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Nontargeted LC/ESI-HRMS Detection of Polyhalogenated Compounds in Marine Mammals Stranded on French Atlantic Coasts

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Ronan Cariou, Paula Méndez-Fernandez, Sébastien Hutinet, Yann Guitton, Florence Caurant, et al.. Nontargeted LC/ESI-HRMS Detection of Polyhalogenated Compounds in Marine Mammals Stranded on French Atlantic Coasts. *ACS ES&T Water*, 2021, 1 (2), pp.309-318. 10.1021/acsestwater.0c00091 . hal-03193843

HAL Id: hal-03193843

<https://hal.science/hal-03193843>

Submitted on 12 Oct 2021

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1 Non-targeted LC/ESI-HRMS detection of
2 polyhalogenated compounds in marine mammals
3 stranded on French Atlantic coasts

4
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12

13

14 ABSTRACT: Up to now, non-targeted analysis (NTA) of halogenated organic compounds in biota
15 has mostly been performed using GC/MS based instruments. We intended to broaden the spectrum
16 of physicochemical properties of amenable substances by taking advantage of liquid
17 chromatography–high resolution mass spectrometry coupling fitted with electrospray ionisation
18 source. Thus, a NTA strategy was applied to a set of twelve blubber samples belonging to five
19 marine mammal sentinel species stranded on the French Atlantic coasts. It involved specific post-
20 acquisition data interpretation using open-source software HaloSeeker 1.0 for annotating chemical
21 formulas. A total of 135 distinct unequivocal molecular formulas were assigned to 466
22 polyhalogenated ion clusters of interest. The most intense representative ions were identified as
23 bioaccumulative heptachloro-1,2'-bipyrrole (Cl_7 -BP), α -hexabromocyclododecane and
24 (1*R*,2*S*,4*R*,5*R*,1'*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2'-chloroethenyl)-5-
25 methylcyclohexane (MHC-1) in the $\mu\text{g/g}$ lipids weight range. To the best of our knowledge, it is
26 the first time that Cl_7 -BP, the most intense signal observed, is reported in biota. A dozen other
27 compound families will require further in-depth work to gain structural information.

28

29

30 KEYWORDS: Halogenated organic compounds; Non-targeted screening; Marine top predator;
31 Environmental contaminant; Halogenated Natural Product; Heptachloro-1,2'-bipyrrole.

32

33 SYNOPSIS: A non-targeted strategy allowed to highlight halogenated compound series amenable
34 by ESI-HRMS, and to report heptachloro-1,2'-bipyrrole for the first time in biota.

35

36 INTRODUCTION

37 The Stockholm Convention is a global treaty aiming at protecting human health and the
38 environment from Persistent Organic Pollutants (POPs).¹ So far, about 30 anthropogenic
39 polyhalogenated substances are listed in its annexes although (poly)halogenation is not a
40 prerequisite for classification as a POP. Assumedly, many other substances might deserve more
41 consideration.² Knowing what to investigate turns out to be a crucial question to guide future
42 research and policy priorities, especially when considering potential (bio)transformation products.
43 For instance, the European Marine Strategy Framework Directive aims at implementing a
44 precautionary approach for managing pressures on marine waters.³ However, regarding
45 contaminants, the Directive focuses on legacy pollutants. The future challenge will be the
46 monitoring of main compounds of emerging concern. Before prioritising the compounds to focus
47 on, based notably on their adverse effect, it would be ideal to have a holistic overview of the
48 chemical exposome.⁴

49 Due to their high trophic position, longevity and low capacity to biodegrade POPs,^{5,6} marine
50 mammals bio-accumulate and bio-magnify large amounts of these contaminants and serve as
51 sentinels for assessing the quality of marine environments.^{7,8} The occurrence of high levels of POPs
52 has been associated with pathological uterus lesions, a disease complex including lesions on skin,
53 claws, intestines, kidneys and adrenal glands, as well as immunosuppression.⁹⁻¹¹ Therefore,
54 identifying halogenated organic compounds (HOCs) of emerging concern, seeking degradation
55 products of POPs and understanding their fate have become a major scientific activity in
56 contaminants monitoring.¹²

57 In addition to anthropogenic pollutants, halogenated natural products (HNPs) are also of special
58 concern. Over 5000 HNPs, mainly produced by marine sponges, algae and bacteria, have been

59 described,¹³ some of which are persistent, bioaccumulative and/or bioactive.¹⁴ Several classes of
60 environmental relevance have been repeatedly detected over the past 25 years. They contain
61 mostly up to seven halogens, mostly brominated or Cl/Br-mixed.¹⁴

62 Targeted analysis remains state of the art for monitoring known contaminants of concern.
63 However, harmful but unknown compounds are inevitably missed.¹² Arising from the advent of
64 modern high-resolution mass spectrometry (HRMS) technologies, non-targeted analysis (NTA)
65 workflows aim at broadening the scope of analysis by taking advantage of selective full scan mass
66 spectra. While their advantages (e.g. accelerating the rate of contaminant discoveries) and
67 weaknesses (e.g. reproducibility) are still controversially discussed,¹⁵⁻¹⁸ practices are rapidly
68 evolving. Besides analytical chemist choices as regards sample preparation and data acquisition
69 technique, the bottleneck shifted to post-acquisition data interpretation, requiring sophisticated
70 cheminformatics tools to process huge datasets. Thus, human expertise remains essential to
71 critically review and discuss the results.

72 Since most HOC of environmental relevance are non-polar, gas chromatography-based
73 techniques (GC) initially appeared as the most suited strategy for tracking them.¹⁴ GC was either
74 combined with electron capture and nitrogen-phosphorus detectors (ECD and NPD), electron
75 capture negative ion mass spectrometry (ECNI-MS), electron ionization mass spectrometry (EI-
76 MS), or more recently comprehensive two-dimensional GC coupled to time-of-flight mass
77 spectrometry (GC×GC/EI-TOF/LRMS).^{12,14,19} However, monitored HOCs excluded chemicals of
78 intermediate polarity. Haraguchi et al.²⁰ previously developed a targeted method dedicated to the
79 heptachlorinated 1'-methyl-1,2'-bipyrrole (MBP) and a mixed hexahalogenated 1,1'-dimethyl-
80 2,2'-bipyrrole (DBP) by liquid chromatography coupled to tandem mass spectrometry (QqQ) with
81 atmospheric pressure chemical ionisation (LC/APCI-MS/MS), showing that atmospheric pressure

82 ionisation coupled to LC was applicable to such compounds. More recently, Liu et al.²¹ applied a
83 non-targeted LC/ESI-HRMS method to investigate halogenated compounds in polar bear serum.
84 In order to better understand the fate of transformation products or to discover undescribed HOCs,
85 whether of anthropogenic or natural in origin, we intended to further broadening the spectrum of
86 physicochemical properties of amenable substances by taking advantage of liquid
87 chromatography–mass spectrometry couplings (LC/MS) fitted with atmospheric pressure
88 ionisation techniques.

