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

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

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

EXPERIMENTAL

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

Chemicals. Hexabromocyclododecane (HBCDD) labelled isomers ($^{13}C_{12}$-γ and $^{2}H_{18}$-β), Dechlorane 603 and five polychlorinated hydroxyl-biphenyls (OH-PCBs: 4-OH-pentaCB-107, 4-OH-hexaCB-130, 3-OH-hexaCB-137, 4-OH-hexaCB-146, 3-OH-hexaCB-153) were purchased from Wellington Laboratories (Guelph, Ontario, Canada). Three other OH-PCBs (3-OH-pentaCB-101, 4-OH-pentaCB-101, 5-OH-hexaCB-138) were obtained from AccuStandard (New Haven, CT, USA). Tetradecyl and hexadecyl sulphate sodium salts were obtained from Sigma-Aldrich (Saint-Louis, MO, USA). The 2-endo,3-exo,5-endo,6-exo,8,8,9,10,10-nonachlorobornane (Parlar 50 or B9-1679) and the 2,2,5,5,8,9,9,10,10-nonachlorobornane (Parlar 62 or B9-1025) were provided by Cambridge Isotope Laboratories (Tewksbury, MA, USA). Pure (1*R*,2*S*,4*R*,5*R*,1'*E*)-2-bromo-1-bromomethyl-1,4-dichloro-5-(2'-chloroethenyl)-5-methylcyclohexane (MHC-1) was previously isolated from red seaweed *Plocamium cartilagineum*.[28] The 2,3,3´,4,4´,5,5´-heptachloro-MBP (Q1 or Cl_7-MBP) was synthesized according to Wu et al.,[29] while the 3,3´,4,4´,5,5´-hexachloro-DBP (Cl_6-DBP) and the 3,3´,4,4´-tetrabromo-5,5´-dichloro-DBP (BC-10 or Br_4Cl_2-DBP) were synthesized according to Martin et al.[30] A synthesis raw product (purity ~70%) of 2,3,3´,4,4´,5,5´-heptachloro-1,2′-bipyrrole (desmethyl-Q1, DQ1 or Cl_7-BP), was produced by silylation of pyrrole, coupling with *N*-chlorosuccinimide, chlorination with $POCl_3/PCl_5$, removal of the protection group and initial chromatographic purification (Wu et al., in prep). Solvents and other chemicals used for sample preparation are described in the Supporting Information.

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

not considered in order to avoid any bias linked to the transfer of POPs to their offspring during gestation and especially lactation. Therefore, for this pilot study, two adult males from five species with very different feeding ecologies were selected, without any previous knowledge on the body burden of environmental contaminants: *Phocoena phocoena* (Pp, harbour porpoise), *Balaenoptera physalus* (Bp, fin whale), *Tursiops truncatus* (Tt, bottlenose dolphin), *Phoca vitulina* (Pv, harbour seal) and *Physeter macrocephalus* (Pm, sperm whale) (Table 1). Additionally, two harbour porpoise individuals (Pp_2 and Pp_3) exhibiting relatively high levels of legacy POPs were included to enrich the analytical fingerprints. These two samples belonged to another set of samples (harbour porpoise, n = 66) for which a set of POPs were previously quantified in blubber (unpublished results) according to ISO 17025 accredited methods. Harbour porpoise Pp_2 exhibited the highest level of dioxin-like polychlorinated biphenyls (dl-PCBs, OMS-TEQ2005 = 216 pg/g lipids weight, lw) and non-dioxin-like PCBs ($\sum_6$ndl-PCBs = 199 μg/g lw) while harbour porpoise Pp_3 the highest level of polybromobiphenyl 153 (PBB-153, 83 ng/g lw). Details concerning stranding and animal sizes are provided in Table S1.

