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Lipid-free tuna muscle samples are suitable for total mercury analysis

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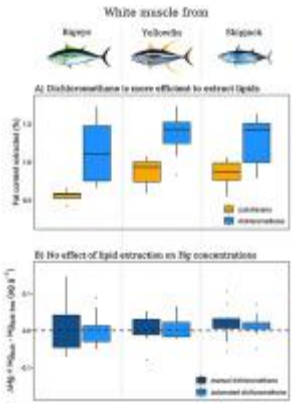
Abstract :

Tropical tunas are largely consumed worldwide, providing major nutritional benefits to humans, but also representing the main exposure to methylmercury, a potent neurotoxin that biomagnifies along food webs. The combination of ecological tracers (nitrogen and carbon stable isotopes, δ15N and δ13C) to mercury concentrations in tunas is scarce yet crucial to better characterise the influence of tuna foraging ecology on mercury exposure and bioaccumulation. Given the difficulties to get modern and historical tuna samples, analyses have to be done on available and unique samples. However, δ13C values are often analysed on lipid-free samples to avoid bias related to lipid content. While lipid extraction with non-polar solvents is known to have no effect on δ15N values, its impact on mercury concentrations is still unclear. We used white muscle tissues of three tropical tuna species to evaluate the efficiency and repeatability of different lipid extraction protocols commonly used in δ13C and δ15N analysis. Dichloromethane was more efficient than cyclohexane in extracting lipids in tuna muscle, while the automated method appeared more efficient but as repeatable as the manual method. Lipid extraction with dichloromethane had no effect on mercury concentrations. This may result from i) the affinity of methylmercury to proteins in tuna flesh, ii) the low lipid content in tropical tuna muscle samples, and iii) the non-polar nature of dichloromethane. Our study suggests that lipid-free samples, usually prepared for tropical tuna foraging ecology research, can be used equivalently to bulk samples to document in parallel mercury concentrations at a global scale.

Graphical abstract



Highlights

► Scarcity of tuna samples makes essential to get the most out of a single sample. ► Dichloromethane is more efficient than cyclohexane to extract lipids. ► Dichloromethane extraction has no effect on Hg levels. ► Bulk and lipid-free tropical tuna samples can be used jointly to infer Hg levels.

Keywords : methylmercury, fat content, delipidation, yellowfin, bigeye, skipjack

1. Introduction

Mercury (Hg) is a widespread heavy metal of particular concern to wildlife and human health. In oceans, it is naturally converted into methylmercury (MeHg), its organic and highest neurotoxic form, well known for its persistence and unique bioaccumulation properties in food webs (Hintelmann, 2010). Consumption of contaminated seafood is considered as the main route of human exposure to MeHg. Top predators like tunas are known to display relatively high MeHg concentrations, sometimes exceeding food safety guidelines (1 µg g^{-1} fresh tissue) (WHO and UNEP Chemicals, 2008) depending on the oceanic basin and the tuna species. Yet, tunas are also among the most popular marine species consumed worldwide, particularly tropical species that account for more than 90% of the global tuna fishery (FAO, 2018). In terms of food and nutrition security, they provide a major source of proteins, essential fatty acids, vitamins and minerals (Di Bella et al., 2015; Sirot et al., 2012).

Knowing their economic importance, nutritional benefits and potential impact on human health, tropical tunas have been studied broadly but at relatively small spatial scale to document their Hg exposure and characterize the processes driving Hg biomagnification along food webs (Bodin et al., 2017; Chouvelon et al., 2017; Houssard et al., 2019; Kojadinovic et al., 2006; Nicklisch et al., 2017). Complex regional interplay between physical (e.g. light intensity), geochemical (e.g. redox status), physiological (e.g. organism's length and age), and ecological factors (e.g. tuna's foraging depth) have been identified to govern Hg concentrations in these top predators (Choy et al., 2009; Houssard et al., 2019; Kojadinovic et al., 2006; Médieu et al., 2021; Wang et al., 2019). Nevertheless, key global aspects remain poorly understood, in particular biogeochemical methylation/demethylation mechanisms controlling MeHg bioavailability at the base of the food web, as well as factors driving both fate and accumulation through the food web. Global studies combining Hg concentrations and ecological tracers are therefore needed to clarify these points, especially in the context of the

UNEP Minamata Convention since monitoring studies in marine biota have become essential the better characterize Hg cycle and fate in oceans.