89 In the present work, a robust NTA strategy involving LC/HRMS fitted with electrospray
90 ionisation (ESI) was applied to characterise blubber samples of twelve individuals from five
91 marine mammal species, including cetaceans and seals stranded on the French Atlantic coasts.
92 Because several biological (e.g. age and sex) and ecological factors (e.g. trophic level and prey
93 type) affect the pollutant burden in marine mammals,^{22–25} the species were selected with different
94 feeding strategies (e.g. fish vs. cephalopods eaters) and habitats (e.g. inshore, offshore) meaning
95 different environmental pollution exposures. Then, the objective was to characterize in these
96 samples amenable non-conventional and known polyhalogenated organic compounds, using a
97 recently made publicly available open-source software, HaloSeeker 1.0, for annotating chemical
98 formulas and select signals worth further investigation.^{26,27} The perspective is to enlarge the
99 knowledge of the HOC exposome.

100

101 EXPERIMENTAL

102 A glossary of selected terms, abbreviations and chemicals is available in the Supporting
103 Information.

104 **Chemicals.** Hexabromocyclododecane (HBCDD) labelled isomers ($^{13}\text{C}_{12}\text{-}\gamma$ and $^2\text{H}_{18}\text{-}\beta$),
105 Dechlorane 603 and five polychlorinated hydroxyl-biphenyls (OH-PCBs: 4-OH-pentaCB-107, 4-
106 OH-hexaCB-130, 3-OH-hexaCB-137, 4-OH-hexaCB-146, 3-OH-hexaCB-153) were purchased
107 from Wellington Laboratories (Guelph, Ontario, Canada). Three other OH-PCBs (3-OH-pentaCB-
108 101, 4-OH-pentaCB-101, 5-OH-hexaCB-138) were obtained from AccuStandard (New Haven,
109 CT, USA). Tetradecyl and hexadecyl sulphate sodium salts were obtained from Sigma-Aldrich
110 (Saint-Louis, MO, USA). The 2-endo,3-exo,5-endo,6-exo,8,8,9,10,10-nonachlorobornane
111 (Parlar 50 or B9-1679) and the 2,2,5,5,8,9,9,10,10-nonachlorobornane (Parlar 62 or B9-1025)
112 were provided by Cambridge Isotope Laboratories (Tewksbury, MA, USA). Pure
113 (1*R*,2*S*,4*R*,5*R*,1'*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2'-chloroethenyl)-5-
114 methylcyclohexane (MHC-1) was previously isolated from red seaweed *Plocamium*
115 *cartilagineum*.²⁸ The 2,3,3',4,4',5,5'-heptachloro-MBP (Q1 or Cl₇-MBP) was synthesized
116 according to Wu et al.,²⁹ while the 3,3',4,4',5,5'-hexachloro-DBP (Cl₆-DBP) and the 3,3',4,4'-
117 tetrabromo-5,5'-dichloro-DBP (BC-10 or Br₄Cl₂-DBP) were synthesized according to Martin et
118 al.³⁰ A synthesis raw product (purity ~70%) of 2,3,3',4,4',5,5'-heptachloro-1,2'-bipyrrole
119 (desmethyl-Q1, DQ1 or Cl₇-BP), was produced by silylation of pyrrole, coupling with
120 *N*-chlorosuccinimide, chlorination with POCl₃/PCl₅, removal of the protection group and initial
121 chromatographic purification (Wu et al., in prep). Solvents and other chemicals used for sample
122 preparation are described in the Supporting Information.

123

124 **Samples.** Blubber samples of twelve male (individual) marine mammals stranded on the Atlantic
125 coast (English Channel or Bay of Biscay) between 2001 and 2018 were selected within the French
126 sample bank of the national stranded marine mammals network (stored at -20 °C). Females were

127 not considered in order to avoid any bias linked to the transfer of POPs to their offspring during
 128 gestation and especially lactation. Therefore, for this pilot study, two adult males from five species
 129 with very different feeding ecologies were selected, without any previous knowledge on the body
 130 burden of environmental contaminants: *Phocoena phocoena* (Pp, harbour porpoise), *Balaenoptera*
 131 *physalus* (Bp, fin whale), *Tursiops truncatus* (Tt, bottlenose dolphin), *Phoca vitulina* (Pv, harbour
 132 seal) and *Physeter macrocephalus* (Pm, sperm whale) (Table 1). Additionally, two harbour
 133 porpoise individuals (Pp_2 and Pp_3) exhibiting relatively high levels of legacy POPs were
 134 included to enrich the analytical fingerprints. These two samples belonged to another set of
 135 samples (harbour porpoise, n = 66) for which a set of POPs were previously quantified in blubber
 136 (unpublished results) according to ISO 17025 accredited methods. Harbour porpoise Pp_2
 137 exhibited the highest level of dioxin-like polychlorinated biphenyls (dl-PCBs, OMS-TEQ2005 =
 138 216 pg/g lipids weight, lw) and non-dioxin-like PCBs ($\Sigma_{\text{ndl-PCBs}}$ = 199 $\mu\text{g/g}$ lw) while harbour
 139 porpoise Pp_3 the highest level of polybromobiphenyl 153 (PBB-153, 83 ng/g lw). Details
 140 concerning stranding and animal sizes are provided in Table S1.

141

142 **Table 1.** Description of samples. BB: Bay of Biscay; EC: English Channel; Length in m. Previous
 143 analyses revealed relatively high levels of legacy POPs for harbour porpoise Pp_2 and Pp_3.

Species	Acronym	Collection year	Zone	Municipality	Length
<i>Phocoena phocoena</i>	Pp_1	2012	BB	Tarnos	1.62
	Pp_2	2013	EC	Villiers-sur-Mer	1.54
	Pp_3	2015	EC	Dieppe	1.43

	Pp_4	2016	EC	Villiers-sur-Mer	1.55
<i>Balaenoptera physalus</i>	Bp_1	2010	BB	Moliets-et-Maa	7.73
	Bp_2	2018	BB	Naujac-sur-Mer	16.92
<i>Tursiops truncatus</i>	Tt_1	2009	BB	Pornichet	1.85
	Tt_2	2018	BB	La Teste-de-Buch	3.23
<i>Phoca vitulina</i>	Pv_1	2017	EC	Grandcamp-Maisy	1.58
	Pv_2	2016	EC	Saint-Vaast-la-Hougue	1.18
<i>Physeter macrocephalus</i>	Pm_1	2001	BB	Mimizan	10.45
	Pm_2	2016	EC	Marck	13.85

