

KILL SPILL



Kill•Spill project: Enhancement of biodegradation techniques



**TECHNICAL
UNIVERSITY
of CRETE**

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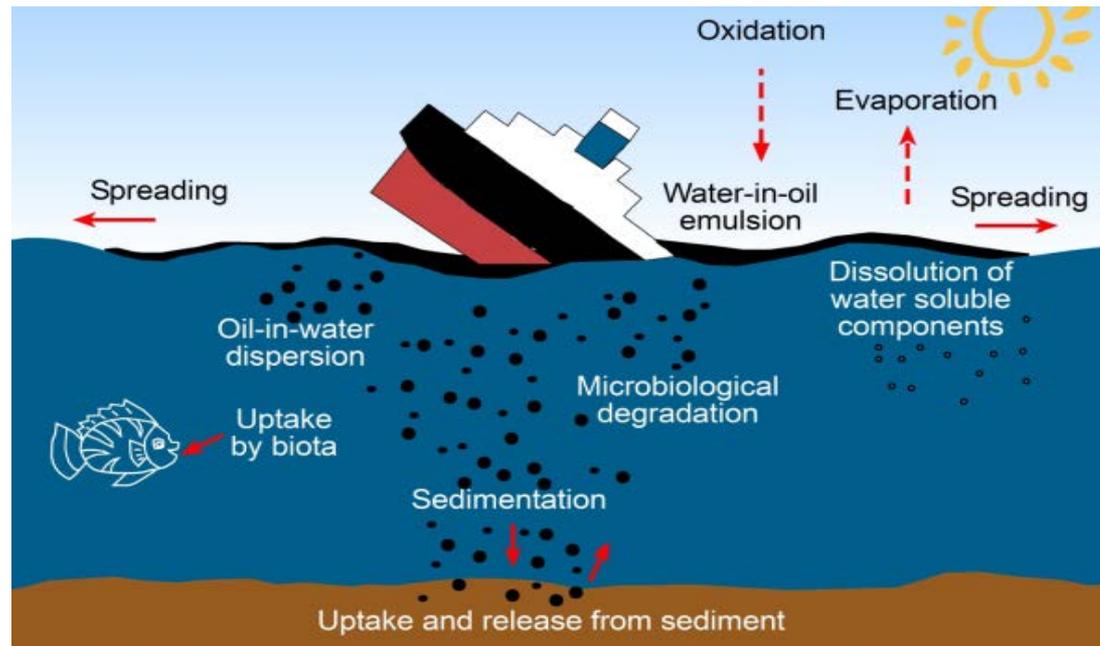
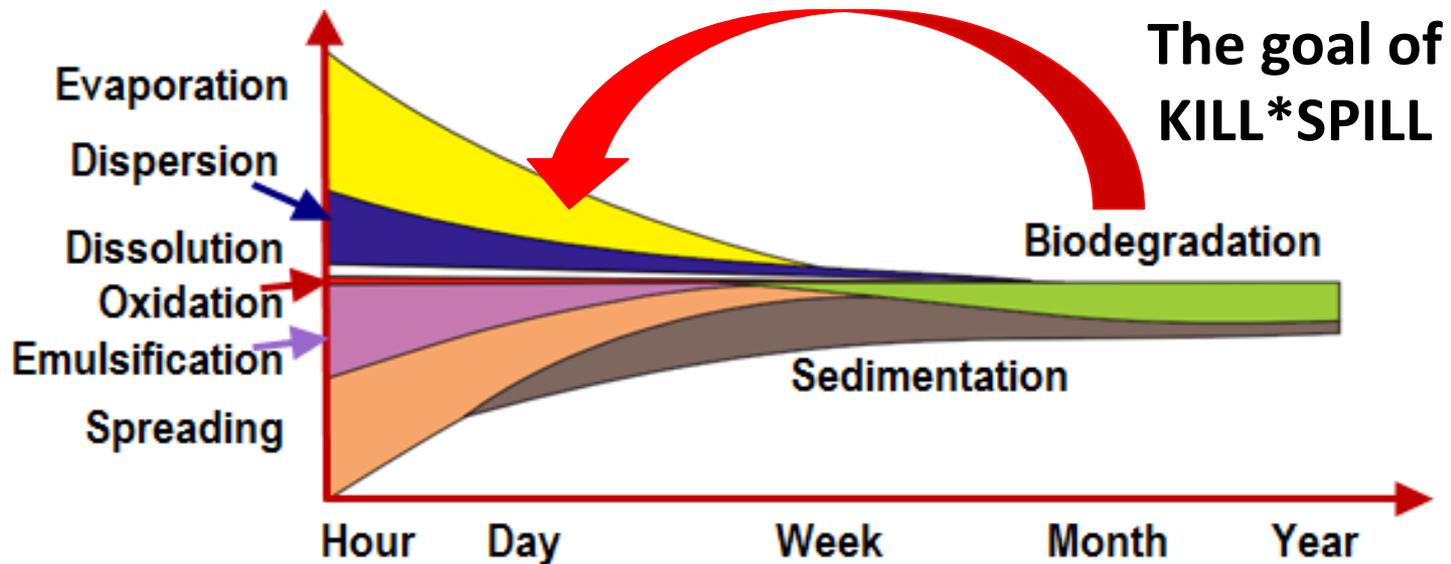


Marine oil spills: Remain a serious environmental problem





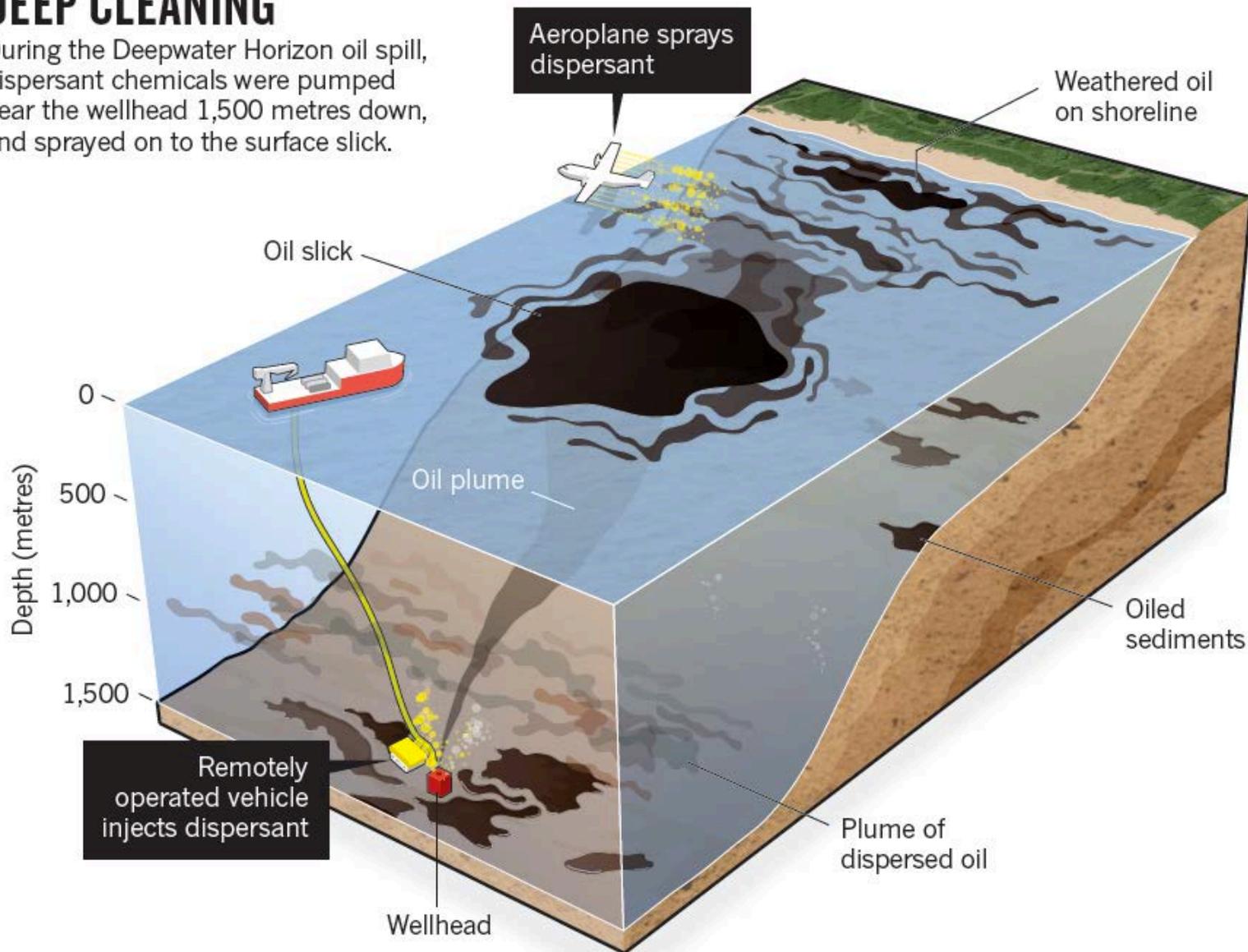
Weathering processes & period of activation



Well blow-out / Deep sea releases

DEEP CLEANING

During the Deepwater Horizon oil spill, dispersant chemicals were pumped near the wellhead 1,500 metres down, and sprayed on to the surface slick.





FP-7 Topic Addressed

KBBE.2012.3.5-01

Innovative biotechnologies for tackling oil spill disasters (The Ocean of Tomorrow)

Kill•Spill project:

Total cost: 12,483,643.40 €

EU contribution: 8,996,599.00 €

with 27.38% to SMEs



Project Partners

KILL SPILL TEAM
1 TECHNICAL UNIVERSITY OF CRETE (TUC) - Greece
2 FACHHOCHSCHULE NORDWESTSCHWEIZ (FHNW) - Switzerland
3 ALMA MATER STUDIORUM-UNIVERSITA DI BOLOGNA (UNIBO) - Italy
4 UNIVERSITY OF NEWCASTLE UPON TYNE (UNEW) - UK
5 The Geological Survey of Denmark and Greenland (GEUS) - Denmark
6 UNIVERSITA DEGLI STUDI DI ROMA LA SAPIENZA (UNIRM) - Italy
7 AGENCIA ESTATAL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS (CSIC) - Spain
8 UNIVERSITY OF ULSTER (Ulster) - UK
9 CONSIGLIO NAZIONALE DELLE RICERCHE (CNR) - Italy
10 UNIVERSITA DEGLI STUDI DI MILANO (UMIL) - Italy
11 UNIVERSITEIT GENT (Ugent) - Belgium
12 VYSOKA SKOLA CHEMICKO-TECHNOLOGICKA V PRAZE (ICTP) - Czech Rep.
13 Københavns Universitet (UCPH) - Denmark
14 BANGOR UNIVERSITY (Bangor) - UK
15 HELMHOLTZ ZENTRUM MUENCHEN DEUTSCHES FORSCHUNGSZENTRUM FUER GESUNDHEIT UND UMWELT GMBH (HMGU) - Germany
16 MARINE BIOLOGICAL ASSOCIATION OF THE UNITED KINGDOM (MBA) - UK
17 UNIVERSITE CATHOLIQUE DE LOUVAIN (UCL) - Belgium

KILL SPILL TEAM
18 NATIONAL UNIVERSITY OF IRELAND, GALWAY (NUIG) - Ireland
19 BIOBASED EUROPE LIMITED (BBE) - UK
20 BIOREM ENGINEERING BVBA (BIOREM) - Belgium
21 GORTON CONSULTANCY LTD (GCL) - UK
22 CREATIVE RESEARCH SOLUTIONS BVBA (CRS) - Belgium
23 ENVIRONMENTAL PROTECTION ENGINEERING SA (EPE) - Greece
24 MADEP SA (MADEP) - Switzerland
25 HEIQ MATERIALS AG (HeiQ) - Switzerland
26 MARITIM MILJO BEREDSKAP AS (MMB) - Norway
27 INSTITUT ZA FIZIKALNO BIOLOGIJO D.O.O. (IFB) - Slovenia
28 EcoTechSystems Srl (ECOTS) - Italy
29 UK SPILL LIMITED (UKSpill) - UK
30 VERMICON AKTIENGESELLSCHAFT (VER) - Germany
31 ACTYGEA SRL (ACTY) - Italy
32 OMYA DEVELOPMENT AG (OMYA) - Switzerland
33 STATE UNIVERSITY OF NEW YORK AT BUFFALO (UB) - USA



Project Focus / Objectives

1. Review **State-of-the-Art** & identify technology gaps
2. Develop techniques (**biosensors & chemical indices**) to monitor hydrocarbon degraders & hydrocarbon degradation
3. Develop novel **bio-based dispersants**
4. Develop novel **bioremediation agents**
5. Develop **solutions for sediments**
6. Develop **multi-functional bioremediation agents**
7. Test their **toxicity**

