# DR-CALUX SCREENING OF STRANDED CETACEAN BLUBBER

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## Introduction

Since 1988, dead cetaceans found stranded around the coast of the UK have been collected and used for collaborative research. One aspect of this collaborative programme has been the analytical measurement of selected contaminants that biomagnify in aquatic food chains.[1] Studies conducted in the UK and in other countries show that persistent organic compounds such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) organochlorine pesticides (OCs), chlorobiphenyls (PCBs) and brominated diphenylethers (BDEs) have been shown to accumulate in the blubber of cetaceans. Traditionally, instrumental analytical techniques have been used to analyse for targeted compounds, however screening for the presence of stable *in vitro* aryl hydrocarbon receptor (AhR) agonists, using a bioanalytical technique such as DR-CALUX, can also provide valuable information on the complex mixture of organic compounds that can biomagnify through aquatic food webs. Due to the complexity of contaminant mixtures it is likely that AhR agonists other than those targeted through instrumental techniques may biomagnify. Here we report the analysis of stranded cetacean blubber using the DR-CALUX assay and subsequent attempts to try and identify the AhR agonists responsible for the measured activity.

## Methods and Materials

The blubber from a harbour porpoise (*Phocoena phocoena*) stranded at Newport, Wales, UK (1998) and a white beaked dolphin (*Lagenorhynchus albirostris*), stranded in the Humber estuary, UK in 1999 were obtained from R. Law (Cefas, UK). Homogenised wet tissue (20 g) was extracted using hexane/cyclohexane/dichloromethane by steam distillation. This yielded 4 fractions which were reduced to 1 ml and stored at –20 °C. **DR-CALUX® analysis.** The rat hepatoma H4IIE cell line stably transfected with luciferase reporter gene under the control of dioxin-responsive enhancers (pGudLuc 1.1) was used to screen for compounds eliciting AhR-mediated gene expression.[2] Cells for assay were seeded into the central 60 wells of a 96-well culture plate (100 µl per well, 90-100 % confluence), 24 hours before exposure. Samples in DMSO were diluted in α –MEM, and 100 µl added to each well to give a maximum DMSO concentration of 0.4 %. Samples were assayed in triplicate and compared to a 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). **Isolation of 'dioxin-like' substances**. An aliquot of each extract underwent a simple clean up procedure to remove all the labile compounds unstable to oxidative breakdown, effectively isolating the most stable compounds. The extracts were passed through a deactivated silica column that also contained $AgNO_3$ and anhydrous $Na_2SO_4$. **Normal Phase HPLC fractionation**. Normal phase HPLC was performed using a Nitro ($NO_2$)-bonded silica column that separates aromatic compounds and compounds with double bonds.[3] **GC-MS Analysis**. HPLC fractions giving a positive response when bioassay tested were analysed by gas chromatography-electron impact (EI) mass spectrometry (GC-MS), GC-Time of Flight (TOF) mass spectrometry and GC coupled to negative chemical ionisation mass spectrometry (GC-NCIMS) using methane as the reagent gas.

## Results and Discussion

The blubber extract AhR agonist potencies are shown in Table 1. The porpoise sample obtained from the Humber had an AhR agonist potency of 4.5 pg TCDD $g^{-1}$ which was present in Fraction D, whilst activity was detected in Fractions A, B and C of the white-beaked dolphin blubber at a total AhR potency of 15.2 pg TCDD $g^{-1}$ (Table 1).

Table 1 AhR agonist potency (pg TCDD $g^{-1}$) of blubber extracts

| Species | Position | Fraction | AhR agonist potency (pg TCDD $g^{-1}$) |
|---|---|---|---|
| Harbour porpoise | 52° 00.88'N 04° 50.52'W | A | <4.3 |
| | | B | <4.3 |
| | | C | <4.3 |
| | | D | 4.5 |
| | | Total | **4.5** |
| White-beaked dolphin | 53° 34.080'N 00° 05.95'E | A | 6.9 |
| | | B | 2.4 |
| | | C | 6.0 |
| | | D | <3.3 |
| | | Total | **15.3** |

The data show that the AhR agonists occurring in the blubber of these two cetaceans are chemically different. In an attempt to further characterise the AhR agonists present the sample extracts were fractionated using a selective normal phase high performance liquid chromatography (HPLC) and the fractions tested for the presence of stable AhR agonists (Figure 1). The most notable effect of fractionation was a reduction in the total AhR activity of the sample following fractionation probably due to the poor recovery of AhR agonists. Further work is required in order to improve on these recoveries in order to ensure that all classes of stable AhR agonists are fractioned with high recoveries. There are major differences between the types of compounds present in the two blubber samples, as shown by the different HPLC profiles in Figure 1. The nitrophenylpropyl bonded silica HPLC phase used, fractionates compounds by the number of double bonds that the compounds have (associated with increasing size and the $\pi$-electron density of the aromatic system). Other factors such the electron-drawing effects of halogenated susbstituents also affect fractionation. The system does allow group type fractionation of AhR agonists based on the degree of halogenation and number of double bonds (Figure 1). For example dioxins are eluted in Fraction 2, whilst Fraction 3 contains aromatic compounds with < 3 aromatic rings. Fraction 2 also contains the majority of the halogenated aromatics in the extract suggesting that PCBs, PCDDs and PCDFs are not the major group of AhR agonists present. The HPLC fractions showing AhR activity were analysed by GC-(EI)MS in an attempt to identify the AhR agonists present. Automated processing of these data failed to identify any potential AhR agonists in the isolated fractions. Further analysis by GC-negative ion chemical ionisation MS also failed to identify any known AhR agonists suggesting that they are present in the form of mixtures which are below the limits of detection of these techniques.

