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Lipid-extracted muscle and liver tissues: Can they reveal mercury exposure of pelagic sharks?

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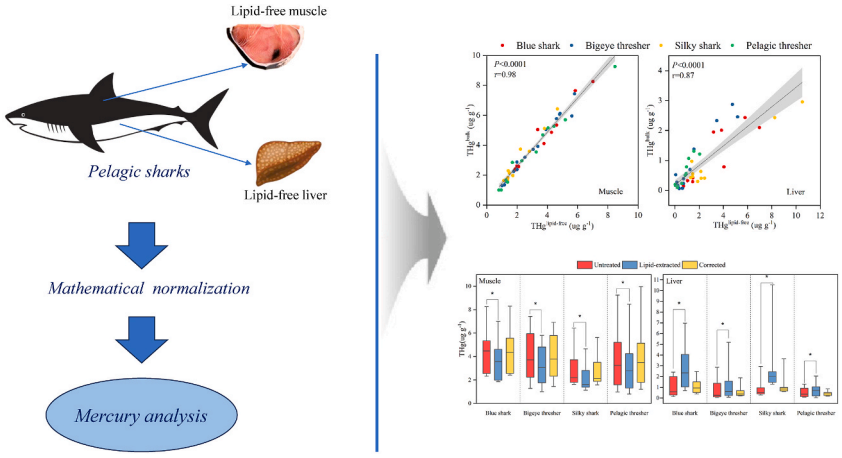
HIGHLIGHTS

- Lipid extraction with dichloromethane-methanol significantly altered the total mercury concentrations in sharks' muscle and liver.
- The impact of lipid extraction on THg concentrations is not related to distinct TLC among species and tissues.
- Lipid-extracted tissues are suitable for mercury analysis of pelagic sharks by mathematical normalization simplifies.

GRAPHICAL ABSTRACT



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ABSTRACT

Pelagic sharks are apex predators in oceanic ecosystems and tend to accumulate high amounts of mercury (Hg). The conventional method for assessing Hg exposure in sharks involves analyzing tissue samples without any chemical treatment. However, a substantial number of chemically treated tissue samples are still being preserved in laboratories or museums. It is critical to maximize the utilization of existing samples to reduce the need for additional sampling of pelagic sharks, especially endangered species. Lipid extraction is a widely employed pretreatment process for carbon isotope analysis in shark trophic ecology, while its impact on Hg quantification remains uncertain. Here, we evaluated the feasibility of using lipid-free muscle and liver tissues for investigation of Hg exposure in four endangered pelagic sharks inhabiting the eastern Pacific, including bigeye thresher (*Alopias superciliosus*), pelagic thresher (*A. pelagicus*), blue shark (*Prionace glauca*) and silky shark (*Carcharhinus falciformis*). Results showed that total Hg concentrations (THg) differed between untreated (THg^{bulk}) and lipid-

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free ($THg^{lipid\text{-}free}$) samples for each tissue type of each species. In addition, dichloromethane-methanol extractions significantly altered the amount of Hg. This may result from the removal of lipoprotein compounds that vary between tissues and species. The THg^{bulk} can be calculated by $THg^{lipid\text{-}free}$ using the following formulas, $THg^{bulk} = 1.14 \times THg^{lipid\text{-}free} + 0.30$ and $THg^{bulk} = 0.33 \times THg^{lipid\text{-}free} + 0.18$, for muscle and liver tissues, respectively. These findings emphasize the applications of lipid-free tissues in THg analysis. This study may have important implications for improving evaluation of Hg exposure in endangered pelagic sharks.

