

## Kill•Spill Report Summary

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### Final Report Summary - KILL•SPILL (Integrated Biotechnological Solutions for Combating Marine Oil Spills)

Executive Summary:

Oil spill disasters remain a worldwide problem despite the fact that the number of large oil spills incidents have steadily decreased over the last five decades. It is important to consider that current emergency response technologies with booms and skimmers seldom go beyond an oil recovery of 10 %. Dispersants represent the classical remediation approach for open seas where the spilled oil is converted to billions of micron-sized droplets that are subsequently removed by intrinsic bioremediation. When an oil spill reaches the shoreline, the ecological and economic damaged is significantly increased. On the other hand, heavier agglomerates of oil and particulate matter often reach the sediments where bioremediation rates are practically negligible due to insufficient oxygenation. The problem of hydrocarbon-contaminated sediments (especially with polycyclic aromatic hydrocarbons and heavy components of crude oil) is often overlooked by clean up teams and policy makers. The Kill•Spill project offers an integrated approach that considers metabolic requirements of biodegrading organisms alongside the properties of the oil, environmental limitations on oil biodegradation and innovative delivery mechanisms for agents that intensify bioremediation.

Kill•Spill delivered innovative and efficient (bio)technologies for use immediately following recovery operations or as a first response option when the use of dispersants is warranted. In general, once crude oil is spilled, it takes at least one week before biodegradation processes begin to take effect. Kill•Spill aimed to shorten this start up period to the absolute minimum by providing technologies for example, that provide the necessary nutrients together with hydrocarbon degrading consortia and/or enhancing compounds (biosurfactants) to both accelerate and maximize bioremediation rates from the time of application. In addition, when the use of dispersants is recommended, the previously mentioned biostimulation and bioaugmentation formulations will be applied together with specific compounds acting as dispersants that further enhance bioremediation by dispersing the oil into tiny droplets. Kill•Spill also developed and tested successfully plant-based biosurfactants to be used as bio-based dispersants.

Once the heavier fractions of the spilled oil reach the sediments, bioremediation rates are substantially reduced due to the prevailing near anoxic conditions. Kill•Spill provided a series of highly innovative technologies (e.g., "Kill•Spill snorkel", "Kill•Spill ElectrO2", "Kill•Spill Sed-Cleaner") that overcome this problem and enhance biodegradation rates in the sediments. These technologies can also be used for the remediation of recurrently polluted sediments (from old oil spills) in all types of environments from the Eastern Mediterranean to Disko Bay in Greenland.

Verification of biodegradation can be obtained by the Kill•Spill biosensors and chemical protocols developed for monitoring in-situ biodegradation in the marine environment. Assessment of the ecotoxicological effects of bioremediation products developed by the Kill•Spill project were performed on five target species of the marine food web, according to recognised best practice and experimental implementations.

The Kill•Spill project involved 14 SMEs active in complementary areas that contributed to the development of innovative and integrated solutions and tailored strategies for the oil spill clean-up market. In addition, the Kill•Spill project has contributed to the Marine Strategy Framework Directive (MSFD). For example, all the technologies developed for hydrocarbon polluted sediments can be part of the mitigation measures to return marine environments to Good Environmental Status (GES). Furthermore, the monitoring tools can be used by Member States in the requested initial assessment to identify current

environmental status. Moreover, many of the Kill•Spill biostimulation strategies can be applied to sea areas faced with chronic pollution.

## Project Context and Objectives:

### 2 Summary description of project context and objectives

#### 2.1 Project context

Oil spill disasters are a worldwide problem and current technologies do not satisfactorily address the problem. It is important to recognize that “miracle microorganisms” and “magic elixirs” will not do the job and an integrated approach that considers metabolic requirements of biodegrading organisms alongside the properties of the oil, environmental limitations on oil biodegradation and innovative delivery mechanisms for agents that alleviate these bottlenecks is essential. This is the essence of Kill•Spill. Response time is of utmost importance facing an oil spill and therefore Kill•Spill offers several innovative technologies to speed up bioremediation. It represents a European initiative to tackle oil spill disasters in an integrated and interdisciplinary fashion to cover a range of foreseeable circumstances including remediation of contaminated sediments – an issue often overlooked by clean up teams and policy makers. Figure 1 relates the Kill•Spill goals and technologies to the various phases of oil spill response actions.

#### Figure 1 Overview of Kill•Spill innovations and approach

The working hypothesis of the Kill•Spill project is that each oil spill is unique and hence, a toolbox of highly innovative (bio)technologies should be developed. Those shall be made readily available to all emergency response personnel subject to final approval by the European Marine Safety Agency and related member state agencies. The oil spill industry is fairly conservative and looks at any remediation method only if it has been field-tested, Kill•Spill therefore planned to carry out field-testing of each technological solution under development to establish its performance. For this reason, the Kill•Spill consortium includes two emergency response companies as partners (MMB S.A. operating in the Norwegian Sea/Baltic Sea area and EPE S.A. responsible for emergency response in the Eastern Mediterranean Sea). All promising remediation (bio)technologies and (bio)monitoring tools will be tested under various conditions at the large pilot scale facilities in Messina (IAMC-CNR) which is equipped with complete monitoring capabilities.

Kill•Spill aimed at delivering highly efficient integrated biotechnological solutions to significantly shorten the remediation period and deal with sunken oil. At present emergency response in many European sea regions (e.g., Baltic Sea, Mediterranean Sea) constitutes in the use of booms and skimmers to contain and recover as much as possible of the spilled oil. However, in open sea the use of dispersants is encouraged with particular emphasis on a new generation of non-toxic compounds to remove the oil from the surface and sink it before the oil spill reaches the shoreline. Natural attenuation is allowed only in open seas and under high wave energy conditions. In situ burning is not an option for the EU.

If one looks at the weathering processes and the typical period of activation of the various mechanisms that act on spilled oil, it becomes obvious that it takes at least one week before biodegradation processes take effect. Kill•Spill aims to shorten this start up period to the absolute minimum by providing technologies for example, that provide the necessary nutrients together with hydrocarbon degrading consortia and/or enhancing compounds (biosurfactants) to both accelerate and maximize bioremediation rates from the time of application. In addition, when the use of dispersants is recommended, the previously mentioned biostimulation and bioaugmentation formulations will be applied together with specific compounds acting as dispersants that take the oil from the surface to the water column and ultimately to the sea floor. Realizing that as we move down the water column the amount of dissolved oxygen is more difficult to replenish by diffusion, Kill•Spill also offers specific novel technologies (Oxygel™ and Aerobeads™) that release oxygen over prolonged periods of time and hence, maintain enhanced bioremediation rates of the dispersed oil in the water column and even when it reaches the sediments. In situations where this is not feasible we will complement this with development of processes to stimulate oil biodegradation anaerobically in anoxic sediments.

Once the dispersed oil reaches the sediments, bioremediation rates are substantially reduced due to the prevailing anoxic conditions. Kill•Spill provides a series of highly innovative technologies that overcome this problem and enhance biodegradation rates in the sediments. These technologies can also be used for the remediation of recurrently polluted sediments (from old oil spills) in all types of environments from the Eastern Mediterranean to Disko Bay in Greenland.

Since claims of enhanced biodegradation without scientific verification are of little value, Kill•Spill has devoted sufficient resources to develop biosensors and chemical-based monitoring tools that will enable the quantitative monitoring of biodegradation rates. Although the development of such tools does not increase the efficiency of proposed remediation technologies, it plays a significant and prominent role in fine-tuning the application rates and most importantly to verify claims of achieved enhanced biodegradation rates. Finally, Kill•Spill will provide a detailed ecotoxicological assessment of all these technologies.

## 2.2 Primary objectives of the project

The principal objective of Kill•Spill is to develop highly efficient economically and environmentally viable solutions for the clean-up of oil spills caused by maritime transport or offshore oil exploration and related processes. Solutions should have been fully validated in large mesocosm facilities under controlled conditions and by potential application to real life oil spills in the Eastern Mediterranean Sea or in the North Sea/Norwegian Sea.

The Kill•Spill primary goals are the on-site validation of comprehensively assessed (bio)technologies developed in the program, and ensuring that these are adapted for an eco-efficient, reliable and predictable remediation of oil spills. Kill•Spill works according to three principles that are synonyms with good technological practice and quality:

**Effectiveness** – Develop highly effective products / technologies based on comprehensive laboratory tests under diverse conditions. There are numerous commercially available products that simply fail to meet their expected performance once the prevailing conditions change – a usual field test observation.

**In-field applicability** – Determine to what extent the effectiveness of the developed technologies to enhance biodegradation rates that has been achieved in laboratory and mesocosm tests, is also maintained in large scale, real life, oil spills and under diverse conditions.

**Eco-friendliness** – (Bio)materials or minerals destined to be used in large quantities in the marine environment should be assessed for the impact on the environment prior to their widespread use. This is an integral part of the Kill•Spill project.

To achieve these ambitious objectives, Kill•Spill was structured into ten interrelated Work Packages (corresponding PERT chart shown in Figure 2), which had the following objectives and concepts:

### Figure 2 Work package structure in Kill•Spill project (PERT chart)

#### WP1- Review and in depth analysis of the current knowledge in the field

The objective is to re-examine the scientific literature, relevant patents and commercially available products, with the main objective to identify knowledge gaps, technological gaps, and current technology trends in an effort to ensure that Kill•Spill's deliverables cover all the existing technological gaps and offer highly efficient – complete solutions for combating all types of oil spills.

#### WP2- Development of biosensors and in-situ monitoring tools to determine biodegradation efficiency

We aspired to design and evaluate efficient and cost-effective tools and methods to allow the in-situ monitoring of hydrocarbon pollutants during and after the appropriate decontamination treatments have taken place. This will allow to closely monitor both biodegradation rates and potentials by the microbial communities present at a contaminated site.

#### WP3- Development of novel dispersants and sorbent materials

The aim was to explore the availability and efficiency of specialized biological surface-active compounds ((bio)surfactants, (bio)dispersants, (bio)emulsifiers) and other suitable sorbent materials to accelerate oil dispersion, emulsification, sorption and ultimately hydrocarbon bioavailability to microbial degradation leading to complete mineralization.

#### WP4- Microbial and additive formulations for enhanced bioremediation

The transfer of the capabilities of microorganisms into innovative formulations for bioaugmentation will serve as the basis for further developments in WP6. The activities were directed to the enrichment, isolation and improvement of novel degraders (single strains and mixed culture consortia). Those were to be combined in biostimulants formulations to achieve highly enhanced bioremediation rates. A special focus was on biosurfactant producing microorganisms as ingredient of bioaugmentation formulations and their positive role on the efficiency of hydrocarbon degradation.