What these limited data do demonstrate is that a bioanalytical approach to assessing the occurrence of specific groups of contaminants has the potential to complement conventional hazard assessment and targeted chemical analysis in the identification of substances that contaminate the marine environment. The analysis of tissues allows those compounds that persist and bioaccumulate to be targeted. However, the limited success in identifying the substances responsible for the observed effects suggests that the complex matrix of tissue samples poses a greater challenge than that of other environmental matrices, such as effluents. Further development in this field will focus on the isolation of the AhR agonists shown to be present and is currently ongoing as part of the European Union Frame Work VI project MODELKEY (Models for Assessing and Forecasting the Impact of Environmental Key Pollutants on Marine and Freshwater Ecosystems and Biodiversity; www.modelkey.org).

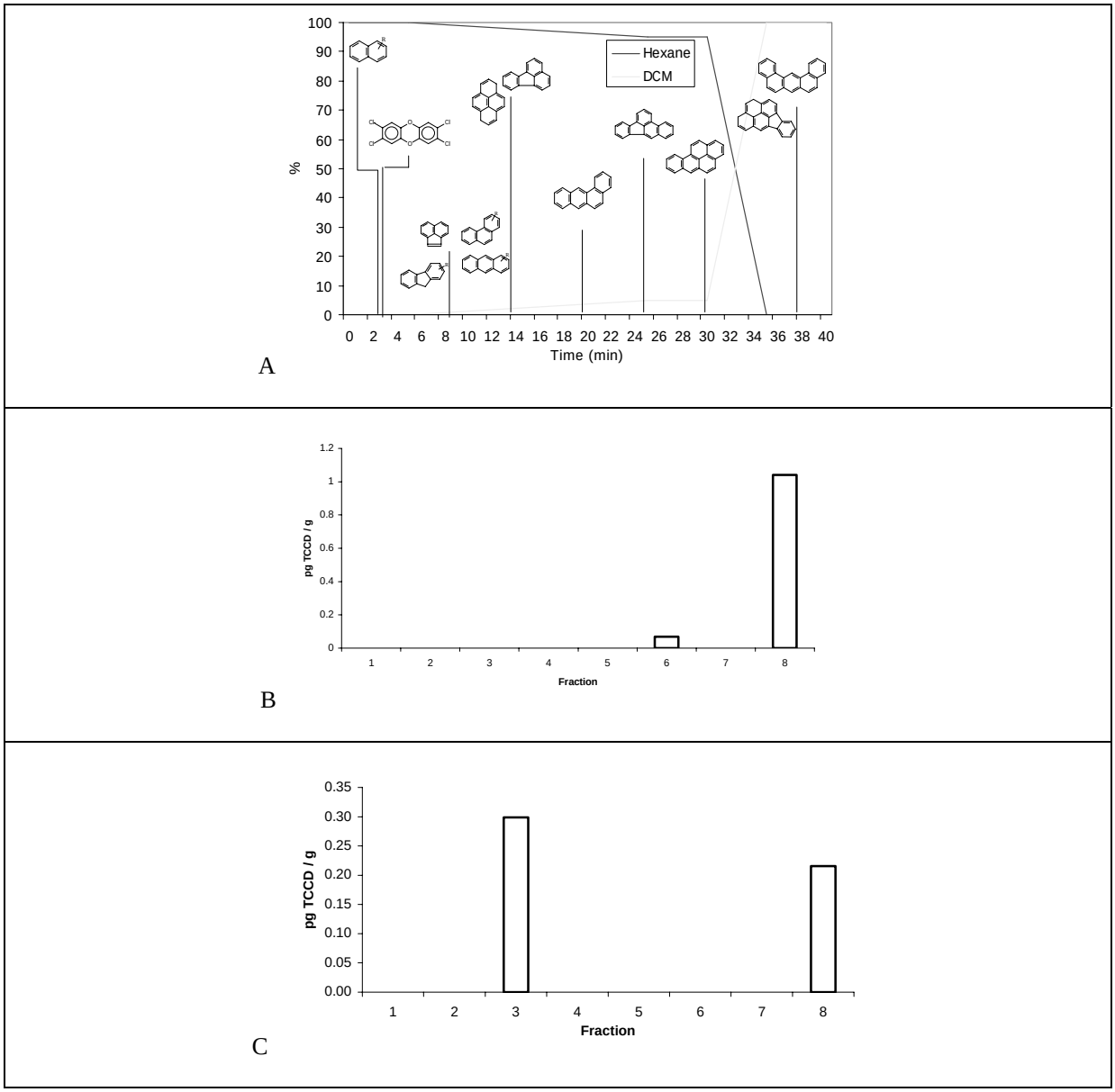


Figure 1 HPLC fractionation of blubber extracts. A Elution of reference compounds, B *in vitro* AhR activity of white beaked dolphin extract and C. AhR activity of Harbour Propoise extract.

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**References**

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