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

Species	Acronym	Collection year	Zone	Municipality	Length
Phocoena phocoena	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
Balaenoptera physalus	Bp_1	2010	BB	Moliets-et-Maa	7.73
	Bp_2	2018	BB	Naujac-sur-Mer	16.92
Tursiops truncatus	Tt_1	2009	BB	Pornichet	1.85
	Tt_2	2018	BB	La Teste-de-Buch	3.23
Phoca vitulina	Pv_1	2017	EC	Grandcamp-Maisy	1.58
	Pv_2	2016	EC	Saint-Vaast-la-Hougue	1.18
Physeter macrocephalus	Pm_1	2001	BB	Mimizan	10.45
	Pm_2	2016	EC	Marck	13.85

Sample preparation. Glassware (flasks, tubes, vials, Pasteur pipettes) was baked at 400 °C for 4 h and Teflon tube caps rinsed with dichloromethane prior to use to minimize procedural contamination. The entire blubber thickness was extracted by Pressurized Liquid Extraction (SpeedExtractor, E-914, Büchi) with toluene/acetone 7:3 (*v*/*v*). An aliquot of extracted lipids (500 mg) was suspended in 10 mL *n*-hexane and fortified with 20 μL toluene containing 20 ng $^{13}C_{12}$-γ-HBCDD as internal standard (IS). The extract was partitioned with 1.5 mL concentrated sulphuric acid added dropwise to remove lipids. Although it was previously found that removal of lipids by gel permeation chromatography (GPC) only, as opposed to acid treatment, resulted in the 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 μL toluene with 10 ng
160 $^{2}H_{18}$-β-HBCDD as recovery standard (RS). Finally, the extract was reconstituted in methanol/water
161 4:1 (*v*/*v*, 62.5 μL). 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 μL) 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 C_{18}-like analytical column (Hypersil Gold, 100 mm × 2.1 mm,
169 1.9 µm, 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 ($[CH_3COO\cdot(NaCH_3CO_2)_3]^-$) 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-MS^2 mode
180 according to the same chromatographic and ionisation parameters, with adjusted injection volume.
181 Precursor ions were selected at ± 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 × 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-programing environment avoids any interactions with the coding component while allowing

interactions with the data, including peak detection, deconvolution, and comprehensive manual review for chemical formula annotation. Briefly, proprietary raw data were converted in an *mzXML* open format using the MSConvert[32] software (proteowizard version 3.0.9810). Then, the application proceeded to the peak picking step using *xcms* 3.2.0 package[33] (mzTol=3, snthresh=10, prefilter step=3, peakwidth=10-60, prefilter level=20,000, noise=0, mzdiff=0.001). Obtained features were paired according to precise mass differences between naturally occurring C, Cl and Br isotopes, according to a script adapted from Cariou et al.[34], reconstituting halogenated isotopic patterns in so-called clusters (tolerances: t_R=1 s, *m/z*=0.5 mDa). H/Cl-scale mass defect plots (MD-plot) were drawn, using the data filters. Indeed, HaloSeeker 1.0 classifies features and clusters according to four filters. Among all features (F0 filter), those which are paired according to precise mass differences between naturally occurring C, Cl and Br isotopes belong to the F1 filter. Considering the feature triplets including the base peak A and the adjacent A-2 and A+2 isotopologues, additional ion ratio rules restrict to F2 and F2+ filters, for halogenated and polyhalogenated clusters, respectively.[26] A graphical representation of the successive filters is available in the glossary of the Supporting Information. Cumulative intensity of features paired in a cluster ($C_{\sum Int}$) was used for comparisons. Finally, the clusters of interest were investigated using the interactive pop-up window offering a formula decomposition script adapted from the Rdisop package[35] to consider either the peak of lowest *m/z* (likely the monoisotopic peak) or preferentially the most abundant isotopologue (likely the base peak). Chemical formulas were annotated by the user considering H, C, N, O, S, Cl and Br up to 50, 30, 10, 10, 10, 15 and 10 elements, respectively. The double bond equivalent (DBE) was deduced considering neutralisation with an additional proton. Ion ratio tolerance of the scoring tool was set at 20%. Theoretical centroid isotopic patterns

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

RESULTS AND DISCUSSION

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

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

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

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

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

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

Among the 466 clusters, no cluster of interest other than labelled standards was detected in procedural blanks. In each blubber dataset, 4 to 94 clusters of interest were annotated with a polyhalogenated compound (Table 2). Compared to the total intensity of features (31-683×10^6 AU of cumulative $C_{\Sigma Int}$), they represented only 0.04 to 1.67% in sperm whale, harbour seal, fin whale and the two harbour porpoises randomly selected, compared to 4-8% in bottlenose dolphins and 12-24% in the two harbour porpoises contaminated with relatively high levels of legacy POPs. Although more samples are required to refine such levels and proportions, these differences support species-specific variabilities in the levels of polyhalogenated substances amenable by LC/ESI in blubber after acidic degradation.[29] They also support that high contamination levels with legacy POPs correlate with multi-exposures.[40,41]

Table 2. Enumeration of features and clusters according to HaloSeeker filters. Feature and cluster intensities ($C_{\Sigma Int}$) as ×10^6 AU; $C_{\Sigma Int}$ threshold: 10^6 AU; t_R range: 7-37 min; F0: all features; F2+: paired clusters complying polyhalogenated ion ratio rules.

