GENERAL INTEREST PAPER

https://doi.org/10.4315/FPT-25-023

Commercial Tuna Fishing as a Source of Environmental DNA (eDNA)

John DeBeer,[1*] Christopher W. Lord,[2] and Patrick Larkin[4] (formerly of [3])

[1]Retired from Chicken of the Sea International, 2150 E. Grand Ave, El Segundo, CA 90245, USA

[2]Eurofish S.A., La Pradera, Manta, Ecuador

[3]Applied Food Technologies, 12085 Research Dr., Alachua, FL 32615, USA

[4]University of Florida College of Veterinary Medicine, 2015 SW 16th Ave., Gainesville, FL 32610, USA

SUMMARY

This case study was prompted by reports that DNA (deoxyribonucleic acid) of undeclared tuna species had been found in cans labeled skipjack. Only one species of tuna is allowed in a can sold in the European Union if the muscle structure of the tuna is visible. This finding implied mislabeling in these processed tuna products. Given these issues, mislabeling may be considered a food fraud and/or food protection issue. The purpose of this study was to determine the source of undeclared DNA in canned tuna. Samples from several steps of the cannery process were sent to several independent laboratories and analyzed by several molecular methods including Sanger and NextGen sequencing. Theses analysis found commingled environmental DNA (eDNA) from a variety of fish including *Thunnus* spp. in the catcher vessel brine or factory thaw water during processing. At least four species of *Thunnini* were found in the brine and five species of *Thunnini* were found in the thaw water by PCR and Sanger sequencing. Nine genera of fish, including four species of *Thunnini*, were found in the brine, and eight genera of fish and three species of *Thunnini* were found in the thawing water by next-generation sequencing (NGS). The DNA from these species or genera could easily accompany the muscle meat of the target species into the can or pouch. As such, unless a quantitative genetic analysis test is run that can identify how much DNA of various organisms is present in a sample, DNA should not be used as a proxy for tuna muscle tissue in retorted canned tuna processed in commercial tuna factory, because of the distinct possibility of adventitious eDNA from other species of tuna.

INTRODUCTION

This paper is the first of a two-paper series about the DNA (deoxyribonucleic acid) from a species of tuna in retorted canned tuna that is not listed on the label. Eurofish S.A. is a tuna cannery in Manta, Ecuador that has received complaints from its European customers of DNA from unlabeled species being present in cans of tuna labeled "Skipjack" (*Katsuwonus pelamis*) (SJ). This finding could be misconstrued to be that unlabeled tuna muscle meat was packed in those cans tested. Nevertheless, this case may be considered a possible food fraud and/or food protection issue.



In the European Union (EU), only a single species of tuna can be packed in a can if the muscle structure is visible *(18)*. In contrast, the United States (U.S.) Food and Drug Administration (FDA) allows one or more *Thunnus*, *Katsuwonus*, *Allothunnus*, *Auxis*, and *Euthynnus* genera, or a combination to be canned as tuna but with species and color restrictions for labeling *(50)*: these are the genera that make up the tribe *Thunnini (23)*.

Seafood substitution by accident or for economic gain and subsequent fraud is common around the world and is generally illegal based on truth-in-labeling laws *(15, 27, 34, 40, 44, 51, 58)*. Besides economic fraud, there is also a potential danger of an allergen or poison reaction in consumers to a certain type of seafood, or violations of religious restrictions *(60)*: thus, mislabeling is a food protection issue as well as a fraud.

*Author for correspondence: Phone: +1 760.670.6141; Email: jdebeer2005@gmail.com; ORCID: 0000-0002-2735-8649

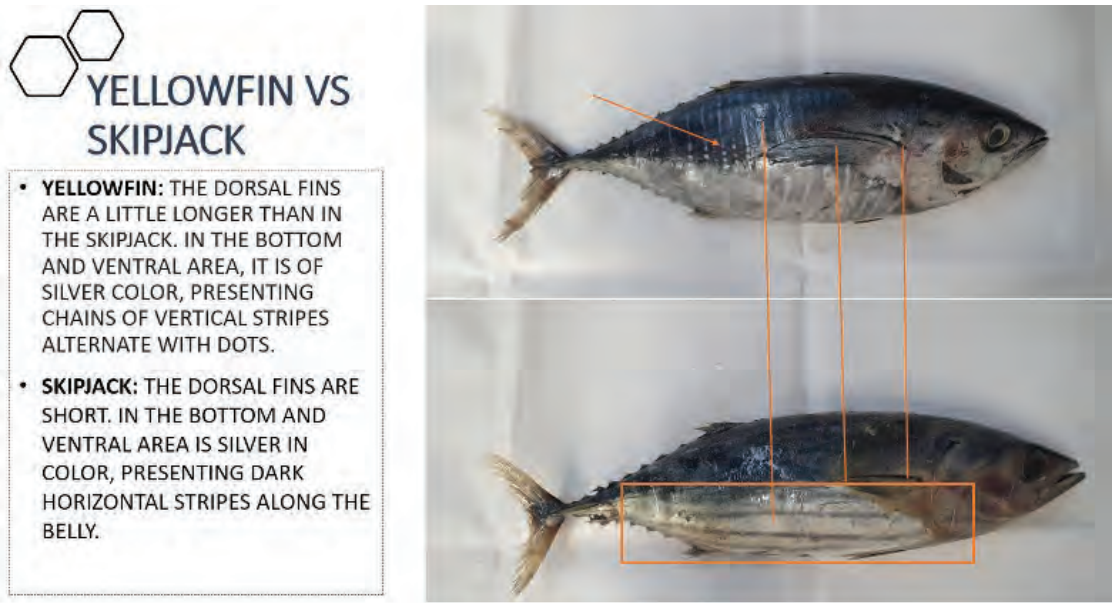

Figure 1. Photos of yellowfin (YF) versus skipjack (SJ) tuna.
Note: These are very realistic photos of yellowfin and skipjack the unloaders, fish sorters, or butchers will see every day.
Source: EuroFish S.A.

In this case, the mislabeled species has been identified as yellowfin tuna (YF, *Thunnus albacares*). YF and SJ are very easy to identify and differentiate between each other *(Fig. 1)*. Adult YF are much larger than adult SJ and large YF have long tails *(19)*. YF, SJ, and *Thunnus obesus* (Bigeye, BE) are easy to identify just after capture; even while frozen and butchered, or further processed *(31, 32)*. YF and SJ generally have different processing times because of size or destination markets. Thus, any mixing of the tuna species muscle meat (if done) in a can is generally intentional, for example, in the U.S. canned tuna market.

