A guide to toxicity assessment and monitoring effects at lower levels of biological organization following marine oil spills in European waters

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The usefulness of applying biological-effects techniques (bioassays and biomarkers) as tools to assist in evaluating damage to the health of marine ecosystems produced by oil spills has been demonstrated clearly during recent decades. Guidelines are provided for the use of biological-effects techniques in oil spill pollution monitoring for the NE Atlantic coasts and the NW Mediterranean Sea. The emphasis is on fish and invertebrates and on methods at lower levels of organization (*in vitro*, suborganismal, and individual). Guidance is provided to researchers and environmental managers on: hazard identification of the fuel oil released; selection of appropriate bioassays and biomarkers for environmental risk assessment; selection of sentinel species; the design of spatial and temporal surveys; and the control of potential confounding factors in the sampling and interpretation of biological-effects data. It is proposed that after an oil spill incident, a monitoring programme using integrated chemical and biological techniques be initiated as soon as possible for ecological risk assessment, pollution control, and monitoring the efficacy of remediation. This can be done by developing new biomonitoring programmes or by adding appropriate biological-effects methods to the existing monitoring programmes.

Keywords: bioassays, biological effects, biomarkers, oil spill, PAH, petroleum, risk assessment.

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Introduction

Marine pollution caused by liquid petroleum (crude oil and products refined from it) may cause serious environmental impacts when released into the marine environment, whether as catastrophic spills or chronic discharges. Such pollution will therefore pose a significant risk to marine life and to the coastal environments where spills most often occur, especially near marine oil-producing regions, along the main oil-tanker routes, and close to major petroleum handling facilities (ports, refineries, etc.). The global energy crisis arising from the exhaustion of existing oil-producing fields implies increasing use of alternative energy sources in the coming decades. Meanwhile, the world's demand for oil is rising and, combined with a potential shortfall in supply, may lead to volatility in prices. Consequently, investments will be made to find new oil fields, most of which are likely to be offshore. Although operational and accidental discharge of oil from vessels has been reduced in the past decades and new regulations concerning tanker safety and the prevention of pollution by oil spills in the marine environment are continuously being developed and adopted, the increasing demand for oil and associated transport by sea continues to pose a risk of oil pollution and, hence, potential damage to coastal and marine ecosystems.

The North Sea is a major oil and gas production area with intense transport of oil by tankers and pipelines; new investment there is continuing. Each year, 800 million tonnes of oil are transported to or from European ports. About 70% of oil-tanker routes in the EU are found along the Atlantic and North Sea coasts (the remaining 30% being via the Mediterranean Sea), making those zones the most vulnerable to oil spills (EU, 2007). In addition, European waters contain major shipping routes for the transport of petroleum products to/from other countries outside the EU, e.g. in the Mediterranean Sea, where thousands of oil tankers

coming from the Middle East via the Suez Canal, or transiting around South Africa, pass the Straits of Gibraltar each year carrying crude oil to Europe and North America. As a consequence of the volume of traffic, major oil spills such as the "Amoco Cadiz" (Brittany, France, 1978), "Haven" (Genoa, Italy, 1991), "Braer" (Scotland, UK, 1993), "Sea Empress" (Wales, UK, 1996), "Erika" (Brittany, France, 1999), and the "Prestige" (Galicia, Spain, 2002) do take place occasionally and receive considerable public attention owing to the obvious acute environmental impacts, e.g. oil-coated shorelines and wildlife mortalities.

After a marine oil spill, monitoring and impact assessment is commonly based on the chemical measurements of petroleum-related hydrocarbons in different biota and marine compartments (sediments, water). However, those measurements fail to give information on the bioavailability and bioactivity of the compounds, so ecotoxicological methods are needed as a complement to chemical analyses. The incorporation of an effective suite of bioassays and biomarkers of exposure and effect can provide insights into the causality of any higher-level adverse effects that may be observed. During recent decades, many studies of oil spills in European waters and elsewhere have clearly demonstrated the potential and usefulness of applying biological-effects techniques in oil spill impact assessments, particularly concerning sublethal and long-term impacts at low levels of biological organization in organisms, and monitoring the efficacy of remediation (e.g. Stott *et al.*, 1983; Berthou *et al.*, 1987; Solé *et al.*, 1996; Davies and Topping, 1997; Lyons *et al.*, 1997; Edwards and Sime, 1998; Harvey *et al.*, 1999; Fernley *et al.*, 2000; Jewett *et al.*, 2002; Peterson *et al.*, 2003; Auffret *et al.*, 2004; Bocquené *et al.*, 2004; Budzinski *et al.*, 2004; Geffard *et al.*, 2004; Laubiert *et al.*, 2004; Lee and Anderson, 2005; Beiras and Saco-Alvarez, 2006; Cajaraville *et al.*, 2006; Marigómez *et al.*, 2006; Martínez-Gómez *et al.*, 2006, 2009; Ordas *et al.*, 2007). However, our understanding of the risk posed to the marine environment by chronic releases of petroleum and especially the cumulative effects of petroleum-related toxic compounds is still limited (NRC, 2003).

The ultimate purpose of toxicity assessment and environmental monitoring is to protect ecosystems from anthropogenic alterations. Using suborganism assays, the modes of action of substances can be detected and, if basal cytotoxicity or key functions are affected, they can give valuable information on possible consequences for populations and communities. The strength of the use of bioassays and biomarkers at low levels of biological organization is that these endpoints can provide reliable indications of the degree of exposure and of the resulting effects on the test organism. The use of such biological-effects techniques in ecotoxicology and ecological risk assessment has been criticized as a result of the lack of a clear ecological relevance, because their linkage to population and community-level effects often remains tenuous (Forbes *et al.*, 2006; Hagger *et al.*, 2006). Thus far, it has not been realistic to state that biological techniques proposed for use following marine oil spills offer enough information by themselves to provide predictions of broader ecological effects, because it is not obvious usually how such information can be conceptually linked with effects at these levels (Thain *et al.*, 2008). However, it is recognized that detecting effects before they become serious requires monitoring at lower levels of organization (Moore, 1998). The use of bioassay/biomarkers offers invaluable early warning information to be used to improve the processes of hazard assessment for populations (Esler *et al.*, 2002; Moore *et al.*, 2006) and ecological risk assessment (Eason and ÓHalloran, 2002; Hagger *et al.*, 2006). Therefore, their use substantially contributes to the three main aims of environmental managers after an oil spill incident:

(i) measurement of the toxicity of the spilled petroleum-related compounds as part of the hazard assessment;

(ii) spatial estimation of the extent and the magnitude of the damage on the marine ecosystems affected;

(iii) evaluation of the time to recovery after the oil spill and/or the effectiveness of any policy measures taken.

