



## Original Article

# Species- and size-related patterns in stable isotopes and mercury concentrations in fish help refine marine ecosystem indicators and provide evidence for distinct management units for hake in the Northeast Atlantic

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Chouvelon, T., Caurant, F., Cherel, Y., Simon-Bouhet, B., Spitz, J., and Bustamante, P. 2014. Species- and size-related patterns in stable isotopes and mercury concentrations in fish help refine marine ecosystem indicators and provide evidence for distinct management units for hake in the Northeast Atlantic. – ICES Journal of Marine Science, 71: 1073–1087.

Received 11 July 2013; accepted 25 October 2013; advance access publication 2 January 2014.

Recent European environmental policies argue for the development of indicators of the ecological status of ecosystems that are easy to implement and powerful enough to detect changes quickly. For instance, some indicators that are currently proposed for monitoring foodweb structure and functioning are based on the size of organisms, using size as a proxy for trophic level. However, these indicators do not necessarily accurately reflect the underlying trophic structure and dynamics to follow. Ecological tracers (i.e. chemical parameters measured in consumer tissues to infer the trophic ecology of organisms) may serve as complementary indicators of trophic level, and may also help distinguish different populations of a species when more commonly used methods (e.g. genetic, age determination) present their own limitations. Here, we analysed the potential of muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and of muscle mercury (Hg) concentrations to depict size-related trophic habits of different fish species. We expected that intra- and interspecific variation in these ecological tracers could be helpful in refining currently proposed indicators of marine ecosystems, and also help in discriminating management units for some species. Four fish species were selected for their economical and/or ecological importance in the Bay of Biscay (Northeast Atlantic): whiting *Merlangius merlangus*, European hake *Merluccius merluccius*, Atlantic horse mackerel *Trachurus trachurus* and anglerfish *Lophius piscatorius*. Muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values segregated the species and enabled us to discriminate species-specific feeding strategies with increasing size of individuals. Fish body size was not always linearly correlated with  $\delta^{15}\text{N}$  or trophic level. In contrast, Hg concentrations and size-related Hg patterns were more similar from species to species. Interestingly, muscle  $\delta^{15}\text{N}$  values together with Hg concentrations segregated the two putative stocks of European hake within the Bay of Biscay. Hence, we propose the combined use of ecological tracers as a promising method for accurately assigning a consumer to a trophic guild, investigating Hg trophodynamics in foodwebs, and helping discriminate distinct ecological populations within a species when defining management units.

## Introduction

Trophic linkages between organisms take a central place in the general structure and functioning of ecosystems (e.g. Lindeman, 1942; Odum, 1969; Cury *et al.*, 2003). Recently, the European Union has adopted the Marine Strategy Framework Directive

(MSFD) that aims at implementing an ecosystem-based management of European marine ecosystems (European Commission, 2008). The descriptor “Foodwebs” is one of the 11 qualitative descriptors proposed to define and to monitor the “good environmental status” of these ecosystems. Within this descriptor, the

three following indicators are proposed: (i) productivity/performance of key predator species using their productivity per unit biomass; (ii) proportion of large fish (by mass) or Large Fish Indicator (LFI); (iii) abundance trends of functionally important selected groups/species (European Commission, 2010; Rombouts *et al.*, 2013). However, the relevance of these indicators is discussed for some geographical areas and countries affected by the MSFD (e.g. for the Bay of Biscay area in the Northeast Atlantic, see Borja *et al.*, 2011; more generally, see Rombouts *et al.*, 2013). They effectively present significant general limitations to their applicability for depicting the ecological status of foodwebs. For instance, the LFI [indicator (ii)] is based on the common assumption that fish body size is related to fish trophic level, so that changes in the size-spectrum of a fish community should reflect changes in its trophic structure. The LFI was primarily developed to assess the effect of fishing on the fish community (e.g. Shin *et al.*, 2005; Greenstreet *et al.*, 2011), as many fisheries target the largest fish considered as *a priori* highest trophic level organisms within a given ecosystem. However, fish body size is not always well correlated with trophic level (e.g. Jennings *et al.*, 2001, 2002). This limits the use of fish size as a proxy for fish trophic level and consequently the use of the LFI as a reliable indicator to assess foodweb status over (Rombouts *et al.*, 2013).

In recent decades, stable isotope analysis (SIA) of carbon ( $\delta^{13}\text{C}$ ) and nitrogen ( $\delta^{15}\text{N}$ ) in consumers' tissues has proven to be a powerful tool for describing the trophic ecology of organisms (e.g. Kelly, 2000; Bearhop *et al.*, 2004; Michener and Kaufman, 2007). Because the difference in  $\delta^{13}\text{C}$  between a source and its consumer (i.e. between two trophic levels) is generally low ( $\leq 1\%$ ; De Niro and Epstein, 1978; Post 2002),  $\delta^{13}\text{C}$  values have often been considered to be a powerful tracer of the primary producer at the base of the foodweb supporting consumers, and consequently as a tracer of their foraging habitat (e.g. France, 1995; Hobson, 1999; Chérel and Hobson, 2007). Alternatively, consumers generally present 2.5–5‰ higher  $\delta^{15}\text{N}$  values relative to their sources (De Niro and Epstein, 1981; Vanderklift and Ponsard, 2003), so  $\delta^{15}\text{N}$  values have been widely used as a proxy for their trophic position or to calculate their trophic level (e.g. Vander Zanden *et al.*, 1997; Post, 2002; Chérel *et al.*, 2010; Navarro *et al.*, 2011). Temporal changes in  $\delta^{15}\text{N}$  values (or derived-trophic levels) have then already been used as indicators of the responses of biological communities to environmental changes (e.g. for the North Sea fish community under fishing pressure, based on the same assumption as for the LFI indicator commented above; Jennings *et al.*, 2002; Nicholson and Jennings, 2004; Greenstreet and Rogers, 2006). The isotopic compositions of organisms (notably  $\delta^{15}\text{N}$  values) have also proved to be powerful tools for understanding pollutants bioaccumulation and pathways throughout foodwebs (e.g. Cabana and Rasmussen, 1994). Naturally occurring stable isotopes have even been able to disentangle individuals' trophic positions, feeding habits and foraging habitats exploited for explaining trace metal concentrations such as mercury (Hg) in top predators (e.g. Cossa *et al.*, 2012; Ramos *et al.*, 2013).

Hg is a non-essential metal released into the environment from both natural and anthropogenic sources, reaching the ocean through river inputs and atmospheric depositions (Fitzgerald *et al.*, 2007). Trophic transfer is then the main pathway for the intake of Hg by marine organisms, bioaccumulating in higher trophic level consumers (Eisler, 1987; Boening, 2000; Fitzgerald *et al.*, 2007). As for  $\delta^{15}\text{N}$  values, Hg may potentially be another good tracer of the trophic level of aquatic organisms (e.g. Cabana

and Rasmussen, 1994), given its proven biomagnification within foodwebs (Gray, 2002). Due to methylation processes by microorganisms in marine sediments on the shelf and in the low oxygen zone of oceanic waters (Bacci, 1989; Fitzgerald *et al.*, 2007), enhanced Hg concentrations in biota from meso-pelagic and deep-water environments have been already highlighted in several ecosystems (Monteiro *et al.*, 1996; Thompson *et al.*, 1998; Choy *et al.*, 2009; Chauvelon *et al.*, 2012a). However, the age of organisms (or their size, as a proxy of age) remains a major factor driving Hg bioaccumulation, principally in its methylated form (Fitzgerald *et al.*, 2007) in the muscle tissue of numerous marine organisms (e.g. Monteiro and Lopes, 1990; Cronin *et al.*, 1998; Bustamante *et al.*, 2006).

Finally, approaches using ecological tracers (e.g. stable isotopes, trace metals, or fatty acid analysis) can provide information on the diet of individuals on different time-scales, depending on the tracer and its turnover in the analysed tissue. Therefore, in addition to segregating species of different trophic ecology, the combination of these approaches may be used to distinguish a different use of resources and/or habitats by the different populations of the same species, and help then in defining management units. Indeed, management units defined by the ecological tracers or other tools may be more appropriate for response to actual management issues rather than those defined by genetic differences alone, which often provide a picture on a broader spatio-temporal scale (e.g. Taylor and Dizon, 1999; Cope and Punt, 2009). When compared with genetic data, other tools providing biological or ecological information at the individual scale (e.g. tagging/mark-recapture data) can effectively lead to questioning of the validity of the stock assessment units used by the ICES study groups concerning exploited fish species (e.g. Fritsch *et al.*, 2007; Pawson *et al.*, 2007 for the European sea bass *Dicentrarchus labrax* in the Northeast Atlantic). For the European hake *Merluccius merluccius* in the Northeast Atlantic in particular, the ICES recognizes the existence of two putative stocks since the 1970s: a northern stock extending from the northern Bay of Biscay to the west of Norway, and a southern stock from the southern Bay of Biscay to the Iberian Peninsula (ICES, 2008; Murua, 2010). However, there is still no clear biological (e.g. Piñeiro and Sainza, 2003) or genetic evidence (Roldan *et al.*, 1998; Castillo *et al.*, 2004; Cimmaruta *et al.*, 2005) that the two are distinct populations, and more generally the knowledge of the biology and ecology of the European hake in the Northeast Atlantic is still quite limited (Murua, 2010).

