

1 Inter-species differences in polychlorinated biphenyls patterns from five sympatric species of
2 odontocetes: can PCBs be used as tracers of feeding ecology?

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34

35 **Abstract**

36 Concentrations of thirty two polychlorinated biphenyls (PCBs) were determined in the
37 blubber of five sympatric species of odontocetes stranded or by-caught along the Northwest
38 coast of the Iberian Peninsula: common dolphin (*Delphinus delphis*), long-finned pilot whale
39 (*Globicephala melas*), harbour porpoise (*Phocoena phocoena*), striped dolphin (*Stenella*
40 *coeruleoalba*) and bottlenose dolphin (*Tursiops truncatus*). Multivariate analyses were
41 applied to evaluate the ability of PCB patterns to discriminate these sympatric species and to
42 determine which eco-biological factors influence these patterns, thus evaluating the relevance
43 of PCB concentrations as biogeochemical tracers of feeding ecology. The five species could
44 be separated according to their PCB patterns. Different exposure to these contaminants, a
45 consequence of their different dietary preferences or habitats, together with **potentially**
46 **dissimilar** metabolic capacities, likely explain these results; sex, age, habitat and the type of
47 prey eaten were the most important eco-biological parameters of those tested. Although, no
48 single congener has been specifically identified as a tracer of feeding ecology, 4 congeners
49 from the 22 analysed seemed to be the most useful and around 12 congeners appear to be
50 enough to achieve good discrimination of the cetaceans studied. Therefore, this study
51 suggests that PCB patterns can be used as tracers for studying the feeding ecology, sources of
52 contamination or even population structure of cetacean species from the **Northwest Iberian**
53 **Peninsula**.

54

55 *Keywords:* Persistent organic pollutants; Biogeochemical tracers; Multivariate analysis;
56 Cetaceans; Northwest Iberian Peninsula.

57 **1. Introduction**

58 Marine mammals, occupying the upper trophic levels of marine food webs and possessing
59 large lipid reserves, are susceptible to considerable bioaccumulation of lipophilic (**fat-**
60 **soluble**) pollutants (Tanabe et al., 1988, 1994; Boon et al., 1994; Niimi, 1996), such as the
61 polychlorinated biphenyls (PCBs). Commercial PCB mixtures have been produced for a wide
62 range of industrial applications **because of their properties, which include resistance to**
63 **breakdown** by other chemicals (WHO, 1976, 1993). These properties have greatly
64 contributed to the ubiquitous distribution of PCBs in the atmospheric, terrestrial and aquatic
65 environments (Niimi, 1996). In the aquatic environment, overall PCB profiles change from
66 those of the industrial products as a result of distinct congener behaviour and relative rate of
67 degradation through physico-chemical processes in the environment, and/or the metabolic
68 action of organisms which ingest the contaminants from the food chain or acquire them
69 directly from the water column and marine sediments (Danis et al., 2003, 2005a, 2005b).

70 Due to the variable number and position of the chlorine atoms on the biphenyl nucleus,
71 **individual PCB congeners** follow different metabolic pathways. This results in the formation
72 of diverse metabolites (Letcher et al., 2000) and different accumulation patterns (Boon et al.,
73 1994). Thus, the PCB concentration patterns observed in marine mammals differ not only
74 from the patterns seen in the technical formulations originally released to the environment
75 (e.g. Arochlor, Clophen, Kanechlor, Pyralene) but also from those in their prey (Muir et al.,
76 1988). Moreover, PCB patterns also differ among areas as a function of the type and of their
77 distance from the source. This is a result of heavier congeners (i.e. those with higher degrees
78 of chlorination) from regional sources adhering to organic particles and remaining closer to
79 the source. The long-range signature includes a higher proportion of lighter congeners as a
80 consequence of these being the more volatile PCBs and thus subject to atmospheric transport
81 taking them some distance from their source (e.g. Staudinger and Roberts, 1996; Wania et al.,
82 2001). Therefore, PCBs can be used as a regional signature with coastal species being
83 enriched in heavier congeners relative to oceanic species, which should present a greater
84 proportion of the lighter compounds (e.g. Ross et al., 2004).

85 For seabirds and marine mammals, exposure to persistent organic pollutants through diet is
86 the only relevant exposure pathway (Borgå et al., 2004). Thus, variation in prey preferences
87 and/or in location of feeding grounds will result in varying tissue concentrations and patterns
88 among different species, and among different individuals of the same species. In the same
89 way, individual and species differences in the capacity to metabolize the different PCB
90 congeners will result in intra- and inter-specific differences in PCB profiles in marine

91 mammals (Boon et al., 1987; Tanabe et al., 1988; Wells and McKenzie, 1994). Previous
92 studies, which investigated the relative metabolic degradation of PCBs in marine mammals,
93 demonstrated that pinnipeds had a higher capacity to metabolize PCBs, especially the highly
94 chlorinated congeners, than cetaceans (Boon et al., 1992, 1997; Weijs et al., 2009). Such
95 differences seem to be the consequence of differences in cytochrome P450-mediated mono-
96 oxygenase activities. Indeed, differences were not only found between pinnipeds and
97 cetaceans, but also among different species of the same taxonomic group (Goksøyr et al.,
98 1992; Wells and Echarri, 1992).

99 There are sex-related differences in metabolism and transfer of PCBs during pregnancy and
100 lactation that may also affect contaminant patterns in marine mammals. It is generally
101 accepted that maternal transfer of lipophilic contaminants to the foetus and calves (during
102 pregnancy and lactation) reduces the concentrations of these compounds in adult female
103 marine mammals relative to males (e.g. Aguilar and Borrell, 1994). Indeed, several studies
104 suggested that lesser chlorinated PCB congeners were preferentially eliminated from females
105 to calves through lactation (e.g. Subramanian et al., 1986; Desforges et al., 2012), resulting in
106 a different PCB profile in females compared to males.

107 These ecological, biological and physiological factors lead to different PCB signatures in
108 individual marine mammal species (Aguilar, 1987; Aguilar and Borrell, 1994). This
109 potentially allows a set of persistent organic pollutants to be used as a tool for the
110 identification of different ecological features of marine mammal species or for identifying
111 populations within the species (Aguilar, 1987; Litz et al., 2007; Yunker et al., 2011).

112 In this context, the first objective of the present study was to assess whether the variation in
113 feeding preferences and sources of contamination among five sympatric odontocete species is
114 reflected in their PCB profiles. The second objective was to assess whether or not there is a
115 minimum number of PCB congeners that together efficiently discriminated between species
116 and can thus potentially be used as ecological tracers. To meet these objectives, we used
117 previously published data on PCB concentrations, lipid content, stable isotope ratios ($\delta^{13}\text{C}$
118 and $\delta^{15}\text{N}$), cadmium (Cd) concentrations, age and reproductive status (Méndez-Fernandez et
119 al., 2012, 2013, 2014) from 120 individuals of five odontocete species (i.e. common dolphin
120 *Delphinus delphis*, harbour porpoise *Phocoena phocoena*, bottlenose dolphin *Tursiops*
121 *truncatus*, striped dolphin *Stenella coeruleoalba* and long-finned pilot whale *Globicephala*
122 *melas*) from the Northwest Iberian Peninsula. Stable isotope and Cd values were used as
123 proxies of habitat and diet. $\delta^{13}\text{C}$ values are widely used in the literature as a proxy of feeding
124 habitat, and more specifically neritic/benthic vs oceanic/pelagic (e.g. DeNiro and Epstein,

125 1978; Fry, 2006). $\delta^{15}\text{N}$ values indicate trophic level (e.g. DeNiro and Epstein, 1978; Fry,
126 2006) and also vary with feeding habitat (e.g. Chouvelon et al., 2012; Ruiz-Cooley et al.,
127 2012). Cadmium concentrations can be used as a tracer of the type of prey eaten, specifically
128 fish vs cephalopod consumption, since cephalopods concentrate more cadmium (Honda et al.,
129 1983; Bustamante et al., 1998; Lahaye et al., 2005).

