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### ► To cite this version:

Ciara Baines, Richard Meitern, Randel Kreitsberg, Jérôme Fort, Jörn Peter Scharsack, et al.. Correlations between oxidative DNA damage and formation of hepatic tumours in two flatfish species from contaminated environments. *Biology Letters*, 2023, 19 (5), 10.1098/rsbl.2022.0583 . hal-04111579

**HAL Id: hal-04111579**

**<https://hal.science/hal-04111579>**

Submitted on 27 Nov 2023

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1 Correlations between oxidative DNA damage and formation of hepatic tumours in two flatfish species  
2 from contaminated environments

3

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13

#### 14 **Abstract**

15 Many species in aquatic environments face increased exposure to oncogenic pollution due to  
16 anthropogenic environmental change which can lead to higher cancer prevalence. The  
17 mechanistic relationship connecting environmental pollution and cancer is multifactorial and  
18 poorly understood, and the specific mechanisms are so far still uncharacterized. One potential  
19 mediator between pollutant exposure and cancer is oxidative damage to DNA. We conducted  
20 a study in the field with two flatfish species, European flounder (*Platichthys flesus* L.) and  
21 common dab (*Limanda limanda* L.) with overlapping distribution and similar ecological niche,  
22 to investigate if the link between oncogenic pollutants and cancer described in ecotoxicological  
23 literature could be mediated by oxidative DNA damage. This was not the case for flounders as  
24 neither PAH bile metabolites nor metallic trace element concentrations were related to  
25 oxidative DNA damage measurements. However, dabs with higher PAH metabolite

26 concentrations did exhibit increased oxidative damage. High oxidative DNA damage also did  
27 not predict neoplasm occurrence, rather, healthy individuals tended to have higher oxidative  
28 damage measurements compared to fishes with pre-neoplastic tumours. Our analyses showed  
29 that flounders had lower concentrations of PAH bile metabolites, suggesting that compared to  
30 dab, this species is less exposed or better at eliminating these contaminants.

31 Keywords: Oxidative stress, hepatic tumours, marine contamination, European flounder,  
32 *Limanda limanda*, wildlife cancer

33

## 34 **Introduction**

35 Cancer is a disease that arose with multicellularity, potentially affecting all  
36 multicellular organisms [1]. Increasing evidence suggests some species have evolved stronger  
37 tumour suppressor mechanisms than others [2,3]. Whilst these mechanisms are the result of  
38 species'-specific life-history and/or environmental exposure to natural oncogenic substances  
39 [4,5], many species face increasing pressure from anthropogenic activities that could affect  
40 cancer prevalence in natural populations [6,7].

41 There is evidence that some species can adapt to increased concentrations of oncogenic  
42 pollutants, especially if the selection pressures are high and pollution is present for extended  
43 time periods [8,9]. One step towards understanding the mechanisms behind evolved cancer  
44 defence mechanisms in natural populations is to focus on specific physiological pathways to  
45 explore why cancer prevalence varies between populations and species living in environments  
46 contaminated with hazardous chemicals. One potential mediator between pollutant exposure  
47 and cancer is oxidative DNA damage, as both organic pollutants [10] and metallic trace  
48 elements [11] increase it. This can lead to cancer initiation and progression by increasing DNA  
49 mutations or inducing genome instability and cell proliferation [12]. However, relatively little  
50 is known about how the amount of oxidative DNA damage impacts cancer risk in pollution-

51 exposed animal populations, as current studies published mostly focus on changes in  
52 antioxidants in response to a specific pollutant in laboratory settings [13-15].

53 Flatfish, such as European flounders (*Platichthys flesus* L.) and dabs (*Limanda limanda*  
54 *L.*) have been studied for both pollution exposure and hepatic tumours [16, 17]. These benthic  
55 species are regularly used in biomonitoring surveys throughout the North Sea and Baltic Sea  
56 marine areas, which have been subjected to high concentrations of pollution for over a hundred  
57 years [18,19], through atmospheric and riverine inputs, and industrial point-sources (e.g. oil  
58 and gas platforms, [20-22]). Histopathological analyses have indicated that dabs have  
59 approximately 10 times higher liver neoplasia prevalence than flounders [23-28]. However, the  
60 causes behind this species-specific difference are not known.

