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Toxicological characterisation of the aqueous soluble phase of the *Prestige* fuel-oil using the sea-urchin embryo bioassay

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Abstract The soluble components of fuel oil are generally assumed to be the fraction that is toxic for organisms living in the water column. We have used a liquid phase bioassay with embryos of sea urchin to assess the toxicity of the water-soluble fraction (elutriate) of the fuel oil spilled when the tanker Prestige sank on 13 November 2002. Two methodologies to obtain elutriates were carried out in order to compare the effect of the extraction method on the measured toxicity. Analyses of Σ16PAHs (naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, Indeno(1,2,3-c-d)pyrene, benzo(a)pyrene, dibenz(a,h)anthracene and benzo(ghi)perylene) and four metals (copper, cadmium, lead and zinc) were conducted and linked to the biological response. The effective concentration that provoked a delay in the successful embryogenesis of 50% of population (EC_{50}) was 2.3% of fuel oil. No differences in final toxicity between the two elutriation treatments were found, although the rotated extraction seemed to be more effective than magnetic stirring in transferring contaminants from the fuel oil to the water. Toxicity was mainly associated with the low-weight PAHs (2–4 benzene rings).

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Introduction

The *Prestige* oil spill on the 13th of November 2002 represented one of the worst ecological disasters to impact the Iberian North Atlantic Coast in recent years. A total amount of 64,000 t of fuel-oil were spilled into the sea and spread along hundreds of miles of coast from Portugal to France, but the area suffering the heaviest impact was the coast of Galicia. Once fuel oil has been spilled into the ocean and has reached the coastline, it looses volatile and soluble components, and becomes relatively solid weathered oil that remains as a coating on the sediment where it can persist for long periods of time. In that almost permanent condition, it becomes bioavailable for organisms feeding directly from sediments or through the soluble components removed to the surrounding marine water.

Because of the peculiarity of the compounds comprising this fuel oil and the wide area and diversity of habitats affected, an integrated assessment including different weight of evidence (WOE) approaches would be necessary to assess correctly the effects of the Prestige oil-spill (Carballeira 2003). One of these WOE approaches involves toxicity bioassays to determine the biological effects by exposing organisms under laboratory conditions to samples collected in the impacted areas. Because the soluble components of fuel oil are assumed to be the fraction that causes toxicity, we have conducted a liquid phase bioassay to assess the potential toxicity of fuel oil to organisms living or feeding in the surrounding water column.

One of the tests most frequently used in environmental quality assessment is the sea-urchin embryo larval bioassay (Kobayashi 1991, 1995; Beiras et al. 2003) because in vitro fertilisation and generation of gametes are relatively easy, results can be obtained in a short-period of time (48 h), and it is sensitive to several kinds of organic and inorganic micropollutants (Kobayashi 1981; Fernández and Beiras 2001; Bellas et al. 2005). Because the *Paracentrotus lividus* sea urchin is very common in the areas involved, we have used this species to evaluate the toxicity of the soluble fraction of fuel-oil spilled from the tanker Prestige. The main objectives of this work were to assess the potential biological effects caused by the fuel oil spill in the water column, to determine the effective concentration of fuel oil that is toxic to sea urchin embryos, and to identify the components of the fuel oil (among PAHs and metals) that are potentially responsible for this toxicity.

The general aim of this work is to establish the risk associated with sediment-bound contaminants to the organisms living in the water column, and to validate the results previously obtained from applying the sea-urchin embryological bioassay in the assessment of sediment quality (by using elutriates) after the impact of the Prestige oil spill (Fernández et al. 2006). For this reason elutriate of fuel-oil was used instead of the usual water accommodated fraction (WAF).

Materials and methods

Fuel oil description

The fuel transported by the tanker *Prestige* corresponds to the category ''M-100'' or ''fuel oil no 6'' according to the Russian and English classifications, respectively. It can be characterized by a high content of sulphur (2.58%) and high viscosity and insolubility. It has a content of 22% saturated hydrocarbons, 50% PAHs and 28% asfaltenes and resins (CSIC 2003a).

