The integrated use of chemical and biochemical markers for assessing the effects of the Aegean Sea oil spill in the Galicia coast (NW Spain)

Department of Environmental Chemistry, CID-CSIC, Barcelona, Spain.

Abstract

The spatial distribution and temporal evolution of petrogenic and pyrolytic hydrocarbons in the Galicia coast (NW Spain), following the Aegean Sea oil spill, were investigated through a detailed study of hydrocarbon (fossil) markers in surface sediments and bivalves (mussels and clams). Sublethal responses in mussels were also assessed by the determination of several biomarkers such as the cytochrome P450 system, antioxidant enzymes and lipid peroxidation.

Triterpane and sterane distributions were useful in tracing, respectively, the oil source and weathering (degradation) along the survey period (3, 6, 9, 12 and 34 months after the accident). Within the aromatic fraction, aromatized steranes were also useful source indicators and oil degradation was also evidenced by the decrease of certain methyl dibenzothiophene isomers. Combustion derived polycyclic aromatic hydrocarbons, produced in the tanker wreck, were widely distributed in the area and found to be more persistent than the petrogenic ones, although less bioavailable. A significant decline of the hydrocarbon contents was evident from 3 to 6 months. However, one year later, an incidental increase was observed in some bivalves, probably due to the resuspension of the subsurface polluted sediments by the winter stormy weather.

Among the studied biomarkers, a significant induction of the cytochrome P450 content and lipid peroxidation was detected in mussels collected near the wreck point six months after the spillage. Besides the strong seasonality observed in some biomarkers, oxidative damage still persisted one year later.

1 Introduction

On December 3, 1992, the Aegean Sea tanker, transporting 79,000 tons of a light crude oil (Brent type, North Sea), ran aground off La Coruña on the Galicia coast
(NW Spain) (Figure 1). The tanker immediately leaked oil and a series of explosions set it on fire, that lasted during six days. The winds and sea-currents drove the petrogenic and pyrolytic hydrocarbons towards the shore, affecting 200 km of coastline. This is highly broken, forming a large suite of estuaries (rias) which provide advantageous conditions for shellfish growing and culturing.

When cleaning operations came to the end, a survey program on the mid- and long-term effects of the oil spill on the marine ecosystem was established. This mainly involved the assessment of the spatial distribution and temporal evolution of the oil and combustion products in the area. To this end, the characterization of the spilled oil and its identification in intertidal sediments and bivalve tissues was carried out. The adopted system should be able to unambiguously identify the oil in both compartments and distinguish it from other possible inputs that occurred in the area during the survey period. Therefore, an extensive use of chemical (fossil) markers, that can easily be traced by mass-fragmentography [1], was made. Moreover, the occurrence of pyrolytic products and their fate was investigated for their harmful effects on biota and the quality of edible products.

The biological effects were measured by the determination of potential biomarkers in mussels, such as the cytochrome P450-dependent monooxygenase (MFO) system, and the antioxidant enzyme superoxide dismutase (SOD). The saturation of the protective defences (phase II and antioxidant enzymes) which may

Figure 1: Map of the area of study showing the grounding site of the Aegean Sea (*), the movements of the oil spill and the pyrolytic material, and the sediment (o) and bivalve (●) sampling stations.
result in membrane oxidative damage was measured in terms of lipid peroxidation. These biochemical responses have been considered as useful early warning systems of exposure of bivalves to organic pollutants [2].

2 Experimental

2.1 Sampling

Sampling stations are indicated in Figure 1 and included reference sites, situated north, south and off-shore from the tanker wreck (Nos. 1, 2, 3 and 13), sites directly affected by the oil spill, according to visual observations (Nos. 5, 6, 9, 10 and 12), and stations located inside the estuaries (Nos. 4, 7, 8 and 11).

Superficial sediments were sampled with a conventional box corer, placed in glass jars and kept frozen (-20°C) until they were freeze-dried and sieved at 1mm to remove coarse sands and organic debris.

Mussels (Mytilus edulis) and clams (Tapes semidecussata) were collected from indigenous populations along the coast. The whole tissues were separated from the shells, wrapped in clean aluminium foil and stored at -20°C before being homogenized and freeze-dried for chemical analysis. Digestive glands were immediately dissected, frozen in liquid nitrogen and stored at -80°C, for biochemical measurements.

