Universitat Autònoma de Barcelona

Enrichment and characterization of anaerobic bacteria degrading organohalide compounds

Alba María Trueba Santiso

Universitat Autònoma de Barcelona

Escola d'Enginyeria

Departament d'Enginyeria Química, Biològica i Ambiental

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PhD Thesis

Alba María Trueba Santiso

Doctorat en Biotecnologia

Supervised by

Dr. Ernest Marco Urrea

Dra. Teresa Vicent Huguet

Dra. Lucía Martín González

Bellaterra, Cerdanyola del Vallès, Barcelona

February 2018

Title: Enrichment and characterization of anaerobic bacteria degrading organohalide compounds

Presented by: Alba María Trueba Santiso

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PhD program in Biotechnology

Departament d'Enginyeria Química, Biològica i Ambiental

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This work was supported by the Spanish Ministry of Economy and Competitiveness and FEDER (projects CTM2013-48545-C2 and CTM2016-75587-C2) and supported by the Generalitat de Catalunya (Consolidated Resarch Group 2014-SGR-476). The author acknowledges the predoctoral fellowship from the Ministry of Economy and Competitiveness (FPI BES-2014-070817), and the financial support for the research stay performed (EEBB-I-17-12318).

Part of this work was done in collaboration with the Department of Isotope Biogeochemistry, Helmholzt Centre for Environmental Research, UFZ, Germany (Dr. Lorenz Adrian), Department of Genetics and Microbiology of the Universitat Autònoma de Barcelona (Dra. N. Gaju, Dra. M. Martínez, E. Parladé), Departament de Cristal-lografia, Mineralogia i Depòsits Minerals, Facultat de Geologia of the Universitat de Barcelona (Dra. Mónica Rosell).

Departament d'Enginyeria Química, Biològica i Ambiental

Escola d'Enginyeria

ERNEST MARCO URREA, Professor Agregat Interí del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, TERESA VICENT HUGUET, Professora Titular del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona, i LUCíA MARTíN GONZÁLEZ, investigadora postdoctoral del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autònoma de Barcelona,

CERTIFIQUEM,

Que la Llicenciada en Biologia Alba María Trueba Santiso ha realitzat sota la nostra direcció, en els laboratoris del Departament d'Enginyeria Química, Biològica i Ambiental de la Universitat Autónoma de Barcelona el treball que amb el títol "Enrichment and characterization of anaerobic bacteria degrading organohalide compounds", es presenta en aquesta memòria, la qual constitueix la seva tesi per optar al Grau de Doctor per la Universitat Autònoma de Barcelona.

I perquè en prengueu coneixement i consti als efectes oportuns, presentem a l'Escola de Postgrau de la Universitat Autònoma de Barcelona l'esmentada tesi, signant el present certificat a Bellaterra, Febrer de 2018,

Dr. Ernest Marco Dra. Teresa Vicent Dra. Lucía Martín

Agradecimientos

Al Ministerio de Economía y Competitividad por la financiación de los proyectos marco de esta tesis (CTM2013-48545-C2 y CTM2016-75587-C2), por la financiación económica de este doctorado (FPI BES-2014-070817), y la estancia de investigación realizada durante el mismo (EEBB-I-17-12318).

Mi mayor agradecimiento es para mis directores de tesis Teresa Vicent, Ernest Marco y Lucía Martín. Os agradezco la oportunidad de haber disfrutado de este doctorado bajo vuestra supervisión. Gracias por vuestra especial dedicación, vuestro continuo apoyo, y por haberme transmitido tanto conocimiento en un amplio sentido de la palabra. Tengo el orgullo de haber trabajado con tres grandes personas durante estos tres años.

Gracias a todos los miembros del grupo de investigación BioRem UAB: Montse, Gloria, Paqui, Jose, Patón, Andrea, Cesc, Marina, Guillem, Natàlia, Romina, Kaidi, David. Un agradecimiento especial es para Lucía Delgado por tu soporte técnico, y por ser como eres.

Quiero transmitir también mi agradecimiento a todas las personas que han colaborado de alguna manera en el trabajo que aquí se presenta. Nùria Gaju, Maira Alonso, Eloi Parladé, Mònica Rosell y David Fernández, gracias por vuestro trabajo y por todo lo que he aprendido con vosotros. A todas las personas que hacen posible nuestro trabajo día a día: personal de limpieza, mantenimiento, secretaría y gestión académica. Gracias especialmente a Manuel Plaza, Pili Martín y Rosi Tello por vuestra asistencia técnica. Extiendo este agradecimiento a todos los miembros del DEQBA, por vuestro buen hacer, y el gran ambiente que se respira en estos pasillos y laboratorios.

Special thanks to Lorenz Adrian from the Department of Isotope and Biogeochemistry of UFZ (Leipzig, Germany) for giving me the wonderful opportunity to perform my research stay in your labs. Thank you for all the knowledge you have transmitted to me and for encouraging me to trust more on my inner power. I also want to transmit my gratitude to Benjamin Scheer for his technical support and for being such a helpful and efficient person. Special thanks also to Katja, Rohit, Chang, Shahab, Camelia, Weiying, Philip, Max, Jan, Joanna, Francis, Stephi and Feli, members of this research group. Thanks for all the nice scientific debates and discussions and for the nice moments we have shared as well. Thank you Sarah and Maria Elena for all the nice moments. Gracias Esther, porque hay cosas que nunca se olvidan!

I also want to express my gratitude to Kenneth Wasmund (University of Vienna) for his magnific work done on the genome-sequencing of the *Dehalogenimonas* strain. Special thanks

for the scientific discussions, and all the knowledge he shared with me during this beautiful work.

A todos los que habéis pasado por el “laboratorio de arriba”: Rober, Marta, Francesc, Diego, Ysabel, Jaime, Jan y Oriol. Al enseñaros, yo también he aprendido mucho. Gracias también por cuidar tan bien a las bebés anaerobias en mi ausencia. A Natália gracias por haberme enseñado otro punto de vista de las cosas, y por lo que nos queda. A David por tu esfuerzo, y tu actitud tan positiva. Mucho ánimo para lo que os queda de camino. Miss Hatijah, thank you for teaching me so much about these anaerobic rebel teenagers. I have so many treasure memories with you. Thank you for being such a special person, for listening all my “anaerobic problems” and offering me your wise advices.

A todos mis compañeros de doctorado. No hubiera sido lo mismo sin vosotros. Gracias por las conversaciones y las risas a la hora de comer, los aguantáaa, las salidas a la montaña, los partidos de voley, la ruta mortal en kayak, los pepetes, las bajadas por despeñaderos, los mercadillos de ropa, las bolas o los papeles doblados, los post-it con mensajitos, las excursiones, cenas, rosas de Sant Jordi, dimecres pastisser, las caras de después de comer, los pasos de baile, las fiestas temáticas, por tener pelazo, y cerebro debajo, por los cafés y tesitos, las conversaciones de cromatógrafo a cromatógrafo, los workshops, las fotos de pájaros, los hey! Como va esa tesis? y por vuestros regalitos cuando me fui a Leipzig. Por todos estos momentos, y muchos más, GRACIAS! Siento no poder poner nombres, pero es que sóis much@s!! Me emociona pensar a cuánta gente increíble he conocido durante estos tres años, y saber que me llevo amigos de verdad! A los que no estáis ya: nos vemos en la vida, amigos! Y los que seguís: nos vemos a las 13:00 en el Chema’s.

Pedro, gracias por integrarme desde el día cero. Gracias por tu alegría, y por miles de momentos. Es eso que tú haces que te hace único. A Jose, compañera de despacho, de grupo, de aventuras, de lo que viene siendo la vida, gracias capa y diva! A los dos, y especialmente por estos meses de escritura, glasias, glasias!

Es muy cierto que pasito a pasito se hace un caminito. En cada pasito que he dado en la vida he tenido la suerte de haber aprendido de gente muy grande, y cada uno de esos pasitos me ha llevado a donde estoy hoy, depositando mi tesis doctoral. Quiero agradecer a Pedro Galán, Damián Romay, María Guillén, Ana López Méndez, Mari Carmen Veiga, Vero Torrijos, Isabel Ruíz y Manuel Soto todo lo que me habéis enseñado, porque a todos vosotros os he considerado mis maestras o maestros en algún momento de mi vida.

Gracias también a toda la gente que ha hecho que Barcelona se haya convertido en mi segundo hogar. Gracias a Laura, Hugo, Daniel, y Enric por literalmente haber compartido hogar conmigo. A Laura, Migue y Ottavia. Al BCN Team, por ser mi familia catalana (aunque seamos mayoría galeguiña) Porque entre todos, hacéis que Barcelona sea un hogar. A Clarita, Enric, Natalia, Luis, Ale, Elías, Víctor, Marc, Javivi, Helena, Bruc, Pedro, y Jose. A todos, os quiero. Sóis lo más bonito de Barcelona.

A Marta, Paula y Sonia. Gracias por vuestro cariño, me acuerdo mucho de vosotras. Seguid mandándome esos vídeos, que me encantan. A Mari Carmen, Ana, Juan, Óscar y Mari, por acogerme desde el primer día. Alejandro, te llevo 20 años. Sin embargo, no sabes cuánto me has enseñado, y no sabes cuánto te admiro. Eres una persona especial. Me gustaría que nunca olvidaras eso. Admiro tu constancia, tu serenidad, tu inteligencia, tu madurez, tu alegría y tu fuerza interior. No tengo ninguna duda de que vas a ser muy grande, en realidad, ya lo eres! No cambies nunca. Te quiero.

A Silvia, Oly, My, Marlén, Bea, Cris, Rafa y Albert, os quiero y os echo siempre de menos.

A Marité, Ángel, Marta, Millán, Antoñito y Ana. A mi abuela Teresa, y a mi abuelo Antonio. A Mari Carmen, Neil, Xacobe y Emma. A Fernando y Pili, a Andrés, a Pepucha, a Manel y a madrina. Tío Comino y María Eugenia (algún día os volveré a explicar qué hacen estas bacterias tan raras). Gracias!!

A Chechu, por haberte venido a Barcelona para compartir la vida conmigo. Por estar siempre a mi lado, incluso cuando estamos lejos. Por tu alegría, tu fuerza, y por tu corazón inmenso. También por escuchar todas mis historias sobre *Dehalobacterium*, el cell harvesting, los picos del cromatógrafo, mis ensayos de presentaciones, e incluso leerte esta tesis de principio a fin. Te amo.

A Adrián y Helvia, hermanos no biológicos. Adrián, eres único, pura fantasía! A Helvia, gracias por encontrar la manera de estar siempre, siempre aquí conmigo, da igual donde estemos.Os quiero.

A mi abuela Pilar, la persona más especial de mi mundo, el norte en mi brújula. Siempre eres fuente inagotable de cariño, de sabiduría, de historias, de fuerza, y de comida también. Te quiero.

A mamá, papá, Roi, abuelo y abuela. A vosotros os agradezco lo más grande que tengo. Gracias por haberme hecho como soy y por apoyarme en cada paso que he dado. Estoy muy orgullosa de mi familia, y a vosotros os dedico el trabajo de esta tesis.

Abstract

The widespread groundwater contamination by organohalide compounds is of a major concern due to the human and ecological risks derived from it. Bioremediation is a sustainable technology that overcomes some limitations of the physical-chemical remediation techniques on these water bodies.

In this study, we aimed to obtain and characterize cultures containing anaerobic bacteria capable of degrading organohalide compounds of environmental concern with potential for *in situ* groundwater bioremediation.

In previous work carried out in our laboratory a highly enriched culture containing organohalide-respiring bacteria from the genus *Dehalogenimonas* degrading vicinally halogenated alkanes was obtained from sediments of the river Besós estuary (Barcelona). In this thesis, the reductive dehalogenase (RDase) from this *Dehalogenimonas* strain responsible for the catalysis of ethylene dibromide (EDB) to the innocuous ethene was identified combining gel-based proteomic techniques, specific enzymatic tests and nano-scale liquid chromatography tandem mass spectrometry (nLC-MS/MS). This RDase is therefore designated as EdbA, for ethylene dibromide RDase subunit A. EdbA is the first RDase identified for debrominating catalytic activity among species of this genus. Moreover, it is the first RDase shown to be functional for respiration without an adjacent membrane-anchoring subunit B encoded on the genome. Additionally, combining ultracentrifugation, gel electrophoresis and nLC-MS/MS, an orthologous enzyme of the dichloropropane-to-propene RDase (DcpA) was the only RDase detected in 1,2,3-trichloropropane-to-allyl chloride dehalogenating cultures. This DcpA was detected in the membrane fraction of the crude protein extract, in accordance to its predicted subcellular localization by bioinformatics tools and it is also not co-localised with an *rdhB* gene. The membrane-anchoring mechanisms of these RDases remains not known and may rely in yet-unidentified proteins.

A second stable bacterial consortium was obtained in the present work from slurry samples of an industrial wastewater treatment plant with a combination of enrichment culture strategies and the dilution-to-extinction technique. This culture was demonstrated to ferment dichloromethane (DCM) and dibromomethane (DBM) into

acetate and formate. The *Dehalobacterium* sp. present in this culture was shown to be the responsible for the dihalomethanes fermentation, and the isolation of this strain was attempted. However, the synergic interactions existing among the different accompanying species present in the bacterial consortia impeded the isolation. Despite a pure culture was not achieved via picking up colonies from semisolid agar cultures, changes in the medium composition, and the application of selected antibiotics, a final relative abundance of *Dehalobacterium* sp. of 67 % was attained. As determined by clone library analysis, bacteria from the genera *Acetobacterium* and *Desulfovibrio* remained present in the culture.

The carbon isotope fractionation during DCM fermentation by this culture was determined by compound-specific stable isotope analysis (CSIA). The value obtained was -27 ± 2‰ and differs from the previously published value of -15.5 ± 1.5‰ of a *Dehalobacter* sp. performing also DCM fermentation. These values are yet significantly different from those reported for facultative methylotrophic bacteria degrading DCM (ranging from -45 to -61‰), and this would allow for further differentiation of these degradation pathways during *in situ* bioremediation works.

Finally, the potential inhibitory effect of selected frequent groundwater co-contaminants over DCM degradation by the *Dehalobacterium*-containing culture was assessed for further *in situ* bioremediation applications. Trichloroethylene (TCE), 1,2-dichloroethane (1,2-DCA), *cis*-dichloroethylene (*cis*-DCE), 1,1,2-trichloroethane (1,1,2-TCA), perfluorooctanoic acid (PFOA), and 3,4-dichloroaniline (3,4-DCA) did not show significant inhibitory effects at the concentrations tested. Differently, a total inhibition was caused with a chloroform concentration of 100 mg/L. Also, the presence of 200 mg/L of perfluorooctanesulfonic acid (PFOS), as well as concentrations higher than 25 mg/L of the pesticide diuron caused a severe inhibitory effect, preventing the full depletion of DCM. Nevertheless, DCM degrading activity was recovered when inhibited cultures were transferred to co-contaminant free medium.

Resumen

La frecuente contaminación de las aguas subterráneas por compuestos organohalogenados es un grave problema ambiental debido a los riesgos ecológicos y para la salud humana de ella derivados. La bioremediación es una tecnología sostenible que evita algunos inconvenientes que presentan los tratamientos físico-químicos.

En este estudio nos proponemos obtener y caracterizar cultivos que contengan bacterias anaerobias que degraden compuestos organohalogenados ambientalmente peligrosos con potencial para la bioremediación *in situ* de aguas subterráneas.

En trabajos previos de nuestro grupo de investigación, se obtuvo un cultivo enriquecido en bacterias del género *Dehalogenimonas* procedente de sedimentos del estuario del río Besós (Barcelona) que degrada alcanos con halógenos situados en carbonos adyacentes. En esta tesis se ha identificado la dehalogenasa reductora (RDasa) de esta cepa de *Dehalogenimonas* implicada en la conversión del dibromuro de etileno (EDB) al compuesto inocuo eteno combinando técnicas de proteómica basadas en geles de electroforesis, ensayos enzimáticos y nano-cromatografía líquida de alta resolución (nLC-MS/MS). Esta RDasa es designada EdbA, y constituye la primera RDasa identificada en este género bacteriano que cataliza una reacción de debromación. Además, es también la primera RDasa en ser demostrada funcional sin una subunidad B de anclaje a la membrana codificada de forma adyacente en el genoma. Adicionalmente, se ha detectado una única RDasa en cultivos que transforman 1,2,3-tricloropropano a cloruro de alilo combinando técnicas de ultracentrifugación, geles de electroforesis y nLC-MS/MS. Esta enzima ortóloga a DcpA, la responsable de la degradación de 1,2-dicloropropano a propeno, ha sido detectada en la fracción proteica de membrana, lo cual concuerta con las predicciones realizadas mediante herramientas bioinformáticas. El mecanismo por el cual EdbA y esta DcpA se anclan a la membrana citoplasmática es desconocido, atribuyéndose a proteínas todavía no descritas.

En este trabajo se ha obtenido un segundo consorcio bacteriano estable a partir de lodos de una planta de tratamiento de aguas residuales industriales aplicando técnicas de cultivo de enriquecimiento y dilución por extinción. Este cultivo fermenta diclorometano (DCM) y dibromometano (DBM) a acetato y formato. Se ha demostrado

que la bacteria responsable de la fermentación pertenece al género *Dehalobacterium*, y se ha procedido a su aislamiento. Sin embargo, las interacciones sinérgicas existentes entre las especies del consorcio han impedido obtener un cultivo puro. Seleccionando colonias en medio de cultivo semisólido, aplicando antibióticos y cambios en la composición del medio, se ha obtenido una abundancia relativa de *Dehalobacterium* del 67%. Le acompañan bacterias de los géneros *Acetobacterium* y *Desulfovibrio*, tal y como se detectó mediante análisis de genotecas.

El fraccionamiento isotópico del carbono durante la fermentación del DCM por este cultivo fue determinado mediante análisis de isótopos estables de compuestos específicos (CSIA). El valor obtenido, -27 ± 2‰, difiere del publicado previamente para una cepa de *Dehalobacter* que también fermenta el DCM (-15.5 ± 1.5‰). Estos valores son significativamente diferentes de los obtenidos con bacterias metilotróficas degradadoras de DCM (-45 a -61‰), y podrían permitir diferenciar vías de degradación de DCM en trabajos de bioremediación *in situ*.

Finalmente, se ha demostrado que la presencia de co-contaminantes que se detectan frecuentemente con el DCM, como el tricloroetileno (TCE), 1,2-dicloroetano (1,2-DCA), *cis*-dicloroetileno (*cis*-DCE), 1,1,2-tricloroetano (1,1,2-TCA), ácido perfluorooctanoico (PFOA) y 3,4-dicloroanilina (3,4-DCA) no provocan una inhibición significativa en la degradación de DCM por parte del cultivo de *Dehalobacterium,* a las concentraciones estudiadas. Una concentración de cloroformo de 100 mg/L provoca una inhibición total. De manera similar, 200 mg/L de sulfonato de perfluoroctano (PFOS), y ≥ 25 mg/L de diuron provocan una inhibición severa, impidiendo la degradación completa del DCM. Sin embargo, la actividad degradadora de DCM se recupera cuando los cultivos inhibidos se transfieren a medio libre de co-contaminantes.

Resum

La freqüent contaminació d'aigua subterrània per compostos organohalogenats és un greu problema degut als riscs humans i ecològics que se'n deriven. La bioremediació és un tècnica sostenible que permet superar algunes de les limitacions que presenten els tractaments fisicoquímics.

En aquest estudi ens proposem obtenir i caracteritzar cultius que contenen bacteris anaerobis capaços de degradar compostos organohalogenats ambientalment perillosos i que es puguin aplicar per a la bioremediació d'aqüífers *in situ*.

En treballs previs realitzats al nostre laboratori es va obtenir un cultiu enriquit que contenia un bacteri dehalorespirador del gènere *Dehalogenimonas* a partir de sediments de la desembocadura del riu Besòs (Barcelona) que degrada alcans amb halògens situats en carbons adjacents. En aquesta tesis, s'ha identificat la dehalogenasa reductora (RDasa) d'aquesta *Dehalogenimona*s implicada en la conversió de dibromur d'etilè (EDB) al compost innocu etilè combinant tècniques de proteòmica basades en gels d'electroforesis, tests enzimàtics i nano-cromatografia líquida acoblada a espectrometria de masses (nLC-MS/MS). Aquesta RDasa es va designar com a EdbA. EdbA és la primera RDasa identificada entre les espècies d'aquest gènere bacterià que catalitza una reacció de debromació. A més, és la primera RDasa que s'ha demostrat funcional i que no té cap subunitat B de fixació a la membrana citoplasmàtica codificada de forma adjacent en el seu genoma. Addicionalment, s'ha detectat un enzim ortolog a l'enzim responsable de la degradació de 1,2-diclorpropà a propé (DcpA) com a única RDasa en cultius que transformen 1,2,3-triclorpropà a clorur d'alil mitjançant la combinació de tècniques d'ultracentrifugació, gels d'electroforesis i nLC-MS/MS. Aquesta DcpA es va detectar en la fracció de la membrana tal i com predeien les eines bioinformàtiques emprades. El mecanisme pel qual aquestes dues RDases identificades es fixen a les membranes és encara desconegut.

En aquesta treball s'ha obtingut un segon consorci bacterià estable provinent de llots d'una planta de tractament d'aigües residuals industrials i aplicant estratègies de d'enriquiment del cultiu i tècniques de dilució fins a l'extinció. Aquest cultiu fermenta diclorometà (DCM) i dibromometà (DBM) en acetat i format. S'ha demostrat que el

bacteri responsable de la fermentació d'aquests dihalometans és un *Dehalobacterium* i s'ha procedit al seu aïllament. Tanmateix, les interaccions sinèrgiques entre les espècies del consorci han impedit el seu aïllament. Mitjançant la selecció de colònies en cultius semi sòlids, canvis en la composició del medi i l'ús de antibiòtics, s'ha assolit un cultiu on l'abundància de *Dehalobacterium* és del 67%. L´acompanyen bacteris dels gèneres *Acetobacterium* i *Desulfovibrio*, tal i com revelen els anàlisis de genoteques.

El fraccionament dels isòtops de carboni durant la fermentació de DCM per aquest cultiu s'ha determinat mitjançant l'anàlisi d'isòtops estables de compostos específics (CSIA). El valor obtingut de -27 ± 2‰ difereix del prèviament publicat per una soca de *Dehalobacter* (-15.5 ± 1.5‰) que també fermentava DCM. Aquests valors són significativament diferent dels obtinguts per bacteris metilotròfics degradadors de DCM (que varien de -45 a -61‰) i podria permetre la distinció entre vies de degradació de DCM en treballs de bioremediació *in situ*.

Finalment, s'ha demostrat que la presència de co-contaminants que es detecten freqüentment amb DCM, tals com tricloroetilè (TCE), 1,2-dicloroetà (1,2-DCA), *cis*-dicloroetilè (*cis*-DCE), 1,1,2-tricloroetà (1,1,2-TCA), àcid perfluorooctanoic (PFOA) i 3,4-dicloroanilina (3,4-DCA) no provoca una inhibició significativa en la degradació de DCM pel cultiu amb *Dehalobacterium* a les concentracions testades. La concentració de cloroform de 100 mg/L provoca una total inhibició. De manera similar, la presència de 200 mg/L d'àcid perfluorooctanosulfonic (PFOS) i ≥ 25 mg/L de diuron provoquen una inhibició severa, impedint la degradació completa de DCM. Tanmateix, l'activitat degradadora de DCM es recupera quan els cultius inhibits es transfereixen a medi fresc sense co-contaminants.

Resumo

A frecuente contaminación das augas subterráneas por compostos organo-haloxenados é un grave problema medioambiental debido ao risco humano e ecolóxico dela derivada. A bioremediación é unha tecnoloxía sostible que evita algún dos inconvenientes que presentan os tratamentos físico-químicos.

Neste estudio, nos propoñemos obter e caracterizar cultivos que conteñan bacterias anaerobias que degraden compostos organo-haloxenados ambientalmente perigosos con potencial para a bioremediación *in situ* das augas subterráneas.

En traballos previos do noso grupo de investigación, obtense un cultivo enriquecido nunha bacteria do xénero *Dehalogenimonas* que degrada alcanos con halóxenos situados en carbonos adxacentes procedente de sedimentos do río Bésos (Barcelona). Nesta tese identifícase a dehaloxenasa reductora (RDasa) desta cepa de *Dehalogenimonas* envolta na conversión do dibromuro de etileno (EDB) ao composto inocuo eteno combinando técnicas de proteómica baseadas en xeles de electroforesis, ensaios enzimáticos e nanocromatografía líquida de alta resolución (nLC-MS/MS). Esta RDasa desígnase como EdbA, e constitúe a primeira RDasa identificada neste xénero bacteriano que cataliza unha reacción de debromación. Ademais, é tamén a primeira RDasa que se amosa funcional sen presentar unha subunidade de ancoraxe á membrana do tipo RdhB codificada de forma adxacente no xenoma. Adicionalmente, detectouse unha única RDasa en cultivos transformadores de 1,2,3-tricloropropano a cloruro de alilo combinando técnicas de ultracentrifugación, xeles de electroforesis e nLC/MS-MS. Esta RDasa é ortóloga á DcpA, responsable da degradación do 1,2-dicloropropano a propeno e foi detectada na fracción proteica da membrana, o cal é acorde ás predicións realizadas mediante ferramentas bioinformáticas. O mecanismo de ancoraxe á membrana citoplasmática da EdbA e desta DcpA é descoñecido, atribuíndose a proteínas aínda non descritas.

Neste traballo obtense un segundo consorcio bacteriano estable a partires de lodos dunha planta de tratamento de augas residuais industriais, e aplicando técnicas de cultivo de enriquecemento e dilución por extinción. Este cultivo fermenta diclorometano (DCM) e dibromometano (DBM) a acetato e formato. Demostrouse que

a bacteria responsable da fermentación pertence ao xénero *Dehalobacterium*, e procedeuse ao seu illamento. Non osbtante, as interaccións sinérxicas entre as especies que conforman este consorcio impediron a obtención dun cultivo puro. Seleccionando colonias en medio semisólido, aplicando antibióticos e cambios na composición do medio, obtense unha abundancia relativa de *Dehalobacterium* do 67 %. Acompáñana bacterias dos xéneros *Acetobacterium* e *Desulfovibrio*, tal e como se detectou mediante análise de xenotecas.

O fraccionamento isotópico do carbono durante a fermentación do DCM por este cultivo foi determinado mediante análises de isótopos estables de compostos específicos (CSIA). O valor obtido, -27 ± 2‰, difire do publicado previamente para unha cepa de *Dehalobacter* que tamén fermenta o DCM (-15.5 ± 1.5‰). Estes valores son significativamente diferentes dos obtidos con bacterias metilotróficas degradadoras do DCM (-45 a -61‰), e poderían permitir a diferenciación entre vías de degradación do DCM en traballos de bioremediación *in situ*.

Finalmente, demostrouse que a presencia de co-contaminantes frecuentemente detectados xunto co DCM, como o tricloroetileno (TCE), 1,2-dicloroetano (1,2-DCA), *cis*-dicloroetileno (*cis*-DCE), 1,1,2-tricloroetano (1,1,2-TCA), ácido perfluorooctanoico (PFOA) e 3,4-dicloroanilina (3,4-DCA) non provocan unha inhibición significativa na degradación do DCM por parte do cultivo de *Dehalobacterium*, nas concentracións estudiadas. Unha concentración de cloroformo de 100 mg/L provoca unha inhibición total. Dunha maneira similar, 200 mg/L de sulfonato de perfluoroctano (PFOS), e ≥ 25 mg/L de diuron provocan unha inhibición severa, impedindo a completa degradación do DCM. Nembargantes, a actividade degradadora do DCM recupérase cando os cultivos inhibidos se transfiren a medio libre de co-contaminantes. Estes resultados son de grande valor de cara a avaliar o potencial deste cultivo de *Dehalobacterium* para unha aplicación real de bioremediación *in situ*.

LIST OF CONTENTS

LIST OF ACRONYMS

1,1,2-TCA	1,1,2-trichloroethane
1,2,3-TCP	1,2,3-trichloropropane
1,2,4-TCB	1,2,4-trichlorobenzene
1,2-DCA	1,2-dichloroethane
1,2-DCP	1,2-dichloropropane
16S rRNA	16S ribosomal ribonucleic acid
3,4-DCA	3,4-dichloroaniline
AKIE	Apparent kinetic isotopic effect
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BES	2-bromoethanesulfonate
BLASTn	Basic Local alignment search tool nucleotides database
BLASTp	Basic Local alignment search tool proteins database
BN-PAGE	Blue native polyacrylamide gel electrophoresis
CB	Chlorobenzene
C-Cl	Carbon-chlorine bond
CerA	Chloroethene reductase subunit A
C-F	Carbon-fluorine bond
CF	Chloroform
CFCs	Chlorofluorocarbons
***cis*-DCE**	*cis*-dichloroethylene
CISM	Complex ison-sulfur molybdoenzyme
CM	Chloromethane
CSIA	Compound specific isotope analysis
DBM	Dibromomethane
DCM	Dichloromethane
DcpA	Dichloropropane-to-propene dehalogenase subunit A
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
DNAPL	Dense non-aqueous phase liquid
EA	Elemental analyzer
EDB	Ethylene dibromide
EdbA	Ethylene dibromide dehalogenase subunit A
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
EU	European Union
Ɛc	Carbon isotopic enrichment factor
FASTA	Text-based format representing nucleotide or peptide sequences, in which using single-letter codes
FID	Flame ionization detector
GC	Gas chromatography
HPLC	High pressure liquid chromatography
HS-SPME	Head-space solid-phase microextraction

IARC	International Agency for Research on Cancer
IRMS	Isotope ratio mass spectrometry
kDa	Kilodaltons
KIE	Kinetic isotopic effect
LE	Lag time extension
MBR	Membrane bioreactor
MIC	Minimum inhibitory concentration
MS/MS	Tandem mass spectrometry
NCBI	National Center for biotechnology information
OHRB	Organohalide respiring bacteria
OTU	Operational taxonomic unit
PBS	Phosphate-buffered saline
PCB	Polychlorinated biphenyl
PCE	Tetrachloroethylene
PceA	Tetrachloroethylene reductive dehalogenase
PCR	Polymerase chain reaction
PES	Polyethersulfone
PFASs	Poly and perfluoroalkyl substances
PFOA	Perfluoroctanoic acid
PFOS	Perfluorooctanesulfonic acid
PSMs	Peptide-to-spectrum matches
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene fluoride
RAST	Rapid Annotation using subsystems categories
RDase	Characterized reductive dehalogenase
Rdh	Uncharacterized reductive dehalogenase
RdhA	Reductive dehalogenase subunit A
RdhB	Reductive dehalogenase subunit B
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Tat	Twin-arginine translocation
TCD	Thermal conductivity detector
TCE	Trichloroethylene
TceA	Trichloroethylene reductive dehalogenase subunit A
TdrA	Trans-dichloroethene reductive dehalogenase subunit A
TOC	Total organic carbon
trans-DCE	Trans-dichloroethylene
U.S	United States of America
UV	Ultraviolet
VC	Vinyl chloride
VcrA	Vinyl chloride reductive dehalogenase subunit A
VOC	Volatile organic compound
VPDB	Vienna Pee Dee Belemnite
WWTP	Wastewater treatment plant
ʎ	Lag time

Thesis overview

This thesis is divided in nine chapters.

Chapter 1 is the general introduction to the research topic. Here, the main characteristics, origins and problematics of groundwater pollution by organohalide compounds are presented. Also, a brief introduction on the remediation technologies for this type of pollution are introduced. The main groups of strictly anaerobic bacteria with potential for *in situ* bioremediation of groundwater are described and categorized based on their degradation metabolism. Finally, the main challenges for research of bioremediation of aquifers impacted with organohalides are discussed.

In **Chapter 2** the overall objective of the thesis is presented, as well as the specific objectives derived from it.

Chapter 3 presents the general materials and methods used in most of the experimental works presented in the following chapters.

Chapter 4, 5, 6, 7 and 8 detail the main results derived from this thesis, being each chapter divided into five sections: a specific introduction, specific materials and methods, results and discussion, conclusions and references cited through the chapter.

A stable anaerobic culture containing a bacteria from the genus *Dehalogenimonas* was previously obtained from sediments of the river Besós estuary (Barcelona) during the doctoral thesis of Siti Hatijah Mortan, which constituted the first thesis on this research line in our research group. The aim of **Chapter 4** was the identification of the reductive dehalogenases involved in the transformation of ethylene dibromide [EDB] and 1,2,3-trichloropropane [1,2,3-TCP] in this bacterium using different proteomics techniques and a draft genome sequence of this bacterium. The genome annotation of this strain is currently undergoing and will be deposited in public databases. The proteomic work was done during a research stay at the Helmholtz-Centre for Environmental Research (UFZ) under the supervision of Dr. Lorenz Adrian from May to September of 2017.

In **Chapter 5**, inoculum from different sediment rivers and sludge from wastewater treatment plants were tested to transform anoxically different selected halogenated compounds of emerging concern. DCM fermentation to acetate and formate was

detected in one cultures set and the microbial composition of the consortium was analysed.

In **Chapter 6**, the bacterium responsible for this DCM degradation process was identified, being affiliated with the genus *Dehalobacterium*. The isolation attempt was performed using different approaches and the impact of the different treatments on the bacteria composition was assessed by molecular techniques.

Chapter 7 presents the carbon stable isotope analysis (CSIA) performed with this *Dehalobacterium*-containing culture fermenting DCM, and the potential of the carbon isotopic fractionation value obtained to distinguish DCM degradation pathways was assessed. CSIA analyses were performed at the facilities of Universitat de Barcelona (UB).

Chapter 8 presents the study of the potential inhibitory effect of selected organohalide compounds frequently detected as co-contaminants in DCM impacted aquifers over the DCM fermentation capacities of the *Dehalobacterium*-containing culture.

In **Chapter 9** the main conclusions of the present thesis are presented, being an overview of the main achievements accomplished. Also, the future work derived from the results obtained is discussed in this Chapter.

Finally, the CV of the author, including her list of publications and contributions is included at the end of this thesis document.

Chapter 1

General Introduction

1.1 Human and ecological risks derived from oganohalide compounds

The uses of organohalide compounds in anthropogenic activities are varied and include their industrial applications as solvents, adhesives, plastics, cross-linking agents, plasticizers, metal degreasing agents or intermediates for chemical synthesis. Also several lead scavengers in fuels, pesticides, herbicides, flame-retardants and pharmaceuticals include organohalogens on their formulations (Bhatt *et al.*, 2007; Jeschke, 2017). Substitutions with chlorine, bromine, or fluorine are the most commonly occurring and produce organochlorine, organobromine, and organofluorine compounds, respectively.

The same physical and chemical properties that have made these compounds so attractive for industrial uses, including being highly stable and resistant to degradation, have also made them extremely dangerous to the environment and the human population. Organohalide compounds tend to have very long half-lives, allowing them to persist in the environment and in animal tissue (EPA, 2000).

According to Nikel *et al.*, (2013), the organohalides are recalcitrant to aerobic biodegradation for several reasons: (i) they are highly toxic for microbes, (ii) most complex organohalides are unnatural compounds recently introduced into the environment by human activities, and microorganisms are unlikely to possess complete catabolic pathways for their mineralization, and (iii) if degradation is initiated by a dehalogenation reaction catalysed by dehalogenases or oxidases, the lack of enzymes for the rapid conversion of the resulting halogenated alcohols or aldehydes would lead to the accumulation of toxic and/or highly reactive metabolic intermediates.

In regards of the ecosystem fitness, organohalides are known to affect the reproduction of aquatic animals, bioconcentrate in the food chain, accumulate in estuary sediments, affect the soils microbiota populations, cause unintended effects on non-target species, and impact local wildlife to the point of pushing to the extinction endangered species (Naidoo et al., 2009; Imfeld and Vuilleumier, 2012; Soto Castiñeira, 2013; Koenig *et al.*, 2014).

Many halogenated compounds are known to be toxic, mutagenic, carcinogenic or endocrine disruptors, among other risks to human health (ATSDR, 2000; Diamanti-Kandarakis *et al*., 2009; ATSDR, 2009). It has also been shown that continuous exposure to low, subtoxic concentrations of certain halogenated pharmaceuticals and personal care products (PPCPs) can cause undesirable effects on humans (Sui *et al.*, 2015). Derived from these risks, the halogenated compounds 1,2-dichloroethane (1,2-DCA), trichloroethylene (TCE), tetrachloroethylene (PCE), vinyl chloride (VC), the total sum of trihalomethanes and chloroform (CF) are regulated in the sanitary criteria for drinking water by the Spanish government (RD 140/2003).

