EXPERT IN GENOTOXICOLOGY

ANALYTICAL REPORT

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Title of report:

ANALYSIS OF BULKY DNA ADDUCT PATTERNS IN THE LIVER OF FISH (DIFFERENT SPECIES) HARVESTED IN TWO SITES (ONE CONTAMINATED WITH PETROLEUM HYDROCARBONS) IN THE NORTHEAST ATLANTIC OCEAN IN 2014 (WATER COLUMN MONITORING PROGRAM)

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1 PURPOSE OF THE STUDY

This analytical report consists of the analysis of the bulky DNA adduct patterns obtained in the liver of 103 fish from different species (Tusk, Ling, Saithe, Redfish), caught in two distinct sites of the Northeast Atlantic Ocean (Reference site and Oil Platform area) in 2014. This genotoxicity study forms part of the Water Column Monitoring program. The detection of the DNA adducts is realised by a nuclease P1 version of the ^{32}P post-labelling method which detailed protocol is provided in an annexe.

The document contains

1) The raw data from the 103 samples (all quantitative results and autoradiographic patterns of interest),
2) A statistical analysis based on the available information associated to the 103 samples (species, gender and site effects on DNA adduct patterns).
3) A first discussion of the results in the light of some published studies in the field.

The analysis of the overall samples was spread over a four months period (October 2014-January 2015).

2 PREPARATION OF THE DNA SOLUTIONS

After receipt, the samples were stored at -80°C until their handling of DNA extraction. Small pieces of tissue (about 100 mg per sample) were taken for the DNA extraction, an important preliminary step before the DNA adduct detection.

For each sample, a purified DNA solution was obtained by a method of phenol-chloroform / liquid-liquid extraction, after the crushing of liver pieces (tissue-lyser, Qiagen ®), isolation of cell nuclei (in sucrose 0.32M) and sample treatment with RNases A, T1 and proteinase K (**Annexe 2a**).

The DNA concentrations of extraction solutions were deduced from the absorbance (optical density) at the wavelength of 260 nm (A_{260}) (Nanodrop Technology, Thermo Scientific ®). The absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} coupled with the absorbance profiles between 230 nm and 300 nm were used to check the quality of the DNA solutions (more especially the absence of contamination by RNA and/or proteins).

In order to always work on DNA freshly extracted, the extraction of DNA was separated in time. The extracted samples were systematically analysed in ^{32}P post-labelling in the next two weeks.

The absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} obtained on the whole 103 DNA samples are of 1.90 *± 0.12* and 2.06*± 0.10* respectively. These experimental ratios are satisfying enough in regards to the usual requirements of the ^{32}P-postlabelling method.

3 ANALYSIS OF DNA ADDUCT PATTERNS BY THE ^{32}P POSTLABELLING METHOD

3.1 Materials and methods

3.1.1 The ^{32}P-postlabelling method

The detailed protocol used by ADn'tox is described in the **Annex 2b**. It is suitable for the research of so-called "bulky" DNA adducts, some additional compounds in DNA that are associated to numerous complex molecules such as certain polycyclic aromatic hydrocarbons (PAHs). Each analysis is realised from 5 micrograms DNA.

Ten manipulations (sets of analysis) were necessary in order to analyse the DNA adduct patterns of the overall **103 samples**. Two independent adduct measurements have been realised for each DNA sample. For the study, the limit of detection (LOD) is fixed to half the smallest DNA adduct level (Relative adduct level=RAL) calculated for an observed spot in a pattern, i.e. ½ x 0.02 = 0.01 adducts per 10^8 nucleotides (RAL x 10^{-8}). For analysis without detectable adducts ("null" results), the concentration in adducts is then defined as <0.01 x 10^{-8} nucleotides (**Table 1**).

In each set of analysis, DNA from both positive and negative controls are systematically included. Positive control is a calf thymus DNA exposed to benzo[a]pyrene dioepoxide (BPDE) kindly provided by F.A Beland (National Center for Toxicology Research, USA). This sample was used as a standard in large interlaboratory trials. The DNA damage level was 110.70 adducts per 10^8 normal nucleotides (according to F.A. Beland, in Philips and Castegnaro, 1999; see Divi et al., 2002 and Zhan et al., 1995 for more details). The negative control was a plasmid DNA.

The autoradiographic patterns from both positive and negative controls are provided in **the Annexe 1**. These results assure the smooth technical functioning, by the absence first of nonspecific signals (a source of false positives, frequently due to improper disposal of certain reagents/impurities used during handling) and then a correct ^{32}P labelling on a reference / standard sample. The good labelling efficiency is checked on the base of the direct level of radioactivity (Cerenkov radiation) in the major spot of the positive control, expressed in radioactive counts per minute (cpm).

3.1.2 Statistical analysis associated to DNA adduct patterns

Usually, the DNA adduct levels measured in the overall samples of a study (expressed as Relative Adduct level per 10^8 normal nucleotides (RAL x 10^{-8})) do not respect the classical normal distribution (Gaussian distribution), due mainly to the large proportion of samples without detectable adducts. This distribution leads to the inability to properly use the parametric tests for statistical analysis of the DNA adduct data, especially the very common analysis of variance (ANOVA). Then, all results are initially treated by nonparametric statistics.
The logarithmic with base 10 transformation (results in Log10) tends to standardise the data distribution for nonzero results (and outliers, i.e. sample values with extreme deviations from the mean) and authorizes the use of parametric statistics on these truncated data.

An analysis is made taking into account the presence or absence of adducts for each sample (qualitative approach of DNA adduct patterns). This approach is of interest given the semi-quantitative aspect of technique, especially in the context of measuring low levels of adducts.
The statistical analysis presented in the report is based on the use of SAS® software by Mr. Didier Pottier, engineer biostatistician at the University of Caen (EA 4651 ABTE-TOXEMAC, France).

3.2 Results from DNA adduct measurements on the 103 samples

The samples in the current study were analysed in duplicate in two separate manipulations (sets), including a total of ten ^{32}P postlabelling manipulations (sets) and 206 analyses. One positive control (benzo[*a*]pyrene diol epoxide (BPDE) + calf thymus DNA) and one negative control (plasmid DNA without detectable adducts) were added in each manipulation.

The proper conduct of each independent manipulation is validated according to the qualitative and quantitative results in the positive control (DNA rich in adducts of benzo[*a*]pyrene diol epoxide (BPDE), **Annexe 1**): pattern of adducts and direct level of radioactivity in the major spot (routinely near 17,000 cpm ± 15%). The reproducible clean pattern of the negative control (DNA without detectable adducts) confirm the absence of unwanted interfering signals that could be misattributed to adducts (prevention of false positive).

The **Table 1** and **Figure 1** present the DNA adduct results per species and per site for the 103 fish sampled during the summer 2014.

Overall, apart from a few exceptions, the measured signals attributed to DNA adducts are quantitatively low and associated to patterns with relatively few spots, whatever the site. The adduct levels or concentrations (expressed as a relative adduct level, i.e. the number of detected adducts per 10^8 normal nucleotides (RAL x 10^{-8})) are consistent with published data from environmental studies (see discussion & conclusion).
Predictably, the DNA adduct levels measured in the overall samples, considered by species and site, do not respect the classical Normal distribution, even after the logarithmic with base 10 transformation (Shapiro-Wilk test, results not shown). Therefore, all of the following statistical analyses are above all based on some non-parametric tests. Parametric tests are used in order to complete (or reinforce) the statistical results.

3.2.1 Qualitative analysis of the DNA adduct patterns

3.2.1.1 Proportion of samples without detectable DNA adducts

In this field study based on two distinct sites and four fish species, **the proportion of samples without any detectable DNA adducts in liver** is 42%, i.e. 42 samples out of 103 (**Figure 5**). The value is rather in accordance with previous results (see reports of earlier studies), probably a little higher. Interestingly, this proportion varies very significantly between the two explored sites, all species combined ($p<0.0001$, one sided Fisher's Exact Test). This proportion reaches two thirds of samples at Reference site, (REF: 32 samples out of 46, 69% of samples), while this is only 17 % of the samples at Platform (PF: 10 samples out of 57).

At each of both sites, the proportion of samples without detectable DNA adduct seems to vary among species. At the Reference site, this proportion ranges from 60% for Tusk (9 out of 15 samples) to 100% for Ling (with only 4 samples). Intermediate results are obtained for Saithe (75%, 9 out of 12 samples) and Redfish (67%, 10 out of 15 samples). At this site, the inter-species difference is not statistically significant ($p=0.5540$, Fisher's exact test). It is to note that this proportion of samples without detectable DNA adducts is consistently well above half the samples, whatever the species.

At platform site, the proportion ranges from 0% for Saithe to 40% for Ling (6 out of 15 samples without detectable adducts). Intermediate results are obtained for Redfish (7%, 1 out of 15 samples) and Tusk (20%, 3 out of 15 samples). At this site, the inter-species differences are statistically significant ($p=0.027$; Fisher's Exact test). It is to note that this proportion of samples without detectable DNA adducts is consistently well under half the samples, whatever the species.

For each of the four species, the intersite difference in the proportion of samples without detectable DNA adducts has been explored. The intersite difference is statistically significant for Redfish (67% of the samples without DNA adducts at REF, 7% at PF; $p=0.0008$, one-sided Fisher's Exact Test), Saithe (75% of the samples without DNA adducts at REF, 0% at PF; $p=0.0002$, one-sided Fisher's Exact Test) and Tusk (60% of the samples without DNA adducts at REF, 20% at PF; $p=0.0302$, one-sided Fisher's Exact Test). Because of the low number of samples at reference site, statistical analysis has not been realized for Ling (100% of the samples without DNA adducts at REF, 40% at PF).

Interestingly, the highest proportions of samples without detectable DNA adducts are associated to Ling at both sites (100% (but only 4 samples) and 40% of the samples at Reference site and Platform, respectively).

The potential **gender effect** has been evaluated on the proportion of samples without detectable DNA adducts at Platform by pooling together Saithe and Tusk samples (in order to compare 13 females and 14 males; Ling and Redfish has been disregarded due to the low numbers of males). The proportion is 0% for females and 21% (3 samples among 14) for males. This difference is not significant (p=0.2222; Fisher's Exact test).

3.2.1.2 Proportion of samples with high concentrations of DNA adducts

The samples that exhibit **mean DNA adduct concentrations greater than or equal to 0.4 adduct per 10^8 normal nucleotides (RAL ≥ $0.4x10^{-8}$)** are of particular interest (see chapter Discussion). On the overall study, these highest concentrations in DNA damages have been measured in about one third of the samples (30 samples out of the 103, i.e. 29% of all samples). Interestingly, the proportion of samples with elevated concentrations in DNA adducts is significantly different from one site to the other one, all species combined ($p<0.0001$, one sided Fisher's Exact Test). About half of the samples (27 among 57, 47%) exhibit DNA adduct levels above 0.4 adduct per 10^8 nucleotides at Platform, while this proportion is only 7% (3 samples among 46) at reference site.

At each of both sites, the proportion of samples with DNA adduct concentrations higher than 0.4 adducts x 10^{-8} seems to vary among species.

At the reference site, the proportion ranges from 0% for Tusk and Ling to 13% for Redfish (2 out of 15 samples). Intermediate results are obtained for Saithe (8%, 1 out of 12 samples). However, this interspecies difference is not statistically significant (p=0.6146; Fisher's exact test).

At platform site, the proportion of samples with elevated concentrations in DNA adducts ranges from 40% for Tusk and Ling (6 out of 15 samples for both species) to 67% for Redfish (10 out of 15 samples). Intermediate results are obtained for Saithe (42%, 5 out of 12 samples). However, at this site, the interspecies variability in proportion of samples with high liver DNA damages is not statistically significant (p=0.426, Fisher's exact test).

For each of the four species, the intersite difference in the proportion of samples that exhibit high hepatic DNA adduct concentrations has been explored. The intersite difference is statistically significant for Redfish (13% of the samples with high DNA adduct levels at REF, 67% at PF; p=0.0039, one-sided Fisher's Exact Test) and Tusk (0% of the samples without DNA adducts at REF, 40% at PF; p=0.0084, one-sided Fisher's Exact Test). The difference is at the limit of significance for Saithe (8% of the samples with high DNA adduct levels at REF, 42% at PF; p=0.0775, one-sided Fisher's Exact Test). Because of the low number of samples at reference site, statistical analysis has not been realized for Ling (0% of the samples with high DNA adduct level at REF, 40% at PF).

Interestingly, the highest proportions of samples with elevated DNA adducts levels are associated to Redfish at both sites. The lowest proportions are observed with Ling.

The potential **gender effect** has been evaluated on the proportion of samples with elevated DNA adduct levels at Platform by pooling together Saithe and Tusk samples (in order to compare 13 females and 14 males; Ling and Redfish has been disregarded due to the low numbers of males). The proportion is 54% (7 out of 13 samples) for female and 28% (4 out of 14 samples) for male. This difference is not significant (p=0.2519; Fisher's Exact test).

3.2.1.3 Qualitative analysis of DNA adduct patterns by the individual number of spots

The calculated mean number of different spots per sample (i.e. different DNA adducts, as one distinct spot equal to one distinct adduct) is 0.45 ± 0.78 at Reference site, all the species combined. It reaches 1.56 ± 1.21 for all fish sampled at Platform. This observed difference between sites is statistically significant (p<0.0001, one-sided Wilcoxon test). The number of distinct spots per sample is generally low, as 82% of the samples (47 out of 57 samples) exhibit 2 spots or less at platform. This proportion even reaches 98% (45 out of 46 samples) of the samples at reference site.

At each of both sites, the mean number of spots per sample seems to vary among species.
At the reference site, the mean number of spots per individual ranges from zero spot for Ling (n=4) to 1.1 ± 1.4 spots for Tusk. Intermediate results are obtained for Saithe (0.5 ± 0.9 spots in average) and Redfish (0.9 ± 1.5 spots in average). However, at this site, the interspecies variability in the mean number of different spots per sample is not statistically significant (p=0.4558, Kruskal-Wallis test).
At platform site, the mean number of spots per individual ranges from 1.1 ± 1.2 spots for Ling to 1.9 ± 1.3 spots for Redfish. Intermediate results are obtained for Tusk (1.5 ± 1.3 spots in average) and Saithe (1.7 ± 1.5 spots in average). However, at this site, the interspecies variability in mean number of different spots per sample is not statistically significant (p=0.230, Kruskal-Wallis test).

