Detailed description of the project

Objectives

Context - During the last years, there is been major accidental spill of oil and chemical at sea that hit strongly the coastal zone of European countries. These events have been accompanied by significant socio-economical consequences and large environmental effects that need to be addressed among the Member States. Both local and national authorities have to answer rapidly to sea industrials and the public opinion who inquire about the impact on the health of the marine environment and also the status of recovery. Because spill events are transboundary by nature, debates should be promoted within the scientific community of the Member States in view to help in political and environmental decision making. Beyond the immediate actions and emergency responses undertaken to clean up and recover as much as possible the substances spilled at sea, some of the central questions address the criteria to use in the assessment of the impact of the pollution on sea life and also how to safely determine restoration of the impacted zone. These questions are particularly important for example for the removal of fisheries restrictions post-spill. In some cases, these restrictions may need to be maintained for years following a spill because of persistent contamination or taint. Also, there is always the threat of serious long-term biological damage to the marine ecosystem (Law and Campbell, 1998).

National agencies are launching monitoring programmes with the view to address the environmental impact of spill. A review of past or existing monitoring programmes shows that environmental issues are divided into different sections addressing several themes i.e. the characterisation and quantification of toxic substances in water, sediment and biota; the acute toxicity of the spilled substances and the long term toxicity on different species which together allow the estimation of the "ecological" impact; the assessment of cleaning activities/operations on the fauna and on the flora.

The direct acute adverse effects are immediate and related to smearing of body surfaces, ingestion, clogging of respiratory organs etc, causing death of marine organisms. The long-term toxic potential of spill is more complex to evaluate. It relates to the release of chronic toxic components in the water phase, and long-term disturbances of habitats which can affect marine populations and ultimately biodiversity. Parameters related to the physico-chemical fate and aging processes of the spilled substances are important to consider in the long-term environmental impact. They relate directly to the hazard identification, the first step in the environmental risk management process.

In this project, we will address issues that relate to the evaluation of the long lasting environmental impact of spill related to aging processes of substances from past accidents along the EU costal zone. Currently, several ways are used for the evaluation of long term impacts within the Member States. Even though each spill is characterized by unique features, a more uniform approach to spill monitoring including a greater homogenisation of methods and tools would benefit EU regulators and environmental decision making. Biodiversity study of benthic organisms or/and the comparison between contaminant levels measured in water, sediment and biota to threshold levels established by international (e.g. the ecotoxicological assessment criteria – EAC - defined by OSPAR in 1997) or national agencies have traditionally been used as the main decision criteria. Biodiversity is ecologically very relevant but this requires years of observation because significant changes in population might only be visible on a long temporal scale. Also, it is not easily applicable for organisms living in the water column. The chemical data include sensitive and accurate measurements that provide both qualitative and quantitative figures of the presence of toxic substances and hence assess the hazard of exposure to the marine biota. The threshold levels which are defined according to the EU-Technical Guidance Document (EC, 2003) allow defining the risk based on the PEC/PNEC ratio but are not an expression of the actual impacts. Also, on the long term, the analytical chemistry-based methodologies may be insufficient to detect the presence of substances because the levels are too low. One of the main problems, more specifically related to heavy oil, remains the repeated contamination of sub-lethal levels due to substances becoming trapped within sediments in low-energy costal environments, resulting in recontamination when the substances are remobilized by storms or tides. This results in potential long lasting effects that can only be perceived within organism because, due to the continuous or successive chronic exposures following remobilization and weathering processes, they may integrate durable damages. Hence, other methods based on the integration of biological change measurements should be incorporated in the existing approach to actually estimate the actual bioavailability of the toxic substances and their consequent effects on biota. More recently, criteria based on the measurements of biological markers ("biomarkers") capable of identifying subtle changes with important consequences for the organisms sampled in polluted zone have been proposed, tested in large environmental programmes (BEEP, BEQUALM) and used extensively in monitoring of spill in real cases (e.g. the *"Erika"* oil spill monitoring programme of the ecological and ecotoxicological consequences, (Laubier et al., 2004)). A suite of different parameters can be examined using different analytical methodologies in order to assess exposure to or effects of environmental pollutants on aquatic ecosystems (figure 1). Some of these methodologies can be specifically related to a type of pollutant or rather general establishing then the health status of organisms under investigations. Compared to the measurement of the presence of chemicals in the water or sediment that pose a risk for biota, this approach allows to actually assess the impacts. To date, the biomarker approach seems to provide sensitive and sound protocols which could serve as a common platform for all EU Member States and complement existing regulations for spill monitoring.

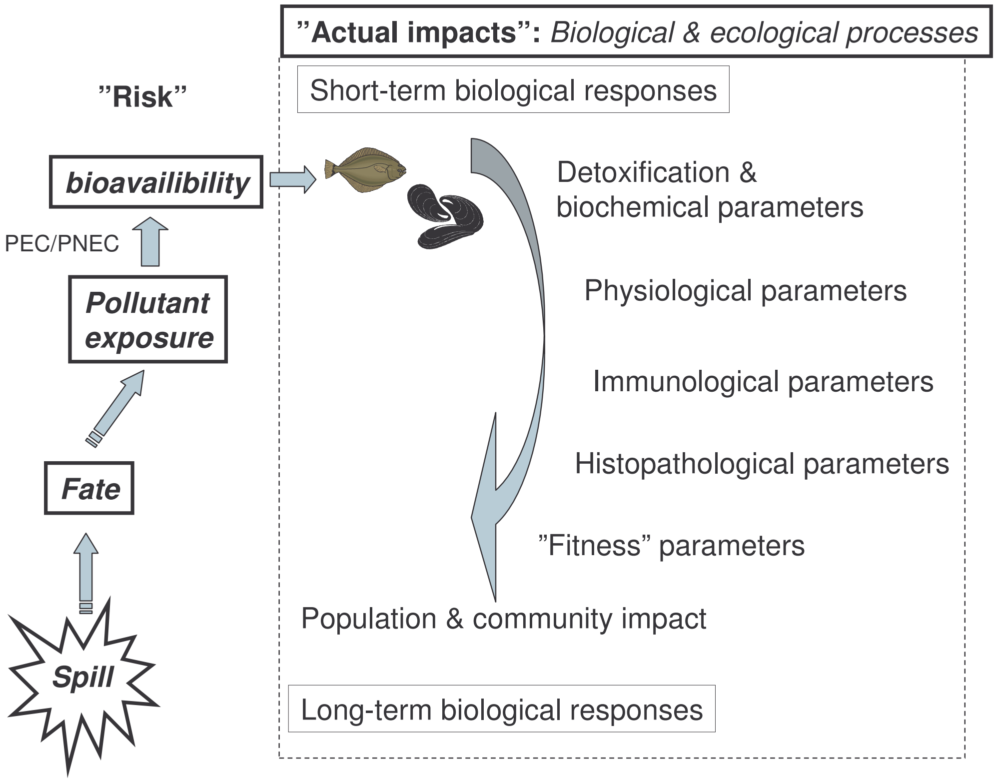


Figure 1 – The "biomarker" approach as a way to assess actual impacts to marine ecosystem

