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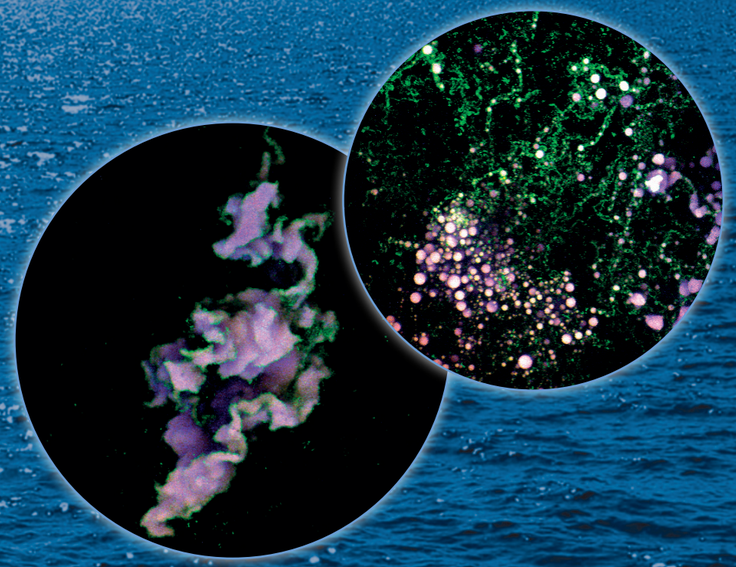

"Oil spills bioremediation in marine environment – biofilm characterization around oil droplets"

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Maria P. Nikolopoulou
Environmental Engineer

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Oil spills bioremediation in marine environment-biofilm characterization around oil droplets

Βιοαποδόμηση πετρελαιοειδών σε θαλάσσιο περιβάλλον - Χαρακτηρισμός σχηματισμού βιοστιβάδας σε σταγονίδια υδρογονανθράκων

Examination Committee

Nicolas Kalogerakis – Supervisor

Professor, Division II: Environmental Process Design and Analysis, School of Environmental Engineering, Technical University of Crete, Chania, Greece

Nikos Pasadakis – Advisory Committee

Associate Professor
School of Mineral Resources Engineering, Technical University of Crete, Chania, Greece

Dr. Thomas R. Neu – Advisory Committee

Head of Group - Microbiology of Interfaces, Department River Ecology, Helmholtz Centre for Environmental Research – UFZ, Magdeburg, Germany

Evangelos Gidarakos

Professor, Division I: Environmental Management
School of Environmental Engineering, Technical University of Crete, Chania, Greece

Elia Psillakis

Associate Professor, Division II: Environmental Process Design and Analysis
School of Environmental Engineering, Technical University of Crete, Chania, Greece

Danae Venieri

Assistant Professor, Division II: Environmental Process Design and Analysis
School of Environmental Engineering, Technical University of Crete, Chania, Greece

Marina Pantazidou

Associate Professor
Department of Geotechnical Engineering, School of Civil Engineering, National Technical University of Athens, Athens, Greece

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Abstract

Oil spills are considered a widespread problem after the last accident at the Mexico Gulf that poses great threat for any ecosystem. Specifically Mediterranean coastal regions are exposed to oil pollution due to extensive industrialization and urbanization and transport of crude and refined oil to and from refineries. The impact of an oil spill in this closed sea and particularly in the Greek coastline and sea that are popular touristic destinations and shelter of many marine species can be disastrous. First response options, such as physical removal (e.g. booms, skimmers, adsorbents, etc.) and dispersants whenever applicable due to their potential toxicity rarely achieve complete cleanup of oil spills.

Nevertheless, major oil spills highlight the need for environmentally responsible and cost-effective mitigation technologies. Bioremediation through bioaugmentation (addition of oil-degrading bacteria) and/or biostimulation (addition of nutrients N&P) constitutes a promising strategy for combatting oil spills following first response actions and recent technological advances could promote bioremediation to a priority option in combating oil spills. However, bioaugmentation is one of the most controversial issues in bioremediation since nutrient addition alone has been found to have a greater effect on oil biodegradation than the addition of microbial products that are highly dependent on environmental conditions. There is increasing evidence that the best way to overcome the above barriers is to exclusively use microorganisms indigenous to the sites (soil, sand, and water) to be decontaminated, an approach termed "autochthonous bioaugmentation" (ABA).

The specific aims of the present work were to investigate possible methods to enhance the rate of biodegradation of oil in a contaminated marine environment (both seawater and shoreline). Hence we investigated the capability of either acclimated indigenous microbial consortium or hydrocarbon degraders consortium enriched from seawater samples taken from Hellenic Petroleum Refinery (Athens, Greece) a site exposed to chronic pollution with crude oil (ABA) in the presence or absence of other rate limiting factors like nutrients and biosurfactants (biostimulation) as a potential strategy for the successful remediation of polluted marine environments. In addition the effectiveness of these certain acclimated consortia (ABA) was compared to indigenous population activity (biostimulation) on the bioremediation of oil spills.

Specifically the effects of the lipophilic nutrients (uric acid and lecithin) and inorganic nutrients (KNO_3, K_2HPO_4) with or without biosurfactants on the degradation of crude oil hydrocarbons in both seawater and sand matrix were also examined. While bioremediation in liquid matrices (seawater) is implemented in a more direct way, in the soil matrix (sand) is achieved through landfarming which is both simple and cost-effective to implement compared with other treatment technologies.

Thus the outcome approaches include 4 sets of experiments:

1. Autochthonous bioaugmentation and/or biostimulation of seawater microcosm (i.e. Seawater 1)
2. Autochthonous bioaugmentation & biostimulation with isolated hydrocarbon degraders consortium of seawater microcosm (i.e. Seawater 2)
3. Landfarming of oil polluted beach sand through biostimulation (i.e. Sand 1)
4. Landfarming of oil polluted beach sand through autochthonous bioaugmentation & biostimulation (i.e. Sand 2)

The method which has been used for the evaluation of these bioremediation methods is based on a modified bioremediation agent effectiveness testing protocol by EPA

(40 CFR Ch. I, Pt 300, App. C, 2003). The protocol tests for microbial activity by Most Probable Number (MPN) determination and quantifies the disappearance of saturated hydrocarbons and polynuclear aromatic hydrocarbons (PAHs) by GC-MS analysis. Moreover the disappearance extent (degradation rate) of certain compounds from both saturated fraction (n-alkanes) and aromatic fraction (PAHs was also investigated through biodegradation kinetics analysis (Batch reactor). Furthermore identification of the key microorganisms in oil biodegradation community response and composition changes among different amendments was also performed through molecular analysis (PCR, RT-PCR, pyrotag Sequencing) of DNA extracts from each treatment.

Hydrocarbon degrading bacteria as shown by several studies have a high affinity to oil droplets which indicates that oil bioremediation is a complex process that involves interactions between oil and microorganisms under certain environmental conditions. Therefore in this study another set of experiments investigating and characterizing the interaction of hydrocarbon degraders consortia on oil and eicosane droplets (cellular level) by means of confocal laser scanning microscopy (CLSM) was also included. Moreover their response after the addition of certain commercial dispersants just like those that have been or could be used in the event of a real oil spill (Corexit, S200, Marichem) but also of more environmentally friendly biosurfactants (Rhamnolipids) was also examined.

Investigation through chemical, microbiological and kinetics analysis (specific degradation rate, q_s) has revealed that Seawater 1 experimental set in which acclimated or not indigenous microorganisms were used performed far better than Sand 1 experimental set in which degradation was induced only by indigenous microbial populations and despite the fact that combined Rhamnolipids and lipophilic nutrients were used still microbial response was not as strong as in Seawater 1 experimental set. Bioavailability of oil hydrocarbons is the critical factor that affects the efficiency of bioremediation in oil contaminated environments and It can be concluded that in Seawater 1 experimental set biosurfactants, in particular rhamnolipids, accelerated the biodegradation of crude oil by making it more available to microorganisms as expected in the two ABA treatments (ULRM-Uric acid, Lecithin, Rhamnolipinds and preadapted indigenous Microorganisms & NPKMR-Inorganic nutrients, Rhamnolipinds and preadapted indigenous Microorganisms) and the biostimulation treatment (ULR- Uric acid, Lecithin, Rhamnolipinds).

On the other hand Sand 2 experimental set seemed more successful than Seawater 2 experimental set in which both experimental sets the same consortium (Eb8) was used. However it should be noted that despite the fact that Rhamnolipids were added lipophilic nutrients were not included in Seawater 2 experimental set, which possibly had contributed to the less successful performance of NPKMR treatment of Seawater 2 experimental set compared to ULRM treatment of Sand 2 experimental set.

Combined application of Rhamnolipids and lipophilic nutrients could be beneficial in liquid matrix (seawater), however when applied to solid matrix their performance is questioned compared to one in the liquid matrix. On the contrary inorganic nutrients usually being washed out when applied in seawater perform better when applied to sand almost equally to ULR combined performance. Inconsistent behaviour of ULR (Sand 1 experimental set) and ULRM (Sand 2 experimental set) treatments between the two oil fractions (alkanes-PAHs) compared to NPK (Sand 1 experimental set) and NPKM (Sand 2 experimental set) treatments respectively could support this conclusion. Still overall ULR treatment performance suggests that the presence of

biosurfactant could possibly have contributed to utilization of lipophilic nutrients by making them more available to soil microorganisms.
Nonetheless especially in the soil matrix (sand) bioavailability of hydrophobic compounds is often the rate-limiting step in the process and the efficiency of biosurfactants or other rate limiting co-substrates mainly could be attributed to the interactions between target organic compounds, bacterial species, water content and surfactants.
Different types of amendments provoke different structures in the resulting biodegradation communities. Thus *Alcanivoracaceae* as investigated in the Seawater 1 experimental set was the dominant family in treatments with inorganic nutrients, when biosurfactant is applied (rhamnolipids-treatments NPKMR, ULR & ULRM) community shifts to the family of *Pseudomonadaceae,* which was also the dominant family at the late stage (30 days) in the Seawater 2 experimental set (treatments NPKM and NPKMR), whereas at early stage of the same experimental set *Alcanivoracaceae* was the dominant family as well. Regarding this observation, *Pseudomonas* sp. can be considered as exceptional biocatalysts utilizing either metabolic by-products or other more recalcitrant hydrocarbons and thus can accelerate bioremediation when other species stop.
Thus mixed consortia are advantageous over single species consortia on hydrocarbons degradation and CLSM investigation has revealed that bacteria of mixed consortia are organized into clusters forming strings, star and grape like shapes of bacteria and fine oil droplets bridging each other with EPS. However it should be noted that contrary to what so far was proposed as potential mechanism for the interaction between oil droplets and bacteria biofilm was not the preferred interaction between the tested consortia and the oil droplets.
This new organization and structure between oil and microbial consortia has brought up a new perspective-mechanism in which mixed consortia utilize oil hydrocarbons and could provide a new dimension for the study of coaggregation and biofilm microbial communities in the marine ecosystem. Moreover understanding the interactions between oil-degrading microorganisms is essential, not only when predicting the fate of hydrocarbons in the environment but also for the development of new improved surfactants formulations or biosurfactans that can be used under different environmental conditions.
Nonetheless highly sensitive coastline environment and oil toxicity that fluctuates depending on the amount released to the environment constitute the impact of an oil spill exceptionally evident. Therefore immediate (bio)remediation is vital in order to decrease oil concentration below critical level and by that diminish marine ecosystem disturbance.
This work has demonstrated that in the absence of essential nutrients, inoculation only with autochthonous hydrocarbon degraders is not an effective treatment, however when the needed nutrients or other biostimulants are supplemented the advantages of such combination are obvious and result in accelerated hydrocarbon consumption by the added autochthonous consortium. Thus we strongly believe that the combination of autochthonous bioaugmentation and biostimulation is a promising strategy to speed up bioremediation in cases where there is lack of both nutrients and indigenous degraders. This technique has a number of advantages like shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support. Thus future research that would define the carrying capacities of various environments and the mechanisms that control them could be fruitful in this regard.

Εκτενής Περίληψη

Παρά τους αυστηρότερους περιβαλλοντικούς κανονισμούς που έχουν θεσπιστεί και έχουν υιοθετηθεί από τις περισσότερες χώρες ατυχήματα όπως του Exxon Valdez στην Αλάσκα, ένα από τα πρώτα που έχουν καταγραφεί, μέχρι το πιο πρόσφατο του Deep Horizon στον κόλπο του Μεξικό, καταδεικνύουν για ακόμη μια φορά την επικινδυνότητα των θαλάσσιων πετρελαιοκηλίδων προς το θαλάσσιο οικοσύστημα.
Οι Μεσογειακές ακτές είναι ιδιαιτέρως εκτεθειμένες σε κάθε πιθανή ρύπανση από πετρελαιοειδή λογω της εκβιομηχάνισης και της αστικοποίησης των περιοχών και της μεταφοράς του πετρελαίου και των ραφιναρισμένων προϊόντων του από τα διυλιστήρια. Υπολογίζεται ότι το ένα τρίτο της παγκόσμιας παραγωγής πετρελαίου που φορτώνεται ετησίως σε δεξαμενόπλοια, μεταφέρεται μέσω της Μεσογείου και από αυτή κατ' εκτίμηση 330.000 τόνοι αποβάλλονται σκόπιμα σε αυτήν την κλειστή θάλασσα, ενώ τα ατυχήματα αποτελούν μια πρόσθετη πηγή ρύπανσης 1.000.000 τόνων ετησίως. Οι επιπτώσεις από ένα τέτοιο θαλάσσιο ατύχημα ιδιαιτέρως σε αυτή την κλειστή θάλασσα είναι αρκετά καταστροφικές για το θαλάσσιο οικοσύστημα οδηγώντας στη θνησιμότητα χιλιάδων θαλασσοπουλιών και θαλάσσιων θηλαστικών, σε σημαντική μείωση του πληθυσμού πολλών οργανισμών, υποβάθμιση του βενθικού συστήματος με πολλές μακροπρόθεσμες κοινωνικοοικονομικές επιπτώσεις π.χ. στην αλιεία και στον τουρισμό. Δεδομένης λοιπόν της ευαισθησίας και σπουδαιότητας της Μεσογείου και δη των ελληνικών ακτών και θαλασσών που αποτελούν δημοφιλή τουριστικό προορισμό αλλά και αλιευτικό καταφύγιο πολλών ειδών κρίνεται απαραίτητη η άμεση αντιμετώπιση αλλά και αποκατάσταση του θαλάσσιου περιβάλλοντος από ρύπανση με πετρελαιοειδή.
Οι συμβατικές μέθοδοι, όπως η φυσική απομάκρυνση (π.χ., πλωτά φράγματα - μηχανική συλλογή) που ανήκουν στους άμεσους τρόπους αντιμετώπισης μιας πετρελαιοκηλίδας, σπάνια επιτυγχάνουν τον πλήρη καθαρισμό των πετρελαιοκηλίδων, ενώ τα χημικά διασκορπιστικά εφ’όσον επιτρέπεται και δύναται να χρησιμοποιηθούν κάτω από προϋποθέσεις είναι τοξικά για τη θαλάσσια βιοποικιλότητα.
Επομένως και εφ’όσον η επικινδυνότητα για θαλάσσιες πετρελαιοκηλίδες εξακολουθεί να υπάρχει, είναι επιτακτική ανάγκη να βρεθούν και να εξεταστούν νέοι αποτελεσματικότεροι, πιο φιλικοί περιβαλλοντικά και πιο οικονομικοί τρόποι – μέθοδοι αποκατάστασης και αντιμετώπισης ενός θαλάσσιου περιβάλλοντος ρυπασμένου με πετρελαιοειδή. Η βιοεξυγίανση μέσω της βιοενίσχυσης (προσθήκη βακτηρίων που αποδομούν το πετρέλαιο) και της βιοδιέγερσης (προσθήκη θρεπτικών N&P ή άλλων περιοριστικών παραγόντων) αποτελεί μια πολλά υποσχόμενη στρατηγική στην αντιμετώπιση πετρελαιοκηλίδων μετά την χρήση συμβατικών μεθόδων άμεσης αντιμετώπισης, ενώ σύμφωνα με πρόσφατες τεχνολογικές εξελίξεις μπορεί και να χρησιμοποιηθεί ως αμέσως τρόπος αποκατάστασης. Εντούτοις η βιοενίσχυση ως μέθοδος βιοεξυγίανσης είναι αρκετά αμφιλεγόμενη ως προς την αποτελεσματικότητα της δεδομένου ότι η προσθήκη μόνο θρεπτικών (βιοδιέγερση) είχε μεγαλύτερη επίδραση στην βιοαποδόμηση του πετρελαίου από ότι η προσθήκη μικροβιακών προϊόντων που ουσιαστικά εξαρτώνται άμεσα από τις περιβαλλοντικές συνθήκες. Ολοένα και περισσότερες έρευνες καταδεικνύουν ότι ο καλύτερος τρόπος να ξεπεραστούν οι παραπάνω περιορισμοί είναι η αποκλειστική χρήση μικροοργανισμών αυτοχθόνων με την περιοχή που θα αποκατασταθεί (έδαφος,

αμμουδιά, νερό), μια προσέγγιση που έχει προταθεί ως βιοενίσχυση με αυτόχθονες μικροοργανισμούς-αυτόχθονη βιοενίσχυση (autochthonous bioaugmentation-ABA).
Οι πετρελαιοκηλίδες ανάλογα με τις ιδιάζουσες συνθήκες του προς αποκατάσταση περιβάλλοντος διακρίνονται σε θαλάσσιες (ανοιχτής θαλάσσης) και παράκτιες (ή αλλιώς χερσαίες), έτσι λοιπόν και η αποκατάσταση του ρυπασμένου περιβάλλοντος προσεγγίζεται διαφορετικά. Ενώ λοιπόν η βιοξυγίανση των πετρελαιοκηλίδων στο θαλασσινό νερό (υγρή μήτρα) αντιμετωπίζεται με άμεσο τρόπο, στο παράκτιο περιβάλλον (στερεή μήτρα) προσεγγίζεται μέσω της τεχνικής αποκατάστασης ρυπασμένων εδαφών γνωστή ως «τεχνική επεξεργασίας στερεάς φάσης»-landfarming, η οποία είναι παράλληλα απλή και χαμηλού κόστους συγκρινόμενη με άλλες τεχνικές αποκατάστασης ρυπασμένων εδαφών. Η επιτυχία της βιοεξυγίανσης των πετρελαιοκηλίδων εξαρτάται από τη δυνατότητά να καθιερωθούν και να διατηρηθούν οι συνθήκες (φυσικές, χημικές και βιολογικές) που ευνοούν τους ρυθμούς ενισχυμένης βιοδιάσπασης του πετρελαίου στο ρυπασμένο περιβάλλον. Αξίζει να σημειωθεί ότι η περιοχή επιφάνειας του πετρελαίου είναι σημαντική επειδή η αύξηση των βιοαποδομητών πετρελαίου εμφανίζεται σχεδόν αποκλειστικά στη διεπιφάνεια πετρελαίου-ύδατος όπου λαμβάνει χώρα η βιοαποδόμηση, έτσι η διασπορά του πετρελαίου στην υδάτινη στήλη αυξάνει την περιοχή επιφάνειας του πετρελαίου και επομένως τη διαθεσιμότητά του για μικροβιακή επίθεση.
Οι πιο κοινοί τύποι θρεπτικών που έχουν χρησιμοποιηθεί στην βιοεξυγίανση περιοχών ρυπασμένων με πετρελαιοειδή αποτελούν τα υδατοδιαλυτά ανόργανα θρεπτικά (water-soluble nutrients), τα στερεά βραδείας απελευθέρωσης (slow-release) θρεπτικά και τα ολεοφιλικά/λιπόφιλα θρεπτικά (oleophilic/lipophilic nutrients). Κάθε είδος θρεπτικής ουσίας έχει τα πλεονεκτήματα και τους περιορισμούς του. Τα ποικίλα αποτελέσματα από διάφορες εργαστηριακές μελέτες υποδηλώνουν ότι η αποτελεσματικότητα τους επηρεάζεται από το ρυθμό απελευθέρωσης τους στο περιβάλλον και από τις επικείμενες περιβαλλοντικές συνθήκες (κύματα, ρεύματα, παλίρροιες κ.α.). Εν περιλήψει, τα λιπάσματα βραδείας απελευθέρωσης μπορούν να αποτελέσουν ιδανικές πηγές θρεπτικών εάν οι ρυθμοί απελευθέρωσης των θρεπτικών μπορούν να ελεγχθούν καλά. Τα υδατοδιαλυτά λιπάσματα (water -soluble fertilizers) είναι οικονομικότερα και αποδοτικότερα σε λεπτόκοκκες χαμηλής ενέργειας ακτές όπου η μεταφορά ύδατος είναι περιορισμένη. Τα ολεοφιλικά λιπάσματα είναι καταλληλότερα για χρήση σε χονδρόκοκκες υψηλής ενέργειας παραλίες. Πέραν της προσθήκης θρεπτικών σκευασμάτων για την βιοαποδόμηση των πετρελαϊκών υδρογονανθράκων η συνδυασμένη εφαρμογή θρεπτικών με επιφανειοδραστικές ενώσεις βοηθά στην διασκόρπιση της πετρελαιοκηλίδας αυξάνοντας έτσι τη διεπιφάνεια ύδατος-πετρελαίου και άρα την περιοχή δράσης των μικροοργανισμών. Οι βιολογικές αυτές επιφανειοδραστικές ουσίες (biosurfactants) προτιμώνται έναντι των χημικών διότι είναι πολύ λιγότερο τοξικές και πολύ ευκολότερα αποδομήσιμες.
Η λογική στην στρατηγική αυτή είναι ότι η βιοδιάσπαση του πετρελαίου εμφανίζεται κυρίως στη διεπιφάνεια πετρελαίου-ύδατος, δεδομένου ότι οι βιογενείς επιφανειοδραστικές ενώσεις (biosurfactants) αυξάνουν την διεπιφάνεια αυτή και ότι τα ολεοφιλικά λιπάσματα είναι σε θέση να εμμείνουν στο πετρέλαιο και να παρέχουν τις θρεπτικές ουσίες στη διεπιφάνεια πετρελαίου-ύδατος, μπορεί να υφίστανται ενισχυμένη βιοδιάσπαση χωρίς την ανάγκη να αυξηθούν οι συγκεντρώσεις των θρεπτικών στο νερό. Εντούτοις, για την επιτυχή εφαρμογή των προϊόντων βιοεξυγίανσης απαιτούνται πάντα κατάλληλες δοκιμές για την αξιολόγηση τους βασισμένες στις συγκεκριμένες συνθήκες κάθε ρυπασμένης περιοχής.

Σκοπός της παρούσας διατριβής ήταν να μελετηθούν πιθανές μέθοδοι που θα ενίσχυαν τον ρυθμό βιοαποδόμησης του πετρελαίου στο ρυπασμένο θαλάσσιο περιβάλλον (ανοιχτής θαλάσσης και παράκτιο) μειώνοντας έτσι τον χρόνο που απαιτείται για να αποκατασταθεί.

Ως εκ τούτου εξετάστηκε η ικανότητα δυο κονσόρτσια είτε με εγκλιματισμένους (σε συνθήκες ρυπασμένης θαλάσσης με πετρέλαιο) αυτόχθονες θαλάσσιους μικροοργανισμούς (παραλία αγ. Ονουφρίου) είτε με απομονωμένους μικροοργανισμούς που αποδομούν πετρελαϊκούς υδρογονάνθρακες (αυτόχθονη βιοενίσχυση - ABA) ως επιτυχής στρατηγική στην εξυγίανση ρυπασμένου θαλάσσιου περιβάλλοντος παρουσία ή μη άλλων περιοριστικών παραγόντων όπως θρεπτικά οι βιογενείς επιφανειοδραστικές ενώσεις- biosurfactants (βιοδιέγερση).

Οι αποδομητές πετρελαίου προήλθαν από διαδοχικούς εμπλουτισμούς και απομονώσεις θαλάσσιων δειγμάτων νερού που συλλέχθηκαν από την περιοχή του κόλπου της Ελευσίνας κοντά στα διυλιστήρια των Ελληνικών Πετρελαίων (ΕΛΠΕ), μια περιοχή που εκτίθεται σε χρόνια ρύπανση από πετρελαιοειδή. Επιπλέον η ικανότητα των κονσόρτσια με τους ήδη προσαρμοσμένους αποδομητές πετρελαίου (ABA) αντιπαρατέθηκε με την ικανότητα των αυτόχθονων μικροοργανισμών (βιοδιέγερση) να αποδομήσουν τους πετρελαϊκούς υδρογονάνθρακες. Συγκεκριμένα μελετήθηκε η επίδραση λιπόφιλων οργανικών θρεπτικών (ουρικό οξύ- uric acid, λεκιθίνη-lecithin) και ανόργανων θρεπτικών (KNO_3 και K_2HPO_4,-NPK treatment) παρουσία ή μη βιογενών επιφανειοδραστικών ενώσεων- biosurfactants στην βιοαποδόμηση πετρελαϊκών υδρογονανθράκων τόσο σε περιβάλλον ανοιχτής θαλάσσης όσο και σε παράκτιο.

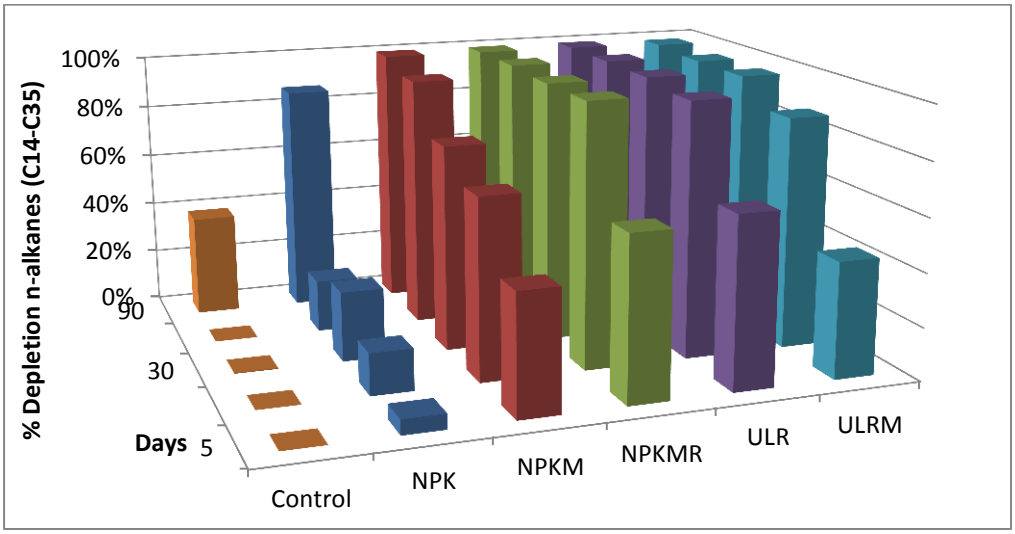
Έτσι προέκυψαν 4 σειρές πειραμάτων και περιλαμβάνουν:

1. Αυτόχθονη βιοενίσχυση και/ή βιοδιέγερση σε θαλάσσιο μικρόκοσμο (π.χ. Seawater 1)
2. Αυτόχθονη βιοενίσχυση & βιοδιέγερση με κονσόρτσια απομονωμένων μικροοργανισμών που αποδομούν τους πετρελαϊκούς υδρογονάνθρακες σε θαλάσσιο μικρόκοσμο (π.χ. Seawater 2)
3. βιοδιέγερση σε ρυπασμένη με πετρέλαιο άμμο μέσω της τεχνικής landfarming (π.χ. Sand 1)
4. Αυτόχθονη βιοενίσχυση & βιοδιέγερση σε ρυπασμένη με πετρέλαιο άμμο μέσω της τεχνικής landfarming (π.χ. Sand 2)

Η μέθοδος που χρησιμοποιήθηκε για την εξέταση των παραγόντων βιοεξυγίανσης βασίζεται στο τροποποιημένο πρωτόκολλο δοκιμής της αποτελεσματικότητας των παραγόντων βιοεξυγίανσης της EPA (40 CFR Ch. I, Pt 300, App. C, 2003). Το πρωτόκολλο δοκιμής της αποτελεσματικότητας των παραγόντων βιοεξυγίανσης σχεδιάστηκε για να καθορίσει την ικανότητα ενός προϊόντος να βιοδιασπάσει το πετρέλαιο ποσοτικοποιώντας τις αλλαγές στη σύνθεση του πετρελαίου ως αποτέλεσμα της βιοδιάσπασης. Το πρωτόκολλο εξετάζει τη μικροβιακή δραστηριότητα με την Ανάλυση του πιο Πιθανού Αριθμού (Most Propable Number) και ποσοτικοποιεί την απομάκρυνση των κορεσμένων υδρογονανθράκων και των πολυαρωματικών υδρογονανθράκων (PAHs) με τη χρήση GC-MS.

Επιπρόσθετα ο βαθμός απομάκρυνσης (ρυθμός αποδόμησης) επιλεγμένων συστατικών (C15, C20, C25, C30, Pristane, Phytane, Fluorene, Dibenzothiothene, Phenanthrene και Chrysene) και από τα δυο κλάσματα των κορεσμένων (ν-αλκάνια) και των αρωματικών (PAHs) εξετάστηκε μέσω βιοχημικής κινητικής ανάλυσης λαμβάνοντας υπόψη τις αρχές που διέπουν βιοαντιδραστήρα διαλείποντος έργου (Batch reactor). Ενώ η ταυτοποίηση των μικροοργανισμών που αποδομούν τους πετρελαϊκούς υδρογονάνθρακες, καθώς και η απόκριση και αλλαγή της δομής των

κονσόρτσια που χρησιμοποιήθηκαν μεταξύ των διαφορετικών πειραμάτων αλλά και επεξεργασιών επετεύχθηκε με μοριακή ανάλυση (PCR, RT-PCR, pyrotag Sequencing) δειγμάτων γενετικού υλικού που ελήφθησαν από κάθε επεξεργασία.
Προηγούμενες μελέτες που αφορούσαν τη βιοδιάσπαση υδρογονανθράκων οδήγησαν στην παρατήρηση ότι τα βακτήρια που αποδομούν υδρογονάνθρακες παρουσιάζουν υψηλή έλξη προς τα σταγονίδια πετρελαίου και δεδομένου ότι η βιοδιάσπαση του πετρελαίου εμφανίζεται κυρίως στη διεπιφάνεια πετρελαίου-ύδατος, καθιστούν τη βιοαποδόμηση πετρελαίου μια πολύπλοκη διεργασία η οποία περιλαμβάνει αλληλεπιδράσεις μεταξύ των μικροοργανισμών και του πετρελαίου, οι οποίες επηρεάζονται άμεσα από τις επικρατούσες περιβαλλοντικές συνθήκες. Έτσι ένα μεγάλο ερώτημα που προκύπτει στην βιοδιάσπαση των υδρογονανθράκων είναι πώς οι μικροοργανισμοί ουσιαστικά έρχονται σε επαφή με το υπόστρωμα. Η κατανάλωση των υδατοδιαλυτών υποστρωμάτων από τους μικροοργανισμούς φαίνεται να μην παρουσιάζει πρόβλημα, αλλά πώς οι μικροοργανισμοί αλληλεπιδρούν με υλικά τα οποία είναι αδιάλυτα όπως τα μεγάλου μοριακού βάρους αλκάνια παραμένει πρόκληση. Έτσι λοιπόν δημιουργήθηκε ακόμη μια σειρά πειραμάτων που στόχο είχε τη διερεύνηση και χαρακτηρισμό των αλληλεπιδράσεων των κονσόρτσια απομονωμένων μικροοργανισμών που αποδομούν πετρελαϊκούς υδρογονάνθρακες με σταγονίδια πετρελαίου άλλα και εικοσανίου (στερεό συστατικό του πετρελαίου που χρησιμοποιήθηκε ως πρότυπο), καθώς επίσης και τη διερεύνηση της πιθανότητας ανάπτυξης –παρακολούθησης βιοστιβάδας γύρω από σταγονίδια πετρελαιοειδών και χαρακτηρισμό αυτής με τη χρήση μικροσκοπίας ομοεστιακής δέσμης λέιζερ (confocal microscopy- CLSM). Επιπλέων εξετάστηκε η επίδραση των εμπορικών διασκορσπιστικών ενώσεων (Corexit, S200 και Marichem) αλλά και της βιολογικής προέλευσης επιφανειοδραστικής ένωσης-biosurfactant (ραμνολιπίδια-rhamnolipids) στο σχηματισμό βιοστιβάδας αλλά και στη γενικότερη συσχέτιση των κονσόρτσια με τα σταγονίδια πετρελαίου και εικοσανίου.

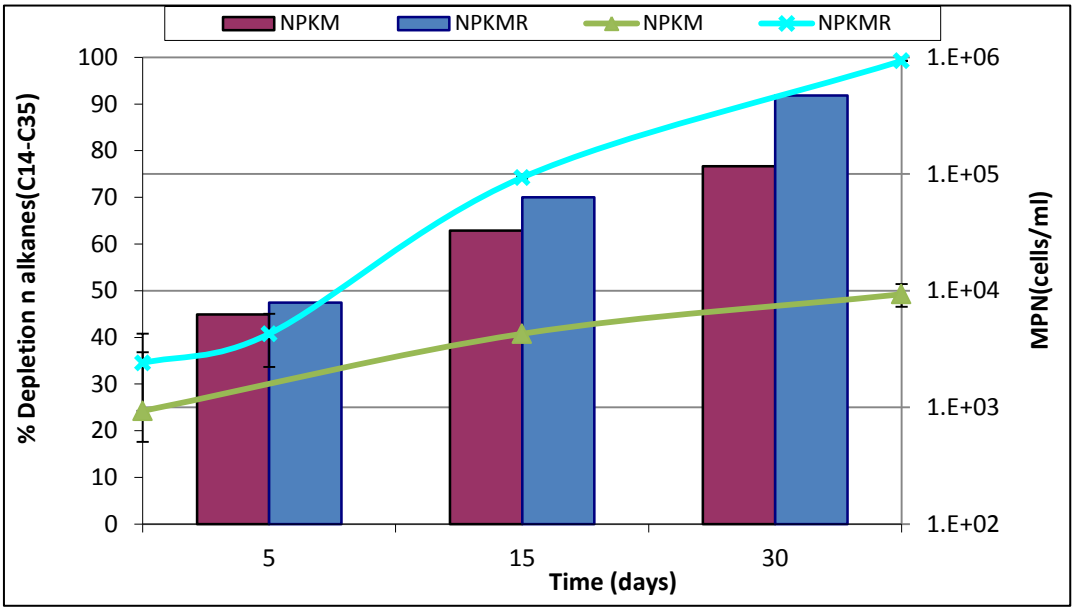


Εικόνα 1: % απομάκρυνση των αλκανίων μετα από 0, 5, 15, 30, 60 και 90 ημέρες παρακολούθησης μεταξύ των Control, NPK, NPKM, NPKMR, ULR και ULRM διαφορετικών επεξεργασιών της πειραματικής σειράς Seawater 1.

Η επεξεργασία των αποτελεσμάτων των χημικών και μικροβιολογικών αναλύσεων αλλά και η κινητική επεξεργασία των δεδομένων τους (ειδικός ρυθμός αποδόμησης-qs) απεκάλυψαν ότι στην πειραματική σειρά Seawater 1, στην οποία χρησιμοποιήθηκαν αυτόχθονες μικροβιακοί πληθυσμοί εγκλιματισμένοι ή μη απέδωσε πολύ καλύτερα σε σχέση με την πειραματική σειρά Sand 1, στην οποία η

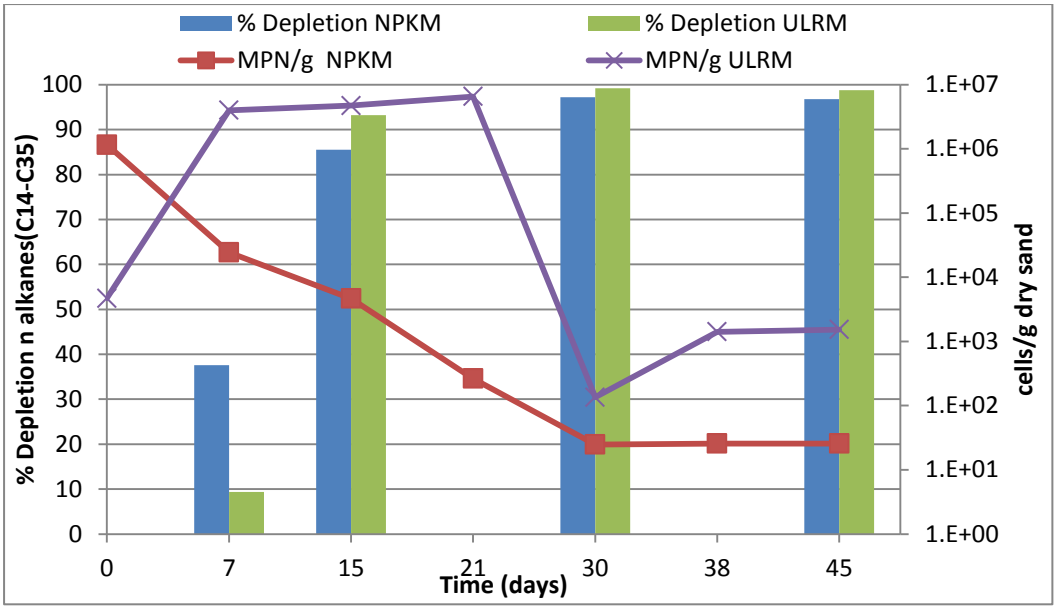
αποδόμηση προερχόταν αποκλειστικά από τους αυτόχθονες (μη εγκλιματισμένους) μικροοργανισμούς και παρά το γεγονός ότι προστέθηκαν συνδυαστικά ραμνολιπίδια (rhamnolipids) και λιπόφιλα θρεπτικά, η απόκριση τους ήταν χαμηλή σχετικά με αυτήν της πειραματικής σειράς Seawater 1.

Η βιοδιαθεσιμότητα των πετρελαϊκών υδρογονανθράκων αποτελεί κρίσιμη παράμετρο που επηρεάζει την αποδοτικότητα της βιοεξυγίανσης σε ρυπασμένα περιβάλλοντα και επομένως εξάγεται το συμπέρασμα ότι στην πειραματική σειρά Seawater 1 (Εικόνα 1), οι βιογενείς επιφανειοδραστικές ενώσεις, συγκεκριμένα τα ραμνολιπίδια (rhamnolipids), επιτάχυναν τη βιοαποδόμηση του πετρελαίου κάνοντας το πιο διαθέσιμο προς τους μικροοργανισμούς, όπως φάνηκε στις 2 επεξεργασίες αυτόχθονης βιοενίσχυσης ABA ULRM & NPKMR (απομάκρυνση 88% και 99% αντιστοίχως από τι πρώτες κιόλας 2 εβδομάδες εφαρμογής τους) και στην επεξεργασία με βιοδιέγερση ULR (απομάκρυνση 97% σε 15 μέρες εφαρμογής της) της συγκεκριμένης σειράς πειραμάτων. Ο συνδυασμός θρεπτικών με τις βιογενείς επιφανειοδραστικές ενώσεις σε αυτές τις 3 επεξεργασίες (ULR, ULRM & NPKMR) έδειξε μεγάλη αποδόμηση τόσο σε κανονικά αλκάνια όσο και σε πολυκυκλικούς αρωματικούς υδρογονάνθρακες, ενώ παράλληλα είχε την μεγαλύτερη ανάπτυξη αποδομητών πετρελαίου σε διάρκεια μόλις 15 ημερών από την αρχή του πειράματος.



Εικόνα 2: % απομάκρυνση των αλκανίων και μικροβιακή ανάπτυξη μεταξύ των διαφορετικών επεξεργασιών (NPKM και NPKMR) στην πειραματική σειρά Seawater 2.

Απεναντίας η πειραματική σειρά Sand 2 ήταν πολύ πιο αποτελεσματική σε σχέση με την πειραματική σειρά Seawater 2 (Εικόνα 2) παρόλο που και στις δυο σειρές χρησιμοποιήθηκε το ίδιο κονσόρτσιο μικροοργανισμών που αποδομούν πετρελαϊκούς υδρογονάνθρακες (Eb8). Μολοταύτα αξίζει να σημειωθεί ότι παρά το γεγονός ότι προστέθηκαν ραμνολιπίδια δεν συνδυάστηκαν με λιπόφιλα θρεπτικά στην πειραματική σειρά Seawater 2, γεγονός το οποίο πιθανότατα συνέβαλε στην όχι και τόσο επιτυχημένη απόδοση της NPKMR επεξεργασίας της πειραματικής σειράς Seawater 2 σε σχέση με την ULRM επεξεργασία της πειραματικής σειράς Sand 2.



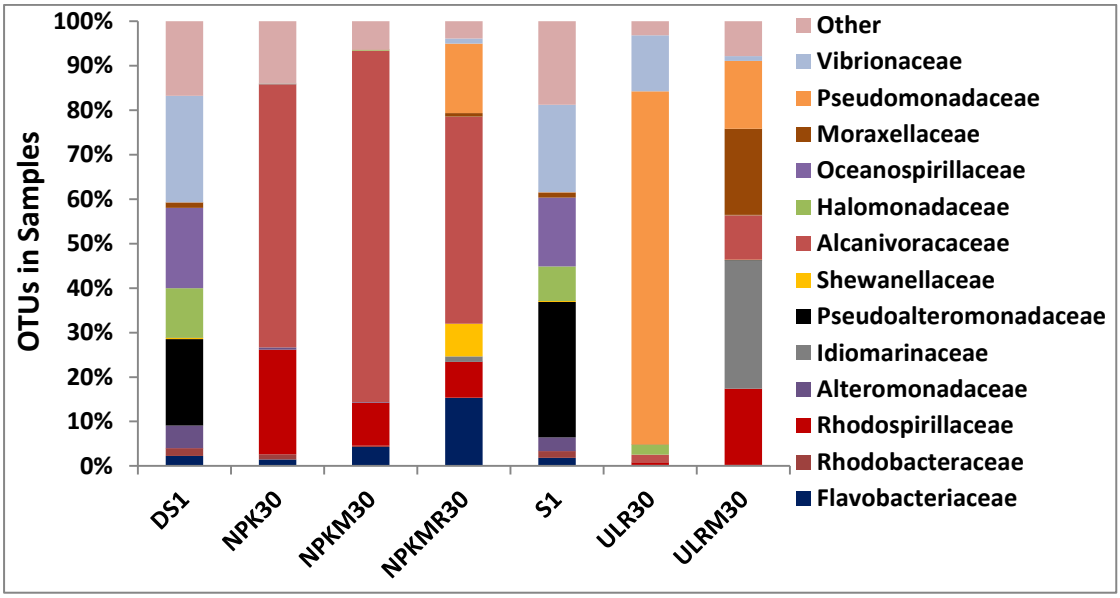
Εικόνα 3: % απομάκρυνση των αλκανίων και μικροβιακή ανάπτυξη μεταξύ των διαφορετικών επεξεργασιών (NPKM και ULRM) στην πειραματική σειρά Landfarming 2.

Η συνδυασμένη εφαρμογή ραμνολιπιδίων και λιπόφιλων θρεπτικών είναι αρκετά επωφελής σε υγρή μήτρα (θαλασσινό νερό), ωστόσο όταν γίνει εφαρμογή τους σε στερεή μήτρα (άμμος) η αποδοτικότητα τους είναι μέτρια και πολλές φορές αμφισβητείται. Εν αντιθέσει τα ανόργανα θρεπτικά τα οποία εκπλένονται γρήγορα στο θαλασσινό νερό αποδίδουν πολύ καλύτερα όταν εφαρμοστούν σε αμμώδη ακτή, εξίσου αποδοτικά με το ULR (ραμνολιπίδια - λιπόφιλα θρεπτικά) συνδυασμό. Η αντιφατική συμπεριφορά των επεξεργασιών ULR (πειραματική σειρά Sand 1) και ULRM (πειραματική σειρά Sand 2) ως προς τα δύο κλάσματα (αλκανίων-αρωματικών) σε σύγκριση με τις επεξεργασίες NPK (πειραματική σειρά Sand 1) και NPKM (πειραματική σειρά Sand 2) αντιστοίχως συντείνει προς αυτό το συμπέρασμα. Πάραυτα η συνολική εικόνα της επεξεργασίας ULR δείχνει ότι η παρουσία βιογενών επιφανειοδραστικών ενώσεων συνεισέφερε στην χρησιμοποίηση των λιπόφιλων θρεπτικών αυξάνοντας τη βιοδιαθεσιμότητα τους προς τους μικροργανισμούς που βρίσκονται στην άμμο.

Τα αποτελέσματα των χημικών αναλύσεων και από τις 4 σειρές πειραμάτων κατέδειξε ότι οι μικρής αλυσίδας υδρογονάνθρακες βιοαποδομούνται πιο εύκολα από ότι η μεγάλης μοριακή αλυσίδας υδρογονάνθρακες. Έτσι το κλάσμα των κορεσμένων συστατικών αποδομείται περισσότερο σε σχέση με το κλάσμα των αρωματικών και ακολουθείται η σειρά: C15> C20> (Pristane, Phytane)>C25 > C30> C35 >(PAHs).

Τα Pristane και Phytane δεν μπορούν να χρησιμοποιηθούν ως βιοδείκτες καθώς και αυτά βιοαποδομούνται.

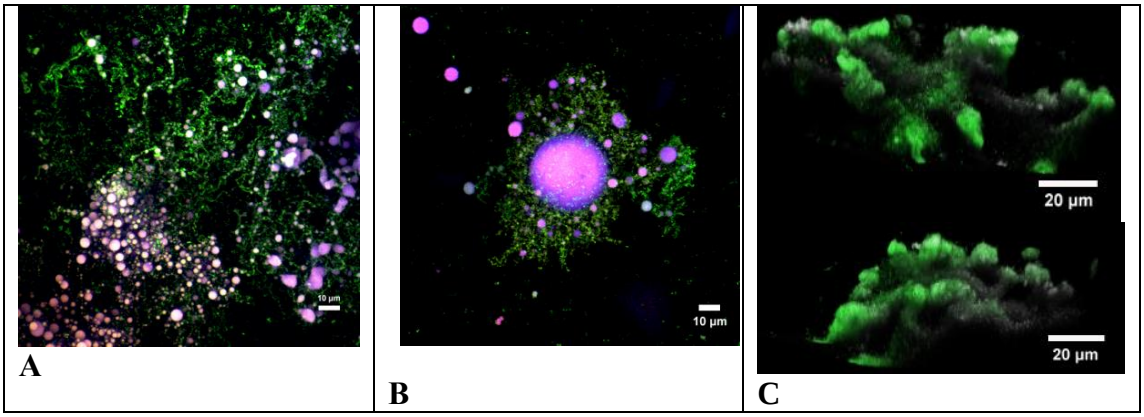
Η μικροβιακή ανάπτυξη μπορεί να συσχετιστεί με την απομάκρυνση συστατικών του πετρελαίου από το διάλυμα.



Εικόνα 4: Σχετική αφθονία των διαφορετικών οικογενειών εκφρασμένων ως operational taxonomic units (OTUs) ανάμεσα στις διαφορετικές επεξεργασίες της πειραματικής σειράς Seawater 1 μετα από 30 μέρες έναρξης του πειράματος. Οπου S1 και DS1 είναι τα αρχικά δειγματα των αυτόχθονων και εγκλιματισμένων μικροοργανισμών των δειγμάτων ελέγχου αντίστοιχα.

Μέχρι στιγμής τα στελέχη του *alcanivorax* θεωρούνται από τα επικρατέστερα OHCB (obligate hydrocarbonoclastic bacteria) βακτήρια και απαντώνται σε όλα τα ρυπασμένα με πετρελαιοειδή περιβάλλοντα, τα οποία παίζουν σημαντικό ρόλο στην αποδόμηση των πετρελαϊκών υδρογονανθράκων. Ωστόσο αξίζει να σημειωθεί ότι παρόλο που η οικογένεια των *Alcanivoracaceae* κυριαρχεί στις επεξεργασίες με ανόργανα θρεπτικά της πειραματικής σειράς Seawater 1 (Εικόνα 4), όταν εφαρμοστούν βιογενείς επιφανειοδραστικές ενώσεις (ραμνολιπίδια -επεξεργασίες NPKMR, ULR & ULRM) η σύνθεση της βακτηριακής κοινότητας αλλάζει προς την οικογένεια των *Pseudomonadaceae* (15.6%, 79.3% & 15.3% για τις NPKMR, ULR & ULRM επεξεργασίες αντιστοίχως). Συγκεκριμένα στη ULR επεξεργασία που έχουν προστεθεί λιπόφιλα θρεπτικά, ο αυτόχθων μικροβιακός πληθυσμός απαρτίζεται κυρίως από τις οικογένειες *Pseudomonadaceae* και *Vibrionaceae* family (12.6%). Μολοταύτα ο συνδυασμός που απαρτίζεται από τις οικογένειες των *Alcanivoracaceae* και *Rhodospirillaceae* (NPK, NPKM) δεν θεωρείται ο πιο επιτυχής όσων αφορά το ρυθμό βιοαποδόμησης στη συγκεκριμένη χρονική περίοδο (30 ημέρες).
Η οικογένεια των *Pseudomonadaceae* είναι η επικρατέστερη στο τελευταίο στάδιο (30 ημέρες) της πειραματικής σειράς Seawater 2, ενώ στο πρώιμο στάδιο η οικογένεια των *Alcanivoracaceae* είναι και πάλι η επικρατέστερη.
Είναι γνωστό ότι τα είδη του *Alcanivorax* ανήκουν στα OHCB βακτήρια και μπορούν να οξειδώσουν τα C_5-C_{16} *n*-αλκάνια και τα διακλαδισμένα αλκάνια. Αντίθετα τα είδη του *Pseudomonas* αντέχουν και μπορούν να μεταβολίσουν συστατικά που θεωρούνται τοξικά για άλλα βακτήρια, καθιστώντας τα έτσι σημαντικούς βιοκαταλύτες οι οποίοι επιταχύνουν την βιοαποδόμηση εκεί που τα άλλα είδη σταματούν. Τέτοια είναι και η περίπτωση των επεξεργασιών NPKM και NKPMR, όπου στην αρχή του πειράματος η μικροβιακή κοινότητα αποτελείται κυρίως από στελέχη που μπορούν να μεταβολίσουν υδρογονάνθρακες (αλκανια και κάποιες αρωματικές ενώσεις) ενώ στο τέλος του πειράματος όπου και οι περισσότεροι

υδρογονάνθρακες έχουν καταναλωθεί τα στελέχη του *Pseudomonas* μεταβολίζουν παραπροϊόντα της βιοαποδόμησης ή άλλους πιο ανθεκτικούς υδρογονάνθρακες.
Η παραπάνω ανάλυση απέδειξε ότι οι διαφορετικές επεξεργασίες προκαλούν διαφορετική απόκριση και σύνθεση των μικροβιακών κοινοτήτων καταδεικνύοντας το πλεονέκτημα των μεικτών κονσόρτσια σε σχέση με κονσορσια που αποτελούνται από στελέχη ενός είδους μόνο.



Εικόνα 5: Σχηματιζόμενες δομές μικροοργανισμών (πρασινο χρώμα) γυρω από σταγονιδια πετρελαίου A&B (λιλά χρώμα) και εικόσάνιο C

Σε αυτό το συμπέρασμα συντείνουν και τα αποτελέσματα των παρατηρήσεων με μικροσκόπιο CLSM (Εικόνα 5) όπου απεκάλυψαν ότι οι μικροοργανισμοί οργανώνονται σε συμπλέγματα μεταξύ τους και με τα διασκορπισμένα σταγονίδια πετρελαίου. Τα σταγονίδια πετρελαίου που είναι διασπαρμένα σε διάφορα μεγέθη καλύπτονται πλήρως από βακτήρια και σχηματίζουν πολύπλοκες δομές τύπου σταφυλιού και αστερία (grape style shapes- star-like patterns) πιθανότατα λόγω μεγάλης βιοαποδόμησης του, οι οποίες ενώνονται μεταξύ τους με γέφυρες από βακτήρια και βιοπολυμερή όπως είναι οι εξωκυτταρικοί πολυσακχαρίτες (EPS).
Η παραπάνω έρευνα κατέληξε σε πρωτοποριακά συμπεράσματα για τον τρόπο δράσης των θαλάσσιων μικροοργανισμών σε πετρελαιοκηλίδες. Έδειξε ότι ο μηχανισμός δράσης των μικροοργανισμών είναι πολύ διαφορετικός και πολυπλοκότερος από αυτόν που οι μέχρι τώρα μελέτες με συγκεκριμένα στελέχη είχαν δείξει (σχηματισμός βιοστιβάδας μέσω της οποίας αποδομούν το πετρέλαιο). Η πιθανότερη εκδοχή με βάση τις μέχρι τώρα παρατηρήσεις, είναι ότι οι μικροοργανισμοί μέσω της παραγωγής επιφανειοδραστικών ενώσεων διαχωρίζουν το πετρέλαιο σε πολύ μικρότερα σταγονίδια (δημιουργώντας μία ομάδα εξαιρετικά μικρών σταγονιδίων που βρίσκονται στην υδατική φάση σαν «τσαμπί σταφυλιού») ώστε να μπορούν πιο εύκολα να το αποδομήσουν και δεν προτιμούν να οργανώνονται σε βιοστιβάδες γύρω από τα σταγονίδια. Το καινοτόμο αυτό συμπέρασμα που καταρρίπτει τα έως τώρα δεδομένα οδηγεί σε εντελώς διαφορετική θεώρηση στον τρόπο δράσης των θαλάσσιων μικροοργανισμών όπου οδηγούν στη διαλυτοποίηση ακόμα και στερεών υποστρωμάτων όπως είναι το εικοσάνιο. Η εξέταση της επίδρασης διάφορων διασκορπιστικών ενώσεων έδειξε ότι ο μηχανισμός δράσης τους εφ'όσον δεν έχει τοξική επίδραση στους μικροοργανισμούς μπορεί να παραλληριστεί με αυτόν των επιφανειοδραστικών ενώσεων που παράγουν οι ίδιοι οι μικροοργανισμοί (π.χ. rhamnolipids) και ίσως να μπορούσε να αποτελέσει μέρος ενός ευρύτερου στρατηγικού πλαισίου στο σχεδιασμό διασκορπιστών πετρελαίου (dispersants) νέας γενιάς.
Εν κατακλείδι εξετάζοντας τα παραπάνω αποτελέσματα και γνωρίζοντας ότι απελευθέρωση πετρελαιοειδών στο θαλάσσιο περιβάλλον αποτελεί μόνιμη απειλή για

το θαλάσσιο οικοσύστημα κρίνεται απαραίτητη η εφαρμογή της βιοεξυγίανσης σαν μέθοδος αποκατάστασης του θαλάσσιου περιβάλλοντος.
Συγκεκριμένα η παραπάνω διερεύνηση απέδειξε ότι απουσία στοιχειωδών θρεπτικών, ο εμβολιασμός μονάχα με πληθυσμό αυτόχθονων μικροοργανισμών ικανών να αποδομήσουν τους πετρελαϊκούς υδρογονάνθρακες δεν θεωρείται επαρκής. Ωστόσο ο συνδυασμός απαραίτητων θρεπτικών ή άλλων περιοριστικών παραγόντων (π.χ. ραμνολιπίδια) μαζι με τον αυτόχθονο πληθυσμό των αποδομητών υδρογοναθράκων πλεονεκτεί με αποτέλεσμα την αυξημένη κατανάλωση των υδρογονανθράκων από τον πληθυσμό αυτόχθονων μικροοργανισμών που προστέθηκε. Έτσι λοιπόν πιστεύουμε ότι ο συνδυασμός αυτόχθονης βιοενίσχυσης και βιοδιέγερσης μπορεί να αποτελέσει μελλοντική στρατηγική που θα μπορούσε να επιταχύνει την βιοαποδόμηση σε περιπτώσεις όπου υπάρχει έλλειψη θρεπτικών και μικροοργανισμών. Μια τέτοια προσέγγιση γίνεται πιο επιτακτική κυρίως όταν μια πετρελαιοκηλίδα πλησιάζει απειλητικά την ακτή και απαιτείται γρήγορη και άμεση αποδόμηση των πετρελαϊκών υδρογονανθράκων.
Μελλοντικά η περαιτέρω εκτενής και διεξοδική μελέτη των βασικών μηχανισμών αλληλεπίδρασης μεταξύ μικροοργανισμών και ρυπαντή (πετρελαιοειδή) μπορεί να βοηθήσει στην κατανόηση όχι μόνο της τύχης του πετρελαίου στο περιβάλλον αλλά και να συντελέσει στην δημιουργία βελτιωμένων παραγόντων βιοεξυγίανσης (θρεπτικών-διασκορπιστικών ουσιών κ.α.), ενώ κυρίως μπορεί να βοηθήσει στην κατάστρωση στρατηγικού σχεδίου δράσης προσαρμοσμένου στις συνθήκες του προς αποκατάσταση ρυπασμένου περιβάλλοντος.

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Chapter 1- Introduction

1. INTRODUCTION

1.1. Addressing the problem

The recent event of Deep Horizon oil spill accident in the Gulf of Mexico still alerted us on the occasion of such event and reminded us that despite the stricter environmental regulations that have been adopted by most countries oil spills still remain a serious risk to marine ecosystems. The majority of spills are small (i.e. less than 7 tonnes) and data on numbers and amounts is incomplete; however, they make a relatively small contribution to the total quantity of oil spilled into the marine environment as a result of tanker accidents. During the period 1970 to 2012 more than 5.75 million tonnes of oil have been released into the sea as a result of oil tanker incidents. Crude oil and its products in the course of the exploration, production, refining and transportation operations can still pose great threat to marine environment with many significant short-term and long-term ecological and economic impacts (ITOPF, 2013). In particular Mediterranean coastal regions are exposed to oil pollution due to extensive industrialization and urbanization and transport of crude and refined oil to and from refineries. Thus the impact of an oil spill in this closed sea specifically, to the marine environment can be very significant and includes loss of species richness in some areas, downgraded sediment quality, a negative impact on offshore fish, crustacean fisheries (Kirby and Law, 2008) and also on the touristic sector with many socioeconomical side effects. Additionally a wide variety of organic contaminants tend to sink to marine sediments and the materials that are occasionally dredged from harbors and marinas often containing many contaminants. Dredged sediments as well as beach sand contaminated by oil spills are also of major concern (Rulkens and Bruning, 2005; NRC Committee, 1997; US EPA, 2012).

Figure 1: Disastrous consequences following oil spills.

Many countries have become signatories to the International Convention on Oil Pollution Preparedness, Response and Co-operation (OPRC) which requires that measures for dealing with pollution incidents as part of a national contingency plan

are produced including the use of oil spill treatment products where appropriate (Kirby and Law, 2008).
Strategies for cleaning up an oil spill both physico-chemical and biological are greatly affected by a variety of factors, such as the type of oil, the characteristics of the spill site, and occasionally political considerations (Zhu et al., 2001).

1.2. Oil Chemical Composition and Physical Properties

The chemical composition of petroleum products is complex and will change over time when released into the marine environment, which makes difficult to select the appropriate either countermeasure or analytical method to evaluate the environmental impact. Thus it is important to understand and evaluate the properties of petroleum and petroleum products to choose the appropriate response option.

