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Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled *Prestige* crude oil

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Abstract

We analyzed the hydrocarbon composition of the *Prestige* oil as it reached the shores, its solubility in sea water, its bioaccumulation, and the genotoxic damage associated to oil exposure, using *Mytilus galloprovincialis* as sentinel organism. Mussels were exposed to two oil volumetric ratios (1:500 and 2:500) for 12 days. Great concentrations of total polycyclic aromatic hydrocarbons (TPAH) have been obtained, being in general higher in the samples from the dose of 1:500, both in sea water (55.14 vs. 41.96 μg/l) and mussel tissue (16,993.80 vs. 17,033.00 μg/kg), probably due to the great tendency of these compounds to link to particles in water. Comet assay results reflected an increase in the DNA damage associated to oil exposure, higher in the mussels exposed to the higher aqueous TPAH content. In the view of our results, the importance of the evaluation of biodisponibility, bioaccumulation and DNA damage in the assessment of the effects of xenobiotic pollutants to marine environments could be highlighted.

Keywords: *Mytilus galloprovincialis*; *Prestige* spill; Comet assay; Genotoxicity; DNA damage; Polycyclic aromatic hydrocarbons; Benzo[*a*]pyrene; Biodisponibility

1. Introduction

In November 2002, a large oil tanker, the *Prestige*, broke up in heavy seas off Galicia, on the Northwest coast of Spain, spilling more than 77,000 tons of crude oil. This disaster led to one of the largest spills in maritime history. The oil formed a large slick on the surface of the water that then washed onto the beaches and rocks all along the coast and also affected the sea bed, a process that was accelerated by tide movements and ocean currents. This oil was classified as fuel oil No. 6 by the USEPA, and the International Agency for Research on Cancer regards this grade of oil as a possible carcinogen (group 2B) (IARC, 1989). Its composition, according to the analysis made by the Superior Center of Scientific Research of Spain (CSIC), can be described as 50% aromatic hydrocarbons, 20% saturated hydrocarbons and 35% resins and asphalthene. It is worth noting that six of its polycyclic aromatic hydrocarbons (PAH) (naphthalene, benzo[*a*]anthracene, benzo[*b*]-fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene and dibenz[*ah*]anthracene) are categorized as probable and possible human carcinogens (groups 2A and 2B), and are included in the 16 PAHs designated by the United States Environmental Protection Agency (USEPA) as primary contaminants. There is one group among the oil members specially important from a genotoxic point of view: PAH, particularly benzo[*a*]pyrene (BaP), which is mutagenic both in prokaryotic and eukaryotic test systems (Micic et al., 2002) and it is involved in the generation of strand breaks via free radical mechanisms (Mitchelmore et al., 1998). Furthermore, this agent is known to be activated by mutagens in the genus *Mytilus* (Livingstone et al., 2000).

In the last decades, a large number of ecosystems have been subjected to the growing influence of human activity,

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and consequently, many people have become aware of the need to protect ecosystems and to measure the damage caused by active sources of contamination. To this end, the application of biomarkers is of special relevance, since molecular/biochemical alterations may reveal the occurrence of environmental disturbance at an early stage, before a more integrated form of toxicity appears at higher levels of the biological organization (Moore, 1988). Among the varieties developed, the interest in genetic biomarkers is increasing day by day, mainly due to the fact that DNA represents one of the main targets in stressed ecosystems (Frenzilli et al., 1999). One of the species most extensively used as a sentinel organism to detect environmental damage or stress is the mussel. Marine mussels (genus *Mytilus*) have a broad distribution in both hemispheres. They live throughout the middle and lower inter-tidal zones and are common on rocky shores and other hard substratum environments. Their distribution, abundance, suitable size and filter-feeding activity have made them a popular sample organism for toxicological studies since they are considered great bioaccumulators of pollutants (Kadhim, 1990).

