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## Mercury in the tissues of five cephalopods species: first data on the nervous system

MINET Antoine<sup>1</sup>, MANCEAU Alain<sup>2</sup>, VALADA-MENNUNI Anaïs<sup>1</sup>, BRAULT-FAVROU Maud<sup>1</sup>, CHURLAUD Carine<sup>1</sup>, FORT Jérôme<sup>1</sup>, NGUYEN Thành<sup>1,3</sup>, SPITZ Jérôme<sup>4,5</sup>, BUSTAMANTE Paco<sup>1,6</sup>, LACOUÉ-LABARTHE Thomas<sup>1,†</sup>

<sup>1</sup> *Littoral Environnement et Sociétés (LIENSs), UMR 7266 CNRS - Université de la Rochelle, 2 rue Olympe de Gouges, 17000 La Rochelle, France*

<sup>2</sup> Univ. Grenoble Alpes, ISTERre, CNRS, CS 40700, 38058 Grenoble, France

<sup>3</sup> Université des Sciences et des Technologies de Hanoï (USTH), 18 Hoàng Quốc Việt, Nghĩa Đô, Cầu Giấy, Hà Nội, Vietnam

<sup>4</sup> *Centre d'Études Biologiques de Chizé (CEBC), UMR 7372 CNRS - Université de la Rochelle, 5 Allée de l'Océan, 17000 La Rochelle, France*

<sup>5</sup> *Observatoire PELAGIS, UMS 3462 CNRS - Université de la Rochelle, 5 Allée de l'Océan, 17000 La Rochelle, France*

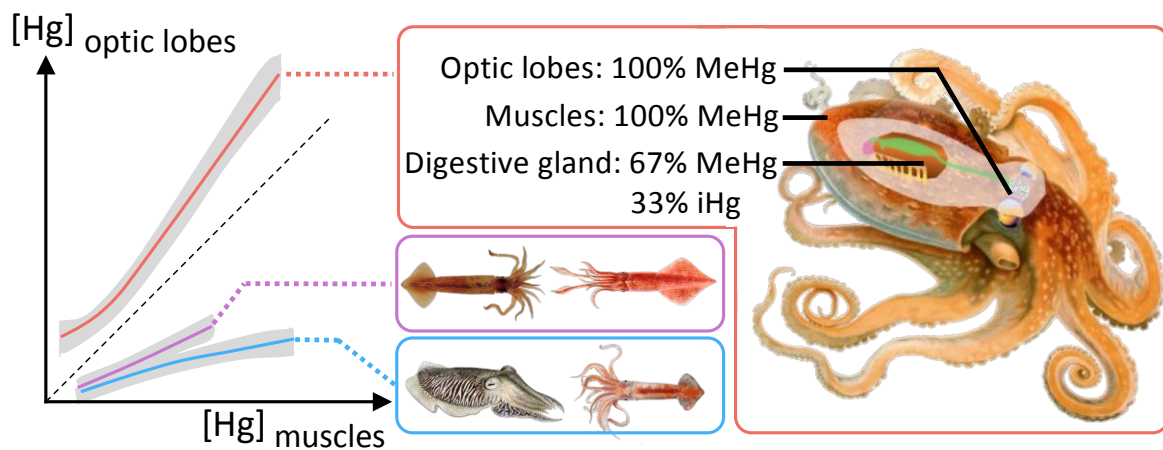
<sup>6</sup> *Institut Universitaire de France (IUF), 1 rue Descartes 75005 Paris, France*

† Corresponding author: UMR 7266 CNRS-Université de La Rochelle, Institut du Littoral et Environnement, 2 rue Olympe de Gouges, 17000 La Rochelle, France, +33 5 46 45 83 88, [tlacouel@univ-lr.fr](mailto:tlacouel@univ-lr.fr)

## Highlights

- Hg levels were measured in digestive gland, muscle and brain in 5 cephalopod species
- Hg concentrations in the optic lobes can be predicted from muscle ones
- Highest Hg concentrations were found in the optic lobes of the octopus *E. cirrhosa*
- XANES spectrometry showed 33% of iHg in octopus digestive gland suggesting demethylation
- 100% of MeHg in brain and muscle implies dietary MeHg storage in these tissues

## Graphical Abstract



## Abstract

Mercury (Hg), one of the elements most toxic to biota, accumulates within organisms throughout their lifespans and biomagnifies along trophic chain. Due to their key role in marine systems, cephalopods constitute a major vector of Hg in predators. Further, they grow rapidly and display complex behaviours, which can be altered by neurotoxic Hg. This study investigated Hg concentrations within 80 cephalopod specimens sampled in the Bay of Biscay, which belonged to five species: *Eledone cirrhosa*, *Sepia officinalis*, *Loligo vulgaris*, *Todaropsis eblanae* and *Illex coindetii*. Hg concentrations were measured in the digestive gland, the mantle muscle and optic lobes of the brain. The digestive gland and the mantle were tissues with the most concentrated Hg among all species considered (up to  $1.50 \mu\text{g}\cdot\text{g}^{-1} \text{dw}$ ), except *E. cirrhosa*. This benthic cephalopod had 1.3-fold higher Hg concentrations in the brain (up to  $1.89 \mu\text{g}\cdot\text{g}^{-1} \text{dw}$ ) than the mantle, while other species had 2-fold lower concentrations of Hg in the brain than in the mantle. Brain-Hg concentrations can be predicted from muscle-Hg concentrations for a given species, which facilitates the assessment of Hg toxicokinetics in cephalopods. In the most contaminated *E. cirrhosa* individual, the chemical form of Hg in its digestive gland, mantle muscle, and optic lobes, was determined using HR XANES spectroscopy. In the digestive gland,  $33 \pm 11 \%$  of total Hg was inorganic Hg speciated as a dicysteinate complex ( $\text{Hg}(\text{Cys})_2$ ), which suggested that the demethylation of dietary MeHg occurs in the organ. All Hg found in the mantle muscle and the optic lobes is methylated and bound to one cysteinyl group (MeHgCys complex), which implies that dietary MeHg is distributed to these tissues via the bloodstream. These results raised questions regarding interspecific differences observed regarding Hg brain concentrations and the possible effect of Hg on cephalopod functional brain plasticity and behaviour.

**Keywords:** Organotropism, mantle muscle, digestive gland, optic lobes, Hg, speciation, HR-XANES

## Introduction

Mercury (Hg) is a globally distributed pollutant that poses considerable risk to the health of humans and wildlife (Scheuhammer et al., 2007). Special attention to this contaminant within the marine environment, a final receptor for a large proportion of atmospheric, freshwater and terrestrial Hg contamination is needed (Driscoll et al., 2013). Mercury is mostly derived from anthropogenic sources and is principally found in inorganic (iHg) free forms and complexed ions, and as organic forms, which include methylmercury (MeHg) (Mason and Benoit, 2003). In fact, under anaerobic conditions in sediments or deep waters, microorganisms such as iron and sulphate reducing bacteria convert iHg to organic MeHg (Benoit et al., 2002; Hammerschmidt and Fitzgerald, 2004). Metagenome and metatranscriptome analyses have revealed that aerobic microorganisms are also potentially involved in MeHg production in subsurface oxic seawater (Villar et al., 2020). Hg mobility and toxicity depend on its molecular speciation (Weiner and Suchanek, 2008), which confers strong neurotoxic properties to MeHg against biota including humans (Farina et al., 2011; Eagles-Smith et al., 2018). Furthermore, MeHg accumulates in marine organisms throughout their lifespans and biomagnifies along trophic chains to reach concentrations well above its toxicity threshold in top predators (Lemos Bisi et al., 2012; Van der Velden et al., 2013; Chauvelon et al., 2018; López-Berenguer et al., 2020).

