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Genotoxic and CYP 1A enzyme effects consecutive to the food transfer of oil spill contaminants from mussels to mammals

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Abstract – The transfer of polycyclic aromatic hydrocarbons (PAHs) from bivalves, through the food chain to vertebrates was of concern. Our research aimed at estimating potential effects for consumers resulting from the ingestion of seafood contaminated by oil spill pollutants. After the "*Erika*" wreck, mussels (*Mytilus* sp.) were collected from sites of the Atlantic coast impacted to various degrees by the oil slick and constituted contaminated food for rats during 2 and 4 weeks. Genotoxic damage were studied in rats by means of COMET assay carried out in liver, bone marrow and peripheral blood. Biochemical and genomic effects such as the induction of CYP 1A1 and the expression of cytochrome genes were measured in rat livers. The most sensitive biological parameter reflecting the transfer of contaminants *via* the food appeared to be DNA breaks studied by means of the COMET assay. Genotoxic damage, observed mainly in the liver, were rather moderate and remained not persistent. This study underlined the bioavailability of pollutants in fuel oil contaminated mussels for consumers, and the complexity of the contamination consecutive to the oil spill. The occurrence of related PAH compounds in addition to non-substituted PAHs in fuel oils and mussels raised the question of their implication in the registered effects.

Key words: Polycyclic aromatic hydrocarbons / Oil spill / COMET assay / DNA damage / EROD activity / CYP 1A / Food transfer

Résumé – Effets génotoxiques et au niveau du CYP 1A, consécutifs au transfert des contaminants du pétrole de la marée noire par voie alimentaire, des moules aux mammifères. Nos préoccupations venaient du transfert des hydrocarbures aromatiques polycycliques (HAPs) des bivalves aux mammifères, par la voie alimentaire. Notre recherche visait à estimer les effets potentiels pour des consommateurs résultant de l'ingestion de fruits de mer contaminés par les polluants de marée noire. Suite au naufrage de l'« *Erika* », des moules (*Mytilus* sp.) ont été échantillonnées sur des stations de la côte atlantique plus ou moins polluées par la marée noire. Des rats ont reçu pendant 2 et 4 semaines des rations de cette nourriture contaminée. La génotoxicité a été étudiée au moyen du test des comètes au niveau du foie, de la moelle osseuse et du sang périphérique des rats. Les effets biochimiques et génomiques tels que l'induction du CYP 1A1 et l'expression des gènes du cytochrome, ont été recherchés au niveau du foie. Le paramètre biologique le plus sensible, reflétant un transfert des contaminants par la voie alimentaire, apparaît être l'étude des cassures à l'ADN par le test des comètes. Les dommages génotoxiques, observés principalement dans le foie, restent plutôt modérés et non persistants. Cette étude a souligné la biodisponibilité des polluants dans les moules pour le consommateur, et la complexité de la contamination engendrée par la marée noire. La présence de composés apparentés aux HAPs, en plus des HAPs non-substitués dans les fiouls lourds libérés et dans les moules, soulève la question de leur implication dans les effets observés.

1 Introduction

On December 12, 1999, the wreck of the oil tanker *Erika* caused the most important pollution by hydrocarbons occurring in France since the oil spill of the Amoco-Cadiz in 1978. This Maltese tanker sunk along the coasts of Brittany (at 75 km of Belle-Ile and at 65 km of the Pointe of Penmarch). 20 000 tons of fuel would have been released in the marine environment. The oil slick spoiled more than 400 km of the French Atlantic coasts. The fuel oil consisted in a type No 2 heavy fuel, a residue of distillation of crude oil. This petroleum fraction contained toxic compounds including PAHs, many heterocyclic hydrocarbons, sulphur organic derivatives, solvents, toxic metals (molybdenum, vanadium, mercury).

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The accident had dramatic consequences on the marine fauna and birds.

As Invertebrates could bioaccumulate polycyclic aromatic hydrocarbons (PAHs) (Meador et al. 1995; Solé et al. 1996; Law et al. 2002), the transfer of these pollutants through the food chain to vertebrates was of concern (Nunn et al. 1996). Indeed, PAHs were present at no negligible levels in heavy fuel oils, released in the marine environment after the *Erika* oil spill on the Atlantic French coast.