1. Introduction

According to the 2018 report of the United Nations Environment Programme (UNEP) (UNEP, n.d.), approximately 3700 tons of mercury (Hg) are released into the atmosphere each year, subsequently settling into the ocean. Hg is a highly toxic environmental contaminant of global concern and poses a significant threat to marine ecosystems. Since the industrial revolution, Hg emissions are estimated to have tripled total Hg concentrations in the ocean (Lamborg et al., 2014). Methylmercury (MeHg) is the most prevalent form of Hg and highly bioavailable by marine organisms (Hong et al., 2012; Hilgendag et al., 2022). Sharks are accumulating large amounts of Hg, of which more than 90% of total mercury (THg) is composed of highly toxic MeHg (Pethybridge et al., 2010; Tiktak et al., 2020; Li et al., 2022). The metabolism and degradation of MeHg in organisms are very slow, making it highly susceptible to accumulate within organisms. Several studies have confirmed that MeHg can produce a series of toxicological effects such as oxidative stress, excitotoxicity, and developmental disorders in a wide range of marine taxa (Branco et al., 2012; Wu and Wang, 2014; Furness, 2018).

Pelagic sharks are often the apex predators in the oceanic ecosystem and tend to be exposed accumulating high levels of Hg throughout their lifetime, owing to their longevity, low metabolic rate, and high trophic position (Gelsleichter and Walker, 2010; Mull et al., 2012). Unfortunately, shark tissues are consumed by humans, such as fin, muscle and liver oil, the last has been frequently utilized as a nutritional supplement for children, pregnant women and elders (Palmieri et al., 2014; EPA, 2022). The present pattern of consumption serves to aggravate the risk of MeHg exposure in humans, potentially leading to serious health consequences (Maurice et al., 2021). In addition, muscle and liver are important tissue matrix in feeding ecology studies of pelagic sharks which commonly used in stable isotope analysis (Shipley et al., 2022; Riveron et al., 2022; Alves et al., 2023; Li et al., 2016a).

There is a burgeoning literature documenting the foraging ecology of pelagic sharks using stable isotope analysis to better understand their ecological role (Li et al., 2016b; Velez N et al., 2021; Besnard et al., 2021). Lipid extraction is a widely employed pretreatment approach for carbon isotope analysis to remove the potential impacts of ^{13}C-depleted lipid (Sheng et al., 2023; Li et al., 2022). Thereafter, a large number of lipid-free tissue samples are still preserved. The majority of these pelagic sharks are categorized as endangered (Gong et al., 2023; Pacoureau et al., 2021). Exploring whether existing samples can be used for Hg analysis can thus maximize utilization and reduce the need for additional pelagic shark sampling.

In this study, we investigated total Hg concentrations (THg) of untreated (THg^{bulk}) and lipid-free ($THg^{lipid\text{-}free}$) muscle and liver tissue samples of four endangered pelagic sharks, i.e., the bigeye thresher (*Alopias superciliosus*), pelagic thresher (*A. pelagicus*), blue shark (*Prionace glauca*), and silky shark (*Carcharhinus falciformis*) from the eastern Pacific. As top oceanic predators, these shark species play important roles in the pelagic ecosystem (Clarke et al., 2006; Joung et al., 2005). However, blue shark has been listed as Vulnerable Near-threatened (NT) species on the international Union for Conservation of Nature (IUCN) Red List (Stevens et al., 2000), the other three sharks have been listed as vulnerable (VU) (Dulvy et al., 2008; Amorim et al., 2009; Li et al., 2022). It is critical to maximize the utilization of existing samples preserved in laboratories or museums to reduce the need for additional sampling of endangered pelagic sharks.

The aims of this study were to (i) assess the effect of the lipid extraction on THg quantification and (ii) test a Hg normalizing approach to be able to estimate THg^{bulk} from $THg^{lipid\text{-}free}$ concentrations.

2. Materials & methods

2.1. Sampling and sample preparation

A total of 47 specimens of four pelagic sharks were found in the eastern Pacific region (Fig. 1). They were obtained as bycatch from tuna longline fishing operations between September 2019 to January 2020. Specimens were frozen on board at −20°Cand transported to the laboratory. Muscle and liver tissues were collected in the front dorsal

