MICROBIOLOGIA BALKANICA 2011

7th BALKAN CONGRESS OF MICROBIOLOGY

8th CONGRESS OF SERBIAN MICROBIOLOGISTS

MICROBIAL CONSORTIA DIVERSITY IN BIODEGRADATION OF PETROLEUM POLLUTANTS DURING BIOREMEDIATION

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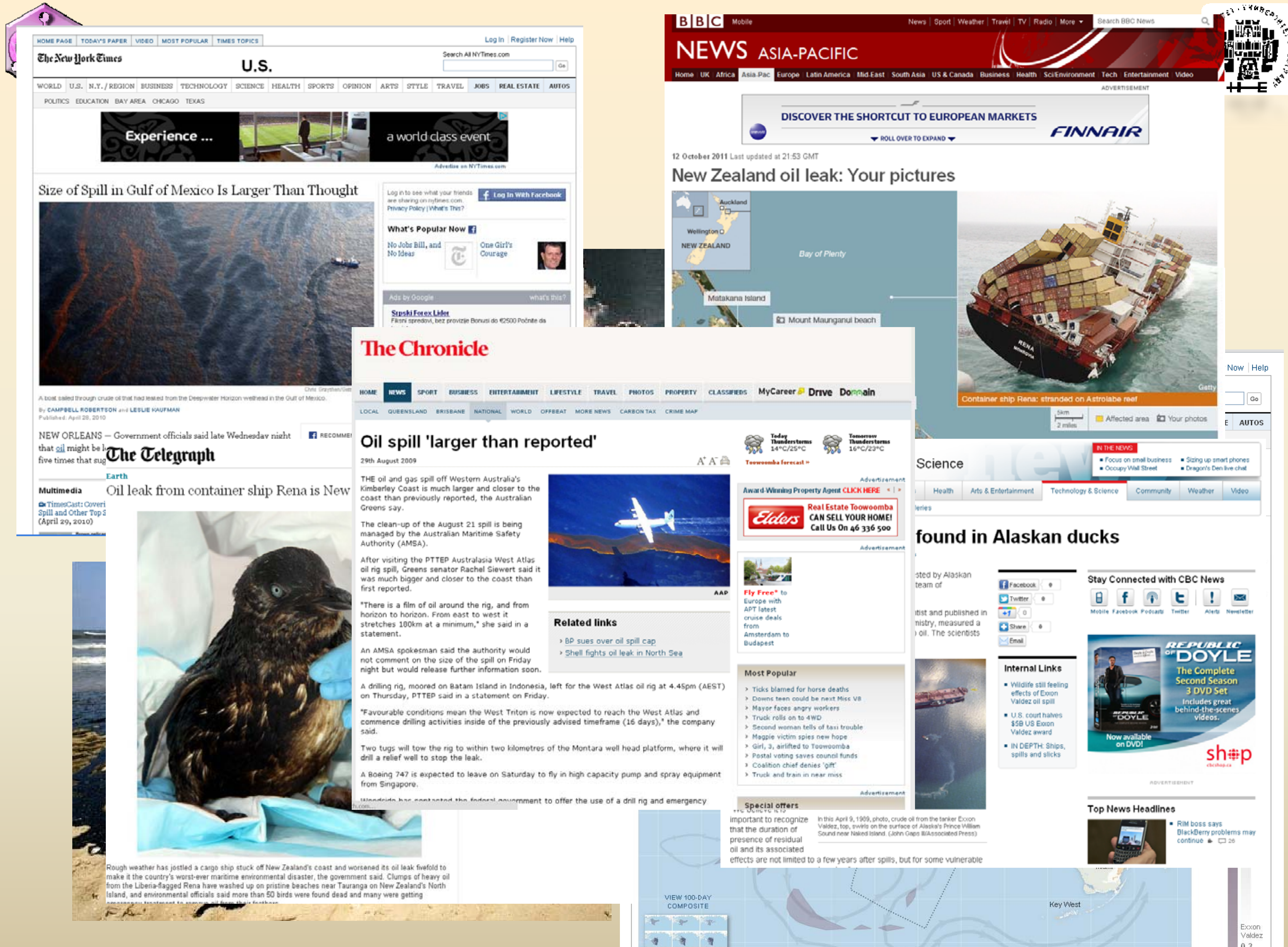
Continental Hotel, Belgrade, Serbia



The New York Times
U.S.

Size of Spill in Gulf of Mexico Is Larger Than Thought

A boat sailed through crude oil that had leaked from the Deepwater Horizon wellhead in the Gulf of Mexico.

By CAMPBELL ROBERTSON and LESLIE KAUFMAN

Published: April 28, 2010

NEW ORLEANS — Government officials said late Wednesday night that oil might be l… five times that sug…

BBC NEWS ASIA-PACIFIC

12 October 2011 Last updated at 21:53 GMT

New Zealand oil leak: Your pictures

The Telegraph

Earth

Oil leak from container ship Rena is New…

Rough weather has jostled a cargo ship stuck off New Zealand's coast and worsened its oil leak fivefold to make it the country's worst-ever maritime environmental disaster, the government said. Clumps of heavy oil from the Liberia-flagged Rena have washed up on pristine beaches near Tauranga on New Zealand's North Island, and environmental officials said more than 50 birds were found dead and many were getting…

The Chronicle

Oil spill 'larger than reported'

29th August 2009

THE oil and gas spill off Western Australia's Kimberley Coast is much larger and closer to the coast than previously reported, the Australian Greens say.

The clean-up of the August 21 spill is being managed by the Australian Maritime Safety Authority (AMSA).

After visiting the PTTEP Australasia West Atlas oil rig spill, Greens senator Rachel Siewert said it was much bigger and closer to the coast than first reported.

"There is a film of oil around the rig, and from horizon to horizon. From east to west it stretches 180km at a minimum," she said in a statement.

An AMSA spokesman said the authority would not comment on the size of the spill on Friday night but would release further information soon.

A drilling rig, moored on Batam Island in Indonesia, left for the West Atlas oil rig at 4.45pm (AEST) on Thursday, PTTEP said in a statement on Friday.

"Favourable conditions mean the West Triton is now expected to reach the West Atlas and commence drilling activities inside of the previously advised timeframe (16 days)," the company said.

Two tugs will tow the rig to within two kilometres of the Montara well head platform, where it will drill a relief well to stop the leak.

A Boeing 747 is expected to leave on Saturday to fly in high capacity pump and spray equipment from Singapore.

Related links

- BP sues over oil spill cap
- Shell fights oil leak in North Sea

CBC News — Science

…found in Alaskan ducks

Internal Links

- Wildlife still feeling effects of Exxon Valdez oil spill
- U.S. court halves $5B US Exxon Valdez award
- IN DEPTH: Ships, spills and slicks

In this April 9, 1989, photo, crude oil from the tanker Exxon Valdez, top, swirls on the surface of Alaska's Prince William Sound near Naked Island. (John Gaps III/Associated Press)

…important to recognize that the duration of presence of residual oil and its associated effects are not limited to a few years after spills, but for some vulnerable…

Gulf of Mexico“Deepwater Horizon“ 2010

Volume of oil: 780,000 m^3;
Area : 6,500 to 180,000 km²

~800,000 x

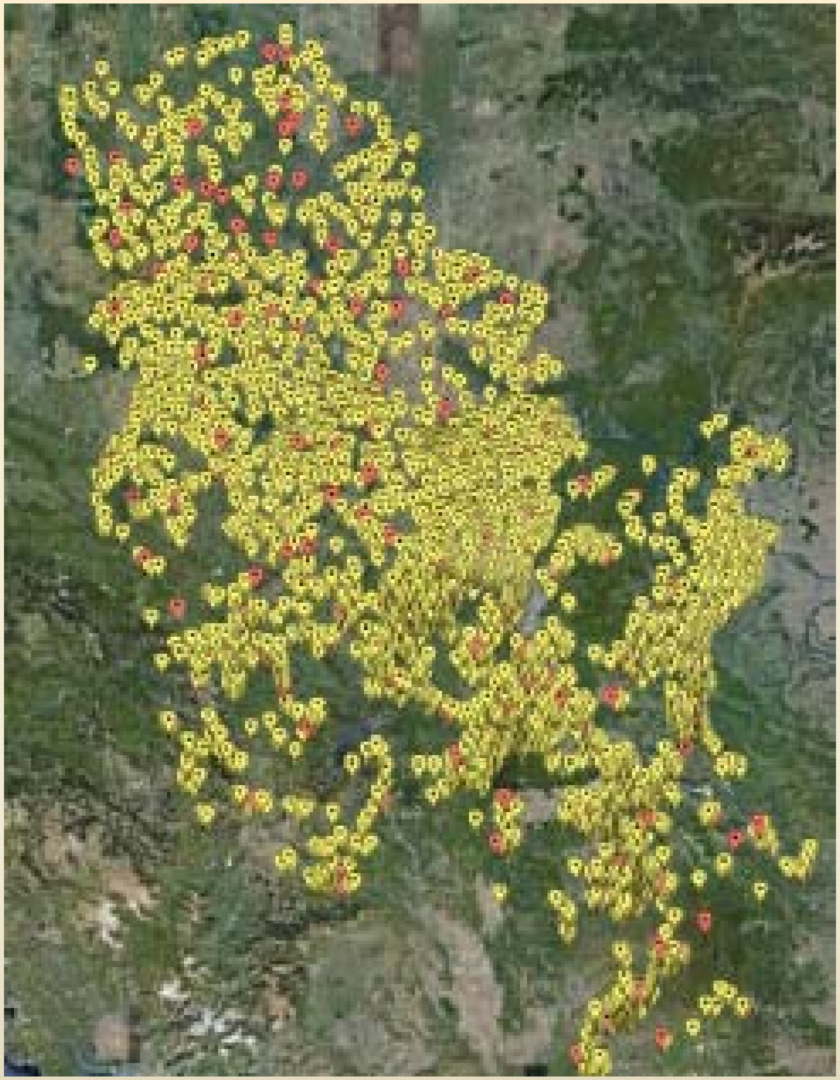
October 2011, Oil spill in New Zealand, 1,700 tonnes of heavy fuel oil

The biggest spill ever occurred during the 1991 Persian Gulf war when about 900 million litters spilled from **oil** terminals and tankers off the coast of Saudi Arabia.