#### WP5- Efficient cleanup of contaminated sediments due to oil spills - emphasis on biotechnological solutions

The objective was to develop novel or improved biotechnological solutions, which enable the acceleration of hydrocarbon degradation in contaminated sediments, either “freshly” contaminated sediments or chronically polluted.

#### WP6- Development of multifunctional remediation agents for oil spills

Combinations of agents were supposed to result in novel and innovative multifunctional agents. At least two modes of actions were to be provided (e.g., dispersing oil and providing nutrients or microbes, or absorbent materials with encapsulated nutrients & hydrocarbon degrading microbes, or dispersants with continuous oxygen supply).

#### WP7- Impact assessment of best developed technologies

The eco-compatibility of dispersant, biostimulants and other oil-degrading products had to be proven, based on standard ecotoxicological assays. Field assessment involved a standard BACI (Before and After Control Impact) survey design. This approach was to favour the development of environmentally-friendly treatments for the degradation of petroleum compounds.

#### WP8- Field Testing of Most Promising Technologies and Benchmarking with existing products

The quantitative evaluation of the efficiency of the developed technologies was performed in mesocosm studies where they were also benchmarked with existing products. Most importantly, the best technologies were to be field tested in real life oil spills normally tackled by our participating SMEs (EPE in Mediterranean Sea and MMB in Norwegian Sea). This was to identify best strategies to tackle oil spills depending on type of oil and prevailing environmental conditions.

#### WP9- Dissemination, policy contributions & exploitation

Strategies were implemented for (1) the dissemination of our developed novel technologies and integrated solutions for combating oil spills to policy-makers, scientific community and industry, the general public, including policy contributions to the EU Marine Strategy Framework Directive & the EU Thematic Strategy on the Sustainable Use of Natural Resources; and (2) the exploitation of results following the EC funded period.

#### Project Results:

##### 3 Description of main S & T results/foregrounds

The main tangible results of the Kill•Spill project are novel products for the application in oil spill remediation. To make real advance in the field of oil spill emergency response, Kill•Spill developed these novel products and technologies on an analysis of tools and techniques currently applied in spill remediation (WP1). Various innovative and reliable analytical methods have been developed in WP2 in order to evaluate and validate the effectiveness of products and technologies. These analytical methods comprise biosensors and isotope or compound specific chemical analyses. The products and technologies of Kill•Spill are: biosurfactants as alternative to chemical dispersants and sorbent materials, microbial formulations to intensify biodegradation potential, technologies and materials for the treatment of sediments and multifunctional products to intensify biodegradation. In most cases the innocuousness of these products and technologies was verified at an early stage using a large set of ecotoxicological tests developed in WP7. The performance of most promising products and technologies of Kill•Spill have been tested in real conditions or in environments nearing the natural ones.

Overall, the Kill•Spill project aspired to develop a number of innovative products and technologies for potential exploitation in the marine emergency response market, as listed in Table 1

Table 1 Kill•Spill products and technologies

##### WP 2

###### Monitoring tools

(biological & chemical) "Kill•Spill Biosensor" A set of biosensors for HC-monitoring in the water column adapted to the high salinity and suitable for on-site monitoring of oil degradation (alkanes, aromatics & PAHs)

"Kill•Spill FISH-Kit" & "Kill•Spill FCM-Kit" two cultivation-independent microbial diagnostic kits.

"Kill•Spill Chip" a microarray chip enabling the quick determination of the presence of hydrocarbon degraders in the water column.

"CHEMSIC" a chemical analysis based method to monitoring of oil biodegradation versus other abiotic removal processes.

##### WP 3

Biosurfactants and novel absorbents Development of polymer-based non-woven fabrics for use as sorbent materials to collect floating hydrocarbons (shoreline and near-shore application)

Development of mineral-based powders as sorbent materials with the additional goal to accelerate bioremediation

Development of oxygen-releasing dispersants for seawater applications accelerating bioremediation in anoxic environments as a gel formulation or porous granular sorbent.

Plant-based biosurfactant blends as surface active agents for efficient dispersion of hydrocarbons in the water column accelerating bioremediation (e.g. for sand washing).

Development of cost effective and efficient production of microbial biosurfactants and emulsifiers for mobilization of oil, sand washing and accelerated bioremediation.

#### WP4

Microbial formulations for enhanced bioremediation Development of formulations of hydrocarbon-degrading microbes and consortia for the in situ bioaugmentation (including autochthonous bioaugmentation approaches) for enhancing bioremediation

Design and operation of a High-Pressure bioreactor (>100 bars) emulating in the laboratory the deep-sea environment and hydrocarbon releases at great depths.

High RPM bioreactor - Isolation of hydrocarbon degrading microorganisms that can cope at increased hydrodynamic stress.

Low cost biostimulant formulations - examination of alternative oleophilic sources for N & P supplementation to accelerate bioremediation rates

#### WP 5

Clean-up of contaminated sediments "Kill•Spill ElectrO2" An electrode-based technology for in situ oxygen supply in anoxic sediments to enhance aerobic bioremediation of petroleum hydrocarbons

"Kill•Spill snorkel" An in situ microbial electrochemical "snorkel" to enhance bioremediation in hydrocarbon contaminated sediments.

In-situ sediment cleanup through infauna accelerated biodegradation of petroleum hydrocarbons.

A novel capping technology with sequestering sorbents: to prevent leaking of contaminants via diffusion to the water column

#### WP 6

Multifunctional remediation agents "Kill•Spill Deep-sea" Multi-functional bioremediation agents developed in the high pressure bioreactor for enhanced bioremediation of hydrocarbon-"clouds" formed in deep-sea oil releases

"Kill•Spill Mesoporous" A novel technology to enhance bioremediation through biostimulation with mesoporous silica (nano)particles with smart gates to control the release of nutrients.

"Kill•Spill SlowRelease" Development of slow release microparticles that enhance bioremediation through bioaugmentation and slow-release of fertilizers in lipophilic carriers

"Kill•Spill All-in-One" Development of a multifunctional carrier as a first response measure for enhanced bioremediation, oil dispersion and nutrient supplementation.

"Kill•Spill MineralSorb" Development of multifunctional sorbent materials based on mineral sinking agents that enhance bioremediation as they sink

### 3.1 In depth analysis of current knowledge and identification of technological gaps (WP1)

We reviewed technologies used in past oil spill incidents and identified technological gaps as well as future trends in combating oil spill. The findings are summarised in a number of reports shortly summarised below.

#### Oil containment and recovery

Oil containment and recovery is the primary response option of choice to mitigate environmental damages. Mechanical methods to combat oil spills make use of booms, skimmers, heavy oil skimmers, skimmer vessels, specialized response vessels and sorbent. Low viscosity oil and high currents can limit the efficiency of these physical barriers. Additional limitations to the deployment of boom are their anchoring in deep water areas, limited volume of oil they can contain, hydrodynamic conditions, and the need for trained users. Skimmers can appropriately be used to recover the contained oil but oil recovery is not always feasible, e.g. presence of ice or debris. One promising approach is the use of sweeping arm oil recovery system, which combines containment and recovery systems. Past oil spill incident reports on Sea Empress, Erika, Baltic Carrier, Prestige, Godafoss and Deepwater Horizon showed that containment and recovery can make a contribution (2 to 50 % recovery of released oil), provided the weather and sea conditions are calm and stable.

#### Chemicals

WP 1 studies included chemicals, in particular dispersants, which have been routinely used in many countries as a response option. The use of dispersants (mixtures of surfactants and solvents) is a “sensitive” subject due to the ecological damage it may cause. Dispersants break oil slick into fine droplets, which are further distributed in the water column. The use of dispersants is not adapted to certain environments e.g. shallow waters and its efficiency is limited by the nature of oil and physico-chemical parameters of the contaminated areas. Further chemical combination can include demulsifiers, solidifiers, surface film chemicals, gelling agents, bioremediation chemicals; burning agents; neutralizing agents; sinking agents; other (e.g. viscoelastic additives). Literature suggest that biosurfactants could play an important role in remediation processes due to their efficacy as dispersion agents and enhancers of oil bioavailability to oil microbial degraders as well as their environmentally friendly characteristics, such as low toxicity and high biodegradability. Review of application of dispersants in past oil spills Sea Empress, Katja, Erika, Prestige, Tasman Spirit, Deepwater Horizon showed variable efficiency ranging from none for heavy HFO180 to 16 % of released oil.

#### In-situ burning

In-situ burning is an oil spill clean-up technique is suitable for massive spills and for remote areas. Fire-resistant booms are used to concentrate the oil into thicker slicks prior to oil ignition. The most obvious disadvantage of burning oil is concerns about toxic emissions from the large black smoke plume produced. The rapid weathering and spreading of oil limit oil in-situ burning. In-situ burning was successfully carried out at Deepwater Horizon were 42,000 m<sup>3</sup> of oil to be burnt (5 % of the estimated total). Observations showed that the combustion residues sank rapidly.

#### Shoreline cleaning

Oil that has not been contained, recovered, dispersed or burned reaches shorelines, where its fate and behaviour is influenced by many factors, i.e. type and amount of oil, degree of weathering of the oil, temperature, tide, type of beach substrate, biota on the beach, and the steepness of the shore. Taking this into account the responders can select response options, which include physical (e.g. manual removal, sorbents, debris removal, pressure washing), and chemical (e.g. elastomers) methods as well as alternative technologies such as nutrients and microbial addition. Shoreline clean-up effectiveness is probably best assessed by how soon the area can be returned to the pre-spill state. In all cases when oil came ashore the primary method of clean up was manual recovery followed in some cases by mechanical, physico-chemical and even biological cleaning methods.

#### Bioremediation

Bioremediation encompasses the addition of oil degrading microorganisms or nutrients to sustain/accelerate biodegradation appears to be an attractive solution. In bioaugmentation, the addition of oil-degrading bacteria boosts biodegradation rates whereas in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of nutrients (mainly N & P) or other growth-limiting nutrients. First option biological methods have gained importance and acceptance mainly due to the low environmental impact, the costs and the capability to degrade a wide variety of organic contaminants.

Bioremediation through its first successful application on the Exxon Valdez spill has motivated many researchers to investigate physical, chemical and biological factors that could produce favourable conditions for in-situ and ex-situ treatments. However, experience made in real marine environment is missing.

#### Surveillance and monitoring

The documents produced in the frame of WP11 showed that surveillance and monitoring are essential to make adequate response decision. Overall there is ‘good’ aerial surveillance coverage within Europe. However there is a real need for marine pollution aerial surveillance, visual observation and oil spill quantification training in the Eastern Mediterranean and Black Sea. Remote sensing sensors can be used successfully in combination with visual observation. Information Gathering Systems are used by all parties to build up a ‘good’ surface picture overall. Information from other sources, particularly the general public, is an invaluable complement. Satellite Surveillance Systems for pollution monitoring are commonly used throughout the area.