144

145 **Sample preparation.** Glassware (flasks, tubes, vials, Pasteur pipettes) was baked at 400 °C for
146 4 h and Teflon tube caps rinsed with dichloromethane prior to use to minimize procedural
147 contamination. The entire blubber thickness was extracted by Pressurized Liquid Extraction
148 (SpeedExtractor, E-914, Büchi) with toluene/acetone 7:3 (v/v). An aliquot of extracted lipids
149 (500 mg) was suspended in 10 mL *n*-hexane and fortified with 20 µL toluene containing 20 ng
150 ¹³C₁₂-γ-HBCDD as internal standard (IS). The extract was partitioned with 1.5 mL concentrated
151 sulphuric acid added dropwise to remove lipids. Although it was previously found that removal of
152 lipids by gel permeation chromatography (GPC) only, as opposed to acid treatment, resulted in the
153 maximum number of detected compounds,¹² we applied acid treatment in order (i) to minimise

154 blank levels and (ii) to maintain the heaviest and most recalcitrant compounds.³¹ In turn, one must
155 bear in mind that the observed signals correspond either to acid-insensitive or to degradation
156 reaction products. After centrifugation (350 g, 10 min), the organic layer was separated and the
157 procedure repeated three times. The limpid organic layer was neutralized twice with 2 mL water
158 (up to pH = 3) and dried with anhydrous sodium sulphate. The extract was concentrated at 40 °C
159 under a gentle stream of nitrogen and transferred to a vial containing 20 μ L toluene with 10 ng
160 ²H₁₈- β -HBCDD as recovery standard (RS). Finally, the extract was reconstituted in methanol/water
161 4:1 (v/v, 62.5 μ L). Three procedural blanks were performed in parallel and, in addition, three vials
162 containing IS and RS mixture were injected as well within the analytical sequence.

163

164 **Data acquisition.** Extracts (7 μ L) were analysed in a single sequence with an UltiMate 3000
165 UHPLC pumping system coupled to an Orbitrap Q-Exactive mass spectrometer equipped with a
166 heated ESI source (Thermo Fisher Scientific, San Jose, CA, USA). The instrument was controlled
167 with Chromeleon Xpress and Xcalibur software (Thermo Fisher Scientific). Chromatographic
168 separation was performed on a C₁₈-like analytical column (Hypersil Gold, 100 mm \times 2.1 mm,
169 1.9 μ m, Thermo Fisher Scientific) kept at 45 °C. A mobile phase consisting of acetonitrile/water
170 99:1 (v/v, A) and water (B), both containing 10 mM ammonium acetate, was used. The gradient
171 started with 20% A (0 to 2 min), was then increased linearly to 60% (10 min), 100% (40 to 46
172 min) and returned to the initial conditions (48 to 52 min). The flow rate was set at 0.4 mL/min.
173 Data were recorded in negative mode with ESI parameters as follows: sheath gas flow, 50 arbitrary
174 unit (AU); auxiliary gas flow, 5 AU; capillary temperature, 350 °C; source temperature, 150 °C;
175 spray voltage, 2.5 kV; s-lens radio frequency, 50 AU. One-dimensional HRMS data were acquired
176 in full scan mode within the m/z range 120–1000 at a resolving power of 140,000 at m/z 200, using

177 m/z 305.02307 ($[\text{CH}_3\text{COO}\cdot(\text{NaCH}_3\text{CO}_2)_3]^-$) as lock mass. The automatic gain control (AGC target)
178 was set at 5×10^5 , and the maximum injection time (Max IT) was set at 250 ms.

179 LC/ESI(-)-MS/HRMS fragmentation experiments were performed in the targeted-MS² mode
180 according to the same chromatographic and ionisation parameters, with adjusted injection volume.
181 Precursor ions were selected at $\pm 0.4 m/z$ and fragmented in the HCD cell at 10, 20, 40 and 60%
182 of normalised collision energy (NCE). Mass spectra of fragments were recorded from m/z 50 on,
183 with the following parameters: AGC target: 1×10^5 ; Max IT: 100 ms; resolution: 35,000 at m/z 200.

184 GC/EI(+)-HRMS complimentary assays were performed on an Orbitrap Q-Exactive mass
185 spectrometer coupled to a Trace 1310 GC equipped with a HT-5MS column (30 m \times 0.25 mm,
186 0.25 μm , Restek, Bellefonte, PA, USA), controlled with Xcalibur software (Thermo Fisher
187 Scientific). Solutions (2 μL toluene) were injected in the splitless mode at 300 °C. Helium was
188 used as carrier gas at 1 mL/min. The oven temperature program ramped from 100 °C (2 min) to
189 325 °C (6 min) at 15 °C/min. Auxiliary and source temperatures were set at 325 and 300 °C,
190 respectively, the electron energy at 70 eV. One-dimensional HRMS data were acquired in full scan
191 mode (m/z 120–800) at a resolving power of 120,000 at m/z 200. AGC target was set at 5×10^5 and
192 Max IT at 200 ms.

193
194 **Post-acquisition data-treatment.** Targeted peak integration of labelled IS and RS as well as
195 MS/HRMS data interpretations were performed using Xcalibur software (Thermo Fisher
196 Scientific). Non-targeted data mining on one-dimensional LC-HRMS datasets was performed
197 using HaloSeeker 1.0, an open source software aiming to seek halogenated signatures in full scan
198 HRMS fingerprints described by Léon et al.²⁶ The ergonomic web user interface in the
199 R-programming environment avoids any interactions with the coding component while allowing

200 interactions with the data, including peak detection, deconvolution, and comprehensive manual
201 review for chemical formula annotation. Briefly, proprietary raw data were converted in an *mzXML*
202 open format using the MSConvert³² software (proteowizard version 3.0.9810). Then, the
203 application proceeded to the peak picking step using *xcms* 3.2.0 package³³ ($mzTol=3$, $snthresh=10$,
204 $prefilter\ step=3$, $peakwidth=10-60$, $prefilter\ level=20,000$, $noise=0$, $mzdiff=0.001$). Obtained
205 features were paired according to precise mass differences between naturally occurring C, Cl and
206 Br isotopes, according to a script adapted from Cariou et al.³⁴, reconstituting halogenated isotopic
207 patterns in so-called clusters (tolerances: $t_R=1\ s$, $m/z=0.5\ mDa$). H/Cl-scale mass defect plots (MD-
208 plot) were drawn, using the data filters. Indeed, HaloSeeker 1.0 classifies features and clusters
209 according to four filters. Among all features (F0 filter), those which are paired according to precise
210 mass differences between naturally occurring C, Cl and Br isotopes belong to the F1 filter.
211 Considering the feature triplets including the base peak A and the adjacent A-2 and A+2
212 isotopologues, additional ion ratio rules restrict to F2 and F2+ filters, for halogenated and
213 polyhalogenated clusters, respectively.²⁶ A graphical representation of the successive filters is
214 available in the glossary of the Supporting Information. Cumulative intensity of features paired in
215 a cluster ($C_{\Sigma Int}$) was used for comparisons. Finally, the clusters of interest were investigated using
216 the interactive pop-up window offering a formula decomposition script adapted from the Rdisop
217 package³⁵ to consider either the peak of lowest m/z (likely the monoisotopic peak) or preferentially
218 the most abundant isotopologue (likely the base peak). Chemical formulas were annotated by the
219 user considering H, C, N, O, S, Cl and Br up to 50, 30, 10, 10, 10, 15 and 10 elements, respectively.
220 The double bond equivalent (DBE) was deduced considering neutralisation with an additional
221 proton. Ion ratio tolerance of the scoring tool was set at 20%. Theoretical centroid isotopic patterns

222 were calculated according to Loos et al.,³⁶ either through the package embedded in HaloSeeker or
223 through enviPat Web 2.4,³⁷ using the ExactivePlus_R140000@200 resolution parameter.