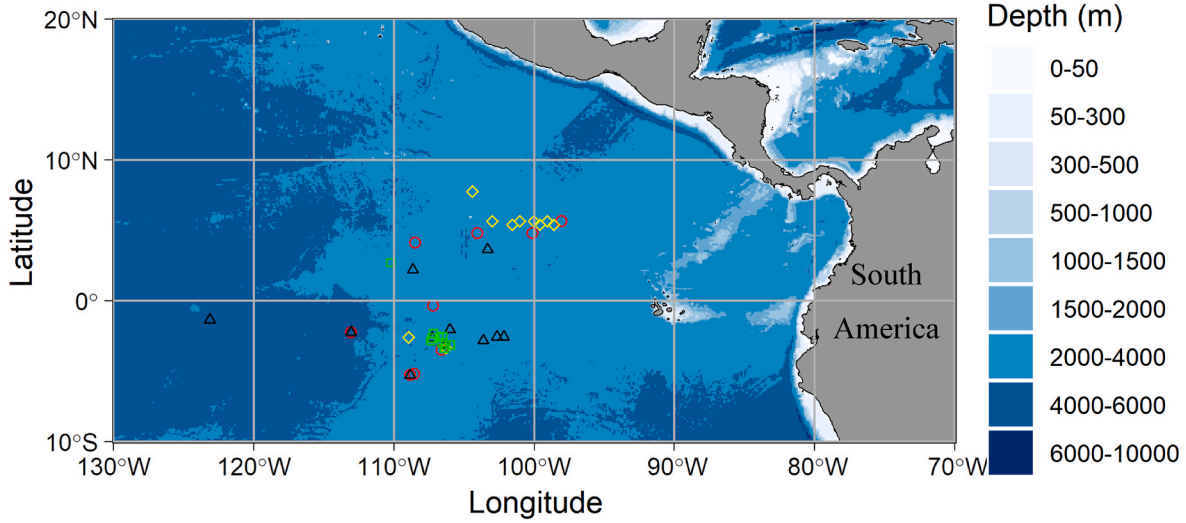

Fig. 1. Sampling locations of four shark species, (○): blue shark *Prionace glauca*; (Δ): bigeye thresher *Alopias superciliosus*; (◇): silky shark *Carcharhinus falciformis*; (□): pelagic thresher *Alopias pelagicus*.

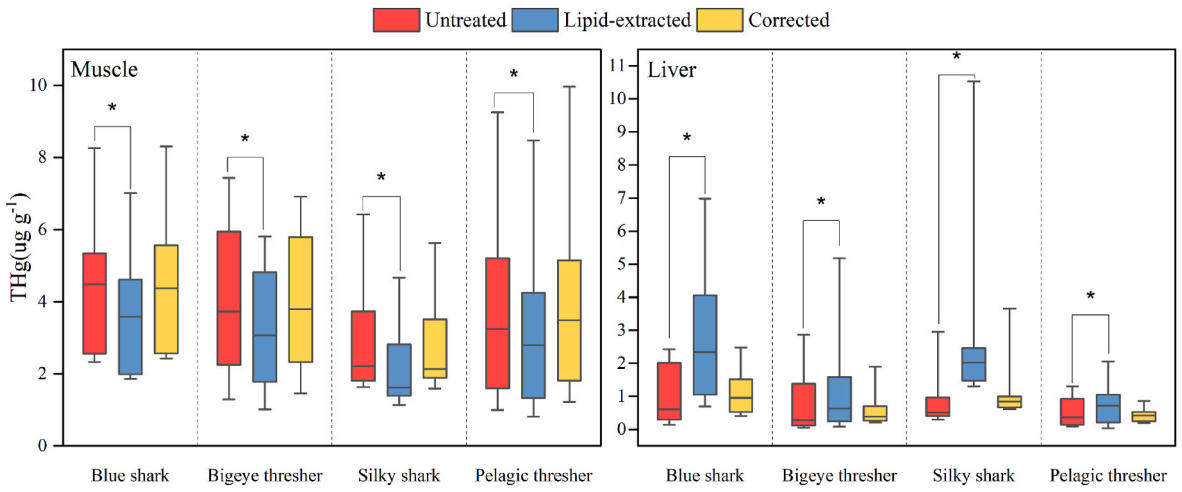

Fig. 2. Total mercury (THg) concentrations of untreated, lipid-extracted and corrected values of four shark species. The boxes represent the interquartile ranges and the whiskers indicate minimum and maximum values and the asterisk indicates the significant differences ($P < 0.05$, paired *t*-test).

position and the front of any lobe of the liver tissue, respectively. In order to minimize the risk of contamination, tissue samples were placed onto a polyethylene plate, and plastic utensils were used and rinsed with ultrapure water (Milli-Q water). Muscle and liver tissue samples were freeze-dried for 48 h, and finally ground to a fine homogeneous powder (Mixer Mill MM 400, Retsch, Haan, Germany) prior to lipid extraction and THg analysis.

