FACEiT

Fast Advanced Cellular and Ecosystems Information Technologies

Contract: 018391

6th Framework Programme priority 3

Global Change and Ecosystems

Final Summary: Sept 2005-February 2009

FACEiT Final summary report of activities

September 2005-February 2009.

Lausanne, April 10th 2009

Jan Roelof van der Meer, Coordinator

Executive Summary

project FACEiT

Recapitulation of FACEiT's objectives

The consortium FACEiT officially started working on September 1st, 2005 with twelve partner institutions from 7 European countries. The focus of FACEiT was Pollution disaster management and more in particular pollution disasters resulting from petroleum oil spills, either in marine or freshwater aquatic systems. The FACEiT project was carried out in the Thematic Sub-Priority Area 6.3 Global Change and Ecosystems, with particular relevance to area III.3 Risk assessment, management, conservation and rehabilitation options in relation to terrestrial and marine ecosystems. The main position of the FACEiT consortium in the area of pollution disaster management was that of a strong biological actor.

Disaster management involves integrating different lines of information from and among different societal actors, such as pollution source, localization, nature of chemicals involved, type of environment, responsible persons, or casualties. Essential elements of disaster management must include the rapid determination of the magnitude of the catastrophy, the protection of (aquatic) ecosystems, goods and persons from further damage and the suggestion of remediation measures which will restore the diversity and functioning of the affected ecosystem. The main focus of the FACEiT project was to provide a biomonitoring approach that can contribute to these three essential elements.

The overall objectives of FACEiT within disaster management were hereto,

-to develop adequate and effective biological methods to detect the presence, nature and magnitude of the pollution and its effects on aquatic living beings

-to predict the medium and the long-term consequences for the aquatic ecosystem and the self regeneration capacity.

-to link different biological, physico-chemical and modeling approaches in order to achieve an integrated measurement and prediction.

-to disseminate the scientific and technical outcomes of the project to the different main actors of disaster management.

Biomonitoring tools.

One of the key concepts of FACEiT was to use organisms, cell lines or biological molecules (DNA, RNA, proteins) to monitor pollution disasters and predict long-term effects and restoration. The importance of biomarkers has long been recognized and different endpoint markers are effectively used in monitoring programs. However, many

List of FACEiT participants

Participant name	Country	Lead responsible
Université de Lausanne	CH	Jan R. van der Meer
University of Essex	UK	Terry McGenity
Royal Netherlands Institute for Sea Research	NL	Corina Brussaard
Université de Pau et des Pays de l'Adour	F	Robert Duran
Swiss Federal Institute for Environmental Science and Technology	CH	Rik Eggen /Marc Suter
UFZ Centre for Environmental Research	D	Hauke Harms
Bioclear B.V.	NL	Janneke Krooneman/Marlea Wagelmans
Centro Nacional de Biotecnologia	ES	Victor de Lorenzo
University of Plymouth Ecotoxicology and Stress Biology Research Centre /University of Exeter	GB	Tamara Galloway
Cybersense Biosystems Ltd	GB	Marcus Yeo
Institute of Biochemistry	Lt	Rolandas Meskys
Biodetection Systems BV	NL	Bart van der Burg

biomarker tests were not sufficiently rapid or ethical and one of the goals of FACEiT was to redesign such tests by new combinations of methods or new concepts, such as by flow cytometric analyses of cell viability. In addition, FACEiT proposed to increasingly replace biomonitoring tests with unicellular organisms or cell-lines, which have the primary advantage of being much more rapid than tests with whole complex organisms and potentially reduce measurement costs. However, by proposing this, FACEiT should demonstrate that responses measured in unicellular organisms or cell lines can be extrapolated and validated to effects on whole organisms and ecosystems, which is one of the most important research objectives of the program.

Essentially no tests existed at the starting date which monitor and predict medium- and long-term consequences of a pollution disaster for ecosystems. FACEiT therefore proposed a few key areas for new method developments. These were: biological diversity, multifunctionality or self-regeneration capacity, specifically focused on petroleum hydrocarbon pollution and its bioavailability, microbial communities, and their biodegradation potential.

Potential impact of FACEiT.