Our research aimed at estimating potential effects for consumers resulting from the ingestion of seafood contaminated with the oil pollutants. The genotoxic, mutagenic and carcinogenic properties of PAHs, and especially of the congeners with five and six aromatic rings, are known in vertebrates (IARC 1987). PAHs are also inducers of cytochrome P450 1A1 (CYP 1A1) in mammals (Nordqvist et al. 1979).

Therefore, we studied the effects of repeated ingestions of oil-contaminated mussels on rats by investigating genotoxic damage using single-cell gel electrophoresis (COMET) assay (Singh et al. 1988) and by measuring the induction of ethoxyresorufin-o-deethylase (EROD) activity (AFNOR 2001; Burke and Mayer 1974), and the expression of CYP1A1 gene by means of Reverse Transcription – Polymerase Chain Reaction (RT-PCR). One of our objectives was to establish a threshold of contamination devoid of toxic effects for mammals exposed to PAHs through their food.

2 Material and methods

Food preparation:

After the *Erika* wreck, mussels (*Mytilus* sp.) were collected from sites of the Atlantic coast impacted to various degrees by the oil slick. The samples were obtained from the IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) Centre in Nantes and kept at −20 °C. Lots of mussels were constituted in order to study the effects at different levels of PAH contamination, between 50 and 1000 μg total PAHs kg^{-1} dry weight (d.w.). Food batches were prepared by pooling mussels from impacted sites as follows: frozen mussels were opened, soft tissues were mixed, homogenized in a grinder and conditioned in pools of 12−15 g (each constituting one meal). The 16 PAH congeners listed as priority pollutants by the US-EPA for environmental biomonitoring (OFR 1982) were analyzed in mussels after preparation of the rations.

PAH analyses:

The quantification of PAHs in food was performed as follows. Samples were dried at 40 °C until constant mass. Extraction of PAHs was conducted by ASE extractor (Accelerated Solvent Extractor 200, from DIONEX, Sunnyvale, USA), allowing a solid/liquid extraction at 100 °C and under a pressure of 136 bars, with dichloromethane and hexane as extraction solvents (ratio 50:50; v:v). After solvent evaporation, extracts were dissolved in acetonitrile. PAHs were separated by reversed high performance liquid chromatography (HPLC) and detected by a spectrofluorimeter coupled with a UV/diode array detector. In addition, the methylated and related PAHs, such as thiophenes, were measured in mussels by gas chromatography coupled to mass spectrometry (GC-MS) after lyophylisation of samples and extraction under warmth and pressure.

Animal treatment:

Rats were fed with contaminated mussels during two and four weeks. Two main studies were conducted. In the first experiment, rats received one mussel ration of 13.5 g fresh weight every two days, and in the second experiment, once a day. The experimental design (Fig. 1) corresponded to a regular and important consumption of contaminated food. Five levels of contamination were studied: around 85, 310, 480, 570, 870 μg total PAHs (sum of the sixteen US-EPA) kg^{-1} of food d.w. Control rats in both experiments received a standard food for laboratory animals. In all cases, the animals (six per lot) were sacrificed 3 days after the last meal of contaminated mussels. Mussels (B1) sampled in a non-impacted site on the North Brittany coast (Les Saumonards, France), were used as additional controls. For biochemical parameters, dissected tissues were immersed in a 5 M guanidium isothiocyanate buffer to avoid RNA degradation and frozen at −196 °C until RNA extraction. Other pieces of tissues were placed in a 50 mM phosphate buffer containing 0.15 mM KCl and 20% glycerol, and frozen at −196 °C. For the COMET assay, all the collected tissues (liver and peripheral blood) were kept at −196 °C in liquid nitrogen except for intact femurs which were kept at −20 °C before bone marrow extraction. Cellular suspensions of bone marrow were prepared by scraping and washing the inside of the femur with phosphate-buffered saline solution (Ca^{++} and Mg^{++} free, pH 7.4) (PBS), with 10% dimethylsulfoxide (DMSO) added. For this tissue, suspensions were kept at −196 °C after progressive freezing (4 hours at −20 °C, one night at −80 °C and −196 °C in liquid nitrogen).