The fuel oil used in this work was provided by the ''Oficina Técnica de Vertidos Marinos'' (OTVM, Universidad de Vigo, Spain) and was recovered directly from the tanker Prestige at a depth of 3,000 m some 2 years after the spill occurred. The fuel oil was transported to the laboratory in a 25 L hermetic tank. Its humidity content was 25% and it was kept in hermetic conditions and at room temperature until use not more than three days after receipt.

Chemical analysis

The $\sum$16 PAHs recommended by the US EPA (Keith and Telliard 1979) were analysed in samples of fuel oil and in elutriates resulting from two types of elutriation method: rotation and magnetic stirring. The PAHs analysed were naphthalene, acenaphtylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, Indeno(1,2,3-c-d)pyrene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(ghi)-perylene. The concentrations of PAHs were determined by gas chromatography with flame ionization detection (FID) after extraction with hexane and the addition of 1,3,5-triphenylbenzene as internal standard (Cymit Química L20500000CY). The PAH mix 9 of Dr. Ehrestofern (Dr. Ehrestofern GmbH- Cymit Química L20950900CY) was used as reference material. The analyses were performed in accordance with the EPA protocols for the preparation and extraction of samples (US-EPA 1996a), for the alumina column (US-EPA 1996b) and for the analysis of petroleum hydrocarbons by gas chromatography (US-EPA 1986). Analyses of Zn, Cd, Pb and Cu were carried out by differential pulse anodic stripping voltammetry using Metrohm equipment, after keeping samples in an acid environment (Campana et al. 2005). Panreac (AA quality) products were used as reference material.

The concentration of PAHs in elutriates was measured at the beginning of the test and after the exposure period (48 h at 20°C) in order to check the stability of solutions in the incubation containers and the actual concentrations of PAHs before and after the test. The stability of the dissolved metals in the test containers is known (Fernández and Beiras 2001), so only the concentration of the metals in elutriates was checked at the beginning of the test.

Elutriation and bioassay development

The elutriates were obtained by mixing fuel oil and control natural filtered seawater in proportion 1:10 (w : w) in 2 L glass containers (Barron et al. 1999).

Magnetic stirring is usually conducted to obtain the soluble fraction of fuel oils (Couillard et al. 2005) whereas rotator elutriation is used to obtain the bioavailable fraction of contaminated sediment samples (Beiras et al. 2003). Because the method of extracting may affect the final toxicity of samples (Beiras 2002) these two different approaches were performed to compare the effect of the extraction method on the toxicity: magnetic stirring and rotator elutriation. One mixture was strongly stirred and another was rotated at

50 rpm. Both methods were performed for 30 min and the mixture was left undisturbed 18 h to allow separation of phases. The liquid phase containing soluble fuel oil components was removed by siphoning and tested for toxicity.

For both types of elutriate, bioassays were conducted with the undiluted elutriates (100%) and with six dilutions with control seawater, containing 87.5, 75, 50, 25, 12.5 and 6.25% of elutriate. Therefore, the actual fuel oil concentrations tested, after correction for initial dilution (1:10) and humidity of fuel oil (25%), were 7.5, 6.6, 5.6, 3.8, 1.9, 0.9 and 0.5%.

Adults of *P. lividus* were collected in a clean area of the Atlantic coast of Cádiz (South of the Iberian Peninsula) and kept in tanks at 15°C with continuous aeration. Gametes from one male and one female of *P. lividus* were directly extracted from gonads after dissection of organisms. In vitro fertilization was conducted following methodology described by Fernández and Beiras (2001). Embryos were added to the test solutions in glass containers and then incubated at 20°C for 48 h. Four replicates per dilution, including negative controls containing only control seawater, were tested for each type of elutriate. Hermetic 20 mL glass containers with caps protected with Parafilm were used for incubations. After the incubation period organisms were fixed with formalin and the percentage of normal pluteus, defined as larvae with the four arms well developed (see Fig. 2a), were recorded over 100 individuals for each replicate.