Among the oceanic trophic compartments, cephalopods are a central link in food webs and play a role as vector for many contaminants such as cadmium (Cd) and Hg (Clark, 1996; Bustamante et al., 1998; Maz-Courrau et al., 2012). Our knowledge of Hg in cephalopods is essentially limited to the species of interest to fisheries (*e.g.* Penicaud et al., 2017) and levels are generally measured in edible portions of organisms (*i.e.* muscles of the mantle and arms of squids and octopods, respectively) that store more than 70% whole body Hg burden (Seixas et al., 2005; Bustamante et al., 2006; Lacoue-Labarthe et al., 2009). Most of the Hg found in cephalopod muscles is in its organic form (Bustamante

et al., 2006; Seco et al., 2020), and the speciation of Hg in tissues other than muscles has not been well documented to date (see below).

Hg concentrations reported in cephalopod muscles vary by two orders of magnitude (Bustamante et al., 2006) among species. Overall, median Hg concentrations in cuttlefish, octopods and squids range from 0.2 and 0.5  $\mu\text{g}\cdot\text{g}^{-1}$  dry weight (dw) (Penicaud et al., 2017). These levels are moderately high with respect to their central trophic position and their short life span (an average of two years) that limits the Hg bioaccumulation within their tissues (Chouvelon et al., 2011). Higher concentrations were reported in some species or individuals, up to 1.76  $\mu\text{g}\cdot\text{g}^{-1}$  dw in the curled octopus, *Eledone cirrhosa*, from Mediterranean Sea (*i.e.* 0.44  $\mu\text{g}\cdot\text{g}^{-1}$  wet weight (ww) reported by Storelli et al., 2010 assuming 75% water content as reported in Spitz et al., 2010). Hg concentrations in mantle muscle and the digestive gland have been used to infer Hg environmental contamination, since Hg mostly accumulates in its organic form in muscles and is highly concentrated in the digestive gland, where Hg detoxification has been reported to occur (Bustamante et al., 2006; Lacoue-Labarthe et al., 2009). However, reports of Hg concentrations in tissues that are likely sensitive to Hg, such as the nervous system, are scarce.

Although Hg neurotoxicity has been recognised, information regarding Hg brain tropism in marine organisms are scattered (Berntssen et al., 2004). Previous studies have focused on fish provided nervous system Hg based on concentrations present in the environment. Alternatively, they assessed classically targeted organs such as muscle and liver. Hg concentrations in the tissues of bluefish, *Pomatomus saltatrix* from New Jersey showed Hg levels one order of magnitude lower in the brain than muscles and kidneys (Burger et al., 2013). In more contaminated fish such as the spotted seatrout, *Cynoscion nebulosus* and European bass, *Dicentrarchus labrax* that were isolated from polluted areas, brain Hg concentrations were less than 3-fold lower than those reported in muscles and liver (Adams et al., 2010; Mieiro et al., 2012). In addition, Hg in brain of the golden grey mullet, *Liza aurata*, from different areas of the Portuguese coastline faithfully reflected local water and

sediment contamination variability (Pereira et al., 2014). Through an experimental approach, autoradiography revealed a specific affinity of  $^{203}\text{Hg}$  in the brain of brown trout *Salmo trutta* and rainbow trout *Oncorhynchus mykiss* following waterborne exposure, suggesting that the transport of this metal occurs from sensory or motor systems to the brain (Rouleau et al., 1999). Hg exposure and subsequent Hg accumulation in the nervous system can then lead to oxidative stress, metallothionein production, brain lesions, morphometric alterations and consequently affected swimming behaviour in Atlantic salmon *Salmo salar*, European bass *Dicentrarchus labrax*, zebrafish *Danio rerio*, and white seabream *Diplodus sargus* (Berntssen et al., 2003; Mieirol et al., 2011; Gentès et al., 2015; Pereira et al., 2016).

Cephalopods are known as an alternative model to vertebrates for the evolution of complex brains and high intelligence, and they are renowned for their advanced cognitive performances (O'Brien et al., 2018) and complex behaviour (Hanlon & Messenger, 2018). Such abilities require a much more evolved and developed brain than is needed in mollusks like bivalves and gastropods (Hanlon and Messenger, 2018). The cephalopod nervous system is composed of the central brain (around 40 interconnected ganglia), two optic lobes and the peripheral nervous system. Their large-sized brain, compared to other invertebrates, is involved in behavioural plasticity, cognitive capacity as well as skin control (Shigeno et al., 2018) and is of increased concern when considering impacts of neurotoxicants (e.g. Chabenat et al., 2019). Very few studies have examined the accumulation of Hg in tissues of the nervous system, and Hg levels in the muscular mantle, branchial hearts, kidneys, gills, ink sack and the digestive gland (Seixas et al., 2005; Pereira et al., 2009). In fact, to the best of our knowledge, only one study that examined a giant squid, *Architeuthis dux*, individual ( $1.25 \mu\text{g}\cdot\text{g}^{-1}$  dw; Bustamante et al., 2008) in which its brain Hg concentration was in the same order of magnitude as that of other tissues. The extent to which Hg bioaccumulation occurs and speciation in the brains of cephalopods requires further examination.

In this context, the current study aims to investigate levels of Hg contamination in five different species of cephalopods from the Bay of Biscay including *Eledone cirrhosa*, *Sepia officinalis*, *Loligo vulgaris*, *Todaropsis eblanae* and *Illex coindetii*. Hg concentrations were measured in the tissues with major roles in Hg bioaccumulation and detoxification including the mantle muscles, which are responsible for storing most the majority of the Hg burden, and the digestive gland, which can demethylate and depurate Hg from the body. Hg levels were also determined in the optic lobes of the brain, since it is a tissue that is potentially sensitive to Hg toxicity. In addition, the molecular form of Hg in the optic lobes, digestive gland and mantle muscles of some curled octopus, *E. cirrhosa*, individuals were investigated via high energy-resolution XANES spectroscopy.

## **Material and methods**

### *Sample collection and sample preparation*

In this study, 80 cephalopods were sampled along the continental shelf of the Bay of Biscay throughout EVHOE surveys conducted by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER) in autumn of 2016 and 2017 (Leaute et al., 2016; Duhamel et al., 2017). Collected organisms belonged to five cephalopod species from four different families (see Table 1), as follows: curled octopus, *E. cirrhosa* (n = 20); common cuttlefish, *S. officinalis* (n = 21); common squid, *L. vulgaris* (n = 20); the lesser flying squid, *T. eblanae* (n = 9); and southern shortfin squid, *I. coindetii* (n = 11). Specimens were frozen at -20°C on board and then stored in the laboratory prior to dissection and sample preparation.

In the laboratory, individuals were thawed and weighed (g) before measurements obtained. Dorsal mantle length (DML; mm) was measured as a reference for measuring the size of the cephalopod. Whenever possible, sex was determined by visual observation of gonads and hectocotylised arms (Table 1). During dissection, the digestive gland, mantle and the optic lobes were collected and wet weighed (g ww). The sampled tissues were frozen and freeze-dried for 48 hours. Samples were dry



weighed (g dw), ground manually with a porcelain pestle and mortar into a fine powder, and hermetically stored in 40 mL plastic vials.