For each of the four species, the intersite differences in the mean number of different spots per sample has been explored. The intersite difference is statistically significant for Redfish (0.9 ± 1.5 spots in average at REF, 1.9 ± 1.3 spots at PF; p=0.0015, one-sided Wilcoxon Test), Saithe (0.5 ± 0.9 spots in average at REF, 1.7 ± 1.5 spots at PF; p=0.0010, one-sided Wilcoxon Test) and Tusk (1.1 ± 1.4 spots in average at REF, 1.5 ± 1.3 spots at PF; p=0.0204, one-sided Wilcoxon Test). Because of the low number of samples at reference site, statistical analysis has not been realized for Ling.

The potential **gender effect** has been evaluated on the mean number of spots per individual at Platform by pooling together Saithe and Tusk samples (in order to compare 13 females and 14 males; Ling and Redfish has been disregarded due to the low numbers of males). This mean number is 1.9 ± 1.2 spots for female and 1.3 ± 0.9 spots for male. This difference is not significant (p=0.2580; Wilcoxon test).

3.2.1.4 Qualitative analysis by spots

On the overall study, 10 distinct spots (and one specific area called DRZ for Diagonale Radioactive Zone) were isolated from their different 2D chromatographic migration on the PEI cellulose sheets (numbered 1 to 10 and DRZ, according to the chronological order of their first detection, **Figure 5**). On the overall samples, no spot can be considered as particularly predominant according to the frequency of occurrence. Four spots were present in at least 15% of the samples, the two sites combined: spots n°1 (28% of the samples, i.e. 29 samples among 103 analysed), n°2 (17%), n°3 (17%), and n°4 (18%). Almost three-quarters of the overall detected spots belong to one of these four spots (82 spots among 110 detected). For these four quite frequent spots, a statistical analysis of the intersite distribution in the samples has been realised (see below and **Table 2**). The spot n°7 and n°8 were detected in 8% and 6% of the samples respectively. All other spots were detected in less than 5% of the samples. The DRZ was occasionally observed for only one sample.

No spot among the more frequent (≥ 15 % of samples) appears to be strictly limited to a particular site (site-specificity). However, some interesting variations of the DNA adduct patterns can be noted, first **between both explored sites** (see **table 2**). All species combined, the proportions of samples that exhibit spots 1, 2, 3 and 4 are significantly different from a site to the other one, with higher values at Platform, systematically ($p<0.05$, one sided Fisher's Exact test). For example, spot 1 is observed in 40% of the samples at platform (23 samples among 57) but only in 13% of the samples at Reference site (6 samples among 46). This difference is statistically significant (p=0.0018, one sided Fisher's Exact test). Other results are compiled in **Table 2**.

Within each species, some differences in the occurrence frequency of the four major spots are observed.
At Reference site, the difference of spot occurrence between the four studied species is not statistically significant, whatever the spot (spot1: p=0.9223; spot2: p=0.2978; spot3: p=0.7684; spot4: p=0.1640; Fisher's exact Test, see **Table 2** for occurrence frequencies).
At Platform, the difference of spot occurrence between the four studied species is only significant for spot 2 (spot1: p=0.2961; spot2: p=0.0063; spot3: p=0.7976; spot4: p=0.3965; Fisher's exact Test, see **Table 2** for

occurrence frequencies). For this spot at this site, the occurrence frequency ranges from 0% (0 sample among 15) of Ling to 40% of Tusk and Redfish (6 samples among 15 for both species).

Interestingly, spot 2 is particularly common in Tusk, on the two sites. In other words, the highest frequencies of occurrence of the spot 2 at Reference site and Platform are associated to Tusk.
In addition, the less frequent spots n° 5 and 7 (4% and 8% of the overall samples, respectively) are observed only at platform. At this site, 3 Tusk among 15 (20%) exhibit the infrequent spot n°5, while 4 Saithe among 12 (33%) exhibit the infrequent spot n°7.

The potential **gender effect** has been evaluated on the frequency of occurrence of the four major spots at Platform by pooling together Saithe and Tusk samples (in order to compare 13 females and 14 males; Ling and Redfish has been disregarded due to the low numbers of males). The frequency of occurrence of spot 1 is 54% (7 out of 13 samples) for females and 21% (3 out of 14 samples) for males. The frequency of occurrence of spot 2 is 46% (6 out of 13 samples) for females and 7% (1 out of 14 samples) for males. The frequency of occurrence of spot 3 is 23% (3 out of 13 samples) for females and 28% (4 out of 14 samples) for males. The frequency of occurrence of spot 4 is 23% (3 out of 13 samples) for females and 28% (4 out of 14 samples) for males. The gender difference is significant only for spot 2 ($p=0.0329$; Fisher's Exact test).

3.2.2 Quantitative analysis of the DNA adduct patterns

The overall results indicate that the DNA adduct concentrations (expressed in relative adduct level per 10^8 normal nucleotides (RALx10^{-8})) are low for the most part, combined with large interindividual differences, even within groups of supposed similar conditions of exposure to (potential) genotoxicants (same site of the field study and same species).
Quantitatively, the mean relative adduct levels (RAL) per sample (mean of two independent measurements) were measured between <0.01 (no detectable adducts) and 3.81 adducts per 10^8 normal nucleotides. It is to note that only 2 individual means (2% of the samples) exceed 2 adducts per 10^8 nucleotides (One Tusk sample at platform and one Redfish sample at platform too). 9 individual means (9% of the samples) are over 1 adduct per 10^8 nucleotides. All these samples are from fish that has been caught in the Platform area. 30 individual means (29% of the overall samples) are over 0.4 adduct per 10^8 nucleotides, an interesting cut-off value, with high difference of this proportion between the two sites (see discussion).

The mean DNA adduct concentration per site, all species combined, is 0.12 *± 0.18* x10^{-8} adducts at Reference site and 0.50 *± 0.62* x10^{-8} adducts at Platform. **(Figure 1a).** The intersite difference of a factor of four of DNA

adduct concentrations is statistically very significant (p<0.0001, one sided Wilcoxon test; p=0.016, anova on log transformed non-zero values).

At the reference site, the mean DNA adduct concentration per individual and species ranges from <0.01 *± 0.16* x10^{-8} adducts for Ling (n=4) to 0.14 *± 0.16* x10^{-8} and 0.14 *± 0.22* x10^{-8} adducts for Tusk and Redfish respectively. Intermediate results are obtained for Saithe (0.10 *± 0.17* x10^{-8} adducts in average). This interspecies difference is not statistically significant (p=0.5378, Kruskal-Wallis test).

At Platform, the mean DNA adduct concentration per individual and species ranges from 0.34 *± 0.38* x10^{-8} adducts for Ling to 0.73 *± 0.89* x10^{-8} adducts for Redfish. Intermediate results are obtained for Tusk (0.39 *± 0.56* x10^{-8} adducts in average) and Saithe (0.55 *± 0.47* x10^{-8} adducts in average). This interspecies difference is not statistically significant (p=0.1199, Kruskal-Wallis test).

Within each species, the mean DNA adduct concentrations raise significantly from Reference site to Platform (**Figure 1b**). For Tusk, the mean DNA adduct concentration is 0.14 *± 0.16* x10^{-8} adducts at Reference site to reach 0.39 *± 0.56* x10^{-8} adducts at Platform (2.5-fold increase, p=0.033, one sided Wilcoxon test). For Redfish, the mean DNA adduct concentration is 0.14 *± 0.22* x10^{-8} adducts at Reference site to reach 0.73 *± 0.89* x10^{-8} adducts at Platform (5-fold increase, p=0.0008, one sided Wilcoxon test). For Ling, the mean DNA adduct concentration is 0.01 *± 0.00* x10^{-8} adducts at Reference site to reach 0.34 *± 0.38* x10^{-8} adducts at Platform (34-fold increase, p=0.037, one sided Wilcoxon test). For Saithe, the mean DNA adduct concentration is 0.10 *± 0.17* x10^{-8} adducts at Reference site to reach 0.55 *± 0.47* x10^{-8} adducts at Platform (5.5-fold increase, p=0.001, one sided Wilcoxon test).

Finally, the potential **gender effect** has been evaluated on the DNA adduct concentration at Platform by pooling together Saithe and Tusk samples (in order to compare 13 females and 14 males; Ling and Redfish has been disregarded due to the low numbers of males). The mean DNA adduct concentration is 0.53 *± 0.60* x10^{-8} adducts for female and 0.38 *± 0.44* x10^{-8} adducts for male. This difference is not significant (p=0.4728; Wilcoxon test).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_01	110	Tusk	F		1.84	2.29	**2.07**	
IRIS_2014_02	101	Tusk	F		0.72	0.45	**0.59**	
IRIS_2014_03	107	Tusk	F		0.66	0.50	**0.58**	
IRIS_2014_04	114	Tusk	F		0.74	0.52	**0.63**	
IRIS_2014_05	118	Tusk	F		0.09	0.01	**0.05**	
IRIS_2014_06	132	Tusk	F		0.01	0.13	**0.07**	
IRIS_2014_07	133	Tusk	M		1.27	0.87	**1.07**	**0.39**
IRIS_2014_08	134	Tusk	M	PF[2]	0.51	0.36	**0.44**	*(± 0.56)*
IRIS_2014_09	135	Tusk	F		0.01[3]	0.12	**0.07**	
IRIS_2014_10	141	Tusk	F		0.01	0.03	**0.02**	
IRIS_2014_11	142	Tusk	M		0.01	0.01	***0.01***	
IRIS_2014_12	143	Tusk	M		0.14	0.18	**0.16**	
IRIS_2014_13	154	Tusk	M		0.01	0.01	**0.01**	
IRIS_2014_14	172	Tusk	M		0.14	0.01	**0.08**	
IRIS_2014_15	173	Tusk	M		0.01	0.01	**0.01**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] PF=Platform
[3] Analysis without any detectable spot/adduct

Table 1: Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species)

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_16	102	Ling	F	PF[2]	0.01 [3]	0.01	***0.01***	
IRIS_2014_17	103	Ling	F		0.01	0.01	**0.01**	
IRIS_2014_18	104	Ling	F		0.01	0.01	***0.01***	
IRIS_2014_19	111	Ling	F		0.01	0.01	***0.01***	
IRIS_2014_20	112	Ling	F		0.90	0.64	**0.77**	
IRIS_2014_21	119	Ling	F		0.02	0.05	**0.04**	
IRIS_2014_22	122	Ling	F		0.45	0.37	**0.41**	**0.34**
IRIS_2014_23	130	Ling	F		0.27	0.42	**0.35**	*(± 0.38)*
IRIS_2014_24	136	Ling	F		0.01	0.01	**0.01**	
IRIS_2014_25	137	Ling	F		0.96	*1.18*	**1.07**	
IRIS_2014_26	144	Ling	F		0.56	0.48	**0.52**	
IRIS_2014_27	147	Ling	F		0.85	0.91	**0.88**	
IRIS_2014_28	148	Ling	F		0.15	0.24	**0.20**	
IRIS_2014_29	150	Ling	F		0.63	0.92	**0.78**	
IRIS_2014_30	153	Ling	F		0.01	0.01	**0.01**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] PF=Platform
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_31	160	Saithe	F		1.09	0.96	**1.03**	
IRIS_2014_32	161	Saithe	M		0.34	0.30	**0.32**	
IRIS_2014_33	162	Saithe	M		1.25	1.00	**1.13**	
IRIS_2014_34	163	Saithe	F		1.39	1.01	**1.20**	
IRIS_2014_35	164	Saithe	M		0.21	0.30	**0.26**	
IRIS_2014_36	165	Saithe	F	PF[2]	0.01 [3]	0.08	**0.05**	**0.55**
IRIS_2014_37	166	Saithe	F		0.56	0.39	**0.48**	*(± 0.47)*
IRIS_2014_38	167	Saithe	M		0.28	0.42	**0.35**	
IRIS_2014_39	168	Saithe	M		0.01	0.08	**0.05**	
IRIS_2014_40	169	Saithe	M		*0.27*	*0.28*	***0.28***	
IRIS_2014_41	170	Saithe	M		0.96	1.57	**1.27**	
IRIS_2014_42	171	Saithe	F		*0.20*	*0.13*	**0.17**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] PF=Platform
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± *SD*) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_43	105	Redfish	F		0.60	0.78	**0.69**	
IRIS_2014_44	108	Redfish	F		0.54	0.72	**0.63**	
IRIS_2014_45	109	Redfish	F		*0.57*	*0.72*	**0.65**	
IRIS_2014_46	113	Redfish	F		4.08	3.53	**3.81**	
IRIS_2014_47	115	Redfish	F		0.84	1.15	**1.00**	
IRIS_2014_48	116	Redfish	F		0.36	0.28	**0.32**	
IRIS_2014_49	117	Redfish	F		0.53	*0.39*	**0.46**	**0.73**
IRIS_2014_50	120	Redfish	F	**PF**[2]	*0.86*	*0.75*	**0.81**	*(± 0.89)*
IRIS_2014_51	121	Redfish	F		*0.70*	*1.00*	**0.85**	
IRIS_2014_52	123	Redfish	F		0.32	*0.21*	**0.27**	
IRIS_2014_53	124	Redfish	F		0.01[3]	0.01	**0.01**	
IRIS_2014_54	125	Redfish	F		0.01	0.10	**0.06**	
IRIS_2014_55	126	Redfish	F		0.56	0.63	**0.60**	
IRIS_2014_56	127	Redfish	F		0.48	0.63	**0.56**	
IRIS_2014_57	128	Redfish	F		0.28	0.29	**0.29**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] PF=Platform
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_58	**201**	Ling			0.01 [3]	0.01	**0.01**	
IRIS_2014_59	**202**	Ling		**REF** [2]	0.01	0.01	**0.01**	**0.01**
IRIS_2014_60	**240**	Ling			0.01	0.01	**0.01**	*(± 0.00)*
IRIS_2014_61	**244**	Ling			0.01	0.01	**0.01**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] REF=Reference site
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_62	228	Redfish		REF [2]	0.01 [3]	0.01	**0.01**	
IRIS_2014_63	229	Redfish			0.52	0.37	**0.45**	
IRIS_2014_64	230	Redfish			0.01	0.01	**0.01**	
IRIS_2014_65	231	Redfish			*0.24*	*0.27*	**0.26**	
IRIS_2014_66	232	Redfish			0.77	*0.67*	**0.72**	
IRIS_2014_67	233	Redfish			0.01	0.01	**0.01**	
IRIS_2014_68	234	Redfish			0.01	0.01	**0.01**	**0.14**
IRIS_2014_69	235	Redfish			0.21	0.12	**0.17**	*(± 0.22)*
IRIS_2014_70	236	Redfish			0.01	0.01	**0.01**	
IRIS_2014_71	237	Redfish			0.01	0.01	**0.01**	
IRIS_2014_72	238	Redfish			0.01	0.01	**0.01**	
IRIS_2014_73	245	Redfish			0.01	0.01	**0.01**	
IRIS_2014_74	246	Redfish			0.01	0.01	**0.01**	
IRIS_2014_75	247	Redfish			0.27	0.44	**0.36**	
IRIS_2014_76	248	Redfish			0.01	0.01	**0.01**	

