Seattle-Kobe International Academic Collaboration Initiative

SKIACI

The 1st Workshop

Development of Environmental DNA Research

March 1, 2021, 4:00 pm - 7:00 pm PST
(March 2, 2021, 9:00 - 12:00 JST)
This workshop is held online via Zoom.

Organized by the Kobe University Science Shop
Supported by the Assistance Grant for Activities to Promote International Exchange, Kobe University
Cooperated by Prof. Toshifumi Minamoto, Kobe University Graduate School of Human Development and Environment, and Prof. Ryan Kelly, University of Washington College of the Environment

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Seattle-Kobe International Academic Collaboration Initiative (SKIACI)

The 1st Workshop:
Development of Environmental DNA Research

March 1, 2021, 4:00 pm - 7:00 pm PST (March 2, 2021, 9:00 - 12:00 JST)

Opening 4:00 pm - 4:15 pm (9:00 - 9:15 JST)
Message from Prof. Hideyuki Yamamoto (Executive Director, KULOS), Introduction of participants

Part 1 4:15 - 5:15 pm

Talk 1 4:15 pm - 4:30 pm (9:15 - 9:30 JST)
Estimation of the spawning period of Japanese seacucumber, Apostichopus japonicus
Daiki Takeshita (Kobe U)

Talk 2 4:30 pm - 4:45 pm (9:30 - 9:45 JST)
Fish community dynamics over the past two decades revealed through DNA metabarcoding
Zack Gold (UW)

Talk 3 4:45 pm - 5:00 pm (9:45 - 10:00 JST)
Performance of benzalkonium chloride (BAC) as the preservative of environmental DNA in seawater targeting longer and nuclear DNA fragments and fish community
Toshiaki Jo (Kobe U)

Discussion 1 5:00 pm - 5:15 pm (10:00 - 10:15 JST)

Part 2 5:30 - 6:50 pm

Talk 4 5:30 pm - 5:45 pm (10:30 - 10:45 JST)
Reconstructing past biological information in Lake Biwa using sedimentary environmental DNA approach
Masayuki K. Sakata (Kobe U)

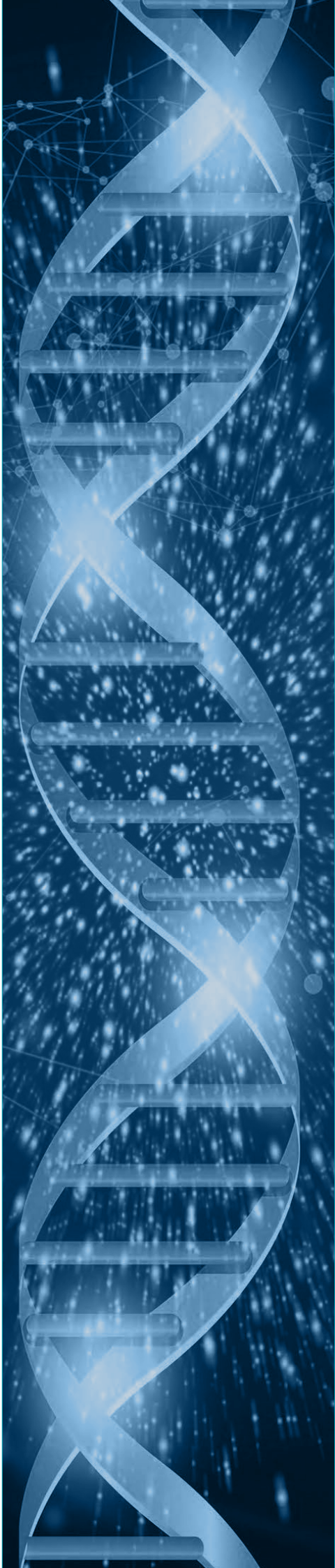
Talk 5 5:45 pm - 6:00 pm (10:45 - 11:00 JST)
Tracking a marine invasion front using molecular surveys
Abigail Keller (UW)

Talk 6 6:00 pm - 6:15 pm (11:00 - 11:15 JST)
Assessing nutritional status of carp by environmental nucleic acid analysis
Mingyang Jiang (Kobe U)

Discussion 2 6:15 pm - 6:30 pm (11:15 - 10:30 JST)

Communication 6:30 pm - 6:50 pm (11:30 - 10:50 JST)

Closing 6:50 pm - 7:00 pm (11:50 - 12:00 JST)



Seattle-Kobe International Academic Collaboration Initiative

The 1st Workshop

"Development of Environmental DNA Research"

March 2, 2021

(Ebina) I would like to start the workshop. I am Kuniyoshi Ebina of Kobe University's Science Shop. The Science Shop usually works to connect ordinary people and experts, but today we are going to connect experts in Seattle and Kobe.

At the start, we would like to have a message from the Executive Director of the Kobe University Liaison Office in Seattle (KULOS), Prof. Yamamoto.

Opening Message

Hideyuki Yamamoto (Executive Director, Kobe University Liaison Office in Seattle)

Hello, everyone. My name is Hideyuki Yamamoto, Director of the Office of the Americas and Executive Director of KULOS. It is my pleasure to give the opening speech for the Seattle-Kobe International Academic Collaboration Initiative (SKIACI) 1st Workshop "Development of Environmental DNA Research," in conjunction with the Research Oriented On-site Training (ROOT) Program in Kobe University. I am very glad that it can be held online, despite the pandemic situation of COVID-19.

I would like to express my gratitude to our guest speakers from the University of Washington, as well as the speakers from Kobe University. I am also glad to hear that Kevan Yamahara of the Monterey Bay Aquarium Research Institute (MBARI) will join us. I would like to extend my gratitude to all the people involved in the workshop, especially the organizer, Prof. Ebina, for putting everything together and for inviting me as a guest speaker.

Before starting today's workshop, I would like to take advantage of this opportunity to briefly introduce Kobe University's Office of the Americas and the recently established KULOS. Kobe University has partnership agreements with 31 North American universities and eight Latin American universities for academic collaboration and student exchange. The Office of the Americas serves as a hub for developing the international network of academic exchange between Kobe University and its counterparts in the Americas. One of our recent major initiatives is the establishment of KULOS in 2020. It is our first liaison office in the mainland U.S., located in the Hyogo Business and Cultural Center in Downtown Seattle.

Like Hyogo Prefecture, the State of Washington has developed an international port city by actively incorporating the cultures of numerous foreign countries into its own. It is a pleasure to have our liaison office in Seattle, which is a sister city of Kobe. I wish that KULOS will contribute to the globalization of research and education in Kobe University by providing venues for international symposia and workshops for faculty members, scholars, and students in various academic fields, as well as global educational activities for students of various levels. KULOS is highly expected to increase international partnerships between Kobe University and its counterparts in the West Coast, especially the University of Washington.

I hope today's workshop will be successful and meaningful for everyone involved in it, and beneficial in enhancing academic interest and developing academic collaboration between Kobe University and the University of Washington.

(Ebina) Thank you, Prof. Yamamoto. Now, I would like to outline today's meeting. We would like to start with self-introductions. Let us start from the Japanese side with Prof. Minamoto.

Self-introductions

(Minamoto) I am Toshifumi Minamoto from Kobe University. I am the PI of the eDNA laboratory in Kobe University. It is a great opportunity for us to have this workshop between our lab and Ryan's lab.

(Jo) I am Toshiaki Jo from Minamoto Lab. I am a PhD student, but I might get my PhD this month. My research interest is the characteristics and dynamics of eDNA.

(Sakata) I am Masayuki Sakata from Minamoto Lab. I am a PhD student, but I will graduate soon. I am interested in eDNA in sediment.

(Yasashimoto) I am Tetsu Yasashimoto. My study subject is eDNA metabarcoding for Odonata.

(Takeshita) I am Daiki Takeshita. I am a Master's student.

(Wu) I am Luhan Wu. I am a second-year doctoral student studying the spawning period of fish by using eDNA.

(Jiang) My name is Mingyang Jiang. I was born in China. I am a second-year Master's student in Kobe University, doing research on eRNA.

(Kihara) I am Natsumi Kihara. I am a fourth-year undergraduate student.

(Higashisaka) I am Hayato Higashisaka. I am a junior in Kobe University.

(Ebina) Next, let us move to Prof. Kelly's lab.

(Kelly) I am Ryan Kelly, a professor at the University of Washington. I am excited to be here. Thank you for inviting us. It is an honor to meet all the people making all of the amazing papers. It is too much work for us to keep up with.

(Keller) I am Abby Keller. I am a Master's student working with Ryan. Today, I will be presenting about using eDNA to manage invasive species.

(Ramon-Laca) I am Ana Ramon-Laca. I am a technician at the National Oceanic and Atmospheric Administration (NOAA), specialized in running basic DNA diagnostics. I am involved in various eDNA projects, mainly on fisheries and surveys using targeted eDNA quantification.

(Allan) I am Eily Allan. I am a postdoc with Ryan at the University of Washington. I am also very excited to hear all of your new and exciting work.

(Kelly) Kevan Yamahara is not in our lab, but he is a friend and I invited him to join us because he is doing related work.

(Yamahara) My name is Kevan Yamahara. I work at MBARI. Most of my research is focused on the design, research, and development of autonomous sampling instruments focused mostly on applications for eDNA monitoring. I have known Ryan Kelly for a long time and it is great to be here.

(Gallego) I am Ramon Gallego. I am a postdoc at NOAA Fisheries in Seattle. I was previously a postdoc in Ryan's lab. I do mostly eDNA metabarcoding. I am interested in both the technical aspects of metabarcoding, decontamination and quantification, and also the correlation between eDNA surveys and environmental evaluations.

(D'Agnese) I am Erin D'Agnese. I am a postdoc in Ryan's lab. I am very excited to be here and learn what everybody is doing.

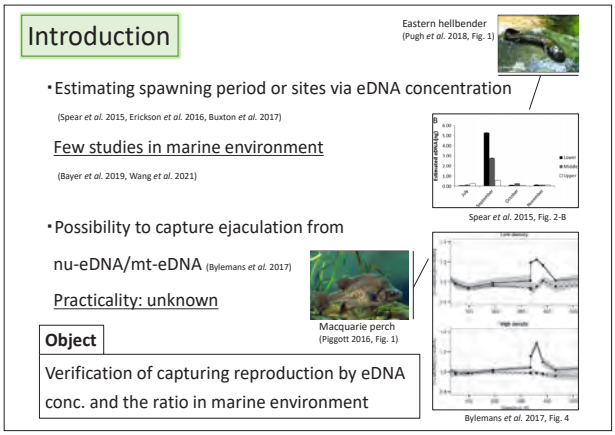
(Gold) I am Zack Gold. I am a postdoc in Ryan's lab. I am at NOAA's Northwest Fisheries Science Center. I work on a variety of eDNA projects, but broadly on how to use eDNA metabarcoding to reconstruct marine ecosystems.

(Ebina) There are some other guests, but we would like to start the first talk. The first speaker is Daiki Takeshita. "Estimation of the spawning period of Japanese sea cucumber, *Apostichopus japonicus.*"

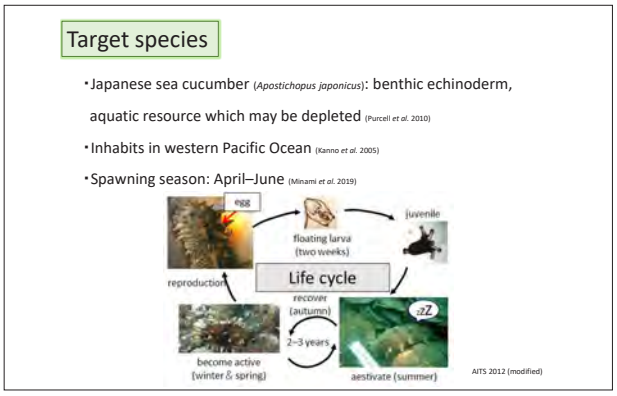
Talk 1

Estimation of the spawning period of Japanese sea cucumber, *Apostichopus japonicus*

Daiki Takeshita (Kobe University)

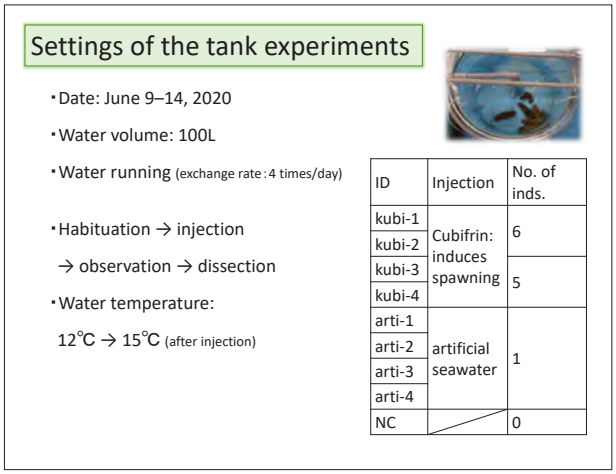


Estimating the spawning period or sites via eDNA concentration has been one of the purposes of eDNA analysis, however there have been few studies in a marine environment. According to a previous study, the possibility to capture ejaculation from nuclear eDNA concentration to mitochondrial eDNA concentration was suggested, however the practicality of the method has been unknown. The object of this study was the verification of capturing reproduction by eDNA concentration and the ratio in a marine environment.