EROD enzymatic activity measurement:

Each liver was homogenized in phosphate buffer, with an antiprotease cocktail added, by means of a manual Potter. The hepatic microsomes were prepared by two differential centrifugations (9000 g for 15 min, and 100 000 g for 60 min at 4 °C). Microsomal EROD activity was measured according to the AFNOR Norm NF T 90-385 (2001) using a fluorimetric microplate reader. Protein concentration determination was performed according to Bradford (1976).

Expression of CYP 1A1 gene:

Total RNAs were extracted from the livers using the GenElute Mammalian Total RNA kit (Sigma) and reverse-transcribed using the RevertAid™ M-MuLV reverse transcriptase (MBI Fermentas). PCRs were performed in a MJ Research PTC-100 thermal cycler (Global Medical Instrumentation Inc.). The PCR reactions were conducted on cDNA from control or treated animals using primers chosen in highly conserved coding sequences of rat CYP1A1 gene. PCR amplification conditions were described in Chaty (2003). Amplimers were separated by electrophoresis on 1.5% agarose gel in TAE buffer (TRIS 40 mM, acetic acid 1 mM, EDTA 40 mM); the gels were stained with ethidium bromide and the PCR products were visualized under UV light. The intensity of PCR products was quantified with Molecular Analyst™ (version 1.1.1., Biorad). The statistical analyses for EROD activity measurement and CYP1A1 expression consisted in two-tailed Mann-Whitney U tests.

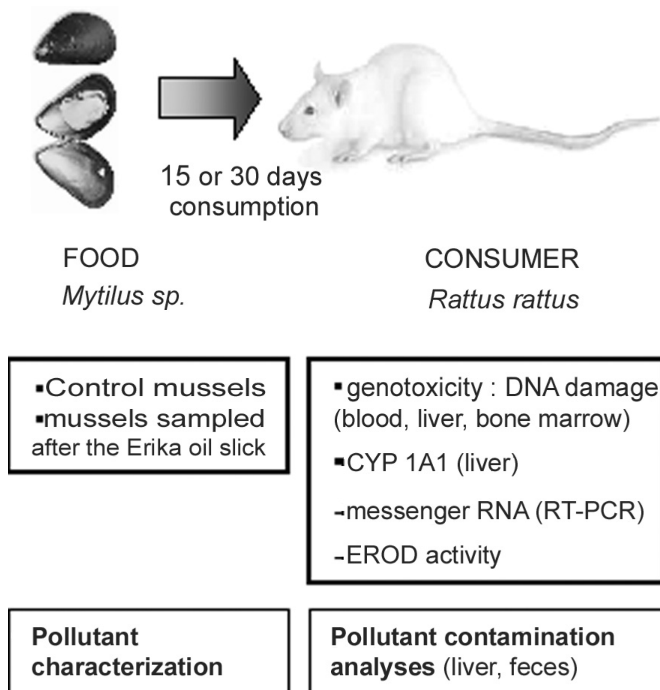


Fig. 1. Experimental design.

Alkaline single cell gel electrophoresis (COMET) assay:

DNA damage were studied by means of the COMET assay as described by Singh et al. (1988) with minor modifications. Briefly, the assays were performed under inactinic light at 4 °C and in triplicate for each sample. The cellular suspensions were included between two layers of agarose on microscope slides and submitted to lysis for 2 h at 4 °C. Thereafter, slides were placed in an electrophoresis tanker, covered with the electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA) to allow DNA unwinding and then electrophoresed (300 mA, 21 min, 20 V). The slides were batched twice in the neutralizing buffer (0.4 M Tris, pH 7.5) before the dehydratation step in absolute ethanol for 10 min. Nuclear DNA was stained with 25 μl ethidium bromide (20 μg ml^{-1}) and observed using an epifluorescence microscope (BX60 Olympus) connected to an image analysis system (Komet 3:1, Kinetic Imaging Ltd). The percentage of DNA in the tail was chosen to express DNA damage and non-parametric statistical tests (the Kruskal-Wallis test, the Median test and the Chi-square test) were used to compare the results.

