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Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish

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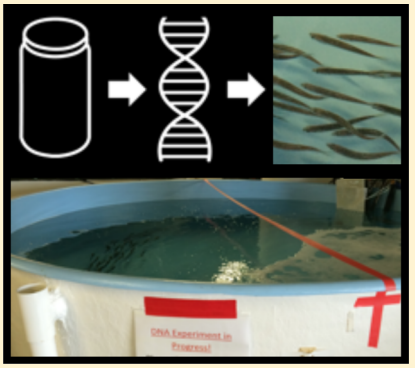
Supporting Information

ABSTRACT: Analysis of environmental DNA (eDNA) to identify macroorganisms and biodiversity has the potential to significantly augment spatial and temporal biological monitoring in aquatic ecosystems. Current monitoring methods relying on the physical identification of organisms can be time consuming, expensive, and invasive. Measuring eDNA shed from organisms provides detailed information on the presence and abundance of communities of organisms. However, little is known about eDNA shedding and decay in aquatic environments. In the present study, we designed novel Taqman qPCR assays for three ecologically and economically important marine fish—*Engraulis mordax* (Northern Anchovy), *Sardinops sagax* (Pacific Sardine), and *Scomber japonicas* (Pacific Chub Mackerel). We subsequently measured fish eDNA shedding and decay rates in seawater mesocosms. eDNA shedding rates ranged from 165 to 3368 pg of DNA per hour per gram of biomass. First-order decay rate constants ranged from 0.055 to 0.101 per hour. We also examined the size fractionation of eDNA and concluded eDNA is both intra- and extracellular. Finally, we derived a simple mass-balance model to estimate fish abundance from eDNA concentration. The mesocosm-derived shedding and decay rates inform the interpretation of eDNA concentrations measured in environmental samples and future use of eDNA as a monitoring tool.



INTRODUCTION

Marine biodiversity is threatened by climate change, pollution, human population growth, overfishing, invasive species, and habitat loss/alteration.[1,2] Monitoring and quantifying the impacts of these threats, however, is challenging due to a lack of biological data describing the abundance and spatial and temporal distribution of organisms in the marine realm.[1] A number of techniques are currently employed to monitor marine organisms including underwater visual counts by divers or video, trawls or netting, tagging, and traditional fishing.[3] These techniques rely on the physical identification and counting of organisms. They can therefore be time-consuming, expensive when taxonomic experts and shiptime are required, and invasive or destructive when netting, trapping or electrofishing methods are used. Moreover, they are dependent on chance encounters with potentially rare organisms and limited to waters accessible by ships or divers.

To address limitations associated with traditional biological monitoring, researchers have proposed the use of molecular techniques to analyze DNA extracted from environmental samples (termed environmental DNA or eDNA) to census the presence of fish and other aquatic organisms.[4−11] Organisms shed DNA into their environments in the form of sloughed tissue or cells, waste products, gametes, saliva, or other secretions. eDNA can be isolated from water samples and used in qPCR assays targeting species-specific DNA sequences or in next-generation sequencing (NGS) that identifies communities of organisms.[7] The concentration of eDNA in a water sample is controlled by a number of environmental processes. As shown in Figure 1, the eDNA concentration depends on its sources (e.g., shedding from organisms) and sinks (e.g., decay) in a given parcel of water and the advection and dispersion of that water parcel in the aquatic system. Thus, the first step to infer organism presence and population densities from eDNA concentrations is obtaining information on eDNA shedding and decay rates. This information can then inform the use of eDNA for biological monitoring, biodiversity assessments, and ultimately policy decisions.

The eDNA shedding rates of aquatic macroorganisms depend on a number of factors: (1) type of organism or species,[5,12] (2) organism size,[13] (3) number of organisms,[5,13−15] (4) life stage,[5,12,16] (5) skin/scale properties, (6) stress an organism is under,[17] and (7) water temperature.[15] Few

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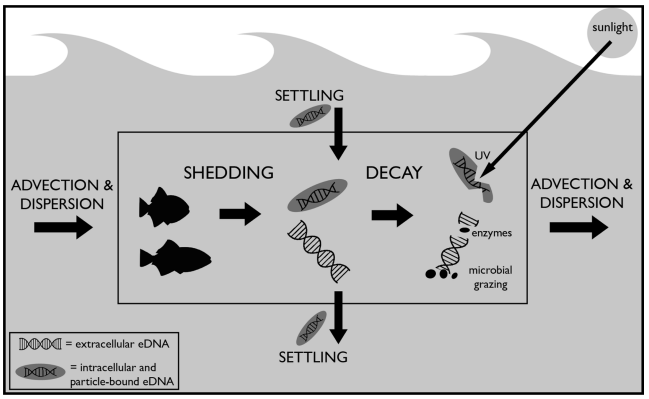

Figure 1. Conceptual model of the sources, transport, and fate of eDNA in aquatic environments. Processes illustrated here include eDNA shedding, decay (due to sunlight, grazing, or enzymatic activity), settling (of eDNA attached to particles), and advection and dispersion.