A diverse array of techniques has been used for detecting adverse genotoxic effects of several exposures in marine mussels. These include micronucleus test (Wrisberg et al., 1992; Bolognesi et al., 1996; Venier et al., 1997), sister-chromatid exchanges (Harrison and Jones, 1982; Brunetti et al., 1986), and chromosomal aberrations (Al-Sabti and Kurelec, 1985). The main problem that these toxicological methodologies using mussels present is their low rate of cell division, which causes difficulties in their application. DNA strand breakage has been shown to be a relatively sensitive and broad specificity indicator of genotoxic pollutant exposure (Mitchelmore and Chipman, 1998), and it does not require cells to be in a dividing state. In this regard, the alkaline comet assay is a suitable technique for measuring DNA strand breaks. Furthermore, it may be applied to any kind of nucleated cell, does not require a large amount of tissue, and it is also quick and cost effective. The most appropriate tissue as target for damage detection is the gill, since it is easy to obtain, and in nature it comes into contact with relatively large volumes of seawater compared with the rest of the animal (the average filtering rate for an adult mussel is approximately 2 l/h at 20 °C), which makes it a particularly suitable target tissue for mutagen exposure. Furthermore, the mussel gill is relatively homogenous with regard to cell type, and can easily be made into a single-cell suspension (Wilson et al., 1998).

Several studies have been reported following previous oil spills (*Sea Empress*, *Exxon Valdez*, *Erika*) in some aquatic organisms, evaluating PAH contents in tissue (Boehm et al., 1996; Carls et al., 2001; Carls et al., 2002), total hydrocarbon levels and immune response (total haemocyte number, superoxide generation and phagocytosis) (Dyrynda et al., 1997), and oil toxicity (mortality test in *Tigriopsus brevicornis* and chlorophyll content in *Enteromorpha intestinalis*) (Pavillon et al., 2002). The purpose of this study was to measure the toxic effects of the collected samples of oil from the spill from the *Prestige* break-up by evaluating its solubility in the sea water, its bioaccumulation and the genetic damage (by means of the comet assay) produced in the mussel *Mytilus galloprovincialis* under laboratory conditions. These three parameters can be considered a first step in the assessment of the damage caused by the oil spill to this rich coastal environment that, thanks to its natural conditions especially to the estuaries, produces near 270,000 metric tons of aquaculture products per year, with mussel the most important.

2. Material and methods

2.1. Sample collection

Oil was collected from Muros–Noia estuary (West coast of Galicia, especially affected by the oil spill, see Fig. 1) on February 2003, and stored in a hermetic container at 4 °C until analysis and use less than 1 week later.

Mussels (*M. galloprovincialis*) were collected from Betanzos estuary (North coast of Galicia), not affected by the oil spill. In order to minimize possible variations, each mussel measured from 6 to 8 cm in length. After collection they were immediately transported to the laboratory (less than 1 h), divided in three groups and placed in highly aerated tanks with filtered sea water (ratio 2.5 animals/l). They were placed in a photoperiod chamber at a controlled temperature of 18 °C in cycles of 12 h light–darkness. The mussels were daily fed with two species of microalgae (*Isocrisis galbana* and *Tetraselmis suecica*). Acclimatization of animals to laboratory conditions lasted for 48 h.

2.2. Conditions of exposure

To prepare the two oil volumetric ratios (1:500 and 2:500) used in this study, the respective quantity of oil was weighed and put into a glass container with 0.5 l of filtered sea water. To facilitate the dispersion and emulsion of the oil in the sea water, it was shaken strongly for half an hour prior to being added to the aquarium. The whole content of the container was added to the corresponding aquarium with a total volume of 4 l. Mussels were not in direct contact with the solid fraction of oil because they were placed on the bottom of the basin linked by their byssal threads, while the oil remained on the water surface and upper part of the walls of the aquarium due to its lower density. Individuals were exposed to oil during 12 days. The selection of the time periods and rates of exposure was based on previous experiments (Neff and Anderson, 1981; Hamoutene et al., 2002). After the treatment, an aliquot of sea water from the

