Doctoral Thesis

Modeling and simulation of environmental DNA dynamics

by

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Summary

In the natural environment, there exist complex relationships between organisms and the environment as well as among organisms. To conserve biodiversity and ecosystems with the development of human society, we must understand organism distributions and biomass/abundance to simulate and predict their dynamics.

Environmental DNA (eDNA) methods have been developed to detect the distribution, abundance, and biomass of organisms in various environments. eDNA methods can be used to detect target species as well as to estimate their biomass and abundance. However, various factors such as sampling season, temperature, pH, salinity, and UV can influence biomass and abundance estimations from eDNA. eDNA generally degrades quickly in the environment; thus, understanding eDNA degradation is critical for eDNA evaluation. Several aspects of the general behavior of eDNA in water remain unclear, especially the state and degradation of eDNA. Many experiments have been conducted on the different states and degradation rates of eDNA under various conditions. In most cases, the eDNA degradation curves declined exponentially, and the degradation rates were fast, but occurred in less than a week. However, other studies have found that eDNA concentrations can decay below the detection limit in less than a week. Additionally, water conditions such as salinity, temperature, and pH can influence the eDNA degradation rate.

Simulation techniques have been used in ecology, particularly for estimating ecological population and community dynamics. The abovementioned issues related to eDNA surveys can improved through modelling and simulation. For example, simulating the spread of eDNA in water may provide a more detailed estimate of the habitat of

released species. Additionally, by predicting the amount of eDNA degradation, we can estimate, for example, how much eDNA will be degraded by the time the water sample reaches the laboratory.

Understanding eDNA states and degradation is essential for the effective sampling and storage of eDNA, and may provide pertinent information to better interpret the results of species distribution, abundance, and biomass estimations. In some cases, eDNA has not been detected, despite confirmation of the habitat of organisms. It has been indicated that false negatives may involve eDNA degradation in the environment and eDNA sample processing, such as water sample transport.

In this study, the characteristics and dynamics of eDNA released by organisms into the environment were verified through experiments and simulations. The aim of this doctoral thesis was to refine the relationship between the characteristics and degradation of eDNA from macroorganisms and to obtain clues to mitigate and eliminate uncertainties related to eDNA detection and quantification. In this thesis, I studied the degradation of eDNA from multiple perspectives, using a meta-analytical approach, which is described in Chapters 2. I also used an experimental approach, which is described in Chapters 3 and 4.

I investigated eDNA degradation using a meta-analytic approach based on previous data and performed eDNA degradation prediction simulations. Our meta-analysis results showed that eDNA degradation was accelerated at higher water temperatures and longer amplicon lengths. Previous studies have assumed that water temperature does not directly affect eDNA degradation, rather indirectly affecting it through enzymatic hydrolysis by microbes and extracellular nucleases. At high temperatures, with increasing activity of microorganisms and extracellular enzymes, eDNA in water degraded more quickly.

I found that the eDNA derived from cells and fragmented DNA declined exponentially after being added to both seawater and pond water samples. The eDNA from resident species showed similar behavior to the eDNA derived from cells. In most cases, a simple exponential model can be used to evaluate the degradation. Increased salinity in the saline sample had no effect on the DNA detection. The degradation rates of the saline pond and pond samples were significantly different. Furthermore, the degradation rates of the diluted pond and pond samples were significantly different. For all sources, the reduction in degradation rate preserved more DNA than the disadvantage caused by reducing the initial concentration to 1/10.

In this study, I examined, validated, and discussed eDNA analysis from multiple perspectives using simulation and modelling through meta-analysis and degradation experiments. Although studies relating to eDNA characteristics and dynamics increase yearly, their number is small compared to the number of overall eDNA studies. However, its practical applicability is still in its infancy, both because of the uncertainty of detection sensitivity and quantification accuracy of eDNA in the field, as well as the lack of information on populations other than presence/absence and/or abundance. Elucidating these features will improve the usefulness, practicality, and reliability of eDNA analysis as a tool for biodiversity and ecosystem monitoring and stock assessment in the future. More, and a deeper understanding of, basic information about eDNA will improve eDNA analysis methods and enable researchers to maximize the potential of future eDNA methods. The outcomes of this study, especially our simulation and models, will add to the basic information on eDNA and contribute to the elucidation of the characteristics and degradation mechanism of eDNA. The findings presented in this thesis provide important groundwork for innovating eDNA analysis for biodiversity monitoring, ecological assessment, and resource management in the future.

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Chapter 3. Degradation Modeling of Water Environmental DNA: Experiments on multiple DNA Sources in Pond and Seawater

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Chapter 1. General Introduction

Ecology is the study of understanding the interactions between organisms and their environments, or between organisms and each other. Organisms are influenced by their environments (Begon & Townsend, 2020), including factors such as climate and topographical changes. However, organisms also affect each other, for example through competition within a species or competition with other species. Human influences, such as global warming, deforestation, land development, overhunting of species, and the release of alien species, also impact organisms. These complex interactions among organisms, the environment, and humans comprise the ecosystem. These are complex structures, and their relationships are difficult to understand. Therefore, I focus on a certain environment and organism, and conduct biological monitoring, such as confirming the presence or absence of inhabitants and the number of individuals to accumulate information. By comparing the data from various organisms, we can discover their common laws and mechanisms, and conversely, their unique laws and mechanisms. Furthermore, we may be able to construct predictive simulations using accumulated data. Recently, molecular ecology studies have been conducted at the intersections of population demography, individual behavior, geography, landscape, history, and molecular data, as well as genetic processes such as inbreeding and adaptation (Begon and Townsend 2020, Rewe et al. 2017).

Simulation studies also contribute to ecology by elucidating species-specific natural history, such as dispersal and mating patterns (Brekke et al. 2011; Puebla et al. 2012), or landscape barriers. Furthermore, the potential future outcomes of management actions (e.g., translocations) and environmental change simulations can be evaluated probabilistically using computer simulations (Bruford et al. 2010). Simulation studies have also recently advanced the

theoretical understanding of range expansion (Travis et al. 2007) and retention of adaptive diversity after bottlenecks or fragmentation (Ejsmond & Radwan 2011). Lastly, simulators help evaluate population genetics tools and methods (e.g., estimators of effective population size) by quantifying their performance in real-world conditions (Antao et al. 2011; Paz-Vinas et al. 2013), and can inform development of optimal sampling strategies (Gapare et al. 2008; Whiteley et al. 2012; Hoban et al. 2012). The population demographic and genetic simulators developed over the past several decades are unique tools for simultaneously modelling these forces across temporal and spatial scales (Caughley 1994; Epperson et al. 2010; Balkenhol & Landguth 2011), as they allow flexible parameterization of relevant processes (e.g., population sizes, migration, recombination, and selection). Parameter-rich and customizable population demographic-genetic simulation software, featuring long-awaited realistic modelling of these relevant processes, are now facilitating a variety of simulation-based investigations at several study stages. They help to reveal ecological patterns and processes, such as estimating the timing, degree, and cause (e.g., exploitation, climate change) of population declines and pre-decline population sizes (Alter et al. 2012), the timing of geneflow cessation (Marino et al. 2013) and factors underlying the extent of observed admixture (Perrier et al. 2012). Moreover, simulations can help fully utilize large-scale genetic, geographical, pedigree, historical, and ecological datasets, including ancient DNA (Campos et al. 2010), and can help provide the front-line advice and information that is increasingly sought by conservationists and natural resource policy makers (Cook et al. 2013).

The eDNA analysis is an innovative method for monitoring distribution, abundance, and diversity of organisms in the environment (Ficetola et al., 2008; Minamoto et al., 2012; Taberlet et al., 2012; Takahara et al., 2012; Ushio et al., 2018; Kakuda et al., 2019; Tsuji et al., 2019). eDNA, which is comprised of DNA fragments released by organisms into environments

such as water or soil, is thought to be derived from mixtures of organism feces (Martellini et al., 2005), skin cells (Ficetola et al., 2008), mucus (Merkes et al., 2014), and secretions (Bylemans et al., 2018). Previous studies have suggested that eDNA is mainly derived from fractions of cells or cellular organs (i.e., mitochondria and nuclei), but it can also be derived from fragmented DNA (degraded DNA) in the water (Turner et al., 2014; Minamoto et al., 2016).

Since the first eDNA surveys were used to detect invasive American bullfrogs in French wetlands (Ficetola et al., 2008), eDNA methods have become common in studies seeking to detect an array of aquatic taxa, including fish (Jerde et al., 2013; Doi, Inui, et al., 2017), reptiles (Piaggio et al., 2014), crustaceans (Tréguier et al., 2014), amphibians (Ficetola et al., 2008), aquatic insects (Doi, Katano, et al., 2017), and mollusks (Egan et al., 2013, 2015; Goldberg et al., 2013), in various habitats, including ponds (Takahara et al., 2013; Tréguier et al., 2014), rivers (Doi, Inui, et al., 2017; Jerde et al., 2013; Yamanaka & Minamoto, 2016), lakes (Eichmiller et al., 2014; Takahara et al., 2012), swaps (Doi, Katano, et al., 2017), and marine habitats (Boussarie et al., 2018; Lacoursière-Roussel et al., 2018; Yamamoto et al., 2017). eDNA analysis (i) has little or no damage to individuals and their habitats; (ii) substantially reduces the effort and cost in the field; (iii) enables species identification based on nucleotide sequence information without high morphological expertise, and (iv) produces fewer variable results among researchers (Darling & Mahon, 2011; Takahara et al., 2016). eDNA methods can be used to detect target species and to estimate their biomass and abundance (Doi, Inui, et al., 2017; Doi, Katano, et al., 2017; Takahara et al., 2012). However, various factors such as sampling season, temperature, pH, salinity, and UV can influence biomass and abundance estimations using eDNA (Doi, Inui, et al., 2017; Doi, Katano, et al., 2017).

The general behavior of eDNA in water (reviewed in Barnes and Turner, 2016) is still

unclear, especially the state (fragment length) and degradation of eDNA (Turner et al., 2015; reviewed in Barnes and Turner, 2016). Understanding eDNA states and degradation is essential for effectively sampling and storing eDNA, and may provide pertinent information for better interpretation of species distribution, abundance, and biomass estimation results. This may be especially problematic for rare and endangered species, which are thought to have small populations and small amounts (or concentrations) of DNA (Fukumoto et al., 2015; Sigsgaard et al., 2015; Pfleger et al., 2016; Doi et al., 2017; Sakata et al., 2017). In some cases, eDNA has not been detected even when the habitat of organisms has been confirmed. It has been suggested that false negatives may involve eDNA degradation in the environment and eDNA sample processing, such as water sample transport (Barnes and Turner 2016).

These eDNA survey issues can be improved by modelling and simulation. For example, simulating the spread of eDNA in water may provide a more detailed estimate of the habitat of the released species. Additionally, by predicting the amount of eDNA degradation, we can estimate, for example, how much eDNA will be degraded by the time the water sample has been transported to the laboratory. If the amount of such degraded eDNA is not taken into consideration, species distribution and abundance/biomass may be underestimated, especially for low-density species such as rare and endangered species. Thus, we can apply the understanding and suppression of eDNA degradation to the detection of trace amounts of eDNA. Similarly, we can apply the understanding of invasive distribution by eDNA because it is important to detect invasive species in the early stages of invasion, when their abundance may be low. Considering the rapid eDNA degradation in water, it is important to suppress any decomposition after obtaining a water sample.

The aim of this doctoral thesis is to refine the relationship between the characteristics and degradation of eDNA from macroorganisms and to obtain clues to mitigate and eliminate

the uncertainties related to eDNA detection and quantification. First, in Chapter 2, I conducted a novel meta-analysis and simulation to model and predict the effects of water conditions and DNA amplicon length on the eDNA degradation rate using data generated in previous eDNA degradation studies. Using this approach, I aimed to evaluate the effects of water conditions (i.e., ecosystem, source, temperature, and pH) and target DNA region on eDNA degradation in previously published data. I also tested the relationship between DNA amplicon length and eDNA degradation, because degradation may differ with amplicon length. Specifically, I conducted a simulation to predict the maximum degradation rate using quantile regression modeling with temperature and DNA amplicon length. Second, in Chapter 3, I observed and compared the degradation of various samples of fragmented eDNA and free cell-derived eDNA. In this experiment, I used seawater and pondwater to understand how water conditions, especially salinity, affect degradation. Based on the results, I discussed the most effective sampling and storage methods for eDNA to maximize the correct interpretation of eDNA results from field surveys. Third, in Chapter 4, I observed and compared the effects of salinity and water dilution on the eDNA degradation rate in freshwater environments. To understand the degradation of each DNA source, such as individually derived, cell-derived, and fragmented DNA, I evaluated the effects of salinity and dilution on eDNA detection while considering the fragmented eDNA, free cell-derived eDNA, and eDNA derived from the resident species of the pond.

Throughout the study, the characteristics and degradation of eDNA released from macro-organisms were investigated, new eDNA basic information was unveiled, and perspectives for the innovation of eDNA analysis were provided.

Chapter 2. A Model and Simulation of Environmental DNA Dynamics and Character : Meta-analysis Approach

2.1. Introduction

The eDNA methods are innovative methods developed for monitoring macroorganisms, especially aquatic species (Ficetola et al., 2008; Minamoto et al., 2012; Taberlet et al., 2012; Takahara et al., 2012; Ushio et al., 2018; Kakuda et al., 2019; Tsuji et al., 2019). The eDNA method is used to investigate species distribution. It is less invasive to organisms, and is especially useful for rare and endangered species, which generally have low tolerance to sampling disturbance and may be difficult to detect. Consequently, eDNA methods have been used to detect rare and endangered species in various taxa, such as fish, salamander, and aquatic insects (Fukumoto et al., 2015; Sigsgaard et al., 2015; Pfleger et al., 2016; Doi et al., 2017; Sakata et al., 2017).

The eDNA, which is compromised of DNA fragments released by organisms into environments such as water or soil, is thought to be derived from mixtures of feces (Martellini et al., 2005), skin cells (Ficetola et al., 2008), mucus (Merkes et al., 2014), and secretions (Bylemans et al., 2018) of organisms. Previous studies have suggested that eDNA is mainly derived from fractions of cells or cellular organs (i.e., mitochondria and nuclei), but it can also be derived from fragmented DNA (degraded DNA) in the water (Turner et al., 2014; Minamoto et al., 2016).

Many points regarding the general behavior of eDNA in water (reviewed in Barnes and Turner, 2016) are still unclear, especially the state (fragment length) and degradation of eDNA (Turner et al., 2015; reviewed in Barnes and Turner, 2016). Understanding eDNA

states and degradation is essential for the effective sampling and storage of eDNA, and may provide pertinent information to better interpret the results of species distribution and abundance and biomass estimations. This may be especially problematic for rare and endangered species, which are thought to have small populations and small amounts (or concentrations) of DNA (Fukumoto et al., 2015; Sigsgaard et al., 2015; Pfleger et al., 2016; Doi et al., 2017; Sakata et al., 2017). Both factors can influence eDNA persistence, potentially inducing false negatives which impact accuracy in occurrence and distribution data.

Many experiments have been conducted to reveal the detailed states and degradation rates of eDNA under various conditions (Thomsen et al., 2012; Barnes et al., 2014; Maruyama et al., 2014; Tsuji et al., 2017; Jo et al., 2019). In most cases, the eDNA degradation curves declined exponentially and quickly, often in less than a week (Thomsen et al., 2012; Barnes et al., 2014). Earlier meta-analyses for eDNA degradation (Collins et al., 2018) found that water conditions, such as salinity (Collins et al., 2018), water temperature (Tsuji et al., 2017; Jo et al., 2019), and pH (Barnes et al., 2014; Tsuji et al., 2017), influenced the eDNA degradation rate. In addition, the characteristics of DNA itself, such as its measured amplicon length, affected the eDNA degradation rate (Bylemans et al., 2018; Jo et al., 2019). From the data so far (temperature and amplification length), it seems possible to predict the approximate degradation rate and estimate the state of eDNA. Therefore, I conducted a novel meta-analysis to model the effects of water conditions and DNA amplicon length on the eDNA degradation rate using data generated in previous eDNA degradation studies. The previous meta-analysis (Collins et al., 2018) used the half-life of the degradation curve as an index of degradation. Although half-life has the advantage of being more intuitively meaningful, I instead used here the degradation rate constants “k” because the provided model uses the degradation rate, not half-life.

Using this approach, I aimed to evaluate the effects of water conditions (i.e., ecosystem, source, temperature, and pH), and target DNA region on eDNA degradation in previously published data. Also, I tested the relationship between DNA amplicon length and eDNA degradation because degradation may differ with amplicon length. Specifically, I conducted a simulation to predict the maximum degradation rate using quantile regression modeling with temperature and DNA amplicon length.

2.2 Materials and Methods

2.2.1 Search Strategy

A Google Scholar search on September 9, 2020, using the search terms “eDNA” OR “environmental DNA” AND “degradation” OR “decay” OR “decomposition,” returned 11,300 hits. The initial filtering of the articles was based on their title; any articles that obviously had no relevance to eDNA degradation were discarded. After title screening, 1,000 articles remained. After abstract screening, 42 articles remained. I manually inspected these remaining articles and selected papers describing the degradation rate of eDNA using experiments or field settings (Supplementary Table 2.1). Upon completion of the screening process, I obtained relevant eDNA data from 28 articles (Table 2.1 and Supplementary Table 2.1) for the meta-analysis.

Table 2.1. The organisms, ecosystem types (Ecosystem), water source (Source), and PCR-amplified DNA regions by quantitative PCR (Region) for all papers analyzed in this meta-analysis.