The ability of these methods to distinguish between natural variations and anthropogenic change after a spill is an important issue. This requires the use of not only sensitive methods but observations made at the correct temporal and spatial resolution. The development of an integrated chemical-and biological based approach to the management and monitoring of marine pollution is leading to a requirement for data to be collected with great frequency and spatial resolution in order to describe variability and temporal change, and assess properly the environmental status. One way is to increase the number of sample analyses but the cost of "classical" analytical work has a load which hinder the inclusion of several sites and frequent sampling. One alternative is to deploy devices that can gather data rapidly and at low cost and hence provide a rapid mapping of pollution at a better spatial and temporal scale. Lately, a range of sensors based on physical, chemical and biological responses is being developed that can have direct application for marine environmental purposes (Kroger et al., 2002; Lucarelli et al., 2003). A combination of such sensors may in future marine monitoring programmes complement significantly and at a reduced cost, the current way of conducting impact assessment of spill and other aquatic pollution events. This could represent the future challenge for field monitoring studies of discrete samples and also in situ at remote sites for real-time observations (Kroger and Law, 2005b; 2005a).

Here, we would like to evaluate a battery of tools based on well-known biological methodologies and others promising tools based on biosensors that could be implemented in current EU guidelines following spill events and be proposed to monitor the environmental effect of spills. A two-step pragmatic approach using a combination of these methodologies will be proposed to complement these guidelines. Based on this approach, a contingency plan could be prepared and distributed among the EU Member States to define immediate criteria regarding the environmental impact of spill at sea. Also, this would contribute to the preparation of environmental monitoring programmes by defining adequate practises.

Based on past or existing monitoring programmes, a selection of sound protocols and manageable methodologies will be made and their outcome could be incorporated in regulatory guidelines used as a common platform at the EU level. Hence, additional analyses based on biological criteria could complement the existing environmental guidelines based on chemical analysis and be proposed for the monitoring and the impact assessment of spill.
More generally, this project will serve as a platform where scientists from European costal countries exchange views and highlight some important aspects of accidental marine pollution related to the environmental impact. This will be promoted through their participation in pilot studies aiming at implementing technical and scientific expertise in the field of marine pollution and a final workshop (see specific application for the workshop: project leader Cedre) including the partners, external scientists, regulators and industrials that will address the outcomes of the project to the current EU regulatory guidelines.

Specific link to the present EU call -The behaviour, fate and impact on biological system are topics that are embraced in monitoring programmes following a spill (see for example (Laubier et al., 2004), concerning the "*Erika*" oil spill monitoring programme; (Law and Kelly, 2004), concerning the impact of the "*Sea Empress"* oil spill; the "*Baltic Carrier*" oil spill monitoring program, (Pecseli et al., 2002); Law et al., (2003) concerning the "*Ieovil Sun*" chemical spill). Indeed, in all accidental discharges, it is clearly shown that the behaviour of the substances in the sea is very much dependent on different factors and each spill is unique in that way. That influences the fate of spilled substances at sea and consequently the environmental impact. From a risk assessment point of view, the determination of the bioavailable fraction is an important issue that needs further insights. In past accidents, some substances posed clearly a higher hazard for the environment than others. Heavy oils are fuels that can reside long in the sea in view of their specific physico-chemical nature. Typically they are characterised by low biodegradability and a high content of potentially toxic compounds like polycyclic aromatic hydrocarbons (PAH) and heavy metals that associate readily with particle matters and sediment. Hence, these substances can be significant and persistent sources of chemical contamination of the marine ecosystems after oil spill event as this was shown after the *Erika* and the *Prestige* oil spill. By this mean, they cause a threat for benthic organisms living in or close to the bottom ground, and for other organisms feeding on them, long after the spill event. Chronic levels of these compounds can be re-mobilized after tide or major storm events, thereby being further bioavailable to species including also those living in the water column. In many recent examples by heavy oil spills, it was shown that the re-mobilization of fuel from the oiled sediment can generate relatively elevated concentrations of PAH in the surroundings of organism's habitat at spilled site over long period of time (Peterson et al., 2003; Tronczynski et al., 2004). That causes a major long term environmental hazard. As weathered oil slick and patch can persist long after the spill into the sediment, substances can be released gradually or sporadically post-spill causing bioaccumulation of PAH and effects that can be comparable to chronic levels from offshore installations. This repeated type of exposure over long period of time can prolong significantly the recovery phase. Ultimately, this will depend on environmental conditions prevailing at the spilled site and the extent of the shoreline and bottom contamination by the fuel. The re-mobilization processes can be particularly important in relative shallow waters where both water-soluble PAHs and possibly PAHs transformation products like those created by photo-oxidation, may constitute a repeated threat to both benthic and pelagic species (Barron et al., 2003; Geffard et al., 2004). Shallow waters are also nursery area for many sensitive embryos and larvae that develop in the upper photic water. Hence, they can readily be exposed to several toxic parental or by-products of oil-related substances that dissolved in the water. This view may also be mitigated by the fact that a large fraction of oil products may bind firmly to the sediment and thereby sequestrate fast on the bottom and consequently decrease its actual bioavailiability to marine species (Neff, 2002).
Likewise, the behaviour and toxicity of chemicals transported at sea is generally documented but it is often based on data obtained from freshwater environment. However, this may significantly deviate in the seawater. This was illustrated for example in the case of the *Ievoli Sun* in the English Channel where a major chemical spill of mixture styrene, methyl ethyl ketone and isopropyl alcohol occurred (Law et al., 2003). In their study, Law *et al.* found only low levels of styrene contamination in edible tissues of crabs caught in the vicinity of the wreck site. However no measurements of effects were done. The GESAMP (Group of Experts on the Scientific Aspects of Marine Pollution) that carries out hazard evaluations of chemicals carried in bulk by ships has actually classified styrene as a MARPOL B because of its low potential for bioaccumulation but the recognition that it can potentially have severe toxic effects like carcinogenicity. Compared to oil-related compounds, fewer investigations about chemicals substances or their biotransformed products exist. Indeed many chemicals can be more potent than oil-related compounds but seldom have their effects