[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] REF=Reference site
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_77	**203**	Saithe			0.01 [3]	0.01	**0.01**	
IRIS_2014_78	**204**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_79	**205**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_80	**206**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_81	**207**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_82	**208**	Saithe		**REF** [2]	0.31	0.47	**0.39**	**0.10**
IRIS_2014_83	**209**	Saithe			0.34	0.52	**0.43**	*(± 0.17)*
IRIS_2014_84	**210**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_85	**211**	Saithe			0.40	0.24	**0.32**	
IRIS_2014_86	**212**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_87	**220**	Saithe			0.01	0.01	**0.01**	
IRIS_2014_88	**221**	Saithe			0.01	0.01	**0.01**	

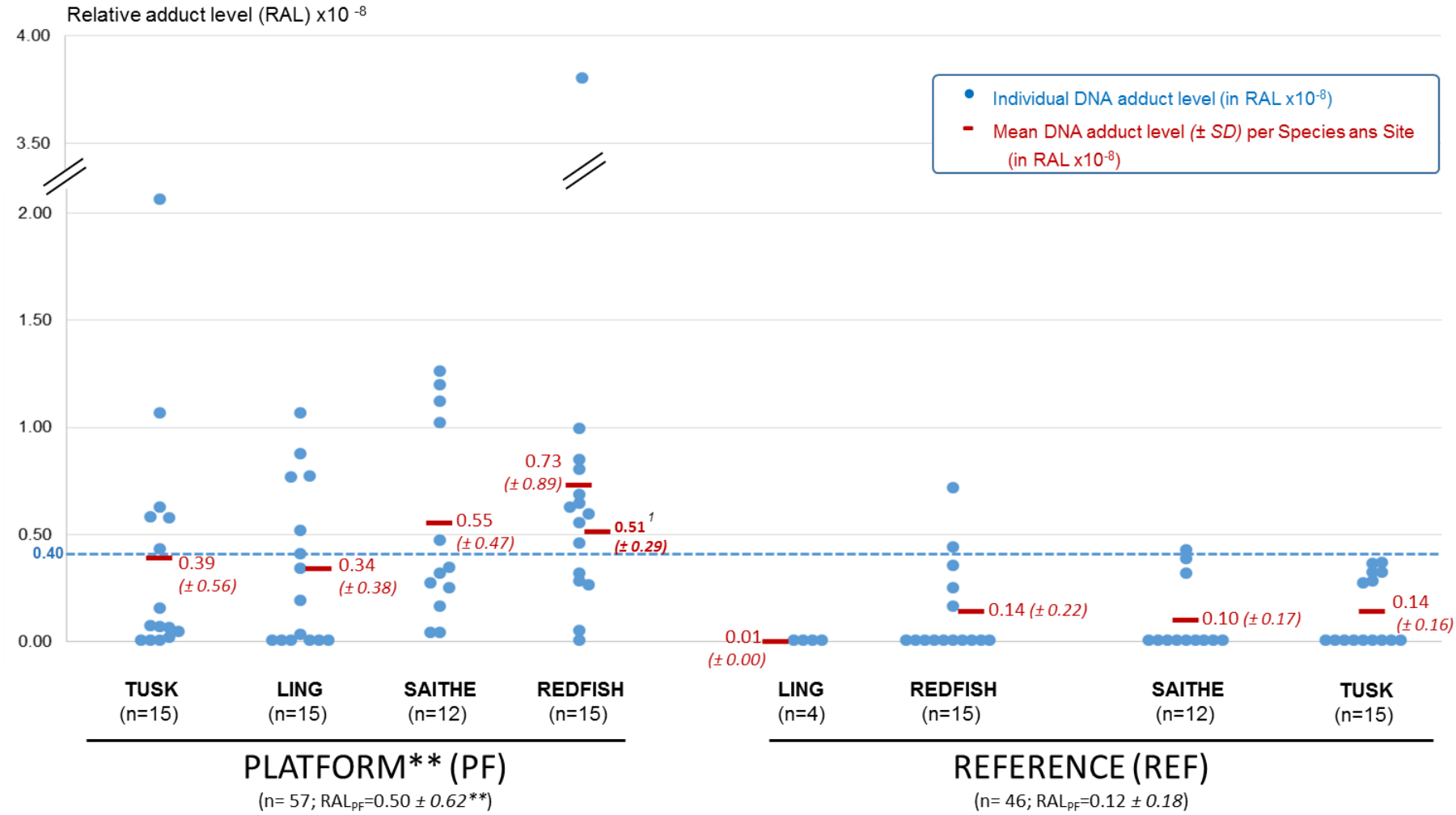
[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] REF=Reference site
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).

Sample identification		Species	Sex	Site	Relative Adduct Level (RAL) x 10^{-8}		Mean RAL per sample (x10^{-8})	Mean RAL(± SD) per Species / per Site (x10^{-8})
ADN'TOX	IRIS				Run 1[1]	Run 2		
IRIS_2014_89	222	Tusk		REF[2]	0.01 [3]	0.01	**0.01**	
IRIS_2014_90	223	Tusk			0.01	0.01	**0.01**	
IRIS_2014_91	224	Tusk			0.01	0.01	**0.01**	
IRIS_2014_92	225	Tusk			0.34	0.21	**0.28**	
IRIS_2014_93	226	Tusk			0.01	0.01	**0.01**	
IRIS_2014_94	227	Tusk			0.01	0.01	**0.01**	
IRIS_2014_95	242	Tusk			0.35	0.22	**0.29**	**0.14**
IRIS_2014_96	243	Tusk			0.36	0.29	**0.33**	*(± 0.16)*
IRIS_2014_97	256	Tusk			0.45	0.29	**0.37**	
IRIS_2014_98	257	Tusk			0.41	0.32	**0.37**	
IRIS_2014_99	270	Tusk			0.01	0.01	**0.01**	
IRIS_2014_100	271	Tusk			0.43	0.22	**0.33**	
IRIS_2014_101	272	Tusk			0.01	0.01	**0.01**	
IRIS_2014_102	273	Tusk			0.01	0.01	**0.01**	
IRIS_2014_103	274	Tusk			0.01	0.01	**0.01**	

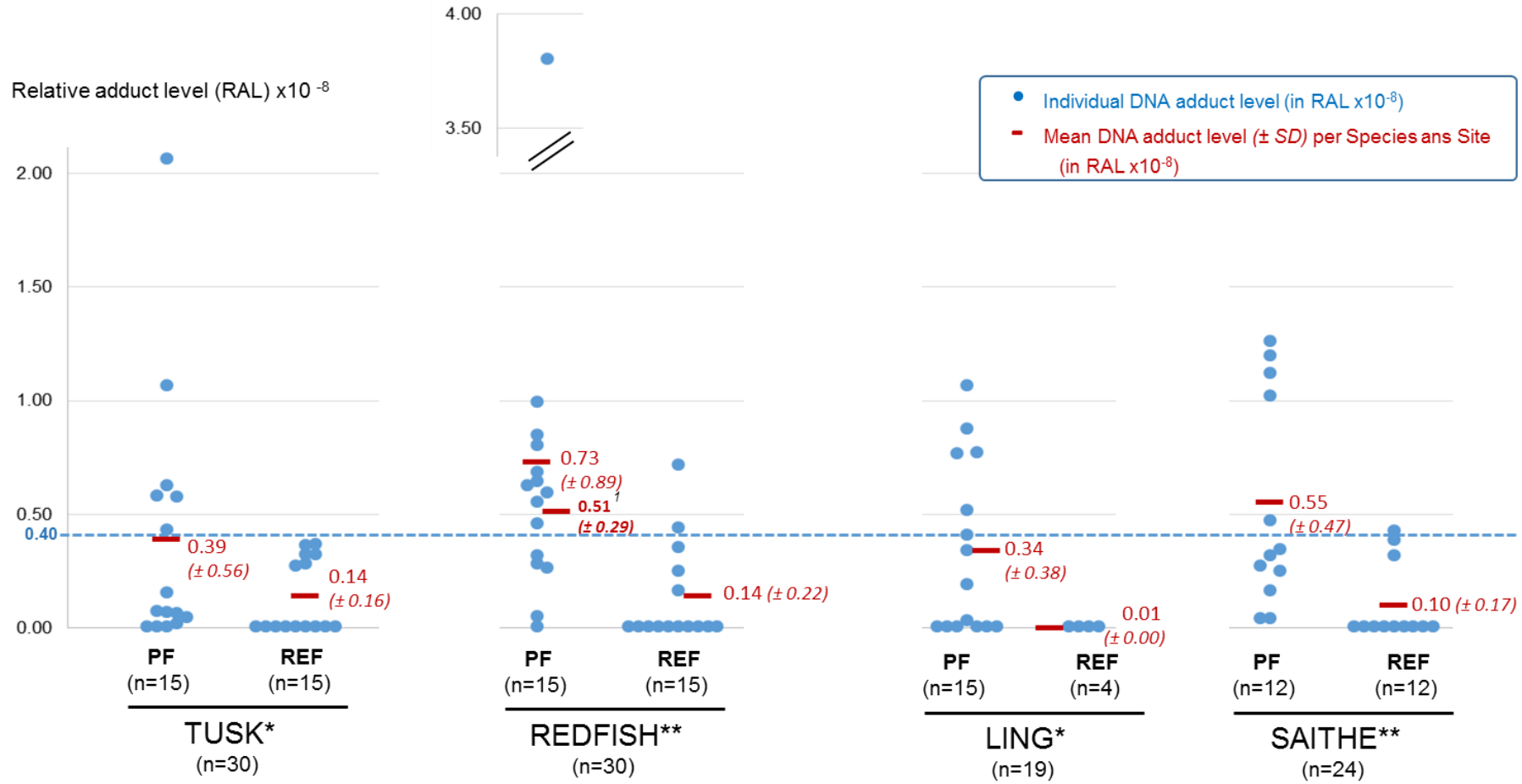
[1] Run 1 and Run 2: two independent measurements of DNA adducts per sample.
[2] REF=Reference site
[3] Analysis without any detectable spot/adduct

Table 1 (continued): Concentrations in bulky DNA adducts (expressed in Relative Adduct Level per 10^8 normal nucleotides (RAL x 10^{-8})) measured on the 103 fish included in the field study, classified according the sampling conditions (Site and Species).



[1]: without outlier n°46 (RAL_{46}=3.81x10^{-8})

Figure 1a: Mean concentrations of DNA adducts (in RAL x 10^{-8}) measured in the liver of 103 fish (different species), classed by sampling site.
Each point of the graph corresponds to the individual mean RAL (mean of two independent adduct measurements)
The dashed line in blue indicates the cut-off value of 0.40 adducts per 10^8 normal nucleotides (see discussion)
** Very significant difference of the mean DNA adduct level per site between REF and PF, all species combined (*see the text for more detail*; p<0.0001, one sided Wilcoxon test)



[1]: without outlier n°46 (RAL_{46}=3.81x10^{-8})

Figure 1b: Mean concentrations of DNA adducts (in RAL x 10^{-8}) measured in the liver of 103 fish of the WCM 2014 program (different species), classed by species.

Each point of the graph corresponds to the individual mean RAL (mean of two independent adduct measurements)

The dashed line in blue indicates the cut-off value of 0.40 adducts per 10^8 normal nucleotides (see discussion)

* Significant difference of the mean DNA adduct level between REF and PF for given species (*see the text for more detail*; p<0.05, one sided Wilcoxon test)

** Very significant difference of the mean DNA adduct level between REF and PF for given species (*see the text for more detail*; p<0.01, one sided Wilcoxon test)

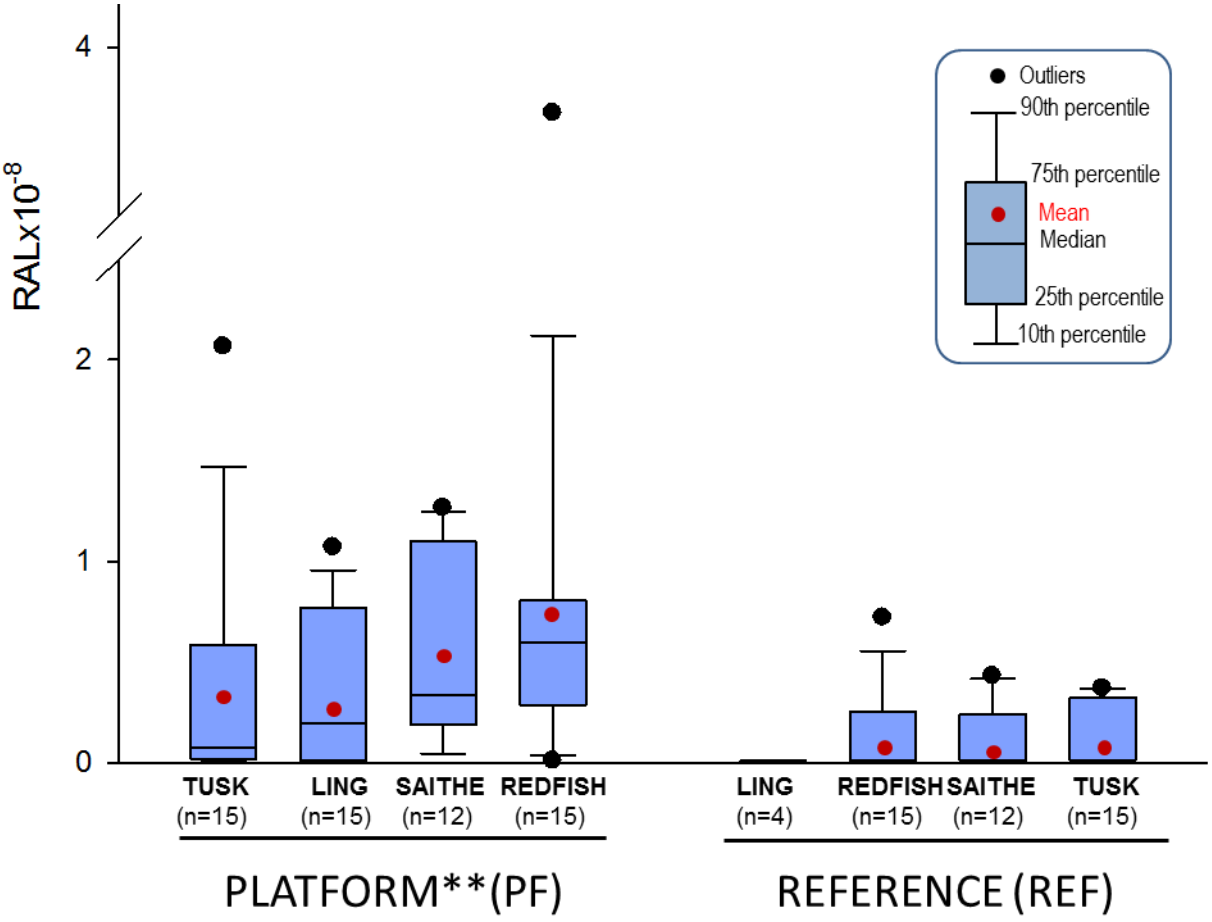


Figure 2a: Mean concentrations in DNA adducts (in RAL x 10^{-8}) measured in the 103 fish of the WCM 2014 program, classed by sampling site.
Representation in box plot (see legend)
** Very significant difference of the mean DNA adduct level per site between REF and PF, all species combined (*see the text for more detail*; p<0.0001, one sided Wilcoxon test)