Organism	Ecosystem	Source	Region	References	Year
Gasterosteus aculeatus	Marine	Marine	CytB	Thomsen et al.	2012
Platichthys flesus	Marine	Marine	CytB	Thomsen et al.	2012
Lepomis macrochirus	Freshwater	Tap	CytB	Maruyama et al.	2014
Cyprinus carpio	Freshwater	Well	CytB	Barnes et al.	2014
Lithobates catesbeianus	Freshwater	Tap	CytB	Strickler et al.	2015
Cyprinus carpio	Freshwater	Well	CytB	Eichmiller et al.	2016
Cyprinus carpio	Freshwater	Lake	CytB	Eichmiller et al.	2016
Engraulis mordax	Marine	Marine	D-loop	Sassoubre et al.	2016
Sardinops sagax	Marine	Marine	D-loop	Sassoubre et al.	2016
Scomber japonicus	Marine	Marine	COI	Sassoubre et al.	2016
Scomber japonicus	Marine	Marine	COI	Andruszkiewicz et al.	2017
Zearaja maugeana	Marine	Marine	ND4	Weltz et al.	2017
Chrysaora pacifica	Marine	Marine	COI	Minamoto et al.	2017
Trachurus japonicus	Marine	Marine	CytB	Jo et al.	2017
Plecoglossus altivelis	Freshwater	River	CytB	Tsuji et al.	2017
Cyprinus carpio	Freshwater	River	CytB	Tsuji et al.	2017
Margaritifera margaritifera	Freshwater	River	NADH	Sansom and Sassoubre	2017
Carcinus maenas	Marine	Marine	COI	Collins et al.	2018
Lipophrys pholis	Marine	Marine	COI	Collins et al.	2018
Hypophthalmichthys nobilis	Freshwater	Deionized	D-loop	Lance et al.	2017
Chionodraco rastrospinosus	Marine	Marine	ND2	Cowart et al.	2018
Carassius auratus	Freshwater	Tap	ITS	Bylemans et al.	2018
Neogobius melanostomus	Freshwater	Lake	COI	Nevers et al.	2018
Cyprinus carpio	Freshwater	River	CytB	Nukazawa et al.	2018
Grandidierella japonica	Marine	Artificial seawater	COI	Wei et al.	2018
Trachurus japonicus	Marine	Marine	CytB	Jo et al.	2019
Daphnia magna	Freshwater	Tap	COI	Moushomi et al.	2019
Daphnia magna	Freshwater	Tap	18S	Moushomi et al.	2019
cyanobacterial	Freshwater	Lake	16S	Zulkefli et al.	2019
Schistosoma mansoni	Freshwater	Tap	COI	Sengupta et al.	2019
Trachurus japonicus	Marine	Marine	CytB	Jo et al.	2020
Trachurus japonicus	Marine	Marine	ITS	Jo et al.	2020
Styela clava	Marine	Marine	COI	Wood et al.	2020
Spirographis spallanzani	Marine	Marine	COI	Wood et al.	2020
Styela clava	Marine	Marine	RNA	Wood et al.	2020
Spirographis spallanzani	Marine	Marine	RNA	Wood et al.	2020
Anguilla japonica	Freshwater	Tap	D-loop	Kasai et al.	2020
Rhinella marina	Freshwater	Tap	16S	Villacorta-Rath et al.	2020
Trachurus japonicus	Marine	Marine	CytB	Saito and Doi	2020
Cyprinus carpio	Freshwater	Pond	CytB	Saito and Doi	2020

2.2.2 Data Extraction

From the selected publications, I assembled a list of factors for eDNA degradation (Supplementary Table 2.1). I collected the following factors and categories: “Ecosystem” was divided into marine and freshwater. “Source” was categorized into water sources (Freshwater: river, lake, well water, pond, tap water, and deionized water; Marine: marine and artificial seawater). “Temperature” and “pH” refer to the water temperature and pH of the water sample for each experiment, respectively. “Region” and “Amplicon length” refer to the amplified DNA region used for quantitative PCR (qPCR) and the number of amplified-DNA bases targeted by the qPCR reaction (bp). “Region” was divided into mtDNA (COI, CytB, 16s, 18s, D-loop, NADH, ND2, ND4), nuDNA (ITS), and RNA. “DNA type” was divided into spike (i.e., the DNA contained in the environment water) and organism. “Experiment type” was divided into “in tank” and “in field.”

I extracted the simple exponential slope (hereafter referred to as “degradation rate”) from the article contents and/or plots according to the simple exponential equation (Motulsky and Christopoulos, 2003) in each experiment:

$$C = C_0 e^{kt}$$

where C_0 is the eDNA concentration at time 0 (i.e., the initial eDNA concentration), and k is the degradation slope (rate) constant per hour. I used the standardized degradation rate per hour. The degradation rate by day was divided by 24 to calculate the degradation rate per hour.

2.2.3 Statistical Analysis and Simulation

I performed the statistical analysis and graphics using R ver. 4.0.2 (R Core Team, 2020). I tested the differences in the eDNA degradation rate in measured DNA regions and water resources using a linear mixed-effect model (LMM) using "lme4" ver. 1.1.23 package with "lmerTest" ver. 3.1.2 package in R. I excluded data points without temperature information in the statistical analyses. I set each study as a random effect. Jo et al. (2020) compared the degradation of mtDNA and nuDNA and found the difference. However, I could not analyze mtDNA and nuDNA due to the limited data.

I performed quantile models (QM) for 0.1, 0.5, and 0.95 quantiles for the regression. By performing 0.95 and 0.1 quantiles for the regression, I evaluated the maximum and minimum degradation rate. The 0.5-quantile used median for the regression, so almost similar to simple linear regression. I employed the Bayesian mixed-effect quantile model using the "lqmm" function of "lqmm" package ver. 1.5.5 in R. In the QM, I set water temperature and amplicon length as explanatory effects and each study as the random effect. I performed the Nelder–Mead algorithm using 10000 MCMC permutations with the Gauss–Hermite quadrature approach. I set the statistical alpha as 0.05 for parameter evaluation. I did not find a significant interaction ($p > 0.1$) between water temperature and amplicon length, so I used the model excluding the interaction, i.e., eDNA degradation rate = water temperature + amplicon length. I evaluated the QM models using the Akaike information criteria (AIC), in which the best QM is identified by having the lowest AIC.

I simulated the combined effects of water temperature and amplicon length, using the obtained 0.95-quantile QM. I generated 100,000 random values for the combination of water temperature (ranging in published values from −1 to 35 °C; see the results) and amplicon length used for the experiments (ranging in published values from 70 to 719) using "runif"

function in R, which generates a random number from the Mersenne-Twister method. I used 100,000 random values to predict the eDNA degradation rate from the 0.95-quantile QM.

2.3 Results

2.3.1 Degradation Rate Experiments from Literature

The number of obtained time points for the eDNA degradation data ranged from 3 to 25 (mean: 8.3, median: 8.0, Supplemental Table 2.1). Details of the sites are listed as water sources (Table 2.1). In total there were 21 marine sites, 1 artificial marine site, and 19 freshwater sites. Within the freshwater sites, there were 9 experiments that used tap or deionized water, 4 river sites, 3 lake sites, 2 well water sites, and 1 pond site. The temperature for the experiments ranged from −1 to 35 °C (mean: 19, median: 20, Supplemental Table 2.1). The amplicon length used for the experiments ranged from 70 to 719 bp (mean: 150, median: 131, Supplemental Table 2.1), and the DNA fragment regions used were mainly Cyt B or COI regions in mtDNA (Supplemental Table 2.1). Degradation experiments of nuDNA and RNA were very few data compared to mtDNA.

2.3.2 Degradation Rate

The observed degradation rate for the previously published eDNA data ranged from 0.0005 to 0.7010 (mean: 0.1317, median: 0.0440, Supplemental Table 2.1). Differences in PCR regions did not affect the rate of DNA degradation, nor did differences in water sources (Figures 2.1A,B). Although the degradation rates for Tap and Marine water sources appear much higher than that observed for other sources, there were no significant differences among water sources, nor among taxa or PCR regions (LMM, $t < 1.859$, $p > 0.07$, Figure 2.1 and Supplemental Figure 2.1, respectively). With the limited data excluded, such as ND2, ND4 for PCR region and pond for water source, there were no significant differences among water

sources (LMM, $t < 1.965$, $p > 0.06$, Supplemental Figure 2.2, respectively), but significant differences among PCR region (LMM, $t = -3.414$, $p = 0.002538$, Supplemental Figure 2.2).

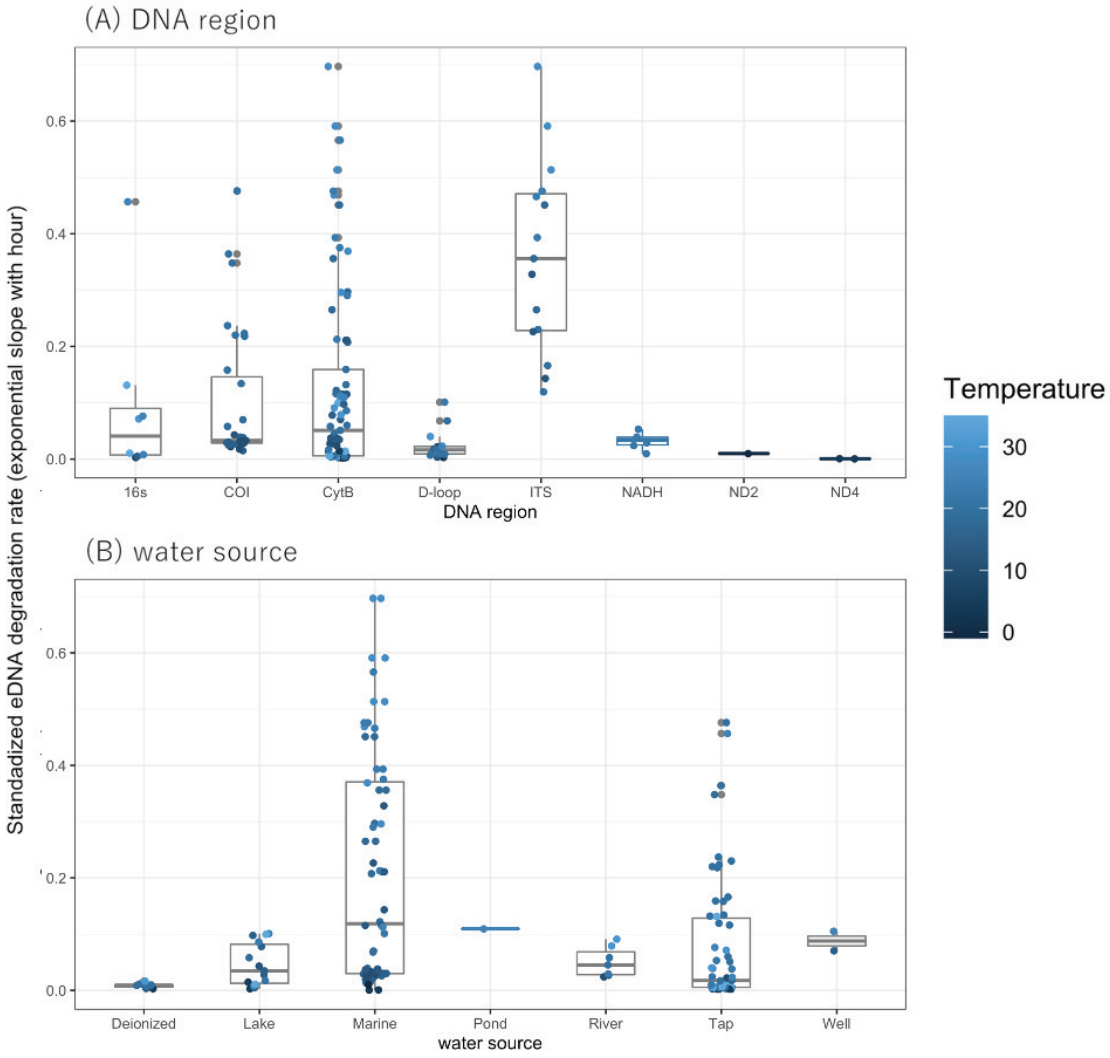

Figure 2.1. The eDNA degradation rate (simple exponential slope) with (A) DNA region and (B) water source. The degradation rate without temperature data in the experiment were excluded in the plot. The dots indicate the individual eDNA degradation rate in each experiment in different ecosystems. The boxes and bars in the box plot indicate median ± inter-quartiles and ±1.5 × inter-quartiles, respectively.

2.3.3 Quantile Model for Temperature and Amplicon Length

The relationship between eDNA degradation rate and water temperature was significant in 0.95-quantile and showed that higher water temperatures accelerated eDNA degradation (Figure 2.2A, $p = 0.02004$ and 0.5761 for 0.95- and 0.5- quantiles, respectively). Upon comparing the QM of 0. 1-, 0. 5-, and 0.95- quantiles, the QM with 0.95-quantile was observed to have the lowest AIC value (0.1-quantile: 41.82, 0.5-quantile: −120.78, and 0.95-quantile: −161.26), indicating that the best model for the relationship. Therefore, I simulated these data using the QM with a 0.95-quantile with a positive slope (slope = 0.020, Figure 2.2A). The relationship between eDNA degradation rate and amplicon length suggests that longer amplicon length undergo greater eDNA degradation (Figure 2.2B). For amplicon length, as for water temperature, the QM with 0.95-quantile had the lowest AIC value (0.1-quantile: 155.1, 0.5-quantile: −110.2, and 0.95-quantile: −145.6). Therefore, I simulated and discussed these data using the QM with a 0.95-quantile with a positive slope (slope = 0.225). I also showed the categories of water temperature range (divided into four levels: −1, 0–10, 11–20, and > 21 °C) and amplicon length (divided into three levels: 0–100, 101–200, and > 201 bp) with eDNA degradation rate (Supplemental Figures 2.3 and 2.4 respectively) with similar trends of Figure 2.2.

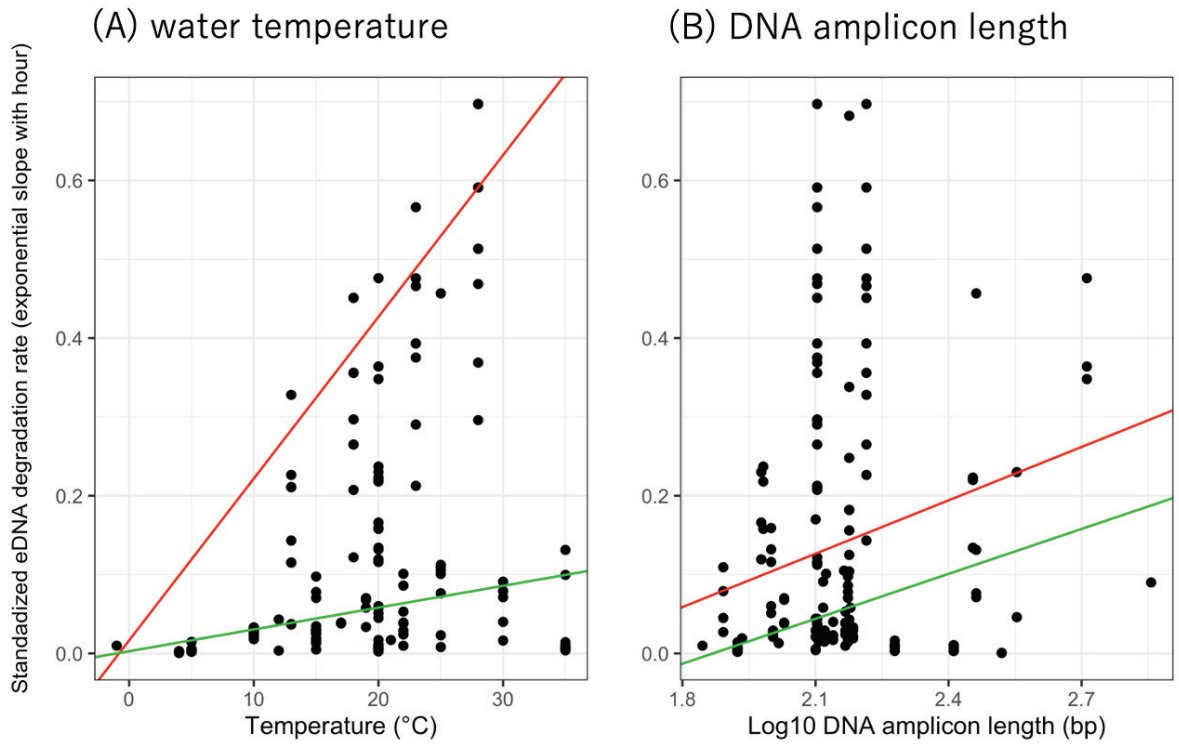

Figure 2.2. The relationship between standardized eDNA degradation rate per hour (simple exponential slope) with (A) water temperature and (B) DNA amplicon length. The red and green lines show 0.95 and 0.5- quantile mixed-effect quantile models for each factor.

2.3.4 eDNA Degradation Simulation

The QM simulation lead to plotting the eDNA degradation on a matrix of water temperature and amplicon length (Figure 2.3), which showed that the water temperature had a great influence on the eDNA degradation rate. At lower (e.g., −1 to 5 °C) and higher (e.g., 15 to 35 °C) water temperatures, the model predicted that amplicon length would have a smaller effect on the eDNA degradation rate, while at moderate (e.g., 5 to 15 °C) water temperatures, the prediction more clearly showed that the longer amplicon length would have a faster degradation rate. Thus, at moderate water temperatures, the amplicon length should also be considered in evaluating eDNA degradation.

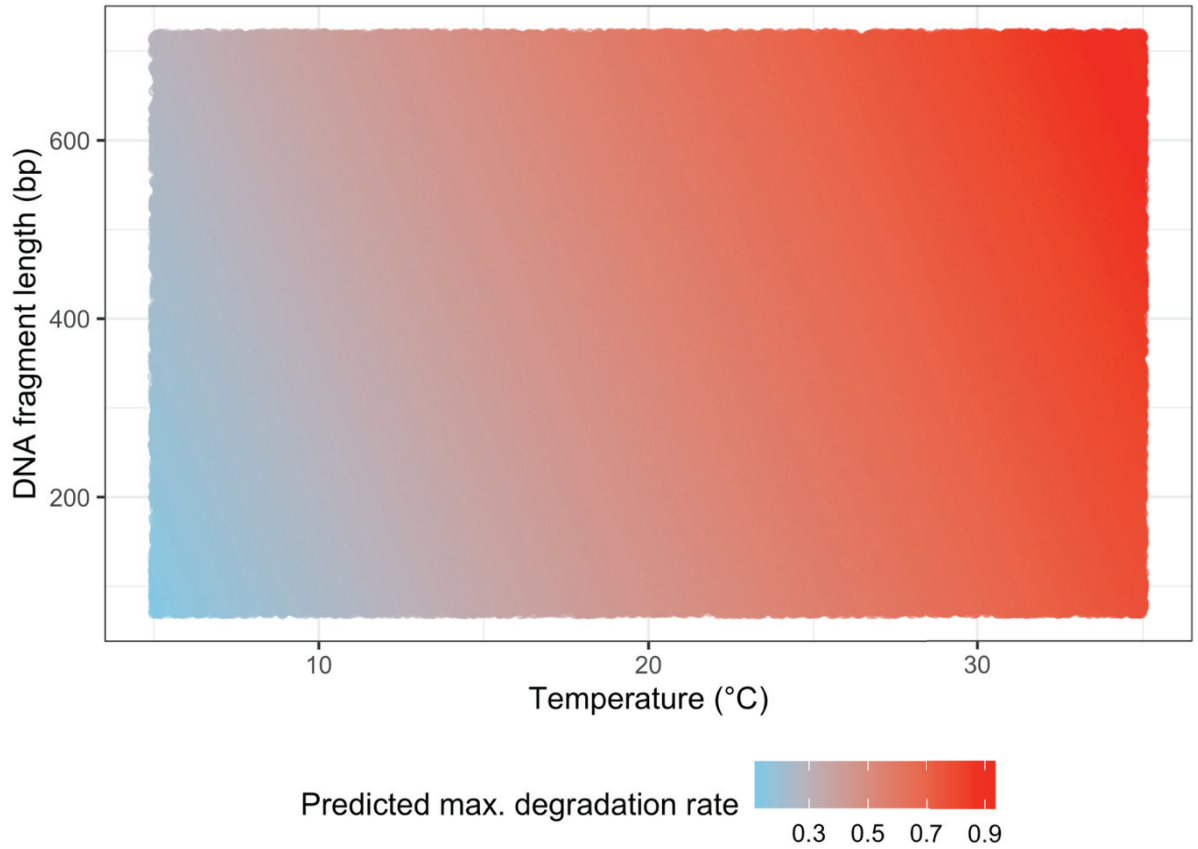

Figure 2.3. The simulation result for predicting eDNA degradation rate on the matrix of water temperature and amplicon length.