1.4.1. Chemical Composition

Crude oil is an extremely complex mixture of tens of thousands of individual hydrocarbons (aliphatics and aromatics) and nonhydrocarbons (containing sulfur, nitrogen, oxygen, and various trace metals). The hydrocarbon content may be as high as 97% by weight in a conventional (lighter) paraffinic crude oil, or about 50% by weight in a heavy crude oil and less than 30% by weight in tar sand bitumen. These compounds range from small, simple, volatile, and distinct compounds (e.g., methane) to extremely large, complex, nonvolatile, colloidally dispersed macromolecules (e.g., asphaltenes). Some representative organic compounds found in crude oil are illustrated in Figure 2. The distribution of these compounds imparts certain physical properties on the oil, and it is these physical properties (e.g., density or viscosity) by which crude oils are generally classified, bought, and sold. Conventional crude oils can be generally classified based upon the predominance of the major hydrocarbon classes — paraffins, naphthenes, and aromatics.
The proportions of aliphatics, aromatics hydrocarbons and non-hydrocarbons occur in varying amounts, depend on the source and character of the oil and can be classified into five major groups:
1 Saturated hydrocarbons: Include normal and branched alkanes (also called n-paraffins) and cyclic alkanes or cycloparaffins (also called naphthenes) which include sesquit-, di-, tri-, tetra-, and pentacyclic terpanes and sterane biomarkers. Saturates usually are the most abundant constituents in crude oils and are markedly reduced due to biodegradation in heavy crude oils.
2 Unsaturated hydrocarbons: Include linear and branched alkenes (also called olefins). Alkenes are not generally found in crude oil, but are common in thermally-produced products, such as naphtha.
3 Aromatic hydrocarbons: Include single-ring aromatics (also called monoaromatics e.g., benzene, toluene, and xylenes) which are considered to be the most acutely toxic components of crude oil and potential carcinogens, and multi-ring aromatics also known as polycyclic aromatic hydrocarbons (PAHs) (e.g., naphthalene, anthracene, and phenanthrene), which have two or more fused aromatic rings. A typical crude oil contains approximately 1% polycyclic aromatic hydrocarbons.
Nonhydrocarbons include polars, resins, and asphaltenes.
4 Resins: Include polar compounds containing nitrogen, sulfur, and oxygen atoms that impart a “polarity” to the compounds and are often referred to as NSO

compounds. Nitrogen-containing compounds include benzocarbazoles, quinolines, and porphyrins, sulfhur-containing compounds include benzo-, dibenzo-, and polynuclear thiophenes, and oxygen-containing compounds include furans, phenols, and acids.

5 Asphaltenes: Consist of poorly characterized high molecular weight compounds that include both high molecular weight and poorly characterized hydrocarbons and NSOs. Metals such as nickel, vanadium, and iron are also associated with asphaltenes.

Heavy crude oils contain higher percentages of aromatic hydrocarbons, predominantly PAH, and nonhydrocarbons (NSOs) than conventional crude oils. (Zhu et al., 2001; Speight and Arjoon, 2012; Wang and Stout, 2007).

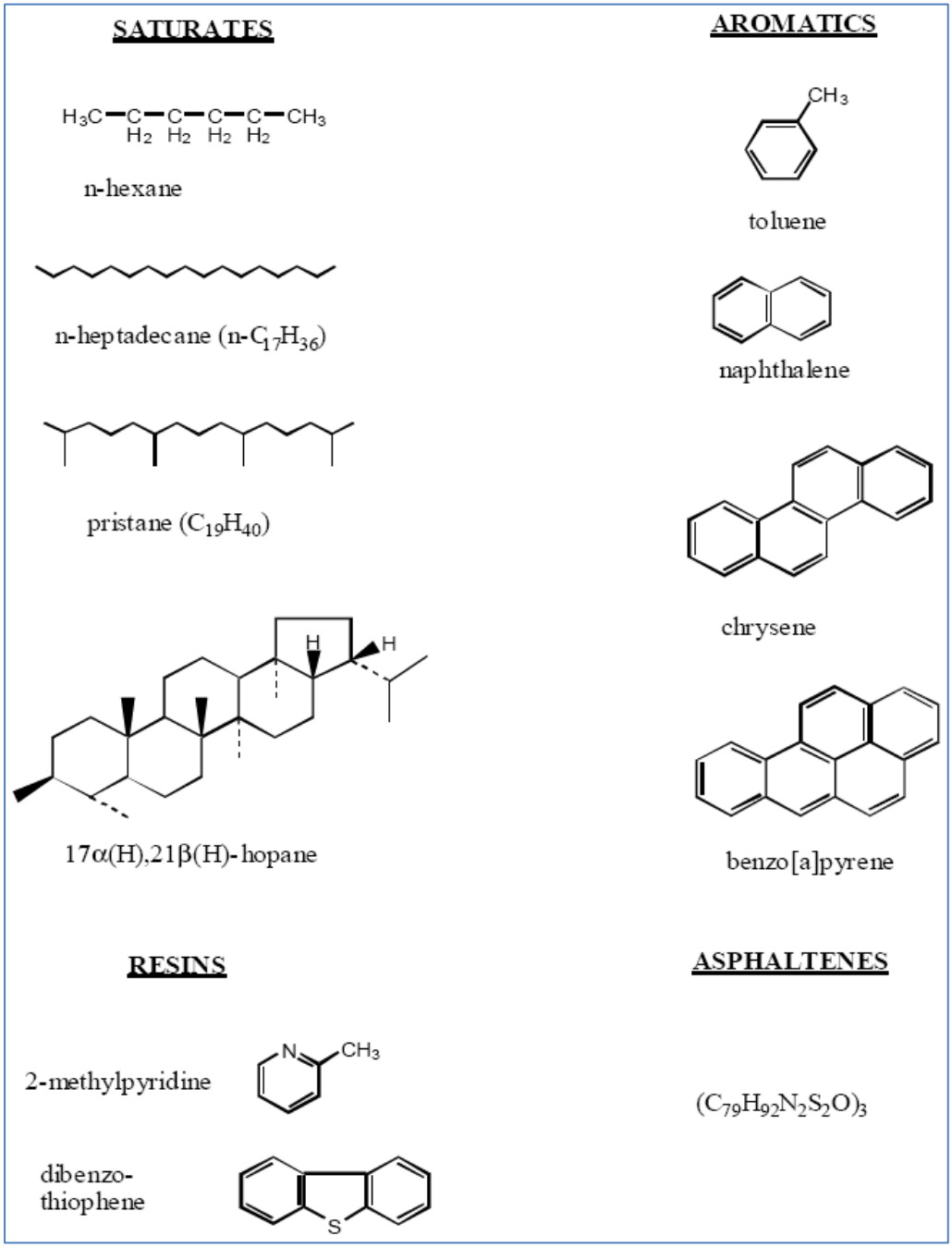


Figure 2: Examples of representative organic compounds found in crude oils.

1.4.2. Physical Properties of Oil

Important properties for characterizing the behaviour of petroleum and its products when released to the environment include:

Density is the mass of a unit volume of material at a specified temperature and has the the dimensions of g/cm^3. Density is a determinant as to whether or not the crude petroleum or its product will float on water and therefore remain susceptible to aerial oxidation and subsequent emulsion formation. :

Two types of density expressions for oils are often used: specific gravity and American Petroleum Institute (API) gravity. Specific gravity is the ratio of the mass of a volume of the substance to the mass of the same volume of water at a specified temperature. The API gravity arbitrarily assigns a value of 10 to pure water at 10°C (60°F). The API gravity can be calculated from the specific gravity using the formula:

$$API\ Gravity\ (\circ) = \frac{141.5}{Specific\ Gravity\ (16\ ^{o}C / 60\ ^{o}F)} - 131.5$$

Oils with low densities or low specify gravities have high API gravities. The specific gravity of petroleum usually ranges from about 0.8 (45.3 API) for the light and heavy crude oil to over 1.0 (< 10 API) for tar sand bitumen. Oil density is an important index of oil composition that is frequently used to predict its fate in water.

2. Viscosity: Viscosity is the property of a fluid that describes how it resists a change in shape or movement. The lower the viscosity a fluid has, the more easily it flows. The viscosity of petroleum is related to oil compositions and the ambient temperature. It is an important index of the spreading rate of spilled oil.

3. Pour Point: The pour point of an oil is the temperature at which it becomes semi-solid or stops flowing. The pour point of crude oils varies from –57°C to 32°C. It is another important characteristic with respect to oil fate and cleanup strategies.

4. Solubility in water: The solubility of oil in water is extremely low and depends on the chemical composition of the petroleum hydrocarbon in question and temperature. For a typical crude oil, solubility is around 30 mg/L. The most soluble oil components are the low molecular weight aromatics such as benzene, toluene and xylene. This property is important with respect to oil fate, oil toxicity and bioremediation processes.

Other important physical properties of oils include flash point, vapor pressure, surface tension, emulsion formation and adhesion (Zhu et al., 2001; Speight and Arjoon, 2012).

1.3. Fate (Weathering) of Oil Spills in the Environment

Oil is a generic term used for petroleum products that consist mainly of hydrocarbons. Crude oils constitute of a wide variety of hydrocarbons ranging from very volatile such as propane and benzene to more complex heavy compounds such as bitumens, asphaltenes, resins and waxes. Refined petroleum products such as diesel or jet oil are composed of smaller and more specific ranges of hydrocarbons.

When oil is spilled in a marine environment, it will break up and be dissipated or scattered over time. This dissipation is the result of a number of processes (chemical, physical or biological) that change the composition of the originally spilled oil. These processes are collectively known as weathering (ITOPF, 2013). As bioremediation is

a rather slow process used after conventional cleanup has been applied, the residual oil is often highly weathered before enhanced bioremediation strategies are applied.

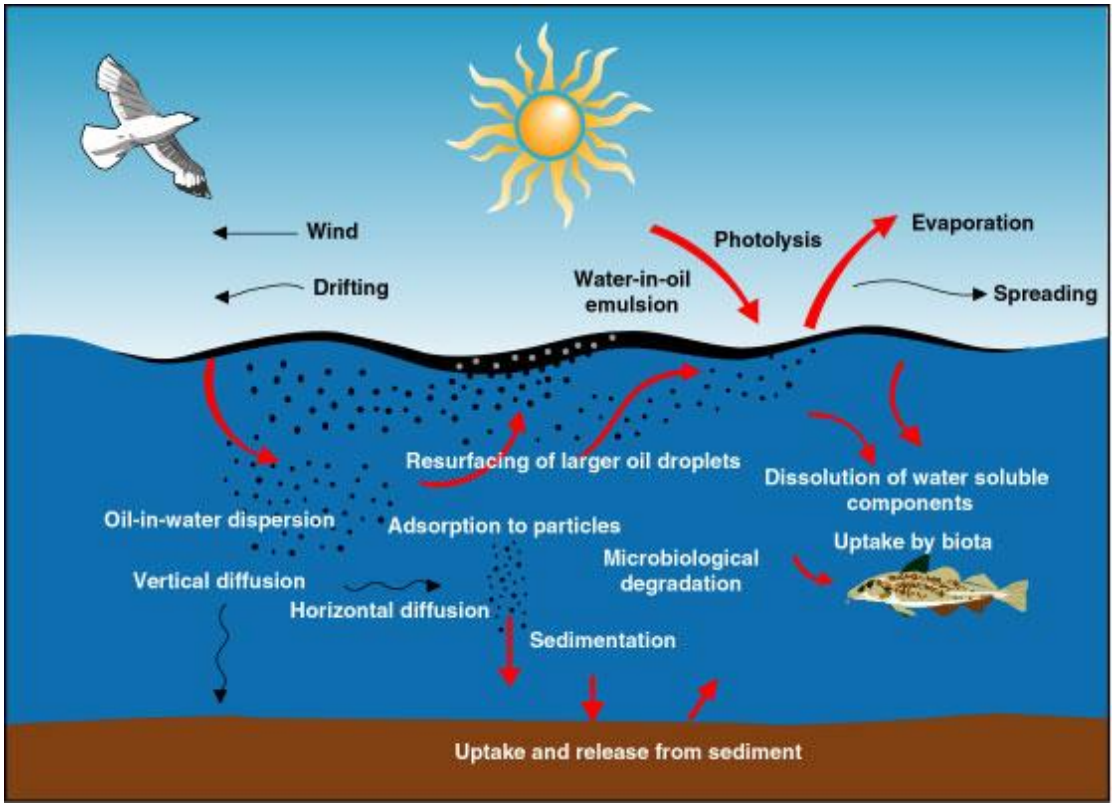


Figure 3: Oil spill weathering processes (http://www.sintef.no).

The physicochemical and biological processes that take place in the weathering of crude oil are: spreading, evaporation, dissolution, photo-oxidation, dispersion, emulsification, biodegradation as well as adsorption onto suspended particulate matter, sedimentation and tar ball formation. During the early stages of a spill, spreading, evaporation, dispersion, emulsification and dissolution are the most important weathering processes whilst oxidation, sedimentation and biodegradation are more important later on and determine the ultimate fate of the oil (ITOPF, 2013). In the long run, it is biodegradation that eventually removes the spilled oil from the environment. The combined effect of these processes in addition to action of the waves creates high variability in field studies and difficulties in the evaluation of the efficacy of bioremediation enhancing agents.

Biomarkers are often used to overcome the latter problem. These are non-biodegradable components present in crude oil. The extent of biodegradation is estimated by comparing the ratio of a target hydrocarbon concentration to the concentration of any of these recalcitrant biomarkers. Several substances have been proposed as biomarkers (e.g., pristane & phytane; hopanes and alkylated PAHs isomers) although hopanes have emerged as the best choice (Prince et al., 1994). Hopane normalization is an effective way to distinguish biodegradation from the effects of the physical washout and sand/sediment exchange (Venosa et al., 1996).

1.4. Mechanism of Oil Biodegradation: A Microbiological Perspective

1.4.1. Distribution of Hydrocarbon-Degrading Microorganisms

Hydrocarbons as part of the organic carbon cycle exist for years in the environment and consequently hydrocarbon degrading microorganisms are present globally but in very low abundance. However fossil fuels exploitation with serious amounts of hydrocarbons either deliberately or accidentally released to the marine environment are interfering to the carbon cycle and are causing substantial changes in the hydrocarbon degraders community's composition and activity. Oil inputs to the marine environment can be highly toxic to the marine microbial communities but on the other hand this selective pressure can considerably increase the number of the hydrocarbon discriminating microbial communities in the particular oil contaminated areas (Harayama et al., 2004).

Hydrocarbon degraders have a very versatile metabolism, so that petroleum hydrocarbons are one amongst many other substrate classes that can serve as carbon sources and are not the first ones which are preferred as substrates (Harayama et al., 2004; Margesin et al., 2003). However there is a newly characterized class of bacteria that uses exclusively hydrocarbons as substrates and has been categorized as obligate hydrocarbonoclastic bacteria (OHCB) (Harayama et al., 2004; Head et al., 2006; Yakimov et al., 2007). These so called hydrocarbonoclastic bacteria are of great importance since they belong to the key players in oil removal from contaminated marine sites.

Seawater bacteria cannot be readily retrieved by culture-dependent methods and those few that can be cultured are generally quite different from those identified by the culture-independent molecular techniques rRNA approaches.

Marine microbial communities response to oil pollution has been extensively investigated with molecular techniques like Polymerase Chain Reaction (PCR) in combination with methods that generate fingerprints such as Denaturing Gradient Gel Electrophoresis (DGGE) the last few years with effective characterization and isolation of hydrocarbon degrading bacteria that usually belong to the genera of Alcanivorax , Cycloclasticus, Marinobacter, Thallassolituus, Neptunomonas, Oleiphilus and Oleispira within the γ-Proteobacteria, and of the genus Planococcus within Gram-positive bacteria (Harayama et al., 2004; Yakimov et al., 2004). Among these Alcanivorax, Marinobacter, Thallassolituus, Cycloclasticus, Oleispira are the most characteristic representatives of the hydrocarbonoclastic bacteria (OHCB) due to their universal distribution and frequent domination on HC-degrading communities that have been fully investigated (Yakimov et al., 2007).

Alcanivorax borkumensis, which is ubiquitous in oil polluted marine environment is able to metabolize linear and branched alkanes, but unable to use aromatic hydrocarbons, sugars, amino acids, fatty acids and most other common substrates as the carbon source (Schneiker et al., 2006; Yakimov et al., 1998). Thallassolituus oleivorans is highly specialized in aliphatic hydrocarbons from C7 to C20 carbons (Yakimov et al., 2004). On the contrary Cycloclasticus strains are exclusively able to grow with several PAHs like naphthalene, dibenzothiophenes, phenanthrenes, anthracene, pyrene and fluorenes with or without alkyl substitution (Staley, 2010)

On the other hand Oleiphilus and Oleispira strains grow on the aliphatic hydrocarbons, alkanoles and alkanoates (Harayama et al., 2004) whereas Marinobacter strains are able to degrade efficiently hydrocarbon and petroleum compounds. This ability to use either aliphatic (i.e. C14-C18, pristine) or aromatic

hydrocarbons (i.e. fluoranthene) as sole carbon sources is a significant characteristic since this ability is not described for other true marine hydrocarbon-degrading strains (Duran, 2010).

Other 'non-professional' hydrocarbonoclastic bacteria that have been isolated include: Vibrio, Pseudoalteromonas, Marinomonas and Halomonas which are marine bacteria capable of degrading phenanthrene or chrysene, naphthalene-degrading bacteria Staphylococcus and Micrococcus, 2-methylphenanthrene- degrading Sphingomonas and alkane-degrading Geobacillus (Harayama et al., 2004).

In general, the trend in biodegradation of petroleum hydrocarbons rate follows the order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. However, this pattern is not consistent since it is highly influenced by the compositional heterogeneity among different petroleum and petroleum products nonetheless resin and asphaltenes are the most recalcitrant compounds of the petroleum hydrocarbons.

Alkane degradation is induced by alkane hydroxylases which introduce oxygen atoms derived from molecular oxygen into the alkane substrate and convert alkanes to alkanols. According to van Beilen and Funhoff, 2007 depending on the chain-length of the alkane substrate three categories of alkane hydroxylases that act on short-, medium- and long-chain alkanes can be distinguished. Short-chain-length alkanes (C2–C4) are oxidized by methane monooxygenase-like enzymes. Medium-chain-length alkanes (C5–C16) are oxidized by integral membrane non-heme iron monooxygenases, related to the well-characterized Pseudomonas putida GPo1 AlkB alkane hydroxylase, or by cytochrome P450 monooxygenases. Finally, longer (C17+) chain alkanes contain other types of alkane hydroxylases that only recently have started to be characterized (van Beilen and Funhoff, 2007).

Furthermore aromatic hydrocarbons besides their recalcitrant nature can be metabolized by specific microorganisms that possess the suitable genes encoding the initial enzymes mono- and dioxygenases that catalyze hydroxylation reactions that activate aromatic rings by addition of the atoms of molecular oxygen to one or more carbon atoms of the substrate. Cyclic aromatic products containing two hydroxyl substituents on adjacent carbon atoms of the ring are then converted to noncyclic products by ring-cleavage dioxygenases that cleave the C–C bond either between the hydroxylated carbons (ortho cleavage) or between a hydroxylated and nonhydroxylated carbon (meta cleavage). The pathways for degradation of diverse aromatic compounds often converge by channeling the intermediates towards production of a few common hydroxylated ring-cleavage substrates such as (methyl)catechols and protocatechuate. Following ring cleavage, subsequent reactions then yield products such as acetate, pyruvate and succinate that enter the Krebs Tricarboxylic Acid cycle or are used for biosynthesis (Haddock, 2010).

In this sense and due to practical reasons and most likely because functional genes like alkBs (alkane hydroxylases) have a broad spectrum, it is informative to quantify the spatial and temporal distribution of the alkB genes with PCR (van Beilen et al., 2003). Thus gene alkB could possibly be used as a marker to predict the potential of different environments for oil degradation (van Beilen and Funhoff, 2005). In the same sense functional genes like PAH dioxygenases could be used as markers for the prediction of the contaminated environment's capacity for aromatic hydrocarbons degradation.

Additionally as has been reported by Yakimov et al., 2007 studies have shown that an influx of oil in a marine site causes population densities of OHCB to transiently increase up to 90% of the total microbial community. Among them (OHCB)

Alcanivorax borkumensis, Thallassolituus oleivorans and Cycloclasticus spp. are recognized as key players in the clean-up of petroleum-contaminated marine environments due to their global distribution by, respectively, degrading alkanes and aromatic compounds of petroleum. Hence, these universal hydrocarbon degraders which appear to be reliable bio-indicators could be used as model organisms for monitoring the age and type of hydrocarbon contamination occurred in marine environment by tracking there functional genes responsible for alkanes and PAHs degradation.

1.4.2. Bacterial Affinity-Interaction to Oil

Bioremediation studies have depicted the ability of certain bacteria to degrade petroleum hydrocarbons as was discussed above, however the mechanisms of interaction between bacteria and oil still are not completely clear -haven't been studied extensively.
However observation by microscopical apparatus (means) has demonstrated that hydrocarbon degrading bacteria have a high affinity to oil droplets and Kennedy et al, 1975 was the first that indicated direct contact between bacterial cells and oil droplets while observing hexadecane spheres densely covered by Acinetobacter sp. cells. Examination by microscopy has shown the ability of bacteria to grow on several single compounds of oil HCs both liquid and solid in the saturated and in the aromatic fraction as well forming aggregates, clusters and biofilm around HCs droplets. Biofilms are increasingly being recognised as the preferred mode of growth of microbes in a wide range of interfaces liquid–liquid, solid–liquid, gas–liquid or solid–gas. These spatially structured communities of microbes whose function is dependent upon a complex web of symbiotic interactions, are held together by sugary molecular strands, collectively termed "extracellular polymeric substances" or "EPS." The cells produce EPS and are held together by these strands, allowing them to develop complex three-dimensional, resilient, attached communities. Biofilms can be as thin as a few cell layers or many inches thick, depending on environmental conditions (CBE, 2013; Neu and Lawrence, 2009). EPS are organic polymers of microbiological origin which, in biofilm systems, are responsible for the interaction with interfaces, as well as with dissolved, colloidal and particulate compounds (after T.R. Neu and J.R. Lawrence) and these organic polymers include polysaccharides (PSs), proteins, nucleic acids, amphiphilic polymers and bacterial refractory compounds expressing different functionalities (EPS can be constructive, adsorptive, active, surface-active, informative, nutritive, locomotive and redox-active) (Neu and Lawrence, 2009).
Biofilm growth follows a stepwise pattern of development involving cell differentiation and collective behavior of the cells; however studies of bacteria growing at oil hydrocarbons interfaces have not yet gone far enough to say whether they share all the characteristics of extensively studied model biofilms. Moreover in this particular type of biofilms, oil droplets can serve both as substrate and substratum for bacteria that can be found embedded within the matrix or growing at the oil–water interface utilizing these compounds as energy source. This specificity distinguishes this type of biofilms among others and so far hasn't been studied extensively (Grimaud et al, 2010).
Since the time of first observation of cells around Hexadecane droplets by Kennedy et al, 1975, analogous examinations have been repeated by diverse alkane degrading strains like, Rhodococcus sp. Q15 on C16, C28 (Whyte et al., 1999), Acinetobacter

venetianus RAG-1 on C16, nC12-C28 (Baldi et al., 1999), Oleiphilus messinensis on C16 (Golyshin et al., 2002), Pseudomonas UP-2 on C24 (Zilber Kirschner et al., 1980), Acinetobacter calcoaceticus MM5 on C14 (Marin et al., 1996), Halomonas sp. ANT-3b on C16 (Pepi et al., 2005), Marinobacter hydrocarbonoclasticus SP17 on C16, nC8-C28 (Klein et al., 2008) and *Acinetobacter oleivorans* DR1 on C16 (Jung et al., 2011).

Attachment to solid surfaces like polycyclic aromatic hydrocarbons (PAHs) has also been described for Pseudomonas sp. on pyrene, naphthalene, fluorene and phenanthrene (Eriksson et al., 2002; Mulder et al., 1998, Rodrigues et al., 2005, Seo and Bishop 2007), Sphingomonas sp.CHY-1 on chrysene (Willison, 2004) and Mycobacterium sp. LB501T on fluorene and phenanthrene (Bastiaens et al., 2000).

Few studies focused on attachment to more complex HCs mixtures like crude oil, diesel fuel, and heating oil as has been described for Acinetobacter calcoaceticus MM5 (Marin et al., 1996) and Alcanivorax borkumensis SK2 (Martins dos Santos et al., 2010).

However biofilms in nature most often consist of multi-species consortia. In these consortia, individual species interact with the community in both synergistic and antagonistic ways. Relationships between species can either be mutualistic, where both organisms benefit, commensal, where one benefits and the other is unaffected, or parasitic, where one organism benefits at the expense of another. Nonetheless only few studies have been conducted for the observation of multispecies biofilm development on HCs. Deppe et al., 2005 has observed biofilm forming on oil droplets by a consortium that was enriched on crude oil from Arctic Sea ice and seawater from Spitzbergen. This consortium effectively utilized n-c24-c34, pristine, xylenes and crude oil as mixed culture on the contrary single or combination of strains weren't able to adapt and degrade crude oil successfully. Similar investigations were conducted by other researchers like Li et al., 2000 using 16 different kinds of hydrocarbon degrading bacteria on liquid paraffin and naphthalene, Southam et al., 2001 on waste engine oil with an unknown consortium and Oliveira et al., 2009 using 2 species consortium on oil. Finally Stach and Burns, 2002 compared diversity of planktonic cells to biofilm cells developing on PAHs (naphthalene-phenanthrene) which exhibited 3 times higher diversity from planktonic cultures.

Although attachment and growth of bacterial cells on several hydrocarbon compounds has been demonstrated, only few studies examined actual consumption i.e. degradation of these hydrocarbon compounds (Efroymson and Alexander, 1991; Wick et al., 2003; Zilber Kirschner et al., 1980; Rodrigues et al., 2005;, Macebo et al., 2005;, Seo and Bishop 2007;, Klein et al., 2008;, Jung et al., 2011).

By experience and knowledge, immediate response actions in the event of an oil spill include first of all mechanical containment and collection of the oil spill and most likely afterwards the application if it is environmentally accepted of dispersants in order to dissolve the remaining oil into the water column which subsequently will be drifted away by the waves and the currents.

Studies have shown that there is great variety of bacteria that either have affinity, can metabolize hydrocarbons or produce biosurfactants or similar chemicals that is induced in a hydrocarbon polluted environment. Nonetheless single strain bacteria have been thoroughly tested as has been described in detail above on their ability to degrade a variety of single components of petroleum but not so many on mixtures of hydrocarbons such as crude oil or any other petroleum products, little work has been conducted on multispecies effect and action on degradation of either single components nor complex mixtures of hydrocarbons

Although in the real environment numerous bacteria are organized and grouped in an interactive way in between them and become associated to the pollutant, studies haven't yet focused on multispecies consortia mechanisms to degrade hydrocarbons and most preferably oil since real accidents include release of oil and its products.
Every possible means of Microscopy have been used for the observation of biofilm community developing at Hydrocarbon - water interfaces. However a major question that arises from investigations on hydrocarbons biodegradation is how microorganisms contact the substrate.

1.5. Response to Oil Spills in Marine Shorelines

A number of approaches and technologies have been developed for controlling oil spills in marine shorelines. These methods have been reviewed and described extensively in a number of technical documents and the most commonly used shoreline cleanup options are summarized in Table 1.

Table 1: Conventional shoreline clean-up options

Category of Response Options	Example Technology
Physical Method	Booming Skimming Manual removal (Wiping) Mechanical removal Washing Sediment relocation/Surf-washing Tiling In-situ burning
Chemical Method	Dispersants Demulsifiers Solidifiers Surface film chemicals

Although conventional response actions, such as physical removal with booms, skimmers and absorbent materials (Figure 4), are the first option, they rarely achieve complete cleanup of oil spills (10-15% of spilled oil is recovered) and must be deployed soon after the spill occurs. Chemical methods, particularly dispersants, although they have been routinely used in many countries as a response action, are only allowed when the coastline depth is more than 15 m. Chemical dispersants, a mixture of solvents and surfactants, reduce the interfacial tension between the water and oil phases and, hence, the oil is easily dispersed into small droplets carried away by natural seawater movement. They offer immediate and significant relief to sea-surface wildlife (marine birds and mammals) and shoreline protection although these benefits are realized to the detriment of the prevailing environmental quality in the water column. Due to their potential toxicity effects on marine organisms, chemical dispersants can be applied only under certain conditions and after they have undergone significant testing before their use is approved (Kirby and Law, 2008). The dispersion of oil into small droplets increases its bioavailability to naturally occurring microorganisms and thus if the chemical dispersants are not very toxic, they are generally expected to enhance biodegradation rates. This expected increase can only

be realized if other potentially limiting substrates are also present in the water column, i.e., N, P and dissolved oxygen (Nikolopoulou and Kalogerakis, 2009).
On the other hand biological methods have gain importance and acceptance mainly due to the low environmental impact, the costs and the capability to degrade a wide variety of organic contaminants (Rulkens and Bruning, 2005). Bioremediation through its first successful application on Exxon Valdez spill (Zhu et al., 2001), has motivated many researchers to investigate physical, chemical and biological factors that could produce favorable conditions for in-situ and ex-situ treatments.

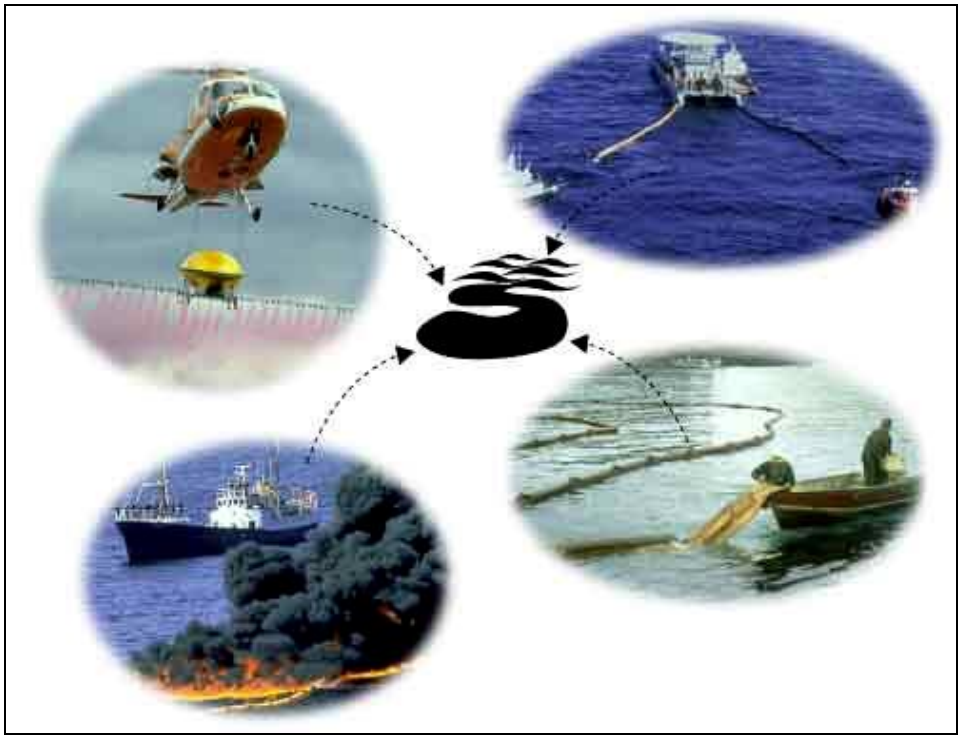


Figure 4: Most common first response options to an oil spill.

1.6. Alternative Technologies to Combat Oil Spills

Over the past few years enhanced bioremediation has emerged as a promising technology for combating marine oil spills following first response actions in the case that decontaminated area is accessible and not sensitive. By now it is a well known fact that diverse oil-degrading bacteria inhabit marine environments around the globe. The natural cleaning action by indigenous hydrocarbon degraders, known as intrinsic bioremediation, can be enhanced by the following two complementary approaches: bioaugmentation and biostimulation. In bioaugmentation, the addition of oil-degrading bacteria boosts bioremediation rates whereas in biostimulation, the growth of indigenous hydrocarbon degraders is stimulated by the addition of nutrients (mainly N & P) or other growth-limiting nutrients.
Oil spill incidents are approached differently due to the case specific conditions that can be encountered near Shore and off shore. So oil spill problems are divided in two categories open water problems and Shoreline problems. While bioremediation in liquid matrices (seawater) is implemented in a more direct way, in the soil matrix is achieved through landfarming systems. The common strategy that is being followed in the seawater matrix is to design bioremediation agents which target the oil droplets in the sea water and are not readily diluted or washed out by the wave action.

1.6.1. Landfarming

Landfarming, typically used for remediating refinery petroleum sludges, is among the bioremediation technologies that have been also used for the remediation of crude oil contaminated marine soil and sediments (Nikolopoulou and Kalogerakis, 2011).
Landfarming, also known as land treatment or land application, is an above-ground remediation technology for soils that reduces concentrations of petroleum constituents through volatilization and biodegradation (Figure 5). This technology usually involves spreading excavated contaminated soils in a thin layer on the ground surface and stimulating aerobic microbial activity within the soils through aeration and/or the addition of minerals, nutrients, and moisture (US EPA, 2004). Landfarming has been successfully practiced for over 100 years in treating mostly hydrocarbon contaminated soils. Lighter petroleum hydrocarbons are mainly removed from soil through volatilization and to a lesser extent due to microbial degradation. On the other hand, heavier petroleum hydrocarbons like lubricating oils and diesel fuel do not evaporate and their removal is due to microbial breakdown, which takes longer (Khan et al., 2004). Nonetheless it has become more attractive than other soil remediation methods because it has low cost, energy consumption, risk of contaminant migration and low environmental impact, but most importantly landfarming complies with government regulations and is very versatile to any climate and location (Besaltatpour et al., 2011). Major factors influencing landfarming performance are summarized in Table 2. Landfarming can be in situ or ex situ; if contaminated soils are shallow (i.e., < 1 m below ground surface), it may be possible to effectively stimulate microbial activity without excavating the soils, if petroleum contaminated soil is deeper than 1.7 m, the soils should be excavated and reapplied on the ground surface (US EPA, 2004).

Figure 5: Typical landfarming treatment unit

Soil conditions that are often controlled to optimize the rate of contaminant degradation include:

- Moisture content (usually by irrigation or spraying).
- Aeration (by tilling the soil with a predetermined frequency, the soil is mixed and aerated).
- pH (buffered near neutral pH by adding crushed limestone or agricultural lime).
- Other amendments (e.g., Soil bulking agents, nutrients, etc.) (US FRTR, 2007).

Petroleum hydrocarbon compounds bind to soil components and are more difficult to remove and degrade compared to oil in seawater. According to Harmsen et al., 2007

microorganisms that exist within pores in the soil or the sediment matrix cannot inhabit pores that are smaller than their own size. Under such conditions of pore size distribution, the bioavailability of the absorbed contaminants is limited (Harmsen et al., 2007). The stimulation of the indigenous microbial population is often beneficial since they can produce oil dispersive compounds like biosurfactants that could accelerate hydrocarbon degradation processes (Kosaric, 2001; Ron and Rosenberg, 2002). Biosurfactants which are more effective and environmentally friendlier than chemical surfactants consist of a hydrophilic moiety and a hydrophobic moiety, structure that allows them to enhance water solubility of hydrocarbons and increase the displacement of oil molecules from soil particles (Calvo et al., 2009; Banat et al., 2010; Ron and Rosenberg, 2010). For these reasons, the application of biosurfactants in a bioremediation treatment of a hydrocarbon polluted environment could be really advantageous. In addition, successful landfarming operation requires beside the addition of N & P based-nutrients and other growth limiting co-substrates (biosurfactants), the addition of specialized cultures typically comprising of allochthonous degrading prokaryotes especially at the startup phase of the landfarming process (Kalogerakis, 2005).

Table 2: Advantages and Disadvantages of Landfarming (US EPA, 2004; Maila and Cloete, 2004).

Advantages	Disadvantages
Technology is simple to design and implement	Reductions of concentration greater than 95% and concentrations lower than 0.1 ppm are difficult to achieve
Short treatment times (6-24 months under optimal conditions)	May not be effective for high constituent concentrations (greater than 50,000 ppm total petroleum hydrocarbons)
Very low capital and operation input required	Applicable only to biodegradable pollutants
Large soil volumes can be treated	Large treatment area is needed
Can be applied ex-situ	Volatile constituents tend to evaporate rather than biodegrade during treatment
Effective on organic constituents with slow biodegradation rates	Involves risk of pollutant exposure
Has small environmental impact	Adsorbents like clay and organic matter can decrease the bioavailability and therefore lower biodegradation efficiency as contaminants are tightly bound to the soil matrix
Energy efficient	Substantial cost can be incurred during excavation

Existing bioremediation approaches and current research for open sea or near shore polluted marine environments by petroleum hydrocarbons are reviewed in the following section.

1.7. Types of Bioremediation Amendments and Considerations in Their Application

1.7.1. Biostimulation

In marine ecosystems, spilled petroleum hydrocarbons represent a large carbon source for the microorganisms whereas in most cases the presence of nitrogen and phosphorous is limited. Biostimulation refers to the addition of one or more rate-limiting nutrients to accelerate contaminant biodegradation rates. In most shoreline ecosystems that have been heavily contaminated with hydrocarbons, nutrients are likely the limiting factors in oil biodegradation. Oxygen represents another very significant and potentially rate-limiting nutrient that should be kept in mind before embarking on a biostimulation program in the field (Nikolopoulou and Kalogerakis, 2009).

1.7.1.1. Water Soluble Inorganic Nutrients

From laboratory experiments it has been shown that the addition of growth limiting nutrients, namely nitrogen and phosphorus, enhances the rate of oil biodegradation and the optimum ratio of carbon to nitrogen to phosphorus is about 100:10:1 (Evers et al., 2004). The actual amount of N and P needed for biodegradation of the released hydrocarbons is site-specific as it is associated with the type of oil and the background value of nutrients in the marine environment.
Xia et al., 2006 studied the effects of different forms of N in seawater polluted by diesel. They found that the addition of NO_3-N was more successful than that of NH_4-N in accordance to previous studies by Wrenn et al., 1994 where in poorly buffered seawater polluted with Arabian light crude oil, nitrate was found as a better nitrogen source compared to ammonia. This is attributed to acid production associated with ammonia metabolism which inhibits oil biodegradation. When the pH was controlled, the performance of oil biodegradation was similar for both amendments with a shorter lag time for ammonia. With no control of pH, nitrate was found to have the most pronounced effect in stimulating oil degradation when using pristane as a biomarker (Ramstad and Sveum, 1995).
Prevailing seawater temperature affects oil biodegradation. Coulon et al., 2007 found that when temperature was increased from 4°C to 20°C, it had a significant effect in all microcosm treatments and the maximum degradation of TPH was observed at 20°C. Furthermore, addition of N and P resulted in the greatest hydrocarbon degradation. However, these results do not exclude bioremediation as a treatment in polluted arctic environments, as Wrabel and Peckol, 2000 showed the effectiveness of nutrients application at coastline temperatures of the western North Atlantic. Biostimulation has been tested and applied successfully to enhance oil biodegradation in cold arctic, alpine, and Antarctic environments where psychrophilic bacteria are plentiful (Margesin and Schinner, 1999). Recently, Garcia-Blanco et al., 2007 assessed the effectiveness of biostimulation in remediating an oil-contaminated

coastal marsh dominated by *Spartina alterniflora* under north-temperate conditions (Nova Scotia, Canada).

Commonly used water-soluble nutrients include mineral nutrient salts (e.g. KNO_3, $NaNO_3$, NH_3NO_3, K_2HPO_4, $MgNH_4PO_4$, $Ca(H_2PO_4)_2$, $Na_5P_3O_{10}$), and many commercial inorganic fertilizers (e.g., the 23:2 N:P garden fertilizer used in the Exxon Valdez case). Typically, they are applied in the field by spraying aqueous nutrient solutions or by spreading dry granules. This approach was used in many field trials and was found to be effective in enhancing oil biodegradation (Roling et al., 2004; Swannell et al., 1996; Venosa et al., 1996) including arctic environments (Prince et al., 2003). However, the problem that still remains is that water-soluble nutrients are easily washed by wave and tide action and thus enhanced biodegradation is difficult to achieve in non sheltered marine environments or medium to high energy shorelines.

1.7.1.2. Slow Release Fertilizers

Considerable effort has been devoted to the development of nutrient delivery systems that overcome the washout problems characteristic of open sea and intertidal environments. Use of slow release fertilizers can provide a continuous source of nutrients to oil contaminated areas overcoming the requirement for multiple nutrient applications in the field and resulting in cost benefits compared to water-soluble nutrients due to less frequent application. Slow release fertilizers consist typically of inorganic nutrients in solid form coated with a hydrophobic compound like paraffin or vegetable oil (Nikolopoulou and Kalogerakis 2009). The best known slow-release fertilizer, Customblen (vegetable oil coated calcium phosphate, ammonium phosphate, and ammonium nitrate) performed well on some of the shorelines of Prince William Sound, particularly in combination with an oleophilic fertilizer (Atlas, 1995; Swannell et al., 1996).

Kasai et al., 2002 investigated the effects of slow release fertilizers -solid granular nitrogen fertilizer (Super IB) and slow-release solid granular phosphorous fertilizer (Linstar 30)- on the biodegradation of crude oil. The addition of fertilizers promoted the degradation of certain components of crude oil: more than 90% of *n*-alkanes (C15–C30) and more than 60% of (alkyl)naphthalenes were degraded within 30 days, whereas the degradation of three-ring aromatics (phenanthrene, anthracene, fluorene and their alkylsubstituted derivatives) was less extensive, being between 30% and 40%. On the other hand, Maki et al., 2002; 2003 found that alkanes were degraded to a lesser extent than naphthalenes or fluorenes and to almost the same extent as dibenzothiophenes and phenanthrenes in field experiments performed in sand and cobblestone beaches in Japan after the Nakhodka oil spill. However, in both laboratory and field experiments the final degradation efficiencies for each oil component in the fertilized sections were not significantly different from those in the unfertilized sections, and the degradation of each oil component had almost ceased after 6 weeks. It was concluded that excessive amounts of macronutrients are required to accelerate oil biodegradation and under these conditions fertilization is only effective in the early stages.

Xu et al., 2004 conducted a 105-d field experiment to determine the potential of Osmocote (Scotts, Marysville, OH), a slow-release fertilizer, to stimulate biodegradation of petroleum hydrocarbons in an oil-spiked beach sediment at an intertidal foreshore in Singapore. Triplicate microcosms containing 80 kg of weathered sediment, spiked with 5% (w/w) Arabian light crude oil and 1.2% (w/w) Osmocote pellets, were established, together with control microcosms without

fertilizers. The concentration of Osmocote used in this study was previously optimized in the laboratory (Xu et al., 2003). Relative to the control, the presence of the Osmocote sustained a significantly higher level of nutrients and metabolic activity of the indigenous microbial biomass in the sediment pore water over the duration of the experiment. The loss of total recoverable petroleum hydrocarbons (TRPH) and biodegradation of total *n*-alkanes (C10–C33), branched alkanes (pristane and phytane), as well as total target polycyclic aromatic hydrocarbons (PAHs) (two- to six-ring), in both the control and Osmocote-amended sediments, could be described by a first-order biodegradation model. The first-order loss rate was 2.57 times greater than that of the control. The hopane-normalized rate constants for total *n*-alkanes, branched alkanes, and total target PAHs biodegradation in the Osmocote-treated sediments were 3.95-, 5.50-, and 2.45-fold higher than the control, respectively. Overall, the presence of Osmocote was able to significantly enhance and accelerate the biodegradation of aliphatics and PAHs in oil-contaminated sediments under natural field conditions (Xu et al., 2004).

Xu et al., 2005a; 2005b also investigated the effect of the slow-release fertilizer Osmocote as well as two biopolymers, chitin and chitosan, on the bioremediation of oil-spiked beach sediments over a 56-day period under laboratory conditions. Osmocote was found to be effective in sustaining a high level of nutrients in leached sediments, as well as elevated levels of microbial activity resulting in elevated rates of hydrocarbon biodegradation. Chitin was more biodegradable than chitosan and gradually released nitrogen into the sediment. The addition of chitin or chitosan to the Osmocote amended sediments enhanced biodegradation rates of the alkanes relative to the presence of Osmocote alone, where chitosan was more effective than chitin due to its greater oil sorption capacity. Furthermore, chitosan significantly enhanced the biodegradation rates of all target polycyclic aromatic hydrocarbons (Xu et al., 2005a; 2005b).

Oh et al., 2001 studied the effect of a slow release fertilizer to stimulate the indigenous microbial biodegradation of petroleum hydrocarbons in an oil-contaminated microcosm (3% v/v) which simulated intertidal environmental systems. Results in this study suggested that nutrient amendment in a high dose (microcosm I, 144.4 mg C/Kg sand/day, versus microcosm II, 8.5 mg C/Kg sand/day) can accelerate initial oil degradation rates and this in turn may shorten the treatment period for cleaning up the contaminated site (Oh et al., 2001).

Nonetheless, the challenge that still remains in applying slow release fertilizers is to control the release rates so that suitable 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. For example, Sveum and Ramstad, 1995 tested Max Bac, a slow release fertilizer similar to Customblen, and found that it failed to enhance oil biodegradation significantly due to its slow release rate. On the other hand, if one uses a mixture of water soluble and slow release fertilizers in one application better results can be obtained.

1.7.1.3. Oleophilic Biostimulants

A successful alternative that overcomes the problem of quick dilution and wash out of water-soluble nutrients containing nitrogen and phosphorus is oleophilic biostimulants. 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 (Santas and Santas, 2000).

The best-known oleophilic fertilizer is Inipol EAP22, a 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. 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 (Swannell et al., 1996; Zhu et al., 2001). Another oleophilic fertilizer that was used extensively at the Prestige heavy fuel oil spill is S200 which differs from Inipol EAP22 only in the formulation of the surfactant component (Díez et al., 2005; Jiménez et al., 2006). Díez et al., 2005 observed enhanced biodegradation of the Prestige fuel oil in microcosms containing S200 compared with those containing inorganic phosphorous and nitrogenous salts. These results led to a bioremediation field assay at a cobblestone mixed with sand and gravel beach on the Cantabrian coast (north Spain) using S200. A rigorous control of biodegradation of aliphatic and aromatic hydrocarbons using internal conservative molecular markers for 220 days showed an acceleration of biodegradation at 30–60 days and an enhancement of biodegradation, especially of the heavier n-alkanes (C25–C35) and the alkylated PAHs (Jiménez et al., 2006). 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.

As an alternative to the chemical surfactants present in most commercial oleophilic biostimulant formulations, biosurfactants can be employed. Biosurfactants are surface-active compounds produced by microorganisms. Glycolipids, lipopolysaccharides, oligosaccharides, and lipopeptides, produced by diverse bacterial genera, have received considerable attention for environmental applications including bioremediation, soil washing, and soil flushing. Biosurfactants seem to enhance biodegradation by increasing the bioavailability of organic pollutants including crude oil components. Due to their biodegradability and low toxicity they are very promising for use in remediation technologies (Mulligan, 2005). However, successful commercialization of biosurfactants can be accomplished, only if their production costs are low and their efficacy as dispersion and remediation agents is proven in the field. Research efforts for the development of novel biosurfactants with diverse environmental applications are continuing (Mulligan, 2005; Saeki et al., 2009).

The effectiveness of oleophilic biostimulant formulations depends on the characteristics of the site such as type of sediment or high/low energy wave action and tide. From early on it was shown that oleophilic fertilizers can be more effective than water-soluble fertilizers when the spilled oil resided in the intertidal zone (Sveum et al., 1994); however, no enhancement of biodegradation rates was observed in zones of limited water transport. Variable results have also been produced regarding the persistence of oleophilic fertilizers. Some studies showed that Inipol EAP22 can persist in a sandy beach for a long time under simulated tide and wave actions (Swannell et al., 1995; Santas and Santas, 2000); however, experience from very high energy shorelines suggests that even oleophilic fertilizers can be rapidly washed out. It is noted that addition of rhamnolipid biosurfactants alone had little effect on biodegradation; however, in combination with water soluble nutrient additions, provoked a significant increase (McKew et al., 2007). Sole biosurfactant addition is warranted only to increase bioavailability of weathered petroleum components in situations where background levels of N & P are sufficiently high to sustain increased biodegradation rates.

Many researches (Atlas, 1995; Coulon et al., 2007; Díez et al., 2005; Garcia-Blanco et al., 2007; Jiménez et al., 2006; Kasai et al., 2002; Maki et al., 2002; Maki et al., 2003; Margesin and Schinner, 1999; McKew et al., 2007; Prince et al., 2003; Ramstad and Sveum, 1995; Roling et al., 2004; Santas and Santas, 2000; Swannell et al., 1995; Swannell et al., 1996; Sveum et al., 1994; Sveum and Ramstad, 1995; Venosa et al., 1996; Wrabel and Peckol, 2000; Wrenn et al., 1994; Xia et al., 2006; Zhu et al., 2001) have compared the effectiveness of these nutrient products to stimulate oil biodegradation rates. 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 (Nikolopoulou et al., 2007; Nikolopoulou and Kalogerakis, 2008), thus making it an effective tool for combating oil spills. In Table 3 the major nutrient types used in biostimulation of oil spills are shown.

Table 3: 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_3NO_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 (Pritchard et al., 1992) Delaware (Venosa et al., 1996)
Slow release (e.g., Customblen, IBDU, Max-Bac)	○ Provides a continuous sources of nutrients ○ More cost effective than other types of nutrients	○ Maintaining optimal nutrient release rates could be a challenge	Alaska (Pritchard et al., 1992) Nova Scotia (Lee and Trembley, 1993) Japan (Maki et al., 2003)
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 (Pritchard et al., 1992) Nova Scotia (Lee and Levy, 1987; Lee and Levy, 1989; Lee et al., 1995a; Lee et al., 1995b) Cantabrian Coast, Spain (Jiménez et al., 2006)

* adapted from Zhu et al., 2001 and updated with recent studies.

1.7.1.4. Oxygen Limitations

Despite the apparent effectiveness of oleophilic fertilizers or mixed products, no enhancement of oil biodegradation rates should be expected if they are added to an anoxic marine environment. In several instances the concentration of dissolved oxygen can be close to zero leading to practically zero aerobic biodegradation rates. It should be noted that although anaerobic biodegradation of hydrocarbons has been documented in marine environments, the actual rate is very low. Although oxygen can be successfully delivered (in various forms) to hydrocarbon-contaminated soils and groundwater enhancing biodegradation rates, this is not the case in marine environments as it is very difficult to implement such technologies in the field. Tiling is essentially the only option in aerating the top layers of contaminated sediments during low tide. All the above criteria for the successful biostimulation of oil spills are summarized in Tables 4 and 5.

Table 4: Additional criteria for successful bioremediation of contaminated shorelines

Criterion	Condition
Oil type	Medium – heavy oil
Treatment volume	< 4000 L
Nutrients	Limitation
Prevailing temperatures	> +5^{o}C

Table 5: Situations where biostimulation is recommended for the bioremediation of contaminated shorelines

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 limitations)

[+] NR: Biostimulation is Not Recommended

It should be noted that when we are faced with a chronically polluted marine site, we should be careful and examine more carefully the conditions that inhibit bioremediation prior to proceeding with the addition of slow release or oleophilic fertilizers (Nikolopoulou and Kalogerakis, 2009).

1.7.2. Bioaugmentation

Bioaugmentation is defined as the technique for improving the biodegradation capacity within a contaminated site to remove pollution by the introduction of specific competent strains or consortia of microorganisms (El Fantroussi and Agathos, 2005).

1.7.2.1. 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 potential substrates present in complex mixtures such as crude oil (Leahy and Colwell, 1990).

Many vendors offer microbial agents claiming to enhance oil biodegradation (Prince, 1993). However, laboratory studies on bioaugmentation have produced mixed results.

Aldrett et al., 1997 tested thirteen different bioremediation products for their effectiveness in biodegrading petroleum hydrocarbons. All 13 products tested in this 28-day period experiment were listed on the NCP (US EPA National Contingency Plan) product schedule. Of these 13 products, 12 were bioaugmentation agents and one was a biostimulation agent. This experiment revealed that the petroleum hydrocarbons were biodegraded to an extent significantly greater than that achieved by the naturally occurring microorganisms. After 28 days, some products reduced the total saturated petroleum hydrocarbons fraction to 60% of its initial weight and the total aromatic petroleum hydrocarbons fraction to 65%. Three of the 13 products tested enhanced microbial degradation of the petroleum to a degree significantly better than the nutrient control treatments (Aldrett et al., 1997).

Hozumi et al., 2000 tested the effectiveness of the bioaugmentation agent TerraZyme^{TM} in treating heavy oil spilled from Nakhodka. The results exhibited a high potential for biodegradation of oil. Approximately 35% of the Nakhodka oil was degraded in 100 ml of test samples containing 1000 ppm of the initial concentration of the oil during the three-week test period and biodegradation extended to the hardest material in this contained heavy oil, the asphaltene fraction (Hozumi et al., 2000).

In another shaker flask experiment ten oil spill bioremediation products were tested for their ability to enhance biodegradation of weathered Alaskan North Slope crude oil in both freshwater and saltwater media. The products included nutrients to stimulate inoculated microorganisms, nutrients plus an oil-degrading inoculum, nutrients plus compounds intended to stimulate oil degrading activity, or other compounds intended to enhance microbial activity. The product tests were undertaken to evaluate significant modifications in the existing official United States Environmental Protection Agency (EPA) protocol used for qualifying commercial bioremediation agents for use in oil spills. In saltwater tests, six products demonstrated various degrees of biodegradative activity against the alkane fraction of the crude oil and three degraded the aromatic hydrocarbons by more than 10% (2 of them were oleophilic ferilizers and one was microorganisms with nutrients). In general, little evidence of significant growth of either alkane- or PAH-degraders occurred among any of the ten products in the saltwater. With respect to the seven products containing microbial cultures, only one product was able to significantly biodegrade both oil fractions (Haines et al., 2005).

Vinas et al., 2002 investigated the capacity of three microbial consortia that were obtained by sequential enrichment using three different oil products on degrading crude oil. Consortium F1AA was obtained on a heavily saturated fraction of a degraded crude oil; consortium TD, by enrichment on diesel and consortium AM, on a mixture of five polycyclic aromatic hydrocarbons. The three consortia were incubated for 10 and 20 days at 25 ^{o}C on a rotary shaker with Casablanca crude oil in order to investigate possible differences in the biodegradation of these complex hydrocarbon mixtures in relation to their origin. The extent of the attack on the different fractions of a crude oil by the different consortia was consistent with their origin. Consortium F1AA was more efficient in degradation of the saturated fraction (60%); consortium TD attacked the aliphatic fraction to a high degree (48%) but also degraded the polyaromatic fraction (11%); and consortium AM was the most efficient in the degradation of the polyaromatic fraction (19%). Consortia F1AA and TD removed 100% of n-alkanes and branched alkanes, whereas with consortium AM, 91% of branched alkanes remained. The 500-fold amplification of the inocula from the consortia by subculturing in rich media, necessary for use of the consortia in bioremediation experiments, showed no significant decrease in their degradation capability. They came to the conclusion that enrichment on selected PAHs does not make mixed populations more efficient in degrading the aromatic fractions of crude oils than crude oil fractions such as diesel (Vinas et al., 2002).

Zrafi-Nouira et al., 2009 examined the ability of the indigenous microbiota of polluted coastal seawater in Tunisia that was enriched by increasing the concentration of zarzatine crude oil to degrade this oil. The results of the present study showed that the heavy zarzatine crude oil was significantly biodegraded after 28 days of incubation with the adapted microbiota, with non-aromatic and aromatic hydrocarbon degradation rates having reached 92.6 and 68.7%, respectively. This suggests that the native microflora could have a positive effect on hydrocarbon degradation (Zrafi-Nouira et al., 2009).

Gertler et al., 2009 tested in a series of microcosms, 25 different treatments including nutrient amendment, bioaugmentation with *Alcanivorax borkumensis* and application of sorbent. The amount of transformed oil in microcosms containing non sterile seawater in general was higher than in pure cultures of *A. borkumensis*. Moreover, the relative concentrations of all components measured after 36 days of the experiment were generally lower than those measured after 7 days. Interestingly, the relative concentration of polyaromatic hydrocarbons detected after 7 days was relatively similar between 58% and 45%, but decreased in microcosms containing the original seawater community within the following 29 days. Aliphatic compounds on the other hand decreased in a different pattern depending on the type of the microcosm. After 36 days, more than 95% of the aliphatic compounds were transformed in the augmented microcosms, whereas the seawater-based microcosm still contained 18% and with the pure culture of *A. borkumensis* more than 30% of the residual hydrocarbons were remaining.

Results of this study indicate that owing to its exceptional adaptation to oil-polluted marine environments and its strong dominance in case of adequate nutrient supply, *A. borkumensis* is a major organism initiating and mainly conducting the degradation of aliphatic hydrocarbons. As it is not able to degrade all components of the oil type used in the experiment, it obviously promotes the growth of other microbes possibly by providing better access to the substrate through oil emulsification using extracellular polysaccharides (Gertler et al., 2009).

The role of biosurfactants and microorganisms that promote the production of biosurfactants on oil degradation is also stressed in another study conducted by Zhang et al., 2005. They found that *Pseudomonas aeruginosa* could degrade more than 58% of crude oil with direct or indirect addition of rhamnolipids that were used to improve the emulsification of the crude oil and thus make it more accessible to microbes (Zhang et al., 2005).
This conclusion was supported by another study from Perfumo et al., 2006 with strain *Pseudomonas aeruginosa* AP02-1 that was isolated from a hot spring environment on hydrocarbon containing mineral salts media and based on its ability to utilize a range of hydrocarbons both n-alkanes and polycyclic aromatic hydrocarbons as sole carbon source. Strain AP02- 1 had an optimum growth temperature of 45°C and degraded 99% of crude oil 1% (v/v) and diesel oil 2% (v/v) when added to a basal mineral medium within 7 days of incubation. Surface activity measurements indicated that biosurfactants, mainly glycolipids in nature, were produced during the microbial growth on hydrocarbons as well as on both water-soluble and insoluble substrates (Perfumo et al., 2006).
Abalos et al., 2004 found also that the addition of rhamnolipids accelerated the biodegradation of total petroleum hydrocarbons from 32% to 61% at 10 days of incubation. Nevertheless, the enhancement by biosurfactant addition was more noticeable in the case of the group of isoprenoids from the aliphatic fraction and the alkylated polycyclic aromatic hydrocarbons (PHAS) from the aromatic fraction. The biodegradation of some targeted isoprenoids increased from 16% to 70% and for some alkylated PAHs from 9% to 44%, indicating that the solubilization of these complex hydrophobic compounds was effective (Abalos et al., 2004).