Technologies (products) to be Delivered

No.	Technology	Application
1	"Kill•Spill Biosensor" (Biosensors for HC-monitoring)	On-site monitoring of oil degradation
2	"Kill•Spill FISH-Kit" (Cultivation-independent microbial diagnostic kits)	CARD-FISH diagnostic kit for on-site monitoring of microbial communities
3	"Kill•Spill FCM-Kit" (Cultivation-independent microbial diagnostic kits)	FISH + FCM diagnostic kit for on-site monitoring of microbial communities
4	"Kill•Spill Chip" (Microarray chip)	On-site monitoring of microbial communities
5	CHEMSIC	Monitoring of oil degradation
6	Polymer-based non-woven fabrics	Sorbent material (shoreline and near-shore)
7	Mineral-based powders	Sorbent material, accelerated bioremediation (oxic and anoxic environments)
8	Oxygen-releasing dispersants (OXYGEL™)	Dispersant, accelerated bioremediation (oxic and anoxic environments)
9	Porous granular sorbent (AEROBEADS™)	Sorbent (floating oil), accelerated bioremediation (oxic and anoxic environments)
10	Plant-based biosurfactant blends (SC1000™, SUPERSOLV™, EASYSOLV™)	Emulsification and mobilization of oil, sand washing, accelerated bioremediation
11	Microbial biosurfactants and emulsifiers	Emulsification and mobilization of oil, sand washing, accelerated bioremediation
12	Formulated HC-degrading MOs and consortia	In-situ bioaugmentation (incl. ABA), further technology development
13	High-pressure reactor	Lab-scale testing environment for deep-sea cases
14	Microdroplet reactor	Improvement/isolation of degrading MOs



Technologies (products) to be Delivered

No.	Technology	Application
15	Low cost biostimulant formulations	Accelerated bioremediation, further technology development
16	"Kill•Spill ElectrO ₂ " (Electrode-based oxygen supply)	In-situ sediment cleanup
17	"Kill•Spill snorkel" (Microbial electrochemical snorkel)	In-situ sediment cleanup
18	"Kill•Spill Robot" (Bio-electro-chemical roaming system)	In-situ sediment cleanup
19	Infauna accelerated degradation	In-situ sediment cleanup
20	"Kill•Spill Sed-Cleaner" (Modular system for enhanced biodegradation)	In-situ bioaugmentation and biostimulation for sediments
21	Sequestering sorbents	Sorbent material for oil sequestration in sediments
22	"Kill•Spill Deep-sea" (Multi-functional bioremediation agents)	Enhanced bioremediation of HC-"clouds" formed in deep-sea oil releases
23	"Kill•Spill Mesoporous" (Mesoporous silica (nano)particles)	Enhanced bioremediation though bioaugmentation and biostimulation on silica
24	"Kill•Spill SlowRelease" (Slow release microparticles)	Enhanced bioremediation though bioaugmentation and slow-release fertilizers in lipophilic carriers
25	"Kill•Spill All-in-One" ("All-in-One" multifunctional carrier)	First response measure for enhanced bioremediation and oil dispersion
26	"Kill•Spill MineralSorb" (Multifunctional sorbent materials)	Mineral based Sinking and agent (Oil transferred to sediments) and enhanced bioremediation
27	"Kill•Spill Bio-carriers" (Porous bio-carriers)	Biomaterials for immobilization of HC degraders and biostimulants for sea water & sediments
28	"Kill•Spill Bio-boom" (Improved biodegrading booms)	Oil barriers (booms) and with enhanced sorbent & bioremediation capabilities.



“Mandatory” Field Testing of Technologies

- Field tests of developed technologies during 3rd & 4th year:
 1. At CNR-IAMC pilot testing facilities
 2. **In Aegean Sea (EPE)**
 3. **In North Sea/Norwegian Sea (MMB)**
 4. At Sea DIAMOND wreck (EPE)
 5. In Disko Bay / Greenland (GEUS)

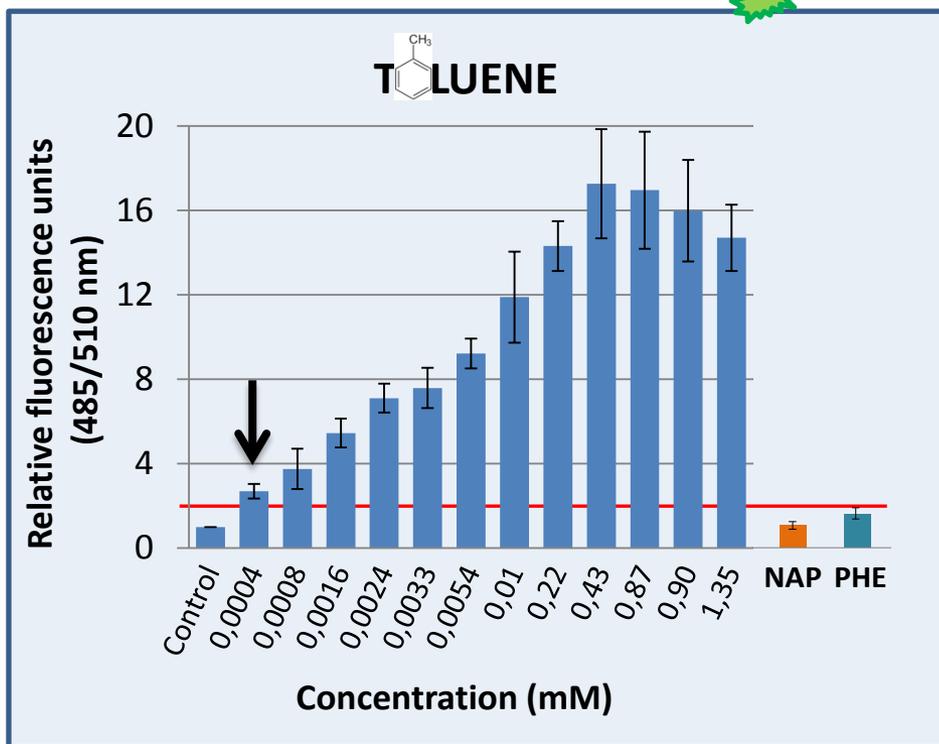
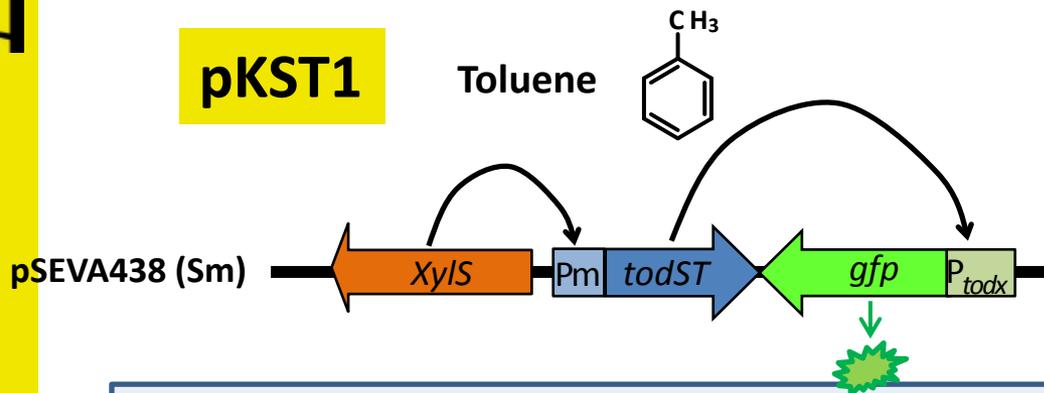
and at

 6. TUC landfarming mesocosms
 7. At ship wreck (CNR-IAMC)

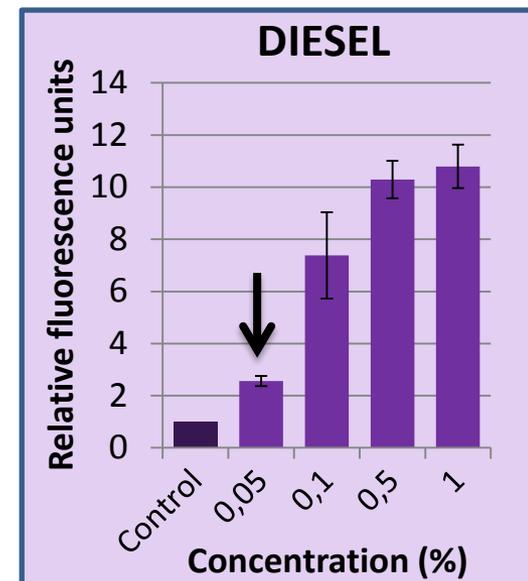
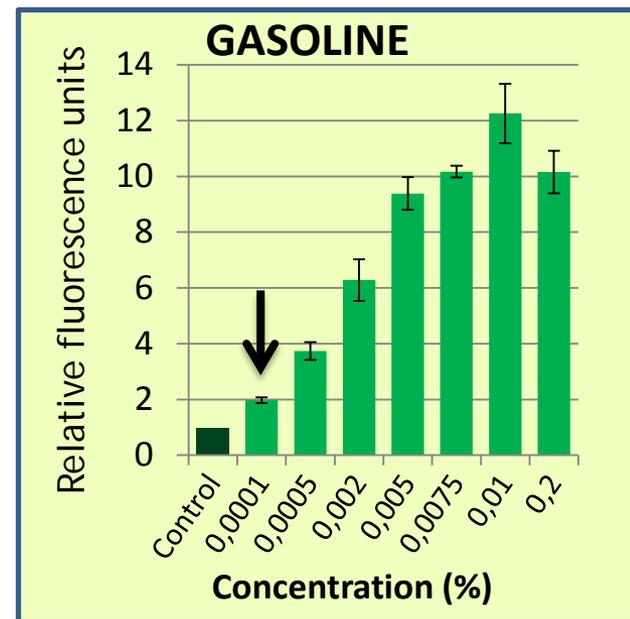




pKST-1 in *Alcanivorax borkumensis* SK2



↓ Indicates the detection limit of this biosensor





KILL•SPILL Biosensor

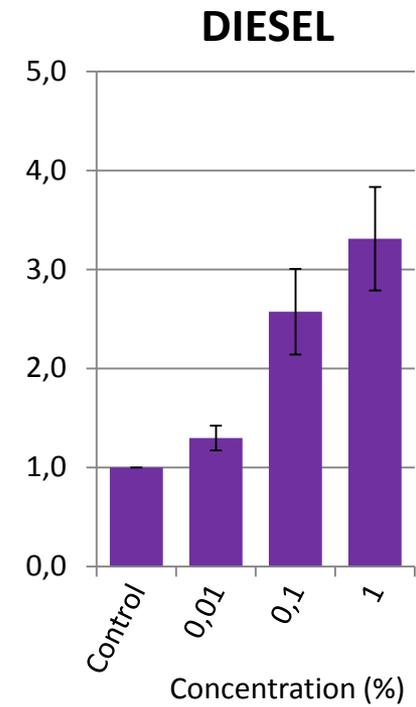
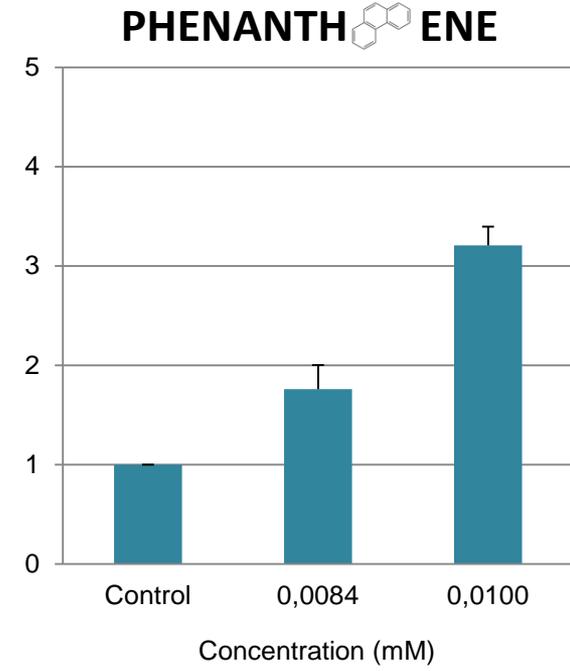
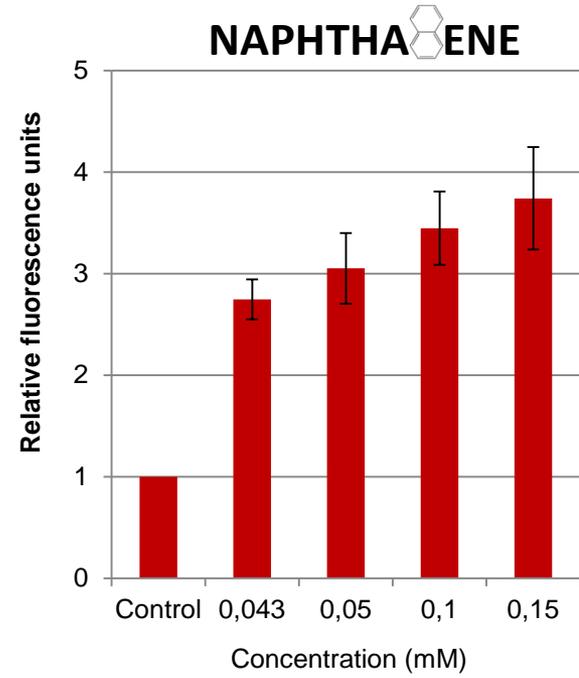
PAH biosensor (pKSPA-1)



Novosphingobium sp. HR1a



No induction with BTEX



Detection limits

- c1ccc2ccccc2c1 Naphthalene: **40 μM** Fuel: **0.1%**
 - c1ccc2cc3ccccc3cc2c1 Phenanthrene: **8.5 μM**
 - O=C(O)c1ccccc1O Salicylic acid: **0.1 μM**
- Experiments done in marine media