2.4 Discussion

The meta-analysis results showed that eDNA degradation was accelerated in higher water temperatures and in longer amplicon length. These generally supported the effect of water temperature on the eDNA degradation rate in previous hypotheses for each condition and species (e. g., Strickler et al., 2015; Eichmiller et al., 2016; Lance et al., 2017; Tsuji et al., 2017; Jo et al., 2019; Kasai et al., 2020). Previous studies have assumed that water temperature does not directly affect eDNA degradation, but indirectly affects it through enzymatic hydrolysis by microbes and extracellular nucleases (reviewed in Barnes and Turner, 2016). At high temperatures, with increasing activity of microorganisms and extracellular enzymes, the eDNA in water would degrade more quickly (reviewed in Barnes and Turner, 2016). The meta-analysis results showed that there were no significant differences between laboratory water (purified or tap water) and environmental water (seawater or freshwater). This may indicate the enzymes and bacteria possessed by experimental organisms affected the eDNA degradation. In fact, the degradation experiment, which intracellular DNA and fragmented DNA were added to purified water, showed that intracellular and fragments DNA were not degraded in the water for a week (see Chapter 3).

Evidence from previous studies suggested that, in eDNA samples, long amplicon length are less likely to be detected than short amplicon (Jo et al., 2017). The meta-analysis supports these previous results. A possible explanation is provided by Jo et al. (2017), in which it was suggested that the DNA degradation rate was higher in longer amplicon length (719 bp) than in shorter amplicon (127 bp). The simulation by QM indicated that shorter amplicon lengths were more likely to be detected when eDNA degradation was less affected by water temperature. When the eDNA degradation rates were very fast or very slow due to

water temperature (e.g., 15 to 35 °C or 0 to 5°C, respectively), the amplicon length had a smaller effect on eDNA degradation than at other water temperature ranges. In higher temperatures, microbial activity that breaks down DNA is occurring fast on both large and short DNA fragments, such that both classes of fragments are not detectable by either a large or small fragment amplicon assay at a similar rate. Whereas in colder temperatures, both fragment classes are degraded at lower rates, and thus it is possible that the longer fragments are able to last longer than under warmer conditions, thus remaining detectable for longer (suggesting a slower decay rate).

In the meta-analysis, I evaluated amplicon lengths ranging from 70 to 719 bp, but there were no experiments in which longer amplicon were measured. Recently, however, long range PCR was used to amplify full mitogenomes from eDNA samples (Deiner et al., 2017a,b). Additional investigation is needed to better understand retention of such extremely long DNA (>16,000 bps), and the role of degradation in these cases.

In conclusion, the meta-analysis results should that eDNA degradation was accelerated in higher water temperatures and in longer DNA amplicon. I predicted the combined effects of water temperature and amplicon length on the maximum eDNA degradation rate. The meta-analysis and simulation provided new insights for future eDNA studies. I should note the limitations: The number of papers used for the meta-analysis was limited to 28 studies, and the data was limited especially for other environmental factors, such as UV, pH, and salinity, which are important factors for eDNA degradation (Barnes et al., 2014; Lance et al., 2017; Tsuji et al., 2017; Collins et al., 2018; Mächler et al., 2018). When data such as UV, pH, and salinity are obtained in addition to water temperature, more complex phenomena can be evaluated to determine the eDNA degradation rate in water. A greater

understanding and accumulation of eDNA degradation data would improve future eDNA methods.

2.5 Supplementary Materials

Supplemental Table 2.1

Supplemental Figure 2.1, 2.2, 2.3, and 2.4

https://www.frontiersin.org/articles/10.3389/fevo.2021.623831/full

Chapter 3. Degradation Modeling of Water Environmental DNA: Experiments on Multiple DNA Sources in Pond and Seawater

3.1. Introduction

The eDNA analysis methods have been newly developed to monitor macro-organisms and manage aquatic ecosystems (Ficetola et al., 2008; Minamoto et al., 2012; Takahara et al., 2012; Tsuji et al., 2019). eDNA is DNA released by an organism into an environment, such as water or soil. eDNA is thought to be derived from the feces (Martellini et al., 2005), skin cells (Ficetola et al., 2008), mucus (Merkes et al., 2014), and secretions (Bylemans et al., 2017) of organisms. eDNA can be collected in aquatic systems (Ficetola et al., 2008; Turner et al., 2015). It is mainly derived from fractions of cells or organelles but can also be derived from free DNA suspended in water (Minamoto et al., 2016; Turner et al., 2014).

Since the first eDNA surveys were used to detect invasive American bullfrogs in French wetlands (Ficetola et al., 2008), eDNA methods have become common in studies seeking to detect an array of aquatic taxa, including fish (Jerde et al., 2013; Doi, Inui, et al., 2017), reptiles (Piaggio et al., 2014), crustaceans (Tréguier et al., 2014), amphibians (Ficetola et al., 2008), aquatic insects (Doi, Katano, et al., 2017), and mollusks (Egan et al., 2013, 2015; Goldberg et al., 2013), in various habitats, including ponds (Takahara et al., 2013; Tréguier et al., 2014), rivers (Doi, Inui, et al., 2017; Jerde et al., 2013; Yamanaka & Minamoto, 2016), lakes (Eichmiller et al., 2014; Takahara et al., 2012), swaps (Doi, Katano, et al., 2017), and marine habitats (Boussarie et al., 2018; Lacoursière-Roussel et al., 2018; Yamamoto et al., 2017). eDNA methods can be used to detect target species, but also potentially to estimate their biomass and abundance (Doi, Inui, et al., 2017; Doi, Katano, et

al., 2017; Takahara et al., 2012). However, various factors such as sampling season, temperature, pH, salinity, and UV can influence biomass and abundance estimations using eDNA (Doi, Inui, et al., 2017; Doi, Katano, et al., 2017). eDNA analysis methods can be performed quickly and noninvasively without the need for capturing individuals and carrying out biomonitoring surveys (Katano et al., 2017; Takahara et al., 2013; Tréguier et al., 2014).

Many experiments have been conducted on the different states and degradation rates of eDNA under various conditions (Barnes et al., 2014; Thomsen et al., 2012). In most studies, a target organism is placed in an aquarium and then removed. The eDNA is measured over an experimental period to evaluate eDNA release and degradation (Barnes et al., 2014; Maruyama et al., 2014; Thomsen et al., 2012). In most cases, the eDNA degradation curves declined exponentially and the degradation rates were fast, but less than a week. However, other studies have found that eDNA concentrations can decay below the limit of detection in less than a week (Barnes et al., 2014; Thomsen et al., 2012). In addition, water conditions, such as salinity (Collins et al., 2018), temperature (Jo et al., 2019; Tsuji, Ushio, et al., 2017), and pH (Barnes et al., 2014; Tsuji, Yamanaka, et al., 2017), can influence the eDNA degradation rate. To understand the details of how eDNA degrades in nature, an experiment separating these eDNA sources was required, but, prior to this study, was never before attempted.

The aim of this study was to observe and compare the degradation of various samples of fragmental eDNA and free cell-derived eDNA. In this experiment, I used water from the sea and a pond to understand how water conditions, especially salinity, affect degradation. Based on the results, I discuss the most effective sampling and storage methods for eDNA so as to maximize the correct interpretation of eDNA results from field surveys.

3.2. Materials and Methods

3.2.1 Experiment Outline

I used sea (from the Seto Inland Sea), pond (from an artificial pond in Kobe), and purified water (as DNA-free samples, A300, AS ONE) (Figure 3.1) and divided each water sample into 12 bottles. A solution of isolated cells (from *Oncorhynchus kisutch*) and fragmental DNA (from an internal positive control, IPC [Internal PCR control (IPC), Nippon Gene; 1.5×10^5 copies], Figure 3.1) was added to each bottle. The seawater and pond water contained the eDNA of Japanese jack mackerel (*Trachurus japonicus)* and common carp (*Cyprinus carpio*) as free-living individuals, respectively. I used *O. kisutch* tissue for the isolation of cells because this species is not distributed in the sampling region. I conducted the experiment for 7 days. A Sterivex filter (Merck Millipore) was used to filter 500 mL samples of water and 1.5 mL of the filtrate from each bottle was collected (Figure 3.1). After extracting eDNA from the filtrate and the Sterivex filter, the copy number of each type of DNA contained in the Sterivex samples and filtrate was estimated by qPCR (Figure 3.1).

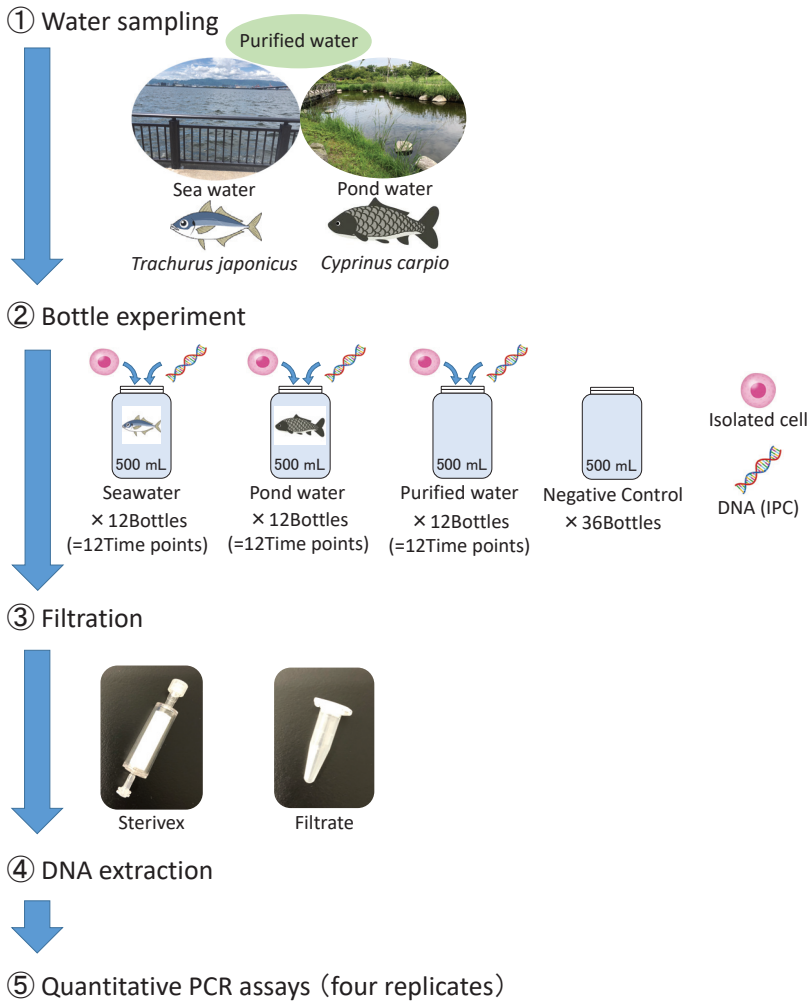

Figure 3.1. Experimental overview for the bottle experiments. I used sea, pond, and purified water and divided each into 12 bottles. Each bottle was added a solution of isolated cells (from *Oncorhynchus kisutch*) and fragmental DNA (IPC). The seawater and pond water were expected to contain the eDNA of *Trachurus japonicus* and *Cyprinus carpio*, respectively. I used *O. kisutch* tissue for the isolation of cells. I conducted the experiment for 7 days. A Sterivex filter was used to filter 500-mL samples of water and 1.5 mL of the filtrate from each bottle was collected. After extracting eDNA, the copy number of each type of DNA were estimated by qPCR.

3.2.2 Preparation of Isolated Cells

Oncorhynchus kisutch tissue was isolated using a Single Cell Isolation Kit (Cosmo Bio). I obtained the muscle of farmed *O. kisutch* in Miyazaki, Japan. I placed 29 mg of *O. kisutch* epidermal muscle samples into the filter unit of the kit and added 100 μL of cold buffer A from the Single Cell Isolation Kit, according to the manufacturer's protocols. The tissue was then ground 60 times using a plastic rod. I added 400 μL of buffer to the filter unit, then inverted it a few times, and centrifuged it at 4000 g for 4 min (Eppendorf, Hamburg, Deutschland). The filter unit was vortexed and then centrifuged at 2000 g for 5 min. I repeated the above procedure three times to prepare 1500 μL of isolated cells. The isolated cells were immediately used for the following experiments.

3.2.3 Bottle Experiment

I collected the seawater from the Seto Inland Sea on July 29, 2019, and the pond water from an artificial pond in Kobe, Japan on July 25, 2019 (34°38′28″N, 135°13′37″E and 34°39′40″N, 135°13′02″E, respectively) using bleached tanks. Measurements were obtained using a salinity meter (CD-4307SD, Mother Tool), thermometer (ProODO, YSI), and pH meter (Twin pH, HORIBA); the salt concentration, temperature, and pH at time of collection were 2.80%, 25.8°C, and 6.7 for the sea and 0.04%, 27.7°C, and 6.2 for the pond, respectively.

I bought the purified water (A300, AS ONE) for the experiment. The sea, pond, and purified water were each divided into 12 bottles. Bottles and equipment were sterilized with 10% commercial bleach (ca. 0.6% hypochlorous acid) (KAO) and washed with DNA-free distilled water. Bottles were maintained in the laboratory at about 25°C.

Each bottle received 100 µL of a solution of isolated cells (equivalent to 1.0×10^5 copies of cells) and DNA (1.0×10^5 copies) (IPC). I collected and filtered 500 mL of water from each bottle using 0.45 µm Sterivex filters (Merck Millipore) at 0, 0.5, 1, 3, 6, 12, 18, 24 (day 1), 48 (day 2), 72 (day 3), 120 (day 5), and 168 (day 7) h after the introduction of the cells and DNA, and then collected 1.5 mL samples of the filtered water. After filtration, approximately 2 mL of RNAlater (Thermo Fisher Scientific) was injected into the Sterivex. As a filtration blank, the 500 mL DNA-free water was filtered in the same manner after filtration of the samples to monitor cross-contamination. The Sterivex filters and filtrate were immediately stored at −20°C until further analysis.

3.2.4 DNA Extraction

The Sterivex filter was extracted using a DNeasy Blood and Tissue Kit (Qiagen) following Miya et al. (2016). The RNAlater was removed using a 50 mL syringe, and 440 µL of the mixture (220 µL of phosphate-buffered saline [PBS], 200 µL of buffer AL, and 20 µL of proteinase K [Qiagen]) was added to the Sterivex filter. I incubated the filters on a rotary shaker (at 20 rpm) at 56°C for 20 min (AS ONE). I transferred the incubated mixture into a new 1.5-mL tube by centrifugation at 5000 g for 5 min. I added 200 µL of 99.5% ethanol to the mixture and vortexed it for 2–3 s. I then purified the mixture using the DNeasy Blood and Tissue Kit, and finally eluted the DNA in 100 µL of buffer AE from the kit.

The filtrate was extracted using a DNeasy Blood and Tissue Kit, according to Uchii et al. (2016). I added 440 µL of the mixture (220 µL of PBS, 200 µL of buffer AL, and 20 µL of proteinase K with 600 mAU mL^{-1}) to 500 µL of the filtrate. I incubated the filtrate at 56°C for 30 min, then added 200 µL of 99.5% ethanol to the mixture, and vortexed it for 2–3 s. I transferred the total amount of mixture into a new 1.5-mL tube and then centrifuged it at 5000

g for 5 min. I then purified the mixture using the DNeasy Blood and Tissue Kit, and finally eluted the DNA in 100 μL of the AE buffer from the kit. The extracted DNA from both methods was stored at −20°C until qPCR analysis.

3.2.5 Primer-Probe Design for *O. kisutch*

To detect and quantify the DNA of *O. kisutch* using qPCR, the forward and reverse primers for a 120-bp fragment of the COI region of the mitochondrial DNA were prepared according to Chalde et al. (2019) and I designed a TaqMan probe using Primer3Plus (http://www.bioinformatics.nl/primer3plus). The sequences of the real-time PCR primers and TaqMan probes are shown in Table 3.1.

