Deliverable 1.4

Report on Patent and Literature Review on Bioremediation Agents

Kill●Spill

Integrated solutions for combating marine oil spills

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1 Introduction (FHNW)

Although conventional oil spill response techniques, i.e. physical/mechanical removal with booms, skimmers and adsorbent materials are usually applied as an emergency response, they rarely lead to a complete clean-up of the spill area. In addition, the use of chemicals, such as dispersants, is authorized in specific conditions only i.e. at a coastline depth of minimum 15m, and their potential toxicity on marine organisms have to be considered. Bioremediation is now seen as a key technology to clean up the polluted areas after first response actions. Indeed, a certain number of indigenous bacterial species are known hydrocarbon degraders; among them, strains belonging to the genera *Alcanivorax* [1] [2], *Cycloclasticus* [3], *Marinobacter* [4], *Neptunomonas* [5].
Nevertheless, intrinsic bioremediation is effective if the required nutrients are present in sufficient quantity in the water column, especially nitrogen, phosphorus and oxygen.
As a response to those potentially limiting factors, and in order to encourage intrinsic bioremediation, specific products have been designed based on two approaches: biostimulation and bioaugmentation. On one hand, biostimulation encourages the growth of indigenous hydrocarbon degraders by providing the limiting nutrients or electron acceptor (mostly N, P and O_2), on the other hand, bioaugmentation supports intrinsic bioremediation by dosing hydrocarbons degraders. Recently, more complex bioremediation agents combining both nutrients and microorganisms have been produced.

This report aims at providing an overview on existing, patented and/or approved biostimulation and bioaugmentation industrial products that are used during oil spills response action.

2 List of approved and patented biostimulation and bioaugmentation agents

The National Contingency Plan (NCP) published by the U.S. Environmental Protection Agency (EPA) on 18/11/ 2013 lists 26 registered bioremediation agents; 43 products previously listed were removed from the listing.
Among these 26 products, 7 are Nutrient Additives (NA), 15 Microbiological Cultures (MC), 1 is an Enzyme Additive (EA), 2 combine Nutrient Additives and Microbiological Cultures (MC/NA) and 1 combines Nutrient Additive, Microbiological Culture and Enzyme Additive (MC/EA/NA). All these products and their specification are listed in Table 1 and Table 2. In addition, 3 products that have been removed from the NCP listing (as of November 2013) are also presented in Table 3 as they were extensively used in the past. Finally 9 of them, patented in the U.S., are quoted in Table 4.

All the listed products have passed the minimum EPA or EU member state (e.g. CEDRE, FEPA, ROPME, Greek, Norwegian government agencies) acceptability tests.

2.1 NCP bioremediation agents by U.S. EPA

This list summarizes the products for which required data have been submitted to EPA as required by Subpart J of the National Oil and Hazardous Substances Pollution Contingency Plan, Section 300.915. (Source: 40 CFR §300.920 (e)). It does not mean that EPA approves, recommends, licenses, certifies or authorizes the use of the products on an oil discharge.

For bioremediation agents, toxicity tests are not required and provided only if submitted by the manufacturer; effectiveness test of the listed products is performed according to the standardized protocols "Swirling flask" described in Appendix C to 40 CFR part 300, p. 233-243.

"*The bioremediation agent effectiveness testing protocol is designed to determine a product's ability to biodegrade oil by quantifying changes in the oil composition resulting from biodegradation. The protocol tests for microbial activity and quantifies the disappearance of saturated hydrocarbons and polynuclear aromatic hydrocarbons (PAHs). The sample preparation procedure extracts the oil phase into dichloromethane (DCM), with a subsequent solvent exchange into hexane. To effectively accomplish the goals of the testing protocol, it is necessary to normalize the concentration of the various analytes in oil to a non-biodegradable marker, either* C_2*-*C_3*-phenanthrene,* C_2*-chrysene or hopane. The test method targets the relatively easy to degrade normal alkanes and the more resistant and toxic PAHs. It normalizes their concentrations to* C_2*-*C_3*-phenanthrene,* C_2*-chrysene, or* C_{30}*17α(H), 21β (H)-hopane on an oil weight basis (mg marker/kg oil, mg target analyte/kg oil). The analytical technique uses a high resolution gas chromatograph/mass spectrometer (GC/MS) […]. For quantitative analyses, the instrument is operated in the selective ion detection (SIM) mode at a scan rate greater than 1.5 scans per second to maximize the linear quantitative range and precision of the instrument. The sample preparation method does not exclude analysis of selected samples by GC/MS in the full scanning mode of operation to qualitatively assess changes in the oil not accounted for by the SIM approach. Performed concurrently with the chemical analysis described above is a microbiological analysis. The microbiological analysis is performed to determine and monitor the viability of the microbial cultures being studied. Under this procedure, microbial enumerations of hydrocarbon degraders are performed at each sampling event using a microtiter Most Probable Number (MPN) determination.*"

100mL of seawater enriched with 0.5 g of oil are inoculated with the tested bioremediation agent, prepared according to the manufacturer's instructions and incubated at 20°C on a rotary shaker over 28 days. Three samples are collected, at 0, 7 and 28 days of incubation.

2.1.1 Biostimulation agents

Table 1: Characteristics of Bioremediation Agents Listed on the NCP Product Schedule (as of December, 2013) - Biostimulation agents (NA)

	REMEDIADE ™	**VELITE**	**INIPOL EAP 22**	**JE1058BS**
General description	Liquid RemediAde™ is a blend of plant extracts and other organic substances designed to promote rapid *in situ* bacteria growth for bio-remediation of hydrocarbons	Powder	Oleophilic liquid Microemulsion containing urea as N-source, lauryl phosphate as P-source, 2-butoxy-1-ethanol as a surfactant, and oleic acid to give the mixture its hydrophobicity	hygroscopic powder JE1058BS contains biosurfactant and has an ability to stimulate the biodegradation of oil by indigenous microorganisms.
Active Ingredients	Nutrients	Nutrients	C:N:P ratio of 62:5:1. N-source: urea	Biosurfactant produced by *Gordonia* sp. strain JE-1058
Nutrient Composition	NP*	NP	Microemulsion	N/A
How does it change the behaviour	NP	Immediate protection to flora and fauna; Changes oil from a liquid to a non-sticking solid	Softens the oil; can cause oil to lift off substrates	Disperses oil
NCP Listed - Number	NA B-66	NA B-55	NA (No longer manufactured) B-10	NA B-58
Application Rate	Soil - 1gal per $30yd^3$ (20 000 TPH) Water - 1gal per 30 ft^2	1:3 product to oil	1:10 product to oil	1:10 product to oil

	REMEDIADE ™	VELITE	INIPOL EAP 22	JE1058BS
Application Method	Spray from boats, aircraft, fire eductor systems on boats, helicopter buckets, hand-held or backpack sprayers, or from hoses attached to small pumps, water trucks and aerial spray, including typical spreading systems.	On water, spread over contaminated area at 1 to 3 ratio. On soil, blend to depth equivalent to contamination level.	Spray product neat onto oiled surfaces	Use conventional spraying equipment, for example a powder mist duster attached with boom type multi-hole head.
Temperature Limitation	N/A	32 to 135°F; optimal is 77-86oF for microbial activity	>52°F (11.1 °C)	NP
EPA Efficacy Test**	Alkanes: 99%, Aromatics: 94%, Gravimetric weight decrease: 36%	Alkanes: 43% , Aromatics: 32%, Gravimetric weight decrease: 25%	Alkanes: 94%, Aromatics: 23%, Gravimetric weight decrease: 50%	Alkanes: 93%, Aromatics: 39%, Gravimetric weight decrease: 82%
Use in Fresh/Salt Water?	Yes	Yes	Yes	Yes
Other information	NP	Optimum pH of 6-8	NP	NP
Application Assistance Information	JDMV Holdings, LP (281) 558-3433 Fax: (281) 870-1200 Email:msuttle@jdmvholdings.com Web Site:www.jdmvholdingslp.com (Mr. Michael Suttle)	Land and Sea Restoration LLC (210) 650-5556 Fax: (210) 650-5567 (Mr. T. Shawn Parker)	CECA S.A.- ARKEMA GROUP (Paris, France) 011.33.1.47.96.92.91 Fax: 011.33.1.47.96.92.33 Email:serge.kuchto@ceca.fr (Mr. Serge Kuchto)	Japan Energy Corporation (81) 48-433-2191 Fax: (81) 48-444-3223 Email:saeki@j-energy.co.jp (Hisashi Saeki)