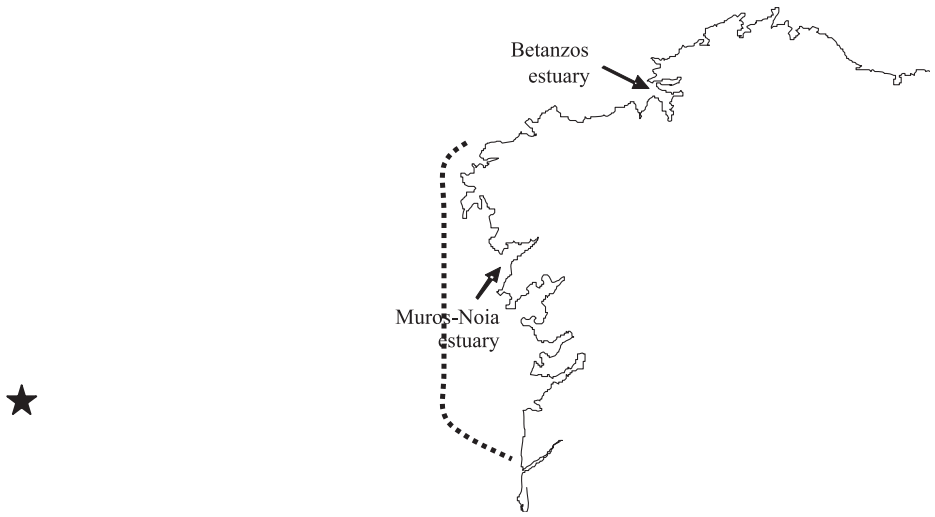

Fig. 1. Map of Galicia coast indicating oil and mussels sampling sites. The dotted line represents the coastal area affected by the spill, and the black star shows the place of the Prestige break-up (133 nautical miles from the coast).

aquarium and 3 individuals per dose were randomly selected for hydrocarbon content determination, and 7 individuals per dose for DNA damage evaluation.

2.3. Determination of hydrocarbon content of oil, water and tissue

Analyses of oil, sea water and mussel tissue were carried out by means of a gas chromatography-mass spectrometry methodology following Baumard et al. (1999). For each treatment, analyses were performed in triplicate. The PAH determined were: naphthalene (N), 2-methylnaphthalene (MN), biphenyle (BP), 2,6-dimethylnaphthalene (2,6DMN), 1,2-dimethylnaphthalene (1,2DMN), acenaphthylene (ANL), acenaphthene (AN), 2,3,5-trimethylnaphthalene (TMN), fluorine (F), 1-methylfluorene (MF), dibenzothiophene (DBT), phenanthrene (PH), 1-methyllphenanthrene (MPH), 2-methylanthracene (MA), fluoranthene (FL), pyrene (P), 11H-benzofluorene (BF), 1,2-benzodiphenylensulfure (BDS), anthracene (A), benz[*a*]anthracene (BaA), triphenylene (T), chrysene (C), benzo[*b*]fluoranthene (BbFL), benzo[*j*]fluoranthene (BjFL), benzo[*k*]fluoranthene (BkFL), benzo[*e*]pyrene (BeP), benzo[*a*]pyrene (BaP), perylene (PL), benzo[*ghi*]perylene (BghiPL), dibenz[*ah*]anthracene (DahA), indopyrene (IP), 2-methyldibenzothiophene (MDBT), 2,4-dimethyldibenzothiophene (DDBT), 2-propylbenzothiophene (PBT) and 2-butylbenzothiophene (BBT). The sum of the total quantity of all these compounds was considered as the index total PAH (TPAH).

2.4. Comet assay

2.4.1. Gill extraction and preparation of the cell suspension

The sample gills (2 gills per individual) were excised and sliced, using dissection scissors and tweezers, and placed in a tube containing 8 ml of calcium magnesium-free saline solution (CMFS: 20 mM HEPES, 500 mM NaCl, 12.5 mM KCl, 5 mM EDTA) and left for an hour shaking slowly horizontally. They were then placed in a vertical position for 5 min in order to allow the pieces of tissue to settle. The supernatant containing the dissociated cells was collected with a pipette, transferred to another tube and centrifuged at $500\times g$ for 5 min at 4 °C. Having removed the supernatant, the pellet was re-suspended in 500 μl of Kenny's salt solution (KSS: 0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$) and viability was tested using the Trypan Blue exclusion technique.

