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Task 7.1.1b: Develop Protocols for Type of Monitoring and Seasonality

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PROTOCOL FOR SELECTION OF TYPE OF MONITORING AND SEASONALITY

Introduction

Monitoring involves the survey of a specific area during a certain period of time to establish a baseline situation or to detect alterations relative to a pre-existing baseline or to a predicted status (Hiscock, 1998). An appropriate monitoring plan should be selected after the careful consideration of the questions being addressed and taking into consideration the available resources. Scale and resolution, frequency and timing of sampling, and number and type of parameters to be measured, among other questions, should be considered.

Wherever possible, monitoring should be undertaken in a hypothesis test framework to disprove no change if testing for alterations (i.e. impact assessment studies), or to disprove change when seeking to demonstrate no alteration (i.e. baseline monitoring, surveillance). The obvious requirement of such schemes is repeated sampling. It is important to select the time of the year and the frequency of the survey so as to be suitable to attain the objectives of the study, and to repeat surveys at the same time each year to avoid confounding factors (e.g. seasonal changes and/or periodic natural cycles). For various purposes a single annual monitoring effort is enough to answer the questions posed. When seasonal patterns are to be described, monthly or bimonthly sampling is needed. A sampling frequency as low as four to six times a year would give some idea of the seasonal differences between years (Hill *et al.*, 1998). Good designs are of paramount importance to detect deviations from natural cycles: random sampling within seasonal periods (Underwood, 1997). While surveillance (condition monitoring) of the biotopes may be desirable at 3 to 5 year intervals, the high cost of monitoring may dictate that further surveys, and especially compliance monitoring, is undertaken only following changes in the area (Elliot *et al.*, 1998).

An appropriate sampling design and the performance of adequate analytical procedures and calibration methods should reduce natural variability within samples. Length stratified sampling may be maintained where it has been applied successfully in the past; however, for new time series, it may be more appropriate to sample with a view to minimising natural variability within a sample (OSPAR, 1997a). Numbers and size of individuals collected should be constant from year to year at each station, or they should at least fall in a narrow range. To reflect recent levels of contamination, young individuals should be chosen. Of course, samples should be representative of the studied populations. Existing data should be thoroughly examined where possible to reduce survey costs, and information obtained should be maximally used to minimise redundant or unnecessary sampling.

A quality assurance scheme (QA) should be planned to ensure data quality. All procedures must be evaluated and controlled on a regular basis. This includes regular intercalibration proficiency exercises within each laboratory and among laboratories, the use of reference materials and/or organisms, standard operation procedures (SOPs) and analytic quality control (AQC). To avoid contamination or loss of determinands during sampling, storage, pre-treatment and analysis, a QA scheme should be applied to the samples from the first contact to the final measurement and data reporting, following standard procedures (Anon, 1997a,b; HELCOM, 2000; ICES, 1987a, 1996, 1998a, 2002, 2004a,b; OECD, 1997; OSPAR, 1997a, 2002).

1. Sediment characterisation

Sediment is an important compartment when assessing environmental health since it may serve as a sink and secondary source for many persistent chemicals. Re-suspension caused by bioturbation, turbulence, storms, tides and/or anthropogenic activity is a means of releasing pollutants from sediments (Watanabe *et al*., 1997). Parameters such as pH, salinity, oxygen concentration, redox potential, organic carbon content and sediment mineral constituents dictate the chemical species present, the sorption behaviour and the bioavailability of many pollutants (Chapman and Wang, 2001).

In relation to spilled oil, some heavy refined products have densities greater than one and, therefore, they will sink in fresh or brackish water. However, seawater has a density of approximately 1.025 and only a few types of petrochemical products are dense enough or weather sufficiently so that their residues sink into the sediment. Sinking usually occurs due to the adhesion of particles or organic matter to the oil. Shallow waters are often laden with suspended solids providing favourable conditions for sedimentation. Oil stranded on sandy shorelines often becomes mixed with sediments. If this mixture is subsequently washed off the beach back into the sea, it may then sink to the bottom. In addition, if the oil catches fire after it has been spilled, the residues formed can be sufficiently dense to sink. Moreover, after treatment with dispersants, oil sinks to the bottom, contaminating the sediments and being more bioavailable to benthic communities. The Sediment Quality Guidelines (SQG; Long and McDonald, 1998) approach provides concurrent assessment of sediment contamination, community structure and health of individual species by using bioassays. SQG is used to differentiate sediment contaminant concentrations of little concern from those predicted to have adverse biological effects. Sediment is often heterogeneous in nature such that multiple sampling is necessary for the physic-chemical characterisation of an area. Separate samples may be pooled but this will remove any indication of structural differences within the site.

1.1. Visual assessment

A report of the condition of the sediment describing its colour, its physical consistency and texture should be performed. The presence of oil, tar residues or other alien materials should be noted.

1.2. Granulometry

Grain size should be analysed because it determines to a large measure the structure of the community present. In addition, oil penetration and persistence increases with sediment grain size. Spills of viscous heavy oils, such as some crudes and heavy fuel oils, if not cleaned up, may incorporate sand, gravel and stones and harden into relatively persistent asphalt pavements. Buried oil may persist for decades. A Procedural Guideline for a rapid particle size determination of sediments is provided in the present report (PG No. 1).

1.3. Total organic matter

Sediment organic matter is derived from biotic detritus and other natural and anthropogenic sources. Total organic carbon (TOC) dramatically increases during spills owing to the high carbon content of the hydrocarbons. TOC refers to the amount of organic matter preserved within the sediment and may be determined using PG No. 2. Organic matter in sediment consists of carbon and nutrients in the form of carbohydrates, proteins, fats and nucleic acids. Bacteria quickly degrade the less resistant molecules, such as the nucleic acids and many of the proteins.

1.4. Redox potential

Redox potential (Eh) measures the intensity of the reducing conditions in sediment. In general, the more negative the Eh, the lower the ability of the sediment to exchange electrons and, thus, there is an impairment of the chemical reactions vital to sustain life, being a limiting parameter for oil microbial degradation and bioremediation treatments (Bodennec *et al.*, 1987). Redox can also be used as an indicator of sulphites in sediments and anoxic conditions. A number of redox acceptors (i.e. Fe (III), nitrates, sulphate, manganese (IV)) can be utilised by bacteria to mediate the oxidation of hydrocarbons in the absence of oxygen;

however, the rates of degradation can significantly vary under different electron-accepting conditions (Krumholz *et al.*, 1996). Redox potential may range from –19 to –114 mV in polluted sediments and from 112 to 164 mV in unpolluted sediments. The Redox potential has been found to be negatively correlated with the amount of total petroleum hydrocarbon (TPH) content at four estuarine sites in the Galician coast, after the *Prestige* oil spill (Andrade *et al.*, 2004).

1.5. Oxygen

Formation of solid sediment–oil mixtures and clogging of pores prevents oxygen from entering into the sediment. Anaerobic biodegradation of oil is slower than aerobic degradation, but aerobic degradation rate declines only below 1% saturation (Berthe-Corti and Hopner, 2005).

1.6. Pigments

Pigments in the sediment provide useful information concerning epibenthic plant and microbial communities giving an estimation of primary productivity. Chlorophyll *a* provides an estimate of overall biomass, while carotenoid pigments provide taxonomic biomarkers characteristic of phytoplankton. High pigment and chlorophyll concentrations in areas with low oxygen levels indicate high eutrophication, hypoxia conditions or both. Bodin and Boucher (1982) found little quantitative effect on chlorophyll levels on the Brittany beaches oiled by the wreck of the *Amoco Cadiz*. Microphytobenthos and meiofauna generally exhibited normal seasonal fluctuations and, therefore, it is difficult to detect deviations from this normal fluctuation caused by oil spills. Because so many factors and interactions are involved, it is impossible to assign unambiguously the cause and effect in such uncontrolled observations. A procedural guideline on chlorophyll quantification in the sediments is provided in annex PG No. 3.

1.7. Other physic-chemical parameters

Physic-chemical parameters should be monitored because they may influence biological parameters, oil behaviour and weathering processes.

Temperature controls every physic-chemical reaction. It affects evaporation, which is the most important parameter in the weathering process in the first few days of an oil spill. The influence of temperature depends also on oil composition (Atlas, 1975). For light refined products such as gasoline, evaporation will remove 100% of the spill within a short period of time. For heavy refined products such as No. 6 fuel oil, or Bunker C, evaporation will only remove 5–10% of the spill (Michel *et al.*, 1992). In a slurry sediment system, hexadecane degradation follows the general temperature/reaction rate rule, the rate doubling every 10ºC rise in temperature, reaching zero at about 0ºC, but slowing at temperatures higher than 30ºC (Dalyan *et al*., 1990). Oil classifications are dynamic for spilled oils because weather conditions and water temperature greatly influence the behaviour of oil and refined petroleum products in the environment. For example, as volatiles evaporate from Class B oil, it may become Class C oil. If a significant temperature drop occurs (e.g. at night), a Class C oil may solidify and resemble a Class D oil. Upon warming, the Class D oil may revert back to Class C oil (U.S. EPA, 2006).

Dissolution and emulsification of oil are also controlled by physic-chemical variables. Solubilities of alkanes, cycloalkanes and aromatic hydrocarbons are about 70% lower in seawater than in freshwater (Michel *et al.*, 1992).

Light is also important since it determines the photodegradation of oil and its toxicity. For example, photoenhanced toxicity of spilled oil in the presence of ultraviolet light was described in the *Exxon Valdez* oil spill (Barron, 2000).

Some essential physic-chemical parameters to consider in monitoring programmes are:

- Temperature
- Salinity
- Dissolved oxygen
- Turbidity
- Ammonia
- Nitrites
- Nitrates
- Phosphates
- Chlorophylls
- Carotenoid pigments

2. Concentration of chemical agents in sediments, water and biota

To improve the power of a programme, samples for chemical analysis should be collected from areas characterised by low natural variability. In addition, the spatial representativeness of the area should be known. For the monitoring of metals and polycyclic aromatic hydrocarbons (PAHs) in water, pore water, sediments and suspended particulate matter, a large number of recommended procedures for sample collection, storage, extraction, analysis and quality control are available in publications and guidelines of international institutions (ICES, 1987a–d, 1990a,b, 1991a; Loring, 1991; OSPAR, 1989, 1995a, 1997a,b, 1998, 2001, 2002).

According to OSPAR (Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic) guidelines for contaminant monitoring in the biota, sampling should occur when specimens are in a more stable physiological state and in any case during a period before spawning. In the case of mussels, on Atlantic coasts gametogenesis and spawning generally occur from spring to early summer, when individuals may lose up to 50% of their soft tissue weight. The number of mussels collected should be at least three pools of 20 individuals per station and 50 individuals for spatial distribution sampling (OSPAR, 1997a). For fish, a single sex, preferably females, of 2–3 years should be collected. The number should be at least 25 individuals ±10% for length-stratified sampling or spatial distribution sampling, and at least 12 if individuals of a narrow size range interval are collected to minimise natural variability (OSPAR, 1997a). These parameters may be adjusted according to the biomarker being monitored. Sea bird eggs should be sampled during species-specific, year-specific and site-specific peaks of the first laying cycle during the year. This generally occurs in May/June. Only fresh eggs should be taken from full clutches. For each species, site and year, 10 eggs should be sampled with one egg taken randomly from each of the 10 clutches (OSPAR, 1997a). Other technical reports on sampling contaminants in the biota (ICES, 1991b,c, 1996; OSPAR, 1997d,j) and sediment preparation and analysis (ASTM, 2003a, 2005) are available.

Crude oil and its products are highly complex and variable mixtures; consequently, constituent-specific chemical analysis of these mixtures are challenging. The principal constituents are carbon and hydrogen, which in their combined form are hydrocarbons. In the refining process petroleum products are strongly enriched with hydrocarbons, leaving the most crude-based inorganic materials and other types of organic components containing sulphur, nitrogen and oxygen in the residual material. Petroleum hydrocarbons are divided into two families, aliphatics and aromatics. Aliphatics are further divided into three main classes, alkanes, alkenes and cycloalkanes. Alkynes are not commonly found in petroleum hydrocarbons (Potter and Simmons, 1998).

Aromatic molecules have ring structures and are basically flat and symmetric with clouds of electrons above and below the plane of the molecule. Aromatic carbon–carbon bonds are termed resonance bonds in that electrons are shared between multiple carbon atoms. In this sense, the electrons are “delocalised” (participating in several bonds). This imparts chemical stability. The bonding pattern of aromatic structures contributes to their moderate polarity. The electron clouds surrounding the molecules can be deformed by the charge on adjacent molecules. This results in the development of partial positive and negative charge sites on the molecule. Aromatic hydrocarbons have one or more benzene rings as structural components (Potter and Simmons, 1998).

Aliphatic structures have highly directional bonds, in which carbon atoms share electrons only with adjacent carbons. The molecules are essentially free to rotate around these bonds and thus the aliphatic structures can assume many different conformations. Aliphatics are non-polar or only slightly polar since their bonding pattern does not permit non-uniform distribution of charge to the same degree as the aromatic molecules. Alkanes contain only single carbon–carbon bonds. Alkenes are hydrocarbons that contain less hydrogen, carbon for carbon, than the corresponding alkane owing to the occurrence of one or more double bonds between carbon atoms in the alkene structure. An alkene is the unsaturated form of the corresponding

saturated alkane. Cycloalkanes are alkanes where carbon atoms form cyclic structures (Potter and Simmons, 1998).

The polarity of hydrocarbon structures governs the degree to which molecules interact with themselves and with water. Generally, as polarity increases, water solubility (i.e. the interaction with water) and boiling points increase. It follows that aromatics are more water soluble and less volatile than alkanes with a corresponding number of carbons. In general, the alkane fraction is the most biodegradable, whereas the polar fraction is resistant to biological degradation. The aromatic compounds, especially the PAHs, are of intermediate biodegradability. Trends in degradation rates according to structure are: (1) n-alkanes, especially in the C10 to C25 range, are degraded readily; (2) isoalkanes are degraded more slowly; (3) alkenes degrade more slowly than alkanes; (4) benzene, toluene, ethylbenzene and xylene (BTEX) are metabolised when present in concentrations that are not toxic to the microorganisms; (5) PAHs degrade more slowly than monoaromatics; and (6) degradation of higher molecular weight cycloalkanes may be very slow. These trends typically result in the depletion of the more readily degradable compounds and the accumulation of the most resistant in residues (Potter and Simmons, 1998).

Petroleum fuel mixtures are produced from crude oil through a variety of refining and blending processes. After treatment to remove dissolved gas, dirt and water, crude oil is distilled and a variety of petroleum product fractions result. The fractions can be used directly or their hydrocarbon composition can be altered through cracking and/or reforming. *Cracking* is a process that converts long-chain alkanes into smaller alkanes, alkenes and some hydrogen. It is this process which accounts for the occurrence of alkenes in petroleum fuel mixtures. Alkenes are not abundant in crude oil. *Reforming* is a process that converts aliphatics into aromatics. Composition of a distillation fraction can also be altered through treatment that involves removal or conversion of undesirable components, or addition of desired components (Potter and Simmons, 1998).

Organic compounds containing sulphur, nitrogen and oxygen may be encountered at significant concentrations in crude oil and in some heavier fuels such as No. 6 fuel oil. Sulphur-containing heterocyclic aromatic compounds are the major constituents. They are mainly in the form of thiophenes and thiophene derivatives. Nitrogen heterocyclics are present as derivatives of thiazole and quinoline, although they are present at much lower concentrations than the sulphur derivatives. Metals are also encountered in petroleum fuel mixtures in the form of salts of carboxylic acids, or as porphyrin chelates. A wide variety of metals are also present in crude oils and some are added to refined products to achieve desired properties.

2.1. Total hydrocarbon content (THC)

Quantification of THC in sediments is a method to estimate the degree of contamination of a spilled area (ASTM, 2006d). Records of THC taken seasonally enhance our ability to confirm the extent of pollution, especially by comparing with data from non-polluted areas or available baseline data. It is a useful parameter to indicate hydrocarbon contamination, but is of limited use in the assessment of health and ecological risk, since it refers to the concentration of a complex mixture of compounds that do no exhibit toxicological or fate and transport properties. THC may be expressed in terms of the concentrations of hydrocarbon compounds with various carbon ranges, e.g. C6 to C9 (New Zealand Ministry of the Environment, 1999). The Massachusetts Department of Environmental Protection (MDEP) adopted a health-based alternative to the TPH parameter, considering the information available regarding the toxicity and fate and transport of whole products (e.g. unleaded gasoline) and individual key components (e.g. benzene). The approach was based on developing specific acceptance criteria for the contaminants of primary concern (e.g. benzene, benzo(a)pyrene) and the development of acceptance criteria based on indicator chemicals for each of the TPH fractions considered. A surrogate chemical was assigned to each of the fractions considered and then each fraction assessed as if it were comprised entirely of the surrogate compound, e.g. the C6 to C9 fraction was assessed as if it were all n-hexane. In practice, this approach is conservative as the C7 to C9 compounds are less toxic than n-hexane. TPHCWG has another approach with the advantage of considering the properties of each range of

chemicals included in each fraction. The toxicological and fate and transport properties assumed for each of the fractions are then characterised.

2.2. Polycyclic aromatic hydrocarbons (PAHs)

PAHs are taken as an indicator of petroleum persistence since they weather more slowly than aliphatic hydrocarbons and are associated with chronic toxicity of petroleum in the environment. A conservative threshold measure of the potential of the PAH toxicity in the biota is the "effects range low" (ER-L) value of 4022 ppb for the total PAH (TPAH) concentration in sediment (Long *et al.,* 1995).

Aromatic hydrocarbons are the most toxic components of oil for the marine biota. Among them, those with lowest molecular weight (BTEX) present the highest acute toxicity and those with highest molecular weight the highest chronic toxicity. PAHs with low molecular weight are very volatile and disappear from the water within a few days after a spill. Intermediate molecular weight PAHs, such as those having two to four benzene rings, are more persistent and their solubility in water is high enough to cause toxicity to organisms living in the water column. Higher molecular weight PAHs are well-known pro-carcinogenic compounds. However, in general, they do not pose a short-term risk to water column organisms, but tend to accumulate and to biomagnify in the trophic chain. PAHs vary in their carcinogenic potencies; the more potent carcinogenic PAHs are 3-methylcolanthrene and 7,12-dimethyl-benz(a)anthracene (Klaassen, 2001).

The 16 PAHs prioritised by the U.S. Environmental Protection Agency (U.S. EPA) are:

- Naphthalene
- Acenaphthylene
- Acenaphthene
- Fluorene
- Phenanthrene
- Anthracene
- Fluoranthene
- Pyrene
- Benzo[a]anthracene
- Chrysene
- Benzo(b)fluoranthene
- Benzo(k)fluoranthene
- Benzo(a)pyrene
- Dibenzo(ah)anthracene
- Benzo(ghi)perylene
- Indeno(123-cd)pyrene

Procedures to analyse contaminants in the biota include homogenisation, drying, extraction with organic solvents, removal and destruction of lipids, clean-up, fractionation and gas chromatographic separation and electron capture or mass-spectrophotometric detection, and results should be reported on a wet weight basis (OSPAR, 1999). The International Council for the Exploitation of the Seas and Helsinki Commission (ICES/HELCOM) guidelines do not request data to be reported on a wet weight basis, but the fat content has to be reported (ICES, 2002). Other standard methods are available for PAH analysis in water, pore water, other aqueous matrices and sediments (ASTM, 1998a, 2006a,b,d; ICES, 1991a; OSPAR, 1998), as well as for quantification of complex PAH mixtures or petroleum oils in water (ASTM, 2000).

2.3. BTEX

BTEX are abundant components in spilled petroleum and gasoline. BTEX are non-polar monoaromatic organic compounds that have non-specific toxicity, causing non-specific narcosis in tissues above certain levels, acting particularly on lipidic membranes, leading to alterations of permeability especially in the gills (Meyerhoff, 1975; Morrow *et al*., 1975). Moreover, benzene is confirmed to be carcinogenic to humans and presents danger of absorption through the skin; toluene also presents danger of absorption through the skin (Klaassen, 2001).

BTEX compounds have a moderate affinity for partitioning into lipid tissues of aquatic organisms and to sorption to organic matter (values of log K_{ow}, the octanol–water partition

coefficient, range from 2.13 to 3.2). BTEX are the most concerning monoaromatic hydrocarbons in the environment due to their toxicity, although they are not persistent in seawater and have a low potential for bioaccumulation. Predicted LC50, the median lethal concentration, for benzene is 48 mg/l and that for *m*-xylene is ~7.2 mg/l, according to the regression relationship between aquatic organisms and the physical/chemical properties of non-polar organic chemicals with non-specific toxicity of narcosis (McCarthy *et al*., 1992).

The main removal mechanisms for BTEX from seawater are evaporation, adsorption to particles and sedimentation, biodegradation and photolysis. Volatilisation is quantitatively the most important process.