	S-200	SHAMANTRA GREEN	VB591™, VB997™, BINUTRIX®
General description	Light amber liquid	Liquid Shamantra is a natural product made using humic acid.	Powder VB591™, VB997™, and BINUTRIX® is a powder Patented, partial encapsulated oleophilic (oil-loving) nutrient
Active Ingredients	Nutrients	Nutrients	Oleophilic compounds
Nutrient Composition	Contains: 7.9% N and 0.6% P. Microemulsion of saturated solution of urea (nitrogen source) in oleic acid as a carrier, an oleophilic phosphate ester (phosphorus source and surfactant), and a viscosity reducer	NP	NP
How does it change the oil behaviour?	Bioremediation accelerator	Like a synthetic sorbent, encapsulates the oil through a chemical and physical process. Once encapsulated, the spilled oil is captured within the pore matrix contained within the carbon structure.	sticks to oil and micronutrients are always available on a time-release basis in useful concentrations for microbes
NCP Listed - Number	NA B-56	NA B-68	NA B-42
Application Rate	1:10 product to oil; 1 lb/sq. yard of surface area	1:5–1:30 product to oil	5 - 15 pounds of product per barrel of spilled oil Do not exceed 250 pounds/acre

	S-200	SHAMANTRA GREEN	VB591™, VB997™, BINUTRIX®
Application Method	Applied with pressurized sprayers or back pack sprayers	Apply with hand-held sprayers or sand-blasting equipment, fire hoses or pneumatic spreading equipment. Can be used in booms, sacks, and pillows.	Apply with hand held pressurized dust blowers or boat mounted dust blowers. Follow up application recommended after 48 hours.
Temperature Limitation	50-120°F; optimal is 86°F	40 to 140°F	None
EPA Efficacy Test**	Alkanes: 98% Aromatics: 10% Gravimetric weight decrease:28%	Alkanes: 99.7% Aromatics: 95% Gravimetric weight decrease: 25%	Alkanes: 97% Aromatics: 73% Gravimetric weight decrease: 18%
Use in Fresh/Salt Water	Yes	Yes	Yes
Other information	pH 4.5-10	NP	NP
Application Assistance Information	International Environmental Products, LLC (610) 520-7665 Fax: (610) 520-7663 Email:info@iepusa.com (Mr. Jim Lynn)	Molecular Mediation LLC (302) 861-0400 Fax: (302) 861-0410 Email:info@molecularmediation.co m (Mr. Ronen Hazarika, Director)	BioNutraTech Inc. (281) 354-5900 Mobile: (713) 301-0254 Fax: (281) 354-1997 Email: shruza@bionutratech.com Web Site: http://www.bionutratech.com Mobile: (713) 301-0254 (Ms. Sandra Hruza)

*NP: Information non-provided

**Reports % of reduction of components over a 28 days period

2.1.2 Bioaugmentation agents

Table 2: Characteristics of Bioremediation Agents Listed on the NCP Product Schedule (as of December, 2013) - Bioaugmentation agents (MC)

	BET BIOPETRO	Oil Spill Eater II	WMI-2000	Oppenheimer Formula
General Description	Powder Powder containing granules of bacterial product formulated to provide performance in the bioremediation of heavy refined and crude hydrocarbon contaminants in both soil and water environments.	Amber liquid, ferment smell	Tan powder, with yeast odor that contains microbial cultures specifically selected for remediation of petroleum products and other contaminants	Powder
Active Ingredients	NP	Nutrients, Enzymes, and Surfactants	Microbes	Microbes (Highly concentrated (10^{11} cells/g); oil absorbing clay mixed with hydrophobic, aerobic and microaerophilic Archaeobacteri a
Nutrient Composition	Contact BET for specific nutrient dosing requirements. Contact BET for specific nutrient dosing requirements.	Nutrient enhancement product with nitrogen, phosphorus, and readily available carbon and vitamins	None; product requires nutrient supplements	NP
How does it change the oil behavior?	NP	OSE II breaks down the adhesion properties of hydrocarbons and causes hydrocarbons to float. complete bioremediation occurs in 3-30 days	No immediate change	NP

	BET BIOPETRO	Oil Spill Eater II	WMI-2000	Oppenheimer Formula
NCP Listed - Number	MC B-48	EA B-53	MC B-19	Yes
Application Rate	Varies. Contact BET for specific technical advice	1 gal product/50 gal crude oil, as a 2% solution; 1 gal product /100 gal light oil at 1% solution	1.4 lb/1000ft^2 inoculation concentration of 5-9 billion spores per gram	10 lbs per acre surface on open water; 100 lbs per acre on estuaries.
Application Method	Contact BET for specific technical advice.	Use surface spray apparatus, such as small hand held tanks, back pack, large mixing tanks with mechanical pumping devices, vessels with booms for spraying wide paths, or spray devices on airplanes or helicopters. OSE II can be applied by eductor systems from vessels, fire trucks, etc. Set the eductor system to 2% and apply 1 gallon of mixed OSE II to each spilled gallon of hydrocarbon.	Activate culture in water for 2 h, then spray or inject, mix in nutrients, and till/aerate	Spray dry powder directly or as a water mix with nutrients
Temperature Limitations	45-100^{o}F	28°F to 120°F; bioremediation slows below 40°F	35-100°F, optimal at 45-90°F	32-140°F; optimal is 82°F
EPA Efficacy Test	Alkanes: 99% Aromatics: 67% Gravimetric weight decrease: 30%	Alkanes: 90% Aromatics: 90% Gravimetric weight decrease: 85%	Alkanes: 60% Aromatics: 33% Gravimetric weight decrease: 44%	Alkanes: 89% Aromatics: 38% Gravimetric weight decrease: 10%
Use in Fresh/Salt Water?	Yes	Yes	Yes	Yes /to 24% salt, optimal is 0.5-3.5%

	BET BIOPETRO	Oil Spill Eater II	WMI-2000	Oppenheimer Formula
Other Information	Product works at pH 5.5-8.5 and dissolved oxygen level of 3 to 5 mg/l.	Optimum pH: 3.5 - 8.0	Optimum pH 7.0-8.0	pH of 5.5 – 10
Application Assistance Information*	BioEnviroTech (Tomball, Texas) 281-351-5594 800-758-3253	OSEI Corp., Dallas, Texas (972) 669-3390 Email:oseicorp@msn.com www.osei.us	WMI International, Inc. (Houston, Texas) (713) 956-4001 Fax: (713) 956-7305 Email:wmiintlinc@yahoo.com	Oppenheimer Biotechnology, Inc. (512) 474-1016 Fax: (512) 681-0367 Email:jen.neve@obio.com Web Site:www.obio.com (Ms. Jen Neve)

