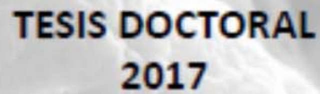

TESIS DOCTORAL
2017

RESPONSE OF THE BACTERIAL COMMUNITIES IN OXYGEN LIMITED ENVIRONMENTS TO THE PRESENCE OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS).

Sophie Marie Martirani Von Abercron
Doctorado en Biología Fundamental y de Sistemas

Universidad de Granada

Programa de doctorado en Biología Fundamental y de Sistemas

Response of the bacterial communities in oxygen limited environments to the presence of polycyclic aromatic hydrocarbons (PAHs).

TESIS DOCTORAL

Sophie Marie Martirani Von Abercron

Granada, 2017

Universidad de Granada
EEZ-CSIC

Programa de doctorado en Biología Fundamental y de Sistemas

Response of the bacterial communities in oxygen limited environments to the presence of polycyclic aromatic hydrocarbons (PAHs).

Tesis doctoral presentada por la Licenciada en Ciencias Ambientales
Sophie Marie Martirani Von Abercron
para optar al Título de Doctora

Fdo.: Sophie Marie Martirani Von Abercron

VºBº de la directora de Tesis

Dra. Silvia Marqués Martín
Investigadora Científica CSIC,
Estación Experimental del Zaidín

Editor: Universidad de Granada. Tesis Doctorales
Autora: Sophie Marie Martirani Von Abercron
ISBN: 978-84-9163-694-6
URI: http://hdl.handle.net/10481/48911

El doctorando / The *doctoral candidate* **[Sophie Marie Martirani Von Abercron]** y los directores de la tesis / and the thesis supervisor/s: **[Silvia Marques Martin]**

Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

/

Guarantee, by signing this doctoral thesis, that the work has been done by the doctoral candidate under the direction of the thesis supervisor/s and, as far as our knowledge reaches, in the performance of the work, the rights of other authors to be cited (when their results or publications have been used) have been respected.

Lugar y fecha / Place and date:

Granada, 14 de Junio de 2017

Director/es de la Tesis / *Thesis supervisor/s;* Doctorando / *Doctoral candidate*:

Firma / Signed Firma / Signed

Esta tesis doctoral ha sido realizada en el Grupo de Microbiología Ambiental y Biodegradación perteneciente al Departamento de Protección Ambiental de la Estación Experimental del Zaidín (CSIC), gracias a una beca JAE Predoc del CSIC concedida a Sophie Marie Martirani Von Abercron, cuya Investigación ha sido financiada por los proyectos BIO-2011-23615 del MICINN y P08-CVI03591 de la Junta de Andalucía.

Parte de los resultados de este trabajo han sido presentados en los siguientes congresos y publicaciones:

Publicaciones

Acosta-González A, Martirani-Von Abercron SM, Rosselló-Mora R, Wittich RM & Marqués S. The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the *Prestige* oil spill. *Env Sci Pollut Res*, **22**:15200-15214 (2015). DOI: 10.1007/s11356-015-4458-y

Martirani-Von Abercron SM, Pacheco D, Benito-Santano P, Marín P and Marqués S. Polycyclic Aromatic Hydrocarbon-Induced Changes in Bacterial Community Structure under Anoxic Nitrate Reducing Conditions. *Front Microbiol* **7:**1775 (2016)**.** DOI:10.3389/fmicb.2016.01775.

Martirani-Von Abercron SM, Marín P, Solsona-ferraz M, Castañeda-Cataña MA and Marqués S. Naphthalene biodegradation under oxygen limiting conditions: changes in the bacterial community and relevance of biofilm formation capacity. *Microb Biotechnol*, 2017 DOI: 10.1111/1751-7915.12842

Congresos

Martirani Von Abercron SM, Acosta González A, Rosselló-Mora R, Rodríguez-Garcia I and S Marqués. Anaerobic PAHs degradation: diversity of naphthalene degrading bacteria from marine polluted sediments. FEMS, 4th Congress of European Microbiologists, Geneva, Suiza, 26-30 de junio de 2011.

Martirani Von Abercron SM, Acosta González A, Rosselló-Mora R and S Marqués. Microbial diversity in polluted sediment focusing on nitrate-reducing communities. Environment workshops 2011, Metagenomics and Environmental Microbiology, UNIA, Baeza, Spain. 3-5 October 2011

Rama-Garda R, Pacheco D, Martirani Von Abercron SM, Marín P, Acosta-González A & Marqués S. Occurrence and diversity of the novel oxidative pathway involved in the anaerobic degradation of dihydroxylated aromatic compounds. FEMS, 5th Congress of European Microbiologists, Leipzig, Germany, July 21-25, 2013.

Martirani Von Abercron SM, Marín, P, Solsona-Ferraz, M and Marqués, S. Characterization of a naphthalene-degrading multi-species biofilm isolated from oil-contaminated ground water. FEMS, 6th Congress of European Microbiologists, Maastricht, the Netherlands, June 7-11, 2015.

Martirani Von Abercron SM, Marqués S and Wittich RM. Towards the anaerobic-aerobic bacterial mineralization of chlorendic acid. FEMS, 6th Congress of European Microbiologists, Maastricht, the Netherlands, June 7-11, 2015.

Marqués S, Martirani Von Abercron SM, Marín P, Solsona M y Castañeda M. Biodegradación de hidrocarburos aromáticos policíclicos en condiciones de limitación de oxígeno. VI Congreso De Microbiología Industrial Y Biotecnología Microbiana (CMIBM). León (España) 12-14 de septiembre de 2016.

Martirani von Abercron SM, Marín P, Solsona M, Castañeda M and Marqués S. Production of bacterial added-value polymers from polycyclic aromatic hydrocarbons (PAHs). BioRemid 2017, International Meeting on New Strategies in Bioremediation Processes, supported by the Group of Biodegradation, Bioremediation and Biodeterioration (BBB) of the Spanish Society of Microbiology (SEM), Granada, Spain, 9-10 March 2017.

A mi queridísima y especial familia
Giuliana, Charles, Giovanna, Martin, Elena y Zia Maria

A mí 'familia personal', mis amores
Gaetano, Jonathan y Christian

No te deseo un regalo cualquiera,
te deseo aquello que la mayoría no tiene,
te deseo tiempo, para reír y divertirte.
Si lo usas adecuadamente, podrás obtener lo que quieras.

Te deseo tiempo para tu quehacer y tu pensar,
no sólo para ti mismo,
sino también para dedicárselo a los demás.

Te deseo tiempo, no para apurarte y andar con prisas,
sino para que siempre estés contento.

Te deseo tiempo, no sólo para que transcurra, sino para que se quede:
tiempo para asombrarte y tiempo para tener confianza,
y no sólo para que lo veas en el reloj.

Te deseo tiempo para tener esperanza otra vez,
y para amar; no tiene sentido añorar.

Te deseo tiempo para que te encuentres contigo mismo.
Para vivir cada día, cada hora, cada minuto, como un regalo.

También te deseo tiempo para perdonar y aceptar.

Te deseo de corazón que tengas tiempo,
tiempo para la vida, y para tu vida.

Elli Michler

Agradecimientos

Esta aventura empezó hace algunos años, cuando decidí seguir con la investigación después de la carrera. Aunque no muchos entendían porqué quería seguir investigando, porqué quería seguir siendo un ‘estudiante’, y no encontrar un trabajo ‘normal’ en mi ciudad, en mi país, yo deseaba seguir descubriendo, seguir aprendiendo, seguir buscando respuestas, seguir mi pasión. Al final no sé cuántas respuestas concretas he encontrado, muy pocas probablemente, más bien tengo muchas más preguntas e incertidumbres. Pero sí he crecido como persona, y ¡mi corazón se ha enriquecido gracias a todas las personas que he encontrado por el camino!

La primera fue Silvia…un sábado durante las vacaciones de navidad recibió mi CV y el lunes me contestó con algunas preguntas y luego: ¡venga vamos a probar las becas! Me diste tu confianza en seguida junto con un proyecto que yo veía mucho más grande que mis posibilidades, porque no sabía nada, tenía que aprender mucho, partiendo del idioma. He hecho muchos errores, pero tú me dejaste hacerlos, me dejaste pensar con mi cabeza, sin decirme nunca que tenía que hacer. A veces me has dicho, también un poco enfadada: ‘¡Sophie tienes que pensar!’. Alguna vez me pregunté: ‘¿porque no me dice que tengo que hacer y punto?’, porque han sido muchos los momentos que me sentía perdida…hubiera sido más fácil ¡pero no lo correcto! Eres una persona muy justa y siempre dices lo que piensas, y esto lo hace todo más fácil, aunque también más duro, porque lo más difícil es aceptar y mejorar lo que somos. Tu nos enseñas a reflexionar con nuestras cabezas, a hacer nuestros errores, nos dejas caer así que podamos levantarnos más fuertes...y tú estás ahí desde fuera, y cuando acudimos a ti por ayuda siempre nos la das...también desde lejos, cuando me he tenido que ir por trabajo y por mi familia has estado ahí dándome caña y ánimo y sobre todo toda la ayuda que he necesitado. ¡¡Gracias!!

Luego he entrado en el laboratorio 316 donde he tenido la suerte de encontrar una ¡FAMILIA!
El primero que he conocido fue Alejo en Sevilla. ¡Alejo te he querido y te quiero muchísimo! Has sido mi referencia por mucho tiempo…no sé cuantas preguntas te echo, y tú siempre has tenido la paciencia de contestarme y de enseñarme todo lo que sabías. ¡Con tu Sofí y Mija me habéis acogido en vuestra casa como una hermana! Águeda tu amistad, tus risas y alegría todavía me llenan el corazón, has estado ahí en mis momentos de bajón dándome animo con tu positividad. Moha…el hombre HPLC…que decirte…has sido mi amigo, compañero, hermano, me has acompañado en todos momentos en lab: ayudándome en el trabajo, discutiendo juntos de los bichos anaerobios y del naftaleno, haciéndome partir de las risas, con tus billetitos de buenos días en todos lados y tus ’Ispecciónes’…jajajaja…¡no sabes cuánto te hemos echado de menos en el lab! ¡Eres una de las personas más lista que conozco, espero verte pronto!

Y luego están ellos…lo que han estado conmigo desde el primer día hasta el último, y siguen en mi vida, en mi corazón cada día: ¡Patri y Pacheco! No creo que unas pocas líneas puedan expresar lo que tengo en mi corazón. Me habéis acogido juntos con Antonio y Mónica en vuestras casas como una más de vuestra familia. ¡Os Quiero con locura! Patri he tardado en conocerte, eres reservada. Nuestra amistad ha ido intensificándose poco a poco, hasta que entraste en mi corazón y yo te di el mío, muchas veces un poco roto para que me lo arreglaras. Me siento privilegiada al haberte conocido, tu generosidad e inteligencia me sorprenden cada día. ¡No he visto a nadie trabajar como tú en lab! Eres mi amiga, hermana y consejera. Muchas veces me pregunto ¿que haría Patri? Sobre todo cuando no sé que hacer con mis niños :)

Pachequito mío, hemos empezado juntos esta aventura y seguimos en ello. Has estado conmigo desde el primer día, cuando no conocía a nadie ni sabía el idioma ni nada y me hiciste sentir como en casa

abriéndome la tuya. En seguida me quisiste, aunque decías que no querías a nadie. :D Eres mi hermano, mi sabio hermano mayor, tus palabras y tus consejos los tengo bien guardados. Me has dicho siempre lo que piensas sin reservas, y también nos hemos peleado a veces, ¿pero acaso no es esto lo que hacen las personas que se quieren? Patri y Pacheco gracias por cuidar de mí incluso en la distancia.

No puedo olvidarme de todos los 'pollitos' que han pasado en el lab. Ruth, me has acompañado en mi primer año, juntas hemos empezado los experimentos de anaerobios. Ramon, no olvidaré la ayuda que me diste cuando estaba con mi barriguita, y sobre todo las tartas de chocolate que hiciste para mis antojos :) ¿¡¡¿ah y recuerdas de los NMP y DGGE?!!? Mi Patri chica: ¡gracias por tu ayuda sin fin y tu cariño! Eres una trabajadora única. Alberto te quiero muchísimo, me acuerdo todavía cuando llorabas como un niño chico y un poco borracho en mi boda porque te ibas del lab. ¡Eres especial! Marta, tu fuerza, tu sonrisa y determinación a pesar de las pruebas que la vida te ha dado me siguen asombrando, ¡eres muy valiente!

Quiero agradecer también a todo el grupo de degradación de tóxicos, y el resto de la EEZ. Si me olvido de algunos ¡perdonadme!

A Miriam gracias por tu amistad, tu cariño, tus abrazos, tu sonrisa, tu fuerza, tu apoyo en todo momento, por nuestras charlas sobre la vida, muchas veces necesitaba otro punto de vista. ¡Te echo de menos y te quiero! A Patri B. por abrirme las puertas de tu casa y acogerme, por tu amistad, tu afecto, tu dulzura y por compartir tantos momentos conmigo. ¡Te he extrañado mucho cuándo te fuiste a Londres! A Carlos por tu amistad desde el primer momento, por tu disponibilidad a ayudarme siempre y por tu animo tan gentil. A Ali por tu sonrisa contagiosa en todos momentos, y sobre todo por tu sonrisa, optimismo y energía en los momentos más difíciles. A Joaquín… has llegado más tarde, pero hemos compartido tantos momentos. Gracias por tu amistad, tu cariño, tu alegría y por las palabras bonitas que me has dedicado. A Cristi y Josemi por nuestros desayunos llenos de risas que me han recargado cada día y por vuestra disposición a ayudar. A Jesús por ayudarme y por enseñarme tantas cosas de ciencia y no. A Oscar por tu energía, fuerza y sonrisa siempre en la cara. A Laura por tu amistad y por escucharme muchas veces. A Silvia por estar siempre disponible y por hacerme reír tanto. A María por tu simpatía y cariño, eres muy fuerte y paciente como no he visto nunca. A Miguel que he conocido en los últimos meses y que me ha regalado tanta alegría.

A Zulema, Saray, Marta, Vero, Mati, Lázaro, Alicia, Álvaro, Andrés, Jose, Nené, Marta, Sara, Bertrand, Sandy, Ana María, Adela por todos los momentos que he tenido la suerte de compartir con vosotros, cada uno me ha dejado su huella.

A M. Angustias por tener el cariño de una mamá y por hacer cada día más fácil y ligero.

A los jefes: Juan Luis, no he visto jefe más listo y carismático. A pesar de tu poco tiempo siempre has tenido unas palabras para dedicarnos, de ciencia o de apoyo. Estrella, tu cariño y atenciones me han acompañado desde mi primer día en Granada. Regina, gracias por tu tiempo y consejos sobre los anaerobios y sus rutas y por preocuparte por mí. Pieter, gracias por ser siempre disponible a enseñarme todo lo que sabes sobre la piro y el QIIME y por darme animo cuando lo he necesitado. Echaré de menos nuestras charlas en el servidor :) Marian, eres un ejemplo de mujer, madre e investigadora. Gracias por darme fuerza y apoyo. Tino, gracias por tus consejos y confianza. Ana gracias por tu ayuda y sugerencias. Maribel y Manolo gracias por vuestras sugerencias y por compartir vuestro conocimiento.

A Bea por tu amistad y afecto. Por escucharme y apoyarme todas las veces que lo he necesitado.

A Pao, la persona más dulce que he conocido nunca. Eres encantadora y me has regalado tu cariño y afecto desde el primer momento que nos conocimos. No pierdas nunca esta sonrisa tierna que te hace una persona maravillosa.

A Rafa mil gracias por estar siempre disponible a explicarme con paciencia una y otra vez y con una sonrisa todo sobre el GC-MS, para darme tantas ideas diferentes y para dedicarme tanto tiempo. Haces tu trabajo con tanta dedición e ilusión.

A Javi y César por ser siempre disponibles con todas mis dudas y problemas informáticos.

A mi familia 'napolitana' en Granada: Chiara y Salvatore. Chiara, mí Amiga, mi alma gemela, ya son 16 años de amistad pura y sincera, contigo no necesito decir una palabra, porque tienes la capacidad de leerme dentro. Sin ti nunca habría venido a Granada, y haberte tenido a mi lado ha sido un privilegio. Salvatore se dice 'quien encuentra un amigo encuentra un tesoro'. Pues contigo ¡ya soy la mujer más rica del mundo! Has estado y estas siempre, y digo siempre ahí todas las veces que hemos necesitado ayuda. Las veces me ha hecho reír de corazón son infinitas. Estar lejos de la familia, sobre todo con un niño y trabajando, es duro, pero vosotros lo habéis hecho mucho más llevadero. ¡Chicos os quiero con toda mi alma y no sabéis cuanto os echo de menos!

A mi familia tan querida y tan especial. A mi mamá por ser la persona más valiente que he conocido, por ser un modelo que seguir, por no rendirte nunca, por darme tu amor incondicionado siempre, por transformarme en la mujer que soy, por confiar siempre en mí, por dejarme soñar mis sueños, por apoyarme en todo momento…¡¡Gracias!! A mi hermano, la persona más generosa y lista que conozco, gracias por quererme a tu lado, por amarme tanto, por confiar ciegamente en mí, por hacerme ver el mundo desde otra perspectiva, por hacerme reflexionar tanto y por estimularme siempre a mejorar…eres mi orgullo, mi fuerza… contigo a mi lado siempre me sentiré segura y protegida…¡te quiero y te cuidaré siempre! A Giovanna, Martin y Elena por vuestro amor tan infinito, por la alegría y por toda la felicidad que me regaláis cada vez que os veo. ¡Ha sido muy difícil estar lejos de vosotros! A mi tía María, eres una madre y una confidente, la persona más buena del mundo. No hay día sin ti, sin tu voz, sin tu dulce sonrisa impresa en mi mente…estarás siempre en mi corazón, en un lugar especial y seguro.

A mis amores, mis hombres, mi todo…Gaetano son 13 años que estas a mi lado, en cualquier lugar del mundo tu estas ahí…primero en Nápoles, y luego en Australia, en Suecia, en Granada, en Albania, en Ibiza y dondequiera sé que tu estarás. Tu prioridad ha sido siempre amarme y crecer juntos, por esto viniste a Granada donde hemos construido nuestro hogar, donde hemos iniciado nuestra familia con el don más grande que la vida nos podía regalar: Jonathan. Gracias por ayudarme tanto a seguir adelante con la tesis, porque si este año he parido dos veces es también gracias a ti. Gracias por darme fuerza cada día, gracias por aceptarme por lo que soy con todas mis debilidades, gracias por alejar de mi la tentación de rendirme, gracias por ser la constante en mi vida, gracias por tu comprensión, gracias por hacer mi vida aún más maravillosa. Y a ti, mi Principito, este año han sido demasiadas las veces que te he tenido que decir que no, sea por el trabajo, por la tesis o por el embarazo…pero tú estabas siempre con una sonrisa diciéndome que estabas enamorado de mí, que la cosa más bella soy yo, que lo más bonito es darme abracitos y besitos. Gracias por enseñarme tanto cada día, por demostrarme que realmente el amor puede ser infinito y que una sonrisa basta para ser feliz. Y por último a ti, mi pequeño gordito, que me has acompañado más que nadie en la escritura de la tesis, y que ya sabes muchas cosas sobre el naftaleno, gracias por recordarme con tus pataditas que la vida es un milagro y hay que cuidarlo.

Contents

FIGURE INDEX IV
TABLE INDEX IV
LIST OF ABBREVIATIONS VII
RESUMEN IX
SUMMARY XI

I. GENERAL INTRODUCTION 1

1. THE ENVIRONMENTAL QUESTION 3
2. POLYCYCLIC AROMATIC HYDROCARBONS 4
   2.1. General properties 4
   2.2. Physicochemical properties 5
   2.3. Toxicity 6
3. PAHS IN THE ENVIRONMENT 7
4. BIOREMEDIATION OF PAHS 9
   4.1. Novel bioremediation strategies 11
5. PAH DEGRADATION BY BACTERIA 12
   5.1. Aerobic PAH degradation 13
   5.2. Anaerobic PAH degradation 18
   5.2.1. Anaerobic naphthalene degrading microorganisms. 21
   5.2.2. Anaerobic naphthalene biodegradation mechanisms 22
6. MICROBIAL DIVERSITY 25
7. MICROBIAL COMMUNITY ANALYSIS 27
   7.1. Culture dependent methods 27
   7.2. Culture independent methods 28
8. REFERENCES 31

II. OBJECTIVES 41

III. GENERAL METHODS 45

1. CULTURE CONDITIONS, ENRICHMENT AND ISOLATION PROCEDURES 47
2. MOST PROBABLE NUMBER ENUMERATION OF DENITRIFYING BACTERIA 48
3. CHEMICAL ANALYSIS OF HYDROCARBON CONTENT 48
4. TOTAL DNA EXTRACTION, PCR AMPLIFICATION AND PYROSEQUENCING LIBRARY CONSTRUCTION 49
5. 16S RRNA GENE AMPLIFICATION 50
6. DATA ANALYSIS 50
7. REFERENCES 51

IV. RESULTS AND DISCUSSION 53

CHAPTER 1 55

POLYCYCLIC AROMATIC HYDROCARBON-INDUCED CHANGES IN BACTERIAL COMMUNITY STRUCTURE UNDER ANOXIC NITRATE REDUCING CONDITIONS. 55

1.1. INTRODUCTION 59
1.2. MATERIALS AND METHODS 61
   1.2.1. Sample collection and experimental design 61
   1.2.2. Culture conditions, enrichment and isolation procedures 61
   1.2.3. Most probable number enumeration of denitrifying bacteria 63
   1.2.4. Chemical analysis 64
   1.2.5. Total DNA extraction, PCR amplification and pyrosequencing library construction 65
   1.2.6. Functional gene amplification 65
   1.2.7. 16S rRNA gene amplification 66
   1.2.8. Data Analysis 66
   1.2.9. Nucleotide sequence accession numbers 67
1.3. RESULTS AND DISCUSSION 68
   1.3.1. Presence of a nitrate-reducing, PAH-degrading bacterial community in environmental samples 68
   1.3.2. Bacterial community structure in the selected environments 70

1.3.3. *General effect of PAHs on the bacterial community structure* ... 74
1.3.4. *Effect of PAHs on specific bacterial populations* ... 77
1.3.5. *Isolation of nitrate reducing bacteria from PAH enrichments* ... 81
1.3.6. *Aromatic anaerobic degradation genes* ... 82
1.4. CONCLUDING REMARKS ... 83
1.5. REFERENCES ... 84
1.6. SUPPLEMENTARY MATERIAL ... 89
1.6.1. *Supplementary tables* ... 89
1.6.2. *Supplementary figures* ... 97

CHAPTER 2 ... 103

MICROBIAL COMMUNITIES IN FUENTE DE PIEDRA ATHALASSOHALINE LAGOON SEDIMENTS: IMPACT OF THE CONTAMINATION WITH NAPHTHALENE. ... 103

2.1. INTRODUCTION ... 107
2.2. MATERIALS AND METHODS ... 109
2.2.1. *Site description and Sampling* ... 109
2.2.2. *Microcosm experiment* ... 109
2.2.3. *Culture conditions and anaerobic enrichment* ... 111
2.2.4. *Total DNA extraction* ... 112
2.2.5. *Illumina sequencing and Data Analysis* ... 114
2.3. RESULTS AND DISCUSSION ... 115
2.3.1. *Effect of naphthalene on bacterial community structure and diversity in the microcosm.* ... 115
2.3.2. *Microcosms bacterial community structure* ... 117
2.3.3. *Effect of microcosm incubation on bacterial communities* ... 119
2.3.4. *Bacterial community evolution in naphthalene enrichments* ... 122
2.3.5. *Patterns of microbial diversity in the anaerobic enrichments* ... 125
2.3.5.1. *First sampling campaign: enrichments with PAHs dissolved in HMN* ... 125
2.3.5.2. *Second sampling campaign: enrichment with naphthalene provided as crystals.* ... 127
2.4. CONCLUDING REMARKS ... 130
2.5. REFERENCES ... 131
2.6. SUPPLEMENTARY MATERIAL ... 136

CHAPTER 3: ... 137

NAPHTHALENE BIODEGRADATION UNDER OXYGEN LIMITING CONDITIONS: COMMUNITY DYNAMICS AND THE RELEVANCE OF BIOFILM FORMATION CAPACITY ... 137

3.1. INTRODUCTION ... 141
3.2. EXPERIMENTAL PROCEDURES ... 143
3.2.1. *Sampling and site description.* ... 143
3.2.2. *Most Probable Number enumeration of bacteria.* ... 143
3.2.3. *Culture conditions, enrichment, and isolation procedures.* ... 144
3.2.4. *Measurement of oxygen concentration in the culture bottles.* ... 145
3.2.5. *Synthetic microbial community.* ... 146
3.2.6. *Chemical analysis.* ... 146
3.2.7. *Total DNA extraction, 16S rRNA gene 454-Pyrosequencing and data analysis.* ... 146
3.2.8. *Full-length 16S rRNA gene amplification and clone library construction.* ... 147
3.2.9. *Functional gene amplification.* ... 147
3.2.10. *RT-PCR assays.* ... 148
3.2.11. *Biofilm quantification in multi-well plates (crystal violet assay).* ... 148
3.2.12. *Scanning electron microscopy (SEM).* ... 149
3.2.13. *Nucleotide sequence accession numbers.* ... 149
3.3. RESULTS AND DISCUSSION ... 150
3.3.1. *Betaproteobacteria are dominant in a hydrocarbon polluted aquifer* ... 150
3.3.2. *Changes in the community structure under strictly anoxic denitrifying conditions.* ... 154
3.3.3. *Microaerophilic conditions strongly select for two dominant strains.* ... 156
3.3.4. *A pathway with high affinity for oxygen is favoured under microaerophilic conditions.* ... 160
3.3.5. *Evolution of a synthetic community under microaerophilic conditions* ... 164

3.3.6. Aerobic growth allows higher bacterial diversity ... 164
3.3.7. Starkeya novella strain N1B is responsible for biofilm formation and naphthalene degradation. ... 165
3.4. Concluding remarks ... 166
3.5. References ... 167
3.6. Supplementary material ... 171
3.6.1. Supplementary tables ... 171
3.6.2. Supplementary figures ... 175

V. GENERAL DISCUSSION ... 179

1. Anaerobic naphthalene degradation: the problem. ... 182
2. Changes in the microbial communities: effect of the culture conditions. ... 184
3. Changes in the microbial communities: effect of PAHs. ... 186
4. Naphthalene-degrading communities under microaerophilic conditions. ... 188
5. References ... 189

VI. CONCLUSIONES ... 193

Figure index

GENERAL INTRODUCTION

FIGURE 1. STRUCTURES AND NOMENCLATURES OF THE 16 PAHS ON THE EPA PRIORITY POLLUTANT LIST 5
FIGURE 2. GENE ORGANIZATION OF THE NAPHTHALENE DEGRADATION PATHWAY IN NAH7 PLASMID OF PSEUDOMONAS PUTIDA G7 14
FIGURE 3. PROPOSED CATABOLIC PATHWAYS OF NAPHTHALENE BY AEROBIC BACTERIA 15
FIGURE 4. PATHWAY OF ANAEROBIC TOLUENE AND BENZOATE METABOLISM TO BENZOYL-COA IN THAUERA AROMATICA 20
FIGURE 5. GENE PRODUCTS INVOLVED IN THE BENZOYL-COA DEGRADATION PATHWAY IN FACULTATIVE AND OBLIGATE ANAEROBES 21
FIGURE 6. PROPOSED PATHWAYS FOR ANAEROBIC NAPHTHALENE AND 2-METHYLNAPHTHALENE DEGRADATION IN THE ENRICHMENT CULTURE N47........ 24
FIGURE 7. 16S RIBOSOMAL RNA SECONDARY STRUCTURE MODEL AND ITS VARIABLE REGIONS. 29

RESULTS AND DISCUSSION

Chapter 1

FIGURE 1. 1. MPN ENUMERATION IN THE ENVIRONMENTAL SAMPLES OF NITRATE REDUCING BACTERIA ABLE TO GROW ON DIFFERENT PAHS........ 69
FIGURE 1. 2. CUMULATIVE PLOT OF BACTERIAL PHYLA DETECTED IN THE INITIAL ENVIRONMENTAL SAMPLES 72
FIGURE 1. 3. CUMULATIVE PLOT OF BACTERIAL PHYLA DETECTED IN THE DIFFERENT ENRICHMENTS. 75
FIGURE 1. 4. PRINCIPAL COORDINATE ANALYSIS (PCOA) AND UNWEIGHTED PAIR GROUP METHOD WITH ARITHMETIC AVERAGE (UPGMA IN THE INITIAL ENVIRONMENTAL SAMPLES AND THE ENRICHMENTS OBTAINED FROM THEM 76
FIGURE 1. 5. EFFECT OF PAHS ON THE RELATIVE ABUNDANCE OF THE MOST RELEVANT GROUPS IN THE COMPOST PILE (CP) SAMPLE 77

Chapter 2

FIGURE 2. 1. CORES USED IN THIS WORK FOR THE MICROCOSM EXPERIMENT, ENRICHMENT CULTURES AND ILLUMINA-SEQUENCING AT THE START OF THE EXPERIMENT 110
FIGURE 2. 2. SCHEME OF EXPERIMENTAL SET-UP 113
FIGURE 2. 3. BOXPLOTS OF THE OBSERVED OTUS PER RANDOMIZED SAMPLE IN THE CONTROL AND NAPHTHALENE CORES AT DIFFERENT INCUBATION TIMES 116
FIGURE 2. 4. ANALYSIS OF MICROCOSM MICROBIAL COMMUNITIES WITH WEIGHTED AND UNWEIGHTED UNIFRAC DISTANCE.. 117
FIGURE 2. 5. RELATIVE ABUNDANCE OF BACTERIAL PHYLA OR CLASS (ONLY FOR *PROTEOBACTERIA*) IN THE FUENTE DE PIEDRA SEDIMENT MICROCOSMS 119
FIGURE 2. 6. EFFECT OF MICROCOSM INCUBATION ON SPECIFIC BACTERIAL POPULATION AT ORDER LEVEL. 120
FIGURE 2. 7. MICROBIAL COMMUNITY ANALYSIS OF THE SEDIMENT SAMPLES AND ENRICHMENT CULTURES USING WEIGHTED AND UNWEIGHTED UNIFRAC DISTANCE 124
FIGURE 2. 8. RELATIVE ABUNDANCE OF BACTERIAL PHYLA OR CLASS (ONLY FOR *PROTEOBACTERIA*) IN THE FUENTE DE PIEDRA ENRICHMENTS.. 126

Chapter 3

FIGURE 3. 1. AQUIFER SAMPLE SHOWING A VISIBLE BIOFILM GROWING AT THE INTERFACE BETWEEN THE OIL AND THE WATER LAYERS. 143
FIGURE 3. 2. OXYGEN CONCENTRATION IN THE MICROAEROPHILIC CULTURE BOTTLES USED IN THIS STUDY 145
FIGURE 3. 3. ALIPHATIC HYDROCARBON DISTRIBUTION (%) IN THE INITIAL CONTAMINATED AQUIFER SAMPLE 151
FIGURE 3. 4. MPN ENUMERATION IN THE AQUIFER INITIAL SAMPLE OF NITRATE REDUCING AND AEROBIC BACTERIA ABLE TO GROW ON DIFFERENT PAHS AS CARBON SOURCE. 152
FIGURE 3. 5. CUMULATIVE PLOT OF BACTERIAL PHYLA DETECTED IN THE INITIAL ENVIRONMENTAL SAMPLES AND IN THE ANAEROBIC ENRICHMENTS AMENDED WITH NAPHTHALENE (NAP), 2-METHYLNAPHTHALENE (2MN) AND HEPTAMETHYLNONANE (HMN). 155
FIGURE 3. 6. BACTERIAL BIOFILM DEVELOPED AROUND THE NAPHTHALENE CRYSTAL.: ENRICHMENT CULTURE BOTTLE AND SCANNING ELECTRON MICROSCOPY (SEM) MICROGRAPH 157
FIGURE 3. 7. CUMULATIVE PLOT OF BACTERIAL TAXONS PRESENT AT MORE THAN 1% OF THE COMMUNITY DETECTED IN THE MICROAEROPHILIC, AEROBIC AND SYNTHETIC MICROAEROPHILIC ENRICHMENTS AMENDED WITH CRYSTALS OF NAPHTHALENE AS CARBON SOURCE.. 158
FIGURE 3. 8. NEIGHBOUR-JOINING TREE OF THE 16S RRNA GENE OF THE BACTERIAL STRAINS ISOLATED IN THIS STUDY AND THEIR CLOSEST RELATIVES IN THE DATABASES 159
FIGURE 3. 9. PHYLOGENY OF THE ALPHA SUBUNIT OXYGENASE COMPONENT OF HYDROXYLATING NAPHTHALENE DIOXYGENASE AND GENTISATE 1,2-DIOXYGENASE PARTIAL AMINO ACID SEQUENCE RETRIEVED FROM SUBCULTURE 5 AND DIFFERENT ISOLATES OBTAINED IN THIS STUDY. 163
FIGURE 3. 10. RT-PCR ANALYSIS OF TOTAL RNA EXTRACTED FROM SUBCULTURE 15 163
FIGURE 3. 11. BIOFILM FORMATION AT THE AIR/LIQUID INTERPHASE BY DIFFERENT ISOLATES IN LB OR LB SUPPLEMENTED WITH A NAPHTHALENE CRYSTAL 165

Table index

GENERAL INTRODUCTION

TABLE 1. PHYSICOCHEMICAL AND CANCEROGENIC PROPERTIES OF 16 PAHS ENLISTED AS PRIORITY POLLUTANTS BY US EPA 6
TABLE 2. POSSIBLE FATE OF PAHS IN THE ENVIRONMENT 8
TABLE 3. PROKARYOTIC ABUNDANCE DETERMINED BY TOTAL GENOMIC DIVERSITY IN PROKARYOTIC COMMUNITIES CALCULATED FROM THE REASSOCIATION RATE OF DNA ISOLATED FROM THE COMMUNITY.... 26

RESULTS AND DISCUSSION

Chapter 1

TABLE 1. 1. CHARACTERIZATION OF THE SAMPLES USED IN THIS STUDY. 62
TABLE 1. 2. COMPARISON OF OTU NUMBER, DIVERSITY, EVENNESS INDICES AND COVERAGE FOR THE DIFFERENT SAMPLES. 71
TABLE 1. 3. RELATIVE ABUNDANCE OF *ACIDOBACTERIA* IN THE INITIAL SAMPLES AND ENRICHMENT CULTURES. 78

Chapter 2

TABLE 2. 1. PROFILE DESCRIPTION OF THE CORES COLLECTED IN THE FIRST CAMPAIGN. 110
TABLE 2. 2. GRAVIMETRIC WATER CONTENT IN THE DIFFERENT CORES LAYERS (P1 TO P4) AND IN THE MIXED LAYER (P1 TO P4).... 111
TABLE 2. 3. COMPARISON OF OTU NUMBER, DIVERSITY, EVENNESS INDICES AND COVERAGE FOR THE DIFFERENT CORES SAMPLES.... 115
TABLE 2. 4. COMPARISON OF OTU NUMBER, DIVERSITY, EVENNESS INDICES AND COVERAGE FOR THE ENRICHMENT CULTURE OF THE FIRST AND SECOND SAMPLING EVENT.... 122

Chapter 3

TABLE 3. 1. AROMATIC HYDROCARBON COMPOSITION AND ABUNDANCE IN THE CONTAMINATED AQUIFER INITIAL SAMPLES. 150
TABLE 3. 2. CHARACTERIZATION OF THE INITIAL POLLUTED AQUIFER SAMPLE. 150
TABLE 3. 3. COMPARISON OF OTU NUMBER, DIVERSITY, EVENNESS INDICES AND COVERAGE FOR THE DIFFERENT SAMPLES. 153
TABLE 3. 4. STRAINS ISOLATED FROM THE MICROAEROPHILIC ENRICHMENT CULTURES.... 161

List of abbreviations

2MN	2-methylnaphthalene
2NA	2-naphtoic acid
Ace	Acetate
ANT	Anthracene
AS	Activated sludge
Bss	Benzylsuccinate synthase
CODEHOP	Consensus degenerate hybrid oligonucleotide primers
CP	Composting pile
DHNCR	5,6-dihydro-2-naphthyl-CoA reductase
EEA	European environment agency
EPA	Environmental protection agency
EPS	Extracellular polymeric substances
FdP	Fuente de Piedra
FISH	Fluorescence *in situ* hybridization
GMOs	Genetically modified organisms
HMN	2,2,4,4,6,8,8-heptamethylnonane
HMW	High molecular weight
LB	Luria-Bertani medium
LMW	Low molecular weight
MID	Multiplex identifier
MOPS	3-(N-morpholino)propanesulfonic acid
MPN	Most probable number
MS	Marine sediment sample
NCR	2-naphthoyl-CoA reductase
Nms	**N**aphthyl-2-methyl succinate synthase
NRB	Nitrate reducing bacteria
OM	Organic matter
OUT	Operational taxonomic unit
OYE	Old yellow enzymes
PAHs	Polycyclic aromatic hydrocarbons
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
QIIME	quantitative insights into microbial ecology
RPCal	Rice-paddy in Calasparra
RPS	Rice-Paddy Soil
RPW	Rice-Paddy Water
RT-PCR	Reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
SMM	Seawater minimal medium
SRB	Sulphate reducing bacteria
TCA	Tricarboxylic acid cycle
THNCR	5,6,7,8-tetrahydro-2-naphthoyl-CoA
UPGMA	Unweighted pair group method with arithmetic mean

Resumen

El uso masivo del petróleo como fuente de energía y como materia prima ha generado serios problemas de contaminación ambiental. Entre los principales contaminantes ambientales, los compuestos aromáticos generan gran preocupación porque son relativamente persistentes en el medio ambiente debido a la alta estabilidad termodinámica del anillo de benceno. El benceno y muchos hidrocarburos aromáticos policíclicos (HAPs) presentan efectos tóxicos, carcinogénicos, mutagénicos y teratogénicos y su eliminación del medio ambiente es importante para proteger el entorno y la salud humana. Una medida que ha tenido un éxito significativo es el empleo de microorganismos para la eliminación de contaminantes con la aplicación de técnicas de biorremediación. Los procesos aerobios de degradación de HAPs se han caracterizado exhaustivamente durante los últimos años; sin embargo, en muchos ambientes naturales la concentración de oxígeno es limitante y, en estas condiciones, los procesos activos son esencialmente anaerobios. La degradación anaeróbica bacteriana de los compuestos monoaromáticos se ha caracterizado en profundidad; no obstante, la degradación de los hidrocarburos aromáticos policíclicos (HAPs) como el naftaleno sólo ha comenzado a ser comprendida en las bacterias reductoras de sulfato, y se sabe muy poco sobre la degradación anaeróbica de los HAPs en bacterias nitrato reductoras. Partiendo de una serie de ambientes que habían sufrido diferentes grados de contaminación por hidrocarburos, utilizamos la técnica del número más probable (NMP) para detectar y cuantificar la presencia de comunidades bacterianas capaces de degradar varios HAPs usando el nitrato como aceptor de electrones. Con el NMP se detectó la presencia de una comunidad nitrato reductora capaz de degradar naftaleno, 2-metilnaftaleno (2MN), y antraceno en algunos de los sitios muestreados. Además, con el fin de aislar cepas capaces de degradar los HAPs en condiciones desnitrificantes, establecimos una serie de cultivos de enriquecimiento con nitrato como aceptor terminal de electrones y HAP como única fuente de carbono y seguimos los cambios en las comunidades bacterianas a lo largo del proceso. Los resultados evidenciaron cambios atribuibles al régimen de respiración de nitrato impuesto, que en varias muestras se exacerbó en presencia de los HAPs. La presencia de naftaleno o 2MN enriqueció a la comunidad en grupos de organismos no cultivados y mal caracterizados, y notablemente en el grupo no cultivado de *Acidobacteria iii1-8*, que en algunos casos era sólo un componente menor de las muestras iniciales. Otros taxa seleccionados por HAPs en estas condiciones incluyeron *Bacilli*, que fueron enriquecidos en los cultivos con naftaleno. Varias cepas nitrato reductoras han sido aisladas en medio solido con naftaleno y 2MN como única fuente de carbono, aunque el fenotipo no pudo reproducirse en cultivos líquidos. El análisis de los genes funcionales de degradación anaeróbica de HAPs en las muestras originales y los cultivos de enriquecimiento no revelaron la presencia de secuencias del gen *nmsA* para la degradación anaerobia de 2MN, pero confirmaron la presencia de genes de *bssA* relacionados con la degradación anaeróbica de tolueno. En conjunto, estos resultados sugieren que la degradación de las HAPs por las bacterias reductoras de nitrato puede requerir la contribución de diferentes cepas, en condiciones de cultivo que todavía necesitan ser definidas.

Los resultados de los NMP de una de las muestras analizadas, los sedimentos anóxicos de Fuente de Piedra mostraron una comunidad importante de nitrato reductores degradadores de naftaleno y, en menor parte, de 2MN. Quisimos entonces tomar muestras para ver la respuesta de la microbiota autóctona de los sedimentos de la laguna ante la presencia de naftaleno, y además

iniciar cultivos de sulfato reductores, al ser los sedimentos ricos en sulfatos, para enriquecer degradadores potenciales de HAPs. Las comunidades bacterianas y sus cambios se analizaron mediante secuenciación masiva Illumina MiSeq. *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes* y *Firmicutes* fueron los grupos más abundantes de todos los microcosmos y también los más frecuentemente encontrados en ambientes hipersalinos. Después de 4 y 12 meses de incubación con naftaleno, las comunidades bacterianas de los microcosmos no sufrieron cambios relevantes. El factor estructurante de las comunidades no fue la presencia del contaminante sino el tiempo de incubación de los cores. Sin embargo, se observaron cambios significativos en los enriquecimientos. En particular en el medio mínimo salino diseñado para sulfato reductores hubo un aumento significativo de la familia de las *Desulfobulbaceae* (*Deltaproteobacteria*), previamente identificadas como degradadoras anaerobias de tolueno y naftaleno. En los cultivos preparados con el agua filtrada de Fuente de Piedra los *Firmicutes* fueron el taxa más enriquecido con el dominio del genero *Dethiosulfatibacter*, un grupo de bacteria probablemente relacionado con la degradación de HAPs. El uso de condiciones diferentes de cultivo ha permitido seleccionar poblaciones diversas, y en particular, el empleo de un medio natural se ha revelado una opción valida para enriquecer microorganismos procedentes de un ambiente extremo como es el de Fuente de Piedra. En cualquier caso, serían convenientes ensayos adicionales para determinar la actividad degradadora de las poblaciones seleccionadas, y detectar los posibles genes implicados en la ruta.

Además de condiciones estrictamente anaerobias para nitrato y sulfato reductores, quisimos testar condiciones de microaerofilia para aislar cepas degradadoras de HAPs. Estas condiciones son la que más se acercan, en muchos casos, a las condiciones ambientales reales, donde hay gradientes de concentración de oxígeno. Establecimos cultivos de enriquecimiento a partir de una muestra de acuífero contaminado con petróleo, utilizando condiciones anóxicas o microaerofílicas y HAPs como única fuente de carbono. A pesar de la presencia de una comunidad significativa de bacterias nitrato reductoras detectada con el NMP, la comunidad inicial, dominada por *Betaproteobacteria*, era incapaz de degradar la HAPs en condiciones anóxicas estrictas, aunque se observó un claro cambio en la estructura de la comunidad hacia un aumento en la *Alphaproteobacteria* (*Sphingomonadaceae*), *Actinobacteria* y un grupo no cultivable de *Acidobacteria* en los enriquecimientos. Por otro lado, el crecimiento en condiciones microaerofílicas con naftaleno como fuente de carbono evidenció el desarrollo de una estructura de biofilm alrededor del cristal de naftaleno. Después de varios pases, el 97% de la comunidad bacteriana y del biofilm estaba compuesto por solo dos géneros: *Variovorax* spp. (54%, *Betaproteobacteria*) y *Starkeya* sp. (43%, *Xanthobacteraceae*). Las dos cepas eran capaces de crecer con naftaleno, aunque sólo *Starkeya* tenía la habilidad de reproducir la estructura del biofilm alrededor del cristal de naftaleno. También se identificó la ruta para la degradación del naftaleno, que incluía dioxigenasas con alta afinidad por el oxígeno y que mostraba una identidad del 99% con el grupo de *dbd* de *Xanthobacter polyaromaticivorans* para la degradación de HAPs. Dada la presencia en nuestros primeros pases de los enriquecimientos de *Xanthobacter polyaromaticivorans* probablemente la ruta de degradación del naftaleno haya sido adquirida por *Starkeya* por transferencia horizontal. Por otro lado, la capacidad de formar biofilm de *Starkeya* ha favorecido la cepa en el cultivo, proporcionando una estructura compleja para situarse a una distancia apropiada de la fuente de carbono tóxico y crear un ambiente favorable al crecimiento.

En conjunto, hemos estudiado las respuestas de las comunidades bacterianas a los HAPs en las condiciones limitantes de oxígeno más frecuentemente encontradas en la naturaleza (nitrato reductoras, sulfatoreductoras y microaerofilicas) pero menos investigadas tanto por las dificultades de cultivos de estas bacterias como por los largos tiempos de crecimiento que requieren estos microorganismos. No hemos logrado aislar cepas anaerobias estrictas capaces de degradar HAPs, pero sí de individualizar poblaciones bacterianas presentes en la naturaleza y probablemente involucradas en la eliminación de contaminantes en el medio ambiente. También hay bacterias no cultivables (*v.g. Acidobacteria,* iii1-8) que se han enriquecido, de las cuales todavía desconocemos el papel ecológico, pero que podrían ser importantes en la biodegradación anaerobia. En condiciones de microaerofilia, sí hemos logrado cepas degradadoras, y además capaces de formar biofilm a partir de HAPs. El crecimiento en forma de biofilm es probablemente una manera de crecer que las bacterias adoptan en condiciones de estrés y que le ayuda a hacer frente a los factores adversos. Esto posiblemente nos indique que las condiciones de cultivo que imponemos en el laboratorio son demasiado restrictivas y se alejan de la variabilidad que hay en la naturaleza.

Summary

Polycyclic aromatic hydrocarbons (PAHs) are chemicals of particular environmental concern because of their stability, persistence in the environment and resistance to degradation. Many of them are known to be toxic to various organisms and dangerous for health. They are frequently released into the environment either from natural sources (e.g. hydrocarbon seeps) or as consequence of industrial activities such as the massive transport or the synthesis of added value chemicals. Several strategies are currently available for the remediation of aromatics contaminated sites. Among them, bacterial bioremediation stands out because of its low cost and little physical alteration of the environment, and seems especially suitable for low molecular weight aromatic compounds. The aerobic aromatic metabolism in bacteria has been studied for decades and numerous bacterial strains able to degrade aromatics in the presence of oxygen have been isolated and characterized. However, oxygen is not always available in contaminated sites, such as marine sediments, or as in initially oxic sites turned anoxic due to the high oxygen demand for aerobic degradation processes. Although bacterial anaerobic degradation of mono-aromatic compounds has been characterized in depth, the degradation PAHs such as naphthalene has only started to be understood in sulphate-reducing bacteria, and little is known about the anaerobic degradation of PAHs in nitrate-reducing bacteria. Starting from a series of environments, which had suffered different degrees of hydrocarbon pollution, we used most probable number (MPN) enumeration to detect and quantify the presence of bacterial communities able to degrade several PAHs using nitrate as electron acceptor. We detected the presence of a substantial nitrate-reducing community able to degrade naphthalene, 2-methylnaphthalene (2MN) and anthracene in some of the sites. With the aim of isolating strains able to degrade PAHs under denitrifying conditions, we set up a series of enrichment cultures with nitrate as terminal electron acceptor and PAHs as the only carbon source and followed the changes in the bacterial communities throughout the process. Results evidenced changes attributable to the imposed nitrate respiration regime, which in several samples were exacerbated

in the presence of the PAHs. The presence of naphthalene or 2MN enriched the community in groups of uncultured and poorly characterized organisms, and notably in the *Acidobacteria* uncultured group iii1-8, which in some cases was only a minor component of the initial samples. Other phylotypes selected by PAHs in these conditions included *Bacilli*, which were enriched in naphthalene enrichments. Several nitrate-reducing strains showing the capacity to grow on PAHs could be isolated on solid media, although the phenotype could not be reproduced in liquid cultures. Analysis of known PAH anaerobic degradation genes in the original samples and enrichment cultures did not reveal the presence of PAH-related *nmsA*-like sequences but confirmed the presence of *bssA*-like genes related to anaerobic toluene degradation. Altogether, our results suggest that PAH degradation by nitrate-reducing bacteria may require the contribution of different strains, under culture conditions that still need to be defined.

The MPN results of one of the Fuente de Piedra lagoon samples evidenced an important community of nitrate-reducing bacteria able to degrade naphthalene and to a lesser extent 2MN. We decided to collect new samples to follow the response of the sediment bacterial community to the presence of naphthalene. Since these sediments were rich in sulphate, we also to initiated enrichment cultures of sulphate-reducing bacteria to enrich potential PAHs degraders. The bacterial communities and the changes produced during enrichment were analyzed using Illumina MiSeq. *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes* y and *Firmicutes* were the most abundant groups in all the microcosms, as normally found in hypersaline environments. After 4 and 12 months incubation with naphthalene, the changes in the bacterial community were not significant. However, significant changes were observed during enrichment. In particular, the use of mineral salt medium designed for the selection of sulphate-reducing bacteria produced a significant increase in the *Desulfobulbaceae* family (*Deltaproteobacteria*), previously identified as toluene and naphthalene degraders. In the cultures prepared with filtered water from the lagoon, *Firmicutes* was the most enriched taxon, where the genus *Dethiosulfatibacter*, a bacterial group probably related to PAH degradation, was dominant. The use of different culturing procedures has allowed the enrichment of different communities. In particular, the use of a natural medium, such as lagoon water, as proved to be a valid approach to enrich the community in organisms originating from extreme habitats such as the Fuente de Piedra lagoon. Further assays will be required to assess the degrading capacity of the selected communities, and to detect the genes involved in naphthalene degradation.

Besides strict anoxic conditions, we also wanted to test microaerophilic conditions to isolate PAHs degrading strains. These conditions are closer to the real situation in environments polluted with hydrocarbons, where oxygen gradients are generally established. We set up a series of enrichment cultures starting from samples from a hydrocarbon polluted aquifer, using PAHs as sole carbon source. Despite the presence of a significant population of nitrate-reducing bacteria, the initial community dominated by *Betaproteobacteria* was incapable of PAH degradation under strict anoxic conditions, although a clear shift in the community towards an increase in *Alphaproteobacteria* (*Sphingomonadaceae*), *Actinobacteria* and a group of uncultured *Acidobacteria* was observed. In contrast, growth under microaerophilic conditions with naphthalene as carbon source evidenced the development of a biofilm structure around the naphthalene crystal. After several enrichment steps, 97% of the community in the biofilm was exclusively composed of two strains: *Variovorax* spp. (54%, *Betaproteobacteria*) and *Starkeya*

sp. (43%, *Xanthobacteraceae*). The two strains were able to grow on naphthalene, although only *Starkeya* was capable of reproducing the biofilm structure around the naphthalene crystal. Its pathway for naphthalene degradation was identified, which included as essential steps dioxygenases with high affinity for oxygen showing 99% identity with *Xanthobacter polyaromaticivorans dbd* cluster for PAH degradation. Our results suggest that the biofilm formation capacity of *Starkeya* favoured horizontal gene acquisition of the pathway while providing a structure to allocate its cells at an appropriated distance from the toxic carbon source.

Altogether, we have analyzed the response of the bacterial communities to the presence of PAHs in oxygen limiting conditions, which are the conditions more frequently found in real polluted environments (sulphate-reducing, nitrate-reducing and microaerophilic), but which have been less investigated both because of the difficulty to cultivate these organisms and their slow growth rates. In this study, we have been unable to isolate strict anaerobes able to degrade PAHs, although we have identified certain groups potentially involved in the elimination of pollutants in the environment. We also were able to enrich the cultures in previously uncultured groups (*i.e. Acidobacteria,* iii1-8), which could play a relevant role in biodegradation. Under microaerophilic conditions we were able to select the most efficient degraders, which in addition were able to produce a biofilm structure from PAHs. Biofilm structure are possibly a response of the bacteria to the presence of stressors, which would help them to face these conditions. This might be an indication that the culture conditions imposed in our experimental set-ups are too restrictive to recover the diversity present in natural samples.

I. GENERAL INTRODUCTION

1. The environmental question

Human activities have had an important impact on the environment, especially during the last two centuries with the increase of industrial activities. Nowadays there is a general awareness about the harmful effects of certain products and sub-products from industrial processes, and their release and disposal into the environment should be controlled or even prevented.

The oil industry is one of the most important industrial activities of our era. In fact, oil is our main source of energy, both industrial and domestic, accounting for the largest share in total primary energy supply with 36.1%, followed by natural gas (26%) and coal (18%) in 2015 (IEA, 2016a). Every day about 96 million barrels of oil and liquid fuels are produced and consumed (IEA, 2016b), and the global demand is constantly growing. About 15% of the oil is used as raw material for the synthesis of other compounds, including plastics (polyalkenes, polystyrenes and polyvinyl chloride), pharmaceuticals, solvents, fertilizers, pesticides and synthetic fragrances. The remaining heaviest and least valuable fractions of oil, called asphaltenes, are used as constituents of pavement.

The massive use of oil as a source of energy and as raw material has generated serious environmental pollution problems. It is estimated that there are more than 250.000 contaminated sites in Europe, among which 20% would include aromatic compounds, especially polyaromatic hydrocarbons (PAHs), as contaminants (EEA, 2007).

Aromatic compounds are toxic and potentially carcinogenic and have been classified as dangerous for health and environment by the EC (Pedersen and Falck, 1997). The European Environment Agency (EEA) recommends increasing the countries' efforts devoted to remediation policies, both in the short term, by implementing direct remediation protocols, and in the long term, by stimulating research on remediation strategies. Several strategies are currently available for the remediation of aromatics contaminated sites. Among them, bacterial bioremediation stands out because of its low cost and little physical alteration of the environment. Bioremediation uses the metabolic versatility of microorganisms to degrade hazardous contaminants. A viable recovery technology requires microorganisms able of fast adaptation and of efficient use of pollutants in a reasonable time interval (Seo *et al.*, 2009). Bacteria have developed a striking adaptive capacity to degrade natural and synthetic aromatics to CO_2 and water.

Initially, research on the field focussed towards the engineering of recombinant bacterial strains improved in their degradation competence, especially against extremely recalcitrant compounds. However, the limited efficiency of this approach in natural environments (Cases and de Lorenzo, 2005), and especially the EC restrictive policies on the use of genetically modified organisms (GMOs) has encouraged the development of strategies based on the use of natural

autochthonous bacterial populations, a process known as natural attenuation. Natural attenuation can be improved either through the addition of specific nutrients to promote microbial communities' growth and activity (biostimulation), or through the inoculation of previously selected and enriched autochthonous microorganisms (bioaugmentation) (El Fantroussi and Agathos, 2005). Many factors influence the use of pollutants as substrates or their cometabolism by the autochthonous microbial communities. Therefore, understanding the molecular mechanisms underlying the capacity of these bacteria to degrade aromatic compounds is essential for the development and improvement of bioremediation tools (Ramos *et al.*, 2011).

2. Polycyclic aromatic hydrocarbons

2.1. General properties

Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic compounds composed by two or more fused benzene rings in linear, angular or clustered arrangements. By definition, they only contain carbon and hydrogen atoms in their structure, although nitrogen, sulphur and oxygen atoms may substitute some carbons of the benzene ring to form heterocyclic aromatic compound. PAHs are classified according to the number of rings, the type of ring and the atom composition. The low molecular weight (LMW) PAHs contain two or three aromatic rings and the high molecular weight (HMW) ones more than three.

In 1976 the Environmental Protection Agency (EPA) of the United States has recorded 16 PAHs as priority pollutants based on their occurrence and persistence in the environment and their toxicity (Keith and Telliard, 1979) (Figure 1). They have been used by regulatory authorities to identify contamination sites and to specify monitoring guidelines. Despite PAHs are a group of compounds that consists of more than one hundred individual homologues and isomers, only the 16 EPA PAHs have been routinely investigated in many environmental situations in the last 40 years, and nowadays there is a debate on the completeness of the list. In fact, the currently state of knowledge is very different from that of the 80s and questions are being asked whether the presence of some of these compounds in the list is still useful or whether others might be included (Keith, 2015). For example, there are PAHs of considerably higher toxicity than that of the priority PAHs in environmental samples such as alkylated PAHs, higher molecular weight PAHs and substituted PACs (amino-PAHs and cyano-PAHs). However, more studies are needed about the toxicity, presence in the environment and chemical analysis of these compounds (Andersson and Achten, 2015).

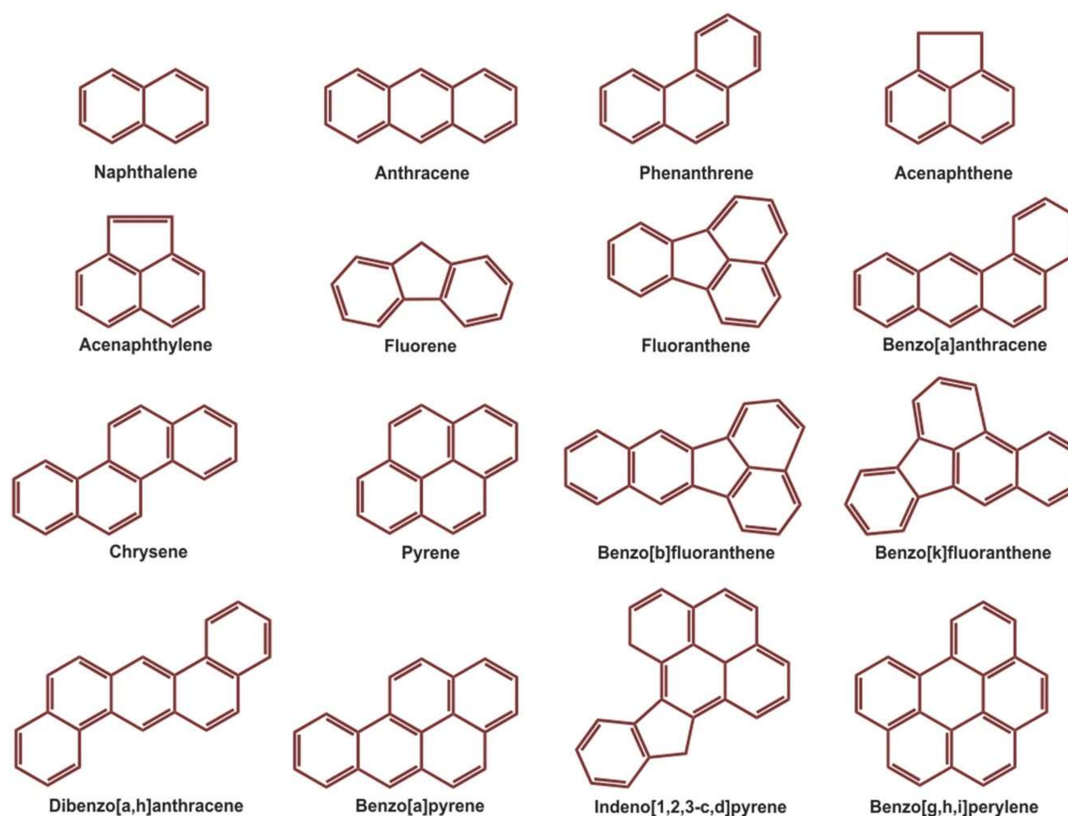

Figure 1. Structures and nomenclatures of the 16 PAHs on the EPA priority pollutant list. Taken from (Ghosal *et al.*, 2016).

2.2. Physicochemical properties

PAHs have a flat structure, are crystalline solid, from colourless to white or pale yellow-green colour in appearance. Physicochemical data of PAHs are used to predict their distribution and effect in the different environmental compartment (air, water, soil and sediment). Aqueous solubility, octanol-water partition coefficient and vapour pressure are their most important physicochemical properties (Table 1). The physical and chemical characteristics vary with both the number of aromatic rings and the pattern of ring linkage (de Maagd *et al.*, 1998). The aqueous solubility of PAHs is very low and decreases for each additional ring (Masih *et al.*, 2010). The hydrophobicity is expressed as the *n*-octanol/water partition coefficient (K_{ow}) and increases with the molecular weight. Their hydrophobic nature and very low water solubility make them very persistent in the environment. The persistence of PAHs is also due to the presence of dense π electrons on both sides of the ring structure that makes them more resistant to nucleophilic attack (Haritash and Kaushik, 2009). Most PAHs are soluble in non-polar organic solvents. The vapour pressure of PAHs is low; therefore, most of them are not volatile. An exception are the PAHs with two aromatic rings, like naphthalenes, which show a tendency to volatilize.

Table 1. Physicochemical and cancerogenic properties of 16 PAHs enlisted as priority pollutants by US EPA (Ghosal *et al.*, 2016; Gupte *et al.*, 2016).

PAHs	Molecular formula	Molecular weight (g/mol)	Solubility (mg/L)	K_{ow}	Vapor pressure (Pa)	EPA[a]
Naphthalene	$C_{10}H_8$	128.17	31	3.37	11.86	C
Acenaphthene	$C_{12}H_{10}$	154.21	3.8	3.92	0.50	D
Acenaphthylene	$C_{12}H_8$	152.2	16.1	4.00	3.86	D
Anthracene	$C_{14}H_{10}$	178.23	0.045	4.54	$3.40x10^{-3}$	D
Phenanthrene	$C_{14}H_{10}$	178.23	1.1	4.57	$9.07x10^{-2}$	D
Fluorene	$C_{13}H_{10}$	166.22	1.9	4.18	0.432	D
Fluoranthene	$C_{16}H_{10}$	202.26	0.26	5.22	$1.08x10^{-3}$	D
Benzo[a]anthracene	$C_{18}H_{12}$	228.29	0.011	5.91	$2.05x10^{-5}$	B2
Chrysene	$C_{18}H_{12}$	228.29	0.0015	5.91	$1.04x10^{-6}$	B2
Pyrene	$C_{16}H_{10}$	202.26	0.132	5.18	$5.67x10^{-4}$	D
Benzo[a]pyrene	$C_{20}H_{12}$	252.32	0.0038	5.91	$6.52x10^{-7}$	B2
Benzo[b]fluoranthene	$C_{20}H_{12}$	252.32	0.0015	5.80	$1.07x10^{-5}$	B2
Benzo[k]fluoranthene	$C_{20}H_{12}$	252.32	0.0008	6.00	$1.28x10^{-8}$	B2
Dibenzo[a,h]anthracene	$C_{22}H_{14}$	278.35	0.0005	6.75	$2.80x10^{-9}$	B2
Benzo[g,h,i]perylene	$C_{22}H_{12}$	276.34	0.00026	6.50	$1.33x10^{-8}$	D
Indenol[1,2,3-cd]pyrene	$C_{22}H_{12}$	276.34	0.062	6.50	$1.87x10\text{-}^{8}$	B2

[a]EPA carcinogenic classification: A, human carcinogenic; B1 and B2: probable human carcinogenic; C, possible human carcinogenic; D, not classifiable as to human carcinogenicity; E, evidence of non-carcinogenicity for humans.

2.3. Toxicity

The toxicity of PAHs was first recognized in the 18th century by the physician John Hill, who documented a high incidence of nasal cancer in tobacco snuff consumers, and then by the surgeon Percival Pott that reported a high rate of scrotal skin cancer in chimney sweeps (Cerniglia, 1984). The impact of PAHs on human health depends mainly on time exposure, concentration, and subjective factors (i.e. health status and age). Generally, the LMW PAHs are considered acutely toxic, causing short-term effect like skin irritation and inflammation, whilst HMW PAHs are largely considered as genotoxic (Cerniglia, 1992). Genotoxicity plays an important role in the carcinogenicity process. PAHs can induce carcinogenesis by the enzymatic activation of the PAH into metabolites, the covalent binding of the PAH metabolites to DNA, RNA and proteins and the induction of mutations as a result of the formation of PAH-DNA adducts (Marston *et al.*, 2001). In addition, some PAH-derived products are more toxic than their parent PAHs and can lead to critical cellular effects (Schnitz *et al.*, 1993). Naphthalene, chrysene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, dibenz(ah)-anthracene, and indeno(1,2,3-cd)pyrene are some of the PAHs classified as probable human carcinogens by the EPA and the International Agency for Research on Cancer (Abdel-Shafy and Mansour, 2016).

PAHs can induce immune suppression, although the mechanisms involved are still not clear. Burchiel and Luster (2001) concluded that most of the immunotoxic effects of PAHs are correlated with aromatic hydrocarbon receptor binding and the formation of reactive metabolites that exert genotoxicity and/or produce oxidative stress. PAHs can also act as a teratogen agent. Embryotoxic effects of benzo(a)anthracene, benzo(a)pyrene, and naphthalene have been described in animal (Kristensen *et al.*, 1995; Wassenberg and Di Giulio, 2004). Moreover, it has been established that exposure to PAHs can have adverse effects during pregnancy and childhood (Perera *et al.*, 2005; Edwards *et al.*, 2010).

Living organisms can undergo additional toxicity mechanisms such as phototoxicity and nonpolar narcosis. Phototoxicity is based on the property of some PAHs (*i.e.* anthracene, pyrene, chrysene, benzo(*a*)pyrene) to adsorb UV light. The photo-excited PAH molecules transfer the UV energy to an oxygen molecule, creating an oxygen radical that can disrupt cell membranes via lipid peroxidation. This phenomenon has been described in freshwater and marine environment (McDonald and Chapman, 2002). However, the ecological relevance of PAH phototoxicity remains questionable, and environmental management decisions should be based on more factors. Narcosis is the prevalent mechanism of PAH toxicity in sediments which results in the alteration of cell membrane function and structure. This toxicity is inversely correlated to the octanol-water partition coefficient (K_{ow}) that determines the accumulation of toxicant in the biological membranes (Vaes *et al.*, 1998). Naphthalene, fluorene and phenanthrene are some of the PAHs that can exert narcosis effect.

3. PAHs in the environment

The PAHs of natural origin are produced by petrogenic, pyrogenic and biogenic processes. Petrogenic PAHs are produced during crude oil maturation and similar processes, and are often composed of alkyl-substituted PAHs. Pyrogenic PAHs are formed from incomplete combustion of organic matter such as fossil fuels, wood and coal and are principally composed of unsubstituted PAHs. Biogenetic aromatics are the result of biotransformation processes mainly derived from plants (*i.e.* aromatic amino acids, lignin compounds, etc.) and bacteria (Seo *et al.*, 2009).

PAHs are released into the environment either from natural or anthropogenic sources. Natural sources include volcano eruptions, burning of vegetation in forests and bush fires, plant and bacterial reactions and thermal and geological reactions associated with fossil fuel (Suess, 1976). However, the main sources of aromatic in the environment are anthropogenic and mostly originate from petroleum refining industries, combustion of fossil fuels, coal gasification and liquefaction

processes, accidental oil spills, transport activities and waste incineration, and from wood-treatment processes and wood-preservative production (Cerniglia, 1992).

Natural and anthropogenic sources of PAHs, in combination with global transport phenomena, result in their worldwide distribution (Ghosal *et al.*, 2016). Their ubiquity together with their marked stability, persistence and resistance to degradation make them of particular environmental concern. Additionally, their physical-chemical properties such as lipophilic nature and hydrophobicity leads to their bioconcentration, bioaccumulation and bioamplification, reaching relevant toxic concentrations in living organisms (Jones and de Voogt, 1999).

The possible fates of PAHs in the environment are volatilization, photooxidation, chemical oxidation, bioaccumulation, adsorption to soil particles, leaching and microbial degradation (Cerniglia, 1992) (Table 2). The importance of these processes varies depending on the environment compartment.

Table 2. Possible fate of PAHs in the environment (Gupte *et al.*, 2016).