#### Technological gaps

It was concluded that technological gaps exist as for more effective clean-up techniques, e.g. ways to recover or directly degrade a larger portion of spill oil. This also calls for more alternative cost-effective and environmentally more acceptable techniques such as bioremediation as an oil response technology. In addition, also lack of a sufficient and integrated decision making plan and oil spill contingency plans or countermeasures hamper effective spill response.

### 3.2 Development of biosensors and in-situ monitoring tools to determine biodegradation Efficiency (WP2)

While information on oil pollution can be obtained nowadays with considerable accuracy, the technology needed requires heavy and/or sophisticated equipment, time-consuming procedures, and transfer of samples to specialised laboratories (it cannot be used in-situ). Several monitoring tools have been developed or optimized within the Kill•Spill project that, when used in a combined way, allow obtaining a very complete picture of the extent, impact and fate of the contamination. The tools developed within the project Kill•Spill are: i) biosensors designed to detect the presence of hydrocarbons in seawater; fluorescence in situ hybridization (FISH kits) to detect bacteria known for their oil degradation capacity, DNA micro-array enabling the detection of the presence genes related to the biodegradation of oil components, chemical analyses

#### Whole-cell based biosensors

In the frame of Kill•Spill, whole cell biosensors have been developed to detect the presence of hydrocarbons in seawater. Whole-cell biosensors consist in a bioreporter system that is introduced into an appropriate host. The bioreporter is formed by a regulatory element and a reporter element. The regulatory element is based on a natural regulatory circuit composed of a transcriptional regulator and a promoter that controls the expression of the reporter. The reporter gene encodes an easily measurable protein. The bioreporter systems have to be introduced into a host that should at least i) allow expression of the regulatory circuit; ii) be tolerant towards relatively high concentrations of contaminants; iii) transport the contaminant inside the cell. CSIC partner has developed several biosensors that can efficiently detect the presence of key hydrocarbons in seawater samples. The biosensors and their sensing performances are summarized in Table 2.

[TABLE 2]

The performances and characteristics of the biosensors developed within the Kill•Spill project can be summarised as follows. All biosensors developed were highly specific for the compounds to be detected, e.g. the biosensor for n-alkanes was not able to detect aromatic hydrocarbons. All biosensors gave positive signals with samples from environments contaminated with the specific pollutants, and gave no signals when these pollutants were not present (no false positives). Several biosensors were able to detect lower quantities of one specific contaminant in complex mixtures than when pure substances were tested. For example, the amount of toluene detected by *Alcanivorax* SK2 (pKST1) in petrol (calculated to be 0.03 mg L<sup>-1</sup> in samples with 0.0001 % (v/v) of petrol) is lower than the detection limit for toluene (0.04 mg L<sup>-1</sup>). It is important to keep in mind that biosensors measure the amount of contaminants bioavailable to the cell. These biosensors can be used for the determination of the presence/absence of contaminants in seawaters; although there is a correlation between the contaminant concentration and the biosensor response, the biosensor cannot be used for the quantification of the contaminants in environmental samples.

#### Kill•Spill FISH kits

Kits to directly detect bacteria that are well-known hydrocarbons degraders within a complex microbial community have been developed within the Kill•Spill project. These kits are designed to evaluate the biodegradation potential of the microbial communities present at the contaminated sites. They are based on fluorescence in situ hybridization (FISH) methodology. This tool is cultivation-independent and enables a fast, easy and effective way of analysing microbes within numerous environmental samples such as hydrocarbon-contaminated water. By transferring the FISH technology to an industrialized format, the so-called VIT® kits, the experiments are user-friendly and are applicable even for on-site use. They can be used either with a fluorescence microscope or in combination with semi-automated flow cytometry measurements. For the Kill-Spill FISH kits, gene probes were designed to specifically target several well-known marine hydrocarbon-degrading bacteria, namely *Alcanivorax* spp. (including *A. borkumensis* and *A. dieselolei*), *Cycloclasticus* spp., *Marinobacter* spp., *Oleibacter marinus*, *Oleiphilus messinensis*, *Oleispira* spp. and *Thalassolituus oleivorans*. The basic protocol to use this kit to detect aforementioned microorganisms is made up of only five steps: (I) specific gene probes are applied to the sample, (II) gene probes penetrate all microorganisms during hybridization, (III) the gene probes bind specifically only within living target organisms, (IV) unbound gene probes are washed off and (V) target microorganisms can be visualized and quantified in relation to the total viable flora with a fluorescence microscope or as absolute numbers per millilitre of water sample. All kits were tested with pure and mixed cultures of the target strains, with samples from micro- and mesocosm experiments conducted within the frame of the Kill•Spill project as well as with real samples from naturally contaminated marine sites. Each batch produced is routinely checked for functionality and specificity. The test kits were validated for different applications: (I) Quality control of cultured hydrocarbon degrading bacteria prior to their use in oil spill accidents. Here, the kits

allow identification and quantification of target microorganisms as well as the detection of putative contaminants. (II) Identification of target microorganisms in environmental samples, e.g. seawater collected from oil contaminated sites. (III) The bacterial populations of interest can also be quantified.

#### Kill•Spill microarray

A DNA microarray designed to detect genes known to be involved in the degradation of hydrocarbons has been developed in the frame of Kill•Spill. The information provided by this kind of technology serves as a useful signature of the hydrocarbon degradation potential of the microbial populations present in contaminated sites. The Kill•Spill chip is a microarray system designed from curated key aromatic catabolic gene families and key alkane degradation genes. It contains probes designed from curated 10 key aromatic catabolic gene families and key alkane degradation genes. The microarray contains 3605 probes representing catabolic gene subfamilies encoding key activities in hydrocarbon degradation pathways, e.g. extradiol dioxygenases. Most probes are derived from genomes from cultivable bacteria of at least 182 different species, distributed among 70 genera that included. The experimental procedure to use the Kill•Spill micro-array can be summarized as follows. Total DNA of the sample to be tested (contaminated site at different times and locations) is extracted, fragmented and labelled prior to hybridization to the microarray and subsequent reading.

The Kill•Spill chip developed was thoroughly tested by using DNA material of *Amycolatopsis tucumanensis* DSM 45259 and *Cycloclasticus* sp. ME-7 in order to ensure that signals measured are consistent with the hydrocarbon metabolic capacities of these microorganisms. Finally the Kill•Spill chip was tested using total DNA from contaminated sediments before and after oil spill accident in Gela (Sicily, Italy). The number of probes that hybridized using DNA samples collected one month after the spill was approximately 4-fold higher to that before the oil spill occurred. This strongly indicated an improved capacity of the microbial communities developing in this sample to support a higher number of degradation reactions in the presence of a high concentration of petroleum hydrocarbons. Similar observation has been reported previously during the Deepwater Horizon oil spill.

To summarize, the Kill•Spill microarray is a gene detection system that allows disentangling degradation phenotypes in any organism or microbial community, without the need of genome or metagenome sequencing. It contains probes designed from curated 10 key aromatic catabolic gene families and key alkane degradation genes.

#### Analytical method for the reliable detection of hydrocarbons

Efficient extraction, pre-concentration and clean-up methods are indispensable prior to the determination of PAHs in water, soil and sediments. Methods for the quantification of petroleum hydrocarbons from sediment and water samples were developed and used to assess the performances of the Kill•Spill remediation products and technologies. These methods are designed to detect changes in the composition of crude oil that allow searching for signatures that are indicative of biodegradation of hydrocarbons.

For extraction of oil from soil and sediment, two different methods were applied, i.e. a simple technique with stepwise extraction by means of ultrasonication and clean-up, and a more sophisticated approach with simultaneous extraction and clean-up using pressurized liquid extraction (PLE). For oil in water, simple liquid/liquid extraction was applied. In all cases, the adequate deuterated standards (internal and external) were selected according to the analytical platform employed and the purpose of the analysis, i.e. GC-FID analysis for TPH quantification, GC-MS analysis for PAC and biomarker quantification and GC-MS analysis for weathering specific semi-quantitative diagnostic ratio calculations. It was possible to perform a chemical fingerprinting of oil hydrocarbons using GC-MS and GC-FID and in some cases to discriminate between hydrocarbon isomers that are assimilated faster by microorganisms from those that are assimilated more slowly. Thus it was possible to deduce that a sample had been exposed to biodegradation when this oil sample was enriched in hydrocarbon isomers that are metabolized more slowly.

#### Compound Specific Isotope Analysis

Compound-specific isotope analysis (CSIA) has become an established approach to better understand transformation reactions of organic compounds. CSIA is based on the observation that microbial enzymes typically act faster on chemical bonds containing a light isotope ( $^{12}\text{C}$  or  $^1\text{H}$ ) than on those containing the corresponding heavier isotope ( $^{13}\text{C}$  or  $^2\text{H}$ , respectively). Therefore, those hydrocarbons containing the heavier stable isotopes are progressively enriched in a sample along the biodegradation process. Determining the isotope enrichment factors for specific compounds allows deducing whether a sample has been exposed to biodegradation processes or not. Furthermore, CSIA enables the allocation of types and sources of



contamination as well as the identification and quantification of bioremediation processes by measuring the relative abundance of stable isotopes of an element within single organic compound at its natural abundance – even at very low concentrations ( $\mu\text{g/L}$ ).

Gas chromatography-isotope ratio mass spectrometry (GC-IRMS) was developed and applied to analyse the isotope ratios of individual compounds of complex mixtures of hydrocarbons in various samples of the Kill•Spill project.

In conclusion, CSIA by GC-IRMS enabled the observation of carbon and hydrogen isotope shifts within monoaromatic and polyaromatic hydrocarbons, giving evidence for biodegradation processes within oil spills. Thus, CSIA can be used on one hand to monitor bioremediation of oil spills under natural conditions and on the other hand for quality control of applied bioremediation techniques and strategies (e.g., application of nutrients, surfactants) enhancing degradation of oil contaminants in the environment.