Pelagic food web structuration and functioning have been broadly investigated, mainly through the use of carbon and nitrogen stable isotopes data ($\delta^{13}C$ and $\delta^{15}N$ values) (Fry, 2006). Recently, collaborative and global studies relying upon $\delta^{13}C$ and $\delta^{15}N$ values enabled identifying broad-scale patterns of trophic structure, movements and trophodynamics of tunas in relation to environmental conditions (Logan et al., 2020; Lorrain et al., 2020; Pethybridge et al., 2018). Based on individual records with associated metadata (i.e. fish length, fishing date and position) (Bodin et al., 2020), these collaborative and global studies also represent a gold mine of already collected and preserved samples to characterize spatial and/or temporal Hg trends in tunas.

An issue with global modern and historical datasets is that samples from different laboratories are not always processed the same way. To account for the influence of lipids on $\delta^{13}C$ values (DeNiro and Epstein, 1977) while making a single analysis for both $\delta^{13}C$ and $\delta^{15}N$ values, $\delta^{13}C$ values are either produced from i) bulk tissue and a mathematical correction (Sardenne et al., 2015), or from ii) lipid-free tissue, with lipids removed through various methods and solvents selected not to alter $\delta^{15}N$ values. In the latter case, manual or automated (high temperature and pressure) methods are generally applied with solvents of low polarity such as dichloromethane and cyclohexane (Bodin et al., 2009; Ménard et al., 2007). While these methods do not affect $\delta^{15}N$ values and provide valuable data on lipid content, nothing is known regarding their effect on Hg content, restricting the development of global studies on tuna's Hg concentration with preserved samples prepared for tuna foraging ecology research.

Here, we investigated i) the efficiency and repeatability of two common lipid extraction methods (manual and automated) and two neutral solvents (dichloromethane and cyclohexane) on lipid content determination (experiment A), and ii) the influence of the most

efficient solvent for lipid extraction on total Hg concentrations (experiment B). Experiments were carried out in three tropical tuna species, i.e. bigeye (*Thunnus obesus*), yellowfin (*T. albacares*) and skipjack (*Katsuwonus pelamis*), and on white muscle tissues, the commonly used tissue in studies investigating both trophic ecology and Hg bioaccumulation and the final storage for MeHg in fish. .

2. Material & methods

2.1. Sample collection

All bigeye, yellowfin, and skipjack tuna samples (n_{total} = 33) were collected in the western Indian Ocean during the unloading of commercial vessels (purse seine) at Victoria port (Seychelles). To test for efficiency and repeatability of different lipid extraction protocols (experiment A), we used three individuals, one per tuna species ($n_{exp\ A}$ = 3). To test for the effect of lipid extraction on Hg concentrations (experiment B), we used 10 other individuals per tuna species ($n_{exp\ B}$ = 30), all collected from the same fishing set. For these 30 individuals, fork length (FL) was measured to the lowest cm and ranged respectively for bigeye, yellowfin and skipjack from 44 to 91 cm (62 ± 17 cm), 35 to 129 cm (70 ± 35 cm), and 34 to 64 cm (45 ± 10 cm) (Supplementary Information Appendix S1). For both experiments, white muscle samples of around 20 g (wet weight) were collected in the front dorsal position, stored frozen at -20°C, freeze-dried for 48 hours with an Alpha 1-4 LD freeze-dryer (Christ, Coueron, France) and finally ground to a fine homogeneous powder using a MM400 grinder (Retsch, Eragny sur Oise, France) prior to lipid extraction and total Hg analyses.