been tested on marine species and in conditions simulating spill. This lack of documentation needs to be addressed and revised so that Member States are better prepared to evaluate and respond to the possible environmental effects of toxic substances transported on sea.
The concentration levels of spilled substance are usually too low to be analytically detected in seawater samples some months after the spill. In sediments some compounds like PAH can be accumulated to a higher extent but their distribution is often patchy and thereby may vary with the locality of the sampling. In water, quality standard are decided by regulatory guidelines currently based on total concentration but studies have demonstrated that PAH composition of oil is as important. Also, the analytical detection of substances do not inform about their actual bioavailability to biota and thereby their effects on marine life. Alternatively, bioavailability of substances can be estimated by the bioaccumulation and the effects they have on biota. Organisms accumulate and integrate pollutants via several routes of uptake depending on factors like habitat, feeding habit and other biochemical-related mechanisms like metabolism. Thereby, the monitoring of environmental impact should include biological measurements made on key species including sensitive stages rather than rely solely on chemical measurements of pollutant. In particular, a better link between pollutant levels actually bioaccumulated in tissue and effects should be established. This link between dose and response in organisms is currently used in environmental risk assessment modelling which in turn help to foresee the impacts and take important environmental decisions. Some species like bivalves have been extensively used as sentinels to evaluate environmental impacts of pollution. Mollusc bivalves do accumulate a substantial amount of pollutant which they keep long in their tissues because of their relatively low metabolism. Hence, they tend to release accumulated PAH slowly compared to other organisms. Also they are particularly exposed because of the multiple route of exposure through water, inorganic and organic particles uptake. However, massive acute mortality is seldom observed in these organisms shortly after a spill. Even though they seem to tolerate relatively high exposure levels, the possible prolonged effects of repeated exposure on for example growth or reproduction is more uncertain. Other species like fish are able to metabolize and excrete accumulated substances rapidly. Thereby, their burdens do not reflect necessarily their exposure and toxic molecules have often non-detectable concentrations of toxic pollutants. Hence, tissue levels are not adequate indicators of contamination in fish. Rather, biotransformed metabolites can trigger several types of effects and metabolisation of spilled substances may on a long-term have an energetic cost solved at the expense of growth or reproduction. In bottom-living fish, it seems that the main route of contamination is through low level of dissolved compounds of pore water rather than direct contact from the sediment. Percolation of seawater through the contaminated sediment represents a source of chronic, low level exposure than can impair functional integrity and decrease larval production long after the spill event.
Essential parameters related to oil weathering like fuel solubility should be evaluated in the level of risk and assess in relation to their effects on organisms. There is recognition that mortality is not an adequate means to evaluate the environmental impact of spilled oil. Mortality is the most visible effect but other type of effects, more subtle, may have even larger impairments. These can affect essential functions of individuals like those associated with reproduction, thereby leading to long reaching ecological consequences for example to offspring.

It follows that there is a need i) to investigate better the fate, bioavailability and transformation of contaminants, ii) to use sound and cost-effective methodologies and define criteria able to document biological impairments, their relation with the bioavailable fraction and to long lasting ecological effects.
These issues will be addressed in pilot studies with the different partners of the project to give better insights in weathering of substances and their effects on selected species. These analyses will be based on substances accidentally discharged to the sea during past accidents including oil and chemicals, thereby helping to define important parameters for regulatory guidelines used for the monitoring of spill.

An integrated and pragmatic approach – Regulatory guidelines should be based on a better understanding of the fate of spilled substances and their toxicity towards key species and sensitive life stages. Laboratory-simulated exposures on which they rely, should aim at integrating as much as possible some of the important features and effects related to spill weathering. A choice of relevant species and sensitive stages should be made. Natural seasonal variations should also be included to take into consideration important natural biological variations and distinguish them from the effect of accidental spill. This might be particularly true as aging of the spill progresses.
This view relies on the implementations of methods that are able to measure biological changes in the organisms. The use of biological markers or so called biomarkers measured at the molecular,

biochemical, cellular or physiological level in individuals is becoming more and more integrated in current individual Member States environmental programmes. Most of the recent monitoring programmes that were launched following spill events integrate both chemical and biological markers. Yet, there seems to be a lack of harmonisation at the EU level and a better rationalisation of methods ought to be proposed in current decision criteria and technical guidelines among the Member States. Biomarkers have been proposed as sensitive biological tools for effect measurement, pollution monitoring and assessment environmental impact. In the North Sea, for example, they are being implemented in the assessment of chronic pollution from oil and gas industries within OSPAR (e.g. JAMP[1] programme, 1998; see also (Børseth et al., 2000)) and a draft document has recently been issued to provide guidance on the way to conduct monitoring programme (OIC, 2004). One important feature of biomarkers is that they may be indicative of long lasting effects and as such shall have a predictive value which is not the case with the measures of seawater levels only. However, these levels can be used to predict potential biological effects if prior investigations have clearly established a link between contaminant levels and responses in biological systems from ecotoxicological laboratory-based observations.

Monitoring programmes following recent spills have served to implement various biological markers that can be used to measure these responses. These are typically classified in biomarkers of exposure i.e. that indicate bioavailability, uptake and biotransformation of the toxic substances - and biomarkers of effect i.e. than can be related to disruption of important physiological functions or damage in tissues with an established or possible health impairment or disease to the organisms (Van Der Oost et al., 2003).

The common problem facing the scientists of the member states is to determine what response strategies to use, define the methodologies and select the markers that are most likely associated with actual biological effect and their ecological meaning. There are hundreds of biomarkers and it is neither cost-effective nor sound to include all of them in the monitoring of spill. Obviously, the best approach is to use a set of biomarkers that indicate changes and impairments in different functions, and also establish a link with ecologically relevant parameters indicative of long lasting effect (Moore et al., 2004). Some of the basic knowledge has to be established in laboratory-controlled conditions based on different key species representative of different niche. Finally, the full potential of using a biomarker-based monitoring approach is relying on analyses that integrate the individual variations of each biomarker in a global index of environmental quality (Cajaraville et al., 2000; Beliaeff and Burgeot, 2002). By taking into account chemical and biological criteria, this index can be used in the evaluation of environmental impact of spill and regulatory decision by governmental agencies at the level of the Member States.

The ongoing research done within national programmes and studies launched after some recent spills constitute a good platform to select the methods and techniques available, and assess their value and applicability for future monitoring programmes. This can help to prepare a contingency plan as a response to spill incident. At the EU's member level, a pragmatic framework of biomarkers ought to be defined based on state-of-the-art techniques used on past or existing monitoring programmes following spill event. The members should establish a network in order to develop and use agreed testing protocols that could form the nucleus for sustainable co-operations. Ideally, at least some of the methods selected should be cost-effective, rapid and user-friendly so that all members may be able to use them with no requirements of sophisticated facilities. For example, a development towards simple environmental analytical tools with cost-effective environmental performance should also be envisaged. Today, a promising technology may help to achieve this goal by the implementation of easy-to-use, portable devices based on "lab-on-the-chip" techniques like biosensors. Biosensors are analytical devices that closely couple a biological sensing element with a transducer that analyses within minutes the degree of interaction, affinity or effect on cells, enzymes, DNA molecules in the presence of toxicants. Finally, it is important to disseminate the knowledge and among other to improve co-operation of Member States initiative that will add scientific value and cost efficiency to each national programmes concerning accidental marine pollution.