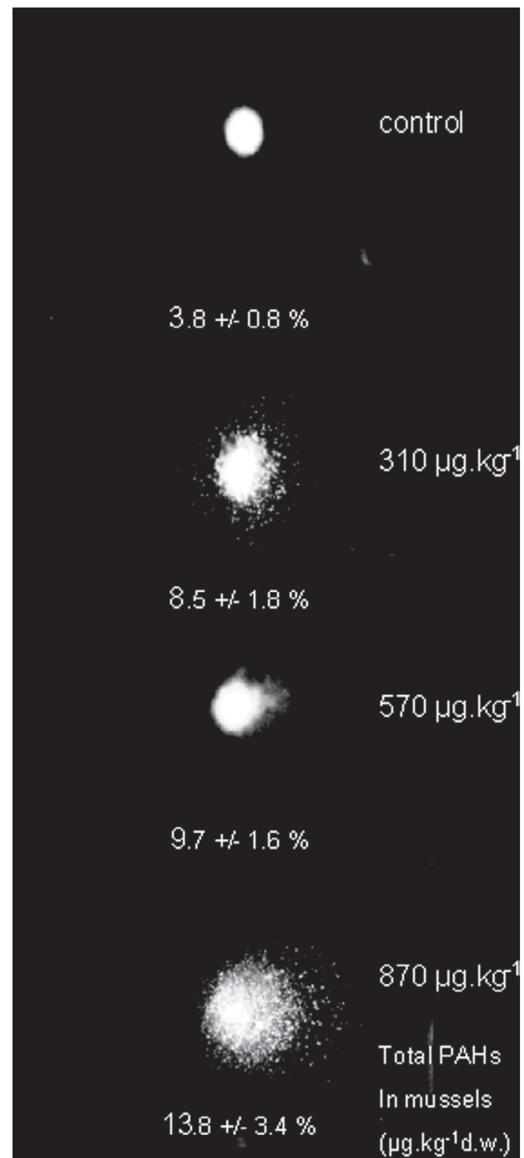


Fig. 2. Individual average nuclei of hepatocytes visualized by the COMET assay in livers of rats fed during 15 days with either standard food (control) or mussels sampled in contaminated areas (second experiment, 6 animals per treatment); Averaged percentages of DNA in the tail of nuclei with standard deviation.

3 Results

Those studies with repeated meals of contaminated food on 15 and 30 days, showed that (Table 1):

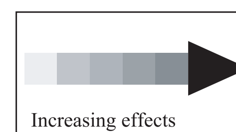
1. The biochemical effects (induction and the expression of the gene of the CYP 1A1) were transient: a kinetic study showed that the induction was maximum in the 12 hours following the ingestion and was no more detectable after 3 days. In the 2 and 4 weeks experiments, no induction was observed in rats 3 days after the last ingestion, although rats had been fed during 15 and 30 days with contaminated mussels, even heavily contaminated mussels (870 μg total PAHs kg^{-1} of mussels dry weight).
2. Genotoxic effects were registered in the liver and the bone marrow, 3 days after the last ingestion (Fig. 2). The genotoxicity was more persistent than the biochemical effects. The intensity of DNA damage increased with mussel contamination level but remained moderate. The threshold of contamination of the mussels from which a genotoxicity was observed, was 310 μg total PAHs kg^{-1} mussels (dry weight), below the threshold recommended by AFSSA (500 μg total PAHs kg^{-1} mussels (dry weight)) (AFSSA 2000). Yet, the genotoxicity did not increase over time, beyond 15 days. Indeed, effects after 30 days of exposure with fuel oil contaminated food were equivalent to

Table 1. Synthesis of the biological effects (EROD activity (liver), levels of CYP 1A1 messenger RNA (by RT-PCR) (liver) and DNA damage (COMET assay) (hepatocytes, bone marrow cells and peripheral blood nucleated cells) studied in rats fed with mussels sampled in areas impacted to various degrees by the oil slick, during 15 or 30 days: relative scale of increasing effects (abbreviations: d.w. dry weight; + significant effect).