Our consortium is strongly convinced that biological monitoring tools for affected ecosystems are an essential element of disaster management, and should be used by other actors in the landscape of disaster management to aid in decision making and in developing remediation strategies. We realize therefore, that the tools and methods developed within the realm of FACEiT can only make the transition to management and application if they were sufficiently supported by research findings, validation and training.

In order to promote the use of biomonitoring tools – which could lead to their testing and validation by third persons, FACEiT attempted to actively inform different communities of actors in the disaster management 'landscape' or via web-based resources. Apart from various brochures, press releases, smaller smeetings, this has culminated in one 'hands-on' training course in July 2008, a final two-day Symposium in January 2009 and a new handbook on Microbiology of Hydrocarbons, Oils and Lipids, which will appear Summer 2009 with Springer publishers.

Key scientific objectives

WP1. To test and develop a multiwell, multistrain platform of fluorescent and luminescent bioreporter microorganisms covering the oil-related pollutants in aquatic systems, to rapidly, cost effectively and reliably measure pollutant exposure concentrations

WP2. To test and develop a highly sensitive first warning standalone semi-continuously operating bacterial bioreporter system with a general response against toxicity

WP3. To identify and mine microbial responses to oil-pollutant shocks

WP4. To test and link rapid microbial diversity tools to consequence prediction for aquatic ecosystems

WP5. To identify microbial community functional markers and use them to rapidly analyse oil pollution induced ecosystem response

WP6. To develop fast flow cytometric approaches on native planktonic unicellular algae and microbial populations to detect pollutant stress by means of cellular integrity and viability losses

WP7. To develop rapid fish embryo and human cell bioassays and identify key responsive elements in the toxicity chain based on transcriptome analyses

WP8. To validate multibiomarker approaches for organism exposition with biosensor, bioassays and other unicellular methods to link unicellular informative tools to organisms higher in the foodweb

WP9. To model the biodegradative and toxicologal effects of pollutants in any community with an interactive bioinformatic database and design of a selective biochip

WP10. To model the fate of pollutant classes with respect to their bioavailability, ecosystems' risk, biodegradative potential, and biodiversity loss on the basis of bio-tools

WP11. To test and validate existing and newly developed measurement tools on selected pristine and contaminated aquatic sites, develop of protocols and recommendations for tool usage and their importance in pollution prevention control

WP12. To transfer prototype technologies, biotool concepts and protocols to communities of potential endusers

WP13. To ensure and assess the proper project's progress, dissemination, public relations, to provide contact with the Commission and essential scientific and financial reports.

Research Summary

One of the main technological developments in FACEiT was the application of **bacterial reporter assays**. Such assays consist of relatively simple incubations in aqueous media with genetically modified bacteria, engineered to produce bioluminescence or fluorescence in contact to a set of target chemicals. A large number of reporter strains was developed targeting key components of oil (e.g., alkanes, BTEX, PAHs). Overall, this work package has been very successful and most of its goals could be realized. A concise set of bioreporter strains with the same chassis and reporter output for use in assays to detect key oil-borne pollutants was developed. Different assay formats were designed, extensively calibrated and tested by various users, which makes us confident that we have realized a very simple and rapid set of assays to quantitatively analyze oil pollution in water with bacterial bioreporters. The assays were also validated on a real-life scenario of an open sea spill and were robust to be carried on board and perform analysis in the first two hours after a spill. We thus feel very confident that we have produced a potentially very useful tool for spill analysis that can find its place among a biotool set.

The concept of bioreporter assays was further taken up in instrumentation and product development. A semi-continuous standalone bacterial bioreporter system for on-line analysis of general inducible stress was instrumentalized. A functional 8 chamber system was developed based upon the so-called ROTAS system. Within this system freezedried bioreporter strains were capable of being rehydrated and functioning effectively in response to contaminated samples. A limitation of the work was the propensity of the peizoactuated microfluidic pumps to block and the need for upfront filter development, however a few effective prototypes were generated.