224

225 RESULTS AND DISCUSSION

226 **Total ion chromatograms and QA/QC.** Aside from peaks of solvent front and of return to
227 initial conditions, no signals were visible in the total ion chromatograms (TICs) of standards and
228 procedural blanks injections (Figure S1). Unlike other studied species, sperm whale TICs
229 exhibited intense signals. This is consistent with the lower purification degree revealed by the
230 observed milky precipitate in the final vial requiring an additional centrifugation and separation
231 step prior to injection. These interferences corresponded to $[M - H]^-$ ions of saturated
232 alkylsulphates ($[C_nH_{2n+1}O_4S]^-$, $9 \leq n \leq 38$) (Figures S2), as confirmed by standards of sodium
233 *n*-tetradecyl- and *n*-hexadecylsulphate. Regardless of the chain length, MS² fragmentation featured
234 the unique $[HSO_4]^-$ ion at m/z 96.9601 (± 2 ppm). EICs of this fragment ion showed the
235 predominance of branched isomers which eluted prior to the corresponding *n*-alkyl sulphates
236 (Figure S3). The singular presence of alkyl sulphates in sperm whale could be due to the very high
237 level of wax (sterols and esters), the main lipid class in the blubber of this cetacean species³⁸, and
238 result from the incomplete oxidation by concentrated sulphuric acid.

239 IS and RS were not considered as representative of all amenable compound but rather as
240 indicators of sample preparation and instrumental performances. Retention times (t_R) for IS and
241 RS were stable along the LC-HRMS sequence. Mass deviations for both IS and RS were in the
242 range 0.23–0.51 mDa (0.40–0.81 ppm) (Table S2), allowing for sub-ppm formula searches during
243 the formula annotation step. IS recoveries in sample and blank extracts ranged from 50 to 93%,
244 except for sperm whales (44–46%). These results were consistent with the fact that losses occurred

245 at each partitioning, depending on the visual discernibility of the interface between *n*-hexane and
246 concentrated sulphuric acid. Regarding sperm whales, lower recoveries are in agreement with the
247 occurrence of the milky precipitates. Considering such losses and the ion suppression
248 phenomenon, results were not considered quantitatively but qualitatively only. However, semi-
249 quantitative assumptions could be performed, assuming a recovery and a response factor similar
250 to IS.

251
252 **Enumeration of clusters of interest and manual formula annotation.** Using HaloSeeker 1.0
253 (see experimental), LC-HRMS datasets of marine mammals exhibited between 761 and 4,023
254 features above a threshold of 10^6 AU for $C_{\Sigma\text{Int}}$. Procedural blanks and standards were quite variable,
255 possibly due to the lack of initial sequence equilibration, lower ion suppression phenomenon
256 and/or a threshold effect during the peak picking step. Corresponding MD-plots appeared
257 consequently overloaded when considering all features (F0 filter) but pairing drastically decreased
258 plots passing filter F1, as illustrated in [Figure S4](#). Eventually, 47 to 98% of features and 74 to
259 99.9% of clusters were discarded between F0 (entire set of features) and F2+ (paired clusters
260 passing the polyhalogenated ion ratio rules) filters, permitting the realistic manual review of 888
261 remaining polyhalogenated clusters we chose to focus on. Further details on enumeration of
262 clusters of interest are available in the Supporting Information.

263 All F2+ clusters were reviewed through the HaloSeeker interface and either discarded ($n = 422$,
264 IS- or RS-related adducts or obviously non halogenated when considering extracted ion
265 chromatograms – EICs, cluster slope, ion ratios) or annotated with an ion formula ($n = 466$,
266 [Table S4](#)) according to all available information, including EICs, cluster's pattern and slope,
267 pattern score and m/z deviation with theoretical suggestions. All this information allowed to reach

268 level 4 of identification confidence (*unequivocal molecular formula*) according to the scale
 269 developed by Schymanski et al.³⁹

270 Among the 466 clusters, no cluster of interest other than labelled standards was detected in
 271 procedural blanks. In each blubber dataset, 4 to 94 clusters of interest were annotated with a
 272 polyhalogenated compound (Table 2). Compared to the total intensity of features (31-683×10⁶ AU
 273 of cumulative C_{ΣInt}), they represented only 0.04 to 1.67% in sperm whale, harbour seal, fin whale
 274 and the two harbour porpoises randomly selected, compared to 4-8% in bottlenose dolphins and
 275 12-24% in the two harbour porpoises contaminated with relatively high levels of legacy POPs.
 276 Although more samples are required to refine such levels and proportions, these differences
 277 support species-specific variabilities in the levels of polyhalogenated substances amenable by
 278 LC/ESI in blubber after acidic degradation.²⁹ They also support that high contamination levels with
 279 legacy POPs correlate with multi-exposures.^{40,41}

280
 281 **Table 2.** Enumeration of features and clusters according to HaloSeeker filters. Feature and cluster
 282 intensities (C_{ΣInt}) as ×10⁶ AU; C_{ΣInt} threshold: 10⁶ AU; t_R range: 7-37 min; F0: all features; F2+:
 283 paired clusters complying polyhalogenated ion ratio rules.