SJ is an open ocean schooling tuna primarily caught with purse seiners or pole-and-line baitboats using live bait as an attractant. The unlabeled canned tuna species reported is generally yellowfin (YF). Mixing YF with SJ in the can does not make economic sense because YF has more value in the raw *(Fig. 2)* *(38)* and targets a higher value market than SJ. This first paper will briefly describe tuna fishing and known sources of environmental DNA (eDNA) or adventitious DNA *(43)*.

Environmental DNA (eDNA)

All the cells of animals and plants contain DNA and as these cells are shed from the organism in various ways, the DNA these items contain becomes environmental (eDNA). This eDNA is everywhere, and scientists use it extensively to determine what plants or animals were or are in the area. For example, Andruszkiewicz et al., *(2)* found the DNA from 72 marine vertebrates and Closek et al., *(11)* found the eDNA from 80 taxa in the water near/from Monterey Bay, California, USA. Willette et al., *(59)* found eDNA from numerous finfish in the brine and meltwater in the fish holds of tuna vessels fishing off Ecuador, including *Katsuwonus* and *Thunnus* species. Bakker et al., *(4)* used eDNA to monitor shark species in several areas in two oceans.

Most tuna are schooling fish when younger *(37)*. Schools of SJ, YF, and BE are commonly caught by purse seiners in the tropical oceans when these schools are at or near the ocean surface *(13)*. The large purse seiners may carry nets 2000 meters long and 200 meters deep. At the top of the net are corks to keep the net afloat, and at the bottom is the lead-line, chain and rings, and the purse cable. The lead-line, chain and rings help the net to sink quickly and capture the fish. The cable is used to purse-up the net at the bottom, to close the net and keep the fish from escaping. In the Eastern Tropical Pacific (ETP), the lead-line and purse rings of the net need to get below the thermocline as quickly as possible to keep the tuna in the net; otherwise, the tuna will dive below the lead-line to escape. Thus, the thermocline acts as the bottom of the net until the net is pursed up *(47)*. After the net is set and closed, the net is rolled aboard the tuna seiner until the tuna are concentrated in the "sack" so the tuna can be brailed aboard the seiner using large net "buckets". The fish are put immediately into fish wells with refrigerated seawater (RSW) or cold dense salt brine (CDSB) held at freezing temperatures to prevent decomposition, the development of honeycomb (a physical sign of decomposition) *(20)*, and/or elevated histamine levels *(9)*.

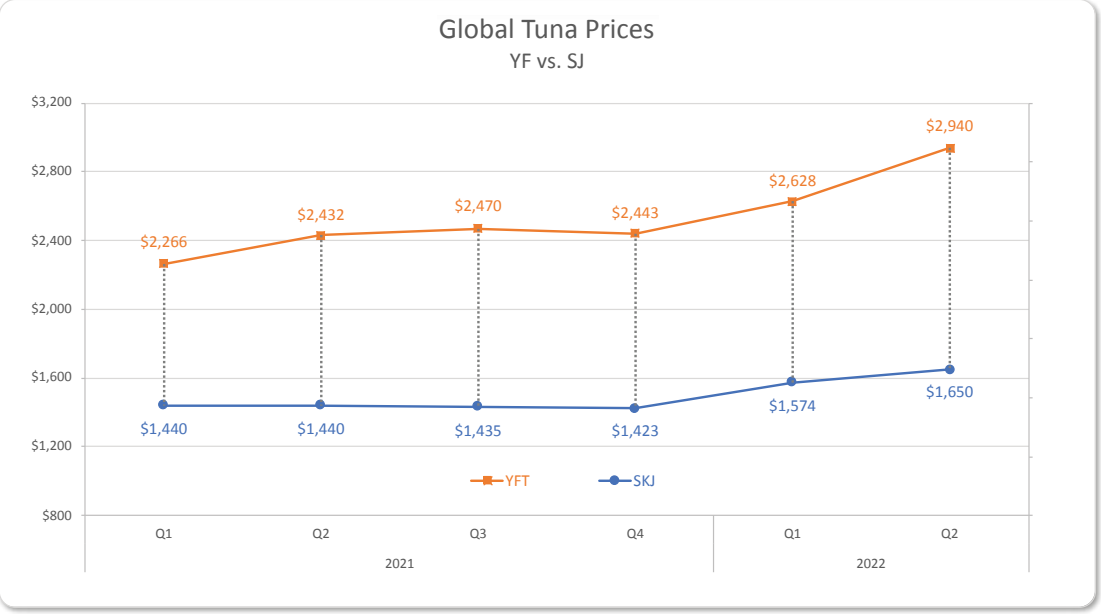

Figure 2. Global tuna prices for 2021 and 2022.
Source: Christopher W. Lord - Data from European Pricing Report

The top speed or cruising speed of tuna depends on their size (length); the larger fish can swim faster than the smaller fish *(25)*. There are several types of schools of tuna species *(46)*. With SJ, there are free swimming schools *(55)*, and there are schools of multiple fish species associated with flotsam of some type *(29)*. There are free swimming schools of YF in all the tropical oceans and schools of YF associated with dolphins (*Stenella attenuata* or *S. longirostris*) in the ETP *(42)*. Each of these schools are caught with different capture techniques to keep the tuna in the net while the net is being set and pursed. The success of the set depends on the skill of the captain to manage the crew, speedboats, tow boats, and net in synchrony *(13)*.

The free-swimming schools of SJ are generally free of other species. The yellowfin and dolphins are generally the only species captured in the dolphin-associated set, but in a set associated with flotsam or floating aggregating devices (FADs), there may be many, many species including sharks *(10, 30)*. All the while, these schools of marine animals are moving through the water, they are shedding DNA, and when the tuna seiners make up the RSW or CDSB *(9)*, they use plain seawater containing this eDNA *(53)*.

The frozen tuna from the fishing boats can be unloaded directly onto a dock at a port and transported by truck or forklift to a cannery. The tuna also can be transshipped in refrigerated containers or more commonly, in bulk carriers that can hold the tuna frozen for the trip from the catcher boat to the port with the cannery. A prime example is transshipping tuna from Majuro, in the Marshall Islands in the Central Pacific, to Bangkok, Thailand for processing *(5)*.

The eDNA remains with or on the fish as it is unloaded at the factory or on the frozen carrier and stays on the target fish as it is unloaded, as well as through processing. Willette et al., 2021 *(59)* found the DNA from both target and non-target fish in brine or melt water from the purse seiners unloading in the tuna port of Manta, Ecuador. They found the DNA from four species of target fish and from four non-target species in the brine or meltwater, meaning that that DNA from non-target fish must have gotten onto the tuna boat either along with the target fish, or was pumped aboard in the seawater used to make up the RSW and CDSB. A schematic of the travels or presence of eDNA from the ocean into a retorted can is shown in *Figure 3*.