	F0		F2+ with annotated formula		
Sample	**Features**	**Intensity**	**Features**	**Clusters**	**Total $C_{\Sigma Int}$**
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

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

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

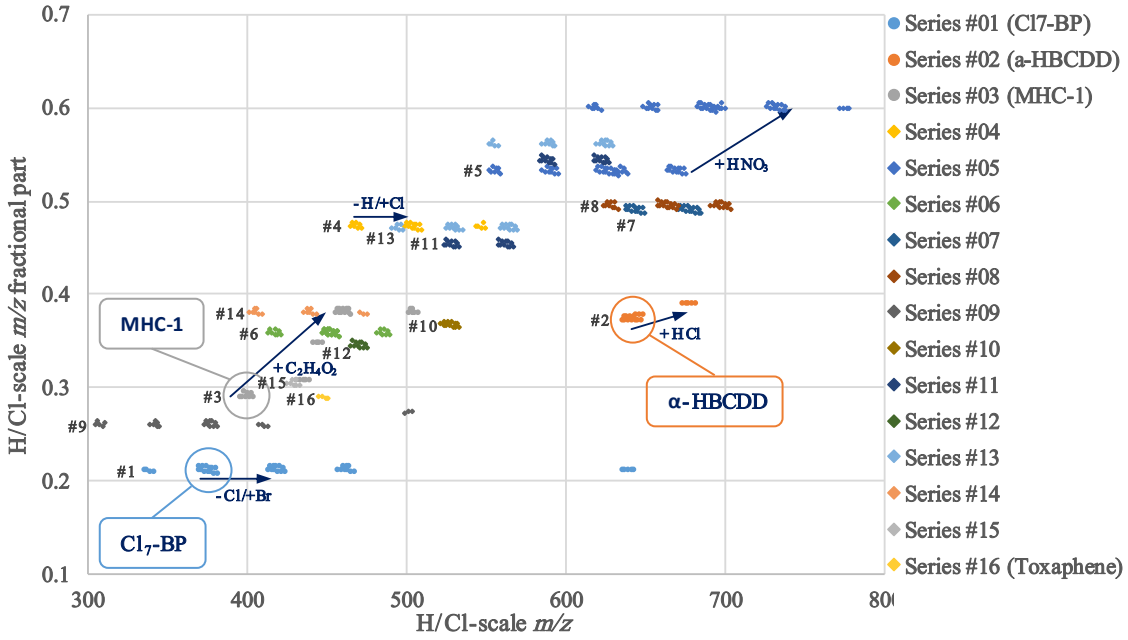
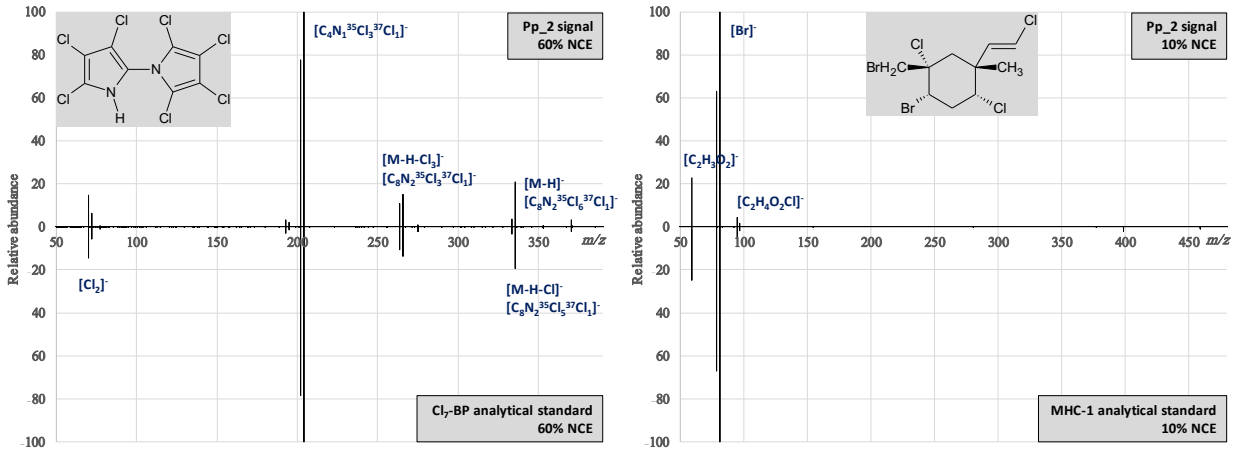