Sample	F0		F2+ with annotated formula		
	Features	Intensity	Features	Clusters	Total C _{ΣInt}
IS-RS_1	4,015	63,156	-	-	-
IS-RS_2	671	9,933	-	-	-
Blk_1	3,355	54,463	-	-	-

Blk_2	3,524	53,963	-	-	-
Blk_3	697	10,308	-	-	-
Pp_1	4,023	61,520	306	49	1,028
Pp_2	1,187	12,411	518	83	2,931
Pp_3	1,634	20,988	682	94	2,470
Pp_4	3,719	51,906	302	46	823
Bp_1	761	16,448	40	7	34
Bp_2	785	15,457	31	4	32
Tt_1	999	14,072	336	52	593
Tt_2	2,612	24,899	551	77	1,966
Pv_1	771	14,374	107	15	111
Pv_2	801	20,475	102	14	110
Pm_1	1,928	169,952	61	13	88
Pm_2	2,030	244,503	58	12	96
IS-RS_3	385	9,436	-	-	-

284

285 Among these 466 annotated ion formulas, only 135 were unique compounds (from one sample

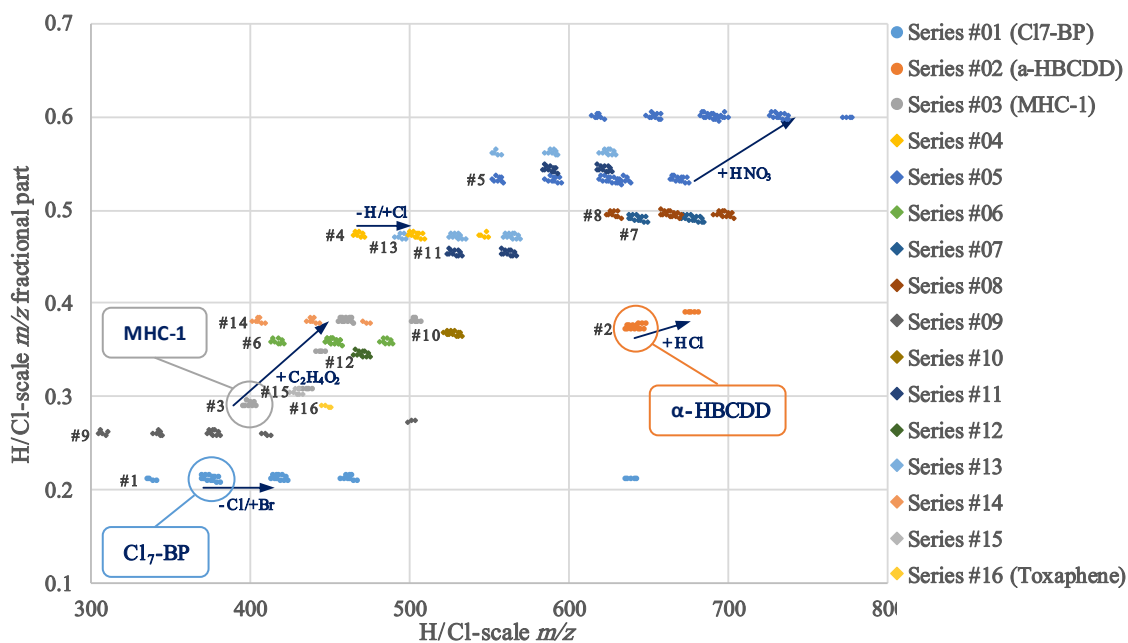
286 to another), most being observed in several samples and some being isomers as well. The masses

287 detected by LC/ESI-MS ranged from m/z 203 to m/z 809. The compounds either primarily eluted
288 from 8.4-21.3 min (less nonpolar range, 60-75% organic mobile phase) or from 29.6-35.0 min
289 (most nonpolar compounds, 85-95% of organic mobile phase) (Figure S5), for cumulative $C_{\Sigma Int}$ of
290 9.0 and 1.3×10^9 AU, respectively. Mass deviations for known compounds and annotated molecular
291 formulas varied from 0 to 0.9 mDa but the observed distribution according to m/z suggested a
292 lower confidence level for those formulas with mass deviation above 0.6 mDa (erroneous or
293 suffering from interference) (Figure S6).

294

295 **Remarkable cluster series.** After formula annotation, an exported data table was manually
296 reviewed for grouping clusters mainly based on congeners, homologues, isomers, and adduct ions.
297 More than 300 out of the 466 clusters, corresponding to >90% of cumulative $C_{\Sigma Int}$ for each dataset,
298 were grouped into 16 cluster series of decreasing intensity (Tables S5) gathering congeners and
299 homologues. For each series, the most intense clusters among the datasets are displayed as MD-
300 plots in Figure 1, revealing patterns described hereafter.

301

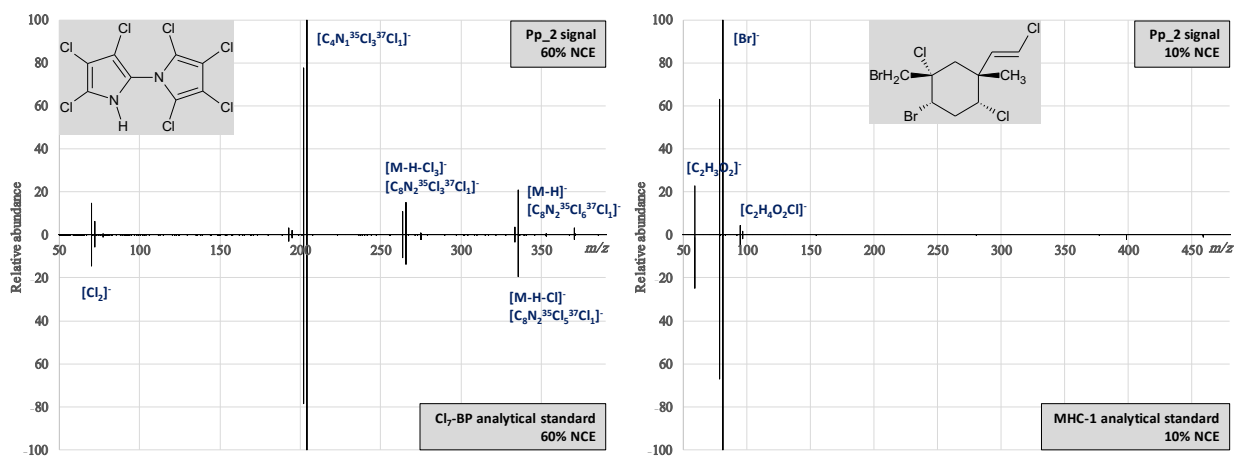


302
 303 **Figure 1.** H/Cl scale mass defect plot of most intense representative clusters among all marine
 304 mammal datasets for each series. Arrows: adduct or homologue vectors. Each dot represents an
 305 isotopologue (feature), clusters of dots represent paired isotopologues (isotopic pattern),
 306 horizontally aligned clusters represent homologue series.