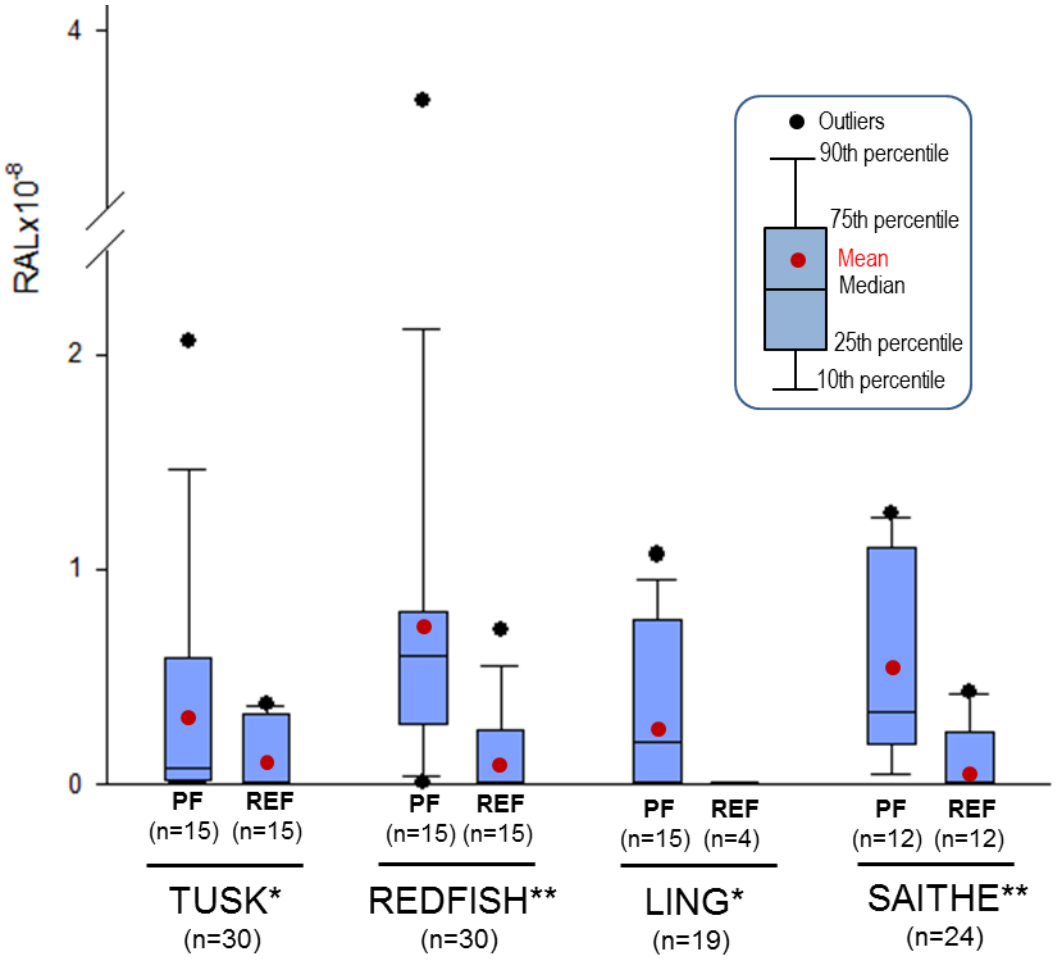


Figure 2b: Mean concentrations in DNA adducts (in RAL x 10^{-8}) measured in the 103 fish of the WCM 2014 program, classed by sampling site and species.

Representation in box plot (see legend)

* Significant difference of the mean DNA adduct level between REF and PF for given species (*see the text for more detail*; p<0.05, one sided Wilcoxon test)

** Very significant difference of the mean DNA adduct level between REF and PF for given species (*see the text for more detail*; p<0.01, one sided Wilcoxon test)

Figure 3.a:
Representative hepatic DNA adduct patterns associated to **Tusk at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

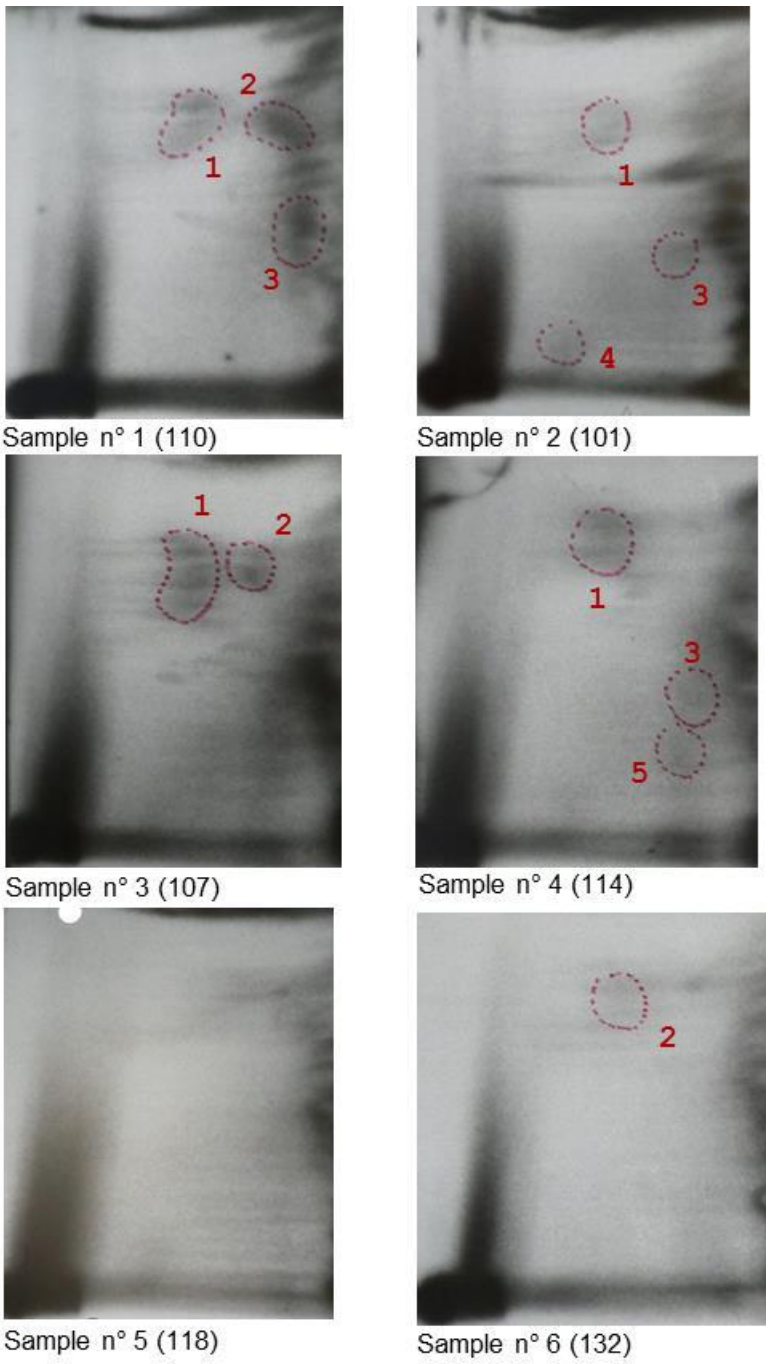
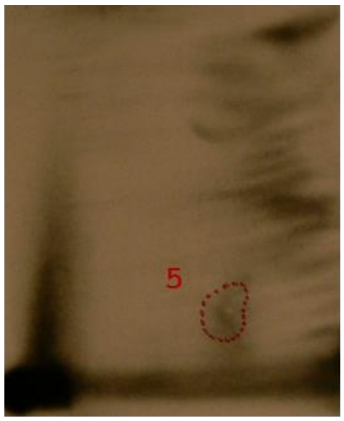


Figure 3.a *(continued)*:
Representative hepatic DNA adduct patterns associated to **Tusk at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).



Sample n° 12 (143)

Sample n° 13 (154)

Sample n° 15 (173)

Figure 3.b:
Representative hepatic DNA adduct patterns associated to **Ling at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

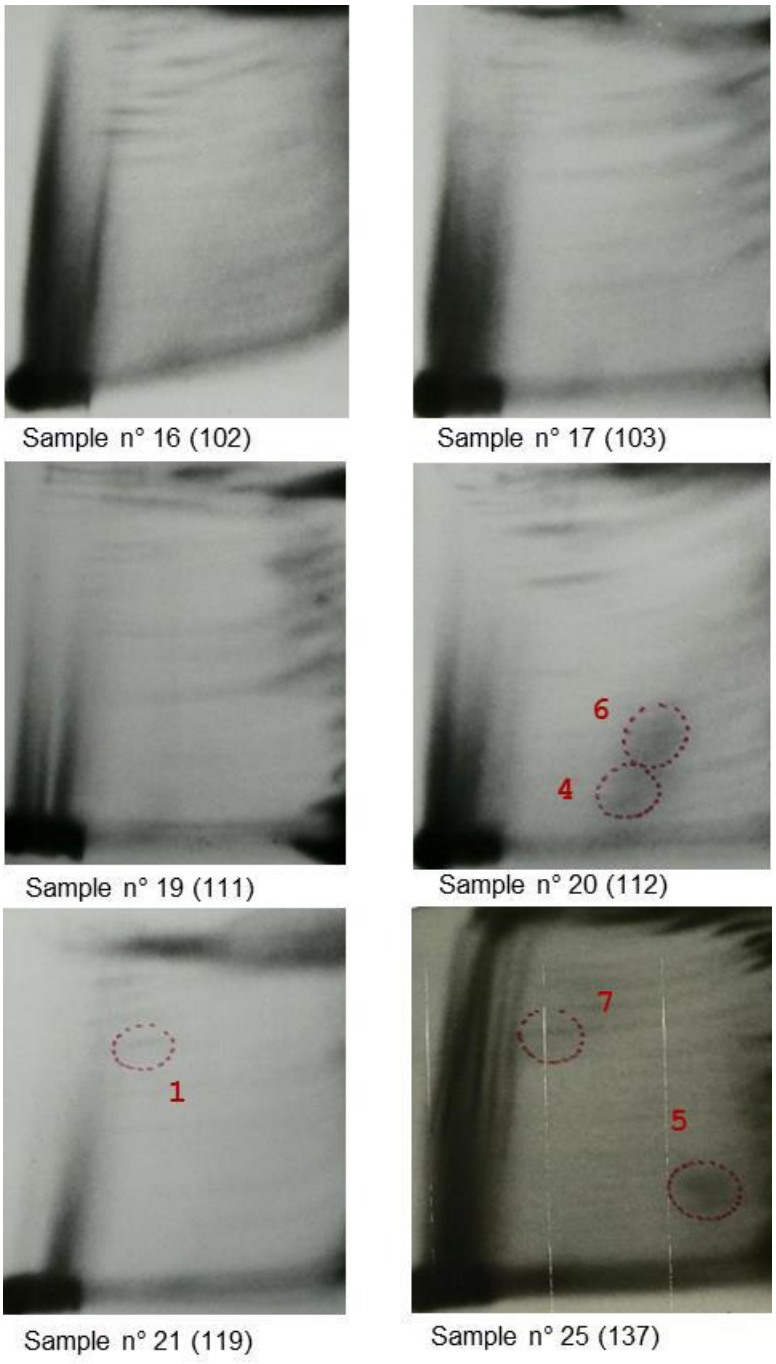
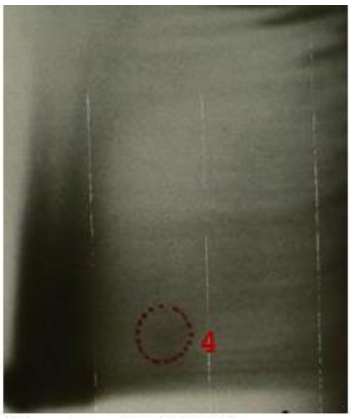
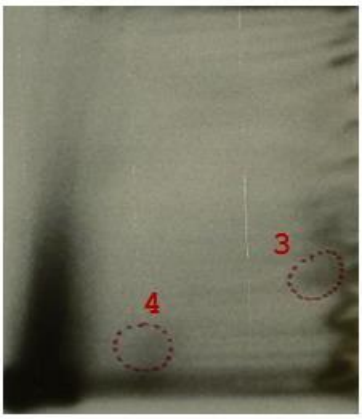


Figure 3.b *(continued)*:
Representative hepatic DNA adduct patterns associated to **Ling at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).