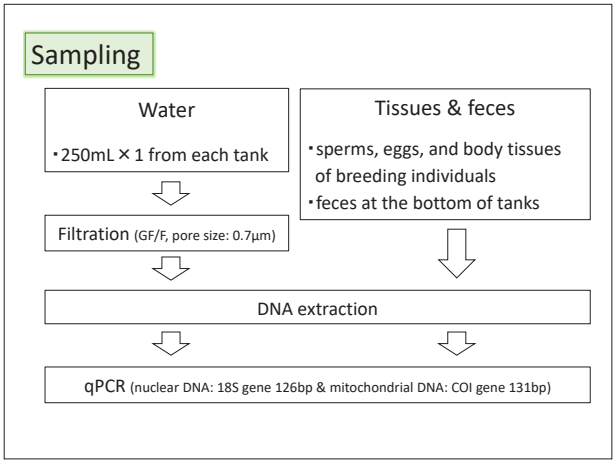
The target species was the Japanese sea cucumber, a species of benthic echinoderm which is an aquatic resource of Japan which may be depleted. This species inhabits the western Pacific Ocean. The spawning season is April to June.

The lifecycle is as follows. First, they hatch from eggs, then they spend two weeks as floating larvae, and then they move to benthic life and spend two to three years until sexual maturity. They aestivate in summer, recover from aestivation in autumn, and become active in winter and spring.



ID	Injection	No. of inds.
kubi-1	Cubifrin: induces spawning	6
kubi-2		
kubi-3		5
kubi-4		
arti-1	artificial seawater	1
arti-2		
arti-3		
arti-4		
NC		0

Using individuals of this species, tank experiments were conducted from June 9 to 14, 2020. The water volume was 100 L and the water condition was running with an exchange rate of four times per day. The outline of this experiment was habituation, injection, observation, and then dissection. The water temperature was raised from 12°C to 15°C after injection. Nine tanks were prepared for this experiment, with one tank without individuals as a negative control. Four of the tanks were for injecting cubifrin which induces spawning, and the others were for injecting artificial seawater.



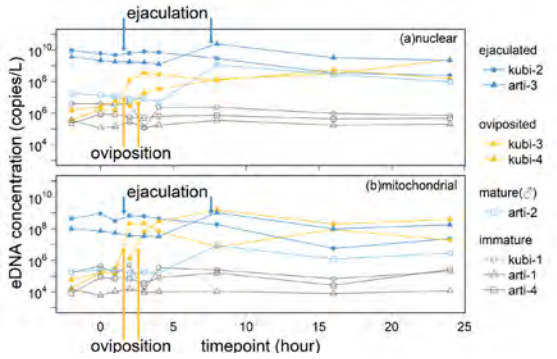
For water samples, the volume was 250 mL from each tank. They were filtered, and then DNA was extracted and qPCR was performed. Sperm, eggs, and body tissues of breeding individuals were collected, as well as feces at the bottom of tanks with breeding individuals.

Results of observation and dissection

ID	Injection	No. of inds.	Repro-duction	Sexual maturity	Type
kubi-1	Cubifrin: induces spawning	6			immature
kubi-2			♂ × 1	♂ × 1	ejaculated
kubi-3		5	♀ × 1	♀ × 1	oviposited
kubi-4			♀ × 1	♀ × 1	oviposited
arti-1	artificial seawater	1			immature
arti-2				♂ × 1	mature (♂)
arti-3			♂ × 1	♂ × 1	ejaculated
arti-4					immature

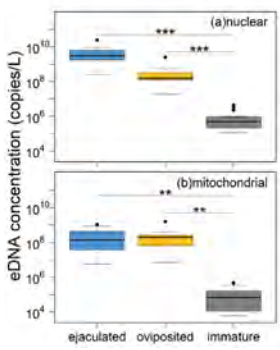
These are the results of observation and dissection. For "kubi-2" and "arti-3," one of the individuals ejaculated. Sexual maturity was confirmed from dissection, so these tanks were considered as "ejaculated." For "kubi-3" and "kubi-4," one of the individuals oviposited. Sexual maturity was confirmed from dissection, so these tanks were considered as "oviposited." For "arti-2," remarkable ejaculation was not observed, however sexual maturity was confirmed, so this tank was regarded as "mature." The others were regarded as "immature."

eDNA concentration in tanks



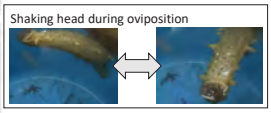
These are the results of eDNA concentration. The horizontal axis indicates time after injection, with time 0 indicating immediately after injection. The vertical axis indicates eDNA concentration.

Comparison of eDNA concentration



・"ejaculated" and "oviposited" ＞"immature" due to gametes or increased activity during reproduction?

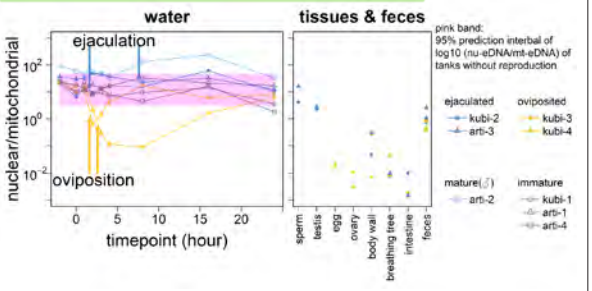
Shaking head during oviposition



・Injection and water temperature did not affect significantly.

From the results of the statistical analysis, the eDNA concentrations of "ejaculated" and "oviposited" were significantly higher than "immature." This could be due to gametes or increased activity during reproduction, observed as head shaking during oviposition. On the other hand, injection and water temperature did not have a significant effect.

The ratio of (e)DNA concentration



・Eggs with small nu-DNA/mt-DNA caused the values below the PI?

The decrease in ratio can be used to capture oviposition.

These are the results of the ratio of nu-eDNA concentration over mt-eDNA concentration. The pink band indicates a 95% prediction interval of the ratio converted into a common logarithm of tanks without reproduction. After oviposition, the values lower than this prediction interval were recorded. This could be caused by eggs because they have a low ratio value. This study newly suggested that a decrease in the ratio can be used to capture oviposition.

A series of field surveys

・Site: Maizuru Fishery Research Station of Kyoto University, Japan

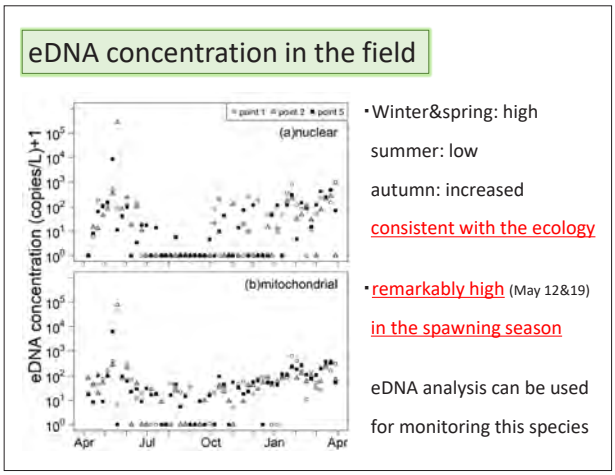
・Period: April 2015–March 2016

・Sample: 1L × 1 for each point (P1, P2, P5)

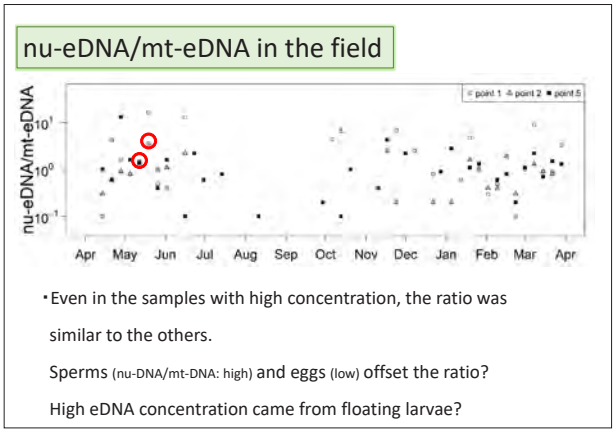
・Frequency: once a week

Next is about a series of field surveys. The study site was the Maizuru Fishery Station of Kyoto University. The study period was April 2015 to March 2016. The volume of samples was 1 L, with one replication for each point (P1, P2, and P5). The sampling frequency was once a week.

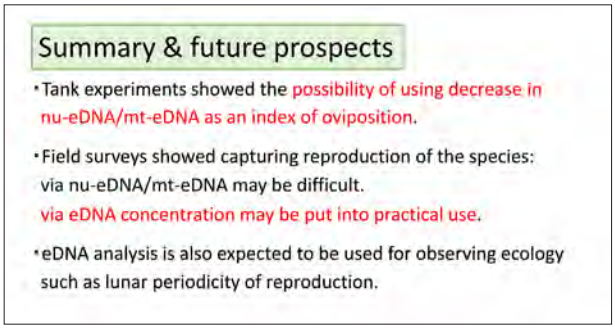
These are the results of eDNA concentration. The vertical axis indicates eDNA concentration, added one



copy. In winter and spring, eDNA concentration was high. Individuals become active in these seasons. In summer, eDNA was rarely detected or low. Individuals aestivate in this season. In autumn, eDNA concentration increased. Individuals recover from aestivation in this season. The fluctuation of eDNA concentration was consistent with the ecology of the Japanese sea cucumber. In addition, eDNA concentration was remarkably high in the spawning season. From this, eDNA analysis can be used for the monitoring of this species.



However, for nuclear eDNA concentration to mitochondrial eDNA concentration, the ratio was similar to others, even in samples with high concentration. This could be because sperm and eggs can offset the ratio or because the high eDNA concentration might have come from floating larvae.



To summarize, tank experiments showed the possibility of using a decrease in the ratio as an index of oviposition. Field surveys showed capturing reproduction of the species via the ratio may be difficult, but it may be put into practical use via eDNA concentration. Of course, eDNA analysis is expected to be used in reproduction, but it is also expected to be used for observing ecology such as the lunar periodicity of reproduction.

(Ebina) Thank you very much, Daiki. Any questions or comments?

(Kelly) The use of two different loci to make the inferences about the species is really interesting. I think the idea of using them in combination is very powerful. Did you measure inhibition? Did you see different inhibition between the two different loci, 18S and COI?

(Takeshita) I used Environmental Master Mix. It is strong for inhibition, so in this study, I assumed there was no inhibition of detecting eDNA.

(Allan) What filter pore size did you use?

(Takeshita) 0.7 µm.

(Allan) For the tissues and feces, did you just use a blood and tissue kit and a standardized mass of DNA?

(Takeshita) I used the QIAGEN blood and tissue kit. The kit was used to extract eDNA for water samples, and the same kit was used for tissues and feces.

(Allan) I like the plot where you compared the water samples to the tissues and feces. I wanted to see the pore size, thinking about what you might be capturing in the water. It is probably a combination of those things.

(Takeshita) In this study, the filter pore size was 0.7 µm. For this filter, eDNA can be captured in or out of organelles, so maybe a little eDNA out of the organelles may not be detected in some cases, but I did not care about the pore size of the filters in this study.

(Ebina) Thank you, Daiki. The next speaker is Zack Gold from the University of Washington. "Fish community dynamics over the past two decades revealed through DNA metabarcoding."

Talk 2

Fish community dynamics over the past two decades revealed through DNA metabarcoding

Zack Gold (University of Washington)

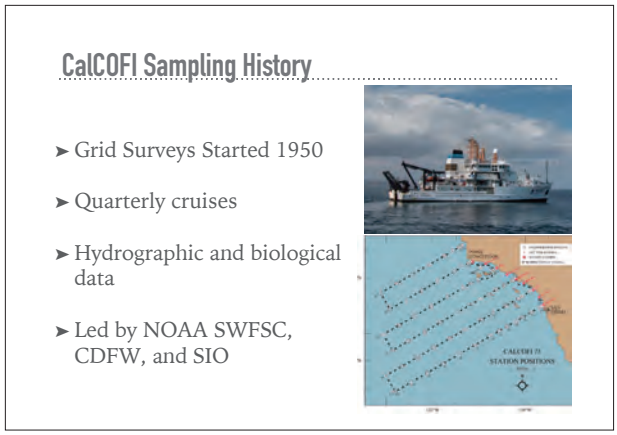
I am going to be talking about marine heatwave-driven shifts in larval fish community dynamics over the past two decades revealed through DNA metabarcoding.

I want to acknowledge all of the coauthors and funders. There are a lot of people who have helped move this project along, so I would like to thank them for their support.

In the 1940s and 1950s, in both Japan and California, there was a dramatic crash in sardine fisheries. In California in particular, this led to a really huge economic crisis.

Cannery Row in Monterey was economically devastated by the loss of the fishery. It was the largest fishery in the United States at the time. At both the federal and state governments, they had no idea how this happened because a fishery had never been overfished before.

At the time, scientists pointed fingers at each other, claiming either the environment or overfishing as the cause. No one knew the answer, so the California Cooperative Oceanic Fisheries Investigations (CalCOFI) program was developed.



The idea was to get independent information from fisheries on fish recruitment and ichthyoplankton data using a grid survey. It is actually one of the longest marine ecosystem surveys taken continuously to date. They combine hydrographic and biological data. They do net tows that capture both zooplankton and ichthyoplankton, and they get every hydrographic variable that you could want. It is currently run by NOAA and state agencies in the State of California.