	Micro-Blaze	SPILLREMED (MARINE)	BIOREM-2000 OIL DIGESTER	Step One
General Description	Concentrated, white liquid; perfumed; when mixing, add product to water or solution will foam. Micro-Blaze is a liquid formulation of several microbial strains (Bacillus spores), surfactants, and nutrients designed to metabolize organics and hydrocarbons in soil and water	Liquid	Liquid Products are a mixture of 12 naturally-occurring microbes and several types of enzymes coupled with a nutrient package that accelerates the digestion process.	Liquid Components of the product STEP ONE (BC101) and STEP ONE (MSE2.5) are only sold together. B&S Research will make application recommendations based on contamination and ppm of contamination at time of purchase order and employ specified application methods (spray, plowing, agitation, etc.) appropriate for a particular situation.
Active Ingredients	Nutrients, Microbes, and Surfactants	Microbes	Microbes, Nutrients and Enzymes	Microbes, Nutrients

	Micro-Blaze	SPILLREMED (MARINE)	BIOREM-2000 OIL DIGESTER	Step One
Nutrient Composition	NP	NP	NP	Phosphoric acid
How does it change the oil behavior?	Surfactant cleaves oil droplets into molecules small enough for microbes to effectively digest.	NP	NP	NP Starts digesting oil particles immediately
NCP Listed - Number	MC B-41	MC B-57	MC B-63	MC B-43
Application Rate	Spills-1:10, product to oil, as 3-6% solution; Soil- 1 gal per 10 yd^3 at 3-6% solution	1:10, product to oil Rate of application is one gallon per 10 minutes.	Diluted with five (5) parts water and use 1 gal/ 1.500 ft^2 contaminated soil. apply directly undiluted onto the surface for oil spills in water	Provided by vendor at time of purchase
Application Method	Mix in hand-held sprayers; educt into spray systems; pour concentrate directly on oil**;** in all cases, use broom or pressurized water stream to agitate the solution; then rinse clean with water and vacuum up liquids; **do not** discharge untreated solution to water bodies.	SPILLREMED (MARINE)® is a ready to use liquid product. It can be sprayed undiluted over the spill in open water conditions.	Spray from boats, aircraft, fire eductor systems on boats, helicopter buckets, hand-held or backpack sprayers, or from hoses attached to small pumps, water trucks and aerial spray, including typical spreading systems.	Provided by vendor at time of purchase
Temperature Limitations	35ºF - 180ºF	optimal at 40°F – 90°F	35-125°F	50-135°F; optimal is 70-90°F
EPA Efficacy Test	Alkanes: 94% Aromatics: 48% Gravimetric weight decrease: 12%	Alkanes: 97% Aromatics: 47% Gravimetric weight decrease: 85%	Alkanes: 99.8% Aromatics: 71% Gravimetric weight decrease: 67%	Alkanes: 44% Aromatics: 55% Gravimetric weight decrease: 51%

	Micro-Blaze	SPILLREMED (MARINE)	BIOREM-2000 OIL DIGESTER	Step One
Use in Fresh/Salt Water?	Can be mixed with any fresh, brackish or brine. However, brine reduces the effectiveness by 10%.	SPILLREMED (MARINE)® is a marine salt formulation and is not effective in fresh water conditions. Optimal effectiveness is in the salinity range of 10 to 35 ppt.	Yes	Fresh or salt (ocean < 110 ppm)
Other Information	Optimum pH of 4 - 11.5	pH 6.5 – 6.9	Optimum pH: 3 - 11.5	Optimal pH 5.0-9.0
Application Assistance Information *	Verde Environmental, Inc. 713-691-6468 800-626-6598 Email: bscogin@micro-blaze.com (Mr. William Scogin) www.micro-blaze.com	Sarva Bio Remed, LLC (717) 779-0040 (877) 717-2782 Fax: (419) 710-5831 Email: sales@sarvabioremed.com Web Site: http://sarvabioremed.com	Clift Industries, Inc. (800) 996-9901 (704) 752-0031 Fax: (704) 544-2532 Email:matt@cliftindustries.com (Mr. Matt Barnhill)	B & S Research, Inc. (218) 984-3757 Fax: (218) 984-3212 Email: farmforprofit@frontier.com (Mr. H.W. Lashmett)

	SOIL RX	BioWorld BHTP	DRYLET™ MB BIOREMEDIATION	ERGOFIT MICROMIX AQUA
General Description	Liquid concentrated liquid formulation of activated humic acid, highly concentrated hydrocarbon-oxidizing bacteria, and a readily biodegradable natural amino acid complex consisting of a nutrient-rich extract with a broad-spectrum package of identifiable amino acids, surfactants, and other proteins	BioWorld BHTP is a two part product 1) Bioremediation Enhancer liquid and 2) Hydrocarbon Digesting Microbes in a dry form	Dried powder MB Bioremediation™ is a patented dry product (**Patent # US 8,409,822 B2)** that provides millions of nanobioreactors that contain billions of live microorganisms that consume oil, gasoline and diesel fuel along with other petroleum-based products.	Liquid with fermentation and yeast smell. It is an organic fluid oil remediator utilizing natural ingredients derived from glucides and essential amino acids which form powerful decomposing agents
Active Ingredients	Microbes, Nutrients and Surfactants	Microbes and Surfactants	Microbes, Nutrients and Surfactants	Microbes, Nutrients and Enzymes
Nutrient Composition	NP	NP	NP > (10^{11}) cfu/g microbes	NP
How does it change the oil behavior?	NP	NP	NP	Breaks down the adhesive properties of the hydrocarbons, preventing oils from attaching to animals, vegetation, rocks, and earth.
NCP Listed - Number	MC/NA B-61	MC B-59	MC B-64	MC/EA/NA B-67
Application Rate	Soil - 1 gal (10:1 diluted product) per 1 yd^3. Water-3 gal (10:1 diluted product) per 1000 ft^2	40 gal Bioremediation Enhancer liquid and 4 lbs Hydrocarbon Microbes per acre	soil - 0.4 pounds per 1 yd^3 Water-50 pounds per acre	As a rule, 2 kg ERGOFIT MICROMIX AQUA is required per metric ton of oil spilled. Dilution varies depending on oil thickness or simple sheen floating on water surface.

	SOIL RX	BioWorld BHTP	DRYLET™ MB BIOREMEDIATION	ERGOFIT MICROMIX AQUA
Application Method	The product can be sprayed after dilution using standard spray application equipment, including but not limited to hand sprayers, mechanical sprayers, water trucks, fire or emergency response equipment, pressure washers, etc. Mix or saturate concentrate/water mixture with contaminated soils thoroughly for maximum performance.	Prior to use the microbes are rehydrated in 100ºF water for 10 to 300 minutes. Example for small volume applications – best results are obtained by applying Bioremediation Enhancer first followed by the rehydrated Hydrocarbon Microbe solution in the same location. Example for large volume applications – the two parts of BioWorld BHTP can be mixed with additional water and applied together to spray over the perimeter and over the top of the spill. Example for soil– evenly apply to soils and mix with till or discing equipment.	DRYLET™ MB BIOREMEDIATION may be applied by the usual methods of aerial or manual broadcast spreading.	Spray from boats, aircraft, fire eductor systems on boats, helicopter buckets, hand-held or backpack sprayers, or from hoses attached to small pumps, water trucks and aerial spray, including typical spreading systems.
Temperature Limitations	34-125°F; optimal is 42-100°F	33-120°F; optimal is 80-100°F	35-180°F; optimal is 85-110°F	32-104°F; optimal is 57-97°F
EPA Efficacy Test	Alkanes: 50% Aromatics: 74% Gravimetric weight decrease: 30%	Alkanes: 97% Aromatics: 88% Gravimetric weight decrease: 96%	Alkanes: 99% Aromatics: 85% Gravimetric weight decrease: 85%	Alkanes: 99% Aromatics: 82% Gravimetric weight decrease: 46%

