Kill•Spill

Integrated Biotechnological Solutions for Combating Marine Oil Spills

Deliverable D4.5

Delivery of a Metabolic Atlas of the best-performing experimental setup and the high pressure reactor

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1   About this Deliverable

Work in WP4 relates to the analysis of the catabolic capacities as consequence of application of bio-augmentation and/or bio-stimulant formulations and high pressure for enhanced bioremediation. WP4 contemplated the microcosm experiments to ensure efficiency and optimal composition of developed formulations and nutrients and pressure.

This deliverable refers to the usage of metagenomics/transcriptomic data in combination with Kill•Spill Microarray Chip data, to draft the metabolic atlas (herein referrred to as catabolic networks) in the experimental samples and to understand key degrading catabolic variations associated to different formulations and identify best formulations and conditions.

2   Introduction

Strategies such as *i*) bio-stimulation with different nitrogen sources to improve microbial growth and bio-surfactants to improve oil solubility and *ii*) bio-augmentation with oil-degrading microorganisms to speed up the rate of oil degradation, are often regarded as strategies of choice in combating oil spills in marine environments. It is fundamental to analyze the consequences of the application of different nutrients, bio-surfactants and microbial formulations under different conditions (e.g. those representing deep sea conditions such as under pressure) to design and identify optimal formulations for oil spill mitigation in the sea. Following on from this, through D4.5 we deliver a ready-to-use bioinformatics tools that allows producing "metabolic atlas" to examine:

- Effect of bio-stimulants (TUC, Bangor) on degradation pathways.
- The effect of oil-degrader bio-augmented set-ups (with strains from UMIL and UCL, e.g. fungi) on degradation pathways.
- Effect of bio-surfactant producing strains (UNIBO, MADEP) on degradation pathways.
- The effect of pressure on oil degradation.

The tools (summarized in Table 1) used are:

- (a) The web-based AromaDeg resource that based on the utilization of shotgun DNA meta-sequences from the community organisms that develop under any experimental setup, allows identifying genes involved in degradation pathway.
- (b) The Kill•Spill Microarray Chip that, based on the utilization of labelled DNA from the community organisms that develop under any experimental setup, allows identifying genes involved in degradation pathway.
- (c) A computation method that allows the automatic reconstruction of degradation networks (referred to as metabolic atlas) in a graphical format. The complete workflow, including the scripts and commands, which has been reported (Bargiela et al., 2015), allows visualization of the alterations on degradation pathways under any experimental condition or setup.
- (d) Transcriptomics tools for evaluating alterations on alkane degradation pathways in high pressure reactor.
- Tools (a) to (c) have been used to deliver, as validated case-of-study, the metabolic atlas that allows examining the effect of a bio-stimulant formulation based on the addition of 0.7 g per liter of the natural fertilizer uric acid (UA) for improving oil degradation. This nutrient was found in the frame of Kill Spill investigations (Gertler et al., 2015) to be one of the most cost-efficient bio-stimulants for enhancing bacterial growth in polluted sediments and for enhancing oil degradation, as compared to other commonly used bio-stimulants such as ammonium. As study cases, microcosms set up with sediments from chronically crude oil-contaminated marine sediments from Ancona harbor were used. Tool (d) has been used to

deliver, as validated case-of-study, the metabolic atlas that allows examining the effect of pressure in 3 axenic alkane-degrader cultures provided with single alkanes (dodecane).

Table 1   Monitoring tools and formulation/conditions tested

Monitoring tool	Formulation/condition tested	Providing Partner
The web-based AromaDeg resource and Kill•Spill Microarray Chip targeting genes involved in degradation pathway	Best bio-stimulant formulation based on the addition of the natural fertilizer uric acid	Bangor, IAMC-CNR, ICP-CSIC (Partners 14, 9, 7)
A computational workflow allowing visualization of the alterations on degradation pathways under any experimental condition or setup	Best bio-stimulant formulation based on the addition of the natural fertilizer uric acid, tested in microcosms set up with crude oil	ICP-CSIC (partner 7)
Transcriptomics tools for evaluating alterations on alkane degradation pathways in high pressure reactor	Pressure in cultures provided with single alkanes	UGent (partner 11)

Table 2   Tests performed

Monitoring tool	Sample	Partner
The web-based AromaDeg resource and Kill•Spill Microarray Chip and computational workflow targeting genes involved in degradation pathway	Microcosms set up with sediments from chronically crude oil-contaminated marine sediments from Ancona harbor (Italy) and the natural fertilizer uric acid, which was found as a best bio-stimulant for promoting oil degradation. Microcosms set up with ammonium were used as controls.	Bangor, IAMC-CNR, ICP-CSIC (Partners 14, 9, 7)
Transcriptomics tools for evaluating alterations on alkane degradation pathways in high pressure reactor	3 axenic alkane-degrader cultures provided with single alkanes (dodecane)	UGent (partner 11)