Organohalide compounds are not only artificially produced. Also, several natural processes were identified to originate these compounds. Around 5000 chlorinated compounds, and other halogenated chemicals are known to be produced in natural processes such as geothermal activities, volcano emissions, or forest fires (Frische *et al.*, 2006; Field, 2016). Likewise, living organisms release these compounds via their metabolic processes. For instance, marine algae are important sources of brominated compounds, as well as insects, fungi or mammals that are also contributing to organohalide presence in nature (Quack *et al.*, 2007; Field, 2016). The real extent of the natural production of organohalides is still not totally understood.

1.2 Halogenated compounds as groundwater pollutants

Roughly, the 70 % of the Earth is covered by water, with fresh water constituting less than the 3% of the total amount of water available, including ice caps. From all the existing fresh water, less than 1% is usable in a renewable way (or 0.01 % of all water). Discarding glaciers and ice caps, groundwater is estimated to represent 98% of the available fresh water on Earth (NASA Earth Observatory, 2003). Fifty percent of the total population relies on groundwater for daily drinking water, and 37 % of agricultural water use depends on this water body (Zehnder, 2005). Groundwater, according to Directive 2006/118/EC, is not only the largest fresh water body in the European Union, but also the most sensitive to pollution.

Over the last 150 years, the number of organic chemicals released into the environment has increased markedly, leaving an unprecedented chemical footprint on Earth (Loganathan, 2012). Regarding groundwater, many contaminations originates from point sources, derived from industrial emissions, accidents or deliberated spills. For instance, around an 80% of the impacted soil and groundwater sites detected so far in Catalonia were originated from industrial and commercial practices (Fernández-García *et al.*, 2014). These contaminations typically form plumes with high concentrations of pollutants (µg/L to mg/L range). Alternatively, chemicals may enter groundwater via widespread applications derived from agriculture or release from sewage treatment into rivers. Here, pesticides, pharmaceuticals, or consumer care products are introduced as nonpoint sources and typically occur in much smaller concentrations (micropollutants in ng/L to µg/L range) (Meckenstock *et al.*, 2015).

More than 50% of the top fifty compounds included in the ATSDR List of Hazardous compounds are halogenated pollutants. This list ranks the substances in regards of their toxicity, frequency of detection at the monitored facilities of the U.S National Priorities List (NPL), and potential for human exposure (ATSDR, 2017). The halogenated compounds present in this list include industrial solvents (e.g. TCE or dichloromethane [DCM]), lead scavengers (e.g. ethylene dibromide [EDB]) or pesticides (e.g. diuron), among other families of contaminants. Additionally, halogenated volatile organic compounds are the most frequently occurring type of soil and groundwater contaminants in Europe, and particularly in Spain (Scheidleder *et al*., 1999), as well as at Superfund and other hazardous waste sites in the U.S (EPA, 2000). The presence of organochlorine compounds was detected in 95 of the soil and groundwater polluted sites detected in Catalonia up to 2014 (Fernández-García, D., Carles Brangarí, 2014) .

The majority of the halogenated pollutants are chlorinated, however also fluorinated compounds, such as flame retardants (e.g. PFOS, PFOA), or chlorofluorocarbons (CFCs) have been extensively used and released into the environment, posing a high risk to the environment and the human health (Campo *et al.*, 2014; Bartolomé *et al.*, 2017). Regarding the iodinated compounds, mainly X-ray contrast agents as iopromide or iopamidol, due to their daily use in medical practices

during the last decades have also reached the groundwater (Cabeza *et al.*, 2012; Sui *et al.*, 2015).

1.3 Remediation technologies for groundwater restoration

The removal of contaminants from groundwater present some constraints, as for instance, the low accessibility. Also, groundwater ecosystems are much more heterogeneous and dynamic than currently perceived. Therefore, a variety of factors including the hydrology, the characteristics of the contamination plume, the existence of heterogeneous flow paths, or limitation factors related with the diffusion should be taken into account in detoxification projects (Meckenstock *et al.*, 2015).

Moreover, many chlorinated solvents can occur as dense non-aqueous phase liquids (DNAPLs). Once released, DNAPL can migrate through the soil and reach the groundwater table. An aliquot of DNAPL (referred to as 'free' or 'mobile' DNAPL) then moves downward through the aquifer and eventually forms 'pools' on low permeability surfaces, such as clay layers. DNAPL movement is more often controlled by gravity than by hydraulic head in the groundwater. Therefore, an in-depth understanding of site geology and hydrogeology is needed before locations and pathways of DNAPL movement can be identified. Both the residual and mobile DNAPL act as slow releaser sources of groundwater contamination that continuously dissolve into the water flow, often generating large contamination plumes. These DNAPLs represent a major challenge to site remediation due to their persistence and relative inaccessibility (Majone *et al.*, 2015).

Remediation technologies can be divided into biological, chemical or physical treatments, yet sometimes combinations of technologies are applied inside the same remediation project to reduce the contamination to safe and acceptable levels. The most common cleanup method for groundwater remediation is the pump and treat (Majone *et al.*, 2015). This technology involves the installation of wells for pumping the groundwater to the ground surface, directly into a treatment system, or into a storing tank until treatment begins. Treatment can be for instance activated carbon adsorption or chemical oxidation (Rivett *et al.*, 2006; Spira *et al.*, 2006).

In the same way as ecosystems produces halogenated compounds, ecosystems also harbour microorganisms with potential for the degradation of these compounds (Nadalig *et al.*, 2014). However, the role of these microorganisms in the recycling of halogenated compounds is crucial, but still poorly understood (Zanaroli *et al.*, 2015). The nature of these microorganisms is the basis for organohalide compounds bioremediation.

Bioremediation is the exploitation of the ability of microorganisms to degrade and/or detoxify organic contamination, and it presents several advantages over more conventional technologies (Zehnder, 2005; Majone *et al.*, 2015). In many cases, pollutants can be treated at the site (*in sit*u), thus reducing the risk of exposure. Furthermore, bioremediation techniques usually can be expected to have minimal environmental impact, since the aquifer is not dewatered, and bioremediation is a natural process. Also, waste products are frequently not generated, as bioremediation often results in the conversion of contaminants to innocuous products. Unlike many physicochemical treatment processes that mainly transfer the pollutants from one phase (or location) to another, bioremediation offers a terminal solution. These characteristics make bioremediation techniques potentially ideal for the detoxification of organohalide pollutants (EPA, 2000). The less intrusive bioremediation approach is the monitored natural attenuation (MNA) also named intrinsic bioremediation, which is a technology based on monitoring the progress of natural, non-engineered processes that often include biodegradation, but abiotic processes as well (e.g. chemical reactions with natural occurring iron). Alternatively, enhanced *in situ* bioremediation is based on the manipulation of environmental conditions within the contaminated aquifer to increase the microbial activity comprising also the increase of contaminant availability. This includes the biostimulation and the bioaugmentation. Biostimulation can be achieved through the delivery of nutrients or electron donors/acceptors and/or control of pH, redox state, or temperature (Majone *et al.*, 2015) into the subsurface. An anaerobic groundwater treatment zone is then created, and hydrogen is generated through fermentation reactions. These conditions are conducive to anaerobic biodegradation of chlorinated solvents dissolved in groundwater. In some cases, microbial amendments of non-native bacteria are done

when the native microbiota is incapable of performing the required transformations, and this is called bioaugmentation (Leeson *et al.*, 2004).

A main advantage of *in situ* bioremediation technologies for groundwater relies on its cost-effectiveness when compared to more conventional physical-chemical treatments. The major cost of *in situ* groundwater bioremediation relies on the installation of monitoring and injection wells, and the prize of amendments to the aquifer, e.g. lactate injections in bioestimulation or extrinsic microbiota delivery in bioaugmentation. However, in general terms, an *in situ* bioremediation work save energy costs derived from extraction pumping, transportation of the contaminated groundwater if required, and further thermal treatment for the activated carbon, if compared to a conventional pump and treat technique (Majone *et al.*, 2015).

1.4 Bacteria capable of degrading organohalides

Microbially mediated reductive dechlorination usually occurs through hydrogenolysis or dichloroelimination. In hydrogenolysis, a chlorine atom is replaced by a hydrogen atom, with a net input of two electrons (Bhatt *et al.*, 2007). As an example, the reductive dechlorination of PCE proceeds to ethene through a sequence of hydrogenolysis steps, involving the intermediate formation of TCE, *cis*-dichloroethene (*cis*-DCE) and VC (McCarty, 2016). Dihaloelimination is the concomitant removal of two halogen atoms from vicinal carbon atoms, which results in the formation of a double bond between the two carbon atoms, requiring a net input of two electrons (Dolfing, 2016). Haloalkanes can also undergo dehydrochlorination, that is the concomitant removal of a halogen and a hydrogen atoms from adjacent carbons, so converting an (n) chlorinated alkane into the (n - 1) chlorinated alkene. Dehydrochlorination is an abiotic reaction which does not require the input of electrons (Leeson *et al.*, 2004).

The full understanding of factors controlling the relative extent of these different reaction pathways as for instance the field conditions is required because intermediate daughter products can have different persistence, mobility, and toxicity. As an example, the dechlorination of tetrachloroethane can occur via hydrogenolysis,

dichloroelimination and dehydrochlorination, and depending on the pathway, the toxic VC may either be formed or not (Majone *et al.*, 2015).

The strong electronegativity of chlorine atoms gives an oxidising character to polychlorinated aliphatic compounds, so that they can be reductively dechlorinated by serving as respiratory electron acceptors under the anaerobic conditions usually found in contaminated groundwater. These conditions pose the strictly anaerobic bacteria as the best candidates for organohalide bioremediation projects on these locations. However, organohalide compounds can be also transformed in aquifers by no strictly anaerobic bacteria. For instance, facultative methylotrophic bacteria were found to transform dichloromethane in groundwater (Muller *et al.*, 2011). Additionally, some facultative anaerobes were found to degrade organohalides in cometabolic processes. For instance, bacteria from the genus *Pseudomonas* can degrade TCE or tetrachloromethane, when using toluene or fumarate. The also facultative anaerobes from *Xanthobacter* genus can degrade TCE, VC, *cis*- and *trans*-DCE, as well as chlorophenols in cometabolic proceses (Bhatt *et al.*, 2007). Interestingly, *Xanthobacter* strain EL8 was found to grow on 1,2-DCA under denitrifying conditions. This strain was detected at a DCA impacted site together with organohalide-respiring bacteria from different genera of organohalide-respiring bacteria (Munro *et al*., 2017). Regarding the strictly anaerobic organohalide transforming bacteria, they can be roughly divided into two groups according to the mechanism employed for the degradation process, and here we will focus on these two groups presenting their main characteristics.

1.4.1 Organohalide-respiring bacteria

Organohalide-respiring bacteria (OHRB) harbour the capability to couple reductive dehalogenation with energy conservation and growth (Adrian and Löffler, 2016). During organohalide respiration, hydrogen is tipically used as electron donor and the organohalide compound as electron acceptor. In this processes, reductive dehalogenation is catalysed by reductive dehalogenase enzymes, which are denotated as RDases if they are characterized, and Rdh when they are not (Leys *et al.*, 2013). This is a diverse protein family with low sequence similarity and a punctuated distribution across the tree of life. The diversity, environmental distribution, and evolution of the

Rdh proteins remain open questions. The evolutionary history of the reductive dehalogenases includes vertical inheritance combined with numerous mechanisms for lateral transfer, including integrases, circularizing transposable elements, and, possibly, phage-mediated transfer. The Rdh remain incompletely characterized from the perspectives of sequence diversity, substrate specificities, global distribution, and modes of inheritance (Hug, 2016).

Most so far characterised RDases proven to be functional in respiration are encoded by operons composed mainly by two genes: *rdhA*, encoding for the catalytic subunit A and *rdhB*, putatively encoding a small membrane anchoring subunit B, as well as other associated genes (Hug *et al.*, 2013), as exemplified in Fig.1.1. Additionally, other RdhA homologs are known to function in a non-respiratory manner, i.e., they may act to dehalogenate halogenated organic molecules, so microorganisms can access the non-dehalogenated daughter molecules for subsequent catabolism. The so far characterised 'non-respiratory' reductive dehalogenases are seemingly not encoded with associated *rdhB* genes (Chen *et al.*, 2013; Payne *et al.*, 2015).

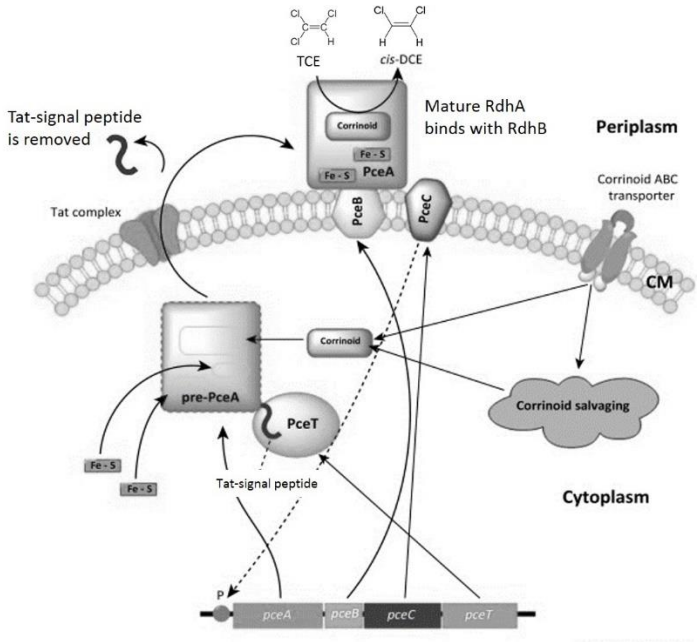

Figure 1.1 A proposed model of reductive dehalogenase maturation in *Dehalobacter restrictus*. The overall steps involved in the RDases biosynthesis are shown. First, the translation of a catalytic subunit (PceA), membrane-anchoring subunit (PceB), a chaperone protein (PceT), and a transcriptional regulator (PceC) from their corresponding genes, and constitution of cofactors (two Fe–S clusters and a corrinoid). The pre-PceA protein is exported to the periplasm via the Tat secretion pathway, losing the Tat-signal peptide and is then binded to the corresponding membrane anchoring protein PceB. Modified from Jugder *et al.*, 2015).

RdhA proteins contain two Fe–S clusters (either two 4Fe–4S or one 4Fe–4S and one 3Fe–4S) in the C-terminus, and an intervening sequence of 450–500 amino acids. Many, but not all RdhA, present a twin-arginine translocation (Tat) signal sequence (RRXFXK) and a hydrophobic stretch at the N-terminus (Jugder *et al.*, 2015). The Tat pathway serves the role of transporting folded proteins across energy-transducing membranes and acts separately from the general secretory (Sec) pathway, which transports proteins in an unfolded state (Patel *et al.*, 2014). Therefore, RdhAs containing Tat-signal peptide are transported to the outer side of the cytoplasmic membrane, losing the Tat-signal sequence in a post-translational modification and becoming into their mature state (Fig. 1.1). Thus, mature RdhA may require membrane anchoring subunits, presumably RdhB homologs (Kruse *et al.*, 2016). Additionally, the Tat signal is also used for recognition by chaperones (Jugder *et al.*, 2015).

Among all OHRB genus characterized to date, the obligate OHRB *Dehalococcoides* constitute the most studied one. Bacteria from this genus were first discovered in an enrichment culture from sewage sludge that reductively dechlorinated the groundwater pollutants PCE and TCE to VC and ethene, in contrast to other organohalide-respiring bacteria that dechlorinated PCE and TCE only as far as dichloroethenes (DCEs). The presence of *D. mccartyi* at chloroethene-contaminated groundwater sites is linked to dechlorination of PCE and TCE past DCEs, and molecular analysis targeting these bacteria and its associated *rdhAB* genes have become part of contaminated site characterization (Zinder, 2016). Moreover, *D. mccartyi*-containing cultures have been commercially developed for bioaugmentation, especially strains BAV1 and VS that can efficiently convert VC to nontoxic ethene (Ritalahti *et al.*, 2006). Members of this genus use a variety of halogenated aliphatic and aromatic compounds as electron acceptors, including chlorobenzenes, dioxins, pentachlorophenol, bromophenol or PCBs (Zinder, 2016).

Another interesting obligate OHRB are those belonging to the genus *Dehalogenimonas*. In contrast to the wide array of electron acceptors suitable for *Dehalococcoides* strains, some bacteria from the genus *Dehalogenimonas* are only capable of transforming vicinally halogenated alkanes via dihaloelimination, such as 1,2-dichloropropane, 1,2-dichloroethane, or ethylene dibromide, presenting therefore

a higher substrate specialization (Moe *et al.*, 2016). However, the WBC-2 strain was reported to transform *trans*-DCE into VC via hydrogenolysis (Molenda *et al.*, 2016) and 'Candidatus *Dehalogenimonas etheniformans*' was shown to grow respiring chlorinated ethenes, including VC into ethene (Yang *et al.*, 2017) expanding the substrate range of this genus.

Other bacteria from the genera *Dehalobacter* (Maillard and Holliger, 2016), *Desulfitobacterium* (Ding *et al.*, 2014; Zhao *et al.*, 2015), or *Sulfurospirillum* (Goris and Diekert, 2016) are facultative OHRB capable of growing using a wide array of organohalide compounds as electron acceptors, but also harbour other energy-gaining metabolisms.

Despite of the broad spectrum of halogenated compounds shown to be used by OHRB, to date no organohalide-respiration process has been described for the case of the widespread pollutant DCM. Thus, in case of aquifer pollution with DCM, other groups of bacteria should be considered for bioremediation projects.

1.4.2 Organohalide-fermenting bacteria

Many organohalogens are considered to be fermentable. Fermentations are disproportionation reactions, which means these are redox reactions in which a portion of a compound of intermediate oxidation is oxidized while the remainder is reduced and no external electron acceptor is involved (Madsen, 2015). An example of organohalogen that in theory can be fermented is trichloroethane. At least two pathways can be predicted: fermentation of trichloroethane to acetate, and fermentation of trichloroethane to ethane. Also, chlorophenol fermentation to acetate and HCl is thermodynamically possible. However, these reactions were only theoretically calculated, and as stated in Dolfing, (2016), they have not been reported in nature to date. The only example of organohalogenated compound that have been shown to be fermented to date is dichloromethane (DCM, methylene dichloride).

DCM is one of the halogenated solvents most frequently detected in groundwater. Also, it is considered to be more persistent in anoxic water than in aerobic water, as exemplified in Fig.1.3. DCM is toxic and probable carcinogen (ATSDR,

2000), and it was recently proposed to be included in the Montreal Protocol due to its increasing threat to the stratospheric ozone (Hossaini *et al.*, 2017).

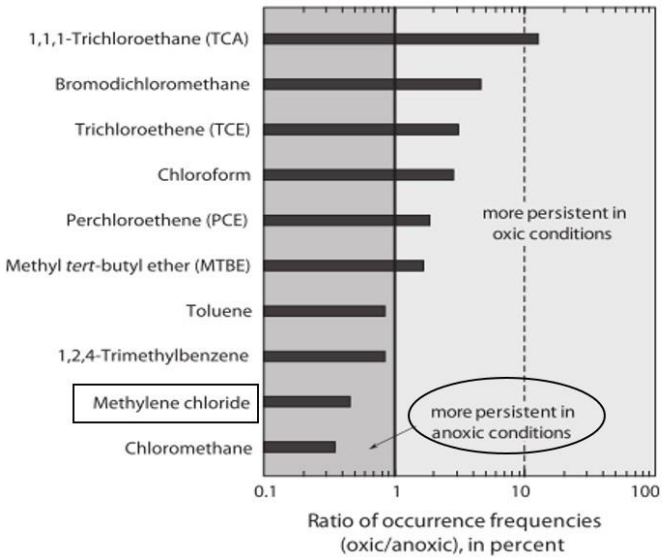

Figure 1.3 Ratio of oxic occurrence frequency to anoxic occurrence frequency from 1996 – 2002 of 10 halogenated compounds in aquifers of U.S. Assessment level of 0.02 µg ⁄ L was considered. Oxic conditions are considered when groundwater presents a dissolved-oxygen concentration ≥ 0.5 mg⁄L, and anoxic conditions with dissolved-oxygen concentration < 0.5 mg⁄ L. Modified from Carter *et al.*, (2008).

Regarding strictly anaerobic bacteria, to date, only three genera affiliated with the family Peptococcaceae have been described to metabolize DCM, all of them performing fermentation processes. *Dehalobacterium formicoaceticum* strain DMC was the first strictly anaerobic bacterium isolated and characterized. Since then, there have been no other *DCM* fermentators isolated described in the literature (Dolfing, 2016).

D. formicoaceticum was able to ferment DCM, producing formate and acetate in a molar ratio of 2:1, respectively. This first step of this DCM degradation pathway is the transference of the methylene group of DCM onto methylene tetrahydrofolate (Fig.1.4). This key reaction of anoxic DCM utilization was demonstrated to occur *in vitro* and it is dependent on the presence of ATP, hydrogen and methyl viologen in the incubation mixture (Mägli *et al.*, 1998). However, the removal mechanism of the chlorine substituents remains unclear. Reductive dechlorination is apparently not involved, as chloromethane is not produced and no reductive dehalogensases are encoded in the genome of *D. formicoaceticum* strain DMC (Chen *et al.*, 2017). This

requirement of hydrogen and the electron carrier methyl viologen in the incubation mixture point the role of a reduced corrinoid in the DCM dehalogenation step, since electrons from hydrogen may be required to keep the catalyst in the reduced state. The light-reversible inhibition of this step by propyl iodide, as well as the strong sensitivity of the system to the oxygen, support the role of a Co-(I)-corrinoid (Mägli *et al.*, 1998).

The further conversion of two third parts of the methylene tetrahydrofolate produced is hypothesized to proceed via oxidation to formate by enzymes from the acetyl coenzyme A pathway (Mägli *et al.*, 1998)(Fig.1.4). Accordingly, the complete set of enzymes involved in the Wood-Ljungdahl pathway with a featured core acetyl coenzyme A synthase (acs) gene cluster are encoded in *D. formicoaceticum* strain DMC genome (Chen *et al.*, 2017). The reducing equivalents of this oxidation are used by the methylene tetrahydrofolate reductase and the CO_2 dehydrogenase, with the incorporation of CO_2 from the medium, to produce acetate (Fig.1.4). The carboxyl group of acetate is originated from CO_2, while the methyl group derives from the DCM. The activity of methylene tetrahydrofolate reductase was detected only in desalted crude protein extracts, and was not depending on the utilization of methyl viologen o NADH, relying on a yet unidentified endogenous electron donor (Mägli *et al.*, 1998).

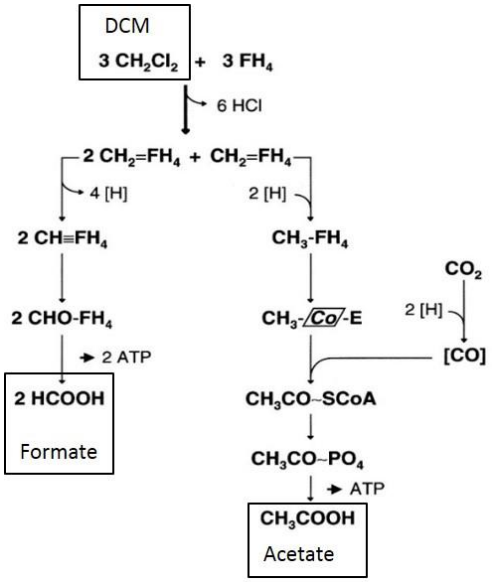

Figure 1.4 Proposed pathway for the metabolism of dichloromethane by *Dehalobacterium formicoaceticum* strain DMC, showing the production of formate and acetate. Modified from Mägli *et al.*, 1998.

D. formicoaceticum strain DMC does not grow by using the typical acetogenic substrates, such as CO_2 and H_2 or formate. This was at first hypothesized to be caused by the lack of ATP gaining by a chemiosmotic mechanism in this bacteria. At substrate level there is no ATP production during acetogenesis from CO_2 and H_2, or formate, and the growth on these substrates therefore depends on energy conservation via chemiosmotic mechanisms. DCM fermentation, differently results in ATP generation by acetate kinase and the reverse formyl tetrahydrofolate synthase reaction. ATP synthase activity was detected in *D. formicoaceticum* cell extracts, and attributed to a F_0-F_1-type ATP synthase, as hypothesized in (Mägli *et al.*, 1998). This protein was later shown to be encoded in the genome of this strain (Chen *et al.*, 2017). Energy conservation appears to be sodium independent. *D. formicoaceticum* therefore was suggested to belong to the group of acetogenic bacteria generating a proton diffusion potential for ATP synthesis during acetogenesis. Cytochromes may be then involved in the delivery of electrons to methylene tetrahydrofolate reductase, and accordingly genes encoding cytochromes were revealed in the genome of *D. formicoaceticum* strain DMC (Chen *et al.*, 2017).

The majority of the enzymes involved in the DCM fermentation pathway of *D. formicoaceticum* were demonstrated in the impressive works published in Mägli *et al.*, 1996 and Mägli *et al.*, 1998. Nevertheless, there is no empirical demonstration for certain reactions involved, the protein responsible for the DCM dehalogenation remains unknown, and more detailed study may be required to completely develop the unsolved steps.

Two different strains of the genus *Dehalobacter* have also shown ability to ferment DCM, expanding the metabolic versatility of this genus beyond organohalide respiration (Justicia-Leon *et al.*, 2012; Lee *et al.*, 2012). DCM fermentation in the non-methanogenic culture described in Justicia-Leon *et al.*, 2012 which contained *Dehalobacter* and *Acetobacterium* members yielded acetate and biomass. The achieved partial mass balance suggests a 2:1 DCM/acetate ratio and concurs with a previous report on DCM fermentation in the original acetogenic mixed culture from *Dehalobacterium formicoaceticum* and *Desulfovibrio* sp. (Justicia-Leon *et al.*, 2012). The production of acetate, methane and biomass mirrored the cumulative

consumption of DCM while formate was detectable only in trace amounts in the *Dehalobacter*-containing culture described in Lee *et al.*, 2012 in which also other taxa were detected (e.g. *Desulfovibrio*).

More recently, the novel DCM-fermenting species 'Candidatus *Dichloromethanomonas elyunquensis'* was identified and genome-sequenced. The genome encodes the Wood-Ljungdahl pathway, hydrogenases and, interestingly reductive dehalogenases (Kleindienst *et al.*, 2017). In this case, no methane was produced in these cultures with inhibited methanogenesis, and 97.1 ± 1.9% of the DCM-carbon could be accounted for in the amount of acetate produced. Hydrogen production was observed prior to acetate and methane formation, suggesting that hydrogen was generated during DCM degradation and supported H_2/CO_2-consuming methanogens and acetogens (Kleindienst *et al.*, 2017).

The sequencing of new genomes of DCM-degrader strains belonging to Peptococcaceae family would permit the better understanding of this peculiar group of bacteria. Also, it would be important to develop tools that allow to distinguish DCM degradation pathways taking part in aquifers during *in situ* bioremediation projects.

1.5 Current challenges on the groundwater bioremediation of halogenated compounds.

As exemplified above, the nature of anaerobic microorganisms capable of transforming halogenated pollutants into innocuous or less toxic compounds pose a solution for the negative impacts of these contaminants. Biodegradation is one of the most favoured and sustainable means of removing organic pollutants from contaminated aquifers, yet there is insufficient knowledge on certain aspects of this microbial-mediated processes (Meckenstock *et al.*, 2015).

Research efforts over the last two decades have substantially improved the understanding of OHRB, and this progress has supported successful bioremediation applications in aquifers contaminated with chlorinated solvents. Yet, major knowledge gaps remain, and detailed research on biochemical, genetic, regulatory, evolutionary, taxonomic, and ecological aspects is required to answer the unsolved questions

regarding organohalide respiration, to better define the roles of OHRB in natural microbial communities, and to fully exploit their activities for contaminated site cleanup (Adrian and Löffler, 2016).

The presence and/or expression of specific genes catalysing dehalogenation reactions can be applied as biomarkers for evaluating the potential or activity of organohalide-degrading bacteria in bioremedial sites. For instance, PCR targeting the *vcrA* gene from *Dehalococcoides* strain VS can be used as a biomarker to predict *in situ* reductive VC dehalogenation (Müller *et al.*, 2004). Despite the broad diversity of Rdh that are known to exist, only very few have been biochemically investigated to date and this is a major limitation for this application.

The characterization of groundwater presents some constraints, as the expensive installation of monitoring wells, or the difficulties for closing mass balances to discern if biodegradation is the process responsible for the elimination of contaminants. Measurement of isotope values of organic contaminants in groundwater samples is a valuable tool to demonstrate with a reasonable number of sampling wells that organic contaminants are naturally broken down (Elsner, 2010). For the monitoring of groundwater contaminations, the most immediate added values of compound-specific isotope analysis by GC-IRMS have been (i) the possibility of isotopic fingerprinting to distinguish contamination sources and (ii) the ability to demonstrate the occurrence of natural transformation reactions. The latter aspect is of particular interest in the management of contaminated sites as legislation requires a direct line of evidence of natural attenuation (Elsner, 2010; Höhener and Aelion, 2010; Nadalig *et al.*, 2013).

The presence of complex mixtures of pollutants at impacted aquifers is a frequent scenario. Co-contaminants may compromise the performance of bioremediation processes, as there are known inhibitory effects of certain organohalogens over bacteria candidate for detoxification processes (Duhamel *et al.*, 2002; Mayer-Blackwell *et al.*, 2016). More research effort is required in this regard, to assess the potential effects of co-contaminants over bioremediation projects.

1.6 References

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Chapter 2:

Objectives of the thesis

The main objective of this thesis is **to obtain and characterize cultures containing anaerobic bacteria capable of performing biodegradation processes of organohalide compounds of environmental concern**. To achieve its overall objective, the present work has the following specific objectives:

- To identify the reductive dehalogenase(s) used by the population of *Dehalogenimonas* growing in a mixed culture to respire selected halogenated compounds using different proteomic techniques and a draft of this *Dehalogenimonas* genome.
- To test the capability of anaerobic bacteria derived from different sources to transform an array of recalcitrant halogenated compounds.
- To enrich and isolate the bacterial population catalyzing the biodegradation of the organohalides previously tested.
- To determine the carbon stable isotope fractionation during the fermentation of dichloromethane (DCM) by the obtained *Dehalobacterium*-containing culture to test whether this tool can be used to distinguish DCM degradation pathways in contaminated groundwater.
- To assess the potential inhibitory effect of selected halogenated compounds frequently detected in groundwater over the DCM-fermenting performance of a mixed culture containing a *Dehalobacterium* sp.

Chapter 3

General materials and methods

3.1 Materials

Chemicals

All chemicals used were analytical grade reagents with a purity higher than 98%. In Table 3.1 are listed the chemicals used, the abbreviations used along the text, their corresponding brands, and their state of matter.

Table 3.1: List of chemicals used during this thesis

Chemicals	Acronym	Brand	State of matter
1,1,2-Trichloroethane	1,1,2-TCA	J.T Baker	Liquid
1,2,4-Trichlorobenzene	1,2,4-TCB	Sigma	Liquid
1,2-Dichloroethane	1,2-DCA	Sigma	Liquid
1,2-Dichloropropane	1,2-DCP	Sigma	Liquid
1-Chloropropane	1-CP	Sigma	Liquid
2,3-Dichloro-1-propene		Sigma	Liquid
2-Chloropropane	2-CP	Sigma	Liquid
4-Aminobenzoic acid		Sigma	Solid
4-chlorophenol		Sigma	Solid
Acetic acid		Sigma	Liquid
Acetone		Sigma	Liquid
Allyl chloride		Sigma	Liquid
Allyl disulfide		Sigma	Liquid
Allyl sulfide		Sigma	Liquid
Ammonium chloride		Panreac	Liquid
Ampicillin		Sigma	Solid
Biotin		Sigma	Solid
Boric acid		Panreac	Solid
Calcium chloride		Panreac	Solid
Calcium pantothenate		Sigma	Solid
Chloramphenicol		Sigma	Solid
Chlorobenzene	CB	Sigma	Liquid
Chloroform		Sigma	Liquid
Chloromethane		Sigma	Liquid
cis-1,2-Dichlorethene	*cis*-DCE	Chem Service	Liquid
Cobalt chloride 6-hydrate		VWR	Solid
Copper(II) chloride dihydrate		Sigma	Solid
Cyanocobalamin		Sigma	Solid
Dibromomethane	DBM	Sigma	Liquid
Dichloromethane	DCM	Sigma	Liquid
Diclofenac sodium salt		Sigma	Solid
Ethanol		Fisher	Liquid
Ethylene		Sigma	Gas
Ethylene dibromide	EDB	Sigma	Liquid
Formic acid		Merck	Liquid
Gas mix CO_2/N_2		Carburos metálicos	Gas
HEPES sodium salt		Merck	Solid

Table 3.1: Continuation. List of chemicals used during this thesis

Hydrogen		Carburos metálicos	*Gas*
Iron(II) chloride tetrahydrate		Sigma	Solid
Kanamycin sulfate		Sigma	Solid
L-Cysteine		Sigma	Liquid
Low melting point agarose		Lonza	Solid
Magnesium chloride hexahydrate		Sigma	Solid
Manganese (II) chloride 4- hydrate		Panreac	Solid
Methyl viologen dichloride hydrate		Sigma	Liquid
Metronidazole		Sigma	Solid
Nalidixic acid		Sigma	Solid
Nickel (II) chloride		Sigma	Solid
Nicotinic acid		Sigma	Solid
Nitrilotriacetic acid		Sigma	Liquid
Nitrogen		Carburos metálicos	Gas
Perfluorooctanoic acid		Sigma	Solid
Perfluorooctanosulfonate		Sigma	Solid
Potassium chloride		Panreac	Solid
Potassium dihydrogen phosphate		VWR	Solid
Propene		Chem Service	Gas
Propionic acid		Panreac	Liquid
Pyridoxine hydrochloride		Sigma	Solid
Resazurin sodium salt		Sigma	Solid
Sodium 2-bromoethanesulfonate	BES	Sigma	Solid
Sodium bicarbonate		Sigma	Solid
Sodium chloride		Panreac	Solid
Sodium DL-lactate		Sigma	Solid
Sodium formate		Alfa Aesar	Solid
Sodium fumarate		Alfa Aesar	Solid
Sodium hydroxide		Panreac	Solid
Sodium molybdenum oxide dihydrate		VWR	Solid
Sodium pyruvate		VWR	Solid
Sodium sulfide nonahydrate	$Na_2S.9H_2O$	Sigma	Solid
Streptomycin sulfate salt		Sigma	Solid
Thiaminchloride- hydrochloride		Merck	Solid
trans-1,2-Dichloroethylene	*trans*-DCE	Sigma	Liquid
Triclocarban		Sigma	Solid
Triclosan		Sigma	Solid
Vancomycin hydrochloride hydrate		Sigma	Solid
Vinyl chloride	VC	Chem Service	Liquid
Vitamin B12		Sigma	Solid
Yeast extract		Scharlau	Solid
Zinc chloride		VWR	Solid

3.2 Methods

3.2.1 Culture medium preparation

Anoxic basal media was prepared as described elsewhere (Adrian *et al.*, 2000). The basal medium contained the following constituents (per liter): KH_2PO_4, 0.2 g; NH_4Cl, 0.27 g; NaCl, 1 g; $MgCl_2.6H_2O$, 0.41 g; KCl, 0.52 g; $CaCl_2.2H_2O$, 0.15 g; ZnCl2, 0.7 mg; $MnCl_2.4H_2O$, 0.8 mg; H_3BO_3, 0.06 mg; $CoCl_2.6H_2O$, 0.19 mg; $CuCl_2.2H_2O$, 0.02 mg; $NiCl_2.6H_2O$, 0.24 mg; $Na_2MoO_4.2H_2O$, 0.36 mg; $FeCl_2.4H_2O$, 20 mg; nitriloacetic acid, 128 mg. When the medium mixture is prepared, it is connected to a vacuum pump for 45 min and then bubbled with nitrogen for 20 min to remove oxygen. After that, medium is aliquoted in 65 mL inside an anaerobic glovebox (Coy) in 120 mL glass serum bottles. Bottles are then sealed with Teflon-coated butyl rubber septa and aluminium crimp caps. All bottles were autoclaved at 121 °C for 40 min (Fig.3.1). Once autoclaved and cooled, the following components were added aseptically from sterile and anoxic stock solutions: $NaHCO_3$ solution (1 g/L), $Na_2S.9H_2O$ + L-cysteine solution (25 mg/L), vitamin solution containing (40 mg/L 4-aminobenzoic acid, 10 mg/L D(+)-biotin, 100 mg/L nicotinic acid, 50 mg/L calcium D (+)-pantothenate, 150 mg/L pyridoxine dihydrochloride, 0.1 mL thiamin thiamine chloride.