Process	Consequence	Factors
Transfer (*processes that relocate PAHs without altering their structure*)		
Volatilization	Loss of PAHs due to evaporation from soil, plant, or aquatic ecosystems.	Vapor pressure, wind speed, temperature.
Absorption	Uptake of PAHs by plant roots or animal ingestion. Polycyclic aromatic hydrocarbons usually do no transfer into aboveground biomass from soil.	Cell membrane transport, contact time, susceptibility, plant species.
Leaching	Translocation of PAHs either laterally or downward through soils.	Water content, macropores, soil texture, clay and organic matter content, rainfall intensity, irrigation.
Erosion	Movement of PAHs by water or wind action.	Rainfall, wind speed, size of clay and organic matter particles with adsorbed PAHs on them.
Degradation (*processes that alter the PAH structure*)		
Biological	Degradation of PAHs by microorganisms, biodegradation and cometabolism.	Environmental factors (pH, moisture, temperature, oxygen), nutrient status organic matter content, PAH bioavailability, microbial community present, molecular weight of the PAHs (LMW or HMW).
Chemical	Alteration of PAHs by chemical processes such as photochemical (*i.e.* UV light) and oxidation-reduction reactions.	High and low pH, structure of PAHs, intensity and duration of sunlight, exposure to sunlight, and same factors as for microbial degradation.
Sequestration *(processes that relocate PAHs into long-term storage without altering structure)*		
Adsorption	Removal of PAHs from bioavailable pools through interaction with soils and sediments.	Clay and organic matter content, clay type, moisture.
Diffusion	Diffusion of PAHs into soil micropores where it is unavailable for microbial degradation.	Hydrophobic nature of micropores and PAHs.

The atmosphere is the first dispersion vehicle of PAHs. PAHs are emitted to the atmosphere primarily from the burning of biomass, including both biofuel combustion and wildfires. Then they can be transported in a vapor phase or adsorbed onto particulate matter and be deposited into the different environmental compartment by dry or wet deposition processes (Zhang and Tao, 2009). In soil, abiotic reactions such as photooxidation and chemical oxidation account for loss of two- and three-ring PAH compounds. Naphthalene and methylnaphthalenes are partially lost by volatilization; for the remaining compounds, volatilization is negligible (Park *et al.*, 1990). Furthermore, aromatic compounds can be sequestered in the soil matrix. Aquatic systems also receive PAHs from industrial, urban, and sanitary effluents as well as roadway runoff. Because of their non-polar structures and low solubility, most of them are incorporated into sediments, and the lower molecular weight PAHs such as naphthalene and alkyl-derivatives can be partially dissolved in water (Abdel-Shafy and Mansour, 2016). Some PAHs can be photo-oxidized and volatilized. However, despite PAHs may undergo adsorption, volatilization, photolysis, and chemical degradation in the different environments, microbial degradation is the major degradation process (Cerniglia, 1992).

4. Bioremediation of PAHs

In the last years several physical, chemical, thermal and biological technologies have been developed to clean-up PAH polluted sites. Unfortunately, many of these physicochemical-thermal techniques, such as incineration, thermal conduction, and chemical oxidation have a high economical cost and can have potentially destructive ecological consequences (Kuppusamy *et al.*, 2017). Thus, there is considerable interest in the use of biological system to remediate contaminated environments. Biological remediation procedures have been developed for some hazardous organic pollutants and have shown to be successful, in terms of pollutant elimination efficiency, economic feasibility, and eco-friendly approach.

Bioremediation relies on biological mechanisms to reduce (degrade, detoxify, mineralize, or transform) the concentration of pollutants to an innocuous state. In this process microorganisms play a fundamental role for their innate ability to degrade many types of pollutants (Azubuike *et al.*, 2016). Indeed, many bacterial, fungal, and algal strains have been shown to degrade a wide variety of PAHs (Ghosal *et al.*, 2016). Bioremediation of PAH contaminated sites includes both *ex-situ* (*i.e* bioreactors, biopiles) and *in-situ* (*i.e* biostimulation, bioaugmentation, composting, phytoremediation, biosparging) treatment options. *Ex-situ* techniques involve excavation and transport of contaminants from the polluted sites to the treatment site. *In-situ* bioremediation

allows treating polluted sites at the place of pollution, avoiding any excavation and transport of contaminants, which implies little or no disturbance of the habitat.

As mentioned before there are two major approaches to enhance *in-situ* microbial communities' degradation activities in contaminated sites: biostimulation and bioaugmentation. Biostimulation processes stimulate the autochthonous microorganisms to use the pollutants as a carbon source providing oxygen, nutrients, co-susbstrates or surfactants to the indigenous community. Bioaugmentation methods introduce exogenous microorganisms with degradative capabilities into the contaminated environment. This process might proceed without any form of stimulation (Straube *et al.*, 2003). Moreover, instead of treatments with a single microorganism, the decontamination could be carried out by a microbial consortium. In fact, it has been reported that microbial consortia may degrade target pollutants more efficiently than a single strain, and give better results for the remediation of a contaminated site (Silva-Castro *et al.*, 2012). This is probably due to the higher genetic and metabolic diversity associated with microbial consortia as compared to pure cultures. The effectiveness of biostimulation and bioaugmentation have been observed in many PAH contaminated sites, and the efficiency of both approaches have been compared. In general, better performances are obtained using a combination of both methods, improving the remediation results for both LMW and HMW PAHs. This has been shown in the recovery of refinery waste waters using a PAH-degrading *Rhodococcus ruber* isolate (Sun *et al.*, 2012) and in landfarming experiments with the aid of a biosurfactant-producing *Pseudomonas aeruginosa* strain (Straube *et al.*, 2003), among many other examples. However, the convenience of the addition of microbes to natural environments is still a matter of debate. Competition between endogenous and exogenous microbial populations, the risk of introducing pathogenic organisms into an ecosystem, and the possibility that the inoculated microorganisms may not survive are aspects to consider when a bioaumentation method is being applied.

Ex- or *in-situ* bioremediation are usually evaluated on the basis of the cost of treatment, depth and degree of pollution, type of pollutant, geographical location, and geology of the polluted site (Frutos *et al.*, 2012). *Ex-situ* bioremediation techniques may be used to treat a wide range of pollutants in a controlled manner and generally is considered to be more expensive due to the excavation and transportation costs. *In-situ* bioremediation does not include costs of excavation and transportation, and is generally considered economically favourable. However, in some cases the costs due to the on-site installation equipment can be higher. Therefore, the costs of remediation should not be the major aspect determining the bioremediation technique to be applied to any polluted site. In fact, additional factors such as those mentioned above should be taken into account and lead to the decision on the most suitable and efficient method to effectively treat

polluted sites. Furthermore, the application of combined remediation techniques in a polluted site has been proven to increase the decontamination efficiency, reducing the weakness of individual methods (Cassidy *et al.*, 2015; Martínez-Pascual *et al.*, 2015).

4.1. Novel bioremediation strategies

In addition to the studied and documented PAHs remediation methods mentioned above, there are several promising sustainable systems and technologies in full development, like mixed cell culture systems, biosurfactant flushing, transgenic approaches, nanoremediation and electrode-based biodegradation.

Mixed cell culture systems are consortia of different organisms, usually bacteria, algae and fungi, which are used for environmental remediation. They are developed to overcome the possible limitation arising when only one type of microorganism is used in a contaminated site. It has been observed, for example, that bacteria-algae consortia are more efficient than bacterial consortia in the degradation of PAHs. Microalgae can produce a variety of compounds (*i.e.* nucleic acids, lipids, proteins, excretion and products) and oxygen that improve the degradation of contaminants (Kirkwood *et al.*, 2006).

The use of GMOs was a promising approach in the bioremediation field. In fact, with the advances in protein and genetic engineering and molecular biological techniques it was possible to include in a single host the desirable enzymes and degradation pathways, to increase the substrate specificity and to improve the degradation rate (Timmis and Pieper, 1999). However, as mentioned previously, the fitness and performance of these system was limited under natural conditions (Cases and de Lorenzo, 2005). Also, the use of genes encoding biosynthetic pathways for biosurfactants or conferring resistance to environmental stress could improve the efficiency of the degradation of pollutants (Dua *et al.*, 2002). However, before the releasing of any GMO, it is critical to ensure its stability, and also the stability of any recombinant plasmid introduced in the bacteria. Furthermore, the current legal limitations to the use of GMOs in natural ecosystems, due to the limited predictability of the side effect of these organisms in the environment, is the major burden to their use in bioremediation (Ang *et al.*, 2005).

Althoug it cannot be considered a bioremediation strategy *senso stricto*, nanoremediation has emerged as a new promising technology in contaminated site remediation. It involves the use of nanomaterial with two major types of strategies: adsorptive and reactive remediation. The adsorptive remediation removes contaminant by sequestration on nanoparticles (*i.e.* dendritic material), while the reactive remediation (i.e. photooxidation) transforms the pollutant into innocuous compounds. These remediation techniques could be applied *ex-situ* and *in-situ*

(Tratnyek and Johnson, 2006). However, the use of *in-situ* nanotechnologies is controversial because of the unknown effect that this technology might have to human or ecological health. In fact, to date, few studies have investigated the toxicological and environmental effects of direct and indirect exposure to nanomaterials and no clear guidelines exist to quantify these effects (Colvin, 2003). For this reason, research seeks on the development of 'eco-friendly' nanomaterial, for the future environmental nanotechnologies applications. Some examples that could be used in PAH removal are nanofertilizer (for biostimulation and bioaugmentation), nanominerals (for biostimulation) or green synthesized nanooxidizers (PAH oxidation) (Kuppusamy *et al.*, 2017).

Electrode-based bioremediation applies the capacity of many bacterial strains to use conductive graphite electrodes as electron acceptor, either by direct contact or through indirect mechanisms, for the degradation of contaminants, thus overcoming the limitation of electron acceptors (Zhang *et al.*, 2010; Daghio *et al.*, 2017). The effectiveness of this methodology for the remediation of polluted sites is being evaluated for an increasing number of pollutants and conditions, with promising results (Domínguez-Garay *et al.*, 2016).

Currently, we can say that a standard bioremediation method does not exist, and for each polluted site a strategy should be designed based on the environmental conditions, the nature of the contaminant and the presence of the suitable degrading microorganisms. Also, the simultaneous application of different remediation methods could lead to a more efficient decontamination. However, despite the great progress and knowledge about the physiology, ecology, biochemistry of PAHs degrading microorganisms and catabolism of organic pollutants more research is required to have a more comprehensive understanding of PAHs contaminated sites. Moreover, still very little is known about the microrganisms, genes and catabolic pathway of PAHs degradation in high salinity environments or in anaerobic environments.

5. PAH degradation by bacteria

Bacteria have been evolving since more than three billion years and have developed strategies to obtain energy from nearly every compound available in nature. The abundance of microorganisms, together with their great genetic diversity and the ability for horizontal gene transfer allows them to evolve quickly and to adapt to many different environmental conditions, even to extreme environments. The presence of PAHs in the biosphere since the primitive earth explains the metabolic versatility and the evolution of microbial catabolic pathways to utilize these compounds as energy and carbon sources (Diaz, 2004). Microorganisms play, in fact, a crucial role in the recycling of carbon from the aromatic ring. Various bacteria have been found to degrade

PAHs, and aerobic degradation of naphthalene and phenanthrene has been most widely studied for decades (Davies and Evans, 1964; Kiyohara *et al.*, 1976; Foght and Westlake, 1988; Churchill *et al.*, 1999; Kim *et al.*, 2003; Pumphrey and Madsen, 2007).

Aromatic compounds have limited chemical reactivity and two key steps are involved in their degradation pathway: the destabilization of the resonance energy that stabilizes the ring structure, and its subsequent cleavage (Fuchs *et al.*, 2011). This is also true for the degradation of polycyclic aromatic hydrocarbons (PAHs). The aerobic and anaerobic degradation of these compounds, and the methods that bacteria use in the corresponding PAHs degradation pathways, differ fundamentally. There are two major strategies to degrade PAHs depending on the availability of oxygen. These strategies are based on different ring activation mechanisms that consist of either oxidation to produce dihydroxylated derivatives under aerobic conditions, or reduction of the aromatic ring under anoxic conditions. An additional strategy is functional in some organisms under low oxygen concentrations: oxidation to a non-aromatic ring-epoxide, which suffers rearrangement followed by hydrolytic cleavage (Fuchs *et al.*, 2011). In the aerobic PAH catabolism pathways, the first activation step involves direct incorporation of oxygen by the activity of multicomponent dioxygenases. The key enzymes are ring-hydroxylating dioxygenases (RHDs) belonging to the Rieske-type non-heme iron oxygenase family (Pieper *et al.*, 2004), and the key central intermediates are dihydroxylated PAHs. In anaerobic conditions two main mechanisms were initially proposed for PAH molecule activation: i) ring carboxylation and ii) ring methylation followed by addition to fumarate. However, methylation as initial step in PAH degradation has been discarded and currently only the carboxylation pathway, in which the key enzyme is a carboxylase belonging to the UbiD-like proteins family (Rabus *et al.*, 2016), is considered (see below).

5.1. Aerobic PAH degradation

Principally, microbial communities prefer aerobic conditions for the degradation of PAHs because oxygen provide a higher amount of energy to the cells (Field *et al.*, 1995). The biodegradation of aromatic compounds carried out by aerobic microorganisms is well understood and numerous bacterial strains able to degrade PAHs have been isolated and characterized (Korda *et al.*, 1997; Gibson and Parales, 2000; Diaz, 2004; Cao *et al.*, 2009). A wide phylogenetic diversity of bacteria, such as species of the genus *Alcaligenes*, *Acinetobacter*, *Pseudomonas*, *Rhodococcus*, and *Nocardia*, is known to aerobically degrade PAHs. Most of them are isolated from contaminated soil or sediments (Cao *et al.*, 2009). *Pseudomonas* species and closely related

organisms have been the most extensively studied due to their ability to degrade many different aromatic compounds, from benzene to benzo(pyrene) (Balashova *et al.*, 1999; Wackett, 2003).

The enzymatic pathways for the degradation of a variety of PAHs have been elucidated, the genes characterized, their regulation, distribution and diversity have been analysed, so that the current cumulative knowledge allows a thorough and deep view of the processes involved (Harayama *et al.*, 1992; Gibson and Parales, 2000; Diaz, 2004). Naphthalene has often been used as a model compound to investigate the ability of bacteria to degrade PAHs because it is the simplest and most soluble PAH. Naphthalene degrading bacteria are ubiquitous in nature and there are enormous numbers of reports documenting the bacterial degradation of naphthalene including the elucidation of the biochemical pathways, enzymatic mechanisms, and genetic regulations (Cerniglia, 1992; Peng *et al.*, 2008). Therefore, information of the bacterial degradation of naphthalene has been used to understand and predict pathways in the degradation of three- or more ring PAHs. Furthermore, the bacterial naphthalene dioxygenase system is particularly useful for the oxidation of bi- and tri-cyclic PAH substrates, such as phenanthrene and anthracene. The bacterial degradation of naphthalene has been well characterized for the catabolic enzyme system encoded by the plasmid NAH7 of *Pseudomonas putida* G7. The plasmid NAH7 has two operons that contain the structural genes for naphthalene degradation (*nah*): one of them contains the genes for the so-called upper pathway encoding the enzymes involved in the conversion of naphthalene to salicylate, and the other operon contains the genes for the lower pathway encoding the enzymes involved in the conversion of salicylate to pyruvate and acetaldehyde (Simon *et al.*, 1993) (Figure2). The *nahR* coding gene for the LysR family regulator that controls the expression of the pathway constitutes a third operon (Wagner and Haider, 2012).

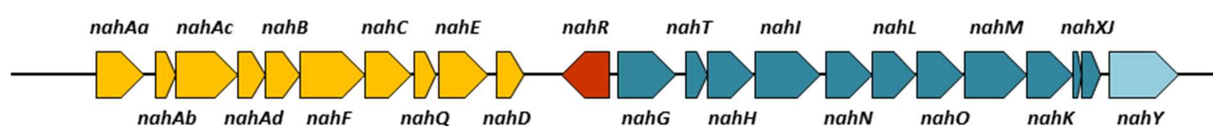

Figure 2. Gene organization of the naphthalene degradation pathway in NAH7 plasmid of *Pseudomonas putida* G7. The genes for the upper pathway are shown in yellow, those for the lower pathway in blue, and the *nahR* regulator in red. A receptor involved in naphthalene chemotaxis is shown in pale blue. Adapted from (Yeates *et al.*, 2003).

In the upper pathway, naphthalene degradation is initiated by the activation of one of the aromatic rings by a naphthalene dioxygenase to form *cis*-1,2-dihidroxy-1,2-dihydronaphthalene, which is further transformed to 1,2-dihydroxy-naphthalene by the activity of a cis-dihydrodiol dehydrogenase (Figure 3). The resulting activated dihydroxynaphthalene is cleaved in meta position by a 1,2-dihydroxynaphthalene dioxygenase to produce 2-hydroxy-2*H*-chromene-2-

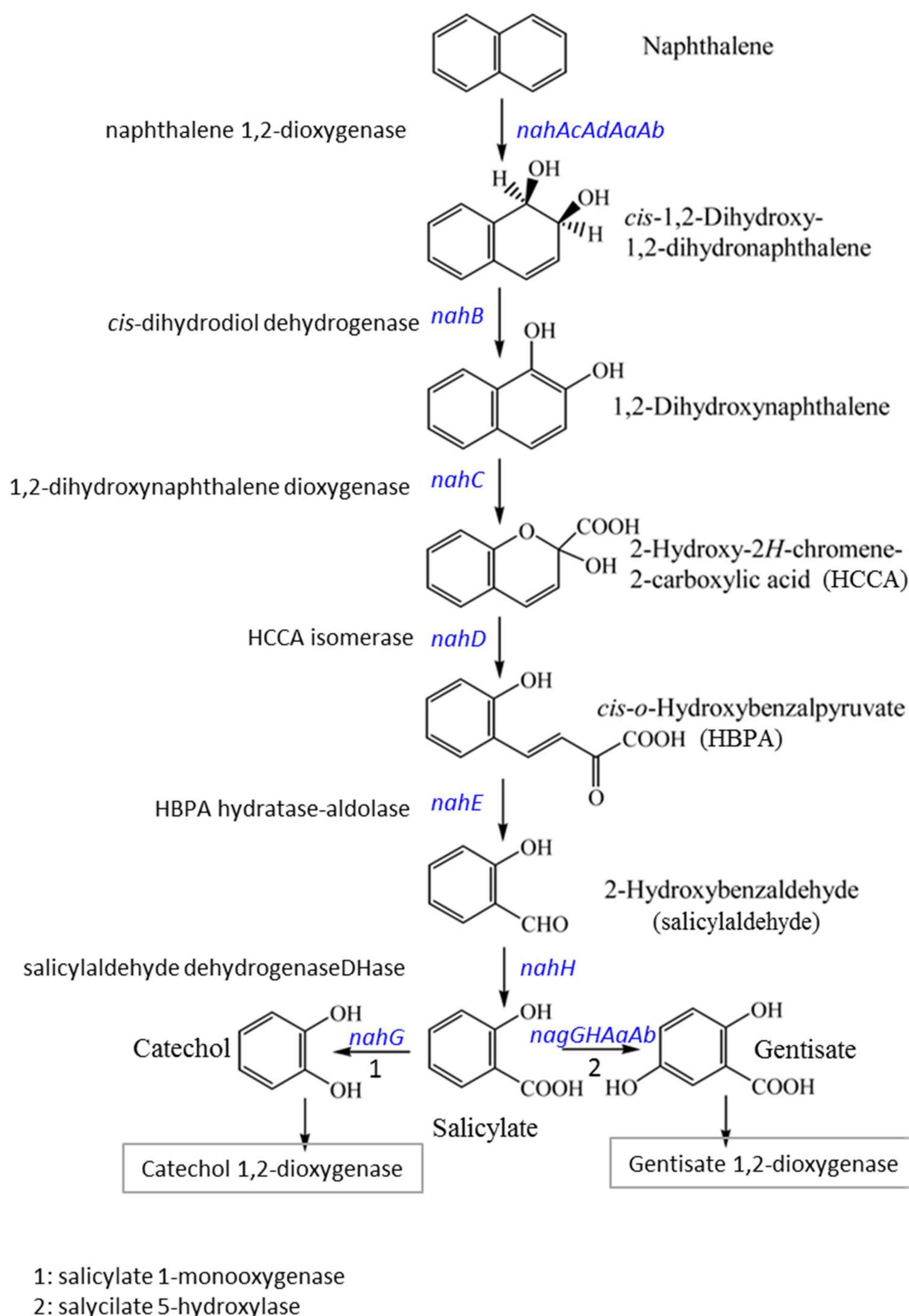

Figure 3. Proposed catabolic pathways of naphthalene by aerobic bacteria (Peng *et al.*, 2008). The genes involved in the different steps are written in blue.

carboxylic acid, which is successively transformed to trans-*o*-hydroxy-benzylidenepyruvic acid, salicylaldehyde and finally salicylic acid. These steps constitute the upper pathway. In the so-called lower pathway, salicylic acid is further metabolized through either catechol or gentisate to tricarboxylic acid (TCA) cycle intermediates (Cerniglia, 1992; Gibson and Parales, 2000; Wagner and Haider, 2012) (Figure 3).

The multicomponent naphthalene dioxygenase is the key step in naphthalene degradation and includes an NADH oxidoreductase, a ferredoxin and an oxygenase component, which contains the active site and is composed of large and small subunits in an $\alpha3\beta3$ organization (Peng *et al.*, 2008).

In the different naphthalene-degrading *Pseudomonas* strains, sequences of genes encoding the upper pathway enzymes are organized as operons and are very conserved (more than 90% identical) (Peng *et al.*, 2008; Wagner and Haider, 2012). Although the paradigm of PAH degradation is based upon *Pseudomonas* pathways, an increasing number of non-pseudomonads PAH degraders are described. The discovery in different strains of distantly-related dioxygenase suggests a higher diversity than initially thought (Larkin *et al.*, 2005). However, it is interesting to note that despite a significant sequence divergence from classical *Pseudomonas* sequences, there seems to be a substantial structural conservation of subunit arrangement (Ahn *et al.*, 1999). *Ralstonia sp.* U2, originally classified as a *Pseudomonas* sp., has the naphthalene upper pathway genes similar to the classical *nah* genes of *Pseudomonas* strains placed in the same order (Zhou *et al.*, 2001). Also, *Comamonas testeroni* strain GZ42 has a similar genetic pathway to that of *Ralstonia sp.* U2, although more divergent from the typical *nah* genes (Goyal and Zylstra, 1997).

Different strains have also been reported to carry naphthalene degradation pathways, such as *Burkholderia sp*. RP007, *Alcaligenes faecalis* AFK2 and *Nocardioides sp.* KP7 (Kiyohara *et al.*, 1982; Iwabuchi and Harayama, 1997; Laurie and Lloyd-Jones, 1999), although the sequence similarity and gene organization of the pathways may greatly vary between strains. The genus *Sphingomonas*, Gram-negative bacteria within the *Alphaproteobacteria*, includes a number of strains able to degrade a wide range of natural and xenobiotic compounds including PAHs like naphthalene, phenanthrene, and anthracene, using common pathways found in other Gram-negative bacteria. It has been shown that their degradative capabilities rely on the large plasmids present in these bacteria. The catabolic versatility of this genus does not seem to depend on the significant differences in degradative pathways with other bacteria, but on the unusual catabolic gene organization, and possibly on the presence of high-affinity uptake systems. The degradative genes are not organized in regulated operons, and this ‘flexible’ gene organization seems to be responsible of their quick and efficient adaptation to novel compounds (Basta *et al.*, 2005). The Gram-positive *Rhodococcus* strains are another very diverse group of the order *Actinomycetales*

that possesses the ability to degrade many recalcitrant and toxic organic compounds. *Rhodococcus* species are common in many environmental niches from soils to industrial waste water (Gürtler *et al.*, 2004). The ability of rhodococci to degrade numerous organic compounds depends largely on their tolerance to solvent and toxic substrate, and on their large genome that hide an enormous gene diversity. For example, many oxygenases, including at least six ring-hydroxylating dioxygenases and ten cytochromes P450, are encoded in the 9.7 Mb genome of *Rhodococcus* sp. strain RHA1 (Larkin *et al.*, 2005).

Phenanthrene, a PAH containing three condensed rings, is often used as a model substrate for studies on the metabolism of carcinogenic PAHs. The enzymes involved in the conversion of naphthalene to salicylate can also degrade phenanthrene via deoxygenation at the 3,4 position, to finally render 1-hydroxy-2-naphtoate. This intermediate is further channelled through two possible pathways, according to the host: i) by hydroxylation to 1,2-dihydroxynaphthalene, which would then enter the regular naphthalene degradation pathway, and ii) by direct ring cleavage by a dioxygenase and further transformation to *o*-phthalate, which is then metabolized via protocatechuate (Wagner and Haider, 2012). Many Gram-positive and Gram-negative bacteria have been isolated that utilize phenanthrene as sole source of carbon and energy (Peng *et al.*, 2008; Seo *et al.*, 2009; Mallick *et al.*, 2011). We mention some: a *Staphylococcus* sp. strain with a novel phenanthrene degradation pathway was isolated from petroleum-contaminated soil. The genes involved in phenanthrene assimilation were found to be plasmid-encoded, probably acquired by horizontal transfer (Mallick *et al.*, 2007). Ghosal *et al.* (2010) documented mineralization of phenanthrene by *Ochrobactrum* sp. strain PWTJD, isolated from municipal waste-contaminated soil. It was the first report describing an *Ochrobactrum* species with PAH biodegradation abilities and the involvement of the meta-cleavage pathway of 2-hydroxy-1-naphthoic acid in phenanthrene assimilation in a Gram-negative species. *Novosphingobium* sp. HS2a isolated from soil was described as a good phenanthrene and naphthalene degrader (Rodriguez-Conde *et al.*, 2016). Other bacterial strains able to catabolize phenanthrene are member of the genera *Acidovorax*, *Arthrobacter*, *Brevibacterium*, *Burkholderia*, *Comamonas*, *Mycobacterium*, and *Pseudomonas* (Seo *et al.*, 2009).

Other PAHs like anthracene, fluorene, acenaphthene and acenaphthylene are also present in high amounts in PAH-contaminated environments, and many bacterial species can mineralize these compounds. Among these the *Actinobacteria Mycobacterium* sp. strain LB501T was isolated from enrichments of PAH-contaminated soil and sludge samples, where PAHs were adsorbed on hydrophobic membranes. LB501T grows primarily as a biofilm on solid anthracene, probably a

response to optimize the bioavailability of the substrate (Bastiaens *et al.*, 2000). Generally, the anthracene degradation pathway proceeds through 2,3-dihydroxynaphthalene and further through the naphthalene degradation pathway to form salicylate. Nevertheless, the transient accumulation of o-phthalic acid during the *Mycobacterium sp*. cultivation on anthracene suggests the metabolism goes through *o*-phthalic acid and protocatechuic acid (van Herwijnen *et al.*, 2003). Many *Mycobacterium* species have been reported to degrade also other PAHs such as pyrene, fluoranthene, benz[a]anthracene, and benzo[a]pyrene (Heitkamp and Cerniglia, 1988; Schneider *et al.*, 1996; López *et al.*, 2006). The degradation of acenaphthene and acenaphthylene by an *Acinetobacter* sp. was first reported by Ghosal *et al.* (2013). The strain was isolated from municipal waste-contaminated soil. The characterization of metabolites suggested the hydroxylation of acenaphthene to 1-acenaphthenol, which was subsequently transformed to catechol before entering into the TCA cycle.

In addition to the cited strains, many other species are involved in LMW and HMW PAHs aerobic biodegradation. Some examples are *Bacillus, Cycloclasticus* and *Marinobacter* (Peng *et al.*, 2008; Seo *et al.*, 2009). However, the degradation of HMW PAHs and its regulatory mechanisms still need further investigations.

5.2. Anaerobic PAH degradation

While PAH oxidation by aerobic bacteria has been extensively studied for years, the anaerobic catabolism of aromatic compounds has only recently attracted the general interest and still needs a deeper understanding (Widdel and Rabus, 2001; Jones *et al.*, 2008; Meckenstock and Mouttaki, 2011). The rate of PAH anaerobic degradation was initially considered very slow and its ecological importance in most environments and biodegradation strategies non-significant. However, the availability of oxygen is often limited in natural environments. In many polluted sites, oxygen can be rapidly consumed in the aerobic biodegradation processes, resulting in a decreasing oxygen concentration gradient, so that reduction of other electron acceptors becomes energetically favourable (Rivett *et al.*, 2008a). A similar pattern is observed in habitats that are under permanent anoxic conditions, such as flooded sediments in marine and fresh-water environments (Brune *et al.*, 2000). First, microorganisms consume the oxygen that penetrates the first millimetres/centimetres of the sediments (Braker *et al.*, 2001). Once the oxygen consumption exceeds its supply, anoxic conditions are established. Below the zone of oxygen-influence, anoxic processes are stimulated: nitrate, manganese, iron and sulphate are then sequentially used for anaerobic respiration, if available (Sørensen, 1978; Canfield *et al.*, 1993). The theoretical yield of

energy obtained from the oxidation of organic matter using each of the electron acceptors is the driving force for microbes to use a specific metabolism when oxygen is absent.

Thus, anoxic conditions dominate in many natural habitats and contaminated sites, where anaerobes, using alternative final electron acceptors, metabolize organic compound that would otherwise persist in nature (Foght, 2008; Fuchs *et al.*, 2011). Field studies indicate that in many cases contaminant degradation under natural conditions is dominated by anaerobic processes (Meckenstock *et al.*, 2004b). In sites where anaerobic processes are established, biostimulation approaches maintaining the intrinsic anoxic conditions could enhance the active and acclimated natural bacterial population and promote efficient contaminant degradation (Coates and Anderson, 2000).

Although the anaerobic degradation of aromatic compounds in bacteria has been extensively studied in the past decades, our current knowledge on the biochemistry, genetics and physiology of the anaerobic pathways is essentially centred on the degradation of model monoaromatics such as benzoate, toluene and substituted derivatives in bacterial strains with different respiratory metabolisms (e.g. iron reducing, denitrifying, sulfate reducing bacteria, etc.), and of the model PAHs naphthalene and 2-methylnaphthalene (2MN) in sulphate-reducing bacteria (SRB). Progresses in the field are generally linked to the availability of bacterial isolates able to carry out anaerobic biodegradation of specific compounds (Dolfing *et al.*, 1990; Lovley and Lonergan, 1990; Evans *et al.*, 1991; Rabus *et al.*, 1993; Fries *et al.*, 1994; Beller *et al.*, 1996; Galushko *et al.*, 1999a; Meckenstock *et al.*, 2000a). Besides the overall conservation of the main known central pathways, different strategies may be employed depending on the electron acceptors used and the metabolic efficiency of the organism (Boll *et al.*, 2014). As for aerobic degradation, activation of a broad range of aromatic compounds converges into a few major central metabolites, which are further dearomatized and channelled to the central cell metabolism (Heider and Fuchs, 1997; Schink *et al.*, 2000; Foght, 2008; Carmona *et al.*, 2009).

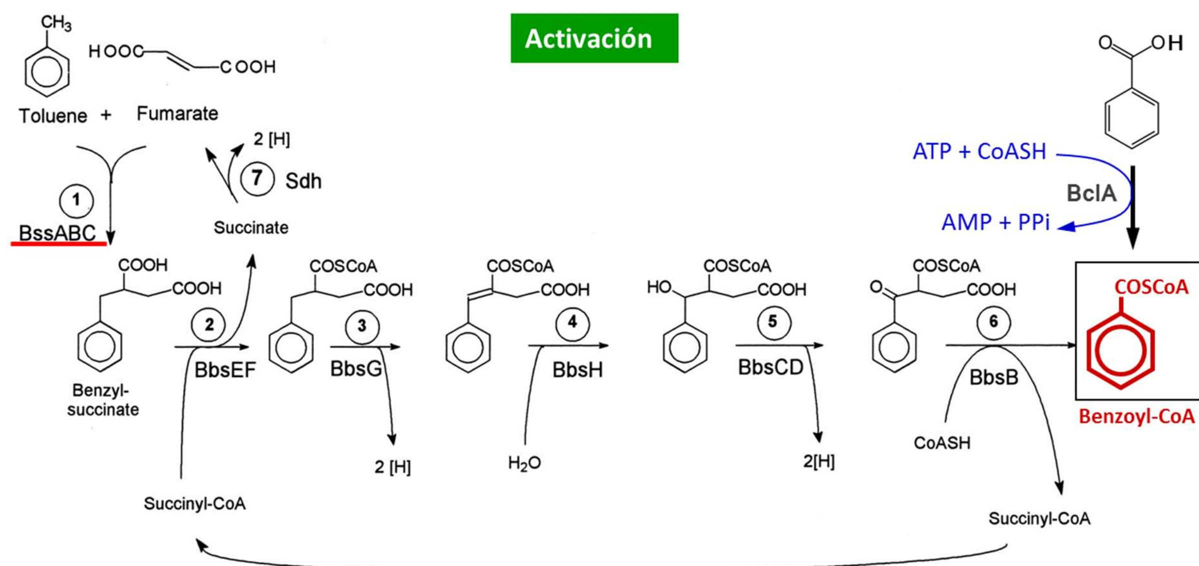

Figure 4. Pathway of anaerobic toluene and benzoate metabolism to benzoyl-CoA in Thauera aromatica. The enzymes involved are indicated by their gene names: (1) benzylsuccinate synthase, BssABC; (2) succinyl-CoA: benzylsuccinate CoA-transferase, BbsEF; (3) benzylsuccinyl-CoA dehydrogenase, BbsG; (4) phenylitaconyl-CoA hydratase, BbsH; (5) 3-hydroxyacyl-CoA dehydrogenase, BbsCD; (6) benzoylsuccinyl-CoA thiolase, BbsB; (7) succinate dehydrogenase, Sdh. BclA, benzoate-CoA ligase. Modified from (Leuthner and Heider, 2000).

The most common of the central intermediates used by bacteria in the absence of oxygen is benzoyl-CoA, in which the carboxy-thioester group acts as an electron-withdrawing substituent that facilitates reduction of the aromatic ring (Fuchs *et al.*, 2011). Under anoxic conditions, compounds such as those belonging to the BTEX group, benzoate, phenol, cresols or phenylacetate are transformed through different peripheral pathway to benzoyl-CoA. Figure 4 shows as example the metabolism of toluene to benzoyl-CoA in *Thauera aromatica*. The essential step is carried out by the glycyl radical enzyme benzylsuccinate synthase (*bssABC* genes). Benzyl-succinate synthase catalyses the addition of the methyl group of toluene to the double bond of fumarate to produce benzyl-succinate, which is then further metabolized to benzoyl-CoA through a pathway similar to β-oxidation. Benzoate is directly activated to benzoyl-CoA by the activity of an AMP forming benzoate-CoA ligase (Schühle *et al.*, 2003). In most denitrifying organisms, the central intermediate benzoyl-CoA is dearomatized by an ATP-dependent (type I) benzoyl-CoA reductase, while in low energy yielding fermenting, sulphate-reducing and most iron-reducing bacteria, ring reduction is carried out by an ATP-independent (type II) benzoyl-CoA reductase through a recently proposed electron bifurcation mechanism (Kung *et al.*, 2010; Buckel and Thauer, 2013; Boll *et al.*, 2014) (Figure 5). In both cases the resulting product 1,5-dienoyl-CoA suffers a further reduction and ring opening through a series of reactions similar to a modified β-oxidation (Heider, 2007).

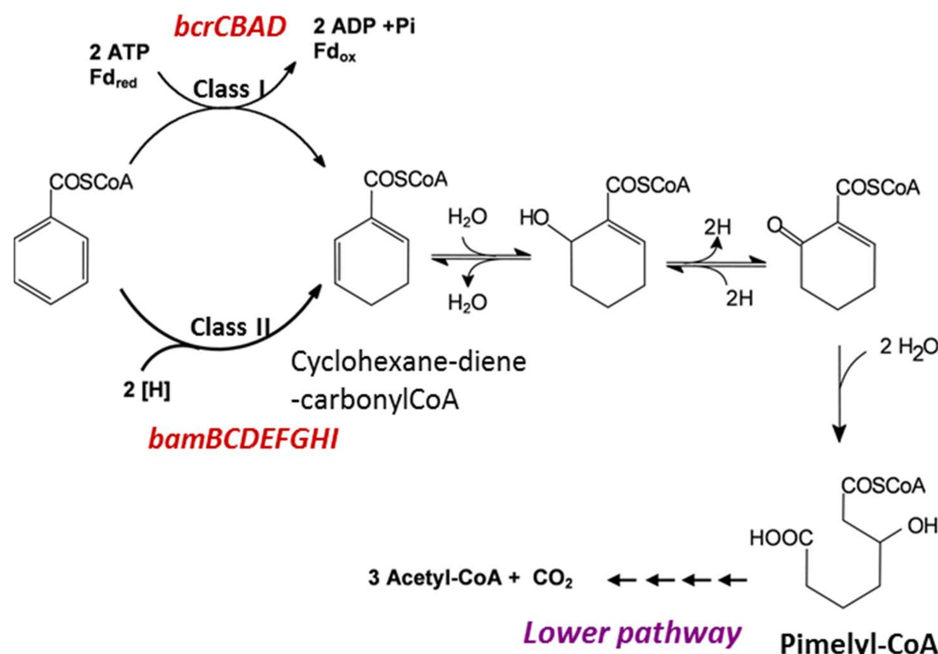

Figure 5. Gene products involved in the benzoyl-CoA degradation pathway in facultative and obligate anaerobes. Bcr, benzoyl-CoA reductase (ATP-dependent); Bam, benzoic acid metabolism. Modified from (Heintz *et al.*, 2009).

5.2.1. Anaerobic naphthalene degrading microorganisms.

Anaerobic naphthalene degradation under nitrate-reducing conditions was first reported by Mihelcic and Luthy (1988). Rockne *et al.* (2000) reported the isolation of three naphthalene degrading NRB, but the activity of these strains could never be confirmed (Meckenstock and Mouttaki, 2011). Despite a number of studies documenting naphthalene degradation with nitrate as electron acceptor (Rockne and Strand, 2001; Eriksson *et al.*, 2003b; Dou *et al.*, 2009, Acosta-González et al., 2013b), to date there is no isolate available able to degrade naphthalene using nitrate as terminal electron acceptor (Meckenstock *et al.*, 2016).

Naphthalene degradation was later described with other electron acceptors, including sulphate (Coates *et al.*, 1996a; Bedessem *et al.*, 1997; Zhang and Young, 1997; Galushko *et al.*, 1999a; Meckenstock *et al.*, 2000a), ferric iron (Coates *et al.*, 1996b; Anderson and Lovley, 1999; Kleemann and Meckenstock, 2011), and manganese (Langenhoff *et al.*, 1996). Zhang and Young (1997) described the first consortium, isolated from heavily contaminated marine sediments, capable of mineralizing naphthalene under sulphate-reducing conditions. Furthermore, this was the first report providing evidence for carboxylation as initial activation reaction in naphthalene anaerobic degradation. Isolation of pure cultures of naphthalene degrading anaerobes from marine sediment has only been possible for two sulphate-reducing marine *Delsulfobacteriaceae*, the NaphS2 and NaphS3 strains (Galushko *et al.*, 1999a; Musat *et al.*, 2009), although only strain

NaphS2 has been characterized in depth. In addition, a bacterial consortium, enriched from a contaminated aquifer and composed mainly of the *Desulfobacterium* strain N47 and a *Spirochaetes* as partner organism has been stably maintained, although the naphthalene degrading partner N47 strain was unable to grow on its own on naphthalene (Meckenstock *et al.*, 2000a; Selesi *et al.*, 2010)

Although ferric iron is an important and highly abundant electron acceptor in anoxic environments, few report documented PAH degradation with this electron acceptor. Anderson and Lovley (1999) first observed anaerobic oxidation of benzene and naphthalene to CO_2 in sediments collected from a petroleum-contaminated aquifer under Fe(III)-reducing conditions. Later, an anaerobic naphthalene-degrading culture (N49) was enriched from an aquifer contaminated with tar oil with ferric iron as electron acceptor (Kleemann and Meckenstock, 2011). The culture was mainly composed of bacteria belonging to the family *Peptococcaceae* within the phylum *Firmicutes*. In this enrichment culture, production of 2-naphthoic acid was observed, suggesting carboxylation as the initial activation reaction of naphthalene mineralization. Langenhoff *et al.* (1996) studied the degradation of naphthalene in anaerobic sediment columns supplemented with different electron acceptors, among which manganese. They observed that naphthalene was only partly transformed when nitrate or manganese were used as electron acceptor, but most of the added naphthalene disappeared under sulphate-reducing conditions, indicating a favourable redox condition for sulphate-reducing naphthalene degradation.

Naphthalene degradation under methanogenic conditions was also reported, although in some cases the naphthalene biodegradation was only partial (Christensen *et al.*, 2004; Chang *et al.*, 2006; Dolfing *et al.*, 2009; Maillacheruvu and Pathan, 2009). Christensen *et al.* (2004) observed naphthalene removal from sewage sludge enrichment cultures under methanogenic conditions, in the presence of hydrogen utilizing methanogens. Cultures were a mixture of bacteria, oxidizing the naphthalene, and archaea, generally identified as *Methanobacteriales*, converting the hydrogen produced by oxidation to methane. Later, Dolfing *et al.* (2009) evaluated the thermodynamic constraints on methanogenic PAH degradation, demonstrating that PAH-based methanogenesis is an exergonic reaction and therefore energetically favourable.

5.2.2. Anaerobic naphthalene biodegradation mechanisms

The extensive knowledge on anaerobic degradation of substituted aromatics summarized above is much scanter when we consider non-substituted aromatics such as benzene and naphthalene. The special interest of these compounds relies on the difficulty to activate the non-substituted aromatic ring in the absence of oxygen (Meckenstock and Mouttaki, 2011). Several lines of evidence suggest that in both cases activation proceeds through direct carboxylation of the

aromatic ring to render benzoic or naphthoic acid, respectively (Zhang and Young, 1997; Musat *et al.*, 2009; Abu Laban *et al.*, 2010; DiDonato *et al.*, 2010; Selesi *et al.*, 2010; Luo *et al.*, 2014) (Figure 6). Candidate genes for a naphthalene carboxylase have been identified in the metagenomes of the N47 enrichment and in NaphS2 genome (DiDonato *et al.*, 2010; Bergmann *et al.*, 2011). Further activation of these compounds would involve a specific CoA ligase, as already shown for benzoic acid, followed by reductive opening of the aromatic ring (Meckenstock and Mouttaki, 2011). Specifically, naphthalene is degraded through the corresponding 2-naphtoil-CoA intermediate. Further dearomatization of 2-naphtoil-CoA appears to proceed sequentially for the two aromatic rings by means of two different sets of enzymes (DiDonato *et al.*, 2010; Selesi *et al.*, 2010; Meckenstock and Mouttaki, 2011; Boll *et al.*, 2014). In N47 enrichment a 2-naphthoyl-CoA reductase (NCR) belonging to a new class of dearomatizing arylcarboxyl-CoA reductases showing similarity to the family of "old yellow enzymes" (OYE), was initially thought to catalyse the four-electron reduction of the non-activated ring of 2-naphtoyl-CoA to render 5,6,7,8-tetrahydro-2-naphthoyl-CoA (THNCoA) (Eberlein *et al.*, 2013a). However, further analysis demonstrated that two specific reductases belonging to the OYE family were involved in dearomatization of naphtoyl-CoA: one was the previously mentioned NCR, producing 5,6-DHNCoA, which was further transformed by a 5,6-DHNCoA reductase (DHNCR) to THNCoA (Estelmann *et al.*, 2015). In the naphthalene degrading sulphate-reducing strain NaphS2, an *ncr* gene with homology with this reductase was found close to the gene for a naphtoyl-CoA ligase responsible for the previous step in the degradation pathway. The next step in the pathway is the ATP-dependent reduction of 5,6,7,8-tetrahydro-2-naphthoyl-CoA to a hexahydro-2-NCoA compound by a class I (ATP-dependent) arylcarboxyl-CoA reductase (Figure 6) (Eberlein *et al.*, 2013b). It is worth noting that most of the genes for naphthalene degradation identified in one of the strains had its homologue in the other strain (DiDonato *et al.*, 2010).

In the case of naphthalene, it should be noted that all anaerobic naphthalene-degrading cultures isolated so far were also able to degrade 2-methylnaphthalene (2MN), which was initially taken as an indication that degradation could proceed via methylation (Annweiler *et al.*, 2000). However, this hypothesis, as well as the hydroxylation pathway, has been discarded (Meckenstock *et al.*, 2016). Degradation of 2MN would follow a pathway similar to anaerobic degradation of toluene: activation through the addition of the methyl group to fumarate through a naphthyl-2-methyl succinate synthase (*nmsABC* genes), followed by β-oxidation-like steps to finally render

naphtoyl-CoA, which then suffers the sequential ring reduction steps (DiDonato et al., 2010; Meckenstock *et al.*, 2016) (Figure 6).

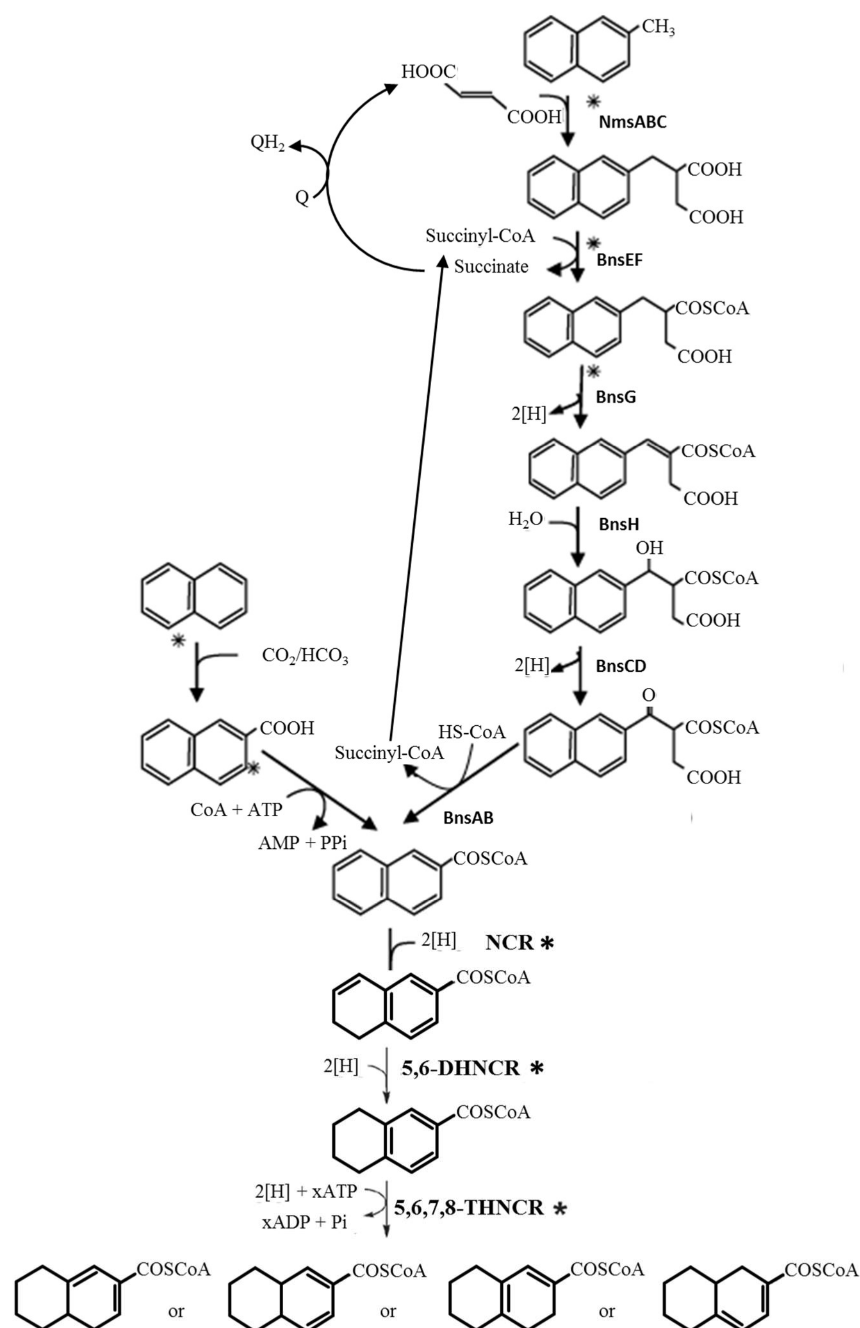

Figure 6. Proposed pathways for anaerobic naphthalene and 2-methylnaphthalene degradation in the enrichment culture N47. NmsABC = Naphthyl-2-methyl-succinate synthase; BnsEF = naphthyl-2-methyl-succinate-CoA transferase; BnsG = naphthyl- 2-methyl-succinyl-CoA dehydrogenase; BnsH = naphthyl-2-methylene-succinyl-CoA hydratase; BnsCD = naphthyl-2-hydroxymethyl- succinyl-CoA dehydrogenase; BnsAB = naphthyl- 2-oxomethyl-succinyl-CoA thiolase; NCR (N47_G38220) = 2-naphthoyl-CoA reductase; 5,6-DHNCR (N47_G38210) = 5,6-dihydro-2-naphthyl-CoA reductase; 5,6,7,8-THNCR = 5,6,7,8-tetrahydro-2-naphthoyl-CoA reductase. Reactions which have been identified and the activity measured in N47 cells grown with naphthalene or methylnaphthalene, respectively, are marked with an asterisk (Meckenstock *et al.*, 2016).

6. Microbial diversity

Prokaryotic life on earth exists since at least 3.8 billion years ago, approximately 750 million years after the Earth was formed. So, they dominated life prior to the appearance of eukaryotes, 1 to 1.5 billion years later, and this is probably the reason why the prokaryotic evolution led to its great diversity (Cooper, 2000). Also, prokaryotes are ubiquitous organism and have adapted to almost any environmental condition, even the most extreme one. They are a key component of the biosphere. It has been estimated that about one-half of the "living protoplasm" on earth is microbial (Kluyver and van Niel, 2014). The number of prokaryotes cells was initially estimated to be 4-6 × 10^{30} and their biomass to be comparable to the total carbon found in plants (Whitman *et al.*, 1998). However, these figures have recently been revised and reduced by almost one order of magnitude (Kallmeyer *et al.*, 2012). The enormous abundance, the ubiquity and the genetic diversity of prokaryotic microorganisms make them play crucial roles in the geochemical cycles that process the major elements of life, including C, N, and S (Falkowski *et al.*, 2008).

Despite the great interest to evaluate the magnitude of prokaryotic diversity, to date there are no definite estimates of the total number of prokaryotic species on earth (Whitman, 2009). Up to a few years ago the estimates were based on cultivable organisms, but only an average of 1% of the microbes can be cultured in the laboratory (Whitfield, 2005), so these efforts had led to a great underestimation of microbial diversity. Nowadays, with the development of the molecular techniques and massive sequencing possibilities, scientists are trying to get a more accurate answer. Torsvik *et al.*, (2002) calculated the prokaryotic diversity with the DNA reassociation method. This technique uses the reassociation of denatured DNA to measure the genome size and complexity of prokaryotes and to estimate the total community genetic diversity. This method has revealed a great diversity in high organic content soil and pristine sediment. In contrast, aquatic and extreme environments seem to host much less prokaryotic genetic complexity (Table 3). However, recent analysis of the environmental 16S rRNA sequences revealed that hypersaline ecosystems have a diversity comparable to that of soil (Lozupone and Knight, 2007). Other authors using different approaches have estimated the number of microbial species in the range from 10^6 to 10^9 (Dykhuizen, 1998; Curtis *et al.*, 2002), but other authors consider this number to be overestimated (Finlay, 2002; Pedrós-Alió, 2006). Then, despite a growing knowledge of the magnitude of prokaryote diversity, a precise estimation is not available yet. This is probably due to the lack of a unique concept of prokaryote species (Rossello-Mora and Amann, 2001; Tamames *et al.*, 2010). Also, the methods to study microbial communities' diversity allow a better knowledge of the abundant taxa, while the rare species are often underestimated (Lynch and Neufeld, 2015). However, in the last few years new sequencing technologies are allowing a deeper

Table 3. Prokaryotic abundance determined by total genomic diversity in prokaryotic communities calculated from the reassociation rate of DNA isolated from the community. Community genome complexity is described as numbers of base pairs (bp). Genome equivalents are given relative to the *Escherichia coli* genome (4.1x10^6 bp) (Torsvik *et al.,* 2002).

DNA source	Abundance (cells x cm^{-3})	Community genome complexity (pb)	Genome equivalents
Forest soil	4.8x10^9	2.5x10^{10}	6000
Forest soil, cultivated prokaryotes	1.4x10^7	1.4x10^8	35
Pasture soil	1.8x10^{10}	(1.5x10^{10})-(3.5x10^{10})	3500-8800
Arable soil		(5.7x10^8)-(1.4x109)	140-350
Pristine marine sediment	3.1x10^9	4.8x10^{10}	11400
Marine fish-farm sediment	7.7x10^9	2x10^8	50
Salt-crystallizing pond, 22% salinity	6.0x10^7	2.9x10^7	7

sampling and a more complete analysis of the different species in a given ecosystem, and probably in the next few years we will be able to have a good approximation to overall diversity values (Yarza *et al.*, 2014).

Diversity is one of the emerging properties of the study of community ecology. Two main characteristics can be determined in the microbial ecology exploration: the taxon richness and the relative abundance of different taxa in the community (Konopka, 2009). Bacterial population are normally governed by the few most abundant taxa, also defined as the core taxa. These species are responsible for carbon and energy flow in the ecosystem. On the other hand, there are the low abundant species that are considered as genetic seed bank of the habitat (Pedrós-Alió, 2006). The rare taxa seem to contribute significantly to the nitrogen and sulphur cycling. Although the role of the 'rare biosphere' is not well understood, there is a growing attention in knowing its ecological function and the reason why the ecosystem supports so many species in low numbers. Probably they constitute the immense genetic and functional diversity reserve of the ecosystem, that can be activated when environmental conditions change, in agreement with the view 'everything is everywhere; the environment selects' (Whitfield, 2005; Lynch and Neufeld, 2015). If fact, some studies are supporting the idea that environmental factors (i.e. temperature, pH, salinity, etc.) are the driving forces of the microbial diversity pattern (Lozupone and Knight, 2007; Tamames *et al.*, 2010; Jeffries *et al.*, 2011).

However, though the scientific community still has the debate about microbial biogeography and diversity open, they agree about the importance to understand how microbes contribute to the ecological and biogeochemical functioning of our ecosystems and how they respond to stresses such as climate change and contamination (Torsvik *et al.*, 2002; Whitfield, 2005; Falkowski *et al.*,

2008). Microorganisms represent near half of the biomass of our planet and we still know little of the prokaryotic diversity on the biosphere, which reveals the largely unexplored genetic pool of the microbial world (Diaz, 2004). In addition, they are considered a crucial component of our efforts to restore degraded ecosystem. In fact, as discussed above, the enormous versatility and genetic diversity of microorganisms offer interesting strategies to reduce environmental pollution (Whitfield, 2005).

7. Microbial community analysis

A systematic analysis of microbial communities may help towards the understanding of how microorganism diversity can be managed for bioremediation purposes or for biotechnological applications. Both, the exploration of the microbial diversity and the genetic content of the communities will lead to this purpose. After an environmental stress factor in a specific habitat, like contamination by PAHs, the initial community is disturbed. Microbial communities' structure, diversity and dynamics are important to identify the key population and their probable interaction to be able to enhance microbial activities during biodegradation. There is not a single method to study the shifts of microbial communities, and a combination of different approaches can give a more comprehensive overview. Generally, culture-independent and-dependent methods for microbial community analysis have been used simultaneously to describe bacterial communities.

7.1. Culture dependent methods

Culture-dependent approaches rely on the cultivation of prokaryotes. In this group, we find the classic isolation and culture methods in solid and liquid media and the most probable number (MPN) techniques. Culture-based methods have been extremely useful to have a first view of the microbial communities' diversity and to understand the physiological potential of isolated organisms, although only about 0.1%-1% of the total bacteria could be enumerated or isolated (Staley and Konopka, 1985; Amann *et al.*, 1995). Those techniques have, in fact limits and restrictions. For example, differences of several orders of magnitude have been observed between direct microscopic and viable-cell counts of bacteria for oligotrophic to mesotrophic aquatic ecosystems. Staley and Konopka (1985) called this phenomenon 'the great plate count anomaly'. Conventional culture media could not satisfy specific requirements (nutrients, pH conditions, incubation temperatures or levels of oxygen) for the growth of a bacterium (Vartoukian *et al.*, 2010). Most culture media used to date are rich in nutrients and promote the growth of faster-growing bacteria at the expense of slow-growing species, some of which can only thrive in

nutrient-poor environments (Connon and Giovannoni, 2002). Also, some bacteria could not grow in monoculture, because of their need of bacterial interactions and signalling molecules. Due to this documented disparity between cultivatable and *in-situ* diversity, it is often difficult to assess the significance of cultured members in resident microbial communities. Some studies, for example, have employed culture-independent molecular methods to show that cultivated microorganisms often may represent very minor components of the microbial community (Ward *et al.*, 1990; Suzuki *et al.*, 1997). Despite the potential biases of the culture-dependent methods, it is only through pure cultures that we can fully characterize bacteria. Therefore, it is important to use both culture-dependent and -independent approaches to understand the composition and function of natural microbial communities and to uncover the unknown diversity in the various ecosystems.

7.2. Culture independent methods

Culture-independent approaches are based on molecular methods to study microbes within their environments. These techniques give a more representative view of whole communities and are not subject to the limits of pure-culture techniques. Among them we find methods based on polymerase chain reaction (PCR), fluorescent *in situ* hybridization (FISH) and metagenomic and proteomic analysis. We briefly described some. Many communities' analyses studies use PCR techniques to amplify the small subunit (SSU) of ribosomal 16S RNA (16S rRNA) or highly conserved functional genes. The 16S rRNA genes has been widely used as molecular marker to phylogenetically characterize microbial communities (Woese and Fox, 1977). The SSU rRNA has both highly conserved and species-dependent variable regions (Figure 7), which evolved at different time rates and can be used to unravel old phylogenetic relationships (domains or phyla) and more recent evolutionary relationships between closely related organisms (genera or species) (Yarza *et al.*, 2014). The differently conserved regions of 16 rRNAs provide target sites for specific probes that are a powerful tool to study microbes and their taxonomic relationships. Conserved regions in the 16S rRNA sequence can be useful for the design of universal primers, for sequence alignment and for the search of homologous regions in different bacteria sequences, while the variable regions can be used for species-specific primers or hybridization probes (Van de Peer *et al.*, 1996). The shift from Sanger sequencing to the massive sequencing technologies (the so-called next generation sequencing) have opened new opportunities in microbial community analysis. There is a growing number of environmental 16S rRNA gene sequences available at 'low cost' that can be used to study the unexplored biodiversity and the ecological characteristics of either whole communities or individual microbial taxa (Sogin *et al.*, 2006; Caporaso *et al.*, 2011).

Perhaps, in the next years, we will be able to detect most of the existing taxa using the high-throughput sequencing techniques. However, we should consider the information limits that SSU rRNA gene shorter sequence lengths provide. In fact, only near full-length 16s rRNA gene can give accurate richness estimations and taxa classifications (Yarza *et al.*, 2014).

An important tool to study the microbial communities' genetic potential is the functional gene analysis. Commonly, there is a positive correlation between the abundance of biodegradation genes and natural attenuation and bioremediation processes. Catabolic genes from aerobic and anaerobic PAHs degradation pathways can be used as genetic biomarker to investigate microbial activity both in field and *in vitro* studies. Functional genes can be detected by PCR with degenerated primer designed based on the homology of the amino acid sequences of relevant and conserved proteins in the degradation pathways. Generally, the target genes of oligonucleotides

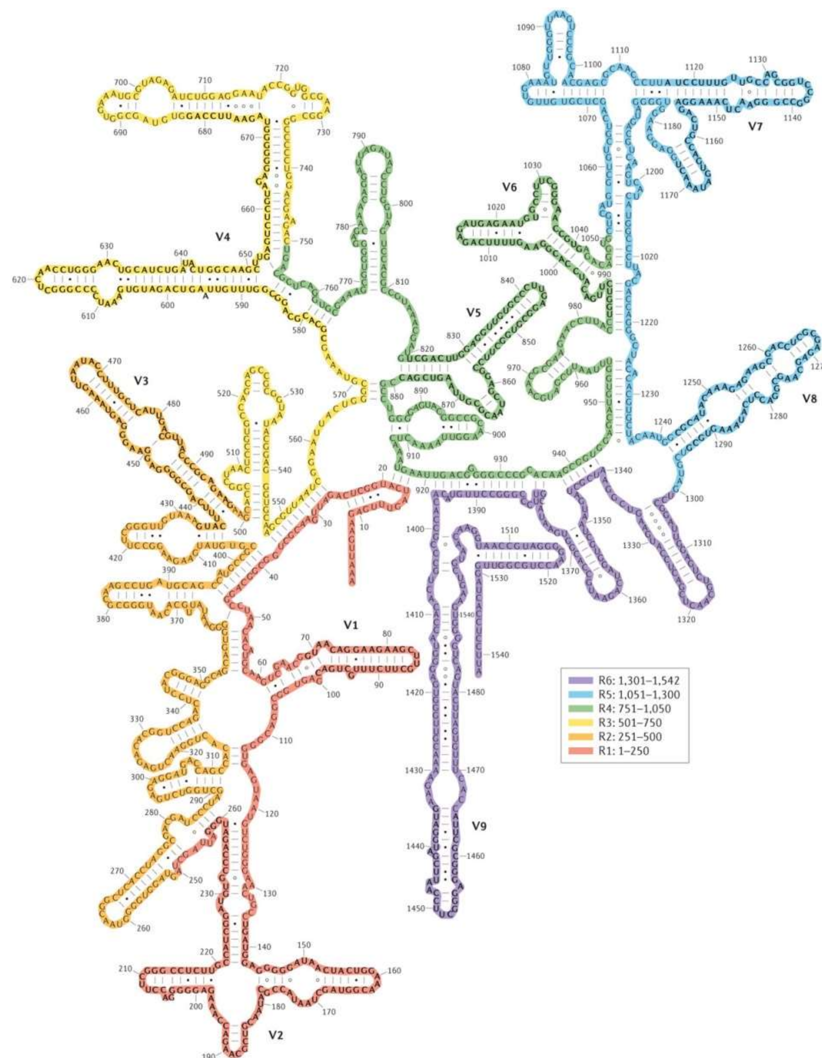

Figure 7. 16S ribosomal RNA secondary structure model and its variable regions. In red, fragment R1 including regions V1 and V2; in orange, fragment R2 including region V3; in yellow, fragment R3 including region V4; in green, fragment R4 including regions V5 and V6; in blue, fragment R5 including regions V7 and V8; and in purple, fragment R6 including region V9. Taken from (Yarza *et al.*, 2014).

are those involved in the first activation step of a determined degradation pathway. For the aerobic aromatic degradation pathway, many primers have been designed targeting the α-subunit of aromatic dioxygenases (Iwai *et al.*, 2011). Among these, the naphthalene dioxygenase genes (*nah*) are of particular interest as a marker for PAH degradation because they are highly conserved and the enzyme encoded by this gene could also mediate degradation of phenanthrene, anthracene, dibenzothiophene, fluorine, and methylated naphthalenes (Ahn *et al.*, 1999). For the PAH anaerobic degradation pathway, there is much less information available. Genetic biomarkers targeting genes encoding benzylsuccinate synthase-like enzymes (*bssA*), which are known to catalyse anaerobic biodegradation of many mono-aromatic substrates, such as toluene, *o*-, *m*-, and *p*-xylene and ethylbenezene, have been used to determine the potential hydrocarbon biodegradation in contaminated sites (Winderl *et al.*, 2007; Acosta-González *et al.*, 2013a). Recently, another fumarate-adding enzyme, naphthyl-2-methyl succinate synthase (*nmsA*) involved in the first step of the degradation of 2MN by SRB (Selesi *et al.*, 2010), has been established as specific functional marker genes for anaerobic hydrocarbon degraders and different sets of primers have been designed (von Netzer *et al.*, 2013). Morris *et al.* (2014) were the first to design degenerate oligonucleotide probes specifically targeting the gene encoding 2-naphthoyl-CoA reductase (*ncr*), responsible for the dearomatization of the nonsubstituted ring of the central intermediate in PAH degradation 2-naphthoyl-CoA (Eberlein *et al.*, 2013a) (Figure 6). However, the paucity of anaerobic PAH degradation gene sequences makes difficult the development of universal functional marker genes. In addition, it has to be considered that the genes associated with aromatic removal can be present but not expressed. So, there is an increasing interest in quantifying the mRNA of key catabolic genes via real-time PCR (Debruyn and Sayler, 2009).

Fluorescence *in situ* hybridization (FISH), is another useful technique that provides a rapid phylogenetic identification and quantification of microorganisms in natural environment (Amann *et al.*, 2001). Intact fixed cells are hybridized with fluorescently labelled probes that target 16S rRNA. Recently FISH method has been used to target also functional genes (Wagner and Haider, 2012). Even though FISH is a powerful technique in which PCR biases are avoided, this method can have limits. In fact, application of FISH probes requires optimization of the hybridization conditions to avoid non-specific results, the probe targeting a taxonomic group could be non-specific, and cells can have limited permeability to the specific probes (Yeates *et al.*, 2003).

8. References

Abdel-Shafy, H.I., and Mansour, M.S.M. (2016). A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egypt J Petrol* **25**: 107-123.

Abu Laban, N., Selesi, D., Rattei, T., Tischler, P., and Meckenstock, R.U. (2010). Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. *Environ Microbiol* **12**: 2783-2796.

Acosta-González, A., Rosselló-Mora, R., and Marqués, S. (2013a). Diversity of benzylsuccinate synthase-like (*bssA*) genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering. *Appl Environ Microbiol* **79**: 3667-3676.

Acosta-González, A., Rosselló-Mora, R., and Marqués, S. (2013b). Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the Prestige oil spill. *Environ Microbiol* 15, 77-92.

Ahn, Y., Sanseverino, J., and Sayler, G.S. (1999). Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. *Biodegradation* **10**: 149-157.

Amann, R., Fuchs, B.M., and Behrens, S. (2001). The identification of microorganisms by fluorescence in situ hybridisation. *Curr Opin Biotechnol* **12**: 231-236.

Amann, R.I., Ludwig, W., and Schleifer, K.H. (1995). Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59**: 143-169.

Anderson, R.T., and Lovley, D.R. (1999). Naphthalene and Benzene Degradation under Fe(III)-Reducing Conditions in Petroleum-Contaminated Aquifers. *Bioremediation J* **3**: 121-135.

Andersson, J.T., and Achten, C. (2015). Time to Say Goodbye to the 16 EPA PAHs? Toward an Up-to-Date Use of PACs for Environmental Purposes. *Polycycl Aromat Compd* **35**: 330-354.

Ang, E.L., Zhao, H., and Obbard, J.P. (2005). Recent advances in the bioremediation of persistent organic pollutants via biomolecular engineering. *Enzyme Microb Technol* **37**: 487-496.

Annweiler, E., Richnow, H.H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., Francke, W., and Michaelis, W. (2000). Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. *Appl Environ Microbiol* **66**: 518-523.

Azubuike, C.C., Chikere, C.B., and Okpokwasili, G.C. (2016). Bioremediation techniques–classification based on site of application: principles, advantages, limitations and prospects. *World J Microbiol Biotechnol* **32**: 180.

Balashova, N.V., Kosheleva, I.A., Golovchenko, N.P., and Boronin, A.M. (1999). Phenanthrene metabolism by *Pseudomonas* and *Burkholderia* strains. *Process Biochem* **35**: 291-296.

Basta, T., Buerger, S., and Stolz, A. (2005). Structural and replicative diversity of large plasmids from sphingomonads that degrade polycyclic aromatic compounds and xenobiotics. *Microbiology* **151**: 2025-2037.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., Dewachter, R., Verachtert, H., and Diels, L. (2000). Isolation of Adherent Polycyclic Aromatic Hydrocarbon (PAH)-Degrading Bacteria Using PAH-Sorbing Carriers. *Appl Environ Microbiol* **66**: 1834-1843.