No induction with Gasoline



KILL•SPILL chip microarray

On-site monitoring of microbial communities

Kill Spill chip: analysis pipeline



Design of Chip

Sequences analysis

Probe design

Printing of chip probes

Preparation of Environmental samples

DNA extraction

DNA amplification

DNA Labelling

Hybridization

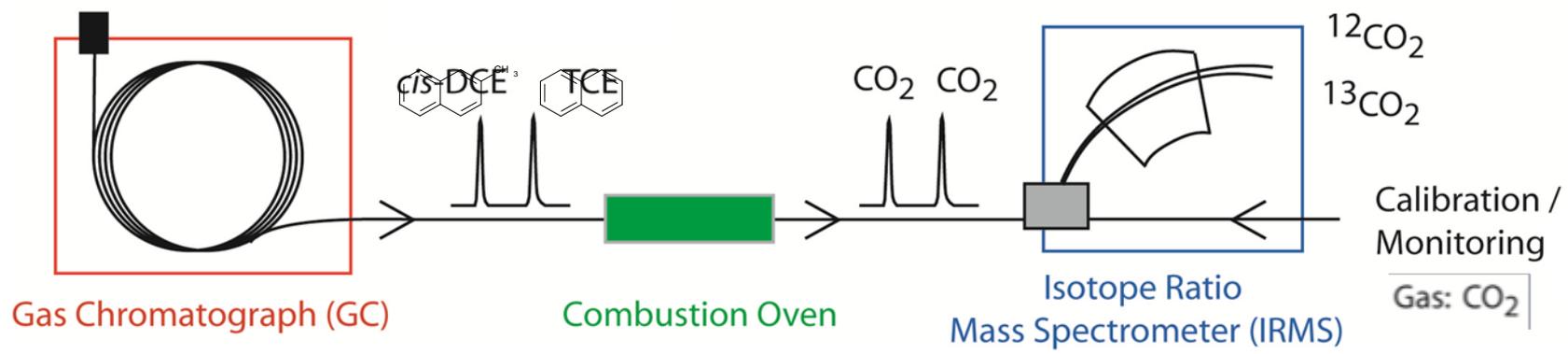
Data processing

Scanning and image analysis

Data analysis and network reconstruction

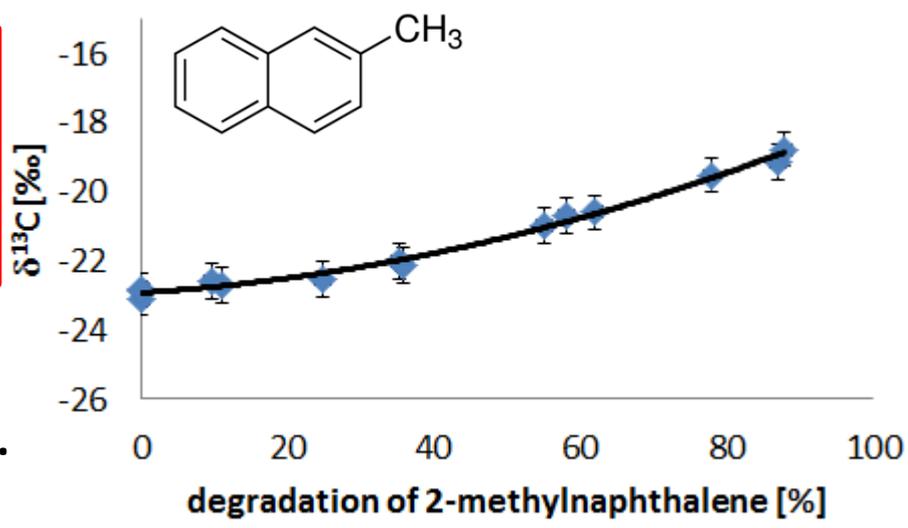
Development of **molecular tools for the rapid detection of microbial degradation** of oil compounds in the marine environment

Compound Specific Isotope Analysis: Identification of in-situ Degradation of Polycyclic Aromatic Hydrocarbons



✓ Degradation is associated with an enrichment of ¹³C in the remaining contaminant pool → increasing δ¹³C

✓ Differentiation between degradation and physical processes (isotope insensitive) like sorption and dilution.



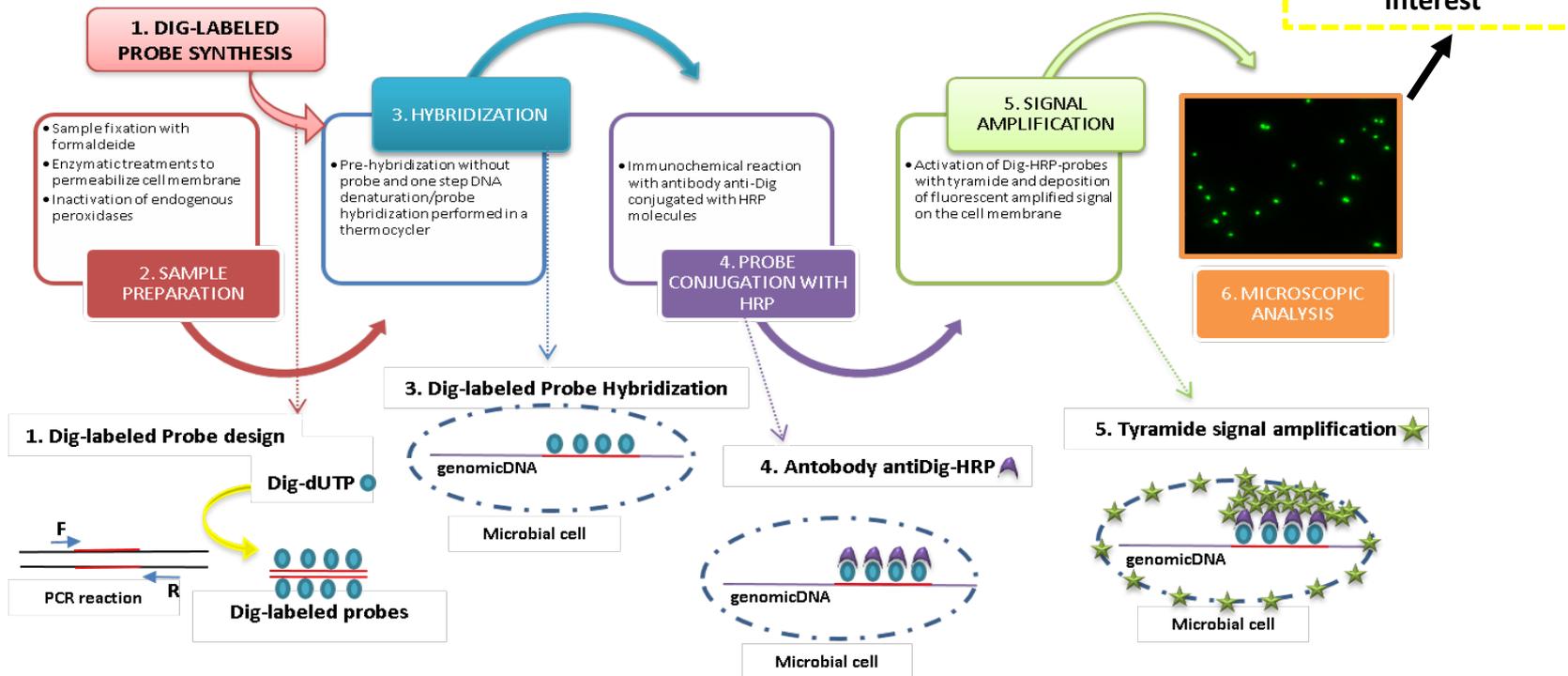
geneCARD-FISH assay: detection of *alkB* genes involved in the hydrocarbons degradation

A NOVEL BIOMONITORING TOOL:

- Detection of whole cells carrying specific genes involved in hydrocarbons degradation processes
- Fluorescence *in situ* hybridization assay based on the fluorescent signal amplification system
- Accurate and rapid quantification of cells also at very low concentration

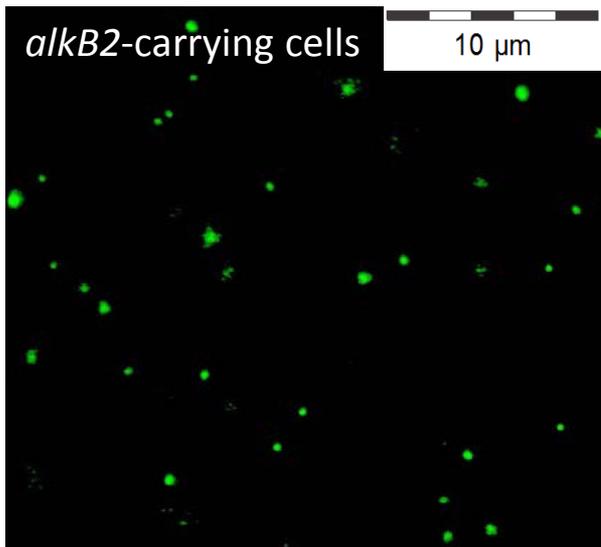
geneCARD-FISH PROCEDURE:

- Preparation of the molecular probe
- Hybridization probe/DNA
- Fluorescence signal amplification
- Detection of cells carrying *alkB* genes



geneCARD-FISH assay: detection of *alkB* genes involved in the hydrocarbons degradation

geneCARD-FISH: application on *Alcanivorax borkumensis* SK2^T pure culture



- No DNA extraction required
- Specific hybridization
- Maintenance of cellular morphology
- Fluorescent signal well distributed on the cellular membrane
- Low background noise during the microscopic analysis
- Good detection efficiencies
- Assay suitable for the application on environmental samples

First Kill•Spill FISH kits are now commercially available ...

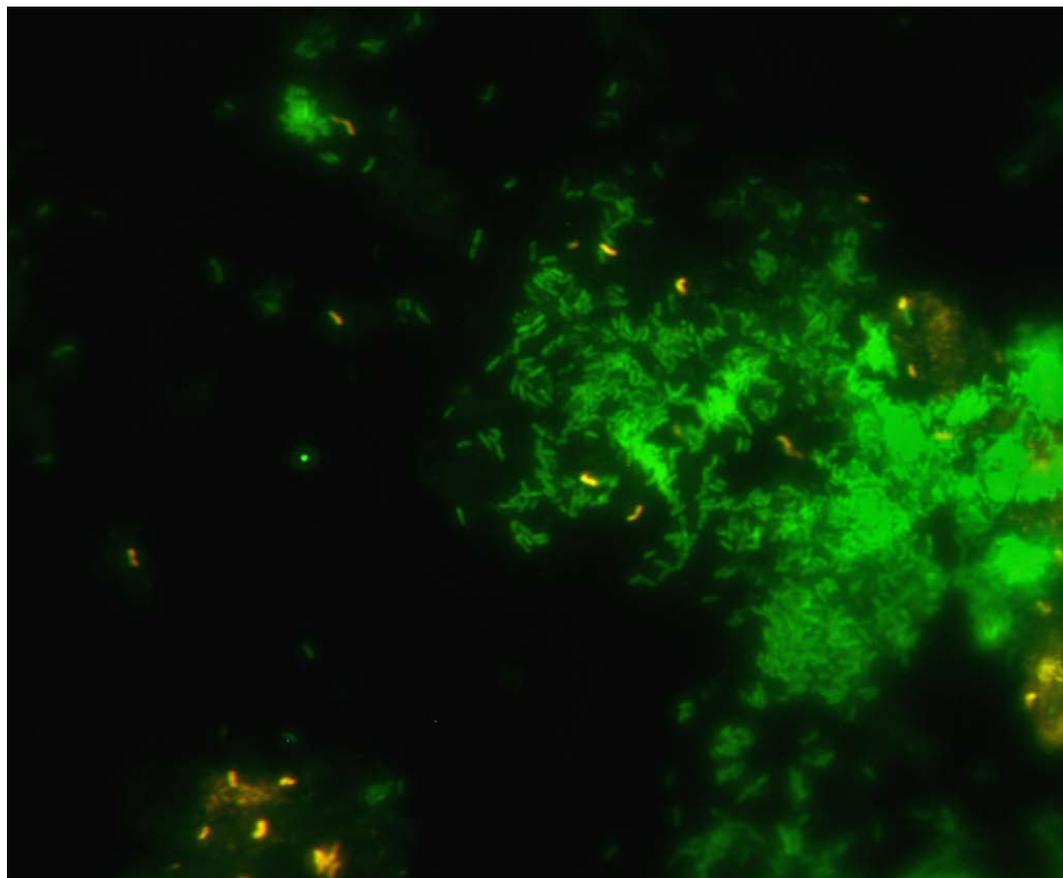
- FISH protocols adapted to easy and standardized VIT® format allows fast and specific on-site monitoring
- Identification and quantification of various hydrocarbon-degrading bacteria using developed test kits:



- *Alcanivorax borkumensis*
- *Cycloclasticus pugetii*
- *Marinobacter* sp.
- *Oleispira antarctica*
- *Oleiphilus messinensis*
- *Thalassolituus oleivorans*

Kill•Spill FISH kit

- Example for the analysis of hydrocarbon-degrading bacteria in natural sample using fluorescence microscopy

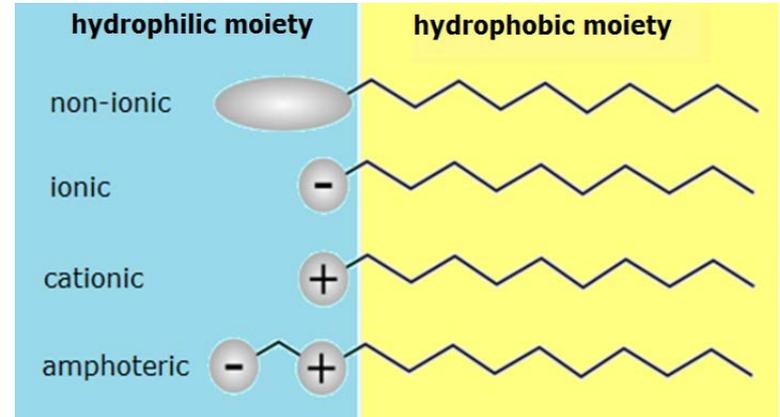


Green: *Alcanivorax borkumensis*, **Yellow:** *Oleispira antarctica*;
Total magnification **1000 x**

Available surface active products to date

- **Microbial Biosurfactants:**

- Rhamnolipids
- Sophorolipids
- Biosurfactants + Oxygel (O₂ delivery) + treated CaCO₃ (adsorption/sinking agent) + hydrocarbon degrading bacteria.