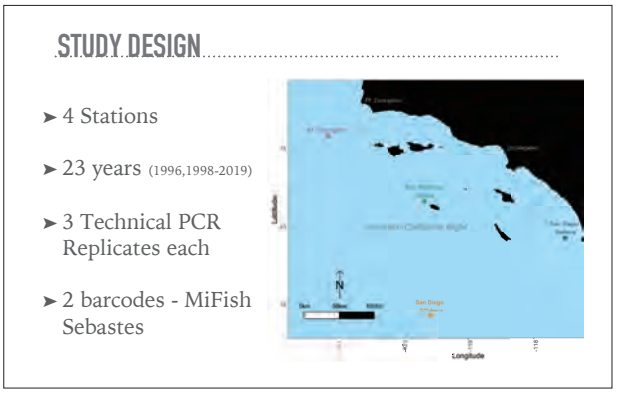
With this data of 70 years, we now know the answer. It was actually both the environment and overfishing which led to the collapse of sardines. This was highlighted by Francisco Chavez's work at MBARI in 2003, but also by some work at the University of Washington from Tim Essington, a professor in the School of Aquatic and Fishery Sciences (SAFS). We now know what caused the shift, but it is too late to go back and prevent the collapse of the sardine fishery.

That brings up the limitations of the CalCOFI survey. It is really time and labor-intensive. Oftentimes, this leads to a four to five-year delay between when they capture a net sample and when they actually identify all the species of fish and invertebrates in the sample. It also requires the highest level of taxonomic expertise. It is very difficult to identify fish eggs and larvae. A lot of the eggs do not have any morphological characteristics, so you cannot identify them at the species level.

That leaves us largely blind to real-time ecosystem dynamics, and so if we have a marine heatwave like we did in the Northeast Pacific from 2014 to 2016, we will not know what happened to those ecosystems until 2019 when we finally get the data turned around. By then, we have already been fishing for five years.

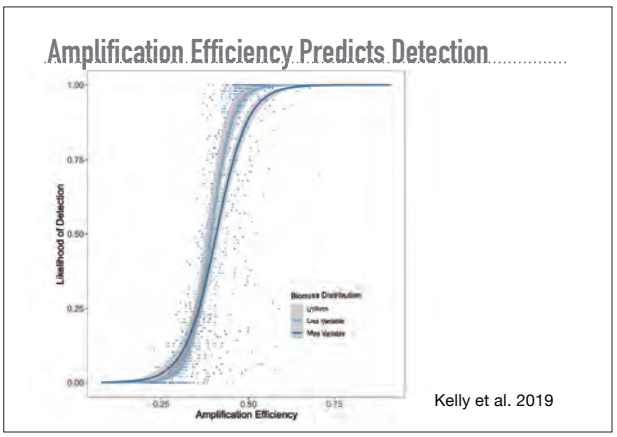
What that means is we need a new monitoring tool that is rapid, reliable, standardized, accurate, and cost-effective. That is the promise of eDNA and DNA metabarcoding technologies.

What we did here is to compare DNA metabarcoding with microscopy in a non-destructive way. There is a picture of a CalCOFI net tow sample preserved in ethanol. We do not touch or disturb the sample. We filter the ethanol and do DNA metabarcoding side-by-side with microscopy work so that we can have a comparison of what we are find under a microscope with what we get with eDNA.



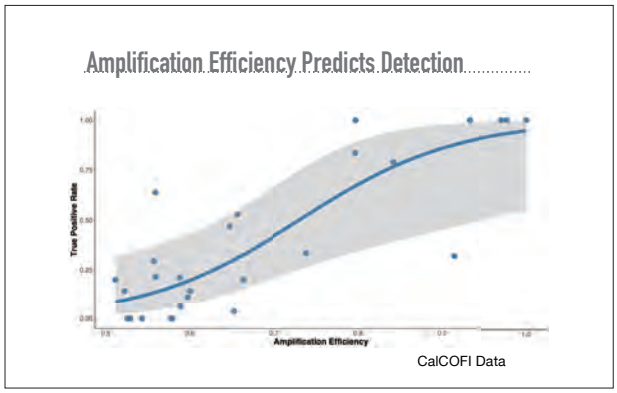
This is the study design. We picked four stations that are representative of the main habitats that we have: the California Current, the countercurrent, and more variable sites. We have 23 years of data, dating back to 1996. We have three technical PCR replicates for two MiFish universal primers, as well as the MiSebastes, a rockfish-specific primer that I helped develop with a PhD student at the University of Washington.

Before jumping into the results, I think it is important to understand PCR bias. Ryan, Ramon, and Andrew Shelton (2019) have a great theoretical framework for understanding how the propagation of small biases of certain species amplify slightly better than other species for the same barcode results in driving the ratios that we see of different species, basically leading to some species amplifying much better than others for the same metabarcoding assay.

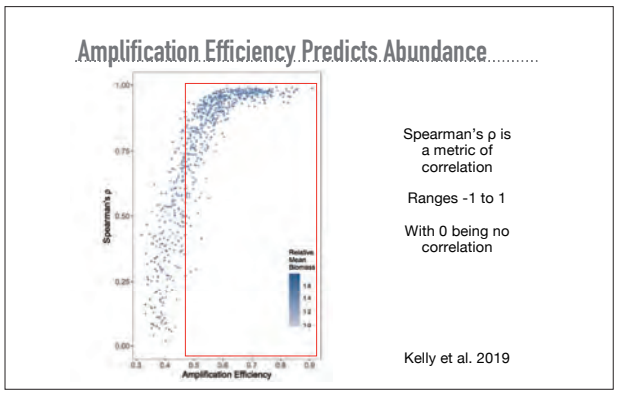


What they were able to demonstrate with modeling efforts is that for species with high amplification efficiency, we expect there to be a strong likelihood of detection. We can detect a species that has good amplification efficiency, but our ability to detect a species with low amplification efficiency in a given assay is going to be quite low.

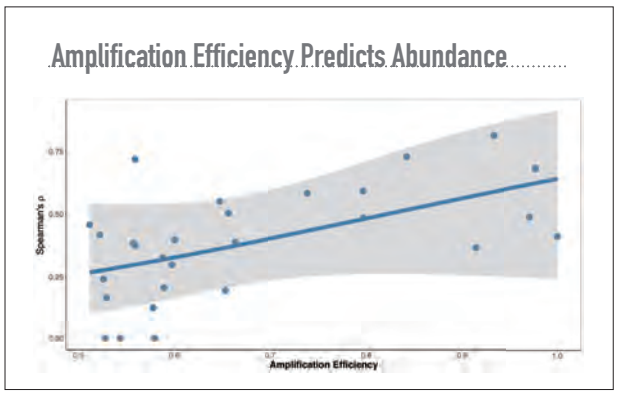
We compared that to the data we generated from our CalCOFI study and we actually found very similar results.



We are finding that species with high amplification efficiency have the highest detection or true positive rate.



At the same time, the modeling efforts also looked at how well we can track biomass for a species. For a species with high amplification efficiency, again, we expect that to correlate quite well.

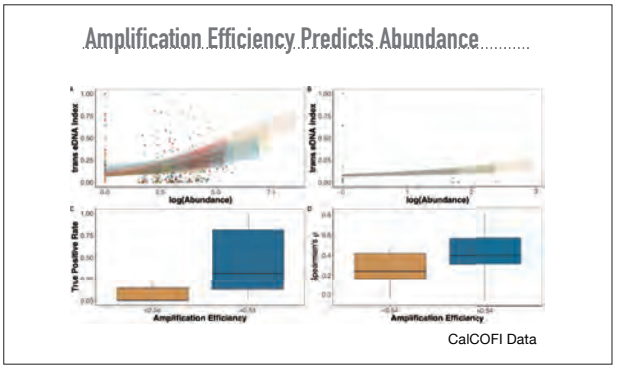


We actually showed similar results with the CalCOFI data. The species with high amplification efficiency had the strongest correlation in biomass. This is exciting because it ties the theory to what we expect from empirical data. This demonstrates that understanding amplification efficiency is important for interpreting eDNA metabarcoding data for ecological results.

Why are the real fits not as good as the theory? There are a couple of reasons. One is these are paired bongo nets, so the ethanol and formaldehyde are coming from

different sides of the nets, and it is possible that they are not the exact same ichthyoplankton communities and that there is microspatial structuring. We are also comparing two imperfect methods. Microscopy cannot identify very many species, and many eggs are hard to identify. Also, extracellular DNA does not really matter. With DNA metabarcoding, it does not matter what the source is. It can amplify it. As long as there is mitochondrial DNA floating around in the ethanol, the DNA metabarcoding will pick it up. However, DNA metabarcoding is not a perfect method either.

What we are able to show is that the amplification efficiency is the key threshold. We can use this information then to consider if these are species we can trust. If we trust the true positive rate and the correlation with biomass, we can use these species to focus the ecological analyses because we know that our method is working well for these species.



These are a couple of examples. One is Pacific sardine. We show that the eDNA index tracks quite well with the log of abundance. We see the same thing for anchovy. These are two of the forage fish species that are the targets of the CalCOFI work. This is really exciting because it is showing that we can actually get estimates of relative abundance from eDNA data, which is going to be important for ecological analyses.

How do you use amplification efficiency? We were able to show that amplification efficiencies track well with biomass and detection rates, but you can calculate these amplification efficiencies from mock community estimates. This is done in a paper by McLaren, Willis, and Callahan(2019). They did very good analyses demonstrating that from different extraction methods, as well as PCR biases, you can then correct data to use this. It is demonstrating that amplification efficiency is important for the interpretation of metabarcoding results. Also, they are easy to do because you just need to get tissues and set up mock community experiments ahead of time to be able to know how well each species amplifies for a given locus.

How do we use this towards accurate reconstruction of marine ecosystems, going back to the bigger question of how marine ecosystems are changing in response to marine heatwaves?

What we were able to show is that eDNA is able to identify two main groups of species. We have warm and cold-associated species assemblage groups. The vast majority of the species are subtropical. A lot of them are myctophids. We are seeing these species showing up in marine heatwave events, whereas a lot of the fishery targets like hake, sardine, anchovy, and some of the main rockfish taxa are recruiting much better in colder years.

Looking at the whole ecosystem, we can see the 1998 El Nino, which was one of the warmest years on record, with the marine heatwaves which were a little bit cooler, but still three of the top seven hottest years ever recorded in the California Current. Those are all clustering together and we are getting a lot of tropical and subtropical species showing up in our coastal waters associated with these marine heatwave events.

However, it is not an easy and straightforward story. We also got unexpectedly high anchovy recruitment in the California Current during some of the warmest years ever recorded. That helped break the idea that has been longstanding in the literature that sardines are warm-associated and anchovies are cold-associated taxa. eDNA metabarcoding was able to pick that up and track this interesting phenomenon that happened. Basically, climate change and marine heatwaves that are becoming more frequent are going to impact our fisheries and our ecosystems in unexpected and non-linear ways, so we need a tool that gives us the biggest and fullest picture of marine ecosystems, and eDNA can provide that.

With eDNA, we can get a much broader view of biodiversity. We are getting about three times as many species as we are identifying via microscopy. A lot of them are important fishery targets. eDNA can provide quantitative estimates which are going to be important for a lot of ecological analyses. In addition, it is much cheaper and automatable, so it is scalable to something like the size of the CalCOFI where they are collecting upwards of 1,000

samples in net tows per year. Also, we can get faster results which are going to be needed for dynamic management. Realistically, we can expect results in a couple of months instead of four to five years, with enough investment. One interesting advantage is that we can access archived samples from 23 years ago without damaging them, so future studies can still look at the eggs or larvae and use the ethanol preservative to get ecosystems reconstructed.

(Ebina) Thank you very much, Zack. Do you have any questions or comments?

(Jo) In real-world eDNA metabarcoding experiments, how can we know the amplification efficiency?

(Gold) It is something that you cannot directly calculate from a water sample collected from seawater, but it is something that you can calculate by doing a mock community experiment. For example, if you took all the fish species that show up in Maizuru Bay and you put them in a mock community, you could see what the relative amplification efficiencies are, especially if you put them all at known starting concentrations. That number is static. It is inherent to the method you are using, the marker you are using, and the species that are in the sample. So you could do a little mock community experiment and go back to samples that you have already collected or analyses that you have already done, and you can correct the data once you know what the amplification efficiencies are.

Something that Ryan and I have been working on is that you can estimate the amplification efficiency. Basically, you find ones that have high amplification efficiency. It is fairly easy because they are usually obvious and show up in many samples and have high read counts. Where it becomes difficult is if you have a rare species. It might have really high amplification efficiency, but if they only show up in one sample, you cannot distinguish them from the background noise of other species. Therefore, I think a mock community is ultimately the best way to get at that, but you can also figure out which species are doing well if they are showing up in all of your samples with high reads. Those species clearly amplify quite well for your metabarcoding locus of choice.

(Kelly) The equation we have been using is amplicons ~ [biomass] * $(1+a)^{N_{PCR\text{-}cycles}}$, so if you have some information about biomass and some information about the number of amplicons, then you can estimate the amplification efficiency, "*a*."

Reading the Cusick paper, there are many options to think about how to improve these kinds of quantitative questions with metabarcoding.

(Yamanaka) How do you obtain basic information on the amplification efficiency for each species by using a mock community? I believe the results will be affected by the biomass ratio between species in the mock samples. How do you treat the issue related to these variations?

(Gold) I recommend the Mclaren, Willis, Callahan (2019) paper. It has really good examples of three different mock communities and how they take information and calculate the amplification efficiency. Even if you make all the species of interest even and add the same concentrations of extracted tissue sample to the mock community, you can at least know that they are all even starting concentrations. You would expect with perfect metabarcoding, the same number of reads from every single species put in a mock community. We know that never happens. So you can start even, look at the relative abundance of each different species, and calculate the amplification efficiencies. The paper takes three microbiome examples with multiple different mock communities, and then demonstrates how to do those calculations.