2.2. Lipid extraction and quantification

Lipid extraction was performed according to the modified procedure outlined following the methods of Xu et al. (2022) Specifically, samples of each tissue type were placed into stoppered centrifuge tubes and homogenized in 12 mL of 2:1 (v/v) dichloromethane–methanol solvent for approximately 20 h at near-room temperature. After centrifuging 6000 r·min^{-1} for 10 min, the liquid supernatant was drained into a pre-weighed aluminum dish. The process was repeated 3 times. The contents were evaporated at 70 °C using a vacuum drying oven, then cooled to room temperature.

Untreated (W_1) and lipid extracted (W_2) samples were weighted on an analytical micro-balance to the nearest 0.0001 g. The total lipid content (TLC) was calculated based on the following equation:

$$TLC\ (\%) = (W_2 - W_1)\ /\ W_1 \times 100\%$$

2.3. Total mercury analysis

THg concentrations, including THg^{bulk} and $THg^{lipid\text{-}free}$, were measured on powdered, dried, and homogenized tissue by thermal decomposition (combustion), amalgamation, and atomic absorption spectrometry using a calibrated DMA-80 Direct Mercury Analyzer (Milestone, Italy). Approximately 0.02 g of crushed sample was loaded into the DMA-80, dried and burned at a temperature of 650 °C in an oxygen atmosphere (Li et al., 2022). Quality control procedures included analysis of laboratory method blanks, duplicate for all tissue samples, and certified reference materials (DORM-4) (O'Bryhim et al., 2017). The precision of duplicate samples averaged 16.56%, and percentage recovery for the certified reference materials ranged from 95% to 108%. THg concentrations are expressed in μg·g^{-1}.

2.4. Mercury normalizing approach

In addition, we used $THg^{lipid\text{-}free}$ and the TLC to calculate an estimated THg ($THg^{estimated}$) of untreated tissues using the following formula, which hypothesized that the lipid extractions had no effect on the THg analysis.

$$THg^{estimated} = THg^{lipid-free} \times (1 - TLC)$$

2.5. Statistical analysis

Comparisons of TLC values among species were performed using the Kruskal-Wallis test since the assumptions of normality and homogeneity of the variances were not met. The paired *t*-test was used to detect the differences between values of THg^{bulk} and $THg^{lipid\text{-}free}$, and THg^{bulk} and $THg^{estimated}$ in the two tissue types for each species. To evaluate the degree of associations between inter-specific THg^{bulk} and $THg^{lipid\text{-}free}$, and $\Delta THg = (THg^{bulk} - THg^{estimated})$ and THg^{bulk}, Spearman's rank correlation was applied to test for the presence of any relationships between these values (where r_s is Spearman's rank correlation coefficient) for each species. To evaluate the impacts of shark species on THg more objectively, analysis of covariance (ANCOVA) was used to remove the inter-specific effects, $THg^{lipid\text{-}free}$ and $THg^{estimated}$ as variables, THg^{bulk} as the covariate, and shark species as the categorical variable. All statistical analyses and graphics were carried out in software OriginPro Version 2022 (OriginPro, n.d.).