3   Results

3.1   Catabolic atlas/network delivered for microcosms set up with best bio-stimulants [Bangor, IAMC-CNR, ICP-CSIC]

We have recently delivered data, in the form of a delivered publication, which demonstrated that biodegradable natural fertilizers like uric acid can be used as most cost-efficient bio-stimulant formulation, as compared to the low soluble ammonium, for enhancing bacterial growth in polluted sediments and for increasing crude oil degradation (Gertler et al., 2015). To prove this, we have applied the web-based AromaDeg resource to deliver the metabolic atlas (herein referred to as catabolic networks) of the microcosm set up with sediments from chronically crude oil-contaminated marine sediments from Ancona harbor (Italy) and the natural fertilizer UA; microcosms set up with same sediment but with AMM as nitrogen source were used as control (Gertler et al., 2015). In both cases, crude oil was used to create the microcosms. Ancona sediments were selected because Ancona harbour is very close to the urban area and hosts a multi-purpose port receiving cruise liners,

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passenger ferries, transport liners and fishing boats. A minor part of the related airborne pollutants is due to the vessels calling at the port while the main contribution comes from road traffic and other human activities. Furthermore, sediments in Ancona harbour are heavily contaminated due to its role as a major ferry terminal and industrial port on the Adriatic Sea.

Basically, after microcosms were produced, the resulting microbial communities from microcosms were destructively sampled, the isolated DNA subjected to both paired-end sequencing (Illumina HiSeq 2000) (CSIC-ICP) and fragmentation and labelling to perform hybridization with the Kill•Spill Microarray Chip (Bangor). Further, the web-based AromaDeg resource (see details in Bargiela et al., 2015a,b) was used for *metabolic atlas* (or catabolic network) reconstruction. Briefly, potential protein-coding genes (≥ 20 amino acids long) obtained by direct Illumina HiSeq sequencing of DNA material of the corresponding microcosms (delivered in the publication Gertler et al., 2015) or obtained from probes that hybridized with the Kill•Spill Microarray Chip, constituted the input information.

AromaDeg is a web-based resource with an up-to-date and manually curated database that includes an associated query system which exploits phylogenomic analysis of the degradation of aromatic compounds. This database addresses systematic errors produced by standard methods of protein function prediction by improving the accuracy of functional classification of key genes, particularly those encoding proteins of aromatic compounds' degradation. In brief, each query sequence corresponding to each of the hybridized oligonucleotide that matches a given protein family of AromaDeg is associated with an experimentally validated catabolic enzyme performing an aromatic compound degradation reaction. Individual reactions, and thus the corresponding substrate pollutants and intermediate degradation products, can be linked to reconstruct catabolic networks. We have recently designed and delivered an in-house script allowing the automatic reconstruction of such networks in a graphical format, which was used in present work. The script allows visualization and comparison potential pollutant being degraded and the pathways implicated in their degradation. The complete workflow, including the scripts and commands used for catabolic network reconstruction has been delivered in two recent publications (Bargiela et al., 2015a, 2015b).