(Kelly) The evidence we have so far is that the composition of the community does not change amplification efficiency very much. It seems there are more primers than DNA, so maybe there is no real competition.

(Minamoto) I could not catch the methodology. Did you extract DNA from the museum samples of individuals or from ethanol?

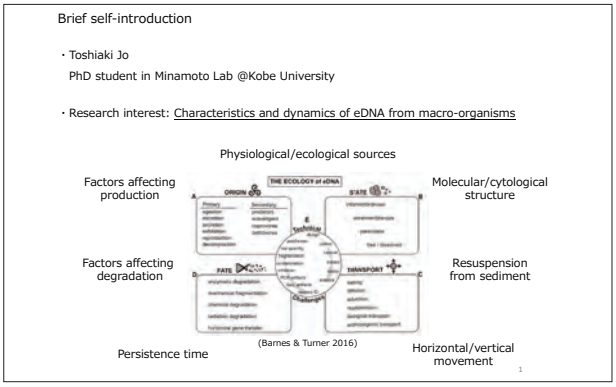
(Gold) Basically, it is the cod end of a plankton tow that got stored in ethanol, so there are hundreds of species in each jar. Also, they have been untouched for 23 years. They just put them in the museum. We took a large pipette and pipetted the ethanol off the top of the sample, and then put it into a filter and did the same as we would with seawater eDNA. I think on average it was 125 mL of ethanol that went into the sample, and then we got the filter and did a QIAGEN DNeasy extraction kit. We then amplified them for the MiFish universal primer, as well as the MiSebastes rockfish primer, and used the CALeDNA protocols. They are very similar to the ones that Kevan uses at MBARI.

(Ebina) Thank you very much, Zack. We would like to move to the next speaker, Toshiaki Jo. "Performance of benzalkonium chloride as the preservative of environmental DNA in seawater targeting longer and nuclear DNA fragments and fish community."

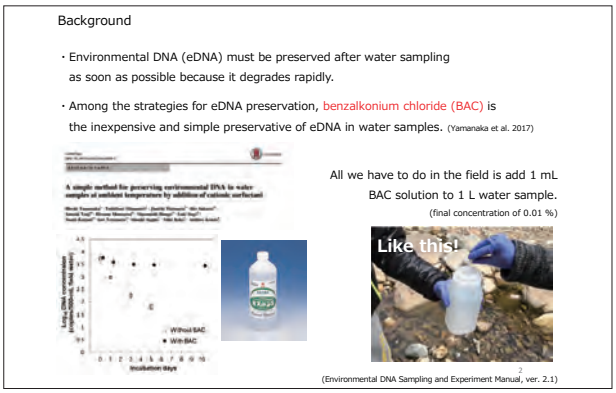
Talk 3

Performance of benzalkonium chloride as the preservative of environmental DNA in seawater targeting longer and nuclear DNA fragments and fish community

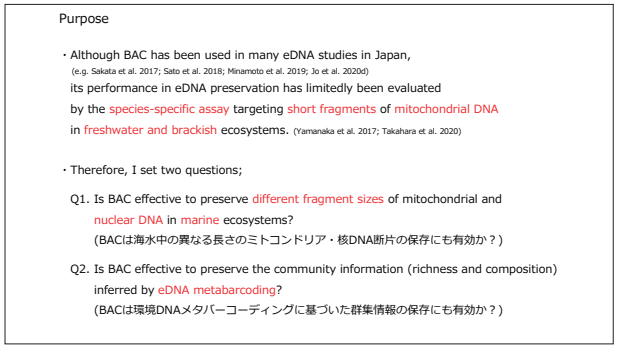
Toshiaki Jo (Kobe University)



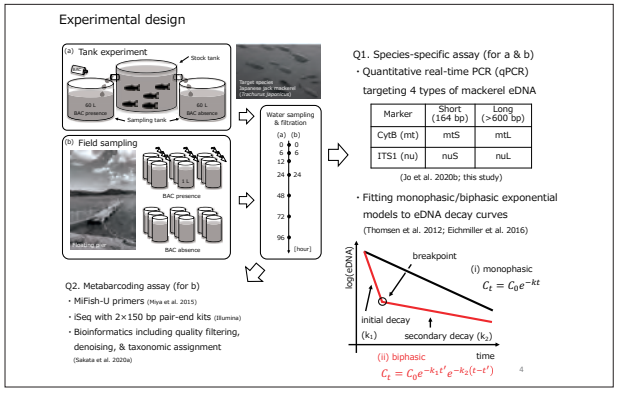
As I introduced before, I am a PhD student in Minamoto Lab at Kobe University. My primary research interest is the characteristics and dynamics of eDNA from macro-organisms such as fish and vertebrates. This is sometimes referred to as the ecology of eDNA. It includes source, state, transport, and fate.



Recently, I have also been engaged in eDNA-based monitoring of invasive and endangered species in freshwater ecosystems where eDNA in water samples must be preserved after sampling as soon as possible because it degrades rapidly. Among the strategies for eDNA preservation, benzalkonium chloride (BAC) is known as an inexpensive and simple preservative of eDNA in water samples. This preservative was used for the first time in Yamanaka et al. (2017), which showed that the addition of BAC substantially suppresses the degradation of mitochondrial eDNA from freshwater fish in water samples. In the field, we only need to add 1 mL of BAC solution to 1 L of water sample and mix it.

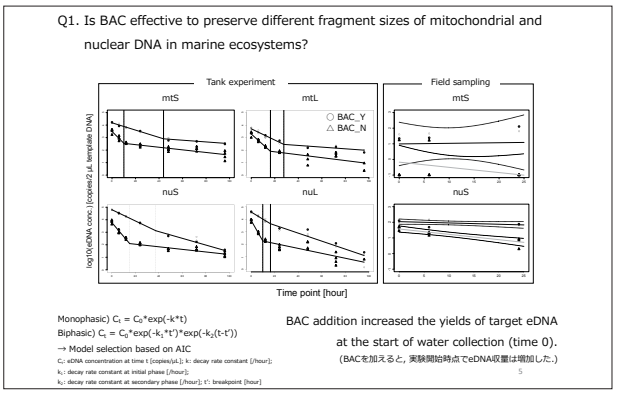


Because of its high cost-performance, BAC has been used in many eDNA studies, particularly in Japan, however the performance of eDNA preservation has been limitedly evaluated by species-specific assays targeting short fragments of mitochondrial DNA in freshwater and brackish ecosystems. Therefore, I set two questions. Q1: Is BAC effective to preserve different fragment sizes of mitochondrial and nuclear DNA in marine ecosystems? Q2: Is BAC effective to preserve the community information, such as richness and composition, inferred by eDNA metabarcoding?

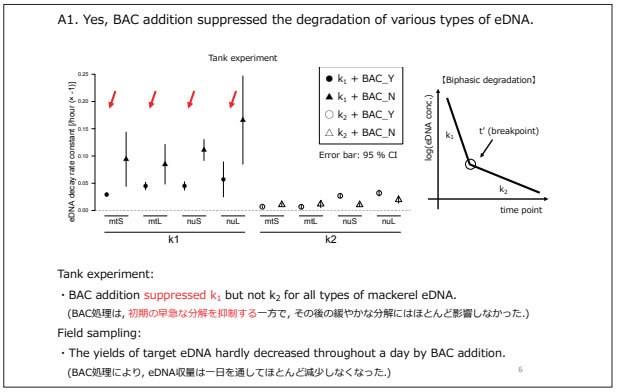


I will briefly explain the design of the experiment. In the tank experiment, rearing seawater of the stock tank with Japanese jack mackerel was transported into two sampling tanks. BAC was then added to one of the sampling tanks, and rearing water was collected and filtered for 60 hours. In the field sampling, seawater samples were collected from Maizuru Bay in Kyoto Prefecture. BAC was added to half of them, and then water samples were filtered after water collection, after six hours, and after a day.

To answer Q1, four types of mackerel eDNA were quantified by real-time PCR. I targeted 164 base pair fragments of cytochrome *b* gene and nuclear internal transcribed spacer 1 region, and more than 600 base pair fragments of both genes. Monophasic or biphasic exponential models were then fitted to each eDNA decay curve to estimate decay rate constants. In the monophasic model, the eDNA decay rate is consistent during time passage. On the other hand, in the biphasic model, rapid eDNA degradation occurs which is followed by a slower degradation, divided by a breakpoint. To answer Q2, eDNA metabarcoding was performed targeting the fish community in Maizuru Bay. MiFish-U primers were used, and iSeq raw reads were preprocessed and analyzed.

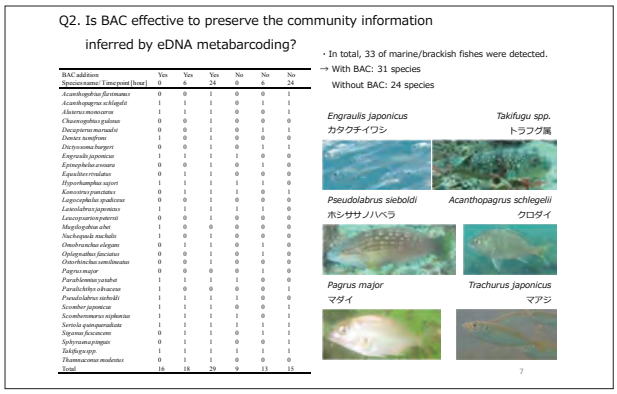


These are the results for Q1. The decay rate curves of each type of mackerel eDNA in the tank experiment and field sampling are shown. The x-axis shows the sampling time points and the y-axis shows the log-transformed eDNA concentrations. The circular plots in each box represent the datasets from the treatment with BAC, and the triangular plots represent the treatment without BAC. Biphasic degradation models were supported in the tank experiment, while monophasic models were supported in the field sampling. The addition of BAC increased the yields of all types of target eDNA at the start of experiment.

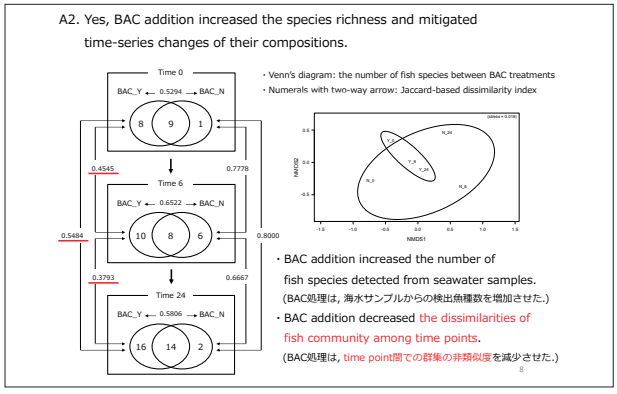


The decay rate constants derived from the model fittings in the tank experiment are shown. Black and white plots represent the decay rate constants in the initial and secondary phases in biphasic degradation. In the tank experiment, the addition of BAC suppressed the initial rapid degradation, but not the following slower degradation for all types of mackerel eDNA.

In addition, the yields of target eDNA from the field samples hardly decreased throughout a day by the addition of BAC.

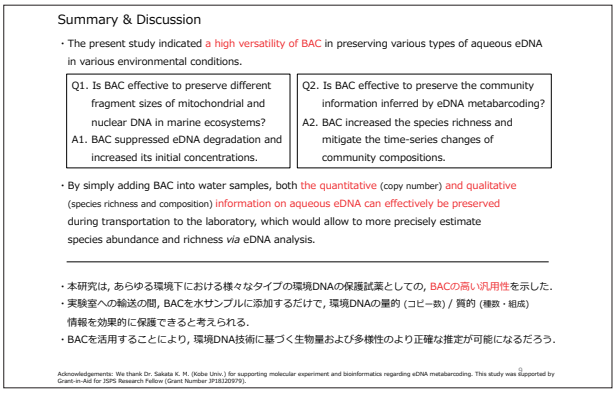


These are the results for Q2. The list represents all marine and brackish fish species detected from seawater samples. In total, 33 fishes were detected. 31 species were detected from the samples with the addition of BAC, and 24 species were detected from those without the addition of BAC. I then compared the number of detected fish species between BAC treatments and compared the similarities of community compositions between BAC treatments and sampling time points.



The left figure shows the summary of species richness and composition among BAC treatments in sampling time points. Each Venn diagram represents a comparison of the number of fish species detected from seawater samples between BAC treatments. Each numeral with a two-way arrow represents the Jaccard-based dissimilarity indices where a lower value indicates that the compositions are more similar between treatments. In addition, the right figure represents the NMDS product

based on the Jaccard-based index for the fish community among each BAC treatment and time point. At all time points, the addition of BAC increased the number of fish species from seawater samples. Moreover, the addition of BAC decreased the dissimilarities of the fish community among time points, implying that time series changes of community compositions were mitigated by the addition of BAC.


Summary & Discussion

・The present study indicated a high versatility of BAC in preserving various types of aqueous eDNA in various environmental conditions.

Q1. Is BAC effective to preserve different fragment sizes of mitochondrial and nuclear DNA in marine ecosystems?
A1. BAC suppressed eDNA degradation and increased its initial concentrations.

Q2. Is BAC effective to preserve the community information inferred by eDNA metabarcoding?
A2. BAC increased the species richness and mitigate the time-series changes of community compositions.