2.2. Lipid extraction protocols

Two lipid extraction methods (manual and automated) and neutral solvents (dichloromethane and cyclohexane) were first tested on five replicates of three individuals, one per tropical tuna species, to identify the most efficient and repeatable method and solvent in extracting lipids (experiment A, $n_{exp\ A}$ = 3, Fig. 1A). The use of one single individual per tuna species allowed overcoming the potential bias related to inter-individual variability when estimating both efficiency and repeatability of the lipid extraction methods, as recommended and done in

other studies testing for different lipid extraction methods (AOCS, 2017; JCGM, 2012; Sardenne et al., 2019a). All lipid extractions were done at LEMAR (Plouzané, France).

2.2.1. Manual extraction: Following the method of Ménard et al. (2007), 100 ± 4 mg of each powdered and dried aliquot were mixed with 10 mL of solvent for 1 hour using a rotary shaker. The mixture of the powder and the solvent was then separated by centrifugation at 2500 rpm for 10 min at 10°C. The lipid-free powders were stored in a dry-room until total Hg analysis, while the lipid extracts were collected in a pre-weighted vial and evaporated to dryness under N_2 flow with a N-evap 111 nitrogen evaporator (OA-SYS, Berlin, USA).

2.2.2. Automated extraction: 150 ± 10 mg of homogenized dried aliquot were extracted with 20 mL of solvent at 100°C under 1400 psi for 10 min using a ASE 350 Accelerated Solvent Extractor (Dionex, Voisins de Bretonneux, France), following the method of Bodin et al. (2009). The lipid-free powders were stored in a dry-room until total Hg analysis, while the lipid extracts were collected in a pre-weighted vial and evaporated to dryness under N_2 flow with a N-evap 111 nitrogen evaporator (OA-SYS, Berlin, USA).

2.2.3. Total lipid content: For both extraction methods, lipid residuals were weighted on a Mettler Toledo MX5 analytical micro-balance (Mettler Toledo, Colombus, Ohio) to the nearest 0.001 mg to determine the total lipid content (TLC, %) of the samples, expressed on a dry weight basis (dw).

2.3. Total mercury analysis

The potential impact of lipid extraction methods on Hg concentrations was assessed on 10 individuals per tuna species, with Hg concentrations determined on both bulk and lipid-free powders (Experiment B, $n_{exp\ B}$ = 30, Fig. 1B). Total Hg concentrations were measured on powdered, dried and homogenized tissue by thermal decomposition, gold amalgamation and

atomic adsorption (AMA 254, Altec, Czech Republic, Száková et al., 2004) at GET (Toulouse, France). Blanks and two biological materials, TORT-3 (lobster hepatopancreas; Hg = 292 ± 22 ng g^{-1} dw) and BCR-464 (tuna fish; Hg = 5240 ± 100 ng g^{-1} dw), covering a wide range of Hg concentrations, were routinely used every 5-10 samples in each analytical batch to check Hg measurement accuracy. Recovery rates (defined as the ratios of observed value by certified value) were calculated for the two certified reference materials (TORT-3: 100 ± 3%; and BCR-464: 100 ± 2%) and variability was below 4%. Hg concentrations are expressed in µg g^{-1} (equivalent to part per million, ppm), on a dw basis.