studies have directly measured how much eDNA an organism sheds into a water body over time,[17−19] and these studies focused on freshwater amphibian larvae, salamanders, and carp. A microcosm study with amphibian larvae found that eDNA concentrations increased over time and were higher in microcosms with more larval species; however, shedding rates differed between larval species.[5]

eDNA decay is influenced by (1) DNA characteristics (e.g., whether it is extracellular or cellular),[20] (2) abiotic factors (e.g., sunlight, temperature, pH, salinity, flow rate, or residence time),[21−23] and (3) biotic factors (e.g., extracellular enzymes and microorganisms).[22,24] The majority of research on eDNA decay has focused on freshwater fish and amphibians. These studies reported eDNA concentrations falling below detection limits in 4.2−54 days.[5,14,17,21−23,25,26] Thus far, eDNA reportedly decays faster in marine waters than freshwater;[27,28] however, very few eDNA decay studies have been carried out in marine waters. Studies conducted with marine organisms in experimental tanks report eDNA decaying below detection limits in 0.9 days for European Flounder (*Platichthys flesus*)[27] and 6.7 days for Three-spined Stickleback (*Gasterosteus aculeatus*)[27] and a decay rate constant of 0.104 ± 0.047 per hour for bluegill sunfish (*Lepomis macrochirus*).[16]

The implementation of eDNA in biological monitoring and management practices will likely require eDNA abundance to be representative of organismal abundance. Several studies have been published revealing a positive relationship between biomass or species abundance and eDNA concentration or sequence abundance.[5,13,15,19,24,29−31] However, there are limited studies in the marine environment and none that present an empirical equation for estimating fish abundance from eDNA concentrations that incorporate the fate and transport of eDNA in the marine environment.

The goal of the present study is to determine eDNA shedding and decay rates for three ecologically and economically important marine fish in the temperate waters of the eastern Pacific Ocean.[32−34] We developed a novel set of sensitive and specific Taqman qPCR assays for Northern Anchovy (*Engraulis mordax*), Pacific Sardine (*Sardinops sagax*), and Pacific Chub Mackerel (*Scomber japonicas*). We then conducted seawater mesocosm experiments to quantify fish eDNA shedding and decay rates. We also investigated eDNA size fractionation to gain insight into the source of eDNA (e.g., intracellular or extracellular). Finally, we utilized the eDNA shedding rates and decay rate constants for Northern Anchovy in a simplified mass balance model that estimates fish abundance from eDNA concentrations. The results of this research are critical to inform the use of eDNA as a biological monitoring tool for fisheries management and marine conservation efforts.

MATERIALS AND METHODS

Genus-Specific Assay Design and qPCR Optimization. Genus-specific primers and Taqman probes were designed to amplify Northern Anchovy (*E. mordax*), Pacific Sardine (*S. sagax*), and Pacific Chub Mackerel (*S. japonicas*). Primers and probes were designed using PrimerBlast[35] (see SI). The specificity of potential primers/probes sequences was assessed in silico using PrimerBlast and using an alignment of closely related sequences in NCBI. Primer/probe sequences showing specificity in silico were obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) and tested for cross-reactivity using genomic DNA (1−4 ng) extracted from the tissue samples of organisms found in the same marine habitats as anchovies, sardines, and mackerel. Genomic DNA was extracted using the Qiagen DNeasy Blood and Tissue extraction kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Genomic DNA from the following fish and one cephalopod was tested against each primer set: sardines (*Sardinops*), anchovies (*Engraulis*), mackerel (*Scomber*), herring (*Clupea*), rockfish (*Sebastes*), tuna (*Thunnus*), and squid (*Doryteuthis*). Primer/probe sets were considered specific if no amplification (within 40 cycles) was observed for any of the nontarget genomic DNA. The final primer and probe sequences and concentrations, DNA targets, and primer annealing temperatures are shown in Table 1.

qPCR standards were constructed using genomic DNA (gDNA) extracted from target fish tissue samples and quantified using a QUBIT fluorometer 2.0 (Life Technologies, Grand Island, NY). Standard curves consisted of 1:10 dilutions of gDNA from 1 ng/μL to 0.01 pg/μL and were run in triplicate alongside samples in each 96-well plate. Standard curves were pooled across plates to calculate concentrations of unknown samples.[36] All unknowns were amplified in triplicate 20 μL qPCR reactions, and each qPCR plate contained triplicate no template controls (NTCs). Reaction chemistry consisted of 1X Taqman Universal Mastermix II, 0.2 mg/mL bovine serum album (BSA), a Taqman probe, and forward and reverse primers in optimized concentrations. Cycle quantification (Ct) thresholds were set at 0.02 for anchovy, 0.01 for sardine, and 0.01 for mackerel.