	SOIL RX	BioWorld BHTP	DRYLET™ MB BIOREMEDIATION	ERGOFIT MICROMIX AQUA
Use in Fresh/Salt Water?	Can be mixed with any fresh, brackish or brine. However, brine reduces performance or increase overall remediation time.	Yes	Salinity: 0.5-4.0 (above 4.5 reduces effectiveness by half)	Fresh water to salt water up to 4.9%
Other Information	Optimum pH of 4 – 8	pH of 3 – 10	pH 4 – 11.5	Optimum pH: 4.5 - 8.5
Application Assistance Information*	3 Tier Technologies LLC (877) 226-7498 Fax: (877) 570-0072 Email: dburdette@3tiertech.com Website: http://www.3tiertech.com	BioWorld Products International (559) 651-2042 Fax: (559) 651-9041 Email:mail@bioworldusa.com Website: www.bioworldusa.com	DryLet Technologies, Inc. (972) 347-2341 Fax: (972) 347-2816 Email: sellis@drylet.com (Mr. Steve Ellis)	ERGOFIT USA LLC (302) 235-3085 Fax: (801) 846-8043 Email:info@micromix-usa.com Website: www.micromix-usa.com

	MUNOX SR	PRO-ACT	Pristine Sea II	SUMP SAFE BIO-RECLAIM
General Description	Powder or liquid bacterial mixture MUNOX SR® is a consortium of robust organisms that tolerate the natural salinity of seawater up to approximately 5% and water temperatures below 100°F blend of naturally occurring stabilized *Pseudomonas* strains of bacteria with exceptionally high degradation capabilities	Liquid PRO-ACT consists of two parts: liquid microbes and activator (i.e., nutrient additive).	Powder or liquid bacterial mixture	Dry powder

	MUNOX SR	PRO-ACT	Pristine Sea II	SUMP SAFE BIO-RECLAIM
Active Ingredients	Microbes, Nutrients	Microbes, Nutrients	Microbes, Nutrients	Microbes, Nutrients and Surfactants
Nutrient Composition	Soy Peptone – 2.2%, Yeast Extract – 2.2%, Dextrose – 0.2%, Sodium Nitrate – 0.2%, Di-potassium Phosphate – 0.2%, Sodium Thiosulfate – 0.01% microbial density of $1x10^{8}$cfu/mL	NP	NP	NP sodium nitrite A combination of 70 percent live active yeast mixed with 30 percent fine ground corn cob is used as a nutrient
How does it change the oil behavior?	NP	NP	NP	NP
NCP Listed - Number	MC B-60	MC/NA B-62	MC (no longer manufactured) B-54	MC B-69
Application Rate	Water-55 gals per 22000 gals of impacted water soil – 275 gals per acre	1:10 liquid microbe/water ratio activator portion is applied in such way to maintain nutrient levels at the optimal concentration for microbial action. 1 gal undiluted liquid microbes is good for up to 5 gals of spilled oil	Varies. Contact vendor for assistance.	6 gal (25:1 dilution rate with water) per 1 m^3
Application Method	Apply using in situ, hand sprayer, pump, pressure washer, etc.	The product can be applied with a sprayer, either pump or pneumatic.	"Soak at a rate of 1kg to 4L influent waste and 4L tap-water, or add directly to your system."	Apply by a drum pump with sprayer or other usual spraying methods. Mix with an auger based soil homogenizer.

	MUNOX SR	PRO-ACT	Pristine Sea II	SUMP SAFE BIO-RECLAIM
Temperature Limitations	optimal is 4-35°C	15-45°C	40°F to 120°F; bioremediation slows below 50ºF	40-120°F; optimal is 70-90°F
EPA Efficacy Test	Alkanes: 95% Aromatics: 75% Gravimetric weight decrease: 14%	Alkanes: 99.8% Aromatics: 94% Gravimetric weight decrease: 65%	Alkanes: 96% (20 d tests) Aromatics: 90% (20 d tests) Gravimetric weight decrease: NP	Alkanes: 47% Aromatics: 49% Gravimetric weight decrease: 3%
Use in Fresh/Salt water?	Yes	Yes	Yes	Yes
Other Information	Optimal pH 6.0-8.0	pH of 6 – 9	NP	pH of 4.8 – 9
Application Assistance Information*	Osprey Biotechnics (941) 351-2700 Fax: (941) 351-0026 Email: ldanielson@ospreybiotechnics.com (Lauren Danielson, President and CEO) Email: kbrattan@ospreybiotechnics.com (Ms. Kate Brattan, Director of Technical Sales)	Pro-Act Microbial, Inc. (401) 245-7004 Fax: (401) 633-6270 Email:bd@proactbiotech.com Web Site:www.proactbiotech.com (Mr. William Donohue)	Fluid Tech, Inc. (702) 871-1884 Fax: (702) 871-3269 (Mr. Stan True)	Teamwork Distributing (780) 968-5367 Mobile: (780) 238-2741 Fax: (780) 958-9070 Email:marlin@xplornet.com Email:marlin@teamwrk.ca (Mr. Marlin Rudolph) (Mr. Michael Suttle)

	System E.T. 20	WASTE AWAY	DUALZORB
General Description	Brown powder SYSTEM E.T. 20 is an oleophilic, non-water soluble nutrient, which releases nitrogen and phosphorus enzymatically	Liquid	dehydrated product
Active Ingredients	Microbes, Nutrients	Microbes, Enzymes	Microbiological Culture
Nutrient Composition	NP	NP A broad and diverse population of live naturally occurring beneficial water/soil microorganisms including: *Bacillus* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Rhodococcus* sp., *Chlorobium* sp., *Cyanbacteria* sp., and *Actinomycetes* sp	NP
How does it change the oil behavior?	NP	NP	absorbs oil
NCP Listed - Number	MC B-45	MC B-70	MC B-65
Application Rate	Varies	Water- 1:10-1:30 product to impacted water soil – 1 gal/10 yd^3	Water- 4 pounds per gallon of oil soil – 50 pounds of hydrated DUALZORB (1:1 with water) per yd^3

	System E.T. 20	WASTE AWAY	DUALZORB
Application Method	Spray reconstituted organisms, broadcast nutrients, mix into affected soils	Apply using liquid delivery system (i.e., boom sprayers, spray pumps).	The product can be applied by hand, mechanical spreaders, portable mixer or blown onto a surface using an air conveyor
Temperature Limitations	41-95°F ; optimal is 39-95°F	40-125°F	45-105°F ; optimal is 60-100°F
EPA Efficacy Test (Reports % reduction of components over a 28 day period)	Alkanes: 99% Aromatics: 77% Gravimetric weight decrease: 18%	Alkanes: 99% Aromatics: 61% Gravimetric weight decrease: 50%	Alkanes: 99% Aromatics: 92% Gravimetric weight decrease: 30%
Use in Fresh/salt Water?	Yes	Salinity up to 110 ppt	Yes
Other Information	pH: 6.5 - 8.5	Optimal pH 4.0-11.0	pH of 5.5 – 9; Optimal 6.5-7.5
Application Assistance Information*	Environmental Restoration Services Email:ERS.BTI@gmail.com (619) 253-0664 (Mr. John Chase) (760) 746-5145 Fax: (760) 746-2034 (Mr. Jack Roberts)	Chem–X International, LLC (aka, CXI) (972) 471-7775 Fax: (972) 393-2011 Email:david@cxinternational.com Website:www.cxinternational.com (Mr. David Howard)	LBI Renewable (307) 684-9340 Fax: (307) 684-5815 Email:info@lbirenewable.com (Mr. Dale Lee)

Table 3: Characteristics of 3 bioaugmentation agents removed from the U.S. EPA NCP listing but extensively used in the past