2.4. Metals

Vanadium (Va), nickel (Ni), chromium (Cr), copper (Cu), lead (Pb), zinc (Zn), barium (Ba), arsenic (As), iron (Fe), cadmium (Cd), manganese (Mn) and molybdenum (Mo) are, in general, components of petrochemical products, although Va and Ni are predominant, occurring at highest concentration in crude oil and residual fuel oils. Altered levels have been observed after oil spill contamination or around oil platforms and drilling mud deposits. Cr, Ni, Cu, Fe, Pb and Va contents of polluted sediments affected by the *Prestige* oil spill were 2 to 2,500 times higher than in unpolluted sediments and the background concentrations of Galician coastal sediments. In the cases of Cr, Cu, Ni, Pb and V, their origin in the polluting oil was corroborated by the high correlation ($r \geq 0.74$) between the concentrations of these metals and the TPH content of the polluted sediments (Andrade *et al*., 2004). A high concentration of Zn was also detected in water samples collected over the place where the *Prestige* sank. These samples contained levels of Zn two to three orders of magnitude higher than in uncontaminated water (CSIC, 2003).

Analyses of trace metals in the biota generally include homogenisation, drying decomposition, dissolution, matrix separation and detection using element-specific spectrometric instrumental procedures (i.e. Atomic absorption spectrophotometry – AAS, Inductively-coupled plasma – ICP, X-ray fluorescence technology – XRF, Neutron activation analysis – NAA). The results should be reported on a wet weight basis. It is important to also report dry weight (OSPAR, 1997a). There is a technical report from ICES concerning the determination of Ca concentration in organic matrices using electrothermal furnace atomic absorption spectrophotometry (ICES, 1987d). Other guidelines related to metals analyses in sediments are available from ASTM (2001, 2003b, 2006c).

2.5. Blending agents and additives

The blending agents and additives used for various petroleum fuel mixtures are shown in table 1. Of these, only lead, barium, methyl-ter t-butylether (MTBE) and ethylene dibromide (EDB) are likely to be detected in environmental samples. Analytical methods for these substances are well developed and are routinely performed in environmental laboratories (Potter and Simmons, 1998).

Gasoline	
Type	**Compound**
Anti-knock	2,2,4-trimethylpentane
	tetraethyl lead
	tetramethyl lead
	ter t-butyl alcohol
	methyl-ter t-butylether
Anti-oxidants	ortho-alkylated phenols
	p-phenylenediamine
	aminophenols
	2,6-di-ter t-butyl-p-cresol
Metal activators	N,N-disalicylidene-1,2-diaminopropane
Lead scavengers	ethylene dibromide
	ethylene dichloride
Anti-rust agents	fatty acid amines
	sulphonates
Anti-icing	agents alcohols
	glycols
	amides
	amines
	organophosphate salts
Upper-cylinder lubricants	cycloalkane distillates
Detergents	aminohydroxy amide
Dyes	alkyl derivatives of azobenzene-4-azo-2-naphthol
	benzene-azo-2-naphthol
	p-diethylaminoazobenzene
	1,4-di-isopropylaminoanthraquinone

Diesel	
Type	**Compound**
Ignition improvers	alkyl nitrates, alkyl nitrites
	nitro-, nitroso- compounds, peroxides
Combustion catalysts/ deposit modifiers	organometallics of Ba, Ca, Mn, Fe
	Mn, MnO
	Mg, MgO, MgO_2
	Al_2O_3
Anti-oxidants	N,N-dialkylphenylenediamines
	2,6-dialkyphenols, 2,4,6-trialkylphenols
Cold flow improvers	ethylene vinyl acetate copolymers
	ethylene vinyl chloride copolymers
	polyolefins
	chlorinated hydrocarbons
Metal deactivators	N,N-disalicylidene-alkyldiamines
	detergents/ alcohols
	dispersants amines
	alkylphenols
	carboxylic acids
	sulphonates
	succinamides
JP-4 fuel	
Anti-oxidants	alkyl phenols
	N,N-di-sec-butyl-p-phenylenediamine
Metal deactivators	N,N-di-disalicylidene-butyl-p-propanediamine
	N,N-disalicylidene-1,2-cyclohexanediamine
	N,N-disalicylidene-1,2-ethanediamine
Icing inhibitors	carboxylates
	alcohols
	dimethylformamide
	ammonium dinonylnaphthalene

Table 1. Types of blending agents and additives for petroleum fuel mixtures (from Potter and Simmons, 1998).

3. Biomonitoring

Monitoring the effects of environmental contaminants on the biota is an important element in monitoring programmes since it can demonstrate links between contamination and effects at several levels of biological organisation, according to the approach chosen and the measured parameters. In the last few decades, biomonitoring based on parameters commonly known as biomarkers has been widely used.

Biomarkers are measurements in body fluids, cells or tissues indicating molecular, biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response (NRC, 1987). In an environmental context, biomarkers offer promise as sensitive indicators, demonstrating that toxicants have entered organisms, and have been distributed between tissues, eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990).

The use of biomarkers involves the necessity of a better knowledge of their seasonal and natural variability. It is fundamental to define the range of normal values in sentinel species before implementing the use of these biomarkers in field studies since other factors, apart from toxic concentration, such as temperature, season or sexual hormones, can also modulate their response.

Biomarkers have been used to detect harmful effects caused by oil spills. For example, biomarker responses were measured in the liver of two demersal fish species (*Lepidorhombus boscii* and *Callionymus lyra*) from the northern Iberian shelf five months after the *Prestige* oil spill. The biomarkers selected were 7-ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), glutathione reductase (GR), catalase (CAT) and DNA integrity. Interspecies differences and spatial variations in biomarker responses were observed along the shelf. GST, GR and CAT activities were significantly elevated in *L. boscii* in the most oil-impacted area (Finisterre) and positively correlated with tar aggregate densities. The lack of previous data from the area together with the existence of chronic background pollution of the shelf implies that the observed biomarker responses cannot be solely attributed to the petroleum hydrocarbon components of the spilled oil (Martínez-Gómez *et al*., 2006), highlighting the need for baseline monitoring programmes.

Pollutants may damage organisms with lethal consequences or cause sub-lethal effects on the organism at various levels, i.e. biochemical, sub-cellular, cellular, organs and whole organism. Effects may also be at higher levels of biological organisation (i.e. population, community, ecosystems). In the following part of this section, some biomarkers and bioindicators that have been commonly used in biomonitoring studies will be described, giving special emphasis to those that have been used in oil spill biomonitoring.

3.1. Functional effects on individual organisms

When contaminants enter into an organism they and/or their metabolites may cause a variety of effects on functions that are determinant for the survival and/or performance of the individuals. Below, a list of biomarkers that have been shown to be altered by components of petrochemical products is given, grouping them by physiological functions.

3.1.1. Nervous system

Some contaminants interfere with nervous system function, causing direct effects on it and indirect effects on several other physiological functions such as breathing, feeding, behaviour and reproduction. Acetylcholinesterase (AChE) is an enzyme belonging to the family of cholinesterases (ChEs), which has a determinant role in the transmission of nervous impulses across cholinergic synapses of both vertebrates and invertebrates, being responsible for the degradation of the neurotransmitter acetylcholine (PG No. 4). In the presence of inhibitors of this enzyme, acetylcholine is not degraded and accumulates in the synapse, causing the continuous stimulation of cholinergic receptors located in the pos-synaptic membrane, processes that may cause death. In addition to AChE, ChEs also include pseudocholinesterase or butyrylcholinesterase, which is a less specialised enzyme that is responsible for the degradation of some xenobiotics and that seems to have a protective role towards the function of cholinergic synapses by binding anticholinesterase agents and

decreasing the number of them able to reach AChE. With exception of the nervous system, both enzymes usually occur in the tissues of animals. Owing to their sensitivity to both organophosphorous (OP) and carbamate (CB) pesticides, ChEs have been used as a specific biomarker for these compounds. However, ChEs are also sensitive to other environmental contaminants such as some metals, surfactants, petroleum-derived products and undetermined components of complex mixtures of pollutants (Cunha *et al*., 2005; Frasco *et al*., 2005; Galgani *et al.*, 1992; Guilhermino *et al*., 1998, 2000; Labrot *et al*., 1996; Moreira *et al*., 2004; Payne *et al.*, 1996) and a more general use for this biomarker has been proposed. They may be determined in blood and used in a non-destructive way or in other tissues, such as brain and muscle.

3.1.2. Detoxification

After entering in the organism, lipophilic xenobiotics are metabolised in more water-soluble compounds. This process is usually called biotransformation and might occur by different ways depending on the properties of the initial compound. Biotransformation of a considerable number of such xenobiotics occurs in two phases: phase I and phase II of biotransformation. Phase I enzymatic reactions add a functional group to the parent compound or expose an already existing one, allowing the resulting metabolite(s), which is (are) in general more water soluble, to be eliminated or to enter into phase II. One of the most important enzymatic systems of phase I is the monooxygenase system of the cytochrome P450 (P450). Phase I biotransformation may also be performed by esterases, amilases, epoxide hydrolase and other enzymes or enzymatic systems. In phase II, the parent compound or its metabolites produced in phase I are conjugated with endogenous molecules (e.g. glutathione or UDP-glucoronic acid) producing water-soluble conjugates that can be more easily eliminated from the organism. These reactions require energy and are carried out by enzymes such as GSTs, glucuronyl transferases, sulphotransferases and others. Organisms also have mechanisms for dealing with non-lipophilic compounds. For example, some endogenous molecules such as albumin and metallothioneins may bind to some inorganic metals decreasing their amount in blood circulation, promoting their storage in specific organs or facilitating their elimination.

P450 1A induction in fish liver has been associated with exposure to several hydrophobic organic compounds such as some PAHs, but also with polychlorinated biphenyls (PCBs), dioxins, benzofurans, pesticides and various drugs (Antunes *et al*., 2005; Ferreira *et al*., 2004, 2006; Frasco and Guilhermino, 2002). Nevertheless, one cannot always expect to observe a linear dose–response relationship between the concentration of certain chemical and CYP1A content or activity in the natural environment, where a mixture of both inducers and inhibitors may act simultaneously (Pluta, 1993). Fish collected after oil spills often show increases in cytochrome P450 system activity, content and bile fluorescent aromatic compounds (FAC) that are correlated with exposure to PAHs in the oil. There is also some evidence for increases in bile FAC and induction of cytochrome P450 in marine birds and mammals after oil spills. However, increases are transitory and generally they return to background levels within 1 year after the exposure. Laboratory studies have shown induction of the cytochrome P450 system after exposure of fish to oils in water, sediment or food. Most of the PAH found in crude oil (dominantly 2- and 3-ring PAHs) are not strong inducers of cytochrome P450. Exposure to the 4-ring chrysenes or the photo-oxidised products of the PAH may account for cytochrome P450 responses in fish collected from oil-spill sites (Lee and Anderson, 2005). Three biomarkers of hydrocarbon exposure, CYP1A in the liver, liver ethoxyresorufin O-deethylase (EROD) and biliary FACs, were examined in the near-shore fish, masked greenling (*Hexagrammos octogrammus*) and crescent gunnel (*Pholis laeta*), collected in Prince William Sound, Alaska, 7–10 years after the Exxon Valdez oil spill. All biomarkers were elevated in fish from sites originally oiled, in comparison to fish from unoiled sites (Jewett *et al*., 2002). In invertebrates, the activity of CYP1A is generally very low and this makes it difficult to measure induction levels. EROD catalytic enzyme activity assay (PG No. 5) is the technique recommended for monitoring CYP1A activity (OSPAR, 1997i). Other methodological techniques are also available from the International Council for the Exploration of the Sea (ICES, 1991d, 1998b). In biological tissues, PAHs are difficult to determine using traditional chemical analysis because they are rapidly metabolised in the liver and excreted into the gall bladder, where they are temporarily stored. Thus, the determination of bile PAH metabolites by Fixed Wavelength Fluorescence can be an alternative and

sensitive method for screening PAH contamination in fish (PG No. 6) (Ferreira *et al*., 2006). This fluorimetric procedure is simple, requiring little amounts of bile, and may be performed with common laboratory equipment. More sophisticated techniques such as that recommended by OSPAR (1997d) couple high-precision liquid chromatography (HPLC) and fluorimetric procedures. A review of methods for bile metabolite quantification was published by ICES (2005).

GSTs are a well-known family of iso-enzymes, involved in the detoxification of both xenobiotics and endogenous substances with electrophilic centres, by catalysing their conjugation with glutathione (PG No. 7). Over the last few decades, they have been widely used as effect criteria in toxicity assays and as biomarkers in different scenarios of environmental contamination, including contamination by petrochemical products which have been shown to induce GSTs of several species (Cheung *et al*., 2001; Cunha *et al*., 2005; Ferreira *et al*., 2006; Gowland *et al*., 2002; Moreira *et al*., 2004; Moreira and Guilhermino, 2005). The activity of GSTs may also be altered (induced or inhibited) by exposure to other types of environmental contaminants such as metals (Almeida *et al*., 2004; Canesi *et al*., 1999; Mosleh *et al*., 2006; Regoli and Principato, 1995; Tang *et al*., 1996), pesticides (Frasco and Guilhermino, 2002) and pharmaceuticals (Nunes *et al*., 2006). GSTs also play an important role in lipid peroxidation processes.

Stress proteins (e.g. heat shock proteins, metallothioneins) comprise a set of abundant and inducible proteins involved in the protection and repair of the cell against stress and harmful conditions (Sanders, 1993). A special group of stress proteins are metallothioneins that are present in the tissue of both vertebrates and invertebrates and have an important role in the control of levels of several metals, including both essential (such as Cu and Zn) and non-essential ions (e.g. Cd and Hg). The OSPAR Commission for the Protection of the Marine Environment of the North-East Atlantic recommends three methods for their determination: an immunochemical method (RIA/ELISA) (Hogstrand and Haux, 1990), a pulse polarography technique (Olafson and Sim, 1979) and a spectrophotometric method (Viarengo *et al*., 1997). There is also a technical report from ICES describing methods for measuring these proteins (ICES, 1999a).

Anti-oxidative stress parameters and lipid peroxidation: several environmental compounds, including metals and other components of petrochemical products, may induce oxidative stress through the formation of reactive oxygen species (ROS) in the cell. When the ROS levels surpass the capability that the anti-oxidative stress defences can cope with, several toxic effects may be induced including lipid peroxidation of cell membranes, cell death and DNA alterations. Anti-oxidant enzymes are responsible for reducing active ROS, superoxide (O_2^-) and hydrogen peroxide (H_2O_2) and for recycling glutathione. Lipids and especially polyunsaturated fatty acids are abundant in marine organisms and highly prone to oxidative attack by ROS, metals and other compounds, giving origin to lipid peroxidation.

Several of these parameters, including the enzymes superoxide dismutase (SOD – PG No. 8), catalase (CAT – PG No. 9), glutathione peroxidases (GPx – PG No. 10), glutathione reductase (GR – PG No. 11), oxidised/reduced glutathione ratio, lipid peroxidation (LPO – PG No.12) have been widely used in ecotoxicology to investigate the effects of several environmental contaminants, including petrochemical products and their components, under both laboratory and field conditions (Ferreira *et al*., 2006; Figueiredo-Fernandes *et al.,* 2006; Moreira *et al*., 2006; Nunes *et al*., 2004, 2006).

3.1.3 Genotoxicity

The exposure of organisms to genotoxic chemicals may induce cascade events (Shugart *et al*., 1992): structural alterations in DNA, procession of DNA damage and subsequent expression in mutant gene products, and disease (i.e. cancer) resulting from genetic damage. The detection and quantification of various events in this sequence may be employed as biomarkers of exposure and effects in organisms exposed to genotoxic substances in the environment. In the literature, there are examples of the use of such parameters for the evaluation of genotoxicity after exposure to petrochemical products. For example, the measurement of DNA adduct levels in selected invertebrate and vertebrate species after the *Sea Empress* oil spill indicated no effects on two invertebrate species (*Halichondria panicea*

and *Mytilus edulis*) but induction of DNA adducts was found in teleost fish (*Lipophrys pholis*, *Pleuronectes platessa* and *Limanda limanda)* (Harvey *et al*., 1999). Data obtained 12–17 months after the spill suggested that the affected species recovered from the oil contamination. While the studies indicate that the genetic impact of the oil contamination was less severe than might have been expected, it remains possible that the DNA adducts detected in the teleosts could lead to genetic changes in these species in the future (Harvey *et al*., 1999). The most widely used technique for quantification of DNA adducts is the P-32 post labelling technique, owing to its high sensitivity, the requirement of small amounts of DNA and its ability to detect carcinogenic DNA adducts of unknown structure (ICES, 1999b; Micael *et al.*, in press; OSPAR, 1997d).

3.1.4. Energy production

In hypoxia or under chemical stress, animals may need additional energy in a short period of time, increasing the use of the anaerobic pathway for energy production (Diamantino *et al*., 2001). The increase of lactate dehydrogenase (LDH) activity, which is the enzyme responsible for the inter-conversion of pyruvate to lactate in glycolysis (PG No. 13), has been used as a biomarker indicative of an increase of anaerobic energy production under chemical stress (Castro *et al.*, 2004; De Coen *et al.*, 2006; Guilhermino *et al*., 1994; Moreira *et al*., 2006; Nunes *et al*., 2004). However, toxicants may also induce a decrease of LDH activity, for example, by binding to the enzymatic molecule and inactivating it, and by blocking the enzyme synthesis (Mishra and Shukla, 1997). Dispersants seem to have an effect on LDH activity that was significantly stimulated in the liver of fish exposed to dispersed crude oil water accommodation fraction (WAF), compared to the crude oil WAF alone (Cohen *et al*., 2001). After the *Exxon Valdez* oil spill, LDH in the blood of guillemots was significantly higher in birds from oiled sites versus those from unoiled sites (Golet *et al*., 2002).

3.1.5. Disruption of the haem biosynthesis – ALAD

There are only two currently available biomarkers that are regarded as specific for metal contamination: metallothioneins in the liver and δ-aminolevulinic acid dehydratase (ALAD) inhibition in blood (specific for lead). Lead (Pb) causes a dose-dependent inhibition of ALAD, which is an enzyme essential for the synthesis of haemoglobin in the haemopoetic tissue, although Fe is also known to affect this enzyme. OSPAR (1997j) and ICES (2004c) propose a spectrophotometric method for ALAD activity quantification.

3.1.6. Cellular integrity

Lysosomes are very important cellular organelles, as they accumulate substances such as metals and organic chemicals. When the amount of these substances exceeds the lysosomes' capability to accumulate them, the membranes of these organelles are greatly affected leading to the release of their content in hydrolytic enzymes into the cytoplasm of the cell, which causes severe cell damage. Lysosomal stability has been so widely applied and validated as a measure of cellular integrity that it has been indicated by OSPAR/JAMP (Joint Assessment and Monitoring Programme) for use as an indicator of general health in national monitoring programmes (OSPAR, 1997c). Neutral red retention (NRR) time is an assay to measure the lysosomal integrity, and thus haemocyte condition, reflecting the exposure of animals to contaminants (PG No. 14). However, several factors may interfere with the technical performance of this assay. For example, results may present a high variability if measured in organisms at different stages of the reproductive cycle, especially during the spawning season (Castro *et al.*, 2004; Galloway *et al.*, 2004).

The combination of three biomarker techniques is recommended by OSPAR to describe the impact of PAHs on biota at the biochemical level (OSPAR, 1997d): CYP1A activity, bulky aromatic-DNA adducts and PAH metabolites in bile. PAHs induce CYP1A activity that then transforms these lipophilic compounds to more water-soluble metabolites, towards excretion or phase II detoxification. Some metabolites that result from this biotransformation are reactive epoxides that may form DNA and protein adducts, linked to mutagenesis and carcinogenesis. Determination of PAH metabolites in the bile are not indicative of biological effects but can provide a sensitive marker of exposure to the bioavailable levels of these xenobiotics in the environment. Histopathological examination of the liver is subsequently

recommended to give additional information on the deleterious effects of PAH exposure (OSPAR, 1997g,h).

3.2. Toxicity assays

The relation between the quantity of a chemical to which an organism is exposed and the nature of consequent harmful (toxic) effects is of central importance in ecotoxicology (Walker *et al*., 2001). Dose–response relationships provide the basis for assessment of hazards and risks presented by environmental chemicals. An effort has been made towards standardising both biological and analytical methods used to produce acute toxicity estimates of complex mixtures, such as oil, dispersants and dispersed oil. This standardisation will provide guidelines so that future investigations can be conducted in a sufficiently rigorous manner in order for both inter- and intra-laboratory datasets to be comparable, thus providing a more coherent and robust database from which to derive response guidance (Singer *et al*., 2000).