This process will contribute in the preparation of monitoring programmes in case of other incidents.

Definition of objectives The following objectives will be aimed in the project:

- To evaluate existing methodologies based on biologically-important effect markers used in monitoring programmes and studies following some recent case studies with oil spill and chemical spill along the costal zone of the Member States
- To propose the methodologies that can be used as a common platform in environmental monitoring of spills and contingency plans

[1] Joint Assessment and Monitoring Programme developed by the Oslo and Paris Commissions (OSPAR)

- To incorporate simple, cost-efficient and manageable analytical tools in future pollution monitoring programme within the member states.
- To promote exchange of scientific and technical expertise in the field of environmental pollution monitoring
- To run pilot studies in the participants laboratories with the aim to

a. Analyse the behaviour and fate of heavy oil and styrene spilled in the marine environment, in particular with aging of the spill, taking case studies from recent accidental discharges
b. Test the selected set of biomarkers using both key species and sensitive stages in conjunction with analyses of chemical measurements as input for regulatory guidelines, and integrate the responses in an environmental index used to manage environmental spill.
c. Test simple protocols, based on rapid low-cost analytical tools for routine application of biological and chemical monitoring applicable on field

- To disseminate the protocols, research results at international/European level

Actions and means involved

Organisation of the project
The coordination of the whole project will be done formally by RF/Akvamiljø who will also have the main responsibilities for the:

- management of the project and contact with the EU commission
- technical organisation of the project
- pilot study with mussels and heavy oil/chemicals
- synthesis of project data and reporting to the EU commission
- enhancement of results dissemination

The head and directors of each applicant/partners will have the administrative responsibility of the project and contractual relationship with the commission. A technical and scientific expert group consisting of the contact person from the applicant and each partner institute/university will constitute the core management of the project. The same person will delegate and organize the work with the other staff inside their institute/university.
Each partner will be attributed tasks within the 4 main work packages of the project. In each of them, the EU partners will share the responsibility of different activities.
Cedre will be responsible for the pilot study with fish and heavy oil/chemicals that will be conducted in their research centre. They will also have the responsibility for the organisation of a workshop at the end of the project from which input to regulatory guidelines in accidental spill monitoring will be the main outcome. However, this workshop will be submitted under a separate proposal to this call and consequently the present pilot project does not include the cost related to the workshop organisation.
Both universities (UPV/EHU and UBO/LBPC) will participate with their scientific expertise in the sampling, analyses and reporting of the data from the pilot studies with marine organisms.
The University of Firenze (Unifi; contact person: Prof. Marco Mascini) will be subcontracted by RF/Akvamiljø to perform the analytical work related to the biosensors.
There will be regular updates between the project partners through exchange of information and data. An internet site will be created under the applicant website at www.rf.no/akvamiljo. A kick-off meeting will be organised at the RF/Akvamiljø shortly after the funding agreement by the EU and a final meeting will be held at Cedre. (see figures 2 and 3).