Experimental Design. Experiments were conducted at the Tuna Research Conservation Center (TRCC) at Hopkins Marine Laboratory of Stanford University, in Pacific Grove, CA. We performed four seawater mesocosm experiments where we examined the shedding and decay rates of (1) anchovies only, (2) sardines only, (3) mackerel only, and (4) mackerel plus sardines. The mesocosm consisted of an indoor, circular fiberglass tank with a capacity of ∼5200 L and a water depth of 1 m. Seawater from Monterey Bay, CA, was used for all experiments. For experiments examining shedding and decay of anchovy and mackerel eDNA, the tank was filled with seawater circulating through the TRCC (see description in SI). For the sardine-only and the mackerel plus sardine experiment, the tank was filled with seawater from Monterey Bay rather than TRCC seawater. TRCC seawater was not used for the experiments

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Table 1. Genus-Specific Primers and Probes Designed for This Study: Primer and Probe Sequences, Gene Target, Fragment Size, Optimized Primer, and Probe Concentration and Annealing

target organism	forward primer reverse primer probe	gene target	fragment size	final primers/probe concentrations	annealing temperature	slope	intercept	limit of quantification based on standard curve (pg/μL)	assay efficiency
Engraulis (anchovy)	109F- 5′TTCACTTGGCATTTGACGGG 3′	control region d-loop	133	0.2 μm/0.15 μm	60 °C	−3.5	27.9	0.01	92%
	241R- 5′TGCTCCTGAGATCACTTATGC 3′								
	153P-5′-FAM- AGGTTGAACATTTTCCTTGCTTGCGA-BHQ								
Sardinops (sardine)	89F-5′ TCAGAGCAAGGAAACTG 3′	control region d-loop	107	0.8 μm/0.1 μm	60 °C	−3.6	30.5	0.1	91%
	195R-5′ CCGACGGTATACATAAAGC 3′								
	133P-5′-FAM- ATTTACCGGGTGCCCTTCATT -BHQ								
Scomber (mackerel)	324F-5′ GCTGAACAGTTTATCCTCCCCTCG 3′	COI	107	0.6 μm/0.1 μm	60 °C	−3.4	34.4	0.1	95%
	430R-5′ CCCAAGGATTGAGGAAACACCTGCTAG 3′								
	349P-5′-FAM- TGGGAACCTGGCACACGCCGGG-BHQ								

with sardines to minimize the potential background sardine eDNA signal that may result due to the fact the TRCC tuna are fed sardines. Once the experimental tank was filled, seawater was circulated in a closed loop system to aerate the water throughout the experiments. For the mackerel and sardine experiments, additional aeration was supplied by bubbling oxygen into the tank due to the high biomass content in the tank. Tank water temperatures ranged from 22.0 ± 1 °C during the anchovy experiment (due to a malfunction of the water chilling device) to 18.7 ± 1 °C for the sardine-only, mackerel-only, and mackerel plus sardine experiments. The temperatures were within a range suitable for the fish and are similar to temperatures within Monterey Bay, CA (10−15 °C).[39]

Fish were added to the tank and fasted during the experiments (∼3 days) to control for potential eDNA input resulting from feeding. The experiment with only anchovies contained 43 anchovies with an average weight of 20 ± 2 g per fish (determined by displacement). The experiment with only sardines contained 27 sardines with an average weight of 88 ± 11 g per fish. The experiment with only mackerel contained 20 mackerel with an average weight of 424 ± 4 g per fish. The experiment with sardines and mackerel contained 30 sardines and 15 mackerel with average weights of 110 ± 29 and 449 ± 90 g per fish, respectively. The number of fish used in the experiments was based on the number of fish available and the capacity of the tank.

Sampling Procedure. All water samples were collected in two 500 mL acid-washed (10% HCl) polypropylene bottles at each time point as biological replicates. Two samples were collected from the tank before fish were added ($t = 0$). The fish were then added to the tank. Water was sampled frequently while the fish were in the tank (3−8 times per day depending on the experiment and results from previous experiments). On the third day of the experiment, the fish were removed from the tanks and the water was sampled 2−3 times per day for 3−4 more days (Table S1). Each day, a filtration blank was created with 50 mL of molecular grade water (Sigma-Aldrich, St. Louis, MO) to test for contamination during sample filtration. New gloves were worn during each sample collection. Samples were placed on ice and immediately processed at the laboratory. A 250−500 mL amount was filtered in duplicate through 0.2 μm pore size 47 mm diameter track-etched polycarbonate filters (Nucleopore Track-Etch Membrane, Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA) in disposable filter funnels (ThermoScientific, Waltham, MA). Filters were stored at −20 °C until DNA extraction (approximately 1−4 weeks after the experiment).