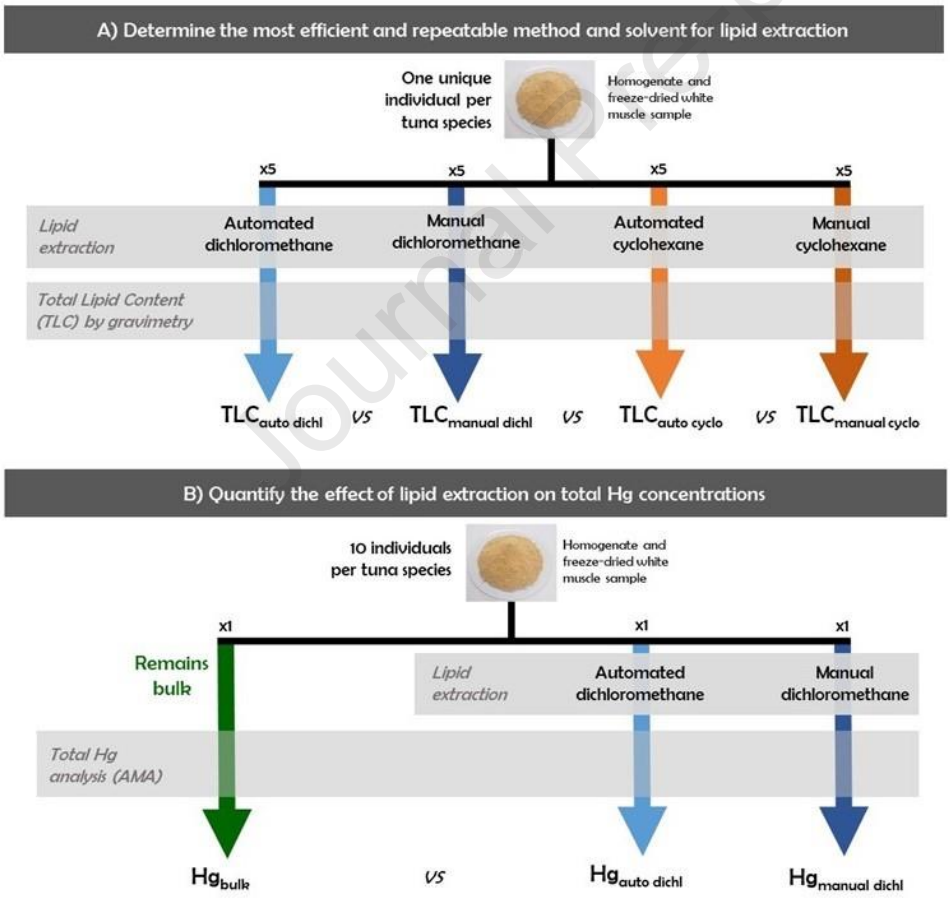


Figure 1: Outline of the two experiments testing for A) the efficiency and repeatability of each lipid extraction protocol (manual and automated method, and dichloromethane and cyclohexane solvent) using five replicates of the same individual per tropical tuna species ; and for B) the influence of manual and automated method with

dichloromethane on total Hg concentrations ($n_{exp\ B}$ = 30 individuals). TLC=Total Lipid Content. See Material and methods for details.

2.4. Statistical analysis

All statistical analyses were performed with the statistical open source R software 3.6.1 (R Core Team, 2018). We first evaluated the efficiency of both solvents (i.e. dichloromethane and cyclohexane) and methods (i.e. manual and automated) in extracting lipids with an analysis of variance (ANOVA) on TLC values (experiment A). The higher the TLC values, the higher the efficiency. Repeatability of each extraction experiment was assessed per tropical tuna species using the coefficient of variation (CV, defined as the ratio of standard deviation by mean) of TLC values measured on the five replicate samples. The lower the CV, the higher the repeatability. Then, we assessed the influence of manual and automated extraction methods on total Hg concentrations with two separated paired t-test on ΔHg values (with $\Delta Hg = Hg_{bulk} - Hg_{lipid\text{-}free}$) (experiment B). For these two tests, we considered all individuals ($n_{exp\ B}$ = 30 pairs of observations), no matter the species, as ΔHg values do not significantly differ between species (ANOVA, p>0.05 for both methods). TLC, ΔHg values, and residuals of ANOVA were tested for normality with a Shapiro test. Observations were considered as outliers according to the "1.5 rule", i.e. if they were less that Q1 – 1.5*IQR, or greater than Q3 + 1.5*IQR, where Q1, Q3 and IQR are the lower quantile, upper quantile and inter-quantile range (defined as Q3-Q1), respectively.

3. Results & discussion

3.1. Efficiency and repeatability of lipid extraction methods and solvents: TLC values measured in white muscle varied between 0.4 and 1.7% dw (Supplementary Information Appendix S1). These values are similar to levels reported in same tropical tuna species from the Indian (~3%, Sardenne et al., 2016), Atlantic (~6%, Sardenne et al., 2019) and Pacific (~2%, Peng et al., 2013) Oceans, which confirms tropical tunas are lean species.