	PRP	Vita-Bugg	BioGee HC
General Description	Granular, yellow powder (0.25 to 500 micrometers) with a wax coating that makes it float, oleophilic, and hydrophobic	Powder	Liquid
Active Ingredients	Enzymes	Nutrients	Microbes
Nutrient Composition	Enzyme names: oxidoreductases, transferases, hydrolases, lyases, isomerases, and lipases	Oleophilic	NP
How does it change the oil behavior?	Immediate change – binds the oil. Does not allow the oil to sink or emulsify. Reduces stickiness	No immediate change	No immediate change
NCP Listed	Removed B-29		Removed B-35
Application Rate	1:2 product to oil; 50 lb/1,000 ft2 of contaminated surface, 1 ton of PRP covers 40,000 ft2 to a depth of ¼ inch	5-15 lb/bbl oil; 6 lb/1,000ft2	1 gal/yd3 soil; 0.25 gal/1,000 ft2 water surface
Application Method	Apply dry powder to small spills; for large spills and in open waters, mix or educt with water and spray affected area.	Use conventional powder spraying equipment to apply product; additional applications at 48-72 h as needed	Spray
Temperature Limitations	Wax is sensitive to heat at 85°F, melts at 120°F	None	34-140°F; optimal is 83°F
EPA Efficacy Test	Alkanes: 12% Aromatics: 3% Gravimetric weight decrease: 1%	Alkanes: 97% Aromatics: 73% Gravimetric weight decrease: 18%	Alkanes: NP Aromatics: NP Gravimetric weight decrease: 13%

	PRP	Vita-Bugg	BioGee HC
Use in Fresh/Salt Water?	Yes	Yes	Yes, salinity may have slight effect
Other Information	NP	NP	NP
Application Assistance Information*	Petro Rem, Inc. 412-279-9745 http://www.unireminc.com/	BioNutraTech, Inc. 281-894-7371 http://www.bionutratech.com/	RMC Bioremediation 318-219-3929 Fax: 318-219-3920

2.2 Patented bioremediation agents

Table 4: Bioremediation agents with identified patent(s)

Name	Country	Patent No.	Publication date	Name
DRYLET™ MB BIOREMEDIATION	USA	US 8409822 B2	2013	Composition of porous silica granules for delivering microorganisms
		EP 2493334 A1	2012	Composition and method for delivery of substances in a dry mode
		US 20110082040 A1	2011	Composition and method for delivery of substances in a dry mode
		WO 2011044145 A1	2011	Composition and method for delivery of substances in a dry mode
		CA 2784273 A1	2011	Composition and method for delivery of substances in a dry mode
INIPOL EAP 22	USA	EP 0721499 B1	1998	Method for the rehabilitation of soil contaminated by hydrocarbons and other biodegradable substances
		US 5707857	1998	Process for the rehabilitation of soils contaminated by hydrocarbons and other biodegradable substances
		WO 1995006715 A1	1995	Method for the rehabilitation of soil contaminated by hydrocarbons and other biodegradable substances
		CA 1156574 (A1)	1983	Micro-emulsified nutrients
		US 4460692 A	1983	Microemulsion of nutrient substances
		US 4401762 A	1983	Process of culturing microorganisms using a microemulsion
		DE 3137020 (C2)	1983	Nutrient compositions for microorganism culture
		DE 3137020 (A1)	1982	Nutrient compositions for microorganism culture

Name	Country	Patent No.	Publication date	Name
MICRO-BLAZE®	USA	US 6527970 B1	2003	Microbiological aqueous film forming foam (AFFF) fire-fighting formulation
		US 6225362 B1	2001	Microbiological fire-fighting formulation
		US 5942552 A	1999	Microbiological fire-fighting formulation
		US 5658961 A	1997	Microbiological fire-fighting formulation
MUNOX SR®	USA	US6245552 B1	2001	Method for waste degradation
OIL SPILL EATER II	USA	US 5160525 A	1992	Bioremediation enzymatic composition
PRO-ACT (also OILCLEAN w/ACTIVATOR)	USA	US 7267766	2007	Method and apparatus for aerating a surface layer in a stratified liquid body
		US 20060027496 A1	2006	Microbial manure treatment system
		WO 2006007363 A2	2006	Microbial manure treatment system
		US 20050087894 A1	2005	Method and apparatus for aerating a surface layer in a stratified liquid body
S-200 (also BILGE CLEAR or OIL GONE EASY or S-200C or S-200 OIL CLEAN or S-200 OIL GONE or SHEEBCLEAN or Z-11)	USA	US 7005133 B2	2006	Composition and process for treating pollution

Name	Country	Patent No.	Publication date	Name
STEP ONE (also B&S INDUSTRIAL)	USA	CA 2196284 C	1999	Method and Apparatus for Remediating Contaminated Material
		US 5772782 A	1998	Method for remediating contaminated material
		US 5709234 A	1998	Method and apparatus for remediating contaminated material
		US 5589004 A	1996	Method for remediating contaminated material
		WO 1996004083 A1	1996	Method and apparatus for remediating contaminated material
		US 5492139 A	1996	Method and apparatus for remediating contaminated material
		CA 2196284 A1	1996	Method and Apparatus for Remediating Contaminated Material
VB591TM, VB997TM, BINUTRIX®	USA	CN 1107655 C	2003	Coating layer for enhancing bioremediation
		DE 69423249 T2	2000	Composition for enhanced bioremediation of oil
		EP 0809612 B1	2000	Method and composition for enhanced bioremediation of petroleum
		WO 1999046210 A1	1999	Method and composition for enhanced bioremediation of oil
		US 5954868 A	1999	Method and composition for enhanced bioremediation of oil
		US 5725885 A	1998	Composition for enhanced bioremediation of oil
		EP 0809612 A1	1997	Method and composition for enhanced bioremediation of petroleum
		CN 1121703 A	1996	Method and composition for enhanced bioremediation of petroleum
		US 5443845 A	1995	Composition for enhanced bioremediation of petroleum
		WO 1994024057 A1	1994	Method and composition for enhanced bioremediation of petroleum

3 Literature review on bioremediation agents

This section refers to experimental data related to the application of biostimulation and/or bioaugmentation agents for the treatment of oil spills, either at laboratory scale or in the field, of the commercial products cited in the previous section.

3.1 Biostimulation

3.1.1 Water-soluble Inorganic Nutrients

Biostimulation through the addition of water soluble mostly inorganic nutrients-fertilizers was the first, direct and economically effective strategy to deal with oil contamination in the marine environment. Commercially available inorganic fertilizers which include mineral nutrient salts (e.g. KNO_3, $NaNO_3$, NH_4NO_3, K_2HPO_4, $MgNH_4PO_4$, $Ca(H_2PO_4)_2$, $Na_5P_3O_{10}$) have been extensively used in treatments in the lab but also in the field which were effectively applied at Nova Scotia, Canada and Delaware Bay, US with contradictory results sometimes. Among other parameters the form of N in seawater effects the rate of bioremediation and as was reported [6][7] the addition of NO_3-N was more successful than that of NH_4-N.
Their application is simple even in the field using conventional spraying equipment for liquids and powders, and proved to be effective in enhancing oil degradation [8][9][10] when environmental conditions favored their bioavailability and microbial exploitation. Usually controlling their release rate is extremely difficult since these type of fertilizers are water soluble and can easily be washed away by wave and tide action making it difficult to sustain enhanced biodegradation in rough marine environments like medium to high energy shorelines.