130

131 **2. Material and methods**

132 *2.1. Study area and sampling*

133 Fieldwork was carried out in the Northwest Iberian Peninsula (NWIP), from the Northern
134 limit of the Galician coast in Spain (43° 31' N, 7° 2' W) to Nazaré on the Portuguese coast
135 (39° 36' N, 9° 3' W; Fig. 1). Experienced members of the Spanish (*Coordinadora para o*
136 *Estudo dos Mamíferos Mariños, CEMMA*) and Portuguese (*Sociedade Portuguesa de Vida*
137 *Salvagem, SPVS*) stranding networks **have been collecting stranded and by-caught** cetaceans
138 for over twenty-five years and over fifteen years, respectively. Animals were identified to
139 species, measured, sexed and, if the decomposition state of the carcass allowed, full
140 necropsies were performed and samples collected whenever possible. However, only animals
141 recovered in a “fresh” state (**a score of between 1 and 3 from the European Cetacean Society**
142 **protocol: stranded alive, freshly dead or mildly decomposed**; Kuiken and García Hartmann,
143 1991) were selected for analyses.

144 The age data are from Méndez-Fernandez et al. (2013, 2014). The procedure for age
145 determination consisted of counting Growth Layer Groups (GLGs) from tooth sections,
146 assuming that one GLG equals 1 year (as described by Lockyer, 1993; Hohn and Lockyer,
147 1995).

148 *2.2. Lipid determination and polychlorinated biphenyl (PCB) analysis*

149 Blubber samples were previously analysed for polychlorinated biphenyls and for lipid content
150 (Méndez-Fernandez et al., 2014). Briefly, approximately 200 mg of blubber was cut (i.e. a
151 vertical section of the full thickness), homogenised and mixed with pre-cleaned sodium
152 sulphate (~ 20 g) and then extracted by Pressurised Liquid Extraction (PLE). Following PLE,
153 lipid content was determined by gravimetry and the rest of the extract was concentrated by
154 Syncore (fitted with a flushback module) to ~ 0.5 mL and passed through silica columns,
155 before transferring, with washings, to amber glass gas chromatography (GC) vials. Then, the
156 concentrations of 32 PCB congeners (International Union of Pure and Applied Chemistry
157 PCB Nos 28, 31, 52, 49, 44, 74, 70, 101, 99, 97, 110, 123, 118, 105, 114, 149, 153, 132, 137,
158 138, 158, 128, 156, 167, 157, 187, 183, 180, 170, 189, 194 and 209) were determined by Gas

159 Chromatography Electron Impact Mass Spectrometry (GC–EIMS) using an HP6890 series
160 gas chromatograph interfaced with an HP5975 MSD.

161 The methods employed were validated by the replicate analysis of standards and samples,
162 regular blank controls, and through spiking experiments or analysis of certified and
163 laboratory reference materials (LRM; cod liver oil), following the same procedure as in
164 Méndez-Fernandez et al. (2014).

165 *2.3. Data treatment*

166 The **similarities/differences** in PCB patterns for different species and/or different groups of
167 individuals are commonly difficult to discern in graphical output arising directly from the
168 chemical analysis. Data reduction (e.g. dimensionality reduction) is a way of identifying
169 patterns more readily and to provide a descriptive overview. Multivariate analyses were used
170 to infer patterns and differences in PCB profiles. These techniques (e.g. principal component,
171 redundancy and discriminant function analysis) have been used in previous studies for
172 detecting differences in organochlorine compound patterns between species of marine
173 mammals, birds and other vertebrates as well as in marine invertebrates (e.g. Schwartz and
174 Stalling, 1991; Storr-Hansen et al., 1995; Boon et al., 1997; Borrell and Aguilar, 2005).

175 All the PCB congener concentrations were normalized to the lipid-based content of the
176 blubber and thus expressed in µg/g lipid weight (lw). For statistical analyses only those
177 congeners for which the concentrations contributed more than 10% of the total sum of the 32
178 congeners were selected. Thus, a total of 22 PCBs were included: CB-28, 31, 52, 49, 44, 70,
179 101, 99, 110, 118, 105, 149, 132, 138, 156, 187, 183, 180, 170, 189 and 194.

180 *2.3.1. Differences in PCB patterns among species*

181 Discriminant analysis (DA) was used to examine differences among the PCB patterns of the
182 species using data from the 22 PCB congeners. However, in order to avoid problems due to
183 collinearity of variables (i.e. concentrations of different congeners) we carried out a DA on
184 principal component analysis (PCA) scores rather than on raw data. In addition, to better
185 show the differences in the accumulation pattern of each PCB congener and to remove
186 differences in absolute values of concentration between samples, PCBs were normalized to
187 the concentration of CB-153. CB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) was used to
188 **calculate** these ratios (i.e. CB ratios) since it is the dominant congener in the great majority of
189 aquatic mammals, and because it has a molecular structure that makes it highly resistant to
190 biotransformation (Duinker et al., 1989; Wells and Echarri, 1992; Boon et al., 1992).

191 Thus, the CB ratio was calculated as follows (values vary from 0 to 1):

$$192 \text{CB ratio}_x = [\text{CB}_x] / [\text{CB-153}]$$

193 Where $[CB_x]$ is the concentration of an individual CB congener x and

194 $[CB-153]$ is the concentration of CB-153.

195 2.3.2. *Can we identify the most suitable PCBs to be used as tracers?*

196 In order to identify the CB ratios that together efficiently discriminated between species and
197 may thus be used as tracers; a re-sampling procedure was implemented based on the DA
198 results. With this aim, first the most relevant principal components from the PCA that were
199 used as input data in the DA were selected using the “elbow method” (i.e. the principal
200 components on the left of the visual break or elbow were selected). Then, a threshold of 10%
201 of absolute contribution was used to select the CB ratios that contributed most to each
202 principal component. Thus, our initial DA on principal components indicated that 9 CBs were
203 most influential. Subsequently every possible DA using a combination of 9 CB ratios out of
204 the 22 available (i.e. 497,420 combinations) was computed, and for each DA the same
205 computation of assignment errors was made. Afterwards, an assignment errors histogram was
206 plotted to compare the original assignment error with the distribution obtained.

207 Finally, in order to identify if there was a minimum number of congeners needed to
208 efficiently discriminate pairs of species based on DA results, a resampling procedure was
209 implemented. For this, a discriminant analysis was produced with all CB ratios, and from 1 to
210 20 congeners were sequentially removed. Subsequently, for each random removal (i.e. from 1
211 to 20 congeners) the DA was repeated 1,000 times. For each DA, the mean Euclidean
212 distance was calculated over the first 4 axes of the DA between the centroids of all pairs of
213 species. Finally, the mean Euclidean distance of the pairs of species were plotted with their
214 corresponding 95% confidence intervals as a function of the number of CB ratios used.

215 2.3.3. *Factors influencing PCB patterns*

216 To examine the relationship between CB ratios and the set of potential ecological and
217 biological explanatory factors, redundancy analysis (RDA) was used. Redundancy analysis is
218 a principal component analysis in which the axes are restricted to be linear combinations of
219 explanatory variables (Zuur et al., 2007). **A RDA was performed using the combination of
220 CB ratios leading to smallest numbers of assignment errors in the DA as response variables.**
221 Moreover, RDA requires the number of samples to exceed the number of explanatory
222 variables, preferably by a factor of two. In total, for the RDA we analysed 118 samples and
223 we had eleven explanatory variables, which is an acceptable ratio. Those variables were:
224 species, sex, age, individual reproductive status, lipid content, habitat (i.e. neritic/benthic vs
225 oceanic/pelagic; represented by $\delta^{13}C$ values), trophic level (i.e. high vs low trophic level;
226 represented by $\delta^{15}N$ values) and type of prey eaten (i.e. fish vs cephalopods; represented by

227 renal cadmium concentrations). Sex and reproductive status were considered as categorical
228 variables and species identity was coded as a series of dummy variables. Data for the
229 ecological and biological variables used to perform the RDA were obtained from published
230 studies conducted on the same individuals (Méndez-Fernandez et al., 2012, 2013, 2014).
231 Significance testing in RDA is based on a permutation test so no assumption of normality is
232 required and collinearity between explanatory variables is not an important issue (Zuur et al.,
233 2007).