All primer specificities for the three fish species were confirmed by each study that developed the primer-probe set (Chalde et al., 2019; Takahara et al., 2012; Yamamoto et al., 2017). While I tested the specificity of the designed *O. kisutch* probes, I also checked them in silico using homologous sequences from other Oncorhynchus that inhabit Japan (National Center for Biotechnology Information, NCBI; http://www.ncbi.nlm.nih.gov/). No other Oncorhynchus genus were observed during the in silico screening for specificity, which was performed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 3.1. The primers and probes for the targeted DNA used in the experiment

Target	Primer/Probe	Sequence
Cyprinus carpio	primer F	5’-GGTGGGTTCTCAGTAGACAATGC-3’
	primer R	5’-GGCGGCAATAACAAATGGTAGT-3’
	probe	5’-(FAM)-CACTAACACGTTCCACTTCC-(TAMRA)-3’
Trachurus japonicus	primer F	5’-CAGATATCGCAACCGCCTTT-3’
	primer R	5’-CCGATGTGAAGGTAAATGCAAA-3’
	probe	5’-(FAM)-TATGCACGCCAACGGCGCCT-(TAMRA)-3’
Oncorhynchus kisutch	primer F	5’-GCACCGGAAGCACTGTTATA-3’
	primer R	5’-CTTTGTGCTCGTGGGACTTT-3’
	probe	5'-(FAM)-CCACTGCTGGCGTAGCTTA-(TAMRA)-3'
IPC	primer F	5'-CCGAGCTTACAAGGCAGGTT-3'
	primer R	5'-TGGCTCGTACACCAGCATACTAG-3'
	probe	5'-(FAM)-TAGCTTCAAGGGCTGTCGGC-(TAMRA)-3'

3.2.6 Quantitative PCR Assays

Quantitative PCRs for *C. carpio* (Takahara et al., 2012), *T. japonicus* (Jo et al., 2017), *O. kisutch*, and the IPC were performed. The DNA concentrations were quantified by qPCR using a PikoReal™ qPCR system (Thermo Fisher Scientific). Each TaqMan reaction contained 900 nM of forward and reverse primers and 125 nM of a TaqMan probe in the 1× TaqPath™ qPCR master mix (Thermo Fisher Scientific). To this, 2 µL of sample template was added to reach a final volume of 10 µL. A four-step dilution series containing 1.5×10^1 to 1.5×10^4 copies per PCR plate was prepared and used as quantification standards. A quantitative PCR was performed with the following conditions: 2 min at 50°C, 10 min at 95°C, and 55 cycles of 15 s at 95°C and 1 min at 60°C. Four replicates were performed for each sample, and four replicate negative non-template controls containing DNA-free water instead of template DNA were included in all PCR plates. I performed the real-time PCR procedures according to the MIQE checklist (Bustin et al., 2009). The PCR and qPCR were set up in two separate rooms to avoid PCR-amplicon contamination.

The qPCR results were analyzed using PikoReal software ver. 2.2.248.601 (Thermo Fisher Scientific). For the standard curve, I used target DNA cloned into a plasmid. The R2 values of the standard curves ranged from 0.985 to 0.998 (Table 3.S1 and Figure 3.S1) and the PCR efficiency varied from 91.07% to 101.68%. The concentration of DNA in the water collected (DNA copies mL^{-1}) was calculated based on the volume of filtered water. DNA copy numbers were evaluated including negative amplifications set as zero values. I performed a limited of detection (LOD) test for the PCR assay, measured the concentration (ng μL^{-1}) of the extracted DNA using a Qubit dsDNA HS assay kit with Qubit 4.0 (Thermo Fisher Scientific), and calculated the copy number from the weight of mitochondrial DNA, then diluted each copy number (1, 2, 4, and 8 copies) for the PCR. I evaluated the LOD of the

qPCR with four replicates for all the primers/probes used to detect *C. carpio*, *T. japonicus*, *O. kisutch*, and the IPC.

3.2.7 Statistical Analysis

All statistical analysis and data plotting were performed using R version 3.6.0 (R Core Team, 2019). Degradation models were formulated to determine a first-order rate constant from biotransformation/degradation studies, including standard biotic studies conducted using soil, water, or mixed media (Boesten et al., 2005; Motulsky & Christopoulos, 2003). The degradation rates in this study were estimated from the DNA degradation curves obtained from three models: the Single First-Order rate model (SFO), First-Order Multi-Compartment model (FOMC), and Double First-Order in Parallel model (DFOP) (Boesten et al., 2005; Motulsky & Christopoulos, 2003). The SFO establishes a simple procedure for determining a first-order rate constant from the degradation. The FOMC establishes a procedure for determining how fast the degradation rate declines with decreasing concentration owing to the degradation of DNA, as well as determining a first-order rate constant from the degradation of DNA. The DFOP establishes a procedure for determining two first-order rate constants from the degradation of DNA. The model equations are as follows:

$$C = C_0 e^{kt} \text{ (model 1, SFO)}, \tag{1}$$

$$C = C_0\left(\frac{t}{\beta} + 1\right)^{-\alpha} \text{ and } \alpha = \frac{1}{n-1} \text{ and } \beta = \frac{C_0^{1-n}}{k(n-1)} \text{ (model 2, FOMC)}, \tag{2}$$

$$C = C_0 g^{-k_1 t} + C_0(1-g)^{-k_2 t} \text{ (model 3, DFOP)}$$

where C is the eDNA concentration at time t; C_0 is the eDNA concentration at time 0 (i.e., the initial eDNA concentration); k is the degradation rate constant per hour; n determines how fast the degradation rate declines with decreasing concentration and is an indicator of how far the data deviate from a first-order model (where n = 1); and α, β, and g are constants, which are estimated by analyzing the nonlinear least-squares regression. I performed all modeling using the 'mkin' package version 0.9.49.8 in R. I evaluated the fitting of the models using the chi-squared error level (Boesten et al., 2005). The chi-squared error considers the deviations between observed and calculated values in relation to the uncertainty of the measurements (denominator) and describes the measurement error (Boesten et al., 2005). Significant differences in model coefficients were evaluated by overlapping the 95% confidential intervals (CIs) of the coefficients (i.e., α = 0.05).

3.3 Results

3.3.1 Primers and Probe Testing, and LOD

I confirmed that the DNA from isolated cells was amplified using the primers and designed probe. I found the LODs of the qPCR were one copy per PCR for all the primers/probes used to detect *C. carpio*, *T. japonicus*, *O. kisutch*, and the IPC.

3.3.2 eDNA Detection from Sterivex Filter

I detected all the targeted DNA of *C. carpio*, *T. japonicus*, the *O. kisutch* cells, and the IPC using qPCR (Figure 3.2, Supplemental Table 3.1). While I could not detect the eDNA of *C. carpio* and *T. japonicus* on day 2, I detected the IPC DNA and *O. kisutch* cells in the purified water and seawater up to day 7, and the IPC DNA and *O. kisutch* cells in pond water up to

days 5 and 3, respectively. The DNA concentrations of the cells and IPC DNA decreased exponentially after they were added (day 0). I observed these trends in both the sea and pond samples that were not observed in the purified water samples. Each real-time PCR assay included four no template controls (NTC), and there were no amplifications from the NTCs. Also, there were no amplifications from the filter and extraction blanks.

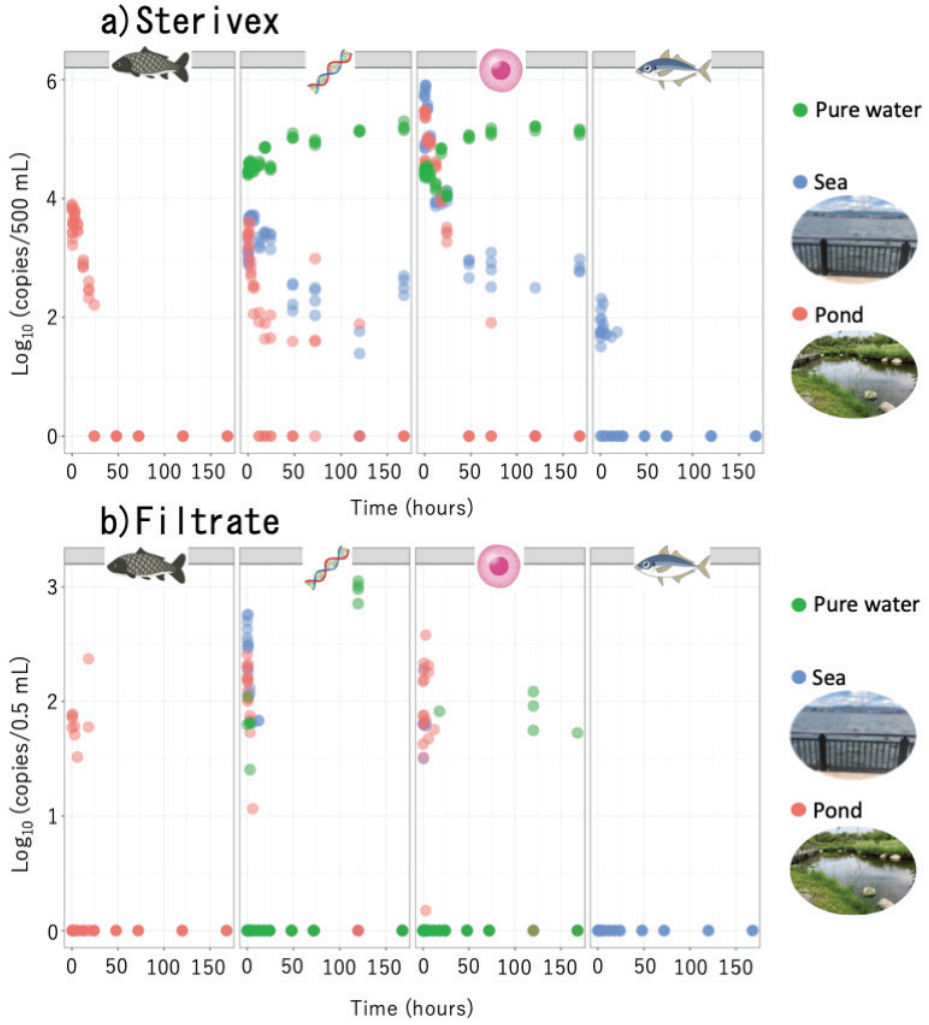

Figure 3.2. The relationship between the eDNA concentrations of the targets (*Cyprinus carpio*, the internal positive control, *Oncorhynchus kisutch* cells, and *Trachurus japonicus*), and the sampling timing of the experiment. The eDNA were extracted from a) the Sterivex filter and b) the filtrate. The dots indicate the eDNA concentrations of the targets at each time point under different water conditions: purified water, green; sea, blue; pond, red (N = 4 for each time point).

The SFO model was the most suitable model among the three degradation models after comparing their chi-squared error levels (Table 3.2). The SFO model showed the lowest chi-squared error levels between the three models (SFO, FOMC, and DFOP), except for a few samples. This result indicates that the efficiency of eDNA degradation did not decrease with time. However, the *O. kisutch* cells and *C. carpio* in the pond water had slightly lower chi-squared error levels for the DFOP than for the other two models. This result shows that the *O. kisutch* cells and *C. carpio* in the pond water had two different degradation rates: A faster rate was observed during the early part of the degradation curve and a slower rate was observed later on. The degradation rate constant of the IPC was significantly different between the sea and pond samples when comparing the 95% CIs (Table 3.3, Figure 3.3). This indicates that pond–sea differences significantly affected the eDNA degradation rate. The degradation constants of the *O. kisutch* cells, *C. carpio,* and *T. japonicus* were not significantly different between the sea and pond samples (Figure 3.3). The degradation constants of *C. carpio* and *T. japonicus* were not significantly different from that of the *O. kisutch* cells but were significantly different from that of the IPC (Figure 3.3).

Table 3.2 The fitting of the three models (SFO, FOMC, and DFOP) by chi-squared error level. The error level in the table indicates the chi-squared error level. Bold names indicate the model with lowest chi-squared error level. In "Target," IPC, Tja, Okis, and Cyca indicate the fragment DNA of the IPC, the DNA from *Oncorhynchus kisutch* cells, the eDNA of *Cyprinus carpio*, and the eDNA of *Trachurus japonicus*, respectively.

Water	Method	Target	Model	Error level	Water	Method	Target	Model	Error level
			SFO	0.515574				**SFO**	0.33812
Sea	Sterivex	IPC	FOMC	0.536307	Sea	Filtrate	IPC	FOMC	0.351498
			DFOP	0.560186				DFOP	0.365194
			SFO	0.789092				**SFO**	1.285069
Sea	Sterivex	Okis	FOMC	0.820822	Sea	Filtrate	Okis	FOMC	1.336745
			DFOP	0.857371				DFOP	1.396263
			SFO	0.35171				SFO	non-detect
Sea	Sterivex	Tja	FOMC	0.365853	Sea	Filtrate	Tja	FOMC	non-detect
			DFOP	0.382142				DFOP	non-detect
			SFO	0.647998				**SFO**	0.14903
Pond	Sterivex	IPC	FOMC	0.674056	Pond	Filtrate	IPC	FOMC	0.155024
			DFOP	0.703742				DFOP	0.161925
			SFO	0.657318				**SFO**	0.493445
Pond	Sterivex	Okis	FOMC	0.680103	Pond	Filtrate	Okis	FOMC	0.513288
			DFOP	0.613436				DFOP	0.536142
			SFO	0.476753				**SFO**	1.023674
Pond	Sterivex	Cyca	FOMC	0.495924	Pond	Filtrate	Cyca	FOMC	1.064839
			DFOP	0.41902				DFOP	1.100387
			SFO	0.543533				**SFO**	2.459865
Purified water	Sterivex	IPC	FOMC	0.56539	Purified water	Filtrate	IPC	FOMC	2.558782
			DFOP	0.590564				DFOP	2.672711
			SFO	0.654886				**SFO**	1.814764
Purified water	Sterivex	Okis	FOMC	0.68122	Purified water	Filtrate	Okis	FOMC	1.88774
			DFOP	0.711552				DFOP	1.971791

Table 3.3 The degradation rate constant of SFO models. The values were slope k and the values in parentheses were lower and upper confidential intervals of slope k.

Method	Status	IPC	Okis	Cyca	Tja
Sterivex filter	Seawater	0.024 (0.0138, 0.0422)	0.181 (0.105, 0.312)	-	0.112 (0.0511, 0.2474)
	Pond	0.279 (0.177, 0.4422)	0.171 (0.0946, 0.309)	0.109 (0.0759, 0.1573)	-
filtrate	Seawater	0.509 (0.3455, 0.7511)	0.151 (0.04317, 0.5294)	-	-
	Pond	0.439 (0.3373, 0.5709)	0.082 (0.03435, 0.1974)	0.022 (0.002708, 0.1758)	-

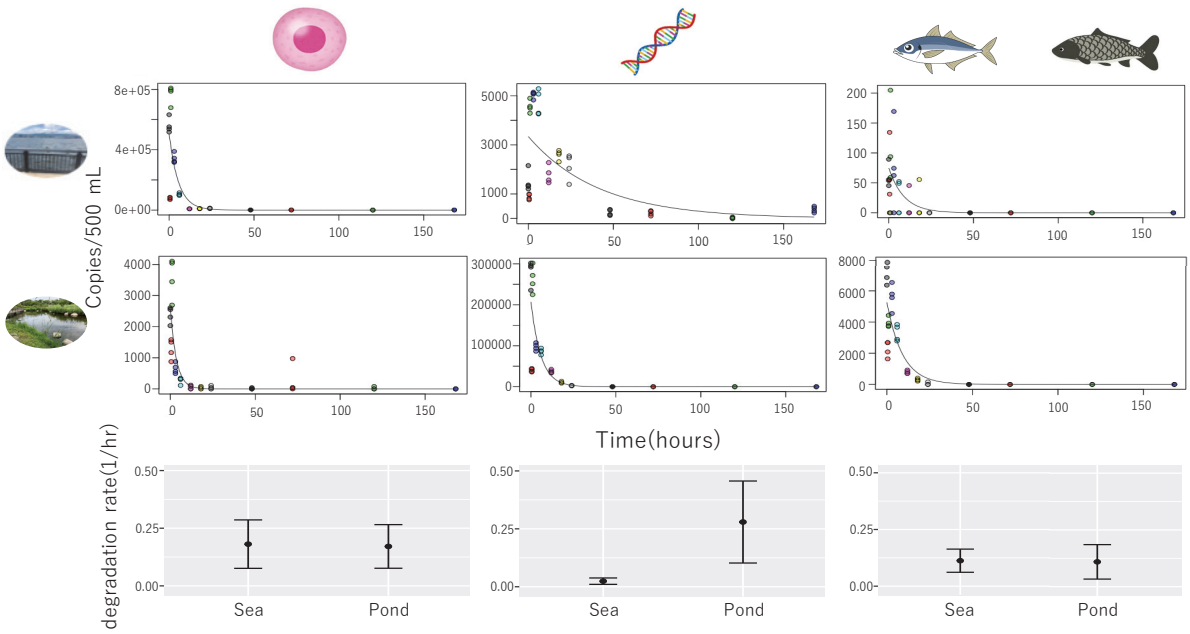

Figure 3.3. Degradation curves of the SFO and the rate constant for the bottle experiments using the Sterivex. The dots indicate the eDNA concentrations of the targets (*Cyprinus carpio*, the IPC, *Oncorhynchus kisutch* cells, and *Trachurus japonicus*) at each time point with different colors (N = 4 for each time point). The upper degradation curves show each target (*O. kisutch* cells, the IPC, and *T. japonicus*) in sea samples. The lower decay curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in pond samples. The slope (k) of each target (*O. kisutch* cells, the IPC, *C. carpio*, and *T. japonicus*) is shown with 95% confidential intervals.

3.3.3 eDNA Detection from Filtrate

I detected three of the targeted DNA types, *C. carpio*, *O. kisutch* DNA from the cells, and the IPC, using qPCR (Figure 3.2, Supplemental Table 3.1). However, I could not detect the IPC, *O. kisutch* cells, and the eDNA of *C. carpio* on day 2 in both the sea and pond samples. In the purified water, only the DNA of the IPC and *O. kisutch* cells were detected on day 5. I could not detect the eDNA of *T. japonicus* in any of the filtrate samples. The DNA concentrations of the cells (1.5×10^5 copies) and IPC decreased exponentially immediately after they were added (Figure 3.2). I observed these trends in both the sea and pond samples, while they were not observed in the samples with purified water.

The SFO model was also the most suitable for modeling the degradation of filtrate, except for samples of Sterivex in pond (*C. carpio* and *O. kisutch*) (Table 3.2), indicating that the efficiency of eDNA degradation did not decrease with time. The degradation constant of the IPC was not significantly different between the sea and pond samples, while the degradation constant of *O. kisutch* cells was significantly different between the sea and pond samples (Figure 3.4).