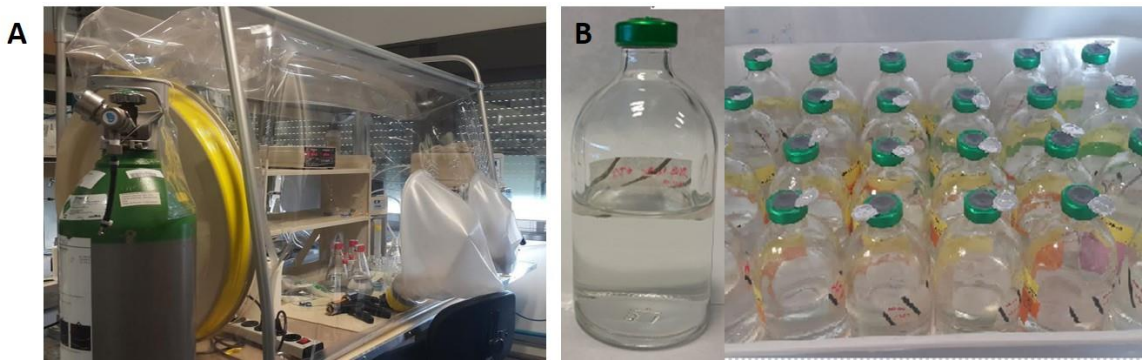

Figure 3.1 Panel A: Anaerobic glovebox used for preparation of the medium-containing serum bottles. Panel B: Aspect of the glass serum bottles once filled with the anoxic medium inside the anaerobic glovebox, crimped with tefflon rubber septums and aluminium crimps and autoclaved.

3.2.2 Gas chromatography (GC)

All gas chromatography quantifications were done by analysing gas samples taken directly from the headspace of culture bottles or vials. Volumes of 0.5 mL were analysed for quantifying volatile halogenated compounds, ethene, propene and

methane, while 0.1 mL were analysed for hydrogen quantification. Samples were taken with gas tight pressure-lock precision analytical syringes (Vici) and the septum surfaces were sterilised by ethanol burning prior to the sampling.

Ethene and propene

Ethene and propene were analysed using a gas chromatograph (GC) model 6890N (Agilent) with flame ionization detector (FID) equipped with a HP Plot Q column (30 m × 0.53 mm, Agilent). Helium was used as the carrier gas (0.9 mL/min). The oven temperature was fixed at 150 °C, the injector temperature at 250 °C and the detector temperature at 260 °C. Run time lasted 6 min.

Volatile halogenated compounds

The same GC equipped with FID described above with a DB-624 column (30 m × 0.32 mm, Agilent) was used to analyse DCM, DBM, CF, CM, PCE, TCE, EDB, 1,2-DCP, 1,2,3-TCP, 1,1,2-TCA, 1,2-DCA and *cis*-1,2-DCE, *trans*-DCE, allyl chloride, allyl alcohol and allyl sulphide. Helium was used as the carrier gas (0.9 mL/min). The injector and detector temperatures were set at 250 °C and 300 °C respectively. After the injection of the sample (split ratio 1:2), the initial oven temperature (35°C) was held for 3 min and then ramped at 10 °C/min to 240 °C, which was held for 4 min. Run time lasted 27 min.

During the research stay at the Helmholtz-Centre for Environmental Research (UFZ), the compounds 1,2,3-TCP, allyl chloride (3-chloro-1-propene), allyl alcohol, allyl sulphide, EDB and ethene were analysed from headspace samples with a gas chromatograph (GC) model 6890N (Agilent) equipped with FID and a HP-5 column (30 m × 0.32 mm with). Helium was used as the carrier gas (1.5 mL/min). The injector and detector temperatures were both set at 250 °C. After the injection of the sample (split ratio 1:2), the initial oven temperature (40 °C), ramped at 10 °C/min to 50 °C, and then ramped at 20 °C/min to 120 °C. Run time lasted 7 min.

Methane

Methane concentration was analysed using a GC HP 5890 (Agilent) with a thermal conductivity detector (TCD) equipped with a Porapack Q column (3 m x 2.1 mm, Agilent) using helium at 338 kPa as the carrier gas. The oven temperature was fixed at 70 °C, the injector temperature at 150 °C and the detector temperature at 180 °C. Run time was 3 min.

Hydrogen

Hydrogen concentration was measured using an Agilent 7820A GC equipped with a TCD and separation was achieved using a MolSieve 5A 60/80 SS (1.82 m x 2 mm, Agilent) and a Porapak Q 60/80 UM columns (1.82 m x 2 mm, Agilent). Nitrogen was the carrier gas at 138 kPa. Oven temperature was held isothermal at 40 ºC, the injector temperature at 200 ºC and the detector temperature at 250 ºC. Run time lasted 5 min.

Standard curves and stock solution preparation

Calibration was based on aqueous standards, with the same liquid and headspace volumes as in the microcosms. In the cases of ethane, propene, methane and hydrogen, calibration was done by injecting different known volumes of gas standards into glass bottles or vials sealed with Teflon-coated butyl rubber septa and aluminium crimp caps. The gas stocks were prepared diluting 1 mL of the pure gas in 1 L nitrogen Tedlar gas sampling bags. Liquid standard solutions were prepared by dissolving known volumes of neat compounds or acetone stocks in 70 mL of miliQ water bottled in 120 mL serum bottles sealed with Teflon-coated butyl rubber septa and aluminum crimp caps. The solutions were allowed to equilibrate overnight, and then 0.5 mL aliquots were analyzed in the gas chromatography as described above. Calibration curves were prepared by plotting peak areas versus known concentrations. Peak areas were calculated using Millenium/Empower software (Waters). Results are presented as nominal concentrations expressed in µmols in the case of ethane and propene, in µM for methane and halogenated compounds and in percentage (%) in the case of hydrogen.

3.2.3 High performance liquid chromatography (HPLC)

Triclosan, diclofenac and triclocarban

For triclosan (irgasan) and triclocarban (3,4,4-trichlorocarbanilide) analysis, 2 mL liquid samples were taken from the microcosms. Then, a simple acetone extraction was performed by dilution of the sample 1:2 in pure acetone. The mixture was shaken for 5 min, and then 1 mL of the mixture was filtered filtered (Millex-GV, PVDF, 0.22 µm, Millipore). For diclofenac analysis, liquid samples (1 mL) were taken, and centrifuged on a Heraens PICO21 centrifuge (ThermoScientific) at 6000 ×g for 10 min. Then the supernatant was filtered (Millex-GV, PVDF, 0.22 µm, Millipore).

Triclosan, diclofenac and triclocarban were measured using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 277 nm. Chromatographic separation was achieved using a GraceSmart RP 18 5u column (250 × 4.6 mm, Grace). For triclosan and diclofenac, the column temperature was 30 °C. The mobile phase consisted of 70 % pure acetonitrile and 30 % of a 0.1% formic acid solution in ultra-pure water. The analysis were performed isocratically with a 1 mL/min flux. In the case of triclocarban, the column temperature was 35 °C. The mobile phase consisted of 72 % pure methanol and 28 % of a 0.01 M Na_2HPO_4 phosphoric acid solution in ultra-pure water acidified to pH=3 with HCl. The analysis were performed isocratically with a 1 mL/min flux. In all cases, a sample volume of 10 µL was injected from a Dionex autosampler.

Volatile fatty acids

Liquid samples (1 mL) were taken, and centrifuged on a Heraens PICO21 centrifuge (ThermoScientific) at 6000 g for 10 min. Then the supernatant was filtered (Millex-GV, PVDF, 0.22 µm, Millipore). Formate, acetate and other organic acids were measured using a Dionex 3000 Ultimate HPLC equipped with a UV detector at 210 nm. Chromatographic separation was achieved using a ICE-COREGEL 87H3 column (300 x 4.6 mm, Transgenomic). The mobile phase consisted of an acidic solution with 320 µL H_2SO_4 per liter of ultrapure water. The analysis was performed isocratically with a 0.5 mL/min flux. The column temperature was 40°C, and a sample volume of 20 µL was injected from a Dionex autosampler.

4-chlorophenol and phenol

Liquid samples (1 mL) were centrifuged on a Heraeus PICO21 centrifuge (ThermoScientific) at 6000 g for 10 min, then the supernatant was filtered (Millex-GV, PVDF, 0.22 µm, Millipore). Phenol and 4-chlorophenol concentrations were measured using a Dionex 3000 Ultimate (Barcelona, Spain) HPLC equipped with a UV detector at 210 nm. Chromatographic separation was achieved using a Zorbax SB-C18 column (100 × 4.6 mm, Agilent). The mobile phase consisted of an acidic solution (pH=1.41) with 1120 µL H_2SO_4 per liter of ultra-pure water. The analysis was performed isocratically with a 1.9 mL/min flux. The column temperature was 30 °C, and a sample volume of 20 µL was injected from a Dionex autosampler.

Standard curves and stock solution preparation

Standards were prepared as aqueous stock solutions in the case of volatile fatty acids, 4-chlorophenol and phenol and in acetone stock solutions in the case of triclosan, triclocarban and diclofenac. Calibration curves were prepared by plotting peak areas versus known concentrations. Peak areas were calculated using Chromeleon software 6.8 (ThermoScientific).

3.2.4 Ionic chromatography

Anionic concentration was analysed from 1 mL liquid samples, filtered prior to the analysis of chloride and bromide with PVDF syringe filters (Millex-GV, 0.22 µm, Millipore) and with PES syringe filters for fluoride (Millex-GV, 0.22 µm, Millipore) to avoid the retention of fluorinated compounds or fluoride in the PVDF filtering matrix. The analysis was performed by ion chromatography with conductivity detection using a Dionex ICS-2000 equipment, and an IonPac AS18 REIC anion-exchange column (4 x 250 mm, Dionex). The column was operated at 30 °C and the potassium hydroxide concentration of the eluent varied from 25 mM to 50 mM along the 10 min analysis at a flow rate of 1 mL/min. The injection volume was 25 µL.

Standard curves and stock solution preparation

Standards were prepared as aqueous stock solutions. Calibration curves were prepared by plotting peak areas versus known concentrations. Peak areas were calculated using Chromeleon software 6.8 (ThermoScientific).

3.3 References

Adrian, L., Szewzyk, U., Wecke, J., and Görisch, H. (2000) Bacterial dehalorespiration with chlorinated benzenes. *Nature* **408**: 580–3.

Chapter 4

Identification of *Dehalogenimonas* reductive dehalogenases using proteomic techniques

Part of this chapter is under preparation for publication as:

"Genome sequence of a Dehalogenimonas alkeniginges strain and identification of the novel EdbA catalysing the dihaloelimination of ethylene dibromide to ethene" in the journal Environmental Microbiology.

The work included in this Chapter was partly performed during a research stay at the Helmholtz-Centre for Environmental Research (UFZ) under the supervision of Dr. Lorenz Adrian, and with the collaboration of Dr. Keneth Wasmund from the University of Vienna, who performed the genome-sequencing of the Dehalogenimonas strain.

Abstract

Reductive dehalogenase enzymes (RDases if characterized, otherwise Rdh) typically show a high substrate specificity, therefore the detection of such genes can be applied as biomarkers for monitoring organohalide respiring activities during bioremediation projects. Bacteria from the genus *Dehalogenimonas* were shown to harbour a high number of Rdh, however, only three of them were function-assigned to date. In our laboratory, a *Dehalogenimonas*-containing culture was obtained from the sediments of the River Besós estuary (Barcelona). The genome of this new strain of *Dehalogenimonas alkenigignens* was recently sequenced, and it encodes for 36 RdhA and only 3 RdhB homologous genes. As the catalytic activity of brominated compounds remains unknown in this genus, we used a combination of BN-PAGE, enzymatic assays, and nLC-MS/MS analysis to identify the Rdh catalysing the dihaloelimination of ethylene dibromide (EDB) into ethene. The novel EdbA identified was shown to be the first Rdh characterised to date in this genus being functional with no adjacent *rdhB*. Additionally, combining ultracentrifugation, SDS-PAGE and nLC-MS/MS with cells grown with 1,2,3-trichloropropane (1,2,3-TCP) as only electron acceptor, we detected only one Rdh in the membrane fraction of the protein extract. This Rdh was shown to be a member of the ortholog group of dichloropropane-to-propene Rdh (DcpA). The sequences of both *edbA* and *dcpA* encoded in our genome include a Tat-signal peptide, indicating these proteins are exported out of the cytoplasmic membrane. The use of bioinformatics tools also predicted their localization at the outer part of the membrane. These results taken together point that yet unidentified proteins may act as membrane-anchoring subunits for EdbA and this DcpA.

4.1 Introduction

Organohalide-respiring bacteria (OHRB) provide a potential solution to decontaminate organohalogens-impacted sites due to their capability to harness energy using halogenated compounds as electron acceptors. During organohalide respiration, reductive dehalogenation is catalysed by Rdh homologous proteins, whereby all so far characterised Rdh proven to be functional in respiration are encoded by operons composed by two genes: *rdhA*, encoding for the catalytic subunit and *rdhB*, putatively encoding a membrane anchor (Hug *et al.*, 2013; Jugder *et al.*, 2015).

Rdh enzymes typically show high substrate specificity and can be of great interest for monitoring bioremediation processes. That is, the occurrence and/or expression of such genes can be applied as biomarkers for evaluating the presence or activity of organohalide-respiring bacteria (OHRB) in bioremedial sites. The identification of *rdhA* genes in the field using specific primers is a more reliable method than targeting 16S rRNA genes of typical ORHB, because of the weak correlation between 16S rRNA gene identities and dehalogenation activities (e.g., not all *Dehalococcoides* isolates can detoxify tetrachloroethylene to ethene) (Hölscher *et al.*, 2003 and Ritalahti *et al.*, 2006).

Despite the broad diversity of Rdh that are known to exist, and the potentially various dehalogenase reactions they may catalyse, only very few of them have been biochemically investigated to date. The growth yields of OHRB on halogenated substrates are usually low, which makes the production of sufficient biomass for enzyme purification arduous. This applies especially to the metabolically restricted OHRB which cannot be cultivated with alternative electron acceptors such as nitrate or fumarate to obtain more biomass. This lack of function-assignment is a major limitation for their use as biomarkers during *in situ* bioremediation projects.

For the biochemical investigation and functional genomic work with Rdh proteins, the first required step is the genome-sequencing and annotation from those bacteria candidate with dehalogenating potential. In regard of the Gram-negative bacteria from the genus *Dehalogenimonas* (phylum Chloroflexi, class

Dehalococcoidetes), five strains were sequenced to date: *D. lykanthroporepellens* type strain BL-DC-9^T (Siddaramappa *et al.*, 2012), *D. alkenigignens* IP3-3 (Key *et al.*, 2016), *D.* WBC-2 (Molenda *et al.*, 2016), *D. formicexedens* (Key *et al.*, 2017) and "Candidatus *Dehalogenimonas etheniformans*" (Yang *et al.*, 2017).

The *Dehalogenimonas* strains that have been genome-sequenced to date, harbour 22-52 *rdhA* genes. In contrast to all other known OHRB, most *rdhA* genes lack membrane anchoring protein cognates (*rdhB*). This suggests that either some of the RDases are cytoplasmic enzymes, or since the majority of them contain one Tat-signal peptide indicating that they are transported out of the cytoplasm, that they are anchored to the membrane by 'common' RdhB (i.e. encoded in other locations in the genomes), or yet-unidentified proteins.

Within the genus *Dehalogenimonas*, three RDases involved in respiration of organochlorines have been characterized: (i) DcpA has been shown to dechlorinate 1,2-dichloropropane (1,2-DCP) to propene (Padilla-Crespo *et al.*, 2014); (ii) TdrA was responsible for the hydrogenolysis of *trans*-dichloroethene (tDCE) to vinyl chloride (Molenda *et al.*, 2016), and more recently, (iii) CerA was shown to catalyse the dechlorination of vinyl chloride to ethene (Yang *et al.*, 2017). It was therefore of great interest to identify the unknown enzymes and underlying genes that are responsible for the reductive dehalogenation of other compounds transformed by *Dehalogenimonas* strains.

Considering the specialization of *Dehalogenimonas* spp. to perform dihaloelimination reactions with vicinally-halogenated alkanes, previous work had focused on enriching unique *Dehalogenimonas* strains with potentially important catalytic properties. In our laboratory, a *Dehalogenimonas*-containing culture was obtained from sediment samples collected from the River Besós estuary (Barcelona, Spain) (Martín-González *et al.*, 2015) and it appeared to transform exclusively vicinally halogenated alkanes via dihaloelimination. The culture was enriched to a final composition of two taxa, *Dehalogenimonas* and *Desulfovibrio,* and showed the ability to transform the chlorinated compounds 1,2-DCP, 1,2,3-trichloropropane (1,2,3-TCP), 1,2-dichloroethane (1,2-DCA), 1,1,2-trichloroethane (1,1,2-TCA), 1,1,2,2-

tetrachloroethane (1,1,2,2-TeCA) and ethylene dibromide (EDB) (Martín-González *et al.*, 2015; Palau *et al.*, 2017a; Palau. *et al.*, 2017b; Mortan *et al.*, 2017).

The brominated alkane EDB is a toxic, recalcitrant and carcinogenic compound and is ranked 39th (out of 275) on the 2017 ATSDR Priority list of hazardous substances (IARC, 1997; EPA, 2005; ATSDR, 2017). EDB was extensively used as a lead scavenger in gasoline, as well as a pesticide, fumigant and soil sterilant. Due to gasoline spills, especially from underground storage tanks, and the direct application on agricultural soils, EDB has reached aquifers in various places (Rowe *et al.*, 2007). Even though the use of EDB was banned in the U.S in the late 1980s and in Europe in 2001, significant concentrations of EDB persist in many water bodies. For instance, EDB was found to exceed the maximum 'safe' concentration level (0.05 µg /L) in 42% of the samples from 102 analysed groundwater sites affected by gasoline leakages across 19 states of U.S (Wilson *et al.*, 2008).

During organohalide respiration process, EDB can be potentially transformed to innocuous ethene via dihaloelimination, or vinyl bromide via hydrogenolysis (Yu *et al.*, 2013). Among all *Dehalogenimonas* strains known to date, *D. lykanthroporepellens* BL-DC-9^{T} was the only one tested before with EDB, yielding ethene and stoichiometric release of bromide ions (Moe *et al.*, 2016). For all of the strains that have been genome sequenced to date, with the exception of *D. alkenigignens* IP3-3, dichloroelimination of the chlorinated EDB analogue 1,2-dichloroethane to ethene was reported (Siddaramappa *et al.*, 2012; Molenda *et al.*, 2016; Key *et al.2017*; Yang *et al.*, 2017).

To date, the only enzyme described to transform EDB was the trichloroethene RDase (TceA) from *Dehalococcoides mccartyi* strain 195, which converted EDB to ethene and minor amounts of vinyl bromide (<1%) in biochemical assays with the purified enzyme (Magnuson *et al.*, 2000). However, it was not investigated if EDB would support the growth of the organisms. Also, it was shown that the number of *Dehalococcoides mccartyi* cells in an anaerobic enrichment culture increased with EDB amendments, also producing predominantly ethene and traces of vinyl bromide (Yu *et al.*, 2013). Crude extracts from *Sulfurospirillum multivorans* transformed EDB to

ethene, but this species was not able to grow with EDB, likely due to toxicity effects as stated in Kuntze *et al.*, (2016).

The chlorinated alkane 1,2,3-trichloropropane (1,2,3-TCP) is a toxic and persistent pollutant in groundwater, classified as "likely to be carcinogenic to humans" by the EPA and included in the 2017 ATSDR Priority list of hazardous substances (ATSDR, 1992; ATSDR, 2011; EPA, 2014; ATSDR, 2017). This non-natural compound today mainly forms a building block in the manufacture of pesticides (e.g the nematicide 1,3-dichloropropene), chlorinated solvents, and cross-linking agents. Its occurrence in groundwater and soil is mainly a result of improper disposal of 1,2,3-TCP-contaminated chemical waste. However, it is not probable to sorb to soil based on its low soil organic carbon water partition coefficient and consequently, it is likely to leach from soil into groundwater, being detected in several aquifers (Rowe *et al.*, 2007).

There are no aerobic bacteria reported to date capable of metabolically degrade 1,2,3-TCP, and regarding anaerobic bacteria, the only strains shown to transform 1,2,3-TCP belongs to the genus *Dehalogenimonas* (Samin and Janssen, 2012). The *D. lykanthroporepellens* strains BL-DC-8 and BL-DC-9 (Yan *et al.*, 2009; Moe *et al.*, 2009) were the first bacteria demonstrated in pure culture to reductively dehalogenate 1,2,3-TCP. The direct product of the dihaloelimination reaction mediated by *D. lykanthroporepellens* is allyl chloride (3-chloro-1-propene), as shown in Fig.4.1.

However, allyl chloride is not stable and is abiotically hydrolyzed to allyl alcohol, while in the presence of cysteine or sulfide, used as reducing agents in the growth medium, allyl chloride was transformed to allyl mercaptan, S-allyl mercaptocysteine and allyl sulphides (Fig.4.1). These final products exhibit a strong garlic aroma, which inspired the species name, composed from Latin and Greek roots and meaning "repelling to werewolves." When grown in media lacking sulfide as a reducing agent, allyl alcohol is the primary product of 1,2,3-TCP dechlorination (Bowman *et al.*, 2013). Among the other *Dehalogenimonas* isolated to date, *D. formicexedens* NSZ-14T (Key *et al.*, 2017), and *D. alkenigignens* IP3-3 (Key *et al.*, 2016) were shown to transform 1,2,3-TCP, while the potential of transforming this compound by *Dehalogenimonas* WBC-2 is not reported (Molenda *et al.*, 2016) and in 'Candidatus *D. etheniformans*' was tested

with a negative result (Yang *et al.*, 2017). In all the 1,2,3-TCP transforming strains, allyl chloride was detected as the main initial dechlorination product. However, the mechanism of the reductive dechlorination reaction is not completely clear, as the enzyme/s responsible for 1,2,3-TCP dechlorination has/have not yet been characterized.

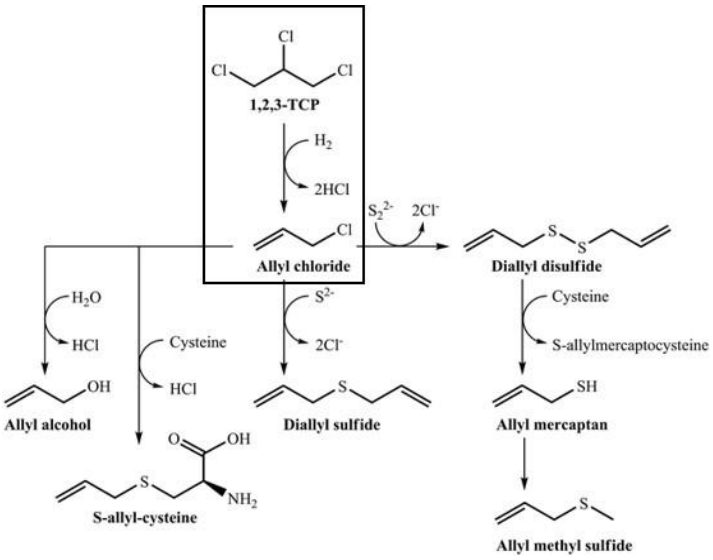

Figure 4.1 Reaction pathways reported for the dechlorination of 1,2,3-trichloropropane with *Dehalogenimonas lykanthroporepellens* strains grown in the presence of cysteine and sulfide as reducing agents. The biologically mediated step is marked with a square (modified from Yan *et al.*, 2009).

The objective of this study was the identification of the Rdh proteins catalysing the dehalogenation of EDB and 1,2,3-TCP in a *Dehalogenimonas* population derived from estuary sediments. To do this, we combined information from a draft genome sequence and proteomic techniques including sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), ultracentrifugation, blue native polyacrylamide gel electrophoresis (BN-PAGE), dehalogenation activity assays, and nano-scale liquid chromatographic tandem mass spectrometry (nLC-MS/MS) analyses.

4.2 Material and methods

4.2.1 Cultivation of the *Dehalogenimonas*-containing culture

A *Dehalogenimonas*-containing sediment free culture transforming 1,2-DCP (50 µM) to propene was maintained for more than five years in our laboratory as described elsewhere (Martín-González *et al.*, 2015). Briefly, each microcosm contained

65 mL of anaerobic bicarbonate-buffered medium (section 3.2.1), reduced with $Na_2S \times 9H_2O$ and L-cysteine (0.2 mM each) with 5 mM sodium acetate as carbon source, and gassed with N_2/CO_2 (4:1, v/v, 0.2 bar overpressure) and H_2 (added to an overpressure of 0.4 bar). All microcosms were cultivated under static conditions in the dark at 25 ºC in a thermostatic chamber.

For genome sequencing, 420 mL of this enriched culture growing on 1,2-DCP as electron acceptor for 34 generations was used. For proteomic studies, cultures growing on 1,2-DCP were transferred in parallel to defined anaerobic medium (i) with EDB (25 µM) and (ii) 1,2,3-TCP (50-75 µM) as unique electron acceptors, in both cases for four consecutive transfers (5% v/v).

4.2.2 Gas chromatography analysis

The compounds EDB, ethene, 1,2,3-TCP, allyl chloride, allyl alcohol and allyl sulphide were quantified by GC, as detailed in Section 3.2.2.

4.2.3 Genomic analysis

DNA extraction for genome sequencing

For cell harvesting, centrifugation was performed at 5000 g and 16 ºC for 1 h. After this step, 50% of the supernatant was removed, and the remaining volume was centrifuged again. This procedure was repeated for 3 times. Then, for the DNA extraction, the NucleoSpin Tissue kit (Machery Nagel) was used following the manufacturer instructions.

Genome sequencing, assembly, binning and refinement

Recently, the genome of the *Dehalogenimonas* strain obtained from sediments of the River Besós estuary in our laboratory was sequenced by Dr. Kenneth Wasmund (University of Vienna) as detailed in this section. The genome annotation is currently undergoing and it will be submitted to public databases.

DNA was sheared to approximately 300-350 bp using a Covaris Micro-Tube and an AFA™ Focused-ultrasonicator. Sheared DNA was prepared as an Ilummina sequencing library using the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®

(New England Biolabs, Austria) following the manufacturer's instructions. The library was sequenced using an Illumina HiSeq 3000 instrument using 2x 150 bp paired-end mode, resulting in 4.3 million read pairs. Sequences were assembled using SPAdes (v. 3.10.1) with pre-assembly read-error correction. Scaffolds less than 1000 bp were removed and since the enrichment culture was not pure, the following steps were taken to retrieve all scaffolds belonging to *Dehalogenimonas* spp. For this, all assembled scaffolds were 'classified' using Kaiji (Menzel *et al.*, 2016). Scaffolds classified as belonging to *Dehalogenimonas* spp. were then retrieved, while additional scaffolds that had similar coverage but remained unclassified were also retrieved. This was possible since *Dehalogenimonas*-related scaffolds had distinctly higher coverage profiles (>200-fold) than scaffolds from other less-abundant co-cultured taxa, e.g., a *Desulfovibrio* sp. was the next highest covered organism with approximately 30-fold coverage. All *Dehalogenimonas*-related scaffolds were then subjected to automatic genome annotation using RAST (Aziz *et al.*, 2008). Predicted genes or proteins were then manually subjected to BLASTn or BLASTp analyses (10.1016/S0022-2836(05)80360-2), respectively, against the BLASTp nr database from NCBI to verify the identities of the scaffolds by confirming best hits to other *Dehalogenimonas* spp. for all scaffolds. Additional analysis by CheckM (Parks *et al.*, 2015) further confirmed no contamination was evident. A draft of the genome was obtained, and a database of the protein encoding genes (pegs) predicted was prepared in a FASTA file for its use in the proteomics studies detailed in this Chapter.

4.2.4 Procedures for proteomic analyses

Cell counting

Cell numbers were quantified by direct cell counting with an epifluorescence microscope after staining of the cells with SYBR-green I (diluted with sterile TE-buffer to 1%) on agarose-coated slides (low-melting SeaPlaque® agarose). For cell counting, 20 µL of culture sample was mixed with 6.5 µL of SYBR-green I 1% solution in a sterile Eppendorf tube and incubated in dark for 10 min. Then, 18 µL of the stained sample was transferred onto a 20 mm × 20 mm cover glass which was then turned over and placed onto an agarose-coated slide. For each sample, 20 random micrographs were

taken by a NikonDS-Ri1 digital camera at 400x magnification. Micrographs were taken by the NIS imaging software (Nikon, Tokyo, Japan) and cell numbers were automatically calculated by ImageJ (http://imagej.nih.gov/ij/) and self-designed macros as described by Adrian *et al.*(2007).

Preparation of the crude protein extract

For cell harvesting, centrifugation of the *Dehalogenimonas* culture actively dechlorinating 1,2,3-TCP, or debrominating EDB was performed at 6000 g and 16 ºC. Cultures actively debrominating EDB (140 – 210 mL) were aliquoted in 35 mL into different 50-mL Falcon tubes under anoxic conditions. Six centrifugation cycles were done: (i) 60 min centrifugation and removal of 7 mL of the supernatant (ii) 50 min centrifugation and removal of 7 mL of the supernatant (iii) 40 min centrifugation and removal of 7 mL of the supernatant (iv) 30 min centrifugation and removal of 7 mL of the supernatant (v) 20 min centrifugation and removal of 1.4 mL of the supernatant. Then, all the supernatants were combined in one Falcon tube and centrifuged again for 20 min. Then, pelleted cells and 5 mL of medium was left, mixed and aliquoted for different analysis. Before each proteomic analysis, 1 mL of aliquoted concentrated cells was centrifuged again at 6000 g for 15 min, 800 µL of supernatant was removed and 1 x PBS buffer (pH 7.2) or 1 × BN-PAGE simple buffer (pH 7.2, Invitrogen) was added. Cells were lysed by a bead beater (Fast Prep FP120, Thermo) at speed 4.0, in six cycles of 40 s beating alternated with 1 min resting in ice. Membrane proteins were solubilized by adding n-dodecyl β-D-maltoside (DDM) to a concentration of 1% w/v, and incubating the samples at 10 ºC for 1 h with gentle shaking under anoxic conditions. The samples were then centrifuged for 20 min at 14000 g and 10 ºC and the supernatant was concentrated by ultrafiltration with Amicon centrifugal filter units with a 3 kDa cut off (Amicon Ultra – 0.5 mL Centrifugal Filters, Millipore) at 14000 g, 30 min and 10 ºC. After centrifugation, filters were turned upside down and the protein extract was spun off for 2 min at 2000 g and 10 ºC.

Protein profile using shot gun approach

Protein expression in cultures grown with EDB was firstly investigated using a shot gun approach with gel pieces. Protein lysates were separated using a 12% SDS-

PAGE, with the one-dimensional PAGE LC–MS/MS procedure in order to improve coverage for in-depth characterization as described elsewhere (Schiffmann *et al.*, 2016). For this, samples after DDM addition were treated with SDS buffer (containing 0.01% bromophenol blue) for 10 min and denatured at 90 ºC. Then, 25 µL per well in triplicate were loaded onto a hand-casted gel. A short electrophoresis was run at 90 V, until the front of the samples had moved 1 cm into the separation gel. At that point, the current was stopped, the gel lane excised according to the blue front of the sample and cut in 3 pieces with different running distances for sample prefractionation. These three gel pieces were then further processed by in-gel digest for nLC-MS/MS as described below.

Ultracentrifugation

Crude extract in aliquots of 1 mL were treated by ultracentrifugation for 1 h at 100 000 × g and 4°C in a Optima MAX-XP Ultracentrifuge (Beckman Coulter) with a rotor MLA-130 130K RPM (Beckman Coulter) to separate the cytosolic and membrane protein fractions derived from *Dehalogenimonas* cells grown with 1,2,3-TCP as unique electron acceptor. After ultracentrifugation, the pellet (containing the membrane fraction) and the supernatant (containing the cytosolic fraction) were separated for further procedure.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

To study the cellular localization of the predicted reductive dehalogenases expressed in the 1,2,3-TCP-transforming cultures, proteins present in the cytosolic and membrane fractions after ultracentrifugation were separated by SDS-PAGE (Laemmli, 1970). Ultracentrifugation supernatant (cytosolic fraction) was first ultrafiltered in 3 kDa cutoff centrifugal filter units to concentrate the sample, and then incubated 1:1 with 15 µL SDS buffer, 10 min at 70 ºC. The ultracentrifugation pellet (membrane fraction) was directly resuspended with 30 µL of SDS by gently pippeting. Then it was incubated first for 10 min at room temperature, followed by 20 min at 70 ºC. After SDS treatment, 30 µL of membrane fraction preparation in duplicate is loaded in gel 1, and 30 µL of cytosolic preparation in duplicate is loaded in gel 2. Both gels were hand-casted 12% SDS-PAGE and electrophoresis was ran at 80 V for 10 min, and then at 90 V

for gel 1. Gel 2 was run at 80 V for 20 min, and then at 90 V. Once the electrophoresis is finished, gels were stained for protein bands visualization by a short silver staining method (Nesterenko *et al*., 1994). Gel pieces of interest were then excised from both gels and further treated for nLC-MS/MS analysis.

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

Crude protein extracts were loaded onto a precast 4-16% gradient Bis-Tris gel (NativePAGE Novex, Invitrogen) and ran inside an anaerobic glovebox, maintaining the BN-PAGE system in contact with ice packs. Light blue cathode buffer and anode buffer were prepared following the manufacturer instructions, degassed and cooled down to 4 ºC before the electrophoresis was started. Crude protein extract (25 µL) was amended with a 0.125% w/v Coomassie G-250 additive and loaded in triplicate wells. NativeMark Unstained Protein Standard (Invitrogen) was used as molecular size marker. Electrophoresis was ran at 150 V for 60 min, and then at 200 V for 30 min more. Once the electrophoresis was finished, one of the replicate lanes and the molecular size marker lane were cut from the gel using a scalpel, and then stained for protein bands visualization by a short silver staining method (Nesterenko *et al*., 1994). The rest of the gel was stored in anode buffer at 4 °C.

Dehalogenase activity assays

To test the reductive dehalogenase enzymes activity, specific assays were set up inside an anaerobic glovebox (Hölscher *et al.*, 2003). Anaerobic 10 mL glass vials were used, containing 2 mL of an assay buffer with 200 mM potassium acetate buffer (pH 5.8), 2 mM methyl viologen, and 2 mM titanium (III) citrate [2 mM in respect to titanium (III)] and amended with 50 µM of EDB in duplicate. For the whole-cell activity assays, 200 µL of a 3 x 10^7 cells/mL concentrate was inoculated to each assay vial.

To measure activity in bands from the BN-PAGE, a non-stained lane from the BN-PAGE was sliced using a scalpel in 4 selected pieces. Each piece was then introduced into an assay vial. Gel pieces were smashed in smaller parts to better expose the proteins to the assay buffer. The vials were closed with Teflon-coated rubber septa and aluminium crimps and amended with EDB from a 100 mM acetone stock solution with a glass syringe (Hamilton) to a final concentration of 50 µM EDB. As

a positive control, 25 µL of the crude protein extract was added to the assay buffer instead of the gel pieces. To control for abiotic transformation of EDB, negative controls without cells and gel pieces from outside the area where proteins were loaded were included (no cell controls). Also, negative controls containing the assay buffer with 25 µL of crude extract but without EDB were included to test production of ethene from other sources than EDB (no-substrate controls). All controls were performed in triplicate. The vials were thoroughly mixed and incubated upside down inside the glovebox, at 30 ºC without shaking. After 48 h, the headspace of each sample was analysed for ethene production by gas chromatography.

Identification of proteins

Silver stained slices from SDS-PAGE or BN-PAGE were washed with ddH_2O and de-stained in a 1:1 (v/v) mixture of 30 mM $K_3[Fe(CN)_6]$ and 100 mM $Na_2O_3S_2$. Non-stained SDS-PAGE and destained SDS-PAGE or BN-PAGE pieces were treated as described previously to reduce and alkylate cysteine residues (Kublik *et al.*, 2016). In gel protein digestion was done overnight at a 37 ºC shaker at 2000 rpm by the addition of 0.1 µg of porcine trypsin (Proteomics Sequencing Grade, Promega). The obtained peptides were extracted from the gel pieces by 10 min incubation in a solution containing formic acid 5% (v/v) and acetonitrile 50% (v/v) three times, and extracts were combined, dried, resolubilized in 0.1% of formic acid solution, and desalted using C18 Zip Tips (Millipore).