Size Fractionation of Environmental DNA. The particle size distributions of mackerel and sardine eDNA were determined during the mackerel plus sardine experiment. Forty-one hours after sardine and mackerel were introduced into the tank, triplicate 20 mL and 1 L seawater samples were collected directly from the tank. Each 1 L sample was sequentially filtered through a 10 μm, followed by a 1 μm, followed by a 0.2 μm pore size, 47 mm diameter polycarbonate filter (Nucleopore Track-Etch Membrane, Whatman, GE Healthcare Bio-Sciences, Pittsburgh, PA). The full 1 L was sequentially filtered through the 10 and 1 μm filters. Due to filter clogging, 250 mL of the 1 μm pore size filtrate was filtered through the 0.2 μm filter. All filters were stored at −20 °C until extracted. The triplicate 20 mL of unfiltered tank seawater sample was prepared for DNA precipitation. The filtrate from the 0.2 μm pore size filter was also prepared for DNA

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precipitation. Briefly, 20 mL samples were combined with 20 mL of molecular grade isopropanol (Fisher Scientific, Fair Lawn, NJ) and 1.5 mL of sodium acetate (3M, pH 5.2, CALBIOCHEM, EMD Biosciences, La Jolla, CA) and saved at −20 °C until extraction.[40] A DNA precipitation blank made with 20 mL of molecular grade water was also prepared and processed alongside the samples. Filter and precipitate DNA extraction methods are described below.

DNA Precipitation and Extraction. DNA was precipitated from unfiltered seawater from the tank, filtrate from the 0.2 μm pore size filter, and the DNA precipitation blank. The samples were centrifuged at 6000*g* for 45 min at room temperature.[39] The supernatant was carefully removed from the pellet. DNA was extracted from pellets and filters using the Qiagen DNeasy Blood and Tissue extraction kit (Qiagen, Valencia, CA) with modifications described in SI. A DNA extraction blank was extracted with each set of samples to test for contamination in the reagents. DNA was eluted in 100 μL of warmed Buffer AE (10 mM Tris-Cl and 0.5 mM EDTA, Qiagen, Valencia, CA) for the filters and 50 μL of warmed Buffer AE for the DNA precipitations.

Inhibition. Samples from each experiment were investigated for inhibition using serial dilutions.[37,38] Briefly, samples were diluted in two series, 5-fold (1:5, 1:25) and 10-fold (1:10, 1:100), to find the minimal dilution needed to dilute out inhibitors but not lower the target concentration below the detection limit. A 5-fold dilution is expected to result in a C_t change of 2.32 cycles ($\log_2(5) = 2.32$), and a 10-fold dilution is expected to result in a C_t change of 3.32 cycles ($\log_2(10) = 3.32$), assuming 100% efficiency. We considered the sample inhibited if the difference between the sample and the dilution was 0.5 cycles less than the expected change. We also added bovine serum album (BSA) to each QPCR to help with inhibition.

Data Analysis. *eDNA Shedding.* The tank with fish was modeled as a completely mixed batch reactor for each experiment

$$V\frac{dC}{dt} = S - kCV \tag{1}$$

where V is the volume of the tank with units of liters, C is the concentration of eDNA with units of pg/mL, t is the time since the start of the experiment with units of hours, S is the eDNA shedding rate with units of pg/h, and k is the first-order decay rate constant with units of per hour (see decay rate constant calculation in eDNA decay section below). Equation 1 assumes that the tank is well mixed and that decay is first order. Given that the fish were constantly swimming in the tank, we believe this is a reasonable assumption. Steady state was reached after 17−25 h depending on the experiment (Table S1) and lasted until the fish were removed from the system. At steady state, $dC/dt = 0$ and $S = kCV$. The error associated with the shedding rate was determined by propagating errors associated with k, C, and V. A z-test was used to compare shedding rates between fish species and experiments, and a significant difference was determined by $p < 0.05$.

eDNA Decay. After the fish were removed from the tanks, $S = 0$ and $dC/dt = -kC$. The first-order decay rate constant, k, and its standard error were calculated for each experiment using data collected after the fish were removed from the tank by fitting a straight line to $\ln(C/C_o)$ versus time using linear regression in Matlab (Mathworks, Natick, MA). For C_o, we used the average eDNA concentration across samples collected while the concentrations in the tank were at steady state. Decay rate constants for different fish were compared using an analysis of covariance (ANOCOVA) that tested the null hypothesis that k values were not statistically different. A statistical difference was determined by $p < 0.05$.