#### MEDSLIK-III software package

Partner TUC has developed a software package, named MEDSLIK-III, for modelling oil spill fate and transport in the marine environment considering factors not included in existing similar packages. MEDSLIK-III is based on its precursor MEDSLIK-II (<http://gnoo.bo.ingv.it/MEDSLIKII/>), which has been modified by adding modules describing biodegradation of oil dispersed or dissolved in the water column and improving existing oil transport and weathering subroutines. The new model predicts the transport and weathering (evaporation, emulsification, dispersion in the water column, biodegradation, adhesion to coast) of an oil spill based on data such as the oil spill characteristics (location, time, extent of the spill), oil properties (density, viscosity, vapour pressure), wind field, sea surface temperature, 3D currents and wave characteristics. Biodegradation is modelled by Monod kinetics, allowing the investigation of several important parameters that can limit biodegradation rate, such as oil composition, microbial population, availability of dissolved oxygen and nutrients, and oil droplets-water interface. The model produces as output the evolution of the concentration and the position of the surface oil slick, the dispersed oil and the oil adhered to coast.

#### 3.3 Development of novel dispersants and sorbent materials (WP3)

The activities within WP 3 dealt with the use of biosurfactants of microbial and plant origin to disperse and accelerate biodegradation and the use of cheap and sustainable sorbent materials to prevent pollution of water by hydrocarbons contained in the spilled oil.

##### Microbial biosurfactants

Most of the surface-active compounds (SACs) in use for oil dispersion are chemically synthesised and are usually toxic to the environment and non-biodegradable. Biosurfactants are SACs produced extracellularly by microorganisms. In the frame of the Kill•Spill project four microbial biosurfactants (sophorolipids, rhamnolipids, surfactin and mannosylerythritol lipids) were produced and tested.

The microbial biosurfactants were produced by several strains, which have been isolated for their capabilities to excrete these SACs (Table 3). Out of those the most important surface-active products were sophorolipids, rhamnolipids, surfactins, and mannosylerythritol lipids.

[TABLE 3]

Within Kill•Spill a large-scale production process was obtained taking into account low cost and sufficient production of selected biosurfactant, which was achieved by use of a database from Actygea partner. This approach was mainly based on the selection of cultivation media for aforementioned microorganisms yielding the highest volumetric amounts of biosurfactants. Selected strains and optimized fermentation media were used for up-scaled production of biosurfactants in pilot scale bioreactors with subsequent downstream processes. The quality and characteristics of the biosurfactants obtained by means of the developed up-scaled processes are summarized in Table 4.

[TABLE 4]

The effect of biosurfactants amendment on oil bioremediation was tested in 50 litres mesocosms at IAMC-CNR (Messina). The action of optimized Kill•Spill biosurfactants (rhamnolipids or sophorolipids) was assessed in combination with microbial consortia (HC degraders). The experimental design enabled the monitoring of following parameters over 15 days:

(i) Dispersion of crude oil in sea water;

- (ii) Enhancement of degradation of crude oil by microorganisms;
- (iii) Influence of the evolution of bacterial populations;
- (iv) Restoration of the pristine pre-pollution situation.

Concerning emulsification effect, significant micelle formation was detected only for homogenized rhamnolipids. Rhamnolipids' emulsification was stable and gravimetric measurements performed after 75 h showed that more than 80 % of added crude oil was dispersed in water column (0.81-0.83 mg.mL<sup>-1</sup>). In concordance with emulsification activity, best degradation rates were detected in the mesocosm supplemented with homogenized rhamnolipids (65 mg per litre of crude oil). At the end of experiment (day 15) approximately 60 % of added petroleum was degraded. Noteworthy, the short-chain alkanes (< C20) disappeared almost completely, while longer hydrocarbons were degraded to a much lesser extent. Sophorolipids also stimulated the biodegradation, although only 15 % of added petroleum was degraded within the duration of the experiment. Additional evidence for microbial degradation of oil components was gained by measuring bacterial colony forming units. Microbial counts showed an increased of total microbial counts of  $3.2 \times 10^5$  cell mL<sup>-1</sup> at the beginning of the experiments to  $1.3 \times 10^7$  cell mL<sup>-1</sup> in the rhamnolipids treated mesocosm after 15 days of incubation. In comparison sophorolipids treatment did not have such a pronounced response with counts being  $9.5 \times 10^5$  cell mL<sup>-1</sup> at day 15. No statistically significant change in microbial population density was observed in control tank during 15 days of incubation.

Overall petroleum contamination level of sediments was detected as 50 ppm (95 % of biodegradation) and 120 ppm (88 % of biodegradation) in rhamnolipid- and sophorolipid-supplemented mesocosms, respectively, indicating very efficient strategy of biosurfactant treatments. Experiments in mesocosms within the Kill•Spill project have shown that biosurfactants can be effective in degrading a significant portion of the hydrocarbons accumulated in the sediment layer.

Contrarily to other industrial applications, biosurfactants can easily be used in an environmental application such as hydrocarbon remediation and, indeed the presence of a mixture of surface active compounds with varying activities could be seen as an advantage particularly in terms of lowering the cost needed for separation or downstream processing.

#### Mineral based powder MBS-2M

Partner microsTECH has developed a new stearic acid-treated calcium carbonate-based product with increased oil-adsorbing properties and sinking properties. The new material combines beneficial properties of inorganic (cost-efficiency, inertness and incombustibility) and organic sorbents (can be applied on the water surface). The product has been produced using ground calcium carbonate (GCC, particle size of 1.5 µm) by grinding white calcite using jaw, cone crushers, and Jet Mill. Molten stearic acid was adsorbed onto the surface of GCC powder using the dry process coating system of a direct melting spray method. Different particle sizes and combinations with different stearic acid percentages were investigated for sorption capacity and sinking performance. These investigations were made in microcosm trials in microsTECH facilities. The results have shown that MBS-2M calcium carbonate powder, if applied on an oil carpet, has a great sorption value, but the material stays completely on the surface. However, if swell is simulated, it begins to sink immediately and within 2 hours the carbonate powder is completely sedimented. The oil absorption number of the surface-treated calcium carbonate powder MBS-M2 (according to the DIN EN 787-5 - Determination of oil absorption value) is 30.5 g•100 g<sup>-1</sup>. To evaluate the best sinking value, different functionalized and non-functionalized calcium carbonate powders have been tested in different product to oil ratios. The best sinking ratio (sedimented mixture/applied oil-carbonate mixture) is achieved with a carbonate:oil ratio of 1.5:1. MBS-2M was tested on the open sea in Kill•Spill field tests at the facilities in Messina and at the Environmental Protection Engineering S.A. (EPE) in Piraeus. In this trials MBS-2M showed good sorbent and sinking performances, both in heavy and light pollution conditions. One day after the application, most of the oil was removed from the surface.

The MBS-2M calcium carbonate powder is ready to be commercialised. The large-scale production is established and microsTECH AG is now able to deliver activated CaCO<sub>3</sub> in bulk volumes. Further development tasks have been carried out to assess if the MBS-2M product can be used as a growth material for hydrocarbon degrading bacteria in order to sorb oil and concomitantly biodegrade its components. Preliminary results have shown that it is possible to immobilize bacteria onto the surface of MBS-2M calcium carbonate powder. The degradation of sorbed crude oil by immobilised bacteria is subject to future investigation.

#### Oilguard 2014

Partner HeiQ further modified an existing oil sorbing fabric towards enhanced properties. The standard product was coated with different wax containing formulations which enhanced the sorption capacity. Eventually Oilguard 2014, wetted with

seawater, was able to adsorb oil up to 12 times its own weight.

#### Oxygel and Aerobeads

Partner Biorem has developed oxygen-releasing formulations combined with oil dispersion or adsorption and sinking properties for the stimulation of oil hydrocarbons biodegradation in anoxic sediments.

The first formulation Oxygel is a gel based on calcium phosphate hydrated by hydrogen peroxide, combined with a mixture of bio based solvents and surfactants. Oxygel is produced by a patented method of precipitating a calcium phosphate salt in the presence of hydrogen peroxide. When the precipitation occurs, hydrogen peroxide is trapped within the CaPO<sub>4</sub> crystalline structure, forming a sol-gel. After a period of maturation, the different additives (extra nutrients, solvent and surfactant) are mixed into the gel to form the final product. Viscosity, particle size and oxygen content was adjusted by changing the ratio between the different salts and hydrogen peroxide. The gel particles have the capacity of releasing oxygen over time (in the form of hydrogen peroxide), thus stimulating natural biodegradation processes in environments where oxygen is lacking.

The efficacy of Oxygel was tested in laboratory and compared to the commercial product Ixper 75C (Solvay), which consists of calcium peroxide in powder form. The oxygen release profile for Oxygel showed the capacity to release oxygen for a period of 30-40 days in a marine environment. In comparison, Ixper 75C showed a lower total amount of oxygen released, but the end-point was difficult to determine (around 30 days). The effect of Oxygel on TPH degradation was tested in Sicily at IAMC-CNR mesocosms facilities for different potential applications. Experiments were run in small tanks (45 L) filled with Messina seawater and sediments. In small tanks, the tests with Oxygel showed a very good degradation in the contaminated sediment, with an overall reduction of 60 % of TPH in the sediment after 90 days.

The second product Aerobeads is a hydrophobic powdered sorbing agent able, when saturated with oil, to sink to the sea floor. Biorem produced Aerobeads starting from Oxygel material, which was dried. With the best drying process, it was possible to obtain a powder that have an oxygen content equivalent to a maximum of 19 % in mass. After this drying step, the obtained powder was mixed with a functionalized CaCO<sub>3</sub> (MicrosTech, Switzerland) at a ratio 50/50 to obtain the final product. It was ensured that the two powders are of similar granulometry as to minimize segregation effects in the final product. Stability studies were carried out and Aerobeads showed no significant decrease in oxygen content over a period of 12 month, which makes it viable as a commercial product.

The efficacy of Aerobeads was tested in laboratory and compared to the commercial product Ixper 75C (Solvay) at lab scale in seawater. The Aerobeads were capable to release oxygen over a period of 100 to 120 days compared to 30-40 days for Ixper 75C. Aerobeads was also tested in mesocosm experiments, in its native form, but also in combination with organisms such as *Cladosporium pseudocladosporioides* for the treatment of contaminated shorelines. Other experiments were also performed for the treatment of bilge water, which is often contaminated with a variety of pollutants (mainly petroleum hydrocarbons). Aerobeads was tested as an internal pre-treatment of bilge water. The product could remove >99 % of TPH in the water, effectively concentrating it all within its matrix.