Figure 1. H/Cl scale mass defect plot of most intense representative clusters among all marine mammal datasets for each series. Arrows: adduct or homologue vectors. Each dot represents an isotopologue (feature), clusters of dots represent paired isotopologues (isotopic pattern), horizontally aligned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

Series #1 – Cl_7-BP, a new non-methylated 1,2′-bipyrrole detected. The most abundant cluster of interest ($C_{\Sigma Int}$ ~1.04×10^9) was detected in harbour porpoise Pp_2 extract. The most abundant isotopologue peak, observed at *m/z* 370.7858, corresponded with the molecular formula $[C_8{}^{35}Cl_6{}^{37}Cl_1N_2]^-$ (ΔmDa=0.06, score=95%, DBE=6). This N_2-containing compound was also detected in other extracts, except sperm whale for which matrix effect from alkyl sulphates may have hampered its ionisation. Assuming $[M - H]^-$, the formula differed by CH_2 from Cl_7-MBP, a HNP. Hence, it was suspected to correspond to the fully chlorinated heptachloro-1,2′-bipyrrole (Cl_7-BP). Analysis of a raw synthesis product of Cl_7-BP resulted in a full match in retention time and MS/HRMS fragmentation patterns (Figure 2). More precisely, fragmentation of the

317 [M – H]$^-$ pseudo-molecular ion of Cl_7-BP was observed as losses of Cl and Cl_3, cleavage of the
318 1,2'-bond with preferential charge retention on the Cl_4-containing pyrrole moiety (C_4Cl_4N,
319 monoisotopic peak *m/z* 201.8790), as well as $[Cl_2]^-$ ions. In addition, mixed halogenated
320 homologues of Cl_7-BP (Br_1Cl_6-, Br_2Cl_5-BPs) were also detected at slightly lower t_R, with 9-14%
321 and 1.6-2.0% LC/ESI-MS abundance relative to Cl_7-BP, respectively. Several unresolved isomers
322 were visible on EICs. This series accounted for more than 40% of cumulative $C_{\Sigma Int}$ 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 Cl_7-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 Cl_7-BP has never been reported before in environmental samples. Instead, the corresponding
332 Cl_7-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 Cl_7-MBP and Br_4Cl_2-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 Cl_7-BP,
340 Cl_7-MBP, Cl_6-DBP and Br_4Cl_2-DBP, which were available as standards. Polyhalogenated *N*-
341 methyl-bipyrroles were unambiguously identified by GC/EI-HRMS, with Cl_7-MBP (~1.7 µg/g lw)
342 being about three order of magnitude more abundant than Cl_6-DBP and Br_4Cl_2-DBP, but Cl_7-BP
343 (the predominant compound in LC/MS) could not be detected. Thus, a fraction centred about the
344 LC retention time of Cl_7-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 Cl_7-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 Cl_7-BP could
348 be relatively sensitive to such a phenomenon. It might explain why Cl_7-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, Cl_7-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, $C_{\sum Int}$
356 ~0.9×10^9 AU in harbour porpoise Pp_3 extract, ΔmDa=0.34). HBCDD is a well-known POP

357 widespread in the global environment[49–51] 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, $C_{\sum Int}$
364 ~0.45×10^9) 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 $[C_{12}H_{16}Br_2Cl_3O_2]^-$ (ΔmDa=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 (~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

environment difficult to predict.[56] Here, MHC-1 concentrations were estimated up to 1.3 µg/g lw in harbour porpoise Pp_2. Interestingly, harbour porpoise Pp_2 and bottlenose dolphin Tt_1 extracts also featured a Br_3Cl_2 homologue (t_R=13.87 min) as acetate adduct at ~1% intensity relative to MHC-1. Both species showed a coastal habitat with a high trophic level among the species studied.