61 This field study investigates correlations between the formation of hepatic neoplastic  
62 lesions, and (a) concentrations of polycyclic aromatic hydrocarbon (PAHs) bile metabolites,  
63 (b) liver concentrations of metallic trace elements (Hg, Cd, As, Pb). We also investigate  
64 whether oxidative DNA damage (measured via 8- hydroxy-2'-deoxyguanosine, 8-OhdG) acts  
65 as a mediator between contaminant exposure and neoplasm occurrence.

66

## 67 **Methods**

### 68 **Field Sampling**

69 Fish were collected using a trawler at ten Baltic and North Sea locations (See Table S1 for  
70 locations and numbers sampled) in August 2019. Fishes were weighed and measured before  
71 being killed by a percussive blow to the head that was followed by destruction of the brain  
72 using a surgical knife. Bile and liver samples were flash frozen in liquid nitrogen and stored at  
73 -80°C until analysis. A 3 mm liver section for histopathology analysis was stored in 4%  
74 formalin for 48 hours, then in 70% ethanol. Otoliths were collected to measure age. Sites were

75 allocated as 'reference' or 'polluted' based on OSPAR/HELCOM data on local anthropogenic  
76 pressures (Table S1, [29-31]).

#### 77 Histopathology

78 Liver samples were processed according to Feist et al [32] for histopathological analysis,  
79 prepared onto slides and analysed using a Nikon Eclipse 80i microscope. Foci of cellular  
80 alteration (FCA) and neoplastic lesions were recorded [32] (see supplementary Table S1 and  
81 figures S1, S2 for further details).

#### 82 PAH bile metabolites

83 PAH bile metabolites were measured using fixed fluorescence methods. Bile samples were  
84 diluted with 48% ethanol (1:1600) [33] and read using a fluorescence spectrophotometer (BMG  
85 Omega Fluostar) measuring fixed wavelength pairs (excitation and emission, respectively).  
86 290/380 nm (2-ring PAH); 256/380 nm (3-ring PAH); 341/383 nm (4-ring PAH); and 380/430  
87 nm (5-ring PAH) [34]. The results were presented as fluorescence units (FU) [35].

#### 88 Trace metal concentrations

89 Liver samples were freeze-dried for 48 hours and ground to powder. Total Hg concentrations  
90 were measured, in duplicate (relative standard deviation for aliquots <10%), in subsamples of  
91 ~1mg of homogenized liver using an advanced Hg analyser spectrophotometer (Altec AMA  
92 254) [36]. Remaining samples were analysed for trace elements arsenic (As), cadmium (Cd),  
93 and lead (Pb). Samples were digested with a mixture of 3 mL HNO<sub>3</sub> and 5 mL HCl Suprapur  
94 quality, heated in a microwave oven and diluted to 50 ml with deionized water, and analysed  
95 by Inductively Coupled Plasma Atomic Emission Spectrometry (Varian Vista-Pro ICP-AES)  
96 and Mass Spectrometry (ICP-MS II Series Thermo Fisher Scientific).

#### 97 Oxidative stress markers

98 Biomarker 8-hydroxy-2'-deoxyguanosine (8-OHdG) was used to measure oxidative lesions  
99 formed in nuclear or mitochondrial DNA induced by free radicals. DNA, extracted using the

100 Purelink Genomic DNA Mini Kit (Invitrogen), was quantified using a Qubit 4 fluorometer.  
101 Standards were read on the dsDNA HS setting to calibrate the machine. 1µL of sample was  
102 added to 199µL of the working solution in triplicates, analysed, and the means calculated in  
103 ng/µL.