234 All the statistical analyses were performed using R version 3.1.3 (R Development Core
235 Team, 2014). The map (Fig. 1) was created using the MARMAP package (Pante and Simon-
236 Bouhet, 2013) and figures were created using the packages MASS and ggplot2 (Venables and
237 Ripley, 2002; Wickham, 2009).

238

239 **3. Results**

240 *3.1. Differences in PCB patterns among species*

241 **The average lipid content of blubber in the five species analysed was higher in females than**
242 **in males (Table 1) although the difference was not statistically significant ($p > 0.05$). Total**
243 **PCB concentrations (i.e. ΣPCB_{32}) were higher in males than in females with the exception of**
244 **harbour porpoises. Moreover, the species that showed the highest ΣPCB_{32} concentrations**
245 **were bottlenose dolphin, pilot whale and harbour porpoise (Table 1).**

246 The first two principal components of the PCA accounted for 84.0% of the CB ratio
247 variability, while the first two-discriminant factors (i.e. LD1 and LD2) of the DA together
248 explained 82.9% of the variance of the CB ratio data (Fig. 2). The DA revealed the existence
249 of consistent differences among the five species, with harbour porpoises (shown in light blue)
250 and striped dolphins (in orange) being clearly separated in this bi-plot. The three pilot whales
251 were also distinctly placed relative to the striped dolphins, common dolphins and harbour
252 porpoises, although there was overlap with one of the seven bottlenose dolphins. Between
253 66.7 and 100% of the individuals of each species were classified in the correct species group
254 (Table 2). Individuals of common dolphin, bottlenose dolphin and harbour porpoise were all
255 well classified to the correct species. However, one striped dolphin out of 15 (6.7%) was
256 assigned to the common dolphins and one pilot whale out of three was assigned as a
257 bottlenose dolphin. For the first discriminant axis, which clearly separated striped dolphin
258 and, to a lesser extent harbour porpoise, from the other species (Fig. 2), the most influential
259 principal components of the PCA were, in decreasing order: 22 and 21 (Fig. S1a). For the
260 second discriminant axis, which clearly separated pilot whales and harbour porpoise from the

261 bottlenose dolphin and most of the common dolphins, the most influential principal
262 components were, in decreasing order: 22, 21, 19, 20, 18 and 17 (Fig. S1b). Finally, CB-183,
263 189 and 70 contributed most to principal components 22 and 21, and CB-138, 149, 70, 189,
264 110, 52, 118 and 99 contributed most to principal components 19, 20, 18 and 17 (Table S1).
265 All these 9 congeners had between 4 and 7 Cl atoms per biphenyl nucleus.

266 *3.2 Can we identify the most suitable PCBs to be used as tracers?*

267 The assignment errors histogram showed that the modal error rate using 9 congeners is
268 around 12% and almost all combinations give error rates less than 20% (Fig. 3). Moreover,
269 error rate of less than 6% was almost never achieved. However, some combinations of CB
270 ratios led to very small numbers of assignment errors, including three combinations that led
271 to only 4 assignment errors (3.3%). **The PCBs present in these best combinations and that**
272 **lead to low assignment errors were CB-105 (3 times), 110 (3), 170 (3), 180 (3), 187 (3), 194**
273 **(3), 49 (3), 189 (2), 31 (2), 138 (1) and 183 (1).**

274 For all **pairs** of species, the mean Euclidean distance **decreased with a decreasing number** of
275 congeners used in the DA (Fig. 4). Considering a specific number of congeners (e.g. 10), the
276 mean Euclidean distances were different according to the pair of species compared, showing
277 that some of them were easier to discriminate than others. As an example, the pairing of
278 harbour porpoise with striped dolphin (Pp-Sc), and pilot whale with harbour porpoise (Gm-
279 Pp), both **exhibited** high mean Euclidean distances. In comparison, the lowest mean
280 Euclidean distances were shown when bottlenose dolphins (**Tt**) were paired with common
281 dolphins, pilot whales or striped dolphins (Dd-Tt, Gm-Tt and Sc-Tt, respectively) and also
282 when common dolphins were compared with striped dolphins (Dd-Sc), showing that these
283 species were more difficult to discriminate. Approximately half of the comparisons presented
284 in Figure 4 showed a clear plateau or at least a point of inflection, indicating that the analysis
285 of more congeners would not result in a better discrimination. Where this was the case (e.g.
286 Dd-Gm, Gm-Tr), the optimal number of congeners (showing the highest mean Euclidean
287 distance) was between 10 and 15, which was consistent with the decreasing number of
288 assignment errors related to the number of congeners used in the DA.

289 *3.3 Factors influencing PCB patterns*

290 The CB ratios that **gave** the best separation of the species and the smallest number of
291 misassignment errors (i.e. CB-31, 49, 105, 110, 138, 170, 180, 183, 187, 189 and 194) were
292 used as response variables to compute a RDA together with the following ecological and
293 biological parameters as explanatory variables: species, sex, age, reproductive status, lipid
294 content, habitat, trophic level and prey type (see material and methods for details of

295 explanatory variables). The first two axes explained 9.8% of the CB ratio variability (Fig. 5).
296 All explanatory variables tested had a significant influence ($p < 0.05$) with the exception of
297 trophic level represented by $\delta^{15}\text{N}$ values (Table 3). Additionally, either **bottlenose dolphin or**
298 **striped dolphin** had no significant influence ($p > 0.05$) (Table 3).

299 The positions of individual animals in the bi-plot, represented by coloured points (Fig. 5),
300 showed that harbour porpoises and bottlenose dolphins were positively correlated with the
301 second axis as well as with lipid content and habitat. Most of the striped dolphins **were**
302 **associated with high** renal cadmium concentrations **and negatively correlated** with habitat.
303 They were also associated with tetra- to hexa- chlorinated congeners (i.e. CB-52, 99, 110,
304 118 and 138).

305 Examination of the bi-plot also highlighted a cluster of individuals in the lower part of the bi-
306 plot **corresponded to old common and striped dolphin females**. These individuals were also
307 **associated** in this part of the bi-plot **with higher concentrations of the hepta- and octa-**
308 **chlorinated congeners** (i.e. CB-183, 187 and 194), as well as with high cadmium
309 concentrations and **high age values**. **Finally, common and striped dolphin individuals showed**
310 **the lowest segregation within the bi-plot (Fig. 5).**

311

312 **4. Discussion**

313 *4.1. Differences in PCB patterns and identification of most suitable PCBs to be used as* 314 *tracers*

315 The PCB profiles of the five odontocete species differed substantially and could be
316 successfully distinguished from each other (Fig. 2), with all the common dolphins, bottlenose
317 dolphins and harbour porpoises being correctly assigned to species (Table 2). Pilot whales
318 exhibited the lowest percentage of successful classification. However, the sample size was
319 very small for this latter species. **As such, one out of three incorrectly classified animals**
320 **resulted in only a 66.7% of well classification (Table 2)**. Harbour porpoise and striped
321 dolphin were the species with the greatest separation on the DA plot and thus **presented** the
322 most significant difference with respect to PCB profiles (Fig. 2). The habitat where these
323 animals feed is likely to be a factor that strongly influences the PCB profiles. **Higher**
324 **pollutant burdens are generally found** in species inhabiting coastal regions due to the close
325 proximity of these animals to possible emissions, discharges and losses of persistent organic
326 pollutants in temperate areas (Storr-Hansen and Spliid, 1993). Moreover, the pattern of the
327 PCBs may differ according to the distance from the source; the lighter congeners are more
328 **volatile, and thus are capable of being transported** over longer distances (Aguilar and Borrell,

2005). Therefore, the proportion of the highly chlorinated congeners decreases with distance from the source. Additionally, the relative abundance of heavy to light carbon isotopes has also been used to discriminate between habitats, pelagic/offshore habitats where phytoplankton is the only source of organic carbon and vegetated inshore/benthic habitats where macrophytes may be an additional source of organic carbon (DeNiro and Epstein, 1978; Fry, 2006). Likewise, $\delta^{15}\text{N}$ values also vary strongly with habitat (between inshore and offshore systems, with latitudes and between oceanic basins) and are used as an indicator of feeding habitat (e.g. Chouvelon et al., 2012; Ruiz-Cooley et al., 2012). Based on the stable isotope composition and direct observations at sea of the five species studied, we can affirm that in the waters off the NWIP the harbour porpoise is a coastal species, while the striped dolphin mainly inhabits offshore waters (Table 4). The discrimination between both species on the basis of their PCB profiles was consistent with the information available on features of their ecology. The other three species can be observed in both coastal and offshore waters, and are therefore likely to feed in both, as indeed may be inferred from their diets in this region (see Table 4 and references therein) and, based on this research, their PCB profiles.