Sample n° 26 (144)



Sample n° 27 (147)

Sample n° 28 (148)

Figure 3.c :
Representative hepatic DNA adduct patterns associated to **Saithe at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

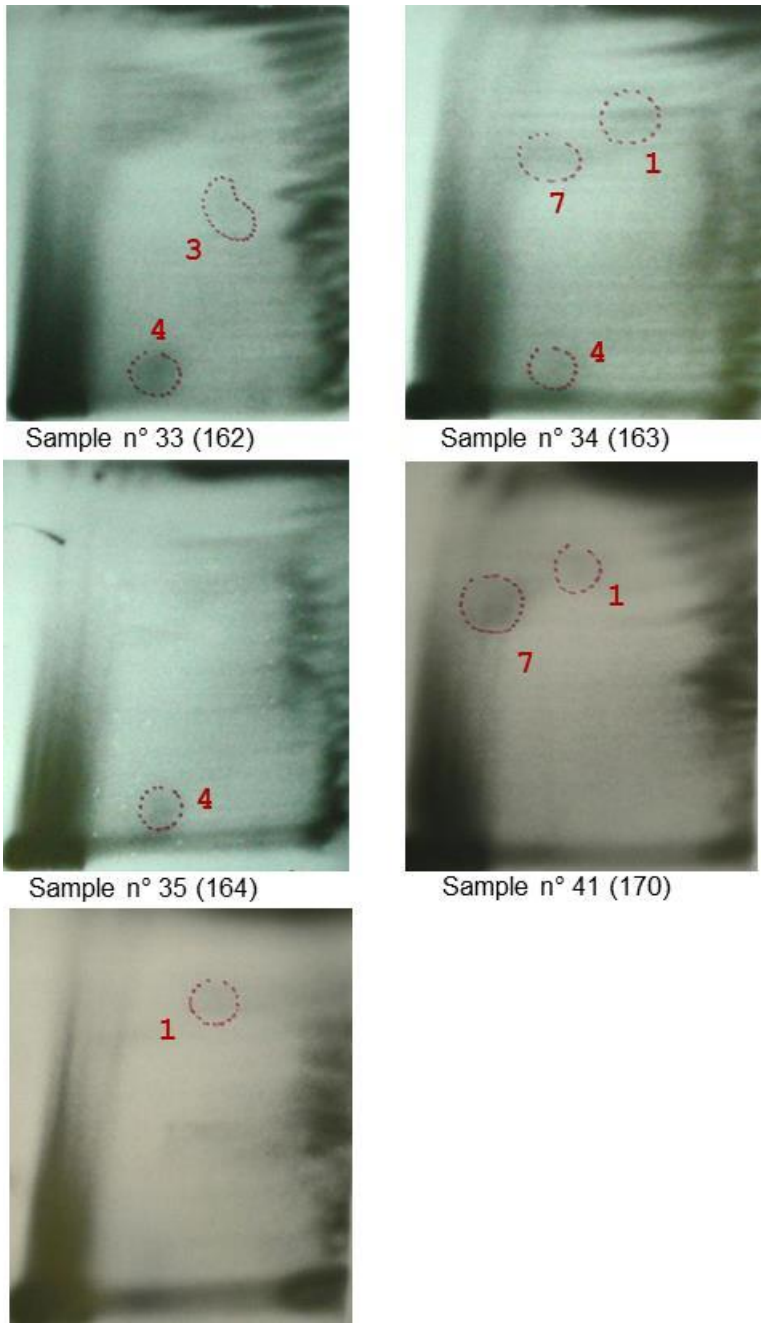


Figure 3.d :
Representative hepatic DNA adduct patterns associated to **Redfish at PLATFORM**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

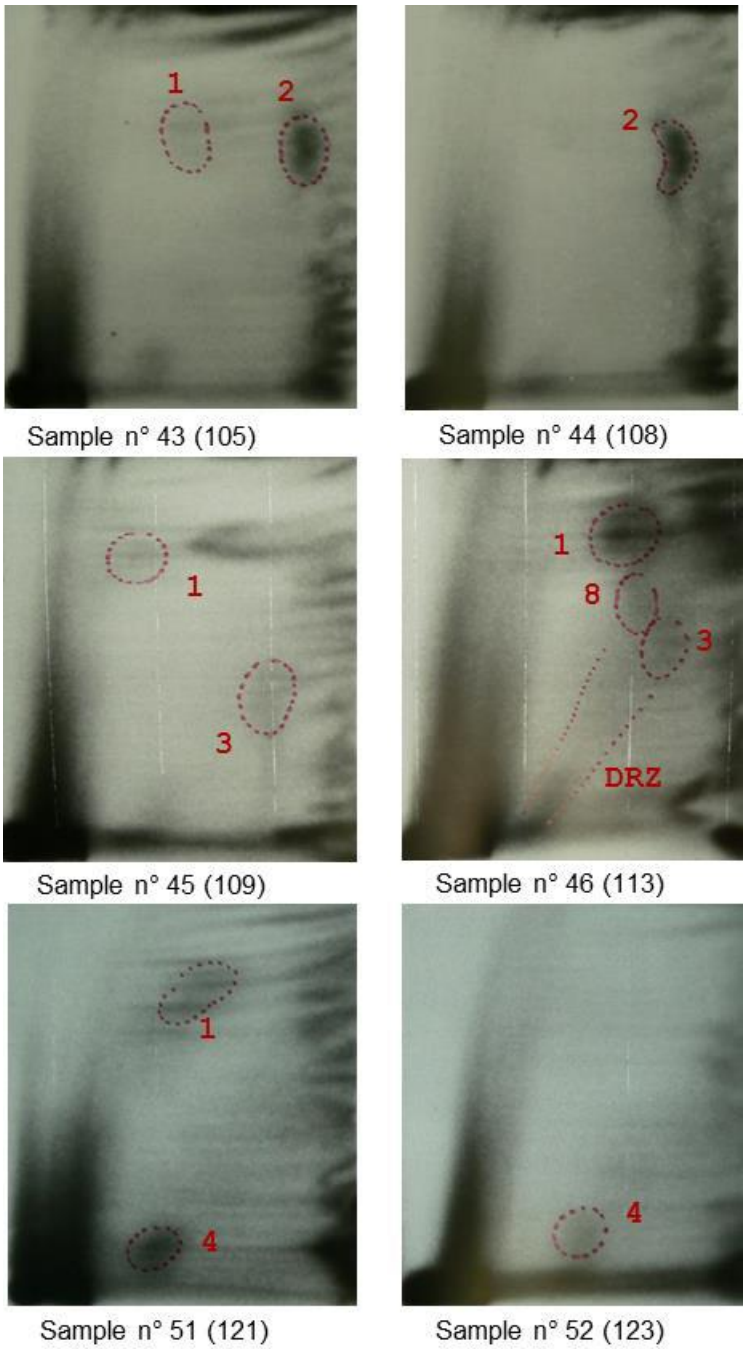
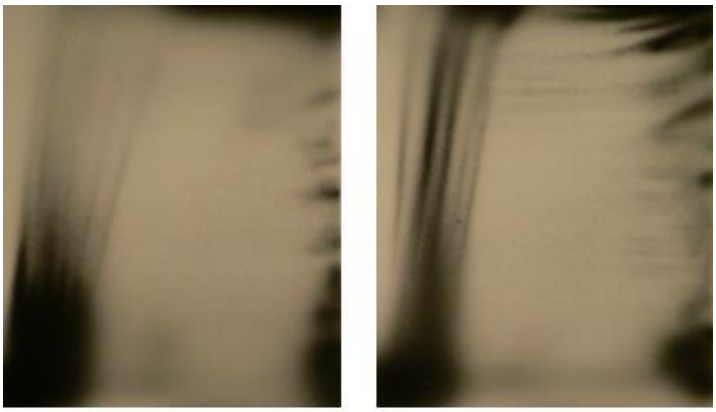


Figure 3.e :
Representative hepatic DNA adduct patterns associated to **ling at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).



Sample n° 60 (240) Sample n° 61 (244)

Figure 3.f :
Representative hepatic DNA adduct patterns associated to **Redfish at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

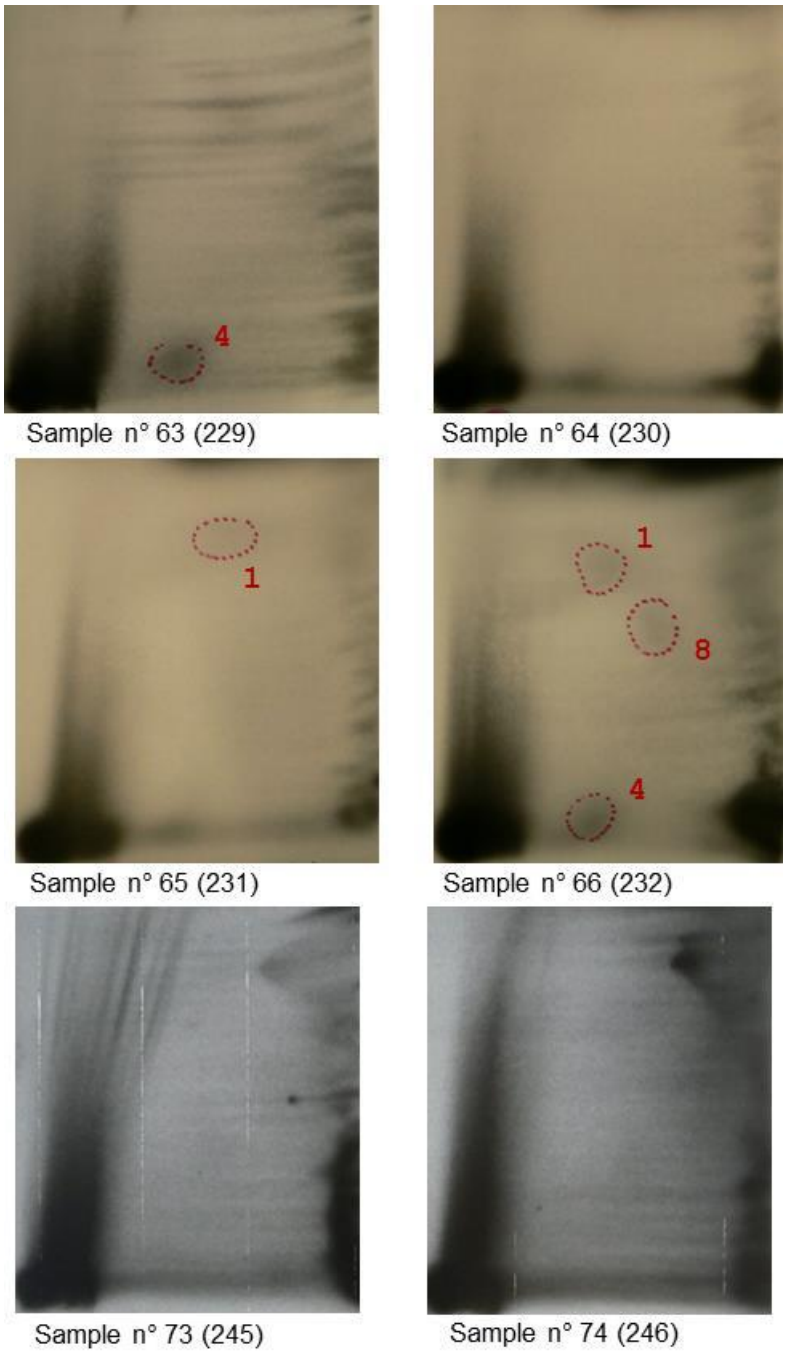
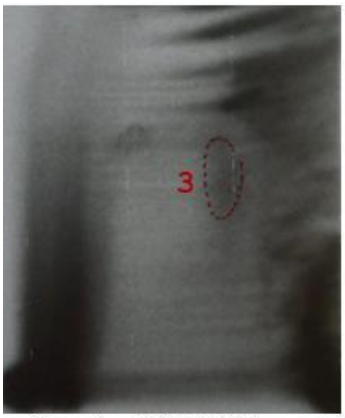


Figure 3.f *(continued)*:
Representative hepatic DNA adduct patterns associated to **Redfish at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).



Sample n° 75 (247)


Sample n° 76 (248)

Figure 3.g :
Representative hepatic DNA adduct patterns associated to **Saithe at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

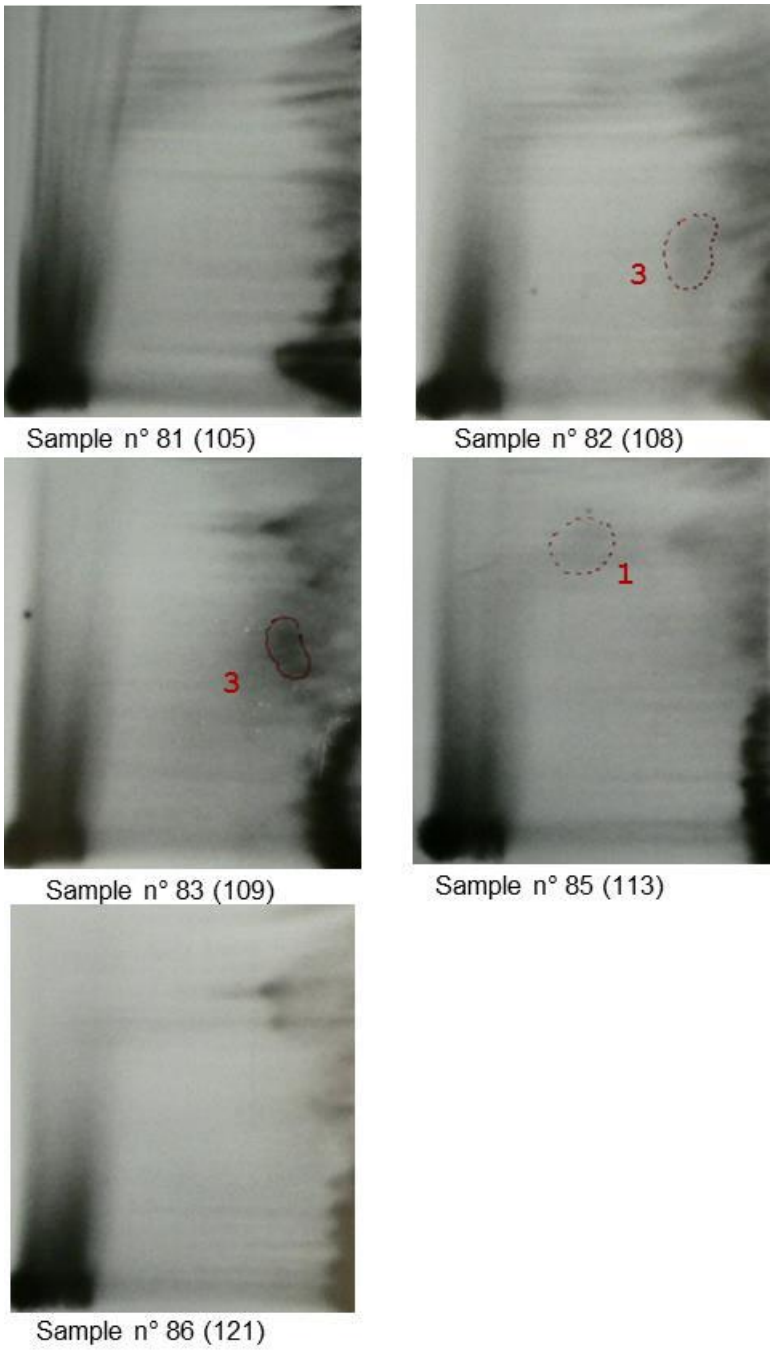


Figure 3.h :
Representative hepatic DNA adduct patterns associated to **Tusk at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).