#### Porous bio-carriers with immobilised microorganisms

Partner ICTP worked on the use of cheap oil sorbing material as a support for the immobilisation of oil-degrading bacteria. Biochar (Burkhard GmbH) was produced as a residue after gasification of pinewood. This biochar was used for passive immobilisation of a hydrocarbon-degrading microorganism (*Alcanivorax jadensis* KS-339) by soaking biochar in the microbial cultures and the colonized material was finally lyophilized. The Effectiveness of biochar with immobilised microorganisms was tested in laboratory scale with freshly prepared material and with material stored for 10 weeks at four different temperatures (20; 4; -18 and -80°C) to optimise storage conditions. Sorption/degradation tests were done with Danish Underground Consortium light crude oil. Mixtures consisted of 0.01 g of light crude oil in 4 mL of artificial seawater; after 24 hours of incubation (28°C; 130 rpm) 0.05 g of biochar with immobilized microorganisms was added. Crude oil disappearance was monitored by means of GC-FID during 14 days by determination of selected aliphatic hydrocarbons (C9; C10; C12; C14; C16; C18; C19; C20; C22; C24; C26; C28; C30 and C36) from crude oil in the seawater.

The performance of biochar with immobilised microorganisms was compared with that of biochar alone (adsorption only) and with free microorganisms (biodegradation only). The obtained results have shown that the extent of oil sorption to biochar without immobilized microorganisms is higher (60 % sorption) than that to biochar with immobilised microorganisms (almost 0 % sorption). However, the biochar with immobilised microorganisms led to better removal (almost 20 %) than microorganisms alone (less than 5 %) or biochar without microbes (less than 5 %). Interestingly the degradation activity of immobilised

microorganisms was only slightly affected by storage temperature. The survival of immobilised microorganisms was good since after 10 weeks of storage amounts of surviving cells was in the order of magnitude of 10<sup>6</sup> CFU. In other words the amount of viable microorganisms decreased only of one logarithmic order during storage.

Despite the encouraging results the use of microbially colonized biochar was only tested in small scale in laboratory conditions. Therefore, evaluation of its usability in real conditions is required as further step towards its release on the market. The estimated price of this product could be around 1 euro per 1 kilogram.

#### 3.4 Microbial and additive formulations for enhanced bioremediation (WP4)

Main results achieved in the frame of WP4 was the production of microbial, i.e. fungal and bacterial formulations to be used for seawater and sediment bioaugmentation in order to achieve accelerated hydrocarbon removal rates of these oil contaminated compartments.

##### Fungal formulations for bioaugmentation

First, research efforts were dedicated to enrich and isolate fungal species of interest for crude oil biodegradation. To this end soil/sand samples were collected as microbial source material from sites chronically exposed to oil contamination, namely the volcanic island of Santorini, (Aegean Sea, Greece) and different sites along the coastal zone of Saronikos Gulf (Agioi Theodoroi, Megara, Skaramangas) in Attica (Greece). The crude oil-enriched cultures were appropriately diluted and plated onto Potato Dextrose Agar (PDA) medium supplemented with penicillin G and streptomycin sulphate to prevent bacterial growth and light crude oil 2 % (v/v). After various incubation periods (up to several weeks) at room temperature, fungal colonies were selected and re-plated on the same medium without antibiotics until pure colonies were obtained. Single fungal colonies were identified by 18S ribosomal DNA sequencing. Fungal spores were produced by means of aerobic, solid state, batch fermentations. The amount of living spores (colony forming units, CFU) was determined by on-plate germination on PDA medium.

Strain maintenance was performed regularly checking colony morphology, optimal spore production (spore counting by microscopic observation) and optimal crude oil biodegradation (in-flask or microcosm tests). Slants or plates with the best clones were used for the production of second-generation slants or plates.

The oil degradation by the strains was tested in liquid cultures contaminated with 2 % (v/v) light crude oil containing different PAH concentrations ranging from 20 to 3,000 mg/L. Linear-alkanes C<sub>14</sub>-C<sub>20</sub> were almost completely removed by *Cladosporium pseudocladosporioides* in the liquid cultures after 27 days of incubation. *Penicillium brevicompactum* and *Amorphotheca resinae* showed a preference for degrading the short aliphatic chain alkanes although *Penicillium brevicompactum* was generally more efficient in the removal of n-alkanes compared to *Amorphotheca resinae*. Long chain n-alkanes C<sub>28</sub>-C<sub>35</sub> were most extensively degraded by *Cladosporium pseudocladosporioides* (6 % to 47 % degradation). In addition, of *C. pseudocladosporioides* efficiently degraded the majority of the PAH tested (74 % removal of total PAHs). *Penicillium brevicompactum* and *Amorphotheca resinae* were able to remove 56 % and 53 % of the total amount of PAH respectively. The produced spores were used for bioaugmentation of oil-contaminated sediments in combination with HeiQ Oilguard™ 2014, a powerful oil sorbing fabric. This material was expected to facilitate the accessibility of oil to the fungi. Dansk/PierE1 petroleum (150 g) was applied to a 7-cm layer of sediments (30 kg) in 200-liter tanks. Simulation of oil contaminated beach conditions was achieved by adding 15 L of seawater to the oil-polluted sediments. The experimental system consisted of three independent compartments: a control untreated section; a section with fungal spores only, and a section where the HeiQ Oilguard™ 2014 was placed on the surface. Suspensions containing the fungal spores of all three selected fungi were spread over the sorbent/contaminated sediments at a concentration of 10<sup>4</sup> CFU cm<sup>-2</sup>. After one-month incubation, the residual petroleum was extracted from the sediments and analyzed by gas chromatography. These experiments performed with *Cladosporium pseudocladosporioides* did not demonstrate statistically significant degradation rates. Thorough analyses of the sediments revealed that below 0.5 cm from the surface reducing conditions (anoxic) with redox potential of Eh -300 mV explaining the poor biodegradation. This circumstance was taken into consideration and to avoid anaerobic conditions, contaminated sediments were placed in contact with permanently circulating seawater. Qualitative analyses of the various HC showed a significant degree of oil disappearance from sediments. Noteworthy, more than 95 % of all petroleum hydrocarbons with molecular masses <C<sub>22</sub> were missing in sandy sediments at the end of of experiment. Under the best performing conditions (the HeiQ Oilguard™ 2014 treated with *Amorphotheca resinae* and *Penicillium brevicompactum*), at the end of the experimental period, the specific removal of petroleum from sediments was estimated as 89 % and 92 %, which corresponded to 550 ppm and 400 ppm of residual petroleum, respectively. These values are over one order of magnitude lower than the

amount of petroleum extracted from the untreated control sediments (4,900 ppm).

Bioaugmentation with bacteria and bacterial communities

Rhodococcus erythropolis HFO-S2B an oil-degrading bacterium was isolated and characterized by the company Madep SA. Actygea and Madep partners worked together in order to achieve economically feasible bioaugmentation product based on Rhodococcus erythropolis HFO-S2B. These partners paid particular attention to following aspects: i) high producer variants in the population; ii) short fermentation downtime; iii) use of low cost materials as ingredients for the fermentation medium. The strain was routinely cultivated on Tryptic Soy Agar (TSA) and Nutrient Agar (NA) medium. Growth of R. erythropolis HFO-S2B was estimated as CFU on TSA/NA medium and by packed cell volume. Emulsifying products excreted by this microorganism was indirectly determined using the Emulsification Index on crude oil or on hexadecane. For bioaugmentation purposes, R. erythropolis HFO-S2B was produced in aerobic, submerged, batch fermentation.

Strain maintenance was performed through subsequent cycles of in-plate isolation of colonies with the typical morphology, replicated on slants or plates of TSA medium and tested in-flask for optimal emulsifying activity. Slants or plates with the highest emulsifying activity were used for the production of second-generation slants or plates. The colonies displaying expected morphology of R. erythropolis HFO-S2B were isolated on plates of TSA medium starting from slants, lyophilized cell vials, frozen vials or from fermentation broths from flasks or bioreactor with good emulsifying activity. Plating of serial dilutions of this starting material was performed and selected colony re-suspended to re-inoculate slants of TSA medium (1st generation slants). When 1st generation slants or plates had the expected morphology, each selected colony was collected from slants to prepare glycerol stocks.

The strain was cultivated on an Actygea proprietary medium. Starter culture was carried out in three-litre bioreactors prior to a production stage performed in 30-liter bioreactors. The 30-liter bioreactors were inoculated under stirring at a concentration from 1 to 10 % of the final working volume. The fermentation process was operated controlling dissolved oxygen (2-100 %), pH (6.7-7.7), agitation (200-350 rpm), aeration (0.5-1.25 VVM) and temperature (28.5-32.5). The strain was grown in aerobic, submerged, batch fermentation mode. Biosurfactants production was used as the indicator of the potential of the strain to degrade crude oil and was monitored using the emulsification test. Fermentation was stopped when the maximum emulsifying activity was observed (about 144-168 hours). The biomass was harvested and stored. For oil degradation purposes, living cells ( $1-3 \times 10^8$  CFU/mL) were dispensed in suitable, sterile plastic containers and stored at a temperature of 4°C for no more than one week.

Concluding remarks on bioaugmentation approach results

- A series of trials on application of bioaugmentation approaches with various formulations of fungi, bacteria have been performed in bioreactors at few millilitres to mesocosms of several cubic meters scale
- Bioaugmentation tests were performed by the amendment of biomass formulations alone and, at the large scale, with the combined application of further Kill•Spill products, e.g. sorbent materials.
- Future studies on the fine-tuning the application protocols are needed to further improve the efficiency of bioaugmentation, in particular, at large or field scale

### 3.5 Efficient cleanup of contaminated sediments (WP5)

The main research efforts made within WP5 were dedicated to the development of technologies addressing the treatment of oil-contaminated sediments. Three bioelectrochemical systems aiming to facilitate biodegradation of hydrocarbons in anoxic sediments and one capping technology based on the use of a cheap and renewable material for oil pollution containment were developed within WP5.

#### Kill•Spill Snorkel

Partner CNR has developed the “Kill•Spill Snorkel”: a fully passive system (no electrical energy and no maintenance required) ideally suited for the long-term treatment of contaminated sediments in remote areas (e.g., at open sea). The system consists of a single conductive material (i.e., the snorkel) positioned suitably to create an electrochemical connection between the anoxic contaminated sediment and the oxic overlying water. The segment of the electrode that is buried within the sediment plays a role of anode, accepting the electrons deriving from the anaerobic oxidation of contaminants and other reduced species. Electrons flow through the conductive material up to the part exposed to the aerobic environment (i.e., the cathode), where they reduce oxygen to form water.

The “Kill•Spill Snorkel” was tested in a microcosm using sandy marine sediments from Messina Harbour (Italy) artificially contaminated with crude oil. Microcosms containing 1 or 3 graphite snorkels and controls (snorkel-free and autoclaved) were monitored for over 400 days. The results confirmed that the snorkels accelerate oxidative reactions taking place within the sediment, as documented by a significant 1.7-fold increase in the cumulative oxygen uptake and 1.4-fold increase in the cumulative CO<sub>2</sub> evolution in the microcosms containing 3 snorkels compared to snorkel-free controls. Accordingly, the initial rate of total petroleum hydrocarbons (TPH) degradation was also enhanced. Indeed, while after 200 days of incubation a negligible degradation of TPH was noticed in snorkel-free controls, a significant reduction of 12±1 % and 21±1 % was observed in microcosms containing 1 and 3 snorkels, respectively.