For the three tropical tuna species, TLC values were significantly higher when lipids were extracted with dichloromethane than with cyclohexane (Fig. 2; ANOVA, $p<0.01$). Dichloromethane and cyclohexane are both non-polar solvents recommended for routine analysis of lipids/fatty acids and/or prior $\delta^{13}C$ and $\delta^{15}N$ analysis (Bodin et al., 2009; Ménard et al., 2007). Here, the higher efficiency of dichloromethane may result from its slightly higher Snyder polarity index (3.1) compared to cyclohexane (0.2) and therefore to the fact that dichloromethane, although a neutral solvent is likely to extract some membrane polar lipids (e.g. phospholipids and sphingolipids) too, increasing TLC values. Nevertheless, this might be of minor importance regarding the protein fraction as this solvent has been shown to have negligible effects on the $\delta^{15}N$ values across tissues and tuna species (Sardenne et al., 2015).

Regarding differences between manual and automated methods, TLC values were significantly higher for bigeye and skipjack replicates for the automated extraction (Fig. 2; ANOVA, $p<0.05$). For bigeye replicates, interaction between solvent and method was also significant, showing that the efficiency of the extraction method depends on the solvent used, with automated extraction with dichloromethane being the most efficient protocol to extract lipids (Fig. 2, ANOVA $p<0.05$). Conversely, TLC values did not differ significantly between the two extraction methods for yellowfin replicates (Fig. 2; ANOVA, $p>0.05$). The higher

198 efficiency of the automated method may be explained by its combined conditions of elevated
199 temperature and pressure that maintain solvents near their supercritical region where they
200 have high extraction properties (Ramos et al., 2002). This suggests that lipid extraction with
201 automated method would be preferred over manual method. On the other hand, this automated
202 method is twice more costly in polluting solvent, and its cleaning steps are longer compared
203 to the manual method. Considering the low difference in extraction efficiency and the fact that
204 is was significant only for the skipjack in-house reference sample, both manual and automated
205 methods are relevant to extract lipids from lean tuna muscle tissues.

206 The repeatability of the different experiments varied between the protocols between 4 and
207 27%, but remained overall lower than 18% (Supplementary Information Appendix 2).
208 Acceptable repeatability often ranges between 5 and 10%, but for lean tissues like white
209 muscle of tropical tunas, repeatability down 20-30% is considered acceptable too, as found in
210 other studies (Bodin et al., 2009; Dodds et al., 2004). As expected, automated method showed
211 generally lower CV values, as ASE 350 has been developed to reduce handling steps and to
212 ensure increased reproducibility. This would be another argument to prefer the automated
213 method; yet given the pros and cons arguments listed above, both manual and automated
214 methods remain acceptable for the lipid extraction from white muscle tissue of tropical tunas.