1.7.2.2. Mesocosm Studies

Although laboratory tests were very positive, numerous mesocosm studies have demonstrated the ineffectiveness of bioaugmentation treatments. For example, Tagger et al., 1983 tested two mesocosms with crude oil. One was inoculated with an acclimated culture, while only indigenous populations were used in the other. Treating an experimental oil spill by adding a large quantity of a mixed culture of hydrocarbon utilizing bacteria did not appear to be effective: five months after the spill, the differences between the treated and untreated basins could not be determined by an uninformed observer. These tests as well as previous work led them to the conclusion that when nitrogen and phosphorous concentrations are the principal limiting factors of bacterial growth rate in an oil spill, it does not seem necessary to treat it with additional bacterial species adapted to hydrocarbon degradation. In addition, none of the five allochthonous species previously selected on crude oil were able to proliferate in the marine environment. However, the natural adaptative capabilities of autochthonous marine microflora suggests that it would be interesting to promote the proliferation of these widespread marine bacteria (Tagger et al., 1983).
Wright and Weaver, 2004 investigated the effect of seeding in salt marsh conditions. Glasshouse experiments were conducted to determine the impacts of fertilization and commercial bioremediation products on crude oil biodegradation and on changes in nutrient concentrations and populations of hydrocarbon-degrading microorganisms in salt marsh mesocosms growing *Spartina alterniflora*. Five commercial bioremediation products were used in this study and were designated by numbers 1 through 5. Product 1 was Oil Spill Eater-II Concentrate (OSEI Corp., Dallas, Texas), which contained enzymes. Product 2 was BioGEE HC Concentrate (BioGEE

International Inc., Houston, Texas), which was a microbial inoculant. Product 3 was Alpha Biosea (Alpha Environmental Inc., Austin, Texas), a microbial inoculant with nutrients. Product 4 was Oppenheimer Formula I (Oppenheimer Environmental Corp., Austin, Texas), a microbial inoculant with enzymes. Product 5 was Micro-Blaze Out (Verde Environmental Inc., Houston, Texas), a microbial inoculant with enzymes and dispersing agents. Experiments were conducted under continuously flooded and alternately-flooded/drained conditions with and without N and P fertilization. MaxBac, a slow-release fertilizer, was applied at a rate of 100 kg N /ha and 20 kg P /ha, while additional P was applied at 20 kg P /ha. Commercial products failed to enhance total oil or total petroleum hydrocarbon (TPH) degradation or the population sizes of total heterotrophs and hydrocarbon degrading microorganisms under either continuously or alternately-flooded conditions. It appears that native microbial populations were capable of degrading oil and TPH without further need of bioaugmentation. Over 50% of total oil and 60% of TPH were degraded within 33 to 41 d. The location of applied oil, either on the water surface or the flooded sediment surface, had little influence on either oil or TPH degradation. However, total oil and TPH degradation was dependent on N and P fertilization under continuously-flooded conditions. Maximum enhancement of oil degradation for continuously-flooded mesocosms occurred when the mesocosms were supplemented with NH_4^+ and P, although N and P fertilization may not be necessary when oil is associated with sediments (Wright and Weaver, 2004).

1.7.2.3. Field Studies

Most field studies have indicated that bioaugmentation is not very effective in enhancing oil biodegradation in marine shorelines, and nutrient addition or biostimulation alone had a greater effect on oil biodegradation than the microbial seeding. Nonetheless, there are some field trials that have demonstrated the effectiveness of commercial bioaugmentation products in enhancing oil biodegradation. Alpha BioSea (Alpha Environmental, Houston, Tex.) was used to treat the Angolan Palanca crude oil spilled from Mega Borg off Texas coast (Mauro and Wynne, 1990) and the catalytic feedstock oil spilled from Apex Barge in the Pelican Island and Marrow Marsh in Texas (Swannell et al., 1996). Terra-Zyme (Oppenheimer Biotechnology) was used in enhancing biodegradation of heavy oil spilled from Nakhodka in Japan in a period of eight weeks (Tsutsumi et al., 2000). Although in these studies it is claimed that bioaugmentation success was supported by either visual observation (i.e., the Mega Borg study and Apex Borg study) or digital photographic image analysis (i.e., the Nakhodka study), there is no concrete evidence to demonstrate that natural biodegradation rates of the oil were enhanced or that bioaugmentation was responsible for the disappearance of the oil.

On the other hand, Venosa et al., 1992 based on a previous laboratory (Venosa et al., 1991) study evaluating the effectiveness of 10 commercial products in stimulating enhanced biodegradation of Alaska North Slope crude oil, chose two of the products that provided significantly greater alkane degradation in closed flasks than indigenous Alaskan bacterial populations supplied only with excess nutrients. These two products, which were microbial in nature, were then taken to a Prince William Sound beach to determine if similar enhancements were achievable in the field. A randomized complete block experiment was designed in which four small plots consisting of a no-nutrient control, a mineral nutrient plot, and two plots receiving mineral nutrients plus the two products were laid out in random order on a beach in

Prince William Sound that had been contaminated 16 months earlier from the Exxon Valdez spill. The results indicated no significant differences ($P < 0.05$) among the four treatments in the 27-day time period of the experiment. A statistical power analysis, however, revealed that the variability in the data prevented a firm conclusion in this regard. Failure to detect significant differences was attributed not only to variability in the data but also to the highly weathered nature of the oil and the lack of dissolved oxygen and sufficient time for biodegradation to take place (Venosa et al., 1992).

Several studies (Lee and Levy, 1987; Venosa et al., 1992; Wright and Weaver, 2004) have noted the advantage of natural bacterial communities over allochthonous microbial inocula.

Rosenberg et al., 1992 after optimizing conditions in the laboratory for the use of F-1 and the selected bacteria for degrading crude oil, a field trial was performed on an oil contaminated sandy beach between Haifa and Acre, Israel, in the summer of 1992. The sand was treated with 5 g F-1 per kg sand and inoculated with the selected bacteria; the plot was watered with sea water and plowed daily. After 28 days the average hydrocarbon content of the sand decreased from 5.1 mg/g sand to 0.6 mg/g sand. Overall, there was an approx. 86% degradation of pentane extractables as demonstrated by dry weight, I.R. and GLC analyses. An untreated control plot showed only a 15% decrease in hydrocarbons. During the winter of 1992, the entire beach (approx. 200 tons of crude oil) was cleaned using the F-1 bacteria technology. The rate of degradation was 0.06 mg/g day (10 °C) compared to 0.13 mg/g day during the summer (25 °C) (Rosenberg et al., 1992). However, conclusions were confounded by the lack of adequate controls in the study (Swannell et al., 1996).

Accordingly Lee at al., 1997 concluded that in both shaker-flask and mesocosm-scale experiments, a commercial oleophilic bioremediation agent containing biostimulation (nutrients) and bioaugmentation (bacterial inocula) properties was more effective in enhancing oil biodegradation rates than that of no treatment and/or periodic inorganic nutrient addition. However, similar results were not obtained from a subsequent 129-day field trial conducted in a sand beach environment. In this case, periodic additions of inorganic nutrients, with and without the commercial bioremediation agent PRP (Petrol Rem, Incorporated, Pittsburgh, Pennsylvania), enhanced the number of heterotrophic bacteria and microbial respiration rates within the oiled sediments. The commercial product appeared to elevate the number of oil-degrading bacteria within the oiled sediment between days 17 and 89. However, the addition of inorganic nutrients alone, on a periodic basis, was the most effective means of enhancing the extent of oil biodegradation within the residual oil and of reducing sediment toxicity. By retaining residual oil and altering the physical and chemical characteristics of the treated sediment, the oleophilic product suppressed both the rate and extent of oil loss by tidal activity and biodegradation. This is not to say that the use of the product was ineffective in protecting the environment or was detrimental to it; the product does enhance natural biodegradation rates, and it limits the transport of beached oil to more sensitive areas (Lee at al., 1997).

In a similar way Simon et al., 1996, 2004 evaluated the performance of two commercial bioaugmentation products used to enhance petroleum bioremediation in a wetland. A 152-day experiment was conducted at a research facility on the San Jacinto River near Houston, TX, USA, using a controlled oil application to reduce heterogeneity normally associated with spilled petroleum. Additional treatments included inorganic nutrients and an oiled control (intrinsic). The biodegradation rates obtained for the bioaugmentation treatments did not show any significant differences

as compared to the oiled control, although the products demonstrated enhanced performance in the laboratory flask experiment (Aldrett et al., 1997). Overall, none of the bioremediation treatments appeared to benefit the wetland recovery in these environmental conditions (Simon et al., 2004).
Fernandez-Alvarez et al., 2006 also conducted a field study to assess the efficiency of several bioremediation products in accelerating the in situ biodegradation of the Prestige heavy fuel oil spill. Trials of bioremediation were conducted in sand, rocks and granite tiles on the beach of Sorrizo (A Coruna, NW Spain) with a water-soluble commercial fertilizer (Nitrophoska® Suprem), two commercial bioaugmentation products B350 and L1800 (Bio-Systems Co., USA) and an autochtonous microbial culture obtained by enrichment of fuel-degrading populations from the beach of Corrubedo (A Coruna, NW Spain) that was polluted by the spill. In contrast to Jimenez et al., 2006 neither the added microorganisms nor the nutrients significantly enhanced the degradation rate of the fuel oil in rocks, granite tiles or sand. Eighteen months after the spill, the rocks of the beach were still coated by a black layer of weathered fuel oil. For this reason an oleophilic product, sunflower biodiesel was tested on a rock. The application of biodiesel accelerated the gradual clean-up of the polluted surface and could also accelerate the degradation of the residual oil (Fernandez-Alvarez et al., 2006).
In summary, bioaugmentation may be effective in bench-scale studies where environmental conditions are well controlled, but this will not guarantee its effectiveness in the field.

1.7.3. Bioaugmentation or Biostimulation?

From all the above we see that even though the addition of microorganisms may be able to enhance oil biodegradation in the laboratory, the effectiveness of bioaugmentation has not been convincingly demonstrated in the field. Most of the field studies indicated that bioaugmentation is not effective in enhancing significantly oil biodegradation in most environments. Generally, it appears that in most environments, indigenous oil-degrading microorganisms can carry out oil the biodegradation if the prevailing environmental conditions do not limit them. Case studies conducted by vendors and research teams still support bioaugmentation potential as a remediation strategy to combat oil spills. The bioaugmentation treatment has been regarded as a promising technology, but is still in the experimental stage (El Fantroussi and Agathos 2005).
Studies comparing the relative performance of bioaugmentation and biostimulation suggest that nutrient addition alone had a greater effect on oil biodegradation than the addition of microbial products (Lee et al., 1997; Venosa et al., 1996) as the survival and degrading ability of microbes introduced to a contaminated site are highly dependent on environmental conditions (Gentry et al., 2004; Pritchard, 1992; Vogel, 1996). Microbial populations grown on rich media under laboratory conditions are stressed when exposed to field conditions where nutrient concentrations are substantially lower.
There are several studies (Lee and Levy, 1987; Venosa et al., 1992; Xia et al., 2006) that have reported the advantage of natural bacterial communities over allochthonous microbial inocula.
The growth of exogenous microorganisms used for bioaugmentation within a contaminated site is affected by biotic and abiotic factors. The factors responsible for

the failure of inocula in degrading contaminants in nature were summarized by Goldstein et al., 1985, Pritchard et al., 1992, Vogel, 1996 and van Veen et al., 1997. Abiotic factors include: (1) low contaminant concentration not capable to support the growth of the inoculated species, (2) the environmental conditions that play a pivotal role in determining biological activity, such as temperature, humidity and ionic strength that reduce the microbial activity and clay and organic-matter content that restrict the mass transfer (mainly by diffusion in sediments, the seeded microorganisms may be unable to move through the pores of the sediment to the contaminants),and mainly the physical removal rate caused by scouring of biomass when breaking waves tumble sand grains, (3) the biodegradability of the pollutants, which is related to chemical structure and its related physico-chemical characteristics, (4) the growth of the inoculated species may be limited by predation (e.g.,by protozoa) or competition and (5) the inoculated microorganisms may use other available substrates instead of the targeted contaminants.

There is increasing evidence that the best way to overcome the above barriers is to use microorganisms from the polluted area. Ueno et al., 2007 have proposed autochthonous bioaugmentation (ABA) defined as the bioaugmentation technology that uses exclusively microorganisms indigenous to the sites (soil, sand, and water) to be decontaminated. Isolated single strains or enriched cultures, which are obtained ‘‘before’’ or ‘‘after’’ the contamination of the target sites, are administered to the sites once contamination occurs. The key idea is to conduct the enrichment of contaminant-degrading bacteria under the same or very similar conditions to those where bioaugmentation will be performed. ABA as defined in this study uses indigenous microbial consortia or isolates that are highly enriched and much better adapted to the historically or artificially contaminated environments (Hosokawa et al., 2009).

The application of ABA in the coastal areas of Hokkaido Prefecture, Japan, was considered by Hosokawa et al., 2009, as Hokkaido is located south of Sakhalin Island, Russia, where development of oil fields is in progress. If oil spills in this region were well characterized in advance, ABA could be a feasible technology in the near future. Crude oil-degrading microbial consortia collected from the seashores of Abashiri, Hokkaido, were enriched using liquid and sea sand-containing solid media supplemented with MSM and crude oil. When no-sterilized sand from the same place was inoculated with two types of microbial consortia, degradation of crude oil was greater in the consortium prepared by cultivation in sand (consortium 2) than in that prepared by liquid cultivation (consortium 1). The extent of degradation of crude oil by consortium 1 was almost the same as that by biostimulation. These results suggested that the proliferation of bacteria indigenous to sea sand is highly dependent on their environment (Hosokawa et al., 2009).

Although, the ABA technique is not a new concept as it has been described above, only a limited number of reports have been published on ABA (Vinas et al., 2002; Zrafi-Nouira et al., 2009) compared to other types of bioaugmentation treatments. This is probably because the practical benefits of this method (ABA) have not been recognized according to Hosokawa et al., 2009.

The question that still arises is bioaugmentation or biostimulation? The answer is not unique. The appropriate strategy is shown in Figure 1 and depends highly on the particular environmental conditions (background nutrients concentration, type of pollutant, indigenous population, etc.). For example, if nutrients are limiting, the rate of oil biodegradation will be less than optimal even if there are many oil-degraders present (case II). In this case, supply of nutrients will enhance bioremediation rates, whereas bioaugmentation is not expected to have a significant effect except only short

term benefits. Cases I & III are rather unrealistic for a marine environment. They represent a oligotrophic (case I) or non-oligotrophic (case III) pristine environment that has never seen oil contamination! Case IV represents situations where both N&P nutrients are present and there is an abundance of oil-degrading microbes. In this case, low bioremediation rates are often due to lack of oxygen (Nikolopoulou and Kalogerakis 2009).

Although biostimulation is considered to be effective because indigenous bacteria are best adapted to the environment of the site that is being treated (Rahman et al., 2003), this is not always effective and a long time (of the order of weeks) may be required to obtain high microbial densities particularly for pristine environments where there is a scarcity of indigenous microbes capable of degrading hydrocarbons or better a limited diversity. In this case, bioaugmentation can clearly provide certain advantages in the short term.

Therefore, in addition to surveying the background nutrient levels at an oil spill site, the indigenous hydrocarbon-degrading populations should also be determined as part of the site assessment.

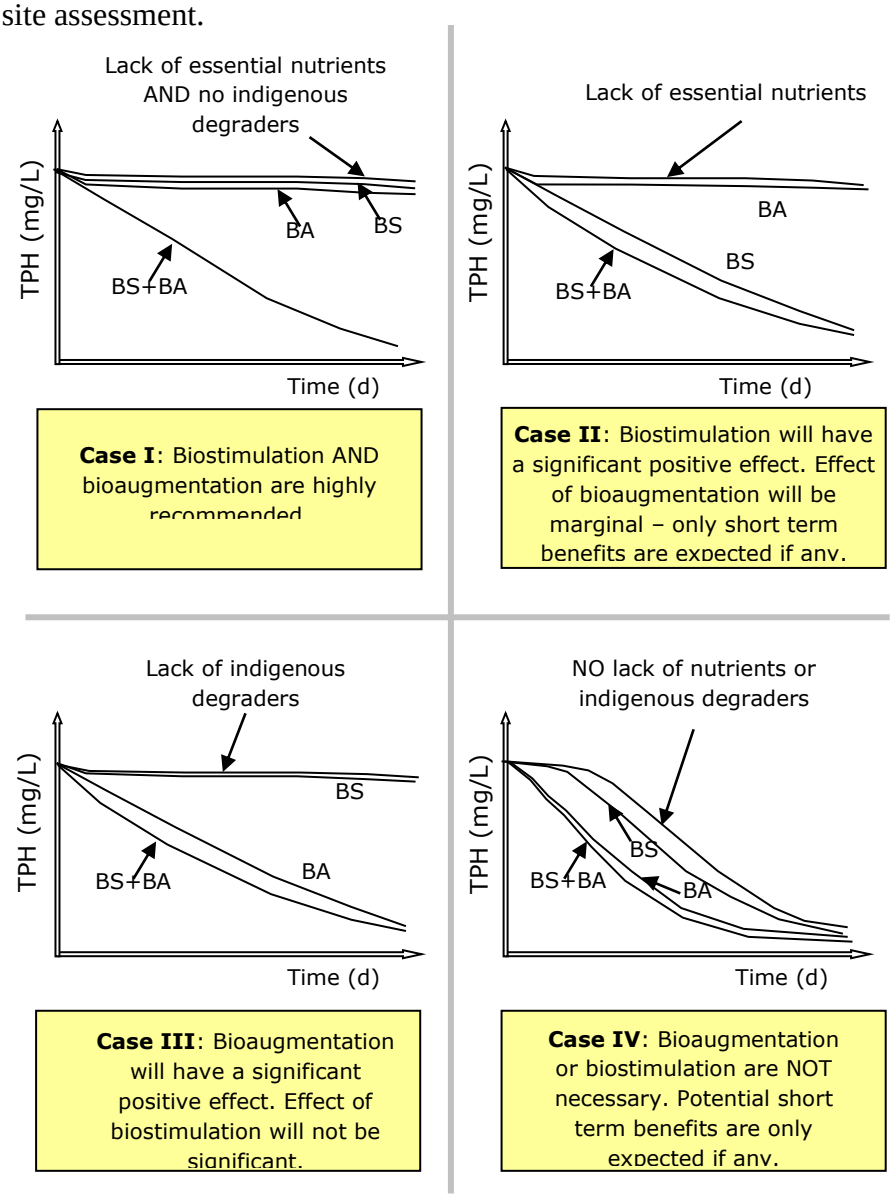


Figure 6: Effect of bioaugmentation and biostimulation on biodegradation of petroleum hydrocarbons (not to scale) for four different cases. Abbreviations of treatments: BA=Bioaugmentation, BS=Biostimulation, BS+BA=Bioaugmentation and biostimulation

Based on the above, it becomes rather obvious that a robust bioremediation strategy should provide suitable stimulation for long term performance and augmentation with oil-degrading consortia for short term benefits. Furthermore, if the augmentation is performed using indigenous populations over allochthonous ones, the bioaugmentation effects will be much more pronounced.

1.8. Contribution of this PhD

As long as society keeps on relying on petroleum hydrocarbons to cover its energy needs, despite the stricter environmental regulations that have been adopted by most countries, oil spills will remain a serious hazard to marine ecosystems. Furthermore Mediterranean Sea sensitivity, importance and especially of the Greek coastline and sea that are popular touristic destinations and shelter of many marine species makes it crucial to act and remediate the marine environment in the threat of oil spills. Accordingly seeking new, alternative, natural methods that could speed up cleaning process with minimum environmental impact could become priority.
In this manner the specific aims of the present work were to investigate possible methods to enhance the rate of biodegradation of oil in a contaminated marine environment (both seawater and shoreline), which could be incorporated in a general contingency plan. Hence we investigated the capability of either acclimated indigenous microbial consortium or enriched consortia from seawater samples taken from Hellenic Petroleum Refinery (Athens, Greece) a site exposed to chronic pollution with crude oil (ABA) in the presence or not of other rate limiting factors like nutrients and biosurfactants (biostimulation) as a potential strategy for the successful remediation of polluted marine environments. In addition the effectiveness of these certain acclimated consortia (ABA) was compared to indigenous population activity (biostimulation) on the bioremediation of oil spills.
Specifically the effects of the oleophilic nutrients (uric acid and lecithin) and inorganic nutrients (KNO_3, K_2HPO_4) with or without biosurfactants on the degradation of crude oil hydrocarbons in both seawater and sand matrix were also examined.
Thus the outcome approaches included 4 sets of experiments:

1. Autochthonous bioaugmentation and/or biostimulation of seawater microcosm (i.e. Seawater 1)
2. Autochthonous bioaugmentation & biostimulation with isolated hydrocarbon degraders consortium of seawater microcosm (i.e. Seawater 2)
3. Landfarming of oil polluted beach sand through biostimulation (i.e. Sand 1)
4. Landfarming of oil polluted beach sand through autochthonous bioaugmentation & biostimulation (i.e. Sand 2)

The method which has been used for the evaluation of these bioremediation methods is based on a modified bioremediation agent effectiveness testing protocol by EPA (40 CFR Ch. I, Pt 300, App. C, 2003). This protocol was 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 by Most Probable Number (MPN) determination and quantifies the disappearance of saturated hydrocarbons and polynuclear aromatic hydrocarbons (PAHs) by GC-MS analysis.
Moreover whether the disappearance extent (degradation rate) of certain compounds from both saturated fraction (n-alkanes) and aromatic fraction (PAHs) is due to biomass increase or due to specific degradation rate increase which implies a different

metabolic pathway was also investigated through biodegradation kinetics analysis (Batch reactor). The degradation kinetics of representative compounds from n-alkanes group and PAHs group were investigated, namely C_{15}, C_{20}, C_{25}, C_{30}, Pristane, Phytane, Fluorene, Dibenzothiothene, Phenanthrene and Chrysene. The average specific degradation rate q_s of any particular compound (S) can be obtained by Least Squares Estimation as the slope in linear plot of S_t versus the integral $I(t)=\int X(t)dt$ where X(t) is the biomass concentration at time t.

Furthermore identification of the key organisms that play roles in pollutant biodegradation is important for understanding, evaluating and developing in situ bioremediation strategies. For this reason, one of the tasks was to characterize bacterial communities, to identify responsible degraders, and to elucidate the catalytic potential of these degraders under different bioremediation methods. In the above experimental sets besides microbial activity and oil chemical analysis, molecular analysis (PCR, RT-PCR, pyrotag sequencing) of DNA extracts to identify community response and composition changes through different amendments was also performed.

Early studies on hydrocarbon biodegradation led to the observation that hydrocarbon degrading bacteria have a high affinity to oil droplets. Oil bioremediation is a complex process that involves interactions between oil and microorganisms under certain environmental conditions. Therefore in this study a 5^{th} set of experiments investigating and characterizing the interaction of hydrocarbon degraders consortia on oil and eicosane droplets (cellular level) by means of confocal laser scanning microscopy (CLSM) was also included. Moreover their response after the addition of certain commercial dispersants just like those that have been or could be used in the event of a real oil spill (Corexit, S200, Marichem) but also of more environmentally friendly biosurfactants (Rhamnolipids) was also examined. Investigation of these interactions that take place between marine bacteria and oil hydrocarbons could improve our understanding on the fate of hydrocarbons in the environment and thus help to develop the most suitable bioremediation strategy.

Chapter 2- Materials and Methods

2. Materials and Methods

For the evaluation of the bioremediation agents used, laboratory and field tests can be conducted. In this study we have run some laboratory tests in a microcosm scale according to a modified EPA bioremediation agent effectiveness test protocol.

The method which has been used for the evaluation of these bioremediation agents is based on a modified bioremediation agent effectiveness testing protocol by EPA (40 CFR Ch. I, Pt 300, App. C, 2003). This protocol was 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 by Most Probable Number (MPN) determination and quantifies the disappearance of saturated hydrocarbons and polynuclear aromatic hydrocarbons (PAHs) by GC-MS analysis.

In some experimental sets besides microbial activity and oil chemical analysis, molecular analysis of DNA extracts to identify community response and composition changes through different amendments was also performed.

2.1. Determing Soil Characteristics

2.1.1. Water Holding Capacity

The water-holding capacity of the soil was determined by placing duplicate 20 g field-moist soil samples in funnels fitted with folded Whatman 2V filter paper on the inside and mounted on preweighed 250 ml flasks as described by Forster, 1995. Percentage water-holding capacity was calculated with the following formula:

$$\%\, Water\ holding\ capacity = \frac{(100 - W_p) + W_i}{d_{wt}} \times 100, \quad (1)$$

where W_p is the weight of the percolated water in grams, W_i is the initial amount of water in grams contained in the sample, and d_{wt} is the soil dry weight in grams (Forster, 1995).

The soil was classified as sandy and its estimated water-holding capacity for the soil was 33.73%. Too much water in the soil will hinder the supply of oxygen and as a result will decrease the rate of biodegradation. On the other hand, too little water will inhibit microbial activities. The optimal soil moisture range for supporting the microbes is between 30 and 60% of the field capacity and as was estimated the optimal soil moisture content should be between 10.12% and 20.24%.

2.1.2. Soil Gravimetric Water Content and Soil Dry Mass

Water content in sand samples was determined gravimetrically after desiccation at 105°C overnight. The differences in masses before and after drying are a measure for the water content of soils. The water content is calculated on gravimetric (*g* water/*g* soil) or on volumetric basis (cm^3 water/cm^3 soil) (Wilke, 2005).

The dry mass content (w_{dm}) or water content (w_{H2O}) on a dry mass basis expressed as percentages by mass to an accuracy of 0.1% (*m/m*) are calculated using the following equations:

$$W_{dm} = \frac{m_2 - m_0}{m_1 - m_0} \times 100 \quad (2), \qquad W_{H_2O} = \frac{m_1 - m_2}{m_2 - m_0} \times 100 \quad (3), \text{ where:}$$

$m_{0=}$ mass of the empty container (g)

m_1 =mass of the container with field-moist soil (g)

$m_{2=}$ mass of the container plus oven-dried soil (g)

2.2. Crude oil weathering

Two types of crude oils were used (A: .Uralsk -light B: light from Iran, both compliments of Hellenic Petroleum Co., Aspropyrgos, Greece) Crude oil is artificially weathered (<C15) by heating to 200°C according to ASTM D86 (Standard Test Method for Distillation of Petroleum Products), which covers the atmospheric distillation of petroleum products using a laboratory batch distillation unit.
The unweathered Iranian light crude oil had a gravity of: 0.8232 g/cm^3 and SG (specific gravity): 0.8247 g/cm^3 at RT. The weathered Iranian light crude oil had a gravity of: 0.8886g/cm^3 and SG (specific gravity): 0.8902 g/cm^3 at RT

2.3. Sand preparation and spiking with crude oil

Sand for the landfarming experiments was collected from Agios Onoufrios beach (Chania), screened to remove particulates greater than 2 mm in size and was spiked with weathered crude oil at 5 g per 1000 g of sand (dry weight equivalent) that was dispersed in 1 L of pentane/DCM (1:20) solution. The soil was then further homogenized. The solvents were allowed to evaporate from the soil by placing the container of spiked soil in a fume hood, thus leaving behind the fuel oil in the sand at a theoretical initial TPH concentration of approximately 5,000 mg/kg of sand after equilibration.
In all treatments, the water content was adjusted to 60% of the field-holding capacity. This moisture content has been used in several studies and lies within the interval recommended by Dibble and Bartha, 1979.

2.4. Types of Amendments

2.4.1. Nutrients

Two types of nutrients have been used and compared: inorganic and lipophilic. Advantages and disadvantages for both types have been described in detail in the Introduction section. In addition the effect of a biosurfactant was also tested.

- Source of nitrogen:

Inorganic form: potassium nitrate (KNO_3- Sigma-Aldrich Co), which is water soluble and basic ingredient of many fertilizers.
Lipophilic form: uric acid (Sigma-Aldrich Co) that is cost effective natural origin waste product of birds etc., it has low solubility in water (it isn't readily washed out) binds to crude oil and is therefore available for bacteria which grow at the hydrocarbon-water interface

- Source of phosphorous:

Inorganic form: dipotassium phosphate (K_2HPO_4- Sigma-Aldrich Co) that is water soluble and basic ingredient of many fertilizers.
Lipophilic form: lecithin (L-a-Phosphatidylcholine (L-a-Lecithin) derived from soybean, Type II-S, with a purity of about 19% was supplied by Sigma-Aldrich Co)

that is a natural phospholipid in fact oil soluble, easy to get at low cost as by-product of oil seeds industry and has good dispersant properties (serves as a biosurfactant).

Nutrients were added to such amount that resulted in a final concentration equivalent to a C:N:P molar ratio of 100:10:1.

2.4.2. Rhamnolipids as Biosurfactant

The biosurfactant JBR210 of microbial origin (rhamnolipid) was a blend of $C_{26}H_{48}O_9$ and $C_{32}H_{58}O_{13}$. It was composed of 10% active ingredient, supplied by Jeneil Biosurfactants Co., USA. The biosurfactant is readily biodegradable and has a very low environmental impact, low toxicity and low skin irritation with excellent wetting and emulsification properties. Rhamnolipids are glycosides of rhamnose (6-deoxymannose) and β-hydroxydecanoic acid.

2.4.3. Dispersants

Corexit, a 2nd generation dispersant approved as Type I, was kindly offered by CHEMO SA (Skaramagas, Piraeus, Greece)
S200 was used at the Prestige oil spill accident in Spain and was kindly offered by IEP EUROPE, S. L. Co. (Madrid-Spain).
Marichem, a 3rd generation dispersant approved as Type II&III dispersant, was kindly offered by EPE S.A. (Piraeus, Greece).

2.4.4. Isolated Consortia

Consortia Eb8 and E4 which have been used in this study have been obtained from successive enrichments and isolations in ONR7 medium with crude oil as the sole hydrocarbon source of seawater samples taken on April 6th, 2011 from the Elefsina Refinery (Hellenic Petroleum), a site exposed to chronic oil pollution in Elefsina gulf near Athens, Greece. This work has been conducted under the FP-7 project ULIXES.

2.5. Culture Media

ONR7 Medium
ONR7 medium, an artificial seawater mineral salts medium, was used for enrichment cultures and isolation of HDB (Dyksterhouse et al., 1995). ONR7 contains (per liter of deionized water) 22.79 g of NaCl, 11.18 g of $MgCl_2 \cdot 6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2 \cdot 2H_2O$, 1.3 g of TAPSO {3-[N-tris(hydroxymethyl) methylamino]-2-hydroxypropanesulfonic acid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HPO_4 \cdot 7H_2O$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCl_2 \cdot 6H_2O$, 2.6 mg of NaF, and 2.0 mg of $FeCl_2 \cdot 4H_2O$.

Bushnell Haas Medium (BHM)
The medium, used to prepare the dilutions for the dilution series to perform the most probable number (MPN) and plate count determination, was prepared by suspending 3.27 g of Bushnell Haas Broth (HiMedia) in 1000 mL deionized H2O.

Marine Agar

ZoBell Marine Agar 2216 (HiMedia) has been used for the CFUs determination of marine heterotrophs by plate count.

2.6. Microbiological Analyses

2.6.1. Plate Counts

Enumeration of total marine heterotrophs was undertaken by spreading aliquots of 100 μL in pre estimated dilutions, taken from the MPN determination, on ZoBell Marine Agar 2216 (HiMedia). Plates were incubated aerobically at 20^{o}C for 48 h.

2.6.2. MPN Procedure

Hydrocarbon degraders in the flasks were estimated by the most probable number (MPN) method according to Wrenn and Venosa, 1996. The growth medium was a Bushnell-Hass minimal salts medium (BHS) supplemented with crude oil as the hydrocarbon substrate. The MPN plates were 96-well microtiter tissue culture plates, with each well containing 180 μl BHS, 5 μL crude oil and 20 μL of the appropriate dilution of sample. For seawater microcosms one milliliter from each microcosm was diluted in a 9 mL Bushnell-Hass solution (pH 7). While for landfarming microcosms an initial 1:10 (w/v) dilution was prepared for each sand sample before setting up the microbial assays. This was done to assure that the sediment-associated microbes were as evenly distributed as possible for all assays. Ten grams of wet sand was added to 90 mL of marine BH medium. The initial dilution bottles were shaken for 30 min at 200 rpm. Once the soil particles settled down, one milliliter from each supernatant was diluted in a 9 mL Bushnell-Hass solution (pH 7).

Then for both type of matrices samples (seawater and sand) the procedure was comprised by tenfold serial dilutions that were carried out to 10^{-10} and the plates were inoculated by adding 20 μL of each dilution to one of the 12 rows of eight wells. The inoculated plates were incubated at 20 ^{o}C for 2 weeks. At the end of the incubation period, 50 μL of p-iodonitrotetrazolium violet dye (INT 3 g/L) was added to each well of the tissue culture plates and allowed to stand at room temperature for 1 hour. The dye turns from colorless to red (when reduced) in the presence of actively respiring microorganisms. The MPNs were calculated using "MPN Calculator" software program by Albert J. Klee, 1993 of the EPA Risk Reduction Engineering Laboratory.

2.7. Chemical Analysis

2.7.1. Reagents, materials and standards

Trace analysis (SupraSolv) dichloromethane (CH_2Cl_2) and *n*-hexane (C_6H_{14}) were obtained from Merck (Darmstadt, Germany). Solid-phase cartridges of silica/cyanopropyl (SiO2/C3–CN, 1.0/0.5 g, 6 ml) were obtained from Interchim (Best Buy Analytical, Greece) and solid phase extraction cartridges "Varian Bond Elut TPH" were obtained from Agilent technologies.

The standard hydrocarbon mix (100 ppm in hexane/DCM, 9:1) for the calibration curve that contained aliphatic hydrocarbons (*n*-C10- *n*-C35, pristane, phytane) and polycyclic aromatic hydrocarbons (PAHs) (naphthalene, phenanthrene, anthracene, fluorene, dibenzothiophene, fluoranthene, pyrene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(e)pyrene, benzo(*a*)pyrene, perylene, indeno(g,h,i)pyrene, dibenzo(*a,h*)anthracene, benzo(1,2,3-cd)perylene) was obtained from Absolute Standards Inc. The semivolatile internal standard mix contained 7 deuterated compounds: d_8-naphthalene, d_{12}-chrysene, d_{12}-perylene, d_{10}-acenaphthene, d_{10}-phenanthrene and d_4-1,4-dichlorobenzene (2000 ppm in DCM) was obtained from Supelco Co. The surrogate standards (d_{10}-anthracene and 5α-androstane 2000 ppm

each in DCM) were obtained from Supelco Co. The biomarker $C_{30}17\alpha(H)$, $21\beta(H)$-hopane was obtained from Chiron, Norway.

2.7.2. Procedure and sample preparation of spilled oil

Quantification of the hydrocarbon target analytes was performed by gas chromatography/mass spectrometry.

- Seawater samples were Liquid-Liquid extracted (Figure 7).

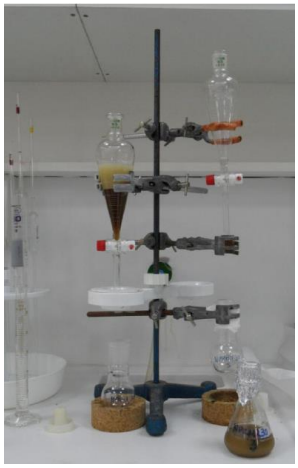


Figure 7: L-L Extraction

Tube contents (20 mL) were extracted by adding approximately 20 mL of dichloromethane spiked with 400 μL of surrogate recovery standard (200ppm of each d_{10}-anthracene and 5α-androstane).

Flask contents (50 mL) were extracted twice by adding approximately 20 mL of dichloromethane spiked with 50 μL of surrogate recovery standard (200ppm of each d_{10}-anthracene and 5α-androstane)

After mixing for several minutes, the flask was set aside to allow the dichloromethane and water layers to partition. The dichloromethane layer was drained by passing through a funnel packed with anhydrous sodium sulfate. Subsequently, the dichloromethane was evaporated in a rotavapor concentrator.

- Sand samples were extracted with soxhlet apparatus (Figure 8).

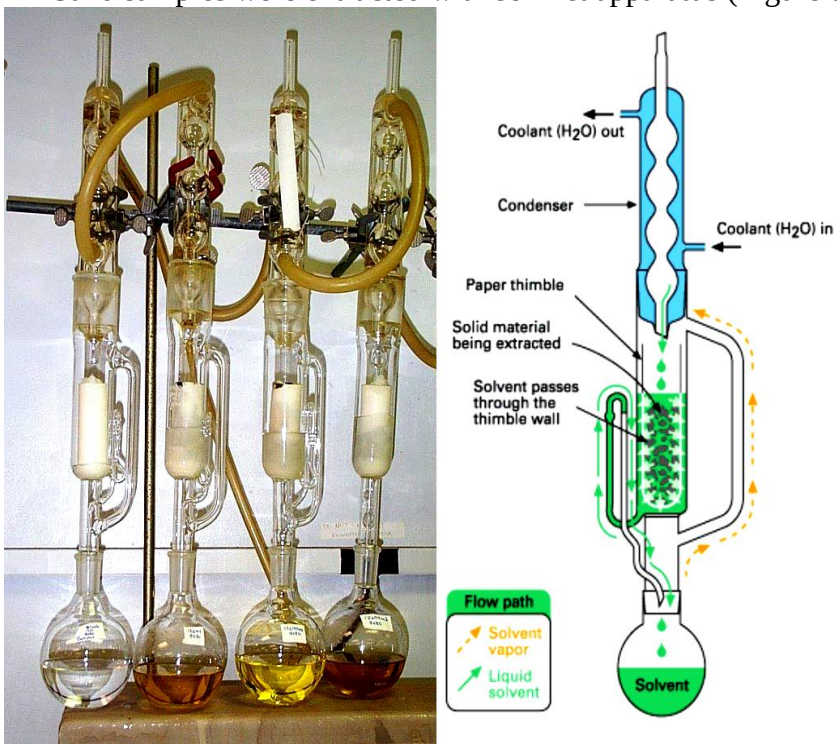


Figure 8: Soxhlet apparatus

Sand samples were dried with anhydrous sodium sulfate, spiked with a surrogate recovery standard (200ppm of each d_{10}-anthracene and 5α-androstane) and finally extracted with soxhlet apparatus using 300 mL of DCM. Afterwards, the dichloromethane was evaporated in a rotavapor concentrator.

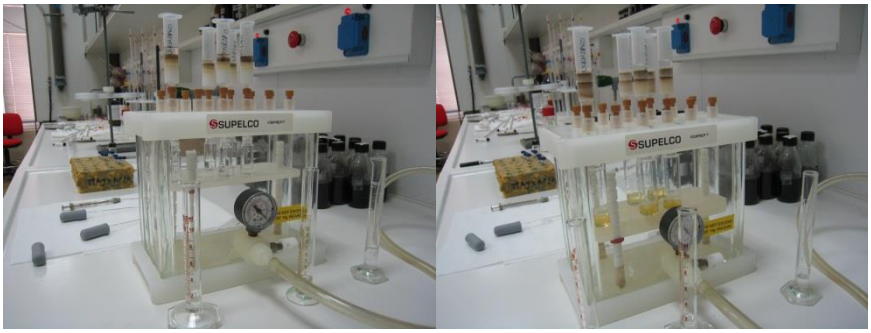


Figure 9: Solid Phase Extraction apparatus used for the fractionation of oil samples.

Recovered oil from experimental set of Seawater 1 and Sand 1 was fractionated using Solid Phase Extraction cartridges as described by Alzaga et al., 2004. According to this method a known weight of 5-10 mg of the dried oil was dissolved in n-hexane and was transferred onto the SiO2/C3–CN SPE cartridge and eluted, under a positive pressure, with 4ml of *n*-C6 (FI- aliphatics) and 5ml of *n*-C6– DCM (1:1) (FII- aromatics). Prior to sample loading, before the SPE fractionation, cartridges were conditioned with 4.0 ml of hexane. The two fractions were blown down to dryness with nitrogen. The weight of FI- aliphatics and FII- aromatics was recorded and redesolved in 1 mL *n*-C6 and 1 mL *n*-C6– DCM (1:1) respectively for use on the autosampler of the GC/MS instrument.

Recovered oil from experimental set 2 and 4 was fractionated with 4mL of n-C6 (FI- aliphatics) and 4mL of DCM (FII- aromatics) using Solid Phase Extraction cartridges "Varian Bond Elut TPH" as suggested by Agilent technologies. The two fractions were dried with nitrogen flow. The weight of FI- aliphatics and FII- aromatics was recorded, redissolved in 1 mL n-C6 and 1 mL DCM respectively and samples were ready to be putted on the autosampler of GC-MS for analysis.

The final concentration of the internal standards added in each fraction right before the injection is 1 ppm. This solution contained 4 deuterated compounds: d_8-naphthalene, d_{10}-phenanthrene, d_{12}-chrysene and d_{12}-perylene.For quantitative analyses, an Agilent HP 7890/5975C GC-MS system with an Agilent HP-5 5% phenyl methyl siloxane column (30m x 250µm x 0.25µm) was operated in Full scan mode (range 50-500 m/z). The initial oven temperature was set at 60 °C, followed by a temperature ramp of 6 °C/min up to 300 °C. The samples (1µL) were injected through a split-splitless injector (pulsed-splitless mode, at 250 °C) by an Agilent 7693A Automatic Liquid Sampler. The transfer line, MS source and quadrupole temperatures were set at 280 °C, 230 °C and 150 °C respectively.

External multilevel calibrations were carried out for both alkanes and PAHs quantification ranging from 1 ppb to 20ppm. Major hydrocarbons in crude oil were identified on the basis of their retention time and by comparing them with those of analytical standards. The repeatability of the whole experimental procedure for each experimental approach is given in the appendix, where in boxplot form the analytical data for the saturated hydrocarbons determined from all the control experiments are shown. For clarity the data are presented after subtraction of their average value. The relative standard deviation (%RSD) of the saturated fraction of *n*-alkanes and of the aromatic fraction of selected compounds ranged from 0.9 to 13.5 for n=5 repetitive analyses which is well below 25% which is the acceptable limit of each compound analysed.

To help ensure that the observed decline in target analytes is caused by biodegradation rather than by physical loss from mishandling or inefficient extraction, it is necessary to normalize the concentrations of the target analytes via a "conserved

internal marker." The conserved internal marker that has been found useful for quantification is $C_{30}17\alpha(H), 21\beta(H)$-hopane. Analytes of crude oil were normalized to the conservative biomarker 17a(H), 21b(H) C_{30}-hopane naturally present in crude oil (Prince et al., 1994). The percent depletion of all analytes within oil was calculated using the equation:

$$\%\text{Depletion} = \frac{\left[A_0/H_0 - A_S/H_S \right]}{A_0/H_0} \times 100\% \quad (4)$$

where A_S is the concentration of target analyte in the sample; A_0 the concentration of target analyte in the initial sample; H_S the concentration of 17a(H), 21b(H) C_{30}-hopane in the sample, and H_0 the concentration of 17a(H), 21b(H) C_{30}-hopane analyte in the initial sample (Prince et al., 2003).

2.8. Biodegradation kinetics

The rate of petroleum hydrocarbons degradation depends on biomass concentration and on the specific degradation rate. Therefore it is essential to investigate whether enhanced degradation of any particular hydrocarbon compound is due to an increase in biomass or due to an increase of the specific degradation rate, which suggests a different metabolic pathway. The degradation kinetics of representative compounds from n-alkanes group and PAHs group were investigated, namely C_{15}, C_{20}, C_{25}, C_{30}, Pristane, Phytane, Fluorene, Dibenzothiothene, Phenanthrene and Chrysene.
In a batch culture the cell growth rate is given by the expression:

$$r_X = \frac{dX}{dt} = \mu X \quad (5)$$

where X (cells/mL) is the biomass concentration and μ is the specific growth rate (1/h). The average specific growth rate can be readily estimated as the slope in the plot of *ln(X)* versus time.
Similarly, the rate of any particular substrate utilization (i.e., removal of a particular hydrocarbon) S (mg-compound/mL or mg-compound/g dry sand) is given by the expression:

$$r_S = \frac{dS}{dt} = -q_s X \quad (6)$$

where q_s is the specific degradation rate (μg/cells h). The estimation of q_s can be more reliably done by the integral method (Englezos and Kalogerakis, 2001). In this approach, we first integrate Equation (6) to yield

$$S_t - S_0 = -\bar{q}_s \int_0^t X dt \quad (7)$$

The average specific degradation rate $\bar{q}_S$ can be obtained by Least Squares Estimation as the slope in linear plot of S_t versus the integral $I(t)=\int X(t)dt$. The latter is readily computed numerically from the experimental data of X versus time (Englezos and Kalogerakis, 2001).

2.9. Molecular Analyses

Identification of the key organisms that play roles in pollutant biodegradation is important for understanding, evaluating and developing in situ bioremediation strategies. For this reason, one of the tasks was to characterize bacterial communities, to identify responsible degraders, and to elucidate the catalytic potential of these degraders under different bioremediation methods.

Molecular fingerprinting techniques were introduced in soil microbial ecology in the past 15 years and allowed the study of the ecology of microorganisms which could not be cultivated in synthetic media yet constitute the majority of soil microorganisms (2). Denaturating gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) are two of the most popular fingerprinting methods used in microbial ecology (3). DGGE and TRFLP alone or in combination with cloning and sequencing have been used in different steps of bioaugmentation strategies (Karpouzas and Singh, 2010).
The ecological role of strains and their impact on the endogenous microbial community of the micro-ecosystems are investigated by fingerprinting PCR-based methods. PCR-based microbial community analyses are widely used in microbial ecology and, for most environments, they give a more realistic picture about community structure than classical techniques based on cultivation. Over the past decades, this technique has helped to identify, taxonomically, microorganisms that have never been cultured. In environmental microbiology, a routine molecular analysis starts with the thorough sampling of the material to be investigated (soil, water, sediment, etc.). Subsequently, isolation and purification of nucleic acids is followed by the corner-stone of the technique: a nucleic acid amplification step using PCR, with primers binding to conserved regions of specific genes containing phylogenetic or functional information. It is a Polymerase enzyme that drives a PCR. A polymerase will synthesize a complementary sequence of bases to any single strand of DNA providing it has a double stranded starting point. The template for the PCR amplification is a mixture of homologous genes; therefore, the objective is to produce adequate amounts of DNA from each taxon present in the sample from which specific taxa can be distinguished qualitatively and quantitatively. The analysis of the heterogeneous PCR products is carried out by cloning and sequence analysis or by different fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), or terminal restriction fragment length polymorphism (T-RFLP) (Sipos et al., 2010).
Real-time PCR on the other hand monitors the progress of a PCR reaction based on the detection and quantification of a fluorescent reporter molecule that binds to the target PCR template. From the amount of fluorescence emitted at each cycle in the exponential phase, it is possible to calculate the initial amount of target template. Real-time PCR is highly sensitive, down to a detection limit of 1–2 genome copies, in contrast to microarrays (see below), which may be 100- to 10,000-fold less sensitive than PCR, a potential problem for sequences of poor abundance. Real-time PCR does not require any tedious post- PCR steps for the quantification of amplicons, as their amount is monitored in real time. Therefore, this is a high-throughput technique with superior analytical sensitivity for the detection and quantification of specific genes in environmental samples (Stenuit et al., 2009).

Real-time PCR is now widely used for measuring 16S rRNA and functional gene abundance and expression in the environment, including many studies of hydrocarbon-degrading bacteria in which specific primers and probes have been developed.

The dominant native microorganisms in the oil contaminated seawater and sand after each treatment processes were determined via various molecular techniques such as: Real Time PCR, polymerase chain reaction, pyrotag sequencing and nucleotide sequence analysis.

2.9.1. Autochthonous Bioaugmentation and/or Biostimulation of Seawater Microcosm (Seawater 1)

Since oil components have been proved to be biodegradable it is therefore of great importance to understand the behavior of microbial populations responsible for the degradation of crude oil. Thus two RT-PCR primer sets were used to detect a wide range of genes encoding alkane hydroxylases, which are the key enzymes catalyzing the first step of alkane degradation. Another set of RT-PCR was used to detect the gene encoding the aromatic ring-hydroxylating dioxygenase which is the key enzyme catalyzing the aromatic ring of PAHs (Table 6).

***DNA extraction from seawater samples*:** DNA was extracted from 2 mL of the seawater microcosms using QIAamp STOOL kit (Qiagen). Extracted DNA was further purified by PureLink PCR Purification kit (Invitrogen).

***RT-PCR analysis*:** Abundance of specific aerobic degradation genes in seawater samples is tracked with Real-time PCR. Primers were based on the Alcanivorax alkane hydroxylase (alkB2) gene, Thalassolituus alkane hydroxylase (alkB) gene and Cycloclasticus aromatic ring-hydroxylating dioxygenase (phnA) gene. Standard curves of targeted genes for RT-PCR were generated by stock solutions of total extracted DNA from pure cultures of A. borkumensis, T.oleivorans, C. pugetti.

DNA standard curves for each gene were then created using dilution series ranging from 5×10^{1} to 5×10^{7} ng dna/µl. DNA isolated from the microcosm samples, together with no-template controls (NTC) were used in RT-PCR amplifications in triplicate with each target gene as standard. Reactions were performed on an ABI StepOnePlus Sequence Detection System (Applied Biosystems) with initial denaturation for 5 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each 20 µl of reaction contained 2 µl of template, 10 µl of 2× SYBR® Green PCR Master Mix (Applied Biosystems) and 100 nM of each primer.

Table 6: Primer sets used for group-specific amplification of alkB and phnA gene fragments

Target genes	Primer	Sequence (5'-3')	Product (bp)	References
alkB2	ALC*alk*B2F867	CGCCGTGTGAATGACAAGGG	132	MacKew et al., 2007
	ALC*alk*B2R999	CGACGCTTGGCGTAAGCATG		
alkB	THALalkBF125	GACGTCGCCACACCTGCC	217	MacKew et al., 2007
	THAL*alk*BR342	GGGCCATACAGAGCAAGCAA		
phnA	CYC*phn*AF243	CGTTGTGCGCATAAAGGTGCGG	145	MacKew et al., 2007
	CYC*phn*-AR388	CTTGCCCTTTCATACCCCGCC		

The number of gene copies per microliter was calculated as follows:

$$\text{Target abundance} = \frac{6.023 \times 10^{23}\ (\text{copies/mol}) \times \text{conc.of target}\ (\text{g/µl})}{\text{MWF}\ (\text{g/mol})} \quad (8)$$

The molecular weight (MWF) of the fragment is determined by multiplying the product size in base pairs with the molecular weight of double-stranded DNA (660 Da).

When changing target concentration to ng/µl then formula converts to:

$$\text{Target abundance} = \frac{6.023 \times 10^{14}\ (\text{Da/ng}) \times \text{conc.of target}\ (\text{ng/µl})}{\text{MWF}\ (\text{Da})} \quad (9)$$

, and target abundance can be expressed as gene copies/µl.

Amplicon numbers were quantified against the standard curve using the ABI Step One sequence detection software (Applied Biosystems) using automatic analysis settings for the Ct values and baseline settings. The limit of detection for all three genes was set at 3.3 cycles lower than the Ct value of the NTC (Smith et al., 2006), which corresponds with a gene abundance of 3×10^4, 5×10^3 and 1×10^4 cells per millilitre of seawater for Thalassolituus alkB, Alcanivorax alkB2 and Cycloclasticus phnA respectively. Detected target genes were converted to cell density (cells/ml) assuming a single copy per genome, as demonstrated for Alcanivorax (Schneiker et al., 2006). This assumption was further indirectly confirmed for all three target genes using the Q-PCR primers for amplification from known amounts of genomic DNA, where the chromosome copy number was calculated from the known genome sizes of 3.12, 2.9 and 2.2 Mb for Alcanivorax (Schneiker et al., 2006), Cycloclasticus (Button et al., 1998) and Thalassolituus (Yakimov et al., 2004) respectively.

Community screening by pyrotag sequencing: PCR and pyrotag sequencing were performed in Research and Testing Laboratory, Lubbock, Texas, for the V4 hypervariable region of the 16S rDNA locus using primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') which are known to have reduced bias for soil and sediment bacterial communities and cover a wide range of bacterial and archaeal phyla (Kuczynski et al., 2012). Noise filtering (using the AmpliconNoise package), chimera removal (using the PerseusD algorithm), Operational Taxonomic Unit (OTU) clustering (at 97% similarity), OTU table construction, phylogenetic assignments using the RDP naïve Bayesian classifier (Wang et al., 2007) and heatmap analysis

were performed using QIIME v1.4 (Caporaso et al., 2010). For the creation of rarefaction curves, the OTU table was rarefied from 100 to 9,000 sequences (the lowest number of clean reads per sample) with a step of 100 sequences ten times at each step and the mean Chao1 and "observed species" diversity indices were calculated at each step. The Chao1/"observed species" Vs the number of sequences plot was performed in QIIME.

For alpha diversity estimates, Shannon's index (log 2), Pielou's evenness and the expected number of species (for the smallest sample size, 9,000) were calculated using Primer 6 software for Windows (PRIMER-E Ltd, Plymouth, UK). Non-metric multidimensional scaling (nMDS) plots, similarity percentages (SIMPER) analysis and analysis of similarity (ANOSIM) tests were also performed with the PRIMER6 software, by transforming the rarefied OTU table into a resemblance matrix using the Bray-Curtis similarity index. A square root transformation was performed prior to all the above analyses in order to down-weight the highly abundant OTUs. The closest known representatives of OTUs of interest were determined by BLASTing the consensus sequences of the OTUs in question against the "nr" nucleotide collection of the NCBI database.

2.9.2. Autochthonous Bioaugmentation & Biostimulation with Isolated Hydrocarbon Degraders Consortium of Seawater Microcosm (Seawater 2)

***DNA extraction from seawater samples*:** Cells were harvested from 2 mL of the seawater microcosms cultures pelleted by centrifugation (16,000 g for 15 min at 4°C) and processed according to "miniprep" method described by Moore et al., 1999.

***PCR analysis*:** The bacterial 16S rDNA fluorescently labelled universal primers (Escherichia coli numbering) used for PCR were 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′- GGTTACCTTGTTACGACTT-3′) which generates a ~1465 bp product. PCR reactions were carried out in a final volume of 50 μL mixture that contained both primers at 0.5 μM, 0.01 mM dNTPs, 0.03U of Taq Polymerase and the buffer supplied with the enzyme (Roche). Amplification was performed using a Mastercycler PCR system 9700 Thermocycler (Eppendorf) as follows: an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. Cycling was completed by a final elongation at 72°C for 10 min (Benito et al., 2004). The fluorescent PCR products were viewed on 1% w/v agarose gels, cleaned with PCR purification kit (Invitrogen) and subsequently were processed to sequencing analysis.

Sequencing analysis of PCR products: Sequencing analysis was performed in an ABI PRISM 3700 automated sequencer (Applied Biosystems) and BIOEDIT software was used to assemble the sequences. The resulting sequences were examined with the BLAST search alignment tool comparison software (BLASTN) to detect the closest bacterial group to each strain from the GenBank database and multiple alignment of this set of sequences was performed with CLUSTALW software. Phylogenetic trees were generated by MEGA 5.0 software using maximum likelihood and neighbour-joining treeing algorithms (Tamura et al., 2007).

2.10. CLSM conditions

Sample droplets from the cultures in the flasks were placed to a specifically designed 1.02mm Deep Chamber stained with Syto 9, a specific nucleic acid dye which can stain bacteria, and were monitored under the CLSM.
PMT properties have been adjusted in a way that the adhered bacteria could be differentiated from the bulk autofluorescence of the oil area that was even stronger after the staining with Syto9 and the corrected adjustment had resulted in lila autofluorescence of oil.
CLSM. Laser scanning microscopy was performed using the model TCS SP1 (Leica, Heidelberg, Germany) attached to an upright microscope. The instrument was controlled by Leica Confocal software, version 2.61, built 1537. The system was equipped with three visible lasers: an Ar laser (458, 476, 488, and 514 nm), a laser diode (561 nm), and a He-Ne laser (633 nm). The spectrophotometer feature allowed flexible and optimal adjustment of sliders on the detector side. The following settings were used for excitation and recording of emission signals, respectively: for Syto9, excitation of 488 and emission from 500 to 550 nm, also for oil autofluoresence excitation of 561 and emission from 575 to 620nm and excitation of 633 with emission from 650 to 700nm. Biofilm samples were observed with 63× 1.2-NA, and 63× 0.9-NA water-immersible lenses (Neu and Lawrence, 2010).
Digital image analysis. Images were visualized by using the microscope software (Leica) for maximum-intensity projections and the free open source software for image post-processing, visualization and analysis BioImageXD, version 1.0, for XYZ projections and isosurface rendering. Images were mounted in Photoshop CS5 (Adobe, San Jose, Calif.) without any image adjustments.

Chapter 3- Experimental Design

3. Experimental Design

In this study we examined the effectiveness of the combined autochthonous bioaugmentation with biostimulation versus biostimulation strategies for the successful remediation of polluted marine environments. Indigenous populations adapted or not to oil pollution and isolated hydrocarbon degrading consortia were examined both in seawater and on shore in the presence or not of other rate limiting factors like nutrients (inorganic or lipophilic) and biosurfactants as a potential strategy for the successful remediation of polluted marine environments. Thus the outcome approaches include:

1. Autochthonous bioaugmentation and/or biostimulation of seawater microcosm (Seawater 1)
2. Autochthonous bioaugmentation & biostimulation with isolated hydrocarbon degraders consortium of seawater microcosm (Seawater 2)
3. Landfarming of oil polluted beach sand through biostimulation (Sand 1)
4. Landfarming of oil polluted beach sand through autochthonous bioaugmentation & biostimulation (Sand 2)
5. Biofilm investigation on oil droplets & Eicosane

3.1. Autochthonous Bioaugmentation and/or Biostimulation of Seawater Microcosm (Seawater 1)

In this study we examined the effectiveness of the combined autochthonous bioaugmentation with biostimulation versus biostimulation strategies for the successful remediation of polluted marine environments. Seawater was collected from a pristine environment in Crete (Agios Onoufrios beach, Chania) and was placed in a batch bioreactor (Autoclavable laboratory fermenter ALF, Bioengineering) with 1% v/v crude oil in order to grow and adapt indigenous population and use this consortium later for bioaugmentation purposes.

Duplicate microcosms were established in sterile 40 ml vial bottles containing 20 ml of seawater and contaminated with 0.5% w/v weathered crude oil (A).

Table 7: 1st Experimental approach Set Up (Seawater 1)

Treatment	Weathered crude oil 0.5% w/v	Nutrients (KNO_3, KH_2PO_4)	Nutrients (uric acid, lecithin)	Rhamnolipid biosurfactant	Preadapted indigenous population
Control	+				
NPK	+	+			
ULR	+		+	+	
NPKM	+	+			+
NPKMR	+	+		+	+
ULRM	+		+	+	+

Three biostimulation treatments: (i) seawater + oil (Control), (ii) seawater + oil supplemented with KNO_3 and K_2HPO_4 (NPK), (iii) seawater + oil supplemented with

Uric Acid, Lecithin and Biosurfactant (Rhamnolipids) (ULR), and three autochthonous bioaugmentation treatments were established as shown in Table 7: (iv) seawater + oil supplemented with KNO_3, K_2HPO_4 and pre-adapted indigenous cultures (NPKM), (v) seawater + oil supplemented with KNO_3, K_2HPO_4, Biosurfactant (Rhamnolipids) and pre-adapted indigenous cultures (NPKMR) and (vi) seawater + oil supplemented with Uric Acid, Lecithin, Biosurfactant (Rhamnolipids) and pre-adapted indigenous cultures (ULRM). Microcosms were incubated under aerobic conditions at 20°C with continuous agitation on an orbital shaker (200 rpm.).
Growth of oil degraders was measured by most probable number (MPN) procedure after 0 7, 15, 21, 30, 37, 45, 60 and 90 days, and hydrocarbons were analysed with chromatographic techniques (solid-phase extraction followed by gas chromatography – mass spectrometry) after 0, 5, 15, 30, 60 and 90 days.
We have investigated the effects of autochthonous bioaugmentation with these organisms upon hydrocarbon degradation in marine waters, and additionally compared the role of bioaugmentation with biostimulation via different types of nutrients (organic and inorganic) with/without a rhamnolipid biosurfactant amendment.
PCR and pyrotag sequencing were performed in samples taken from treatments NPK, NPKM, NPKMR, ULR, and ULRM in the 30th day of the experiment and in samples taken from the initial consortia (indigenous seawater population-S1 and acclimated seawater population-DS1) used for those treatments.