2.4.2. Slide preparation

The technique used was based on the alkaline assay described by Wilson et al. (1998), with minor modifications. Cells were washed twice in KSS and centrifuged for 3 min at $1000\times g$, with each sample being divided in two. The pellet containing $\approx 2.5\times 10^5$ cells was re-suspended in 80 μl of 0.5% low-melting-point agarose (LMA, Gibco BRL, Paisley, Scotland) in KSS and placed on a slide pre-coated with a layer of 0.5% normal-melting-point agarose (Gibco). It was left for 10 min on a metal plate at 4 °C before the addition of a final layer of 80 μl LMA. The slides were placed in a Coplin jar which contained lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 250 mM NaOH, 10 mM Tris–HCl, 1% sarcosyl, pH 10 with 1% Triton X-100 added just before use) for 1 h at 4 °C in darkness.

2.4.3. Electrophoresis and staining

Alkaline unwinding was carried out for 20 min in a horizontal tank filled with electrophoresis solution (1 mM Na_2EDTA, 300 mM NaOH, pH>13). Electrophoresis was conducted at 25 V and 300 mA (0.83 V/cm) for 20 min. The slides were neutralized by soaking in 0.4 M Tris–HCl, pH 7.5, stained with 60 μl of 5 μg/ml 4,6-diamidino-2-phenylindole in antifade solution, covered with a coverslip and stored in a humidified air-tight container to prevent drying of the gel. All procedures were carried out under

Table 1
PAH content of the oil employed

Parameter	Quantity (μg/kg dry mass)
TPAH	802,728.88
BaP	10,004.06

artificial lighting conditions to minimize UV-induced damage.

2.4.4. Imaging and analysis

One hundred randomly selected cells (50 per replicate slide) were examined from each sample by a "blind" scorer using a magnification of 400×. Image capture and analysis were performed using the QWIN Comet software (Leica Imaging Systems, Cambridge, UK). The parameters established as representative of DNA damage are comet tail length (TL), measured from the estimated center of the cell, percentage of DNA in the comet tail (%TDNA), and tail moment (TM), which results from multiplying TL and %TDNA.

2.5. Statistical analysis

All statistical analyses were carried out using the SPSS for Windows statistical package, version 11.0 (Illinois, USA). The distribution of the variables obtained was compared with normal distribution using the Kolmogorov–Smirnov goodness-of-fit test. As the distribution did not depart significantly from normality, parametric tests were deemed adequate for the statistical analysis of these data. Statistical significance was determined by the analysis of variance (ANOVA), followed by Bonferroni's test. The associations between two variables were analyzed by Pearson's correlation. The level of significance was set at 0.01.

Table 2
TPAH and BaP content (μg/l, mean±standard error) in the sea water from the experimental basins

Oil dose	TPAH[a]	BaP[a]
Control	0.004±0.000	<LOQ
1:500	55.142±2.689	1.104±0.051
2:500	41.956±2.021	0.516±0.029

<LOQ: under the limit of quantitation.

3. Results

3.1. Analysis of the oil

The composition in PAH of the oil employed in this experiment is summarized in Table 1, and details of every hydrocarbon analyzed content are shown in Fig. 2. The two parameters gathered in Table 1 (TPAH and BaP content) are considered as the most representative of the oil potential toxicity, as TPAH is usually taken as an indicative index of the presence of PAH in general (Soclo et al., 2000; Magi et al., 2002; Tao et al., 2004), and BaP is one of the most carcinogenic PAH and is considered as reference, since