Toxicity testing methods vary with the objective of the study. In general, they are divided into laboratory bioassays, which are performed under controlled laboratory conditions, and *in situ* assays, which are performed in real scenarios. Both testing methods may be acute if the tested organisms are exposed to high concentrations or doses of the test substance (or mixture) for a short period of time relative to their lifecycle, or chronic if the organisms are exposed to low concentrations or doses of the toxicant(s) for a long period of time relative to their lifecycle. Acute toxicity testings with microalgae are used to assess the effects in primary production. The indicative toxicity parameter is the inhibition of microalgae growth and the result of the assay is expressed as the IC50 value, i.e. the concentration of the toxicant that inhibits 50% of algae growth under the specific conditions of the test. In toxicity assays with animals, the most frequently used toxicity parameter is death and the result of the assay is expressed as LC50, i.e. the concentration of the toxicant that causes the death of 50% of the population tested under the experimental conditions used. However, there is a growing interest in the use of more sophisticated indices (Walker *et al*., 2001) and other effect criteria, such as feeding inhibition, biomarkers and behavioural alterations. In this case, the result of the assay is expressed as EC50, i.e. the concentration of the toxicant causing a 50% inhibition or induction of the selected endpoint. In chronic bioassays with animals, the effect criteria most frequently used are alterations in reproduction and growth. Other endpoints that have been used are, feeding inhibition, biomarkers and behavioural alterations. Here, toxicological endpoints are the no observed effect concentration (NOEC), the lowest observed concentration (LOEC) and the EC50. Individual growth and reproduction are key parameters for the evolution of populations. For example, age and reproductive output of females may be used to calculate population parameters (e.g. population intrinsic growth rate, *r*) and to predict the evolution of the population.

In Europe, the most frequently used toxicity assays are standardised laboratory bioassays (acute and chronic) following OECD guidelines (http://www.oecd.org/) for testing chemicals, which are required by European and national legislations. They are performed with organisms considered representative of different trophic levels of the ecosystem (e.g. producers, primary consumers, secondary consumers). *In situ* assays are very convenient procedures owing to their high ecological relevance (e.g. they eliminate the extrapolation from laboratory conditions to field conditions, they eliminate the potential alterations of water and sediment samples during the transport, and they may be performed with local species, therefore eliminating the extrapolation incertitude related to differences of sensitivity between laboratory and autochthonous organisms). However, they are more expensive and usually they require a higher human effort, thus, they are not performed routinely. Various other standard methodologies are available for determining lethal and sub-lethal effects of contaminants in water and sediments (ASTM, 1990a,b, 1998b–f; ICES, 1991e, 2001b,c; OECD, 1985, 1992a,b, 1998, 2000; OSPAR, 1995a; U.S. EPA, 1994, 1998). Both laboratory and *in situ* assays may be incorporated into monitoring programs providing useful information about the toxicity of water and/or sediments to the biota. Ideally, they should be performed with local organisms and, for comparison purposes, laboratory assays with standard species may also be needed. Of course, to fulfil legislation requirements or legal issues, standard laboratory bioassays should be performed according to accepted guidelines.

In addition to the previously mentioned assays, *in vitro* tests (e.g. with fish cell lines) have also been used. However, since in general they are less sensitive, and they do not consider the effects that occur in the organism as a whole or the biotransformation of toxicants, they have a limited use in ecotoxicology. However, they are important for investigating specific questions and, in certain situations, they may be used as screening assays.

Following the general concern about the use of animals in research, education and experimentation, in ecotoxicology there has also been a tendency for the use of non-invasive techniques and "lower animals". The European Centre for the Validation of Alternative Methods (ECVAM) has been set up, with its main objective being to promote the scientific and regulatory acceptance of alternative methods based on the **3 R's** concept: **R**eduction of the number of animals used in experimentation; **R**eplacement of the current methods by alternatives not using animals; and **R**efinement of the techniques used in order to decrease the pain and stress of the animals used in experiments.

3.3. Effects on populations

When pollutants enter into an ecosystem, populations may be affected in different ways: (i) the number of individuals may decline and become extinct; (ii) the number of individuals may decrease but a new equilibrium level may be attained, so that the population is able to survive in the contaminated environment; or (iii) the population size increases reaching an equilibrium at a higher density because it was able to develop resistance to pollution (Walker *et al*., 2001) and it beneficiates from the reduction of competition owing to the elimination of more sensitive species. These effects may be investigated using quantitative genetics, population dynamics and ecotoxicological approaches. However, it is desirable to detect early signs of potential harmful effects in a precocious phase because this will allow the adoption of mitigation measures before severe effects are induced. Here, the use of biomarkers combined with ecologically relevant parameters (at individual or population levels) may be of great value.

3.3.1. Pelagic and demersal fish

Information on the effects of oil on pelagic communities is very sparse, partly because the dilution factor in open oceans is thought to rapidly reduce toxic concentrations that may be present under an oil spill. Adult fish do not generally experience acute mortalities after an oil spill, especially in open water environments (Scholz *et al.,* 1992) where they can move to less contaminated areas. After the *Amoco Cadiz* and *Exxon Valdez* oil spills, demersal rockfish species were the only fish found dead in significant numbers (Gundlach *et al*., 1983; Khan and Nag, 1993). Other long-term direct or indirect effects on fisheries have also been reported in the case of the *Exxon Valdez*, namely a crash of pacific herring (*Clupea pallasi*; Stokesbury, 1999) and pink salmon (*Oncorhynchus gorbuscha*), whose populations had fallen to very low levels four years after the spill, prompting questions about the long-term effects on ecosystems (Cooney and Allen, 1999). Significant reductions in the abundance of Norway lobster, *Plesionika heterocarpus*, and four-spot megrim were detected in the *Prestige* oil spill maximum impact area, located over the Galician shelf (Sánchez *et al*., 2006). However, it is worth noting that recoveries were observed in the 2004 abundance indices of four-spot megrim and *Plesionika.* On the other hand, no significant effects were detected in the abundance or distribution of hake juveniles even though the tar aggregates were formed by the same oceanographic drift events that normally cause the drift of eggs and larvae from spawning sites to the nursery areas of the Cantabrian Sea during the winter (Navidad current) (Sánchez *et al*., 2006). Feeding patterns of the species analysed did not present apparent modifications (Sánchez *et al*., 2006). Also, a decrease in the densities of several epibenthic indicators was detected the first year after the spill, followed by a noteworthy recovery in 2004. Non-macroscopic toxicity and some oceanographic agents have been suggested as possible causes of these shifts (Serrano *et al.*, 2005).

Different types of gear can be used for sampling fish depending on the scope of the monitoring programme (i.e. qualitative, semi-quantitative and quantitative) and the fish species to be sampled (i.e. pelagic or bottom fish). There is also apparatus to perform acoustic surveys. Estimations of fish abundance and biomass may also be derived from commercial fisheries. Recommended procedures for field operations are available in

publications from international institutions (FAO, 1973, 1975, 1977, 1980, 1981 1982, 1983; ICES, 1991f, 1999c).

3.3.2. Plankton

Effects on planktonic communities are difficult to document, because effects of oil must be separated from the high natural variability and seasonality found. Phytoplankton is generally less sensitive to the effects of oil than zooplankton, with different species having different sensitivities. Phytoplankton communities determine the production of upper trophic levels and it seems that factors affecting the duration, distribution and composition of phytoplankton blooms will produce a bottom-up control of food web dynamics (McRoy *et al*., 1999). After the *Tasman Spirit* oil spill (Pakistan), phytoplankton groups usually inhabiting Manora Channel during July to September were either absent or were rarely present, sometimes having slight cell deformations; moreover, light penetration was negligible and photosynthetic activity was substantially reduced (OCHA/UNEP, 2003). Changes in phytoplankton variables were studied during spring 2003, after the *Prestige* shipwreck, using historical data available for the area. Some minor changes were occasionally observed, but they did not show any clear pattern, and seemed to be related to the natural variability of the ecosystem. The phytoplankton community structure did not change. The higher abundances of a few species were related to the high temperatures measured during 2003 that resulted in an advance of summer-like conditions to spring (Varela *et al*., 2005). Above 50 ng g^{-1}, there is a progressive decrease in photosynthetic activity in algal cultures. There are direct (OSPAR, 1997e) and indirect (ICES, 1987e, 2001a; OSPAR, 1997f) standard methods available for studying phytoplankton abundance and diversity.

Some studies have reported an increase in primary productivity after an oil spill, but it was not clearly demonstrated whether this was caused by a stimulation of photosynthesis or a decrease in zooplankton grazing caused by oil (Johansson *et al*., 1980; Laännergren, 1978). After the *Tsesis* oil spill in Sweden (1977), zooplankton biomass felt dramatically for about 5 days, followed by an increase in phytoplankton and productivity in the next period. Zooplankton and diatoms play a key role in removing both oil particles and emulsions from surface waters. Weathered oil, after loss of volatile and water-soluble components, is not very toxic and copepods have been observed to ingest oil particles and pass them through the gut with no apparent harm (Nuzzi, 1973). Many foraging fish (sand lance, herring, capelin, etc.) feed on zooplankton, on which man, other marine mammals, many seabirds, jellyfish and other fish and marine predators depend for food, and therefore dramatic changes to zooplankton biomass may have effects at higher levels of the food web. After the *Torrey Canyon* spill, 50–90% of the eggs of pilchard were dead, and larvae were rare in plankton samples collected off Cornwall (Smith, 1968). There has also been concern that larval fish, which often concentrate at the ocean surface, may be adversely affected by floating oil, either by toxicity or entrapment (Nelson-Smith, 1972). Oil reduced the hatching rate of fertilised capelin eggs at concentrations of 10–25 ng l^{-1}. Suitable techniques for quantitative and qualitative zooplankton surveys, including ichtyoplankton, include towed net sampling and bottom fixed nets or traps. Recommended procedures for field operations, as well as laboratory procedures, are available in several publications (e.g. Brander and Thomson, 1989, Diéach, 2000; FAO, 1967, 1974; ICES, 2000; Smith and Richardson, 1977; UNESCO, 1968, 1975; Wiebe *et al*., 1985).

Increases in bacterioplankton abundance due to oil spills have been reported in field and mesocosms studies (Johansson *et al*., 1980; Lee and Fuhrman, 1987). In coastal waters of La Coruña, no abundance or increased activity of bacterioplankton could be related to THC increase one year after the *Prestige* oil spill (Bode *et al*., 2005).

3.3.3. Marine mammals

These animals are not particularly abundant along most coastal zones, but concern was expressed for the sea lions and elephant seals of Santa Barbara's Channel Islands, and for migrating endangered grey whales. Marine mammals may be affected in several ways by oil spills. The bodies of sea otters may become covered by oil, which causes a build-up of oil in the otters' air bubbles. These air bubbles are located in the fur and help them to survive the cold oceans acting like a covering for their body and helping them to float. When oil builds up

in the air bubbles, the otters may die due to low body temperature. Oil spills are also dangerous for whales since oil may be eaten or enter the whale's blowhole when whales rise up over the water to take a breath. The blowhole then becomes plugged with oil, impairing breathing. Also, when whales eat fish that swim through the oil, they engulf oil along with the fish. The number of harbour seals (*Phoca vitulina*) in Prince William Sound has decreased by about one-quarter relative to the 1975 population, and the food web dynamics has been investigated as a possible factor (Hirons and Schell, 1999). River otters (*Lutra canadensis*) were reported to be suffering from chronic intoxication from the *Exxon Valdez* oil spill, despite signals of recovery being present in 1997 (Bowyer *et al.*, 1999). Signs of acute respiratory distress were reported in moulting grey seals hauled out on Lady's Helm, Shetland, following the *Braer* oil spill, with the proportion of animals exhibiting a discharge of nasal mucus being significantly higher than the proportion at a reference site. The proportion of animals affected increased for up to one month following the spill; however, without sufficient baseline data on the occurrence of respiratory distress in grey seals it is difficult to determine the proportion attributed to other causes (Hall *et al*., 1996). Controlled laboratory experiments with minks, *Mustela vison,* show that Alaskan North Slope crude oil or bunker C fuel oil exposure alters circulating leukocyte numbers, erythrocyte homeostasis, hepatic metabolism and adrenal physiology (Mazet *et al*., 2000). In another experiment, hepatic cytochrome P4501A1 mRNA was elevated in the fuel oil exposed group (Schwartz *et al*., 2004).

3.3.4. Birds

Seabirds are strongly affected by oil spills. They may be covered by the oil, so that the feathers clump and they are unable to fly. They also eat oil as they clean their feathers and become poisoned, frequently showing inflammation of the digestive tract. The small feather barbules are dishevelled, allowing a disruption of the insulation and buoyancy of the feathers. Birds may sink and drown or may rapidly lose body heat and succumb to cold and several diseases, including pneumonia. Loss of heat induces a very high compensatory metabolic rate that, together with cessation of feeding, may lead to accelerated starvation and death (Boesch *et al.*, 1974).

The most susceptible species are those that are attracted by the slick and land on it. Diving birds that remain submerged for several minutes may surface under oil slicks. In Europe, auks, razorbills, puffins, guillemots and some sea ducks have been especially hard hit by oil spills (Boesch *et al*., 1974). Because of the migratory behaviour of some species, a large proportion of a bird species may often be concentrated in a small area; oil pollution could thus gravely reduce populations. These zones have to be carefully indicated on each country's contingency plans.

In order to quantify bird injury it is necessary to access the numbers and species of oiled birds, the condition of those birds (e.g. dead, alive, entered for rehabilitation, state of decomposition and scavenging) and the sampling effort for the area. However, these estimates often do not include dead birds not reaching the shore. It has been estimated that only 5–15% of those birds killed by oil are actually washed onto the shore (Nelson-Smith, 1972). Nonetheless, 40,000 to 100,000 birds were reported killed by the *Torrey Canyon* oil spill (Smith, 1968), and over 30,000 carcasses were recovered after the *Exxon Valdez* spill (Piatt *et al.*, 1990). There are methodologies available for bird contamination monitoring (Elliott *et al*., 1992; OSPAR, 1997a, 1999; Peakall *et al*., 1986), including some for oiled birds (OSPAR, 1995b).

3.4. Effects on community structure

Oil spills may have dramatic impacts on the structure of marine communities. This impact may be due to the oil itself or due to the cleaning techniques applied (e.g. dispersants, scraping) that sometimes end up being as deleterious as the spilled oil itself or even more severe. Recovery of the community structure and dynamics depends on its resilience and susceptibility to oil. An impact on a particular species can potentially affect the structure of the whole community. This is particularly likely when key species are affected. Animals that are good recruiters and more resistant to contamination rapidly recolonise the bare space created by the oil spill and the subsequent clean-up. Those shores that show the greatest degree of natural fluctuation are easily perturbed and are most likely to suffer the greatest impact from

anthropogenic factors. This creates the problem of detecting anthropogenic effects against a background of biologically induced fluctuations (i.e. competition for space, grazing, predation, recruitment variations).

3.4.1. Intertidal rocky shore

The littoral zone is the most assessable marine habitat. Rocky shore monitoring provides a cheap and efficient means of assessing the condition of coastal ecosystems. Oil spills may have a dramatic acute impact on the shore, which may take years to recover. The effects of removing most of the biota are profound and long lasting. In the case of the *Torrey Canyon* disaster where dispersants were applied, the time scale of recovery seemed to be at least 10 years. If limpet population structure and barnacle densities are used as criteria, then 15 years may be more realistic. In contrast, on a shore that was substantially untreated, recovery was almost complete within three years (Hill *et al.*, 1998).

A good time of the year to monitor rocky shores is between late summer and autumn, by which time the cohort of most species will be settled. However, in general, several sampling periods throughout the year should be selected. A general qualitative description of the shore, including biological (number of zones present, shore levels occupied, dominant space-occupying organisms in each zone) and physical factors (expose/sheltered, bedrock/boulder, size of boulders, sloping/highly broken, rock type), will be useful. Among the qualitative methods, photographs and video will also provide an instant record of the gross features of the shore community (Hill *et al.*, 1998).

All numerical methods aim to provide a description of the abundance of certain species at different positions on the shore. Monitoring aims can often be achieved by restricting surveillance to a number of important species (Lewis, 1976). This species should be easy to identify and play a key role in the community; in this case, the abundance of these species should affect and be affected by the abundance of other species. Recommended species are those that dominate available space such as macroalgae, barnacles and mussels, grazers such as limpets and important predators. It should be possible to assess the abundance of 15 to 30 species in a single shore visit. The higher the number of species sampled, the higher the detail but the fewer the sites sampled per unit of effort. Therefore, since available resources are usually limited, a compromise must be achieved. The key species approach is less time consuming than producing a detailed inventory of all the species present; however, it will not provide information on species richness (Hill *et al.*, 1998).

There are qualitative and quantitative methods for assessing and recording the abundance of chosen species (Crisp and Southward, 1958). Quantitative methods are more suitable for detailed monitoring or impact assessment, being more amenable to statistical analysis. Often time, budget or the characteristics of the zone to be monitored limit the choice of the method to be used. The actual numbers of each species or the area it covers on the bedrocks can be recorded with quadrats. On boulder zones, the use of quadrats is not always possible and a timed search is often used. Personnel with experience on species identification should be able to search an area of 20 m^2 in 15 min. This method is subject to between observer variations and at best provides an index of abundance rather than an absolute measure (Hill *et al.*, 1998). Other methods are available in ecology textbooks.

3.4.2. Intertidal sand and mudflats

Oil spills can cause large-scale deterioration of communities in intertidal systems. Sheltered, low-energy areas such as mudflats, in enclosed bays or estuaries, will be more susceptible to oil spills due to low dispersion rate, in contrast to the higher energy sedimentary biotopes. Oil covering intertidal mud prevents oxygen transport to the substratum and produces anoxia resulting in the death of infauna, whereas tidal-pulsing pushes oil into intertidal sand (Elliot *et al.*,1998). In both biotopes, the change will favour the development of opportunistic communities such as the polychaetes *Capitella capitata,* in sand banks, and *Manayunkia aesturina,* in mudflats. Oil pushed into coarse sand will destabilise the sediment and may produce an oxygen demand in the surface layers and a reduction of degradation processes. In grossly polluted environments, the sediment is defaunated and may be recovered by sulphur-reducing bacteria such as *Beggiatoa* spp. (Majeed, 1987; McLusky, 1982). The

release of a refinery effluent to an intertidal mudflat will result in anoxic sediments, and a degraded infaunal community and change in predator–prey relationship through the possible decrease in the palatability of the prey, impairing the functioning of that marine area (Elliot and Griffiths, 1988). Degraded petroleum hydrocarbons may also cause organic matter enrichment.

In order to determine the community structure of the biotope, intertidal sand and mudflats will require core sampling together with quadrat sampling. Core sampling is required to extract the infauna for identification and enumeration, and surface quadrat sampling will allow surface features to be quantified. Biological samples should be collected concurrently with physic-chemical samples, at the same time and location, otherwise interpretation of biological data will be invalid or not possible. In most areas, macrofauna will provide the best assessment of the biological nature of the biotope and so key macrofaunal species that are indicative of the area should be monitored (Elliot *et al.*, 1998).

3.4.3. Subtidal sand banks

Subtidal sand banks are less at risk from oil spills unless dispersants are used or wave action allows sediment mobility and thus oil to be incorporated into the sediments. Eelgrass beds are of special interest because of their high habitat value for marine organisms, including nursery areas. Other important biotopes of important conservational importance, such as seapen and burrowing megafauna communities and maerl beds, should be given particular importance.

Macrofauna quantitative substratum samples can be obtained by lowering equipment, such as grabs or cores, from vessels. The Van Veen or Day grabs and the Hamon or Shipek grabs are used to collect samples of coarse and compacted sediments, whereas the Craib and Knudson cores are used to collect samples of soft sediment. The Reineck box corer and Forster anchor dredge are used for semi-quantitative and quantitative sampling of mobile megafauna. In areas where the epifauna and demersal fish are important components, small beam trawls fitted with tickler chains can be towed for a fixed time, for example 20 min, to give a semi-quantitative estimation (Elliot *et al*., 1998). For meiofauna (benthos from 1 mm to 0.1 mm long), sample sub-sampling or smaller area instruments ought to be used, since they occur at higher densities. They require cores from 2 to 4 cm in diameter, depending on the nature of the sediment. ICES has produced a technical document on collection, treatment and quality assurance of soft-bottom macrofauna samples (ICES, 1999c).

3.4.4. Subtidal rock

Subtidal rock habitats comprise a wide range of sub-features from the upper infralittoral to the deep circalittoral zone. They are very variable in form and in the types of communities they support. Untreated oil does not present a risk for these communities, particularly for circalittoral ones that are protected by their depth. Infralittoral rock with kelp and the extensive benthic and pelagic communities they support are also rarely impacted on by oil spills, except for those in the very upper infralittoral fringe, where kelp fronds often float on the surface and may become oiled. Spilled oil and clean-up techniques do not seem to cause acute effects on kelp plants nor does the oil stick to fronds, but their physiology may be adversely affected (JNCC, 2004).