Bedessem, M.E., Swoboda-Colberg, N.G., and Colberg, P.J.S. (1997). Naphthalene mineralization coupled to sulfate reduction in aquifer-derived enrichments. *FEMS Microbiol Lett* **152**: 213-218.

Beller, H.R., Spormann, A.M., Sharma, P.K., Cole, J.R., and Reinhard, M. (1996). Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. *Appl Environ Microbiol* **62**: 1188-1196.

Bergmann, F.D., Selesi, D., and Meckenstock, R.U. (2011). Identification of new enzymes potentially involved in anaerobic naphthalene degradation by the sulfate-reducing enrichment culture N47. *Arch Microbiol* **193**: 241-250.

Boll, M., Loffler, C., Morris, B.E., and Kung, J.W. (2014). Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ Microbiol* **16**: 612-627.

Braker, G., Ayala-Del-Rio, H.L., Devol, A.H., Fesefeldt, A., and Tiedje, J.M. (2001). Community structure of denitrifiers, bacteria, and archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. *Appl Environ Microbiol* **67**: 1893-1901.

Brune, A., Frenzel, P., and Cypionka, H. (2000). Life at the oxic-anoxic interface: microbial activities and adaptations. *FEMS Microbiol Rev* **24**: 691-710.

Buckel, W., and Thauer, R.K. (2013). Energy conservation via electron bifurcating ferredoxin reduction and proton/Na(+) translocating ferredoxin oxidation. *Biochim Biophys Acta* **1827**: 94-113.

Burchiel, S.W., and Luster, M.I. (2001). Signaling by Environmental Polycyclic Aromatic Hydrocarbons in Human Lymphocytes. *Clin Immunol* **98**: 2-10.

Canfield, D.E., Jorgensen, B.B., Fossing, H., Glud, R., Gundersen, J., Ramsing, N.B., Thamdrup, B., Hansen, J.W., Nielsen, L.P., and Hall, P.O. (1993). Pathways of organic carbon oxidation in three continental margin sediments. *Mar Geol* **113**: 27-40.

Cao, B., Nagarajan, K., and Loh, K.C. (2009). Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. *Appl Microbiol Biotechnol* **85**: 207-228.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., and Knight, R. (2011). Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proc Natl Acad Sci USA* **108**: 4516-4522.

Carmona, M., Zamarro, M.T., Blázquez, B., Durante-Rodríguez, G., Juárez, J.F., Valderrama, J.A., Barragán, M.J., García, J.L., and Díaz, E. (2009). Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev* **73**: 71-133.

Cases, I., and De Lorenzo, V. (2005). Genetically modified organisms for the environment: stories of success and failure and what we have learned from them. *Int Microbiol* **8**: 213-222.

Cassidy, D.P., Srivastava, V.J., Dombrowski, F.J., and Lingle, J.W. (2015). Combining in situ chemical oxidation, stabilization, and anaerobic bioremediation in a single application to reduce contaminant mass and leachability in soil. *J Hazard Mater* **297**: 347-355.

Cerniglia, C.E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. *Adv Appl Microbiol* **30**: 31-71.

Cerniglia, C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**: 351-368.

Chang, W., Um, Y., and Holoman, T.R. (2006). Polycyclic aromatic hydrocarbon (PAH) degradation coupled to methanogenesis. *Biotechnol Lett* **28**: 425-430.

Christensen, N., Batstone, D.J., He, Z., Angelidaki, I., and Schmidt, J.E. (2004). Removal of polycyclic aromatic hydrocarbons (PAHs) from sewage sludge by anaerobic degradation. *Water Sci Technol* **50**: 237-244.

Churchill, S.A., Harper, J.P., and Churchill, P.F. (1999). Isolation and Characterization of a *Mycobacterium* Species Capable of Degrading Three- and Four-Ring Aromatic and Aliphatic Hydrocarbons. *Appl Environ Microbiol* **65**: 549-552.

Coates, J.D., and Anderson, R.T. (2000). Emerging techniques for anaerobic bioremediation of contaminated environments. *Trends Biotechnol* **18**: 408-412.

Coates, J.D., Anderson, R.T., and Lovley, D.R. (1996a). Oxidation of Polycyclic Aromatic Hydrocarbons under Sulfate-Reducing Conditions. *Appl Environ Microbiol* **62**: 1099-1101.

Coates, J.D., Anderson, R.T., Woodward, J.C., Phillips, E.J.P., and Lovley, D.R. (1996b). Anaerobic Hydrocarbon Degradation in Petroleum-Contaminated Harbor Sediments under Sulfate-Reducing and Artificially Imposed Iron-Reducing Conditions. *Environ Sci Technol* **30**: 2784-2789.

Colvin, V.L. (2003). The potential environmental impact of engineered nanomaterials. *Nat Biotech* **21**: 1166-1170.

Connon, S.A., and Giovannoni, S.J. (2002). High-Throughput Methods for Culturing Microorganisms in Very-Low-Nutrient Media Yield Diverse New Marine Isolates. *Appl Environ Microbiol* **68**: 3878-3885.

Cooper, G. (2000). *The Cell: A Molecular Approach.*

Curtis, T.P., Sloan, W.T., and Scannell, J.W. (2002). Estimating prokaryotic diversity and its limits. *Proc Natl Acad Sci USA* **99**: 10494-10499.

Daghio, M., Aulenta, F., Vaiopoulou, E., Franzetti, A., Arends, J.B.A., Sherry, A., Suárez-Suárez, A., Head, I.M., Bestetti, G., and Rabaey, K. (2017). Electrobioremediation of oil spills. *Water Res* **114**: 351-370.

Davies, J.I., and Evans, W.C. (1964). Oxidative metabolism of naphthalene by soil pseudomonads. The ring-fission mechanism. *Biochem J* **91**: 251-261.

De Maagd, P.G.-J., Ten Hulscher, D.T.E.M., Van Den Heuvel, H., Opperhuizen, A., and Sijm, D.T.H.M. (1998). Physicochemical properties of polycyclic aromatic hydrocarbons: Aqueous solubilities, n-

octanol/water partition coefficients, and Henry's law constants. *Environ Toxicol Chem* **17**: 251-257.

Debruyn, J.M., and Sayler, G.S. (2009). Microbial community structure and biodegradation activity of particle-associated bacteria in a coal tar contaminated creek. *Environ Sci Technol* **43**: 3047-3053.

Diaz, E. (2004). Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *Int Microbiol* **7**: 173-180.

Didonato, R.J., Jr., Young, N.D., Butler, J.E., Chin, K.J., Hixson, K.K., Mouser, P., Lipton, M.S., Deboy, R., and Methe, B.A. (2010). Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. *PLoS One* **5**: e14072.

Dolfing, J., Xu, A., Gray, N.D., Larter, S.R., and Head, I.M. (2009). The thermodynamic landscape of methanogenic PAH degradation. *Microb Biotechnol* **2**: 566-574.

Dolfing, J., Zeyer, J., Binder-Eicher, P., and Schwarzenbach, R.P. (1990). Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. *Arch Microbiol* **154**: 336-341.

Domínguez-Garay, A., Boltes, K., and Esteve-Núñez, A. (2016). Cleaning-up atrazine-polluted soil by using Microbial Electroremediating Cells. *Chemosphere* **161**: 365-371.

Dou, J., Liu, X., and Ding, A. (2009). Anaerobic degradation of naphthalene by the mixed bacteria under nitrate reducing conditions. *J Hazard Mater* **165**: 325-331.

Dua, M., Singh, A., Sethunathan, N., and Johri, A.K. (2002). Biotechnology and bioremediation: successes and limitations. *Appl Microbiol Biotechnol* **59**: 143-152.

Dykhuizen, D.E. (1998). Santa Rosalia revisited: why are there so many species of bacteria? *Antonie Van Leeuwenhoek* **73**: 25-33.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Muller, M., Meckenstock, R.U., and Boll, M. (2013a). Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol Microbiol* **88**: 1032-1039.

Eberlein, C., Johannes, J., Mouttaki, H., Sadeghi, M., Golding, B.T., Boll, M., and Meckenstock, R.U. (2013b). ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. *Environ Microbiol* **15**: 1832-1841.

Edwards, S.C., Jedrychowski, W., Butscher, M., Camann, D., Kieltyka, A., Mroz, E., Flak, E., Li, Z., Wang, S., Rauh, V., and Perera, F. (2010). Prenatal exposure to airborne polycyclic aromatic hydrocarbons and children's intelligence at 5 years of age in a prospective cohort study in Poland. *Environ Health Perspect* **118**: 1326-1331.

EEA (2007). "Progress in management of contaminated sites (CSI 015) - Assessment published Aug 2007," in *http://www.eea.europa.eu/data-and-maps/indicators/progress-in-management-of-contaminated-sites/progress-in-management-of-contaminated-1*.).

El Fantroussi, S., and Agathos, S.N. (2005). Is bioaugmentation a feasible strategy for pollutant removal and site remediation? *Curr Opin Microbiol* **8**: 268-275.

Eriksson, M., Sodersten, E., Yu, Z., Dalhammar, G., and Mohn, W.W. (2003). Degradation of Polycyclic Aromatic Hydrocarbons at Low Temperature under Aerobic and Nitrate-Reducing Conditions in Enrichment Cultures from Northern Soils. *Appl Environ Microbiol* **69**: 275-284.

Estelmann, S., Blank, I., Feldmann, A., and Boll, M. (2015). Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Mol Microbiol* **95**: 162-172.

Evans, P.J., Mang, D.T., Kim, K.S., and Young, L.Y. (1991). Anaerobic degradation of toluene by a denitrifying bacterium. *Appl Environ Microbiol* **57**: 1139-1145.

Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008). The microbial engines that drive Earth's biogeochemical cycles. *Science* **320**: 1034-1039.

Field, J.A., Stams, A.J.M., Kato, M., and Schraa, G. (1995). Enhanced biodegradation of aromatic pollutants in cocultures of anaerobic and aerobic bacterial consortia. *Antonie Van Leeuwenhoek* **67**: 47-77.

Finlay, B.J. (2002). Global Dispersal of Free-Living Microbial Eukaryote Species. *Science* **296**: 1061-1063.

Foght, J. (2008). Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects. *J Mol Microbiol Biotech* **15**: 93-120.

Foght, J.M., and Westlake, D.W. (1988). Degradation of polycyclic aromatic hydrocarbons and aromatic heterocycles by a *Pseudomonas* species. *Can J Microbiol* **34**: 1135-1141.

Fries, M.R., Zhou, J., Chee-Sanford, J., and Tiedje, J.M. (1994). Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Appl Environ Microbiol* **60**: 2802-2810.

Frutos, F.J., Pérez, R., Escolano, O., Rubio, A., Gimeno, A., Fernández, M.D., Carbonell, G., Perucha, C., and Laguna, J. (2012). Remediation trials for hydrocarbon-contaminated sludge from a soil washing process: evaluation of bioremediation technologies. *J Hazard Mater* **199-200**: 262-271.

Fuchs, G., Boll, M., and Heider, J. (2011). Microbial degradation of aromatic compounds - from one strategy to four. *Nat Rev Microbiol* **9**: 803-816.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* **1**: 415-420.

Ghosal, D., Chakraborty, J., Khara, P., and Dutta, T.K. (2010). Degradation of phenanthrene via meta-cleavage of 2-hydroxy-1-naphthoic acid by *Ochrobactrum* sp. strain PWTJD. *FEMS Microbiol Lett* **313**: 103-110.

Ghosal, D., Dutta, A., Chakraborty, J., Basu, S., and Dutta, T.K. (2013). Characterization of the metabolic pathway involved in assimilation of acenaphthene in *Acinetobacter* sp. strain AGAT-W. *Res Microbiol* **164**: 155-163.

Ghosal, D., Ghosh, S., Dutta, T.K., and Ahn, Y. (2016). Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Front Microbiol* **7**: 1369.

Gibson, D.T., and Parales, R.E. (2000). Aromatic hydrocarbon dioxygenases in environmental biotechnology. *Curr Opin Biotechnol* **11**: 236-243.

Goyal, A.K., and Zylstra, G.J. (1997). Genetics of naphthalene and phenanthrene degradation by *Comamonas testosteroni*. *J Ind Microbiol Biotechnol* **19**: 401-407.

Gupte, A., Tripathi, A., Patel, H., Rudakiya, D., and Gupte, S. (2016). Bioremediation of Polycyclic Aromatic Hydrocarbon (PAHs): A Perspective. *The Open Biotechnology Journal* **10**: 363-378.

Gürtler, V., Mayall, B.C., and Seviour, R. (2004). Can whole genome analysis refine the taxonomy of the genus *Rhodococcus*? *FEMS Microbiol Rev* **28**: 377-403.

Harayama, S., Kok, M., and Neidle, E.L. (1992). Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46**: 565-601.

Haritash, A.K., and Kaushik, C.P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *J Hazard Mater* **169**: 1-15.

Heider, J. (2007). Adding handles to unhandy substrates: anaerobic hydrocarbon activation mechanisms. *Curr Opin Chem Biol* **11**: 188-194.

Heider, J., and Fuchs, G. (1997). Anaerobic metabolism of aromatic compounds. *Eur J Biochem* **243**: 577-596.

Heintz, D., Gallien, S., Wischgoll, S., Ullmann, A.K., Schaeffer, C., Kretzschmar, A.K., Van Dorsselaer, A., and Boll, M. (2009). Differential membrane proteome analysis reveals novel proteins involved in the degradation of aromatic compounds in *Geobacter metallireducens*. *Mol Cell Proteomics* **8**: 2159-2169.

Heitkamp, M.A., and Cerniglia, C.E. (1988). Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. *Appl Environ Microbiol* **54**: 1612-1614.

IEA (2016). "Key world energy statistics 2116". International Energy Agency.

IEA (2016). "Oil Market Report". International Energy Agency.

Iwabuchi, T., and Harayama, S. (1997). Biochemical and genetic characterization of 2-carboxybenzaldehyde dehydrogenase, an enzyme involved in phenanthrene degradation by *Nocardioides* sp. strain KP7. *J Bacteriol* **179**: 6488-6494.

Iwai, S., Johnson, T.A., Chai, B., Hashsham, S.A., and Tiedje, J.M. (2011). Comparison of the Specificities and Efficacies of Primers for Aromatic Dioxygenase Gene Analysis of Environmental Samples. *Appl Environ Microbiol* **77**: 3551-3557.

Jeffries, T.C., Seymour, J.R., Gilbert, J.A., Dinsdale, E.A., Newton, K., Leterme, S.S., Roudnew, B., Smith, R.J., Seuront, L., and Mitchell, J.G. (2011). Substrate type determines metagenomic profiles from diverse chemical habitats. *PLoS One* **6**: e25173.

Jones, D.M., Head, I.M., Gray, N.D., Adams, J.J., Rowan, A.K., Aitken, C.M., Bennett, B., Huang, H., Brown, A., Bowler, B.F.J., Oldenburg, T., Erdmann, M., and Larter, S.R. (2008). Crude-oil biodegradation via methanogenesis in subsurface petroleum reservoirs. *Nature* **451**: 176-180.

Jones, K.C., and De Voogt, P. (1999). Persistent organic pollutants (POPs): state of the science. *Environ Pollut* **100**: 209-221.

Kallmeyer, J., Pockalny, R., Adhikari, R.R., Smith, D.C., and D,Äôhondt, S. (2012). Global distribution of microbial abundance and biomass in subseafloor sediment. *Proc Natl Acad Sci USA* **109**: 16213-16216.

Keith, L.H. (2015). The Source of U.S. EPA's Sixteen PAH Priority Pollutants. *Polycyc Aromatic Compounds* **35**: 147-160.

Keith, L.H., and Telliard, W.A. (1979). Priority pollutants I- A perspective view. *Environ Sci Technol* **13**: 416-423.

Kim, T.J., Lee, E.Y., Kim, Y.J., Cho, K.-S., and Ryu, H.W. (2003). Degradation of polyaromatic hydrocarbons by *Burkholderia cepacia* 2A-12. *World J Microbiol Biotechnol* **19**: 411-417.

Kirkwood, A.E., Nalewajko, C., and Fulthorpe, R.R. (2006). The Effects of Cyanobacterial Exudates on Bacterial Growth and Biodegradation of Organic Contaminants. *Microb Ecol* **51**: 4-12.

Kiyohara, H., Nagao, K., Kouno, K., and Yano, K. (1982). Phenanthrene-degrading phenotype of *Alcaligenes faecalis* AFK2. *Appl Environ Microbiol* **43**: 458-461.

Kiyohara, H., Nagao, L., and Nomi, R. (1976). Degradation of Phenanthrene through o-Phthalate by an *Aeromonas* sp. *Agric Biol Chem* **40**: 1075-1082.

Kleemann, R., and Meckenstock, R.U. (2011). Anaerobic naphthalene degradation by Gram-positive, iron-reducing bacteria. *FEMS Microbiol Ecol* **78**: 488-496.

Kluyver, A.J., and Van Niel, C.B. (2014). *The Microbe's Contribution to Biology.*

Konopka, A. (2009). What is microbial community ecology? *ISME J* **3**: 1223-1230.

Korda, A., Santas, P., Tenente, A., and Santas, R. (1997). Petroleum hydrocarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used. *Appl Microbiol Biotechnol* **48**: 677-686.

Kristensen, P., Eilertsen, E., Einarsdottir, E., Haugen, A., Skaug, V., and Ovreba, S. (1995). Fertility in mice after prenatal exposure to benzo[a]pyrene and inorganic lead. *Environ Health Perspect* **103**: 588-590.

Kung, J.W., Baumann, S., Von Bergen, M., Muller, M., Hagedoorn, P.L., Hagen, W.R., and Boll, M. (2010). Reversible biological Birch reduction at an extremely low redox potential. *J Am Chem Soc* **132**: 9850-9856.

Kuppusamy, S., Thavamani, P., Venkateswarlu, K., Lee, Y.B., Naidu, R., and Megharaj, M. (2017). Remediation approaches for polycyclic aromatic hydrocarbons (PAHs) contaminated soils: Technological constraints, emerging trends and future directions. *Chemosphere* **168**: 944-968.

Langenhoff, A.a.M., Zehnder, A.J.B., and Schraa, G. (1996). Behaviour of toluene, benzene and naphthalene under anaerobic conditions in sediment columns. *Biodegradation* **7**: 267-274.

Larkin, M.J., Kulakov, L.A., and Allen, C.C. (2005). Biodegradation and *Rhodococcus* -masters of catabolic versatility. *Curr Opin Biotechnol* **16**: 282-290.

Laurie, A.D., and Lloyd-Jones, G. (1999). The phn genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. *J Bacteriol* **181**: 531-540.

Leuthner, B., and Heider, J. (2000). Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of beta oxidation of the intermediate benzylsuccinate. *J Bacteriol* **182**: 272-277.

López, Z., Vila, J., Minguillón, C., and Grifoll, M. (2006). Metabolism of fluoranthene by *Mycobacterium* sp. strain AP1. *Appl Microbiol Biotechnol* **70**: 747-756.

Lovley, D.R., and Lonergan, D.J. (1990). Anaerobic Oxidation of Toluene, Phenol, and p-Cresol by the Dissimilatory Iron-Reducing Organism, GS-15. *Appl Environ Microbiol* **56**: 1858-1864.

Lozupone, C.A., and Knight, R. (2007). Global patterns in bacterial diversity. *Proc Natl Acad Sci USA* **104**: 11436-11440.

Luo, F., Gitiafroz, R., Devine, C.E., Gong, Y., Hug, L.A., Raskin, L., and Edwards, E.A. (2014). Metatranscriptome of an anaerobic benzene-degrading, nitrate-reducing enrichment culture reveals involvement of carboxylation in benzene ring activation. *Appl Environ Microbiol* **80**: 4095-4107.

Lynch, M.D.J., and Neufeld, J.D. (2015). Ecology and exploration of the rare biosphere. *Nat Rev Micro* **13**: 217-229.

Maillacheruvu, K.Y., and Pathan, I.A. (2009). Biodegradation of naphthalene, phenanthrene, and pyrene under anaerobic conditions. *J Environ Sci Health A Tox Hazard Subst Environ Eng* **44**: 1315-1326.

Mallick, S., Chakraborty, J., and Dutta, T.K. (2011). Role of oxygenases in guiding diverse metabolic pathways in the bacterial degradation of low-molecular-weight polycyclic aromatic hydrocarbons: a review. *Crit Rev Microbiol* **37**: 64-90.

Mallick, S., Chatterjee, S., and Dutta, T.K. (2007). A novel degradation pathway in the assimilation of phenanthrene by *Staphylococcus* sp. strain PN/Y via meta-cleavage of 2-hydroxy-1-naphthoic acid: formation of trans-2,3-dioxo-5-(2'-hydroxyphenyl)-pent-4-enoic acid. *Microbiology* **153**: 2104-2115.

Marston, C.P., Pereira, C., Ferguson, J., Fischer, K., Hedstrom, O., Dashwood, W.-M., and Baird, W.M. (2001). Effect of a complex environmental mixture from coal tar containing polycyclic aromatic hydrocarbons (PAH) on the tumor initiation, PAH–DNA binding and metabolic activation of carcinogenic PAH in mouse epidermis. *Carcinogenesis* **22**: 1077-1086.

Martínez-Pascual, E., Grotenhuis, T., Solanas, A.M., and Viñas, M. (2015). Coupling chemical oxidation and biostimulation: Effects on the natural attenuation capacity and resilience of the native microbial community in alkylbenzene-polluted soil. *J Hazard Mater* **300**: 135-143.

Masih, J., Masih, A., Kulshrestha, A., Singhvi, R., and Taneja, A. (2010). Characteristics of polycyclic aromatic hydrocarbons in indoor and outdoor atmosphere in the North central part of India. *J Hazard Mater* **177**: 190-198.

Mcdonald, B.G., and Chapman, P.M. (2002). PAH phototoxicity-an ecologically irrelevant phenomenon? *Mar Pollut Bull* **44**: 1321-1326.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**: 2743-2747.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A.M. (2016). Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. *J Mol Microbiol Biotechnol* **26**: 92-118.

Meckenstock, R.U., Morasch, B., Griebler, C., and Richnow, H.H. (2004). Stable isotope fractionation analysis as a tool to monitor biodegradation in contaminated acquifers. *J Contam Hydrol* **75**: 215-255.

Meckenstock, R.U., and Mouttaki, H. (2011). Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr Opin Biotechnol* **22**: 406-414.

Mihelcic, J.R., and Luthy, R.G. (1988). Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl Environ Microbiol* **54**: 1182-1187.

Morris, B.E., Gissibl, A., Kummel, S., Richnow, H.H., and Boll, M. (2014). A PCR-based assay for the detection of anaerobic naphthalene degradation. *FEMS Microbiol Lett* **354**: 55-59.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., and Rabus, R. (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ Microbiol* **11**: 209-219.

Park, K.S., Sims, R.C., Dupont, R.R., Doucette, W.J., and Matthews, J.E. (1990). Fate of PAH compounds in two soil types: Influence of volatilization, abiotic loss and biological activity. *Environ Toxicol Chem* **9**: 187-195.

Pedersen, F., and Falck, J. (1997). "Environmental Hazard Classification - Classification of selected substances as dangerous for the environment; NORDBAS2 software program. TemaNord. (Copenhagen, Denmark: Nordic Council of Ministers.), pp. 101.

Pedrós-Alió, C. (2006). Marine microbial diversity: can it be determined? *Trends Microbiol* **14**: 257-263.

Peng, R.H., Xiong, A.S., Xue, Y., Fu, X.Y., Gao, F., Zhao, W., Tian, Y.S., and Yao, Q.H. (2008). Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiol Rev* **32**: 927-955.

Perera, F., Tang, D., Whyatt, R., Lederman, S.A., and Jedrychowski, W. (2005). DNA damage from polycyclic aromatic hydrocarbons measured by benzo[a]pyrene-DNA adducts in mothers and newborns from Northern Manhattan, the World Trade Center Area, Poland, and China. *Cancer Epidemiol Biomarkers Prev* **14**: 709-714.

Pieper, D.H., Martins Dos Santos, V.A., and Golyshin, P.N. (2004). Genomic and mechanistic insights into the biodegradation of organic pollutants. *Curr Opin Biotechnol* **15**: 215-224.

Pumphrey, G.M., and Madsen, E.L. (2007). Naphthalene metabolism and growth inhibition by naphthalene in *Polaromonas naphthalenivorans* strain CJ2. *Microbiology* **153**: 3730-3738.

Rabus, R., Boll, M., Heider, J., Meckenstock, R.U., Buckel, W., Einsle, O., Ermler, U., Golding, B.T., Gunsalus, R.P., Kroneck, P.M., Kruger, M., Lueders, T., Martins, B.M., Musat, F., Richnow, H.H., Schink, B., Seifert, J., Szaleniec, M., Treude, T., Ullmann, G.M., Vogt, C., Von Bergen, M., and Wilkes, H. (2016). Anaerobic Microbial Degradation of Hydrocarbons: From Enzymatic Reactions to the Environment. *J Mol Microbiol Biotechnol* **26**: 5-28.

Rabus, R., Nordhaus, R., Ludwig, W., and Widdel, F. (1993). Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. *Appl Environ Microbiol* **59**: 1444-1451.

Ramos, J.L., Marqués, S., Van Dillewijn, P., Espinosa-Urgel, M., Segura, A., Duque, E., Krell, T., Ramos-González, M.I., Bursakov, S., Roca, A., Solano, J., Fernández, M., Niqui, J.L., Pizarro-Tobias, P., and Wittich, R.M. (2011). Laboratory research aimed at closing the gaps in microbial bioremediation. *Trends Biotechnol* **29**: 641-647.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W., and Bemment, C.D. (2008). Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. *Water Res* **42**: 4215-4232.

Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000). Anaerobic Naphthalene Degradation by Microbial Pure Cultures under Nitrate-Reducing Conditions. *Appl Environ Microbiol* **66**: 1595-1601.

Rockne, K.J., and Strand, S.E. (2001). Anaerobic biodegradation of naphthalene, phenanthrene, and biphenyl by a denitrifying enrichment culture. *Water Res* **35**: 291-299.

biphenyl by a denitrifying enrichment culture. *Water Res* 35**,** 291-299.

Rodriguez-Conde, S., Molina, L., Gonzalez, P., Garcia-Puente, A., and Segura, A. (2016). Degradation of phenanthrene by *Novosphingobium* sp. HS2a improved plant growth in PAHs-contaminated environments. Appl Microbiol Biotechnol 100, 10627-10636.

Rossello-Mora, R., and Amann, R. (2001). The species concept for prokaryotes. FEMS Microbiol Rev 25, 39-67.

Schink, B., Philipp, B., and Muller, J. (2000). Anaerobic degradation of phenolic compounds. *Naturwissenschaften* **87**: 12-23.

Schneider, J., Grosser, R., Jayasimhulu, K., Xue, W., and Warshawsky, D. (1996). Degradation of pyrene, benz[a]anthracene, and benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135, isolated from a former coal gasification site. *Appl Environ Microbiol* **62**: 13-19.

Schnitz, A.R., Squibb, K.S., and O'connor, J.M. (1993). Time-varying conjugation of 7,12-dimethylbenz[a]anthracene metabolites in rainbow trout (*Oncorhynchus mykiss*). *Toxicol Appl Pharmacol* **121**: 58-70.

Schühle, K., Gescher, J., Feil, U., Paul, M., Jahn, M., Schägger, H., and Fuchs, G. (2003). Benzoate-Coenzyme A Ligase from Thauera aromatica: an Enzyme Acting in Anaerobic and Aerobic Pathways. *J Bacteriol* **185**: 4920-4929.

Selesi, D., Jehmlich, N., Von Bergen, M., Schmidt, F., Rattei, T., Tischler, P., Lueders, T., and Meckenstock, R.U. (2010). Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J Bacteriol* **192**: 295-306.

Seo, J.-S., Keum, Y.-S., and Li, Q.X. (2009). Bacterial Degradation of Aromatic Compounds. *Int J Env Res Public Health* **6**: 278-309.

Silva-Castro, G.A., Uad, I., Gónzalez-López, J., Fandiño, C.G., Toledo, F.L., and Calvo, C. (2012). Application of selected microbial consortia combined with inorganic and oleophilic fertilizers to recuperate oil-polluted soil using land farming technology. *Clean Technol. Envir.* **14**: 719-726.

Simon, M.J., Osslund, T.D., Saunders, R., Ensley, B.D., Suggs, S., Harcourt, A., Suen, W.C., Cruden, D.L., Gibson, D.T., and Zylstra, G.J. (1993). Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. *Gene* **127**: 31-37.

Sogin, M.L., Morrison, H.G., Huber, J.A., Mark Welch, D., Huse, S.M., Neal, P.R., Arrieta, J.M., and Herndl, G.J. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc Natl Acad Sci USA* **103**: 12115-12120.

Sørensen, J. (1978). Denitrification Rates in a Marine Sediment as Measured by the Acetylene Inhibition Technique. *Appl Environ Microbiol* **36**: 139-143.

Staley, J.T., and Konopka, A. (1985). Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annu Rev Microbiol* **39**: 321-346.

Straube, W.L., Nestler, C.C., Hansen, L.D., Ringleberg, D., Pritchard, P.H., and Jones-Meehan, J. (2003). Remediation of Polyaromatic Hydrocarbons (PAHs) through Landfarming with Biostimulation and Bioaugmentation. *Acta Biotechnol* **23**: 179-196.

Suess, M.J. (1976). The environmental load and cycle of polycyclic aromatic hydrocarbons. *Sci Total Environ* **6**: 239-250.

Sun, G.-D., Xu, Y., Jin, J.-H., Zhong, Z.-P., Liu, Y., Luo, M., and Liu, Z.-P. (2012). Pilot scale ex-situ bioremediation of heavily PAHs-contaminated soil by indigenous microorganisms and bioaugmentation by a PAHs-degrading and bioemulsifier-producing strain. *J Hazard Mater* **233–234**: 72-78.

Suzuki, M.T., Rappé, M.S., Haimberger, Z.W., Winfield, H., Adair, N., Ströbel, J., and Giovannoni, S.J. (1997). Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. *Appl Environ Microbiol* **63**: 983-989.

Tamames, J., Abellan, J.J., Pignatelli, M., Camacho, A., and Moya, A. (2010). Environmental distribution of prokaryotic taxa. *BMC Microbiol* **10**: 85.

Timmis, K.N., and Pieper, D.H. (1999). Bacteria designed for bioremediation. *Trends Biotechnol* **17**: 201-204.

Torsvik, V., Ovreas, L., and Thingstad, T.F. (2002). Prokaryotic diversity--magnitude, dynamics, and controlling factors. *Science* **296**: 1064-1066.

Tratnyek, P.G., and Johnson, R.L. (2006). Nanotechnologies for environmental cleanup. *Nano Today* **1**: 44-48.

Vaes, W.H.J., Ramos, E.U., Verhaar, H.J.M., and Hermens, J.L.M. (1998). Acute toxicity of nonpolar versus polar narcosis: Is there a difference? *Environ Toxicol Chem* **17**: 1380-1384.

Van De Peer, Y., Chapelle, S., and De Wachter, R. (1996). A quantitative map of nucleotide substitution rates in bacterial rRNA. *Nucleic Acids Res* **24**: 3381-3391.

Van Herwijnen, R., Springael, D., Slot, P., Govers, H.a.J., and Parsons, J.R. (2003). Degradation of Anthracene by *Mycobacterium* sp Strain LB501T Proceeds via a Novel Pathway, through o-Phthalic Acid. *Appl Environ Microbiol* **69**: 186-190.

Vartoukian, S.R., Palmer, R.M., and Wade, W.G. (2010). Strategies for culture of 'unculturable' bacteria. *FEMS Microbiol Lett* **309**: 1-7.

Von Netzer, F., Pilloni, G., Kleindienst, S., Krüger, M., Knittel, K., Gründger, F., and Lueders, T. (2013). Enhanced Gene Detection Assays for Fumarate-Adding Enzymes Allow Uncovering of Anaerobic Hydrocarbon Degraders in Terrestrial and Marine Systems. *Appl Environ Microbiol* **79**: 543-552.

Wackett, L.P. (2003). Pseudomonas putida--a versatile biocatalyst. *Nat Biotechnol* **21**: 136-138.

Wagner, M., and Haider, S. (2012). New trends in fluorescence *in situ* hybridization for identification and functional analyses of microbes. *Curr Opin Biotechnol* **23**: 96-102.

Ward, D.M., Weller, R., and Bateson, M.M. (1990). 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. *Nature* **345**: 63-65.

Wassenberg, D.M., and Di Giulio, R.T. (2004). Synergistic Embryotoxicity of Polycyclic Aromatic Hydrocarbon Aryl Hydrocarbon Receptor Agonists with Cytochrome P4501A Inhibitors in Fundulus heteroclitus. *Environ Health Perspect* **112**: 1658-1664.

Whitfield, J. (2005). Boigeography: Is everything everywhere? *Science* **310**: 960-961.

Whitman, W.B. (2009). The Modern Concept of the Procaryote. *J Bacteriol* **191**: 2000-2005.

Whitman, W.B., Coleman, D.C., and Wiebe, W.J. (1998). Prokaryotes: The unseen majority. *Proc Natl Acad Sci USA* **95**: 6578-6583.

Widdel, F., and Rabus, R. (2001). Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotechnol* **12**: 259-276.

Winderl, C., Schaefer, S., and Lueders, T. (2007). Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. *Environ Microbiol* **9**: 1035-1046.

Woese, C.R., and Fox, G.E. (1977). Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc Natl Acad Sci USA* **74**: 5088-5090.

Yarza, P., Yilmaz, P., Pruesse, E., Glockner, F.O., Ludwig, W., Schleifer, K.-H., Whitman, W.B., Euzeby, J., Amann, R., and Rossello-Mora, R. (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat Rev Micro* **12**: 635-645.

Yeates, C., Saunders, A.M., Crocetti, G.R., and Blackall, L.L. (2003). Limitations of the widely used GAM42a and BET42a probes targeting bacteria in the *Gammaproteobacteria* radiation. *Microbiology* **149**: 1239-1247.

Zhang, T., Gannon, S.M., Nevin, K.P., Franks, A.E., and Lovley, D.R. (2010). Stimulating the anaerobic degradation of aromatic hydrocarbons in contaminated sediments by providing an electrode as the electron acceptor. *Environ Microbiol* **12**: 1011-1020.

Zhang, X., and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* **63**: 4759-4764.

Zhang, Y., and Tao, S. (2009). Global atmospheric emission inventory of polycyclic aromatic hydrocarbons (PAHs) for 2004. *Atmos Environ* **43**: 812-819.

Zhou, N.Y., Fuenmayor, S.L., and Williams, P.A. (2001). *nag* genes of *Ralstonia* (formerly *Pseudomonas*) sp. strain U2 encoding enzymes for gentisate catabolism. *J Bacteriol* **183**: 700-708.

II. OBJECTIVES

Polycyclic aromatic hydrocarbons (PAHs) are among the most prevalent organic pollutants in the environment. While the aerobic degradation of PAHs has been intensively studied, the anaerobic pathways using alternative electron acceptors are much less investigated. The main aim of this work was to study the microbial communities' response to the presence of PAHs in various environments with different degrees of hydrocarbon pollution and in oxygen limited condition. We used culture-dependent and culture-independent methods to characterize the bacterial communities and to enrich microbial populations able to thrive in the presence of PAHs under oxygen limiting conditions, with the following specific objectives:

1. To investigate the effects that incubation with PAHs under nitrate-reducing condition produced on the natural microbial communities' and to identify potential microorganisms involved in PAHs degradation.

2. To study the *in-situ* response of the indigenous microbial community from hypersaline sediments of Fuente de Piedra lagoon (Málaga, Spain) to PAH contamination and to enrich sulphate-reducing population possibly involved in PAHs degradation.

3. To investigate the shift of microbial population from a hydrocarbon polluted aquifer exposed to PAHs under different oxygen limiting conditions: anaerobic nitrate-reducing and microaerophilic conditions.

The results are presented as three independent chapters addressing the three objectives, each including specific sections: Introduction, Material and methods, and Results and Discussion.

III. GENERAL METHODS

We describe here the general methods that are common to all the chapters, which include the cultivation of nitrate reducing bacteria, the most probable number enumeration of culturable communities, the amplification and analysis of 16S rRNA libraries and the chemical analysis of hydrocarbon compounds. Notwithstanding, each chapter describes some specific modifications to these general methods.

1. Culture conditions, enrichment and isolation procedures

For the cultivation of nitrate-reducing bacteria (NRB) 25 g of samples were transferred to 120 ml serum bottles containing 75 ml of non-reduced Widdel mineral medium, modified according to (Darley *et al.*, 2007): the medium was buffered with 30 mM 3-(N-morpholino)propanesulfonic acid (MOPS) instead of bicarbonate, prepared in a nitrogen atmosphere and sealed with Teflon-lined 1 cm thick stoppers. The basal freshwater medium used for the soil and sludge samples contained 1.0 g NaC1 and 0.4 g $MgCl{\cdot}6H_2O$ per liter, whilst the marine medium used for the marine and lagoon sediments contained 20.0 g NaC1 and 3.0 g $MgCl_2$ per liter. Techniques for the preparation of media and for the cultivation of NRB under anoxic conditions were as follows: briefly, after autoclaving, the medium was cooled in a sterile nitrogen atmosphere and supplemented with 5 mM $NaNO_3$ as electron acceptor, 1 mM Na_2SO_4 as the sulphur source, 1 ml of a vitamin solution, 1 ml of the trace element solution SL10, 1 ml selenite-tungstate solution and 30 mM MOPS buffer, pH 7.2 (Darley *et al.*, 2007), and dispensed in 120 ml serum bottles flushed with sterile nitrogen gas. Naphthalene and 2-methylnaphthalene (2MN) were dissolved at 20 g l^{-1} in sterile anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) that served as an inert carrier phase to reduce the PAH concentration in the water phase. The resulting suspension (2 ml) was then added to the serum bottles as an overlay using a nitrogen-flushed syringe. A control bottle without added aromatic compounds in the HMN phase was prepared for each sample. The cultures were incubated in the dark at 28°C without shaking. To determine growth, the cultures were monitored periodically for nitrite produced from nitrate respiration (Snell and Snell, 1949), or for nitrate consumption by ion chromatography. After three months of incubation, the medium above soil/sediment particles and the organic layer were removed and replaced by fresh mineral medium and the corresponding carbon source in HMN. This operation was repeated twice every three months, and after an additional two-month incubation samples were taken for pyrosequencing. Further transfers (every 6 months) were made by inoculating 10% (v/v) of the cultures into fresh medium. At several stages, bacterial strains were isolated under anoxic conditions, either using serial agar dilutions (agar shakes) prepared in a nitrogen atmosphere (Widdel and Bak, 1992) and overlaid with aromatic hydrocarbons in HMN, or in standard agar Petri dishes prepared and

incubated in a nitrogen atmosphere with naphthalene provided in the vapour phase as crystals on the Petri dish cover placed up-side-down.

2. Most probable number enumeration of denitrifying bacteria

Nitrate reducing PAH-degrading bacterial populations were enumerated using the most-probable number (MPN) assay in 10-fold serial dilutions. The medium was prepared and dispensed as described above. Naphthalene, 2-methylnaphthalene, naphtoic acid and anthracene were added to the tubes from stock diethyl ether solutions to reach a final concentration of 0.025‰, 0.028‰, 0.035‰ and 0.035‰, respectively, and the solvent was evaporated before dispensing the medium. Acetate was used as a positive control to a final concentration of 5 mM, and controls with no added carbon source were included. The minimal medium containing 5 mM sodium nitrate as the electron acceptor was purged with sterile nitrogen gas and 9 ml were poured into oxygen-free tubes. Growth of NRB was measured as the nitrite produced from nitrate respiration as described above. For each sample, triplicate tubes were inoculated with 1 ml of the corresponding dilution in 10-fold serial dilutions. The tubes were incubated at 28°C in the dark and growth was checked for 6 months. The data are the result of three replicate per sample within the 95% confidence interval.

3. Chemical analysis of hydrocarbon content

For aliphatic and aromatic hydrocarbon compounds determination, we used duplicate 5 g aliquots of soil/sediment samples. Prior to the extraction 100 ppb of the Mix 37 (manufactured by Dr. Ehrenstorfer) and 15 ppm of the 5-alpha-cholestane (Aldrich) were added to each sample as internal standards. A volume of 10 ml of a hexane/acetone mixture (2:1) was added to the samples, and the mixture was shacked and sonicated twice during 5 min. Next 20 ml of saturated NaCl were added to the suspensions. The supernatants were collected and passed through a BOND ELUT TPH 500 mg Na_2SO_4 column (Varian) previously conditioned with 3 ml of hexane to separate the aliphatic and the aromatic fractions. The aliphatic fraction was first collected and the BOND ELUTE TPH columns were dried completely. The aromatic fraction was next eluted with 6.5 ml of dichloromethane. The extracts were concentrated by evaporation under a gentle nitrogen flow and the hydrocarbons were determined at the Scientific Instrumentation Service of the EEZ (CSIC), Granada, Spain, in a Varian-450 Gas Cromatographer (GC) equipped with the TG-5SilMS column (30 m x 0.25 mm x 0.25 µm) and a CTCGCpal autosampler. Data were acquired and processed using Varian MS Workstation 6.9.1. Helium was used as carrier gas at a flow rate of 1 ml/min. The injected volume was 1 µl (splitless mode). The oven temperature started at 50°C and was raised at 15°C/min up to 150°C. A new ramp of 6°C/min was started up to 300°C, when the

temperature was held for 10 min. Injector and detector temperatures were set at 300°C and 290°C respectively. Compound identification was performed using the NIST08 library included in the MS Workstation software 6.9.1 and the information provided by the standards injected under the same conditions. The standards used in the chromatography were the alkane mixture DRH-008S-R2 y DRH-001S, the PAH mixture Mix 37 y Mix 9 (manufactured by Dr. Ehrenstorfer), 1-Chlorooctadecane and 5-alpha-cholestane, and the pure compounds naphthalene, 1-methylnaphthalene, 2MN, 1-4-5-trimethylnaphthalene, 1-4-6-7-tetramethylnaphthalene, dibenzothiophene, phenanthrene and anthracene.

4. Total DNA extraction, PCR amplification and pyrosequencing library construction

DNA extraction was done following a modified version of the SDS-based method developed by (Zhou *et al.*, 1996) except that in the third extraction three freeze-thaw cycles were included before the incubation at 65°C. Supernatants from the three extractions were combined and extracted first with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v) and then with chloroform-isoamylalcohol (24:1, v/v), and precipitated overnight at room temperature with 0.6 volume of isopropanol. Nucleic acids were recovered by centrifugation (7000 rcf/45 min), washed with cold 70% ethanol, resuspended in 100 µl of sterile deionized water and stored at -20°C. Nucleic acid quantity and purity were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). A multiplex pyrosequencing amplicon approach was used for the characterization of the bacterial communities. The PCR amplifications of the hypervariable V1–V3 region of the 16S rRNA gene were carried out using the bacterial universal primers 6F and 532R containing 5′ tags with multiplex identifier and sequencing adapters. PCR amplifications were performed in 50 µl reactions containing 1x PCR Buffer (Biorad), 200 µM dNTPs (Roche), 0.5 µM of each primer (Roche), 1U of iProof™ High-Fidelity DNA Polymerase (Biorad) and 20 ng of target DNA. The PCR program consisted of an initial denaturation step at 98°C for 30 s, followed by 25 cycles at 98°C for 10 s, 50°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR fragments were purified using the PCR Purification Kit (Qiagen) and checked on 1.5% agarose gel. Amplicon products with adapters and barcodes were quantified using Qubit™ fluorometer (Invitrogen), pooled at an equimolar ratio and sequenced using a 454 Titanium amplicon sequencing kit and a Genome Sequencer FLX 454 at either Citius (University of Seville) or Macrogen (Korea).

5. 16S rRNA gene amplification

PCR amplification of the 16S rRNA gene to identify the isolated strains was performed with the bacterial universal primers GM3F and GM4R (Muyzer *et al.*, 1995). PCR reactions were carried out in 50 µl reactions as previously described (Acosta-González *et al.*, 2013). The amplified fragments were cloned in pGEM-T (Promega) according to the manufacturer instructions. Positive clones were checked by PCR with pUC/M13F and pUC/M13R primers and were Sanger-sequenced as above. The generated chromatograms were analyzed and edited with the Chromas (Technelysium) and DNA Baser (Heracle Biosoft) softwares for quality checking, vector trimming and sequence assembly. Phylogenetic analysis was done with the ARB package (Ludwig *et al.*, 2004) using the online SINA alignment service and Silva database version SSU Ref 119.

6. Data Analysis

The 454 bacterial 16S sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) v. 1.7.0 pipeline (Caporaso *et al.*, 2010). A total of 32 samples were analysed. We first performed the sample demultiplexing, primer removal and quality-filtering. Briefly, sequences with lengths <150 bp, ambiguous bases >0, homopolymers >6, primer mismatches, and average quality scores <50 were removed. Reads were checked for chimeras using the ChimeraSlayer algorithm (Haas *et al.*, 2011). All chimeras and singletons were removed before further analysis. The clustering method was used to assign similar sequences to operational taxonomic units (OTUs) at a 97% similarity threshold. A representative sequence from each OTU was annotated with PyNAST (Caporaso *et al.*, 2010). The taxonomic assignment of OTUs was performed using the RDP Classifier. A variable number of sequences were obtained per sample; to avoid sampling size effects, the number of reads was normalized to 1,500 for each sample. Otherwise the non-rarefied OTU table was used. Alpha and Beta diversity analyses, rarefaction curves, Chao1 richness estimator, Shannon diversity index and Good's sample coverage were calculated using QIIME v. 1.7.0 pipeline. Principal coordinate analysis (PCoA) was performed using the subsampled data to detect microbial community differences on the basis of weighted and unweighted UniFrac distance metrics (Lozupone and Knight, 2005). Jackknife resampling was used to assess the stability of the PCoA analysis. Hierarchical clustering was conducted to group the communities of different samples using the unweighted pair group method with arithmetic mean (UPGMA). Differences in the relative abundance among treatments were calculated by two-sided t-tests. To construct the phylogenetic trees of iii1-8 sequences we used the Neighbour-Joining method. Bootstrap test (1000 replicates) was used to calculate the percentage of replicate

trees in which the associated taxa clustered together. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

7. References

Acosta-González, A., Rossello-Mora, R., and Marqués, S. (2013). Diversity of benzylsuccinate synthase-like (*bssA*) genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering. *Appl. Environ. Microbiol.* **79**: 3667-3676.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat methods* **7**: 335-336.

Darley, P.I., Hellstern, J.A., Medina-Bellver, J.I., Marques, S., Schink, B., and Philipp, B. (2007). Heterologous expression and identification of the genes involved in anaerobic degradation of 1,3-dihydroxybenzene (resorcinol) in Azoarcus anaerobius. *J. Bacteriol.* **189**: 3824-3833.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S.K., Sodergren, E., Methe, B., Desantis, T.Z., Human Microbiome, C., Petrosino, J.F., Knight, R., and Birren, B.W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res.* **21**: 494-504.

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl. Environ. Microbiol.* **71**: 8228-8235.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**: 1363-1371.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannasch, H.W. (1995). Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch. Microbiol.* **164**: 165-172.

Schumacher, B. (2002). *Methods for the determination of total organic carbon (toc) in soil and sediments US EPA las vegas.* US EPA.

Snell, F.D., and Snell, C.T. (1949). *Colorimetric Methods of Analysis.* New York: D. Van Nostrand Reinhold, Co.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol.Evol* **28,** :2731-2739.

Widdel, F., and Bak, F. (1992). "Gram-Negative Mesophilic Sulfate-Reducing Bacteria," in *The Prokaryotes,* eds. A. Balows, H. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Springer New York), 3352-3378.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. *App. Environ Microbiol* **62**: 316-322.

IV. RESULTS AND DISCUSSION

CHAPTER 1

Polycyclic aromatic hydrocarbon-induced changes in bacterial community structure under anoxic nitrate reducing conditions.

This chapter was published as:

Martirani-Von Abercron SM, Pacheco D, Benito-Santano P, Marín P and Marqués S.

Polycyclic Aromatic Hydrocarbon-Induced Changes in Bacterial Community Structure under Anoxic Nitrate Reducing Conditions.

Front Microbiol, 2016 7:1775 , DOI:10.3389/fmicb.2016.01775

If we knew what it was we were doing,
it would not be called research,
would it?

Albert Einstein

1.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are chemicals of particular environmental concern because of their stability, persistence in the environment and resistance to degradation. Many of them are known to be toxic to various organisms and dangerous for health. They are frequently released into the environment either from natural sources (e.g. hydrocarbon seeps) or as consequence of industrial activities such as the massive transport or synthesis of added value chemicals. For most low molecular weight PAHs, microbial biodegradation is the primary process leading to their complete mineralization. The biodegradation of PAHs by aerobic bacteria has been studied extensively and is well understood (Fuchs *et al.*, 2011). In aerobic environments, oxygen is not only the terminal electron acceptor for bacterial respiration, but is also an essential co-substrate for the activation and cleavage of the aromatic ring. Enzymatic steps invariably involve mono-oxygenases and dioxygenases as key activities for the molecular oxygen-dependent initial attack of the aromatic ring (Doyle *et al.*, 2008). However, the availability of oxygen is often limited in natural environments. In many polluted sites, oxygen can be rapidly consumed in the aerobic biodegradation processes, resulting in a decreasing oxygen concentration gradient whereupon reduction of other electron acceptors becomes energetically favourable. Sediments are a good example of such a situation, where initially microorganisms consume the oxygen that penetrates the first millimetres/centimetres of the sediments. Once the oxygen consumption exceeds its supply, anoxic conditions are established. Below the zone of oxygen-influence, anoxic processes are stimulated: nitrate, manganese, iron and sulphate are then sequentially used for anaerobic respiration, if available (Sorensen, 1978; Canfield *et al.*, 1993). A similar pattern is observed in habitats that are under permanent anoxic conditions, such as flooded sediments in marine and fresh-water environments. Bioremediation of PAHs in these conditions becomes challenging. Our current knowledge of the biochemistry of anaerobic degradation of PAHs is restricted to the simplest molecules naphthalene and 2-methylnaphthalene (2MN) in sulphate-reducing bacteria (SRB). To date only two examples of naphthalene degrading SRBs have been described and isolated, one of which is a two-strain consortium (N47) that could not be obtained as a pure isolate (Selesi *et al.*, 2010; DiDonato *et al.*, 2010). In SRB, naphthalene is activated through carboxylation (Zhang and Young, 1997), and the resulting naphtoic acid is further converted to its coenzyme A (CoA) thioester 2-naphtoil-CoA. Further dearomatization of 2-naphtoil-CoA proceeds sequentially for the two aromatic rings: first a 2-naphthoyl CoA reductase, encoded by the *ncr* gene, and then a 5,6-dihydro-2-naphthoyl-CoA reductase, catalyze two sequential 2-electron reduction steps, followed by the final reduction of the resulting 5,6,7,8-tetrahydronaphthoyl-CoA to hexahydro-2-naphthoyl-CoA (DiDonato *et al.*, 2010; Boll *et al.*, 2014; Estelmann *et al.*, 2015).

On the other hand, anaerobic degradation of 2MN by SRB requires initial activation through addition of its methyl group to fumarate to render naphthyl-2-methyl-succinic acid, a reaction carried out by a naphthyl-2-methylsuccinate synthase encoded by the *nmsA* gene (Selesi *et al.*, 2010). Naphthyl-2-methyl-succinic acid is further transformed to 2-naphthoil-CoA. To date, these pathways have been biochemically and genetically verified in only one SRB isolate (DiDonato *et al.*, 2010) and in a sulphate-reducing enrichment (Selesi *et al.*, 2010). Only recently SRB microcosms with naphthalene as carbon source could link the enrichment of *Desulfobacterium* strains related to N47 with naphthalene degradation, although the strains were not isolated (Kummel *et al.*, 2015). Anaerobic degradation of PAHs with nitrate as electron acceptor has been repeatedly observed in environmental samples and microcosm experiments (al-Bashir *et al.*, 1990; Eriksson *et al.*, 2003; Uribe-Jongbloed and Bishop, 2007; Acosta-González *et al.*, 2013a), but little is known about the degradation mechanism in nitrate-reducing bacteria (NRB). Initial reports of naphthalene-degrading isolates closely related to *Pseudomonas stutzeri* and *Vibrio pelagius* (Röckne *et al.*, 2000) could not be reproduced. Despite efforts by different groups to isolate NRB able to degrade PAHs under anoxic conditions, to date no bacterial strain or consortium able to consistently degrade naphthalene using nitrate as terminal electron acceptor has been described.

With the aim of assessing nitrate reduction-dependent PAH degradation in the environment and identifying potential organisms involved in the process, we selected a series of environmental sites that had been exposed to different degrees of hydrocarbon pollution and presented transient or permanent anoxic conditions. Most probable number estimation evidenced the presence of an initial PAH degrading NRB community. Starting from this material, we initiated enrichment cultures under nitrate-reducing conditions with naphthalene and its methylated derivative 2MN as the added carbon source. We used culture-dependent and molecular techniques to investigate the effects that incubation with PAHs produced on the natural microbial communities' present in the selected environments. We anticipated that exposure to PAHs would result in an altered microbial community structure reflecting both the toxicity of the added aromatic and the opportunity for the biodegradation of a new carbon source under the imposed respiration metabolism. We found that shifting to nitrate as terminal electron acceptor strongly affected the structure of the bacterial community. The presence of naphthalene further disturbed microbial communities and produced a general increase in poorly characterized and uncultured groups.

1.2. Materials and methods

1.2.1. Sample collection and experimental design

Samples were collected from 5 different sampling sites (Table 1. 11). Two rice-paddy soil samples with different water content were taken in November 2011 from surface soil (0–15 cm depth) in a rice paddy located in Las Cabezas de San Juan (Sevilla, Spain) (37°01'42N 5°58'49W), close to some fuel oil leaks. One was obtained from the more aqueous top layer (upper 5 cm) (RPW, Rice-Paddy Water) and the other one from the bottom layer (approximately 5-10 cm) (RPS, Rice-Paddy Soil). A third rice-paddy sample (RPCal) was collected in December 2011 from the surface (0–15 cm) of a rice-paddy in Calasparra (Murcia, Spain) (38°14'44N 1°41'32W). The activated sludge (AS) and composting pile (CP) samples were collected in July 2010 from the CEPSA oil refinery in La Rábida, Huelva (37°34'15N 0°55'30W). The marine sediment sample (MS) was collected by scuba divers at 5-6 m below the water surface from Figueiras beach (Atlantic Islands, Spain) in June 2005 (42°13'31N 8°53'59W) using 5 cm diameter cores inserted in the sediment. The sediment column between 2 and 35 cm was mixed and used in this study. Anoxic black sediments (0-10 cm in depth) from the athalassohaline lagoon of Fuente de Piedra (FdP) (Málaga, Spain) were collected close to the shore (50 cm below water surface) in March 2012 (37°05'07N 4°47'05W).

1.2.2. Culture conditions, enrichment and isolation procedures

For the cultivation of nitrate-reducing bacteria (NRB) 25 g of samples were transferred to 120 ml serum bottles containing 75 ml of non-reduced Widdel mineral medium, modified according to Darley *et al.* (2007): the medium was buffered with 30 mM 3-(N-morpholino)propanesulfonic acid (MOPS) instead of bicarbonate, prepared in a nitrogen atmosphere and sealed with Teflon-lined 1 cm thick stoppers. The basal freshwater medium used for the soil and sludge samples contained 1.0 g NaC1 and 0.4 g $MgCl·6H_2O$ per liter, whilst the marine medium used for the marine and lagoon sediments contained 20.0 g NaC1 and 3.0 g $MgCl_2$ per liter. The cultures were set up as follows: briefly, after autoclaving, the medium was cooled in a sterile nitrogen atmosphere by flowing pure nitrogen gas through it for at least 20 min, and supplemented with 5 mM $NaNO_3$ as electron acceptor, 1 mM Na_2SO_4 as the sulphur source, 1 ml of a vitamin solution, 1 ml of the trace element solution SL10, 1 ml selenite-tungstate solution and 30 mM MOPS buffer, pH 7.2 (Darley *et al.*, 2007), and dispensed in 120 ml serum bottles flushed with sterile nitrogen gas. Naphthalene and 2-methylnaphthalene (2MN) were dissolved at 20 g l^{-1} in sterile anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) that served as an inert carrier phase to reduce the PAH

Table 1. 1. Characterization of the samples used in this study.

Sample Name	Sample type	Description	Location	Coordinates	Water[a] content (%)	Organic matter (%)	Nitrate (µM)	Sulphate (mM)	Total hydrocarbons[c] (ug/kg)	
									Aliphatic	Aromatics
RPCal	Rice pad	Soil	Calasparra (Murcia)	38°14'44N 1°41'32W	18.43±0.23	2.50±0.13	416.65±5.2	0.04±0.004	2990	857.0
RPW	Rice pad	Overlaying muddy water	Sevilla	37°01'42N 5°58'49W	74.85[b]	3.37[b]	14.96±5.1	0.32±0.004	3990	521.0
RPS	Rice pad	Soil	Sevilla	37°01'42N 5°58'49W	5.6±0.0039	4.14±0.08	3.00±0.7	0.09±0.009	3890	460.0
AS	Activated sludge	Mud	Huelva	37°34'15N 0°55'30W	1975.04±390	63.59±0.14	12.65±1.4	0.64±0.02	45150	888.5
CP	Compost pile	Soil	Huelva	37°34'15N 0°55'30W	0.68±0.06	0.94±0.03	501.72±8.9	2.37±0.20	1760	745.1
FdP	Athalassohaline lagoon	Sediment	Fuente de Piedra (Málaga)	37°05'07N 4°47'05W	107.58±6.39	5.87±0.59	0	26.48±3.15	26400	117.0
MS	Marine sediments	*Prestige* oil spill-affected sandy sediments	(Pontevedra)	42°13'31N 8°53'59W	24.74±0.22	0.58±0.02	117.79±4.7	3.15±0.14	1740	95.0

[a]Gravimetric, % of dry weight.
[b]The sample duplicate was lost
[c]The detailed hydrocarbon composition of the samples can be found in supplementary tables S3a and S3b

concentration in the water phase. The resulting suspension (2 ml) was then added to the serum bottles as an overlay using a nitrogen-flushed syringe. A control bottle without added aromatic compounds in the HMN phase was prepared for each sample. Then the appropriate amount of inoculum was added and the cultures were sealed with Teflon-lined 1 cm thick stoppers fixed with aluminum crimp seals. The cultures were incubated in the dark at 28°C without shaking. To determine growth, the cultures were monitored periodically for nitrite produced from nitrate respiration (Snell and Snell, 1949), or for nitrate consumption by ion chromatography. After three months of incubation, the medium above soil/sediment particles and the organic layer were removed and replaced by fresh mineral medium and the corresponding carbon source in HMN. This operation was repeated twice every three months, and after an additional two-month incubation samples were taken for pyrosequencing. Further transfers (every 6 months) were made by inoculating 10% (v/v) of the cultures into fresh medium. At several stages, bacterial strains were isolated under anoxic conditions, either using serial agar dilutions (agar shakes) prepared in a nitrogen atmosphere (Widdel and Bak, 1992) and overlaid with aromatic hydrocarbons in HMN, or in standard agar Petri dishes prepared and incubated in a nitrogen atmosphere with naphthalene provided in the vapour phase as crystals on the Petri dish cover placed up-side-down.

1.2.3. Most probable number enumeration of denitrifying bacteria

Nitrate-reducing PAH-degrading bacterial populations were enumerated using the most-probable number (MPN) assay in 10-fold serial dilutions. The medium was prepared and dispensed as described above. Naphthalene, 2-methylnaphthalene, naphtoic acid and anthracene were added to the tubes from stock diethyl ether solutions to reach a final concentration of 0.025‰, 0.028‰, 0.035‰ and 0.035‰, respectively, and the solvent was evaporated before dispensing the medium. Acetate was used as a positive control to a final concentration of 5 mM, and controls with no added carbon source were included. The minimal medium containing 5 mM sodium nitrate as the electron acceptor was purged with sterile nitrogen gas and 9 ml were poured into oxygen-free tubes. Growth of NRB was measured as the nitrite produced from nitrate respiration as described above. For each sample, triplicate tubes were inoculated with 1 ml of the corresponding dilution in 10-fold serial dilutions. The tubes were incubated at 28°C in the dark and growth was checked during 6 months. The data are the result of three replicate per sample within the 95% confidence interval.

1.2.4. Chemical analysis

All determinations were carried out in duplicate just before starting the enrichments. Soil moisture was determined as follows: samples (5 g) were dried to constant mass in an oven at 105°C. The difference in mass before and after the drying process was used to determine the dry matter and the water content. The loss-on-ignition method was used to determine the organic matter content (Schumacher, 2002). The concentration of soil-available nitrate and sulphate was measured by ion chromatography (IC) using a Metrohm-761 Compact Ion Chromatograph with a Metrosep A Supp 4-250 column with chemical suppression (50 mM H_2SO_4). The eluent was an aqueous sodium carbonate/bicarbonate solution prepared by mixing $NaHCO_3$ and $NaCO_3$ to a final concentration of 1.7 mM and 1.8 mM, respectively. Samples (1 g) were extracted with 5 ml of deionized water by mechanical agitation for 16 h and then analyzed by IC. The detection limit for ions was 0.5 ppm. The detection limit for nitrate determination with this method was 0.5 ppm, and the associated error was 5%. For aliphatic and aromatic hydrocarbon compounds determination we used duplicate 5 g aliquots of soil/sediment samples. Prior to the extraction 100 ppb of the Mix 37 (manufactured by Dr. Ehrenstorfer) and 15 ppm of the 5-alpha-cholestane (Aldrich) were added to each sample as internal standards. A volume of 10 ml of a hexane/acetone mixture (2:1) was added to the samples, and the mixture was shacked and sonicated twice during 5 min. Next 20 ml of saturated NaCl were added to the suspensions. The supernatants were collected and passed through a BOND ELUT TPH 500 mg Na_2SO_4 column (Varian) previously conditioned with 3 ml of hexane to separate the aliphatic and the aromatic fractions. The aliphatic fraction was first collected and the BOND ELUTE TPH columns were dried completely. The aromatic fraction was next eluted with 6.5 ml of dichloromethane. The extracts were concentrated by evaporation under a gentle nitrogen flow and the hydrocarbons were determined at the Scientific Instrumentation Service of the EEZ (CSIC), Granada, Spain, in a Varian 450-GC GC gas chromatographer system coupled to a Varian 240-MS ion-trap mass spectrometer with MS Workstation software, equipped with the TG-5SilMS column (30 m x 0.25 mm x 0.25 μm) and a CTCGCpal autosampler. Data were acquired and processed using Varian MS Workstation 6.9.1. Helium was used as carrier gas at a flow rate of 1 ml/min. The injected volume was 1 μl (splitless mode). The oven temperature started at 50°C and was raised at 15°C/min up to 150°C. A new ramp of 6°C/min was started up to 300°C, when the temperature was held for 10 min. Injector and detector temperatures were set at 300°C and 290°C respectively. Compound identification was performed using the NIST08 library included in the MS Workstation software 6.9.1 and the information provided by the standards injected under the same conditions. The standards used in the chromatography were the alkane mixture DRH-008S-R2 y DRH-001S, the PAH mixture Mix 37 y Mix 9 (manufactured by

Dr. Ehrenstorfer), 1-Chlorooctadecane and 5-alpha-cholestane, and the pure compounds naphthalene, 1-methylnaphthalene, 2MN, 1-4-5-trimethylnaphthalene, 1-4-6-7-tetramethylnaphthalene, dibenzothiophene, phenanthrene and anthracene. The average PAH extraction efficiency obtained with our extraction method was 64 (±18) %.

1.2.5. Total DNA extraction, PCR amplification and pyrosequencing library construction

DNA extraction was done following a modified version of the SDS-based method developed by Zhou *et al.* (1996) except that in the third extraction three freeze-thaw cycles were included before the incubation at 65°C. Supernatants from the three extractions were combined and extracted first with an equal volume of phenol-chloroform-isoamylalcohol (25:24:1, v/v) and then with chloroform-isoamylalcohol (24:1, v/v), and precipitated overnight at room temperature with 0.6 volume of isopropanol. Nucleic acids were recovered by centrifugation (7000 rcf/45 min), washed with cold 70% ethanol, resuspended in 100 µl of sterile deionized water and stored at -20°C. Nucleic acid quantity and purity were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). A multiplex pyrosequencing amplicon approach was used for the characterization of the bacterial communities. The PCR amplifications of the hypervariable V1–V3 region of the 16S rRNA gene were carried out using the bacterial universal primers 6F and 532R containing 5′ tags with multiplex identifier and sequencing adapters (Tables S1a and S1b). PCR amplifications were performed in 50 µl reactions containing 1x PCR Buffer (Biorad), 200 µM dNTPs (Roche), 0.5 µM of each primer (Roche), 1U of iProof™ High-Fidelity DNA Polymerase (Biorad) and 20 ng of target DNA. The PCR program consisted of an initial denaturation step at 98°C for 30 s, followed by 25 cycles at 98°C for 10 s, 50°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The PCR fragments were purified using the PCR Purification Kit (Qiagen) and checked on 1.5% agarose gel. Amplicon products with adapters and barcodes were quantified using Qubit™ fluorometer (Invitrogen), pooled at an equimolar ratio and sequenced using a 454 Titanium amplicon sequencing kit and a Genome Sequencer FLX 454 at either Citius (University of Seville) or Macrogen (Korea).

1.2.6. Functional gene amplification

Gene fragments of *bssA*, *ncr*, and *nmsA* were amplified by PCR using available primer sets (Table S2). For *bssA* gene amplification we used 7772f and 8546r primers (Winderl *et al.*, 2007) and cycling conditions as described by Acosta-González *et al.,* 2013b. *Thauera aromatica* K172

DNA was used as positive control. The *nmsA* gene was amplified using different primer sets (7768f, 7363f, 7374f and 8543r; von Netzer *et al.*, 2013) and the following cycling conditions: 1 min of initial denaturation (98°C), 30–35 amplification cycles of (10 s at 98°C, 20 s at 58°C, 30 s at 72°C) and 5 min of final extension (72°C). PCR conditions for the *ncr* gene amplification comprised initial denaturation at 98°C for 1 min, followed by 30 amplification cycles (20 s at 98°C, 20 s at 55°C, 15 s at 72°C), and a final extension step at 72°C for 5 min. Genomic DNA from NaphS2 strain was used as a positive control. All PCR reactions were performed in 50 µl containing 1x PCR Buffer (Biorad), 200 µM dNTPs (Roche), 0.5 µM of each primer (Sigma), 1 U of iProof™ High-Fidelity DNA Polymerase (Biorad) and 10 to 20 ng of target DNA. The appropriately sized amplicons were purified using the Gel Extraction Kit (Qiagen) and cloned in pCR2.1 (TA Cloning Kit, Invitrogen) according to the manufacturer's instructions, and 16 positive clones from each sample were selected for Sanger sequencing (IPBLN López Neyra, CSIC, Granada, Spain). Sequence analysis was performed as described previously (Acosta-González *et al.*, 2013b).

1.2.7. 16S rRNA gene amplification

PCR amplification of the 16S rRNA gene to identify the isolated strains was performed with the bacterial universal primers GM3F and GM4R (Muyzer *et al.*, 1995). PCR reactions were carried out in 50 µl reactions as previously described (Acosta-González *et al.*, 2013a). The amplified fragments were cloned in pGEM-T (Promega) according to the manufacturer instructions. Positive clones were checked by PCR with pUC/M13F and pUC/M13R primers and were Sanger-sequenced as above. The generated chromatograms were analyzed and edited with the Chromas (Technelysium) and DNA Baser (Heracle Biosoft) softwares for quality checking, vector trimming and sequence assembly. Phylogenetic analysis were done with the ARB package (Ludwig *et al.*, 2004) using the online SINA alignment service and Silva database version SSU Ref 119.