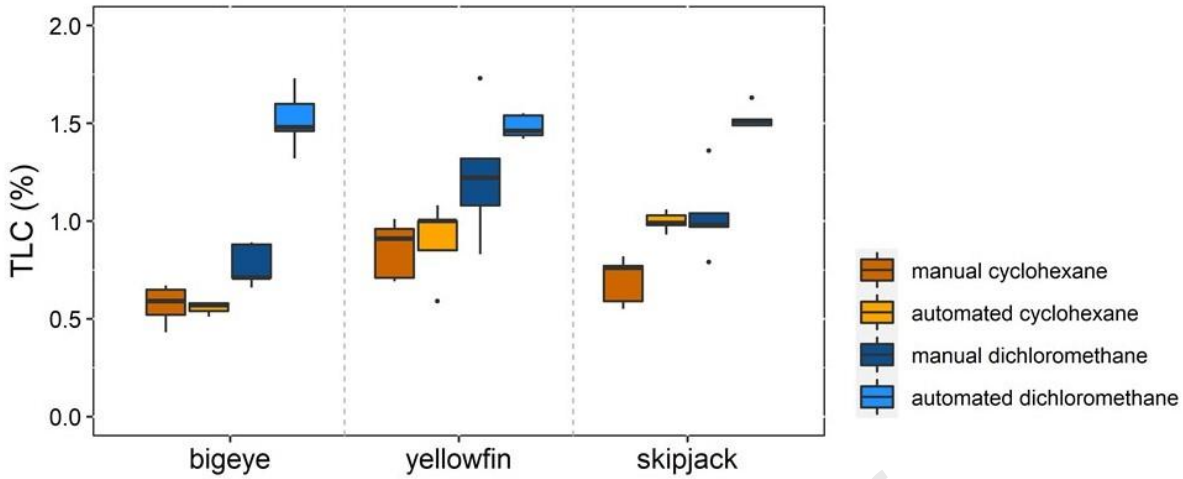
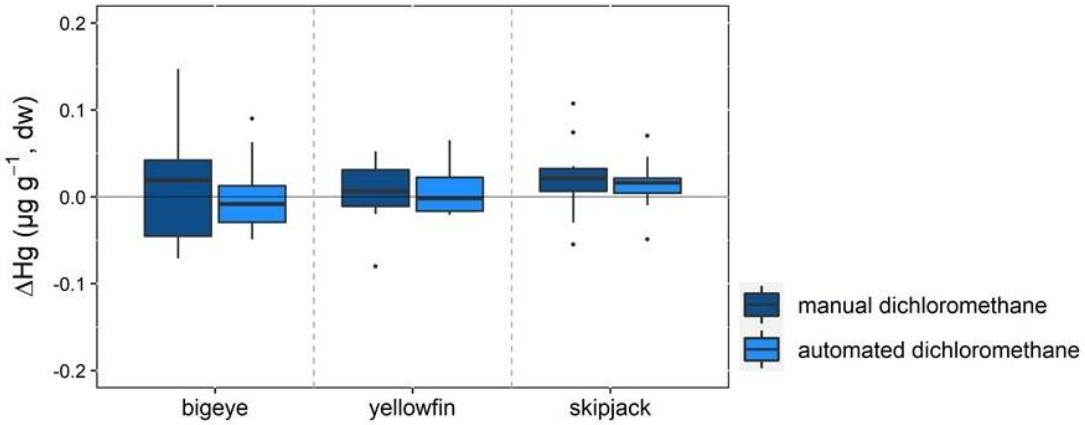


Figure 2: Boxplots of total lipid content (TLC, % dry weight) associated to the four lipid extraction protocols and measured in five replicates of each single individual per tuna species. Thick bar is the median value, points are outliers of five replicates, and the box contains 50% of the data.

3.2. Influence of lipid extraction on total Hg concentrations: Bulk total Hg concentrations (mean ± sd, min-max, dw) varied between tuna species (Kruskal-Wallis, $p<0.05$), with significantly higher levels in bigeye (0.81 ± 0.47 µg g^{-1}, 0.20-1.37 µg g^{-1}; Dunn's test, $p<0.05$) than yellowfin (0.38 ± 0.27 µg g^{-1}, 0.12-1.03 µg g^{-1}) and skipjack (0.30 ± 0.17 µg g^{-1}, 0.12-0.61 µg g^{-1}) (Supplementary Information Appendix S1). Those levels and differences are similar to those reported previously in the western Indian Ocean or in both north and south Pacific Oceans for similar fish size ranges (Bodin et al., 2017; Choy et al., 2009; Médieu et al., 2021). The highest Hg concentrations in bigeye are likely to result from confounding effects: a higher trophic position, a deeper vertical habitat giving access to mesopelagic species with higher Hg concentrations, and a longer lifespan compared to the two other tropical tunas (Choy et al., 2009; Olson et al., 2016).

Manual and automated lipid extractions with dichloromethane did not significantly affect the Hg concentrations in white muscle samples of the three tuna species (Fig. 3; paired t-test, $p>0.05$). This agrees with Cipro et al. (2017) where no difference of Hg levels was found