Figure 1 summarizes the catabolic networks that have been delivered, following the above protocol, to investigate the effect of bio-stimulants on degradation pathways. Particularly, we deliver the metabolic atlas for best bio-stimulant formulations based on the addition of 0.7 g per liter of the natural fertilizer uric acid (Bargiela et al., 2015b), using ammonium as no cost-efficient bio-stimulant. The analysis of the networks revealed that different bio-stimulants (uric acid and ammonium) applied in crude-oil polluted sediments caused significant alteration in degradation capacities. This was demonstrated by showing that uric acid enriched for bacteria with the capability of degrading pollutants otherwise not degraded, or possibly degraded at low level, by those stimulated by the addition of ammonium and vice versa. Therefore, the results of this deliverable show that smart formulations based on the application of multiple nitrogen sources, rather than commonly used single sources (mostly ammonium), for example, may increase the efficiency of the biological removal of the widest diversity of aromatic pollutants and could be essential to support effective biodegradation strategies in response to an oil spill incident or in response to chronical pollution. Thus, as herein demonstrated, the utilization of both ammonium and uric acid in conjunction will have a double aim. In one side, ammonium may most likely enhance the bio-stimulation of bacterial populations capable of degrading heavy oil components such as naphthalene, phenanthrene and dibenzofuran, as well as sulfonated-benzenes and substituted benzoate derivatives such as p-cumate (Figure 1). In other side, uric acid will promote the growth of bacteria most active against benzene, orcinol-, ibuprofen- and phenyl-propionate (Figure 1). This will provoke a significant increase in multiple aromatics consumption in polluted areas.
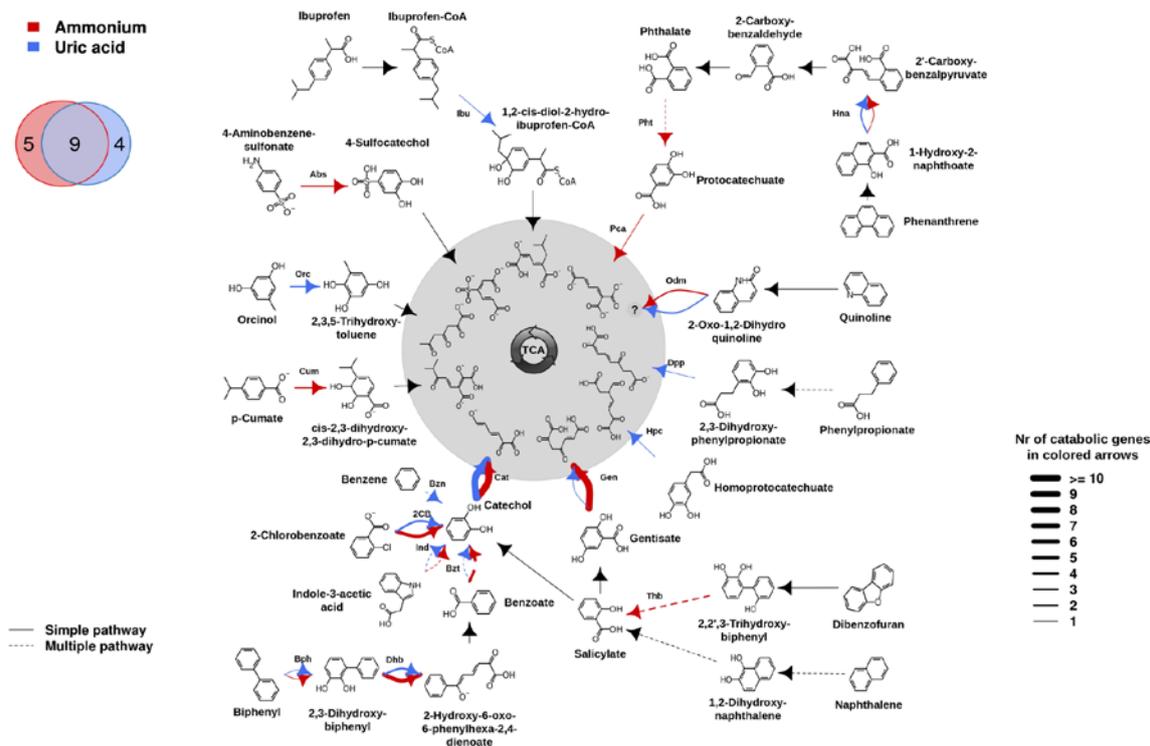
Figure 1 **Potential aromatic catabolic networks in the ammonium and uric acid microcosms (see color code) set up with sediments from crude oil-contaminated marine sediments from Ancona harbor.**

The biodegradation network reconstruction was performed as delivered in Bargiela et al., (2015a,b). For network reconstruction, each catabolic gene sequence subsequently was assigned to a metabolic substrate as well as a product with an assigned code. The putative substrates and products processed in the sample were connected, creating a metabolic network using appropriate scripts and commands (for details, see Bargiela et al. (2015a,b)). The number of each catabolic gene assigned to degradation reactions, is represented by the thickness of the lines in the figure and the complete list of substrates possibly degraded by the communities are summarized. Common and microcosm-specific initial pollutants or intermediates for which presumptive degradation signatures were identified are specifically indicated in the Venn diagram. Solid lines represent single step reactions while dotted lines represent degradation steps where multiple reactions are involved (for details see Bargiela et al., 2015a,b). Codes for proteins encoded by genes as follows: Abs, 4-aminobenzenesulfonate 3,4-dioxygenase; Bph, biphenyl dioxygenase; Bzn, benzene dioxygenase; Bzt, benzoate dioxygenase; Cat, catechol 2,3-dioxygenase; 2CB, 2-chlorobenzoate dioxygenase; Cum, *p*-cumate dioxygenase; Dhb, 2,3-Dihydroxybiphenyl dioxygenase; Dpp, 2,3-dihydroxyphenylpropionate dioxygenase; Gen, gentisate dioxygenase; Hna, 1-hydroxy-2-naphthoate dioxygenase; Hpc, homoprotocatechuate 2,3-dioxygenase; Ibu, ibuprofen-CoA dioxygenase; Ind, Rieske oxygenase involved in indole acetic acid degradation; Odm, 2-oxo-1,2-dihydroxyquinoline monooxygenase; Orc, orcinol hydroxylase; Pca, protocatechuate 3,4-dioxygenase; Pht, phthalate 4,5-dioxygenase; Thb, 2,2',3-trihydroxybiphenyl dioxygenase.