104 Samples were prepared for ELISA kits (Cell Biolabs Inc, cat number STA-320) following the  
105 protocol for cell or tissue DNA samples by converting to single strand DNA, digesting DNA  
106 using nuclease P1 (M0660S, New England Biolabs), adding alkaline phosphatase (CIP,  
107 M0371S New England Biolabs), centrifuging at 6000g for 5 minutes and collecting  
108 supernatants. Plates were washed using BioTek ELx50 microplate washer and absorbance  
109 (wavelength 450nm) measured using a spectrophotometer (Biotek Synergy 2). Results were  
110 normalised against the DNA concentrations, converted from 1 to 4 strand DNA and presented  
111 as ng oxidised DNA bases per mg DNA.

#### 112 Statistical analysis

113 Statistical analyses were run using R 4.0.5[37]. Principle component analysis (PCA), using  
114 packages “stats”[38] and “psych”[39], grouped 2 components (see Figures S3-S4) splitting the  
115 variables into metals (As, Cd, Pb, Hg) and hydrocarbons (2, 3, 4, 5 ring). Generalised linear  
116 models tested whether oxidative stress or tumours could be predicted by trace metals or PAH  
117 metabolite concentrations, age, location (Baltic or North Sea), or whether sites were  
118 polluted/reference (Table S3). Chi-squared tested for a difference between the number of fishes  
119 with or without neoplasms/FCAs (3-levels: no abnormalities, FCA, neoplasm). Finally,  
120 ANOVA and post-hoc Tukey tests tested for differences between oxidative stress and tumours.  
121 Other packages used for plotting and data reorganisation were ggplot2[40] tidyverse [41],  
122 car[42] and various dependencies within them. Additional analysis with subsets of data (fish  
123 aged 3+ as younger fish as less likely to develop cancer and sites where both species were

124 present to exclude the influence of site characteristics on the results) are provided as  
125 supplementary material alongside all code and data (<https://doi.org/10.5281/zenodo.7846187>).

126

## 127 **Results**

128 Histopathology analyses indicated that dabs do not have significantly higher tumour prevalence  
129 (FCA or neoplasm) than flounders (Figure 1a, Chi-squared =2.81, df=2, p=0.2). This result  
130 could, however, be affected by the bias in the sampling, with more younger dabs in the data set  
131 compared to flounders. Linear models found that flounders were older and had higher amounts  
132 of oxidative DNA damage than dabs, dabs had significantly higher concentrations of PAH  
133 metabolites (Table 1). However, these results may be confounded by differences between sites.  
134 When we repeated the species comparison, including only the fish from the three sampling  
135 sites where both species were sampled, we found that only age was significantly different  
136 between dabs and flounders (Table S4). However, all the fishes with the highest contaminant  
137 concentrations were dabs (Figure 1b). There was a significantly higher variation (F-test) in  
138 metals ( $p<0.001$ ) and hydrocarbons ( $p=0.007$ ) in dabs than flounders, whereas age ( $p=0.057$ )  
139 and log-transformed oxidative DNA damage ( $p=0.555$ ) did not vary significantly between the  
140 species.

141 The generalised linear model suggested that neither age, contaminant concentrations, oxidative  
142 stress, or sea (North vs Baltic) influenced the likelihood of a fish developing neoplastic/pre-  
143 neoplastic lesions (both species). However, there was significantly higher proportion of  
144 tumours in fish living in reference sites compared to more polluted sites (Table 2).  
145 Additionally, older flounders had more tumours than younger ones. As age is known to  
146 significantly affect cancer prevalence, and we had an over-representation of young dabs in our  
147 dataset, additional analysis excluding fishes under 3 years is provided in supplementary  
148 materials. Results of these models were the same as for the full dataset (Table S5). In order to

149 check that site characteristics were not confounding our results, additional analysis was run  
150 with site as a predictor (Supplementary materials: Table S8, Figure S5). The model including  
151 site resulted in a singular fit, which suggests preferring a simpler model, as such we chose the  
152 models with "polluted/reference" and "sea" for the main results.