Among the three combinations of PCBs that generated only four assignment errors, eleven of the selected PCBs (i.e. CB-105, 110, 170, 180, 187, 194, 49, 189, 31, 138 and 183) presumably reflect interspecific differences in habitat but also in feeding preferences. In fact, a subsequent DA performed with this combination of PCBs confirm this statement giving a better separation of the species than if we consider the PCBs selected from the first DA (Tables S2 and S3).

Moreover, whatever the pair of species is considered, the mean Euclidean distance separating them decreases with the number of congeners randomly removed (Fig. 4). However, different pairs of species had different mean Euclidean distances between them as might be expected based on the eco-biological differences presented (see Table 4) and PCB profiles. The highest mean Euclidean distances between species correspond to harbour porpoise in comparison with striped dolphin and pilot whale, the species that are the most different from each other in terms of eco-biological traits. The lowest mean Euclidean distances correspond to the ecologically closest species, the common dolphin when compared with striped and bottlenose dolphins.

The assignment errors together with the mean Euclidean distances show that around 12 congeners is enough to well separate the species. This is a good result since PCB analysis requires considerable effort in terms of data reduction, quality assurance, and processing.

362 Potential quality control and comparability issues when applying this approach widely
363 include variability in co-elution patterns, different sets of congeners analysed by different
364 laboratories (although the ICES 7 CB-28, 52, 101, 118, 153, 138, 180 is an internationally
365 recognised set of PCBs), and normal inter-laboratory variation. Hence, the minimum number
366 of congeners needed to allow discrimination of the species is an important issue since the cost
367 of analyses can be greatly reduced by lowering the number of congeners that are analysed.
368 Although it was not possible to identify specific congeners as representing the most useful
369 tracers, it is clear that among the set of 12 congeners selected, those that were more
370 frequently repeated in the discriminant analyses performed (i.e. CB-110, 138, 183, 189)
371 should be included.

372 *4.2. Factors influencing specific PCB patterns*

373 The variability of PCB patterns in marine mammals basically depends on differences in their
374 pollutant intakes, which is a direct consequence of the specific feeding preferences and
375 associated habitats (e.g. Tanabe et al., 1988; Borrell and Aguilar, 2005), as well as capacities
376 to metabolize and/or eliminate (e.g. maternal transfer) some congeners. However, identifying
377 whether the separation is due to differences in PCB metabolism or differences in ecological
378 and/or biological traits of the species is a difficult issue.

379 This research revealed a good separation among species based on PCB profiles, that is in
380 agreement with recent studies, using stable isotopes and other ecological tracers, showing
381 that these species occupy different foraging niches within the NWIP (Fernández et al., 2013;
382 Méndez-Fernandez et al., 2013). Among the set of explanatory variables used to elucidate
383 this issue (Fig. 5), only trophic level, represented by $\delta^{15}\text{N}$ values, had no significant influence
384 on CB ratios. More specifically, in the NWIP food web, these species have the same range of
385 trophic level with no significant differences among them (Table 4). However, occupying the
386 same trophic level does not necessarily mean that these species exploit the same food
387 resource. This is reflected in the RDA since the prey type variable, which is represented by
388 the renal cadmium concentrations of the specimens analysed, has a significant effect on the
389 CB ratio data and is, together with habitat, the most important ecological explanatory variable
390 (see Table 3).

391 Cadmium is a trace element used as an indicator of the type of prey eaten by the various
392 odontocetes. More specifically, it helps distinguish fish vs cephalopod consumption,
393 cephalopods being animals that concentrate more cadmium (Honda et al., 1983; Bustamante
394 et al., 1998; Lahaye et al., 2005). This would explain the positive correlation found between
395 higher cadmium concentrations and the majority of the striped dolphins, some common

396 dolphins and one of the three pilot whales (Fig. 5), which feed on cephalopods in the NWIP
397 (Table 4). Moreover, common dolphins showed the highest dispersion, which is consistent
398 with their mixed diet (Table 4), and the fact that the proportion of the different prey species
399 in their diet can vary according to the oceanic or neritic origin of the individuals (Pusineri et
400 al., 2007).

401 In the same way, Barone et al. (2014) compared PCB profiles of fishery products (i.e. fish,
402 cephalopods and crustaceans) from Southern Italy, and highlighted a species-specific
403 bioaccumulation of contaminants and differences in PCB profiles among the three different
404 groups of seafood. Specifically, invertebrates (i.e. cephalopods and crustaceans) had a high
405 percentage contribution of lower-chlorinated PCBs such as CB-28 and 52 with a slight
406 predominance of **CB-138**, while in fish samples high-chlorinated congeners such as CB-180
407 were more prominent. Similarly, in the RDA, low chlorinated congeners such as CB-105, 49
408 31, 110 and 138 seems to be more **associated** with most of the striped and common dolphins
409 (Fig. 5), and therefore with the mainly cephalopod feeders in the area. On the contrary, the
410 high-chlorinated congeners CB-170 and 180 showed **a positive relationship** with most of the
411 harbour porpoises and bottlenose dolphins, which are largely piscivorous in the NWIP (Fig.
412 5). **Boon et al. (1997) reviewed the types of metabolic behaviour of several PCB congeners in**
413 **five species of mammals (seals, otters and cetaceans) and revealed that CB170 and 180 are**
414 **highly resistant to biotransformation and consequently difficult to metabolise. These**
415 **congeners are also among those exhibiting a high octanol-water partition coefficient (log**
416 **Kow \approx 7) indicating their high liposolubility (Jäntschi and Bolboacă, 2006; Walters et al.,**
417 **2011). The high lipid content exhibited by harbour porpoise and bottlenose dolphin (67.2 and**
418 **66.3% for males and 83.2 and 71.8% for females, respectively, Table 1) compared to the**
419 **other species may thus partly explain the high ratio of these both congeners and the positive**
420 **relationship with lipid content in these individuals (Fig. 5).**

421 With respect to the various biological factors investigated, sex **had** an important effect on
422 PCB accumulation and patterns, primarily due to the maternal transfer during gestation and
423 lactation. The physicochemical characteristics of the congeners govern this transfer, with the
424 less lipophilic and lower molecular weight congeners being those primarily transferred to the
425 foetus (e.g. Greig et al., 2007; Desforges et al., 2012). Thus, the RDA bi-plot revealed a
426 cluster of several individuals positively correlated with age and with kidney cadmium
427 concentration (linked to diet), as well as with hepta- and octa- chlorinated CBs (CB-183, 187
428 and 194) (Fig. 5). Of the individuals belonging to this cluster, there are individuals of
429 common and striped dolphin species, all of them are females and 60% were found to be

430 mature. Thus, CB-183, 187 and 194, which are high-chlorinated congeners, probably
431 accumulates with age in females and may be not well transferred to offspring. Consequently,
432 the older females, which have probably been pregnant and hence eliminated some less-
433 chlorinated PCB congeners, may have high concentrations of these congeners **as was**
434 **previously reported in several marine mammal species (e.g. Desforges et al., 2012; Peterson**
435 **et al., 2014).**

436 Despite the evident effects of eco-biological factors, the effect of metabolism on PCB
437 patterns cannot be overlooked. Differences in metabolic capacities have been demonstrated
438 between pinnipeds and cetaceans (Tanabe et al., 1988; Goksøyr et al., 1992; Wells et al.,
439 1996), in which the iso-enzymes of the cytochrome P450 1A and 2B subfamilies (CYP1A
440 and CYP2B) seem to play a key role in the biotransformation of PCBs. However, there are
441 few comparable studies on odontocete species, and taxonomic proximity should not be taken
442 as an indication of identical metabolic capacities. Indeed, substantial interspecific variation in
443 the capacity to degrade persistent organic pollutants has been observed between common and
444 striped dolphins (Marsili et al., 1996; Borrell and Aguilar, 2005), and between other
445 taxonomically close vertebrates (Fossi et al., 1995). The set of congeners used in the RDA,
446 which resulted in a high segregation among species and low intra-species dispersion, could
447 be involved in the species' metabolic capacities. Nevertheless, with the information currently
448 available, it is impossible to distinguish the contribution of metabolism to the observed PCB
449 patterns.