3.2. Autochthonous Bioaugmentation & Biostimulation with Isolated Hydrocarbon Degraders Consortium of Seawater Microcosm (Seawater 2)

In this study we examined the effectiveness of innovative joined autochthonous bioaugmentation/ biostimulation approaches so that remediation of contaminated marine shorelines is effective. The experimental process is comprised by a shaker flask set up and certain microbiological and chemical analyses of the preceding samples. The reflected experimental arrangement as shown in table 8 is disposed on an orbital shaker in duplicates at day 0.
The consortium (Eb8) used for bioaugmentation was enriched from seawater samples taken from Elefsina Bay (Attica region) near the Hellenic Petroleum Refinery; a site exposed to chronic crude oil pollution. Seawater was collected from Agios Onoufrios beach (Chania-Crete). Microcosms were established in sterile 100 mL flasks containing 50 mL of sterile seawater with salinity of 32.16 g/L contaminated with 0.5% w/v weathered crude oil (B) incubated under aerobic conditions at 20°C with continuous agitation at 150 rpm. In addition, a known quantity of the isolated consortium was added to these flasks so that the initial biomass concentration reached 10^6 cells/mL. Three autochthonous bioaugmentation treatments were established as shown in Table 8: (i) oiled seawater and pre-adapted consortium-(CM) (ii) oiled seawater supplemented with KNO_3, K_2HPO_4 and pre-adapted consortium-(NPKM), and (iii) oiled seawater supplemented with KNO_3, K_2HPO_4, biosurfactant (rhamnolipids) and pre-adapted consortium-(NPKMR). Growth of oil degraders was measured by the most probable number (MPN) procedure and hydrocarbons were analysed with

chromatographic techniques (solid-phase extraction followed with gas chromatography–mass spectrometry) after 0, 5, 15 and 30 days.

Table 8: 2nd Experimental approach Set Up (Seawater 2)

Treatment	Weathered crude oil 0.5% w/v	Nutrients (KNO_3, KH_2PO_4)	Rhamnolipid biosurfactant	Isolated Hydrocarbon Degraders Consortium (Eb8)
Control	+			+
NPKM	+	+		+
NPKMR	+	+	+	+

3.3. Landfarming of Oil Polluted Beach Sand through Biostimulation (Sand 1)

The main objective of this work was to explore possible methods that could enhance the rate of oil biodegradation in contaminated beach sand and consequently reduce the lag phase of indigenous hydrocarbon degraders. Enhancement of biodegradation was achieved through biostimulation and the effectiveness of novel biostimulants for the successful remediation of polluted marine environments was examined.

All landfarming treatments were prepared in duplicate and placed in aerobic stainless steel trays (38 cm long × 27 cm wide × 3 cm high) microcosms (2Kg of sand, dry weight equivalent) at ambient temperature. Water content was adjusted to 60% of the field-holding capacity using seawater before spiking with weathered crude oil (A). Twice a week, the microcosm content was mixed to maintain an aerobic condition and deionized water was added. Three treatments (Table 9) were carried out in duplicate trays during 45 days: (i) sand + oil (Control), (ii) oiled sand + KNO_3 and K_2HPO_4 (NPK), (iii) oiled sand + Uric Acid, Lecithin and Biosurfactant (Rhamnolipids) (ULR Growth of oil degraders was measured by most probable number (MPN) procedure and hydrocarbons were analysed with chromatographic techniques (solid-phase extraction, gas chromatography–mass spectrometry) after 0, 15, 30 and 45 days.
We have investigated the effects of landfarming through biostimulation of oil hydrocarbons in marine oil contaminated soil by comparing the role of different types of nutrients (organic and inorganic) and/or rhamnolipid biosurfactant amendment.

Table 9: 3rd Experimental approach Set Up (Sand 1)

Treatment	Weathered crude oil 0.5% w/v	Nutrients (KNO_3, KH_2PO_4)	Nutrients (uric acid, lecithin)	Rhamnolipid biosurfactant	Indigenous population
Control	+				+
NPK	+	+			+
ULR	+		+	+	+

3.4. Landfarming of Oil Polluted Beach Sand through Autochthonous Bioaugmentation & Biostimulation (Sand 2)

In this study we examined the effectiveness of novel combined autochthonous bioaugmentation/biostimulation strategies for the successful remediation of polluted marine coastlines. For that reason adapted consortium degrading capabilities combined with inorganic or lipophilic nutrients in the presence of biosurfactants were explored.

The consortium was enriched from seawater samples taken from Elefsina Bay (Attica region) near the Hellenic Petroleum Refinery; a site exposed to chronic crude oil pollution. All landfarming treatments were prepared in pyrex trays (20 cm long × 20 cm wide × 6 cm high) microcosms (1Kg of sand, dry weight equivalent) with a quantity of isolated consortium equivalent to 10^6 cells/g and incubated under aerobic conditions at 20°C. Water content was adjusted to 60% of the field-holding capacity using seawater before spiking with weathered crude oil (B). Twice a week, the microcosm content was mixed to maintain an aerobic condition and deionized water added. Three treatments (Table 10) were carried for 45 days: (i) oiled sand + pre-adapted consortium (Control M), (ii) oiled sand + KNO_3 and K_2HPO_4 + pre-adapted consortium (NPKM,), (iii) oiled sand + Uric Acid, Lecithin and Biosurfactant (Rhamnolipids) + pre-adapted consortium (ULRM). Growth of oil degraders was measured by most probable number (MPN) procedure and hydrocarbons were analysed with chromatographic techniques (solid-phase extraction, gas chromatography–mass spectrometry) after 0, 7, 15, 30 and 45 days.

Table 10: 4th Experimental approach Set Up (Sand 2)

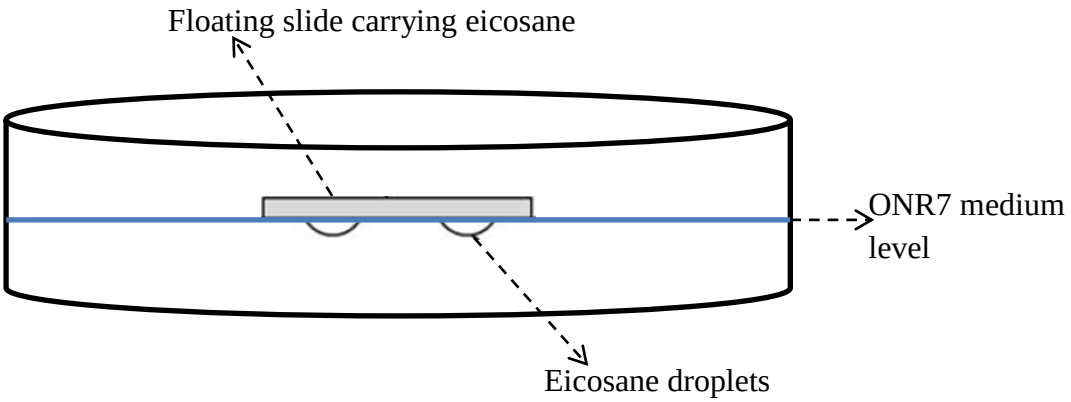
Treatment	Weathered crude oil 0.5% w/v	Nutrients (KNO_3, KH_2PO_4)	Nutrients (uric acid, lecithin)	Rhamnolipid biosurfactant	Isolated Hydrocarbon Degraders Consortium
Control M	+				
NPKM	+	+			+
ULRM	+		+	+	+

3.5. Biofilm Investigation on Oil Droplets & Eicosane

Studies have shown that there is great variety of bacteria that either have affinity, can metabolize hydrocarbons or produce biosurfactants or similar chemicals that is induced in a hydrocarbon polluted environment. Nonetheless single strain bacteria

have been thoroughly tested as has been described (Ch. 1) on their ability to degrade a variety of single components of petroleum but not so many on mixtures of hydrocarbons such as crude oil or any other petroleum products. Although in the real environment numerous bacteria are organized and grouped on the basis of how they interact with each other and become associated to the pollutant, studies haven't yet focused on multispecies consortia mechanisms to degrade hydrocarbons and most preferably oil since real accidents include release of oil and its products. Investigation of these interactions that take place between marine bacteria and oil hydrocarbons could improve our understanding on the fate of hydrocarbons in the environment and thus help to develop the most suitable bioremediation strategy.
Thus the main objective in this study was to observe through Confocal Laser Microscopy the response of consortia Eb8 (20^{o}C) and E4(14 ^{o}C) that were enriched from seawater samples taken from Elefsina Bay (Attica region) near the Hellenic Petroleum Refinery; a site exposed to chronic crude oil pollution, on crude oil contamination. Moreover their response after the addition of certain commercial dispersants just like those that have been or could be used in the event of a real oil spill but also of more environmentally friendly biosurfactants (Rhamnolipids) was also examined.

Enrichments and Investigation of the tested consortia
The consortia were enriched from seawater samples taken from Elefsina Bay (Attica region) near the Hellenic Petroleum Refinery; a site exposed to chronic crude oil pollution. Consortia Eb8 and E4 were enriched in 100ml ONR7 medium with 607μl crude oil under constant agitation (200 rpm) at 20 ^{o}C and at 14 ^{o}C respectively. We haven't further proceed to isolations of the enriched consortium since our primary goal was to use the acclimated consortium as it was and observe how it responds on oil contamination in seawater.



Droplets of crude oil and C20 (500000 ppm, 0.4 μl) were placed on a plastic sterile slide that is a solvent-resistant and is inert against crude oil, although solvents such as dichloromethane cause specific plastic slides to warp. Prepared slides with the C20 droplets were placed downwards on the water surface of a petri dish filled with 100mL of ONR7 medium with the appropriate consortium (Eb8/E4), the appropriate amount of nutrients and/or Rhamnolipids. The flasks of the testing consortia accordingly were filled with 100mL of ONR7 medium with the appropriate consortium (Eb8/E4) and 0.5%w/v of weathered crude oil, the appropriate amount of nutrients and/or Rhamnolipids in the recommended dose dispersant: oil (1:10) by the manufacturer as described in Tables 11&12. Three types of Dispersants were used: Corexit, S200 and Marichem. The petri dish microcosms and prepared flasks were

kept at room temperature (20 ^{o}C) for consortium Eb8 and at 14 ^{o}C for consortium E4 with gentle agitation. After 5, 7, 11, 14 and 18 days, pieces with 2 C20 droplets per slide were cut off and immediately examined by CLSM. Also sample droplets from the cultures in the flasks were placed to a specifically designed 1.02mm Deep Chamber stained with Syto 9, a specific nucleic acid dye which can stain bacteria, and were monitored under the CLSM.
PMT properties have been adjusted in a way that the adhered bacteria could be differentiated from the bulk autofluorescence of the oil area that was even stronger after the staining with Syto9 and the corrected adjustment had resulted in lila autofluorescence of oil (Neu and Lawrence, 2010).

Table 11: Experimental Set up of Selected Consortia and Rhamnolipids on Eicosane Droplets

Treatment with C20	Nutrients (KNO_3, KH_2PO_4)	Rhamnolipid biosurfactant	Consortium Eb8	Consortium E4
Eb8 (20^{o}C)	+		+	
E4 (14^{o}C)	+			+
Eb8R (20^{o}C)	+	+	+	+
E4R (14^{o}C)	+	+		+

Table 12: Experimental Set up of Selected Consortia and Dispersants on Crude oil Droplets

Treatment With Eb8(20^{o}C)/E4(14^{o}C)	Weathered crude oil 0.5% w/v	Nutrients (KNO_3, KH_2PO_4)	Rhamnolipid biosurfactant	Corexit	marichem	S200
Eb8R/ E4R	+		+			
Eb8RNP/ E4RNP	+	+	+			
Eb8C/ E4C	+			+		
Eb8CNP/ E4CNP	+	+		+		
Eb8MNP/ E4MNP	+	+			+	
Eb8SNP/ E4SNP	+	+				+

Chapter 4- Experimental Results and Discussion

4. Experimental Results and Discussion

4.1. Autochthonous Bioaugmentation and/or Biostimulation of Seawater Microcosm (Seawater 1)

Results and Discussion

Evaluation of the effectiveness of each treatment on crude oil biodegradation rate was estimated in terms of alkanes, PAHs and hydrocarbon degraders' compositional changes throughout the period of the experiment. Figure 10 represents total depletion rate of the saturated fraction of *n*-alkanes (C_{14}-C_{35}) of control treatment (C) as well as of the treatments NPK, NPKM, NPKMR, ULR and ULRM at different time intervals of the experiment. Control had no significant effect on the degradation rate as there were no hydrocarbon degraders detected. NPK treatment also was not as successful as the rest ones in terms of time and quantity of hydrocarbons depleted; only about 20% of n-alkanes were removed in 60 days of the experimental period.

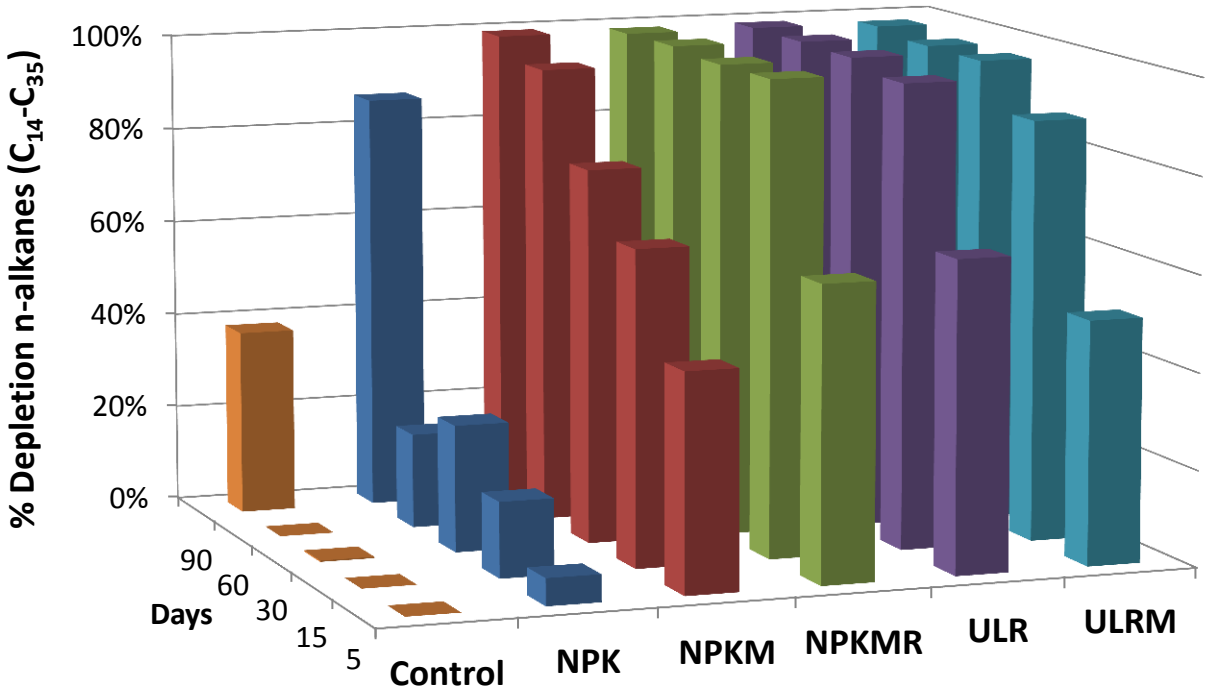


Figure 10: Depletion Rate of C14-C35 n-alkanes after 0, 5, 15, 30, 60 and 90 days of monitoring in Control, NPK, NPKM, NPKMR, ULR and ULRM treatments (Seawater 1).

On the contrary, treatments with ABA and biostimulation (NPKM, NPKMR and ULRM) and treatment of biostimulation with the indigenous population (ULR) were the most effective ones giving a fast degradation rate of n-alkanes above 80% within 30 days of the experiment (Figure 10). Moreover, the degradation in the treatments NPKMR ULR and ULRM reached 99%, 97% and 88% respectively within 15 days.

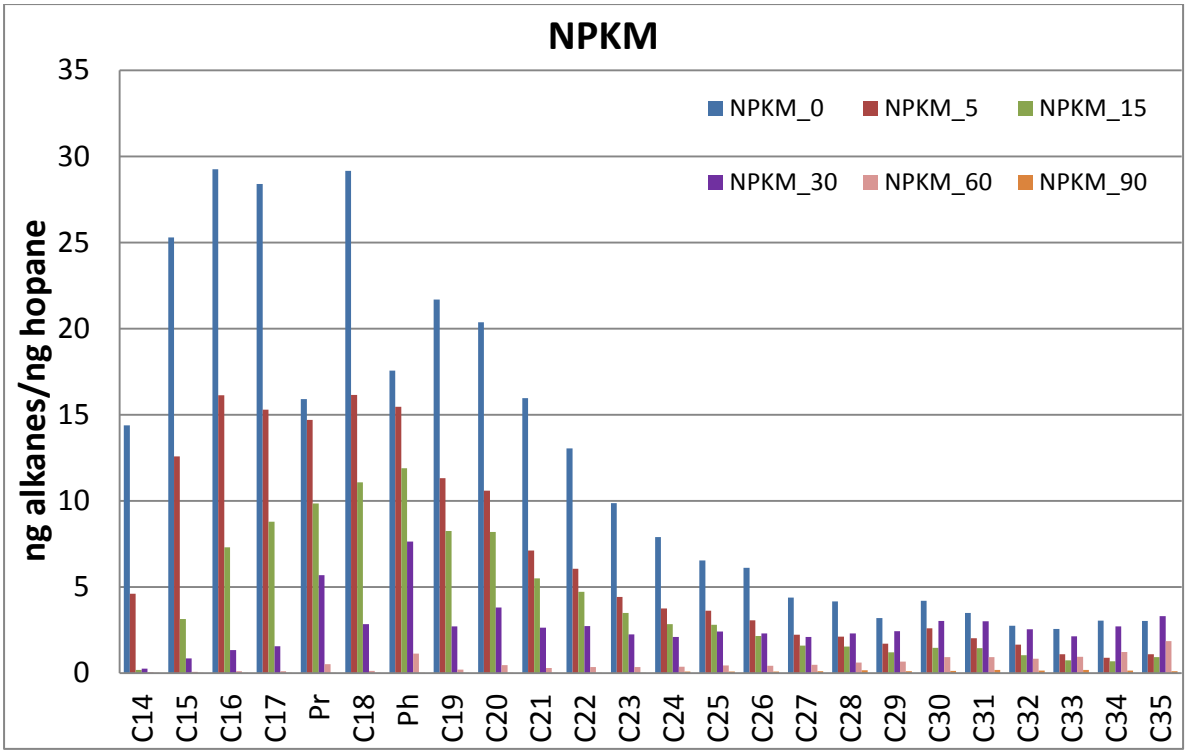


Figure 11: Concentration of C14-C35 n-alkanes after 0, 5, 15, 30, 60 and 90 days of monitoring in NPKM treatment (Seawater 1).

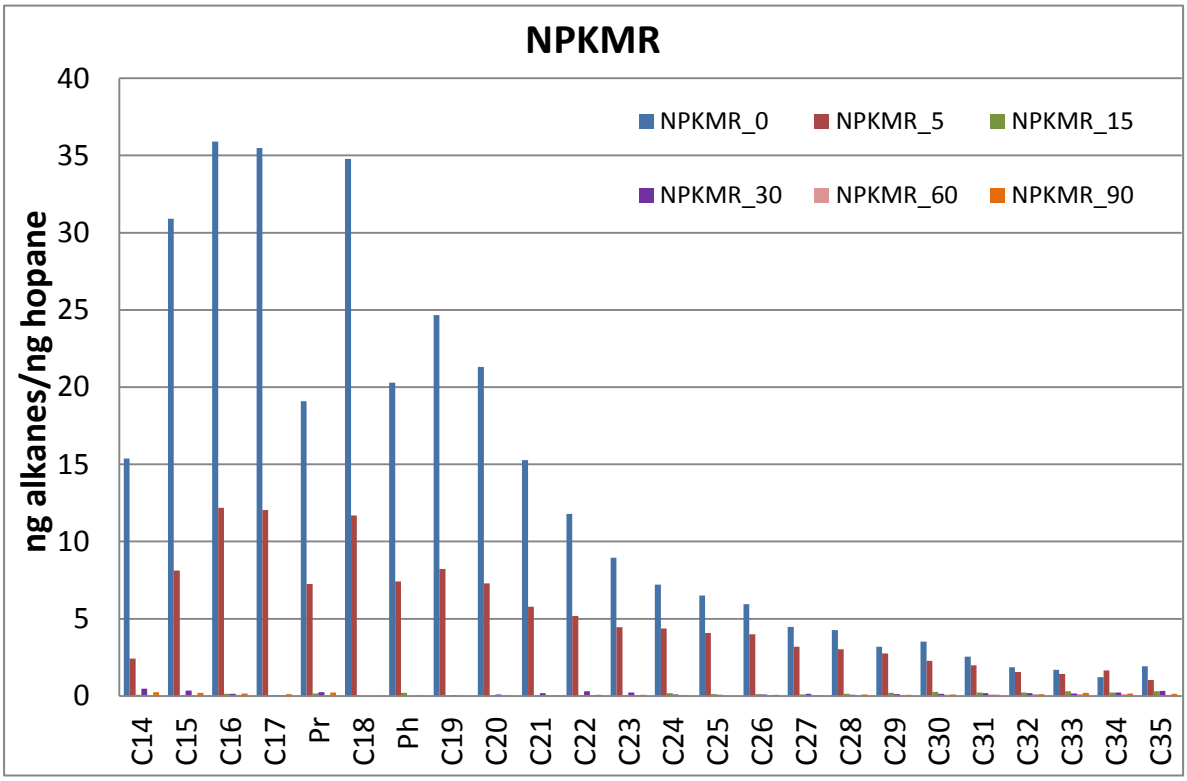


Figure 12: Concentration of C14-C35 n-alkanes after 0, 5, 15, 30, 60 and 90 days of monitoring in NPKMR treatment (Seawater 1).

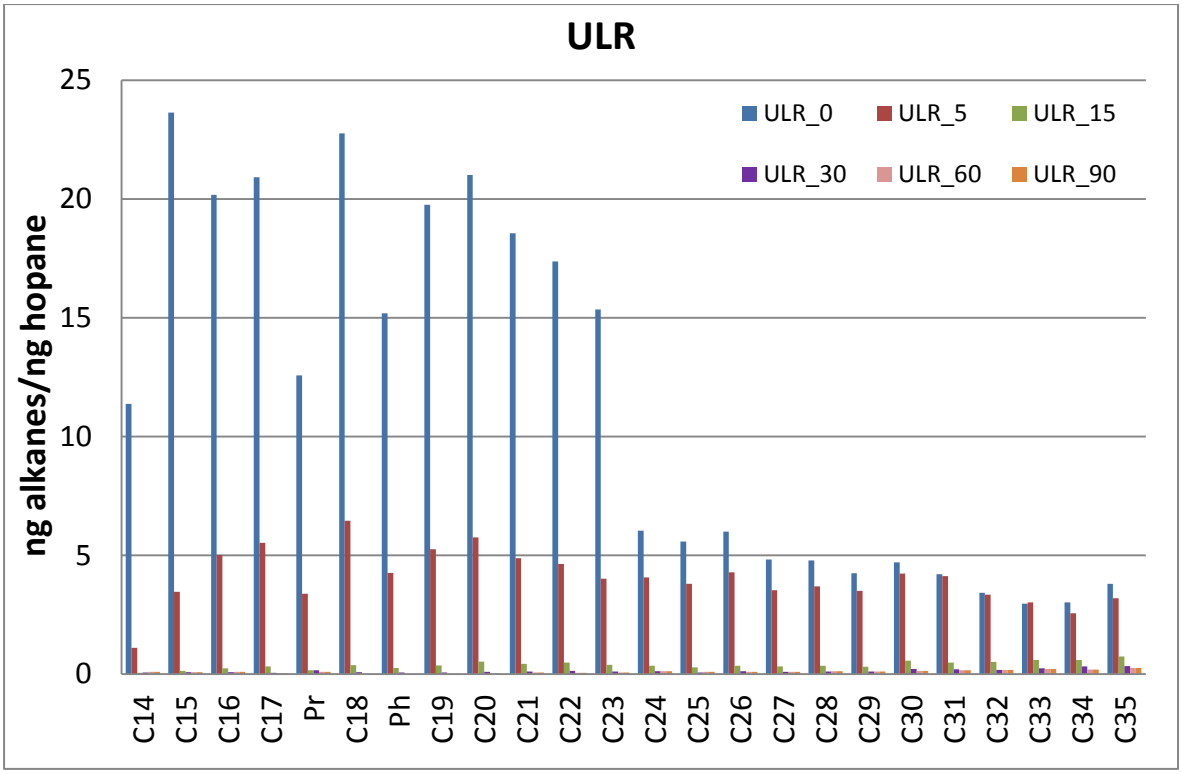


Figure 13: Concentration of C14-C35 n-alkanes after 0, 5, 15, 30, 60 and 90 days of monitoring in ULR treatment (Seawater 1).

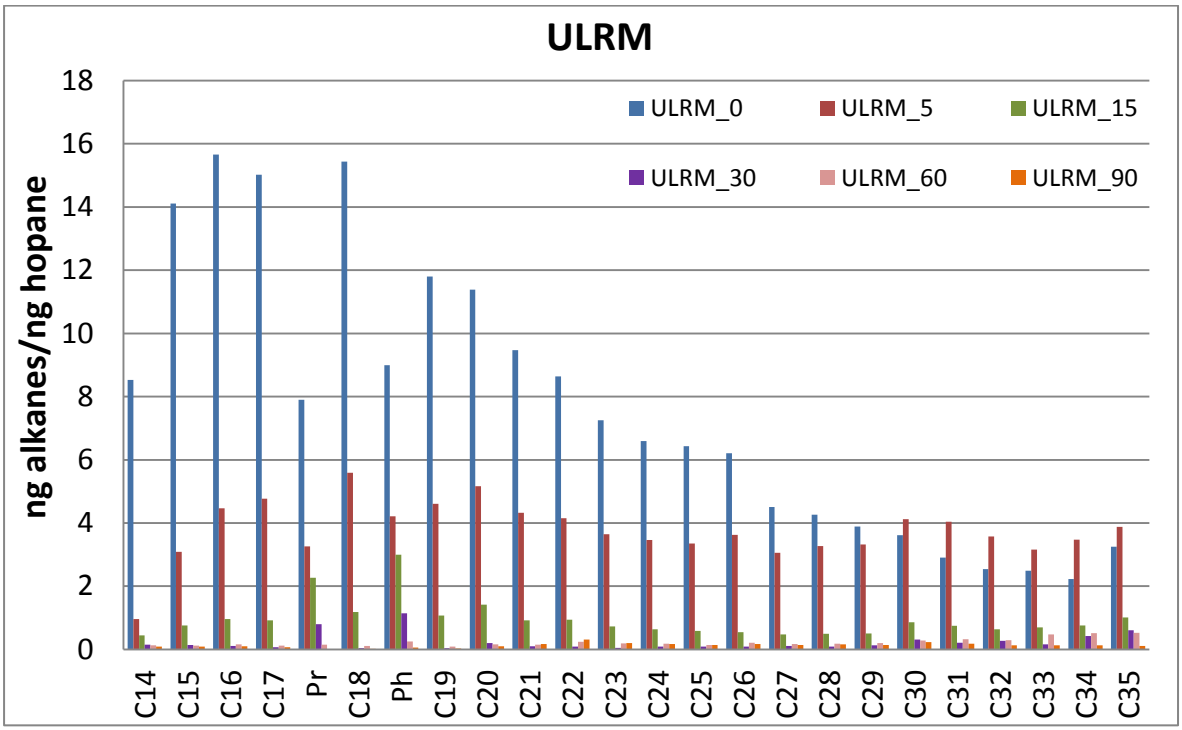


Figure 14: Concentration of C14-C35 n-alkanes after 0, 5, 15, 30, 60 and 90 days of monitoring in ULRM treatment (Seawater 1).

As it is seen from Figures 11-14 in treatments ULR, NPKMR and ULRM (where rhamnolipids were added) there is high decreasing rate for medium chain *n*-alkanes (C_{14} to C_{30}) as well as for high chain alkanes (C_{31} to C_{35}) in almost 15 days of the experiment. In treatment NPKM however, high chain alkanes (C_{31} to C_{35}) remained

stable for the whole duration of the experiment as shown in Figure 11. The average specific consumption (degradation) rate $\bar{q}_S$ was calculated over the time period that degradation occurred, which as can be observed in Figure 12 for NPKMR and ULR is 0 to15 days, for ULRM 0 to 30 days and for NPKM is 0 to 60 days. Although there is nearly no growth detected in the control treatment, we can assume that specific degradation rate in the control treatment is very low in the magnitude of 0.0001. If we compare the specific degradation rate of treatments NPKM, NPKMR, ULR and ULRM (Table 13 and Figure 19) we can estimate that specific degradation rate in those treatments can exceed 10^6 orders of magnitude than the control.

Table 13: Specific growth and degradation rate of selected alkanes (Seawater 1)

Treatment	μ (1/h)	q_s (μg/cells h)						
		C_{15}	C_{20}	C_{25}	C_{30}	C_{35}	Pristane	Phytane
NPKM	0.004	64.9	51.3	15.7	8.4	3	39.6	42.4
NPKMR	0.023	1239.3	854.8	257.3	131.3	65.2	760.6	807.3
ULR	0.024	898.4	783.2	202.3	158.3	116.9	474.6	571
ULRM	0.010	105	84.1	47.7	24.9	19.9	53.4	59

If we make the comparison between treatments we can estimate that specific degradation rate for C_{15} in NPKMR, ULR and ULRM treatments is 18, 13 and 1.5 times higher than that of treatment NPKM. Also the specific degradation rate for C_{20} is approximately 16, 14 and 1.5 times higher for treatments NPKMR, ULR and ULRM than treatment NPKM. Moreover, the specific degradation rate for C_{25} and C_{30} in treatments NPKMR and ULRM is about 16 and 3 times higher than treatment NPKM respectively, where specific degradation rate for C_{25} and C_{30} in treatment ULR is 12 and 18 times higher than treatment NPKM. The specific degradation rate for the heavier components C35 is 21, 37 and 6.5 times higher than treatment NPKM in NPKMR, ULR and ULRM treatments respectively. More specifically, for treatments NPKMR, ULR and ULRM (where the biosurfactant is present) the average specific growth rate μ for NPKMR and ULR equals to approximately 0.02 (1/h), whereas in treatment ULRM the specific degradation rate is not equal to the other two treatments, leading to the conclusion that this consortium in the presence of organic lipophilic nutrients prefers to utilize this carbon source rather than petroleum hydrocarbons. Branched alkanes and formerly used biomarkers like Pristane and Phytane are also degraded by 18, 12 times higher in NPKMR and ULR treatments and 1.5 times higher in ULRM treatment compared to NPKM.

The same behaviour between treatments NPKM-NPKMR-ULR-ULRM in terms of specific degradation rate applies also for selected PAHs that are shown in figures 15-18. Furhtermore, we observe that there is a low but decreasing rate in low molecular weight PAHs (fluorene-dibenzothiothene- phenanthrene) compared to high molecular weight PAH (chrysene) which remains practically stable. Degradation in treatments ULR and NPKMR of low molecular PAHs is achieved within 15 days, while for NPKM and ULRM is achieved within 30 days of the experiment (Figures 15-18).

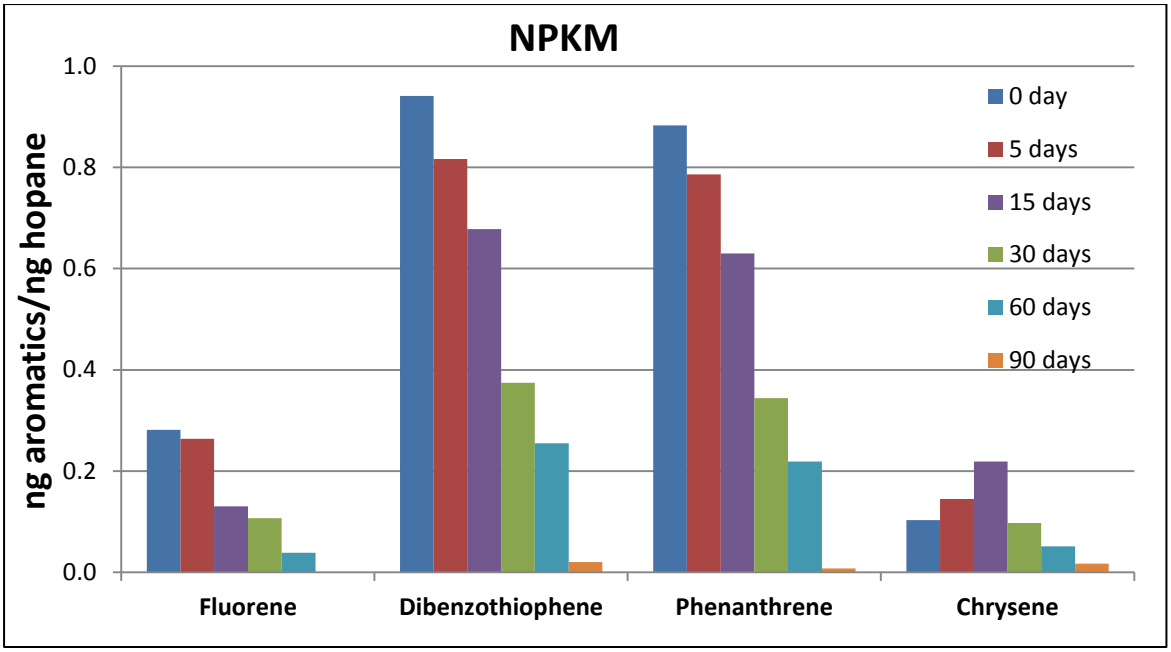


Figure 15: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15, 30, 60 and 90 days of monitoring in NPKM treatment (Seawater 1).

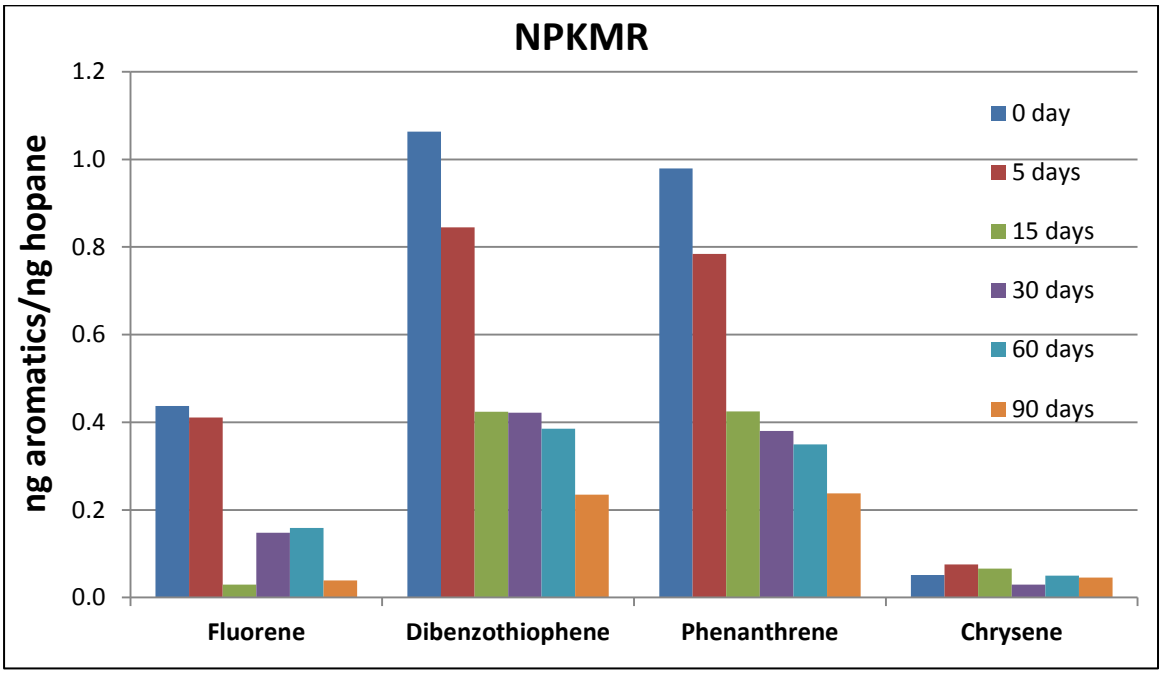


Figure 16: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15, 30, 60 and 90 days of monitoring in NPKMR treatment (Seawater 1).

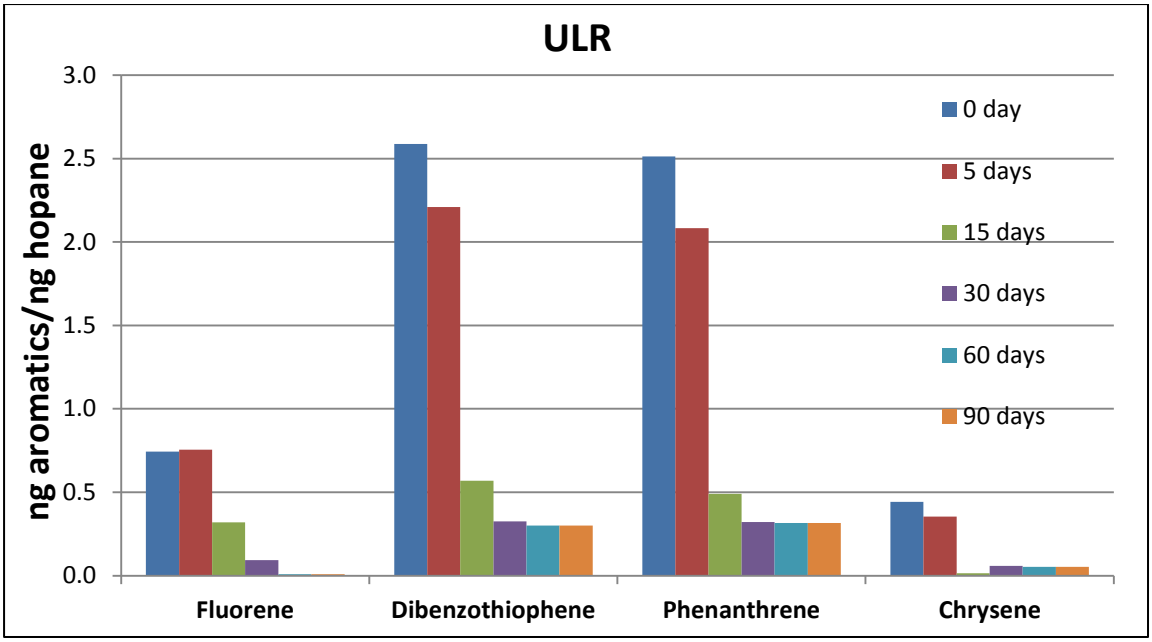


Figure 17: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15, 30, 60 and 90 days of monitoring in ULR treatment (Seawater 1).

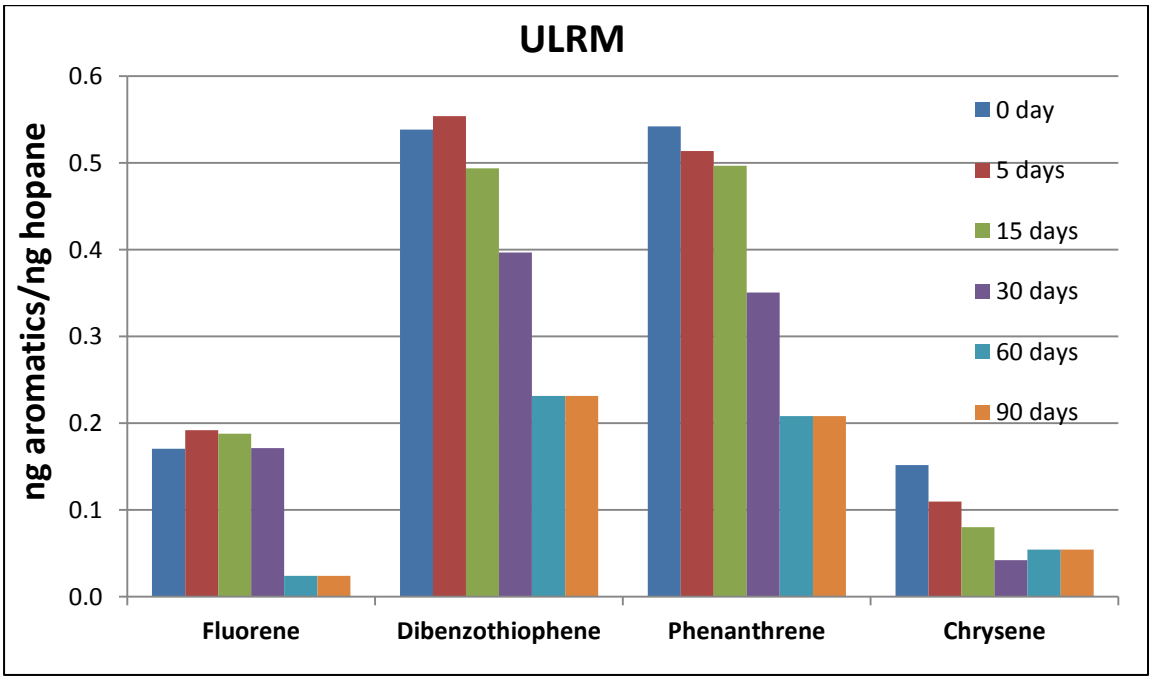


Figure 18: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15, 30, 60 and 90 days of monitoring in ULRM treatment (Seawater 1).

PAHs degradation in the NPKMR treatment reached 60% within 15 days equally for fluorene, dibenzothiothene and phenanthrene, which remained practically stable for the next 45 days and was reduced to 90% (fluorene) and 75% (dibenzothiothene-phenanthrene) by the end of the experiment. Accordingly in ULR treatment degradation rate reached 60% for fluorene and above 75% for dibenzothiothene and phenanthrene during the first 2 weeks of the experiment, then all reached 87% by the 30th day and remained stable until the end of the experiment with only fluorene reaching 98% after 60 days. On the contrary, in the NPKM treatment fluorene and

dibenzothiothene were depleted >50% and phenanthrene to about 35% only after 30 days. Moreover, the depletion for both dibenzothiothene and phenanthrene exceeded 70%, fluorene reached 86% by day 60th and by the end of the experiment all were completely depleted. More or less the same trend is observed also in ULRM treatment where fluorene is decreased by 85% while dibenzothiothene- phenanthrene decreased only to 60% after 60 days of the experiment.

Table 14: Specific degradation rate of the selected PAHs (Seawater 1)

Treatment	q_s (µg/cells h)			
	Fluorene	Dibenzothiothene	Phenanthrene	Chrysene
NPKM	0.28	0.79	0.77	0.06
NPKMR	0.46	0.96	0.86	0.01
ULR	1.72	6.76	6.50	1.16
ULRM	0.29	0.62	0.67	0.20

If we make the comparison between treatments (Table 14) we can estimate that specific degradation rate for dibenzothiothene and phenanthrene in ULR treatment is 7, 8.5 and 11 times higher than that of treatments NPKMR, ULRM and NPKM respectively. Also the specific degradation rate for fluorene in ULR treatment is approximately 4 times higher than that of treatment NPKMR and 6 times higher than that of both treatments NPKM and ULRM. This trend is in accordance to the observed degradation rate in ULR treatment during the first 2 weeks of the experiment.

In NPKM and to lesser extent in the ULRM treatments the late response on PAHs and long chain length alkanes degradation is related to the gradual growth rate of hydrocarbon degraders (30 days) which remained at considerably high level (10^2 orders of magnitude more in cells/mL) compared to those in ULR and NPKMR treatments at the very end of the experiment. Considering the above specific growth rate µ which is the same for both NPKM and ULRM after 30 days of treatment is 4 and 2 times higher than ULR and NPKMR respectively in which most of the hydrocarbon degraders decreased drastically only after most of the oil was consumed within 15 to 30 days. Presumably, the incomplete depletion of PAHs by the end could be attributed to the low number of hydrocarbon degraders, which was caused by the lack of essential nutrients that probably had been already utilized for the depletion of the saturated fraction. Despite late response of microbial community on oil degradation especially in NPKM treatment, the degrading capability of the adapted consortium for both oil fractions has been proved and in the presence of suitable biostimulants it could be accelerated.

The preferred biodegradation of the more easily biodegradable substrates such as the lower-molecular-weight PAHs and small chain length aliphatic hydrocarbons that are found in contaminated areas could also be associated to the difference in aqueous solubility that decreases as the carbon number increases. In terms of chemical composition, the saturated fraction of the residual oil as expected was degraded more extensively than the aromatic fraction. The trend in degradation rate follows the pattern C15> C20> (Pristane, Phytane)>C25 > C30> C35 >(PAHs).

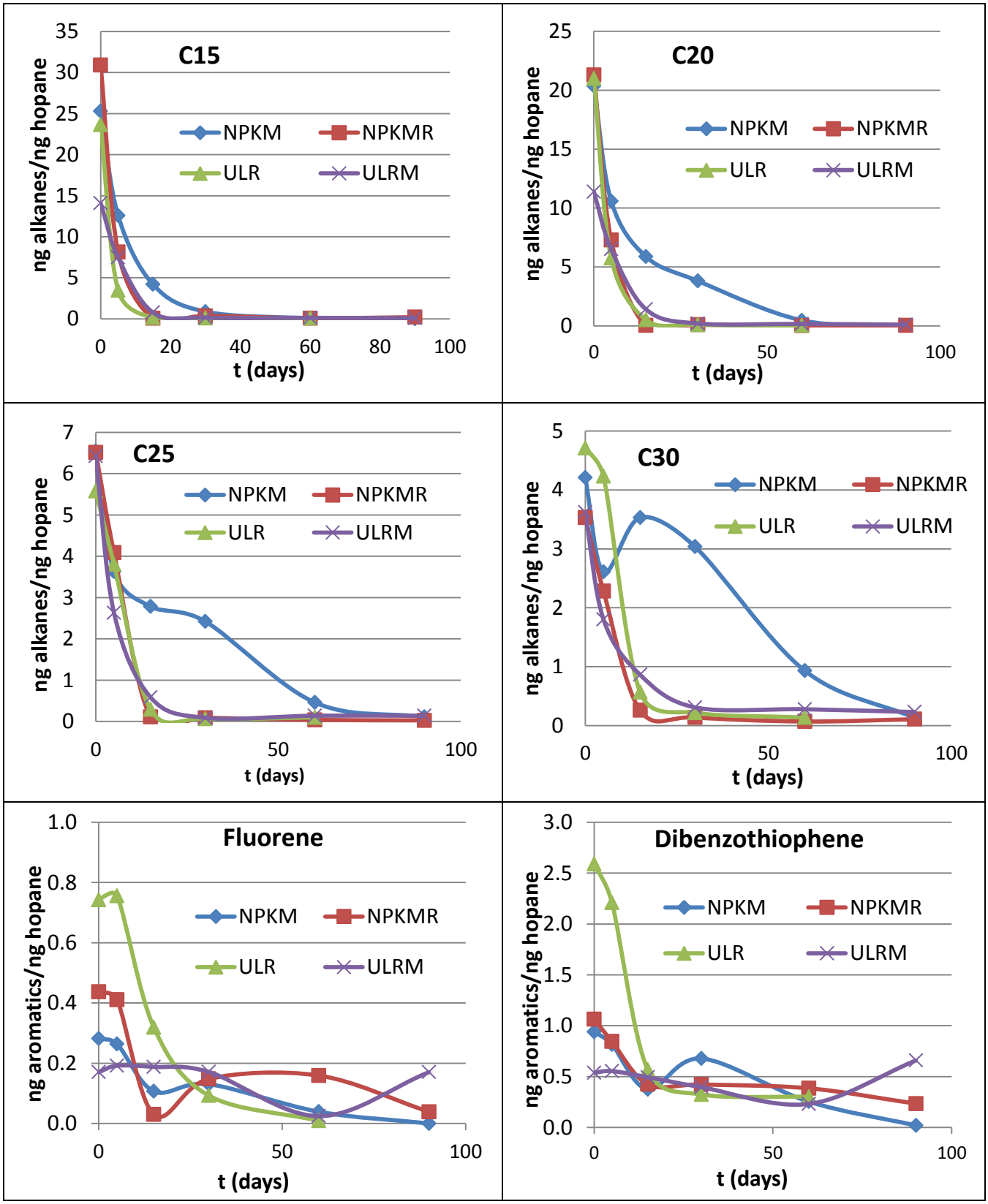


Figure 19: Concentration profiles of selected n-alkanes and PAHs compounds in NPKM, NPKMR, ULR and ULRM treatments (Seawater 1).

Although Pristane and Phytane were considered in the past as conserved internal markers in biodegradation index, as shown in Figure 17 and Table 13 (especially in

ULR treatment) they were completely degraded within 15 days and thus they should be considered unreliable as biodegradation index.

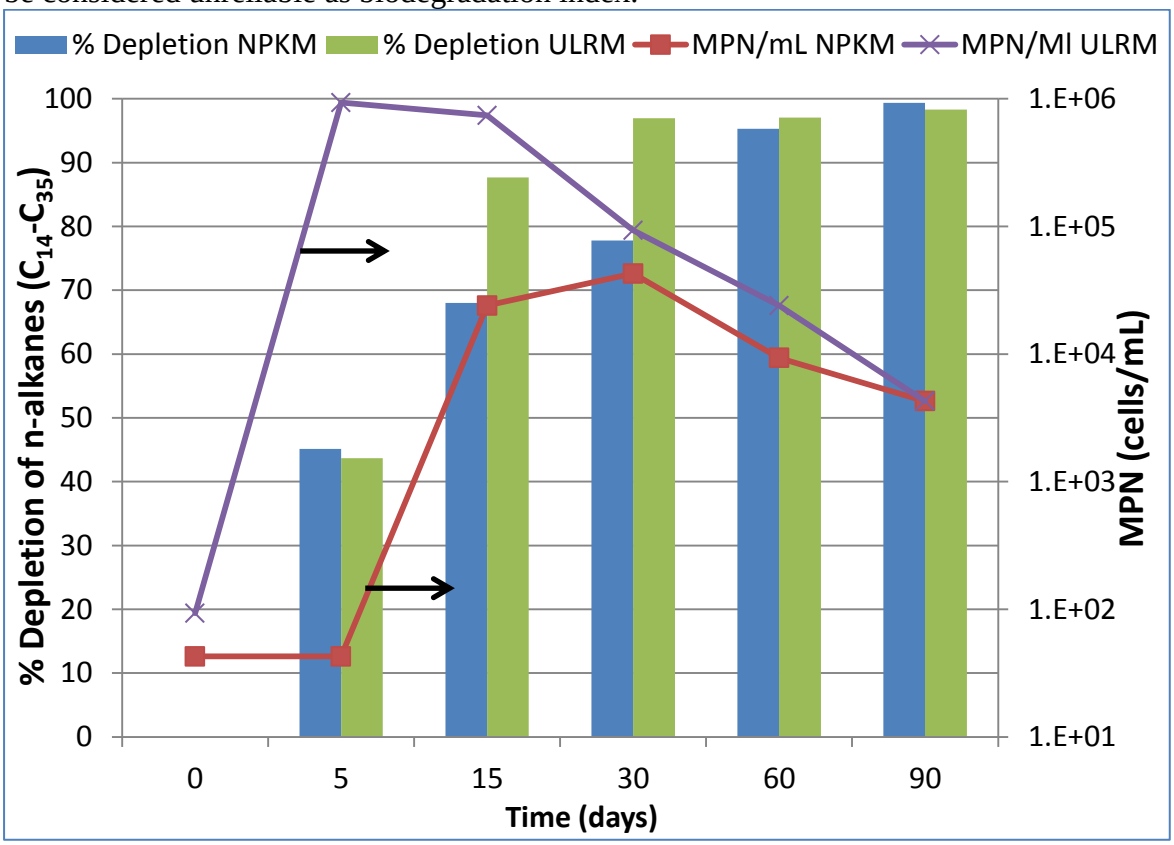


Figure 20: Alkanes % depletion and microbial growth curve between different treatments (NPKM and ULRM) through 90 days of monitoring (Seawater 1).

Comparison of the removal of the saturated fraction and the microbial growth among the NPKM (figure 20) NPKMR and ULR treatments suggests that the removal of the saturated fraction depends on the increase in population of hydrocarbon degraders.

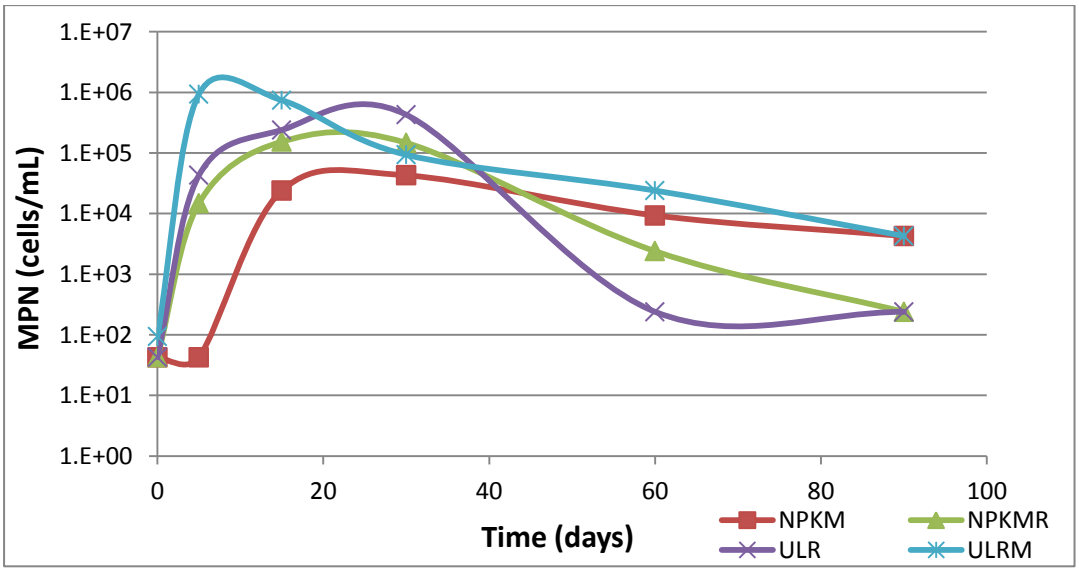


Figure 21: MPN profile in NPKM, NPKMR, ULR and ULRM treatments (Seawater 1).

The application of nutrients in the solutions enhanced the growth of the hydrocarbon degraders as was estimated by the MPN method in comparison with the control

solution where no nutrients were added. Hydrocarbon degraders population in NPKMR, ULR and ULRM microcosms reached 10^6 per mL within 15 days where nearly complete degradation above 90% was achieved (SE for all values ranged from 1.89 to 2.04 for NPKM, NPKMR and ULR treatments and only for ULRM treatment ranged from 1.89 to 2.09). On the contrary, in the NPKM treatment the hydrocarbon degraders population reached 10^5 per mL and degradation reached 65%. The application of biosurfactants in the solutions enhanced the growth of the hydrocarbon degraders within 5 days as it was estimated by the MPN method in NPKMR, ULR and ULRM treatments (Figure 21).

A comparison of the *n*-alkanes and PAHs profiles (Figures 12-18) after oil application revealed that the application of fertilizer plus biosurfactant can favour the degradation of crude oil in communities that are already well adapted but lack essential nutrients. It has been observed in previous study by McKew et al., 2007 that addition of rhamnolipid biosurfactants alone had little effect on biodegradation; however, in combination with water soluble nutrient additions, provoked a significant increase. Sole biosurfactant addition increases only bioavailability of petroleum components; however, if there is lack of essential nutrients (N & P) microbial activity will still be limited.

On the other hand when the added fertilizer is of organic origin it could result in an increased consumption of the organic source rather than consumption of oil. In cases where there is an excess of biosurfactant present, any further production of biosurfactant is stopped while the microbes utilize these nutrients. As it was reported in previous studies on liquid microcosms when these lipophilic nutrients alone (Nikolopoulou et al., 2007) or in combination with rhamnolipids (Nikolopoulou and Kalogerakis, 2008) are applied to the indigenous population, a high degradation of petroleum hydrocarbons can be achieved, which is also confirmed in the present study (ULR treatment). Furthermore, most of the crude oil saturated fractions were totally utilized after 5 days of treatment with the fertilizer plus biosurfactant (NPKMR). The biodegradation reached almost 50 % within 5 days of incubation while 70% was reached after 15 days of incubation (NPKMR). Microbial response to hydrocarbon consumption was remarkable as stated above, however information on the community structure variability and shift mainly affected by the different amendments was investigated also along.

RT-PCR results revealed (Figure 22) that the dominant bacteria in population numbers among the three model markers (*Alcanivorax, Thalossolituus* and *Cycloclasticus*) that were estimated was *Alcanivorax* at the early stage (5days) in the three autochthonous bioaugmentation treatments NPKM, NPKMR and ULRM, which after 15 days becomes the leader in all four treatments. The population numbers of *Alcanivorax* in NPKM especially and NPKMR treatments are particularly high (10^{12}-10^{13} cells/mL) compared to the other 2 treatments during the first 30 days of the experiment. Though the highest number of *Alcanivorax* in NPKM treatment, no significant degradation occurs during this time span that could be correlated in part to *Alcanivorax* dominant presence and as has been investigated through pyrosequencing other species and particularly other *Alcanivorax* strains can be responsible for hydrocarbons degradation in these treatments.