1.2.8. Data Analysis

The 454 bacterial 16S sequences were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME) v. 1.7.0 pipeline (Caporaso *et al.*, 2010). A total of 32 samples were analysed. We first performed the sample demultiplexing, primer removal and quality-filtering. Briefly, sequences with lengths <150 bp, ambiguous bases >0, homopolymers >6, primer mismatches, and average quality scores <50 were removed. Reads were checked for chimeras using the

ChimeraSlayer algorithm (Haas *et al.*, 2011). All chimeras and singletons were removed before further analysis. The clustering method was used to assign similar sequences to operational taxonomic units (OTUs) at a 97% similarity threshold. A representative sequence from each OTU was annotated with PyNAST (Caporaso *et al.*, 2010). The taxonomic assignment of OTUs was performed using the RDP Classifier. A variable number of sequences were obtained per sample; to avoid sampling size effects, the number of reads was normalized to 1,500 for each sample. Otherwise the non-rarefied OTU table was used. Alpha and Beta diversity analyses, rarefaction curves, Chao1 richness estimator, Shannon diversity index and Good's sample coverage were calculated using QIIME v. 1.7.0 pipeline. Principal coordinate analysis (PCoA) was performed using the subsampled data to detect microbial community differences on the basis of weighted and unweighted UniFrac distance metrics (Lozupone and Knight, 2005). Jackknife resampling was used to assess the stability of the PCoA analysis. Hierarchical clustering was conducted to group the communities of different samples using the unweighted pair group method with arithmetic mean (UPGMA). Differences in the relative abundance among treatments were calculated by two-sided t-tests. To construct the phylogenetic trees of iii1-8 sequences we used the Neighbour-Joining method. Bootstrap test (1000 replicates) was used to calculate the percentage of replicate trees in which the associated taxa clustered together. The evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

1.2.9. Nucleotide sequence accession numbers

The 454 pyrosequencing raw reads have been deposited in the NCBI short-reads archive database (accession number SRP077784). The partial *bssA* gene sequences obtained from the clone libraries have been deposited in GenBank under accession numbers KX455933 to KX456036). The 16S rRNA gene clone library sequences have been deposited in GenBank under accession numbers KX417378 to KX KX417406.

1.3. Results and discussion

1.3.1. Presence of a nitrate-reducing, PAH-degrading bacterial community in environmental samples

To assess the presence of PAH-degrading bacterial communities, we selected a range of environments that had been exposed to different degrees of hydrocarbon pollution (Table 1. 1). Water and soil from two different rice-paddy fields (RPCal, RPS, RPW), sediments from the Fuente de Piedra athalassohaline lagoon in southern Spain (FdP), marine sediments affected by an oil spill (MS), activated sludge from an oil refinery (AS) and its resulting composting pile (CP) were sampled and analyzed for total organic matter, electron acceptors and hydrocarbon content, (Table 1. 1; Tables S3a and b). The concentration of PAHs in the initial samples ranged from 95 µg/kg in the MS sample to 888 µg/kg in the AS sample, and was also considerable high in the rice-paddy soil from Calasparra (RPCal) (857 µg/Kg) (Table S3b). These values were in the range of those generally found in industrial areas and agricultural soils (Nadal *et al.*, 2004). MPN enumeration of NRB able to grow with different carbon sources showed that acetate, which was our control as a simple carbon source utilizable by most organisms harbouring a TCA cycle, gave the highest counts of NRB in all samples except FdP (Figure 1. 1).

As expected, values were highest in the soil samples (in the range of 10^8 cells/g) and lowest in the marine and lagoon sediments (10^3-10^5 cells/g). We observed that the intrinsic OM present in the samples allowed the basal growth of a significant bacterial population, as deduced from MPN values of the unamended control cultures (with no added carbon source) included in the analysis. This was especially true in the AS sample (above 10^5 cells/g), which also showed the highest values of OM content (Table 1. 1), and in the rice-paddy samples (10^4-10^5 cells/g), probably because of a better adaptation of the community to nitrate respiration in this habitat. However, above these basal values, we were able to detect the presence of an autochthonous NRB community capable of growing with naphthalene as carbon source in some of the samples, which reached values as high as 10^7 cells/g in RPS and RPW samples, 2 x 10^6 cells/g in the FdP sediments, and almost 10^2 cells/g in the MS sample, as previously observed (Acosta-González *et al.*, 2013a) (Figure 1.1, Table S4). These samples also included important communities able to grow with 2-methylnaphthalene (2MN) (except for MS), 2-naphtoic acid (2NA) and anthracene (ANT). In addition, the presence of a significant anthracene degrading NRB community was detected in the rice-paddy and compost-pile samples. In this latter sample, we were also able to enumerate a 2NA degrading community, although the MPN of naphthalene and 2MN degrading

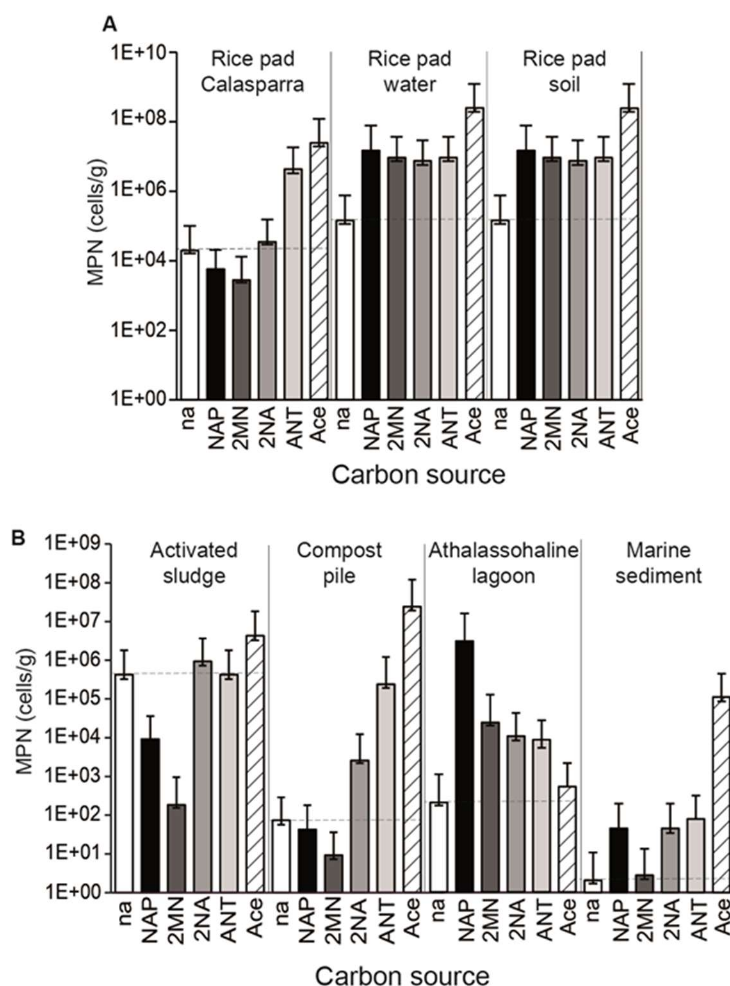

Figure 1. 1. MPN enumeration in the environmental samples of nitrate reducing bacteria able to grow on naphthalene (NAP), 2-methyl-naphthalene (2MN), 2-naphtoic acid (2NA), anthracene (ANT), acetate (Ace) and with no added carbon source (na). Counts were made in triplicate. Numerical values (95% C.I.) can be found in supplementary Table S4. Dotted lines indicate the basal growth with no added carbon source.

NRB were equal or below the control values. Surprisingly, in the activated sludge the bacterial counts on all the carbons sources gave values similar or below the basal growth on the sample intrinsic OM. Especially naphthalene and 2MN seemed to produce a toxic effect on the bacterial communities present in the sludge, which diminished by 2 and 3 orders of magnitude, respectively, with respect to the absence of any added carbon source. Although the presence in natural samples of PAH-degrading nitrate reducing communities has been described in different environments (Mihelcic and Luthy, 1988; al-Bashir *et al.*, 1990; Eriksson *et al.*, 2003; Uribe-Jongbloed and Bishop, 2007), the microorganisms involved in the process have not been identified and the reproducibility of the observations has been questioned (Meckenstock *et al.*, 2016). However, our results suggest that a bacterial community able to utilize PAHs as carbon source with nitrate as terminal electron acceptor was present in some of the samples.

1.3.2. Bacterial community structure in the selected environments

Enrichment cultures were initiated with the above described environmental samples, nitrate as terminal electron acceptor and HMN-dissolved naphthalene or 2MN as carbon sources. Parallel cultures only supplemented with HMN were run as controls. After 6 months enrichment with two culture transfers (See methods section), samples were taken to analyze changes in the bacterial communities using 16S rRNA gene V1-V3 region pyrosequencing. DNA from duplicate samples of the starting material was analyzed in parallel.

A total of 262,601 sequences from the 32 amplicon-libraries were obtained after applying all quality filters and deleting dimers and singletons (Table 1. 2). The read numbers ranged between 1,907 and 16,246, with an average of 8,206 sequences per sample. The rarefaction curves (Figure S1) and the Good's sample coverage estimator, which gave value between 75.54% (RPC-2MN) and 99.19% (CP-2MN), suggested that sampling depth was sufficient to estimate the microbial diversity in all the samples (Table 1. 2). The community structures of the initial samples varied considerably between environment types, as expected, and were in agreement with the bacterial distribution expected for each ecosystem. The observed OTU number and diversity indices were reproducible between replica (Table 1. 2) and indicated that the bacterial richness and diversity were highest in the rice field soils, reflecting the known complexity of soil ecosystems, which are amongst the most diverse environments (Torsvik *et al.*, 2002). The number of OTUs was in the same range in the marine sediment samples, in accordance with global studies showing that sediments are more phylogenetically diverse than any other habitat (Lozupone and Knight, 2007), and reached intermediate values in the remaining samples. Figure 1. 2 shows that the community structure of the initial environments was dominated by the phylum *Proteobacteria* in all cases except FdP, followed, in different proportions, by *Bacteroidetes*, *Chloroflexi* and *Actinobacteria*. Interestingly, the FdP samples were dominated by the uncultured candidate phylum *Parcubacteria* (former OD1 candidate division), and the *Proteobacteria* only represented 15% of this community. The community structure in the three rice-paddy samples was similar: the sequences classified as *Proteobacteria* (26.5-46.6%), *Chloroflexi* (10-17.8%), *Acidobacteria* (4.7-22.8%), *Bacteroidetes* (7.4-17.1%), *Gemmatimonadetes* (2-8.4%), *Planctomycetes* (1.9-4.6%), *Actinobacteria* (1.3-5.2%), *Firmicutes* (1.5-4.9%) and *Nitrospirae* (2-2.1%) accounted for 84–96% of all sequences. Although the abundance of dominant phyla in soil environments has been shown to be highly variable, and many factors can affect phyla distribution, the predominance of these groups in the paddy soils was expected, as these phylotypes have been described as common inhabitants of agricultural soils (Lopes *et al.*, 2014). Members of these groups have been shown to account for

Table 1. 2. Comparison of OTU number, diversity, evenness indices and coverage for the different samples.

Sample[a]	NS[b]	OTUs[c]	OTUs (1500)[d]	Chao1 (1500)[e]	Shannon	Coverage[f]
RPW-I_a	3026	1210	791.6	1748.4	9.5	80.32%
RPW-N	5920	1072	530.5	1065.4	8.4	92.79%
RPW-2MN	7584	1148	490.2	1064.8	8.1	94.04%
RPS-I_a	6425	1706	745.1	1818.2	9.5	89.31%
RPS-I_b	5962	1631	744.7	1763.2	9.5	89.38%
RPS-N	15953	1710	492.2	1146.6	8.0	96.06%
RPS-2MN	8753	1412	550.6	1234.9	8.4	93.51%
RPCal-I_a	4276	1388	786.9	1611.0	9.6	87.11%
RPCal-I_b	4115	1443	809.5	1690.1	9.7	85.95%
RPCal-N	6662	1620	716.4	1569.4	9.4	90.54%
RPCal-2MN	1907	853	738.6	1649.4	9.0	75.54%
RPCal-HMN	3164	1104	712.6	1448.8	9.2	83.67%
AS-I_a	7821	686	317.1	621.0	6.8	96.82%
AS-I_b	6787	635	297.5	570.4	6.4	96.12%
AS-N	16246	966	345.0	609.8	7.5	98.29%
AS-2MN	5781	674	354.7	665.6	7.3	95.37%
AS-HMN	13257	923	358.5	658.5	7.5	97.69%
CP-I_a	9195	694	311.9	584.8	7.0	97.52%
CP-I_b	5029	531	313.1	528.8	7.1	96.11%
CP-N	8278	206	94.3	166.7	3.2	98.99%
CP-2MN	8772	214	103.7	187.6	3.4	99.19%
CP-HMN	8608	302	163.1	237.5	5.2	98.98%
FdP-I_a	6202	591	304.8	592.8	6.3	96.78%
FdP-I_b	3542	468	319.4	537.8	6.7	95.14%
FdP-N	5252	743	386.2	841.2	6.6	94.11%
FdP2-2MN	14660	1097	338.1	798.0	5.7	97.53%
FdP-HMN	13789	1179	381.7	909.5	6.4	97.11%
MS-I_a	7711	1219	500.2	1082.7	8.2	94.18%
MS-I_b	8159	1179	487.9	1140.2	7.8	93.96%
MS-N	10818	414	178.5	299.3	5.9	98.53%
MS-2MN	16260	484	180.7	306.6	5.6	98.92%
MS-HMN	12687	446	176.0	318.4	5.6	98.56%

[a] I_a and I_b refer to the two replica of the initial samples of each environment, except for RPW-I, where one of the replica was lost.
[b]Number of sequences for each library filtered for chimera and singletons.
[c]OTU numbers calculated with all sequences at the 3% distance level.
[d]OTU numbers calculated for a randomized subset of 1500 reads per sample at the 3% distance level.
[e]Chao index calculated with 1500 subsampled sequences.
[f] Good's sample coverage estimator.

90% of the microbial population in these habitats (Janssen, 2006). The AS and CP samples originated from early and final steps, respectively, in the wastewater treatment of an oil refinery. In oil refineries, the wastewater influent can differ daily in terms of concentrations and composition of pollutants, which results in a high variability of the community distribution in these habitats (Hu *et al.*, 2013). In the AS sample, the community was dominated by *Proteobacteria* (39.1%), followed by *Actinobacteria* (18.7%), *Chloroflexi* (10.7%), *Bacteroidetes* (6.2%), *Chlorobi* (5.3%), *Spirochaetes* (2%), *Planctomycetes* (2.3%), *SBR1093* (2.4%) and *GN04* (2.6%), which made up about 90% of the libraries. *Proteobacteria* also represented the predominant phylum in different activated sludge samples, suggesting that these organisms are involved in

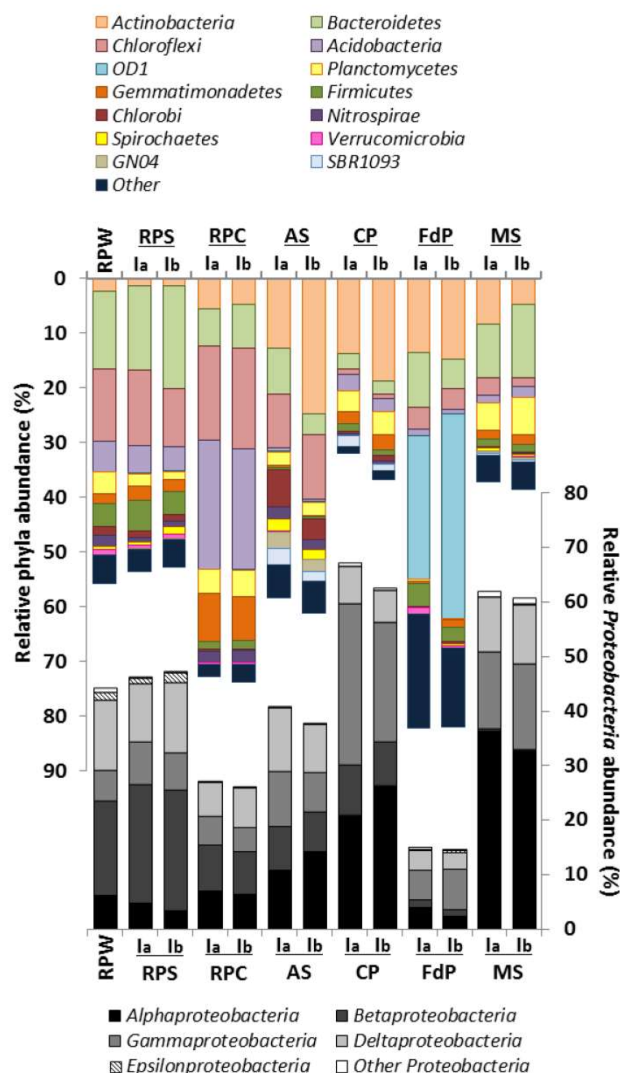

Figure 1. 2. Cumulative plot of bacterial phyla detected in the initial environmental samples. Samples were analyzed in duplicate (labelled Ia and Ib) except for RPW, for which one of the samples was lost. RPS, rice-paddy soil; RPW, rice-paddy water, RPCal, rice-paddy Calasparra; AS, activated sludge; CP, compost pile; MS, marine sediement, FdP, Fuente de Piedra athalassohaline lagoon.

biodegradation processes and in the removal of organic pollutants such as aromatic compounds (Wagner and Loy, 2002; Yang *et al.*, 2011; Silva *et al.*, 2012). In contrast, in the CP sample the *Proteobacteria* were twice as abundant, especially the *Gammaproteobacteria* class. Furthermore, members of the *Planctomycetes* (3.9%) and especially *Acidobacteria* (2.9%), which were below 1% in the AS sample, were more represented in this sample. On the other hand, some groups were less abundant, such as *Bacteroidetes* (2.6%), and especially *Chlorobi* (0.7%) and *Chloroflexi* (0.8%), which were almost absent.

In the FdP sediment samples the most abundant phyla was the candidate division *OD1* (*Parcubacteria*) (31.8%), followed by the *Proteobacteria* (14.9%), *Actinobacteria* (14.1%), *Bacteroidetes* (7.7%) and *Firmicutes* (3.5%). Although this type of environment is of great interest, its prokaryotic diversity and especially that of the anoxic sediments, has been poorly studied. In a number of sites with similar characteristics, the most prominent groups were *Proteobacteria*, *Bacteroidetes* and *Firmicutes* (Demergasso *et al.*, 2004; Jiang *et al.*, 2006; Mesbah *et al.*, 2007). The phylum *Parcubacteria*, with no cultivated member to date, has been detected globally in both aquatic and terrestrial habitats and mostly in anoxic environments (Elshahed *et al.*, 2005; Peura *et al.*, 2012; Rinke *et al.*, 2013). The dominance of *Parcubacteria* in this type of lagoon has not yet been reported and its ecological role is unclear, although members of this phylum are commonly identified in anoxic environments and sediments. The *Parcubacteria* are characterized by the small size of their genome, with a large number of unique genes (Nelson and Stegen, 2015). Analysis of the genomes sequenced so far after single genome amplification suggests poor mechanisms for energy and nutrient conservation (hence the name *Parcubacteria*), the absence of genes for the tricaboxylic acid cycle, electron transport and amino acids, nucleotides, vitamins and lipids biosynthesis (Wrighton *et al.*, 2012). This paucity of metabolic functions led to the proposed description of *Parcubacteria* as symbiontic/parasite organisms (Nelson and Stegen, 2015). Genes supporting an anaerobic lifestyle have also been detected, which appear to be involved in hydrogen and sulphur cycles in anoxic sediments (Elshahed *et al.*, 2005; Wrighton *et al.*, 2012). This would explain their presence in anoxic and sulphur rich sediments such as those of the Fuente de Piedra lagoon under study.

In the MS samples *Proteobacteria* was the most abundant phylum (61%), as reported for most coastal sediments (Zinger *et al.*, 2011). The community was dominated by *Alphaproteobacteria* (34%) followed by *Gammaproteobacteria*, *Bacteroidetes*, *Deltaproteobacteria*, *Actinobacteria* and *Planctomycetes*. *Gammaproteobacteria* and *Alphaproteobacteria* also dominated superficial marine sediments exposed to the Deepwater Horizon spill (Kostka *et al.*, 2011). Under aerobic

conditions, *Gammaproteobacteria* are generally associated to the early stages of aerobic hydrocarbon degradation, when alkanes and easily degradable hydrocarbons are degraded, while the abundance of *Alphaproteobacteria* increases in the later stages of degradation, when more recalcitrant hydrocarbon compounds such as PAHs predominate (Acosta-González *et al.*, 2015; King *et al.*, 2015). Therefore, the high proportion of *Alphaproteobacteria* in our sediment samples, which were a mixture of the oxic superficial layers and the deeper transition and anoxic zones, may be related to the aerobic hydrocarbon biodegradation stage in the community. The abundance of *Actinobacteria* might also be associated to later degradation stages, since they are involved in the degradation of both the long-chain alkane and aromatic fractions (Acosta-González *et al.*, 2015; Acosta-González and Marqués, 2016). Unfortunately, little is known about hydrocarbon degradation in anoxic conditions, which are the prevalent conditions in the deeper layers of the sediment, although members of the *Deltaproteobacteria* seem to play a relevant role in the process (Kimes *et al.*, 2013; Kleindienst *et al.*, 2014; Acosta-González and Marqués, 2016).

1.3.3. General effect of PAHs on the bacterial community structure

To evaluate the possible long-term effect of PAHs on the bacterial richness and diversity, we followed the changes in the community structure in the enrichment cultures (Figure 1. 3) and analysed the OTU richness and Chao diversity index in each case (Table 1. 2). In the paddy soil enrichments, we observed two different trends: whilst the OTU number and Chao index in all the enrichments from Calasparra soil only showed a slight decrease, a significant reduction of the richness estimators in the Seville rice-paddy samples (RPS, RPW) was observed, although in this case the absence of the control culture with HMN hindered us from reaching a clear conclusion on the toxic effect of PAHs. No significant variations in OTU richness and Chao index were detected in the activated sludge samples, suggesting no overall adverse effect of naphthalene and 2MN on the community, as expected from a highly polluted environment. This contrasted with the toxic effect of PAHs on the nitrate reducing community inferred from the MPN results, and suggested that this type of metabolism constituted only a minor fraction of the population. It is worth noting that in the cultures used for the MPN enumeration, PAHs were provided as crystals and probably reached the saturating concentrations (i.e. 200 µm for naphthalene), whilst in the enrichments PAHs were supplied dissolved in HMN to keep concentrations below saturation (approx. 70 µM, (Ghoshal *et al.*, 1996)).

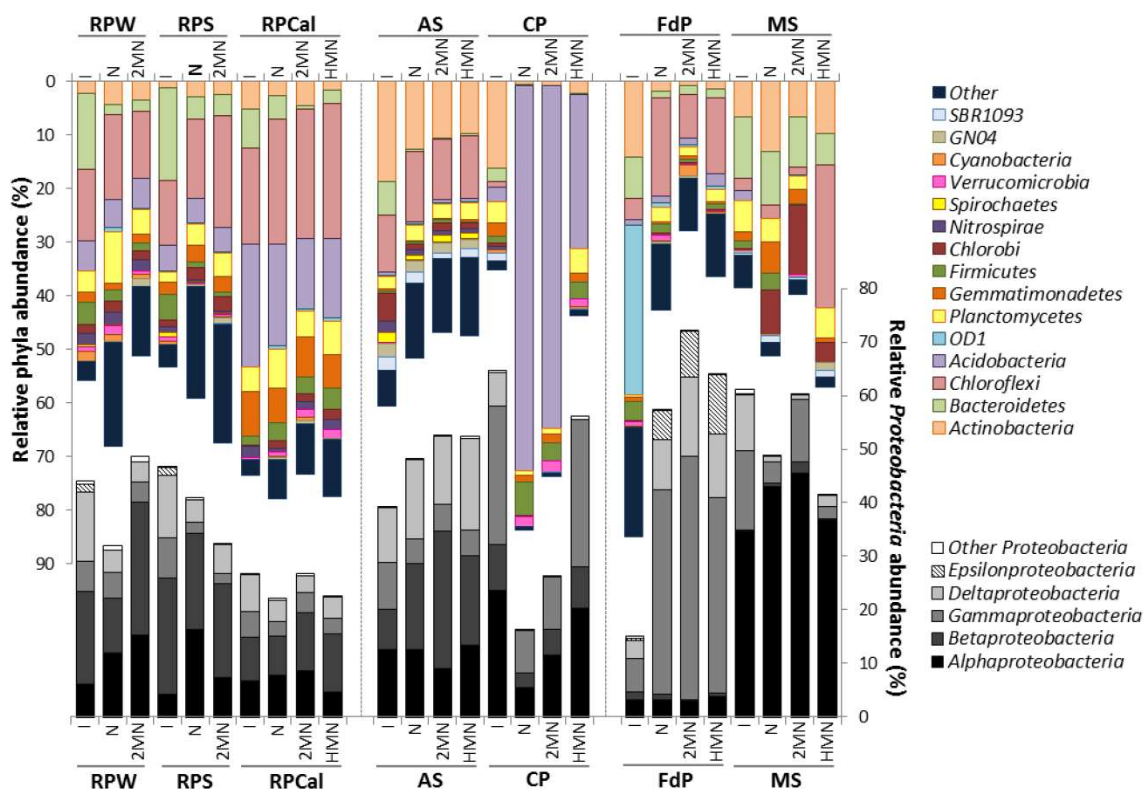

Figure 1. 3. Cumulative plot of bacterial phyla detected in the different enrichments. The average values of the duplicate initial samples (Figure 1.2) are included for comparison, labelled as I. All other labels are as in figure 1. 2.

In contrast, the compost pile cultures were significantly affected by the culture conditions and especially by the presence of PAHs, which produced the lower values of richness estimators and species evenness. In the marine sediment samples, the decrease in the number of OTUs and Chao index was significant in all cases, indicating that changes from the original sulphate-reducing metabolism to nitrate reduction conditions had a determinant detrimental effect on the community. In contrast, in the athalassohaline lagoon sediment samples the richness estimator increased in the three enrichments, suggesting a niche increment in the ecosystem as the samples switched from a sulphate-reducing environment to nitrate reducing conditions. The FdP lagoon is characterized by drastic changes in salinity throughout the year, depending on the local pluviometry, with variations ranging between 30 g/l and 300 g/l in extremely dry years. Furthermore, the lagoon is surrounded by agricultural fields periodically supplemented with nitrogen fertilizers, which in the past drained into the lagoon. Interestingly, neither nitrate nor nitrite were detected in the FdP samples (Table 1. 1), indicating a strong potential for denitrifying activity (Spence *et al.*, 2005), which explains the positive response of the community to the change imposed on the respiratory regime. Principal coordinate analysis (PCoA) of the phylogenetic variation measured via UniFrac distances (Lozupone and Knight, 2005) revealed that an important community structuring factor was the

original environment type where the samples were collected from (Figure 1. 4A). The microbial communities, in fact, were clustered into three groups: rice-paddy original samples and enrichments, activated sludge and compost pile original samples and enrichments, and, marine sediment and athalassohaline lagoon original samples and enrichments. To estimate if the structure of the community was affected by the addition of PAHs, we focused on the environment type subgroups. UPGMA, PCoA and significance analysis (two-sided Student's tests, Table S5) were performed using QIIME.

Within the rice-paddy sample group the differences were minor, although statistically significant changes in RPS and RPW naphthalene and 2MN enrichments ($p \leq 0.05$) were found as compared to the original sample. Naphthalene RPCal enrichment was also more affected than the HMN and 2MN cultures. No consistent differences between the HMN controls and the PAH enrichments were found in the activated sludge communities ($p \geq 0.05$). Furthermore, the compost pile PAH enrichments were significantly distant from the initial sample and HMN control ($p \leq 0.05$), especially the naphthalene culture, indicating an important phylogenetic variation of the community structure due to the presence of this PAH (Figure 1. 4B, Table S5). Finally, in the MS and FdP samples no important differences could be detected between the control and the PAH-

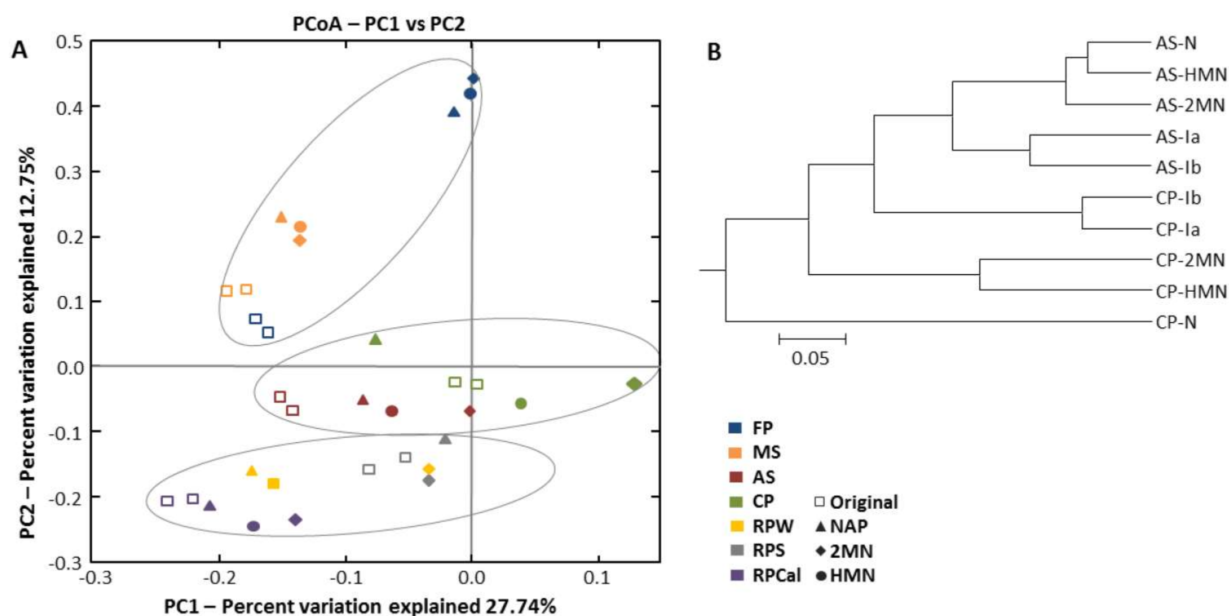

Figure 1. 4. A) Principal coordinate analysis (PCoA) of distances between OTUs present in the initial environmental samples and the enrichments obtained from them. The percentage of the variation between the samples by principal coordinates is indicated on the axes. B) Unweighted pair group method with arithmetic average (UPGMA) cluster analysis of bacterial community structure in the compost pile (CP) and activated sludge (AS) environmental sample and enrichments from them using pyrosequencing analysis data based on Pearson correlation using OTUs (> 97% sequence similarity) as the species data.

amended cultures, indicating that the community shift was mainly due to changes in the culture conditions (i.e., the shift to nitrate as terminal electron acceptor).

1.3.4. Effect of PAHs on specific bacterial populations

Over-imposed upon this general shift, we were able to detect some specific changes ascribable to the presence of PAHs. The compost pile was the sample where the presence of PAHs produced the major changes. In the naphthalene and 2MN enrichments we observed a decrease in the abundance of six phylotypes (*Proteobacteria, Actinobacteria, Bacteroidetes, Gemmatimonadetes, Planctomycetes and SBR1093*). Specifically, *Alpha*- *Beta*- and *Gammaproteobacteria* decreased to a higher extent in naphthalene and 2MN treatments as compared to the HMN control. *Actinobacteria* also decreased significantly (from 16.2% to 1.3%) in all the enrichment cultures. In parallel an important increase of the relative abundance of the phyla *Acidobacteria*, *Firmicutes* and *Verrucomicrobia* was observed. In particular *Acidobacteria* relative abundance increased in all the cultures, but the increase was extreme in naphthalene and 2MN enrichments (from 2.9% to 71.7% and 63.7%, respectively), where they became the dominant phylum (Figure 1. 5A). The enriched *Acidobacteria* community was almost exclusively composed of the uncultured group iii1-8 (order DS18) (Figure 1. 5B).

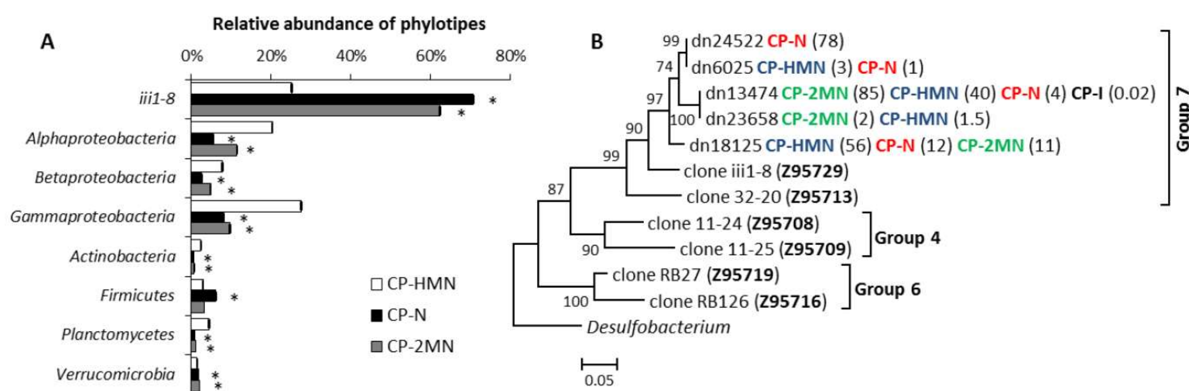

Figure 1. 5. Effect of PAHs on the relative abundance of the most relevant groups in the compost pile (CP) sample. A) Differences in the relative abundance of the most abundant groups between control (HMN), naphthalene (N) and 2-methyl-naphthalene (2MN) enrichments from the compost pile sample. Asterisks (*) indicate significant differences between rarefied samples according to the Mann-Whitney test ($p<0.01$). B) Evolutionary relationships of iii1-8 DS18 sequences retrieved from the compost pile initial sample and enrichments. The optimal Neighbour-Joining tree is shown. The bootstrap (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site. Numbers in brackets indicate the relative abundance (%) of each sequence in the corresponding sample. The sequences of previously described Acidobacteria clones are included (Hugenholtz *et al.*, 1998).

Interestingly, a significant increase in this group was also observed in the PAH enrichments obtained from the rice-paddy samples (Table 1. 3) where members of the order DS18 of the iii1-8 class, which were initially minor components of the community, increased up to 5.2% in the naphthalene enrichments from RPCal. The iii1-8 sequences retrieved from the different enrichments formed different clusters, and separated from the previously described iii1-8 sequences (Figure S2). Notably, the 5840 reads (78% of the sequences) from the CP naphthalene enrichments constituted a single OTU, distinct from the bulk of 2MN-enrichment sequences. Half

Table 1. 3. Relative abundance of *Acidobacteria* in the initial samples and enrichment cultures.

Sample		Taxon
		iii1-8; DS-18
RPW	$initial_A$	0.2
	$initial_B$	0.69
	NAP	**1.05**
	2MN	**1.37**
RPS	$initial_A$	0.76
	$initial_B$	0.49
	NAP	**2.46**
	2MN	**2.07**
RPCal	$initial_A$	1.43
	$initial_B$	1.22
	NAP	**5.18**
	2MN	1.47
	HMN	1.68
CP	$initial_A$	0
	$initial_B$	0.02
	NAP	**70.55**
	2MN	**62.27**
	HMN	**25.2**

of the sequences from the CP HMN control culture formed a distinct branch, whilst the other half belonged to the same OTU as the 2MN-enriched sequences. Furthermore, paddy soil PAH enriched sequences tended to cluster together, and separated from the CP PAH enriched sequences (Figure S2). *Acidobacteria* are among the most dominant phyla present in soils and are believed to play an important role in the ecology of this ecosystem. Unfortunately, the lack of acidobacterial isolates complicates the elucidation of their ecology and significance (Janssen, 2006; Jones *et al.*, 2009). *Acidobacteria* class iii1-8 belongs to the monophyletic subdivisions 7 and was first identified in 1997 (Ludwig *et al.*, 1997). Evidence suggests that pH is the best predictor of *Acidobacteria* abundance and distribution in soils, although the different subgroups are differentially affected by this variable: some subgroups are favoured at lower pH while some others, particularly sub-group 7, are positively correlated with higher pH values (Jones *et al.*, 2009;

Rousk *et al.*, 2010). It is worth noting that in our experiments the pH remained neutral. In addition, a negative correlation with carbon availability and carbon mineralization rates has been observed, leading to the description of this group as slow-growing oligotrophs (Fierer *et al.*, 2007). Furthermore, almost nothing is known about the role of *Acidobacteria* in aromatic degradation, and even less under anoxic conditions. Mukherjee *et al.* (2014) hypothesized that *Acidobacteria* phylum was not affected by hydrocarbon contamination levels and behaved as generalists in hydrocarbon polluted soils; however, a general increase of *Acidobacteria* in soil was observed in the presence of pyrene (Ren *et al.*, 2015), and stable isotope probing of soils amended with ^{13}C-labelled benzene identified a member of the *Acidobacteria* among the strains involved in benzene degradation (Xie *et al.*, 2011). Although the function of *Acidobacteria* class iii1-8 in the communities analysed here is difficult to predict, we could speculate that they are PAH resistant and may play a role in some step of the anaerobic degradation of PAHs, given their enrichment in PAH cultures under nitrate reducing conditions. To a lesser extent but still interesting was the increment of the *Firmicutes* phylum, especially of members of the *Bacilli* class, which increased from 0.7% to 6% in the CP naphthalene enrichments but only to 3.3%, and 3.0%, in the 2MN enrichment and HMN control, respectively. We observed the same trend in other enrichments, as in MS where *Bacilli* class increased from 1% in the original sample to 3% in the naphthalene enrichment, but decreased to 0.05% and 0.06% in the 2MN and HMN cultures, respectively. The same was true for the FdP samples where, despite a general decrease of the *Firmicutes* group, the *Bacilli* phylotypes increased especially in the naphthalene cultures. This increase of *Bacilli* relative abundance is noteworthy, since members of this class have been described as aerobic and anaerobic oil-degrading strains (Van Gestel *et al.*, 2003; da Cruz *et al.*, 2011; Maddela *et al.*, 2016), which suggests a link between the increase of this group and the degradation process.

In the FdP enrichments we also observed a drastic change of the structure of the bacterial community in the enrichment cultures, although in this case the variation may be mainly attributed to the imposed culture conditions rather than to the presence of PAHs. The main changes detected were the great increase in members of the *Gammaproteobacteria*, *Deltaproteobacteri*a and *Epsilonprotebacteria* and the significant decrease of the candidate phylum *OD1*, which almost disappeared (from 31 to 0.33%). The order *Chromatiales* accounted for more than 70% of the increase in the *Gammaproteobacteria.* Members of this group have been shown to contain the gene complement for both sulphur oxidation and nitrate respiration, and the regulation of the two functions seems to be coordinated in these organisms (Baker *et al.*, 2015). They are generally found in shallow sulphate-rich environments where they are thought to play a role in nitrate-dependent sulphur oxidation. The change from the original sulphate-reducing conditions in the

athalassohaline lagoon to the nitrate reducing conditions in the FdP cultures may explain the enrichment of this phylogenetic group. Additionally, a general decrease of the other dominant phyla was observed, except for *Chloroflexi*, *Acidobacteria* and *Planctomycetes*. The increase in members of the *Anaerolineae* of the *Chloroflexi* and of *Planctomycetes* was especially noticeable in the naphthalene enrichments. The increase of *Anaerolineae*, a group which has been retrieved from many natural and artificial ecosystems, among which hypersaline lakes, agriculture soils, waste water treatment plants and microbial fuel cells, is probably not related to nitrate respiration, as all cultured strains of this class do not utilize nitrate as terminal electron acceptor (Yamada *et al.*, 2006). Rather it seems that these organisms, with a fermentative anaerobic metabolism, are using carbohydrate and cells remains produced in the enrichment cultures (Yamada and Sekiguchi, 2009).

In the enrichments from marine sediment, the change from the initial sulphate respiration regime to the imposed nitrate reduction metabolism produced less drastic changes in the community structure than expected. Although the changes in the three enrichments were parallel, differences between the HMN control and the PAH amended enrichments were noticeable. *Alphaproteobacteria* remained the dominant class among the *Proteobacteria* (34.53-45.46%), increasing in all three conditions, although to a higher extent in the naphthalene and 2-MN cultures. Interestingly a higher increase in the relative abundance of *Actinobacteria* was observed in the naphthalene enrichments. Furthermore, the phylotypes *Gemmatimonadetes* and *Firmicutes* increased in the PAH enrichments and decreased in the HMN control cultures (Figure 1. 3). Altogether, these changes in the community may reflect a connection with resistance to PAHs. The decrease of the phyla *Bacteroidetes*, *Planctomycetes*, *Acidobacteria* and *WS3* were similar in all the culture conditions, and thus could not be attributed to the presence of PAHs.

Changes in the remaining enrichments were modest. Incubation of rice-paddy samples with PAHs under nitrate reducing conditions produced only slight changes in the bacterial communities, and most of them were attributable to the new culture medium, since similar shifts were observed in the presence and absence of PAHs. Nevertheless, it is important to point out that communities with a high diversity, such as those present in soil, appear to be more stable when under stress (McCann, 2000). Looking in more details at some of the specific changes, we observed that *Bacteroidetes* relative abundance decreased significantly in the naphthalene and 2MN enrichments, suggesting sensitivity to the presence of PAHs. Although this phylum is widespread in both pristine and contaminated environments, a similar decrease of *Bacteroidetes* abundance has been recently reported in soil microcosms contaminated with PAHs (Sawulski *et al.*, 2014).

Firmicutes, especially members of the *Clostridia*, increased in the presence of naphthalene and 2MN enrichments whilst the changes in the relative abundance of *Bacilli* did not show a clear pattern. Several anaerobic hydrocarbon degraders belonging to the *Clostridia* have been isolated (Morasch *et al.*, 2004), or identified as hydrocarbon degraders in the environment (Kunapuli *et al.*, 2007; Fowler *et al.*, 2012), although most of them are SRB, iron reducers and methanogens. The relative abundance of *Acidobacteria* remained unchanged or slightly reduced in the difference paddy soil enrichments and controls. However, as discussed above, an interesting enrichment of the *Acidobacteria* class iii1-8 was observed in all the samples with N and 2-MN (Table 1. 3).

Finally, a general increase of the *Proteobacteria* relative abundance was observed in AS enrichments, although the final proportion of the different classes was different in the three culture conditions (Figure 1. 3). The *Betaproteobateria* relative abundance increased in all cultures but especially in the 2MN enrichment, reaching 25% of the community. The remaining phyla only showed slight changes in the cultures with respect to their relative abundance in the initial sample, and no clear effect of the presence of PAHs was evidenced. Overall no clear association could be established between the observed changes in the community structure and the presence of PAHs.

1.3.5. Isolation of nitrate reducing bacteria from PAH enrichments

The growth of the different cultures was checked by following the respiratory activity as nitrate consumption. Nitrate respiration was observed in some enrichment cultures on PAHs (data not shown). However, significant differences between the control HMN cultures and the PAH amended cultures were only observed in the CP culture with 2MN as the carbon source. The nitrate consumed after the last transfer was below 3 mM, which would correspond to a maximum consumption of 310 μM of 2MN if full denitrification of nitrate was assumed (Mihelcic and Luthy, 1988). The concentration of PAHs used in the HMN solution was too high to allow accurate determination of PAH consumption at these levels. We therefore attempted to isolate strains able to grow on solid media under denitrifying conditions with naphthalene or 2MN as carbon source. Several strains able to consistently grow with naphthalene as carbon source could be isolated both on agar plates prepared under anoxic conditions and using the agar shakes method (Table S6). These included species of the *Bacillus* and *Pseudomonas* genera, among others. However, the growth phenotype could not be reproduced in liquid medium, where no nitrate respiration was observed in the presence of naphthalene after several months. A possible reason for this discrepancy might be that despite anaerobic preparation and incubation of the solid media cultures, oxygen could be a contaminant and allow microaerophylic growth of the strains. However, no aerobic growth of the isolates in liquid medium was obtained either with the corresponding PAHs

as carbon source. Interestingly, strains of *P. stutzeri* were isolated with both methods, which were also the strain originally described by Röckne *et al.* (2000) as naphthalene degraders. Although some strains of this species are known aerobic naphthalene degraders (Lauber *et al.*, 2009), the strains isolated in this study were unable to grow aerobically with naphthalene as carbon source. It is worth noting that in both isolation methods, no growth on solid medium was observed when naphthalene was omitted from the medium.

1.3.6. Aromatic anaerobic degradation genes

In SRB naphthalene and 2MN degradation pathways have been elucidated and primers for the amplification of the fumarate adding enzyme naphthyl-2-methylsuccinate synthase gene (*nmsA*) have been used to detect the presence of the pathway in environmental samples (Winderl *et al.*, 2007; Acosta-González *et al.*, 2013b; von Netzer *et al.*, 2013). To evaluate the anaerobic hydrocarbon degrading potential in the environmental samples and enrichments we tested different previously designed primer pairs to detect *nmsA* and *bssA*, coding for the homologous toluene pathway degradation enzyme benzylsuccinate synthase. In addition, we used primers for the amplification of naftoil-CoA reductase (*ncr*), the central step in naphthalene degradation (Morris *et al.*, 2014). Only the primer set for the detection of fumarate addition enzymes consistently rendered PCR amplification products. In total, twelve clone libraries were constructed from DNA isolated from the samples giving PCR amplification products, and sixteen clones from each library were sequenced. Finally, we were able to retrieve *bssA* homologues from rice-paddy initial samples (RPW and RPS) and enrichments (RPS-N, RPCal-N, RPCal-2MN and RPCal-HMN), from the AS initial sample and naphthalene and 2-MN enrichments and from the naphthalene enrichment of the marine sediment (Figure S3). The resulting amplified *bssA*-like sequences were translated into amino acid sequences and compared with available sequences in the databases to build a phylogenetic tree (Figure S4). All the sequences clustered within the cluster I of toluene-specific BssA sequences (*bssA sensu stricto*), which included sequences from well-characterized toluene-degrading strains and from environmental samples such as hydrocarbon-polluted aquifers, aquifer sediments, oil fields, sludge and polluted soils. Interestingly, within cluster I the sequences retrieved from marine sediments and activated sludge formed a distinct cluster, which was already observed for sequences of marine origin (Acosta-González *et al.*, 2013b). These results reflect the absence of efficient ‘universal’ primers for *nms* and *ncr* genes, considering the limited number of anaerobic PAH degradation gene sequences available (DiDonato *et al.*, 2010; Selesi *et al.*, 2010). Moreover, the reference genes used to design the probes were obtained from sulphate-reducing organisms; nitrate dependent PAH degradation may follow a different pathway.

1.4. Concluding remarks

In this study, we were able to detect in some environments the presence of a significant nitrate reducing bacteria community able to thrive using PAHs as carbon source. Enrichment cultures under anoxic conditions with nitrate as terminal electron acceptor and naphthalene or 2MN as the carbon source produced shifts in the structure of the community connected to the change imposed on the respiratory regime, from aerobic, microaerophilic and sulphate reducing conditions to nitrate respiration. However, some changes specifically related to the presence of PAHs could be identified. The relative abundance of *Bacteroidetes* and *Actinobacteria* was reduced to different extents in PAH enrichments, which was attributed to the sensitivity of these groups to the toxicity of PAHs. In contrast, some groups increased significantly. Especially noticeable was the selection for the uncultured *Acidobacteria* class iii1-8, particularly. in the compost-pile sample, where the iii1-8 community increased under all culture conditions, but to a significantly higher extent in the naphthalene cultures, and was also clearly observed in all those naphthalene enrichments where this group was present in the initial samples. Unfortunately, a direct connection between enrichment in iii1-8 sequences and PAH degradation under nitrate reducing conditions could not be established, and the ecological role of this group in the enrichments is unclear. Members of the *Bacilli* were also enriched in most PAHs cultures, and strains belonging to this group could be isolated on solid media with naphthalene as carbon source. However, these isolates were unable to grow with naphthalene in liquid media. The failure of our attempts to isolate nitrate reducing PAH-degrading bacteria may suggest that in the environment, PAH degradation is a complex process in which different organisms are involved. Based on the experience of the past decades in the field of biodegradation, both under aerobic and anoxic conditions, this inability to identify and isolate NRBs able to degrade common natural compounds such as the PAHs used here is unexpected, especially considering that NRBs capable of degrading benzene (an unsubstituted monoaromatic with a structure similar to naphthalene) have been identified (Chakraborty *et al.*, 2005; Kasai *et al.*, 2006). However, in this latter case an aerobic metabolism based on internally produce oxygen through the potential dismutation of the nitric oxide produced during respiration has been proposed (Meckenstock *et al.*, 2016). NRBs are generally facultative anaerobes, with the capacity to carry out a mixed oxic/anoxic type of metabolism (Valderrama *et al.*, 2012). It would thus be expected that in some environments processes as complex as PAH degradation would require the coordinated activity of both types of metabolisms. The strict anaerobic conditions imposed in our enrichments would prevent the development of such consortia.

1.5. References

Acosta-González, A., and Marqués, S. (2016). Bacterial diversity in oil-polluted marine coastal sediments. *Curr Opin Biotechnol* **38**: 24-32.

Acosta-González, A., Martirani-Von Abercron, S.M., Rossello-Mora, R., Wittich, R.M., and Marqués, S. (2015). The effect of oil spills on the bacterial diversity and catabolic function in coastal sediments: a case study on the *Prestige* oil spill. *Environ Sci Pollut Res Int* **22**: 15200-15214.

Acosta-González, A., Rossello-Mora, R., and Marqués, S. (2013a). Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the Prestige oil spill. *Environ Microbiol* **15**: 77-92.

Acosta-González, A., Rossello-Mora, R., and Marqués, S. (2013b). Diversity of benzylsuccinate synthase-like (*bssA*) genes in hydrocarbon-polluted marine sediments suggests substrate-dependent clustering. *Appl Environ Microbiol* **79**: 3667-3676.

Al-Bashir, B., Cseh, T., Leduc, R., and Samson, R. (1990). Effect of soil/contaminant interactions on the biodegradation of naphthalene in flooded soil under denitrifying conditions. *Appl Microbiol Biotechnol* **34**: 414-419.

Baker, B.J., Lazar, C.S., Teske, A.P., and Dick, G.J. (2015). Genomic resolution of linkages in carbon, nitrogen, and sulfur cycling among widespread estuary sediment bacteria. *Microbiome* **3**: 14.

Boll, M., Loffler, C., Morris, B.E., and Kung, J.W. (2014). Anaerobic degradation of homocyclic aromatic compounds via arylcarboxyl-coenzyme A esters: organisms, strategies and key enzymes. *Environ Microbiol* **16**: 612-627.

Canfield, D.E., Thamdrup, B., and Hansen, J.W. (1993). The anaerobic degradation of organic matter in Danish coastal sediments: Iron reduction, manganese reduction, and sulfate reduction. *Geochim Cosmochim Acta* **57**: 3867-3883.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Chakraborty R, O'Connor SM, Chan E, Coates JD. (2005). Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Appl Environ Microbiol* **71**: 8649-8655.

Coates, J.D., Chakraborty, R., Lack, J.G., O'connor, S.M., Cole, K.A., Bender, K.S., and Achenbach, L.A. (2001). Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. *Nature* **411**: 1039-1043.

Da Cruz, G.F., De Vasconcellos, S.P., Angolini, C.F., Dellagnezze, B.M., Garcia, I.N., De Oliveira, V.M., Dos Santos Neto, E.V., and Marsaioli, A.J. (2011). Could petroleum biodegradation be a joint achievement of aerobic and anaerobic microrganisms in deep sea reservoirs? *AMB Express* **1**: 47.

Darley, P.I., Hellstern, J.A., Medina-Bellver, J.I., Marques, S., Schink, B., and Philipp, B. (2007). Heterologous expression and identification of the genes involved in anaerobic degradation of 1,3-dihydroxybenzene (resorcinol) in Azoarcus anaerobius. *J Bacteriol* **189**: 3824-3833.

Demergasso, C., Casamayor, E.O., Chong, G., Galleguillos, P., Escudero, L., and Pedros-Alio, C. (2004). Distribution of prokaryotic genetic diversity in athalassohaline lakes of the Atacama Desert, Northern Chile. *FEMS Microbiol Ecol* **48**: 57-69.

Didonato, R.J., Jr., Young, N.D., Butler, J.E., Chin, K.J., Hixson, K.K., Mouser, P., Lipton, M.S., Deboy, R., and Methe, B.A. (2010). Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. *PLoS ONE* **5**: e14072.

Doyle E, Muckian L, Hickey AM, Clipson N. (2008). Microbial PAH degradation. *Adv Appl Microbiol* **65**: 27-66.

Elshahed, M.S., Najar, F.Z., Aycock, M., Qu, C., Roe, B.A., and Krumholz, L.R. (2005). Metagenomic Analysis of the Microbial Community at Zodletone Spring (Oklahoma): Insights into the Genome of a Member of the Novel Candidate Division OD1. *Appl Environ Microbiol* **71**: 7598-7602.

Eriksson, M., Sodersten, E., Yu, Z., Dalhammar, G., and Mohn, W.W. (2003). Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. *Appl Environ Microbiol* **69**: 275-284.

Estelmann, S., Blank, I., Feldmann, A., and Boll, M. (2015). Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Mol Microbiol* **95**: 162-172.

Fierer, N., Bradford, M.A., and Jackson, R.B. (2007). Toward an ecological classification of soil bacteria. *Ecology* **88**: 1354-1364.

Fowler, S.J., Dong, X., Sensen, C.W., Suflita, J.M., and Gieg, L.M. (2012). Methanogenic toluene metabolism: community structure and intermediates. *Environ Microbiol* **14**: 754-764.

Fuchs, G., Boll, M., and Heider, J. (2011). Microbial degradation of aromatic compounds - from one strategy to four. *Nat Rev Microbiol* **9**: 803-816.

Ghoshal, S., Ramaswami, A., and Luthy, R.G. (1996). Biodegradation of Naphthalene from Coal Tar and Heptamethylnonane in Mixed Batch Systems. *Environ Sci Technol* **30**: 1282-1291.

Haas, B.J., Gevers, D., Earl, A.M., Feldgarden, M., Ward, D.V., Giannoukos, G., Ciulla, D., Tabbaa, D., Highlander, S.K., Sodergren, E., Methe, B., Desantis, T.Z., Human Microbiome, C., Petrosino, J.F., Knight, R., and Birren, B.W. (2011). Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. *Genome Res* **21**: 494-504.

Hu, G., Li, J., and Zeng, G. (2013). Recent development in the treatment of oily sludge from petroleum industry: A review. *J Hazard Mater* **261**: 470-490.

Hugenholtz, P., Goebel, B.M., and Pace, N.R. (1998). Impact of Culture-Independent Studies on the Emerging Phylogenetic View of Bacterial Diversity. *J Bacteriol* **180**: 4765-4774.

Janssen, P.H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* **72**: 1719-1728.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L.R., and Fields, M.W. (2006). Microbial Diversity in Water and Sediment of Lake Chaka, an Athalassohaline Lake in Northwestern China. *Appl Environ Microbiol* **72**: 3832-3845.

Jones, R.T., Robeson, M.S., Lauber, C.L., Hamady, M., Knight, R., and Fierer, N. (2009). A comprehensive survey of soil acidobacterial diversity using pyrosequencing and clone library analyses. *ISME J* **3**: 442-453.

Kasai, Y., Takahata, Y., Manefield, M., and Watanabe, K. (2006). RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Appl Environ Microbiol* **72**: 3586-3592.

Kimes, N.E., Callaghan, A.V., Aktas, D.F., Smith, W.L., Sunner, J., Golding, B., Drozdowska, M., Hazen, T.C., Suflita, J.M., and Morris, P.J. (2013). Metagenomic analysis and metabolite profiling of deep-sea sediments from the Gulf of Mexico following the Deepwater Horizon oil spill. *Front Microbiol* **4**: 50.

King, G.M., Kostka, J.E., Hazen, T.C., and Sobecky, P.A. (2015). Microbial responses to the deepwater horizon oil spill: from coastal wetlands to the deep sea. *Ann. Rev. Mar. Sci.* **7**: 377-401.

Kleindienst, S., Herbst, F.A., Stagars, M., Von Netzer, F., Von Bergen, M., Seifert, J., Peplies, J., Amann, R., Musat, F., Lueders, T., and Knittel, K. (2014). Diverse sulfate-reducing bacteria of the *Desulfosarcina*/*Desulfococcus* clade are the key alkane degraders at marine seeps. *ISME J* **8**: 2029-2044.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C., and Huettel, M. (2011). Hydrocarbon-degrading bacteria and the bacterial community response in gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. *Appl Environ Microbiol* **77**: 7962-7974.

Kummel, S., Herbst, F.A., Bahr, A., Duarte, M., Pieper, D.H., Jehmlich, N., Seifert, J., Von Bergen, M., Bombach, P., Richnow, H.H., and Vogt, C. (2015). Anaerobic naphthalene degradation by sulfate-reducing *Desulfobacteraceae* from various anoxic aquifers. *FEMS Microbiol Ecol* **91**.

Kunapuli, U., Lueders, T., and Meckenstock, R.U. (2007). The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* **1**: 643-653.

Lauber, C.L., Hamady, M., Knight, R., and Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl Environ Microbiol* **75**: 5111-5120.

Lopes, A.R., Manaia, C.M., and Nunes, O.C. (2014). Bacterial community variations in an alfalfa-rice rotation system revealed by 16S rRNA gene 454-pyrosequencing. *FEMS Microbiol Ecol* **87**: 650-663.

Lozupone, C., and Knight, R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**: 8228-8235.

Lozupone, C.A., and Knight, R. (2007). Global patterns in bacterial diversity. *Proc Natl Acad Sci USA* **104**: 11436-11440.

Ludwig, W., Bauer, S.H., Bauer, M., Held, I., Kirchhof, G., Schulze, R., Huber, I., Spring, S., Hartmann, A., and Schleifer, K.H. (1997). Detection and in situ identification of representatives of a widely distributed new bacterial phylum. *FEMS Microbiol Lett* **153**: 181-190.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Maddela, N.R., Burgos, R., Kadiyala, V., Carrion, A.R., and Bangeppagari, M. (2016). Removal of petroleum hydrocarbons from crude oil in solid and slurry phase by mixed soil microorganisms isolated from Ecuadorian oil fields. *Int Biodeterior Biodegrad* **108**: 85-90.

Mccann, K.S. (2000). The diversity-stability debate. *Nature* **405**: 228-233.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A.M. (2016). Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. *J Mol Microbiol Biotechnol* **26**: 92-118.

Mesbah, N.M., Abou-El-Ela, S.H., and Wiegel, J. (2007). Novel and unexpected prokaryotic diversity in water and sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. *Microb Ecol* **54**: 598-617.

Mihelcic, J.R., and Luthy, R.G. (1988). Microbial degradation of acenaphthene and naphthalene under denitrification conditions in soil-water systems. *Appl Environ Microbiol* **54**: 1188-1198.

Morasch, B., Schink, B., Tebbe, C.C., and Meckenstock, R.U. (2004). Degradation of o-xylene and m-xylene by a novel sulfate-reducer belonging to the genus *Desulfotomaculum*. *Arch Microbiol* **181**: 407-417.

Morris, B.E., Gissibl, A., Kummel, S., Richnow, H.H., and Boll, M. (2014). A PCR-based assay for the detection of anaerobic naphthalene degradation. *FEMS Microbiol Lett* **354**: 55-59.

Mukherjee, S., Juottonen, H., Siivonen, P., Lloret Quesada, C., Tuomi, P., Pulkkinen, P., and Yrjala, K. (2014). Spatial patterns of microbial diversity and activity in an aged creosote-contaminated site. *ISME J* **8**: 2131-2142.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannasch, H.W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165-172.

Nadal, M., Schuhmacher, M., and Domingo, J.L. (2004). Levels of PAHs in soil and vegetation samples from Tarragona County, Spain. *Environ Pollut* **132**: 1-11.

Nelson, W.C., and Stegen, J.C. (2015). The reduced genomes of *Parcubacteria* (OD1) contain signatures of a symbiotic lifestyle. *Front Microbiol* **6**: 713.

Peura, S., Eiler, A., Bertilsson, S., Nykänen, H., Tiirola, M., and Jones, R.I. (2012). Distinct and diverse anaerobic bacterial communities in boreal lakes dominated by candidate division OD1. *ISME J* **6**: 1640-1652.

Ren, G., Ren, W., Teng, Y., and Li, Z. (2015). Evident bacterial community changes but only slight degradation when polluted with pyrene in a red soil. *Front Microbiol* **6**: 22.

Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N.N., Anderson, I.J., Cheng, J.F., Darling, A., Malfatti, S., Swan, B.K., Gies, E.A., Dodsworth, J.A., Hedlund, B.P., Tsiamis, G., Sievert, S.M., Liu, W.T., Eisen, J.A., Hallam, S.J., Kyrpides, N.C., Stepanauskas, R., Rubin, E.M., Hugenholtz, P., and Woyke, T. (2013). Insights into the phylogeny and coding potential of microbial dark matter. *Nature* **499**: 431-437.

Röckne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000). Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Appl Environ Microbiol* **66**: 1595-1601.

Rousk, J., Baath, E., Brookes, P.C., Lauber, C.L., Lozupone, C., Caporaso, J.G., Knight, R., and Fierer, N. (2010). Soil bacterial and fungal communities across a pH gradient in an arable soil. *ISME J* **4**: 1340-1351.

Sawulski, P., Clipson, N., and Doyle, E. (2014). Effects of polycyclic aromatic hydrocarbons on microbial community structure and PAH ring hydroxylating dioxygenase gene abundance in soil. *Biodegradation* **25**: 835-847.

Schumacher, B. (2002). *Methods for the determination of total organic carbon (toc) in soil and sediments US EPA las vegas.* US EPA.

Selesi, D., Jehmlich, N., Von Bergen, M., Schmidt, F., Rattei, T., Tischler, P., Lueders, T., and Meckenstock, R.U. (2010). Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J Bacteriol* **192**: 295-306.

Silva, C.C., Hayden, H., Sawbridge, T., Mele, P., Kruger, R.H., Rodrigues, M.V., Costa, G.G., Vidal, R.O., Sousa, M.P., Torres, A.P., Santiago, V.M., and Oliveira, V.M. (2012). Phylogenetic and functional diversity of metagenomic libraries of phenol degrading sludge from petroleum refinery wastewater treatment system. *AMB Express* **2**: 18.

Snell, F.D., and Snell, C.T. (1949). *Colorimetric Methods of Analysis.* New York: D. Van Nostrand Reinhold, Co.

Sorensen, J. (1978). Denitrification rates in a marine sediment as measured by the acetylene inhibition technique. *Appl Environ Microbiol* **36**: 139-143.

Spence, M.J., Bottrell, S.H., Thornton, S.F., Richnow, H.H., and Spence, K.H. (2005). Hydrochemical and isotopic effects associated with petroleum fuel biodegradation pathways in a chalk aquifer. *J. Contam. Hydrol.* **79**: 67-88.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731-2739.

Torsvik, V., Ovreas, L., and Thingstad, T.F. (2002). Prokaryotic diversity--magnitude, dynamics, and controlling factors. *Science* **296**: 1064-1066.

Uribe-Jongbloed, A., and Bishop, P. (2007). Comparative study of PAH removal efficiency under absence of molecular oxygen: effect of electron acceptor and hydrodynamic conditions. *J Environ Eng Sci* **6**: 367-376.

Valderrama, J.A., Durante-Rodriguez, G., Blazquez, B., Garcia, J.L., Carmona, M., and Diaz, E. (2012). Bacterial degradation of benzoate: cross-regulation between aerobic and anaerobic pathways. *J Biol Chem* **287**: 10494-10508.

Van Gestel, K., Mergaert, J., Swings, J., Coosemans, J., and Ryckeboer, J. (2003). Bioremediation of diesel oil-contaminated soil by composting with biowaste. *Environ Pollut* **125**: 361-368.

Von Netzer, F., Pilloni, G., Kleindienst, S., Kruger, M., Knittel, K., Grundger, F., and Lueders, T. (2013). Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems. *Appl Environ Microbiol* **79**: 543-552.

Wagner, M., and Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. *Curr Opin Biotechnol* **13**: 218-227.

Widdel, F., and Bak, F. (1992). "Gram-Negative Mesophilic Sulfate-Reducing Bacteria," in *The Prokaryotes,* eds. A. Balows, H. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. Springer New York), 3352-3378.

Winderl, C., Schaefer, S., and Lueders, T. (2007). Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (*bssA*) genes as a functional marker. *Environ Microbiol* **9**: 1035-1046.

Wrighton, K.C., Thomas, B.C., Sharon, I., Miller, C.S., Castelle, C.J., Verberkmoes, N.C., Wilkins, M.J., Hettich, R.L., Lipton, M.S., Williams, K.H., Long, P.E., and Banfield, J.F. (2012). Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. *Science* **337**: 1661-1665.

Xie, S., Sun, W., Luo, C., and Cupples, A.M. (2011). Novel aerobic benzene degrading microorganisms identified in three soils by stable isotope probing. *Biodegradation* **22**: 71-81.

Yamada, T., and Sekiguchi, Y. (2009). Cultivation of uncultured chloroflexi subphyla: significance and ecophysiology of formerly uncultured chloroflexi 'subphylum i' with natural and biotechnological relevance. *Microbes Environ* **24**: 205-216.

Yamada, T., Sekiguchi, Y., Hanada, S., Imachi, H., Ohashi, A., Harada, H., and Kamagata, Y. (2006). *Anaerolinea thermolimosa* sp. nov., *Levilinea saccharolytica* gen. nov., sp. nov. and *Leptolinea tardivitalis* gen. nov., sp. nov., novel filamentous anaerobes, and description of the new classes *Anaerolineae* classis nov. and *Caldilineae* classis nov. in the bacterial phylum *Chloroflexi*. *Int J Syst Evol Microbiol* **56**: 1331-1340.

Yang, C., Zhang, W., Liu, R., Li, Q., Li, B., Wang, S., Song, C., Qiao, C., and Mulchandani, A. (2011). Phylogenetic diversity and metabolic potential of activated sludge microbial communities in full-scale wastewater treatment plants. *Environ Sci Technol* **45**: 7408-7415.

Zhang, X., and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* **63**: 4759-4764.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**: 316-322.

Zinger, L., Amaral-Zettler, L.A., Fuhrman, J.A., Horner-Devine, M.C., Huse, S.M., Welch, D.B., Martiny, J.B., Sogin, M., Boetius, A., and Ramette, A. (2011). Global patterns of bacterial beta-diversity in seafloor and seawater ecosystems. *PLoS One* **6**: e24570.

1.6. Supplementary material

1.6.1. Supplementary tables

Table S1a. Bacterial universal primers 6F and 532R containing 5′ tags with multiplex identifier (MID) and sequencing adapters used in this study for the initial samples.