Types of DNA sequencing analyses

There have been several methods for sequencing DNA developed in the last several decades. Polymerase chain reaction (PCR) was developed by Kerry Mullis in the mid-80s *(41)*. Mullis developed a method of amplifying (making multiple exact copies or amplicons) strands of DNA using unique primers for targeted genes. This PCR combined with Sanger sequencing (PCR/SS) *(45)* allows strands of DNA to be identified by individual nucleotides in the order they occur in the gene fragment. To identify the DNA from a unique species or genus, PCR/SS is considered the gold standard of DNA sequencing *(1, 22)*. The limitation of the technology is in identifying DNA from several different species simultaneously from a pool of DNA since only strands from a single gene from a single species can be amplified and analyzed at one time via SS.

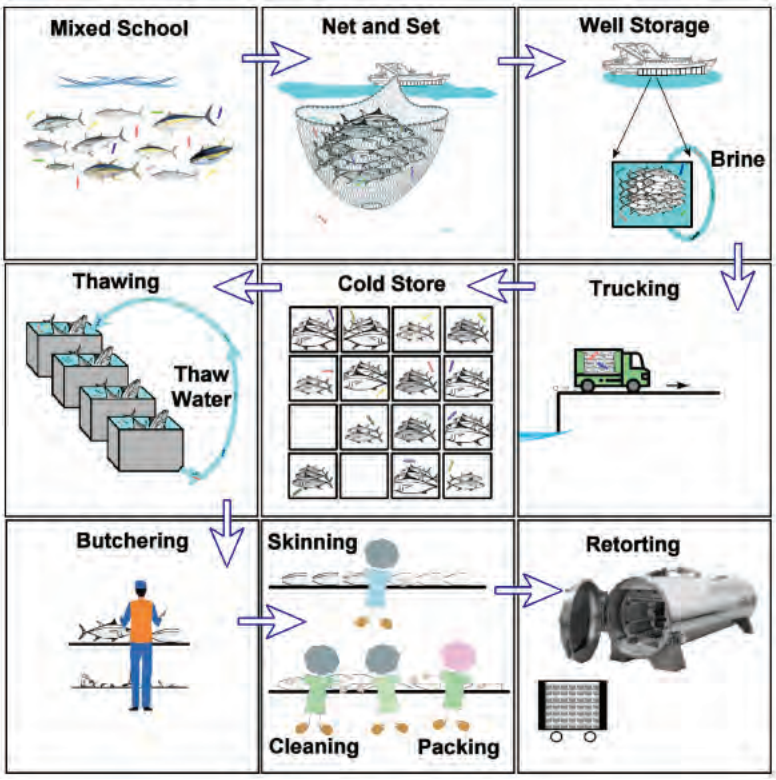

Figure 3. The sources of eDNA from the ocean and through to the retorted can.
Source: Dr. Mona Bumgartel (personal communication)

A typical PCR reaction growth curve is shown in *Fig. 4*. During the PCR reaction there are three growth phases: exponential, log linear, and plateau. SS is typically run using samples obtained from the plateau phase, and because of this, PCR/SS is a qualitative assay, and is not a quantitative test. This procedure works well for a presence/absence responses *(21, 28, 47)*; however, this procedure does not work well to determine trace amounts of DNA in a sample. To determine trace amounts of DNA in a sample, a real time (also referred to as quantitative PCRP) reaction needs to be run, and the samples analyzed in the log linear phase of PCR amplification.

Quantitative PCR (qPCR) is a sensitive and accurate molecular technique that allows for the measurement of the initial amount of DNA present in a sample by monitoring the amplification of the DNA in real-time. This analysis occurs by using fluorescent dyes such as SYBR GREEN or probes that bind to the DNA and emit a signal that is proportional to the amount of DNA being amplified in each PCR cycle *(35, 39, 54)*. Done properly, this technique can identify several species with the proper primers and determine the relative amount of starting DNA from the different species in the sample. Unlike Sanger sequencing reactions, qPCR reactions are optimized and run in the log-linear phase of amplification and can be used in a quantitative manner *(6)*.

Next generation sequencing (NGS) is a method based on developing and using a generic primer for multiple taxa, amplifying the existing DNA, identifying them simultaneously, and then, comparing them to known strands of DNA by using computer databases *(1)*. Dozens of species or genera or families can be identified at the same time. Because of this ability, NGS is very useful in environmental research for identifying whether there are samples of unknown species.

DNA barcoding

DNA analysis for the identification of a single species or genus is usually done using standard DNA barcoding with PCR/SS. DNA barcoding sequencing uses a short section of DNA from a specific gene. For example, the mitochondrial DNA (mtDNA) gene for cytochrome c oxidase I (COI) is over 1300 base pairs (bp) long. Whereas, the standard COI barcode region used for animal DNA is about 658 bp *(3, 26)*.

The standard DNA barcode for most raw seafood is 655 bp of the COI mitochondrial gene *(24)*. In general, a DNA sequence that is longer, i.e., has more bp, is more accurate in identifying the correct species; however, for canned tuna, the retort heat breaks up long DNA strands making them much shorter than 655 bp *(7, 8, 16, 17, 52)*.

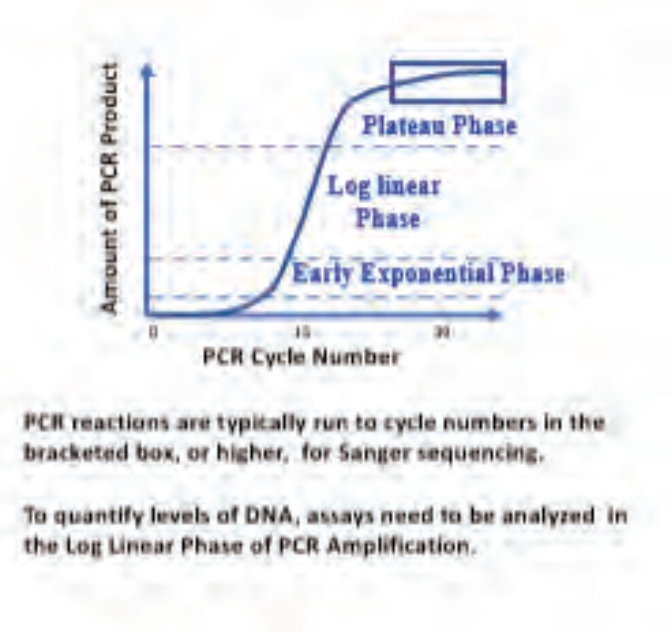

Figure 4. Growth curve during PCR reaction.
Source: Partick Larkin (personal communication)

Use of DNA as a proxy for tuna

According to the EU *(18)*, there cannot be muscle meat from two or more species of tuna in the can when sold. Numerous papers have described DNA analyses from canned tuna, finding DNA from non-labeled tuna in the can, and calling it "mislabeling fraud" because of the single species limitation. In these instances, the researchers are using tuna DNA as a proxy for muscle meat. In many cases, the researchers are finding higher value tuna such as YF in a can labeled SJ, but this approach does not make economic sense *(Fig. 1)*. There are other publications suggesting that shark meat is being packed in petfood in Thailand and marine mammals packed in cans of bluefin tuna (*Thunnus thynnus*) in Mexico. These scenarios also are implausible and will be explained further.