3.1.2 Slow-Release Fertilizers

Instead of using water soluble fertilizers which are more difficult to control and are more susceptible to environmental conditions, slow release fertilizers can be a continuous source of nutrients to oil contaminated areas. Slow release fertilizers can be found normally in solid forms that consist of either relatively insoluble nutrients or water-soluble nutrients coated with hydrophobic materials such as paraffin or vegetable oils [40]. Slow release fertilizers have been widely used in oil bioremediation studies and applications after their first documented application in the Exxon Valdez accident.
The most well-known slow-release fertilizer, Customblen which is a vegetable oil coated calcium phosphate, ammonium phosphate, and ammonium nitrate (N:P:K ratio 28-8-0) - Sierra Chemical Co. performed well on some of the shorelines of Prince William Sound the Exxon Valdez accident, particularly in combination with an oleophilic fertilizer [9][11].
Another granulated slow release fertilizer Osmocote that contains water-soluble N–P–K at concentrations of 18, 4.8, and 8.3% (w/w), respectively, coated with organic resin (Scotts Miracle-Gro Company, Marysville, Ohio) was effectively used to bioremediate an oil contaminated sediment in a mesocosm study [12]. Osmocote 14-14-14 (granules containing 14 % N, 6.1 % P and 11.6 % K; (w/w) Scotts)
On the contrary Nitrophoska Supreme (NPK) which is a granular fertilizer (20% Total Nitrogen (N), 13% ammonia (N), 7%nitrate, 5% Phosphorus (P_2O_5) 10% Potassium (K_2O) 2% Magnesium (MgO), 10% S, 0.3% (Fe) 0.1% (Zn)(w/w)) (Compo, Germany) was not effective when applied at oil contaminated sand, rocks and granite tiles on the beach of Sorrizo (A Coruna, NW Spain) from the Prestige accident [13].
In another study the influence of a slow-release fertilizer Max-Bac (Grace-Sierra International) on the biodegradation rate of an Arabian Light crude oil contaminating an estuarine environment in the bay of Brest, France was evaluated. However there was no significant difference in biodegradation rates observed between fertilised and non-fertilised plots, which was attributed to the high background

level of N and P in the site under study. Max Bac CB24-89 (Grace-Sierra Horticultural Products, Milpitas, CA) is a granular slow-release fertilizer (10% NO_3-N, 12% NH_4-N and 13% P_2O_5 (w/w)) containing NH_4NO_3, $Ca(H_2(PO_4))_2$, $(NH_4)_2HPO_4$, and $(NH_2)_2CO$.
Miracle Gro (30-6-6) has been used to remediate crude oil contaminated marsh grassland in the Seal Beach National Wildlife Refuge. The results of a 35-day monitoring effort showed no differences between the treated and untreated oil plots. According to the manufacturer's description, this slow-release fertilizer contains 30% nitrate, 6% ammonium and 6% phosphorus pentoxide (w/w).
Another type of slow release fertilizers includes isobutyraldehyde diurea (IBDU). IBDU is synthesized by condensing isobutyraldehyde with urea. Soluble phosphorous can also be incorporated in the fertilizer during manufacturing. Many varieties of IBDU fertilizers are available, with release periods ranging from a few weeks to three years, depending on physical form and size. Sheets of briquettes (attached by webbing) can be commercially purchased for application on steep grades. Two commercial IBDU fertilizers that have been used in the field are Super IB (Mitsubishi Chemicals) contains 35% (w/w) nitrogen as isobutylidene diurea (IBDU) and Woodace (Vigoro Industries) briquettes (N–P–K at 14:3:3) containing 14 percent nitrogen (IBDU).
Super IB along with Linstar 30 (Mitsubishi Chemicals) which is a slow-release solid granular phosphorous fertilizer that contains 10% (w/w) phosphorous as calcium and/or magnesium phosphate salts, were used in biodegradation experiment in sand and cobblestone beaches covered with residual oil from Nakhodka oil spill at the northern coastline of Hyogo Prefecture (Japan) [14][15]. Woodace was applied in field trials conducted following the *Exxon Valdez* spill.

It should be noted that ingredients concentrations of these commercial products may differ depending on their production period and before use the manufacturing company should be contacted for further details.

Several recent studies have shown that a slow release nutrient (Max Bac, a product similar to Customblen) failed to demonstrate enhancement of oil degradation because the nutrient release rate was too slow to affect oil biodegradation [16][17].
So despite the fact that washout problems can be overcome by using slow release fertilizers, the challenge that still remains is to control their release rates so that optimal nutrient concentrations can be maintained over longer periods of time in the marine environment. Fast release rates do not provide a long term source of nutrients whereas very slow release rates are insufficient to enhance biodegradation rates, thus optimizing the nutrients release rates could be a promising bioremediation strategy for stimulating oil biodegradation. Moreover, direct comparison of these products is to be carefully considered as not only the type and composition of the slow release fertilizer plays a role but also other parameters such as the type of oil. Indeed, heavy oil may be less readily affected by bioremediation.

3.1.3 Oleophilic Biostimulants

Oleophilic biostimulants could be a strong alternate to overcome the problem of quick dilution and wash out of water-soluble nutrients. The application of N and P sources in oleophilic form is considered to be the most effective nutrient application method, since oleophilic additives remain dissolved in the oil phase and thus are available at the oil-water or oil-sediment interface where they enhance bacterial growth and metabolism.
The most famous oleophilic fertilizer is Inipol EAP22 (CECA S.A.- ARKEMA GROUP, Paris, France), a microemulsion containing urea as N-source, lauryl phosphate as P-source, 2-butoxy-1-ethanol as a surfactant, and oleic acid to give to the mixture its hydrophobicity (C:N:P ratio of 62:5:1). This fertilizer has been subjected to extensive studies under various shoreline conditions and was

successfully used in oil bioremediation on the shorelines of Prince William Sound, Alaska of Exxon Valdez oil spill and in Amoco Cadiz spill on the Brittany coast.
Another famous fertilizer which has been extensively applied and studied at the Prestige heavy fuel oil spill on the Cantabrian coast (north Spain) is S200 (IEP Europe, Madrid, Spain) which differs from Inipol EAP22 only in the formulation of the surfactant component [18][19].
S200 (7.9% N and 0.6% P) is a microemulsion composed of a saturated solution of urea (nitrogen source) in oleic acid as a carrier, and an oleophilic phosphate ester (phosphorus source and surfactant), and a viscosity reducer, identical to INIPOL EAP22, 2-(2-butoxyethoxy)ethanol.

Furthermore some other oleophilic fertilizers include polymerized urea and formaldehyde, and organic fertilizers derived from natural products such as fishmeal and meat meal or from natural byproducts such as guano fertilizer and manure compost; however their composition differs depending on the manufacturing company.
As a substitute to the chemical surfactants present in most commercial oleophilic biostimulant formulations, which are somewhat toxic, biosurfactants can be employed. Biosurfactants by definition are molecules of microbial origin (i.e. bacteria, fungi, plants) which can serve as surface active compounds that are biodegradable and less toxic than synthetic chemical ones. Such types of biosurfactants include: glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides, which have been largely appreciated lately in environmental applications like bioremediation, soil washing, and soil flushing. Biosurfactants enhance oil biodegradation by increasing oil availability to microbial population [20]. Biosurfactants have been successfully applied in oil bioremediation studies however their high production costs limits their use in the field.

Oleophilic biostimulants effectiveness highly depends on the characteristics of the site such as type of sediment or high/low energy wave action and tide. Clearly oleophilic fertilizers can perform better than water-soluble fertilizers when water transport is not the limiting factor like in the case where spilled oil is resided in the intertidal zone [21]. Moreover oleophilic fertilizers can deliver nutrients at the oil-water interface, where bioremediation mainly occurs. However several studies that have been conducted with oleophilic fertilizers have produced mixed results regarding their persistence. Studies conducted with Inipol EAP22 on a sandy beach showed that it is highly persistent over time under simulated tide and wave actions [22][23]; whereas when applied to high energy shorelines it is rapidly washed out. Moreover the combined application of biosurfactants (rhamnolipids) and water soluble nutrients had considerably greater effect in the biodegradation rate of oil compared to sole application of biosurfactants [24]. Sole application of biosurfactants can only promote oil dissolution and transport when oil is entrapped in the soil matrix and thus furthermore can increase oil bioavailability, however in the absence of essential nutrients oil biodegradation cannot be enhanced.