3. Results & discussion

3.1. Total mercury concentrations of untreated and lipid-free tissues

The THg^{bulk} of muscle for all sharks ranged from 1.00 to 9.25 and ranged from 0.06 to 2.96 μg·g^{-1} for liver. Interspecific variations were found in THg^{bulk} values among four species in either tissue type (ANOVA, $P < 0.01$). For muscle, the blue shark (4.51 ± 2.15 μg·g^{-1}) had higher amounts of THg, followed by bigeye thresher (3.93 ± 2.01 μg·g^{-1}), pelagic thresher (3.72 ± 2.43 μg·g^{-1}), and silky shark (3.04 ± 1.65 μg·g^{-1}). For liver, the highest THg^{bulk} was also found in the blue shark (1.06 ± 0.94 μg·g^{-1}), followed by silky shark (0.95 ± 0.94 μg·g^{-1}), bigeye thresher (0.79 ± 0.98 μg·g^{-1}) and pelagic thresher (0.53 ± 0.46 μg·g^{-1}) (Fig. 2). This was consistent with the results of Maurice et al. (2021) which indicated that blue shark contained one of highest THg concentrations among six pelagic shark species. The highest THg^{bulk} values of blue shark probably due to their highest trophic position. Compared to other three species, previous studies of the stomach contents of blue sharks showed that they occasionally feed on prey with high-trophic-levels such as whale carrion, seabirds, and shark species (Markaida and Sosa-Nishizaki et al., 2010; Kitchell et al., 2002), which can lead to a higher trophic position. The interspecific Hg exposure of sympatric shark species has also been reported in several other studies of

Table 1
Sampling information.

Common name	Taxonomic name	N	Fork length range (cm)	Tissue	Untreated (THg^{bulk})	Lipid-extracted ($THg^{lipid\text{-}free}$)	TLC
Blue shark	*Prionace glauca*	10	119.3–194.5	Muscle	4.51 ± 2.15	3.68 ± 1.80	4.72 ± 2.84
				Liver	1.06 ± 0.94	2.94 ± 2.22	76.52 ± 8.14
Bigeye thresher	*Alopias superciliosus*	15	81.3–189.8	Muscle	3.93 ± 2.01	2.95 ± 1.66	4.83 ± 1.65
				Liver	0.79 ± 0.98	1.33 ± 1.71	56.39 ± 13.93
Silky shark	*Carcharhinus falciformis*	10	54.0–153.6	Muscle	3.04 ± 1.65	2.21 ± 1.20	3.86 ± 2.25
				Liver	0.95 ± 0.94	3.31 ± 3.27	78.28 ± 12.65
Pelagic thresher	*Alopias pelagicus*	12	95.4–170.1	Muscle	3.72 ± 2.43	3.13 ± 2.24	5.79 ± 2.19
				Liver	0.53 ± 0.46	0.74 ± 0.64	45.01 ± 12.80

pelagic and demersal sharks. (Pethybridge et al., 2010; Kiszka et al., 2015; Le Croizier et al., 2020).

The TLC values of all muscle samples ranged from 1.0% to 10.0% (dry weight), while in liver tissue, varied from 10.0% to 90.0%. Significant interspecific variations were found in TLC values of each tissue (Kruskal-Wallis test, $P < 0.0001$). For muscle, the TLC values were significantly higher in pelagic thresher (5.79 ± 2.19), followed by the bigeye thresher (4.83 ± 1.65), blue shark (4.72 ± 2.84), and silky shark (3.86 ± 2.25) (Table 1). For liver, silky shark (78.28 ± 12.65) and blue shark (76.52 ± 8.14) showed the higher TLC values than the bigeye thresher (56.39 ± 13.93) and pelagic thresher (45.01 ± 12.80). The results possibly driven by interspecific dietary sources and high energy allocation strategies, which would be consistent with previous results reported for the pelagic sharks (Xu et al., 2022).

The results of paired *t*-test showed that the $THg^{lipid\text{-}free}$ values were significantly lower than those of THg^{bulk} in muscle of each species ($P < 0.0001$), while the $THg^{lipid\text{-}free}$ values of the liver were significantly higher than those of THg^{bulk} ($P < 0.0001$). These findings somewhat contradict the results of studies of Weddell seal *Leptonychotes weddellii* (Cipro et al., 2017) and tropical tuna species (Medieu et al., 2021). These authors reported that there is no effect of lipid extraction on THg analysis. This inconsistency among studies may be relates to differences in the methodology, including the different solvents used for lipid extraction. For example, the lipid extraction of tuna muscle tissues (Medieu et al., 2021) and shark tissues in this study were performed using non-polar (dichloromethane and cyclohexane) and polar (dichloromethane-methanol) solvents, respectively. The effect of lipid extraction with different solvents remains to be explored. Moreover, the impact of lipid extraction on THg concentrations is not related to distinct TLC values among species and tissues. As we mentioned above, despite distinct TLC values among species and tissues were found, $THg^{lipid\text{-}free}$ is significantly different from THg^{bulk} in each tissue in each shark species.