Peptides were analysed by nLC-MS/MS on a nanoUPLC system (nanoAcquity, Waters) hyphenated via a TriVersa NanoMate (Advion, Ltd., Harlow, UK) to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific), as described previously (Hartwig *et al.*, 2017). Glycerinaldehyde-3-phosphate dehydrogenase (GapDH) from *E. coli* was used as internal standard and was added to the gel slices before derivatization.Peptide identification was conducted by Proteome Discoverer (v1.4.1.14, Thermo Fisher Scientific) using SequestHT as a search engine and a FASTA-file of the draft genome of the *Dehalogenimonas* strain in the culture as a database. False discovery rates of 1%, calculated versus a decoy database (percolator, Thermo Fisher Scientific), were set as threshold for peptide identification. Label free quantification of the detected proteins

was performed by semi-quantitatively measurement with the abundance value obtained from Proteome Discoverer, calculated from protein the PSMs (peptide spectral masses).

Bioinformatic tools

The non-redundant protein database from the basic local alignment search tool BLASTp nr from NCBI was used to determine pairwise amino acid identity. InterPro Scan (Jones *et al.*, 2014) and TatP 1.0 server (Bendtsen *et al.,* 2005) were used for Tat-signal peptides search. Prediction of subcellular protein localisation and protein transmembrane helices (TMHs) were performed using the TMHMM server v2.0 and TMMOD tools (Möller *et al.*, 2001). The automatic annotation performed by the RAST server was used for bidirectional best BLASTp hits.

4.3 Results and discussion

4.3.1 Preliminary analysis of the draft genome

Taxonomic affiliation of the *Dehalogenimonas* sp.

The draft genome utilized consisted on 17 contigs with a total size of 1.61 Mb and has a G+C content of 56.3 %. A total of 1695 protein encoding sequences in 237 subsystems were functionally annotated by Rapid Annotation using the Subsystems Technology (RAST), as well as 50 RNAs. A FASTA file database was prepared from this automatic annotation for proteomic analysis.

The 16S rRNA gene comparison of this strain to other *Dehalogenimonas* genomes previously published showed a 100 % gene identity with *Dehalogenimonas alkenigignens* strain IP3-3. Whole genome identity (gANI) for aligned regions of both genomes calculated with JspeciesWS showed a 98.66 ANIb % (blast) identity from 92.24 % aligned and Jspecies 99.37 ANIm % (mummer) from 91.50 % aligned. Reciprocal BLASTp of all proteins from both genomes showed differences in gene content. Therefore, the *Dehalogenimonas* present in our culture must be considered as the same species as *Dehalogenimonas alkenigignens*, yet a different strain than IP3-3.

Reductive dehalogenases encoded on this *Dehalogenimonas* genome

The automatic annotation of the genome draft of this new strain of *Dehalogenimonas alkenigignen*s revealed the presence of 36 *rdhA* homologous genes, while only 3 predicted *rdhB* encoded on it. In a similar way, the *Dehalogenimonas* strains that have been genome-sequenced to date, harbour 22-52 *rdhA* genes (Siddaramappa *et al.*, 2012; Key *et al.*, 2016; Molenda *et al.*, 2016; Key *et al.*, 2017; Yang *et al.*, 2017). In contrast to the high number of *rdhA* genes, the lack of cognates B in this genus is remarkable, and seems to be a frequent feature among bacteria affiliated to the *Dehalogenimonas* genus (Moe *et al.*, 2016).

4.3.2 Identification of the reductive dehalogenase involved in the EDB dihaloelimination by *Dehalogenimonas* sp.

Reductive debromination of EDB

Debromination of EDB to ethene by the *Dehalogenimonas*-containing culture was sustained after 4 consecutive transfers inoculating 5% (v/v) into fresh medium containing EDB (25 µM) as unique electron acceptor. The repeated addition of EDB led to faster debromination rates indicating that this reaction was supporting growth of *Dehalogenimonas* (Fig. 4.2). Ethene production was stoichiometric with EDB transformation (Fig 4.2), discarding vinyl bromide from dehydrohalogenation reactions as described previously in *Dehalococcoides mccartyi* spp. (Yu et al., 2013).

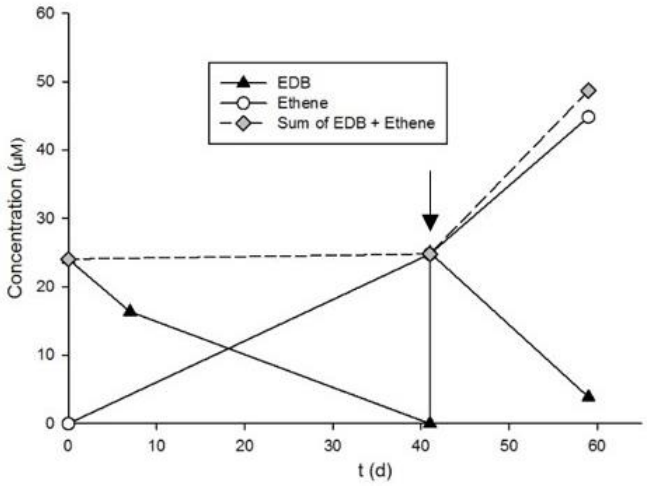

Figure 4.2 Debromination of EDB to ethene from duplicate *Dehalogenimonas*-containing culture. The arrow indicates a second EDB feeding.

Reductive dehalogenation of EDB to ethene via dihaloelimination was corroborated using enzymatic assays with whole cells and methyl viologen as artificial electron donor (Table 4.1). No ethene was produced in the controls without cells. In the controls without EDB, a small production of ethene was detected, derived from the EDB carried with the inoculum source. Production of ethene was, however detected in the experimental vials containing essay buffer, whole cells and EDB.

Table 4.1 Activity test with whole cells from the *Dehalogenimonas*-containing culture. NCC: controls without cells, NSC: controls without EDB, EV: experimental vials.

	EDB (µM)	Ethene (µM)	Sum of EDB and ethene (µM)
NCC_1	47.2	0	47.2
NCC_2	49.8	0	49.8
NSC_1	0	1.9	1.9
NSC_2	0	1.6	1.6
EV_1	33.8	20.9	54.7
EV _2	34.8	15.7	50.5

Identification of the EDB-to-ethene RDase

First, shot gun approach with SDS-PAGE gel pieces was applied in order to investigate the protein expression on EDB growing cultures. The proteomic analysis of the three SDS-PAGE gel slices yielded a total of 13 proteins (Table 4.2).

Two putative Rdh enzymes were identified, peg. 18 and peg. 1385, and the abundance value, as well as the number of the unique peptide hits detected indicated that peg. 1385 was the most abundant protein detected in this culture (Table 4.2).

In order to identify the Rdh involved in EDB dihaloelimination, a combination of BN-PAGE, enzymatic assays and nLC-MS/MS analysis was used. Crude protein extracts were separated on a native PAGE. Then, one lane was excised into four slices covering the region with molecular masses between 45-480 kDa: BN4 (242-480 kDa), BN5 (146-242 kDa), BN6 (66-146 kDa) and BN7 (45-66 kDa) (Fig. 4.3).

Table 4.2 Proteins identified after nLC-MS/MS analysis of the SDS-PAGE loaded with crude protein extracts obtained from cells of the new *Dehalogenimonas alkenigignens* strain cultivated with EDB as unique electron donor. An SDS-PAGE lane was sliced in three pieces corresponding to GS1, GS2 and GS3. Accessions correspond to temporary protein encoding gene numeration from the *Dehalogenimonas* draft genome. Descriptions are the protein function assigned by automatic annotation with RAST server. PSMs means peptide spectrum matches. Values in columns GS1, GS2 and GS3 corresponds to the abundance factors calculated by the Proteome discoverer analysis. Samples are listed in decreasing order of abundance factor.

					Abundance		
Accession	**Description**	**Coverage [%]**	**PSMs**	**Unique Peptides**	**GS1**	**GS2**	**GS3**
peg_1385	**Reductive dehalogenase**	30	18	9	2168.8	9816.9	
peg_574	Protein 60 family chaperone GroEL	15	7	6			4411.8
peg_573	Protein 60 family co-chaperone GroES	35	7	3			2626.2
peg_1242	LSU ribosomal protein L7/L12	22	10	3	46733.4	50465.4	25599.7
peg_18	**Reductive dehalogenase**	4	2	2			1549.3
peg_1116	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	5	4	2		5878.4	
peg_1259	Oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA	3	2	2	2042.2		
peg_202	Formate dehydrogenase alpha subunit	1	1	1	2563.9		
peg_1219	Fructose-1 6-bisphosphatase type V archaeal	3	1	1	3081.8		
peg_961	ATP synthase alpha chain	1	2	1	45211.2	12694.4	
peg_297	Hypothetical protein	12	1	1	8002.0	4414.2	
peg_190	Branched-chain amino acid ABC transporter amino acid-binding protein	2	1	1	31844.9	1319.3	
peg_823	Hypothetical protein	5	3	1		1362.1	

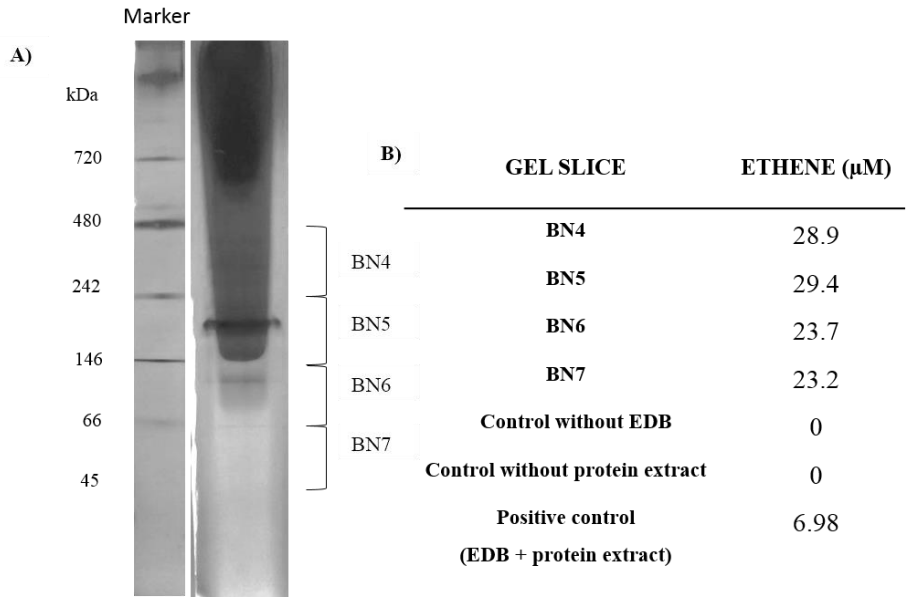

Figure 4.3 Activity assays following BN-PAGE separation of crude protein extracts of *Dehalogenimonas* grown with EDB. Panel A: Silver-stained BN-PAGE showing the predominant proteins and the gel sections that were subjected to dechlorination activity testing with EDB. For enzyme activity assays, gel slices from unstained lanes adjacent to the silver-stained lanes were used. Panel B: Ethene produced in gel regions from 45 kDa to 480 kDa. Gel slices BN4, BN5, BN6 and BN7 were further analysed by nLC-MS/MS.

This section of the gel was selected for debromination activity because the minimum predicted molecular weight of a single subunit of RDase is around 45 kDa and previous BN-PAGE studies with *Dehalogenimonas* and *Dehalococcoides mccartyi* species found maximum dehalogenating activity around 240 kDa (Kublik et al., 2016, Molenda et al., 2016). Dehalogenation activity tests revealed that ethene was produced in the four abovementioned pieces of the gel (Fig. 4.3). Ethene was not produced in any of the negative controls. The presence of RDases was confirmed by LC-MS/MS analysis of the silver stained counterpart of these gel fragments (Table 4.3). Interestingly, peg. 1385 was found in the four gel slices and its abundance corresponds well with the amount of ethene produced in the dehalogenation activity test (Fig. 4.4). A low abundance of peg. 18 was detected in only two of the gel slices tested (BN4 and BN6) (Fig. 4.4). In all, these results strongly support peg. 1385 is the protein primarily responsible for EDB dihaloelimination and is hereby designated as EdbA, for ethylene dibromide reductive dehalogenase, subunit A.

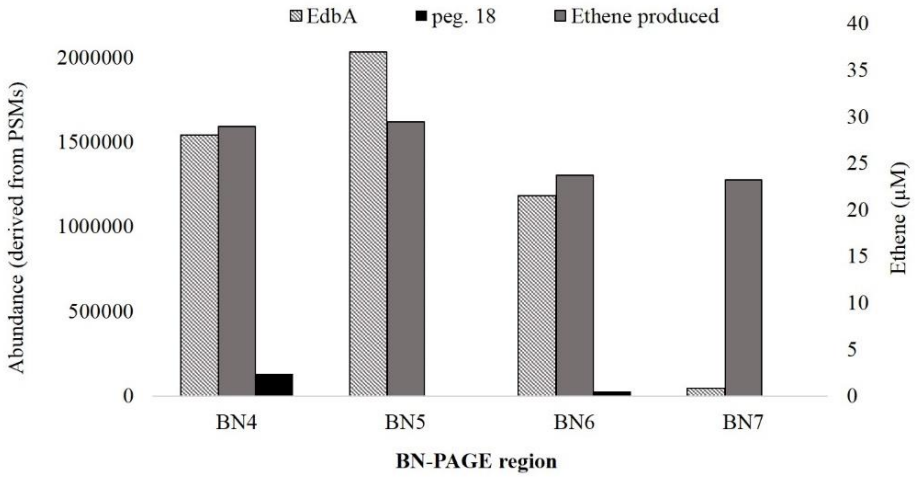

Figure 4.4 Ethene production and abundance of the two RdhA detected on each BN-PAGE piece, derived from the separation of protein extracts from EDB-growing *Dehalogenimonas.*

Table 4.3 Proteins identified after nLC-MS/MS analysis of fours slices from one replicate lane of the BN-PAGE loaded with crude protein extracts obtained from *Dehalogenimonas cells* cultivated with EDB. Accessions correspond to temporary protein encoding gene numeration from the *Dehalogenimonas* draft genome. Descriptions are the protein function assigned by RAST server on the automatic annotation performed with the sequenced genome. PSMs means peptide spectrum matches. Values in columns BN4, BN5, BN6 and BN7 corresponds to the abundance factors calculated by the Proteome discoverer analysis. Samples are listed in decreasing order of abundance factor.

Accession	Description	Coverage [%]	PSMs	Unique Peptides	Abundance (BN4)	Abundance (BN5)	Abundance (BN6)	Abundance (BN7)
peg_1385	**Reductive dehalogenase**	30	18	9	1539737.6	2029524.6	1183117.5	43938.6
peg_574	Protein 60 family chaperone GroEL	15	7	6	85174.0	540778.4	90320.5	44609.7
peg_573	Protein 60 family co-chaperone GroES	35	7	3	600898.6	1400036.7	12668.7	189664.3
peg_1144	Tryptophan synthase beta chain like	9	3	3	88026.3	445741.2	49727.0	
peg_1409	Related to 2-hydroxyglutaryl-CoA dehydratase beta subunit	7	3	3			44409.5	578404.2
peg_1495	Glutamine ABC transporter periplasmic glutamine-binding protein	9	4	2	148052.9	271399.6	32273.2	
peg_1242	LSU ribosomal protein L7/L12	22	10	3	91128.4	163074.7	220591.4	2516036.2
peg_18	**Reductive dehalogenase**	4	2	2	132698.8		24674.4	
peg_1116	NAD-dependent glyceraldehyde-3-phosphate dehydrogenase	5	4	2	33028.1			
peg_1153	Single-stranded DNA-binding protein	8	1	1			45464.8	27018.6
peg_1259	Oligopeptide ABC transporter periplasmic oligopeptide-binding protein OppA	3	2	2	132483.9			2910.4
peg_1581	Type IV pilin PilA	10	4	1	325914.5	608566.3	298273.1	194851.2
peg_729	Hypothetical protein	7	1	1	45831.6	8829.1		
peg_202	Formate dehydrogenase alpha subunit	1	1	1	6400.3	79352.6	82800.5	
peg_723	Serine hydroxymethyltransferase	2	1	1			45863.1	
peg_1219	Fructose-1 6-bisphosphatase typeV archaeal	3	1	1	59282.7			
peg_961	ATP synthase alpha chain	1	2	1	346726.1	135372.3	75849.8	28934.7
peg_607	Mannose-6-phosphate isomerase	11	1	1		7727.7	41067.5	
peg_297	Hypothetical protein	12	1	1	94638.0			
peg_127	Branched-chain amino acid aminotransferase	3	1	1	34480.4			
peg_190	Branched-chain amino acid ABC transporter amino acid-binding protein	2	1	1		21058.3	420535.6	
peg_823	Hypothetical protein	5	3	1	1085008.8	147058.6	98647.7	138192.4

Characteristics of EdbA

The *edbA* gene sequence contained reductive dehalogenase conserved sequences encoding regions. These includes a leader sequence enclosing a twin-arginine translocation (Tat) system signal peptide at the N-terminus, according to TatP and InterPro Scan analysis, as well as two iron-sulfur cluster binding motifs (predicted with InterPro Scan). The Tat-signal peptide is characteristic for proteins that are assembled in the cytoplasm and then transported in their folded status through or into the cytoplasmic membrane via the Tat machinery losing the signal peptide, and

becoming into their mature state. Non-mature EdbA protein is predicted to have a total length of 482 amino acids. Unlike other characterized RDases, its corresponding gene, *edbA,* is not adjacent to an *rdhB* gene. The *rdhB* genes typically encode for the membrane-integral proteins thought to anchor the RdhA subunits to the outer side of the cytoplasmic membrane.

RdhBs are small proteins of ~90 amino acids, mostly with three predicted transmembrane regions, and they can be located up or downstream of the *rdhA* gene (Hug, 2016). The adjacent *edbA* genes, peg. 1386 (86 aa length) and peg. 1384 (42 aa length), both with unknown functions, do not encode transmembrane helices and their predicted subcellular localization is at the outer side of the cytoplasmic membrane, according to the TMHMM tool (Krogh et al., 2001). Moreover, the three consecutive pegs located both upstream and downstream of the peg. 1385 do not contain any transmembrane region, and EdbA is not located at the edge of a contig. The lack of cognate *rdhB* genes associated with *rdhA*s seems to be a frequent feature within the genus *Dehalogenimonas*. Only 6, 3, 8, and 2 of 25, 29, 25, and 22 *rdhA* genes have a cognate *rdhB* on *D. lykanthroporepellens*, *D. alkenigignens*, *D. formicexedens*, and *D.* strain WBC-2, respectively (Siddaramappa *et al*., 2012; Key *et al*., 2016; Key *et al.*, 2017; Molenda *et al*., 2016). More recently, in 'Candidatus *Dehalogenimonas etheniformans*' strain, only 10 out of 52 *rdhA* genes were shown to harbour a corresponding *rdhB* (Yang et al., 2017). In contrast, *Dehalococcoides* strain CBDB1 genome harbour 32 *rdhAB* pairs (Kube *et al.*, 2005), and *Dehalococcoides mccartyi* strain WBC-2, 15 *rdhA* and 13 *rdhB* (Molenda *et al.,* 2016). The three previously characterized RdhA in *Dehalogenimonas* spp. (DcpA, TdrA, and CerA) have a cognate RdhB. EdbA constitutes the first RDase with proposed function of the genus *Dehalogenimonas* without an associated protein subunit B. However, the use of methyl viologen (MV) as artificial electron donor in enzymatic assays with whole cells suggests that the localization of the RDase responsible for EDB debromination was in the outer side of the cytoplasmic membrane (Nijenhuis and Zinder, 2005), as MV is considered unable to cross lipid bilayers (Jones and Garland, 1977). This is supported with the bioinformatics tools analysis, showing the presence of a Tat-signal peptide on its sequence. These results taken together would suggest the requirement of an

unknown membrane-anchoring protein encoded in a different region of the genome. It should be mentioned that potentially related, but non-adjacent RdhB subunits may not have been detected in these analysis due to their nature of small hydrophobic proteins (i.e containing 2-11 transmembrane helices) with a low number of trypsin-cleavage sites. Also, rdhB are thought not to be easily ionized in the mass spectrometer.

BLASTp nr analysis revealed that EdbA shares only 25 % pairwise amino acid identity with TceA from *D. mccartyi*. Although TceA was shown to catalyse the dihaloelimination of EDB to ethene in enzymatic assays (Magnuson et al., 2000), TceA and EdbA are not orthologous genes, according the criteria of Hug (2016). The highest pairwise aminoacid identity with EdbA in BLASTp nr database from NCBI is found with an annotated 'Hypothetical protein' from Multispecies: *Dehalogenimonas* with a 90 % identity (NCBI accession number WP_058439793.1). Second, a reductive dehalogenase from *Dehalogenimonas* strain GP show an 81 % identity (WP_102330580.1). Also, different reductive dehalogenases from *Dehalococcoides* show identities in all cases lower than 40 %.

4.3.3 Identification of the RDases expressed on 1,2,3-TCP transforming cultures

Reductive dechlorination of 1,2,3-TCP

Dechlorination of 1,2,3-TCP to allyl chloride was sustained after 4 consecutive transfers inoculating 5% v/v into fresh medium containing 1,2,3-TCP as unique electron acceptor at nominal concentration of 50 µM as initial dose for each new generation, and consecutive doses of 75 µM. The repeated addition of 1,2,3-TCP led to faster dechlorination rates indicating this reaction was supporting growth of *Dehalogenimonas* (Fig. 4.5). GC monitoring of these cultures showed 1,2,3-TCP degradation, the production of allyl chloride, and afterwards, its abiotic transformation products, allyl alcohol, allyl sulphide and allyl disulphide were detected in variable concentrations over the time.

Cellular localization of reductive dehalogenases expressed in 1,2,3-TCP growing cultures.

To study the localization of the potential RDases involved in the 1,2,3-TCP dechlorination, a combination of ultracentrifugation, SDS-PAGE and nLC-MS/MS analysis was used. In Fig. 4.6 are shown the stained 12% SDS-PAGE used for the separation of two samples corresponding to the membrane proteins fraction (panel A) and two samples corresponding to the cytosolic protein fraction (panel B) after ultracentrifugation treatment of the crude protein extract.

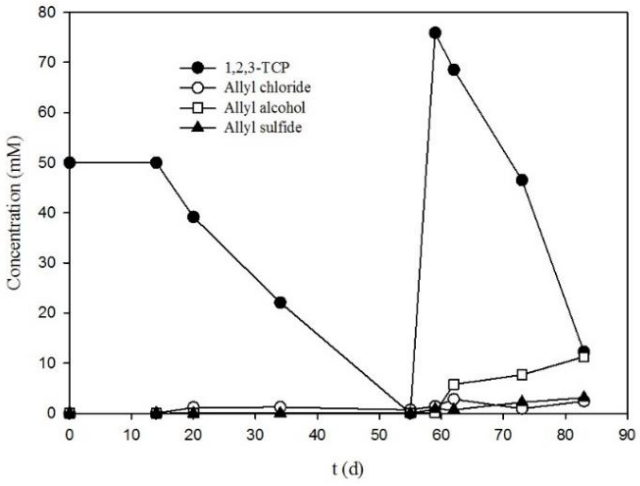

Figure 4.5 Dechlorination of 1,2,3-TCP from duplicate *Dehalogenimonas*-containing culture. The arrow indicates a second 1,2,3-TCP feeding.

The nLC-MS/MS analysis of the gel pieces from the region targeting RdhA (45-70 kDa) and RdhB (7-15 kDa) yielded a total of 38 proteins (Table 4.4). Membrane fraction abundances are the grouped abundances detected in all samples analysed from the ultracentrifugation pellet. Cytosolic fraction abundances are the grouped abundances detected in all samples analysed from the ultracentrifugation supernatant. Only one putative Rdh enzyme was detected on this analysis, the peg. 1317, in both of the samples of the SDS-PAGE gel slices corresponding to the 45-70 kDa region of the gel loaded with the membrane proteins fraction. No Rdh was detected in the SDS-PAGE loaded with the cytosolic protein fraction.

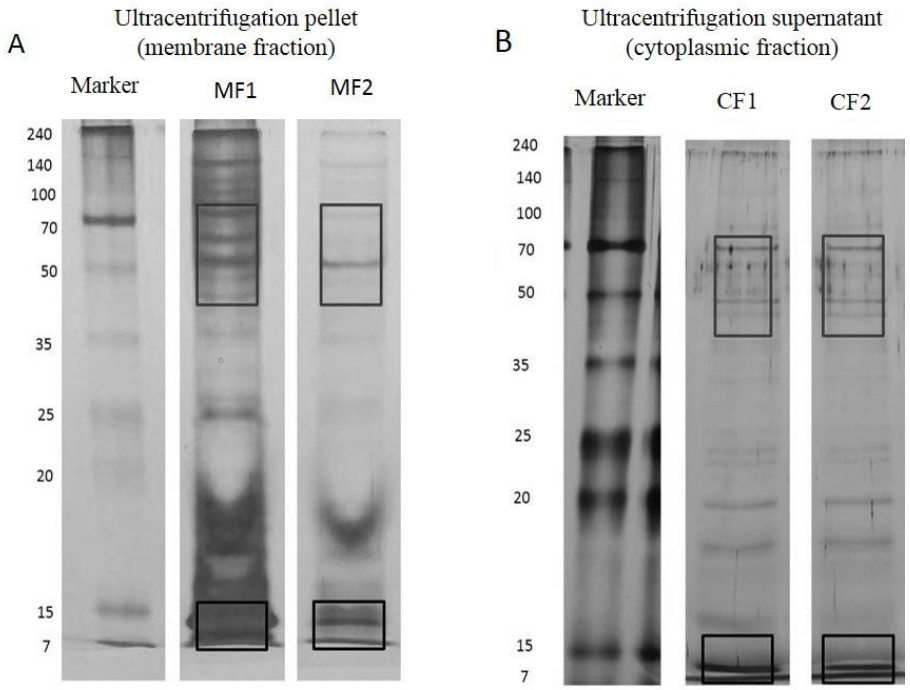

Figure 4.6 Silver stained SDS-PAGE gels of 1,2,3-TCP growing *Dehalogenimonas* cultures. Panel A: Two samples of membrane fraction of the crude protein extract (MF1 and MF2) corresponding to the ultracentrifugation pellet. Panel B: Two samples of cytosolic fraction of the crude protein extract (SF1 and SF2) corresponding to the ultracentrifugation supernatant. Both gels were run separately. Gel slices corresponding to molecular sizes between 45-70 kDa, targeting RdhA subunits, and 7-15 kDa, targeting RdhB, were sliced from each of the gels and further processed for analysis by nLC-MS/MS.

Table 4.4 Proteins identified after nLC-MS/MS analysis of the membrane fraction and the cytosolic fraction of crude protein extracts obtained from *Dehalogenimonas* cells cultivated with 1,2,3-TCP . Protein crude extracts were subjected to ultracentrifugation. Membrane fraction corresponds to ultracentrifugation pellet, while the cytosolic fraction corresponds to the ultracentrifugation supernatant. Accession correspond to temporary protein encoding gene numeration of the *Dehalogenimonas* draft genome. Description is the function assigned by RAST server on the automatic annotation performed with the sequenced genome. PSMs means peptide spectrum matches. Values in columns membrane fraction and cytosolic fraction corresponds to abundance factors calculated for grouped samples. Samples are listed in decreasing order of abundance facto

Accession	Description	Coverage [%]	PSMs	Unique Peptides	Abundance (Membrane Fraction)	Abundance (Cytosolic Fraction)
peg_574	Protein 60 family chaperone GroEL	26	22	13	5146707.1	4016.7
peg_961	ATP synthase alpha chain	13	9	6	495050.0	5482.3
peg_1238	Transcription termination protein NusA	2	1	1	389211.1	
peg_1545	DNA polymerase III beta subunit	5	2	2	249500.7	
peg_1242	LSU ribosomal protein L7/L1	26	20	3	247880.1	83427.3
peg_171	RecA protein	4	1	1	232278.8	6244.2
peg_597	Cytochrome c family protein	6	10	5	217179.2	5682.0
peg_596	Transcription termination factor Rho	5	3	2	211575.0	86367.0
peg_827	Cell division protein FtsA	3	1	1	194184.5	32834.1
peg_1581	Type IV pilin PilA	10	1	1	184092.1	4340.2
peg_81	ATP-dependent Clp protease ATP-binding subunit ClpA	4	2	2	136043.4	2803.8
peg_190	Branched-chain amino acid ABC transporter amino acid-binding protein	16	7	6	131413.6	9447.2
peg_1317	**Reductive dehalogenase**	2	1	1	118387.5	
peg_1409	Related to -hydroxyglutaryl-CoA dehydratase beta subunit	15	8	6	116069.8	4341.2
peg_458	17 SSU ribosomal protein S4p	3	1	1	102878.6	36284.1
peg_1144	Tryptophan synthase beta chain like	3	1	1	98202.0	
peg_437	17 SSU ribosomal protein S3p (	4	1	1	92484.5	
peg_1259	Oligopeptide ABC transporter periplasmic oligopeptide-binding protein	14	6	6	86698.4	5699.1
peg_246	Hypothetical protein	2	1	1	85878.3	
peg_1148	Serine/threonine protein kinase related protein	7	2	2	82024.1	
peg_344	Glutamine synthetase type III GlnN	2	1	1	70612.2	
peg_1357	Pyrophosphate-energized proton pump	3	2	2	63718.1	7026.4
peg_14	Prolyl-tRNA synthetase bacterial type	2	1	1	61051.7	2109.1
peg_1249	Translation elongation factor Tu	4	2	2	59149.5	11000.4
peg_466	S-adenosylmethionine synthetase	4	1	1	53143.3	
peg_985	Anthranilate phosphoribosyltransferase	3	1	1	46075.0	6240.6
peg_1233	Inositol-1-phosphate synthase	4	1	1	44945.0	
peg_202	Formate dehydrogenase O alpha subunit	1	1	1	42418.5	9463.9
peg_309	Ribonucleotide reductase of class II (coenzyme B1-dependent))	1	2	1	36036.4	3963.7
peg_1574	Type IV pilus biogenesis protein PilM	2	1	1	30463.7	
peg_1617	Hypothetical protein	16	2	2	29794.9	
peg_1476	Hypothetical protein	4	1	1	26717.8	9635.4
peg_1047	MarR family transcriptional regulator MJ558	2	1	1	16817.9	
peg_287	Cell division protein FtsZ	3	1	1	14786.2	
peg_1137	Branched-chain amino acid ABC transporter amino acid-binding protein	3	1	1	13517.5	14914.6
peg_573	Protein 60 family co-chaperone GroES	13	1	1	12330.3	31079.2
peg_1302	Chaperone protein DnaJ	3	1	1	8664.6	6574.0

Interestingly, the highest pairwise amino acid identities with peg.1317 in NCBI BLASTp nr database is found with two 1,2-dichloropropane RDases (DcpA) from *Dehaloccocoides mcartyi* (Table 4.5). DcpA was shown to catalyse the reductive dechlorination of 1,2-DCP to propene in different Chloroflexi-containing cultures (Padilla-Crespo *et al.*, 2014). The third, fourth and fifth higher pairwise amino acid identities with peg.1317 correspond to the DcpA from *Dehalogenimonas lykantroporepellens* strain BL-DC-9 (Padilla-Crespo *et al.*, 2014), *Dehalogenimonas alkenigignens* strain IP3-3, and *Dehalogenimonas formicexedens* strain NSZ-14T, respectively (Table 4.5). Additionally, high pairwise sequence identities, over 90% were detected with the DcpA sequences from 'uncultured bacteria' presented in Padilla-Crespo *et al.*, (2014), with accession numbers from AFV32472.1 to AFV32506.1, however, these are partial sequences.

Table 4.5 Highest pairwise amino acid identities with peg. 1317, encoded in the genome of the *Dehalogenimonas alkenigignens* strain obtained from the River Besós estuary among the protein database of NCBI, BlastP 2.8.0, nr version.

Description	Identity (%)	e-value	Query cover (%)	Score-bits	Accession number
1,2-dichloropropane reductive dehalogenase *Dehalococcoides mcartyi*	93	0.0	100	964	AGS15114.1
1,2-dichloropropane reductive dehalogenase *Dehalococcoides mcartyi*	93	0.0	100	961	AGS15112.1
Reductive dehalogenase *Dehalogenimonas lykanthroporepellens* strain BL-DC-9	91	0.0	100	936	WP_013218938.1 Dehly_1524
Reductive dehalogenase *Dehalogenimonas alkenigignens* strain IP3-3	90	0.0	100	935	WP_083496427.1 Dealk_17200
Reductive dehalogenase *Dehalogenimonas formicexedens* strain NSZ-14T	89	0.0	100	920	WP_083635400.1 Dform_1463
1,2-dichloropropane-to-propene reductive dehalogenase, partial [uncultured bacterium]	94	0.0	74	738	AFV32475.1
1,2-dichloropropane-to-propene reductive dehalogenase, partial [uncultured bacterium]	94	0.0	74	738	AFV32473.1

It is worth to mention now that in previous studies, the putative DcpA present in our *Dehalogenimonas* strain amplified by PCR with genomic DNA from 1,2-DCP growing cells when using the primers reported in Padilla-Crespo *et al.*, (2014) for detection and quantification of these genes (Martín-González *et al.*, 2015). Additionally, the bidirectional best BLASTp hits among our *Dehalogenimonas* strain and the genomes of the *Dehalogenimonas* and *Dehalococcoides* presented in Padilla-Crespo *et al.*, (2014) are also found in the DcpA proteins and peg.1317. The group of DcpA proteins described in Padilla-Crespo *et al.*, (2014) is reported to form an ortholog group. According to the criteria of Hug (2016), orthologous RdhAs show greater than

90% pairwise amino acid identity over the full-length of the sequences, and bidirectional best BLASTp hits. Applying the criteria from Hug (2016) to the sequence analysis results explained above, peg.1317 is an ortholog of the previously mentioned DcpA genes, and from now on referred to as DcpA, as well.

Experiments employing end-point reverse transcription-PCR (RT-PCR) in conjunction with primers targeting all 25 *rdhA* genes in *D. lykanthroporepellens* BL-DC-9T revealed that 19 *rdhA* genes, including *dcpA* (Dehly_1524), were consistently expressed in cultures actively dechlorinating three different electron acceptors, 1,2-DCA, 1,2-DCP, and 1,2,3-trichloropropane (Mukherjee *et al.*, 2014). The authors stated that this leaves open the possibility that the same enzyme was responsible for the transformation of all three halogenated alkanes. In our particular case, we only detected one putative reductive dehalogenase by proteomic analysis, the DcpA However, enzymatic activity was not detected in specific enzymatic activity tests using methyl viologen as an artificial donor. Numerous trials were done and activity was neither detected with whole cell concentrates nor with crude protein extracts. The reason for this absence of activity remains unknown. Therefore, it is not possible to link the expression of DcpA directly with the transformation of 1,2,3-TCP, and there is no direct evidence to date of a single RDase responsible for the transformation of 1,2,3-TCP to allyl chloride.

The architecture analysis of the predicted amino acid sequence of the DcpA from our *Dehalogenimonas* strain by InterPro Scan and TatP 1.0 shows a Tat-signal peptide, indicating that this protein is exported to the outer side of the cytoplasmic membrane via the Tat machinery. This is in accordance with our proteomic results that identified this RDase in the membrane fraction of the protein extracts. Also, DcpA is predicted to be located at the outer face of the cytoplasmic membrane analysing its subcellular localization by both TMHMM and TMMOD tools. The *dcpA*, in the same way as *edbA*, is not adjacent to an *rdhB* gene, and is not at the edge of a contig. The adjacent genes to DcpA are: (i) one putative reductive dehalogenase with no Tat-signal sequence nor transmembrane helix (TMMHM) and predicted to have a cytosolic localization (peg. 1316) and (ii) one 'hypothetical protein' (peg. 1318) with 43 amino acid length, without any significant match in the BLASTp nr database from NCBI, no

transmembrane region (TMHMM) and predicted location in the outer side of the cytoplasmic membrane. Moreover, the predicted proteins from the three consecutive loci both upstream and downstream of this DcpA do not contain any transmembrane region. Also, no predicted reductive dehalogenase subunit B (RdhB) was detected in this proteomic analysis. Differently, the previously reported DcpA RDses were shown to be co-localized in the genome with DcpB subunits (Padilla-Crespo *et al.*, 2014). Further research may be performed in order to better understand the anchorage system of the RdhA subunits present in this *Dehalogenimonas* strain to the cell membrane.