The main objectives of this study were thus threefold: (i) to assess size-related feeding strategies through SIA in four different fish species of economical and ecological importance in the Northeast Atlantic (whiting *Merlangius merlangus*, European hake, Atlantic horse mackerel *Trachurus trachurus* and anglerfish *Lophius piscatorius*); (ii) to evaluate whether body size, trophic position or diet and foraging habitat can explain size-related variations in  $\delta^{15}\text{N}$  values and total-Hg (T-Hg) concentrations in fish muscle tissue; and (iii) to evaluate the potential of the combined use of SIA and Hg concentrations to help refine current indicators of marine foodwebs and ecosystems and their contribution to the definition of management units for hake in the Northeast Atlantic. Consequently, our three hypothesis were primarily that: (i) fish body size should be related to fish trophic level, although the relationship between both variables may be not always strictly linear; (ii) bioaccumulation of Hg in fish should be driven by size (as a proxy for age), the trophic level of individuals and their foraging habitat; and (iii) the combined analysis of stable isotopes and Hg concentrations can

contribute to elucidating the existence of two putative stocks of hake in the Bay of Biscay.

## Material and methods

### Selected species

Four fish species were selected according to their phylogeny (i.e. different families), habitat (i.e. coastal vs. offshore area) and vertical distribution in the water column (i.e. pelagic vs. benthic species): whiting and European hake (both belonging to the Gadiform order), Atlantic horse mackerel, and anglerfish. (See species' characteristics summarized in Table 1.) Commercial fisheries target these species in the Northeast Atlantic, notably in the Bay of Biscay (Palomares and Pauly, 2010; Lassalle *et al.*, 2011). They also present different diets, with food items ranging from small planktonic crustaceans to small pelagic or benthic-pelagic fish, and have dietary shifts associated with growth (i.e. juvenile vs. adult fish). Information on the main prey species consumed during ontogenesis by the four selected fishes is detailed in Table 1.

### Sampling

A total of 255 individuals covering a large range in size for each of the four species were collected during the annual EVHOE groundfish survey, conducted by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), from the continental shelf to the shelf edge of the Bay of Biscay in the autumns of 2008 and 2009 (Figure 1, Tables 2 and 3). On board, the total length (TL) of each organism was measured, and a piece of dorsal white muscle (without skin) was removed for stable isotope and Hg analyses. Samples were immediately placed in individual plastic bags and frozen at  $-20^{\circ}\text{C}$ . They were then freeze-dried, ground into a fine powder and stored in individual plastic vials until analysis.

### Isotopic and mercury analyses

Because lipids are depleted in  $^{13}\text{C}$  relative to other tissue components (De Niro and Epstein, 1977), they were extracted from muscle samples using cyclohexane. Most solvents (e.g. a chloroform–methanol mixture) that are currently used for lipid extraction generally affect  $\delta^{15}\text{N}$  values in an unpredictable way (e.g. Post *et al.*, 2007 and references therein). As an alternative, cyclohexane is a non-chlorinated solvent with low toxicity that can rapidly extract lipids from the tissues of marine organisms (Smedes, 1999). Moreover, internal laboratory experiments investigating a range of marine taxa (including fish) have proven that cyclohexane significantly corrects  $\delta^{13}\text{C}$  values for the lipid effect in originally fatty muscle samples only (i.e. samples with bulk C:N ratio  $> 3.5$ ; Post *et al.*, 2007), without affecting  $\delta^{15}\text{N}$  values of any samples (Chouvelon *et al.*, unpublished data). For each sample, an aliquot of  $\sim 100$  mg of fine powder was thus agitated with 4 ml of cyclohexane for 1 h. The samples were then centrifuged for 5 min at 4000g, and the supernatant containing lipids was discarded. The samples were finally dried in an oven at  $45^{\circ}\text{C}$  for 48 h, and subsamples of lipid-free powder were weighed in tin cups ( $0.40 \pm 0.05$  mg). SIAs were performed with a Thermo Scientific Flash EA1112 elemental analyser coupled to a Thermo Scientific Delta V Advantage mass spectrometer (CF IR-MS). The results are presented in the usual  $\delta$  notation relative to the deviation from standards (Vienna Pee Dee Belemnite for  $\delta^{13}\text{C}$ , and atmospheric nitrogen for  $\delta^{15}\text{N}$ ), in parts per thousand (‰). Replicate measurements of internal laboratory

standards (acetanilide) indicate measurement errors of  $< 0.15\text{‰}$  for both  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values.

T-Hg analyses were carried out with an Advanced Mercury Analyser (ALTEC AMA 254) on at least two homogenized dry muscle tissue subsamples (untreated/non-lipid-extracted powder) for each individual. For Hg determination, the metal was evaporated by progressive heating up to  $800^{\circ}\text{C}$ , then held under an oxygen atmosphere for 3 min, and finally amalgamated on a gold net. Afterwards, the net was heated to liberate the collected Hg, which was finally measured by atomic absorption spectrophotometry. Hg analyses were run according to a thorough quality control program including the analysis of a certified reference material (CRM) TORT-2 (lobster hepatopancreas; National Research Council, Canada). CRM aliquots were treated and analysed under the same conditions as the samples. CRM results were in good agreement with the certified values, with an average recovery rate of 95%. The detection limit was  $5 \text{ ng}\cdot\text{g}^{-1}$  dry mass (dm). Hg concentrations in tissues are expressed in  $\text{ng}\cdot\text{g}^{-1}$  dm.

### Data treatment

The variability of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values measured within each species (and consequently, the isotopic niche width of each species investigated) (Newsome *et al.*, 2007) was first described in terms of the species-specific areas on a  $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$  biplot that includes all individual values (Figure 2). The species trophic niche width was estimated by: (i) the total area (TA), which is a measure of the area of the polygon delineated by the line drawn through the most extreme datapoints of a species in the  $\delta^{15}\text{N}$ – $\delta^{13}\text{C}$  space (Layman *et al.*, 2007); and (ii) a sample size-corrected version of the Bayesian estimate of the standard ellipse area (SEAc) proposed by Jackson *et al.* (2011), as an alternative to the TA metric that tends to increase with sample size. The variability of Hg concentrations measured within each species was represented on a boxplot (Figure 3). Since data did not satisfy conditions for the use of parametric statistics (i.e. normality checked by Shapiro–Wilks tests, and homogeneity of variances checked by Bartlett tests), Kruskal–Wallis (KW) tests followed by *post hoc* multiple comparison tests with Holm's adjustment method were performed to test general interspecific differences in muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values and Hg concentrations.