Due to the difficulty and cost of sampling on these habitats, the emphasis should be placed on assessing those biotopes with greater conservational value (e.g. *Sabellaria spinulosa* reefs, *Modiolus modiolus* beds, brittlestar beds). However, these biotopes may be clumped disproportionately in a small section of a larger area and it would be important to include biotopes and sampling locations on the remaining areas (JNCC, 2004). Ideally, a mapping or inventory study of the biotopes should be undertaken. Possible methods for studying community structures on subtidal rocky habitats are *in situ* survey by quadrats using diving techniques, drop-down video recording, *in situ* hand-held video, towed sledge video and still photography.

4. Bibliography

Almeida, E.A., Miyamoto, S., Bainy, A.C.D., Medeiros, M.H. and Mascio, P. (2004). Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. *Mar. Poll. Bull.* **49**, 386–392.

Andrade, M.L., Covelo, E.F., Veja, F.A. and Marcet, P. (2004). Effect of the *Prestige* Oil Spill on Salt Marsh Soils on the Coast of Galicia (Northwestern Spain). *J. Environ. Qual.* **33**, 2103–2110.

Anon (1997a). Quality assurance of fieldwork. *Tema Nord Environment 1997:5955.* Nordic Council of Ministers.

Anon (1997b). Quality assurance in environmental monitoring. A guidance document. *Tema Nord Environment 1997:591*. Nordic Council of Ministers.

Antunes, P., Ferreira, M., Gil, O. And Reis-Henriques, M.A. (2005). Effect of PCBs in selected metabolic enzymes in juvenile and adult sea bass (*Dicentrarchus labrax*). *Proceedings of ECOTOX 2005 – Adv. Trends Ecotox*, 5–7 September, Brno, Czech Republic.

ASTM, American Society for Testing and Materials (1990a). Standard guide for conducting solid phase 10-day static sediment toxicity tests with marine and estuarine infaunal amphipods. *Procedure E 1367-90*. American Society for Testing and Materials, Philadelphia, PA, pp. 1–24.

ASTM, American Society for Testing and Materials (1990b). Standard guide for conducting static 96-hour toxicity tests with microalgae. *Procedure E 1218-90*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (1996). Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. *Standard E 729-96*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998a). Standard test method for Polynuclear Aromatic Hydrocarbons in water. *Standard D 4657-92*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998b). Standard guide for conducting sediment toxicity test with marine and estuarine Polychaetous Annelids. *Standard E 1611*, in *Annual Book of Standards*, Vol. 11.05. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998c). Standard guide for determination of bioaccumulation of sediment-associated contaminants by benthic invertebrates. *Standard E 1688-97a*, in *Annual Book of Standards*, Vol. 11.05. American Society for Testing Materials Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998d). Standard guide for designing biological tests with sediments. *Standard E 1525-94a*, in *Annual Book of Standards*, Vol. 11.05. American Society for Testing Materials Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998e). Standard guide for conducting static sediment toxicity tests with marine and estuarine amphipods. *Standard E 1367-92*, in *Annual Book of Standards*, Vol. 11.05. American Society for Testing Materials Philadelphia, PA.

ASTM, American Society for Testing and Materials (1998f). Standard guide for collection, storage, characterization and manipulation of sediments for toxicological testing. *Standard E*

1391-94, in *Annual Book of Standards*, Vol. 11.05. American Society for Testing Materials Philadelphia, PA.

ASTM, American Society for Testing and Materials (2000). Standard test method for quantification of complex Polycyclic Aromatic Hydrocarbon mixtures or petroleum oils in water. *Standard 5412*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2001). Standard practice for total digestion of sediment samples for chemical analysis of various metals. *Standard D 4698-92*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2003a). Standard practice for development and use (preparation) of samples for collaborative testing of methods for analysis of sediments. *Standard D 3975-93.* American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2003b). Standard practices for extraction of trace elements from sediments. *Standard D 3974-8.* American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2005). Standard practice for preparation of sediment samples for chemical analysis. *Standard D 3976-92*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2006a). Standard test method for pore water analysis of poly-aromatic hydrocarbons (PAHs) using solid phase micro-extraction method. *Standard WK 10122*. American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2006b). Standard test method for determining Polynuclear Aromatic Hydrocarbons in aqueous environmental matrices using UPLC technology. *Standard WK 6647.* American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2006c). Standard practice for acid-extraction of elements from sediments using closed vessel microwave heating. *Standard D 5258-02.* American Society for Testing Materials, Philadelphia, PA.

ASTM, American Society for Testing and Materials (2006d). Standard practice for solvent extraction of total petroleum hydrocarbons from soils and sediments using closed vessel microwave heating. *Standard D 5765-05*. American Society for Testing Materials, Philadelphia, PA.

Atlas, R.M. (1975). Effects of temperature and crude oil composition on petroleum biodegradation. *Appl. Microbiol.* **30**, 396–403.

Bard, S.M., Woodin, B. and Stegeman, J.J. (1998). Induction of the multixenobiotic resistance transporter and cytochrome P450 1A in intertidal fish exposed to environmental contaminants. *Toxicol. Sci.* **42**, 16–26.

Barron, M.G. (2000). Potential for photoenhanced toxicity of spilled oil in the Prince William Sound and Gulf of Alaska Waters. *Final Report*, prepared for Prince William Sound Regional Citizen's Council, Alaska, 29pp.

Berthe-Corti, L. and Hopner, T. (2005). Geo-biological aspects of coastal oil pollution. *Palaeogeo. Palaeoclimat. Palaeoecol.* **219**, 171–189.

Bodin, P. and Boucher, D. (1982). Evolution à moyen-terme du meiobenthos et du microphytobenthos sur quelques plages touchée par la maree noire de 1'Amoco Cadiz, in Gundlach, E.R. and Marchand, M. (eds.), *Ecological Study of the Amoco Cadiz Oil Spill: Report of the NOAA-CNEXO Joint Scientific Commission, Brest, France*, pp. 245–268.

Bohgal, N., Gridon, C., Combes, R. and Balls, M. (2005). Toxicity testing: creating a revolution based on new technologies. *Trends Biotech*. **23**, 299–307.

Bode, A., González, N., Lorenzo, J., Valencia, J., Varela, M. and Varela, M.M. (2005). Bacterioplankton activity off A Coruña (Galicia, NW Spain) after the "Prestige" oil spill in November 2002. *ICES CM 2005/S:29*.

Bodennec, G., Desmarquest, J.P., Jansen, B. and Kantin, R. (1987). Evolution of hydrocarbons and bacterial activity in the marine sediments contaminated by crude oil overflow and treated. *Int. J. Environ. Anal. Chem*. **29**,153–78.

Boesch, H.M., Hershner, A.F. and Milgram, N.J. (1974). Effects of oil on marine ecosystems, in *Oil Spills and the Marine Environment: Papers Prepared for the Energy Policy Project of the Ford Foundation*. Ballinger Publishing Company, United States, 115pp.

Bowyer, R.T., Blundell, G.M., Duffy, L.K., Jewett, S.C. and Ben-David, M. (1999). Chronic effect of the Exxon Valdez Spill in river otters: is recovery complete? *Proceedings of the Symposium on Legacy of an Oil Spill, 10 Years after Exxon Valdez*, 23–26 March, University of Alaska, United States.

Brander, K. and Thomson, A.B. (1989). Diel difference in avoidance of three vertical profile sampling gears by herring larvae. *J. Plankt. Res.* **11**, 775–784.

Cajaraville, M.P., Bebbiano, M.J., Blásco, J., Porte, C., Sarasquete, C. and Vaiarengo, V. (2000). The use of biomarker to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *Sci. Tot. Environ.* **247**, 295–311.

Canesi, L., Viarengo, A., Leonzio, C., Filippelli, M. and Gallo G. (1999). Heavy metals and glutathione metabolism in mussel tissues. *Aquat. Toxicol*. **46**, 67–76.

Castro, B.B., Sobral, O., Guilhermino, L. and Ribeiro, R. (2004). An *in situ* bioassay integrating individual and biochemical responses using small fish species. *Ecotoxicology* **13**(7), 667–681.

Castro, E., Santos, M.M., Monteiro, N. and Vieira, N. (2004). Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. *Marine Environ. Res.* **58**(2-5), 739–743.

Chapman, P., Dexter, R.N. and Long, E.R. (1987). Synoptic measures of sediment contamination, toxicity and infaunal community composition (the sediment quality triad) in San Francisco Bay. *Mar. Ecol. Prog. Ser.* **37**, 75–96.

Chapman, P. and Wang, F.Y. (2001). Assessing sediment contamination in estuaries. *Environ. Toxicol. Chem.* **20**, 3–22.

Cheung, C., Zheng, G., Li, A., Richardson, B. and Lam, P. (2001). Relationships between tissue concentrations of polycyclic aromatic hydrocarbons and antioxidative responses of marine mussels, *Perna viridis*. *Aquat. Toxicol.* **52**,189–203.

Cohen, A., Nugegoda, D. and Gagnon, M.M. (2001). Metabolic responses of fish following exposure to two different oil spill remediation techniques. *Ecotox. Environ. Saf.* **48**, 306–310.

Cooney, R.T. and Allen, J.R. (1999). Sound ecosystem assessment (SEA): ecological controls of pink salmon and herring production in Prince Williams Sound. In *Proceedings of the Symposium on Legacy of an Oil Spill, 10 Years after Exxon Valdez*, 23–26 March, University of Alaska, United States.

Crisp, D.J. and Southward, A.J. (1958). The distribution of intertidal organisms along the coasts of the English Channel. *J. Mar. Biol. Ass. UK* **37**, 157–208.

CSIC, Consejo Superior de Investigaciones Científicas (2003). Presencia de metales pesados en la zona de hundimiento del petrolero Prestige y composición de metales y complejantes del fuel emulsionado de la costa. *Informe Tecnico CSIC "Prestige" 2.*

Cunha, I., García, L.M. and Guilhermino, L. (2005). Sea-urchin (*Paracentrotus lividus*) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. *J. Environ. Monit*. **7**, 1–7.

Dalyan, U., Harder, H. and Hopner, Th. (1990). Hydrocarbon biodegradation in sediments and soils. A systematic examination of physical and chemical conditions – Part III. Temperature. *Erdol und Kohle. Erdgas. Petrochemie* **43**, 435–437.

De Coen, W., Robbens, J. and Janssen, C. (2006). Ecological impact assessment of metallurgic effluents using in situ biomarker assays. *Environ. Pollut.* **141**, 283–294.

Diamantino, T., Almeida, E., Carvalho, A.M.V.M. and Guilhermino, L. (2001). Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. *Chemosphere* **45**, 553–560.

Diéach, L. (2000). Ichtyoplankton sampling, in Goñi, R., Harmelin-Vivien, M., Badalementi, F., Diéarch, L. and Bernard, G. (eds.), *Introductory Guide to Methods for Selected Ecological Studies in Marine Reserves*. GIS Posidonie Publ., France, pp. 23–27.

Elliott, M. and Griffiths, A.H. (1988). Contamination and effects of hydrocarbons on the Forth ecosystem. *Proc. Roy. Soc. Edin.* **93B**, 327–342.

Elliot, M., Nedwell, S., Jones, N.V., Read, S.J., Cutts, N.D. and Hemingway, K.L. (1998). Intertidal sand and mudflats and subtidal mobile sandbanks (Vol. II). *An Overview of Dynamic and Sensitivity Characteristics for Conservation Management of Marine SACs.* Scottish Association for Marine Science (UK Marine SACs Project), 151pp.

Elliott, J.E., Scheuhammer, A.M., Leighton, F.A. and Pearce, P.A. (1992). Heavy metal and metallothionein concentrations in Atlantic Canadian seabirds. *Arch. Environ. Contam. Toxicol*. **22**, 63–73.

FAO, Food and Agriculture Organization (1967). Rapport du groupe de travaile sur méthods de récolte des formes larvaires. *Rapport Pêche 37.*

FAO, Food and Agriculture Organization (1973). Manual of methods for fisheries resource survey and appraisal. Part 3. Standard methods and techniques for demersal fisheries resource surveys, by Mackett, D.J. *FAO Fish. Tech. Pap.* **124**, 39pp.

FAO, Food and Agriculture Organization (1974). International training course on fish eggs and larval studies, La Jolla, CA, 2–29 September 1973. FAO/ MARMAP. *FAO Fish. Tech. Report* **144**, 18pp.

FAO, Food and Agriculture Organization (1975). Manual of methods for fisheries resource survey and appraisal. Part 5. Objectives and basic methods, by Gulland, J.A. *FAO Fish. Tech. Pap.* **145**, 29pp.

FAO, Food and Agriculture Organization (1977). Methods of measuring stock abundance other than by the use of commercial catch and effort data, by Ulltang, O. *FAO Fish. Tech. Pap.* **176**, 23pp.

FAO, Food and Agriculture Organization (1980). Guidelines for sampling fish in inland waters, Backiel, T. and Welcomme, R.L. (eds.). *EIFAC Tech. Pap.* **33**, 176pp.

FAO, Food and Agriculture Organization (1981). A manual on acoustic surveys. Sampling methods for acoustic surveys, Bazigos G.P. (ed.). *CECAF/ECAF Ser*. **80/17**, 137pp.

FAO, Food and Agriculture Organization (1982). Introduction to the use of sonar systems for estimating fish bio mass, by Burczynski, J. *FAO Fish. Tech. Pap.* **191**(Rev 1), 89pp.

FAO, Food and Agriculture Organization (1983). Fisheries acoustics. A practical manual for aquatic biomass estimation, by Johannesson, K.A. and Mitson, R.B. *FAO Fish. Tech. Pap.* **240**, 249pp.

Figueiredo-Fernandes, A., Fontainhas-Fernandes, A., Peixoto, F., Rocha, E. and Reis-Henriques, M.A. (2006). Effects of gender and temperature on oxidative stress enzymes in Nile tilapia Oreochromis niloticus exposed to paraquat. *Pest. Biochem. Physiol.* ***85***, 97–103.

Ferreira, M., Antunes, P., Gil, O., Vale, C. and Reis-Henriques, M.A. (2004). Organochlorine contaminants in flounder (*Platichthys flesus*) and mullet (*Mugil cephalus*) from Douro estuary, and their use as bioindicator species for environmental monitoring. *Aquat. Toxicol*. **69**(4), 347–357.

Ferreira, M., Moradas-Ferreira, P. and Reis-Henriques, M.A. (2006). The effect of long-term depuration on phase I and phase II biotransformation in mullets (*Mugil cephalus*) chronically exposed to pollutants in River Douro Estuary, Portugal. *Mar. Environ. Res.* **61**(3), 326–338.

Frasco, M.F., Fournier, D., Carvalho, F. and Guilhermino, L. (2005). Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. *Biomarkers* **10**, 360–375.

Frasco, M.F. and Guilhermino, L. (2002). Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. *Fish Physiol. Biochem.* **26**(2), 149–156.

Galgani, F., Bocquené, G. and Cadiou, Y. (1992). Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North Sea. *Mar. Ecol. Progr. Ser.* **91**, 77–82.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B. and Depledge, M.H. (2004). Ecosystem management bioindicators: the ECOMAN project – a multibiomarker approach to ecosystem management. *Mar. Environ. Manag.* **58**, 233–237.

Golet, G.H., Seiser, P.E., McGuire, A.D., Roby, D.D., Fischer, J.B., Kuletz, K.J., Irons, D.B., Dean, T.A., Jewett, S.C. and Newman, S.H. (2002). Long-term direct and indirect effects of the 'Exxon Valdez' oil spill on pigeon guillemots in Prince William Sound, Alaska. *Mar. Ecol. Prog. Ser.* **241**, 287–302.

Gowland, B., McIntosh, A., Davies, I., Moffat, C. and Webster, L. (2002). Implications from a field study regarding the relationship between polycyclic aromatic hydrocarbons and glutathione S-transferase activity in mussels. *Mar. Environ. Res.* **54**, 231–235.

Guilhermino, L., Barros, P., Silva, M.C. and Soares, A.M.V.M. (1998). Should use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? *Biomarkers* **3**, 157–163.

Guilhermino, L., Celeste Lopes, M., Donato, A.M., Silveira, L., Carvalho, A. and Soares, A.M.V.M. (1994). Comparative study between the toxicity of 3,4-dichloroaniline and sodium bromide with 21-days chronic test and using lactate dehydrogenase activity of Daphnia magna Straus. *Chemosphere* **28**(11), 2021–2027.

Guilhermino, L., Lacerda, M.N., Nogueira, A.J.A. and Soares, A.M.V.M. (2000). *In vitro* and *in vivo* inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. *Sci. Total Environ.* **247**, 137–141.

Gundlach, E.R., Boehm, P.D., Marchand, M., Atlas, R.M., Ward, D.M. and Wolfe, D.A. (1983). The fate of Amoco Cadiz oil. *Science* **221**, 122–129.

Hall, A.J., Watkins, J. and Hiby, L. (1996). The impact of the 1993 Braer oil spill on grey seals in Shetland. *Sci. Total Environ.* **186**, 119–125.

Harvey, J.S., Lyons, B.P., Page, T.S., Stewart, C. and Parry, J.M. (1999). An assessment of the genotoxic impact of the Sea Empress oil spill by the measurement of DNA adducts levels in selected invertebrate and vertebrate species. *Mut. Res.* **44**, 103–114.

HELCOM, Baltic Marine Environment Protection Commission (2000). *Manual for Marine Monitoring in the Combine Programme of HELCOM. Part B: General Guidelines on Quality Assurance for Monitoring in the Baltic Sea.*

Hill, S., Burrow, M.T. and Hawkins, S. (1998). Intertidal reef biotopes. *UK Marine SACs Project,* Vol. 4.

Hirons, A.C. and Schell, D.M. (1999). A perspective on harbour seal trophic interactions in Prince Williams Sound and the Gulf of Alaska. *Proceedings of the Symposium on Legacy of an Oil Spill, 10 Years after Exxon Valdez,* 23–26 March, University Alaska, United States.

Hiscock, K. (1998). *Biological Monitoring of Marine Special Areas of Conservation: a Handbook of Methods for Detecting Change. Part 1. Review and Description of Methods. Joint* Nature Conservation Committee, Peterborough, UK.

Hogstrand, C. and Haux, C. (1990). A radioimmunoassay for perch (*Perca fluviatilis*) metallothionein. *Toxicol. Appl. Pharmacol*. **103**, 56–65.

ICES, International Council for the Exploration of the Sea (1987a). Control procedures: good laboratory practice and quality assurance, by Vijverberg, F.A.J.M. and Cofino, W.P. *ICES Tech. Mar. Environ. Sci.* **6**, 33pp.

ICES, International Council for the Exploration of the Sea (1987b). Cadmium in marine sediments: determination by graphite furnace atomic absorption spectroscopy, by Rantala, R.T.T. and Loring, D.H. *ICES Tech. Mar. Environ. Sci.* **3**, 9pp.

ICES, International Council for the Exploration of the Sea (1987c). Trace metals in seawater: sampling and storage methods, by Yeats, P.A. *ICES Tech. Mar. Environ. Sci*. **2**.

ICES, International Council for the Exploration of the Sea (1987d). Cadmium and lead: determination in organic matrices with electrothermal furnace atomic absorption spectrophotometry, by Harms, U. *ICES Tech. Mar. Environ. Sci.* **1**, 10pp.

ICES, International Council for the Exploration of the Sea (1987e). Primary production: guidelines for measurement by 14C incorporation, by Richardson, K. *ICES Tech. Mar. Environ. Sci.* **5**, 21pp.

ICES, International Council for the Exploration of the Sea (1990a). Sediments and suspended particulate matter: total and partial methods of digestion, by Loring, D.H. and Rantala, R.T.T. *ICES Tech. Mar. Environ. Sci.* **9**, 14pp.

ICES, International Council for the Exploration of the Sea (1990b). Suspended particulate matter: collection methods for gravimetric and trace metal analysis, by Yeats, P.A. and Brügemann, L. *ICES Tech. Mar. Environ. Sci.* **7**.

ICES, International Council for the Exploration of the Sea (1991a). Hydrocarbons: review of methods for analyses in seawater, biota and sediments, by Erhardt, M., Klungsøyr, J. and Law, R.J. *ICES Tech. Mar. Environ. Sci.* **12**.

ICES, International Council for the Exploration of the Sea (1991b). Temporal trend monitoring: introduction to the study of contaminant levels in marine biota, by Uthe, J.F., Chou, C.L., Misra, R.K., Yeates, P.A., Loring, D.H., Musial, C.J. and Cofino, W. *ICES Tech. Mar. Environ. Sci.* **14**, 18pp.

ICES, International Council for the Exploration of the Sea (1991c). Temporal trend monitoring: contaminant levels in tissues of Atlantic cod, by Uthe, J.F., Misra, R.K., Chou, C.L., Scott, D.P. and Musial, C.J. *ICES Tech. Mar. Environ. Sci.* **15**, 11pp.

ICES, International Council for the Exploration of the Sea (1991d). Biological effects of contaminants: microplate method for measurement of ethoxyresorufin-O-deethylase (EROD) in fish, by Galgani, F. and Payne, J.F. *ICES Tech. Mar. Environ. Sci.* **13**, 11pp.