Mass Balance Model. The shedding rate and decay rate constant calculated based on the controlled experiment with anchovies were used to model anchovy eDNA concentrations in the ocean. A simplified, Lagrangian mass-balance model (eq 2) was developed to model anchovy eDNA concentrations as a function of time

$$C(t) = C(t - \Delta t) + C_{ocean}\alpha\Delta t - C(t - \Delta t)\alpha\Delta t - kC(t - \Delta t)\Delta t \tag{2}$$

Here $C(t)$ is the concentration of eDNA in a water parcel at time t, C_{ocean} is the concentration of eDNA outside of the water parcel, α is the exchange rate of the water within the parcel with water outside the parcel (1/time), k is the decay rate constant of eDNA (1/time), and Δt is the time step in the model (here equal to 1 h). Here water parcel refers to an arbitrary volume of water in the ocean. The model is initialized by assuming F fish swim in the parcel of water for time T (here assumed to be 1 h), shedding eDNA at a rate S_{Fish} (units of mass eDNA per fish per time), and then leave the water parcel. Assuming that $C_{ocean} = 0$ then C can be related to F present at $t = 0$, given t (the time since the eDNA was shed, or the age of the eDNA) ($t = n\Delta t$ where $\Delta t = 1$ hr here) is known (eq 3) (see SI for details)

$$F = CV(1 - \alpha - k)^{-n}/(S_{Fish}T) \tag{3}$$

Here C is the concentration of eDNA measured and V is the volume of the water parcel (here assumed to be 1000 m^3).

RESULTS AND DISCUSSION

Genus-Specific qPCR Assays. The primers and probes designed for this study are genus specific and showed no cross reactivity with nontarget genomic DNA from marine organisms found in the same habitat as anchovies, sardines, and mackerel. The assays are sensitive with limits of quantification, 0.01 pg/μL for anchovy (35 cycles), 0.1 pg/μL (36 cycles) for sardine, and 0.1 pg/μL (38 cycles) for mackerel. The assay efficiencies based on pooled standard curves were 92% for the anchovy-specific assay, 91% for the sardine-specific assay, and 95% for the mackerel-specific assay.

Quality Assurance/Quality Control. All filtration and extraction blanks showed no evidence of contamination. All qPCR no template controls (NTCs) showed no amplification.

eDNA Shedding. After transferring the fish into the experimental tank, the concentration of eDNA increased over the first ∼12−24 h (Figure 2) until it reached a steady state concentration that did not change with time. This increase over the first day might be caused by elevated eDNA shedding rates due to the stress of being handled as observed in a previous study investigating the relationship between common carp biomass and eDNA in freshwater mesocosms.[13] We observed that anchovies and sardines shed scales which sunk to the bottom of the tank. In contrast, the mackerel did not visibly shed scales but produced a visible mucus-like substance that appeared as a film on the water surface. Research is needed to determine the concentration of eDNA in different sources (e.g., tissue, scales, mucus, feces) as well as the effect of different fish physiologies, metabolic rates, and feeding on eDNA shedding.

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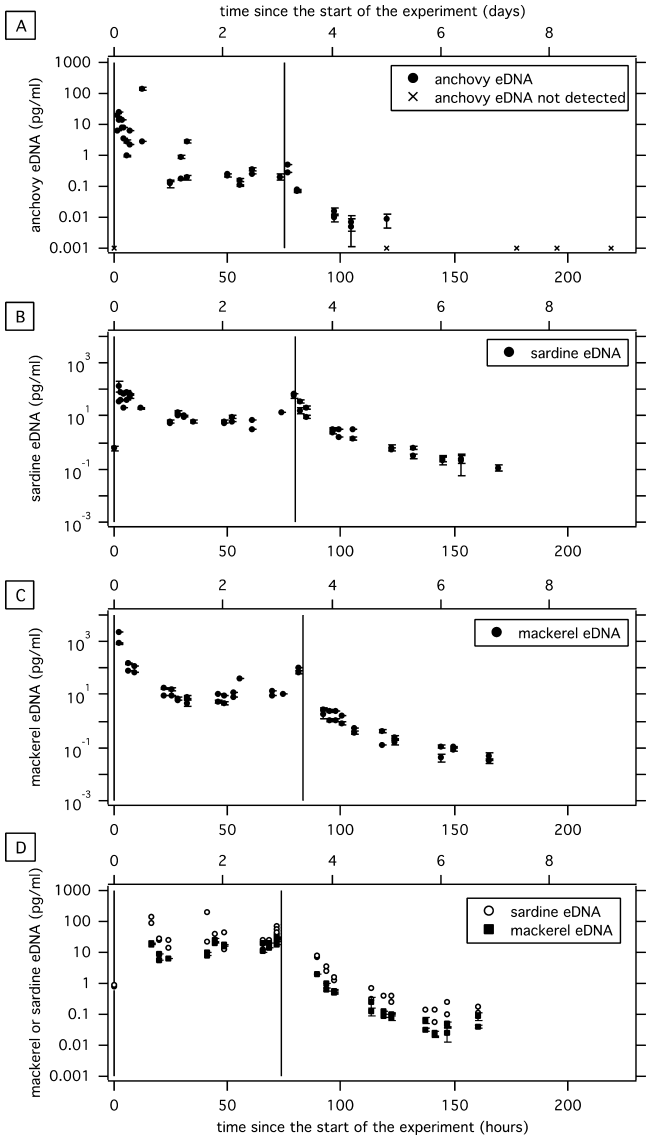

Figure 2. eDNA concentration in seawater mesocosm experiments: anchovies (A), sardines (B), mackerel (C), and mackerel plus sardine (D). *y* axis is the concentration of DNA determined with genomic DNA standards. Bottom *x* axis is time since the start of the experiment in hours, and top *x* axis is equivalent in units of days. Biological duplicates for each time point are represented as separate symbols. Error bars represent the standard deviation of triplicate qPCR measurements. Vertical lines represent when the fish were moved into the tank (first vertical line) and out of the tank (second vertical line).