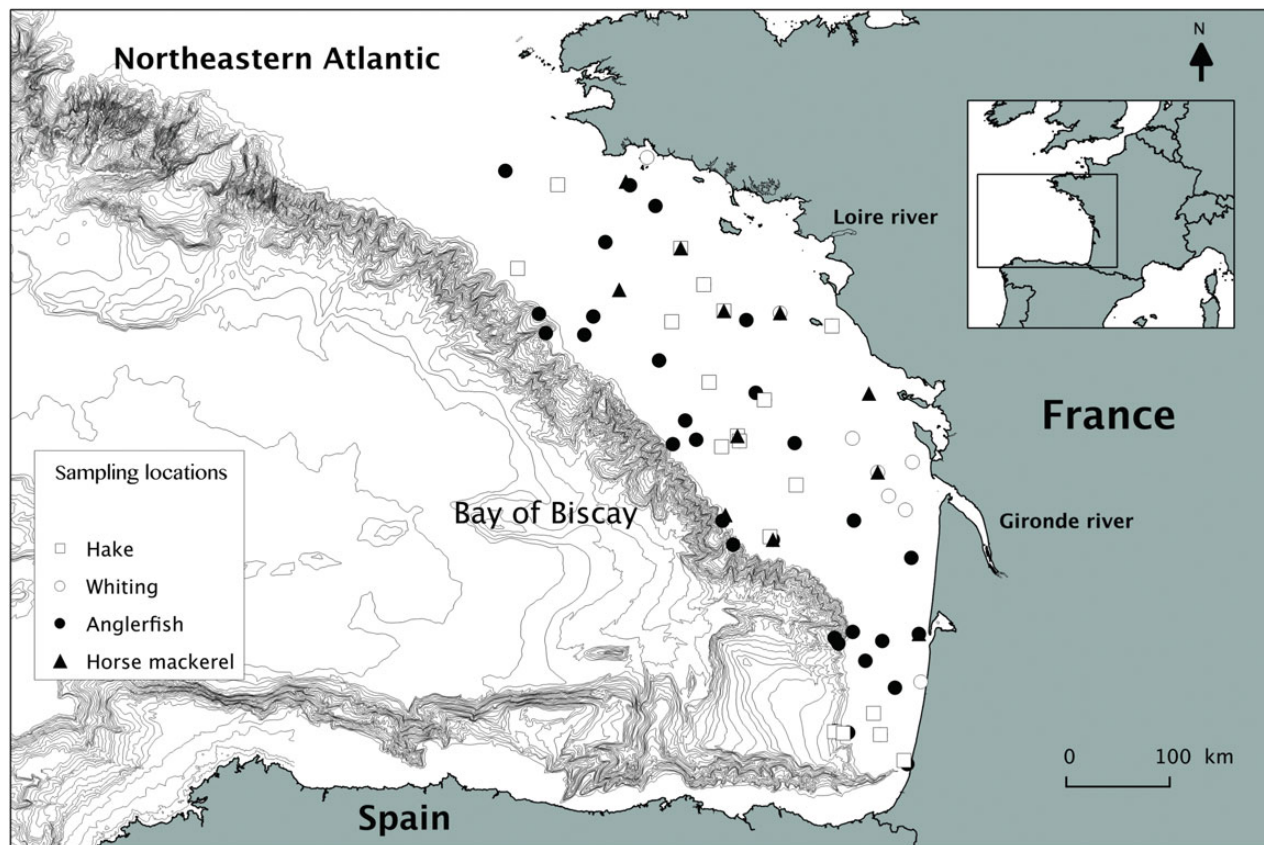
To identify size-related, spatial and/or year-related trends for explaining  $\delta^{15}\text{N}$  variability, Gaussian Generalized Additive Models (GGAMs) were fitted to individual  $\delta^{15}\text{N}$  values (Zuur *et al.*, 2007) using the mgcv package in R (R Development Core Team, 2011). GAMs are non-parametric generalizations of multiple linear regressions; they are particularly helpful in capturing and modelling non-linear relationships (Zuur, 2012). TL of individuals was considered as a continuous explanatory variable in the models, as well as  $\delta^{13}\text{C}$  values. In the Bay of Biscay,  $\delta^{13}\text{C}$  values (and  $\delta^{15}\text{N}$  values) integrate information about the habitat of individuals (i.e. neritic vs. oceanic domain) as they decrease significantly in organisms found further offshore (Chouvelon *et al.*, 2012b; Nerot *et al.*, 2012). Major river plumes of the Bay of Biscay also influence isotopic signatures of the more coastal species (Chouvelon *et al.*, 2012b). Sampling locations of organisms were thus split into two latitudinal zones, the northern area (*a priori* influence of the Loire river plume) vs. southern area (*a priori* influence of the Gironde river plume), as generally defined during EVHOE surveys. The latitudinal zone (LZ) of sampling of individuals was then treated as a possible categorical explanatory variable and added as an interaction term when appropriate (i.e. for the species whose sampling was balanced enough for

**Table 1.** Characteristics of the four fish species from the Bay of Biscay selected for this study (phylogeny, distribution and main dietary items during ontogenesis from literature data—mainly stomach contents data).

Order and family	Species	Common name	Usual depth range <sup>a</sup>	Habitat <sup>a</sup>	General distribution in the water column <sup>a</sup>	Main prey species and feeding habits in the Northeast Atlantic <sup>b</sup>	
						Juveniles	Adults
Gadiform							
Gadidae	<i>Merlangius merlangus</i>	Whiting	0–100 m	Coastal/Shelf	Bentho-pelagic	Small crustaceans such as mysids or the shrimps <i>Crangon</i> sp.	Bentho-pelagic fish such as the Norway pout <i>Trisopterus esmarkii</i> , or pelagic fish such as the sandeel <i>Ammodytes marinus</i> , the sprat <i>Sprattus sprattus</i> and the herring <i>Clupea harengus</i> . Prey size and the proportion of fish in the diet increase with increasing whiting size.
Merlucciidae	<i>Merluccius merluccius</i>	European hake	0–400 m	Shelf/Upper slope	Bentho-pelagic	Crustaceans (mainly euphausiids)	Small pelagic fish such as the horse mackerel <i>Trachurus trachurus</i> , the anchovy <i>Engraulis encrasicolus</i> and the sardine <i>Sardina pilchardus</i> , or the herring <i>C. harengus</i> ; also the bentho-pelagic blue whiting <i>Micromesistius poutassou</i> in larger individuals. Fully piscivorous once adult, with prey size increasing with increasing hake size.
Perciform							
Carangidae	<i>Trachurus trachurus</i>	Atlantic horse mackerel	0–200 m	Coastal/Shelf	Pelagic	Planktonic crustaceans such as copepods and euphausiids	Planktonic crustaceans such as copepods and euphausiids, also small fish (<10 cm) in larger individuals (>30 cm), such as gobiids or small individuals of <i>E. encrasicolus</i> , <i>Gadiculus argenteus</i> and <i>M. poutassou</i> from the shelf edge. Prey size increasing with increasing horse mackerel size.
Lophiiform							
Lophiidae	<i>Lophius piscatorius</i>	Anglerfish	0–1000 m	Shelf/Upper slope	Bentho-pelagic/ Benthic	(not documented)	Variety of prey, ranging from bentho-pelagic fish such as <i>Trisopterus</i> sp., or even pelagic fish such as the sandeel <i>A. marinus</i> , but also cephalopods and large crustaceans such as the lobster <i>Nephrops norvegicus</i> .

<sup>a</sup>References: Quéro, 2003; Trenkel *et al.*, 2009; Palomares and Pauly, 2010.

<sup>b</sup>References: whiting (Moore and Moore, 1976; Hislop *et al.*, 1991; Greenstreet *et al.*, 1998; Pedersen, 1999), European hake (Guichet 1995; Velasco and Olaso, 1998; Mahe *et al.*, 2007), Atlantic horse mackerel (Macer, 1977) and anglerfish (Crozier, 1985; Olaso *et al.*, 1999; Velasco *et al.*, 2001; Cabral and Murta, 2002; Le Loc'h, 2004; Laurenson and Priede, 2005).



**Figure 1.** Map of sampling locations within the Bay of Biscay for whiting *Merlangius merlangus*, European hake *Merluccius merluccius*, Atlantic horse mackerel *Trachurus trachurus* and anglerfish *Lophius piscatorius*. The grey lines are isobaths corresponding to the shelf break (200 m depth and beyond), separating therefore the continental shelf area (where most of the sampling occurred) and deep-water areas. The Loire and Gironde rivers are also indicated.

**Table 2.** Characteristics of the species analysed ( $n$  = sample size, C:N mass ratios after delipidation, muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, muscle Hg concentrations) for four fish species from the Bay of Biscay.

Species	n	Total length (mm) Min, max	C:N mass ratio Mean $\pm$ SD (min, max)	$\delta^{13}\text{C}$ (‰) Mean $\pm$ SD (min, max)	$\delta^{15}\text{N}$ (‰) Mean $\pm$ SD (min, max)	[Hg] ( $\text{ng}\cdot\text{g}^{-1}$ dm) Mean $\pm$ SD (min, max)
Whiting ( <i>Merlangius merlangus</i> )	47	80, 480	3.1 $\pm$ 0.0 (3.1, 3.2)	-16.6 $\pm$ 0.4 (-17.6, -15.9)	14.5 $\pm$ 0.5 (13.2, 15.3)	342 $\pm$ 278 (41, 1065)
Hake ( <i>Merluccius merluccius</i> )	90	65, 720	3.1 $\pm$ 0.0 (3.1, 3.2)	-17.8 $\pm$ 0.5 (-19.0, -16.7)	12.8 $\pm$ 1.0 (10.7, 14.9)	267 $\pm$ 375 (26, 1954)
Horse mackerel ( <i>Trachurus trachurus</i> )	57	80, 410	3.2 $\pm$ 0.1 (3.1, 3.4)	-18.2 $\pm$ 0.9 (-20.2, -17.1)	12.0 $\pm$ 1.2 (8.9, 14.0)	307 $\pm$ 317 (17, 1112)
Anglerfish ( <i>Lophius piscatorius</i> )	61	80, 1020	3.1 $\pm$ 0.0 (3.0, 3.2)	-17.1 $\pm$ 0.5 (-18.3, -15.7)	13.3 $\pm$ 1.2 (9.1, 15.9)	716 $\pm$ 489 (43, 2460)

Values are mean  $\pm$  Standard Deviation (SD), and/or minimum and maximum.

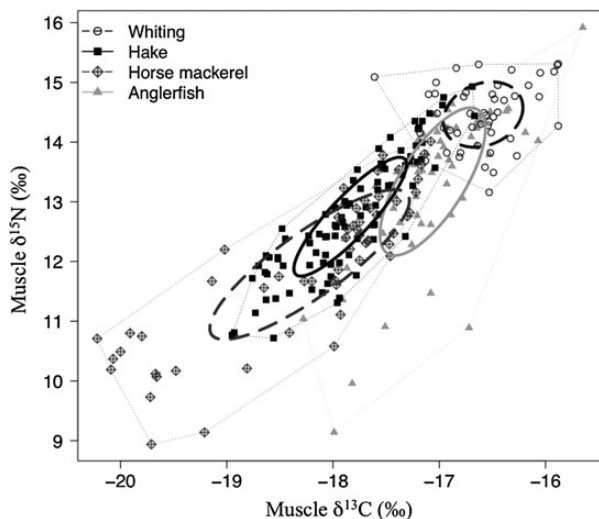
considering this latitudinal effect). As prey assemblages and therefore prey availability may vary from one year to another, and because interannual variations in the isotopic signatures of some prey species from the Bay of Biscay have been shown to occur (Chouvelon *et al.*, 2012b), the year (Y) of sampling of individuals was also treated as a possible categorical explanatory variable and added as an interaction term when appropriate. Details of the original models tested for each species are provided in Table 3.

