

The “*Alcanivorax* paradox”: mild hydrostatic pressure impacts deep-sea oil bioremediation

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The relevance of oil spill contamination for the North Sea and the Belgian coast

Belgium coastline is one of the shortest among North Sea coastal States. Yet, it is subjected to an intense naval traffic and is considered of strategical relevance ecologically and socioeconomically (Schallier and Van Roy, 2016). The presence of several major harbors in the South part of the North Sea together with illegal oil discharge exposed this area to chronic oil pollution. Since policy measures as the MARPOL 73/78 Convention (1983) and the multilateral Bonn Agreement (1969/1983) were established, aerial surveillance and oil spill response sensibly reduced oil pollution of surface and shallow Belgian waters (Lagring et al., 2012), this target met also for other surrounding countries (e.g., The Netherlands [Camphuysen and Vollaard, 2016] and Denmark [Christensen and Carpenter, 2016]). Nonetheless, when one speaks of petroleum and sustainable development, maritime transport with oil cargo will continue to exist along with the risk of accidents involving oil and by-product spills (Barrow and Rothschild, 2002). A quick look at the multiple petroleum reservoirs studding the North Sea indicates that this remains a major threat for Belgium as for other North Sea coastal State.

Alongside naval traffic-related pollution, the recent Deepwater Horizon (DWH) disaster in the Gulf of Mexico (April 2010) opened a new era for oil spills worldwide. The blowout of a well rig at 1500 m below surface level (bsl) resulted in the largest marine oil spill in history (780'000 m³ of spilled oil). Oil deep-sea platforms are defined as those operating in the mesopelagic zone (200 to 1000 m bsl, subjected to mild hydrostatic pressure [HP] of 2 to 10 MPa, or 20 to 100 bar) and further below (bathypelagic zone). There are several deep-sea oil fields in the world, two being located in the North Sea (Troll A and Gullfaks, in the Norwegian sector, 472 and 380 m, respectively). Limited maturity of deep-sea technologies was claimed as a critical factor for the DWH spill (Jernelov, 2010; Thibodeaux et al., 2011) which in fact lasted for 3 months. The implications for the Gulf of Mexico were impressive: dispersant application at the well rig, alongside deep-sea conditions of temperature and HP, formed multiple oil plumes (Kujawinski et al., 2011), with underwater currents enlarging the deep-sea area directly impacted by oil. The estimated deep seafloor contaminated area is 3200 to 8000 km² large (Chanton et al., 2014). The oil reaching the surface was eventually delivered to the coast and directly impacted an area of 180'000 km² and more than 25'000 km of shorelines (Norse and Amos, 2010). While the range of techniques to remove oil on surface and shallow waters is relatively wide (e.g., booms and skimmers, dispersants, oil burning), at deep-sea conditions microbial biodegradation represents the only (bio)technology available.

With continued political and economic pressure to get access to deep-sea oil the risk for future spills in the deep sea is not expected to decrease. A full understanding of the physiological requirements enhancing bioremediation at deep-sea condition is therefore pivotal to provide policymakers with critical information to elaborate proper protocols to combat deep-sea oil spills. This is particularly true when considering the metabolic network entrenched in deep-sea marine life. Following the DWH, the implications for the latter were outstanding: deep-sea sediments were classified as low to moderately polluted (Romero et al., 2015); deep-sea pore-water exerted high toxicity levels and DNA mutagenesis (Paul et al., 2013); primary production and carbon export to the deep sea was reduced (Prouty et al., 2016); in sea-food, concentration of certain petroleum-hydrocarbons was 1000 times above the threshold for human consumption (Sammarco et al., 2013). While another oil spill of the proportions of the Gulf of Mexico is unlikely, the impact of a deep-sea uncontrolled oil release within the much smaller area of the North Sea would expose oil remediation operators to a number of novel different issues, including the lack of meaningful long-term solutions for oil degradation.

Research environment supporting the present project

The text corpus presented here was elaborated in the frame of the 33-partner EU project Kill Spill (grant agreement N. 312139, Integrated Biotechnological Solutions for Combating Marine Oil Spills). Belgian science was represented by the Center for Microbial Ecology and Technology (CMET) of the University of Gent (UGent). The data discussed here refers to the 3-year research period of the present candidate, Alberto Scoma, as postdoctoral researcher at CMET-UGent, which resulted in three research

articles and one review in Q1 international peer-reviewed journals (i.e., Scientific Reports, impact factor [IF] 5.5, and Frontiers in Microbiology, IF 4.2), all published between March and August 2016. Following the “*Alcanivorax paradox*” case we received two invitations for a paper by EMBO Reports (IF 7.7) and Trends in Biotechnology (IF 12.1), currently under preparation. We expect the publication with these four different editors (Nature, Frontiers, EMBO and Cell) to widen the impact of our results. It is worth mentioning that the present body of work is completed by two further manuscripts presently under revision in Frontiers in Microbiology and Environmental Science and Technology (IF 5.4) and one under preparation for ISME Journal (IF 9.3). These latter findings are not discussed here. A complete list of the material published, under revision or preparation is provided in chapter six of my attached CV. The present results put CMET-UGent atop the field of HP microbial oil degradation physiology, an emerging topic with a rising number of competitors, with current developments involving a PhD candidate who is applying synthetic communities and in situ conditions of both HP and low temperature.