153 There was a significantly higher level of oxidative damage in the fishes from the Baltic Sea  
154 than the North Sea for both species ( $t=-4.511$ ,  $p<0.001$ ), flounders ( $t\text{-value}=-2.305$ ,  $p<0.031$ )  
155 but age, contaminant concentrations or whether a site was characterised as polluted/reference  
156 did not affect oxidative damage (Table S6). In dabs, oxidative damage was significantly higher  
157 in the Baltic ( $t\text{-value}=-4.198$ ,  $p<0.001$ ) and in dabs with higher concentrations of PAH  
158 metabolites. In the reduced dataset (only fish aged 3+) oxidative damage was significantly  
159 higher in the Baltic but the relationship, for dabs, with hydrocarbons (Table S7).

160 An ANOVA model indicated that oxidative damage was significantly higher in healthy dabs  
161 compared to those with FCA ( $p=0.027$ ) but not different between healthy individuals and those  
162 with neoplasms or an FCA (Figure 2). There were no significant differences for flounders.

163

## 164 **Discussion**

165 This field study with two flatfish species investigates whether the link between oncogenic  
166 contaminants and hepatic tumours, described in ecotoxicological literature, could be mediated  
167 by oxidative DNA damage. We did not find evidence for this in flounders, as neither PAH nor  
168 metallic trace element concentrations were related to their oxidative DNA damage, whereas in  
169 dabs, higher concentrations of PAH metabolites were related to increased oxidative damage. It  
170 is possible that including other contaminants (e.g., persistent organic pollutants) could provide  
171 a different picture. Furthermore, oxidative DNA damage was not related to neoplasm  
172 occurrence in flounders whereas healthy dabs had increased DNA damage compared to dabs  
173 with FCAs. When comparing the species with each other (previous studies indicated higher



174 pollution-induced neoplasm prevalence in dabs than flounders [eg.23-28]) we found flounders  
175 had more oxidative DNA damage. However, contaminant concentrations varied more in dab  
176 compared to flounders suggesting potential differences in either exposure or pollutant removal  
177 mechanisms (Figure 1b). Additionally, PAH metabolite concentrations in flounders were lower  
178 than in dabs, suggesting more efficient organic pollutant metabolism in flounders. It must be  
179 noted, however, that age structure and sample sizes for age groups differed between the species,  
180 with more younger dab samples compared to flounders (Table S2). While age was included in  
181 the models, this still might confound the results when comparing species. Additionally, it  
182 should be noted that there are fewer 'reference' sites than 'polluted', and it is possible that  
183 adding additional sampling locations could change these results.

184 Contaminants can induce oxidative stress by either enhanced reactive oxygen species  
185 generation and/or weakening of antioxidant/damage repair systems [43]. Fish studies have  
186 indicated that environmental contaminants can affect antioxidant levels (organic pollutants  
187 [44], heavy metals [45,46]). However, limitations in detecting oxidative stress, through  
188 measuring oxidants and antioxidants, occur from time-lagged and hormetic upregulation of  
189 protective mechanisms [47]. Therefore, we assessed the oxidative DNA damage (8-OHdG)  
190 from the liver tissue of fish exposed to contaminated environments. The levels of 8-OHdG in  
191 aquatic species have been studied as a biomarker of oxidative stress caused by contamination,  
192 showing increased levels in populations exposed to higher contamination burdens [48,49]. In  
193 our study, increased PAH metabolite concentrations in dabs, but not flounders, were related to  
194 higher oxidative DNA damage levels, suggesting that PAH metabolism is linked to increased  
195 DNA damage in dabs. The different results for dabs and flounders could arise here from the  
196 differences in sampling sites, as only 3 of the 10 sampling sites included both species. This  
197 result for dabs, whilst unexpected, could be an indication of acclimatization or adaptation  
198 processes, resulting from long-term exposure to oncogenic contamination to reduce oxidative

199 DNA damage. However, a study on killifish (*Fundulus heteroclitus*) suggested that most of the  
200 DNA damage present following PAH exposure was the result of covalent binding of  
201 metabolites to DNA rather than from oxidative damage [50]. Whether this is the case for  
202 flounders and dabs remains to be studied. Interestingly, tumours were not related to the levels  
203 of oxidative DNA damage in the liver, suggesting other processes must be involved that  
204 contribute more to tumour development in wild fish than oxidative DNA damage. Rather, in  
205 dabs with no abnormalities detected, there was less oxidative damage than in dabs with an FCA  
206 (no difference with neoplastic individuals or in flounders).