Shark meat in canned pet food. French and Wainwright *(21)* mentioned that 5 out of 45 pet food products that had been packed in Thailand were made up, at least partially, from silky shark (*Carcharhinus falciformis*) meat. Pet food canning in Thailand is based primarily on the availability of tuna red meat from the dozen or more tuna factories in Thailand. The raw tuna for canned tuna comes from the Western Pacific and Indian Oceans where there are numerous Floating Aggregating Devices (FADs) attracting the tuna and concentrating it for a purse seine capture. Silky sharks are part of the multiple species gathering around these FADS *(46)*. There are several reasons it is implausible for silky shark meat to be part of the make-up of the pet food, but it is entirely plausible that eDNA from silky shark could be found in the can of pet food.

The implausible reasons include the following information:

1. Silky sharks are on a non-retention policy in the Pacific Ocean by both the Western & Central Pacific Fisheries Commission (WCPFC) and the InterAmerican Tropical Tuna Commission (IATTC) so silky sharks should not be purposefully loaded on to carriers in the Pacific. In fact, purse seiners can be and are fined if silky sharks are discovered in their holds during unloading *(30, 33, 54, 56)*.
2. There is 100% observer coverage so the capture and release of silky sharks would be recorded in the observer logs.
3. Most all the canneries in Thailand are certified kosher, and sharks are not kosher *(36)*. The red meat destined for pet food is removed from the tuna during skinning and cleaning the tuna *(14)*. No cannery would allow a shark into the cannery for processing because the cannery and the equipment would have to be certified kosher again.
4. Shark muscle meat can have a strong ammonia like odor. Pets would likely not accept it, nor would the owners who are opening the canned shark meat. There is a 1951 patent *(49)* describing the steps that are taken to rid the ammonia odor from shark meat before canning can occur.
5. Sanger sequencing was used in the aforementioned study, providing a presence/absence result and therefore, is not a quantitative tool.

The study demonstrates that silky shark DNA is present, but at what levels? Being present in pet food at high levels would suggest fraud. Being present in pet food at low levels may not necessarily suggest fraud, but perhaps, eDNA. Police and paternity forensic work, and food authenticity determination differ because for the latter; there is often a

TABLE 1. Results of testing for *Thunnini* DNA in different samples from the tuna process supply chain are presented, along with the method of sequencing. The number of tuna or liquid samples sequenced is indicated, along with the number of different DNA samples found and sequenced from those samples. The number of samples that were positive for the *Thunnini* species is recorded. The species of tuna were shown if identified and if the primers for *Thunnus* spp. were positive

Type of Heating	Type of Sample *	Origin of Sample	Lab	Method of Sequencing	Samples analyzed	Unique DNA sequences analyzed	*Thunnini* spp. found	*Katsuwonus pelamis (SJ)*	*Thunnus* spp.	*Thunnus albacares (YF)*	*Thunnus obesus (BE)*	*Thunnus alalunga (Albacore)*	*Euthynnus* spp.	*Auxis* spp.
Brine	MUS	Brine, tuna vessel	U.S. lab	Sanger seq	3	9	2 spp.	1 seq	3 seq					
	MUS	Brine, tuna vessel	N.Z. lab	NGS-SBS	2	2	5 spp.	2 seq	2 seq				2 seq	3 seq
Thaw Water	MUS	Thaw water	U.S. lab	Sangerseq	10	40	3 spp.	13 seq	17 seq	4 seq	4 seq			
	MUS	Thaw water	N.Z. lab	NGS-SBS	6	6	4 spp.	6 seq	6 seq		1 seq		1 seq	
Total				21	57									

SJ = Skipjack, YF = Yellowfin, BE = Bigeye

NGS-SBS = Next Generation Sequencing, Sequencing by Synthesis

requirement for quantitative analysis to authenticate foods. This requirement stems from the need to distinguish between environmental or adventitious contamination and deliberate fraud. Historically, undeclared species at less than 5% (w/w) level was considered adventitious contamination *(43)*.

Given this information, it appears that adventitious silky shark DNA, not the meat, accompanies the tuna shipped to Bangkok and shows up in the can *(43)*. This analysis was accomplished by PCR amplification of silky shark DNA and Sanger sequencing, using a single species primer analysis *(21)*. It is much more plausible that the eDNA from the silky shark traveled with the SJ and YF through the supply chain from the tuna seiner to the factory storage and thawing stations. There, the DNA is mixed with the other DNA in the recirculated thawing water.

Reports of dolphin meat in canned tuna

There are reports of *Delphinus delphis* DNA in canned tuna from Mexico, implying that dolphin meat was packed in the can with tuna *(28, 48)*. The dolphin meat scenario also is implausible for the following reasons:

1. There is 100% observer coverage in the ETP tuna fishery.
2. Every single dolphin death is recorded.
3. No dolphins are retained for selling to the canneries.
4. Dolphin muscle is very different than tuna muscle, so it would be visualized as non-tuna meat in the can.
5. The canned tuna was labeled as *Thunnus thynnus*, (Atlantic bluefin), a tuna species which does not school with dolphins.
6. *Delphinus delphis* (common dolphin) is not a dolphin that purse seiners chase to set on: it typically does not have tuna following it. The common dolphin is very common in the ETP off Mexico (*13*).
7. The DNA testing was done with PCR and Sanger sequencing, so only one species gene was amplified per PCR run; therefore, a tiny amount of DNA can be amplified.

It is much more plausible that dolphin eDNA accompanied the seawater making the RSW and CDSB, and then the dolphin eDNA accompanied the tuna through the process into the can and was identified with PCR/SS.

As a result of the information provided herein, the objective of this case study was to verify that the DNA from multiple marine species was in the brine of tuna boats delivering frozen tuna to Eurofish, as well as in the thawing water used in the cannery in question.