The Exxon Valdez accident at Bligh Reef in 1989 discharged 40 million litres.

POLLUTED ENVIRONMENT IN SERBIA!!!

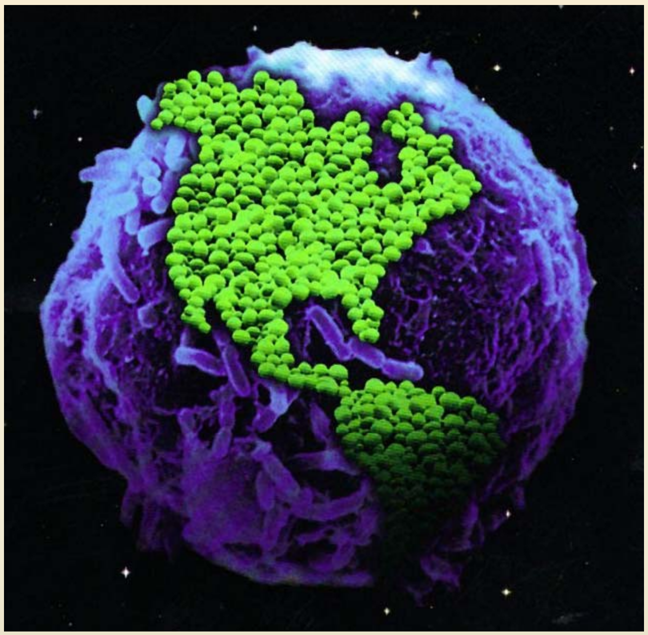
Europe ~ 1,800,000 potentially contaminated sites/240,000 sites require remediation!
Serbia 357 heavy polluted sites!



Locations of **known** **illegal** **dumps** in the territory of the Republic of Serbia,
REPORT ON THE STATE OF THE ENVIRONMENT IN SERBIA 2009,
Ministry of Environment and Spatial Planning

BIO/ REMEDIATION

BIOREMEDIATION



- Microorganisms degrade organic contaminants in the course of using the chemicals for their own **growth** and **reproduction**.
- Organic chemicals provide: **carbon**, source of cell **building** material, **electrons**, source of **energy.**

Biotransformation- reducing the complexity of organic molecules

Biodegradation- degradation to complete mineralization

“EQUATION” OF BIOREMEDIATION

HARMFUL SUBSTANCES —MICROORGANISMS→ **SUBSTANCES THAT ARE WITHOUT HARMFUL EFFECTS ON THE ENVIRONMENT AND HUMANS** + **BIOMASS**

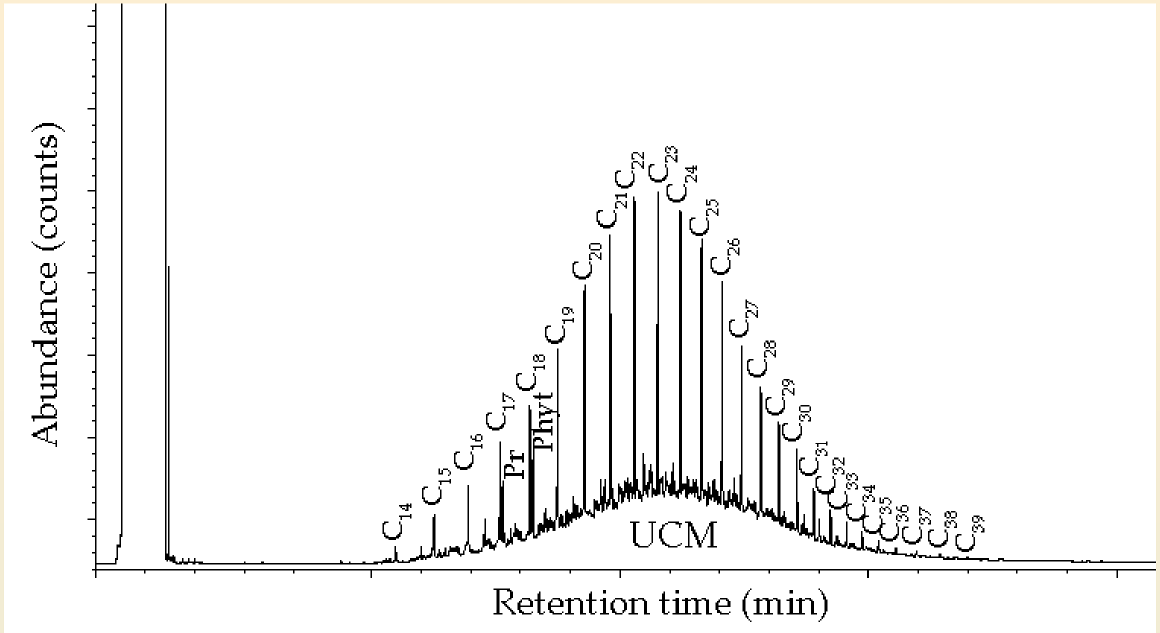
General equation of complete mineralization of organic substances:

$$C_cH_hO_oN_nP_pS_sCl_{cl} \rightarrow cCO_2 + h/_2H_2O + nNH_4^+ + pHPO_4^{2-} + sSO_4^{2-} + clCl^- + Energy$$

What Types of Compounds Can Be Treated Biologically?

- **Petroleum Hydrocarbons**
  - Gasoline
  - Diesel Fuel
  - Gasoline Additives such as MTBE
- **Polycyclic Aromatic Hydrocarbon**
  - Creosote
- **Chlorinated Hydrocarbons**
  - Chlorinated Aliphatics: trichlorethylene
  - Chlorinated Aromatics : PCB’s, Pentachlorophenol
- **Explosives**
  - RDX, TNT
- **Inorganics** via Reduction to a Lower Valence Causing Precipitation
  - Uranium, Technicium
  - Sulfur and Sulfuric Acid
  - Ammonia or Nitrate/Nitrite

CRUDE OIL AS A DOMINANT POLLUTANT



GROUP COMPOSITION

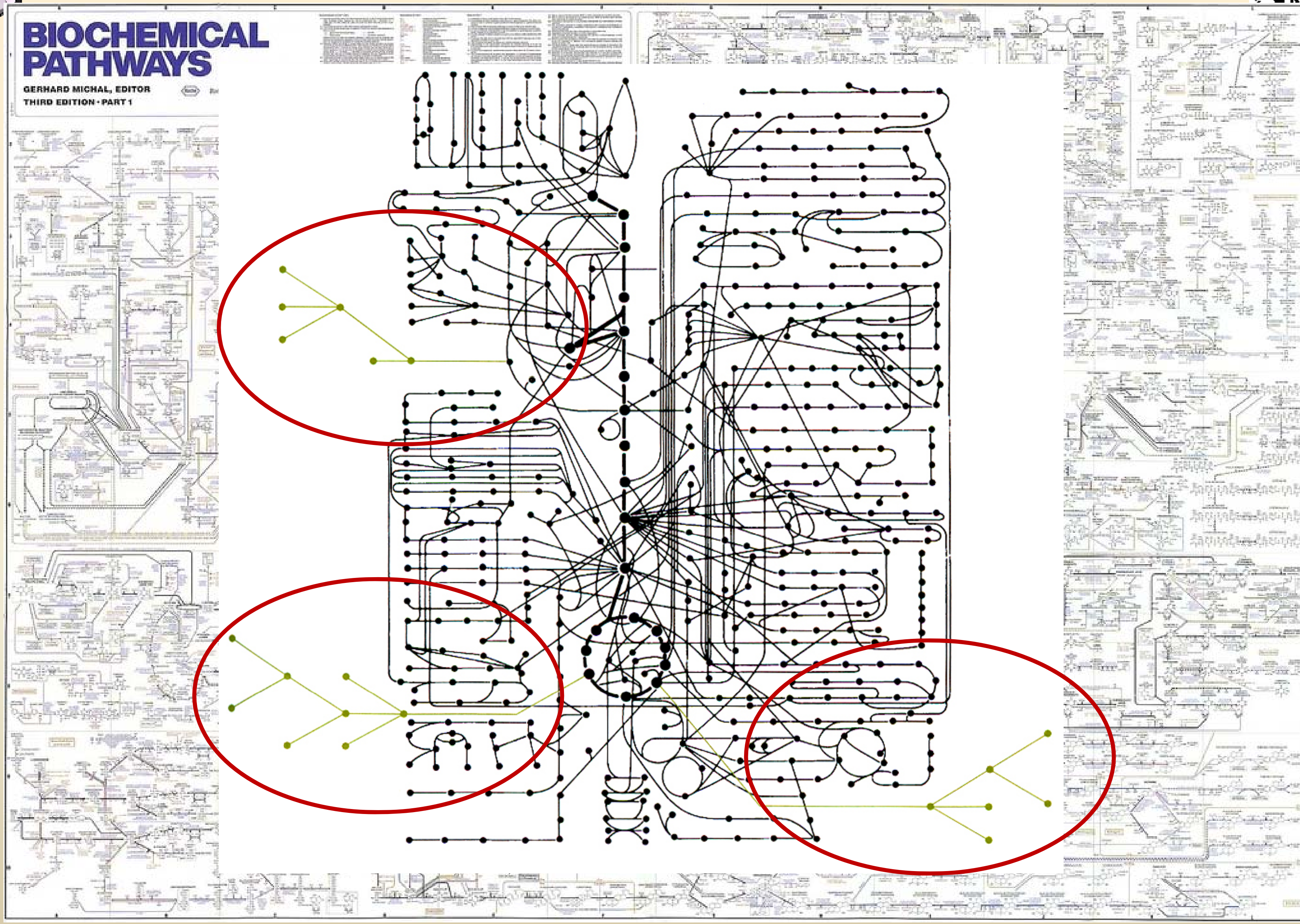
-**S**aturated (*n*-alkanes, iso-alkanes, cyclo alkanes)