ICES, International Council for the Exploration of the Sea (1991e). Biological effects of contaminants: oyster (*Crassostrea gigas*) embryo bioassay, by Thain, J.E. *ICES Tech. Mar. Environ. Sci.* **11**, 12pp.

ICES, International Council for the Exploration of the Sea (1991f). Benthic communities: use in monitoring point source discharges, by Rees, H.L., Heip, C., Vincx, M. and Parker, M.M. *ICES Tech. Mar. Environ. Sci.* **16**, 77pp.

ICES, International Council for the Exploration of the Sea (1996). Contaminants in marine organisms: pooling strategies for monitoring mean concentrations, by Nicholson, M.D. and Fryer, R.J. *ICES Tech. Mar. Environ. Sci.* **18**, 30pp.

ICES, International Council for the Exploration of the Sea (1998a). Temporal trends monitoring: robust method for analysing contaminant trends monitoring data, by Nicholson, M.D. and Fryer, R.J. *ICES Tech Mar Environ Sci* **20**, 22pp.

ICES, International Council for the Exploration of the Sea (1998b). Biological effects of contaminants: determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity, by Stagg, R. and McIntosh, A. *ICES Tech. Mar. Environ. Sci.* **23**, 16pp.

ICES, International Council for the Exploration of the Sea (1998c). Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds, by Bocquené, G. and Galgani, F. *ICES Tech. Mar. Environ. Sci.* **22**, 12pp.

ICES, International Council for the Exploration of the Sea (1999a). Biological effects of contaminants: quantification of metallothionein (MT) in fish liver tissue, by Hylland, K. *ICES Tech. Mar. Environ. Sci.* **26**, 18pp.

ICES, International Council for the Exploration of the Sea (1999b). Biological effects of contaminants: measurement of DNA adducts in fish by 32P-post-labelling, by Reichert, W.L., French, B.L. and Stein, J.E. *ICES Tech. Mar. Environ. Sci.* **25**, 45pp.

ICES, International Council for the Exploration of the Sea (1999c). Soft-bottom macrofauna: collection, treatment, and quality assurance of samples, by Rumohr, H. *ICES Tech. Mar. Environ. Sci.* **27**.

ICES, International Council for the Exploration of the Sea (2000). *Ices Zooplankton Methodology Manual*, Harris, R., Wiebe, P., Lenz, J., Skjoldal, J. and Huntley, M. (eds.). Academic Press, 683pp.

ICES, International Council for the Exploration of the Sea (2001a). Chlorophyll a: determination by spectroscopic methods, by Aminot, A. and Rey, F. *ICES Tech. Mar. Environ. Sci.* **30**, 18pp.

ICES, International Council for the Exploration of the Sea (2001b). Biological effects of contaminants: sediment bioassay using the polychaete *Arenicola marina*, by Thain, J. and Bifield, S. *ICES Tech. Mar. Environ. Sci.* **29**, 16pp.

ICES, International Council for the Exploration of the Sea (2001c). Biological effects of contaminants: *Corophium* sp. sediment bioassay and toxicity test, by Thain, J. and Roddie, B. *ICES Tech. Mar. Environ. Sci.* **28**, 21pp.

ICES, International Council for the Exploration of the Sea (2002). *Report of the ICES/HELCOM Steering Group on Quality Assurance of Chemical Measurements in the Baltic Sea,* Sopot, Poland, 18–21 February 2002.

ICES, International Council for the Exploration of the Sea (2004a). Chemical measurements in the Baltic Sea: guidelines on quality assurance, by Lysiak-Pastuszak, E. and Krysell, M. *ICES Tech. Mar. Environ. Sci.* **35**, 149pp.

ICES, International Council for the Exploration of the Sea (2004b). Biological monitoring: general guidelines for quality assurance, by Rees, H. *ICES Tech. Mar. Environ. Sci.* **32**, 45pp.

ICES, International Council for the Exploration of the Sea (2004c). Biological effects of contaminants: quantification of d-aminolevulinic acid dehydratase (ALA-D) activity in fish blood, by Hylland, K. *ICES Tech. Mar. Environ. Sci.* **34**, 9pp.

ICES, International Council for the Exploration of the Sea (2005). Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile, by Ariese, F., Beyer, J., Jonsson, G., Visa, C.P. and Krahn, M.M. *ICES Tech. Mar. Environ. Sci.* **39**, 41pp.

Jewett, S.C., Dean, T.A., Woodin, B.R., Hoberg, M.K. and Stegeman, J.J. (2002). Exposure to hydrocarbons 10 years after the Exxon Valdez oil spill: evidence from cytochrome P4501A expression and biliary FACs in nearshore demersal fishes. *Mar. Environ. Res.* **54**, 21–48.

JNCC, Joint Nature Conservation Committee (2004). *Common Standard Monitoring Guidance for Monitoring Littoral Rock and Inshore Subtidal Rock Habitats.* Joint Nature Conservation Committee, U.K., 27pp.

Johansson, S., Larsson, U. and Boehm, P. (1980). The Tsesis oil spill. Impact on the pelagic ecosystem. *Mar. Poll. Bull.* **11**, 284–293.

Khan, R.A. and Nag, K. (1993). Estimation of hemosiderosis in seabirds and fish exposed to petroleum. *Bull. Environ. Contam. Toxicol.* **50**, 125–131.

Klaassen, C.D. (ed.) (2001). *Casarett and Doull's Toxicology. The Basic Science of Poisons*. McGraw-Hill, New York.

Krumholz, L., Caldwell, M.E. and Suflita, J.M. (1996). Biodegradation of "BTEX" hydrocarbons under anaerobic conditions, in Crawford, R. and Crawford, D. (eds.), *Bioremediation—Principles and Applications.* Cambridge University Press, Cambridge, U.K., pp. 61–99.

Laännergren, C. (1978). Net- and nanoplankton: effects of an oil spill in the North Sea. *Bot. Mar.* **21**, 353–356.

Labrot, F., Ribera, D., Saint M.D. and Narbonne, J.F. (1996). *In vitro* and *in vivo* studies of potential biomarkers of lead and uranium contamination: lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. *Biomarkers* **1**, 23–30.

Lee, R.F. and Anderson, J.W. (2005). Significance of cytochrome P450 system responses and levels of bile fluorescent aromatic compounds in marine wildlife following oil spills. *Mar. Poll. Bull.* **50**, 705–723.

Lee, S. and Fuhrman, J.A. (1987). Relationships between biovolume and biomass of naturally derived marine bacterioplankton. *Appl. Environ. Microbiol.* **53**,1298–1303.

Lewis, J.R. (1976). Long-term ecological surveillance: practical realities in the rocky littoral. *Oceanogr. Mar. Biol. Ann. Rev.* **14**, 371–390.

Long, E.R. and MacDonald, D.D. (1998). Recommended uses of empirically derived, sediment quality guidelines for marine and estuarine ecosystems. *Hum. Ecol. Risk Assess.* **4**, 1019–1039.

Long, E.R., Macdonald, D.D., Smith, S.L. and Calder, F.D. (1995). Incidence of adverse biological effects within ranges of chemical concentrations in marine estuarine sediments. *Environ. Manag.* **19**, 81–97.

Loring, D.H. (1991). Normalization of heavy-metal data from the estuarine and coastal sediments. *ICES J. Mar. Sci.* **48**, 101–115.

Majeed, S.A. (1987). Organic-matter and biotic indexes on the beaches of North Brittany. *Mar. Poll. Bull.* **18**, 490–495.

Martínez-Gómez, C., Campillo, J.A., Benedicto, J., Fernández, B., Valdéz, J., García, I. and Sánches, F. (2006). Monitoring biomarkers in fish (*Lepidorhombus boscii* and *Callionymus lyra*) from the northern Iberian shelf after the Prestige oil spill. *Mar. Poll. Bull.* **53**, 305–314.

Mazet, J.K., Gardner, I.A., Jessup, D.A., Lowenstine, L.J. and Boyce, W.M. (2000). Evaluation of changes in hematologic and clinical biochemical values after exposure to petroleum products in mink (*Mustela vison*) as a model for assessment of sea otters (*Enhydra lutris*). *Am. J. Vet. Res.* **6**, 1197–203.

McCarthy, L.S., Mackay, D., Smith, A.D., Ozburn, G.W. and Dixon, D.G. (1992). Residue-based interpretation of toxicity and bioconcentration QSARs from aquatic bioassays: neutral narcotic organics. *Environ. Toxicol. Chem*. **11**, 917–930.

McCarthy, J.F. and Shugart LR. (1990). Biological markers of environmental contamination, in McCarthy, J.F. and Shugart, L.R. (eds.), *Biomarkers of Environmental Contamination*. Lewis Publishers, Boca Raton, FL, pp. 3–16.

McLusky, D.S. (1982). The impact of petrochemical effluent on the fauna of an inter-tidal estuarine mudflat. *Estuar. Coast. Shelf. Sci.* **14**, 489–499.

McRoy, C.P., Cooney, R.T., Simpson, P., Ward, A., Tamburello, K. and Cameron, J. (1999). The case for bottom-up control of food web dynamics in Prince Williams Sound. *Proceedings of the Symposium on Legacy of an Oil Spill 10 Years after Exxon Valdez*, 23–26 March, University of Alaska, United States.

Meyerhoff, R.D. (1975). Acute toxicity of benzene, a component of crude oil, to juvenile striped bass (*Morone sexatilis*). *J. Fish. Res. Bd. Can.* **32**, 1864–1866.

Micael, J., Reis-Henriques, M.A., Carvalho, A.P. and Santos, M.M. Genotoxic effects of binary mixtures of xenoandrogens (tributyltin, triphenyltin) and a xenoestrogen (Ethinylestradiol) in a partial life-cycle test with zebrafish (*Danio rerio*). *Environ. Int.* in press.

Michel, J., Hayes, M.O., Hoff, R., Shigenaka, G. and Scholz, D. (1992). Introduction of coastal habitats and biological resources for oil spill response. *HMRAD Report 92-4*, prepared for the Hazardous Materials Response and Assessment Division, NOAA, Seattle, WA, 384pp.

Mishra, R. and Shukla, S.P. (1997). Impact of endosulfan on lactate dehydrogenase from the fresh water catfish *Clarias batrachus*. *Pestic. Biochem. Physiol*. **57**, 220–234.

Moreira, S.M. and Guilhermino, L. (2005). The use of *Mytilus galloprovincialis* acetylcholinesterase and glutathione S-transferases activities as biomarkers of environmental contamination along the northwest Portuguese coast. *Environ. Monit. Assess*. **105**(1-3), 309–325.

Moreira, S.M., Lima, I., Ribeiro, R. and Guilhermino, L. (2006). Effects of estuarine sediment contamination on feeding and on key physiological functions of the polychaete *Hediste diversicolor*: Laboratory and in situ assays. *Aquat. Toxicol.* **78**, 186–201.

Moreira, S.M., Moreira dos Santos, M., Ribeiro, R. and Guilhermino, L. (2004). The 'Coral Bulker' fuel oil spill on the North coast of Portugal: spatial and temporal biomarker responses in *Mytilus galloprovincialis*. *Ecotoxicology* **13**, 619–630.

Morrow, J.E., Gritz, R.L. and Kirton, M.P. (1975). Effects of some components of crude oil on young coho salmon. *Copeia* **2**, 326–331.

Mosleh, Y.Y., Paris-Palacios, S. and Biagianti-Risbourg, S. (2006). Metallothioneins induction and antioxidative response in aquatic worms *Tubifex tubifex* (Oligochaeta, Tubificidae) exposed to copper. *Chemosphere* **64**, 121–128.

Nelson-Smith, A. (1972). *Oil Pollution and Marine Ecology*. Elek Science, London, 260pp.

New Zealand Ministry of the Environment (1999). *Guidelines for Assessing and Managing Petroleum Hydrocarbon Contaminated Sites in New Zealand*.

NRC, Committee on Biological Markers of the National Research Council (1987). Biological markers in environmental health research. *Environ. Health Perspect.* **74**, 3–9.

Nunes, B., Carvalho, F. and Guilhermino, L. (2004). Acute and chronic effects of clofibrate and clofibric acid on the enzymes acetylcholinesterase, lactate dehydrogenase and catalase of the mosquitofish, *Gambusia holbrooki*. *Chemosphere* **57**(11), 1581–1589.

Nunes, B., Carvalho, F. and Guilhermino, L. (2006). Effects of widely used pharmaceuticals and a detergent on oxidative stress biomarkers of the crustacean *Artemia parthenogenetica*. *Chemosphere* **62**(4), 581–594.

Nuzzi, R. (1973). Effects of water-soluble extracts of oil on phytoplankton. *Proc. Joint Conf. on Prevention and Control of Oil Spills*. A.P.I., Washington, DC, pp. 809–813.

OCHA/UNEP, United Nations Office for the Coordination of Humanitarian Affairs/United Nations Environment Programme (2003). *Tasman Spirit Oil Spill Report*, 22pp.

OECD, Organisation for Economic and Co-operation and Development (1985). Guideline for testing of chemicals. *Fish, Prolonged Toxicity Test 14-day Study 204*.

OECD, Organisation for Economic and Co-operation and Development (1992a). Guideline for testing of chemicals. *Fish, Acute Toxicity Test 203*.

OECD, Organisation for Economic and Co-operation and Development (1992b). Guideline for testing of chemicals. *Fish, Early-Life stage Toxicity Test 210*.

OECD, Organisation for Economic and Co-operation and Development (1997). Guidance for good laboratory practices monitoring authorities. *Series on Principles of Good Laboratory Practice and Compliance Monitoring 1,* 41pp.

OECD, Organisation for Economic and Co-operation and Development (1998). Guideline for testing of chemicals. *Fish, Short-term Test on Embryo and Sac-Fry Stages 212*.

OECD, Organisation for Economic and Co-operation and Development (2000). Guideline for testing of chemicals. *Fish, Juvenile Growth Test 215*.

Olafson, R.W. and Sim, R.G. (1979). An electrochemical approach to quantitation and characterization of metallothioneins. *Anal. Biochem*. **100**, 343–351.

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1989). Guidelines for the sampling and analysis of trace metals in sea water under the Joint Assessment Monitoring Program. *Tenth Annual Report on the Activities of the Paris Commission*, Annex 24.

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1995a). *Protocols on Methods for the Testing of Chemicals used in the Offshore Industry.*

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1995b). *JAMP Guidelines on Standard Methodology for the use of Oiled Birds as Indicators of Marine Oil Pollution*. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997a). *JAMP Guidelines for Monitoring Contaminants in Biota,* Annex 2 – determination of metals; Annex 3 – determination of PAH. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997b). *JAMP Guidelines for Monitoring Contaminants in Sediments*. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997c). *JAMP Guidelines for General Biological Effects Monitoring,* Annex 6 – lysosomal stability. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997d). *JAMP Guidelines for Contaminant-Specific Biological Effects Monitoring.* Annex 2 – PAH-specific biological effects monitoring. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997e). *JAMP Eutrophication Monitoring Guidelines – Phytoplankton Species Composition*. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997f). *Jamp Eutrophication Monitoring Guidelines – Chlorophyll a*.. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997g). *JAMP Guidelines for General Biological Effects Monitoring,* Annex 7 – liver neoplasia/hyperplasia. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997h). *JAMP Guidelines for General Biological Effects Monitoring,* Annex 8 – liver nodules. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997i). *JAMP Guidelines for General Biological Effects Monitoring,* Annex 5 – CYP1A. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1997j). *JAMP Guidelines for Contaminant-Specific Biological Effects Monitoring,* Annex 1 – metal-specific biological effects monitoring. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1998). *JAMP Guidelines for Monitoring Contaminants in Sediments,* Annex 3 – determination of PAHs. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (1999). *JAMP Guidelines for Monitoring Contaminants in Biota,* Annex 3 – determination of PAH. Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (2001). *JAMP Guidelines for Environmental Monitoring in the Vicinity of Offshore Oil and Gas Installations.* Joint Assessment and Monitoring Programme (JAMP).

OSPAR, Oslo/Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (2002). *JAMP Guidelines for Monitoring Contaminants in Sediments,* Annex 6 – determination of metals – analytical methods. Joint Assessment and Monitoring Programme (JAMP).

Payne, J., Mathieu, A., Melvin, W. and Fancey, L.L. (1996). Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. *Mar. Pollut. Bull.* **32**, 225–231.

Peakall, D.B., Norstrom, R.J., Rahimtula, A.D. and Butler, R.D. (1986). Characterisation of mixed-function oxidase systems of the nestling Herring gull and its implications for bioeffects monitoring. *Environ. Toxicol. Chem.* **5**, 379–385.

Perkins, R.A., Rhoton, S. and Behr-Andres, C. (2005). Comparative marine toxicity testing: a cold-water species and standard warm-water test species exposed to crude oil and dispersant. *Cold Reg. Sci. Technol.* **42**, 226–236.

Piatt, J.F., Lensink, C.J., Butler, W., Kendziorek, M. and Nysewander, D.R. (1990). Immediate impact of the Exxon Valdez oil spill on marine birds. *The Auk* **107**, 387–397.

Pluta, H.J. (1993). Investigations on biotransformation (mixed function oxygenase activities) in fish liver, in Braunbeck, T., Hanke, W. and Senger, H. (eds.), *Fish Ecotoxicology and Ecophysiology*. VCH Weinheim, pp. 13–33.

Regoli, F. and Principato, G. (1995). Glutathione, glutathione dependent and anti-oxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use as of biochemical biomarkers. *Aquat. Toxicol.* **31**, 143–164.

Robert, A., Perkins, T., Rhoton, S. and Behr-Andres, C. (2005). Comparative marine toxicity testing: a cold-water species and standard warm-water test species exposed to crude oil and dispersant. *Cold Reg. Sci. Technol.* **42**, 226–236.

Sánchez, F., Velasco, F., Cartes, J.E., Olaso, I., Preciado, I., Fanelli, E., Serrano, A. and Gutierrez-Zabala, J.L. (2006) Monitoring the Prestige oil spill impacts on some key species of the Northern Iberian shelf. *Mar. Poll. Bull.* **53**, 332–349.

Sanders, B.M. (1993). Stress proteins in aquatic organisms: an environmental perspective. *Crit. Rev. Toxicol.* **23**, 49–75.

Schwartz, J.A., Aldridge, B.M., Lasley, B.L., Snyder, P.W., Stott, J.L. and Mohr, F.C. (2004). Chronic fuel oil toxicity in American mink (*Mustela vison*): systemic and hematological effects of ingestion of a low-concentration of bunker C fuel oil. *Toxicol. Appl. Pharmacol.* **200**, 146–158.

Serrano, A., Sánchez, F., Preciado, I., Parra, S. and Frutos, I. (2006). Spatial and temporal changes in benthic communities of the Galician continental shelf after the Prestige oil spill. *Mar. Poll. Bull.* **53**, 315–331.

Potter, T.L. and Simmons, K.E. (1998). Composition of petroleum mixtures. *Total Petroleum Hydrocarbon Criteria Working Group Series 2*. Amherst Scientific Publications, Amherst, MA, 114pp.

Scholz, D., Michel, J., Shigenaka, G. and Hoff, R. (1992). Biological resources. Introduction of coastal habitats and biological resources for oil spill response. *HMRAD Report 92-4*, prepared for the Hazardous Materials Response and Assessment Division, NOAA, Seattle, WA, 384pp.

Shugart, L.R., Bickham, J., Jackim, G., McMahon, G., Ridley, W., Stein, J. and Steinert, S. (1992). DNA alterations, in Huggett, R.J., Kimerly, R.A., Mehrle, P.M. Jr and Bergman, H.L. (eds.), *Biomarkers: Biochemical, Physiological and Histological Markers of Anthropogenic Stress.* Lewis Publishers, Chelsea, MI, pp.155–210

Singer, M.M., Auranda, D., Bragin, G.E., Clark, J.R., Coelho, G.M., Sowby, M.L. and Tjeerdema, R.S. (2000). Standardization of the preparation and quantitation of water-accommodated fractions of petroleum for toxicity testing. *Mar. Poll. Bull.* **40**, 1007–1016.

Smith, J.E. (1968). *Torrey Canyon Pollution and Marine Life: A report by the Plymouth Laboratory, Vol. XIV.* Cambridge University Press, Cambridge, U.K., 196pp.

Smith, P.E. and Richardson, S.L. (1977). Standard techniques for pelagic fish eggs and larva surveys. *FAO Fish. Tech. Pap.* **175**, 100pp.

Stokesbury, K.D.E. (1999). A re-examination of events influencing the 1993 Pacific herring population crash in Prince Williams Sound, Alaska. *Proceedings of the Symposium on Legacy of an Oil Spill 10 Years after Exxon Valdez*, 23–26 March, University of Alaska, United States.

Tang, S.S., Lin, C.C. and Chang, G.G. (1996). Metal-catalyzed oxidation and cleavage of octopus glutathione transferase by the CU(II)-ascorbate system. *Free Rad. Biol. Med.* **21**, 955–964.