The second goal stipulated the production of a semi-continuous standalone bacterial bioreporter system for multiple chemical target analytes. Experiments demonstrated that the ROTAS system could direct the reporter assays effectively. Samples for bioluminescent detection and quantification were successful in two of the four biosenors developed by UNIL (alkane and HBP sensor). Response was significant and dose dependant following inducer application. Freeze-drying experiments conducted using a range of parameters on a programmable freeze-drying system successfully developed a freeze-drying protocol for the *E. coli* reporter strains. Freeze-dried reporter cell batches also revealed a dose dependent response and no loss of sensitivity. Long-term viability studies conducted over a 12 month period demonstrated that freeze-dried inducible reagent could be stored with no significant loss of performance. The next step was to load the reagents into blister pack type device for the automated application of the reagents in response to a pollution event. Limited progress was made on this. Further work would have focussed on the miniaturisation of the blister pack applicator into the ROTAS hardware and its remote usage as a standalone system.

At the level of **microbial community analysis**, proteomics of *A. borkumensis* and *M. hydrocarbonoclasticus* revealed some common and species-specific responses to oil pollution. The most represented class of protein over-expressed when grown with n-alkanes compared with acetate or pyruvate are those involved in transport across cell envelopes including, efflux pumps, outer membrane porins and components of ABC transporters, alkane-metabolism genes, and proteins involved in the TCA cycle leading to enhancement of the glyoxylate shunt. Iron uptake and transport proteins are especially represented suggesting an increase in iron demand or iron starvation during growth on hexadecane. These provide potential candidates for reporter strains, and several have been further investigated by real-time quantitative PCR and mutant analysis. The *alkB1* genetic circuit proved to be useful, and the AlkS-PalkB1 construct from *A. borkumensis* was well expressed in *E. coli*, even giving a large response to octane and a slight response with the longer chain alkane, tetradecane.

We have shown that it is critical to consider the physiological status of the cells. For example, when grown with n-alkanes, cells of *M. hydrocarbonoclasticus* form two physiologically distinct cell types, and importantly, we have discovered that that cells detached from the biofilm have a protein expression profile very different from biofilm cells. About 50% of the detected proteins (532 proteins) were expressed at different levels in the two conditions. Considering that biofilm cells are precursors of detached cells, this result is rather surprising and indicates that biofilms undergo dramatic changes before or during detachment from the biofilm.

The response of *A. borkumensis* to diverse stressors was tested when pre-grown on pyruvate or tetradecane. Tetradecane-grown *A. borkumensis* was more tolerant of solvents than pyruvate-grown cells, and the proteomic stress response was strongly determined by the growth substrate more than the stressor. For example, a pilin biogenesis-related protein was found to be in much greater abundance under all stress conditions in pyruvate-grown cells compared with the control. However, its production was much lower in tetradecane-grown cells, and even lower when tetradecane-grown cells were stressed by benzene and phenol. Apart from this protein, there were few 'universal' stress responses detected.

The impact of crude oil contamination on microbial diversity is of great interest since microorganisms play a major role in determining the fate of hydrocarbon compounds in the environment. The modifications of

bacterial communities after crude oil contamination were examined in microcosm experiments thanks to a set of molecular biology methods combined with chemical analysis for hydrocarbon content determination. All the ecosystems tested (estuarine sediments, microbial mats and marine waters) showed large changes in the microbial community structure. In all cases, the analysis showed a correlation between community structure and hydrocarbon content (i.e. contamination level). Similar correlations were obtained with in situ analyses of the Etang de Berre sediments where a gradient of hydrocarbons was observed. Although a detailed examination of bacterial community composition revealed the presence of oil-degrading bacteria in all the studied communities, we could not define a typical signature in response to oil pollution. These results suggested that other parameters may drive the structure and composition of bacterial communities and that the effect of crude oil is probably not restricted to its toxic effect and may involve physical effects as well. Further studies are still required to better understand the complete "hydrocarbon response" of the microbial world.

Functional community analyses were performed as well, targeting the catabolic genes *alk* and *nah* encoding the alkane monooxygenase and the naphthalene dioxygenase, respectively. Both genes had a limited diversity and were found to be present during all the incubation period without loss of diversity. Interestingly the genes were expressed immediately after crude oil addition and their expression was maintained during at least three days. The degradation of hydrocarbons was correlated with the expression of the genes. These observations revealed that the degradation capacities of the bacterial communities could be monitored by following the gene expression of the targeted catabolic genes. This is valuable information for the monitoring of remediation technologies and for the management of microbial activity. In addition, the fact that expression levels are detectable within three days can demonstrate recent pollution histories.

