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Thorough study of persistent organic pollutants and halogenated natural products in sperm whale blubber through preparative sample cleanup followed by fractionation with countercurrent chromatography

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ABSTRACT

Marine mammals are top predators of the marine food chain and thus known to bioaccumulate high concentrations of polyhalogenated compounds. Yet, details of the organohalogen pattern are largely unknown. For this reason, we isolated the polyhalogenated compounds from 750 g blubber of a sperm whale (*Physeter catodon*), which deceased at the German North Sea coast in January 2016. The sample matrix was decomposed by sulfuric acid treatment and the polyhalogenated compounds were then fractionated by countercurrent chromatography (CCC). Seventy-three CCC fractions were taken and analyzed by gas chromatography with electron capture negative ion mass spectrometry (GC/ECNI-MS). The bulk of the polyhalogenated compounds in the sample originated from classic persistent organic pollutants (POPs). Altogether 90 polychlorinated biphenyl (PCB) congeners were detected in the sample including all possible octa- to decachloro congeners except one. The sample also featured 105 toxaphene congeners including 30 chlorobornenes (contribution ~14% of the total toxaphene content) which were only detected after the CCC fractionation. In addition, several chlordane and mirex related compounds were detected which were never or very scarcely described before in biota. Classic POPs (PCBs, DDT, toxaphene, chlordane, mirex) were predominant, while new emerging contaminants were scarcely detected. The sample featured several halogenated natural products (HNPs) some of which were less stable and destroyed during the sample cleanup involving treatment with sulfuric acid. Sample fractionation by means of CCC was crucial for the detection and assignment of many of the uncommon polyhalogenated compounds.

1. Introduction

Many polyhalogenated compounds are persistent, bioaccumulative, and toxic to living organisms [1]. For this reason, 22 compounds and compound classes were classified as persistent organic pollutants (POPs) by the Stockholm Convention on POPs from 2001 to 2017 [2]. This international plenary board aims to ban (or restrict) synthesis and application of compounds eventually classified as POPs [2]. Marine mammals, top predators of the marine food chain, are known to bioaccumulate particularly high POP levels in their blubber tissue [3]. While concentrations of POPs in marine mammals are widely documented, surprisingly little is known about the detailed contaminant pattern of polyhalogenated compounds. For instance, only 130 of the 209 theoretically possible polychlorinated biphenyl (PCB) congeners have been detected in all technical products together [4]. Still, the total number of PCB congeners and those of other POPs in marine top predators is barely known. This is likely due to the fact that GC/MS-SIM analyses do not provide full structure information and tend to focus on selected compounds. Therefore, non-target approaches using GCxGC-TOF-MS [5–7] and thorough GC/MS in the selected ion monitoring (SIM) mode [8,9] were introduced in order to detect previously unknown compounds. Despite the efforts made this way, structures of potentially relevant compounds remained partly undetermined because only analytical amounts of the sample (corresponding with less than 1 g lipids, which is the maximum amount that can be removed by gel-permeation chromatography) were processed.

In this study we aimed to overcome these drawbacks, by the analysis of 750 g blubber (lipid content 64%) of a sperm whale (*Physeter catodon*) which stranded and deceased at the German North Sea coast in January 2016. Lipids were decomposed and removed by repeated treatment with sulfuric acid, and

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the remaining polyhalogenated compounds were fractionated by countercurrent chromatography (CCC). CCC is a preparative, all liquid chromatographic tool predominantly used for the isolation of natural products [9–11], but its merits were also demonstrated in the field of polyhalogenated compounds in recent years [12,13]. CCC pairs good resolution power with high sample load (for non-polar compounds typically in the range of high mg-amounts). In addition, CCC can also be used for the fractionation of complex samples, where compounds with different partition coefficients are distributed in 30 or more small fractions (e.g. each about 5 mL volume or less) [14,15]. Analysis of the resulting CCC fractions by an analytical method with complementary separation characteristics such as GC/MS can be used to get deeper insights in the sample composition. Typically, major compounds in a sample elute into a few fractions, while other fractions feature minor compounds which are not detectable in the unfractionated sample [16]. For instance, this approach was used to describe >400 fatty acids in a butter sample [15] and 170 tocochromanol artefacts in an inadequately produced vitamin E dietary supplement [14]. In the present case, 73 CCC fractions were taken and analyzed by gas chromatography with electron capture negative ion mass spectrometry (GC/ECNI-MS). In combination with additional experiments and measurements in the SIM mode we wished to determine the most relevant substance classes (i.e. POPs and non-POPs including halogenated natural products (HNPs)) in this top predator of the marine food chain.