#### *Mercury analyses*

Total Hg concentrations within samples were analysed at the Littoral ENvironnement et Sociétés laboratory (LIENSs, La Rochelle, France) using an Advanced Mercury Analyser spectrophotometer (Altec AMA 254) as described in Chauvelon et al. (2009). All analyses were carried out in duplicate or triplicate, and had relative standard deviation values < 10%. Hg analyses were run according to a thorough quality control programme that included an analysis of procedural blanks at the beginning of each analytical sessions and a certified reference material (CRM) was assessed every 10 samples. Thus, the accuracy of the analytical procedure was verified via assessing CRM TORT-2 (lobster hepatopancreas, NRC, Canada; Hg concentration of  $0.27 \pm 0.06 \mu\text{g}\cdot\text{g}^{-1}$  dw). Results indicated an average Hg concentration of  $0.257 \pm 0.009 \mu\text{g}\cdot\text{g}^{-1}$  dw, with an average recovery of  $95 \pm 3\%$ , and were in good agreement with certified values. The detection limit of the method was  $0.005 \mu\text{g}\cdot\text{g}^{-1}$  dw. Mercury concentrations will be expressed as  $\mu\text{g}\cdot\text{g}^{-1}$  dw in subsequent portions of the text.

#### *HR-XANES measurements*

Freeze-dried powdered digestive gland, mantle muscle and optic lobe of *E. cirrhosa* were pressed into pellets, and their Hg L<sub>3</sub>-edge XANES (for X-ray Absorption Near Edge Structure) spectra were measured using high energy-resolution (HR-XANES) with high-luminosity analyser crystals on beamline ID26 at the European Synchrotron Radiation Facility (ESRF, Rovezzi et al., 2017). Mantle muscles and the digestive gland of the most contaminated individual were selected for further examination, whereas the optic lobes of the six individuals that were most contaminated were pooled because each lobe had low mass (~50–80 mg). Spectra were collected at a temperature of 10–15 K and a scan time of 15 s to reduce exposure, and repeated at using pristine positions of the sample to increase the signal-to-noise ratio. Scans were monitored carefully for any evidence of

radiation damage. Incident energy was scanned from 12260 to 12360 eV in increment of 0.2 eV and the spectra were normalised to unity at  $E = 12,360$  eV. The identities and relative proportions of Hg species were determined using a spectral database (Bourdineaud et al., 2019). The precision of the fit of components was estimated to be equal to the variation of their best-fit values when the fit residual (*NSS*) was increased by 20%. *NSS* was defined as the normalised sum-squared difference between data and fit, was expressed as follows:  $\Sigma[(y_{\text{data}} - y_{\text{fit}})^2] / \Sigma(y_{\text{data}}^2)$ . Details regarding data acquisition and analysis were performed as previously described (Manceau et al., 2019).

#### *Statistical and data analyses*

Statistics were computed using R version 3.6.1 (R Development Core Team, 2011). The influence of the tissue on Hg concentration was tested by using generalised linear models (GLM) with a gaussian distribution and an identity link function. Model selection was based on Akaike's Information Criteria adjusted for small sample sizes (AICc) (Burnham and Anderson, 2002).

Concentrations of Hg analysed in the mantle, digestive gland and optic lobes of the five species assessed were compared using a one-way ANOVA followed by a Tukey test. Normality and heteroscedasticity of values were tested using a Barlett and Shapiro test, respectively, and each of the cephalopods relative to the others were considered separately. When the conditions of the statistical tests were not met, non-parametric tests of Wilcoxon followed the post-hoc test of Nemenyi were applied. A linear model was applied to describe relationships between Hg concentrations in the optic lobe and mantle muscle and an analysis of covariance (ANCOVA) was used to test differences in the slopes of regression lines for the five species considered.

Hg concentrations reported from the literature were converted when necessary from ww to dw using measured moisture values from the different tissues, as follows: dw represents 20% of the ww

for muscle and brain tissues and 35% of the ww of the digestive gland (Suppes et al., 1967; Arai et al., 2016 and personal data).

## Results

The mantle size and weight of animals varied from 4.2 to 19.0 cm and 19 to 764 g ww, respectively. Measured Hg concentrations were 0.02–1.48  $\mu\text{g.g}^{-1}$  dw, from 0.09–1.35  $\mu\text{g.g}^{-1}$  dw, and 0.03–1.89  $\mu\text{g.g}^{-1}$  dw in mantle muscles, the digestive gland and optic lobes, respectively (Table 1).

GLMs revealed that Hg concentrations differed according to species and tissue assessed (Fig. 1; Table 2). Overall, although concentrations among the three analysed tissues were of the same order of magnitude, Hg concentrations were significantly higher in mantle muscles and the digestive gland than optic lobes in all species assessed, except in *E. cirrhosa*. Relative Hg concentrations in mantle muscles of species assessed were as follows: *E. cirrhosa*  $\approx$  *S. officinalis*  $\approx$  *T. eblanae*  $>$  *L. vulgaris*  $>$  *I. coindetii* (values measured were  $0.52 \pm 0.35$ ,  $0.46 \pm 0.24$ ,  $0.29 \pm 0.06$ ,  $0.16 \pm 0.03$ , and  $0.08 \pm 0.01$   $\mu\text{g.g}^{-1}$  dw, respectively).

In the digestive gland, the same trend was observed, and significantly higher levels of Hg were measured in *E. cirrhosa* and *S. officinalis* than in other squid species. Relative values were as follows: *E. cirrhosa*  $\approx$  *S. officinalis*  $>$  *L. vulgaris*  $\approx$  *T. eblanae*  $>$  *I. coindetii* ( $0.47 \pm 0.34$ ,  $0.43 \pm 0.12$ ,  $0.29 \pm 0.06$ ,  $0.24 \pm 0.06$ , and  $0.12 \pm 0.02$   $\mu\text{g.g}^{-1}$  dw, respectively). In the optic lobes, relative Hg concentrations were similar, however, a greater difference between *E. cirrhosa* and other species was observed, as follows: *E. cirrhosa*  $>$  *S. officinalis*  $\approx$  *T. eblanae*  $>$  *L. vulgaris*  $\approx$  *I. coindetii* ( $0.60 \pm 0.47$ ,  $0.23 \pm 0.09$ ,  $0.18 \pm 0.07$ ,  $0.09 \pm 0.02$ , and  $0.04 \pm 0.01$ ,  $\mu\text{g.g}^{-1}$  dw, respectively). Interestingly, two *E. cirrhosa* individuals (53 and 90 mm mantle size) had elevated Hg concentrations that reached more than 1  $\mu\text{g.g}^{-1}$  in the mantle and the digestive gland and more than 1.5  $\mu\text{g.g}^{-1}$  in the optic lobes, whereas values rarely exceeded 0.5  $\mu\text{g.g}^{-1}$  dw in other species, in any tissue assessed.

The concentrations of Hg in the optic lobes were linearly and strongly associated with those of mantle muscles (Fig. 2, Table 3). ANCOVA revealed that slopes of the regressions were significantly different among when *E. cirrhosa* ( $R^2 = 0.978$ ), *S. officinalis* and *T. eblanae* ( $R^2 = 0.829$ ), and *I. coindetii* and *L. vulgaris* ( $R^2 = 0.663$ ) were compared. This implies that the ratio of brain and muscle Hg concentrations differed among the three groups. Interestingly, *E. cirrhosa* had an optic lobe Hg concentration that was slightly higher (1.15-fold greater) than that of mantle muscles, whereas *S. officinalis*/*T. eblanae* and *I. coindetii*/*L. vulgaris* had optic lobe concentrations that were 0.35 and 0.50-fold lower, respectively, than those of mantle muscles.