Several studies comparing the effectiveness of different type of nutrients on oil biodegradation rate have been conducted in the past [6]–[9], [11], [14], [15], [17]–[19], [21]–[33]. Experimental results from laboratory and field studies indicate the importance of local prevailing conditions. Water-soluble fertilizers are likely more cost-effective in low-energy and fine-grained shorelines and generally sheltered sites where washout is limited. On the other hand, slow-release fertilizers may be ideal nutrient sources if the nutrient release rates can be well controlled and the non-dissolved particles cannot be washed out by the wave action. Finally, oleophilic fertilizers may be more suitable for use in higher-energy, coarse-grained beaches and generally exposed sites and open sea environments. Biostimulation with nutrients and biosurfactants enables naturally occurring microbes to adapt better and faster to the oil spill environment resulting in shorter lag phase and faster crude oil degradation [34], [35], thus making it an effective tool for combating oil spills.

THE POLLUTANT MOBILIZATION AND DIFFUSION ASSOCIATED TO THE SURACTANT USE IS NOT QUOTED. POLLUTION DISTRIBUTION CAN BE A RELEVANT PROBLEM ASSOCIATED TO THE INTERVENTION. In my view, something about this aspect should be mentioned in the text.

In Table 5 the major nutrient types used in biostimulation of oil spills are shown.

Table 5: Major nutrient types used in oil bioremediation

Type of nutrients	Advantages	Disadvantages	Applications in the field or field trials
Water soluble (e.g., KNO_3, $NaNO_3$, NH_4NO_3, K_2HPO_4, $MgNH_4PO_4$)	○ Readily available ○ Easy to manipulate for target nutrient concentrations ○ No complicated effect of organic matter	○ Rapidly washed out by wave and tide ○ Labor-intensive, and physical intrusive applications ○ Potential toxic effect	Alaska [36] Delaware [31] Stert Flats, UK [8]
Slow release (e.g., Customblen, IBDU, Max Bac, Osmocote)	○ Provides a continuous sources of nutrients ○ higher nutrient concentrations can be maintained in the pore water ○ More cost effective than other types of nutrients	○ Maintaining optimal nutrient release rates could be a challenge	Alaska [36] Nova Scotia [37] Japan [27] Stert Flats, UK [8]
Oleophilic (e.g., Inipol EAP22, F1, MM80, S200)	○ Able to adhere to oil ○ Provides nutrients at the oil-water interface	○ Expensive ○ Effectiveness is variable ○ Containing organic carbon, which may compete with oil degradation and result in undesirable anoxic conditions	Alaska [36] Nova Scotia [38]–[41] Cantabrian Coast, Spain [19]

3.1.4 Oxygen Limitations

Biodegradation rates can be limited to anoxic marine environments where the concentration of dissolved oxygen can be close to zero, despite the proved effectiveness of oleophilic fertilizers and other products. Although anaerobic biodegradation of hydrocarbons in marine environment is well documented, the actual rate is very low. Despite that aeration in contaminated soils, groundwater (in many forms) and in surface soils (via tilling) is easy to implement, this is not the case for the marine environment and especially in deep sea or in sediments, where no special oxygen delivering products or systems have been yet produced or proposed.

All the above criteria for the successful biostimulation of oil spills are summarized in Table 6.

Table 6: Situations where biostimulation is recommended for the bioremediation of contaminated shorelines [42]

Type of coast	Type of exposure	
	High energy	Low energy
Cliffs, seawalls and piers	NR	NR
Rock platforms	NR	NR
Pebble beaches	Oleophilic	Oleophilic
Mixed sand and gravel beaches	Oleophilic	Oleophilic
Coarse-grained sand beaches	Oleophilic	Slow release
Fine-grained sand beaches	N/A	Slow release or water soluble (plus tiling if oxygen limitation

N/A: not applicable; NR: biostimulation is not recommended

3.2 Bioaugmentation

Bioaugmentation is defined as the technique in which specific consortia are introduced to a contaminated site in order to improve pollutant biodegradation capacity [43].

3.2.1 Laboratory Studies on Bioremediation of Oil

Bioaugmentation is one of the most controversial issues of bioremediation. Addition of oil-degrading microorganisms has been proposed as a bioremediation strategy. The rationale for adding oil-degrading microorganisms is that indigenous microbial populations may not be capable of degrading the wide range of pollutants present in complex mixtures of hydrocarbons such as crude oil [44].
Bioaugmentation agents manufacturers and commercial distributors claim that their products can enhance oil biodegradation [45]. Nonetheless commercial bioaugmentation agents which are listed on Table 2 have been tested through extensive laboratory studies and have produced diverse results [46], [47]. Nevertheless, although these studies were published, products identities have been kept anonymous.
Despite this fact, the bioremediation agent of microbiological cultures TerraZyme^{TM} (Oppenheimer Biotechnology Inc.) has been effectively used to treat heavy oil spilled from Nakhodka. The results exhibited a high potential for biodegradation of oil, even to the most difficult to degrade fraction of asphaltene [48].
TerraZyme^{TM} (1 g of TerraZyme^{TM} contains approximately 10^8-10^9 cells of bacteria) is made up of various microbiological complexes, which have been collected from many different geographical localities under different environmental conditions, shown extensive clean-up impacts on oil pollution under various environmental conditions, either with the supplementation of specific compounds like biosurfactants or not. The initial bacterial density of the test samples with TerraZyme^{TM} was adjusted to be approximately less than 5 ×107/mL [48].However these products are difficult to manufacture in large quantities and their availability on the market is scarce [49]–[54].

3.2.2 Mesocosm Studies

Upscaling seeding in mesocosm facilities with better simulation of environmental conditions did not demonstrate effectiveness of bioaugmentation treatments compared to lab scale studies.
For instance five commercial bioaugmentation products have been tested for their effectiveness to remediate crude oil in a salt marsh mesocosm under flooded/drained conditions with or without nutrients (Max Bac fertilization).
Among the five commercial bioremediation products that have been used in this study there were three U.S. EPA NCP product schedule currently listed bioremediation agents and two formerly listed bioremediation agents: Oil Spill Eater-II Concentrate (OSEI Corp., Dallas, Texas), which contained

enzymes, Oppenheimer Formula I (Oppenheimer Environmental Corp., Austin, Texas), a microbial inoculant with enzymes, Micro-Blaze Out (Verde Environmental Inc., Houston, Texas), a microbial inoculant with enzymes and dispersing agents, BioGEE HC Concentrate (BioGEE International Inc., Houston, Texas), which was a microbial inoculant and Alpha Biosea (Alpha Environmental Inc., Austin, Texas), a microbial inoculant with nutrients. Disappointingly, these commercial products have failed to promote oil removal as well as to increase population of hydrocarbon degraders [55].

3.2.3 Field Studies

The majority of field studies failed to demonstrate that supplementation only with oil degrading consortia is more efficient than supplementation with nutrients.