450

451 **Conclusion**

452 The results presented in this paper show clear differences in PCB patterns between different
453 sympatric species of odontocetes. These differences are a presumably consequence of the
454 effect of a mixture of metabolism and eco-biological parameters, with sex, age, habitat and
455 the type of prey eaten probably being the most important among all that were tested. No
456 single congener has been identified as a tracer of feeding ecology. However, 4 congeners (i.e.
457 CB-110, 138, 183 and 189) from the 22 analysed seemed to be the prime ones and around 12
458 congeners appear to be enough to efficiently discriminate the species. Further studies are
459 necessary to evaluate the potential generalisation of these results. Thus the same data
460 treatment will have to be applied to other data sets of different species and/or different
461 geographical areas in order to confirm the relevancy and the universality of these results.
462 Moreover, the effectiveness of this data **treatment** for addressing issues associated with the
463 conservation and management of wildlife could be significant. The importance of defining

464 ecological diversity and populations has been the subject of much debate (ICES 2014), and
465 genetic together with ecological segregation is the fundamental basis for identifying true
466 populations. This is all the more important considering that the impact of localized
467 anthropogenic threats, such as contamination, differs among populations. The profile of
468 persistent organic pollutants, such as PCBs, was previously proposed as an additional tool to
469 identify segregation of marine mammal populations (as in Borrell et al., 2006; Herman et al.,
470 2005; Krahn et al., 2007; Pierce et al., 2008). In light of these results, the proposed data
471 treatment can be used as a complementary tool in the identification of marine mammal
472 populations.

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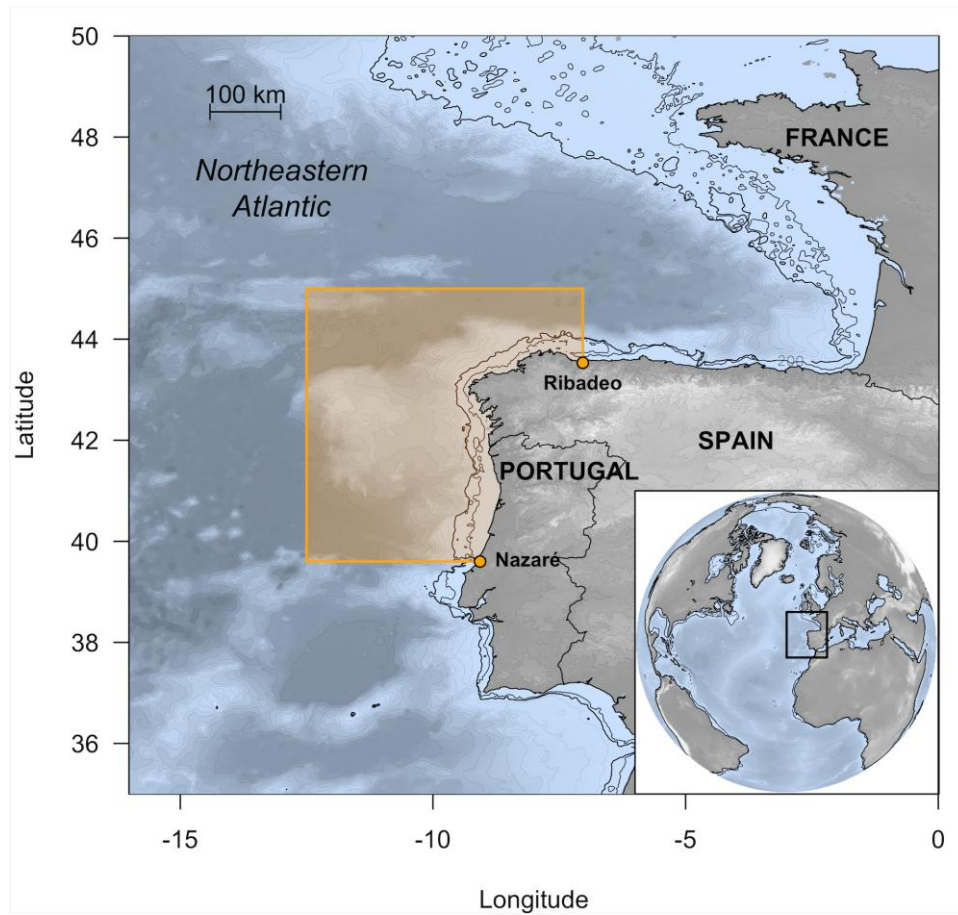
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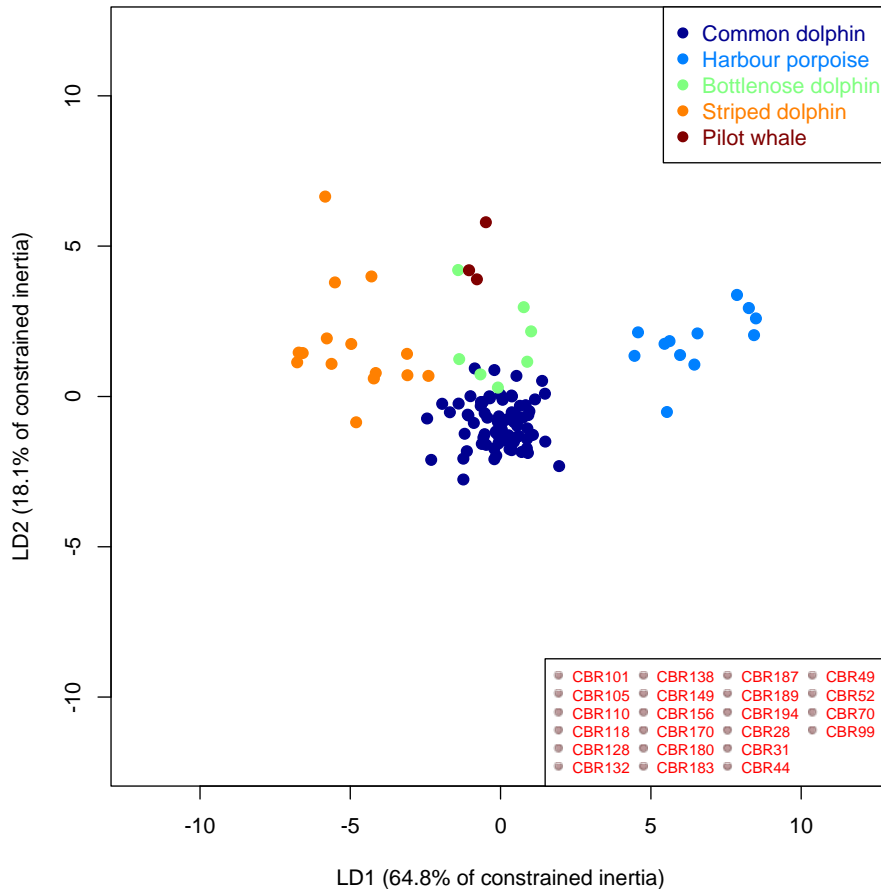
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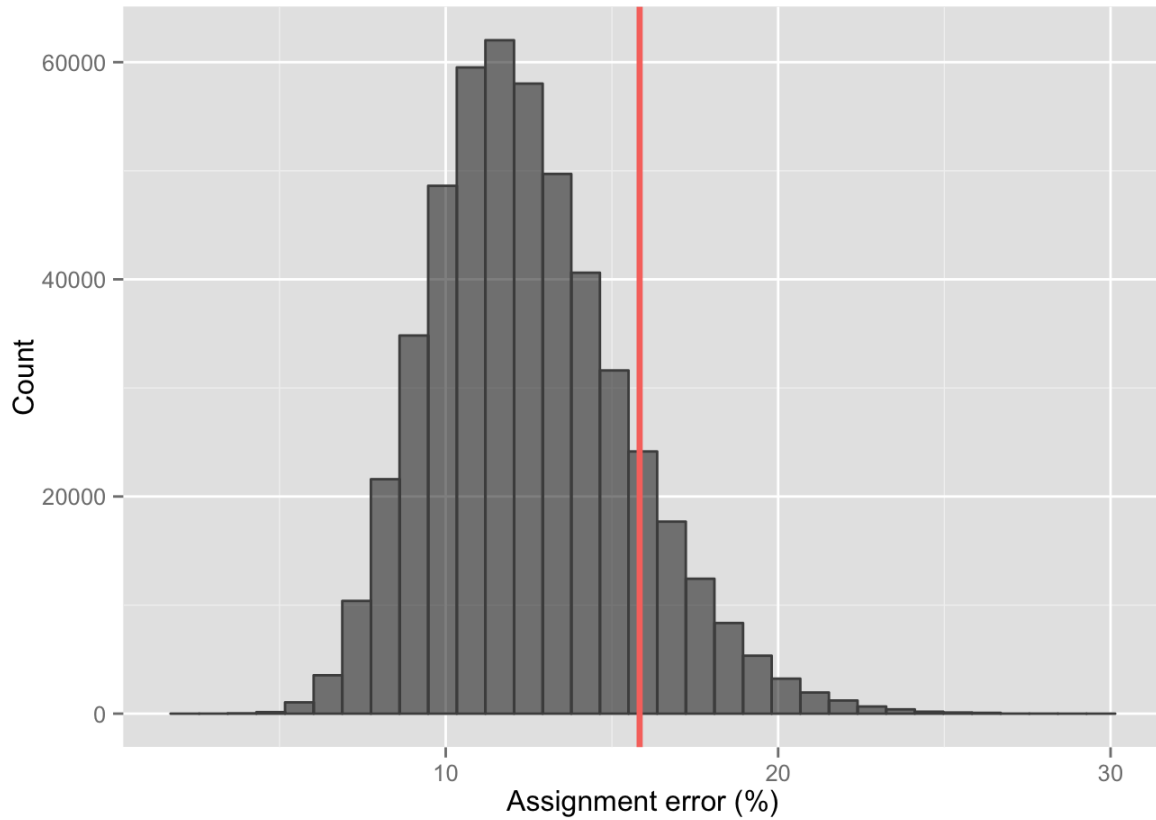