・By simply adding BAC into water samples, both the quantitative (copy number) and qualitative (species richness and composition) information on aqueous eDNA can effectively be preserved during transportation to the laboratory, which would allow to more precisely estimate species abundance and richness *via* eDNA analysis.

・本研究は，あらゆる環境下における様々なタイプの環境DNAの保護試薬としての，BACの高い汎用性を示した．
・実験室への輸送の間，BACを水サンプルに添加するだけで，環境DNAの量的（コピー数）/ 質的（種数・組成）情報を効果的に保護できると考えられる．
・BACを活用することにより，環境DNA技術に基づく生物量および多様性のより正確な推定が可能になるだろう．

Acknowledgements: We thank Dr. Sakata K. M. (Kobe Univ.) for supporting molecular experiment and bioinformatics regarding eDNA metabarcoding. This study was supported by Grant-in-Aid for JSPS Research Fellow (Grant Number JP18J20379).

In summary, the present study indicated a high versatility of BAC in preserving various types of aqueous eDNA in various environmental conditions. In Q1, I found that the addition of BAC suppressed eDNA degradation and increased its initial concentrations for different fragment sizes of mitochondrial and nuclear genes. In Q2, I also found that the addition of BAC increased the species richness in water samples and mitigated the time series changes of community compositions. By simply adding BAC into water samples, both the quantitative and qualitative information on aqueous eDNA can effectively be preserved during transportation to the laboratory, which would allow to more precisely estimate species abundance and biodiversity via eDNA analysis.

(Ebina) Thank you very much, Toshiaki. Do you have any questions or comments?

(Allan) I think the results you found between the biphasic and the monophasic with the field are interesting. Do you have a hypothesis for why you did not see biphasic in the field sampling?

(Jo) The difference can be explained by the lower initial concentration of eDNA or fewer sampling time points in the field sampling relative to the tank experiment.

(Allan) So you are already at the second phase or you are just not getting the resolution between time 0 and six hours?

(Jo) We might have confirmed biphasic degradation if the sampling time points were prolonged or increased.

(Kelly) It seems fantastic. Do you recommend everyone to use BAC or is there any situation in which you would not use BAC?

(Jo) The addition of BAC increased initial concentrations, but depending on water chemistries, maybe the addition of BAC completes not only eDNA but also some PCR inhibition substances. For example, with very turbid water, the addition of BAC would maybe rather cause PCR inhibition.

(Yamahara) Do you think you can modify the BAC protocol to be added to filters instead of water samples?

(Jo) Maybe, but I have not tried it.

(Yamanaka) I can answer the question. I have tried to preserve an eDNA sample in a Sterivex cartridge filter. I added the BAC to water at 0.01% final concentration, filtered the water with Sterivex, capped both ends with luer lock caps, and transferred the sample at room temperature to the lab during a few days of travel back to the university. I did not confirm the efficiency, but it worked.

(Yamahara) That is good news.

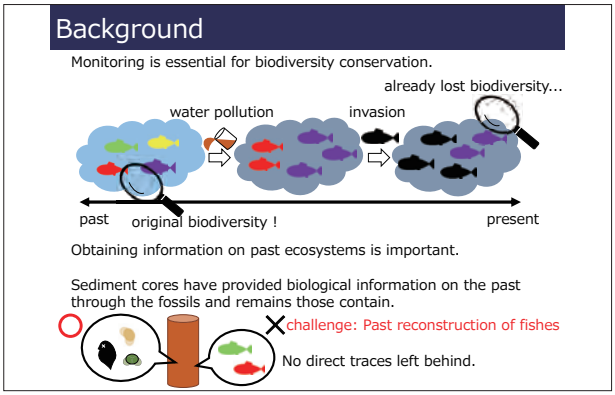
(Ebina) Thank you very much, Toshiaki. The next speaker is Masayuki Sakata. “Reconstructing past biological information in Lake Biwa using sedimentary environmental DNA approach.”

Talk 4

Reconstructing past biological information in Lake Biwa using sedimentary environmental DNA approach

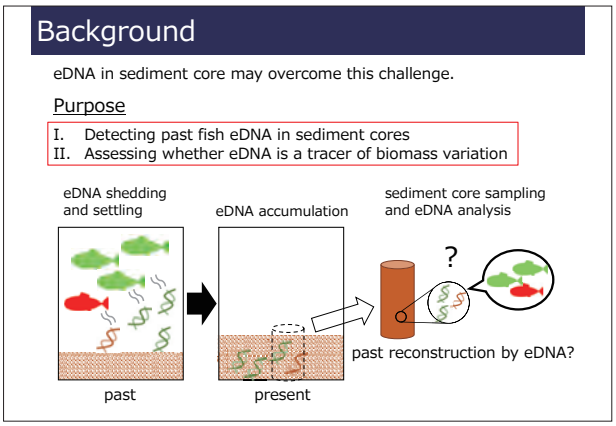
Masayuki Sakata (Kobe University)

My name is Sakata. I am a PhD student in Minamoto Lab. I am mainly in charge of eDNA metabarcoding. I am interested in eDNA in sediment. This is some research related to that.

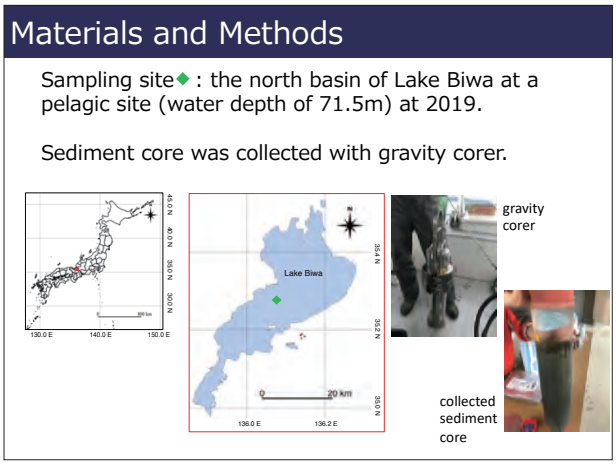


The loss of biodiversity is a serious environmental problem on the planet. It is reported to be particularly serious in freshwater environments. Biodiversity monitoring is important to conserve biodiversity. In monitoring, it is important to understand where, what, and how much species inhabit. However, even if a diversity survey was conducted in an environment, it would not be able to understand the original biodiversity that has already decreased. It is important to know what kind of species originally inhabited an area in order to achieve the goal of ecosystem reconstruction.

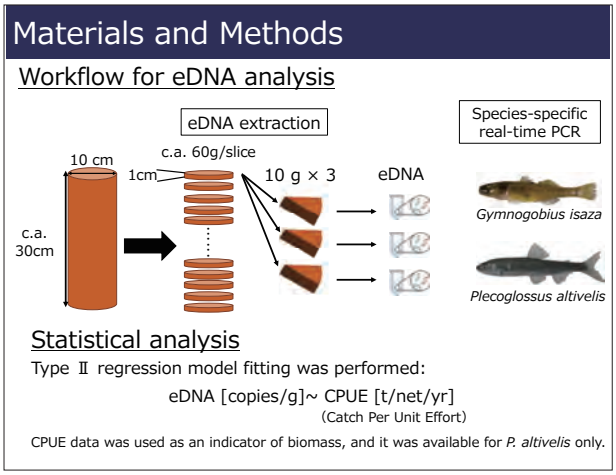
Sediment cores contain the remains and fossils of organisms and have been used to obtain information on past organisms. However, in the case of fishes, despite their importance in freshwater ecosystems, they do not leave direct traces such as remains in sediment, so past reconstruction is a challenge.



However, since eDNA in sediment is preserved for a long time, it can be used to overcome this challenge. Therefore, this research was conducted with the following two purposes. First, an attempt was made to detect past fish eDNA in sediment cores. Second, the temporal variation of eDNA concentrations was tested to see if it could be a tracer of biomass variation.



The sediment cores used in this study were collected off the coast of Lake Biwa in 2019. Lake Biwa is the largest lake in Japan. The sediment cores were collected with a gravity corer. The diameter is about 10 cm and the length is about 30 cm.

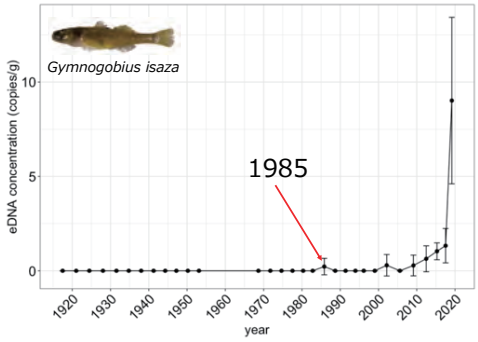


This figure shows the workflow of eDNA analysis in the study. First, the sediment cores were sliced at a thickness of 1 cm. Next, three 10-gram samples were collected from each sediment slice and eDNA was extracted. The extracted eDNA was analyzed by species-specific real-time

PCR for two target fish species, both of which are native to Lake Biwa, to examine whether past fish eDNA could be detected in the sediment cores. Next, type Ⅱ regression model fitting was performed to examine the relationship between eDNA variation and biomass variation. CPUE data was used as an indicator of biomass, but it was only available for *Plecoglossus altivelis*, so this analysis was performed only for one species.

Results

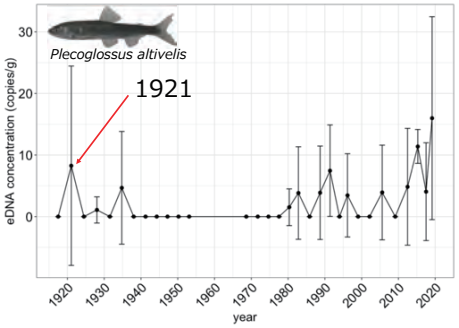
Gymnogobius isaza eDNA was detected in sediment slices corresponding to about 35 years ago at the oldest.



Through dating, it was estimated that the sediment cores contained sediment of about the past 100 years. This graph shows the results for *Gymnogobius isaza*. The vertical axis shows the eDNA concentration and the horizontal axis shows the estimated date of sediment. The results of the analysis showed that the eDNA of this species was detected in sediment slices corresponding to about 35 years ago at the oldest.

Results

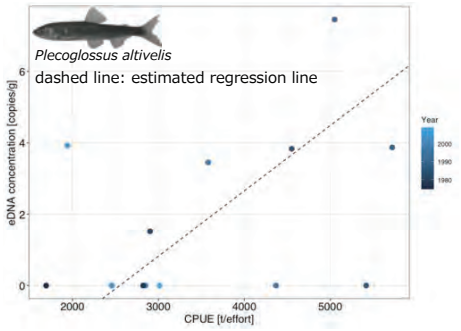
Plecoglossus altivelis eDNA was detected in sediment slices corresponding to about 100 years ago at the oldest.



This graph shows the results for *Plecoglossus altivelis*. The vertical axis shows the eDNA concentration and the horizontal axis shows the estimated date of sediment. The results of the analysis showed that the eDNA of this species was detected in sediment slices corresponding to about 100 years ago at the oldest.

Results

CPUE was not significant for eDNA concentration but had a positive trend.

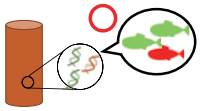


Type Ⅱ regression model: eDNA ~ CPUE (p = 0.16)

Here, the relationship between eDNA concentration and CPUE is shown. In this graph, the vertical axis shows eDNA concentration and the horizontal axis shows CPUE. The analysis was conducted using data from 1975 to 2019 when CPUE data was available. Statistical analysis showed that there was no significant relationship between the two factors, but a positive trend was observed.

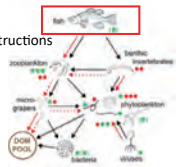
Discussion

- eDNA in sediments can provide information on past fish species.



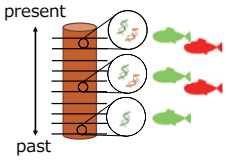
Sedimentary eDNA analysis may have the potential to overcome the gap in past reconstructions.

The gap in past reconstructions



(Ellegaard *et al*. 2020: Fig. 1)

- Temporal detection limits were differed among fish species.



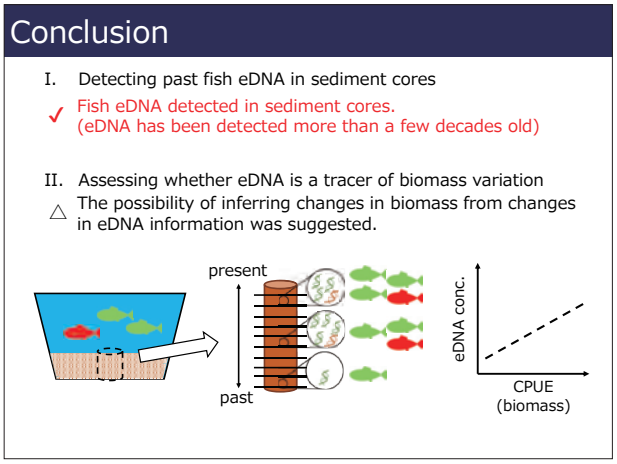
may caused by...
the difference of biomass,

or shedding rate,

or spatially heterogenous distribution of eDNA in sediment.

In this study, fish eDNA was detected in past sediments. Two species of eDNA could be detected in sediment layers corresponding to 35 years ago and 100 years ago, at the oldest, respectively. Fish information was a gap in past reconstructions, especially in freshwater ecosystems, however sedimentary eDNA analysis may have the potential to overcome this gap.