4.3.4 Expression of non-RDase proteins in 1,2,3-TCP and EDB respiring cultures

In the case of EDB-utilizing cultures, molecular chaperones, and more specifically the GroEL-GroES chaperonin system, were detected in SDS-PAGE slices (Table 4.2), as well as in all BN-PAGE slices (Table 4.3), in all cases as the second and third most abundant proteins after EdbA. Among the proteins identified on the SDS-PAGE slices derived from 1,2,3-TCP growing cells also the GroEL-GroES chaperonin system was detected. GroEL (peg. 574) was the most abundant protein detected in the membrane fraction, while GroEs (peg. 573) was detected in higher abundance in the cytosolic fraction (Table 4.4). Similarly, molecular chaperone GroEL was the second most abundant protein detected in *Dehalogenimonas* sp. strain WBC-2 growing with *trans*-DCE in BN-PAGE gel slices (Molenda *et al.*, 2016). GroEL was also detected in SDS-PAGE gel slices derived from *D. lykanthroporepellens* cells growing with 1,2-DCP, together with the DcpA (Padilla-Crespo *et al.*, 2014). Additionally, a chaperon protein DnaJ (peg. 1302) was detected both in the cytosolic and membrane fractions of the crude extracts derived from 1,2,3-TCP growing cells (Table 4.4). Chaperones are proteins related with protein folding. The close link between cobalamin and [4Fe–4S] cluster binding during RdhA maturation may explain why several RdhA are linked with a variety of chaperones (Dobbek and Leys, 2016).

A tryptophan synthase (peg. 1144), which is a lyase involved in amino acid biosynthesis pathways, was detected in BN-PAGE slices from the EDB-growing cultures

(Table 4.3), and also in the membrane fraction of the 1,2,3-TCP growing cells (Table 4.1).

Proteins related with transcription were detected in the SDS-PAGE slices derived from 1,2,3-TCP growing cultures. MarR (multiple antibiotic resistance regulator) transcriptional regulator and the transcription termination proteins NusA and Rho were found only or in higher abundance in the membrane fraction samples (Table 4.4). MarR transcriptional regulator was also found in from *D. mccartyi* strain CBDB1 linked with reductive dehalogenases, as well as in *D. lykantroporepellens*. *In vivo* studies of the interaction of the regulator with the promoter suggested that the MarR-type regulator acts as a transcription repressor (Rupakula *et al.*, 2013). MarR mediates bacterial response to changes in environmental stress and metabolic conditions by functioning as a repressor protein (Wilkinson and Grove, 2006). The binding of a substrate to the MarR protein results in its release from the promoter, thereby initiating transcription (Providenti and Wyndham, 2001; Wagner *et al.*, 2013).

A predicted formate dehydrogenase (FDH) subunit A (peg. 202) was detected in the SDS-PAGE and BN-PAGE slices derived from the EDB-growing cultures (Tables 4.2 and 4.3, respectively). FDH was also detected in both the cytosolic and the membrane fraction derived from 1,2,3-TCP-growing cultures, yet in much higher abundance in the former one (Table 4.4). It is remarkable that cultures used for these experiments were grown in a medium without formate, but hydrogen as an electron donor. The molecular weight of the formate dehydrogenase detected in this study is 112.6 kDa, it does not contain any transmembrane helix and it is predicted to be located at the outer part of the cytoplasmic membrane. In the genomic sequence, there is a predicted FDH subunit B (peg. 201), located adjacently to FDH subunit A. The highest pairwise amino acid identity found among Chloroflexi to this formate dehydrogenase in the BLASTp nr databse from NCBI was with the formate dehydrogenase-N subunit alpha from *Dehalogenimonas alkenigignens* strain IP3-3 (NCBI accession number WP_083496438.1) showing a 99% identity. This strain can use either formate or hydrogen as electron donors, in the same way as *D. lykanthroporepellens*, and *D. formicexedens*. However, the pairwise amino acid identities found with FDH from these species is 77%, and 66%, respectively. Taken together, these results suggest the

ability of this new strain of *Dehalogenimonas alkenigignens* to grow by using formate as electron donor, in absence of hydrogen. However, this ability remains to be empirically confirmed.

Additionally, motility-related proteins were detected both in the EDB and 1,2,3-TCP growing cultures. Peg.1581, corresponding with pilM, type IV pilus assembly protein was detected in the membrane fraction of the 1,2,3-TCP growing cultures, as well as in the BN-PAGE gel slices derived from the EDB growing cultures (Tables 4.4 and 4.3, respectively). Also, peg. 1574 corresponding with a Type IV pilus biogenesis protein appeared in the membrane fraction of the 1,2,3-TCP respiring cells (Table 4.4). Type IV pili are known to mediate twitching motility of non-flagellated Gram-negative bacteria and are also associated with horizontal gene transfer. The corresponding loci for pilM type IV proteins was already reported for *Dehalogenimonas lykanthroporepellens* (Moe et al., 2016).

4.4 Conclusions

The genome-sequencing of a new strain of *Dehalogenimonas alkenigignens* obtained from sediments of the River Besós estuary (Barcelona) revealed the presence of a set of 36 *rdhA*, and 3 *rdhB* homologous genes, and allowed further proteomic work oriented to identify the RdhA protein involved in the transformation of selected organohalide compounds. The novel EdbA (peg. 1385) was identified in this work as responsible for the dehaloelimination of EDB to ethene. EdbA was found to be the first reductive dehalogenase characterised to date among the genus *Dehalogenimonas* to catalyse debromintation activity, as well as the first RdhA shown to be functional with no cognate RdhB adjacently encoded in the genome. Interestingly, peg. 1317 was the only RdhA homologous protein detected in crude protein extracts of *Dehalogenimonas* cells grown on 1,2,3-TCP. This putative reductive dehalogenase was shown to be ortholog to the previously described dichloropropane-to-propene RDases (DcpA) from different strains of *Dehalococoides* and *Dehalogenimonas*. Both EdbA and DcpA contain a Tat-signal peptide that is characteristic of some redox cofactor-containing proteins that must be translocated into or across the cytoplasmic membrane in their native state, and are predicted by bioinformatics tools to be located in the outer face

of the cytoplasmic membrane. However, both do not form the typical *rdhAB* operon, as there is no *rdhB* gene adjacent to them in the genome. Their localization in the outer side of the cytoplasmic membrane, and the lack of adjacent *rdhB* suggests that they might be anchored to the membrane by a yet unidentified membrane anchoring protein, or that one non-adjacent RdhB may serve as an anchor for more than one RdhA.

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Chapter 5

Exploring the capability of anaerobic bacteria from different inoculum sources to degrade selected organohalides

Part of this chapter was published as:

Trueba-Santiso, A., Parladé, E., Rosell, M., Llirós, M., Mortan S.H., Martínez-Alonso, M., Gaju, N., Martín-González, L., Vicent, T. and Marco-Urrea, E. (2017). *Molecular and carbon isotopic characterization of an anaerobic stable enrichment culture containing Dehalobacterium sp. during dichloromethane fermentation.* Science of the Total Environment, 581–582, 640–648. doi: 10.1016/j.scitotenv.2016.12.174.

The molecular analyses presented in this Chapter were performed in collaboration with the Department of Genetics and Microbiology of the Universitat Autònoma de Barcelona.

Abstract

Microcosms established with inocula from different contaminated sources in Catalonia were tested for the anaerobic biodegradation of halogenated pollutants. No biodegradation was found for triclosan, triclocarban, diclofenac and chloroform (CF). Differently, dichloromethane (DCM) biodegradation was detected in microcosms containing slurry samples from a membrane bioreactor operating in an industrial wastewater treatment plant. Methanogens were eliminated from the consortia by the application of CF, and DCM doses consumed were increased over the time. Microbial analysis composition of the enriched consortia by bacterial 16S rRNA gene amplicon paired-end sequencing confirmed the presence of a member of the genus *Dehalobacterium,* together with three additional phylotypes belonging to *Acetobacterium*, *Desulfovibrio*, and *Wolinella*, representing all four operational taxonomic units near 99.9% of the retrieved sequences. The halogenated compounds 1,2-dichloroethane, 1,2-dichlopropane, trichloroethylene, tetrachloroethylene and 4-chlorophenol were tested as substrates for this DCM-degrading culture, with a negative result. Only the brominated analogue dibromomethane (DBM) was transformed, suggesting a strong substrate specialization of the degrader bacterium. Acetate and formate were identified as the biodegradation products for both substrates, indicating that fermentation was the metabolic pathway implied in these transformations.

5.1 Introduction

Halogenated compounds can be found in groundwater derived from their production in several natural processes, but also from industrial emissions. Organohalide-transforming bacteria are believed to play a crucial role in the global halogen cycle and their capability to transform organohalides can be exploited for bioremediation of contaminated aquifers. The widespread occurrence of organohalide respiring bacteria (OHRB) in pristine soil samples has been demonstrated in different studies (Duhamel and Edwards, 2007; Justicia-Leon *et al.*, 2012; Kleindienst *et al.*, 2017; Yang *et al.*, 2017). For instance, the detection of 16S rRNA genes from *Dehalococcoides*-like Chloroflexi was positively correlated with the quantification of naturally occurring organochlorine compounds and total organic carbon (TOC) in 116 pristine soil samples collected from different locations in USA, while no correlation was detected for universal bacterial 16S rRNA genes (Krzmarzick *et al*. 2012). However, the large majority of OHRB have been obtained from contaminated sites all around the world, mainly from polluted sediments or soils (Duhamel and Edwards, 2007; Marzorati, 2007; Key *et al.*, 2017), but from wastewater treatment plants as well (Mägli *et al.*, 1996; Chen *et al.*, 2014; Wang *et al.*, 2014).

More information about distribution, physiological characteristics and requirements of organohalide transforming anaerobic bacteria, is important to extend the knowledge on the roles of these organisms in the ecosystems, and especially in the recycling of organohalogens from both natural and anthropogenic sources. As pointed in Lorenz and Löffler, (2016), certain families of halogenated compounds of environmental concern and present in impacted groundwater have not been sufficiently investigated so far as electron acceptors for OHRB. This is the case for instance of pharmaceuticals, perfluoroalkyl and polyfluoroalkyl substances (PFASs), monohalogenated alkanes, pesticides or halogenated methanes. In this work we selected halogenated compounds for which biodegradation under anaerobic conditions is poorly studied to date. The emerging contaminants diclofenac, triclosan, and triclocarban, as well as the priority substances PFOA, chloroform (CF) and dichloromethane (DCM) were included in this study.

The degradation of the pharmaceuticals diclofenac, triclosan and triclocarban was mostly studied under aerobic conditions. Diclofenac [2-(2,6-dichloranilino) phenylacetic acid] is a non-steroidal antiinflamatory drug widely detected in groundwater (Cabeza *et al.*, 2012; Sui *et al.*, 2015) and harmful to the environment (Naidoo *et al.*, 2009). Its biodegradation was only reported by aerobic bacteria and by the white-rot fungus *Trametes versicolor* (Marco-Urrea *et al.*, 2010; Poirier-Larabie *et al.*, 2016; Domaradzka *et al.*, 2016). The multichlorinated aromatic compounds triclosan [2,4,40-trichloro-20-hydroxydiphenyl ether, or Irgasan] and triclocarban (3,4,40-trichlorocarbanilide) are synthetic broad spectrum antimicrobial agents incorporated into a wide variety of personal care products (e.g. hand soap, toothpastes or acne creams) and other consumer products. Degradation of triclosan in aerobic conditions was shown for wastewater bacteria from the genera *Sphingomonas* or *Nitrosomas* (Hay *et al.*, 2001; Roh *et al.*, 2009).

Perfluorooctanoic acid (PFOA) is a synthetic perfluorinated carboxylic acid, toxic and carcinogenic, used in the industry mainly because of its surfactant properties, for instance as a flame retardant (Steenland *et al.*, 2010). PFOA is considered persistent under aerobic conditions (Parsons *et al.*, 2008). To date, only one study has reported its biodegradation under anoxic conditions. However, no fluorine anions were detected, nor the degradation products, and the bacteria responsible for this process was not identified, thus no clear conclusions could be drawn (Schröder, 2003).

Dehalogenation of chlorinated methanes under anoxic conditions is poorly understood if compared to other compounds (e.g. chloroalkenes). CF and DCM are produced both in natural and anthropogenic processes. Under anoxic conditions, CF is transformed by organohalide-respiring bacteria from the genus *Dehalobacter* and *Desulfitobacterium* strain PR (Grostern *et al.*, 2010; Lee *et al.*, 2012; Justicia-Leon *et al.*, 2014; Ding *et al.*, 2014). DCM was described to be transformed under anoxic conditions by facultative methylotrophic bacteria (Bader and Leisinger, 1994; Muller *et al.,* 2011). Regarding strictly anaerobic bacteria, DCM degradation was only reported by fermentation in a few species affiliated to the Peptococcaceae family: *Dehalobacterium formicoaceticum* (Mägli *et al.*, 1996), *Dehalobacter* spp. (Lee *et al.*,

2012; Justicia-Leon *et al.*, 2012) and 'Candidatus *Dichloromethanomonas elyunquensis*' (Kleindienst *et al.*, 2017). Interestingly, no DCM organohalide-respiration was reported so far.

The main objectives of this work were: i) to explore the capacity of bacteria from different inoculum sources to degrade the selected halogenated pollutants under anaerobic conditions, ii) to enrich the degrading cultures and study their microbial composition, iii) to study the range of halogenated substrates potentially transformed by the degrading cultures, and iv) to identify the byproducts produced from organohalide degradation.

5.2 Materials and methods

5.2.1 Sampling

Sludge and sediments from wastewater treatment plants and rivers, respectively, were collected in order to explore the potential of native bacteria to degrade the selected compounds. The chosen sampling locations were known to be impacted with halogenated compounds and therefore they constituted a potential suitable niche for microbiota able to transform them (Table 5.1). First, slurry samples from the anaerobic digester of the Girona urban wastewater treatment plant (WWTP) were used as inoculum since wastewater from the hospital Josep Trueta (Girona) is discharged on it, containing different halogenated pharmaceuticals (Cruz-Morató *et al.*, 2014). Likewise, slurry samples from a membrane bioreactor (MBR) operating at the centralised industrial WWTP of Distiller S.A, located in Barcelona, were included in this study because this MBR receives influents containing halogenated compounds (e.g. DCM, chloropropane, monochlorobenzene and tetrachloroethylene) from industrial wastewater. Also, black sediments were collected from the margin of the Ebro River Delta (Tarragona), as it was previously reported the presence of a wide array of halogenated compounds, including halogenated flame retardants, in this compartment (Barón *et al.*, 2014; Pignotti *et al.*, 2017). Last, black sediments from the margin of the River Besós estuary (Sant Adriá de Besós, Barcelona) were selected because this coastal area has been historically contaminated with short-chain

chlorinated paraffins and halogenated flame retardants (Castells *et al.*, 2008; Cristale *et al.*, 2013). Additionally, in previous studies of our research group, an OHRB from the genus *Dehalogenimonas* was obtained from this sampling point (Martín-González *et al.*, 2015). The inoculum samples were collected in sterile Falcon tubes maintaining anoxic conditions as possible, sealed with parafilm, transported to the lab at 4 ºC, and placed inside an anaerobic glovebox (Coy) to set up the microcosms in the same day

Table 5.1. Inoculum sources, type of samples collected and summary of the halogenated compounds previously detected in the sampling points of interest for this work.

Inoculum sources	Sample type	Halogenated compounds previously reported	References
Girona urban WWTP (Girona)	Sludge from the anaerobic digester	Halogenated pharmaceuticals from the effluents of the hospital Josep Trueta	(Cruz-Morató *et al.*, 2014)
Centralised industrial WWTP of Distiller S.A (Barcelona)	Sludge from the membrane bioreactor	Industrial wastewater containing DCM, chloropropane, monochlorobenzene and tetrachloroethylene, among others.	Personal communication
Ebro River Delta (Tarragona)	Black sediments from the margin	Halogenated flame retardants	(Barón *et al.*, 2014; Pignotti *et al.*, 2017)
Besós River Estuary (Barcelona)	Black sediments from the margin	Short-chain chlorinated paraffins and halogenated flame retardants	(Castells *et al.*, 2008; Cristale *et al.*, 2013)

5.2.2 Cultivation

Microcosms were prepared into an anaerobic glovebox with either 6 g of homogenized slurry or sediments (in wet weight) in 120 mL glass serum bottles containing 65 mL of the sterilised anaerobic synthetic medium described in Section 3.2.1. Microcosms were then closed with Teflon rubber septa and aluminium crimps. Acetate (5 mM), pyruvate (5 mM), fumarate (5 mM), formate (5 mM), and yeast extract (200 mg/L) were simultaneously amended as potential carbon sources through

the septum from 100 X aqueous anaerobic stock solutions. In the same way, vitamins and bicarbonate solution as buffer (pH = 7) were added to each microcosm. Two reducing solutions were tested in parallel for each combination of inoculum and contaminant: (i) $Na_2S \times 9H_2O$ and L-cysteine at 0.2 mM each, and (ii) Titanium citrate (0.8 mM titanium (III), 1.6 mM citrate). Each microcosm was gassed with N_2/CO_2 (4:1, v/v) to overpressure of 0.2 bar and then with H_2 at 0.4 bar. Contaminants were spiked to the microcosms with a 10 µL glass syringe (Hamilton) from acetone stock solutions. For each inoculum, triclosan, diclofenac, triclocarban, PFOA, DCM and chloroform were spiked in parallel microcosms at two different concentrations (25 and 50 µM).

Abiotic controls consisted of microcosms containing the defined medium spiked with the corresponding halogenated compound without slurry or sediments, and were used to exclude abiotic transformations. Killed controls consisted of microcosms containing the defined medium spiked with the corresponding halogenated compound and inoculated with sediments or slurry, but inactivated with sodium azide (50 µM), and were used to discard that contaminants elimination occurred via adsorption into the solid matrix. Controls without the halogenated compound were also included to determine whether degradation products were produced from sources different from the spiked contaminants. All controls and cultures were set up in triplicate.

The microcosms that depleted the initial dose of organohalide were reamended with the same dose and transferred to fresh medium (10% v/v) during the exponential phase of degradation of at least, the second dose of contaminant. The contaminant dose amended was increased progressively among the serial transferences in order to favour the bacteria responsible for the degradation process as explained elsewhere (Löffler *et al.*, 2005).

5.2.3 Dilution-to-extinction technique in liquid medium

The dilution-to-extinction technique in liquid medium was used to enrich the degrading cultures. This strategy was conducted in 20 mL glass vials containing 12 mL of the anoxic medium described in section 5.2.2, but using as a reducing agent, $Na_2S \times 9H_2O$ and L-cysteine. Vials were set up and cultivated following the procedure

described in section 5.2.2 for those cultures in serum bottles. Under these conditions, sequential dilutions series were generated through serial 1 in 10 dilutions starting from an active culture in the exponential phase of DCM degradation and up to 10^{10} dilution (Fig. 5.1). Each vial was amended with DCM from an acetone stock to a final nominal concentration of 50 µM with a 10 µL glass syringe (Hamilton). Dilution vials were incubated in dark, static conditions at 25 ºC in a thermostatic chamber and monitored weekly for DCM consumption.

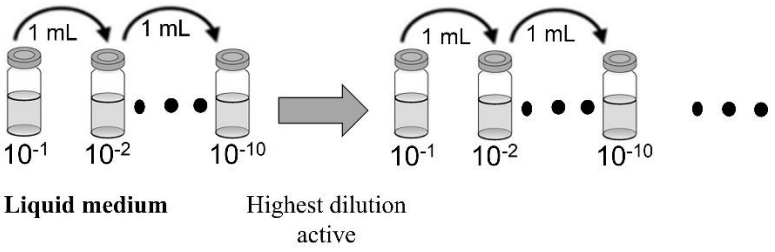

Figure 5.1 Dilution-to-extinction technique.

5.2.4 Analytical methods

Volatile halogenated compounds and methane were quantified from the headspace of the microcosms by GC. Fluoride, chloride and bromide ions were quantified in liquid samples by ionic chromatography. The concentrations of volatile fatty acids (acetate, lactate, formate and pyruvate), triclosan, diclofenac, triclocarban, and 4-chlorophenol were quantified from liquid samples by HPLC. For detailed information see General materials and methods in Chapter 3.

5.2.5 DNA extraction

Microcosms were filtered using 0.22 µm GV Durapore membrane filters (Millipore, Barcelona, Spain) and stored at − 80 °C until they were processed. DNA extraction was conducted using the Power Water DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) following the instructions of the manufacturer.

5.2.6 Illumina sequencing

The V4 region of the bacterial 16S rRNA gene was sequenced through a paired-end approach of 250 bp with primers previously described (Caporaso *et al.*, 2012) and sample identifying barcodes. Samples were sequenced in the latest version of the Illumina MiSeq sequencing platform at external facilities (Sistemas Genómicos, Valencia, Spain). Illumina sequences are available in the Sequence Read Archive from the NCBI under accession number SRR4422954. Raw sequences and statistical analyses were performed using mothur (Schloss *et al.*, 2009) following an analysis procedure described elsewhere (Kozich *et al.*, 2013). The traditionally considered forward and reverse raw reads were aligned to construct a continuous sequence. Non-aligned sequences were removed at this step. Furthermore, sequences were quality trimmed according to the following criteria: ambiguous bases or undetermined nucleotides (N), sequences larger than 300 bp or containing N6 homopolymers were filtered out of the data set. Chimeras were identified and removed from the pool of sequences using Uchime (Edgar *et al.*, 2011). Sequences were aligned against the SILVA reference alignment and were classified using Mothur's Bayesian classifier against the SILVA database (Quast *et al.*, 2013), which was trimmed to the V4 hypervariable region of the 16S rRNA gene to improve classification depth down to genus level (Werner *et al.*, 2011). Those sequences not identified as bacteria were removed from further analysis. Operational taxonomic units (OTUs) were clustered into phylotypes using a database-dependent approach and then subsampled according to Kozich suggestions (Kozich *et al.*, 2013).

5.3 Results and discussion

5.3.1 Degradability of the selected halogenated compounds by inocula from different sources

The set of microcosms prepared was monitored for the biodegradation of the halogenated compounds diclofenac, triclosan, triclocarban, PFOA, chloroform and DCM. In the first-generation cultures inoculated with sludge from the MBR, 50 µM of DCM was consumed in 20–25 d. The consumption of additional amendments lead to

faster degradation rates and removal of DCM was not observed in the abiotic controls, indicating that the reaction was biotically mediated. The microcosms established with the other abovementioned halogenated compounds were monitored each 2-4 weeks. No release of fluoride ions was detected in PFOA-containing cultures. Also, no significant degradation was detected neither in the cultures nor in the controls of the rest of the microcosms. The overall experimental period was 18 months to allow the detection of dehalogenating activity after a prolonged lag phase. Therefore, the DCM-degrading culture line was chosen for further studies.

Our results with diclofenac, triclosan, triclocarban and PFOA are in accordance to previous reports in the literature. Diclofenac is considered persistent under anoxic conditions, as exemplified in Poirier-Larabie *et al.*(2016), where diclofenac was barely degraded 1% in absence of oxygen. In cultures derived from agricultural soil triclocarban and triclosan were degraded in aerobic conditions, but both compounds persisted under anoxic conditions (Ying *et al.*, 2007). Anaerobic degradation of triclosan was only reported so far under methanogenic conditions (Gangadharan Puthiya Veetil *et al.*, 2012; Ying *et al.*, 2007). A recent study showed the increase in the abundance of *Dehalococcoides*-like Chloroflexi 16S rRNA genes in response to triclosan exposition in microcosms from agricultural soil (McNamara *et al.*, 2014). However, no direct evidence of triclosan dehalogenation was provided to date, so further research is necessary to determine if triclosan is a suitable substrate for OHRB. Similarly, to our knowledge, there is no evidence on degradation of triclocarban under anoxic conditions (Ying *et al.*, 2007). PFOA degradation was also not reported under anaerobic conditions. Indeed, organohalide respiration of fluorinated compounds have never been reported. Despite the fact that defluorination reaction is thermodynamically favored and bacteria could obtain energy for growth from reductive defluorination; it seems that this mechanism is hindered by the kinetic stability of the C–F bond, that constitutes the strongest single bond formed by carbon (Parsons *et al.*, 2008; Ochoa-Herrera *et al.*, 2008; Ferrey *et al.*, 2012; Dolfing, 2016).

5.3.2 Enrichment of the DCM-degrading culture

The anaerobic synthetic medium of the DCM-degrading culture initially contained acetate, formate, fumarate, pyruvate and yeast extract as potential carbon sources to support the dehalogenating activity. With the aim of elucidating the carbon source requirements of the DCM-degrading culture, a first set of experiments was conducted to investigate the need for these substrates. The removal of formate, fumarate and pyruvate from the medium in parallel cultivations did not affect DCM degradation after six consecutive transfers (5% v/v) indicating that they were not required in this process. However, different attempts to remove acetate from the medium were done during this initial enrichment process, and after two sequential transfers, DCM dechlorination always stopped. Similarly, the lack of yeast extract prevented DCM degradation. Acetate and yeast extract could be utilized by either the DCM-degrading bacteria itself or other microorganisms acting synergistically with it that provided key nutrients (i.e. cofactors) not supplied in the synthetic medium. Secondly the removal of tungsten and selenium ended DCM degradation after two transfers and, therefore, they were maintained in the medium.

Methane production occurred concomitantly to DCM degradation. To discard a role of methanogens in DCM degradation, 2-bromoethanesulfonate (BES), a structural analogue of the cofactor coenzyme-M involved in the terminal step of methane biosynthesis, was tested as methanogen inhibitor (Bauchop, 1967). After the addition of BES (25 µM), methane production was strongly depressed, but DCM degradation stopped. Alternatively, CF (10 µM) was used as inhibitor of methanogens (Weathers and Parkin, 2000), and production of methane completely ceased with no negative effect on DCM degradation. This is in accordance to several evidences showing that BES can also inhibit non methanogens catalysing dechlorination reactions (Löffler *et al.*, 1997; Ye *et al.*, 1999). Chloroform was maintained as inhibitor for methanogens for 20 transfers, and then eliminated from the medium.

To investigate the effect of antibiotics interfering with bacterial cell wall biosynthesis on degradation of DCM, vancomycin (500 µg/mL) and ampicillin (1

mg/mL) were tested in parallel microcosms by duplicate. DCM degradation was completely inhibited in both cases, suggesting that a Gram-positive bacterium was responsible for the degradation of DCM.

During the enrichment of this culture, the concentration of DCM amended was gradually increased from 50 µM up to 3000 µM. Enrichment cultures in the 15th transfer were considered stable after exhibiting robust and reproducible DCM degradation profiles. From this point, the dilution to extinction technique was applied in this cultures.

5.3.3 Analysis of the bacterial diversity present on the DCM-degrading culture

To get insight about the microbial composition of the DCM-degrading culture, two cultures derived from the most diluted vial (10^{-10}) of the sixth serial dilution tank (T6) were selected for molecular analyses by Illumina MiSeq sequencing (Fig. 5.2).

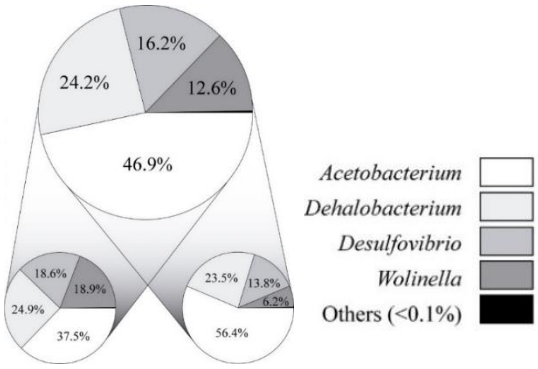

Figure 5.2 Composition of the microbial consortia after six serial dilution tanks by Illumina sequencing. Taxonomic affiliations of retrieved non-singleton OTUs (0.03 cut-off) are represented by pie charts at genus level. Data from both replicates are shown in small pies below the main chart.

The quality criteria applied for reduction of sequencing noise, chimeric sequences and presence of contaminants discarded on average ca. 25.0% of the retrieved reads. Alpha diversity measurements (Shanon and Chao diversity index) of both replicates were within the same range. Interestingly, rank abundance curves for each replicate showed the dominance of 4 shared operational taxonomic units (OTUs) representing near 99.9% of the retrieved sequences in both replicates. The OTUs (in

decreasing order of relative abundance) correspond to the genera *Acetobacterium*, *Dehalobacterium*, *Desulfovibrio*, and *Wolinella* (Fig. 5.2).

5.3.4 Test of alternative substrates for the DCM-degrading culture

The potential of the enriched DCM-degrading culture to transform chloroalkanes (1,2-dichloroethane and 1,2-dichloropropane) and chloroethenes (trichloroethylene and tetrachloroethylene) was assessed replacing DCM with the alternative halogenated compounds in parallel cultures at two different concentrations (25 µM and 100 µM) in duplicate. No biodegradation of these chlorinated compounds was detected after two months of incubation. After confirming the absence of biological degradation, 2400 µM DCM was amended to these cultures to investigate the possible need of DCM as electron donor to proceed with the dechlorination of the tested compounds. Then, DCM was consumed, but the alternative chlorinated substrates remained in the medium.

Recently, an enrichment culture containing a bacteria belonging to the genus *Dehalobacterium* sp. was shown to dechlorinate 4-chlorophenol under iron reducing conditions (Li *et al.*, 2014). In order to assess if these conditions could expand the range of substrates utilized by this culture we reproduced the same iron reducing conditions by applying amorphous ferrous solution and amending 4-chlorophenol in parallel cultivations. However, triplicate cultures amended with and without amorphous ferrous (prepared as explained in Li *et al.*, 2014) did not transform 25 µM of 4-chlorophenol after two months.

Finally, the brominated analogue dibromomethane (DBM) was tested in duplicate at concentrations of 25 and 100 µM, showing debrominating activity in both concentrations after one month of incubation and maintaining this activity in four sequential transfers. These results suggest the high substrate specificity of this bacterium. Bacteria capable of degrading DBM have been described in marine ecosystems (Goodwin *et al.*, 1998), linked with the natural production of this compounds in seawater by macroalgae. In the case of freshwater only few cases of biological removal were reported. DBM was transformed by the aerobic bacteria

Xanthobacter autotrophicus GJ10 in a cometabolic process during 1,2-dichloroethane degradation, detecting formaldehyde and bromide release as the DBM transformation byproducts (Torz *et al.*, 2007). A methylotrophic bacteria isolated from groundwater (strain DM11) was found to use DCM or DBM as sole carbon and energy source (Scholtz *et al.*, 1988). In a packed bed reactor under reducing conditions, a DCM-fermenting culture was described to degrade DBM (De Best *et al.*, 2000). Finally, the isolate *Dehalobacterium formicoaceticum* was able to use only DCM and DBM as sole substrates, but in this case, in contrast to our study, DCM was required to proceed with the DBM debromination process (Mägli *et al.*, 1996).

5.3.5 Identification of DCM and DBM degradation products

As shown in Figure 5.3, this culture transformed DCM to acetate and formate, suggesting that DCM was transformed via a fermentation pathway. Repeated additions led to faster degradation rates, which is an indicator of growth-related processes. Production of acetate was calculated by subtracting the concentration of acetate in the control cultures without DCM from acetate concentration in the experimental bottles containing the culture plus DCM.

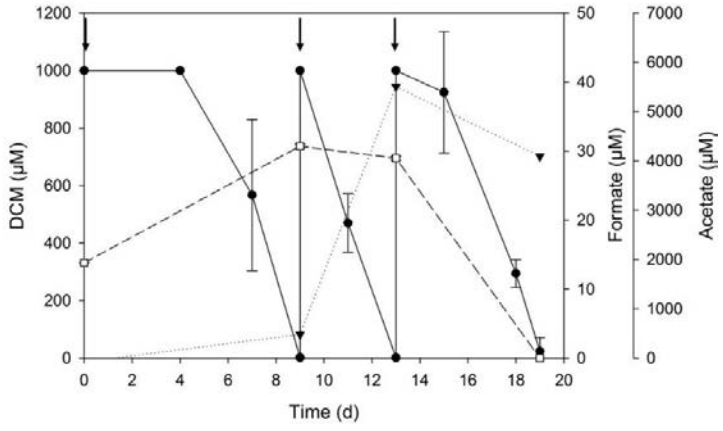

Figure 5.3 Time-course of dichloromethane (DCM) degradation and product formation in the anaerobic *Dehalobacterium*-containing culture. Symbols: DCM (●), acetate (▼), formate (□). Arrows indicate additions of DCM and error bars indicate standard deviations for three experimental bottles.

Traces of chloromethane were detected occasionally during incubation, indicating that hydrogenolysis was negligible. Chloride ions released could not be

quantified due to the salt concentration on the medium that overlapped the chloride peaks in ionic chromatography analysis. The metabolism of DCM according to the previously isolated *Dehalobacterium formicoaceticum* resulted in the following reaction:

$$3CH_2Cl_2 + CO_2 \rightarrow 2HCOO^- + CH_3COO^- + 6Cl^- + 9H^+$$ (Mägli *et al.*, 1996)

The concentration of acetate produced in our culture was higher than the stoichiometric amount expected from DCM degradation in accordance with the previous identification of homoacetogens (i.e. *Acetobacterium*) in the culture, which can produce acetate from carbon dioxide and hydrogen (or other suitable electron donors such as formate) via the Wood-Ljungdahl pathway. Formate was solely produced in the cultures containing DCM, confirming that it derived from DCM, but it did not accumulate over time, probably due to its use as electron donor by other microorganisms.

Time-course experiments of DBM degradation showed that repeated additions of DBM (50 µM) led to faster degradation rates, suggesting that this debromination process was also growth-related (Fig. 5.4). The measured ratio for the micromoles of DBM degraded and bromide ions released was 1:1.7, indicating a near-complete debromination.

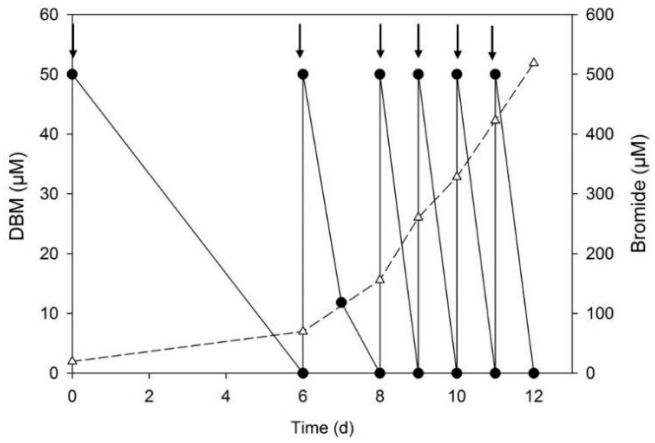

Figure 5.4 Time-course of dibromomethane (DBM) degradation and bromide ions released in the anaerobic *Dehalobacterium*-containing culture. Symbols: DBM (●) and bromide (Δ). Arrows indicate additions of DCM. The experiment was performed in duplicate.

5.4 Conclusions

Diclofenac, triclosan, triclocarban, and PFOA are widely spread halogenated compounds of environmental concern considered to be persistent in groundwater. This recalcitrance is in accordance to the lack of biodegradability observed under the anaerobic conditions tested in our study. Chloroform was also not transformed. Differently, in the case of DCM we obtained an enriched culture derived from slurry samples of MBR of an industrial WWTP capable of fermenting this halomethane. The mixed DCM-degrading consortia was shown to be mainly composed by members of the genus *Dehalobacterium*, *Acetobacterium* and *Desulfovibrio*. Among all alternative substrates tested only DBM was transformed by this culture, pointing a strong substrate specialization. The compounds 1,2-dichloroethane, 1,2-dichloropropane, trichloroethylene, tetrachloroethylene, and 4-chlorophenol, however, were not transformed. Acetate and formate were detected as major products of DCM and DBM fermentation by this culture. To date, only bacteria from the genus *Dehalobacterium*, *Dehalobacter* and *Dichloromethanomonas* have shown the potential to transform anaerobically DCM via fermentative pathway. Therefore it is of a great interest to characterize the new DCM fermenting culture obtained in this study and to identify the bacteria responsible for DCM degradation. Also, it would be important to explore the potential inhibitory effect of other halogenated compounds frequently detected in groundwater over the DCM-fermentation performance of this culture.

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Chapter 6

Identification and isolation attempt of a DCM-degrading anaerobic bacterium

Part of this chapter was published as:

Trueba-Santiso, A., Parladé, E., Rosell, M., Llirós, M., Mortan S.H., Martínez-Alonso, M., Gaju, N., Martín-González, L., Vicent, T. and Marco-Urrea, E. (2017). *Molecular and carbon isotopic characterization of an anaerobic stable enrichment culture containing Dehalobacterium sp. during dichloromethane fermentation.* Science of the Total Environment, 581–582, 640–648. doi: 10.1016/j.scitotenv.2016.12.174.