HR-XANES spectra of the optic lobes and mantle muscles were statistically indistinguishable, indicating similar or identical chemical forms of Hg were present in each tissue (Fig. 3a). The best spectral match with our database of model compounds was obtained with MeHg bound to cysteine residues in fish (Fig. 3b). In this complex, Hg is linearly binds a methyl group and the aliphatic thiol from a cysteinyl group (forming a MeHgCys complex). This geometry gives a characteristic absorption peak at 12,279.8 eV, which is not observed when Hg is coordinated to three or four thiols (Bourdineaud et al., 2019). This peak occurs at 12,279.6 eV in the digestive gland and its intensity was lower (Fig. 3c). These two features are diagnostic of the linear coordination to two thiol groups, as it is observed in an inorganic  $\text{Hg}(\text{Cys})_2$  complex (Manceau et al., 2016). Proportions of methylated and inorganic Hg, as determined by a linear combination fit of the optic lobe spectrum and the  $\text{Hg}(\text{Cys})_2$  standard, were  $68 \pm 11\%$  for MeHgCys and  $33 \pm 11\%$  for  $\text{Hg}(\text{Cys})_2$  (Fig. 3d).

## Discussion

Among marine organisms, most available information regarding Hg contamination has been determined in bivalves, fish and top predators (mammals and birds), mainly due to their importance

as a food source for humans and their roles as bioindicators of Hg contamination of the ocean (Renzoni et al., 1998). In contrast, the information regarding Hg contamination in cephalopod tissues remains scarce, despite the fact that molluscs are known to efficiently accumulate chemical contaminants, including non-essential metals that may be concentrated at very high levels in their tissues (Dorneles et al., 2007; Lischka et al., 2018). Although Hg concentrations are moderate in cephalopods, significant differences between taxons have been observed, and values have been shown to vary by two orders of magnitude among cephalopod families (Penicaud et al., 2017). The cephalopods have a pivotal place in trophic webs, and have a key role in the Hg intake of top predators including humans (Jackson et al., 2007), therefore a better understanding of the Hg bioaccumulation processes in cephalopods is needed. Finally, despite their invertebrate status, cephalopods have an exceptionally developed nervous system and display complex cognitive abilities that confer great adaptive capacity, despite the short lifespan of the animals (Hanlon and Messenger, 2018). However, the effects of Hg in the brains of cephalopods remains unknown, despite the fact that Hg is known for its high degree of toxicity on the nervous system of vertebrates (Syversen and Kaur, 2012).

Mercury concentrations varied among species collected in the same geographical location (*i.e.* the Bay of Biscay) at the same time, suggested that individuals were exposed to Hg to variable extents. More precisely, *E. cirrhosa* and *S. officinalis* had the highest levels of Hg in their tissues, relative to levels in other species assessed. This is congruent with the previous observation that benthic octopus and nektobenthic cuttlefish (*i.e.* Octopodidae and Sepiidae) generally display significantly higher Hg concentrations in their tissues than pelagic squids, including Ommastrephidae and Loliginidae, from the same area (Pénicaud et al., 2017). For example, among seven cephalopods from the Mediterranean Sea, Octopodidae (*E. moschata*, *E. cirrhosa*, and *Octopus salutii*) had the highest Hg concentrations, followed by Sepiidae (*Sepia elegans* and *Sepia orbignyana*). The lowest concentrations were found in Ommastrephidae (*I. coindetii*) and Loliginidae (*L. vulgaris*) (Storelli et al.,

2010). To date, these inter-specific differences have mainly been attributed to ecological factors, such as the proximity of benthic species to sediment where Hg methylation is located (Storelli et al., 2005). Nevertheless, squids generally displayed elevated trophic levels than octopus and cuttlefish, indicating that they likely feed on MeHg-enriched prey such as fish and/or cephalopods (Pierce et al., 1994; Coelho et al., 1997) compared to benthic cephalopods, which prey on invertebrates including crustaceans and bivalves with lower concentrations of Hg and lower proportions of MeHg. This incongruity indicates that species-dependent physiological processes, such as demethylation and elimination capacities, could explain the interspecific Hg variation.

Mercury concentrations in mantle muscles ranged from 0.06 to 1.48  $\mu\text{g}\cdot\text{g}^{-1}$  dw and were similar to those reported by Rjeibi and collaborators (2015) in cephalopods from Tunisia (0.01 to 0.95  $\mu\text{g}\cdot\text{g}^{-1}$  dw). They were also comparable to concentrations reported for cephalopods from the Adriatic Sea (*L. vulgaris* from 0.20 to 1.50  $\mu\text{g}\cdot\text{g}^{-1}$  dw, *O. vulgaris* from 0.12 to 1.30  $\mu\text{g}\cdot\text{g}^{-1}$  dw, *S. officinalis* from 0.25 to 4.78  $\mu\text{g}\cdot\text{g}^{-1}$  dw) and the Atlantic Ocean (*S. officinalis* > 1  $\mu\text{g}\cdot\text{g}^{-1}$  dw) (Brambilla et al., 2013; Pereira et al., 2009), which showed that a homogeneous level of contamination occurs between locations.

Heterogeneity in Hg concentrations among tissues (Lischka et al., 2018; Manso et al., 2007) is driven by uptake, detoxification, storage and/or excretion (including MeHg) differences (Penicaud et al., 2017). Hg concentrations were highest in the digestive gland and in mantle muscles in all species considered, except for the curled octopus, *E. cirrhosa*, for which higher Hg concentrations were found in optic lobes (see discussion thereafter). High Hg concentrations in the digestive gland of cephalopods have previously been documented in several species (Bustamante et al., 2006; Manso, 2007; Rjeibi et al., 2014). Mercury, and notably the MeHg within prey, mainly accumulates via food consumption (Lacoue-Labarthe et al., 2009; Chouvelon et al., 2011), which implies that the physiological functions of digestion and absorption of the digestive gland contribute to the high Hg levels found in the tissue (Boucaud-Camou and Yim, 1980; Boucher-Rodoni et al., 1987).

The digestive gland is also known as the main organ of metal detoxification and storage in cephalopods (Martin and Flegal 1975; Miramand and Bentley 1992; Lacoue-Labarthe et al., 2016; Bustamante et al., 2002). Although the digestive gland represents only 6 to 10% of the mass of an animal, the organ may contain more than 80% of the body burden of some metals such as Ag, Cd, and Co (Penicaud et al., 2017). However, Hg in the digestive gland contributes to far less of the total body burden than muscles (Bustamante et al., 2006; Kojadinovic et al., 2011). Concentrations of Hg were in the same order of magnitude in both tissues, but as muscles accounted for more than 70% of the total body weight, and contained 70 to 90% of the total Hg body burden (Bustamante et al., 2006; Seco et al. 2020). This tissue distribution is likely due to two main mechanisms: 1) translocation and binding of MeHg to muscle proteins because they are rich in sulfhydryl groups with a high affinity for Hg, as shown in fish (Bloom, 1992). Consequently, the muscles constitute a good bioindicator tissue of MeHg exposure in cephalopods as like in many organisms, most of the Hg found in the muscular parts is under organic form (Bustamante et al., 2006; Seco et al., 2020; Chételat et al., 2020). Relatively similar Hg concentrations observed in the digestive gland compared and mantle muscles may also result from 2) the demethylation of MeHg, which facilitates iHg elimination by the digestive gland. Indeed, more than 70% of the Hg within the muscles of cephalopods is methylated, but more than a half of the Hg found in their digestive glands is inorganic (Bustamante et al., 2006; Seco et al. 2020). Here, HR-XANES spectrometry speciation analyses revealed that one third of the total Hg within the digestive gland of *E. cirrhosa* was made up of an inorganic form bound to peptides (*e.g.* glutathione, GSH) or proteins as a dicysteinate complex, which was modeled using the Hg(Cys)<sub>2</sub> reference. This suggests that 1) iHg may come from the environment (Sun et al., 2020) through both dissolved and trophic pathways. Indeed, it has been demonstrated that cuttlefish efficiently bioconcentrate dissolved <sup>i</sup><sup>203</sup>Hg, which is then distributed throughout the digestive gland (Lacoue-Labarthe et al., 2009). Thus, this organ contained 20% of accumulated <sup>i</sup><sup>203</sup>Hg after 10 days of exposure. In addition, ~90% of the <sup>i</sup><sup>203</sup>Hg from prey is assimilated