- **Several plant surface active extracts/oils** from BioBased Europe Ltd (a Scottish company). 

- **Work is in progress** to develop a biocatalyst of rhamnolipids produced by *Burkholderia* cells immobilised on delignified Cellulose for testing in environmental applications.



Available strains with ability to produce biosurfactants

Strain	Biosurfactants (main congener)	Yield (g/L)	Surface tension (mN/m)	EI 24 (%)
<i>Pseudomonas aeruginosa</i> PAO1	Rhamnolipids (Rha-Rha-C ₁₀ -C ₁₀)	2-3	24-29	53-64
<i>Pseudomonas aeruginosa</i> ST5	Rhamnolipids (Rha-Rha-C ₁₀ -C ₁₀)	1.5-2.5	25-29	52-65
<i>Pseudomonas aeruginosa</i> DS10	Rhamnolipids (Rha-Rha-C ₁₀ -C ₁₀)	1.5-3	25-31	52-61
<i>Burkholderia thailandensis</i> E264	Rhamnolipids (Rha-Rha-C ₁₄ -C ₁₄)	2-4	26-33	67-70
<i>Burkholderia glumae</i>	Rhamnolipids (Rha-Rha-C ₁₄ -C ₁₄)	1.3-2.2	28-30	65-72
<i>Burkholderia plantarii</i>	Rhamnolipids (Rha-Rha-C ₁₄ -C ₁₄)	0.8-1.1	26-27	60-67
<i>Burkholderia kururiensis</i>	Rhamnolipids (Rha-Rha-C ₁₄ -C ₁₄)	1.9-3.1	30-33	60-63
<i>Candida bombicola</i>	Sophorolipids	20-100	36-39	48-53
<i>Pseudozyma aphidis</i>	Mannosylerythritol lipids	*	*	*
<i>Bacillus</i>	Surfactin	0.2-0.8	24-31	58-65

* Currently under evaluation



Isolation and selection of novel marine aerobic microbes with biosurfactant production potential



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

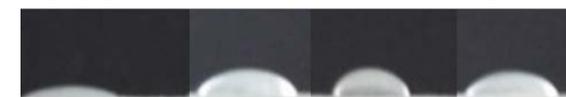
34 fast-growing isolates in liquid medium (1 or 0,5% glucose)

144 pure isolates obtained on mMSM agar among them 79 having different colony morphology selected



Drop Collapse

+++ - - +



TW80
0,5%
NC
G19.30
G3.20

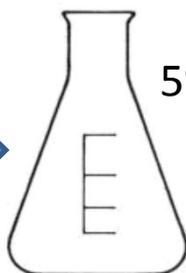
Emulsification Index (EI₂₄)



Bacterial isolate



48-120 h
of growth



5% inoculum

24-120 h
of growth



Culture

Emulsion production and stability under extreme conditions





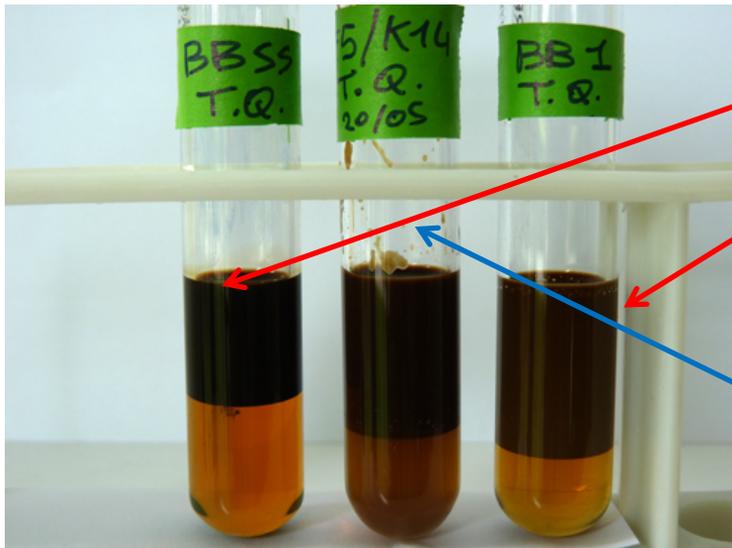
KILL•SPILL pilot scale production

Biosurfactants produced at industrial pilot scale



Sample	Crude Oil EI (%)
Rhamnolipids Actygea	57
Sophorolipids Actygea	63
SC2000 (Commercial emulsifiers from vegetables)	65
SuperSolv (Commercial emulsifiers from vegetables)	50

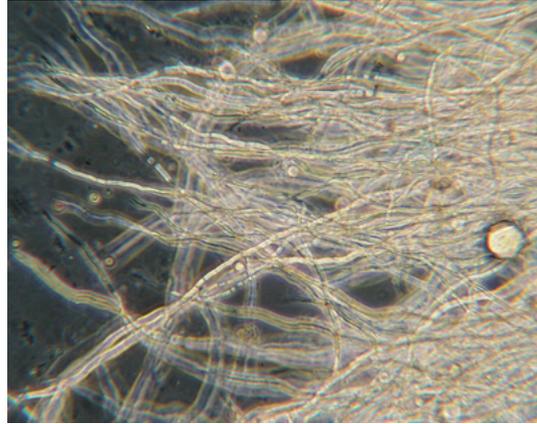
Commercial



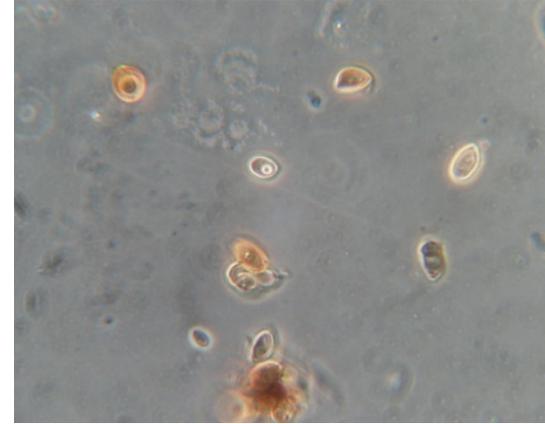
Test



Products: spores of *Cladosporium* sp. (bioaugmentation)



Mycelium



Spores

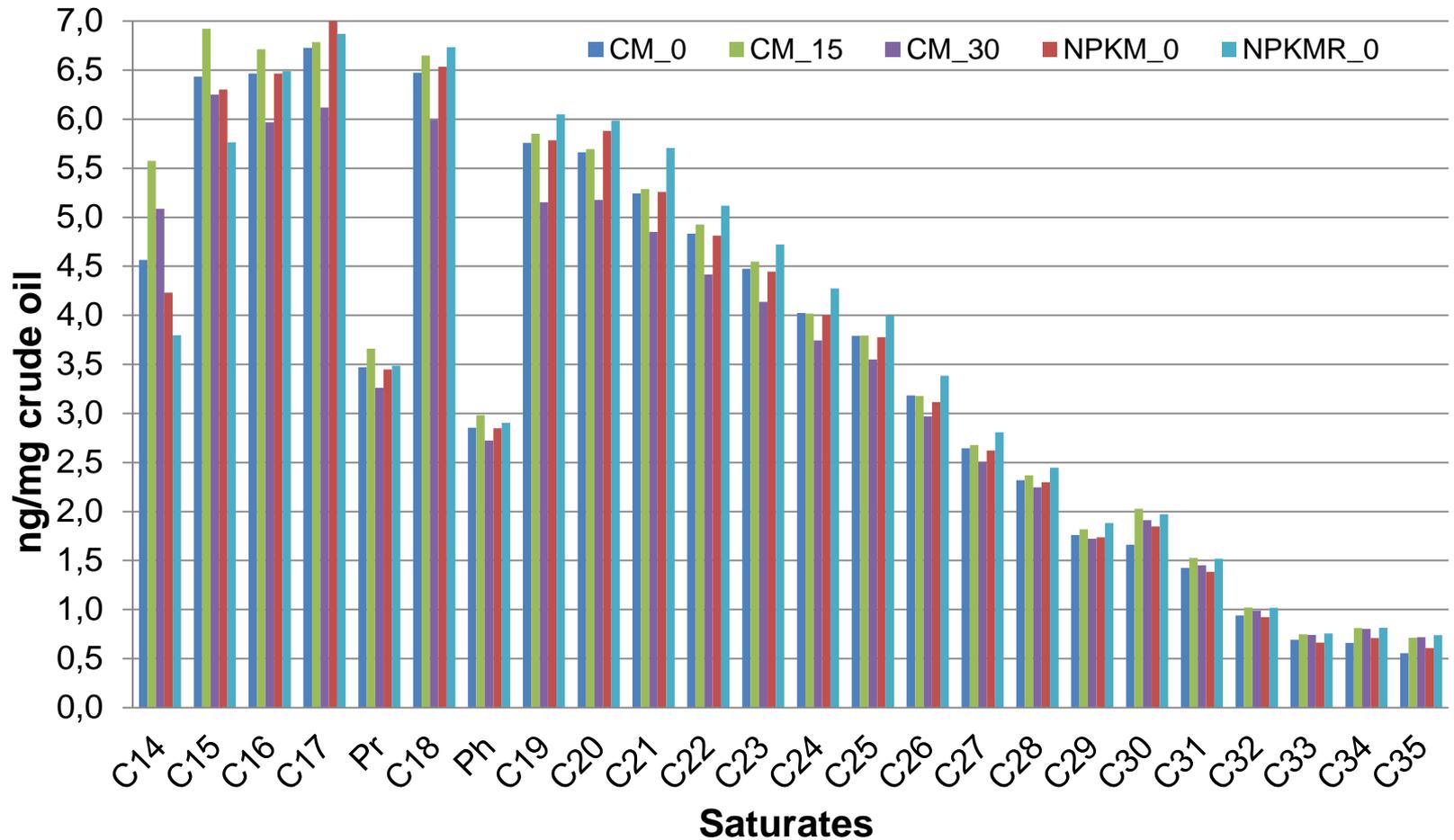
Cladosporium sp. characteristics:

- *Able to degrade 80% of 50-80 g/L crude oil added to liquid cultures or to sea sand microcosms*

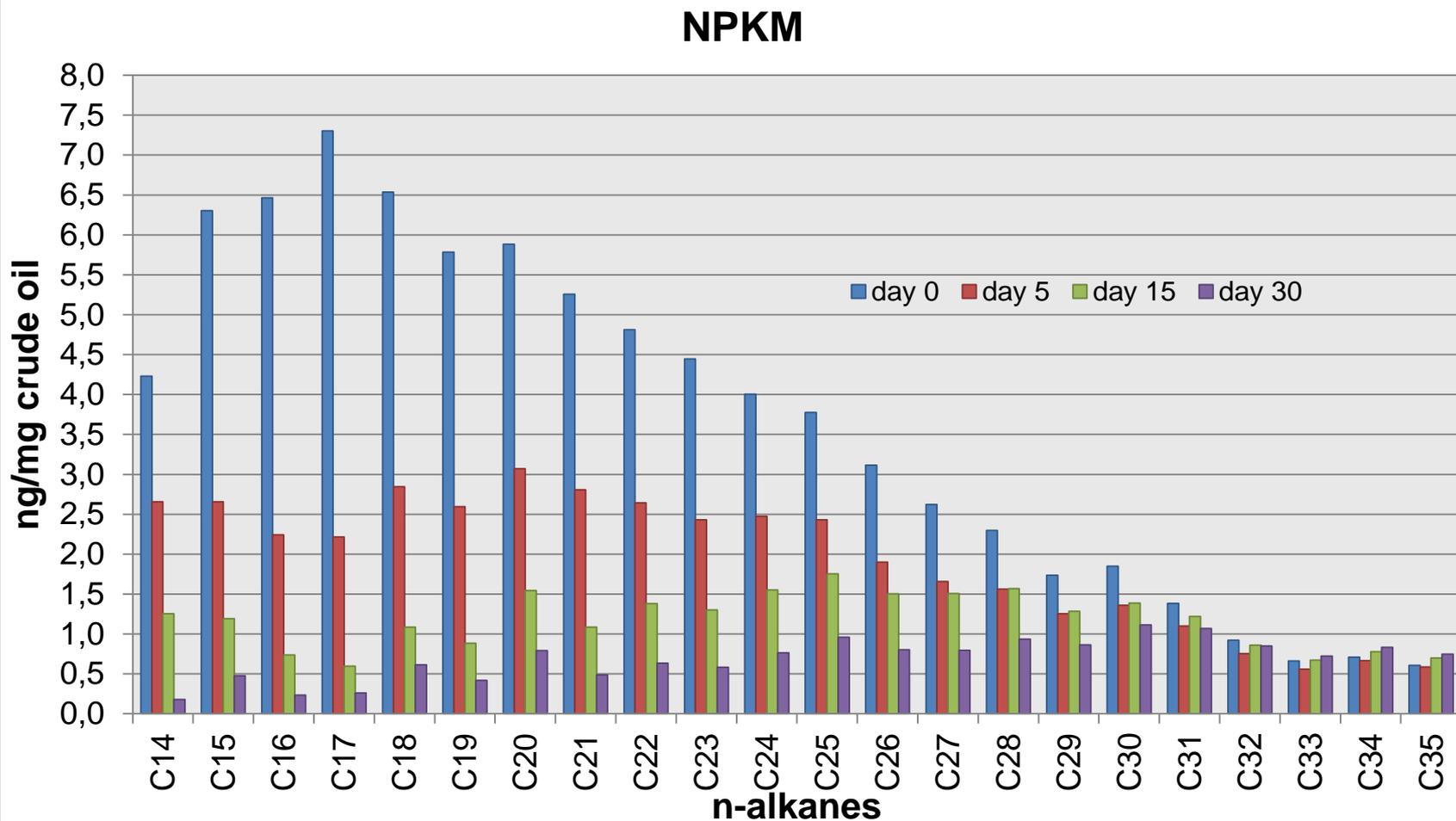
Cladosporium sp. advantages:

- *Spore forming organism;*
- *Easily stored in dry form & easy to blend with nutrients;*
- *Preferentially grows on solid substrates;*

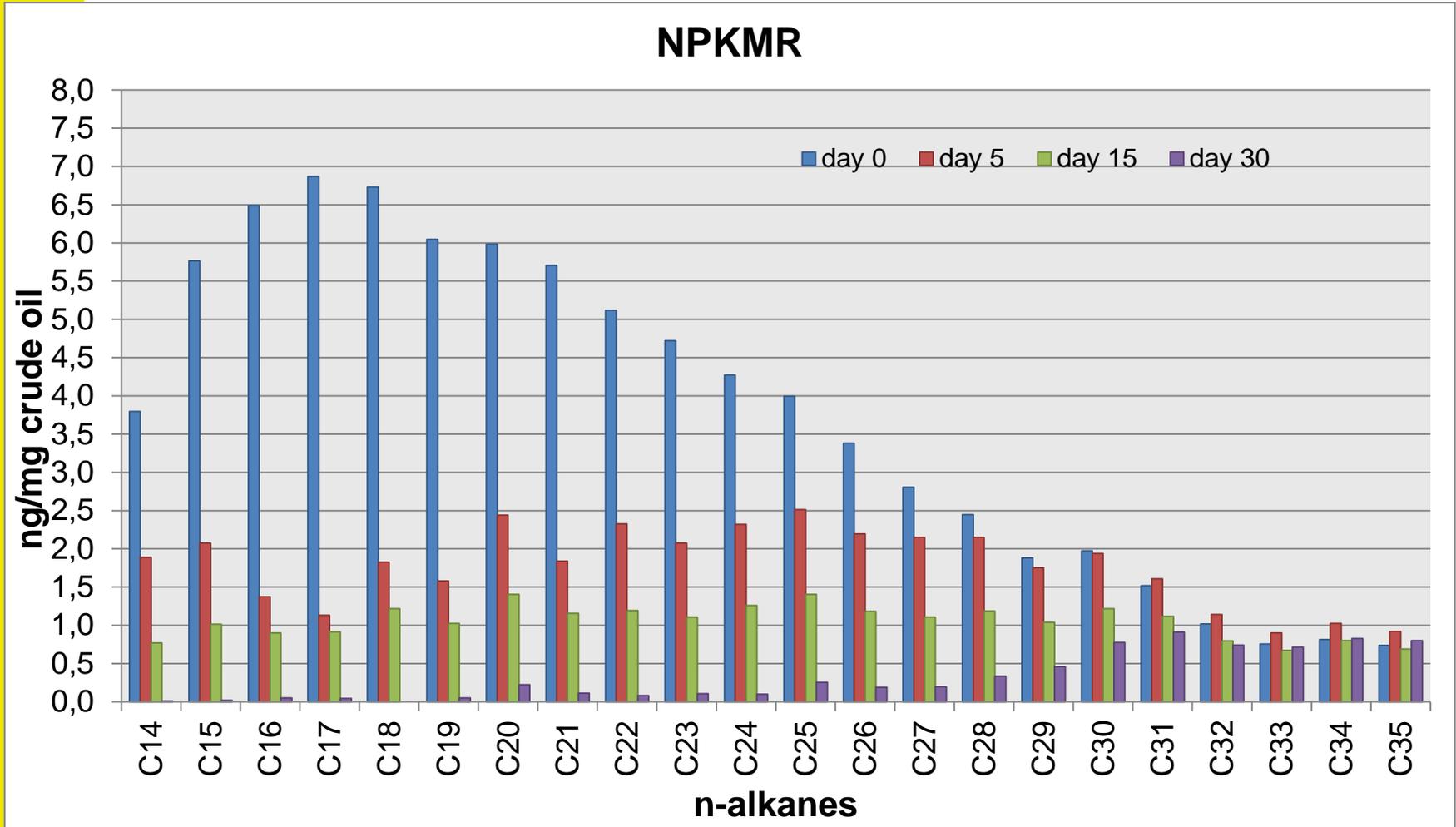
Effect of Bioremediation Agents (biostimulants) Concentration profile of alkanes (Control) at time 0, 15, 30 days



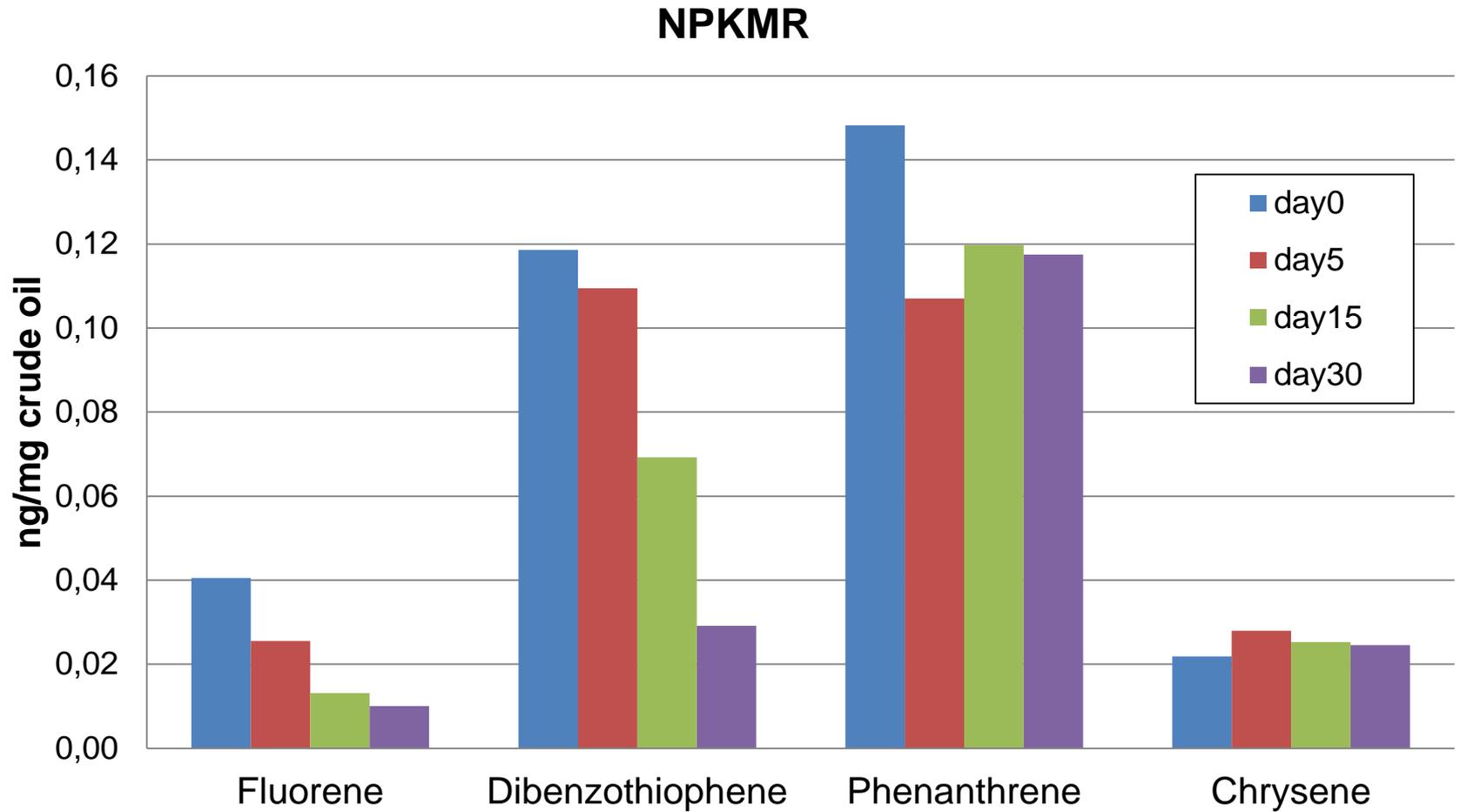
Results of the NPKM Treatment (stimulation with N, P, K & ABA-microbes)



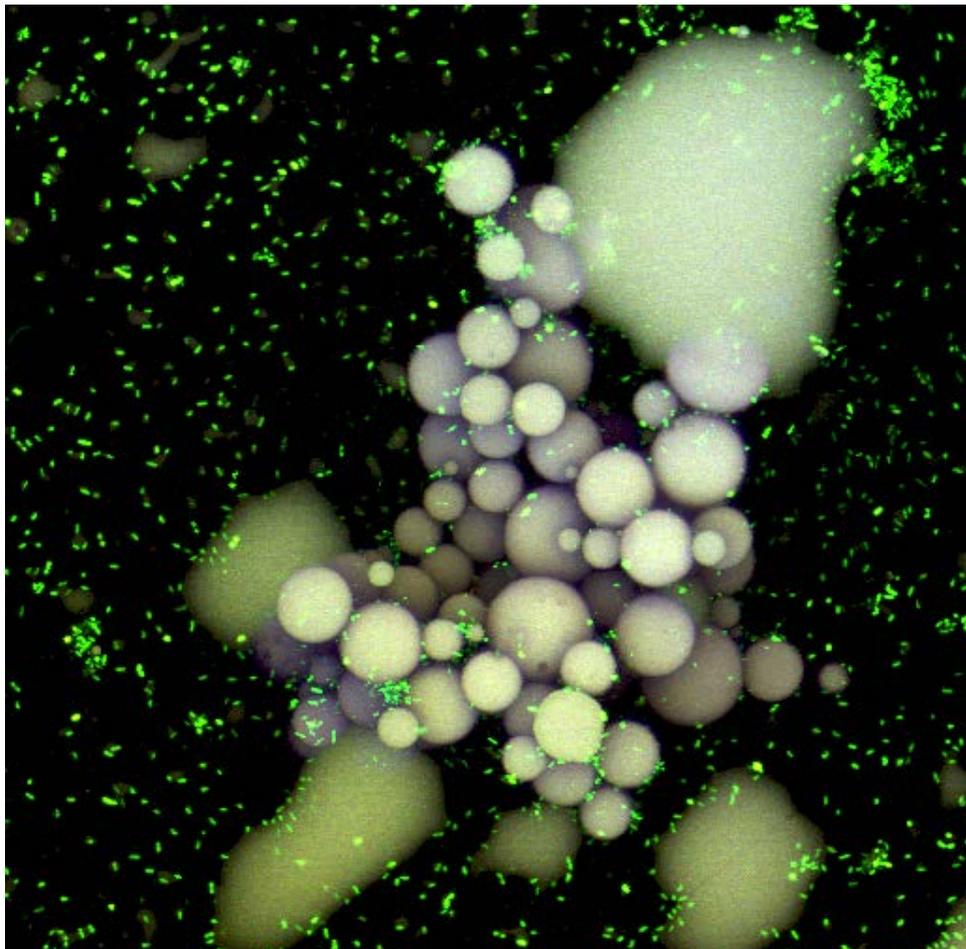
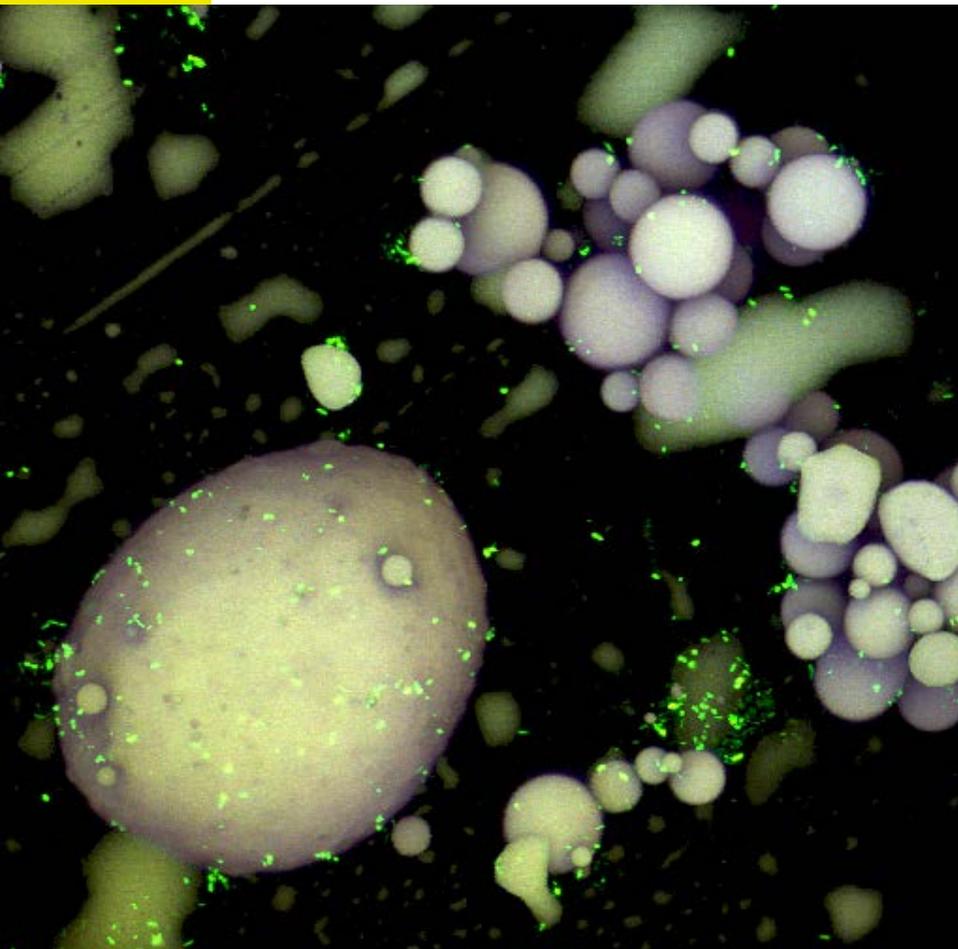
Results of the NPKMR Treatment (plus Rhamnolipids)