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2 Fig. 1. Map of the study area, Northwest Iberian Peninsula. The sampling area is delimited by
3 Nazaré and Ribadeo. Depth contours (-100 m and -200 m) are shown in grey scale.



4

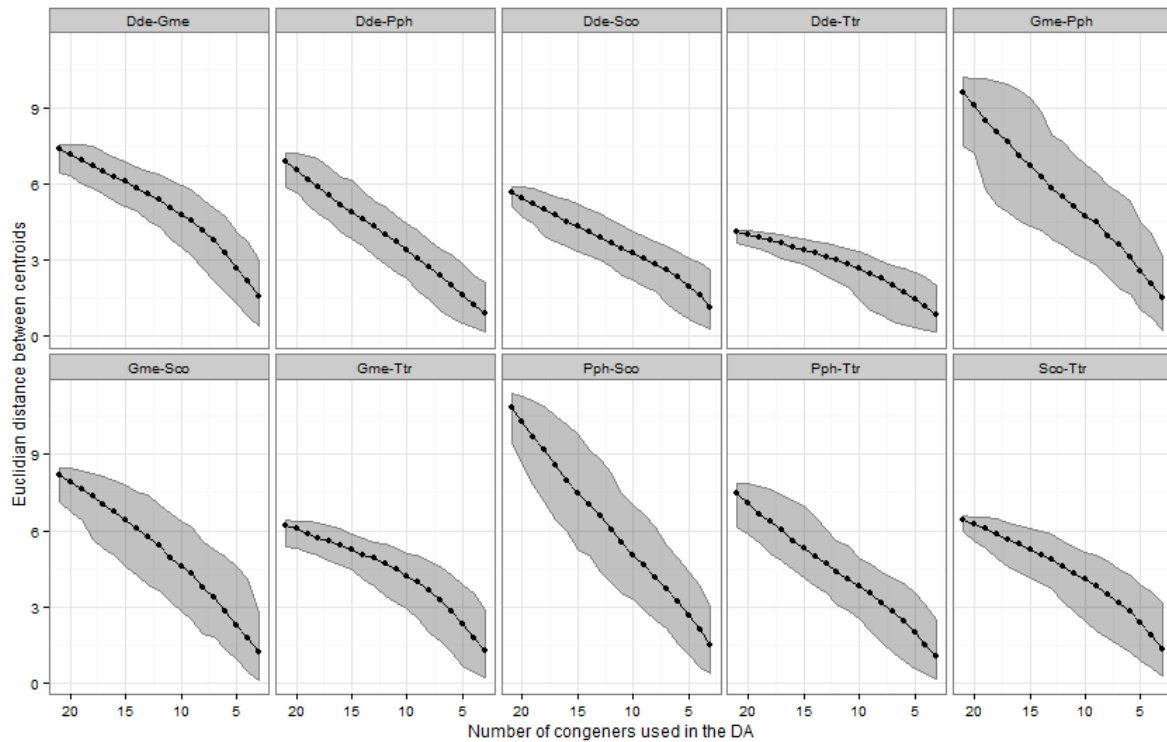
5 Fig. 2. Results of the discriminant analysis (DA) on principal components scores (i.e. LD1
 6 and LD2) of individuals of common dolphin *Delphinus delphis*, harbour porpoise *Phocoena*
 7 *phocoena*, bottlenose dolphin *Tursiops truncatus*, striped dolphin *Stenella coeruleoalba* and
 8 long-finned pilot whale *Globicephala melas* from the Northwest Iberian Peninsula. The 22
 9 CB ratios (CBR) included in the DA are given on the bottom right side of the bi-plot.