To identify size-related, diet-related, spatial and/or year-related trends for explaining Hg concentration variability, GGAMs were fitted to individual log-transformed Hg concentrations. Size and  $\delta^{15}\text{N}$  values of individuals covaried within each species (Figure 2 and results of the previous GGAMS), so these two parameters could not be included in the same model (Zuur *et al.*, 2007). Consequently, TL and  $\delta^{13}\text{C}$  values of individuals were first considered as the continuous explanatory variables in all models, while

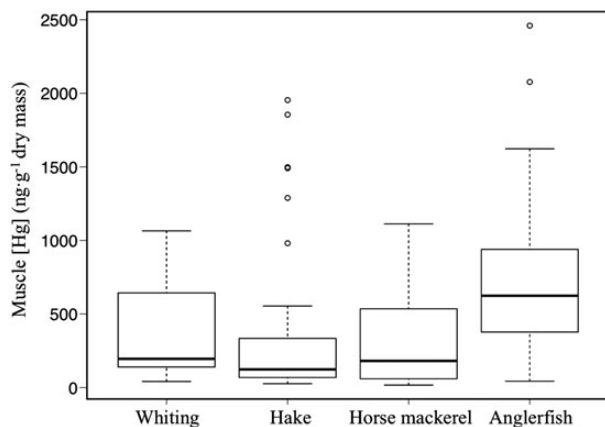
**Table 3.** Details of the sampling performed for each species and of the original models that could be tested for each species. The number of individuals sampled per latitudinal zone (LZ) and per year (Y) is given, as well as the size range in mm (in brackets).

Species	Sampling details				Original models tested	
Whiting ( <i>Merlangius merlangus</i> )			<b>Year (Y) *</b>			
			2008	2009	<b>Total</b>	
	<b>Latitudinal zone (LZ)</b>	North	25 (80, 470)	16 (210, 480)	<b>41</b> (80, 480)	$\delta^{15}\text{N} = s(\text{TL}, \text{by} = \text{Y}) + \text{Y} + s(\delta^{13}\text{C})/$
		South	1 (450)	5 (200, 230)	<b>6</b> (200, 450)	$\log [\text{Hg}] = s(\text{TL}, \text{by} = \text{Y}) + \text{Y} + s(\delta^{13}\text{C})/\log [\text{Hg}] = s(\delta^{15}\text{N}, \text{by} = \text{Y}) + \text{Y}$
	<b>Total</b>		<b>26</b> (80, 470)	<b>21</b> (200, 480)	<b>47</b> (80, 480)	
Hake ( <i>Merluccius merluccius</i> )			<b>Year (Y) *</b>			
			2008	2009	<b>Total</b>	First: $\delta^{15}\text{N} = s(\text{TL}) + \text{LZ} + \text{Y} + s(\delta^{13}\text{C})/$
	<b>Latitudinal zone (LZ)*</b>	North	27 (130, 630)	36 (65, 720)	<b>63</b> (65, 720)	$\log [\text{Hg}] = s(\text{TL}) + \text{LZ} + \text{Y} + s(\delta^{13}\text{C})/\log [\text{Hg}] = s(\delta^{15}\text{N}) + \text{LZ} + \text{Y}$
		South	5 (470, 680)	22 (90, 720)	<b>27</b> (90, 720)	Then: $\delta^{15}\text{N} = s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + \text{Y} + s(\delta^{13}\text{C})/$
	<b>Total</b>		<b>32</b> (130, 680)	<b>58</b> (65, 720)	<b>90</b> (65, 720)	$\log [\text{Hg}] = s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + \text{Y} + s(\delta^{13}\text{C})/\log [\text{Hg}] = s(\delta^{15}\text{N}, \text{by} = \text{LZ}) + \text{LZ} + \text{Y}$
Horse mackerel ( <i>Trachurus trachurus</i> )			<b>Year (Y) *</b>			
			2008	2009	<b>Total</b>	
	<b>Latitudinal zone (LZ)</b>	North	31 (80, 410)	21 (80, 320)	<b>52</b> (80, 410)	$\delta^{15}\text{N} = s(\text{TL}, \text{by} = \text{Y}) + \text{Y} + s(\delta^{13}\text{C})/$
		South	0	5 (80–100)	<b>5</b> (80, 100)	$\log [\text{Hg}] = s(\text{TL}, \text{by} = \text{Y}) + \text{Y} + s(\delta^{13}\text{C})/\log [\text{Hg}] = s(\delta^{15}\text{N}, \text{by} = \text{Y}) + \text{Y}$
	<b>Total</b>		<b>31</b> (80, 410)	<b>26</b> (80, 320)	<b>57</b> (80, 410)	
Anglerfish ( <i>Lophius piscatorius</i> )			<b>Year (Y)</b>			
			2008	2009	<b>Total</b>	
	<b>Latitudinal zone (LZ)*</b>	North	9 (530, 970)	35 (80, 940)	<b>44</b> (80, 970)	$\delta^{15}\text{N} = s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + s(\delta^{13}\text{C})/$
		South	4 (520, 910)	13 (100, 1020)	<b>17</b> (100, 1020)	$\log [\text{Hg}] = s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + s(\delta^{13}\text{C})/\log [\text{Hg}] = s(\delta^{15}\text{N}, \text{by} = \text{LZ}) + \text{LZ}$
	<b>Total</b>		<b>13</b> (520, 970)	<b>48</b> (80, 1020)	<b>61</b> (80, 1020)	

\*Attributed to the factor(s) that could be tested in the GGAM models, depending on the number of individuals and the size range within each category (i.e. sampling balanced enough for considering the latitudinal and/or the year effect).



**Figure 2.** Individual muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values for the four fish species analysed, namely whiting *Merlangius merlangus*, European hake *Merluccius merluccius*, Atlantic horse mackerel *Trachurus trachurus* and anglerfish *Lophius piscatorius*. The total area (TA; clear dotted lines) and the standard ellipse area (SEAc; bold lines) derived from individual  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values are also represented for each species (see sections “Data treatment” and “Species-specific differences and variability in stable isotope ratios and Hg concentrations”).



**Figure 3.** Boxplots of muscle Hg concentrations measured in the four fish species considered, namely whiting *Merlangius merlangus*, European hake *Merluccius merluccius*, Atlantic horse mackerel *Trachurus trachurus* and anglerfish *Lophius piscatorius*. The box length represents the interquartile, the bar length represents the range, and the horizontal lines in bold are median values.

LZ and/or Y were treated as categorical explanatory variables and added as interaction terms when appropriate. In a second step, GGAMS included individual  $\delta^{15}\text{N}$  values (instead of TL) as the continuous explanatory variable while, as previously, LZ and/or Y were treated as categorical explanatory variables and added as interaction terms when appropriate.  $\delta^{13}\text{C}$  values were not included because of their covariation with  $\delta^{15}\text{N}$  values (following Zuur *et al.*, 2007). Details of the original models tested for each species are provided in Table 3.

Finally, a selection of the “best” GGAM was based upon the Akaike Information Criterion (AIC), as the smaller the AIC, the simpler the

model and the more it explains data variability. Also, when the AIC was not significantly different between the two last nested models, the simplest model was preferred (Zuur *et al.*, 2007). A model validation was finally systematically applied by checking normality and homogeneity in models’ residuals with no violation of independence (Zuur *et al.*, 2007; Zuur, 2012). Models not complying with all these assumptions were not presented.

## Results

Analysis of species-specific differences in feeding strategies or Hg bioaccumulation patterns with size would have been more powerful if “species” could have been included as a fixed factor in a single modelling for each parameter (i.e.  $\delta^{15}\text{N}$  values on the one hand and Hg concentrations on the other hand). Such models were tested but not kept because model validation was not good (due to violation of independence, notably because size range is different among the four species).

### Species-specific differences and variability in stable isotope ratios and Hg concentrations

Stable isotope ratios and Hg concentrations varied greatly between and within the four species (Figure 2, Table 2). The species range of  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values was the largest in anglerfish (3.1‰ and 6.8‰, respectively), and the narrowest in whiting (1.7‰ and 2.1‰, respectively) (Table 2). However, TA and SEAc calculated were the highest for horse mackerel (7.6 and 1.8, respectively), followed by anglerfish (7.5 and 1.4) and hake (3.5 and 0.8). Whiting presented the lowest values of TA and SEAc (2.5 and 0.6, respectively) (Figure 2). Overall, Hg ranged from 17–2460  $\text{ng}\cdot\text{g}^{-1}$  dm, with the largest range of values in anglerfish and the narrowest in whiting (Table 2, Figure 3).