Sample name	Primer name	Adaptor	Key	MID	Primer	Primer position
RPW-I$_a$	M3A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGACGCACTC	TCAGAGTTTGATCCTGGCTCAG	6F
	M3B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGACGCACTC	CACCGCGGCKGCTGGCAC	532R
RPW- I$_b$	M4A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGCACTGTAG	TCAGAGTTTGATCCTGGCTCAG	6F
	M4B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGCACTGTAG	CACCGCGGCKGCTGGCAC	532R
RPS- I$_a$	M5A	CGTATCGCCTCCCTCGCGCCA	TCAG	ATCAGACACG	TCAGAGTTTGATCCTGGCTCAG	6F
	M5B	CTATGCGCCTTGCCAGCCCGC	TCAG	ATCAGACACG	CACCGCGGCKGCTGGCAC	532R
RPS- I$_b$	M6A	CGTATCGCCTCCCTCGCGCCA	TCAG	ATATCGCGAG	TCAGAGTTTGATCCTGGCTCAG	6F
	M6B	CTATGCGCCTTGCCAGCCCGC	TCAG	ATATCGCGAG	CACCGCGGCKGCTGGCAC	532R
RPCal- I$_a$	M7A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTGTCTCTA	TCAGAGTTTGATCCTGGCTCAG	6F
	M7B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGTGTCTCTA	CACCGCGGCKGCTGGCAC	532R
RPCal- I$_b$	M8A	CGTATCGCCTCCCTCGCGCCA	TCAG	CTCGCGTGTC	TCAGAGTTTGATCCTGGCTCAG	6F
	M8B	CTATGCGCCTTGCCAGCCCGC	TCAG	CTCGCGTGTC	CACCGCGGCKGCTGGCAC	532R
AS- I$_a$	M9A	CGTATCGCCTCCCTCGCGCCA	TCAG	TAGTATCAGC	TCAGAGTTTGATCCTGGCTCAG	6F
	M9B	CTATGCGCCTTGCCAGCCCGC	TCAG	TAGTATCAGC	CACCGCGGCKGCTGGCAC	532R
AS- I$_b$	M10A	CGTATCGCCTCCCTCGCGCCA	TCAG	TCTCTATGCG	TCAGAGTTTGATCCTGGCTCAG	6F
	M10B	CTATGCGCCTTGCCAGCCCGC	TCAG	TCTCTATGCG	CACCGCGGCKGCTGGCAC	532R
CP- I$_a$	M11A	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	TCAGAGTTTGATCCTGGCTCAG	6F
	M11B	CTATGCGCCTTGCCAGCCCGC	TCAG	TGATACGTCT	CACCGCGGCKGCTGGCAC	532R
CP- I$_b$[1]	M12A	CGTATCGCCTCCCTCGCGCCA	TCAG	TACTGAGCTA	TCAGAGTTTGATCCTGGCTCAG	6F
	M12A	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	CACCGCGGCKGCTGGCAC	532R
FdP- I$_a$	RL90A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGCGCTATACT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL90B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGCGCTATACT	CACCGCGGCKGCTGGCAC	532R
FdP- I$_b$[1]	M12A	CGTATCGCCTCCCTCGCGCCA	TCAG	TACTGAGCTA	TCAGAGTTTGATCCTGGCTCAG	6F
	M12A	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	CACCGCGGCKGCTGGCAC	532R
MS- I$_a$	RL84A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGACACTAT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL84B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGACACTAT	CACCGCGGCKGCTGGCAC	532R
MS- I$_b$	RL85A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGAGTGTGT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL85B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGAGTGTGT	CACCGCGGCKGCTGGCAC	532R

[1]The samples analyzed with identical MID primers were sequenced in different runs.

Table S1b. Bacterial universal primers 6F and 532R containing 5′ tags with multiplex identifier (MID) and sequencing adapters used in this study for the enrichment culture samples.

Sample name	Primer name	Adaptor	Key	MID	Primer	Primer position
RPW-N[1]	M3A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGACGCACTC	TCAGAGTTTGATCCTGGCTCAG	6F
	M3B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGACGCACTC	CACCGCGGCKGCTGGCAC	532R
RPW-2MN[1]	M5A	CGTATCGCCTCCCTCGCGCCA	TCAG	ATCAGACACG	TCAGAGTTTGATCCTGGCTCAG	6F
	M5B	CTATGCGCCTTGCCAGCCCGC	TCAG	ATCAGACACG	CACCGCGGCKGCTGGCAC	532R
RPS-N[1]	M5A	CGTATCGCCTCCCTCGCGCCA	TCAG	ATCAGACACG	TCAGAGTTTGATCCTGGCTCAG	6F
	M5B	CTATGCGCCTTGCCAGCCCGC	TCAG	ATCAGACACG	CACCGCGGCKGCTGGCAC	532R
RPS-2MN	M6A	CGTATCGCCTCCCTCGCGCCA	TCAG	ATATCGCGAG	TCAGAGTTTGATCCTGGCTCAG	6F
	M6B	CTATGCGCCTTGCCAGCCCGC	TCAG	ATATCGCGAG	CACCGCGGCKGCTGGCAC	532R
RPCal-N	M7A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTGTCTCTA	TCAGAGTTTGATCCTGGCTCAG	6F
	M7B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGTGTCTCTA	CACCGCGGCKGCTGGCAC	532R
RPCal-2MN	RL89A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGCGATCGTAT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL89B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGCGATCGTAT	CACCGCGGCKGCTGGCAC	532R
RPCal-HMN	M8A	CGTATCGCCTCCCTCGCGCCA	TCAG	CTCGCGTGTC	TCAGAGTTTGATCCTGGCTCAG	6F
	M8B	CTATGCGCCTTGCCAGCCCGC	TCAG	CTCGCGTGTC	CACCGCGGCKGCTGGCAC	532R
AS-N	M9A	CGTATCGCCTCCCTCGCGCCA	TCAG	TAGTATCAGC	TCAGAGTTTGATCCTGGCTCAG	6F
	M9B	CTATGCGCCTTGCCAGCCCGC	TCAG	TAGTATCAGC	CACCGCGGCKGCTGGCAC	532R
AS-2MN[1]	128A	CGTATCGCCTCCCTCGCGCCA	TCAG	CACTCGCACG	TCAGAGTTTGATCCTGGCTCAG	6F
	128B	CTATGCGCCTTGCCAGCCCGC	TCAG	CACTCGCACG	CACCGCGGCKGCTGGCAC	532R
AS-HMN	M10A	CGTATCGCCTCCCTCGCGCCA	TCAG	TCTCTATGCG	TCAGAGTTTGATCCTGGCTCAG	6F
	M10B	CTATGCGCCTTGCCAGCCCGC	TCAG	TCTCTATGCG	CACCGCGGCKGCTGGCAC	532R
FdP-N[1]	M3A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGACGCACTC	TCAGAGTTTGATCCTGGCTCAG	6F
	M3B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGACGCACTC	CACCGCGGCKGCTGGCAC	532R
FdP-2MN	RL93A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTATAGTGCT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL93B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGTATAGTGCT	CACCGCGGCKGCTGGCAC	532R
FdP-HMN	131A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGACAGCGAG	TCAGAGTTTGATCCTGGCTCAG	6F
	131B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGACAGCGAG	CACCGCGGCKGCTGGCAC	532R
CP-N	M11A	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	TCAGAGTTTGATCCTGGCTCAG	6F
	M11B	CTATGCGCCTTGCCAGCCCGC	TCAG	TGATACGTCT	CACCGCGGCKGCTGGCAC	532R
CP-2MN	M4A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGCACTGTAG	TCAGAGTTTGATCCTGGCTCAG	6F
	M4B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGCACTGTAG	CACCGCGGCKGCTGGCAC	532R
CP-HMN	M12A	CGTATCGCCTCCCTCGCGCCA	TCAG	TACTGAGCTA	TCAGAGTTTGATCCTGGCTCAG	6F
	M12A	CTATGCGCCTTGCCAGCCCGC	TCAG	TACTGAGCTA	CACCGCGGCKGCTGGCAC	532R
MS-N	RL84A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGACACTAT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL84B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGACACTAT	CACCGCGGCKGCTGGCAC	532R
MS-2MN[1]	128A	CGTATCGCCTCCCTCGCGCCA	TCAG	CACTCGCACG	TCAGAGTTTGATCCTGGCTCAG	6F
	128B	CTATGCGCCTTGCCAGCCCGC	TCAG	CACTCGCACG	CACCGCGGCKGCTGGCAC	532R
MS-HMN	RL85A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGAGTGTGT	TCAGAGTTTGATCCTGGCTCAG	6F
	RL85B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGAGTGTGT	CACCGCGGCKGCTGGCAC	532R

[1]The samples analyzed with identical MID primers were sequenced in different runs.

Table S2. Functional gene primer sequences used in this study.

Target gene	Primer set	5′-3′ sequence	Reference
bssA	7772f	GACATGACCGACGCSATYCT	Winderl *et al.*, 2007
	8546r	TCGTCGTCRTTGCCCCAYTT	
bssA, nmsA	7768f	CAAYGATTTAACCRACGCCAT	Von Netzer *et al.*, 2013
	8543r	TCGTCRTTGCCCCAYTTNGG	
nmsA	7363f	TCGCCGAGAATTTCGAYTTG	Von Netzer *et al.*, 2013
	7374f	TTCGAYTTGAGCGACAGCGT	
	8543r	TCGTCRTTGCCCCAYTTNGG	
ncr	Ncr1f	CGTTATWCKCCYTGCCGTG	Morris *et al.*, 2014
	Ncr1r	CGATAAGCCATRCADATRGG	
ncr	Ncr2f	TGGACAAAYAAAMGYACVGAT	Morris *et al.*, 2014
	Ncr2r	GATTCCGGCTTTTTTCCAAVT	

Table S3a. Aliphatic hydrocarbon composition and abundance in the initial samples.

Compound	RPW	RPS	RPCal	AS	CP	FdP	MS
nC9	nd	nd	nd	0.17±0.01	0.17±0.02	0.24±0.04	0.12±0.09
nC10	nd	nd	nd	2.92±0.36	0.04±0.006	0.08±0.04	0.04±0.01
nC11	0.16±0.01	0.15±0.03	0.193±0.007	0.42±0.003	0.08±0.01	0.24±0.02	0.10±0.07
nC12	0.16±0.02	0.16±0.02	0.129±0.04	12.97±1.26	0.05±0.01	0.18±0.03	0.05±0.02
nC13	nd	nd	nd	3.04±0.36	0.04±0.07	nd	0.04±0.01
nC14	0.54±0.15	0.48±0.08	0.41±0.08	14.13±2.24	0.11±0.01	0.54±0.02	0.17±0.09
nC15	0.11±0.02	0.12±0.02	0.11±0.05	nd	0.17±0.04	0.19±0.02	0.04±0.01
nC16	0.62±0.32	0.53±0.06	0.481±0.001	7.12±2.66	0.17±0.03	0.76±0.10	0.24±0.12
nC17	0.26±0.03	0.30±0.04	0.39±0.16	nd	0.40±0.22	0.46±0.04	0.12±0.06
nC18	0.74±0.30	0.72±0.09	0.50±0.03	2.93±1.35	0.51±0.03	0.92±0.16	0.29±0.17
nC19	0.09±0.00	0.10±0.02	0.06±0.01	nd	nd	0.09±0.004	0.02±0.01
nC20	0.68±0.24	0.68±0.09	0.433±0.001	1.45±0.37	nd	0.85±0.11	0.27±0.17
nC21	nd	nd	0.03±0.23	nd	nd	0.13±0.14	nd
nC22	0.46±0.17	0.47±0.09	0.27±0.01	nd	nd	0.79±0.40	0.18±0.19
nC23	0.02±0.001	0.02±0.001	0.006±0.001	nd	nd	0.70±1.18	nd
nC24	0.15±0.12	0.17±0.05	<dl	nd	nd	1.74±2.72	0.08±0.08
nC25	nd	<dl	<dl	nd	nd	2.44±4.65	nd
nC26	<dl	<dl	<dl	nd	nd	3.32±6.15	<dl
nC27	nd	nd	<dl	nd	nd	3.67±6.99	nd
nC28	<dl	nd	nd	nd	nd	3.34±6.37	nd
nC29	nd	nd	<dl	nd	nd	2.52±4.94	nd
nC30	nd	nd	nd	nd	nd	1.69±3.31	nd
nC31	nd	nd	nd	nd	nd	1.01±1.98	nd
nC32	nd	nd	nd	nd	nd	0.43±0.85	nd
nC33	nd	nd	nd	nd	nd	nd	nd
nC34	nd	nd	nd	nd	nd	nd	nd
nC35	nd	nd	nd	nd	nd	nd	nd
nC36	nd	nd	nd	nd	nd	nd	nd
nC37	nd	nd	nd	nd	nd	nd	nd
nC38	nd	nd	nd	nd	nd	nd	nd
nC39	nd	nd	nd	nd	nd	nd	nd

Data in ppm (μg/g)

nd: not detected.

<dl: below the limit of detection

TableS3b. PAH composition and abundance in the initial samples.

Compound	RPW	RPS	RPCal	AS	CP	FdP	MS
N	0.11±0.029	0.097±0.006	0.049±0.009	0.111±0.004	0.27±0.03	0.045±0.006	0.06±0.03
1-MN	<dl	<dl	0.02±0.02	0.02±0.04	0.109±0.001	0.0040±0.0008	0.003±0.007
2-MN	0.005±0.009	0.005±0.008	0.02±0.01	0.02±0.04	0.305±0.003	0.006±0.003	0.005±0.004
N3	nd	nd	0.05±0.03	nd	nd	nd	nd
N4	nd	nd	0.07±0.05	nd	nd	0.025±0.015	0.001±0.002
D	0.09±0.05	0.062±0.001	0.12±0.08	nd	nd	0.005±0.003	0.005±0.0003
Ph	0.12±0.06	0.10±0.01	0.13±0.07	nd	0.020±0.001	0.004±0.003	0.004±0.00004
A	0.12±0.07	0.09±0.01	0.14±0.07	nd	0.024±0.007	0.007±0.002	0.0100±0.0008
Ba	nd	nd	0.060±0.006	nd	nd	nd	nd
Py	0.07±0.05	0.06±0.01	0.06±0.03	nd	nd	0.0047±0.0005	0.006±0.0005
CN	nd	0.009±0.001	0	0.0205±0.0009	0.0077±0.0005	0.012±0.003	nd
Fl	nd	0.03±0.005	0.04±0.08	nd	nd	0.0039±0.0001	nd
C	nd	nd	0.08±0.07	0.71±0.04	nd	nd	nd

Naphthalene (**N**), 1-methylnaphthalene (**1-MN**), 2-methylnaphthalene (**2-MN**), 1,4,5-trimethylnaphthalene (**N3**), 1,4,6,7-tetramethylnaphthalene (**N4**), dibenzothiophene (**D**), Phenanthrene (**Ph**), Anthracene (**A**), Benzo[a]anthracene (**Ba**), Pyrene (**Py**), C2-naphthalene (**CN**), Fluoranthene (**Fl**), Chrysene (**C**)

Data in ppm (µg/g)
nd: not detected.
<dl: below the limit of detection

Table S4. Most probable number counts of nitrate reducing bacteria in the environmental samples. Carbon source are naphthalene (NAP), 2-methylnaphthalene (2MN), 2-naphtoic acid (2NA), anthracene (ANT), acetate (Ace) and no added carbon source (na).

Sample name	C source	MPN counts	95% C.I.	
RPCal	na	1.95E+04	3.28E+03	8.04E+04
	NAP	5.80E+03	1.41E+03	1.47E+04
	2MN	2.79E+03	4.09E+02	1.03E+04
	2NA	3.46E+04	4.98E+03	1.19E+05
	ANT	4.27E+06	1.03E+06	1.38E+07
	Ace	2.40E+07	4.76E+06	9.65E+07
RPW	na	4.27E+04	1.03E+04	1.38E+05
	NAP	2.40E+07	4.76E+06	9.65E+07
	2MN	7.41E+06	1.74E+06	2.15E+07
	2NA	7.41E+06	1.74E+06	2.15E+07
	ANT	2.40E+06	4.76E+05	9.65E+06
	Ace	4.27E+07	1.03E+07	1.38E+08
RPS	na	1.43E+05	2.74E+04	6.07E+05
	NAP	1.47E+07	2.78E+06	6.32E+07
	2MN	9.33E+06	2.07E+06	2.71E+07
	2NA	7.41E+06	1.74E+06	2.15E+07
	ANT	9.33E+06	2.07E+06	2.71E+07
	Ace	2.40E+08	4.76E+07	9.65E+08
AS	na	4.27E+05	1.03E+05	1.38E+06
	NAP	9.18E+03	2.05E+03	2.66E+04
	2MN	1.86E+02	3.20E+01	7.78E+02
	2NA	9.33E+05	2.07E+05	2.71E+06
	ANT	4.27E+05	1.03E+05	1.38E+06
	Ace	4.27E+06	1.03E+06	1.38E+07
CP	na	7.41E+01	1.74E+01	2.15E+02
	NAP	4.27E+01	1.03E+01	1.38E+02
	2MN	9.33E+00	2.07E+00	2.71E+01
	2NA	2.59E+03	3.89E+02	9.76E+03
	ANT	2.40E+05	4.76E+04	9.65E+05
	Ace	2.40E+07	4.76E+06	9.65E+07
FdP	na	1.90E+02	3.24E+01	7.89E+02
	NAP	2.40E+06	4.76E+05	9.65E+06
	2MN	2.04E+04	3.37E+03	8.32E+04
	2NA	9.18E+03	2.05E+03	2.66E+04
	ANT	7.41E+03	1.74E+03	2.15E+04
	Ace	4.78E+02	1.16E+02	1.47E+03
MS	na	2.04E+00	3.70E-01	8.30E+00
	NAP	4.27E+01	1.03E+01	1.38E+02
	2MN	2.75E+00	5.90E-01	1.02E+01
	2NA	4.27E+01	1.03E+01	1.38E+02
	ANT	7.41E+01	1.74E+01	2.15E+02
	Ace	9.33E+04	2.07E+04	2.71E+05

Table S5. Significance tests performed using a two-sided Student's two-sample t-test comparing the Unifrac weighted distance.

Samples	Compared group		p-value
	Group 1	**Group 2**	
RPW/RPS	initial vs. initial	initial vs. NAP	**0.001**
	initial vs. initial	initial vs. 2MN	**0.005**
RPCal	initial vs. initial	initial vs. NAP	**0.05**
	initial vs. initial	initial vs. 2MN	0.10
	initial vs. initial	initial vs. HMN	0.10
AS	initial vs. initial	initial vs. NAP	0.09
	initial vs. initial	initial vs. 2MN	0.13
	initial vs. initial	initial vs. HMN	0.17
CP	initial vs. initial	initial vs. NAP	**0.007**
	initial vs. initial	initial vs. 2MN	**0.02**
	initial vs. initial	initial vs. HMN	**0.01**
FdP	initial vs. initial	initial vs. NAP	**0.02**
	initial vs. initial	initial vs. 2MN	**0.01**
	initial vs. initial	initial vs. HMN	**0.01**
MS	initial vs. initial	initial vs. NAP	0.06
	initial vs. initial	initial vs. 2MN	**0.04**
	initial vs. initial	initial vs. HMN	0.08

Table S6. Taxonomic affiliation of strains isolated on solid medium with PAHs as carbon source.

(GenBank Accession No.)	Isolates (Sample)	Carbon source	Method	Closest type strain relative in GenBank database	Similarity (%)
KX417378	**RPCal-N 2.1**	NAP	Agar shakes	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417379	**RPCal-N 2.2**	NAP	Agar shakes	*Ochrobactrum anthropi, type sp., Brucellaceae*	99
KX417380	**RPCal-N 1.1**	NAP	Agar shakes	*Paenibacillus sp, Paenibacillaceae*	96
KX417381	**RPCal-N 1.2**	NAP	Agar shakes	*Paenibacillus sp, Paenibacillaceae*	96
KX417382	**RPCal-2MN 1.1**	2MN	Agar shakes	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417383	**RPCal-2MN 1.2**	2MN	Agar shakes	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417384	**AS-2MN 1.1**	2MN	Agar shakes	*Bacillus thioparans, Bacillaceae*	99
KX417385	**AS-2MN 1.2**	2MN	Agar shakes	*Bacillus thioparans, Bacillaceae*	99
KX417386	**RPCal-N 10-2-3**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417387	**RPCal-N 10-2-4**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417388	**RPCal-2MN 10-2-2**	2MN	Agar plate	*Paenibacillus graminis, Paenibacillaceae*	97
KX417389	**RPCal-2MN 10-3-3**	2MN	Agar plate	*Bacillus korlensis, Bacillaceae*	98
KX417390	**RPCal-2MN 10-3-4**	2MN	Agar plate	*Paenibacillus xylanilyticus, Paenibacillaceae*	97
KX417391	**RPCal-HMN 10-2-3**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417392	**RPCal-HMN 10-2-4**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417393	**RPCal-HMN 10-2-5**	NAP	Agar plate	*Bacillus korlensis, Bacillaceae*	99
KX417394	**RPCal-HMN10-3-2**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417395	**RPCal-HMN 10-3-4**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417396	**RPW-N 10-2-4**	NAP	Agar plate	*Pseudomonas stutzeri, Pseudomonadaceae*	99
KX417397	**RPS-2MN 10-2-1**	2MN	Agar plate	*Streptomyces sp., Streptomycetaceae*	96
KX417398	**CP-2MN 10-3-4**	2MN	Agar plate	*Bacillus niacini, Bacillaceae*	97
KX417399	**CP-2MN 10-3-5**	2MN	Agar plate	*Bacillus thermocopriae, Bacillaceae*	98
KX417400	**CP-2MN 10-3-6**	2MN	Agar plate	*Bacillus niacini, Bacillaceae*	98
KX417401	**CP-HMN 10-3-3**	NAP	Agar plate	*Pseudomonas balearica, Pseudomonadaceae*	99
KX417402	**CP-HMN 10-3-4**	NAP	Agar plate	*Bacillus korlensis, Bacillaceae*	99
KX417403	**AS-2MN 10-2-1**	2MN	Agar plate	*Bacillus sp., Bacillaceae*	90
KX417404	**FdP-HMN 10-2-2**	NAP	Agar plate	*Marinilabilia sp., Marinilabiliaceae*	98
KX417405	**FdP-2MN 10-2-3**	2MN	Agar plate	*Bacillus sp, Bacillaceae*	92
KX417406	**FdP-HMN 10-2-6**	NAP	Agar plate	*Marinilabilia sp, Marinilabiliaceae*	98

1.6.2. Supplementary figures

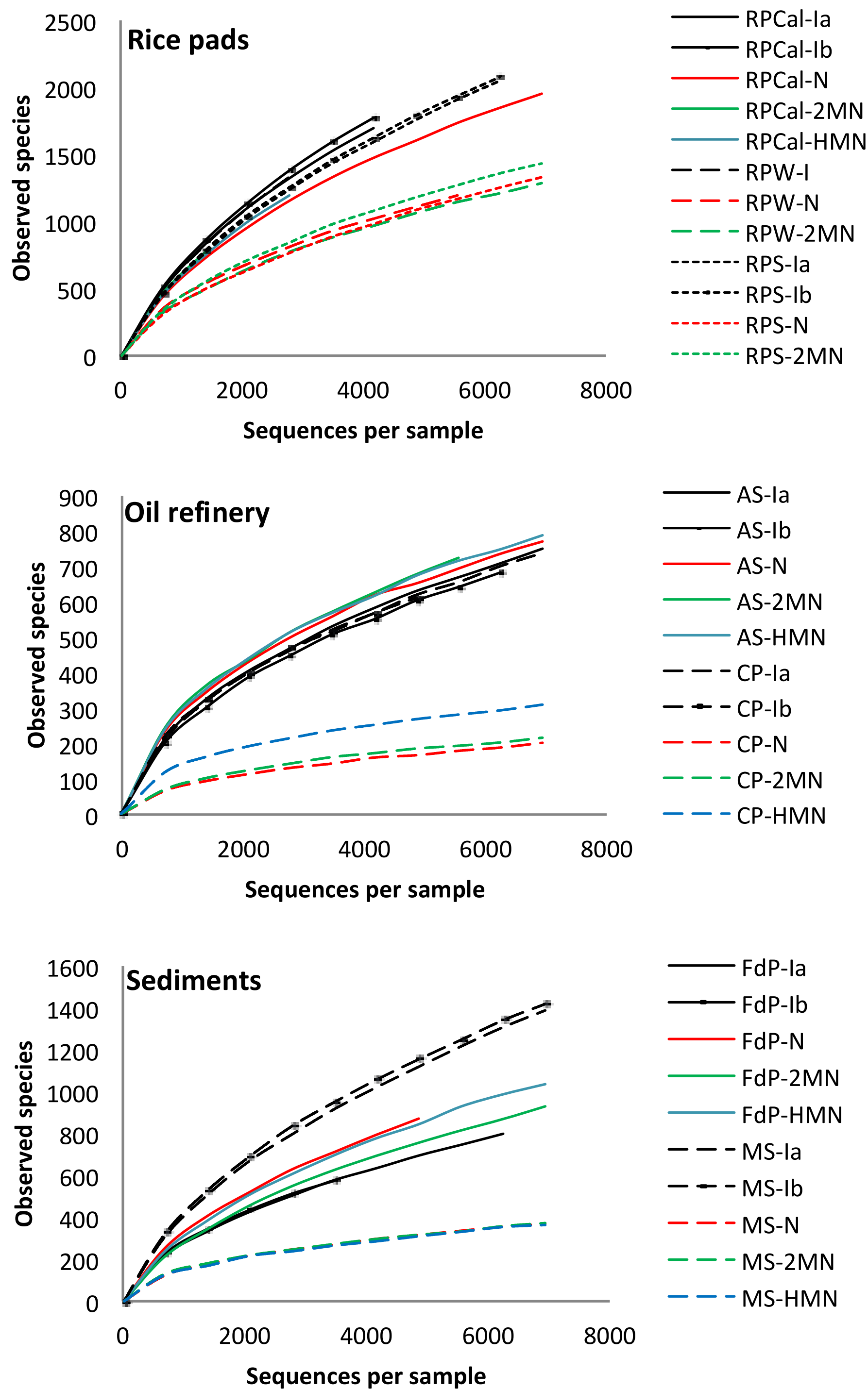

Figure S1. Rarefaction curves based on bacterial OTUs at a dissimilarity level of 3% of the initial samples and enrichments as calculated by Qiime.

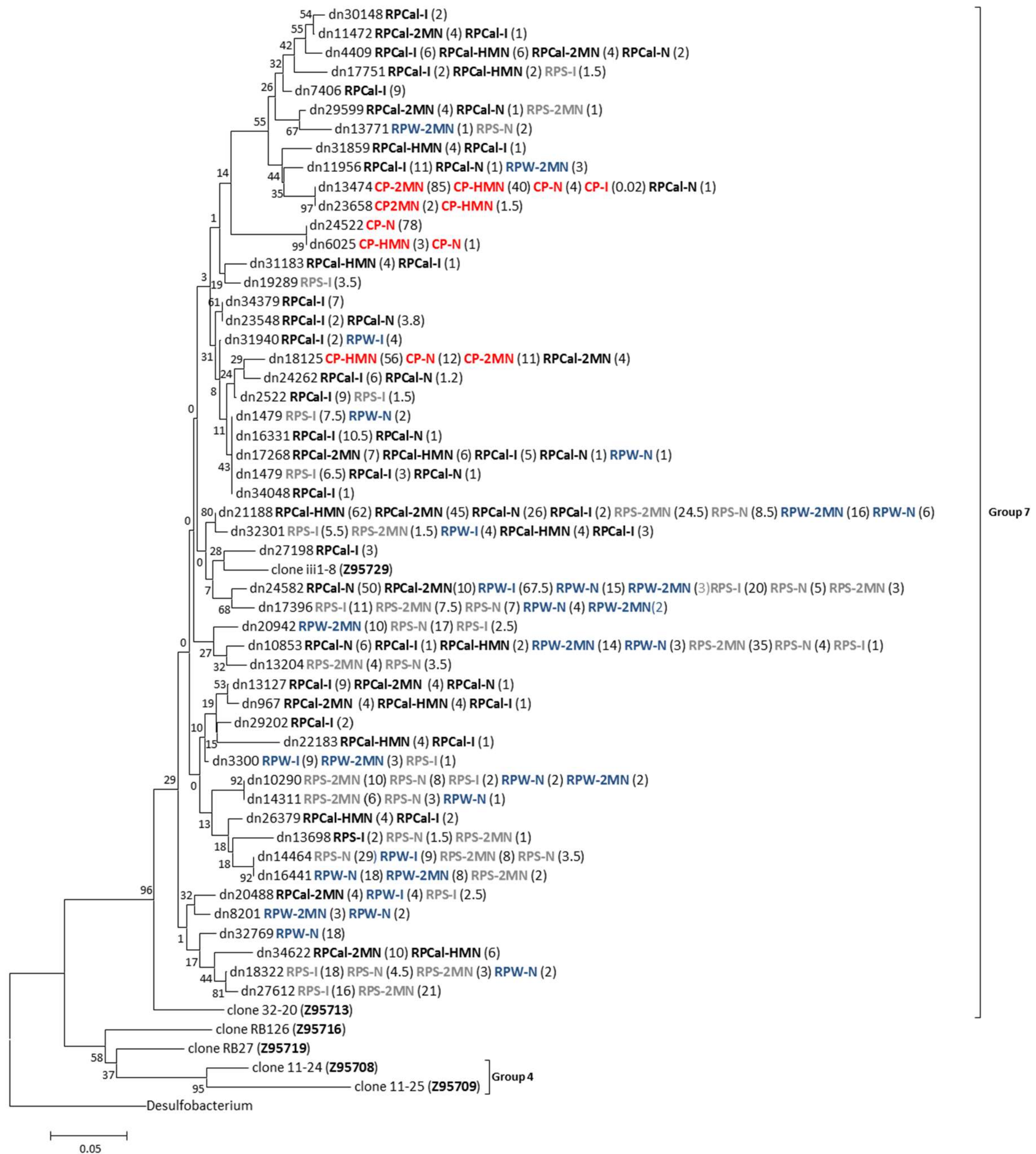

Figure S2. Evolutionary relationships of iii1-8 DS18 sequences retrieved from the rice-paddy and compost-pile initial sample and enrichments. The optimal Neighbour-Joining tree is shown. The bootstrap (1000 replicates) is shown next to the branches. The evolutionary distances are in the units of the number of base substitutions per site. Numbers in brackets are the percentage of each sequence type over the complete set of iii1-8 reads in each sample. The sequences of three different *Acidobacteria* subgroups are included (Hugenholtz *et al.*, 1998). Numbers in brackets indicate the relative abundance (%) of each sequence in the corresponding sample.

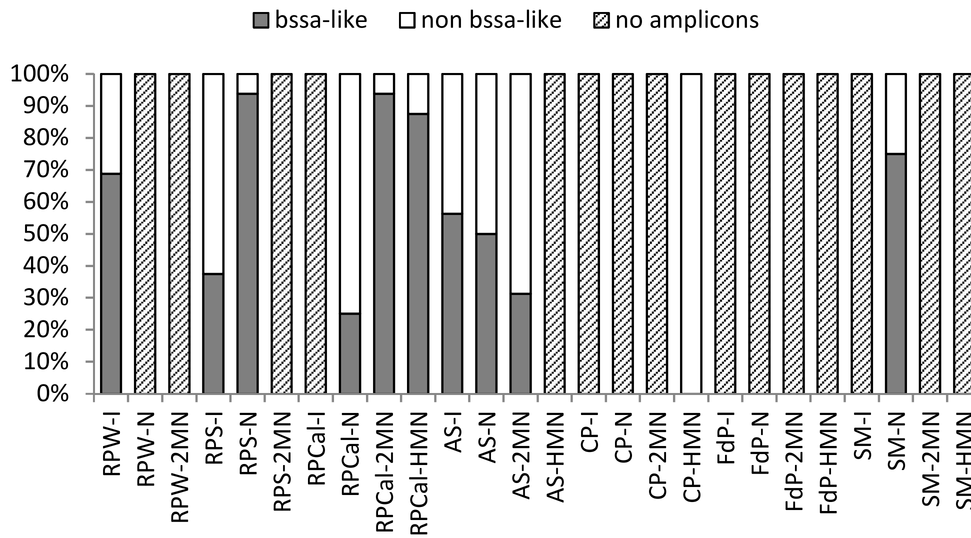

Figure S3. Frequency distribution of *bssA*-like and non *bssA*-like sequence types in the different libraries constructed from the samples.

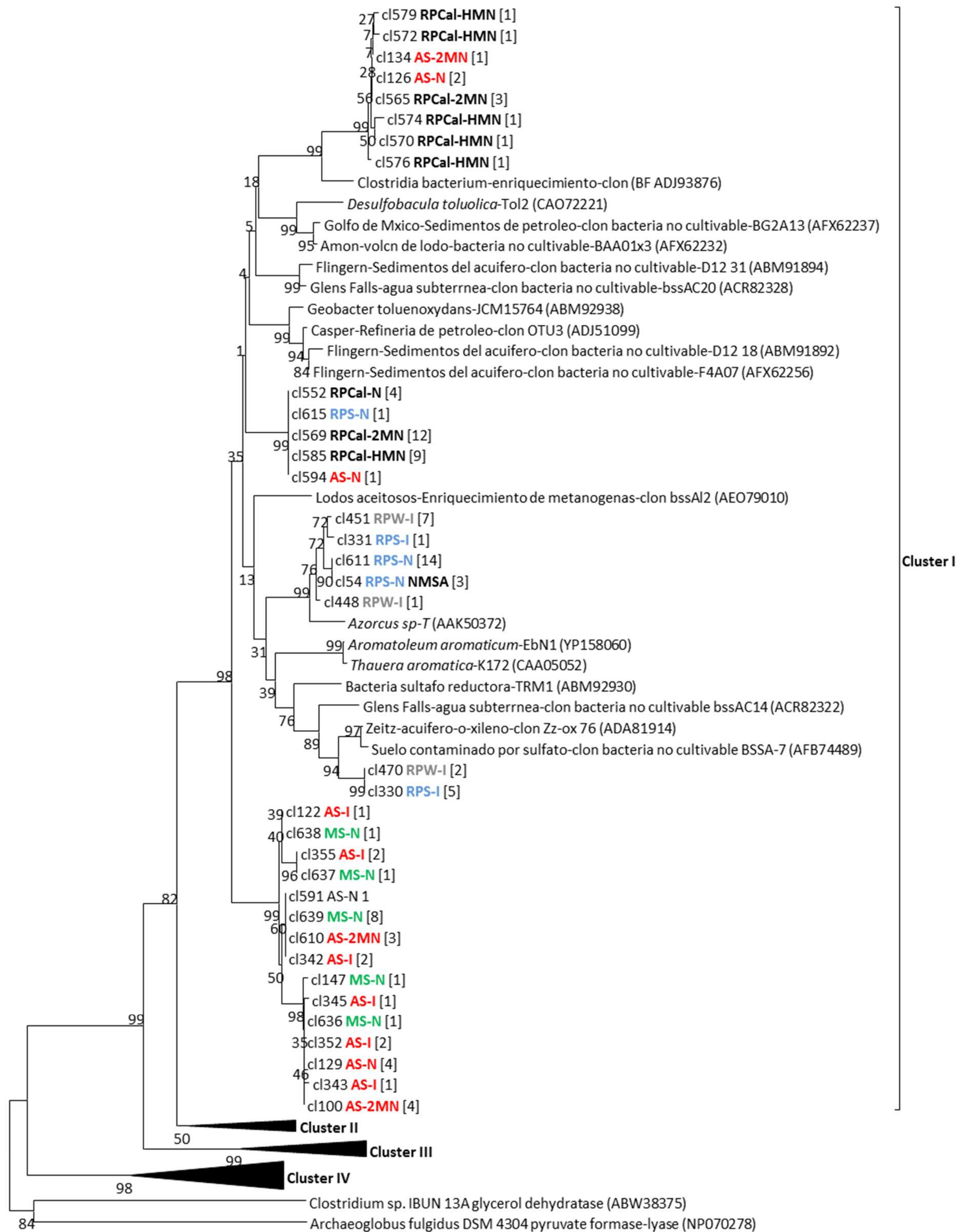

Figure S4. Phylogeny of partial BssA-like amino acid sequences retrieved from initial samples and enrichments. Initial samples were rice-paddy water (RPW-I; grey), rice-paddy soil (RPS-I; blue), and activated sludge (AS-I; red). Enrichments were rice-paddy soil naphthalene (RPS-N; blue), rice-paddy soil Calasparra naphthalene (RPCal-N; black), rice-paddy soil Calasparra 2-MN (RPCal-2MN; black), rice-paddy soil Calasparra HMN (RPCal-HMN; black), activated sludge naphthalene (AS-N; red), activated sludge 2MN (AS-2MN; red) and marine sediments naphthalene (MS-N; green). The closest relatives, detected in anaerobic hydrocarbon-oxidizing strains or enrichments, and

putative environmental sequences available in the databases at the beginning of this study are included. Cluster I include the canonical, toluene-specific BssA sequences; cluster II includes sequences associated with the oxidation of BTEX compounds; cluster III includes the 2-methyl-naphthalene-specific *nmsA* sequences, putative *bssA* gene from the non-proteobacterial *Desulfotomaculum* sp. strain Ox39 and sequences retrieved from enrichments able to grow on o-xylene as the only carbon source; cluster IV comprises *assA* sequences. Codes in brackets are the protein GenBank accession numbers. Numbers in square brackets are the number of sequences found in the corresponding library. The tree was rooted with pyruvate formate lyase paralogues as an out-group.

CHAPTER 2

Microbial communities in Fuente de Piedra athalassohaline lagoon sediments: impact of the contamination with naphthalene.

The important thing is to not stop questioning.
Curiosity has its own reason for existing.

Albert Einstein

2.1. Introduction

Inland waters (e.g. lakes, rivers, wetlands, reservoirs) occupy a small fraction of the earth's surface (~1%), but are of great importance to understand the impact of human activity and climate change on ecosystem structures and functions (Adrian et al., 2009). Saline lakes, characterized by salinities higher than 1 g l^{-1}, account for about 45% of the total inland aquatic systems (Wetzel, 2001). About 75% of saline lakes are located in endorheic watersheds. That is, they are in closed drainage basins that retain water and allow no outflow to other bodies of water such as rivers or oceans. Consequently nutrients, organic matter and salts largely remain within the basin resulting in high dissolved inorganic and organic carbon concentrations and high-water residence times (Anderson, 2007; Ortega-Retuerta et al., 2007). Thus, they are extremely productive systems (Eiler et al., 2003). Microbes are a fundamental component of aquatic ecosystems and play essential roles in global biogeochemical cycles (Newton et al., 2011). Both cultivation dependent and independent techniques have been used to unravel the diversity of bacterial and archaeal communities in saline environments. Several hypersaline lakes from around the world have been studied, including athalassohaline lakes of the Atacama desert, Chile (Demergasso et al., 2004), soda lakes in Mongolia (Sorokin et al., 2004), Mono Lake, California (Scholten et al., 2005), the athalassohaline Lake Chaka, China (Jiang et al., 2006), hypersaline lakes of the Wadi An Natrun, Egypt (Mesbah et al., 2007), athalassohaline lagoon of Tirez, Spain (Montoya et al., 2013) and Soda Saline Crater Lake from Isabel Island, Mexico (Aguirre-Garrido et al., 2016). Notwithstanding the diverse physical and chemical properties of the lakes studied, only a few bacterial groups predominate in these ecosystems, such as *Proteobacteria*, *Bacteroidetes*, *Firmicutes* and some Archea (*Euryarchaeota*) (Sorokin et al., 2014). However, despite the global prevalence and biogeochemical significance of these ecosystems, our understanding of the microbial diversity in hypersaline environments is still limited as compared to marine and freshwater environments. Furthermore, the number of investigations specifically targeting the sediments of hypersaline lakes is small (Kim et al., 2012; Yang et al., 2016).

In the last few years, saline and alkaline lakes have become interesting model systems for studies on microbial diversity and ecosystem functions in extreme environments. In fact, such ecosystems have been a valuable source of novel microorganisms that are economically important for industrial, biotechnological and environmental processes, and many new applications have been and are still being explored (Lizama et al., 2001; Oren, 2010). As all natural ecosystems on our planet, hypersaline environments are subjected to environmental contamination. Industrial and municipal effluents are often discharged into saline and hypersaline depression and intertidal zones. It has been estimated that 5% of the total world effluents are highly saline (Lefebvre and

Moletta, 2006). Therefore, there is great interest in the use of halophilic microorganisms to degrade organic pollutants and to produce biopolymers, biosurfactants, and compatible solutes (Marhuenda-Egea and Bonete, 2002; Le Borgne et al., 2008). Among others, polycyclic aromatic hydrocarbons (PAHs) include a group of priority organic pollutants of significant concern due to their toxic, genotoxic, mutagenic and/or carcinogenic properties (Cerniglia, 1992). PAHs are hydrophobic compounds and their persistence within ecosystems is due to their low water solubility, electrochemical stability and resistance toward biodegradation (Ghosal et al., 2016). PAHs rapidly become associated with sediments, where they may persist and bioaccumulate until degraded or resuspended. Sediments, as many natural habitats, are often anoxic (Brune et al., 2000). In these environments, the anaerobic degradation of aromatic compounds by microorganisms plays a major role in the removal of contaminants, recycling of carbon and sustainable development of the ecosystem. In contrast to aerobic degradation of PAHs, much less is known about the anaerobic degradation of these compounds (Meckenstock et al., 2004a). Whereas the anaerobic degradation of mono-aromatics is well documented (Foght, 2008) the knowledge on anaerobic PAH degradation is still in its infancy. Our current knowledge on anaerobic PAH degradation pathways is limited to the simplest naphthalenes. Isolation of naphthalene and methylnaphthalene degrading anaerobes has only been possible for two pure cultures (NaphS2 and NaphS3 strains) and one bacterial consortium (N47) of marine sulphate-reducing bacteria (Galushko et al., 1999; Meckenstock et al., 2000b; Musat et al., 2009), and to date there is no culture available that degrades naphthalene using nitrate as terminal electron acceptor.

Moreover, our knowledge on anaerobic biodegradation of PAHs in hypersaline anoxic sediments is even scarcer, as compared to other anaerobic systems such as aquifers or marine sediments. A systematic analysis of these environments may help towards understanding the environmental characteristics that drive the architecture of microbial communities. The exploration of the halophile diversity and its response to the presence of PAHs will lead to the understanding on how this diversity can be managed for biorestoration purposes or for biotechnological applications. Our study focused on the anoxic sulphate rich sediment of the athalassohaline lake of Fuente de Piedra located in the north of Malaga (Spain). Athalassohaline systems are saline waters that are rich in anions other than chloride (e.g., sulphate) and frequently in cations such as calcium and magnesium. The best-characterized athalassohaline waters have NaCl as the major salt. Fuente the Piedra lagoon has a salinity ranging from oligo- (ca. 9 g l^{-1}) to hypersaline (> 200 g l^{-1}) conditions depending on the hydrological cycles (García et al., 1997). The lake has an area of 13.5 km^2 and an endorheic basin of 150 km^2 (Heredia et al., 2010) and is

one of the most important breeding sites of aquatic birds, especially the greater flamingo, in the Mediterranean area. It was the third Spanish wetland to be included in the Ramsar convention and it was declared to a nature reserve in 1984 and to a Special Protection Area for Birds in 1987.

Here, we have used Illumina sequencing to characterize the microbial community from hypersaline sediments in Fuente de Piedra. The aim of this work was to assess the diversity and structure of the bacterial communities in the unexplored sediment of the athalassohaline lagoon, to study the effect of PAHs on the bacterial communities and to enrich potential sulphate-reducing-bacteria able to degrade anaerobically the model PAHs naphthalene and 2-methylnaphthalene.

2.2. Materials and methods

2.2.1. Site description and Sampling

Samples were collected from the athalassohaline lagoon of Fuente de Piedra (FdP) (Málaga, Spain) (37°05'07N 4°47'05W) at in two different sampling time campaigns. The first sampling was carried out in February 29th, 2012 and anoxic black sediments (0-10 cm in depth) were collected close to the shore (50 cm below water surface). Water salinity was 70,00‰ and water pH 8.46. The second sampling was carried out on in March 10th, 2014 in the same sampling site using 24 cores (30 cm x 5 cm) sealed with silicone stoppers to maintain anaerobic conditions. Water salinity was 56,50‰ and water pH 8.48. Sediment samples were kept at 4ºC until processed.

2.2.2. Microcosm experiment

Twelve amongst 24 sampled cores were selected based on the similarity of their depth profiles to allow reliable duplicates (Figure 2. 1), and 10 of them were chosen for the microcosm experiment (cores 3 to 12). Cores had an average total sediment width of 19 cm, divided in five different layers (Table 2. 1): P0 (grey colour) was the top oxic layer (average 1 cm thick) in direct contact with the lagoon water; P1 (black coloured) was 5.6 cm thick on average; P2 (average 2.6 cm thick) was the gradient zone between the black and the underlying grey layers; P3 (light grey) was on average 7.8 cm thick; finally, P4 was very hard and light brown and on average 3 cm thick, although it was not present in all the cores due to the difficulty to be sampled (Table 2. 1).

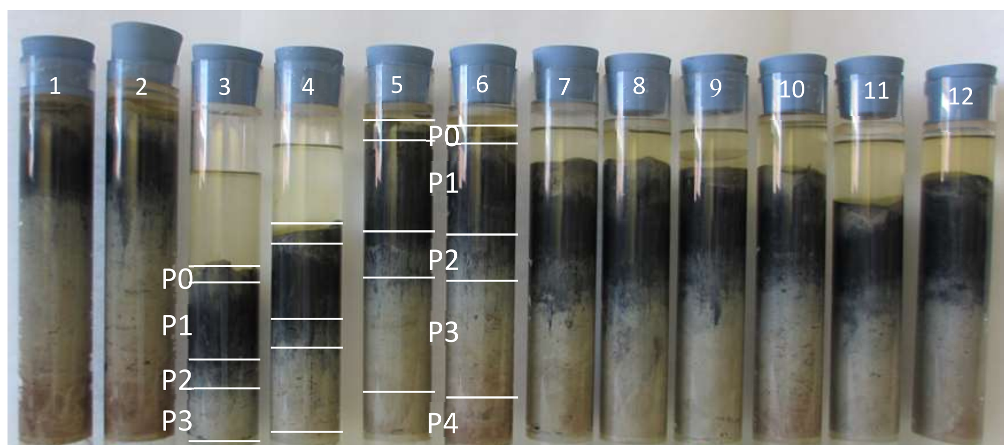

Figure 2. 1. Cores used in this work for the microcosm experiment, enrichment cultures and Illumina-sequencing at the start of the experiment. The cores are numbered from 1 to 12 (labelled in white). The scheme shows examples of the profiles mentioned in Table 2. 1 (labelled in white).

Table 2. 1. Profile description of the cores collected in the first campaign.

Core nº	Label	Sampling time	Treatment	Total sediment thickness (cm)	Profile samples thickness (cm)				
					P0	**P1**	**P2**	**P3**	**P4**
1	Ini	0	Ctrl. layers	23	1-1.2	4.7	1	11.5	5
2	Ini	0	Ctrl. layers	22.5	1	4.7	3	9.5	4
3	Ini	0	Initial	11	1	4.7	2	3.4	/
4	Ini	0	Initial	14.5	0.6-1	5.5	2.9	6	/
5	C1	1	Ctrl	21.5	1	7.5	3	8.5	1.8
6	C1	1	Ctrl	21.5	1	6	2.8	7	4.8
7	C2	2	Ctrl	19.5	1-1.8	5	3.4	6.5	3.2
8	C2	2	Ctrl	19.3	1-1.3	6.3	3.4	7.1	0.9
9	N1	1	Nap	19.5	1	5.5	3	10	/
10	N1	1	Nap	19.2	1	6	1	8.6	2.6
11	N2	2	Nap	17.5	1	5.5	2.9	8	/
12	N2	2	Nap	19	1	6	2.4	7.6	1.9

The water content was analysed for the layers P1 to P4, and also for the mixed layer P1 to P4 (Table 2. 2) as described in Martirani-Von Abercron *et al.*, 2016. Cores 1 to 4 were processed in the lab immediately after sampling. Cores 1 and 2 were fractionated at different depth (P0, P1, P2, P3 and P4; Table 2.1) and stored at -80°C for future analysis. The P4 layer was not included in the DNA analysis. The layers P0, P1, P2 and P3 of cores 3 and 4 were homogenized and part of it was used as Time 0 for the enrichment experiment. The remaining material was stored at -80ºC for future use. Cores 5 to 8 were incubated without any treatment and used as control.

Table 2. 2. Gravimetric water content in the different cores layers (P1 to P4) and in the mixed layer (P1 to P4).

Layer	Water[a] content (%)
P1	74,10±11,59
P2	45,11±0,82
P3	28,72±0,93
P4	20,87±1,11
P1-2-3-4	57,55±3,49

[a]Gravimetric, % of dry weight.

Cores 9 to 12 were penetrated with a 10 ml plastic pipette filled with naphthalene crystals. The pipettes had 1 mm diameter holes every 1 cm around its surface to allow naphthalene diffusion into the sediment. Each core was sealed with silicone stoppers, to maintain anaerobic conditions and water content in the microcosms. All manipulations were carried out in an anaerobic glove box. Duplicate cores were analysed for each condition. Cores 5, 6, 9 and 10 were incubated for 4.5 months (Time 1) and cores 7, 8, 11, and 12 were left for 8 further months (Time 2). All cores were incubated at 18ºC in the darkness. When processed, each core was longitudinally divided in two halves: one was fractionated at different depth and stored at -80ºC, and the remaining half-core was mixed, stored at -80ºC and used for DNA extraction.

2.2.3. Culture conditions and anaerobic enrichment

Black (anoxic) sediment collected in the two sampling campaigns was used as starting material for sulfate-reducing bacteria (SRB) enrichment cultures. For cultivation of SRB we used an artificial seawater minimal medium (SMM) as described elsewhere (Widdel and Bak, 1992) with Na-resazurin solution (0.1% w/v) as redox indicator. Briefly, after autoclaving the medium was flushed with a 80:20 N_2:CO_2 gas mixture for 30 min, then 75 ml of media were dispensed in 120 ml serum bottles containing 25 g of sediment and sealed with Teflon-lined 1 cm thick stoppers. The medium was then supplemented using a nitrogen-flushed syringe with 21 mM of Na_2SO_4, 0.1 ml of trace element solution SL10 (Widdel *et al.*, 1983), 30 mM of CO_2-saturated sodium bicarbonate, 0.1 ml of vitamin solution (Widdel and Bak, 1992), 0.1 µM of $Na_2S_2O_3 \cdot 5H_2O$, 16 mM of $Na_2S \cdot 9H_2O$ and 0.11 mM of $Na_2S_2O_4$. All solutions were prepared under 100% N_2 gas (except SMM and bicarbonate) and autoclaved or filter-sterilized (vitamin and sodium dithionite solutions). The final pH of the medium ranged between 7.2 and 7.4. The enrichment from samples

collected in the first sampling campaign were initiated directly from the collected sediment. In the cultures naphthalene and 2-methylnaphthalene (2MN) were used as carbon sources dissolved at 20 g l^{-1} in 2 ml of sterile anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) that served as insoluble carrier to reduce the PAH concentration in the water phase. A control bottle without added aromatic compounds in the HMN phase was included. Cultures were incubated in the dark at 28°C. Samples of 10% inoculum were transferred periodically to fresh medium. The enrichments from samples collected in the second sampling campaign were started from the sediment cores used in the microcosm experiment, which had been incubated during 4 (core 5-6-9-10) and 8 months (core 7-8-11-12) in the presence and absence of naphthalene. After the incubation period, the cores were processed and 25 g of sediment were homogenised and used as inoculum. Initially, filter-sterilized Fuente de Piedra lagoon water flushed with N_2/CO_2 gas mixture (8:2, v/v) was used to cultivate SRB. When required, naphthalene was provided to the enrichment cultures as crystals, while no carbon source was added to the enrichments from control cores. The second subculture transfers with 10% inoculum were prepared either with filter-sterilized Fuente de Piedra lagoon water or with the SMM medium described above (see scheme in Figure 2. 2). All cultures were incubated in the dark at 18ºC without shaking.

2.2.4. Total DNA extraction

For each sediment sample, total DNA was extracted from four 0.5 g subsamples by the bead-beating method, following the manufacturer's instructions for the MoBio UltraClean Soil DNA Isolation kit (MoBio laboratories, Solana Beach, CA, USA) except for the lysis step. Briefly, cell lysis was achieved adding a heat step at 70°C for 5 min before and after the mechanical lysis with the beads. The extracts were pooled onto the same spin filter. The DNA was then resuspended in 50 µl of sterile deionized water and stored at -20°C until use. DNA extraction for the anaerobic enrichment was performed following a modified version of the SDS-based method developed by (Zhou *et al.*, 1996) as previously described in (Martirani-Von Abercron *et al.*, 2016). DNA quality was assessed by electrophoresis on a 0.8% agarose gel. Nucleic acid quantity and purity were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

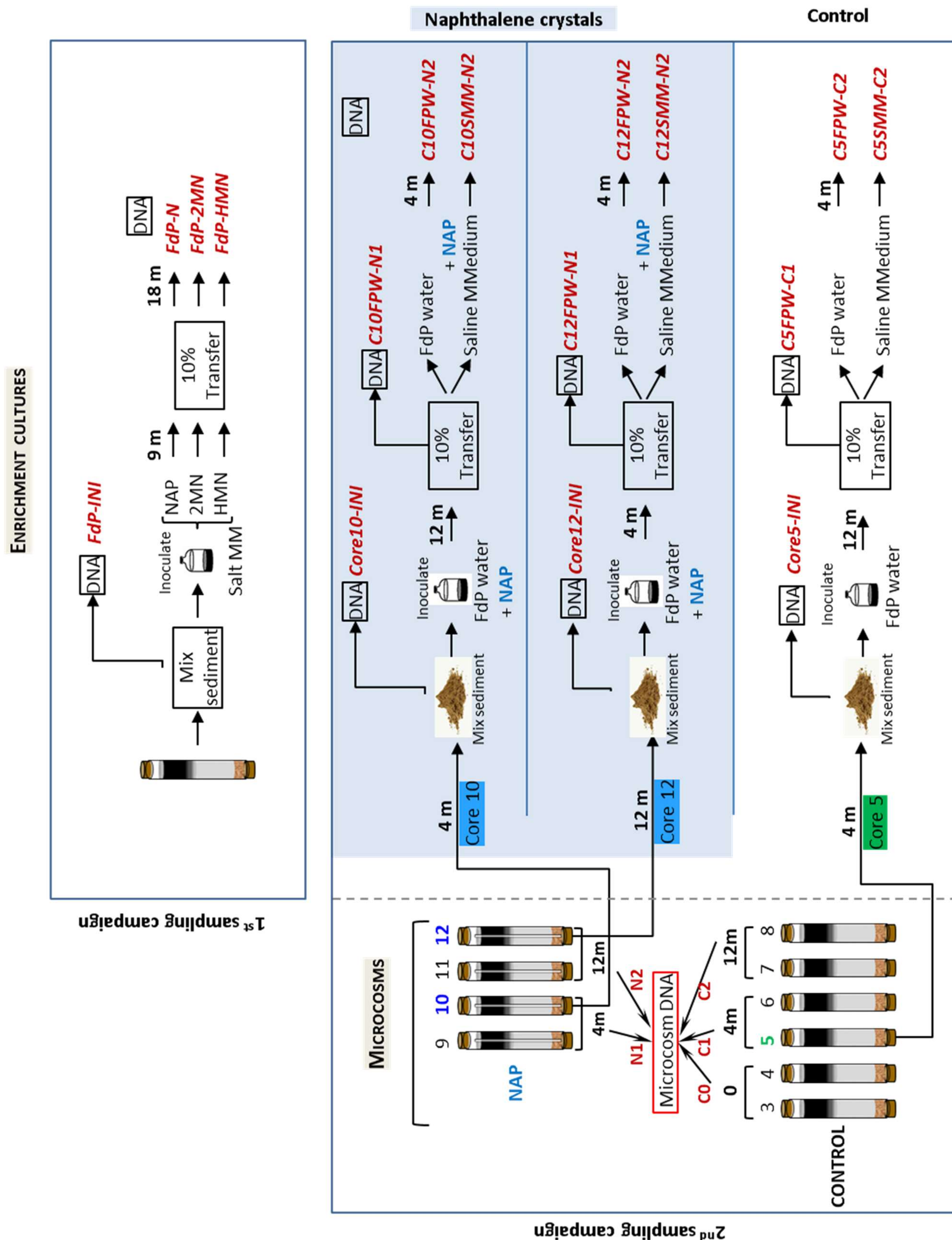

Figure 2. 2. Scheme of experimental set-up. The anaerobic enrichments were prepared starting from the incubated cores and sequenced with Illumina Miseq. A) Cores 5-10 enrichments after the first period of incubation; B) Core 12 enrichment after the second period of incubation.

2.2.5. Illumina sequencing and Data Analysis

A total of 26 samples were analysed in this study. Genomic DNA extracted from soil and enrichment samples were paired-end sequenced (2x300bp) at Era7 Information Technologies S.L with an Illumina Miseq platform (MiSeq run PE 2x300) by analyzing the V3-V4 region of the bacterial 16S rRNA gene. The sequences of forward and reverse primers were 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3' and 5'-TCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3', respectively. The Illumina 16S sequences were processed using the Quantitative Insights Into Microbial Ecology (QIIME, v. 1.9.0) pipeline. (Caporaso *et al.*, 2010b). From the demultiplexed data received from Era7, we merged pair-ends reads into single contigs and proceeded with quality-filtering using a Q25 minimum value (average quality scores <25 were removed). Then, the usearch61 algorithm was used to detect chimeric sequences with de novo method (Edgar *et al.*, 2011). All chimeras and singletons were removed before further analysis. The clustering method was used to assign similar sequences to operational taxonomic units (OTUs) at a 97% similarity threshold. A representative sequence from each OTU was aligned with PyNAST (Caporaso *et al.*, 2010a). A variable number of sequences were obtained per sample. To avoid sampling size effects in the data analysis, sequences were sub-sampled to the same sequence depth (60,000 sequences per sample), otherwise the non-rarefied OTU table was used. Alpha and Beta diversity analyses were calculated. Principal coordinate analysis (PCoA) was done using the subsampled data to measure phylogenetic distance between microbial communities using weighted and unweighted UniFrac distance matrix (Lozupone and Knight, 2005). The unweighted pair group method with arithmetic mean (UPGMA) clustering was conducted to group the communities of different samples with both weighted and unweighted UniFrac distances. To evaluate the robustness of UPGMA clusters and PCoA analysis, Jackknife resampling (100 times) was done.

2.3. Results and Discussion

2.3.1. Effect of naphthalene on bacterial community structure and diversity in the microcosm.

Anoxic sediment microcosms from Fuente de Piedra lagoon were artificially contaminated with naphthalene to study the effect of the PAH on the bacterial community structure and diversity. Unpolluted control cores were included in the analysis. Total DNA from the cores after 4.5 and 12 months incubation was used for Illumina-sequencing of the 16S rRNA gene V3–V4 region. Duplicate cores were analysed for each condition. A total of 794,103 high quality reads (average sequence lengths: 454.27 bp ± 18.27) across the 10 samples were obtained after applying quality filter, chimeras checking, and singletons removal (Table 2. 3). Rarefaction curves (Figure S1) and the Good's sample coverage estimator (Table 2. 3) indicated that the sequencing effort was satisfactory to evaluate microbial diversity in all the samples. The observed OTU number and the Chao diversity and Shannon evenness indices indicated a highly diverse community in the Fuente de Piedra sediments (Table 2. 3). Surprisingly, we observed only a slight decrease of OTU numbers in the naphthalene-spiked microcosms as compared to the unamended controls, indicating only a minor impact of the PAH on the bacterial community (Figure 2. 3). Furthermore, we detected a

Table 2. 3. Comparison of OTU number, diversity, evenness indices and coverage for the different cores samples.

Sample[a]	NS[b]	OTUs[c]	OTUs (60000)[d]	Chao1 (60000)[e]	Shannon	Coverage[f](%)
Core 3-Ini	79762	5448	4903	7287.77	9.52	97.53
Core 4 -Ini	63199	5000	4891	7526.19	9.54	96.86
Core 5-C_1	63877	5074	4922	7161.80	9.48	96.90
Core 6-C_1	71817	5474	5063	7479.57	9.59	97.07
Core 7-C_2	75145	5571	5121	7569.93	9.63	97.26
Core 8-C_2	78177	5658	5093	7437.19	9.59	97.34
Core 9-N_1	89178	5622	4872	7470.69	9.46	97.74
Core 10-N_1	83438	5793	5083	7454.55	9.59	97.38
Core 11-N_2	93886	5986	5051	7565.47	9.57	97.72
Core 12-N_2	95624	5789	4818	7166.42	9.40	97.81

[a] C_1 and C_2 refer to the core control at two different times (4.5 and 12.5 months); N_1 and N_2 refer to the naphthalene core at two different time.
[b]Number of sequences for each library filtered for chimera and singletons.
[c]OTU numbers calculated with all sequences at the 3% distance level.
[d]OTU numbers calculated for a randomized subset of 60,000 reads per sample at the 3% distance level.
[e]Chao index calculated with 1500 subsampled sequences.
[f] Good's sample coverage estimator.

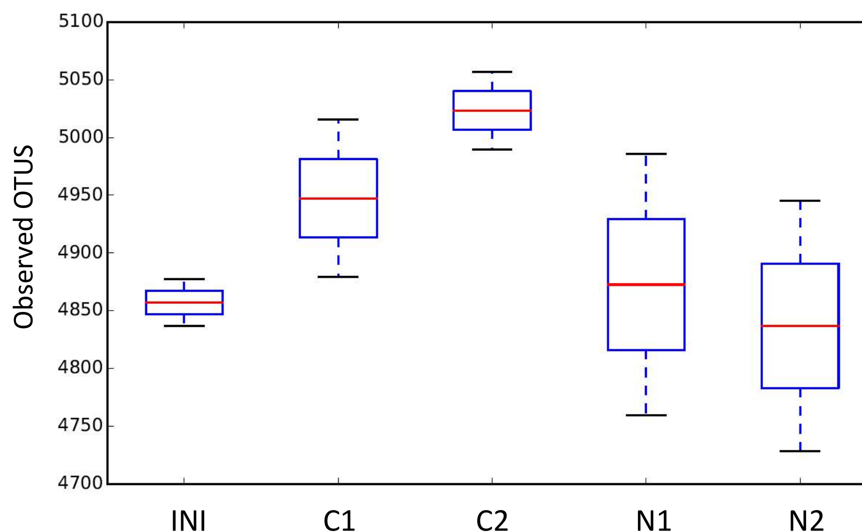

Figure 2. 3. Boxplots of the observed OTUs per randomized sample in the control and naphthalene cores at different incubation times. INI refers to the mean of the control cores 3-4 at time 0; C1 refers to mean of the control cores 5-6 at time 1; C2 refers to mean of the control cores 5-6 at time 2; N1 refers to mean of the naphthalene cores 9-10 at time 1. N2 refers to the mean of the naphthalene cores 11-12 at time 2. Boxplot centre line represents the median. The top and bottom hinges represent 75º and 25º quartiles, respectively.

small increase of microbial diversity in the control microcosms at long incubation times, probably suggesting that the strict anoxic conditions established in the experiment set-up produced a niche increment as found in a previous study in the same ecosystem (Martirani-Von Abercron et al., 2016). We could not observe significant communities' differences with Chao and Shannon index, confirming that naphthalene had a reduced effect on microbial diversity.

To further evaluate the changes in the communities' structure we used PCoA and UPGMA analysis measuring phylogenetic variation via UniFrac distances (Lozupone and Knight, 2005). The weighted PCoA plot evidenced three distinct clusters representing the three sampling times, irrespective of the treatment. However, time could distinguish naphthalene from control samples (Figure 2. 4). We would have expected a more compact clustering of the bacterial community of contaminated microcosms. However, the data suggest that the slight shift in the bacterial community observed might be more related to the time the sediments were exposed to the strict anoxic conditions than to the toxic effects of naphthalene, although the strongest changes were observed after 12 months exposure to naphthalene. So, incubation times, and therefore strictly anoxic conditions, had a more selective pressure on sediment bacterial populations than the contamination.

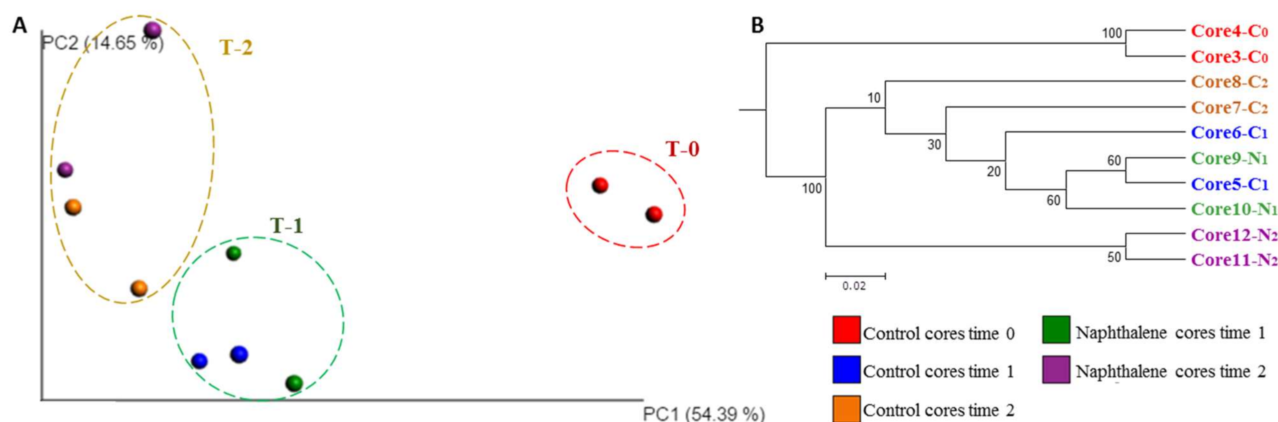

Figure 2. 4. Analysis of microcosm microbial communities with weighted and unweighted UniFrac distance. UniFrac sequences were rarefied at the same sequencing depth (60,000). A) Plot of the weighted Principal coordinate analysis (PCoA), explaining 65.04% of variation. Replicate of the same samples are in the same colour. B) UPGMA clustering of cores samples. The numbers on the nodes indicate bootstrapping values for each node.

To have a more comprehensive understanding of the effect of naphthalene on the composition of the microbial communities, we determined the unweighted UniFrac distances, which are designed to detect differences in the presence/absence of bacterial lineages in different communities. In fact, both quantitative and qualitative measures (weighted/unweighted) of beta-diversity have specific niches in the analysis of microbial communities, and it is recommended to use both approaches to detect the factors that underlie microbial diversity (Lozupone et al., 2007). In the UPGMA tree we found that microcosms grouped in three clusters: 1) initial sediments (time-0); 2) control and naphthalene microcosms (time-1) and control microcosms (time-2); and 3) naphthalene microcosm (time-2). As in the PCoA analysis, control samples at time-0 were distant from all the other samples, indicating that both conditions (control and naphthalene treatment) had a drastic effect on the community structure. Nevertheless, unlike the quantitative analysis, we observed that in this case naphthalene microcosm at time 2 allowed a separate cluster suggesting significant differences in the bacterial composition of the microbial communities.

2.3.2. Microcosms bacterial community structure

The analysis of the Illumina reads grouped the sequences obtained into 66 different phyla, 16 of which belonged to the more abundant phylotypes (>1%). The remaining 50 phylotypes were present at levels below 1% and constituted the “rare” community of this ecosystem. This is an indication of the high diversity of the habitat, likely related to variability of the environmental conditions of the lagoon throughout the seasons, additionally contributed by the microbial

diversity associated to the migrating flamingos. The *Proteobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes* and *Firmicutes* were the most prominent taxa among the sediment samples from all the cores (Figure 2. 5). These five groups accounted for about 69% of all clones in each sediment library, and *Proteobacteria* represented the largest OTU fraction (36%) in all the samples. These results were consistent with those found in sediment and water samples of Tirez lagoon (Spain) (Montoya et al., 2013), but diverged from other characterized athalassohaline systems where the dominant players were members of the *Bacteroidetes*, which in our samples had an average relative abundance of 14.4% (Demergasso et al., 2004; Jiang et al., 2006). Furthermore, the most abundant *Proteobacteria* classes present in extremely alkaline and saline lakes are generally *Gamma* and *Alphaproteobacteria* (Dong et al., 2006; Montoya et al., 2013), whilst in Fuente de Piedra sediment we found a predominance of *Deltaproteobacteria*, which includes a high proportion of SRB. The abundance of this group points to a relevant sulphur cycle within the lagoon, as previously proposed in soda lakes (Sorokin et al., 2011). These findings are in agreement with the high sulphate concentration observed previously in the same ecosystem (Martirani-Von Abercron et al., 2016). *Bacteroidetes* are often found in hypersaline lakes, and in fact, they are one of the most widely distributed and abundant group of bacteria in aquatic systems and their presence in extreme environments is probably due to their capacity to adapt to a wide range of environmental conditions (Kirchman, 2002; Demergasso et al., 2004). *Chloroflexi* was the third most abundant phyla in the anoxic sediment accounting for a total of 9.3%. Member of this group are widely distributed in anaerobic environment for their fermentative activity (Yamada and Sekiguchi, 2009), and their presence in hypersaline environments has already been described (Kambura et al., 2016). Members of *Gemmatimonadetes* accounted for 5.9% of all reads; bacteria belonging to this phylum are frequently observed in soil, but still little is known about their ecology and only a few strains have been isolated and characterized (DeBruyn et al., 2011). Nevertheless, their occurrence in environments with a wide range of nutrient and salinity concentrations and redox states suggests a versatile metabolism (Kim et al., 2012; Yang et al., 2016). Although *Firmicutes* have been described as a predominant phylum along with *Bacteroidetes* in saline and alkaline sediments (Dong et al., 2006; Mesbah et al., 2007; Kim et al., 2012), their relative abundance in our initial samples was about 2.5% (Figure 2. 5).