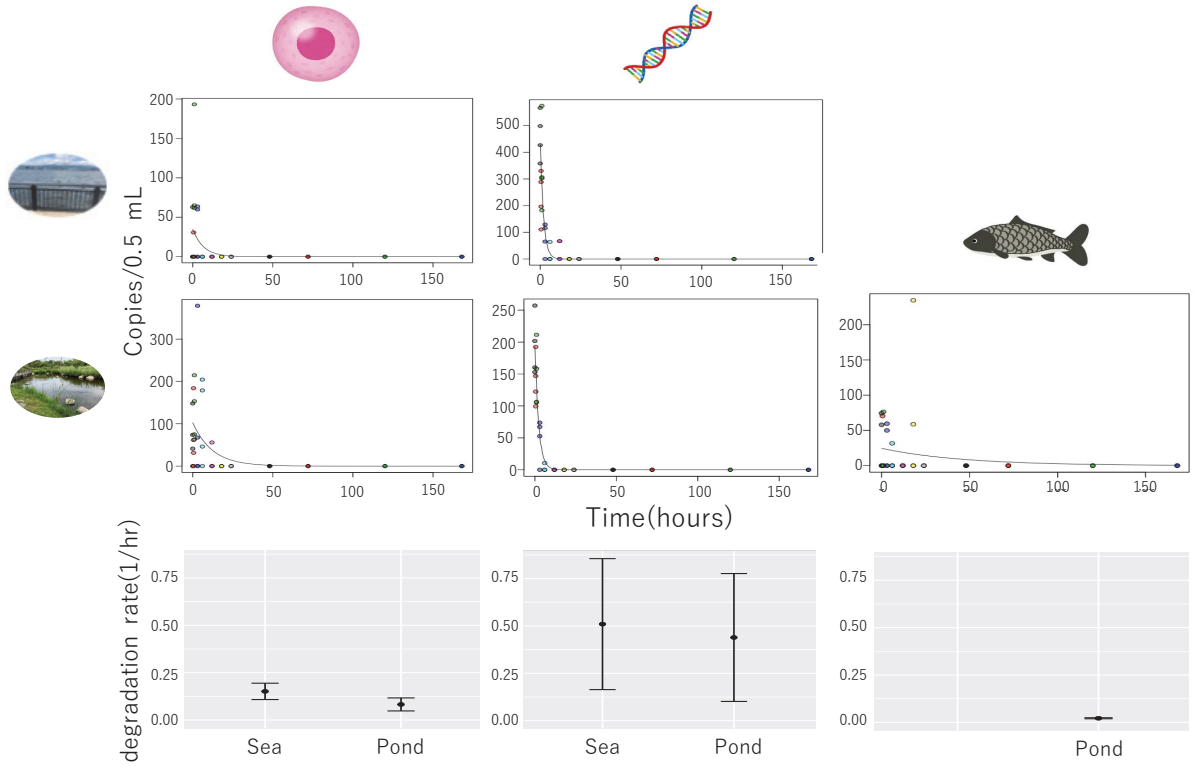

Figure 3.4. Degradation curves of SFO and the rate constant for the bottle experiments using the filtrate. The dots indicate the eDNA concentrations of the targets (*Cyprinus carpio*, the IPC, *Oncorhynchus kisutch* cells, and *Trachurus japonicus*) at each time point with different colors (N = 4 for each time point). The upper degradation curves show each target (*O. kisutch* cells and the IPC) in the sea samples. The lower decay curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in the pond samples. The slope (k) of each target (*O. kisutc*h cells, the IPC, and *C. carpio*) is shown with 95% confidential intervals.

3.4 Discussion

I found that the DNA concentrations of the *O. kisutch* cells and the IPC declined exponentially in both the sea and pond water samples, although this was not the case for purified water. The results of exponential degradation support those of previous studies (Jo et al., 2019; Maruyama et al., 2014; Takahara et al., 2012; Tsuji, Ushio, et al., 2017;). In purified water, the DNA concentrations did not decrease over time. eDNA degradation can be caused by various factors, such as microbes and extracellular enzymes (Barnes & Turner, 2016), and UV radiation (Mächler et al., 2018). I assumed that the purified water contained very low numbers of microbes and extracellular enzymes. When I performed the experiment in the laboratory, the DNA from the cells and the fragmental DNA in the purified water did not decrease over time. This supports the hypothesis that eDNA declines owing to microbes and extracellular enzymes rather than degrading by itself or through UV degradation. For other possibilities of UV, I assumed UV should affect all the bottles because of the same light conditions of the bottles in a room. The detected DNA tended to increase when incubated in purified water. The initial concentration was less than the amount of DNA added, and then, the DNA was detected close to the amount added. Therefore, I speculate that the fragmental DNA was not initially trapped in the Sterivex filter during the first few days, but afterward fragmental DNA aggregated and was trapped in the filter. Further study is needed to confirm how DNA aggregates when incubated in water.

I evaluated eDNA degradation rates using three models (SFO, FOMC, and DFOP) to quantify general degradation processes. Previous studies estimated eDNA degradation rates by fitting simple exponential models (i.e., SFO) (Bylemans et al., 2018; Minamoto et al., 2017; Sansom & Sassoubre, 2017; Sassoubre et al., 2016; Thomsen et al., 2012; Tsuji, Ushio,

et al., 2017;). In addition to the SFO, there are examples of the biphasic model being used (Eichmiller et al., 2016), but few studies have compared the fit of multiple degradation models. The results suggest that the SFO model can be used to evaluate the eDNA degradation rate in most cases. The SFO model assumes that the degradation rate does not change over time. Thus, I can assume that the degradation processes in cell- and fragmental DNA-derived eDNA occur on a similar timescale. This is because both cell decomposition and DNA degradation in water occurred at almost the same rate in the experiments. However, the eDNA degradation of *O. kisutch* cells and *C. carpio* in the pond water fitted the DFOP model better. The DFOP uses two different degradation rates in the model over time. Therefore, the eDNA degradation processes in the pond water might have two stages for the eDNA derived from cells and *C. carpio*. This, I speculate that there were two sequentially occurring degradation processes for cell decomposition and free DNA degradation in the pond water. It is not known why two degradation processes were detected in the pond water but not in the seawater. Thus, further experiments are needed to reveal the details of this phenomenon.

The degradation rates of the eDNA derived from the resident species, *C. carpio* and *T. japonicus*, in each site were not significantly different from that of the *O. kisutch* cells. This result might suggest that the degradation of the organisms' eDNA in the water displays similar behavior to that of eDNA derived from free cells. Previous studies found that the most abundant eDNA size range was from 1 to 10 μm and concluded that eDNA is mainly derived from cells or cellular organs (Minamoto et al., 2016; Turner et al., 2014). The findings indirectly support the possibility that eDNA is mainly derived from cells. This result also suggests that the degradation rate of free cells can represent that of eDNA in nature, rather

than the degradation rate of fragmental DNA. An experimental approach using free cells would be useful to help us understand eDNA behavior in nature.

The fragmental DNA, IPC, degraded in the pond significantly faster than in the sea. Water salinity has been found to be a significant factor in degradation rates. For example, higher salinity sites had slower degradation rates in marine sites with salinity gradients (Collins et al., 2018). This study supports these results of Collins et al. (2018) by experimentally comparing freshwater ponds and seawater. However, there are many differences in environmental factors between the freshwater ponds and seawater, such as microbial abundance, species composition, and the other water quality properties (such as UV radiation and water pH). Further study is needed to understand how differences in environmental factors between freshwater and marine habitats affect the degradation of eDNA derived from fragmental DNA.

These experiments provide new findings on eDNA degradation; however, there were some limitations owing to the experimental design. First, I performed the experiment using only one site each for the sea and pond samples. Therefore, it is unclear whether similar DNA degradation rates exist in other sea and pond habitats. Experiments using a selection of site replicates from various habitats need to be performed to understand eDNA degradation more generally. The evaluation of eDNA degradation while comparing different environmental conditions (e.g., salinity, water temperature, pH, chlorophyll, and microorganism) may reveal what is affecting eDNA degradation in general. Second, I evaluated eDNA derived only from cells and fragmental DNA but other eDNA sources exist, such as organelles (e.g., mitochondria), cells with mucus, and various tissue types (e.g., skin, scales). Experiments using these other sources would provide further information on eDNA degradation.

In conclusion, I found that the eDNA derived from cells and fragmental DNA declined exponentially after being added to both sea and pond water samples. The eDNA from resident species showed similar behaviors to the eDNA derived from cells. A simple exponential model can, in most cases, be used to evaluate degradation. However, for cell-derived eDNA degradation in freshwater ponds, I should consider the possibility of multiple degradation steps, such as cell decomposition and DNA degradation. A greater understanding of and the accumulation of basic information about eDNA would improve eDNA analysis methods and enable researchers to maximize the potential of future eDNA methods.

3.5 Supplementary Materials

Supplemental Table 3.1

https://onlinelibrary.wiley.com/doi/full/10.1002/edn3.192

Chapter 4. Effect of Salinity and Water Dilution on Environmental DNA Degradation in Freshwater Environments

4.1 Introduction

The eDNA evaluation methods have been developed to monitor macroorganism communities and manage aquatic ecosystems (Ficetola et al., 2008; Takahara et al., 2012; Minamoto et al., 2012; Tsuji et al., 2019). eDNA is the DNA released by organisms into an environment, such as water or soil, and derives from the feces (Martellini et al., 2005), skin cells (Ficetola et al., 2008), mucus (Martellini et al., 2014), and secretions (Bylemans et al., 2017) of the organisms. In addition, this eDNA can be collected in aquatic systems (Ficetola et al., 2008; Turner et al., 2015). The DNA sources are mainly fractions of cells or organelles but can also be free DNA fragments suspended in the water (Turner et al., 2014, Minamoto et al., 2016).

An understanding of eDNA degradation, which is a critical eDNA characteristic, is important for eDNA evaluation for both species distribution and abundance/biomass (Chapter 1, Collins et al., 2018). Although processes such as retention within the substrate may contribute to eDNA removal from aquatic systems, a primary route of eDNA decay is the physical degradation of the tissue and particles comprising eDNA. Shed biological tissue will generally begin to degrade immediately from multicellular tissue fragments to whole cells, separate organelles (e.g., mitochondria), and eventually to free (extracellular) DNA, which are then further degraded either by exogenous enzymes or by spontaneous chemical reactions (Harrison et al., 2019). Previous reviews have concluded that the factors influencing eDNA persistence fall into three broad categories: (1) DNA characteristics (i.e., fragment length, and association with cellular/organellar membranes), (2) abiotic environment characteristics (i.e.,

light, oxygen, pH, salinity, and the composition of substrates), and (3) biotic environment characteristics (i.e., the composition and activity of the microbial community and extracellular enzymes) (Barnes et al., 2016). To reveal the states of eDNA, especially its degradation rate, many experiments have been conducted under various conditions (Thomsen et al., 2012; Barnes et al., 2014), such as varied temperature (Tsuji et al., 2017; Jo et al., 2019), pH (Barnes et al., 2014; Tsuji et al., 2017), and salinity (Collins et al., 2018). eDNA is measured over an experimental period to evaluate eDNA release and degradation (Thomsen et al., 2012; Barnes et al., 2014; Maruyama et al., 2014). The degradation curves of the eDNA in most experiments have been observed to have exponentially declined (Chapter 2), and eDNA concentrations can decay below the limit of detection in less than a week (Chapter 3; Thomsen et al., 2012; Barnes et al., 2014).

In previous meta-analyses of eDNA (Chapter 2; Jo et al., 2020), it was similarly shown that no significant difference could be observed in the eDNA degradation rates between freshwater and seawater. However, the results of degradation experiments using sea and pond water have shown that the eDNA degradation rate is slower in the sea (Chapter 3). Collins et al. (2018) showed that eDNA degradation was lower in ocean-influenced offshore waters than in terrestrially influenced inshore environments. Seawater is generally characterized by higher salinity and ionic content, higher pH, and more stable temperatures compared to freshwater, and can promote DNA preservation in water (Collins et al., 2018; Okabe & Shimazu 2007; Schulz & Childers 2011). Furthermore, freshwater and seawater differ in salinity and contents (microorganisms, enzymes, etc.) and are thought to have different degradation factors and mechanisms. Therefore, in this study, I focused on salinity and water content to examine their effect on eDNA degradation. With this aim, I first adjusted the salinity of the pond water by adding artificial seawater powder. Similarly, I tested the

effect of water dilution on eDNA degradation, as this can potentially reduce the factors, such as enzymes and microbes, that lead to eDNA degradation.

The aim of this study was to observe and compare the effects of salinity and water dilution on the eDNA degradation rate in freshwater environments. To understand the degradation of each DNA source, such as individual-derived, cell-derived, and fragmental DNA (Chapter 3), I evaluated the effects of salinity and dilution on eDNA detection while considering the fragmental eDNA, free cell-derived eDNA, and eDNA derived from the resident species of the pond.

4.2 Materials and Methods

4.2.1 Experimental Design

I collected pond water, diluted pond water, and salined pond water and divided each water sample into bottles (Wide-Mouth Bottle, 500 mL; AS ONE, Osaka, Japan) (Figure 4.1). I collected the pond water from an artificial pond in Kobe (the same pond used in Chapter 3). A solution of isolated cells (from *Oncorhynchus kisutch*) and fragmental DNA (from an internal positive control [IPC, 207-bp, 1.5×10^5 copies; Nippon Gene, Tokyo, Japan]) was added to each bottle (Figure 4.1). The pond water contained the eDNA of the resident common carp (*Cyprinus carpio*). I used *O. kisutch* tissue for the isolation of cells because this species is not distributed in the pond. I conducted the experiment for seven days. Water samples (500 mL) from each bottle were filtered and collected using a Sterivex filter (0.45 μm pore size; Merck Millipore, Burlington, MA, USA; Figure 4.1). After extracting eDNA from the Sterivex filter, the copy number of each type of DNA contained in the Sterivex samples and filtrate was estimated by qPCR (Figure 4.1).

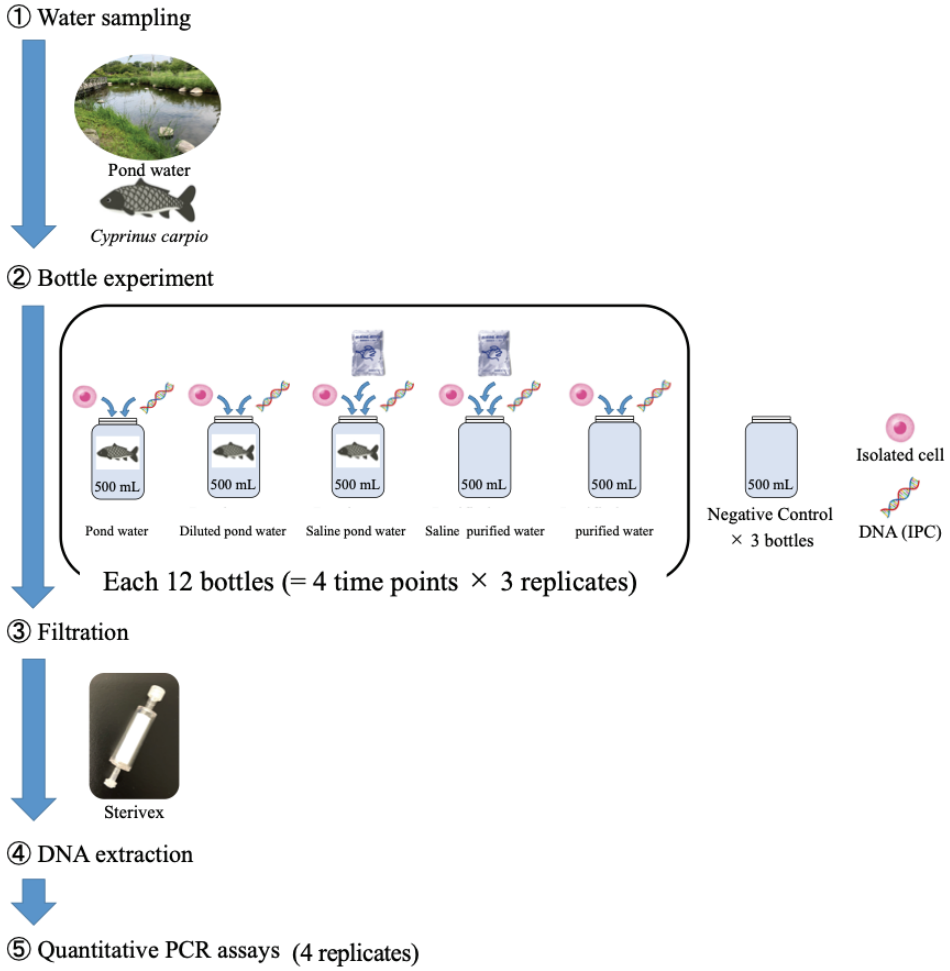

Figure 4.1. Experimental overview of the bottle experiments. I collected pond water, purified water, diluted pond water, saline pond water, and saline purified water and divided each type of water into 12 bottles. A solution of isolated cells (from *Oncorhynchus kisutch*) and fragmental DNA (IPC) was added to each bottle. The pond water was expected to contain the eDNA of *Cyprinus carpio*. I used *O. kisutch* tissue for the isolation of cells. I conducted the experiment for seven days. A Sterivex filter was used to filter 500-mL samples of water from each bottle. After extracting eDNA, the copy number of each type of DNA was estimated by qPCR.

4.2.2 Bottle Experiment

I collected the pond water from an artificial pond in Kobe, Japan (34º39' 40" N, 135º 13' 02" E) on July 16, 2020, using bleached tanks. I measured the salt concentration (salinity) and temperature of the collected water using a salinity meter (CD-4307SD; Mother Tool, Nagano, Japan) and a thermometer (ProODO; YSI, Tokyo, Japan), respectively. The salt concentration (salinity) and water temperature at the time of the water collection were 0.04 and 26.5 ºC, respectively.

For the saline water, artificial seawater powder (Marine Art BR; Osaka Yakken, Osaka, Japan) was added to the pond water and purified water to increase the salinity to 3.3, the mean seawater salinity around Japan. For the diluted pond water, the pond water and purified water (A300; AS ONE) were mixed at a ratio of 1:9. The pond water, purified water, diluted pond water, saline pond water, and saline purified water were each divided into 12 bottles (4 time points × 3 replicates) for 500 mL each. The bottles and equipment were sterilized with 10 % commercial bleach (ca. 0.6 % hypochlorous acid) (KAO, Tokyo, Japan) and washed with DNA-free distilled water to avoid DNA contamination.