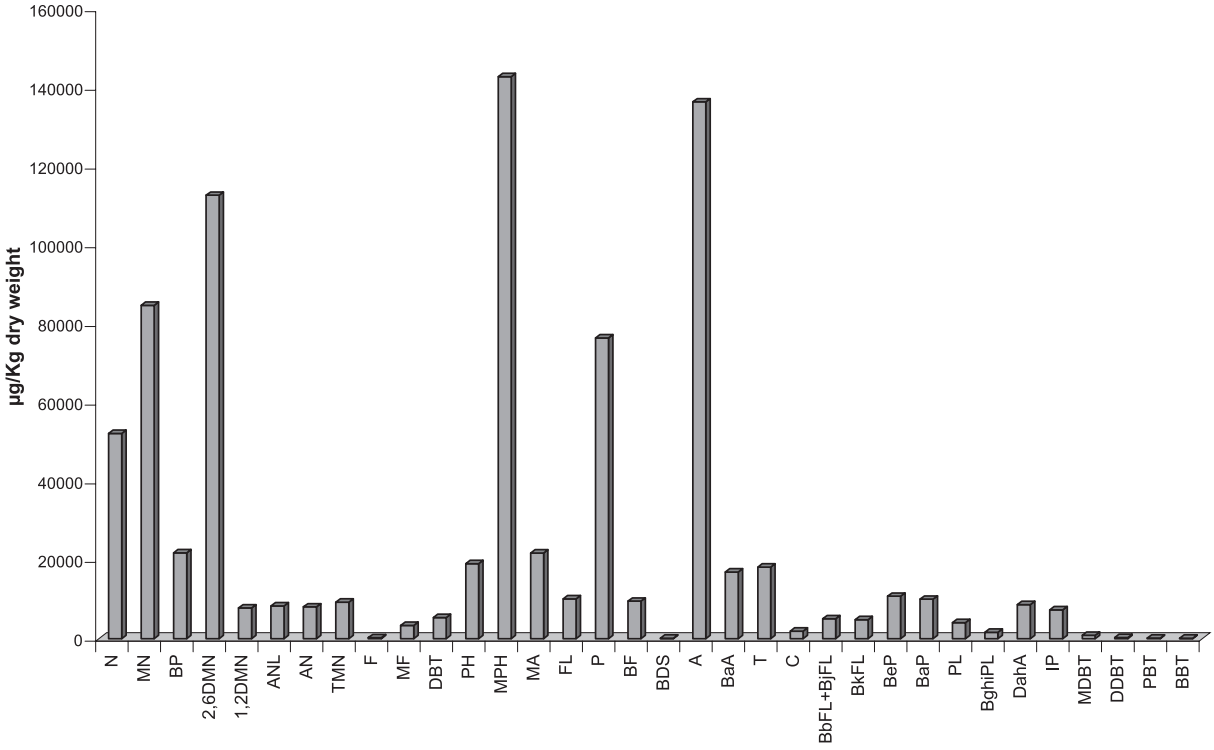

Fig. 2. Detailed PAH composition of the oil employed in the experiments. Acronyms used for PAH are shown in Materials and methods.