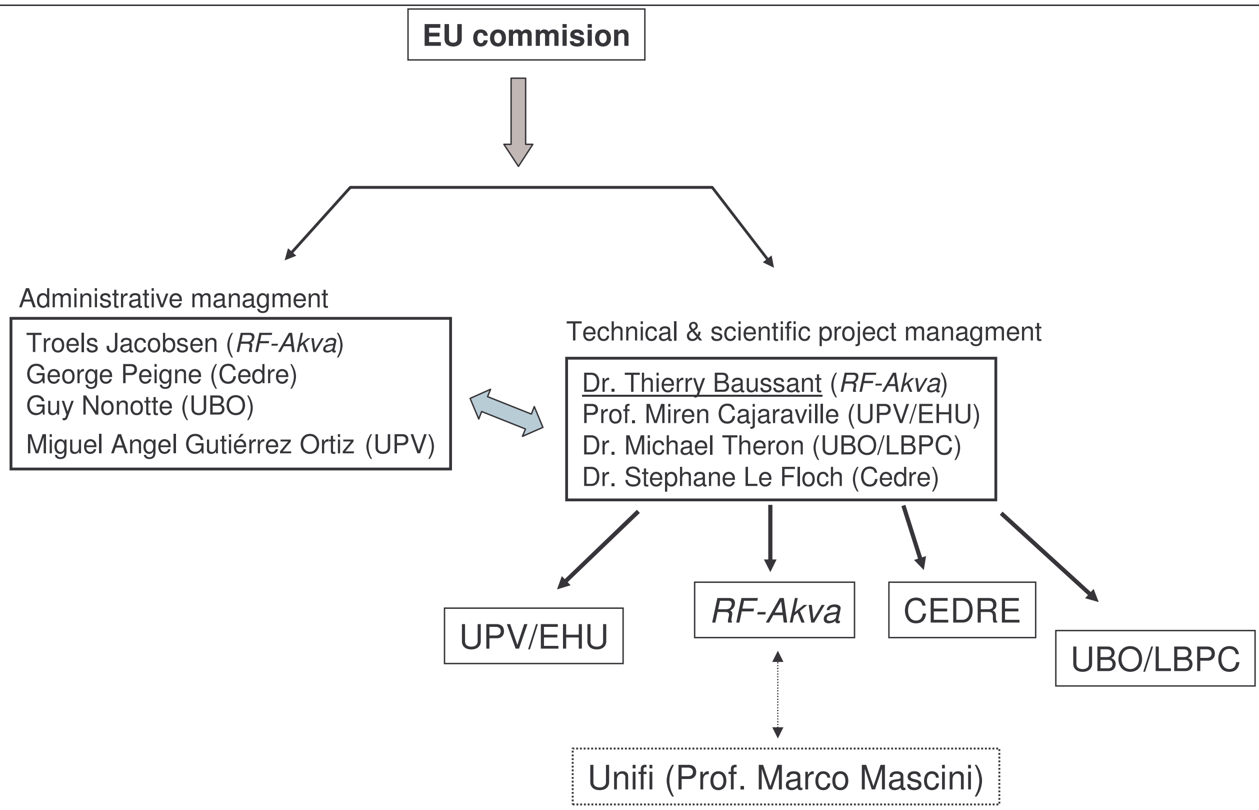


Fig.2 – Project management system

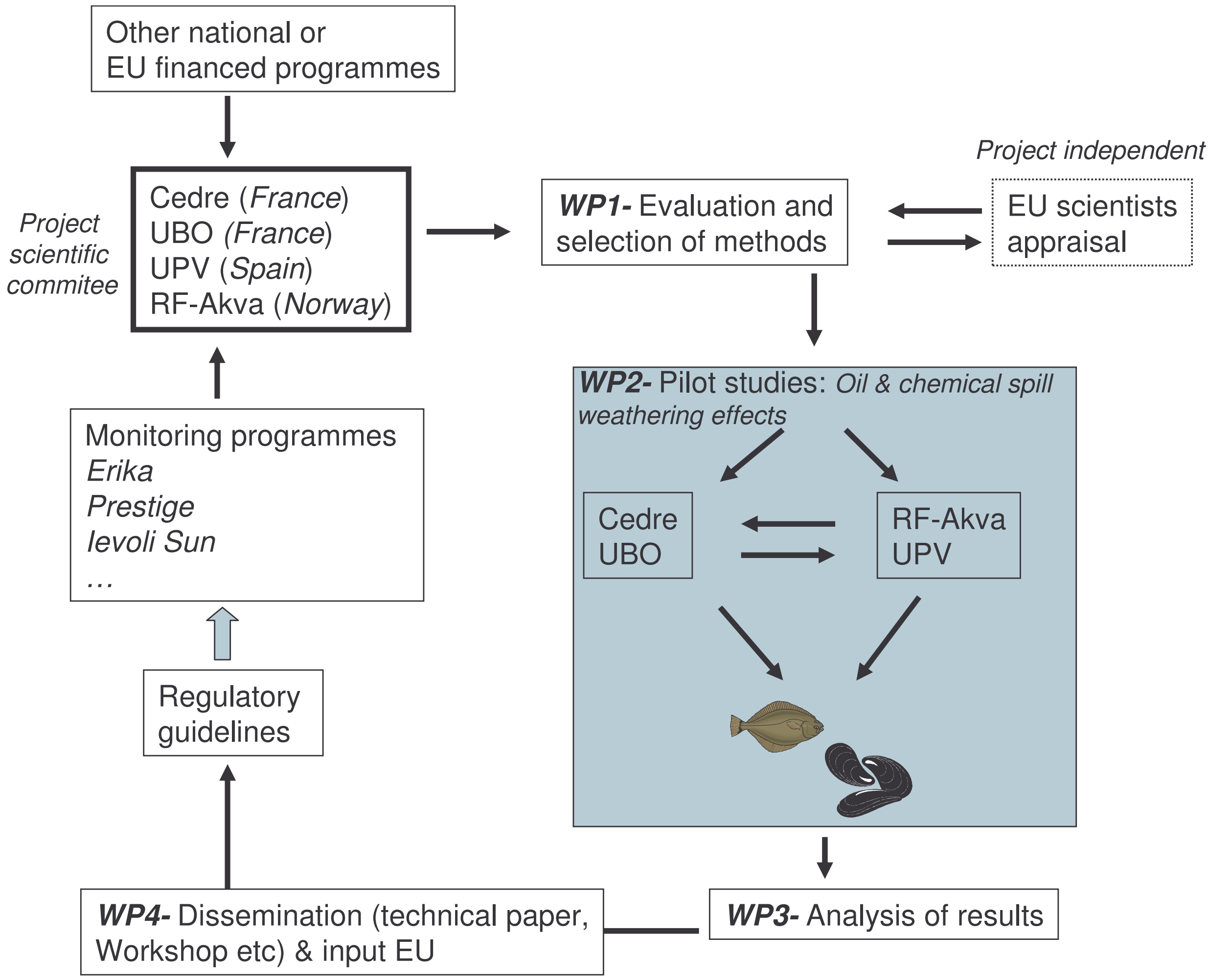


Fig. 3- Diagram of the organisation of the project

WP1. Evaluation and selection of analytical methods

A project scientific committee formed of the applicant/partner's contact persons will select existing methodologies from relevant monitoring programmes following recent accidental oil and chemical spills in the EU coastal zone. The input from other existing/past national or EU programmes relevant for the project will be sought for in order to obtain a synergy and add information. The methods selected will be based on biological markers chosen for their relevance and sensitivity, and other considerations connected to cost efficiency and manageability for their use in monitoring programmes. The advices and appraisal of project independent scientists involved in environmental pollution monitoring will be taken into consideration in the choice of the methods. Also, recommendations and relevant elements found within other organisations like OSPAR, IMO and UNEP will be integrated. The aim will be to select the parameters that can best be related to the exposure dose and for example integrate the effects of several pulse events like after re-mobilization of substances in the water. Biomarkers that give transitory signals or are subject to more biological variation will be avoided (Bocquene et al., 2004). Rather, the methodologies selected will be based on their ability to assess the general health status and impairments that can be connected to a reduced fitness of the organisms under exposure following the spill event. Hence, we will prioritize biomarkers of effects. The proposed biomarkers are not pollutant-specific but indicate rather a general fitness and the state of homeostatic balance in organisms following exposure. However, other more specifically oil-related markers of exposure will be selected to assess the bio-availability of certain organic contaminants including PAH.

Table 1 summarizes the biological markers we would like to address in the study and which are very briefly described below.

- **Biomarker of organic pollution** -.In fish, the induction of a class of proteins (CYP1A proteins) involved in the oxidative metabolism following exposure to certain organic pollutants can be measured by the so-called EROD activity (Van Der Oost et al., 2003). This parameter is used as a biomarker of exposure in several environmental monitoring programmes worldwide. EROD is not suitable for mussel which has a lower oxidative metabolic capacity. Hence, alternatively, we will evaluate exposure using peroxisome proliferation in digestive gland cells of mussels which was proposed a good biomarker of organic pollutant exposure (Cajaraville et al., 2000). Peroxisomes are cytoplasmic organelles involved in the metabolism of lipids and reactive oxygen free radicals. Their proliferation indicates increased bioavailability of PAHs.
- **Parental and metabolites burden** – The contaminant level accumulated in individuals will be measured as a way to estimate the bioavailability of the spilled substances. The bioaccumulated substances will represent the fraction available to organisms and which can generate toxic effects (Neff, 2002). As mussel have low metabolic degradation rate, parental body burden represent a good marker of available substances. On the contrary, in fish, the induction of the metabolic activity shortly after exposure results in a high excretion rate of parental compounds and simultaneously the production of bile metabolites, which can easily be detected by fluorescence-based techniques (Aas et al., 2000; Baussant et al., 2001a; Baussant et al., 2001b; Jonsson et al., 2004; Ariese et al., 2005).
- **Genetic** - The genotoxic markers will evaluate impairments in the genetic material (DNA) of cells. The Comet assay measures alkali-labile strand breaks as a measurement of DNA integrity and the micronuclei test measures genetic aberrations at the chromosome level. These two tests are widely utilised in mussel and fish (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Barsiene et al., 2004; Buschini et al., 2004; Taban et al., 2004).
- **Endocrine** - Endocrine effects will be assessed by the level of vitellogenin (Vtg) or vitellogenin-like (alkali-labile phosphate or ALP) protein. The level of this protein can be analysed in both fish and mussels exposed to different pollutant sources (Van Der Oost et al., 2003; Aarab et al., 2004b; Ortiz-Zarragoitia and Cajaraville, 2005; Pampanin et al., 2005a) and is used as an endocrine biomarker. Vitelogenin is an important protein used in the egg yolk and hence is essential for the development of embryos in invertebrates like bivalve mollusc and vertebrates like fish. In normal condition, vitellogenin is only detected in female individuals and the presence of this protein in male individuals indicates hormonal disruption following exposure to substances with an estrogen mimicking effect on the endocrine system.
- **Physiological** - Several parameters will be envisaged to establish the physiological state of the organisms exposed to spilled pollutants. In mussel, we will use biomarkers of stress like the lysosomal membrane destabilisation of hemolymph cells and digestive gland or liver cells, the survival in air ("stress on stress" analysis) and also ecophysiological measures by the clearance rate (=ability of mussels to filter dense algae culture) and the condition index (ratio of soft meat weight to the weight of the shell) (Lowe and Pipe, 1994; Viarengo et al., 1995; Pampanin et al., 2005b). In fish, we will utilise blood samples to analyse several markers involved in the maintenance of the homeostatic balance and to diagnose the general fitness of individuals.
- **Histopathology** – Histopathological parameters are considered highly ecologically relevant responses usually indicative of irreversible injuries of exposure to a variety of pollutants. Histopathological changes are relatively easy to identify providing that proper reference material and control data exist. Hence, it seems advisable to integrate these parameters in pollution monitoring from spill (Wedderburn et al., 2000). Histopathologic observations will be performed on preparation from gills, digestive gland (or liver) and gonad. The aim will be to detect anomalies at the tissue level that can be related to physiological malfunctions and disease in organisms. These comprise for example the atrophy of the digestive epithelium