Other series. For series #4 to #16, carbon numbers ranged from 10 to 21 and DBE from 2 to 10. All representative ions featured between 6 and 11 Cl, but no Br (Table 3). This could be due to the fact that Br-containing HNPs tend to be unstable in sulphuric acid.[28] Series #4 and #5 were remarkable because of their high number of carbons and masses which are at (or above) the limit of GC analysis. This may explain why no information could be found for these compounds in the consulted scientific literature. Series #4, #5 included mixed Cl/Br-containing homologues. This may indicate that they were also HNPs,[14] while the other series would rather be of anthropogenic origin. Only three series originated from a single compound (#10, #12, #15), while the other indicated compound families of different complexity (e.g. #9 with 4 homologue groups and many isomers). Some series exhibited both pseudo-molecular and acetate adduct ions (#11, #13). MS^2 experiments were performed for selected representative ions (#4-9, #12), revealing that some were acetate adducts (#6-8, #12). Thus, attention must be paid to the fact that, beyond 2 oxygen atoms and considering the mobile phase, observed ions could correspond to acetate adducts, even if no co-eluting ion is observed. Eventually, at least 8 out of 13 series contained 1 or 3 oxygen atoms according to LC/ESI-MS. Members of series #5 eluted between 29.6 and 35.0 min and exhibited pseudo-molecular and nitrate ions at similar relative intensities, with DBE=10. Slight in-source fragmentation was observed for the representative $[C_{21}H_{17}Cl_{10}]^-$ ion. MS^2 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 Dashboard[57] 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 MS^2) 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]

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

Table 3. Summary of cluster series characteristics, including their most intense representative ions. t_R: retention time; DBE: double bond equivalent when considering the neutral mass with an 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_2Cl_5-, Cl_6- and Br_6Cl_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_3Cl_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_2Cl_8- homologues; in-source fragments; MS^2: HCl losses

#6	$[C_{12}H_{10}Cl_7O_3]^-$	11.91	$C_{10}H_7Cl_7O$	DBE=4; isomers; Cl_6- and Cl_8- homologues; MS^2: acetate adduct (chlordene chlorohydrin as tentative candidate)
#7	$[C_{19}H_{10}Cl_{11}O_3]^-$	18.14	$C_{17}H_7Cl_{11}O$	DBE=9; Cl_{10}- homologues; MS^2: acetate adduct (carbonylic derivative of Dechlorane 603 as tentative candidate)
#8	$[C_{19}H_{12}Cl_{11}O_2]^-$	18.61	$C_{17}H_9Cl_{11}$	DBE=8; isomers; Cl_{10}- and Cl_{12}- homologues; MS^2: acetate adduct (monohydro analogue of Dechlorane 603 as tentative candidate)
#9	$[C_{12}H_4Cl_5O]^-$	11.06-13.41	$C_{12}H_5Cl_5O$	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	$[C_{12}H_{11}Cl_{10}O]^-$	15.56	$C_{12}H_{12}Cl_{10}O$	DBE=2; unique; MS^2: HCl losses and C_6 fragments
#11	$[C_{15}H_{15}Cl_8O_3]^-$	13.39	$C_{13}H_{12}Cl_8O$	DBE=4; shows acetate adduct; Cl_9- homologue
#12	$[C_{12}H_9Cl_8O_2]^-$	14.61	$C_{10}H_6Cl_8$	DBEU=4; unique; MS^2: acetate adduct of $C_{10}H_6Cl_8$; most likely chlordane as tentative candidate
#13	$[C_{15}H_{16}Cl_9O_3]^-$	14.57	$C_{13}H_{13}Cl_9O$	DBE=3; shows acetate adduct; Cl_8- and Cl_7- homologues; Likely related to #11 (samples, Cl patterns, 1 unsaturation difference)
#14	$[C_{12}H_{13}Cl_8O_2]^-$	14.94	-	DBE=2; Cl_7- and Cl_6-homologues
#15	$[C_{10}H_9Cl_8O]^-$	14.56	-	DBE=2; unique

#16	$[C_{10}H_8Cl_9]^-$	16.95	$C_{10}H_9Cl_9$	DBE=2; confirmed B9-1679 nonanhloronornane