307
 308 **Series #1 – Cl₇-BP, a new non-methylated 1,2'-bipyrrole detected.** The most abundant cluster
 309 of interest ($C_{\Sigma \text{int}} \sim 1.04 \times 10^9$) was detected in harbour porpoise Pp₂ extract. The most abundant
 310 isotopologue peak, observed at m/z 370.7858, corresponded with the molecular formula
 311 $[C_8^{35}Cl_6^{37}Cl_1N_2]^-$ ($\Delta mDa=0.06$, score=95%, DBE=6). This N₂-containing compound was also
 312 detected in other extracts, except sperm whale for which matrix effect from alkyl sulphates may
 313 have hampered its ionisation. Assuming $[M - H]^-$, the formula differed by CH₂ from Cl₇-MBP, a
 314 HNP. Hence, it was suspected to correspond to the fully chlorinated heptachloro-1,2'-bipyrrole
 315 (Cl₇-BP). Analysis of a raw synthesis product of Cl₇-BP resulted in a full match in retention time
 316 and MS/HRMS fragmentation patterns (Figure 2). More precisely, fragmentation of the

317 [M – H]⁻ pseudo-molecular ion of Cl₇-BP was observed as losses of Cl and Cl₃, cleavage of the
 318 1,2'-bond with preferential charge retention on the Cl₄-containing pyrrole moiety (C₄Cl₄N,
 319 monoisotopic peak *m/z* 201.8790), as well as [Cl₂]⁻ ions. In addition, mixed halogenated
 320 homologues of Cl₇-BP (Br₁Cl₆-, Br₂Cl₅-BPs) were also detected at slightly lower *t_R*, with 9-14%
 321 and 1.6-2.0% LC/ESI-MS abundance relative to Cl₇-BP, respectively. Several unresolved isomers
 322 were visible on EICs. This series accounted for more than 40% of cumulative C_{ΣInt} in 3 out of 4
 323 harbour porpoises.

324



325

326 **Figure 2.** Fragmentation patterns obtained for harbour porpoise Pp_2 base peak signals (Top) of
 327 Cl₇-BP (left, *m/z*=370.79±0.40, 60% NCE) and MHC-1 (right, *m/z*=458.85±0.40, 10% NCE), as
 328 well as corresponding analytical standards (Mirror, same conditions). Subtraction of background
 329 spectra was applied (before and after chromatographic peaks).

330

331 Cl₇-BP has never been reported before in environmental samples. Instead, the corresponding
 332 Cl₇-MBP and mixed brominated/chlorinated 1'-methyl-1,2'-bipyrrole (PMBPs) and the
 333 structurally related 1,1'-dimethyl-2,2'-bipyrroles (PDBPs) were previously reported in the μg/g

334 lw range in marine mammals worldwide.^{12,19,42,43} They have been proven to biomagnify within
335 marine food webs^{44,45} and to be present in the human diet as well as in human milk.⁴⁶⁻⁴⁸ However,
336 none of these HNPs were detected in the samples analysed. A subsequent LC/ESI-HRMS analysis
337 of standards of Cl₇-MBP and Br₄Cl₂-DBP failed to give signals with the present method, obviously
338 due to an inadequate ionisation technique.

339 Therefore, the harbour porpoise Pp_2 extract was further analysed by GC/EI-HRMS on Cl₇-BP,
340 Cl₇-MBP, Cl₆-DBP and Br₄Cl₂-DBP, which were available as standards. Polyhalogenated *N*-
341 methyl-bipyrroles were unambiguously identified by GC/EI-HRMS, with Cl₇-MBP (~1.7 µg/g lw)
342 being about three order of magnitude more abundant than Cl₆-DBP and Br₄Cl₂-DBP, but Cl₇-BP
343 (the predominant compound in LC/MS) could not be detected. Thus, a fraction centred about the
344 LC retention time of Cl₇-BP (±0.2 min) was collected using a switching valve placed between the
345 LC analytical column and the ESI source. Re-analysis of this fraction by GC/EI-HRMS confirmed
346 the presence of Cl₇-BP in the harbour porpoise blubber. Matrix effect likely led to ion suppression
347 by GC/MS in EI mode for the original harbour porpoise Pp_2 extract. It appears that Cl₇-BP could
348 be relatively sensitive to such a phenomenon. It might explain why Cl₇-BP was not previously
349 described in biota in GC-based studies, while ionization via cleavage of the N-H bond during ESI
350 appeared credible. Assuming a response factor similar to IS, Cl₇-BP concentration would reach
351 3.4 µg/g lw in harbour porpoise Pp_2. A pure analytical standard will be required prior to
352 producing quantitative results and comparing with other polyhalogenated bipyrroles and HOCs.

353

354 **Series #2 – α-HBCDD.** The second most abundant compound detected in the investigated
355 extracts was unambiguously identified as α-HBCDD using authentic standard (*t_R*=15.40 min, *C*_{ΣInt}
356 ~0.9×10⁹ AU in harbour porpoise Pp_3 extract, Δ*mDa*=0.34). HBCDD is a well-known POP

357 widespread in the global environment⁴⁹⁻⁵¹ and one of the few POPs preferentially analysed by
358 ESI.⁵² It was detected in all blubber extracts at concentrations in the 0.01 to 4 µg/g lw range. The
359 technical product HBCDD consists of three major isomers with γ-HBCDD being the predominant
360 one.⁴⁷ In agreement with other studies, however, the α-isomer was the most abundant and the only
361 HBCDD isomer detected in the samples.⁴⁸

362
363 **Series #3 – MHC-1.** The third most abundant cluster observed in the study ($t_R=13.51$ min, $C_{\Sigma Int}$
364 $\sim 0.45 \times 10^9$) was detected in the harbour porpoise Pp_2 extract. When neutralised with a proton,
365 the base peak at m/z 458.8542 corresponded to the formula $[C_{12}H_{16}Br_2Cl_3O_2]^-$ ($\Delta mDa=0.16$,
366 score=96%, DBE=2). This mixed pentahalogenated compound was also detected in all other
367 extracts except those from fin whales. The LC/ESI-MS spectrum also featured a $[C_{10}H_{12}Br_2Cl_3]^-$ ion
368 ($\sim 4\%$ relative intensity), suggesting a pair of acetate adduct and pseudo-molecular ions. Therefore,
369 the deduced molecular formula ($C_{10}H_{13}Br_2Cl_3$) of the compound corresponded with the one of
370 MHC-1, a HNP.⁵³ MHC-1, a monoterpene insensitive to concentrated sulphuric acid,²⁸ was
371 previously reported in various marine environmental (including mammals) and food samples, as
372 well as in human milk.^{28,54,55} Subsequent LC/ESI-HRMS analysis of the harbour porpoise Pp_2
373 extract and MHC-1 standard solution resulted in a good match of retention times and MS/HRMS
374 fragmentation patterns (Figure 2). Conversely, only $[C_2H_3O_2]^-$, $[Br]^-$ and $[C_2H_4O_2 + Cl]^-$ ions arose
375 from the LC/ESI-MS/MS fragmentation of the $[M + C_2H_3O_2]^-$ adduct of MHC-1.