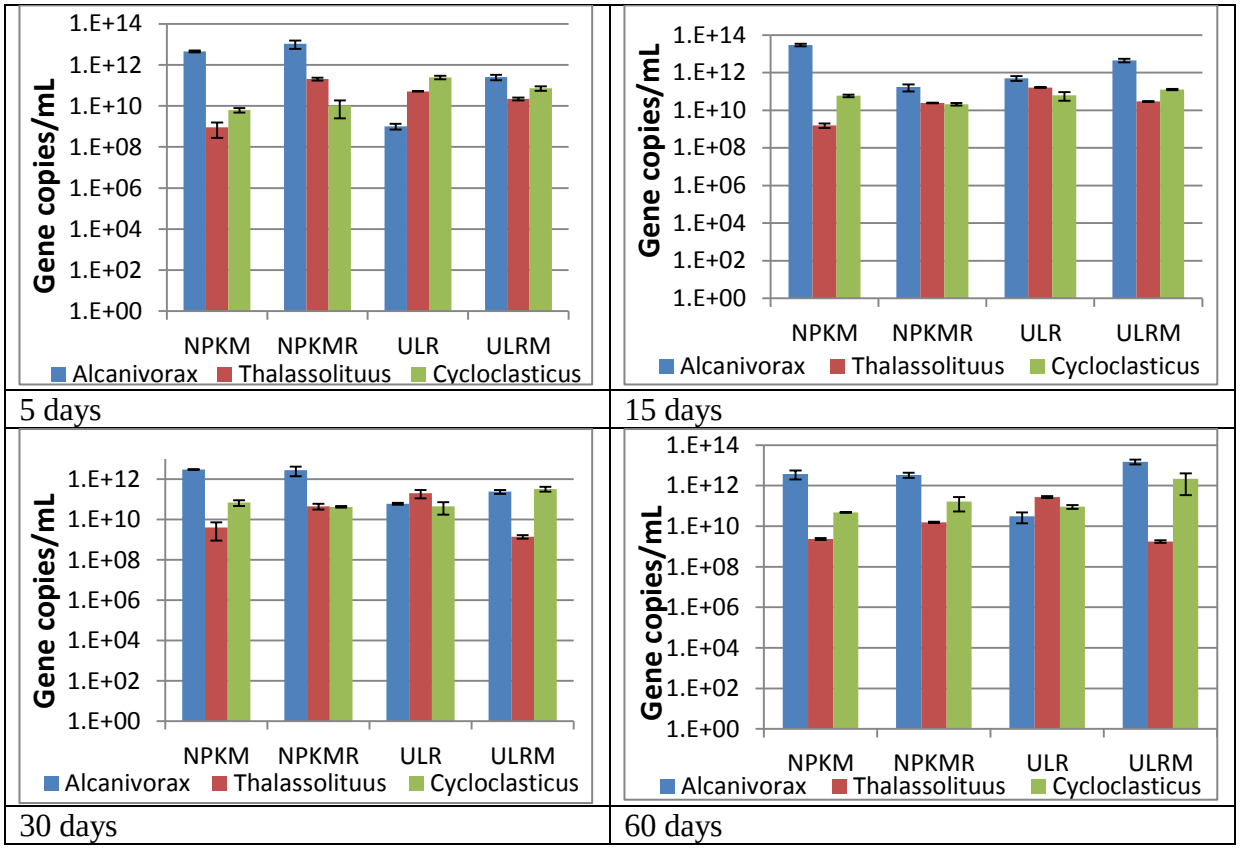


Figure 22: Abundance of alkB2 (Alcanivorax), alkB (Thalassolituus) and phnA (Cycloclasticus) genes in NPKM, NPKMR, ULR and ULRM treatments (Seawater 1).

As it can be observed in the above graphs *Cycloclasticus* numbers have gradually increased, slightly though over time in treatments NPKMR and ULRM, whereas in treatments NPKM and ULR remained practically stable, in the region of 10^{10} cells/mL. It is though remarkable that T*halossolittus* numbers were kept low at most of the experimental time below *Alcanivorax*'s and in some cases *Cycloclasticus'* numbers except for ULR biostimulation treatment the only one that contain indigenous population in which pattern changed after 30 days with T*halossolituus* being the dominant strain among the three that were estimated. As has been described and in detail investigated through pyrosequencing in the samples taken after 30 days of the starting period of the experiments community structure shifts and changes through time depending on the nutrients and the inoculum used in the specific bioremediation strategy.

Specifically twelve families made up the largest part of the community in each sample (84-97% of clean sequences per sample): *Flavobacteriaceae* (0.01-15.3% per sample), *Rhodobacteraceae* (0.04-1.4% per sample), *Rhodospirillaceae* (0.04-23.6% per sample), *Alteromonadaceae* (0-5% per sample), *Idiomarinaceae* (0-28.8% per sample), *Pseudoalteromonadaceae* (0-30.4% per sample), *Shewanellaceae* (0-7.3% per sample), *Alcanivoracaceae* (0.01-79% per sample), *Halomonadaceae* (0-11.1% per sample), *Oceanospirillaceae* (0-18% per sample), *Moraxellaceae* (0.01-19.3% per sample), *Pseudomonadaceae* (0-79.3% per sample) and *Vibrionaceae* (0-24% per sample). The rest (3.2-18.8%) of the community consisted of 69 low abundant (<0.01%) families as well as some OTUs without possible phylogenetic affiliation to the family level. Family distribution is presented in Figure 23.

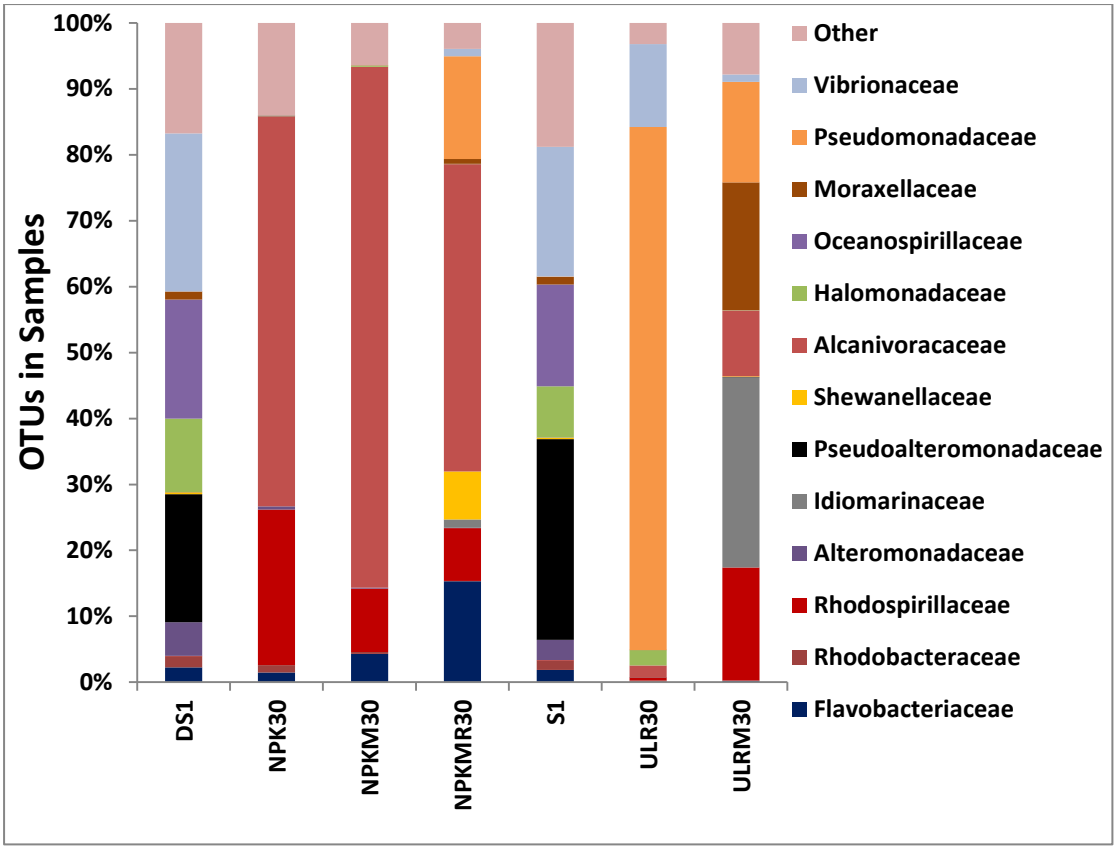


Figure 23: Relative abundance of different family groups based on operational taxonomic units (OTUs) obtained by pyrotag sequencing of amplified 16S rDNA genes (Seawater 1).

S1 represents the initial seawater indigenous population used in biostimulation treatments and DS1 the acclimated consortium in a bioreactor that has been used in the autochthonous bioaugmentation treatments and clearly look similar in terms of species distribution, bacteria belonging mainly to the families of *Pseudoalteromonadaceae* (30.4% & 19.4% for S1 and DS1 respectively), *Halomonadaceae* (7.8% & 11.1%), *Oceanospirillaceae* (15.4% & 18%) and *Vibrionaceae* (19.6% & 24%) per sample). Dominant family groups in NPK, NPKM and NPKMR treatments that inorganic nutrients were used, belong to *Alcanivoracaceae* (59.1%, 79% & 46.6% for NPK, NPKM and NPKMR treatments respectively) and *Rhodospirillaceae* (23.6%, 9.6% & 8% for NPK, NPKM and NPKMR treatments respectively). However in the presence of biosurfactant (rhamnolipids-treatments NPKMR, ULR & ULRM) there is a community shift to the family of *Pseudomonadaceae* (15.6%, 79.3% & 15.3% for NPKMR, ULR & ULRM treatments respectively). Specifically for ULR treatment that lipophilic nutrients added to support indigenous population, indigenous population shifts to consist mainly additionally to *Pseudomonadaceae* family, *Vibrionaceae* family (12.6%). While preadapted consortium that was treated also with lipophilic nutrients besides *Pseudomonadaceae* family also consists mainly of the families of *Idiomarinaceae* (28.8%), *Moraxellaceae* (19.3%), *Rhodospirillaceae* (17.1%) and to lesser extent to the family of *Alcanivoracaceae* (10%).

Total diversity as a mean across all relevant samples (based on the Shannon index, H'log2), species richness (based on the expected number of species corrected for the lowest sample size, ES9000) and/or community evenness (based on Piellou's evenness

indicator, J') were estimated and how these estimates were affected by different nutrients and/or rhamnolipid addition are presented in Table 15.

Table 15: alpha diversity estimates changes between treatments (Seawater 1).

Sample	J'	H'_{log2}	ES_{9000}
DS1	0.5854	4.706	263
NPK30	0.3832	3.072	259
NPKM30	0.2845	2.14	184
NPKMR30	0.4299	3.37	229
S1	0.527	4.274	276
ULR30	0.292	2.128	156
ULRM30	0.4643	3.677	242

Considering the above results and by performing nMDS analysis on the rarefied OTU table, treatments were grouped accordingly (Figure 24). Furthermore ANOSIM tests were then performed to check if there was a significant change in community structure according to different "nutrient" groups (i.e. "no" for samples without any nutrient addition. "npk" for samples with the addition of KNO_3 and K_2HPO_4 and "ul" for samples with the addition of uric acid and lecithin) and “no” or “yes” according to the addition of rhamnolipids. Test results were significant for "nutients" (R=0.85. p=0.01) but not for rhamnolipid addition (R=-0.037. p=0.4), which could mainly be attributed to the lack of data. The individual tests between each "nutrient" group were significant for "no" and “npk” (R=1. p=0.1) but not between “no” and “ul” (R=1. p=0.33) or “np” and “ul” groups (R=0.5. p=0.2).

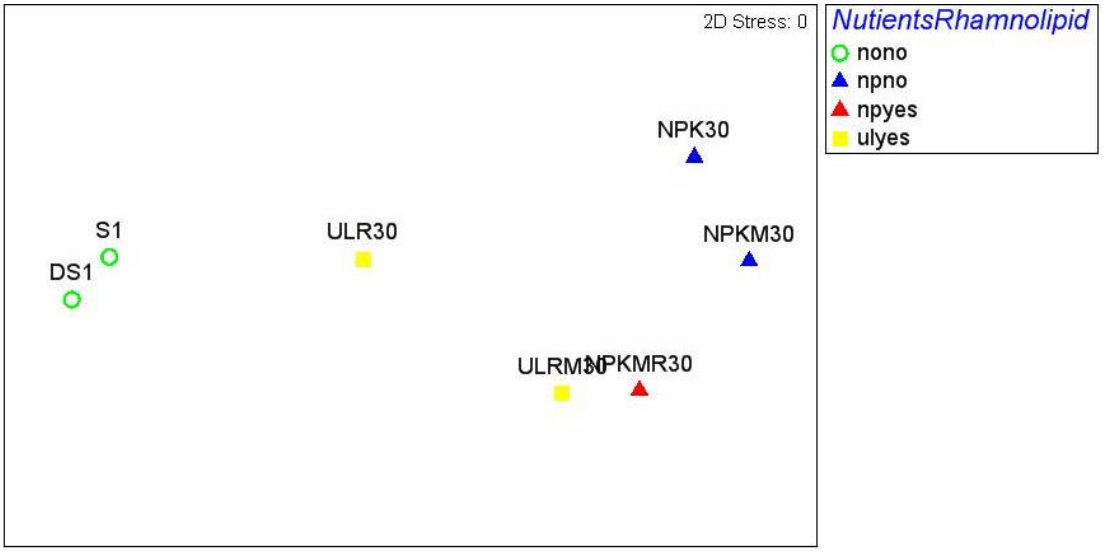


Figure 24: Samples grouping according to Bray-Curtis similarity index (Seawater 1)

Subsequently SIMPER analysis was used to examine which OTUs were important for grouping within and across "no" and "npk" groups. In specific, which OTUs abundances or changes in abundances could explain a significant proportion (>5% each) of community similarity/dissimilarity within and between groups respectively were examined (Table 16 & Table 17). Results showed that within group "no" three OTUs were responsible for 51.9% of total similarity: OTU 1633 (*Pseudoalteromonas sp.*, 23.23% of similarity explained), OTU 449 (*Vibrio sp.*, 15.95% of similarity explained) and OTU 250 (*Marinomonas sp.*, 12.72% of similarity explained). For

group "npk" most (93.78%) of similarity could be attributed to two OTUs: OTU 824 (*Alcanivorax borkumensis*, 80.96% of similarity explained) and OTU 1611 (*Thalassospira lucentensis*, 12.82% of similarity explained). Likewise 60.07% of dissimilarity between "no" and "npk" groups could be attributed to changes in the abundances of the same OTUs: an increase in OTU 824 (*Alcanivorax borkumensis*, 29.4% of similarity explained), a decrease in OTU 1633 (*Pseudoalteromonas sp.*, 12.03% of similarity explained), a decrease in OTU 449 (*Vibrio sp.*, 6.97% of similarity explained), an increase in OTU 1611 (*Thalassospira lucentensis*, 6.38% of similarity explained) and a decrease in OTU 250 (*Marinomonas sp.*, 5.28% of similarity explained) after the addition of nutrients.

Table 16: The most significant OTUs in determining similarity/dissimilarity within or between "no" and "npk" nutrient groups (Seawater 1). The examined group/groups are presented in the first column. The ID number of the most contributing OTUs in each case is shown in the second lane. The third and fourth lanes show the individual and cumulative community similarity or dissimilarity respectively that can be attributed to these OTUs.

Group/Groups	Most contributing OTUs ID	Contribution (%)	Cumulative contribution (%)
"no"	1633	23.23	51.90
	449	15.95	
	250	12.72	
"npk"	824	80.96	93.78
	1611	12.82	
"no" and "npk"	824	29.40	60.07
	1633	12.03	
	449	6.97	
	1611	6.38	
	250	5.28	

Table 17: Phylogenetic affiliation of the OTUs presented in Table 1 based on the BLAST results of the consensus sequence of each OTU against the "nr" nucleotide collection of the NCBI database (Seawater 1).

OTU ID	Phylogenetic affiliation	Accession number	Similarity %
1633	*Pseudoalteromonas sp.*	HQ724506.1	100
449	*Vibrio sp.*	KC737551.1	99
250	*Marinomonas sp.*	JQ409370.1	99
824	*Alcanivorax borkumensis*	KC565664.1	99
1611	*Thalassospira lucentensis*	KC534149	99

Despite the fact that the above statistical analysis of rarefied OTUs was limited only to treatments with inorganic nutrients, due to lack of data especially for the treatments with lipophilic nutrients, has though provided important information on the community structure (grouped in families) based on the treatments applied compared to the initial consortia used for biostimulation and ABA treatments. It needs to be stressed out that although *Alcanivoracaceae* is the dominant family in treatments with inorganic nutrients, when rhamnolipids are applied community shifts to the family of *Pseudomonadaceae*, nonetheless combination of *Alcanivoracaceae* and *Rhodospirillaceae* (NPK, NPKM) is not regarded as the strongest in terms of biodegradation rate at the particular time interval (30 days). On the contrary

combinations of *Pseudomonadaceae* and *Vibrionaceae* families (ULR), of *Alcanivoracaceae*, *Pseudomonadaceae*, *Flavobacteriaceae* and *Rhodospirillaceae* families (NPKMR), of *Pseudomonadaceae*, *Idiomarinaceae*, *Moraxellaceae*, *Rhodospirillaceae* and *Alcanivoracaceae* families (ULRM) have been more effective in terms of hydrocarbons degradation. This proves that different type of amendments and consortia provoke different structures in the resulting biodegradation communities and should be considered when deciding for the suitable bioremediation strategy, however further investigation on the composition of microbial communities with respect to time and interactions with oil hydrocarbons under different conditions should be run in this regard. The advantage of mixed consortia over single species consortia on hydrocarbons degradation has been proved in this study.

It can be concluded that in this study biosurfactants, in particular rhamnolipids, accelerated the biodegradation of crude oil by making it more available to microorganisms as expected in the two ABA treatments (ULRM & NPKMR) and the biostimulation treatment (ULR). Bioavailability of oil hydrocarbons is the critical factor that affects the efficiency of bioremediation in oil contaminated environments. The ability of biosurfactants to emulsify hydrocarbon – water mixtures, to enhance water solubility of hydrocarbons and thus increase the uptake and assimilation of hydrocarbons by the microorganisms is highly recognized (Ron and Rosenberg, 2002; Banat et al., 2010). Kinetics investigation of the specific degradation rate (q_s) support this conclusion since the specific degradation rate is not only growth associated but is also enhanced by intermediate products or biosurfactants activity that possibly affects metabolic pathway. However this was not the case for ULRM treatment in the presence of other organic sources (lipophilic nutrients-rhamnolipids) and leads to the conclusion that either other intermediate products delay the degradation process or this certain community prefers utilizing organic carbon from the organic fertilizers - something that needs to be investigated further.

Nonetheless combination of lipophilic nutrients with/without biosurfactants in combination with a well-adapted indigenous community of hydrocarbon degraders can be a promising tool to be used in cases where immediate response to oil spill incidents is necessary particularly in pristine environments.

4.2. Autochthonous Bioaugmentation & Biostimulation with Isolated Hydrocarbon Degraders Consortium of Seawater Microcosm (Seawater 2)

Results and Discussion

Effectiveness of each treatment in crude oil biodegradation was estimated by observing trends in the compositional changes of alkanes, PAHs and hydrocarbon degraders throughout the period of the experiment. All monitored concentrations of the Control treatment (CM) remained practically unchanged at all times of the experiment as also compared to the initial concentration profile of NPKM and NPKMR treatments (Figure 25) and will not be discussed any further. This outcome clearly demonstrates that the pre-adapted inoculum had marginal to no effect on the degradation of *n*-alkanes throughout the whole experiment in the absence of essential nutrients.

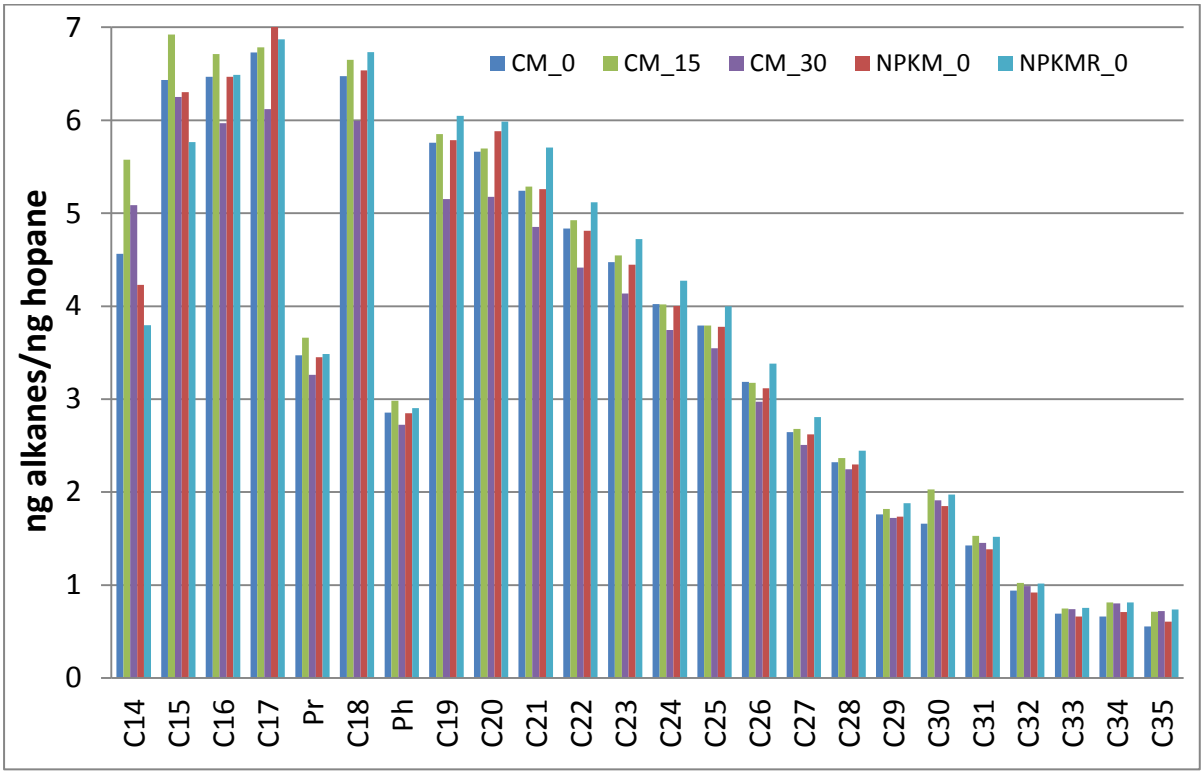


Figure 25: Concentration of C14-C35 n-alkanes after 0, 15 and 30 days of monitoring in Control (CM) treatment and at 0 days of NPKM and NPKMR treatments (Seawater 2).

On the contrary, in both treatments NPKM and NPKMR (where stimulants were present) a decreasing rate was observed of the medium chain *n*-alkanes (C_{14} to C_{30}) while high chain alkanes (C_{31} to C_{35}) remained stable in the 30 days of the experiment as shown in Figures 26 and 27. The average specific consumption (degradation) rate $\bar{q}_S$ was calculated over the time period that degradation occurred, which as can be observed in Figures 26 and 27 is 0 to 30 days, for treatments NPKM and NPKMR. The average specific growth rate μ increased 3 to 8 times more than the control (CM) and the average specific degradation rate increased 28 times and 23 times higher than the control for C_{15} in NPKM and NPKMR treatments respectively, as shown on the Table 18. Similarly, for C_{20} the average specific degradation rate was approximately 20 times higher and for C_{25} about 10 times higher than the control for both treatments. C_{35} remained practically stable throughout the whole experiment in three treatments.

On the contrary biomarkers Pristane and Phytane were also degraded by 3 and 12 times higher than the control, as seen for the treatments NPKM and NPKMR respectively.

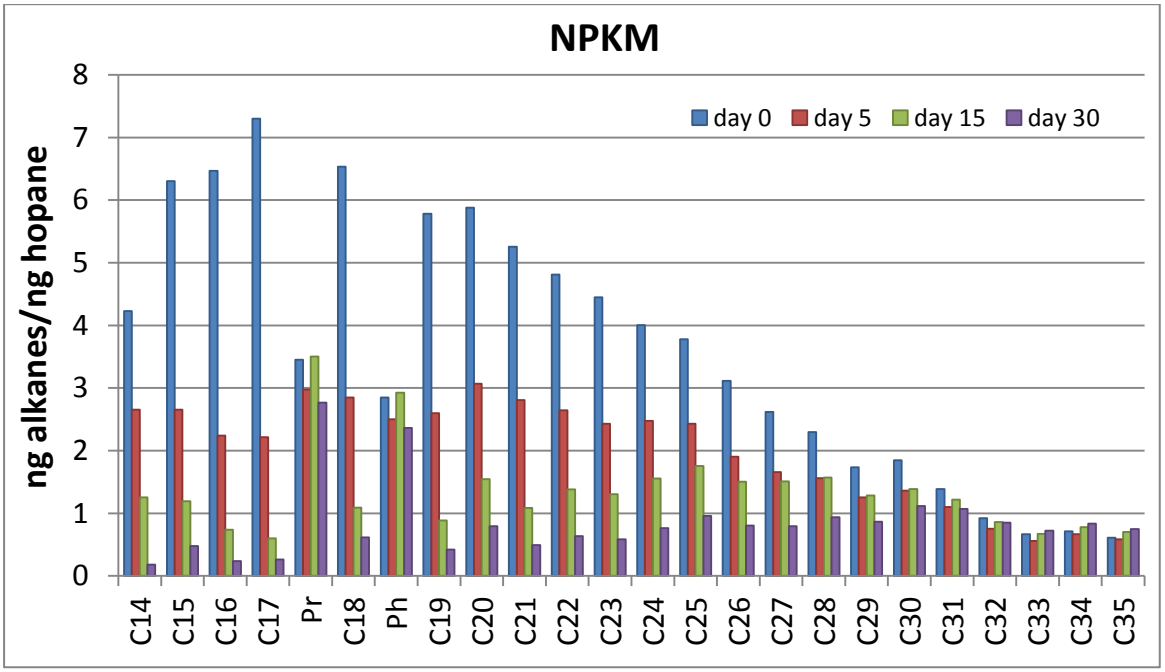


Figure 26: Concentration of C14-C35 n-alkanes after 0, 5, 15 and 30 days of monitoring in NPKM treatment (Seawater 2).

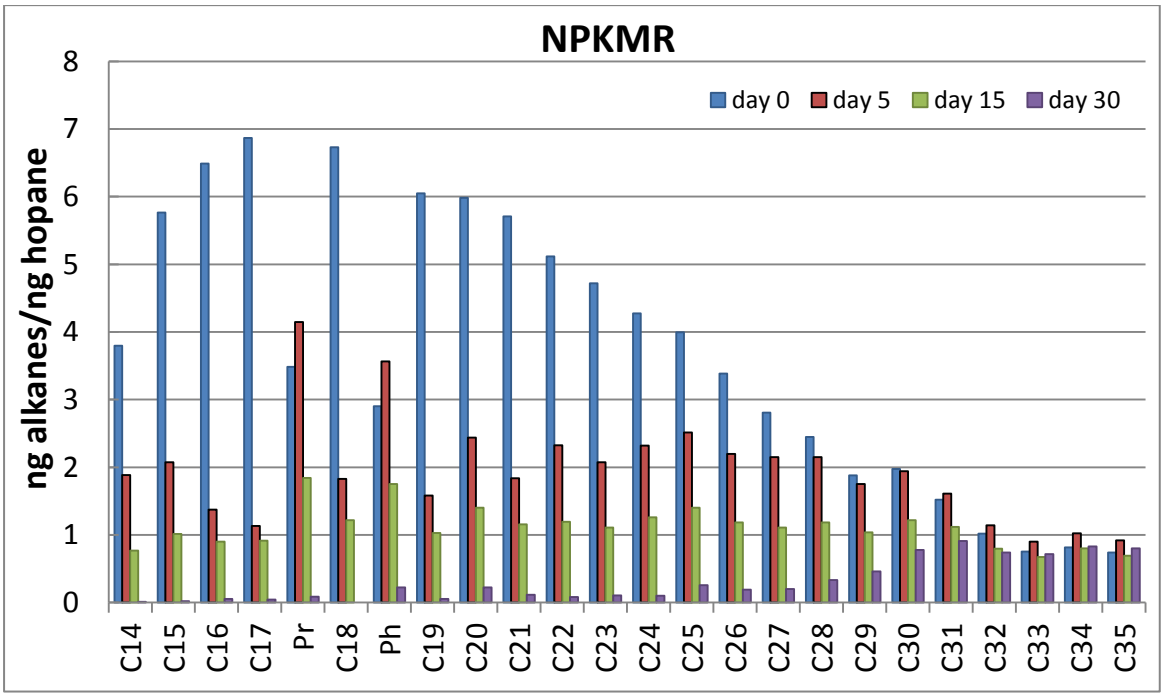


Figure 27: Concentration of C14-C35 n-alkanes after 0, 5, 15 and 30 days of monitoring in NPKMR treatment (Seawater 2).

Low molecular weight PAHs (fluorene & dibenzothiothene) exhibited a lower specific degradation rate compared to n-alkanes whereas the concentration of the

higher molecular weight PAHs (phenanthrene & chrysene) remained practically stable (at least 15 days for phenanthrene) as shown in Figures 28 and 29.

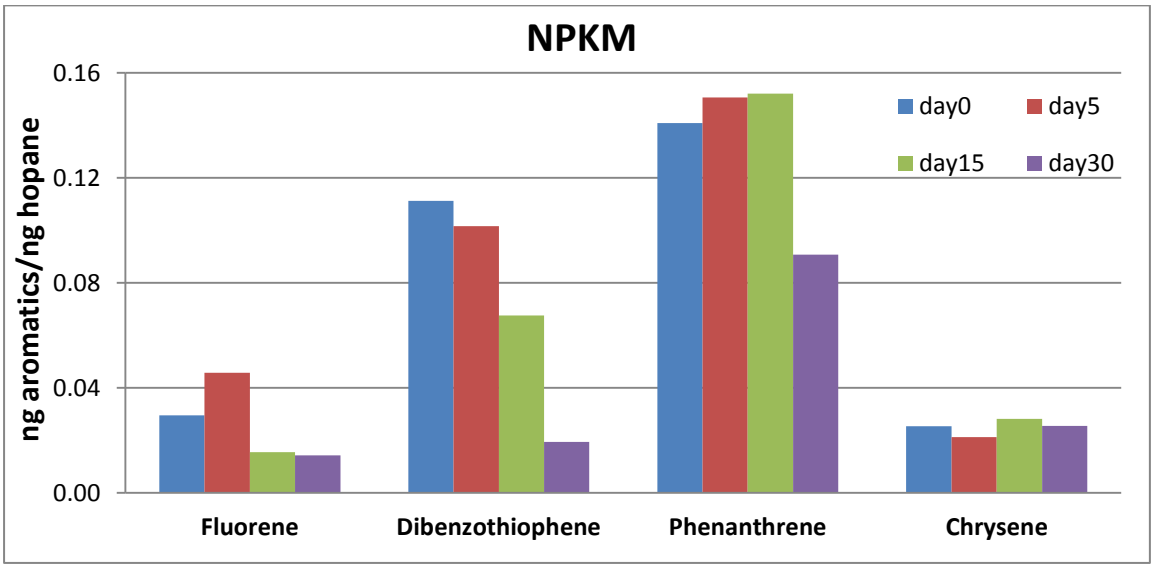


Figure 28: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15 and 30 days of monitoring in NPKM treatment (Seawater 2).

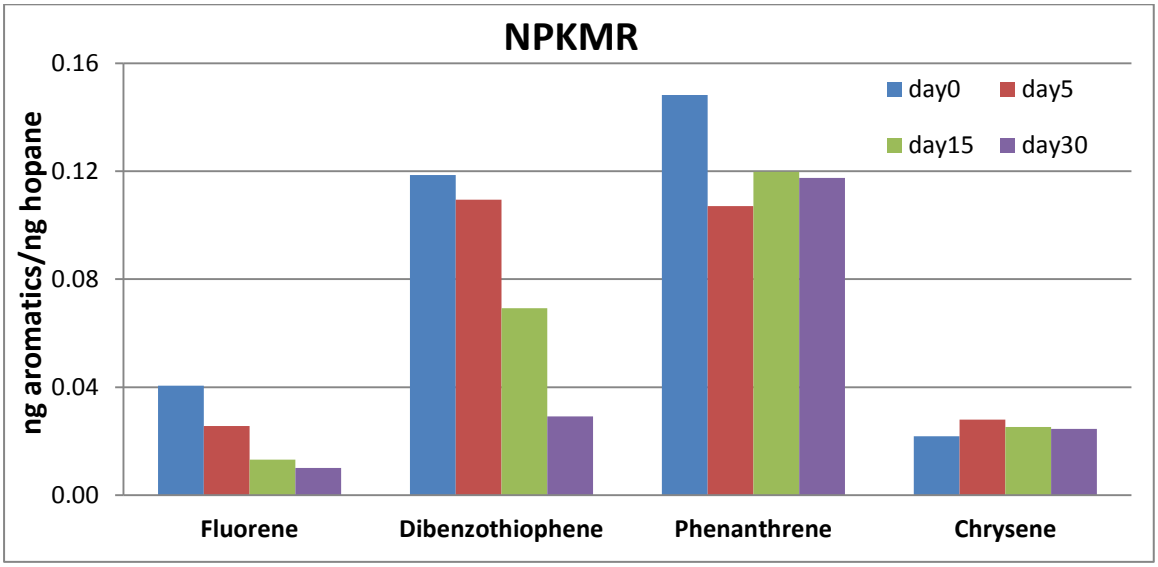


Figure 29: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 5, 15 and 30 days of monitoring in NPKMR treatment (Seawater 2).

Specific degradation rate of PAHs is much slower when compared to n-alkanes as shown in Table 19. Saturated fraction is depleted to greater extent than aromatics fraction and the degradation rate follows the pattern from high to low: C15 > C20 > C25 > (Pristane, Phytane) > C30 > (PAHs).

Pristane and Phytane have often been used as conserved internal markers in biodegradation index; however, especially in the NPKMR treatment, we observe that both compounds were completely degraded within 30 days as shown in Figure 27 and Table 18 and thus, they are unreliable as biodegradation index and should not be used.

Table 18: Specific growth and degradation rate of the selected alkanes (Seawater 2)

Treatment	μ (1/h)	q_s (μg/cells h)					
		C_{15}	C_{20}	C_{25}	C_{30}	Pristane	Phytane
CM	0.011	9	24	12	~0	10	6
NPKM	0.031	250	218	121	31	29	21
NPKMR	0.083	196	197	128	41	116	92

Table 19: Specific degradation rate of the selected PAHs (Seawater 2)

Treatment	q_s (μg/cells h)			
	Fluorene	Dibenzothiothene	Phenanthrene	Chrysene
CM	~0	~0	~0	0
NPKM	0.97	3.94	2.15	0
NPKMR	1.04	3.06	1.05	0

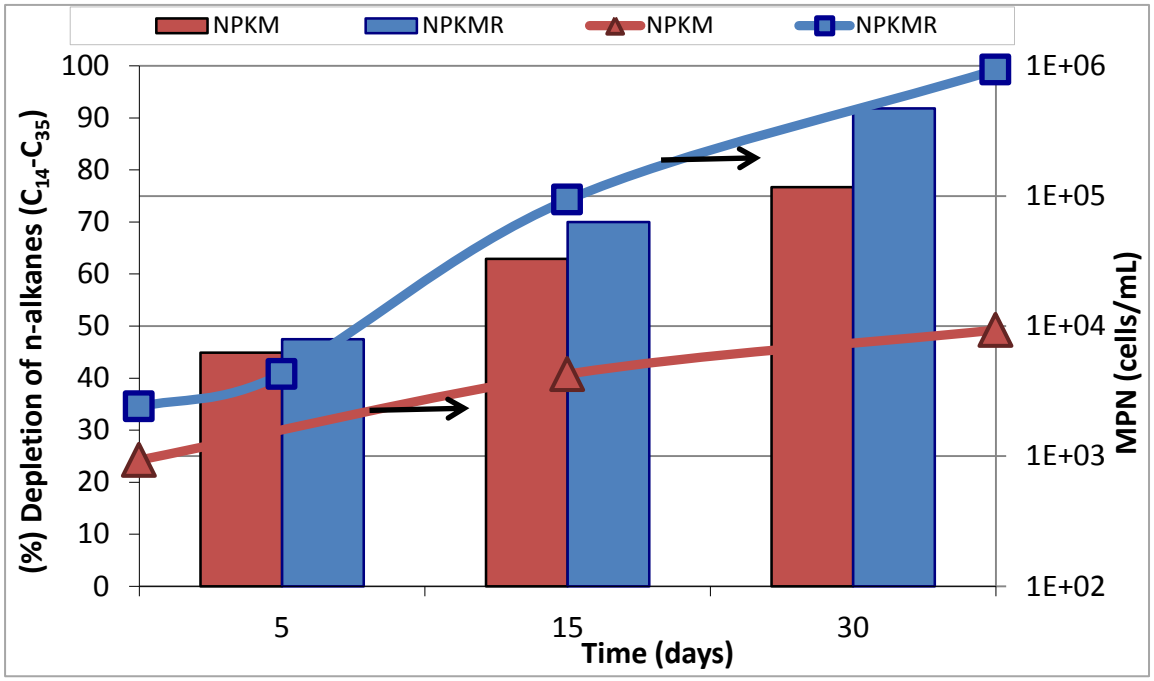


Figure 30: Alkanes % depletion and microbial growth curve between different treatments (NPKM and NPKMR) through 30 days of monitoring (Seawater 2).

Percent depletion of n-alkanes fraction compared to microbial growth between NPKM and NPKMR treatments (as seen in Figure 30) depicts that the depletion of n-alkanes is a function of oil degrading bacteria augmentation. In particular by applying certain nutrients amendments, population of oil degrading bacteria was increased as was verified by the MPN method contrary to the no nutrients amendment-control (reached 10^3 cells/mL at the end of the experiment). Hydrocarbon degraders population in NPKMR microcosms reached 10^6 cells/mL (SE for all values ranged from 1.89 to 2.04) within 30 days where nearly complete degradation above 90% was achieved. On the contrary, in the NPKM microcosm hydrocarbon degraders population reached 10^4

cells/mL (SE for all values ranged from 1.89 to 2.03) and total degradation reached 77%. When biosurfactants were applied in the NPKMR treatment the population of hydrocarbon degrading bacteria was favored within 5 days according to MPN estimations.

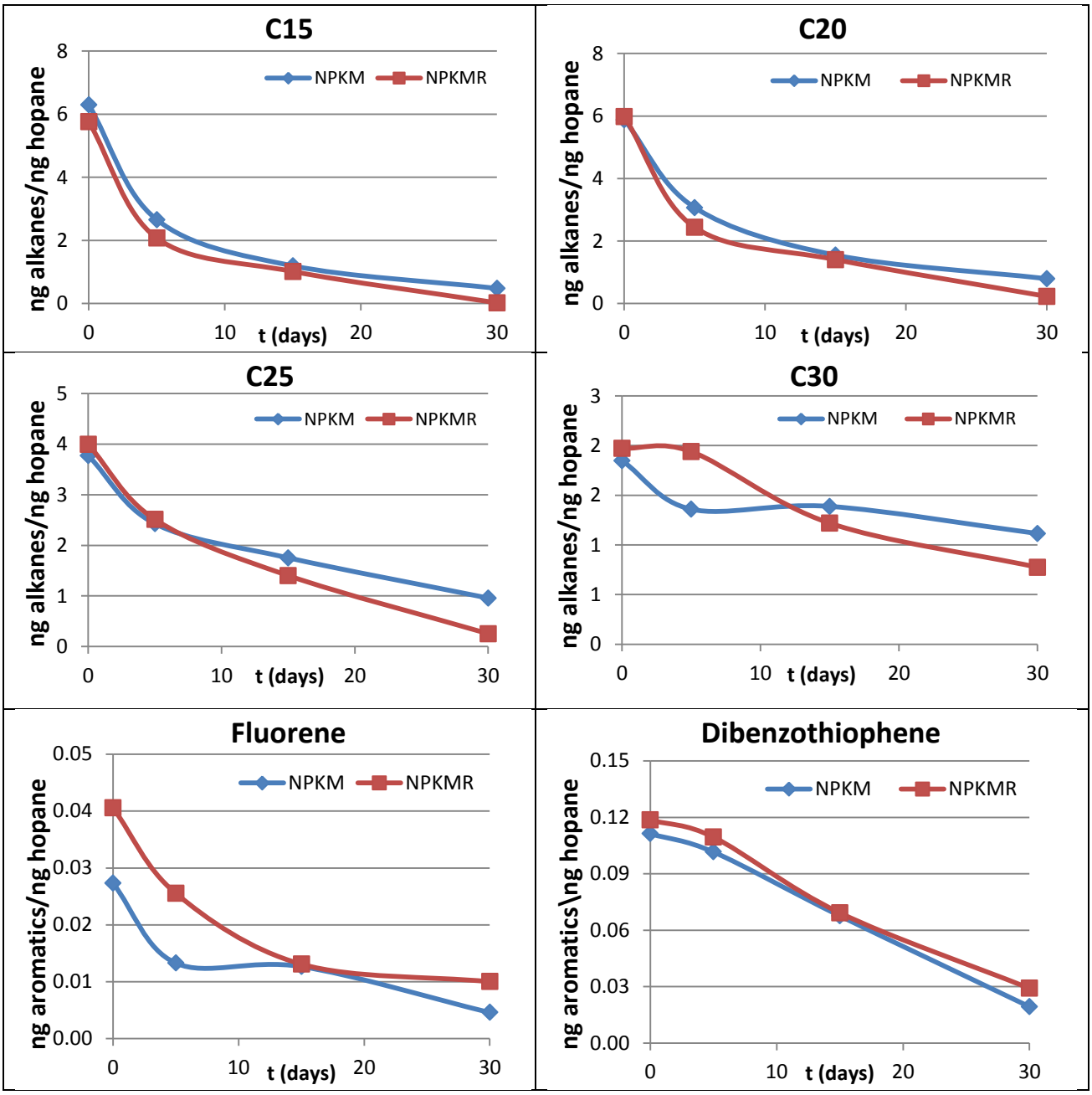


Figure 31: Concentration profiles of selected n-alkanes and PAHs compounds in NPKM and NPKMR treatments (Seawater 2).

A comparison of the n-alkanes and PAHs concentration profiles (Figures 26-29 and 31) after crude oil application confirmed that substrate (oil) consumption rate was considerably higher when nutrients and biosurfactant were jointly applied. Particularly saturated fraction in crude oil was entirely consumed in almost 5 days in the NPKMR amendment that inorganic nutrients and biosurfactant were used simultaneously. The biodegradation almost reached 50% within 5 days after the initial application of biostimulants and 70% after 15 days in the NPKMR amendment.

Hence biosurfactant's capability to speed up oil biodegradation by emulsifying hydrocarbon – water mixtures and thus increasing its bioavailability is demonstrated in the present work (Banat et al., 2000; Southam et al., 2001; Mulligan, 2005; Ron and Rosenberg, 2010). Kinetics investigation of the specific degradation rate q_S supports this conclusion since it is not only growth associated but was also enhanced by intermediate products or biosurfactants activity that possibly affected the metabolic pathway.

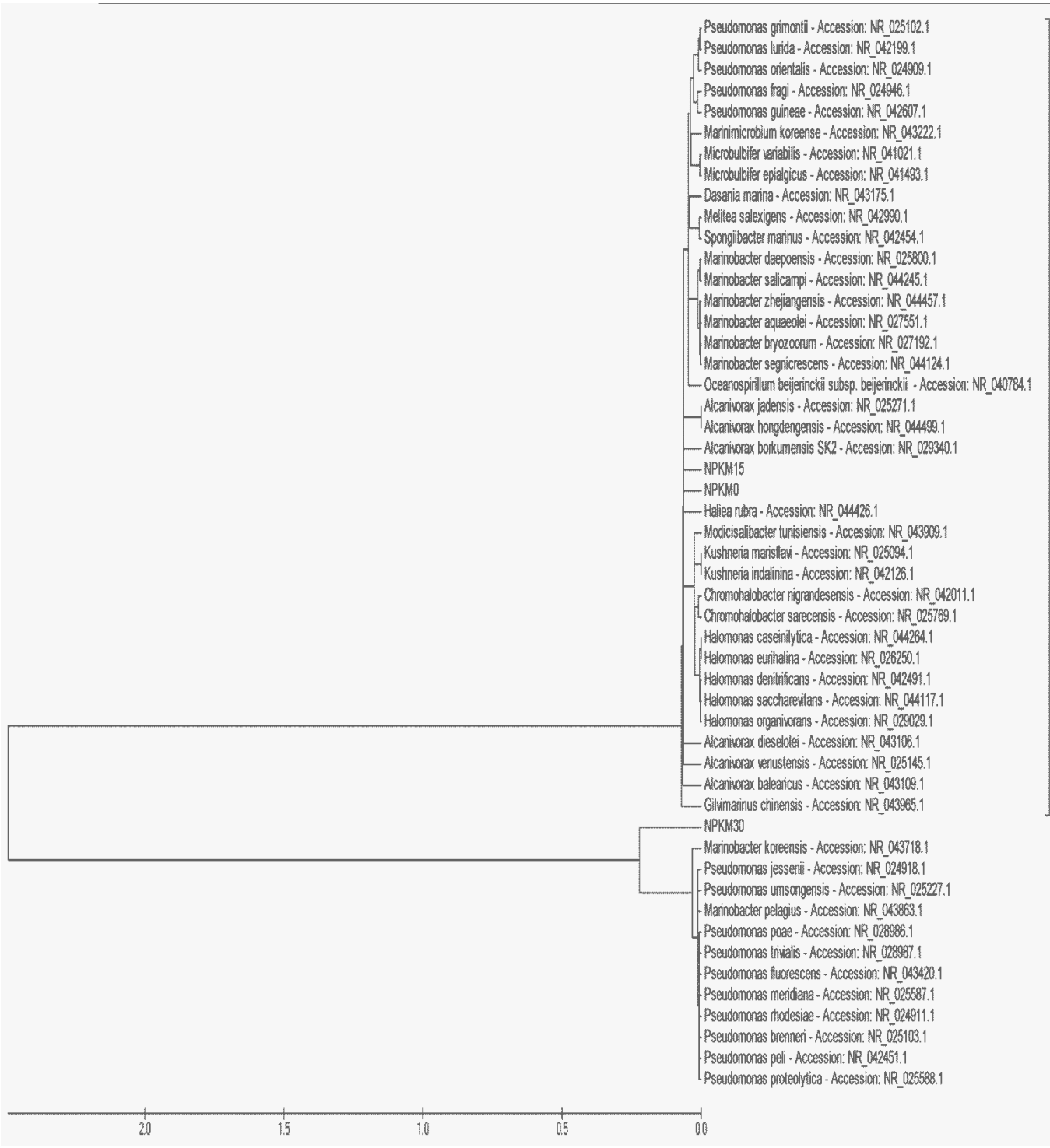


Figure 32: Phylogenetic tree of bacteria isolated during NPKM treatment (Seawater 2).

Early studies have shown that the variety of microorganisms which have great affinity and can survive in oil products hydrocarbons is quite extended (Harayama et al., 2004; Palleroni et al., 2010; Tanaka et al., 2008; Berthe-Corti and Nachtkamp, 2010; Prince et al., 2010). However response of naturally present microorganisms when crude oil s added is still not fully investigated. Sequence analysis of the dominant genera within the 16S rDNA gene library of the adapted microflora revealed a wide range of phylogenetic groups closely related to the genera of *Pseudomonas, Marinobacter, Alcanivorax, Halomonas* and *Microbulbifer* (Harayama et al., 2004). The phylogenetic analysis of the marine bacterial community composition in the NPKM amendment showed that at the early stage of biodegradation (15 days) the dominant bacteria belonged mainly to the genera of *Marinobacter, Microbulbifer, Halomonas, Pseudomonas, Alcanivorax* and *Chromohalobacter.* While after 30 days of treatment the dominant bacteria mainly belonged to the genera of *Pseudomonas* and *Marinobacter*. In the NPKMR treatment the phylogenetic analysis revealed that at the early stage (after 15 days) the dominant bacteria belonged to genera of *Alcanivorax, Marinobacter, Microbulbifer, Melitea* and *Halomonas* whereas after 30 days they mainly belonged to the genus of *Pseudomonas*. In fact, the addition of biosurfactant in NPKMR treatment provoked extensive hydrocarbon degradation and a shift in the community composition in favour of the genera *Alcanivorax, Marinobacter, Microbulbifer* and *Halomonas*, whereas *Pseudomonas* was dominant after 30 days of treatment.

Alcanivorax species are known as hydrocarbonoclastic bacteria that oxidize C_5-C_{16} *n*-alkanes and branched alkanes. *Halomonas* belongs to the marine microflora that can metabolize phenanthrene or Chrysene (Harayama et al., 2004). Species of *Microbulbifer* genus can degrade aromatic compounds like naphthalene and fluoranthene. Bacteria of the *Alcanivorax, Marinobacter, Microbulbifer* and *Halomonas* genera are characterized as halotolerant, however pseudomonas although considered as hydrocarbon degraders (of alkanes and PAHs) are not tolerant to high salinity. On the contrary *Pseudomonas* sp. are able to endure and metabolize contaminants that are considered very toxic to other bacteria. Several studies have proved that *Pseudomonas* sp. can utilize a vast range of contaminants either naturally present or xenobiotic (Palleroni et al., 2010). Regarding the above, *Pseudomonas* sp. can be considered as exceptional biocatalysts and can accelerate bioremediation when other species stop. This is the case in the NPKM and most likely in the NKPMR treatment, at the beginning of the experiment the community is comprised by strains that can utilize hydrocarbons (alkanes and some aromatic compounds) whereas by the end of the experiment where most of the hydrocarbons have been consumed, pseudomonas strains take over utilizing either metabolic by-products or other more recalcitrant hydrocarbons (Palleroni et al., 2010).

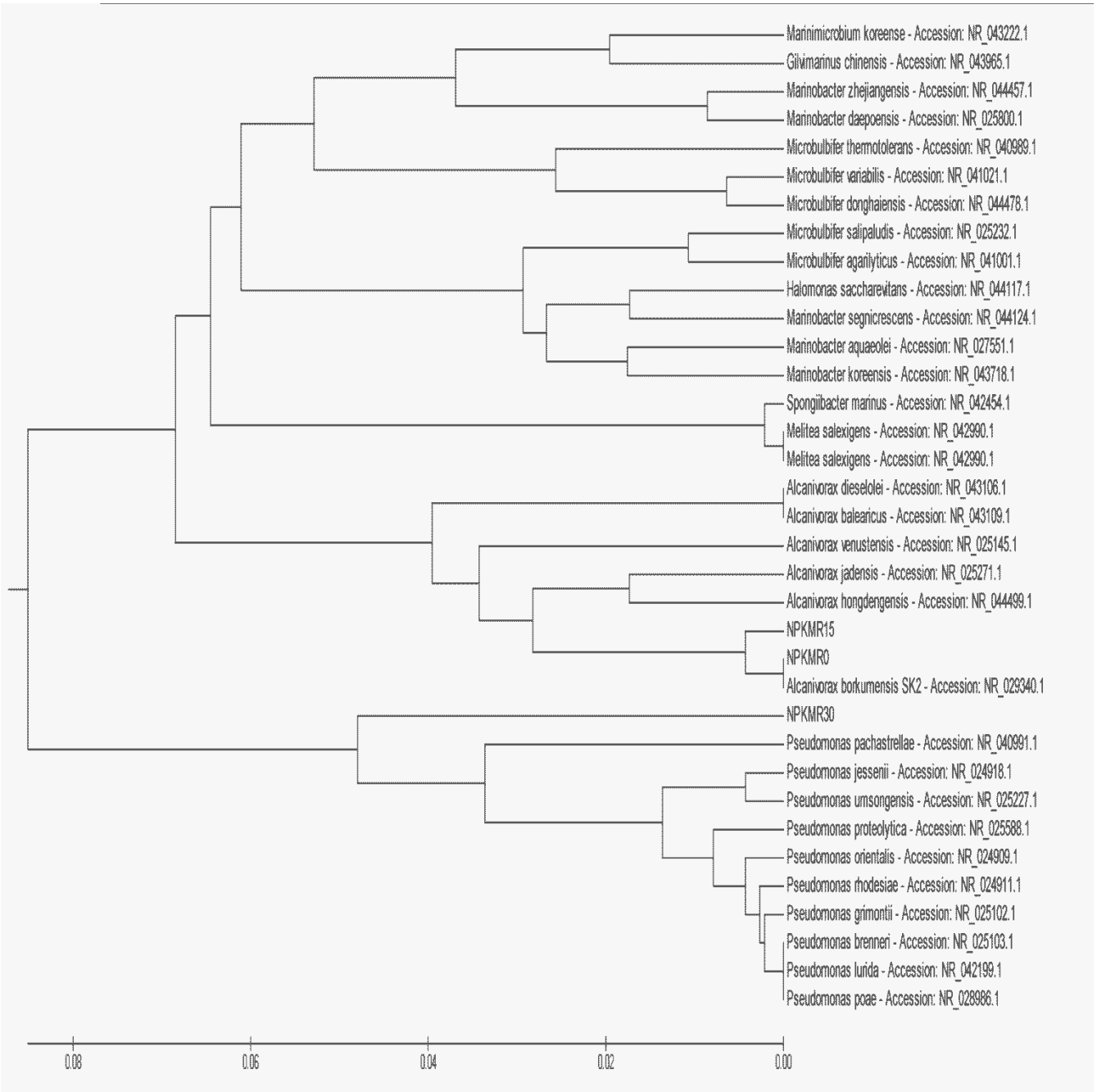


Figure 33: Phylogenetic tree o of bacteria isolated during NPKMR treatment (Seawater 2).

Microbial communities' structure is affected and shifts depending on these interactions between surfactants and other stimulants. Thus identifying the key organisms that play role in different bioremediation treatments is very important for understanding, evaluating and further decide on the best in situ bioremediation strategy.

4.3. Landfarming of Oil Polluted Beach Sand through Biostimulation (Sand 1)

Results and Discussion

Evaluation of the effectiveness of each treatment on crude oil biodegradation rate was estimated in terms of alkanes, PAHs and hydrocarbon degraders compositional changes throughout the whole period of the experiment. Figures 34-36 represent total depletion rate of the saturated fraction of *n*-alkanes (C_{12}-C_{35}) of control treatment (C) as well as for treatments NPK and ULR at different time intervals of the experiment. Control, in which hydrocarbon degraders population was not further increased, had no significant effect on the degradation rate at the first 30 days of treatment (Figure 34). NPK treatment with inorganic nutrients and ULR treatment were more successive in terms of time and quantity of hydrocarbons depleted; about 48% and 58 % of n-alkanes were removed in 15 days of the experimental period respectively, whereas in control treatment depletion of n-alkanes reached 58% after 30 days of treatment. Moreover the degradation rate of NPK and ULR reached equally 97% within 30 days and 99% at the end of the experiment. On the contrary in control treatment depletion of n-alkanes reached 97% after 45 days of treatment.

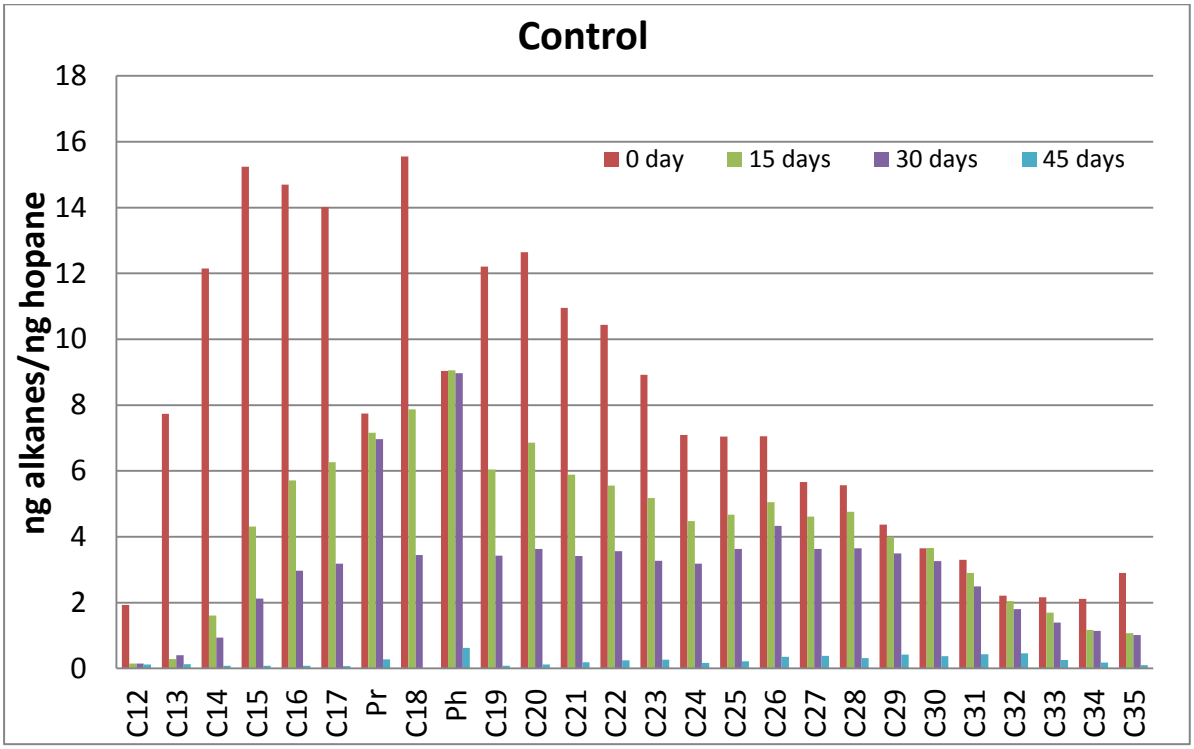


Figure 34: Concentration of C_{12}-C_{35} n-alkanes after 0, 15, 30 and 45 days of monitoring in Control treatment (Sand 1).

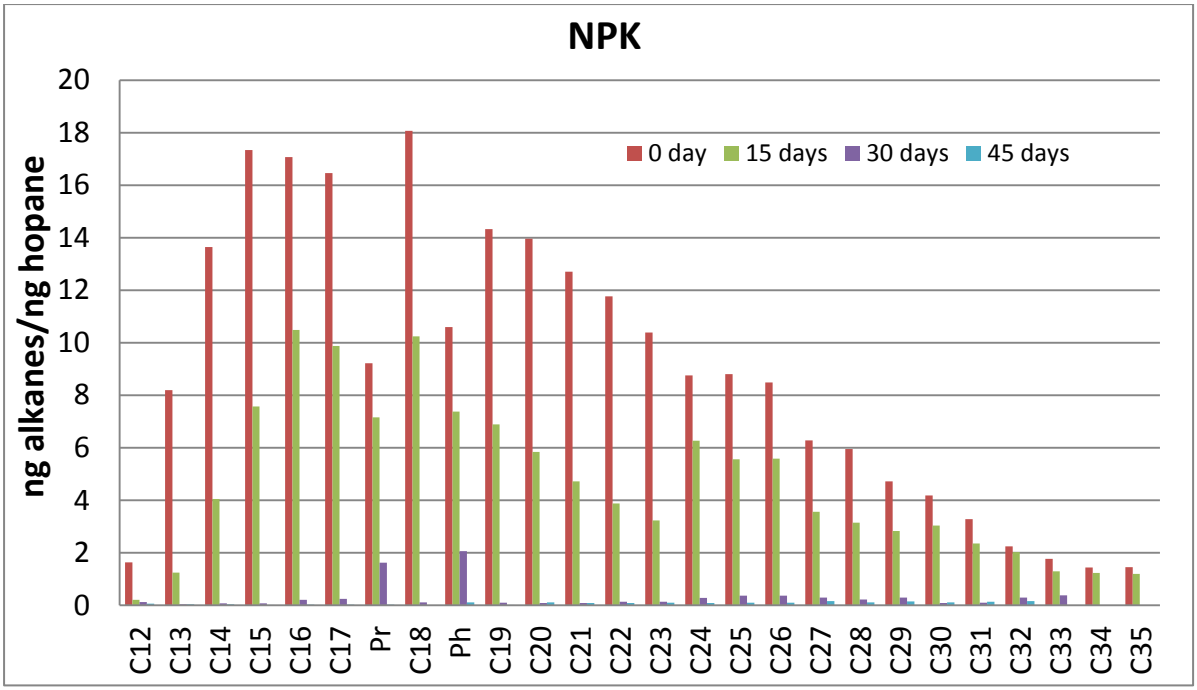


Figure 35: Concentration of C_{12}-C_{35} *n*-alkanes after 0, 15, 30 and 45 days of monitoring in NPK treatment (Sand 1).