The molecular analyses presented in this Chapter were performed in collaboration with the Department of Genetics and Microbiology of the Universitat Autònoma de Barcelona.

Abstract

Previously, an enriched dichloromethane (DCM) fermenting culture was obtained from the slurry samples of a MBR reactor treating industrial wastewater, and it was mainly composed by *Dehalobacterium*, *Acetobacterium, Wolinella*, and *Desulfovibrio* members. To date, only a few strictly anaerobic bacteria were reported to ferment DCM, thus the isolation of this DCM degrader is of a major interest. Comparative analysis of bacterial 16S rDNA-DGGE profiles from colonies-derived cultures degrading DCM contained a predominant band belonging to *Dehalobacterium*, however this band was absent in colonies-derived cultures unable to degrade DCM. The isolation of this *Dehalobacterium* sp., responsible for the DCM degradation, was attempted. Due to the high dilution applied to the cultures, *Wolinella* was eliminated from the consortia. The lack of hydrogen, acetate and CO_2 amendments in the medium led to a marked decrease in the relative abundance of the homoacetogen *Acetobacterium* sp., whereas no effect over *Desulfovibrio* sp. abundance was detected. The application of the antibiotic metronidazole decreased the abundance of *Desulfovibrio* sp., yet it was not completely eliminated. Due to the production of acetate and formate from DCM fermentation and the presence of $NaHCO_3$ and traces of hydrogen in the microcosms, the removal of *Acetobacterium* and *Desulfovibrio* could not be accomplished. However, a relative abundance of 67.0 % of *Dehalobacterium* sp was obtained. This enriched consortia is a suitable candidate for bioremediation projects due to its robustness, and the potential stimulation effect of the DCM-degradation byproducts on native organohalide-respiring bacteria present in the contaminated sites.

6.1 Introduction

DCM has been widely used at many industrial processes as solvent, propellant, and postharvest fumigant. To illustrate this fact, DCM was the third industrial organochloride compound more utilized in Catalonia from 1995 to 2011 (Fernández-García *et al.*, 2014). Due to its extensive use, improper disposal practices and accidental releases, DCM is one of the most frequently encountered subsurface pollutants in industrial areas (Shestakova and Sillanpää, 2013). Exposition to DCM can cause liver and kidney damage and the United States Environmental Protection Agency (EPA) (U.S. EPA, 2000), as well as the International Agency for Research on Cancer (IARC, 1999) have classified this compound as a Group B2, probable human carcinogen. Moreover, it is ranked 11th on the list of priority substances in the framework of water policy of the European Union (Directorate General for Environment of the European Comission, 2011).

Once released in the environment, DCM can migrate through the unsaturated soil column (vadose zone) and tends to accumulate as dense non-aqueous phase liquid (DNAPL) at the bottom of the polluted aquifers (Seyedabbasi *et al*., 2012). A feasible strategy to detoxify contaminated sites is *in situ* bioremediation because it is an environmental friendly technology and relatively cheaper if compared with physical-chemical remediation techniques. Aerobic biodegradation of DCM has been described in several bacterial isolates (Muller and Bringel, 2011), but the main bottleneck for their application is the usually low dissolved oxygen concentration found in contaminated groundwater. This makes anaerobic biodegradation a preferred alternative strategy to remediate DCM-contaminated aquifers.

The use of DCM as the sole carbon source was reported for a mixed culture under methanogenic conditions, yielding acetate, CO_2 and H_2 when methanogenesis was inhibited (Freedman and Gossett, 1991). The degradation of DCM under nitrate-reducing conditions has also been described for an *Acinetobacter* sp. (Freedman *et al.*, 1997). The degradation of DCM by different facultative methylotrophic bacteria has been deeply characterized (Muller and Bringel, 2011; Vuilleumier and Leisinger, 1996; Nikolausz *et al.*, 2006). The enzyme responsible for this reaction is DcmA, a dehalogenase which belongs to the glutathione S-transferases (Vuilleumier, 1997).

DcmA transforms DCM into two molecules of HCl and formaldehyde. DCM degradation by this metabolic pathway has been extensively characterised. The *dcmA* gene is extremely highly conserved and horizontal gene transfer is supported by its genetic context, replete with mobile elements (Muller *et al*., 2011).

Regarding strictly anaerobic bacteria, to date, only three genera have been described to metabolize DCM. These bacteria performs organohalide fermentation. The reported pathway for this process includes the conversion of DCM to methylene tetrahydrofolate, of which two-thirds was oxidized to formate while one-third gave rise to acetate by incorporation of CO_2 from the medium in the acetyl coenzyme A synthase reaction (Mägli *et al.*, 1998). The Gram-positive *Dehalobacterium formicoaceticum* (phylum Firmicutes, class *Bacilli*) was the first strictly anaerobic bacterium isolated and characterized which was able to ferment DCM, producing formate and acetate in a molar ratio of 2:1, respectively (Mägli *et al.*, 1995; Mägli *et al.*, 1998). Since then, there have been no other *Dehalobacterium* isolates described in the literature. Also, two different strains of the genus *Dehalobacter* have shown ability to ferment DCM, expanding the metabolic versatility of this genus beyond organohalide respiration (Justicia-Leon *et al.*, 2012; Lee *et al.*, 2012). More recently, the novel DCM-fermenting species 'Candidatus *Dichloromethanomonas elyunquensis*' was identified (Kleindienst *et al.*, 2017).

Dibromomethane (DBM) is a natural ozone-depleting substance and one of the two most abundant short-lived bromocarbons in the atmosphere, together with bromoform ($CHBr_3$). DBM is mostly emitted by the ocean, as it is produced by phytoplankton and macro algae (Quack *et al.*, 2007). A further process generating DBM is reductive hydrogenolysis of bromoform, which occurs mainly in anoxic environments (Vogel *et al.*, 1987). Bacterial oxidation of DBM was shown for saline and freshwater under aerobic conditions (Goodwin *et al.*, 1998), but in the case of anoxic conditions, very few is known about DBM degrading bacteria. *D. formicoaceticum* was capable of transforming DBM, but only when growing with DCM (Mägli *et al.*, 1996).

Due to the widespread presence of DCM in contaminated groundwater, as well as the limited knowledge available on anaerobic bacteria transforming DBM, the

identification and characterization of bacteria capable of transforming these dihalomethanes to environmentally acceptable products is of major interest.

In Chapter 5, a consortium capable of fermenting DCM and DBM mainly composed by *Dehalobacterium*, *Acetobacterium*, *Desulfovibrio*, and *Wolinella* was obtained. It is common to detect organohalide transforming bacteria in microbial communities containing other anaerobes, but the synthrophic association among them is scarcely known. The interdependencies established among these bacteria are a challenge to isolate the organohalide degrading bacteria of interest.

To illustrate the benefits of these synergistic relations, in co- and tri-cultures of the organohalide-respiring bacteria *Dehalococcoides mccartyi* strain 195, the presence of *Acetobacterium* and *Desulfovibrio* enhanced the growth and dechlorination rate of organochlorines, suggesting that strain 195 benefited from the organic cofactors (e.g. corrinoids) produced by the others strains (He *et al.*, 2007; Men *et al.*, 2011). Also, *Desulfovibrio* was required for *Dehalobium* strain and *Dehalobacter* to dechlorinate polychlorinated biphenyls and 1,1,1-trichloroethane, respectively (May *et al.*, 2008; Grostern and Edwards, 2006). Reductive dechlorination by *Dehalococcoides* was irreversibly inhibited by exposure to oxygen; thus, *Dehalococcoides* may depend on oxygen-scavenging organisms, such as δ-proteobacteria (e.g. *Desulfovibrio*), when present in mixed communities (Hug *et al.*, 2012). *Desulfovibrio* sp. were also found in the initial stages of the enrichment of *D. formicoaceticum*, and it was described to use the formate and acetate (or possibly hydrogen or carbon monoxide) produced by *Dehalobacterium* from DCM (Mägli et al., 1995). A decrease in TCE-respiration performance in a mixed culture containing *Dehalococcoides, Geobacter* and *Acetobacterium* was found with the higher TCE influent concentrations tested. Using molecular analyses and electron balances this was shown to be related with the suppression of *Acetobacterium* sp. when this high TCE concentrations were applied. *Acetobacterium* was supposed to proportionate cofactors to *Dehalococcoides* that are essential for the reductive dechlorination of TCE (Ziv-El *et al.*, 2012).

In some cases, the link among members of bacterial consortia was so strong that in order to isolate the target species, cell extracts from other species were required in the culture medium. For instance, in the case of *D. formicoaceticum*, cell

extracts from *Desulfovibrio* sp. were initially necessary to achieve the isolation. The degradation of 2,3,4,5-tetrachlorobiphenyl by *Dehalobium chlorocoercia* strain DF-1 was prevented when *Desulfovibrio* sp. was removed from the medium. The degradation activity was only restored when adding alive cells or cell-free extracts or *Desulfovibrio* sp. (May *et al.*, 2008).

Due to the widespread presence of DCM in contaminated groundwater, as well as the small knowledge available on anaerobic bacteria transforming DBM, the identification of bacteria capable of fermenting these halomethanes to environmentally acceptable products is of a major interest. The objectives of this work were the identification and isolation attempt of a DCM degrader contained in an enriched culture.

6.2 Materials and methods

6.2.1 Dilution-to-extinction technique in liquid and semisolid medium

The dilution-to-extinction technique in liquid medium is described in section 5.2.4. The dilution-to-extinction technique in semisolid medium was also conducted in 20 mL glass vials containing 12 mL of the anoxic medium described in section 5.2.3, using as a reducing agent $Na_2S \times 9H_2O$ and L-cysteine. The setting up procedure of the vials was as detailed in section 5.2.3, with the exception of the addition of a 1% w/v low melting point agarose in each of the vials prior to its autoclaving. All the elements added after autoclaving, including the inoculum, were amended through the septum, while maintaining the dilution vials in liquid state at 35 ºC in a heated water bath. Under these conditions, sequential dilution-to-extinction series were performed up to 10^{-10} dilution. Each vial was amended with DCM from an acetone stock to a final nominal concentration of 50 µM with a 10 µL glass syringe (Hamilton). Dilution vials were incubated in dark, static conditions at 25 ºC in a thermostatic chamber and monitored weekly for colony formation.

The picking-up of the colonies was performed through the septum of the semisolid dilution vials by using 1 mL one-use syringes and needles containing 0.2 mL of anaerobic water inside. Once the needle was inserted inside the dilution vial containing the colony, 0.1 mL of the anaerobic water was released on the top of the

agar column, to avoid breaking the agar when going through it with the needle towards the colony. Then, the colony was picked up and extracted out of the dilution vial suspended in the 0.1 mL of anaerobic water left, and inoculated into a new fresh liquid medium-containing serum bottle (65 mL) with a small magnetic stirrer inside. The microcosm was then agitated to break the colony, and further cultivated under dark static conditions at 25 ºC.

6.2.2 Analytical methods

Volatile halogenated compounds and hydrogen were quantified from the headspace of the microcosms by GC as described in section 3.2.2.

6.2.3 Denaturing gradient gel electrophoresis (DGGE) analysis

DGGE was used to analyse the microbial community from liquid dilution vials and colonies from semisolid dilution vials. The liquid vials and colonies selected for DGGE were inoculated back to 65-mL serum bottles containing fresh defined medium as described in section 5.2.3 and DNA was subsequently extracted, as detailed in section 5.2.6.

For bacterial DGGE analyses, the primer set 341f/907r (Muyzer *et al*., 1993) was employed in order to amplify a 550 bp DNA fragment from the 16S region of the small subunit rRNA gene. A GC clamp was added at the 5' end of the primer 341f and final concentrations of the PCR reactions were: 1× PCR buffer, 2 mM of $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (Invitrogen), 500 nM of each primer and 2.5 units of Taq DNA polymerase (Invitrogen). The amplification program consisted of: 94 °C for 5 min; 20 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 3 min; 15 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min; and a single final extension of 72 °C for 7 min. Dcode Universal Mutation Detection System (Bio-Rad) was utilized to run the DGGE. Obtained PCR products covering the ca. 550 bp fragment were loaded onto 6% (w/v) polyacrylamide gels (acrylamide/bis solution 37.5:1) containing a denaturing linear chemical gradient ranging from 30 to 70% (100% denaturant corresponding to 7 M urea and 40% (v/v) deionized formamide). A mixed 50-bp ladder DNA markers (Invitrogen and New England Biolabs). Gels were run at 75 V and 60 °C during 16 h in 1× Tris acetate-EDTA. For its visualization, gels were stained with 1 µg/mL ethidium

bromide solution for 25 min, washed with deionized water 25 min and photographed with Universal Hood II (Bio-Rad, Barcelona, Spain) under UV light. Bands of interest were recovered for further amplification using the described PCR protocol. Amplicons obtained from individual bands were placed in 96-well plates and sequenced by Macrogen (Republic of Korea).

6.2.4 Clone libraries

Primers used for clone libraries were 341f-1492R. First of all, amplification of the desired fragment from the DNA extract was carried out and size of amplicons was checked by electrophoresis in 1.5 % agarose gels. Once quality DNA samples were obtained, libraries were performed using the TOPO TA cloning kit (ThermoFisher), following the manufacturer recommendations. One µL of culture was used as template for each clone PCR reaction. Results were checked in 1.5% agarose gel and sent to Macrogen sequencing facilities (Amsterdam, the Netherlands). Clone libraries were kept at 4 ºC during the analysis and stored at –80 ºC indefinitely. Each clone library consisted of a 96 plate experiment, including one plate as a negative control. The final number of plates presented in the results corresponds to those plates amplified that resulted of enough quality to allow BLAST analysis.

6.3 Results and discussion

6.3.1 Evidence of *Dehalobacterium* as responsible for DCM degradation

The DCM-degrading culture was serially diluted in liquid medium, and dechlorinating activity was recovered from a terminal positive vial in the 10^{-10} dilution after five previous consecutive serial dilution series (T5). The whole *Dehalobacterium*-containing culture isolation procedure is presented as a scheme in Fig. 6.1. The culture contained in this vial was used as inoculum in serum bottles analysed by DGGE after consuming 2400 µM of DCM. As observed in Fig. 6.2 (Lane 1), two predominant bands appeared in this culture belonging to *Dehalobacterium* and *Acetobacterium* genus.

In parallel, this inoculum was used in the dilution-to-extinction approach with agar vials. Colonies of two different morphologies appeared in the 10^{-7} dilution vials after 7–10 d in all the shakes performed. The most abundant type (type A) of colony was circular, with an entire margin, punctiform, glistening, opaque and gold to black

pigmented not visible in pictures due to their small size. A second type of colony (type B) shown in Fig. 6.3 appeared in a number of 5–30 in each agar vial and were rhizoid shaped with a filamentous margin, larger than the first type (sizing 1–1.5 mm diameter), non-pigmented, translucent and with a dull appearance. Over 30 colonies from both types were picked up and inoculated back in serum bottles to test their ability to ferment DCM.

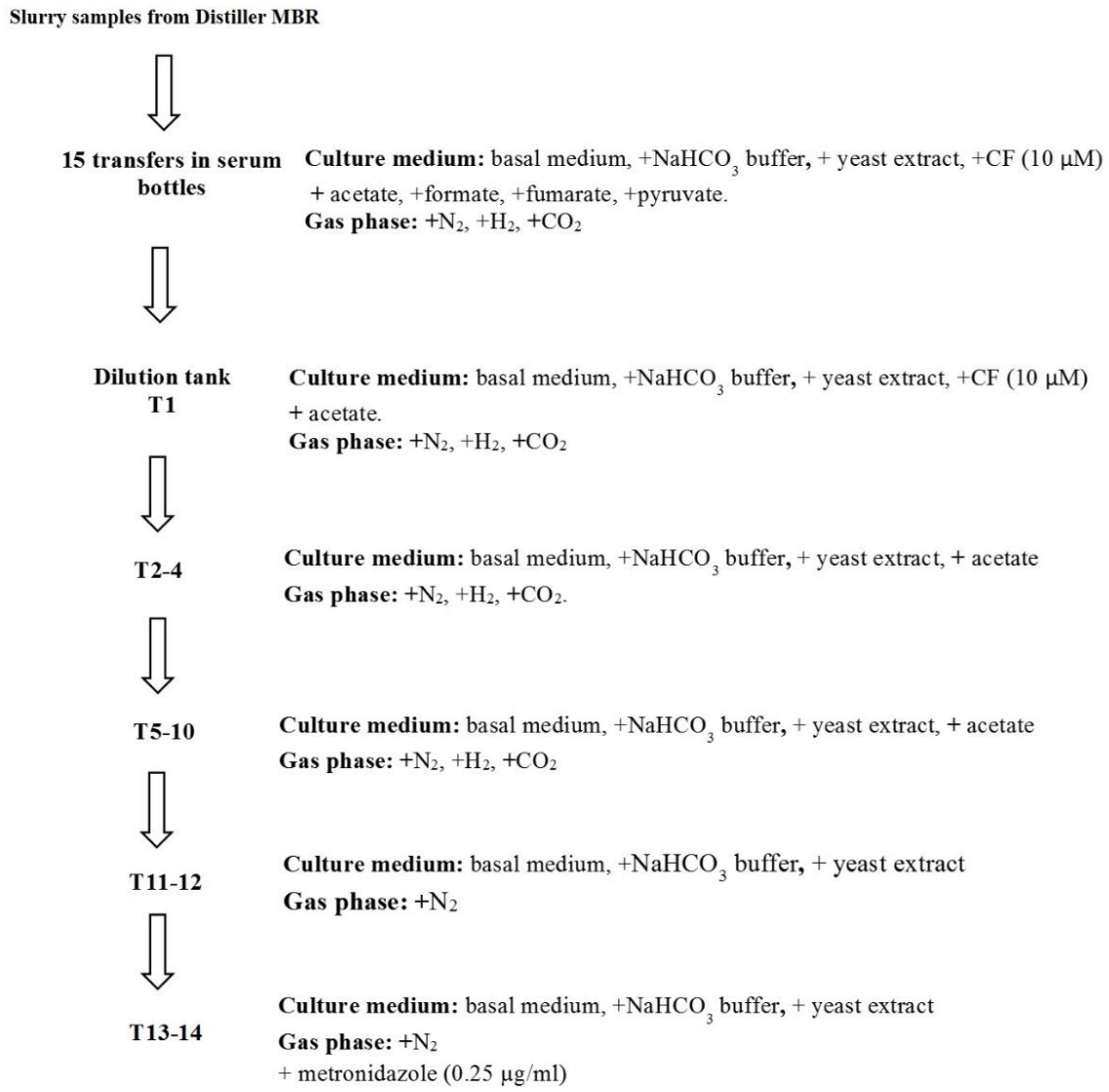

Figure 6.1 Scheme of the isolation procedure indicating composition of the culture medium, and gas phase at the different stages.

Only one of these colonies, which derived from the morphological type B described above, showed DCM degrading activity. This culture was transferred three

consecutive times (5% v/v) and at this point it was subjected to DGGE analysis. The results obtained in this culture indicated again the predominance of *Dehalobacterium* and *Acetobacterium* (Fig. 6.2, Lane 2). Interestingly, the serum bottles inoculated with the colonies that did not show activity against DCM, produced turbidity in the liquid, indicating cell proliferation which was also observed in the DCM degrading cultures. Two of these cultures were analysed by DGGE and the sequence indicate presence of *Acetobacterium* but absence of *Dehalobacterium* (Fig. 6.2, Lanes 3 and 4). In all means, our results indicated that *Acetobacterium* was accompanying *Dehalobacterium* along the different enrichment approaches but the latter was responsible for DCM fermentation.

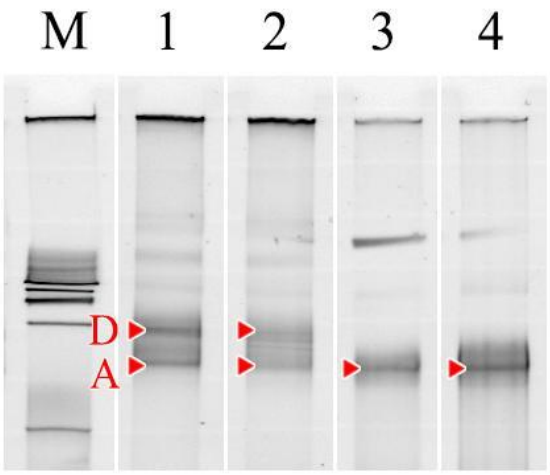

Figure 6.2 DGGE profiles of 16S rRNA gene fragments of DNA extracted from serially diluted cultures of the DCM-degrading enrichment culture. Lane M corresponds to the marker. Lane 1 corresponds to the enrichment culture showing DCM degradation activity from the 10^{-10} diluted terminal positive vial. Lane 2 corresponds to culture derived from a colony showing DCM degradation activity. Lanes 3 and 4 correspond to cultures derived from colonies without DCM degrading activity. The arrows indicate bands excised and sequenced. Band "A" belongs to *Acetobacterium* sp. and band "D" to *Dehalobacterium* sp.

In the literature there are several examples of bacterial consortia degrading halogenated compounds where members of *Acetobacterium* genus were accompanying the degraders. For instance, in enrichment cultures containing *Dehalococcoides ethenogenes* cultivated with CO_2 in the gas phase and ethanol, lactate or hydrogen as electron donors, three different strains of *Acetobacterium* were detected (Duhamel *et al.*, 2002). Also, in the *Dehalobacter*-containing culture fermenting DCM described in Justicia-Leon *et al.*, (2012), *Acetobacterium* sp. was detected, probably favoured by the presence of CO_2, bicarbonate and hydrogen in the

medium. Moreover, *D. formicoaceticum* was shown to be able to grow in co-culture with *Acetobacterium woodii* (Mägli *et al.*, 1995). Bacteria from the genus *Acetobacterium* are considered to supply cofactors to organohalide degrading species.

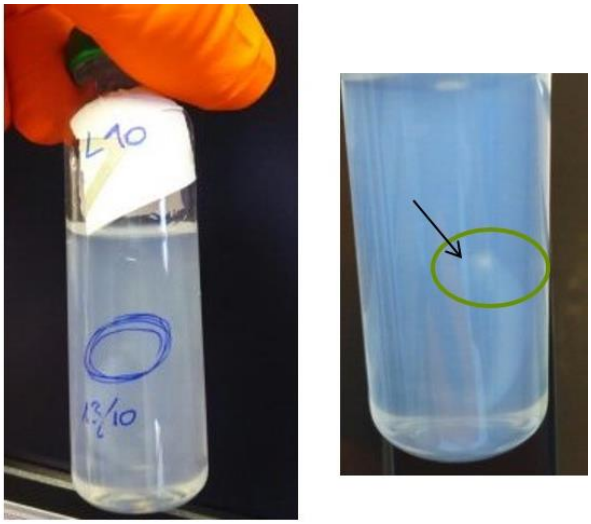

Figure 6.3 Agar shake vials inoculated with the DCM-fermenting culture showing type B colonies.

6.3.2 Isolation attempt

6.3.2.1 Effect of acetate, carbon and hydrogen on the bacterial community composition

Dehalobacterium sp. was shown to be responsible for the DCM degradation, and obtaining a pure culture would allow to perform further biochemical and metabolic analyses. For this purpose, we continued applying the dilution-to-extinction technique to the culture. The microbial composition of the culture was analysed by clone library and Illumina sequencing after four consecutive dilution tanks (from T7 to T10) from a positive tube in the 10^{-10} dilution. Relative abundances of 42.5 % *Acetobacterium*, 27.7 % *Dehalobacterium*, 5.3 % *Desulfovibrio,* 1.1 % *Artrobacter* and 23.4 % of 'other taxa' were determined (Table 6.1). Comparing these results with the previous microbial composition analysis by Illumina (section 5.3.3), *Wolinella* sp. appeared to be absent, and *Desulfovibrio* sp. relative abundance decreased, probably due to the high dilution applied during the consecutive dilution tanks. *Acetobacterium* abundance increased, probably only because of the decrease in other taxa abundance.

Table 6.1 Microbial composition of the clone library performed with the *Dehalobacterium*-containing culture after 10 serial dilution tanks.

OTU	Abundance (%)	Clone numbers	16S rRNA gene copies
Dehalobacterium	27.7	26	5
Acetobacterium	42.5	40	5
Desulfovibrio	5.3	5	3
Arthrobacter	1.1	1	6
Other taxa	23.4	22	
Total	100	94	

In order to eliminate *Acetobacterium* and *Desulfovibrio* members of the DCM-degrading consortia we paid attention into the physiological features and antibiotic sensitivity reported for these genera On the one hand, *Acetobacterium* spp. are homoacetogenic strictly anaerobic bacteria, which catalyse the synthesis of acetate from C_1 units in their catabolism. Most of the homoacetogens are able to grow oxidising H_2 and reducing CO_2 as the sole energy source, according to the following equation: $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$. Thus, the reduction of 2 CO_2 to acetate must be coupled to the synthesis of ATP (Diekert and Wohlfarth, 1994). *Acetobacterium* spp. perform homoacetic fermentations from glucose, lactate, methanol and glycerate, and may be adapted to ferment formate (e.g *Acetobacterium woodii)* (Balch *et al.*, 1977). On the other hand, *Desulfovibrio* spp. are obligate anaerobes and sulphate or nitrate reduction is their respiratory dissimilatory process (Saraiva *et al.*, 2001). Autotrophic growth on hydrogen has not been critically demonstrated for this genus. *D. vulgaris*, model organism for the study of sulphate-reducing bacteria, derives energy only from oxidative phosphorylation by coupling the reduction of sulphate (or other sulphur oxyanions, e.g. sulphite or thiosulfate) to sulphide with the oxidation of hydrogen. Species of this genus can utilize acetate or formate (e.g *D. vulgaris*) as carbon sources, among others (Heidelberg *et al.*, 2004; Postgate and Campbell, 1966).

The effect of removing acetate, hydrogen and CO_2 from the medium over DCM degradation was tested at this point in parallel microcosms derived from T10 (Fig 6.1). Firstly, the lack of acetate amendment produced no negative effect on the DCM degradation performance, thus it was not further included in the culture medium. However, as stated in chapter 5, acetate was hypothesized to be produced from both *Acetobacterium* and *Dehalobacterium*, so the lack of acetate in the medium composition might not be effective to remove *Desulfovibri*o.

Secondly, we tested the effect of not amending H_2 on the microcosms. Parallel cultivations with hydrogen amendment at 0.4 bar (gas phase $N_2/CO_2/H_2$) and without hydrogen amendment (gas phase N_2/CO_2) were tested. However, it should be taken into account that culture medium was always bottled inside an anaerobic glovebox that contains ~ 3% of hydrogen (varying depending on the usage), leading to a small percentage of hydrogen at the headspace of the microcosms (<1.5%). Parallel cultivations amended or not with hydrogen did not show differences regarding the DCM degradation performance, consuming 2000 µM of DCM in 4 d after a lag phase of 4 d. So further cultivations were done without hydrogen amendment. The response of this culture to hydrogen is different to what was reported for the *Dehalobacter* DCM-fermenting species present in Justicia-Leon *et al.* (2012), which was inhibited by hydrogen additions.

The effect of removing CO_2 and/or $NaHCO_3$ on DCM fermentation was also investigated. Parallel cultivations with (i) a gas phase composed by CO_2/N_2, and HEPES buffer in substitution of $NaHCO_3$, (ii) only N_2 in the gas phase, and HEPES buffer in substitution of $NaHCO_3$ (iii) only N_2 in the gas phase with $NaHCO_3$ as buffer were monitored. Cultures on the conditions (i) and (ii) lost the DCM degrading activity at the second transfer, while in conditions (iii), the DCM degradation was not affected. The requirement of $NaHCO_3$ for DCM degrading activity is in accordance to reports for other DCM-fermentators previously investigated. DCM degradation by *Dehalobacter* sp. occurred in both bicarbonate and HEPES-buffered microcosms, but only occurred when amended with CO_2 (Justicia-Leon *et al.*, 2012). Also, in 'Candidatus *Dichloromethanomonas elyunquensis*', omission of bicarbonate from the medium precluded DCM degradation activity, which could not be recovered following transfer

to medium with bicarbonate (Kleindienst *et al.*, 2017). *Dehalobacterium formicoaceticum* required a medium containing carbonate, and according to the proposed metabolism for this species the carboxyl group of acetate originates from CO_2 (Fig. 6.4) (Mägli *et al.*, 1996; Mägli *et al.*, 1998).

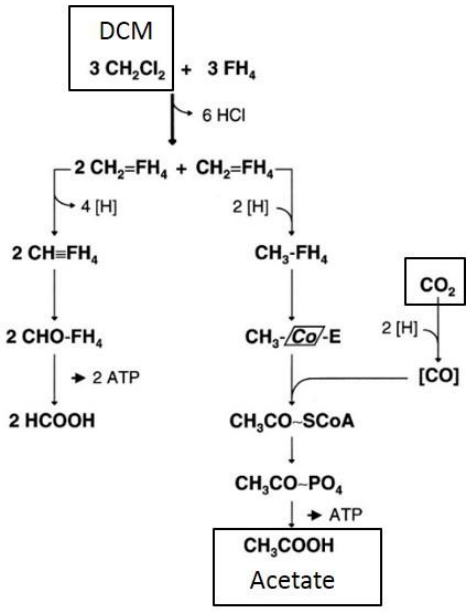

Figure 6.4 Proposed pathway for the metabolism of dichloromethane by *Dehalobacterium formicoaceticum* strain DMC, showing the incorporation of CO_2 from the medium into the carboxyl group of acetate. Modified from Mägli *et al.*, 1998.

The acetyl coenzyme A synthase reaction from the Wood–Ljungdahl pathway is probably used for the CO_2 fixation in the DCM fermentators, as stated in Mägli *et al.*, 1998. The presence of all enzymes involved in the Wood-Ljungdahl pathway with a featured core acetyl coenzyme A synthase (*acs*) gene cluster was corroborated by the genome-sequencing of *D. formicoaceticum* strain DMC (Chen *et al.*, 2017), as well as for 'Candidatus *Dichloromethanomonas elyunquensis*' (Kleindienst *et al.*, 2017).

Taking the information obtained from the previous studies into account, two consecutive dilution tanks without acetate, CO_2 and H_2 amendments in the medium were cultivated (T11 and T12, Fig 6.1). Then, inoculum from the vial corresponding to dilution 10^{-9} from T12 was taken and transferred to a serum bottle. After consumption of two amendments of 2000 µM, the culture was again analysed by clone library and Illumina sequencing (Table 6.2).

Table 6.2 Microbial composition of the clone library performed with the *Dehalobacterium*-containing culture after the 12th serial dilution tank to assess the effect of the lacking of acetate, H_2 and CO_2 amendments on the culture medium. Abundance is presented as relative abundance in %.

OTU	Abundance (%)	Clone numbers	16S rRNA gene copies
Dehalobacterium	52.6	26	5
Acetobacterium	27.4	40	5
Desulfovibrio	6.3	6	3
Other taxa	13.7	13	
Total	100	85	

Relative abundances of the taxa at this point were 27.4 % of *Acetobacterium,* 52.6 % of *Dehalobacterium*, 6.3 % of *Desulfovibrio*, and 13.7 % of 'Other taxa'. These results show a positive effect of the lack of acetate, hydrogen and CO_2 amendment on the microcosms, increasing the relative abundance of *Dehalobacterium,* while decreasing the *Acetobacterium* population. On the contrary, *Desulfovibrio* sp. relative abundance was not affected by these changes in the cultivation conditions. Both *Desulfovibrio* and *Acetobaterium* are described to use H_2 but probably the first one may present a lower hydrogen threshold than *Acetobacterium* to grow, thus its relative abundance was not affected by the changes in the medium.

6.3.2.2 Effect of selected antibiotics on the bacterial community composition

At this point, in an attempt of eliminating *Desulfovibrio* from the consortia, the antibiotics metronidazole and chloramphenicol were selected for their known effectivity against 23 different *Desulfovibrio* strains of clinical interest. Metronidazole and chloramphenicol MIC_{90} (minimum inhibitory concentration at which a 90% of the isolates tested were inhibited) reported for these strains were 0.25 µg/mL, and 16 µg/mL, respectively (Lozniewski *et al.*, 2001).

First, the susceptibility of the *Dehalobacterium* culture to these antibiotics at the MIC_{90} concentrations above-mentioned was tested in parallel dilution tanks derived from T12 (Fig. 6.1). Conditions tested were (i) both antibiotics at once, (ii) single application of chloramphenicol and (iii) single application of metronidazole. Conditions (i) and (ii) prevented DCM degradation, while DCM degradation in condition

(iii) was not affected, leading to the conclusion of *Dehalobacterium* susceptibility to chloramphenicol at 16 µg/mL. Consequently, metronidazole was chosen for further cultivation.

After two consecutive dilution tanks with metronidazole at 0.25 µg/mL in the culture medium (T13 and T14, Fig.6.1), inoculum from the vial corresponding to dilution 10^{-7} from T14 was taken and transferred to a serum bottle. After consumption of two 2000 µM amendments of DCM, the culture was again analysed by clone library and Illumina sequencing (Table 6.3). A decrease in *Desulfovibrio* sp. relative abundance from a 6.3 % to a 1.1 % was detected. Also, the relative abundance of 'other taxa' decreased from a 13.7 % to 4.4 %. On the contrary, *Acetobacterium* sp. relative abundance was not affected by this antibiotic, as its relative abundance was 27.4 % and 27.5 % before and after its application, respectively. These together resulted in an enrichment of *Dehalobacterium* sp. from 52.6 % to 67.0 %.

Table 6.3 Microbial composition of the clone library performed with the *Dehalobacterium*-containing culture after the 14th serial dilution tank to assess the effect of the addition of metronidazole to the culture medium.

OTU	Abundance (%)	Clone numbers	16S rRNA gene copies
Dehalobacterium	67.0	61	5
Acetobacterium	27.5	25	5
Desulfovibrio	1.1	1	3
Other taxa	4.4	4	
Total	100	91	

6.4 Conclusions

The *Dehalobacterium* sp. present in the DCM degrading consortia was shown to be the responsible for DCM degradation. On the one hand, it was shown to require the presence of bicarbonate in the medium, in accordance to previous DCM-degraders investigated. On the other hand, DCM-fermentation was not negatively affected by the presence of hydrogen in the microcosms, differently to a previously described DCM-

fermenting *Dehalobacter* strain. Also, *Dehalobacterium* was shown to be susceptible to chloramphenicol, but not to metronidazole at the concentrations tested. Due to the presence of $NaHCO_3$ and hydrogen in the medium, as well as the production of acetate and formate by the *Dehalobacterium* sp., *Acetobacterium* and *Desulfovibrio* may have developed cooperative metabolic interactions with the DCM degrader that impede the isolation. However, there was a clear enrichment on *Dehalobacterium* sp. through the treatments performed and the presence of the accompanying genera may bring up an advantage on the DCM fermentation capacities of this culture enhancing the robustness of this culture (e.g supplying growth cofactors). In any case, this consortia was shown to be stable on its composition and is a strong candidate for bioremediation purposes, as its major byproducts, i.e acetate and formate, are interesting biostimulants for bacteria (e.g. organohalide-respiring bacteria) relevant to decontaminate halogenated pollutants accompanying DCM in aquifers.

6.5 References

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Chapter 7

Carbon stable isotope fractionation during dichloromethane fermentation by a mixed culture containing a *Dehalobacterium* sp.

Part of this Chapter was published as:

A. Trueba-Santiso, E. Parladé, M. Rosell, M. Llirós, S.H. Mortan, M. Martínez-Alonso, N. Gaju, L. Martín-González, T. Vicent and E. Marco-Urrea (2017). *Molecular and carbon isotopic characterization of an anaerobic stable enrichment culture containing Dehalobacterium sp. during dichloromethane fermentation.* Science of the Total Environment, 581–582, 640–648. doi: 10.1016/j.scitotenv.2016.12.174.

CSIA was performed at the Universitat de Barcelona (UB) at the laboratories of the Grup de Mineralogia Aplicada i Geoquímica de Fluids, Departament de Mineralogia, Petrologia i Geologia Aplicada, Facultat de Ciències de la Terra.

Abstract

Evaluation of the occurrence and extent of biodegradation of volatile organic compounds (VOCs) in impacted aquifers present some constraints. Physical-chemical processes like volatilization, dilution or sorption may lead to the erroneous interpretation of biodegradation taking part if the assessment relies only in pollutant concentration determinations. Additionally, to elucidate the *in situ* transformation pathways of the pollutants is also problematic as mass balances are frequently difficult to close in complex environments. In this regard, the compound stable isotope analysis (CSIA) is a mature and strong tool with potential to overcome the abovementioned constraints. In contrast to the relatively frequent detection of dichloromethane (DCM) in contaminated groundwater, there is a lack of information on isotopic fractionation during DCM degradation compared to other halogenated volatile organic compounds. So far, the only reported isotopic enrichment factor (ε_C) of DCM was determined for methylotrophic strains under both oxic and nitrate-reducing conditions, and regarding strict anaerobes, only in the case of a *Dehalobacter*-containing culture. In this Chapter, ε_C value determined during DCM degradation in a *Dehalobacterium*-containing culture is -27 ±2‰ that differs from the ε previously reported for *Dehalobacter* (-15.5±1.5‰). However, they are both significantly different from those reported for facultative methylotrophic organisms (ranging from -45 to -61‰), which allow to differentiate between hydrolytic transformation of DCM via glutathione-dependent dehalogenases and fermentation pathway.