NPKMR treatment: Profile of PAHs



Understanding biosurfactant/microbial action: Crude oil in seawater



The “grape bunch formation” effect

Advantages of functionalized CaCO_3

- Functionalized CaCO_3 only sinks if sorbed with oil
- Oil carpet is completely removed
- Formation of dense drops

Functionalized CaCO_3



Non-functionalized CaCO_3



Sinking capacity: Marine water, simulated swell (200 RPM), 2h

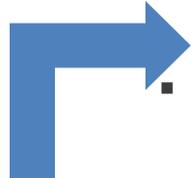
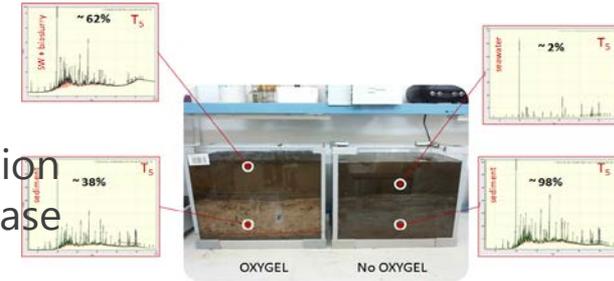


Enhancing aerobic biodegradation with Oxygen releasing compounds: Gel form



Oxygel™

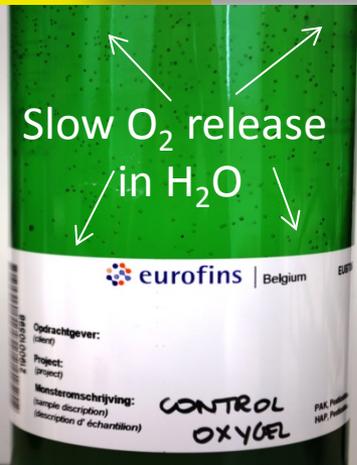
- Without additives:
 - Suitable for injection into sediments
 - Oil desorption & degradation stimulation by oxygen release



- With additives (solvents & surfactants from Bio-Based):
 - Suitable for free oil absorption into its matrix
 - Oxygen release for enhanced degradation.



- With a catalyst (OxygelPLUS™)
 - Release of oxidative radicals
 - Chemical oxidation of recalcitrant pollutants





KILL•SPILL all-in-one

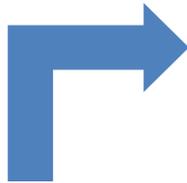
Oxygen releasing compounds: *Dry form*



Aerobeads™

Composition:

- Dried Oxygel™
- Functionalized CaCO₃ (Microstech)



▪ Without additives:

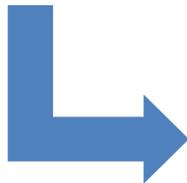
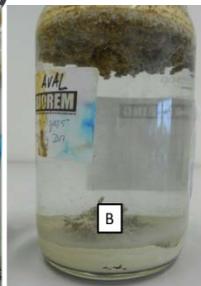
- Adsorption of a floating oil layer
- Slow release of Oxygen over time for enhanced natural attenuation



▪ With additives (Solvents / surfactants, lyophilized bacteria, micro-nutrients)

- 'All-in-one solution'
- Adsorption of a floating oil layer
- Enhanced biodegradation on ocean floor

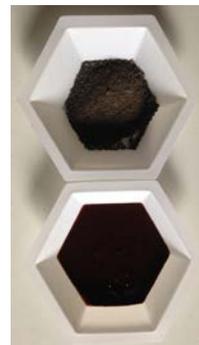
Work in progress



▪ With a catalyst (AerobeadsPLUS™)

- Release of oxidative radicals
- Chemical oxidation of recalcitrant pollutants

Work in progress



SLOW-RELEASE MICROPARTICLES

Gel-Beads - Dry Alginate Beads (DABs)

Immobilized preparation for bioaugmentation and bioremediation of oil-spills in sea-water



Wet
Preparation



Dry
Preparation

What's inside?

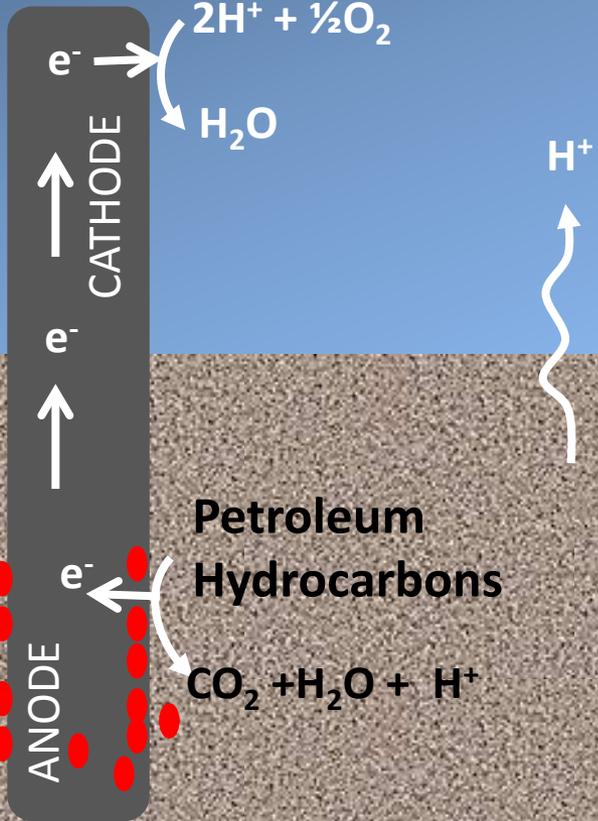
- HC degrading microorganisms
- (Bio)Surfactants
- P,N nutrients
- Other additives

Advantages

- High operational stability
- Low water content - resistance to the biodegradation or microbial contamination
- Immediately ready for “on-field” operations

The Kill•Spill Snorkel: Breathing a “spatially distant” electron acceptor

OVERLAYING AEROBIC ZONE



Hydrocarbon degrading bacteria

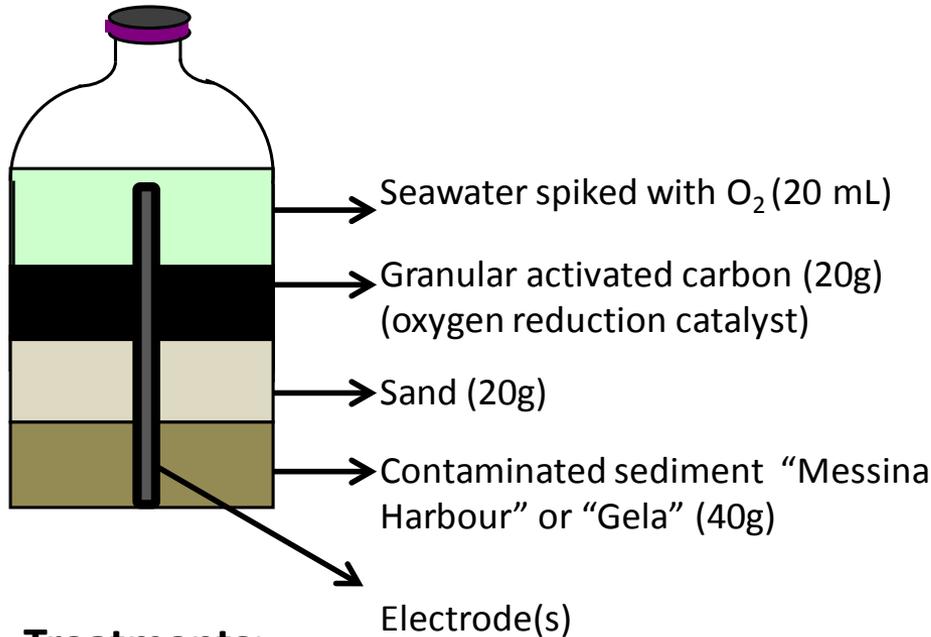
Petroleum Hydrocarbons

ANAEROBIC OIL-CONTAMINATED SEDIMENT

- Oxidative biodegradation processes in sediments are typically **rate-limited** by poor availability of high-potential electron acceptors (e.g., O_2)
- The **Kill•Spill Snorkel** aims to **accelerate biodegradation** by creating an **electrochemical connection** between the anaerobic sediment and the overlaying aerobic water
- Takes advantage of the capability of certain bacteria to anaerobically oxidize organic substrates with an electrode (ANODE) serving as **respiratory electron acceptor**

Experimental setup: The Kill•Spill Snorkel “Proof-of-Principle”

Sacrificial microcosms in 120 mL-serum bottles



Treatments:

1. Snorkel with 3 electrodes
2. Snorkel with 1 electrode
3. Biotic control with no electrodes
4. Abiotic (Autoclaved) Snorkel with 3 electrodes
5. Abiotic (Autoclaved) Snorkel with 1 electrodes

Sediment from **Messina (Italy)**
Harbour spiked with IFO 180

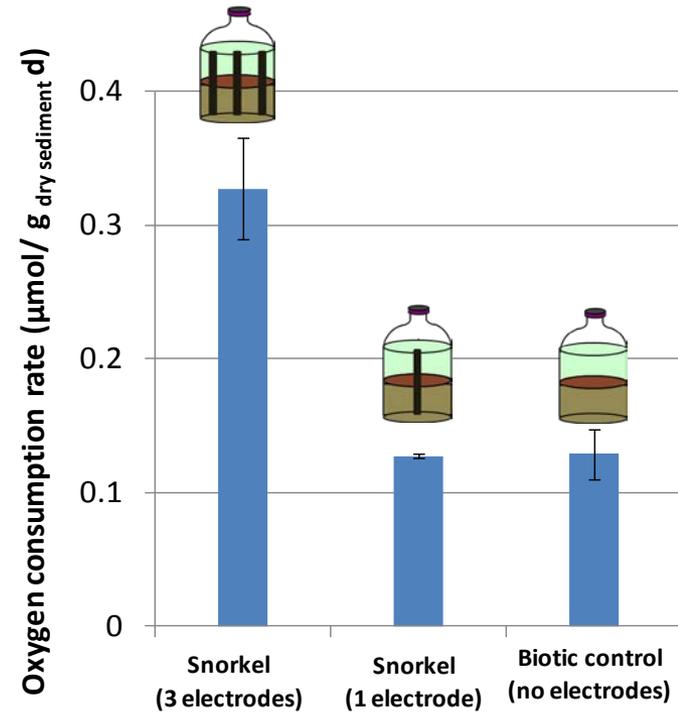
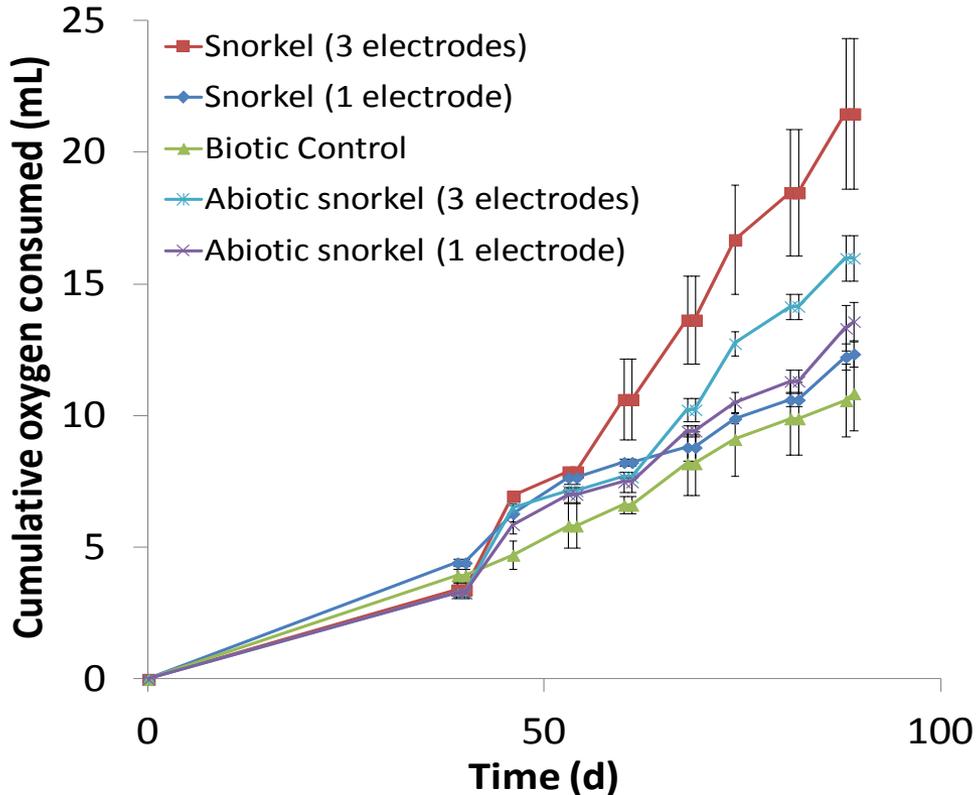