(reduction in epithelial thickness) and cell type replacement in mussels, and prevalences of preneoplastic foci and neoplastic alterations in fish (Kohler et al., 1992; Marigómez et al., 2004).

- **Immunological** - The ability of a species to "defend" against environmental stress from a pollutant relies on various aspects of health status. Under exposure, immune functions can be altered, thereby reducing protection against microbial or parasitic attacks and ultimately the survival of individuals. In mussel, we will use a phagocytosis assay (the cellular ingestion and digestion of particulate matter) performed on haemocyte cells (that play a central role in defence system) or other easy-to-perform tests with a plate reader (Gomez-Mendikute et al., 2002; Gomez-Mendikute and Cajaravillle, 2003) and, in fish, we will analyse simple blood parameters like white blood cell counts and lymphocyte status (Van Der Oost et al., 2003)

Biological markers	***Bivalve***	***Fish***
Exposure		
	peroxisome proliferation body burden	EROD bile metabolites
Effect		
Genotoxicity	Comet assay	Micronuclei
Endocrine	ALP assay	Vtg/Zrp assay
Physiological	Lysosomal stability Clerance rate Survival in air Condition index	Haematology[1] Blood gases (P_{O2}, P_{CO2}) Acid/base status[2] Hydromineral balance[3]
Histo-pathology	Digestive gland Gonad, gills	Liver, gonad Gills
Immunological	Phagocytosis	Haematology
Ecological		
Embryo-larval bioassay	Fecundity succes, Developmental deformities, growth	----

Table 1 – Biological markers and bioassay overview

- **Embryo-larval bioassay** – A proper ecological understanding of the relationships between biomarker responses and survival, growth or reproduction is essential to validate the proposed approach. This is also considered a prerequisite for the use of biomarkers in environmental risk assessment. Here, this bioassay will serve two goals: i) establish a link between exposure of spilled pollutants to adults and long term impacts mediated by parental transfer to embryos and larvae; ii) assess the sensitivity of embryos and larvae in conditions simulating sublethal concentrations following the weathering of spilled substances. Bioassays will be performed on bivalves molluscs only as they can be conducted within relatively short time period. After 48h, the standard end point parameters include the percentage of malformed larvae according to some criteria defined for example in His et al. (1999). Although strictly restricted to the laboratory, experiments run on longer duration will allow an assessment of larval growth which is highly relevant for the long term impact assessment of spill.

- **Biosensor to detect damaging agents** - Rapid and cost-effective analytical methods are also clearly needed in order to reduce the cost compared to existing techniques (Moore et al., 2004). Screening analytical devices based on biosensors technology appear to be a promising way for routine cost-effective analyses of biological and chemicals measurements applicable on field and as "front line" tools in the case of a pollution event. Consequently, there is presently recognition that this type of technique may have important impact on future environmental biomonitoring studies and effective management strategies at sea. The techniques may be useful to pre-screen environmental samples, possibly helping to direct the efforts for further analysis, take immediate actions and hence delimit rapidly the extent of the damage.

Biosensors are constituted by the combination of a biological-.based recognition element retained in contact with a transducer element which converts the biological recognition event into a useable output signal (Kroger et al., 2002; Rodriguez-Mozaz et al., 2005). The potential for environmental applications lies in the ability of biosensors to measure the interaction of pollutants with biological systems through a biomolecular recognition capability without extensive extraction, multi-step procedures and cleanup protocols that usually hinder cost-efficiency of other analytical techniques. In the project, we will include an analysis of a simple

device that can provide an indication of the affinity of substances to the DNA and can be used to further evaluate the potential for genotoxic effects to the contaminated organism (Lucarelli et al., 2003; Bagni, 2004 a-b). Also, enzyme based amperometric biosensor is another type of device that can be used for the detection of low levels of classes of marine pollutants under appropriate conditions and electrode design. For example, amperometric peroxidase-based electrodes have been developed for the detection of a large number of pollutants (Ruzgas et al., 1995; Solna et al., 2005). This type of biosensor represents a potential cost-effective alternative to relatively expensive chemical analysis and can enable to screen substances accumulated in environmental oil-related samples even at relatively low levels (Bulukin *et al.*, 2004).
This work will be established in cooperation with the University of Florence in Italy (contact person: Prof. Marco Mascini).

WP2. Pilot study experiments: laboratory exposure to chemical and oil compounds – measurement of general health parameters and biosensor sensitivity