Each bottle received 100 µL of a solution of isolated cells [equivalent to 1.0×10^5 copies of cells] and DNA (1.0×10^5 copies) (IPC). The bottles were incubated in the laboratory at about 25 ºC for a week. I collected and filtered 500 mL of the water from each bottle using 0.45-µm Sterivex filters (Merck Millipore) at 0, 3, 12, and 168 h (day 7) after the introduction of the cells and DNA. After filtration, approximately 2 mL of RNAlater (Thermo Fisher Scientific, Waltham, MA, USA) was injected into the Sterivex. As a filtration blank, the 500 mL of DNA-free water was filtered in the same manner after filtration of the samples to monitor cross-contamination. The Sterivex filters were immediately stored at –20 ºC until further analysis.

4.2.3 DNA Extraction

DNA was extracted from the Sterivex filter using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following Miya et al. (Miya et al.,2016) and Minamoto et al. (Minamoto et al., 2021). The RNAlater was removed using a 50-mL syringe, and 440 µL of the mixture (220 µL of phosphate-buffered saline, 200 µL of Buffer AL, and 20 µL of proteinase K [Qiagen]) was added to the Sterivex filter. I incubated the filters on a rotary shaker (AS ONE) at 20 rpm for 20 min in a 56 °C dry oven. I transferred the incubated mixture into a new 1.5 mL tube by centrifugation at 5000 g for 5 min. I then purified the mixture using a DNeasy Blood and Tissue Kit, and finally eluted the DNA in 100 µL of buffer AE from the kit. The extracted DNA from both methods was stored at –20 ºC until qPCR analysis.

4.2.4 Quantitative PCR Assays

I performed the qPCR analysis for *C. carpio* (Takahara et al., 2012), *O. kisutch*, and the IPC (Chapter 3) (Table 4.1). I quantified the DNA concentrations by qPCR using a PikoReal™ qPCR system (Thermo Fisher Scientific). Each TaqMan reaction contained 900 nM of forward and reverse primers and 125 nM of TaqMan probe in 1× TaqPath™ qPCR master mix (Thermo Fisher Scientific). To this, 2 µL of the sample template was added to reach a final volume of 10 µL. A four-step dilution series containing 1.5×10^1 to 1.5×10^4 copies was prepared and used as quantification standards. For the standard curves, we used target DNA cloned into a plasmid.

Table 4.1. Primers and probes for the targeted DNA used in the experiment

Target	Primer- probe	Sequence
Cyprinus carpio	primer F	5'-GGTGGGTTCTCAGTAGACAATGC-3'
	primer R	5'-GGCGGCAATAACAAATGGTAGT-3'
	probe	5'-(FAM)-CACTAACACGTTCCACTTCC-(TAMRA)-3'
Oncorhynchus kisutch	primer F	5'-GCACCGGAAGCACTGTTATA-3'
	primer R	5'-TTTGTGCTCGTGGGACTTT-3'
	probe	5'-(FAM)-CCACTGCTGGCGTAGCTTA-(TAMRA)-3'
IPC	primer F	5'-CCGAGCTTACAAGGCAGGTT-3'
	primer R	5'-TGGCTCGTACACCAGCATACTAG-3'
	probe	5'-(FAM)-TAGCTTCAAGGGCTGTCGGC-(TAMRA)-3'

A qPCR was performed with the following conditions: 2 min at 50 °C, 10 min at 95 °C, and 55 cycles of 15 s at 95 °C and 1 min at 60 °C. Four replicates were performed for each sample, and four replicate negative non-template controls (NTC) containing DNA-free water instead of template DNA were included in all PCR plates. I performed the qPCR procedures according to the MIQE checklist (Bustin et al., 2009). The PCR and qPCR were set up in two separate rooms to avoid DNA contamination.

The qPCR results were analyzed using PikoReal software ver. 2.2.248.601; Thermo Fisher Scientific). The R^2 values of the standard curves ranged from 0.985–0.998, and the PCR varied from 91.07–101.68 %. The concentration of DNA in the water collected (DNA copies mL^{-1}) was calculated based on the volume of filtered water. DNA copy numbers were evaluated including negative amplifications set as zero values. In our previous study (Chapter 3), I have already performed a limit of detection (LOD) test for the PCR assay, which resulted in one copy for the LOD.

4.2.5 Statistical Analysis

Statistical analysis and data plotting were performed using R software version 3.6.0 (R Core Team, 2019). I used the Single First-Order rate model (SFO) as the degradation model because the SFO was the most effective model of degradation in Chapter 3. The SFO establishes a simple procedure for determining a first-order rate constant from the degradation. The model equation is as follows:

$$C = C_0 e^{kt} (\text{model 1, SFO}), \quad (1),$$

where C is the eDNA concentration at time t, C0 is the eDNA concentration at time 0 (i.e., the initial eDNA concentration), and k is the degradation rate constant per hour. I performed modeling using the “mkin” package version 0.9.49.8 in the R software. I evaluated the fit of the models using the chi-squared error level (Boesten et al., 2005). In this study, significant differences in the model coefficients were evaluated by overlapping the 95 % confidence intervals (CIs) of the coefficients (i.e., $\alpha = 0.05$). This means that there is a significant difference between the slopes. However, when the 95 % CIs overlap, there is no significant difference.

Table 4.2 Fitting of the SFO model using a chi-squared error level. The error level in the table indicates the chi-squared error level. In "Water" PW, pond, Pond +Salt, and Pond 10% indicate the purified water, pond water, saline pond water, and diluted pond water. In "Target" IPC, Okis, and Cyca indicate the fragment DNA of the IPC, the DNA from *Oncorhynchus kisutch* cells, and the eDNA of *Cyprinus carpio*.

Water	Target	Error level
PW	IPC	0.4198997
PW	Okis	0.1180956
SW	IPC	0.9520691
SW	Okis	0.3286611
Pond	IPC	0.07503389
Pond	Okis	0.00315666
Pond	Cyca	0.3159354
Pond + Salt	IPC	0.632964
Pond + Salt	Okis	0.05493841
Pond + Salt	Cyca	0.365181
Pond 10 %	IPC	0.4563734
Pond 10 %	Okis	0.0555217
Pond 10 %	Cyca	0.1001298

4.3 Results

4.3.1 Degradation of eDNA in Saline Pond Water

I detected all the targeted DNA of *C. carpio*, *O. kisutch* cells, and IPC using qPCR in saline pond water (Figure 4.2, the data in Supplemental Table 4.1). The degradation rates in the saline pond water were significantly lower than those in the regular pond water for all three DNA sources (Figures 4.2 and 4.3). I could not detect the eDNA of *C. carpio*, *O. kisutch* cells, or the IPC on day seven in pond water. However, I detected the eDNA of *C. carpio* and IPC in saline pond water up to day seven. In the IPC results of saline pond water, immediately after the addition of IPC (0 h), DNA was detected at approximately 1/100 of the amount added. However, after 3 and 12 h, DNA equivalent to the amount added was detected. After 168 h, approximately 1,000 copies of DNA were detected, although they were degraded when compared to the amount added. There were no amplifications from the filter, extraction blanks, or NTCs in this experiment or in the following experiments.

The fit of the models was evaluated using the chi-squared error level (Table 4.2) and showed lower error levels in IPC and cell DNA in pond and pond 10 % water. The degradation rate constant (k) of the cells, IPC, and *C. carpio* were significantly different between the saline pond and pond samples when comparing the 95 % CIs (Figure 4.3). The degradation rates of the saline pond were significantly lower than those of the pond water for all three DNA sources.

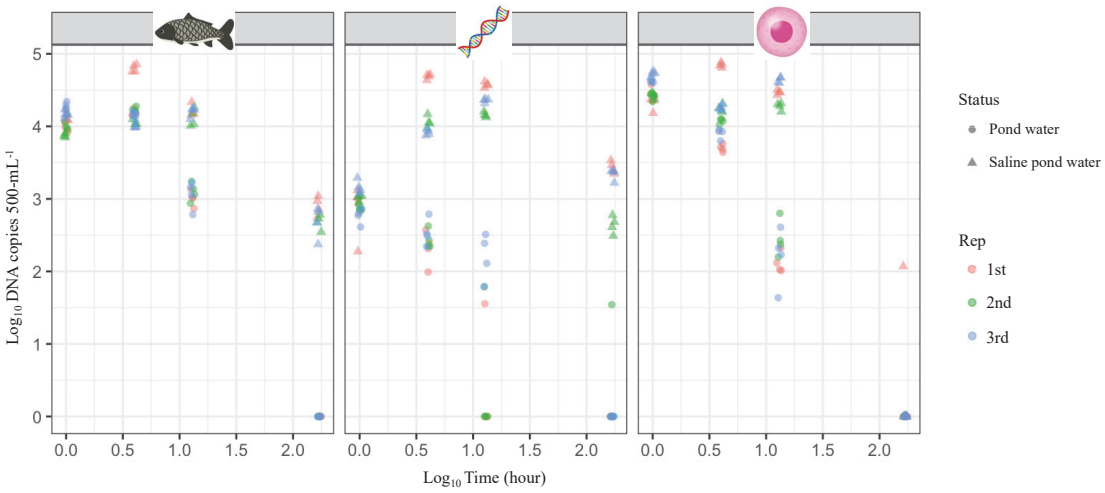

Fig 4.2. Relationship between the eDNA concentrations of the water source (pond water and saline pond water). The dots indicate the eDNA concentrations of the targets at each time point under two water conditions: pond water, circles; saline pond water, triangles (N = 12 for each time point: 1st replication, red; 2nd replication, green; 3rd replication, blue).

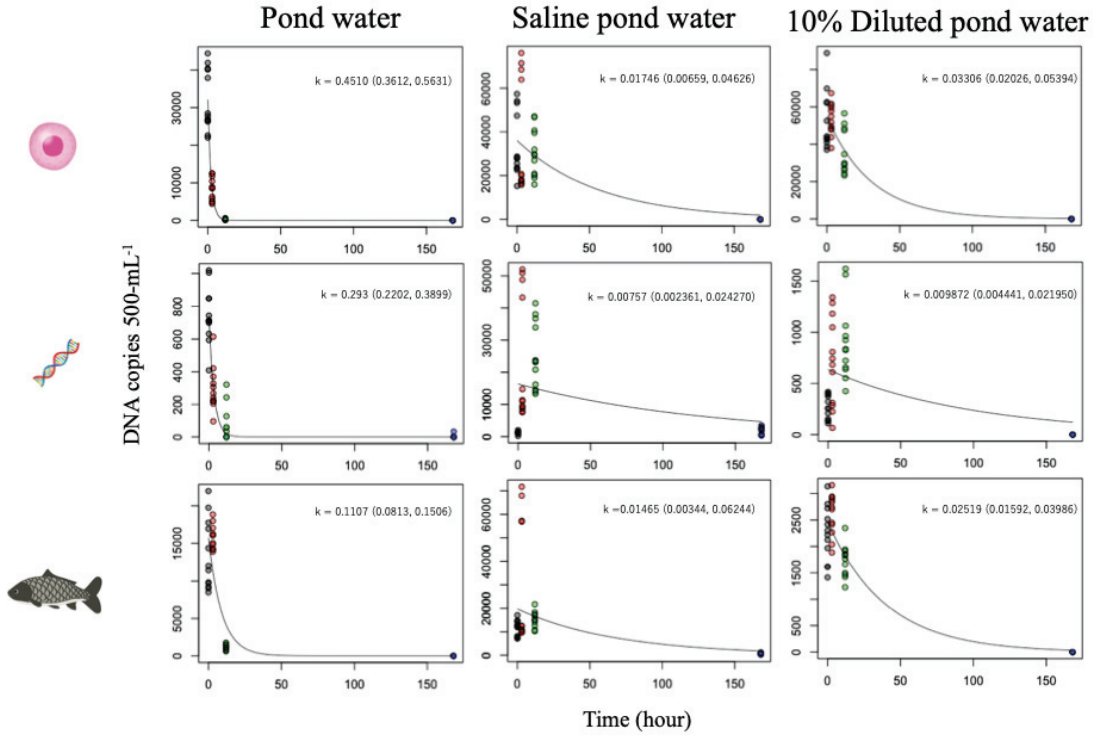

Figure 4.3. Degradation curves of the Single-First Order (SFO) model and the rate constant for the bottle experiments. DNA copies (Y-axis) showed different magnitudes. The dots indicate the eDNA concentrations of the targets (*Cyprinus carpio*, the internal positive control [IPC], and *Oncorhynchus kisutch* cells) at each time point with different colors (Gray: 0 h, Red: 3 h, Green: 12 h, Blue: 168 h, N = 12 for each time point). The left degradation curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in the pond samples. The center decay curves show each target (*O. kisutch* cells, the IPC, and *C. carpio*) in the saline pond samples. The right decay curves show each target (*O. kisutch* cells and the IPC) of the diluted pond samples. The slope k is the degradation rate constant of the Single-First Order model, and the numbers in parentheses are the 95 % confidential intervals below and above the slope k.

4.3.2 Degradation of eDNA in Saline Purified Water

I detected all the targeted DNA of the *O. kisutch* cells and IPC using qPCR in saline purified water (Figure 4.4). I detected *O. kisutch* cells and IPC DNA in purified water and saline purified water, respectively, up to 168 h.

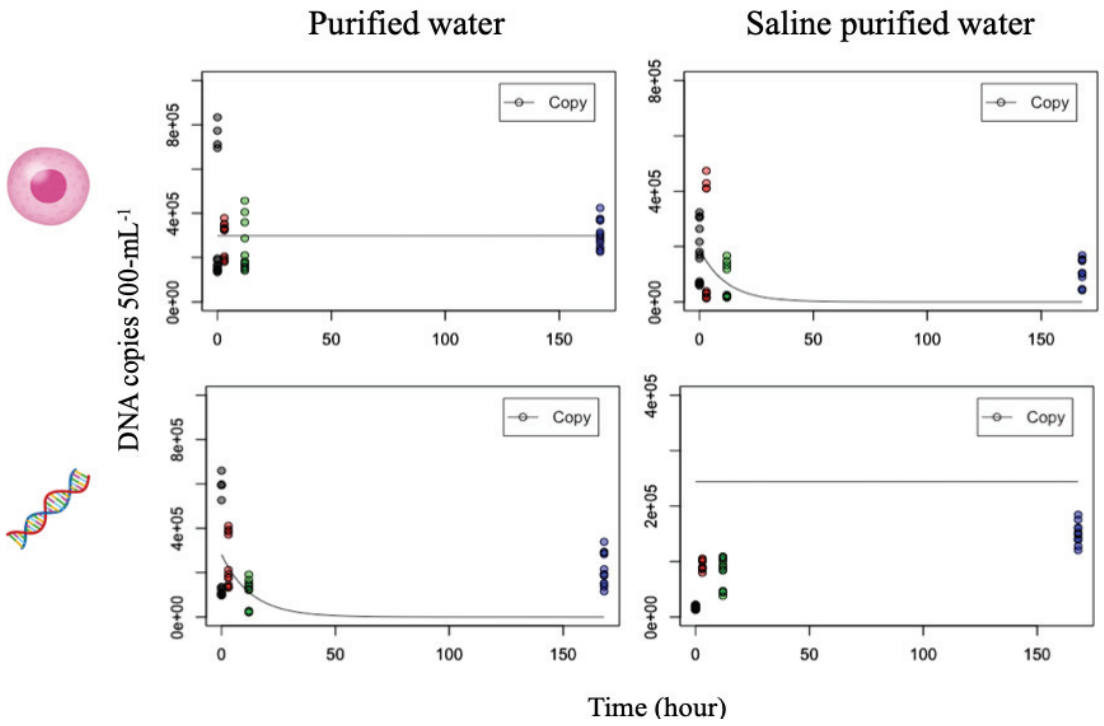

Figure 4.4. Degradation curves of the Single-First Order model (SFO) for the bottle experiments in the saline purified water and purified water. The dots indicate the eDNA concentrations of the targets (the internal positive control [IPC] and *Oncorhynchus kisutch* cells) at each time point with different colors (Gray: 0 h, Red: 3 h, Green: 12 h, Blue: 168 h, N = 12 for each time point). The left degradation curves show each target (*O. kisutch* cells and the IPC) in the purified water samples. The right decay curves show each target (*O. kisutch* cells and the IPC) in the saline purified water samples.

4.3.3 Degradation of eDNA in Diluted Pond Water

I detected three of the targeted DNA types, *C. carpio*, *O. kisutch* DNA from the cells, and the IPC, using qPCR in diluted pond water (Figure 4.2, Supplemental Table 4.2). The degradation rates of the diluted pond were significantly lower than those of the pond water for all three DNA sources (Figure 4.3). For *C. carpio*, results of diluted pond water showed that DNA was reduced to 1/10 of the initial concentration (0 h) compared to that of the pond water. In the results for pond *C. carpio*, DNA copies decreased to approximately 1/10 after 12 hours, whereas in the diluted pond, almost no degradation was observed even after 12 hours. For the diluted pond, the results of IPC and cell showed that little to no degradation occurred after 12 hours of addition. At 168 h, we could not detect the DNA of *C. carpio*, or *O. kisutch* cells, and the IPC in both pond and diluted pond water.

The degradation rate constant (k) of the cells, IPC, and *C. carpio* were significantly different between the diluted pond and pond samples when comparing the 95 % CIs (Figure4.3). The degradation rates in the diluted pond water were significantly lower than those in the pond water for all three DNA sources.

4.4 Discussion

I found that the DNA concentrations of the *C. carpio* and *O. kisutch* cells, and the IPC did not decline exponentially in both the saline purified water and purified water samples. Furthermore, I detected the DNA in the saline purified water. This result showed that the increased salinity in the saline sample did not have any effect on DNA detection. Several studies have observed the degradation of eDNA, but most of them have focused on temperature, and few have obtained data on pH and salinity (Chapter 2; Jo et al., 2020). In this study, I specifically focused on salinity, controlled the salinity, and diluted the environmental water affected the degradation of eDNA.

The degradation rates in the saline pond samples were significantly lower than those in the pond water for all three DNA sources. This result might suggest that salinity suppresses the degradation of eDNA. A previous study (Collins et al., 2018) found salinity to be a better predictor of eDNA decay than pH, with salinity varying between locations. In addition, Collins et al. (Collins et al., 2018) showed that eDNA degradation was lower in ocean-influenced offshore waters than in terrestrially influenced inshore environments. However, salinity itself may not be completely responsible for the differences in degradation rate, but rather may be related to the amount of microorganisms present and their communities. In fact, the characteristics of the microorganisms involved in DNA degradation may vary depending on the environment. For example, microorganisms that live in freshwater are susceptible to salinity and cannot adapt to rapid environmental changes caused by the addition of salt, which is thought to reduce their activity and suppress DNA degradation. Therefore, additional evaluation of the composition of microorganisms and DNA enzymes in salt water is necessary for a better understanding of eDNA degradation from the present results.