Further research efforts are needed to clarify factors and conditions affecting the snorkel-driven biodegradation processes and to identify suitable configurations for field applications.

#### Kill•Spill sulphide scavenger

Partner UGent assessed the application of a potentiostatically controlled (0.0 V to +0.3 V) carbon-based anode positioned within the sediment or in a recirculation loop, which promotes the (bio)electrochemical oxidation of toxic sulphides to elemental sulfur (or eventually sulphate), thereby stimulating the metabolism of sulphate-reducing, hydrocarbon-degrading microbial populations.

Lab-scale bioelectrochemical experiments were carried using toluene, as a model contaminant. The objectives of this study were to (i) investigate the biodegradability of toluene in bioelectrochemical systems inoculated with marine contaminated sediment, (ii) evaluate the effect of anode potential on toluene biodegradation, (iii) identify the most abundant microbial populations involved in bioelectrochemical toluene degradation process, and (iv) investigate the role of the sulfur cycle on toluene biodegradation in bio-electrochemical systems.

Results of bioelectrochemical experiments indicated that electric current (in the range 283 – 431 mA/m<sup>2</sup>) was generated during toluene degradation. This latter proceeded at a rate of 1 mg toluene/L d or 16 mg toluene/kg sediment per day regardless of the applied anode potential (0 and 300 mV). The most abundant microorganisms enriched both in the anodic and in the bulk communities were sulphate-reducing bacteria, some of which were phylogenetically related to known anaerobic hydrocarbon degraders.

This study demonstrated that a potentiostatically-controlled bioanode could be implemented as an effective strategy to remove toluene from marine oil contaminated sediments. Interestingly, results strongly indicated that sulphur metabolism was involved in toluene bioelectrodegradation. Preliminary microbial characterization of the bulk anolyte and anode samples by 16S rRNA-based Illumina sequencing conducted at the end of the experiments confirmed this assumption since sulphate reducing microorganisms were the prevailing bacterial population. Based on these observations further quantitative PCR (qPCR), targeting the 16S rRNA, the  $\alpha$ -subunit of the dissimilatory sulphite reductase (*dsrA*), and the  $\alpha$ -subunit of the benzyl succinate synthase (*bssA*) genes was conducted in the bulk solution and on the anode electrodes of all replicate reactors. The highest *dsrA* and *bssA* gene abundances were observed in the bulk of reactors whose anodes had been polarized at +300 mV. Results of this analysis confirm and stress the role of sulphur metabolism in hydrocarbons degradation.

#### Kill•Spill Electro2

Partner CNR developed a system consisting of a Dimensionally Stable Anode (Ti mesh coated with an iridium and ruthenium oxide mixture) deployed within the contaminated sediment and a Stainless Steel mesh cathode kept in the overlying seawater. A fixed voltage difference (>2 V) or a fixed current is applied with a power supply in order to electrolyze seawater and, by so doing, generate oxygen in the sediment and accordingly stimulate the aerobic degradation of petroleum hydrocarbons by autochthonous hydrocarbon-degrading microbial communities.

The viability of the “Kill•Spill ELECTRO2” system was verified by means of lab-scale bioelectrochemical experiments. The experiments were setup in approximately 1 L wide-mouth jars. The jars were filled with approximately 700 g (on a dry weight basis) of sandy marine sediments from Messina Harbour (Italy) and seawater (approximately 450 mL) from the site. The sediment was artificially contaminated in the laboratory with Danish Underground Consortium light crude oil to a final concentration of approximately 20 g/kg. A DSA electrode (44 cm<sup>2</sup> geometric area; Ti mesh covered with mixed metal oxides, primarily consisting of Ir and Ru) was placed at the bottom of the jars (buried within the sediment) whereas a stainless steel (type 304) 40 mesh woven cathode (44 cm<sup>2</sup> geometric area) was placed in the overlying seawater.

Results showed an increased redox potential and a decreased pH in the vicinity of the anode of “electrified” treatments,

consistent with the occurrence of oxygen generation. Accordingly, hydrocarbons biodegradation was substantially accelerated (up to 3-times) compared to “non-electrified” controls, while sulphate reduction was severely inhibited. An important finding of this study is that the effect of electrolysis on the redox potential extended several centimetres (>5 cm) upward from the anode surface, thereby indicating that the radius of influence of the technology is probably much greater than that reported for other bioelectrochemical TPH remediation technologies based on direct electron transfer to electrodes. For field applications, anodes have to be deployed underneath the contaminated sediment layer. Considering that petroleum hydrocarbons typically remain confined within a superficial layer (5-20 cm) of sediment, the herein estimated radius-of-influence (> 5 cm) already provides some important indications regarding the practical viability of the proposed approach. A precise estimation of the radius of influence of the technology will, however, require conducting experiments with larger scale systems. Such an estimate will ultimately allow determining the geometric size of electrode required to treat e.g., 1 m<sup>2</sup> of contaminated sediments and, ultimately, the actual economical viability of the proposed electrode-based sediment remediation approach.

Intermittent application of electrolysis proved to be an effective strategy to minimize the energy requirements of the process, without adversely affecting degradation performance. At the end of the experimental period, the specific energy consumption was estimated as 0.11 kWh per kg of degraded TPH. This value is over one order of magnitude lower than that reported for competing soil and sediment treatment technologies, such as in situ thermal desorption (typically >5 kWh/kg of removed TPH), notwithstanding the fact that biodegradation results in contaminants degradation and not only in their phase transfer. These low energy requirements make the technology fully compatible with the use of solar panels (photovoltaic modules) as power supply systems. Taken as a whole, this study suggests that electrolysis-driven bioremediation could be a sustainable technology for the management of contaminated sediments.

#### Active capping of oil-contaminated sediments with Biochar

Partner UNIRM developed an in-situ pollution management solution using capping to confine hydrocarbon spreading in oil-contaminated sediment. The material used for capping was biochar, a carbonaceous waste material usually obtained by the pyrolysis of biomass. The performances of the BC obtained by pine wood gasification were compared to common capping materials, such as activated carbon (AC, Filter Carb GBC 830, Carbonitalia®) and organoclay (OC, PM-199, Cetco®). First, the sorbent materials were initially tested in aqueous phase with several organic contaminants, such as toluene (low hydrophobicity HC) and a mixture of polycyclic aromatic hydrocarbons (PAHs) (target compounds of the oil spill contamination in the sediment). Batch tests were carried out to investigate adsorption kinetics and to obtain experimental isotherms for all the materials with all the contaminants. All the tests were initially carried out in deionized water and then in synthetic seawater (pH 7, 25°C). Kinetic tests have shown BC is the sorbent material that faster achieves the equilibrium. In addition, equilibrium tests (adsorption isotherms) were carried out using different contaminant concentrations. From these experiments it can be concluded that the adsorption onto BC is comparable with the target materials, such as AC and OC. Similar observations were made with more hydrophobic contaminants, such as the PAHs.

Capping batch experiments have been started in box models using marine sediment from Messina (Sicily) contaminated with Dansk Blend crude oil and a mixture of PAHs in seawater. The capping efficiency of a 2-cm layer of the material to be tested was experimentally monitored after 1, 2 and 6 months. The PAHs released from the sediments was measured using a passive sampling approach (PDMS fibres). The profiles through the sediment and the capping layer of the porewater concentration of the total PAHs (acenaphthylene, fluorene, anthracene, fluoranthene) and the comparison between AC, OC and BC, after 1 month monitoring could be achieved. The experimental data showed that BC has higher/comparable performances as capping material than AC and OC that are the most common sorbents used in capping technologies. The total PAHs relative concentration (C/C<sub>0</sub>) is indeed 0.44, 0.47 and 0.52 respectively for BC, AC and OC in the last point of the capping layer (-0.5 cm, top of the capping layer). The long-term modelling has shown capping performances of biochar after 12 month are still higher than AC and OC.

The use of BC instead of AC and OC has the advantage of using a product of zero/low cost since biochar is the residual of a gasification process where wood pellet are gasified for making local energy.

#### 3.6 Development of multifunctional remediation agents for oil spills (WP6)

Main efforts made within WP6 were dedicated to the development of multifunctional systems that support/sustain microbial degradation of hydrocarbons by delivering limiting nutrients (mainly N and P) to autochthonous and microorganisms used for

bioaugmentation purpose. Four novel products were developed in the frame of this workpackage: two biostimulation systems for slow release of nitrogen and phosphorous combining either sorptive or bioaugmenting properties, one biostimulating and emulsifying formulation, and one sorbent boom with bioaugmenting, emulsifying and bioaugmenting capacities.

#### SmartGate

In the frame of the Kill•Spill project partner FHNW has developed mesoporous silica nanoparticle systems, which can be applied in open sea environment to intensify the biodegradation of hydrocarbons from crude oil. The particles are designed to sorb/coagulate with oil-water emulsions and remain associated to the hydrophobic oil phase, where they release nutrients needed by microbial oil degraders. The limitation concerning the C:N:P availability for microbial oil degraders and the quick dilution of biostimulants in open sea environment can be circumvented with this product.

Homemade and commercially available mesoporous silica particles were used as carrier material. The mesoporous structure allowed for the easy loading of the desired nitrogen and phosphorus nutrients into the pores. Functionalization of the surface of the particles is carried out by grafting hydrophilic molecules at the surface of silica in order to procure hydrophobic properties to the particles and enable them to target specifically oil phase. This step is a 2-in-1 process where not only the particles are functionalised and becomes hydrophobic but also where "smart gates" are created. In fact, the pores are surrounded by alkyl chains that are collapsing, when transferred in a aqueous phase, creating a hydrophobic barrier maintaining the pores closed thus preventing the release of the nutrients and there rapid dilution in seawater. In contact with an oil phases resulting from oil spills the opposite occurs, the alkyl chains are solvated and unfold, the pores open and nutrients can be released at oil/water interface.

The efficiency of N and P release was tested with a oil degrading model microorganism *Marinobacter hydrocarbonoclasticus* KS-ANU5 in artificial seawater contaminated with crude oil at a final concentration of 0.5 % w/v) over 45 days. The performance of the SmartGate product was compared to a commercially available oleophilic fertilizer, namely S200 and controls (abiotic and biotic containing basal concentration of N and P of seawater). C12 to C15 were entirely degraded in S200 and SmartGate treatments. Interestingly, the addition of SmartGate compared to S200 treatment enabled a faster and complete degradation of long chain alkanes, i.e. octadecane, nonadecane and tetracosane. This fact might be attributed to the oleic acid contained in the formulation of S200, which may slow down the degradation of C18 and longer chain hydrocarbons. Biotic controls demonstrated that basal concentration of N and P in seawater are not sufficient to support rapid and relevant biodegradation of oil components. In these systems inoculated with *Marinobacter*, benzene and naphthalene were markedly degraded when external source of N and P was added, the SmartGate giving better elimination.