207 In this study, older fishes had more oxidative DNA damage than younger ones. Increased DNA  
208 damage in older fish have been recorded in dabs using comet assay methods [51]. Additionally,  
209 a study on three-spined sticklebacks (*Gasterosteus aculeatus*) found that larger fishes have  
210 more DNA damage than smaller ones using comet assay and micronucleus tests [52],  
211 potentially resulting from either the higher metabolic need of larger fish, leading to more  
212 oxidative DNA damage, or DNA damage accumulation with age resulting from the senescence  
213 process.

214 Fishing location had the greatest influence on elevated oxidative damage, demonstrated by  
215 Baltic Sea fish exhibiting a significantly higher oxidative damage than North Sea fish. Changes  
216 in salinity can cause oxidative stress in fish [53] and a study on olive flounder (*Paralichthys*  
217 *olivaceus*) found that hyposaline conditions caused oxidative stress [54] so it is possible that  
218 the low saline Baltic environment is increasing oxidative damage in flounders and dabs.

219 Additionally, a report by the European Environment Agency suggests a larger proportion of  
220 the Baltic (96%) is polluted than the North Sea area (75%) [55] which could cause increased  
221 oxidative damage in the Baltic. Actively removing organic pollutants from the organism is  
222 considered a risk factor for cancer, as the by-products of active pollutant metabolism by the  
223 cytochrome p450 enzymes (CYPs) lead to carcinogenesis via DNA adduct formation [56,57].

224 In this study, fish caught in ‘reference’ sites had more tumours than those from ‘polluted’ sites.  
225 However, it should also be noted that migration of fishes was not accounted for when  
226 categorising sites as ‘reference’ or ‘polluted’ and exposure rates could vary between  
227 individual’s dependant on migratory distances. It is possible that fish living in heavily polluted  
228 environments can adapt/acclimate to cope with contamination [58]. In conclusion, we showed  
229 that in an ecosystem where pollution has persisted for a long time, it is not possible to find a  
230 clear link between contaminants (PAH and metallic trace elements), oxidative DNA damage,  
231 and neoplasms. In the North and Baltic seas, both flounders and dabs have had approximately  
232 50 generations to adapt to high concentrations of environmental contamination. We can suggest  
233 that both species, but especially flounders, could have gone through evolutionary processes,  
234 where stronger cancer defence mechanisms, more efficient pollutant metabolism, and  
235 protection against oxidative DNA damage was selected for.

236

### 237 **Acknowledgements**

238 This work was supported by the Estonian Research council grants PSG653 to Tuul Sepp and  
239 PRG1137 to Peeter Hõrak. We are grateful to C. Churlaud and to M. Brault-Favrou from the  
240 "Plateforme Analyses Élémentaires" of LIENSs for their assistance during trace element  
241 analyses and to the crew of RW Walther Herwig III for all-round help during the fieldwork.

242

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439 Table 1: Linear model results for a difference between oxidative damage, age and contaminant  
440 burdens between species. \* represents p-value <0.05, \*\* represents p<0.01.

	Coefficient	S.E.	Wald Z	Pr(> Z )
Intercept	-18.7452	5.1216	-3.660	0.000252 ***
Oxidative DNA Damage(log)	2.3344	0.8339	2.799	0.005121 **

Metals	-0.5968	0.4578	-1.304	0.1924
Hydrocarbons	-0.9054	0.3677	-2.462	0.0138*
Age	1.1217	0.2451	4.577	<0.0001*
				**

441

442 Table 2: Model results (N=128) of variables influencing the differences in pre-  
443 neoplastic/neoplastic tumours between both species and each species individually. \*  
444 represents p-value <0.05, \*\* represents p<0.01.