On the other hand, there are several issues. One of them is the difference in temporal detection limits among fish species, even though both target species must have inhabited Lake Biwa during the period when the sediments contained them. This can be attributed to several factors. For example, differences in initial concentration due to different biomass and release rates, or spatially heterogeneous distribution of eDNA in sediments. The resolution of such issues is a future challenge.



In conclusion, two purposes were set for this study. The first purpose of detecting past fish eDNA in sediment cores was achieved. On the other hand, the second purpose of tracking biomass variation by eDNA was not completely achieved, but a positive trend was suggested. Therefore, one future issue to achieve this purpose. I am very much looking forward to the future development of past reconstruction using sedimentary eDNA.

(Ebina) Thank you, Masayuki. Do you have any questions or comments?

(Kelly) This is quite exciting. I did not realize that you could get DNA from so long ago in sediment. I was just reading your paper about the comparison of sediment and water DNA, and so this is the logical extension of that work. Of course, there is some degradation over time. Is that a term in the model for CPUE? How are you thinking about degradation over time so that you are not comparing directly 1920 and today?

(Sakata) I understand that problem, but this is a proof of concept. The comparison of CPUE and eDNA concentration is a challenging issue, but a positive trend was suggested. In the future, maybe we will calculate the degradation rate in sediment cores to compare CPUE and eDNA concentration.

(Allan) When you see no DNA in earlier years and then you start to see DNA 30 or so years ago, how would you separate out whether it is an introduction of a species or a detection limit because of decay? Are there other tools or historical data that you could use?

(Sakata) That is a very difficult problem. For example, after the introduction of an alien species, we will collect sediment examples and test for detection or no detection.

(Minamoto) We have records of species in Lake Biwa maybe from 400 or 500 years ago, so no detection would probably mean degradation.

(D'Agnese) I saw that your timeline went back 100 years. Is that as long as the core sample that you took or was it only a portion of it?

(Sakata) In this study, the max age was 100 years ago. Maybe we will collect longer cores and examine older environments.

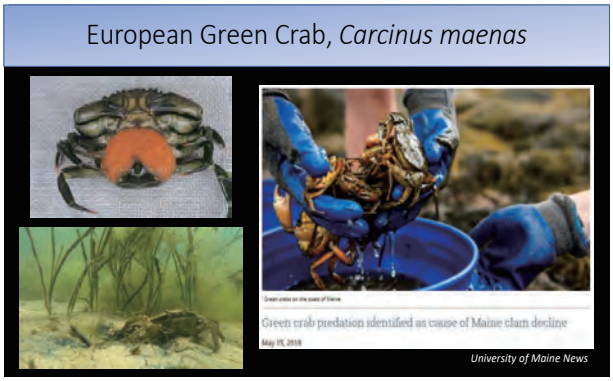
(Ebina) Thank you, Masayuki. The next speaker is Abigail Keller. "Tracking a marine invasion front using molecular surveys."

Talk 5

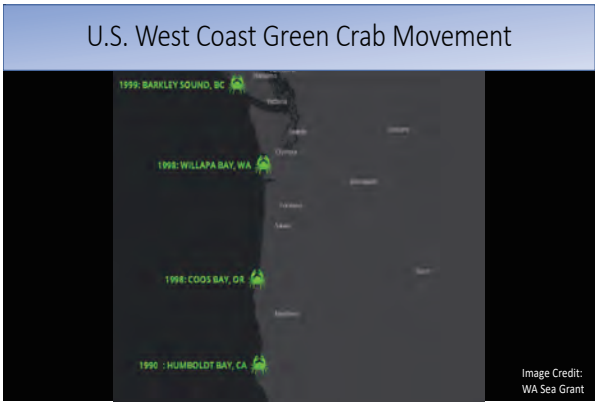
Tracking a marine invasion front using molecular surveys

Abigail Keller (University of Washington)

I am Abby Keller. I am a Master's student at the University of Washington working with Ryan Kelly.

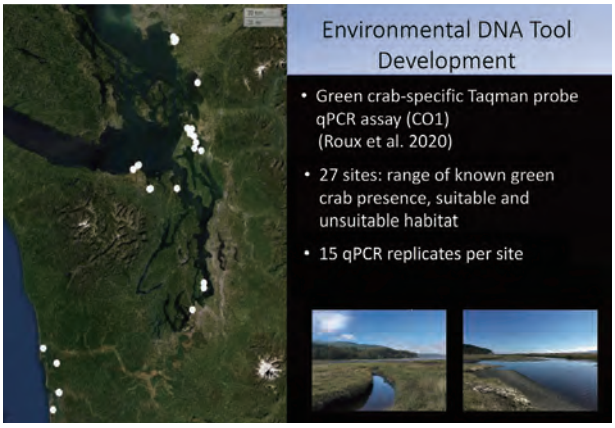


This study focuses on the European green crab. It is one of the most harmful aquatic invasive species in the world. It is also one of the most widespread. It is found on all continents except Antarctica. They are successful invaders because they are robust and can survive a wide range of temperatures and conditions. They also reproduce in large abundances. The picture in the top-left is a female green crab with about 400 eggs. They can negatively impact ecosystems because they dig up eelgrass habitats, outcompete native shore crabs, and are also aggressive and voracious predators. This predation has been designated as the singular main cause of the collapse of a soft-shell clam fishery on the East Coast of the United States. As waters are warming, they are able to expand into new ranges and survive in new locations.



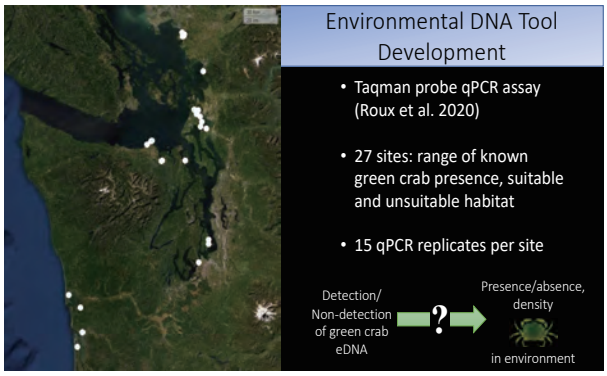
In the 1990s, they were first discovered on the West Coast of the U.S. and have since then been making their way north.

In 2012, they made their way into the Salish Sea which is a shared water body between British Columbia in Canada and Washington in the United States.



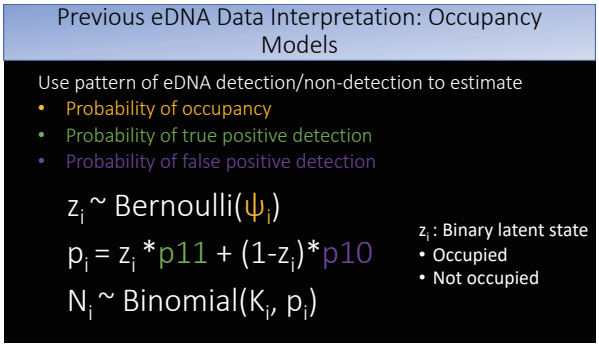
Managers are really concerned about habitat alteration and fishery resources in the Salish Sea, so they were interested in developing an early detection eDNA monitoring tool for the green crab. We used and refined an existing qPCR assay that was developed by scientists in British Columbia, and then to test it in the field, we collected water samples at 27 sites all around the Salish Sea and also along the coast of Washington. Green crabs like marshy, soft-bottom habitats, so 20 of these sites were highly suitable for green crabs and seven of them were unsuitable. Across these sites, we have a range of known presence and abundances of green crabs.

What we are trying to understand is how the detection or non-detection of green crabs relates

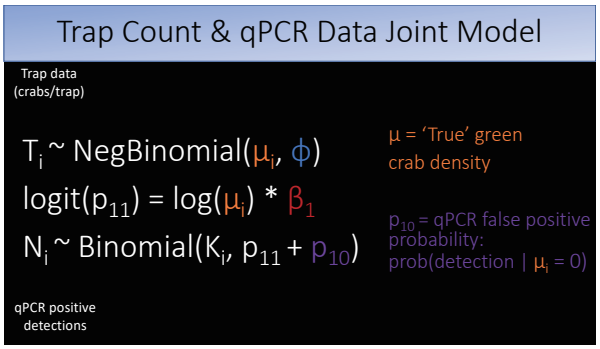


to the presence and absence or density of green crabs in the environment.

A few government groups conduct regular monitoring for green crabs. They put out a baited trap, leave it there overnight, come back the next day, and then remove any green crabs caught in the trap. We have some estimates of green crab densities from these trapping efforts at many of our sites. These trapping efforts are conducted by both the U.S. government and the Washington Department of Fish and Wildlife. In addition, Washington Sea Grant has a community science group called the Crab Team that helps expand monitoring efforts.



Occupancy modeling is a technique that was originally developed for wildlife studies, but recently they have been applied to understand detection histories from single-species eDNA data. These models estimate the probability of occupancy of a species at a site, as well as the probabilities of true positive and false positive detections. These models are contingent upon the latent state, which is binary. It is either truly occupied or truly not occupied by a species. We were finding that this binary latent state was insufficient for our data because each site seemed to have a different underlying abundance of green crabs. The probability of detecting green crabs depends on abundance, so the estimates of occupancy depend on abundance as well. We wanted to find a way to make better use of the quantitative information that we had. We also had information from trapping efforts, so we wanted to find a way to incorporate both the trapping data and the eDNA qPCR data so that they can mutually inform each other.



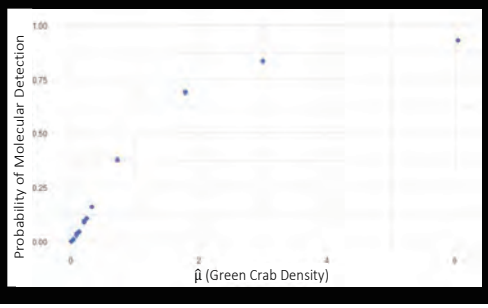
In order to use all available information to estimate green grab density and presence, we developed this joint model that uses both trapping data and qPCR information. The symbols and colors are the parameters estimated by the model. We used trapping data that were collected within a three-week window of when our water samples for eDNA were detected so that they both would capture the same environmental status, but with different forms of sampling.

The number of crabs per trap at site "*i*" is an independent draw from a negative binomial distribution, and the mean of that distribution is the true green crab density. We then incorporated our qPCR false positive detections. The number of detections at site "*i*" is being drawn from a binomial distribution with a probability detection that is broken up into two parts. The first part, p_{11}, is our true positive probability of detection. This is our link between the qPCR data and the trapping data. As the true green crab density increases, so does the true positive probability of detection. False positives are an issue with eDNA surveys, so p_{10} is our false positive probability. This is the probability of a molecular detection, given that the true green crab density is actually zero.

Also, this model is formulated under a Bayesian framework. In this type of statistics, you can incorporate extra information into the distributions that inform the estimated parameters, so we included an informative prior

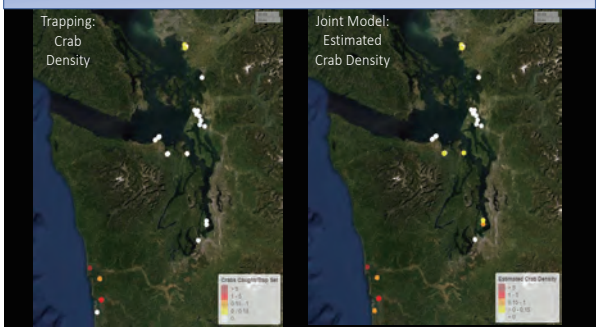
distribution on p_{10} using information from our negative controls. This model is useful because it incorporates two types of data that arise in different ways from the same shared underlying crab density.

Green Crab Density & qPCR Detection Probability: Log-Linear Relationship



As green crab density increases, the probability of detecting them with molecular methods also increases. This is fundamental to the model. This probability of detection then reaches a saturation point at higher densities. Therefore, if you do enough qPCR application, you can get a decent estimate of the probability of molecular detection and then relate it back to the underlying green crab density.

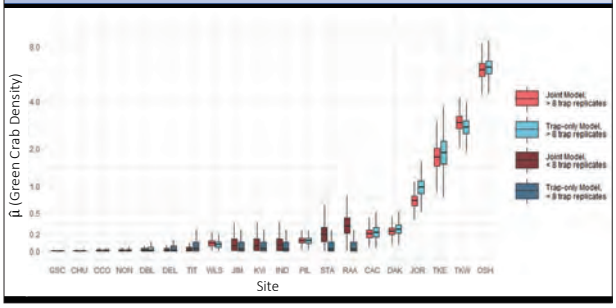
Tracking the Green Crab Invasion Front



Using this joint model of both qPCR and trapping data, we have been able to improve estimates of green crab density and track the invasion front. The map on the left is trapping data over the three-week time period that we used that overlapped with our eDNA collection. This is the number of crabs caught per trap. There has been a historically high abundance of green crabs at the coast, as well as at Drayton Harbor at the border of Canada and the United States.

The map on the right is the output of our model. This is the estimated green crab density that is informed by both eDNA and trapping data. We have some non-zero estimates of green crab density in places that they were trapped. In particular, the two sites in the bottom-right are much farther south than green crabs have ever been seen before.