3.2. Determined and estimated total mercury concentrations of untreated tissues

The $THg^{estimated}$, estimated by the $THg^{lipid\text{-}free}$ and TLC values, of all muscle tissue samples ranged from 0.80 to 7.81 $\mu g \cdot g^{-1}$, while in liver tissues, the $THg^{estimated}$ varied from 2.21 to 27.11 $\mu g \cdot g^{-1}$. Differences between THg^{bulk} and $THg^{estimated}$ values were apparent in both tissues of four shark species (Paired *t*-test, $P < 0.0001$). All samples exhibited positive ΔTHg values (muscle: 0.08 to 2.21 $\mu g \cdot g^{-1}$; liver: 0.01 to 0.99 $\mu g \cdot g^{-1}$), and they were positively correlated with THg^{bulk} values (muscle: $r_s > 0.97$, $P < 0.01$; liver: $r_s > 0.92$, $P < 0.01$), indicating a loss of Hg during lipid extraction. As an alcohol, methanol competes with methylmercury for binding sites, resulting in alterations to its behavior. As a standard practice, methanol is employed for biological lipid extraction, which is the removal of fatty substances from biological samples before analysis. This process may affect THg by dissolving certain types of Hg. Specifically, the majority of Hg (>90%) in shark tissues is present in methylmercury (MeHg) (Pethybridge et al., 2010), which primarily by forming complexes with the amino acid cysteine (Leaner and Mason, 2004; Tiktak et al., 2020). Another possible explanation for the variability between THg^{bulk} and $THg^{estimated}$ values could be attributed to the removal of lipoprotein compounds during lipid extraction, since MeHg is known to bioaccumulate in the protein fraction (Perkins et al., 2017). However, further investigation is required to fully elucidate the mechanism by which dichloromethane-methanol solvents facilitate Hg removal.

3.3. Mathematical normalization

Mathematical normalization does not necessitate an additional analysis step or the sampling of tissue samples from pelagic sharks, as the requisite information for mathematical normalization—$THg^{lipid\text{-}free}$ or TLC in the sample—are typically estimated. Considering the significant correlations between THg^{bulk} and $THg^{lipid\text{-}free}$ (as mentioned

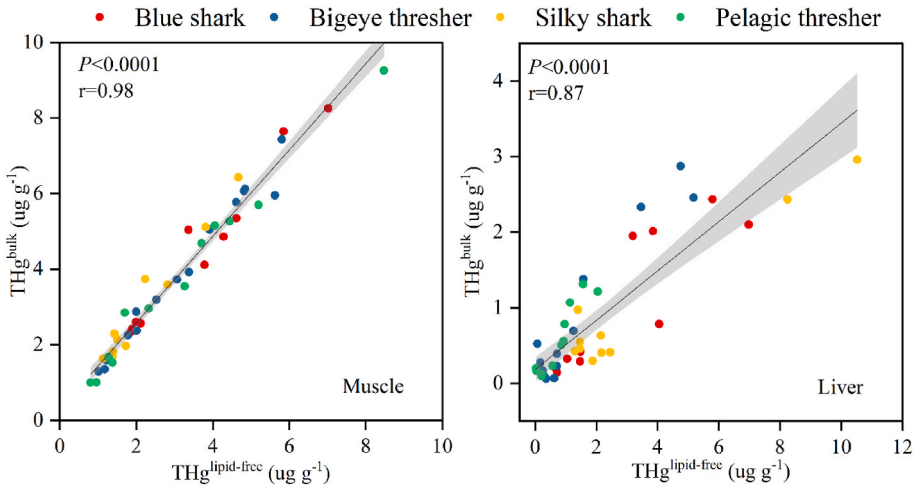

Fig. 3. Linear relationships between the total mercury concentrations of untreated (THg^{bulk}) and lipid-free ($THg^{lipid\text{-}free}$) tissues of four shark species.