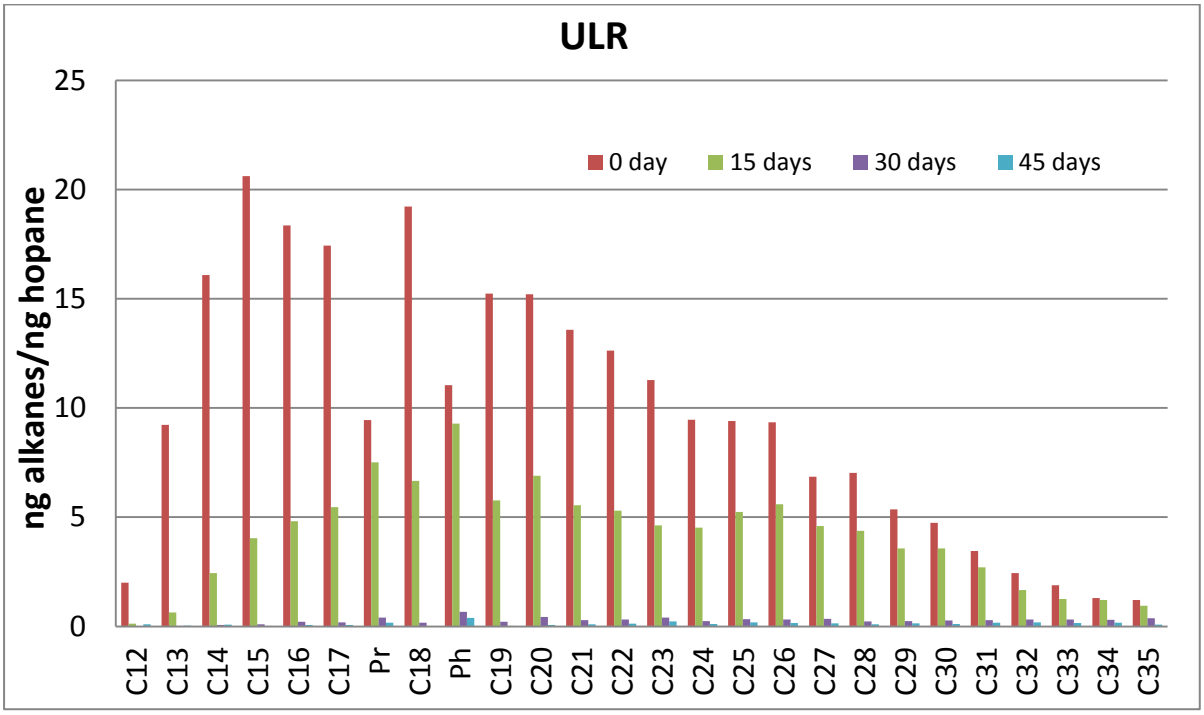


Figure 36: Concentration of C_{12}-C_{35} n-alkanes after 0, 15, 30 and 45 days of monitoring in ULR treatment (Sand 1).

As it is observed from Figures 35&36 in treatment ULR, (where Rhamnolipids were added) and in treatment NPK (inorganic nutrients) there is high decreasing rate for medium chain *n*-alkanes (C_{12} to C_{30}) within 15 days of the experiment as well as for high chain alkanes (C_{31} to C_{35}) in almost 30 days of the experiment. In control treatment instead medium to high chain alkanes (C_{25} to C_{35}) remain stable for the whole duration of the experiment as shown in Figure 34.

Table 20: Specific growth and degradation rate of the selected alkanes (Sand 1)

Treatment	μ (1/h)	q_s (μg/cells h)					
		C_{15}	C_{20}	C_{25}	C_{30}	Pristane	Phytane
Control	0.005	61.3	50.6	27.6	13.3	30.2	34
NPK	0.014	201.1	161.6	98.2	47.7	88.4	99.3
ULR	0.017	202.7	145.9	89.5	44	89.4	102.5

The average specific consumption (degradation) rate $\bar{q}_S$ was calculated over the time period that degradation occurred, which for NPK and ULR is 0→30 days and for Control is 0→45 days. If we make the comparison between treatments (Table 20) we can estimate that specific degradation rate for C_{15}, C_{20}, C_{25} and C_{30} in NPK and ULR treatments is 3-3.5 times higher than control whereas C_{35} remained practically stable. By the end of the experiment specific degradation rates for C_{15}, C_{20}, C_{25} and C_{30} is equal for all treatments, however in the presence of specific nutrients (treatments NPK and ULR) specific degradation rates are accelerated within 15 days. Especially for treatments NPK and ULR (biosurfactant is present) specific growth rate μ equals to 0.014 (1/h) and 0.017(1/h) respectively, and is approximately 3-3.5 times higher than the one of the control treatment, which implies a growth associated degradation rate. Branched alkanes like Pristane and Phytane are also degraded by 3 times higher in both NPK and ULR treatments compared to control. Regarding the above observations, this trend implies that at early stage of the experiment light hydrocarbons, which are more water soluble are depleted at about the same level in all treatments, however depletion rate of medium, high and branched alkanes that are less accessible is accelerated in treatments were nutrients are added later on.

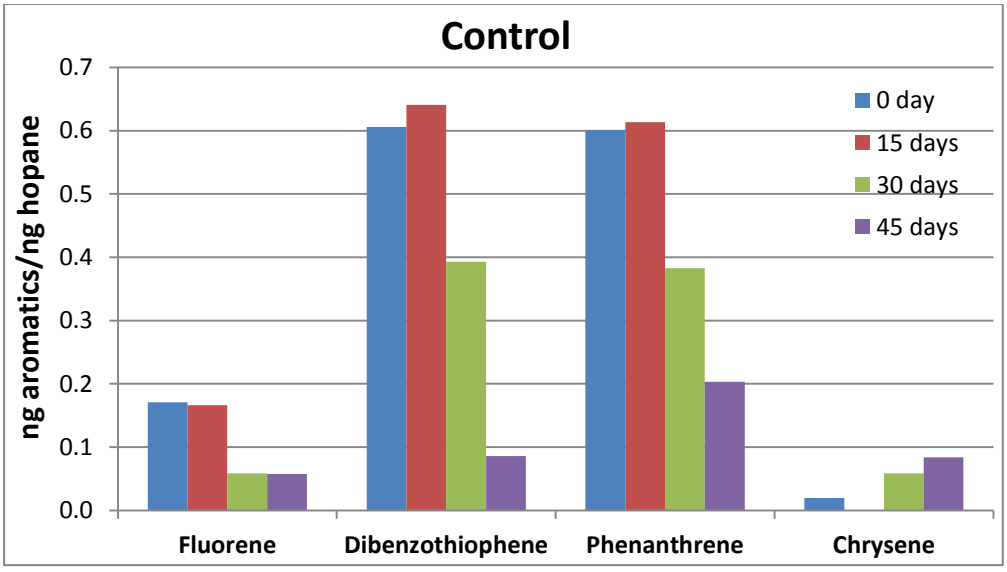


Figure 37: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 15, 30 and 45 days of monitoring in Control treatment (Sand 1).

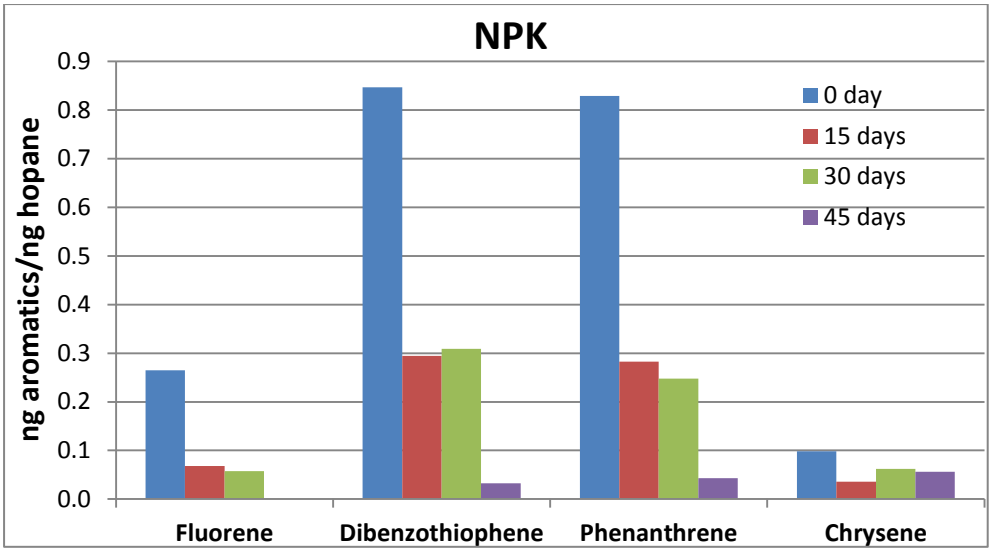


Figure 38: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 15, 30 and 45 days of monitoring in NPK treatment (Sand 1).

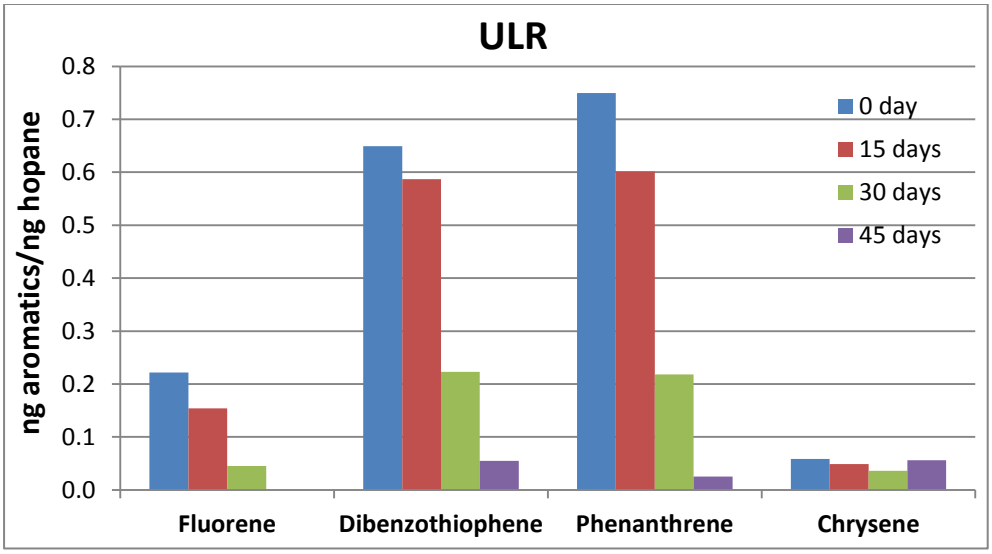


Figure 39: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 15, 30 and 45 days of monitoring in ULR treatment (Sand 1).

The same behaviour of treatments NPK-ULR in terms of specific degradation rate applies also for selected PAHs that are plotted in Figures 37-39. Also there is still low but decreasing rate in low molecular weight PAHs (fluorene-dibenzothiothene-phenanthrene) compared to high molecular weight PAH (chrysene) which remains practically stable. Degradation in treatment NPK of low molecular PAHs is achieved within 15 days, while for control and ULR is achieved within 30 days of the experiment (Figures 37-39). PAHs degradation in NPK treatment reached for fluorene 75% and for dibenzothiothene- phenanthrene was equally 65% within 15 days while in ULR treatment degradation rate was slower, reaching 30% for fluorene, only 10% for dibenzothiothene and almost 20% for phenanthrene. In this sense, estimated specific degradation rate for fluorene was 5 and 4 times higher than control in treatments NPK and ULR respectively. Moreover specific degradation rate for phenanthrene was 4 and 3.3 times higher, while dibenzothiothene was 3 and 2 times higher than control for NPK and ULR treatments respectively. Besides this slower

behaviour of ULR treatment, it catches up and after 30 days of the experiment, degradation rate for both treatments is about the same level; specifically >63% for dibenzothiothene, 70% for phenanthrene and about 80% for fluorene and exceeds 95% for all PAHs by the end of the experiment. On the contrary PAHs depletion in the control treatment does not exceed 85% at the end of the experiment.

Table 21: Specific growth and degradation rate of the selected PAHs (Sand 1)

Treatment	q_s (µg/cells h)		
	Fluorene	**Dibenzothiothene**	**Phenanthrene**
Control	0.46	2.10	1.61
NPK	2.29	6.44	6.36
ULR	1.74	4.21	5.25

The preferred biodegradation of more easily biodegradable substrates such as the lower-molecular-mass PAH and aliphatic hydrocarbons that are found in contaminated soils could also be attributed to the difference in aqueous solubility between the PAHs, since it is known that the aqueous solubility decreases logarithmically as the ring number increases (Deschenes et al., 1996). Solubility of Chrysene is over 1000 times lower compared to fluorene and phenathrene (1,992 & 1.6 mg/L respectively).

Accordingly specific degradation rate of PAHs is much slower when compared to n-alkanes obviously (Table 21) and follows the same trend as mentioned before from lighter hydrocarbons to heavier hydrocarbons. Once again former conserved internal markers like Pristane and Phytane, especially in ULR treatment as shown in Figure 36 and Table 20 are completely degraded within 15 days and thus they cannot be used reliably as biodegradation indexes.

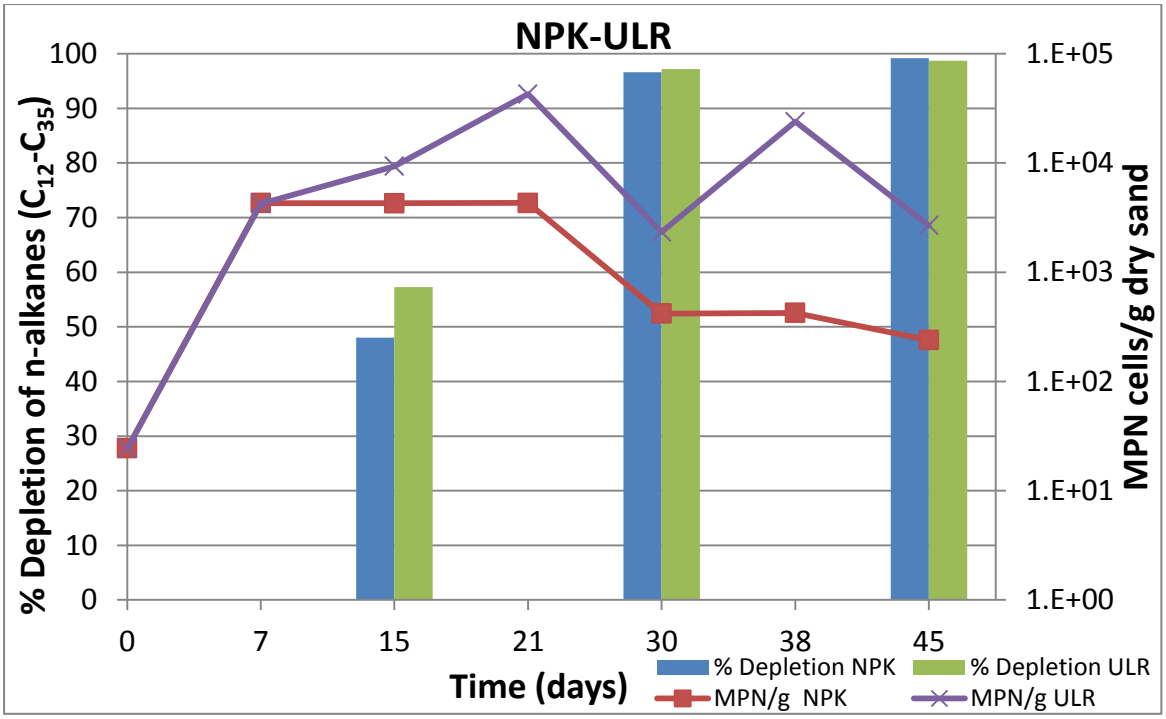


Figure 40: Alkanes % depletion and microbial growth curve between different treatments (NPK and ULR) through 45 days of monitoring (Sand 1).

Comparison of the removal of the saturated fraction and the microbial growth among the NPK and ULR treatments (Figure 40) suggests that the removal of the saturated fraction depends on the increase in population of hydrocarbon degraders. The application of nutrients in the solutions enhanced the growth of the hydrocarbon degraders as was estimated by the MPN method in comparison with the control solution where no nutrients were added. Hydrocarbon degrader's population in NPK and ULR microcosms even though pretty low at the beginning of the experiment (10^2 cells/g dry sand) reached 10^5 cells/g dry sand within 15 days where most of the oil hydrocarbons were utilized.

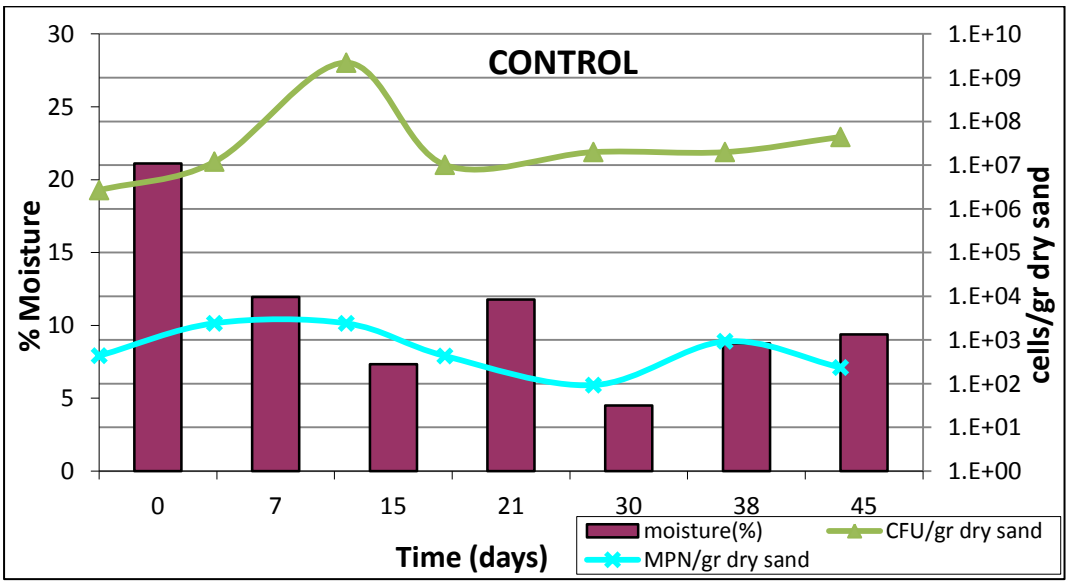


Figure 41: Moisture and microbial growth curve in Control treatment through 45 days of monitoring (Sand 1).

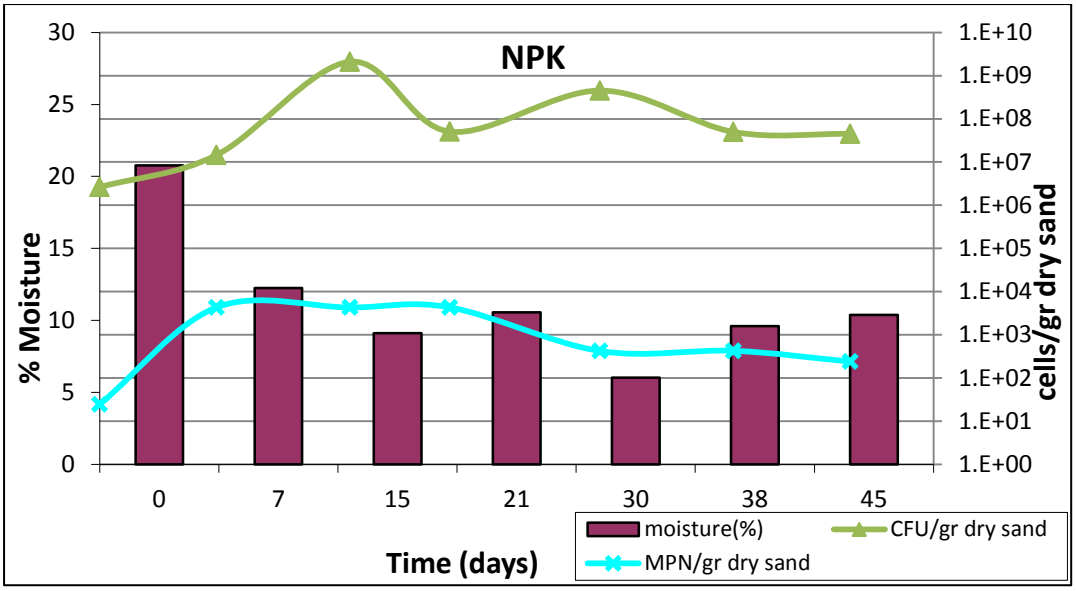


Figure 42: Moisture and microbial growth curve in NPK treatment through 45 days of monitoring (Sand 1).

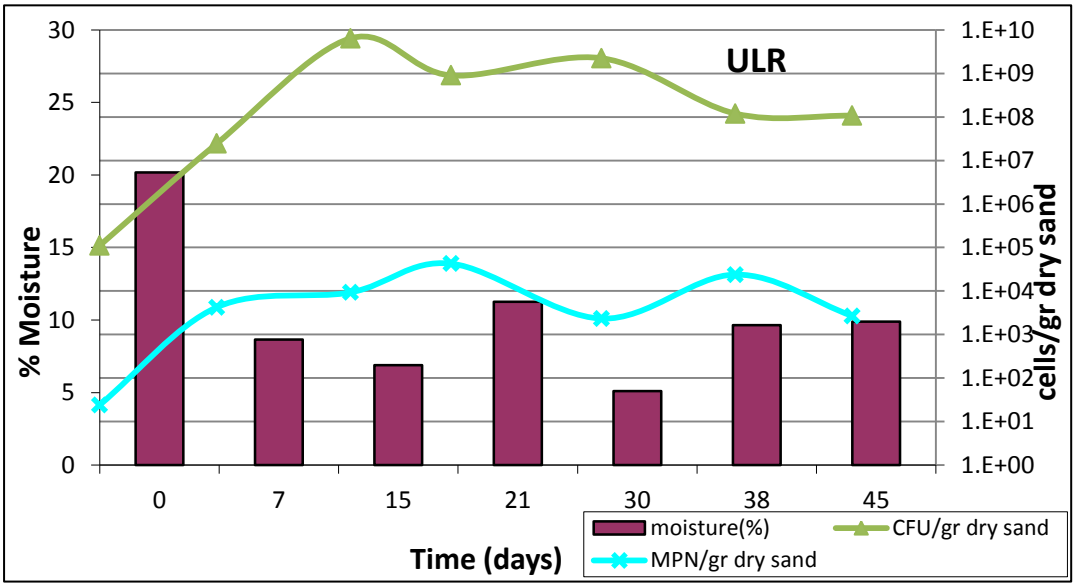


Figure 43: Moisture and microbial growth curve in ULR treatment through 45 days of monitoring (Sand 1).

Water content availability for microbial growth and metabolism can be rate limiting in hydrocarbons landfarming. Even though as reported by Calvo et al., 2009 the addition of surfactant to sandy soil can increase retention of soil moisture over longer time, moisture content in our case dropped below 10% through most of the time of the experiment, thus microbial activity was kept low and finally decreased after 30 days.
The application of nutrients and biosurfactants in the solutions enhanced the growth of the hydrocarbon degraders within 15 days as it was estimated by the MPN method in NPK and ULR treatments even though moisture content was pretty low.
A comparison of the n-alkanes and PAHs profiles (Figures 34-39 and 44) after oil application revealed that application of fertilizer plus biosurfactant can favour the degradation of crude oil in indigenous communities that lack essential nutrients.

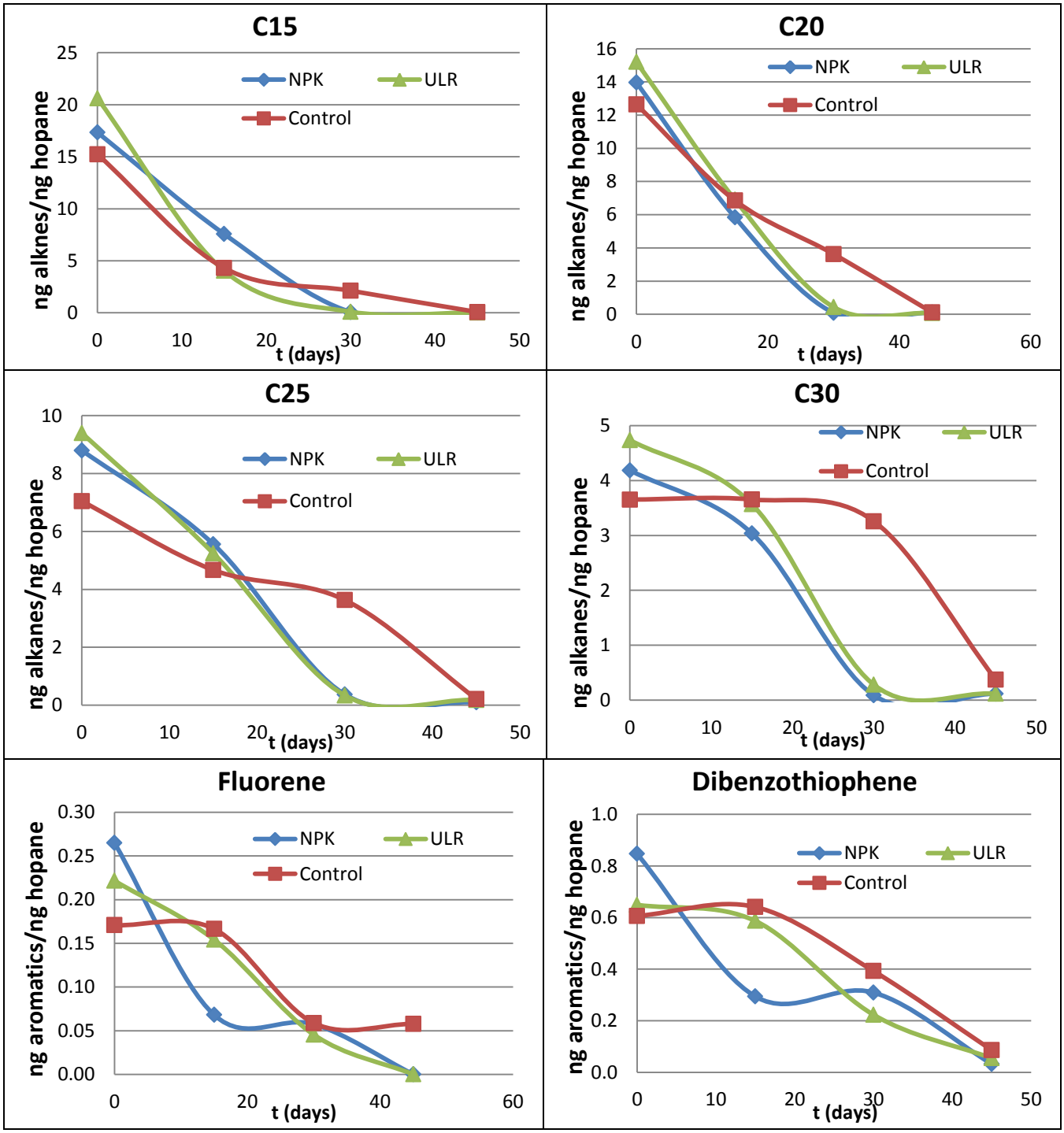


Figure 44: Concentration profiles of selected n-alkanes and PAHs compounds in Control, NPK and ULR treatments (Sand 1).

It can be concluded that in this study biosurfactants especially rhamnolipids (ULR) accelerated the biodegradation of n-alkanes by making them more available to microorganisms as expected but this was not the case for PAHs –NPK treatment with inorganic nutrients had responded better on PAHs depletion. There are numerous factors that could support the poor efficiency of ULR on PAHs degradation.

It is known the ability of biosurfactants to emulsify hydrocarbon – water mixtures and studies in liquid cultures have shown that uptake and assimilation of hydrocarbons is increased. When applied to soils, biosurfactants enhance water solubility of

hydrocarbons and thus increase the displacement of oily substances from soil particles. However, reports of biosurfactants effects on bioremediation in contaminated soils were inconsistent (Kosaric, 2001; Ron and Rosenberg, 2002; Calvo et al., 2009; Banat et al., 2010; Ron and Rosenberg, 2010; Ward, 2010).

Rhamnolipids efficiency in desorption of hydrocarbons has been subjected into many studies with diverse results, while the complex interactions taking place in soil between oil diverse hydrophobic molecules and different types of soil particulates makes difficult to interpret and characterize the roles of biosurfactants in oil bioremediation (Ward, 2010). As mentioned by Noordman et al., 2002 biodegradation process efficiency in soil and the specific mechanism of rhamnolipid's action highly depend on substrates physicochemical interactions within certain matrix. Regarding this, they showed that rhamnolipid and several other surfactants stimulated the degradation of hexadecane to a greater extent when it was entrapped in matrices with pore-sizes larger than 300 nm rather than in matrix with smaller pore-sizes or in sea sand (Noordman et al., 2002).

Chun-jiang et al., 2011 have showed that the variation of desorption of contaminants is also closely related with the soil DOM and the presence of more salt ions made phenanthrene more persistent on the solid phase and adversely affected its desorption from contaminated soil. Moreover many researchers support that long term bioremediation inefficiency of rhamnolipids is caused by the biodegradation of itself (Ward, 2010). On the other hand solubilization of high concentrations of PAH could be toxic to the soil microorganisms (Deschenes et al., 1996) as well as alter the composition of the microbial populations responsible for hydrocarbon mineralization.

As mentioned before soil hydrocarbon degradation may also be limited by the available water for microbial growth and metabolism.

Other studies have confirmed also that inorganic nutrients are more suitable to fine grained shorelines rather than to coarse-grained shorelines that lipophilic nutrients could be more efficient (Nikolopoulou and Kalogerakis, 2011). Inconsistent behaviour of ULR between the two oil fractions (alkanes-PAHs) compared to NPK treatment could support this conclusion although in previous studies lipophilic nutrients have effectively promoted oil degradation in liquid microcosms (Nikolopoulou et al., 2007; Nikolopoulou and Kalogerakis, 2008). Still overall ULR treatment performance suggests that the presence of biosurfactant could possibly have contributed to utilization of lipophilic nutrients by making them more available to soil microorganisms.

As has already been mentioned, bioavailability of hydrophobic compounds in contaminated soils is often the rate-limiting step in the process and the efficiency of biosurfactants or other rate limiting co-substrates mainly could be attributed to the interactions between target organic compounds, bacterial species and surfactants. Hence further investigation should be done in this regard and more possible combinations of different types of nutrients and biosurfactants on bioremediation of a variety of oil contaminated shorelines should be tested.

4.4. Landfarming of Oil Polluted Beach Sand through Autochthonous Bioaugmentation & Biostimulation (Sand 2)

Results and Discussion

Evaluation of the effectiveness of each treatment on crude oil biodegradation rate was estimated in terms of alkanes, PAHs and hydrocarbon degraders compositional changes through the whole period of the experiment. Figures 45-47 represent total depletion rate of the saturated fraction of *n*-alkanes (C_{14}-C_{35}) of control treatment (CM) as well as for treatments NPKM and ULRM at different time intervals of the experiment. Control had no significant effect on the degradation rate at the first 30 days of treatment which was accompanied by gradual decrease in population of hydrocarbon degraders (from 10^6 to 10^4 cells/g of dry sand- Figure 52).

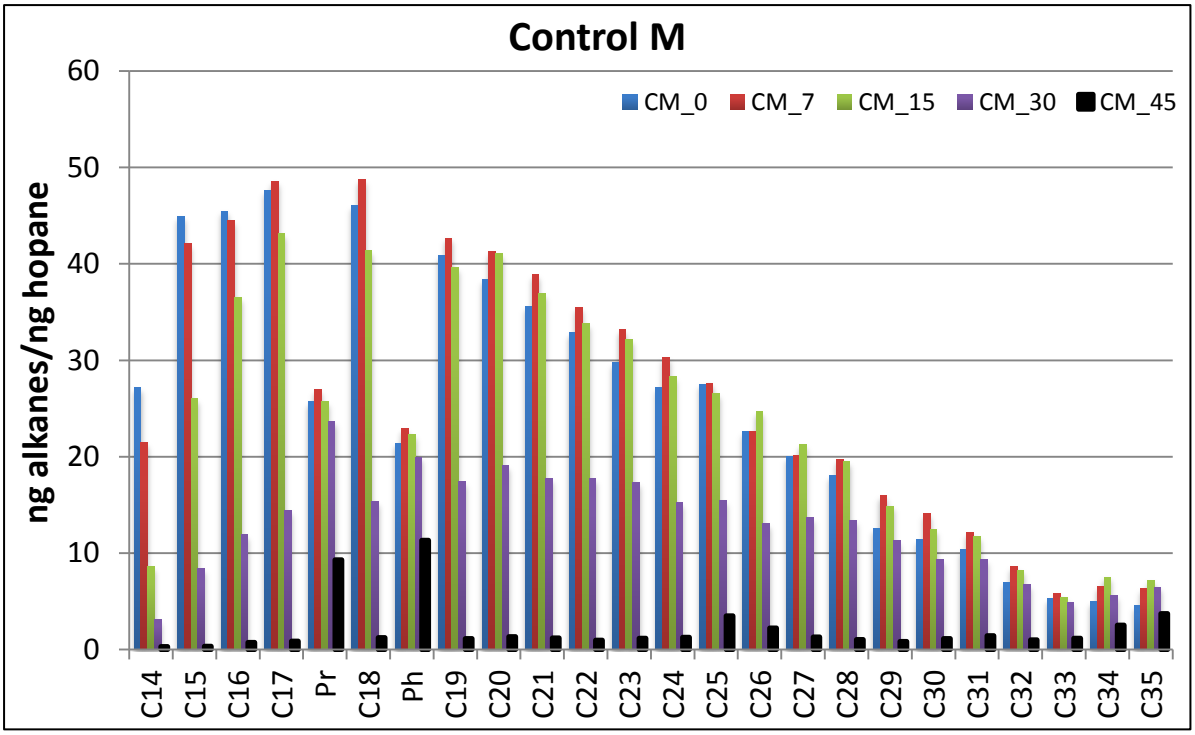


Figure 45: Concentration of C14-C35 n-alkanes after 0, 7, 15, 30 and 45 days of monitoring in Control treatment (Sand 2).

NPKM treatment with inorganic nutrients and ULRM treatment were more successful in terms of time and quantity of hydrocarbons depleted; about 85% and 94 % of n-alkanes were removed in 15 days of the experimental period respectively, whereas in control treatment depletion of n-alkanes reached 50% after 30 days of treatment. Moreover the degradation rate in both NPKM and ULRM amendments exceeded 97% within 30 days and 99% at the end of the experiment, whereas in control treatment depletion of n-alkanes reached 90% after 45 days of treatment.

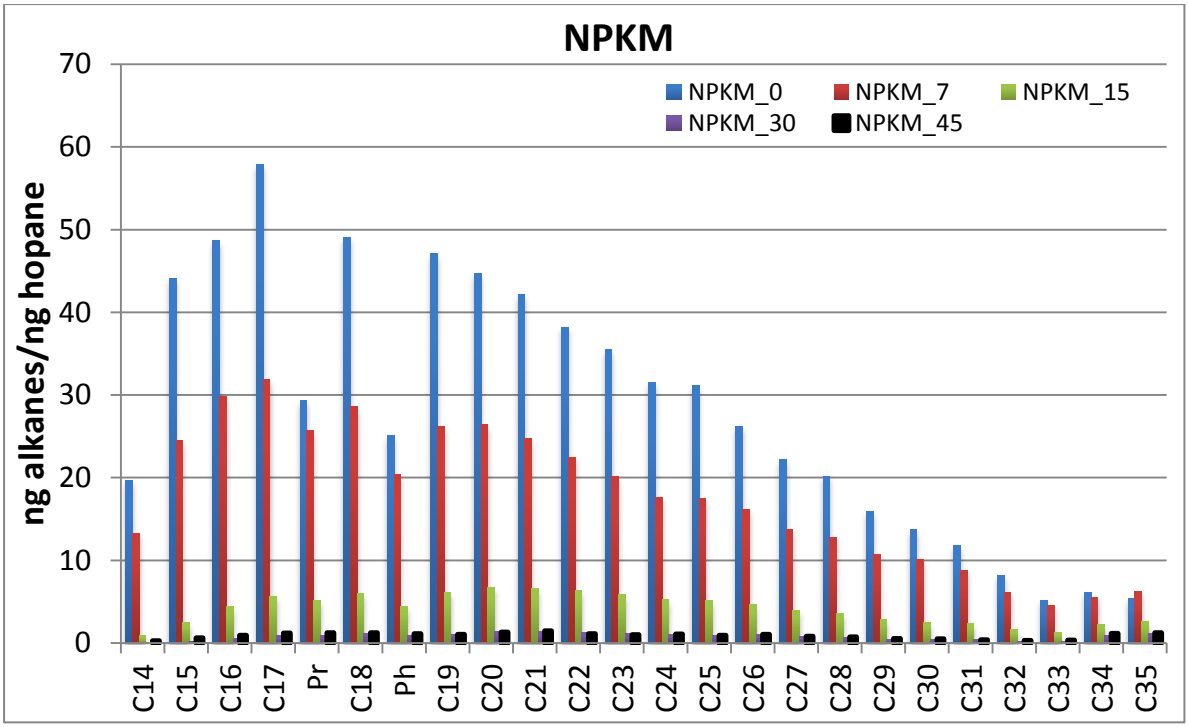


Figure 46: Concentration of C14-C35 n-alkanes after 0, 7, 15, 30 and 45 days of monitoring in NPK treatment (Sand 2).

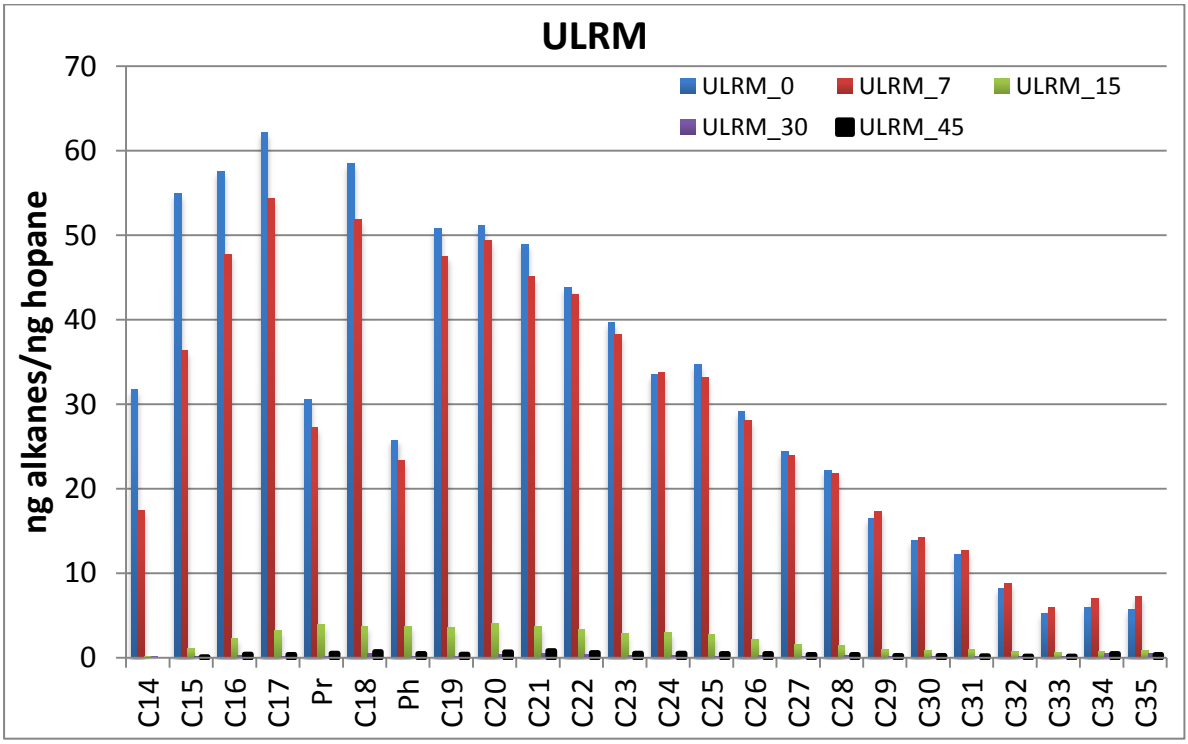


Figure 47: Concentration of C14-C35 n-alkanes after 0, 7, 15, 30 and 45 days of monitoring in ULR treatment (Sand 2).

Medium chain *n*-alkanes (C_{14} to C_{30}) as observed from Figures 46&47 in treatment NPKM (inorganic nutrients) have been decreased to a great extent within almost a week, whereas in treatment ULRM, (where Rhamnolipids were added) are depleted in an even higher rate as well as high chain alkanes (C_{31} to C_{35}) within 2 weeks of the experiment followed by NPKM treatment in efficiency. Most of the n-alkanes fraction was lost after 30 days for both treatments (NPKM-ULRM). On the contrary medium

to high chain alkanes (C_{25} to C_{35}) in the control (Figure 45) remain stable for the most time of the experiment and are only depleted after 45 days of the experiment. In addition past conservative biodegradation indexes like phytane and pristine are severely degraded in amendments NPKM-ULRM within 2 weeks compared to control (Figures 45-47).

Table 22: Specific growth and degradation rate of the selected alkanes (Sand 2)

Treatment	q_s (µg/cells h)						
	C_{15}	C_{20}	C_{25}	C_{30}	C_{35}	Pristane	Phytane
Control M	160.7	133.7	86.6	36.9	3	59.2	36.4
NPKM	1203.8	1096.1	751.9	324.3	82.2	700.9	598.3
ULRM	1252.1	1097.2	745.5	302.7	114.4	621	512.6

When comparing the treatments (Table 22), we can estimate that specific degradation rate for C_{15} in NPKM and ULRM treatments is 7 times higher than control from the first week of the experiment, while specific degradation rate for C_{20}, C_{25} and C_{30} is 8-9 times higher than control for both treatments within 2 weeks. Moreover specific degradation rate for C_{35} in treatments NPKM and ULRM is about 29 and 38.5 times higher than the control respectively. By the end of the experiment specific degradation rates for C_{15}, C_{20}, C_{25} and C_{30} is more or less equal for all treatments, however in the presence of specific nutrients (treatments NPKM and ULRM) specific degradation rates, as were estimated, are accelerated within 15 days. Branched alkanes like Pristane and Phytane are also degraded by 12 and 17 times higher in NPKM and by 10.5 and 14 times higher in ULRM treatments respectively when compared to control.

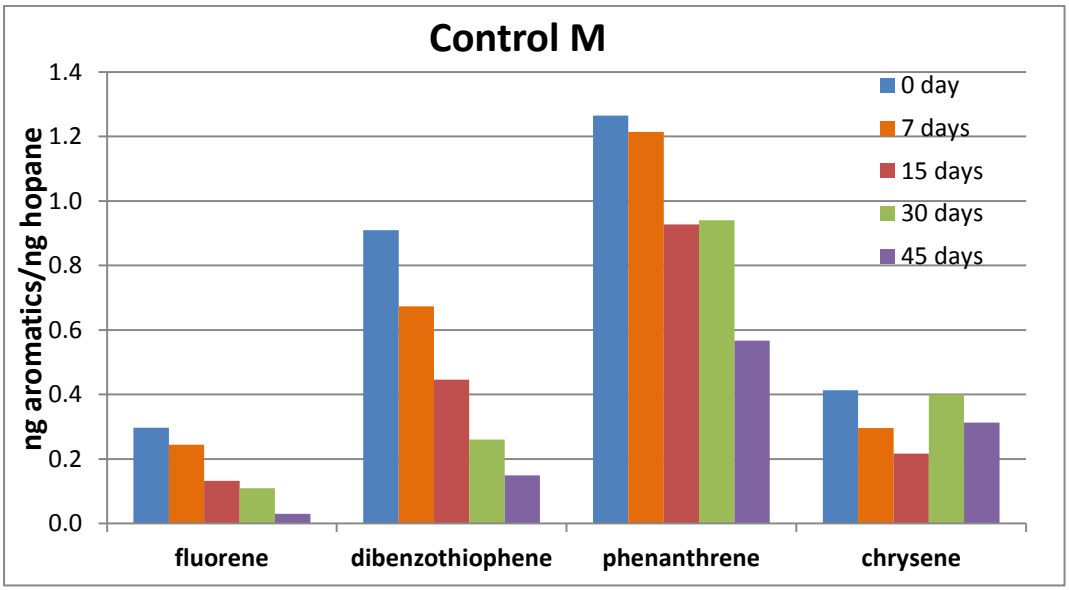


Figure 48: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 7, 15, 30 and 45 days of monitoring in Control M treatment (Sand 2).

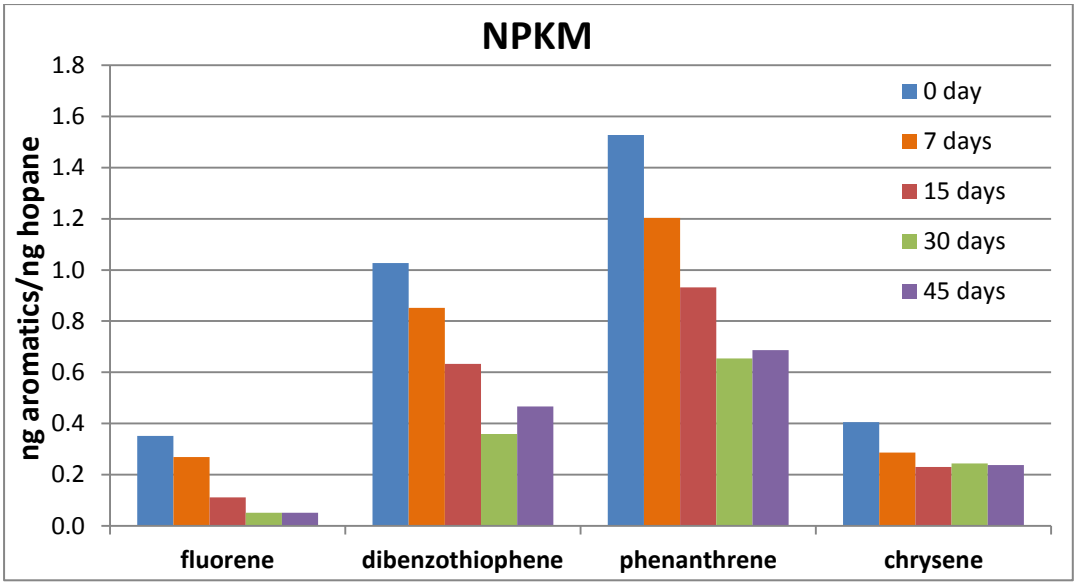


Figure 49: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 7, 15, 30 and 45 days of monitoring in NPKM treatment (Sand 2).

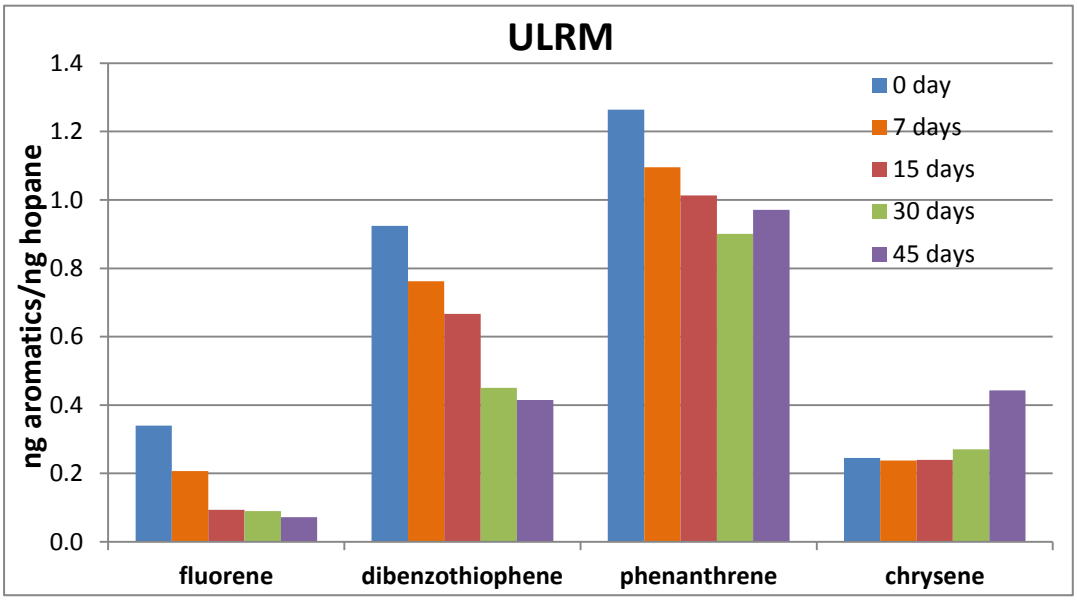


Figure 50: Concentration of fluorene, dibenzothiophene, phenanthrene and chrysene after 0, 7, 15, 30 and 45 days of monitoring in ULRM treatment (Sand 2).

The behaviour of treatments CM, NPKM and ULRM in terms of specific degradation rate for selected PAHs that are plotted in Figures 48-50 is different. Decreasing rate in low molecular weight PAHs (fluorene-dibenzothiothene- phenanthrene) is still low compared to n-alkanes and high molecular weight PAH (chrysene) remains practically stable. PAHs degradation in NPKM treatment reached for fluorene 85% and for dibenzothiothene- phenanthrene was 65% and 60% respectively for over 30 days. In this sense, estimated specific degradation rate in NPKM treatment for fluorene was 3 and 2 times higher than control and ULRM treatments respectively. Moreover specific degradation rate for phenanthrene was 3.3 and 4 times higher than control and ULRM treatments respectively, while dibenzothiothene was equally 2.3 times higher for both treatments (control and ULRM).

Table 23: Specific growth and degradation rate of the selected PAHs (Sand 2)

Treatment	Fluorene	Dibenzothiothene	Phenanthrene
Control M	0.96	2.74	2.51
NPKM	2.86	6.38	8.34
ULRM	1.52	2.89	2.22

However in ULRM treatment degradation rate was slower, reaching 75% for fluorene, only 50% for dibenzothiothene and almost 30% for phenanthrene, which remained at the same level by the end of the experiment for both treatments. Contrary to above treatments, depletion in the control for fluorene was above 60% and for dibenzothiothene- phenanthrene was 70% and almost 30% respectively after 30 days which by the end of the experiment reached 90% for fluorene, 83% for dibenzothiothene and 55% for phenanthrene.

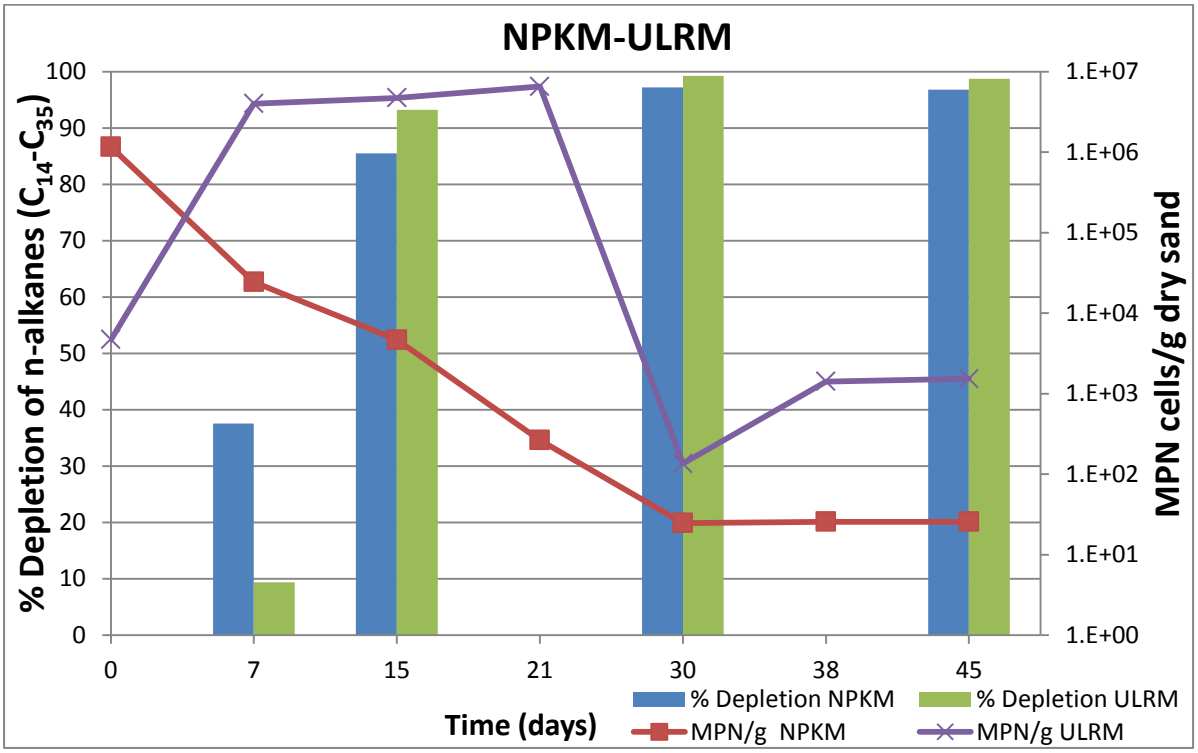


Figure 51: Alkanes % depletion and microbial growth curve between different treatments (NPKM and ULRM) through 45 days of monitoring (Sand 2).

Comparison of the removal of the saturated fraction and the microbial growth in ULRM treatment (Figure 51) suggests that the removal of the saturated fraction depends on the increase in population of hydrocarbon degraders when water content is not rate limiting. Marine heterotrophs remained relatively stable throughout the whole duration of the experiment in the range of 10^8-10^9 cfus/g dry sand for all treatments. However this was not the case for NPKM where utilization of lighter and more accessible hydrocarbons just kept alive hydrocarbon degraders since water content in the sand was pretty low and dropped to below 5% after 20 days (Figure 53), after

which population of hydrocarbon degraders decreased (from 10^6 to 10 cells/g of dry sand) radically as was estimated by the MPN method.

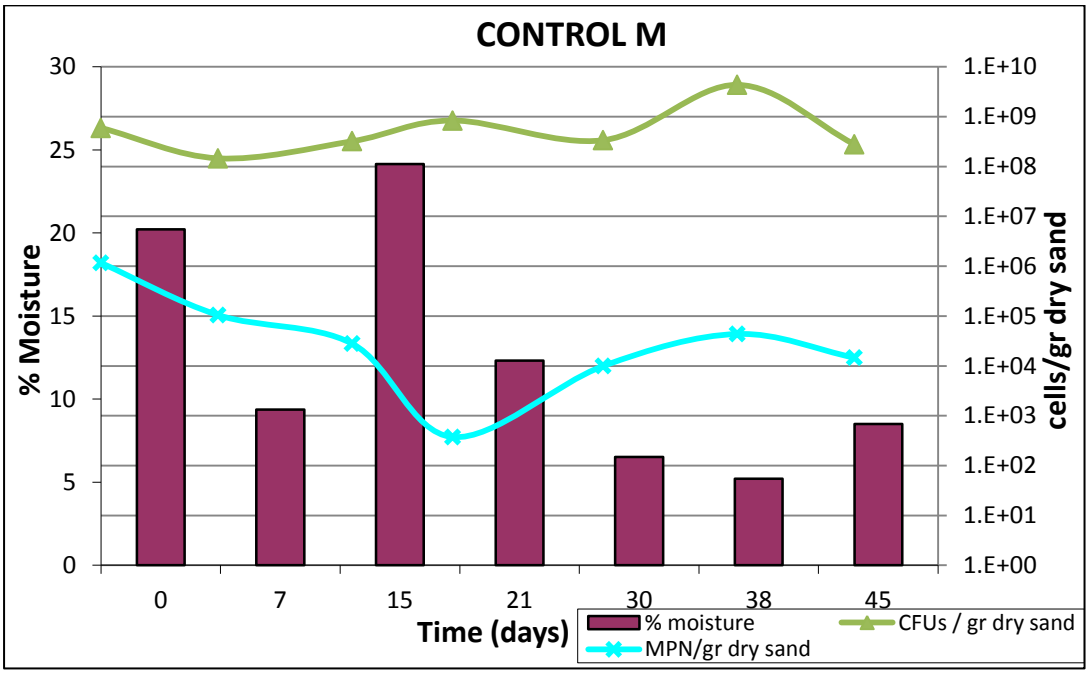


Figure 52: Moisture and microbial growth curve in Control M treatment through 45 days of monitoring (Sand 2).

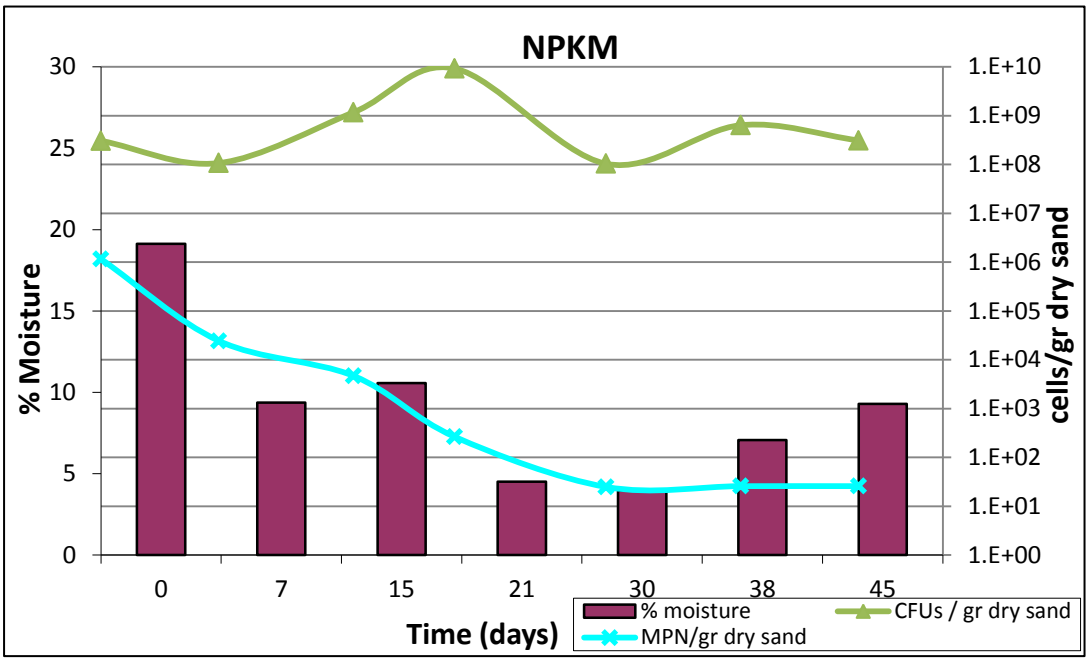


Figure 53: Moisture and microbial growth curve in NPKM treatment through 45 days of monitoring (Sand 2).