2.2 Chemical analysis

2.2.1 Isolation of hydrocarbon fractions

Freeze-dried sediments (≈ 10 g) were Soxhlet extracted during 12 h with a mixture of n-hexane-dichloromethane (4:1). The extracts were collected and treated overnight with recently activated copper for elemental sulfur removal. On the other hand, freeze-dried tissues (≈ 2 g) were saponified with 100 ml of methanolic 6N KOH at 40°C overnight and extracted with 3 x 100 ml of hexane:dichloromethane (4:1). The organic extracts were carefully evaporated near to dryness and dissolved with 0.5 ml of n-hexane for further fractionation into aliphatic and aromatic hydrocarbons. This was performed by column chromatography (5% water-deactivated silica-alumina) by elution with n-hexane (alkanes + alkenes) and n-hexane:dichloromethane (9:1 and 8:2)(mono and polycyclic aromatics, respectively) [3].

2.2.2 GC-MS analysis

The aliphatic fraction was analysed by gas chromatography (GC-FID) using a Mega 5000 instrument (Fisons, Milan, Italy) equipped with a 30m x 0.25 mm i.d. 5% phenyl-methyl-polysiloxane DB5 (J&W, Scientific, USA) under the operational conditions described elsewhere [4]. Resolved hydrocarbons and the unresolved complex mixture (UCM) of hydrocarbons were quantified by comparison with external standards (mixture of n-C14, n-C15, n-C16, n-C22, n-C23, n-C24, n-C28, n-C32, n-C36 and pristane). Samples were spiked with squalane and deuterated pyrene as surrogates for recovery calculations.
Quantification of 13 PAHs, and their alkyl derivatives, was achieved by GC-MS, using a Fisons GC 8000 series chromatograph interfaced to a Fisons MD800 mass spectrometer, operating under the electron impact mode, with full scan and selected ion monitoring (SIM). A standard mixture containing phenanthrene, anthracene, dibenzothiophene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo(b)- and (k)-fluoranthenes, benzo(a)pyrene, indeno(1,2,3-cd)pyrene, dibenz(ah)anthracene and benzo(ghi)perylene, was used for calibration.

2.3 Biochemical analysis

Pooled digestive glands of 4 to 6 mussels were used for each replicate sample, and six replicates were prepared per site. Subcellular samples were prepared at 4°C by differential centrifugation as described by Livingstone [5]. The microsomal pellet was resuspended in 10 mM Tris-HCl pH 7.6, 20 % w/v glycerol at protein concentrations of approximately 10 mg ml-1. Biochemical measurements were carried out either immediately (cytosolic fractions), or after overnight storage in liquid nitrogen (microsomes), as described in Porte et al. [6].

In short, cytochrome P450 components and activities were measured on microsomes by the carbon-monoxide difference spectrum of sodium dithionite reduced samples. SOD activities were measured spectrophotometrically in the cytosolic fraction by inhibition of the reduction of cytochrome c by superoxide anion radical, generated by hypoxanthine/xanthine. Lipid peroxidation was determined in malonaldehyde equivalents by reaction with thiobarbituric acid, and protein contents, using bovine serum albumin as standard. Statistical differences between groups of values were tested by multivariance one way ANOVA analysis, considering P<0.05 as statistically significant.

3 Results and discussion

3.1 Crude oil fingerprinting

When the oil is released into the marine environment a series of physicochemical and biological processes take place that induce compositional changes. The result is a GC profile devoided of light components and resolved peaks, and often showing little more than an unresolved complex mixture (UCM) of hydrocarbons. Therefore, the distinctive features for oil characterization should be found in the heavier components contained in the UCM. Good candidates are those known as fossil markers, and used in organic geochemistry for oil/oil and oil/source rock correlations [7].