The soundness of the proposed approach will be assessed through laboratory experiments which will aim at characterising the aging processes of the selected substances, their influence on the bioavailability to marine organisms as well as the evaluation and manageability of biological impacts measurements for relative long term monitoring. The methodologies described above will serve as a basis to make this evaluation.
Two pilot studies will be performed: one in Cedre and the other one in RF-Akvamiljø. The excellent facilities existing in each of these centres allow the simulation of realistic exposures to marine organisms of toxic substances either individually (single chemical) or in complex mixture (like oil). Here, the exposure system will aim at simulating the release of substances in the water following the dissolution and aging processes of spilled substances coated to the sea-bed (heavy oil) or spill situation with chemical substance (styrene) release after the wrecking to the sea bottom. The heavy oil tested will be either the *Prestige* oil or oil with a composition resembling that of the *Prestige*.
Bottom living organisms representative of relative shallow waters will be chosen. Turbot fish (*Scophthalmus maximus*) and blue mussel (*Mytilus edulis*) will be used as test species. These organisms will be placed in exposure tanks for varying period. For the heavy oil, the experiment will last a period as long as 6 months. For the styrene, we will perform shorter experiments i.e. 5 weeks. The selected biomarkers of effects will then be used to evaluate the health status impairments and these will be related to the aging process of the spilled substances assessed by chemical analyses in seawater and in biota. The biological material collected during the experiments will also be used for implementing measurements done with the biosensors and to assess the sensitivity of the technique as screening device for the rapid detection of organic chemicals in the water and the potential genotoxic effects to organisms at contaminated sites.
Technical advices and exchanges of expertise will be promoted before the start and during the experimental period so that partner's experience in experimental design simulating exposure to toxicants related to marine environmental pollution best benefit to the project.
Cedre will be in charge of the experimental study with fish exposed to heavy oil and styrene. Likewise, a similar experiment with the blue mussels will be performed at the RF-Akvamiljø marine centre.
Each set of experiment will include:

- Experimental setup in flow-trough or semi-continuous flow mode and in open system using controlled conditions.
- Exposure of heavy oil: the system will simulate percolation of water trough oil-coated sediments
- Exposure to styrene simulating release in the seawater from a ship wreck lying on sea-bed
- Chemical characterisation and follow up of the bioavailable substances in seawater and in biota.
- Biological effects measurements using the selected methodologies and analytical measurements with the biosensors

To study the effects of oil bind to sediments and analyse the biovailable fraction of oil to marine organisms, an experimental system consisting of a column filled with oil-coated glass beads will be prepared following a setup based on Carls *et al.* (1999). The effects of the fuel will be analysed by continuously washing fuel-coated beads for a period as long as 6 months. The column will be refilled with newly prepared oil-coated beads after 5 weeks to boost up the exposure level, simulating the re-mobilization of fuel during high tide events or storms. After that period, the exposure will be kept for a following 4 months without modification. This system will simulate the percolation of seawater through contaminated sediment, will enable to determine the bioavailable fraction of oil that can lead to biological effects on the mussels and evaluate the recovery.

In addition, bioassays with early life stages of mussel (embryos and larvae) will be performed to assess the long term impact of the pollutants at the population levels and analyse their relation with the biomarker responses.

WP3. Analysis of results

Following the pilot studies, the data will be gathered and put into the web site so as to communicate results to each partner and allow the commission to see the work progression. The individual responses provided by each biomarker measurements will be diagnosed in connection to the biovailable exposure dose for each organism and in each experiment. However, rather than analysing each biomarker individually, a multivariate biomarker-based approach will be emphasised so as to combine individual markers into an assessment of the overall health status of the organisms. This will be realised by implementing indexes of environmental quality that take into account both chemical and biological criteria, and in order to classify the heath status using a simple scale and identify pollutant-induced change over time. Here, we will select existing methods developed for the monitoring of pollution and that integrate the responses of individual biological responses in an index of pollution. Analytical methods based on discriminant analyses (Chevre et al., 2003; Aarab et al., 2004a; Narbonne et al., 2005) or even simple multivariate graphic visualtion (Beliaeff and Burgeot, 2002) will be used. The IBR (integrated biomarker response) index proposed by Beliaeff and Burgeot (Beliaeff and Burgeot, 2002) is particularly interesting since it is based on a simple and powerful method that allows a visual integration, using star plots, of a set of biological responses. The integrated environmental pollution index is computed as the star plot area and can be compared with the exposure or the dose level assessed by chemical measurements. This methodology has already been applied to follow up the spatial and temporal evolution of the environmental effect following the "*Erika*" oil spill (Bocquene et al., 2004). Using the set of biological responses measured during the pilot studies at RF-Akvamiljø and Cedre, we will evaluate the use of such pollution index for the monitoring of spill. This could comply with the current EU's water framework directive (2000/60/EC) that integrates freshwater and also coastal environments. This index will also be valuable in long-term biomonitoring studies of environmental pollution with oil and chemical spill.

The results of the biosensor measurements will be evaluated for their pragmatic use in real field situation for spill monitoring. Among other, careful considerations of the matrix effects, variability and stability with time will be made. Also, the validation of the technique for its field-applicability requires a close comparison with the other methodologies used in this project. The implementation of standardised protocols, with few protocol steps, easy to use by different personnel among the different countries ("biosensor kit") will be considered although the financial frame of this project will not allow the full development of such concept.

All together, these results will help to prepare a manageable and cost-effective monitoring strategy for assessing the environmental impact of spill. This could consist for example in a two-step analytical phase. Biosensors could be proposed as "front line" instrumental techniques for the rapid screening of signals of pollution by spilled substances in water and in organisms at different sites, and the selected biomarkers analysed in case of significant signal detection (figure 4).

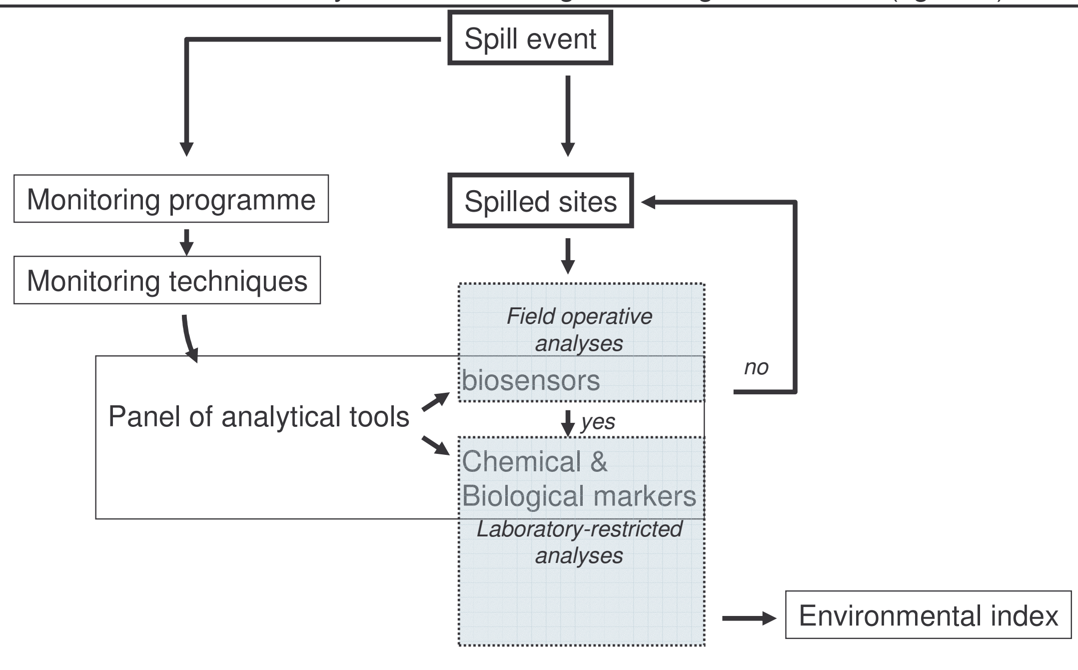
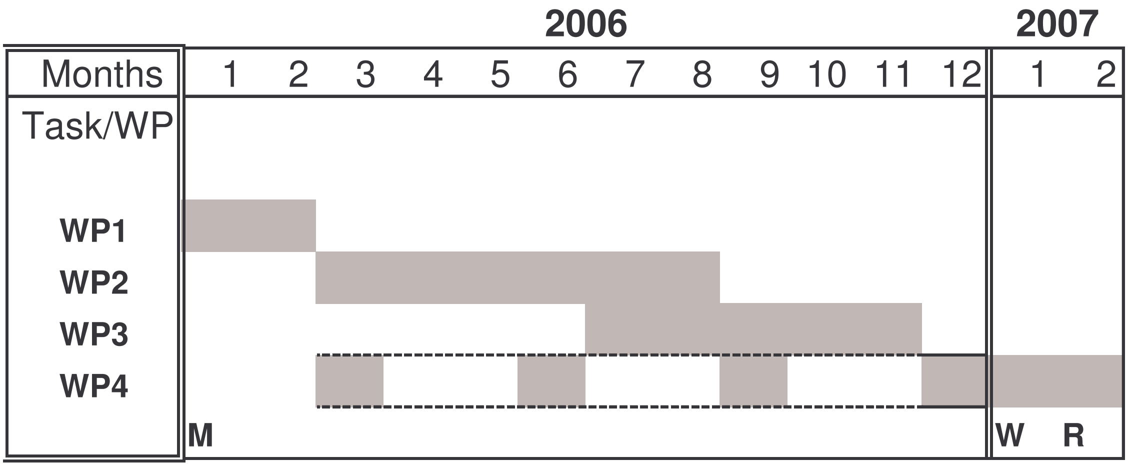