and then mostly (~70%) found in the digestive gland. Alternatively, 2) iHg may result from *in vivo* MeHg demethylation (Sun et al., 2020), which likely occurs in the digestive gland, facilitating the rapid elimination of produced iHg, based on experimental findings in which a short biological half-life (*i.e.* 4 days) of dietary  $^{203}\text{iHg}$  was reported (Lacoue-Labarthe et al., 2009). The biochemical mechanism of demethylation that occurs in the digestive gland has not been determined.

Assuming that cephalopods are exposed to the MeHg form of Hg by prey, remaining MeHg from the pool of ingested Hg entering the bloodstream may become distributed throughout other tissues. Some studies have investigated the binding and transport of Hg(II) to hemocyanin (Brouwer et al., 1982, 1983), but little is known regarding MeHg binding to hemocyanin (blood oxygen transport proteins) in cephalopods. Nevertheless, it can be assumed its transport is facilitated by sulphhydryl or tryptophanyl binding sites of the molecule, which allows MeHg transfer to other tissues (Brouwer et al., 1982).

As mentioned previously, only mercury concentration (*i.e.*  $1.25 \mu\text{g}\cdot\text{g}^{-1} \text{ dw}$ ) has been reported for the brain of the giant squid, *Architeuthis dux*, to our knowledge (Bustamante et al., 2008). In the present study, the Hg concentrations were determined in the optic lobes, which is part of the nervous central system of cephalopods and are responsible for the highest level of coordinated function such as association, learning and visual analysis (Young, 1971). In this tissue, Hg concentrations ranged from 0.03 to  $1.89 \mu\text{g}\cdot\text{g}^{-1} \text{ dw}$  among analysed individuals, which demonstrated that Hg accumulates in this area of the central nervous system. Values were comparable to, even slightly higher than, Hg concentrations reported in fish brains including 0.02 to  $0.80 \mu\text{g}\cdot\text{g}^{-1} \text{ dw}$  in the golden grey mullet, *Liza aurata* (Pereira et al., 2014); 0.10 to  $1.45 \mu\text{g}\cdot\text{g}^{-1} \text{ dw}$  in the bluefish, *Pomatomus saltatrix* (*i.e.* 0.02 to  $0.29 \mu\text{g}\cdot\text{g}^{-1} \text{ ww}$ ; Burger et al., 2013); and 0.10 to  $1.60 \mu\text{g}\cdot\text{g}^{-1} \text{ dw}$  in the seabass, *Dicentrarchus labrax* (*i.e.* 0.02 to  $0.32 \mu\text{g}\cdot\text{g}^{-1} \text{ ww}$ ; Mieiro et al., 2011). In addition to the lack of information regarding



contamination of the central nervous system of cephalopods, such as Hg concentrations in the brain, knowledge regarding Hg neurotoxicity in this Class is also incomplete.

Interestingly, Hg concentrations in the brain are strongly correlated with that of the muscles in different species investigated here, which indicates that Hg concentrations of the optic lobes are not regulated independently from Hg bioaccumulation throughout the organism. In addition,  $R^2$  values ranged from 0.66 to 0.98, depending on the group of species considered (see Fig. 2; Table 3), which indicated that Hg accumulation in the brain may be driven by the Hg concentrations in muscles in accordance with a defined brain/muscle ratio that is determined for each species. *L. vulgaris* and *I. coindetii* had a brain/muscle ratio of 0.35, *S. officinalis* and *T. eblanae* had a brain/muscle ratio of 0.50, and *E. cirrhosa* was the only species that displayed a ratio greater than 1 (about 1.3) as a result of elevated Hg concentrations in the brain than mantle (Fig. 2). A positive correlation between Hg concentrations in the brain and muscles has been reported previously in eight species of catfish (*Brachyplatystoma filamentosum*; *B. platynemum*; *B. rousseauxii*; *B. vaillantii*; *Phractocephalus hemiliopterus*; *Pseudoplatystoma punctifer*; *P. tigrinum*; *Zungaro zungaro*) ( $R^2 = 0.917$ ;  $p < 0.0001$ ; Bastos et al., 2015). More importantly, the brain/muscle ratio never exceeded 0.6 when 21 fish species whose Hg concentrations in muscles ranged from 0.25 to 20.50  $\mu\text{g}\cdot\text{g}^{-1}$  dw were examined (*i.e.* 0.05 to 4.10  $\mu\text{g}\cdot\text{g}^{-1}$  ww; Bastos et al., 2015). Similarly, the brain/muscle Hg ratio (0.054/0.274  $\mu\text{g}\cdot\text{g}^{-1}$  ww) did not exceeded 0.2 in yellow perch, *Perca flavescens* (Khadra et al., 2019). Furthermore, ratios lower than 1 are also common in birds. In the following species, ratios less than one were determined, as follows: 0.43 for the semipalmated sandpiper, *Calidris pusilla* (Burger et al., 2014); 0.60 for the Atlantic puffin, *Fratercula arctica*; 0.68 for the common guillemot, *Uria aalge*; and 0.55 for the black-legged kittiwake, *Rissa tridactyla* (Fort et al., 2015). Thus, ratios observed for the squids and cuttlefish in our study are in accordance with previously reported data and suggest that Hg accumulation in the brain is limited compared to muscle tissue in cephalopods.

Interestingly, the most contaminated species among the cephalopod species panel investigated was in *E. cirrhosa*, which had a brain/muscle Hg ratio of 1.3. This finding suggested that Hg brain-tropism is most significant in *E. cirrhosa*. To the best of our knowledge, a similar ratio not been described in fish, except blackchin tilapia, *Sarotherodon melanotheron*, which had a brain/muscle Hg ratio of 1.5 (Hg concentrations were of  $1.25 \mu\text{g}\cdot\text{g}^{-1}$  dw (*i.e.*  $0.25 \mu\text{g}\cdot\text{g}^{-1}$  ww) in the brain and  $0.85 \mu\text{g}\cdot\text{g}^{-1}$  dw (*i.e.*  $0.17 \mu\text{g}\cdot\text{g}^{-1}$  ww) in muscle (Coulibaly et al., 2012). Similar to seabirds, the razorbill, *Alca torda*, had a Hg brain/muscle ratio close to 1 (*i.e.*  $2.05$  and  $2.04 \mu\text{g}\cdot\text{g}^{-1}$  dw, respectively). Finally, the highest ratios have been determined humans, and have reached 3.6 (*i.e.*  $0.090$  and  $0.025 \mu\text{g}\cdot\text{g}^{-1}$  dw in the occipital cortex and abdominal muscles, respectively; Björkman et al., 2007). Despite these observations, no clear explanation has been provided to enhance our understanding of how Hg accumulation preferentially occurs in the brain versus muscles in these species.