At the current state of scientific development, a range of standardized biological-effects techniques is available that can provide information on the degree of exposure to petroleum-related hydrocarbons and/or their effects upon the individual organisms in a population. We provide guidelines on the selection and use of appropriate batteries of bioassays and biomarkers for toxicity assessment and for monitoring the biological effects at low levels of biological organization in fish and invertebrates associated with oil spills in western European waters in relation to the three management requirements listed above. The guidelines are based largely on advice prepared for the Oslo and Paris Commission (OSPAR) by members of the International Council for Exploration of the Sea (ICES) Working Group on Biological Effects of Contaminants (WGBEC). The batteries of assays recommended by WGBEC have been selected, after critical review and discussion, from the broad range of assays that are applicable at low levels of biological organization (ICES, 2007a). Our aim is to provide guidelines applicable to EU waters, in particular the Northeast Atlantic and western Mediterranean, accompanied by a general overview of the relevant literature in this field.

A general overview on toxicity and environmental effects of petroleum-related hydrocarbons

Liquid petroleum is a complex mixture of tens of thousands of compounds, in which various hydrocarbons are the most abundant classes, usually accounting for >75% of the total oil composition (OSPAR, 2004). Nitrogen-, sulphur-, and oxygen-containing hydrocarbon analogues and other materials such as metals (iron, nickel, vanadium, and arsenic) can be important minor constituents. These hydrocarbon compounds include saturated substances (alkanes and cycloalkanes), unsaturated substances or oleofins, aromatic compounds (mono- and polyaromatic), and polar compounds. Monocyclic aromatic hydrocarbons (benzene, toluene, phenols), but particularly polycyclic aromatic hydrocarbons (PAHs), are possibly the contaminants that have the most serious long-term environmental effects.

The chemical and physical nature of crude oils or refined products determines the fate and effects of these compounds in marine ecosystems. The lightest oils (classes A and B) will spread rapidly on solids or water surfaces and may be acutely toxic to humans, fish, and other biota. Despite having a fast rate of evaporation, these types of oil do not tend to adhere to surfaces, but penetrate porous materials including muddy or sandy sediment, and may persist in such matrices. Therefore, chronic exposure to hydrocarbons may result from the incorporation of spilled oil into sediments in which the breakdown of oil components is retarded. On the other hand, the heavy or non-fluid oils (classes C and D) will attach more strongly to solid materials, but the oil does not readily penetrate porous materials. Those types of oil

are relatively dense and often sink. The acute toxicity of heavy oils is much lower than that of classes A and B, but wildlife can be smothered by such materials. Likewise, heavier compounds of the oil that disperse as droplets can persist in suspension for a long time, many years on occasion, and may be transported thousands of kilometres by water currents, whereas the remaining crude oil partly dissolves in the water and partly forms tar. In contrast to beaches and shallow subtidal habitats, deep-sea benthic habitats do not benefit from the clean-up activities conducted after a spill to reduce any long-term impacts. Moreover, the residual oil on the deep seabeds will be subject to very low physical energy. Dispersion may be relatively weak, and biodegrading and weathering processes relatively slow.

A typical crude oil may contain 0.2 to >7% total PAHs, with four- through six-ring PAHs present at low or trace concentrations (NRC, 2003). PAHs such as naphthalene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, and dibenzo[a,h]anthracene have been classified by the International Agency for Research on Cancer (IARC) as possible or probable human carcinogens (groups 2A and 2B; IARC, 1989) so may pose a risk to humankind and wildlife. As the aromatic hydrocarbon fractions of fuel/crude oil will be the most toxic fractions, chemical characterization and quantification of this group of constituents have been prioritized. For environmental fate and ecotoxicological studies of oil spill situations, however, chemical analysis of the aromatic hydrocarbons in the liquid petroleum (crude oil/refined products) will not be sufficient, because other compounds associated with the spill or generated during weathering processes will contribute to the toxicity under certain field conditions (Burns *et al.*, 2000; Neff *et al.*, 2000). In addition, the use of chemical oil dispersants may be a factor contributing to the toxicity of PAHs (Cotou *et al.*, 2001; Fuller *et al.*, 2004).