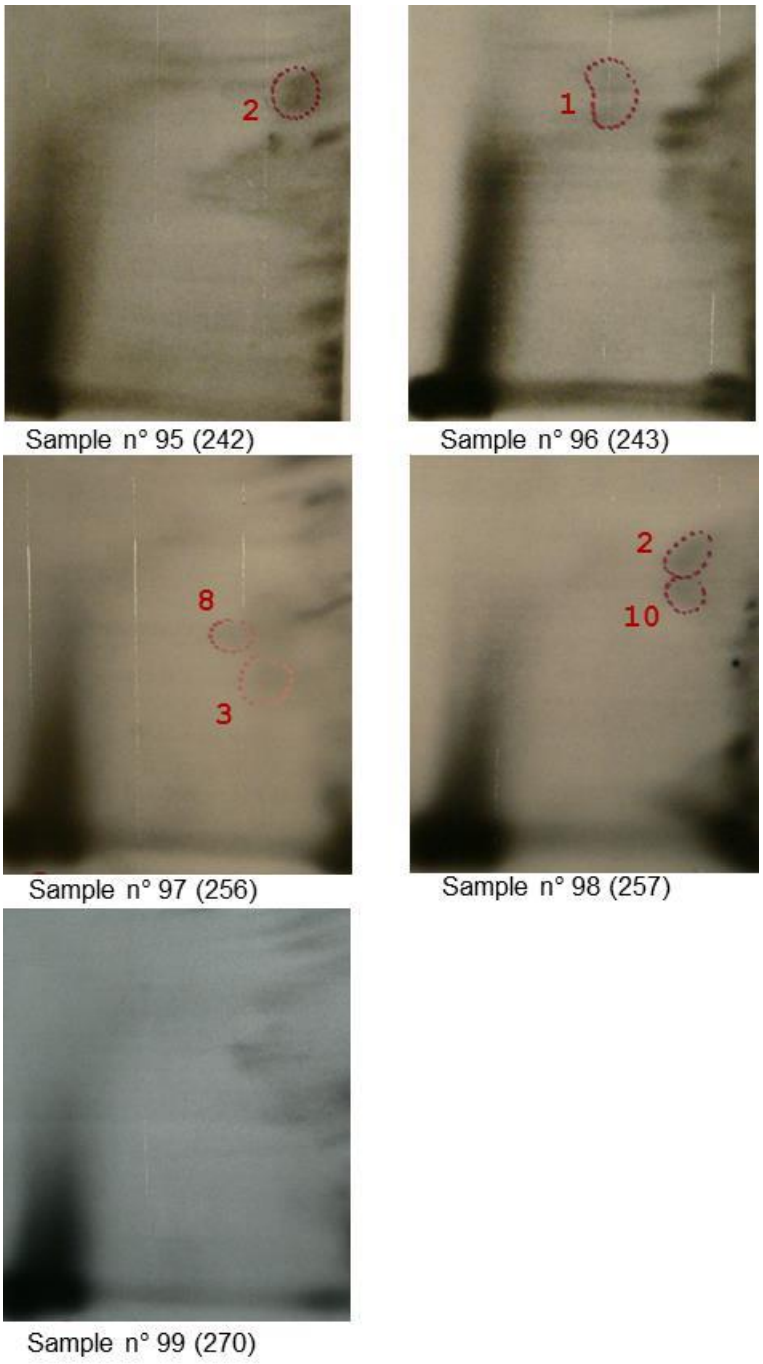
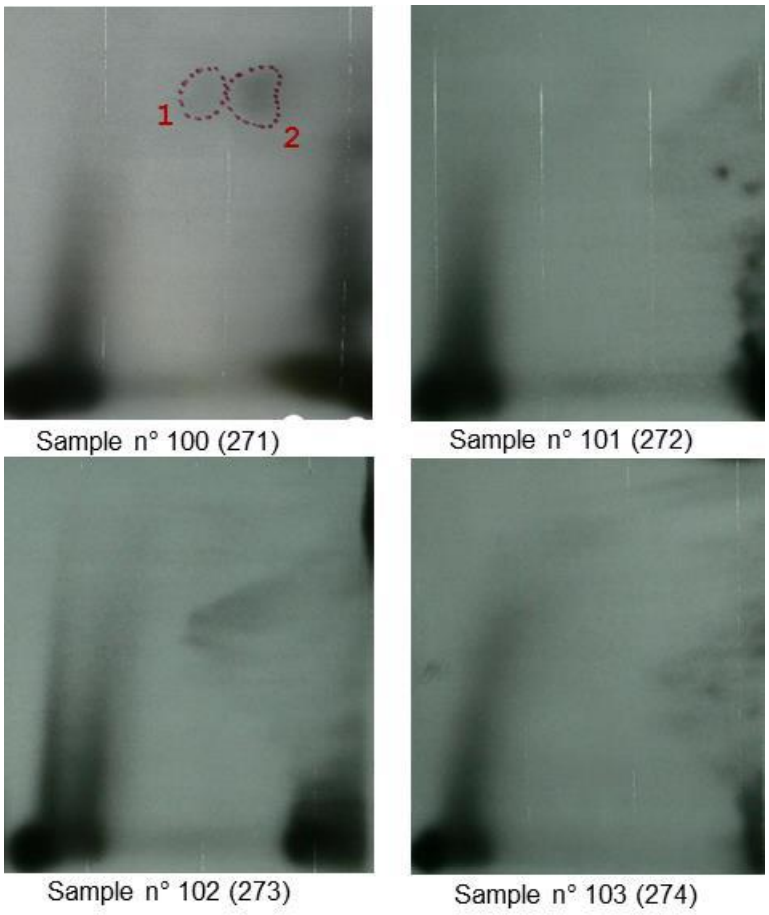


Figure 3.h *(continued)*:
Representative hepatic DNA adduct patterns associated to **Tusk at REFERENCE site**

For represented samples, autoradiographic pattern is one among both realised (two analyses per sample).
Spots are numbered according to their location on PEI-cellulose plates (see template at **Figure 4**)
Exposure conditions: Amersham Hyperfilm MP (GE Healthcare), exposure time: 72 hours (-80°C).



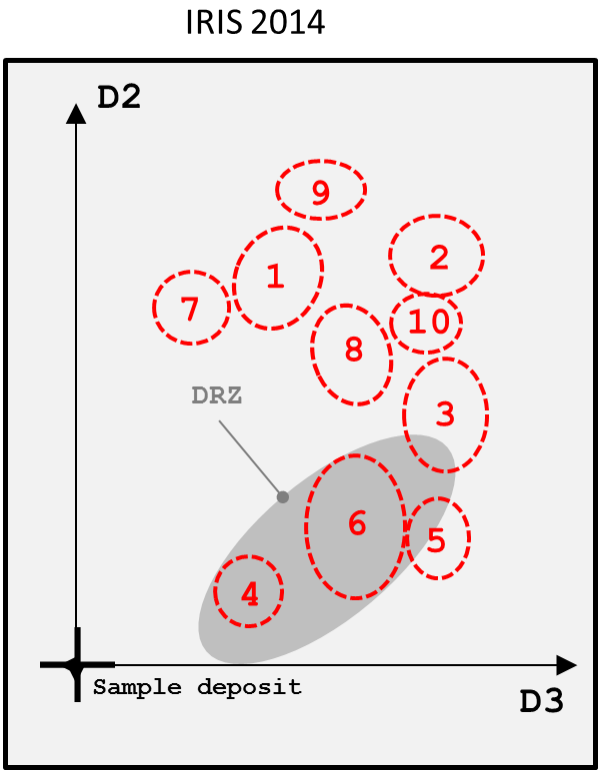


Figure 4: Location template of the different distinct spots attributed to DNA adducts obtained after two-dimensional Thin Layer Chromatography on the overall 206 patterns (103 samples).

D1, D2, D3 and D4 migrations are detailed in annexe 2b.

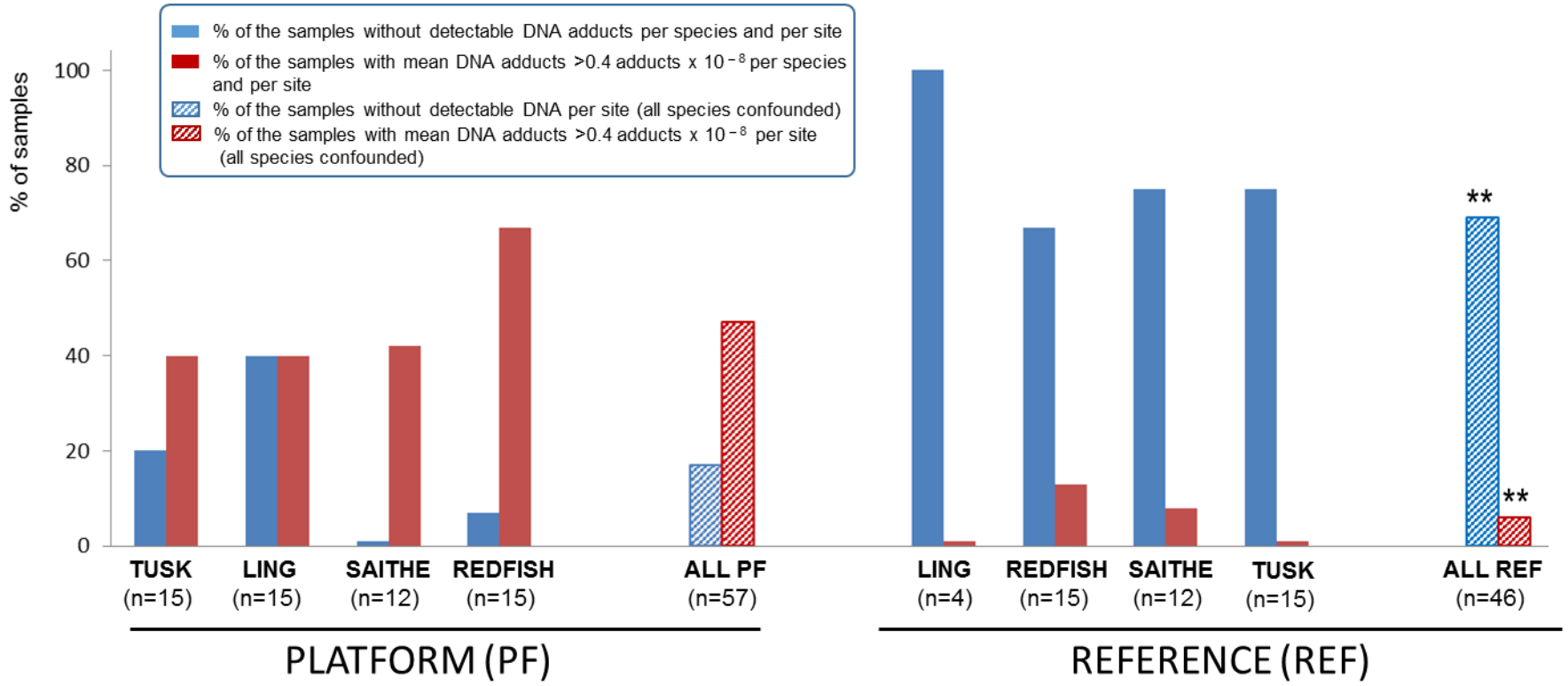


Figure 5: Occurrence frequency of the samples without detectable DNA adducts and samples with higher concentrations in DNA adducts (>0.4 adducts per 10^8 nucleotides) among the 103 fish samples, classed by site.

** Very significant difference between REF and PF, all species combined ($p<0.0001$, one sided Fisher's exact test).

See text for more details

Site	Species	Number of samples	Number of samples with defined spot *(and % of samples with defined spot at the site for each species and all species together)*			
			Spot 1**	**Spot 2***	**Spot 3***	**Spot 4****
PF	Tusk	15	5 *(33%)*	6 *(40%)*	5 *(31%)*	2 *(13%)*
	Ling	15	4 *(27%)*	0 *(0%)*	4 *(27%)*	5 *(33%)*
	Saithe	12	5 *(42%)*	1 *(8%)*	2 *(17%)*	5 *(42%)*
	Redfish	15	9 *(60%)*	6 *(40%)*	3 *(20%)*	4 *(27%)*
	All	*57*	*23 (40%)*	*13 (23%)*	*14 (24%)*	*16 (28%)*
REF	Ling	4	0 *(0%)*	0 *(0%)*	0 *(0%)*	0 *(0%)*
	Redfish	15	2 *(13%)*	0 *(0%)*	1 *(7%)*	3 *(20%)*
	Saithe	12	1 *(8%)*	1 *(8%)*	2 *(17%)*	0 *(0%)*
	Tusk	15	3 *(20%)*	3 *(20%)*	1 *(7%)*	0 *(0%)*
	All	*46*	*6 (13%)*	*4 (9%)*	*4 (9%)*	*3 (6%)*
Total (PF+REF)	*All*	*103*	*29 (28%)*	*17 (17%)*	*18 (17%)*	*19 (18%)*

Table 2: Occurrence frequency of the four major spots n°1, n°2, n°3 and n°4 in fish samples, classed by site and species.

* The proportions of samples with spots 2 and 3 are different from site to site ($p<0.05$, one sided Fisher's Exact Test)
** The proportions of samples with spots 1 and 4 are very different from site to site ($p<0.01$, one sided Fisher's Exact Test)
See text for more details

4 DISCUSSION AND CONCLUSION

The present field study consists in the analysis of the DNA adduct patterns in the liver of 103 individual fish of four different species (Tusk, Redfish, Ling and Saithe), sampled in two distinct collection areas of the North East Atlantic seas in summer 2014. The purpose of the study, associated to the Water Column Monitoring (WCM) program is to contribute to the evaluation of the genotoxic impact of the offshore oil and gas activities on the marine environment of the North East Atlantic seas.

Historically, the analysis of DNA adduct profiles in fish exposed to environmental pollutants represents an important approach in environmental risk assessment since Dawe et al. claimed in 1964 that bottom feeding fish were “useful indicators of environnemental carcinogens”. DNA adducts are now recognized as a crucial biomarker of exposure, especially for there early emergence after a genotoxic exposure, which may play a key role in establishing a mode of action for cancer (Pottenger et al., 2009). Because of its high sensibility and versatility, the method of ^{32}P postlabelling has been applied to environmental fish studies as early as 1980s, few years after the first publication of the method (1981). Thus, in 1987, Dunn et al. measured significant DNA adduct levels in livers of wild Brown bulheads sampled from sites in the Buffalo and Detroit Rivers, in association with exposure of fish to high concentrations of polycyclic aromatic hydrocarbons. Since these early scientific works, a large range of fish species was studied, in a large panel of applications (laboratory and field studies).
In the marine environment, numerous published works are focused on flounder (*Platichthys flesus*), haddock (*Melanogrammus aeglefinus*) or Atlantic cod (*Gadus morhua*). Most of them indicate that DNA adducts are detected in the liver when fish are exposed to environmental genotoxicants. Growing data are available in relation with controlled laboratory exposures and environmental field studies (Harvey et al. 1997, Reynolds et al., 2003, Malmström et al., 2009). The published studies on DNA adduct measurement in other fish species, especially those included in the present study, seem to be less abundant, or even non-existent!