Several experiments were performed in oiled estuary water microcosms supplemented with nutrients, biosurfactant, and/or with hydrocarbonoclastic bacteria. But considering the oiled but otherwise unamended experiment, *Thalassolituus* and *Cycloclasticus* could be detected by day 5, while *Alcanivorax* could not be detected even after 30 days. In another study, this time focussing on the expressed genes (by examining mRNA), only *Thalassolituus* gene expression was detected when hexadecane was supplied; only *Cycloclasticus phnA* mRNA was found when 1-methylnaphthalene was supplied, and *Alkanivorax* gene expression was detected only upon addition of pristane. With crude oil, *Cycloclasticus* and *Thalassolituus* gene expression occurred at day 5, but *Alcanivorax* gene expression was not detected by day 15. Therefore, in estuarine waters, it may be more suitable to use genes from *Thalassolituus* and *Cycloclasticus* or to design primers that encompass a diversity of oil-degrading microbes.

The bacterial response to oil contamination was also assessed by differential display in sediment samples plus or minus oil during 24 h of incubation. The technique proved highly reproducible, and differential expression of DD-PCR fragments was validated by dot-blot analyses. Expression of one gene was 15 times higher in contaminated microcosm. Some differentially expressed genes were typical of stress responses, such as DNA repair proteins (4), chaperonin GroEL protein (1) and a methylene hydrofolate reductase (1).

We have advanced the method of **metatranscriptomics** whereby poly-A tails are added to mRNA from oil-degrading communities to enable amplification and subsequent pyrosequencing. Similarly, protein-extraction techniques from sedimentary environments have been optimised. These methods were tested on the samples from the cruise and on oiled tidal mesocosms. In parallel with identifying gene expression patterns in response to oil pollution we have greatly advanced our understanding of oil-degrading communities in experimental systems at different scales and in different sample types, ranging from bottle experiments, mud-flat sediment tidal mesocosms, to in-situ marine oil spills. In addition to the species indicated above we have shown that many other microbes are implicated in oil degradation.

In relation to the original goals of WP6 the main conclusions are that FACEiT successfully developed and tested 11 rapid **flow cytometric assays** of which 3 assays were finally chosen to be most sensitive markers for oil-pollutant shock exposure. Up to 39 marine phytoplankton species/strains were screened, including representatives of all major phytoplankton groups and including tropical, temperate and cold-water species. Four ecologically relevant algal species were found to be most sensitive, of which *Micromonas pusilla* was chosen as model species (sensitive to oil and easy to grow) with an EC50 value of appr. 0.35 mg oil/L and a detection limit of 50 µg oil/L. Rapid staining techniques were also tested for different bacteria species and using the wild type freshwater bacterium *Pseudomonas fluorescens* was used to further develop an assay to interrogate the potential action of toxic oil components on heterotrophic bacteria. Both algal and bacterial bioassays were successfully used on laboratory provoked pollution accidents to detect environmental stress, as well as used to provide baseline monitoring in pristine and chronically, experimentally and accidentally polluted aquatic environments (mesocosms and natural). The assays were, furthermore, effectively validated with other ecotox markers used within FACEiT.

The rapid flow cytometric assays were also used for analysis of the ecotoxical effects of oil on the trophic interactions using two oil-sensitive algal species (*M. pusilla* and *Phaeocystis globosa*). Grazing by microzooplankton did not seem to be affected by the oil contamination. However, the water accommodated oil-fraction showed a distinct negatively impact on virally infected P. globosa, resulting in 10-fold lower abundance of newly produced viruses per lysed host cell.

Again at the level of new in vitro biological assays, FACEiT developed **rapid fish embryo and human cell bioassays**; a molecular DarT test and two CALUX reporter gene assays. These tests closely match with regulatory requirements and in their original form they are DIN accredited (DarT), accepted in international legislation (DR CALUX), and can be run under ISO17025 and related international quality standards.

Both vertebrates systems were extensively tested in exposure experiments with crude oil, water fractions and representative chemicals from oil. In both systems, the *cyp1A* (zebrafish)/*CYP1A1*(human) gene was identified as key responsive element in the toxicity chain of oil exposed higher vertebrates. This gene is responsive to activation of the dioxin receptor and our results suggest that its activation is mainly due to the carcinogenic poly aromatic hydrocarbon fraction in oil. In zebrafish eggs we could confirm that PAHs alone caused identical morphological and gene expression effects as crude oil. Real time RT-PCR analysis was developed and used to measure expression of this gene in environmental sample-exposed eggs. To be able to measure PAH is environmental samples, a simple PAH CALUX test was designed. In addition, a more broad CALUX screening method for oil-derived carcinogens was designed and is currently under construction.