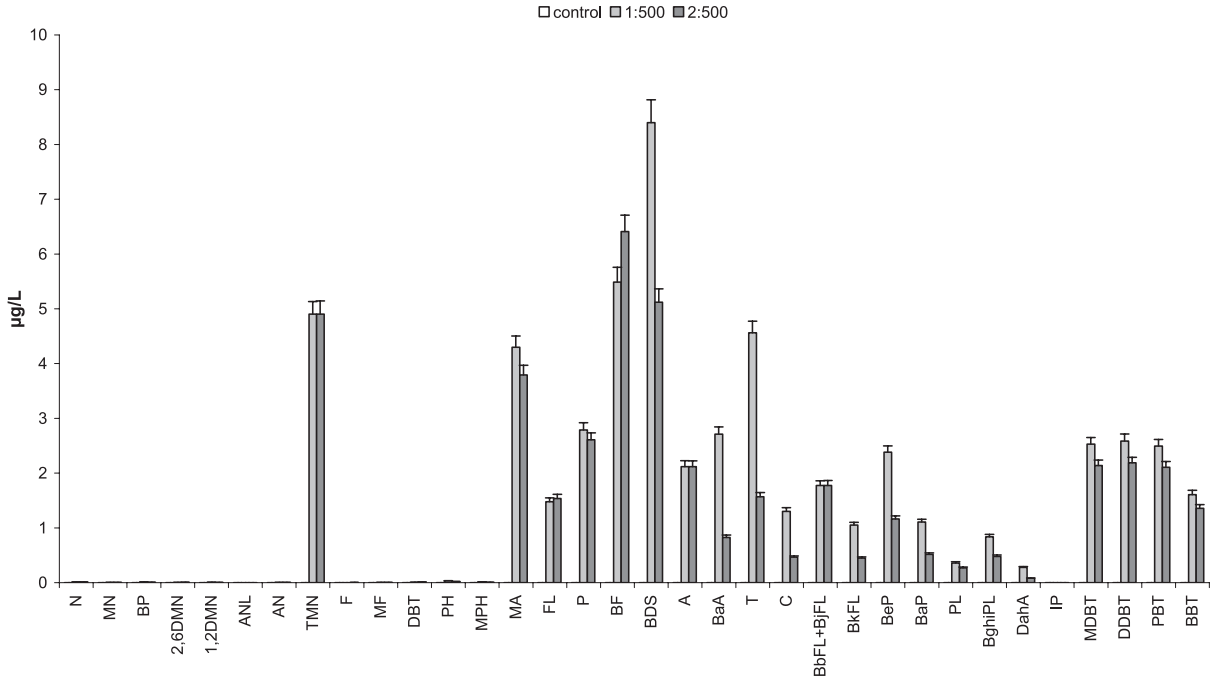

Fig. 3. Detailed PAH composition of the sea water from the experimental basins (results from 3 independent analyses). Acronyms used for PAH are shown in Materials and methods.

mutagenic potencies of all PAH are calculated as relative to it (Machala et al., 2001).

3.2. Hydrocarbon content in sea water and tissue

Results obtained for the concentration of TPAH and BaP in sea water and mussel tissue are featured in Tables 2 and 3, respectively. A more detailed separate PAH content can be found in Figs. 3 and 4, respectively. Table 2 shows the practically total absence of the hydrocarbons analyzed in the control sea water, and the significant presence of both TPAH and BaP in the water from the two treatments, with a higher level being recorded in the aquarium containing the lower oil concentration (1:500) than in the other (2:500).

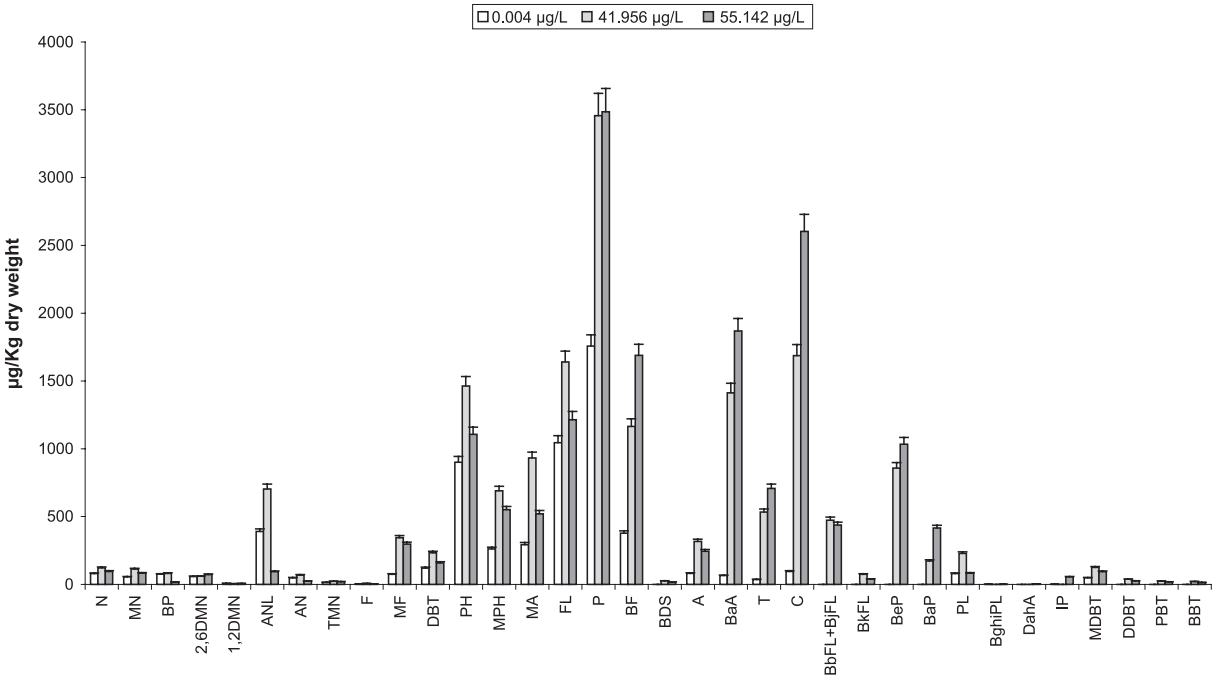

Fig. 4. Detailed PAH composition of the tissues from mussels exposed to the different aqueous TPAH contents (results from 3 independent analyses). Acronyms used for PAH are shown in Materials and methods.