Table 2
Linear regression equations and diagnostic statistics for THg^{bulk} and $THg^{lipid\text{-}free}$, THg^{bulk} and THg^{corr} in two tissues among four sharks.

Tissues	Species		*P* values	Pearson correlation coefficient (r)
Muscle	Blue shark	$THg^{bulk} = 1.17\ THg^{lipid\text{-}free} + 0.19$	$P < 0.01$	0.98
		$THg^{bulk} = 1.26\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.24$	$P < 0.01$	0.97
	Bigeye thresher	$THg^{bulk} = 1.19\ THg^{lipid\text{-}free} + 0.12$	$P < 0.01$	0.99
		$THg^{bulk} = 1.26\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.11$	$P < 0.01$	0.99
	Silky shark	$THg^{bulk} = 1.35\ THg^{lipid\text{-}free} + 0.05$	$P < 0.01$	0.99
		$THg^{bulk} = 1.45\ THg^{lipid\text{-}free} \times (1 - TLC) - 0.03$	$P < 0.01$	0.98
	Pelagic thresher	$THg^{bulk} = 1.08\ THg^{lipid\text{-}free} + 0.34$	$P < 0.01$	0.99
		$THg^{bulk} = 1.18\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.28$	$P < 0.01$	0.99
Liver	Blue shark	$THg^{bulk} = 0.37\ THg^{lipid\text{-}free} - 0.02$	$P < 0.01$	0.87
		$THg^{bulk} = 1.49\ THg^{lipid\text{-}free} \times (1 - TLC) - 0.06$	$P < 0.01$	0.98
	Bigeye thresher	$THg^{bulk} = 0.55\ THg^{lipid\text{-}free} + 0.05$	$P < 0.01$	0.96
		$THg^{bulk} = 1.34\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.05$	$P < 0.01$	0.97
	Silky shark	$THg^{bulk} = 0.28\ THg^{lipid\text{-}free} + 0.03$	$P < 0.01$	0.97
		$THg^{bulk} = 1.48\ THg^{lipid\text{-}free} \times (1 - TLC) - 0.02$	$P < 0.01$	0.99
	Pelagic thresher	$THg^{bulk} = 0.67\ THg^{lipid\text{-}free} + 0.04$	$P < 0.01$	0.94
		$THg^{bulk} = 1.23\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.06$	$P < 0.01$	0.92

above), and THg^{bulk} and $THg^{estimated}$ (muscle: $r_s > 0.98$, $P < 0.01$; liver: $r_s > 0.97$, $P < 0.01$) and no difference was found in the slope of the Pearson fitting lines of the four shark species (ANCOVA, $P > 0.05$) (Fig. 3), the equations (Table 2) provide a reliable method for normalizing estimates of THg^{bulk}. When using a multispecies data set, the equations for shark muscle tissues were:

$$THg^{corrected,\ bulk} = 1.14\ THg^{lipid\text{-}free} + 0.30,$$

$$THg^{corrected,\ bulk} = 1.23\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.24,$$

For shark liver tissues were:

$$THg^{corrected,\ bulk} = 0.33\ THg^{lipid\text{-}free} + 0.18,$$

$$THg^{corrected,\ bulk} = 1.40\ THg^{lipid\text{-}free} \times (1 - TLC) + 0.01,$$

We concluded that mathematical normalization simplifies sample preparation and can make better use of existing samples to reduce the need for additional samples from pelagic sharks, particularly endangered species. The strong relationship between THg^{bulk} and $THg^{lipid\text{-}free}$ and lipid content at the center of the mathematical normalization technique could also provide a useful method for estimating Hg exposure in pelagic sharks.

CRediT authorship contribution statement

Zehao Guo: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft. **Yi Gong:** Resources, Validation, Writing – review & editing, Methodology, Software. **Zezheng Li:** Writing – review & editing, Supervision. **Yongfu Shen:** Writing – review & editing, Supervision. **Yunkai Li:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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