On average, the four fish species segregated by both their  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values ( $p_{\text{KW}}$  and  $p_{\text{post hoc tests}}$  all < 0.001). The coastal and benthic-pelagic whiting presented the highest average  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, while the pelagic horse mackerel presented the lowest values. The benthic-pelagic hake displayed significantly lower  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than whiting, and anglerfish showed intermediate isotopic signatures relative to both species of Gadiforms (Table 2, Figure 2). On the other hand, only Hg concentrations in anglerfish differed significantly from those of the three other species ( $p_{\text{KW}} < 0.001$  and  $p_{\text{post hoc tests}}$  all < 0.001 for anglerfish,  $p_{\text{post hoc tests}}$  all > 0.05 between the three other species) (Table 2, Figure 3).

### Intraspecific variability of muscle $\delta^{15}\text{N}$ values

Taking into account the model with the best statistical value for each species (herein the “best model”), the total explained deviance in  $\delta^{15}\text{N}$  values variability was high (52%, 86%, 90% and 85% for whiting, hake, horse mackerel and anglerfish, respectively) (Table 4). The effect of TL on  $\delta^{15}\text{N}$  values was highly significant for all species (all  $p < 0.0001$ ) and, overall, the relationships between muscle  $\delta^{15}\text{N}$  values and TL showed an increase in  $\delta^{15}\text{N}$  values with increasing size of individuals (Figure 4). The effect of  $\delta^{13}\text{C}$  values was also significant for all species except for whiting. Finally, a significant effect of LZ and of the interaction term was found for hake, but not for anglerfish (Table 4). Individuals of hake sampled in the south of the Bay of Biscay presented significantly lower  $\delta^{15}\text{N}$  values than those from the north, whatever their size (Figure 4).

**Table 4.** Detailed results for "best models" (i.e. significantly smallest Akaike Information Criterion AIC) selected to explain  $\delta^{15}\text{N}$  values or (log-transformed) Hg concentrations variability in the muscle of the four fish species from the Bay of Biscay.

Species	$\delta^{15}\text{N}$ (%)		log [Hg] ( $\text{ng}\cdot\text{g}^{-1}\text{ dm}$ )		log [Hg] as a function of $\delta^{15}\text{N}$	
	$\delta^{15}\text{N}$ as a function of size (TL)		log [Hg] as a function of size (TL)			
	Variables	p-value	Variables	p-value		Variables
Whiting ( <i>Merlangius merlangus</i> )	$\delta^{15}\text{N} = s(\text{TL})$ AIC = 49 Explained deviance = 52%		log [Hg] = $s(\text{TL}, \text{by} = \text{Y}) + \text{Y}$ AIC = -67 Explained deviance = 93%		—	
	s(TL)	<0.0001***	s(TL)*2008	<0.0001***		
			s(TL)*2009	<0.0001***		
			Y	0.576		
Hake ( <i>Merluccius merluccius</i> )	$\delta^{15}\text{N} = s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + s(\delta^{13}\text{C})$ AIC = 89 Explained deviance = 86%		log [Hg] = $s(\text{TL}, \text{by} = \text{LZ}) + \text{LZ} + s(\delta^{13}\text{C})$ AIC = -107 Explained deviance = 93%		log [Hg] = $s(\delta^{15}\text{N}) + \text{LZ}$ AIC = 2 Explained deviance = 72%	
	s(TL)*North	0.0021**	s(TL)*North	<0.0001***	s( $\delta^{15}\text{N}$ )	<0.0001***
	s(TL)*South	<0.0001***	s(TL)*South	<0.0001***	LZ	<0.0001***
	LZ	<0.0001***	LZ	<0.0001***		
	s( $\delta^{13}\text{C}$ )	<0.0001***	s( $\delta^{13}\text{C}$ )	0.0002***		
Horse mackerel ( <i>Trachurus trachurus</i> )	$\delta^{15}\text{N} = s(\text{TL}) + s(\delta^{13}\text{C})$ AIC = 83 Explained deviance = 90%		log [Hg] = $s(\text{TL}, \text{by} = \text{Y}) + \text{Y} + s(\delta^{13}\text{C})$ AIC = -83 Explained deviance = 97%		—	
	s(TL)	<0.0001***	s(TL)	<0.0001***		
	s( $\delta^{13}\text{C}$ )	<0.0001***	s(TL)*2008	<0.0001***		
			s(TL)*2009	<0.0001***		
			Y	0.178		
			s( $\delta^{13}\text{C}$ )	0.0491*		
Anglerfish ( <i>Lophius piscatorius</i> )	$\delta^{15}\text{N} = s(\text{TL}) + s(\delta^{13}\text{C})$ AIC = 108 Explained deviance = 85%		log [Hg] = $s(\text{TL}) + \text{LZ} + s(\delta^{13}\text{C})$ AIC = -58 Explained deviance = 88%		—	
	s(TL)	<0.0001***	s(TL)	<0.0001***		
	s( $\delta^{13}\text{C}$ )	<0.0001***	LZ	0.0289*		
			s( $\delta^{13}\text{C}$ )	0.0023**		

Explanatory variables included in the models are specified [i.e. total length (TL), and/or latitudinal zone (LZ) of sampling, and/or year of sampling (Y), and/or  $\delta^{13}\text{C}$  values, and/or  $\delta^{15}\text{N}$  values when appropriate; see section "Data treatment" and Table 2]. AIC values and the total explained deviance by each best model performed are also indicated. Finally, the  $p$ -value associated with each term of the model applied is given, indicating the degree of significance for the effect of an explanatory variable within each best model. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

### Intraspecific variability of muscle Hg concentrations

All the models run with TL were valid (i.e. assumptions of normality and homogeneity in residuals were respected, with no violation of independence), while most models run with  $\delta^{15}\text{N}$  values were not. Only those for hake was valid (Table 4).

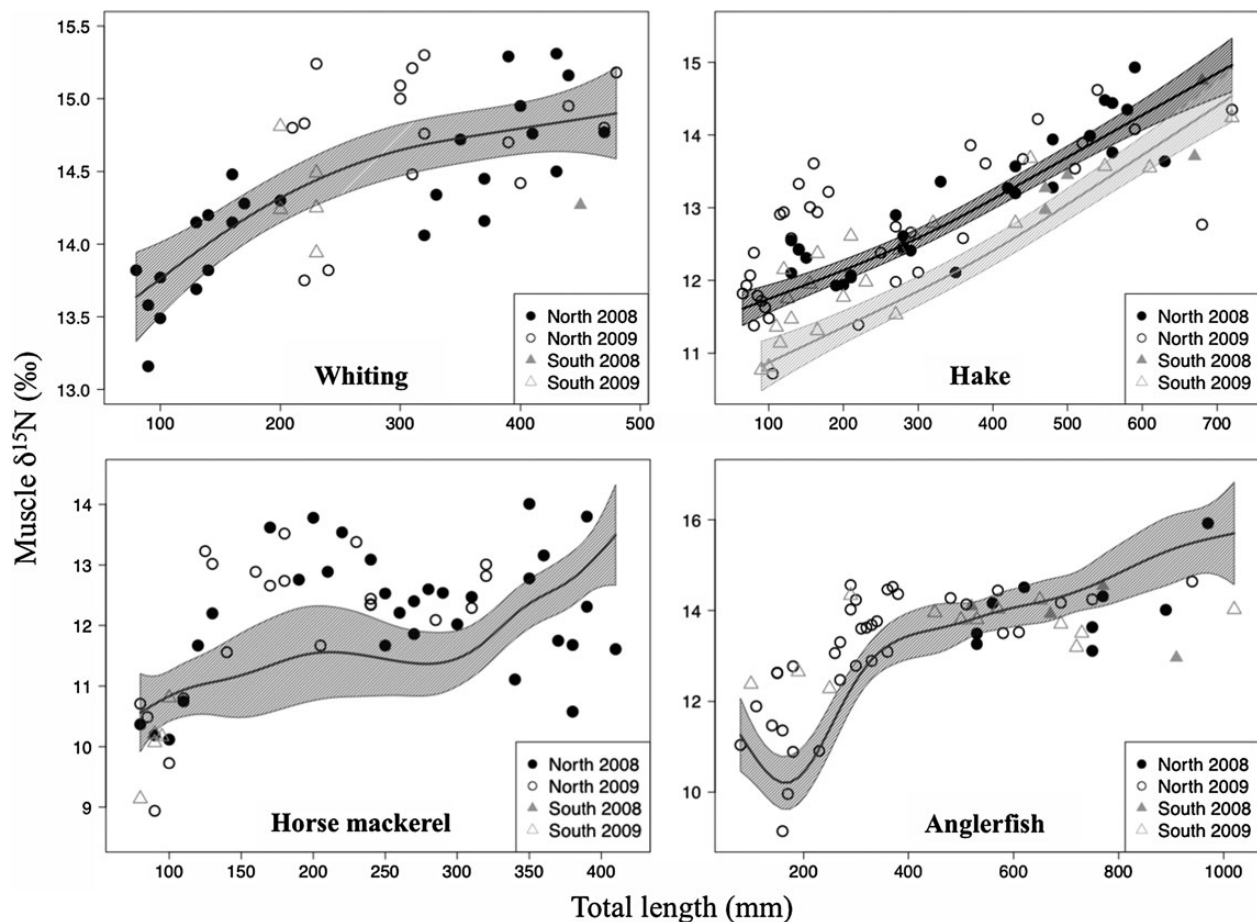
For all species, the total explained deviance in muscle Hg concentration variability was high in the best models run with TL, ranging between 88 and 97% (Table 4). For hake, the total explained deviance was higher when log-transformed Hg concentrations were modelled as a function of TL (93%) than when modelled as a function of  $\delta^{15}\text{N}$  values (72%), and the AIC was accordingly considerably lower (-107 vs. 2; Table 4).