The efficiency of SmartGate particles was compared to the one of S200 in Aegean Sea in Keratsini (Piraeus, Greece) in pools formed by booms on the removal of heavy oil. The product SmartGate gave satisfactory visual results removing heavy oil from the cleaning the surface of seawater. The oil removal performances of the SmartGate particles were at least as good as those obtained with S200. In addition to potential biodegradation (impossible to demonstrate in open sea), two different dissipation phenomena occurred, i.e. transportation in the water column and sinking of oil-SmartGate particle aggregates and emulsification by S200 with transportation in the water column. Ecotoxicological tests with *Vibrio fischeri* have shown that the SmartGate product actually decreases the toxicity of heavy oil IFO 180 to this microorganism and no ecotoxicity of the SmartGate product itself could be demonstrated using various organisms. The SmartGate product is commercially available.

#### Dry alginate beads (DABs)

Another product was developed in order to intensify oil biodegradation by implementing a biostimulation and bioaugmentation approach. The partner UMIL achieved the concomitant microencapsulation of lyophilised or fresh microbial oil degraders with various combinations of K<sub>2</sub>HPO<sub>4</sub>, urea, and sophorolipids/rhamnolipids in calcium-alginate gel. The primary goal was to develop a material that can slowly release bacteria that can degrade hydrocarbons (HC) together with nutrients to adjust C:N:P ratio and biosurfactants, which can increase the bioavailability of oil to the HC degraders. DABs microparticles performances in terms of oil biodegradation were tested in seawater contaminated with 0.1 % of n-dodecane or 0.5 % of crude oil. The following optimal composition of ingredients (g/100g DABs) was determined:

- 46.4 g of K<sub>2</sub>HPO<sub>4</sub>
- 2.32 g of urea
- 0.232 g of rhamnolipids/sophorolipids
- 4.65 g of lyophilized cells of *Marinobacter hydrocarbonoclasticus* KS-ANU5



- 46.4 g alginate

Using this composition in microcosm bioremediation tests, the DABs were able to consume 24 % of n-dodecane and 8 % of crude oil in 5 days experiment. The release and viability *M. Hydrocarbonoclasticus* was studied in these lab-scale microcosms using flow cytometry. DABs allowed for the release of  $4 \times 10^4$  UFC/mL of viable cells in the surrounding mineral medium. The DABs have to be applied at a DABs/oil ratio of 2. The toxicity of the whole preparation has not been tested yet.

DABs can be further improved adapting their composition (in terms of N and P loads but also of microorganisms and biosurfactants) to different kind of contamination (light/heavy oils, other hydrocarbons etc.) or environments (surface spills, sediments). DABs are still far from commercialization but the technology can be easily scaled up industrially. To bring these tools closer to the markets, future efforts will be directed to find a large-scale industrial producer and distributor.

#### Low cost biostimulant formulations

Partner TUC investigated the use of low cost oleophilic or inorganic nutrients (N & P sources) and biosurfactants to enhance bioremediation of crude oil in the marine environment. Procedures followed the EPA bioremediation agent effectiveness test protocol with weathered crude oil over a period of 28 or 56 days with sampling every week for microbiological as well as chemical analysis.

Various sources of nitrogen, phosphorous and biosurfactants were tested in various combinations at lab scale:

Source of nitrogen: inorganic nutrient -KNO<sub>3</sub>, Basfoliar 36- (Liquid foliar nitrogen fertilizer (27 % N) containing magnesium and micronutrients), Avant Natur SL (Liquid foliar NPK-fertilizer which is a biostimulant with high concentration of amino acids (100 % vegetable origin - derived through enzymatic action) and uric acid (oleophilic form with purity of 99 %).

Source of phosphorus: inorganic nutrient - K<sub>2</sub>HPO<sub>4</sub>, Lecithin Bolec (native lecithin, derived from crude soybean oil, is a mixture of phospholipids, glycolipids and carbohydrates dissolved in triglycerides, 1.73 % P), L- α -Lecithin (L-α-phosphatidylcholine from soybean oil, granular with purity of 97 %).

Biosurfactant: Oil Begone Bio, New Cherry, BB1000 (BioBased Europe Ltd; Non toxic, 100 % plant based biosurfactant) and rhamnolipids (Actygea).

The biostimulation-emulsification efficiency of the various formulations was evaluated by determining the Most Probable Number (microbial count), GC-MS analyses of the HC, and calculating biodegradation kinetics. This approach led to the optimization of the formulation respecting the desired C:N:P amendment 100:10:1:

- L-α-Lecithin: 470 mg/g oil
- Avant Natur SL: 1.47 mL/g oil
- Biosurfactant Oil Begone: 500 μL/g oil
- Biosurfactant rhamnolipids: 50 or 100 times diluted

#### Self-regenerating Bio-Boom oil-sorbent with enhanced absorption & bioremediation capabilities

Several self-regenerating sorbent booms have been developed and optimized within the Kill•Spill project. Special emphasis was given on the development of sorbent booms characterized by very high adsorption capacity which enabled the concomitant degradation of adsorbed oil by petroleum-degrading microbial consortia loaded into the sorbent boom cylinder. Self-regenerating boom sorbents with enhanced absorption & bioremediation capabilities consisted of four principal components:

1. Efficient and non-toxic oil-absorbing external part of Bio-Boom: oil-absorbing, water-repelling, nonwoven fabric Oilguard (produced by partner HeiQ).
2. Bacterial and fungal petroleum-degraders immobilized either in chitosan beads (see part above) or in other carriers (see dry alginate beads).
3. Optimized bio-surfactant characterizing by both high efficacy of emulsification and low toxicity. We offer two products: homogenized sophorolipids and homogenized rhamnolipids.
4. Optimized slow-released nutrients (see the benchmark products developed by FHNW).

Different formulations of Bio-Boom were tested during 45 days in open-door mesocosms containing 15,000 litres of seawater contaminated with Dansk/Pier1 crude oil (800 g). Two formulations were tested:

- (i) Bio-Boom filled with 69 % of *Alcanivorax* and 10 % of *Marinobacter* enrichment + 97 % of *Cycloclasticus*); pure isolate *Marinobacter hydrocarbonoclasticus* KS ANU5 cells immobilized in alginate beads and spores of *Cladosporium*

pseudocladosporioides, *Penicillium brevicompactum* and *Amorphotheca resiniae* + enrichment homogenised rhamnolipids (1 % of total weight) + slow releasing Miracle-Gro™ NPK fertiliser (Scotts, Godalming, UK) (20 % of total weight) and

- (ii) Bio-Boom filled with dried alginate beads + *Marinobacter hydrocarbonoclasticus* KS-ANU5 + fertilizers + sophorolipids (formulation DABs4 – University of Milan). The sterile HeiQ OilGuard 2014 sorbent was used as the control.

At the end of the testing period, the residual oil still remaining in these Bio-Boom formulations was equal. Only 4.0 % to 5.6 % of initially adsorbed amount of Dansk/Pier1 crude oil (3,200 mg of oil g<sup>-1</sup> of sorbent) was extracted from the sorbent. All Bio-Boom formulations tested were highly efficient for both the absorption and degradation of immobilized crude oil. Depending on microbial consortia applied in Bio-Boom formulations, the level of degradation varied from 94 to 96 % of applied total petroleum hydrocarbon.

### 3.7 Impact assessment of developed technologies (WP7)

A crucial issue concerning products for the treatment of oil spills, more than their effectiveness, is their eco-compatibility or potential toxicity. Assessment of the ecotoxicological effects of bioremediation products developed in the Kill•Spill project were performed on different target species of the marine food web, according to OSPAR best practice and experimental implementations.

A total of five target species were used. Each target species was used to assess the product toxicity through at least one endpoint. For *Paracentrotus lividus*, three different endpoints were tested. The output of each experiment was the quantification of EC50 or LC50 (i.e. the amount of product needed to observe an effect in 50 % of tested individuals). In general, the results obtained by the ecotoxicity assays performed displayed great differences among products in terms of eco-compatibility and different responses of target organisms to the tested products.

Considering all the results obtained for each product, we can conclude that microbial biosurfactants have a great performance in terms of eco-compatibility, impacting the target species only at very high concentrations (out of the hypothesized range for their use in the field). The best results have been obtained on bacteria, benthic crustaceans and teleostean, whereas they can apparently produce noticeable effects on plankton (microalgae and crustaceans). Very low effects were found on gametes of *P. lividus* and on the development of embryos, suggesting that these products cannot interfere with any of the main phases of the reproduction of this species.

The toxicity of plant biosurfactants were apparently in the same range of commercial surfactants, commonly used as dispersants in oil spills although Supersolv and SC-1000 were tested at concentrations 10 to 100 times higher than those used in the field. Based on the derived dose-response curves, it can be inferred that very low (if any) effects are expected on target organisms.

The target species were also tolerant towards the O<sub>2</sub> releasing products. Oxygel and Oxygel Plus displayed generally higher toxicity than Aerobeads, suggesting that the physical state of this product and the O<sub>2</sub> releasing rate influence the measured toxicity.

Similarly, the effects of the Kill•Spill products during application at an oil spill were also investigated. Such experiments are crucial, especially for products at the final stage of development and almost ready for commercialization. In addition to the field experiment performed in Gela in 2013, a second field experiment was performed in the touristic port of Ancona (Italy). This second experiment was performed by ECOTS in a big mesocosm to be able to study temporal variations of biological communities subjected to oil contamination and treatments. The use of sophorolipids resulted not only in a less severe mortality of benthic organisms, but also in less evident changes of the community structure. The preservation of ratios between taxa is an indication of a less severe impact of the oil contamination on the ecological community, preserving the original ecologic functions.

#### Potential Impact:

4 Potential impact (including the socio-economic impact and the wider societal implications of the project so far) and main dissemination activities and exploitation results

##### 4.1 Scientific and technical impact

The Kill•Spill project through innovative research has significantly contributed to the development of economically and environmentally viable solutions, validated on the basis of field trials, for the clean-up of oil spills caused by maritime

transport and oil exploration and related processes. Kill•Spill addressed fully the requirements of the call by developing a series of innovative products and technologies.