376 Of the five species studied, fin whale has the lowest trophic level. This species feeds almost
377 exclusively on an invertebrate species, the euphausiid krill *Meganyctiphanes norvegica*, which
378 lives in deep waters and hence may not be in proximity of the MHC-1 natural producer, the pelagic
379 red seaweed *Plocamium sp.*²⁸. Previous research indicated that the abundance of MHC-1 in the

380 environment difficult to predict.⁵⁶ Here, MHC-1 concentrations were estimated up to 1.3 µg/g lw
381 in harbour porpoise Pp_2. Interestingly, harbour porpoise Pp_2 and bottlenose dolphin Tt_1
382 extracts also featured a Br₃Cl₂ homologue (t_R=13.87 min) as acetate adduct at ~1% intensity
383 relative to MHC-1. Both species showed a coastal habitat with a high trophic level among the
384 species studied.

385

386 **Other series.** For series #4 to #16, carbon numbers ranged from 10 to 21 and DBE from 2 to 10.
387 All representative ions featured between 6 and 11 Cl, but no Br (Table 3). This could be due to the
388 fact that Br-containing HNPs tend to be unstable in sulphuric acid.²⁸ Series #4 and #5 were
389 remarkable because of their high number of carbons and masses which are at (or above) the limit
390 of GC analysis. This may explain why no information could be found for these compounds in the
391 consulted scientific literature. Series #4, #5 included mixed Cl/Br-containing homologues. This
392 may indicate that they were also HNPs,¹⁴ while the other series would rather be of anthropogenic
393 origin. Only three series originated from a single compound (#10, #12, #15), while the other
394 indicated compound families of different complexity (e.g. #9 with 4 homologue groups and many
395 isomers). Some series exhibited both pseudo-molecular and acetate adduct ions (#11, #13). MS²
396 experiments were performed for selected representative ions (#4-9, #12), revealing that some were
397 acetate adducts (#6-8, #12). Thus, attention must be paid to the fact that, beyond 2 oxygen atoms
398 and considering the mobile phase, observed ions could correspond to acetate adducts, even if no
399 co-eluting ion is observed. Eventually, at least 8 out of 13 series contained 1 or 3 oxygen atoms
400 according to LC/ESI-MS. Members of series #5 eluted between 29.6 and 35.0 min and exhibited
401 pseudo-molecular and nitrate ions at similar relative intensities, with DBE=10. Slight in-source
402 fragmentation was observed for the representative [C₂₁H₁₇Cl₁₀]⁻ ion. MS² experiments confirmed

403 the nitrate adduct and showed successive HCl losses down to $[C_{21}H_8Cl_1]$. Successive HCl losses
404 were observed for other series (#4, #10).

405 Database searches in the CompTox Chemistry Dashboard⁵⁷ and SciFinder returned a few hits,
406 allowing to increase confidence level up to 3 (*tentative candidate*)³⁷ for series #6 (chlordene
407 chlorhydrin), #7 and #8 (carbonylic derivative and monohydro analogue of Dechlorane 603,
408 respectively), #9 (polychlorinated hydroxyl-biphenyls (OH-PCBs) or diphenyl ethers, and a
409 tetrabromo hydroxyl-diphenylether), #12 (chlordane) and #16 (nonachlorobornane). For series #6,
410 Chlordene chlorhydrin has been reported as a biotransformation product of nonachlor and
411 chlordane (see series #12) in terrestrial mammals and fish.⁵⁸⁻⁵⁹ Concerning series #7 and #8, Liu et
412 al.⁶⁰ suggested the presence of a carbonylic derivative and a monohydro analogue of Dechlorane
413 603, using GC-MS instruments, in peregrine falcon eggs and shortfin mako shark livers from the
414 Western North Atlantic regions. Within series #8, a homologue compound observed at 21.18 min
415 (acetate adduct ion) exhibited the same ion formula as an acetate adduct ion of Dechlorane 603.
416 Further experiments (full scan and MS²) showed that Dechlorane 603 standard was amenable by
417 LC/ESI-MS as acetate adduct ions. However, retention times did not match (5 min shift). Still,
418 both compounds remain isomers. Eight authentic standards of OH-PCBs were injected and
419 provided matches with 2 isomers (3-OH-pentaCB-101 and 3-OH-hexaCB-153), showing that
420 series #9 corresponded to chlorinated hydroxyl-biphenyls (OH-tetra- to heptaPCBs). For series
421 #16, nonachlorobornane was confirmed using authentic standard (level 1) in the bottlenose dolphin
422 Tt_1 extract as B9-1679 (nonachlorobornane isomer), a component of the legacy POP toxaphene,
423 by both LC/ESI(-)-HRMS and GC/EI(+)-HRMS. The presence of B9-1025 was also confirmed by
424 GC/EI(+)-HRMS. Series #15 ($C_{10}H_9Cl_9O$) corresponds to a hydroxylated toxaphene metabolite.
425 This substance class, polychlorinated hydroxybornanes, was recently detected in fish.⁵⁸⁶¹

426 Interestingly as well, ten series represented compounds with an even carbon number while six (#4,
 427 #5, #7, #8, #11, #13) had odd carbon numbers. Odd carbon numbers are rather unusual and
 428 uncommon for anthropogenic POPs.

429

430 **Table 3.** Summary of cluster series characteristics, including their most intense representative
 431 ions. t_R : retention time; DBE: double bond equivalent when considering the neutral mass with an
 432 additional proton.

Series	Representative ion	t_R (min)	Proposed molecular formula	Comments
#1	$[C_8Cl_7N_2]^-$	12.53	$C_8HCl_7N_2$	Confirmed Cl ₇ -BP; BrCl ₆ ⁻ , Br ₂ Cl ₅ ⁻ , Cl ₆ ⁻ and Br ₆ Cl ₁ ⁻ homologues
#2	$[C_{12}H_{17}Br_6]^-$	15.40	$C_{12}H_{18}Br_6$	Confirmed α-HBCDD
#3	$[C_{12}H_{16}Br_2Cl_3O_2]^-$	13.51	$C_{10}H_{13}Br_2Cl_3$	Confirmed MHC-1; acetate adduct; Br ₃ Cl ₂ homologue
#4	$[C_{19}H_{13}Cl_6O_3]^-$	19.55	-	DBE=10; Cl ₅ ⁻ and BrCl ₅ ⁻ homologues; MS ² : HCl losses, C ₁₈ and C ₇ fragments
#5	$[C_{21}H_{17}Cl_{10}]^-$	33.28	-	DBE=8; high t_R ; shows HNO ₃ adduct; Cl ₉ ⁻ , Cl ₈ ⁻ , BrCl ₉ ⁻ , BrCl ₈ ⁻ and Br ₂ Cl ₈ ⁻ homologues; in-source fragments; MS ² : HCl losses