-**A**romatic

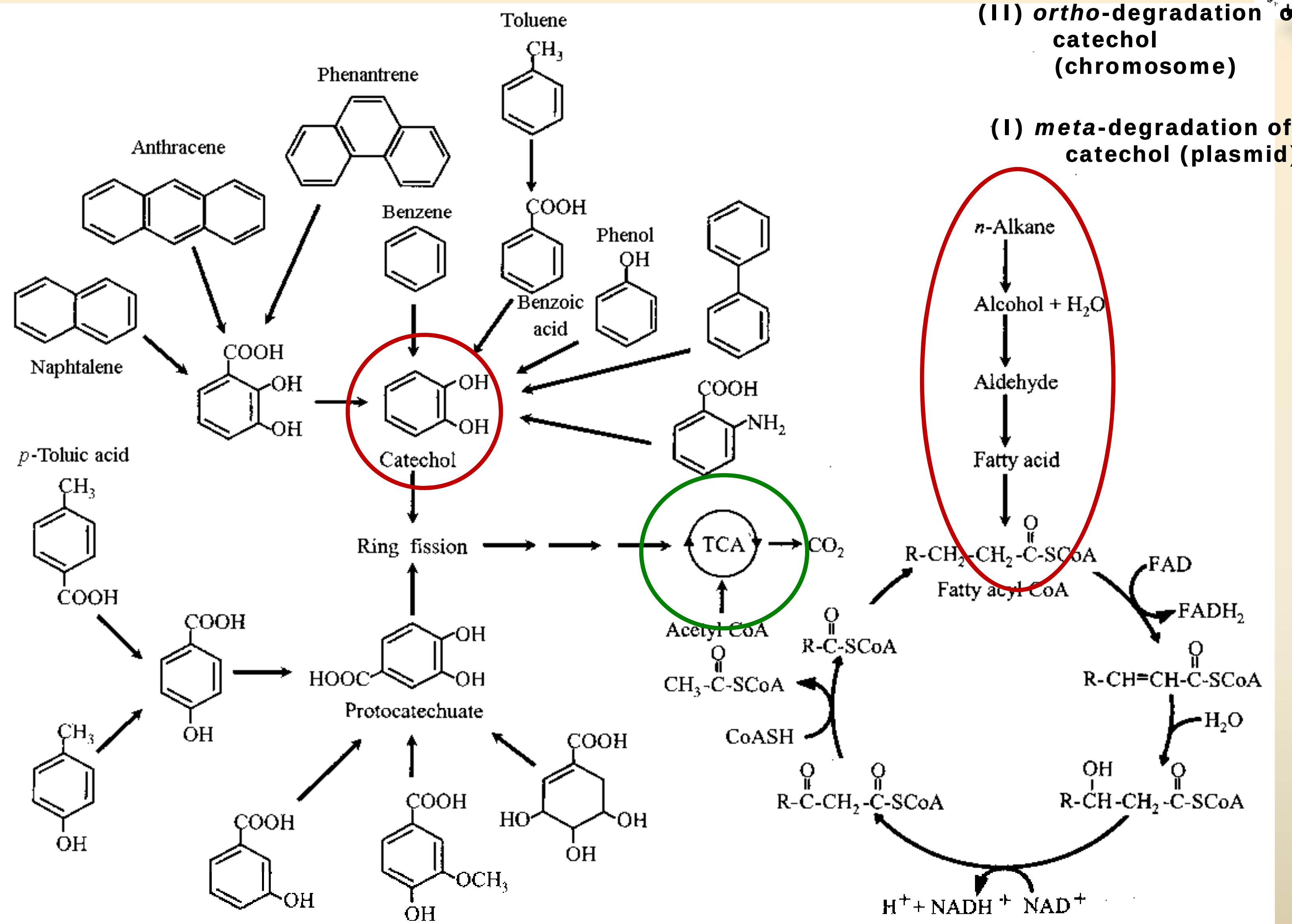
-**R**esins (heterocyclic (NSO) compounds such as acids, bases, phenolics, naturally occurring compounds (humic acids)

-**A**sphaltens (high molecular weight complex matrix)

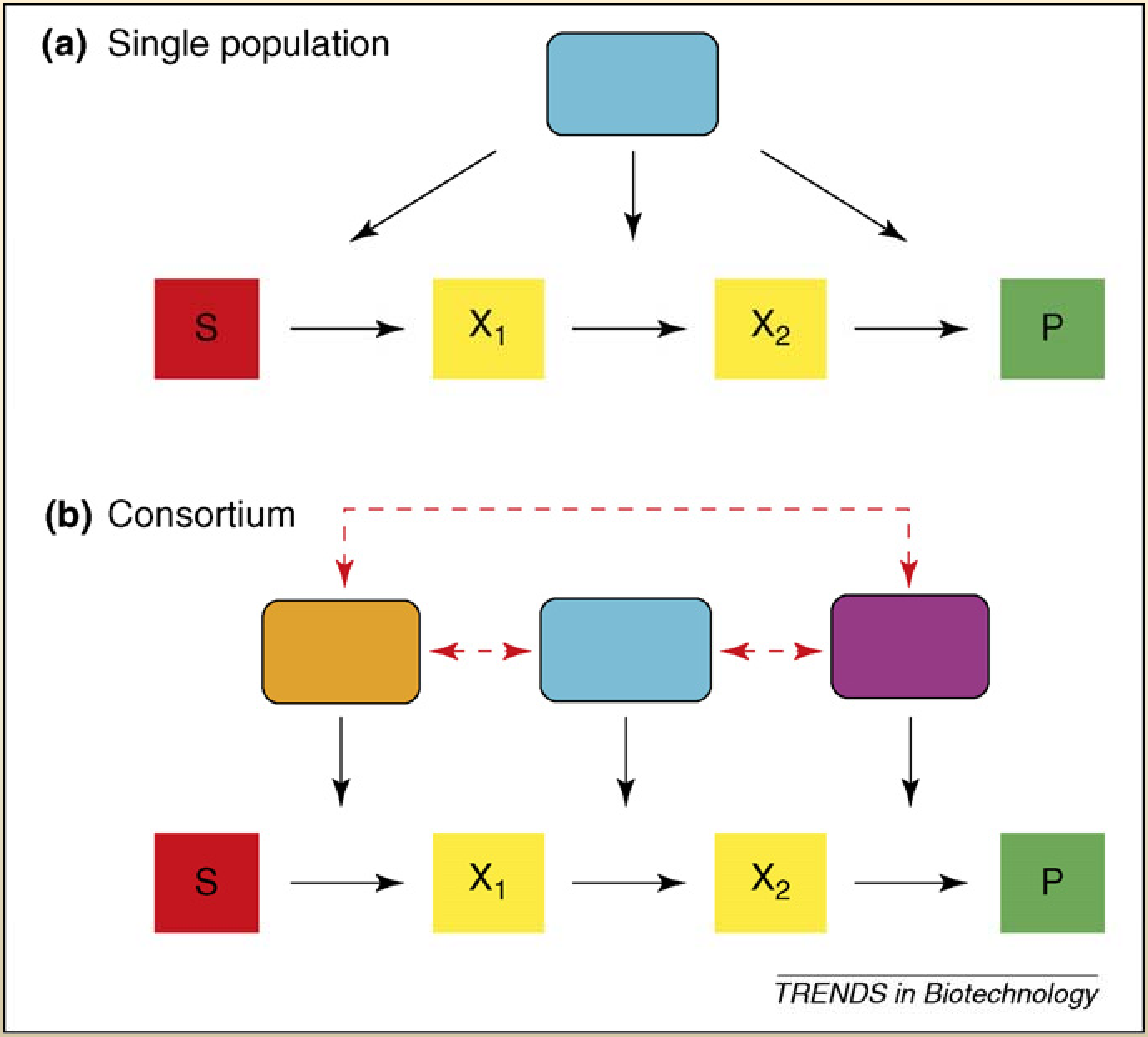
<u>17,000</u>
distinct chemicals!!!