Data analysis

Effective concentrations that delay the embryological success of 50% of the population (EC_{50}) and their corresponding 95% confidence limits were calculated by Probit analysis. The effect of elutriation methodology on toxicity was determined by ANOVA after normalization of response by arsine-transformation. All statistical analyses were performed using the SPSS 11.5 software.

Results

Chemical analysis

Table 1 shows the concentrations of the individual and Σ16PAHs measured in the fuel oil and in elutriates obtained by rotation and magnetic stirring. Σ16PAHs in fuel oil was 1,685 mg kg^{-1} while the averaged concentration of Σ16PAHs in elutriates was 8.3 μg kg^{-1}, meaning that only a very small fraction of these PAHs (5×10^{-6}%) was transferred from the original sample of fuel oil to the aqueous phase by both methods of elutriation.

The concentration of PAHs from two to four rings in the aqueous phase ranged from about 0.96 μg kg^{-1} for acenaphthene to 2.00 μg kg^{-1} for phenanthrene, while PAHs of more than four rings were in general below the detection limit of the methodology (0.1 μg kg^{-1}), associated with the low solubility in water characterizing the high-molecular weight PAHs. Furthermore, naphthalene (two rings) was not detected in aqueous samples probably due to its high volatility at ambient temperature. Low differences ranging from 25 to 31% were observed between the concentration of PAHs at the beginning and the end of incubation period, meaning that containers were suitable for testing the toxicity of the elutriates.

Bioassay

Table 2 includes percentage of normal larvae measured over each replicate (A–D) at each fuel oil concentration, for both type of elutriate (rotation and magnetic stirring), and Fig. 1 plots the average and standard deviations of these percentages of embryological success at each fuel-oil concentration. The embryological success of *P. lividus* sea-urchin was observed to be highly dependent on concentration. The median effective concentrations were 2.32 and 2.24 % of fuel oil for rotated and magnetic stirring elutriation, respectively. No significant differences ($p < 0.05$) in toxicity were observed between the two methods of elutriation when normal larval pluteus was recorded (Table 2). Nevertheless, a qualitative difference between treatments was observed when embryological stage was recorded for toxicity assessment instead of the percentage of embryological success. Fertilized eggs of sea-urchin change into morulae, blastulae, gastrulae and prism embryos before reaching the larvae stage. The higher the toxicity, the earlier the development of the embryo stops. Embryogenesis of fertilized eggs tested in rotated elutriates was observed to have been delayed at earlier stages than in the magnetic stirring elutriates (Table 2 and Fig. 2). In rotated elutriates with 7.5% of fuel oil, embryos stopped their development at the blastulae stage (Fig. 2b(5)), while in the corresponding magnetic stirring elutriate, most of the embryos reached the prism stage (Fig. 2a(5)). Similarly, at 6.6% of fuel oil, embryos incubated in the magnetic stirring elutriates reached the prism stage (Fig. 2a(4)) compared with the earlier gastrulae stage

Table 1 Total (∑16PAHs) and individual PAH concentrations analysed in samples of fuel-oil (mg kg^{-1}) and elutriates (μg kg^{-1}) obtained by rotation (*Rot*) and magnetic stirring (*Magn*) at the beginning (0 h) and the end of the incubation period (48 h), and metal concentrations analysed in the fuel oil (mg kg^{-1}) and in rotated elutriate (μg kg^{-1}) at the beginning of the test