#6	$[\text{C}_{12}\text{H}_{10}\text{Cl}_7\text{O}_3]^-$	11.91	$\text{C}_{10}\text{H}_7\text{Cl}_7\text{O}$	DBE=4; isomers; Cl_6^- and Cl_8^- homologues; MS^2 : acetate adduct (chlordene chlorohydrin as tentative candidate)
#7	$[\text{C}_{19}\text{H}_{10}\text{Cl}_{11}\text{O}_3]^-$	18.14	$\text{C}_{17}\text{H}_7\text{Cl}_{11}\text{O}$	DBE=9; Cl_{10}^- homologues; MS^2 : acetate adduct (carbonylic derivative of Dechlorane 603 as tentative candidate)
#8	$[\text{C}_{19}\text{H}_{12}\text{Cl}_{11}\text{O}_2]^-$	18.61	$\text{C}_{17}\text{H}_9\text{Cl}_{11}$	DBE=8; isomers; Cl_{10}^- and Cl_{12}^- homologues; MS^2 : acetate adduct (monohydro analogue of Dechlorane 603 as tentative candidate)
#9	$[\text{C}_{12}\text{H}_4\text{Cl}_5\text{O}]^-$	11.06- 13.41	$\text{C}_{12}\text{H}_5\text{Cl}_5\text{O}$	DBE=8; isomers; confirmed Cl_5^- and Cl_6^- -OH-PCBs suspected Cl_4^- and Cl_7^- -OH-PCBs, and Br_4^- -OH-BDE
#10	$[\text{C}_{12}\text{H}_{11}\text{Cl}_{10}\text{O}]^-$	15.56	$\text{C}_{12}\text{H}_{12}\text{Cl}_{10}\text{O}$	DBE=2; unique; MS^2 : HCl losses and C_6 fragments
#11	$[\text{C}_{15}\text{H}_{15}\text{Cl}_8\text{O}_3]^-$	13.39	$\text{C}_{13}\text{H}_{12}\text{Cl}_8\text{O}$	DBE=4; shows acetate adduct; Cl_9^- homologue
#12	$[\text{C}_{12}\text{H}_9\text{Cl}_8\text{O}_2]^-$	14.61	$\text{C}_{10}\text{H}_6\text{Cl}_8$	DBEU=4; unique; MS^2 : acetate adduct of $\text{C}_{10}\text{H}_6\text{Cl}_8$; most likely chlordane as tentative candidate
#13	$[\text{C}_{15}\text{H}_{16}\text{Cl}_9\text{O}_3]^-$	14.57	$\text{C}_{13}\text{H}_{13}\text{Cl}_9\text{O}$	DBE=3; shows acetate adduct; Cl_8^- and Cl_7^- homologues; Likely related to #11 (samples, Cl patterns, 1 unsaturation difference)
#14	$[\text{C}_{12}\text{H}_{13}\text{Cl}_8\text{O}_2]^-$	14.94	-	DBE=2; Cl_7^- and Cl_6^- -homologues
#15	$[\text{C}_{10}\text{H}_9\text{Cl}_8\text{O}]^-$	14.56	-	DBE=2; unique

#16	[C ₁₀ H ₈ Cl ₉] ⁻	16.95	C ₁₀ H ₉ Cl ₉	DBE=2; confirmed B9-1679 nonanhloronornane
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433

434 CONCLUDING REMARKS

435 The applied NTA analytical strategy combining LC/ESI(-)-HRMS and an open-source post-
436 acquisition data treatment software proved to be successful in highlighting the presence of
437 abundant polyhalogenated substances in blubber of marine mammals. Precise mass information
438 allowed determining 135 unequivocal molecular formulas for 466 ion clusters of interest, which
439 were further grouped and reduced to 16 consistent series of compound classes. Literature searches
440 allowed to hypothesise a few chemical structures. Interestingly, the compound spectrum detected
441 via LC/ESI-HRMS in marine mammal blubber varied strongly from and was complimentary to
442 the one described by NTA methods via GC/MS.^{12,31,62-63} Despite the knowledge of the molecular
443 formulas, only part of the compounds could be tentatively annotated with known compounds,
444 mostly known from GC analysis such as the B9-1679 nonachlorobornane (toxaphene), chlordan,
445 OH-PCBs or Dechlorane 603 analogues. Eventually, the most intense Cl₇-BP, α-HBCDD, MHC-1
446 and less intense OH-PCBs and B9-1679 were identified at confidence level 1 (*confirmed*
447 *structure*³⁹). On the other hand, compounds like polyhalogenated *N*-methyl-bipyrroles could not
448 be detected by LC/ESI-HRMS. These results confirm that GC- and LC-based NTA methods are
449 complementary, and that a range of polyhalogenated compounds will likely be overlooked when
450 only one method is used. In this line, the water fraction used to neutralise the acidic extract may
451 contain polar HOCs which could be investigated. Similarly, GPC would help investigating acid-
452 sensitive compounds.

453 The results of this study, including the very first reporting of Cl₇-BP which turned out to be the
454 most abundant polyhalogenated compound in the datasets, suggest that implementing this strategy
455 would expand the knowledge of polyhalogenated compounds in the environment. Especially, for
456 series #4 to #15, further in-depth work could help expressing and testing structural hypotheses
457 based on interrogation of other databases as well as performing complimentary MS analyses on
458 collected fractions, and at best comparing them with analytical standards. Also LC fractions could
459 be collected and analysed by bioanalytical methods in order to assess the toxicology.

460 In terms of perspectives for the data mining, HaloSeeker could offer further automation and
461 higher deconvolution by reconstituting mass spectrum (pairing co-eluted ions adducts/fragments)
462 and/or by aligning clusters from one sample to another, opening the way to realistic investigation
463 of long sequences with ionisation mode leading to complex spectra. A blank subtraction filter
464 would also be valuable. Still, the manual review of deconvoluted data remains the best way to
465 guarantee high quality output. Indeed, for example, although the parameters have been optimised
466 to minimise ghost peaks, it is likely that some will always occur and pass the filters. Connexions
467 with internal and external databases would also help in annotating more rapidly signals from
468 known compounds, including spiked standards.

469

470 ASSOCIATED CONTENT

471 **Supporting Information.** The following files are available free of charge (PDF file). Detailed
472 description of targeted POPs in harbour porpoises Pp_2 and Pp_3; additional QA/QC elements;
473 TICs of all samples and EICs of alkyl sulphates; detailed results of enumeration of features and
474 clusters; illustrative H/Cl-scale MD-plots for harbour porpoise Pp_1; complete table of clusters
475 with annotated formula; Qualitative figures related to formula annotation; detailed cluster series
476 tables are provided.

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481 **Author Contributions**

482 The manuscript was written through contributions of all authors. All authors have given approval
483 to the final version of the manuscript.

484 **Funding Sources**

485 This research was funded by the French General Directorate for Food as well as the European
486 Union's Horizon 2020 research and innovation programme HBM4EU under Grant Agreement
487 No. 733032.

488 **Notes**

489 The authors declare no competing financial interest.

490 ACKNOWLEDGMENT

491 The authors want to express their acknowledgments to all the members of the French national
492 stranding network for their assistance in the field and data collection processes. Among
493 reviewers, we particularly acknowledge reviewer #2 for the SciFinder search.

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