Table 3
TPAH and BaP content in mussel tissue samples and oil-induced DNA damage in *M. galloprovincialis* gill cells

Aqueous TPAH (μg/l)	Mussel tissue content (μg/kg dry mass)		DNA damage			
	TPAH[a]	BaP[a]	N	TL[a] (μm)	%TDNA[a]	TM[a,b] (μm)
0.004	5978.80±249.65	<LOQ	7	43.59±0.58	2.22±0.38	130.39±24.43
41.956	17,033.00±827.07	173.50±8.50	7	67.71±0.63*	3.18±0.26	249.39±19.26*
55.142	16,993.80±825.28	416.00±19.90	6	77.08±0.92*	6.17±0.40*	584.47±40.88*

<LOQ: under the limit of quantitation.
[a] Mean±standard error.
[b] TM results from multiplying TL and %TDNA.
* Significant difference ($P<0.01$) with regard to the lowest aqueous TPAH content.

Tissues from 3 individuals per dose were analyzed in order to evaluate the bioaccumulation of hydrocarbons. Although the concentration of TPAH is similar in mussels from both tanks, a greater concentration of BaP has been detected in mussel tissues from the aquarium which contained the higher level of aqueous TPAH. Relationships between water content–tissue content were evaluated, obtaining Pearson's correlation coefficients of $r=0.973$ for TPAH and $r=0.998$ ($P=0.037$) for BaP.

3.3. Genotoxicity

Cell viability, analyzed by means of the Trypan Blue exclusion technique was in all cases and treatments above 75%. The comet assay results are given by three parameters: TL, %TDNA and TM. The mean values of the three parameters are gathered in Table 3. The two treatments assayed caused an increase in the DNA damage regarding to controls, reflected by statistically significantly higher values of the three variables (excepting %TDNA in mussels exposed to the lower aqueous TPAH content), being this increase always more accentuated for those individuals placed in the basin containing higher level of aqueous TPAH. Pearson's correlation coefficients obtained for relationships between aqueous TPAH content and DNA damage were $r=0.999$ for TL ($P=0.028$), $r=0.836$ for %TDNA and $r=0.847$ for TM. Moreover, relationship between TPAH tissue content and DNA damage (evaluated as TL) was investigated and the Pearson's correlation coefficient obtained was $r=0.962$.

4. Discussion

Following the man-made disaster that occurred on the shores of Galicia a year ago, and in view of the great importance that this ecosystem has, its biological richness and even its importance for the food industry, it is extremely important to try to evaluate the damage caused, and to monitor the main consequences and their progress. With this in mind, the aim of this study was to evaluate the genotoxic effects of the spilled *Prestige* oil on the sea water organism *M. galloprovincialis* that is widely distributed along Galicia coasts. In addition, we have examined the hydrocarbon content of the oil spilled as it reached the shores, and its biodisponibility in the mussel organism, as a first step in the assessment of the spill consequences on the marine environment.

Oil components can appear in the sea environment dissolved, constituting the water-soluble fraction (WSF), or particulated. In this study, mussels were exposed to both WSF and particulate oil due to the dosing technique used, designed to simulate environmental conditions of the oil in the sea water. The filtration of contaminants associated to particulate organic matter can be source of contamination for filter-feeding organisms like mussels (Narbonne et al., 1992). On the other hand, Mackay et al. (1980) suggested that the major toxic effects of an oil spill may be from hydrocarbons dissolved from dispersed oil. In fact, WSF has been used in toxicity tests, and Carls et al. (2002) found sufficient evidence to show that it was equivalent to the composition of hydrocarbon fractions present in the water column following the Exxon Valdez spill. Therefore, WSF is clearly important for the determination of PAH content in water. In this study, the determination of PAH content in sea water has shown that they are indeed significant for both treatments assayed regarding to the control. A higher concentration of TPAH, and particularly of BaP, has been found in the tank with an oil concentration of 1:500, compared with the one which contained more oil (2:500). This could be explained by the fact that BaP, as many contaminants, has a great tendency to link to particles present in water (Irwin et al., 1997; Bowman et al., 2002), so that as the total concentration of oil in water increases, its components experience more difficulty in being incorporated to the WSF. On the other hand, the differences obtained in TPAH concentrations in sea water between the two volumetric ratios tested could also be explained in part by solubility, as some of the components of the TPAH index could have reached its solubility limit. Thus, the evaluation of water quality has great importance, due to the combination of this analysis with toxicology tests results provides a broad perspective for assessing the possible adverse biological effects of the oil spill for marine plants and animals (Neff and Stubblefield, 1995).