	MW (g mol^{-1})	Rings	Rot (0 h) (μg kg^{-1})	Rot (48 h) (μg kg^{-1})	Magn (0 h) (μg kg^{-1})	Magn (48 h) (μg kg^{-1})	Fuel oil (mg kg^{-1})
Naphthalene	128.19	2	n.d.	n.d.	n.d.	n.d.	240
Acenaphtylene	152.20	3	1.10	1.30	1.23	1.54	n.d.
Acenaphthene	154.21	3	1.06	n.d.	1.00	0.96	4.6
Fluorene	166.20	3	n.d.	n.d.	n.d.	n.d.	130
Phenanthrene	178.20	3	0.94	1.20	2.00	1.85	310
Anthracene	178.20	3	1.19	1.23	1.46	1.38	40
Fluoranthene	202.26	3	n.d.	n.d.	1.04	1.12	55
Pyrene	202.26	4	2.13	1.63	1.69	1.58	813
Benzo(a)anthracene	228.30	4	n.d.	n.d.	n.d.	3.81	34.1
Chrysene	228.30	4	n.d.	n.d.	n.d.	n.d.	55.1
Benzo(b)fluoranthene	252.32	5	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo(k)fluoranthene	252.32	5	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo(a)pyrene	252.32	5	0.74	n.d.	n.d.	n.d.	9.89
Indeno(1,2,3-c-d)pyrene	276.00	6	n.d.	n.d.	n.d.	n.d.	n.d.
Dibenzo(a,h)anthracene	278.36	5	n.d.	n.d.	n.d.	n.d.	n.d.
Benzo(ghi)perylene	276.34	6	n.d.	n.d.	n.d.	n.d.	n.d.
∑16PAHs			7.16	5.37	8.42	12.23	1685
Metals							
Cu			0.3	n.m.	n.m.	n.m.	6.28
Cd			2.2	n.m.	n.m.	n.m.	0.19
Pb			n.d.	n.m.	n.m.	n.m.	0.55
Zn			1.2	n.m.	n.m.	n.m.	13.7

Also molecular weigh (MW g mol^{-1}) and number of rings are showed for each PAH

n.d. < detection limit

n.m. not measured

Table 2 Percentage of normal larvae registered at each fuel oil concentration and replicate (A–D), for both elutriation methods

	Rotated elutriates				Magnetic stirred elutriates				Significance
	A	B	C	D	A	B	C	D	
Control	96	90	99	97	95	97	98	97	0.277
7.5	0 (b)	0 (f)	0 (b)	0 (b)	0 (g; p)	0 (g; p)	0 (n.r)	0 (n.r)	–
6.6	0 (g)	0 (g)	0 (g)	0 (g)	0 (g; p)	0 (g; p)	0 (g; p)	0 (g; p)	–
5.6	0 (g; p)	0 (p)	0 (g; p)	0 (g; p)	0 (p)	0 (p)	0 (p)	0 (p)	–
3.8*	0 (p)	0 (p)	0 (p)	0 (p)	0 (p)	0 (p)	0 (p)	0 (p)	–
1.9	89	91	86	90	92	83	87	78	0.275
0.9	95	87	81	94	85	79	88	94	0.547
0.5	91	95	91	96	93	93	92	89	0.363

In brackets, embryological stage reached by most of the fertilized eggs after 48 h at 20°C (*f* fertilized egg, *m* morulae, *b* blastulae, *g* gastrulae, *p* prism)

Significance: ANOVA statistical significance ($p < 0.05$) for elutriation methodologies

n.r. not registered

observed in the rotated elutriates (Fig. 2b(4)). This pattern was consistent for all the fuel oil dilutions, with the rotated elutriates showing a slightly higher toxicity than that measured in the magnetic stirred elutriates.