BIOCHEMICAL PATHWAYS
GERHARD MICHAL, EDITOR
THIRD EDITION • PART 1

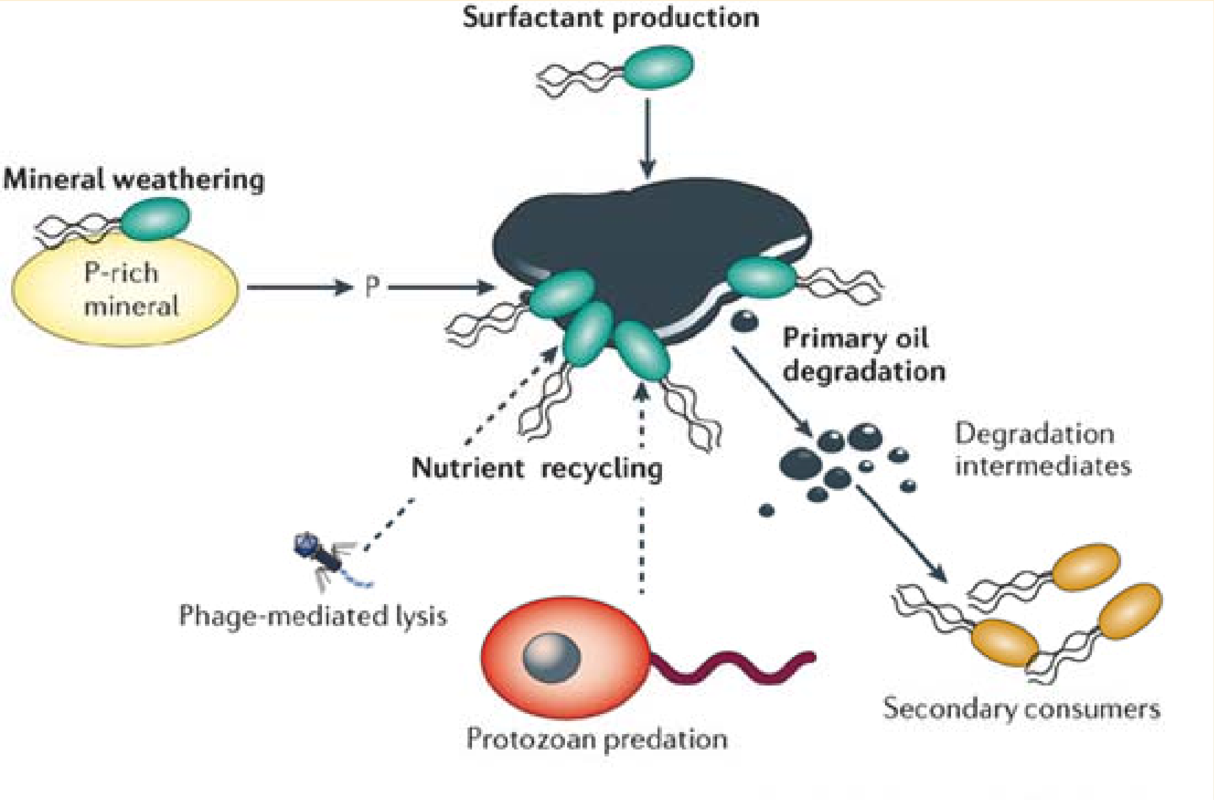
OXYDATION OF HYDROCARBONS



Single Population *vs.* Consortium



A MICROBIAL DEGRADATION NETWORK



Nature Reviews | Microbiology

Some defined bacterial species are able to degrade, to a limited extent, all hydrocarbons present in heavy fuel oil or oil sludge. Some of the polluting components may be degraded only by the coupled metabolic activity of multiple genera of microorganisms. A **CONSORTIUM** (mixed culture) of microorganisms can conduct these complex processes of degradation, while at the same time, being more resistant, on average, to changes in the ecosystem than just a single microbial species.

Microbial populations and the development of catabolism

Average composition of biomass in soil

Component of the soil biomass	Tons per hectare
Bacteria	1-2
Fungi	2-5
Actinomycetes	1-2
Protozoa	up to 0.5
Nenatodes	up to 0.2
Earthworms	0-2.5
Other fauna (insects and arthropods)	up to 0.5

ADAPTATION OF MICROORGANISMS:

- Enzymatic regulation (induction or repression of enzyme)
- The exchange of genetic material (transduction, transformation or conjugation)
- By changing external environment (pH, redox)
- Selective "enrichment"
- Changes in genetic material (mutation, duplication, recombination)

MICROBIAL GENERA KNOWN TO BIODEGRADE ORGANIC POLLUTANTS

Genus or group	Compound
Acinetobacter	Cyclohexanol
Actinomycetes	2,4,6-Trinitrotoluene
Aeromonas	Phenanthrene
Agrobacterium	Glyphosate
Alcaligenes	2-Aminobenzenesulfonate
	2,4-Dichlorobenzoate
	2,4-Dichlorophenoxyacetic acid
	Toluene-4-sulfonate
Ancylobacter	1,3-Dichloro-2-propanol
	2,4-Dichlorobenzoate
	1,2-Dichloroethane
	2,4-Dichlorophenoxyacetic acid
	Glyphosate
	4-Nitrophenol
	Parathion
	Tyrosine
Arthrobacter	2-Aminobenzoate
	1,3-Dichloro-2-propanol
	2,4-Dichlorobenzoate
	2,4-Dichlorophenoxyacetic acid
	Fluorene
	Glyphosate
	Methyl *tert*-butyl ether
	Nicotine
	4-Nitrophenol
	Parathion
	Phenanthrene
	Tyrosine
Aspergillus	2-Aminobenzoate
	Phenanthrene
Azoarcus	Benzoate
	Toluene
Azotobacter	2,4-Dichlorophenoxyacetic acid
	Thiocyanate
Beijerinckia	Xylene
Brevibacterium	Dibenzofuran
Burkholderia	3-Chloroacrylic acid
	2,4-Dichlorophenoxyacetic acid
	Pentachlorophenol
	Phthalate
	Toluene
	1,2,4-Trichlorobenzene
	Trichloroethylene
	2,4,5-Trichlorophenoxyacetic acid
	o-Xylene
Chelatobacter	Nitrilotriacetate
Clostridium	Phenol
Comamonas	3-Methylquinoline
	Nitrobenzene
	Phthalate
	Toluene-4-sulfonate
Corallinus	Atrazine
Corynebacterium	1,3-Dichloro-2-propanol
	2,4-Dichlorobenzoate
Cunninghamella	Phenanthrene
Dehalobacter	Tetrachloroethene
Dehalococcoides	Tetrachloroethene
Dehalospirillum	Tetrachloroethene
Desulfitobacterium	Tetrachloroethene
Dunaliella	DDT[a]
Enterobacter	DDT
	Glyphosate
	Pentaerythritol tetranitrate
Escherichia	Carbazole
	Methionine
	Organomercury
	3-Phenylpropionate
	Threonine
Eubacterium	Gallate
Exophiala	Styrene
Flavobacterium	Bromoxynil
	2,4-Dichlorophenoxyacetic acid
	Glyphosate
	Parathion
	Pentachlorophenol
Fusarium	2-Nitropropane
Hydrogenophaga	4-Carboxy-4′-sulfoazobenzene
Hyphomicrobium	Dichloromethane
Klebsiella	Acetylene
	Benzonitrile
	Bromoxynil
Methanosarcina	Tetrachloroethene