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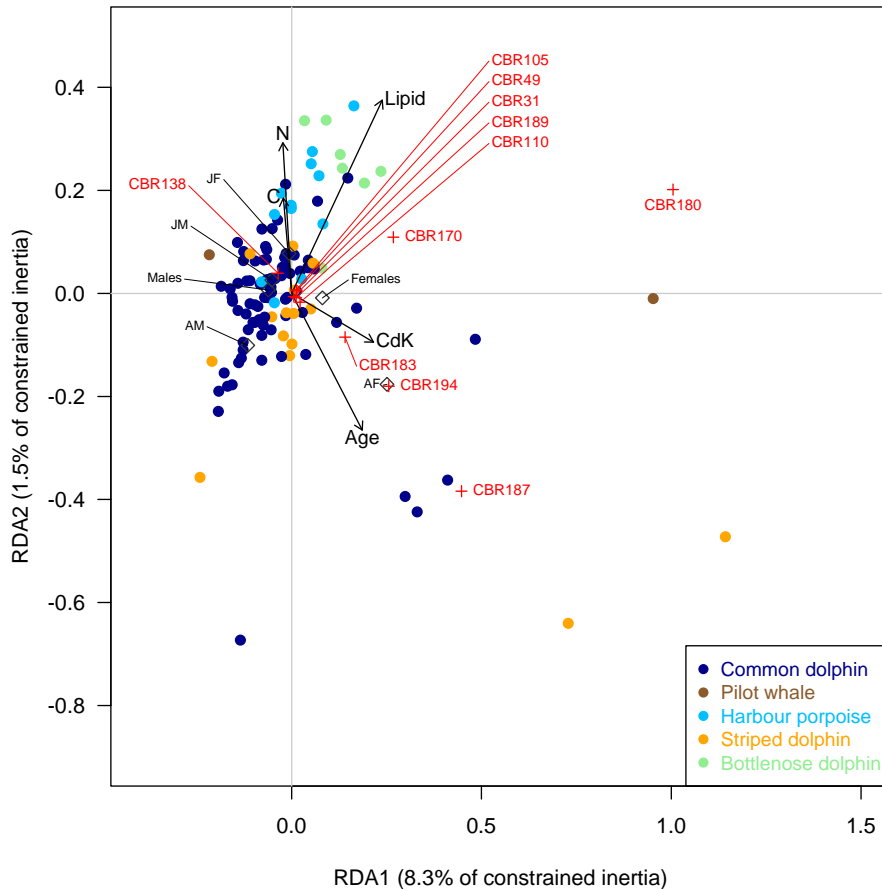
11 Fig. 3. Distribution of the assignment error (in %) obtained from every possible discriminant
12 analysis (DA) using a combination of 9 CB ratios out of the 22 available. The vertical red line
13 represents the original assignment error obtained with the 9 CB ratios selected from the first
14 DA.

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17 Fig. 4. Mean Euclidean distance and 95% confidence interval calculated between the
 18 centroids of all pairs of species when congeners are randomly removed. For each number of
 19 congeners removed we performed 1000 bootstraps. Common dolphin *Delphinus delphis*
 20 (Dde), harbour porpoise *Phocoena phocoena* (Pph), bottlenose dolphin *Tursiops truncatus*
 21 (Tr), striped dolphin *Stenella coeruleoalba* (Sco) and long-finned pilot whale *Globicephala*
 22 *melas* (Gme).



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24 Fig. 5. Results of redundancy analysis (RDA) on the best CB ratios combination (i.e. CB-31,
 25 49, 105, 110, 138, 170, 180, 183, 187, 194 and 189) in blubber of common dolphin *Delphinus*
 26 *delphis*, harbour porpoise *Phocoena phocoena*, bottlenose dolphin *Tursiops truncatus*, striped
 27 dolphin *Stenella coeruleoalba* and long-finned pilot whale *Globicephala melas* from the
 28 Northwest Iberian Peninsula. Bi-plot for axes 1-2 of significant explanatory and response
 29 variables. CdK (cadmium concentrations in kidney), C ($\delta^{13}\text{C}$ values), N ($\delta^{15}\text{N}$ values), AF
 30 (adult female), AM (adult male), JF (juvenile female), JM (juvenile male) and CBR (CB
 31 Ratios). The categorical variables (i.e. sex and reproductive status) are represented by empty
 32 black diamonds and CBR by red crosses.

1 Table 1. Arithmetic means \pm standard deviation (SD) of age (in years), lipid content (%) and
 2 of the sum of 32 congener concentrations (ΣPCB_{32} in $\mu\text{g/g}$ lipid weight (lw) with range in
 3 parenthesis) measured in the blubber of males and females of five odontocete species from
 4 the Northwest Iberian Peninsula. n = sample sizes for each species and sex (total of 67 males
 5 and 51 females).

Species	n	Age	Lipid	ΣPCB_{32}
<i>Male</i>				
Common dolphin <i>Delphinus delphis</i>	50	6.0 \pm 4.9	59.0 \pm 15.9	20.4 \pm 16.1 (3.9 – 77.5)
Harbour porpoise <i>Phocoena phocoena</i>	4	7.2 \pm 7.2	67.2 \pm 11.1	19.8 \pm 20.8 (6.7 – 50.8)
Bottlenose dolphin <i>Tursiops truncatus</i>	4	4.9 \pm 1.6	66.3 \pm 12.4	62.1 \pm 41.8 (36.3 – 124.4)
Striped dolphin <i>Stenella coeruleoalba</i>	8	3.3 \pm 5.1	57.2 \pm 25.7	22.8 \pm 23.3 (3.4 – 68.7)
Long-finned pilot whale <i>Globicephala melas</i>	1	2.0	56.0	38.7
<i>Female</i>				
Common dolphin <i>Delphinus Delphis</i>	31	7.3 \pm 6.3	63.0 \pm 15.6	11.6 \pm 7.2 (1.4 – 27.6)
Harbour porpoise <i>Phocoena phocoena</i>	8	6.9 \pm 6.6	83.2 \pm 12.4	20.8 \pm 21.6 (6.9 – 71.7)
Bottlenose dolphin <i>Tursiops truncatus</i>	3	2.3 \pm 1.6	71.8 \pm 0.8	48.9 \pm 30.9 (13.9 – 72.1)
Striped dolphin <i>Stenella coeruleoalba</i>	7	6.7 \pm 5.7	65.7 \pm 5.7	7.6 \pm 5.9 (1.7 – 15.4)
Long-finned pilot whale <i>Globicephala melas</i>	2	6.7 \pm 6.7	69.8 \pm 10.6	4.9 \pm 4.2 (2.0 – 7.9)

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15 Table 2. Results of the discriminant analysis (DA): classification of the individuals in each
 16 species groups. Results are presented as percentage (%) and well-classified animals are
 17 shown in bold. Each row refers to results for classification of individuals of one species
 18 (summing to 100%). Common dolphin *Delphinus delphis* (Dd), harbour porpoise *Phocoena*
 19 *phocoena* (Pp), bottlenose dolphin *Tursiops truncatus* (Tt), striped dolphin *Stenella*
 20 *coeruleoalba* (Sc) and long-finned pilot whale *Globicephala melas* (Gm).

True Species	Sample size	% classified as:				
		Dd	Pp	Tt	Sc	Gm
Dd	81	100	0	0	0	0
Pp	12	0	100	0	0	0
Tt	7	0	0	100	0	0
Sc	15	6.7	0	0	93.3	0
Gm	3	0	0	33.3	0	66.7

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39 Table 3. Results of redundancy analysis (RDA) on the best CB ratios combination (i.e. CB-
 40 31, 49, 105, 110, 138, 170, 180, 183, 187, 194 and 189) in blubber of common dolphin
 41 *Delphinus delphis* (Dd), harbour porpoise *Phocoena phocoena* (Pp), bottlenose dolphin
 42 *Tursiops truncatus* (Tt), striped dolphin *Stenella coeruleoalba* (Sc) and long-finned pilot
 43 whale *Globicephala melas* (Gm). RS (reproductive status), Lipid (lipid content), CdK
 44 (cadmium concentrations in kidney), $\delta^{13}\text{C}$ (stable isotopes of carbon), $\delta^{15}\text{N}$ (stable isotopes of
 45 nitrogen). Bold values correspond to the significant variables.