In addition, our results revealed that Hg accumulation in the brain with respect to muscles Hg does not depend on contamination level. In other words, the brain/muscle ratio did not increase as the range of Hg muscle concentrations measured for each species increased. Indeed, with the exception of two individuals that were highly contaminated ( $> 1.35 \mu\text{g}\cdot\text{g}^{-1}$  dw), mantle muscle Hg concentrations in *E. cirrhosa* specimens ranged from  $0.24$  to  $0.57 \mu\text{g}\cdot\text{g}^{-1}$  dw, which was similar to the range recorded in *S. officinalis* specimens ( $0.18$ – $0.75 \mu\text{g}\cdot\text{g}^{-1}$  dw). In fish, species reported to contaminated throughout a large range of Hg concentrations (*e.g.* from  $0.25$  to  $20.50 \mu\text{g}\cdot\text{g}^{-1}$  dw *i.e.*  $0.05$  to  $4.10 \mu\text{g}\cdot\text{g}^{-1}$  ww from Bastos et al., 2015) had low brain/muscle ratios that were similar to values determined for cephalopods, with the exception of *E. cirrhosa*. Consequently, Hg accumulation in brain of the octopus may have a species-specific explanation. First, Hg concentrations in the musculature were measured in mantle muscles to facilitate comparison between squids and cuttlefish. However, the majority of muscle mass in octopus species is found in their arms. Not surprisingly, Hg concentrations do not differ between both muscle types (Seixas et al., 2005). This finding indicates the brain/muscle ratio was likely not overestimated as a result of the

analysis of mantle muscles in this study. Further, fostered Hg accumulation in the octopus brain may result from a more permeable tissue to metal intake. Cephalopods have linker junctions between glial cells, which form a blood-brain barrier (BBB) that surrounds the central nervous system (Abbott et al., 1992). Once in the bloodstream, transported MeHg is transferred across the BBB and is capable of reaching the brain thanks to its lipophilicity (Boening, 2000; Holmes et al., 2009). The BBB structure and function are known to vary among different taxa (O'Brown et al. 2018) and may have permeabilities with respect to Hg and/or MeHg (Aschner and Aschner, 1990). However, no findings have indicated that BBB structures differ among coleoid species that would explain the elevated Hg concentrations observed in the optic lobes of *E. cirrhosa*.

Alternatively, elevated levels of Hg accumulation in the brain that result from increased Hg exposure likely are a result of the tight binding between the metal and chemical components of optic lobes. Despite of a lack of comparative data and knowledge, HR-XANES spectrometry analyses performed using optic lobes of *E. cirrhosa* revealed (1) that Hg was entirely methylated, as in mantle muscle in *E. cirrhosa* (Mieiro et al., 2011), and (2) that MeHg is complexed to thiol groups. Importantly, Hg detoxification via with selenium (Se) association can be excluded because no Hg bond signature to Se was observed via HR-XANES spectrometry in the tissues of *E. cirrhosa*. Thus, cysteine residues from peptides (*e.g.* glutathione) or proteins (*e.g.* metallothionein) are the most likely candidate ligands, since they are by far the most predominant biological thiols (Manceau et al., 2019). Nevertheless, it should be noted that it was not possible to determine whether another form of MeHg association occurred in other cephalopod species, since brain concentrations in other species assessed were too low to examine via XANES spectrometry.

### **Conclusions and perspectives**

The present study provided information regarding the bioaccumulation and tissue distribution of Hg in five cephalopod species (*E. cirrhosa*, *S. officinalis*, *L. vulgaris*, *T. eblanae* and *I. coindetii*) from the

Bay of Biscay and has provided new information regarding Hg concentrations and speciation of the digestive gland, muscles and central nervous system. Results showed that efficient Hg accumulation occurred in the optic lobes of cephalopods, which was strongly correlated with Hg concentrations measured of the mantle. The present study also pointed out interspecific differences in Hg concentrations among the five studied species and the unique pattern of Hg bioaccumulation in the brain of *E. cirrhosa*.

The collection of data regarding Hg concentrations and speciation in this specific tissue is essential for evaluating Hg exposure risk in cephalopods, which are characterised by the presence of centralised nervous systems, their high cognitive capabilities, and their sophisticated behaviour. While many studies have demonstrated that MeHg impairs motor function, senses, communication (e.g. Basu et al., 2005; Zahir et al., 2005; Puga et al., 2016), and induces oxidative stress responses (Mieiro et al., 2011), no information has been reported regarding Hg neurotoxicity in cephalopods to date. Since Hg concentrations of some *E. cirrhosa* specimens (i.e.  $1.9 \mu\text{g}\cdot\text{g}^{-1}$  dw) were 3 times above the neurotoxic threshold found for the white seabream, *D. sargus* (Pereira et al., 2016), further research will be needed to investigate how unique features of species may explain the high Hg levels recorded in octopodidae, and more generally, how Hg contamination in cephalopods may affect their functional brain plasticity, behaviour and fitness (Liscovitch-Brauer et al., 2017; Di Cosmo et al., 2018).

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Table 1. Mantle size range and mean ( $\pm$  sd in cm), mantle weight range and mean ( $\pm$  sd in g) and total Hg concentrations (mean  $\pm$  sd in  $\mu\text{g}\cdot\text{g}^{-1}$  dw) in mantle muscles, digestive gland and optic lobes of *Eledone cirrhosa* (n=20), *Illex coindetii* (n=11), *Todaropsis eblanae* (n=9), *Loligo vulgaris* (n=20), *Sepia officinalis* (n=21).

| Tissues<br>Species        | Family         | Mantle size range (cm) | Mantle size mean $\pm$ sd (cm) | Weight range (g) | Weight mean $\pm$ sd (g) | [Hg] mantle muscles | [Hg] digestive gland | [Hg] optic lobes |
|---------------------------|----------------|------------------------|--------------------------------|------------------|--------------------------|---------------------|----------------------|------------------|
| <i>Eledone cirrhosa</i>   | Octopodidae    | 4.2 – 14.9             | 8.3 $\pm$ 2.4                  | 19 -764          | 197 $\pm$ 177            | 0.52 $\pm$ 0.35     | 0.47 $\pm$ 0.34      | 0.60 $\pm$ 0.47  |
| <i>Illex coindetii</i>    | Ommastrephidae | 14.3 - 18.0            | 15.5 $\pm$ 1.4                 | 62 - 131         | 85 $\pm$ 19              | 0.08 $\pm$ 0.01     | 0.12 $\pm$ 0.02      | 0.04 $\pm$ 0.01  |
| <i>Todaropsis eblanae</i> | Ommastrephidae | 7.8 – 13.0             | 10.6 $\pm$ 1.8                 | 43 - 167         | 105 $\pm$ 46             | 0.29 $\pm$ 0.15     | 0.24 $\pm$ 0.06      | 0.18 $\pm$ 0.07  |
| <i>Loligo vulgaris</i>    | Loliginidae    | 13.2 - 19.0            | 16.6 $\pm$ 1.8                 | 83 - 198         | 139 $\pm$ 34             | 0.16 $\pm$ 0.03     | 0.29 $\pm$ 0.06      | 0.09 $\pm$ 0.02  |
| <i>Sepia officinalis</i>  | Sepiidae       | 12.5 – 17.0            | 14.6 $\pm$ 1.2                 | 240 - 544        | 397 $\pm$ 105            | 0.46 $\pm$ 0.24     | 0.43 $\pm$ 0.12      | 0.23 $\pm$ 0.09  |

Table 2. AICc model ranking for log Hg concentrations within the five studied cephalopod species from Bay of Biscay. Models are GLMs with a gaussian distribution and an identity link function. Abbreviations: AICc, Akaike's Information Criteria adjusted for small sample-sizes values;  $w_i$ , AICc weights.

| Models                | N° parameters | AIC <sub>c</sub> | $\Delta$ AIC <sub>c</sub> <sup>a</sup> | $w_i$ |
|-----------------------|---------------|------------------|--|-------|
| Cephalopods (n = 240) |               |                  |  |       |
| Tissue * Species      | 16            | 225              | 0                                      | 1     |
| Tissue + Species      | 8             | 276              | 51                                     | 0     |
| Species               | 6             | 330              | 105                                    | 0     |
| Tissue                | 4             | 499              | 274                                    | 0     |
| Null                  | 2             | 519              | 294                                    | 0     |

<sup>a</sup> Scaled  $\Delta$ AICc;  $\Delta$ AICc = 0.00 is interpreted as the best fit to the data among the models.