between bulk and lipid-free muscle samples of two marine fish (i.e. *Notothenia coriiceps* and *N. rossii*). This may be related to the particular chemical properties of MeHg in fish flesh and the polarity of the solvent used for lipid extraction. In tuna white muscle, most of total Hg (>91%) is in its methylated form (Bloom, 1992; Houssard et al., 2019), which is known to bioaccumulate in the protein fraction of the muscle, mostly by forming complexes with the amino acid cysteine (Amlund et al., 2007; Leaner and Mason, 2004; Manceau et al., 2021). On the other hand, we used dichloromethane, a neutral solvent that does not affect the protein fraction by limiting the removal of proteins bound to membrane lipids (Bodin et al., 2009). Thus, by preserving the Hg-protein fraction in muscle tissues during lipid extraction, we assumed to have preserved MeHg concentrations of the samples. The absence of a significant effect of lipid extraction on Hg concentrations may be due also to the low lipid content of tropical tuna species (<6%), as measured in our experiment and showed in other studies (Peng et al., 2013; Sardenne et al., 2019b, 2016), and therefore to an unchanged mass balance in the samples. Finally, when discussing the effects of the different protocols tested, it is worthwhile mentioning that high temperature and pressure conditions of the automated extraction did not significantly affect total Hg concentrations neither, suggesting no thermal and pressure degradation of the total Hg pool in the samples. This confirms that MeHg is bound up tightly to the protein fraction in fish muscle tissues, where it is known to remain for a long time (Leaner and Mason, 2004)..



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256 **Figure 3:** Boxplots of the differences of Hg concentrations (µg g^{-1}, dry weight) between bulk and lipid-free
257 samples. Box colours display lipid extraction methods (manual and automated). Thick bar is the median value,
258 points are outliers, and the box contains 50% of the data.

259

260 ## 4. Conclusion

261 This study reveals the higher efficiency of dichloromethane compared to cyclohexane to
262 extract lipids in tropical tuna white muscle tissue, which may be attributed to its slightly
263 higher polarity index. Automated method appeared more efficient than manual method to
264 extract lipids, especially when used with dichloromethane, which may be attributed the
265 combined conditions of elevated temperature and pressure. Repeatability of all experiments
266 were in acceptable ranges considering that we were working on lean samples.

267 We show no significant effect of lipid extraction with dichloromethane on total Hg
268 concentrations in tropical tuna white muscle tissue. This may be related to i) the affinity of
269 MeHg (i.e. the most abundant Hg chemical form in tuna muscles) to proteins, ii) the relative
270 low lipid content in white muscle tissue of tropical tuna species, and iii) the non-polar
271 characteristics of dichloromethane, known to efficiently extract storage lipids without
272 affecting the protein fraction. This suggests that white muscle samples that have undergone
273 lipid extraction with dichloromethane can be used equivalently to bulk samples to investigate

Hg bioaccumulation in these pelagic top predators from the western Indian Ocean. Knowing that tropical tunas in the Atlantic, Indian and Pacific Oceans all have relatively low lipid content in white muscle tissue (<6%, Peng et al., 2013; Sardenne et al., 2019, 2016), this suggests that our results might be valid at a global scale, but further studies are needed to confirm this statement. As lipid content can vary between tissues, species and oceanic basins because of differences in diet and/or in energy allocation strategies distributed among metabolic functions (i.e. growth, reproduction and maintenance), we recommend using bulk samples to infer precisely Hg concentrations in marine fish other than tropical tunas. Further studies would be particularly needed for tissues and species with high lipid content (e.g. swordfish white muscle tissue, between 20-40% dw, Young et al., 2010).

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Author contribution: Anaïs Médieu: Conceptualization, Methodology, Investigation, Writing – original draft. Fany Sardenne: Conceptualization, Methodology, Supervision, Writing – review & editing. Anne Lorrain: Conceptualization, Supervision, Writing – review & editing. Nathalie Bodin: Conceptualization, Methodology, Resources, Writing – review & editing. Chloé Pazart: Investigation. Hervé Le Delliou: Investigation, Resources. David Point: Conceptualization, Supervision, Resources, Writing – review & editing.

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Highlights

- Scarcity of tuna samples makes essential to get the most out of a single sample
- Dichloromethane is more efficient than cyclohexane to extract lipids
- Dichloromethane extraction has no effect on Hg levels
- Bulk and lipid-free tropical tuna samples can be used jointly to infer Hg levels

Competing interests

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.