species, it is equally important to investigate whether an absence or low concentration of eDNA indicates the absence or low abundance of target species. This is especially important for applications related to rare, threatened, or endangered species, as well as invasive species as they spread or are eradicated. In these cases, a non-detect using eDNA methods does mean there are no or only a few individuals in the water sampled at that time, location and depth. However, non-detects are still hard to interpret. Future studies should include analysis of more samples, larger volumes of waters samples if possible, and/or utilization of passive samplers that account for pulse inputs of eDNA (Kirtane et al., 2020; Schmidt et al., 2013).

4.5 | Study limitations

While the research presented here represents an important contribution to the development of eDNA tools for fisheries management, certain limitations of this should be highlighted and investigated in future studies. The four species-specific assays (two of which targeted WF) designed in this study were tested extensively in silico to ensure high sensitivity and specificity. However, the assays were tested experimentally for cross-reactivity with only the other fish present in the mesocosm experiments, BSB, WF, SF, and banded killifish. Future studies using these assays should validate their specificity by testing them against DNA extracted from species that are closely related and/or co-occurring in the location being studied. It is also important to note that the mesocosm experiments (like others) may not completely represent the environmental conditions in environmental waters. In this study, the experimental systems were filled with water from Sandy Hook Bay, NJ, in an effort to capture at least certain abiotic environmental conditions such as salinity and pH that reflect environmental waters and the conditions of the waters where the trawl surveys were conducted. However, environmental conditions such as temperature and biological activity were likely altered due to physical, chemical, and biological treatment and water circulation in the facility where the experiments were conducted. This could have affected eDNA decay rates. More research is needed on the effect of microbial activity on eDNA degradation. It is also likely that fish kept in enclosed tanks, especially benthic fish, may behave differently than in their natural environments, which could have affected eDNA shedding estimates. The duration of time the fish were kept in the tanks may also affect the decay rates, as seen by the tailing eDNA signal from SF. SF were present in the system for 4 weeks before removal and decay rate quantification compared with BSB and WF, which were present in the system for only a few days prior to removal and decay rate quantification (Figure 2). Despite these limitations, results from this research suggest the qPCR assays developed are robust and the eDNA shedding and decay rates are reasonable estimates. Due to the lack of eDNA detections in the environmental water samples and very few catches in the bottom trawls, we were unable to thoroughly investigate the relationships between eDNA concentration and physical fish counts by trawling. We were, however, able to show a consistent lack of detection between the two methods for all three fish species. While more studies are needed on whether and how eDNA methods can be used to confirm low abundance or the absence of a target species, this research is one piece of evidence.

Overall, this research presents new, sensitive, and specific qPCR assays for three commercially harvested and economically important marine fish species. These assays are used to quantify eDNA shedding and decay rates that can be directly applied in models relating eDNA concentrations to fish presence and/or abundance in space and time. For these models to be used as complementary tools for traditional fisheries management, knowledge about how to interpret not only positive eDNA quantifications but also a lack of eDNA detection is needed. The research presented here not only supports the development of eDNA-based modeling tools but also adds to the interpretation of eDNA concentrations, specifically the lack thereof, in environmental waters.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

AK and LS contributed to the design of the study, acquisition and analysis of the data, and writing of the manuscript. DW contributed to the design of the study, performed the acquisition and interpretation of the data, and reviewed the manuscript. TN and LB contributed to the design of the study, performed the acquisition of the data, and reviewed the manuscript. CO, RP, AC, and KL contributed to the acquisition of the data and reviewed the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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