Trap/qPCR Joint Model: Useful at Lower Trapping Efforts



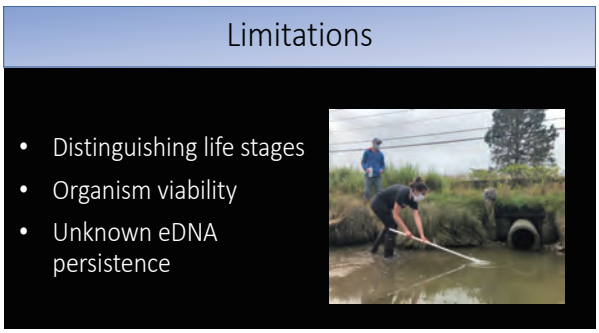
We wanted to directly compare how the addition of eDNA information influences crab density estimates. This is the output of two separate models. One model uses only trapping data and the other is our joint model that uses both qPCR and trapping data. For each paired box plot, the left is the estimated density from the joint model and the right is the estimated density from the trap-only model. Across all of our sites, there were differing trapping efforts. Some of our sites had 60 traps set over the three-week period and some only had three traps set, so I used two different color schemes. The lighter colors represent the sites that had a larger trapping effort and the darker colors represent the sites that had a lower trapping effort.

The sites where the estimates of green crab density diverge between the two models are those where there was a lower trapping effort. For example, with DEL and TIT, by adding qPCR data to the model, it narrows the credibility intervals so that we can more confidently say that green crabs are likely not there. On the other hand, with STA and RAA, by adding qPCR information, we can estimate non-zero estimates of green crab density and say that they are likely at low abundances that cannot be detected using such a low number of traps set.

Conclusions

- eDNA sampling → useful supplement at low trapping efforts
- Two sampling methods: arise from the underlying density in consistent ways

I think the biggest conclusion is that eDNA sampling is useful to supplement low trapping efforts to see if they are there at low abundances or not. What I found interesting is that the two sampling methods of trapping and qPCR eDNA arise from the same underlying crab density in consistent ways.



These are the limitations. At least in the southern sites where we are detecting green crab eDNA where we have never seen them before, maybe we are actually detecting larval DNA because there is not a great way of distinguishing life stages. Maybe we are detecting non-viable organisms, maybe it is a crab molt, or maybe it is eDNA that has been resuspended from the sediment. However, I think from a management perspective, these uncertainties might not matter as much, because this is really most useful to be able to figure out where to increase and direct future management and removal trapping efforts for monitoring the invasion front.

(Ebina) Thank you very much, Abby. Any questions or comments?

(Jo) How did the crab invade the U.S.? Did it move by itself or was it moved by some anthropogenic activity?

(Keller) Most likely anthropogenic activity. I think over longer distances, it is probably ballast water in ships. Over shorter distances, you can have a recreational boat where a crab is accidently stuck in someone's shoe, but I think for the most part, probably ballast water. A lot of the sites that have historically high abundances are harbors where you would expect ballast water exchange to be happening.

(Jo) Are the crabs used for recreation or for eating?

(Keller) Certain areas have been trying to turn them into a culinary delicacy to try to manage them, but they are usually not eaten.

(Jo) You performed real-time PCR to detect the invasive crabs. What is the threshold of detection or non-detection in the 15 PCR replicates?

(Keller) The threshold of detection is about 38 cycle thresholds.

(Jo) Do you mean you considered the target eDNA was detected if one of the 15 replicates was eDNA positive?

(Keller) Yes, I considered a positive detection as any amplification below 38 cycles in any of the replicates.

(Kelly) The model treats each replicate separately, so it would be counted as one detection out of 15 attempts.

(Takeshita) You did qPCR with 15 replications. Why did you choose 15? I feel that it is a lot of replications.

(Keller) I took five water samples at each site, and then for each water sample, I did three replicates. In retrospect, I think you would want so many replicates, especially for green crabs, because they have a relatively low shedding rate, so they are harder to detect than other taxa like fish which are maybe shedding mucus in higher abundances. Therefore, by having the high replication, we were able to better estimate the probability of detection.

(Takeshita) I have another question about qPCR detection. How many copies are the limit of detection and the limit of quantification? The detection may be dependent on the limit of detection.

(Keller) The limit of detection is about 16 copies. I am not sure about quantification. We were finding that most of our positive qPCR had high cycle numbers, so beyond the limit of quantification, we were doing qPCR, but not quantitatively. If we got a hit, it was one. In that sense, we were probably not using qPCR to its full capability.

(Takeshita) 16 copies per 1 L?

(Keller) I think it was 16 copies per 1 µL.

(Minamoto) You incorporated the false positive rate into your model. How did you estimate the false positive rate?

(Keller) We have negative controls at different stages of the process like the qPCR stage and the extraction stage. Given that, we estimated that the probability of a false positive detection was probably less than 0.01, and so we generously set it at 0.01.

(Kelly) The model is a Bayesian framework, so we can use the negative controls to inform our prior distribution on the false positive rate, and then let the model find the posterior distribution for that parameter.

(Ebina) Thank you, Abigail. Let us move to the last talk by Mingyang Jiang. "Assessing nutritional status of carp by environmental nucleic acid analysis."

Talk 6

Assessing nutritional status of carp by environmental nucleic acid analysis

Mingyang Jiang (Kobe University)

My name is Mingyang. I am a second-year Master's student in Kobe University. I am doing research on eRNA.

Introduction ~eDNA

- Lower cost & non-invasiveness (Evans *et al.* 2017).
- No need for morphological taxonomic expertise (Thomsen & Willerslev 2015).
- However, it is difficult to identify the status and developmental stage of an organism with eDNA.

Compared with traditional methods, eDNA has some benefits. For example, we do not have to spend a lot of time and money, and it is not invasive. eDNA detects species by analyzing DNA released by organisms into the environment. It does not require morphological taxonomic expertise because it identifies species based on their DNA sequences. eDNA analysis has been widely used in studies, but it is difficult to identify the status and development stage of an organism with eDNA. In order to solve such problems, eRNA analysis is being considered.

Introduction ~eRNA

- Research to detect eukaryotic RNA in environmental water is on the increase(von Ammon *et al.* 2019; Tsuri *et al.* 2020).
- Because RNA degrades rapidly after cell death, it can be a "living" signal (Pochon *et al.* 2017; Zaiko *et al.* 2018).
- RNA expression patterns change due to physiological conditions (reproduction, etc.) and environmental stress (hypoxia, etc.) (Shahjahan *et al.* 2010; Rytkönen *et al.* 2014; Zhang *et al.* 2015).
- If RNA can be detected from environmental water, it may be possible to extract more detailed biological information from "water" such as the health status of living organisms.
- This research focused on the nutritional status of the organism.

In recent years, research on the detection of eukaryotic RNA from environmental water has increased. RNA degrades rapidly after cell death, so it can be considered a "living" signal of an organism. RNA expression patterns change due to physiological conditions and environmental stress. If RNA can be detected from environmental water, it may be possible to extract more detailed biological information from water such as the health status of living organisms. This research focused on the nutritional status of the organism.

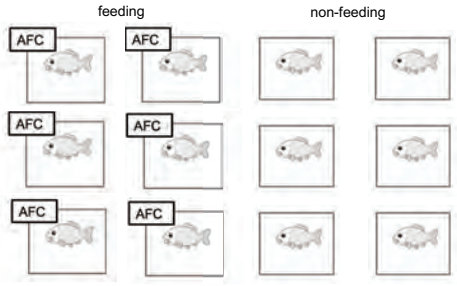
Introduction ~RNA/DNA

- In macro-organisms, the RNA/DNA in tissues is used as an index for growth and health status(Buckley 1979; Buckley 1984).
- RNA/DNA in well-nourished individuals is higher than in poorly-nourished individuals(Caldarone *et al.* 2003; Wu *et al.* 2019).
- However, there is no previous study of evaluating the nutritional status of an organism using eRNA and eDNA.
- Objective: verify whether eRNA/eDNA can be used to evaluate nutritional status.

The RNA and DNA ratio in tissues is used as an index of growth, nutritional status, and condition of macroorganisms. Compared with poorly-nourished individuals, well-nourished individuals have a higher RNA to DNA ratio. However, there is no previous study evaluating the nutritional status of an organism using eRNA and eDNA. This study verifies if the eRNA to eDNA ratio can be used to evaluate nutritional status.

Material & Methods

- Experimental organism: common carp (*Cyprinus carpio*)
- Time: Oct 6~27, 2020 3 weeks



AFC: Auto Feeding Charger

In this experiment, I used common carp as the experimental organism. The experiment was conducted for three weeks. One carp was placed in each of 12 water tanks. Six of the carp as the feeding group were fed daily with an automatic feeder. The other six were not fed as the non-feeding group during the entire experiment.

Material & Methods

- Water sample:

Breeding water volume: 50 L
Frequency of water collection: twice a week
Water sampling: 0.5 L each for eDNA and eRNA
Filtration: 0.7 μm GF/F

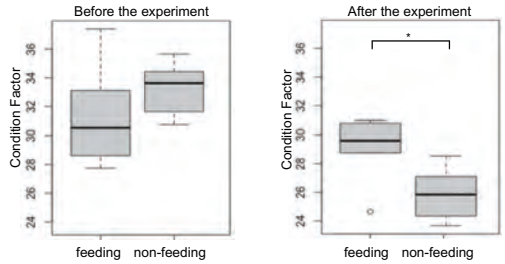
- Tissue sample: muscle tissue (last day)

- DNA extraction: DNeasy Blood & Tissue Kit
- RNA extraction: NucleoSpin RNA Plus kit
- cDNA synthesis: PrimeScript RT reagent kit with gDNA Eraser

- Real time PCR: Nuclear marker ITS1

I took 0.5 L of water samples twice a week each for eDNA and eRNA. I took carp muscle tissue as a tissue sample on the last day of the experiment, then extracted DNA, as well as RNA to synthesize complementary DNA. After that, real-time PCR was performed on the nuclear marker, ITS1.

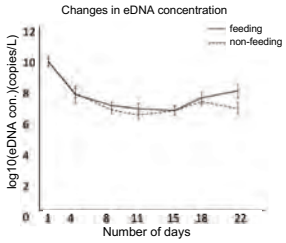
Result ~Condition Factor



- Condition Factor K(CFK)= weight(g) / length(cm)^3×1000
- Before the experiment, there was no significant difference in CFK between the feeding group and the non-feeding group.(Wilcoxon rank sum test, p>0.05)
- After the experiment, there was a significant difference in the CFK between the feeding group and the non-feeding group.(Wilcoxon rank sum test, p<0.05)

These are the results of carp condition factor before and after the experiment. Before the experiment, there was no significant difference in CFK between the feeding group and the non-feeding group. On the other hand, after the experiment, there was a significant difference in CFK between the feeding group and the non-feeding group.

Result ~eDNA



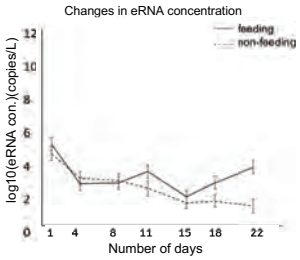
Generalized linear mixed model(GLMM)
(Random variable: water tank)
Response variable: eDNA concentration
(1st day was removed)
Explanatory variable:

- days(p=0.4)
- feeding or non-feeding(p=0.8)
- days*feeding or non-feeding(p=0.6)

- The feeding or non-feeding did not affect the eDNA concentration(p>0.5).

These are the results of changes in eDNA concentration. A generalized linear mixed model was used to test whether the passage of days and the feeding or non-feeding had a significant effect on eDNA concentration. The random variable was the water tank. The response variable was the eDNA concentration. The explanatory variables were the passage of days, feeding or non-feeding, and the interaction between days and feeding or non-feeding. It was found that the feeding or non-feeding did not affect the eDNA concentration.

Result ~eRNA



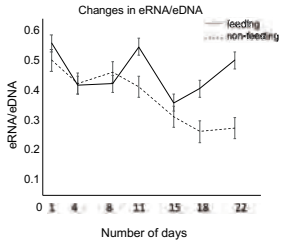
Generalized linear mixed model
(Random variable: water tank)
Response variable: eRNA conentration
(1st day was removed)
Explanatory variable:

- days(p=0.07)
- feeding or non-feeding(p=0.12)
- days* feeding or non-feeding(p<0.05)

- Over time, eRNA concentration decreased in non-feeding condition compared to feeding condition(p<0.5).

These are the results of changes in eRNA concentration. A generalized linear mixed model was used to test whether the passage of days and the feeding or non-feeding had a significant effect on eRNA concentration. It was found that, over time, eRNA concentration decreased in the non-feeding condition compared to the feeding condition.

Result ~eRNA/eDNA



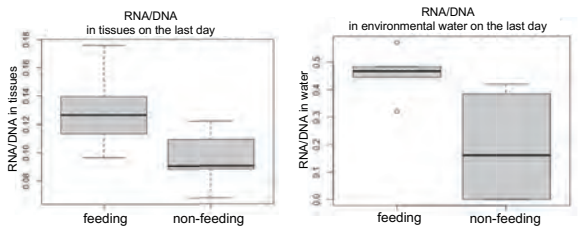
Generalized linear mixed model
(Random variable: water tank)
Response variable:
eRNA/eDNA
(1st day was removed)
Explanatory variable:

- days (p=0.7)
- feeding or non-feeding(p=0.3)
- days*feeding or non-feeding(p<0.05)

- Over time, eRNA/eDNA decreased in non-feeding condition compared to feeding condition(p<0.5).

These are the results of changes in the eRNA to eDNA ratio. A generalized linear mixed model was used to test whether the passage of days and the feeding or non-feeding had a significant effect on the eRNA to eDNA ratio. It was found that, over time, the eRNA to eDNA ratio decreased in the non-feeding condition compared to the feeding condition.