Table 3. Parameters of the linear relationships between Hg concentrations in the optic lobes [Hg]<sub>ol</sub> and the mantle muscles [Hg]<sub>m</sub>. When the slopes between species were not significantly different, only one model was fitted within pooled data. See Figure 2

| Species                                   | Model Equation  | P value     | R <sup>2</sup> |
|---|---|-------------|----------------|
| <i>E. cirrhosa</i>                        | $[\text{Hg}]_{\text{ol}} = 1.32 \times [\text{Hg}]_{\text{m}} - 0.09$ | $p < 0.001$ | 0.978          |
| <i>S. officinalis</i> – <i>T. eblanae</i> | $[\text{Hg}]_{\text{ol}} = 0.35 \times [\text{Hg}]_{\text{m}} + 0.08$ | $p < 0.001$ | 0.829          |
| <i>I. coindetii</i> – <i>L. vulgaris</i>  | $[\text{Hg}]_{\text{ol}} = 0.50 \times [\text{Hg}]_{\text{m}} + 0.01$ | $p < 0.001$ | 0.663          |

Figure

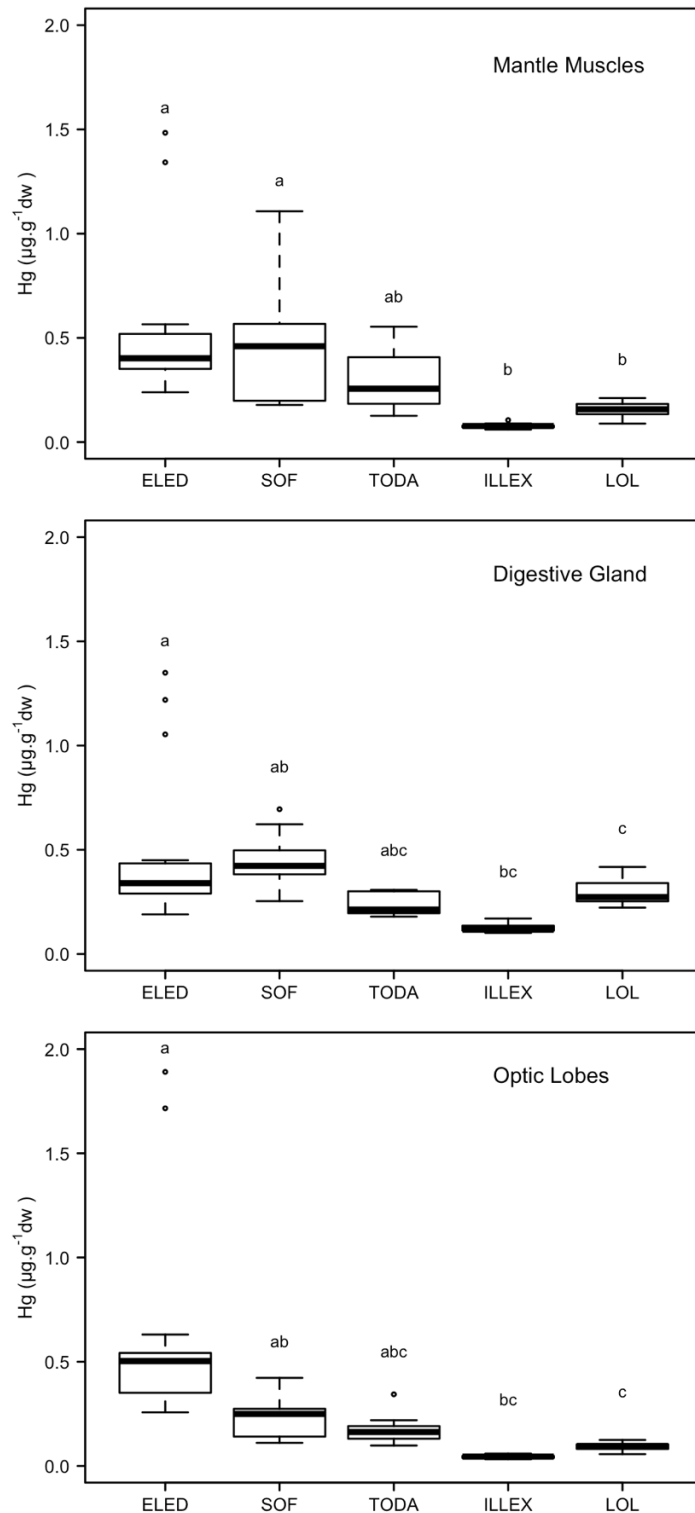


Figure 1. Hg concentrations ( $\mu\text{g.g}^{-1}\text{dw}$ ) in the mantle muscles, digestive gland, and the optic lobes of the five studied cephalopods species: ELED : *Eledone cirrhosa* (n=20); ILLEX : *Illex coindetii* (n=11); LOL : *Loligo vulgaris* (n=20); SOF : *Sepia officinalis* (n=21); TODA : *Todaropsis eblanae* (n=9). Statistical differences are denoted by different letters when significant ( $p\text{-value} < 0.05$ ).