MATERIALS AND METHODS

An industrial case study is presented to better understand how the DNA from more than one species of tuna ends up in cans of tuna and where the muscle meat of only one species was packed intentionally. The hypothesis was tested by reviewing the literature and sampling for eDNA from multiple marine species at critical entry points in the supply chain to answer the following questions:

1. Is the DNA from multiple *Thunnini* species available in the open ocean as eDNA?

TABLE 2. Results of the NGS-SBS analysis of tuna boat brine (the N.Z. lab)

Rank	Group	Scientific Name	Common Name	Count	Percent
Species	Fish	*Katsuwonus pelamis*	Skipjack Tuna	26,998	56.6%
	Fish	*Coryphaena hippurus*	Mahi-Mahi	1,726	3.6%
	Fish	*Vinciguerria lucetia*	Panama Lightfish	339	0.71%
	Fish	*Ophioblennius steindachneri*	Large-Banded Blenny	248	0.52%
	Fish	*Euthynnus lineatus*	Black Skipjack	210	0.44%
	Fish	*Auxis rochei*	BulletTtuna	152	0.32%
	Fish	*Auxis thazard*	Frigate Tuna	131	0.27%
	Fish	*Exocoetus volitans*	Tropical Two-Wing Flying Fish	33	0.07%
	Fish	*Oxyporhamphus micropterus*	Bigwing Halfbeak	24	0.05%
	Fish	*Exocoetus monocirrhus*	Babel Flyingish	22	0.05%
	Fish	*Naucrates ductor*	Pilot Fish	16	0.03%
	Fish	*Canthidermis maculata*	Spotted Oceanic Triggerfish	12	0.03%
	Fish	*Lobotes pacificus*	Pacific Tripletail	10	0.02%
	Fish	*Exocoetus monocirrhus* or *volitans*	Babel or Tropical Two-Winged Flying Fish	8	0.02%
	Fish	*Hemiramphus far*	Blackbarred Halfbeak	7	0.01%
	Fish	*Aluterus monoceros*	Unicorn Leatherjacket	7	0.01%
	Fish	*Remora australis*	Whalesucker	7	0.01%
	Fish	*Lagocephalus lagocephalus*	Oceanic Puffer	7	0.01%
	Fish	*Brama dussumieri*	Lesser Bream	4	0.01%
	Amoebae	*Parvamoeba rugata*	Amoeba	5	0.01%
Genus	Fish	*Thunnus*	Tuna	16,455	34.5%
	Fish	*Vinciguerria*	Bristlemouths	801	1.7%
	Fish	*Euthynnus*	Tuna	84	0.18%
	Fish	*Katsuwonus*	Tuna	62	0.13%
	Fish	*Hirundichthys*	Flying Fish	32	0.07%
	Fish	*Oxyporhamphus*	Halfbeaks	14	0.03%
	Fish	*Cheilopogon*	Flying Fish	8	0.02%
	Fish	*Aluterus*	Filefish	6	0.01%
	Fish	*Naucrates*	Pilotfish	5	0.01%
	Mollusca	*Dosidicus gigas*	Jumbo Flying Squid	106	0.22%
	Mollusca	*Sthenoteuthis oualaniensis*	Purpleback Squid	24	0.05%
	Mollusca	*Tremoctopus violaceus*	Common Blanket Octopus	8	0.02%
	Mollusca	*Argonauta*	Octopus	51	0.11%
	Mollusca	*Tremoctopus*	Octopus	6	0.01%
	Crustaceans	*Artemia*	Brine Shrimp	54	0.11%

TABLE 3. Results of the thaw water analysis by NGS-SBS (the N.Z. lab)

Rank	Group	Scientific Name	Common Name	Count	Percent
Species	Fish	*Katsuwonus pelamis*	Skipjack Tuna	120,625	63.6%
		Thunnus obesus	Bigeye Tuna	62	.003%
		Balistes capricus	Grey Trigger Fish	6	.003%
		Coryphaena hippurus	Mahi-Mahi	4	.002%
		Encrasicholina punctifer	Buccaneer Anchovy	7	.004%
		Euthynnus lineatus	Black Skipjack	7	.004%
		Vinciguerria lucetia	Panama Light Fish	26	.014%
		Dosidicus gigas	Jumbo Flying Squid	5	.003%
		Clogmai albipunctata	Drain Fly	37	.02%
Genus		*Thunnus* spp.	Tuna	6,153	3.3%
		Elagatis spp.	Rainbow Runner	4	.002%
		Euthynnus spp.	Bonito or Black SJ	7	.004%
		Scomber spp.	Mackerels	247	.13%
		Scomberesox spp.	Saury	6	.003%
		Vinciguerria spp.	Bristlemouth	110	.06%
Tribe		*Thunnini*	Tunas	62,045	32.8%
Total				188,991	100%

2. Is that DNA available in the brine of the tuna catcher boats potentially eDNA?
3. Has potential eDNA entered the processing plant?

Sampling eDNA on fishing vessels and in the factory

The salt brine for freezing and possibility storing the frozen fish was sampled from three different purse seine vessels fishing off the coast of Ecuador in the ETP. The brine samples were collected from wells with circulating brine. A clean sampling cup was placed under the discharge pipe and the sample was collected. The sample was transferred to a clean Whirl-Pak sampling bag and transferred on ice to the factory for processing and shipping to the DNA labs.

The thaw water was randomly sampled at the Eurofish factory over a six-day period as the opportunity presented itself, but no attempt was made to include tuna from the same boats from which the brine had been sampled. The thaw water samples were collected from thaw boxes from the overflow. A clean sampling cup was placed under the discharge area and the sample was collected. The sample was transferred to a clean Whirl-Pak sampling bag and transferred on ice to the factory lab for processing and shipping to the DNA labs.

One set of brine and thaw water samples was shipped frozen at -20°C to the U.S. where the samples were analyzed with PCR/SS or PCR/Lateral Flow (Dipstick method). *(Appendix A & B)* Another set of brine and thaw water samples was collected on filters that capture and preserve cellular material in DNA sampling kits sent from the New Zealand (N.Z.) lab, and the filters were sent to that lab for analysis including DNA extraction, PCR, and sequencing. "Wilderlab eDNA kits provide unique syringe-filtration and preservation system that takes the time, effort, cost and contamination out of environmental DNA monitoring. No additional equipment is required, and preserved samples are stable at room temperature for several months while they are transported to the laboratory for analysis" *(57)*.

The DNA from these samples was analyzed by Next Generation Sequencing-Sequencing by Synthesis (NGS-SBS) procedures on an Illumina iSeq 100 platform using the methods outlined in David et al. *(12)*. These analyses give quantitative and semi-quantitative results as well.