In the present study, the measured DNA adduct concentrations in fish livers remain relatively low and generally below the value of 1 adduct per 10^8 normal nucleotides (10 nmole DNA adducts / mole DNA). These concentrations are in accordance with literature associated to field studies, whatever locations and fish species. To go into more detail, it is to note that the low concentrations in DNA adducts observed in average are associated with a large proportion of samples without detectable DNA adducts (nearly between one third and half of all the samples, with large intersite variations), or more precisely below the calculated detection limit of 0.01 adducts for 10^8 normal nucleotides. Interestingly, this proportion reaches two thirds of the samples at the reference site, a marine site presumed to be not contaminated by genotoxic contaminants like PAHs. The measured concentrations are most often about a few adducts for 10^9 normal nucleotides, approaching the limits of detection / quantification accepted

for the method. These values are rather in accordance with previous results (see reports of earlier studies), probably a little higher (for WCM 2013 as example, around one third of the samples without detectable adducts, albeit with some experimental differences compared to the present study). The large proportion of undetectable and very low levels of DNA adducts in tissues of wild fish is of frequent concern in literature. From 98 samples (11 species) caught in presumably pristine areas of the northern Atlantic, DNA adduct levels in liver were below the detection limit of the ^{32}P-postlabelling method in three quarters of cases and just above in the remaining quarter (Aas et al., 2003). The sensitivity of the methods used in the environmental studies focused on genotoxicity is a crucial analytical parameter.

In the quantitative point of view, the mean concentration of DNA adducts measured in fish livers is four fold more elevated in the platform area compared to the reference site. A comparable significant site effect is also observed for each of the four species examined separately. It is interesting to note that at the platform area, the mean concentration in DNA adducts per species is above or just below the value of 0.4 adducts per 10^8 nucleotides (see below for the interest of this cut-off value). Moreover, all the nine samples that exhibit concentration in DNA adducts over $1x10^{-8}$ have been caught at the platform area.

The measurement of more elevated DNA adduct levels in the liver of fish that have been caught in platform areas compared to reference sites is regularly described in literature, and often attributed to the contamination of marine environment by petroleum associated genotoxicants. As an example, a large field study was conducted on haddock (*Melanogrammus aeglefinus*) and atlantic cod (*Gadus morhua*) caught in two areas of the North Sea with extensive oil production: Tampen and Sleipner (Balk et al., 2011). From 2001 to 2004 fish campaigns, Balk et al. revealed significant higher levels of hepatic adducts in haddock from the Tampen area compared to a control site located in southwest Norway (Egersund bank). Similarly, such quantitative differences in DNA adduct levels between reference sites and oil platform areas were observed in previous study conducted in our laboratory from fish caught in the same areas (IMR report, 2011). However, it is to note that results seems to vary significantly from one fishing campaign to the next, resulting in inconsistent intersite differences.

Numerous published studies indicate that DNA adducts are detected in the liver of fish when the fish are exposed to environmental genotoxicants, in particular PAHs. Data are available in relation with controlled laboratory exposures and environmental field studies. For example, the dietary prolonged exposure of flounders to a mixture of four PAHs (5 and 50 mg.kg-1) leads to the appearance of DNA adducts detected by the ^{32}P postlabelling (Reynolds et al., 2003). In a controlled mesocosm system, Harvey et al. (1997) had shown the existence of DNA adducts in the liver of flounders associated to exposure to a mixture of PAHs (and PCBs). The concentrations of adducts measured by ^{32}P post-labelling (between 0 and 1 adduct in 10^8 normal nucleotides) were similar to those in the current study. In 1999, Lyons et al. measured hepatic DNA adducts and PAH metabolites in bile of flounders sampled in different stations of the polluted Tyne Estuary (North East England), while other fish were caught in a clean reference site. Finally, a large difference in DNA adduct levels was observed with higher values for contaminated sites, associated

to large amounts of PAH metabolites in bile. The combination of two biomarkers provides a better estimate of the bioavailability of certain pollutants and indicates that flounders in Tyne Estuary are actually exposed to subsequent sub-lethal genotoxic effects. More recently, a comparable study was conducted on flounders caught in the Baltic Sea (Malmström et al., 2009). Hepatic DNA adduct levels measured in 10 different sites were low, with generally clean autoradiograms (except a few detectable spots and rare faint typical radioactive diagonal zone). The Authors concluded that in the explored areas, flounders are not exposed to significant high concentrations of polycyclic hydrocarbons. Given the results of these numerous fish studies, members of the NOAA's National Marine Fisheries Service had shown the value of using molecular dosimeters like DNA adducts in fish compared to the measurement of pollutants mutagenic and / or carcinogenic in natural environments, since the beginning of the 1990s, (Stein et al., 1994).

In a qualitative point of view, 10 distinct spots assigned to different DNA adducts and a diagonal radioactive zone (DRZ) are counted on the overall study. At the first glance, such qualitative variety can be attributed 1) to the capability for fish from different species to realize enzymatic bioactivations of xenobiotics according different metabolic pathways and/or 2) to the presence of different genotoxic pollutants in fish environment. However, despite the global variety in spots on the overall study, the number of distinct spots per sample remains generally low, whatever the site. This apparent discrepancy could be explained by differences in the metabolic bioactivation of pollutants from one fish to another, probably due in part to interspecies variability. This is consistent with the absence of major spots on the overall study, the most frequent of them involving only 15% to 20% of fish, both sites together. Interestingly, the richness of DNA adduct pattern per fish remains statistically different between Reference site and Platform, with higher distinguished spots per sample at platform. That is in accordance with the higher exposure to genotoxicants at platform.

No spot among the more frequent ones appears to be strictly specific to one site. However, all the four more frequent spots (detected in more than 15% of the overall samples) are observed with significantly higher frequency at platform compared to reference site, all species combined. The lack of site-specificity of some spots for a presumably contaminated area could be attributed to the relative presence of certain genotoxic pollutants in the overall sites including reference ones, in probably very different concentrations and potentials of bioavailability. This result, in association with the very high proportion of samples without detectable adducts in reference site, could reflect the presence of genotoxic pollutants in very low levels (and/or bioavailability) at this site, under an undefined level that contributes to a detectable DNA adduct formation in fish liver. Other hypothesis for the lack of specificity of spots at contaminated sites and the presence of detectable DNA adducts in supposed unpolluted areas are the possible migration of fish from site to site or the revelation of endogenous DNA adducts (Aas et al., 2003; Swenberg et al., 2011). Endogenous DNA adducts are lesions of the DNA that can occur outside of exposure to xenobiotics. In contrast, individuals with higher DNA adduct levels (>0.40 adducts per 10^8 normal nucleotides) are thought to be exposed to higher levels of genotoxicants and/or are especially sensitive to the genotoxicity of certain pollutants

because of the genetic susceptibility combined with environmental exposures during vulnerable periods of development.

The mean adduct levels measured at platform all species combined and for each species are below or somewhat above the value of 0.40 adducts per 10^8 nucleotides. Interestingly, in previous comparable field studies, this cut-off value of 0.40 adduct per 10^8 nucleotides has been proposed by our laboratory as a possible threshold value for the detection of a significant genotoxic effect attributed to environmental pollutants (unpublished data). It is to note that this hypothesised value is equal to the mean DNA adduct concentration measured by Balk et al (2011) in haddock at the control site Egersund bank. In the way of a better environmental risk assessment, the determination of reliable threshold values for biomarkers is now a crucial issue. The question is under discussion, as shown in a report of the study Group on integrated monitoring of contaminants and biological effects dated 14-18 march 2011 (ICES, 2011). The proposed BAC (Background assessment concentrations) and EAC (Environmental Assessment criteria) values for DNA adducts are in accordance with the results obtained in our laboratory since 2009.

Interspecies differences in DNA adduct patterns are generally not statistically significant, except for the proportion of samples without detectable DNA adducts at Platform. The occurrence frequency of one spot among the four most frequent ones has been associated to species at platform too. Since it is not exclusively associated to inter-site differences of pollutants exposure, such qualitative variation could be related to differences in metabolic capabilities (such as absorption, distribution, elimination and/or bioactivation of pollutants like PAHs,), and/or conditions of exposure to genotoxic pollutants (associated to species behaviour: habitat, diet, sexual behaviour and reproduction…). On two closely related species of fish (brown bullhead Ameriurus nebulosus and channel catfish Ictalurus punctatus), the concentrations of hepatic DNA adducts measured after a single injection of BaP (20 mg / kg ip) appear significantly different from one species to another (Ploch et al., 1998). Since the absorption and distribution of BaP were similar for both species, higher rates of adducts in the bullhead livers could be explained by a slower elimination of BaP in this species. DNA adduct results are consistent with the inter-species difference in susceptibility to the PAH-associated induction of tumours. This example illustrates the existence of inter-species variability in metabolism of genotoxicants, even between closely related species. The link with the inter-species sensitivity in term of tumoural incidence is of particular interest to environmental risk assessment.

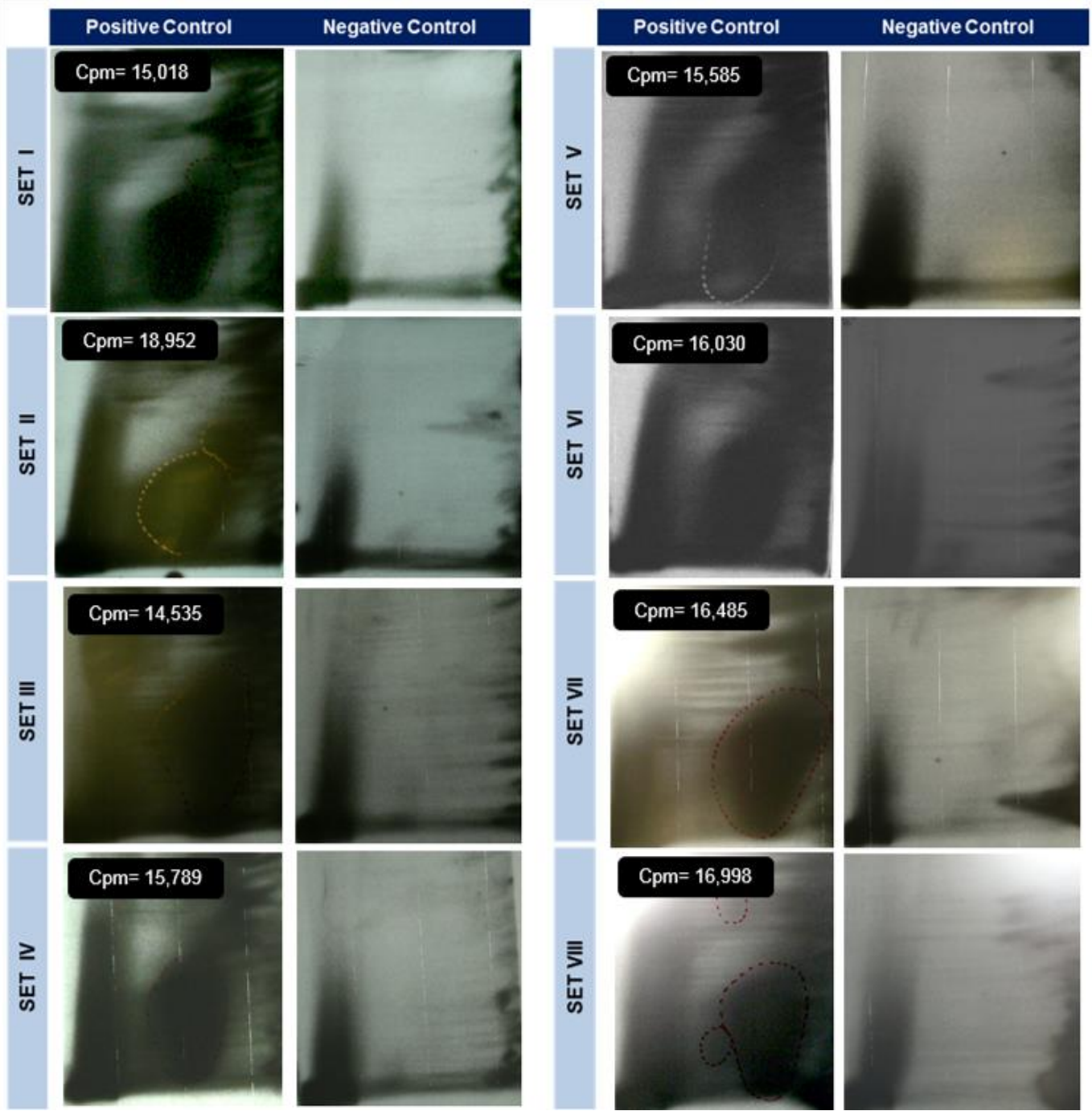
Concerning the potential sex related effect, no significant differences in levels of DNA adducts has been shown between males and females throughout the study, in the platform area. Qualitatively, only one spot among the four more frequent seems to be dependent on the gender. In fact, the involvement of gender in the profiles of DNA adducts is difficult to assess. In 2004, Akcha et al. studied the effect of biotic (age, sex) and abiotic (sampling site and period) on rates of liver adducts in flounder on the Eastern Channel (Bays of Seine and Somme). In a qualitative aspect, the richest patterns were for male adults taken from the site “Antifer” (compared with females and

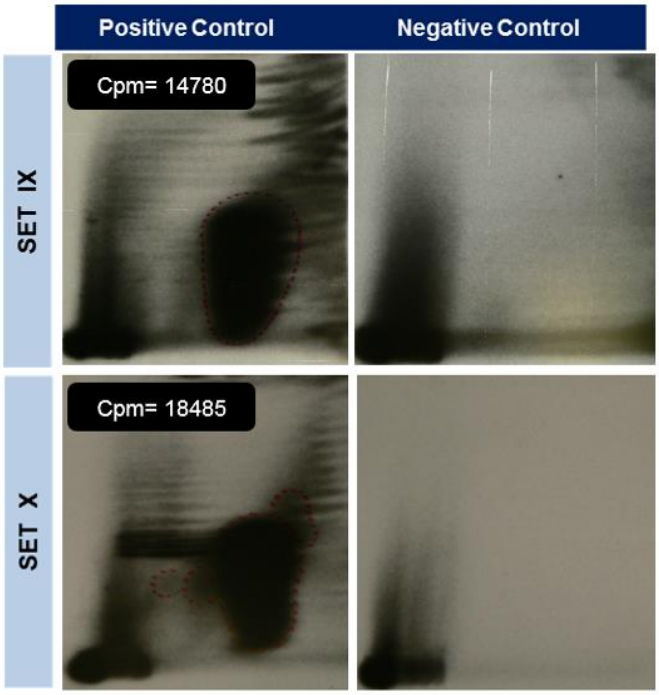
juveniles). However, no quantitative effect was observed in association with gender, age, site of sampling and sampling period (March-September 2001).