Particularly valuable for the characterization of marine oil spills are acyclic isoprenoids, steranes (St) and triterpanes (Tt), that can be easily monitored by mass fragmentography, using the corresponding diagnostic ions [1]. This approach has been used in monitoring oil spills in coastal sediments [8-11], and also in relation with different Brent type oil pollution incidents in the North Sea [12-14], but has rarely been applied to organisms, as it is done in Figure 2.
Distinctive features for the unambiguous characterization of the Aegean Sea oil stranded on the coast were: the C-28 member of the series of pentacyclic triterpanes (m/z 191), the 17α(H),18α(H),21β(H)-28,30-bisnorhopane, with a

Figure 2: Gas chromatogram (aliphatic fraction) and characteristic fossil marker fingerprints of a mussel sample collected in April 1993, in station 6.
C-28:C-29:C-30 hopane distribution of 1:1.7:2.5 [13]; a sterane profile (m/z 218) showing the predominance of the C-27, the depletion of the C-28 and the presence of the extended C-30 homologs, which are all reflected in the mono-(MA) and tri-aromatic (TA) derivatives (m/z 253 and 231); and, finally, a distribution of the C-2 and C-3 alkyl dibenzo thiophenes (DBT) (m/z 212+226), phenanthrene/anthracenes (m/z 206+220) and chrysene/benzo(a)anthracenes (m/z 256+270) exhibiting a relative ratio of 1:2.8:0.2, respectively (Figure 2).

The Aegean Sea oil spill was unique in the sense that a significant part of the spilled oil was burnt, so the identification of pyrogenic hydrocarbons was also an important issue. Although smoke samples from the fire of the tanker were not available for analysis, it is well known that the profiles exhibited by soot from fossil fuels combustion are characterized by a distribution of polycyclic aromatic hydrocarbons (PAHs) dominated by non-alkylated species, and a greater abundance of 4-6 ring components, relative to those of 2-3 rings which are predominant in crude oils.

3.2 The fate of aliphatic hydrocarbons

The GC of the alkane+alkene hydrocarbon fractions of all samples exhibited a more or less pronounced UCM, underlying a series of resolved peaks, mainly consisting of n-alkanes in the C_{15} to C_{35} range, the isoprenoids pristane (Pr) and phytane (Ph), and a number of biogenic hydrocarbons (Figure 2). The GC analysis of the sediments of the area already indicated that oil residues followed predictable weathering patterns. n-Alkanes and isoprenoids were largely represented in the first set of samples collected in April, and were progressively degraded throughout the sampling period, when biogenic alkanes and alkenes were increasingly prominent. Particularly noteworthy was the shift of the maximum of the UCM hump towards higher retention times, the lower fractions being more soluble in water and more easily degraded.

In order to assess the correspondence of these hydrocarbons with the Aegean Sea spilled oil a detailed study of the fossil markers was carried out (Figure 2). The triterpane profiles (m/z 191) contained 28,30-bisnorhopane, the main distinctive feature of the Brent tanker oil, thus confirming the presence of the oil in the sediments and bivalves, although the C-28/C-29 triterpane ratios were usually lower than in the crude, possibly indicating a mixture with a pre-existing (chronic) pollution. In fact, stations 10 and 11 were found polluted by the tanker oil in April 1993, but exhibited a totally different profile at the end of the year, probably as a result of the chronic impact of the industrial city and harbour of El Ferrol. In December 1993 (one year after the accident) only mussels from stations 5 and 6 showed evidence of oil pollution, but stations 10 and 12 exhibited an incidental increase, probably as a result of sediment resuspension in the water column originated by the rough sea conditions. In any case, no evidence of the spill was found in a survey carried out three years later, in October 1995.

Similar results were obtained from the sterane distributions (m/z 218), but the diagnostic markers were less conclusive because they are more indicative of the oil weathering (degradation) than of the source. This degradation was reflected in the depletion of the C_{27} steranes, and, more significantly, in the
increase of the lower $C_{21}$ and $C_{22}$ homologs, a feature that was also common to the aromatized derivatives (m/z 231+253), as shown in Figure 2. Extensive degradation of sterane hydrocarbons were observed in clams collected in the area 3 years later (Figure 3). Whether this is the result of an endogenous process or simply the result of a microbially mediated one in the sedimentary environment is still an open question, although the above features were more largely recognized in those bivalves, such as clams, which live on the sediment.

The concentrations of the UCM of aliphatic hydrocarbons in sediments and bivalves showed the relative severity of oiling in the coastal area, just after the accident and a significant decline in the whole area between three and six months after the spill (Figure 4). Other studies carried out in temperate climate waters have pointed out that most of the petrogenic hydrocarbons have similarly been reduced after this period of time when the oil source was removed [15, 16].