RESULTS & DISCUSSION

Starting at the beginning of the raw tuna supply chain and after capture, both SJ and YF DNA were found in the brine from multiple purse seiners using PCR/SS *(Table 1)*. For PCR/SS, one amplicon can be developed per PCR run. So, if one sample of brine or thaw water might contain four species or genera, the four species using separate primers must be run through four comparable PCR procedures.

NGS-SBS technology found DNA from more than 20 other finned fish including SJ, *Euthynnus*, and *Auxis* in the

brine samples *(Table 2)*. These results are similar to the results found by Willette et al. *(59)*.

In the thaw water, PCR/SS found DNA from three species of *Thunnini* including SJ, YF, and BE *(Table 1)* and the NGS-SBS method found DNA from three species of *Thunnini*: SJ, BE, and *Euthynnus (Table 1)*, *Thunnus* spp., and DNA from 11 species of finfish, flying squid, and other marine species *(Table 3)*.

CONCLUSIONS

eDNA in the open ocean and brine

Researchers have shown that eDNA is common in the open ocean *(2, 4, 11)*. Willette et al. *(59)* found the eDNA from multiple species of scombroid fish in the brine and melt water of the tuna boats in Ecuador including genera of *Auxis*, *Katsuwonus*, *Euthynnus*, and *Thunnus*. The current research found two species of *Thunnini* in the brine using PCR/SS and found five species of *Thunnini* in the brine using NGS-SBS. NGS-SBS found multiple species of other non-target species in the brine as well *(Table 2)*.

eDNA in thaw water

The current research also found SJ, YF, and BE eDNA in the thaw water using PCR/SS. SJ, BE, and *Euthynnus* spp. was found in the thaw water using NGS-SBS *(Table 1)* and many other fish species as well *(Table 3)*.

It is apparent that eDNA from multiple species of many different type of fish can and does enter the cannery from the ocean via the commercial tuna supply chain of fishing, refrigeration on the fishing vessels, frozen transportation (transhipping), unloading, and frozen storage. Unless a quantitative genetic analysis test is run that can identify how much DNA of various organisms is present in a sample, DNA should not be used as a proxy for tuna muscle tissue in retorted canned tuna processed in commercial tuna factory, because of the distinct possibility of adventitious eDNA from other species of tuna.

REFERENCES

1. Alekseyev, Y. O., R. Fazeli, S. Yang, R. Basran, T. Maher, N. S. Miller, and D. Remick. 2018. A next-generation sequencing primer—how does it work and what can it do? *Acad. Pathol.* 5:2374289518766521. https://doi.org/10.1177/2374289518766521.
2. Andruszkiewicz, E. A., H. A. Starks, F. P. Chavez, L. M. Sassoubre, B. A. Block, and A. B. Boehm. 2017. Biomonitoring of marine vertebrates in Monterey Bay using eDNA metabarcoding. *PLOS One.* 12(4):e0176343. https://doi.org/10.1371/journal.pone.0176343.
3. Annunziato, A. 2008. DNA packaging: nucleosomes and chromatin. *Nat. Educ.* 1(1):26. Available at: https://www.nature.com/scitable/topicpage/dna-packaging-nucleosomes-and-chromatin-310/ Accessed 5 October, 2023.
4. Bakker, J., O. S. Wangensteen, D. D. Chapman, G. Boussarie, D. Buddo, T. L. Guttridge, H. Hertler, D. Mouillot, L. Vigliola, and S. Mariani. 2017. Environmental DNA reveals tropical shark diversity in contrasting levels of anthropogenic impact. *Scientific Reports* 7, no. 1 2017: 16886. https://doi.org/10.1038/s41598-017-17150-2.
5. Blaha, F. 2025. Personal communication. April 28, 2025. [Email: franciscoblaha@mac.com].
6. Bojolly, D., P. Doyen, B. Le Fur, U. Christaki, V. Verrez-Bagnis, and T. Grard. 2017. Development of a qPCR method for the identification and quantification of two closely related tuna species, bigeye tuna *(Thunnus obesus)* and yellowfin tuna *(Thunnus albacares)*, in canned tuna. *J. Agric. Food Chem.* 65(4):913–920. https://doi.org/10.1021/acs.jafc.6b04713.
7. Bossier, P. 1999. Authentication of seafood products by DNA patterns. *J. Food Sci.* 64(2):189–193. https://doi.org/10.1111/j.1365-2621.1999.tb15862.x.
8. Botti, S., and E. Giuffra. 2010. Oligonucleotide indexing of DNA barcodes: identification of tuna and other scombrid species in food products. *BMC Biotechnology.* 10(1), 1–7. https://doi.org/10.1186/1472-6750-10-60.
9. Burns, F. D. 1985. Tuna Handling and Refrigeration on Purse Seiners. NOAA Technical Memorandum NMFS. Retrieved from: https://repository.library.noaa.gov/view/noaa/5685 Accessed 3 May, 2023.
10. Chumchuen, W., P. Luesrithawornsin, and S. Wongkeaw. 2019. Fish species around fish aggregating devices and other floating objects used for tuna purse seine fishing in the Eastern Indian Ocean. *J. Fish & Environment* 43(1):33–39.
11. Closek, C. J., J. A. Santora, H. A. Starks, I. D. Schroeder, E. A. Andruszkiewicz, K. M. Sakuma, S. J. Bograd, E. L. Hazen, J. C. Field, and A. B. Boehm. 2019. Marine vertebrate biodiversity and distribution within the central California Current using environmental DNA (eDNA) metabarcoding and ecosystem surveys. *Front Mar. Sci.* 6:732. https://doi.org/10.3389/fmars.2019.00732.
12. David, B.O., D. R. Fake, A. S. Hicks, S. P. Wilkinson, M. Bunce, J. S. Smith, D. W. West, K. E. Collins, and D. M. Gleeson. 2021. Sucked in by eDNA – a promising tool for complementing riverine assessment of freshwater fish communities in Aotearoa New Zealand. *N. Z. J. Zoology.* 48(304):217–244. https://doi.org/10.1080/03014223.2021.1905672.
13. DeBeer, J. 2025. pers. comm. [jdebeer2005@gmail.com].
14. DeBeer, J., F. Nolte, C. W. Lord, J. Colley, G. Correa Gonzalez, J. Cox, and J. W. Bell. 2021. Processing tuna, scombridae, for canning: A review. *Mar. Fish Rev.* 83 (3–4):1–44. https://doi.org/10.7755/MFR.83.3-4.1.
15. Di Pinto, A., P. Di Pinto, V. Terio, G. Bozzo, E. Bonerba, E. Ceci, and G. Tantillo. 2013. DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks. *Food Chem.* 141(3):1757–1762. https://doi.org/10.1016/j.foodchem.2013.05.093.
16. Ebbehøj, K. F., and P. D. Thomsen. 1991. Species differentiation of heated meat products by DNA hybridization. *Meat Sci.* 30(3):221–234. https://doi.org/10.1016/0309-1740(91)90068-2.
17. Emmi A. J., B. Fatusin, and R. S. Hellberg. 2023. Comparison of DNA extraction methods for the detection of canned tuna species with DNA mini-barcoding. *J. Food Quality* 2023(1):7121260. https://doi.org/10.1155/2023/7121260.
18. European Economic Community. 1992. Council Regulation (EEC) No 1536/92 (Common marketing standards for preserved tuna and bonito). Available at: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A31992R1536 Accessed 5 October, 2023.
19. Food & Agriculture Organization. 2023. FAO FishFinder Aquatic Species. Available at: https://www.fao.org/fishery/en/aqspecies/search Accessed May 9, 2025.
20. Frank, H. A., M. E. Rosenfeld, D. H. Yoshinaga, and W. K. Nip. 1984. Relationship between honeycombing and collagen breakdown in skipjack tuna, Katsuwonus pelamis. *Mar. Fish. Rev.*, 46(2): 40–42.
21. French, I., and B. J. Wainwright. 2022. DNA barcoding identifies endangered sharks in pet food sold in Singapore. *Frontiers in Marine Science*, 9, 836941. https://doi.org/10.3389/fmars.2022.836941.