In human cells and particularly in zebrafish additional target genes were identified that may be related to addition effects of oil exposure such as teratogenicity, oxidative stress, developmental disturbances, maintenance of homeostasis. Our results indicate that some of these genes represent markers of activation of pathways independent of dioxin receptor activation.

The work resulting from Work Package 8 has demonstrated the strength and potential of an **integrated multibiomarker and analytical chemical** approach for the rapid assessment of real oil pollution incidents. By combining comprehensive chemical fingerprinting techniques of high precision and accuracy with biomarkers of sublethal effect in a sentinel organism of ecological importance, we have been able to distinguish spilled oil from background pollution and gained a clearer understanding of baseline responses outside the area immediately impacted by the spill. The integration of biological and chemical biomarkers in this way offers huge benefit for responders in providing greater certainty for the decision making process in environmental management practices and directly addresses the policy need of improved risk assessment methodologies for ecological damage assessment.

Additionally work conducted as part of Work Package 8 has led to the development of innovative techniques for combining these biomarker tools with emerging bioreporter technologies for contaminant exposure assessment in the field. The novel application of bioreporter technology to biological matrices of animals from contaminated environments enabled bioassay measurements of both the original contaminant and its metabolites, quantifying bio-concentration factors of up to one hundredfold in crab urine. Our results reveal the substantial potential of using bacterial bioreporter assays in real-time monitoring of biological matrices to determine exposure histories, with wide ranging potential in the in situ measurement of xenobiotics in risk assessments and epidemiology.

The period of time encompassing the development of **molecular and predictive models** has strengthened most of the grounds that were posed as the basis of the work at the time of writing the proposal. Yet, other original premises have been surpassed by the emergence of non-anticipated technologies and new facts, both inside and outside the FACEiT Consortium. Along the way, the outcome of this WP has produced a considerable volume of new knowledge and new technologies, which includes, *inter alia*:

The development, validation an popularization of a consistent computational platform for both simulations of biodegradative processes in the inclusive microbial community (the MetaRouter system) and the prediction of the environmental fate of chemicals even in the absence of background experimental information (the PDB-Server). The interfacing of the predictive system with other toxicity prognosis platforms could not be achieved during the lifetime of FACEiT due to serious incompatibilities in the formats of the data, but this important issue will be followed up in future endeavours.

The WP has framed the maturity of a wide-use approach to the generation of large repertoires of antibodies against extensive mixtures of microbial proteins. Although the initial intention was just the production such antibodies for monitoring expression of key biodegradative enzymes in environmental samples, the method has acquired now a momentum much beyond this somewhat limited initial objective. What is at stake is the possibility to generate antibodies for each protein of a proteome and even have them expressed in different cell compartments in an active form.

Early in the development of this WP, it became clear that the use of DNA arrays for mapping the biodegradative landscape was far less valuable than originally foreseen. Not only was the very concept worked out by competitor groups outside the consortium. The technology in itself has started recently to be supplanted by the massive DNA sequencing possibilities brought about by the 454 pyro-sequencing methods. Looking in retrospect, it was by no means a failure that we spent so little effort in the landscape-wide DNA chip approach, given that the technology is destined for becoming obsolete in the not so distant future.

While the standard DNA chip concept was discarded as a general frame, a considerable progress was made in the side of developing electrochemical methods for detection of key biodegradative activities, either as such or by means of detecting their DNA signatures in environmental DNA. Along this line, a number of amperometric detection of enzyme-labelled DNAs were explored as part of this WP. While the casting of a prototype sensor could not be achieved in time, the methods have become established for a future development.