3.3 The fate of aromatic hydrocarbons

The GC of the aromatic fractions also showed the characteristic UCM of hydrocarbons and prominent resolved peaks corresponding to biogenic sources such as polyunsaturated alkanes, highly branched isoprenoids and squalene. On the other hand, the GC-MS analysis proved the occurrence of a mixture of petrogenic and pyrogenic hydrocarbons, according to the presence of, respectively, low molecular weight (2-3 aromatic ring) compounds together with their alkylated derivatives, and higher mol. wt. (>4 aromatic ring), particularly peri-condensed and non-alkylated components.

Figure 3: Mass fragmentograms of sterane hydrocarbons (m/z 217) and methyl-DBTs (m/z 198) of clams collected in station 9, in April 1993 (A) and December 1995 (B).
The distributions of certain alkylated PAH homologous series have been proposed for fingerprinting crude oils [17]. Thus, the C-2 and C-3 alkyl homologs of DBT and phenanthrene/anthracene (P) and the same homologs of DBT and chrysene/benzo(a)anthracene (C), shown in Figure 2, were found useful in, respectively, differentiating hydrocarbon sources and characterizing the weathering of the spilled oil in sediments and bivalves.

The source ratios (DBT/P) exhibited a slight increase with respect to those of the crude oil, possibly due to the faster degradation of the phenanthrene derivatives. On the other hand, the weathering ratios (DBT/C) were in the sediments clearly below those of the original crude oil and decreased over time, whereas a less defined pattern was observed in bivalves. Although no information exists about the uptake and release of all these compounds in marine biota and particularly in bivalves, and how these processes affect bioaccumulation, it appears that the trimethyl ratios exhibit more consistent values, probably because these compounds are more hydrophobic and less affected by environmental weathering (degradation).

A conclusive evidence of advanced degradation of the oil residues was obtained from the relative distribution of methyl DBT isomers (m/z 198) (Figure 3), as the 2- and 3-methyl substituted isomers are more easily degraded in aerobic conditions than the 4-methyl derivative [19].

Of alternative use for source recognition can be the series of mono- and tri-aromatic steroid hydrocarbons which are not biodegraded until steranes and triterpanes are severely affected, and can be readily detected by GC-MS (m/z 253 and 231) (Figure 2). Evidence of metabolic breakdown of these markers was obtained, as in the case of steranes, from the large abundance of the C-19 – C-21 homologs devoided of the isopranyl side-chain. Whether this degradation has occurred in the sediment or in the bivalves is still to be proven, although the low metabolic capacity of these organisms lean to admit the former process as the more likely. The smaller molecular size of these compounds could also play a role in enhancing bioaccumulation.

Superimposed to this petrogenic signature was found the series of 3 to 6 ring PAHs, considered to be typical of combustion. The data indicate that the whole area has received these pyrogenic inputs, most probably originated in the tanker fire, but differently from the sedimentary profiles, that were clearly enriched in the higher homolgs, bivalve distributions were generally dominated by the alkylated PAHs, consistently with the predominance of the petrogenic components, phenanthrene and chrysene. The only exception were samples from station 11 that contained significant amounts of benzo(a)pyrene, possibly derived from the urban runoff of the area of El Ferrol.

In general, these data support the hypothesis that petrogenic PAHs are more readily available to filter-feeding bivalves, than combustion generated PAHs, which are more strongly adsorbed onto soot particles. We should take into account, however, that bioaccumulation is the result of uptake and metabolism, and it is well known that parent PAHs are more easily metabolised than the alkylated ones, although this process seems to be not very relevant in bivalves.

Despite of this, the pyrogenic fingerprint was more evident in clams than in mussels, according to their benthic habitat. Moreover, this imprint was also
enhanced along the survey period, suggesting a relatively higher stability of pyrogenic components in the bivalve tissues or the progressive degradation of the oil in the environment and, therefore, a reduced uptake by the organisms.

The bivalve concentrations reported in Figure 4 reflected a moderate pollution in the area after the accident, and a sharp decrease few months later. In fall, however, an increase in the PAH body burdens was again detected in different stations, probably as a result of the resuspension of sediments. The range of concentrations found in mussels 3 years after the accident, when the Aegean Sea oil pollution was unperceivable, was similar to those recently found in other European coastal areas [19].