22. GenScript. 2021. Sanger Sequencing vs. Next-Generation Sequencing (NGS). Retrieved from: https://www.genscript.com/gene-news/sanger-sequencing-vs-next-generation-sequencing.html Accessed 3 May, 2023.

23. Graham, J. B., and K. A. Dickson. 2004. Tuna comparative physiology. *J. Exp. Bio.* 207(23):4015–4024. https://doi.org/10.1242/jeb.01267.

24. Handy, S. M., J. R. Deeds, N. V. Ivanova, P. D. Hebert, R. H. Hanner, A. Ormos, L. E. Weigt, M. M. Moore, and H. F. Yancy. 2011. A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *J. of AOAC Inter.* 94(1): 201–210. https://doi.org/10.1093/jaoac/94.1.201.

25. He, P. 1993. Swimming speeds of marine fish in relation to fishing gears. *ICES Mar. Sei. Symp.* 196:183–189.

26. Hebert, P. D. N., A. Cywinska, S. L. Ball, and J. R. DeWaard. 2003. Biological identifications through DNA barcodes. *Proc. R. Soc. Lond. B. Biol. Sci.* 270(1512):313-321. https://doi.org/10.1098/rspb.2002.2218.

27. Hellberg, R. S. R., and M. T. Morrissey. 2011. Advances in DNA-based techniques for the detection of seafood species substitution on the commercial market. *JALA: J. Lab. Auto.* 16(4):308–321. https://doi.org/10.1016/j.jala.2010.07.004.

28. Hernandez Herbert, K. V. 2018. Deteccion de carne de Delfin *(Delphius delphis)* en Atun *(Thunnus Thynnus)* Enlatado, por Medio de PCR. Tesis. Universidad Nacional Autonoma de Mexico. Available at: https://hdl.handle.net/20.500.14330/TES01000775270 Accessed May 9, 2025.

29. Hunter, J. R., and C. T. Mitchell. 1967. Association of fishes with flotsam in the offshore waters of Central America. *U.S. Fish Wildl. Serv., Fish. Bull.* 66:13–29.

30. Hutchinson, M. R., D. G. Itano, J. A. Muir, and K. N. Holland. 2015. Post-release survival of juvenile silky sharks captured in a tropical tuna purse seine fishery. *Marine Ecology Progress Series.* 521:143–154. https://doi.org/10.3354/meps11073.

31. Itano, D. 2004a. A handbook for the identification of yellowfin and bigeye tunas in brine frozen condition. Available at: https://static1.squarespace.com/static/52c1c633e4b035d7c738b56a/t/57ae1b94197aeaa4592427f6/1471028122626/2_BE-YF+ID+Frozen_ENGLISH_v5_logo.pdf Accessed May 5, 2025.

32. Itano, D. 2004b. A handbook for the identification of yellowfin and bigeye tunas in fresh condition. Available at: https://www.spc.int/DigitalLibrary/Doc/FAME/Meetings/SCTB/17/INF_FTWG_5.pdf Accessed May 5, 2025.

33. Itano, David – email dated 4/28/2025.

34. Jacquet, J. L., and D. Pauly. 2008. Trade secrets: renaming and mislabeling of seafood. *Mar. Policy.* 32(3):309–318. https://doi.org/10.1016/j.marpol.2007.06.007.

35. Karapareddy, S. and M. Sreedhar. 2020. Basic guide to real time PCR in the current scenario. Advances in Biotechnology and Bioscience (Vol. 6) chap 6: 99–121. Akinik Publications.

36. Kashrut.com. 2025. Kosher and Non-Kosher Fish. Retrieved from https://www.kashrut.com/articles/fish/ Accessed May 1, 2025.

37. Kasumyan, A. O. and D. S. Pavlov. 2023. Development of schooling behavior in fish ontogeny. *J. Ichthyology*, 63(7), 1348–1361. https://doi.org/10.1134/S0032945223070111.

38. Krčmář, P., Z. Piskata, and E. Servusová. 2019. Identification of tuna species *Thunnus albacares* and *Katsuwonus pelamis* in canned products by real-time PCR method. *Acta Veterinaria Brno.* 88(3):323–328. https://doi.org/10.2754/avb201988030323.

39. Kubista, M., J. M. Andrade, M. Bengtsson, A. Forootan, J. Jonák, K. Lind, R. Sindelka, R. Sjoback, B. Sjogreen, L. Strombom, A. Stahlberg, and N. Zoric. (2006). The real-time polymerase chain reaction. *Mol. Aspects Med*, 27(2–3), 95–125. https://doi.org/10.1016/j.mam.2005.12.007.

40. Lowenstein, J.H., G. Amato, and S-O Kolokotronis. 2009. The real maccoyii: Identifying tuna sushi with DNA barcodes – contrasting characteristic attributes and genetic distances. *PLOS One.* 4(11): e7866. https://doi.org/10.1371/journal.pone.0007866.

41. Mullis, K. B. and F. A. Faloona. 1987. [Chap 21] Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. In *Methods in Enzymology* (Vol. 155, pp. 335–350). Academic Press. https://doi.org/10.1016/0076-6879(87)55023-6.

42. Perrin, W. F. 2009. Historical perspectives. *Aquat Mamm.* 35(2), 292–305. https://doi.org/10.1578/AM.35.2.2009.292.

43. Primrose, S.B. 2019. Chapter 1: The role of DNA analysis in the determination of food authenticity, in DNA techniques to verify food authenticity: Applications in food fraud, pp. 1–11. 2019 e https://doi.org/10.1039/9781788016025.