CONCLUDING REMARKS

The applied NTA analytical strategy combining LC/ESI(-)-HRMS and an open-source post-acquisition data treatment software proved to be successful in highlighting the presence of abundant polyhalogenated substances in blubber of marine mammals. Precise mass information allowed determining 135 unequivocal molecular formulas for 466 ion clusters of interest, which were further grouped and reduced to 16 consistent series of compound classes. Literature searches allowed to hypothesise a few chemical structures. Interestingly, the compound spectrum detected via LC/ESI-HRMS in marine mammal blubber varied strongly from and was complimentary to the one described by NTA methods via GC/MS.[12,31,62-63] Despite the knowledge of the molecular formulas, only part of the compounds could be tentatively annotated with known compounds, mostly known from GC analysis such as the B9-1679 nonachlorobornane (toxaphene), chlordane, OH-PCBs or Dechlorane 603 analogues. Eventually, the most intense Cl_7-BP, α-HBCDD, MHC-1 and less intense OH-PCBs and B9-1679 were identified at confidence level 1 (*confirmed structure*[39]). On the other hand, compounds like polyhalogenated *N*-methyl-bipyrroles could not be detected by LC/ESI-HRMS. These results confirm that GC- and LC-based NTA methods are complementary, and that a range of polyhalogenated compounds will likely be overlooked when only one method is used. In this line, the water fraction used to neutralise the acidic extract may contain polar HOCs which could be investigated. Similarly, GPC would help investigating acid-sensitive compounds.

453 The results of this study, including the very first reporting of Cl_7-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

ASSOCIATED CONTENT

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

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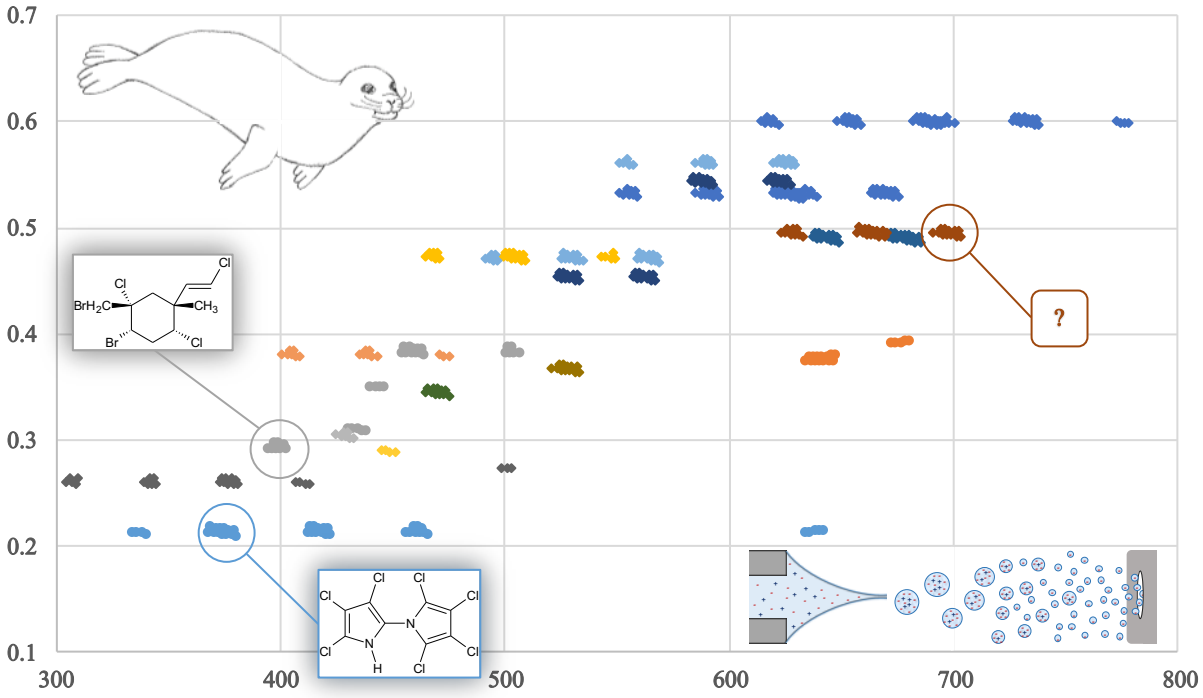

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