Compounds known or proposed to be oxidized by *Pseudomonas putida* F1

Known substrates

1,1-Dichloro-1-propene
1,1-Dichloroethene
1,2-Dichlorobenzene
1,2-Dihydronaphthalene
1,2-Dimethylbenzene
1,2-Methylenedioxybiphenyl
1,3-Dibromobenzene
1,3-Dichlorobenzene
1,3-Dimethylbenzene
1,4-Dichlorobenzene
1,4-Dimethylbenzene
1-Bromo-2,3-difluorobenzene
1-Bromo-4-iodobenzene
1-Chloro-2-methyl-propene
1-Fluoro-4-iodobenzene
2-(2-Bromoethyl)bromobenzene
2,3-Dichloro-1-propene
2,3-Dimethoxybiphenyl
2-Acetoxyethylbenzene
2-Azidoethylbenzene
2-Bromoethylbenzene
2-Bromostyrene
2-Chlorobiphenyl
2-Chlorostyrene
2-Cyanoethylbenzene
2-Indanone
2-Isothiocyanatoethylbenzene
2-Methoxybiphenyl
2-Methoxynaphthalene
2-Methylphenol
2-Nitrotoluene
2-Thiocyanatoethylbenzene
3,4-Dichloro-1-butene
3-Chlorobiphenyl
3-Chlorostyrene
3-Methylphenol
3-Nitrotoluene
4-Bromotoluene
4-Chlorobiphenyl
4-Chlorostyrene
4-Chlorotoluene
4-Fluorotoluene
4-Methylphenol
4-Nitrotoluene
Acetate
Acetophenone
Anisole
Benzene
Benzenenitrile
Benzoate
Benzocyclohept-1-ene
Biphenyl
Bromobenzene
Chlorobenzene
cis-1,2-Dichloroethene
cis-1,4-Dichloro-2-butene
cis-1-Bromo-1-propene
cis-1-Chloro-1-propene
cis-2-Chloro-2-butene
cis-β-Bromostyrene
cis-Dibromoethene
Citrate
Cyanobenzene
D-Glucose
cis-β-Bromostyrene
Ethylbenzene
Ethylphenyl sulfide
Ethynylbenzene
Fluorobenzene
Fumarate
Glucose
Indan
Indene
Indole
L-Arginine
L-Glutamate
m-Bromobenzotrifluoride
Methylphenyl sulfide
Methyl *p*-nitrophenyl sulfide
Methyl *p*-tolyl sulfide
o-Iodotoluene
p-Cumate
p-Cymene
Perdeuteriobenzene
Phenetole
Phenol
Phenylethanol
p-Hydroxybenzoate
p-Iodotoluene
p-Methoxyphenylmethyl sulfide
Propoxybenzene
Protocatechuate
Pyruvate
Styrene
Succinate
Toluene
trans-1,4-Dichloro-2-butene
trans-1-Bromo-1-propene
trans-1-Chloro-1-propene
trans-2-Chloro-2-butene
trans-Cinnamonitrile
trans-Dibromoethene
Trichloroethylene
Trifluoromethoxybenzene
Trifluorotoluene

Proposed substrates

2-Ketogluconate
Betaine
Butylamine
Butyrate
Caprate
Caproate
Caprylate
D-Fructose
Gluconate
Glutarate
Glycerol
Heptanoate
Isovalerate
Lactate
L-Alanine
L-Aspartate
L-Histidine
L-Isoleucine
L-Leucine
L-Malate
L-Ornithine
L-Phenylalanine
L-Proline
L-Tyrosine
L-Valine
Malonate
n-Butanol
Pelargonate
Propionate
Putrescine
Quinate
Saccharate
Sarcosine
Spermine
Tryptamine
Valerate
α-Aminovalerate
α-Ketoglutarate
β-Alanine
β-Hydroxybutyrate
δ-Aminovalerate

>100 known substrates

> 40 proposed substrates

BIOREMEDIATION

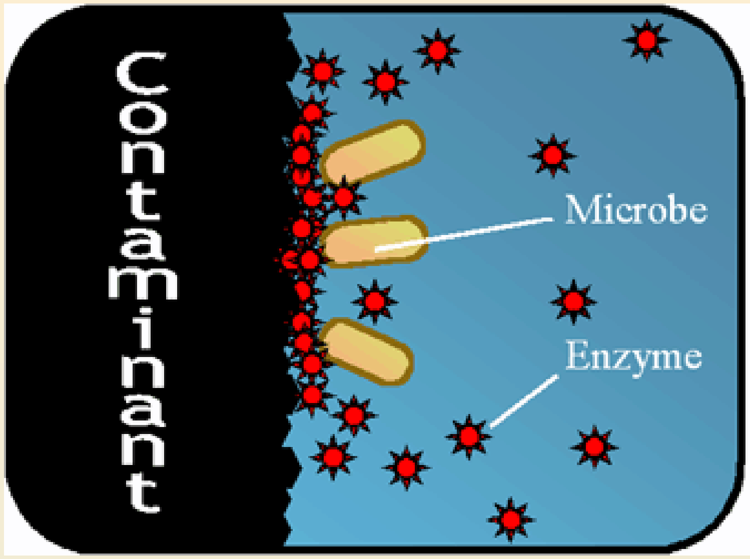
In situ/Ex situ

- **BIOSTIMULATION (N, P)**
- **BIOVENTILATION/AERATION**
- **SURFACTANTS**
- **BIOAUGMENTATION/REINOCULATION**
  - Pure cultures
  - Mixed cultures
  - Alochtonous (from the world market) (poor survival and low activity)
  - Genetically engineered microorganisms
  - Plasmid catabolic genes
  - Zymogenous consortium (isolation, selection and adaptation)

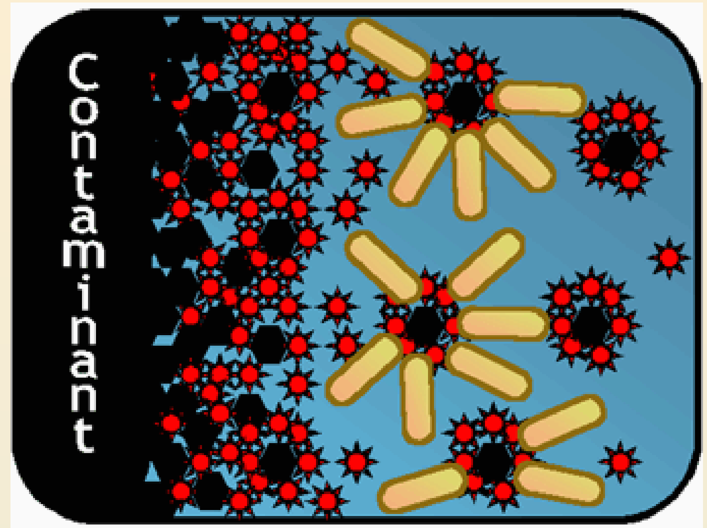
1-2% CULTURABLE MICROORGANISMS

1-10% of microorganisms can use hydrocarbons as a source of carbon and energy. These are called **ZYMOGENOUS** microorganisms. When the oil spill occurs only the zymogenous microorganisms will increase in numbers but not all autochtonous.

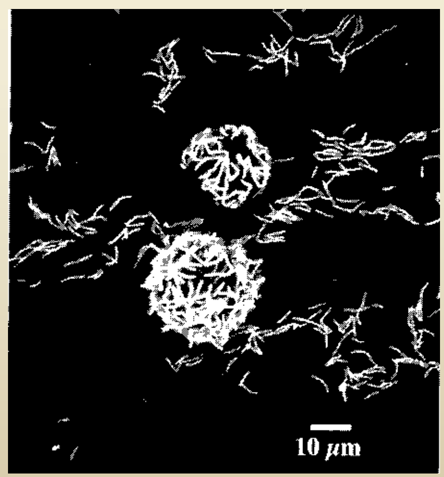
Access to Contaminants



No surfactants

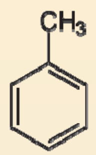


With surfactants

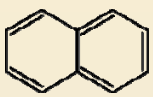


Photomicrography of bacterial cells adhering to an oil droplet

DETECTION OF CATABOLIC GENES IN INDIGENOUS MICROBIAL CONSORTIA ISOLATED FROM A DIESEL-CONTAMINATED SOIL

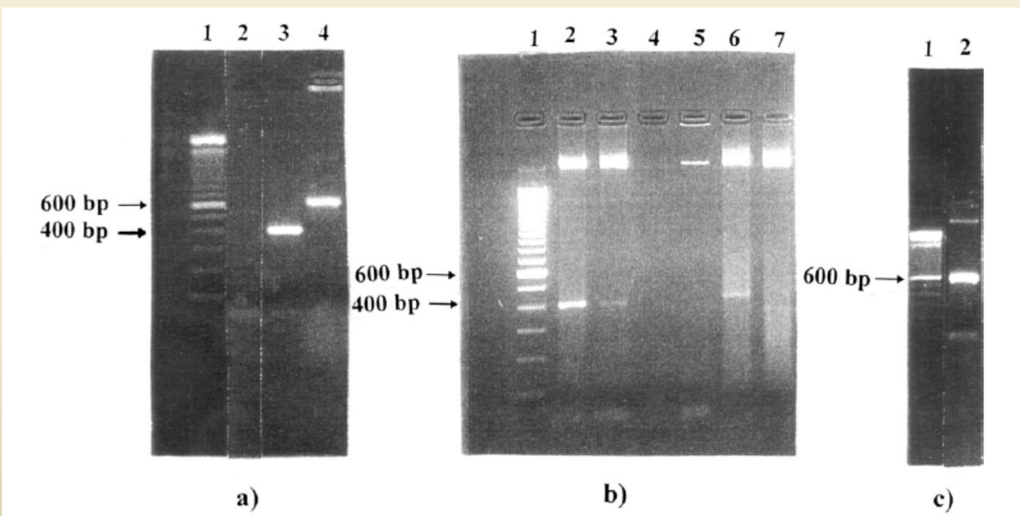


Toluene



Naphtalene

Total bacterial counts	Contaminated soil (CFU/g)	Pristine soil (CFU/g)
AODC	$6.1 \pm 1.9 \times 10^{12}$	$3.4 \pm 1.2 \times 10^{12}$
Heterotrophic	$2.2 \pm 0.4 \times 10^{7}$	$3.6 \pm 0.7 \times 10^{10}$
Diesel – degrading	$8.3 \pm 1.7 \times 10^{5}$	not detected
Toluene – degrading	$7.3 \pm 0.7 \times 10^{4}$	not detected
Naphthalene – degrading	$5.4 \pm 1.0 \times 10^{4}$	not detected