The degradation rates in the diluted pond samples were significantly lower than those in the pond water for all three DNA sources. In the pond water diluted 10 times, the initial DNA concentration was reduced to one-tenth, and the degradation rate was slower than that in the pond water. In the pond samples, the amount of DNA was reduced to at least 1/10 after 12 hours, whereas in the diluted pond samples, very little degradation occurred even after 12 hours. The reduction in the rate of degradation preserved more DNA than the disadvantage of reducing the initial concentration to 1/10 for all sources. This is thought to be due to the dilution of the degradation factors as well as the eDNA. Takasaki et al. (2021) showed that pre-filters that remove humic substances, such as humic acid and fulvic acid, are effective in detecting eDNA. The results showed that simply diluting eDNA without removing its degraders and inhibitors was effective. However, if the amount of DNA in the environmental water is already low, such as in the case of endangered or rare species, dilution may be non-detectable. In addition, DNA was not detected after seven days. Therefore, it was found that even if the concentration of the degradation factor is low, the degradation progresses over time.

In this study, insights into eDNA degradation were achieved, but additional analyses need to be performed. In particular, DNA metabarcoding can reveal how the water contents (microorganisms and enzymes) in the environment are influenced by the addition of salt, and whether there is a change in the composition of microorganisms. Based on the changes in microbial composition, it may be possible to estimate which microorganisms are involved in the degradation of eDNA. In addition, it is unclear whether similar DNA degradation rates exist seasonally and in other aquatic habitats, such as rivers and wetlands. Experiments using a selection of site replicates from various habitats need to be performed to achieve a more generalized understanding of eDNA degradation. The evaluation of eDNA degradation while

comparing different environmental conditions (e.g., salinity, water temperature, pH, chlorophyll, and microorganism population) may reveal what affects eDNA degradation in general.

In conclusion, increased salinity in the saline sample had no effect on DNA detection. The degradation rates of the saline pond and pond samples were significantly different. Furthermore, the degradation rates of the diluted pond and pond samples were significantly different. The reduction in the rate of degradation preserved more DNA than the disadvantage of reducing the initial concentration to 1/10 for all sources.

The evaluation of eDNA under different environmental conditions (e.g., salinity, water temperature, pH, chlorophyll, and microorganism community) can reveal the causes of its degradation (Chapter 2; Collins et al., 2018; Thomsen et al., 2012; Barnes et al., 2014; Tsuji et al., 2017; Jo et al., 2019; Tsuji et al., 2017; Maruyama et al., 2014; Jo et al., 2020). A greater understanding of and the accumulation of basic information about eDNA would improve eDNA analysis methods and enable researchers to maximize their potential.

4.5 Supplementary Materials

Supplementary Table 4.1 and 4.2

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Chapter 5. General discussion

Over the last decade, the application of eDNA for biological monitoring of various species and environments has flourished (Taberlet et al., 2012). However, false positive/negative detection and various errors in experimental procedures hindered the reliability of eDNA detection in the field (Darling & Mahon, 2011; Furlan et al. 2016; Dorazio & Erickson, 2018; Doi et al. 2019). Errors related to eDNA detection can mislead inferences regarding the presence or absence of species in the field. Those related to eDNA quantification may weaken the correlation between eDNA concentration and species biomass/abundance, leading to uncertain inferences (Yates et al., 2019). To overcome these uncertainties in eDNA analysis, including the physicochemical and molecular states and processes of production, transport, and degradation, the characteristics and degradation of eDNA must be better understood (Strickler et al., 2015; Barnes & Turner, 2016; Hansen et al., 2018). In this thesis, I studied degradation of eDNA from multiple perspectives using a meta-analytical approach, described in Chapters 2, and an experimental approach, described in Chapters 3 and 4. In Chapter 3, I added the multiple DNA sources, i.e., cells and fragmented DNA, to the bottle and observed their DNA degradations. Such experiment containing multiple DNA sources has not been performed before, and by comparing the environmental DNA, fragment DNA, and cell-derived DNA, the detailed state of the eDNA can be inferred. As a result, the basic understanding of the state and degradation of eDNA was deepened, and new clues to resolve the uncertainty in the detection of eDNA were obtained.

In Chapter 2, I investigated eDNA degradation from a meta-analytical approach; I then performed eDNA degradation prediction simulation based on the results. The meta-analysis results showed that eDNA degradation was accelerated at higher water temperatures and longer

amplicon lengths. These results generally supported the effect of water temperature on the eDNA degradation rate in previous hypotheses for each condition and species (e. g., Strickler et al., 2015; Eichmiller et al., 2016; Lance et al., 2017; Tsuji et al., 2017; Jo et al., 2019; Kasai et al., 2020). Previous studies have assumed that water temperature does not directly affect eDNA degradation, rather indirectly affecting it through enzymatic hydrolysis by microbes and extracellular nucleases (reviewed in Barnes and Turner, 2016). At high temperatures, with increasing activity of microorganisms and extracellular enzymes, eDNA in water degrades more quickly (reviewed in Barnes and Turner, 2016). The meta-analysis results showed no significant differences between laboratory water (purified or tap) and environmental water (seawater or freshwater). This may indicate that the enzymes and bacteria of the experimental organisms affected the eDNA degradation. Evidence from previous studies suggests that long amplicon lengths are less likely to be detected in eDNA samples than in short amplicons (Jo et al., 2017). The meta-analysis supported these results. A possible explanation is provided by Jo et al. (2017), who suggested that the DNA degradation rate was higher for longer amplicon lengths (719 bp) than for shorter amplicons (127 bp). The simulation by QM indicated that shorter amplicon lengths were more likely to be detected when eDNA degradation was less affected by water temperature. When the eDNA degradation rates were very fast or very slow due to water temperature (e.g., 15–35 °C or 0–5 °C, respectively), the amplicon length had a smaller effect on eDNA degradation than in other water temperature ranges. At higher temperatures, microbial activity for DNA degradation occurred rapidly on both large and short DNA fragments, such that both classes of fragments were not detectable by either a large or small fragment amplicon assay at a similar rate. However, at colder temperatures, both fragment classes are degraded at lower rates, and thus it is possible that the longer fragments can last longer than under warmer conditions, thus remaining detectable for longer (suggesting a slower

decay rate).

Based on these results, I performed a simulation to predict the eDNA degradation. This allowed us to understand in advance how much degradation would occur based on water temperature at the time of field sampling and the amplicon length of the primers used. In this study, only water temperature and DNA amplicon length were analyzed, but as research on eDNA degradation progresses and more data are accumulated, it may become possible to conduct simulations that take various factors into account.

In Chapter 3, I found that the eDNA derived from cells and fragmented DNA declined exponentially after being added to both sea and pond water samples. The eDNA from resident species showed similar behavior to the eDNA derived from cells. In most cases, a simple exponential model could be used to evaluate the degradation. The degradation rates of the eDNA derived from the resident species at each site, *C. carpio* and *T. japonicus*, were not significantly different from those of the *O. kisutch* cells. This result suggests that the degradation of the organisms' eDNA in water displayed similar behavior to that of eDNA derived from free cells. Previous studies found that the most abundant eDNA size ranged from 1 to 10 μm and concluded that eDNA is mainly derived from cells or cellular organs (Minamoto et al., 2016; Turner et al., 2014). These findings indirectly support the possibility that eDNA is mainly derived from cells. The fragmented DNA, IPC, degraded significantly faster in the pond than in the sea. Water salinity was found to be a significant factor affecting degradation rates. For example, higher salinity sites had slower degradation rates in marine sites with salinity gradients (Collins et al., 2018). The present study supports the results of Collins et al. (2018) by experimentally comparing freshwater ponds and seawater.

In Chapter 4, I observed and compared the effects of salinity and water dilution on the eDNA degradation rate in freshwater environments. I found that the DNA concentrations of the

C. carpio and *O. kisutch* cells and IPC did not decline exponentially in both the saline-purified water and purified water samples. Furthermore, I detected DNA in saline-purified water. This result showed that increased salinity in the saline sample did not affect DNA detection. The degradation rates in the saline pond samples were significantly lower than those in the pond water for all three DNA sources. These results suggest that salinity suppressed eDNA degradation. A previous study (Collins et al., 2018) found salinity to be a better predictor of eDNA decay than pH, with salinity varying between locations. Additionally, Collins et al. (Collins et al., 2018) showed that eDNA degradation was lower in ocean-influenced offshore waters than in terrestrially influenced inshore environments. However, salinity itself may not be completely responsible for the differences in degradation rates, but may be related to the amount of microorganisms present and their communities. The degradation rates in the diluted pond samples were significantly lower than those in the pond water for all three DNA sources. In the pond water diluted 10 times, the initial DNA concentration was reduced to one-tenth, and the degradation rate was slower than it was in the pond water. In the pond samples, the amount of DNA was reduced to at least 1/10 after 12 h, whereas in the diluted pond samples, very little degradation occurred even after 12 h. The reduction in the degradation rate preserved more DNA than the disadvantage of reducing the initial concentration to 1/10 for all sources. This is thought to have been caused by the dilution of the degradation factors as well as the eDNA.

In this study, I examined, validated, and analyzed eDNA from multiple perspectives through meta-analysis and degradation experiments. Although studies relating to eDNA characteristics and dynamics increase yearly, there are still few relative to eDNA studies overall. However, its practical applicability is still in its infancy because of the uncertainty of detection sensitivity and quantification accuracy of eDNA in the field (Yates et al., 2019), as well as because of the lack of information on populations other than presence/absence and/or

abundance (Hansen et al., 2018). Elucidating these features will enable eDNA analysis to be a more useful, reliable, and practical tool for biodiversity and ecosystem monitoring and stock assessment in the future. Better understanding of and more basic information about eDNA will improve eDNA analysis methods and enable researchers to maximize the potential of future eDNA methods. As eDNA data accumulate, I will be able to simulate various ecosystem events. This simulation will be updated by adding the accumulated data over a few years, and there is a possibility that new findings will be obtained. The results obtained in this study will lead to the accumulation of basic information on eDNA and contribute to the elucidation of the characteristics and degradation mechanism of eDNA. The findings of this thesis provide important groundwork for innovating eDNA analysis for biodiversity monitoring, ecological assessment, and resource management in the future.

In conclusion, I investigated the degradation of eDNA using a meta-analytical approach, and based on these results, I performed simulation and modelling to predict eDNA degradation. My meta-analysis results showed that eDNA degradation was accelerated at higher water temperatures and longer amplicon lengths. I found that the eDNA derived from cells and fragmented DNA declined exponentially after being added to both seawater and pond water samples. The eDNA from resident species showed similar behavior to the eDNA derived from cells. In most cases, a simple exponential model can be used to evaluate the degradation. Increased salinity in the saline sample had no effect on DNA detection. The degradation rates of the saline pond and pond samples were significantly different. Furthermore, the degradation rates of the diluted pond and pond samples were significantly different. The reduced degradation rate preserved more DNA than the disadvantage of reducing the initial concentration to 1/10 for all sources. Understanding eDNA states and degradation is essential for the effective sampling and storage of eDNA, and may provide pertinent information for

better interpretation of species distribution, abundance, and biomass estimation results. By predicting the amount of eDNA degradation, I can estimate, for example, how much eDNA will be degraded by the time the water sample has been transported to the laboratory. If the amount of such degraded eDNA is not taken into consideration, species distribution and abundance/biomass may be underestimated, especially for low-density species such as rare and endangered species. Thus, I can apply the understanding and suppression of eDNA degradation to the detection of trace amounts of eDNA. Similarly, I can apply the understanding of invasive distribution by eDNA because it is important to detect alien species in the early stages of invasion, when their abundance, that is, eDNA concentration, may be low. Considering the rapid eDNA degradation in water, it is important to suppress any decomposition after obtaining the water sample. Understanding the characteristics and dynamics of multiple eDNA in this thesis contribute to the further enrichment of basic information on eDNA analysis. Moreover, the new applicability of the analyses of multiple eDNA will be important for the development of future eDNA analyses for population and community ecology.

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References

Alter S.E., Newsome S.D., Palumbi S.R. (2012). Pre-whaling genetic diversity and population ecology in Eastern Pacific gray whales: insights from ancient DNA and stable isotopes. PLOS ONE, 7, e35039.

Andruszkiewicz, E.A., Sassoubre, L.M., Boehm, A.B. (2017). Persistence of marine fish environmental DNA and the influence of sunlight. PLOS ONE, 12, e0185043.

Antao, T., Perez-Figueroa, A., Luikart, G. (2011). Early detection of population declines: high power of genetic monitoring using effective population size estimators. Evolutionary Applications, 4, 144–154.

Balkenhol, N., Landguth, E.L. (2011). Simulation modelling in landscape genetics: on the need to go further. Molecular Ecology, 20, 667–670.

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L., Lodge, D.M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. Environmental Science and Technology, 48, 1819–1827.

Barnes, M.A., Turner, C.R. (2016). The ecology of environmental DNA and implications for conservation genetics. Conservation Genetics, 17, 1–17.

Begon, M., Townsend, C.R. (2020). Ecology: from Individuals to Ecosystems. John Wiley & Sons.

Boesten, J.J.T.I., Aden, K., Beigel, C., Beulke, S., Dust, M., Dyson, J.S., Fomsgaard, I.S., Jones, R.L., Karlsson, S., Van der Linden, A.M.A., Richter, O., Magrans, J.O., Soulas, G. (2005). Guidance document on estimating persistence and degradation kinetics from environmental fate studies on pesticides in EU registration. Report of the FOCUS Work Group on Degradation

Kinetics, EC Doc. Ref. Sanco/10058/2005, version 1.

Boussarie, G., Bakker, J., Wangensteen, O.S., Mariani, S., Bonnin, L., Juhel, J.B., Kiszka, J.J., Kulbicki, M., Manel, S., Robbins, W.D., Vigliola, L. (2018). Environmental DNA illuminates the dark diversity of sharks. Scientific Advances 4, eaap9661.

Brekke, P., Bennett, P.M., Santure, A.W., Ewen, J.G. (2011). High genetic diversity in the remnant island population of hihiand the genetic consequences of re-introduction. Molecular Ecology, 20, 29–45.

Bruford, M.W., Ancrenaz, M., Chikhi, L. Lackman-Ancrenaz, I., Andau, M., Ambu, L., Goossens, B. (2010). Projecting genetic diversity and population viability for the fragmented orangutan population in the Kinabatangan floodplain, Sabah, Malaysia. Endangered Species Research 12, 249–261.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J. (2009). The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry 55, 611– 622.

Bylemans, J., Furlan, E.M., Hardy, C.M., McGuffie, P., Lintermans, M., Gleeson, D.M. (2017). An environmental DNA-based method for monitoring spawning activity: A case study, using the endangered Macquarie perch (*Macquaria australasica*). Methods in Ecology and Evolution 8, 646–655.

Bylemans, J., Furlan, E.M., Gleeson, D.M., Hardy, C.M., Duncan, R.P. (2018). Does size matter? An experimental evaluation of the relative abundance and decay rates of aquatic environmental DNA. Environmental Science and Technology, 52, 6408-6416.

Campos, P.F., Kristensen, T., Orlando, L. Sher, A., Kholodova, M.V., Götherström, A., Hofreiter, M., Drucker, D.G., Kosintsev, P., Tikhonov, A., Baryshnikov, G.F. (2010). Ancient DNA sequences point to a large loss of mitochondrial genetic diversity in the saiga antelope (*Saiga*

tatarica) since the Pleisocene. Molecular Ecology 19, 4863–4875.

Caughley, G. (1994). Directions in conservation biology. Journal of Animal Ecology, 63, 215–244.

Chalde, T., Nardi, C.F., Fernandez, D.A. (2019). Early warning: detection of exotic coho salmon (*Oncorhynchus kisutch*) by environmental DNA and evidence of establishment at the extreme south of Patagonia. Canadian Journal of Fisheries and Aquatic Sciences, 76, 2343–2349

Collins, R.A., Wangensteen, O.S., O'Gorman, E.J., Mariani, S., Sims, D.W., Genner, M.J. (2018). Persistence of environmental DNA in marine systems. Communications Biology, 1, 185.

Cook, C.N., Mascia, M.B., Schwartz, M.W., Possingham, H.P., Fuller, R.A. (2013). Achieving conservation science that bridges the knowledge-action boundary. Conservation Biology, 27, 669–678.

Cowart, D.A., Murphy, K.R., Cheng, C.H.C. (2018). Metagenomic sequencing of environmental DNA reveals marine faunal assemblages from the West Antarctic Peninsula. Mar. Genomics. 37, 148-160.

Darling, J.A., Mahon, A.R. (2011). From molecules to management: adopting a DNA-based methods for monitoring biological invasions in aquatic environments. Environmental Research, 111, 978-988.

Deiner, K., Bik, H.M., Mächler, E., Seymour, M., Lacoursière‐Roussel, A., Altermatt, F., Creer, S., Bista, I., Lodge, D.M., De Vere, N., Pfrender, M.E. (2017). Environmental DNA metabarcoding: Transforming how we survey animal and plant communities. Molecular

Ecology, 26, 5872-5895.

Deiner, K., Renshaw, M.A., Li, Y., Olds, B.P., Lodge, D.M., Pfrender, M.E. (2017). Long-range PCR allows sequencing of mitochondrial genomes from environmental DNA. Methods in Ecology and Evolution, 8, 1888-1898.

Doi, H., Inui, R., Akamatsu, Y., Kanno, K., Yamanaka, H., Takahara, T., Minamoto, T. (2017). Environmental DNA analysis for estimating the abundance and biomass of stream fish. Freshwater Biology 62, 30–39.

Doi, H., Katano, I., Sakata, Y., Souma, R., Kosuge, T., Nagano, M., Ikeda, K., Yano, K., Tojo, K. (2017). Detection of an endangered aquatic heteropteran using environmental DNA in a wetland ecosystem. Royal Society Open Science 4, 170568.

Doi, H., Uchii, K., Matsuhashi, S., Takahara, T., Yamanaka, H., Minamoto, T. (2017). Isopropanol precipitation method for collecting fish environmental DNA. Limnology and Oceanography Methods, 15, 212-218.

Doi, H., Fukaya, K., Oka, S. I., Sato, K., Kondoh, M., Miya, M. (2019). Evaluationof detection probabilities at the water-filtering and initial PCR steps in environmental DNA metabarcoding using a multispecies site occupancy model. Scientific Reports, 9, 3581.