3.4 Sublethal effects on mussels

Biomarkers of exposure/effect were determined in mussels from stations severely affected by the spill (5, 6, 8 and 12) and from one considered as reference (13). Total cytochrome P450 content was significantly elevated in station 5 at the first sampling period, six months after the accident. A 10-fold PAH gradient was observed between this station and the reference site (Figure 4), and this gradient seemed to be high enough to induce sublethal responses in mussels. In subsequent samplings (9 and 12 months after the spill), no site dependent differences were observed.

Beside this, levels of cytochrome P450 steadily decreased over the year, and a statistically significant decrease was recorded 12 months after the spill in mussels from all the stations, except those from the reference site. The decrease was particularly evident in mussels from station 5, suggesting the concurrence of both, a seasonal effect and a decrease of P450 inducing agents.

Figure 4: Temporal trends of aliphatic (UCM) and aromatic (PAHs) hydrocarbon concentrations and biomarkers in mussels.
Stimulation of reactive oxygen species is reported to be a PAH mediated mechanism of toxicity in mussels [20]. For that reason, antioxidant enzymes (SOD) and oxidative damage (lipid peroxidation) were also included in the study as possible biomarkers of pollutants impact. A significant increase in oxidative damage was detected at the tanker wreck site six months after the accident (Figure 4), and lipid peroxidation was related to total PAHs body burden ($R = 0.64$). Thereafter, no site related differences were seen, though lipid peroxidation increased throughout the year. Results on oxidative stress might also be affected by the existence of a seasonal effect. Viarengo et al. [21] reported a reduction of the antioxidant defence systems and an enhanced susceptibility of mussels to oxidative stress during winter. On the other hand, the increase in tissue PAH levels observed in December may also lead to increased lipid peroxidation. Hence, both pollution and seasonal effects appear to be superimposed in terms of oxidative stress, and this can certainly have negative effects on mussels health long after the accident.

In addition, it is worth mentioning the increase in SOD activity observed in autumn, 9 months after the spill (data not shown). This increase was statistically significant in mussels from stations 5, 6 and 8, and suggests an increased oxidative stress in those organisms, despite the fact that hydrocarbons had decreased in mussel tissues. A seasonal variation in SOD activity has also been reported, but this activity was low throughout the autumn-winter period, increasing in spring and maximizing in June [22]. Hence, the high SOD activities detected in autumn-winter in the present study, further suggests the existence of pollutants-mediated oxidative stress in the area closest to the accident. A similar trend was observed in clams. This “delayed” response in antioxidant enzymes may well reflect the existence in the environment, and particularly in the sedimentary compartment, of oxidized hydrocarbons formed through chemical and biochemical reactions, which have not been considered in the survey.

4 Conclusions

The present study has shown the helpfulness of the chemical marker approach for assessing the spatial and temporal evolution of an oil spill in coastal sediments and bivalves. From all the above it can be concluded that despite the extensive contamination observed in the Galicia coast after the Aegean Sea oil spill, six months after the accident bivalves were able to show a significant decline of the oil pollution, and based on the occurrence of specific fossil markers, one year later the oil was almost undetectable within the background hydrocarbon load.

The distribution of parent 3-6 ring PAHs reflected the concurrence of oil residues and combustion products, possibly from the tanker fire. These distributions, however, were depleted in bivalves in higher ring isomers with respect to those found in sediments, indicating a lower bioavailability of the pyrogenic components, but a longer persistence in time because this imprint was enhanced in mussels along the survey period. However, levels of total cytochrome P450 and lipid peroxidation still evidenced exposure to/effects of petrogenic hydrocarbons.
In summary, sediments and bivalves respond to oil spills differently, both qualitatively and quantitatively, being the later more useful in assessing spatial and temporal pollution trends, although bioaccumulation is a complex process not yet fully understood.

Acknowledgements

This study was supported by the Spanish Plan for Research (PLANICYT) under Project Ref. AMB-93-0693, the Spanish Ministry of Public Works and Environment (MOPT) and the Spanish Institute of Oceanography (IEO). Special thanks are due to J.J. Gonzalez (IEO, Vigo) for his assistance in field sampling.

References