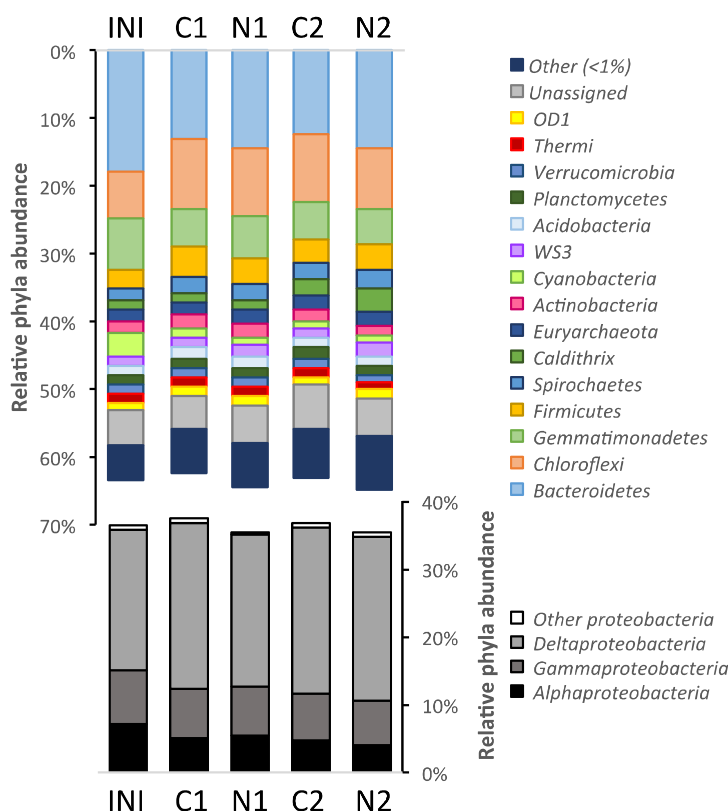

Figure 2. 5. Relative abundance of bacterial phyla or class (only for *Proteobacteria*) in the Fuente de Piedra sediment microcosms. Data are the average of duplicate samples. Labels are as in Figure 2. 3.

As mentioned, a very high number of rare phylotypes have been detected in the anoxic sediment of the lagoon, highlighting the great genetic diversity of this ecosystem. It is remarkable that all microcosms studied harboured very high OTU's numbers (~5500), considering their high pH and salinity and the prevalent anoxic conditions. This observation suggests that extreme environmental conditions are not a limit to microbial richness, but rather hide new opportunities for niche colonization under changing conditions. The great variability of pH and salinity in the athalassohaline lakes throughout the seasons, the high primary productivity rate, and the presence of microhabitats in the sediments could play an important role and explain in part this notable diversity (Lanzen et al., 2013).

2.3.3. Effect of microcosm incubation on bacterial communities

As discussed above, the presence of naphthalene had little or no effect on the overall distribution of the different phyla. To identify specific bacterial groups that may be affected by naphthalene addition we further analysed the relative abundance of the dominant taxa at deeper

taxonomic levels (Figure 2. 6). As mentioned previously, the *Proteobacteria* were the most abundant phyla in all the samples, clearly dominated by the *Deltaproteobacteria*. A general increase of this class was observed in all conditions, which could be ascribed to the cumulative slight increments of the orders *Desulfobacterales*, *Desulfuromonadales* and *Desulfovibrionales*. This, together with the decrease in *Cyanobacteria*, reflected the dark and strict anoxic conditions established in the microcosms, suggesting that sulphate reduction may be one of the most important processes involved in carbon mineralization in Fuente de Piedra lagoon, as described in other alkaline lakes (Scholten et al., 2005; Foti et al., 2007).

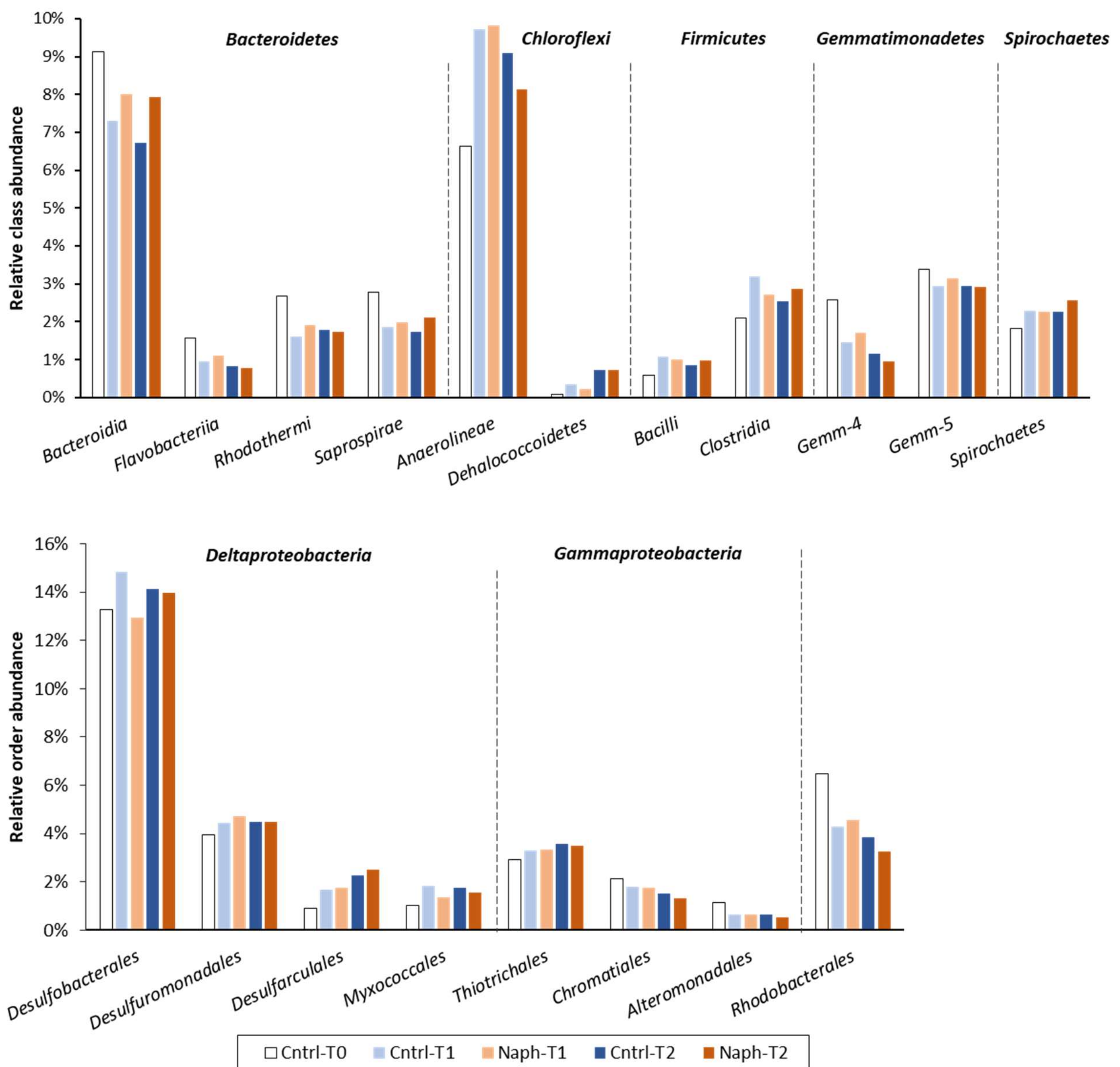

Figure 2. 6. Effect of microcosm incubation on specific bacterial population at order level. Each sample was analysed in duplicate. Labels are as in Figure 2. 3. *Alpha*. is for *Alphaproteobacteria.*

Myxococcales abundance also showed a positive trend during incubation, especially in the non-polluted controls. Members of the *Myxobacteria* are generally found in soil ecosystem, and few recent studies revealed their presence in hypersaline environment (Kambura et al., 2016; Mohr et al., 2016). These organisms have been well studied because of their enormous potential to produce bioactive secondary metabolites, their unique cooperative social behaviour and their ability to survive in nutrient deficient environments (Mohr et al., 2016). *Myxobacteria* have been considered obligate aerobic bacteria (Dawid, 2000), although anaerobic strains have been isolated (Sanford et al., 2002), and their finding in anoxic sediments have been described (Acosta-Gonzalez et al., 2013). Some authors hypothesised the possibility of an ancestral anaerobic lifestyle for this bacterial group (Shimkets and Woese, 1992) given their phylogenetic proximity with the *Deltaproteobacteria*, thus their occurrence in anaerobic environment it is not surprising.

The abundance of gammaproteobacterial sequences related to the *Thiotrichales* and *Chromatiales* is consistent with previous studies (Mesbah et al., 2007). These two groups are common inhabitants of soda lakes and are involved in the oxidation of the sulphide produced by sulphate reducers in the sediment. Most members of the *Chromatiales* are capable of anoxic photosynthesis, whilst *Thiotrichales* are not. This may explain the slight increase in the *Thiotrichales*/*Chromatiales* ratio, since incubations were carried out in the dark. *Rhodobacterales* sequences dominated the *Alpharoteobacteria* in all libraries as would be expected in a hypersaline environment (Sorokin et al., 2014), although we observed a net decrease in the abundance of this order in all the sample, especially after long-term incubation. The phylum *Bacteroidetes* was mainly composed by *Bacteroidia*, *Flavobacteriia*, *Rhodothermi* and *Saprospirae* classes. All these phylotypes showed a tendency to decrease in both microcosm conditions (control and naphthalene), although the decrement of the *Bacteroidia* was less important in the PAH amended cores, which could reflect a greater resistance of these organisms to hydrocarbon contamination, or even an active role in hydrocarbon degradation. The most important family within the *Chloroflexi* was the *Anaerolineae*, which showed a marked increment as compared to the original sediment, especially after four months of incubation; however, no significant correlation with the presence of PAHs could be found. Within the *Firmicutes*, *Bacilli* and *Clostridia* were majority, and both classes increased in all the samples with incubation time. Members of both phylotypes include aerobic and anaerobic oil-degrading strains (Kunapuli et al., 2007; da Cruz et al., 2011).

2.3.4. Bacterial community evolution in naphthalene enrichments

We initiated a series of enrichment cultures starting from sediments collected during the first sampling campaign (enrichments in SMM medium with HMN-dissolved naphthalene and 2MN as carbon source) and from the pre-enriched naphthalene microcosms described above (cores 10 and 12, plus core 5 as unamended control) (enrichments in SMM medium and in filtered Fuente de Piedra lagoon water) with naphthalene provided as crystals; see enrichment set-up scheme in figure 2. 2). The microbial communities in the enrichment cultures at different time points were analysed through massive sequencing of the bacterial V3-V4 16S rRNA gene region using the Illumina-MiSeq platform. After processing and chimera removal, a total of 1,718,618 sequences were obtained from the 16 libraries. An average of 107,414 reads were generated for each sample (Table 2. 4). The sequences were grouped into OTUs at 97% identity. The rarefaction curves reached the asymptote (supplementary Figure S1), suggesting that only a small fraction of the ecosystem biodiversity remained unexplored. Moreover, the Good's coverage estimator reached an average value of 97.92%, indicating the sequencing depth was enough to evaluate bacterial

Table 2. 4. Comparison of OTU number, diversity, evenness indices and coverage for the enrichment culture of the first and second sampling event.

Sample[a]	Sampling campaign	NS[b]	OTUs[c]	OTUs (60000)[d]	Chao1 (60000)[e]	Shannon	Coverage[f] (%)
Fdp-I	1	104864	5695	4549	7029.57	9.15	97.96
FdP-N	1	115640	4746	3582	5820.26	8.19	98.37
FdP-2MN	1	116683	4838	3667	5958.83	8.33	98.39
FdP-HMN	1	102655	4391	3496	5720.78	8.04	98.25
Core5-I	2	127020	6763	4953	7975.76	9.20	97.98
Core5-FPW-C1	2	101583	6123	4970	7584.59	9.61	97.76
Core5-FPW-C2	2	84313	5785	5051	7801.57	9.76	97.34
Core5-SMM-C2	2	94050	5363	4424	7307.70	8.80	97.59
Core10-I	2	114852	6470	4923	7931.54	9.05	97.78
Core10-FPW-N1	2	85036	5616	4853	7807.92	9.18	97.29
Core10-FPW-N2	2	102156	5664	4503	7536.37	8.41	97.66
Core10-SMM-N2	2	110607	5828	4500	7440.79	9.12	97.86
Core12-I	2	117092	6062	4603	7501.59	9.00	97.99
Core12-FPW-N1	2	98989	6112	4942	7933.36	9.36	97.49
Core12-FPW-N2	2	129043	4215	3003	5036.21	6.21	98.64
Core12-SMM-N2	2	114035	4101	3091	5200.54	7.40	98.48

[a] I refer to the initial sample of each enrichment; 1 and 2 refer to the subcultures at different time (0,1 and 2); FPW refer to Fuente de Piedra water used as culture media; SMM refer to SRB artificial see water media.
[b]Number of sequences for each library filtered for chimera and singletons.
[c]OTU numbers calculated with all sequences at the 3% distance level.
[d]OTU numbers calculated for a randomized subset of 60000 reads per sample at the 3% distance level.
[e]Chao index calculated with 60,000 subsampled sequences.
f Good's sample coverage estimator.

diversity in all the samples. The results showed that the initial samples had similar richness index values. In the enrichments from the first sampling campaign we observed a similar decrease of the diversity index in the three cultures (naphthalene, 2MN and HMN), suggesting that culture media had a stronger impact on the microbial diversity than exposure to PAHs. In contrast, the enrichments originating from the sediment microcosms did not show changes in OTU richness and Chao index when only Fuente de Piedra's water was used as culture medium, except for the Core12-FPW-N2 sample. Interestingly, richness indices decreased when and artificial salt medium (SRB-SMM) was used (Table 2. 4). These findings highlight the importance of the choice of culture media and conditions for microbial enrichments, where different factors as pH, salinity and temperature could influence bacterial community diversity.

Naphthalene addition to the cultures resulted in only a slight decrease of the alpha diversity indices of the enrichments from Core-10. In contrast, we found significant variation in the enrichments from Core-12, especially after long time incubation: the Core12-FPW-N2 and Core12-SMM-N2 samples showed the lowest OTU number and Chao index values of all samples. The only difference between the enrichments from both cores was that Core-12 was exposed to naphthalene for a longer time (12 months) as compared to Core-10 (4 months) before starting the enrichments cultures, which was compensated by a shorter incubation time of the enrichment before the first transfer (Figure 2. 2). In fact, Core-12 microcosm was the most affected by naphthalene incubation, showing the lowest richness values (Figure 2. 3). Successive subcultures of this sediment led to a further decrease in OTU number and Chao index.

The analysis of the of PCoA, based on weighted UniFrac distance metrics, of all the enrichments and initial sediment samples provided an overview of the differences between bacterial community structures resulting from the enrichment process (β-diversity). Figure 2. 7A shows that the PCoA plot captured 53.21% of the observed variation between samples. Samples could be grouped in four clusters: all the sediment samples used as starting material for the enrichments clustered together (INI), which suggested similar patterns of community distribution in the sediments collected in the two sampling campaigns and also after core pre-enrichment, as shown previously (Figure 2. 5). The enrichments from the first sampling campaign constituted a separated cluster from all the other samples, suggesting a significantly different community structure of these cultures. Since the initial sediment used in these enrichments was close to the remaining initial samples, this divergence was probably not due to the sampling time, but may rather reflect the culture medium used, and especially the way in which the PAHs were added to

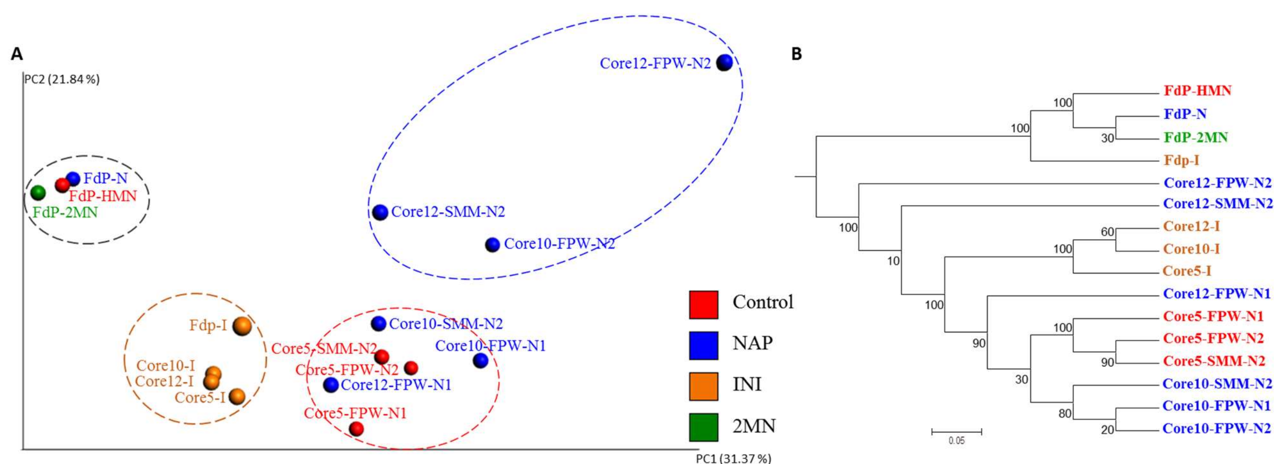

Figure 2. 7. Microbial community analysis of the sediment samples and enrichment cultures using weighted and unweighted UniFrac distance. UniFrac sequences were rarefied at the same sequencing depth (60,000). A) Plot of the weighted Principal Coordinate Analysis (PCoA), explaining 53.21% of variation. Colours indicate the different type of samples: orange, initial sediment samples used for the enrichments; red, control cultures; blue, naphthalene enrichments; green, 2MN enrichments. B) UPGMA clustering of original samples and the different enrichments. The bootstrapping values are indicated in each node.

the cultures (provided dissolved in HMN instead of as crystals). Moreover, these samples had been incubated under strict anoxic culture conditions for the longest periods (27 months overall). However, the community response to the presence of PAHs was similar to that observed in the HMN control.

The third group comprised all the microcosm control cultures (i.e., unamended cultures) together with the naphthalene-amended cultures after the first incubation time. Finally, the longest incubation times sampling of naphthalene microcosm enrichments clustered together. These samples did not form a very compact group, and in fact the Core10-SMM-N2 sample clustered with the control and incubation time 1 samples. Still, a gradual shift from the original samples and control cultures towards the longer incubation times could be observed. The furthermost sample was Core12-FPW-N2, suggesting that naphthalene significantly affected the bacterial community composition and abundance of this culture.

We further analysed β-diversity using the UPGMA clustering method considering unweighted UniFrac distances (thus considering only the OTU presence/absence). Figure 2. 7 shows a slightly different clustering of the samples in the UPGMA tree to the one obtained in the PCoA plot: in this case, the first sampling campaign initial sediment clustered together with the enrichments derived from it. Enrichments were grouped according to the core they originated from, except for

cultures from core 12, which grouped apart from all the other samples, indicating a significantly different bacterial composition both in Fuente de Piedra water and in SMM artificial culture medium.

Altogether these findings evidenced that three major factors structured the microbial communities: composition of the culture media, incubation time and exposure to naphthalene.

2.3.5. Patterns of microbial diversity in the anaerobic enrichments

2.3.5.1. First sampling campaign: enrichments with PAHs dissolved in HMN.

To identify the bacterial taxa responsible for the shifts in bacterial community structure attributable to the PAHs during the enrichment, we investigated the relative abundance of the phylotypes present at the phylum and lower taxonomic levels (Figure 2. 8). *Proteobacteria* was the most abundant phylum in the three cultures from the first campaign. The group was mainly composed by *Deltaproteobacteria*, followed by *Gamma*- *Alpha*- and *Epsilonproteobacteria*. Interestingly, the *Betaproteobacteria* were nearly absent from these sediments. The *Deltaproteobacteria* were dominated by the order *Desulfobacterales*, which is exclusively composed of sulphate-reducing bacteria. A marked decrease of *Gamma*- and *Epsilonproteobacteria* was observed in the three culture conditions as compared to the original sediment, emphasizing the possible media effect on these phylotypes (Figure 2. 8, left). Proteobacterial classes did not show major differences with respect to the control enrichment, except for the *Deltaproteobacteria*, which in the naphthalene culture had a lower relative abundance (19.80%) than in the control HMN culture (27.77%) suggesting a possible toxic effect of the PAH on this group. This result contrasts with the previous finding that strains of the order *Desulfobacterales* were the only pure culture of SRB obtained that anaerobically degraded naphthalene (Galushko et al., 1999). Although *Bacteroidetes* was the second phylum in abundance in the initial sample, as expected in an athalassohaline lake, we observed a remarkable reduction of this phylum in the enrichment cultures. The decrease was even more evident in the PAH enrichments, suggesting a toxic effect on this population. *Chloroflexi* presented the major variation as compared to the original sample, increasing from 5.5% to 25% in the naphthalene enrichment, but no significant differences were observed between the PAH enrichment and the control. As for the microcosms, *Chloroflexi* population was dominated by *Anaerolineae*, fermentative organisms mainly retrieved from anoxic environments and often present in anaerobic culture (Martirani-Von Abercron et al., 2016). The group has been frequently found in activated sludge where they are

associated to sludge granulation. Although *Anaerolineaceae* have been recovered from hydrocarbon-impacted environment (Sherry et al., 2013), in our enrichments they are not likely to be directly linked to hydrocarbons metabolism, but they probably grow on carbohydrates, peptides and/or cells debris (Yamada and Sekiguchi, 2009).

Intriguingly, some phylotypes including *Firmicutes*, *Actinobacteria*, *Thermi* and *Gemmatimonadetes* were enriched, as revealed by the significant increase in relative abundance in the naphthalene and 2MN cultures. *Firmicutes*, in which both *Clostria* and *Bacilli* dominated, and *Actinobacteria* were often associated to hydrocarbon tolerance and/or degradation (Sun et al., 2014; Yang et al., 2014). *Thermus* (*Deinococci*) group increased in all the culture as compared to the original sample. The increase was more important in the naphthalene enrichment, where the relative abundance shifted from 1.4% (FdP-I) to 6.24% (FdP-N). The *Deinococcus*-*Thermus*

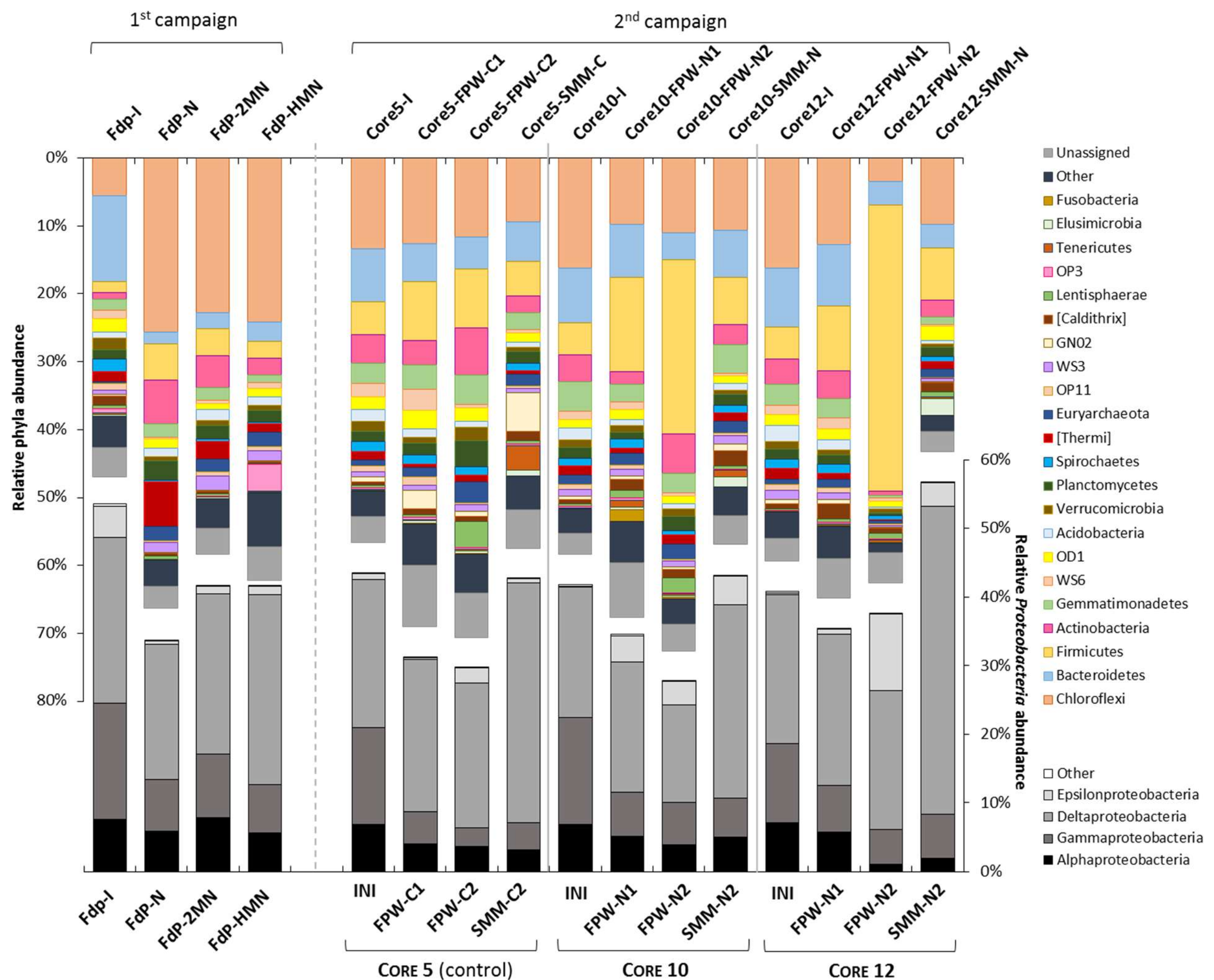

Figure 2. 8. Relative abundance of bacterial phyla or class (only for Proteobacteria) in the Fuente de Piedra enrichments. Data from the first and second sampling campaigns are separated by a doted line.

phylum comprises organisms with a high resistance to radiation, oxidizing agents and desiccating conditions which has made them suitable to be used in the bioremediation of radiation-contaminated sites (Misra et al., 2006; Griffiths and Gupta, 2007; Ho et al., 2016). The greater increase in the PAH cultures, primarily in the naphthalene one, suggests a further interesting ability of this phylotypes such as hydrocarbon resistance. To date, only one study has reported the capacity of this group to transform crude oils (Hao et al., 2004).

2.3.5.2. Second sampling campaign: enrichment with naphthalene provided as crystals.

In the enrichment cultures with the Fuente de Piedra sediments from the microcosms described in 2.3.3 (Figure 2. 2), pure naphthalene crystals were used as sole carbon source (Core10 and Core12) after the two incubation times. A culture initiated from the control microcosm (Core5) was included as control, in which no carbon source was added. Naphthalene and control enrichments were prepared in filtered FdP water. The use of FdP water as culture medium was designed to promote the growth of natural and unexplored microbial populations. In fact, athalassoaline lakes are ecosystems characterized by specific ranges of salinity, pH and nutrient which are difficult to reproduce in the laboratory, so that artificial media could select bacterial communities different from those naturally dominant in the habitat (Connon and Giovannoni, 2002). After the first incubation period, parallel subcultures in SRB SMM medium were included in the experiment (Figure 2. 2).

Although the relative abundance of the main taxa varied significantly under the different treatments, five phyla including *Proteobacteria* (27.8%-56.7%), *Chloroflexi* (3.4%-16.2%), *Bacteroidetes* (3.3%-9.0%), *Firmicutes* (4.6%-42.1%) and *Actinobacteria* (0.6%-6.9%) were the most abundant, accounting for a total of 61.7%-87.2% of the whole community (Figure 2. 8, right). These phylotypes are considered the main bacterial inhabitants of hypersaline environments (Mesbah et al., 2007; Sorokin et al., 2014).

In our enrichments, the *Proteobacteria* phyla showed a tendency to decrease in the cultures with FdP lagoon water (FdPW-enrichments) and to increase in the cultures with SMM, reaching 56.7% of the community in the Core12-SMM-N2 sample. *Alpha* and especially *Gammaproteobacteria* decreased in all enrichments. The *Deltaproteobacteria* class did not show significant changes during the enrichment in FdP water, but we observed that the SMM medium clearly enriched this group in both control and naphthalene amended cultures, although the increase was especially remarkable in Core12-SMM-N2 sample, where *Deltaproteobacteria* reached up to 45% of the community. The SMM medium is specifically designed for the

cultivation of SRB, and has been previously used to isolate pure cultures of *Deltaproteobacteria* able to degrade naphthalene (Galushko et al., 1999). The increment of members of this class was especially notable for the order of *Desulfobacterales*, a sulfate-reducing group commonly found in soda lake (Scholten et al., 2005). Within this order, the abundance of an OTU affiliated to the *Desulfobulbaceae* significantly increased in all the cultures prepared in SMM medium, although especially in Core12-SMM-N2 sample, where the values were 10 times higher (21% of the community) than in the initial sample (as compared to the increase in the control Core5-SMM-C2 sample, where it reached 11% of the community) (Table S1). Members of the *Desulfobulbaceae* have been previously described as toluene and naphthalene degraders under sulfate-reducing condition (Muller et al., 2009; Pilloni et al., 2011; Kümmel et al., 2015) suggesting that this taxon could be directly involved in naphthalene degradation. In the Core 12 samples, the *Desulfovibrionales* was a second *Deltaproteobacteria* class positively influenced by the presence of naphthalene, increasing from values below 0.01% in the initial core sample to 3 and 8% of the community after 12 months incubation in FdP water and SMM medium, respectively. Two OTUs belonging to the *Desulfomicrobiaceae* were mainly responsible for the increment. Surprisingly the *Epsilonproteobacteria* were also favoured in naphthalene enrichments, especially in the Core12-FPW-N2 sample, where it increased from 0.17% in the initial Core sample to 11.2%. This was essentially due to an unclassified OTU belonging to the *Campylobacteriaceae* that dominated the *Campylobacteriales* class in the naphthalene-amended cultures. Sequence reads belonging to the *Campylobacteriales* have been retrieved from municipal wastewater (Ruiz et al., 2014), in reactors with multiple electron acceptors under sulfur-cycling conditions (Zhao et al., 2013) and recently they have been associated to higher concentrations of PAHs in soil (Ma et al., 2016). Unfortunately, little is known about the possible function of the members of this class detected in environmental samples.

Firmicutes was the phylum that showed the most dramatic changes directly related to the presence of naphthalene in the enrichments. The phylotype was enriched in the naphthalene cultures with FdP water as culture medium in both Core10 (FPW-N1: 13.8%; FPW-N2: 25.5%) and Core12 (FPW-N1: 9.6%; FPW-N2: 42.16%) cultures, whilst the relative abundance in the control culture in the same medium remained around 8% in both subcultures. Members of *Bacilli* and *Clostridia* classes were the principal component of the *Firmicutes* in all the enrichments, although the *Clostridia* showed the major increases. Particularly, the genus *Dethiosulfatibacter* was strongly selected for in FdP water cultures with naphthalene, reaching a relative abundance of 18% in Core10-FPW-N2 and Core12-FPW-N2 (its abundance was below 0.1% in the initial core samples, and remaining at this level in the unamended cultures from the control Core5).

Dethiosulfatibacter aminovorans is the only cultivated strain of this genus and was first isolated from coastal marine sediments of the eutrophic Tokyo Bay, Japan (Takii et al., 2007). The strain is a halophilic, thiosulfate-reducing, strict anaerobe. Previous studies suggested that *Dethiosulfatibacter* was among the main players in PAH degradation in mangrove sediments (Muangchinda et al., 2013). This strain has also been detected in crude oil microbial communities, although at very low abundance (Cai et al., 2015). The strong enrichment of *Dethiosulfatibacter* in our naphthalene enrichments is remarkable and could likely be associated to the anaerobic degradation of naphthalene. Interestingly two other members of *Clostridiales*, *Halanaerobiaceae* and *Acidaminobacteraceae*, were strongly enriched with naphthalene, although in this case only in the Core12-FPW-N2 culture. Unclassified members of the *Halanaerobiaceae* family accounted for the 11.2% of the whole community. Representatives of this family are obligate halophilic anaerobes with a fermentative metabolism, and have been found and isolated from a wide variety of anaerobic hypersaline environments (Kivisto and Karp, 2011). They have also been reported to reduce nitro-substituted aromatic compounds to the corresponding amine, although they were not incorporated by the cells (Oren et al., 1991). We have repeatedly observed the selection of fermentative organisms in the presence of PAHs, which we generally attribute to PAH toxicity resulting in cell lysis, the debris of which could be used as substrate by fermenters that are resistant to the PAHs. The relative abundance of *Acidaminobacteraceae* (mainly represented by the uncultured genus NP25) in the enrichments from Core-12 increased progressively from 0.2% in the original sample up to 10.8% in Core12-FPW-N2 culture. Unfortunately, very little is known about this group. They have been detected in oil-amended salt-marsh sediment microcosms, where their belonged to the so-called “late-responders” to crude oil. The relative abundance of unclassified *Acidaminobacteraceae* in these conditions only increased three weeks after oil treatment, and further increased with the incubation time (Koo et al., 2015). They have also been detected in biogas digesters (Müller et al., 2016). It is thus difficult to predict their ecological role, although the strong increase of this group in the naphthalene amended Core-12 enrichments in FdP water medium suggests a possible role in anaerobic naphthalene degradation. A similar increase was not observed in the parallel enrichments from Core-10, probably because of the almost undetectable levels of this genus in the initial sample (below 0.1%). Interestingly, this increase was not observed in the enrichment in SMM medium, which started from the 4 months enrichment in FdP water, where the *Acidaminobacteraceae* NP25 community had already reached 2.2% of the community. Additional 8 months incubation in SMM medium reduced the abundance of this genus back to levels similar to Core-12 initial values. This is a further example of the dramatic influence of the medium composition on the community structure evolution, and of the importance to use

media as close to natural conditions as possible, as it is the case of the FdP water used here. In fact, often the change from natural environment to artificial media could de-structure the community in such a way as to render its degradative capacities less efficient, or support bacteria that do not have these abilities.

2.4. Concluding remarks

Most ecological studies on hypersaline ecosystems to date have focused on the water bodies. Therefore, the data about bacterial communities present in hypersaline sediments provides relevant information as to the community structures in these ecosystems. Overall, we observed remarkable levels of diversity, which were amongst the highest described for microbial communities and in the range of marine and freshwater sediments (Wang et al., 2012). The structural complexity of the soil and sediment matrix favours spatial resource partitioning, thus creating a variety of microhabitats. This provides a distinct mechanism for shaping diversity that differs essentially from the situation in aqueous environments (Torsvik et al., 2002), which probably contributes to the high diversity observed in this ecosystem. In fact, the differences in sample clustering observed when comparing weighted and unweighted community analysis suggest an important contribution of the so-called “rare” community to the overall diversity. Rare taxa are considered as “seed bank” organisms which could become activated upon changes in the environmental conditions (Pedrós-Alió, 2006).

In our microcosm experiments, naphthalene treatment did not produce a clear shift of the bacterial community. It must be noted that the communities were dominated by *Proteobacteria*, *Bacteroidetes, Firmicutes* and *Chloroflexi*, taxa of which members are capable of metabolizing or tolerant to complex hydrocarbons (An *et al.*, 2013; Sherry *et al.*, 2013; Yang *et al.*, 2014). The resulting bacterial structure might be more dependent on the strict anoxic conditions imposed in the microcosms. Still, the changes after 12 months of anoxic incubation were small, and probably reflect the anoxic nature of these sediments below the upper oxic layer, which allowed the establishment of a community already adapted to strict anoxic conditions. It is worth noting that FdP sediment already had a basal level of hydrocarbons contamination (aliphatic and aromatics) (Martirani-Von Abercron *et al.*, 2016), which could explain why further addition of PAHs may not have had the expected toxic effect, especially if the community structure is resilient.

In contrast, enrichment cultures showed that the presence of PAHs induced a clear shift in the bacterial community structure. Members of *Firmicutes*, *Actinobacteria* and *Thermi* increased in

the naphthalene and 2MN cultures initiated after the first sampling campaign and set up with the PAHs dissolved in HMN, supporting previous studies that identified these organisms as related to PAH degradation. More interesting were the changes produced by naphthalene in the enrichments initiated from microcosms that had been pre-treated with naphthalene. We found that the cultivation-dependent approach enriched a few specific populations that might be involved in the aromatic biodegradation process. Specifically, members of *Desulfobulbaceae*, detected in the SMM medium, and members of *Dethiosulfatibacter*, *Halanaerobiaceae* and *Acidaminobacteraceae*, in the FdP water, were clearly selected for in the presence of naphthalene, reaching important relative abundances. The actual capacity of these strain to degrade PAHs deserve further analysis. Finally, we verified that natural media, such as FdP water, could be the best option to enrich bacteria potentially useful in bioremediation.

2.5. References

Acosta-González, A., Rosselló-Mora, R., and Marqués, S. (2013). Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the *Prestige* oil spill. *Environ Microbiol* **15**: 77-92.

Adrian, R., O'reilly, C.M., Zagarese, H., Baines, S.B., Hessen, D.O., Keller, W., Livingstone, D.M., Sommaruga, R., Straile, D., Van Donk, E., Weyhenmeyer, G.A., and Winder, M. (2009). Lakes as sentinels of climate change. *Limnol Oceanogr* **54**: 2283-2297.

Aguirre-Garrido, J.F., Ramirez-Saad, H.C., Toro, N., and Martinez-Abarca, F. (2016). Bacterial Diversity in the Soda Saline Crater Lake from Isabel Island, Mexico. *Microb Ecol* **71**: 68-77.

An, D., Brown, D., Chatterjee, I., Dong, X., Ramos-Padron, E., Wilson, S., Bordenave, S., Caffrey, S.M., Gieg, L.M., Sensen, C.W., and Voordouw, G. (2013). Microbial community and potential functional gene diversity involved in anaerobic hydrocarbon degradation and methanogenesis in an oil sands tailings pond. *Genome* **56**: 612-618.

Anderson, N.J. (2007). The effect of evapoconcentration on dissolved organic carbon concentration and quality in lakes of SW Greenland. *Freshwat Biol* **v. 52**: pp. 280-289-2007 v.2052 no.2002.

Brune, A., Frenzel, P., and Cypionka, H. (2000). Life at the oxic-anoxic interface: microbial activities and adaptations. *FEMS Microbiol Rev* **24**: 691-710.

Cai, M., Nie, Y., Chi, C.-Q., Tang, Y.-Q., Li, Y., Wang, X.-B., Liu, Z.-S., Yang, Y., Zhou, J., and Wu, X.-L. (2015). Crude oil as a microbial seed bank with unexpected functional potentials. *Sci Rep* **5**: 16057.

Caporaso, J.G., Bittinger, K., Bushman, F.D., Desantis, T.Z., Andersen, G.L., and Knight, R. (2010). PyNAST: a flexible tool for aligning sequences to a template alignment. *Bioinformatics* **26**: 266-267.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Cerniglia, C.E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**: 351-368.

Connon, S.A., and Giovannoni, S.J. (2002). High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microbiol* **68**: 3878-3885.

Da Cruz, G.F., De Vasconcellos, S.P., Angolini, C.F.F., Dellagnezze, B.M., Garcia, I.N.S., De Oliveira, V.M., Dos Santos Neto, E.V., and Marsaioli, A.J. (2011). Could petroleum biodegradation be a

joint achievement of aerobic and anaerobic microrganisms in deep sea reservoirs? *AMB Express* **1**: 47-47.

Dawid, W. (2000). Biology and global distribution of myxobacteria in soils. *FEMS Microbiol Rev* **24**: 403-427.

Debruyn, J.M., Nixon, L.T., Fawaz, M.N., Johnson, A.M., and Radosevich, M. (2011). Global Biogeography and Quantitative Seasonal Dynamics of *Gemmatimonadetes* in Soil. *Appl Environ Microbiol* **77**: 6295-6300.

Demergasso, C., Casamayor, E.O., Chong, G., Galleguillos, P., Escudero, L., and Pedros-Alio, C. (2004). Distribution of prokaryotic genetic diversity in athalassohaline lakes of the Atacama Desert, Northern Chile. *FEMS Microbiol Ecol* **48**: 57-69.

Dong, H., Zhang, G., Jiang, H., Yu, B., Chapman, L.R., Lucas, C.R., and Fields, M.W. (2006). Microbial Diversity in Sediments of Saline Qinghai Lake, China: Linking Geochemical Controls to Microbial Ecology. *Microb Ecol* **51**: 65-82.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**: 2194-2200.

Eiler, A., Farnleitner, A.H., Zechmeister, T.C., Herzig, A., Hurban, C., Wesner, W., Krachler, R., Velimirov, B., and Kirschner, A.K. (2003). Factors controlling extremely productive heterotrophic bacterial communities in shallow soda pools. *Microb Ecol* **46**: 43-54.

Foght, J. (2008). Anaerobic biodegradation of aromatic hydrocarbons: Pathways and prospects. *J. Mol. Microbiol. Biotech.* **15**: 93-120.

Foti, M., Sorokin, D.Y., Lomans, B., Mussman, M., Zacharova, E.E., Pimenov, N.V., Kuenen, J.G., and Muyzer, G. (2007). Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Appl Environ Microbiol* **73**: 2093-2100.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* **1**: 415-420.

García, C.M., García-Ruiz, R., Rendón, M., Niell, F.X., and Lucena, J. (1997). Hydrological cycle and interannual variability of the aquatic community in a temporary saline lake (Fuente de Piedra, Southern Spain). *Hydrobiologia* **345**: 131-141.

Ghosal, D., Ghosh, S., Dutta, T.K., and Ahn, Y. (2016). Current State of Knowledge in Microbial Degradation of Polycyclic Aromatic Hydrocarbons (PAHs): A Review. *Front Microbiol* **7**: 1369.

Griffiths, E., and Gupta, R.S. (2007). Identification of signature proteins that are distinctive of the *Deinococcus-Thermus* phylum. *Int Microbiol* **10**: 201-208.

Hao, R., Lu, A., and Wang, G. (2004). Crude-oil-degrading thermophilic bacterium isolated from an oil field. *Can J Microbiol* **50**: 175-182.

Heredia, J., García De Domingo, A., Ruiz, J.M., and Araguás, L. (2010). Fuente de piedra lagoon (Málaga, Spain): A deep karstic flow discharge point of a regional hydrogeological system. *Environ Earth Sci*: 231-236.

Ho, J., Adeolu, M., Khadka, B., and Gupta, R.S. (2016). Identification of distinctive molecular traits that are characteristic of the phylum "*Deinococcus-Thermus*" and distinguish its main constituent groups. *Syst Appl Microbiol* **39**: 453-463.

Jiang, H., Dong, H., Zhang, G., Yu, B., Chapman, L.R., and Fields, M.W. (2006). Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. *Appl Environ Microbiol* **72**: 3832-3845.

Kambura, A.K., Mwirichia, R.K., Kasili, R.W., Karanja, E.N., Makonde, H.M., and Boga, H.I. (2016). Bacteria and Archaea diversity within the hot springs of Lake Magadi and Little Magadi in Kenya. *BMC Microbiol* **16**: 136.

Kim, J.-S., Makama, M., Petito, J., Park, N.-H., Cohan, F.M., and Dungan, R.S. (2012). Diversity of Bacteria and Archaea in hypersaline sediment from Death Valley National Park, California. *MicrobiologyOpen* **1**: 135-148.

Kirchman, D.L. (2002). The ecology of *Cytophaga-Flavobacteria* in aquatic environments. *FEMS Microbiol Ecol* **39**: 91-100.

Kivisto, A.T., and Karp, M.T. (2011). Halophilic anaerobic fermentative bacteria. *J Biotechnol* **152**: 114-124.

Koo, H., Mojib, N., Huang, J.P., Donahoe, R.J., and Bej, A.K. (2015). Bacterial community shift in the coastal Gulf of Mexico salt-marsh sediment microcosm in vitro following exposure to the Mississippi Canyon Block 252 oil (MC252). *3 Biotech* **5**: 379-392.

Kummel, S., Herbst, F.A., Bahr, A., Duarte, M., Pieper, D.H., Jehmlich, N., Seifert, J., Von Bergen, M., Bombach, P., Richnow, H.H., and Vogt, C. (2015). Anaerobic naphthalene degradation by sulfate-reducing *Desulfobacteraceae* from various anoxic aquifers. *FEMS Microbiol Ecol* **91**.

Kunapuli, U., Lueders, T., and Meckenstock, R.U. (2007). The use of stable isotope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J* **1**: 643-653.

Lanzen, A., Simachew, A., Gessesse, A., Chmolowska, D., Jonassen, I., and Ovreas, L. (2013). Surprising prokaryotic and eukaryotic diversity, community structure and biogeography of Ethiopian soda lakes. *PLoS One* **8**: e72577.

Le Borgne, S., Paniagua, D., and Vazquez-Duhalt, R. (2008). Biodegradation of organic pollutants by halophilic bacteria and archaea. *J Mol Microbiol Biotechnol* **15**: 74-92.

Lefebvre, O., and Moletta, R. (2006). Treatment of organic pollution in industrial saline wastewater: A literature review. *Water Res* **40**: 3671-3682.

Lizama, C., Monteoliva-Sanchez, M., Prado, B., Ramos-Cormenzana, A., Weckesser, J., and Campos, V. (2001). Taxonomic study of extreme halophilic archaea isolated from the "Salar de Atacama", Chile. *Syst Appl Microbiol* **24**: 464-474.

Lozupone, C., and Knight, R. (2005). UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Appl Environ Microbiol* **71**: 8228-8235.

Lozupone, C.A., Hamady, M., Kelley, S.T., and Knight, R. (2007). Quantitative and Qualitative β Diversity Measures Lead to Different Insights into Factors That Structure Microbial Communities. *Appl Environ Microbiol* **73**: 1576-1585.

Ma, J., Zhang, W., Chen, Y., Zhang, S., Feng, Q., Hou, H., and Chen, F. (2016). Spatial Variability of PAHs and Microbial Community Structure in Surrounding Surficial Soil of Coal-Fired Power Plants in Xuzhou, China. *Int J Env Res Public Health* **13**: 878.

Marhuenda-Egea, F.C., and Bonete, M.J. (2002). Extreme halophilic enzymes in organic solvents. *Curr Opin Biotechnol* **13**: 385-389.

Martirani-Von Abercron, S.-M., Pacheco, D., Benito-Santano, P., Marín, P., and Marqués, S. (2016). Polycyclic Aromatic Hydrocarbon-Induced Changes in Bacterial Community Structure under Anoxic Nitrate Reducing Conditions. *Front Microbiol* **7**: 1775.

Meckenstock, R., Safinowski, M., and Griebler, C. (2004). Anaerobic degradation of polycyclic aromatic hydrocarbons. *FEMS Microbiol Ecol* **29**: 27-36.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**: 2743-2747.

Mesbah, N.M., Abou-El-Ela, S.H., and Wiegel, J. (2007). Novel and unexpected prokaryotic diversity in water and sediments of the alkaline, hypersaline lakes of the Wadi An Natrun, Egypt. *Microb Ecol* **54**: 598-617.

Misra, H.S., Khairnar, N.P., Kota, S., Shrivastava, S., Joshi, V.P., and Apte, S.K. (2006). An exonuclease I-sensitive DNA repair pathway in *Deinococcus radiodurans*: a major determinant of radiation resistance. *Mol Microbiol* **59**: 1308-1316.

Mohr, K.I., Stechling, M., Wink, J., Wilharm, E., and Stadler, M. (2016). Comparison of myxobacterial diversity and evaluation of isolation success in two niches: Kiritimati Island and German compost. *MicrobiologyOpen* **5**: 268-278.

Montoya, L., Vizioli, C., Rodriguez, N., Rastoll, M.J., Amils, R., and Marin, I. (2013). Microbial community composition of Tirez lagoon (Spain), a highly sulfated athalassohaline environment. *Aquat Biosyst* **9**: 19.

Muangchinda, C., Pansri, R., Wongwongsee, W., and Pinyakong, O. (2013). Assessment of polycyclic aromatic hydrocarbon biodegradation potential in mangrove sediment from Don Hoi Lot, Samut Songkram Province, Thailand. *J Appl Microbiol* **114**: 1311-1324.

Müller, B., Sun, L., Westerholm, M., and Schnürer, A. (2016). Bacterial community composition and fhs profiles of low- and high-ammonia biogas digesters reveal novel syntrophic acetate-oxidising bacteria. *Biotechnol Biofuels* **9**: 48.

Muller, S., Vogt, C., Laube, M., Harms, H., and Kleinsteuber, S. (2009). Community dynamics within a bacterial consortium during growth on toluene under sulfate-reducing conditions. *FEMS Microbiol Ecol* **70**: 586-596.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., and Rabus, R. (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ Microbiol* **11**: 209-219.

Newton, R.J., Jones, S.E., Eiler, A., Mcmahon, K.D., and Bertilsson, S. (2011). A guide to the natural history of freshwater lake bacteria. *Microbiol Mol Biol Rev* **75**: 14-49.

Oren, A. (2010). Industrial and environmental applications of halophilic microorganisms. *Environ Technol* **31**: 825-834.

Oren, A., Gurevich, P., and Henis, Y. (1991). Reduction of nitrosubstituted aromatic compounds by the halophilic anaerobic eubacteria *Haloanaerobium praevalens* and *Sporohalobacter marismortui*. *Appl Environ Microbiol* **57**: 3367-3370.

Ortega-Retuerta, E., Pulido-Villena, E., and Reche, I. (2007). Effects of Dissolved Organic Matter Photoproducts and Mineral Nutrient Supply on Bacterial Growth in Mediterranean Inland Waters. *Microb Ecol* **54**: 161-169.

Pedrós-Alió, C. (2006). Marine microbial diversity: can it be determined? *Trends Microbiol* **14**: 257-263.

Pilloni, G., Von Netzer, F., Engel, M., and Lueders, T. (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. *FEMS Microbiol Ecol* **78**: 165-175.

Ruiz, V., Ilhan, Z.E., Kang, D.-W., Krajmalnik-Brown, R., and Buitrón, G. (2014). The source of inoculum plays a defining role in the development of MEC microbial consortia fed with acetic and propionic acid mixtures. *J Biotechnol* **182–183**: 11-18.

Sanford, R.A., Cole, J.R., and Tiedje, J.M. (2002). Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. *Appl Environ Microbiol* **68**: 893-900.

Scholten, J.C., Joye, S.B., Hollibaugh, J.T., and Murrell, J.C. (2005). Molecular analysis of the sulfate reducing and archaeal community in a meromictic soda lake (Mono Lake, California) by targeting 16S rRNA, *mcrA*, *apsA*, and *dsrAB* genes. *Microb Ecol* **50**: 29-39.

Sherry, A., Gray, N.D., Ditchfield, A.K., Aitken, C.M., Jones, D.M., Röling, W.F.M., Hallmann, C., Larter, S.R., Bowler, B.F.J., and Head, I.M. (2013). Anaerobic biodegradation of crude oil under sulphate-reducing conditions leads to only modest enrichment of recognized sulphate-reducing taxa. *Int Biodeterior Biodegrad* **81**: 105-113.

Shimkets, L., and Woese, C.R. (1992). A phylogenetic analysis of the myxobacteria: basis for their classification. *Proc Natl Acad Sci USA* **89**: 9459-9463.

Sorokin, D.Y., Berben, T., Melton, E.D., Overmars, L., Vavourakis, C.D., and Muyzer, G. (2014). Microbial diversity and biogeochemical cycling in soda lakes. *Extremophiles* **18**: 791-809.

Sorokin, D.Y., Gorlenko, V.M., Namsaraev, B.B., Namsaraev, Z.B., Lysenko, A.M., Eshinimaev, B.T., Khmelenina, V.N., Trotsenko, Y.A., and Kuenen, J.G. (2004). Prokaryotic communities of the north-eastern Mongolian soda lakes. *Hydrobiologia* **522**: 235-248.

Sorokin, D.Y., Kuenen, J.G., and Muyzer, G. (2011). The microbial sulfur cycle at extremely haloalkaline conditions of soda lakes. *Front Microbiol* **2**: 44.

Sun, G.-D., Xu, Y., Liu, Y., and Liu, Z.-P. (2014). Microbial community dynamics of soil mesocosms using Orychophragmus violaceus combined with *Rhodococcus* ruber Em1 for bioremediation of highly PAH-contaminated soil. *Appl Microbiol Biotechnol* **98**: 10243-10253.

Takii, S., Hanada, S., Tamaki, H., Ueno, Y., Sekiguchi, Y., Ibe, A., and Matsuura, K. (2007). *Dethiosulfatibacter aminovorans* gen. nov., sp. nov., a novel thiosulfate-reducing bacterium isolated from coastal marine sediment via sulfate-reducing enrichment with Casamino acids. *Int J Syst Evol Microbiol* **57**: 2320-2326.

Torsvik, V., Ovreas, L., and Thingstad, T.F. (2002). Prokaryotic diversity-magnitude, dynamics, and controlling factors. *Science* **296**: 1064-1066.

Wang, Y., Sheng, H.F., He, Y., Wu, J.Y., Jiang, Y.X., Tam, N.F., and Zhou, H.W. (2012). Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl Environ Microbiol* **78**: 8264-8271.

Wetzel, R.G. (2001). "Rivers and lakes — Their distribution, origins, and forms," in *Limnology (Third Edition)*. (San Diego: Academic Press), 15-42.

Widdel, F., and Bak, F. (1992). "Gram-Negative Mesophilic Sulfate-Reducing Bacteria," in *The Prokaryotes: A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification,*

Applications, eds. A. Balows, H.G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. (New York, NY: Springer New York), 3352-3378.

Widdel, F., Kohring, G.-W., and Mayer, F. (1983). Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. *Arch Microbiol* **134**: 286-294.

Yamada, T., and Sekiguchi, Y. (2009). Cultivation of uncultured *Chloroflexi* subphyla: significance and ecophysiology of formerly uncultured chloroflexi 'subphylum i' with natural and biotechnological relevance. *Microbes Environ* **24**: 205-216.

Yang, J., Ma, L.A., Jiang, H., Wu, G., and Dong, H. (2016). Salinity shapes microbial diversity and community structure in surface sediments of the Qinghai-Tibetan Lakes. *Sci Rep* **6**: 25078.

Yang, S., Wen, X., Zhao, L., Shi, Y., and Jin, H. (2014). Crude Oil Treatment Leads to Shift of Bacterial Communities in Soils from the Deep Active Layer and Upper Permafrost along the China-Russia Crude Oil Pipeline Route. *PLoS ONE* **9**: e96552.

Zhao, H.-P., Ilhan, Z.E., Ontiveros-Valencia, A., Tang, Y., Rittmann, B.E., and Krajmalnik-Brown, R. (2013). Effects of Multiple Electron Acceptors on Microbial Interactions in a Hydrogen-Based Biofilm. *Environ Sci Technol* **47**: 7396-7403.

Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996). DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**: 316-322.

2.6. Supplementary material

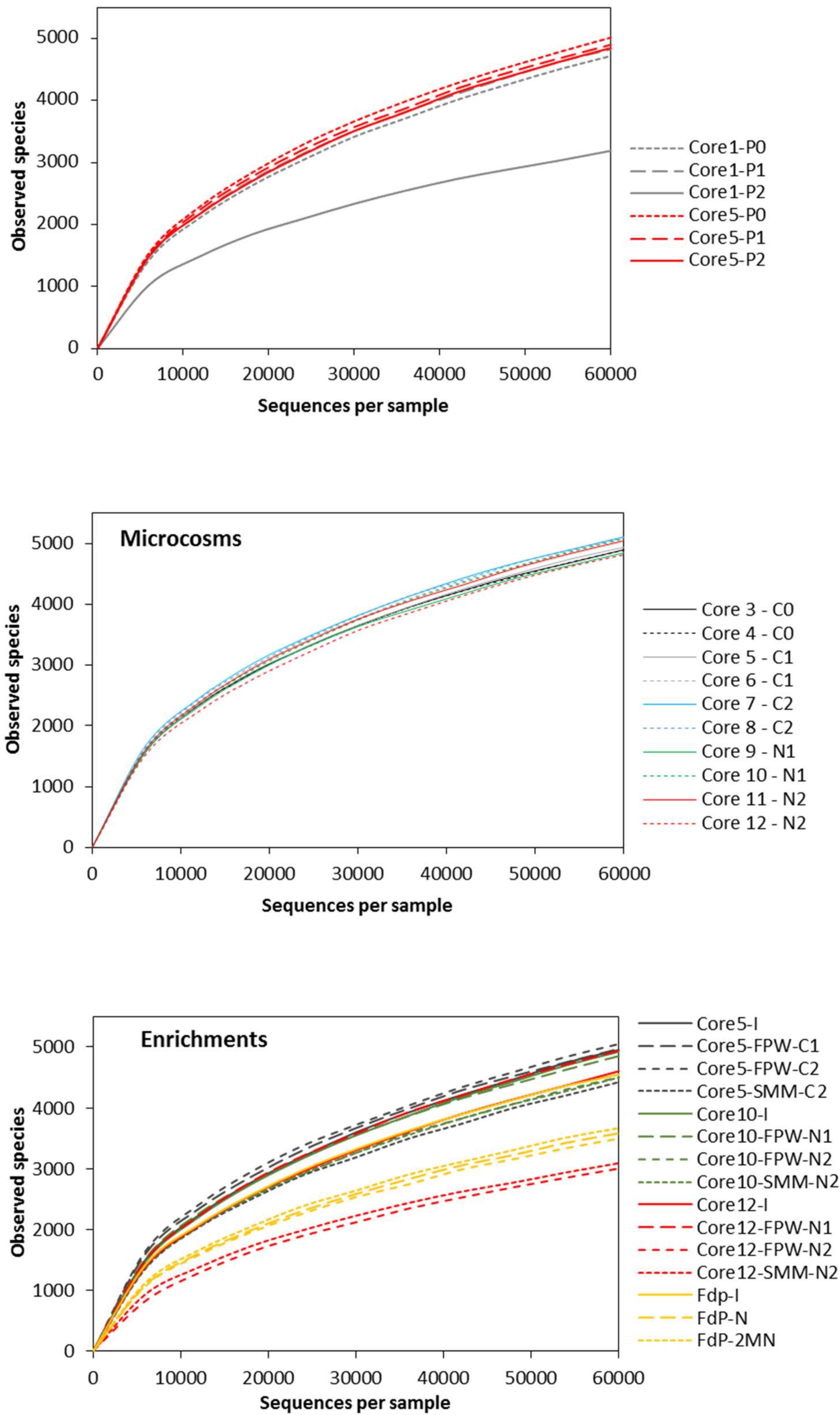

Figure S1. Rarefaction curves at a phylogenetic distance of 0.03 for partial (V3-V4) 16S rRNA genes of microbial populations of the different samples analysed in this study.

CHAPTER 3:

Naphthalene biodegradation under oxygen limiting conditions: community dynamics and the relevance of biofilm formation capacity

This chapter was published as:

Martirani-Von Abercron SM, Marín P, Solsona-Ferraz M, Castañeda-Cataña MA & Marqués S.
Naphthalene biodegradation under oxygen-limiting conditions: community dynamics and the relevance of biofilm formation capacity.
Microb Biotechnol, 2017 DOI: 10.1111/1751-7915.12842

Anyone who has never made a mistake
has never tried anything new.

Albert Einstein

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are toxic compounds of particular environmental concern because of their marked stability and resistance to degradation. They are released into the environment either from natural sources or as a result of industrial activities. The simplest PAH naphthalene is included among the 16 PAHs considered to be priority pollutants by the US EPA. International efforts to improve remediation strategies for hydrocarbon polluted sites focus on biological processes mediated by bacteria. Bacteria have developed a striking adaptive capacity for degrading natural and synthetic aromatics to CO_2 and water, which is the basis for the use of bioremediation approaches to clean aromatics polluted areas.

Bacteria able to degrade PAHs under strict aerobic conditions are widespread and have been widely described. The biodegradation pathway involves the deeply characterized aromatic mono- and dioxygenases for ring activation (hydroxylation) and ring cleavage (Vaillancourt *et al.*, 2006; Ullrich and Hofrichter, 2007). The genes and gene organization of PAH degradation operons are reasonably conserved among PAH degrading isolates, especially among the *Pseudomonads*, which are predominant in the databases of PAH degraders. However, the availability of oxygen is often limited in natural environments because oxygen can be rapidly consumed in the aerobic biodegradation processes, resulting in a decreasing oxygen concentration gradient whereupon reduction of other electron acceptors such as nitrate, iron or sulphate becomes energetically favourable (Rivett *et al.*, 2008b). A similar pattern is observed in habitats that are under permanent anoxic conditions, such as flooded sediments in marine and fresh-water environments (Brune *et al.*, 2000). Because nitrate respiration is not a strictly anoxic process, aerobic and nitrate reducing metabolisms generally coexist at the boundaries of polluted sites, where microaerophilic conditions are generally found (Wilson and Bouwer, 1997).

Under strict anoxic conditions PAH biodegradation has only been reliably proved in sulphate-reducing bacteria (SRB), although only one *Deltaproteobacteria* strain and a two-strain consortium able to degrade naphthalene have been isolated and characterized to date (Meckenstock *et al.*, 2016). Anaerobes use a reductive strategy to overcome the stability of the aromatic ring. Naphthalene must be converted to 2-naphtoic acid (NA) (Zhang and Young, 1997) previous to its activation to naphthoyl-CoA, which is then dearomatized, starting from the non-activated ring, by the activity of two specific reductases: a naphthoyl-CoA reductase (*ncr* gene) followed by a 5,6-dihydro-2-naphthyl-CoA reductase (DHNCR) (Eberlein *et al.*, 2013a, Estelmann *et al.*, 2015). The subsequent dearomatization of the activated ring requires the activity of a 5,6,7,8-tetrahydro-2-naphthoic acid reductase (THNCR) (Eberlein *et al.*, 2013b). Degradation of 2-methylnaphthalene (2MN) requires

the addition of its methyl group to fumarate through a naphthyl-2-methyl succinate synthase (*nmsABC* genes), followed by β-oxidation-like steps to finally render naphtoyl-CoA (Meckenstock *et al.*, 2016). The degradation of PAHs under nitrate reducing conditions has been detected in the environment and the presence of nitrate reducing bacteria (NRB) communities able to degrade PAHS in environmental samples has been quantified in a number of sites (Eriksson *et al.*, 2003a; Uribe-Jongbloed and Bishop, 2007; Acosta-González *et al.*, 2013a; Martirani-Von Abercron *et al.*, 2016). However, to date no NRB isolate able to reproducibly degrade PAHs under strict anoxic conditions has been reported. Recently, the description of intracellular oxygen production from nitrate respiration through a putative nitric oxide dismutase (Zedelius *et al.*, 2011; Ettwig *et al.*, 2012) and the discovery of a high abundance of the corresponding *nod* gene in the environment (Zhu *et al.*, 2017a), have opened up the possibility of the utilization of aerobic degradation pathways under anoxic, denitrifying conditions. However, the actual functioning of this supposed oxygen-producing pathway under real conditions for the degradation of hydrocarbons and other pollutants has not been proven. Furthermore, most NRB are facultative anaerobes that may alternate nitrate and oxygen respiration according to the availability of terminal electron acceptors, which is likely to fluctuate in polluted environments. Under microaerophilic prevailing conditions, minute amounts of oxygen suffice to promote the aerobic degradation of hydrocarbons (Yerushalmi *et al.*, 2002; Táncsics *et al.*, 2013).

Experimental approaches in the lab have mainly focussed on PAH degradation by organisms in the planktonic state. However, in the environment, most microbial processes occur through the development of multispecies biofilm where several groups of bacteria coexist and interact. Natural biofilms are complex supracellular structures composed of different species that are self-organized and coordinated to generate an appropriated habitat for the community (Flemming *et al.*, 2016). Growth in biofilm structures provides a number of advantages for the members of the consortium as compared to growth in the planktonic state (Roder *et al.*, 2016). Fundamental to these biofilm properties is the ability to produce extracellular polymeric substances (EPS), which support the architecture of the biofilm and provides mechanical stability, physical proximity, protection against stressors and predators, water retention, and passive sorption of solutes, amongst other benefits. Furthermore, interaction between species, which are favoured in complex biofilms, may lead to the development of new capabilities resulting either from the joint activity of members of the consortium (i.e. synergy (Ren *et al.*, 2015)), or from the transfer and combination of functions in a single strain (i.e. horizontal gene transfer (Madsen *et al.*, 2012; Roberts and Kreth, 2014)).

Here, we investigate the fate of microbial populations exposed to PAHs under conditions resembling natural situations. To this end, we started from a natural bacterial biofilm sample collected

at the oil-water interface of a hydrocarbon polluted aquifer, and we used parallel approaches under different oxygen limiting conditions that bacteria may face in the environment: complete anoxia with nitrate as electron acceptor, which are found when the available oxygen is completely consumed and there is no replenishment, and microaerophilic conditions, which are those generally found at the boundaries of contaminated plumes. We followed changes in the microbial communities during enrichment with PAHs in static setups. In these conditions, we expected active enrichment of those strains adapted to thrive more efficiently under a respiratory regime that resembles the conditions found in natural polluted sites using the provided PAH as carbon source.

3.2. Experimental Procedures

3.2.1. Sampling and site description.

The ground water sample used in this study was collected in sterile bottles at 25 m depth from a hydrocarbon contaminated aquifer located under an oil refinery (REPSOL) in Valle de Escombreras (Murcia, Spain) (37°34'20.6"N 0°55'24.7"W). The bottles were sealed under nitrogen gas, and stored at 4 °C until used. The aerobic and anaerobic enrichment culture of this work started from the biofilm present at the interface between the aquifer water and the hydrocarbon layer (Figure 3. 1).

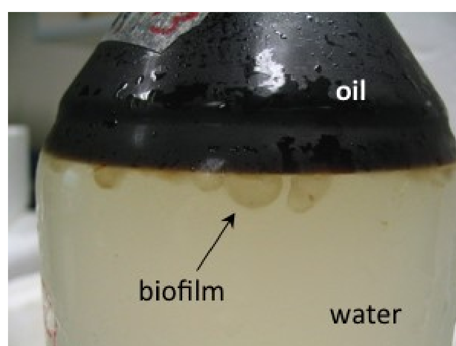

Figure 3. 1. Aquifer sample showing a visible biofilm growing at the interface between the oil and the water layers.

3.2.2. Most Probable Number enumeration of bacteria.

Aerobic and nitrate reducing PAH-degrading bacterial populations were enumerated using the most probable number (MPN) technique. The aerobic and the anaerobic media composition were similar and were prepared as described previously (Martirani-Von Abercron *et al.*, 2016). The medium (9 ml) was distributed in 12 ml vials (LABCO Limited, UK) where previously naphthalene,

2-methylnaphthalene, naphtoic acid and anthracene had been added to the tubes from stock diethyl ether solutions to reach a final concentration of 0.025‰, 0.028‰, 0.035‰ and 0.035‰, respectively, and the solvent had been evaporated before dispensing the medium. Acetate was used as a positive control to a final concentration of 5 mM, and controls with no added carbon source were included. For each sample, triplicate tubes were inoculated with 1 ml of the corresponding dilution in 10-fold serial dilutions. For the NRB enumeration the minimal medium containing 5 mM sodium nitrate as the electron acceptor was purged with sterile nitrogen gas and 9 ml were poured into oxygen-free tubes. Cultures were incubated at 28°C in the dark and anaerobic growth of NRB was checked during 6 months as the nitrite produced from nitrate respiration as described (Martirani-Von Abercron *et al.*, 2016). Aerobic cultures were incubated with slow shaking (100 rpm) during 1 month and growth was determined as turbidity. The data are the result of three replicate per sample within the 95% confidence interval.