Considering the best models with TL as the continuous explanatory variable, the effect of size was highly significant for all species (all  $p < 0.0001$ ). Overall, GGAM smoothers revealed a continuous Hg bioaccumulation with increasing individual size (Figure 5). A significant effect of the interaction term associated with Y was found in whiting and horse mackerel, highlighting a slightly different form of the relationship between log-transformed Hg concentrations and TL over the two years of sampling (Figure 5).

A significant effect of LZ was found for hake and anglerfish (Table 4), with individuals sampled in the south of the Bay of Biscay presenting significantly higher Hg concentrations than those sampled in the northern part of the area for similar size ( $p < 0.0001$  and  $p = 0.0289$  for hake and anglerfish, respectively). However, the interaction term associated with LZ was only significant for hake (Table 4), highlighting some difference in the shape of the relationship between log-transformed Hg concentrations and TL between individuals from the two zones (especially in larger individuals; Figure 5).

Considering the best model using  $\delta^{15}\text{N}$  values as the continuous explanatory variable (instead of TL) for explaining Hg concentration variability in hake, the effects of  $\delta^{15}\text{N}$  values and of LZ were also highly significant ( $p < 0.0001$  for both variables; Table 3). For similar  $\delta^{15}\text{N}$  value, individuals sampled in the south of the Bay of Biscay presented with significantly higher Hg concentrations than those sampled in the northern part of the area. However, the interaction term was not significant, suggesting an identical relationship of Hg concentrations with  $\delta^{15}\text{N}$  values for individuals from the two zones (Figure 6).





**Figure 4.** Relationships between muscle  $\delta^{15}\text{N}$  values (‰) and total length (TL, mm) for the four fish species from the Bay of Biscay. Results from the GGAM models (fitted to individual  $\delta^{15}\text{N}$  values to identify size-related and spatial and/or interannual trends explaining the variability of values within each species, see text) are plotted on raw  $\delta^{15}\text{N}$  and TL data in the form of a smoother (i.e. polygon including 95% confidence interval), illustrating the modelled relationship between both parameters. For the species for which the latitudinal zone (LZ) and the interaction term were significant in the GGAM (i.e. hake; see Table 3), two smoothers are then presented (one in black for northern individuals, one in grey for southern individuals).

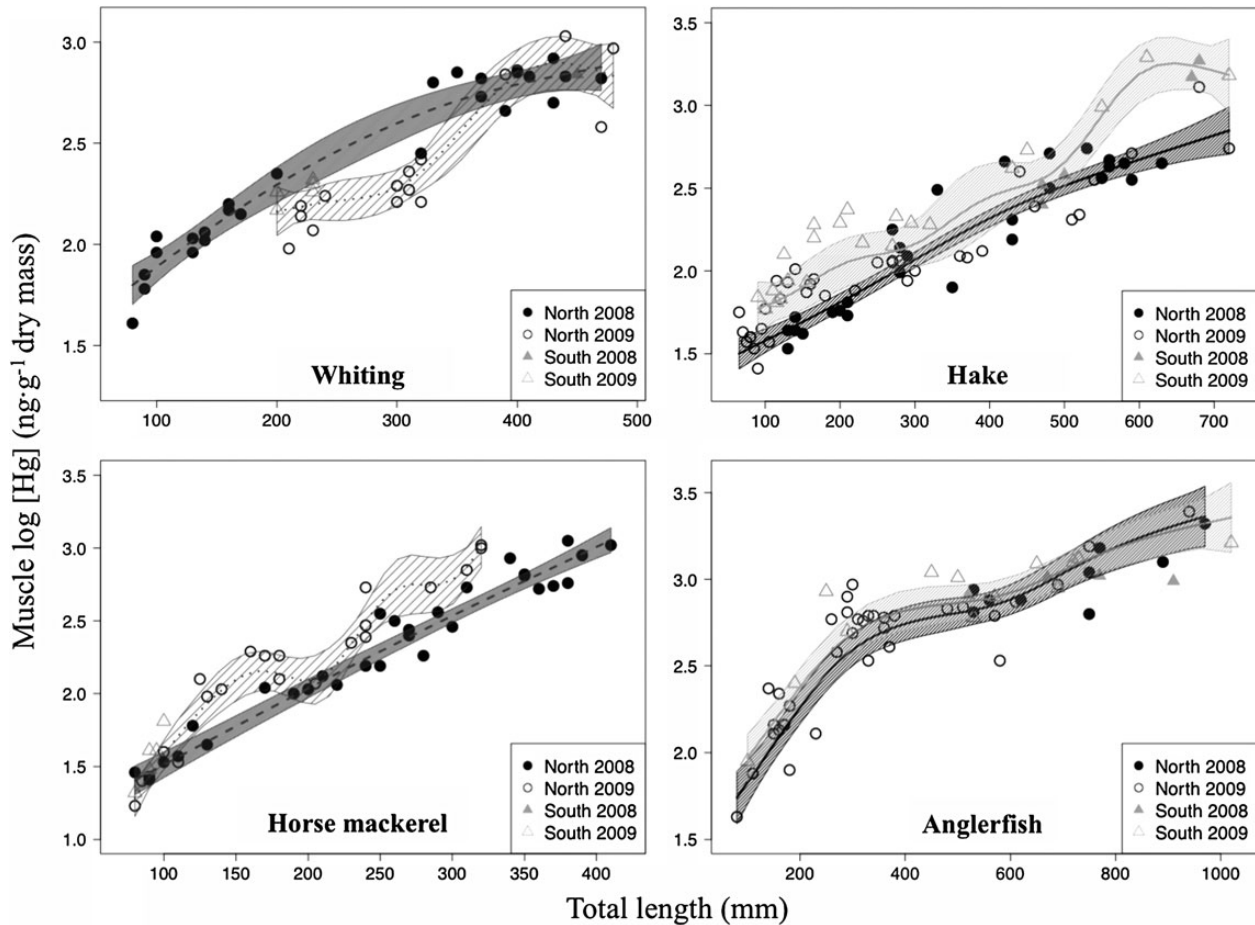
## Discussion

Muscle  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were good proxies for the different feeding ecology of fishes, both between species (segregation) and within species (size-related changes). In contrast, muscle Hg concentrations alone did not segregate the four fish species. As expected, Hg concentrations increased with size (i.e. age) and trophic level ( $\delta^{15}\text{N}$  values) within each species. Finally, hakes sampled in the southern part of the Bay of Biscay presented significantly lower muscle  $\delta^{15}\text{N}$  values and higher Hg concentrations than those sampled in the northern part, whatever their size. This study therefore provides crucial information for the combined use of these ecological tracers (stable isotope ratios and Hg concentrations in marine organisms) to refine indicators for monitoring the ecological status of marine foodwebs and ecosystems. It also highlights their potential usefulness for segregating populations, notably those of hake in European waters, by working at a more relevant time-scale than the genetic to define management units.

### Stable isotopes document species- and size-related feeding strategies

Average isotope values followed the decreasing order: whiting > anglerfish > hake > horse mackerel. Therefore, compared with

the habitat and general distribution assigned to each species in the literature, the isotopic segregation of the four species agreed relatively well with the isoscapes previously described for the Bay of Biscay (Chouvelon *et al.*, 2012b; Nerot *et al.*, 2012). Higher  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values were effectively recorded by these authors for coastal and/or benthic to benthopelagic organisms (e.g. whiting), when compared with more offshore and/or pelagic organisms (e.g. horse mackerel) in this ecosystem. Such isotopic trends were related to a variety of potentially co-occurring factors: (i) river plumes may be a vector of  $^{15}\text{N}$ -enriched organic matter into coastal waters (McClelland *et al.*, 1997; Vizzini and Mazzola, 2006), which is then diluted in offshore areas; (ii) benthic algae were reported to be enriched by 5‰ on average relative to phytoplankton in marine coastal environments, due to the differential carbon fixation and greater diffusion resistance by benthic algae (France, 1995); (iii) fractionation processes occurring at the dissolved inorganic nitrogen level, and differential nutrient availability during phytoplankton blooms in coastal waters or in upwelling areas may enrich or deplete in  $^{15}\text{N}$  the nitrogen sources available for primary producers in different environments (see detailed review by Montoya, 2007); the associated “isotopic perturbation” may then propagate in the different food chains; etc. As such, the