Discussion

The chemical analysis of fuel oil samples shows a concentration of about 1,600 mg kg^{-1} for Σ16PAHs. The content in low-molecular weight PAHs was higher

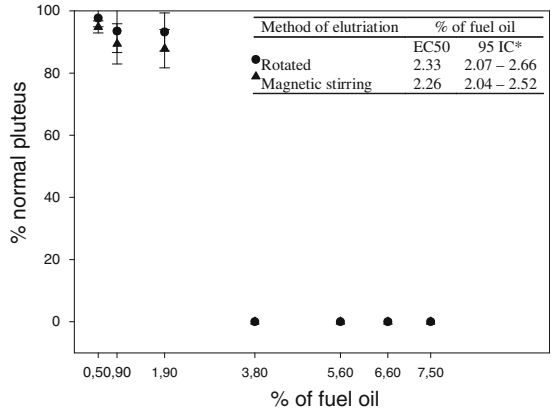

Fig. 1 Percentage of embryological success (% of normal pluteus) for each type of elutriate versus fuel-oil concentrations. The table enclosed shows the median effective concentrations (EC_{50}) and corresponding confidence intervals (95% CI) calculated by Probit analysis for both elutriation methodologies

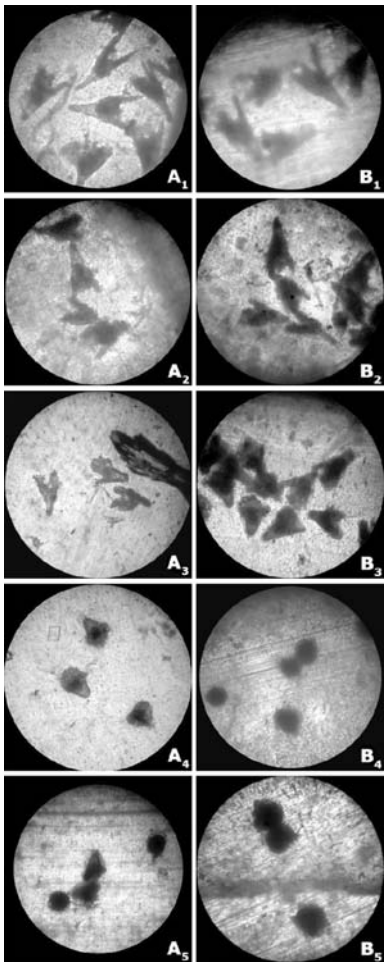

Fig. 2 Comparison of stages reached by the embryos after incubation period (48 h at 20°C) in elutriates obtained by magnetic stirring (**A**) and rotation (**B**), in different fuel-oil concentrations: control seawater ($_1$); 1.9% ($_2$); 3.8% ($_3$); 6.6% ($_4$); 7.5% ($_5$)

than the concentrations of the high-molecular weight compounds. These results are in agreement with those reported by the 'Centre de Documentation, de Recherche et d'Expérimentations sur les Pollutions Accidentelles des Eaux' (CEDRE) http://www.lecedre.fr/fr/prestige/z_produit.htm, Institute Français de Recherche pour l'exploitation de la Mer (IFREMER) http://www.ifremer.fr/envlit/prestige/prestigefioul-ana_sp.htm) and with the technical report of Consejo Superior de Investigaciones Científicas (CSIC 2003a) which reported concentrations ranging from 1,200 to 2,110 mg kg^{-1} or ppm.

Analyses performed on the elutriates showed the presence of only low-molecular weight PAHs; this was thus the only fraction of PAHs transferred to the aqueous phase with both elutriation methods. The solubility in water of low-molecular weight PAHs (two to four rings) ranges from about 3 mg L^{-1} for acenaphthylene to 0.3 mg L^{-1} for fluorantene, while the solubility of higher PAHs is much lower, from 4×10^{-3} mg L^{-1} (for the B(a)P with five rings) to practically nil for highest molecular weight PAHs (May et al. 1978; Futoma et al. 1981): this explains the results reported in our study.

The analysis of elutriates shows differences in the total concentration of the Σ16PAHs ranging from 25 to 31% at the beginning and at the end of the incubation period (48 h at 20°C). No tendency of increasing or decreasing concentration was observed with time, so differences measured between concentrations at the beginning and the end of the bioassay may be related more to the analytical error than to the instability of solutions in the test containers. Consequently we assumed there was no loss of chemicals by adsorption processes during the incubation period.