3.2.3. Culture conditions, enrichment, and isolation procedures.

For the cultivation of nitrate-reducing bacteria (NRB) under strict anoxic conditions 25 ml of sample were transferred to 120 ml serum bottles containing 75 ml of modified non-reduced Widdel mineral medium. Medium preparation and composition was as described previously (Martirani-Von Abercron *et al.*, 2016). Naphthalene (NAP) and 2-methylnaphthalene (2MN) prepared in 2 ml sterile anoxic 2,2,4,4,6,8,8-heptamethylnonane (HMN) (20 g l^{-1}) as carrier were added in each serum bottle. A control with only HMN was included. The cultures were prepared under nitrogen atmosphere and sealed with Teflon-lined 1 cm thick stoppers. All culture manipulations were carried out under nitrogen atmosphere. To determine growth, the cultures were monitored periodically for nitrite produced from nitrate respiration or for nitrate consumption by ion chromatography as described (Martirani-Von Abercron *et al.*, 2016). Transfers of NRB cultures were made every three months by inoculating 10% (v/v) of the cultures into fresh medium. This operation was repeated three times, and after an additional two-month incubation samples were taken for pyrosequencing. For the microaerophilic enrichments, the medium composition and the preparation were the same as for the anaerobic one, except that naphthalene provided as crystals was used as the sole carbon source and the rubber-stoppers were completely wrapped in a Teflon tape. Teflon tape is slightly permeable to air and thus allows a slow rate oxygen flow-through into the culture vessels (see below). In the absence of inoculum, oxygen only reached saturation after 5-7 days (Figure 3. 2). A similar oxygen evolution rate could be obtained by inserting a 30 gauge needle through the rubber stopper instead of wrapping it with teflon tape (not shown). Microaerophilic enrichment cultures were incubated at 28°C

without shaking. Every month 10% (v/v) of the cultures were inoculated into fresh medium. Samples for pyrosequencing analysis were taken after five (Micro5-N) and fifteen (Micro15-N) transfers. Aerobic enrichments were set up starting from the fifth transfer of the microaerophilic culture. Briefly 5 mL of the fifth microaerophilic enrichment were inoculated in 250 mL shake flasks containing 50 mL of fresh Widdel minimal medium with naphthalene crystals as the sole carbon source. Flasks were incubated in an orbital shaker at 100 rpm and at 28 °C. Transfers (10%) were made every 15 days. Total DNA for pyrosequencing analysis was isolated after the fifteenth (Aer15-N) and nineteenth (Aer19-N) transfer. Isolation of aerobic strains was performed at 28 °C in Widdel mineral medium supplemented with either naphthalene crystals on the Petri dish cover, which was wrapped with Teflon tape, or 10% Luria-Bertani medium (LB). Isolates were selected by colony morphology and re-streaked until pure cultures were obtained. Aerobic growth of the isolates on naphthalene was carried out at 28°C on Widdel mineral medium supplemented with naphthalene crystals

3.2.4. Measurement of oxygen concentration in the culture bottles.

The set-up used to measure the oxygen concentration inside the bottles is shown in Figure 3. 2A. Temperature-compensated oxygen concentration of the medium was continuously recorded using oxygen-sensitive optodes (5 mm diameter spots; OXSP5, Pyro Science GmbH, Aachen, Germany) glued to the bottle inner wall and connected to a Firesting Optical Oxygen Meter via fiber-optic cables (SPFIB-BARE, Pyro Science. K., Aachen, Germany) fixed outside the wall in front of the sensor spot

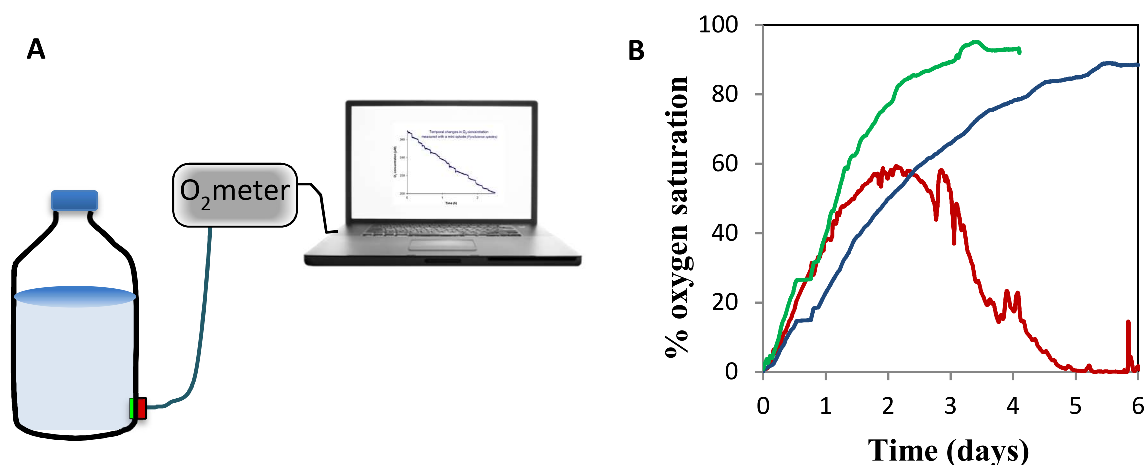

Figure 3. 2. Oxygen concentration in the microaerophilic culture bottles used in this study. Initially, 50-100 ml serum bottles filled-in with medium were flushed with nitrogen gas until oxygen concentration was zero and were then sealed with 1 cm-thick butyl stopper wrapped up with Teflon tape. An oxygen sensor spot glued at the bottom and connected to a Firesting Optical Oxygen Meter via fibre-optic cables (Pyro Science. K., Aachen, Germany) allowed continuous recording of oxygen concentration. A) Scheme of the bottle and oxygen meter set-up. B) Oxygen concentration evolution at the bottom of the bottle bottom: blue and green lines: examples of two non-inoculated controls; red line, inoculated culture.

with the aid of a Basic Spot Adapter (SPADBAS). The fiber was connected to a fiber-optic oxygen meter (Fire Sting O2, Pyro Science GmbH, Aachen, Germany) linked to a computer with Pyro Oxygen Logger software. Incubations were carried out at 28ºC. A bottle filled with water and fitted with a temperature sensor was placed in the same chamber for temperature record. The temperature-compensated dissolved oxygen tension was recorded continuously and expressed as percentage of air saturation. Figure 3. 2B shows the oxygen evolution in sterile and inoculated culture bottles equipped with an oxygen-sensitive optode placed at the bottom of the bottle wall.

3.2.5. Synthetic microbial community.

Strains were first picked from colony into Widdel mineral medium supplemented with 1/10 LB. Late–exponential-phase cells were harvested and washed twice with fresh medium without any carbon source. Cell densities were determined based on OD_{600} readings. Co-culture growth was initiated by inoculating 0.02 OD_{660} units of each strain in N_2 flushed Widdel mineral medium supplemented with a naphthalene crystal as the sole carbon source. Serum bottles were sealed with Teflon tape-wrapped rubber-stoppers as for the microaerophilic enrichments. Culture was incubated at 28ºC without shaking.

3.2.6. Chemical analysis.

For the analysis of nitrate and sulfate we used duplicate 5 ml aliquots of the original aquifer sample, and quantification was done by ion chromatography (IC) using a Metrohm-761 Compact Ion Chromatograph equipped with a Metrosep A Supp 4-250 column with chemical suppression (50 mM H_2SO_4) as described previously (Martirani-Von Abercron *et al.*, 2016). Duplicate samples of the initial contaminated aquifer (5 ml) were used to extract aliphatic and aromatic hydrocarbon compounds and the deuterated Mix 37 (manufactured by Dr. Ehrenstorfer) and 5-alpha-cholestane (Aldrich) were added as internal standards before the extraction. Hydrocarbon analysis was carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (CSIC), Granada, Spain by GC-MS as in (Martirani-Von Abercron *et al.*, 2016).

3.2.7. Total DNA extraction, 16S rRNA gene 454-Pyrosequencing and data analysis.

DNA extraction for the initial environmental sample was performed as described (Martirani-Von Abercron *et al.*, 2016). For the enrichment cultures and isolated strains, genomic DNA was purified using the Wizard Genomic DNA Purification Kit as recommended by the manufacturer (Promega,

Madison, WI, USA). Nucleic acid quantity and purity were determined with a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). A multiplex pyrosequencing amplicon approach was used for the characterization of the bacterial communities. The PCR amplifications of the 16S rRNA gene V1–V3 hypervariable region were carried out using the bacterial universal primers 6F and 532R containing 5′ tags with multiplex identifier and sequencing adapters (Tables S1). PCR amplifications were performed in 50 µl reactions containing 1 x PCR Buffer (Biorad), 200 µM dNTPs (Roche), 0.5 µM of each primer, 1U of iProof™ High-Fidelity DNA Polymerase (Biorad) and 20 ng of target DNA. The PCR program consisted of an initial denaturation step at 98°C for 30 s, followed by 25 cycles at 98°C for 10 s, 50°C for 20 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR reactions were cleaned up using the Qiaquick MiniElute columns (Qiagen) and checked in 1.5% agarose gel. Amplicons were quantified using Qubit™ fluorometer (Invitrogen), pooled at an equimolar ratio and sequenced using a 454 Titanium amplicon sequencing kit and a Genome Sequencer FLX 454 at either Citius (University of Seville) or Macrogen (Korea). Analysis of the 16S 454 pyrosequencing data was performed following the Quantitative Insights Into Microbial Ecology (QIIME v. 1.9.0) pipeline (Caporaso *et al.*, 2010b). Demultiplexing, primer removal, quality-filtering, chimeras and singletons removal, and alpha and beta diversity studies were done as defined in (Martirani-Von Abercron *et al.*, 2016).

3.2.8. Full-length 16S rRNA gene amplification and clone library construction.

Near-full-length 16S rRNA genes were amplified using bacterial universal primers GM3F and GM4R (Muyzer *et al.*, 1995). PCR were performed in 50 µl reactions as previously described (Acosta-González *et al.*, 2013a) and the products (~1500-bp) were cloned in pGEMT (Invitrogen). Positive clones were checked by PCR with vector primers prior to Sanger sequencing. Phylogenetic analysis was done with the ARB package (Ludwig *et al.*, 2004) using the online SINA alignment service and Silva database version SSU Ref 119. OTU's assignation was done at ≥ 97% sequence similarity. Phylogenetic tree was estimated by the neighbour-joining method.

3.2.9. Functional gene amplification.

Gene fragments of *bssA*, *ncr*, *nmsA*, *nahA*, *dbd* and *nod* were amplified by PCR using available primer sets and the cycling conditions originally described (Table S2). Genomic DNA from NaphS2 and *Thauera aromatica* K172 strains was used as a positive control for *nmsA, ncrA*, and *bssA* amplification, respectively. All PCR reactions were performed in 50 µl containing 1 x PCR Buffer (Biorad), 200 µM dNTPs (Roche), 0.5 µM of each primer (Sigma), 1 U of iProof™ High-Fidelity

DNA Polymerase (Biorad) and 10 to 20 ng of target DNA. The appropriately sized amplicons were purified and cloned in pCR2.1 (TA Cloning Kit, Invitrogen), and 16 positive clones for each gene were Sanger sequenced (IPBLN, CSIC, Granada, Spain). In addition to the available primers, six naphthalene dioxygenase (*ndo*) degenerated primers (Table S2) were designed using the CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primers) software (http://blocks.fhcrc.org/codehop.html) (Rose *et al.*, 2003) based on the alignment of 29 sequences of dioxygenase proteins of different species (Table S3). A gradient PCR was performed with annealing temperatures ranging from 50°C to 63°C for all primer combinations to determine optimal annealing temperatures. Touchdown PCR was used to avoid amplifying nonspecific sequences: after 1 min of denaturation at 98°C, the first 10 thermal cycles were 10 s at 98ºC, 15 s at 63ºC to 53ºC (the annealing temperature was reduced 1°C per cycle from 63ºC to 53ºC) and 20 s at 72ºC. The remaining 20 cycles were 98ºC for 10 s, 55ºC for 15 s and 72ºC for 20 s, with the last cycle followed by a 5 min extension at 72°C. Genomic DNA from *P. putida* KT2440 (NAH7) (Fernández *et al.*, 2012) was used as positive control.

3.2.10. RT-PCR assays.

Reverse transcriptase PCR (RT-PCR) of a 200 bp fragment of *dbdCa* was done with 75 ng of total RNA in a final volume of 50 µl using the Titan OneTube RT-PCR system according to the manufacturer’s instructions (Roche Laboratories). Positive (*S. novella* strain N1B DNA) and negative (absence of reverse transcriptase in the assay) controls were included. The primers used were Xdbd and XdbdI (Table S2).

3.2.11. Biofilm quantification in multi-well plates (crystal violet assay).

Biofilm formation at the air/liquid interphase was analysed in LB medium or LB medium saturated with naphthalene under static conditions in polystyrene 96-well microtiter plates using a modification of the method of Barahona *et al.*, 2010. Overnight cultures of each isolate grown in LB medium were diluted in the corresponding medium to an OD_{600} of 0.05. Then, 200 µl of each culture were dispensed in triplicate into the multi-well plates and incubated at 30°C without shaking. Because the biofilm formation kinetics varied for each strain and condition, biofilms were quantified at different time points for 50 hours as follows: the cell suspension was removed, the wells were washed with 200 µl of sterile water, the same volume of crystal violet solution (0.4%, w/v) was added to each well and incubated for 20 min to allow staining of adhered cells. Excess stain was eliminated by rinsing with water. Plates were air dried and 200 µl of 95% ethanol (v/v) were added to each well to

extract the crystal violet from cells. Distaining was performed overnight with shaking (40 rpm) after which the DO_{540} was measured on a microplate reader. The values when biofilm formation was highest were selected for each strain.

3.2.12. Scanning electron microscopy (SEM).

Specimen preparation and SEM were performed at the Scientific Instrumentation Centre (CIC) of the University of Granada. Biofilm samples from microaerophilic cultures growing on naphthalene were fixed for 2 h at 4ºC in 2.5% glutaraldehyde prepared in cacodylate buffer, pH 7.4. The fixed samples were rinsed three times (15 min each) with the same buffer at 4ºC and incubated for 1 h with 1% osmium tetroxide at room temperature, rinsed three times (5min) with water and dehydrated in an increasing ethanol concentration gradient from 50% to 100%. Samples were further desiccated with carbon dioxide in a Leica EM CPD300 critical point dryer according to Anderson (1951). Samples were carbon coated in an EMTECH K975X evaporator and examined in a Zeiss SUPRA40VP scanning electron microscope equipped with a Schottky type emission gun.

3.2.13. Nucleotide sequence accession numbers.

The 454 pyrosequencing raw reads have been deposited in the NCBI short-reads archive database (accession number SRR5417958 to SRR5417967). The partial *dbdB* gene sequences obtained from the clone libraries have been deposited in GenBank under accession numbers MF537652 to MF537654. The 16S rRNA gene clone library sequences have been deposited in GenBank under accession numbers MF156538 to MF156549.

3.3. Results and discussion

3.3.1. *Betaproteobacteria* are dominant in a hydrocarbon polluted aquifer

In the initial sample collected from a 25 m-deep well of a hydrocarbon polluted aquifer, a biofilm spreading between the water phase and the upper 10 cm oil layer was clearly visible (Figure 3. 1). The most abundant PAHs in the water phase were two-ring aromatics (0,14 ± 0.082 ppm of naphthalene, 0,20 ± 0.119 ppm of 2-methylnapthalene) followed by one order of magnitude lower levels of aromatics with three or more rings (Table 3. 1). The alkane distribution was coherent with contamination with a mixture of diesel and gasolines (Figure 3. 3). The concentration of nitrate and sulphate in the aqueous phase was in the range of natural background levels in oligotrophic groundwaters (Einsiedl and Mayer, 2005; Arauzo and Martínez-Bastida, 2015) (Table 3. 2).

Table 3. 1. Aromatic hydrocarbon composition and abundance in the contaminated aquifer initial samples.

Compound	ppm (µg/g)
Naphthalene	0.1377±0.082
1-methylnaphthalene	0.1868±0.081
2-methylnaphthalene	0.2002±0.119
1,4,5-trimethylnaphthalene	nd
1,4,6,7-tetramethylnaphthalene	0.0145±0.009
Dibenzothiophene	0.0299±0.026
Phenanthrene	0.0244±0.015
Anthracene	0.0245±0.005
Benzo[a]anthracene	nd
Pyrene	0.0066±0.0007
C2-naphthalene	0.0294±0.018
Fluoranthene	nd
Chrysene	nd
Total	0.654

nd: not detected.

Table 3. 2. Characterization of the initial polluted aquifer sample. The detailed hydrocarbon composition of the sample can be found in Table 3. 1 and Figure 3. 3.

Sample Name	Description	Location	Coordinates	Nitrate (µM)	Sulphate (µM)	Total hydrocarbons (µg/kg)	
						Aliphatic	Aromatics
INI	Aquifer below an oil refinery, 25 m depth	Valle de Escombreras, Murcia (Spain)	37°34'20.6"N 0°55'24.7"W	84.64±0.24	19.27±0.72	2130.80	654.00

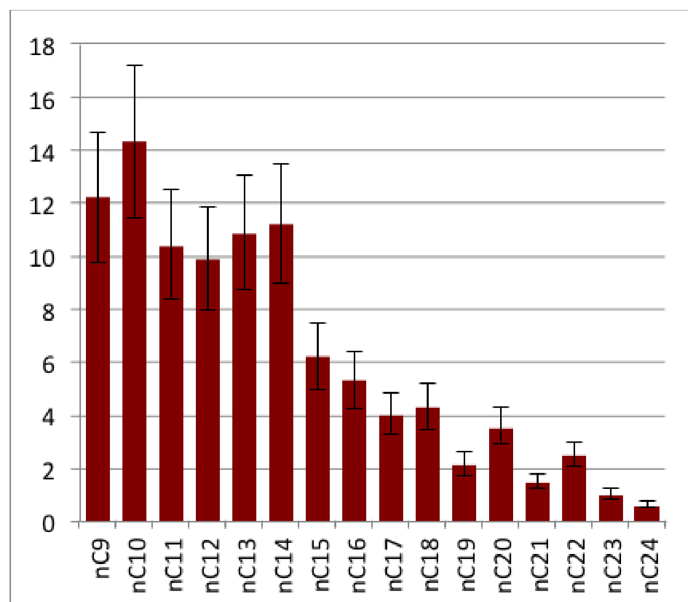

Figure 3. 3. Aliphatic hydrocarbon distribution (%) in the initial contaminated aquifer sample. The total amount of aliphatic hydrocarbons in the sample was 0.654 µg/ml.

The potential of the initial biofilm community to degrade PAHs was determined using the MPN enumeration of aerobic and nitrate reducing bacteria. Naphthalene (NAP), 2-methylnaphthalene (2MN), 2-naphtoic acid (2NA, a known intermediate in anaerobic naphthalene degradation) and anthracene (ANT) were used as carbon sources. A positive control with acetate as carbon source, and a negative control with no addition, were included in the analysis to estimate the size of the nitrate reducing community and the basal growth on the sample's intrinsic organic matter, respectively.

Under anoxic conditions, the high counts (10^5 cells/ml) of NRB able to grow with acetate revealed the presence of a significant community of NRB in the biofilm (Figure 3. 4, Table S4). However, no growth above the basal level was observed with any of the aromatics used, except for a minor increase with 2MN, indicative of the absence of NRB able to use naphthalenes or anthracene. This contrasted with the results obtained under aerobic conditions; substantial growth on the three aromatics was observed, which ranged between 4.3 x 10^2 cells/ml of naphthalene degraders and 2.4 x 10^4 cells/ml of 2MN degraders, consistent with the higher levels of 2MN in the polluted aquifer (Table 3. 1). These values represented only a minor fraction of the culturable aerobic community present, which reached 1.4 x 10^7 cells/ml when acetate was used as the carbon source.

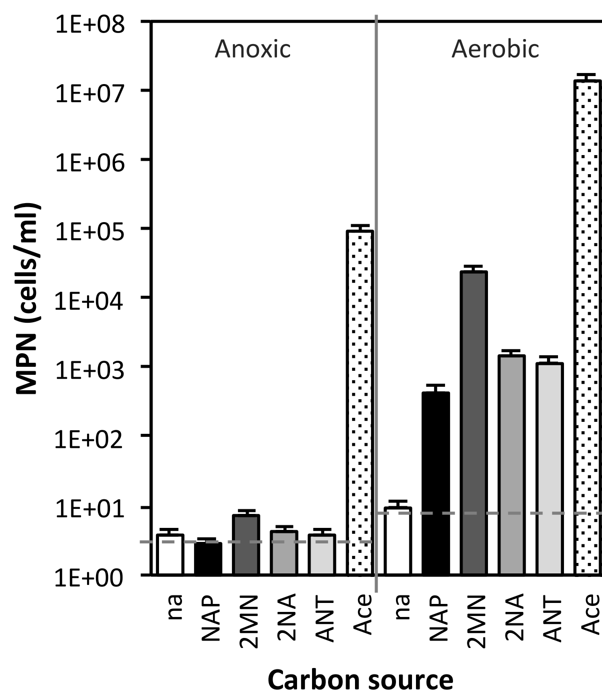

Figure 3. 4. MPN enumeration in the aquifer initial sample of nitrate reducing and aerobic bacteria able to grow on naphthalene (NAP), 2-methyl-naphthalene (2MN), 2-naphtoic acid (2NA), anthracene (ANT), acetate (Ace) and with no added carbon source (na). Counts were made in triplicate. Numerical values (95% C.I.) can be found in supplementary Table S4. Dotted lines indicate the basal growth with no added carbon source.

We isolated the total DNA from duplicate biofilm samples taken at the oil-water interphase to determine the structure of the community present using 16S rRNA gene V1-V3 region pyrosequencing. Total read numbers generated from all the samples analysed in this study and the corresponding diversity indices are summarized in Table 3. 3. The rarefaction curves (Figure S1) and the Good's sample coverage estimator suggested that sampling depth was sufficient in all cases to estimate the microbial diversity (Table 3. 3). The observed OTU number, diversity indices and OTU distribution of the initial sample were reproducible between replica (weighted Unifrac significant test P value 0.33).

Figure 3. 5 shows that the biofilm community was clearly dominated by the *Proteobacteria*, where the *Betaproteobacteria* accounted for more than 60% of the bacterial population. *Bacteroidetes*, *Chloroflexi* and the uncultured lineage SAR406 (also referred to as Marine Group A (Wright *et al.*, 2014)) were also present at substantial levels. The major presence of *Betaproteobacteria*, *Bacteroidetes* and *Chloroflexi* in this hydrocarbon polluted site is in agreement with the strains frequently found and isolated from aquifers and groundwater environments (Griebler

Table 3. 3. Comparison of OTU number, diversity, evenness indices and coverage for the different samples.

Sample[a]	NS[b]	OTUs[c]	OTUs (1500)[d]	Chao1 (1500)[e]	Shannon	Coverage[f]
INI_a	1807	167	158	259.08	4.08	95.87%
INI_b	1762	174	168	248.50	4.29	96.19%
Anox-NAP	3611	138	101	207.25	4.24	98.22%
Anox-2MN	6778	161	84	189.86	4.48	99.13%
Anox-HMN	3992	148	104	198.23	4.57	98.57%
Micro5-N	34611	115	42	61.13	2.66	99.94%
Micro15-N	22599	69	26	65.00	1.35	99.91%
Micro-12s-N	23347	101	43	58.00	2.33	99.91%
Aer15-N	29889	111	37	64.20	1.58	99.89%
Aer19-N	20685	116	45	129.33	2.46	99.79%

[a] INI_a and INI_b refer to the two replica of the initial samples.
[b]Number of sequences for each library filtered for chimera and singletons.
[c]OTU numbers calculated with all sequences at the 3% distance level.
[d]OTU numbers calculated for a randomized subset of 1500 reads per sample at the 3% distance level.
[e]Chao index calculated with 1500 subsampled sequences.
[f] Good's sample coverage estimator.

and Lueders, 2009), especially when carbon source availability is high (Fierer et al., 2007). The *Betaproteobacteria* community in the sample was relatively simple, dominated by two operational taxonomic units (OTUs): one belonging to the *Comamonadaceae* (29.5%) and a *Rhodocyclaceae* closely related to *Azovibrio* (30.6%) (Figure 3. 5B). *Betaproteobacteria* belonging to the *Comamonadaceae* occur in diverse pristine and polluted habitats such as soil, freshwater, groundwater, and industrial processing water, and can use a variety of substrates, including hydrocarbons, as carbon source (Willems, 2014). Interestingly, members of this group were also highly prevalent in toluene-polluted model wetlands and in BTEX-contaminated groundwater (Táncsics *et al.*, 2013; Martinez-Lavanchy *et al.*, 2015). The *Gammaproteobacteria* were almost exclusively composed of a single OTU closely related to *Pseudoxanthomonas* (3.8%) . Using samples from this biofilm layer, we set-up a series of enrichment cultures under two different oxygen limitation regimes to select for PAH degrading communities.

3.3.2. Changes in the community structure under strictly anoxic denitrifying conditions.

Enrichment cultures with nitrate as the electron acceptor and naphthalene and 2MN as the only carbon source were prepared under strict anoxic conditions. To reduce the PAH concentration in the water phase, which might limit growth of anaerobes (Galushko et al., 1999a), the PAHs were provided dissolved in HMN. After 12 months and 3 subsequent subcultures, DNA from the anoxic cultures was isolated and analysed as above by 16S rRNA 454 pyrosequencing to determine changes in the community structure (samples Anox-NAP, Anox-2MN, Anox-HMN) (Table 3. 3). A moderate loss of diversity was observed in all the nitrate reducing cultures, together with a clear shift in the community structure that may be attributed to the imposed change in the respiratory regime. Overall, the changes were comparable in the presence of the PAHs and when only the carrier HMN was added to the cultures (Figure 3. 5A). Whilst the dominant *Commamonadaceae* detected in the starting material was still present at high levels in the NAP, HMN, and to a lesser extent in the 2MN cultures, the dominant *Azovibrio* OTU disappeared almost completely in the three enrichments, partially balanced by an increase in *Betaproteobacteria* representatives belonging to the *Burkholderiaceae*, *Rhodoferax* and especially to the *Rhodocyclaceae* genus Dok59 in the case of the 2MN cultures (Figure 3. 5B). Members of the *Bacteroidetes*, which constituted more than 3% of the initial community, decreased to levels below 0.2% in all conditions. The strong decrease of some groups in all the enrichments suggests their high sensitivity to the new culture conditions, i.e., strict anoxic nitrate respiration and presence of PAHs. In contrast, an increase of the *Alphaproteobacteria* was observed in all conditions, especially represented by the *Sphingomonadaceae*, *Acetobacteraceae* and *Rhizobiaceae*. This might be explained by the denitrifying capacity of some members of the *Sphinomonadaceae* and *Rhizobiaceae*, in addition to their ability to tolerate the presence of toxic hydrocarbons (Carareto Alves et al., 2014; Cua and Stein, 2014). The *Anaerolineae* within the *Chloroflexi* also increased significantly in the 2MN and HMN enrichments. Since members of this class seem incapable of nitrate respiration, enrichment of this group is probably a consequence of their capacity for fermentative metabolism on cell remains (Yamada and Sekiguchi, 2009). Some specific changes were observed that could be attributed to the presence of PAHs: the *Actinobacteria*, which were almost undetectable in the initial biofilm sample, increased up to 18% in the HMN and 2MN cultures, and to almost 30% of the community in the NAP enrichment. Although an increase in the abundance of *Actinobacteria* has been related to the degradation of PAHs in aerobic conditions (Muangchinda et al., 2017; Zhu et al., 2017b), there is no evidence of the contribution of this phylum to anaerobic degradation. Interestingly, an increase in the *Acidobacteria* candidate class iii1-8 (order DS-18) from undetectable levels in the initial biofilm sample to more than 9% of the community in

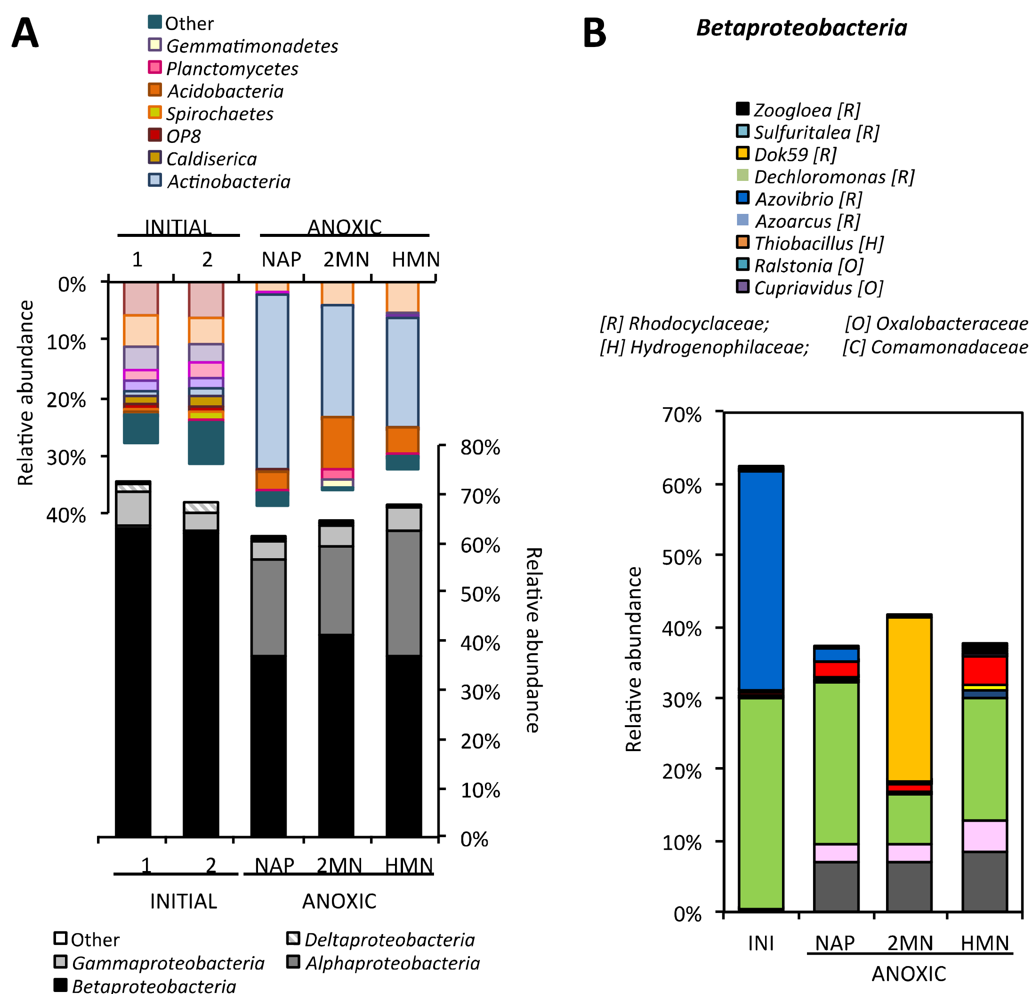

Figure 3. 5. A) Cumulative plot of bacterial phyla detected in the initial environmental samples and in the anaerobic enrichments amended with naphthalene (NAP), 2-methylnaphthalene (2MN) and heptamethylnonane (HMN). The *Proteobacteria* are described at the class level. The initial samples were analyzed in duplicate (labelled 1 and 2). B) Detailed cumulative plot of the *Betaproteobacteria* detected in the same samples. The average values of the duplicate initial samples shown in A are included for comparison, labelled as INI. *[R], Rhodocyclaceae; [O] Oxalobacteraceae; [H] Hydrogenophilaceae; [C] Comamonadaceae.*

the 2MN enrichment was also observed (Figure 3. 5A), in agreement with the previous observation of a dramatic increase of this poorly characterized group under nitrate reducing conditions, and especially in the presence of PAHs (Martirani-Von Abercron et al., 2016). This supports the suggestion that the group is composed of slow-growing oligotrophs (Fierer et al., 2007), probably highly resistant to PAHs (Martirani-Von Abercron et al., 2016). During the enrichment process under anoxic conditions, the minor changes in PAH concentration were not statistically significant (not shown), which reflects the absence of anaerobic PAH degraders determined with the MPN

enumeration. Furthermore, our attempts to detect genes involved in anaerobic PAH degradation in the initial sample and in the enrichment cultures using primers against *ncr* and *nmsA* gave negative results. Neither was any amplification product obtained with primers against nitric oxide dismutase (*nod*). To date, no growth of NRB isolates with PAHs as carbon source has been observed under strict anoxic conditions, and in most cases laboratory conditions can only reproduce aerobic degradation (Yagi et al., 2010). The observed shift in the bacterial community describes the adaptation to the new respiratory regime. However, NRBs are facultative anaerobes prepared to thrive in the oxic-anoxic transition zone, and in these environments anaerobic respiration and PAH degradation (aerobic) may be spatially separated concomitant processes.

3.3.3. Microaerophilic conditions strongly select for two dominant strains.

To mimic the natural habitat of most NRB, which are facultative anaerobes that can thrive at low oxygen concentrations, we set-up microaerophilic enrichment cultures. To this end, culture bottles were initially flushed with nitrogen gas to reach anoxic conditions; the caps of the bottles were prepared to allow a slow influx of air (see Experimental Procedures section) so that in the absence of inoculum oxygen saturation in the medium could be reached after approximately one week (Fig. 3. 2B). In this setup, inoculation of the medium resulted in oxygen consumption, which increased gradually to reach rates at equilibrium with the oxygen influx rate. Further growth of the culture caused oxygen consumption at higher rates than diffusion, leading to a progressive decay in the oxygen saturation level, which finally reached undetectable values in spite of the constant oxygen influx. We initiated an enrichment culture using naphthalene as the representative PAH, provided as the sole carbon source in the form of crystals. A fraction of 10% of the culture was regularly transferred to fresh medium. After three months, a fully developed biofilm was clearly visible at 0.5-1 cm around the naphthalene crystal in all the cultures of the enrichment process (Figure 3. 6A). The subsequent subcultures were characterized by the formation of a similar biofilm structure at a short distance from the naphthalene crystal, which was progressively consumed until it completely disappeared. Oxygen concentration at the biofilm level remained close to zero once the biofilm had developed, as previously shown for single species bacterial biofilms (Staal et al., 2011). Scanning electron microscopy (SEM) of the developed biofilm revealed the presence of a mixed community of bacteria with different sizes and shapes, adsorbed or attached onto filamentous, sometimes amorphous, EPS material (Figure 3. 6B). From subcultures 5 (8 months, 5 transfers) and 15 (26 months, 15 transfers), total DNA was isolated and the structure of the community in the enrichments

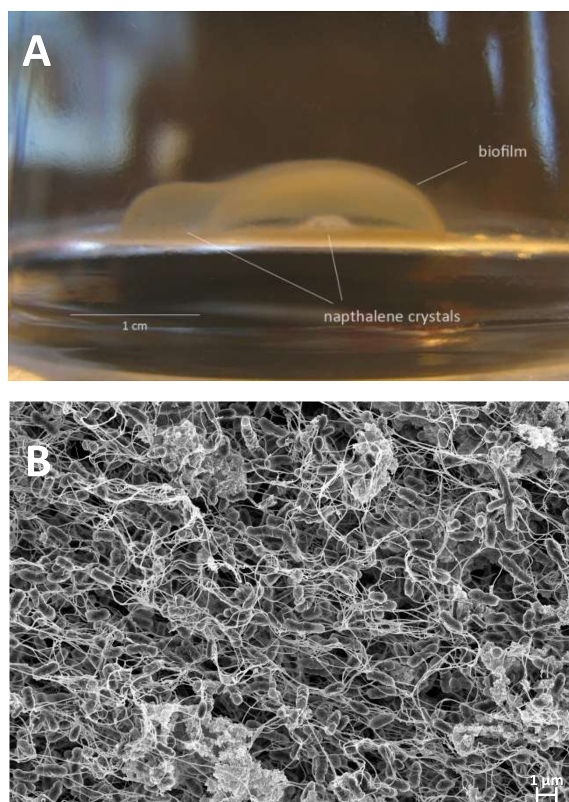

Figure 3. 6. A) Enrichment culture bottle under microaerophilic conditions showing the characteristic bacterial biofilm developed around the naphthalene crystal. B) Scanning electron microscopy (SEM) micrograph showing details of the multispecies biofilm formed around the naphthalene crystal during growth under microaerophilic conditions. Scale bar = 1 µm.

was analysed as above (samples Micro5-N and Micro15-N, Table 3. 3). Chao indices and rarefaction curves indicated that during microaerophilic enrichment, the diversity of the bacterial community was gradually reduced, reaching the lowest values in Subculture 15 (Figure S1). Accordingly, a progressive selection for specific groups was observed in the community: the enrichment process initially selected for four dominant *Proteobacteria* in Subculture 5, which in some cases could be affiliated to the genus level after comparing the pyrosequencing reads with the 16S rRNA sequence of strains isolated from the enrichments (see below) (Figure 3. 7). The four selected taxa belong to the *Xanthobacteraceae* (*Xanthobacter* and *Starkeya*), the *Commamonadaceae* (*Variovorax*), and the *Nocardiaceae*. Further enrichment under these conditions (Subculture 15) finally selected for two co-dominant strains which constituted 97% of the community: *Variovorax* (54%) and *Starkeya* (43%), and consequently the slowest Shannon evenness index characterized this community (Table 3. 3). It is worth noting that the two enriched strains were almost undetectable in the initial sample. From Subculture 5 we were able to isolate a number of strains on rich medium and on minimal medium with naphthalene as carbon source that were further characterized (Table 3.4; Figure 3.8). Taxonomic

affiliation of the strains matched those taxa which were among the most abundant in the enrichment, although we were not able to isolate some groups, such as *Actinotalea* and the *Nocardiaceae.* Because pyrosequencing only targeted the V1-V3 fragment of 16S rRNA, several OTUs in the pyrosequencing data were assigned to the taxon "*Xanthobacteraceae*", resulting in an ambiguous linking of the OTUs to the isolates. This ambiguity was solved by constructing a phylogenetic tree based on the V1-V3 region of all *Xanthobacteraceae* present in the pyrosequencing data and of the isolates (Fig. S2). Results assigned the most abundant *Xanthobacteraceae* in the final enrichment (Subculture 15) to *Starkeya novella*, which corresponded to strain 964 isolated on solid medium with naphthalene as the sole carbon source and was designated as *S. novella* strain N1B. The most abundant strain in the

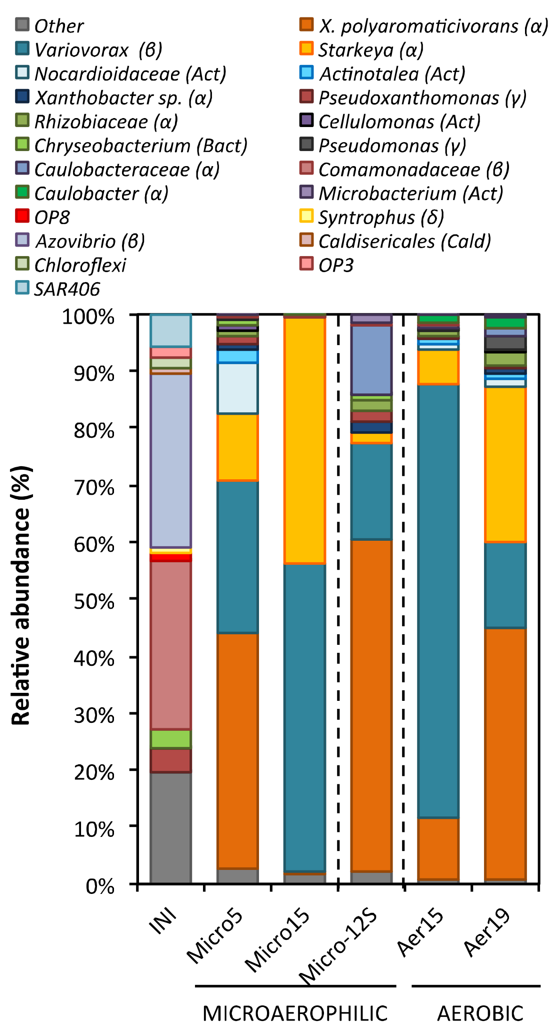

Figure 3. 7. Cumulative plot of bacterial taxons present at more than 1% of the community detected in the microaerophilic, aerobic and synthetic microaerophilic enrichments amended with crystals of naphthalene as carbon source. The deepest taxonomic affiliation reached (phyla indicated in brackets) is shown. The average values of the duplicate initial samples (Figure 3. 4A) are included for comparison, labelled as INI.

enriched community was affiliated to *Variovorax paradoxus* (Figure 3. 8), which corresponded to strain 968 isolated on rich medium and was designated as *V. paradoxus* strain N2.

Interestingly, the isolate 966, which constituted almost 12% of the Subculture 5 community, disappeared almost completely in the final enrichment. This strain was finally assigned to *Xanthobacter polyaromaticivorans* (99% 16S rRNA gene identity) based on the *Xanthobacteracea* phylogenetic tree, although it was also closely related to *Aquabacter spiritensis* (98%) (Figure 3. 8). The remaining isolates represented strains that were present at levels below 1% in the final Micro15-N enrichment (Table 3. 4).

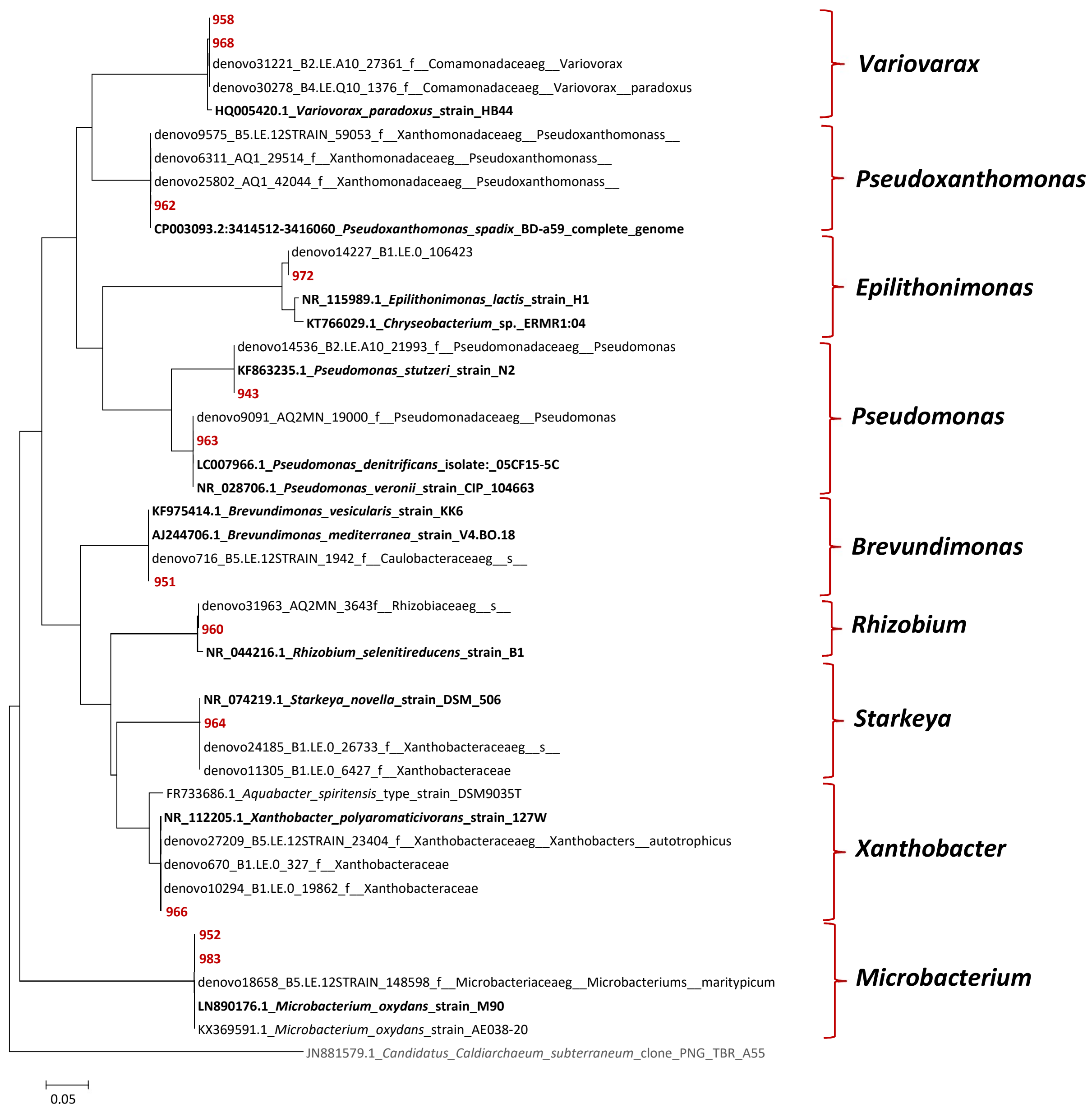

Figure 3. 8. Neighbour-joining tree of the 16S rRNA gene of the bacterial strains isolated in this study and their closest relatives in the databases. Representative closest QIIME OTUs for each strain (labelled as "denovo") are included. The bar represents 0.05 substitutions per site.

3.3.4. A pathway with high affinity for oxygen is favoured under microaerophilic conditions.

Eight strains amongst the isolates, including the dominant *Variovorax* and *Starkeya* strains, were able to grow aerobically with naphthalene as the carbon source (Table 3. 4). To identify the genes involved in naphthalene degradation, we used previously described primers and newly designed degenerated primers based on the alignment of 29 *nahA* sequences for naphthalene dioxygenases belonging to different taxonomic groups (Table S3, see the Experimental Procedure section). PCR analysis with several sets of primers to amplify naphthalene dioxygenase gene were carried out with DNA from the enrichments and from all the isolates. Only *Pseudomonas veronii* and the total DNA from Subculture 5 gave positive results with *nahA* primers. The *P. veronii nahA* gene product clustered with genes for class A ring-hydroxylating dioxygenases an was predicted to utilize alkyl substituted monoaromatics as well as PAHs as substrate, whilst Subculture 5 product was predicted to prefer hetero-polycyclic hydrocarbons and arylbenzenes, according to the ring-hydroxylating oxygenase database (Chakraborty *et al.*, 2014) (Figure 3. 9). As *X. polyaromaticivorans* was present among the isolates, and was the predominant strains in the Subculture 5 community, we designed and assayed primers for the *dbdB* gene coding for a dioxygenase from *X. polyaromaticivorans* strain 127W naphthalene/dibenzothiophene degradation pathway (Hirano *et al.*, 2004). Only the isolates belonging to the *Xanthobacteraceae*, *S. novella* and *X. polyaromaticivorans*, produced the corresponding PCR amplification products, which showed 99% identity with the *X. polyaromaticivorans dbdB* sequence (Figure 3. 9). We further investigated the presence in *S. novella* strain N1B of the complete PAH degradation cluster identified in *X. polyaromaticivorans*. We were able to amplify the genes for *dbdABCaCbCcDE* from the *S. novella* strain N1B DNA (Table 3. 5) and showed a remarkable gene identity (99%) with the described *X. polyaromaticivorans* PAH degradation gene cluster, including a *traA* conjugation gene located upstream *dbdA*.

The high percentage of identity of the sequences at the DNA level and the presence of flanking transfer genes suggest that *S. novella* strain N1B could have acquired the naphthalene degradation pathway through a recent horizontal gene transfer event, favoured by the stable physical environment for cell-to-cell contact provided by the biofilm matrix. In fact, we were still able to detect *X. polyaromaticivorans* in a gene library of whole-length 16S rRNA gene constructed with DNA from subculture 9 (not shown), and this could even be isolated on naphthalene plates from subculture 14. It is worth noting that no plasmid was detected in *S. novella* strain N1B genome. The DbdCa protein, which was shown to code for the alpha subunit of a naphthalene hydroxylating dioxygenase, is closer

Table 3. 4. Strains isolated from the microaerophilic enrichment cultures.

Clon #	Isolated from Subculture	Closest relatives (Class)[1]	% ident.	Pyroseq QIIME assigned taxon	% in the Subcultures		Growth on NAP[2]	Biofilm[3]	*dbdB*	*nahA*
					Sub 5	Sub 15				
964	5, 15	*Starkeya novella* (B)	99%	Xanthobacteraceae, Other	26.71	43.19	++	++	+	-
966	5	*Xanthobacter polyaromaticivorans* (B)	99%	Xanthobacteraceae, g_	11.77	0.38	+++	-	+	-
960	5	*Rhizobium naphthalenivorans* (A)	99%	Rhizobiaceae, g_	1.13	<0.01	+	-	-	-
951	5	*Brevundimonas mediterranea* (A)	100%	Caulobacteraceae	0.34	<0.01	++	-	-	-
952	5	*Microbacterium paraoxydans* (Act)	99%	Microbacterium	0.04	<0.01	±	-	-	-
983	5	*Microbacterium* sp. (Act)	99%	Microbacterium	<0.01	<0.01	++	-	-	-
958	5, 15	*Variovorax ginsengisoli* (B)	99%	Variovorax	41.46	54.22	-	-	-	-
968	5, 15	*Variovorax paradoxus* (B)	99%	Variovorax			++	-	-	-
962	5	*Pseudoxanthomonas spadix* (G)	99%	Pseudoxanthomonas	1.13	<0.01	+	-	-	-
943	5	*Pseudomonas nitroreducens* (G)	99%	Pseudomonas	0.37?	<0.01	-	-	-	-
963	5	*Pseudomonas veronii* (G)	99%	Pseudomonas			±	-	-	+
972	5	*Chryseobacterium* sp. (Bact)	98%	Flavobacteriaceae, Other	0.07	<0.01	-	-	-	-

(B), *Betaproteobacteria*; (A), *Alphaproteobacteria*; (Act), *Actinobacteria*; (G); *Gammaproteobacteria*; (Bact), *Bacteroidetes*.
[2] +++ OD>0.9 ; ++ OD>0.4; + OD>0.3; ± OD<0.1.
[3] Biofilm developed around the naphthalene crystal.

Table 3. 5. Naphthalene degradation gene sequences identified in *S. novella* strain N1B.

Genes	Amplicon Size	% Id.	Function
dbdAB'	994	98%	ferredoxin (*dbdA*); gentisate 1,2-dioxygenase (*dbdB*) (preceded by a partial *traA* gene coding for a conjugative transfer relaxase)
dbdCa	1398	99%	naphthalene 1,2-dioxygenase alpha subunit
dbdCbCc	1556	99%	naphthalene 1,2-dioxygenase beta subunit (*dbdCb*) Ferredoxin (*dbdCc*)
dbdD	1078	99%	dihydrodiol dehydrogenase

to group IV dioxygenases utilizing benzene/toluene/biphenyl as substrate, although it showed a preference for larger substrates and was functional at extremely low oxygen concentrations (Hirano *et al.*, 2006).

The *dbdB* gene codes for a gentisate1,2-dioxygenase with high affinity for oxygen (Hirano *et al.*, 2007). This suggests that the microaerophilic conditions were selecting for a degradation pathway that included enzymes with high affinity for oxygen. The selection for organisms bearing specific aromatic degradation enzymes observed under microaerophilic conditions both in competition experiments and in natural environments has been attributed the high affinity for oxygen of the selected mono- and dioxygenases (Balcke *et al.*, 2008; Kiesel *et al.*, 2008; Táncsics *et al.*, 2013; Martinez-Lavanchy *et al.*, 2015). This supports the idea that high oxygen tensions (i.e., "absolute" aerobic conditions) are not essential for aromatic degradation as long as the appropriate organisms are present. RT-PCR amplification of a *dbdCa* gene fragment from Subculture 15 showed that the gene was expressed in these conditions (Figure 3. 10). The naphthalene degradation genes of *Variovorax* and of the remaining strains except *Pseudomonas* could not be identified with the different sets of primers tested. *Variovorax* strains are frequently found in polluted environments and some of them, which bore genes for aromatic ring-hydroxylating dioxygenases with diverse suggested functions, were capable of aromatic degradation (Satola *et al.*, 2013; Posman *et al.*, 2017). However, a specific gene for naphthalene degradation has not been described in this genus. Previous studies have shown that the diversity of the aromatic dioxygenases alpha subunit is underrepresented in the databases, which are dominated by *Pseudomonas*-related sequences (Gomes *et al.*, 2007; Iwai *et al.*, 2011), and the use of primers designed to select for substrate specificity do not guaranty the detection of the targeted specific activities (Witzig *et al.*, 2006).

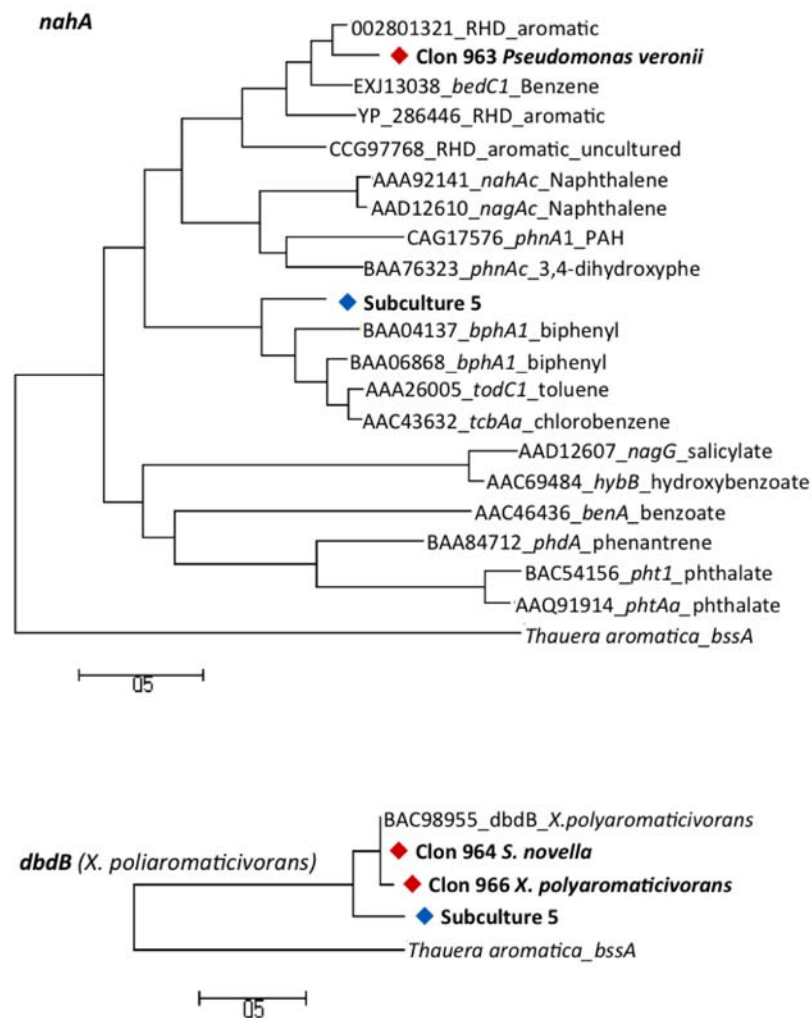

Figure 3. 9. Phylogeny of the alpha subunit oxygenase component of hydroxylating naphthalene dioxygenase (upper panel) and gentisate 1,2-dioxygenase (lower panel) partial amino acid sequence retrieved from Subculture 5 and different isolates obtained in this study. The trees were rooted with unrelated *bssA* gene product as out-group.

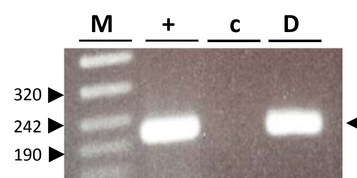

Figure 3. 10. RT-PCR analysis of total RNA extracted from subculture 15 (+). A negative control (c) with RNA where the reverse transcription step had been omitted and a positive control with DNA (D) from *S. novella* strain N1B were included. M, molecular weight marker.

3.3.5. Evolution of a synthetic community under microaerophilic conditions

To gain further insight into the community structure dynamics, we reproduced an artificial microbial community by mixing equal proportions of the twelve isolates (Table 3. 4) in a single microaerophilic culture, which was then subjected to a similar selective pressure using naphthalene as the sole carbon source for 12 weeks. The structure of the resulting community was analysed by 16S rRNA pyrosequencing as above (Table 3. 3, sample Micro-12s-N). Because the only possible strains present in this culture were the twelve isolates, we used the pyrosequencing data from this community to build the phylogenetic tree of the different taxons to establish the link between QIIME affiliation and strain isolates (Figure 3. 8 and not shown). Figure 3. 7 (sample Micro-12s) shows that the community had evolved from an initially even situation with equally abundant strains, to a community where the naphthalene degrading strains *X. polyaromaticivorans*, and to a lesser extent *Brevundimonas* and *Variovorax* dominated. Some *Brevundimonas* species are characterised for their dependence on microaerophilic conditions for growth (Buczolits *et al.*, 2001). In contrast the abundance of *Microbacterium*, *Starkeya*, *Pseudoxanthomonas* and *Rhizobium*, which were also capable of naphthalene degradation, was reduced to between 1.5 and 2% of the community. Finally, the two *Pseudomonas* strains and *Chryseobacterium* sp., unable to used naphthalene as a carbon source, almost disappeared, in accordance with their low abundance in the enriched subcultures 5 and 15. Overall, the distribution resembled the community structure in Subculture 5, and probably represented an intermediate step in the evolution of the community.

3.3.6. Aerobic growth allows higher bacterial diversity

To analyze the impact of oxygen tension on the evolution of the community, Subculture 5 of the microaerophilic enrichment was used as starting material to set-up enrichment cultures under higher oxygen concentrations provided through continuous shaking at 100 rpm. The oxygen saturation level in the cultures in these conditions was 92%. Samples were taken for community analysis as above after 5 months (10 transfers, sample Aer15-N) and 9 months (14 transfers, Aer19-N). Enrichment under aerobic conditions initially favoured *Variovorax* to the detriment of *X. polyaromaticivorans*, and further evolved to a structure closer to Subculture 5 as regards the dominant OTUs, though with a higher *Starkeya* to *Variovorax* ratio (Figure 3. 7). In addition, the final sample Aer19-N also showed a clear increase in overall bacterial diversity (Table 3. 3), where the previously least favoured groups such as *Pseudomonas*, *Rhizobiaceae*, *Caulobacteraceae* and *Microbacterium* recovered levels well above 1% of the community. Despite the constant shaking of the flaks, the cells showed a strong tendency to form clumps in the medium, which probably creates microhabitats of different oxygen

concentration, which may explain the fluctuations observed in the structure of the community with time.

3.3.7. *Starkeya novella* strain N1B is responsible for biofilm formation and naphthalene degradation.

We analyzed the capacity of the different isolates to reproduce the biofilm structure around the naphthalene crystal. Almost all the isolates were capable to certain extent of forming biofilm at the air/liquid interphase in rich medium, as determined with the standard crystal violet assay (Figure 3. 11), both in the presence and absence of naphthalene crystals. However, only *Starkeya novella* strain N1B was able to develop a well-defined biofilm structure in minimal medium around the naphthalene crystal similar to the one developed by the bacterial community in the microaerophilic enrichments. No growth was observed in these conditions when the naphthalene crystal was omitted from the medium. Interestingly, *X. polyaromaticivorans* was among those strains that were less efficient in standard biofilm formation, together with *Pseudoxanthomonas* and *Rhizobium*. In contrast, the two *Variovorax* isolates were characterized by the formation of clumps during growth, both in the presence and absence of naphthalene, indicative of the production of an EPS matrix, which characterizes some *Variovorax* strains (Jamieson *et al.*, 2009). However, none of these strains were

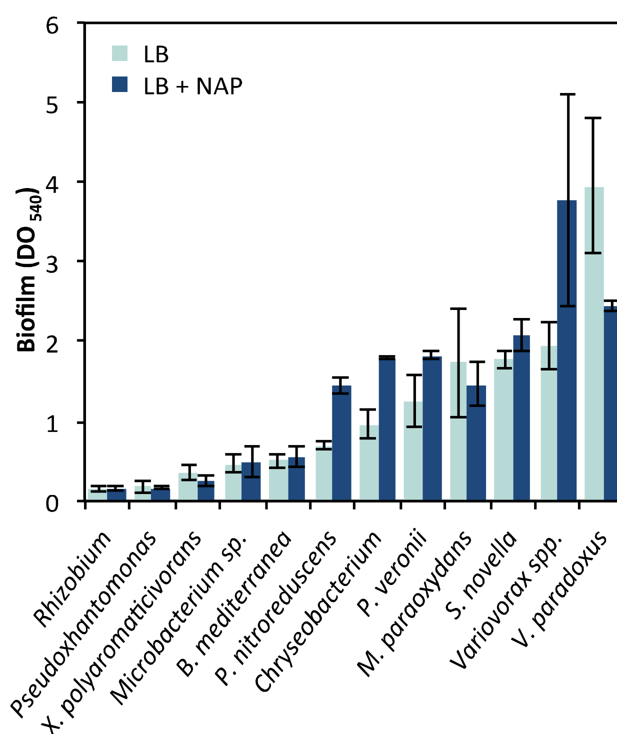

Figure 3. 11. Biofilm formation at the air/liquid interphase by different isolates in LB (light blue) or LB supplemented with a naphthalene crystal (dark blue). Assays were carried out in triplicate in microtiter plates as described in the Experimental procedures section.

capable of developing a structured biofilm around the naphthalene crystal. The closest relative of *S. novella* strain N1B is *S. novella* type strain (99% identity of 16S rRNA gene) (Kappler *et al.*, 2001), which is a strict aerobe incapable of naphthalene degradation and lacking any naphthalene degradation gene within its genome (Kappler *et al.*, 2012). In contrast, *S. novella* N1B harbors a naphthalene degradation pathway which is almost identical to the *X. polyaromaticivorans* pathway, characterized by the presence of key dioxygenases with high affinity for oxygen. *X. polyaromaticivorans* was originally described as capable of growth and PAH degradation under extremely low oxygen tensions (below 0.2 ppm) (Hirano *et al.*, 2004). However, in our microaerophilic enrichments, the *S. novella* strain N1B was able to totally outcompete *X. polyaromaticivorans*, probably after having horizontally acquired its *dbd* pathway. Thus, it seems that the capacity of the *S. novella* strain N1B to develop a structured biofilm in close proximity to the naphthalene crystal is a key factor that provides a strong advantage to the strain for growth with naphthalene under these oxygen limiting conditions. In fact, it has been hypothesized from simulated biofilm competition experiments that polymer producers are evolutionary favoured at the expenses of non-producers (Xavier and Foster, 2007). The ecological benefit driving this selection in our enrichment may be to provide a better competition for the substrate by locating the biofilm producers at the optimum distance from the carbon source to allow efficient mass transfer, simultaneously maintaining a sufficient distance to avoid a possible toxic effect of naphthalene. The stable presence of *V. paradoxus* in the biofilm suggests that its EPS production capacity favors its presence in the community, probably contributing to the structure of the biofilm.

3.4. Concluding remarks

Mixed bacterial biofilms are increasingly being considered as attractive tools in bioremediation protocols for their increased stability and resistance (Demeter *et al.*, 2015). In fact, bacteria in the sessile state which form part of biofilms are more resistant to pollutants (Khoei *et al.*, 2016) and more effective in pollutant degradation (Edwards and Kjellerup, 2013) than as free-living cells. In this study, the initial sample collected at the oil-water interphase had a medium to low phylogenetic diversity, numerically dominated by *Betaproteobacteria*, which represented more than 60% of the community. Enrichment under microaerophilic conditions resembling those naturally found in real environmental polluted sites produced a drastic change in the structure of the community, pointing to oxygen limitation as the main factor driving the structure of the community, selecting for strains that were undetectable in the starting material. Community members bearing degradation pathway enzymes with high affinity for oxygen were selected for, whilst additionally the capacity to produce

EPS and to develop biofilms was crucial for the final selection. Interestingly, this enrichment evidenced the function of biofilm structure to favour gene transfer, thus generating a naphthalene degrading strain able to grow at very low oxygen tensions and with a striking capacity to develop a physical structure to locate the cells at the appropriate distance from the toxic carbon source. Non-polar substances such as hydrocarbons can accumulate in the EPS matrix (Späth *et al.*, 1998), which would help increase the local concentration of the substrate in proximity to the biofilm producers and biofilm associated strains. Finally, no growth was observed when oxygen was completely omitted from the cultures and nitrate was the only terminal electron acceptor (anoxic enrichments), which supports the general view that NRB are actually adapted to microaerophilic conditions, where the different reactions involved in hydrocarbon degradation could be spatial and/or temporally separated in the bacterial communities **(**Yagi *et al.*, 2010**).**

3.5. References

Acosta-González, A., Rossello-Mora, R., and Marqués, S. (2013). Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the Prestige oil spill. *Environ Microbiol* **15**: 77-92.

Anderson, T. (1951). Techniques for the preservation of three dimensional structure in preparing specimens for the electron microscope. *Trans NY Acad Sci* **13**: 130-133.

Arauzo, M., and Martínez-Bastida, J.J. (2015). Environmental factors affecting diffuse nitrate pollution in the major aquifers of central Spain: groundwater vulnerability vs. groundwater pollution. *Environ Earth Sci* **73**: 8271-8286.

Balcke, G.U., Wegener, S., Kiesel, B., Benndorf, D., Schlomann, M., and Vogt, C. (2008). Kinetics of chlorobenzene biodegradation under reduced oxygen levels. *Biodegradation* **19**: 507-518.

Barahona, E., Navazo, A., Yousef-Coronado, F., Aguirre De Carcer, D., Martinez-Granero, F., Espinosa-Urgel, M., Martin, M., and Rivilla, R. (2010). Efficient rhizosphere colonization by *Pseudomonas fluorescens* f113 mutants unable to form biofilms on abiotic surfaces. *Environ Microbiol* **12**: 3185-3195.

Brune, A., Frenzel, P., and Cypionka, H. (2000). Life at the oxic-anoxic interface: microbial activities and adaptations. *FEMS Microbiol Rev* **24**: 691-710.

Buczolits, S., Rosengarten, R., Hirt, R., and Busse, H.J. (2001). Classification of a *Brevundimonas* strain detectable after PCR with a *Helicobacter*-specific primer pair. *Syst Appl Microbiol* **24**: 368-376.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., Mcdonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335-336.

Carareto Alves, L.M., De Souza, J.a.M., Varani, A.D.M., and Lemos, E.G.D.M. (2014). "The Family *Rhizobiaceae*," in *The Prokaryotes: Alphaproteobacteria and Betaproteobacteria,* eds. E. Rosenberg, E.F. Delong, S. Lory, E. Stackebrandt & F. Thompson. (Berlin, Heidelberg: Springer Berlin Heidelberg), 419-437.

Chakraborty, J., Jana, T., Saha, S., and Dutta, T.K. (2014). Ring-Hydroxylating Oxygenase database: a database of bacterial aromatic ring-hydroxylating oxygenases in the management of bioremediation and biocatalysis of aromatic compounds. *Environ Microbiol Rep* **6**: 519-523.

Cua, L.S., and Stein, L.Y. (2014). Characterization of denitrifying activity by the alphaproteobacterium, *Sphingomonas wittichii* RW1. *Front Microbiol* **5**: 404.

Demeter, M.A., Lemire, J.A., Yue, G., Ceri, H., and Turner, R.J. (2015). Culturing oil sands microbes as mixed species communities enhances ex situ model naphthenic acid degradation. *Front Microbiol* **6**: 936.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Muller, M., Meckenstock, R.U., and Boll, M. (2013). Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol Microbiol* **88**: 1032-1039.

Eberlein, C., Johannes, J., Mouttaki, H., Sadeghi, M., Golding, B.T., Boll, M., and Meckenstock, R.U. (2013). ATP-dependent/-independent enzymatic ring reductions involved in the anaerobic catabolism of naphthalene. *Environ Microbiol* **15**: 1832-1841.

Edwards, S.J., and Kjellerup, B.V. (2013). Applications of biofilms in bioremediation and biotransformation of persistent organic pollutants, pharmaceuticals/personal care products, and heavy metals. *Appl Microbiol Biotechnol* **97**: 9909-9921.