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		Estimate	Std. Error	t value	Pr(> t )	
Both	(Intercept)	3.11513	4.5926	0.678	0.49759	
	Metals	-0.20913	0.21477	-0.974	0.33019	
	Hydrocarbons	0.12996	0.22643	0.574	0.56601	
	Age	-0.02367	0.1649	-0.144	0.88584	
	Oxidative Stress	-0.56179	0.75214	-0.747	0.45511	
	Sea (North)	1.18170	0.71741	1.647	0.09927	
	Polluted	-1.4411	0.55388	-2.602	0.00927	**
Dab	(Intercept)	9.92253	5.94056	1.670	0.0949	
	Metals	-0.20196	0.22902	-0.882	0.3778	
	Hydrocarbons	0.24261	0.28230	0.859	0.3901	
	Age	-0.03751	0.34256	-0.110	0.9128	
	Oxidative Stress	-1.73457	0.96487	-1.798	0.0722	
	Sea (North)	1.14057	1.03903	1.714	0.2723	
	Polluted	-1.56631	0.75596	-2.142	0.0383	*
Flounder	(Intercept)	-23.9688	12.3031	-1.948	0.0514	
	Metals	-2.0686	1.4950	-1.384	0.1665	
	Hydrocarbons	-0.5449	0.6646	-1.820	0.4123	
	Age	0.9142	0.4362	2.096	0.0361	*
	Oxidative Stress	3.1787	1.9040	1.669	0.0950	
	Sea (North)	1.6869	1.5987	1.055	0.2913	
	Polluted	-3.1224	1.4183	-2.201	0.0277	*

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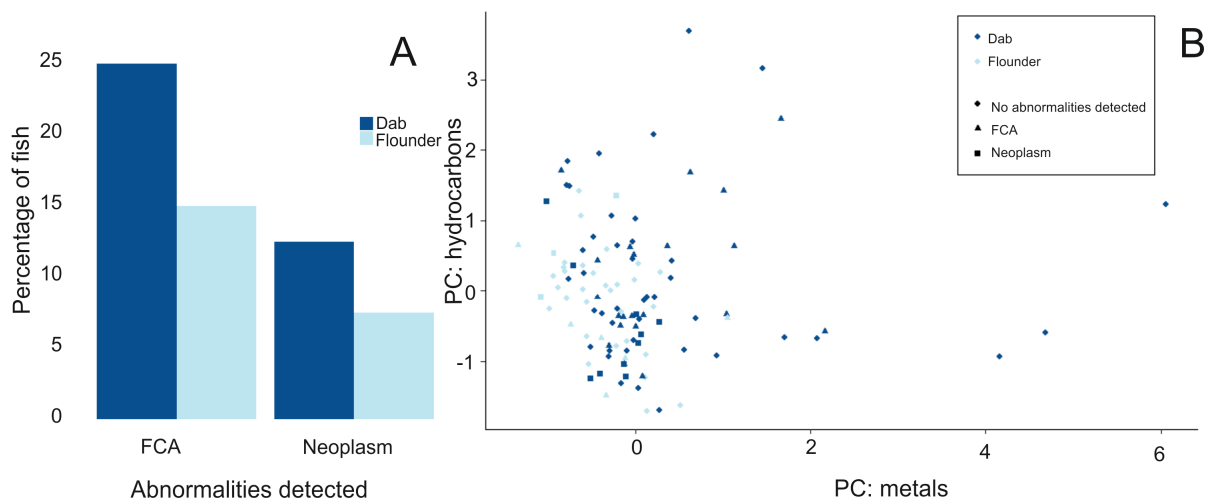
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451 Figure 1: A: The prevalence of cancer and foci of cellular alterations (FCA) in flounder (n=40)

452 and dab (n=88) livers. Individual numbers on bars. B: PCA loadings for metallic trace elements

453 and polycyclic aromatic hydrocarbons (PAHs).

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456

457 Figure 2: Mean (log-transformed) oxidative DNA damage between fish with neoplasms, foci

458 of cellular alterations (FCA) or neither. Asterix (\*) represents  $p < 0.05$ . Bars represent mean  $\pm$

459 standard deviation. Dots are individual data points.

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