Sediment from **Gela (Italy)** oil-spill



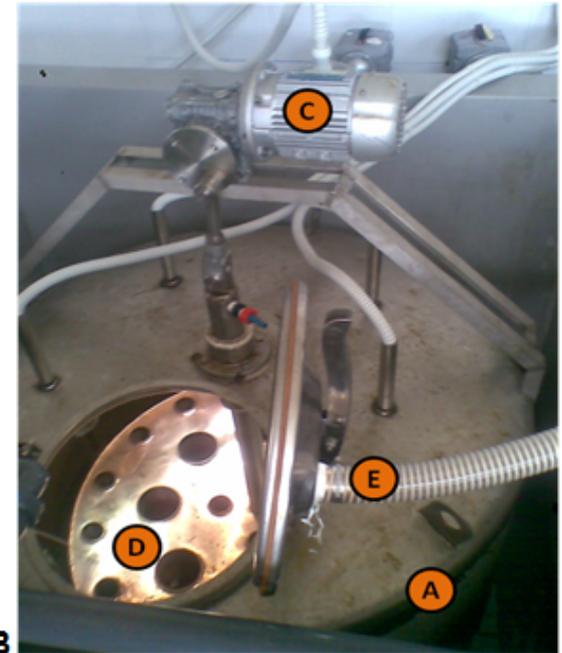
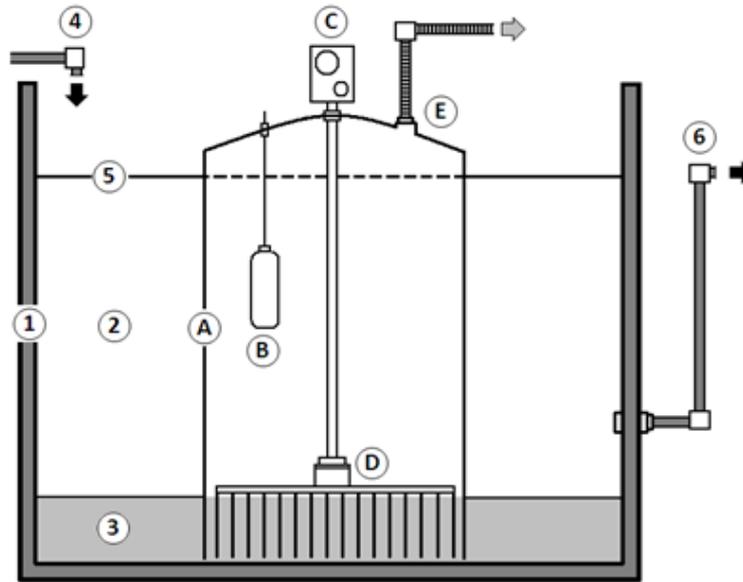


Preliminary results: Oxygen Consumption Rate by “Gela Sediments”



- Oxygen consumption rate (OCR) in the **Kill•Spill Snorkel** (3-electrodes setup) is **2.5 higher** than in the corresponding biotic control (identical setup but with no electrodes)
- **Petroleum hydrocarbon analyses** are in progress to verify if the enhanced OCR corresponded to enhanced biodegradation
- Enhanced OCR also in the abiotic snorkel (3-electrode setup), possibly due to electrochemical oxidation of inorganic species in the sediment (e.g., S^{2-} ; Fe^{2+})

Sediment cleaner prototype: Modular Slurry System (MSS)



A

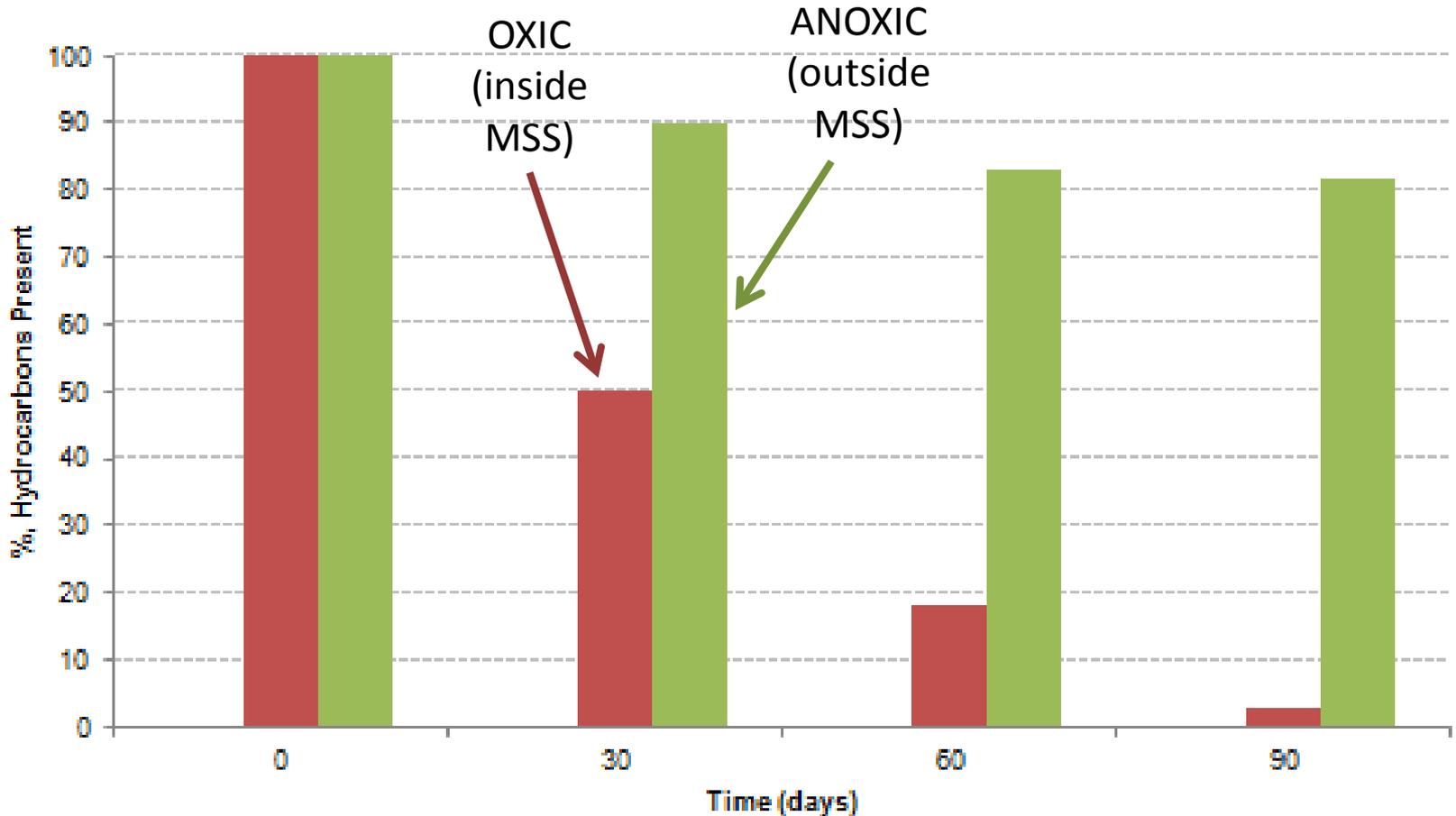
B

Schematic representation (A) and picture (B) of “Biostimulation System” proposed in this work. In this “open” system, the seawater is filled *in continuous* in reaction tank (mesocosm) during the all experimental period. 1, Mesocosm tank; 2, seawater; 3 marine artificially polluted sediments; 4. fill line; 5, level of seawater; 6 “too full” system and line of discharge of water. A, “Biostimulation System”, thermostat system; C, external engine whit air pump; D, steel plate of insufflation air system; E, line for filter air system.



KILL•SPILL sediment cleaner

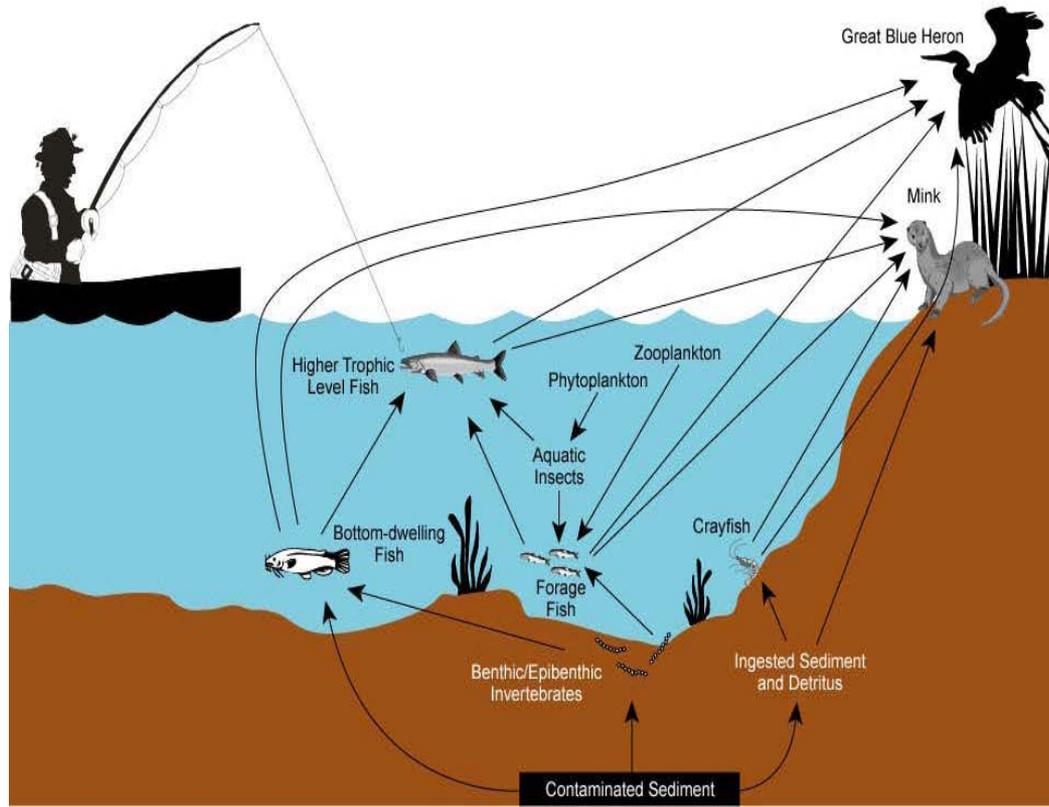
Sediment Cleaner prototype: Modular Slurry System (MSS)



Total Extracted Hydrophobic Fractions	OXIC	AN
90 days	780 ± 80 mg Kg ⁻¹	5400 ± 120 mg Kg ⁻¹

PASSIVE SAMPLING OF HYDROPHOBIC ORGANIC COMPOUNDS (Sediment capping)

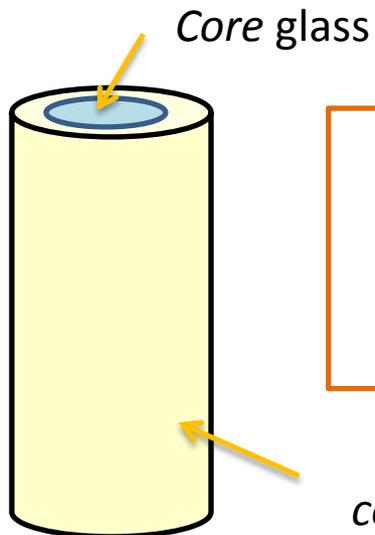
- **Evaluate** the amount of Hydrophobic Organic Compounds (**HOCS**) in water and sediment porewater.
- **HOCS sorbed** amount onto the **fiber** correlated to the **mobile contaminants** concentration.
- Evaluation of **[HOC]_{mobile}** strongly related to the **bioavailable concentration**.
- Provide a measure of the **HOCS availability** and their migration properties