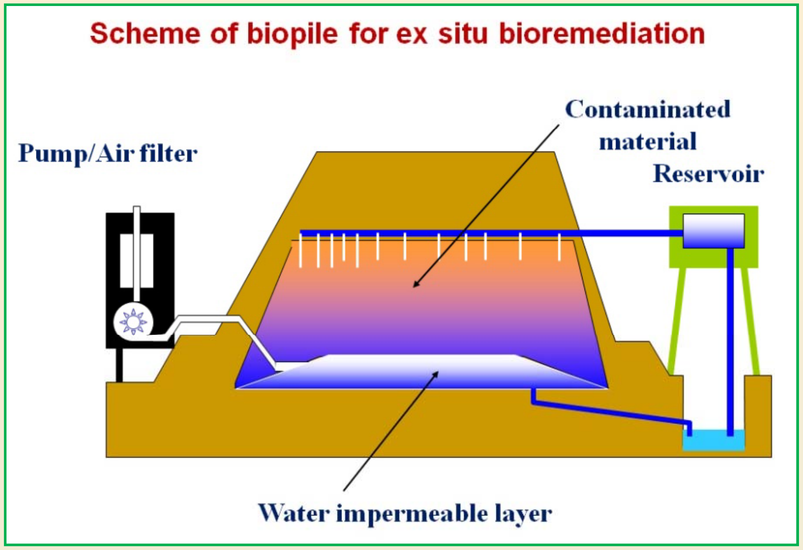
xylE - genes for aromatic hydrocarbon degradation
ndoB – genes for PAH degradation

- 74% of the colonies from the **diesel-consortium** possessed the *xylE* gene, and the *ndoB* gene (78%);
- Minority (29%) of the **toluene-consortium** harbored the *xylE* gene and no *ndoB* gene;
- 59% of the colonies from the **naphthalene-consortium** had the *ndoB* gene, and did not have the *xylE* gene;

These results indicate that the microbial population has been naturally enriched in organisms carrying genes for aromatic hydrocarbon degradation and that significant aromatic biodegradative potential exists at the site.

1. J. Milčić-Terzić, Y. Lopez-Vidal, M.M. Vrvić, S. Saval, Biodegradation potential assessment of microbial consortia isolated from a diesel-contaminated soil, *Water Sci. Technol.* 42 (2000) 403-406.
2. J. Milcic-Terzic, Y. Lopez-Vidal, M.M. Vrvic, S. Saval, Detection of catabolic genes in indigenous microbial consortia isolated from diesel-contamined soil, *Bioresource Technol.* 78 (2001) 47-54.

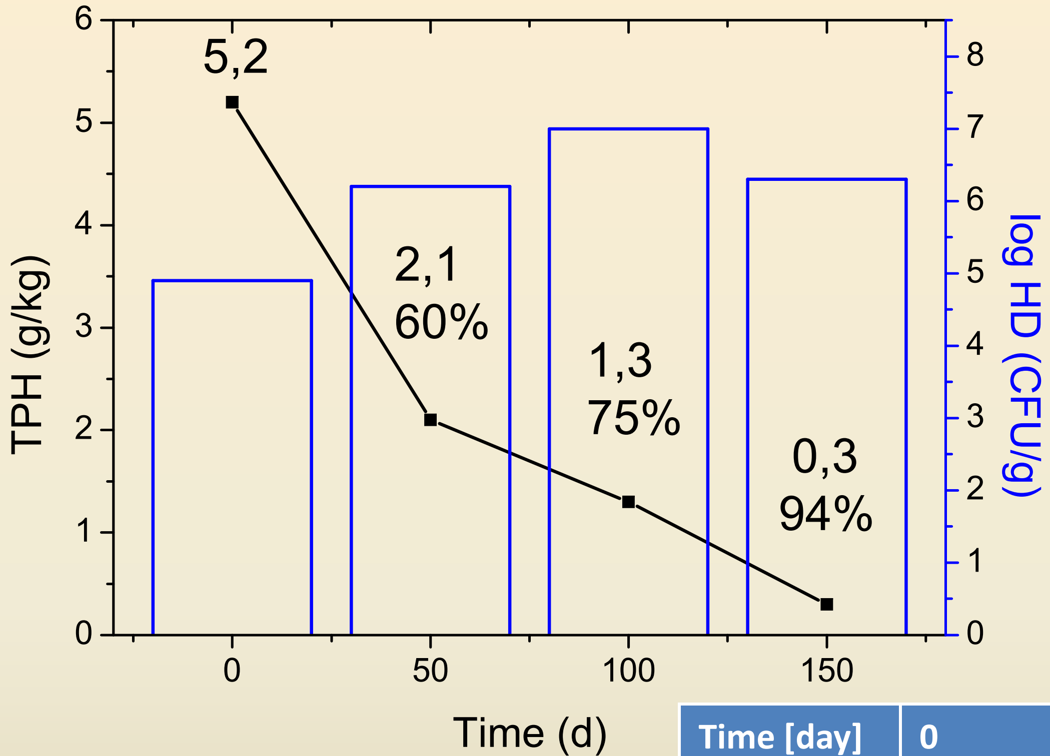
EX SITU BIOREMEDIATION OF <u>WASTE MAZUT (150t</u>) FROM BELGRADE POWER PLANTS ON 600 m^3 BIOPILE




Mixing of soil


Biostimulation and bioaugmentation

CHANGES IN TOTAL PETROLEUM HYDROCARBON CONTENT AND NUMBER OF HYDROCARBON DEGRADERS DURING BIOREMEDIATION ON INDUSTRIAL LEVEL



Average rate of TPH degradation:
0-50 day: **62** mg/kg/day
0-100 day: **16** mg/kg/day
100-150 day: **20** mg/kg/day

Reduced biodiversity at the expense of increasing the number of HD within the number of TC!

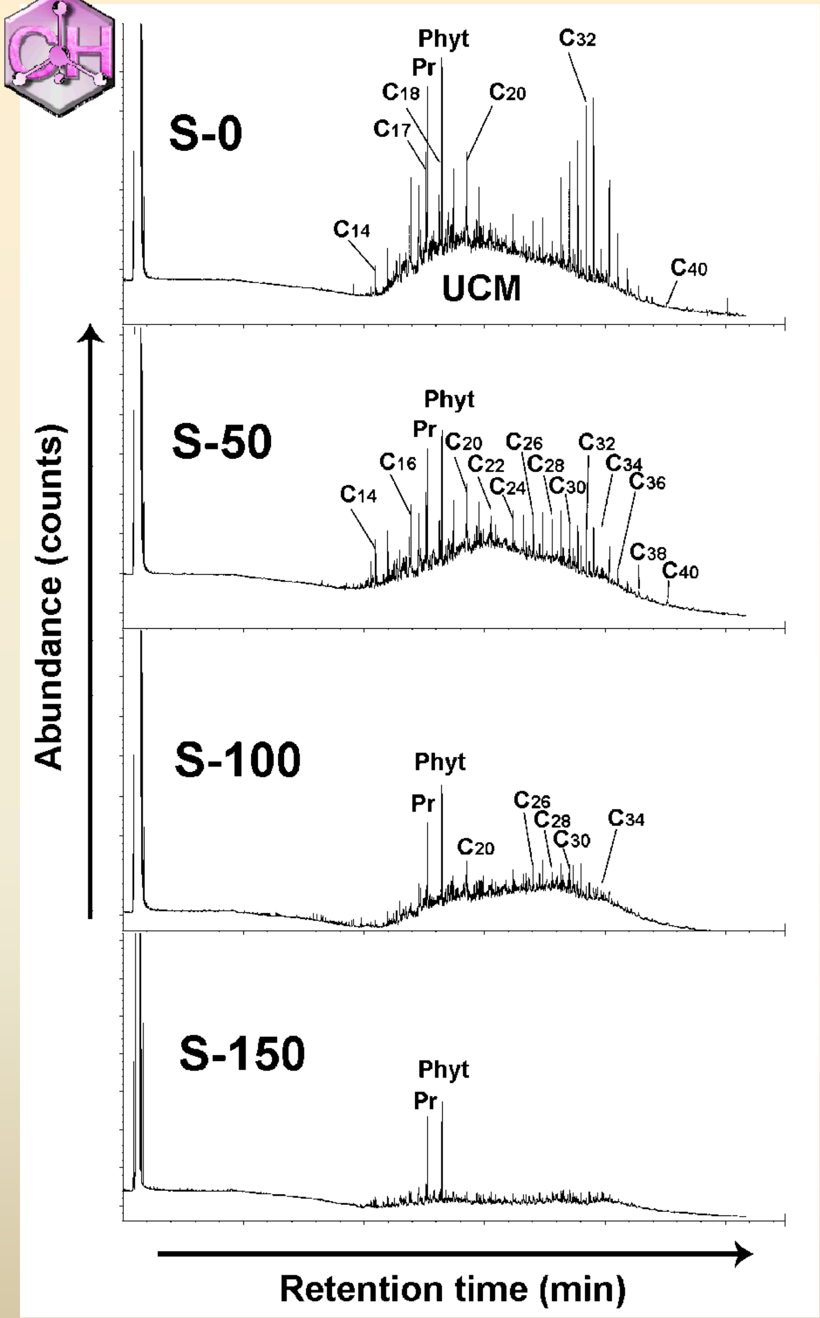
Time [day]	0	50	100	150
TC [CFU/g]	2.0×10^6	2.2×10^6	1.3×10^7	8.0×10^6
HD [CFU/g]	7.2×10^4	1.5×10^6	9.9×10^6	2.0×10^6
HD [%]	4	68	76	25

V.P. Beškoski, G. Gojgić-Cvijović, J. Milić, M. Ilić, S. Miletić, T. Šolević, M.M. Vrvić, *Ex situ* bioremediation of a soil contaminated by mazut (heavy residual fuel oil) – A field experiment, *Chemosphere,* 83, (2011) p. 34-40.