A close relationship between the percentage of embryological success of *P. lividus* and the concentration of fuel oil was observed. Embryo development is strongly inhibited when associated with a fuel oil content in water of more than 3.8% (Fig. 2a(4,5), b(4,5) showing blastulae, gastrulae and prism stages) whereas the toxic effect disappears completely when water content of fuel oil is below 1.9% (Fig. 2a(2), b(2) showing normal pluteus larvae). No significant difference ($p < 0.05$) in toxicity was observed between types of elutriation method (Table 2) although rotated elutriation seemed to be a more effective treatment in extracting the compounds that provoke embryogenesis delay, as shown qualitatively by the earlier stage of embryos incubated in this type of elutriate (Fig. 2a(1–5) compared with Fig. 2b(1–5)).

The toxicity observed could be associated with the low-molecular weight PAHs transferred from the fuel

oil to the liquid phase, especially acenaphtylene, acenaphthene, phenanthrene and anthracene. Anderson et al. (1974) identified the low-molecular weight aromatic hydrocarbons as the primary contributors to oil toxicity by testing a water-soluble fraction of the oil. These PAHs are known to cause acute toxicity in marine organisms (Kennish 1997) and in particular to delay embryological development of *P. lividus* sea-urchin (Fernández 2002; Pérez 2004). However, the above-cited studies show that individual PAHs are not toxic enough to delay the embryo development of *P. lividus*, so an integrated effect involving all the low-molecular weight PAHs must be operating.

Nevertheless, Barron et al. (1999) have observed that PAHs are not the major determinant of the toxicity of some weathered middle distillate oils, so the contribution to toxicity of other chemicals cannot be excluded. The emulsified fuel oil is enriched in metals complexed by sulphur atoms bound to aromatic molecules that could be transferred from the fuel oil to the water fraction. In fact, studies of the composition of samples collected in the oil spill area showed an increase of Zn, Cu and Pb concentration in sea-water after the spill (CSIC 2003b). Metals are known to cause toxicity at low concentrations in embryos of marine invertebrates and particularly in *P. lividus* embryos (Kobayashi 1981; Fernández and Beiras 2001); although the metal concentrations measured in the fuel oil elutriate were below the reported median effective concentrations, a synergistic effect could not be ruled out. The toxicity of a chemical can be enhanced by the presence of another chemical with similar mode of action, and this has been widely observed for different chemical mixtures and marine organisms including the *P. lividus* sea-urchin (Vanegas et al. 1997; Fernández and Beiras 2001).

Other methodologies have been developed to test the toxicity of fuel oils by using the WAF in which water and oil are mixed for a period of time sufficient to achieve an equilibrated concentration of dissolved and dispersed or emulsified components (OSPAR 2002). In this respect, Beiras et al. (2005) also reported toxicity in *P. lividus* when embryos were exposed to WAF of the Prestige-fuel, observing a reduction of 10% in early larval growth at WAF dilutions of 131-fold, whereas the fish larvae of *Cyprinodon variegatus* were found to be very tolerant. Toxicity of WAF of different fuel oils has been reported for several marine invertebrates (Cajaraville et al. 1992; Pelletier et al. 1997; Geffard et al. 2004) and in most of the cases toxicity has been associated with the PAH content of fuel oil.

Results from this work also agree with those obtained using elutriates of natural sediments collected in different areas affected by the *Prestige* oil spill, by exposing them to larvae of *P. lividus* (Fernández et al. 2006). High toxicity of elutriates was observed for some sediments containing a high concentration of PAHs, indicating that the soluble fraction of fuel oil can be transferred to the water phase. Mariño-Balsa et al. (2003) have also observed that elutriates from sediments highly contaminated by the *Prestige* fuel oil caused toxicity (under laboratory conditions) to embryos of clam during the first month after the spill. They have also observed that, sometimes, sediments containing high levels of PAHs did not cause toxicity, whereas seawater collected at the same sampled sites certainly showed toxicity to microalgae. This confirms that the toxic fraction of fuel oil resides more in the aqueous phase than in the solid fraction of fuel oil.