7.1 Introduction

The evaluation of the occurrence and extent of *in situ* biodegradation processes in contaminated groundwater is a crucial issue for the optimal design of remediation strategies. In this regard, a classical approach based on the quantification of the pollutants and their reaction partners throughout the contamination plume presents some drawbacks. If a decrease in the pollutant concentration was detected, then it could be interpreted as biodegradation occurring. However, this decrease may be also caused by physical-chemical processes, as for instance dilution, volatilization, adsorption, chemical transformation or dispersion (Meckenstock *et al.*, 2015). Improper sampling techniques may also lead to the loss of pollutants, especially in the case of gases and volatile compounds (Höhener and Aelion, 2010). Therefore, biodegradation could be wrongly assumed to be happening. Additionally, mass balances are frequently difficult to be closed in complex environments, becoming also problematic to elucidate the occurrence of potential transformation pathways taking part.

In the last decades, the advances made on the use of isotope techniques for the study of environmental processes positioned compound-specific stable isotope analysis (CSIA) as an innovative, but mature tool to overcome the limitations of other classical approaches.

The word isotope was used for the first time by Frederick Soddy in 1913 and is composed by the combination of the Greek roots *isos* (meaning equal) and *topos* (meaning place) in reference to the fact that different isotopes of the same element occupied the same place on the periodic table of elements. Elements differ in their number of protons in their nucleus (Z). The atomic weight (A) of an element is the result of summing the number of protons (Z) and neutrons (N) in the nucleus (A= N+Z). The periodic table of elements organizes the elements according to their atomic weight. Differences in the number of neutrons in the nucleus lead to different atomic weights for the same element, and the corresponding different atoms are named nuclides. Nuclides with the same number of protons (Z) but different number of neutrons (N), thus different atomic weights (A) are called isotopes. Today, more than

2200 isotopes of the 92 naturally occurring elements are known (Höhener and Aelion, 2010). Commonly, for each element one of its isotopes is present in more abundance in global means in nature. In the case of carbon, for instance, 98.93% of this element in nature is C^{12}, while 1.07% is C^{13} (Höhener and Aelion, 2010). Chlorine isotopes in nature are distributed in a 75.76% of Cl^{35} and 24.25% of Cl^{37}. Isotopes can be divided into stable or no radioactive (e.g C^{12} and C^{13}) and unstable or radioactive (e.g C^{14}). Generally, isotopes with a similar number of protons and neutrons ($N/Z \leq 1.5$) tend to be stable and do not decay to other element. In isotope techniques, the stable isotopes are taken into account. The abundance of the heavy isotope relative to the abundance of the light isotope (e.g C^{13}/ C^{12} for carbon) is denoted as isotope ratio, and frequently reported as ratio R. For instance, the mean global carbon isotope ratio C^{13}/ C^{12} is R = 0.011237.

Heavier isotopes tend to form shorter and more stable chemical bonds, because of their larger masses (Urey, 1946)(Urey, 1946)(Elsner *et al*., 2005). Generally, the chemical bonds are broken more easily when containing a light isotope compared to a heavier one (Melander and Saunders, 1980). Consequently, light isotopes have lower activation energies and normally react faster (Elsner, 2010b). Accordingly, the rate at which heavy and light stable isotopes react during degradation differs (isotope fractionation). Organic contaminant molecules containing heavy isotopes are degraded more slowly, and the fraction remaining when natural transformation occurred will be therefore enriched in the heavier isotopes (Elsner, 2010). Thus, biochemical processes involving the breakage of the chemical bonds, such as microbial transformation or photosynthesis present differences in regard to the reaction with different isotopes (Höhener and Aelion, 2010) leading to small but measurable variations in the isotope ratios among pools of elements and compounds in nature. Physical processes such as gas phase diffusion, sorption and volatilization can also generate fractionation, however is assumed to be much smaller and is generally neglected (Hunkeler and Elsner, 2010; Elsner, 2010a). These variations in the isotope ratios are large enough compared to the analytical uncertainty of modern mass spectrometers allowing consistent determinations. The CSIA overcomes some of the bottlenecks mentioned before for mass-balance approaches. The quantification of isotope ratios is less

affected by the abovementioned limitations of contaminant concentration measurement. Especially when studying volatile compounds, as isotope ratios are not affected by dilution, or sampling artefacts such as volatilization or sorption, equally affecting both heavy and light isotopes (Höhener and Aelion, 2010).

CSIA is the powerful analytical tool utilized to quantify the stable isotope ratio for a specific compounds. For CSIA analysis of sample mixtures, most commonly gas chromatography (GC) separation is coupled to an isotope ratio mass spectrometer (IRMS). After separation on the GC column, and prior to the IRMS analysis, the analytes are converted to the corresponding molecule of measurement usually in online mode. For measuring the isotopic composition of a certain element (e.g carbon) in a compound (e.g dichloromethane), the compound needs to be first transformed to the corresponding molecule of measurement (e.g. CO_2) in order to optimize the mass spectrometer only for a few molecules leading to a high precision in the measurements of the isotopes (e.g C^{13}/C^{12}). This transformation is done by combustion in the case of carbon (GC-C-IRMS), or pyrolysis in the case of hydrogen (Merritt *et al.*, 1995; Hunkeler and Bernasconi, 2010).

After the transformation to the molecule of measurement, samples enter in the IRMS. Here, the molecules of interest already in gaseous form are ionized when passing through the ion source and start moving through a magnetic field. Ions are then deflected with different radius depending on the mass/charge ratio and separated in the detector collectors, commonly Faraday cups. Therefore, what is measured is not directly the abundance of isotopes of an element but the abundance of small molecules with different mass containing the element of interest.

To allow the comparison of isotope analysis most laboratories refer their measurements to international standards. For carbon analysis, the standard is a calcite structure of a marine fossil *Belemnitella americana* commonly referred to as Vienna Pee Dee Belemnite (VPDB) (Coplen *et al.*, 2006a). For IRMS analysis of carbon, a CO_2 reference gas standard that has been cross-calibrated against VPDB is used for daily standardization of sample injections (Hunkeler and Bernasconi, 2010).

Variations in stable isotope ratios towards the reference material are normally reported in the delta (δ) notation in parts per thousand (‰, per mil) following Coplen, (2011), although recently, the term urey (1‰= 1 mUr) has been proposed (Brand and Coplen, 2012). Delta values are not absolute isotope abundances but differences between sample and the international standard.

The isotope ratios of one contaminant provide qualitative indication for biodegradation, yet the quantitative evaluation of field isotope data can be based on the Rayleigh equation (Meckenstock *et al.*, 2004; Elsner *et al,* 2005; Schmidt *et al.*, 2014). The extent of isotope fractionation is usually expressed as the isotopic enrichment factor ε or the isotope fractionation factor α, however ε is nowadays the most frequently used, and it can be obtained applying the linearized logarithmic form of the Rayleigh equation (Renpenning and Nijenhuis, 2016).

CSIA offers a number of interesting applications on the field of groundwater bioremediation: (i) identify the source of a pollutant discharge, (ii) detect the origin of a compound formed in a natural environment from all the potential precursors, (iii) verify reactive transport models to predict duration and length of contamination plumes, (iv) chemical or molecular tracing by using labelled compounds, (v) differentiate between abiotic or phase transport processes and biochemical transformations, (vi) elucidate transformation pathways taking part on natural attenuation processes (vii) quantify the extent of the degradation by applying the Rayleigh equation, and (viii) understand the enzymatic reaction mechanisms to provide evidences of *in situ* microbial transformations (Höhener and Aelion, 2010; Renpenning and Nijenhuis, 2016).

In contrast to the relatively frequent detection of DCM in groundwater, the information on isotopic fractionation during DCM degradation is scarce compared to other halogenated volatile organic compounds such as the chlorinated ethenes (Renpenning and Nijenhuis, 2016; Nijenhuis and Richnow, 2016). So far, the only reported carbon isotopic enrichment factor (ε_C) of DCM was determined for a methanogenic enrichment culture containing a *Dehalobacter* sp. with and without

zerovalent iron (Fe^0) (Lee *et al.*, 2015) and for different methylotrophic strains under both oxic and nitrate-reducing conditions (Nikolausz *et al.*, 2006; Heraty *et al.*, 1999).

The aim of this work is to determine the carbon isotopic enrichment factor value for the *Dehalobacterium*-containing culture to assess whether it can be useful to distinguish different metabolic pathways implied in DCM degradation.

7.2 Materials and methods

7.2.1 Biodegradation experiment

For isotopic analysis, 12 parallel batch cultures were inoculated with the 10^{-10} dilution vial from the sixth consecutive dilution series analysed by Illumina sequencing as explained in section 5.3.3. This consortia contained 4 predominant OTUs: *Acetobacterium*, *Dehalobacterium Desulfovibrio* and *Wolinella*. Culture medium contained mineral and trace elements, vitamins, tungsten and selenium, acetate, yeast extract, Na_2S + L-cysteine as a reducing agent, and $N_2/CO_2/H_2$ in the gas phase (as described in section 5.2.3). All cultures were fed with a dose of 2400 µM of neat DCM (nominal concentration). To stop the biological transformations at different percentages of DCM degradation (determined by FID-GC measurement), 1.5 mL of orthophosphoric acid (50%) was added to the cultures, after stored at 4 °C until analysis (Nikolausz *et al.*, 2006). Two types of controls were included in triplicate: (i) abiotic controls containing the growth medium with DCM without inoculum to control volatile losses, abiotic transformations, potential impurities from the stock solution and the carbon initial isotopic composition of the DCM used as substrate, and (ii) live controls without DCM to analyse the impact on the isotopic fractionation of the DCM transferred with the inoculum source.

7.2.2 Carbon stable isotope analysis

To determine the carbon isotopic fractionation of DCM, liquid aliquots were removed from the sacrificed experimental bottles and placed in 20 mL vials filled with 10 mL aqueous phase (samples were diluted or not in ultrapure Milli-Q water depending on the DCM concentration) and containing a 30 mm PTFE-coated stir bar. This solution was stirred at room temperature and DCM was extracted during 20 min by headspace solid-phase micro-extraction (HS-SPME) using a manual sampler holder equipped with a 75 µM Carboxen- PDMS fiber (Supelco, Bellefonte, USA). For DCM, an Agilent 6890 gas chromatograph (Palo Alto, USA) equipped with a split/splitless injector, coupled to a Delta Plus isotope ratio mass spectrometer through a GCCombustion III interface (ThermoFinnigan, Bremen, Germany). The GC was equipped with a Supelco SPB-624 column (60 m × 0.32 mm, 1.8 µm film thickness; Bellefonte, USA). The oven temperature program was kept at 60 °C for 2 min, heated to 220 °C at a rate of 8 °C/min and finally held at 220 °C for 5 min. Helium was used as a carrier gas with a gas flow rate of 1.8 mL/min. Several DCM aqueous control standards were prepared daily from a pure standard which δC^{13} was known (see below) and analysed on the same days as the samples to ensure accuracy of the isotopic measurements and to correct potential carbon isotopic fractionation induced by the HS-SPME preconcentration technique.

The carbon isotopic composition of this pure DCM standard (−39.6 ± 0.1‰) was determined previously using a Flash EA1112 (Carlo-Erba, Milano, Italy) elemental analyser (EA) coupled to a Delta C isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) through a Conflo III interface (Thermo Finnigan, Bremen, Germany) using six international reference materials (NBS 19, IAEA-CH-6, USGS40, IAEA-600, IAEA-CH-7, L-SVEC) with respect to the Vienna Pee Dee Belemnite (VPDB) standard, according to (Coplen *et al.*, 2006b). All the controls injected in different replicates, days and concentrations (200 µg/L to 10 mg/L) had an average DCM-$\delta^{13}C$ values of −39.9 ± 0.4‰ (n = 18) which is not statistically different from the EA value.

7.2.3 Calculation of the enrichment factor

Carbon isotope ratios are reported in delta notation ($\delta^{13}C$) relative to the international standard VPDB. Following the nomenclature stated by Coplen (2011), results are expressed in parts per mil (‰) by the formula:

$$\delta C^{13}\ (‰) = (R_{sample}/\ R_{std} - 1) \times 1000$$

R_{sample} and R_{std} representing the isotope ratios (C^{13}/C^{12}) of the sample and the standard, respectively. A simplified Rayleigh equation for a closed system was used to quantify the isotopic fractionation:

$$\ln (R_t/R_0) = (Ɛ/1000) \ln (C_t/C_0)$$

where the isotopic enrichment factor (ε_C) describes the relationship between changes in carbon isotopic composition:

$$R_t/R_0 = (\delta C^{13}{}_t + 1000)/(\delta C^{13}{}_0 + 1000)$$

and the concentrations (C_t) along the time course (t) with respect to the initial concentration (C_0). Most of the measurements were run in duplicate, and the one standard deviation (1σ) of the δC^{13} values obtained was below or equal to ±0.5‰ (Lollar *et al.*, 2007). The apparent kinetic carbon isotope effect (AKIE) was calculated according to

$$AKIE = 1/(1\ (z * n/x\varepsilon\ /1000))$$

where *n* is the number of carbon atoms of the element considered that are present in the molecule, *x* of them are located at the reactive site and *z* of which are in intramolecular isotopic competition. To facilitate comparison of measured enrichment factors (ε) between different compounds, it is helpful to express the magnitude of fractionation in terms of apparent kinetic isotope effects (AKIE). AKIE is the position-specific kinetic isotope effect (KIE) accounting for differences in the number of carbon atoms in the molecule (n), the number of carbon atoms in ε value reacting positions (x) and the number of carbon atoms in chemically equivalent positions (z) (Elsner *et al.*,

2005). However, in the case of DCM with only one carbon atom, *n, x* and *z* = 1 (Elsner *et al.*, 2005).

7.3 Results and discussion

The *Dehalobacterium*-containing cultures started consuming the DCM dose after a lag phase of 3 d and DCM was completely depleted in 11 d. The $\delta^{13}C$ of DCM during its fermentation by the *Dehalobacterium*-containing culture is plotted against the degraded fraction of DCM in Fig. 6.1.A. The δC^{13} of DCM increased as transformation took part from an initial value of -38.4 to 54.1‰, indicating a significant enrichment of C^{13} in the residual fraction of DCM. This strong fractionation is in accordance to the fact that DCM molecules only have one carbon atom and it is known that the higher the number of carbons, the smaller the magnitude of carbon isotope fractionation. This decrease is attributed to the higher probability of the heavy isotope to be located at a non-reacting position with bigger molecule sizes (Abe *et al.*, 2009).

The carbon isotopic composition of spiked DCM was -39.6 ± 0.1 in the elemental analyser determination and -39.9 ± 0.4 is the value obtained from aqueous control standards injected in the samples batch. This difference is minimum and derived from the sample processing and the GC-C-IRMS analysis itself. In abiotic controls without inoculum, the DCM concentration was constant during the experiment discarding abiotic transformation, and also no isotope fractionation occurred, as there is no significant differences between time zero value for the experimental bottles and value for the abiotic controls (- 38.4 ± 0.4 and - 38.4 ± 2 ‰, respectively).

In live controls without DCM, the $\delta^{13}C$ measured was + 33.01 ±1 ‰ and it corresponds to the residual C^{13}-enriched DCM transferred with the inoculum. The effect of this enriched DCM is noted as the time zero value for experimental bottles spiked with DCM was -38.40 ± 0.1 ‰, slightly more positive than the value for DCM in the standards -39.9 ± 0.4 ‰.

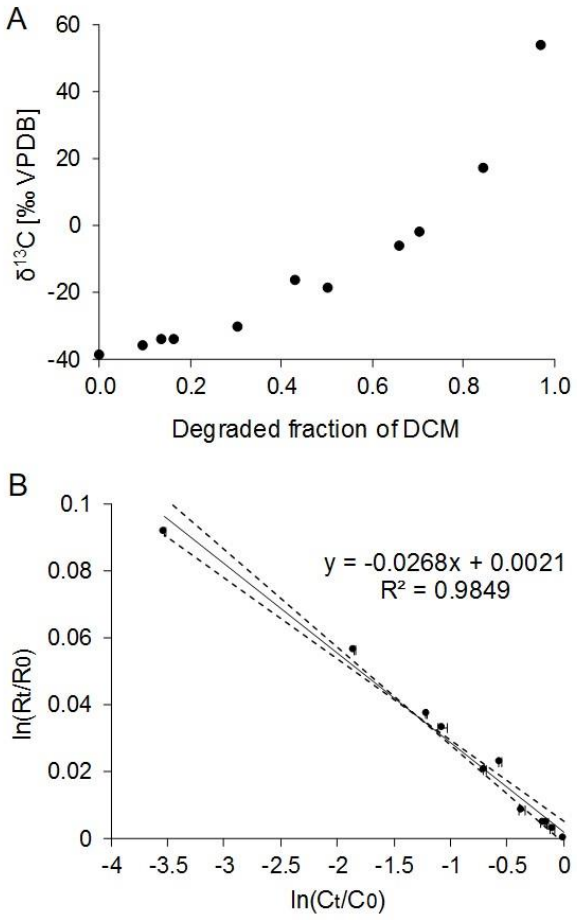

Figure 6.1 Panel A: Carbon isotopic composition of DCM (●) in enrichment cultures during DCM degradation. The error bars showing the one standard deviation (1σ) for duplicate measurements are smaller than the symbols. Panel B: Double logarithmic plot according to the Rayleigh equation of the carbon isotope ratio versus the residual concentration of DCM during fermentation by the enrichment culture. The solid line corresponds to a linear regression model for total combined data and dashed lines to its associated 95% confidence intervals. Data points show the error bars related to duplicate analysis.

The experimental bottles results are plotted in Fig. 6.1.B and showed a good linear correlation factor (R^2= 0.985), indicating that DCM degradation is well described by the Rayleigh model. The carbon isotopic enrichment factor for DCM in this culture resulted in a value of -27 ±2‰.

The apparent kinetic isotope effect (AKIE) obtained from this ε_C was 1.027, which is lower than the theoretical KIE ("semiclassical Streitwieser Limits") for the breaking of the cleavage C-Cl, 1.057. However, considering the indication of Elsner *et al.* (2007), realistic values with transition states at about 50% bond cleavage can be expected to be half as pronounced (AKIE = 1.03), so this reaction was possible.

To date, carbon isotopic enrichment factor for DCM degradation has been only determined for methylotrophic bacteria and one *Dehalobacter* strain present in a methanogenic mixed culture (Freedman *et al.*, 1997; Lee *et al.*, 2015). Carbon isotopic fractionation of DCM by individual facultative methylotrophic organisms showed no consistent differences when they were grown under oxic or nitrate-reducing conditions, but ε_C values significantly differ among the different bacteria tested from −46 to −61‰ (calculated by the transformation of the isotope fractionation factor αC presented on Nikolausz *et al.*, 2006 to ε_C by using the following equation $\varepsilon = 1000 \times ((1/\alpha) - 1)$). The variability in isotopic fractionation among these strains was attributed to differences in the amino acid sequences in DCM dehalogenases (Nikolausz *et al.*, 2006)

The isotopic fractionation of DCM by *Dehalobacter* and *Dehalobacterium* was expected to be within the same order because both strains were fermenting DCM, but the value previously obtained for *Dehalobacter* was significantly weaker ($\varepsilon = -15.5 \pm 1.5$‰). In *Dehalobacter* sp. and *Dehalobacterium formicoaceticum*, DCM has been proved to be directly inserted into the methyl group of acetate, which is yielded after condensing with the carboxyl group derived from the reduction of the carbon dioxide in the medium (Mägli *et al.*, 1998; Lee *et al.*, 2012). Besides they may share the same metabolic pathway, variability in DCM carbon isotopic enrichment factors in *Dehalobacter* and *Dehalobacterium* might be a result of isotope masking due to differences in their cell envelope characteristics or enzyme location (McNamara and Krzmarzick, 2013). Isotope masking in bacteria is a result of rate-limiting events prior to the actual catalytic reaction, as for instance, extracellular and intracellular mass transfers (Elsner, 2010).

The unique *Dehalobacterium* isolated to date (*Dehalobacterium formicoaceticum*) is Gram-positive (Mägli *et al.*, 1996) whereas the Gram staining was mostly negative for the described *Dehalobacter* strains (Maillard and Holliger, 2016). However, a detailed study of the cell envelope of *Dehalobacter restrictus* PERK-23 showed that possesses a proteinaceous surface layer instead of an outer membrane typical for the cell envelope of Gram negative bacteria (Mägli *et al.*, 1996). This surface layer is hypothesized to hinder the Gram stain in *Dehalobacter* and might act as a rate-

limiting barrier for DCM, which would explain the stronger isotope fractionation observed in *Dehalobacterium*. Knowledge of the localization, structure and properties of the reacting enzymes involved in DCM fermentation might also help to understand this significant difference in the isotope fractionation between *Dehalobacterium* and *Dehalobacter*. Interestingly, very different ε_C values have been found for reductive dechlorination of chloroform by different *Dehalobacter*-containing cultures (from −4.3 to −27.5 ± 0.9‰, this last one with an AKIE for C-Cl cleavage equal to that determined in this research) which may reflect the complexity of this genus (Lee *et al.*, 2015; Chan *et al.*, 2012).

Furthermore, the physicochemical characteristics of the chlorinated compound can also influence the extent of rate limitation observed, as evidenced by Renpenning *et al.*, (2015) comparing sorption differences of PCE between gram-positive and gram-negative bacteria. Nevertheless, DCM is less hydrophobic than PCE or TCE and for the latter compound no significant effects on observed isotope fractionation were detected, so this hypothesis could be discarded. In this case, it might be more relevant the localization or typology of the reacting enzyme, but the enzymatic mechanism involved in the anaerobic degradation of DCM by this *Dehalobacterium* is still unknown.

7.4 Conclusions

Despite the uncertainties, the isotopic fractionation of *Dehalobacter* and *Dehalobacterium* are significantly smaller than that for methylotrophic bacteria. This difference can permit to distinguish between anaerobic fermentation and hydrolytic conversion of DCM in field-derived microcosms. The generation of new data of isotopic fractionation for different species, under different conditions is precious for the progress in the understanding and application of CSIA for bioremediation projects. Especially in the DCM bioremediation in underground waters a lot remains to be known, and it is of a major interest to increase the knowledge in this field. The observed strong fractionation also provides the foundation for using CSIA to monitor DCM biodegradation in field applications.

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Chapter 8

Inhibition studies of dichloromethane fermentation by *Dehalobacterium* sp. with halogenated contaminants

Part of this Chapter was submitted for publication as:

"Inhibition studies of dichloromethane fermentation by *Dehalobacterium* sp. with halogenated contaminants". Authors: Trueba-Santiso, A., Fernández-Verdejo, D., Martín-González, L., Vicent, T. and Marco-Urrea, E. (2017) to Journal of Hazardous Materials.

The work presented in this Chapter was done in collaboration with David Juan Fernández Verdejo as a part of his master thesis.

Abstract

The heterogeneity of pollution sources, *in situ* transformation processes of primary pollutants, as well as the recalcitrant character of some chemicals, among other factors, lead to the frequent occurrence of pollutant mixtures at impacted aquifers. The interactions of these co-contaminants with microorganisms can confound bioremediation efforts. In this work, the potential inhibitory effect of selected priority halogenated pollutants frequently detected in groundwater was tested towards the anaerobic DCM degradation performance of a *Dehalobacterium*-containing culture. The increasing concentrations tested of trichloroethylene (TCE), 1,2-dichloroethane (1,2-DCA), *cis*-dichloroethylene (*cis*-DCE), 1,1,2-trichloroethane (1,1,2-TCA), perfluorooctanoic acid (PFOA) and 3,4-dichloroaniline (3,4-DCA) did not show significant inhibitory effects on dichloromethane (DCM) degradation. Differently, a total inhibition was caused with a chloroform concentration of 100 mg/L. Also, the presence of 200 mg/L of perfluorooctanesulfonic acid (PFOS), as well as concentrations higher than 25 mg/L of diuron caused a severe inhibitory effect, impeding the full depletion of DCM. Our results showed that resilience of *Dehalobacterium* was not sensitive to the inhibitory concentration of the co-contaminants tested, and DCM degrading activity was reversible when transferred to fresh medium with DCM alone. Findings derived from this work are of a great interest on predicting the effect of inhibitors during anaerobic DCM bioremediation in groundwaters.

8.1 Introduction

The improper release of hazardous substances from industrial, urban or agricultural practices, among others, often coexist within the same areas, generating residues that may reach the subsurface level (Cabeza *et al.,* 2012; Meckenstock *et al.*, 2015). In addition, groundwater contaminants may undergo biological or abiotic transformations leading to the co-existence of the primary pollutant and its transformation products. For instance, the stepwise reductive dechlorination of perchloroethylene (PCE) involves its conversion to the less chlorinated compounds trichloroethylene (TCE), *cis*-dichloroethylene (*cis*-DCE), vinyl chloride (VC), and the innocuous ethene by organohalide respiring bacteria (OHRB). Frequently, TCE and PCE transformation leads to *cis*-DCE accumulation, in a phenomenon known as "DCE stall", impeding the detoxification of the aquifer and producing complex contaminant mixtures (Maymó-Gatell *et al.*, 1999; Futagami *et al.*, 2008). Co-contaminants may have an inhibitory or toxic effect over those bacteria interesting for bioremediation, understanding inhibition is a biostatic effect that results in reversible damage of biomass function, and toxicity as a biocidal effect that is usually irreversible, but not necessarily lethal (Astals *et al.*, 2015). Elongation of the lag phase is an acclimatization effect previously observed in several bacterial cultures as a response to stress conditions derived from e.g. salt concentration in the growth medium, increase in the temperature, pH or antibiotic applications (Van Impe *et al.*, 1992; Francois *et al.*, 2006).

To date, limited data are available on the potential inhibitory effect of frequently detected co-contaminants over those bacteria of interest for organohalide bioremediation. Most of the works investigating the effect of co-contaminants focused on chlorinated ethenes (e.g., PCE and TCE), chlorinated ethanes (trichloroethanes and dichloroethanes) and chloromethanes (chloroform [CF] and dichloromethane [DCM]) on OHRB bacteria from the genus *Dehalococcoides* (Adamson, *et al.*, 2000; Weathers *et al.*, 2016; Mayer-Blackwell *et al.*, 2016), *Dehalobacter* (Grostern and Edwards 2006; Grostern, 2009) or *Desulfitobacterium* (Futagami *et al.*, 2013).

Dichloromethane (DCM) is a frequently detected groundwater contaminant that typically occurs in mixtures in either disposal sites or even in domestic well samples (Dieter and Kerndorff 1993; Rowe *et al.*, 2007). Under anoxic conditions, DCM can be anaerobically metabolized via a fermentative pathway by bacteria from the genus *Dehalobacterium*, *Dehalobacter* and *Dichloromethanomonas* (Mägli *et al.*, 1996; Lee *et al.*, 2012; Justicia-Leon *et al.*, 2012; Kleindienst *et al.*, 2017). To date, only a few information has been published about the inhibitory effect of certain chlorinated solvents over DCM-fermenting bacteria. For instance, l,1-dichloroethane, PCE and TCE did not affect DCM degradation by *Dehalobacterium formicoaceticum* (Mägli *et al.*, 1998) but tetrachloromethane, CF, and chloromethane, however, caused an immediate and complete inhibition of DCM degradation and bacterial growth. Also, CF was shown to be inhibitory towards a DCM-fermenting mixed culture containing *Dehalobacter* spp. (Justicia-Leon *et al.*, 2012).

Perfluoroalkyl and polyfluoroalkyl substances (PFASs) are a relevant group of halogenated contaminants raising concern based on its toxicity, persistence and widespread presence (Lau *et al.*, 2007; Steenland *et al.*, 2010; Pignotti *et al.*, 2017). Their stability to chemical and biological degradation due to the strong carbon–fluorine bonds lead to widespread buildup and bioaccumulation (ATSDR, 2009). Related to the aim of this study, mixtures of fuels, chlorinated solvents and fire-extinguishing agents such as PFASs were found in aquifers due to their extensive use until the 1990s in firefighter training labours. These activities implied the direct release of such compounds into the environment without any treatment, ending up on the infiltration of this cocktail of pollutants to the groundwater (Moody and Field, 2000; McGuire *et al.*, 2014). The importance of this group of contaminants led to the study of the effect of a mixture of ten PFASs over the OHRB *Dehalococcoides* sp. that caused the inhibition of TCE reductive dechlorination (Weathers *et al.*, 2016).

Special attention should be also payed to pesticides, as an important amount of the chemicals applied to the agricultural soil may reach the underground level and may affect microbial communities (Imfeld and Vuilleumier, 2012). Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a widespread herbicide frequently detected in groundwater (Cabeza *et al.*, 2012; Herrero-Hernández *et al.*, 2013; Köck-Schulmeyer *et*

al., 2014). This compound shows potential for persistence and transport in the environment due to its resistance to decomposition in soil and water and moderate sorption (Stork *et al.*, 2008). Diuron was reported to be transformed into 3,4-dichloroaniline (3,4-DCA) in abiotic reactions (Salvestrini *et al.*, 2002), but also by aerobic aquatic microorganisms and soil bacteria cultures (Ellis and Camper, 1982). 3,4-DCA is described to be more toxic than its precursor for microalgae, protozoa, phytoplankton and bacteria (Giacomazzi and Cochet, 2004; Tixier *et al.*, 2001; Osano *et al*., 2002). 3,4-DCA is a persistent pollutant in groundwater and considered toxic to humans by inhalation and skin contact (UE Institute for Health and Consumer Protection, 2006)

The work presented in this Chapter aims to assess the impact of selected halogenated contaminants over the DCM-fermenting performance of a mixed culture containing a *Dehalobacterium* sp. The selected compounds are among the most frequently detected in contaminated groundwater and included in the 2017 ATSDR Priority List of Hazardous Substances. The list of tested compounds comprises chloroalkanes, chloroalkenes, CF, PFASs, as well as the halogenated pesticide diuron. Also, the transformation product of diuron, 3,4-DCA, was included in the study.

8.2 Materials and methods

8.2.1 *Dehalobacterium*-containing culture

The experiments were performed with a stable consortia composed predominantly by bacteria from the genus *Dehalobacterium*, *Acetobacterium* and *Desulfovibrio* (see section 6.3.2). The consortia was maintained active by transferring the microcosms (3% v/v inoculum) into fresh medium during the exponential degradation phase of 2000 µM DCM, and this culture line was used to inoculate all the experiments described here. Culture medium and cultivation conditions were described in Section 5.3.2, but not hydrogen was added, since it was shown to be not required for DCM degradation (see Chapter 6). Selected co-contaminants were amended to the microcosms as acetone stock solutions to achieve the desired concentration, while DCM was fed as a neat compound. Both were fed through the

septum with 5-1000 µL glass syringes (Hamilton) 24 hours before the bacterial inoculation, to allow the gas-liquid phase equilibrium.

8.2.2 Analytical methods

DCM and other volatile compounds 1,2-DCA, 1,1,2-TCA, *cis*-DCE, TCE and acetone) were monitored by GC as described in Section 3.2.2.

8.2.3 Inhibition tests

Each co-contaminant inhibition test consisted on two groups of experimental bottles prepared in triplicates at the same time: (i) controls amended only with neat DCM and (ii) parallel sets of experimental bottles amended with different concentrations of the co-contaminant object of study plus neat DCM. At time zero, all experimental bottles were inoculated with 3 % v/v inoculum from a *Dehalobacterium*-containing culture at the exponential phase of DCM degradation. Then, a time course experiment was performed, monitoring DCM concentration daily by GC in all experimental bottles. To determine whether an inhibitory or toxic effect was occurring we attended to the following parameters: (i) the duration of the lag phase and (ii) the DCM degradation rates. All experimental bottles amended with co-contaminants were compared to their corresponding controls. In order to quantitatively evaluate the effect of the tested co-contaminants on the lag phase, the parameter 'lag time extension' (LE) was calculated as described elsewhere (Li *et al.*, 2016), following the equation LE= λ_c/λ_0, where λ_c is the lag time of the culture at the concentration C of a co-contaminant, and λ_0 is that at controls without the co-contaminant. When bacterial activity is completely inhibited, λ_c is considered as infinite. To calculate the DCM degradation rates, the linear part of the plot representing DCM fermentation over the time was considered.

8.2.4 Microscope observation

Fresh aliquots of 20 µL of *Dehalobacterium*-containing cultures at different DCM degradation phases were placed in slides, covered with a cover slip, and observed

under a light microscope (Zeiss Axioscop) at 40 x or 100 x (adding immersion oil) objectives.

8.3 Results and discussion

8.3.1 Inhibition tests

Chloroalkanes

We evaluated the potential inhibitory effect of two of the most commonly detected chloroalkanes in groundwater: 1,1,2-TCA and 1,2-DCA at the concentrations of 100, 250, 500 and 850 µM. The results of the inhibition test with 1,1,2-TCA are presented in Fig. 8.1.A, representing the DCM concentration over the time for the different sets of concentrations tested.

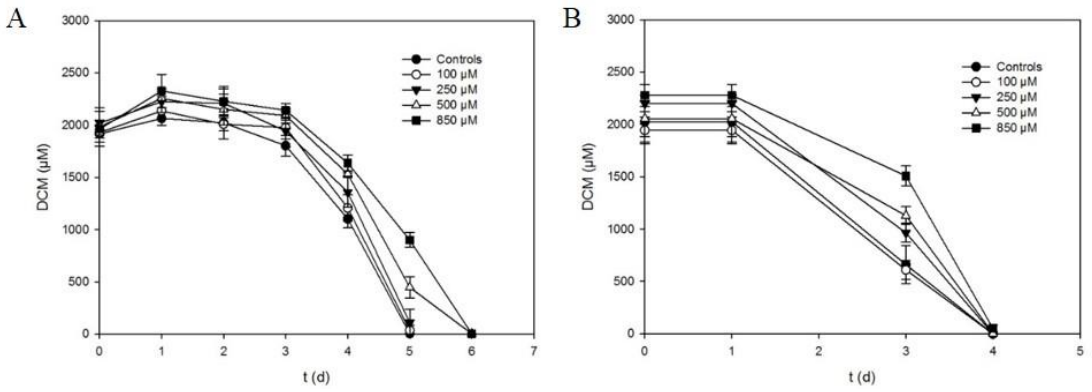

Figure 8.1 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of 1,1,2-TCA (Panel A) and 1,2-DCA (Panel B). Error bars indicates standard deviation of triplicates.

No effect was detected on the duration of the lag phase with the different concentrations of 1,1,2-TCA tested. A value of LE=1 was determined in all cases, as shown in Table 8.1, which presents the LE values and the DCM degradation rates calculated for all the inhibition tests performed in this work. DCM degradation rates were similar in cultures spiked with 100 and 250 µM when compared to the controls. Those cultures amended with 500 and 850 µM, however, depleted the DCM dose one day later, due to a lower DCM degradation rate (Table 8.1). To our knowledge, it was the first study assessing the inhibitory effect of 1,1,2-TCA in the bioremediation of

chlorinated solvents. In the case of 1,2-DCA, as presented in Fig. 8.1.B, all sets of cultures depleted the DCM dose at the same day, however, in this case, it is not possible to determine the LE, nor the DCM degradation rate, as values from day 2 were not analysed. In a recent study, 1,2-DCA was shown to markedly reduce the vinyl chloride respiring capacities of a *Dehalococcoides*-containing population (Mayer-Blackwell *et al.*, 2016).

Table 8.1 Lag time extension factors (LE) and DCM degradation rates for the different concentrations of co-contaminants tested in this study with a *Dehalobacterium*-containing culture.