Einsiedl, F., and Mayer, B. (2005). Sources and Processes Affecting Sulfate in a Karstic Groundwater System of the Franconian Alb, Southern Germany. *Environ Sci Technol* **39**: 7118-7125.

Eriksson, M., Sodersten, E., Yu, Z., Dalhammar, G., and Mohn, W.W. (2003). Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. *Appl Environ Microbiol* **69**: 275-284.

Estelmann, S., Blank, I., Feldmann, A., and Boll, M. (2015). Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Mol Microbiol* **95**: 162-172.

Ettwig, K.F., Speth, D.R., Reimann, J., Wu, M.L., Jetten, M.S., and Keltjens, J.T. (2012). Bacterial oxygen production in the dark. *Front Microbiol* **3**: 273.

Fernández, M., Niqui-Arroyo, J.L., Conde, S., Ramos, J.L., and Duque, E. (2012). Enhanced tolerance to naphthalene and enhanced rhizoremediation performance for *Pseudomonas putida* KT2440 via the NAH7 catabolic plasmid. *Appl Environ Microbiol* **78**: 5104-5110.

Fierer, N., Bradford, M.A., and Jackson, R.B. (2007). Toward an ecological classification of soil bacteria. *Ecology* **88**: 1354-1364.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* **14**: 563-575.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* **1**: 415-420.

Gomes, N.C.M., Borges, L.R., Paranhos, R., Pinto, F.N., Krögerrecklenfort, E., Mendonça-Hagler, L.C.S., and Smalla, K. (2007). Diversity of *ndo* Genes in Mangrove Sediments Exposed to Different Sources of Polycyclic Aromatic Hydrocarbon Pollution. *Appl Environ Microbiol* **73**: 7392-7399.

Griebler, C., and Lueders, T. (2009). Microbial biodiversity in groundwater ecosystems. *Freshwat Biol* **54**: 649-677.

Hirano, S., Haruki, M., Takano, K., Imanaka, T., Morikawa, M., and Kanaya, S. (2006). Gene cloning and in vivo characterization of a dibenzothiophene dioxygenase from *Xanthobacter polyaromaticivorans*. *Appl Microbiol Biotechnol* **69**: 672-681.

Hirano, S., Kitauchi, F., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (2004). Isolation and characterization of *Xanthobacter polyaromaticivorans* sp. nov. 127W that degrades polycyclic and heterocyclic aromatic compounds under extremely low oxygen conditions. *Biosci Biotechnol Biochem* **68**: 557-564.

Hirano, S., Morikawa, M., Takano, K., Imanaka, T., and Kanaya, S. (2007). Gentisate 1,2-dioxygenase from *Xanthobacter polyaromaticivorans* 127W. *Biosci Biotechnol Biochem* **71**: 192-199.

Iwai, S., Johnson, T.A., Chai, B., Hashsham, S.A., and Tiedje, J.M. (2011). Comparison of the specificities and efficacies of primers for aromatic dioxygenase gene analysis of environmental samples. *Appl Environ Microbiol* **77**: 3551-3557.

Jamieson, W.D., Pehl, M.J., Gregory, G.A., and Orwin, P.M. (2009). Coordinated surface activities in *Variovorax paradoxus* EPS. *BMC Microbiol* **9**: 124.

Kappler, U., Davenport, K., Beatson, S., Lucas, S., Lapidus, A., Copeland, A., Berry, K.W., Glavina Del Rio, T., Hammon, N., Dalin, E., Tice, H., Pitluck, S., Richardson, P., Bruce, D., Goodwin, L.A., Han, C., Tapia, R., Detter, J.C., Chang, Y.J., Jeffries, C.D., Land, M., Hauser, L., Kyrpides, N.C., Goker, M., Ivanova, N., Klenk, H.P., and Woyke, T. (2012). Complete genome sequence of the facultatively chemolithoautotrophic and methylotrophic alpha Proteobacterium *Starkeya novella* type strain (ATCC 8093(T)). *Stand Genomic Sci* **7**: 44-58.

Kappler, U., Friedrich, C.G., Truper, H.G., and Dahl, C. (2001). Evidence for two pathways of thiosulfate oxidation in *Starkeya novella* (formerly Thiobacillus novellus). *Arch Microbiol* **175**: 102-111.

Khoei, N.S., Andreolli, M., Lampis, S., Vallini, G., and Turner, R.J. (2016). A comparison of the response of two *Burkholderia fungorum* strains grown as planktonic cells versus biofilm to dibenzothiophene and select polycyclic aromatic hydrocarbons. *Can J Microbiol* **62**: 851-860.

Kiesel, B., Balcke, G.U., Dietrich, J., Vogt, C., and Geyer, R. (2008). Microbial community shifts as a response to efficient degradation of chlorobenzene under hypoxic conditions. *Biodegradation* **19**: 435-446.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.H. (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363-1371.

Madsen, J.S., Burmolle, M., Hansen, L.H., and Sorensen, S.J. (2012). The interconnection between biofilm formation and horizontal gene transfer. *FEMS Immunol Med Microbiol* **65**: 183-195.

Martinez-Lavanchy, P.M., Chen, Z., Lunsmann, V., Marin-Cevada, V., Vilchez-Vargas, R., Pieper, D.H., Reiche, N., Kappelmeyer, U., Imparato, V., Junca, H., Nijenhuis, I., Muller, J.A., Kuschk, P., and Heipieper, H.J. (2015). Microbial Toluene Removal in Hypoxic Model Constructed Wetlands Occurs Predominantly via the Ring Monooxygenation Pathway. *Appl Environ Microbiol* **81**: 6241-6252.

Martirani-Von Abercron, S.-M., Pacheco, D., Benito-Santano, P., Marín, P., and Marqués, S. (2016). Polycyclic Aromatic Hydrocarbon-Induced Changes in Bacterial Community Structure under Anoxic Nitrate Reducing Conditions. *Front Microbiol* **7**: 1775.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A.M. (2016). Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. *J Mol Microbiol Biotechnol* **26**: 92-118.

Muangchinda, C., Yamazoe, A., Polrit, D., Thoetkiattikul, H., Mhuantong, W., Champreda, V., and Pinyakong, O. (2017). Biodegradation of high concentrations of mixed polycyclic aromatic hydrocarbons by indigenous bacteria from a river sediment: a microcosm study and bacterial community analysis. *Environ Sci Pollut R* **24**: 4591-4602.

Muyzer, G., Teske, A., Wirsen, C.O., and Jannasch, H.W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165-172.

Posman, K.M., Derito, C.M., and Madsen, E.L. (2017). Benzene Degradation by a Variovorax Species within a Coal Tar-Contaminated Groundwater Microbial Community. *Appl Environ Microbiol* **83**.

Ren, D., Madsen, J.S., Sorensen, S.J., and Burmolle, M. (2015). High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. *ISME J* **9**: 81-89.

Rivett, M.O., Buss, S.R., Morgan, P., Smith, J.W., and Bemment, C.D. (2008). Nitrate attenuation in groundwater: a review of biogeochemical controlling processes. *Water Res* **42**: 4215-4232.

Roberts, A.P., and Kreth, J. (2014). The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome. *Front Cell Infect Microbiol* **4**: 124.

Roder, H.L., Sorensen, S.J., and Burmolle, M. (2016). Studying Bacterial Multispecies Biofilms: Where to Start? *Trends Microbiol* **24**: 503-513.

Rose, T.M., Henikoff, J.G., and Henikoff, S. (2003). CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. *Nucleic Acids Res* **31**: 3763-3766.

Satola, B., Wübbeler, J.H., and Steinbüchel, A. (2013). Metabolic characteristics of the species *Variovorax paradoxus*. *Appl Microbiol Biotechnol* **97**: 541-560.

Späth, R., Flemming, H.-C., and Wuertz, S. (1998). Sorption properties of biofilms. *Water Science &Technology* **37**: 207-210.

Staal, M., Borisov, S.M., Rickelt, L.F., Klimant, I., and Kuhl, M. (2011). Ultrabright planar optodes for luminescence life-time based microscopic imaging of O_2 dynamics in biofilms. *J Microbiol Methods* **85**: 67-74.

Táncsics, A., Farkas, M., Szoboszlay, S., Szabó, I., Kukolya, J., Vajna, B., Kovács, B., Benedek, T., and Kriszt, B. (2013). One-year monitoring of meta-cleavage dioxygenase gene expression and microbial community dynamics reveals the relevance of subfamily I.2.C extradiol dioxygenases in hypoxic, BTEX-contaminated groundwater. *Syst Appl Microbiol* **36**: 339-350.

Ullrich, R., and Hofrichter, M. (2007). Enzymatic hydroxylation of aromatic compounds. *Cell Mol Life Sci* **64**: 271-293.

Uribe-Jongbloed, A., and Bishop, P. (2007). Comparative study of PAH removal efficiency under absence of molecular oxygen: effect of electron acceptor and hydrodynamic conditions. *J Environ Eng Sci* **6**: 367-376.

Vaillancourt, F.H., Bolin, J.T., and Eltis, L.D. (2006). The ins and outs of ring-cleaving dioxygenases. *Crit Rev Biochem Mol Biol* **41**: 241-267.

Willems, A. (2014). "The Family *Comamonadaceae*," in *The Prokaryotes – Alphaproteobacteria and Betaproteobacteria,* ed. E.R.E.A. (Eds.). (Berlin Heidelberg: Springer-Verlag), 777-851.

Wilson, L.P., and Bouwer, E.J. (1997). Biodegradation of aromatic compounds under mixed oxygen/denitrifying conditions: a review. *J Ind Microbiol Biotechnol* **18**: 116-130.

Witzig, R., Junca, H., Hecht, H.J., and Pieper, D.H. (2006). Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl Environ Microbiol* **72**: 3504-3514.

Wright, J.J., Mewis, K., Hanson, N.W., Konwar, K.M., Maas, K.R., and Hallam, S.J. (2014). Genomic properties of Marine Group A bacteria indicate a role in the marine sulfur cycle. *ISME J* **8**: 455-468.

Xavier, J.B., and Foster, K.R. (2007). Cooperation and conflict in microbial biofilms. *Proc Natl Acad Sci USA* **104**: 876-881.

Yagi, J.M., Suflita, J.M., Gieg, L.M., Derito, C.M., Jeon, C.O., and Madsen, E.L. (2010). Subsurface cycling of nitrogen and anaerobic aromatic hydrocarbon biodegradation revealed by nucleic Acid and metabolic biomarkers. *Appl Environ Microbiol* **76**: 3124-3134.

Yamada, T., and Sekiguchi, Y. (2009). Cultivation of uncultured chloroflexi subphyla: significance and ecophysiology of formerly uncultured chloroflexi 'subphylum i' with natural and biotechnological relevance. *Microbes Environ* **24**: 205-216.

Yerushalmi, L., Lascourreges, J.F., and Guiot, S.R. (2002). Kinetics of benzene biotransformation under microaerophilic and oxygen-limited conditions. *Biotechnol Bioeng* **79**: 347-355.

Zedelius, J., Rabus, R., Grundmann, O., Werner, I., Brodkorb, D., Schreiber, F., Ehrenreich, P., Behrends, A., Wilkes, H., Kube, M., Reinhardt, R., and Widdel, F. (2011). Alkane degradation under anoxic conditions by a nitrate-reducing bacterium with possible involvement of the electron acceptor in substrate activation. *Environ Microbiol Rep* **3**: 125-135.

Zhang, X., and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* **63**: 4759-4764.

Zhu, B., Bradford, L., Huang, S., Szalay, A., Leix, C., Weissbach, M., Tancsics, A., Drewes, J.E., and Lueders, T. (2017a). Unexpected Diversity and High Abundance of Putative Nitric Oxide Dismutase (*nod*) Genes in Contaminated Aquifers and Wastewater Treatment Systems. *Appl Environ Microbiol* **83**.

Zhu, F.X., Storey, S., Ashaari, M.M., Clipson, N., and Doyle, E. (2017b). Benzo(a)pyrene degradation and microbial community responses in composted soil. *Environ Sci Pollut R* **24**: 5404-5414.

3.6. Supplementary material

3.6.1. Supplementary tables

Table S1. 16S rRNA primers 6F and 532R targeting V1-V3 region containing 5′ tags with multiplex identifier (MID) and sequencing adapters used for pyrosequencing analysis.

Sample name	Primer name	Adaptor	Key	MID	Primer	Primer position
Ia	M1A	CGTATCGCCTCCCTCGCGCCA	TCAG	ACGAGTGCGT	TCAGAGTTTGATCCTGGCTCAG	6F
	M1B	CTATGCGCC TTGCCAGCCCGC	TCAG	ACGAGTGCGT	CACCGCGGCKGCTGGCAC	532R
Ib	M2A	CGTATCGCCTCCCTCGCGCCA	TCAG	ACGCTCGACA	TCAGAGTTTGATCCTGGCTCAG	6F
	M2B	CTATGCGCCTTGCCAGCCCGC	TCAG	ACGCTCGACA	CACCGCGGCKGCTGGCAC	532R
Anox-N	11A	CGTATCGCCTCCCTCGCGCCA	TCAG	TGATACGTCT	TCAGAGTTTGATCCTGGCTCAG	6F
	11B	CTATGCGCCTTGCCAGCCCGC	TCAG	TGATACGTCT	CACCGCGGCKGCTGGCAC	532R
Anox-2MN	84A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGACACTAT	TCAGAGTTTGATCCTGGCTCAG	6F
	84B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGACACTAT	CACCGCGGCKGCTGGCAC	532R
Anox-HMN	M2A	CGTATCGCCTCCCTCGCGCCA	TCAG	ACGCTCGACA	TCAGAGTTTGATCCTGGCTCAG	6F
	M2B	CTATGCGCCTTGCCAGCCCGC	TCAG	ACGCTCGACA	CACCGCGGCKGCTGGCAC	532R
Micro5-N	86A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGAGTCATCGT	TCAGAGTTTGATCCTGGCTCAG	6F
	86B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGAGTCATCGT	CACCGCGGCKGCTGGCAC	532R
Micro15-N	92A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTAGCTCTCT	TCAGAGTTTGATCCTGGCTCAG	6F
	92B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGTAGCTCTCT	CACCGCGGCKGCTGGCAC	532R
Micro12s-N	M4A	CGTATCGCCTCCCTCGCGCCA	TCAG	AGCACTGTAG	TCAGAGTTTGATCCTGGCTCAG	6F
	M4B	CTATGCGCCTTGCCAGCCCGC	TCAG	AGCACTGTAG	CACCGCGGCKGCTGGCAC	532R
Aer15-N	91A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGTACAGATAT	TCAGAGTTTGATCCTGGCTCAG	6F
	91B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGTACAGATAT	CACCGCGGCKGCTGGCAC	532R
Aer19-N	87A	CGTATCGCCTCCCTCGCGCCA	TCAG	CGATCGTATAT	TCAGAGTTTGATCCTGGCTCAG	6F
	87B	CTATGCGCCTTGCCAGCCCGC	TCAG	CGATCGTATAT	CACCGCGGCKGCTGGCAC	532R

Table S2. Functional gene primers used in this study.

Target gene	Primer set	5′-3′ sequence	Reference
bssA	7772f 8546r	GACATGACCGACGCSATYCT TCGTCGTCRTTGCCCCAYTT	(Winderl *et al.*, 2007)
bssA, nmsA	7768f 8543r	CAAYGATTTAACCRACGCCAT TCGTCRTTGCCCCAYTTNGG	(von Netzer *et al.*, 2013)
nmsA	7363f 7374f 8543r	TCGCCGAGAATTTCGAYTTG TTCGAYTTGAGCGACAGCGT TCGTCRTTGCCCCAYTTNGG	(von Netzer *et al.*, 2013)
ncr	Ncr1f Ncr1r	CGTTATWCKCCYTGCCGTG CGATAAGCCATRCADATRGG	(Morris *et al.*, 2014)
	Ncr2f Ncr2r	TGGACAAAYAAAMGYACVGAT GATTCCGGCTTTTTTCCAAVT	(Morris *et al.*, 2014)
nahA	pPAH-F pPAH-NR700	GGYAAYGCNAAAGAATTCGTNTGYWSHTAYCAYGGITGGG CCAGAATTCNGTNGTRTTHGCATCRATSGGRTKCCA	(Hedlund *et al.*, 1999)
	DP1Rieske_f ARHD2R	TGYMGNCAYMGNGG AANTKYTCNGCNGSNRMYTTCCA	(Iwai *et al.*, 2011)
ndo	NAPH-1F NAPH-1R	TGGCTTTTCYTSACBCATG DGRCATSTCTTTTTCBAC	(Gomes *et al.*, 2007)
ndo	NAH-306F NAH-621F NAH-627F NAH-612R NAH-633R NAH-1110R	AAGGGCTTCGTGTGCAACTAYCAYGGNTG CGTGGGCGACGCCTAYCAYRTNGG GACGCCTACCACGTGGGNTGGRMNCA TGGTAGGCGTCGCCCRYRAARTTYTC GGCGTGGGTCCAGCCNAYRTGRTA TGTTCATCTGGGTCACGGTCNHCATRTTNTC	This study
dbdB	dbdB-F dbdB-R	GAGGGCCACATATGTCATTCGCG CCGAAGCGGGTCGACTTTCAC	(Hirano *et al.*, 2007)
dbdAB	XdbA_up Dbd_R_280	GTAGAGAAGGAGAAGGCCAT GTCGTGATCCGCGATTGTCC	This study
dbdCa	XdbdIII XdbdI	GGACCGCGGCAATCAGGAGTGA GCCGGACTCTTCGGCGTAGG	
dbdCaCbCc	Xdbd XdbdI-Rev	ATGGTCCGCACCTTCTCGGC CCGGCGATCGAGGCGTTGTA	
dbdDE	XdbdII XdbdII-Rev	AGGCGATCGTCGAGCGGTTC CAGCAGCATGCGCCGATGAC	
dbdCa	XdbdIII XdbdI	GGACCGCGGCAATCAGGAGTGA GCCGGACTCTTCGGCGTAGG	
nod	nod684Fv2 nod1706Rv2	GGCTTSGCRATCCAGTAGAAG TGYMGNCAYMGNGG	(Zhu *et al.*, 2017)

Table S3. Naphthalene dioxygenase sequences used to design the CODEHOP primers used in this study.

Strain	Accession number
Pseudomonas fluorescens	AAL07262
Pseudomonas putida	WP_011117400
***Burkholderia* sp.**	ACT53249
Pseudomonas stutzeri	AAD02136
Paraburkholderia sartisoli	AAD09872
Marinomonas profundimaris	WP_024024133
Nevskia ramosa	WP_022978279
***Polaromonas naphthalenivorans* CJ2**	AAZ93388
Alteromonas naphthalenivorans	WP_013785992
***Diaphorobacter* sp. DS2**	AGH09226
Comamonas testosterone	AAF72976
***Delftia* sp. Cs1-4**	WP_013801305
***Acidovorax* sp. JS42**	AAB40383
***Acidovorax* sp. NA3**	ACG70971
***Sphingomonas* sp. VKM B-2434**	AHF58581
bacterium enrichment pahAc3	AFH77961
Paraburkholderia fungorum	AGN90996
Novosphingobium aromaticivorans	WP_011906634
Cycloclasticus zancles	WP_016389425
***Cycloclasticus* sp. NY93E**	ADD10619
Sphingomonas polyaromaticivorans	PhnA 2CKF_A
***Novosphingobium* sp. PP1Y**	WP_013834067
***Sphingomonas* sp.**	A4 BAD34447
***Comamonas* sp. MQ**	AEV91670
Pseudomonas chlororaphis	ADM26645
Burkholderia cepacia	AAL50021
Variovorax paradoxus	WP_041942906.

Table S4. Most probable number counts of nitrate reducing and aerobic bacteria in the initial contaminated aquifer sample. Carbon source are naphthalene (NAP), 2-methylnaphthalene (2MN), 2-naphtoic acid (2NA), anthracene (ANT), acetate (Ace) and no added carbon source (na).

Sample name	C source	MPN counts	95% C.I.	
Anoxic	na	3,79E+00	9,00E-01	1,27E+01
	NAP	2,86E+00	6,30E-01	1,05E+01
	2MN	7,28E+00	1,71E+00	2,12E+01
	2NA	4,27E+00	1,03E+00	1,39E+01
	ANT	3,79E+00	9,00E-01	1,27E+01
	Ace	9,33E+04	2,07E+04	2,71E+05
Aerobic	na	9,30E+00	2,07E+00	2,71E+01
	NAP	4,27E+02	1,03E+02	1,38E+03
	2MN	2,40E+04	4,76E+03	9,65E+04
	2NA	1,47E+03	2,78E+02	6,32E+03
	ANT	1,14E+03	2,37E+02	3,56E+03
	Ace	1,38E+07	2,68E+06	6,32E+07

3.6.2. Supplementary figures

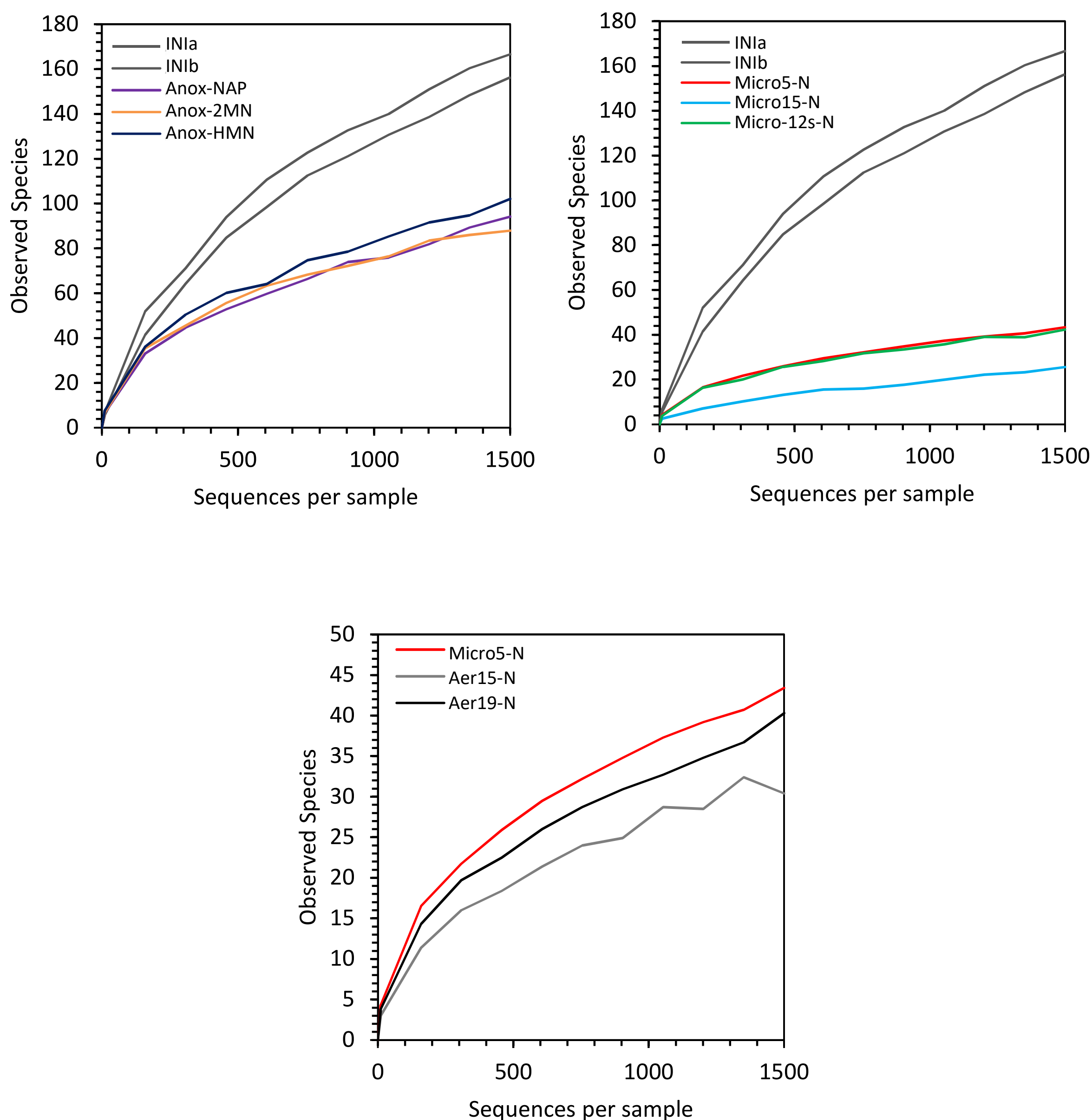

Figure S1. Rarefaction curves for 16S rRNA genes of microbial communities from the initial sample and enrichment cultures. Upper panel, anoxic enrichments; Middle panel, microaerophilic enrichments; Lower panel, aerobic enrichments. Labels are as in Table 2. Clones were grouped into phylotypes at a level of ≥ 97% sequence similarity.

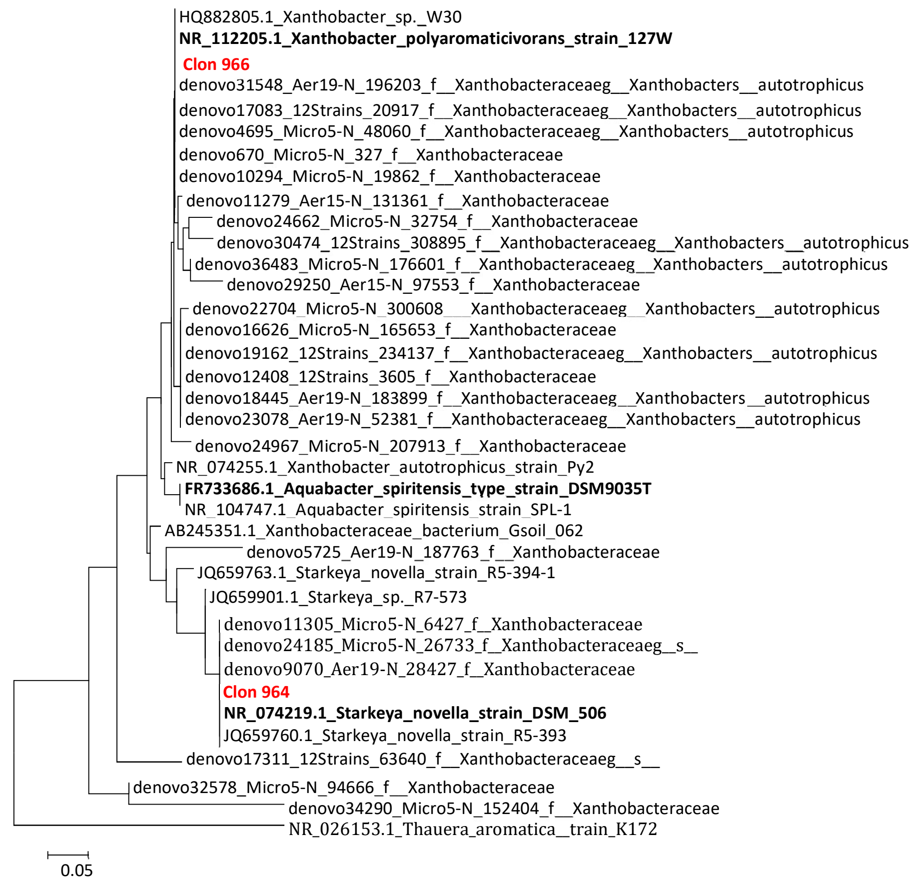

Figure S2. *Xanthobacteraceae* phylogenetic tree using the 16S rRNA V1-V3 region of the isolates and pyrosequencing reads in the microaerophilic, aerobic and synthetic enrichments. Sequences labelled with "denovo" are the pyrosequencing read OTUs retrieved from each dataset, as they are classified by QIIME. The closest type strains are highlighted in bold.

References

Gomes, N.C.M., Borges, L.R., Paranhos, R., Pinto, F.N., Krögerrecklenfort, E., Mendonça-Hagler, L.C.S., and Smalla, K. (2007) Diversity of ndo Genes in Mangrove Sediments Exposed to Different Sources of Polycyclic Aromatic Hydrocarbon Pollution, *Appl Environ Microbiol* **73**: 7392-7399.

Hedlund, B.P., Geiselbrecht, A.D., Bair, T.J., and Staley, J.T. (1999) Polycyclic Aromatic Hydrocarbon Degradation by a New Marine Bacterium, Neptunomonas naphthovorans gen. nov., sp. nov, *Appl Environ Microbiol* **65**: 251-259.

Hirano, S., Morikawa, M., Takano, K., Imanaka, T., and Kanaya, S. (2007) Gentisate 1,2-dioxygenase from Xanthobacter polyaromaticivorans 127W, *Biosci Biotechnol Biochem* **71**: 192-199.

Iwai, S., Johnson, T.A., Chai, B., Hashsham, S.A., and Tiedje, J.M. (2011) Comparison of the specificities and efficacies of primers for aromatic dioxygenase gene analysis of environmental samples, *Appl Environ Microbiol* **77**: 3551-3557.

Morris, B.E., Gissibl, A., Kummel, S., Richnow, H.H., and Boll, M. (2014) A PCR-based assay for the detection of anaerobic naphthalene degradation, *FEMS microbiol lett* **354**: 55-59.

von Netzer, F., Pilloni, G., Kleindienst, S., Kruger, M., Knittel, K., Grundger, F., and Lueders, T. (2013) Enhanced gene detection assays for fumarate-adding enzymes allow uncovering of anaerobic hydrocarbon degraders in terrestrial and marine systems, *Appl Environ Microbiol* **79**: 543-552.

Winderl, C., Schaefer, S., and Lueders, T. (2007) Detection of anaerobic toluene and hydrocarbon degraders in contaminated aquifers using benzylsuccinate synthase (bssA) genes as a functional marker, *Environ microbiol* **9**: 1035-1046.

Zhu, B., Bradford, L., Huang, S., Szalay, A., Leix, C., Weissbach, M., *et al.* (2017) Unexpected Diversity and High Abundance of Putative Nitric Oxide Dismutase (Nod) Genes in Contaminated Aquifers and Wastewater Treatment Systems, *Appl Environ Microbiol* **83**.

V. GENERAL DISCUSSION

Overall this thesis investigated the microbial diversity related to PAH degradation in many different environments under oxygen limiting condition. NRB and SRB communities were analysed, including those from the unexplored hypersaline anoxic sediment of the athalassoaline lake of Fuente de Piedra. Furthemore, a study of a multispecies biofilm in microaerophilic condition was carried out. Anaerobic bacteria, extreme habitats and multispecies biofilms are very common in nature although they have been poorly studied, probably due to the difficulties to investigate these organisms and ecosystems. Anyhow, understanding the bacterial diversity and the PAHs biodegradation mechanisms in these conditions is crutial for the development of bioremediation tools.

Biodegradation mediated by bacteria is one of the most popular technologies for the recovery of aromatic polluted sites. It is currently based on the diversity of aerobic bacteria and their capacity to degrade almost any natural compound and many man-made aromatic structures to CO_2 and water. The use of natural communities for the remediation processes requires a deep knowledge of the diversity of the organisms available, their performance under different conditions, and the mechanisms that drive their adaptation to changing environments such as those imposed by the pollutant. The use of aerobic processes requires the polluted areas be under permanent aerobic conditions. It is known that in many polluted sites, the initial consumption/degradation of the contaminant by aerobic microorganisms results in the exhaustion of the available oxygen, which then limits aerobic bacterial performance for the removal of the pollutants. When oxygen is consumed, only those strains able to use alternative compounds as terminal electron acceptors can be effective in the degradation of the contaminant. The anaerobic processes related to aromatic degradation are less understood than their aerobic counterpart, and only in recent years has their actual performance in the field started to be addressed (Kümmel *et al.*, 2016; Rabus *et al.*, 2016). Extensive research on the biochemistry and genetics of the pathways, the availability of an increasing number of isolates, sequenced genomes and metagenomes, and more recently transcriptomics and proteomics approaches have contributed to improve our picture of the anaerobic aromatic degradation in bacteria and its diversity (Kube *et al.*, 2005; Butler *et al.*, 2007; DiDonato *et al.*, 2010; Selesi *et al.*, 2010; Bergmann *et al.*, 2011; Holmes *et al.*, 2012; Rabus *et al.*, 2014; Sierra-Garcia *et al.*, 2014). Whilst this is essentially true for most monoaromatic compounds in bacteria with different anaerobic respiratory metabolisms, the anoxic degradation of PAHs is still an important bottleneck in our knowledge of anaerobic aromatic degradation by microorganisms.

1. Anaerobic naphthalene degradation: the problem.

As mentioned in the different chapters of this thesis, it is remarkable that to date only two examples of naphthalene degrading SRBs have been described and isolated, one of which is a two-strain consortium (N47) that cannot be obtained as a pure culture (Galushko *et al.*, 1999; Meckenstock *et al.*, 2000; Musat *et al.*, 2009). The pathways involved in naphthalene and 2MN degradation in the two isolates have been almost completely identified, showing that the general strategy resembles that used for monoaromatic compounds (Zhang and Young, 1997; Musat *et al.*, 2009; DiDonato *et al.*, 2010; Selesi *et al.*, 2010), although some singularities are also present (Eberlein *et al.*, 2013; Estelmann *et al.*, 2015; Meckenstock *et al.*, 2016; Weyrauch *et al.*, 2017). The actual functioning of the pathway in the environment was only proven recently by analysing microcosms amended with naphthalene, which linked the enrichment of *Desulfobacterium* strains related to N47 with naphthalene degradation under sulphate reducing conditions. However, the strains responsible for naphthalene metabolism were not isolated (Kümmel *et al.*, 2015). Sulphate reduction-dependent anaerobic degradation of PAHs is relevant in ecosystems where sulphate reduction conditions prevail, such as marine and brackish sediments (Foti *et al.*, 2007; Muyzer and Stams, 2008; Leloup *et al.*, 2009; Enning *et al.*, 2012), and it has also been observed in groundwater, freshwater sediments (Kümmel *et al.*, 2015) in addition to marine sediments (Coates *et al.*, 1996; Rothermich *et al.*, 2002). However, this process is slow as compared to the aerobic degradation. Furthermore, in many polluted sites other types of respiratory metabolism are more relevant. Among them, denitrification is probably the most efficient energy metabolism after aerobic respiration, and the possibility of nitrate-reducing PAH degradation processes would be of special interest for the restoration of these ecosystems.

Although naphthalene degradation under nitrate-reducing conditions was reported as early as in 1988 (Mihelcic and Luthy, 1988), to date there is no culture available capable of naphthalene degradation using nitrate as terminal electron acceptor, despite attempts made in different laboratories. The main aim of this thesis was to characterize the changes of the natural bacterial communities when exposed to PAHs under anoxic conditions, focusing on the simplest model compound naphthalene and its methylated derivative in 2 position. An underlying goal in this approach was the enrichment and possible isolation of new PAH-degrading anaerobes, especially under nitrate-reducing conditions, which has been elusive for years. Rockne *et al.* (2000) reported the isolation of three naphthalene degrading NRB, although the actual anaerobic naphthalene degradation capacity of these strains could never be reproduced (Rockne *et al.*, 2000; Meckenstock and Mouttaki, 2011; Meckenstock *et al.*, 2016). The difficulty to isolate PAH degrading NRB is

unexpected, considering that naphthalene is a natural compound with which bacteria have coexisted for a long time. Furthermore SRB, which have a lower energy-yielding metabolism than NRB, are able to use naphthalene and 2MN as carbon source under strict anoxic conditions. This contrasts the situation for benzene degradation, a non-substituted mono-aromatic with a structure similar to that of naphthalene. The special interest of both compounds relies on the difficulty to activate the non-substituted aromatic ring in the absence of oxygen (Meckenstock and Mouttaki, 2011). In both cases activation proceeds through direct carboxylation of the aromatic ring to render benzoic or naphthoic acid, respectively (Zhang and Young, 1997; Abu Laban *et al.*, 2010; DiDonato *et al.*, 2010). The first isolated strains capable of anaerobic benzene degradation were in fact nitrate-reducing bacteria (Coates *et al.*, 2001; Chakraborty *et al.*, 2005; Kasai *et al.*, 2006; Vogt *et al.*, 2011), although recently, the actual anaerobicity of benzene degradation by these strains was put into question (Meckenstock *et al.*, 2016), and still needs to be unequivocally proven. The recently discovered oxygenic dismutation into N2 and O2 of the NO produced during nitrate respiration was suggested to account for methane and alkane oxidation in an anaerobic methanotrophic bacterium and in an alkane-oxidizing gammaproteobacterium, respectively (Ettwig et al., 2010; Zhu et al., 2016). The presence of high abundance and diversity of the putative *nod* gene coding for the nitric oxide dismutase in contaminated aquifer and waster waters (Zhu *et al.*, 2016), opened up the possibility of the functioning of this pathway with hydrocarbons other than alkane, which would allow their aerobic degradation (i.e., using aerobic pathways) under anoxic conditions. Still, there is no biochemical evidence of the oxygenic activity of the proposed dismutase, and this must be clarified before further speculation on the relevance of this process in the environment.

As shown in the first chapter of this study, the efforts in our laboratory to isolate NRB able to grow on naphthalene were unsuccessful. A set of samples initially selected based on the presence of a nitrate-reducing community able to grow on naphthalene, as determined by the MPN (Acosta-González *et al.*, 2013; Chapter 1) were used to set up enrichment cultures using different approaches and culture media, to isolate candidate strains able to degrade PAHs under nitrate-reducing conditions. The analysis of a number of candidates selected on solid media following different approaches revealed that the PAH degradation capacity could not be reproduced in liquid media. Furthermore, tests performed with ^{13}C-labelled naphthalene with the most promising isolates confirmed the absence of degradation (Martirani-Von Abercron, unpublished). The reason for the contradictory results in the two culture conditions is unknown.

Thus, under our enrichment conditions, we could not cultivate neither select the initially detected community able to use naphthalene and 2MN as carbon source with nitrate as terminal

electron acceptor. PAH degradation is a complex process that implies a high number of genes, and produces a high number of intermediates. In the environment, such complex processes are likely to be shared by different members of the community, which would hamper the possibility to obtain a single isolate as anaerobic naphthalene degrader. We cannot discard that there might be an intrinsic hindrance for the complete anaerobic degradation of unsubstituted aromatics under nitrate-reducing conditions. It has been suggested that the failure to isolate naphthalene-degrading NRB could be due to the production of highly reactive nitrogen species such as NO during nitrate respiration, which could react with intermediates in the degradation pathway and block further metabolism, or even be toxic to the cell. This possibility needs to be seriously evaluated. On the other hand, NRB are generally facultative anaerobes adapted to live in conditions of fluctuating oxic/anoxic conditions, and in these conditions PAH degradation would take advantage of the coordinated activity of both types of metabolisms as described for other aromatics (Valderrama *et al.*, 2012), somehow "preventing" the need for a natural selection for naphthalene degradation pathways under strict nitrate-reducing conditions. In fact, we have observed by following $^{13}CO_2$ evolution from ^{13}C-labellet naphthalene that under microaerophilic conditions, PAH biodegradation occurs concomitantly with a significant nitrate respiration activity (Martirani-von Abercron, unpublished).

A second approach of this thesis focused the isolation of SRB able to grow on naphthalene starting from sulphate-rich sediments where basal levels of PAHs had been detected. We used a pre-enrichments strategy by incubating the undisturbed sediments with naphthalene crystals prior to the initiation of the enrichment cultures (Chapter 2). The shifts observed in the community structure attributable to the presence of naphthalene suggested the enrichment of a naphthalene degrading community. A main problem when studying anaerobic PAH degradation is the extremely slow growth rates obtained, especially with SRB, which show doubling times in the range of several weeks to months and low biomass yield (Meckenstock and Mouttaki, 2011). Unfortunately, in this study we were not able to come to the isolation phase, but these enrichments are a promising possibility for the future isolation of new naphthalene degrading SRB.

2. Changes in the microbial communities: effect of the culture conditions.

In our hands, a recurring observation during enrichment incubations using different media (under both nitrate and sulphate reducing conditions) was the strong effect that the incubation conditions exerted by themselves on the community structure. In environments that are not under permanent anoxic conditions, anoxia is a transient state and the communities are probably adapted to changing oxygen concentrations. We observed that after the establishment in the laboratory of

strict anoxic conditions in samples collected from different environments, a notable shift of the communities was observed, which was independent of the presence of PAHs, suggesting that either the imposed anoxic conditions or the composition of the culture media were exerting a strong selective pressure on the community. The effect was less evident when the samples originated from environments that were closer to strict and permanent anoxic conditions, such as marine and athalassohaline lagoon sediments. However, a dramatic shift in the community composition was observed when the Fuente de Piedra sediments were transferred from the original sulphate-reducing conditions to a nitrate-reducing medium, suggesting that the drastic shift in respiratory metabolism was increasing the niche possibilities of the community, allowing previously dormant populations to emerge, confirming the resilient nature of these highly diverse assemblages.

The effect of the incubation conditions was further evidenced when we compared the community structure of Fuente de Piedra sediments in the control enrichments using an artificial mineral medium, with that obtained when only filtered lagoon water was used as basal medium. Whilst incubation in an artificial medium produced a marked change in the community structure and a decrease in diversity, parallel incubation in FdP water resulted in less pronounced changes in the community structure and conservation of diversity values. This highlights the importance of selecting appropriated media when searching for specific bacterial traits, and supports the idea that culturing in the laboratory with artificial media implies an unavoidable bias towards already known, culturable microbes for which the media were originally designed, with the consequent loss in culturability. In an attempt to improve culturability, we assayed media as close as possible to the natural conditions, an approach that has already given interesting results (Pascual *et al.*, 2016). The use of FdP water as basal medium in the enrichments with PAHs as substrate resulted in the significant selection of some bacterial groups that could be good candidates for future isolation of PAH-degrading SRB. This confirms that the use of culture media reproducing natural conditions, especially emphasizing the presence of low nutrient concentrations, is an efficient approach for the isolation of previously uncultured organisms (Zengler *et al.*, 2002).

The methods used to provide the poorly soluble PAHs to the media were an additional factor influencing the final community composition. We observed that HMN, used as solvent to provide PAHs at lower than saturating concentrations, produced a strong shift in the community even in the absence of PAHs. These changes were essentially different to those observed when naphthalene was provided by directly adding crystals of the compound to the culture media. Whilst there is no doubt that HMN produces and effect on the communities, the saturating naphthalene concentrations reached when naphthalene is provided directly may results in a higher toxicity for

certain communities, which may also decrease the overall culturability. Furthermore, the use of saturating concentrations of PAHs hampers the experimental determination of low consumption rates, which would be masked by the constant dissolution of the compound when consumed. It must be noted than after prolonged incubations, the size of the naphthalene crystals in the sulphate-reducing FdP cultures decreased or even disappeared.

3. Changes in the microbial communities: effect of PAHs.

As mentioned above, the different incubations of samples from different origins under denitrifying conditions in the presence of PAHs produced some changes in the bacterial community structure. In most cases, the differences between the control and the presence of PAHs were subtle. Moreover, the groups selected for and against varied according to the particular community composition in the initial samples, so that the response was different in each case, and different groups were enriched from the different environments. An exception to this observation was the candidate class iii1-8 of the *Acidobacteria*, with no cultured representative, which was significantly and repetitively enriched in denitrifying cultures in the presence of PAHs from at least three types of environment (compost pile, different rice-paddy soils and water, and water from a polluted aquifer). The available information on this bacterial group is scarce, their respiratory metabolism is unknown, thus little conclusions can be extracted from this observation. The approach used is this study and the slow growth rates obtained did not allow us to link the presence of this group to PAH degradation, and other hypothesis, such as a high resistance of the group to PAH toxicity, could be considered to explain the enrichment of organisms of this class (Mukherjee *et al.*, 2014; Ren *et al.*, 2015). Not so striking but also relevant was the increase in the abundance of *Bacilli* in NRB enrichments from different origin in the presence of PAHs. Members of the *Bacilli* constitute a very diverse and versatile community, which highly diverse metabolic capabilities, including the capacity to degrade oil components both under aerobic and anaerobic conditions. As above, a direct linked between the increases in this population and anaerobic PAH degradation needs to be determined, and alternative explanations cannot be discarded (da Cruz *et al.*, 2011).

In contrast, some other groups were drastically reduced in the presence of PAHs under denitrifying conditions, such as *Bacteroidetes* and *Actinobacteria*, which we attributed to sensitivity of members of these groups to the PAH toxicity. The disappearance of certain groups from the community, sometimes to undetectable levels, implies cell mortality with the consequent destruction of cell structures and release of cell debris and metabolic compounds into the medium. This probably explains the selection under our conditions of certain bacterial groups such as

Chloroflexi, known to be incapable of nitrate respiration and able to carry out a fermentative metabolism, which are probably using the cell remains as carbon source (Yamada and Sekiguchi, 2009). Furthermore, some denitrifying groups could also be efficiently using the cell remains as carbon source, thus increasing their relative abundance in the community.

The enrichments under sulphate-reducing conditions produced a quite different picture. These experiments were carried out starting from samples with an expected high activity of SRB. We used sediments from the Fuente de Piedra athalassohaline lagoon as starting material, and implemented two different approaches. Whilst in the experiment performed in microcosm set-ups, keeping the sediment structures as undisturbed as possible, little changes were observed after PAH amendment, the cultures initiated thereafter from this material using different mineral media evidenced progressive changes in the community that were attributable to the presence of naphthalene. The changes were essentially observed when naphthalene was provided as crystals. An important increase in the relative abundance of certain groups known to carry out a sulphate-reducing type of respiration was observed. Members of the *Firmicutes*, and especially the genus *Dethiosulfatibacter*, were progressively and significantly enriched in the presence of naphthalene. Two other groups were also favoured by the imposed culture conditions: the relative abundance of members of the *Acidaminobacteraceae*, ascribed to the uncultured genus NP25, increased notably in the presence of naphthalene. This group had previously been associated to the response of salt-marsh communities to the presence of crude-oil (Koo *et al.*, 2015). The clear increase of these two groups could have a direct link to naphthalene degradation in these conditions, which need to be further analysed. Members of the *Halanaerobiaceae* also showed a significant increase in their relative abundance. However, members of this family have been described as obligate fermentative anaerobes (Kivisto and Karp, 2011), and are probably linked to the metabolism of cell remains.

The above-described changes were observed when filtered FdP water was used as culture medium. However, when an artificial mineral medium designed for the cultivation of sulphate-reducing bacteria belonging to the *Deltaproteobacteria* was used instead, the community shifted towards different groups. Certain groups of the *Deltaproteobacteria* accumulated the major changes, which was not unexpected. Notably, members of the family *Desulfobulbaceae* showed a significant increase in their relative abundance when naphthalene was present in the cultures. *Desulfobulbaceae* were identified in the analysis of the community dynamics of SRB related to the presence and degradation of PAHs and monoaromatics as toluene in environmental samples (Pilloni *et al.*, 2011; Kümmel *et al.*, 2015), and could probably be playing a similar role in the enrichments on artificial medium described here. Unfortunately, growth of SRB is slow, and in

the frame of this thesis we could not reach a point to initiate the isolation of naphthalene degraders. Furthermore, actual naphthalene degradation needs to be assessed in these enrichments. To that end, new culture conditions should be set up in order to be able to precisely measure small changes in naphthalene concentration. As an alternative, following $^{13}CO2$ evolution in cultures spiked with stable isotope-labelled naphthalene would provide evidence of anaerobic naphthalene degradation.

4. Naphthalene-degrading communities under microaerophilic conditions.

As it has been repeatedly mentioned throughout this thesis, the natural niche for NRB is probably an environment with fluctuating oxygen concentrations in the low range, probably under almost permanent microaerophilic conditions. We therefore set up a series of enrichments to analyse the community response to naphthalene under very low oxygen tensions, which were established when oxygen consumption by the growing populations overcame oxygen inflow through the permeable stopper or through a narrow-gauge needle. With this experimental set-up, and starting from samples from a gasoline-polluted aquifer, we observed that growth was much faster and with a higher biomass yield than under anoxic conditions, as expected. Interestingly the community was able to develop a similar biofilm structure around the naphthalene crystal, which had a similar appearance in each enrichment step. The initial sample was water from the aquifer collected at the boundary between the water phase and the overlaying oil phase, where a natural biofilm had developed. Biofilms are among the most distributed and effective ways of microbial life in nature, and have colonized all kinds of habitats. They are defined as complex sessile aggregations, encapsulated in an exopolymeric matrix (Flemming *et al.*, 2016). The bacterial assemblage constituting the initial biofilm community had probably been selected for their cooperative efficiency in the development of such structures. Analysis of the community in the biofilm during the enrichment on naphthalene evidenced a dramatic shift in the predominant populations during cultivation, which finally reached a structure with two co-dominant strains: *Starkeya novella* strain N1B and *Variovorax* sp. strain N2. Our results suggest that the two strains were selected for their capacity to use naphthalene as growth substrate (not all the strains present in the biofilm were capable of naphthalene degradation), and *S. novella* because it was competitive at low oxygen concentrations. Furthermore, this strain had acquired, through a probably recent horizontal gene transfer event, a naphthalene degradation pathway bearing enzymes with high affinity for oxygen. Interestingly, *Xanthobacter aromaticivorans*, a strain with similar features with respect to oxygen affinity for both respiration and naphthalene degradation (Hirano *et al.*, 2004), was also present in the initial culture and was the probable donor of the degradation pathway. However, this strain was outcompeted during enrichment. A relevant characteristic that

distinguished the two strains was that *S. novella* was able on its own to develop a biofilm structure that placed the cells around and at a distance from the naphthalene crystal, whilst *X. polyaromaticivorans* was inefficient in the formation of biofilm structures.

Since *S. novella* strain N1B was capable on its own to efficiently grow on naphthalene and develop a consistent biofilm around the crystal, the reason for the co-selection of *Variovorax* sp. strain N2 at the same level during enrichment is unclear, but it is assumable that it should provide some benefit to the community. We have shown that among the strains isolated from the enrichment cultures, *Variovorax* sp. was amongst the most efficient in the formation of biofilm in the air-water interphase, which implies that it is capable of producing some kind of extracellular polymeric substances at least under certain conditions. We could thus assume that in the multispecies biofilm, *Variovorax* sp. is somehow contributing to the formation of the biofilm structure. It is worth noting that this structure in the multispecies biofilm (Subculture-15) was more consistent and harder than the biofilm formed by pure cultures of *S. novella* strain N1B, suggesting that additional components not synthesized by this strain were present in matrix that contributed to biofilm texture. *Variovorax* would be one of the candidates to produce such substances.

5. References

Abu Laban, N., Selesi, D., Rattei, T., Tischler, P., and Meckenstock, R.U. (2010). Identification of enzymes involved in anaerobic benzene degradation by a strictly anaerobic iron-reducing enrichment culture. *Environ Microbiol* **12**: 2783-2796.

Acosta-González, A., Rosselló-Mora, R., and Marqués, S. (2013). Characterization of the anaerobic microbial community in oil-polluted subtidal sediments: aromatic biodegradation potential after the *Prestige oil* spill. *Environ Microbiol* **15:** 77-92.

Bergmann, F., Selesi, D., Weinmaier, T., Tischler, P., Rattei, T., and Meckenstock, R.U. (2011). Genomic insights into the metabolic potential of the polycyclic aromatic hydrocarbon degrading sulfate-reducing Deltaproteobacterium N47. *Environ Microbiol* **13:** 1125-1137.

Butler, J.E., He, Q., Nevin, K.P., He, Z., Zhou, J., and Lovley, D.R. (2007). Genomic and microarray analysis of aromatics degradation in Geobacter metallireducens and comparison to a Geobacter isolate from a contaminated field site. *BMC Genomics* **8**: 180.

Chakraborty, R., O'connor, S.M., Chan, E., and Coates, J.D. (2005). Anaerobic degradation of benzene, toluene, ethylbenzene, and xylene compounds by *Dechloromonas* strain RCB. *Appl Environ Microbiol* **71**: 8649-8655.

Coates, J.D., Anderson, R.T., and Lovley, D.R. (1996). Oxidation of Polycyclic Aromatic Hydrocarbons under Sulfate-Reducing Conditions. *Appl Environ Microbiol* **62**: 1099-1101.

Coates, J.D., Chakraborty, R., Lack, J.G., O'connor, S.M., Cole, K.A., Bender, K.S., and Achenbach, L.A. (2001). Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of Dechloromonas. *Nature* **411**: 1039-1043.

Da Cruz, G.F., De Vasconcellos, S.P., Angolini, C.F., Dellagnezze, B.M., Garcia, I.N., De Oliveira, V.M., Dos Santos Neto, E.V., and Marsaioli, A.J. (2011). Could petroleum biodegradation be a joint achievement of aerobic and anaerobic microrganisms in deep sea reservoirs? *AMB Express* **1**: 47.

Didonato, R.J., Jr., Young, N.D., Butler, J.E., Chin, K.J., Hixson, K.K., Mouser, P., Lipton, M.S., Deboy, R., and Methe, B.A. (2010). Genome sequence of the deltaproteobacterial strain NaphS2 and analysis of differential gene expression during anaerobic growth on naphthalene. *PLoS One* **5**: e14072.

Eberlein, C., Estelmann, S., Seifert, J., Von Bergen, M., Muller, M., Meckenstock, R.U., and Boll, M. (2013). Identification and characterization of 2-naphthoyl-coenzyme A reductase, the prototype of a novel class of dearomatizing reductases. *Mol Microbiol* **88**: 1032-1039.

Enning, D., Venzlaff, H., Garrelfs, J., Dinh, H.T., Meyer, V., Mayrhofer, K., Hassel, A.W., Stratmann, M., and Widdel, F. (2012). Marine sulfate-reducing bacteria cause serious corrosion of iron under electroconductive biogenic mineral crust. *Environ Microbiol* **14**: 1772-1787.

Estelmann, S., Blank, I., Feldmann, A., and Boll, M. (2015). Two distinct old yellow enzymes are involved in naphthyl ring reduction during anaerobic naphthalene degradation. *Mol Microbiol* **95**: 162-172.

Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., De Beer, D., Gloerich, J., Wessels, H.J., Van Alen, T., Luesken, F., Wu, M.L., Van De Pas-Schoonen, K.T., Op Den Camp, H.J., Janssen-Megens, E.M., Francoijs, K.J., Stunnenberg, H., Weissenbach, J., Jetten, M.S., and Strous, M. (2010). Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543-548.

Flemming, H.C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S.A., and Kjelleberg, S. (2016). Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* **14**: 563-575.

Foti, M., Sorokin, D.Y., Lomans, B., Mussman, M., Zacharova, E.E., Pimenov, N.V., Kuenen, J.G., and Muyzer, G. (2007). Diversity, activity, and abundance of sulfate-reducing bacteria in saline and hypersaline soda lakes. *Appl Environ Microbiol* **73**: 2093-2100.

Galushko, A., Minz, D., Schink, B., and Widdel, F. (1999). Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. *Environ Microbiol* **1**: 415-420.

Hirano, S., Kitauchi, F., Haruki, M., Imanaka, T., Morikawa, M., and Kanaya, S. (2004). Isolation and characterization of *Xanthobacter polyaromaticivorans* sp. nov. 127W that degrades polycyclic and heterocyclic aromatic compounds under extremely low oxygen conditions. *Biosci Biotechnol Biochem* **68**: 557-564.

Holmes, D.E., Risso, C., Smith, J.A., and Lovley, D.R. (2012). Genome-scale analysis of anaerobic benzoate and phenol metabolism in the hyperthermophilic archaeon Ferroglobus placidus. *ISME J* **6**: 146-157.

Kasai, Y., Takahata, Y., Manefield, M., and Watanabe, K. (2006). RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Appl Environ Microbiol* **72**: 3586-3592.

Kivisto, A.T., and Karp, M.T. (2011). Halophilic anaerobic fermentative bacteria. *J Biotechnol* **152**: 114-124.

Koo, H., Mojib, N., Huang, J.P., Donahoe, R.J., and Bej, A.K. (2015). Bacterial community shift in the coastal Gulf of Mexico salt-marsh sediment microcosm in vitro following exposure to the Mississippi Canyon Block 252 oil (MC252). *3 Biotech* **5**: 379-392.

Kube, M., Beck, A., Meyerdierks, A., Amann, R., Reinhardt, R., and Rabus, R. (2005). A catabolic gene cluster for anaerobic benzoate degradation in methanotrophic microbial Black Sea mats. *Syst Appl Microbiol* **28**: 287-294.

Kümmel, S., Herbst, F.A., Bahr, A., Duarte, M., Pieper, D.H., Jehmlich, N., Seifert, J., Von Bergen, M., Bombach, P., Richnow, H.H., and Vogt, C. (2015). Anaerobic naphthalene degradation by sulfate-reducing *Desulfobacteraceae* from various anoxic aquifers. *FEMS Microbiol Ecol* 91.

Kümmel, S., Starke, R., Chen, G., Musat, F., Richnow, H.H., and Vogt, C. (2016). Hydrogen Isotope Fractionation As a Tool to Identify Aerobic and Anaerobic PAH Biodegradation. *Environ Sci Technol* **50**: 3091-3100.

Leloup, J., Fossing, H., Kohls, K., Holmkvist, L., Borowski, C., and Jørgensen, B.B. (2009). Sulfate-reducing bacteria in marine sediment (Aarhus Bay, Denmark): abundance and diversity related to geochemical zonation. *Environ Microbiol* **11**: 1278-1291.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B. (2000). Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. *Appl Environ Microbiol* **66**: 2743-2747.

Meckenstock, R.U., Boll, M., Mouttaki, H., Koelschbach, J.S., Cunha Tarouco, P., Weyrauch, P., Dong, X., and Himmelberg, A.M. (2016). Anaerobic Degradation of Benzene and Polycyclic Aromatic Hydrocarbons. *J Mol Microbiol Biotechnol* **26**: 92-118.

Meckenstock, R.U., and Mouttaki, H. (2011). Anaerobic degradation of non-substituted aromatic hydrocarbons. *Curr Opin Biotechnol* **22**: 406-414.

Mihelcic, J.R., and Luthy, R.G. (1988). Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. *Appl Environ Microbiol* **54**: 1182-1187.

Mukherjee, S., Juottonen, H., Siivonen, P., Lloret Quesada, C., Tuomi, P., Pulkkinen, P., and Yrjala, K. (2014). Spatial patterns of microbial diversity and activity in an aged creosote-contaminated site. *ISME J* **8**: 2131-2142.

Musat, F., Galushko, A., Jacob, J., Widdel, F., Kube, M., Reinhardt, R., Wilkes, H., Schink, B., and Rabus, R. (2009). Anaerobic degradation of naphthalene and 2-methylnaphthalene by strains of marine sulfate-reducing bacteria. *Environ Microbiol* **11**: 209-219.

Muyzer, G., and Stams, A.J.M. (2008). The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Micro* **6**: 441-454.

Pascual, J., García-López, M., Bills, G.F., and Genilloud, O. (2016). Longimicrobium terrae gen. nov., sp. nov., an oligotrophic bacterium of the under-represented phylum *Gemmatimonadetes* isolated through a system of miniaturized diffusion chambers. *Int J Syst Evol Microbiol* **66**: 1976-1985.

Pilloni, G., Von Netzer, F., Engel, M., and Lueders, T. (2011). Electron acceptor-dependent identification of key anaerobic toluene degraders at a tar-oil-contaminated aquifer by Pyro-SIP. *FEMS Microbiol Ecol* **78**: 165-175.

Rabus, R., Boll, M., Heider, J., Meckenstock, R.U., Buckel, W., Einsle, O., Ermler, U., Golding, B.T., Gunsalus, R.P., Kroneck, P.M., Kruger, M., Lueders, T., Martins, B.M., Musat, F., Richnow, H.H., Schink, B., Seifert, J., Szaleniec, M., Treude, T., Ullmann, G.M., Vogt, C., Von Bergen, M., and Wilkes, H. (2016). Anaerobic Microbial Degradation of Hydrocarbons: From Enzymatic Reactions to the Environment. *J Mol Microbiol Biotechnol* **26**: 5-28.

Rabus, R., Trautwein, K., and Wohlbrand, L. (2014). Towards habitat-oriented systems biology of "*Aromatoleum aromaticum*" EbN1: chemical sensing, catabolic network modulation and growth control in anaerobic aromatic compound degradation. *Appl microbiol biotechnol* **98**: 3371-3388.

Ren, G., Ren, W., Teng, Y., and Li, Z. (2015). Evident bacterial community changes but only slight degradation when polluted with pyrene in a red soil. *Front Microbiol* **6**: 22.

Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E. (2000). Anaerobic Naphthalene Degradation by Microbial Pure Cultures under Nitrate-Reducing Conditions. *Appl Environ Microbiol* **66**: 1595-1601.

Rothermich, M.M., Hayes, L.A., and Lovley, D.R. (2002). Anaerobic, Sulfate-Dependent Degradation of Polycyclic Aromatic Hydrocarbons in Petroleum-Contaminated Harbor Sediment. *Environ Sci Technol* **36**: 4811-4817.

Selesi, D., Jehmlich, N., Von Bergen, M., Schmidt, F., Rattei, T., Tischler, P., Lueders, T., and Meckenstock, R.U. (2010). Combined genomic and proteomic approaches identify gene clusters involved in anaerobic 2-methylnaphthalene degradation in the sulfate-reducing enrichment culture N47. *J Bacteriol* **192**: 295-306.

Sierra-Garcia, I.N., Correa Alvarez, J., De Vasconcellos, S.P., Pereira De Souza, A., Dos Santos Neto, E.V., and De Oliveira, V.M. (2014). New hydrocarbon degradation pathways in the microbial metagenome from Brazilian petroleum reservoirs. *PloS one* **9**: e90087.

Valderrama, J.A., Durante-Rodriguez, G., Blazquez, B., Garcia, J.L., Carmona, M., and Diaz, E. (2012). Bacterial degradation of benzoate: cross-regulation between aerobic and anaerobic pathways. *J Biol Chem* **287**: 10494-10508.

Vogt, C., Kleinsteuber, S., and Richnow, H.H. (2011). Anaerobic benzene degradation by bacteria. *Microb biotechnol* **4**:710-724.

Weyrauch, P., Zaytsev, A.V., Stephan, S., Kocks, L., Schmitz, O.J., Golding, B.T., and Meckenstock, R.U. (2017). Conversion of cis-2-carboxycyclohexylacetyl-CoA in the downstream pathway of anaerobic naphthalene degradation. *Environ Microbiol, in press*.

Yamada, T., and Sekiguchi, Y. (2009). Cultivation of uncultured chloroflexi subphyla: significance and ecophysiology of formerly uncultured C*hloroflexi* 'subphylum i' with natural and biotechnological relevance. *Microbes Environ* **24**: 205-216.

Zengler, K., Toledo, G., Rappe, M., Elkins, J., Mathur, E.J., Short, J.M., and Keller, M. (2002). Cultivating the uncultured. *Proc Natl Acad Sci U S A* **99**: 15681-15686.

Zhang, X., and Young, L.Y. (1997). Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. *Appl Environ Microbiol* **63**: 4759-4764.

Zhu, B., Bradford, L., Huang, S., Szalay, A., Leix, C., Weissbach, M., Táncsics, A.S., Drewes, J.R.E., and Lueders, T. (2017). Unexpected diversity and high abundance of putative nitric oxide dismutase (Nod) genes in contaminated aquifers and wastewater treatment systems. *Appl Environ Microbiol* **83**.

VI. CONCLUSIONES

1. El medio utilizado para los enriquecimientos a partir de muestras ambientales es un factor clave para la selección de cepas con características de interés, que hay tener en cuenta antes de establecer los protocolos experimentales. El uso de aguas procedentes del sitio de muestreo como medio de cultivo ha permitido el enriquecimiento de grupos que se pierden utilizando medios artificiales.

2. El análisis de las comunidades bacterianas durante los procesos de enriquecimiento de bacterias anaerobias degradadoras de PAHs muestra el enriquecimiento recurrente de diversos grupos bacterianos, algunos sin representante cultivado, a pesar de el origen muy diverso de las muestras. Esto nos indica que la mayoría de los taxa se pueden encontrar en muchos tipos diferentes del ambiente, y destaca la dificultad para el aislamiento de bacterias con las características deseadas.

3. La abundancia relativa de miembros de la clase candidata iii1-8 dentro de las *Acidobacteria* aumentó de forma recurrente en presencia de naftaleno o 2MN en cultivos de enriquecimiento preparados a partir de muestras de origen muy diverso, lo que podría indicar una alta resistencia a la presencia de estos compuestos, o un papel relevante en la respuesta de la comunidad bacteriana a la presencia de PAHs.

4. El cultivo de muestras de sedimentos de la laguna Fuente de Piedra en condiciones de sulfato-reducción en presencia de naftaleno ha permitido enriquecer de forma progresiva y significativa grupos específicos pertenecientes a *Firmicutes* y *Deltaproteobacteria*, que probablemente participen en la degradación de los PAHs.

5. En la selección en condiciones microaerófilas estáticas de la comunidad bacteriana co-dominada por los géneros *Starkeya* y *Variovorax* a partir de muestras de acuífero contaminado con gasolinas, los factores determinantes fueron la capacidad de crecer a bajas tasas de oxígeno, de portar una ruta de degradación de naftaleno con alta afinidad por el oxígeno, y ser eficientes en la producción de sustancias poliméricas extracitoplásmicas.

6. *Starkeya novella* N1B*,* seleccionada a partir de muestras de acuífero contaminado, es la cepa de la comunidad bacteriana responsable de la formación de la biopelícula de sustancias poliméricas que se sitúa de forma regular a 0,5-1 cm de distancia alrededor del cristal de naftaleno.

Imagen de portada: Micrografía electrónica de barrido (SEM) de la biopelícula formada por un consorcio microbiano alrededor de un cristal de naftaleno (se estudia en el capítulo 3).

El uso masivo del petróleo como fuente de energía y como materia prima ha generado serios problemas de contaminación ambiental. Entre los principales contaminantes los hidrocarburos aromáticos policíclicos (HAPs) generan gran preocupación porque son relativamente persistentes en el medio ambiente y presentan efectos tóxicos, carcinogénicos, mutagénicos y teratogénicos, por lo tanto su eliminación es importante. Una medida que ha tenido bastante éxito es el empleo de microorganismos para la eliminación de contaminantes con la aplicación de técnicas de biorremediación. Los procesos aerobios de degradación de HAPs se han caracterizado exhaustivamente, sin embargo en muchos ambientes naturales la concentración de oxígeno es limitante y se activan procesos de degradación anaeróbica sobre los cuales todavía se sabe muy poco.

Partiendo de una serie de ambientes que habían sufrido diferentes grados de contaminación por hidrocarburos, utilizamos la técnica del número más probable (NMP) para detectar y cuantificar la presencia de comunidades bacterianas capaces de degradar anaeróbicamente varios HAPs, establecimos una serie de cultivos de enriquecimiento con nitrato y sulfato como aceptor terminal de electrones y HAP como única fuente de carbono y seguimos los cambios en las comunidades bacterianas a lo largo del proceso. Además quisimos testar condiciones de microaerofilia para aislar cepas degradadoras de HAPs haciendo enriquecimiento a partir de una muestra de biofilm de un acuífero contaminado con petróleo, utilizando HAPs como única fuente de carbono.

En conjunto, hemos estudiado las respuestas de las comunidades bacterianas a los HAPs en las condiciones limitantes de oxígeno más frecuentemente encontradas en la naturaleza (nitrato reductoras, sulfatoreductoras y microaerofilicas) pero menos investigadas tanto por las dificultades de cultivo de estas bacterias como por los largos tiempos de crecimiento que requieren estos microorganismos. No hemos logrado aislar cepas anaerobias estrictas capaces de degradar HAPs, pero sí de individualizar poblaciones bacterianas presentes en la naturaleza y probablemente involucradas en la eliminación de contaminantes en el medio ambiente. También hay bacterias no cultivables (*v.g. Acidobacteria,* iii1-8) que se han enriquecido, de las cuales todavía desconocemos el papel ecológico, pero que podrían ser importantes en la biodegradación anaerobia. En condiciones de microaerofilia, sí hemos logrado cepas degradadoras, y además capaces de formar biofilm a partir de HAPs. El crecimiento en forma de biofilm es probablemente una manera de crecer que las bacterias adoptan en condiciones de estrés y que le ayuda a hacer frente a los factores adversos. Todo esto posiblemente nos indique que las condiciones de cultivo que imponemos en el laboratorio son demasiado restrictivas y se alejan de la variabilidad que hay en la naturaleza.

ugr Universidad
eez
CSIC