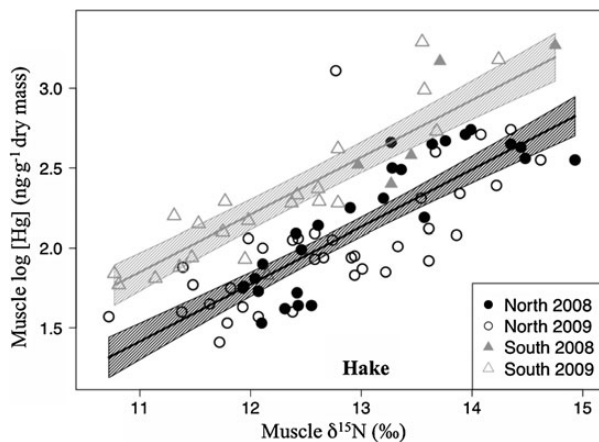


**Figure 5.** Relationships between muscle log-transformed Hg concentrations ( $\text{ng}\cdot\text{g}^{-1}$  dry mass) and total length (TL, mm) for the four fish species from the Bay of Biscay. Results from the GGAM models (fitted to individual log-transformed Hg concentrations to identify size-related and spatial and/or interannual trends explaining the variability of concentrations within each species, see text) are plotted on raw log-transformed Hg concentrations and TL data in the form of a smoother (i.e. polygon including 95% confidence interval), illustrating the modelled relationship between both parameters. For the species for which the latitudinal zone (LZ) and/or the interaction term were significant in the GGAM (i.e. hake and anglerfish; see Table 3), two smoothers are then presented (one in black for northern individuals, one in grey for southern individuals). For the species for which the interaction term and/or the year of sampling (Y) was significant in the GGAM (i.e. whiting and horse mackerel; see Table 3), two smoothers are then presented (one with dense dark grey lines for 2008 individuals, one with lighter grey lines for 2009 individuals).

anglerfish (*a priori* a deeper species occurring more offshore than the hake) should have presented lower average  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values, which is not the case. A higher consumption of benthic to benthic-pelagic prey by anglerfish may thus explain higher average isotope values by the anglerfish in comparison with the hake (which feeds mostly on small pelagic fish when adult). Moreover, adult hakes are known to inhabit shelf-edge areas as well (Quéro, 2003; Trenkel et al., 2009; Palomares and Pauly, 2010), where they would prey upon blue whiting *Micromesistius poutassou* (Guichet, 1995; Mahe et al., 2007). Finally, several biotic and abiotic parameters such as species, age, type and quality of the consumer's food, temperature, etc. may also impact the trophic enrichment factors between prey and consumers (e.g. Vanderklift and Ponsard, 2003; Caut et al., 2009; Martínez del Rio et al., 2009). This may in turn influence the isotopic differences measured between individuals and consumer species analysed, calling for caution in the interpretation of isotopic data.

The positive and significant within-species relationships between  $\delta^{15}\text{N}$  values and TL agree with the common expectation that the trophic level of organisms increases with growth, because

body size generally drives prey sizes (Cohen et al., 1993). However, the shape of the relationship was species-specific, confirming the potential of the isotopic tool to depict different size-related feeding patterns. This has already been shown in other taxa, such as crustaceans, cephalopods, marine mammals (Le Loc'h and Hily, 2005; Chauvelon et al., 2011; Meissner et al., 2012) and fish (Jennings et al., 2001, 2002). For whiting, the progressive transition to piscivory deduced from stomach contents data was well illustrated by the changing isotopic signatures from smaller to larger individuals. For hake,  $\delta^{15}\text{N}$  values increased linearly with size, and this pattern was also consistent with that described for individuals of this species from the Great Mud Bank area in the Bay of Biscay (Le Loc'h and Hily, 2005). The continuous increase in  $\delta^{15}\text{N}$  values with predator size therefore suggests progressive dietary shifts with size for both species of Gadiforms, namely an increase in the trophic level of both species throughout growth/mass increase of the individuals. The diets of horse mackerel and anglerfish, and their potential changes with growth are poorly documented in the Northeast Atlantic. Hence, stable isotopes offer an opportunity to obtain new information on their size-related feeding strategies.



**Figure 6.** Relationship between muscle log-transformed Hg concentrations ( $\text{ng}\cdot\text{g}^{-1}$  dry mass) and  $\delta^{15}\text{N}$  values (in ‰) for hake from the Bay of Biscay. Results from the GGAM model (fitted to individual log-transformed Hg concentrations to identify diet-related and spatial trends explaining the variability of concentrations within the species, see text) are plotted on raw log-transformed Hg concentrations and TL data in the form of a smoother (i.e. polygon including 95% confidence interval), illustrating the modelled relationship between both parameters. As the latitudinal zone (LZ) (but not the interaction) was significant in the GGAM (see Table 3), two smoothers are presented (one in black for northern individuals, one in grey for southern individuals).

Neither species showed the clear increase in  $\delta^{15}\text{N}$  values with predator size, as did whiting and hake. In horse mackerel, only the largest individuals ( $>30$  cm) are reported to possibly prey on small fish ( $<10$  cm). Before reaching such sizes, and along a large part of their life cycle, planktonic crustaceans effectively remain the main prey items for this species. This could explain the very slight slope observed between individuals of  $\sim 150$ – $300$  mm TL in horse mackerel. In anglerfish, a very small increase in  $\delta^{15}\text{N}$  values with predator size was also observed for individuals from  $\sim 350$ – $1000$  mm TL. This is further evidence that body size is not always well correlated with trophic level (e.g. Jennings *et al.*, 2002, for fish) and, hence, a change in the size structure of a community does not necessarily mean that there is a change in its trophic level structure.

With regard to the significant role of  $\delta^{13}\text{C}$  value (and not only TL) in explaining  $\delta^{15}\text{N}$  value variability in three out of the four species analysed (i.e. hake, horse mackerel, anglerfish), it is undoubtedly inherent that there is both  $^{13}\text{C}$  and  $^{15}\text{N}$  enrichment between sources and consumers, even if this is less important for  $^{13}\text{C}$  than for  $^{15}\text{N}$  (De Niro and Epstein 1978, 1981; Post, 2002). On the other hand, we cannot exclude a possible effect of age-dependent or length-based migrations that can occur in pelagic fish (e.g. Nøttestad *et al.*, 1999) such as horse mackerel. The feeding of some individuals and/or part of the populations in different areas [presenting different baseline signatures in  $\delta^{13}\text{C}$  (and  $\delta^{15}\text{N}$ ) in the Bay of Biscay (Chouvelon *et al.*, 2012b)] may then also explain part of the interindividual variation in  $\delta^{15}\text{N}$  values. Furthermore,  $\delta^{13}\text{C}$  values were not significant in explaining  $\delta^{15}\text{N}$  values variability in whiting, probably because of the relatively low range of spatial distribution of this species (and consequently the narrowest range in  $\delta^{13}\text{C}$  values) in comparison with the others. Indeed, the range of isotope values, TA and SEAc values all followed the decreasing order: anglerfish or horse mackerel  $>$  hake  $>$  whiting. This is quite consistent with the usual depth range described, the range of sizes sampled, and the reported

variety of the types of prey captured at the different stages within the four species analysed. However, parts of the whole isotopic niche width of each species are probably missing here because (i) some size classes could not be sampled (especially the smallest ones), and (ii) sampling occurred in autumn, thus missing a possible seasonality of the diet within species.

Finally, the spatial parameter LZ was only significant in hake for explaining intraspecific  $\delta^{15}\text{N}$  values variability. Southern individuals presented lower  $\delta^{15}\text{N}$  values than northern individuals. However, the shape of the relationship between  $\delta^{15}\text{N}$  values and TL was quite similar for the two groups, despite the significance of the interaction term in the model. This phenomenon of distinct  $\delta^{15}\text{N}$  values between southern and northern individuals has previously been reported for coastal species of fish and cephalopods from the Bay of Biscay (Chouvelon *et al.*, 2012b). This result confirms that hakes from the southern part of the area do not feed on the same food sources as those from the northern part (e.g. different prey composition, or the same prey with different isotopic signatures in the different environments). This also suggests that there is no significant feeding migration of individuals between the areas during growth.

### Size-related, diet-related and spatial patterns of Hg bioaccumulation in muscle

As previously found in cephalopods from the Bay of Biscay (Chouvelon *et al.*, 2011), muscle Hg concentrations poorly discriminated fish species. Size-related patterns for muscular Hg concentrations were quite uniform between species, with Hg concentrations increasing continuously with increasing size. This confirms that age is a key factor determining Hg bioaccumulation in fish muscle (e.g. Monteiro and Lopes, 1990; Cronin *et al.*, 1998; Cossa *et al.*, 2012). Indeed, Hg mainly accumulates as methyl-Hg, and this organic form likely binds with sulfhydryl groups of muscular proteins (Bloom, 1992; Konovalov, 1994; Harris *et al.*, 2003). Methyl-Hg then shows very slow elimination rates (Amlund *et al.*, 2007; Dang and Wang, 2012), leading to Hg bioaccumulation with age in the muscle. On the other hand, muscle Hg concentrations were linked to the trophic level ( $\delta^{15}\text{N}$ ) of organisms as well. The second model kept for hake for explaining variability in hake Hg concentrations (using  $\delta^{15}\text{N}$  values instead of TL as the main explanatory covariable) effectively highlighted a positive and significant relationship between Hg concentrations and  $\delta^{15}\text{N}$  values. However, the AIC of this second model was higher and the total explained deviance lower than for the first model with TL.