From the results obtained in our study it is concluded that the compounds contained in the Prestige fuel oil caused toxicity in the earliest stages of the sea urchin *P. lividus* through the soluble fraction, the effective median concentration of fuel oil being 2.3%. The interaction of low-molecular weight PAHs, especially acenaphtylene, acenaphthene, phenanthrene and anthracene, and possibly other compounds like metals, could be the main toxic agents. The sea-urchin embryo bioassay is a shown to be good tool for screening and monitoring the toxicity of seawaters and sediments impacted by the *Prestige* oil spill.

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References

Anderson JW, Neff JM, Cox BA, Tatum HE, Hightower GH (1974) Characteristics of dispersions and water soluble extracts of crude and refined oil and their toxicity to estuarine crustaceans and fish. Mar Biol 27:75–88

Barron MG, Podrabsky T, Ogle S, Ricker RW (1999) Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? Aquat Toxicol 46:253–268

Beiras R (2002) Comparison of methods to obtain a liquid phase in marine sediment toxicity bioassays with Paracentrotus lividus sea urchin embryos. Arch Environ Contam Toxicol 42:23–28

Beiras R, Fernández N, Bellas J, Besada V, González-Quijano A, Nunes T (2003) Integrative assessment of marine pollution in galician estuaries using sediment chemistry, mussell bioaccumulation and embryo-larval toxicity bioassays. Chemosphere 52:1209–1222

Beiras R, Bellas J, Saco-Álvarez L, Mariño-Balsa JC, Pérez M, Fernández N (2005) (Personal communication). Acute toxicity of individual polycyclic aromatic hydrocarbons (PAH), and Prestige-fuel water accommodated fractions (WAF) to early life stages of commercial marine organisms, zooplankton and phytoplankton. In: Proceedings of the symposium for monitoring of accidental oil spills projects in marine environment. Vigo (Pontevedra), Spain, 13–16 July 2005

Bellas J, Beiras R, Mariño JC, Fernández N (2005) Toxicity of organic compounds to marine invertebrates embryos and larvae: a comparison between sea urchin embryogenesis bioassay and alternative test species. Ecotoxicology 14:337–353

Cajaraville MP, Marigomez JA, Angulo E (1992) Comparative effects of the water accomodated fraction of three oils on mussels. 1. Survival, growth and gonad development. Comp Biochem Physiol C 102(1):103–112

Campana O, Rodríguez A, Blasco J (2005) Bioavailability of heavy metals in the Guadalete River Estuary (SW Iberian Peninsula). Cienc Mar 31(1B):135–147

Carballeira A (2003) Consideraciones para el diseño de un programa de monitorización de los efectos biológicos del vertido del Pretige. Cienc Mar 29(1):123–139

CEDRE (Centre de Documentation, de Recherche et d'Expérimentations sur les Pollutions Accidentelles des Eaux). Connaissance du produit. Analices et étude du comportement du fuel du Pretige. Technical report. http://www.lecedre.fr/fr/prestige/z_produit.htm

Couillard CM, Lee K, Légaré B, Kinf TL (2005) Effect of dispersant on the composition of water accommodated fraction of crude oil and its toxicity to larval marine fish. Environ Toxicol Chem 24(6):1496–1504

CSIC (Consejo Superior de Investigaciones Científicas) (2003a) Caracterización del vertido y evolución preliminar en el medio. Tech Rep 01, pp. 1–6

CSIC (Consejo Superior de Investigaciones Científicas) (2003b) Presencia de metales pesados en la zona de hundimiento del petrolero Prestige y composición de metales y complejantes del fuel emulsionado en la costa. Tech rep 02:1–2

Fernández N, Beiras R (2001) Combined toxicity of dissolved mercury with copper, lead and cadmium on embryogenesis and early larval growth of the *Paracentrotus lividus* sea-urchin. Ecotoxicology 10:263–271