After 17−25 h, depending on the experiment, the eDNA concentration remained relatively constant for approximately 2 days (between approximately 20 and 70 h after the fish were added to the tank) at which point the fish were removed from the tank (Figure 2). At steady state, shedding balanced decay, and thus data from this period was used to calculate the DNA shedding rates using eq 1. To account for differences in fish biomass and fish numbers, the shedding rates are presented as picogram (pg) of DNA per hour, pg of DNA per hour per fish, and pg of DNA per hour per gram of fish (Table 2). The shedding rates were on the order of 10^2 and 10^3 pg/h/g: anchovy (165 pg/h/g), sardine (1275 pg/h/g only sardine, 3368 pg/h/g sardine with mackerel), and mackerel (500 pg/h/g only mackerel, 737 pg/h/g mackerel with sardines) (Table 2). There was no statistical difference between anchovy, sardine, and mackerel shedding rates on a per gram basis in the experiments conducted with only one species. The anchovy shedding rate on a per gram basis, however, was statistically different from the sardine and mackerel shedding rates on a per gram basis in the mackerel plus sardine experiment (p = 0.05 and 0.04 for anchovy and sardine and anchovy and mackerel respectively). There was not a statistically significant difference in the shedding rates on a per gram basis of mackerel and sardines observed when experiments were conducted with just one species of fish versus when experiments were conducted with two species of fish (p = 0.20 and 0.85 for sardine and mackerel, respectively).

The eDNA shedding rates are among the first reported eDNA shedding rates for marine fish and support previous research suggesting that the amount of eDNA a fish sheds is related to its mass and varies among species.[5,15,16,19,24,29,30,41] Our results demonstrate the importance of reporting eDNA shedding on a per mass (grams) basis to account for differences in fish size. Our results also suggest that eDNA shedding rates on a per gram basis for mackerel and sardines are not affected by the presence of the other fish species. The shedding rate (pg/h/g) of 15 mackerel in the mackerel plus sardine experiment is similar to the shedding rate (pg/h/g) of the 20 mackerel in the mackerel-only experiment. Similarly, the shedding rate on a per gram basis of 30 sardines in the mackerel plus sardine experiment is similar to the shedding rate on a per gram basis of the 27 sardines in the sardine-only experiment. Additional research on whether the number of fish in a school influences the concentration of eDNA shed into the environment would be useful.

eDNA Decay. Decay of eDNA from anchovies, sardines, and mackerel was well modeled as a first-order process as evidenced by exponential decline in eDNA concentration with

Table 2. Shedding Rates and Decay Rate Constants for Anchovies, Sardines, and Mackerel[a]

	shedding rates			
	pg/h ± propagated standard deviation	pg/h/fish	pg/h/g	decay rate constant per hour ± standard error
E. mordax (Northern Anchovy)	$1.39 \times 10^5 \pm 1.08 \times 10^5$	3.24×10^3	165	0.101 ± 0.011
S. sagax (Pacific Sardine)	$3.04 \times 10^6 \pm 2.24 \times 10^6$	1.13×10^5	1275	0.068 ± 0.004
S. japonicas (Pacific Chub Mackerel)	$4.24 \times 10^6 \pm 3.13 \times 10^6$	2.12×10^5	500	0.070 ± 0.004
S. sagax (Pacific Sardine) in the mackerel plus sardine experiment	$1.11 \times 10^7 \pm 4.96 \times 10^6$	3.69×10^5	3368	0.057 ± 0.005
S. japonicas (Pacific Chub Mackerel) in the mackerel plus sardine experiment	$4.96 \times 10^6 \pm 2.29 \times 10^6$	3.31×10^5	737	0.055 ± 0.005

[a]The errors for the decay rate constants represent the standard error. The shedding rate (in pg/h) has a propagated standard deviation based on the error associated with the average eDNA concentration at steady state, the tank volume, and the decay rate constant.

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time and the good linear fit between $\ln(C/C_o)$ and time (R-squared values ranged from 0.76 to 0.88). First-order decay has been used to model eDNA decay in other studies[42,43] but is likely a simplification for modeling complex eDNA destruction mechanisms.