Based on the data delivered above, it has been demonstrated that the *metabolic atlas* generated allows investigating the effect of different formulation in the context of effective degradation efficiency. Accordingly, using the approach delivered in D4.5, we plan to further reconstruct the *metabolic atlas* for best bio-augmented set-ups (with strains from UMIL and UCL, e.g. fungi) and set-ups with bio-surfactant producing strains (UNIBO, MADEP). Such formulations have been just

selected and micro- and mesocosms will be produced and the corresponding material (DNA) will be used to evaluate the effect of such best formulation on degradation pathways.

3.2   Catabolic atlas/network delivered for anexic cultures set up with alkanes [UGent]

UGent tested under 0.1, 5 and 10MPa (equivalent to surface waters, 500 and 1000 m depth) 3 axenic strains of the ubiquitous, hydrocarbonoclastic *Alcanivorax* genus, namely *A. jadensis* KS 339, *A. dieselolei* KS 293 and *A. borkumensis* SK2. This genus is known for dominating bacterial blooms following oil spills on surface waters and grows rapidly at sites where they were previously absent (Hara et al., 2003).

A recognized limitation for oil bioremediation is its tendency to create tar balls and droplets, which eventually sink to the seafloor together with bacterial biomass belonging to the surface. Overwhelming oil release to the environment enhances also marine snow formation, which eventually drives hydrocarbons to the seafloor (Passow et al., 2010). These phenomena postulate that microbial oil degraders at the sea surface, such as *Alcanivorax*, will eventually deal with increased hydrostatic pressure. The latter is a physical state that typically features sub-surface waters and deep sea, as every 10 meters of seawater pressure increases about 0.1MPa. However, oil may contaminate the deep sea also as a result of the use of dispersants, by adsorption to heavier particulate or non-miscible components, or due to problems encountered at deep-sea drilling sites (as in the case of the Deepwater Horizon spill in April 2010).

In order to identify the role played by the abundant *Alcanivorax* in oil bioremediation under mild hydrostatic pressure as that experienced at low depth, we have axenically cultivated the 3 aforementioned strains of *Alcanivorax* up to 10MPa using the *n*-alkane dodecane (C12) as sole carbon source.

All strains had a piezosensitive profile, with lower growth rates at increasing pressures. While in *A. jadensis* KS 339 both cell number and integrity decreased upon increasing pressure, *A. dieselolei* KS 293 showed comparable cell integrity at 0.1 and 10MPa, indicating a certain structural resistance to hydrostatic pressure. In *A. borkumensis* SK2, cell survival was essentially compromised under 5MPa, but a further increase to 10MPa indicated that pressure-resistance mechanisms were activated, as cell number and integrity were significantly higher than at 5MPa ($P <0.05$).

Transcriptomic response in *A. dieselolei* and *A. borkumensis* revealed a response to pressure which was similar only in part. Common network of upregulated pathways involved energy production, as ATP synthase and respiration chain related genes were upregulated under 10MPa as compared to 0.1MPa. Also, almost all the subunits of the multimeric ribosome complex were highly upregulated, suggesting that protein synthesis was the most compromised biological process impaired by mild hydrostatic pressure. As concerns alkane degradation, genes related with enzymes involved in hydrocarbons activation, such as cytochrome P450 and alkane 1 mono-oxygenase, were generally upregulated. However, the expression of none of the genes involved in fatty acid catabolism was upregulated in either *A. dieselolei* or *A. borkumensis*. On the contrary, in *A. borkumensis* SK2 cells, where a clear pressure-resistance mechanism was triggered, genes involved in the fatty acids biosynthesis pathway were either upregulated or unaffected by pressure.

These results support the hypothesis that C12 or its derivatives were used as building blocks rather than energy source by microbes under mild pressure. Consistently with this observation, the TCA cycle and the glyoxylate cycle, alternative pathways using fatty acids with 2 or 3 carbons to generate energy, were either upregulated or unaffected in *A. borkumensis* SK2 under pressure. Introduction of an oxygen atom into saturated hydrocarbons may proceed through a terminal or subterminal pathway, which can coexist within the same microorganism (Rojo, 2009). In the case of C12, this

would eventually result into the generation of dodecanoyl-CoA (terminal oxidation) and/or decanoyl-CoA and acetyl-CoA (subterminal oxidation). As the latter would both generate acetyl-CoA to feed TCA and glyoxylate cycle and provide building blocks for fatty acids biosynthesis, our hypothesis is that under such stressing conditions *Alcanivorax* cells would prefer this pathway.

In conclusion, energy saving strategies and efficient culture growth mechanisms appears to be crucial for piezosensitive hydrocarbonoclastic bacteria provided with oil as sole carbon source.

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