Regarding the **modeling behaviour of hydrocarbon mixtures** a strong focus was laid on the formerly overlooked aspect of carrier functions of dissolved organic matter (DOM) and biomass formed in the course of oil-biodegradation. The research led to the counterintuitive conclusion that these potent sorbents may increase the bioavailability of separate phase oil. Sorbents are commonly seen as obstacles to biodegradation, as they reduce the freely dissolved fraction of the contaminant. Oil pollution scenarios constitute an exception as (i) dissolution of floating oil constantly fills the aqueous and the DOM pool, (ii) the DOM pool is mobile and acts as a vector to (iii) oil-degrading bacteria that seem to have direct access to DOM-sorbed oil constituents. Inherent to this mechanism is that DOM does not exert such a positive effect in cases of below-solubility degrees of pollution, i.e. the lack of a reservoir of per se unavailable pollutants. Sorption to and carrier function of the DOM, however, was found to be strongly dependent on the octanol-water partitioning coefficients of the oil constituents studied.

With respect the question of **hydrocarbon bioavailability** to microbes degrading the oil and to toxicity response a hypothesized bioavailability factor was proposed, namely the development of diffusion barriers around oil but could not be experimentally simulated. Quite interesting observations were made regarding the sequential biodegradation of oil constituents in the aqueous phase. The typical metabolization of alkanes (including quite insoluble ones) ahead of PAHs cannot be explained by physical rules and appears to be determined by biological factors (ecological/ecotoxicological and/or specifics of the bioacquisition/uptake). It is important to note that the outcomes of this work package have been integrated and are in good agreement with corresponding results from different types of biological assays in other WPs.

The most important development regarding the establishment of **ageing fingerprints** was the extension of the original idea of a purely oil-composition based weathering index, i.e. use of oil composition changes induced by physical processes and biological degradation. We added a second fingerprint criterion that is based on stable isotope shifts as an independent indicator for the contribution of biodegradation, i.e. the most desirable process, to the fate of the oil. As this requires additional analyses of samples stored during FACEiT lab experiments and the FACEiT cruise, the realization of the two-criteria weathering index is still pending, but its accomplishment is very likely.

For overall and general **site-specific ecological risk assessment** of polluted soils, sediment and waters the consortium propose to apply the Triad approach. This method combines environmental chemistry, toxicity tests and ecological field observations. Triad is an effective combination for ecological risk assessment of polluted environments and many of the newer tools developed in FACEiT would have a rightful place in such an analysis. However some bottlenecks still remain. In the current ecological risk assessment it is not always clear which pollutant or other stressor is causing negative effects, especially when a site is polluted with a cocktail of pollutants. Although the strength of the Triad is that it takes all present pollutants and stressors into account, in case of remediation or redevelopment one wants to know at which pollutant remediation efforts have to be directed. Also, traditional tests can be expensive and time-consuming, although biomarkers can resolve parts of those drawbacks. Genomics and reporter gene based tools, like the ones developed within FACEiT play an important role in resolving these bottlenecks and they add enormous value to the current risk assessments (for instance by being fast or pollutant specific).

FACEiT was biologically and ecologically oriented, no hard chemical analyses were developed within this project. However, most bioreporter assays can be complementary to chemical analyses because they were shown to give quantitative analysis of the bioavailability of specific chemical compounds. Also modelling is traditionally considered as chemical part of the Triad. Some of the tools are operating in a sort of twilight zone between two of the three Triad components (e.g. chemistry and toxicology for the Calux system or toxicology and ecology for biomarkers).

For the tests developed within FACEiT already a corre-

lation between effects and hydrocarbon concentration has been shown both in lab and field studies (Etang the Berre). Of specific value was the open sea oil spill experiment on the North Sea in May 2008, which enabled to test many of the FACEiT tools and concepts in a direct scenario.

Depending on the pollutant, the type of environment and it's characteristics, site use and research question a site specific set of tools will be chosen to assess the ecological risk of the pollutant (or cocktail of pollutants). Usually the most sensitive model organisms and processes are chosen. It is also important to select representative model organisms for the polluted site under investigation. A combination of different organisms at different trophic levels is preferred because this gives the best impression of risks at the entire ecosystem. Both chronic (with endpoints such as growth, reproduction and genomic responses) and acute tests (short term with endpoints such as survival) can be used as well as functionality tests (for instance nitrification rate and capacity).

FACEiT has added a number of useful tools and concepts for disaster management strategies. However, not one tool will hold all, and regulatory agencies or policy and decision makers often require a single integrated value to assess the risks of contamination. FACEiT hereto favors integrative approaches, which would lead to 'color scales' or a single numbering system for quality assessment.