First-order rate constants were similar among fish species (k on the order of 10^{-1} per hour) (Figure 2). Decay rate constants (±standard error) measured in the anchovy-only, sardine-only, and mackerel-only experiments were 0.101 ± 0.011, 0.068 ± 0.004, and 0.070 ± 0.004 per hour, respectively. Decay rate constants measured during the mackerel plus sardine experiment were 0.055 ± 0.005 per hour for mackerel and 0.057 ± 0.005 per hour for sardine (Table 2). Anchovy eDNA k was statistically larger than sardine eDNA k in the sardine-only experiment and sardine and mackerel eDNA k values from the mackerel plus sardine experiment (ANOCOVA, $p < 0.05$). The anchovy eDNA k was not different than the mackerel eDNA k in the mackerel-only experiment (ANOCOVA, $p = 0.059$). The mackerel and sardine decay rate constants in the mackerel-only and sardine-only experiments were not different than those obtained from the mackerel plus sardine experiment (ANOCOVA, $p < 0.05$).

The similarity in k values across fish species suggests consistent eDNA decay mechanisms despite potentially different eDNA sources (e.g., fecal waste, scales, mucus). Decay rate constants also did not significantly change when two fish species were in the same tank, suggesting that decay rates are not strongly influenced by the presence of other fish or their eDNA in water.

The first-order eDNA decay rate constants here are among the first reported for marine fish and are consistent with two other studies done with marine fish. Maruyama et al.[16] reported a decay rate constant of 0.104 ± 0.047 per hour for bluegill sunfish. Thomsen et al.[27] reported that initial concentrations of 48 and 214 molecules eDNA per 400 mL of decayed below detection in 0.9−6.7 days for European Flounder and Three-spined Stickleback, respectively.[27] The present study in addition to the two previous studies completed in marine waters support the conclusion that eDNA decay is faster in marine versus fresh waters. eDNA k from a freshwater common carp was reported to be between 0.35 and 2.42 per day (0.015 and 0.1 per hour).[23] Other studies report eDNA from freshwater organisms decaying below detection limits in 0.9 days (*Platichthys flesus*[27]), 4.2 days (common carp[22]), 6.7 days (*Gasterosteus aculeatus*[27]), 8−18 days (salamander[17]), 7−14 days (amphibian larvae[5]), 14 days (sturgeon[44]), 25 days (tadpoles[44]), 21−44 days (mudsnails[25]), <1−54 days (bullfrog[21]), and >28 days (silver carp[26]). The eDNA signal in our experiments decayed to below or close to the assay detection limit within 3−4 days, which is consistent with the studies listed above when comparing time until eDNA concentration is below the detection limit. Reporting the number of days until the eDNA signal is below the detection limit is problematic because the length of time the eDNA signal persists is dependent on the starting concentration[26] and the detection limit of the assay. Future studies should report decay rate constants rather than days of detection. First-order rate constants should be comparable across studies and can be directly used in eDNA modeling.

Size Fractionation. eDNA was detected on all filters, in 0.2 μm pore size filter filtrate, and in the unfiltered tank water for the size fraction analysis (Table S2). The average eDNA concentrations and standard deviations (from biological replicates) measured in the unfiltered tank seawater samples extracted by DNA precipitation were 153 ± 41 pg/mL for sardines and 138 ± 66 pg/mL for mackerel. For both the sardines and the mackerel, the total mass of eDNA in the unfiltered tank seawater samples was larger than the sum of the eDNA masses captured on the filters and in the 0.2 μm pore size filtrate, suggesting methodological inefficiencies in sequential filtering and DNA extractions. The total sardine and mackerel eDNA captured by the sequential filtering was 55% and 26%, respectively, of the sardine and mackerel eDNA measured in the unfiltered tank seawater samples. Of the eDNA captured on the filters, the largest proportion of both sardine and mackerel eDNA was captured on the 10 μm pore size filter followed by the 1 μm pore size filters (Table S2). The smallest proportion of both sardine and mackerel eDNA was captured on the 0.2 μm pore size filter. For sardine eDNA, the mass captured on the 10 μm filter was significantly larger than that captured on the 1 μm pore size filter, 0.2 μm pore size filter, and 0.2 μm filtrate (ANOVA with posthoc Tukey−Kramer test, $p < 0.05$). For mackerel eDNA, the mass captured on the 10 μm filter was significantly larger than that captured on the 0.2 μm pore size filter, and the 1 μm filter was significantly different than that captured on the 0.2 μm pore size filter (ANOVA with posthoc Tukey−Kramer test, $p < 0.5$).

The results from the size fractionation experiments demonstrate the importance of carefully considering eDNA capture methods in experiment designs. Currently, studies show that there is no ideal sample capture method (filter vs precipitate) or volume combination that captures all eDNA in a sample.[45,46] We found that more sardine and mackerel eDNA was quantified in the unfiltered tank seawater samples (20 mL precipitated) than on the 10 μm filter (1 L filtered). The precipitated unfiltered tank seawater sample presumably captured both intracellular and extracellular DNA, while the 10 μm pore size filter captured only intact tissue and cells (assuming the extracellular DNA attached to particles or attracted to the filter is negligible). This finding illustrates that intracellular and extracellular DNA are both important contributions to organism eDNA concentrations measured in seawater. The relative importance of intra- and extracellular eDNA for the detection of rare organisms (e.g., invasive or endangered species) or a community of organisms in aquatic environments is not known. A previous study with common carp found that carp eDNA was most abundant in the 1−10 μm size fraction.[20] Both the study with common carp and the present study suggest that fish eDNA is mostly associated with cells or particles larger than 1 μm when fish are present in the water. However, it is possible that the size fraction of eDNA may change over time as the tissue, cells, and particles break down. More mesocosm experiments are needed to determine how the size fraction capturing eDNA changes as eDNA ages. More information about the age and size fraction of eDNA could inform the best possible sample volume and capture method given a specific research question or biological monitoring application.