Underwood, A.J. (1997). Experiments in ecology: their logical design and interpretation using analysis of variance. *Ecological Experiments*. Cambridge University Press, Cambridge.

UNESCO, United Nations Educational, Scientific and Cultural Organization (1968). Zooplankton sampling. *Mono. Oceanogr. Methodol.* **2**, 174pp.

UNESCO, United Nations Educational, Scientific and Cultural Organization (1975). Ictioplancton. Informe del seminario de las CICAR sobre ictyoplancton. Mexico, 17–26 July, 1974. *Document Technique UNESCO Ciencias del Mar 20*, 44pp.

U.S. EPA, United Sates Environmental Protection Agency (1991). *Evaluation of Dredged Material Proposed for Ocean Disposal Testing Manual*, Chapter 11, Table 11-1. United States Environmental Protection Agency and Department of The Army, U.S. Army Corps of Engineers, EPA 503/8-91/001, Duluth, MN.

U.S. EPA, United Sates Environmental Protection (1994). *Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Estuarine and Marine Invertebrates.* Environmental Protection Agency, EPA 600/R-94/025, Duluth, MN.

U.S. EPA, United Sates Environmental Protection (1998). *Methods for Assessing the Chronic Toxicity of Sediment-Associated Contaminants with Leptocheirus plumulosus.* Environmental Protection Agency, EPA/600/R-94/025, Duluth, MN.

U.S. EPA, United Sates Environmental Protection (2006). http://www.epa.gov/oilspill/crude.htm.

Varela, M., Bode, A., Alvares-Ossorio, M.T., Anadón, R., Llope, M. and Valdés, L. (2005). The effect of the Prestige oil spill on the phytoplankton in the N-NW Spanish coast during spring. *Symposium on Marine Accidental Oil Spills – VERTIMAR*, 13–16 July, Vigo, Spain.

Viarengo, A., Ponzano, E., Dondero, F. and Fabbri, R. (1997). A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Mar. Environ. Res*. **44**, 69–84.

Walker, C.H., Hopkin, S.P., Silby, R.M. and Peakall, D.B. (2001). *Principles of Ecotoxicology*. Taylor & Francis, London.

Watanabe, N., Takatsuki, H. and Sakai, S. (1997). Desorption of tributyltin, dibutyltin and zinc from resuspended sediment. *Appl. Organometal. Chem.* **11**, 273–279.

Wiebe, P.H., Morton, A.W., Bradley, A.M., Backus, R.H., Craddock, J.E., Barber, V., Cowles, T.J. and Flierl, G.R. (1985). New development in MOCNESS, an apparatus for sampling zooplankton and microneckton. *Mar. Biol.* **87**, 313–323.

Wrenn, B.A. and Venosa, A.D. (1996). Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most probable number procedure. *Can. J. Microbiol.* **42**, 252–258.

Zhang, X., Wu, R.J., Zhang, Z.H. and Dong, Z.F. (2005). Benzene toxicity to the scallop, *Chlamys farreri*, and the shrimp, *Penaeus japonicus*. *Ecological Effects of Offshore Oil and Gas Development Oil Spills.* The North Pacific Marine Science Organization – PICES XIV S9.

5. Procedural guidelines

The following Procedural Guidelines (PGs) consist of the methodologies validated and used by the laboratories of CIIMAR in the scope of EROCIPS. They are described in detail here, so that other partners can use them.

A. PGs on Sediment Characterisation

1. Particle Size Determination Assay
2. Organic Matter Assay
3. Chlorophyll *a*, *b* and *c* and Phaeopigments Assay in the Sediment

B. PGs on Biological Effects Monitoring

4. Cholinesterase Activity Assay in Fish (*Pomatoschistus microps*) Heads
5. Activity of the CYP1A – EROD Assay in Fish Liver
6. Fixed Wavelength Fluorescence for Detection of Polycyclic Aromatic Hydrocarbon Metabolites in Fish Bile
7. Glutathione S-Transferase Activity Assay in Fish (*Pomatoschistus microps*) gills
8. Superoxide Dismutase Activity Assay in Fish (*Pomatoschistus microps*) Liver
9. Catalase Activity Assay in Fish (*Pomatoschistus microps*) Liver
10. Glutathione Peroxidase Activity Assay in Fish (*Pomatoschistus microps*) Liver
11. Glutathione Reductase Activity Assay in Fish (*Pomatoschistus microps*) Liver
12. Lipid Peroxidation in Fish (*Pomatoschistus microps*) Liver
13. Lactate Dehydrogenase Activity Assay in Fish (*Pomatoschistus microps*) Muscle
14. The Neutral Red Retention Assay in the Lysosomes of Mussel (*Mytilus* sp.) Haemocytes

Procedural Guideline No. 1
PARTICLE SIZE DETERMINATION ASSAY
Rapid partial analysis of sediment

1. INTRODUCTION

1.1. USES
In conjunction with more extensive benthic surveys, sufficient information can often be derived from a measurement of the combined silt and clay content of the sediment.

1.2. PRINCIPLE OF THE METHOD
The particle size determination involves an initial splitting of the sediment into a sand fraction (particles greater than 63 µm) and a silt-clay fraction (particles less than 63 µm). The sand fraction may be further divided through a series of graded sieves, but the initial splitting is achieved with the 63 µm sieve employing a wet sieving method. If several 63 µm sieves are available, the method is rapid and large numbers of samples can be processed in a short time.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT
- Centrifuge (KUBOTA 5400)
- Sieves: 63 µm + base (RETSCH)
- Analytical balance (KERN 770)
- Drying oven (RAYPA mod. DO-90)

2.2. MATERIALS
- 100 ml syringe
- Glass jars 100 ml
- Pasteur pipette

2.3. REAGENTS
- Calgon $(NaPO_3)_6$ (SIGMA 71600) solution (6.2 g/l in ddH_2O)

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD
- Take 10 samples from the first 1 cm of soil with a 100 ml syringe.
- Once in the laboratory, store the samples at –20ºC until analysis.

3.2. SAMPLE COLLECTION IN THE LABORATORY
- Weigh a 100 ml glass jar using an analytical balance (0.0001 g). Record the number of the jar.
- Put one sample in each glass jar and dry the sample in an oven at 100–105ºC.
- Weigh the jar and sample to estimate the sample dry weight.
- Make up to 80 ml of distilled water.
- Gently break up the sample.
- Add 200 µl of $(NaPO_3)_6$ solution.
- Break up the sediment with a glass rod and then stir (400 rpm) for about 15 min and allow the sediment to soak overnight.
- Re-stir (400 rpm) for 15 min. This will disperse soil aggregates.
- Gently pour the sample through a 63 µm sieve. Use caution not to lose any of the samples by spillage. Thoroughly wash all silt and clay through the 63 µm sieve using tap water. The entire sand fraction (very fine to very coarse) is now in the sieve.

- Carefully transfer all of the sand to the corresponding 100 ml glass jar. Leave for decantation for about 15 min. Take the excess water out using a Pasteur pipette. Dry the sand in an oven (105ºC).
- Remove the sand fraction from the oven, weigh and calculate the weight of the sand fraction. Express as a percentage of the original weight.
- If wanted, correct the percentage of the original weight considering the amount of organic matter measured in the other set of samples (organic matter).

4. REFERENCES

Holme, N.A. and McIntyre, A.D. (1971). *Methods for the Study of Marine Benthos (Blackwell Scientific International Biological Programme).* Blackwell Science, pp. 263–265.

Procedural Guideline No. 2
ORGANIC MATTER ASSAY

1. INTRODUCTION

1.1. USES

Organic matter in sediment consists of carbon and nutrients from plant and animal detritus derived from natural and anthropogenic sources. The amount of organic matter in sediment depends on the amount of source material reaching the sediment surface and the rates at which organic matter is degraded by microbial processes during burial.

1.2. PRINCIPLE OF THE METHOD

The organic matter content is estimated by combustion of previously dried samples. After the combustion of the organic matter, the ash-free dry weight of the samples can be estimated using the formulas proposed by Strickland and Parsons (1972).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Drying oven (RAYPA mod. DO-90)
- Muffler combustion oven (NABERTHERM L3/C6)
- Analytical balance (KERN 770)

2.2. MATERIALS

- 100 ml syringe
- Aluminium foil cups (48 ml)

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- Take 10 replicates (2 m distance) from the first 1 cm of soil with a 100 ml syringe.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- Each sample is allocated in the aluminium foil cup.
- Dry the samples in an oven at 60ºC for 24 h and weight (A).
- Put the samples in a muffler combustion oven (550ºC for 2 h) and thereafter weight again (B).
- Estimations of organic matter content are made with the formulas suggested by Strickland and Parsons (1972):

$$\% \text{ Organic matter} = 100 - ((A - B)/A) \times 100$$

4. REFERENCES

Holme, N.A. and McIntyre, A.D. (1971). *Methods for the Study of Marine Benthos (Blackwell Scientific International Biological Programme).* Blackwell Science, pp. 263–265.

Strickland, J.D.H. and Parsons, T.R. (1972). *A Practical Handbook of Seawater Analysis*. Fisheries Research Board of Canada.

Procedural Guideline No. 3
CHLOROPHYLL *a*, *b* AND *c* AND PHAEOPIGMENTS ASSAY IN THE SEDIMENT

1. INTRODUCTION

1.1. USES
The determination of chlorophyll concentration in sediment samples gives an estimation of primary productivity.

1.2. PRINCIPLE OF THE METHOD
Chlorophyll concentration is estimated spectrophotometrically by measuring absorbance at different wavelengths.

The plant pigments are extracted with 90% acetone. After reading the absorbance at several wavelengths, it is possible to determine the concentration of chlorophylls using the formulas suggested by Strickland and Parsons (1972).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT
- Centrifuge (KUBOTA 5400)
- Spectrophotometer (JENWAY UV/VIS Spectrophotometer)
- Analytical balance (KERN 770)
- Drying oven (RAYPA mod. DO-90)

2.2. MATERIALS
- 100 ml syringe
- 50 ml polyethylene centrifuge tubes
- Aluminium foil
- Cuvettes (HELLMA optical glass, 10 mm)
- Micropipette (LABMATE 20–200 µl)
- Petri dishes (6 cm diameter)

2.3. REAGENTS
- Acetone (MERCK 1.00014.1011) solution (90% in ddH_2O)
- Magnesium carbonate (SIGMA 13118) solution (1 g/100 ml in ddH_2O)
- HCl (MERCK 1.00319.1000) solution (1 N in ddH_2O)

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD
- Take 10 samples from the first 1 cm of soil with a 100 ml syringe.
- Add the sediment to a 50 ml polyethylene centrifuge tube and add 30 ml of 90% acetone.
- Add 0.2 ml of magnesium carbonate (1 g/100 ml).
- Mix thoroughly and cover with tin foil.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- Once in the laboratory, shake again and keep in the fridge for 24 h.
- After 24 h centrifuge the tube for 3 min at 3500 rpm.
- Shake again and centrifuge again for 10 min.
- Remove 3 ml of the supernatant and put it in a glass spectrophotometer cuvette.
- Read the samples in the spectrophotometer at 480, 630, 645, 647 664, 665 and 750 nm.
- Add 0.2 ml of HCl 1 N directly to the cuvette and agitate. Wait 4 min and then re-measure the absorbance at 665 and 750 nm.
- Put the remaining sediment in Petri dishes, dry at 60ºC until constant weight is reached.

$$C \text{ (chlorophyll } a) = 11.6\ E_{665} - 1.31\ E_{645} - 0.14\ E_{630}$$
$$C \text{ (chlorophyll } b) = 20.7\ E_{645} - 4.34\ E_{665} - 4.42\ E_{630}$$
$$C \text{ (chlorophyll } c) = 55\ E_{630} - 4.64\ E_{665} - 16.3\ E_{645}$$

$$\text{Phaeopigments (mg/m}^3) = (26.7\ (1.7\ (E_{665a}) - E_{665o})\ \text{x}\ v)/(V\ \text{x}\ l)$$

where

E_{665} = absorbance at the wavelength of 665 nm
E_{645} = absorbance at the wavelength of 645 nm
E_{630} = absorbance at the wavelength of 630 nm
E_{665a} = absorbance at the wavelength of 665 nm after acidification
E_{665o} = absorbance at the wavelength of 665 nm before acidification
v = volume of acetone used for extraction (ml)
V = volume of sediment (cm^3)
l = path length of the cuvette (cm)

4. REFERENCES

Strickland, J.D.H. and Parsons, T.R. (1972). *A Practical Handbook of Seawater Analysis*. Fisheries Research Board of Canada.

Procedural Guideline No. 4
CHOLINESTERASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) HEADS

1. INTRODUCTION

1.1. USES

Cholinesterase (ChE) inhibition has been widely used as an environmental biomarker allowing the diagnosis of exposure to anticholinesterase agents and the assessment of its effects. ChEs have been shown to be sensitive to organophosphate and carbamate insecticides, some metals, some surfactants and detergents, and to some petrochemical products.

1.2. PRINCIPLE OF THE METHOD

The method most frequently used for ChE determination in ecotoxicology is Ellman's method (Ellman *et al*., 1961). The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accomplished by the continuous reaction of the thiol with the 5:5-dithiobis-2-nitrobenzoate ion to produce the yellow anion of 5-thio-2-nitro-benzoic acid. The rate of colour production may be measured at 412 nm in a microplate reader (ε = 13.6 mM^{-1} cm^{-1}). The technique described here is an adaptation to a microplate of Ellman's method (Guilhermino *et al.*, 1996) with minor modifications.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Microplate reader

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- Acetylthiocholine
- 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB)
- Sodium hydrogen carbonate ($NaHCO_3$)
- Bovin γ-globulines
- Bio-Rad protein reagent

Phosphate buffer 0.1 M, pH 7.2

Prepare a solution of K_2HPO_4 0.1 M in ultra-pure (u.p.) water.
Prepare a solution of KH_2PO_4 0.1 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.2 (keep at –4ºC).

Stable as long as no bacterial contamination occurs.

Acetyltiocholine solution 0.075 M in u.p. H_2O
(protect from light and keep at –4ºC)

DTNB 10 mM solution with $NaHCO_3$ 17.855 mM in phosphate buffer
(protect from light and keep at –4ºC)

Stable for about 5 days.

Reaction solution
0.2 ml acetyltiocholine solution + 1 ml DTNB solution + 30 ml phosphate buffer.

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared (on the day and just before the assay).

Standard protein solution
1 mg of bovin γ-globulines in 1 ml of u.p. water (store at –20ºC).

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. TISSUE PREPARATION IN THE LABORATORY

- One fish head is sufficient for this assay.
- Fish is sacrificed by decapitation.
- Remove the gills from the head.
- Homogenise the head in 1 ml of phosphate buffer.
- Centrifuge the homogenised material at 6000 rpm, 4ºC, for 3 min.
- Collect the supernadant in a 1.5 ml microtube and store it at –80ºC (for no more than 2 weeks).

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. ChE ASSAY IN MICROPLATE

- Quantify the protein content of the supernatant and adjust it to 0.3 mg/ml.
- Perform four replicates for each sample.

	Blank	**Sample**
Phosphate buffer	50 µl	-
Reaction solution	250 µl	250 µl
Supernatant	-	50 µl

- *After 10 min, read the absorbance at 412 nm every 20 s for 5 min.*
- **Keep the temperature at 25ºC.**

3.3.2. PROTEIN ASSAY (Bradford, 1976)

Standards	V standard (µl)	V H_2O (µl)	V Bio-Rad (µl)
P0	0	10	250
P1	2	8	250
P2	5	5	250
P3	10	0	250
Sample	**V sample (µl)**		
X	10	0	250

- Protein standard solution: 1 mg/ml bovin γ-globulin.
- Bio-Rad solution (1:4; Biorad: u.p. H_2O).
- Perform four replicates for each sample.
- Read at 600 nm after 15 min.

3.3.3. DETERMINATION OF ChE ACTIVITY

- ChE activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(13.6 \times 0.9)(VA/VT)} \times \frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

13.6 = extinction coefficient for DTNB (mM^{-1} cm^{-1}) with the unit conversion required so that the final result of enzymatic activity is expressed in nmol of substrate per minute per mg protein

0.9 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (50 µl)

VT = total volume placed in each plate well (300 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample (mg/ml)

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilising the principle of protein dye binding. *Anal. Biochem.* **7**, 248–254.

Ellman, G., Courtney, K., Andres, V. and Featherstone, R. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.

Guilhermino, L., Lopes, M., Carvalho, A. and Soares, A.M.V.M. (1996). Acetylcholinesterase activity in juveniles of *Daphnia magna* Straus. *Bull. Environ. Contamin. Toxicol.* **57**, 979–985.

Procedural Guideline No. 5
ACTIVITY OF THE CYP1A – EROD ASSAY IN FISH LIVER

1. INTRODUCTION

1.1 USES

The ethoxyresorufin-*O*-deethylase (EROD) assay is a biomarker that can be used to measure the catalytic activity of CYP1A (a member of the P450-monooxygenase system family involved in the biotransformation of endogenous and exogenous compounds). The induction of CYP1A has been the most widely used biomarker to evaluate the exposure of vertebrates to organic contaminants. The activity of this enzyme tends to be increased when vertebrates are exposed to contaminants such as planar halogenated compounds, certain polycyclic aromatic hydrocarbons (PAHs) or polychlorinated biphenyls (PCBs).

1.2 PRINCIPLE OF THE METHOD

EROD measures the production of resorufin from ethoxyresorufin, in the presence of NADPH (β-nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt).

The resorufin production is then determined fluorimetrically at 530 and 585 nm for excitation and emission spectra, respectively. The catalytic activity of the enzyme is an indication of the amount of enzyme present and is expressed as the concentration of resorufin produced per mg protein per minute (pmol/mg/min).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Analytical balance
- Centrifuge
- Water bath
- Fluorimeter
- Microplate reader

2.2. MATERIALS

- Forceps
- Micropipettes
- Eppendorffs
- Quartz cuvette
- Glass tubes
- Microplates

2.3. REAGENTS

- Tris HCl
- KCl
- $Na_2EDTA.2H_2O$
- DL-Dithiothreitol
- Glycerol
- Ethoxyresorufin
- Resorufin sodium salt
- DMSO
- B-NADPH, Na_4

- Acetone
- Ethanol
- NaCl
- Sodium azide
- Bovine serum albumin
- Na_2CO_3
- Sodium-potassium tartarate
- NaOH
- H_2SO_4
- $CuSO_4$
- Folin reagent

Tris buffer (50 mM, pH 7.4)
3.94 g of Tris HCl + ddH_2O (complete to a 500 ml volume, pH 7.4).

Buffer A
1.12 g KCl + Tris buffer (complete to a 100 ml volume).

Buffer B
0.0372 g $Na_2EDTA.2H_2O$ + 0.0154 g dithiothreitol + 20 ml glycerol, complete to a 100 ml volume with Tris buffer.

Buffer C
0.0154 g dithiothreitol + 20 ml glycerol, complete to 100 ml with Tris buffer.

Buffer D (Tris HCl 0.05 M, pH 8)
0.788 g Tris HCl + 1.12 g KCl + 0.0154 g dithiothreitol + ddH_2O (complete to a volume of 100 ml, pH 8.0).

7-Ethoxiresorufin (7-ER) 400 µM
0.9 mg 7-ER + DMSO (complete to a 10 ml volume).
Store in aliquots at 4^{o}C, light protected.

NADPH 4.5 mM
3.7 mg NADPH/ml Buffer D.
Fresh made every day and kept on ice.

Reaction mixture
500 µl NADPH + 100 µl 7-ER solution + EROD buffer (complete to a 10 ml volume).
Keep light protected.

Resorufin stock solution (1 mM)
5.9 mg resorufin + 25 ml ethanol 100%.
Store at 4^{o}C, light protected.

Resorufin standards
0.1 mM, 1 µM, 0.1 µM, 0.05 µM, 0.025 µM, 0, 0125 µM, 0.006 µM, 0.003 µM in Buffer D.

NaCl solution (0.9%)
0.9 g NaCl + 0.1 g sodium azide + ddH_2O (complete to a 100 ml volume).

BSA stock
0.0625 g Bovine serum albumine + NaCl solution (complete to a 25 ml volume).

NaOH 0.1 N
4 g NaOH/L ddH_2O.

Solution A
20 g Na_2CO_3 + 0.2 g sodium-potassium tartarate + NaOH 0.1 N (complete to a 1000 ml volume).

Solution B
Add one drop of concentrated H_2SO_4 to 0.5 g $CuSO_4$ to 100 ml ddH_2O.

Solution A+B
5 ml solution A + 0.1 ml solution B.

Folin reagent working solution
1 ml Folin reagent: 1 ml H_2O.