Physiology of hydrocarbons degradation at mild hydrostatic pressure

Due to its unique nature, the DWH spill was extensively investigated either through in situ studies employing next-generation sequencing techniques or by ex situ experiments simulating microbial biodegradation in laboratory conditions. Despite supplying unprecedented information, both approaches failed to describe the exact metabolic routes and constraints in deep-sea bioremediation. In situ studies using molecular techniques can only provide information on potential biological activities while, with no exception, ex situ studies neglected the impact of one of the major drivers for biodiversity in marine environments, i.e., HP (Ghiglione et al., 2012).

Pressure increases linearly with depth about 0.1 MPa (or 1 bar) every 10 m of seawater column. The relative HP increase experienced in the mesopelagic zone (2 to 10 MPa) is much higher with respect to deeper zones, as HP doubles 5 times within 800 m, suggesting this might be a transition area for HP resistance. Other critical factors such as pH and salinity are not as remarkably affected by depth as HP, while temperature varies significantly in this zone although to a lower extent with respect to pressure and depending on latitude and season (Garrison, 2015). Very little is known about the physiological requirements and metabolic features of HP-adapted oil-degraders, with only four papers in the whole literature, two of which dating back to the 1970's (Schwarz et al., 1974; Schwarz et al., 1975; Grossi et al., 2010; Schedler et al., 2014). To investigate the impact of HP on oil biodegradation we tested three independent axenic cultures of the marine obligate oil degrader (i.e., hydrocarbonoclastic) *Alcanivorax* genus, namely *A. jadensis* KS_339, *A. dieselolei* KS_239 and the reference strain *A. borkumensis* SK2, using three HPs (0.1, 5 and 10 MPa, equivalent to surface water, 500 m and 1000 m bsl) and n-dodecane as sole carbon source. Sequences belonging to these species are ubiquitously detected in a wide range of oil-contaminated environments, from surface to deep sea (Lai et al., 2011), in both seawater and sediments (Wang et al., 2010; Kostka et al., 2011). In particular, *A. borkumensis* typically dominates oil-contaminated surface marine waters worldwide (up to 90% of the whole population within 30 days after the oil spill, Yakimov et al., 2007; Head et al., 2006), background to why it is adopted as a model organism to investigate hydrocarbon degradation pathways in marine environments (Kasai et al., 2002). In our experiments, HP significantly reduced cell replication in all *Alcanivorax* strains to a different extent (ranging from a factor 3 to 5), with the most severe effects observed for the predominant *A. borkumensis* where 5 MPa was sufficient to suppress culture growth (i.e., cell number at the end of the incubation was lower than at the onset). This was mirrored by a lowered CO₂ production by the cultures, a proxy for hydrocarbon degradation capacity, as reflected in a reduced pH acidification owe to CO₂ hydration in water as HCO₃⁻ + H⁺ (there are no fermentation products affecting pH under aerobic hydrocarbons degradation). Cell damage increased at increasing HP, with the major impact being observed in *A. borkumensis*, where intact cells were reduced to 10% already at 5 MPa. In this strain, a further HP increase to 10 MPa triggered some resistance mechanism, as cell intact and final number significantly improved with respect to 5 MPa, while remaining largely lower than at surface water pressure. Such a dramatic effect on hydrocarbon degradation and culture growth imposed by mild HP was confirmed by transcriptome analysis, with large downregulation affecting the whole genome expression at 10 MPa as compared to surface pressure in the examined *A. dieselolei* KS_293 and *A. borkumensis* SK2. While a genomic-based approach has been proposed for piezophilic adaptation (i.e., increased microbial growth at increasing HP) (Simonato et al., 2006), piezosensitive and piezotolerant bacteria might cope with HP through a physiological response. Given the piezosensitive nature of *Alcanivorax*, the few upregulated genes highlighted by transcriptomic analysis were indicative of the major physiological requirements imposed by mild HP shifts. The most interesting results concerned the hydrocarbon degradation pathway. Following alkanes activation by introduction of an oxygen atom, the generated fatty acid normally enters beta-oxidation to be fully mineralized to CO₂. None of the genes related to beta-oxidation was upregulated at HP, contrary to several biosynthetic pathways related with fatty acid elongation. Lack of upregulation of beta-oxidation genes does not entail that this was not active to some extent, provided that n-dodecane was the sole carbon and energy source. However, the capacity to withdrawn oxidized n-alkanes from the