The toxicity of hydrocarbons is the result of the uptake of dissolved hydrocarbons by aquatic organisms, and it can lead to a wide variety of physiological responses (see the review by NRC, 2003). The overall picture is often complex because a range of processes, such as biodegradation, bioaccumulation, and biotransformation, will determine the bioavailability and toxic potency of PAHs and other petroleum-related compounds in the field. Moreover, marine organisms, even from the same taxonomic group, may vary greatly in their sensitivity to the same compound (NRC, 2003; van der Oost *et al.*, 2003). During the past few decades, a large body of research has been published on the biological effects and impact of petroleum-related hydrocarbons, such as PAHs, in the marine environment (see the review by NRC, 2003; Hylland, 2006). PAHs with higher molecular weight (more than three aromatic rings) are less water-soluble than two- or three-ring PAHs and tend to be bound to particles (algae, faecal pellets, soot particles) or dissolved organic compounds, resulting in lower degradability and consequently higher potential bioaccumulation in pelagic foodwebs or sedimentation and accumulation in sediments. Chronic exposure to petroleum hydrocarbons can affect feeding, growth, and reproduction and cause irreversible tissue damage. Marine organisms such as filter-feeding molluscs have an outstanding ability to bioaccumulate hydrocarbons in their tissues, whereas other organisms, particularly vertebrates, readily metabolize and excrete them. However, the degradation pathways may create reactive intermediates with toxic effects (deBethizy and Hayes, 1994; van Brummelen *et al.*, 1998). Biotransformation of PAHs and metabolic activation of the carcinogenic PAHs are thought to take place predominantly through the aryl hydrocarbon receptor (AhR)-mediated induction of the CYP1 family of P450 monooxygenases. CYP1A induction can also increase the metabolism of oestrogens, and there may be interactions between Ah and oestrogen receptors, suggesting the potential to interfere with endocrine processes (Wenger *et al.*, 2009). Several studies have demonstrated that PAH exposure in polluted environments results in reproductive and developmental effects in fish (reviewed in Nicolas, 1999; Monteiro *et al.*, 2000; Patel *et al.*, 2006). Other mechanisms of toxicity that may be involved are nonpolar narcosis and photoxicity (van Brummelen *et al.*, 1998). In particular, their mutagenic and carcinogenic potential, in addition to their cytotoxic properties (Berthou *et al.*, 1987; Varanasi *et al.*, 1987; Vethaak *et al.*, 1996; Lyons *et al.*, 1997), pose serious threats to marine organisms, especially during embryogenesis and early stages of development (Geffard *et al.*, 2002; Pollino and Holdway, 2002; Incardona *et al.*, 2005). Apart from directly affecting the reproductive status and the growth of aquatic biota, PAHs compromise the immune system of fish and other aquatic organisms (Wester *et al.*, 1994; Wootton *et al.*, 2003; Auffret *et al.*, 2004). These alterations can reach higher levels of biological organization, where they may alter vital functions that affect the survival of organisms and cause damage in populations and communities (Capuzzo, 1990; de Maagd and Vethaak, 1998; Peterson *et al.*, 2003).

Toxicity assessment of the spilled petroleum-related compounds

An essential initial stage in a risk analysis of an oil spill is to gain understanding of the hazard that the spill presents to the environment. In some cases, published data may be sufficient to provide initial guidance to managers and fulfil their need for a hazard assessment, but it is likely that additional information about the specific material comprising the spill will be required.

Hazard assessment of petroleum-related compounds should be based on a set of relevant *in vitro*/*in vivo* bioassays conducted on water or sediment extracts. This may be undertaken rapidly and in a relatively cheap and easy way by analysing field samples or laboratory exposure experiments (for details see below). Multiple bioassays can be selected to allow identification of the toxicity profile of the specific petroleum-related hydrocarbons concerned. *In vitro* bioassays comprise bacterial tests of acute toxicity (e.g. Microtox®, *Vibrio fischeri* assay), genotoxicity (e.g. Umu test, Ames test), and reporter gene assays for measuring dioxin-receptor activation (DR-CALUX). In addition to *in vitro* bioassays, the standard *in vivo* laboratory toxicity tests (i.e. algae, crustaceans, sea-urchins, molluscs, fish) with lethal or sublethal biological endpoints may be performed (Table 1).

The application of 3–4 *in vitro* bioassays covering different modes of action (AhR-mediated toxicity, acute toxicity, genotoxicity) of the toxicants is recommended, particularly including the DR-CALUX (Table 1). For a more detailed toxicity assessment, reporter gene assays for oestrogenicity screening can be included (YES, ER-CALUX). In addition and because of the differences in sensitivity of test organisms, a test set of *in vivo* bioassays with different species and different parameters has to be applied. A small set (3–4) of *in vivo* bioassays should be conducted representing different animal classes and different trophic levels, including at least one embryo larval test (Table 1). *In vivo* bioassays should preferably be applied to marine species for which methods are

Table 1. Appropriate *in vitro* and *in vivo* bioassays for toxicity profiling to be used after an oil spill.

Method	Time-scale of response by method/expected effect in field	Organism	Action mode	Purpose and biological significance	Source of method
In vitro toxicity profiling using ambient water, WAF, CEWAF, sediment, or biota extracts					
Dioxin receptor (AhR)-mediated chemical-activated luciferase gene expression assay (DR CALUX)	Hours/days to months depending on conditions	H4IIE genetically modified rat hepatoma cells	Induction of xenobiotic detoxification system	Measure of exposure to planar organic compounds including PAHs	Murk *et al.* (1996) and Klamer *et al.* (2005)
Acute toxicity: Microtox®, *Vibrio fischeri* assay	Hours/days to weeks depending on conditions	Genetically modified bacteria *Photobacterium phosphoreum, Vibrio fischeri*	Toxic stress: reduction in intensity of light emitted from the bacteria	Measure of acute toxicity	Environment Canada (1992), Stronkhorst *et al.* (2003), Klamer *et al.* (2005), and Morales-Caselles *et al.* (2008)
Genotoxicity test: Umu test, Ames test	Hours/days to weeks depending on conditions	Genetically modified bacteria, *Salmonella typhimurium*	Expression of SOS response, umu-C genes induced by genotoxic compounds	Measure of reverse mutation induced by genotoxic compounds	Maron and Ames (1983), Oda *et al.* (1985), and Klamer *et al.* (2005)
Oestrogen receptor (ER)-mediated chemical-activated luciferase gene expression assay (ER CALUX), also using fish bile	Hours/days to months depending on conditions	T47D genetically modified human breast cancer cells	Alteration of endocrine system	Measure of exposure to oestrogenic compounds	Legler *et al.* (1999, 2002a, b), Klamer *et al.* (2005), and Houtman *et al.* (2007)
Yeast [o]estrogen screen (YES) (also using fish bile)	Hours/days to weeks depending on conditions	Genetically modified yeast cells	Alteration of endocrine system	Measure of exposure to oestrogenic compounds	Routledge and Sumpter (1996) and Gibson *et al.* (2005)
			Purpose and biological significance		
In vivo bioassays and experimental exposures (whole organisms), using ambient water, WAF, CEWAF, elutriates, or sediments					
Scope for growth	Days/years	Mussels	Physiological impairment		
Embryo larval test	Days/days to weeks	Sea urchins, mussels, oysters, and clams	Mortality and deformity in embryos		Thain (1991), ASTM (1993), Mariño-Balsa *et al.* (2003), Beiras and Saco-Alvarez (2006), and Widdows and Staff (2006)
Algal growth inhibition test	Days/days to weeks depending on conditions	Unicellular algae	Mortality		Mariño-Balsa *et al.* (2003) and Navas *et al.* (2006)
Water/WAF bioassays	Hours/days to weeks depending on conditions	Copepods and invertebrates	Mortality and behaviour, reproductive impairment		Barata *et al.* (2005) and Navas *et al.* (2006)
Sediment bioassays	Weeks to months depending on conditions	Amphipods, polychaetes, clams, and fish	Mortality and behaviour, induction of detoxification mechanisms and tissue damage (fish)		Thain and Bifield (2001), Thain and Roddie (2001), Mariño-Balsa *et al.* (2003), Beiras and Saco-Alvarez (2006), and Morales-Caselles *et al.* (2006)