In conclusion, higher DNA adducts concentrations in the liver of fish are consistently observed on the platform compared to the reference site, species by species or all species combined, with large interindividual differences. These results are in accordance with a probable higher genotoxic exposure for fishes at platform. The DNA adduct concentrations are low but often near or above the possible threshold value for the detection of a significant genotoxic effect attributed to environmental pollutants, especially at platform. Qualitative results are more difficult to interpret, especially because of the lack of platform specific spot(s). However, the four more frequent spots are mostly detected at platform and could be probably attributed to genotoxicants exposure. The fact that these spots are also observed in few fish caught at reference site could be explained by the ubiquitous nature of pollutants (that's the case for PAH and especially for benzo[a]pyrene) and/or the migration of fish.

ANNEXES

ANNEXE 1: Autoradiographic patterns of the negative and positive controls included in each set of 32P-postlabelling (sets I to X).





Cpm= count per minute= direct radioactivity measured in the major spot (MS) in the positive control (after subtraction of background noise), for each set of analyses.

Autoradiography is realised after the specific ^{32}P labelling of DNA adducts and 2D-chromatographic separation on PEI-cellulose sheet. Time of exposure is to 72 hours.
Spot radioactivity is measured on PEI cellulose sheet with a scintillation counter (Cerenkov mode).
Positive control: calf thymus DNA treated by benzo[a]pyrene dioepoxide (BPDE) with a final concentration of 110.70 adducts for 10^8 normal nucleotides (according to F.A. Beland, in Philips and Castegnaro, 1999)
Negative control: plasmid DNA.

ANNEXE 2: Procedures

ANNEXE 2a: DNA extraction

The procedure is to extract purified DNA after isolation of the cell nuclei in the samples. It is applicable to any type of biological sample containing DNA, from 50 to 100 mg of tissue (such as "liver") or any cell pellet.

- Process for tissues treatment
- On the ice, finely cut tissue (take 70 to 80 mg)
- Add 1.5 ml of sucrose 0.32 M and mix thoroughly to lyse tissue (Tissue lyser, Qiagen: 20 Hz, 2 minutes)
- Centrifuge at 800G for 10 Minutes, at +4 °C

- Dissolve the pellet with 1.2 ml of EDTA / Tris (1 / 20 mM. pH 7.4)
Add 100 µl of 10% SDS solution and vortex for 1 minute.

- Incubate 30 minutes at 37 °C with:
0.2 mg / ml RNase A
33.4 U RNase T1

- Incubate 2.5 hours at 37 °C with 0.50 mg / ml proteinase K
(Until complete digestion of samples)

- Add 0.5 volume (0.7 ml) of saturated phenol and vortex 1 minute
Centrifuge 5 minutes at 5000 rpm.

- Remove the upper phase (aqueous phase) and transfer it to a clean tube
Add 0.5 volume (0.7 ml) of CIP (phenol + Sevag 1 / 1) and vortex 1 minute
Centrifuge 5 minutes at 5000 rpm (+4 ° C)

- Remove the upper phase and transfer it to a clean tube
Add 0.5 volume of Sevag (chloroform + isoamyl alcohol (1 / 24)) and vortex 1 minute
Centrifuge 5 minutes at 5000 rpm (+4 ° C)

- Remove the upper phase

- **Precipitation of DNA:**

Add to the aqueous phase 0.1 volumes of a solution of NaCl 5 M and 2 volumes of cold ethanol (stored at -20 ° C)
Shake and vortex lightly manually

- Allow to air dry the DNA. Add 150 µl of ultra pure water.

- **Spectrophotometric quantification of DNA solutions** (Nanodrop, Thermo Scientific)

- Spectrophotometric assay:
Principle: 1 unit of absorbance at 260 nm corresponds to a double-stranded DNA solution concentration equal to 50 µg / ml

- Quality criteria selected:
1.85 <A260 / A280 <1.95
A260 / A230> 2.00

- Prepare solutions close to 2 µg / µl
- Keep these solutions at -80 °C in glass vials (type 2 ml)

ANNEXE 2b. ^{32}P Post-labeling protocol used for the present study

«^{32}P Post-labeling protocol for detection of DNA adducts from environmental exposures to PAHs [(1)].»

[(1)] Polycyclic aromatic hydrocarbons

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2. Procedure for DNA adduct detection

2.1 Biological material

In order to allow a search of DNA adducts by the described ^{32}P post-labelling protocol, biological material supplied must meet requirements in both quantitative (2 x 5 mg DNA about 15 to 25 mg tissue and / or $5x10^6$ to 10^7 cells) and qualitative aspects (cell richness of the tissue samples)

2.2 Procedure for ^{32}P post-labelling

As result of the technical variability classically described with the ^{32}P post-labelling method, each sample was analysed twice in two independent manipulations (runs). Four controls are systematically added to the manipulations to check the successful completion of the manipulation. The two first control samples are one negative in adducts (cell DNA free of adducts) and the second positive in adducts (DNA rich in adducts of benzo [a] pyrene) with known quantity of adducts according to Philips and Castegnaro, 1999. The third and fourth controls are realised by 32P-labelling of 1) normal nucleotides (deoxyadenosine 3'phosphate, control of labelling by polynucleotide kinase) and 2) a small fraction of DNA (1 µg) coming from the negative control (verification of DNA hydrolysis efficiency).

2.2.1 Hydrolysis

- Prepare 5 µg of DNA / analyse
- Dry sample (Speed Vac SV, 15 minutes)
- Hydrolyse of DNA : MN : 0.7 µg / 5 µg DNA
SPDE : 10 mU / 5 µg DNA
+ Buffer solutions } 3.5 hours / 37°C

MN= micrococcal nuclease (Sigma); SPDE: spleen phosphodiesterase (Calbiochem)

2.2.2 Enzymatic enrichment with NP1

- Dry sample (SV) after hydrolysis
- NP1: 5 µg / 5 µg DNA
+ Buffer solutions } 30 minutes / 37°C
- Stop incubation with a tris base solution (1.8 µl/sample)

NP1= Nuclease P1 (Sigma)

2.2.3 ^{32}P radioactive labelling

-Add to sample:
- PNK : 10U/5µg DNA
- PNK buffer A 1X
- ^{32}P-ATP : 25 µCi / 5 µg DNA

} 30 minutes / 37°C

PNK : polynucleotide kinase (+ buffer A 10X ; Fermentas)

2.2.4 Chromatographic separation

Separation of radiolabeled adducts in the previous step is performed by bidirectional thin layer chromatography on polyethyleneimine (PEI) cellulose sheet (12 x 10 cm), by using D1 to D4 successive migrations (D1 and D4 being "clean-up" migrations). Solvent (mobile phase) composition is provided for each migration.

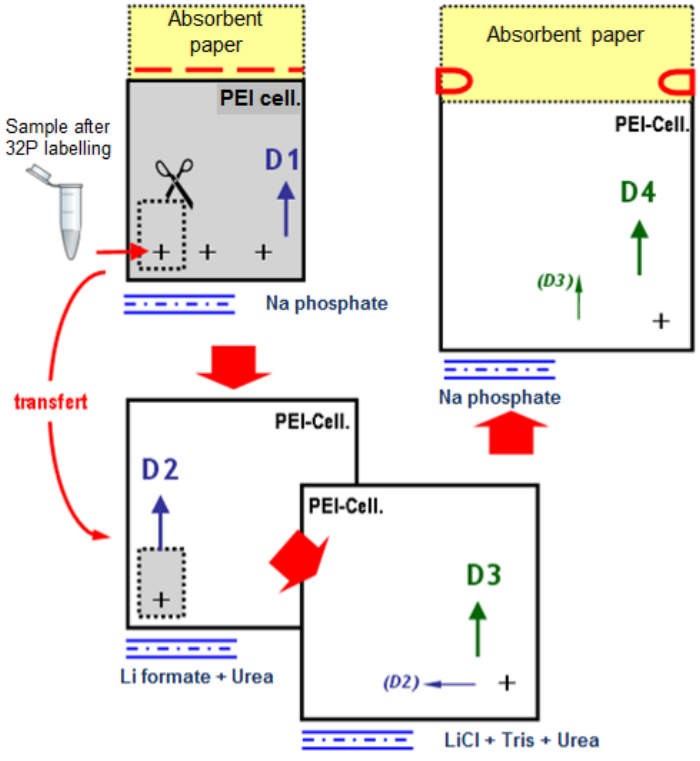
PEI-cellulose sheet (Macherey-Nagel)

• D1:
- Mobile phase: Na Phosphate 1 M. pH 6
- Wash sheet in deionized H2O after D1
- Dry sheet
- Cut up PEI Cellulose Sheet (transfer step)

• D2:
- Mobile phase:
Li Formate 4.5 M
Urea 8.5 M } pH 3.5
- Wash sheet in deionized H2O
- Dry sheet

• D3:
- Mobile phase:
Li chloride 1.6 M
Tris 0.5 M } pH 8
Urea 8.5 M
- Wash sheet in deionized H2O
- Dry sheet

• D4:
- Mobile phase:
Na Phosphate 1 M. pH 6.8
- Dry sheet



2.2.5 Revelation of DNA adducts

DNA adduct patterns are revealed by autoradiography (Kodak X-OMAT® / BIOMAX®). The optimum exposure time is a function of radioactive signal strength (exposure time at -80°C: from 12 to 72 hours).

2.2.6 Quantification / results analysis

The quantification is performed using the scintillation counting of spots cut on chromatographic sheets, by Cerenkov mode, and on the basis of the radioactive signal associated to the labeling of a known quantity of DNA adducts (positive control: 5 µg of a DNA which contains 110.7 adducts for 10^8 normal nucleotides, according to Phillips and Castegnaro, 1999, kindly provided by F.A. Beland, FDA, USA).

The results are given in two complementary approaches:

• Quantitative Approach:
- Results in relative levels of adducts (= RAL)
- By interest: Results per spot or per sample.
- Statistical Analysis

• Qualitative approach:
- Analysis of spots of interest in potential patterns
- Statistical analysis (presence / absence of a spot under the experimental conditions ...)

The exploitation of the results is made on the basis of two analysis per sample in two different manipulations.

ANNEXE 3: Qualitative raw data.

Qualitative results on the 103 samples according to the presence/absence of spots.

When spot (n°1 to 10 + DRZ) is present for only one of both analyses, the number 1 is indicated

When spot (n°1 to 10 + DRZ) is present for both analyses, the number 2 is indicated

Sample number	Study variables			Spot number										
	Site	Species	Sex	1	2	3	4	5	6	7	8	9	10	DRZ
1	PF	Tusk	F	2	1	1			1	1				
2	PF	Tusk	F	2		1	2							
3	PF	Tusk	F	1	2									
4	PF	Tusk	F	2		1		1						
5	PF	Tusk	F	1										
6	PF	Tusk	F		1									
7	PF	Tusk	M			2		1						
8	PF	Tusk	M		1	1								
9	PF	Tusk	F		1									
10	PF	Tusk	F		1									
11	PF	Tusk	M											
12	PF	Tusk	M					2						
13	PF	Tusk	M											
14	PF	Tusk	M				1							
15	PF	Tusk	M											
16	PF	Ling	F											
17	PF	Ling	F											
18	PF	Ling	F											
19	PF	Ling	F											
20	PF	Ling	F				2		2					
21	PF	Ling	F	2										
22	PF	Ling	F				2							
23	PF	Ling	F	1		1								
24	PF	Ling	F											
25	PF	Ling	F	1		1		1		1				
26	PF	Ling	F				2							
27	PF	Ling	F			2	2							
28	PF	Ling	F								2			
29	PF	Ling	F	2		1	2							
30	PF	Ling	M											
31	PF	Saithe	F		2									
32	PF	Saithe	M	1					1					
33	PF	Saithe	M			2	1							
34	PF	Saithe	F	1			2			1				
35	PF	Saithe	M				1			1				
36	PF	Saithe	F							1				
37	PF	Saithe	F				2							
38	PF	Saithe	M								2			
39	PF	Saithe	M				1							
40	PF	Saithe	M	2										
41	PF	Saithe	M	1		1				1				
42	PF	Saithe	F	1					1					

Sample number	Study variables			Spot number										
	Site	Species	Sex	1	2	3	4	5	6	7	8	9	10	DRZ
43	PF	Redfish	F	1	2									
44	PF	Redfish	F		2									
45	PF	Redfish	F	2		2								
46	PF	Redfish	F	2		2				1	2			2
47	PF	Redfish	F	2			2							
48	PF	Redfish	F	2										
49	PF	Redfish	F	2			2							
50	PF	Redfish	F	2	1						2	1		
51	PF	Redfish	F	1	1					1				
52	PF	Redfish	F				2							
53	PF	Redfish	F											
54	PF	Redfish	F									1		
55	PF	Redfish	F		2	2								
56	PF	Redfish	F		2		2							
57	PF	Redfish	F	2										
58	REF	Ling												
59	REF	Ling												
60	REF	Ling												
61	REF	Ling												
62	REF	Redfish												
63	REF	Redfish					2							
64	REF	Redfish												
65	REF	Redfish		2										
66	REF	Redfish		2			2				1			
67	REF	Redfish												
68	REF	Redfish												
69	REF	Redfish					1		1					
70	REF	Redfish												
71	REF	Redfish												
72	REF	Redfish												
73	REF	Redfish												
74	REF	Redfish												
75	REF	Redfish				2								
76	REF	Redfish												
77	REF	Saithe												
78	REF	Saithe												
79	REF	Saithe												
80	REF	Saithe												
81	REF	Saithe												
82	REF	Saithe			1	1								
83	REF	Saithe				2								
84	REF	Saithe												
85	REF	Saithe		2										
86	REF	Saithe												
87	REF	Saithe												
88	REF	Saithe												

Sample number	Study variables			Spot number										
	Site	Species	Sex	1	2	3	4	5	6	7	8	9	10	DRZ
89	REF	Tusk												
90	REF	Tusk												
91	REF	Tusk												
92	REF	Tusk		2										
93	REF	Tusk												
94	REF	Tusk												
95	REF	Tusk			2									
96	REF	Tusk		2										
97	REF	Tusk				2					1			
98	REF	Tusk			2								1	
99	REF	Tusk												
100	REF	Tusk		2	2									
101	REF	Tusk												
102	REF	Tusk												
103	REF	Tusk												

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