Explanatory variables	<i>F</i>	<i>p-value</i>
Sex	29.5422	0.001
Age	22.3346	0.001
RS	13.7578	0.001
Lipid	39.5943	0.001
CdK	18.2575	0.002
Pp	12.4306	0.002
Dd	4.8600	0.023
$\delta^{13}\text{C}$	4.7597	0.024
Tt	2.3000	0.123
Sc	1.0594	0.268
$\delta^{15}\text{N}$	1.1529	0.299

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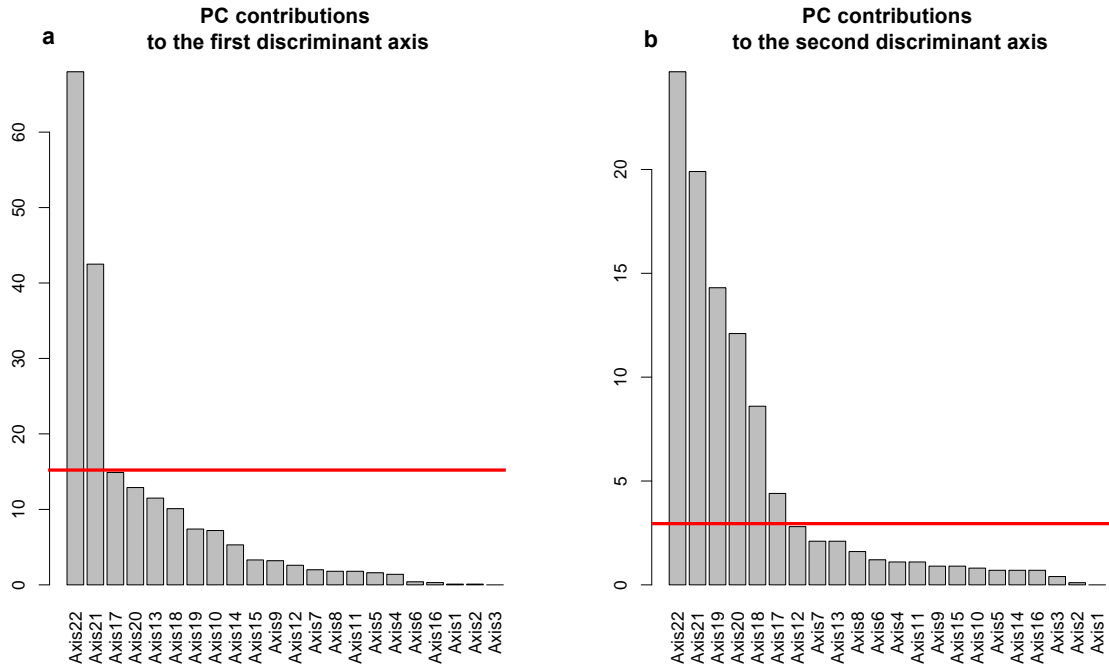
48 Table 4. Background on the feeding ecology of the odontocetes studied in the Northwest Iberian Peninsula, obtained from previous studies on:
 49 stomach contents analysis, land-based and sea surveys and chemical analyses (stable isotopes of carbon and nitrogen and renal cadmium
 50 concentrations). Trophic levels were calculated following Vander Zanden and Rasmussen (2001) in Méndez-Fernandez et al. (2012).

Species	Habitat	Trophic level	Food source
Common dolphin <i>Delphinus delphis</i>	Oceanic/neritic	4.7	Mixed feeder (blue whiting, sardine, hake and sand smelt, but also sepiolids, common and curled octopus)
Harbour porpoise <i>Phocoena phocoena</i>	Neritic	5.3	Piscivorous (pouting, scad and blue whiting)
Bottlenose dolphin <i>Tursiops truncatus</i>	Neritic (and offshore ecotype)	5.1	Mainly piscivorous (blue whiting and hake)
Striped dolphin <i>Stenella coeruleoalba</i>	Oceanic	4.3	Mainly teuthophagous (cephalopods and crustaceans, but also blue whiting, sand smelt and scad)
Long-finned pilot whale <i>Globicephala melas</i>	Oceanic/neritic	4.9	Teuthophagous (common and curled octopus)

51 Adapted from Santos et al., 2007, 2013, 2014; Spitz et al., 2006, 2011; Pierce et al., 2010; Fernández et al., 2011a; Spyarakos et al., 2011;
 52 Méndez-Fernandez et al., 2012, 2013.

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Fig. S1. Histogram representing the principal component (i.e. axes) contributions to the first-
two discriminant axes of the discriminant analyses. The red horizontal lines correspond to the
“elbow criterion” for each discriminant axis.

6 Table S1. Contribution of CB ratios (i.e. CBR) on the selected principal components (PCs) of
 7 the PCA. The most influential CB ratios are shown in bold (i.e. the one with an absolute
 8 contribution above 10%).

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CB ratios	PC 17	PC 18	PC 19	PC 20	PC 21	PC 22
CBR 101	0.0	0.0	0.1	0.0	0.0	0.0
CBR 105	5.9	0.1	0.4	0.3	0.0	0.0
CBR 110	1.9	57.8	2.2	0.4	0.5	0.0
CBR 118	43.1	0.1	2.7	0.5	0.0	0.0
CBR 128	0.1	0.2	1.0	0.0	0.0	0.0
CBR 132	0.0	0.2	0.1	0.1	0.0	0.0
CBR 138	0.3	9.0	53.5	10.7	0.0	0.0
CBR 149	1.0	1.7	18.7	43.9	5.3	4.2
CBR 156	1.5	0.4	0.3	0.2	0.0	0.0
CBR 170	0.2	0.4	0.0	0.0	0.0	0.0
CBR 180	1.1	1.2	0.0	0.1	0.0	0.0
CBR 183	0.2	2.8	6.7	0.2	21.9	50.0
CBR 187	0.0	1.7	3.7	0.7	0.0	0.0
CBR 189	0.6	2.8	3.5	14.7	16.0	43.2
CBR 194	0.1	0.0	0.0	0.0	0.0	0.0
CBR 28	0.5	0.1	0.1	0.0	0.0	0.0
CBR 31	0.0	0.1	0.0	0.0	0.0	0.0
CBR 44	1.1	2.3	0.2	0.1	0.0	0.0
CBR 49	2.3	2.6	0.0	0.0	0.0	0.0
CBR 52	5.7	16.0	1.6	0.7	0.0	0.0
CBR 70	0.8	0.4	1.6	19.1	56.2	2.4
CBR 99	33.5	0.3	3.7	8.1	0.0	0.1

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11 Table S2. Results of the discriminant analysis (DA) of the selected CB ratios: CB-52, 70, 99,
 12 110, 118, 138, 149, 183 and 189: classification of the individuals in each species group.
 13 Results are presented as percentage (%) and well-classified animals are shown in bold. Each
 14 row refers to results for classification of individuals of one species (summing to 100%).
 15 Common dolphin *Delphinus delphis* (Dd), harbour porpoise *Phocoena phocoena* (Pp),
 16 bottlenose dolphin *Tursiops truncatus* (Tt), striped dolphin *Stenella coeruleoalba* (Sc) and
 17 long-finned pilot whale *Globicephala melas* (Gm) from the Northwest Iberian Peninsula.
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True Species	Sample size	% classified as:				
		Dd	Pp	Tt	Sc	Gm
Dd	81	97.6	1.2	0.0	1.2	0.0
Pp	12	25.0	75.0	0.0	0.0	0.0
Tt	7	57.1	0.0	42.9	0.0	0.0
Sc	15	40.0	0.0	0.0	40.0	20.0
Gm	3	0.0	0.0	33.3	0.0	66.7

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21 Table S3. Results of the discriminant analysis (DA) of the best CB ratios combination: CB-
 22 31, 49, 105, 110, 138, 170, 180, 183, 187, 194 and 189: classification of the individuals in
 23 each species group. Results are presented as percentage (%) and well-classified animals are
 24 shown in bold. Each row refers to results for classification of individuals of one species
 25 (summing to 100%). Common dolphin *Delphinus delphis* (Dd), harbour porpoise *Phocoena*
 26 *phocoena* (Pp), bottlenose dolphin *Tursiops truncatus* (Tt), striped dolphin *Stenella*
 27 *coeruleoalba* (Sc) and long-finned pilot whale *Globicephala melas* (Gm) from the Northwest
 28 Iberian Peninsula.
 29

True Species	Sample size	% classified as:				
		Dd	Pp	Tt	Sc	Gm
Dd	81	98.8	0.0	1.2	0.0	0.0
Pp	12	8.3	91.7	0.0	0.0	0.0
Tt	7	14.3	0.0	71.4	14.3	0.0
Sc	15	6.7	0.0	0.0	93.3	0.0
Gm	3	33.3	0.0	0.0	0.0	66.7

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