well-documented (e.g. certain bivalves *Mytilus* sp. and *Crassostrea gigas*, sea urchins *Paracentrotus lividus*, crustaceans *Corophium volutator*, *Acartia* sp., and *Tisbe battagliai*, polychaetes *Arenicola marina*, fish *Scophthalmus maximus*, *Platichthys flesus*, and *Gadus morhua*, and the algae *Isochrysis galbana*, *Chlorella vulgaris*, *Skeletonema costatum*, *Fucus* sp., and *Ulva* sp.), although biogeographical differences may require the use of alternative species (cf. Pollino and Holdway, 2002; Mariño-Balsa *et al.*, 2003; Stronkhorst *et al.*, 2003; Budzinski *et al.*, 2004; Siu *et al.*, 2004; Barata *et al.*, 2005; Morales-Caselles *et al.*, 2006; Patel *et al.*, 2006).

The *in vitro* and *in vivo* bioassays for the toxicity assessment of petroleum-related hydrocarbons in Table 1 can be conducted using ambient water, the water-accommodated fraction (WAF), the chemically enhanced WAF (CEWAF), elutriates, sediments or their extracts, and also using fish bile for some *in vitro* bioassays (ER-CALUX and YES). The use of ambient water and WAF is recommended for toxicological profiling soon after the spill, although when chemical oil dispersants have been applied, the bioassays on CEWAF also offer valuable relevant information (Ramachandran *et al.*, 2004, 2006; Casado *et al.*, 2006; Navas *et al.*, 2006). *In vitro* bioassays can be conducted in an integrated way with *in vivo* bioassays using fish, i.e. by assessing exposure to oestrogenic compounds through levels in fish bile.

As spill response develops, it will become more important to direct the hazard assessment towards the environmental conditions at the site of the spill. Exposure concentrations used to perform toxicity tests should be similar to those expected or measured in the field. Salinity and temperature of the ambient water, particularly when chemical oil dispersants have been used, also have an influence on the toxicity (Ramachandran *et al.*, 2004, 2006). With weathering processes and loss of the monoaromatic compounds, the PAHs become more significant contributors to the toxicity of weathered oils. Therefore, these factors must be taken into account in the sampling design of the experiments. The use of local seawater is recommended, but if that is not possible, salinity and temperature should be similar to those existing in field conditions. Some studies have demonstrated the impact of natural light (UV) on the toxicity of certain environmental contaminants (including PAHs) in elutriates and WAF, through photodegradation and photoactivation processes (Garret *et al.*, 1998; Mallakin *et al.*, 1999; Little *et al.*, 2000; Lyons *et al.*, 2002, 2006; Kirby *et al.*, 2007). The potential influence of the UV factor on the toxicity can be considered by the establishment of two treatments (with or without UV light exposure) in the experiments, but also using fresh and artificially weathered oil for the experiments (for details, see Aurand and Coelho, 2005).

Monitoring the biological effects of spilled petroleum-related compounds

General considerations

Although hazard assessment describes the toxic potential of the petroleum-related compounds, the relevant risk assessment considers the effects of such compounds when released into the environment through an oil spill. Monitoring after spills is normally required to determine whether contaminant exposure results in ecologically relevant harm to living resources, and a study of organisms collected from their natural habitats is required. This provides a validation of the conclusions of the hazard assessment, and it is essential for the environmental risk analysis and environmental management. The sampling design of such a biological-effects monitoring programme is related to the scientific and management objectives of the study. Spatial estimation of the extent and the magnitude of the damage on the marine ecosystems affected will be assessed through spatial monitoring programmes and the evaluation of the time to recovery after the oil spill, and/or the effectiveness of any policy measures taken will be conducted through a temporal monitoring strategy. Although such distinction is used in these guidelines, there is nothing to prevent the two activities from being carried out simultaneously, as long as this is recognized in the design of the programme and appropriate to the selected statistical analysis of the data. The objectives and sampling design for both monitoring objectives are closely related, and they should be developed simultaneously. Note that biogeographical differences between countries and the local requirements for oil spill responses and activities may require adaptations to this general guidance.

The contribution of non-spill background PAH concentrations, particularly combustion-derived (pyrogenic) PAH, can be a confounding factor when evaluating biomarker results in oil-spill monitoring programmes. It is essential that a set of biomarkers be used in conjunction with supporting analytical chemistry data from samples of water, sediment and/or biota, and other biological measurements. Appropriate integration of sampling for biological effects and chemistry can be conducted according to the specific or integrated guidelines developed by OSPAR (2004) and ICES (2006, 2007b) as part of existing programmes. The study of PAH ratios (i.e. fluoranthene/pyrene and chrysene/benzofluoranthenes) and fingerprinting analysis using molecular markers can provide signatures for identifying hydrocarbon sources in the environmental matrices (Soriano *et al.*, 2006). Certain PAH metabolites (1-pyrenol and 1-naphthol) in fish bile also can add valuable information about the origin (petrogenic/pyrogenic) of the PAH exposure in fish biomarker monitoring (Fernandes *et al.*, 2008).