Dorazio, R.M., Erickson, R.A. (2018). EDNAOCCUPANCY: An R package for multiscale occupancy modelling of environmental DNA data. Molecular Ecology Resources, 18, 368–380.

Egan, S.P., Barnes, M.A., Hwang, C.T., Mahon, A.R., Feder, J.L., Ruggiero, S.T., Tanner, C.E., Lodge, D.M. (2013). Rapid invasive species detection by combining environmental DNA with light transmission spectroscopy. Conservation Letters, 6, 402–409.

Egan, S.P., Grey, E., Olds, B, Feder, J. L., Ruggiero, S. T., Tanner, C. E., Lodge, D. M. (2015). Rapid molecular detection of invasive species in ballast and harbor water by integrating environmental DNA and light transmission spectroscopy. Environment Science and Technology 49, 4113–4121.

Eichmiller, J.J., Bajer, P.G., Sorensen, P.W. (2014). The relationship between the distribution of common carp and their environmental DNA in a small lake. PLOS ONE, 9, e112611.

Eichmiller, J.J., Miller, L.M., Sorensen, P.W. (2015). Optimizing techniques to capture and extract environmental DNA for detection and quantification of fish. Molecular Ecology Resources, 16, 56– 68.

Eichmiller, J.J., Best, S.E., Sorensen, P.W. (2016). Effects of temperature and trophic state on degradation of environmental DNA in lake water. Environment Science and Technology, 50, 1859–1867.

Ejsmond, M.J., Radwan, J. (2011). MHC diversity in bottlenecked populations: a simulation model. Conservation Genetics, 12, 129–137.

Epperson, B.K., McRae, B.H., Scribner, K.I.M., Cushman, S.A., Rosenberg, M.S., FORTIN, M.J., James, P.M., Murphy, M., Manel, S., Legendre, P., Dale, M.R., (2010). Utility of computer simulations in landscape genetics. Molecular Ecology, 19, 3549–3564.

Ficetola, G.F., Miaud, C., Pompanon, F., Taberlet, P. (2008). Species detection using environmental DNA from water samples. Biology Letters 4, 423–425.

Fukumoto, S., Ushimaru, A., Minamoto, T. (2015). Basin-scale application of the environmental DNA assessment for rare endemic species and closely related exotic species in rivers: A case study of giant salamanders in Japan. Journal of Applied Ecology, 52, 358–365.

Furlan, E.M., Gleeson, D., Hardy, C.M., Duncan, R.P. (2016). A framework for estimating the sensitivity of eDNA surveys. Molecular Ecology Resources, 16, 641–654.

Gapare, W.J., Yanchuk, A.D., Aitken, S.N. (2008). Optimal sampling strategies for capture of genetic diversity differ between core and peripheral populations of *Picea sitchensis* (Bong.) Carr. Conservation Genetics, 9, 411–418.

Goldberg, C.S., Sepulveda, A., Ray, A., Baumgardt, J., Waits, L.P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus antipodarum*). Freshwater Science, 32, 792–800.

Hansen, B.K., Bekkevold, D., Clausen, L.W., Nielsen, E.E. (2018). The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. Fish and Fisheries, 19, 751–768.

Hoban, S.M., Schlarbaum, S.E., Brosi, S.L., Romero-Severson, J. (2012). A rare case of natural regeneration in butternut, a threatened forest tree, is parent and space limited. Conservation Genetics, 13, 1447–1457.

Jerde, C.L., Chadderton, W.L., Mahon, A.R., Renshaw, M.A., Corush, J., Budny, M.L., Mysorekar, S., Lodge, D.M. (2013). Detection of Asian carp DNA as part of a Great Lakes basin-wide surveillance program. Canadian Journal of Fisheries and Aquatic Sciences, 70, 522–526.

Jo, T., Murakami, H., Masuda, R., Sakata, M.K., Yamamoto, S., Minamoto, T. (2017). Rapid degradation of longer DNA fragments enables the improved estimation of distribution and biomass using environmental DNA. Molecular Ecology Resources, 17, e25–e33.

Jo, T., Murakami, H., Yamamoto, S., Masuda, R., Minamoto, T. (2019). Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. Ecology and Evolution, 9, 1135–1146.

Jo, T., Arimoto, M., Murakami, H., Masuda, R. Minamoto, T. (2020). Estimating shedding and

decay rates of environmental nuclear DNA with relation to water temperature and biomass. Environmental DNA, 2, 140-151.

Jo, T., Minamoto, T. (2021) Complex interactions between environmental DNA (eDNA) state and water chemistries on eDNA persistence suggested by meta-analyses. Molecular Ecology Resources, 21, 1490-1503.

Kakuda, A., Doi, H., Souma, R., Nagano, M., Minamoto, T., Katano, I. (2019). Detection of environmental DNA of the invasive red-eared slider in ponds for evaluating their distribution in comparison to water quality. PeerJ, 7, e8155.

Kasai, A., Takada, S., Yamazaki, A., Masuda, R., Yamanaka, H. (2020). The effect of temperature on environmental DNA degradation of Japanese eel. Fisheries Research 86, 465–471.

Katano, I., Harada, K., Doi, H., Souma, R., Minamoto, T. (2017). Environmental DNA method for estimating salamander distribution in headwater streams, and a comparison of water sampling methods. PLOS ONE, 12, e0176541.

Lacoursière-Roussel, A., Howland, K., Normandeau, E., Grey, E.K., Archambault, P., Deiner, K., Lodge, D.M., Hernandez, C., Leduc, N., Bernatchez, L. (2018). eDNA metabarcoding as a new surveillance approach for coastal Arctic biodiversity. Ecology and Evolution, 8, 7763–7777.

Lance, R.F., Klymus, K.E., Richter, C.A., Guan, X., Farrington, H.L., Carr, M.R., Thompson, N., Chapman, D.C., Baerwaldt, K.L. (2017). Experimental observations on the decay of environmental DNA from bighead and silver carps. Management of Biological Invasions, 8, 343.

Mächler, E., Osathanunkul, M., Altermatt, F. (2018). Shedding light on eDNA: neither natural levels of UV radiation nor the presence of a filter feeder affect eDNA-based detection of aquatic

organisms. PLOS ONE, 13, e0195529.

Marino, I.A.M., Benazzo, A., Agostin,i C. Mezzavilla, M., Hoban, S.M., Patarnello, T., Zane, L., Bertorelle, G. (2013). Evidence for past and present hybridization in three Antarctic icefish species provides new perspectives on an evolutionary radiation. Molecular Ecology, 22, 5148–5161.

Martellini, A., Payment, P., Villemur, R. (2005). Use of eukaryotic mitochondrial DNA to differentiate human, bovine, porcine and ovine sources in fecally contaminated surface water. Water Research, 39, 541–548.

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., Minamoto, T. (2014). The release rate of environmental DNA from juvenile and adult fish. PLOS ONE, 9, e114639.

Merkes, C.M., McCalla, S.G., Jensen, N.R., Gaikowski, M.P., Amberg, J.J. (2014). Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. PLOS ONE, 9, e113346

Minamoto, T., Yamanaka, H., Takahara, T., Honjo, M. N., Kawabata, Z. (2012). Surveillance of fish species composition using environmental DNA. Limnology, 13, 193–197.

Minamoto, T., Naka, T., Moji, K., Maruyama, A. (2016). Techniques for the practical collection of environmental DNA: filter selection, preservation, and extraction. Limnology, 17, 23–32.

Minamoto, T., Fukuda, M., Katsuhara, K. R., Fujiwara, A., Hidaka, S., Yamamoto, S., Takahashi, K., Masuda, R. (2017). Environmental DNA reflects spatial and temporal jellyfish distribution. PLOS ONE, 12, e0173073.

Minamoto, T., Miya, M., Sado, T., Seino, S., Doi, H., Kondoh, M., Nakamura, K., Takahara, T., Yamamoto, S., Yamanaka, H. Araki, H. (2021). An illustrated manual for environmental DNA research: Water sampling guidelines and experimental protocols. Environmental DNA, 3, 8-13.

Miya, M., Minamoto, T., Yamanaka, H., Oka, S. I., Sato, K., Yamamoto, S., Sado, T., Doi, H. (2016). Use of a filter cartridge for filtration of water samples and extraction of environmental DNA. Journal of Visualized Experiments, 117, e54741.

Motulsky, H.J., Christopoulos, A. (2003). *Fitting models to biological data using linear and nonlinear regression: a practical guide to curve fitting.* GraphPad Software. Inc.: San Diego, CA.

Moushomi, R., Wilgar, G., Carvalho, G., Creer, S., Seymour, M. (2019). Environmental DNA size sorting and degradation experiment indicates the state of Daphnia magna mitochondrial and nuclear eDNA is subcellular. Scientific Reports, 9, 12500.

Nevers, M.B., Byappanahalli, M.N., Morris, C.C., Shively, D., Przybyla-Kelly, K., Spoljaric, A.M., Dickey, J., Roseman, E.F. (2018). Environmental DNA (eDNA): A tool for quantifying the abundant but elusive round goby (*Neogobius melanostomus*). PLOS ONE, 13, e0191720.

Nukazawa, K., Hamasuna, Y., Suzuki, Y. (2018). Simulating the advection and degradation of the environmental DNA of common carp along a river. Environmental Science and Technology 52, 10562-10570.

Paz-Vinas, I., Quemere, E., Chikhi, L., Loot, G., Blanchet, S. (2013). The demographic history of populations experiencing asymmetric gene flow: combining simulated and empirical data. Molecular Ecology, 22, 3279–3291.

Perrier, C., Bagliniere, J-L., Evanno, G. (2012). Understanding admixture patterns in supplemented populations: combining molecular analyses and temporally explicit simulations in Atlantic salmon. Evolutionary Applications, 6, 218–230.

Pfleger, M.O., Rider, S.J., Johnston, C.E., Janosik, A.M. (2016). Saving the doomed: using eDNA to aid in the detection of rare sturgeons for conservation (Acipenseridae). Global Ecology and Conservation, 8, 99–107.

Piaggio, A.J., Engeman, R.M., Hopken, M.W., Humphrey, J.S., Keacher, K.L., Bruce, W.E., Avery, M.L. (2014). Detecting an elusive invasive species: a diagnostic PCR to detect Burmese python in Florida waters and an assessment of persistence of environmental DNA. Molecular Ecology Resources, 14, 374–380.

Puebla, O., Bermingham, E., McMillan, W.O. (2012). On the spatial scale of dispersal in coral reef fishes. Molecular Ecology, 21, 5675–5688.

R Core Team. R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing. 2020

Rowe, G., Sweet, M., Beebee, T.J.C., 2017. Introduction to Molecular Ecology Oxford: Oxford University Press.

Sakata, M.K., Maki, N., Sugiyama, H., Minamoto, T. (2017). Identifying the breeding habitat of a critically endangered fish, *Acheilognathus typus*, in a natural river in Japan. The Science of Nature, 104, 100

Sansom, B.J., Sassoubre, L.M. (2017). Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. Environmental Science and Technology, 51, 14244–14253.

Sassoubre, L.M., Yamahara, K.M., Gardner, L.D., Block, B.A. (2016). Boehm, A. B. Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. Environmental Science and Technology, 50, 10456–10464.

Sengupta, M.E., Hellström, M., Kariuki, H.C., Olsen, A., Thomsen, P.F., Mejer, H., Willerslev, E., Mwanje, M.T., Madsen, H., Kristensen, T.K., Stensgaard, A.S. (2019). Environmental DNA for improved detection and environmental surveillance of schistosomiasis. Proceedings of the National Academy of Sciences of the United States of America, 116, 8931-8940.

Sigsgaard, E.E., Carl, H., Møller, P.R., Thomsen, P.F. (2015). Monitoring the near-extinct

European weather loach in Denmark based on environmental DNA from water samples Biological Conservation, 183, 46–52.

Strickler, K.M., Fremier, A.K., Goldberg, C.S. (2015). Quantifying effects of UV-B, temperature, and pH on eDNA degradation in aquatic microcosms. Biological Conservation, 183, 85–92.

Taberlet, P., Coissac, E., Pompanon, F., Brochmann, C., Willerslev, E. (2012). Towards next-generation biodiversity assessment using DNA metabarcoding. Molecular Ecology, 21, 2045–2050.

Takahara, T., Minamoto, T., Yamanaka, H., Doi, H., Kawabata, Z. (2012). Estimation of fish biomass using environmental DNA. PLOS ONE, 7, e35868

Takahara, T., Minamoto, T., Doi, H. (2013). Using environmental DNA to estimate the distribution of an invasive fish species in ponds. PLOS ONE, 8, e56584.

Takahara, T., Yamanaka, H., Minamoto, T., Doi, H., Uchii, K. (2016). Current state of biomonitoring method using environmental DNA analysis. Japanese Journal of Ecology, 66, 583–599. (in Japanese)

Takahara, T., Taguchi, J., Yamagishi, S., Doi, H., Ogata, S., Yamanaka, H., Minamoto, T. (2020). Suppression of environmental DNA degradation in water samples associated with different storage temperature and period using benzalkonium chloride. Limnology and Oceanography Methods, 18, 437-445.

Takasaki, K., Aihara, H., Imanaka, T., Matsudaira, T., Tsukahara, K., Usui, A., Osaki, S., Doi, H. (2021). Water pre-filtration methods to improve environmental DNA detection by real-time PCR and metabarcoding. PLOS One, 16, e0250162.

Thomsen, P.F., Kielgast, J., Iversen, L.L., Møller, P.R., Rasmussen, M., Willerslev, E. (2012). Detection of a diverse marine fish fauna using environmental DNA from seawater samples.

PLOS ONE, 7, e41732.

Travis J.M.J., Munkemuller T., Burton O.J. Best, A., Dytham, C., Johst, K. (2007). Deleterious mutations can surf to high densities on the wave front of an expanding population. Molecular Biology and Evolution, 24, 2334–2343.

Tréguier, A., Paillisson, J.M., Dejean, T., Valentini, A., Schlaepfer, M.A., Roussel, J.M. (2014). Environmental DNA surveillance for invertebrate species: advantages and technical limitations to detect invasive crayfish *Procambarus clarkii* in freshwater ponds. Journal of Applied Ecology, 51, 871–879.

Tsuji, S., Ushio, M., Sakurai, S., Minamoto, T., Yamanaka, H. (2017). Water temperature-dependent degradation of environmental DNA and its relation to bacterial abundance. PLOS ONE, 12, e0176608.

Tsuji, S., Yamanaka, H., Minamoto, T. (2017). Effects of water pH and proteinase K treatment on the yield of environmental DNA from water samples. Limnology, 18, 1–7.

Tsuji, S., Takahara, T., Doi, H., Shibata, N., Yamanaka, H. (2019). The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. Environmental DNA, 1, 99–108.

Turner, C.R., Miller, D.J., Coyne, K.J., Corush, J. (2014). Improved methods for capture, extraction, and quantitative assay of environmental DNA from Asian bigheaded carp (*Hypophthalmichthys* spp.) PLOS ONE, 9, e114329.

Turner, C.R., Uy, K.L., Everhart, R.C. (2015). Fish environmental DNA is more concentrated in aquatic sediments than surface water. Biological Conservation, 183, 93–102.

Uchii, K., Doi, H., Minamoto, T. (2016). A novel environmental DNA approach to quantify the cryptic invasion of non-native genotypes. Molecular Ecology Resources, 16, 415–422.

Ushio, M., Murakami, H., Masuda, R., Sado, T., Miya, M., Sakurai, S., Yamanaka, H., Minamoto, T., Kondoh, M. (2018). Quantitative monitoring of environmental DNA in multispecies fish using high-throughput sequencing Metabarcoding and Metagenomics, 2, e23297.

Villacorta-Rath, C., Adekunle, A.I., Edmunds, R.C., Strugnell, J.M., Schwarzkopf, L., Burrows, D. (2020). Can environmental DNA be used to detect first arrivals of the cane toad, Rhinella marina, into novel locations? Environmental DNA, 2, 635-646.

Wei, N., Nakajima, F., Tobino, T. (2018). A microcosm study of surface sediment environmental DNA: decay observation, abundance estimation, and fragment length comparison. Environmental Science and Technology, 52, 12428-12435.

Weltz, K., Lyle, J.M., Ovenden, J., Morgan, J.A., Moreno, D.A., Semmens, J.M. (2017). Application of environmental DNA to detect an endangered marine skate species in the wild. PLOS ONE, 12, e178124

Whiteley, A.R., Coombs, J.A., Hudy, M., Robinson, Z., Nislow, K.H., Letcher, B.H. (2012). Sampling strategies for estimating brook trout effective population size. Conservation Genetics, 13, 625–637.

Wilcox, T.M., McKelvey, K.S., Young, M.K., Lowe, W.H., Schwartz, M.K. (2015). Environmental DNA particle size distribution from Brook Trout (*Salvelinus fontinalis*). Conservation Genetics Resources, 7, 639-641.

Wood, S.A., Biessy, L., Latchford, J.L., Zaiko, A., von Ammon, U., Audrezet, F., Cristescu, M.E., Pochon, X. (2020). Release and degradation of environmental DNA and RNA in a marine system. Science of the Total Environment, 704, 135314.

Yamanaka, H., Minamoto, T. (2016). The use of environmental DNA of fishes as an efficient method of determining habitat connectivity. Ecological Indicators, 62, 147–153.

Yamanaka, H., Minamoto, T., Matsuura, J., Sakurai, S., Tsuji, S., Motozawa, H., Hongo, M., Sogo, Y., Kakimi, N., Teramura, I., Sugita, M. (2017). A simple method for preserving environmental DNA in water samples at ambient temperature by addition of cationic surfactant. Limnology, 18, 233-241.

Yamamoto, S., Masuda, R., Sato, Y., Sado, T., Araki, H., Kondoh, M., Minamoto, T., Miya, M. (2017). Environmental DNA metabarcoding reveals local fish communities in a species-rich coastal sea. Scientific Reports, 7, 1, 1–12.

Yates, M.C., Fraser, D.J., Derry, A.M. (2019). Meta-analysis supports further refinement of eDNA for monitoring aquatic species-specific abundance in nature. Environmental DNA, 1, 5–13.

Zulkefli, N.S., Kim, K.H., Hwang, S.J. (2019). Effects of microbial activity and environmental parameters on the degradation of extracellular environmental DNA from a eutrophic lake. International Journal of Environmental Research and Public Health, 16, 3339.