Co-contaminant	**Set of cultures**	**(LE)**	**DCM degradation rate (µM/d)**
1,1,2-TCA	Controls	-	873.8 ± 31.4
	100 µM	1	859.0 ± 61.7
	250 µM	1	917.3 ± 70.0
	500 µM	1	696.2 ± 14.6
	850 µM	1	713.3 ± 18.7
TCE	Controls	*	889.4 ± 21.6
	100 µM	*	841.7 ± 113.0
	500 µM	*	884.2 ± 36.2
	850 µM	*	604.6 ± 35.8
cis-1,2-DCE	Controls	-	991.9 ± 26.4
	100 µM	1	1047.4 ± 38.2
	250 µM	1	1038.2 ± 31.9
	500 µM	1	992.9 ± 25.2
	850 µM	1	993.1 ± 41.3
Chloroform	Controls	-	515.0 ± 39.1
	2 mg/L	1	492.0 ± 47.0
	8 mg/L	1.5	366.7± 106.1
	10 mg/L	3.5	314.64 **
	100 mg/L	∞	-
PFOA	Controls	-	1249.7 ± 93.1
	50 mg/L	1	1140.5 ± 168.2
	100 mg/L	1	829.9 **
	200 mg/L	3	774.5 ± 40.1
PFOS	Controls	-	751.2 ± 16.8
	10 mg/L	1	713.3 ± 36.0
	50 mg/L	3	693.6 ± 94.6
	200 mg/L	7	-

Diuron	Controls	-	890.4 ± 70.1
	10 mg/L	3	276.0 ± 76.6
	25 mg/L	8	-
	75 mg/L	8	-
3,4-DCA	Controls	*	858.2 ± 18.0
	10 mg/L	*	833.5 ± 19.9
	25 mg/L	*	812.2 ± 45.6
	75 mg/L	*	753.6 ± 14.6
Acetone	Controls	-	940.6 ± 89.5
	7.78 µM	1	1002.7 ± 122.6
	20.03 µM	1	993.1 ± 62.9
	32.10 µM	1	990.7 ± 122.2
	77.82 µM	1	993.6 ± 60.0

*In the experiments corresponding to TCE and 3,4-DCA, no lag phase was detected in the controls, therefore LE value for these sets of cultures was not calculated.

**Cultures spiked with 10 mg/L of CF and 100 mg/L of PFOA correspond to duplicates, therefore standard deviation is not presented.

Chloroalkenes

The solvent TCE and the product of its organohalide-respiration, *cis*-DCE were included in this study. Concentrations of 100, 500 and 850 µM of TCE were tested towards our DCM-degrading mixed culture. In this case, controls presented no lag phase, as shown in Fig. 8.2.A, thus is not possible to calculate the LE factor. In cultures amended with TCE at a concentration of 100 µM DCM, degradation occurred similarly to the controls without TCE, as no lag phase occurred, and DCM degradation rates were similar (Table 8.1). Concentrations of 500 and 850 µM led to a longer period of time required to deplete the amended DCM dose and also to a slower DCM degradation rate in the case of 850 µM. Nevertheless, all cultures consumed the full dose of DCM.

The concentrations tested of *cis*-DCE were 100, 250, 500 and 850 µM. As shown in Fig. 8.2.B, no inhibitory effect was found, as all investigated concentrations produced no elongation in the lag phase (LE values of 1) and DCM degradation rates were similar between the different culture sets (Table 8.1).

The inhibitory response to TCE and *cis*-DCE is different among different species tested in the literature. In accordance with our results, DCM degradation by *D. formicoaceticum* was not inhbited by TCE at a concentration of 1 mM (Mägli *et al.*, 1996). However, the negative effect of TCE (38.5 µM) over the dechlorination of 1,1,1-TCA by *Dehalobacter* sp was reported (Grostern and Edwards, 2006). Additionally, the production of *cis*-DCE (concentrations of 25 and 65 µM) via TCE reductive dechlorination lead to a strong inhibition of 1,2-DCA respiration in a consortia composed by different *Dehalococcoides* strains (Mayer-Blackwell *et al.*, 2016).

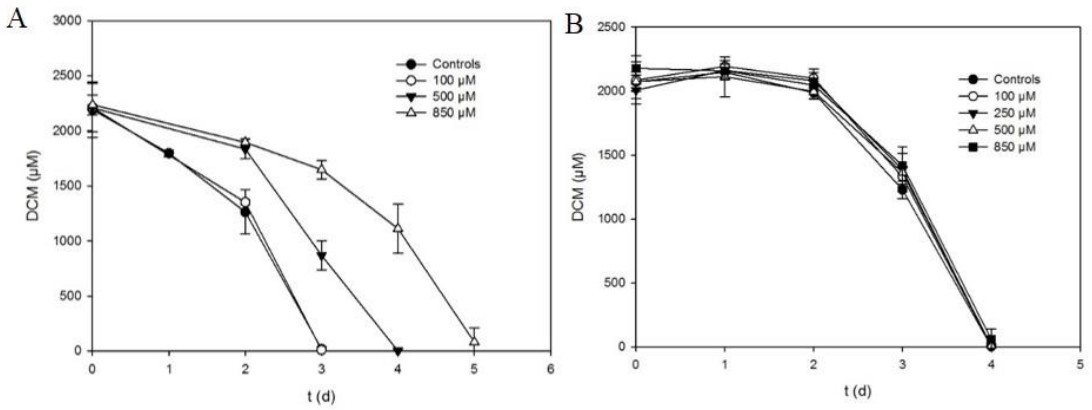

Figure 8.2 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of TCE (Panel A), and *cis*-DCE (Panel B). Error bars indicates standard deviation of triplicates.

Chloroform

The co-contamination of groundwater with CF and DCM is a specially important scenario for this study, due to the existence of anaerobic bacteria belonging to the genus *Dehalobacter* capable to transform CF into DCM via reductive dechlorination (Grostern *et al.*, 2010; Justicia-Leon *et al.*, 2012). In addition, some physical-chemical remediation treatments, as for instance the application of nano-scale zinc particles can cause the transformation of CF into DCM (Teik-Thye Lim, 2005). Both processes lead to the co-occurrence of both compounds.

Chloroform concentrations tested in this study were: 2, 8, 10 and 100 mg/L (16.7, 67.0 83.8 and 837.7 µM, respectively). As shown in Fig. 8.3, cultures amended with CF at 2 mg/L showed a similar DCM degradation profile compared to the controls

without CF, with a LE of 1 and similar DCM degradation rates. Concentrations of 8 and 10 mg/L provoked an inhibitory effect, extending the lag phase (LE) to 1.5 and 3.5, respectively, and decreasing the degradation rates (Table 8.1). Differently, cultures spiked with 100 mg/L showed a total inhibition response, without DCM-degradation activity after 90 d of exposition.

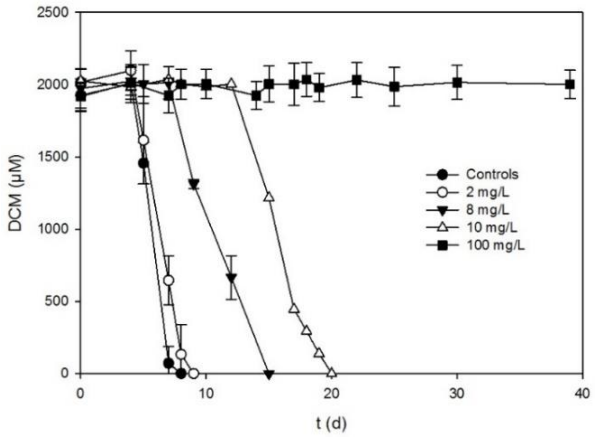

Figure 8.3 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of CF. All points correspond to triplicates except for cultures amended with 10 mg/L of CF that are duplicates. Error bars indicates standard deviation of triplicates.

An inhibitory effect of CF was already reported for *Dehalobacterium formicoaceticum* in pure culture at a concentration of 1 mM (119.5 mg/L) (Mägli *et al.*, 1996), and in the case of the *Dehalobacter*-containing culture reported in Justicia-Leon *et al.*, (2012) at a concentration of 5 mg/L (41.8 µM). CF is also a known strong inhibitor for many bacterial processes, such as methanogenesis (Bauchop, 1967; Weathers and Parkin, 2000) and reductive dechlorination of PCE (Adamson, *et al.*, 2000; Bagley *et al.*, 2000; Duhamel *et al.*, 2002) or orto-chlorophenol (Futagami *et al.*, 2013).

Egli *et al.* (1988) pointed that dechlorination of chlorinated methanes is frequently reported for bacteria harbouring the acetyl coenzyme A (acetyl-CoA) pathway, that involves the coenzyme B12 as a carrier for methyl groups. This pathway is utilized by *Dehalobacterium formicoaceticum* to insert CO_2 into the carboxyl group of acetate during DCM fermentation (Mägli *et al.*, 1998; Chen *et al.*, 2017). CF is considered an inhibitor for coenzyme B12, thus, the presumed benefit of adding

vitamin B12 to cultures exposed to this co-contaminant would be to mitigate its toxic effect. Therefore, we tested if the addition of 10 µM of vitamin B12 was effective to reactivate the DCM degradation in cultures after 37 d of exposition to 100 mg /L of CF, but DCM was not degraded after 40 d of cultivation (data not shown).

Perfluoroalkyl and polyfluoroalkyl substances

Both PFOA and PFOS were tested in this study at concentrations up to 200 mg/L. In the case of PFOA (Fig. 8.4.A), DCM degradation occurred similarly in cultures amended with 50 mg/L in comparison to the controls. No significant difference was detected on the duration of the lag phase (LE=1), nor in DCM degradation rate. Cultures amended with 100 mg/L show an LE of 1, but a slower DCM degradation rate, therefore the DCM dose was depleted one day later. In cultures spiked with the concentration of 200 mg/L a marked elongation in the lag phase (LE=3) was produced, as well as a strong decrease in the DCM degradation rate. Consequently, a longer time was required to consume the DCM dose (Table 8.1). However, cultures depleted the DCM dose in all cases.

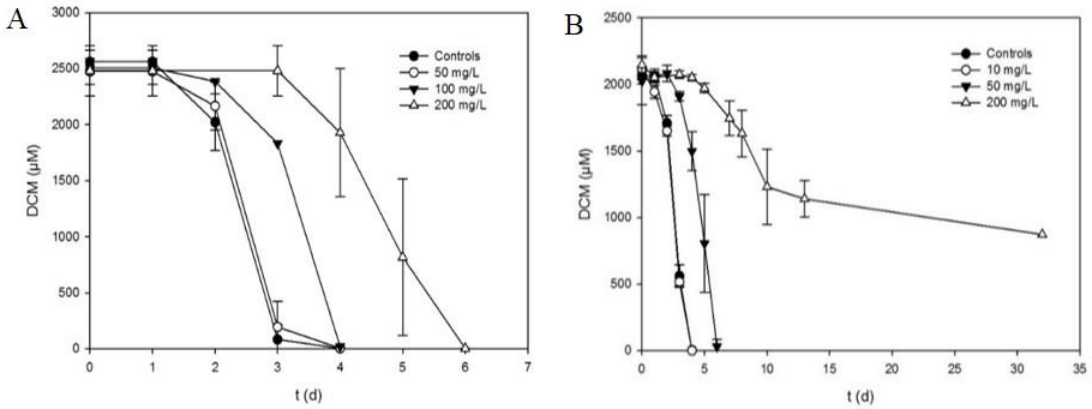

Figure 8.4 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of PFOA (Panel A) and PFOS (Panel B). All points correspond to triplicate cultures except for 100 mg/L of PFOA that are duplicates. Error bars indicate standard deviation.

In the case of PFOS, cultures spiked with 10 mg/L showed no differences in lag phase duration (LE=1) or DCM degradation rates compared to the controls (Table 8.1). Those cultures spiked with 50 mg/L of PFOS showed an elongation in the lag phase (LE=3) but no significant difference in the DCM degradation rate, and, accordingly a longer time was required to deplete the DCM dose. In the presence of 200 mg/L of

PFOS, a marked elongation of the lag phase occurred (LE=7), then DCM degradation was initiated, but stopped at day 13 after the consumption of 1000 µM DCM (Fig. 8.4.B).

Our results showed a severe inhibitory effect of PFOS over the DCM degradation fitness of the *Dehalobacterium*-containing culture that did not occur with PFOA at the same concentration (200 mg/L). This is in contrast with a recent published study showing that PFOA was more toxic than PFOS for the model organism *Escherichia coli*. Results from this study also indicated potential differences in the toxicity mechanisms of these compounds, pointing that PFOS provoked changes at the cell surface properties more acute than PFOA (Liu *et al.*, 2016). Also a slight negative effect of PFOS over methanogenic bacteria was reported (Ochoa-Herrera *et al.*, 2016).

With regards to bacteria of interest for groundwater bioremediation of chlorinated solvents, a mixture of ten PFASs (including PFOA and PFOS) in a total concentration of 110 mg/L was shown to prevent TCE reductive dechlorination by a *Dehalococcoides*-containing culture (Weathers *et al.*, 2016). This resulted in the accumulation of vinyl chloride, which is highly toxic and could potentially affect other dechlorinators existing in impacted sites. However, it is not possible to compare the single effect of PFOS or PFOA with this PFASs mixture. More information would be required to assess whether PFOS alone is toxic to *Dehalococcoides* spp. or other OHRB, as well as the effect of PFASs mixtures over *Dehalobacterium* sp.

It should be mentioned that the average concentrations of PFOA and PFOS in groundwater are in the order of ng/L-mg/L. Exceptionally, a concentration of 47 mg/L of PFOA was found in sediments beneath a landfill, being the highest concentration reported in the literature to date to our knowledge (Ferrey *et al.*, 2012). We found no inhibitory effect at concentration of 50 mg/L PFOA, so it could be concluded that only in a worst-case scenario, PFOS would affect DCM degradation by this *Dehalobacterium*-containing culture.

Diuron and 3,4-DCA

We tested the inhibitory effect of the herbicide diuron and its degradation product 3,4-DCA in separate inhibition tests. As presented in Fig. 8.5.A, diuron amended at 10 mg/L caused an elongation in the lag phase (LE=3), and a clear decrease in the DCM degradation rate (Table 8.1). In those cultures spiked with diuron concentrations of 25 and 40 mg/L, elongation in the lag phase was more marked (LE=8), approximately 500 µM of DCM were consumed at day 14, but then degradation activity stopped. After 32 d of cultivation, the DCM dose was not depleted.

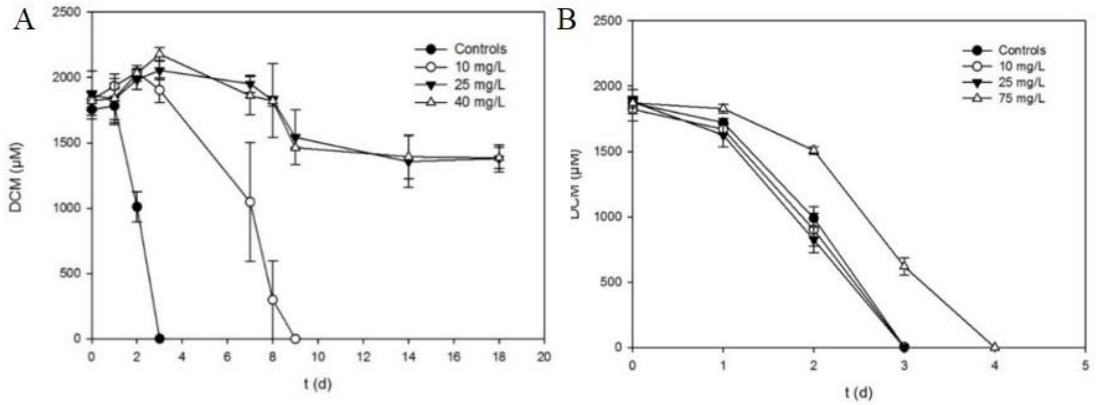

Figure 8.5 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of diuron (Panel A) and 3,4-DCA (Panel B). All points correspond to triplicates cultures. Error bars indicates standard deviation of triplicates.

In the inhibition test with 3,4-DCA, controls presented no lag phase, thus is not possible to calculate the LE factor (Fig. 8.5.B). No significant differences in the lag phase duration or the DCM degradation rates in concentrations of 10 and 25 mg/L were found. With the concentration of 75 mg/L of 3,4-DCA, the DCM degradation rate was slightly lower than in controls, and DCM dose was depleted one day later (Table 8.1).

The concentrations reported for diuron in the literature in groundwater samples are in the range of ng/L, with maximum values ranging from 178 ng/L to 8.55 µg/L depending the environmental matrix (Köck-Schulmeyer *et al.*, 2014; Cabeza *et al.*, 2012; Herrero-Hernández *et al.*, 2013). The concentrations tested in our study are considerably higher than those typically found in the environment, discarding

therefore the potential inhibitory effect of the herbicide diuron and its transformation product 3,4-DCA on DCM transformation by *Dehalobacterium*.

Acetone

All previous co-contaminants studied in this Chapter were added in acetone stock solutions. Therefore, in order to confirm that the inhibitory effects detected in the experiments were caused by the co-contaminants and not by the solvent used, we performed an inhibition study following the same approach. Different concentrations of acetone were included in this study, corresponding to those added with the contaminants stock, in triplicate cultures. Additionally, this study has a second objective, as acetone is also included in the 2017 Priority List of Hazardous Substances, ranked 189th out of 275 and it was detected in 652 of the Sites monitored in the frame of the ATSDR national priority list. Acetone is widely used to make plastic, fibres, drugs or other chemicals, and as solvent in several industrial processes. It can cause toxic effects on the respiratory system as well as alterations in the nervous system, among other negative effects on human health (ATSDR, 2007). As shown in Fig. 8.6, cultures with all the investigated concentrations presented a similar degradation profile as the controls. No elongation on the lag phases occurred, and no significant differences between the DCM degradation rates were detected (Table 8.1). Thus, acetone was found to have no inhibitory effect at the concentrations added in the experiments.

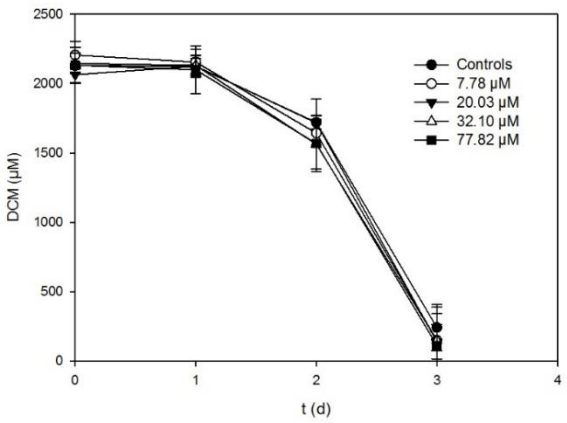

Figure 8.6 Fermentation of DCM in a *Dehalobacterium*-containing culture amended with different concentrations of acetone. All points correspond to triplicate cultures. Error bars indicate standard deviation.

8.3.2 Recovery of the DCM degradation activity after the exposition to inhibitors

The resilience of the *Dehalobacterium*-containing culture to ferment DCM after CF exposure was assessed. Cultures inhibited with 100 mg/L CF for 37 d were purged with nitrogen in both liquid and gas phase for 5 min to remove CF, and 3.5 mL was transferred as 5% (v/v) into fresh medium (n=6) spiked with 1200 µM DCM. The lack of CF in these microcosms was confirmed by GC-measurements (data not shown). This new line of cultures growing in a CF-free medium started degrading DCM after a lag phase of 4 d, and after 13 d of cultivation, the DCM dose was depleted in all six replicates (Fig. 8.7.A). The same procedure was repeated after 90 d of exposition to 100 mg/L CF. In this case, after 32 d of lag phase, and after 36 d of cultivation in CF-free medium, DCM dose was depleted (Fig. 8.7.A). In both cases the degradation rate of DCM was similar but the lag phase was 30 d longer in the cultures exposed for 90 d to CF rather than those exposed for 37 d. This is probably due to the decrease of viable cells in the first case due a longer exposition to the inhibitor concentration of CF.

In a similar way, the resilience of DCM fermentation to diuron exposure was tested. After 32 d of exposition, 3.5 mL of inoculum was taken from cultures affected by both 25 and 40 mg/L of diuron and transferred in parallel to diuron-free microcosms (5% v/v). In both cases, the inoculum recovered the DCM degrading activity after 7 d of lag phase, and DCM dose was depleted after 9 d of cultivation (Fig. 8.7.B). Comparing these results to the abovementioned case of recovery after two different times of exposure to the same concentration of CF, it suggests that the duration of inhibitors exposure is affecting the ability of the culture to recover DCM degrading activity rather than the concentration of the inhibitor.

The resilience of DCM fermentation to PFOS exposure was also tested transferring 5% (v/v) inoculum from microcosms exposed to 200 mg/L PFOS for 30 d to fresh medium with DCM alone. The culture recovered the DCM-degrading activity after a lag phase of 7 d, and DCM dose was depleted after 16 d of cultivation (Fig. 8.7.C).

The reason for this recovery capacities may rely on the fact that *Dehalobacterium sp.* are endospore-forming bacteria (Mägli *et al.*, 1995; Chen *et al.*, 2017), which are known to provide bacteria with a higher degree of resistance to

several physical or chemical damage, as for instance those derived from wet and dry heat, UV and gamma radiation, extreme desiccation or oxidizing agents (Nicholson *et al.*, 2000; Madigan *et al.,* 2004). Accordingly, endospores were also observed in optical microscope observations in our DCM-degrading culture (data not shown). It should be mentioned that in this mixed consortia *Dehalobacterium* is present together with bacteria from the genus *Acetobacterium* and *Desulfovibrio* mainly, and these are described as non-sporulating bacteria (Balch *et al.*, 1977; Postgate and Campbell, 1966), thus the observed endospores might belong to *Dehalobacterium* cells. This hypothesis seems a potential explanation especially for the recovery after PFOS inhibition, as it is described to disrupt bacterial cell membranes, among other citotoxical effects, ending up with the cell inactivation or cell death. However, another possibility is that these pollutants act as reversible enzyme inhibitors binding via weak noncovalent bonds to *Dehalobacterium* enzymes modulating the enzyme activity by competing either directly or indirectly with the substrate. Further research is required to explore the nature of this inhibitory responses and the recovery capacities of *Dehalobacterium* sp.

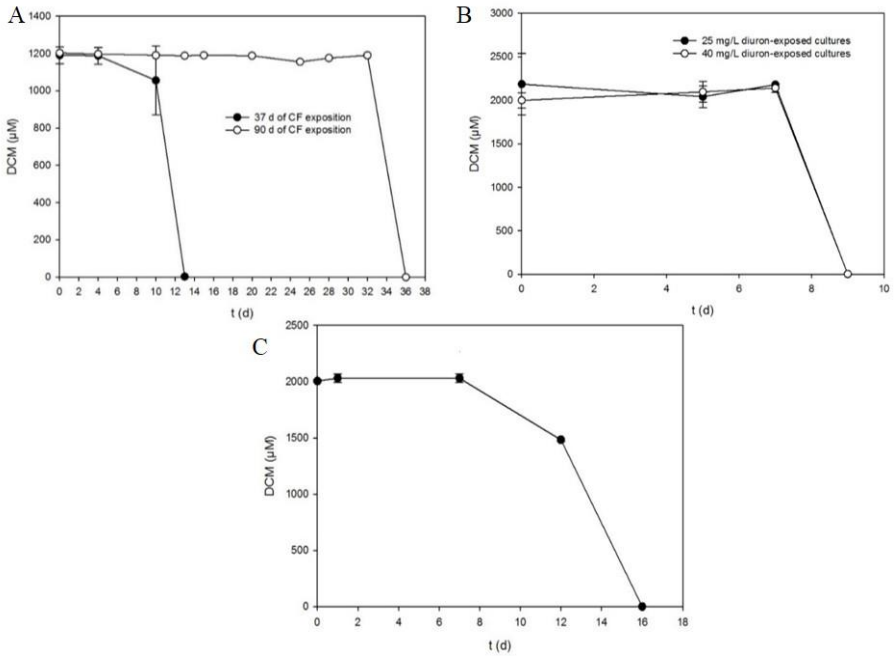

Figure 8.7 Fermentation of DCM in a *Dehalobacterium*-containing culture transferred to fresh medium without co-contaminant after 37 and 90 d of exposition to the inhibitory concentration of 100 mg/L of chloroform (Panel A), 32 d of exposition to 25 and 40 mg/L of diuron (Panel B), and 30 d of exposition to 200 mg/L of PFOS (Panel C). Experiments with CF and PFOS were duplicates. Error bars indicates standard deviation of triplicates.

8.4 Conclusions

No severe inhibition was found over the DCM-degradation performance of this *Dehalobacterium*-containing culture when testing the effect of 1,2-DCA, 1,1,2-TCA, TCE and *cis*-DCE at concentrations up to 850 µM, CF up to 10 mg/L, PFOA up to 200 mg/L, PFOS up to 50 mg/L, diuron at 10 mg/L, and 3,4-dichloroaniline up to 75 mg/L. Also, the inability of this culture to degrade 1,2-DCA, 1,1,2-TCA, TCE, *cis*-DCE, diuron and 3,4-dichloroaniline was confirmed during the previously described inhibition tests. Differently, CF, PFOS and diuron at higher concentrations can be severe inhibitors to DCM degradation by *Dehalobacterium* sp. However, *Dehalobacterium* resilience following inhibitory concentrations of co-contaminants was demonstrated after transferring the exposed culture into fresh medium. The recovery capacity may relay on the fact that *Dehalobacterium* sp. are endospore-forming bacteria. Taken together, the results of this study demonstrate that this DCM-fermenting culture is not inhibited in the concentrations tested of some of the most frequently detected halogenated pollutants in groundwater. Results of this work are useful to ensure the fitness of DCM degradation by *Dehalobacterium* sp in the presence of the studied co-contaminants in the frame of monitoring natural attenuation, enhanced bioremediation efforts.

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Chapter 9

General conclusions and future work

9.1 General conclusions

The main objective of this thesis was to obtain and characterize cultures containing anaerobic bacteria capable of performing biodegradation processes of organohalide compounds of environmental concern. A combination of enrichment culture strategies, dilution to extinction techniques, analytical methods, proteomic techniques, molecular analyses, use of selected antibiotics and carbon stable isotope analyses was used throughout the thesis to achieve the specific objectives of this work.

The main achievements and conclusions that can be drawn from this thesis are summarized below:

- A novel reductive dehalogenase from a *Dehalogenimonas* strain previously obtained from sediments of the River Besós estuary (Barcelona) was identified as responsible for the dihaloelimination of ethylene dibromide to ethene and named as EdbA. Debromination catalytic activity remained unknown among this genus of organohalide-respiring bacteria. This enzyme resulted to be the first reductive dehalogenase shown to be functional without a co-localised membrane anchoring cognate *rdhB* in the genome.
- A stable bacterial consortia containing a *Dehalobacterium* sp. was obtained from slurry samples of the membrane bioreactor of an industrial wastewater treatment plant. This consortia was shown to ferment dichloromethane (DCM) and dibromomethane (DBM) into acetate and formate.
- The carbon isotopic fractionation was determined during DCM fermentation by this mixed *Dehalobacterium*-containing culture. The value obtained was significantly different from the previous values reported for facultative methylotrophic bacteria degrading DCM, but similar to the value determined for a *Dehalobacter*-containing culture fermenting DCM. This difference can permit to distinguish between anaerobic fermentation and hydrolytic conversion of DCM in field-derived microcosms. The observed strong fractionation also provides the foundation for using CSIA to monitor DCM biodegradation in field applications.
- DCM degradation was not inhibited by the presence of high concentrations of frequently detected groundwater co-contaminants indicating the noticeable

robustness of this *Dehalobacterium*-containing culture. In those cases where inhibition occurred, the DCM degradation capacity was recovered when the co-contaminants were absent from the culture medium showing the resilience and recovery capacities of DCM degradation by this culture.

9.2 Future work

- Further proteomic research is required to understand the anchoring mechanisms of the EdbA identified in this thesis that do not have a cognate RdhB.
- A novel aspect to be studied within the *Dehalogenimonas* genus is the structure and composition of its unknown respiratory complex.
- The genome-sequencing of the obtained *Dehalobacterium* strain would allow to achieve a better knowledge on the metabolism repertoire within the Peptococcaceae family and would stablish the basis to compare the metabolism of DCM with bacteria belonging to other families.
- A better knowledge on the cell envelope characteristics of *Dehalobacterium,* as well as isotope studies with growing cells versus crude extracts or purified enzymes, might permit a deeper comprehension of the degradation mechanisms involved in the DCM fermentation.
- Multielemental stable isotope analysis (carbon, chlorine, hydrogen) during DCM degradation by *Dehalobacterium* would be of enormous value to elucidate variations for the same metabolic mechanism previously masked in a single-element isotope analysis and characterize degradation processes in the field.
- Considering the already mentioned heterogeneity of contaminants present in impacted aquifers, more investigation are required to assess the potential additive effect of more than one co-contaminant in mixtures towards the DCM activity of this *Dehalobacterium*-containing culture.

Alba María Trueba Santiso

Contact information: albamaría.trueba@uab.cat / albatrueba@live.com

PhD Candidate

MINECO Predoctoral Grant FPI, 2014. Starting date: 1st May 2015. BioREM Research Group. Department of Chemical Biological and Environemental Engineering at the Escola Técnica Superior de Enginyeria (ETSE) of the Universitat Autonoma de Barcelona. Cerdanyola del Vallés, Barcelona (Spain).

PhD student of the Biotechnology Phd Program. Thesis title: "Enrichment and characterization of anaerobic bacteria degrading organohalide compounds". Work included within the H2OPHARMA Project, funded by the MINECO. Project reference: CTM2013-48545-C2-1-R.

SUPERVISORS: Dr. Ernest Marco Urrea, Dra. Teresa Vicent Huguet, and Dra. Lucía Martín González.

PREVIOUS WORK

October 2013 – December 2014. **Researcher** at the Environmental Engineering Research Team EnQA UDC – ECOSAN of the Faculty of Sciences of the University of A Coruña. Operation of a hybrid lab-scale constructed wetland for the natural treatment of high organic load wastewater. Hired researcher within the project *Respirometric techniques and anaerobic essays for design and control of constructed wetlands*, funded by the MINECO.

September 2011 – February 2012. **Master Thesis** of the Master Degree in Advanced Biotechnology. Environmental Engineering Research Team. Faculty of Sciences of the University of A Coruña. Thesis title: "*Operation of Biological Reactors for Wastewater Treatment*". Qualification acquired: With honours.

September 2011 – February 2012. **Training period of the Master Degree** in Advanced Biotechnology. Aquagest S.A (Production Department of the Northern Galician Area, Spain). Gas measurement campaigns in Betanzos WWTP (A Coruña, Spain). Analysis and production control on different WWTP located in the geographical area of Northern Galicia.

July 2010 – August 2010. **Training period of the Bachelor Degree** in Biology. Food and environment lab of the company EPTISA Engineering Services S. L. in Vilaboa (A Coruña). Microbiological and chemical analysis of water, food, soil and surfaces.

ACADEMIC EDUCATION

2012. Master in Advanced Biotechnology, specialty in Environmental Biotechnology. University of A Coruña (90 ECTS)

2010. Bachelor degree on Biology, University of A Coruña.

LIST OF PUBLICATIONS

Torrijos, V, Gonzalo, O.G, Trueba-Santiso, A., Ruiz, I., Soto, M. (2016). "*Effect of by-pass and effluent recirculation on nitrogen removal in hybrid constructed wetlands for domestic and industrial wastewater treatment*". *Water Research, Volume 103, 15 October 2016, Pages 92–100.*

Trueba-Santiso, A., Parladé, E., Rosell, M., Llirós, M., Mortan S.H., Martínez-Alonso, M., Gaju, N., Martín-González, L., Vicent, T. and Marco-Urrea, E. (2017). "*Molecular and carbon isotopic characterization of an anaerobic stable enrichment culture containing Dehalobacterium sp. during dichloromethane fermentation"*. Science of the Total Environment, 581–582, 640–648.

Trueba-Santiso, A., Fernández-Verdejo, D., Martín-González, L., Vicent, T. and Marco-Urrea, E. "*Inhibition studies of dichloromethane fermentation by Dehalobacterium sp. with halogenated contaminants*". **Submitted** to Journal of Hazardous Materials.

Trueba-Santiso, A., Wasmund, K., Vicent, T., Adrian, L. and Marco-Urrea, E. "*Genome sequence of a Dehalogenimonas alkeniginges strain and identification of the novel EdbA catalysing the dihaloelimination of ethylene dibromide to ethene*". **Under preparation** to be submitted to the journal Environmental Microbiology.

PARTICIPATION ON SCIENTIFIC CONFERENCES

AquaConSoil 14th International Conference. **Oral presentation**: "*Characterization of a Dehalobacterium-containing culture fermenting high concentrations of dichloromethane under reducing conditions: microbial composition, isotope analysis, and potential inhibitory effect of co-contaminants*". 26th – 30th of June, 2017. Lyon, France.

DehaloCon II, A conference on Anaerobic Biological Dehalogenation. **Flash poster presentation**: "*Dichloromethane fermentation by an enrichment culture containing a Dehalobacterium sp: molecular and carbon isotopic analysis*". 26th-29th March, 2017. Leipzig, Germany (UFZ).

2º Seminar REGATA NETWORK (Galician Network of Water Treatment). A Coruña, 27th February 2015, ETSICCP. **Oral presentation**: "*Integral treatment of wastewater by constructed wetlands, EnQAUDC-ECOSAN group*". Presented by V. Torrijos and A. Trueba-Santiso. Authors: V. Torrijos, A. Trueba-Santiso, D. de la Varga, I. Ruiz and M. Soto.

WETPOL 2015 International symposium on wetland pollutant dynamics and control. "*Investigating different hybrid configurations of constructed wetlands for nitrogen removal from domestic and industrial wastewaters*". **Oral presentation**. Authors: V. Torrijos, A. Trueba-Santiso, I. Ruiz, M. Soto. Organized by the Constructed Wetland Association and Cranfield University. York (Reino Unido).

ASSISTANCE TO SCIENTIFIC CONFERENCES

Technical Seminar "*Jornada R+D+i a l'Aplicació de tecnologies de descontaminació del subsòl. Casos pràctics*", 9th and 10th March 2017. Agència de Residus de Catalunya. CosmoCaixa de Barcelona.

Conference: "*Distribution of Dehalococcoides in marine sediments and strategies for their enrichment*" Camelia Algora Gallardo from the Isotope Biogeochemistry Department of the UFZ Helmholzt Institut of Leipzig (Germany), 13th April 2016. ETSE (UAB).

Conference: "*Advancement in the application of CSIA in chlorinated solvent contamination studies*". Orfan Shouakar-Stash, Ph.D. Director and Research and Development Manager of Isotope Tracer Technologies Inc., Waterloo, Canada; School of Engineering, University of Guelph, Canada; Department of Earth and Environmental Sciences, University of Waterloo, Canada. 26th May 2015. Earth Science Institute Jaume Almera, Universitat de Barcelona (UB) and CSIC.

Technical seminar: "*Engineered nanoparticles (ENP) in water and wastewater*". Technical School of Engineer, Santiago de Compostela, 25th November 2014. REGATA NETWORK.

Technical seminar: "*Successful cases of industrial wastewater treatment*". Technical School of Engineer, Santiago de Compostela, 14th November 2014. REGATA NETWORK.

4th International Conference on Biotechniques for Air Pollution Control (Biotechniques 2011). Universidade da Coruña and Federación Europea de Biotecnología (EFB). A Coruña. 11th-14th October2011.

1st Congress of Industrial Microbiology and microbial biotechnology. Spanish Society of Microbiology (CMIBM-SEM). A Coruña. November, 2006.

OTHER PROFESSIONAL EXPERIENCES

September 2017 – January 2018. **Co-director** of the **Masther Thesis** of David *Fernández-Verdejo, titled "Inhibition Studies of Halogenated Contaminants with an Enrichment Culture Containing Dichloromethane-Fermenting Dehalobacterium sp." Màster en Enginyeria Biològica i Ambiental.*

12th June 2017. **Lecture on TU Berlin** on the lecture series on "Functional Genomics of Prokaryotes" directed by Dr. Lorenz Adrian for the Masther students of Biotecnology course and titled *"Dehalogenimonas sp. genome annotation and its role in the frame of an in situ groundwater bioremediation project".*

April 2012 and March 2013. **Teacher** of the subject called "Environment" in the Recreational Activities Instructor Course of the Educational Centre for Recreational Activities in the University of A Coruña, Spain.

September 2012 – June 2013. **Environmental educator** and environment interpretation specialist on Terranova Environmental Interpretation and Management, S.L.

LANGUAGE CERTIFICATES

Certificate in Advanced English, grade B (C1 level on the European Language Reference), University of Cambridge. November 2013.

ABROAD EXPERIENCES

May - September 2017. Research stay at the Department of Isotope and Biogeochemistry of UFZ (Leipzig, Germany), under the supervision of Dr. Lorenz Adrian.

September – October 2010. English language programme at the Canadian as a Second Language Institute in Vancouver, Canada.

Acedemic year 2008/2009. Erasmus scholarship at the Faculdade de Ciências of the Universidade de Lisboa.