Selection of appropriate sentinel species

Biological techniques will generally be applied to representative organisms from the marine environment where the petroleum-related hydrocarbons have been spilled or have accumulated. Whenever possible, one should use monitoring species for which biological-effects techniques are well-documented. That is the case for mussels and for certain demersal fish species (European flounder, dab, Atlantic cod, and red mullet), which are routinely used in biomonitoring programmes for assessing contamination along western European marine waters and for which background data are available (Table 2). Methods will have to be adapted for alternative sentinel species using site-specific monitoring criteria, including a wide distribution in the affected area, benthic/demersal life style, close contact with sediment or active filter-feeders, and low migratory activity, as well as relative ease of sample collection (Harvey *et al.*, 1999; Pietrapiana *et al.*, 2002; Budzinski *et al.*, 2004; Marigómez *et al.*, 2006; Martínez-Gómez *et al.*, 2006; Joly-Turquin *et al.*, 2009).

Biomarkers of short- and long-term responses

Tables 3 and 4 provide summaries of the most practical and useful biomarkers for oil spill situations, with special reference to the type of response measured, the time-scale of response of each method, the time-scale of expected effect in the field, the organism used, and the target tissue/organ. For certain biomarkers, several techniques are available, and final selection should be based on the

Table 2. Field organisms recommended as target monitoring species in oil spill situations.

Target organisms	Species	Geographic area used for monitoring	Marine environment for monitoring
Demersal fish	European flounder (*Platichthys flesus*)	Northeast Atlantic: North Sea	Estuaries/coastal areas/inner shelf
	Dab (*Limanda limanda*)	Northeast Atlantic: North Sea, Irish Sea	Inner/middle shelf
	Plaice (*Pleuronectes platessa*)	Northeast Atlantic: North Sea	Estuaries/coastal areas/inner shelf
	Four-spotted megrim (*Lepidorhombus boscii*)	Galician and Cantabrian shelf, Mediterranean Sea	Middle and outer shelf
	Dragonet (*Callionymus lyra*)	Northeast Atlantic: North Spain and France	Estuaries/coastal areas/inner shelf
	Sole (*Solea solea*)	Northeast Atlantic: France	Estuaries/coastal areas/inner shelf
	Eelpout (*Zoarces viviparus*)	Northeast Atlantic: North Sea,	Estuaries/coastal areas
	Red mullet (*Mullus barbatus*)	Mediterranean Sea	Coastal areas/inner shelf
	Haddock (*Melanogrammus aeglefinus*)	Northeast Atlantic: North Sea	Inner/middle shelf
Demersal/ pelagic fish	Atlantic cod (*Gadus morhua*)	Northeast Atlantic: North Sea	Middle shelf
Molluscs	Mussel (*Mytilus edulis* and *Mytilus galloprovincialis*)	Northeast Atlantic: North Sea, Bay of Biscay, Cantabrian Sea, Mediterranean Sea	Estuaries/coastal areas

capacity and experience of the research group involved. Some biomarkers reflect an acute response to a short-term exposure directly after the oil spill (days to months); others reflect a chronic response after a long-term exposure (months to years). For oil-spill biomonitoring programmes, 3–4 biomarkers of the short-term effects (Table 3) and 3–4 of the long-term effects (Table 4) should be included. Acute biomarker responses include induction of the 1A family of cytochrome P450 enzymes (CYP1A). The activity of 7-ethoxyresorufin-*O*-deethylase (EROD) appears to be the most sensitive catalytic probe for determining the inductive response of CYP1A1 in vertebrates. Acute biomarker responses further include induction of other biotransformation enzymes, e.g. glutathione-conjugating and antioxidant enzymes, formation of PAH metabolites, and general markers of physiological status, e.g. lysosomal membrane stability (LMS). Chronic biomarker responses measuring the long-term effects putatively resulting in carcinogenesis and reproductive failure include prolonged oxidative stress, DNA damage, and enzymes of steroid metabolism. Histopathological changes, in particular liver disease, are also important indicators of chronic effects of PAH exposure. LMS reflects the whole range of time-scale in its response from very early to long-term effects.

The selection of acute biomarker responses will depend on the target organisms used: for fish, PAH bile metabolites, EROD activity, antioxidant activities, and LMS are the recommended methods. For mussels, LMS, antioxidant activities, and acetylcholinesterase (AChE) inhibition are recommended. Selection of chronic biomarker responses preferably should include the measurement of DNA damage and histopathology in liver/digestive gland, lipid peroxidation/oxidative stress, and LMS. Liver lesions cause degenerative changes that impair the cytochrome P450-dependent enzymes of the mixed-function oxygenase detoxification system (Köhler and Pluta, 1995). Measurements of LMS in fish as a long-term effect reflect the impact on liver function. This biomarker provides useful supporting information if EROD measurements are also being made. Examination for histological changes in the gonads of both mussels and fish also will offer valuable information (Stott *et al.*, 1983) and is essential in those cases where endocrine disruption effects may be expected according to the results of the biological toxicity profile (YES and ER-CALUX). If evidence of oestrogenic endocrine disruption effects is observed in gonadal fish tissue, such as intersex in male fish (Gercken and Sordyl, 2002; Vethaak *et al.*, 2002), measurement of other biomarkers, e.g. vitellogenin in male and juvenile fish blood plasma, should be added to the monitoring programme (Scott and Hylland, 2002).