In particular, Kill•Spill provided sustainable, industrially driven and tested strategies for mitigating oil spills in the marine environment by advancing the knowledge in the biotechnological eco-efficient degradation of petroleum hydrocarbons released in the marine environment through innovative biostimulation and bioaugmentation protocols in addition to biosurfactant-based dispersants and novel multifunctional agents. In addition, it may significant advances in the technologies for the remediation of contaminated sediments.

#### 4.2 Policy relevance and impact

The development of a sustainable European Knowledge-based Bio-Economy (KBBE) offers a convergent and coordinated activity to address the challenges facing today's society as highlighted in the Europe 2020 Strategy. The Kill•Spill project has specifically addressed "the needs to detect, monitor, prevent, treat and remove pollution".

Following the provisions of the 6th Environmental Action Programme, EC adopted the Thematic Strategy on the Sustainable Use of Natural Resources (COM(2005) 670). The objective of the thematic strategy is to reduce the environmental impacts associated with resource use and to do so in a growing economy. Petroleum hydrocarbons represent an important resource that EU is heavily depending on despite the shift towards renewable resources. The Kill•Spill project is related to the petroleum hydrocarbons transportation, off shore exploration, production and completion in EU sea regions. By providing better means to combat potential hydrocarbon releases to the environment, enables policy makers to accept more easily "high risk exploration" sites at depths beyond the 2000 m from the sea surface. As the Commission proposed that each EU Member State develops national measures and programmes on the sustainable use of natural resources to achieve the strategy's objectives, these measures and programmes should focus on resource use, which has the most significant environmental impacts. Should petroleum hydrocarbons become the resource under scrutiny, Kill•Spill could aid Member States to include mechanisms to monitor progress and, where possible, develop targets.

The Kill•Spill project contributed also to the Marine Strategy Framework Directive [MSFD]. For example, all the technologies developed for hydrocarbon polluted sediments can be part of the mitigation measures to return marine environments to Good Environmental Status (GES). Moreover, many of the developed Kill•Spill biostimulation strategies could be readily applied to sea areas of chronic pollution. Furthermore, as the project did not focus on a single remediation method, several promising technologies and applications were developed. As anticipated from the call (KBBE2012.3.5-01: Innovative biotechnologies for tackling oil spill disasters –The Ocean of Tomorrow), "oil spill products and technologies champions" have emerged from the Kill•Spill project with the highest potential impact in terms of removal efficiency, robustness and cost-effectiveness besides the eco-toxicological assessment of the developed innovative technologies.

#### 4.3 Socio-economic impact

As the Europe 2020 Strategy puts a strong emphasis on integrating research and industry to favour innovation and technology transfer, with a particular attention to SMEs, the Kill•Spill project involved 14 SMEs active in complementary areas, contributing to the development of innovative and integrated solutions and tailored strategies for oil spill clean-up market.

#### 4.4 Successful dissemination and exploitation.

A crucial step in the realisation of impact was a well defined dissemination and exploitation of project results. A general awareness of public and experts for the contents and results of the project was created by the public website, the project flyer, and dedicated stakeholder events.

##### 4.4.1 Dissemination to the general public

We reached out to the general public particularly by two video productions.

- Kill the Spill by Euronews and
- Microbes clean up ocean by Youris

The movies conveyed the project ideas and approaches in an illustrative and concise manner. Also newspaper or magazine articles helped to flag our activities..

##### 4.4.2 Education

Kill•Spill took the opportunity to familiarise pupils and students with the concept of oilspills and their bioremediation during exhibitions also open to the general public (e.g Ecomondo, Oceanology). Dedicated lecture series or contribution ot summer schools linked the Kill•Spill approaches to general education of students.

#### 4.4.3 Dissemination of results to peers and end-users

To achieve a significant outreach to a wider group of stakeholders and end-users interested in further utilizing the project results, findings were spread among the research community, authorities and the spill industry.

A total of 44 peer reviewed journal papers were published. They form the science-based evidence for the viability and applicability of certain tools and technologies. They were majorly related to analytical methods and findings from their testing in spill related environments. Additional 88 conference talks and posters added to the visibility of Kill•Spill work and results.

Contacts and exchange with stakeholders and particular the spill industry were made in dedicated events.

A total of about 120 participants attended three Kill•Spill workshops held in

- Piraeus (GR) organised by partner EPE on 29 June 2016 (45 participants)
- Stavanger (NO) organised by partner MMB on 29 September 2016 (17 participants)
- Southampton (UK) organised by partner UKSPILL on 18 October 2016 (55 participants)

Participants represented the Coast Guard, Governmental agencies, Non-Governmental Organizations, Protection & Indemnity Clubs (P&I), Marine Consulting Companies, Maritime Lawyer Agencies, the oil industry and research.

An exchange about the potential of bioremediation in spill response in general and the applicability of Kill•Spill technologies in particular was triggered. As the use of dispersants is viewed highly critically by (European) marine authorities, the development of bio-based and eco-toxicologically safe products would be rewarding. In that respect we also made alliance with CEDRE, the 'Centre of Documentation, Research and Experimentation on Accidental Water Pollution', staff of which was also part of the project Advisory Board. Kill•Spill contributed to their events to disseminate to authorities and the spill industry. Additional direct contacts with marine authorities were sought, especially by partner IAMC-CNR, who participated in a spill response / emergency exercise of the Italian Navy.

#### 4.4.4 Generation of knowledge, product development and exploitation

Toward exploitation, commercial partners further developed existing products while improving e.g. the properties of sorbents for the application in shore protection or new types of booms (HeiQ, microsTECH). Other extended their product portfolio to oil spill remediation applications, such as the tailoring of FISH test kits for the detection of oil degrading bacteria (VER) or the use of oxygen releasing compounds for enhanced biodegradation (BIOREM).

The potential and applicability of bio-electrochemical sediment remediation approaches were significantly progressed during the project by research partner CNR-IRSA.

The Kill•Spill Handbook constitutes a focused, end-user targeted summary of the status of development and applicability of a range of products for oil dispersing, stimulation of biodegradation, oil sorbents, electro-chemical treatments and monitoring approaches. It highlights the principles and performance of the different Kill•Spill products pointing out their range and field of application. The Handbook provides easy access to the major project results especially for the expert (authorities, response companies).

A summary of the most progressed and important Kill•Spill products is provided in the Table 5.

Table 5 Selected Kill•Spill products and state of development

Kill•Spill Biosensor (CNB-CSIC, EEZ-CSIC): Reporter strains engineered to detect specific oil hydrocarbons. Prototype. Works well in laboratory tests, several environmental samples. Detection of hydrocarbons in the  $\mu\text{M}$  range.

Electronic version Kill•Spill Biosensor (CRS): Optoelectronic device to detect the fluorescence emitted by biosensors in response to specific oil hydrocarbons. Installation of the opto-electronic sensing system in two different commercial sensor buoys.

CHEMSIC (UCPH, HMGU): Protocols for extraction, clean-up and analysis of petroleum hydrocarbons from samples. Allow determining isomers and isotopes enrichment factors. Serve to assess the extent of oil biodegradation. Application protocols are available. Analytical services provided for a fee.

Kill•Spill FISH Kit (VER): Kits for cultivation-independent on-site identification and quantification of selected hydrocarbon degrading bacteria (e.g. *Alcanivorax* spp., *Cycloclasticus pugetii*, *Marinobacter* sp.) in microbial communities. Also suited for

quality control purposes during fermentation of those bacteria. Based on FISH / 16S rRNA. Kits commercially available.

Kill•Spill FCM Kit (VER): Diagnostic kits for monitoring of target microorganisms. Allows identification and quantification. Combination with a low-cost flow cytometer allows automatic read-out of results. Based on FISH / 16S rRNA. Tested with pure cultures and mixtures of microorganisms.

Multifunctional sorbent materials (Microstech AG): Mineral-based sorbent (CaCO<sub>3</sub>) with *Psychrobacter glacincola*, edible oil, PEG, and glycerol in the formulation. Sorption and degradation of crude oil

Oxygel™ (BIOREM): Oxygel™ is an inorganic gel that slowly releases oxygen in water. Its main technology is based on encapsulation of hydrogen peroxide in a 3D microstructure based on an inorganic compound such as calcium phosphate or Calcium sulphate. H<sub>2</sub>O<sub>2</sub> can then be used as a source of oxygen for enhanced aerobic stimulation or for chemical degradation of mineral oil (with an added catalyst). Oil adsorption (TPH Vs Product) : 0.75:1, O<sub>2</sub> content: 12-15 %, O<sub>2</sub> release time: 40-60 days. Dispersion of floating oil: With the addition of bio-solvents & surfactants, Oxygel™ can be used as a dispersant for floating oil layer, with the added value of also serving as an oxygen source for enhanced biostimulation. The standard version of Oxygel™ can also be used for remediation of contaminated sediments.

Aerobead (BIOREM): Aerobead™ is a porous media designed for the adsorption of floating layer of mineral oil, to address the problem of oil slick in marine environment. Once applied on the oil layer, it absorbs the oil in its matrix, then sinks to the bottom of the water column. Afterwards, the product slowly releases Oxygen or hydrogen peroxide to start up some aerobic degradation of the trapped oil, or chemical oxidation. Oil adsorption (TPH Vs Product) :0.9 to 1:1, O<sub>2</sub> content: 6-10 %, O<sub>2</sub> release time: 100 - 110 days. Field of application: Dispersion of floating oil. Oxygen source for enhanced aerobic degradation. In Situ Chemical oxidation agent (with the addition of a catalyst)

Kill•Spill Slow Release/ DABs (UMIL): Calcium Alginate Dry Beads immobilizing HC-degrading microorganisms, P and N fertilizers, biosurfactants. Prototype. Works well in laboratory tests.

SmartGate (FHNW): Mesoporous particles loaded with urea and phosphate. Surface of mesoporous modified using alkylated organo-silane, which enables sorption to oil slick and controlled release of N and P for degrading microorganisms. Lab tests and open sea (Fall 2016). *Marinobacter* as model degrader, 0.5 % crude oil above 1 month incubation, RT in artificial seawater.

Kill•Spill Chip Microarray (Bangor, ICP-CSIC, IAMC-CNR): Monitoring tool to detect genes (2375) relevant for the degradation of alkanes and aromatic hydrocarbons and to assess microbial activity in oil-contaminated environmental samples. Validated with samples of known composition. Tested with seawater, sediments and enrichment cultures using samples from experimental sites. Detects the gene homologues from known biodegradation pathways from as low as 100 ng of DNA material derived from environmental samples or pure cultures.

List of Websites:

[www.killspill.eu](http://www.killspill.eu)

## Related information

### Result In Brief

[Using microbes for oil spill clean-up](#)

### Documents and Publications

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