CHARACTERIZATION OF HYDROCARBON DEGRADING STRAINS

- Biochemical
- Microbiological
- Molecular

During bioremediation of hydrocarbon contaminated soil a stable microbial community had been formed after initial fluctuations, and the microorganisms which decompose hydrocarbons were the dominant microbial population at the end of the *ex situ* bioremediation process.



Pseudomonas, Achromobacter, Sphingomonas, Acinetobacter, *Bacillus*, *Micrococcus*, *Mycobacterium*, *Micromonospora*, *Rhodococcus* (9)

Pseudomonas, Achromobacter, Sphingomonas, Bacillus, *Mycobacterium*, *Micromonospora, Rhodococcus* (7)

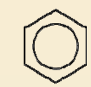
Pseudomonas, Achromobacter, Bacillus, Micromonospora, Rhodococcus (5)

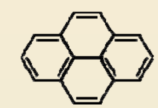
Pseudomonas, Achromobacter, Sphingomonas, Acinetobacter, *Bacillus*, *Staphylococcus*, *Micrococcus*, *Mycobacterium*, *Micromonospora*, *Rhodococcus*, *Penicillium*, *Aspergillus* (12)

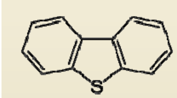
Growth of isolated strains on selected hydrocarbon as the sole C source

	Bacillus sp. NS026	*Bacillus* sp. NS032	*Micromonospora* sp. NS094	*Pseudomonas* sp. NS009	*Achromobacter* sp. NS014
Diesel fuel	+[a]	+	+	+	+
n-Hexane	+	+	+	+	+
n-Hexadecane	+	+	+	+	+
n-Octadecane	+	+	+	+	+
Benzene	+	+	+	+	+
Toluene	+	+	–	–	–
Xylene	–	+	–	–	–
Phenantrene	–	+	+	–	–
Pyrene	+	+	+	+	+
Etylbenzene	+	+	–	–	–
Octylbenzene	+	+	+	+	+
Hexadecylbenzene	+	+	+	+	+
Sodium-benzoate	+	+	+	–	+
2-Phenylphenol	+	+	+	+	–
Dibenzothiophene	+	+	+	+	+

[a] +viable growth observed in 1×10^{-5} dilution







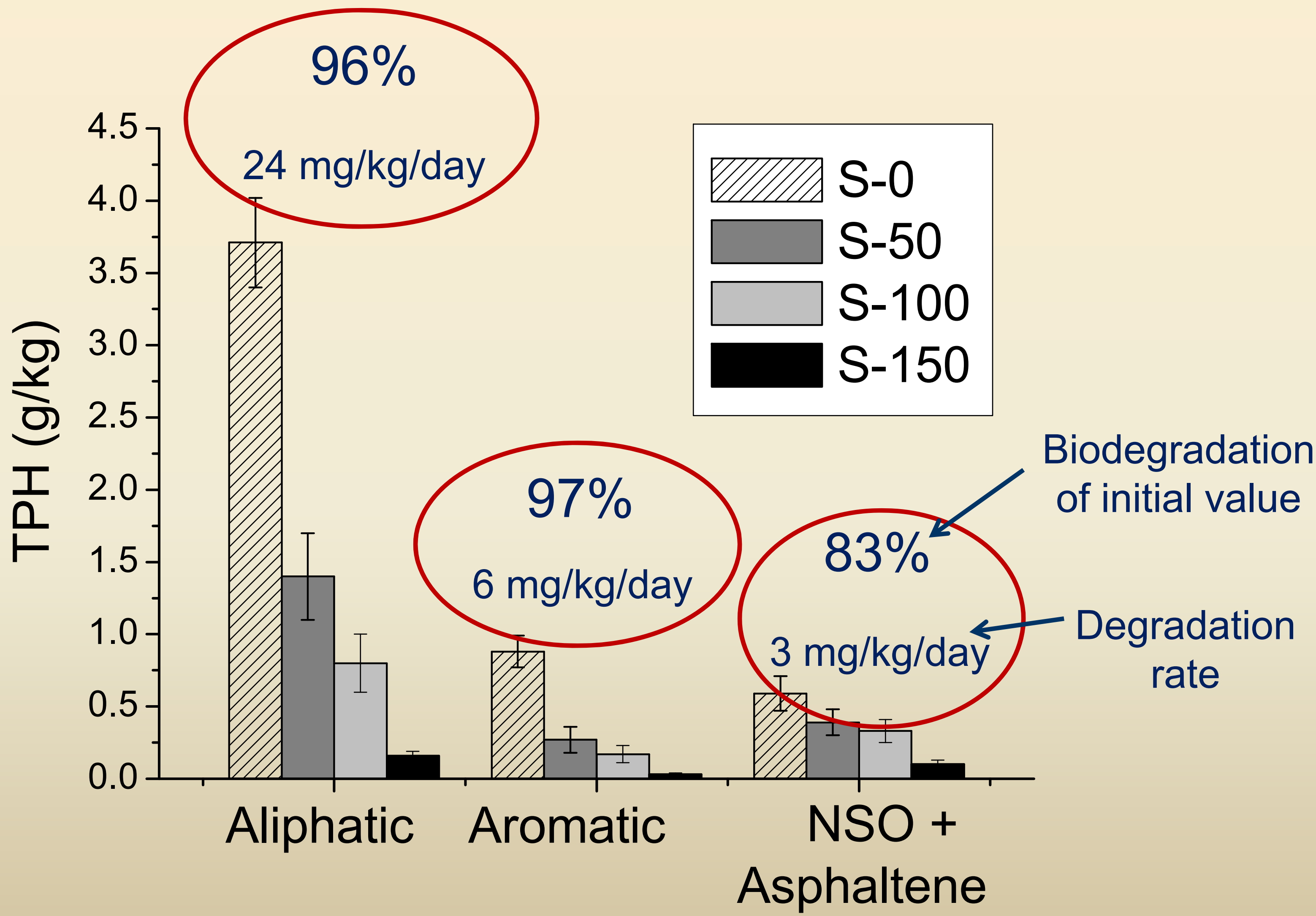
Tolerance to metal ions, mmol l^{-1}

	Ni^{2+}	Cu^{2+}	Cr^{3+}	Cd^{2+}
Bacillus sp. NS026	25	2.5	25	1.25
Bacillus sp. NS032	5	2.5	2.5	1.25
Micromonospora sp. NS094	5	2.5	5	2.5
Pseudomonas sp. NS009	5	25	5	25
Achromobacter sp. NS014	12.5	12.5	2.5	12.5

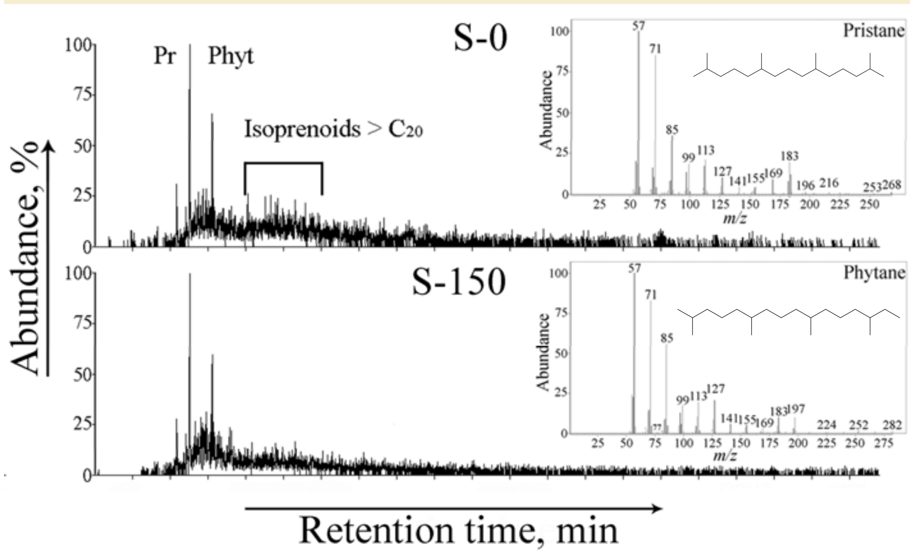
Broad capacity for the degradation and ability to survive!!!