degradation pathway and incorporate them as their corresponding fatty acids in the membrane has been shown for *Alcanivorax* (Naether et al., 2013) and other hydrocarbon-degrading microbes (e.g., *Marinobacter hydrocarbonoclasticus* [Doumenq et al., 2001] and *Rhodococcus erythropolis* [de Carvalho et al., 2009]). Alternative hydrocarbon utilization pathways may be explained as a way to counterbalance the impacted energy-production metabolism. The multicomponent ATPase complex was largely upregulated indicating that generation of ATP was affected by HP along with cytochromes, whose nature shifted from oxidases to reductases. The latter resembles a strategy adopted by *Shewanella piezophila* which possesses two respiratory chains for ambient and high HP (Kato and Qureshi, 1999). The central role of electron and proton transport at HP is majorly highlighted by the model piezophile *Photobacterium profundum* SS9 which possesses two complete operons for the F_0F_1 ATPase and multiple cytochrome sets (Vezi et al., 2005). Finally, the effect of concomitant stresses imposed by increased osmotic and HP on the model organism *A. borkumensis* SK2 was tested. A synergistic effect enhancing cell activity at HP was noted, but this resulted into a dramatically lowered culture performance, confirming the piezosensitive nature of this strain (Scoma and Boon, 2016).

The environmental significance of the *Alcanivorax* paradox

While predominating in oil-polluted surface waters worldwide, the bioremediation capacity of the hydrocarbonoclastic genus *Alcanivorax* is reduced by mild HP equivalent to only 500 m bsl (Scoma et al., 2016a,b). When *Alcanivorax* species were isolated from up to 5000 m bsl (up to 50 MPa, Liu and Shao, 2005; Lai et al., 2011) they could not grow <10°C, i.e., at much higher temperatures than those registered for these depths (<4°C). As such isolation protocols did not apply HP, one could conclude that HP relief reestablishes *Alcanivorax* capacity to grow. As a matter of fact, *Alcanivorax* isolates were obtained from decompressed DWH water samples (Gutierrez et al., 2013), resembling results for DWH surface water and sediment samples where *Alcanivorax* enrichment in situ was noted based on sequence analysis (Kostka et al., 2011; Liu and Liu, 2013). On the contrary, in situ *Alcanivorax* abundance in bathypelagic water (Gutierrez et al., 2013), oil plume (Hazen et al., 2010; Dubinsky et al., 2013; Yang et al., 2014) and deep-sea sediments was low (Kimes et al., 2013), unrelated to hydrocarbons (Kimes et al., 2013) and its contribution to deep-sea bioremediation considered negligible (Gutierrez et al., 2013). Upon reviewing the literature related to the DWH, we noted that the *Alcanivorax* paradox actually extends to all hydrocarbonoclastic bacteria (Scoma et al., 2016c) such as *Marinobacter* or the several *Oceanospirillales* genera as *Thalassolituus*, *Oleiphilus*, *Neptunomonas* and *Oleispira*, which were not enriched in deep-sea oil contaminated samples (Hazen et al., 2010; Dubinsky et al., 2013; Kimes et al., 2013; Mason et al., 2014). Low temperature was proposed to account for this (Redmond and Valentine, 2012), but species as *O. antarctica* are psychrophilic (Yakimov et al., 2003) and sequences belonging to these genera have been retrieved from hydrocarbon-seeps in the Gulf of Mexico (King et al., 2013).

Most importantly, impacted beta-oxidation at deep-sea HP was indicated by in situ studies on DWH water and sediment samples based on metabarcoding and other metaomics approaches (Hazen et al., 2010; Lu et al., 2012; Kimes et al., 2013; Mason et al., 2012; 2014; Rivers et al., 2013). Upregulation of alkane-1 mono-oxygenases responsible for n-alkane activation was detected in all studies, consistent with our experiments with *Alcanivorax* (Scoma et al., 2016a,b). However, we argued that genes related to beta-oxidation were only partially upregulated in two studies out of six (reviewed in Scoma et al., 2016c), in sharp contrast with the high concentration of n-alkanes detected in plume and sediment DWH samples up to 1.5 year after the spill.

While the reason why predominant hydrocarbonoclastic genera and beta-oxidation are impaired by deep-sea HP remains unknown, we showed that HP represents a major factor driving microbial community structures and pathways associated with oil-degradation, both distinct from those occurring at surface water pressure. The physiology of microbial oil degradation is still in its infancy, with the present published data by CMET-UGent already constituting 50% of the whole literature on this topic. Understanding the metabolic routes governing oil degradation at HP is key to develop successful bioremediation strategies in the deep sea. This would improve understanding of bioremediation pathways and assist policymaker in assessing protocols challenging biodegradation at deep-sea HP.