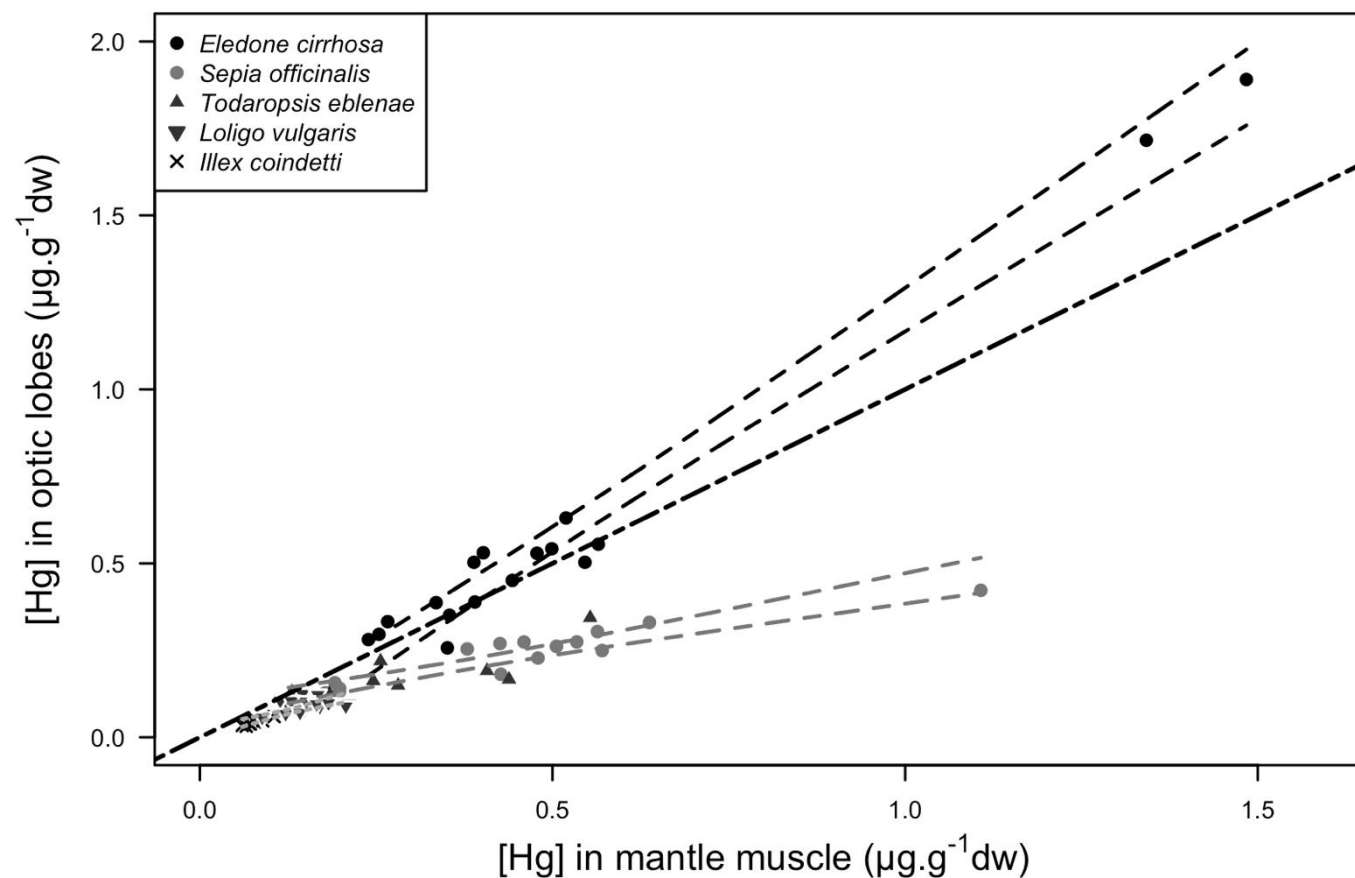


Figure 2. Hg concentrations ( $\mu\text{g}\cdot\text{g}^{-1}\text{dw}$ ) In optic lobes function of Hg concentration in mantle muscle of the five cephalopods species: *Eledone cirrhosa* (black points), *Sepia officinalis* (grey points), *Todaropsis eblanae* (black triangles), *Loligo vulgaris* (inversed black triangles), *Illex coindetii* (black crosses). Linear models for *E. cirrhosa*, *S. officinalis* and *T. eblanae*, and *I. coindetii* and *L. vulgaris* are represented by the 95% confidence intervals (black, grey and light grey curves, respectively). The black dashed-dotted line is the bisector of the plan. See parameters of the linear models on the Table 3.

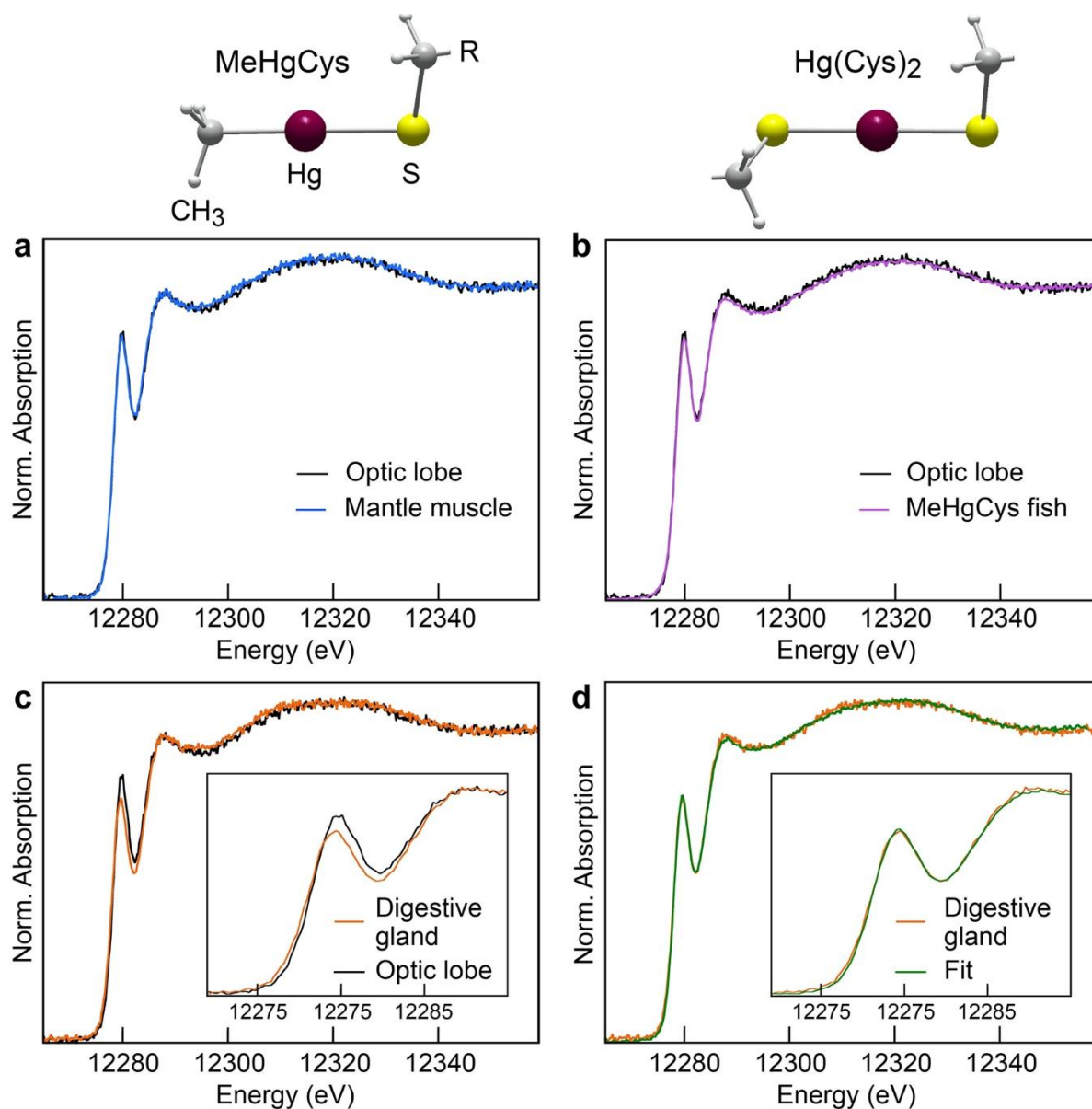


Figure 3. Chemical forms of Hg determined by Hg  $L_3$ -edge HR-XANES spectroscopy and ball-and-stick representation of the MeHgCys and Hg(Cys)<sub>2</sub> complexes. a-c) Comparison of the spectrum for the optic lobes with the spectra (a) for the mantle muscle, (b) for MeHgCys in fish muscle, and (c) for the digestive gland. d) Linear combination fit of the spectrum for the digestive gland with  $68 \pm 11\%$  of the optic lobes spectrum and  $33 \pm 11\%$  of the dicysteinate complex Hg(Cys)<sub>2</sub>.  $NSS = 1.50 \times 10^{-4}$ .