44. Roungchun, J.B., A. M. Tabb, and R. S. Hellberg. 2022. Identification of tuna species in raw and processed products using DNA mini-barcoding of the mitochondrial control region. *Food Control.* 134, 108752. https://doi.org/10.1016/j.foodcont.2021.108752.

45. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America (PNAS)*, 74(12), 5463–5467. https://doi.org/10.1073/pnas.74.12.5463.

46. Scott, J. M., and G. A. Flittner. 1972. Behavior of bluefin tuna schools in the eastern north Pacific Ocean as inferred from fishermen's logbooks, 1960–67. *Fish. Bull.* 70(3):915–927.

47. Scott, M. D., S. J., Chivers, R. J Olson, P. C Fiedler, and K. Holland. 2012. Pelagic predator associations: tuna and dolphins in the eastern tropical Pacific Ocean. *Marine Ecology Progress Series*, 458, 283–302. https://doi.org/10.3354/meps09740.

48. Seafoodsource. 2020. Study finds evidence of dolphin meat in Mexican canned tuna. Retrieved from https://www.seafoodsource.com/news/environment-sustainability/study-finds-evidence-of-dolphin-meat-in-mexican-canned-tuna Accessed May 1 2025.

49. U.S. Patent Office. 1951. U.S. Patent 2,564,487A. Method of preparing shark meat for canning. Available at: https://patentimages.storage.googleapis.com/fb/86/a1/0bed8d2294fb9a/US2564487.pdf Accessed June 2, 2025.

50. U.S. Food and Drug Administration. 2001. 21CFR§161.190. Canned tuna standard of identity. Available at: https://www.govinfo.gov/content/pkg/CFR-2012-title21-vol2/pdf/CFR-2012-title21-vol2-sec161-190.pdf Accessed 5 October, 2023.

51. U.S. Food and Drug Administration. 2012. Guidance for industry: The seafood list. Available at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-seafood-list Accessed 5 October, 2023.

52. Unseld, M., B. Beyermann, P. Brandt, and R. Hiesel. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *Genome Res.* 4(4):241–243. https://doi.org/10.1101/gr.4.4.241.

53. Valsecchi, E., A. Arcangeli, R. Lombardi, E. Boyse, I. M. Carr, P. Galli, and S. J. Goodman. 2021. Ferries and environmental DNA: underway sampling from commercial vessels provides new opportunities for systematic genetic surveys of marine biodiversity. *Front. Mar. Sci.* 1136. https://doi.org/10.3389/fmars.2021.704786.

54. VanGuilder, H. D., E. Vrana, and W. M. Freeman. 2008. Twenty-five years of quantitative PCR for gene expression analysis, *BioTechniques*, 44:5, 619-626, https://doi.org/10.2144/000112776.

55. Wang, X., L. Xu, Y, Chen, G. Zhu, S. Tian and J. Zhu. 2012. Impacts of fish aggregation devices on size structures of skipjack tuna *(Katsuwonus pelamis)*. *Aquat Ecol* 46, 343–352. https://doi.org/10.1007/s10452-012-9405-0.

56. Western and Central Pacific Fisheries Commission. 2025. Conservation and management measure for sharks. Retrieved from https://cmm.wcpfc.int/sites/default/files/cmm_attachments/CMM%202024-05%20CMM%20on%20Sharks.pdf?_dl=1 Accessed May 2, 2025.

57. Wilderlab. (2025). eDNA kits, tests and accessories. Available at https://wilderlab.co/consumables Accessed June 4, 2025.

.

58. Willette, D. A., S. R. Simmond, S. H. Cheng, S. Esteves, T. L. Kane, H. Nuetzel, N. Pilaud, R. Rachmawati, and P. H. Barber. 2017. Using DNA barcoding to track seafood mislabeling in Los Angeles restaurants. *Conserv Biol.* 31(5):1076–1085. https://doi.org/10.1111/cobi.12888.

59. Willette, D.A., G. Navarrete-Forero, Z. Gold, A. M. D. Lizano, L. Gonzalez-Smith, and G. Sotil. 2021. Characterizing industrial and artisanal fishing vessel catch composition using environmental DNA and satellite-based tracking data. *Foods* 10(6):1425. https://doi.org/10.3390/foods10061425.

60. Yancy, H. F., T. S. Zemlak, J. A. Mason, J. D. Washington, B. J. Tenge, N. L. T Nguyen, J. D. Barnett, W. E. Savary, W.E. Hill, M. M. Moore, F. S. Fry, S. C. Randolph, P. L. Rogers, and P. D. Hebert. 2008. Potential use of DNA barcodes in regulatory science: applications of the Regulatory Fish Encyclopedia. *J. Food Protect.* 71(1):210–217. https://doi.org/10.4315/0362-028X-71.1.210.

APPENDIX A. POLYMERASE CHAIN REACTIONS/ AGAROSE GELS / SANGER SEQUENCING

1. The American lab has a suite of primers that are used to identify and distinguish various species.
2. Genomic DNA (gDNA) is extracted from tissue samples using a phenol-chloroform method of extraction; gDNA is quantitated with a Nanodrop Spectrophotometer; and PCR reactions are set up and run under sterile conditions. Aliquots of all PCR reactions are visualized using agarose gels and/or lateral flow dipsticks. Another aliquot from positive PCR reactions are purified using ExoSap.
3. PCR purified reactions are sent to the University of Chicago, Cancer Research Center, DNA sequencing core for Sanger Sequencing.
4. The obtained sequences results are then compared to vouchered sequences for species identification.

APPENDIX B. LATERAL FLOW PROCEDURE AT THE AMERICAN LAB

Adapted from Partick Larkin, Ph.D. (personal communication)

Lateral flow dipsticks utilized in this study were developed based on the following platform (Milenia Biotec, https://www.milenia-biotec.com/en/product/hybridetect/). A suite of primers specific to various tuna species were used in these diagnostics. Briefly, 1 µL each of forward primers labelled with biotin at the 5' end (10µM) and reverse primers labelled with fluorescein at the 5' end were added to a 25 µL PCR reaction that also contained 2 µLs of gDNA, 21 µLs deionized H_2O, and ready to go beads (Fisher Scientific). PCR reactions were run for 30 cycles. Following a PCR reaction, 100 µLs of dipstick buffer was placed in a well in a 96 well plate, 10 µLs of the PCR reaction was then add to this well, and briefly mixed. A lateral flow strip was then added upright to the well, and the results were observed and recorded via a digital image after 2–3 minutes.