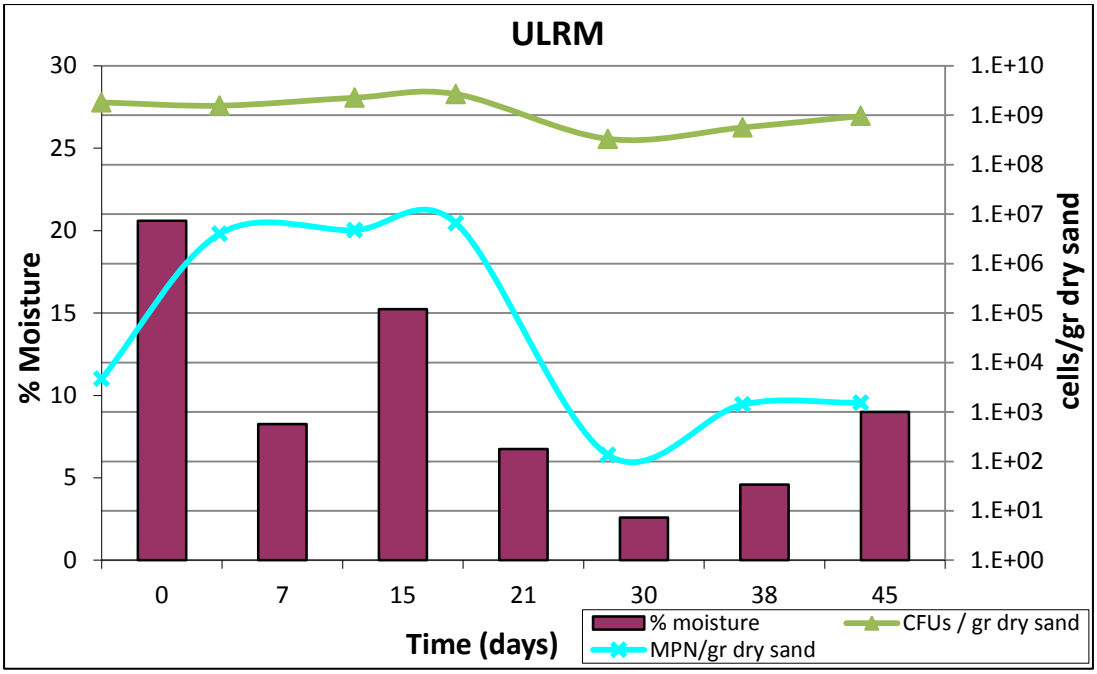


Figure 54: Moisture and microbial growth curve in ULRM treatment through 45 days of monitoring (Sand 2).

Hydrocarbon degraders' population profile between all treatments follows moisture pattern (Figures 52-54). Hydrocarbon degraders were sustained for longer in ULRM treatment especially because as reported by Calvo et al., 2009 the addition of surfactant to sandy soil can increase retention of soil moisture over longer time. In this situation moisture content dropped below 5% after 30 days and affected consortium survival which led to a radical decrease in the population of hydrocarbon degraders (10^7 to 10^2 cells /g of dry sand), however later moisture content was raised above 10% but microbial population couldn't recover (it was increased only to 10^3 cells /g of dry sand) by the end of the experiment. Moisture was reserved for longer time above 10% in control treatment which had a better response on hydrocarbons degraders population and even when only dropped for a while in the middle of the process (20 days), population even in the end remained in the range of 10^4 cells /g of dry sand. The results support that microbial growth and activity are consistent with moisture content in soils and can be rate limiting in hydrocarbons landfarming.

As already has been mentioned biodegradation index of hydrocarbons present in contaminated soils is a function of their aqueous solubility and increases when aqueous solubility increases, i.e. when ring number decreases (Deschenes et al., 1996). Water content in soil also influences solubility of adsorbed hydrocarbons from the soil matter to the bulk liquid and further their availability to soil microorganisms. In the case of NPKM and ULRM treatments soil moisture was the critical parameter that inhibited hydrocarbon degraders growth and succession of alkane degradation by more recalcitrant hydrocarbons like PAHs after 2 weeks of treatment, where most of the n-alkanes were completely depleted. However control treatment that moisture was retained to an accepted level surprisingly exhibited a better performance the last 2 weeks of the experiment in terms of n-alkanes and PAHs consumption, which shows the high potential and dynamic of the added consortium to degrade even more recalcitrant compounds like PAHs.

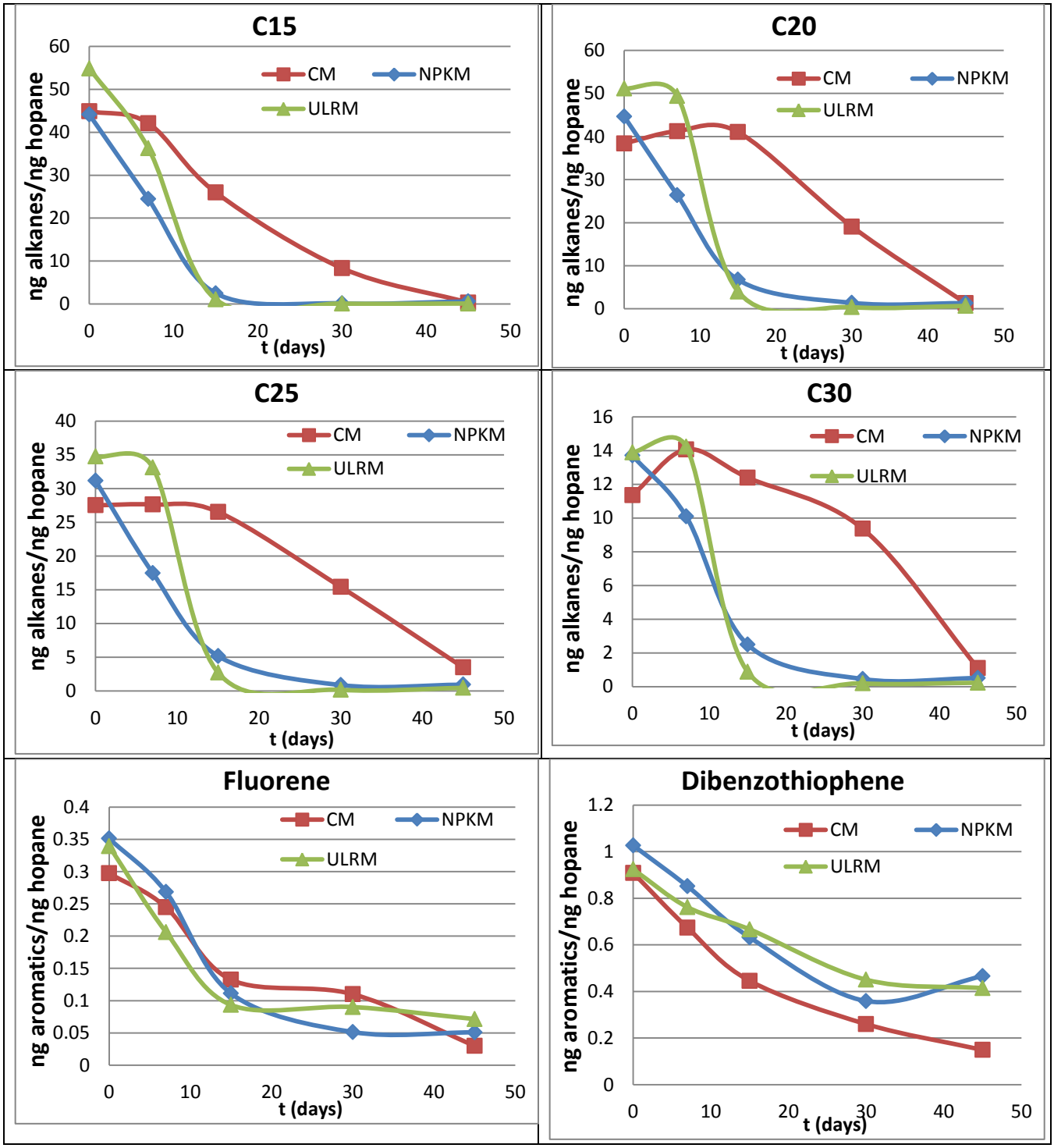


Figure 55: Concentration profiles of selected n-alkanes and PAHs compounds in Control M, NPKM and ULRM treatments (Sand 2).

In this sense specific degradation rate of PAHs is much slower when compared to n-alkanes obviously (Table 23) and follows the same pattern as mentioned before, i.e. C15> C20> (Pristane, Phytane)>C25 > C30> C35 >(PAHs).

The comparison of the n-alkanes and PAHs profiles (Figures 45-50 and 55) after oil application revealed that application of fertilizer plus biosurfactant can favour the degradation of crude oil by the adapted indigenous consortium that lacks essential nutrients.

Despite the known ability of biosurfactants to emulsify hydrocarbon – water mixtures which when applied specifically to soils enhance water solubility of hydrocarbons and increase the displacement of oily substances from soil particles, reports of biosurfactants effects on bioremediation in contaminated soils were inconsistent (Kosaric, 2001; Ron and Rosenberg, 2002; Calvo et al., 2009; Banat et al., 2010; Ron and Rosenberg, 2010; Ward, 2010).

It can be concluded that in this study biosurfactants especially rhamnolipids (ULRM) accelerated the biodegradation of n-alkanes by making them more available to microorganisms as expected but this was not the case for PAHs even though partially could be attributed to the low water content of soil as described above –still NPKM treatment with inorganic nutrients had responded better on PAHs depletion. There are numerous factors that could support the pour efficiency of ULRM on PAHs degradation.

Rhamnolipid's efficiency on biodegradation process depends on the pore size of the matrix, the smaller the pore size the less efficient rhamnolipids will be (Noordman et al., 2002). Furthermore poor long term bioremediation efficiency of rhamnolipids (biosurfactant) is caused by the biodegradation of itself (Ward, 2010), While solubilization of high concentrations of PAH could be toxic to the soil microorganisms (Deschenes et al., 1996) as well as affecting the distribution and the composition of the hydrocarbon degraders populations.

Moreover as has been reported lipohlilic nutrients were found to be more effective in to coarse grained shorelines than in fine grained shorelines due to the difficulty in penetration for the lipohlilic nutrients in fine grained shorelines. By contrast, inorganic nutrients are recommended for hydrocarbon biodegradation into fine grained shorelines (Nikolopoulou and Kalogerakis, 2011). Accordingly inconsistent behaviour of ULRM amendment between the two fractions (alkanes-PAHs) of oil could have also be influenced by the addition of lipophilic nutrients, despite the fact that previous studies in liquid micocosms proved that lipophlic nutrients have effectively contributed in oil degradation (Nikolopoulou et al., 2007; Nikolopoulou and Kalogerakis, 2008).

On the other hand overall ULRM treatment performance suggests that utilization of lipophilic nutrients could possibly have been favoured by the presence of biosurfactant which increased their bioavailability to soil microorganisms. Rhamnolipids efficiency in desorption of hydrocarbons from soil matrix is imposed to complex interactions existing in soil between oil diverse hydrophobic molecules and various types of soil particulates (Ward, 2010).

As has been mentioned bioavailability of hydrophobic compounds in contaminated soils is often the rate-limiting step in the process and the efficiency of biosurfactants or other rate limiting cosubstrates mainly could be attributed to the interactions between target organic compounds, bacterial species and surfactants. Hence further investigation should be done in this regard and more possible combinations of different types of nutrients and/or biosurfactants on bioremediation of a variety of oil contaminated shorelines should be tested, since elsewhere it was found that biosurfactants alone are capable of promoting biodegradation to a large extent without added fertilizers (Thavasi et al., 2011).

This work denotes that inoculation only with autochthonous hydrocarbon degraders without any additional nutrients is not an effective treatment, however when the needed nutrients or other biostimulants are supplemented the advantages of such combination are obvious and result in accelerated hydrocarbon consumption by the added autochthonous consortium. Thus the combination of autochthonous

bioaugmentation with biostimulation could be really beneficial in this regard, however further tests regarding new formulations application to a variety of different type of shorelines should be carried out, in order to establish the parameters that could enhance oil bioremediation in particular marine environments and thus lead to a detailed contingency plan to high risk areas.

Nonetheless combination of autochthonous bioaugmentation with biostimulation is still a very promising strategy that could speed up the natural biodegradation process as long as crucial parameters for microbial sustainability are well monitored and controlled.

4.5. Cluster analysis

Much chemistry involves using data to determine patterns. For example can a chromatograph be used to decide on the origin of a treatment and if so, what main features in the chromatograph distinguish different treatments?
Exploratory data analysis techniques are often quite helpful in elucidating the complex nature of multivariate relationships and determine general relationships between data. Sometimes more complex questions need to be answered, such as, do the samples fall into groups? Cluster analysis is a well established approach that was developed to determine similarities between samples.
A major problem in cluster analysis is defining a cluster. There is no measure of cluster validity that can serve as a reliable indicator of the quality of a proposed partitioning of the data. Clusters are defined intuitively, depending on the context of the problem, and not mathematically, which limits their utility. Therefore, prior knowledge about the problem is essential when using these methods. Because the threshold value for similarity is developed directly from the data, criteria for similarity are often subjective and depend to a large degree on the nature of the problem investigated, the goals of the study, the number of clusters in the data sought, and previous experience.
Cluster analysis is based on the principle that distances between pairs of points (i.e., samples) in the measurement space are inversely related to their degree of similarity. Although several different types of clustering algorithms exist, by far the most popular is hierarchical clustering, which is the focus here. The starting point for a hierarchical clustering experiment is the similarity matrix, which is formed by first computing the distances between all pairs of points in the data set. Each distance is then converted into a similarity value

$$s_{ik} = 1 - \frac{d_{ik}}{d_{\max}} \qquad (10)$$

Where S_{ik} is the measure of similarity between samples i and k, d_{ik} is the Euclidean distance between samples i and k, and d_{max} is the distance between the two most dissimilar samples, which is also the largest distance in the data set. The smaller is the value the more similar are the samples.
The next step is to link the objects. The most common approach is called agglomerative clustering whereby single objects are gradually connected to each other in groups. There is a variety of ways to compute distances between data points and clusters in hierarchical clustering such as, single-linkage, complete-linkage and average linkage method. In this analysis we used the average linkage method which assesses similarity by computing the distance between all pairs of points where a member of each pair belongs to the cluster, with the average of these distances being a measure of similarity between a cluster and a data point.
The result of this procedure is a diagram called a dendogram, which is a visual representation of the relationships between samples in the data set. Interpretation of the results is intuitive, which is the major reason for the popularity of these methods (Davidson and Lavine, 2006).

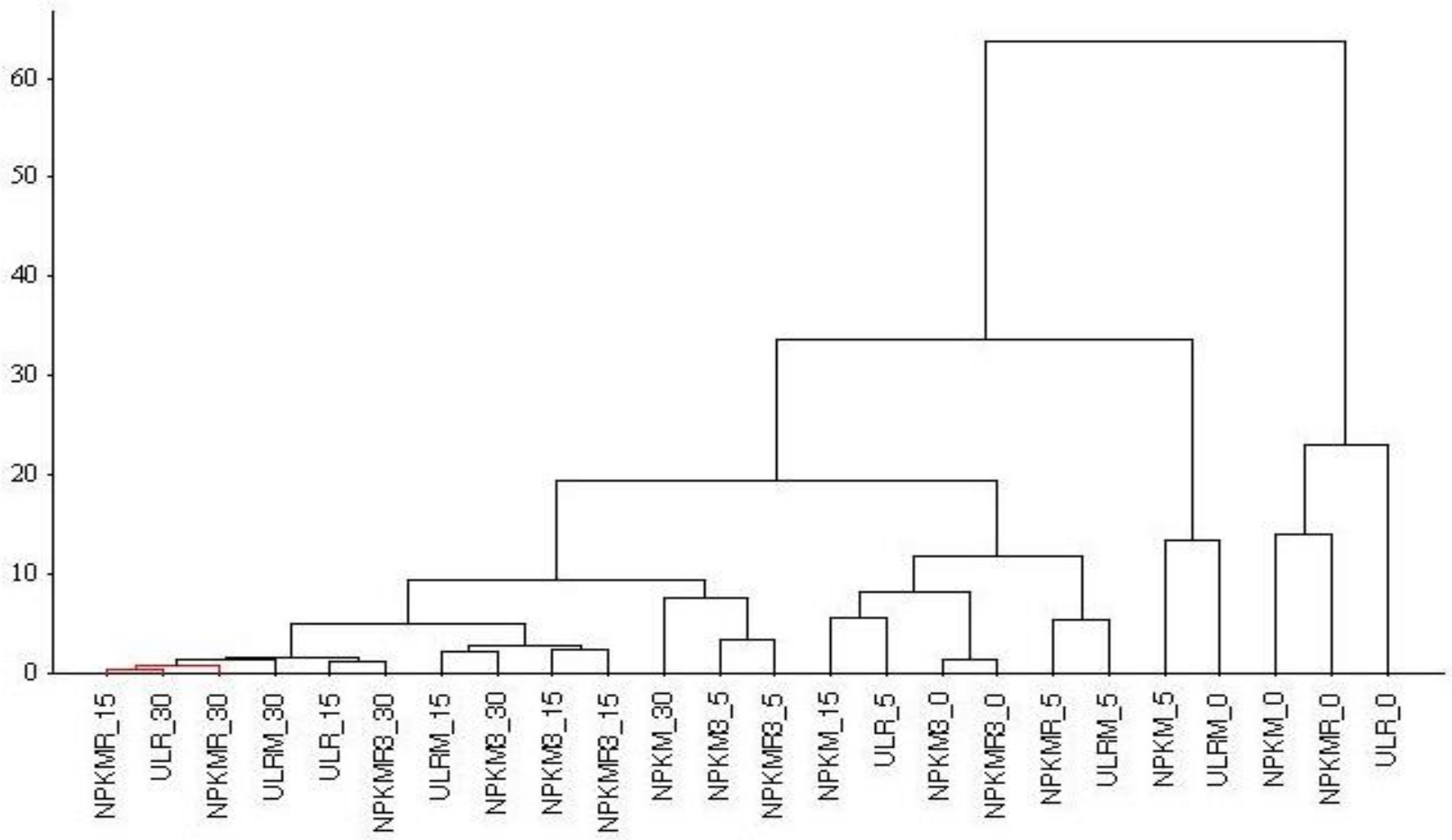


Figure 56: Dendogram developed from gas chromatograms of the two seawater experimental data sets.

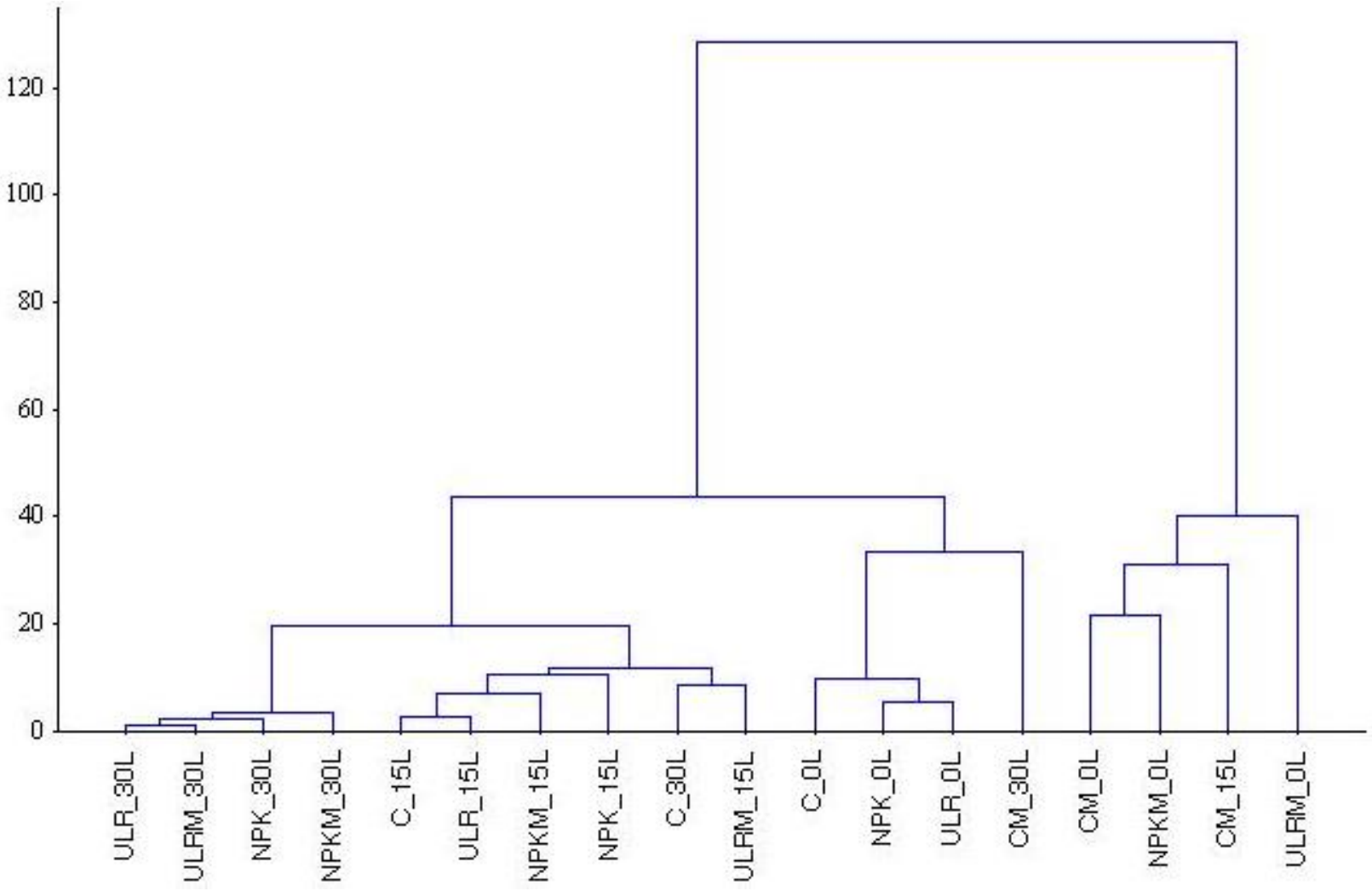


Figure 57: Dendogram developed from gas chromatograms of the two Sand experimental data sets.

Cluster analysis has been used to group samples from the two Seawater experimental data sets and from the two Sand data sets computing Euclidean distance and average linkage. In order to establish a common comparison base in terms of mutual time sampling periods, data of n-alkanes (C14-C35) concentration profiles up to 30 days of treatment have been used for all experimental data sets. The dendogram shown in Figure 56 suggests that gas chromatographs from Seawater experimental data set can be divided into 4 main groups (Cluster 1: NPKMR_15, ULR_30, NPKMR_30, ULRM_30, ULR_15, NPKMR3_30; cluster 2: ULRM_15, NPKM3_30, NPKM3_15, NPKMR3_15; cluster 3: NPKM_30, NPKM3_5, NPKMR3_5; cluster 4: NPKM_15, ULR_5, NPKM3_0, NPKMR3_0, NPKMR_5, ULRM_5). Moreover dendogram shown in Figure 57 indicates that gas chromatographs from Sand experimental data set can be divided also into 4 main groups (Cluster 1:, ULR_30L, ULRM_30L, NPK_30L, NPKM_30L; cluster 2: C_15L, ULR_15L, NPKM_15L, NPK_15L, C_30L, ULRM_15L; cluster 3: C_0L, NPK_0L, ULR_0L, CM_30L; cluster 4: CM_0L, NPKM_0L, CM_15L, ULRM_0L).

Cluster 1 from both data sets (Seawater & Sand) is the visualization of the best performance treatments expressed in biodegradation index which have been already shown. However it should be noted that any differences in the pattern of dendogram which doesn't follows overall degradation rate between treatments could be attributed to the different initial composition (type of crude oil) and concentration of crude oil used between the experimental data sets. Nonetheless it is clear those treatments that exhibited more or less same performance in terms of degradation index can be grouped and grouping follows chromatograms concentration profile.

Furthermore changes in profile concentrations of selected compounds (n-alkanes C14-C35) in the chromatograms due to different treatments applied could be only examined into data sets that contained the same type (composition) of crude oil. Thus two groups of data sets depending on the type of crude oil used have been recognized and these are:

Group A→ Seawater 1 experimental data set and Sand 1 experimental data set and

Group B→ Seawater 2 experimental data set and Sand 2 experimental data set

Weighted concentrations (to their sum) have been used to overcome differences arising from the amount of the initial concentration used in each experimental set.

Insight on the behavior of crude oil components due to different treatments applied was investigated and dendogram of Goup A experimental data sets (Figure 58) revealed 4 main clusters (Cluster 1: NPKMR_15, ULR_30, NPKMR_30, ULR_15; cluster 2: C_30L, NPKM_30, ULRM_15, ULRM_30, NPK_30L, ULR_30L; cluster 3: C_15L, ULR_15L, NPKM_15L; cluster 4: C_0L, NPK_0L, ULR_0L, ULR_0, NPK_15L NPKM_0, NPKMR_0, ULRM_0, NPKM_5, NPKMR_5, ULRM_5).

Each cluster represents different distribution of oil components (concentration profile) which in other words mean different response in terms of oil components depletion to various amendments. Cluster 1 represents the profile of oil light, heavy and branched hydrocarbons (pristine-phytane) which have been severely depleted in NPKMR and ULR treatments after 15 days. Cluster 2 represents the profile of oil components in all three treatments of Sand 1 experimental data set (Control, NPK &ULR) after 30 days

and also for NPKM and ULRM treatments of Seawater 2 experimental set after 30 and 15 days respectively. Particularly in this cluster light and heavy hydrocarbons of crude oil are also depleted but to a lesser extent compared to cluster 1, on the other hand branched hydrocarbons (pristine-phytane) are not depleted at the same level as the rest hydrocarbons. Moreover in cluster 3 treatments of control and ULR from Sand 1 experimental data set and treatment NPKM from Seawater 2experimental set exhibit the same trend after 15 days, light to medium chain hydrocarbon are depleted to some point compared to cluster 4 which comprises mostly by treatments of starting day 0 for both experimental sets (Sand 1 and Seawater 2). In addition branched alkanes are depleted to even lesser extent compared to treatments of cluster 1 and heavy chain hydrocarbons remain stable. In the same manner but to lesser extent depletion of oil hydrocarbons somewhat which have been severely depleted in NPKMR and ULR treatments after 15 days.

In the same manner behavior of crude oil components among different amendments was investigated and dendogram of Goup B experimental data sets (Figure 59) revealed 3 main clusters (Cluster 1: CM_0L, NPKM_0L, CM_15L, ULRM_0L, NPKM3_0, NPKMR3_0, NPKM3_5; cluster 2: CM_30L, NPKM_15L, ULRM_15L, NPKM_30L, ULRM_30L; cluster 3: NPKM3_30, NPKM3_15, NPKMR3_5, NPKMR3_15, NPKMR3_30). Each cluster demonstrates a different oil concentration pattern (concentration profile) that corresponds to different response in terms of oil components depletion of each group of amendments. Cluster 1 represents the profile of oil initial concentration which consequently is the same for both Seawater 1 and Sand 2 experimental set, however classification in cluster 2 and 3 matches samples according to the matrix in which amendments have been applied, i.e. cluster 2 for solid matrix (Sand 2) and cluster 3 for liquid matrix (Seawater 1). Nonetheless this denotes that in each matrix concentrations profile of oil components follow different patterns. Specifically as has also been verified by previous examination through biodegradation kinetics in cluster 2 long chain alkanes (above C25) and branched alkanes (pristine-phytane) are severely depleted compared to cluster 3 in which instead, long chain alkanes and branched alkanes remain practically stable or are slowly depleted (NPKMR3) [*].

[*] Number 3 at the end of each treatment name has been used to distinguish samples with same titles that come from different experimental data set, so treatments that include 3 come from 2nd seawater experimental data set. Letter L stands for Landfarming and has been used to distinguish common treatments titles between sand data sets and seawater data sets. The number after the underscore symbol (_) though implies the treatment days of each sample.

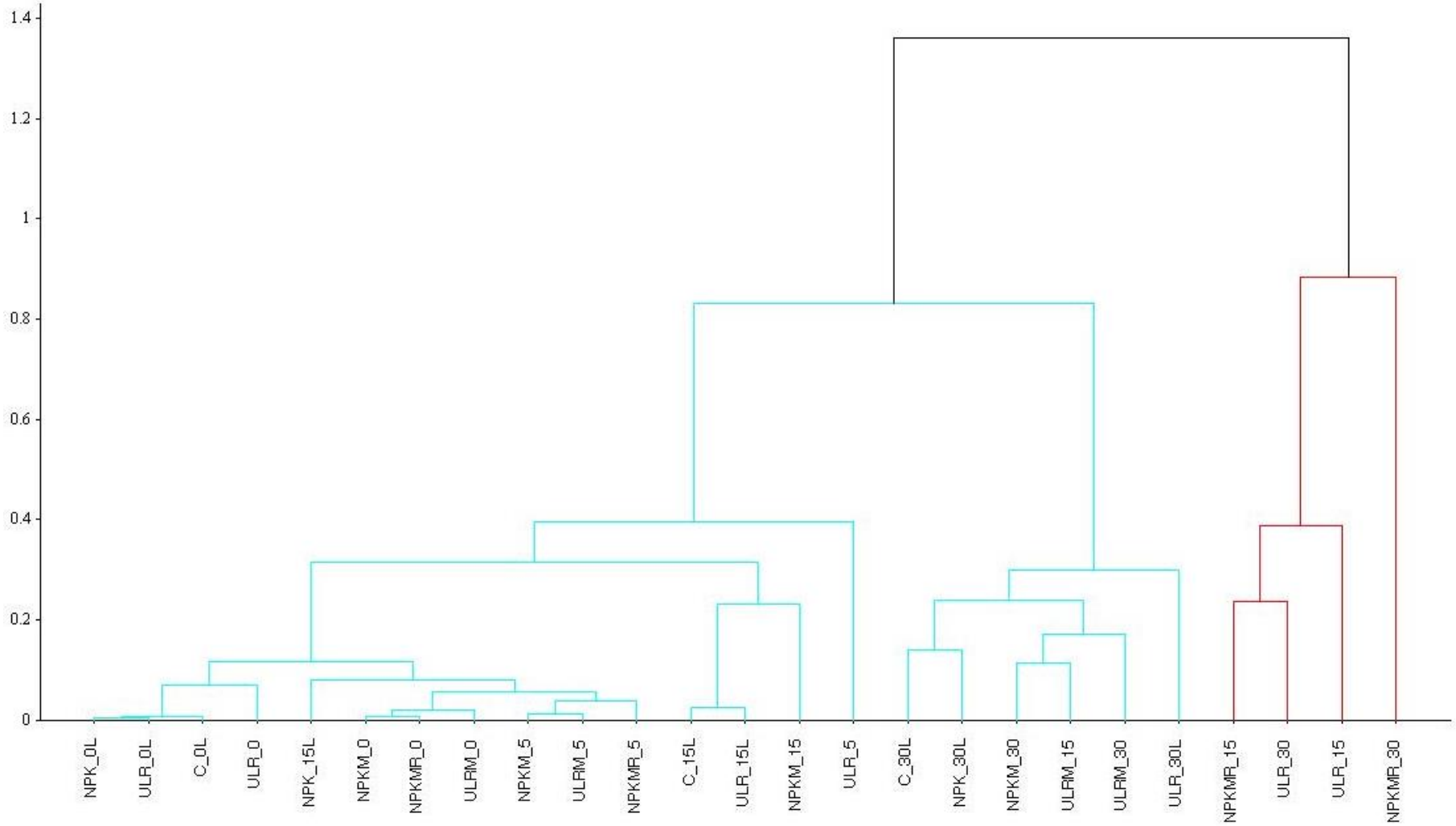


Figure 58: Dendogram developed from gas chromatograms of Seawater 1 and Sand 1 experimental data sets.

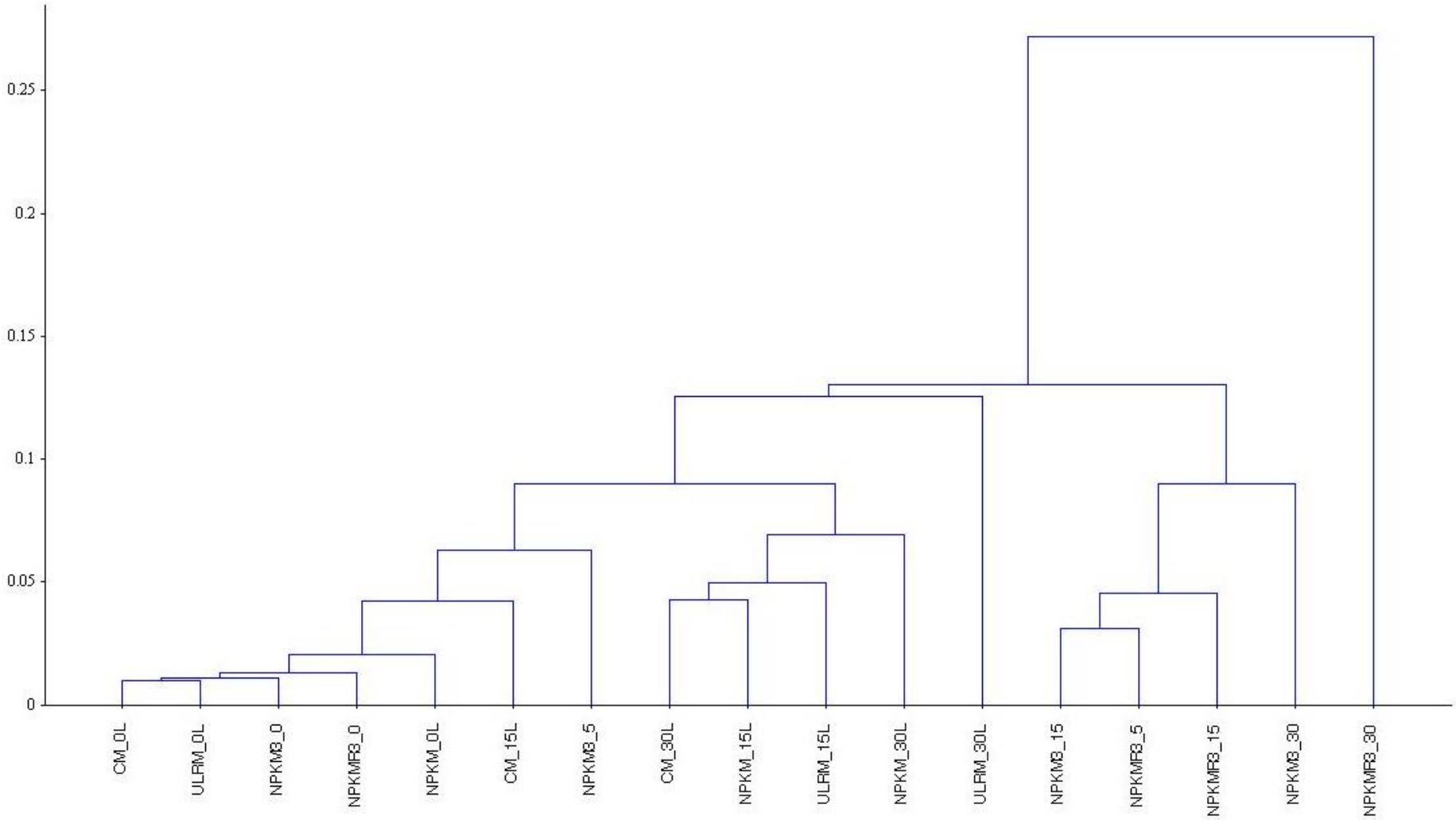


Figure 59: Dendogram developed from gas chromatograms of Seawater 2 and Sand 2 experimental data sets.

4.6. Biofilm Investigation on Oil Droplets&C20

Results and Discussion

Preliminary tests of the tested consortia

Preliminary tests were conducted on consortia Eb8 and E4 (samples taken from Elefsina Bay -Attica region near the Hellenic Petroleum Refinery; a site exposed to chronic crude oil pollution), that were enriched in 100ml ONR7 medium with 607μl crude oil and consequently there development within 10 days was monitored with CLSM. The response of consortia was surprisingly at the beginning (2nd day) very promising with bacteria covering dispersed oil droplets (Figure 60A) which evolved further after a week of incubation (Figures 60B&C). Analysis of the samples with consortium Eb8 showed scattered small oil droplets forming clusters with bacteria of grape, star style shapes with strings of cells and oil droplets, probably due to heavy degradation. Strings are composed by larger and also fine oil droplets connected to bacteria, probably due to EPS bridging between the bacteria (Figures 60D&E).

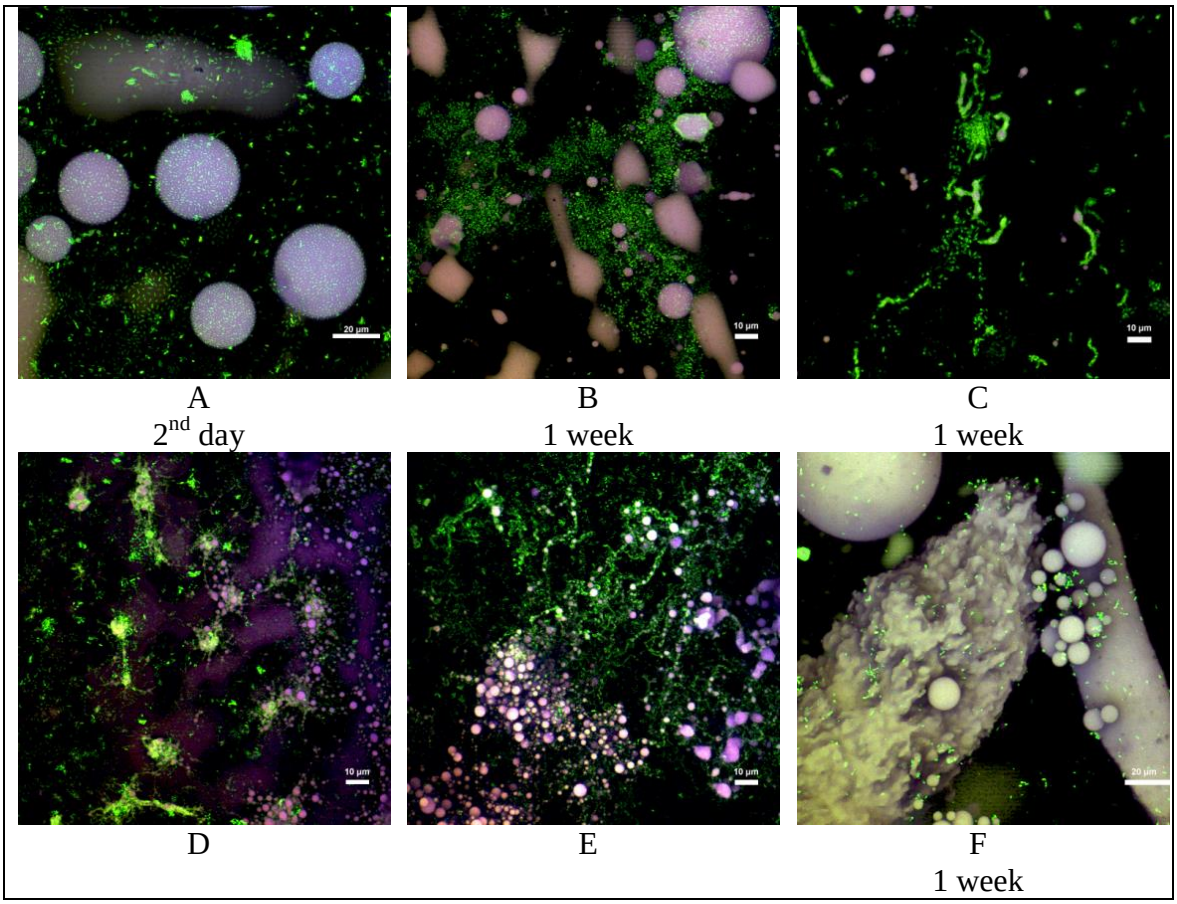


Figure 60: Structures formed between oil droplets and bacteria from consortia Eb8 (A-E) & S (F). Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

Consortium from the pristine area also adapted well and formed various size of oil droplets covered with the adapted indigenous seawater bacteria. Also as detected (Figure 60F) there were interesting spots of spherical, dissolved like a sponge roll shapes but not

visibly covered with many bacteria but hypothesis of bacteria in between the sponge like figure. Possibly oil droplets were connected to each other with bacteria forming a biggest sponge or roll like shape with bacteria in between. To a lesser extend compared to consortium Eb8, grape like shapes formed between various sizes of oil droplets and bacteria are also observed after 10 days of incubation.
This unexpected fast response of consortia on oil contamination intrigued us to further examine their behavior under different conditions as described in Material and Methods section, which are as follows:

- *Consortia Eb8 and E4 with Rha ±NP (Nutrients)*

Unfortunately in treatments with Rha with or without the nutrients we couldn't capture the clustering effect between the bacteria and oil droplets since the effect of Rhamnolipids was immediate and enhanced by the already observed degrading capability of the consortia (Eb8-E4) in even earlier stage of 5 days – 1 week. Oil is dispersed into fine tiny droplets (<10μm) floating around within 1 week and sample seemed completely disintegrated into fine tiny oil droplets although bacteria weren't obviously present or their signal was too weak implying that had already passed to death phase and even addition of nutrients wasn't adequate since the critical point of getting to death phase has been exceeded. Evidence of the clustering effect around oil droplets it is been shown in the following graphs (Figure 61) that include some of the remains which, in parts of the sample dispersed oil droplets produced interesting grape like shapes (microcolonies of bacteria in connection with oil droplets). However the signal from oil is too strong covering the weak signal of bacteria and thus it could be assumed that possibly aggregates formed could have been there from previous time however it is not clear if the bacteria surrounding them are still alive. Data from previous studies verify the above observations since in tests with the consortium Eb8 on oil degradation that have been conducted in our lab showed that most of the saturated fraction of crude oil is depleted within the first week (Section 4.2).

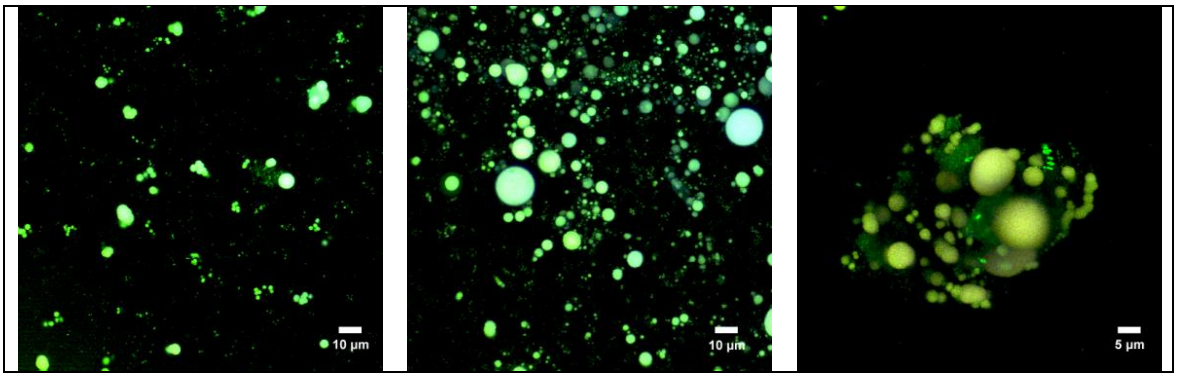


Figure 61: Rhamnolipids effects on the interaction between oil droplets and bacteria from consortia Eb8 & E4. Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections

- *Consortia Eb8 and E4 with Corexit ±NP (Nutrients)*

In the case of corexit at the first 5-7 days (Figures 62A, B&D), samples of both consortia analyzed showed that there are not so many bacteria which are scattered in the medium and even fewer colonizing oil droplets due to possible toxicity from the dispersant-population not so high
However in the case of consortium Eb8 (Figures 62E&F) oil is emulsified and even though this is not representative of the whole sample there are some sponge like shapes of emulsified oil which are covered at the edge by some bacteria. On the contrary in

consortium E4 there are some isolated spots of very small microcolonies and aggregates but most bacteria are free. More bacteria in microcolonies rather colonizing oil droplets (Figure 62C).

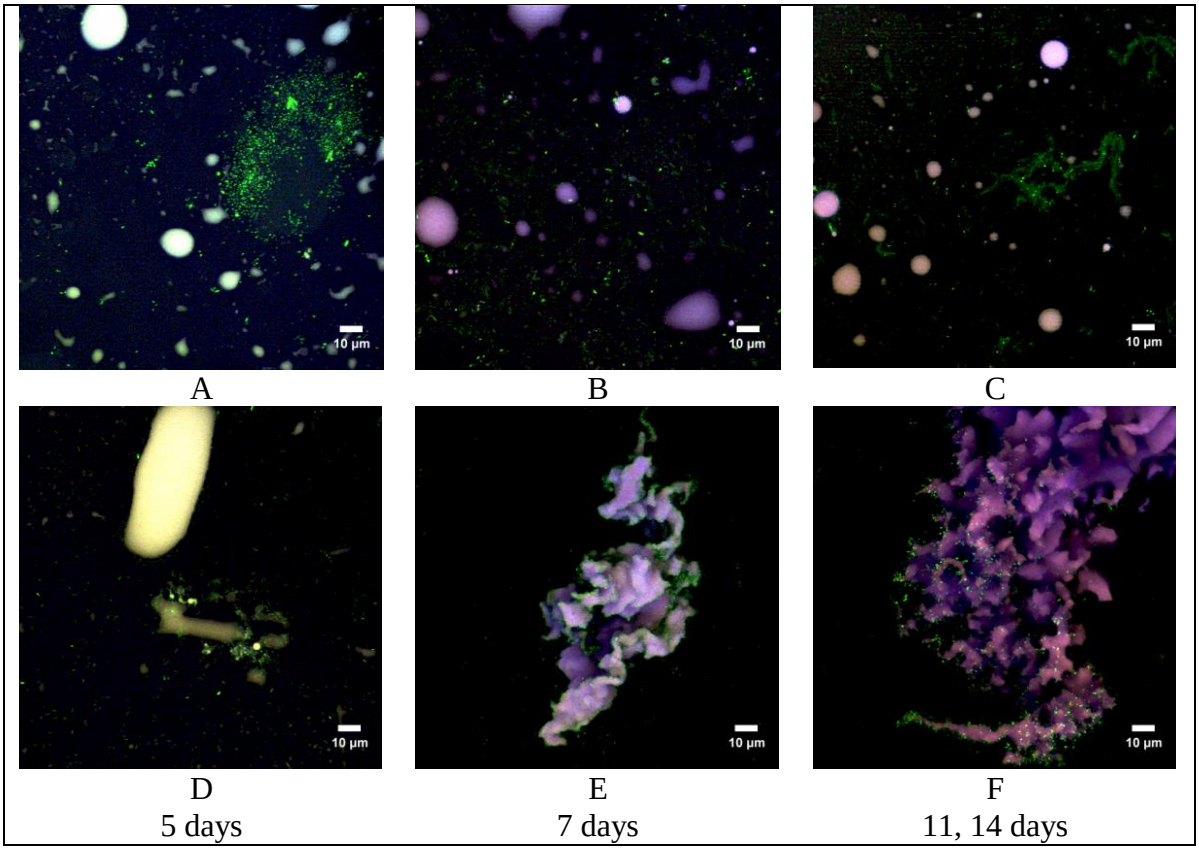


Figure 62: Corexit effects on the interaction between oil droplets and bacteria from consortia Eb8 (D-F) & E4 (A-C). Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

After the addition of nutrients bacteria seemed to recover and made some progress in associating with oil droplets, however this wasn't so effective and still many of the bacteria prefer to stay at free state in the water phase. Bacteria don't seem to like the environment they look tiny and few and their weak signal is covered by the strong signal of the oil which makes difficult to differentiate

- *Consortia Eb8 and E4 with S200 +NP (Nutrients)*

S200 has a similar behavior as corexit and tends also to emulsify oil. At the first 5 days as shown in Figures 63A&D below bacteria from both consortia are swimming around and only few are just starting colonizing oil droplets (>10μm). Sample of consortium Eb8 is more populated than sample of consortium E4 which implies that Eb8 is less affected by the presence of S200. Improvement on colonization of oil droplets has been observed for both consortia forming strands with each other and oil droplets within a week (Figures 63B&E) but to greater extent for consortium Eb8, however after 11 days although there are some remains and isolated spots of clusters formed between oil droplets-bacteria in the samples, which are not indicative of the whole sample's impression, the number of bacteria has decreased which implies that already passed to death phase most likely due to lack of essential nutrients that help them keep the balance with crude oil (Figures 63C&E). Therefore nutrients were added to both consortia and bacteria population has recovered but not as anticipated and although bacteria have started again to colonize oil droplets, this trait was more or less restricted to some

isolated spots in the sample with star like shapes of bacteria microcolonies around oil droplets (Figure 64).

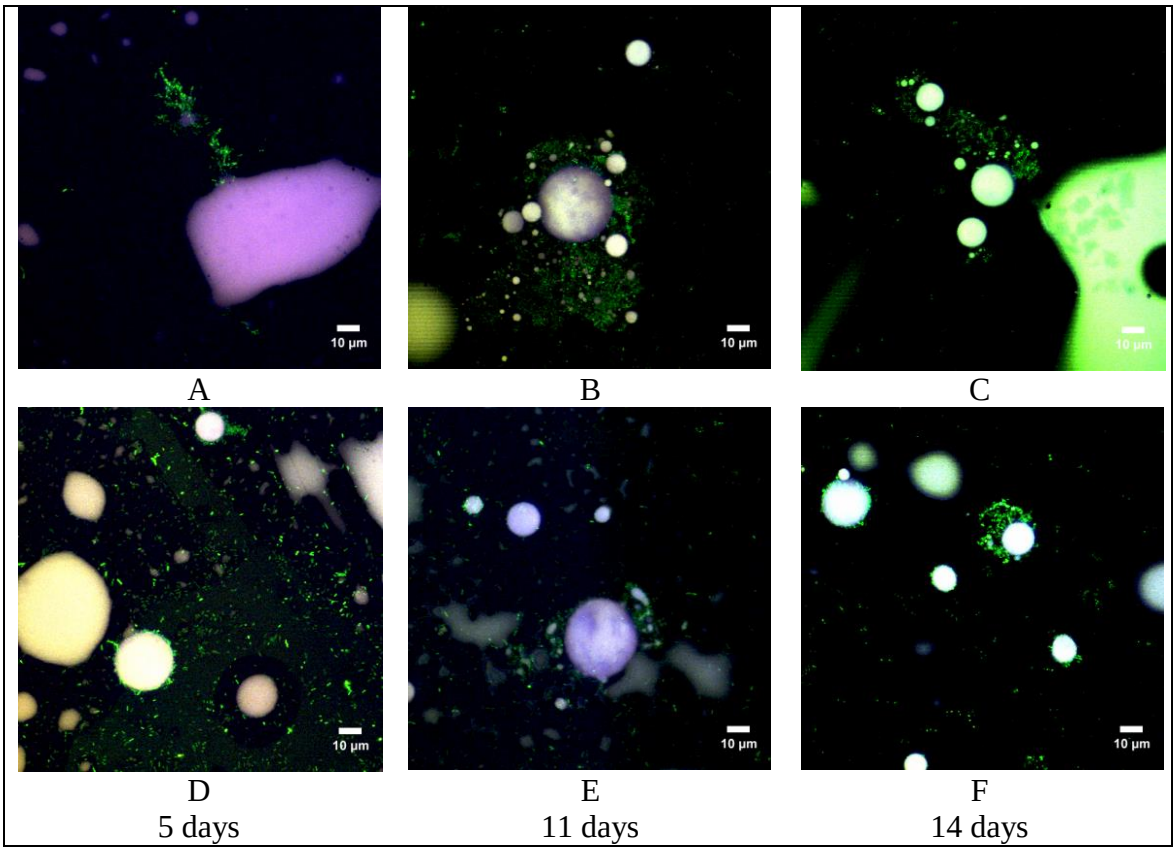


Figure 63: S200 effects on the interaction between oil droplets and bacteria from consortia Eb8 (D-F) & E4 (A-C). Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

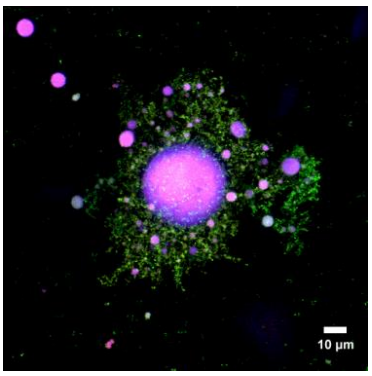


Figure 64: Grape like structure of bacteria on oil droplets after 18 days in sample with S200 and consortium Eb8. Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

The recovery of bacteria population was more limited especially for consortium E4 in which no traces of bacteria were detected after 18 days of incubation. The fact that S200 emulsifies oil rather than disperses, has limited the colonization of oil droplets by bacteria and possible biochemical interactions between dispersant and consortia have prevailed bacteria from producing surfactant like biopolymers or adapting their cell hydrophobicity. In addition possible dispersant toxicity probably has affected consortia survival, even planktonic bacteria were few.

➢ *Consortia Eb8 and E4 with Marichem +NP (Nutrients)*

Surprisingly for a dispersant like behavior Marichem did effectively disperse crude oil into fine droplets but also promoted growth in both consortia compared to the other two dispersants which proved to be toxic, particularly Corexit, for bacteria. Especially in the case of consortium Eb8 it was even faster (less than the initial starting date-5 days), which made the lack of essential nutrients the main reason of getting earlier to the death

phase (11 days) and even after the addition of nutrients this was inevitable. But this was not the case for consortium E4 which had a later response compared to Eb8 and re-addition of nutrients prevented bacteria of passing to death phase. Nonetheless same features and structures apply for both consortia which demonstrate the effectiveness of Marichem dispersant in enhancing both growth of bacteria and colonization of oil droplets (Figures 65&66).

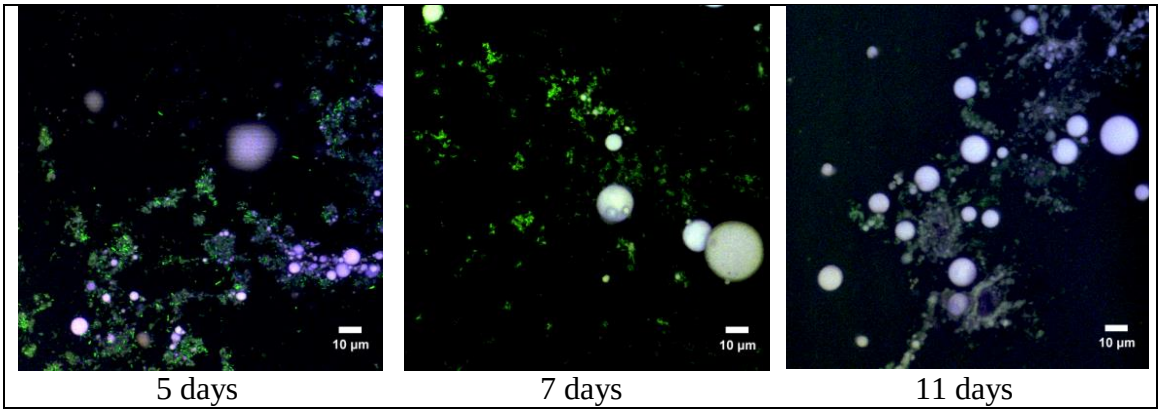


Figure 65: Marichem effects on the interaction between oil droplets and bacteria from consortia Eb8& E4 thought time. Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

At the beginning some of the bacteria have already covered effectively oil droplets forming clusters between them and the oil, however evolution through time showed that star like shapes of bacteria and oil interconnected to each other were the dominant features in the samples. Oil was highly colonized by bacteria forming grape style shapes with oil droplets and bacteria in between which helped to assume that a lot of EPS is formed connecting each other. Series of oil droplets were covered with bacteria forming filamentous structures and bacteria were oriented in a directed way.

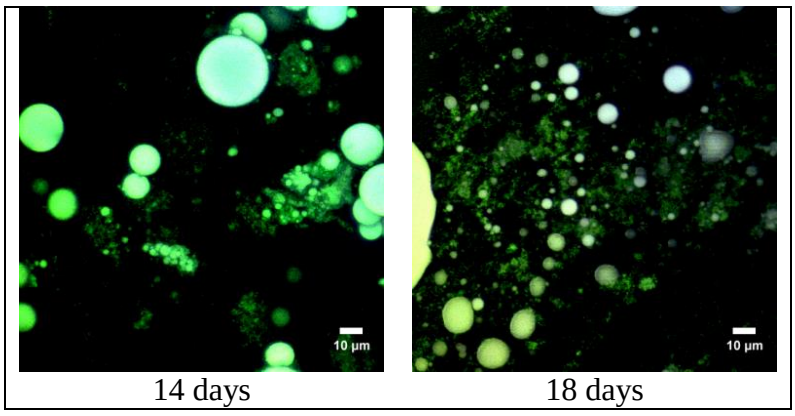


Figure 66: Marichem effects on the interaction between oil droplets and bacteria from consortia Eb8& E4 thought time. Photos represent Maximum Intensity Projections (MIPs) of data sets consisting of several optical sections.

➢ *Consortia Eb8 and E4 with C20 and NP (Nutrients)*

In the case of C20 both consortia responded well and covered the edge and center of C20 droplet from the first 5 days of incubation and especially for Eb8 evolution was even faster. Even though C20 has irregular structure that hides bacteria an obvious thick film, particularly for consortium Eb8 is very thick, has been formed all over this C20 droplet within a week in either consortia. After 11 days of incubation the C20 is fully

covered by bacteria which have already dissolved -degraded some parts of C20 that look floating from the rest part of C20. Dissolution of C20 droplet is even more intense in consortium Eb8 in which a strong biofilm of bacteria with lots of EPS is formed minimizing drastically the radius of the droplet, while the resulting reflection signal of C20 becomes weak. C20 droplet got a more rift form full of canyons and openings that resulted into smaller pieces of C20 that have been cut off from the main one possibly due to bacteria action after 14-18 days of incubation. This cracked form of C20 with many isolated parts of C20 like a complex of islands makes it difficult to define anymore the edge or the center of the original droplet. More severe dissolution is observed in Eb8 consortium.