Confounding factors

When biomarkers are used in monitoring programmes, it is important to be aware of potential confounding factors (Thain *et al.*, 2008). Seasonal variations in temperature affect the biological activities of marine organisms (especially EROD activity) and may also affect the behaviour, toxicity, and bioavailability of the oil. To make a critical assessment of the field data, parameters such as temperature and salinity of the ambient water in the sampling period must be recorded. The age/size, sex, maturation, and reproductive cycle of the sentinel species may affect the biomarker responses. To reduce the possible effect of these biological factors, samples should be as homogeneous as possible. Specimens have to be sampled within a limited size range, which may vary in terms of function of the species selected and the availability within the geographical area studied. For fish, data from males and females should not be mixed before analyses if significant differences attributable to this factor cannot be ruled out. Sampling time should be outside the spawning season to avoid any influence of spawning activity on certain biomarker responses, although for certain biomarkers such as histology in gonads, the sampling of mature animals in prespawning condition can be necessary. Sampling period and frequency would be related to the purposes of the monitoring, as discussed below. In general, parallel sampling in a similar control or reference area can assist greatly in interpreting monitoring data.

Spatial estimation of the extent and the magnitude of the damage to marine ecosystems

In any oil spill situation, it is important to assess the spatial extent of the area in which organisms have been affected by the spill. If a spatial impact is to be detected, special attention has to be directed towards the sampling design, e.g. the BACI (Before, After, Control, Impact) sampling design (Kingsford, 1998). However, in the

Table 3. Major short-term (acute) biomarkers recommended for use after an oil spill.

Acute-effect biomarkers	Time-scale of response by method/time-scale of response expected in field	Organism	Target tissue	Purpose/biological significance	Source of method
PAH metabolites	Hours/days to months depending on conditions	Fish	Bile	Indicates exposure to PAHs	Ariese *et al.* (2005)
Lysosomal stability	Hours/days to months depending on conditions	Mussels and fish	Blood cells, digestive gland, or liver	Subcellular damage	Köhler *et al.* (2002) and Moore *et al.* (2004)
EROD activity	Days/days to months depending on conditions	Fish	Liver	Induction of detoxification mechanisms	Galgani and Payne (1991) and Stagg and McIntosh (1998)
AChE inhibition	Hours/months	Mussels	Gills	Inhibition of AchE activity as general indicator of physiological status	Bocquené and Galgani (1998) and Bocquené *et al.* (2004)
Antioxidant activities: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), gluthatione reductase (GR), DT-diaphorase (DT-D)	Days/days to months depending on conditions	Mussels and fish	Digestive gland or liver	Induction of oxidative stress responses enzymes	Ramos-Martínez *et al.* (1983), Greenwald (1985), and Livingstone *et al.* (1992)

Table 4. Major long-term (chronic) biomarkers recommended to be used after an oil spill.

Biomarker of long-term effects	Time-scale of response by method/time-scale of response expected in field	Organism	Target tissue	Purpose/biological significance	Source of method
Lipid peroxidation/oxidative stress: total oxidant scavenging, capacity (TOSC), malondialdehyde (MDA), lipofucsin content	Hours-days/months	Molluscs, fish	Digestive gland, liver	Cellular response to oxidative stress	Buege and Aust (1978), Krishnakumar *et al.* (1994), Winston *et al.* (1998), Regoli and Winston (1999), Köhler *et al.* (2002), Pampanin *et al.* (2005), and Kaloyianni *et al.* (2009)
LMS	Hours/days to months depending on conditions	Mussels, fish	Blood cells or digestive gland, liver	Interference with steroid metabolism/effects of reproductive performance/ endocrine disruption	Köhler *et al.* (2002) and Moore *et al.* (2004)
DNA damage: micronuclei frequency (MN), comet assay, DNA adducts, alkaline unwinding, alkaline elution	Hours/days to months depending on conditions	Mussels, fish	Blood cells, gills, digestive gland, liver	Genotoxic damage	Kohn *et al.* (1981), Shugart (1988), MacGregor (1991), Nacci *et al.* (1992), Everaarts (1995), UNEP/RAMOGE (1999), Aas *et al.* (2000, 2002), Dolcetti *et al.* (2002), Pietrapiana *et al.* (2002), Akcha *et al.* (2004), Lyons *et al.* (2004), Siu *et al.* (2004), and Taban *et al.* (2004)
Histopathology	Months/1+ years	Mussels, fish	Target organs including liver, digestive gland, gills, and gonads	Tissue damage/neoplastic diseases	Winzer *et al.* (2001), Vethaak *et al.* (2002), and Feist *et al.* (2004, 2006)

particular cases of accidental oil spills, a BACI sampling design is not fully realistic because of the unpredictable character of such events. Therefore, the existence of pre-oil-spill data in the study areas is a critical point in the risk and impact analysis process. The statistical methods used to analyse data should be chosen as part of the programme design activity. Every level of sampling needs to be appropriately replicated (sample units, sex, localities, areas, control/impact, etc.). In field studies, as a rule, the aim should be to collect a random and independent sample for each species under examination and to conduct the biological-effects measurements according to internationally agreed recommended protocols. Two general sampling protocols have been widely used for monitoring of biomarkers in fish and mussels. The first was set out by OSPAR JAMP (2003) and is particularly applicable to OSPAR maritime waters. The second protocol was developed by UNEP/RAMOGE (1999) for use in the Mediterranean Sea. It is recommended that samples for histological assessment be collected from fish samples that have also been sampled for biomarker studies and chemistry. Fish should be captured by trawling, using appropriate fishing gear. The duration of individual trawls should be minimized, however, to reduce the effects of stress and possible mechanical damage to the fish. As soon as possible, fish should be transferred to aerated flow-through seawater tanks. Only live fish exhibiting normal opercular movement should be selected for necropsy (OSPAR JAMP, 2003; Feist *et al.*, 2004).