However there a few field trials that have claimed success in demonstrating the effectiveness of bioaugmentation for oil biodegradation, such as a field evaluation of Alpha BioSea^{TM} (Alpha Environmental, Inc.) to treat the Angolan Palanca crude oil spilled from Mega Borg off the Texas coast [56] and the catalytic feedstock oil spilled from the Apex Barge in Pelican Island and Marrow Marsh in Texas [9] and using TerraZymeTM (Oppenheimer Biotechnology) for enhancing biodegradation of a heavy oil spilled from Nakhodka in Japan [57]. Nonetheless, the success of these studies was based on either visual observation (i.e. the Mega Borg study) or digital photographic image analysis (i.e., the Nakhodka study). No comprehensive monitoring program was used to verify the oil was indeed removed through enhanced biodegradation. The two products basically contain the same bacterial cultures and nutrients. Alpha BioSea is a product composed of a lyophilized bacterial mixture and inorganic phosphorus and nitrogen nutrients in a cornstarch carrier and was formerly included as a bioremediation agent in the US EPA NCP product schedule. Despite this, there are few studies showing that bioaugmentation can contribute to the degradation of oil hydrocarbons.

Another product, previously listed in the US EPA NCP product schedule, bioremediation agent PRP (Petrol Rem, Incorporated, Pittsburgh, Pennsylvania), was evaluated in an oiled sandy beach at Nova Scotia, Canada and showed that adding the bioremediation product did not perform better in terms of enhancing alkane degradation than applying inorganic agricultural fertilizers alone within the oiled sediments. Nonetheless PRP appeared to limit the transport of beached oil to more sensitive areas. PRP consists of tiny spheres of plant derived natural products (beeswax), which contain nutrients and nonpathogenic bacteria and it can absorb twice its weight of oil and form a physical matrix that floats on water, thereby preventing the pollutants from sinking and limiting the transport of oils. The matrix then provides an environment that uses naturally occurring microorganisms in the water to degrade the pollutant as well as PRP itself.

Additionally Petrol Rem provided two real-world case studies: one case involved fuel oil contaminated beaches and a mangrove along the Persian Gulf in Abu Dhabi (1998) and the other case involved heavy oil spilled into a lagoon in Mexico (1994). Vendors claim that PRP perform well in both contaminated sites, however in both cases, only visual observation was used to monitor the performance of PRP treatment on site without any reliable or scientifically approved methods of assessing the treatability of the site, to support their claims.

In another study bioaugmentation products B350 and L1800 (Bio-Systems Co., USA) failed to enhance oil degradation from the heavy fuel oil spill of the Prestige in sand, rocks and granite tiles on the beach of Sorrizo (A Coruna, NW Spain) [13]. According to Bio-Systems Co. B350 contains a high concentration of naturally-occurring microorganisms that have been selected for their ability to degrade a wide range of petroleum-based and other hydrocarbon chemicals. L1800 contains biodegradable surfactants.

The above case studies show that even though seeding can enhance oil biodegradation in the laboratory, the effectiveness of bioaugmentation has not been convincingly demonstrated in the field. Actually, most field studies indicated that bioaugmentation is not effective in enhancing oil biodegradation in most environments. It appears that in most environments, indigenous oil degrading microorganisms are more than sufficient to carry out oil biodegradation if nutrient levels and other adverse environmental conditions do not limit them.

4 Contributions of the Kill●Spill project to progress beyond the state of the art

4.1 Constraints for successful biostimulation and bioaugmentation

4.1.1 Nutrients limitation

It is fundamental, yet only scarcely accounted for, to consider the whole microbial biodegradation network, including "helper" microorganisms that support the degrading microbes to attack the oil molecules increasing their bioavailability or by increasing availability of limiting nutrients. In marine systems iron is a limiting factor, while bioavailability of hydrophobic oil components is a general constraint for successful biodegradation. So far, no attempts have been made to explicitly include microorganisms capable of overcoming such constraints (e.g., release of siderophores to enhance iron availability or biosurfactants to increase bioavailability of hydrophobic compounds) into bioaugmentation formulations. The Kill●Spill project proposed to investigate the effect of micro-nutrients limitations.

Biostimulation is regarded as an excellent strategy for combating oil spills to mitigate nitrogen and phosphorus limitations. However, supply of these two essential elements as water-soluble salts presents problems of quick dilution and leads to wash out due to wave action and currents. Hence, they are only recommended for low energy beaches. A safe approach is the use of oleophilic biostimulants that remain preferentially at the oil phase / seawater interface and thus are available to HC degraders for enhancing bacterial growth and metabolism. Attempts have been made to use less toxic, biodegradable and of natural origin fertilizers like uric acid and lecithin. Uric acid has a low solubility in water and is the major component of guano fertilizer and hennery wastes. Natural phospholipids such as lecithin are in fact oil soluble, easy to get at low cost as by-products of the oil seeds industry and have good dispersant properties. The development of bioremediation agents using natural, low costs nutrient sources such as oleophilic fertilizers will be studied. In parallel, biosurfactant producing microbes will also be used to supplement bioremediation agents with biosurfactants capabilities.

4.1.2 Hydrocarbon degraders

Bioaugmentation strategies include the addition of pure or mixed microbial cultures capable of degrading the pollutants. This approach provides best results when using autochthonous microbial strains and/or consortia. Previous studies showed that hydrocarbon (HC)-degrading microbial consortia are characterized by the presence of a number of ubiquitous highly specialized obligate hydrocarbon-degrading microbes (OHCM) that form stable communities with relatively high reliability. Although such consortia can be complex, a core set of oil-degrading microbes is constant. Prokaryotes as well as eukaryotic organisms such as fungi have the capability to degrade HC and they are frequently observed in oil-impacted environments. Population dynamics, community species interactions and function in such habitats however remain largely unknown. Stable small populations in micro-environments or micro-compartments are ubiquitous in the environment. Bio-aerosols are one such environment where bacteria are condensed with atmospheric water, particles and volatile organic compounds, e.g. arising from crude oil. This fact has not been accounted for in traditional methods for obtaining high-efficiency HC-degraders. Pressure is an important thermodynamic parameter, especially at higher depths in the marine environment. The largest fraction of the ocean is at depths of more than 200 m and 75% of the marine biosphere is located below 1 km depth. Marine microorganisms can live up to about 110 MPa hydrostatic pressure, however it is unknown what effect pressure has on HC degradation and strain selection.

The Kill●Spill project will overcome these issues by isolating OHCM to exploit them for autochthonous bioaugmentation, investigating the potential of fungi in marine environment and their interaction with prokaryotes communities. In parallel, novel methods for population size controlled evolution in microdroplet bioreactors will be used to improve HC degrading abilities of existing strains. Finally, the effect of high pressure on hydrocarbon-degrading microorganisms will be investigated in a high pressure rector.

4.2 Monitoring

The belief that increasing the number of microorganisms within a system should increase the rate of removal of hydrocarbons has proven to be simplistic. Despite the selection of strains with great catabolic potential, the organisms have often failed to survive in competitive environments like seawater, thus highlighting the necessity of monitoring and field-validation of bioaugmentation and biostimulation approaches.

The Kill●Spill project will develop monitoring tools to follow microbial community shifts, especially by combining ARISA fingerprinting and *in silico* interpretation, and HC degradation by applying OMICS techniques, paired with powerful *in silico* processing.

4.3 Integration of approaches

It is anticipated that a successful bioremediation technique should lead to changes in the composition of oil-impacted microbial communities in favour of oil-degrading bacteria and fungi, stimulating the indigenous microorganisms or providing advantage to the augmented ones. A variety of limiting factors, both abiotic (e.g., Dissolved Oxygen, pH and lack of inorganic nutrients) and biotic (e.g., competition with indigenous microbial populations), as mentioned above, have to be taken into consideration. Hence, there is currently little agreement on bioaugmentation strategies, delivery systems, inoculum size, strain selection or other strain factors to be taken into account.

The Kill●Spill project will deliver integrated approaches, combining competencies on the physiology and genetics of microorganisms, their interaction and driving forces for an efficient degradation of hydrocarbons in contaminated environments. This includes acquisition of competent microbes and consortia beyond solely focusing on metabolic capabilities, also integrating approaches for delimiting metabolic constraints in such environments.

5 References

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