Protein standards

Standard	Protein content (µg)	Volume standard (ml)	Volume NaCl solution (ml)
St8	250.00	5.0 BSA stock	0
St7	200.00	4.00 St8	1.00
St6	150.00	3.75 St7	1.25
St5	100.00	3.30 St6	2.70
St4	50.00	2.5 St5	2.50
St3	25.00	2.5 St4	2.50
St2	12.50	2.5 St3	2.50
St1	6.25	2.5 St2	2.50

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- Fish should be collected in the field preferentially by a non-invasive method, and transported to the laboratory to be acclimatised and aerated. Alternatively, fish can be sacrificed in the field, and the liver immediately frozen in liquid nitrogen.

3.2. SAMPLES COLLECTION IN THE LABORATORY

- Sacrifice the animals after anaesthetising in saline water and ice.
- Remove the liver from the fish, weigh it and immediately freeze about 100 mg (for most species this is enough for EROD determinations) of liver in liquid nitrogen.
- Store at –80^{o}C.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. EXTRACTION OF LIVER MICROSOMES (KEEP ALL PROCEDURES AT 4ºC)

- Weight a portion of liver (about 100 mg) and place it in a glass tube containing 800 µl of Buffer A, kept on ice.
- Homogenise the liver for a few seconds, maintaining the glass tube on ice.
- Transfer the homogenisation product to an eppendorf and centrifuge at 9,000*g* for 30min, at a temperature of 4^{o}C.
- Remove the supernatant to a new eppendorf.
- Centrifuge the supernatant at 36,000*g* for 90 min.
- Remove the supernatant and re-suspend the pellet in 800 µl of Buffer B.
- Centrifuge at 36,000*g* for 120 min.
- Remove the supernatant and re-suspend the pellet in Buffer C (1 mg liver: 3 µl Buffer C).
- Divide in aliquots and store at –80^{o}C.

3.3.2. EROD ACTIVITY DETERMINATION

- Prepare three glass tubes per sample (blank + two replicates).
- Add 1 ml of cold acetone to all the blanks.
- Add 30 µl of Buffer D and 20 µl of sample to all the tubes.
- Add 450 µl of reaction mixture to the tubes (with a 15 s interval), and place them in the water bath → the reaction begins.
- Incubate at 25°C for 10 min, and add 1 ml of cold acetone to each tube (with a 15 s interval) → the reaction stops.
- Centrifuge for 10 min at 400*g*, 4°C.
- Collect the supernatant, place it in a quartz cuvette and read the fluorescence at λ emission = 530 nm and λ excitation = 585 nm.
- Read the fluorescence of the resorufin standards and make a calibration curve.
- Confirm the concentration of the standard 1 µM by reading its absorbance in the spectrophotometer at 572 nm (it should be about 0.073).
- The resorufin calibration curve is made using the fluorescence values of the standards, and thus the resorufin concentration in the sample calculated.
- The EROD activity can be calculated through the following equation:

$$\frac{\text{resorufin sample (nM)} \times \text{reaction volume (l)}}{\text{vol. sample (ml)} \times \text{protein sample (mg/ml)} \times \text{time (min)}} = X \text{ nmol/min/mg}$$

3.3.3. PROTEIN DETERMINATION

- Place 100 µl of ddH_2O in the microplate first column wells.
- Place 100 µl of protein standards solution (three replicates for each standard) in the microplate.
- Dilute the samples in water (60 µl sample: 300 µl ddH_2O) and place 100 µl in the microplate (three replicates for each sample).
- Add to every well 80 µl of solution A+B.
- Incubate for 10 min in the dark, with agitation, at room temperature.
- Add to every well 40 µl of Folin reagent working solution.
- Incubate for 30 min in the dark, with agitation.
- Read the absorbance at 690 nm.

4. REFERENCES

Fent, K. and Bucheli, T.D. (1994). Inhibition of hepatic microsomal monooxygenase system by organotins *in vitro* in freshwater fish. *Aquat. Toxicol.* **28**, 107–126.

Machala, M., Nezvedaa, K., Petrivalsky, M., Jarosová, A., Piacka, B. and Svobodová, V. (1997). Monooxygenase activities in carp as biochemical markers of pollution by polycyclic and polyhalogenated aromatic hydrocarbons: choice of substrates and effects of temperature, gender and capture stress. *Aquat. Toxicol.* **37**, 113–123.

Pacheco, M. and Santos, M.A. (1998). Induction of Liver EROD and erythrocytic nuclear abnormalities by cyclophosphamide and PAHs in *Anguilla anguilla* L. *Ecotoxicol. Environ. Saf.* **40**, 71–76.

Peters, L.D. and Livingstone, D.R. (1995). Studies on cytochrome P4501A in early and adult life stages of turbot (*Scophthalmus maximus* L.). *Mar. Environ. Res.* **39**, 5–9.

Siroká, Z. and Drastichová, J. (2004). Biochemical markers of aquatic environment contamination – Cytochrome P450 in fish. A review. *Acta Vet. BRNO* **73**, 123–132.

Whyte, J.J., Jung, R.E., Schmitt, C.J. and Tillitt, D.E. (2000). Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Crit. Rev. Toxicol.* **30**(4), 347–570.

Procedural Guideline No. 6
FIXED WAVELENGTH FLUORESCENCE FOR DETECTION OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES IN FISH BILE

1. INTRODUCTION

1.1. USES

Polycyclic aromatic hydrocarbons (PAHs) are the most hazard components of oil, and have been shown to display carcinogenic and mutagenic properties. In biological tissues, PAHs are difficult to determine using traditional chemical analysis because they are rapidly metabolised in the liver and excreted into the gall bladder, where they are temporarily stored. Thus, the determination of bile PAH metabolites by Fixed Wavelength Fluorescence can be an alternative and sensitive method for screening PAH contamination in fish.

1.2. PRINCIPLE OF THE METHOD

Many PAHs and subsequent metabolites have strong fluorescence properties and characteristic excitation/emission spectra. This feature allows us to detect them through Fixed Wavelength fluorescence.

The technique consists of diluting the bile sample in ethanol 48%, and reading its fluorescence in a spectrofluorimeter at wavelength pairs of 260/380 nm, 290/335 nm, 341/383 nm and 380/430 nm for the phenanthrene-type, naphthalene-type, pyrene-type and benzo(a)pyrene-type (B(a)P-type) metabolites, respectively. The term metabolite-type is used because this method detects groups of compounds that fluoresce at those specific wavelengths.

It is also necessary to make a calibration curve using 1-OH pyrene standards, to take into account the natural variability of the instrument and to allow the samples to be compared. It is worth noting that this technique gives an estimative, rather than the absolute concentration of the metabolite-type, as the results are expressed in terms of 1-HO pyrene equivalents.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Spectrofluorimeter
- Vortex

2.2. MATERIALS

- Micropipettes
- Quartz cuvette
- Glass tube

2.3. REAGENTS

- Ethanol 48%
- 1-HO pyrene

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- Fish should be collected in the field by a non-invasive method, and transported to the laboratory to be acclimatised and aerated.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- Sacrifice animals after anaesthetising them in saline water and ice.
- Remove the gall bladder from the fish, and immediately freeze in liquid nitrogen.
- Store at –80^{o}C.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. MEASUREMENT OF PAH METABOLITE FLUORESCENCE

- Dilute the bile (5 µl in 5 ml ethanol 48%) in glass tubes and agitate in the vortex.
- Calibrate the spectrofluorimeter for phenanthrene-type metabolite determination, using ethanol 48% as blank, and set the wavelength pairs for emission and excitation at 260 nm and 380 nm, respectively.
- Read the fluorescence of all the samples for this metabolite.
- Repeat this procedure for the other metabolites, setting the wavelength pairs at 290/335 nm (naphthalene), 341/383 nm (pyrene) and 380/430 nm (B(a)P).
- Repeat all the readings.
- To avoid fluctuations of the spectrofluorimeter and to allow the results to be comparable, it is necessary to make a standard curve using 1-HO pyrene, read at 341/383 nm excitation/emission wavelengths and at the following concentrations: 10, 5, 2.5, 0.625 and 0.156 µg/l (for other type of samples, the concentration of the standards might have to be adjusted).

4. REFERENCES

Gorbi, S. and Regoli, F. (2004). Induction of cytochrome P450 1A and biliary PAH metabolites in European eel *Anguilla anguilla*: seasonal, dose- and time-response variability in field and laboratory conditions. *Mar. Environ. Res.* **58**, 511–515.

Lin, E.L.C., Cormier, S.M. and Torsella, J.A. (1996). Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: comparison with HPLC-fluorescent detection. *Ecotoxicol. Environ. Saf.* **35**, 16–23.

Vuontisjarvi, H., Keinanen, M., Vuorinen, P.J. and Peltonen, K. (2004). A comparison of HPLC with fluorescence detection and fixed wavelength fluorescence methods for the determination of polycyclic aromatic hydrocarbon metabolites in fish bile. *Polycyclic Aromatic Compounds* **24**, 333–342.

Watson, G.M., Andersen, O.K., Galloway, T.S. and Depledge, M.H. (2004). Rapid assessment of polycyclic aromatic hydrocarbon (PAH) exposure in decapod crustaceans by fluorimetric analysis of urine and haemolymph. *Aquat. Toxicol.* **67**, 127–142.

Webb, D., Gagnon, M.M. and Rose, T. (2005). Interseasonal variability in biomarkers of exposure in fish inhabiting a southwestern Australian estuary. *Environ. Toxicol.* **20**, 522–532.

Yang, X.A., Peterson, D.S., Baumann, P.C. and Lin, E.L.C. (2003). Fish biliary PAH metabolites estimated by fixed-wavelength fluorescence as an indicator of environmental exposure and effects. *J. Great Lakes Res.* **29**, 116–123.

Procedural Guideline No. 7
GLUTATHIONE S-TRANSFERASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) GILLS

1. INTRODUCTION

1.1. USES

Glutathione S-transferases (GSTs) are a family of multifunctional enzymes involved in Phase II of biotransformation where they catalyse the conjugation of xenobiotics with glutathione. They also play other important functions in the organism, including anti-oxidative stress defences. GSTs activity induction has been widely used to assess the exposure to environmental contaminants with electrophilic centres. They have been successfully used in situations of contamination by petrochemical products.

1.2. PRINCIPLE OF THE METHOD

GST activity may be measured by the method described by Habig *et al.* (1974). This method measures the conjugation of the 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH), forming a thioeter (ε = 9.6 mM^{-1} cm^{-1}). This reaction is catalysed by GSTs and increases the absorbance at 340 nm, which can be monitored. Here the Habig method is used, adapted to a microplate (Frasco and Guilhermino, 2002) with minor modifications.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Spectrophotometer (microplate reader)

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- L-Glutathione reduced (GSH)
- 1-Chloro-2,4-Dinitro-Benzene (CDNB)
- Ethanol
- Bovin γ-globuline
- Bio-Rad protein reagent

Phosphate buffer 0.1 M, pH 6.5

Prepare a solution of K_2HPO_4 0.1 M in u.p. water.
Prepare a solution of KH_2PO_4 0.1 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 6.5 (keep at 4ºC).

Stable as long as no bacterial contamination occurs.

GSH solution 10 mM in phosphate buffer
(protect from light and keep at 4ºC)

CDNB Solution 60 mM in ethanol
(protect from light and keep at 4ºC)

Reaction solution
150 µl CDNB solution + 900 µl GSH solution + 4950 µl phosphate buffer (protect from light and keep at 4ºC).

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globuline in u.p. water.
Store at –20ºC.

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- Three fish are needed for this assay.
- Fish is sacrificed by decapitation.
- Remove the gills from the head.
- Homogenise all gills in 1.0 ml of phosphate buffer.
- Centrifuge the homogenised material at 9,000*g*, 4ºC, for 30 min.
- Store the supernatant at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. GST ASSAY IN MICROPLATE

- Quantify the protein content of the supernatant and adjust for 0.3 mg/ml.
- Perform four replicates for each sample.

	Blank 1	**Sample**
Phosphate buffer	100 µl	-
Reaction solution	200 µl	200 µl
Supernatant	-	100 µl

- *Immediately read the absorbance at 340 nm every 20 s for 5 min.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See procedure for ChE determination.

3.3.3. DETERMINATION OF GST ACTIVITY

- GST activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(9.6\times 0.9)(VA/VT)}\times\frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

9.6 = extinction coefficient for CDNB (mM^{-1} cm^{-1}) with required unit conversion

0.9 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (100 µl)

VT = total volume placed in each plate well (300 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample (mg/ml)

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

Frasco, M. and Guilhermino, L. (2002). Effects of dimetoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata. Fish Physiol. Biochem.* **26**, 149–156.

Habig, W., Pabst, M.J. and Jakoby, W.B. (1974). Glutathione S-Transferases, the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**(22), 7130–7139.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 57–149.

Procedural Guideline No. 8
SUPEROXIDE DISMUTASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) LIVER

1. INTRODUCTION

1.1. USES

Superoxide dismutase (SOD) is a metalloenzyme that catalyses the dismutation of superoxide anion radicals (O_2^-) to hydrogen peroxide, which is an important reactive oxygen species. Hydrogen peroxide is subsequently detoxified by two types of enzymes, catalase and selenium-dependent glutathione peroxidase.

SOD together with other oxidative stress parameters have been used as biomarkers in biomonitoring studies and also as effect criteria in toxicity assays.

1.2. PRINCIPLE OF THE METHOD

The method described here is that of McCord and Fridovich (1969) adapted to a microplate (Lima *et al.*, 2007). SOD catalyses the dismutation of reactive superoxide anions to hydrogen peroxide. The xanthine–xanthine oxidase system produces superoxide radicals. The cytochrome c is reduced by the superoxide anion. This reaction is monitored at 550 nm. SOD activity is given in SOD units (1 SOD unit = 50% inhibition of the reduction of cytochrome c) per mg protein.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Microplate reader

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- TRITON X-100
- di-Sodium hydrogen phosphate dodecahydrate ($Na_2HPO_4.12H_2O$)
- Sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$)
- Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2-EDTA)
- Sodium hydroxide (NaOH)
- Xanthine
- Cytochrome c
- Xanthine oxidase
- Superoxide dismutase (SOD)
- Bovin γ-globulines
- Bio-Rad protein reagent

Homogenisation buffer (Phosphate buffer 0.05 M, pH 7.0 with 0.1% TRITON X-100)
Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at 4ºC).
The TRITON X-100 must be added gently and in a final concentration of 0.1%.

Phosphate buffer 0.05 M, pH 7.8 with 1 mM Na_2-EDTA
Prepare a solution of $Na_2HPO_4.12H_2O$ 0.05 M with 1 mM Na_2-EDTA in u.p. water.
Prepare a solution of $NaH_2PO_4.2H_2O$ 0.05 M with 1 mM Na_2-EDTA in u.p. water.

Add the $Na_2HPO_4.12H_2O$ solution to the $NaH_2PO_4.2H_2O$ solution to obtain pH 7.8 (keep at 4ºC).

Stable as long as no bacterial contamination occurs.

NaOH solution 0.001N in u.p. water H_2O

Xanthine solution 0.7 mM in NaOH solution
(carefully heat the solution to dissolve the xanthine)

Cytochrome c solution 0.03 mM in phosphate buffer
(protect from light and keep at 4ºC)

Na_2-EDTA solution 0.1 mM in u.p. H_2O

Reaction solution
5 ml Xanthine solution + 20 ml cytochrome c solution (protect from light and keep at 4ºC).

Xanthine oxidase solution (0.3 U/ml) in Na_2-EDTA solution
(protect from light and keep at 4ºC)

Diluted Bio-Rad solution 1:4 (v:v) in u.p.H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globuline in u.p. water (store at –20ºC).

SOD standard (30 U/ml)
(store at –80ºC)

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm long) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- At least three fish (that is three livers) are needed for this assay.
- Remove the liver from each fish.
- Homogenise the three livers in 1:10 (w:v) homogenisation buffer.
- Centrifuge the homogenised material at 15,000*g*, 4ºC, for 15 min.
- Store the supernatant at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. SOD ASSAY IN A MICROPLATE

- Quantify the protein content of the supernatant and adjust it to 1.0 mg/ml.
- Use a 1:20 dilution of SOD standard.
- Perform four replicates for each sample.

	P0	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**
Phosphate buffer	50 µl	42 µl	33 µl	25 µl	17 µl	8 µl	0 µl
SOD	0 µl	8 µl	17 µl	25 µl	33 µl	42 µl	50 µl

	P0….P6	**Sample**
Reaction solution	200 µl	200 µl
Supernatant	-	50 µl
Xanthine oxidase	50 µl	50 µl

- *Read absorbance values at* 550 nm *every 10 s for 2 min.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See protocol for ChE determination.

3.3.3. DETERMINATION OF SOD ACTIVITY

- SOD activity is obtained by extrapolation from the standard SOD curve (power 2) and the results are expressed in U/mg protein.

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

McCord, J. and Fridovich, I. (1969). Superoxide Dismutase, an enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* **244**(22), 6049–6055.

Lima, I., Moreira, S.M., Soares, A.M.V.M. and Guilhermino L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* **66**, 1230–1242.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 57–149.

Procedural Guideline No. 9
CATALASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) LIVER

1. INTRODUCTION

1.1. USES

Catalase (CAT) is an enzyme that facilitates the removal of hydrogen peroxide, which is metabolised to molecular oxygen and water, and therefore it is an important component of anti-oxidative stress defences. In the last few years it has been used as a biomarker in biomonitoring studies and in effect criteria in toxicity tests.

1.2. PRINCIPLE OF THE METHOD

CAT decomposes the hydrogen peroxide in molecular oxygen and water. In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm (ε = 0.04 $mM^{-1}\,cm^{-1}$). The method described here is that of Aebi (1984).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Spectrophotometer

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Cuvettes
- Microplates
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- TRITON X-100
- Hydrogen peroxide (H_2O_2)
- Bovin γ-globuline
- Bio-Rad protein assay

Homogenisation buffer (Phosphate buffer 0.05 M, pH 7.0 with 0.1% TRITON X-100)

Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at 4ºC).
The TRITON X-100 must be added gently and in a final concentration of 0.1%.

Phosphate buffer 0.05 M, pH 7.0

Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at 4ºC).

Stable as long as no bacterial contamination occurs.

H_2O_2 solution 30 mM in phosphate buffer
(protect from light and keep at 4ºC)

Freshly prepared.

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globuline in u.p. water (store at –20ºC).

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm long) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- At least three livers are required for this assay.
- Remove the liver from each fish.
- Homogenise three livers in 1:10 (w:v) homogenisation buffer.
- Centrifuge the homogenised at 15,000*g*, 4ºC, for 15 min.
- Store the supernatant at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. CAT ASSAY IN CUVETTE

- Quantify the protein content of the supernatant and adjust it to 1.0 mg/ml.

	Blank	**Sample**
Phosphate buffer	200 µl	-
Supernatant	400 µl	400 µl
H_2O_2 solution	-	200 µl

- *Start reaction with H_2O_2 and read absorbance values at 240 nm for 30 s.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See protocol for ChE determination.

3.3.3. Determination of CAT activity

- CAT activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(0.04 \times 1.0)(VA/VT)} \times \frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

0.04 = extinction coefficient for H_2O_2 (mM^{-1} cm^{-1})

1.0 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (200 µl)

VT = total volume placed in each plate well (600 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample in mg/ml

4. REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol.* **108**, 121–126.

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 57–149.

Procedural Guideline No. 10
GLUTATHIONE PEROXIDASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) LIVER

1. INTRODUCTION

1.1. USES

Glutathione peroxidase (GPx) catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH (reduced glutathione) to its oxidised form (GSSG, oxidised disulphide glutathione). The main difference between catase and peroxidases is that CAT uses one molecule of H_2O_2 as donor in the reduction of another H_2O_2, while peroxidase employs other reductants. The principal peroxidase in fish is the selenium-dependent tetrameric cytosolic enzyme that employs GSH as a cofactor. GPx has an important role in protecting membranes from damage due to lipid peroxidation. It has been used in biomonitoring studies and as effect criteria in toxicity assays.

1.2. PRINCIPLE OF THE METHOD

GPx catalyses the reduction of H_2O_2 to water and the oxidation of GSH to GSSG. GPx activity is measured indirectly using another enzyme, glutathione reductase. Glutathione reductase catalyses the reduction of GSSG (produced in the previous reaction by GPx) to GSH with the concomitant oxidation of NADPH to $NADP^+$. GR activity is measured by monitoring the decrease in NADPH levels at 340 nm (ε = 6.2 $mM^{-1}cm^{-1}$). In the scope of the EROCIPS Project, the method of Flohé and Günzler (1984) adapted to a microplate (Lima *et al*., 2007) has been used.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Microplate reader

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- TRITON X-100
- Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2-EDTA)
- Dithiothreitol (DTT)
- Sodium azide (NaN_3)
- Tris(hydroxymethyl)-aminomethane (Tris)
- Hydrochloric acid (HCL) 32%
- L-Glutathione reduced (GSH)
- Glutathione reductase (GR)
- β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH)
- Hydrogen peroxide (H_2O_2)

- Bovin γ-globuline
- Bio-Rad protein reagent

Homogenisation buffer (Phosphate buffer 0.05 M, pH 7.0 with 0.1% TRITON X-100)
Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at 4ºC).
The TRITON X-100 must be added gently and in a final concentration of 0.1%.