As a rule, a minimum of 12 individuals of each sex for each area and sampling occasion should be sampled for fish biomarker analyses, and a minimum of 10–20 specimens should be sampled for mussels (depending on the method). An exception is liver and gonad histology, where a minimal sample size of 30 is required, and larger numbers are required to detect disease at lower prevalence rates, i.e. for 5% disease prevalence ($n = 60$) and for 2% disease prevalence ($n = 150$; Feist *et al.*, 2004). Within each of the defined areas of the study, sampling should be carried out at multiple locations randomly distributed over the whole area under study. Detailed information about procedures for establishing an impact study and analytical aspects of the sampling design can be found in Kingsford (1998). After each sampling event, all results should be evaluated and assessed to identify weak points and inconsistencies that can be corrected to increase the quality and statistical robustness of the programme. A good example of how sampling design may affect conclusions is provided by Peterson *et al.* (2001) in relation to environmental effects and recovery following the "Exxon Valdez" oil spill (AK, USA, 1989).

It is clear from previous oil spill scenarios that the assessment of the impact produced by an oil spill using biological-effects techniques can be most successfully established in those areas where previous biological effect and chemical data exist. This is rarely the case, however, and assessments of short- and long-term impacts will therefore nearly always be hampered by a lack of pre-spill baseline data, hydrocarbon levels in the marine compartments, background levels of biomarker responses, or reference values for biological measurements. If previous data do not exist, the only option is to conduct an impact assessment inferred from spatial patterns, where comparisons are made between areas in which there would be suspicions of pollution effects arising from the spill and post-spill control areas. All information available concerning areas where oil or related petroleum hydrocarbon has been found or accumulated, as well as predictions of the oil drift and its fate, need to be used to predict the potential spatial extent of an impact. For the "Prestige" oil spill, chemical characterization of the tar aggregates found on the deep-sea floor allowed a definitive association with the "Prestige" and contributed to the design of a fish biomarker monitoring programme (Martínez-Gómez *et al.*, 2009). If there are no appropriate control areas, however, the impact can only be inferred from temporal changes in the measured parameters.

An exploratory spatial monitoring using the above suggested biomarkers of the short-term biological effects can be used soon after an oil spill to provide an initial estimation of the spatial extent of the impact on organisms. That implies performing the field sampling at any time of the year, not necessarily in the non-spawning season. In cases where there is a lack of knowledge of biomarker responses in the selected target species in the areas of concern, but also in those cases where the oil is spilled over a long period, i.e. for the "Prestige", it was suggested to perform a spatial-seasonal study for at least the first 2 years after the incident (Cajaraville *et al.*, 2006) to obtain sufficient information about biomarker patterns and to estimate variations of the impact on marine ecosystems. Relying on the short-term biomarkers alone may mask the long-term adverse effects. Spatial estimation of the severity of damage or effects or organism health after an oil spill will also include an assessment of biomarkers of the long-term biological effects. Such parameters should be measured as soon as possible if there is a lack of previous data to describe the spatial situation immediately following the oil spill. The biomarkers of the long-term effects proposed above should be measured during the exploratory spatial monitoring, and the monitoring maintained for some years in some areas, particularly in those where acute effects were severe, and also in the control areas. If background information on long-term biomarkers in the species and in the study areas is available, these biomarkers may be added later to the programme, once temporal monitoring has been clearly established.

Evaluation of the time to recovery after an oil spill and/or the effectiveness of any policy measures taken

To design a temporal-trend monitoring programme using the biological-effects techniques, it is necessary to have prior knowledge of the variability of each biomarker response. As most of the biomarker responses have a seasonal pattern, a detailed annual field sampling programme with rigorous statistical analyses is necessary to be able to demonstrate the environmental persistence or long-term biological recovery. The temporal trend programme will therefore be designed using background information available, and/or the results of the exploratory spatial monitoring. The locations/areas should be sampled at the same time of year (preferably within 2 weeks) for each annual sampling cycle, and organisms sampled outside the spawning season to avoid any influence of spawning activity on biomarker responses. As a rule, that sampling should take place at least 1 month after spawning. Within the framework of spatial monitoring, fewer sampling locations/areas should be selected for annual monitoring. As mentioned above, temporal sampling should be conducted at the locations/areas where the greatest concentrations of petroleum-related compounds, and/or acute effects have been already identified, but also in the control locations/area to allow recognition and control for confounding influences.

As mentioned earlier, the release of equal amounts of the same oil substance at different times or locations may have dramatically different environmental impacts, making each spill a unique event. It is understandable, therefore, that the time-scale of the monitoring programme cannot be set *a priori*. Biomarker responses will have recovered when measured levels/responses are similar to those found in control areas (in the case that all areas impacted are considered to have a homogeneous environmental quality status), or those existing in the impacted areas before the oil spill. The power of the monitoring programme to detect some given pattern of change in every chronic biomarker response will depend on the number of years, the annual biomarker value, and its variance (for more details, see Nicholson *et al.*, 1997). Usually, recovery of acute biomarker responses can be observed in the space of days to months and tested using time-to-time variation analysis. However, the interpretation and recovery of chronic biomarker responses will take years and can be hampered by the occurrence of chronic hydrocarbon pollution from other anthropogenic sources. As an annual sampling frequency is the most practical and convenient, at least 5 years of annual sampling is required to perform a robust statistical treatment of the temporal data.