G.D. Gojgic-Cvijovic, J. S. Milic, T. M. Solevic, V. P. Beskoski, M. V. Ilic, L. S. Djokic, T. M. Narancic, M. M. Vrvic, Biodegradation of petroleum sludge and petroleum polluted soil by a bacterial consortium: a laboratory study, *Biodegradation,* (2011) DOI 10.1007/s10532-011-9481-1.

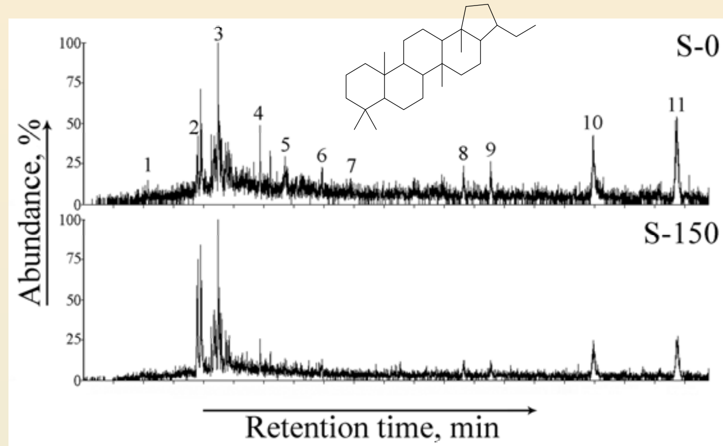
CHAGES IN GROUP COMPOSITION CONTENT DURING *EX SITU* BIOREMEDIATION ON INDUSTRIAL LEVEL (600m^3)



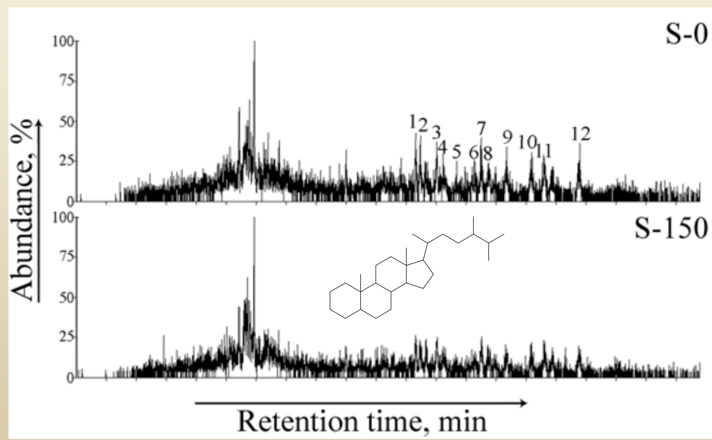
BIODEGRADATION OF ISOPRENOIDS, TERPANES AND STERANES DURING *EX SITU* BIOREMEDIATION ON INDUSTRIAL LEVEL (600m^3)



Fragmentograms of isoprenoids (SIM, *m/z* 183)



Fragmentograms of terpanes (SIM, *m/z* 191)

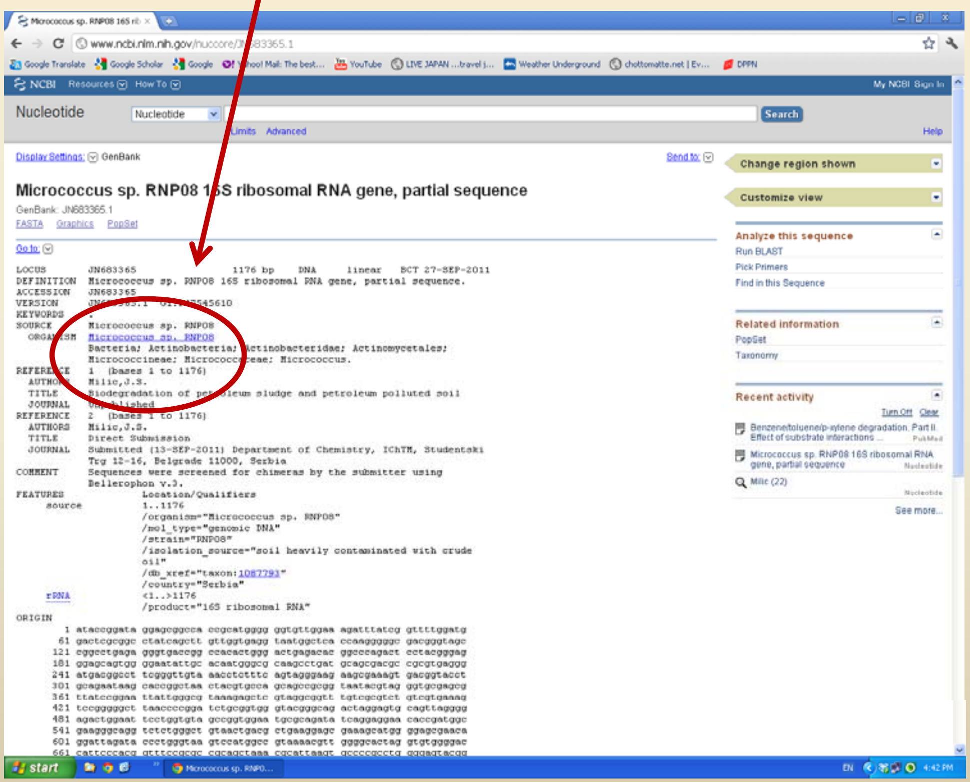


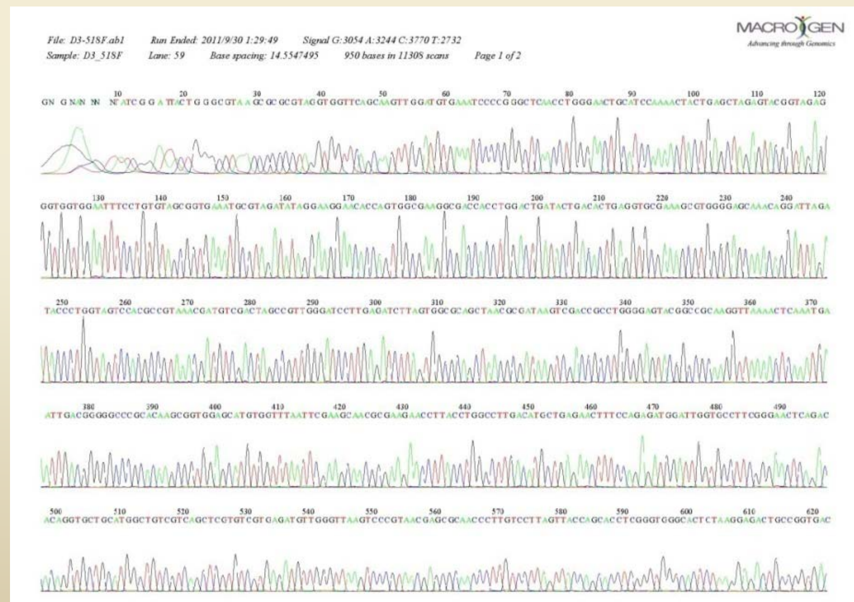
Fragmentograms of steranes (SIM, *m/z* 217)

Reduction and loss of resolution of individual signals!!!

V.P. Beškoski, M.Takić, J.Milić, M.Ilić, G.Gojgić-Cvijović, B. Jovančićević and M. M. Vrvić, Change of isoprenoids, steranes and terpanes during *ex situ* bioremediation of mazut on industrial level, *J. Serb. Chem. Soc.* **75 (11),** (2010) 1605–1616.

Deposition of 16s RNA sequence of microorganisms in GENBANK





J.S. Milic, V.P. Beskoski, M.V. Ilic, S.A. M. Ali, G.Dj. Gojgic-Cvijovic and M.M. Vrvic, Bioremediation of soil heavily contaminated with crude oil and its products: composition of the microbial consortium, *J. Serb. Chem. Soc.* 74 (4), (2009), p. 455-460.

CONCLUSIONS

1. *Ex situ* bioremediation of soil contaminated with high concentrations of oil and its derivatives on industrial-scale can be effectively implemented.

2. Decrease in biodiversity of microbial communities of polluted soil goes at the expense of increasing the share of the zymogenous population able to degrade petroleum hydrocarbons.

3. Key to the success in bioremediation is isolation, selection and adaptation of zymogenous microorganisms from the polluted soil, which is the subject of bioremediation.

4. Biostimulation and bioventilation/aeration, are indispensable and compatible elements of successful bioremediation.

5. None of the analyzed oil fractions is non-degradable, but only vary the rate of degradation!

6. Bioremediation of mazut polluted soil for the first time on an industrial level!

RESEARCH GROUP

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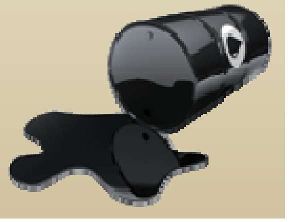

THANK YOU FOR YOUR ATTENTION!