2. Materials and methods

2.1. Chemicals and standards

Bidistilled water was prepared with a Purelab Classic system (Elga, Berkfeld, Germany). Methanol (HPLC grade, >95%), acetonitrile (HPLC grade, 99.9%) and *n*-hexane for residue analysis (>99%) were from Th. Geyer (Renningen, Germany). Sigma-Aldrich delivered benzotrifluoride (BTF, >99%; Taufkirchen, Germany) as well as cyclohexane and ethyl acetate (both >99.5%, Seelze, Germany) which were azeotropically distilled (46:54, w/w). Toluene (99.5%) and sodium sulfate (>99%, water free) were from Carl Roth (Karlsruhe, Germany) while *iso*-octane (for pesticide residue analysis) and silica gel 60 (for column chromatography) were from Fluka (Taufkirchen, Germany). Acetone (distilled before use) and sulfuric acid (concentrated, mass fraction 98%) were from BASF (Ludwigshafen, Germany). Helium (purity 5.0) and nitrogen (purity 4.6) were from Westfalen (Münster, Germany) and methane (purity 5.5) was from Air Liquide (Bopfingen, Germany). Chemical names and abbreviations of all organohalogen compounds in this article are shown in Tables S1 and S2 (Supplementary information). Sources of organohalogen standards and composition of standard mixtures are reported in Tables S3–S6 (Supplementary information).

2.2. Preparation of the sperm whale sample

The sperm whale (*Physeter catodon*) stranded and deceased at Wangerooge (German North Sea coast) in January 2016. About 1 kg sample (mostly blubber with bloody parts) was provided by Nationalpark Wattenmeer (Lower Saxony, Germany). The animal was about 10–15 years old and it weighed ~12–18 t. It was of good health condition and well-nourished [17]. Autopsy indicated that it mainly consumed squid and species occurring in the Norwegian Sea (i.e. the water body north of the North Sea bordered by Norway in the east, Great Britain and Iceland in the south, Spitsbergen in the north and the Greenland Sea in the west). This area was most likely the last residence of the sperm whale [17]. Three cleanup procedures with different sample amounts were prepared for different purposes (Fig. S1, Supplementary information).

2.2.1. Macro sample (750 g blubber) for CCC fractionation

The blubber sample was cut into pieces with a multi-cutter for 3 min. The resulting solid/liquid sample was distributed into nine 500 mL separation flasks and each flask was supplemented with 200 mL *n*-hexane. Then, 50 mL sulfuric acid was carefully added to each flask (both phases immediately turned black) [18]. After one day, further 50 mL *n*-hexane and 40 mL sulfuric acid were added to support phase separation. Two days later, the acidic phases were discarded and re-newed with 40 mL sulfuric acid which again turned brown/black. This procedure was repeated with constantly smaller amounts of sulfuric acid and increasing residence time. After 16 days, the *n*-hexane phases were clear and colorless. Then, the *n*-hexane phases were combined, evaporated (~270 mbar, 35 °C) to 300 mL (further concentration could not be achieved due to boiling retardation) and treated again for 5 days with a total of 100 mL sulfuric acid. The *n*-hexane phase was separated and concentrated to 30 mL and treated with 40 mL sulfuric acid again. The total consumption of sulfuric acid was ~2.5 L. Finally, the *n*-hexane extract was washed three times with 10 mL demineralized water and set to a final volume of 10 mL. From this sample, 1 μL was removed, diluted 1:100, the internal standard 6′-methoxy-2,3′,4,4′-tetrabromodiphenyl ether (BC-IS) was added and quantification of polyhalogenated compounds was carried out by GC/ECNI-MS (Section 2.5). The remaining volume was used for CCC separation (Section 2.3.2).

2.2.2. Micro sample (8 g blubber) for the determination of partition coefficients

An 8 g-aliquot of the sperm whale sample was purified in the same way as the macro sample and finally concentrated to 1 mL. This sample solution was additionally purified by adsorption chromatography (3 g silica gel deactivated with 30% water, elution of polyhalogenated compounds with 60 mL *n*-hexane) [19]. The final volume was set to 1 mL. This sample was used for shake flask experiments (Section 2.3.1).

2.2.3. Analytical sample (0.5 g lipids) for quantification

The analytical sample was initially freeze-dried, followed by accelerated solvent extraction (ASE) [20]. Perdeuterated 1,2,3,4,5,6-aaeeee-hexachlorocyclohexane (α-PDHCH) was used as IS for recovery control. The solvent was removed, the sample was weighed and 0.5 g lipids were placed in a 5 mL flask and solved in 5 mL cyclohexane/ethyl acetate (azeotrope 46/54, w/w). Lipid removal was performed with gel-permeation chromatography (GPC). The GPC fraction was condensed to ~2 mL and the solvent was changed to *n*-hexane. The resulting solution was further purified by adsorption chromatography using deactivated silica gel (Section 2.2.2) and the volume was set to 1 mL. One half of the sample (500 μL) was used for direct GC/MS analysis while the other half was additionally fractionated on 8 g activated silica before GC/MS analysis [20]. Fraction 1 (target compounds: PCBs) was collected with 48 mL *n*-hexane, fraction 2 with 50 mL *n*-hexane/ethyl acetate, 9:1 v/v (target compounds: Chloropesticides, HNPs (except Q1) and brominated flame retardants), and fraction 3 with 50 mL ethyl acetate (target compounds: Phenolic polyhalogenated compounds). The solvents of the fractions were changed to *iso*-octane (1.0 mL final volume).

2.2.4. Lipid content determination

One gram of the sperm whale sample was cut to small pieces and extracted with 80 mL cyclohexane/ethyl acetate, 46/54 (w/w), by open-vessel microwave-assisted extraction using a *Star System* 2 (CEM Corporation, NC) modified with a nitrogen gas supply and a

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