Fernández N (2002) Evaluación biológica de la calidad de sedimentos marinos costeros mediante bioensayos com embriones del erizo de mar *Paracentrotus lividus*. Universidade de Vigo, Vigo, Spain, p 211

Fernández N, Cesar A, González M, DelValls TA (2006) Nivel de contaminación de sedimentos afectados por el vertido del *Prestige* y sus efectos sobre el desarrollo embrionario del erizo de mar. Cienc Mar 32(2B): 421–427

Futoma DJ, Smith SR, Smith TE, Tanaka J (1981) Polycyclic aromatic hydrocarbons in water systems. CRC Press, Boca Raton

Geffard O, Budkinski H, LeMenach K (2004) Chemical and ecotoxicological characterization of the ''Erika'' petroleum: bio-tests applied to petroleum water-accommodated fractions and natural contaminated samples. Aquat Living Resour 17:289–296

IFREMER. Analyses du fioul Prestige. Technical report. http://www.ifremer.fr/envlit/prestige/prestigefioulana_sp.htm)

Keith LH, Telliard WA (1979) Priority pollutants. I-A perspective view. Environ Sci Technol 13:416–423

Kennish M (1997) Practical handbook of estuarine and marine pollution. CRC Press, Boca Raton, London

Kobayashi N (1981) Comparative toxicity of various chemicals, oil extracts and oil dispersant extracts to Canadian and Japanese sea urchin eggs. Publ Seton Mar Biol Lab XXVI(1/3):123–133

Kobayashi N (1991) Marine pollution bioassay by using sea urchin eggs in Tabane Bay, Wakayama Prefecture, Japan, 1970–1987. Mar Poll Bull 23:709–713

Kobayashi N (1995) Bioassay data for marine pollution using echinoderms. In: Cheremisinoff PN (eds) Encyclopaedia of environmental control technology. Gulf. Publ. Co., Houston

Mariño-Balsa JC, Pérez P, Estévez-Blanco P, Saco-Álvarez L, Fernández E, Beiras R (2003) Evaluación de la toxicidad de sedimento y agua de mar contaminados por el vertido de fuel del *Prestige*, mediante el uso de bioensayos con las almejas *Venerupis pullastra*, *Tappes decussatus* y *Venerupis rhomboideus* y la microalga *Skeletonema costatum*. Cien Mar 29(1):115–122

May WE, Wasik SP, Freeman DH (1978) Determination of the solubility behaviour of some polycyclic aromatic hydrocarbons in water. Anal Chem 50(7):997–1000

OSPAR (2002). OSPAR guidelines for toxicity testing of substances and preparations used and discharged offshore. Annex 7. Ref. 2002–3. Meeting of the Offshore Industry Committee (OIC). Cadiz: 11–15 February 2002, pp. 4

Pelletier MC, Burgess RM, Ho KT, Kuhn A, Mckinney RA, Rybe SA (1997) Phototoxicity of individual polycyclic aromatic hydrocarbons and petroleum to marine invertebrate larvae and juveniles. Environ Toxicol Chem 16:2190–2199

Pérez MI (2004) Estudio de los niveles de contaminación por hidrocarburos aromáticos policíclicos en la Ría de Pontevedra y evaluación de su toxicidad sobre la embriogénesis del erizo de mar Paracentrotus lividus. Universidade de Vigo, Vigo, Spain

US Environmental protection (1986) Method 8100. SW-846 Ch 4.2.1. Polynuclear Aromatic Hydrocarbons

US Environmental protection (1996a) Method 3500B. SW-846 Ch 4.2.1. Organic extracting and sample preparation

US Environmental protection (1996b) Method 3910B. SW-846 Ch 4.2.1. Alumina Cleanup

Vanegas C, Espina S, Botello AV, Villanueva S (1997) Acute toxicity and synergism of cadmium and zinc in white shrimp, *Penaeus setiferus*, juveniles. Bull Environ Cotam Toxicol 58:87–92