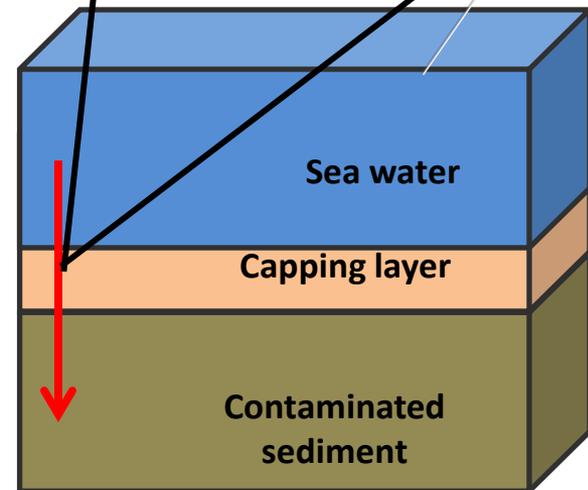
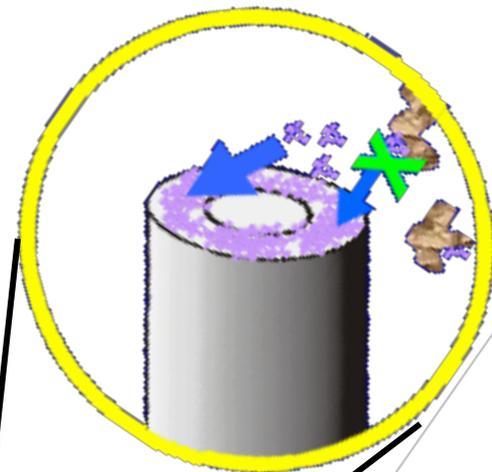
Passive Sampler Devices can be placed in situ for **evaluating** HOCS amount and/or for **monitoring** a remediation process effectiveness

PDSM-SPME

PolyDimethylSiloxane-Solid Phase Micro Extraction



PDMS fiber absorb the PAHs concentration in the water column and in the porewater

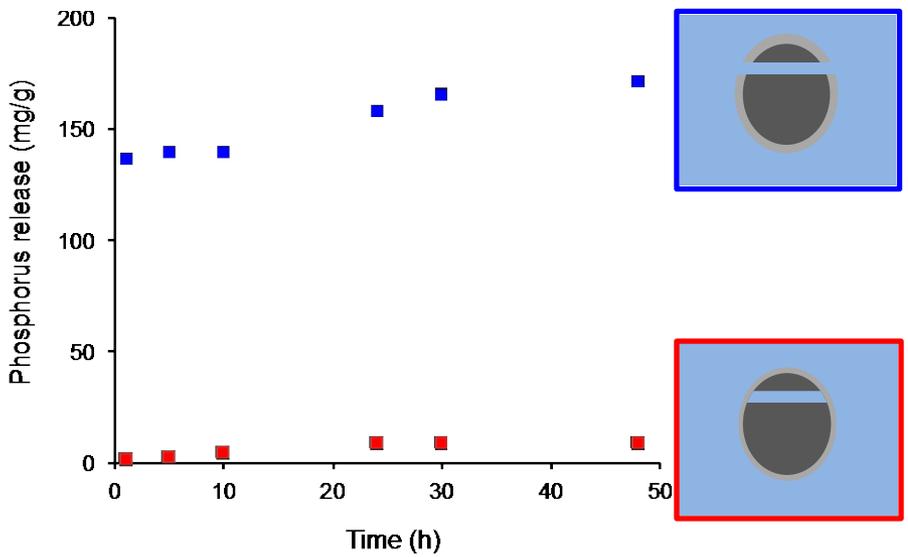
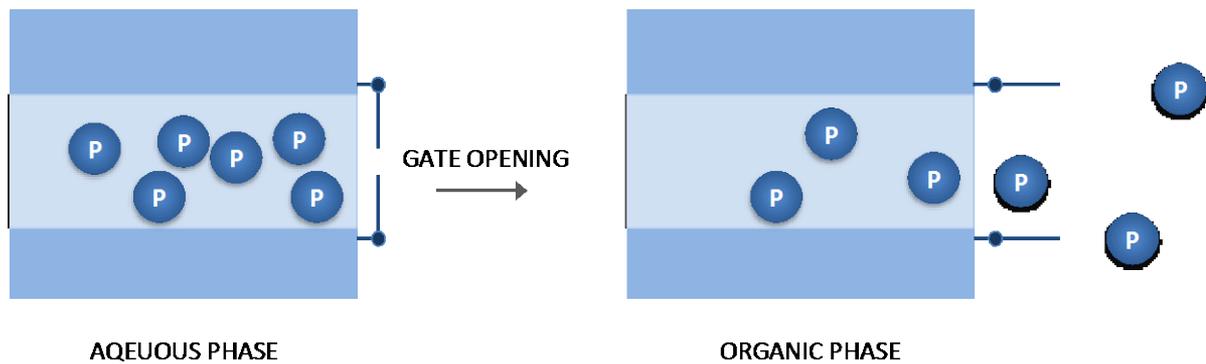


To assess **capping efficiency** the fibers are put into the capping layer and collected after a fixed time for evaluating the PAHs released through the capping layer.

Mesoporous SiO₂ nanoparticles: Formulations to intensify natural (biostimulation) and/or bioaugmentation processes

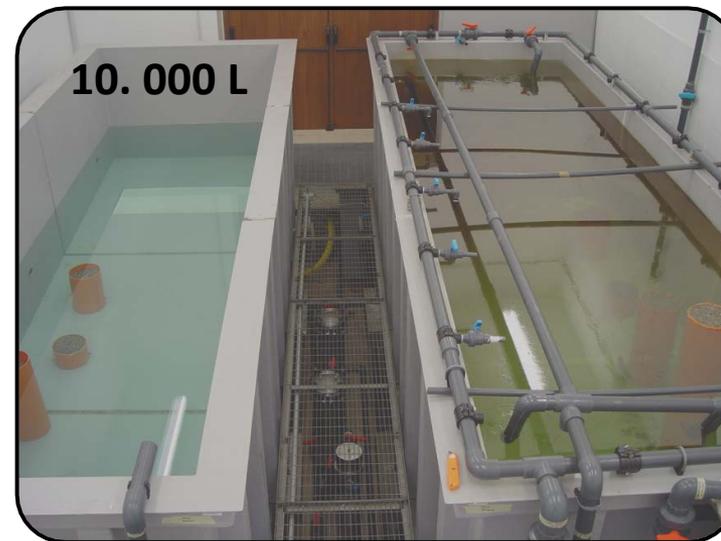
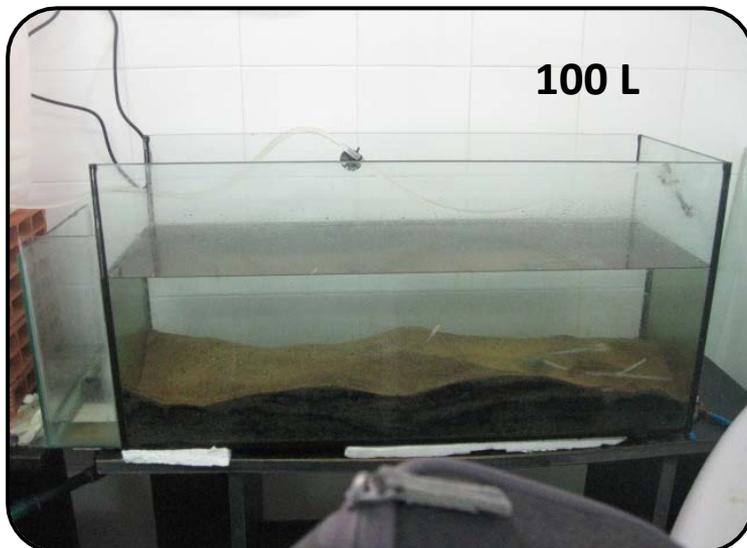


Development of a "smart gate" system allowing the release of the nutrient in the contact of an organic phase



Phosphorous release assayed in the presence of petroleum (10 μL/mL of water)

DEMONSTRATION Facilities at IAMC/CNR: Micro-, & Mesocosms Scale Systems



Indoor Mesocosm Facility



Outdoor Mesocosm Facility

Gela (SR, Sicily, Italy)



**One of the biggest Refinery of Europe
Refining capacity of 100 kbbl/d**

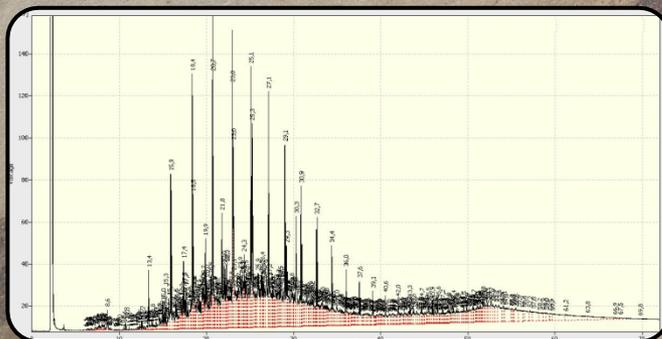
On 4th June 2013, a spill from pipeline of “Topping 1” installation of ENI Refinery of Gela (5 tonnes Crude Oil)

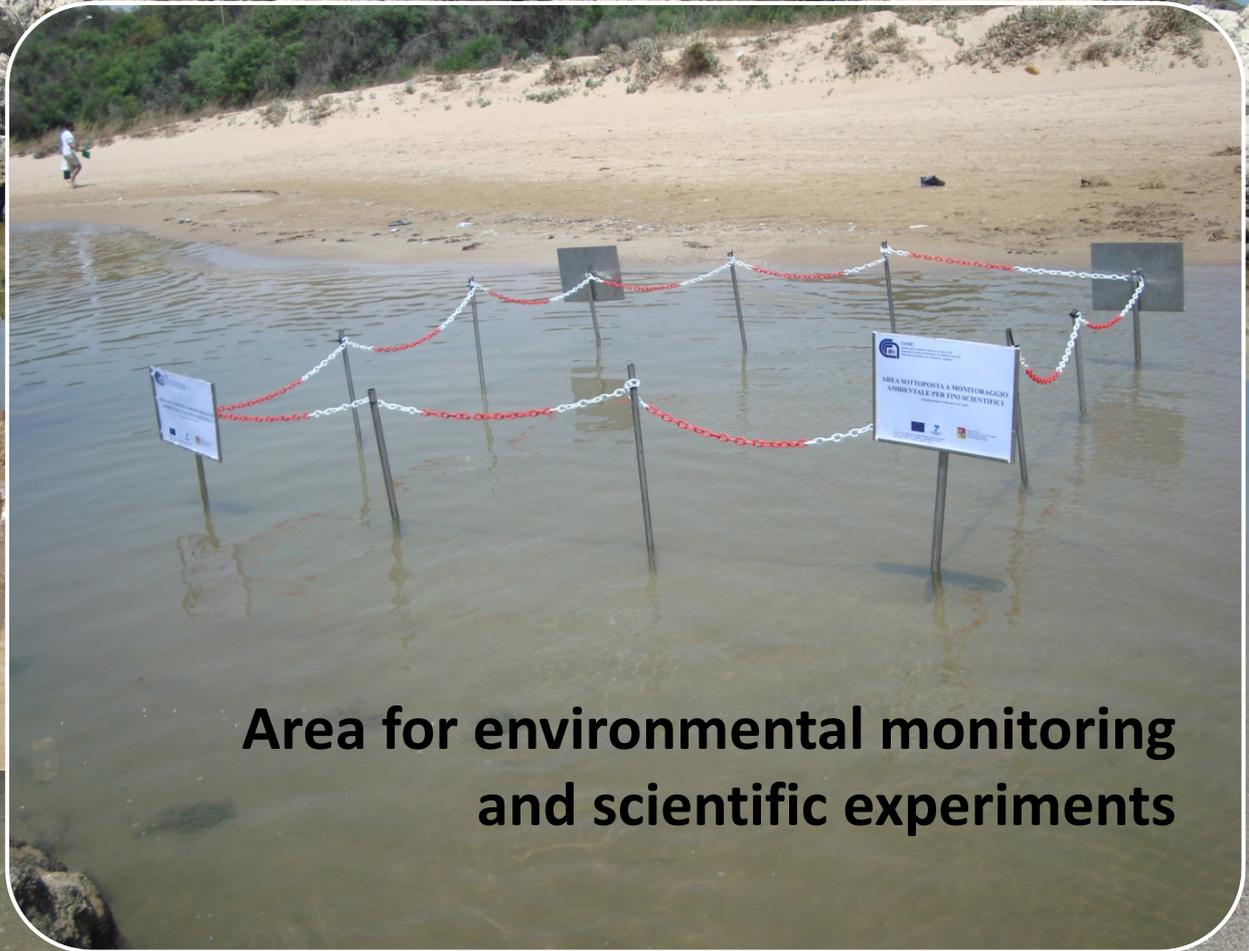


...quick action by Italian Naval Authority and ENI S.p.A to contain the spill...



However... an opportunity for us!





**Area for environmental monitoring
and scientific experiments**



Concluding Remarks

- Kill*Spill is developing several bio-based products for combating marine oil spills.
- By the end of the project, it is expected that at least 15 distinct products will be available for mitigation activities.
- The Oil Spill Response Industry is starting to accept bio-based technologies for marine oil spills.
- Many challenges remain to be addressed!

Thank you for your attention!



Kill•Spill - Integrated Biotechnological Solutions for Combating Marine Oil Spills

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www.killspill.eu