Phosphate buffer (A) 0.1 M, pH 7.5 with 2 mM Na_2-EDTA and 1 mM DTT
Prepare a solution of K_2HPO_4 0.1 M with 2 mM Na_2-EDTA and 1 mM DTT in u.p. water.
Prepare a solution of KH_2PO_4 0.1 M with 2 mM Na_2-EDTA and 1 mM DTT in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.5 (keep at 4ºC).

Phosphate buffer (B) 0.1 M, pH 7.5 with 2 mM Na_2-EDTA and 1 mM DTT with NaN_3
0.0065 g NaN_3 + 100 ml phosphate buffer (A).

Tris buffer 0.01 M, pH 7.0 in u.p.H_2O
(keep at 4ºC)

Add HCl to obtain pH 7.0 (keep at 4ºC).

Stable as long as no bacterial contamination occurs.

NADPH solution 1 mM in Tris buffer
(protect from light and keep at 4ºC)

GSH solution 0.2 mM in phosphate buffer (A)
(protect from light and keep at 4ºC)

GR solution 30 U/ml in phosphate buffer (A)
(protect from light and keep at 4ºC).

H_2O_2 solution 6 mM in phosphate buffer (A)
(protect from light and keep at 4ºC)

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globulines in u.p. water (store at –20ºC).

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm long) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- At least three livers are required for this assay.
- Fish are sacrificed by decapitation.
- Remove the liver from each fish.
- Homogenise three livers in 1:10 (w:v) homogenisation buffer.
- Centrifuge the homogenised material at 15,000*g*, 4ºC, for 15 min.
- Store the supernatant at –80ºC.

3.3. PARAMETERS MEASUREMENT METHOD

3.3.1. GPx ASSAY IN MICROPLATE

- Quantify the protein content of the supernatant and adjust for 1.0 mg/ml.
- Perform four replicates for each sample.

	Blank 1	**Blank 2**	**Sample**
Phosphate buffer	180 µl	160 µl	130 µl
Supernatant	-	50 µl	50 µl
GSH	30 µl	30 µl	30 µl
GR	30 µl	30 µl	30 µl

- Incubate microplate for 10 min.

NADPH	30 µl	30 µl	30 µl
H_2O_2	30 µl	-	30 µl

- *Start reaction with H_2O_2 and read absorbance values at 340 nm for 5 min, with readings between 10 s.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See protocol for ChE determination.

3.3.3. DETERMINATION OF GPX ACTIVITY

- GPx activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(6.2\times 0.9)(VA/VT)}\times\frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

6.2 = extinction coefficient for NADPH (mM^{-1} cm^{-1})

0.9 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (50 µl)

VT = total volume placed in each plate well (300 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample in mg/ml

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilising the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

Flohé, L. and Günzler, W.A. (1984). Assays of glutathione peroxidase. *Methods Enzimol.* **105**, 114–121.

Lima, I., Moreira, S.M., Soares, A.M.V.M. and Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* **66**, 1230–1242.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 7–149.

Procedural Guideline No. 11
GLUTATHIONE REDUCTASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) LIVER

1. INTRODUCTION

1.1. USES

Glutathione reductase (GR) is a very important enzyme of the antioxidant system, because it maintains the GSH (reduced glutathione) /GSSG (oxidised disulphide glutathione) homeostasis under stress conditions, reducing the GSSG to GSH. This ratio between GSH and GSSG has been suggested as a potential biomarker of oxidative stress itself and the quantification of glutathione reductase activity may be important in this case. It has been used as biomarker in biomonitoring programs and as effect criteria in toxicity tests.

1.2. PRINCIPLE OF THE METHOD

GR catalyses the reduction of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^+$. GR activity is measured by monitoring the decrease in NADPH levels at 340 nm (ε = 6.2 mM^{-1} cm^{-1}). Here the method of Carlberg and Mannervik (1975) adapted to a microplate (Lima *et al*., 2007) is used.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Microplate reader

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- TRITON X-100
- Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2-EDTA)
- Tris(hydroxymethyl)-aminomethane (Tris)
- Hydrochloric acid (HCl) 32%
- β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH)
- L-Glutathione oxidised (GSSG)
- Bovin γ-globuline
- Bio-Rad protein reagent

Homogenisation buffer (Phosphate buffer 0.05 M, pH 7.0 with 0.1% TRITON X-100)

Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at 4ºC).
The TRITON X-100 must be added gently and in a final concentration of 0.1%.

Phosphate buffer 0.1 M, pH 7.5
Prepare a solution of K_2HPO_4 0.1 M with 2 mM Na_2-EDTA in u.p. water.
Prepare a solution of KH_2PO_4 0.1 M with 2 mM Na_2-EDTA in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.2 (keep at 4ºC).

Stable as long as no bacterial contamination occurs.

Tris solution 10 mM in u.p. H_2O
(protect from light and keep at 4ºC)

NADPH solution 1 mM in Tris solution
(protect from light and keep at 4ºC)

GSSG solution 5 mM in u.p. H_2O
(protect from light and keep at 4ºC)

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globulines in u.p. water (store at –20ºC).

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm long) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- At least three fish (three livers) are required for this assay.
- Fish are sacrificed by decapitation.
- Remove the liver from each fish.
- Homogenise three livers in 1:10 (w:v) homogenisation buffer.
- Centrifuge the homogenised material at 15,000*g*, 4ºC, for 15 min.
- Store the supernatant at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. GR ASSAY IN A MICROPLATE

- Quantify the protein content of the supernatant and adjust it to 1.0 mg/ml.
- Perform four replicates for each sample.

	Blank	**Sample**
Phosphate buffer	240 µl	190 µl
GSSG	30 µl	30 µl
Supernatant	-	50 µl

- Incubate the microplate for 10 min.

- *Start reaction with 30 µl NADPH and read absorbance values at 340 nm every 10 s for 5 min.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See protocol for ChE determination.

3.3.3. DETERMINATION OF GR ACTIVITY

- GR activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(6.2 \times 0.81)(VA/VT)} \times \frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

6.2 = extinction coefficient for NADPH (mM^{-1} cm^{-1})

0.81 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (50 µl)

VT = total volume placed in each plate well (270 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample in mg/ml

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

Carlberg, I. and Mannervik, B. (1974). Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* **250**(14), 5475–5480.

Lima, I., Moreira, S.M., Soares, A.M.V.M. and Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* **66**, 1230–1242.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 57–149.

Procedural Guideline No. 12
LIPID PEROXIDATION IN FISH (*Pomatoschistus microps*) LIVER

1. INTRODUCTION

1.1. USES

In spite of the existence of physiological mechanisms of control of reactive oxygen species (ROS), sometimes oxidative damage occurs. Lipid peroxidation (LPO) (oxidation of polyunsaturated fatty acids) is a very important consequence of oxidative stress due to extensive production and/or reduced protective capacity of the organisms. LPO has been used in biomonitoring programs and in toxicity assays.

1.2. PRINCIPLE OF THE METHOD

LPO may be measured by the quantification of thiobarbituric acid reactive substances (TBARS). This method is based on the reaction of compounds such as malondialdehyde (MDA), formed by degradation of initial products of free radical attack, with 2-thio-barbituric acid (TBA). The amount of TBARS is measured by reading the absorbance at 535 nm (ε = 1.56 x 10^5 M^{-1} cm^{-1}), and results are expressed as nmol of TBARS per g of tissue. The methodology described here is that of Ohkawa *et al.* (1979).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Spectrophotometer

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- 15 ml tubes (+100ºC resistant)
- Cuvettes
- Micropipettes

2.3. REAGENTS

- di-Potassium hydrogen phosphate (K_2HPO_4)
- Potassium dihydrogen phosphate (KH_2PO_4)
- TRITON X-100
- Tris(hydroxymethyl)-aminomethane (Tris)
- Hydrochloric acid (HCl) 32%
- Butylated hydroxytoluene (BHT)
- Trichloroacetic acid (TCA)
- Diethylenetriaminepentacetic acid (DTPA)
- 2-Thiobarbituric acid (TBA)

Homogenisation buffer (Phosphate buffer 0.05 M, pH 7.0 with 0.1% TRITON X-100)

Prepare a solution of K_2HPO_4 0.05 M in u.p. water.
Prepare a solution of KH_2PO_4 0.05 M in u.p. water.

Add the KH_2PO_4 solution to the K_2HPO_4 solution to obtain pH 7.0 (keep at –4ºC).
The TRITON X-100 must be added gently and in a final concentration of 0.1%.

Tris buffer 60 mM, pH 7.4 with 0.1 mM DTPA in u.p. H_2O
Add HCl to obtain pH 7.4.

Stable as long as no bacterial contamination occurs.

BHT 4 % solution in methanol
(protect from light and keep at 4ºC)

TCA 12% solution in u.p. H_2O
(protect from light and keep at 4ºC)

Stable for one month.

TBA 0.73% solution in u.p. H_2O
(carefully heat the solution to dissolve the TBA, protect from light and keep at 4ºC)

Freshly prepared.

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- At least three fish (three livers) are required for this assay.
- Remove the liver from each fish.
- Homogenise three livers in 1:10 (w:v) homogenisation buffer.
- Centrifuge the homogenised material at 15,000*g*, 4ºC, for 15 min.
- Store the supernatant at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. LPO ASSAY

- In a 15 ml tube.

	Blank	Sample
Supernatant	-	100 µl
TCA	1000 µl	1000 µl
Tris buffer	1000 µl	900 µl
TBA	1000 µl	1000 µl

- Incubate at 100ºC for 60 min.
- Remove 1.5 ml from the 15 ml tube to an eppendorf.
- Centrifuge at 12,000 rpm, at room temperature, for 5 min.
- Transfer the supernatant to a cuvette and read the absorbance at 535 nm.

3.3.2. DETERMINATION OF TBARS CONCENTRATION

- TBARS concentration (nmol of TBARS per g of tissue):

$$\frac{\text{Abs}}{1.56\times10^{5}\times1}\times3{,}000\times10^{5}$$

where

Abs = absorbance at 535 nm

1.56×10^5 = extinction coefficient for TBARS (M^{-1} cm^{-1})

1 = depth of plate well to be crossed by light (cm)

3000×10^5 = correction factor in order to express results in nmol/g tissue

4. REFERENCES

Lima, I., Moreira, S.M., Soares, A.M.V.M. and Guilhermino, L. (2007). Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. *Chemosphere* **66**, 1230–1242.

Ohkawa, H., Ohishi, N. and Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**, 351–358.

Van der Oost, R., Beyer, J. and Vermeulen, N.P.E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* **13**(2), 57–149.

Procedural Guideline No. 13
LACTATE DEHYDROGENASE ACTIVITY ASSAY IN FISH (*Pomatoschistus microps*) MUSCLE

1. INTRODUCTION

1.1. USES

Lactate dehydrogenase (LDH) is the terminal enzyme of anaerobic glycolysis. It is a key enzyme in muscular physiology, particularly in conditions of chemical stress, when high levels of energy are required in short periods of time. It has been used in biomonitoring programs and in toxicity assays.

1.2. PRINCIPLE OF THE METHOD

LDH reduce pyruvate and oxidise NADH forming lactate and NAD^+. It also catalyses the reversal reaction. LDH activity may be calculated from the amount of pyruvate consumed, by continuously monitoring the decrease in absorbance due to the oxidation of NADH at 339 nm (ε = 6.3 mM^{-1} cm^{-1}), following the method of Vassault (1983) adapted to a microplate (Diamantino *et al*., 2001).

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Homogeniser
- Centrifuge
- Microplate reader
- Micropipettes

2.2. MATERIALS

- Eppendorfs (1.5 ml)
- Microplates

2.3. REAGENTS

- Tris(hydroxymethyl)-aminomethane (Tris)
- Sodium chloride (NaCl)
- Pyruvate
- β-nicotinamide adenine dinucleotide reduced form (NADH)
- Hydrochloric acid (HCl) 32%
- Bovin γ-globulines
- Bio-Rad protein reagent

Tris/NaCl buffer (Tris 81.3 mM; NaCl 203.3 mM) pH 7.2

Adjust to pH 7.2 with HCl 32%. Make up to 500 ml with u.p. water (keep at –4ºC).

Stable as long as no bacterial contamination occurs.

Tris/NaCl/NADH solution (NADH 0.244 mM in Tris/NaCl buffer)

(keep at –4ºC)

Can be frozen and stored for about 1 month.

Tris/NaCl/Pyruvate solution (Pyruvate 9.76 mM in Tris/NaCl buffer)
(keep at –4ºC)

Prepare on the day of use.

Diluted Bio-Rad solution 1:4 (v:v) in u.p. H_2O

Freshly prepared.

Standard protein solution
1 mg/ml of bovin γ-globulines in u.p. water (store at –20ºC).

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- *Pomatoschistus microps* (2.5–3 cm long) are collected with a hand-operated net, during low tide, and immediately transported to the laboratory.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- One fish (one dorsal muscle is in general enough) is required for this assay.
- Remove the one dorsal muscle from the fish.
- Homogenise the muscle portion in 1 ml of phosphate buffer.
- Move the samples from –80ºC to –4ºC until they defrost.
- Perform two freeze/unfreeze cycles.
- Centrifuge the homogenised material at 6,000 rpm, 4ºC, for 3 min.
- Store the samples at –80ºC.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. LDH ASSAY IN MICROPLATE

- Quantify the protein content of the supernatant and adjust it to 0.9 mg/ml.
- Perform four replicates for each sample.

	Blank 1	**Sample**
Tris/NaCl Buffer	40 µl	-
Supernatant	-	40 µl
Tris/NaCl/NADH	250 µl	250 µl
Tris/NaCl/Pyruvate	40 µl	40 µl

- *Start reaction with 40 µl of Tris/NaCl/Pyruvate and **immediately** read absorbance at 339 or 340 nm every 10 s for 3 min.*
- ***Keep temperature at 25ºC.***

3.3.2. PROTEIN ASSAY (Bradford, 1976)

- See protocol for ChE determination.

3.3.3. DETERMINATION OF LDH ACTIVITY

- LDH activity (nmol of substrate hydrolised per min per mg of protein):

$$\frac{1}{(6.3\times 0.99)(VA/VT)}\times\frac{OD}{\text{sample protein content}}$$

where

OD = optical density (mOD min^{-1})

6.3 = extinction coefficient for NADH (mM^{-1} cm^{-1})

0.99 = depth of plate well to be crossed by light (cm)

VA = sample volume placed in each plate well for the enzymatic assay (40 µl)

VT = total volume placed in each plate well (330 µl)

Sample protein content = mean protein in the sample (average of four replicates) for each sample in mg/ml.

4. REFERENCES

Bradford, M. (1976). A rapid and sensitive method for the quantification of microgram quantities of proteins, utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248–254.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M. and Guilhermino, L. (2001). Lactate dehydrogenase activity as an effect criterion in toxicity tests with *Daphnia magna* Straus. *Chemosphere* **45**, 553–560.

Vassault, A. (1983). Lactate dehydrogenase, in Bergmeyer, H.O. (ed.), *Methods of Enzymatic Analysis, vol. III, Enzymes: Oxireductases, Transeferases*. Academic Press, New York, pp. 118–126.

Procedural Guideline No. 14
THE NEUTRAL RED RETENTION ASSAY IN THE LYSOSOMES OF MUSSEL (*Mytilus* sp.) HAEMOCYTES

1. INTRODUCTION

1.1. USES

Because marine mussels are filter feeders, and have the ability to accumulate a great variety of contaminants, they have been widely used in monitoring programs. Mussel haemocytes are involved in a variety of functions, such as defence, and have a well-developed lysosomal system. Lysosomes are very important organelles, as they accumulate substances such as toxic metals and organic chemicals. When the amount of these substances exceeds the lysosome's capability to accumulate them, its membrane is affected and releases hydrolytic enzymes into the cytoplasm, leading to severe cell damage.

The neutral red retention (NRRT) assay measures the lysosomal integrity, and thus haemocyte condition, reflecting the exposure of animals to contaminants.

1.2. PRINCIPLE OF THE METHOD

Neutral red is a weak base that in the unprotonated form permeates the lysosomal membrane, where it becomes protonated and, consequently, trapped. In healthy cells, lysosomes can take up and retain neutral red, but when cells are exposed to contaminants, the membrane is damaged and lysosome starts leaking into the cytosol.

This technique consists of measuring the retention time of neutral red, by observing mussel haemocytes under a transmitted light microscope for 1 min, every 30 min after the dye application. A high retention time reflects an undamaged lysosomal membrane.

2. EQUIPMENT, MATERIALS, REAGENTS

2.1. EQUIPMENT

- Transmitted blue light optical microscope

2.2. MATERIALS

- Micropipettes
- Syringes (1 ml)
- Needles (0.8 x 40 mm)
- Scissors
- Forceps
- Humidified dark chamber
- Light proof jars
- Siliconised microtubes
- Slides
- Cover slips

2.3. REAGENTS

- HEPES
- NaCl
- $MgSO_4$
- KCl

- $CaCl_2$
- NaOH
- Neutral red

Physiological saline solution
To ensure haemocyte integrity during the assay, a saline solution must be prepared:
4.77g HEPES + 25.48 g NaCl+ 13.06 $MgSO_4$ + 0.75g KCl + 1.47g $CaCl_2$ (dissolved in 1000 ml volume, pH 7.3, adjusted with NaOH solution).

Store at 4^{o}C until required, and then allow the solution to reach room temperature and re-adjust the pH if necessary.

Neutral red (stock solution)
28.8 mg neutral red/1 ml DMSO.
Store at 4^{o}C, in a lightproof container for up to three weeks.

Neutral red (working solution)
10 µl neutral red stock solution + 5 ml saline solution.
A fresh solution must be prepared for each experiment and maintained in a lightproof bottle.

3. PROCEDURES

3.1. SAMPLE COLLECTION IN THE FIELD

- Collect 2.5–3.5 cm mussels during the low ebb tides, carefully, and using a knife not to harm the animal's byssus.
- During transport to the laboratory, keep them in a thermo box at about 10 ± 1ºC.
- Place the mussels in a recipient containing 5 l artificial saline water for 1 day.

3.2. SAMPLE COLLECTION IN THE LABORATORY

- Open partially the mussel valves, keeping them apart with the help of forceps, and expose the posterior adductor muscle (this is a white, dense structure that attaches the upper and lower valves).
- Put about 100 µl of saline solution in the syringe and withdraw the same volume of haemolymph from the muscle (keep the valves slightly opened).
- Remove the needle carefully and transfer the syringe content to a siliconised microtube.
- Invert the microtube and place it at 4^{o}C for 30 min.
- After this period, invert each microtube and pipette 40 µl of haemolymph/saline solution onto the centre of a slide.
- Store the slide in a humidified dark chamber for 15 min, allowing the haemocytes to settle down.
- Remove the excess water by gently shaking the slide.
- Add 40 µl of neutral red working solution by placing the pipette tip onto the slide and applying the dye gently onto it.
- Cover the slide with a cover slit and place it in the humid chamber for 15 min incubation.

3.3. PARAMETER MEASUREMENT METHOD

3.3.1. MEASUREMENT OF LYSOSOMAL STABILITY

- Carry out the haemocyte observation using an optical transmission microscope equipped with a blue filter.
- Use the 20x lens to locate the cells and the 40x lens to evaluate lysosomal integrity.
- Maintain the same light intensity in all observations, at a minimum workable level.
- Observe the cells just for 1 min and then transfer them back to the dark recipient.
- 15 min later, carry out a new analysis.

- Repeat this procedure every 30 min, up to a maximum of 180 min (usually, all haemocytes, even from non-contaminated sites, are damaged 180 min after neutral red exposure) and record the time when the slide is altered.
- A slide is considered altered when 50% or more of the haemocytes present increased lysosomal volume, or when the lysosomal membrane is damaged and the neutral red solution is leaking into the citosol.
- The average neutral red retention time is calculated using the results from at least ten individuals per site.

4. REFERENCES

Camus, L., Grosvik, B.E., Borseth, J.F., Jones, M.B. and Depledge, M.H. (2000). Stability of lysosomal and cell membranes in haemocytes of the common mussel (*Mytilus edulis*): effect of low temperatures. *Mar. Environ. Res.* **50**, 325–329.

Castro, M., Santos, M.M., Monteiro, N.M. and Vieira, N. (2004). Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. *Mar. Environ. Res.* **58**, 741–745.

Dailianisa, S., Domouhtsidoub, G.P., Raftopouloub, E., Kaloyiannia, M. and Dimitriadisb, V.K. (2003). Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. *Mar. Environ. Res.* **56**, 443–470.

Lowe, D.M. and Pipe, R.K. (1994). Contaminant induced lysosomal membrane damage in marine mussel digestive cells: an *in vitro* study. *Aquat. Toxicol.* **30**, 357–365.

Lowe, D.M., Soverchia, C. and Moore, M.N. (1995). Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquat. Toxicol.* **33**, 105-112.