Inhibition. As a result of inhibition testing, samples from the anchovy experiment were run at a 1:5 dilution, and sardine samples were run at a 1:10 dilution. Previous research has underscored the importance of considering PCR inhibition in environmental samples.[24,47] Future eDNA studies in coastal waters should test for inhibition and consider strategies to address inhibition such as dilution and PCR additives such as bovine serum albumin (BSA).

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Environ. Sci. Technol. 2016, 50, 10456−10464

Estimation of Fish Abundance in Marine Water. We used eq 3 to illustrate complexities of interpreting fish eDNA concentrations in the ocean using anchovy as an example. Assuming we measure 0.02 fg of anchovy DNA per mL (the limit of quantification for the anchovy assay assuming 1 L of seawater was filtered) in a marine sample, the equation can be used to deduce the abundance of fish initially present in the water parcel. That number depends on the age of the eDNA measured in the water (t), the first-order decay rate constant of eDNA (k), as well as mixing rate (α) provided other parameters in eq 3 are fixed (Figure 3). For $\alpha < k$, mixing

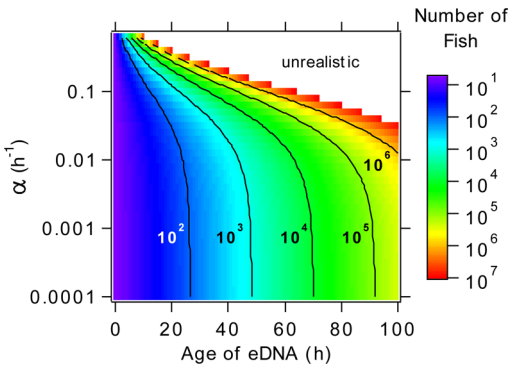

Figure 3. Anchovy abundance in water volume at $t = 0$ as a function of anchovy eDNA age and mixing parameter α for a water sample containing 0.02 fg of anchovy DNA/mL of seawater. White portion of the graph gives unrealistically high fish counts meaning that it is unlikely to find 0.02 fg/mL after a specific time given a high degree of mixing. Black contours show lines of constant fish numbers.

does not affect the estimation of fish abundance. However, as α approaches k, mixing becomes important. In the limit that α approaches 1 h^{-1} then the water parcel is completely flushed within an hour and thus no eDNA can be present in the water parcel after the initial time step. For small α ($\alpha < k$), as the age (t) of the eDNA increases the number of anchovies present at $t = 0$ required to produce the measured 0.02 fg/mL increases from ~6 at $t = 0$ to over 10^5 at $t = 100$ h.

Previous studies have estimated fish biomass, relative abundance, or rank abundance using linear or exponential regressions; however, this model is the first attempt to move beyond correlative and predictive studies to provide an analytical equation that relates eDNA concentrations to fish number.[5,13,15,17,19,31] While there are many assumptions utilized within the model, these assumptions are reminders that many environmental parameters (mixing/dispersivity, fish residence time in a water parcel, etc.) and biotic parameters (shedding rates and decay rate constants) need to be measured to relate eDNA concentrations to actual fish biomass and abundance. Future modeling efforts should try to better understand and incorporate the uncertainty associated with measured eDNA concentrations.

Implications for eDNA as a Biological Monitoring Tool. The development of anchovy-, sardine-, and mackerel-specific qPCR assays, the fish shedding rates, decay rate constants, and the simple mass-balance model are important tools and parameters for using eDNA to monitor these marine fish. To be a useful biological monitoring tool, eDNA should indicate the presence of an organism, and it should not persist long in the environment after the organism is gone. The finding that eDNA shed from a specific organism can be detected and persists on short time scales (e.g., days) suggests that it could be a promising monitoring tool for abundance estimates and biodiversity. However, more information about eDNA persistence and the factors affecting persistence are needed to improve the usefulness of eDNA as a monitoring tool. In the future, organism-specific assays could be adapted to in situ autonomous monitoring devices, advancing the feasibility of spatially and temporally intensive biological monitoring with eDNA.[48] Monitoring ecologically and economically important species using eDNA will ultimately enhance efforts to measure and protect marine biodiversity on local and global scales.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org/. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03114.

Details of methods and results (PDF)

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Notes

The authors declare no competing financial interest.

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