Body size was not the only covariable explaining Hg concentration variability; spatial parameters in particular (i.e.  $\delta^{13}\text{C}$  values and/or LZ) were also highly significant in most of the models performed. Muscle  $\delta^{13}\text{C}$  values were significantly and positively related to Hg concentrations in three out of the four species (i.e. whiting, hake, anglerfish; relationships not shown). This is likely to have resulted from the enrichment in  $^{13}\text{C}$  with trophic level rather than potentially enhanced bioaccumulation of Hg in more coastal organisms (Chouvelon *et al.*, 2012b). Indeed, Hg rather tends to bioaccumulate more in organisms living in meso-pelagic and deep-water environments than in those living in shallower waters (Monteiro *et al.*, 1996; Thompson *et al.*, 1998; Choy *et al.*, 2009; Chouvelon *et al.*, 2012a). This has been related to an enhanced production of methyl-Hg and of monomethyl-Hg in particular in subthermocline, low-oxygen waters; this organic form of Hg is very stable and the most bioavailable form for marine organisms (Bacci, 1989; Fitzgerald *et al.*, 2007). Moreover, as mentioned

above, Hg accumulates with age, and deep-sea fauna are thought to include very long-lived species; even though uncertainties still exist in the age determination of these organisms (e.g. Allain and Lorance, 2000; Cailliet *et al.*, 2001).

The effect of Y was minimal (i.e. significant interaction only) or negligible (i.e. not significant) in all models where this factor could be tested. As such, the slightly different form of the relationship between log-transformed Hg concentrations and size between the two years of sampling for whiting and horse mackerel may be due to different prey availability and thus variability in the diet of these species between years. On the other hand, a significant effect of LZ was detected in hake and anglerfish. Southern individuals presented higher muscle Hg concentrations than northern individuals for similar size, and this effect was particularly pronounced in hake. Such differences between southern and northern individuals can be explained by both biotic (i.e. growth rate) and abiotic factors (i.e. methyl-Hg exposure through food), as recently described for hake from the Gulf of Lion (northwestern Mediterranean) vs. hake from the northern part of the Bay of Biscay (Cossa *et al.*, 2012). If individuals from the southern stock would grow at higher rates and mature earlier than previously considered (Piñeiro and Sainza, 2003), the possible slower growth rate of southern hakes could then explain, at least in part, the higher Hg bioaccumulation by this population. Indeed, a hake from the northern part of the Bay of Biscay and with a TL of ca. 60 cm would be ~ 3 years old (De Pontual *et al.*, 2006) compared with ~ 7 years old for a similar-sized individual from the southern stock (Piñeiro and Sainza, 2003). Alternatively, the continental shelf in the southern Bay of Biscay is significantly narrower than in the northern part, so individuals feeding in the southern area may have a greater access to methyl-Hg-enhanced meso-pelagic prey (Chauvelon *et al.*, 2012a for the Bay of Biscay case study). This hypothesis is also supported by the fact that the two groups display different  $\delta^{15}\text{N}$  values, with southern hake always presenting lower  $\delta^{15}\text{N}$  values characteristic of more oceanic feeding in the Bay of Biscay (Chauvelon *et al.*, 2012a). Besides, lower  $\delta^{15}\text{N}$  values of southern individuals relative to northern individuals may be also due to different nitrogen inputs (with different isotopic composition) from both major river plumes in the Bay of Biscay that are then reflected in local food-webs. For some coastal species from this ecosystem, lower  $\delta^{15}\text{N}$  values have effectively been reported in individuals collected in the southern Bay of Biscay in comparison with those collected in the northern part (Chauvelon *et al.*, 2012b).

### Stable isotopes and Hg concentrations as tools for refining ecological indicators of marine ecosystems and defining managements units for the European hake

Our isotopic results highlighted species-specific feeding strategies during ontogenesis. In particular, in some species (i.e. horse mackerel and anglerfish), individuals could show similar  $\delta^{15}\text{N}$  values (commonly used as a proxy for the trophic level) while they were of very different sizes. This could lessen the accuracy of using size-based indicators to evaluate the ecological state of foodwebs, because it implies that the size structure of a community does not necessarily reflect its trophic level structure, and consequently its underlying trophic linkages and dynamics (for a complete review see Rombouts *et al.*, 2013). Other potential limitations of size-based indicators include that they generally only take into account demersal fish species because they are monitored for fisheries purposes. Also, short-term variations in recruitment processes may cause a

bias in interpretation by directly affecting the size spectrum (e.g. Nicholson and Jennings, 2004; Greenstreet and Rogers, 2006; Greenstreet *et al.*, 2011). All these issues strongly suggest that using size-based metrics casually is not ideal, despite apparent advantages such as the routine collection of these data (i.e. size spectrum of the species caught) during scientific surveys (Greenstreet and Rogers, 2006). On the other hand, Hg concentrations increased continuously with increasing size of individuals within all species due to bioaccumulation with both age and/or with trophic level. In fact, it appears that the coupling of both profiles (i.e.  $\delta^{15}\text{N}$  values not transformed in trophic levels and Hg concentrations as a function of size of individuals, taking into account their sampling zone) is likely to give accurate information on the transition of individuals to another trophic level from a certain size. From its size and from these isotopic and mercury profiles, a consumer could therefore be assigned quite accurately to a trophic guild. Moreover, using both these metrics in significant species of an ecosystem (e.g. those contributing to 90% of the biomass, excluding plankton) could enable a regular assessment of Hg contamination of the food-webs studied. As the Bay of Biscay continental shelf foodweb is mainly controlled by lower trophic levels and detrital biomass (Lassalle *et al.*, 2011), this would mainly include benthic invertebrate species and fish (demersal but also small pelagic fish) that could be sampled for SIA and Hg analyses in this specific area.

In the case of hake frequenting the Bay of Biscay, this study showed that both parameters (isotopic composition and Hg concentrations) measured in different-sized individuals can help the discrimination of distinct hake populations and management units in the Northeast Atlantic. Indeed, difficulties in age estimation for this species have hampered the assessment of stocks for a long time (Piñeiro and Sainza, 2003). Here, we assume the definition of management units as “demographically independent populations whose population dynamics depend largely on local birth and death rates rather than on immigration” (Palsbøll *et al.*, 2007), or again as “geographical areas with restricted interchange of the individuals of interest with adjacent areas” (Taylor and Dizon, 1999). A recent pilot study has already explored the potential of combining different tools and notably ecological tracers for discriminating common dolphin populations and defining such management units in the Northeast Atlantic (Caurant *et al.*, 2009). In the area, the common dolphin is a protected species subjected to bycatch due to interactions with fisheries (Morizur *et al.*, 1999; Lassalle *et al.*, 2012; Spitz *et al.*, 2013). By confronting at a interregional scale the results inferred from stomach contents analysis, ecological tracers analysis (i.e. trace metals, stable isotopes, fatty acids) and genetic analysis, three possible management units have been proposed for this small cetacean species in European Atlantic waters (Caurant *et al.*, 2009). Our study confirms that ecological tracers (i.e. chemical parameters such as stable isotopes or trace metal concentrations used to infer the trophic ecology of individuals) can help in discriminating different populations/stocks and defining (short-term) management units of a species, when other methods (e.g. genetic and age determination) present their own limitations (e.g. Taylor and Dizon, 1999). One must keep in mind that when using ecological tracers, only the differences are effectively informative, as different prey compositions can lead to similar isotopic signatures or trace metal concentrations in a predator’s tissues (e.g. Bearhop *et al.*, 2004). Overall, migrations of hake are estimated to be low, even within a stock (e.g. between the Bay of Biscay and the Celtic Sea; Drouineau *et al.*, 2010). Here, the clear segregation for both isotopic signatures (notably  $\delta^{15}\text{N}$

values) and Hg concentration between southern and northern hakes sampled in the Bay of Biscay, whatever the size of individuals, confirms the existence of two stocks exploiting different trophic resources in these waters. The interchange of individuals between northern and southern areas of the Bay of Biscay cannot be excluded, however it seems clearly restricted.

### Acknowledgements

The authors are very grateful to JP Léauté, R Bellail, M Salaun and JC Mahé from IFREMER for facilitating the sampling during the EVHOE cruises. They also thank P Richard, G Guillou and C Churlaud (UMR LIENSs) for assistance in stable isotope and metal analysis, and C Pignon-Mussaud (UMR LIENSs) and Y Coupeau (IFREMER) for their help in the production of the map. Finally, authors are grateful to B Mialet (UMR LIENSs) and N Niquil (BOREA, UMR 7208 CNRS-IRD-UPMC-MNHN, and BIOMEA, CNRS- FRE 3484) for helpful discussions about the descriptor “Foodwebs” of the European Marine Strategy Framework Directive.

### Funding

This work was supported through the PhD grant of TC from the Conseil Régional de Poitou-Charentes. Part of the work was also supported by the CPER (Contrat de Projet Etat-Région).

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