Fig.4- Possible way of conducting monitoring programme following spill events

WP4. Dissemination of knowledge and input to EU guidelines
Different levels of dissemination will be emphasised. Technical and scientific results of the pilot project will be disseminated through peer-reviewed articles in international journals. A final technical and scientific report including detailed results from the project will be issued to the European Community according to the grant agreement between the commission and the applicant.
A three-days workshop will be organised by Cedre (see separate proposal for that action: workshop on "**Pollutant Monitoring and Ecological Impact Assessment** following an Accidental Oil or other Chemichal Spill in Marine Waters") where the main outcomes of the project and their implication for EU regulatory guidelines will be discussed. The project scientific committee will be represented in the workshop. Project independent scientists, industrials and regulators will also be invited. The results of the project will be presented and this will initiate a discussion forum where the different participants will exchange their opinion. This will also constitute a way to quality ensure the work done during the project by external advisers.
At the community level, common future procedures will be identified enabling good operational practises for pollution monitoring. This initiative may help to define European research priorities and for example enhance the implementation of the water directive framework within EU members by establishing a network that improve trans-national co-operation. By that mean, the project will also contribute to technical and scientific exchanges between nations.

Time schedule



M: Meeting W: Workshop R: Final report

Expected results

**Implementation of analytical methodologies and tools in pollution monitoring programmes – ** The implementation of innovative environmental monitoring and surveillance techniques is needed to understand the extent of impact after a pollution incident. This requires the selection of analytical techniques that can be prognostic for responses to environmental change and help to assess the status of the impacted ecosystem. This implies supporting research into understanding between the physical, chemical, biological and ecological processes. The implementation and incorporation of individual biomarkers, together with chemical measurements, into programmes that monitor the quality of the environment following spill events could be recommended in some selected groups of sentinel species defined in the different geographical areas. At the same time, this implementation has to be pragmatic and, for example, there is a clear evidence for new rapid cost-effective analytical methods that can be applied routinely in the field in order to reduce the economical charge in environmental monitoring programmes launched by national or EU initiatives. Biosensors may correspond to that need and will be proposed in the panel of analytical tools. A development towards a "sensor kit" assembling a simple instrumentation, a set of electrodes and user-friendly procedures will be envisaged. We can imagine that such "sensor kit" could be useful for several scenarios in spill situation, like simply in the detection and mapping of contaminant in a spilled area or evaluating in a cost-efficient way the restoration following the spill event.

This project will propose a pragmatic way to prepare and run monitoring programmes including scientific, organizational and economical considerations.

Integrated approach – The current EU guidelines and decision making criteria for pollutant are mainly established on the basis of chemical measurements performed in seawater, sediment or biota but lack the integration of actual effect parameters measured in organisms. The use of several biomarkers can help to determine the health status of organisms and relate that with the exposure level or the dose of pollutant measured by chemical analysis. However, beyond the implementation of individual biomarkers that can indicate deleterious effect at different levels, it can be difficult to combine these individual effects into an assessment of the overall health status of organisms. Hence, it seems essential to develop simple and manageable decision making methods that integrate individual biological markers in one pollution index and that can serve as a tool for environmental managers to evaluate the relative environmental hazard at various spilled aquatic sites. This could be conceived as a rational and simple way that could be proposed for new EU environmental directives and decision-making systems.

Definition of criteria to analyse pollution impact of hazardous substances in marine water- Clearly, the impact assessment of oil and chemical spill is a major issue which needs to be fed by the involvement of Member States scientists, industrials and the public. Many of the compounds spilled at sea during past or recent accident are included in the list of priority substances elaborated in the COMMPS (combined monitoring-based and modelling-based priority setting scheme) procedure used in the EU's water framework directive to evaluate the concentrations of substances in water or in sediments which are considered not to have any effects (PNEC). The identification of hazardous substances is based on their impact assessment currently identified from acute and chronic data, mainly obtained from freshwater environmental study, where mortality is the end point parameter. Hence, we feel there is room for improvement to ensure that the current criteria are well appropriated to the marine pollution issues. As a continuous improvement of views and development of ideas, discussions and practical studies should be promoted in order to assess the ability of selected methods to detect the actual environmental impacts of these compounds. The proposed project will contribute to implement operative protocols and methodologies that can be used in individual Member States monitoring programmes for assessment of environmental impacts of spill. Moreover, at the Community level, this could serve as guidelines to prepare good monitoring practises available to all countries to be used in contingency plan. This harmonisation could be beneficial to co-ordinate efforts, enhance the effective and efficient use of existing national and European capacities, and implement methodological instruments in EU policies related to accidental marine pollution. Thereby, this should comply with the general EU's spirit of improving co-ordination and co-operation of Member States to better respond to accidental contingencies.

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Form A3

Detailed description of the projectSUMMARY OF THE PROJECT (Maximum 1 page)

Objectives

Actions and means involved

Expected results