Result ~RNA/DNA(last day)



- RNA/DNA of ITS1 in tissues: higher in feeding group than non-feeding group(Wilcoxon rank sum test, $p<0.05$).
- RNA/DNA in environmental water: higher in feeding group than non-feeding group(Wilcoxon rank sum test, $p<0.05$).

These are the results of the RNA and DNA ratio on the last day. It was found that the ratio of RNA to DNA of ITS1 in tissues in the feeding group was higher than in the non-feeding group. Similarly, the ratio of RNA to DNA in the environmental water was higher in the feeding group than in the non-feeding group.

Discussion

- My study suggests that the nutritional status of fish can be estimated by the eRNA/eDNA.
- eRNA concentration decreased with non-feeding condition. Non-feeding may cause the deteriorated nutritional status and decrease of RNA expression.
- It is necessary to examine whether the nucleic acid ratio of ITS1 corresponds to the total nucleic acid ratio generally used as an index of nutritional status.

My study suggested that the nutritional status of fish can be estimated by the ratio of eRNA to eDNA. eRNA concentration decreased with the non-feeding condition. Non-feeding may cause the deteriorated nutritional status and decreased RNA expression. In this experiment, ITS1 was used to compare the nucleic acid ratio in tissues. It is necessary to examine whether the nucleic acid ratio of ITS1 corresponds to the total nucleic acid ratio generally used as an index of nutritional status.

Conclusion

- It was suggested that the nutritional status of fish can be estimated from the eRNA and eDNA.

it is possible to monitor and improve the growth and mortality of fish using eRNA/eDNA in the process of protection of rare species and aquaculture.

In conclusion, my study suggested that the nutritional status of fish can be estimated from the eRNA to eDNA ratio. It is possible to monitor and improve the growth and mortality of fish using the eRNA to eDNA ratio in the process of protecting rare species and aquaculture.

(Ebina) Thank you, Mingyang. Do you have any questions or comments?

(D'Agnese) How did you take the muscle tissue? Did you euthanize the fish and excise muscle?

(Jiang) Yes.

(D'Agnese) Did you do a post-mortem necropsy with pathology to see if there were any underlying health issues or were these fish previously known to be healthy? How did you verify that the lack of RNA expression was nutritional stress and not potentially other underlying confounding health issues?

(Jiang) Sorry, I do not understand what you are talking about.

(D'Agnese) Were the fish verified as healthy to start with?

(Jiang) Yes.

(D'Agnese) So you did not necessarily do a necropsy with any pathology post-mortem?

(Jiang) Yes.

(D'Agnese) If they were healthy, that is fine.

(Kelly) This is very exciting because I think it is a way of telling live animals from dead animals. You compared good condition animals and poor condition animals, but live and dead would be even bigger. It would be an even bigger difference and that is very exciting for people who want to know if they found a live fish or a dead fish. I think this is a way to do that.

(Gold) If you were taking a sample in the field and not from an aquarium, what kind of sampling do you think you would need to take to be able to distinguish the status of a fish, whether it is fed or not fed, to a fish that was maybe there from a couple of days ago? You would expect the eRNA ratio to be lower because DNA lasts longer than RNA.

(Jiang) I think it is difficult to distinguish between good condition fish and bad condition fish because of the RNA degradation, but it is a problem that we are going to solve.

(Minamoto) I think our method can be used in aquaculture or some limited conditions.

(Yamanaka) I want to know the validity. You used the RNA and DNA concentrations in tissue samples of carp as an indicator of the condition of fish, but I am not sure it is the best indicator for your study. In your experiment, you used starvation as the trigger to change the health status of fish, but is there any other better indicator to measure the condition of fish in this case? Maybe using a different tissue would give you a better relationship. What do you think about that?

(Jiang) You mean if I use other tissues I can get better results?

(Yamanaka) Yes. I am not sure, but maybe different types of stress will affect different tissues. It could be a future experiment theme. Please think about that.

(Ebina) Thank you very much, Mingyang. Now, we would like to move to the overall discussion.

Discussion

(Ebina) First, I would like to ask anybody who did not make a self-introduction yet to do so now.

(Yamanaka) I am Hiroki Yamanaka, an associate professor at Ryukoku University.

(Doi) I am Hideyuki Doi from the University of Hyogo. I am working on eDNA things also.

(Saito) I am Tatsuya Saito. I am studying the degradation of eDNA at the University of Hyogo.

(Ebina) Okay, now we would like to move to the overall discussion. Do you have any comments or questions?

(Yamanaka) I want to discuss the future usage of sedimentary eDNA. Does anyone have any idea of how to calibrate the eDNA concentration in each layer of the core?

(Minamoto) We can sometimes estimate the degradation of the eDNA in sediment from the concentration of the profile or organic materials.

(Kelly) You could maybe do a one or two-year study. If there is an exponential decay, most of the decay happens quickly in the time that you do the study, and so you could bury some fish extracts in the sediment at different levels for a time and then calibrate the degradation with a known sample over the first year or two. If the exponential decay is consistent, you could carry it out over time. You cannot go back in time to 100 years ago, but you might be able to constrain the possible values of 100 years ago from more recent samples.

(Gold) Another way you could calibrate it is if there was a well-studied system. For example, a river that we have been keeping track of species abundance for a really long time and we know a lot about the abundance. It could even be better if you picked a system where there was a complete loss of a species or an introduction of a new species. There could be a natural experiment somewhere. A dam removal could be a useful experiment to calibrate the degradation rates.

(D'Agnese) I have an off-the-wall idea. You could maybe partner it with some DNA and use stable isotope metrics of measuring the half-life of DNA molecules in samples like you would with other organic molecules in cores or older samples.

(Kelly) You could use eels, Hiroki. In your new paper, I think you had examples of places eels have been introduced, with known abundances over time.

(Gallego) You could try mRNA, cDNA, and metabarcoding in that order to see if you also see a change in the mRNA with time.

(Kelly) I do not have anything particularly intelligent to say, but it is so nice to see people's faces when we read your papers and to try to make friends across an ocean. This is real treat for all of us and I would just like to say thank you very much. I think it is exciting to see all of the different approaches and technological advances come together. I think it is a particularly exciting time to see the science, imagination, and creativity that are just now coming out of the lab, being used by government agencies to make decisions in the real world.

(Ramon-Laca) Jo, can you explain what BAC does? How does it preserve the sample?

(Jo) BAC is a kind of cationic surfactant which adsorbs the cell surface of microbes and inactivates them.

(Ramon-Laca) So it prevents degradation by microbes?

(Jo) Yes, it is considered that BAC can indirectly preserve eDNA by suppressing enzymatic degradation. A detailed explanation can be seen in Yamanaka et al. (2017) and some citations in it.

(Yamahara) Back to Hiroki's question, I was wondering if there was a way to use the ratio of eRNA to eDNA to look at degradation over time. In the individual slices, if you are looking for one given species, you may presume that there should be both eRNA and eDNA in each slice. That is a chronological record that you already have based on the slices and depth, but if you also look at the eRNA to eDNA ratio, there are going to be some degradation and differences between each individual slice. There could be a way to get an approximation of eDNA age from looking at that ratio as it changes chronologically in the sediment core, because one would expect the eDNA to stick around

much longer than the eRNA.

(Kelly) The size fractionation of the DNA is also a possibility for looking at age. They are maybe independent ways of assessing age. If you have a few independent lines of evidence, you can constrain the possible age.

(Yamanaka) Thank you for your ideas. I think we can use some of your ideas to develop new techniques to calibrate the data. I am not an expert, but probably multiple factors like pressure and oxygen concentration will affect the degradation in each slice of the core, so it will be hard work, but I think we have to do it.

(Gold) I have a question for everyone. I was really fascinated by reading one of Hideyuki Doi's papers about the number of water bottle replicates and technical PCR replicates needed to saturate species diversity in an aquarium. Going forward, what would you recommend in terms of taking samples from coastal marine ecosystems? How many water bottle replicates and how many PCR replicates do you expect we should be taking to saturate diversity?

(Doi) We actually tested how many filters and PCR replicates affect the detection of the community using MiFish metabarcoding and we found PRC replicates to be more important than filter replicates. That means that we should increase PCR replicates more than filter replicates, but I am not sure how we should take water bottle replicates for different habitats. I would like to mention that we should keep many PCR replicates – for example, eight or 16. We can then maybe take more species richness – probably 85%. We usually use eight replicates for the metabarcoding, but for field volume or field replicates, it is a difficult question. We usually use 1 L or 10 L in marine habitats and only one site per coastal regional, but I think it depends on the aim of the study or survey.

(Minamoto) We have some data about the sampling design and the number of replicates. It depends on the habitat. In our experience, in lakes or rivers, we do not have to take so many field replicates, but in the case of dam reservoirs, we have to take many field replicates to detect enough species, so it highly depends on the habitat.

(Kelly) I was interested about the qSeq paper from Prof. Minamoto and Prof. Doi. It seems like a big development. Where will you be taking the quantification next?

(Doi) We just tested in mesocosms. It is a kind of mock community. Actually, this technique is very difficult to directly apply to field samples because it needs many DNA copies in the PCR templates, but Dr. Hoshino, a coauthor of this paper, and I are now thinking about a more developed qSeq technique that needs much less DNA copies in the PCR templates. I am not sure, but we should probably provide a more sensitive technique for qSeq in the near future.

(Kelly) I heard from Hiroki about the potential for a Japanese eDNA meeting at some point in the future. Is there any update? Is there a possibility of that meeting?

(Yamanaka) Thank you for asking. We actually postponed the meeting to next year, 2022. We are still not sure if we can make it an in-person meeting or not, but the place has already been decided. It will be held in Kyoto if it is in person. It could be a good attraction for empowering researchers, so I want to make it an in-person meeting, but we are still not sure. We are looking at the situation related to COVID-19. I will let you know when we decide how to deal with it.

(Kelly) In the United States, different people in management agencies and the government are thinking about what to do with this new technology and this new way of understanding the world, and how it relates to our old way of understanding the world. In Japan, have the government science and management agencies adopted eDNA as a tool? How is that going?

(Minamoto) I think it is the very early stage of usage of eDNA in Japan, especially for the Japanese government. Yamanaka-san, please help me.

(Yamanaka) Hideyuki and Toshifumi are board members of a working group hosted by some ministries of the Japanese government. They are talking about how to go forward with the new techniques of eDNA and eRNA. The Japanese government has big interest in the new technology, but they are still watching how it will be because there are many black boxes. This is my personal opinion, but I think most of the governmental officers want to use

it as a quantification method of fishery resources. There are unknown factors affecting the eDNA concentrations, so they are now looking at the progress of the research to use it as a quantification tool for the fishery species, but we are still talking frequently with government side to promote the usage of this new technique.

(Kelly) It is the same here. The government is interested in this as a technology, but there are people who only want to count fish. They need quantification, but there are many other uses as well such as finding endangered species or invasive species, or even being able to understand water pollution and water quality in a practical way. I think right now it is with the academic scientists and engineers like Kevan to try and make this a tool that works and to demonstrate that, and then as it becomes easy to use, the government will become happier with using it.

(Yamanaka) Kevan, I am very interested in the progress of your research work on the robot behind you. What kind of development are you working on right now? I remember that your robot is working on species-specific detection. How about metabarcoding? Are you trying to develop a robot for that?

(Yamahara) Yes. We have two instruments, the second-generation ESP and the third-generation ESP. On the second-generation ESP, we are not developing any new detection technologies. What we have now is our probe arrays and qPCR, and I think that those will be the main detection technologies that we will focus with on that. On the third-generation ESP, we are always investigating new technologies to do detection, whether it is specific like qPCR or digital PCR. We are also working on a thing called surface plasmon resonance.

We are also working with Oxford Nanopore MinION for onboard sequencing. The difficulty that we are facing with that is data because it is gigabytes of data. How do you send that over a telecommunications network? When you are out in the middle of the ocean, you are sending everything by satellite. It is very expensive and you can only transfer kilobytes of data, so we are working on data reduction. Right now, we have a prototype system that we are working on that goes from sample selection all the way through to MinION sequencing, but the data reduction is a bottleneck that we are having issues with, as well as the sequencing part, and so there are some trade-offs. We are still trying to figure that out, but we are going in that direction.

(Yamanaka) How is the sequencing quality of the MinION? I have heard that it is far lower than the benchtop machine, so how do you deal with it?

(Yamahara) There still are problems with their base calling, but it is getting better and better. I think when you are looking for very specific taxonomic groups, they have been developing some algorithms to look at all the individual sequences and aligning them so that you can get some confidence in your error for your base calls individually. That is just an issue with MinION and I think it will get better. In my personal opinion, I do not like using it, but it is the only format that will allow us to do in situ sequencing at the moment.

(Ebina) Thank you very much, everybody. Prof. Itoh, would you please make the closing remarks?

Closing

(Itoh) I am Masayuki Itoh, the Director of Kobe University Science Shop, the host of today's workshop. My background is astrophysics, but I still enjoyed the talks today. I am very happy to see this event happen because I have been observing Prof. Ebina working so hard to prepare. One of the objectives of the Science Shop is to promote dialogue and collaboration between citizens and scientists. From that point of view, eDNA seems to have very high potential. I hope to see another meeting to discuss such aspects of this wonderful technique. To conclude, I would like to express my gratitude to Prof. Kelly, Prof. Minamoto, Prof. Ebina, and all the other participants today.

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