Some examples

With a few exceptions, biomarker data collection has extended for a period of some years after a spill event. After the "Erika" oil spill in December 1999, a 3-year survey was made of several biological markers in mussels (*Mytilus edulis*) exposed *in situ* to the oil. DNA adduct concentrations and MDA activity were high during the 6 months immediately following the incident, and AChE activity was significantly lower during the first year of the survey, suggesting a general stress syndrome in the mussels (Bocquené *et al.*, 2004). For the "Sea Empress" oil spill of February 1996, mussels fortuitously sampled before the oil spill from the impacted coastline had low levels of DNA adducts. Post-spill mussels were transplanted to the oil-exposed shore. DNA adducts were not observed until 110 d after the spill, a delay thought to reflect the seasonal capacity of *M. edulis* to metabolize PAH to genotoxic metabolites. In fish, levels of DNA adducts were elevated in the shanny (*Lipophrys pholis*) along the impacted shoreline during 1996, but had returned to background levels 1 year after the spill. In dab (*Limanda limanda*), DNA adducts were elevated 4 months after the spill and remained elevated when sampled 12 months after the spill (Harvey *et al.*, 1999). Biomarker monitoring conducted every fourth month in 2003 and 2004 using wild mussels (*Mytilus galloprovincialis*) along the northern Iberian coastline detected the greatest degree of disturbance in coastal areas most impacted by the "Prestige" oil spill (Galicia, Spain, November 2002) and showed a recovery during 2004 related to a decrease in total PAH concentrations in mussel (Cajaraville *et al.*, 2006). Significantly lower biomarker activity (EROD and antioxidant enzymes) 2 and 3 years after the "Prestige" oil spill was detected in four-spotted megrim (*Lepidorhombus boscii*) and dragonet (*Callionymus lyra*) sampled from the most impacted areas along the northern Iberian shelf, indicating a decreasing level of exposure of the fish to residual hydrocarbons associated with the spill. The monitoring results also showed that the spatial extent of the "Prestige" oil spill had a differential impact on sublethal responses in fish from several offshore areas (Martínez-Gómez *et al.*, 2009). Biological-effects monitoring was conducted for 10 years after the "Braer" oil spill of March 1993, measuring the levels of EROD in the liver of dab sampled in the impacted area. Three years after the accident and on subsequent occasions, levels had fallen to those found at remote sites (Topping *et al.*, 1997). Interestingly, EROD activity and PAH metabolites measured 7–10 years after the "Exxon Valdez" oil spill of March 1989 were elevated in fish collected from sites originally oiled, compared with fish from unoiled sites (Jewett *et al.*, 2002) indicating a long recovery time for fish populations there.

Final considerations and proposals

Here, we have provided a guide to toxicity assessment and monitoring effects at lower levels of biological organization following marine oil spills within a structured, but flexible, framework. The batteries of biological-effects techniques (*in vitro*/*in vivo* bioassays and biomarkers) recommended in these guidelines are, in our opinion, the most practical and useful for use in managing oil spill situations and assessing the impact of marine oil spills.

For field surveys, the final selection of target species and assays (bioassays/biomarkers) from the whole range proposed here will require a case-by-case approach, depending on the ecosystem affected (offshore, coastal, estuaries, etc.), and the economic feasibility. In any case, their use will contribute to assessments of the toxicity hazard presented by the spill as well as the spatial and temporal extents of impact caused on organisms and the time to recovery. Their application can be used not only to determine the impact, but also to evaluate the effectiveness of any remediation measures. Robust conclusions can be obtained if the assessments are supported by the availability of pre-spill chemical and biological data, and if data collection of chronic-effect biomarkers is extended for a minimum of 5 years after the spill. Clearly, a monitoring programme using chemical measurements and biological techniques (short- and long-term biomarkers) should be initiated as soon as possible after an oil spill.

Quality control and assurance of chemical- and biological-effects measurements is achieved through the use of internal control procedures and by participating in inter-laboratory performance testing schemes at national or regional levels, such as those organized by the International Atomic Energy Agency (IAEA), Quality Assurance of Information for Marine Environmental Monitoring in Europe (QUASIMEME), the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM), and the Programme for the Assessment and Control of Pollution in the Mediterranean Region (MED POL).

Biomarker responses can offer reliable early warnings of potential adverse effects, but there remains only limited evidence that quantitatively links biomarker responses with adverse effects at higher levels of biological organization. However, such biomarkers of exposure provide an opportunity to assess whether intervention or further investigation is necessary. The direct assessment of ecological risk and population/community responses normally requires data from measurements made at these higher organizational levels. Determination of the effects of marine oil spills on marine populations and ecosystems implies the monitoring of functional and structural endpoints in populations and communities, which are beyond the scope of these guidelines. Clear knowledge gaps exist in understanding the complex effects that environmental contamination has on whole organisms and populations. Therefore, future work needs to focus on the integration of a range of types of monitoring data.

Experience obtained from previous incidents shows that it is desirable to develop a biomonitoring programme or to add

appropriate biological-effects methods to the existing programmes establishing pre-spill baseline data on relevant contaminants and biological-effects responses. These biomonitoring programmes will be especially useful in areas with economically important fisheries and/or sensitive environments that are located near major oil ports or with a high risk of shipping accidents (based on historical data). In this context, within the framework of their contingency plans, many EU countries have developed environmental sensitive index maps, or oil spill vulnerability atlases. It is important to act proactively to potential oil spill accidents by determining chemical and biological baseline levels in the various marine regions corresponding to the bodies of EU waters. Whenever possible, temporal monitoring to evaluate the time to recovery after the oil spill and/or the effectiveness of policy management measures should be undertaken within the framework of the existing monitoring programmes, such as those conducted under the OSPAR (JAMP/CEMP) and Barcelona Conventions (MED POL), coordinating the sampling and adding the appropriate biological-effects methods, to perform an integrated assessment, to avoid duplication, and to ensure cost-effectiveness. A tendency to develop and integrate chemical and biological data from routine national or regional biomonitoring programmes of the marine environment (HELCOM, OSPAR, MED POL) is growing. The existence of such integrated programmes makes it easier to conduct an assessment of any impact caused by an oil spill, because interpretation of the results is supported by the availability of chemical and biological pre-spill data. In addition, detection and interpretation of long-term biological impacts are enhanced by the continuous updating of knowledge.

For the major oil types transported in a given region, toxicological profiling/hazard identification using biomarkers/bioassays should be conducted to determine the responses to the given oil types (e.g. Nigerian light, Siberian light, Ekofisk, and Arabian light oils in EU waters). This would contribute proactively to the hazard assessment element of risk analyses after oil spills and help to optimize a biomonitoring programme for the particular type of oil. A central repository for information on the toxicological characteristics of various types of oil transported either in bulk or in the fuel bunkers of large vessels would be a valuable resource for managers and scientists seeking to develop appropriate responses to oil spills, often in situations where urgency is appropriate. Similarly, the creation of an archive of documents describing the responses that have been adopted, and their relative success, would permit rapid access to information and ensure that the best practice was adopted.

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