Given that the exposure of mussels to dissolved PAH at a level of 5 ng/l causes both the rapid uptake of xenobiotics (bioaccumulation) as well as an increase in oxygenase

enzymes involved in their biotransformation (D'Adamo et al., 1997), the next factor to take into account is the bioaccumulation of these compounds. Our results show a significant increase in the levels of TPAH and BaP in mussel tissues for both aqueous TPAH contents, when compared with control individuals. Furthermore, they show a higher amount of bioaccumulated BaP in mussels from the basin containing the higher aqueous TPAH levels. This finding is consistent with the content of this compound in the sea water of the aquarium. There are many studies that support the bioaccumulation of PAH in mussels. Some of them maintain that the relationship between TPAH values in water and bioaccumulation is very strong (Steinert et al., 1998). Indeed, it is known that the hydrophobic character of PAH causes an accumulation in organisms beyond their concentration levels in the environment (Oliver and Niimi, 1988; Spies et al., 1988; Van der Oost et al., 1988). In addition, their low biodegradation in mussels (Livingstone, 1998) may contribute to this fact during certain periods of time. Our results are in the same range as those reported by Carls et al. (2002), who detected levels of 9.9–15.5 μg/g TPAH in mussels exposed to oil from the *Exxon Valdez*. This is alarming, not only from the genotoxic perspective, but also with regards to human nutrition. Since mussels are a common component in the human diet, and as they are known to be efficient bioconcentrators, their regular consumption could represent a serious risk to human health (D'Adamo et al., 1997).

On another point, the extent of genotoxic effects evaluated in mussel gills could indicate the quality of their respective environments (Bolognesi et al., 1996). In fact, studies show that bivalves at oil-affected sites undergo both oxidative and xenobiotic stress, and oxidative stress by itself can cause the severe debilitation and inhibition of enzymatic activity as well as providing an origin for mutagenesis, decreased reproductive fitness, accelerated senescence, etc. (Downs et al., 2002). PAH, and particularly BaP, are genotoxic agents capable of forming reactive oxygen species (ROS) via redox cycling, and binding to DNA via CYP transformation to arene oxides, forming DNA adducts, that are more effective sites for the production of stand breaks. Alkaline comet assay was applied as a technique to determine DNA damage, measuring the amount of single strand breaks and alkali-labile sites. Results from the comet assay reveal an increase in DNA damage in both doses assayed when compared to the control group. The increase is more marked in the group of individuals exposed to the higher aqueous TPAH content. This is consistent with the levels of PAH and BaP obtained in the mussel tissue samples of this group, and in keeping with other studies that detected a significant production of strand breaks (Venier et al., 1997), DNA adducts (Dolcetti et al., 2002), chromosomal aberrations (Al-Sabti and Kurelec, 1985), and micronuclei (Scarpato et al., 1990, Venier et al., 1997) in mussels associated to BaP exposure. It could be noted that background levels of DNA damage are somewhat elevated, and this point has already been shown and discussed, with some authors believing that this elevated control response is related to DNA packaging and background alkali-labile sites rather than endogenous strand breaks (Mitchelmore and Chipman, 1998). Although endogenous strand break formation is shown to exist, another explanation lies on the fact that different cell types may have very different background levels of DNA strand breaks, due to variations in excision repair activity, metabolic activity, anti-oxidant concentration or other factors (Lee and Steinert, 2003).

In summary, in this study mussels have been exposed to *Prestige* oil, presence of PAH in the sea water tanks and mussel tissues has been determined, and genotoxicity in mussel gills has been evaluated by means of comet assay. Results have shown a significant increase in DNA damage in exposed mussels, according with aqueous and mussel tissue TPAH contents. In view of our results, the importance of the evaluation of biodisponibility, bioaccumulation and DNA damage in the assessment of the effects of xenobiotic pollutants to marine environments could be highlighted.

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