	Food lots	**B1**		**A2**		**A1**		**B2**		**A3**		**B3**		**B4**	
	Length of exposure in days	15	30	15	30	15	30	15	30	15	30	15	30	15	30
	Sum of PAHs (in μg kg^{-1} d.w.)	**70**		**85**		**135**		**310**		**480**		**570**		**870**	
Biological parameters	**EROD Activity (liver)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Biological parameters	**CYP 1A1 mRNA (liver)**	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Biological parameters – DNA damage	**Liver**	-	-	-	-	-	-	++	++	++++	++++	++	++	+++	+++
Biological parameters – DNA damage	**Bone marrow**	-	-	±	-	-	-	+	-	±	±	+	+	++	++
Biological parameters – DNA damage	**Blood**	-	-	-	-	-	-	-	-	-	-	-	-	-	-

➢Induction of the CYP 1A1 was transitory, undetectable 3 days after the last ingestion

➢Genotoxicity in liver and bone marrow: moderated and transitory
-threshold around 300 μg total PAHs kg^{-1} mussels (dry weight)
-return to normality 17 days after the last ingestion



those measured after 15 days. This confirmed the fast elimination of PAHs in mammals, and the capacities of vertebrates to repair DNA. In the present case, DNA repairing systems appeared unsaturated after 30 days, as showed by the return to normality in all cases within 14 days. Recovery of DNA integrity was even registered after oral exposure with mussels containing 570 μg kg^{-1} d.w. (total PAHs).

4 Discussion

Marine mussels had often been used to follow the impact of oil slick pollutions (Solé et al. 1996; Glegg et al. 1999; Harvey et al. 1999; Thomas et al. 1999a,b; Fernley et al. 2000), but had never been studied as a vector of contamination to mammals.

One interest of our work was to study samples representative of the environmental pollution by PAHs and not artificially polluted samples.

This work raised questions about the biological consequences of DNA damage, which may modify the expression of crucial genes.

Moreover, the repair process itself may increase the probability of secondary mutations (Christmann et al. 2003; Sarasin 2003), the latter being undetectable with the COMET assay.

Finally, the questions of the genotoxic properties of methylated PAHs, PAHs related compounds such as thiophenes, and other derivatives, and their implications in registered effects remained to define, as well as the effects of nickel and vanadium likely to contribute to genotoxicity.

Other PAH derivatives, such as methyl chrysene and thiophenes found in mussels, could also be responsible for the observed effects. The methylated PAH sum could reach 1900 μg kg^{-1} d.w. in the mussels A3, and should be considered for their genotoxic effects. Few studies had been conducted to study the genotoxic potentials of these compounds. Their general toxicity was poorly known although their co-occurrence with PAHs in the environment may be suspected. It would be advised to study their toxic potentialities and considered them in regulations. Borosky (1999) underlined that a methyl substitution could lead to an increase in carcinogenicity for PAHs. In addition, 5-methyl chrysene was classified as a carcinogen 2B by IARC whereas chrysene was only in the class 3.

The thiophene compounds might possess mutagen properties similar to benzo(a)pyrene (Sinsheimer et al. 1992). Studies of Poon et al. (1997, 1998), showed histological abnormalities and hepatoxic effects in the rat consecutive to a contaminated diet with benzothiophene.

Gilroy (2000) underlined the difficulty of the health risk evaluation from contaminated seafood following an oil spill. Their work highlighted the necessity of standardized protocols for this concern. In our study, the most sensitive biological parameter reflecting the PAH transfer *via* the food appeared to be DNA breaks studied with the COMET assay. The CYP1A1 induction studied by means of EROD activity appeared to be

more sensitive than the measurement of gene expression by RT-PCR, but responses of these parameters were transitory.

5 Conclusion

The DNA breaks studied with the COMET assay appeared the most sensitive biological parameter to study the impact of food PAH contamination in mammals. Genotoxic damage were observed mainly in the liver. DNA injuries were rather moderate and not persistent. The threshold of mussel contamination below which no genotoxicity was recorded, was 310 μg total PAHs kg^{-1} mussels (dry weight). The measurement of the EROD activity seemed more sensitive than the study of the genic expression to investigate the CYP 1A1 induction.

This study underlined the bioavailability of pollutants in fuel oil contaminated mussels for consumers, and the complexity of the contamination of the invertebrates consecutive to the *Erika* oil slick. The occurrence of substituted PAHs and related compounds such as benzothiophenes in addition to non-substituted PAHs in fuel oils and mussels raised the question of their implication in the registered effects.

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