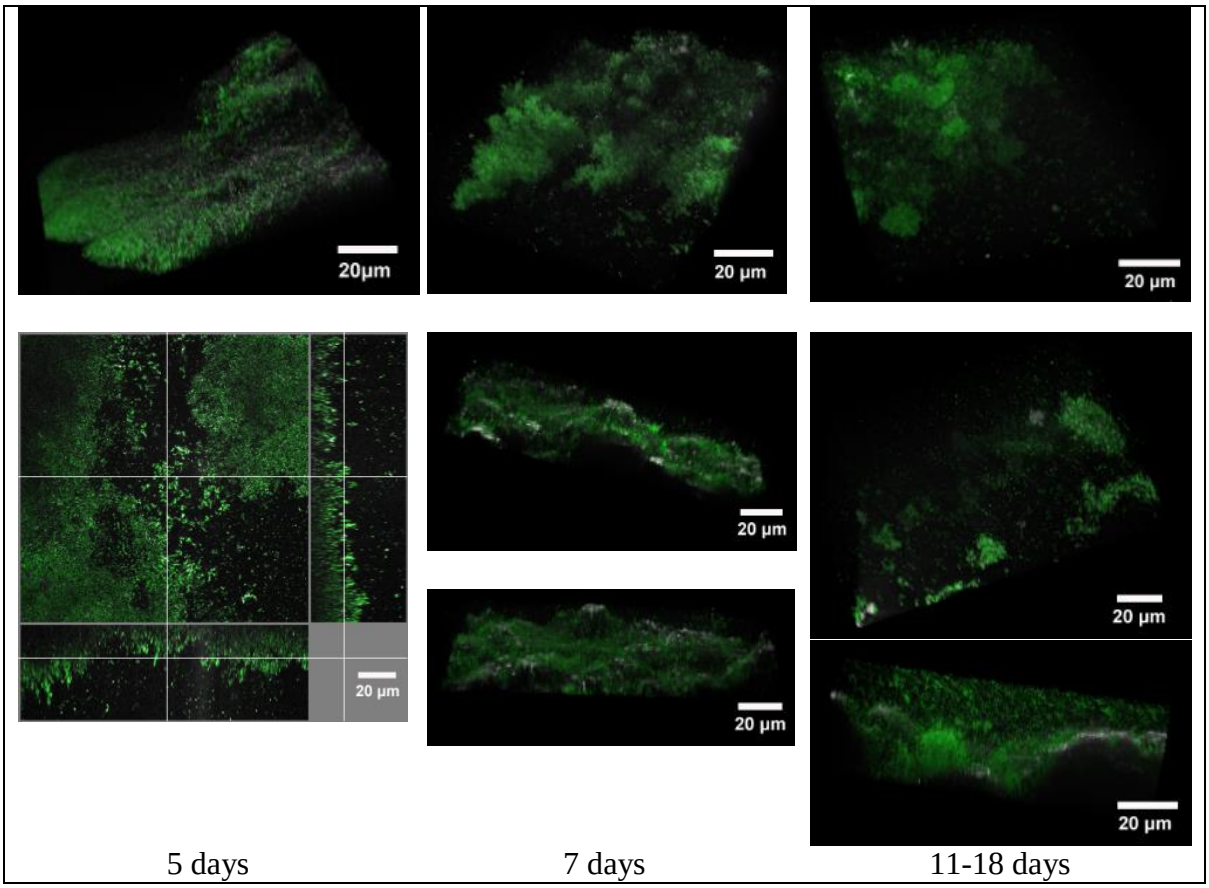


Figure 67: Evolution of bacteria development on eicosane droplets through time for consortia Eb8& E4 shown as XYZ projections.

- *Consortia Eb8 and E4 with C20 with NP (Nutrients)+Rha*

In the presence of biosurfactant Rha the structure of C20 droplet is affected in both consortia and more drastically in consortium Eb8. Same pattern (thick biofilm) as described previously at the beginning (5 days) is observed in C20 (Figures 68A&D)- is fully inhabited by bacteria that form mushroom pattern after 11 days that seems to be ought to Rha dispersive action still fully covered by bacteria (Figures 68B&E). After 14 days C20 is completely disintegrated and it is difficult to distinguish edge from center part with many pieces of C20 dislocated from the rest structure.by the end (18 days) whatever left from the original droplet has been extensively emulsified and degraded (Figures 68C&F).

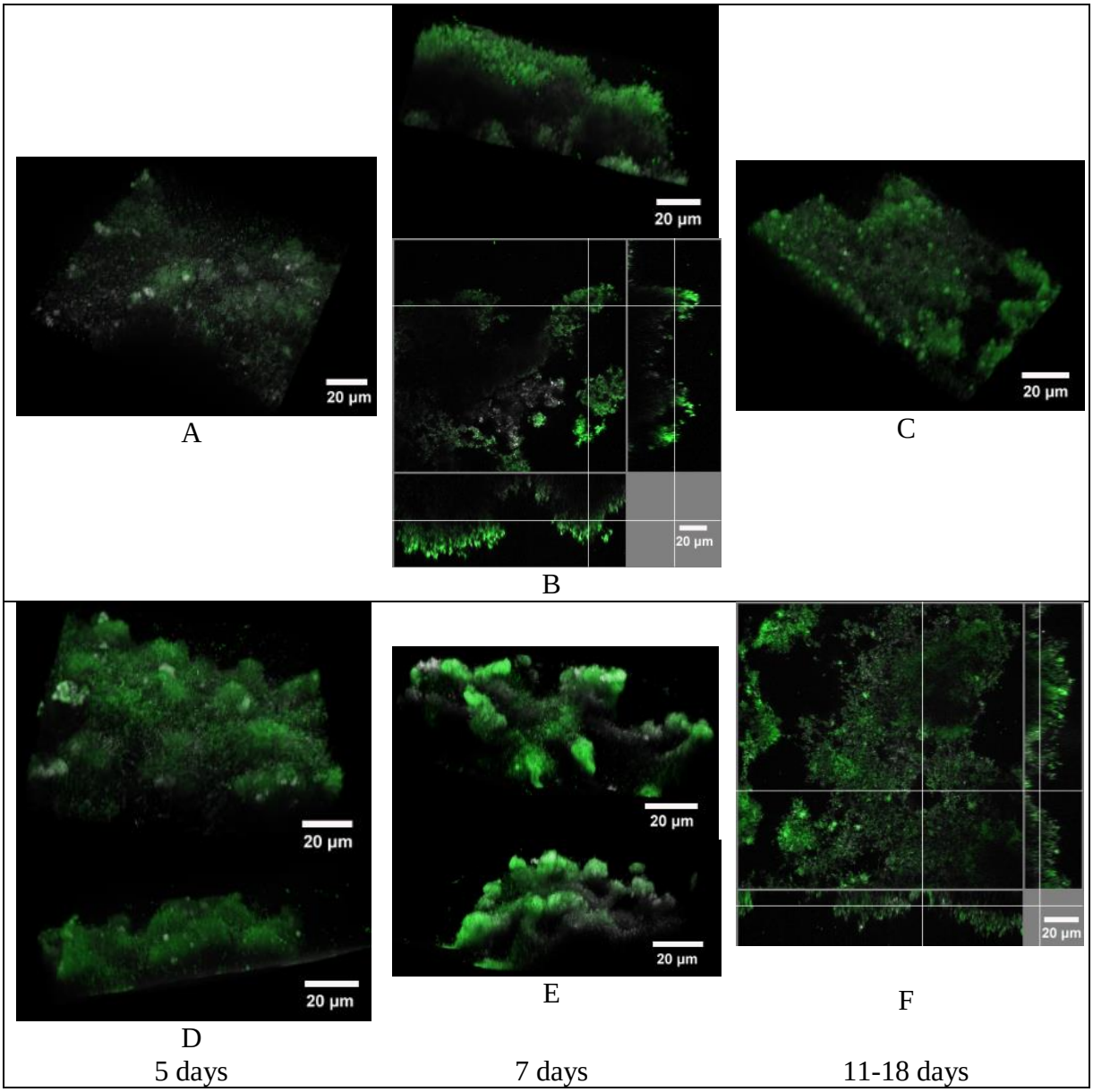


Figure 68: Evolution of bacteria development on eicosane droplets through time in the presence of rhamnolipids for consortia Eb8 (D, E) & E4 (A-C, F) shown as XYZ projections.

Moreover C20 in Eb8 consortium was completely dissolved and disintegrated within 1 week highlighting once more the degrading and dispersive capacity of consortium Eb8 as have been investigated in previously (Section 4.2). As shown in Figure 68 rhamnolipids favored the growth of bacteria as well as dissolution of C20. Heavy thick biofilm has been already formed in the first 5 days. Lots of bacteria cover all over the droplet and sagittal sectioning revealed the typical mushroom-shaped biofilm. Within a week this pattern is most evident which leads to abruption of small parts of the C20 that after 11 days is completely disintegrated and C20 has disappeared.

Conclusions-Discussion

One major limiting factor in terms of the bioavailability of HCs is their limited solubility in water. Enhancing the solubility of the contaminants e.g. by the use of surfactants can significantly improve the efficiency of biodegradation. Thus the main objective in this study was to observe the response of this particular consortium with high dispersion

characteristics in the case of adding certain commercial dispersants just like those that have been or could be used in the event of a real oil spill. Due to the high toxicity of commercial dispersants, there are certain rules on how and when they should be applied in the marine environment.

On the other hand, for the purpose of bioremediation only those surfactants should be used that are themselves completely biodegradable. In addition, they have to be compatible to the surfaces of the bacteria. Physical properties of the macromolecules found on cells membranes and bridges constructed by EPS are the main factors that enable bacterial cells to adhere on HCs (Neu, 1996; Rodrigues et al., 2005). Controlled by the growth conditions extracellular polymers can either be binded on the cell surface or released into the surrounding medium (Osterreicher-Ravid et al. 2000). Moreover, many biosurfactants/bioemulsifiers that have been examined are just complex structures of polysaccharides and proteins, which are known to increase the apparent solubility of hydrophobic compounds (Rodrigues et al., 2005).

Undoubtedly, EPS promotes direct contact between the bacteria cells surfaces and the hydrocarbon substrate and consequently cell surface-associated biosurfactant(s) can favor substrate cellular uptake by solubilizing the hydrocarbon substrate. In this sense EPS have primary role in floc formation between bacteria cells and the hydrocarbon substrate, keeping bacteria cells in close contact to the hydrocarbons (Whyte et al., 1999).

Therefore, the biosynthesis and release of polymeric substances can be a bacterial strategy to promote bioavailability of less water-soluble compounds and/or adhesion to hydrophobic surfaces (Neu, 1996; Rodrigues et al., 2005).

According to Al-Tahhan et al., 2000 there are two main mechanisms by which biosurfactants enhance biodegradation. First, biosurfactants can solubilize hydrophobic compounds within micelle structures, effectively increasing the apparent aqueous solubility of the organic compound and its availability for uptake by a cell. Second, biosurfactants can cause the cell surface to become more hydrophobic, thereby increasing the association of the cell with the slightly soluble substrate. Since the second mechanism requires very low levels of added biosurfactant, it is the more intriguing of the two mechanisms from the perspective of enhancing the biodegradation process.

Regarding the above, the requirements of biocompatibility and biodegradability are met by biosurfactants and by those surfactants that contain structures comparable to naturally occurring microbial surfactants and for that reason they were also tested in this certain consortia (Eb8 &E4).

Microscopy observations of biofilm community developing at Hydrocarbon - water interfaces so far have provided evidence of a stepwise development pattern of the biofilm and typical production of extracellular matrix which is close to the characteristics of the proposed model biofilms. Contrary to what was believed up to now in this work biofilm formation as has been proposed by various stepwise models was not obvious. Special characteristics of this biofilm formation in which hydrocarbons serve as substrate and substratum simultaneously makes difficult to characterize and also to simulate this type of biofilm. Single species –single compound (substrate) interactions have been fully investigated as has been described above and revealed a gradient development of cells aggregates around hydrocarbon droplets forming a biofilm.

Nevertheless in this work the impression we got is completely different to what so far has been observed or proposed as a stepwise pattern of biofilm formation and maybe be a special type of biofilm at the water-oil interface is formed between the tested consortia and the oil droplets. As has been shown in the above figures bacteria are organized into clusters forming strings, star and grape like shapes of bacteria and fine oil droplets

bridging each other with EPS. However it should be noted that speculation of EPS production arises mainly by the fact that EPS of any kind is atypical trait in biofilms and that specificity of Syto9 dye to nucleic acids could also be responsible for staining informative type of EPS (nucleic acids). The chemical and in situ analysis of EPS constituents is still a challenge as the different types of polymers cannot be analysed by using a simple and straightforward analytical approach. In fact the analysis is even more difficult if environmental biofilms containing a huge variety of different cell types and thus different EPS compounds have to be analysed. One part of the EPS matrix is represented by glycoconjugates. It has been demonstrated that lectins are a very useful probe for the in situ characterization of glycoconjugate fraction of the EPS by means of fluorescence lectin-binding analysis (Neu and Lawrence, 2005; Neu et al., 2010). In order to select the appropriate lectin or a selection thereof, a lectin screening of all commercially available lectins (~70) is necessary usually in combination with a nucleic acid counterstain. By means of this in situ technique, an estimate of EPS-specific glycoconjugates can be made and even multiple types of glycoconjugates can be differentiated (Neu et al., 2010). However extracellular matrix although mentioned, was not measured since lectin approach is time consuming, especially when there are no indications of the possible EPS composition, but most of all it requires flushing/destaining which in our case would have changed or disturbed the sample completely. In addition sample's nature, which is in liquid form, and highly dispersed oil droplets, which were difficult to either sample them or retain them especially after flushing, made it difficult for us to further investigate the nature of EPS formed.

As observed dispersion of crude oil is promoted by both consortia exhibiting the capability of mixed consortia over single species cultures to solubilize HCs. Substrate preference by specific strains (Whyte et al., 1999; Pepi et al., 2005; Klein et al., 2008) could be overcome by the combined application of mixed species inoculum that is able to utilize substrates that other strains can't. Therefore combined species consortia are most preferable as they can use a broader range of substrates; besides in a real case scenario of an accidental oil spill in the marine environment mixed populations of indigenous bacteria confront the diverse compounds found in crude oil.

Furthermore as has been mentioned by Osterreicher-Ravid et al., 2000 and Olivera et al., 2009 biopolymers produced by a single strain of mixed consortium can favor the rest strains of the consortium to adapt faster and get into contact with the HCs. Specifically Alasan, the exocellular polymeric emulsifier produced by *Acinetobacter radioresistens* KA53, could bind to the surface of *Sphingomonas paucimobilis* EPA505 and *Acinetobacter calcoaceticus* RAG-1, but not *to Escherchia coli* B, E. coli C600, *Staphlococcus albus* and *Seratia marsescens*, the last of which has a high cell-surface hydrophobicity. Despite Alasan's specificity in binding to bacteria, this horizontal transfer of exopolymers from one bacterial species to another could be highly significant for natural mixed populations grown in close proximity, such as coaggregation and biofilms. By an environmental aspect this transfer of amphipathic polymers between cells has significant implications in oil biodegradation from natural microbial communities by changing their surface properties, which subsequently could alter the way of interaction between cells, their adherence or desorption from surfaces, consumption of substrates, and resistance to bacteriophage infection and other environmental factors (Osterreicher-Ravid et al., 2000).

The case where dispersion of oil is promoted by the use of commercial dispersants like those used in accidental oil spills of the Mexico Gulf (Corexit) and the Galician coast of Spain (S200) has been examined in the present study for their effect on microbial communities response. It could be expected that since dispersants used promote the

dissolution of oil to droplets, bacteria would immediate associate to the oil droplets, on the contrary bacteria population decreased due to dispersants possible toxicity as has been already mentioned by other studies.
Seo and Bishop, 2007: It has been reported that nonionic surfactants like Triton X-100 and Tween 20 interfered with the biofilm structure due to hydrophobic interactions affecting initial biofilm formation and enabling already established biofilm removal. It could be assumed that surfactants interference is mainly caused by the prevention of suspended cells flocculation and EPS bridging mechanisms and finally by EPS dissolution (Seo and Bishop, 2007).
Martienssen et al., 2007 compared different conventional synthetic surfactants (Triton X 100, Tween 20, sodium dodecyl sulfonate) and the close-to-nature surfactant composition (Bioversals FW) with respect to micelle formation and biocompatibility and found that bacterial growth resulting in oil aggregates and their degradation was detected only in the biosurfactant (Bioversals FW).
This manner of bacteria reaction to dispersants with an exception of Marichem compared to Rhamnolipids has also been confirmed in this study. And as has also been reported by Al-Tahhan et al., 2000, Rhamnolipids even at sub-CMC concentration resulted in increased degradation rates of the hydrophobic substrates. Still it is surprising the reaction of bacteria to Marichem dispersant that produced a more close to biosurfactants biocompatibility, which implies that as more investigation is made on creating structures more compatible to bacteria then degradation rates of hydrocarbons would be favored even with the cheaper solution of the chemical dispersants. Additionally in a recent study on a consortium enriched from deep, uncontaminated waters of the Mexico Gulf with oil (Macondo MC252) and dispersant used during the spill (COREXIT 9500) aggregates of bacteria on oil droplets forming flocs have been observed, denoting the advantage of next generation dispersants to overcome possible toxicity to indigenous marine bacteria (Bælum et al., 2012).
In this sense as has been already proved biosurfactants below cmc concentrations could favor degradation rates of HCs with considerable reduced cost ought to the reduced amounts of biosurfactants been applied.
Nonetheless biopolymers are a specific trait of microbial consortia to increase solubility of less water-immiscible compounds and/or adhesion to hydrophobic surfaces.
The broad variety of substrates in crude oil intrigues different kinds of strains and studies have shown (Pepi et al., 2005; Rodrigues et al., 2005; Osterreicher-Ravid et al., 2000; Whyte et al., 1999; Olivera et al., 2009) that EPS or any biopolymers production and physicochemical characteristics are highly influenced by the substrate type, temperature, growth state, species variation and interaction to each other.
Moreover the advantage of combined species consortia over single species has been proved in this study as they can been organized in a manner that through horizontal genes and EPS transfer can metabolize a broader range of substrates; besides in a real case scenario of an accidental oil spill to the marine environment mixed populations of indigenous bacteria confront the diverse compounds found in crude oil.
Furthermore in a marine system that is highly influenced by environmental conditions like tides, waves and currents it is extremely difficult and rare for indigenous populations to form biofilms as has been believed till now over long periods on hydrocarbons, it is more preferable for them to disperse oil into fine droplets by producing biopolymers and then get organized into strands and coaggregates with the oil droplets.
This new mechanism of mixed consortia on hydrocarbons utilization would provide a new dimension for the study of coaggregation and biofilm microbial communities in the

marine ecosystem and needs further investigation in the sense of new improved surfactants formulations or biosurfactans under different environmental conditions.
Understanding the interactions between oil-degrading microorganisms is essential, not only when predicting the fate of hydrocarbons in the environment but also for the development of successful bioremediation techniques. However EPS investigation and characterization by the lectin approach remains a challenge, especially in such kind of samples that are in liquid form and can be severly damaged or disturbed by this approach, thus further work should be done in this regard that could help us better understand and characterize the type of biofilm which is formed at the oil-water interface.

4.7. General Conclusions

Thorough investigation through chemical, microbiological and kinetics analysis (specific degradation rate, q_s) has revealed that Seawater 1 experimental set in which acclimated or not indigenous microorganisms were used performed far better than Sand 1 experimental set in which degradation was induced only by indigenous microbial populations and despite the fact that combined Rhamnolipids and lipophilic nutrients were used still microbial response was not as strong as in Seawater 1 experimental set. Bioavailability of oil hydrocarbons is the critical factor that affects the efficiency of bioremediation in oil contaminated environments and It can be concluded that in Seawater 1 experimental set biosurfactants, in particular rhamnolipids, accelerated the biodegradation of crude oil by making it more available to microorganisms as expected in the two ABA treatments (ULRM & NPKMR) and the biostimulation treatment (ULR).

On the other hand Sand 2 experimental set seemed more successful than Seawater 2 experimental set despite the fact that in both experimental sets, the same Eb8 consortium has been used. However it should be noted that despite the fact that Rhamnolipids were added lipophilic nutrients were not included in Seawater 2 experimental set, which possibly had contributed to the less successful performance of NPKMR treatment of Seawater 2 experimental set compared to ULRM treatment of Sand 2 experimental set.

Combined application of Rhamnolipids and lipophilic nutrients could be beneficial in liquid matrix (seawater), however when applied to solid matrix their performance is modest compared to one in the liquid matrix. On the contrary inorganic nutrients usually being washed out when applied in seawater perform better when applied to sand almost equally to ULR combined performance. In addition other studies have confirmed also that inorganic nutrients are more suitable to fine grained shorelines rather than to coarse-grained shorelines that lipophilic nutrients could be more efficient (Nikolopoulou and Kalogerakis, 2011). Inconsistent behaviour of ULR (Sand 1 experimental set) and ULRM (Sand 2 experimental set) treatments between the two oil fractions (alkanes-PAHs) compared to NPK (Sand 1 experimental set) and NPKM (Sand 2 experimental set) treatments respectively could support this conclusion. Still overall ULR treatment performance suggests that the presence of biosurfactant could possibly have contributed to utilization of lipophilic nutrients by making them more available to soil microorganisms.

Nonetheless especially in the soil matrix (sand) bioavailability of hydrophobic compounds is often the rate-limiting step in the process and the efficiency of biosurfactants or other rate limiting co-substrates mainly could be attributed to the interactions between target organic compounds, bacterial species, water content and surfactants.

Chemical analysis results for all 4 experimental approaches has demonstrated the preferred biodegradation of the more easily biodegradable substrates such as the lower-molecular-weight PAHs and small chain length aliphatic hydrocarbons which follows the pattern C15> C20> (Pristane, Phytane)>C25 > C30> C35 > (PAHs). Also these results confirmed that although Pristane and Phytane were considered in the past as conserved internal markers in biodegradation index yet they could not be used anymore as biodegradation indexes.

So far *alcanivorax* strains are considered to be the dominant OHCB bacteria found in any oil contaminated environment and in which have a primary role in oil biodegradation. It needs to be stressed out that although *Alcanivoracaceae* as investigaterd in the Seawater 1 experimental set is the dominant family in treatments with inorganic nutrients, when biosurfactant is applied (rhamnolipids-treatments NPKMR, ULR & ULRM) community shifts to the family of *Pseudomonadaceae* (15.6%, 79.3% & 15.3% for NPKMR, ULR & ULRM treatments respectively). Specifically for ULR treatment that lipophilic nutrients added to support indigenous population, indigenous population shifts to consist mainly additionally to *Pseudomonadaceae* family, *Vibrionaceae* family (12.6%). Nevertheless combination of *Alcanivoracaceae* and *Rhodospirillaceae* (NPK, NPKM) is not regarded as the strongest in terms of biodegradation rate at the particular time interval (30 days).
Pseudomonadaceae family was also the dominant family at the late stage (30 days) in the Seawater 2 experimental set, whereas at early stage of the experiment *Alcanivoracaceae* was the dominant family as well.
Alcanivorax species are known as hydrocarbonoclastic bacteria that oxidize C_5-C_{16} *n*-alkanes and branched alkanes. On the contrary *Pseudomonas* sp. are able to endure and metabolize contaminants that are considered very toxic to other bacteria. Several studies have proved that *Pseudomonas* sp. can utilize a vast range of contaminants either naturally present or xenobiotic (Palleroni et al., 2010). Regarding the above, *Pseudomonas* sp. can be considered as exceptional biocatalysts and can accelerate bioremediation when other species stop. This is the case in the NPKM and most likely in the NKPMR treatment, at the beginning of the experiment the community is comprised by strains that can utilize hydrocarbons (alkanes and some aromatic compounds) whereas by the end of the experiment where most of the hydrocarbons have been consumed, pseudomonas strains take over utilizing either metabolic by-products or other more recalcitrant hydrocarbons (Palleroni et al., 2010).
This proves that different type of amendments and consortia provoke different structures in the resulting biodegradation communities and should be considered when deciding for the suitable bioremediation strategy, however further investigation on the composition of microbial communities with respect to time and interactions with oil hydrocarbons under different conditions should be run in this regard.
The advantage of mixed consortia over single species consortia on hydrocarbons degradation has been proved in this study which revealed alterations in consortia species composition developing different degrading capabilities provoked by the application of different amendments.
CLSM investigation has contributed into this and revealed that bacteria are organized into clusters forming strings, star and grape like shapes of bacteria and fine oil droplets bridging each other with EPS. Dispersion of crude oil is promoted by both consortia exhibiting the capability of mixed consortia over single species cultures to solubilize HCs. Moreover gradual dissolution of C20 droplet is encouraged in both consortia (especially in the presence of rhamnolipids) but more intense in consortium Eb8, in which a strong biofilm of bacteria with lots of EPS is formed minimizing drastically the radius of the droplet resulting in a more rift form full of canyons and openings. However it should be noted that contrary to what so far was proposed as potential mechanism for the interaction between oil droplets and bacteria, biofilm was not the preferred interaction between the tested consortia and the oil droplets.
This new organization and structure between oil and microbial consortia has brought up a new perspective-mechanism in which mixed consortia utilize oil hydrocarbons and could provide a new dimension for the study of coaggregation and biofilm

microbial communities in the marine ecosystem. In addition it could be expected that since dispersants used promote the dissolution of oil to droplets, bacteria would immediate associate to the oil droplets, on the contrary bacteria population decreased due to dispersants possible toxicity as has been already mentioned by other studies with an exception to Marichem dispersant which exhibited the same organization between bacteria and oil droplets into clusters as when consortia have been tested alone without any additional amendement only more intense.
Thus understanding the interactions between oil-degrading microorganisms is essential, not only when predicting the fate of hydrocarbons in the environment but also for the development of new improved surfactants formulations or biosurfactans that can be used under different environmental conditions.

Still highly sensitive coastline environment and oil toxicity that fluctuates depending on the amount released to the environment constitute the impact of an oil spill exceptionally evident. Therefore immediate bioremediation is vital in order to decrease oil concentration below critical level and by that diminish marine ecosystem disturbance. Although biostimulation of the indigenous bacteria is often considered the best bioremediation strategy, this may not always be very effective particularly if time is of essence, namely, if we do not have the luxury to wait for the indigenous hydrocarbon degraders to reach high densities. Thus, bioaugmentation coupled with biostimulation is obviously beneficial over biostimulation alone under circumstances in which quick response is required or pollutant toxicity or even the absence of necessary consortia is of great importance. On the other hand, autochthonous bioaugmentation (ABA) is advantageous over biostimulation coupled with allochthonous bioaugmentation. Therefore, the best way to overcome the lag phase of indigenous population adjustment and thus reduce the time needed for bioremediation is to combine both techniques. Major determinants that restrain efficiency of oil biodegradation and affect the population of hydrocarbon degraders have been stressed out in the present work throughout different experimental sets conducted in both seawater and sand matrix. Despite the fact that supplementation with nutrients leads to very fast degradation of the saturated fraction which renders pointless any additional supplementation with other biostimulants in the long run, biosurfactants addition may play a significant part on oil degradation especially during the first critical days in the event of an oil spill. Kinetics investigation of the specific degradation rate (q_s) support this conclusion since the specific degradation rate is not only growth associated but is also enhanced by intermediate products or biosurfactants activity that possibly affects metabolic pathway. However biodegradation process efficiency in soil and the specific mechanism of rhamnolipid's action highly depend on substrates physicochemical interactions within certain matrix. Hence further investigation should be done in this regard (response of HCB communities) and more possible combinations of different types of nutrients and biosurfactants on bioremediation of a variety of oil contaminated shorelines should be tested.
This work has demonstrated that in the absence of essential nutrients, inoculation only with autochthonous hydrocarbon degraders is not an effective treatment, however when the needed nutrients or other biostimulants are supplemented the advantages of such combination are obvious and result in accelerated hydrocarbon consumption by the added autochthonous consortium. Thus we strongly believe that the combination of autochthonous bioaugmentation and biostimulation is a promising strategy to speed up bioremediation in cases where there is lack of both nutrients and indigenous

degraders. This technique has a number of advantages like shorter treatment time, greater potential efficiency, lower impact on the environment, and relative ease in obtaining public support. Thus future research that would define the carrying capacities of various environments and the mechanisms that control them could be fruitful in this regard.

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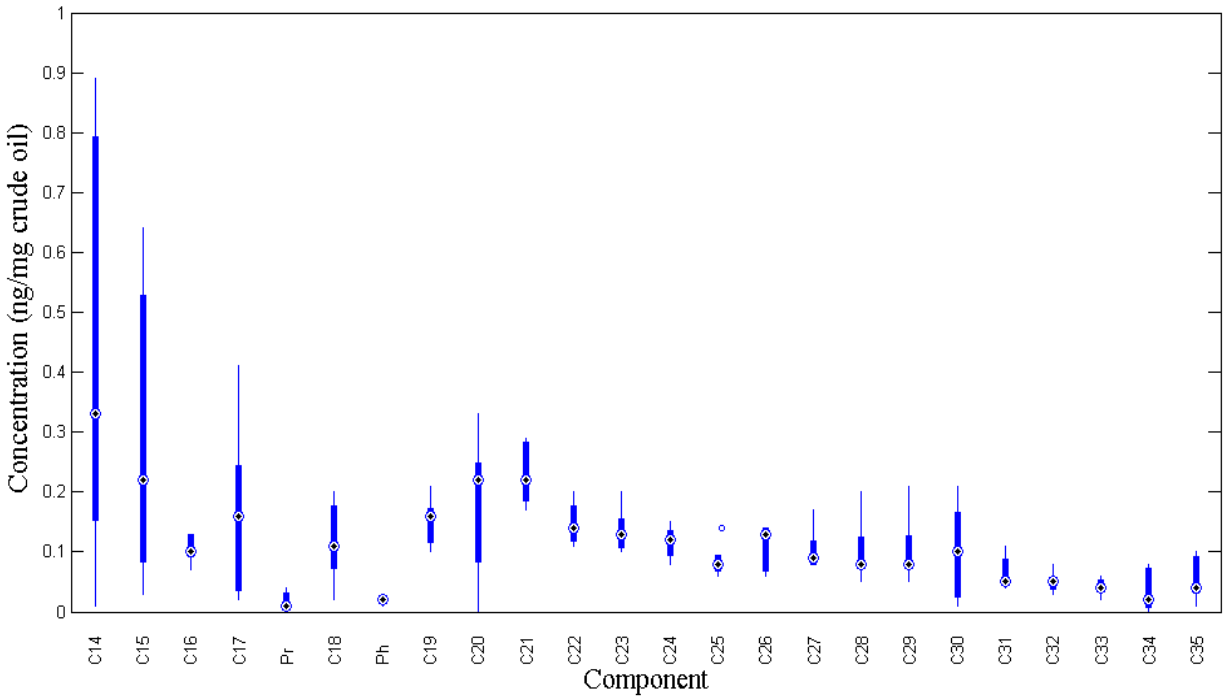
Appendix

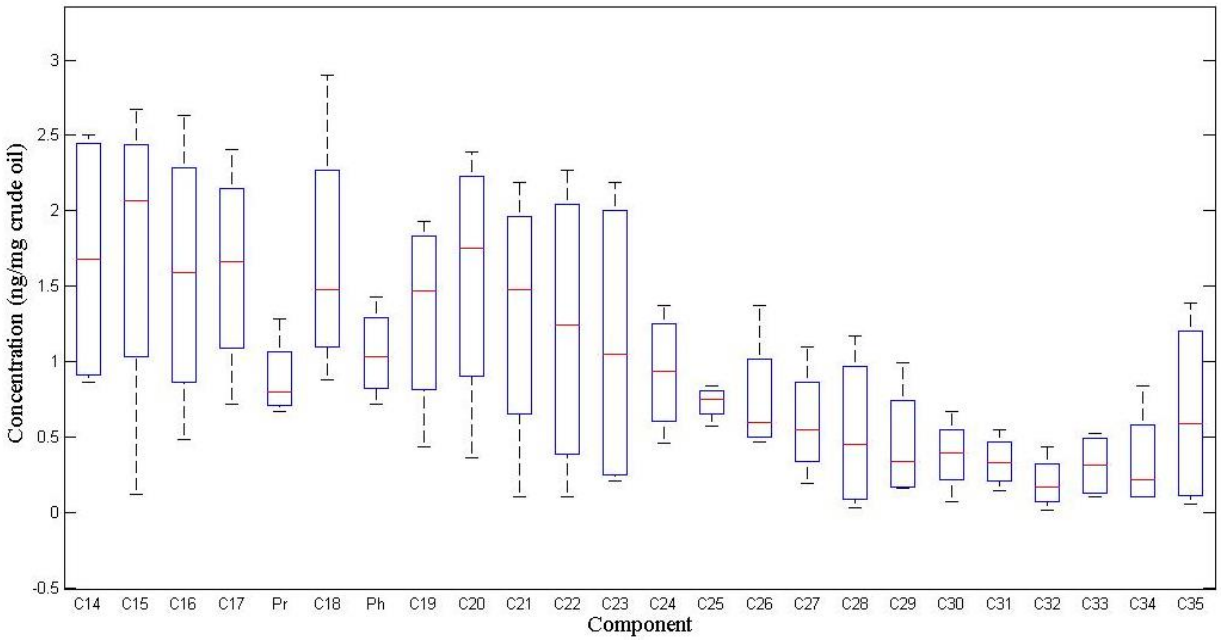
Box Plots

Box-plot showing the distributions of n-alkanes concentrations in oil samples obtained at the 0-day of each experimental data set.

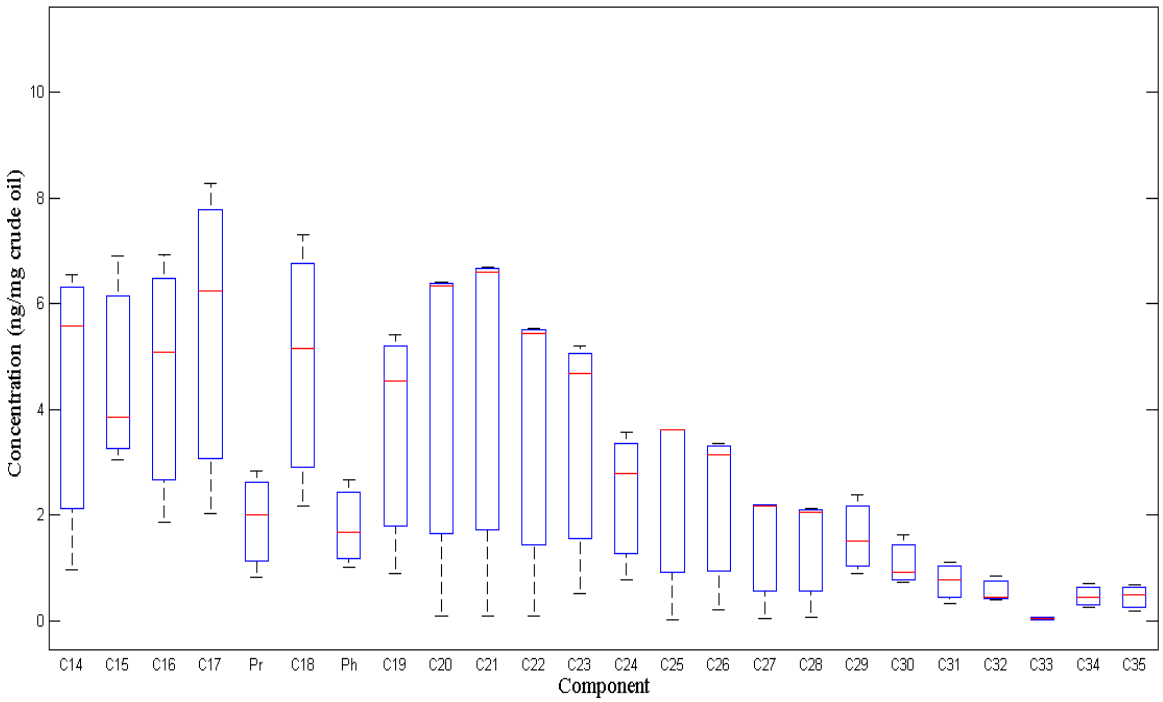
Seawater 1&2



Landfarming 1

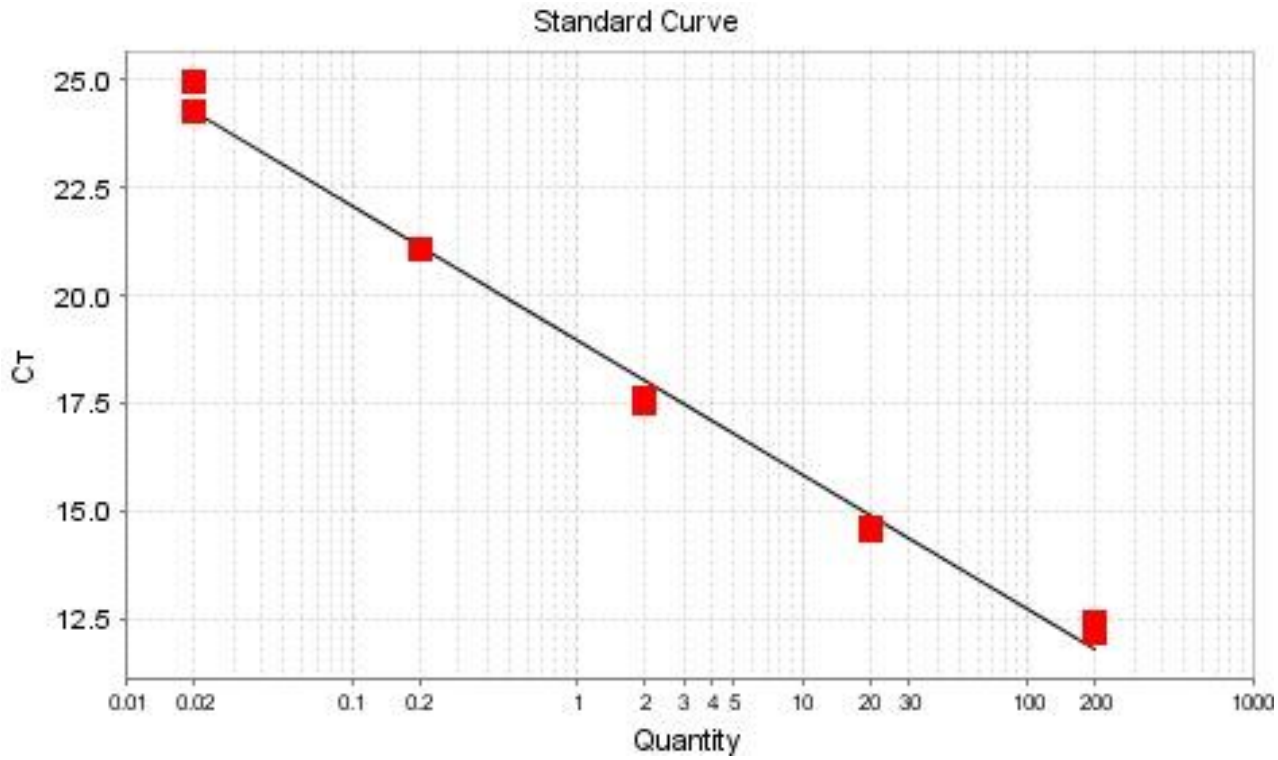


Landfarming 2

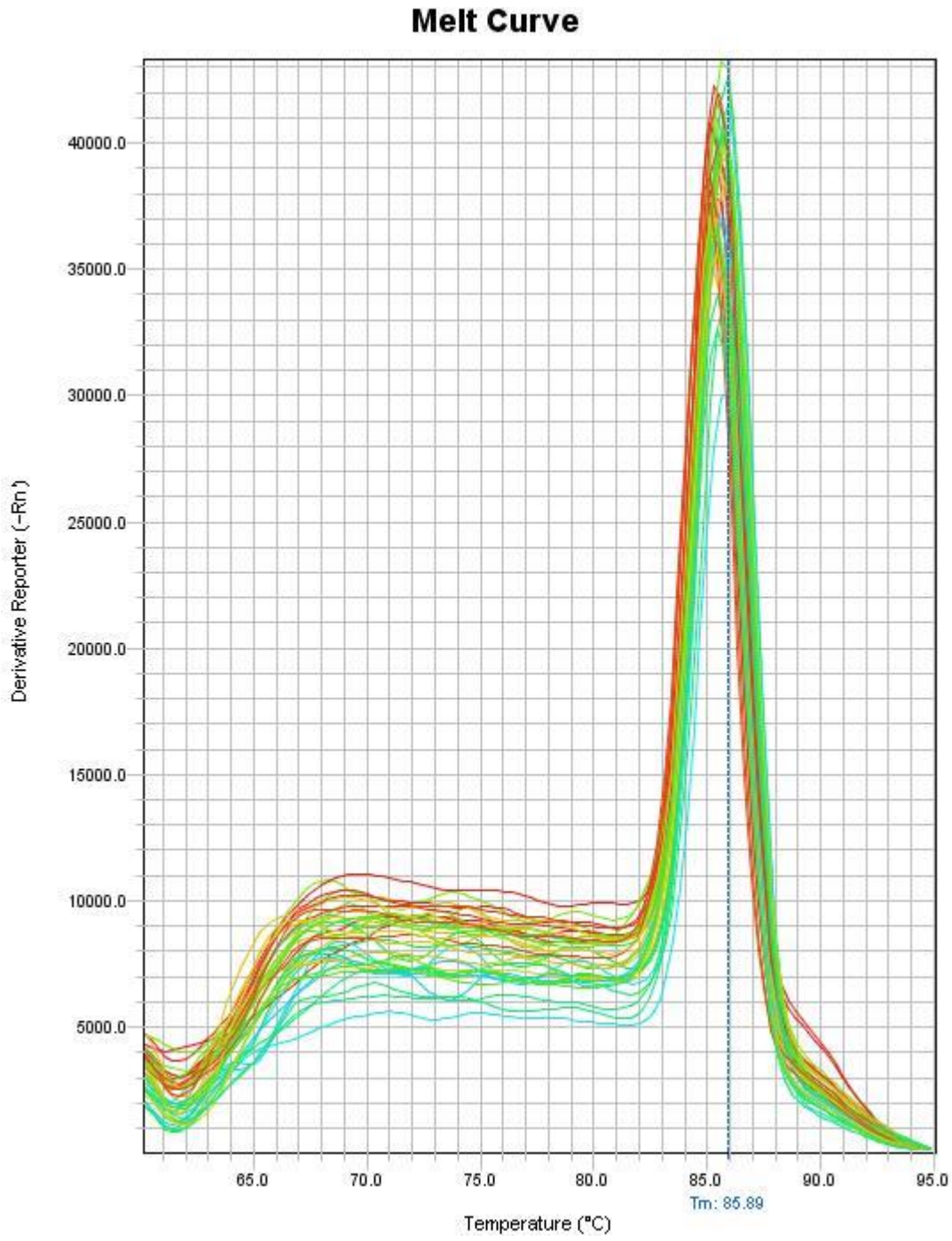


Standard curves and Melting curves of RT-PCR analysis

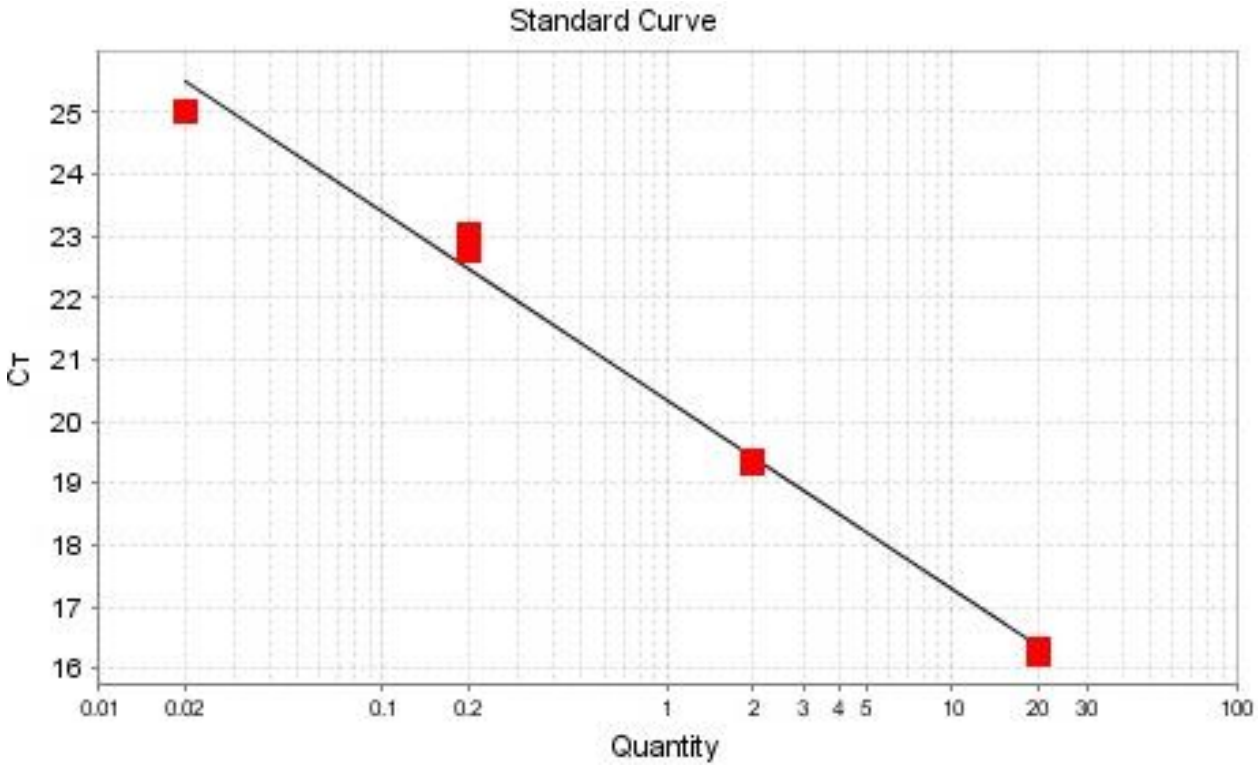
Standard curve for A.borkumensis



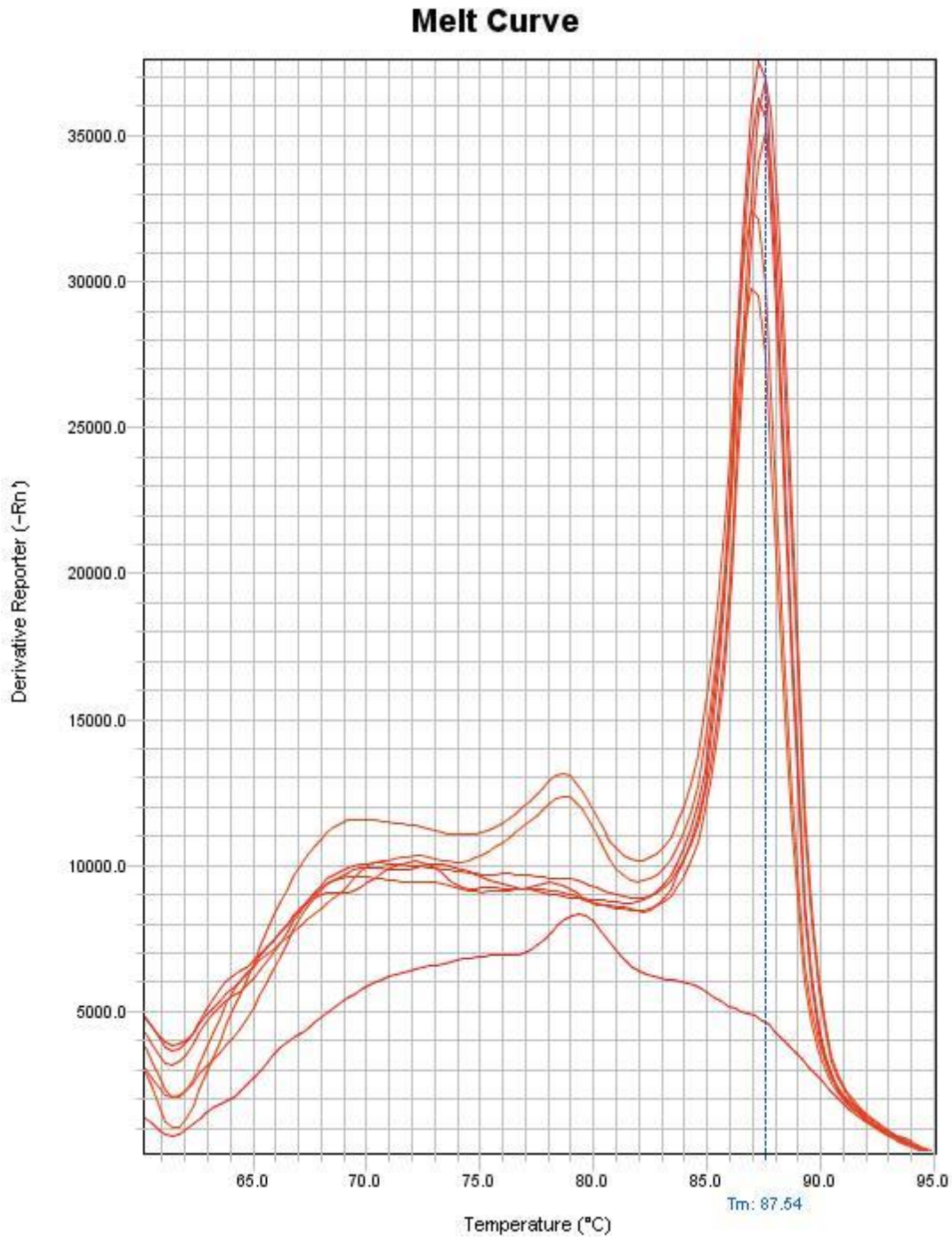
S: -3.118 R^2: 0.991 Y: 18.963


Melt Curve
Derivative Reporter (-Rn')
40000.0
35000.0
30000.0
25000.0
20000.0
15000.0
10000.0
5000.0
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Tm: 85.89
Temperature (°C)

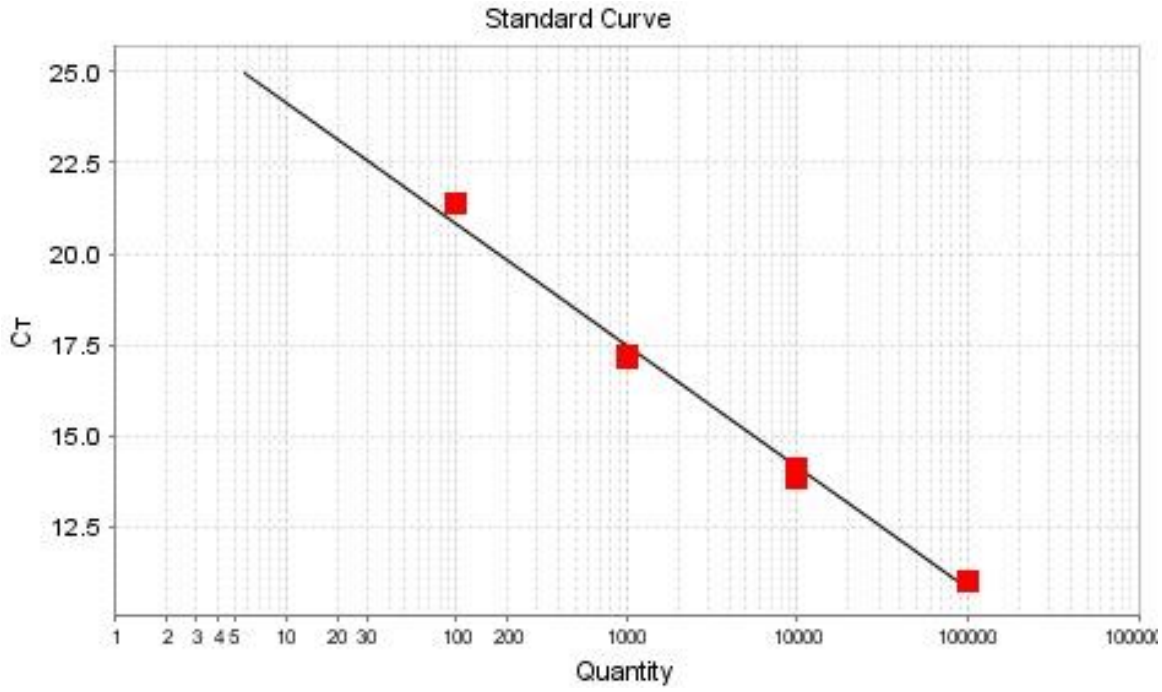
Standard curve for T. Oleivorans.



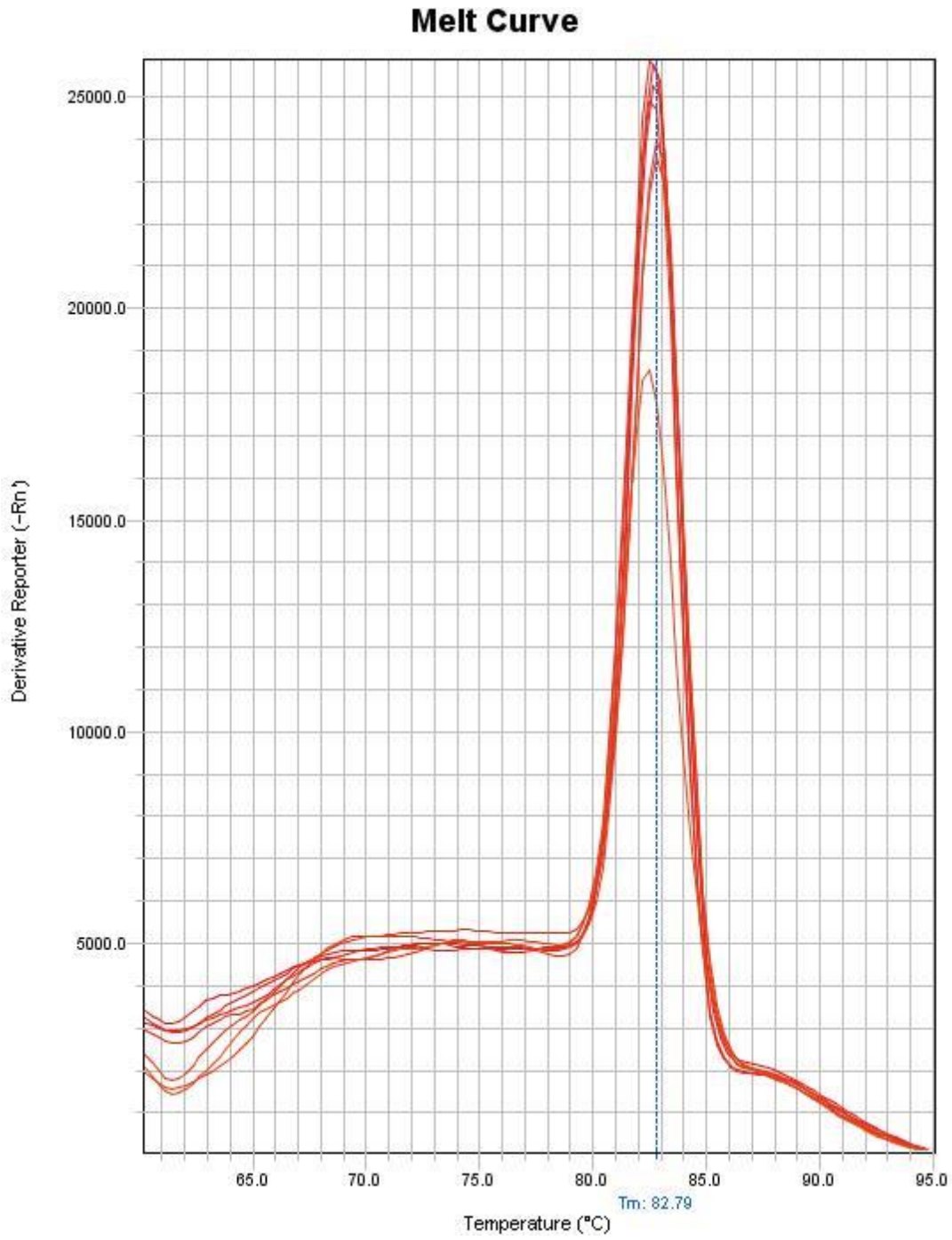
S: -3.052 R^2: 0.99 Y: 20.328


Melt Curve
35000.0
30000.0
25000.0
20000.0
15000.0
10000.0
5000.0
Derivative Reporter (-Rn')
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Tm: 87.54
Temperature (°C)

Standard curve for Cycloclasticus.

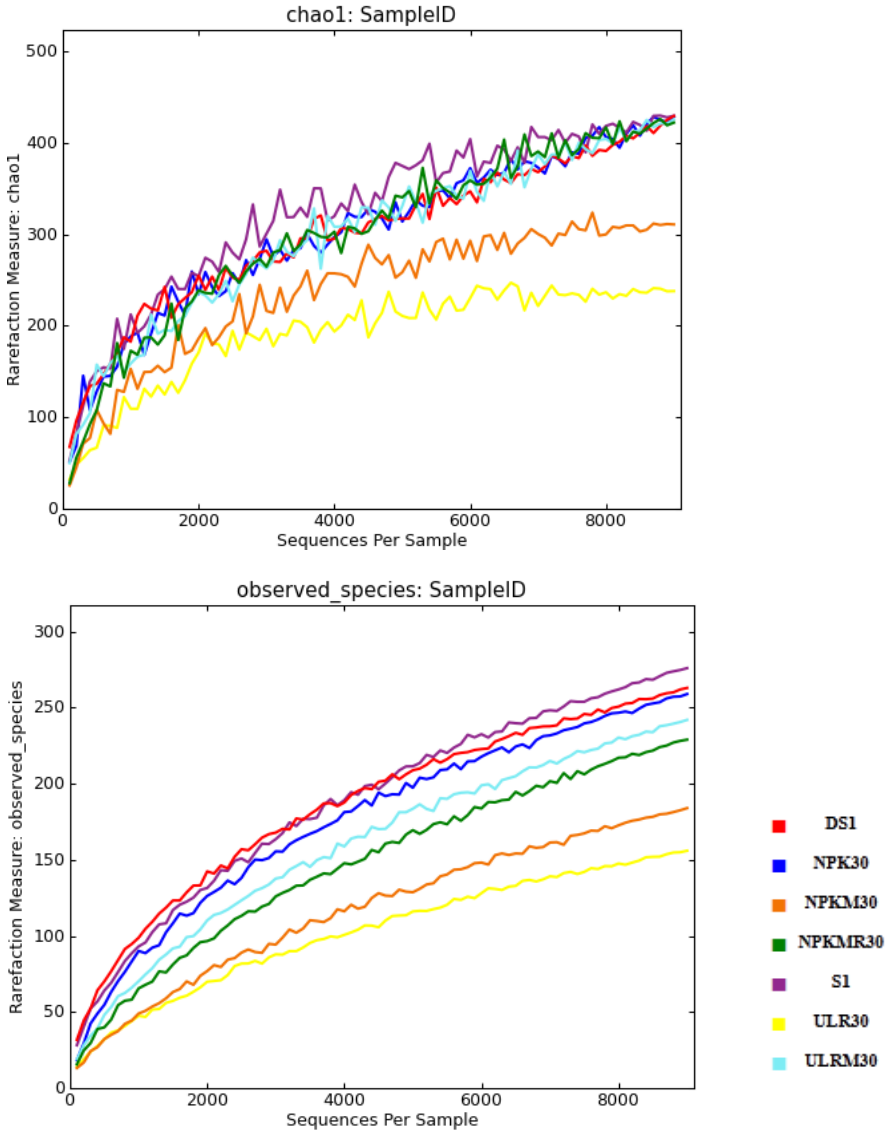


S: -3.352 R^2: 0.992 Y: 27.54


Melt Curve
25000.0
20000.0
15000.0
10000.0
5000.0
Derivative Reporter (-Rn')
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Tm: 82.79
Temperature (°C)

Rarefaction curves

After the removal of "noisy" and chimeric sequences, we ended up with a total of 84,529 sequences. The number of sequences per sample varied from 9,000 to 19,949. In order to have comparable sample sizes, we rarefied the OTU table at the lowest sample size depth, i.e. 9,000. This rarefied OTU table was used for all subsequent community screening analyses. Rarefaction curves were very close to reaching the horizontal asymptote, indicating that sampling was enough to capture the largest fraction of bacterial diversity within samples.



Rarefaction curves of the 97% OTUs for different samples of the 16S rRNA molecule

Noise filtering (using the AmpliconNoise package), chimera removal (using the PerseusD algorithm), Operational Taxonomic Unit (OTU) clustering (at 97% similarity), OTU table construction, phylogenetic assignments using the RDP naïve Bayesian classifier [3] and heatmap analysis were performed using QIIME v1.4 [4]. For the creation of rarefaction curves, the OTU table was rarefied from 100 to 9,000 sequences (the lowest number of clean reads per sample) with a step of 100 sequences

ten times at each step and the mean Chao1 and "observed species" diversity indices were calculated at each step. The Chao1/"observed species" Vs the number of sequences plot was performed in QIIME.